ISOLATION, IDENTIFICATION AND CHARACTERISATION OF THREE VIRUSES FROM *PISUM SATIVUM* IN SOUTH AFRICA

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A thesis submitted to the Faculty of Science, Department of Microbiology, University of Cape Town, in fulfillment of the requirements for the degree Master of Science

February 2000

ACKNOWLEDGEMENTS

I would like to thank:

- ✞ My Creator and Saviour for His endless love and presence in my life
- ❤ My precious family for their love, understanding and support
- ✳ Gerhard Pietersen for the opportunity to complete this study, his inputs in this study and his motivation during the past years
- ✏ Ed Rybicki for all his valuable inputs and motivation to complete this study
- $ The PPRI for financial assistance and time allowed spending on this project
- ❀ My colleques at the PPRI for assistance and moral support, especially the Virologists Glynnis Cook, for proofreading the thesis, Kassie Kasdorf, Marika van der Merwe, Ravini Pillay and Dariusz Goszczynski.
- ☆ Kassie Kasdorf, Hendrik van Tonder and Flip van der Merwe responsible for the electron microscopy
- ❁ Elsa van Niekerk and Susan Koch who helped with the pictures

ABSTRACT

Three virus isolates from pea plants (*Pisum sativum)* from different regions in South Africa were identified and are discussed in this thesis. A number of techniques were used to identify the viruses including serology, electron microscopy, cytopathological studies, aphid transmission tests, host range studies and molecular-based techniques such as PCR, cloning and sequencing and sequence comparisons.

During a survey on peas by researchers of the ARC-PPRI in the George district of the Western Cape, several plants with virus-like symptoms were collected. One of the plant samples, 91/0394, was unique in the symptoms produced by the plant. The virus was initially identified as *Pea enation mosaic virus* (PEMV) based on serological results. Isometric particles were detected in the sample and further characterisation of the virus was completed.

A second pea plant came from a pea producer in the Brits district of the North West Province. The plant showed severe mosaic symptoms and electron microscopy revealed particles typical of *Broad bean wilt virus* (BBWV). Antiserum to a known isolate of BBWV was used to positively identify this virus. As in the case of PEMV, our isolate of BBWV was further characterised.

The third unknown virus from pea was collected at the Vegetable and Ornamental Institute at Roodeplaat, Pretoria. The plant - with a yellow vein clearing - showed flexuous particles under the electron microscope.

Preliminary serological tests indicated that the virus was related to *Bean yellow mosaic virus* (BYMV). The close serological relationships between the different *Potyviruses* prompted the characterisation of this virus on a molecular level. The 3' NCR and part of the CP gene were amplified as cDNA by PCR: a PCR product of the correct size for BYMV was obtained. This was cloned and the nucleic acid sequence was compared to those of other *Potyviruses*. The virus was positively identified as a strain of BYMV.

The knowledge of the virus status in peas in South Africa is limited and the aim of this study was to identify unknown viruses from pea. The results of this study added to the knowledge of viruses occurring on peas in South Africa.

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1.1 INTRODUCTION

Knowledge of the viruses occurring in green peas (*Pisum sativum*) in South Africa is limited. Initial studies were conducted in the 1960s, when Klesser (1960) identified *Tomato spotted wilt virus* (TSWV). Other viruses recorded as occurring on peas in this report are Pea mosaic virus (PMV), Pea stunt virus, a strain of Lucerne mosaic virus, "Pea virus 2", "Pea mosaic virus 4" and "Pea wilt virus". In a report listing plant pathogens in cultivated plants in South Africa, published in 1977, viruses listed as occurring on peas were *Pea mosaic virus* (PMV), Pea stunt virus (*Clover red vein mosaic virus*), Pea wilt virus, *Alfalfa mosaic virus* (AMV) and TSWV (Gorter, 1977). At the time these records were made the structure of an accepted system for naming pathogenic viruses was undeveloped and names that were assigned to viruses then are no longer used in the present virus classification system. The techniques then used for identification of viruses involved host range determinations, determination of thermal inactivation point, longevity *in vitro*, and dilution end point determinations, and confirmation of these earlier reports using more modern techniques for identification and characterisation is therefore necessary.

Members of the Plant Protection Research Institute (PPRI) collected pea samples during a survey of peas in the Western Cape Province. Another two pea samples for identification were submitted to the PPRI by pea growers in the Brits district of the North West Province and from Roodeplaat in the Gauteng Province. The aim of the study was to determine the identity of the viruses collected as samples 91/0394, 94/1969 and 95/0931 and to develop ELISA detection systems to the viruses in our laboratory if such systems were not available.

Preliminary identification of the three samples collected showed that they were similar to known viruses described in the literature. One of the plant samples collected from *P. sativum* cv. Cape Freeze plants during the survey in 1991 on peas in the George district displayed symptoms such as interveinal chlorosis of the upper leaves, with chlorotic flecking and downward leafcurl.

Mechanical inoculation onto *P. sativum* cv. Green Feast plants from this source plant produced the same symptoms as displayed by the original plant. An isometric virus was observed with electron microscopy and was isolated from sample 91/0394. Preliminary serological results with ELISA (enzyme-linked immunosorbent assay) indicated that the virus was PEMV. Observations such as symptoms and particle morphology correlated with those described for PEMV (Hull, 1981).

The second sample (94/1969) was obtained from peas grown in the Brits district and showed severe mosaic. Two types of isometric particles were noted: these were apparently empty particles and complete particles. The phenomenon of some particles containing nucleic acid and others without is characteristic of BBWV (Taylor and Stubbs, 1972) and many other virus groups, eg. tymoviruses and further characterisation was necessary to identify the virus.

A pea plant with yellow mosaic and vein clearing collected from Roodeplaat in Pretoria (sample 95/0391) contained flexuous rod-shaped particles as observed by EM studies. Preliminary ELISA results indicated that this virus could be related to BYMV.

This report is therefore devoted to the more complete characterisation of the three virus isolates preliminarily identified as PEMV, BBWV and BYMV.

1.2 PROJECT PROPOSAL FOR STUDY

The three putative virus isolates obtained (91/0394, 94/1969, 95/0931) had to be established in the greenhouses and isolated according to isolation procedures previously described. Once the viruses were isolated, the characterisation of all three by electron microscopy, cytopathology, host range studies, biophysical properties and serology was undertaken. ELISA detection systems were not available for two of the viruses (91/0394 and 94/1969) and had to be established. Polyclonal antisera were to be raised against these viruses. Molecular approaches needed to be used with the third virus isolate (95/0931) as more definitive identification was necessary due to the close resemblance of different potyviruses. Polymerase chain reaction (PCR) was to be used to amplify the 3' NCR and a portion of the coat protein gene of the virus. The amplified PCR product was to be cloned and sequenced and the sequence compared with those of other known potyviruses.

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2.1 INTRODUCTION

2.1.1 Peas and their production areas

The pea (*Pisum sativum*) is one of the oldest cultivated vegetables and cultivation goes back so far that the wild ancestor is unknown (Bosch and Coertze, 1995). The pea plant played an important role in the breeding experiments of Austrian monk Gregor Mendel, who in the 1860s crossed pea plants of differing morphologies and revealed the discreteness of genes (Watson *et al.,* 1997). The crop plant belongs to the legume family *Fabaceae* and is an important vegetable in South Africa. Peas are divided into two main groups, the green peas, and the dry pea. The green and yellow dry pea each constitutes 50% of the demand in South Africa (Bosch and Coertze, 1995). Peas are used for freezing, canning, dehydration, fresh market; dry peas are used for the making of soup, the yellow dry peas are an important ingredient of Indian dishes (Bosch and Coertze, 1995).

The main production regions for green peas in South Africa are the Western Cape (George, Knysna, Humansdorp area and the Gamtoos River Valley), Northern Cape (Vaalharts area where canning peas are grown in rotation with wheat), and the Northern Provinces (Groblersdal, Marble Hall area). Smaller plantings also occur in the Pretoria, Cullinan, and Bronkhorstspruit area and along the eastern slopes of the Drakensberg Mountains in the Barberton, Nelspruit, Schagen areas. In the Free State peas are grown along the Orange River in the Hartswater area. Dry peas are mainly produced in the Upington area (Bosch and Coertze, 1995).

Peas are the fourth most important grain legume crop world-wide. Green pea production is 4.8 million tons grown on 0.76 million ha, and dry bean production is 16.3 million tons grown on 9.5 million ha (Ali and Randles, 1997). Wild and primitive forms are found world wide in a vast geographic area stretching from the western Mediterranean to

Afghanistan as well as in the high elevations of Ethiopia. Peas are grown as a crop the world over. The USSR and China account for most of the estimated world production of peas (Hagedorn, 1984).

2.1.2 Viruses infecting peas worldwide

Numerous viruses are known to infect peas. In South Africa, P.J. Klesser did research on viruses of legumes such as lupins, cowpeas, peas, sweet peas and broad beans in the 1960s. The report describes eight viruses occurring naturally on *P. sativum* (peas) and *Lathyrus odoratus* (sweet peas) including PMV, "pea virus 2", "pea stunt virus", Pea wilt virus, Lucerne mosaic virus, Bean local chlorosis virus and BYMV (necrotic strain) (Klesser, 1960). In a recent report of the Vegetable and Ornamental Institute of the Agricultural Research Council in South Africa, the only known virus diseases on peas were the so-called spotted wilt and mosaic (Boelema and Coertze, 1995). Subsequent to the above reports no further studies were done on the occurrence of viruses on peas in South Africa. The reasons for this statement were described in the introduction of Chapter 1.

Thirty-five viruses representing 15 virus genera infect peas worldwide naturally or experimentally (Ali and Randles, 1997; Brunt *et al.*, 1996; Maury *et al.*, 1987). Pea viruses are transmitted from plant to plant by aphid vectors or transmission by seed (Brunt *et al.*, 1996). With this literature survey it is shown that viruses of the *Potyviridae* and *Luteoviridae* families are the most prominent viruses occurring on peas worldwide. In total six potyviruses and five luteoviruses are recorded (Brunt *et al.*, 1996). The other virus genera represented are *Comovirus*, *Nanovirus*, *Cucumovirus*, *Nepovirus*, *Tobravirus*, *Enamovirus*, *Alfamovirus*, *Fabavirus*, *Potexvirus*, *Cytorhabdovirus*, *Tospovirus* and *Ilarvirus*. A summary of these viruses is given in Table 2.1.

VIRUS SPECIES	GENUS	FAMILY
Alfalfa mosaic virus (AMV)	*Alfamovirus*	*Bromoviridae*
Bean leaf roll virus (BLRV)	*Luteovirus*	*Luteoviridae*
Bean yellow mosaic virus (BYMV)	*Potyvirus*	*Potyviridae*

Beet western yellows virus (BWYV)	*Luteovirus*	*Luteoviridae*
Broad bean wilt virus (BBWV)	*Fabavirus*	*Comoviridae*
Broad bean stain virus (BBSV)	*Comovirus*	*Comoviridae*
Chickpea stunt virus (CpSV)	*Luteovirus*	*Luteoviridae*
Clover yellow mosaic virus (ClYMV)	*Potexvirus*	-
Clover yellow vein virus (ClYVV)	*Potyvirus*	*Potyviridae*
Cucumber mosaic virus (CMV)	*Cucumovirus*	*Bromoviridae*
Lettuce mosaic virus (LMV)	*Potyvirus*	*Potyviridae*
Milk vetch dwarf virus (MVDV)	*Nanovirus*	*Circoviridae*
Muskmelon vein necrosis virus (MuVNV)	*Carlavirus*	-
Pea early browning virus (PEBV)	*Tobravirus*	-
Pea enation mosaic virus (PEMV)	*Enamovirus*	-
Pea mild mosaic virus (PMiMV)	*Comovirus*	*Comoviridae*
Pea mosaic virus (PMV)	*Potyvirus*	*Potyviridae*
Pea seed-borne mosaic virus (PSbMV)	*Potyvirus*	*Potyviridae*
Pea stem necrosis virus	*Nepovirus*	*Comoviridae*
Pea streak virus (PeSV)	*Carlavirus*	-
Peanut mottle virus (PeMoV)	*Potyvirus*	*Potyviridae*
Peanut stunt virus (PSV)	*Cucumovirus*	*Bromoviridae*
Red clover vein mosaic virus (RCVMV)	*Carlavirus*	-
Soybean dwarf virus (SbDV)	*Luteovirus*	*Luteoviridae*
Subterranean clover red leaf virus	*Luteovirus*	*Luteoviridae*
Subterranean clover stunt virus (SCSV)	*Nanovirus*	*Circoviridae*
Tobacco ringspot virus (TRSV)	*Nepovirus*	*Comoviridae*
Tobacco streak virus (TSV)	*Ilarvirus*	*Bromoviridae*
Turnip mosaic virus (TuMV)	*Potyvirus*	*Potyviridae*
Tomato spotted wilt virus (TSWV)	*Tospovirus*	*Bunyaviridae*
Watermelon mosaic virus (WMV)	*Potyvirus*	*Potyviridae*

Table 2.1. Viruses occurring naturally on peas in the world (Hagedorn, 1984, Brunt *et.al.* 1996 and Brunt, *et. al.* 1990; Pringle, 1999)

The viruses in Table 2.1 are all recorded to occur naturally on peas worldwide. A report from Hagedorn (1984) lists viruses occurring naturally on peas in the USA in order of estimated economic importance. According to this report PEMV is the most important virus infecting peas in the USA followed by *Bean leafroll* (BLRV), *Pea seed-borne mosaic* (PSbMV), *Pea streak* (PeSV), *Red clover vein mosaic* (RCVMV), BYMV,

Alfalfa mosaic (AMV), *Watermelon mosaic* (WMV), *Lettuce mosaic* (LMV), *Cucumber mosaic* (CMV) and *Turnip mosaic virus* (TuMV) (Hagedorn, 1984).

In the United States, PEMV is important in the northeast and northwest pea-growing areas. Disease incidences of 90% on peas are reported from New York State. Severe disease caused by PEMV is reported in the Netherlands, Switzerland, West Germany, and England (Hagedorn, 1996). The incidence of PEMV in South Africa is unknown. PEMV is transmitted by two aphid vectors, *Acyrthosiphon pisum* and *Myzus persicae* (Hull, 1981; Brunt *et al.,* 1996). A more in depth description of PEMV will be discussed later in the chapter.

PSbMV is a continuing threat to the pea seed and processing industry in the United States (Kraft and Hampton, 1980). The virus is found in germplasm collections and had a noticeable incidence on field production as in the North American pea growing areas (Maury *et al.,* 1987). The virus was first reported in Czechoslovakia and has also been found in Japan (Inouye, 1967), the Netherlands (Bos, 1970), and Canada (Hamilton *et al.,* 1975) and may be present in every country were peas are grown. The virus is reported from 30 countries (Ali and Randles, 1997). PSbMV is one of the four viruses on peas that are seed-borne and is efficiently transmitted by at least six aphid species, in particular *Acyrthosiphon pisum, Aphis crassivora, Aphis fabae, Dactynotus escalanti, Macrosiphum crataegarius* and *Rhopalosiphum padi* (Maury *et al.,* 1987; Masmoudi *et al.,* 1994; Brunt *et al.,* 1996).

The involvement of alfalfa in the natural occurrence and spread of *Pea streak virus* (PSV) was not recognised before, but evidence indicates that the pea aphid *Acyrthosiphum pisum* is involved in the spread of PSV (Hampton, 1983). In Washington State (USA) there were reports of pea aphids invading pea fields which resulted in 70-100% of the plants showing apical or whole-plant necrosis (Hampton, 1983). PSV occurs mainly in the Eurasian region, Canada and the USA (Brunt *et al.,* 1996).

RCVMV is found and spreads naturally in the North American region, Germany and the Netherlands. The virus was found with no evidence of spread in the UK, Sweden and Switzerland (Brunt *et al.*, 1996).

BYMV is another important crop on legumes and is distributed worldwide (Falk and Duffus, 1984; Brunt *et al.*, 1996). PMV was isolated from pea (Xiao *et al.*, 1994) and differed from BYMV in the symptoms produced on pea. Later it was established that PMV is a strain of BYMV (Xiao *et al.*, 1994). BYMV is transmitted by more than 20 aphid species such as *Acyrthosiphum pisum, Macrosiphum euphorbiae, Myzus persicae,* and *Aphis fabae.* The percentage seed transmission reported for BYMV is not very high, but seed transmission was observed in *Lupinus luteus* (6%), *Melilotus alba* (3-5%) and a "low" percentage in *V. faba* (Hollings and Brunt, 1981). Literature on the seed transmission of BYMV in *P. sativum* was not found.

BBWV spreads naturally in the African and Eurasian region, the Middle East, the North American and Pacific region, Australia and China (Brunt *et al.*, 1996). The virus was isolated from *Vitis vinifera* from South Africa (Castrovilli *et al.*, 1985). Various aphid vectors such as *Acyrthosiphum pisum, Aphis crassivora, Aphis fabae, Aphis nasturtii, Macrosiphum euphorbiae, Macrosiphum solanifolii and Myzus persicae* transmit the virus (Brunt *et al.*, 1996).

AMV is probably distributed worldwide (Brunt *et al.*, 1996). The virus causes diseases of economic importance in alfalfa, clover, pea, potato, tobacco, pepper, tomato and celery (Van Regenmortel and Pinck, 1981). In the USA forage legumes have been implicated as reservoirs of AMV (Hampton, 1967). The virus is transmitted by at least 13 species of aphid vectors in the nonpersistant manner, especially *Myzus persicae* (Van Regenmortel and Pinck, 1981). The virus is also transmitted by seed, grafting and via pollen to seed (Brunt *et al.*, 1996).

LMV is one of the most damaging viral diseases of lettuce in California (Zerbini *et al.*, 1997) and is also found naturally on peas and is probably distributed worldwide. The

virus is transmitted by seed and by aphid vector *Aphis gossypii* (Brunt *et al.,* 1996) as well as *M. persicae, M. euphorbiae* and *Acyrthosiphon scariolae barri* (Tomlinson, 1970). LMV has a relatively wide host range that includes a variety of common weeds (Zerbini *et al.,* 1997) and also occurs naturally on peas inducing a chlorotic mottling symptom (Brunt *et al.,* 1996).

BWYV is one of the most wide spread and economically important plant viruses in the world (Falk and Duffus, 1984). Various aphid vectors such as the natural vector *Myzus persicae* transmit the virus and the BWYV occurs naturally on peas (Brunt *et al.,* 1996).

PEBV is reported from peas in the Netherlands (Bos and van der Want, 1962). The virus spreads in Belgium, Italy, Morocco, the Netherlands, Sweden, and the UK (Harrison, 1973; Brunt *et al.,* 1996). A nematode vector (*Trichodorus* spp.) spreads the virus (Harrison, 1973; Wang *et al.,* 1997) and 37% seed transmission was observed in *P. sativum* cv. Rondo plants (Brunt *et al.,* 1996). The viral genes affecting PEBV seed transmission have not been investigated (Wang *et al.,* 1997).

The most important virus diseases on peas, their worldwide distribution and possible yield losses were discussed briefly above. The initial tests performed on the three pea samples indicated similarity with three viruses in the literature, therefore a literature overview was compiled specifically on PEMV, BBWV, and BYMV and related viruses.

2.2 *PEA ENATION MOSAIC VIRUS* (PEMV)

2.2.1 INTRODUCTION

Osborn (1935) first described the disease induced by PEMV. He showed that the virus is aphid-transmitted in a persistent manner. The virus has been reported as being a common disease of broad beans, peas, and other legumes in other countries. Yield losses of up to 50% have been reported on peas (Hull, 1981). Studies have shown that PEMV has unique properties and it has been assigned to a taxonomic group of its own, the *Enamovirus* genus (Pringle, 1999). PEMV has traditionally been associated with the *Como-*, *Nepo-*, and *Dianthovirus* genera, but an examination of the PEMV and its hosts suggests a closer affiliation with members of the *Luteoviridae* (Demler and de Zoeten, 1991). There are some similarities but also some differences between the PEMV and the *Luteoviridae*. The *Luteoviridae* are transmitted persistently by aphid vectors as is the case with PEMV (Brunt *et al.,* 1996). A difference is that there are two sedimenting components in purified PEMV preparations, whereas with luteoviruses, one sedimenting component is found (Brunt *et al.,* 1996). The size of the PEMV genome is 9.96 kb and the *Luteovirus* genome size is 5.6-6.9 kb (Brunt *et al.,* 1996). In both cases non-genomic nucleic acid is found in the virions. Satellite RNA is found in the PEMV genome and a subgenomic mRNA or satellite RNA in the *Luteovirus* genome (Brunt *et al.,* 1996). The capsid protein sizes differ from each other. The properties of the PEMV particle, host-virus interactions and epidemiology will be discussed. PEMV has not previously been reported in South Africa.

2.2.2 PARTICLE PROPERTIES AND STRUCTURE OF VIRUS

The particles of PEMV are isometric, not enveloped, and are 25 and 28 nm in diameter. The two different sizes represent the two particle fractions which sediment in density centrifugation. In purified preparations of the virus, two sedimenting components are found – namely, top (T) and bottom (B) components with sedimentation coefficients of 99S (T) and 112S (B) (Brunt *et al.,* 1996). The B components are often hexagonal or pentagonal in outline, whereas T components are less regular in shape (Francki *et al.,* 1985). The components also differ in stability; T components are degraded in CsCl (high salt concentrations) and at high pH (Smith 1972) whereas the density of B components in

CsCl is 1.436g cm^{-3} (Brunt *et al.,* 1996). The density of T and B particles in Cs_2SO_4 is 1.380g cm^{-3} (Brunt *et al.,* 1996). Strains of PEMV differ in component composition. In most strains there is more of the bottom component than the top component (Hull, 1977). Earlier results indicate that the separated components are not infective, but a mixture of the two are infective (Peters, 1982). More recent results suggest that PEMV is not strictly phloem-limited, but shows a strong association with host phloem tissue (Demler and de Zoeten, 1991). According to Demler (1994) PEMV has an advantage in that both phases of the complex can be mechanically transmitted with the ability to expand systemic invasion beyond the phloem.

PEMV particles have three proteins. The Mr of the largest but minor protein is 54 000 Da. This protein facilitates aphid transmission. The Mr of the 2nd largest protein is 21 000 Da and the Mr of the 3rd protein is 17 500 Da (Brunt *et al.*, 1996). The protein constitutes 72% of the particle by weight (Shepherd, 1970).

The isoelectric point of PEMV is between pH 5-6 and the $A_{260/280}$ ratio is 1.63 (Brunt *et al.,* 1996). The specific absorbance E_{260} (1 mg/ml) is 7.2 in a 1 cm light path (Peters, 1982). The base composition of the PEMV genome is G=26.6%, C=24.5%, A=24.1% and U=24.8% (Brunt *et al.,* 1996)

The PEMV genome consists of two positive-strand ssRNAs of Mr 1.7 x 10^6 and 1.4 x 10^6 (Demler and de Zoeten, 1991). The genome of PEMV is composed of a *Luteovirus*-like RNA (RNA-1) and an RNA (RNA-2) that is *Umbravirus*-like (Demler *et al.,* 1996). The nucleotide sequence and genomic organisation of RNA 1 has strong similarities with the *Potato leafroll virus* (PLRV) and BWYV. It appears that much of the basic biology associated with PEMV infection correlates with the *Luteovirus*-like nature of RNA-1 (Demler *et al.,* 1993). Many strains of PEMV also contain a third encapsidated RNA of Mr 0.3 x 10^6 (Demler and de Zoeten, 1989). Studies done by Demler and de Zoeten (1989) confirmed that the RNA-3 is not infective and therefore is a satellite RNA of PEMV. The satellite RNA is dependent on the *Luteovirus* or *Luteovirus*-like component for encapsidation and vector transmission (Demler *et al.,* 1996). Studies also suggested

that RNA-2 is related to the *Tombus-* and *Carmovirus* genera (Demler *et al.,* 1994). RNA-2 was found to be fully capable of autonomous replication in the absence of RNA-1 (Demler *et al.,* 1993).

2.2.3 BEHAVIOUR IN HOST PLANTS

2.2.3.1 Symptoms

PEMV is mechanically transmissible though with some difficulty. PEMV is one of the few circulative aphid transmitted viruses which can be manually transmitted (Hull, 1981). Early infections in cultivars sensitive to PEMV may cause plant distortion and death before bloom. The first symptoms of infection of susceptible pea or broad bean cultivars are a light vein clearing on the young leaves about 7-8 days after inoculation (Hull, 1981). Later infections cause plant stunting, chlorotic flecks, leaf and pod distortion and reduced seed size and quality. The term enations refer to tissue proliferations on pods and along leaf veins. On infected pea plants PEMV produces characteristic unique hyperplasia or translucent "windows"(fleck-like windows). PEMV seems to be the only legume virus that produces this hyperplasia of the epidermis of the veins (McWhorther and Cook, 1958). The severity of symptoms varies according to pea cultivar and the strain of the virus. The symptomatology of five PEMV isolates was studied revealing a variation of symptoms (Rupel and Hagedorn, 1963). These varied from severe vein clearing with stunted and malformed plants to a mottle symptom accompanied by degrees of vein clearing, chlorotic vein banding, and interveinal translucent windows.

2.2.3.2 Host range

Species of at least 10 genera (*Cicer, Lathyrus, Lens, Lupinus, Medicago, Melilotus, Phaseolus, Pisum, Trifolium* and *Vicia*) of legumes are infected by PEMV naturally. No non-leguminous plants are known to be natural hosts of PEMV and few (eg. *Chenopodium spp.* and *Gomphrena globosa*) are susceptible when inoculated experimentally (Hagedorn, 1974). The virus occurs mainly in Canada, China, Iran, the U.K. and the USA (Brunt *et al.*, 1996). The host range of the virus is narrow (Peters, 1970). PEMV is mainly maintained in *P. sativum* because of its limited host range and

also in *V. faba*. It was stated in Brunt *et al.* (1996) that *V. faba* was not a satisfactory host for maintaining the virus. Evidence for this statement could not be found.

A list of the natural-, assay-, and susceptible dicotyledonous hosts (other hosts) are summarised in Table 2.2.

NATURAL HOSTS	ASSAY HOSTS	OTHER HOSTS
Cicer arietinum	*Chenopodium album*	*Anthyllis vulneraria*
Lathyrus odoratus	*Chenopodium amaranticolor*	*Astragalus rubyi*
Lens esculenta	*Chenopodium quinoa*	*Glycine max*
Medicago arabica		*Hedysarum coronarium*
Pisum sativum		*Lathyrus cicera*
Trifolium incarnatum		*Lathyrus hirsutus*
Vicia faba		*Lathyrus latifolius*
Vicia sativa		*Lathyrus tingitanus*
		Lens culinaris
		Lespedeza stipulacea
		Lotus tetragonolobus
		Lupinus albus
		Lupinus angustifolius
		Lupinus luteus
		Medicago hispida
		Medicago lupulina
		Medicago sativa
		Melillotus officinalis
		Melilotus alba
		Melilotus indica
		Nicotiana clevelandii
		Phaseolus acutifolius
		Phaseolus vulgaris
		Trifolium glomeratum
		Trifolium hybridum
		Trifolium repens
		Trifolium subterraneum
		Vicia dasycarpa

	Vicia narbonensis
	Vicia pannonica
	Vicia villosa

Table 2.2. The natural-, assay-, and susceptable dicotyledonous hosts of PEMV (Brunt *et al.,* 1996, Hagedorn, 1996, McWhorter and Cook,1958, Hagedorn *et al.*1964)

2.2.3.3 Cytopathology

PEMV was the first circulative aphid-borne virus detected by electron microscopy in plants and insects (Smith, 1972). The PEMV particle was first observed in the nuclei of sectioned plant cells (Shikata and Maramorosch, 1966). In plants, large accumulations of virus occur in cell nuclei and the virus may multiply there; fewer viruses are found in the cytoplasm and vacuoles (Shepherd, 1970; Hull, 1982). Virus particles were not found in the mitochondrion (Smith, 1972). During early stages of infection synthesis of virus-specific RNA occurs in the nucleus and in later stages of infection, parts of the nuclei of infected cells are destroyed and the particles move into the cytoplasm (Peters, 1982). Structures with a high electron density protrude from the plasmodesmata into the cytoplasm of infected and healthy companion cells and sieve elements of infected plants. These structures, which are found between the 4^{th} and 7^{th} day after inoculation, are thought to play a role in the systemic invasion of the plant by the virus (Peters, 1982). The virus particles in the nucleus form crystalline arrays (Hull, 1982).

Biochemical studies support the fact that PEMV replicates in the nucleus (Francki *et al.,* 1985). PEMV-specific double stranded RNA and virus-induced RNA-dependent RNA polymerases are located in nuclei of infected cells. The RNA-dependent RNA polymerase was detected in the nuclei after priming with PEMV RNA, and some of the product of the enzyme activity hybridised to viral RNA 1 (Francki *et al.,* 1985).

2.2.4 EPIDEMIOLOGY

2.2.4.1 Transmission of virus

Vector transmission: PEMV is transmitted by several aphid species: pea aphid (*Acyrthosiphon pisum)*, cotton aphid (*Aphis gossypii*), potato aphid (*Macrosyphum euphorbiae*), ornate aphid (*Myzus ornatus*) and the green peach aphid (*Myzus persicae*) (Hagedorn, 1996). PEMV is retained in the insect vector after having been acquired and is placed in the persistent group of aphid-borne viruses (Simons, 1954). *Acyrthosiphum pisum* is the most efficient vector in the field (Peters, 1982). The transmission efficiency varies with the strain of *A. pisum*. The acquisition of virus from infected plants may depend on the titre of the virus in the plant. Strains of the virus exist that are not transmissible by *A. pisum* but the aphid could transmit a non-transmissible strain from plants that were also infected by a transmissible strain (Peters, 1982). The virus can be transmitted by all instars of *A. pisum*, the nymphs acquiring the virus more efficiently than adults (Peters, 1982; Hull, 1981). Nymphs can acquire the virus in 15 min and the adults in 1-2 hours (Peters, 1981) although other studies indicate that adults acquire the virus after 8 hours feeding time (Simons, 1954). Nymphs have a higher metabolism rate than the adults and feeding time is higher in the nymphal stage (Simons, 1954). The aphids are able to transmit the virus after a latent period (Hull, 1982). Nymphs exhibit a latent period of 10-11 hours at 20°C (Peters, 1982; Hull, 1982). After the latent period aphids can infect plants during feeds of 1-2 minutes (Hull, 1982). The length of the latent period increases and the frequency of transmission declines with the age of the aphid (Peters, 1982). Toros *et al.* (1978) illustrated that the efficiency of virus transmission by aphids given short inoculation access periods is higher per unit of time than that of aphids given long ones. Thin sections of the aphid *A. pisum* fed on infected leaves shows that virus-like particles accumulate in the stomach and hindgut lumen and that particles are found in the salivary system (Hull, 1981).

Virions of aphid-transmissible strains of PEMV are composed of two structural proteins, a predominant protein of 21 K and a protein of 55 K (Demler, *et al.,* 1991). If these strains are repeatedly mechanically inoculated, the aphid-transmissibility phenotype is

lost along with the protein suggesting that the 55 K protein represents a determinant of circulative aphid transmission (Demler *et al.,* 1991).

Seed transmission: PEMV is not commonly known to be seed-borne (Shepherd, 1970) but a low frequency (1.5%) of seed transmission is reported in *P. sativum* (Peters, 1982).

2.2.4.2 Control of virus

As with most plant virus diseases there are several approaches to controlling the spread of PEMV. Spraying of insecticides will reduce the incidence of the virus by controlling the vector. In a study done by McWhorther and Cook (1958), pea growers and processors organised a community program to control aphids on irrigated alfalfa in the early spring before the aphids reach the peas. Field studies proved that irrigated alfalfa is the principal source of the aphids. During the next growing season they controlled the aphid while they were still in the alfalfa and before they could reach the pea fields. This proved to be a big success. The use of modern herbicides and cultural practices kept the roadsides free from weeds that could be sources of inoculum. This effectively controlled the disease.

Another control measure is breeding for PEMV resistance. Researchers evaluated forty-three pea lines for PEMV resistance (Hagedorn and Hampton, 1975). They found an important characteristic of the PF 60 pea cultivar in its ability to recover from initial disease symptoms. This resistance is not immunity but rather tolerance as the plant is able to grow and reproduce normally even though it may be systemically infected with PEMV.

2.3 *BROAD BEAN WILT VIRUS* (BBWV)

2.3.1 INTRODUCTION

BBWV was first isolated from broad bean (*V. faba*) in Victoria, Australia by Stubbs in 1947 (Makkouk *et al.,* 1990). BBWV is the type member of the genus *Fabavirus.* The fabaviruses have the closest affinities with the comoviruses and the nepoviruses that also have bipartite ssRNA genomes. These three genera are placed together in the family *Comoviridae* (Pringle, 1999). Fabaviruses are aphid-transmitted viruses infecting a wide range of plants. BBWV is transmitted non-persistently by aphids. The virus is reported to occur in Europe, Asia, and North and West Africa. The virus was found in Argentina but no spread of the virus was recorded there (Brunt *et al.*, 1996). Uyemoto and Provvidenti (1974) divided BBWV isolates in two serotypes (I and II). The type strain belongs to serotype I. BBWV was at one time considered a possible *Comovirus*, although it is not serologically related to comoviruses and is transmitted by aphids and not by beetles (Lisa and Boccardo, 1996).

Synonyms used for BBWV in older literature are *Catalpa chlorotic leaf spot virus*, *Tropaeolum ringspot virus*, *Nasturtium ringspot virus*, Ringmosaik der Kapuzinerkresse, *Petunia rinspot virus*, P.O., PeSV and Parsley virus 3 (Brunt *et al.*, 1996).

2.3.2 PARTICLE PROPERTIES AND COMPOSITION

BBWV particles are isometric, not enveloped, and are 25 nm in diameter (Brunt *et al.,* 1996). Particles of BBWV are very stable (Milne, 1991). The genome of BBWV consists of two species of RNA (RNA-1 and RNA-2) which are both necessary for infectivity. The two RNA species are associated with the M (RNA-2) and B (RNA-1) components discussed below. Studies by Doel (1975) showed that M and B components each induced local lesions when inoculated on *C. quinoa* and a mixture of the two components induces more local lesions than the individual components. He states that there was slight residual cross-contamination of the separated M and B components, since no replication of RNA-2 in the absence of RNA-1 has ever been reported within the *Comoviridae*. The genetic functions of the two RNA species of fabaviruses have not been studied, and it is not known whether they are fully interdependent or whether RNA-

1 possesses limited independence from RNA-2 (Lisa and Boccardo, 1996). In the case of comoviruses RNA-2 replication depends on RNA-1. To my knowledge this has not been studied in fabaviruses.

Purified BBWV particles sediment as three components; top (T), middle (M), and bottom (B) components. Sedimentation coefficients of these components range from 56-63 S (T), 93-100 S (M), and 113-126 S (B) for different BBWV isolates (Lisa and Boccardo, 1996). The T component contains empty protein shells without RNA, and the M and B components are two sorts of nucleoprotein particles containing different amounts of RNA (Taylor and Stubbs, 1972). BBWV M component contains 25-26% RNA (RNA-2) of 4.5 kb and the B component contains 35% RNA (RNA-1) of 6.3 kb (Taylor and Stubbs, 1972; Brunt *et al.*, 1996). The protein shells of BBWV are built of two distinct polypeptides with a molecular weight of about 42 000 Da and 26 000 Da (Milne., 1991).

The specific absorbance (at 260 nm) is 8.0 for 1 mg/ml solution in a 1 cm light path (Xu, *et al.*, 1988). The $A_{260/280}$ values for the different components are 1.32 (T), 1.64 (M), and 1.75 (B) (Taylor and Stubbs, 1972).

2.3.3 BEHAVIOUR IN HOST PLANTS

2.3.3.1 Symptoms

The symptoms on *Vicia faba* of an isolate from Syria are distinct vein clearing two weeks after inoculation. Two to three weeks later a severe systemic mottle develops. The leaf size was also reduced (Makkouk *et al.*, 1990). Experimentally infected plants show necrotic local lesions, ringspots, mosaic, and systemic necrosis (Brunt *et al.*, 1996). An isolate from China produces systemic mosaic symptoms on pea (Xu *et al.*, 1988).

2.3.3.2 Host range

The host range of BBWV is wide. The virus is known to infect 43 species in 14 dicotyledonous families by sap inoculation (Taylor and Stubbs, 1972). The natural host range of BBWV includes a few monocotyledons and many dicotyledons (Lisa and Boccardo, 1996). The majority hosts are herbaceous annuals but some are woody

perennials, for example, *Vitis vinifera*. BBWV was isolated from South African grapevine in 1985 (Castrovilli *et al.*, 1985). BBWV has also been isolated from overwintering perennial ornamentals, which can be important virus reservoirs together with wild plants and weeds. BBWV most frequently occurs in horticultural crops but also in ornamentals. BBWV has been considered a virus of minor economic importance (Lisa and Boccardo, 1996) however, BBWV causes serious epidemics in restricted areas and in some crops such as lettuce and spinach. Total crop losses were reported from Germany and France on these crops as well as New York State. Losses caused by BBWV ranged from 2-25% in broad bean (Makkouk *et al.*, 1990). BBWV has a wide host range but seems to cause serious diseases on certain crops under certain climatic conditions. The virus causes localised epidemics and is therefore graded as a virus of minor economic importance.

A summary of the host range of BBWV is given in Table 2.3

NATURAL HOSTS	ASSAY HOSTS	SUSCEPTIBLE DICOTYLEDONOUS HOSTS	
Petroselinum crispum	*Chenopodium amaranticolor*	*Ajuga reptans*	*Lupinus angustifolius*
Petunia hybrida	*Chenopodium quinoa*	*Antirrhinum majus*	*Lycopersicon esculentum*
Pisum sativum	*Datura stramonium*	*Beta vulgaris*	*Lactuca sativa*
Plantago lanceolata	*Phaseolus vulgaris*	*Begonia semperflorens*	*Limonium sinuatum*
Spinacia oleracea	*Vigna unguiculata cv. Blackeye*	*Brassica campestris*	*Matthiola incana*
Tropaeolum majus		*Bouvardia spp.*	*Narcissus tazetta*
Vicia faba		*Calendula offinalis*	*Nicandra physaloides*
Vitis vinifera		*Capsella bursa-pastoris*	*Nicotiana clevelandii*
		Capsicum annuum	*Nicotiana edwardsonii*
		Capsicum frutescens	*Nicotiana debneyi*
		Catharanthus roseus	*Nicotiana glutinosa*
		Chenopodium hybridum	*Nicotiana rustica*
		Cornus florida	*Nicotiana sylvestris*
		Cucumis sativus	*Nicotiana tabacum*

NATURAL HOSTS	ASSAY HOSTS	SUSCEPTIBLE DICOTYLEDONOUS HOSTS	
		Cucurbita pepo	*Ocimum basilicum*
		Cyamopsis tetragonoloba	*Physalis floridana*
		Cynara scolymus	*Physalis peruviana*
		Datura metel	*Phytolacca americana*
		Daucus carota	*Phaseolus vulgaris*
		Gomphrena globosa	*Pogostemon patchouli*
		Glycine max	*Solanum nigrum*
		Helleborus vesicarius	*Tropaeolum majus*
		Lathyrus odoratus	*Vigna sinensis*

Table 2.3. The natural-, assay-, and susceptible dicotyledonous hosts of BBWV (Brunt *et al.,* 1996 and Lisa and Boccardo, 1996)

2.3.4 SEROLOGY OF BBWV

BBWV isolates are divided into two serotypes (I and II) by Uyemoto and Provvidenti (1974); the original virus belongs to serotype I and the variants to serotype II. Differences between the two serotypes refer to serological properties. Seven BBWV isolates were divided into two distinct serological types (serotypes). Serotype I included isolates from pea, spinach, broad bean, nasturtium and *Plantago* I, whereas isolates from lettuce and *Plantago* II were members of the second group (Uyemoto and Provvidenti, 1974). A possible third serotype is suggested. *Artichoke french latent virus* (AFLV), isolated from artichoke in France was reported to differ from BBWV isolates belonging to serotypes I and II (Lisa and Boccardo, 1996). More properties of the other viruses, like AFLV, have to be determined before they can be considered separate fabaviruses or related to BBWV.

2.3.5 EPIDEMIOLOGY

2.3.5.1 Transmission of virus

Fabaviruses, including BBWV, are transmitted non-persistently by aphids (Lisa and Boccardo, 1996). About 20 aphid species have been reported as vectors of BBWV (including NRSV strains) (Lisa and Boccardo, 1996, Taylor and Stubbs, 1972). (Stubbs

(1960) compared the transmission efficiency of three aphid vectors. *M. persicae* was found to be the most efficient vector of BBWV. The acquisition time of the virus by the aphid varies from 15 sec to 10 min and inoculation time varies from 30 sec to 24 hr (Lisa and Boccardo, 1996). BBWV is not transmitted by seed (Brunt *et al.*, 1996).

2.3.5.2 Control of virus

BBWV has a wide host range and different aphid vectors play a role in transmitting the virus. The virus is not retained for long in the vector and survival of BBWV depends on infection of perennial hosts. Wild hosts of BBWV may have epidemiological importance by acting as sources of virus for infection of crops (Lisa and Boccardo, 1996). Elimination of weeds, spraying of insecticide and avoidance of sowing during periods when plants are infested with aphids are all control strategies for BBWV in broad bean crops in Australia (Lisa and Boccardo, 1996). In New York CMV and BBWV are a big problem on commercial lettuce and suggestions of removal of weed hosts were impractical because of the numerous host species around the lettuce fields (Rist and Lorbeer, 1989). The use of resistant cultivars is also a viable control measure.

2.4 *POTYVIRUSES*

2.4.1 INTRODUCTION

Potyvirus virions are flexuous and rod-shaped, 680-900 nm long and 11-15 nm wide (Riechmann *et al.,* 1992). The *Potyvirus* genus is the largest of the 50 plant virus genera currently recognised (Brunt *et al.,* 1996) and contains at least 180 definitive and possible members which cause significant losses in agriculture, pasture, horticultural and ornamental crops (Riechmann *et al.,* 1992). A breakthrough in *Potyvirus* studies was achieved in 1986 when the complete genome sequence of two members of this genus (TEV and TVMV) were reported (Riechmann *et al.,* 1992). A second breakthrough was the isolation of full-length cDNA clones of three potyviruses from which infectious transcripts can be synthesised (Riechmann *et al.,* 1992).

2.4.2 GENOME ORGANISATION

The *Potyvirus* genome consists of a single-stranded, positive sense RNA of approximately 10 000 nucleotides (Shukla *et al.,* 1994; Lindbo *et al.,* 1993). It resembles the genomes of como-, nepo-, and picornaviruses (Shukla *et al.,* 1991; Domier, *et al.,* 1987). The genome of potyviruses has a protein (VPg) covalently attached at its 5'-end, a poly(A) tail at the 3'-end and a single open reading frame coding for a large polyprotein precursor. There is a poly(A) tail located at the 3'-OH end whose length is variable (Hari, *et al.,*1979; Shukla and Ward, 1989). The polyprotein is cleaved by three virus-encoded proteinases into at least eight viral proteins (Dougherty and Carrington, 1988; Allison *et al.,* 1985b). The different gene products into which the polyprotein are cleaved (from the N to the C terminus) are a) P1 protein, b) HC-Pro, c) P3 protein, d) $6K_1$ protein, e) CI protein, f) $6K_2$ protein g) NIa protein h) NIb protein, and i) Coat protein (CP) (Riechmann, *et. al.,* 1992). VPg and CP are the only gene products detected in virus particles (Shukla *et. al.,* 1991).

2.4.3 THE PROTEINS AND THEIR FUNCTIONS

P1 protein-This protein may be involved in specific virus-hosts interactions (Shukla *et. al.*1991) and is involved in cell to cell transport of virus in plants (Arbatova *et al.,* 1998).

The P1 protein is localised in association with cytoplasmic inclusion bodies in the cytoplasm of infected cells (Arbatova *et al.,* 1998).

HC-Pro-The Helper component (HC) has been purified and identified as a protein with a molecular weight of 53 to 58 kDa (depending on the virus) (Blanc *et al.,* 1997). Evidence of the interaction between the coat protein and the helper component was shown with TVMV. These two proteins are involved in the aphid transmission of potyviruses (Blanc, *et al.,* 1997; Maia *et al.,* 1996). The second function of this protein is that it acts as a proteinase for autolytic cleavage of the HC-Pro-P3 junction (Shukla *et. al.*1991). Because of a cluster of cysteine residues in four potyviruses it was suggested that the HC-Pro plays a role in the complex responsible for the cell-to-cell movement (Shukla *et. al.*1991; Maia *et al.,* 1996). The involvement of HC-Pro in virus replication and symptom expression is also a possibility (Maia *et al.,* 1996).

P3 protein- No specific function has been assigned to this protein (Shukla *et. al.* 1991). The P3 protein and N-terminal of the CP are the most variable regions of the polyprotein (Aleman-Verdaguer *et al.,* 1997).

CI protein-This protein forms a characteristic cytoplasmic or 'pinwheel' structures found in the cytoplasm of infected cells of all potyviruses. It also has a possible involvement in the cell-to-cell movement, as virus particles have been found to attach to these pinwheel aggregates (Shukla *et. al.*,1991). The CI protein also contains the so-called nucleoside triphosphate-binding (NTP) motif encoded by most positive strand RNA viruses. In studies performed with *Plum pox potyvirus*, the CI protein interacts with RNA and ATP and co-purifies with a nucleic acid –stimulated ATPase activity (Laín *et al.,* 1991). Recent studies shows CI and CP in cellwall-associated structures and suggestions are that the CI protein may be involved in movement of complexes (Rodriguez - Cerezo, 1997).

NIa protein-This is the small nuclear inclusion protein, forms a complex with the NIb and contains two functional domains, the 22 kDa genome-linked protein (VPg) domain at the N-terminus and 27 kDa protease domain at the C-terminus (Kim *et al.,* 1998). NIa

acts as a proteinase for the cleavage of sites in the C-terminal two-thirds of the *Potyvirus* polyprotein (Shukla *et. al.*1991; Carrington and Dougherty, 1987) and plays an essential role in viral replication (Kim *et al.*, 1996; Hajimorad *et al.*, 1996). The NIa protein can direct the transport of reporter proteins to the nucleus (Hajimorad *et al.*, 1996).

NIb protein-This is the large nuclear inclusion protein and it acts as an RNA-dependent RNA polymerase (Shukla *et. al.*1991; Hajimorad *et al.*, 1996).

Coat protein(CP)-From biochemical and genetic analysis it is clear that each *Potyvirus* contains only a single type of capsid protein (Carrington and Dougherty, 1988). Studies were performed to determine the form and mechanism by which Potyviruses move from cell to cell (Rojas et al., 1997). They concluded both the CP and HC-Pro may be involved in *Potyvirus* cell to cell movement and that these proteins interact with viral RNA to form a nucleoprotein that moves through plasmodesmata (Rojas et al., 1997). A certain net charge near the CP N-terminus is necessary for systemic movement from cell to cell (Lòpez-Moja and Pirone, 1998). The N-terminus is the only large region of the coat protein that is unique to a *Potyvirus*: virus specific antibodies are generated to epitopes located in this region (Allison *et al.*, 1985a; Richter *et al.*, 1995; Shukla *et. al.* 1988a). The N terminal regions of the coat proteins of different potyviruses vary in sequence and strains of the same virus have coat proteins of the same length with highly homologous N-terminal sequences. This means that the N-terminus encode epitopes which distinguish it from other members of the *Potyviridae*.

$6K_1$ and $6K_2$ proteins-The two small proteins may play a role in RNA replication (Riechmann *et. al.*, 1992).

2.4.4 5'AND 3' NON-CODING REGIONS

5' non-coding region (5' NCR)-This region varies from 85 nucleotides in PRSV to 205 in TVMV (Shukla *et al.*, 1994). It is rich in adenine residues with a few guanine residues (Riechmann *et. al.*, 1992). Studies showed that the alignment of the 5' NCR of four potyviruses revealed some nucleotide blocks conserved in the four viruses. These

conserved sequences could play an important role in the virus life cycle (Riechmann *et. al.,* 1992). It has been shown that the TEV 5' NCR can function as an enhancer of translation.

3' non-coding region (3' NCR)-These regions are much more heterogeneous in size and sequence, contain AU-rich sequences, can be predicted to fold into stable secondary structures, and contain a poly (A) tail (Shukla *et al.,* 1994). High sequence diversity is found among the 3' NCRs of different potyviruses (Shukla *et al.,* 1994).

2.4.5 IDENTIFICATION AND CLASSIFICATION OF *POTYVIRUSES*

As mentioned before, the N-terminus is the only region in the coat protein which is unique to a *Potyvirus* , and virus-specific antibodies are generated to epitopes located in this region. Serology has been proven to be useful for the identification of other plant virus groups, but has proven to be unsatisfactory when applied to potyviruses (Shukla and Ward, 1989). The use of monoclonal antibodies has allowed different potyviruses to be distinguished. As with polyclonal antiserum that cross-react, monoclonal antibodies were also raised that cross-react between the potyviruses (Scott *et al,* 1989; Jordan and Hammond, 1991). Monoclonal antibodies generated against a mixture of 12 potyviruses helped to identify 25 distinct epitopes and the distribution of these epitopes between virus isolates can be used to detect different potyviruses (Jordan and Hammond, 1991). Studies indicated that HPLC profiles of tryptic digests of the coat proteins of potyviruses could be useful criteria for the identification and classification of potyviruses (Shukla *et al.,* 1988b).

Nucleic acid hybridisation with the 3' NCR of the *Potyvirus* genome as the probe was shown to be a relatively simple means of distinguishing between distinct potyviruses and their strains (Frenkel *et al.,* 1992).

The coat protein has a unique amino acid composition that is characteristic of the genus. It is also the major gene product of the virion, and comprises 95% of *Potyvirus* particles. The nucleic acid sequence of the 3' NCR of the *Potyvirus* genome and the nucleic acid

and amino acid sequences of potyviral coat proteins can be used to identify and classify potyviruses (Frenkel *et al.,* 1989; Brand *et al.,* 1993; Frenkel, *et al.,* 1992; Luis-Artaga *et al.,* 1996; Siaz *et al.,*1994; Rybicki and Shukla, 1992).

The availability of sequence data of coat proteins of potyviruses made it possible to develop a method for *Potyvirus* identification based upon the amplification of cDNA by the polymerase chain reaction (PCR) method. This requires the design of degenerate primers for amplification of the conserved and unconserved regions of the *Potyvirus* genome (Langeveld *et al.,* 1991). Use of one degenerate primer for a sequence within the CP gene (MVWCIEN box) and a terminally degenerate oligo-dT primer allowed wide-spectrum amplification of potyviruses (Pappu *et al.,* 1993). A pair of degenerate oligonucleotide primers was designed that amplifies a 1.6-2.1 kbp fragment from the 3'-end of the genome of 17 species of the *Potyviridae* (Gibbs and Mackenzie, 1997). A RT-PCR (reverse transcriptase polymerase chain reaction) was also developed to detect five seedborne legume viruses, including BYMV and ClYVV (Bariana, *et al.,* 1994). This technique is valuable in the rapid diagnosis of potyviruses and is used for detection of a range of viruses (Wetzel *et al.,* 1991; Ko *et al.,* 1994; Pappu *et al.,* 1993).

2.5 *BEAN YELLOW MOSAIC VIRUS, PEA MOSAIC VIRUS* AND *CLOVER YELLOW VEIN VIRUS*

2.5.1 *Bean yellow mosaic virus* (BYMV)

BYMV was first isolated from French bean (*Phaseolus vulgaris*) in the USA by Doolittle and Jones (1925) as cited in Shukla, 1994. The virus is known to occur worldwide and infects leguminous and non-leguminous species. BYMV has a very wide host range, infecting 233 species in 71 genera of 21 families (Shukla, 1994). BYMV is transmitted by several aphid species in a non-persistent manner. It is the second most important filamentous virus in beans in South America, and also infects broad beans and peas (Salazar, 1988). In Europe BYMV leads to yield losses of 25-60% in *P. vulgaris*, 25% in *P. sativum*, and 38% in *V. faba* (Lecoq *et. al.,* 1988). In the Mediterranean 75% infection rates and yield losses up to 44% have been recorded. Sources of resistance to BYMV have been successfully introduced in some pea and bean lines (Martelli, 1988). In the

Middle East BYMV is also a problem, particularly on broad beans. Up to 100% infection was recorded after 22 weeks (Cook, 1988). In the Indian subcontinent BYMV is not rated among the top ten viruses, but is noted as causing problems in legumes (Varma, 1988). In Japan a necrotic strain of BYMV is so destructive that it causes 30-60% yield losses in bean. In Southeast Asia BYMV also infects pea, broad beans and clovers (Inouye, *et.al.*, 1988).

Cytological studies revealed that BYMV induced cytoplasmic, cylindrical inclusions and crystalline nuclear inclusions in many hosts (Shukla, 1994). There are reports of resistant pea cultivars and resistant genes in pea to BYMV and PMV infection where this resistance is controlled by separate genes (Shukla, 1994).

2.5.2 *Clover yellow vein virus* (ClYVV)

ClYVV was first isolated from white clover (*Trifolium repens*) in the UK by Hollings and Nariani (1965) as cited in (Shukla, 1994). Symptoms induced by ClYVV are yellow and green mosaic, mottle, vein cleaving, veinal yellowing, ringspots, necrosis and stunting (Shukla, 1994). ClYVV occurs in several countries including Canada, Japan, The Netherlands, New Zealand, UK and USA and in Tasmania and Victoria (Mclean, 1988). ClYVV is transmitted to a large number of hosts (59 species in 29 genera of 9 families) and is spread naturally by aphids (Shukla, 1994).

ClYVV induces cytoplasmic cylindrical and crystalline nuclear inclusions in infected tissues, similar to BYMV strains. ClYVV produces characteristic nuclear enlargements that are different to inclusions produced by BYMV (Shukla, 1994). Resistant cultivars and genes have been identified in bean, pea, clover and cucurbits (Shukla, 1994).

The close serological relationship between BYMV and ClYVV virions is due to common epitopes located throughout the coat proteins of the two viruses including the surface-exposed, virus-specific amino-terminal region (Shukla, 1994).

mosaic caused by BYMV in peas. PMV is also unable to infect bean (Xiao *et. al.,* 1994). Based on weak hybridisation between BYMV-S and PMV-1 using a cDNA probe corresponding to the 3' NCR of BYMV-S RNA, Tracy *et. al.,* (1992) concluded that PMV is a distinct *Potyvirus*. However, recent research by Xiao *et. al.,* (1994) compared peptide profiles and sequences of selected peptides from coat proteins of ten strains of BYMV, three strains of PMV and one strain of *Sweetpea mosaic virus* (SPMV) and *White lupin mosaic virus* (WLMV). This indicated that PMV and WLMV are strains of BYMV.

2.5.4 Particle properties of BYMV and ClYVV

For BYMV and ClYVV the virions are filamentous, flexuous with a length of 750 nm by 12-15 nm wide. The sedimentation coefficients of purified virions differ slightly. For BYMV it is 151 S and for ClYVV it is 159.5 S. Virions of both viruses contain 5% nucleic acid, 95% protein and 0% lipid. The genome of both viruses is ssRNA of which BYMV is the largest at 10 kb and that of ClYVV is 9.5 kb (Brunt *et al.,* 1996).

2.5.5 Sequence comparisons, peptide profiles and hybridisation studies

Recently, nucleotide sequences of the coat protein gene have been determined for many potyviruses. Analysis of these derived nucleotide and amino acid sequences has been shown to be a useful approach to clarify the taxonomic status of viruses and strains in the *Potyvirus* genus (Shukla *et al.,* 1988c). If potyviruses are distinct viruses they share 30-60% homology in nucleic acid comparisons, whereas the homology of strains of the same virus is more than 95% (Shukla *et al.,* 1994). In a study peptides obtained by tryptic digestion of the coat proteins of ten strains of BYMV, five strains of ClYVV, three strains of PMV and one each of WLMV and SPMV were compared (McKern *et al.,* 1993). The peptide profiles of strains of PMV and WLMV were very similar to those of a BYMV profile, indicating the relationship between these viruses. PMV was shown to be a strain of BYMV (Xiao *et al.,* 1994). Tracy *et. al.* (1992) investigated the relationship between ClYVV-B, BYMV-S and PMV-I . Extracts of plants infected with these viruses were hybridized with radiolabeled, amplified 3' NCR of ClYVV-B and BYMV-S. The ClYVV probe hybridized only with the sample containing ClYVV-B,

relationship between ClYVV-B, BYMV-S and PMV-I . Extracts of plants infected with these viruses were hybridized with radiolabeled, amplified 3' NCR of ClYVV-B and BYMV-S. The ClYVV probe hybridized only with the sample containing ClYVV-B, while the BYMV probe hybridized with a BYMV-F and BYMV-S infected plant extract, and hybridized weekly with the ClYVV-B sample. They concluded that the three viruses are distinct potyviruses, but two years later Xiao *et al.* (1994) confirmed that PMV is a strain of BYMV.

The approach of analysing the nucleotide and amino acid sequences of potyviruses was used in this study to determine whether isolate 95/0931 is a strain of BYMV or closely related to PMV or possibly ClYVV. The 3' NCR and part of the coat protein gene was amplified as described by Pappu *et al.,* 1993. Sequence comparisons of these PCR products revealed the identity of our isolate as described later.

In the following chapters of this study the methods used to identify and characterise three isolates 91/0394, 94/1969 and 95/0931 collected from *P. sativum* will be discussed.

CHAPTER 3
IDENTIFICATION OF ISOLATE 91/0394

CHAPTER 3

IDENTIFICATION OF ISOLATE 91/0394

3.1 INTRODUCTION

The Plant Protection Research Institute (PPRI) of the ARC in Pretoria launched a virus survey on *P. sativum* (pea) in 1991. The survey was conducted in the George district of the Western Cape Province on farms that produce peas for the local frozen vegetable industry, 'Table Top'. A plant (sample 91/0394) was collected from *P. sativum* cv. Cape Freeze plants. The plant displayed virus-like symptoms, such as interveinal chlorosis of the upper leaves with chlorotic flecking and downward leafcurl. The symptoms displayed by the plants were similar to those described in the literature for PEMV. To identify the virus several properties of the virus were investigated. A spherical virus was isolated from the plant and initial enzyme-linked immunosorbent assay (ELISA) tests detected PEMV in the sample. Electron microscopy and cytopathological studies assisted to identify the virus. The molecular weight of the capsid protein was determined. An ELISA detection system was developed, and serological studies were performed where the virus was compared with an isolate from Germany.

3.2 MATERIALS AND METHODS

3.2.1 Source of virus and initial identification

Sample 91/0394 was collected from a pea field (*P. sativum* cv. Cape Freeze) in the George district, Western Cape. Dr. D. Goszczynski isolated the virus through two local lesion transfers (See Appendix A). Freeze-dried, desiccated and cryopreserved infected material of the isolate was deposited in the culture collection of the PPRI. A $F(ab')_2$ enzyme-linked immunosorbent assay (ELISA) test (Barbara and Clark, 1982) was performed on sample 91/0394 and the other samples collected from this field survey. A PEMV antiserum, which was produced to the PEMV 3 LM-strain collected by D.J. Hagedorn of the University of Wisconsin in the U.S.A, was deposited in the antiserum collection of PPRI in 1985 and was used to initially identify sample 91/0394.

3.2.2 Symptomatology

The isolate was established, maintained and propagated on *P. sativum* cv. Green Feast plants in the greenhouses of the Plant Protection Research Institute in Pretoria. The temperature of the greenhouses was 20-22 °C. The virus was re-inoculated every ten to twelve days to maintain the isolate. The virus was propagated by sap transmission of virus-infected leaf material macerated in 0.01M sodium phosphate buffer, pH 7.1, containing 0.02M sodium sulphite and a small amount of Celite to healthy *P. sativum* cv. Green Feast plants.

A host range study was performed with isolate 91/0394. The plants were mechanically sap-inoculated to 26 plant species. The study was performed under controlled conditions in a growth room with a temperature of 20°C, 16-hour light period. An ELISA test was performed to confirm the visual symptoms (Appendix A).

3.2.3 Electron microscopy and cytopathological studies

Infected plant material was macerated in 0.1M phosphate buffer, pH 7.1, and examined for the presence of virus particles by negative staining with 2% potassium phosphotungstate (PTA), pH 7, aqueous uranyl acetate (UA), pH 4.3 or 2% ammonium molybdate (AM), pH 5.3, using a ABT-ISI 002A transmission electron microscope. Mr. K. Kasdorf performed the electron microscopy work.

Ultrathin sections were prepared as described in Appendix 1 and viewed with an electron microscope. Mr. H. van Tonder and Mr. F. van der Merwe did the sectioning and viewing of ultrathin sections.

3.2.4 Purification of virus

Leaves of PEMV-91/0394 infected *P. sativum* cv. Green Feast plants were harvested ten to twelve days after inoculation and stored at -80° C. Frozen leaves were used as the source from which PEMV was purified. The purification used was a combination of methods previously described (Mahmood and Peters, 1979; Harrison, 1984). Homogenisation of plant material was in 0.15M sodium acetate, pH 6.1, containing

0.015M magnesium chloride with addition of 0.5% 2- mercaptoethanol. The buffer/plant ratio was 2 ml/g-plant material. The homogenate was emulsified with a mixture of chloroform:n-butanol (1:1) and centrifuged at 10 000 rpm for 20 min in a Beckman JA 14 rotor. The aqueous phase was collected and polyethylene glycol (6000) and NaCl were added to a final concentration of 8% (w/v) and 0.2M respectively. It was stirred at 4°C for 1 hr, incubated at room temperature for 1 hr and centrifuged at 10 000 rpm for 20 min using a Beckman JA 14 rotor. The pellet was resuspended in 0.15M acetate buffer overnight and centrifuged at 10 000 rpm for 5 min in a Beckman JA 20 rotor. The supernatant was loaded onto a 20% sucrose cushion (3 ml) and centrifuged at 26 000 rpm for 170 min in a Beckman TY 30 rotor. The pellet was diluted in a small volume (1-2 ml) of acetate buffer, centrifuged at 5000 rpm for 5 min, loaded onto a 10-40% sucrose gradient and centrifuged at 24 000 rpm for 150 min in a SW 28 rotor. The zones were collected, dialysed against acetate buffer overnight and concentrated by ultracentrifugation as described with the sucrose cushion. During the purification procedure samples of each step of the purification were monitored with the electron microscope.

3.2.5 Serology

3.2.5.1 Antiserum production

Antiserum was raised to purified PEMV-91/0394 in a New Zealand white rabbit immunised with four weekly intramuscular injections of 0.2-1.1 mg virus in Freund's complete adjuvant, used with the first injection, and Freund's incomplete adjuvant for the successive injections (Appendix A). Blood was collected weekly from the rabbit one week after the last injection. The bleed with the highest specific titer was identified by dilution end point determinations in PAS-ELISA (Protein A Sandwich-ELISA) (Edwards and Cooper, 1985). Immunoglobulins were purified, $F(ab')_2$ fragments prepared (Cambell *et al.*, 1970) and a $F(ab')_2$ ELISA developed (Barbara and Clark, 1982). The ELISA was performed as described in Appendix A where the coat anti rabbit-fc portion was conjugated to alkaline phosphatase and the colour reaction developed with 1 mg/ml ρ-nitrophenyl phosphate substrate tablets. The absorbance was measured at 405 nm with a Multiskan MC spectrophotometer after 60 minutes. The

optimum conditions for using the $F(ab')_2$ ELISA were determined by an optimising ELISA as described in Appendix A.

3.2.5.2 Serological relatedness

Serological relatedness of PEMV-91/0394 was determined by DAS-ELISA using antisera to PEMV-Tü (AS-0017) obtained from Dr. S. Winter, DSM, Germany. PEMV-Tü (AS-0017) IgG was used to coat plates. Homologous antiserum was conjugated and used to detect the virus.

3.2.6 Molecular weight determination of capsid protein

Purified virus was used for determining the molecular weight of the capsid protein of this isolate. A purification of PEMV was performed as described above. 20-30 µl of 1.12 mg of purified virus was run on the SDS-polyacrylamide gel as described in Appendix A. The size of the protein band was determined using the UVP Gelworks for Windows program supplied by Ultra Violet Products, Nonlinear Dynamics Ltd.

3.3 RESULTS AND DISCUSSION

3.3.1 Source of virus, initial identification, and maintenance of virus

In an ELISA, PEMV antiserum reacted positively with the sample indicating it was PEMV. Three replicas of the sample were tested in ELISA and the average was used to interpret the results. No positive control was available for use in the test but the absorbance values of the 91/0394-infected samples were considerably higher than twice the value obtained for the healthy control used (healthy *P. sativum*). The result of this ELISA is shown in Figure 3.1.

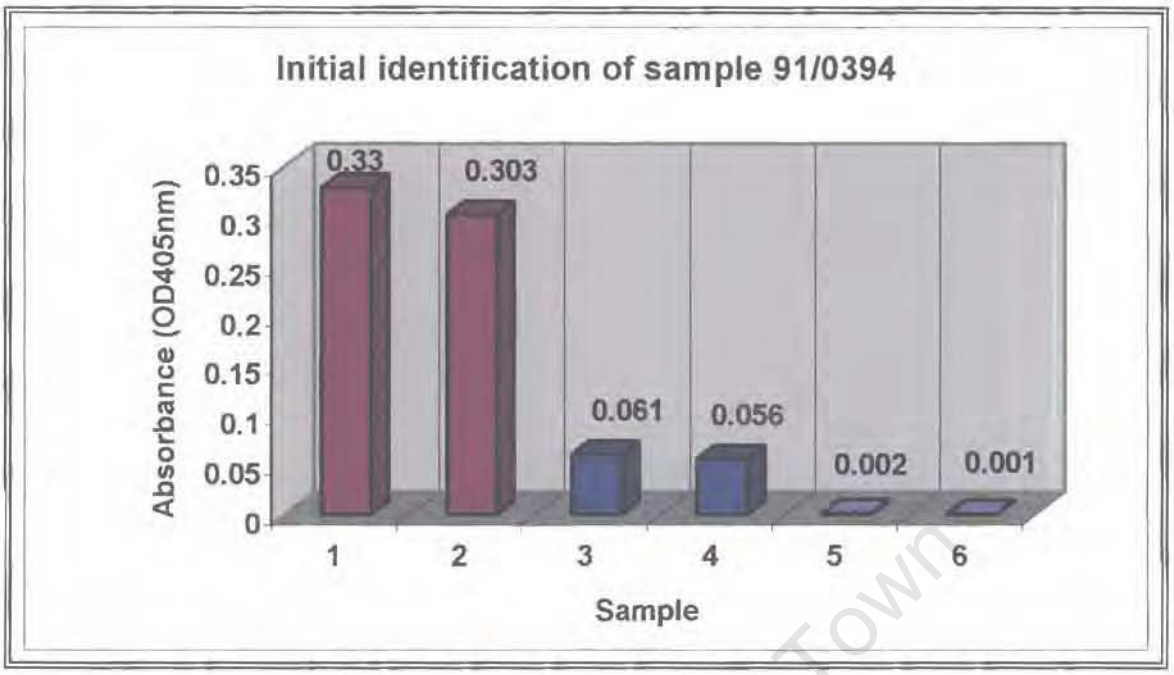

Figure 3.1. Results of the initial ELISA performed to identify sample 91/0394 where HC indicates healthy control. Lane 1 and 2, sample 91/0394; Lane 3 and 4, healthy *P.sativum*; Lane 5 and 6, buffer control

3.3.2 Symptomatology

The isolated virus was maintained by weekly mechanical inoculation and infected *P. sativum* cv. Green Feast plants were harvested and stored at - 80°C. Symptoms on inoculated *P. sativum* plants appeared 6 days after inoculation. The first symptom visible on *P. sativum* cv. Green Feast plants was a systemic chlorotic vein clearing followed by chlorotic flecking, and a downward leafcurl. Symptoms of PEMV 91/0394 are shown in Figure 3.2.

Figure 3.2. Symptoms of 91/0394 on *P. sativum* cv. Green Feast plants

A total of 26 different host plants were used for host range studies. The results of mechanical inoculations are summarised in Table 3.1. From the visual monitoring of symptoms only a few plants showed symptoms. All the plants were tested by ELISA to confirm the visual symptoms. According to ELISA results only the *P. sativum* species tested positive. However, the *C. quinoa* plants displayed chlorotic local lesions, as reported in the literature. *G. max* Rampage and *G. max* Edgar plants showed systemic vein clearing. ISEM performed on these two plants showed no spherical particles. It might be that the virus concentration in the plants was too low to detect with ELISA or ISEM.

HOST PLANTS	ELISA	SYMPTOMS
Arachis hypogaea	-	-
Chenopodium murale	-	-
Chenopodium quinoa	-	Chlorotic local lesions
Cucumis sativus Volcan	-	-
Glycine max B66S10	-	-
Glycine max Edgar	-	Chlorotic mottle, systemic vein clearing
Glycine max Forrest	-	-
Glycine max Rampage	-	Systemic vein clearing
Gomphrena globosa	-	-
Lupinus albus Kiev	-	-
Nicotiana benthamiana	-	-
Nicotiana clevelandii	-	-
Nicotiana glutinosa	-	-
Nicotiana tabacum Samsun	-	-
Phaseolus vulgaris Black Turtle Soup	-	-
Phaseolus vulgaris Bonus	-	-
Phaseolus vulgaris Bountifull	-	-
Phaseolus vulgaris Top Crop	-	-
Pisum sativum Cape Freeze	+	Systemic veinal chlorosis, chlorotic flecks
Pisum sativum Green Feast	+	Systemic vein clearing, chlorotic spots, downward leafcurl
Pisum sativum Sugar Snap	+	Systemic vein clearing, chlorotic spots
Pisum sativum Winter Green	+	Systemic vein clearing, chlorotic spots
Vicia faba	-	-
Vigna unguiculata Blue purple	-	-
Vigna unguiculata Caloona	-	Chlorotic local lesions

Table 3.1. Results of host range studies of isolate 91/0394 where (-) indicates no visual symptoms observed or a negative result

3.3.3 Electron microscopy and cytopathological studies

Negative staining of ultrathin sections showed the presence of isometric particles. During studies of ultrathin sections with the electron microscope isometric particles were observed in the nuclei of PEMV-infected leaves. An electron micrograph of a section is shown Figure 3.3.

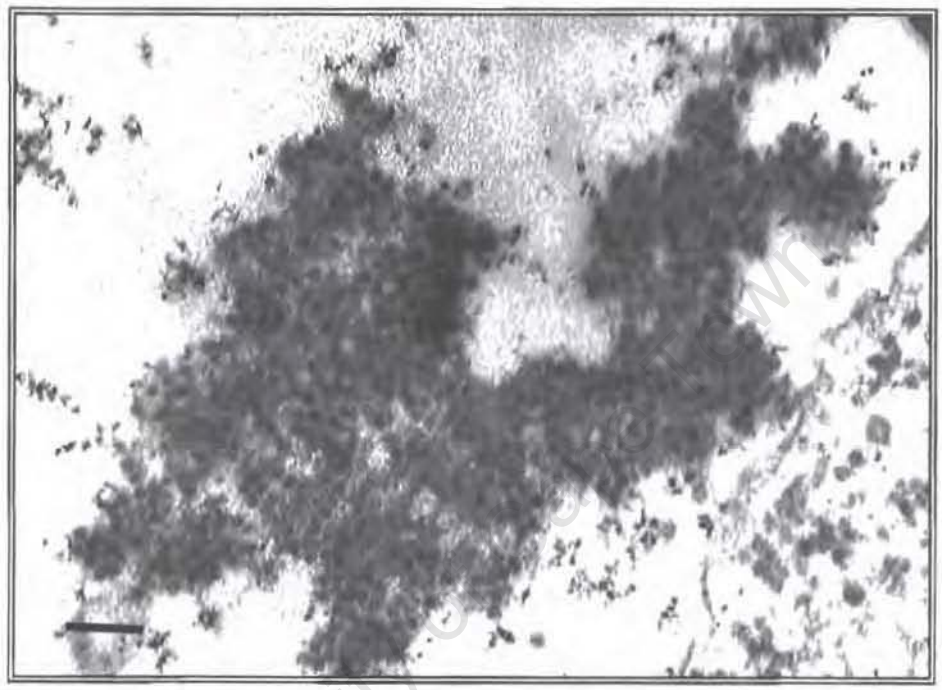

Figure 3.3. The occurrence of PEMV particles in the nucleus of PEMV-infected *P. sativum* Green Feast plants observed in ultrathin sections stained with uranyl acetate and lead citrate and viewed with the electron microscope. Scale bar represents 250 nm.

3.3.4 Purification results

PEMV 91/0394 was successfully purified according to the method described above. One prominent zone was obtained from the sucrose gradient. The sucrose gradient with the visible band is shown in Figure 3.4. The band was monitored with the electron microscope. Virus yield from the purifications was 0.99-1.12 mg virus/0.1 kg plant material and was used to immunise a rabbit four times intramuscularly described previously. Spectrophotometric analysis of purified virus preparations yielded absorbance ratios of A_{260}:A_{280} nm=1.5-1.6. Purified virus particles are shown in Figure 3.5.

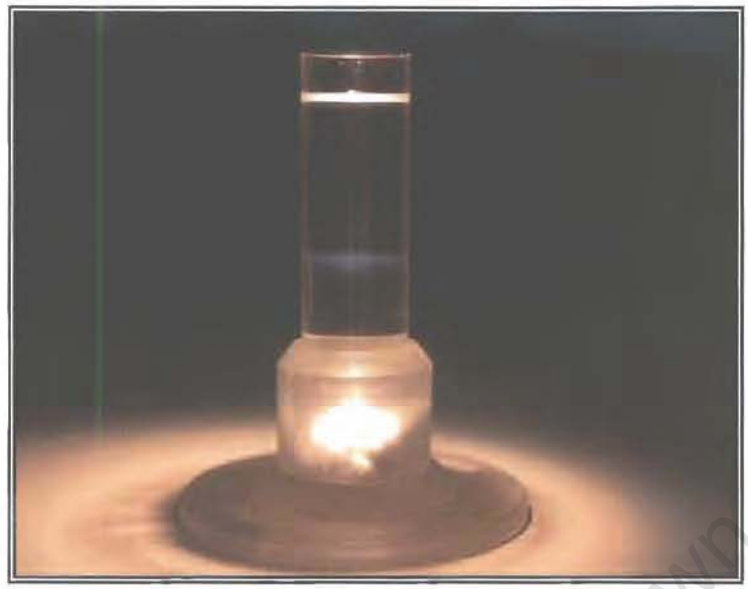

Figure 3.4. A sucrose gradient obtained during the purification procedure of PEMV 91/0394. The blue zone was collected and used to immunise a rabbit.

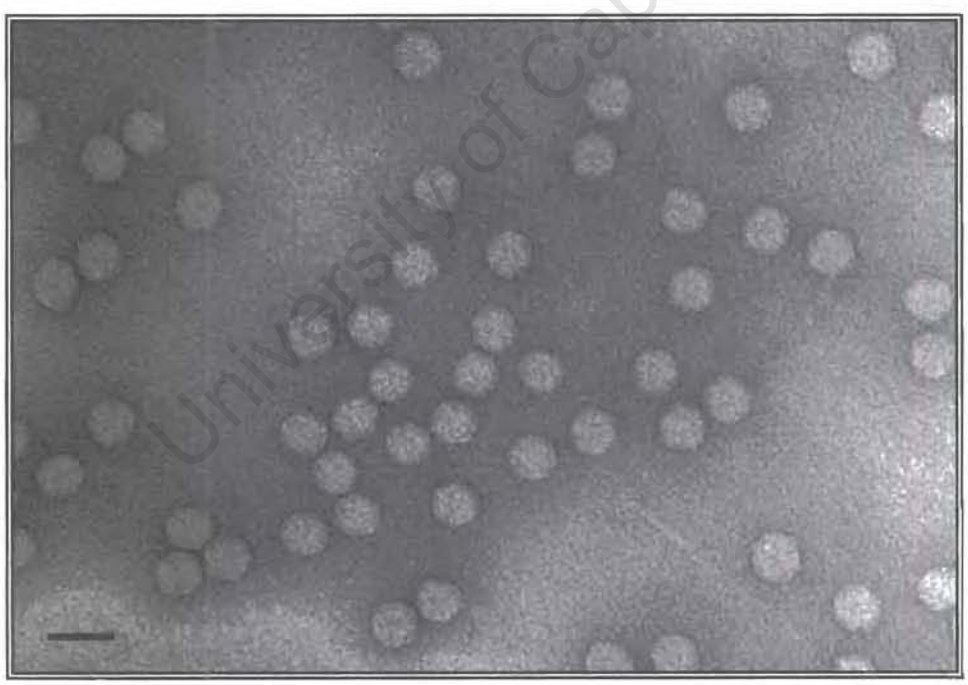

Figure 3.5. Purified PEMV 91/0394 particles with a magnification of 300 000, stained with 2% ammonium molybdate, pH 5.3. Scale bar represents 40.5 nm.

3.3.5 Serology

3.3.5.1 Antiserum production and development of F(ab')$_2$ ELISA

Nine weekly bleeds were collected. According to dilution end point determinations using the PAS-ELISA system, bleed six had the highest titer at a dilution of 1:512 000. The absorbance values of infected plant material vs. healthy plant material were used to determine the best bleed at the highest dilution. The bleed giving the highest differential ratio was bleed 6 (See Figure 3.6). The nine antisera were diluted from 1:1000 to 1:512 000.

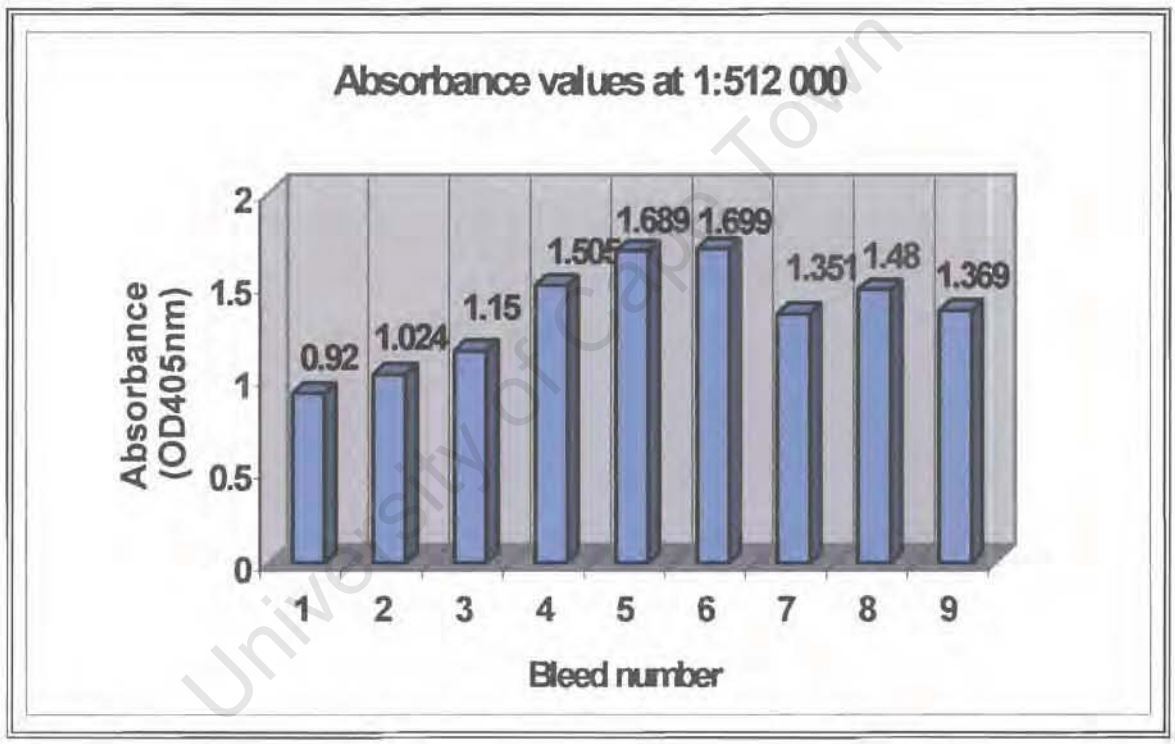

Figure 3.6. Absorbance values of different bleeds at 1:512 000

A F(ab')$_2$ ELISA was developed from bleed six and the optimum dilution for the use of F(ab')$_2$ fragments was determined to be 1:10 000 and that of the IgG, 1:200. Results of the optimising ELISA are shown in Figure 3.7.

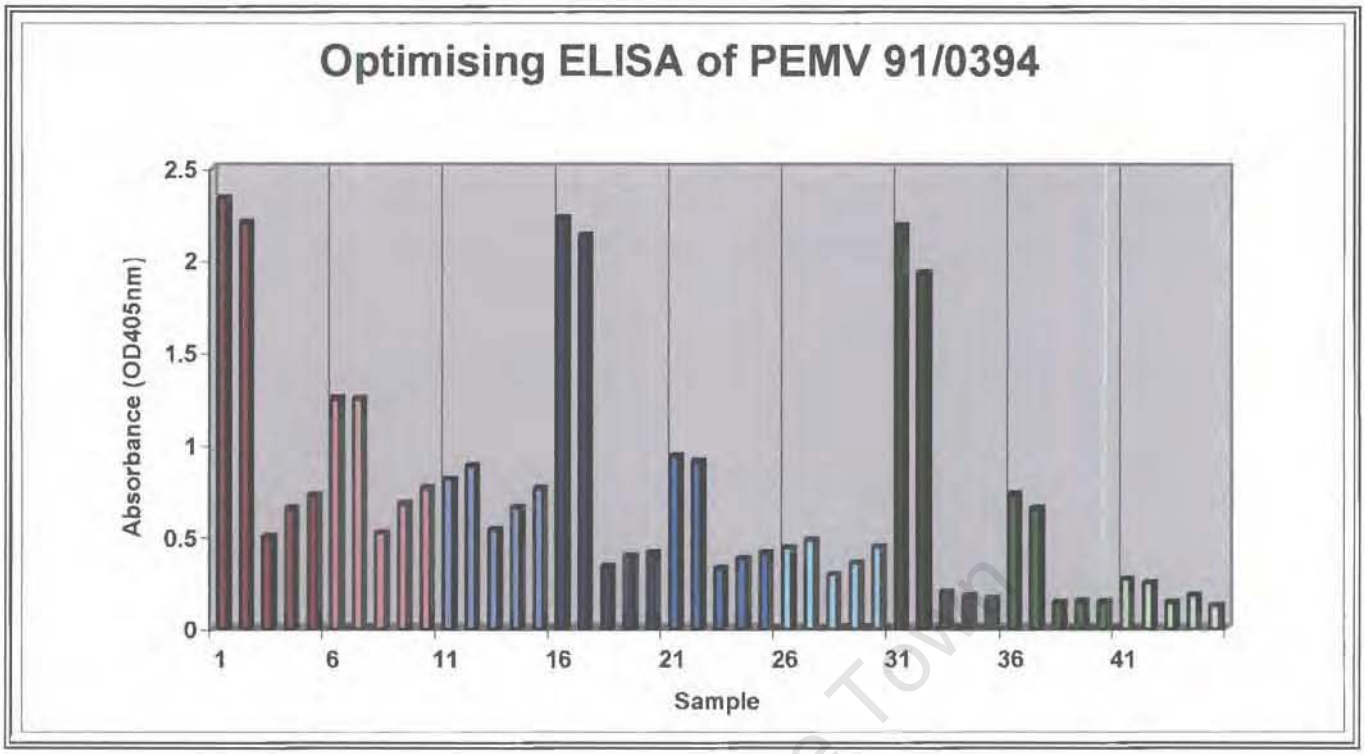

Figure 3.7 Results of optimising ELISA of PEMV 91/0394. Lane 1, 91/0394 1:100; lane 2, 91/0394 1:1000; lane 3, healthy *P. sativum* 1:10; lane 4, healthy *P. sativum* 1:100; lane 5, buffer control. The order was repeated for each seroreagent combination tested:

- $F(ab')_2$ 1:100 and IgG 1:200
- $F(ab')_2$ 1:100 and IgG 1:800
- $F(ab')_2$ 1:100 and IgG 1:3200
- $F(ab')_2$ 1:1000 and IgG 1:200
- $F(ab')_2$ 1:1000 and IgG 1:800
- $F(ab')_2$ 1:1000 and IgG 1:3200
- $F(ab')_2$ 1: 10 000 and IgG 1:200
- $F(ab')_2$ 1: 10 000 and IgG 1:800
- $F(ab')_2$ 1: 10 000 and IgG 1:3200

3.3.5.2 Serological relatedness

Positive serological reaction was obtained with PEMV-91/0394 against antiserum PEMV-Tü (AS-0017) in DAS-ELISA. This test confirmed the identity of our isolate. The results of the absorbance values of the DAS-ELISA are shown in Figure 3.8.

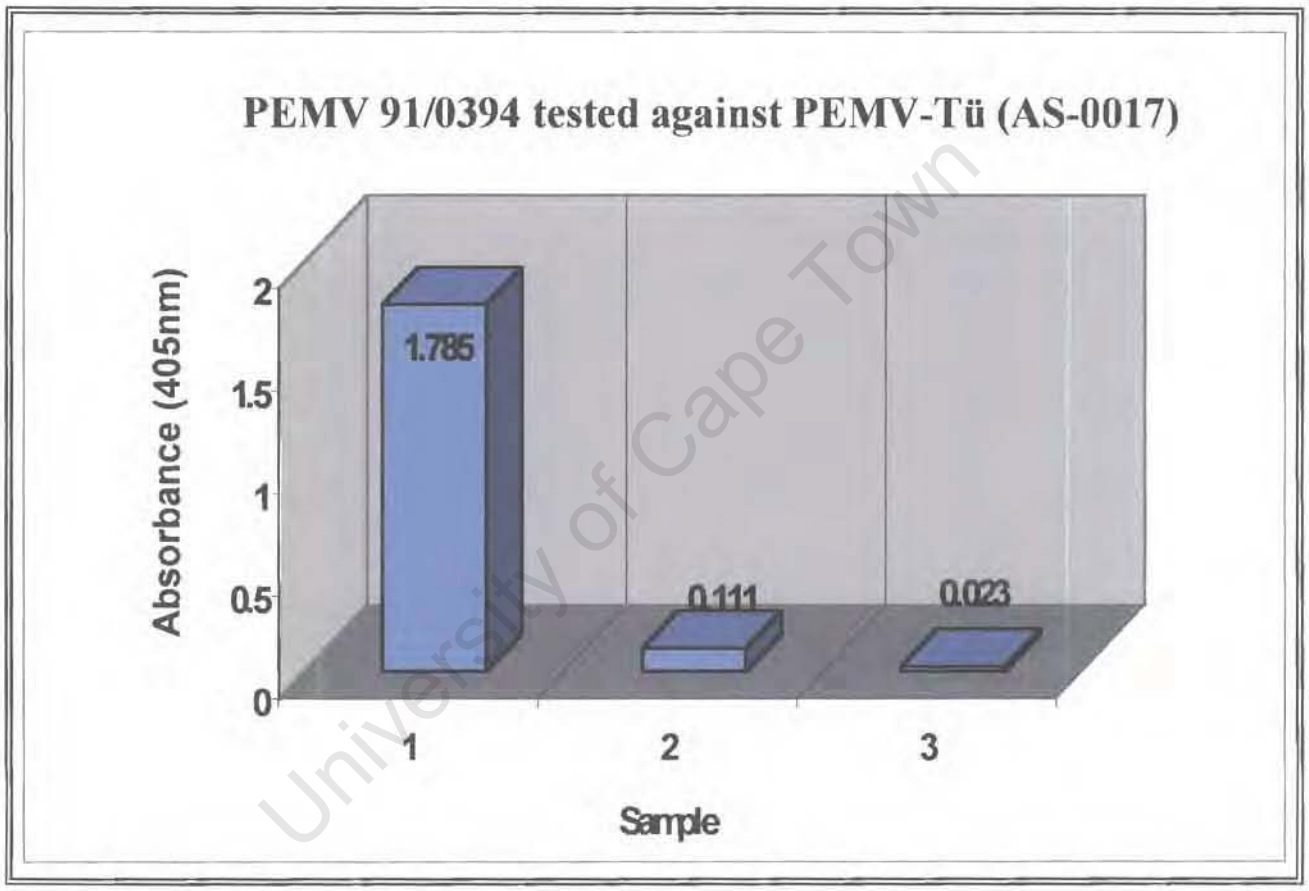

Figure 3.8. Absorbance values of PEMV 91/0394 tested against PEMV-Tü (AS-0017) antiserum where HC indicates healthy control and BC, Buffer control

3.3.6. Molecular weight determination of capsid protein

Only one protein band was detected from purified particles on a SDS-PAGE gel. The virions of aphid-transmissible strains of PEMV are composed of two structural proteins (21000 Da and 55000 Da). If these strains are repeatedly mechanically inoculated, the aphid-transmissibility phenotype is lost because of the 55000 Da protein that is lost. This is probably the reason that one protein band was detected. The protein size was determined to be 22 700 Da using the Gelworks 1D Advanced UVP computer program (See Figure 3.9)

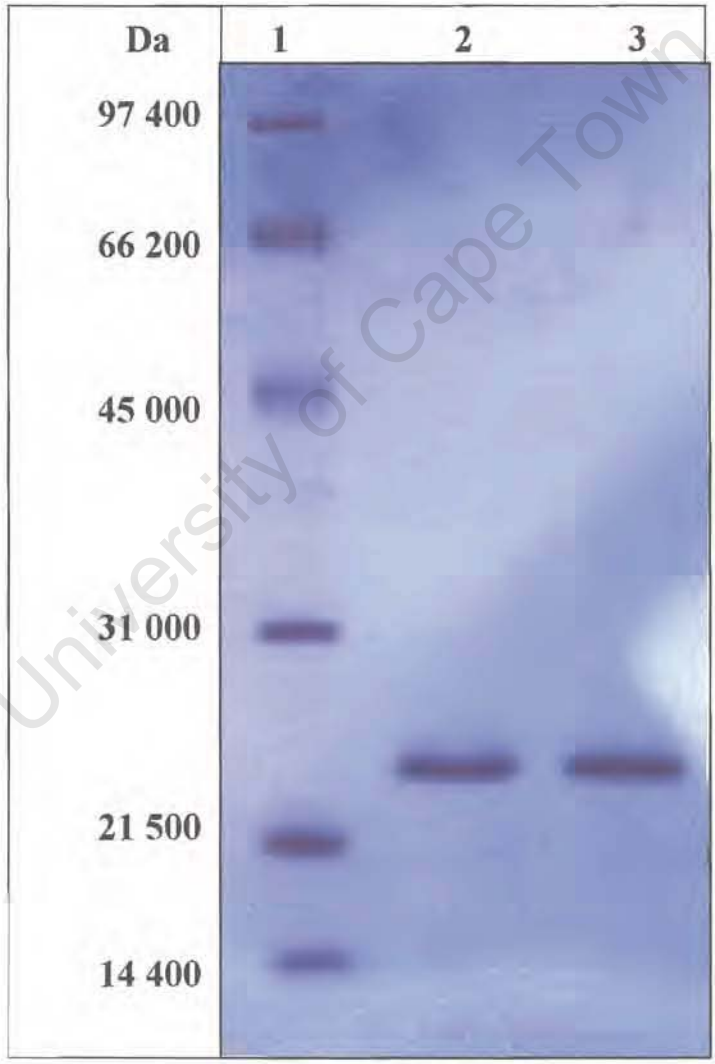

Figure 3.9. SDS-PAGE of capsid protein of isolate 91/0394. Lane 1, low molecular weight protein marker (BioRad), Lane 2 and 3, isolate 91/0394.

3.4. CONCLUSION

Isolate 91/0394 was identified as PEMV by initial ELISA tests with antiserum in the antiserum collection of PPRI. PEMV has unique properties and is the only member of the *Enamovirus* genus (Brunt *et al.*, 1996). Studies with the transmission electron microscope showed spherical particles with negative staining and spherical particles were visible in the cell nuclei during studies of ultrathin sections of infected plants. That virus particles could be observed in the nuclei of cells is described in the literature by Shikata and Maramorosch (1966).

The symptoms described for PEMV are a slight vein clearing on the young leaves about 7-8 days after inoculation followed by chlorotic flecks and enations or tissue proliferations on the pods and along leaf veins (Hull, 1981). According to Rupel and Hagedorn (1963), a variety of symptoms are possible depending on the cultivar and strain of virus. The first symptom that was observed on *P. sativum* cv. Green Feast plants inoculated with isolate 91/0394 was vein clearing followed by chlorotic flecks and downward leafcurl. The host range indicated that the *P. sativum* cultivars were positive for virus infection. Although chlorotic lesions were noted on *C. quinoa*, the ELISA test was negative. In the literature *C. quinoa* is used as assay host (Brunt *et al.*, 1996). *Glycine max* was recorded to be a host of PEMV (McWhorter and Cook, 1958), and based on symptoms the *G. max* Rampage and *G max* Edgar plants had systemic vein clearing. These plants were negative in ELISA and it is possible that the ELISA test used was not sensitive enough. Host plants used in the PEMV 91/0394-host range which showed no symptoms and which are recorded as hosts in the literature are *N. clevelandii, P. vulgaris* spp., and *V. faba*. The host range of PEMV is narrow and would explain the results of the host range studies where only the *P. sativum* species were infected as assayed by ELISA.

A single protein band was detected by SDS-PAGE and the size was determined to be 22 700 Da. This band is the same size as one of the bands described in the literature for aphid-transmissible strains of PEMV (Demler *et al.*, 1991). Aphid-transmissible strains of PEMV have two proteins, a 21 000 Da protein and a 55 000 Da protein. If these

strains are repeatedly mechanically inoculated, the 55 000 Da protein is lost and with it the aphid-transmissibility phenotype. The aphid transmissibility of the original infected plant was not tested.

The isolate was successfully purified and an antiserum was raised to PEMV 91/0394, which could be used to detect PEMV in the future. PEMV 91/0394 was serologically related to another PEMV isolate [PEMV-Tü (AS-0017)] and the serological relatedness of the two isolates was used as the final confirming test. Isolate 91/0394 has therefore been identified as PEMV based on particle morphology, cytopathology, molecular weight of capsid protein, host range studies and serology.

CHAPTER 4
ISOLATION AND IDENTIFICATION OF SAMPLE 94/1969

CHAPTER 4

ISOLATION AND IDENTIFICATION OF SAMPLE 94/1969

4.1 INTRODUCTION

Sample 94/1969 was collected from the Brits district in the North West Province of South Africa. The *P. sativum* plant, with severe mosaic symptoms, was submitted for diagnosis to our laboratories. Electron microscopic analysis showed two types of spherical particles: apparently empty ones and filled particles. The host range of the virus was determined with the available indicator plants and aphid transmission tests were performed. The size of the genomic RNA was determined and an ELISA detection system was developed to the virus.

4.2 MATERIALS AND METHODS

4.2.1 Source of virus, initial identification and isolation of virus

Sample 94/1969 was brought to the laboratories of the PPRI for identification. The virus was isolated from the original pea plant by inoculation to *Chenopodium quinoa*. Two consecutive local lesion transfers were performed as described in Appendix A. The original as well as the isolated sample was stored according to the three methods described in Appendix A. The stored plant material was deposited in the culture collection of the PPRI. The original pea plant was inoculated to *N. benthamiana, V. faba* and *P. sativum* cv. Green Feast plants. The first test performed to identify the virus was ISEM using antiserum (E229) raised to a BBWV isolate from pea produced at IPO, Wageningen in The Netherlands (Makkouk *et al.,* 1990). In the ISEM performed the E229 antiserum was diluted 1:1000 and the infected plant material was macerated 1:10 in 0.1M phosphate buffer, pH 7.1. (See Appendix A). An initial host range study was conducted with *C. quinoa. N. rustica* and *N. benthamiana* plants.

4.2.2 Symptomatology

The isolate was established, maintained, and propagated in the greenhouses of the PPRI, Pretoria. The temperature of the greenhouses was 24-27°C. The virus was propagated by sap-transmission of virus-infected leaf material macerated in 0.01M sodium phosphate buffer, pH 7.1, containing 0.02M sodium sulphite and a pinch of Celite to healthy *Nicotiana benthamiana* plants. Virus symptoms were monitored regularly and the isolate maintained weekly.

A host range study was performed with isolate 94/1969. Thirty-four indicator plants were used in the host range study and the study was performed under the same conditions described above. An ELISA test was performed on local as well as systemically infected leaves to confirm the visual symptoms.

4.2.3 Electron microscopy

Infected plant material was macerated in 0.1M phosphate buffer, pH 7.1, and examined for the presence of virus particles by negative staining with 2% potassium phosphotungstate (PTA), pH 7, aqueous uranyl acetate (UA), pH 4.3 or 2% ammonium molybdate (AM), pH 5.3, using a ABT-ISI 002A transmission electron microscope. (See Appendix 1)

Electron microscopy was also used to monitor the purification procedure of isolate 94/1969.

4.2.4 Purification of virus

Leaves of BBWV-94/1969 infected *N. benthamiana* plants were inoculated and harvested a month prior to purification and stored at –80°C. The purification procedure was a combination of two methods previously described (Xu *et al.*, 1988 and Makkouk *et al.*, 1990).

The infected leaves from *N. benthamiana* from the –80°C freezer were macerated in 0.5M potassium phosphate buffer, pH 7.5, containing 0.01M EDTA, 0.1% thioglycolic

acid (TGA), 0.01%Triton X-100 and 2% (PVP) polyvinylpyrrolidine. The homogenate was left at room temperature for 60 min. It was filtered through cheesecloth and emulsified with 0.25 volumes of chloroform. The solution was stirred at room temperature for 30 min, then centrifuged at 7000 rpm in a Beckman JA20 rotor for 30 min. The supernatant was ultracentrifuged at 24 421 rpm for 2 hours in a Beckman TY 30 rotor and the pellets resuspended overnight at 4°C in 0.5M potassium phosphate buffer, pH 7.5, containing 0.01M sodium sulphite. The suspension was given a low speed centrifugation, 7000 rpm for 20 min and the supernatant was layered on a 8ml 20% sucrose cushion in 0.5M phosphate buffer, pH 7.5, and centrifuged for 2 hours at 24 421 rpm in a Beckman TY 30 rotor. The pellet was resuspended overnight and given a low speed centrifugation for 20 min at 7000 rpm. The supernatant was loaded onto a 10-40% sucrose gradient and centrifuged at 15 000 rpm for 2 hours in a Beckman SW28 rotor. The samples were collected with the ISCO density gradient fractionator. The fractions collected were monitored with the electron microscope and those fractions containing the most filled particles were pooled and concentrated by ultracentrifugation for 4 hours at 24 421 rpm in a TY 30 rotor.

Virus yields were determined with the spectrophotometer using the specific absorbance value at 260 nm for BBWV which is 8.0 absorbance units per mg/ml in a 1cm light path (Xu, *et al.*, 1988).

4.2.5 Serology

4.2.5.1 Antiserum production

An antiserum was raised to purified BBWV isolate-94/1969 in a New Zealand white rabbit as described in Appendix A. Four intramuscular injections emulsified in Freunds complete adjuvant, for the first injection and Freunds incomplete adjuvant for the successive injections were given at weekly intervals. Blood was collected weekly from the rabbit one week after the last injection. The optimal dilution of bleed 5 and infected plant material was determined by PAS-ELISA (Edwards and Cooper, 1985). These dilutions were used in the titer determinations of all the bleeds. The bleed with the highest specific titer was identified by dilution end point determinations in PAS-ELISA.

Immunoglobulins were purified from antiserum that was absorbed against healthy plant material (See Appendix A); $F(ab')_2$ fragments were prepared and a $F(ab')_2$ ELISA developed. (See Appendix A)

4.2.5.2 Serotype of virus

To determine the serotype of the virus ISEM studies were done (See Appendix A) on isolate 94/1969. Antiserum to 94/1969 produced at PPRI and antiserum E229 from the Netherlands (Makkouk *et al.,* 1990) as well as antisera to serotype 1 and II (Uyemoto and Provvidenti, 1974) were used in this test. The antiserum, which is able to distinguish between serotype I and serotype II of BBWV, was produced and supplied by Dr. Uyemoto from USDS-ARS of the University of California in the USA. The antiserum (E229) from the Netherlands (Makkouk *et al.,* 1990), was supplied by IPO in Wageningen in the Netherlands. Virus particles of isolate 94/1969 were trapped with antiserum of 94/1969 at a dilution of 1:1000 and decorated with the four antisera described above at a dilution of 1:10.

The serotype of isolate 94/1969 was also determined serologically with a $F(ab')_2$ ELISA (See Appendix A). $F(ab')_2$ fraction prepared from the antiserum raised to isolate 94/1969 was used at a dilution of 1:1000 as the coating antibody. *N. benthamiana* plants infected with 94/1969 were used as positive control and healthy *N. benthamiana* plants as negative controls. IgG (to 94/1969) and antisera to serotype I and II were used as the detecting antibodies at dilutions of 1:100. The ELISA was performed as described in Appendix A.

4.2.6 RNA size determination

An RNA extraction was performed as described in Appendix A. A purification of BBWV was done using 80 g of previously frozen *N. benthamiana* plants as described in 4.2.4. Fractions of 0.5 ml were collected with the ISCO fractionator from the sucrose gradient. Fractions 1-7 were pooled and called Fraction A and fractions 8-10 were pooled and called Fraction B. The concentration of the purified virus used for the RNA extraction was 0.06 mg/ml (fraction A) and 0.1 mg/ml (fraction B). The extracted RNA

was loaded on a 1% agarose gel and gel electrophoresis was performed as described in Appendix A. A 2.4 -9.5 kb RNA ladder supplied by Gibco BRL was used as marker. The sizes were determined with the UVP Gelworks for Windows program and according to the method of Schaffer and Sederoff (1981). The determination of sizes according to the method described by Schaffer and Sederoff (1981), was done by graphing the logarithm of length against the mobility of standards. A computer program (Seqaid II) was used to do this determinations.

4.2.7 Aphid transmission tests

Aphid transmission tests were performed on isolate 94/1969 using the aphids *Macrosyphum euphorbiae* and *Myzus persicae*. These aphid species were maintained on *Capsicum sp.* plants in insect-tight cages. In transmission tests 15-20 adult insects were fasted for 1 hour and then placed in Petri dishes for acquisition feeding on BBWV-infected *P. sativum* leaves for 5-10 minutes. The aphids were transferred to a healthy *P. sativum* plant and were allowed to feed for 24 hours. The aphids were killed with Garden Gun (Sumitomo Chemical Co. Osaka Japan). The appearance of symptoms was monitored to correlate them with aphid transmission.

4.3 RESULTS AND DISCUSSION

4.3.1 Initial identification and isolation of virus

ISEM studies indicated that isolate 94/1969 is BBWV. Trapping and decoration of isolate 94/1969 particles with E229 antiserum was observed from inoculated *N. benthamiana, V. faba* and *P. sativum* plants. The virus was isolated from local lesions that developed on *C. quinoa* five days after mechanical inoculation. After two consecutive local lesion transfers the isolate was established on *N. benthamiana* plants for propagation of the virus.

4.3.2 Symptomatology

Severe mosaic symptoms were displayed by the original sample 94/1969. The isolated virus was inoculated on *P. sativum* cv Green Feast plants and the symptoms observed were vein clearing and systemic mosaic (See Figure 4.1). Vein clearing on the *P. sativum*

cv. Green Feast plant developed 14 days after inoculation followed by systemic mosaic. On inoculated *N. benthamiana* plants vein clearing was visible five days after inoculation and systemic mosaic symptoms a week later.

Figure 4.1. Symptoms on *P. sativum* cv Green Feast plants inoculated with isolate 94/1969

The results of the host range studies indicated that the virus had a wide host range. The results are shown in Table 4.1. The *Chenopodium* spp. inoculated all developed chlorotic local lesions that became systemic in the plants (See Figure 4.2. and 4.3.). Of the *Glycine max* spp. inoculated, all cultivars were insusceptible except *G. max* York that developed systemic mosaic. Four of the *Nicotiana* spp. inoculated showed mosaic symptoms (See Figure 4.4. and 4.5.). The *Phaseolus vulgaris* spp. inoculated developed local lesions (See Figure 4.6) and a chlorotic spread into the veins (Figure 4.7.). The *V. faba* plant displayed systemic mosaic as seen in Figure 4.8. All the plants with systemic symptoms tested positive in ELISA, but some local infections were negative. This could be attributed to local lesions becoming necrotic and the virus being localised and its multiplication inhibited, and therefore not detected in ELISA in leaf sites away from the lesions.

HOST PLANT	ELISA	SYMPTOM
Arachis hypogaea	-	-
Beta vulgaris	-	local chlorotic local lesions
Chenopodium amaranticolor	+	systemic chlorotic local lesions
Chenopodium murale	+	systemic chlorotic local lesions
Chenopodium quinoa	+	systemic chlorotic local lesions
Datura stramonium	+	systemic mild mosaic, spread into veins
Glycine max B66S10	-	-
Glycine max Edgar	-	-
Glycine max Forrest	-	-
Glycine max Ibis	-	-
Glycine max York	+	systemic mosaic
Gomphrena globosa	+	systemic chlorotic local lesions, mosaic
Gossypium hirsutum	-	-
Lablab purpureus	-	local necrotic lesions
Lupinus albus Kiev	+	systemic severe mottle, smaller leaves
Lycopersicon esculentum	-	-
Medicago sativa	-	-
Nicotiana benthamiana	+	systemic mosaic
Nicotiana clevelandii	+	systemic mild mosaic
Nicotiana glutinosa	-	-
Nicotiana langsdorfii	+	systemic mosaic on young leaves
Nicotiana occidentalis	+	systemic severe mosaic
Nicotiana tabacum Samsun	-	-
Phaseolus vulgaris Black Turtle Soup	-	-
Phaseolus vulgaris Bonus	-	local chlorotic lesions, spread into veins
Phaseolus vulgaris Bountifull	-	local chlorotic lesions
Phaseolus vulgaris Redlands Greenleaf	-	local chlorotic lesions, spread into veins
Phaseolus vulgaris The Prince	-	necrosis along veins
Phaseolus vulgaris Top Crop	-	-
Pisum sativum Green Feast	+	systemic vein clearing, mosaic
Triticum aestivum	-	-
Vicia faba	+	systemic severe mosaic
Vigna unguiculata	-	-
Zea mays	-	-

Table 4.1. Results of host range of BBWV 94/1969 where the +/- column indicates a positive or negative ELISA reaction or no symptoms

Figure 4.2. Local lesions caused by BBWV on *C. amaranticolor*

Figure 4.3. Chlorotic local lesions becoming necrotic on *C. murale* with a BBWV infection

Figure 4.4. Systemic mosaic of BBWV on *N. benthamiana*

Figure 4.5. Systemic mosaic of BBWV on *N. occidentalis*

Figure 4.6. Chlorotic local lesions spreading into veins of *P. vulgaris* Redlands Greenleaf with a BBWV infection

Figure 4.7. Chlorotic local lesions spreading into veins of *P. vulgaris* Bonus with a BBWV infection

Figure 4.8. BBWV causing systemic mosaic in *V. faba*

4.3.3 Electron microscopy

In the initial identification of the virus with a limited host range study, it was observed that specific host plants had different ratios of empty and full particles. More empty particles were observed in leafdips of infected *C. quinoa* and *N. rustica* plants than in the infected *N. benthamiana* plant which had more filled particles. The reason for certain plants containing more empty or more filled particles was not found in literature studies. It might be that the virus replicates better in certain plant species and that more coat protein is made in these. The RNA species of fabaviruses have not been studied, but in the case of comoviruses no replication of RNA-2 has been reported in the absence of RNA-1.

4.3.4 Purification of virus

During the purification procedure no prominent bands were detected in the sucrose gradient. One whitish band was detected approximately 1 cm from the meniscus. Below the meniscus was a blue area of ± 3 cm in length. Fourteen to twenty-seven fractions of 0.5 ml each were collected and monitored with the electron microscope (as described under 4.2.3). The fractions were categorised depending on the amount of virus and if

there was more filled than empty particles. The fractions with the highest amount of filled particles were pooled and concentrated for immunisation.

Virus yields of 0.045 mg-0.49 mg virus were obtained from an average of 120 g of leaf material.

Purified BBWV particles sediment as three components; top (T), middle (M), and bottom (B) components. The T component contains empty protein shells without RNA, and the M and B components contain two sorts of nucleoprotein particles containing different amounts of RNA (Taylor and Stubbs, 1972). The $A_{260/280}$ values for the different components are 1.32 for top components, 1.64 for middle components, and 1.75 for bottom components (Taylor and Stubbs, 1972). The A_{260}:A_{280} absorbance ratios obtained with the four purifications were 1.2-1.3. This reduced A_{260}:A_{280} ratio may be due to the presence of host proteins. No clear bands were visible on the sucrose gradient and fractions were collected with the ISCO fractionator. It is possible that other proteins were collected with the viral proteins. Analysis of the fractions was mainly done with the electron microscope and fractions containing the highest amount of full particles were used for immunisation. Purified particles were photographed after staining with 2% uranyl acetate (See Figure 4.9).

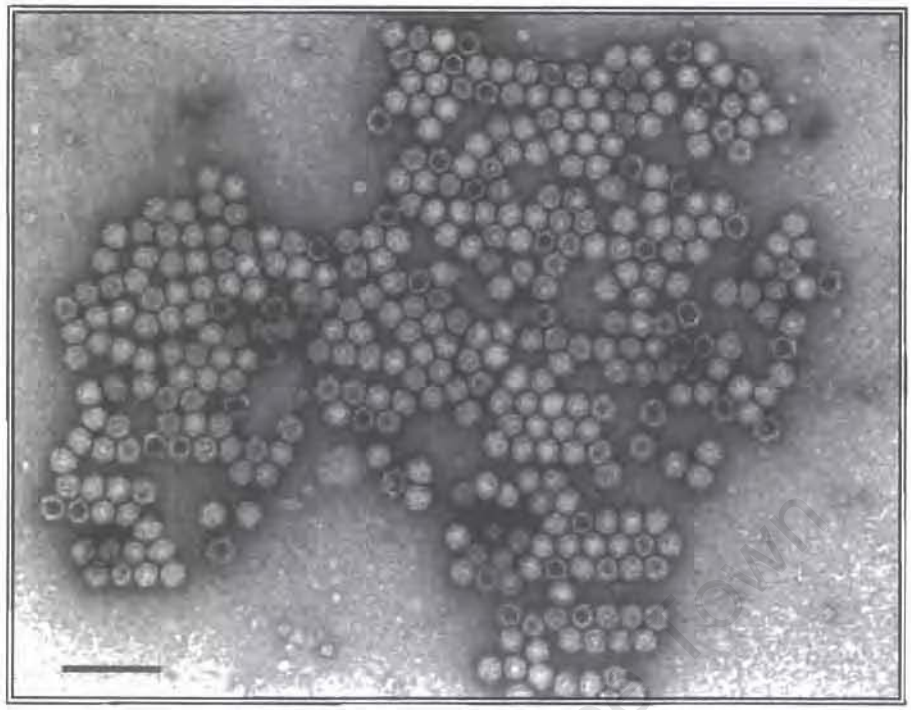

Figure 4.9. Purified BBWV 94/1969 particles, stained with 2%uranyl acetate and enlarged 200 000 times. Scale bar represents 100 nm.

4.3.5 Serology

4.3.5.1 Antiserum production

Eight weekly bleeds were collected from the immunised rabbit. The optimal dilution of Bleed 5 was determined at 1:100 and was used as coating antibody. The optimal dilution for the infected plant material was determined to be 1:10 (plant material:buffer). The absorbance values of infected plant material vs. healthy plant material were used to determine the best bleed at the highest dilution. The results of the titer determinations of the eight bleeds at dilutions of 1:512 000 are shown in Figure 4.10. Bleed 5 was chosen because at the 1:1000 dilution it had the highest virus-infected:healthy plant material ratios and at the highest dilution, 1:512 000, it showed a peak after which the ratios decreased. IgG and $F(ab')_2$ were prepared from bleed 5.

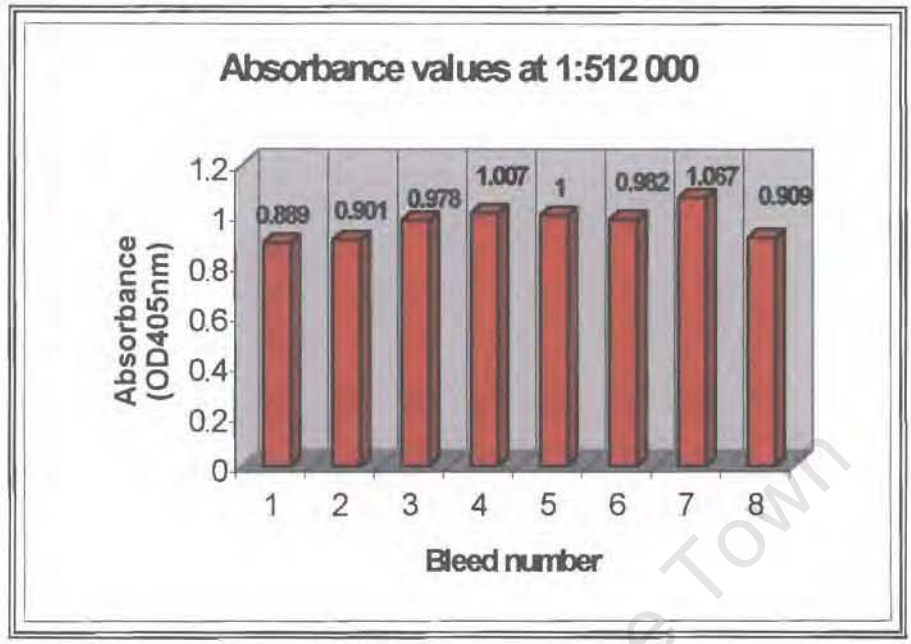

Figure 4.10. Results of titer determinations using PAS-ELISA where absorbance values of healthy plant material vs. infected plant material of eight bleeds.

Optimal concentrations of the $F(ab')_2$ and IgG were shown to be 1:1000 and 1:100 respectively. The optimised absorbance values of the BBWV 94/1969 ELISA are shown in Figure 4.11.

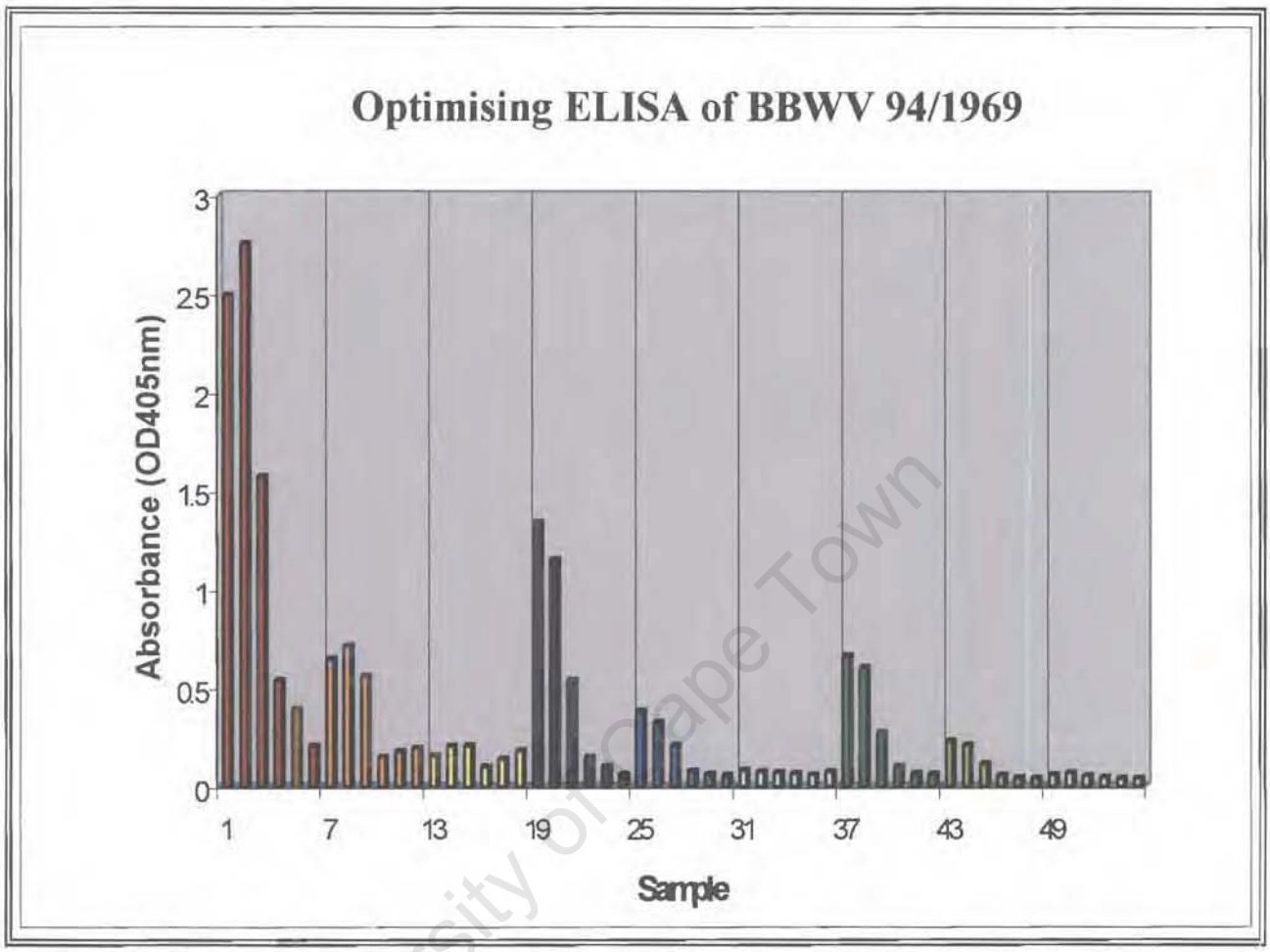

Figure 4.11. Results of the optimising ELISA of BBWV 94/1969. Lane 1, 94/1969 1:10, lane 2, 94/1969 1:100, lane 3, 94/1969 1:1000, lane 4, healthy *N. benthamiana* 1:10, lane 5, healthy *N. benthamiana* 1:100, lane 6, buffer control. The order was repeated for each seroreagent combination tested:

- $F(ab')_2$ 1:100 and IgG 1:100
- $F(ab')_2$ 1:100 and IgG 1:1000
- $F(ab')_2$ 1:100 and IgG 1:2000
- $F(ab')_2$ 1:1000 and IgG 1:100
- $F(ab')_2$ 1:1000 and IgG 1:1000
- $F(ab')_2$ 1:1000 and IgG 1:2000
- $F(ab')_2$ 1:2000 and IgG 1:100
- $F(ab')_2$ 1:2000 and IgG 1:1000
- $F(ab')_2$ 1:2000 and IgG 1:2000

4.3.5.2 Serotype of virus

Results of ISEM tests to determine the serotype of our isolate are summarised in Table 4.2.

ANTISERUM USED FOR TRAPPING	ANTISERUM USED FOR DECORATION	RESULT
94/1969 Bleed 5	94/1969 Bleed 5	Positive trapping and strongly decorated
94/1969 Bleed 5	E229	Positive trapping and decoration
94/1969 Bleed 5	Serotype I	Positive trapping and lightly decorated
94/1969 Bleed 5	Serotype II	Positive trapping and strongly decorated

Table 4.2. Results of ISEM to determine the serotype of 94/1969

The same degree of decoration was obtained using the homologous antiserum of 94/1969 and the antiserum to serotype II. A lighter decoration was obtained with antiserum to serotype I.

ELISA results shown that serotype II antiserum reacted similarly to the 94/1969 antiserum. ELISA results in Figure 4.12. The sequence of the plant material on the ELISA plate was 94/1969-infected *N. benthamiana*, negative control *N. benthamiana* and buffer control. Two replicates of each combination are shown on the graph (Figure 4.12). The serotype I antiserum gave a high background reaction. The antiserum produced to isolate 94/1969 was used to coat the plate and IgG from a serotype I isolate used for detecting. High background reaction occurred because serotype I antibodies didn't recognise the antigen, or protein, of isolate 94/1969 and unspecific binding occurred.

In the serotype determination test of isolate 94/1969 I regard the ISEM tests where decorated particles were detected, described in Table 4.2, as the accurate test (photos not shown here).

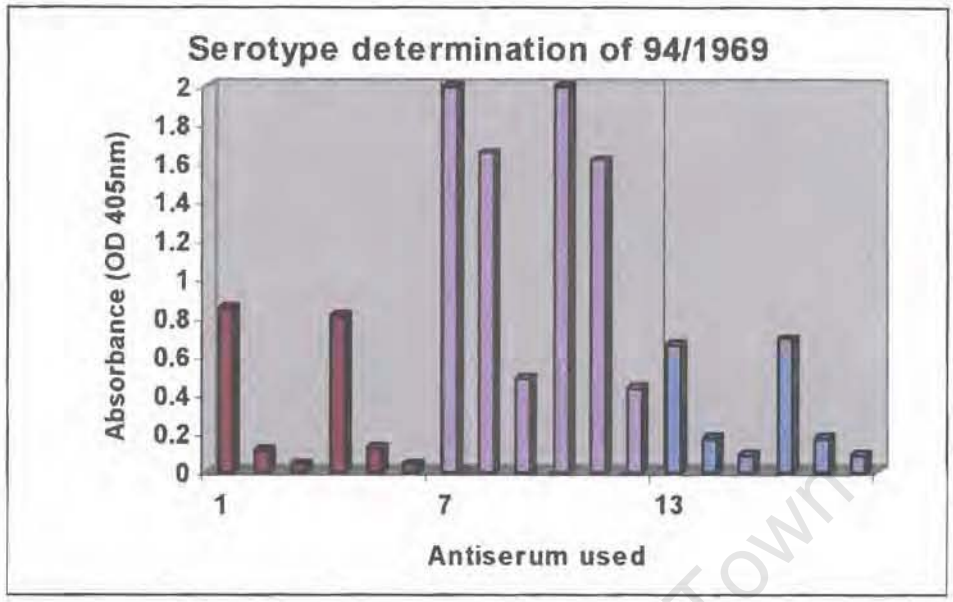

Figure 4.12. Serotype determination of 94/1969.

94/1969 antiserum Serotype I antiserum Serotype II antiserum

4.3.6 RNA size determination

The electrophoretic mobility of nucleic acids depends on both their molecular weight and conformation (McMaster and Carmichael, 1977). Removing secondary and tertiary structure should make the electrophoretic mobility a function of molecular weight. Gels containing denaturing agents like formaldehyde, formamide, methylmercuric hydroxide, glyoxal and urea have been used for molecular weight determinations (McMaster and Carmichael, 1977; Sambrook *et al.*, 1989).

Two RNA bands were detected on non-denaturing agarose gels. The genome of BBWV consists of two species of RNA (1 and 2) which are both necessary for infectivity (Lisa and Boccardo, 1996). The average molecular weights of the RNA bands were determined to be 2.1 x 10^6 Da and 1.42 x 10^6 Da (See Figure 4.13). The sizes were determined by assuming 1 kb of single-stranded RNA to have an molecular weight of $3.4x10^5$ Da. The results are shown in Table 4.3. The estimated molecular weight of RNA-1 is $2.0\text{-}2.6x10^6$ Da and that of RNA-2 is $1.3\text{-}1.7x10^6$ Da (Taylor and Stubbs,

1972). Although agarose gels were used without denaturing the RNA, the results obtained in this study were similar to the data in the literature. The molecular weights of the detected RNA in this study were just an indication of the different molecular weights and to obtain accurate results denaturing gels should have been used.

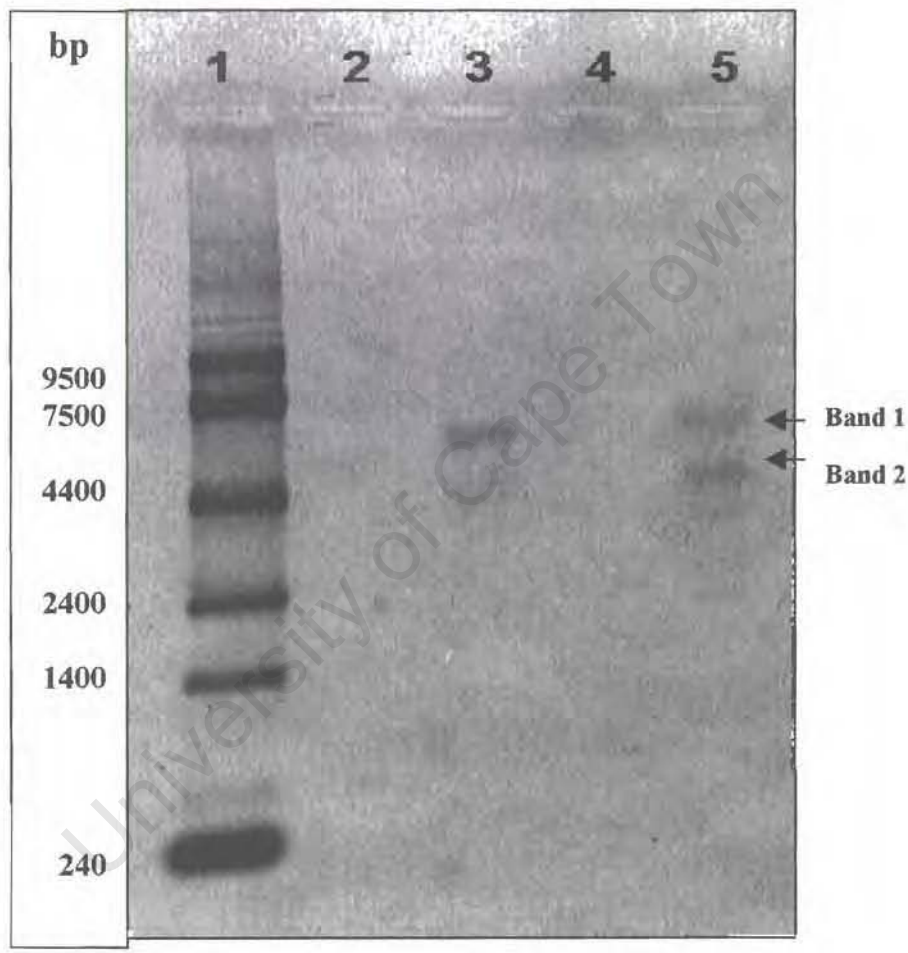

Figure 4.13. Agarose gel of 94/1969 RNA. Lane 1, RNA ladder; Lane 3 and 5, extracted RNA of 94/1969. Two RNA bands detected on agarose gels.

METHOD USED TO DETERMINE SIZE	RNA SIZES OF GEL 1	RNA SIZES OF GEL 2	AVERAGE RNA SIZE
Gelworks program	*Band 1*: 6.7 kb *Band 2*: 4.2 kb	*Band 1*: 5.7 kb *Band 2*: 3.9 kb	*Band 1*: 6.2 kb *Band 2*: 4.1 kb
According to Schaffer and Sederoff(1981)	*Band 1*: 6.5 kb *Band 2*: 4.6 kb	*Band 1*: 5.7 kb *Band 2*: 3.9 kb	*Band 1*: 6.1 kb *Band 2*: 4.3 kb

Table 4.3. RNA size determination of BBWV 94/1969

The average RNA sizes of the RNA bands determined by the methods, Gelworks and Schaffer and Sederhoff (1981), were calculated to be 6.2 kb for RNA band 1 and 4.2 kb for RNA band 2.

4.3.7 Aphid transmission

Aphid transmission was obtained with *M. persicae* and *M. euphorbiae*. Chlorotic mosaic symptoms appeared on *P. sativum* 13 days after transmission. On *N. benthamiana* a veinal clearing developed. Four *P. sativum* plants and two *N. benthamiana* plants were tested in ELISA to confirm the transmission. One of the four *P. sativum* plants tested positive in ELISA but the *N. benthamiana* plants were negative in ELISA (See Figure 4.14).

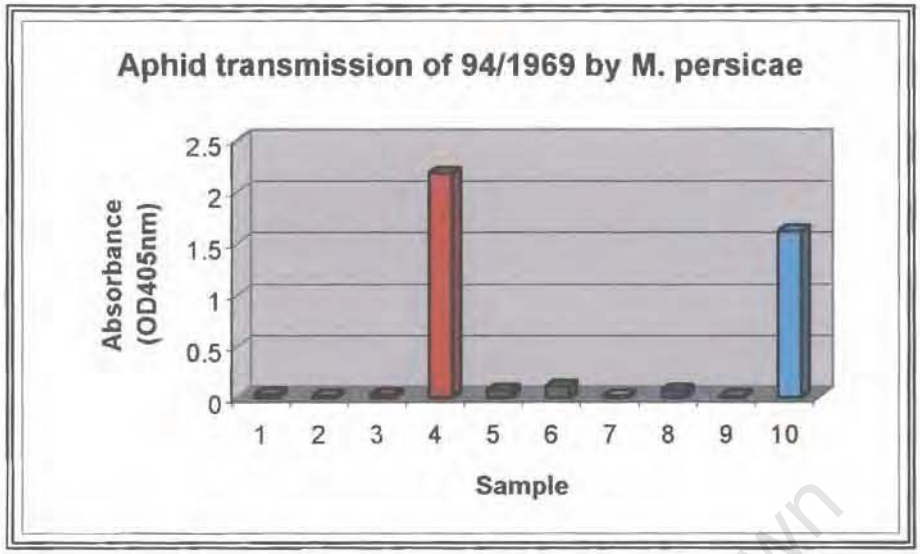

Figure 4.14. ELISA result of aphid transmission of 94/1969 by *M. persicae* aphids.

- *P. sativum* plants tested
- Healthy control of *P. sativum*
- Buffer control
- *N. benthamiana* plants tested
- Healthy control of *N. benthamiana*
- Positive control of BBWV.

4.4 CONCLUSION

According to morphological, serological and host range properties, isolate 94/1969 was similar to BBWV isolates described in literature (Brunt *et al.,* 1996; Stubbs, 1947; Uyemoto and Provvidenti, 1974). Isolate 94/1969 was confirmed with ISEM to be broad bean wilt virus using antiserum to a BBWV isolate from pea (E229) (Makkouk *et al.,* 1990). Particles of isolate 94/1969 were both trapped and decorated with the E229 antiserum. Antiserum that can distinguish between BBWV serotype I and II were used in the serotype determination. We concluded that BBWV 94/1969 belongs to serotype II. A Chinese isolate is reported to belong to serotype II (Xu *et al.,* 1988) and isolates reacting with serotype I were recorded from Syria and Egypt (Makkouk *et al.,* 1990). From Minnesota an isolate from *Begonia semperflorens* reacted with serotype I (Lockhart and Betzgold, 1982). It is not easy to correlate host reactions with serotyping and there is no set of host plants that will distinguish between the serotypes (Makkouk *et al.,* 1990).

An antiserum was produced and an ELISA developed to isolate 94/1969. The optimal antibody dilutions of the 94/1969 ELISA were determined to be 1:1000 for the $F(ab')_2$ fractions and 1:100 for the IgG fractions. The ELISA test was used in confirming tests of host range studies and serotype determination.

Electron microscopy showed two types of particles, apparently empty ones and filled particles. This phenomenon is characteristic of BBWV and was used in the identification. The electron microscope was used in serotype determination with ISEM. This test confirmed that BBWV 94/1969 belonged to serotype II. Results with the electron microscope determined the purification procedure.

Two RNA bands were detected on non-denaturing agarose gels. The genome of BBWV consists of two species of RNA (1 and 2) which are both necessary for infectivity (Lisa and Boccardo, 1996). The molecular weights of the RNA of the isolate were determined to be 6.2 kb and 4.2 kb. Although agarose gels were used without denaturing the RNA, the results obtained in this study were similar to the data in the literature (Taylor and Stubbs, 1972).

The host range study with BBWV 94/1969 revealed a wide host range similar to those reported earlier (Makkouk *et al.,* 1990; Xu *et al.,* 1988; Brunt *et al.,* 1996; Kishtah, *et al.,* 1978). Systemic mosaic symptoms were displayed on *P. sativum.* One of the soybean cultivars, *G. max* York, was positive. *G. max* is reported to be a host of BBWV (Brunt *et al.,* 1996). Characteristic of the virus infection on *P. vulgaris* was that the reaction was local chlorotic lesions and no systemic infection was noted. Host plants that were negative in the study but were previously recorded as hosts are *B. vulgaris, L. esculentum, N. glutinosa,* and *V. unguiculata* (Brunt *et al.,* 1996).

Aphid transmission was proved with *M. persicae* and *M. euphorbiae*. *M. persicae* is reported to be the most efficient vector of BBWV (Stubbs, 1960; Lockhart and Betzgold, 1982). Naturally BBWV is transmitted by a number of aphids, but only *M. persicae* and *M. euphorbiae* were available for the tests.

This is the first report of BBWV on *P. sativum* in South Africa. The virus was previously recorded on *Vitis vinifera* from South Africa (Castrovilli *et al.,* 1985).

CHAPTER 5
ISOLATION AND IDENTIFICATION OF SAMPLE 95/0931

CHAPTER 5

ISOLATION AND IDENTIFICATION OF SAMPLE 95/0931

5.1 INTRODUCTION

A pea plant with yellow mosaic and vein clearing was collected at Roodeplaat at the Vegetable and Ornamental Institute of the ARC. Electron microscopic analysis showed flexuous particles similar to *Potyviruses*. The plant was tested by ELISA for five different potyviruses, and tested positive for BYMV. PMV and ClYVV belong to the same subgroup of potyviruses as BYMV and are serologically related. According to phylogenetic data based on the nucleic acid sequence of the 3' NCR and coat protein by Berger *et. al.* (1997), there are two distinct clusters or subgroups of legume-infecting potyviruses. These include BYMV, ClYVV and PMV in one subgroup, with the other comprising the BCMV subgroup. BYMV, PMV and ClYVV belong to subgroup 1 of the aphid-transmitted potyviruses (Milne, 1988). Recent research by Xiao *et. al.* (1994) comparing peptide profiles and sequences of selected peptides from coat proteins of ten strains of BYMV, three strains of PMV, one strain of *Sweetpea mosaic virus* (SPMV) and *White lupin mosaic virus* (WLMV) shows that PMV and WLMV are probably strains of BYMV.

Because of the existence of so many members of the genus *Potyvirus*, it is important to be able to classify viruses in the genus and to distinguish between distinct viruses and strains of a virus. Coat protein sequence data (Shukla and Ward, 1988), comparative HPLC profiles and serology with polyclonal antibodies directed towards virus-specific N-termini of the coat proteins can be used to discriminate between distinct potyviruses and strains (Frenkel *et al.*, 1989). Pappu *et al.* (1993) described a PCR and sequencing approach for broad spectrum detection and identification, which involved amplification of part of the coat protein gene and the whole 3' NCR. This method was used in this study to determine the identity of our isolate (95/0931). According to Frenkel *et al.* (1989) the sequence of the 3' NCR could serve as an aid to identify and classify potyviruses. Characterisation of isolate 95/0931 was done to determine whether it was

BYMV or a virus serologically related to BYMV. Sequence data from our isolate was compared with different potyviruses.

5.2 MATERIALS AND METHODS

5.2.1 Source of virus, initial identification and isolation of virus

The sample was collected at Roodeplaat at the Vegetable and Ornamental Institute of the ARC. The sample was initially tested in a $F(ab')_2$ ELISA system with antisera to a number of potyviruses including BYMV-92/0751, *Potato virus Y* (PVY-CB3), *Soybean mosaic virus* (SMV-86/20), *Cowpea aphid-borne mosaic virus* (CABMV-87/14), and *Peanut mottle virus* (PeMoV-88/63). The $F(ab')_2$ ELISA was performed according to the method described in Appendix A and included positive, healthy and buffer controls.

An initial host range study was done with the original plant inoculated onto seven indicator plants including *Nicotiana benthamiana, Nicotiana glutinosa, Phaseolus vulgaris* Top Crop, *Nicotiana tabacum* Samsun, *Pisum sativum, Gomphrena globosa,* and *Chenopodium quinoa.*

The virus was established, maintained and propagated on *C. quinoa* plants in the greenhouses of the PPRI, Pretoria. The temperature of the greenhouses was 24-27°C. The virus was propagated by sap transmission of virus-infected leaf material macerated in 0.01M sodium phosphate buffer, pH 7.1, containing 0.02M sodium sulphite and a small amount of Celite to healthy *C. quinoa* plants. Virus symptoms were monitored regularly and the isolate was reinoculated weekly.

Two local lesion transfers on *C. quinoa* (Appendix A) were used to isolate the virus. This isolate and the original field sample were stored (Appendix A) in the collection of PPRI.

5.2.2 Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR)

The 3' NCR and part of the coat protein gene was amplified by IC-RT-PCR using the CN 48 and degenerate oligo-dT primers of Pappu *et al.,* 1993. One primer was directed at the poly A tail at the 3' end of the genome and the other primer at the mid-region of the coat protein gene (WCIEN block). The oligo dT primer consisted of 21 T residues with a terminal degeneracy of A, C, G or T. The nucleotide sequences of the primers are as follows:

Oligo dT primer: 5' TTTTTTTTTTTTTTTTTTTTN 3'

CN48 primer: 5' TGGTGWATHGANAATGG 3'

The ambiguity codes used are

N= A, C, G or T

W= A or T

H= A, C or T

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

The PCR was performed according to the method described by Cook *et al.* (1998) (Appendix A). The potyvirus common epitope Mab (Agdia) was used to coat the tubes. Negative and positive controls were included in the PCR. A clone which contains the entire coat protein and 3' NCR of CABMV-SAP (obtained from Dr. Reon Brand, ARC-Infruitec), was used as positive control for the PCR (Brand *et al.,* 1993). The amplification was performed in a Hybaid thermocycler.

The PCR products were run on a 1% agarose gel in TAE buffer for 30 min at 100 V. Ethidium bromide (10 mg/ml) was added to the gel and this was viewed on the 325 nm wavelength UV transilluminator. The size of the amplified PCR product was determined with the UVPGrab and Gelworks for Windows Program. The PCR product was used for cloning and sequencing.

5.2.3 Ligation and Transformation

The PCR product was purified according to the "Double Gene Clean" procedure (Appendix A) and blunt-end ligated into *Sma*I-cut and dephosphorylated pBluescript IIKS plasmid. The ligation was transformed into competent *E. coli* DH5α cells (Appendix A) and clones screened using the blue/white selection. The preparation of competent cells, preparation of the vector, including large scale plasmid purification, and preparation of the blunt-ended vector are described in Appendix A.

5.2.4 Selection of positive clones

The white colonies resulting from the insertional inactivation of the *lacZ* gene product (β-galactosidase) of the vector were selected from the plates. These colonies were screened for inserts by PCR using the M13 forward and M13 reverse primers (Strategies in Molecular Biology-Stratagene) that are situated on either side of the cloning site. The nucleotide sequences of the primers are as follows:

M13 forward primer: 5' GTAAAACGACGGCCAGT 3'

M13 reverse primer: 5' CAGGAAACAGCTATGAC 3'

The primers were synthesised by the Biochemistry Department, UCT, Cape Town, South Africa.

The PCR conditions were as follows: a denaturation step at 94°C for 2 min followed by 35 cycles of 93 °C for 45 s, 43 °C for 40 s and 72 °C for 60 s. The PCR products were run on a 1% agarose gel in TAE buffer for 60 min at 120 V. Marker VI (Boehringer Mannheim) was used as molecular weight marker. The CABMV-SAP clone was used as positive control in the PCR (Brand *et al.*, 1993).

5.2.5 Sequencing of clones

Sequencing of the clones was done with "Alf-Express Sequencing Service" at the Department of Microbiology, University of Cape Town. Three clones were selected for sequencing.

5.2.6 Sequence analysis

Sequences were compared by BLAST (basic local alignment search tool) searches of the Genbank and EMBL databases (Altschul *et al.,* 1990). Nucleic acid sequence data retrieved from the Genbank and EMBL were aligned using DNAMAN (Lynnon Biosoft, 1996). Homology and phylogenetic trees were drawn. The homology trees is setup with the distance matrix using UPGMA method of Sneath and Sokal (1973) and the phylogenetic trees using the Neigbour-Joining method of Saitou and Nei (1987) (Lynnon Biosoft, 1996).

5.3 RESULTS AND DISCUSSION

5.3.1 Source of virus, initial identification and isolation of virus

The original *P. sativum* plant showed severe yellow mosaic symptoms (See Figure 5.1).

Figure 5.1. Symptoms on original *P. sativum* plant

The ELISA results indicated that the virus was BYMV-related. Graphic representation of the absorbance values obtained in ELISA when testing isolate 95/0931 against various potyvirus antisera are given in figures 5.2, 5.3, 5.4, 5.5 and 5.6. The set-up of the ELISAs was the same for all five i.e. sample 1, 95/0931; sample 2, healthy control; sample 3, buffer control; and sample 4, the positive control.

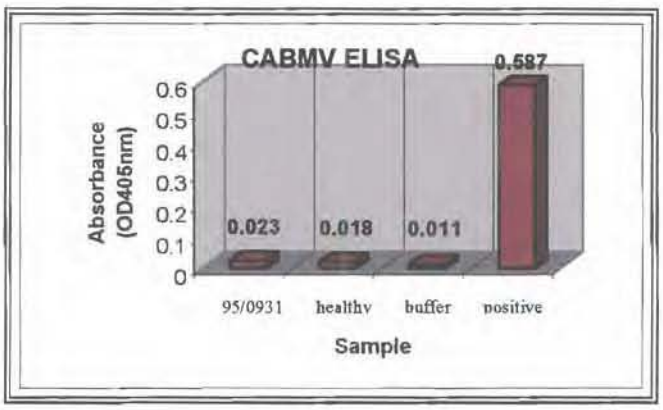

Figure 5.2 95/0931 tested to CABMV antiserum

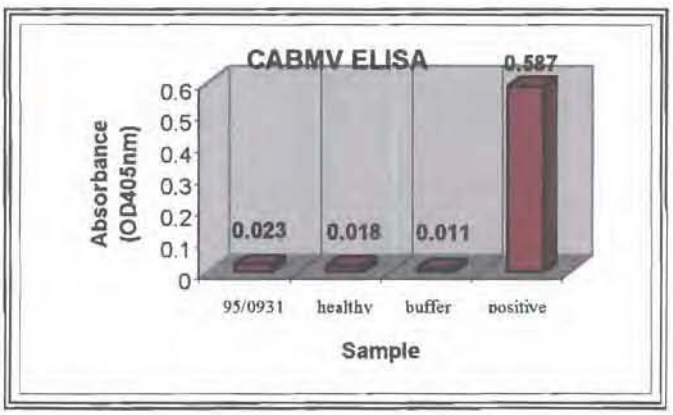

Figure 5.3 95/0931 tested to SMV antiserum

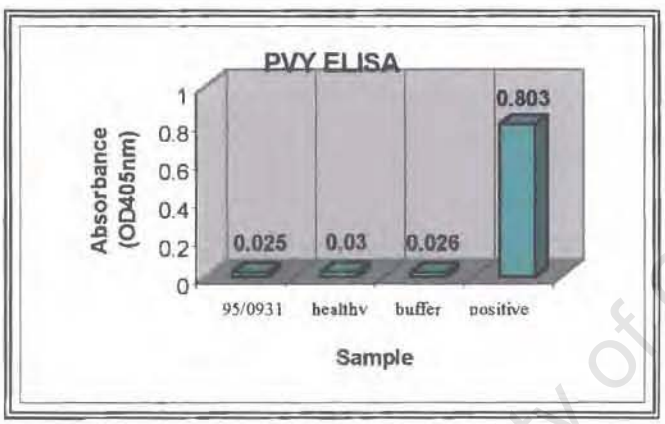

Figure 5.4 95/0931 tested to PVY antiserum

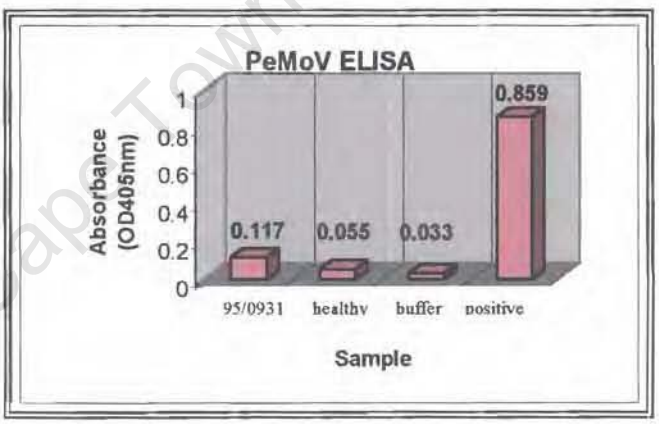

Figure 5.5 95/0931 tested to PeMoV antiserum

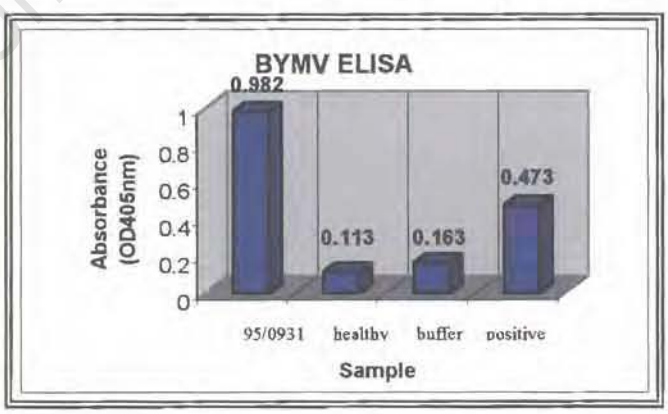

Figure 5.6 95/0931 tested to BYMV antiserum

All the positive controls of the five viruses reacted in the ELISA. Isolate 95/0931 only reacted significantly in the BYMV ELISA. The BYMV ELISA had more background reaction than the other ELISAs but the 95/0931 sample reacted strongest as seen in Figure 5.6. The absorbance value of sample 95/0931 was more than twice the value of the healthy control and therefore considered positive.

The results of the initial host range study with the original sample gave the following results shown in Table 5.1.

HOST PLANT	SYMPTOM
Chenopodium quinoa	Chlorotic local lesions
Gomphrena globosa	Red local lesion with necrotic spots
Nicotiana benthamiana	-
Nicotiana glutinosa	-
Nicotiana tabacum Samsun	-
Phaseolus vulgaris Top Crop	Chlorotic local lesions and systemic mosaic
Pisum sativum	-

Table 5.1 Preliminary host range study from the original *P. sativum* 95/0931-infected plant. (-) indicates no symptoms

The virus was isolated from the chlorotic local lesions produced on *C. quinoa*. *G. globosa* and *C. quinoa* are commonly used as experimental hosts for BYMV (Brunt *et al.*, 1996) and typical symptoms of BYMV-infection were produced. The symptoms described on *Phaseolus vulgaris* Top Crop are similar to the symptoms described for BYMV on *P. vulgaris* (Brunt *et al.*, 1996). *Nicotiana* spp. are listed as hosts of BYMV but were not infected in this preliminary study. During this preliminary host range study the *P. sativum* plants did not display any symptoms. However, the plants inoculated were old and not susceptible to infection: *P sativum* plants are usually mechanically inoculated when the primary two leaves are displayed. The virus was isolated from *C.quinoa* and was maintained in these plants for use in IC-RT-PCR. No wider host range study was conducted, as the host range for BYMV is well determined.

5.3.2 IC-RT-PCR

The CN48/oligo dT primers allowed amplification of a DNA product of approximately 714 bp. No amplification was observed from a healthy *C. quinoa* extract or the buffer control. The CABMV-SAP clone resulted in a product of 740 bp. The expected size of the product when using the CN48/oligo dT primer combination is approximately 700 bp, according to Pappu *et al* (1993). The PCR products can be seen in Figure 5.7.

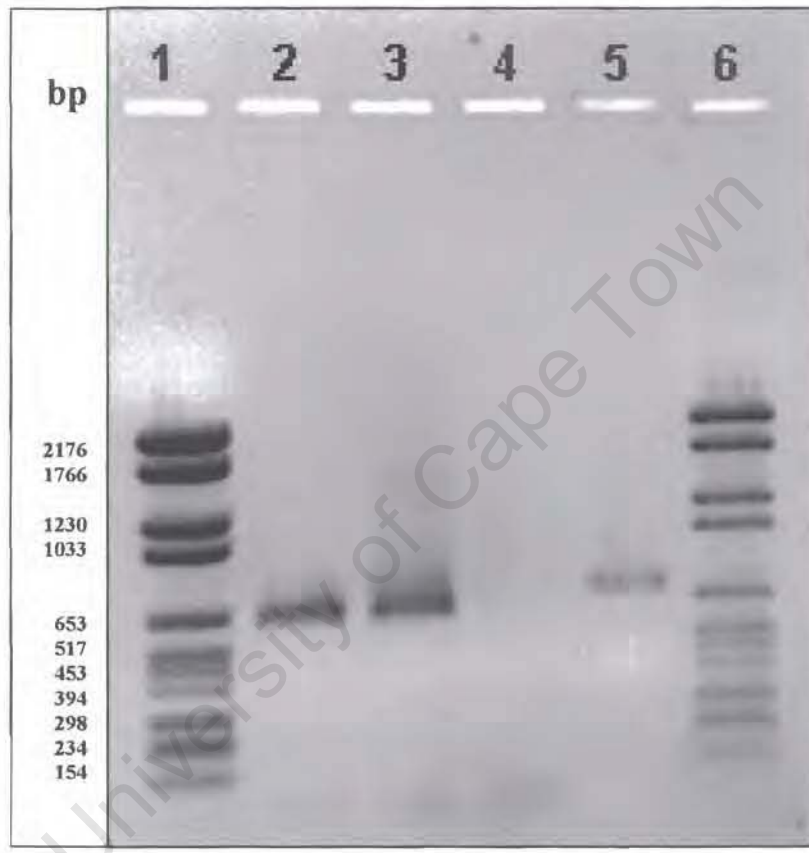

Figure 5.7 PCR products from amplification with potyvirus-specific degenerate primers. Lane 1 and 6, marker VI (Boehringer Mannheim); Lane 2 and 3, isolate 95/0931; Lane 4, negative control (*C.quinoa)*; Lane 5, positive control (CABMV-SAP clone).

5.3.3 Selection of positive clones

Eleven white colonies were obtained after transforming *E. coli* DH5α cells. The clones had inserts that varied from 430 to 911 bp. Three clones, 95931.5, 95931.7 and 95931.10 were selected for sequencing. The inserts of these clones were 755 bp for clones 95931.5 and 95931.10 and 803 bp for clone 95931.7. Figure 5.8 shows the results of the PCR performed to determine which clones contain inserts of the correct size.

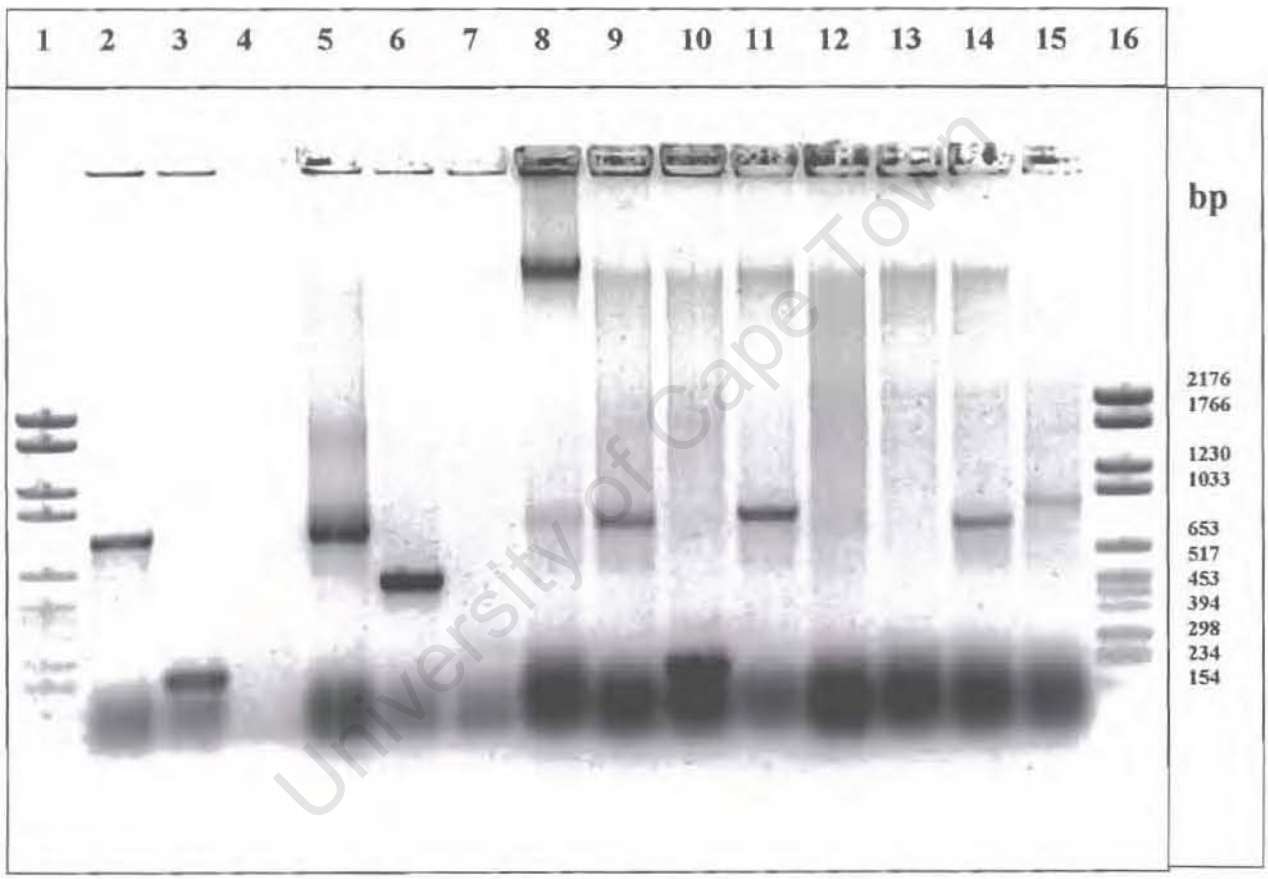

Figure 5.8. PCR on selected white colonies with M13 forward and reverse primers. Lane 1, Marker VI (Boehringer Mannheim); Lane 2, positive control (CIYVV, clone 93/1.3), Lane 3, negative control (blue colony); Lane 4, buffer control; Lane 5, clone 95931.1; Lane 6, 95931.2; Lane 7, 95931.3; Lane 8, 95931.4; Lane 9, 95931.5; Lane 10, 95931.6, Lane 11, 95931.7; Lane 12, 95931.8; Lane 13, 95931.9; Lane 14, 95931.10; Lane 15, 95931.11, Lane 16, Marker VI (Boehringer Mannheim). Clones, 95931.5, 95931.7 and 95931.10 were selected for sequencing.

5.3.4 Sequence analysis

Three clones were sequenced in the forward and reverse direction. The sequence was compiled using the forward and reverse reactions of clones 95931.5 and 95931.7 and the forward reaction of clone 95931.10. All clones were full-length clones of the PCR product as primer sequences (forward and reverse) were present in all 5 clones. When the 5 sequences were aligned a region of 610 bp was similar. Discrepancies (different bases) between the 5 sequences were detected at 2 places. Uncertainty of base identification was noted at 12 sites and extra bases detected at 5 sites. A minimum of 3 sequences were similar and specifying the correct sequence.

Isolate 95/0931 showed strong similarity with several BYMV strains as determined from a BLAST search in Genbank. This result correlates with the preliminary serological tests performed on this isolate. The sequence data and the single putative translation product are shown in Figure 5.9. The 3' NCR of isolate 95/0931 is 172 nucleotides in length. This result is similar to previous findings where the 3' NCR of the BYMV-S strain was shown to contain 173 nucleotides (Tracy *et al.,* 1992). A sequenced PMV-I clone contains 174 nucleotides in the 3' NCR and the PMV-I and BYMV-CS 3' NCR sequences were almost identical and differed at only eight positions (Xiao *et al.,* 1994). The 3' NCR can be used as a probe for detection of related strains of a given potyvirus, but 3' NCR sequences are not useful for showing relationships between distinct viruses (Rybicki and Shukla, 1992). The coat protein region of potyviruses can be used for accurate and detailed classification of viruses. Phylogenetic and homology trees were drawn to view the relationships between different potyviruses and isolate 95/0931.

The nucleic acid sequence of the 3' NCR and part of the CP gene of 95/0931 was aligned to cognate sequences of several potyviruses. Isolate 95/0931 was closely related to different strains of BYMV and showed a 99% homology with the nucleotide sequence of PMV-I. Our isolate also showed a close resemblance with the BYMV-S described by Tracy *et al* (1992). A 91% similarity was detected between isolate 95/0931 and the BYMV-S strain. Isolate 95/0931 was not identical to any one of the other BYMV strains in the database. Phylogenetic and homology trees of nucleic acid comparisons of the 3'

NCR and part of the CP gene of different potyviruses are shown in Figure 5.10 and 5.11. The cluster of BYMV viruses showed a 74% similarity with the ClYVV group of viruses.

The amino acid comparisons revealed that 95/0931 had a 100% identity with the PMV-I strain described by Xiao *et al.* (1994). The amino acid alignments can be seen in Figure 5.12 and 5.13. A list of the nucleic acid and amino acid sequences used in the comparisons can be seen in Appendix B.

Comparisons of protein sequences give a far better idea of distant relationships than comparisons of nucleotide sequences (Rybicki and Shukla, 1992). In this study comparisons of the amino acid sequences showed a closer relationship between isolate 95/0931 and PMV-I than the nucleic acid sequence comparisons. When constructing the trees sequence data from isolates of the BYMV subgroup of viruses were selected (BYMV, PMV, ClYVV). Some members of the BCMV subgroup of viruses were also selected to construct the trees and to show sequence relationships between these viruses. These include BCMV, BCMNV, BlCMV, AzBMV, CABMV. Other viruses distantly related to the BYMV group of viruses were included to show a wide range of sequence relationships. Berger *et al.* (1997) describes phylogenetic trees of different potyviruses based on two methods: maximum parsimony and the least squares FM method. These two methods were used to examine the relationships of different genera of potyviruses. The sequence data of the 3' NCR of potyviruses is not always sufficient to indicate differences between virus strains and its necessary to examine more regions of the genome as more sequence data becomes available.

Potyvirus coat protein sequence analyses have indicated that identities of about 90% and above are an indication of potyviral strains, while identities of less than 70% indicate distinct virus species (Xiao *et. al.,* 1994). The nucleic acid and amino acid sequences of isolate 95/0931 were 99% and 100% similar to PMV-I, while the PMV group shared 90% and 96% similarity with BYMV-S at the nucleotide and amino acid level, respectively. It is therefore confirmed that PMV is a strain of BYMV (Xiao *et al.,* 1994), and that isolate 95/0931 is an isolate of PMV.

Translation of 95931(1-610)

Total amino acid number: 195, MW=22307

Max ORF: 1-438, 146 AA, MW=16715

```
1   GAGAATGGAACATCAGGTGACTTACAAGGTGAGTGGACCATGATGGATGGTGATGAACAA
1     E   N   G   T   S   G   D   L   Q   G   E   W   T   M   M   D   G   D   E   Q

61  GTGACATACCCCTTGAAACCCATCTTAGATAATGCAAAGCCAACATTTCGCCAGATAATG
21    V   T   Y   P   L   K   P   I   L   D   N   A   K   P   T   F   R   Q   I   M

121 TCGCACTTTTCACAGGTTGCCGAAGCTTATATAGAGAAGAGGAATGCAACTGAGAGGTAT
41    S   H   F   S   Q   V   A   E   A   Y   I   E   K   R   N   A   T   E   R   Y

181 ATGCCGCGTTATGGCCTCCAGAGGAACTTAACTGACTATGGTTTGGCTAGATATGCTTTT
61    M   P   R   R   Y   G   L   Q   R   N   L   T   D   Y   G   L   A   R   Y   A   F

241 GATTTCTACAGGCTAACTTCGAGAACTCCTGTGCGTGCTAGGGAAGCACATATGCAGATG
81    D   F   Y   R   L   T   S   R   T   P   V   R   A   R   E   A   H   M   Q   M

301 AAGGCAGCAGCAATTAGAGGCAAGTCAAACCGATTATTTGGTCTTGATGGAAATGTTGGA
101   K   A   A   A   I   R   G   K   S   N   R   L   F   G   L   D   G   N   V   G

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

421 ATGCTTGGTGTTCGTATTTAGAGTATCCGTCTATAAATTCTCTGAAATTTGGCGTTACAT
141   M   L   G   V   R   I

481 TACTTAATACTATGTACTAGCGAGGTTTTACCTCCAGCATTTTAAATTCAGTATGTGTTT
161

541 CATTCTCTCTACTCTGACAGGGTAAGCTGTTAGTGAGGTTACCTCGAGTGGGCCTGATCT
181

601 TTGTAGAGCG
201
```

Figure 5.9 Translation of a portion of the coat protein gene of isolate 95/0931 with DNAMAN

5.4 CONCLUSION

The initial identification of the virus was based on serology and showed that isolate 95/0931 was related to BYMV. PMV and ClYVV belong to the same subgroup of potyviruses as BYMV and are serologically related. PMV was isolated from pea and induced a bright yellow mosaic on peas (Xiao *et al.*, 1994) and the original pea plant of isolate 95/0931 showed the same yellow mosaic symptoms on peas. Sample 95/0931 was isolated from *C. quinoa* and not inoculated back to *P. sativum*.

Previous reports on PMV suggest that the virus is a distinct virus based on weak hybridisation between BYMV-S and PMV-I (Tracy *et al.*, 1992) but an alignment of the 3' NCR sequences of four BYMV strains and PMV-I shows that these viruses share a very high sequence similarity with each other. It was concluded that PMV-I is a strain of BYMV (Xiao *et al.*, 1994). In this study isolate 95/0931 showed a 99% identity with PMV-I based on nucleic acid sequences and the amino acid sequence was 100% similar. Sequence similarities between 3' NCR of distinct potyviruses are reported to be 39%-53% and those between virus strains from 83% to 99% (Frenkel *et al.*, 1989).

We concluded that isolate 95/0931 is an isolate of PMV, and confirmed that it is a strain of BYMV based on nucleic and amino acid sequences.

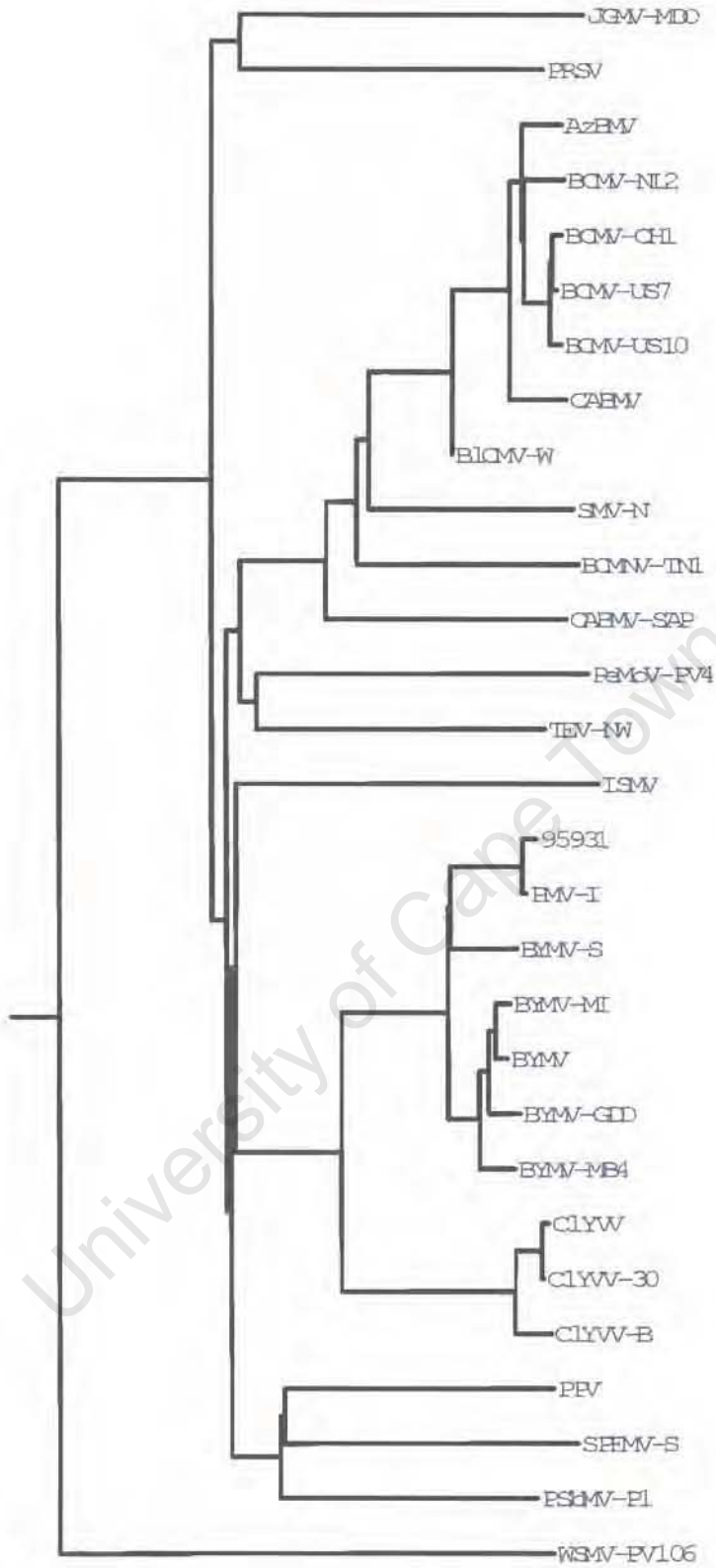

Figure 5.10 Phylogenetic tree of the relationships between nucleic acid sequences of part of the CP gene and the 3' non-coding region of several potyviruses that were aligned using DNAMAN. Horizontal distances are proportional to sequence differences, vertical distances are arbitrary. 95931=virus sequence from this work.

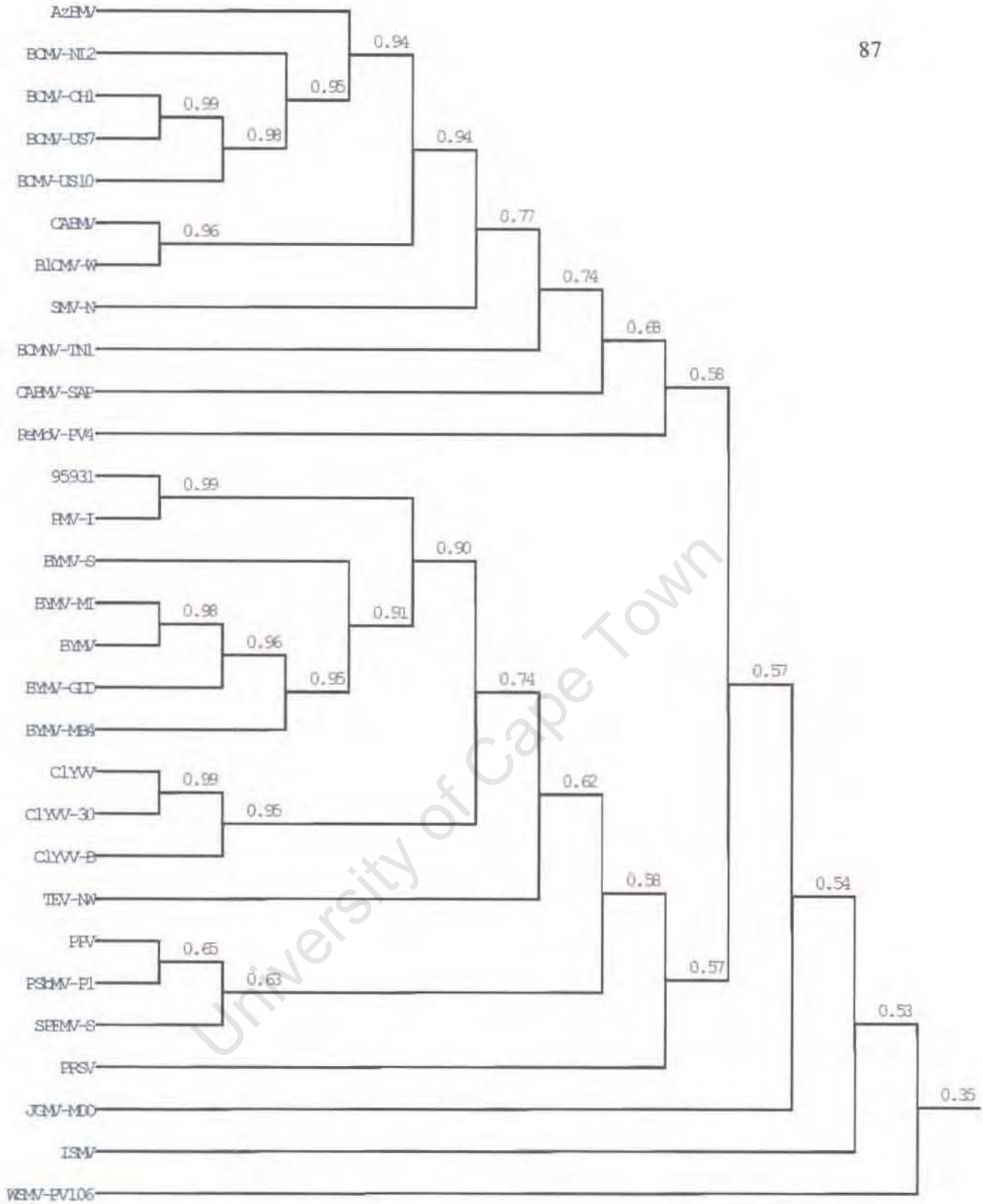

Figure 5.11 Homology tree of the relationships between nucleic acid sequences of part of the CP gene and the 3' non-coding region of several potyviruses that were aligned using DNAMAN. Numbers in red indicate homology, where 1 equals 100% homology. Horizontal distances are not proportional to sequence differences. 95931=virus sequence from this work.

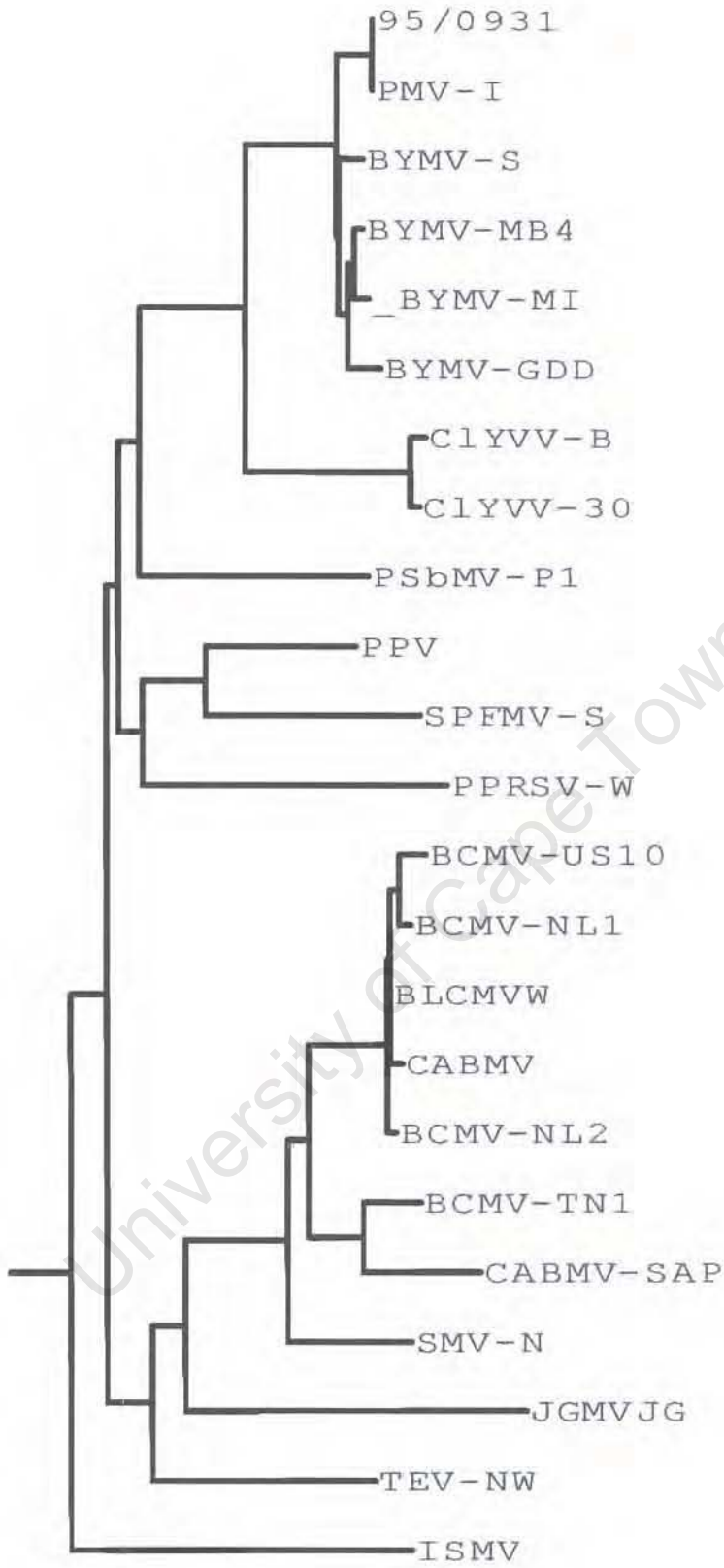

Figure 5.12 Phylogenetic tree calculated using DNAMAN of the relationships of the amino acid sequences of part of the CP of several potyviruses. Branch lengths are proportional to sequence similarities. 95/0931=virus sequence from this work..

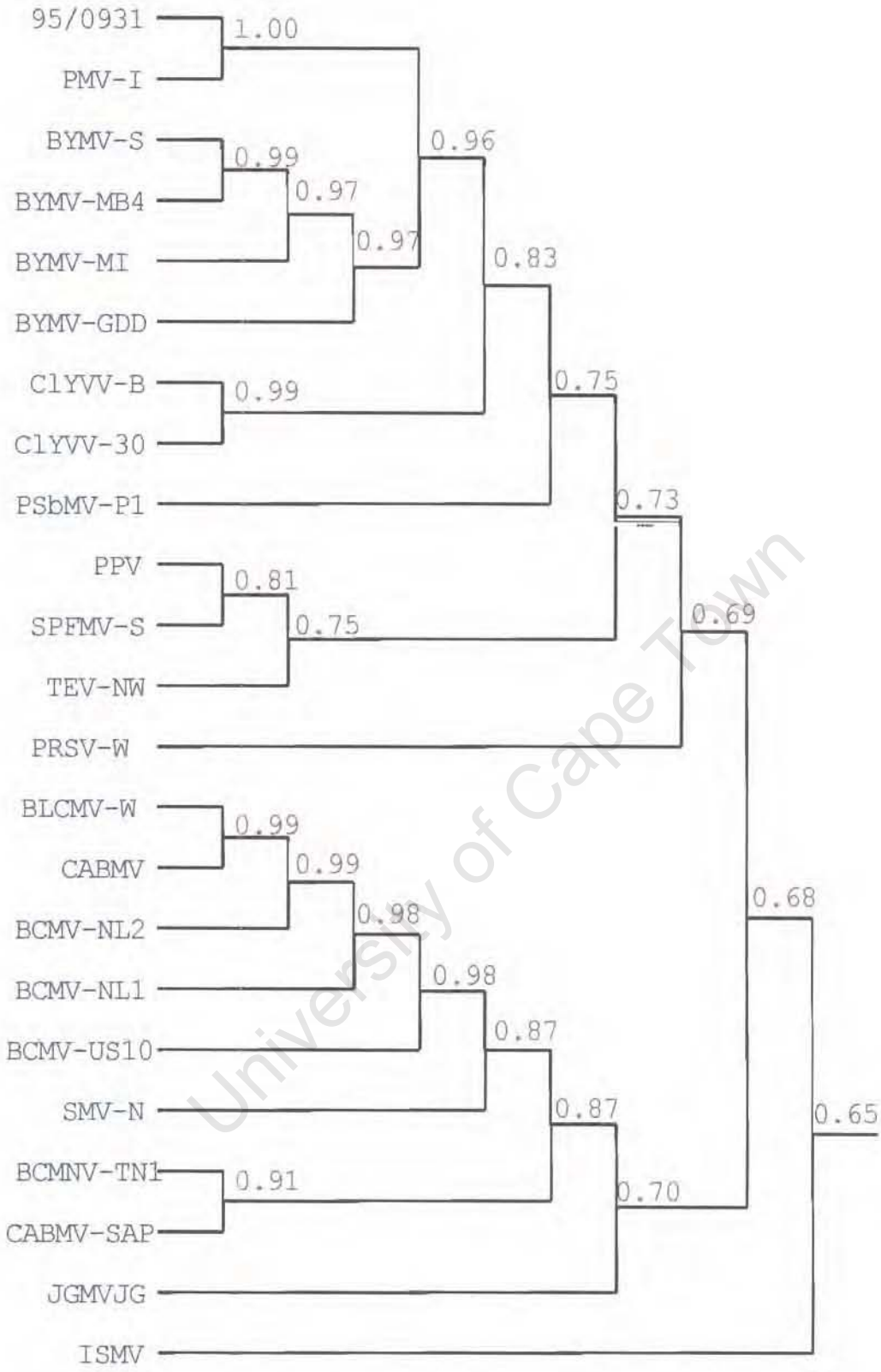

Figure 5.13 Homology tree of the realationships of the amino acid sequences of part of the CP of several potyviruses. Numbers represent homology, with 1 indicating 100% homology. Horizontal distances are not proportional to sequence differences. 95/0931=virus sequence from this work.

CHAPTER 6
CONCLUSION

Three different viruses were isolated from diseased *P. sativum* plants that were collected from different localities in South Africa. These viruses were identified and partly characterised during this study. The three viruses were initially identified as PEMV, BBWV and BYMV using known antisera to various pea viruses. Further characterisation of PEMV and BBWV included host range studies, electron microscopy, serological studies, molecular weight determination, RNA size determination and aphid transmission tests. Confirmation of the identity of BYMV was obtained by sequence analysis of the 3' NCR and of a portion of the coat protein gene. Our isolate shared a 99% sequence similarity with a PMV-I strain previously described (Xiao *et al.*, 1994). The properties determined in this study for the three viruses are summarised in Table 6.1.

Isolate 91/0394 showed similar properties as described for PEMV in the literature (Table 6.2). The same isometric particles were seen with the electron microscope and cytopathological studies showed virus particles in the nuclei of infected plants. The aphid transmissibility of PEMV-91/0394 was not tested on the original sample and further transmission studies were unsuccessful. The probable reason for this being that with repeated mechanical inoculation the larger protein (54 000 Da), which determines aphid transmissibility, is lost (Demler *et al.*, 1991). The size of the capsid protein (22 700 Da) was in the same range as the second largest protein described in the literature (21 000 Da). The host range of our isolate was narrow as expected and all four *P. sativum* cultivars tested were infected. PEMV is known to occur in low concentrations in certain plant species and the severity of symptoms varies according to cultivar and strain of virus. The most important aim of this part of the study was to identify the virus and to produce an ELISA detection method to the virus. The isolate was successfully purified and serological relatedness of our isolate to another PEMV isolate [PEMV-Tü (AS-0017)] was used as the final confirming test.

Table 6.3 summarises the similarities between the properties determined for isolate 94/1969 and BBWV. Isometric particles were seen in the original infected pea plant. ISEM studies with known antiserum revealed that the virus was BBWV and further characterisation of the virus commenced as described in Chapter 4. It was important to determine the serotype of the virus and it was confirmed with ISEM studies that our isolate belongs to serotype II. The RNA size of isolate 94/1969 was in the same range as the published data (Taylor and Stubbs, 1972). *M. persicae* and *M. euphorbiae* transmitted BBWV-94/1969 in a nonpersistant way to healthy *P. sativum* plants. BBWV is known to be transmitted by many aphid species (Lisa and Boccardo, 1996). An ELISA detection method was developed to BBWV-94/1969 and this can be used in future tests.

The identification of the third virus from peas (95/0931) was initially done serologically. Subsequent sequencing of the 3' NCR and a portion of the coat protein gene showed that this isolate is a strain of BYMV, most closely related to PMV. No further characterisation studies of the properties of this virus were done.

Control strategies to minimize the effects of virus infections include quarantine measures, propagation of virus-free plants, use of resistant cultivars, reduction of inoculum, adjustment of cropping practices and the control of vectors. However, control can only be implemented when the disease agent is known. This study has identified three viruses from peas in South Africa, which were hitherto undescribed. ELISA detection systems have since been developed for the identification of PEMV and BBWV and are a valuable addition to the antiserum collection of PPRI. Should outbreaks of any of these viruses occur, the disease agent can be rapidly identified and control strategies implemented.

Virus	Genus	Symptoms on pea	Host range	Cytopathology	Antiserum produced	Capsid protein	RNA size	Aphid transmitted	Sequence analysis
PEMV	*Enamovirus*	Systemic chlorotic vein clearing, chlorotic flecking, downward leafcurl	Narrow	Virus particles observed in nuclei	YES	22 700 Da	-	-	-
BBWV	*Fabavirus*	Severe mosaic	Wide	-	YES	-	6.2 kb, 4.2 kb	*M. persicae*	-
BYMV	*Potyvirus*	Chlorotic vein clearing, mosaic	-	-	NO	-	-	-	Strain of BYMV

Table 6.1 A summary of the properties determined for PEMV, BBWV and BYMV in this study

Properties	Current study	Published data
Particle structure	isometric particles (EM)	isometric particles *
Particle size	-	25-28 nm *
Host range	narrow	narrow ★
Cytopathology	virus particles found in nuclei	virus particles in nuclei, cytoplasm and vacuoles
Capsid protein	22 700 Da	54 000 Da, 21 000 Da, 17 500 Da *
RNA size	-	RNA-1, 5.71 kb; RNA-2, 4.25 kb *
Aphid transmission	-	persistantly by aphids ✿

Table 6.2 Comparison of similarities between properties determined for PEMV in this study and in published data. (–) indicates not determined (*Brunt *et al.*, 1996; ★Peters, 1970; ✿ Hagedorn, 1996).

Properties	Current study	Published data
Particle structure	isometric particles (EM)	isometric *
Particle size	–	25 nm *
Host range	wide	wide ★
Cytopathology	-	virus particles in mesophyll, epidermis and cytoplasm *
Capsid protein	-	42 000 Da, 26 000 Da ✿
RNA size	RNA-1, 6.2 kb; RNA-2, 4.2 kb	RNA-1, 6.3 kb; RNA-2, 4.5 kb ★
Aphid transmission	*M. persicae, M. euphorbiae*	nonpersistently by aphids ♣
Serotype	serotype II	serotype I and II ♦

Table 6.3 Comparison of similarities between properties determined for BBWV in this study and in published data.
(–) indicates not determined (*Brunt *et al.*, 1996; ★Taylor and Stubbs, 1972; ✿ Milne, 1991; ♣ Lisa and Boccardo, 1996; ♦ Uyemoto and Provvidenti, 1974).

APPENDIX A
STANDARD METHODS

APPENDIX A
STANDARD METHODS

Methods given below were used in the studies with the three samples collected. Methods not described here are described in the individual Chapters.

1. Accessing viruses

The three samples obtained through collection or requests for laboratory analysis were accessed according to the method used by the Virology section of the PPRI. The year the sample was collected as well as the number assigned to the sample is represented in the accession number.

2. Storing of samples

It is important to store all virus-infected plant material for future reference. In the event of culture contamination, mutation or loss it is important to have stored material from which a culture can be re-established. For the long-term storage of viruses three methods are mainly used, and are discussed below.

2.1. Desiccation over $CaCl_2$

Two to five leaves of the infected plant material were wrapped in tissue paper and labeled, and placed in a desiccator-vessel containing $CaCl_2$. The samples were removed after one month and placed in polytop bottles. The polytop bottle contained $CaCl_2$, cotton wool, plant material with a layer of cotton wool and $CaCl_2$ on top. The bottles were sealed with wax and stored at 4°C.

2.2. Cryovial preservation at -80 °C

Pieces of the infected plant material were placed in labeled Nunc cryovial tubes and stored at -80 °C.

2.3. Freeze-drying

Infected plant material was freeze-dried according to the method described by Hollings and Lelliot (1960). The plant material was macerated in a solution of 7% glucose and 7% peptone. After pre-freezing the samples, they were freeze-dried with a Virtis freeze-drying apparatus overnight and the glass tubes sealed off under vacuum the next morning.

The stored plant material was deposited in the culture collection of PPRI.

3. Electron microscopy

The electron microscope was used as the first step towards virus diagnosis, using virus morphology as the main criterion

3.1. Negative staining

Negative staining of a leaf dip is a quick method to detect viruses with the electron microscope. Just as certain viruses are more stable in certain buffers during purification procedures, different staining methods and pH ranges need to be used with EM techniques. There are three main stains used in electron microscopy of plant viruses; namely, potassium phosphotungstate (PTA), uranyl acetate (UA), and ammonium molybdate (AM).

Infected plant material was macerated in 0.1M phosphate buffer, pH 7.1, and examined for the presence of virus particles by negative staining with 2% potassium phosphotungstate (PTA), pH 7, aqueous uranyl acetate (UA), pH 4.3, or 2% ammonium molybdate (AM), pH 5.3, using a ABT-ISI 002A transmission electron microscope. Electron micrographs were taken of the purified virus preparations.

3.2. Ultrathin sections

Virus-infected leaves were double-fixed in 2.5% glutaraldehyde and 1% osmium tetroxide, block stained with uranyl acetate, dehydrated in graded acetone series and embedded in Epson-Araldite resin (Goszczynski *et al.*, 1996). Ultrathin sections were made with a Reichert-Jung Ultracut-E microtome and post stained with uranyl acetate

and lead citrate (Reynolds, 1963) and viewed with the transmission electron microscope (JEOL JEM-100C).

3.3. Immunosorbent electron microscopy (ISEM)

Immune electron microscope techniques combine the techniques of electron microscopy and serology, involving the detection of complexes of antigen (virus) using a specific antibody (Hill, 1984). For ISEM, infected plant material was macerated in 0.1M phosphate buffer, pH 7.1, and put onto 400-200 mesh copper grids coated with antiserum for 1 hour at 37°C. The grids were supported with Nicoloidine and evaporated with carbon. The plant material was incubated for 4 hours at 37°C. The grids were placed on antiserum for 1 hour 37°C. Between these steps the grids were washed with buffer. The grids were stained with uranyl acetate and viewed with an ABT-ISI 002A transmission electron microscope.

4. Antiserum production

By injecting purified virus into a rabbit, an immune response is induced that produces antibodies in response to the presence of the foreign molecules in the body. Antibodies can be characterised by their ability to bind both to antigens and to specialised cells or proteins of the immune system (Harlow and Lane, 1988). Antibodies can be visualised as forming an Y shape. IgG molecules have three protein domains. Two of the domains are identical and form the arms of the Y. These contain the antigen binding sites and are known as Fab fragments. The fc fragment is the other segment of the Y structure. (Harlow and Lane, 1988). The region of an antigen that binds to an antibody is called an epitope. Antibodies can recognise small regions of antigens and occasionally they can find similar epitopes on other molecules. This forms the molecular basis for cross-reaction between related viruses. Using the tool of immune response of animals detection methods can be developed to detect plant viruses.

4.1. Immunisation of rabbits

Purified virus was injected into New Zealand white rabbits. Four intramuscular injections were given at weekly intervals. The virus suspension was mixed in a 1:1 ratio

with Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for the last three injections.

4.2. Collection of blood

Blood was collected after the last injection. The blood was allowed to clot at 37°C and then placed at 4°C overnight to obtain serum. The serum was centrifuged at 8000 rpm for 10 min to separate any erythrocytes trapped in the serum. The antiserum was mixed with glycerol in a 1:1 ratio and stored at 4°C.

5. Developing F(ab')$_2$ ELISA (Enzyme-linked immunosorbent assay)

An indirect ELISA using F(ab')$_2$ fragments was developed as described by Barbara and Clark (1982). Virus particles were trapped with F(ab')$_2$ fragments and then detected with whole immunoglobulin (as second antibody) and a conjugate reacting only with the Fc portion of the immunoglobulin.

5.1. Titer determinations

The bleed with the highest specific titer was identified by dilution end point determinations in PAS-ELISA (Protein A Sandwich ELISA) (Edwards and Cooper, 1985).

Protein A is a 42 000 Da polypeptide that is a normal constituent of the cell wall of *Staphylococcus aureus*. Protein A not only binds to antibodies isolated from animals that have been immunised with protein A, but also bind to the antibodies from animals that have never been exposed to this protein. This high affinity for antibodies makes them useful tools in immunological tests (Harlow and Lane, 1988). Protein A has four potential binding sites for antibodies (only two of them can be used simultaneously) and therefore protein A is clearly bifunctional, allowing multimeric complexes to be formed.

The method uses protein A in two applications to sandwich antibody-antigen-antibody layers. The first applied layer of protein A prepares the plate for the coating antibody

layer. The second layer of protein A is conjugated to the enzyme and detects the second antibody layer. (Edwards and Cooper, 1985).

Nunc ELISA plates were coated with Protein A (Sigma) and incubated at 37 °C for 2 hours. The plates were washed three times with PBS-Tween and then blocked with 2% milk powder for 1 hour at 37 °C. Antiserum from a chosen bleed (whose saturation point was determined previously by dilutions in PAS-ELISA) was used to coat the plates. After incubation of 2 hours at 37 °C, the plant material was macerated in extraction buffer (0.02M PBS-Tween, 2% PVP and 0.2% ovalbumin, pH7.4) and placed on the plates for overnight incubation at 4 °C. The same antiserum from the previous antiserum step (with the same dilution) was then placed on the plate. Incubation was for 2 hours at 37 °C and coating with Protein A alkaline phosphatase conjugate followed. Between each step the plates were washed with PBS-Tween. The ELISA was developed with enzyme substrate (1mg/ml ρ-nitrophenyl phosphate in 10% diethanolamine, pH 9.8) (Sigma).

5.2. Purification of immunoglobulins

Immunoglobulins (IgG) were prepared from the bleed with the highest specific reaction determined by PAS-ELISA. Carboxy methyl (CM) Affi-Gel Blue (BioRad Technical Bulletin) columns were used to purify IgG. A mixture of 10 ml Affi-Gel Blue and 200 ml 0.01M KPO_4 was used to pack the column (method performed in PPRI-Virology division). The packed column was washed with a further 50 ml buffer. Antiserum (2 ml) was applied to the equilibrated column. The effluent was collected in marked tubes and the unabsorbed fraction was obtained by monitoring the absorbance of fractions of the eluent at 280 nm. The fractions of the IgG with absorbance values higher than 1.4 were pooled. The concentration of the immunoglobulin fractions was adjusted to 1 mg/ml with 0.01M KPO_4.

5.3. Preparation of F(ab')$_2$ fragments

The preparation of F(ab')$_2$ fragments from immunoglobulins was performed using the pepsin digestion procedure (Campbell and Garvey, 1970). According to this method the IgG molecule is split into subunits by enzyme digestion. Three fragments result from one

IgG molecule (two Fab fractions and the Fc portion). Fragments are separated by elution of the IgG through a carboxymethylcellulose column (PD 10). $F(ab')_2$ is consists of two Fab fragments which are held together by a disulphide bond. Pepsin is used to digest the $F(ab')_2$ fractions. Upon reduction of the $F(ab')_2$ fragments two Fab fragments result. Pepsin split the chain on opposite sides of a disulpide bond at the portion where the two segments of the chain are joined.

A PD-10 column was equilibrated with 100 ml of 0.07M sodium acetate buffer, pH 4.0, containing 0.05M sodium chloride. A volume of 2.5 ml of the 1 mg/ml IgG preparation was allowed to run through the column. A 3.5 ml volume of the sodium acetate buffer was run through the column and the effluent was collected. Pepsin (45 µg/mg) was added to the effluent and incubated overnight at 37°C. The solution was dialysed at 4°C in PBS buffer (0.01M potassium phosphate buffer, pH 7.25, containing 0.15M sodium chloride and 0.02% sodium azide).

5.4. Optimising the $F(ab')_2$ ELISA

The optimal concentrations of the seroreagents of the $F(ab')_2$ ELISA were determined with the titration protocol described by Voller *et al.* (1979). The $F(ab')_2$ fraction used for coating was tested at dilutions of 1:100, 1:1000 and 1:10 000 in coating buffer. Virus-infected plant material was tested at 1:10, 1:100 and 1:1000 and healthy plant material at 1:10 and 1:100. A buffer control was also included. The immunoglobulins (IgG) were tested at dilutions of 1:200, 1:800 and 1:3200. The optimum dilutions of $F(ab')_2$ and IgG were taken as those giving the highest ratio of absorbance value if infected material : absorbance value of healthy material.

6. Absorption of antiserum to healthy plant material

If a reaction against healthy plant material is obtained with antiserum against a specific virus, the antiserum can be absorbed against healthy plant material. The unwanted antibodies not directed against the virus will then absorb to the healthy plant material and be removed from the serum. Equal volumes of antiserum and healthy plant material were mixed. The plant material was ground with a mortar and pestle without adding any

buffer and then expressed through cheesecloth. 0.5 g/100ml sodium chloride was added and incubated for 2 hours at 37°C. The mixture was allowed to cool down and then centrifuged for 30 min at 12 500 rpm in a JA 20 rotor. The pellet was discarded and the supernatant was frozen. The solution was subjected to a low speed centrifugation again and the volume of the supernatant determined. The antiserum was concentrated by adding 40% (w/v) ammonium sulphate and stirred for 2 hours. After low speed centrifugation the pellet was resuspended in distilled water and dialysed against 0.85% sodium chloride. $F(ab')_2$ and IgG were prepared as usual.

7. $F(ab')_2$ ELISA (Enzyme-linked immunosorbent assay)

ELISA plates (Nunc plates) were coated with $F(ab')_2$ fractions diluted in coating buffer (0.05M sodium bicarbonate, pH 9.6). The centre 60 wells of the plate were used. The plates were incubated for 3-4 hours at 37°C and then soaked once and washed three times for three minutes with PBS-Tween (0.02M PBS and 0.5 ml/l Tween 20). Infected and healthy plant material were macerated in a 1:10 ratio in extraction buffer (0.02M PBS-Tween, 2% PVP and 0.2% ovalbumin, pH7.4) and placed on the ELISA plate according to a planned set up. A positive control, negative control and buffer control was included. The plates were incubated overnight at 4 °C and then soaked once and washed five times for three minutes with PBS-Tween. The IgG was diluted in extraction buffer. The plates were incubated three to four hours at 37°C then soaked once and washed three times for three minutes with PBS-Tween. Goat-antirabbit Fc alkaline phosphatase (GAR-fc-AP) was diluted in extraction buffer and placed on the plate. The plates were incubated for three to four hours at 37°C or overnight at 4 °C, then soaked once and washed five times for three minutes with PBS-Tween. ELISAs were developed with substrate tablets (1mg/ml ρ-nitrophenyl phosphate) in substrate buffer (10% diethanolamine, pH 9.8). The plates were incubated for 30 min at 37 °C and the absorbance was measured at 405 nm with the Multiskan MC spectrophotometer.

8. Isolation of viruses

Plant viruses are often isolated from the original infected host plants by two local lesion transfers or with two single aphid transmissions. In this study the local lesion method for

isolating the viruses was used. Sample 94/1969 and sample 95/0931 was isolated by two consecutive local lesion transfers on *Chenopodium quinoa.* After the appearance of chlorotic local lesions on *C. quinoa* one lesion was excised and macerated in approximately 500 µl of 0.01M phosphate buffer, pH 7.1. The lesion was sap-inoculated to a healthy *C. quinoa* plant. On appearance of local lesions the procedure was repeated.

9. Purification of viruses

To obtain information on many biochemical and physical properties and to produce antiserum for serological studies it is necessary to separate the virus particles from the host plant. This process is referred to as purification and the purified virus should be undamaged by the purification procedure and free from plant protein. Procedures for the purification of most known plant viruses are available but different individual viruses may require specifically different treatments at various stages of their purification.

The first stage of the purification process is to extract the virus particles from the host cells. The choice of the buffer used for extraction will depend on the virus being purified and should be optimised. In the case of sample 91/0394 it was observed that 0.5% mercaptoethanol added to the extraction buffer increased virus yield. The mercapto-ethanol prevents virus inactivation by oxidation. The pH of the buffer is also important. For every virus there is a pH at which the particles have no charge (the isoelectric point) and at this pH the particles may precipitate (Walkey, 1991). The next step is to separate the virus from the plant material and to clarify the virus. Subjecting the sap to low speed centrifugation will ensure sedimentation of the larger plant material, but not the virus particles. Solvents such as ethanol, butanol, chloroform, or ether can be used to further clarify the virus suspension. The homogenate of extracted sap and solvent is subjected to another low speed centrifugation. The aqueous phase contains the virus.

Following the separation of the virus from the plant material, the virus is concentrated by ultracentrifigation. To obtain pure virus preparations the virus should be subjected to, for example, density gradient centrifugation. This technique involves high-speed centrifugation in a 10-40% sucrose gradient in a swing out bucket rotor. The virus

particles then separate from other components according to the different sedimentation rates that are determined by the mass and shape of the components. The viral proteins are then visible as a blue band when the gradient is viewed under a white light. This is not always the case and fractions of the gradient have to be collected with an ISCO fractionator and scanned at UV wavelengths.

The purification method for sample 91/0394 was developed from a combination of a method described by Mahmood and Peters (1979) and Harrison (1984). For the purification of sample 94/1969 a combination of purification methods by Xu *et al* (1988) and Makkouk *et al* (1990) were used. These methods are described in Chapter 3 and Chapter 4.

10. Molecular weight determination of capsid protein

SDS-PAGE gel electrophoresis (Laemmli, 1970) was used to determine the molecular weight of the capsid protein of sample 91/0394. Electrophoresis was performed in a LKB 2001 vertical slab apparatus at 4°C. The resolving gel (12% acrylamide) and a stacking gel (4% acrylamide) were cast. Both gels also contained 2.6% bisacrylamide. Samples were mixed in an equal volume of disruption buffer (0.05M Tris-HCL pH 6.8, 9%(v/v) glycerol, 4.3% (v/v) ß-mercaptoethanol, 1.7% (w/v) SDS, and 0.009% bromophenol blue) and boiled for two minutes before loading onto gel. Cold electrophoresis buffer (0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3) was used in the reservoir. The gel was run at constant voltage of 60 V for 20 hours overnight. The gel was stained overnight in 1% Coomassie brilliant blue (in 45% methanol and 10% acetic acid) and destained the next day by repeated changes of 45% methanol and 10% acetic acid. A low molecular weight marker of 94-14.4 kDa (Bio Rad) was used as marker to calculate the CP size.

11. RNA size determination

11.1. RNA extraction

Purified virus was used for RNA extractions. All glassware and buffers were treated with diethyl pyrocarbonate (DEPC, Sigma) and autoclaved to remove possible RNAses. The virus was dissociated by addition of 1:1 (v/v) 200 mM ammonium carbonate buffer, pH 9.0 containing 2.0% SDS and 2 mM EDTA with Proteinase K at ratio of 10 µg enzyme /mg virus. The solution was incubated for 15 min at 20°C. RNA was extracted from the virus suspension using equal volume of a 1:1 (v/v) mix of TE-saturated phenol:chloroform / isopropanol mix. The phenol was prepared by saturating it with TE-buffer (10mM Tris-HCL and 1mM EDTA) and adding 0.1% 8-hydroxyquinoline. The chloroform/isopropanol mix is 96% chloroform and 4% isopropanol. This solution was shaken at room temperature for 10 min. It was centrifuged at 12000 g for 0.5 min and the aqueous phase was collected. The remainder of the organic and interphase was back extracted by the addition of an equal volume of TE buffer. It was mixed well and centrifuged again to collect the aqueous phase and combine it with the first collected sample. The RNA was precipitated by the addition of 1/20 the volume of 3M NaAcetate, pH 5.5. Cold ethanol was added (2.5 volumes) and the sample was stored at -20 °C

11.2. Agarose gel electrophoresis

A Bio-Rad mini-sub DNA cell was used in the electrophoresis. The casting mold was soaked overnight in a 1% SDS and 0.01M EDTA solution. A 1% agarose gel (using RNA-grade agarose) was cast and allowed to polymerise. The electrophoresis buffer used was TBE buffer (Tris-Borate buffer with 0.5M EDTA added, pH 8.0). The RNA precipitate was centrifuged, dried and dissolved in DEPC water. Sample loading buffer (0.25% bromophenol blue and 40% sucrose) was added to the sample and the gel was run at a constant voltage of 60 V for 50 min. The gel was stained in ethidium bromide (0.5µg/ml) and viewed on a UV transilluminator with a wavelength of 325 nm. A 0.24-9.5 kDa RNA marker (Gibco-BRL) was used.

12. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR)

The IC-RT-PCR was used to obtain a PCR product for cloning. The 3' NCR and part of the coat protein gene was amplified by immunocapture RT-PCR using the CN 48 and oligo-dT primers of Pappu *et al.,* 1993. The method as described by Cook *et al.* (1998) was used. PCR tubes (0.6 ml) (Whitehead Scientific, South Africa) were coated with 100 µl universal potyvirus monoclonal antibodies (Agdia, Inc. USA) at a dilution of 1:10 000 diluted in coating buffer (0.05M sodium bicarbonate, pH 9.6). The tubes were incubated for 3 hours at 37°C and washed twice with PBS-Tween (0.01M phosphate buffer, 0.15M NaCl and 0.1% (v/v) Tween 20, pH 7.4). Infected leaf material was macerated in 1:10 (w/v) of extraction buffer (0.02M PBS-Tween, 2% PVP and 0.2% ovalbumin, pH7.4) and microcentrifuged for a few seconds. 100 µl of the supernatant was added to the tubes and incubated at 37°C overnight. The tube was washed twice with PBS-Tween. A reaction mix was prepared to add to the vial that contains the virus template. The reaction mix included 350 µM of each dNTP (Boehringer Mannheim, Germany), 1 µM of each primer, 36 units of HPRI RNAse Inhibitor (Amersham, UK), 10mM DTT, 50mM KCL, 10mM Tris-HCL pH 8.0, 1.76% Triton X-100, 2.5mM $MgCl_2$, 20 units of M-MLV Reverse Transcriptase (Promega, USA), 1 unit of Dynazyme *Taq* polymerase (Finnzymes, Finland). A reaction volume of 600 µl was prepared and 100 µl were added to each tube. 100 µl liquid paraffin was added to each tube to prevent evaporation during PCR. The reverse transcriptase reaction was at 37°C for 45 min. PCR conditions used were: 94 °C for 2 min (1 cycle), and 35 cycles of 94 °C, 1 min; 42 °C, 1 min; 72 °C, 2 min followed by one cycle of elongation at 72 °C for 10 min. The amplification was performed in a 96 well Hybaid thermocycler.

13. Storing bacterial cultures

Bacterial cultures were grown at 37°C overnight by inoculating it in L-Broth (10g bacto-tryptone, 5g yeast extract, 10g NaCl, 950ml H_2O, pH 7.4 adjusted with NaOH, made up to 1 L and autoclaved), containing 100 µg/µl ampicillin. The overnight culture was stored by adding 15% sterile glycerol and freezing at -80°C.

14. Preparation of competent cells

Competent *Escherichia coli* DH5α cells were prepared with the $CaCl_2$ method (Sambrook *et al.*, 1989). An overnight culture of DH5α cells in L-Broth was prepared. The following day 100 ml L-Broth was prewarmed and inoculated with 2 ml of the overnight culture. It was incubated at 37°C with shaking. The spectrophotometer was blanked on L-Broth at 550 nm wavelength and 1 ml of the culture was measured at 30 min intervals until the optical density value was 0.5. The value was reached within 2 hours. The cells were collected by centrifugation of 20 ml of the suspension at 5000 rpm for 5 min at 4°C in a JA 20 rotor. The supernatant was discarded and the cells resuspended in 10 ml ice-cold 50mM $CaCl_2$. The cells were centrifuged again at 5000 rpm for 5 min at 4°C in a JA 20 rotor, the supernatant discarded and the cells resuspended in 1/20 volume if ice-cold $CaCl_2$. The cells were left on ice overnight and a test transformation was done to determine transformation efficiency.

15. Preparation of the vector

15.1 Large scale plasmid extraction

100-300 ml of L-Broth containing the appropriate antibiotic was inoculated with 200 µl of an overnight bacterial culture of DH5∝. The cells were collected (3x35 ml quantities) by low speed centrifugation of 10 000 rpm for 5 min in a JA20 rotor. The pellets from the 3 tubes were pooled by suspending it in 8 ml of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCL, pH 8.0 adjusted with HCL and stored at 4°C). The solution was left on ice for 30 min and 16 ml solution II (0.2N NaOH, 1% SDS) was added, mixed gently and kept on ice for 5 min. 12 ml of solution III (3M NaAc, pH4.8) was added and left on ice for 30-60 min. The solution was centrifuged for 10 min at 15 000rpm in a Beckman JA20 rotor, the supernatant was collected, transferred to a glass tube and precipitated with 72 ml 96% ethanol, and left at -20°C for at least 30 min. The precipitate was centrifuged for 10 min at 8000rpm and resuspended in 6 ml 1x TE buffer (Prepared a stock solution of 10x TE buffer;100mM Tris-HCL and 10mM EDTA, pH8.0). 2.5 ml of 7.5M ammonium acetate was added to the precipitate and kept on ice for 30 min. The RNA precipitate was removed by low speed centrifugation at 8000 rpm for 10 min in a JA20 rotor. The cleared supernatant with the DNA was precipitated with

2 volumes of 96% ethanol and then collected by centrifugation at 8000 rpm for 10 min. The pellet was washed with 80% ethanol and spinned down for 5 min. The precipitate was dried and resuspended in 400 µl 1x TE buffer. The plasmid was stored at -20°C.

15.2 CsCl density centrifugation of plasmid DNA

The volume of the plasmid solution was adjusted to 3 ml with 1x TE buffer. A CsCl gradient was prepared by adding 1.078 g/ml CsCl and 300 µl ethidium bromide to the plasmid solution. The solution was centrifuged for 4 hours at 45 000 rpm in a Beckman TY65 rotor. After centrifugation the tube was viewed under UV light and the lower fluorescent band was removed from the gradient and transferred to a small glass tube where it was diluted with 3 volumes of 1x TE buffer. To extract the ethidium bromide from the DNA, 4 ml of n-butanol were added to the extracted band. The solution was mixed and the phases allowed to separate. The butanol phase was removed. The extraction was repeated twice to remove all ethidium bromide from the DNA solution. The lower aqueous phase was transferred to a siliconised corex tube and 2 volumes of ethanol were added and the solution placed on ice for 60 min. It was then centrifuged at 10 000 rpm for 10 min at 4°C in a Beckman JA20 rotor, the pellet then resuspended in 350 µl TE buffer. The solution was transferred to an eppendorf tube and the corex tube rinsed with an additional 100 µl TE buffer and added to sample in eppendorf tube. 50 µl of 3M sodium acetate, pH 7.0 was added and mixed well. To precipitate the DNA, 1 ml ethanol was added and left at -20°C for 30 min. This was centrifuged in a microcentrifuge for 10 min and the pellet washed with 500 µl of 80% ethanol. The pellet was dried after centrifugation and resuspended in 500 µl of TE buffer. The DNA concentration was determined and stored at -20°C.

15.3 Preparation of blunt-ended vector

Removal of the 5'-phosphate groups with alkaline phosphatase is frequently used to suppress self-ligation and circularisation of the plasmid DNA. Calf intestinal alkaline phosphatase (CIP) was used in this study.

Plasmid pBluescript KS^+ was digested with *Sma*I restriction enzyme and dephosporylated according to the method of Sambrook *et al.* (1989). 10-20 µg of closed circular plasmid DNA was digested with a twofold excess of *Sma*I (Boehringer Mannheim) for 1.5 hours at 25°C in buffer A (33mM Tris-acetate, 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, pH 7.9). An aliquot of plasmid was run on an 1% agarose gel to check if digestion was complete. When digestion was complete, the sample was extracted with phenol:chloroform and the DNA was precipitated with 2 volumes of ethanol. The DNA was recovered by centrifugation at 12 000 g for 10 min at 4°C and the pellet dissolved in 90 µl TE buffer, pH 8.3. An aliquot was removed for test transformation and stored at –20°C.

The dephosphorylation reaction was a mix of 10 µl 10x calf intestinal alkaline phospatase dephosphorylation buffer (CIP) (10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris.Cl, pH 8.3), 5 µl of CIP (Promega) (1 unit per 2 pmoles of termini) added to the *Sma*I digested plasmid. The reaction was incubated at 37°C for 15 min after which 2 µl of 0.25M EDTA was added and the reaction heated at 75°C for 10 min to inactivate the CIP. A phenol:chloroform extraction and ethanol precipitation was performed. 0.1 volume of 3M sodium acetate (pH 7.0) was added. The DNA was recovered by microcentrifugation as described previously. The precipitated DNA was redissolved in TE buffer, pH 7.6.

16. Ligation reaction between foreign DNA and plasmid vectors

Ligation of a segment of foreign DNA to a linearised plasmid vector involves the formation of new bonds between phosphate residues located at the 5' termini of double-stranded DNA and 3'-hydroxyl residues. When the plasmid DNA has been dephosphorylated two new phosphodiester bonds can be formed instead of the four new phosphodiester bonds where both strands of the plasmid vector carry 5'-phosphate

residues (Sambrook *et al.*, 1989). The formation of phosphodiester bonds can be catalysed in vitro by two different DNA ligases. *E. coli* DNA ligase and bacteriophage T4 DNA ligase are used where the T4 DNA ligase are used in all cloning purposes because it will join blunt-ended DNA fragments under normal reaction conditions (Sambrook *et al.*, 1989).

PCR products were purified according to the Double GeneClean Kit (*BIO 101 Inc*). The PCR product was transferred to a new tube leaving the oil behind. To GeneClean for the first time 3 volumes of NaI and 15 µl of glassmilk was added to the product and adsorbed for 5 minutes on ice. The solution was centrifuged for 5 seconds and the supernatant was removed. The pellet was washed with 10-50 volumes of ice-cold NEW (NaCl, ethanol, water) WASH. The pellet was resuspended by pipetting, spinned for 5 seconds and the supernatant removed. The pellet was resuspended in 30 µl of ddH_2O and incubated at 45-55°C for 2-3 minutes. The solution was centrifuged for 30 seconds and the eluted DNA was removed carefully without removing the glassmilk. The glassmilk pellet was resuspended in 20 µl ddH_2O, incubated at 45-55°C for 2-3 minutes and centrifuged as previously described. The supernatant was added to the previously eluted DNA.

To make ends flush for blunt-end cloning, 25 µl of the cleaned product was treated at 37°C for 1 hour with 10 units of DNA polymerase I (Amersham International) and 10 units of T4 polynucleotide kinase (Amersham International) in 10x polymerase buffer (0.5Mtris pH7.5, 0.01M $MgCl_2$, 10mM DTT, 0.5 mg/ml BSA, 200µM dNTPs) containing 0.001M ATP. The volume of the reaction was 100 µl. The reaction was stopped by adding 1 µl of 0.5M EDTA pH 8.0.

The PCR product was ligated to the dephosphorylated vector with 5 units of T4 DNA ligase (Amersham International) in ligation buffer (0.066M Tris, 0.005M $MgCl_2$, 1mM DTT, 1mM ATP) in a reaction volume of 15 µl. The mixture was incubated at 22°C for 16 hours, before transforming into competent *E. coli* DH5α cells.

17. Transformation of *E. coli*

100 µl competent cells were added to a glass tube on ice and mixed with 20 µl of the ligated mixture. It was left on ice for 30 min and given a heatshock for 90 s at 42°C. The solution was cooled on ice for 2 min and 1 ml pre-warmed L-Broth was added after which it was incubated for 60 min at 37 °C. The total amount of the transformed solution was plated out on plates containing ampicillin (100 µg/µl) with the addition of 10 µl IPTG (2.4% w/v in water) and 40 µl X-Gal (2% w/v in N,N-dimethylformamide). The plates were incubated at 37 °C for 16 hours.

APPENDIX B

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

VIRUS	STRAIN	DATABASE ACCESSION NUMBER	NUCLEIC ACID COMPARISON	AMINO ACID COMPARISONS
AzBMV	standard	U60100	*	*
BCMV	NL2	L19472	*	*
BCMV	US10	U37072	*	*
BCMV	CH1	L19539	*	
BCMV	US7	L19474	*	*
BlCMV	W	JQ2364		*
BCMNV	TN1	U37076	*	*
BYMV	GDD	D00490	*	*
BYMV	MB4	D28819	*	*
BYMV	S	S77515	*	*
BYMV	MI	X81124	*	*
BYMV	Standard	X53684	*	
CABMV		U72204	*	*
CABMV	SAP	D10053	*	*
ClYVV	Pns129	D00605	*	
ClYVV	B	S77521	*	*
ClYVV	30	D86044/D00605	*	*
ISMV		X75939	*	*
JGMV	MDO	U07217	*	*
PeMoV	PV4	L32959	*	
PMV	I	S71232	*	*
PPV	Standard	X81081	*	*
PRSV	Standard	X67672	*	*
PSbMV	P1	D10453/D01118	*	*
SMV	N	D00507	*	*
SPFMV	S	D38543	*	*
TEV	NW	L38714	*	*
WSMV	PV106	U54569	*	

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