THE CHARACTERISATION OF ORNITHOGALUM MOSAIC VIRUS

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CERTIFICATION OF SUPERVISOR

In terms of paragraph GP 8 in " General rules for the Degree of Doctor of Philosophy (PhD)", I as supervisor of the candidate, Johan Theodorus Burger, certify that I approve of the incorporation in this dissertation of material that has already been submitted or accepted for publication.

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ABBREVIATIONS

A	adenine / adenosine
AI	amorphous inclusion
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
β-gal	beta-galactosidase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bis	N,N'-methylene-bisacrylamide
bp	base pair
BSA	bovine serum albumin
C	cytidine / cytosine
Ci	curie
CI	cytoplasmic inclusion
CIP	calf intestinal alkaline phosphatase
CP	coat protein
cpm	counts per minute
Da	dalton
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytidine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddNTP	dideoxynucleoside triphosphate
ddTTP	dideoxythymidine triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dpm	disintegrations per minute
DTE	dithioerythritol
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
g	gravitational acceleration
G	guanine / guanosine
HC	helper component
HPLC	high performance liquid chromatography
IEB	immunoelectroblotting
IgG	gamma-immunoglobulin
ISEM	immunosorbent electron microscopy
Kb	kilobase
MAb	monoclonal antibody
MOPS	morpholinepropanesulphonic acid
M_r	relative molecular weight
NBT	nitro-blue tetrazolium chloride
NI	nuclear inclusion
OD	optical density
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PBS	phosphate-buffered saline
PEG	polyethylene glycol
poly(A)	polyadenylic acid
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
spp.	species
T	thymine / thymidine

TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

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ABSTRACT

Ornithogalum mosaic virus (OMV) is the most serious pathogen of commercially grown *Ornithogalum* and *Lachenalia* species in South Africa. Although ornithogalum mosaic disease was first reported as early as 1940, attempts to purify or characterise the virus(es) were not successful. The extremely mucilaginous nature of ornithogalum and lachenalia plant extracts severely hampered virus purification from these hosts. No alternative propagation host for OMV is known: a virus purification protocol for systemically infected ornithogalum and lachenalia was therefore developed. This method eliminated the mucilage in leaf extracts by hemicellulase digestion.

Physicochemical characterisation of purified particles suggested that a single virus was present: it had elongated, filamentous particles with a modal length in the range 720-760 nm; a single major coat protein of M_r 30 000, and a single genomic ssRNA of M_r 2.90 x 10^6 daltons. Oligo(dT)-cellulose chromatography confirmed that the genomic RNA was polyadenylated.

Serological interrelatedness of OMV isolates and the serological relationships between OMV and other potyviruses were studied. The three South African OMV isolates were found to belong to one strain. Antiserum to a Dutch OMV isolate reacted with the South African isolates. OMV was found to be serologically related to hyacinth mosaic virus, bean yellow mosaic virus, potato virus Y, watermelon mosaic virus (strains 1 and morocco), zucchini yellow mosaic virus, sugarcane mosaic virus, soybean mosaic virus and passionfruit woodiness virus, but not related to maize dwarf mosaic virus A or -B, watermelon mosaic virus (strain 2), lettuce mosaic virus or wheat streak mosaic virus. An OMV diagnostic test - which combined a simple, single-vial sap extraction method with DAS-ELISA - was developed. OMV could be reliably detected in as little as 0.1 g leaf tissue from sprouting bulbs.

An investigation of the biological properties of OMV confirmed that it was the causal agent for the mosaic disease of *Ornithogalum* and *Lachenalia* spp. in South Africa.

Typical mosaic symptoms could be produced in virus-free ornithogalum and lachenalia plants by mechanical inoculation with sap from infected plants or purified OMV, or by transmission of the virus using the aphid vector *Myzus persicae* (Sulz.). OMV was not sap or aphid transmissible to any of a wide range of possible propagation hosts, or to a variety of ornamental bulbous plants. Viruses infecting some of these ornamentals, were not transmissible to ornithogalum. In a field survey of the regions to which ornithogalum and lachenalia are indigenous, OMV-infected ornithogalum plants were found in some remote uncultivated and undisturbed localities, indicating that OMV is probably an indigenous viral pathogen of ornithogalum in South Africa.

DNA complementary to the 3'-terminal 3684 nucleotides of the OMV genome was cloned and sequenced. The sequence consisted of a single large open reading frame which probably starts upstream of the cloned region. By comparison to other sequenced potyviruses, it was estimated that the clone contained the 3' non-coding (3'-NC) region, the coat protein gene, the large nuclear inclusion protein (NIb) gene, as well as approximately 85% of the small nuclear inclusion protein (NIa) gene. The 3'-NC region of 274 nucleotides showed 38% to 45% similarity to corresponding regions of other potyviruses. The putative CP gene could encode a 253 amino acid coat protein with a calculated M_r of 28 807. Analysis of predicted amino acid sequences of OMV and those of other potyviruses showed similarities of 66% to 77% for the CP, 72% to 73% for the NIb and 63% to 71% for the partial NIa proteins. These data as well as phylogenetic analysis of the CP sequences confirmed that OMV is a typical but taxonomically distinct potyvirus.

The OMV coat protein was expressed in *Escherichia coli* as a first step towards expressing the gene in ornithogalum and lachenalia plants for possible virus resistance. A 1243 bp fragment of a cDNA clone, containing the complete OMV CP-encoding sequence, was subcloned in the pUEX bacterial expression vectors. OMV CP was expressed in *E. coli* as a β-galactosidase fusion protein after an in-frame fusion with the *lacZ* gene of the pUEX2 vector. Expression was temperature-controlled and could be induced by incubation at 42°C for 2 hours. Expressed β-gal::OMV CP fusion protein could be detected in appropriately-transformed *E. coli* colonies by monospecific anti-OMV antibodies in an immuno-colony blot assay. Large quantities of the insoluble

fusion protein was partially purified from sonicated bacterial cells. It was confirmed that the β-gal fusion protein was indeed CP-specific by its positive reaction with a monospecific anti-OMV antibody and a monoclonal anti-potyvirus CP antibody in immunoelectroblot assays. A specific anti-OMV antiserum was prepared in rabbits using the partially-purified fusion protein as antigen. This antiserum was used to specifically detect OMV in immunoelectroblotting assays.

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 HISTORICAL BACKGROUND

The history of virus diseases in ornamental bulbous plants is much older than the history of plant virology itself: infected tulips, with their characteristic symptoms, were regularly captured on the canvases of the Dutch masters in the early seventeenth century, for instance in a still life painting by Ambrosius Bosschaert in 1619. One of the earliest records of a plant virus disease is of flower breaking caused by the potyvirus tulip breaking virus (TBV) in tulips (Arber, 1940).

The aesthetic value of *Ornithogalum* and *Lachenalia* spp. has been appreciated for a long time: both genera were described for the first time in the seventeenth century (Obermeyer, 1978, Duncan, 1988). The horticultural potential of these bulbs prompted the establishment of breeding programmes for both genera at the Vegetable and Ornamental Plant Research Institute (VOPRI), Pretoria.

The first record of what became known as ornithogalum mosaic virus (OMV) was by Nance in 1940 (according to Smith & Brierley, 1944a), who reported a mosaic disease in *Ornithogalum aureum* in Oklahoma, USA. Smith & Brierley (1944a) described the symptoms of OMV in *O. thyrsoides* in Oregon, USA. These authors further reported the mechanical and aphid transmissibility of OMV to ornithogalum seedlings.

The occurrence of OMV in ornithogalum and lachenalia plants, which were imported from South Africa, was reported in the Netherlands by Derks in 1979. Derks and co-workers also described the transmissibility and serological relationships of OMV (Derks & Vink-van den Abeele, 1980, Derks *et al.*, 1983).

After plant improvement programmes for ornithogalum and lachenalia were started at the VOPRI, an investigation of the virus diseases of these plants led to the first report of OMV in South Africa in 1976 (Klesser & Nel, 1976). Up to the time of this study no attempts were made to purify and characterise the virus which was becoming a serious

threat to the success of these programmes. Some of the cultivars which have been commercially released are currently being re-evaluated; partly as a result of the high virus incidence in the bulbs.

1.2 PROJECT OUTLINE

The ultimate goal of this project (like so many other virus projects) was to "solve the virus problem" in ornithogalum and lachenalia. The initial aim was to find a reliable detection method for OMV in ornithogalum and lachenalia hybrids, which were produced in the VOPRI breeding programmes. This required the availability of a regular supply of purified virus for antiserum preparation. The difficulties experienced in purification of the virus led to the thorough physicochemical, serological and biological characterisation of OMV.

The project was expanded to include a comprehensive molecular characterisation of the virus for the following reasons: first, to confirm physicochemical, serological and biological data which indicated that OMV is a definite but distinct potyvirus; and second, to clone and express regions of the OMV genome for future use as a possible means of engineering virus resistance in ornithogalum and lachenalia.

1.3 LITERATURE REVIEW

1.3.1 Introduction

Viruses from at least 14 plant virus taxonomic groups have been found in ornamental bulbous crops. Additionally, many viruses have not been characterised well enough to be classified to any existing group. To illustrate the diversity of these viruses, an overview of the viruses and the bulbous plants they infect is presented. The intention of this is not to provide a complete list of virus pathogens of ornamental bulbs, but to concentrate on viruses currently under investigation around the world and which have been reported on in the literature.

This study describes the characterisation of OMV, a member of the potyvirus group. The general characteristics of this group are therefore reviewed in detail.

1.3.2 Classification of plant viruses

Viruses are classified into 54 families and groups by the International Committee on Taxonomy of Viruses (ICTV, Matthews, 1982). Biological, physicochemical and serological properties, as well as replication strategies are used to divide viruses into vertebrate, invertebrate, plant, fungal and bacterial virus groups (Matthews, 1982). Most viruses infecting ornamental bulbous crops have been classified into the 28 existing plant virus groups which are currently recognised (Matthews, 1982, Table 1.1).

1.3.3 Viruses infecting ornamental bulbous crops

In this study the terms "bulbous" and "bulb" are used in a broad rather than strict sense, and refer to perennial geophytic plants which can be propagated by subterranean buds. These include true bulbs (e.g. *Ornithogalum* and *Lachenalia* spp.), corms (e.g. *Gladiolus* and *Freesia* spp.) and tuberous rootstocks (e.g. *Bulbine* and *Bulbinella* spp.). Throughout this thesis correct capitalization and italic print will be used when full generic names of plants and insects are given, e.g. *Ornithogalum thyrsoides*. When genera are generally referred to, lower-case letters will be used, e.g. lachenalia, gladiolus, narcissus, etc.

1.3.3.1 Bromovirus group

A new bromovirus, melandrium yellow fleck virus, which caused vein clearing and yellow flecks in *Melandrium album* was described by Hollings & Horvath (1981).

1.3.3.2 Broad bean wilt virus group

Broad bean wilt virus, which was previously considered a possible comovirus (Matthews, 1982), was reported to infect narcissi in Japan (Iwaki & Komuro, 1972).

Table 1.1 Some plant virus pathogens of ornamental bulbs

Virus group	Typical member [a]	Ornamental host
Bromovirus group	MYFV	melandrium
Broad bean wilt virus group	BBWV	narcissus
Carlavirus group	CLV, NLV, NeLV, LSV, KV-1, KV-2, GRSV	narcissus, iris, nerine, lily, tulip, alstroemeria, kalanchoe, gladiolus
Caulimovirus group	CERV, DMV	saponaria, dianthus, silene, dahlia
Closterovirus group	IrV-2, TGV	rhizomatous iris, freesia, tulip
Cucumovirus group	CMV	tulip, lily, narcissus, gladiolus, dahlia, nerine, ranunculus
Ilarvirus group	TSV	gladiolus, alstroemeria, dahlia
Nepovirus group	TRSV, ArMV, TBRV, SLRV, ToRSV, RRV	iris, lily, gladiolus, narcissus, nerine, tulip
Potexvirus group	NaMV, NeVX, LVX, TuVX	lily, narcissus, nerine, agapanthus, tulip
Tobamovirus group	TMV	lily, iris, gladiolus
Tobravirus group	TRV	lily, iris, narcissus, gladiolus, alstroemeria, nerine, ranunculus
Tomato spotted wilt virus group	TSWV	alstroemeria, ranunculus
Tombusvirus group	NTNV	narcissus
Potyvirus group	BYMV, IMMV, ISMV, TuMV, IYMV, BIMV, IFMV, IrV-1, BlIMV, TBV, TCBV, WaTV, TTBV, NDV, NLSYV, NYSV, NWSV, OYDV, NeYSV, NeVY, FMV, FSV, AlMV, HMV,TiLV, TiMV, LSSMV, RaMV	gladiolus, freesia, iris, babiana, ixia, sparaxis, tritonia, crocosmia, montbretia, rhizomatous iris, tulip, lily, narcissus, nerine, alstroemeria, tigrida, hyacinth, ranunculus

[a] See Appendix A for virus names.

1.3.3.3 Carlavirus group

The type member of this group, carnation latent virus, was found in *Narcissus tazetta* in Israel (Brunt, 1980). Narcissus latent virus (iris mild yellow virus) infected narcissi, irises and nerines (Brunt, 1980, Hammond *et al.*, 1985, Balasingam *et al.*, 1988). Nerines were also infected by nerine latent virus (hippeastrum latent virus, Balasingam *et al.*, 1988). Lily symptomless virus (LSV) was reported to infect lilies, tulips and alstroemerias (Allen, 1972, Asjes *et al.*, 1973, Lawson, 1981, Phillips & Brunt, 1983). Two strains of kalanchoe latent virus (KV-1 and KV-2) were found in infected *Kalanchoe blossfeldiana* (Hearon, 1985). In the Netherlands a possible carlavirus - tentatively named gladiolus ringspot virus - infected gladioli (Asjes, 1976).

1.3.3.4 Caulimovirus group

Carnation etched ring virus has been purified from *Saponaria vaccaria* (Lawson & Civerolo, 1976), and according to these authors it was previously also purified from *Dianthus* and *Silene* spp. by Fujisawa and co-workers. In France, 30-100% of the commercial dahlia crops - comprising more than 400 cultivars - are infected by dahlia mosaic virus (Wang *et al.*, 1988).

1.3.3.5 Closterovirus group

Lisa (1980) described iris virus 2 (IrV-2) from rhizomatous iris in Italy, which was serologically related to the known closterovirus lilac chlorotic leafspot virus. An unknown closterovirus, which caused severe leaf necrosis in freesias, was described by Foxe & Wilson (1985). Asjes & Segers (1980) reported a possible closterovirus, tulip grey virus, in tulips in the Netherlands.

1.3.3.6 Cucumovirus group

Cucumber mosaic virus (CMV) has been reported in the following ornamental bulbous crops: tulip (Van Slogteren, 1966), lily (Asjes *et al.*, 1974), narcissus (Iwaki & Komuro,

1972), gladiolus (Logan & Zettler, 1985, Stein *et al.*, 1988), dahlia (Wang *et al.*, 1988), nerine (Hakkaart *et al.*, 1975) and ranunculus (Elliot *et al.*, 1988).

1.3.3.7 Ilarvirus group

According to Brunt (1988), tobacco streak virus was reported in gladioli by Vicchi & Bellardi, and Bellardi and co-workers; in alstroemerias by Phillips & Brunt; and in dahlias by Brunt, and by Bellardi and his colleaques.

1.3.3.8 Nepovirus group

Tobacco ringspot virus, the type member of this group, was reported to infect tulip (Asjes,1972b), iris (Asjes, 1979), lily (Allen & Anderson, 1980), gladiolus (Bellardi & Marani, 1985) and narcissus (Brunt, 1980). Arabis mosaic virus infected lily (Asjes *et al.*, 1974), narcissus (Brunt, 1980), gladiolus (Bellardi & Marani, 1985) and nerine (Balasingam *et al.*, 1988). According to Brunt (1988), tomato black ring virus was reported to infect lilies and tulips. The same virus was earlier reported in narcissus (Brunt, 1980). Strawberry latent ringspot was mentioned as a viral pathogen of narcissus (Brunt, 1980) and gladiolus (Brunt, 1988). Both tomato ringspot virus (ToRV) and raspberry ringspot virus infected narcissus (Brunt, 1980). ToRV is also a pathogen of gladiolus (Lawson, 1980).

1.3.3.9 Potexvirus group

Narcissus mosaic virus is known to infect lily (Bellardi *et al.*, 1988), narcissus (Brunt 1980) and nerine (Koenig *et al.*, 1973, Maat, 1976). Maat (1976) also described nerine virus X in *Nerine* spp., while Phillips & Brunt (1980) reported the virus in *Agapanthus praecox*. Other potexviruses reported in ornamental bulbs are lily virus X in lilies (Stone, 1980) and tulip virus X in tulips (Mowat, 1982).

1.3.3.10 Tobamovirus group

Tobacco mosaic virus (TMV) infections in lily (Allen & Anderson, 1980) and iris (Asjes, 1979) were reported. A tobamovirus, presumably TMV, was found infecting gladioli in the Netherlands (Asjes, 1976).

1.3.3.11 Tobravirus group

The type member, tobacco rattle virus, is known to infect the following bulbous crops: lily (Asjes, 1972a), iris (Asjes, 1979), narcissus (Brunt, 1980), gladiolus (Lawson, 1980), alstroemeria (Hakkaart & Versluijs, 1985, Phillips & Brunt, 1986), nerine (Balasingam *et al.*, 1988) and ranunculus (Elliot *et al.*, 1988).

1.3.3.12 Tomato spotted wilt virus group

Tomato spotted wilt virus was detected in alstroemeria plants in the Netherlands by Hakkaart & Versluijs (1985), and in ranunculus by Bond *et al.* (1983).

1.3.3.13 Tombusvirus group

Narcissus tip necrosis virus was detected in plants of 21 narcissus cultivars in the Netherlands and the United Kingdom (Mowat *et al.*, 1976).

1.3.3.14 Potyvirus group

The potyvirus group constitutes the largest and economically most important group of virus pathogens of ornamental bulbous crops, as has also been shown for most other plants of economic importance (Edwardson, 1974b, Tomlinson, 1987, Milne, 1988).

Bean yellow mosaic virus (BYMV) seems to be the most common potyvirus in ornamental bulbous crops: it has been shown to infect gladiolus (Smith & Brierley, 1944b), freesia (Van Koot *et al.*, 1954) and iris (Brierley & Smith, 1948). According to Derks *et al.* (1980), Brierley & Smith showed that BYMV could infect *Babiana, Ixia,*

Sparaxis and *Tritonia* spp. Derks *et al.* (1980) reported the occurrence of BYMV in *Crocosmia, Montbretia* and *Tritonia* spp., and in bulbous irises in the Netherlands.

Iris mild mosaic virus (IMMV), iris severe mosaic virus (ISMV) and BYMV are well known pathogens of bulbous irises and have been characterised on the basis of serology and symptomatology (Derks *et al.*, 1985). According to Derks & Hollinger (1986), a fourth potyvirus, turnip mosaic virus (TuMV), has been isolated from bulbous irises by Inouye & Mitsahata in Japan. Brunt & Atkey (1970) also reported the occurrence of iris yellow mosaic virus in bulbous irises. The following potyviruses were reported in rhizomatous irises: bearded iris mosaic virus (BIMV, Barnett *et al.*, 1971), iris fulva mosaic virus (Barnett & Alper, 1977), iris virus 1 (Lisa, 1980) and beardless iris mosaic virus (Brierley & Smith, 1948). Barnett & Brunt (1975) suggested that the latter might be a strain of BIMV. Barnett (according to Derks & Hollinger, 1986) recently detected IMMV in rhizomatous iris. The occurrence of BYMV and ISMV in rhizomatous iris in the Netherlands was reported by Derks & Hollinger (1986). After partial characterisation of the viruses, these authors suggested that ISMV and BIMV might be synonymous.

Tulip breaking virus was reported to infect tulip and lily (Van Slogteren, 1971, Asjes *et al.*, 1973). Other potyviruses infecting tulip include the following: tulip chlorotic blotch virus (Mowat, 1985), TuMV and "Wa tulip virus" (Hammond & Chastagner, 1988), and tulip tip breaking virus (Asjes & Segers, 1982).

The occurrence of several potyviruses in *Narcissus* spp. was listed by Brunt (1980): narcissus degeneration virus (NDV), narcissus late season yellows virus (NLSYV, previously known as jonquil mild mosaic virus), narcissus yellow stripe, narcissus white streak virus and onion yellow dwarf virus. In their characterisation of NLSYV, Mowat *et al.* (1988) confirmed that NDV is a distinct virus, but mentioned the possibility that the yellow stripe and white streak symptoms might be related to latent infections of NLSYV.

According to Balasingam *et al.* (1988), two potyviruses have been reported in *Nerine* species in England and the Netherlands: a 750 nm filamentous particle known as nerine

yellow stripe, and an unnamed virus with 800 nm particles. These authors confirmed the occurrence of both these viruses in nerines in New Zealand and suggested the name nerine virus Y for the hitherto unnamed virus.

Freesia mosaic and freesia streak viruses are two potyviruses which were reported to infect freesia (Van Koot *et al.*, 1954, Brunt, 1967b). However, based on symptomatology and particle size, Van Dorst (1973) suggested that these two viruses might be identical.

Alstroemeria mosaic virus and another unknown potyvirus infecting alstroemeria were reported (Hakkaart & Versluijs, 1985, Phillips & Brunt, 1986). Other potyvirus or possible potyvirus pathogens of ornamental bulbs are tigrida latent and tigrida mosaic viruses in *Tigrida pavonia* (Brunt, 1967a), *Lilium speciosum* streak mottle virus (Elser & Allen, 1969), hyacinth mosaic virus (HMV) in hyacinths (Asjes *et al.*, 1974), and ranunculus mottle virus in *Ranunculus asiaticus* (Elliot *et al.*, 1988).

1.3.4 General characteristics of the potyvirus group

1.3.4.1 Potyvirus taxonomy

The potyvirus group, the largest and economically most important of the 28 plant virus groups currently recognised (Matthews, 1982), derives its name from potato virus Y (PVY), the type member. The group was established in 1959 and included 14 members (Brandes & Wetter, 1959), but has since grown to contain 175 definite and possible members (Milne, 1988). However, many viruses are considered synonymous (Francki, 1983), some are considered to be strains of distinct potyviruses (e.g. pepper mottle virus (PeMV) a strain of PVY, Shukla *et al.*, 1988a, Frenkel *et al.*, 1989), while others like soybean mosaic virus (SMV) and sugarcane mosaic virus (SCMV) are believed to each consist of more than one virus (Shukla & Ward, 1988, Shukla *et al.*, 1989c).

The three most generally accepted criteria for classification as a potyvirus are: the virus must have the characteristic particle morphology, it must be able to induce the typical cytoplasmic inclusions, and must be transmitted non-persistently by aphids (Hollings & Brunt, 1981a,b). However, certain viruses which do not conform to all these criteria, are

recognised by the ICTV as possible members of the potyvirus group (Matthews, 1982). For example, some potyviruses are transmitted by soil fungi, mites or white flies (Hollings & Brunt, 1981a,b, Francki *et al.*, 1985), while maclura mosaic virus is shorter than the typical potyvirus length of approximately 750 nm (Plese *et al.*, 1979).

The one aspect of potyvirus taxonomy that virologists do agree on, is that it is in an unsatisfactory state: the differentiation of strains and distinct viruses being one of the major problems. To try and resolve this, the following approaches are being used:

Symptomatology and host range

Virus symptomatology and host range have previously been an important criterion for the classification of potyviruses and, although not considered very precise or reliable, these methods are still used for the recognition of strains (Edwardson, 1974b, Hamilton *et al.*, 1981).

Cross-protection

The use of cross-protection in virus taxonomy is based on the principle that infection by one strain of a virus can protect against secondary infection by related strains of the same virus, but not against infection by distinct non-related viruses. This has been shown for strains of BYMV and SCMV (Bos, 1970, Latorre & Flores, 1985). Cross-protection has limited use in taxonomy though; results do not always correlate with those of other taxonomic parameters (Matthews, 1981).

Particle modal length

Most potyviruses have modal lengths around 750 nm. Although many of the reported differences in modal length can be attributed to the composition of the buffers and stains used (Govier & Woods, 1971, Moghal & Francki, 1981), some differences seem real (Moghal & Francki, 1981). These authors did not, however, find a correlation between differences in modal length and serology of BYMV isolates. Thus the taxonomic value of this parameter must be doubted.

Cytoplasmic inclusions

All potyviruses induce characteristic cytoplasmic inclusions (CIs) in infected plant cells (Edwardson, 1974a). Edwardson and co-workers divided potyviruses into four subgroups, based on inclusion morphology (Edwardson, 1974a, Edwardson *et al.*, 1984). Subgroup I contains viruses that produce tubular scroll-like CIs, subgroup II those that form laminated aggregates, members of subgroup III form both scrolls and laminated aggregates, and subgroup IV contains viruses that produce scrolls and short, curved, laminated aggregates. Although the presence of inclusions is considered one of the more important criteria for assigning a virus to the potyvirus group, its use within the group is still contentious. For example, Francki *et al.* (1985), have shown that strains of the same virus (ryegrass mosaic virus and SCMV) can belong to different inclusion body subgroups; similarly, a number of other closely related viruses belonged to different subgroups (e.g. bean common mosaic virus (BCMV) in subgroup I, BYMV in II, and SMV in III), while distinct non-related viruses were assigned to the same subgroup (Francki *et al.*, 1985).

Nucleic acid hybridisation and sequence homology

Nucleic acid hybridisation has been used to identify and compare a number of potyviruses, for example BYMV (Abu-Samah & Randles, 1981, 1983, Barnett *et al.*, 1987) and PVY (Baulcombe & Fernandez-Northcote, 1988). Reddick & Barnett (1983) found this to be a reliable method to distinguish between potyvirus strains, but could only detect minor homologies between BYMV, pea mosaic virus and clover yellow vein virus (ClYVV). The fact that only seven regions of perfect homology - of at least 15 nucleotides in length - could be found when the complete nucleotide sequences of tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV) were compared, led Shukla & Ward (1989b) to suggest that nucleic acid hybridisation does not seem to be useful for establishing hierarchical relationships among potyviruses.

Nucleic acid sequence comparisons, however, might be useful to distinguish between distinct potyviruses and their strains. Frenkel *et al.* (1989) noticed that the nucleotide sequence of the 3' non-coding (3'-NC) region of distinct potyviruses varied considerably, both in length and in sequence, whereas both these were very conserved in strains of the same virus. Comparing the 3' non-coding nucleotide sequences of

watermelon mosaic virus (WMV, strain 2) and several other potyviruses (for which these sequences had been determined, Table 5.1) these authors determined that percent direct sequence similarities between accepted virus strains ranged from 83% to 99%, while similarities between distinct viruses ranged between 39% and 53%.

Viral coat protein

In a study involving 25 strains of 11 distinct potyviruses Shukla & Ward (1988, 1989a,b) recently demonstrated the use of coat protein (CP) amino acid sequence comparisons to differentiate potyviruses and their strains. Amino (N)-terminal regions of the CPs of distinct potyviruses were shown to vary considerably in length and amino acid sequence homology, whereas the carboxyl (C)-terminal three-quarters of these sequences were highly conserved. Strains of the same virus generally had N-termini of the same length and had strong sequence homology throughout the entire CP. Average amino acid sequence homologies of 54% and 95%, for distinct viruses and strains of one virus, respectively, were observed after pairwise comparisons of 17 strains of 8 distinct potyviruses (Shukla & Ward, 1988).

Shukla *et al.* (1988c) demonstrated that the HPLC peptide profiles of the tryptic digests of a number of potyvirus strains differed markedly from the profiles of distinct viruses, making it another useful means of differentiation. This technique requires the use of specialised and expensive equipment, which probably makes it impractical for many workers.

Fauquet *et al.* (1986a,b) showed that the amino acid composition of the coat protein of a virus is apparently characteristic of that particular group. This technique was also used to differentiate members of the potyvirus group (Moghal & Francki, 1976, Gough & Shukla, 1981)

Serology

Ever since the discovery of the antigenic properties of plant viruses, serology has been one of the most practical and most extensively used tools in virus taxonomy (Moghal & Francki, 1976, Hollings & Brunt, 1981b, Van Regenmortel, 1982). However, relationships among distinct members of the group are complex and often in conflict

with biological properties (Hollings and Brunt, 1981b). The many conflicting reports on serological relationships have been attributed to the use of different laboratory techniques and conditions, the use of antisera of different titres, and partial proteolytic degradation of antigens during purification and storage (Francki *et al.*, 1985, Koenig, 1988). Based on the sequence data of several potyviruses, Allison *et al.* (1985a), Shukla & Ward (1988, 1989a,b), Shukla *et al.* (1988d, 1989a,d) showed that the N- and C-terminal regions of potyvirus CPs are surface-located, and that the N-terminus is the immunodominant part of the virion. Shukla *et al.* (1988d) suggested that antibodies directed against these N- and C-terminal regions should be very specific, while antibodies against the conserved core protein region should be a good broad spectrum probe. A method to remove cross-reacting antibodies - directed against contaminating conserved core protein region - from antiserum preparations was developed (Shukla *et al.*, 1989a), and successfully used to resolve some potyvirus taxonomic problems (Shukla *et al.*, 1989b,c).

Monoclonal antibodies (MAbs) have been produced against several potyviruses. Their potential use in potyviral taxonomy is underlined by the fact that some MAbs are extremely specific and react only with their homospecific viruses while others detect most potyviruses (Jordan & Hammond, 1986).

In spite of the use of all these different methods, most of the problems of potyvirus taxonomy are far from being resolved. The appeal by many virologists for the thorough characterisation of new virus isolates before their classification as distinct viruses, must be strongly supported.

1.3.4.2 Particle structure

Potyvirus morphology is well defined: virions are flexuous rods 680-900 nm long and 11-12 nm wide. Few of the reported differences in particle length (e.g. Edwardson, 1974a) seem real and of taxonomic value. It is believed that much of these differences can be attributed to the composition of the virus suspending medium (Govier & Woods, 1971, Moghal & Francki, 1981). For example, Govier & Woods (1971) reported that

pepper veinal mottle virus particles were 750 nm long, and flexuous when extracted in EDTA buffer; and straight and 850 nm long in extraction buffers containing $MgCl_2$.

Although little fine structure is discernable, particles with a narrow central canal have been observed (Hollings & Brunt, 1981a).

Chemical analysis indicated that potyvirus particles contain 5% nucleic acid and 95% protein. Virions consist of one copy of positive-sense single-stranded RNA of M_r 2.9 - 3.5 x 10^6 (Hill & Benner, 1976, Hollings & Brunt, 1981a,b), and approximately 2000 protein subunits of M_r ranging from 28 000 to 40 000 (Shukla & Ward, 1989a). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of dissociated potyvirus coat proteins indicated the presence of a single protein species. However, faster migrating protein bands were observed for several potyviruses (Hiebert & McDonald, 1976, Hiebert *et al.*, 1984a). These are believed to be products of partial proteolytic degradation (Moghal & Francki, 1976). Optical diffraction studies showed a helical arrangement for protein subunits with a pitch of 3.3 for PVY, 3.4 for BYMV, and 3.5 for ClYVV (Varma *et al.*, 1968).

1.3.4.3 Cytopathology

Potyviral infections cause characteristic morphological changes in host cell cytoplasm and sometimes also in nuclei.

Cytoplasmic inclusions

CIs are the best-documented group of inclusions and are induced by all potyviruses (Edwardson, 1974a). In their review on the cytopathology of the potyvirus group, Francki *et al.* (1985) listed more than 10 descriptive terms that have been used for these inclusions, but conclude that the most commonly used term is cylindrical inclusion. Ultrastructural studies of CIs showed that they consist of a central core tubule and 5-15 radially attached plates, which are usually curved (McDonald & Hiebert, 1974).

The exact function of CIs is unknown; however, since they form 48 h after inoculation, it has been suggested that they are involved in the early infection process (Christie &

Edwardson, 1977). CIs develop at the cell periphery in association with the plasmalemma, and with the central tubules over the plasmodesmata (Lawson & Hearon, 1971). During later stages of infection the CIs seem to detach from the cell membranes to form complex inclusions which are scattered throughout the cytoplasm (Francki *et al.*, 1985, Lesemann, 1988).

Analysis of purified CIs by SDS-PAGE showed one protein of M_r 67-70 x 10^3 (Hiebert & McDonald, 1973), which was not antigenically related to the CP of the same virus or to any other host plant protein (Hiebert *et al.*, 1971). Serological relatedness between CIs of different potyviruses have been reported (Purcifull *et al.*, 1973). Although there is no doubt that CIs are virus encoded, similar pinwheel structures have been observed in apparently virus-free carrot tissue (Wilson *et al.*, 1976).

Several other structures, often less conspicuous than CIs , have been reported in association with potyvirus infection (Edwardson, 1974a, Christie & Edwardson, 1977). Potyvirus particles, when aggregated in the cytoplasm, can form bundles which are often associated with pinwheel inclusions (Weintraub *et al.*, 1974). Virus particles have also been found in association with plasmodesmata, (Weintraub *et al.*, 1974, 1976), tonoplasts (Chamberlain *et al.*, 1977), mitochondria (Kitajima & Lovisolo, 1972) and chloroplasts (Kitajima & Costa, 1973). Potyvirus infection also induces increased numbers of vesicles and vacuoles in the cytoplasm (Martelli & Russo, 1976). Some potyviruses, notably members of the fungus-transmitted subgroup like wheat spindle streak mosaic virus, were reported to induce large membraneous bodies in the cytoplasm (Langenberg & Schroeder, 1973). Electron-dense crystalline inclusions are produced in the cytoplasm following infection by BYMV and a few other potyviruses (Weintraub & Ragetli, 1966, Edwardson 1974a).

Amorphous inclusions (AIs) are non-crystalline masses of fine tubules (Edwardson, 1974a), and consist of one protein which is serologically related to potyvirus helper component (HC, De Mejia *et al.*, 1985a,b).

Nuclear inclusions

Nuclear inclusions (NIs) are induced only by certain members of the potyvirus group. Of these, the crystalline inclusions produced by TEV are the best-studied. Analysis of purified TEV NIs showed that they were composed of two distinct polypeptides of M_r 49 and 54 kDa (Knuhtsen *et al.*, 1974) which were not serologically related to CP or CI proteins (Shepard *et al.*, 1974). NIs are virus encoded and were shown to be the products of two distinct virus genes (Dougherty & Hiebert, 1980b,c, Hellmann *et al.*, 1983). The latter authors also reported positive serological reactions of antisera to TEV NIs with the translation products of TVMV and papaya ringspot virus (PRSV).

1.3.4.4 Host range and mode of transmission

Although members of the potyvirus group infect a wide variety of crops, many potyviruses have extremely narrow host ranges (Edwardson, 1974a, Hollings & Brunt, 1981a,b). Potyviruses are economically the most important viral pathogens of many agricultural crops. In a survey of 10 major world regions, 74 out of 102 cited viruses were potyviruses (Milne, 1988). After a survey of viruses infecting field-grown vegetables, Tomlinson (1987) concluded that the five most important viral pathogens of these crops were CMV, TuMV, PVY, lettuce mosaic virus (LMV) and PRSV: i.e. four out of five were potyviruses.

The large majority of potyviruses are aphid transmitted in the non-persistent manner, which is considered by some workers as one of the three criteria for inclusion in the potyvirus group (Hollings & Brunt, 1981a,b). However, at least 10 poty-like viruses are transmitted by other means and have been included as possible potyviruses in the fourth report of the ICTV (Matthews, 1982). Murant *et al.* (1988) divided these viruses into three groups: the wheat streak mosaic virus (WSMV) cluster, which consists of four mite-transmitted members; the barley yellow mosaic (BaYMV) cluster is fungus-transmitted and has 5 members; and sweet potato mild mottle virus which is unique in that it is transmitted by the whitefly *Bemisia tabaci* (Hollings *et al.*, 1976). Shukla *et al.* (1989b) confirmed the taxonomic status of the mite- and white-fly-transmitted viruses as potyviruses when they showed these viruses reacted positively with antibodies to the conserved CP core region of the recognised potyvirus, johnsongrass mosaic virus

(JGMV). Studies on aphid transmission of potyviruses have shown that vector specificity is very low, and as many as 49, 16 and 28 different vector species have been reported for TuMV, PVY and beet mosaic virus, respectively (Kennedy *et al.*, 1962).

1.3.4.5 Serology

Serological techniques are among the most practical and efficient ways to identify and characterise plant viruses; and therefore also among the most widely used in this field. The role that serology plays in potyvirus taxonomy has been discussed earlier, so this section will be limited to a brief review of serological techniques and their applications in potyvirus characterisation.

Precipitin reactions in liquid medium

These tests are well suited to filamentous viruses because reactions form easily detectable flocculant precipitates, compared to the granular precipitates formed by isometric viruses (Koenig, 1988). Tube, slide, and microprecipitin, ring interface tests, and density gradient centrifugation with antiserum are some of the different variations of liquid precipitation tests that exist. In her review on the serology of the filamentous viruses, Koenig (1988) listed the following applications for these precipitin tests: identification and routine detection of viruses, estimation of virus concentrations based on antigen titres, differentiation and classification of viruses using the average serological differentiating indices (SDIs), differentiation of closely related virus isolates in cross-absorption tests, and purification of viruses or antibodies by precipitation in liquid.

Precipitin reactions in gels

Although precipitation reactions in gels have the advantage that they can differentiate virus and contaminating plant protein reactions, the techniques have limited use for potyviruses, because intact filamentous particles do not diffuse readily into the gel media. Some success was achieved when particles were sonicated (Moghal & Francki, 1976), or subjected to chemical degradation using pyrrolidine or SDS (Shepard, 1972, Purcifull & Batchelor, 1977). According to Koenig (1988) the double-diffusion test with depolymerised coat proteins has been used for the routine detection of several

potyviruses. Double-diffusion gel precipitation tests have also been used for the detection and characterisation of virus-specific nonstructural proteins such as helper component (Thornbury & Pirone, 1983) and cylindrical, amorphous, and nuclear inclusions (Hiebert *et al.*, 1984b).

Agglutination tests

Particles such as latex, bentonite, bacteria and red blood cells have been used in agglutination tests to increase the sensitivity of detection (Abu Salih *et al.*, 1968, Chirkov *et al.*, 1984, Fribourg & Nakashima, 1984). Agglutination tests have been used successfully for the detection of a number of potyviruses e.g. PVY (Fribourg & Nakashima, 1984), plum pox virus (PPV, Torrance, 1980), and BYMV, ClYVV and peanut mottle virus (Demski, *et al.*, 1986).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assay (ELISA) is one of the most widely used techniques for virus detection. Advantages such as its sensitivity, the small quantities of reagents needed, relative simplicity of the procedure, and the fact that the test is not dependent on radioactive materials, make ELISA the preferred detection method in many laboratories, especially in smaller regional facilities which are not well equipped.

The double antibody sandwich (DAS) ELISA in particular, is extensively used and many modifications to increase detection have been reported. These include pretreatment of plant extracts, as well as modifications to buffers, detection systems and incubation times and temperatures (Koenig, 1988). Some of these modifications, and the use of DAS-ELISA in potyvirus detection and relationship studies will be discussed in greater detail in Chapter 3 of this thesis.

In indirect ELISA the detecting anti-virus antibody is not enzyme-labelled, but is in turn detected by an enzyme-labelled anti-globulin antibody from a second animal species (Van Regenmortel & Burckard, 1980, Koenig, 1981, Rybicki & Von Wechmar, 1985). This means that they are usually more sensitive and detect a wider range of serological relatives, but are more laborious because of an additional incubation step. The use of antibodies from a second animal species have been eliminated by directly absorbing

virus onto plates (Koenig, 1981, Rybicki & Von Wechmar, 1981) or by coating plates with the $F(ab')_2$ fragments of virus-specific antibodies (Koenig & Paul, 1982a,b). Torrance (1981) described a modification of indirect ELISA using the properties of bovine Clq (a component of complement, with which plates were precoated) to trap antigen-antibody complexes in microtitre wells. The use of indirect ELISA in the determination of serological relationships among potyviruses is described in Section 3.2.6.2.

Immunoblotting

This technique entails the adsorption of antigens to a membrane with high protein-binding capacity (nitrocellulose or nylon) and the subsequent immuno-detection thereof using specific antibodies.

In the dot-immunoblot (DOT-ELISA) antigens are applied to the membrane by suction, using a multiwell filtration apparatus. Membranes may or may not be precoated with antigen-specific antibodies (Banttari & Goodwin, 1985, Berger *et al.*, 1984). This technique is less labour-intensive than conventional ELISA since only antigens need to be applied separately, but has the problem of various types of non-specific reactions (Koenig & Burgermeister, 1986).

In immunoelectroblotting (IEB, Western blotting), denatured virus proteins are separated in SDS-PAGE, electrophoretically transferred to membranes, and then detected by enzyme immuno-assay. The identification of viral antigens by both molecular weight and serological specificity, makes virus detection by IEB more reliable than methods based on antigenic activity only. The fact that denatured potyvirus proteins immobilised on nitrocellulose react very well with antisera to intact viruses has been attributed to renaturation of the viral proteins on the nitrocellulose after SDS was removed (O'Donnell *et al.*, 1982). However, Shukla & Ward (1989a) showed that antiserum preparations raised against intact viruses contain antibodies to the conserved core region of coat proteins. These antibodies are reactive to denatured coat proteins.

Other applications for IEB have been: the small-scale affinity purification of antibodies to viral proteins (Rybicki, 1986, Rybicki *et al.*, 1990), epitope mapping using monoclonal

antibodies (Dougherty *et al.*, 1985a), and the identification of *in vitro* translation products (e.g. De Mejia *et al.*, 1985b) and complementary DNA (cDNA) clones which express potyviral coat protein (Nagel & Hiebert, 1985, Hammond & Hammond, 1989).

Immunofluorescent techniques

Fluorescent dye-labelled antibodies are used for the *in situ* detection of virus proteins in plant cells and tissues. The *in situ* identification of AIs of PeMV and WMV-1 (De Mejia *et al.*, 1985a) and of NIs of BYMV (Chang et al, 1985) has been demonstrated.

Immunosorbent electron microscopy

Immunosorbent electron microscopy (ISEM), often in combination with the decoration test, is regularly used for the detection and identification of viruses. The procedures and application of ISEM have been reviewed in detail by Milne & Lesemann (1984). According to these authors ISEM has the advantage of minimal false-positive reactions, because viral antigen-antibody complexes can also be distinguished by the virus morphology. High serum dilutions mean that small quantities are used; moreover, because of the visualisation of antigen-antibody complexes, antiserum quality need not be very good. Long incubation times are not essential and results can be obtained in relatively short periods.

Contrast can be enhanced dramatically by decorating the trapped virions with virus-specific antisera. The decoration technique enables the rapid and reliable identification of viruses (Milne, 1986). Unfortunately ISEM requires expensive and specialised equipment; it is also labour-intensive, and therefore not suitable for routine detection of viruses. However, at centres where the infrastructure exists, routine testing by ISEM is often used.

Monoclonal antibodies

The advent of MAbs brought about a marriage between classical serology and molecular biology. Monoclonal antibody technology is a fast expanding discipline and has been extensively reviewed (e.g. Sander & Dietzgen, 1984, Van Regenmortel, 1986). From these studies it is clear that MAbs are superior to polyclonal antibodies in many respects. MAbs can, because of their absolute specificity, differentiate between

otherwise indistinguishable virus strains. They can be raised from small quantities of low quality virus preparations, after which selected hybridoma lines can produce large quantities of the desired antibodies. MAbs have been valuable in the epitope mapping of potyviruses (Allison *et al.*, 1985a, Dougherty *et al.*, 1985a), and for the affinity purification of viruses (Sander & Dietzgen, 1984). MAbs to several potyviruses have been produced; including PVY (Gugerli & Fries, 1983), SMV, LMV and MDMV (Hill *et al.*, 1984) and TEV (Dougherty *et al.*, 1985a). Jordan & Hammond (1986) produced MAb (PTY-1), a monoclonal which was reactive against the coat protein of BYMV, as well as with most other potyvirus coat proteins. Such an antibody is potentially a very useful tool for the general diagnosis of potyviruses.

1.3.4.6 Genome organisation and polyprotein processing

Genome structure

The potyviral genome consists of one molecule of single-stranded (ss) RNA of about 10 000 nucleotides (Brakke & Van Pelt, 1970, Hill & Benner, 1976, Hari *et al.*, 1979). A small genome-linked viral protein (VPg) is covalently attached to the 5' terminus of the genome (Hari, 1981, Siaw *et al.*, 1985), while the 3' terminus consists of a poly(A) stretch of between 20 and 140 nucleotides in length (Allison *et al.*, 1985b, 1986, Dougherty *et al.*, 1985b).

Genome organisation

Cell-free *in vitro* translation analyses of several potyviral RNAs have been reported (e.g. Dougherty & Hiebert, 1980a,b,c, Hellmann *et al.*, 1980, Koziel *et al.*, 1980, Vance & Beachy, 1984, Yeh & Gonsalves, 1985). From results of immunoprecipitation reactions with TEV and PeMV RNA translation products, Dougherty & Hiebert (1980c) proposed a potyvirus genetic map; however, conflicting opinions as to the locations of the CP and NI genes existed (Dougherty & Hiebert, 1980c, Hellmann *et al.*, 1980, Vance & Beachy, 1984). The CP gene was subsequently mapped just upstream of the 3'non-coding region, with the two NI genes adjacent to each other, and flanking the CP gene on the 5' side (Allison *et al.*, 1986, Domier *et al.*, 1986). Fig. 1.1 represents the current potyvirus genetic map as proposed by Dougherty & Carrington (1988).

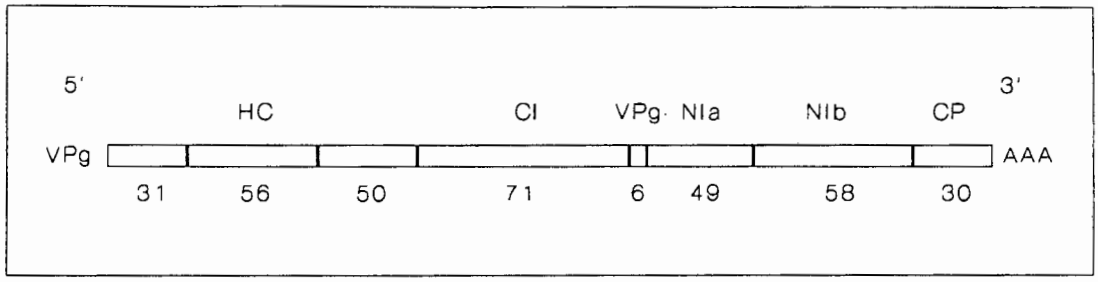

Fig. 1.1. The genetic map of tobacco etch virus (Dougherty & Carrington, 1988).

The genome of sequenced potyviruses has a single large open reading frame (ORF) which theoretically translates into an approximately 350 kDa polyprotein (Allison *et al.*, 1986, Domier *et al.*, 1986). However, cell-free translation of TEV and TVMV RNAs yielded proteins of 87 and 75 kDa respectively, which were coded for by sequences at the 5' termini of the respective genomes (Dougherty & Hiebert, 1980c, Hellmann *et al.*, 1980). These two proteins are subsequently processed to mature proteins of 31 and 56 kDa, and 28 and 53 kDa, respectively (Thornbury *et al.*, 1985, De Mejia *et al.*, 1985b). The 5'-terminal 31 kDa protein showed sequence similarity with the 30 kDa movement protein of TMV, which may indicate a role in cell-to-cell movement for this protein (Domier *et al.*, 1987).

The next gene (see Fig. 1.1) codes for the 56 kDa amorphous inclusion protein (Allison *et al.*, 1986). Co-migration in SDS-PAGE and serological similarity indicate that this protein is the same as the potyvirus HC (De Mejia *et al.*, 1985a,b). HC is a virus-encoded multifunctional protein: it is essential for aphid transmission (Thornbury *et al.*, 1985, Berger & Pirone, 1986), and was recently shown to contain a proteolytically active domain (HC-Pro) on the carboxyl-terminal half of the protein (Carrington *et al.*, 1989a).

The gene 3' to the 56 kDa locus encodes a protein of 50 kDa for TEV and 42 kDa for TVMV (Dougherty & Hiebert, 1980c, Domier *et al.*, 1987). This protein has never been observed in any potyvirus infected plants and its function is not known. However, based on amino acid sequence similarity, a proteolytic function analogous to that of the P2A proteinase of poliovirus has been suggested (Toyoda *et al.*, 1986, Domier *et al.*, 1987).

The next gene on the potyvirus genome encodes the 70 kDa CI protein (Dougherty & Hiebert, 1980b). No definite function has yet been attributed to this protein, but based on amino acid sequence similarity to the picornavirus P2C protein, a role in replication was suggested (Domier *et al.*, 1987). The facts that virions were found to be associated with CIs (Langenberg, 1986) and that these inclusions appear to be associated with the plasmodesmata (Lawson & Hearon, 1971) led Langenberg (1986) to suggest a possible role in cell-to-cell movement.

The sequence that codes for the genome-linked VPg is located 3' to the CI locus (Allison *et al.*, 1986). A potential 6 kDa protein could be translated from this gene, which has cleavage sites 58 amino acids apart on the polyprotein (Carrington *et al.*, 1988). A potential role in virus replication has been proposed for the VPg (Baron & Baltimore, 1982, Vartapetian *et al.*, 1984).

The next two genes encode the "nuclear inclusion proteins" in all potyviruses, although such inclusions are induced by certain potyviruses only (Dougherty & Hiebert, 1980b). The small nuclear inclusion (NIa) protein, which in the case of TEV has an M_r of 49 kDa, has been shown to be the proteinase responsible for at least five post-translational cleavages, all in the 3'-terminal two-thirds of the polyprotein sequence (Carrington & Dougherty, 1987a,b).

The gene that codes for the large nuclear inclusion (NIb) protein is located 3' of the NIa sequence (Allison *et al.*, 1986, Domier *et al.*, 1986). Both TEV and TVMV NIbs, with molecular weights of 58 and 56 kDa respectively, share amino acid sequence similarity with RNA-dependent RNA polymerases of other virus groups (Argos *et al.*, 1984, Domier *et al.*, 1987).

The potyvirus CP gene is located at the 3' terminus of the genome. For TEV, this structural protein has an M_r of 29 622 Da (Allison *et al.*, 1986). Nucleotide and/or amino acid sequences have been determined for several potyvirus CPs (see Table 5.1).

Polyprotein processing

The potyvirus polyprotein of approximately 350 kDa is post-translationally cleaved by at least two virus-encoded proteinases to yield eight mature proteins (Dougherty & Carrington, 1988).

The proteinase responsible for at least five of these processing events is the NIa protein (Carrington & Dougherty, 1987a, Hellmann *et al.*, 1988). Carrington & Dougherty (1987a) demonstrated that the TEV-NIa had proteolytic activity which could be inhibited by immunoprecipitation with antiserum to the 49 kDa protein. A site-directed mutagenesis-induced change in this protein also abolished proteolytic activity (Carrington & Dougherty, 1987b). Garcia *et al.* (1989) showed that the PPV NIa proteinase recognised an artificial NIb-CP cleavage site in an *in vivo Escherichia coli* expression system. Potyviral NIa proteinases showed significant amino acid sequence similarity with the 3C proteinases of several picornavirus and the 24 kDa proteinase from cowpea mosaic virus (Allison *et al.*, 1986, Domier *et al.*, 1987). Bazan & Fletterick (1988) suggested that the proteinases of these viruses originated from the trypsin-like serine proteinases.

The proteolytic active domain of the potyviral 49 kDa NIa proteinases is highly conserved and has been mapped to within a C-terminal 27 kDa segment of the protein (Dougherty & Carrington, 1988). A conserved recognition sequence of ExxYxQ/G or S for the 49 kDa proteinase has been determined, following comparisons of the amino acid sequences of several potyviruses (Dougherty *et al.*, 1989a). These authors demonstrated that substitutions of the conserved E, Y and Q residues reduced or completely inhibited cleavage. Substitutions at the other positions had no effect.

The helper component proteinase (HC-Pro, 56 kDa) is the second proteinase believed to be involved in polyprotein processing. This proteinase, which showed similarity to the trypsin-like cysteine proteinases (Oh & Carrington, 1989), appears to be involved in only one proteolytic reaction (Carrington *et al.*, 1989a,b). Site-directed mutagenesis of the TEV HC-Pro revealed that the proteolytic active domain is located on a 20 kDa carboxyl-terminal segment of the 87 kDa precursor protein (Carrington *et al.*, 1989a,b). HC-Pro cleaves between a G/G dipeptide, but no definite conserved recognition

sequence - as was shown for the 49 kDa proteinase - could be identified for this proteinase (Oh & Carrington, 1989).

A diagrammatic representation of the polyprotein processing of TEV as proposed by Carrington *et al.* (1989a) is shown in Fig. 1.2. *In vitro* studies of the 49 kDa and HC proteinases showed that the polyprotein is autocatalytically cleaved at four positions to yield three polyprotein precursor proteins, of 87, 121 and 88 kDa, and two mature proteins of 6 kDa (VPg) and 49 kDa (NIa proteinase). The 121 and 88 kDa precursors are further cleaved *in trans* by the 49 kDa proteinase, and the 87 kDa precursor in an unknown manner (Carrington *et al.*, 1989a).

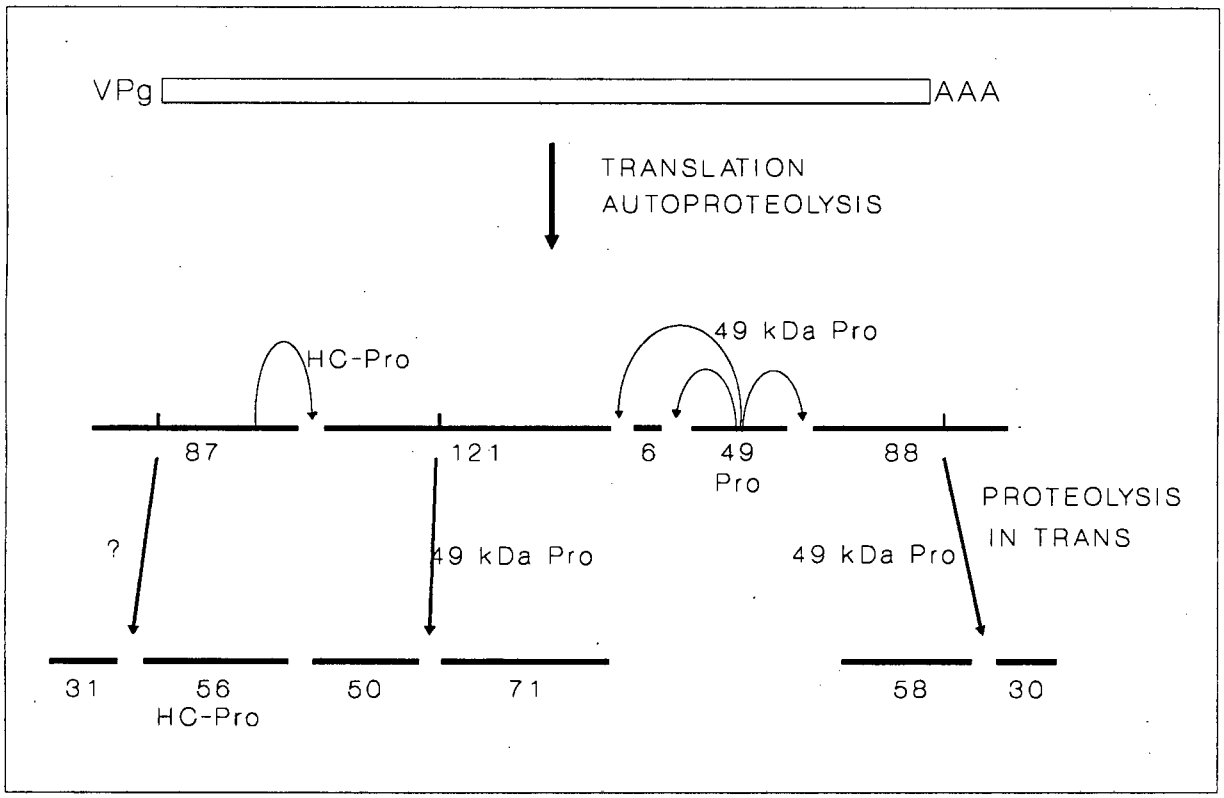

Fig. 1.2. Polyprotein processing of tobacco etch virus (adapted from Carrington *et al.*, 1989a).

1.3.5 Ornithogalum mosaic virus hosts

1.3.5.1 Ornithogalum

According to Obermeyer (1978), the oldest description of a South African *Ornithogalum* species is that of Clusius in 1611, who wrote in the Curae Posteriores: "Bulbs of the genus *Ornithogalum* were brought to Amsterdam by Dutch sailors, who had collected them where they grew, in some bay situated to the west of that extreme and celebrated Promontory of Aethiopia commonly called the Cape of Good Hope, where they had rested for some days to refresh."

The genus *Ornithogalum* belongs to the family Hyacinthaceae of the order Asparagales (Dahlgren & Clifford, 1982) and consists of approximately 245 species in Africa, Europe and western Asia: 120 of these are indigenous to South Africa (Ferreira & Hancke, 1986). In South Africa, where ornithogalum is commonly known as the chinkerinchee, the greatest concentration of species is found in the south-western Cape (Fig. 1.3) in habitats that vary from arid, rocky areas with great extremes in temperature, to sandy flats, marshes, river banks and lower mountain slopes. The genus includes several horticulturally important species and a few that are extremely poisonous to livestock (Watt & Breyer-Brandwijk, 1962).

Ornithogalum has excellent potential as an ornamental cut flower crop: it is resistant to extreme temperatures; longevity of flowers after cutting is exceptionally high (both in cold storage before distribution, and in the vase); minimal pigment change occur in flowers after cutting; flower stalks are long and sturdy; flowering periods can be manipulated, and plants are easily and rapidly vegetatively propagated.

During the 1960's, a breeding programme with *Ornithogalum* spp. was undertaken at Stellenbosch University by Pienaar and co-workers (Pienaar, 1963, Pienaar & Roos, 1966, Pienaar & Van Niekerk, 1968). The breeding programme has been continued at the VOPRI since 1974, using mainly *O. thyrsoides* Jacq., *O. conicum* Jacq., *O. dubium* Houtt., *O. maculatum* Jacq. and *O. pruinosum* Leighton. To date, the VOPRI has released seven cultivars for commercial cultivation (Fig. 1.4).

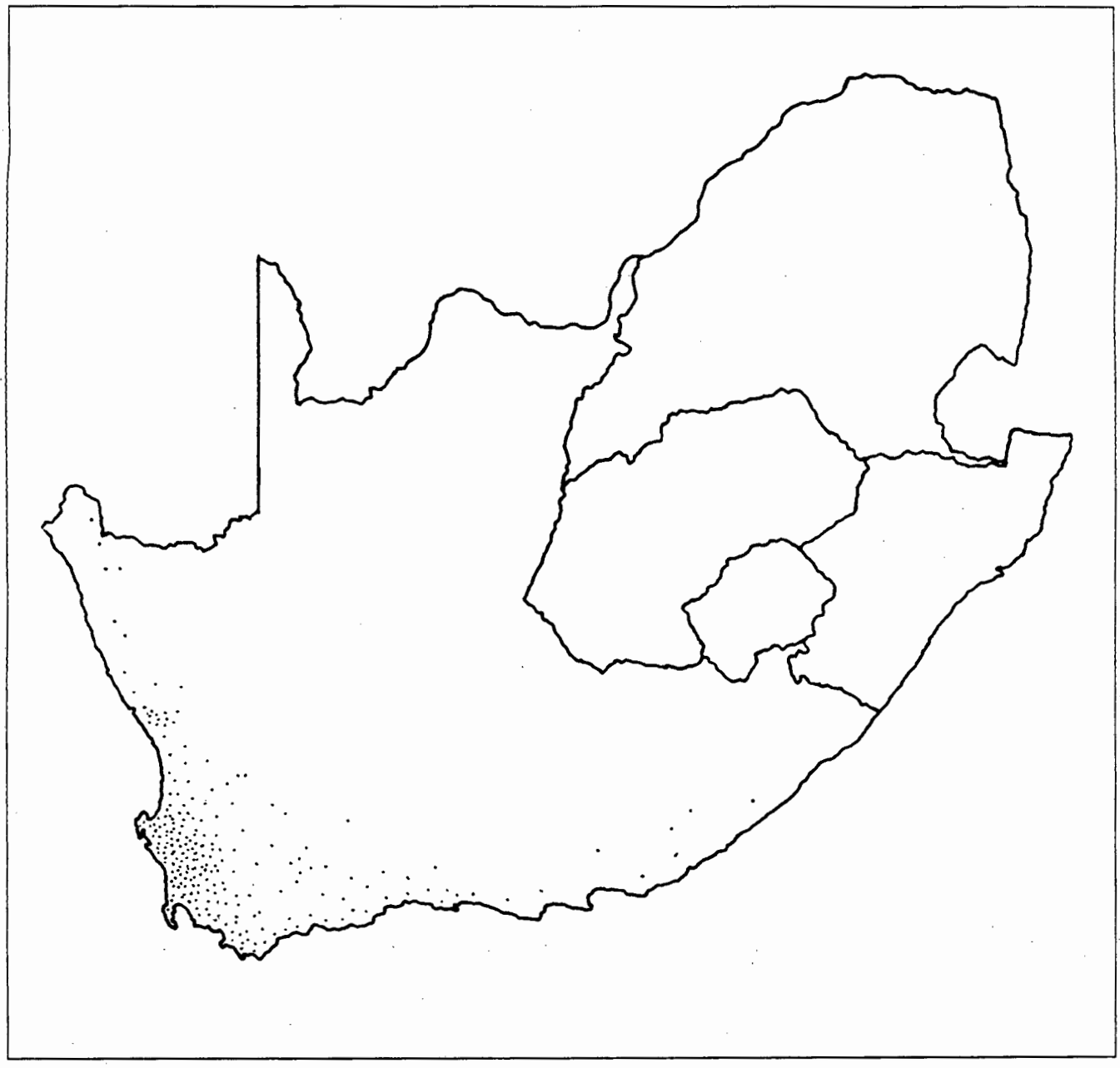

Fig. 1.3. Map of the areas where most South African *Ornithogalum* spp. grow naturally.

Fig. 1.4. An ornithogalum cultivar produced in the VOPRI breeding program (Photograph: A. Coertze, VOPRI).

1.3.5.2 Lachenalia

The earliest record of the genus *Lachenalia* is a sketch of *L. hirta* from 1685. The genus was described, and named after Werner de Lachenal, a Swiss botanist, in 1787. Ninety definite and approximately 20 possible species in five subgenera are currently recognised (Duncan, 1988). Like *Ornithogalum*, the genus *Lachenalia* belongs to the family Hyacinthaceae (Dahlgren & Clifford, 1982) and most species are endemic to South Africa, with the largest concentration of species in the arid regions of south-western Cape (Fig. 1.5). The typical habitat of lachenalia is sandy or rocky locations.

Breeding and cultivation of this genus started in Europe nearly two decades ago, and (according to Moore, 1905) the first hybrid was made by a Rev. John Nelson in 1878. Since then different breeding programmes yielded several hybrids (Hancke, personal communication), but until recently only one cultivar (*L. aloides* cv. "Pearsonii"), which was a New Zealand hybrid, was commercially available (Lubbinge, 1980).

Lachenalia has exceptional potential as an ornamental pot plant: it flowers in winter when other flowering ornamentals are scarce; it is not sensitive to extreme temperatures; it has a long flowering period (from April to December); longevity of individual plants and flowers is good; flowering periods can be manipulated; plants are easily vegetatively propagated, and there is great genetic variety within the genus (Hancke, personal communication).

A lachenalia breeding programme was initiated at the VOPRI in 1966. *Lachenalia* spp. used in this programme were: *L. aloides* (L.f) Engl., *L. orchioides* (L.) Ait., *L. splendida* Diels., *L. viridiflora* W. F. Baker, *L. bulbifera* (Cyrillo) Engl., *L. reflexa* Thunb. and *L. mutabilis* Sweet (Hancke, personal communication). Currently the gene pool consists of 40 species. To date 51 hybrids were selected as potential cultivars; 12 of these were released by the VOPRI as commercial cultivars in 1988 (Fig. 1.6).

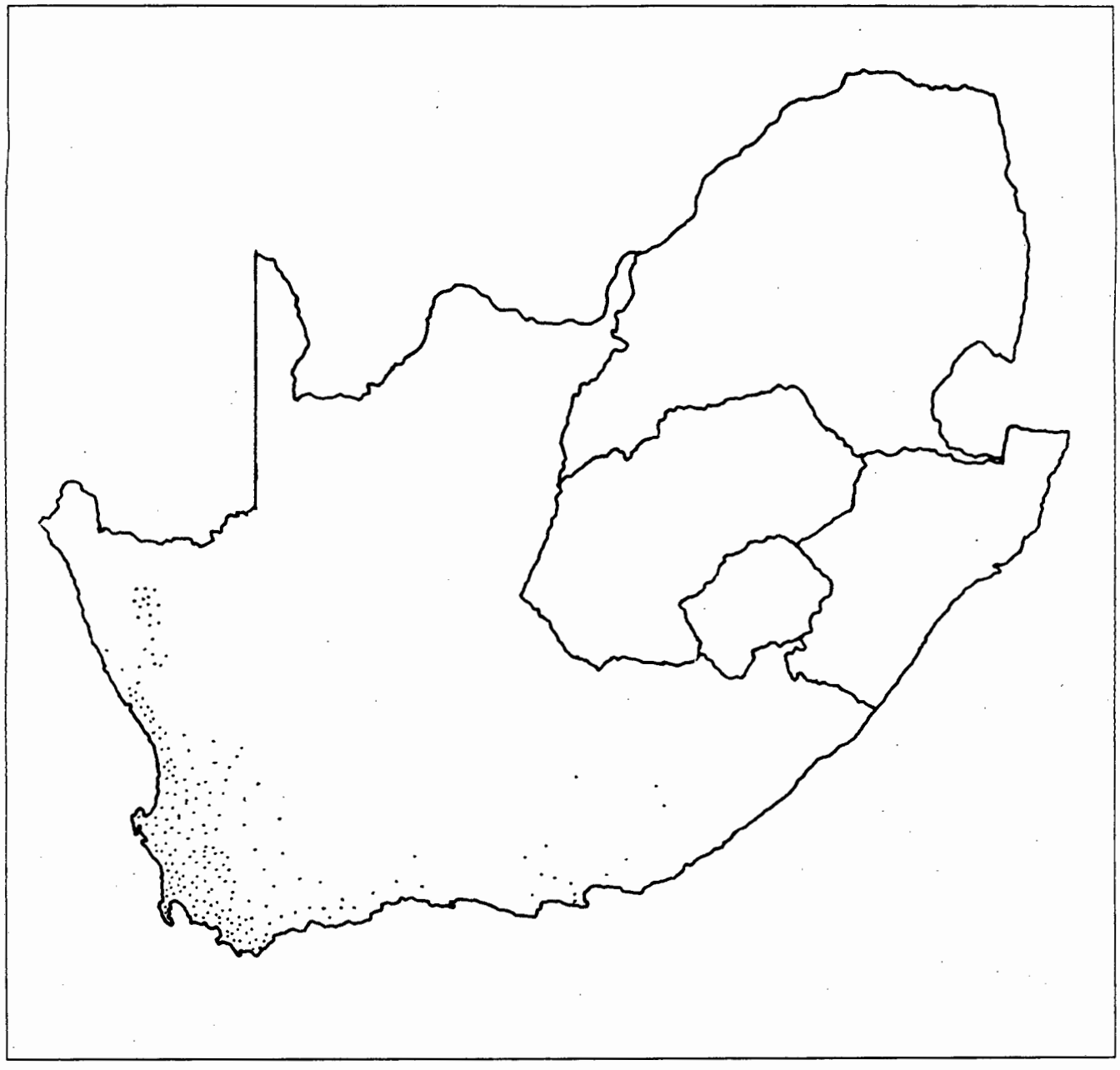

Fig. 1.5. Map of the areas where *Lachenalia* spp. grow naturally.

Fig. 1.6. A lachenalia cultivar from the VOPRI breeding programme (Photograph: A. Coertze, VOPRI).

CHAPTER 2

THE PURIFICATION AND PHYSICOCHEMICAL CHARACTERISATION OF ORNITHOGALUM MOSAIC VIRUS

THE PURIFICATION AND PHYSICOCHEMICAL CHARACTERISATION OF ORNITHOGALUM MOSAIC VIRUS

SUMMARY

Although the first report of a viral disease of *Ornithogalum* and *Lachenalia* species was as early as 1940, all attempts to purify OMV or possible other viruses and to determine physicochemical properties, were unsuccessful, probably due to the extremely mucilaginous nature of ornithogalum and lachenalia sap. Since no alternative propagation host was available, a virus purification protocol was developed for systemically infected ornithogalum and lachenalia. In this method, the mucilage was eliminated by hemicellulase digestion, the virus was precipitated with PEG/NaCl, and concentrated by ultracentrifugation. The virus was further purified by rate zonal centrifugation in sucrose gradients. Physicochemical characterisation of purified particles strongly suggested that a single virus was present: it had elongated, filamentous particles with a modal length in the range 720-760 nm; a single major coat protein of M_r 30 000, and a single genomic ssRNA of M_r of 2.90×10^6 daltons. Oligo(dT)-cellulose chromatography confirmed that the genomic RNA was polyadenylated. These characteristics are typical of the potyvirus taxonomic group.

2.1 INTRODUCTION

In their pioneering work on OMV, Smith & Brierley (1944a) reported the occurrence of the virus in the USA, and its mechanical and aphid transmissibility. The low efficiency of mechanical transmission of OMV - probably due to the mucilaginous nature of ornithogalum plant sap - prevented these authors from characterising the virus. Subsequent publications on OMV reported its occurrence, symptomatology and transmissibility (Klesser & Nel, 1976, Derks, 1979, Derks *et al.*, 1983), and its serological relatedness to HMV (Derks & Vink-van den Abeele, 1980).

The physicochemical characterisation of OMV has never been successfully attempted. Probable reasons for this are that there was no reliable purification method from ornithogalum or lachenalia plants, and no alternative propagation host was known from which the virus could be more easily purified. Results of OMV transmission experiments were inconclusive and failed to produce a suitable alternative host for OMV purification (Smith & Brierley, 1944a, Derks & Vink-van den Abeele, 1980). Purification from ornithogalum and lachenalia was hindered by the extremely mucilaginous nature of their sap: the inhibitory effects of mucilaginous substances in serological assays and on virus purification have previously been reported (Huttinga, 1975, Beijersbergen & Van der Hulst, 1980, Rodoni & Moran, 1988). The use of enzymes was found to reduce the inhibitory effects of lily bulb extracts in ELISA (Beijersbergen & Van der Hulst, 1980). However, enzymatic digestion did not reduce the viscosity of leek (*Allium porrum*) leaf extracts in the purification of leek yellow stripe virus (Huttinga, 1975).

This chapter reports the development of an effective and reliable OMV purification protocol, which employed hemicellulase to liquefy the mucilaginous substances in ornithogalum and lachenalia plant sap. It further reports the determination of physicochemical properties of the virus, and its probable taxonomic assignment. The work described in this chapter was previously published (Burger & Von Wechmar, 1989).

2.2 MATERIALS AND METHODS

2.2.1 Virus sources

Preliminary work with cultivated ornithogalum and lachenalia hybrids indicated that the two OMV isolates purified from them - designated OMV-O and OMV-L respectively - were very similar to one another, and were serologically identical (Section 3.3.1). During a survey in the southern and western Cape regions of South Africa, where most *Ornithogalum* and *Lachenalia* species are indigenous, several ornithogalum plants with typical mosaic symptoms were collected (see Fig. 4.1). A virus designated OMV-W, which was morphologically similar to OMV-O and OMV-L, was isolated from these plants. The OMV-W isolate differed serologically slightly from OMV-O and OMV-L (Fig 3.2). However, differences did not warrant "strain" status: it was therefore decided to characterise the OMV-O isolate only.

Virus isolates OMV-O and OMV-L were originally purified from ornithogalum and lachenalia hybrids, obtained from the VOPRI. OMV-W were purified from ornithogalum plants growing in the wild on the slopes of Table Mountain, Cape Town. OMV isolates were maintained in, and purified from systemically infected ornithogalum and lachenalia hybrids.

2.2.2 Plant sources

The origin and maintenance of OMV-infected ornithogalum and lachenalia plants are described in Appendix B.1.1. Virus-free callus tissue of ornithogalum and lachenalia hybrid lines, which are used in the plant improvement programmes at the VOPRI, was used to regenerate virus-free ornithogalum and lachenalia (Appendix B.1.2).

2.2.3 Virus Purification

Several preliminary purification attempts, using a variety of conventional methods, failed due to the mucilage produced by both ornithogalum and lachenalia plants. Mechanical homogenisations using a mortar and pestle, a commercial coffee grinder, a

Waring blender, and a Pollaehne roller press (Sew-Eurodrive, West Germany) were done, with acetate, borate, phosphate and Tris buffer systems at different buffer to leaf mass ratios. Buffer additives such as 2-mercaptoethanol, Na_2SO_3, thioglycollic acid, DTT and PVP M_r 44 000, the detergent Triton X-100, and organic solvents such as chloroform, n-butanol, diethyl ether and carbon tetrachloride were used in different combinations for virus extraction. Elimination of the mucilage by digestion with β-glucosidase, cellulase, Driselase (a mixture of cellulase and pectinase) and hemicellulase were attempted.

The following method was the most successful in eliminating the mucilage, and resulted in the highest virus yields. Leaves from infected plants were homogenised in a Waring blender with 0.1 M sodium acetate buffer, pH 5.5 (1/3; w/v), containing 1 mg hemicellulase (Sigma Chemical Company, USA) per gram leaf tissue. The mucilaginous extract was liquefied by incubation with the enzyme at room temperature (22°C) for a minimum of 4 h (usually overnight). After separation by low speed centrifugation (10 000 *g* for 10 min) the supernatant was discarded, and solid material re-homogenised with two volumes of 0.1 M Tris-Cl pH 9.0, containing 1% (w/v) Na_2SO_3 and 0.1% (v/v) thioglycollic acid. To the supernatant, obtained from low speed centrifugation of the above extract, were added 2% (v/v) Triton X-100, 5% (w/v) PEG M_r 6 000 and 1.5% (w/v) NaCl. The mixture was gently stirred at 4°C for 60 min to simultaneously solubilise the green plant pigment and precipitate the virus, which was collected by low speed centrifugation. Pellets were resuspended in 0.1 M Tris-Cl pH 9.0, and concentrated by ultracentrifugation (64 000 *g* for 90 min). Virus was usually further purified by rate zonal centrifugation in 10-40% (w/v) sucrose gradients (in 0.1 M Tris-Cl pH 9.0) at 27 000 rpm (96 000 *g*) for 2 h in a Beckman SW28 rotor. Virion-containing fractions were collected using an ISCO model 640 density gradient fractionator and a model UA-5 UV-analyser with a 254 nm filter. Sucrose was removed by overnight dialysis against 0.05 M Tris-Cl pH 9.0, or by ultracentrifugation after diluting the virion-containing fractions 1:4 with 0.05 M Tris-Cl pH 9.0. Virus was concentrated by ultracentrifugation and final pellets resuspended in 0.1 M Tris-Cl pH 9.0. Virus preparations were kept at 4°C.

2.2.4 Electron Microscopy

Virus preparations were adsorbed onto 300 mesh carbon/Ne-collodion-coated copper grids and negatively stained with 2% (w/v) phosphotungstic acid, pH 6.2, or 2% (w/v) ammonium molybdate, pH 6.5, or 2% uranyl acetate, pH 4.5. Immunosorbent electron microscopy was performed by adsorbing 1/10-, 1/100- or 1/1000-dilutions of anti-OMV antiserum onto grids for 5 min at room temperature. The grids were washed by rinsing with 20 drops of 0.1 M phosphate buffer pH 7.0, before floating grids on droplets of diluted virus for 60 min at room temperature. After another wash, bound virus was "decorated" by floating the grids on droplets of 0.1 or 0.01 mg/ml anti-OMV IgG for 30 min at room temperature. The grids were washed again and negatively stained as before.

Samples were viewed and photographed in a Zeiss EM 109, or a Jeol 200 CX, or a Philips EM 201C transmission electron microscope. Particle length measurements were made from electron micrographs of partially purified virus preparations by tracing virions on a Summagraphics tablet coupled to a Tektronix 4051 microcomputer. Freshly purified TMV was used as an internal standard for calibration.

To investigate the presence of inclusion bodies, freshly harvested healthy and OMV-infected ornithogalum and lachenalia leaves were prepared for ultrathin sectioning. Leaf tissue was cut in 1 mm squares, pre-fixed in 4% glutaraldehyde and fixed in 1% osmium tetroxide. The leaf tissue was dehydrated by sequential incubations in baths of increasing ethanol concentration, and in propylene oxide. The leaf squares were embedded in moulds of Araldite CY 212 resin (Polaron Equipment Ltd, England) and ultrathin sections made using a Reichert OM U2 ultramicrotome. The sections were viewed in a Jeol 200 CX transmission electron microscope.

2.2.5 SDS-Polyacrylamide gel electrophoresis

The molecular weight of OMV coat protein was determined by discontinuous SDS-PAGE as described in Appendix B.3.1.

2.2.6 RNA extractions

RNA was extracted from purified virus preparations by a modification of the method of Gallitelli *et al.* (1985). The virus preparation was heated at 60°C for 5 min in 10 mM Tris-Cl pH 8.25; 1% (w/v) SDS; 1 mM EDTA, emulsified by vortexing with an equal volume of a buffer-saturated phenol/chloroform/isoamyl alcohol mixture (25:24:1), and the aqueous phase separated by centrifugation. The aqueous phase was re-extracted twice more with phenol/chloroform, and traces of phenol removed by three cycles of ether extraction. RNA was precipitated from suspension by the addition of 2.5 volumes of ice-cold 96% ethanol, and sodium acetate pH 5.5 to 120 mM. RNA was pelleted by centrifugation and resuspended in sterile distilled water to 1 mg/ml, and stored as small aliquots at -70°C. Precautionary measures taken for RNA manipulations included the treatment of all glassware, microfuge tubes and micropipette tips with 0.1% (v/v) DEPC for 16 h at 37°C before being autoclaved, the use of double distilled, DEPC-treated water and new analytical grade chemicals, and the wearing of gloves throughout all procedures.

The authenticity of OMV genomic ssRNA was investigated by incubating an agarose gel containing a fractionated genomic RNA band in TBE buffer (see Appendix B.4.5) containing 50 µg/ml RNase A.

2.2.7 RNA molecular weight determination

The size of OMV genomic RNA was determined by agarose gel electrophoresis under denaturing conditions. Formaldehyde denaturing gels were run as described by Maniatis *et al.* (1982).

RNA was prepared by heating at 60°C for 10 min in 20 mM MOPS; 5 mM NaOAc; 1 mM EDTA (electrophoresis buffer), containing 50% (v/v) deionised formamide and 6% (v/v) formaldehyde. Denaturing gels were prepared in a fume hood by melting agarose (1%, w/v) in electrophoresis buffer, and adding formaldehyde (to 6%, v/v) after cooling to 60°C. The sample loading buffer was 50% (v/v) glycerol; 0.4% (w/v) bromophenol blue; 0.4% (w/v) xylene cyanol; 1 mM EDTA. Large analytical gels (20 x

15 x 0.7 cm) were run at 50 V for 16 h. RNA sequences derived from bacteriophage T7, yeast 2μ plasmid and bacteriophage lambda DNA ("RNA ladder", Bethesda Research Laboratories, USA) were used as molecular weight markers. Marker sizes were 9.5, 7.5, 4.4, 2.4, 1.4 and 0.3 Kb. OMV RNA molecular weight was determined graphically by reference to marker RNA mobilities.

2.2.8 Oligo(dT)-cellulose chromatography

Oligo(dT)-cellulose chromatography was performed as recommended by the manufacturers (Collaborative Research Inc., Waltham, MA). A column was prepared by pouring oligo(dT)-cellulose, suspended in 10 mM Tris-Cl pH 7.5; 0.05% (w/v) SDS; 1 mM EDTA (low salt elution buffer), in a sterile 1-ml syringe. The column was equilibrated by washing with 10 bed volumes of 10 mM Tris-Cl pH 7.5; 0.5 M NaCl; 0.5% (w/v) SDS; 1 mM EDTA (high salt binding buffer). RNA was resuspended in high salt binding buffer and applied to the column. Non-adsorbed material was eluted by washing with at least 10 bed volumes of high salt binding buffer before poly(A)$^+$ RNA was eluted with the low salt elution buffer. Fractions of 300 μl were collected and RNA-containing fractions identified by UV spectroscopy at 260 nm.

2.3 RESULTS

2.3.1 Virus purification

The very mucilaginous nature of ornithogalum and lachenalia plant sap caused problems in preliminary attempts at virus purification. Homogenisation of leaves resulted in an extremely viscous mucilage even when extractions were made in large buffer volumes. Several approaches to overcome this problem were investigated. Homogenisation in liquid nitrogen or from frozen leaf tissue, as well as using several mechanical methods, failed to eliminate the mucilage effectively. None of the buffer additives, detergents, or organic solvents which were used, reduced the viscosity of extracts significantly. Enzymatic digestion of the mucilage with four different enzymes was attempted: hemicellulase was found to be the most effective. Incubation of 1 mg enzyme per gram leaf tissue in three volumes of 0.1 M NaOAc pH 5.5, for 4 to 16 h at

room temperature, effectively liquefied the plant sap. Solid plant material was separated by centrifugation and virus extracted from it with 0.1 M Tris-Cl pH 9.0, containing 1% (w/v) Na_2SO_3 and 0.1% (v/v) thioglycollic acid. A second extraction of the solid leaf material (after the mucilage digestion step) was not necessary when purifications were made from lachenalia. In this case virus was precipitated directly from the supernatant using PEG/NaCl after the first centrifugation step.

In developing the purification protocol, electron microscopy and ISEM were used to monitor the presence of virus through all the stages of the purification. Approximate virus yields of 4-6 mg/kg infected ornithogalum leaves and 1-2 mg/kg of infected lachenalia leaves were routinely obtained. Comparable yields were obtained from cultivated and wild ornithogalum plants, but lachenalia yielded less virus. Although virus could be purified from infected leaves kept at 4°C for 4 months, better yields were obtained from freshly harvested leaves. Virus yield from ornithogalum could be increased to approximately 6-10 mg/kg if leaves were harvested two to four weeks before plants started flowering. Very little virus was purified from leaves which were kept frozen for a few months.

2.3.2 Electron microscopy

Filamentous, elongated particles were consistently observed in leaf extracts from plants showing mosaic symptoms (Fig. 2.1). Antiserum to an OMV isolate from the Netherlands (Table 3.2) reacted with these particles in ISEM (Fig. 2.2). Length measurements were made of 356 particles from partially purified virus preparations. The modal length was in the range 720-760 nm (Fig. 2.3) with an average length of 688 ± 193.8 nm. The virus width was 11 nm. A few end-to-end aggregates were observed.

The presence of viral inclusion proteins in infected ornithogalum and lachenalia leaf material was investigated. Ultramicrotomy of embedded material was difficult, and although several sections were made and viewed, typical inclusion bodies were not observed (results not shown).

Fig. 2.1. Purified preparation of OMV stained with 2% (w/v) ammonium molybdate, pH 6.5. Scale bar = 100 nm.

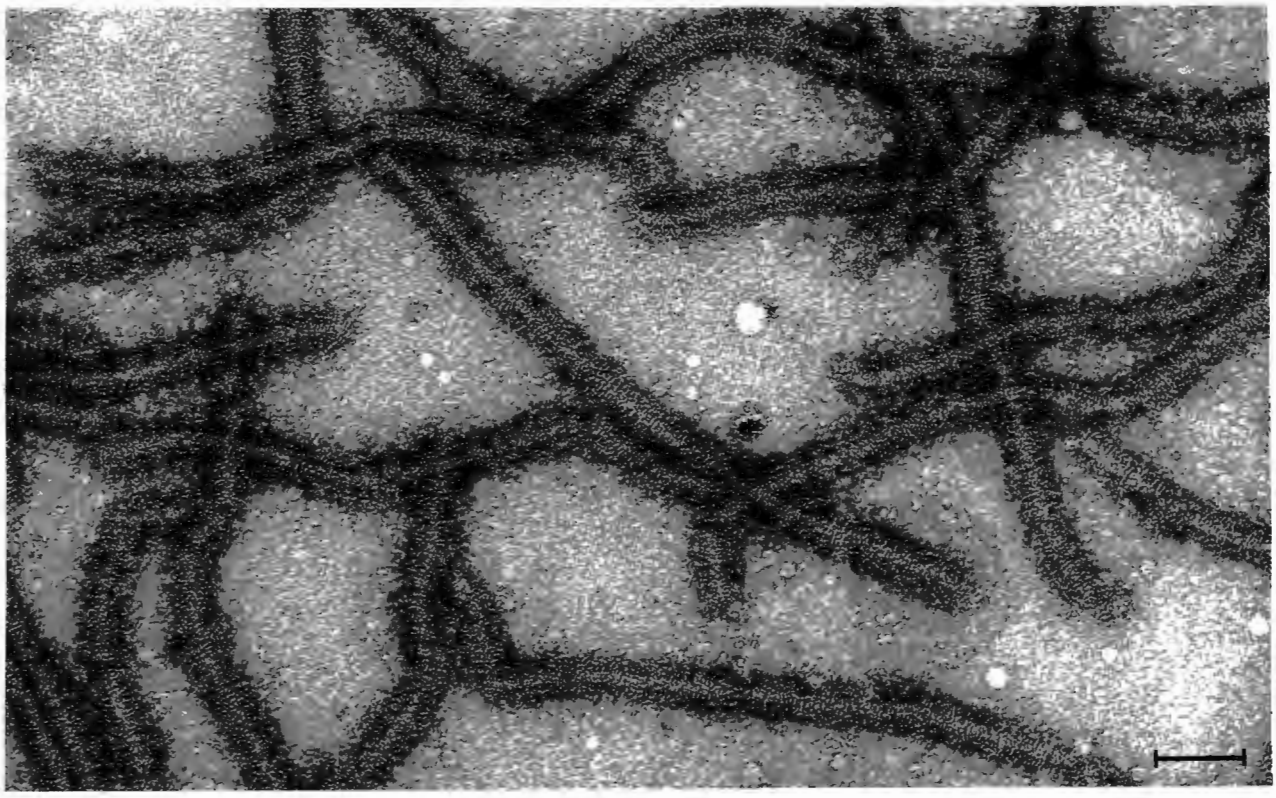

Fig. 2.2. ISEM of OMV particles with antiserum directed against a Dutch isolate of OMV. Grids were negative stained with 2% (w/v) uranyl acetate, pH 4.5. Scale bar = 100 nm.

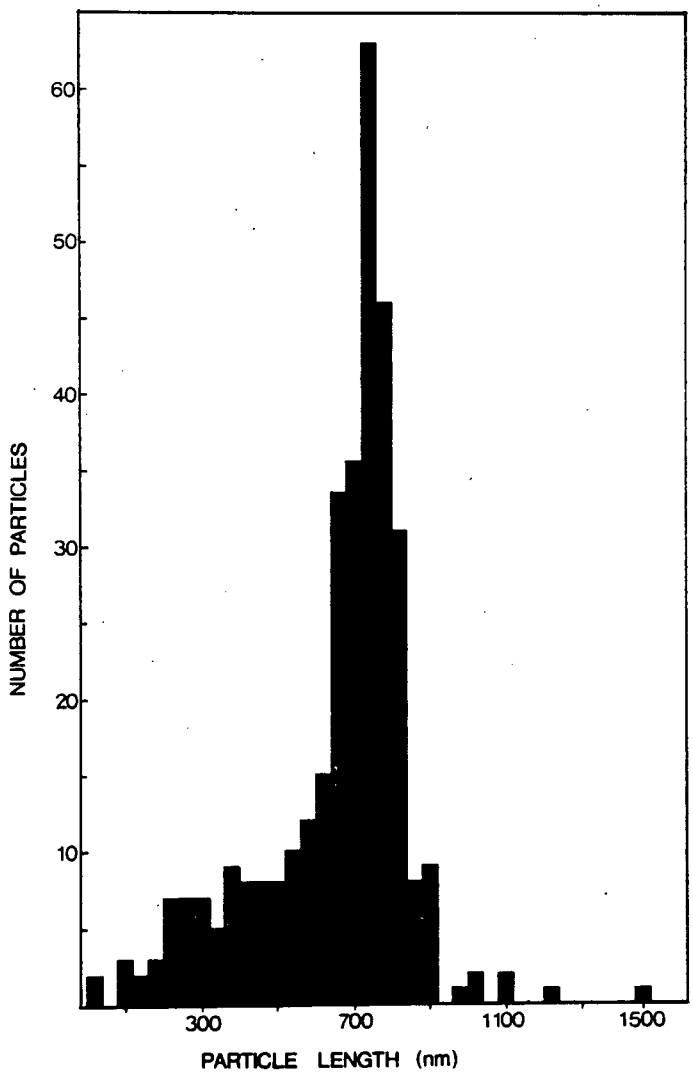

Fig. 2.3. Particle length distribution of OMV from a partially purified preparation stained in 2% (w/v) phosphotungstic acid, pH 6.2. The modal length is in the range 720-760 nm.

2.3.3 PAGE analysis of viral coat protein

A single major protein band of M_r 30 000 (29 971.3 ± 80.6) was observed for SDS-denatured OMV preparations in polyacrylamide gels (Fig. 2.4). The molecular weight was determined graphically (5 repeats) by reference to commercial markers. Minor bands of lower molecular weight were occasionally observed. These are believed to be products of partial proteolysis of the coat protein, as have been observed with other potyviruses (Hiebert & McDonald, 1976, Hiebert *et al.*, 1984a).

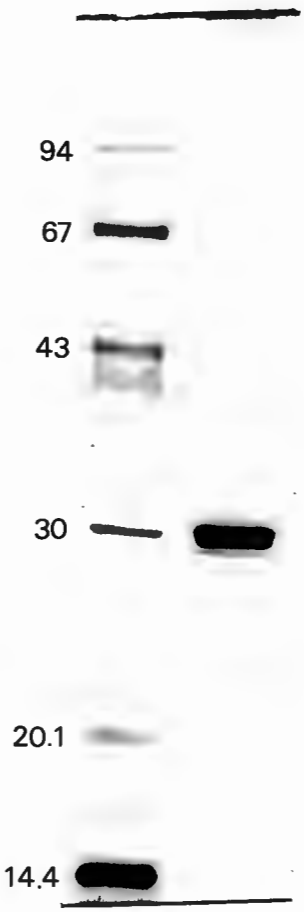

Fig. 2.4. SDS-PAGE of OMV CP. Lane A, molecular weight markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). Lane B, OMV-O (30 kDa).

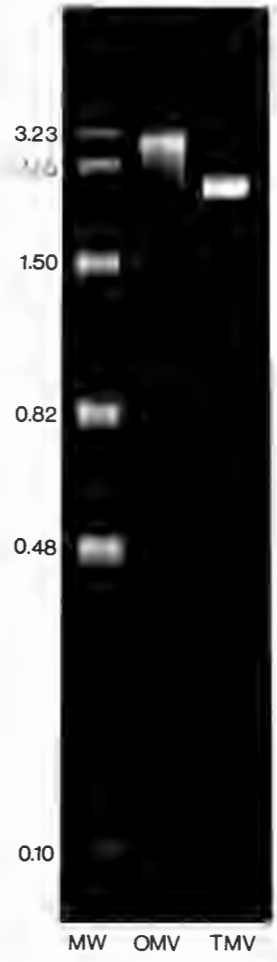

Fig. 2.5. Molecular weight determination of OMV RNA in formaldehyde agarose gels. Lane A, molecular weight markers (Kb); lane B, OMV RNA (M_r 2.9 x 10^6); lane C, TMV RNA (M_r 2.11 x 10^6).

2.3.4 RNA characterisation

The authenticity of OMV genomic ssRNA was confirmed when a fractionated RNA band disappeared from an agarose gel after a 30 min incubation in RNase A. The molecular weight of OMV RNA was calculated from the relative position of RNA bands in formaldehyde agarose gels (7 repeats). The average RNA size was 8.54 ± 0.075 Kb (M_r 2.90 x 10^6) (Fig. 2.5). An average size of 6.22 ± 0.102 Kb (M_r 2.11 x 10^6) was obtained

for TMV RNA, which was included as an alternative marker: this compares well to the value of 2.1 x 10^6 obtained from total genomic sequencing of TMV (Goelet *et al.*, 1982).

The presence of a poly(A) tract on the viral RNA was investigated by passing RNA over an oligo(dT)-cellulose column. Sixty seven percent of the input RNA bound to the oligo(dT)-cellulose, while only 4% of TMV RNA, which served as negative control, bound to oligo(dT)-cellulose. This confirms that OMV RNA is polyadenylated (Fig. 2.6).

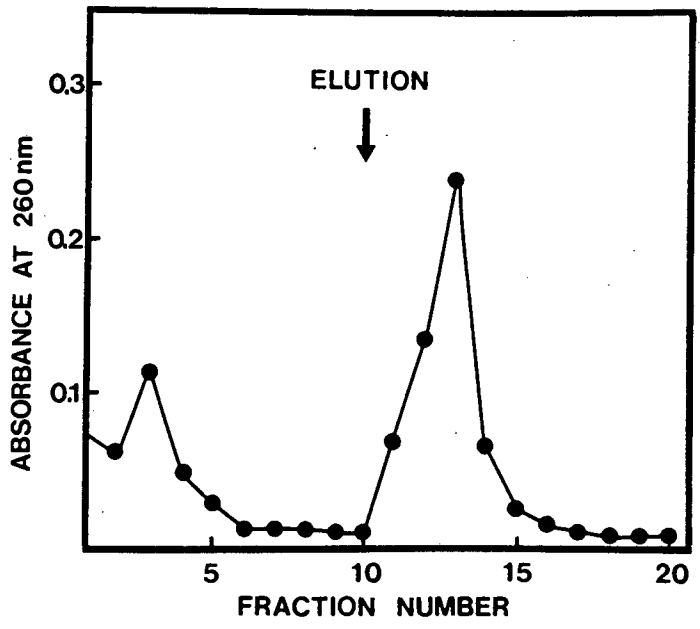

Fig. 2.6. Elution profile of OMV RNA in oligo(dT)-cellulose chromatography. The arrow indicates the beginning of poly(A)$^+$ RNA elution.

2.4 DISCUSSION

The physicochemical characterisation of OMV depended on the availability of purified virus. In developing the purification protocol, the primary problem was the elimination of the highly viscous mucilage in ornithogalum and lachenalia sap extracts. Of all the mechanical and chemical methods employed, only the dilution effect of using very high buffer to leaf mass ratios (20:1) seemed to have some measure of success. However, this

approach was not practical, as low virus yields meant very large volumes of liquid would have to be handled in routine extractions.

The mucilage was isolated and chemically analysed in the Organic Chemistry Department of the University of Cape Town. The water-soluble acidic polysaccharide was identified as an arabinoglucuronomannoglycan, with a structure which resembled those of the mucilaginous polysaccharides isolated from *Drosera capensis*, *D. binata*, and from a suspension culture of *Nicotiana tabacum* cells (Mabusela & Stephen, 1990). The pH of ornithogalum and lachenalia plant sap (pH 5.9 and 5.6, respectively; own observation) was close to that of the optimum working pH (pH 5.5) of hemicellulase, hence the use of 0.1 M NaOAc pH 5.5 as digestion buffer. The fact that the isoelectrical point of OMV is probably between pH 4 and 5, may explain why a re-extraction of solid plant material after the first low speed centrifugation (in high pH buffer), was necessary. In purifications from lachenalia, the virus was extracted in the low pH buffer and only negligible amounts of virus were recovered after a second extraction in a high pH buffer. The reason for this is unknown.

The modal length of 720-760 nm determined for OMV falls in the "recognised" potyvirus range of 680-900 nm (Francki *et al.*, 1985). However, viral modal length cannot always be considered a reliable parameter, as different physiological conditions, especially the presence of Mg^{2+} ions, have been shown to affect the length of certain potyviruses. Govier & Woods (1971) reported modal lengths of 850 and 750 nm for PVMV in the presence or absence of Mg^{2+} ions, respectively. The difference between the 720-760 nm modal length and the average particle length (688 nm) must be attributed to a large percentage of broken particles in the virus preparation.

It is generally accepted that all potyviruses produce cytoplasmic inclusions. They normally are cylindrically shaped and consist of a single protein of M_r 67-70 kDa (Hiebert & McDonald, 1973). The presence of inclusions in infected ornithogalum and lachenalia leaf tissue was investigated, but without success. The difficulties experienced in making ultrathin sections of embedded ornithogalum and lachenalia leaf tissue were probably due to improper dehydration, fixing and embedding of tissue, as a result of the high mucilage content of the leaves.

Potyvirus coat protein molecular weights of 28-40 kDa have been determined in SDS-PAGE (Shukla & Ward, 1989a). Although these authors found molecular weight differences with different acrylamide concentrations, SDS-PAGE is still the most widely used and generally accepted method of coat protein molecular weight determination. The M_r of 30 000 is in reasonable agreement with the 28 807 Da calculated from OMV sequencing data (Section 5.3.7).

The presence of ssRNA in OMV particles, and its characterisation suggested that OMV is a potyvirus. An M_r of 2.9×10^6 was calculated for OMV RNA, compared to the 3.07×10^6, 3.04×10^6 and 2.9×10^6 reported for the definitive potyviruses TuMV, TEV and maize dwarf mosaic virus (MDMV), respectively (Hill & Benner, 1976). The presence of a poly(A) tract on the RNA is typical of potyviruses and was first reported by Hari *et al.* (1979) for TEV.

CHAPTER 3

SEROLOGY OF ORNITHOGALUM MOSAIC VIRUS

SEROLOGY OF ORNITHOGALUM MOSAIC VIRUS

SUMMARY

The serological interrelatedness of OMV isolates and the serological relationships between OMV and other potyviruses were studied by ELISA and immunoelectroblotting. The three South African OMV isolates were found to belong to one strain. Antiserum to a Dutch OMV isolate reacted with the South African isolates. OMV was found to be serologically related to HMV, BYMV, PVY, WMV-1, WMV-Mor, zucchini yellow mosaic virus (ZYMV), SCMV, SMV and passionfruit woodiness virus (PWV), but not related to MDMV-A, MDMV-B, WMV-2, LMV or WSMV. A reliable method for the detection of OMV in ornithogalum and lachenalia plantlets was developed. A simple single-vial sap extraction method followed by DAS-ELISA enabled the detection of OMV in as little as 0.1 g of tissue from one of the first leaves of sprouting bulbs.

3.1 INTRODUCTION

Serology has been used extensively for the detection, classification and characterisation of plant viruses ever since the discovery of the antigenic properties of TMV in 1929 (Purdy, 1929).

Serological methods are still considered the most practical means of establishing relationships among potyviruses (Van Regenmortel, 1982, Koenig, 1988). However, the many conflicting reports in the literature are clear evidence that serological relationships among potyviruses are very complex and do not necessarily correlate with biological properties (Hollings & Brunt, 1981a). Close serological relationships have been reported for potyviruses infecting completely non-related hosts (some even across the monocotyledon/dicotyledon borders), e.g. TEV related to BCMV; pokeweed mosaic virus, TuMV, PVY and WMV related to MDMV (Shepard *et al.*, 1974); LMV related to BYMV (Alba & Oliveira, 1976); and JGMV related to WMV-2 (Shukla *et al.*, 1988d). Several explanations for these contradictory results in potyvirus serology have been proposed (Francki *et al.*, 1985, Koenig, 1988). However, it was only after sequence data for several potyvirus coat proteins became available that the molecular basis of their serology could be unravelled (Allison *et al.*, 1985a, Shukla & Ward, 1988, 1989a,b, Shukla *et al.*, 1988d, 1989a,d). These workers showed that the N- and C-terminal regions of potyvirus coat proteins are surface-located, and that the N-terminus is the immunodominant part of the virus particle. Since the N- and C-terminal regions are highly virus specific, and the core protein regions very conserved, antibodies directed against these regions should be excellent narrow and broad spectrum probes respectively (Shukla *et al.*, 1988d). Shukla *et al.* (1989a) developed a method to remove cross-reacting antibodies (directed against the conserved core protein region) from antiserum preparations. This approach successfully resolved some potyvirus taxonomic problems (Shukla *et al.*, 1989b,c); however, some unexpected paired relationships were still observed (Shukla *et al.*, 1989a).

The importance of a reliable virus detection method in plant disease diagnosis is obvious. Traditional methods of mechanical inoculations and grafting onto indicator plants, linked with symptomatology, are largely outdated. Similarly, traditional

serological methods which were based on immunoprecipitin and agglutination reactions in liquid or gel media (Van Slogteren, 1955, Purcifull & Batchelor, 1977, Van Regenmortel, 1966, 1982), have limited use and have largely been superseded in recent years by amplified immunosorbent assays and nucleic acid hybridisation tests. These newer techniques are more sensitive and require less material.

DAS-ELISA is very specific and has been used to differentiate between isolates of the same virus (Clark & Adams, 1977, Koenig, 1978, Rochow & Carmichael, 1979, Rybicki & Von Wechmar, 1981). Indirect ELISA is well suited to detect distant relationships and has been widely applied in studies of serological relationships (Van Regenmortel & Burckard, 1980, Koenig, 1981, Rybicki & Von Wechmar, 1981, Koenig & Paul, 1982b).

Immunoelectroblotting has more recently been established as a reliable technique for the detection of distant serological relationships (Rybicki & Von Wechmar, 1982, Burgermeister & Koenig, 1984). This technique entails the separation of viral proteins by SDS-PAGE, electrophoretic transfer of these proteins to a nitrocellulose filter, and the detection of the proteins by enzyme immuno-assay with an insoluble substrate product. The identification of viral proteins by both their molecular weight and serological specificity, makes IEB a good discriminatory technique for serological relationship studies.

DAS-ELISA has been used routinely in virus diagnosis. Sample preparation is a critical parameter for reliable detection: the pre-treatment of sample tissue as well as modifications to extraction procedures and buffers were reported to increase virus detectability. Pre-treatments include the artificial breaking of dormancy in potato tubers (Vetten *et al.*, 1983, Reust & Gugerli, 1984), the cutting of gladiolus corms and iris bulbs (Stein *et al.*, 1986, Van der Vlugt *et al.*, 1988); modifications of extraction protocols include the use of additives in extraction buffers to neutralise inhibitory substances in plant extracts (Clark & Adams, 1977, Beijersbergen & Van der Hulst, 1980). Immunoblotting techniques are also routinely used in virus diagnosis (Koenig & Burgermeister, 1986). The immuno-dot blot assay has great potential as a large scale routine testing method (Koenig, 1988). IEB is more labour intensive, but is a more

reliable technique since antigens are evaluated on both their molecular weight and antigenic properties.

Nucleic acid hybridisation tests were initially used for the detection of pathogens in plants, for which serological methods were not applicable, such as viroids and naked RNAs of certain viruses (Owens & Diener, 1981, Harrison & Robinson, 1982). Early hybridisation tests mostly involved liquid-liquid hybridisation reactions, which were labour-intensive and thus not practical for large-scale testing (Palukaitis *et al.*, 1981, Harrison & Robinson, 1982). Since the development of the much more practical dot-blot hybridisation test, which is a solid phase hybridisation technique, this test has been applied for routine virus detection (Maule *et al.*, 1983, Baulcombe *et al.*, 1984). Dot-blot hybridisation tests were effective in detecting several viruses in ornamental plants (Hammond & Hammond, 1985, Linthorst & Bol, 1986, Balasingam *et al.*, 1988, Wang *et al.*, 1988).

Nucleic acid hybridisation is based on the ability of two complementary nucleic acid strands to hybridise under appropriate conditions to form a double stranded structure. In dot-blot tests, the nucleic acid to be tested is immobilised onto a solid support (e.g. nitrocellulose or nylon membrane), and hybridised to the probe nucleic acid, which is labelled to enable detection of hybrids. Radioactive, biotin/photobiotin, avidin/streptavidin and digoxygenin labels have been used for the detection of hybridisation reactions. However, this technology requires technical proficiency and materials (like radio-isotopes) which may be beyond the resources and/or expertise of regional laboratories outside the main centres, and may thus not always be a feasible alternative.

This chapter describes an investigation of the serological interrelationships of OMV isolates, and the serological relatedness of OMV and other potyviruses, as determined by ELISA and IEB. One of the primary objectives of this study was to establish a reliable diagnostic test for OMV, which was to be used for large-scale indexing of ornithogalum and lachenalia propagation material; both at the VOPRI and in industry. A few serological assays and a nucleic acid hybridisation test were evaluated as possible methods of OMV diagnosis. This chapter describes a simple serological assay,

which combines a single-vial sap extraction procedure with DAS-ELISA, to reliably detect OMV in sprouting bulbs. Parts of the work described here were previously published (Burger & Von Wechmar, 1988, 1989).

3.2 MATERIALS AND METHODS

3.2.1 Virus sources

The origins of all potyviruses used in this study are summarised in Table 3.1. The purification of OMV-O, OMV-L and OMV-W is described in Section 2.2.1. All other potyviruses used, are Departmental reference isolates (M. B. von Wechmar, Department of Microbiology, University of Cape Town).

Table 3.1 Virus isolates used in serological relationship studies

Virus [a]	Origin
OMV-O, -L, -W	This study, Section 2.2.1
BYMV-G	J. Burger, UCT collection (gladiolus isolate)
PVY-1	G. Thompson, VOPRI (potato isolate)
PVY-2	M. van Halderen, UCT collection (tobacco isolate)
MDMV-A	M. von Wechmar, UCT collection (johnsongrass isolate)
MDMV-B	M. von Wechmar, UCT collection (maize isolate)
WMV-Mor	F. van der Meer, VOPRI (cucurbit isolate)

[a] See Appendix A for virus names.

3.2.2 Antisera

Antisera to OMV-O, -L and -W were prepared in rabbits as described in Appendix B.2.1. Antisera to BYMV-Scott and SMV were donated by G. Pietersen, Plant Protection Research Institute, Pretoria, South Africa. A monoclonal antibody (MAb PTY-1) which reacts with the coat protein of BYMV and many other potyviruses, was kindly supplied

by R. L. Jordan, USDA Agricultural Research Service, Beltsville, MD. All other antisera used in this study, were obtained from the Departmental collection (M. B. von Wechmar, Department of Microbiology, University of Cape Town). Origins of antisera are summarised in Table 3.2.

Table 3.2 Antisera used in serological relationship studies

Antiserum	Origin
OMV-O, -L, -W [a]	This study, Appendix B.2.1
OMV-D	A. F. L. M. Derks, Lisse, The Netherlands
BYMV-G	J. Burger, UCT collection
BYMV-Scott	R. O. Hampton, Oregon State University
PVY-1	G. Thompson, VOPRI
PVY-2	M. van Halderen, UCT collection
MDMV-A, -B	M. B. von Wechmar, UCT collection
WMV-Mor	F. W. van der Meer, VOPRI
WMV-1	H. Lecoq, Montfavet, France
WMV-2	M. B. von Wechmar, UCT collection
ZYMV	H. Lecoq, Montfavet, France
SCMV	A. G. Gillaspie, Beltsville, MD
WSMV	M. Brakke, Lincoln, NE
PWV	E. W. Kitajima, University of Brazil
SMV	R. M. Lister, Purdue University, IN
HMV	A. F. L. M. Derks, Lisse, The Netherlands
MAb PTY-1	R. L. Jordan, USDA-ARS, Beltsville, MD

[a] See Appendix A for virus names.

3.2.3 Preparation of IgG and monospecific antibodies

The gamma-immunoglobulin fraction of anti-OMV antiserum was prepared by ammonium sulphate precipitation and DEAE-cellulose filtration (Whatman DE-52) as

described by Clark & Bar-Joseph (1984). Final IgG concentration was adjusted to 1 mg/ml in half-strength PBS (5 mM sodium phosphate; 75 mM NaCl pH 7.4) and stored at -20°C.

In order to obtain maximum specificity and to minimise background reactions in serological assays, host-absorbed antisera were used to prepare monospecific antibodies against OMV-O (Appendix B.2.2).

3.2.4 Conjugation of alkaline phosphatase to antibodies

Alkaline phosphatase (Seravac, Cape Town, South Africa, 1.133 U/mg) was conjugated to IgG or to monospecific antibody preparations using glutaraldehyde (Clark & Bar-Joseph, 1984). Goat anti-rabbit and goat anti-mouse alkaline phosphatase conjugates were purchased from Bio-Yeda, Israel and the Sigma Chemical Company, St. Louis, MO, respectively.

3.2.5 Microprecipitin tests

Microprecipitin tests were used to determine anti-OMV antiserum titres. Tests (with polyclonal antisera) were performed in petri dishes as described by Noordam (1973).

3.2.6 Enzyme-linked immunosorbent assay

3.2.6.1 DAS-ELISA

DAS-ELISA was used to determine the interrelatedness of OMV isolates, the serological relatedness of OMV to other potyviruses, and also as a serological detection method for virus diagnosis. DAS-ELISA was done according to Clark & Bar-Joseph, 1984. Microwell plates (Nunc, Denmark) were coated with IgG, which was diluted to 2 µg/ml in 0.05 M sodium carbonate buffer pH 9.6 (coating buffer). This was followed by incubations of 10 mM sodium phosphate; 150 mM NaCl pH 7.4, containing 0.1% (v/v) Tween 20 and 0.2% (w/v) skimmed milk powder (blocking buffer), antigen, and enzyme conjugate (diluted 1/500 in blocking buffer). Three washes of three minutes

each with 10 mM sodium phosphate; 150 mM NaCl pH 7.4, containing 0.1% (v/v) Tween 20 (PBS-T washing buffer) were done between all incubations steps. Incubations were at 37°C for 90 min, except for the antigen, which was incubated overnight at room temperature. The enzyme substrate was a freshly prepared 1 mg/ml solution of p-nitrophenyl phosphate in 10% (v/v) diethanolamine. Colour intensities of the enzyme-substrate reactions were measured at 405 nm with a Titertek Multiskan ELISA Reader (Flow Laboratories, Sweden).

Calibration titrations with dilution series of antigen, coating antibody and conjugate were performed to obtain sensitive virus detection with minimum background. Purified OMV and virus-free ornithogalum sap were always included as controls. To avoid possible temperature related non-specific reactions, the outside wells of microtitre plates were not used (Clark & Adams, 1977).

3.2.6.2 Indirect ELISA

Indirect ELISA was used in serological relationship studies. Microwell plates were coated with a five-fold dilution series of OMV. Antisera to be tested (Table 3.3) were used at dilutions of 1/125, 1/250 and 1/500 (of the polyclonal antisera), and the goat anti-rabbit alkaline phosphatase conjugate at 1/3000. Plates were incubated, washed and reacted with substrate as described for DAS-ELISA.

3.2.6.3 Immuno-dot blot (DOT-) ELISA

The use of DOT-ELISA as a detection method for OMV in ornithogalum and lachenalia was investigated. The assay was performed in a Schleicher & Schuell "Minifold" microwell filtration apparatus (Schleicher & Schuell, Keene, NH). Nitrocellulose filters (BA 85, 0.45 µ pore, Schleicher & Schuell) were coated in anti-OMV IgG-preparation (diluted to 4 µg/ml in coating buffer); then immersed in blocking buffer to saturate protein adsorption sites. Antigens were adsorbed by suction onto the nitrocellulose filters in the "Minifold", and filters were then incubated in a 1/500 dilution of the alkaline phosphatase conjugate. Three 5 min washes in PBS-T washing buffer were

done between each incubation step. Filters were finally reacted with the NBT and BCIP substrate as described for IEB (Appendix B.2.3).

3.2.7 Immunoelectroblotting

IEB was used to determine the serological relatedness of OMV to other potyviruses. Purified OMV was electrophoresed in small acrylamide gels, and electroblotted onto nitrocellulose membranes which were then blocked in a milk powder suspension as described in Appendix B.2.3. Nitrocellulose filters were then cut in strips, each strip corresponding to a single lane on the gel. Individual strips were incubated with 1/50- and 1/100-dilutions of potyvirus antisera in small plastic tubes (Sterilin 142AS). The strips were then further incubated with enzyme conjugate and reacted with NBT/BCIP substrate as described in Appendix B.2.3.

3.2.8 Nucleic acid hybridisation tests

DNA from pOM16Δ18, an OMV clone containing the coat protein encoding sequence (see Section 6.2.1), was labelled with ^{32}P by nick-translation (Appendix B.4.8). Sample preparation was by one of two methods: crude sap preparations were made as for serological assays (see below), or nucleic acid fractions were prepared by phenol/chloroform extraction of bulb tissue which had been homogenised in liquid nitrogen. Samples were adsorbed onto nylon membranes (Hybond N, Amersham, UK) in a "Minifold" apparatus. Hybridisation reactions were performed as described in Appendix B.4.10.

3.2.9 Sample preparation

3.2.9.1 Bulb tissue

Bulbs were tested for virus presence during dormancy and just after dormancy was broken. To establish where in the bulb the virus concentration was highest, bulb storage tissue and growth points were compared by DOT- and DAS-ELISA. Numbered batches of OMV-infected and virus-free ornithogalum and lachenalia bulbs were used

in these experiments. Small wedge-shaped tissue sections were cut from the outer bulb scales, carefully avoiding the inner growth points (see Fig. 3.1). After wounds on these bulbs were treated with agricultural lime to prevent bacterial or fungal infection, the bulbs were planted to determine whether they could survive this treatment. In a parallel test the growth points from another batch of bulbs were excised. Remains of these bulbs were discarded. Sap extractions were made by homogenising tissue directly in microfuge vials with a glass rod, in the presence of 1 ml PBS, containing 0.1% (v/v) Tween-20, 2% (w/v) PVP M_r 44 000, 0.2% skimmed milk powder, and 1 mg/ml hemicellulase. The resulting mucilaginous extracts were incubated overnight at room temperature. Solid material was separated by low speed centrifugation in a microfuge and the supernatants used undiluted as antigens in serological tests.

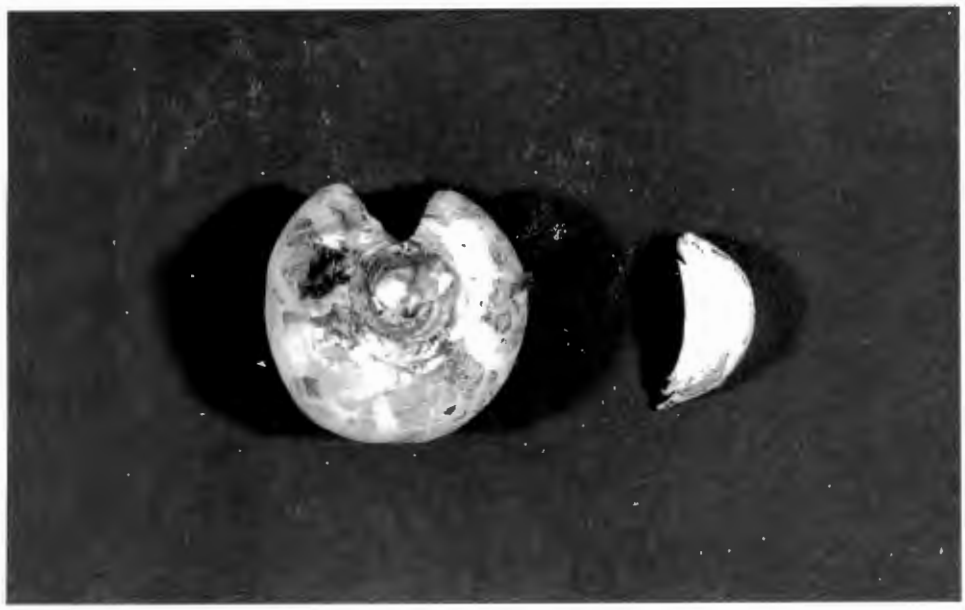

Fig. 3.1. Excision of bulb scale tissue sections without damaging growth points.

3.2.9.2 Leaf tissue

To evaluate DAS- and DOT-ELISA for virus detection in ornithogalum and lachenalia leaves, 0.1 g sections from one of the first leaves of young plantlets were used. Leaf samples were taken 3-4 weeks after planting and extractions made as described for bulb tissue.

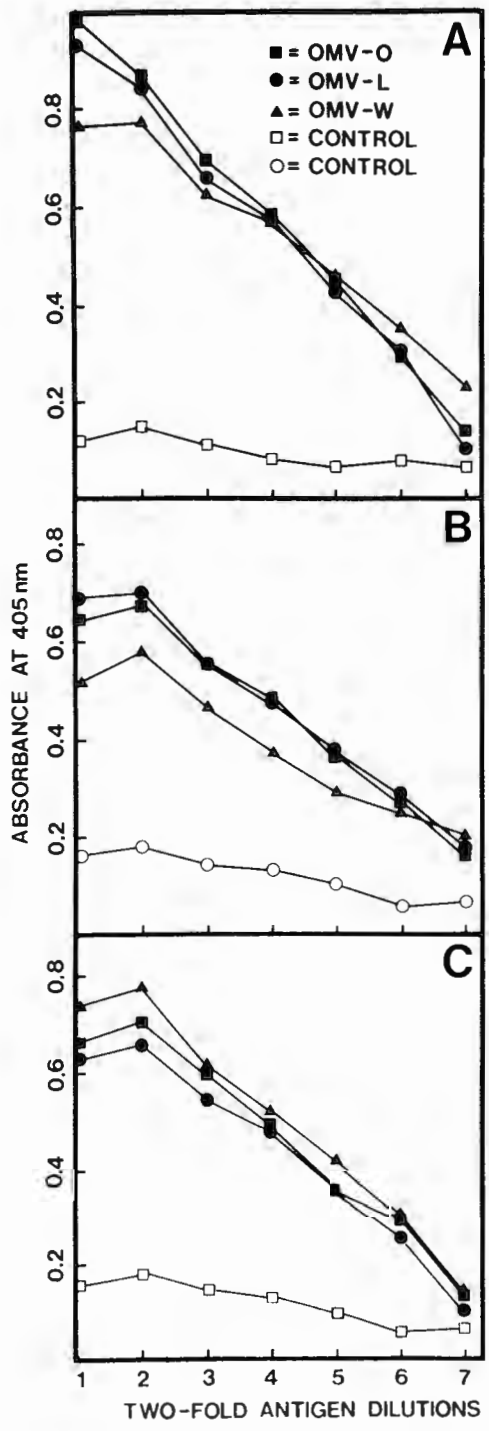

Fig. 3.2. Direct DAS-ELISA showing the serological interrelationships among three South African isolates of OMV, using antiserum to OMV-O (A), antiserum to OMV-L (B), and antiserum to OMV-W (C). Negative controls were virus-free plant sap from ornithogalum (□) and lachenalia (○).

Fig. 3.3. Immunoelectroblot of three South African isolates of OMV probed with antiserum to OMV-D. A, OMV-O, B, OMV-L, C, OMV-W.

3.3 RESULTS

3.3.1 Serological interrelationships of OMV isolates

Homologous end-point titres of antisera to OMV-O, OMV-L and OMV-W in microprecipitin tests were 1/1024, 1/512 and 1/1024, respectively. OMV-O and OMV-L were serologically indistinguishable in DAS-ELISA, while OMV-W differed slightly from both. In reciprocal titrations with all three isolates and their respective homologous antisera, OMV-W reacted less than OMV-O or OMV-L with antisera to the latter two, and more than OMV-O or OMV-L with its homologous antiserum (Fig. 3.2). Antiserum to an OMV isolate from the Netherlands reacted with the local isolates in indirect ELISA (Table 3.3), immunoelectroblots (Fig. 3.3) and ISEM (Fig. 2.2).

3.3.2 Serological relationships with other potyviruses

The relatedness of OMV to other potyviruses was investigated by ELISA and IEB. It was not economically feasible to prepare antiserum conjugates from all the potyvirus antisera which were tested; DAS-ELISA was therefore only used with anti-OMV conjugate in tests with those potyviruses for which purified preparations were available (Table 3.3). Of these, only BYMV-G reacted positively. Indirect ELISA with purified OMV and antisera to other viruses showed serological reactions with antisera to HMV, both the BYMV isolates, both PVY isolates, two out of three WMV isolates, ZYMV, SCMV, and SMV (Table 3.3). Results obtained using IEB correlated well with those from indirect ELISA (Fig. 3.4). OMV reacted positively with MAb PTY-1, but did not react with antisera to CMV, brome mosaic virus, TMV, or maize streak virus, which were included as negative controls (results not shown).

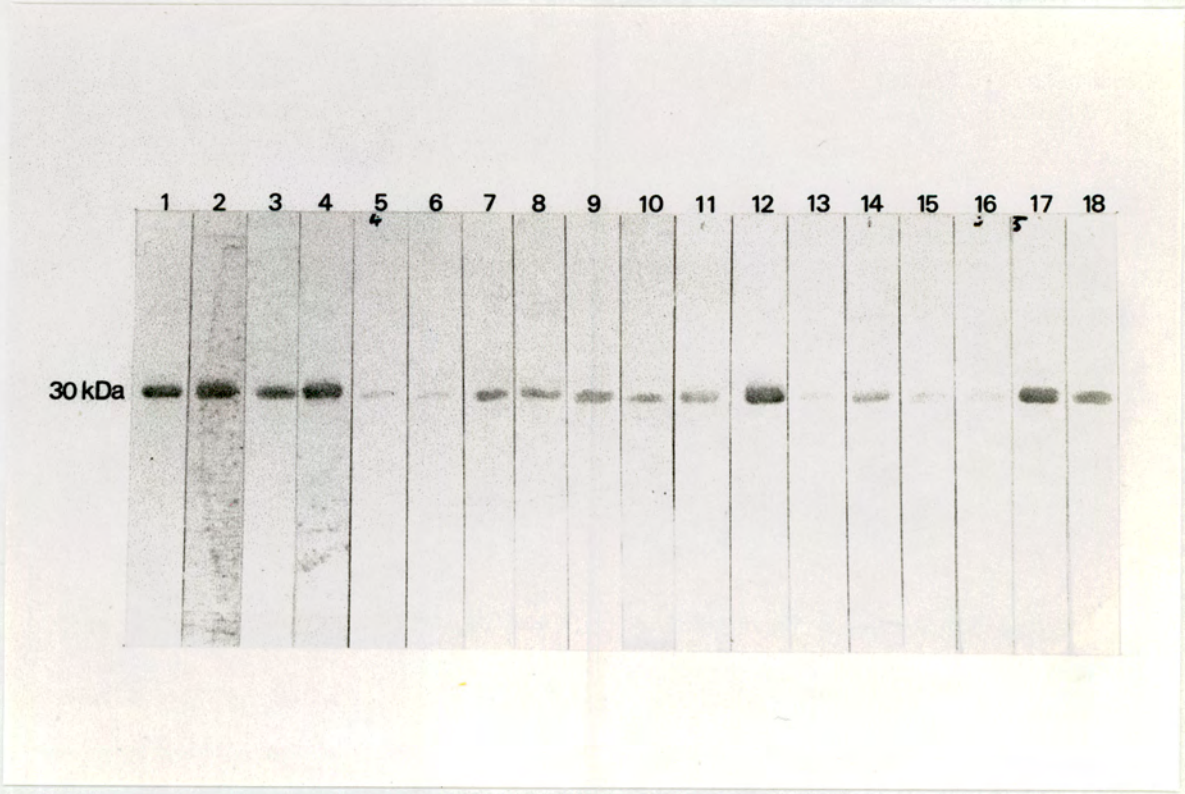

Fig. 3.4. Immunoelectroblot of OMV probed with preparations of different potyvirus polyclonal antisera. 1, OMV; 2, BYMV-G; 3, BYMV-Scott; 4, PWV-SA; 5, WSMV; 6, WMV-2; 7, WMV-1; 8, WMV-Mor; 9, SMV; 10, SMV; 11, SCMV; 12, ZYMV; 13, LMV; 14, HMV; 15, MDMV-A; 16, MDMV-B; 17, PVY-1; 18, PVY-2. See Appendix A for virus names and Table 3.2 for details of strains.

Table 3.3 Serological relationships between OMV and other potyviruses determined by DAS-ELISA, Indirect ELISA and IEB

Virus [a]	Antiserum [a]	DAS-ELISA [b]	Ind-ELISA [b]	IEB [c]
OMV-O	OMV-O	0.89	0.76	++
BYMV-G		0.53	0.41	++
PVY-1		0.03	0.40	+
PVY-2		0.06	0.46	+
MDMV-A		0.00	0.00	-
MDMV-B		0.00	0.00	+
WMV-Mor		0.00	0.00	+
OMV-O	OMV-D	ND [d]	0.56	++
	HMV	ND	0.21	+
	BYMV-G	ND	0.52	++
	BYMV-Scott	ND	0.34	++
	PVY-1	ND	0.42	++
	PVY-2	ND	0.46	++
	WMV-1	ND	0.42	+
	WMV-2	ND	0.00	-
	WMV-Mor	ND	0.18	-
	ZYMV	ND	0.50	++
	SCMV	ND	0.20	+
	WSMV	ND	0.00	-
	SMV	ND	0.33	+
	PWV	ND	ND	++
	MDMV-A	ND	0.00	-
	MDMV-B	ND	0.00	-
	LMV	ND	ND	-

[a] See Tables 3.1 and 3.2 for details of virus strains.

[b] Optical density at 405 nm, readings of healthy plant reactions were subtracted from corresponding reactions with sap from infected plants.

[c] ++ = strong reaction, + = slight reaction, - = no reaction.

[d] Not determined.

3.3.3 Serological virus indexing

3.3.3.1 Bulb tissue

The value and practicability of screening dormant and post-dormant bulbs for virus presence before planting, was investigated by testing bulb storage tissue samples by DAS- and DOT-ELISA. Sections of scale tissue were cut from 36 individually-labelled dormant and 72 post-dormant bulbs. Seventy of the post-dormant bulbs (97%) which were planted after sections were removed, survived this treatment. Table 3.4 summarises ELISA results for OMV-infected and virus-free ornithogalum and lachenalia bulbs. DOT-ELISA of virus-free and OMV-infected ornithogalum and lachenalia bulb scale tissue and growth points (18 samples each) were visually evaluated and are summarised in Table 3.5. Results from both these tests clearly indicated that OMV could not be reliably detected in dormant or post-dormant bulbs by these methods. Results from the parallel tests, where growth points from post-dormant bulbs were used, indicated that virus was concentrated in the growth points during the post-dormancy period (Table 3.4).

Table 3.4 DAS-ELISA absorbance values (405 nm) of bulb scale tissue and bulb growth points

	Dormant Bulbs	Post-dormant Bulbs	
	Scale Tissue	Scale Tissue	Growth Points
Infected ornithogalum	0.313 ± 0.10	0.360 ± 0.09	0.731 ± 0.03
Virus-free ornithogalum	0.138 ± 0.07	0.129 ± 0.08	0.074 ± 0.01
Infected lachenalia	0.257 ± 0.10	0.248 ± 0.08	0.522 ± 0.04
Virus-free lachenalia	0.124 ± 0.06	0.111 ± 0.04	0.103 ± 0.02

Table 3.5 Results of DOT-ELISA on virus-free and OMV-infected ornithogalum and lachenalia bulb scale tissue and growth points (visual assessment of 18 samples of each)

	Scale Tissue			Growth Points		
	Strong-positive	Positive	Negative	Strong-positive	Positive	Negative
Infected ornithogalum	2[a]	9	7	10	8	0
Virus-free ornithogalum	0	5	13	0	6	12
Infected lachenalia	0	10	8	5	11	2
Virus-free lachenalia	0	3	15	0	1	17

[a] Results are expressed as fractions of 18.

3.3.3.2 Leaf tissue

ELISA results from tests of leaf extracts from 90 young plantlets are summarised in Table 3.6. The evaluation of DOT-ELISA results was very difficult due to the non-specific adsorption of leaf pigments onto nitrocellulose filters (Fig. 3.5). Chloroform extraction of samples eliminated some of these pigments, but a degree of non-specific colour adsorption persisted which made evaluation unreliable. The DAS-ELISA results show that virus could readily and reliably be detected in small sections of leaf tissue. Plant growth was not affected by the removal of leaf sections.

Table 3.6 DAS-ELISA absorbance values (405 nm) of leaf extracts

	Infected Plantlets	Virus-free Plantlets
ornithogalum	0.912 ± 0.02	0.151 ± 0.03
lachenalia	0.335 ± 0.04	0.085 ± 0.01

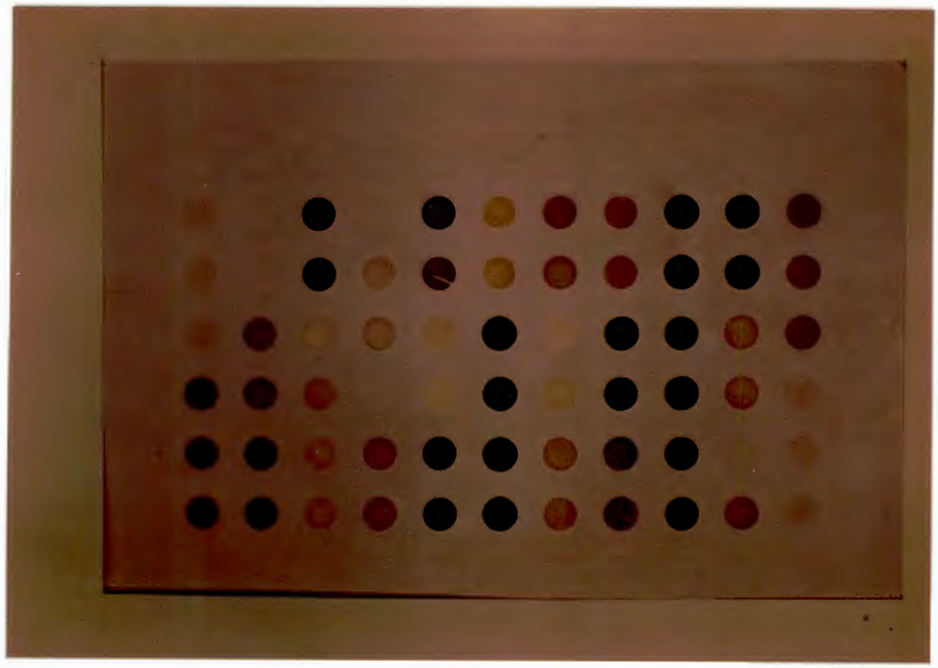

Fig. 3.5. DOT-ELISA of the leaf extracts of young ornithogalum and lachenalia plantlets. The non-specific adsorption of the green chlorophyll pigment made assessment unreliable.

Dot-blot nucleic acid hybridisation assays were not reliable in detecting OMV in phenol-extracted or crude plant sap samples. None of the virus-free or virus infected ornithogalum and lachenalia samples hybridised significantly with the nick-translated OMV coat protein-encoding DNA fragment. However, both purified OMV and isolated OMV RNA, which were included as positive controls, gave strong positive hybridisation signals (results not shown).

3.4 DISCUSSION

In characterising a virus it is essential to determine whether strains of the particular virus exist: differences in virulence, host range, aphid transmissibility and antigenicity of strains can be useful in planning possible control strategies. Serological differentiation by direct DAS-ELISA is a very specific method for demonstrating serological differences between isolates of the same virus (Clark & Adams, 1977, Koenig, 1978, Rochow & Carmichael, 1979, Rybicki & Von Wechmar, 1981). This technique was used to show that OMV-O and OMV-L isolates were indistinguishable,

but that a slight serological difference existed between these and the OMV-W isolate. However, these differences were not considered significant enough to warrant "strain" status for these isolates. Similarly, antiserum to a Dutch isolate of OMV reacted with all three of the South African isolates in indirect ELISA and IEB, meaning that it is at least a related strain if not the same strain. This result is not surprising if it is considered that ornithogalum and lachenalia in the Netherlands were originally imported from South Africa.

DAS-ELISA was of limited use in determining the serological relationships of OMV: first, because of limited quantities available it was not feasible to make conjugates of all the donated potyvirus antisera which were to be tested; and second, only a few virus preparations were available for reciprocal tests with anti-OMV antiserum. A local BYMV isolate from gladiolus (BYMV-G) gave the only positive reaction in DAS-ELISA. Unlike many of the other antisera (of unknown quality) which were used in this relationship study, antiserum to BYMV-G was prepared from freshly purified virus under similar conditions as for OMV. The close serological relationship between OMV and BYMV-G therefore seems real and not the result of non-specific antisera. BYMV has been reported to infect a wide variety of ornamental bulbs (Smith & Brierley, 1944b, Brierley & Smith, 1948, Van Koot *et al.*, 1954, Derks *et al.*, 1980). The possibility that the virus infecting ornithogalum and lachenalia is in fact a strain of BYMV was investigated. Transmission studies (Chapter 4), SDS-PAGE of the viral coat proteins (not shown), and nucleotide sequence comparisons (Chapter 5) proved that OMV is not a strain of BYMV.

Results from indirect ELISA (Table 3.3) and IEB (Fig. 3.4) indicated that OMV is serologically related to BYMV-G and -Scott, PVY-1 and -2, and ZYMV; weakly related to HMV, WMV, SCMV, SMV, and PWV, and not detectably related to MDMV-A or -B, LMV and WSMV. A serological relationship between OMV and HMV, demonstrated using microprecipitin tests, was previously described (Derks & Vink-van den Abeele, 1980). IEB proved to be the preferred method to determine serological relatedness. The testing of viral coat protein (immobilised on strips of nitrocellulose) with several different antisera in small tubes were extremely economical: very little antiserum was needed, and the diluted antisera were frozen in the tubes and were later re-used to

determine relationships for two other potyviruses (South African isolates of PWV and LMV; results not shown). In reciprocal tests, IEB has the advantage that the viruses tested, are evaluated on both their serological specificity and the molecular weight of their coat proteins.

In evaluating different diagnostic tests for OMV in ornithogalum and lachenalia propagation material, the following considerations were important: the earliest possible virus detection; the tissue type to be tested; reliability; simplicity, both in terms of materials and technical skill required; and the cost of such a test. OMV detection in dormant bulb tissue even before planting seems the ultimate objective in virus indexing: both serological and nucleic acid hybridisation assays were evaluated to achieve this.

Serological detection of OMV in ornithogalum and lachenalia bulbs during dormancy was not reliable, probably because virus concentrations were too low. DOT- and DAS-ELISA results of bulbs tested just after dormancy was broken, indicated that virus was concentrated in bulb growth points, and could still not be reliably detected in bulb scale tissue during the post-dormancy period. Similar problems have been experienced with the detection in potato tubers of PVY (De Bokx & Maat, 1979), potato virus X (De Bokx *et al.*, 1980), and potato leafroll virus (PLRV, Tamada & Harrison, 1980). Another factor potentially influencing virus detection is the presence of inhibitory substances in the very mucilaginous bulb extracts, as has been experienced with the detection of LSV in lily bulbs (Beijersbergen & Van der Hulst, 1980), and of BYMV in gladiolus corms (Stein *et al.*, 1986). Stein *et al.* (1986) reported an increased efficiency in the detection of BYMV in gladiolus corms after incisions were made in the corms, prior to testing. Similar results were reported for the detection of ISMV in iris bulbs (Van der Vlugt *et al.*, 1988). Preliminary investigations of the effects of cutting ornithogalum and lachenalia bulbs during dormancy, showed that this treatment failed to significantly increase OMV detectability (results not shown). Unlike gladiolus corms, where it has been suggested that the increased detectability is associated with the physiological processes involved in wound healing (Stein *et al.*, 1986), OMV detectability appeared to be associated with the breaking of dormancy, as has also been shown for PLRV in potato tubers (Vetten *et al.*, 1983, Reust & Gugerli, 1984).

The use of nucleic acid probes in hybridisation assays was very effective in detecting several viruses in ornamental plants (Hammond & Hammond, 1985, Balasingam *et al.*, 1988, Wang *et al.*, 1988). Results of dot-blot hybridisation assays with ^{32}P-labelled DNA from an OMV clone, however, were unreliable. Bulb tissue samples, extracted with phenol/chloroform after homogenisation in liquid nitrogen, were too viscous to be adsorbed onto nylon membranes in a minifold apparatus. When samples were liquefied using the extraction procedure for ELISA, hybridisation seemed not sensitive enough to reliably detect OMV. This problem could probably be solved by concentrating viral RNA in samples by ethanol precipitation, but the practicability of such a tedious extraction procedure becomes doubtful, especially if the success of the DAS-ELISA is considered.

Serological indexing of individual bulbs without sacrificing them does not seem feasible. The concentration of virus in post-dormant bulb growth points, however, suggested that OMV should be easily detected in the first young leaves emerging from bulbs. This was confirmed with the described assay, which is also done at an early stage, often when symptoms are not yet visible. Secondary virus spread can thus be prevented by the early removal of infected plantlets. OMV could reliably be detected in 0.1 g of leaf tissue, the removal of which did not adversely affect plant growth. Comparison of DOT- and DAS-ELISA results, and the interference of leaf pigments in DOT-ELISA, suggested that DAS-ELISA was the more efficient serological detection method. Similar non-specific reactions in DOT-ELISA has previously been observed (Koenig & Burgermeister, 1986). DAS-ELISA has the further advantage that it is optically quantifiable. When compared with the nucleic acid hybridisation test, DAS-ELISA was more reliable, cheaper, easier to perform, and not dependant on hazardous radioactive materials. The reliability, simplicity and relatively low cost of the described assay make it suitable for large-scale routine testing, even in less sophisticated laboratories such as those of larger bulb growers.

Although serological techniques are considered to be the most practical approach to determine relationships among potyviruses (Van Regenmortel, 1982, Koenig, 1988), results are inconsistent and relationships seem very complex. The elimination of non-

specific cross-reacting antibodies from antiserum preparations by affinity chromatography, however, largely resolved potyvirus taxonomic problems (Shukla *et al.*, 1989a,b,c). Shukla *et al.* (1989a) argued that - because of the gradual removal of the virus-specific N- and C-termini of viral coat protein during virus purification and storage - antisera raised from freshly purified virus are especially well suited for distinguishing potyviruses, whereas antisera against aged preparations will detect more distant relationships. Although the value of conventional serological relationships studies - especially in determining broader relationships - are not denied, the abovementioned argument is supported. For this reason, and because of the limited availability of antisera, it was decided to determine the relatedness of OMV with other potyviruses more accurately by the comparison of coat protein nucleotide and deduced amino acid sequence data (Chapter 5).

CHAPTER 4

BIOLOGICAL ASPECTS OF ORNITHOGALUM MOSAIC VIRUS

BIOLOGICAL ASPECTS OF ORNITHOGALUM MOSAIC VIRUS

SUMMARY

An investigation of the biological properties of OMV confirmed that it was the causal agent for the mosaic disease of *Ornithogalum* and *Lachenalia* spp. in South Africa. Typical mosaic symptoms could be produced in virus-free ornithogalum and lachenalia plants by mechanical inoculation with infected plant sap or purified OMV, or by transmission of the virus using the aphid vector *Myzus persicae* (Sulz.). OMV was, however, not sap or aphid transmissible to any of a wide range of possible propagation hosts, or to a variety of ornamental bulbous plants. Similarly, viruses infecting some of these ornamentals, were not transmissible to ornithogalum. In a field survey of the regions to which ornithogalum and lachenalia are indigenous, OMV-infected ornithogalum plants were found in some remote uncultivated and undisturbed localities. This might be an indication that OMV is an indigenous viral pathogen of ornithogalum.

4.1 INTRODUCTION

Earlier publications on OMV reported mainly on biological aspects of the virus. Smith & Brierley (1944a) described fine green mottling on leaves, light- and dark-green blotches on flower stalks, and longitudinal streaks in flowers of *Ornithogalum thyrsoides*, grown in Oregon, USA. They further reported mechanical and aphid transmission of OMV to ornithogalum seedlings. Aphid transmissions from virus-infected lachenalia, galtonias and hyacinths resulted in OMV-like symptoms in ornithogalum seedlings. However, reciprocal transmissions to several members of the Amaryllidaceae, Iridaceae and Liliaceae were unsuccessful.

Klesser & Nel (1976) described at least ten different types of symptoms in virus-infected ornithogalum and lachenalia in South Africa. They did not, however, attribute all of these to OMV infection, but suggested that four possible viruses might be involved. These authors found OMV to be transmissible to ornithogalum seedlings by sap inoculation, aphid transmission and leaf grafts, but not by seed transmission. Their attempts to mechanically infect ornithogalum seedlings with the viruses from irises, daffodils, gladioli, hyacinths, tulips and ranunculi, were not successful.

Mosaic symptoms on leaves, and light- and dark-green blotches and ringspots on flower stalks of OMV-infected ornithogalum and lachenalia were described from the Netherlands by Derks (1979). Derks *et al.* (1983) reported the transmission of OMV (from infected plants imported from South Africa) to *Chenopodium quinoa, Tetragonium tetragonioides* and *Nicotiana clevelandii.*

The biological aspects of OMV were investigated for the following reasons:

1. to establish whether it is indeed the causal agent of the mosaic disease of ornithogalum and lachenalia, and if so, whether it was the only virus involved;
2. to find a possible alternative propagation host from which virus could be more efficiently extracted than the cumbersome method (from ornithogalum and lachenalia) which is currently used;

3. to determine whether OMV could infect other ornamental bulbous plants, and whether viruses from these plants could infect ornithogalum, i.e. to determine whether ornithogalum can be safely co-cultivated with other ornamental bulbs;
4. to determine the current geographical distribution of OMV in the southern and western Cape regions, to which most *Ornithogalum* and *Lachenalia* spp. are indigenous;
5. to establish whether OMV originated in South Africa or whether it entered the country in other imported bulbous plants.

4.2 MATERIALS AND METHODS

4.2.1 Plants

The origin and maintenance of OMV-infected ornithogalum and lachenalia plants are described in Appendix B.1.1.

Virus-free ornithogalum and lachenalia plants were regenerated from callus tissue obtained from the VOPRI (Appendix B.1.2). These symptomless plants were kept in aphid-proof cages and their virus-free status was periodically confirmed by DAS-ELISA.

Species tested as possible propagation hosts were *Chenopodium quinoa* Willd., *C. giganteum* Don., *C. album* L., *C. schraderanum* Roem. and Schult., *C. capitatum* (L.) Aschers., *Nicotiana tabacum* L. cv. Xanthi, *N. clevelandii* Gray, *N. benthamiana* Domin, *Tetragonia tetragonioides* (Pallas) Kuntz, *Phaseolus vulgaris* L. cv. Bonus, *Pisum sativum* L. cv. Greenfeast, *Petunia hybrida* (Hort.) Vilm.- Andr. and *Tropaeolum majus* L. Seedlings were grown from virus-free seed from Departmental stocks.

Amarine, nerine, Dutch iris, brodiaea, gladiolus, narcissus, sparaxis, freesia, tritonia, ixia and hyacinth bulbs and corms used in an epidemiological study, were bought from two local commercial nurseries.

4.2.2 Virus sources

OMV was purified as described in Section 2.2.3. Alternatively, sap extractions were made from OMV-infected ornithogalum and lachenalia plants by homogenising leaves in five volumes of 0.05 M Tris-Cl, pH 9.0.

4.2.3 Aphid origin and maintenance

A colony of an unidentified aphid species was found feeding spontaneously on wild-growing ornithogalum. These aphids died when they were brought into the laboratory. A colony of *Myzus persicae* (Sulz.), originating from the departmental stock, was starved for a day and transferred to virus-free ornithogalum plants. The plants were kept in pots in gauze-covered wooden cages (50 x 50 x 50 cm), which were placed on custom made trolleys fitted with VHO Gro-lux fluorescent lights. The cages were kept at 24-26°C on a 12 h day/night cycle. Barley plants were placed around cages to "catch" stray aphids (Von Wechmar, 1990).

4.2.4 Transmission tests

Two sets of transmission experiments were undertaken; the first to a variety of conventional virus propagation hosts in order to find possible alternative hosts for virus maintenance and purification, and the second to several commercially available ornamental bulbs as part of an epidemiological study.

4.2.4.1 Mechanical inoculations

Leaves of test plants were inoculated with leaf sap or freshly purified virus in 0.05 M Tris-Cl pH 9.0, with Celite as an abrasive. Inoculations to virus-free ornithogalum and lachenalia were included as positive controls; as negative controls, four specimens of each test species were inoculated with buffer and Celite only.

4.2.4.2 Aphid transmission

Aphids from maintenance colonies of *M. persicae* were starved for 4-6 h, then allowed an acquisition feeding period of 5-10 min on freshly cut infected ornithogalum leaves. Aphids were then transferred to virus-free test plants, and allowed to feed for 2 days before being exterminated by spraying with oxydemeton-methyl. Acquisition feeding on virus-free ornithogalum tissue culture explants was included as control.

4.2.5 Field collections

Leaves from individual ornithogalum and lachenalia plants were sampled during field surveys of the western and southern Cape coastal regions (see Fig. 4.1). Surveys were done during the flowering season in early spring.

4.2.6 Virus diagnosis

The presence of OMV in test plants and field-collected samples was assessed by symptomatology, DAS-ELISA and/or IEB. Leaves were visually evaluated for the presence of typical mosaic symptoms. DAS-ELISA and IEB were performed as described in Sections 3.2.6 and 3.2.7.

Sap from the leaves of 11 different ornamental bulbous plants were extracted and concentrated before serological testing. Approximately 10 g leaf material was homogenised in 5 volumes of 0.1 M phosphate buffer, pH 8.0. Solid material was removed by centrifugation, the supernatant was clarified by the addition of 1% Triton X-100, and concentrated by ultracentrifugation.

Field-collected ornithogalum and lachenalia samples were processed by homogenising 2-5 g of leaf material with a Pollaehne roller press in 5 volumes of PBS pH 7.4, containing 0.1% (v/v) Tween 20, 2% (w/v) PVP (M_r 44 000), 0.2% (w/v) skimmed milk powder, and 1 mg/ml hemicellulase. Homogenates were incubated overnight at room temperature, the solid material removed by centrifugation, and supernatants used undiluted in serological assays.

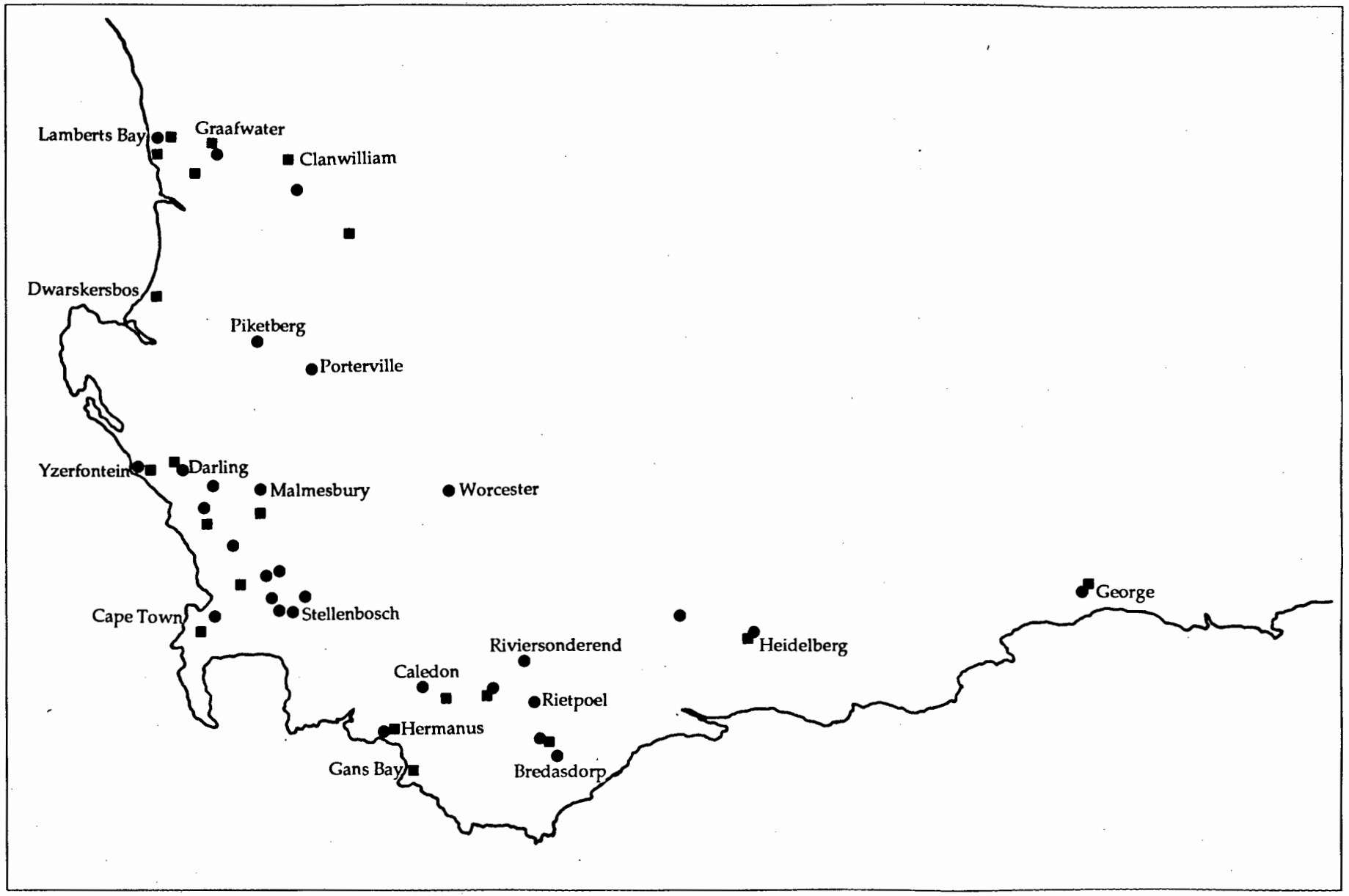

Fig. 4.1. Localities from where ornithogalum (●) and lachenalia (■) leaf samples were collected.

4.3 RESULTS

4.3.1 Symptomatology and reproduction of disease

OMV produced very distinct symptoms in ornithogalum and lachenalia. Infected leaves showed typical mosaic patterns of light- and dark-green mottling (Fig. 4.2). Symptoms on flower stalks were generally more pronounced and consisted of larger light- and dark-green blotches, with the lighter green areas often sunk into surrounding tissue (Fig. 4.3). In cases of severe infection, usually associated with agricultural cultivation, flower stunting and deformation occurred (Fig. 4.4).

Fig. 4.2. Ornithogalum mosaic virus symptoms on ornithogalum leaves.

In compliance with Koch's postulates, typical mosaic symptoms were reproduced in virus-free ornithogalum and lachenalia plants by mechanical inoculation with freshly-

purified OMV. Sucrose gradient-purified OMV was checked in an electron microscope for contaminating viruses before inoculation.

Fig. 4.3. Ornithogalum mosaic virus symptoms on ornithogalum flower stalks.

Fig. 4.4. Flower stunting and deformation in ornithogalum caused by severe OMV infection. Plant on the left is a healthy control.

4.3.2 Transmission to alternative propagation hosts

Results of transmission experiments are summarised in Table 4.1. Mechanical transmission with neither sap nor purified virus could be demonstrated to *T. tetragonioides, P. vulgaris, P. sativum, P. hybrida, T. majus* or any of the *Chenopodium* and *Nicotiana* spp. tested. OMV isolates were readily mechanically transmissible to virus-free ornithogalum and lachenalia explants. Purified virus preparations and sap from infected plants were equally infective. Typical systemic mosaic symptoms appeared 2-3 weeks post-inoculation. Virus in infected leaves kept at 4°C for 4 months were shown to remain infective when inoculated to virus-free plants.

It was assumed that - like most other potyviruses - OMV is aphid transmitted in the non-persistent manner. This was confirmed when *M. persicae* transmitted OMV in a manner consistent with non-persistent transmission to both ornithogalum and lachenalia. Aphid transmission experiments failed to produce typical virus symptoms in any of the other plant species tested.

4.3.3 Transmission to other ornamental bulbs

Some of the plants grown from the commercial bulbs - which were intended for transmission experiments - exhibited symptoms typical of virus disease. This prompted an investigation to determine the serological relationships of these unknown viruses with OMV. Sap extractions were made from individual plants with, and others without symptoms. The sap samples were tested in SDS-PAGE, IEB and DAS-ELISA (Appendices B.3.1, B.2.3 and Section 3.2.6.1). Possible virus coat protein bands in the 20-40 kDa range were observed in polyacrylamide gels for nerines, Dutch irises, hyacinths, narcissi, freesias and gladioli (not shown). Both sap inoculations and aphid transmissions of the virus(es) from these plants failed to produce symptoms in virus-free ornithogalum (Table 4.2). Only the virus(es) in Dutch iris and gladiolus were serologically related to OMV (Table 4.3).

Individual plants without any visible symptoms, and which did not produce any potential virus coat protein band in SDS-PAGE, were considered to be virus-free and were selected for reciprocal transmission tests. Aphid transmissions of OMV to these plants were unsuccessful (Table 4.1). However, OMV was successfully transmitted to virus-free ornithogalum controls.

Table 4.1 OMV transmission to potential propagation hosts

Host	Mechanical inoculation	Aphid transmission
C. quinoa	0/12 [a]	ND [b]
C. giganteum	0/8	ND
C. album	0/8	ND
C. schaderanum	0/8	ND
C. capitatum	0/8	ND
N. tabacum	0/8	ND
N. clevelandii	0/8	0/4
N. benthamiana	0/8	0/4
T. tetragonioides	0/6	ND
P. vulgaris	0/12	0/8
P. sativum	0/8	0/4
P. hybrida	0/8	ND
T. majus	0/8	ND
Ornithogalum	7/12	12/12
Lachenalia	4/8	8/8

[a] Number of plants with symptoms out of total inoculated.

[b] Not determined.

Table 4.2 Transmissibility of OMV and viruses infecting other bulbous ornamentals [a]

	Other viruses to ornithogalum		OMV to other bulbous ornamentals
Bulbs	Mechanical	Aphid	Aphid
Amarine	-	-	-
Nerine	-	-	ND [b]
Dutch iris	-	-	ND
Brodiaea	-	-	-
Hyacinth	-	-	ND
Narcissus	-	-	-
Sparaxis	-	-	-
Freesia	-	+/- [c]	-
Tritonia	-	-	-
Ixia	-	-	-
Gladiolus	-	-	-
Lachenalia	+	+	+
Ornithogalum	+	+	+

[a] Transmissions evaluated by symptom expression.

[b] Not determined, because all available plants were virus infected.

[c] Faint mosaic symptoms developed in one of the test plants only. This result is not considered a true positive transmission.

4.3.4 Geographical distribution of OMV in South Africa

Ornithogalum and lachenalia leaf samples were collected from 62 and 19 locations, respectively, in the western and southern Cape coastal regions (Fig. 4.1). Results of visual assessments and serological assays are projected in Table 4.4. OMV was detected in 26 of the ornithogalum samples, but was never found in any wild-growing lachenalias. Eighteen of the ornithogalum samples were taken from remote locations

with no or very limited agricultural activities in the vicinity. Of these, six were infected with OMV.

Table 4.3 The occurrence of viruses in a variety of ornamental bulbous plants and their serological relatedness to OMV

	Virus presence [a]		Relatedness [b]	
Bulbs	Symptoms	SDS-PAGE	DAS-ELISA	IEB
Amarine	-	-	-	-
Nerine	++	+	-	-
Dutch iris	++	+	+	++
Brodiaea	-	-	-	-
Hyacinth	+	+	-	+
Narcissus	++	+	-	-
Sparaxis	+	-	-	+
Freesia	+	+	-	+
Tritonia	+	-	-	-
Ixia	-	-	-	-
Gladiolus	++	+	+	++

[a] Plants were evaluated for virus infection by symptom expression (++ = severe symptoms, + = slight symptoms, - = no symptoms) and the presence of potential virus-specific bands in SDS-PAGE (+ = presence of a band in the 20-40 kDa range, - = no potential virus bands visible).

[b] Serological relatedness to OMV as tested by DAS-ELISA and IEB (++ = strong reaction, + = slight reaction, - = no reaction).

Table 4.4 Summary of OMV infection in field-collected ornithogalum and lachenalia [a]

	Symptoms [b]	DAS-ELISA [c]
Ornithogalum	27/62	26/62
Lachenalia	0/19	0/19

[a] See Fig. 4.1 for distribution of field collections.

[b] Number of symptom-bearing plants out of total number sampled.

[c] Number of plants reacting positively out of total number sampled. A sample was considered positive when its absorbance value was higher than the mean of the negative controls plus two standard deviations.

4.4 DISCUSSION

Most of the biological properties of plant viruses are not reliable indicators of their identity (Hamilton *et al.*, 1981, Moghal & Francki, 1981). Serological and physicochemical methods have largely taken their place as tools in virus identification and classification (Francki, 1980, Moghal & Francki, 1981). However, symptomatology, host range and mode of transmission are still widely used as guidelines for classification, especially in distinguishing strains of a particular virus (Jones & Diachun, 1977, Shukla *et al.*, 1988a,b). The contradictory results already published on OMV symptomatology, host range and mode of transmission (Smith & Brierley, 1944a, Klesser & Nel, 1976, Derks, 1979, Derks *et al.*, 1983), and those reported here, could be because of differences in experimental conditions and procedures, and are further proof that biological properties cannot be considered conclusive for virus classification.

In the present study, biological aspects of OMV infections were investigated to answer specific questions. First, OMV was confirmed as a plausible sole causal agent for what Smith and Brierley in 1944 called "Ornithogalum mosaic" disease: typical disease symptoms could be reproduced in virus-free ornithogalum and lachenalia by mechanical inoculation of highly-purified OMV. Symptoms were similar to those described for OMV by Smith & Brierley (1944a) and Derks (1979). However, streaking,

leafroll, leaf enations, necrotic specks and scorch, and ring patterns, as reported by Klesser & Nel (1976), were never observed.

If a second virus was involved in the mosaic disease, one would have expected symptoms in one or more of the alternative propagation host plants which were sap inoculated. This, however, did not occur. A second unidentified virus was discovered in OMV-infected ornithogalum and lachenalia plants which were kept in one of the large outdoor cages, during the growing season of 1987. Characterisation of this virus was beyond the scope of this study, but preliminary investigations revealed the following: the virus did not effect existing symptoms in ornithogalum and lachenalia in any way; the icosahedral virions had a diameter of approximately 28 nm (Fig. 4.5), and a coat protein of approximately 25 kDa, and the virus was not related to CMV, which was considered the most likely contaminant (Burger, unpublished results). Because this virus was only found in a single isolated cage - in which it had not been noticed during the previous growing season - it is believed that this was a secondary infection by an opportunist viral pathogen, and that this virus does not contribute to the natural mosaic disease of ornithogalum and lachenalia. However, this shows that ornithogalum and lachenalia are susceptible to infection by other viruses which may be responsible for symptoms similar to those observed by Klesser & Nel (1976).

Second, all attempts to find alternative propagation hosts failed. Derks *et al.* (1983) reported the successful transmission of OMV to *C. quinoa*, *T. tetragonioides* and *N. clevelandii* with sap from ornithogalum plants imported from South Africa. Transmissions to a wide variety of probable propagation hosts, including *C. quinoa*, *T. tetragonioides* and *N. clevelandii*, were all unsuccessful. The differences in OMV transmissibility reported earlier (Derks *et al.*, 1983), and those reported here can possibly be attributed to different experimental conditions and procedures. However, results confirmed a very narrow host range for OMV, which is typical of many potyviruses (Shukla *et al.*, 1989a).

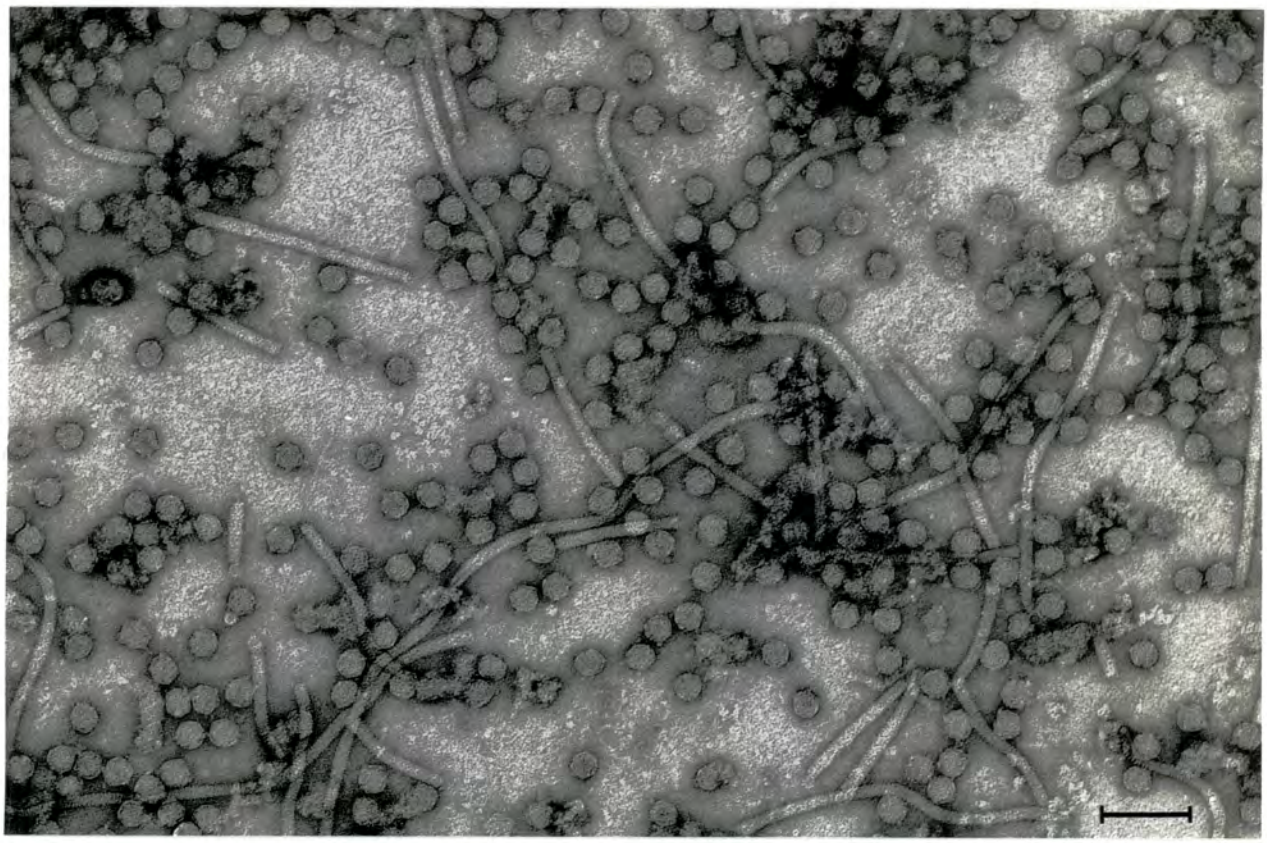

Fig. 4.5. The unknown icosahedral virus which infected ornithogalum and lachenalia. This virus banded with broken fragments of OMV in sucrose gradients.
Scale bar = 100 nm.

Third, the possibilities that OMV could be a pathogen of other ornamental bulbs, and that viruses from other ornamental bulbs could infect ornithogalum, were investigated. It was not possible, either by sap inoculation or by aphid transmission, to transmit OMV to any of a variety of bulbous plants. Moreover, in reciprocal experiments, viruses infecting these plants failed to infect virus-free ornithogalum. This suggests the virus has an extremely restricted host range, which makes ornithogalum and lachenalia fairly "safe" crops for co-cultivation with other ornamental bulbs.

Fourth, the current geographical distribution of OMV in wild-growing *Ornithogalum* and *Lachenalia* spp. was investigated. The majority of *Ornithogalum* and *Lachenalia* spp. are indigenous to the southern and western Cape regions of South Africa. OMV-infected ornithogalum plants were regularly found, usually at locations close to cultivated areas. However, the virus was also found in a few very remote semi-arid locations where agricultural activities are limited to stock farming. Wild-growing OMV-infected lachenalia plants were never found, indicating that perhaps the virus

adventitiously infects this genus in cultivation only, and that *Lachenalia* spp. are not one of the natural hosts of the virus.

Finally, there appear to be two possibilities for the origin of OMV. The first is that OMV is a virus native to *Ornithogalum* spp. in South Africa. The second is that OMV came from foreign ornamental bulbous plants, as a result of the co-cultivation of ornithogalum and lachenalia with these plants in countries like the Netherlands.

Most apparently virus-free ornithogalum and lachenalia plants which are introduced from their natural habitat into a cultivated environment, become virus-infected within one or two growing seasons. This would suggest that these plants become infected with a virus or viruses from other plants. However, transmission studies showed that viruses from a few other ornamental bulbs do not readily infect ornithogalum. A possible explanation for the appearance of symptoms might be latent OMV infections in wild-growing plants which are triggered to become virulent by the milder conditions of cultivation. The extremely dry and hot climatic conditions under which these plants naturally occur, might be responsible for such low virus concentrations in plants that virus presence and symptom expression cannot be detected.

If the restricted host range of OMV and the occurrence of OMV-infected ornithogalum plants in remote virgin land locations are taken into consideration, it is proposed that OMV is native to ornithogalum and originated in South Africa. OMV probably reached Europe through the early export trade of ornithogalum and lachenalia bulbs and flowers.

CHAPTER 5

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCING OF ORNITHOGALUM MOSAIC VIRUS

MOLECULAR CLONING AND NUCLEOTIDE SEQUENCING OF ORNITHOGALUM MOSAIC VIRUS

SUMMARY

DNA complementary to the 3'-terminal 3684 nucleotides of the OMV genome was cloned and sequenced. The sequence consisted of a single large open reading frame which probably starts upstream of the cloned region. By comparison to other sequenced potyviruses, it was estimated that the clone contained the 3' non-coding region, the coat protein gene, the large nuclear inclusion protein (NIb) gene, as well as approximately 85% of the small nuclear inclusion protein (NIa) gene. The 3'-NC region of 274 nucleotides showed 38% to 45% similarity to corresponding regions of other potyviruses. The putative CP gene could encode a 253 amino acid coat protein with a calculated M_r of 28 807. Analysis of predicted amino acid sequences of OMV and those of other potyviruses showed similarities of 66% to 77% for the CP, 72% to 73% for the NIb and 63% to 71% for the partial NIa proteins. These data as well as phylogenetic analysis of the CP sequences confirmed that OMV is a typical but taxonomically distinct potyvirus.

5.1 INTRODUCTION

All earlier characterisation studies on OMV strongly suggest that it is a typical potyvirus; however, as there are at present over 175 definitive and possible potyviruses listed (Milne, 1988), it becomes increasingly important to establish good criteria for deciding whether new viruses are taxonomically distinct from established viruses. While there is increasing evidence that certain "distinct" potyviruses should actually be considered as strains of others - for example, PeMV can be considered a strain of PVY (Shukla *et al.*, 1988a, Frenkel *et al.*, 1989, Hay *et al.*, 1989, Van der Vlugt *et al.*, 1989) - certain other viruses like SMV and SCMV were shown to each consist of more than one distinct virus (Shukla & Ward, 1988, Shukla *et al.*, 1989c).

Molecular characterisation of potyviruses has shown that they share certain fundamental properties: the genomes are monopartite, plus-sense, single stranded RNA molecules of approximately 10 Kb, with a small genome-linked protein covalently linked at the 5' end of the genome (Hari, 1981, Siaw *et al.*, 1985, Shahabuddin *et al.*, 1988, Riechmann *et al.*, 1989), while the 3' end is polyadenylated (Hari *et al.*, 1979). *In vitro* translation studies on a number of potyviruses have shown that the genomic RNA is translated to a single large polyprotein precursor of about 350 kDa, which is subsequently post-translationally cleaved by at least two virus-encoded proteinases into functional, mature viral proteins (Vance & Beachy, 1984, Allison *et al.*, 1985b, Allison *et al.*, 1986, Carrington & Dougherty, 1987b, Chang *et al.*, 1988, Hellmann *et al.*, 1988, Carrington *et al.*, 1989a). Genomic sequencing of TEV and TVMV has enabled the mapping of eight potentially functional proteins on the potyviral polyprotein (Dougherty & Carrington, 1988). Both the amino acid sequence of the CP gene (Shukla & Ward, 1988, 1989a,b) and the nucleotide sequence of the 3'-NC region (Frenkel *et al.*, 1989) have recently been proposed as tools for potyvirus classification: Shukla & Ward (1989b) in particular have demonstrated that alignment of CP sequences of 25 strains of 11 distinct potyviruses gives a phylogenetic tree consistent with other observations on the grouping of potyviruses.

The molecular cloning and nucleotide sequencing of OMV was undertaken to confirm that OMV is indeed a distinct potyvirus. The availability of OMV clones also provided tools for other potential practical applications in this research: for instance,

radiolabelled DNA from cloned viral cDNA can be used in virus diagnostics. Subcloning and expression in plants of virus genes (e.g. the coat protein gene) can be employed as a strategy to protect plants against virus infection (Powell-Abel *et al.*, 1986, Loesch-Fries *et al.*, 1987, Stark & Beachy, 1989).

This chapter reports the cloning and sequencing of the 3'-terminal 3684 nucleotides of the OMV genome, including putative CP and NIb genes, as well as the largest part of a NIa gene homologue. Analyses of CP and other sequence data, and their relevance to the classification of OMV as a distinct potyvirus, are discussed.

The work described in this chapter was previously published (Burger *et al.*, 1990). The sequence data reported here appear in the DNA Databank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL) and Genbank Nucleotide Sequence Databases under the accession number D00615.

5.2 MATERIALS AND METHODS

5.2.1 Virus purification and RNA extraction

OMV was purified from systemically infected *O. thyrsoides* , and RNA extracted from the virions, as described in Sections 2.2.3 and 2.2.6.

5.2.2 Synthesis and cloning of complementary DNA

The detailed cDNA synthesis and cloning protocols are included in Appendix B.4.12. Briefly, cDNA was synthesized from freshly prepared OMV genomic RNA using M-MulV reverse transcriptase, and oligo $d(T)_{12-18}$ as primer. The second DNA strand was synthesized according to the method of Gubler & Hoffman (1983), using RNase H, DNA polymerase I and T4 DNA polymerase. Both first and second strand synthesis reactions were assayed by the incorporation of [α-^{32}P]dCTP. Blunt-ended double-stranded DNA was cloned into the *Sma* I site of the pUC19 polylinker (Yanisch-Perron *et al.*, 1985, see Fig 5.1 for cloning strategy).

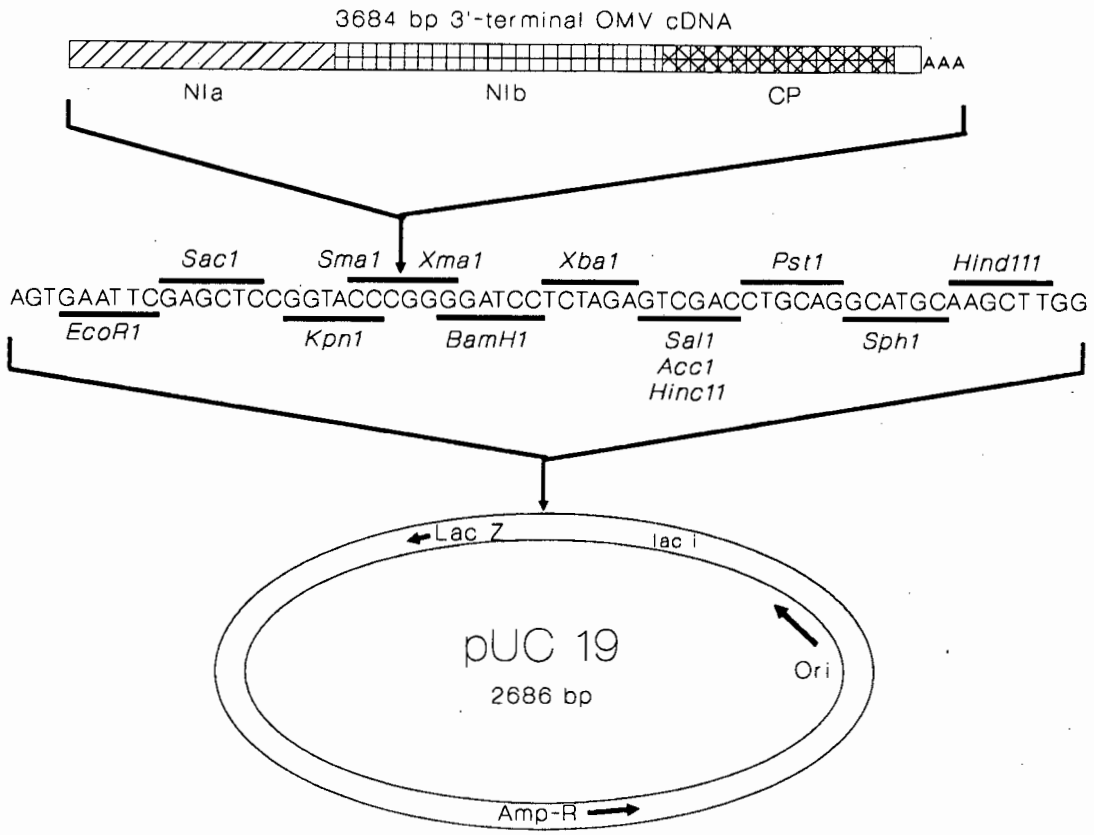

Fig. 5.1. cDNA cloning strategy for pOM16. The 3'-terminal 3684 nucleotides of the OMV genome was inserted in the *Sma* I site of the pUC19 polylinker.

Recombinant plasmids were used to transform *E. coli* LK-111 competent cells (Appendix B.4.13). White colonies, indicating insertional inactivation of the β-galactosidase gene of the vector (Vieira & Messing, 1982), were selected for colony hybridisation assays (Appendix B.4.9). 5'-end-labelled OMV genomic RNA was used as probe in colony hybridisations. RNA was end-labelled according to Chaconas & Van de Sande (1980): 1 μg RNA was mixed with 25 μCi [γ-^{32}P]dATP, 10 U polynucleotide kinase (Boehringer Mannheim) and 5 μl 4X labelling buffer (50 mM Tris-Cl pH 9.5; 45 mM $MgCl_2$; 5 mM DTT) in a 20 μl reaction volume, and incubated at 37°C for 60 min. The reaction was stopped on ice by the addition of 20 μl 0.5 M EDTA, and unincorporated nucleotides removed by Sephadex column chromatography (Appendix B.4.4). RNA end-labelling typically yielded probes of specific activity 1 x 10^6 to 1 x 10^7 dpm/μg.

5.2.3 Characterisation of OMV clones

Clones which were positive in colony hybridisation assays were analysed by restriction enzyme digestion and agarose gel electrophoresis to determine relative insert orientations and sizes, and to find restriction sites for subcloning and exonuclease III shortening experiments.

Small-scale plasmid DNA preparations of all positive clones were made (Appendix B.4.1), and digested with a number of restriction enzymes to find a unique site for plasmid linearisation (Appendix B.4.2). Plasmids were eventually linearised with *Pst* I and sized in a large 0.8% agarose gel. OMV-specific inserts from all clones were excised by *Pst* I/*Kpn* I digestion, fractionated by agarose gel electrophoresis, and transferred to nylon membranes by Southern blotting (Appendix B.4.7). Plasmid DNA from two of the smaller clones (pOM2 and pOM8) was ^{32}P-labelled by nick-translation (Appendix B.4.8) and used as probe to confirm cross-hybridisation amongst clones (Appendix B.4.10).

The largest non-repetitive clone (pOM16, 3.7 Kb) was digested with *Eco* RI, *Sac* I, *Kpn* I, *Bam* HI, *Xba* I, *Acc* I, *Hinc* II, *Sal* I, *Pst* I, *Sph* I and *Hind* III (all with a unique site in the pUC18/19 polylinker) to find enzymes without any internal sites in the OMV insert, for use in subcloning and shortening experiments.

5.2.4 Subcloning of the OMV insert in pUC18

In order to completely sequence both strands, the OMV-specific insert from the largest non-repetitive cDNA clone (pOM16) was excised using *Bam* HI and *Kpn* I. Insert and vector DNA were separated by agarose gel electrophoresis (Appendix B.4.5); the insert was purified by adsorption to and elution from DEAE cellulose membrane (Appendix B.4.6), and ligated into *Bam* HI/*Kpn* I digested pUC18, to create pOM21 (Fig. 5.2). Ligation reactions were carried out by incubating equimolar quantities of vector and insert (100 ng vector : 140 ng insert) at 15°C for 16 h, in a 20 µl ligation buffer (50 mM Tris-Cl pH 7.6; 10 mM $MgCl_2$; 1 mM DTT; 1 mM ATP) reaction volume containing 1 U T4 DNA ligase.

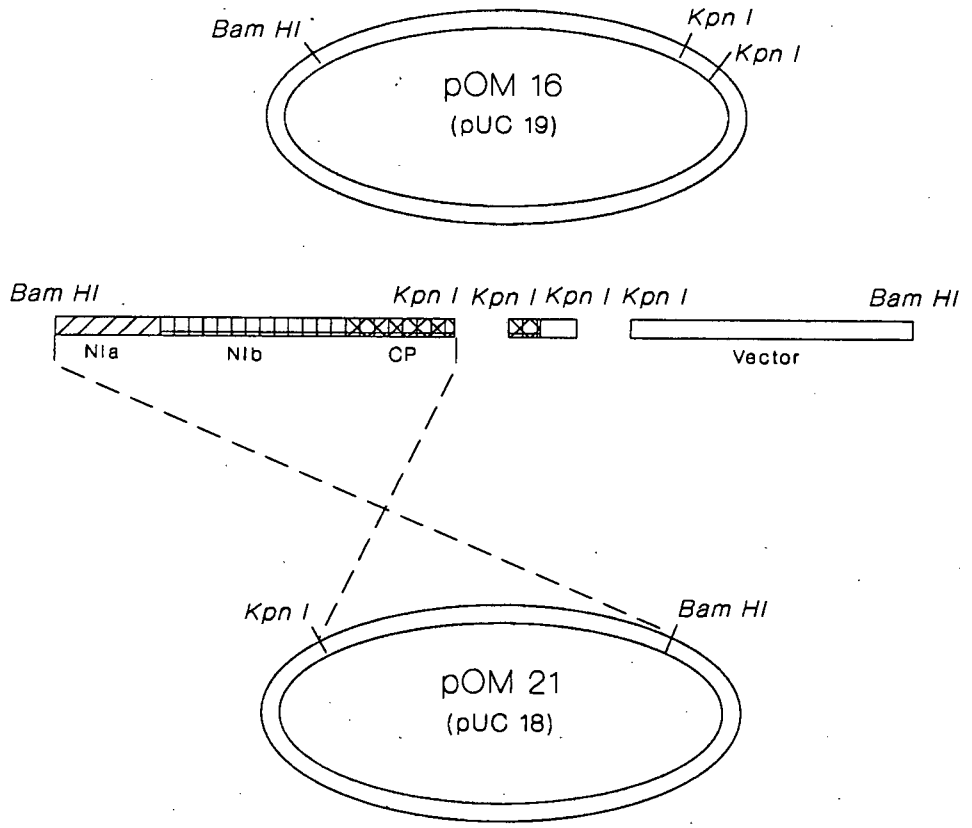

Fig. 5.2. Subcloning strategy for pOM21. A *Bam* HI/*Kpn* I fragment was excised from pOM16 and cloned in the opposite orientation into pUC18. The resultant plasmid (pOM21) lacked the 3'-terminal 372 bp of pOM16 because of an internal pOM16 *Kpn* I site.

5.2.5 Exonuclease III/S1 nuclease shortening

Sets of overlapping deletion mutants were created for both pOM16 and pOM21 by unidirectional shortening using exonuclease III and S1 nuclease in a protocol adapted from Henikoff (1984) (see Appendix B.4.14 for the detailed protocol). Both pOM16 and pOM21 were digested with *Pst* I to create a 5' overhang in the pUC18/19 polylinker for exonuclease III digestion, and with *Bam* HI to create a 3' overhang for protection against exonuclease III digestion in two directions. After ligation, transformation and selection, three bacterial colonies were selected for each time interval. Plasmid DNA was isolated from each and digested with *Hind* III and *Eco* RI - which have restriction sites on the 5'- and 3'-termini of the polylinker - to ensure that the flanking sequencing primer binding sites had not been destroyed during the shortening reactions.

5.2.6 Nucleotide sequence determination

Nucleotide sequencing was performed by the dideoxy chain termination method of Sanger *et al.*, (1977) using modified T7 DNA polymerase (Tabor and Richardson, 1987) from a Sequenase version 2.0 kit (US Biochemicals). The universal sequencing primers 5'-CAGGAAACAGCTATGAC-3' (Amersham) and 5'-GTTTTCCCAGTCACGAC-3' (US Biochemicals) were used for pOM16 and pOM21 respectively. Labelling and termination reactions were carried out in the presence of 10% DMSO (Winship, 1989).

Double-stranded template DNA used in sequencing reactions was purified by caesium chloride density centrifugation (Appendix B.4.1), and treated with RNase A. Primers (1 pmol) were annealed to 5 µg alkaline denatured template DNA in 10 µl 40 mM Tris-Cl pH 7.5; 20 mM $MgCl_2$; 50 mM NaCl, by heating to 40°C for 5 min followed by slow cooling (15 min) to room temperature. To the 10 µl reaction mix were added the following: 1 µl 0.1 M DTT, 2 µl labelling mix (1.5 µM each of dCTP, dGTP and dTTP), 1.75 µl DMSO, 0.5 µl [α-^{35}S]dATP (1000 Ci/mmol; 10 µCi/µl) and 2 µl Sequenase (1/8-dilution in ice-cold 10 mM Tris-Cl pH 7.5; 5 mM DTT; 0.5 mg/ml BSA). Labelling reactions were thoroughly mixed and incubated at 20°C for 3 min. Aliquots (4.3 µl) were then removed to four prewarmed microfuge tubes, each containing one of the four ddNTP termination mixes. The ddATP, ddCTP, ddGTP and ddTTP termination mixes each contained 80 µM of dATP, dCTP, dGTP and dTTP, 50 mM NaCl, and 8 µM of the appropriate ddNTP. Termination reactions were incubated at 45°C for 5 min before being stopped by the addition of 5 µl stop buffer (95% formamide; 20 mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol). Samples were denatured at 80°C for 2 min just before loading onto gels. Denaturing acrylamide gels (40 cm x 30 cm x 0.26 mm) of 4.8% and 6% (w/v) polyacrylamide (BDH Electran) containing 7 M urea (ICN Biomedicals Inc., Cleveland, OH) were run between siliconised glass plates in a vertical sequencing apparatus (Omeg Scientific, South Africa). "Short" and "long" runs were performed at 96 W for approximately 60 min, and 92 W for 3-4 h, using 6% and 4.8% gels respectively. Gels were dried onto filter paper and exposed to Curix RP 1 X-ray film (Appendix B.4.11).

5.2.7 Sequence analysis

Computer analysis of sequence data and compilation of the partial genomic sequence were done using Genepro version 4.0 software (Riverside Scientific, Ca., USA) on an IBM-compatible microcomputer. This software was also used for the preliminary comparison of nucleotide and predicted amino acid sequences of OMV and other potyviruses, which were obtained from the Genbank sequence database and from recently published papers (for references see Table 5.1). Pairwise alignments of the putative CP, NIb and NIa-homologous amino acid sequences of several sequenced potyviruses were done using the Gap programme of the University of Wisconsin Genetics Computer Group (GCG) package version 6.1 (Devereux *et al.*, 1984) run on a DEC\VAX 6000-330 mainframe computer. The LineUp and Distances programmes of the same package were used for multiple sequence alignment and presentation, and for pairwise percent sequence similarity determination, respectively. The KITSCH programme of the Phylogeny Inference Package (PHYLIP) version 3.1 (J. Felsenstein, Department of Genetics, University of Washington, Seattle) was used for the calculation of a phylogenetic tree from a distance data matrix derived from the GCG Distances output. This programme assumes a molecular clock and contemporaneous tip species (Felsenstein, 1988).

5.3 RESULTS AND DISCUSSION

5.3.1 cDNA cloning

The first strand synthesis reaction yielded approximately 173 ng RNA/cDNA hybrid which suggests that 8.7% of input RNA was transcribed into first strand cDNA. During the second strand synthesis reaction 158 ng (91%) of the RNA/cDNA hybrid was converted into ds-cDNA. A blunt-end ligation reaction using 100 ng cDNA and 50 ng *Sma* I-digested pUC19 yielded several hundred transformants from which approximately 100 were selected for colony hybridisation assays. Eighteen of these hybridised positively when probed with [γ-^{32}P]dATP-labelled OMV RNA (Fig. 5.3).

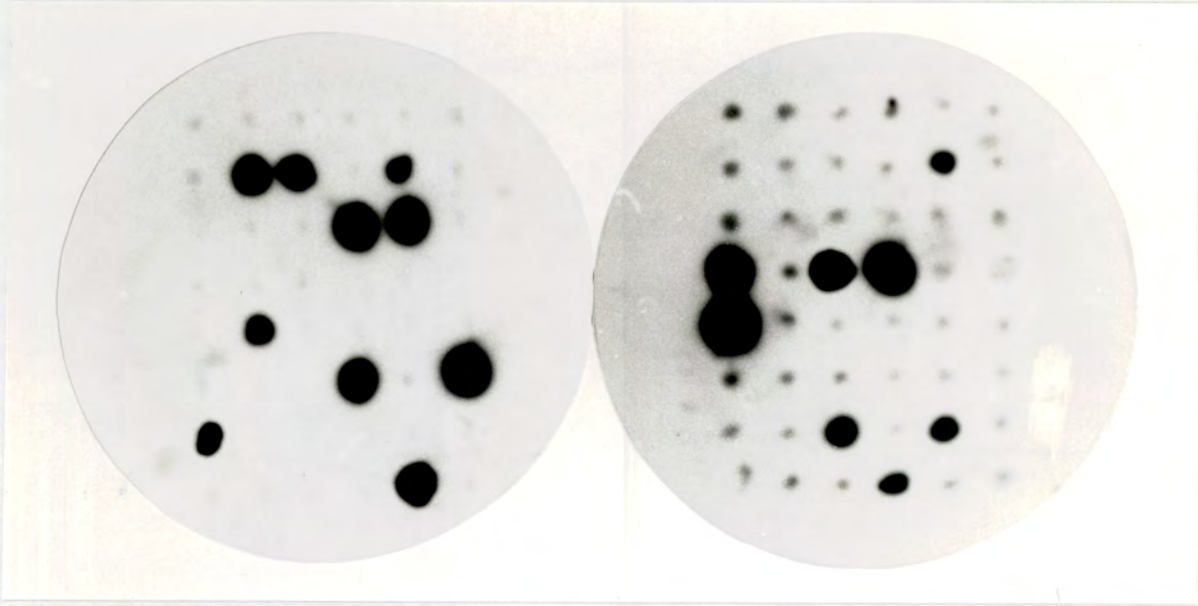

Fig. 5.3. Colony hybridisation of OMV cDNA clones. [γ-^{32}P]dATP-labelled OMV RNA was used as probe.

5.3.2 Characterisation of OMV clones

All 18 positively identified clones (pOM1 to pOM18) were linearised with *Pst* I and sized in a large 0.8% agarose gel (Fig. 5.4). Insert sizes varied from approximately 200 bp (pOM17, Fig. 5.4, lane 19) to one apparently of 4.2 Kb (pOM3, Fig. 5.4, lane 5). Nucleotide sequencing of the 5'- and 3'-termini of this large clone indicated that it was a concatamer of smaller sequences, since both its termini were contained within the sequence of the largest non-repetitive clone (pOM16), which had a 3.7 Kb insert (see below).

Cross-hybridisation amongst clones was confirmed when excised inserts from all 18 clones hybridised positively to labelled DNA from two of the smallest clones in Southern blot analysis (Fig. 5.5).

To find unique restriction sites for subcloning and exonuclease III shortening experiments, pOM16 was digested with all the pUC18/19 polylinker restriction enzymes. Only *Bam* HI, *Xba* I, *Sal* I and *Pst* I had no internal sites in the pOM16 insert sequence (Fig. 5.6) and could therefore be used for these experiments.

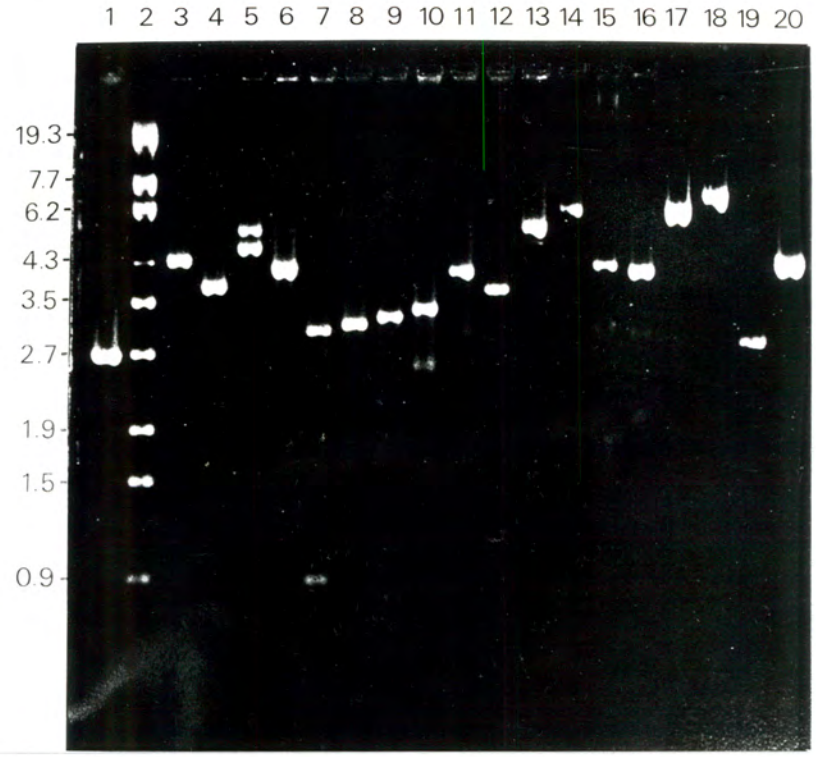

Fig. 5.4. OMV cDNA clone sizes. Plasmids were linearised with *Pst* I and electrophoresed in 0.8% agarose gels. Lane 1, pUC19; lane 2, M_r marker DNA (λ DNA digested with *Sty* I, sizes in Kb); lanes 3 to 20, pOM1 to pOM18.

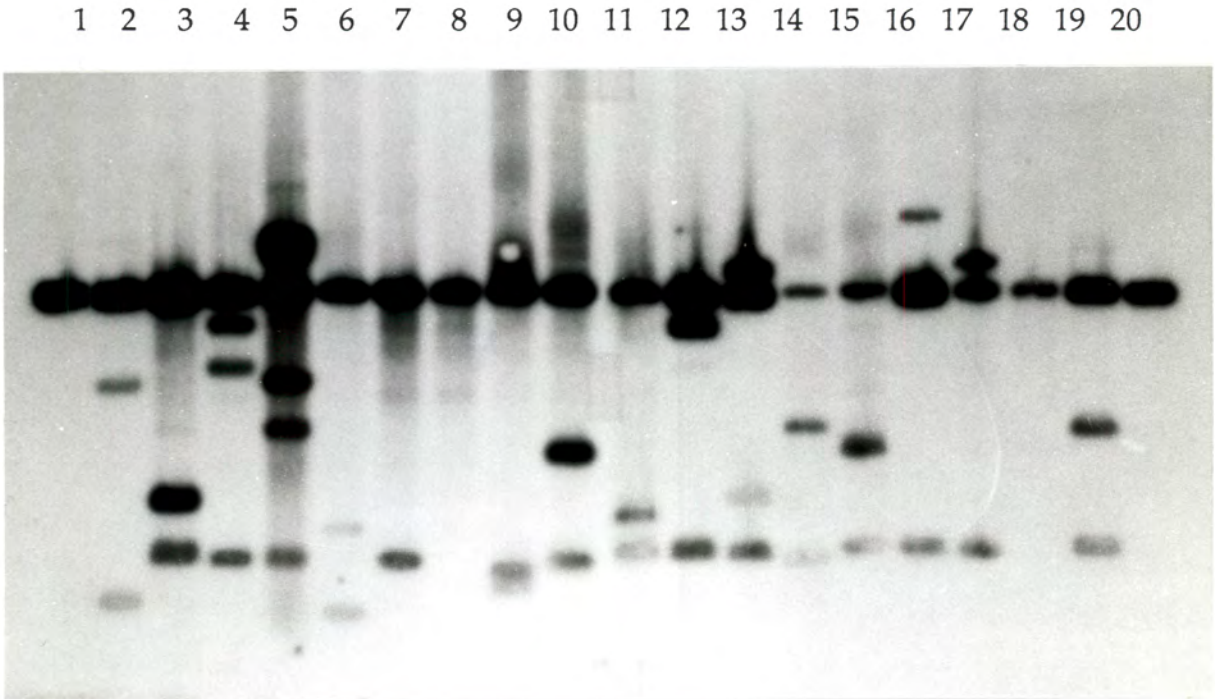

Fig. 5.5. Southern blot hybridisation of eighteen OMV clones. Inserts were excised from the pUC19 vector by *Pst* I/*Kpn* I digestion and probed with nick-translated linearised DNA from one of the smaller clones, pOM2 (1.2 Kb). Lane 1, linearised pUC19, lanes 2-19, pOM1 - pOM18, lane 20, linearised pUC19.

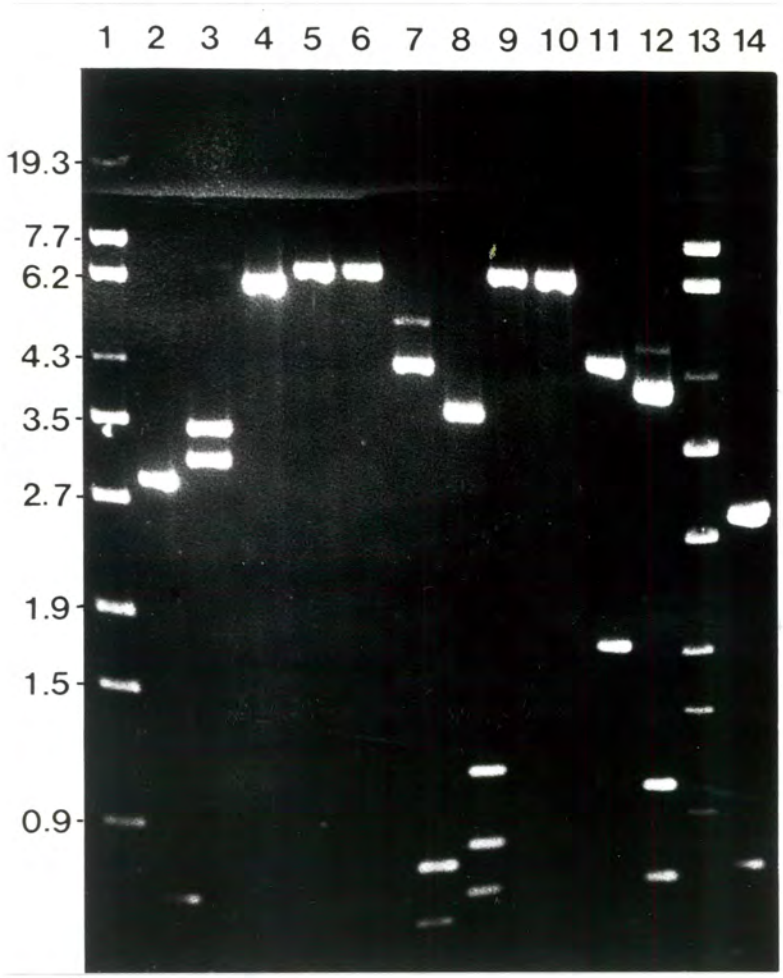

Fig. 5.6. pOM16 digested with the pUC18/pUC19 polylinker restriction enzymes in order to find enzymes without internal sites for use in subcloning and shortening. Lane 1, λ/*Sty* I marker DNA; lane 2, *EcoR* I; lane 3, *Sac* I; lane 4, *Kpn* I; lane 5, *BamH* I; lane 6, *Xba* I; lane 7, *Acc* I; lane 8, *Hinc* II; lane 9, *Sal* I; lane 10, *Pst* I; lane 11, *Sph* I; lane 12, *Hind* III; lane 13, λ/*Sty* I marker DNA, lane 14, pUC19 digested with *Pst* I.

5.3.3 Subcloning in pUC18

Since there were not enough unique restriction sites in the pUC19 polylinker to do Henikoff shortening reactions in both directions, the OMV-specific insert was excised from pOM16 using *Bam* HI and *Kpn* I and ligated into pUC18 which was digested with the same two enzymes (see Fig. 5.2). The resultant plasmid, pOM21, lacked the 3'-terminal 372 base pairs of pOM16 because of an internal pOM16 *Kpn* I site.

5.3.4 Exonuclease III/S1 shortening

Sixteen aliquots (T=0 to T=15) were removed at 30 sec intervals during the shortening reaction, and fractions of these were electrophoresed in an agarose gel. The shortening

reactions extended through the entire OMV-specific inserts of both pOM16 and pOM21 (Fig. 5.7). After they were tested for the integrity of their flanking sequencing primer binding regions, 16 overlapping deletion mutants were selected for the sequencing of pOM16, and 18 for pOM21, respectively. These decreased in size by 200-500 bp increments. See Fig. 5.8 for clone orientations and sequencing strategy.

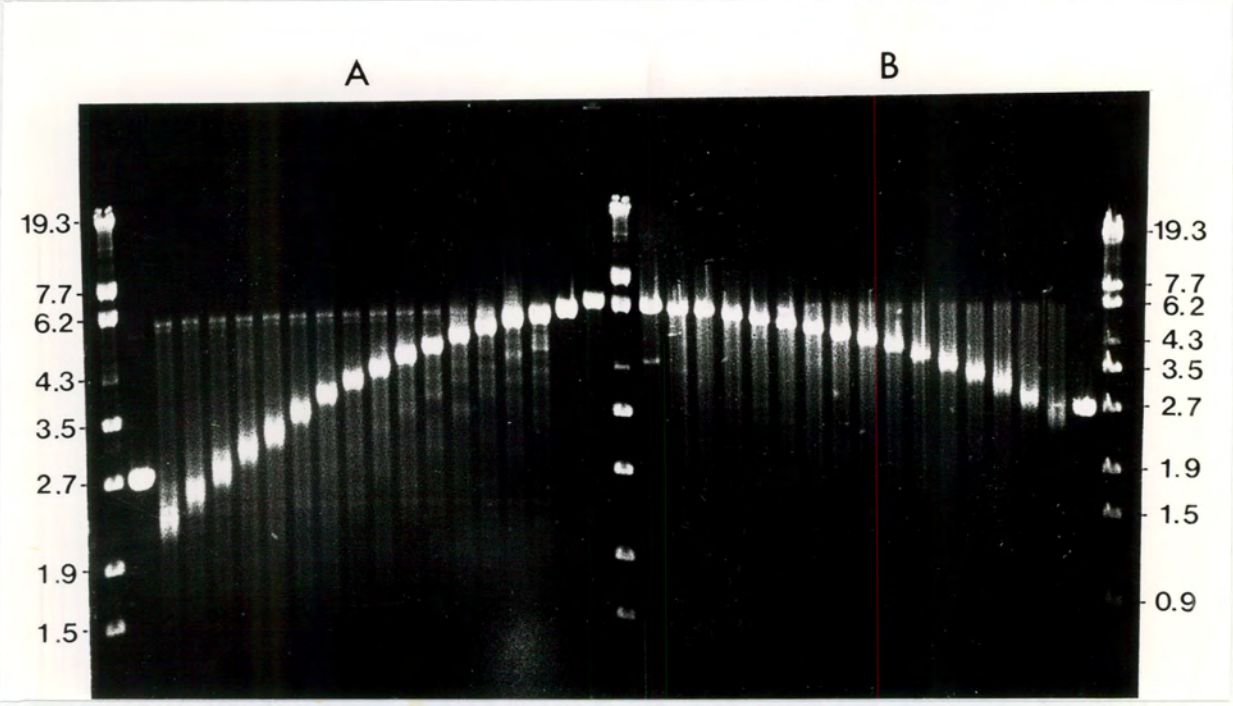

Fig. 5.7. Exonuclease III/S1 nuclease shortening of pOM16 (A), and pOM21 (B). The two lanes on either side of the central marker DNA (λ/*Sty* I) lane contain linearised pOM16 and pOM21, while the two lanes inside the two outer marker DNA lanes contain linearised pUC19.

5.3.5 Nucleotide sequencing

The inclusion of 10% DMSO in labelling and termination reactions resulted in generally darker and sharper bands; it also greatly reduced GC compressions in the gels. Typically, 200-250 bases could be read from a 6% acrylamide denaturing gel during a short run, whilst sequences up to 550 bases away from the primer could be resolved in 4.8% gels after 3-4 h runs. The entire sequence was assembled from alignment of overlapping fragments on both strands, as shown in Fig. 5.8.

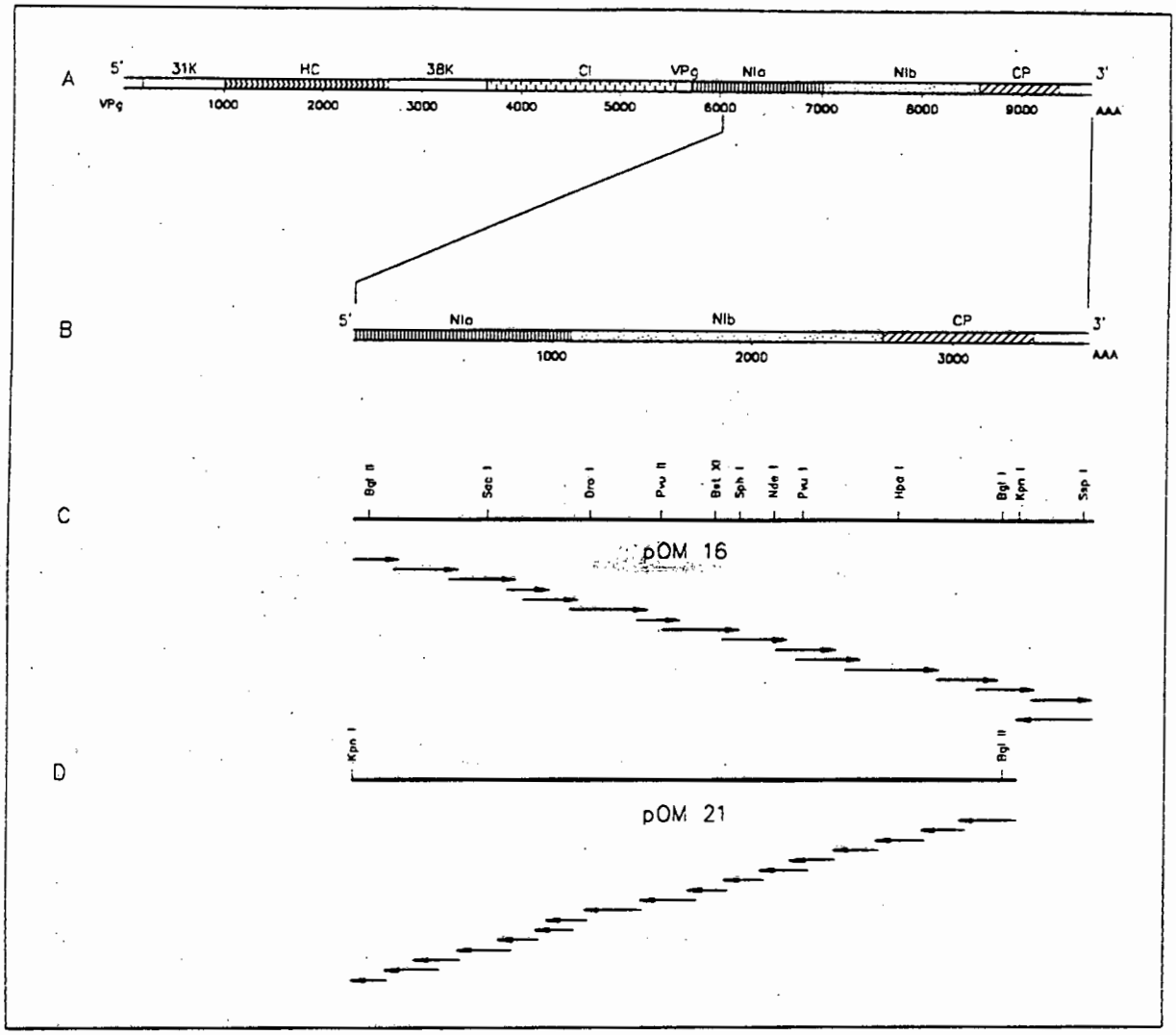

Fig. 5.8. OMV clone orientation and sequencing strategy. A, Genome map of PVY showing positions of putative genes (adapted from Robaglia *et al.*, 1989); B, Relative genomic position and extent of pOM16; C, Deduced restriction map and orientation of pOM16; D, Orientation and relative size of pOM21. The arrows show the location, direction and length of nucleotide sequence obtained from each deletion mutant generated by Henikoff shortening of pOM16 and pOM21.

The nucleotide sequence of the 3684 OMV-specific bases of pOM16, and the deduced amino acid sequences coded for by this sequence, are presented in Fig. 5.9. Computer analysis revealed a single large ORF of 3408 nucleotides in reading frame 3 of the virion-sense cDNA (Fig. 5.10). No other significant ORFs were observed in any of the other 5 possible reading frames. A TGA termination codon was identified 274 nucleotides upstream of a 23 residue 3' polyadenylate tract, and was followed by seven other termination codons in the same reading frame (Fig. 5.10).

```
GAA TAC ACC ATT GTT CGA TAT GTT GAC CCA CTC ACT GGA GCG ACG CAA GAT GAG AAT CCT    62
 E   Y   T   I   V   R   Y   V   D   P   L   T   G   A   T   Q   D   E   N   P   -1117

TTG ATG GCA ATA GAT CTG GTC CAG GAG TAT TTC GCT AAA ATT CGT AGC CAA TTA GTG TCT   122
 L   M   A   I   D   L   V   Q   E   Y   F   A   K   I   R   S   Q   L   V   S   -1097

GAA GAA AAA CTG GAA ACT CAA AAC ATC ATT GCG AAT CCA GGA ATT CAA GCT TAT TAC ATG   182
 E   E   K   L   E   T   Q   N   I   I   A   N   P   G   I   Q   A   Y   Y   M   -1077

AAG AAC AGA GGA GAT GCT GCA CTA AAA GTT GAT TTA ACT CCG CAT AAT CCT CTG TTA GTT   242
 K   N   R   G   D   A   A   L   K   V   D   L   T   P   H   N   P   L   L   V   -1057

ACG AAG ACT GGA ACC ATT GCA GGT TTT CCT GAG AAT GAG TTC ATT CTC CGA CAA ACA GGC   302
 T   K   T   G   T   I   A   G   F   P   E   N   E   F   I   L   R   Q   T   G   -1037

AAG GCA GTT AAT GTT AAA ATG TCG GAA GTG CCA GTG GAG AAT GAA CTT GAA GAA GTT GAG   362
 K   A   V   N   V   K   M   S   E   V   P   V   E   N   E   L   E   E   V   E   -1017

CAT GAA GGG AAG AAC TTG AAT AGA GGA CTT CGA GAC TAC AAC GTG GTT TCA AAT GTG GTG   422
 H   E   G   K   N   L   N   R   G   L   R   D   Y   N   V   V   S   N   V   V   -997

TGC CGC CTT ACA AAT GAA TCC GAT GGC CAC TCG GCC TCG CTC TTT GGT TTG GGT TAT GGT   482
 C   R   L   T   N   E   S   D   G   H   S   A   S   L   F   G   L   G   Y   G   -977

GGA TAC ATC ATC ACG AAC AGA CAC CTG TTC AAG AAC AAC AAC GGT ACT CTC AAA GTT CAA   542
 G   Y   I   I   T   N   R   H   L   F   K   N   N   N   G   T   L   K   V   Q   -957
                           ▲

TCA CAA CAT GGG GAC TTC ATT GTT AAA AAC ACA ACA CAA CTC AAA ATG GTG CCA GTT GGA   602
 S   Q   H   G   D   F   I   V   K   N   T   T   Q   L   K   M   V   P   V   G   -937

AAG ACA GAT ATC TTA ATA ATT CGT ATG CCA AAG GAT TTT CCA GTC CTT CCC CAG AAA CTT   662
 K   T   D   I   L   I   I   R   M   P   K   D   F   P   V   L   P   Q   K   L   -917
         ▲

CGA TTT CGA GCT CCT GCA AAC GAG GAC AAA GTC TGT CTA ATA GCT TCG AAT TTT CAA GAG   722
 R   F   R   A   P   A   N   E   D   K   V   C   L   I   A   S   N   F   Q   E   -897

AGA TAC GTT TCT AGT CTC GTG TCT GAG ACA AGT TCC GTA TAC CCA GTT GGC AAC GGA GAG   782
 R   Y   V   S   S   L   V   S   E   T   S   S   V   Y   P   V   G   N   G   E   -877

TTT TGG CAG CAT TGG ATA TCA ACG AAA GAC GGA CAT TGT GGG CTG CCA TTA ACC TCT ACG   842
 F   W   Q   H   W   I   S   T   K   D   G   H   C   G   L   P   L   T   S   T   -857
                                               ▲

AAG GAC GGA TTC ATT GTT GGA ATT CAC AGT TTG TCA ACA ATT ACG AAT TCA AAG AAC TTT   902
 K   D   G   F   I   V   G   I   H   S   L   S   T   I   T   N   S   K   N   F   -837
                               ▲
```

Fig. 5.9. Nucleotide and deduced amino acid sequence of the OMV virion-sense cDNA. Predicted NIa/NIb and NIb/CP polyprotein cleavage sites are indicated by a /. Nucleotide numbering starts at the cDNA 5' end whilst deduced amino acid sequence from the single large ORF are numbered to the left from the 3'-terminal stop codon, which is indicated by an *. Amino acid residues marked with a ▲ in the NIa and NIb regions were reported to play important roles at the active sites of viral proteinases and RNA polymerases, respectively (Argos *et al.*, 1984; Domier *et al.*, 1987; Dougherty *et al.*, 1989b).

```
TTC GCC TCA ATA CCA GCA AAT TTT GAA GAG CAG TAC CTT GCA AAG CTG GAC CAG CAA GAT    962
 F   A   S   I   P   A   N   F   E   E   Q   Y   L   A   K   L   D   Q   Q   D   -817

TGG ACT GCT AAT TGG AAG TAC AAT CCA AAT GAA GTG AGT TGG AAT GGT TTA CGA TTA CAG   1022
 W   T   A   N   W   K   Y   N   P   N   E   V   S   W   N   G   L   R   L   Q   -797

GAA AAT AAA CCA GGG AGA ATC TTC CAA GCA GTC AAG GAG GTA TCA GCC CTG TTT TCT GAC   1082
 E   N   K   P   G   R   I   F   Q   A   V   K   E   V   S   A   L   F   S   D   -777

GCT GTT TAC GAA CAA GGG CAG GAA GTG GGA TGG CTT TTC CGA GAA CTC AAA GAT AAT CTT   1142
 A   V   Y   E   Q / G   Q   E   V   G   W   L   F   R   E   L   K   D   N   L   -757
             NIa / NIb

AAA GCG GTG GCC GTG CTT CCA AAT CAA TTG GTC ACG AAA CAT GTT GTG AAA GGT CCT TGT   1202
 K   A   V   A   V   L   P   N   Q   L   V   T   K   H   V   V   K   G   P   C   -737

CAG TGT TTC ATT CAA TAT CTT AAC GAA TCG CCA GAA GCT TCT GCA TTC TTT AAG CCA CTC   1262
 Q   C   F   I   Q   Y   L   N   E   S   P   E   A   S   A   F   F   K   P   L   -717

ATG GGC CAA TAC GGG AAA AGT ATT CTA AGC AAA GAA GCG TTC GTC AAG GAT ATT ATG AAA   1322
 M   G   Q   Y   G   K   S   I   L   S   K   E   A   F   V   K   D   I   M   K   -697

TAC AGT AAA CCA ATT GTT CTT GGA GAG GTA GAC TTT ATA AAA TTC GAG GAA GGG TAC AAC   1382
 Y   S   K   P   I   V   L   G   E   V   D   F   I   K   F   E   E   G   Y   N   -677

AAC GTG CTC AGA ATG TTT CAT GAT ATT GGT TTT GAG AAA TGT GAG TAC GTT ACA GAC AGC   1442
 N   V   L   R   M   F   H   D   I   G   F   E   K   C   E   Y   V   T   D   S   -657

ATG GAA GTG TAC AAG AAT CTC AAT TTG AAA GCG GCA GTT GGG GCA ATG TAC ACA GGC AAG   1502
 M   E   V   Y   K   N   L   N   L   K   A   A   V   G   A   M   Y   T   G   K   -637

AAG CAA CAG TAC TTT GAA GGA ATG AGT GAG GAC GAA ATT CAC CAG CTG GTT ATT GCC AGT   1562
 K   Q   Q   Y   F   E   G   M   S   E   D   E   I   H   Q   L   V   I   A   S   -617

TGC TTC CGA CTT TGG TCT GGT AAG TTT GGA GTC TGG AAT GGG TCA CTC AAA GCC GAA CTA   1622
 C   F   R   L   W   S   G   K   F   G   V   W   N   G   S   L   K   A   E   L   -597

CGA CCA TTA GAG AAA GTG CAA GCT TGC AAA ACG CGA ACA TTT ACA GCC GCG CCG CTG GAC   1682
 R   P   L   E   K   V   Q   A   C   K   T   R   T   F   T   A   A   P   L   D   -577

ACG TTA CTT GGA GCA AAG GTT TGT GTT GAT GAT TTT AAC GCT CAA TTT TAT GAC AAA CAC   1742
 T   L   L   G   A   K   V   C   V   D   D   F   N   A   Q   F   Y   D   K   H   -557

TTA ACG GCA CCA TGG ACA GTG GGT ATC TGC AAA TAC TAC AAA GGT TGG GAC ACT TTC ATG   1802
 L   T   A   P   W   T   V   G   I   C   K   Y   Y   K   G   W   D   T   F   M   -537

AAC AAA CTT CCA GAA GGT TGG TTA TAT TGC GAT GCT GAC GGC TCA CAG TTC GAT AGC TCT   1862
 N   K   L   P   E   G   W   L   Y   C   D   A   D   G   S   Q   F   D   S   S   -517

TTA ACA CCT TTC CTT ATT AAT AGT GTT TTG AGA CTT CGG TTG GAA TTT ATG GAG GAT TGG   1922
 L   T   P   F   L   I   N   S   V   L   R   L   R   L   E   F   M   E   D   W   -497
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Fig. 5.9. Nucleotide and deduced amino acid sequence of the OMV virion-sense cDNA, continued.

```
GAT ATA GGA GCA CGC ATG CTG TCA AAT CTT TAT ACT GAA ATA ATT TAT ACA CCC ATA GCG    1982
 D   I   G   A   R   M   L   S   N   L   Y   T   E   I   I   Y   T   P   I   A   -477

ACA CCT GAT GGC ACA GTC GTG AAG AAG TTT CGT GGT AAC AAT AGC GGG CAA CCA TCC ACC    2042
 T   P   D   G   T   V   V   K   K   F   R   G   N   N   S   G   Q   P   S   T   -457
                                                             ▲               ▲

GTT GTT GAC AAT ACA TTG ATG GTT GTT TTG GCT ATG AAC TAT GCG TTG GCT AAA CTT TCA    2102
 V   V   D   N   T   L   M   V   V   L   A   M   N   Y   A   L   A   K   L   S   -437
             ▲

ATA CCA TAT GAA GAG ATG GAT TCA CGT ATA CGC TAT TTT GCA AAT GGT GAC GAT CTT CTG    2162
 I   P   Y   E   E   M   D   S   R   I   R   Y   F   A   N   G   D   D   L   L   -417
                                                         ▲   ▲   ▲

GTT GCA GTT GAG CCT ACC AAA GGA GGC GAA ATT CTT GAC TCT CTC CAA GCC TCT TTT TCT    2222
 V   A   V   E   P   T   K   G   G   E   I   L   D   S   L   Q   A   S   F   S   -397

GAG TTG GGG CTC ATT TAC GAC TTC AAC GAT CGA ACT TTT GAC AAA ACC CAA CTT AGT TTC    2282
 E   L   G   L   I   Y   D   F   N   D   R   T   F   D   K   T   Q   L   S   F   -377

ATG TCC CAT CAA GCT TTG TGG GAT GGT GAT ATG TTT ATC CCG AAG ATC AAG CAA GAA CGT    2342
 M   S   H   Q   A   L   W   D   G   D   M   F   I   P   K   I   K   Q   E   R   -357

GTG GTT TCA ATC TTA GAG TGG GAT CGG AGC ACG CAA CCT GAA CAC CGA ATT GAA GCT GTC    2402
 V   V   S   I   L   E   W   D   R   S   T   Q   P   E   H   R   I   E   A   V   -337

TGT GCA GCT ATG ATT GAA GCG TGG GGT TAT CCT GAA CTA CTC CAA GAG ATT AGA AAG TTT    2462
 C   A   A   M   I   E   A   W   G   Y   P   E   L   L   Q   E   I   R   K   F   -317

TAT GCA TTC ATG GTG ACT CAA GAA CCG TAT AGT GCA ATA CAC GCA CAA GGC AAA ACG AGA    2522
 Y   A   F   M   V   T   Q   E   P   Y   S   A   I   H   A   Q   G   K   T   R   -297

TAT ATA TCT GAG CGT GCA CTT GTT ACA CTA TAC AAA GAC GAA AAG GTT GTG CTA AGT GAC    2582
 Y   I   S   E   R   A   L   V   T   L   Y   K   D   E   K   V   V   L   S   D   -277

ATT GGA CCT TAC ATC CAG AAG CTG GCC GAG ATG AGC CTG GGA TGT GTC GAT GAA GTG GTT    2642
 I   G   P   Y   I   Q   K   L   A   E   M   S   L   G   C   V   D   E   V   V   -257

ATG CAT CAA GCA GAT TCT ATG GAT GCA GGA GGA TCG AGC AGA CCA CCA GCG CCG TTG GTG    2702
 M   H   Q / A   D   S   M   D   A   G   G   S   S   R   P   P   A   P   L   V   -237
       NIb / CP

AGA CAA CAA GAT CAA GAT GTT AAC GTT GGA ACG TTT TCT GTT GCG CGA GTG AAG GCG TTG    2762
 R   Q   Q   D   Q   D   V   N   V   G   T   F   S   V   A   R   V   K   A   L   -217

AGC GAT AAA ATG ATG TTA CCC AAG GTG CGC GGT AAA ACG GTG CTT AAT TTA CAG CAT CTG    2822
 S   D   K   M   M   L   P   K   V   R   G   K   T   V   L   N   L   Q   H   L   -197

GTG CAG TAC AAC CCT GAG CAA ACT GAA ATC TCA AAC ACT CGT GCC ACA CGA ACA CAG TTC    2882
 V   Q   Y   N   P   E   Q   T   E   I   S   N   T   R   A   T   R   T   Q   F   -177

AAC AAT TGG TAC GAT AGG GTT AGA GAT AGT TAT GGG GTT ACA GAT GAC CAA ATG GCT GTT    2942
 N   N   W   Y   D   R   V   R   D   S   Y   G   V   T   D   D   Q   M   A   V   -157
```

Fig. 5.9. Nucleotide and deduced amino acid sequence of the OMV virion-sense cDNA, continued.

```
ATC CTA AAT GGT TTG ATG GTG TGG TGC ATC GAG AAT GGC ACT TCA CCA AAT TTG AAT GGT    3002
 I   L   N   G   L   M   V   W   C   I   E   N   G   T   S   P   N   L   N   G   -137

AAT TGG ACG ATG ATG GAT GGC GAT GAG CAG ATC GAA TAT CCT TTG CAA CCA GTC CTT GAG    3062
 N   W   T   M   M   D   G   D   E   Q   I   E   Y   P   L   Q   P   V   L   E   -117

AAT GCT CAG CCA ACA TTC AGA CAA ATT ATG GCG CAT TTC TCA AAC GCA GCC GAG GCG TAC    3122
 N   A   Q   P   T   F   R   Q   I   M   A   H   F   S   N   A   A   E   A   Y   -97

ATC GAA AAG AGA AAC TCG GAA CAA AGG TAC ATG CCA AGG TAC GGC AGC CAA CGA AAT CTG    3182
 I   E   K   R   N   S   E   Q   R   Y   M   P   R   Y   G   S   Q   R   N   L   -77

AAC GAC TAC AGC TTG GCC CGC TAT GCA TTT GAC TTT TAT GAA ATG ACA TCC CGA ACC GCC    3242
 N   D   Y   S   L   A   R   Y   A   F   D   F   Y   E   M   T   S   R   T   A   -57

AAC AGG GCC AGG GAA GCA CAT ATA CAA ATG AAA GCG GCA GCT CTT CGG AAC ACC AAA ACG    3302
 N   R   A   R   E   A   H   I   Q   M   K   A   A   A   L   R   N   T   K   T   -37

AAG TTG TTT GGT TTA GAT GGG AAA GTG GGT ACC GAG GAA GAG GAC ACA GAA CGG CAT GTT    3362
 K   L   F   G   L   D   G   K   V   G   T   E   E   E   D   T   E   R   H   V   -17

GCA AGC GAT GTC AAT CGC AAC ATG CAT TCA CTA CTT GGT GTT AAT ATG TGA TTT AGT GAA    3422
 A   S   D   V   N   R   N   M   H   S   L   L   G   V   N   M   *               -1

AGTGAACTTGACTCAAGCGCGAGTTAGTTTACTCTGTTAGCATCCTTTCCTTTGAATTATGCGAAGTATCATTTACCTTA   3502

GTGACCAACACAGTCAAGTATTGGGTTGGGTGATAAGGCAAGTGAGTATTATAGTGTGATTTCGAACTTATTATTGTTGT   3582

ATTGTACTCGTTAGATTAGGTGACTGTTCTAGTTTGCTTGGCGAATTACAACAGGTGACTACTCTAGCAAATATTATAAT   3662

ATCTCAATAGTGTGTTAGAGAC Poly (A)                                                    3684
```

Fig. 5.9. Nucleotide and deduced amino acid sequence of the OMV virion-sense cDNA, continued.

5.3.6 Analysis of the 3' non-coding region

The length of 274 nucleotides determined for the OMV 3'-NC region was well within the reported potyvirus range of 169 to 475 nucleotides (Hammond & Hammond, 1989, Gough *et al.*, 1987). The potential eukaryotic polyadenylation signals AAUAAA (Nevins, 1983) or UAUGU (Zaret & Sherman, 1982) could not be found in this region. The inconsistent occurrence and location of these sequences - the AAUAAA sequence was found in the 3'-NC regions of TVMV, PeMV and SMV-N, and UAUGU in those of BYMV, PVY-N, SMV-N, WMV-2, PeMV, PPV-NAT, PPV-D, TEV-NAT, TEV-HAT and TVMV at locations that varied from 13 to 258 nucleotides upstream of the polyadenylate tract - led me to support the hypothesis of Hammond & Hammond,

(1989), which is that these "signals" are fortuitous and not functional. No significant direct nucleotide repeats, as were reported for TEV-NAT (Allison *et al.*, 1985b), PeMV (Dougherty *et al.*, 1985b) TVMV (Domier *et al.*, 1986), and PVY-N (Hay *et al.*, 1989, Van der Vlugt *et al.*, 1989), were found in the OMV 3'-NC region.

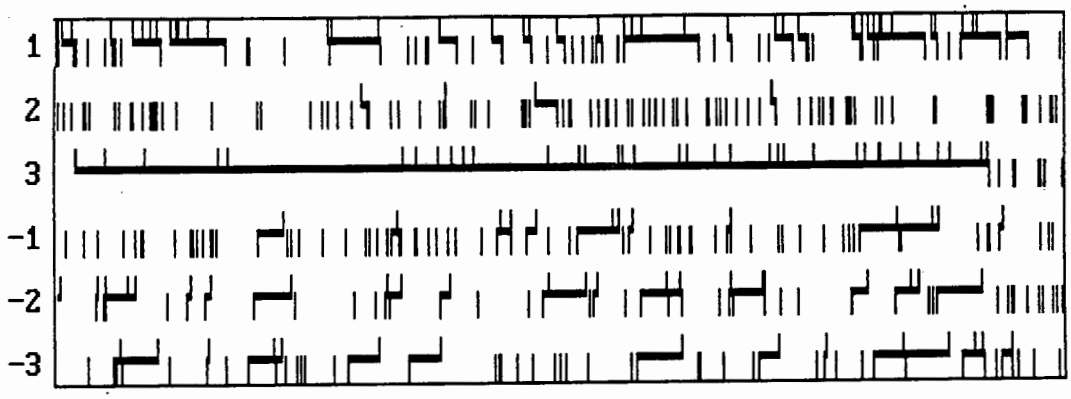

Fig. 5.10. The single large ORF in which the OMV sequence translated. The sequence is probably in frame with the rest of the genome which extends to the 5' end, which is not covered by pOM16.

Frenkel *et al.* (1989) have proposed that the nucleotide sequence similarities between the 3'-NC regions of potyviral genomes are useful for taxonomic purposes: they determined that percent direct sequence similarities between accepted virus strains ranged from 83% to 99%, while similarities between distinct viruses ranged between 39% and 53%. Pairwise comparisons of the OMV 3'-terminal non-coding sequence and eleven other sequenced or partially-sequenced potyviruses, comprising eight distinct viruses, were done using the Gap option of the GCG package (Rybicki & Burger, unpublished results). Apparent sequence similarities between OMV and other viruses ranged from 38% to 45%, with an average of 41.00 ± 2.59%; the similarities between other viruses were very similar to those determined by Frenkel *et al.* (1989), (Table 5.1). The relevance of this similarity becomes doubtful, however, when the value is compared to the 39.21 ± 3.64% "similarity" shared between a 441 bp sequence derived from the CP gene of human papillomavirus type 18, and the same potyvirus sequences (Rybicki & Burger, unpublished). This proves that the sequence similarities between distinct potyviruses are actually in the range of coincidental alignments, and 3'

sequence similarities may only reliably be used to distinguish between virus strains, as suggested by Frenkel *et al.* (1989).

Table 5.1 Percentage sequence similarity of several potyviruses [a] with respect to OMV

Virus	Potyviral Genome Regions [b]				References
	NIa	NIb	CP	3'-NC	
BYMV-GDD	NA [c]	NA	76.28	41.67	Hammond & Hammond, (1989)
SMV-N	NA	NA	71.94	44.36	Eggenberger *et al.*, (1989)
SMV-V	NA	NA	70.75	NC [d]	Gunyuzlu *et al.*, (1987)
WMV-2	NA	NA	71.94	39.44	Frenkel *et al.*, (1989)
PVY-D	NA	NA	76.28	NA	Shukla *et al.*, (1986)
PVY-N	63.29	72.59	76.28	38.69	Robaglia *et al.*, (1989)
PeMV	NA	NA	74.70	41.76	Dougherty *et al.*, (1985)
TEV-NAT	NA	NA	73.91	44.44	Allison *et al.*, (1985)
TEV-HAT	65.21	72.46	73.52	44.97	Allison *et al.*, (1986)
PWV-TB	NA	NA	71.15	NA	Shukla *et al.*, (1988b)
TVMV	62.74	71.71	65.61	38.96	Domier *et al.*, (1986)
SCMV-JG	NA	NA	73.52	39.05	Gough *et al.*, (1987)
PPV-NAT	70.96	72.59	76.68	37.79	Maiss *et al.*, (1989)
PPV-D	NA	NA	75.10	39.91	Ravelonandro *et al.*, (1988)

[a] See Appendix A for virus names.

[b] Amino acid sequence similarities for NIa, NIb and CP proteins were calculated using the Distances programme of the GCG package after they were aligned with the Gap and LineUp programmes. Pairwise nucleotide sequence similarities for the 3' non-coding regions were calculated using the Gap programme.

[c] NA, Sequence not available.

[d] NC, Not calculated.

5.3.7 Analysis of coding regions

5.3.7.1 The coat protein region

The location of the OMV CP gene was determined by comparison of the deduced OMV partial polyprotein amino acid sequence, and those of several other potyviruses. Using the proposed consensus polyprotein cleavage sites V(R or K)FQ / (G or S) for TVMV (Domier *et al.* 1986), ExxYxQ / (G or S) for TEV (Carrington & Dougherty, 1987a,b; Dougherty *et al.*, 1989a), and Vx(H or E)Q / (G or S or A) for PVY-N (Robaglia *et al.*, 1989) as indicators, OMV CP is predicted to be cleaved from the putative polyprotein between the Q/A dipeptide sequence at amino acid position -253/-254 (see Fig. 5.9). Another potential cleavage site in this region was the Q/G dipeptide at position -300/-301; however cleavage at this point would result in an N-terminal extension not shared by other potyviral coat proteins (see Fig. 5.11). An M_r of 28 807 was calculated for the 253 amino acid putative CP, which is in reasonable agreement with the 30 000 Da determined experimentally by SDS-PAGE (Section 2.2.5).

The putative OMV CP sequence was aligned with and compared to those of several other potyviruses (Fig. 5.11): the C-terminus showed far greater similarity than the N-terminus, as is true for all known potyviruses (Shukla & Ward, 1988, 1989a,b).

The amino acid sequence DAG - residues 5-7 in the putative OMV CP - is found within the first 13 N-terminal residues in the CP regions of several aphid transmissible potyviruses, whilst being absent in most aphid non-transmissible potyviruses (Fig. 5.9). Harrison & Robinson (1988) have suggested that the DAG triplet might be involved in aphid transmissibility by playing a role in binding to helper component. Otherwise, sequence similarity between OMV and other potyviruses varied between 66% and 77% (Table 5.1), with comparisons among other potyviruses appearing very similar to values determined by Shukla & Ward (1988, 1989a,b). These authors concluded that sequence similarity between distinct potyviruses ranged from 38% to 71% while that between strains of one virus ranged from 90% to 99%. Our data suggests that OMV is clearly a distinct potyvirus by these criteria.

```
           1                                                                                                  90
      OMV  .....AD.SM DAGGSS.... RP..... PAPL..... .......... .......... .......... .......... ..........
  BYMV-GDD  ... SDQEQL NAGEEKKDKR KKNEGNPNKD SEGQSVR.QI VP..... .......... .......... .......... ..........
    SMV-N  .SGKEKEGDM DADKDPKKST SSSKGAGTS. .......... .......... .......... .......... .......... ..........
    SMV-V  .......SNL QEVGDVKASA KKHQEY..TN PALHP..... .......... .......... .......... .......... ..........
    WMV-2  .SGKEAVENL DTGKDSKKDT SGKGDKPQNS QTGQGSKEQT KIGTVS.... .......... .......... .......... ..........
    PVY-D  .....ANDTI DAGESSKKDA RPEQGSIQVN P......... .......... .......... .......... .......... ..........
    PVY-N  .....ANDTI DAGGSNKKDA KPEQGSIQPN P......... .......... .......... .......... .......... ..........
     PeMV  .... ANDTI DTGGNSKKDV KPEQGSIQPS S......... .......... .......... .......... .......... ..........
  TEV-NAT  ......GGTV DASADVGKKK DQKDDKVAEQ A......... .......... .......... .......... .......... ..........
  TEV-HAT  ......SGTV DAGADAGKKK DQKDDKVAEQ A......... .......... .......... .......... .......... ..........
   PWV-TB  .....KDEII DVGAD.GKKV VSKKDTQDAG EVNK..... .......... .......... .......... .......... ..........
     TVMV  ......SDTV DAGKDKARDQ KLA.DKPTLA .......... .......... .......... .......... .......... ..........
   SCMV-JG  ..... SGNE DAGKQKSAT. PAANQTASGD GKPV..... .......... ..QTTATADN KPSSDNTSNA QGTSQTKGGG ESGGTN.ATA
   PPV-NAT  ADEREDEEEV DA........ ........LQ PPPVIQPAPR TTAPMLNPIF TPATTQPA.T KPVSQVSGPQ LQTFGTYSHE DASPSN.SNA
    PPV-D  ADEREDEEEV DAGKPIVVTA PAATSPI.LQ PPPVIQPAPR TTAPMLNPIF TPATTQPA.T KPVSQVPGPQ LQTFGTYGNE DASPSN.SNA
Consensus  *****a*etv dagkdskk*a kpkq*ki*lq p********* ********** ********** ********** ********** **********

           91                                                                                                 180
      OMV  ..VRQQDQDV NV...GTFSV ARVKALSDKM MLPKVRGKTV LNLQHLVQYN PEQTEISNTR ATRTQFNNWY DRVRDSYGVT DDQMAVILNG
  BYMV-GDD  ......DRDV NAGTVGTFSV PRLKKIAGKL NIPKIGGKIV FNLDHLLKYN PPQDDISNVI ATQEQFEAWY NGVKQAYEVE DSRMGIILNG
    SMV-N  ....S..KDV NVGSKGK.VV PRLQKITRKM NLPMVEGKII LSLDHLLEYK PNQVDLFNTR ATRTQFEAWY NAVKDEYELD DEQMGVVMNG
    SMV-V  ....RKDKDV NAGTSGTFSP PRIKIAAPKI TYPKINGPPV VNLDHKLEYK PQQIDLSNTR ATHSQFKAWH AAVMDAYGIN EEDMKIVLNG
    WMV-2  .......KDV NVGSKGK.EV PRLQKITKKM NLPTVGGKII LSLDHLLEYK PNQVDLFNTR ATKTEFESWY SAVKIEYDLN DEQMGVIMNG
    PVY-D  ..NKGKDKDV NAGTSGTHTV PRIKAITAKM RMPRSKGATV LHLEHLLEYA PQQIDISNTR ATQSQFDTWY EAVRMAYDIG ETEMPTVMDG
    PVY-N  ..NKGKDKDV NAGTSGTHTV PRIKAITSKM RMPTSKGATV LNLEHLLEYA PQQIDISNTR ATQSQFDTWY EAVRMAYDIG ETEMPTVMNG
     PeMV  ..NKGKEKDV NAGTSGTHTV PRIKAITAKM RMPKSKGAAV LKLDHLLEYA PQQIDISNTR ATQSQFDTWY EAVRVAYDIG ETEMPTVMDG
  TEV-NAT  ....SKDRDV NAGTSGTFSV PRINAMATKL QYPRMKGEVV VNLNHLLGYK PQQIDLSNAR ATHEQFAAWH QAVMTAYGVN EEQMKILLNG
  TEV-HAT  ....SKDRDV NAGTSGTFSV PRINAMATKL QYPRMRGEVV VNLNHLLGYK PQQIDLSNAR ATHEQFAAWH QAVMTAYGVN EEQMKILLNG
   PWV-TB  ..GKEKDKDV NAGSKGS.GV PRLQKITKKM NLPMVKGNMV LDLDHLIEYK PDQTKLFNTR ATDAQFATWY EGVKAEYELS DDQMGVIMNP
     TVMV  .IDRTKDKDV NTGTSGTFSI PRLKKAAMNM KLPKVGGSSV VNLDHLLTYK PAQEFVVNTR ATHSQFKAWH TNVMAELELN EEQMKIVLNG
   SCMV-JG  ...TKKDKDV DVGSTGTFVI PKLKKVSPKM RLPMVSNKAI LNLDHLIQYK PDQRDISNAR ATHTQFQFWY NRVKKEYDVD DEQMRILMNG
   PPV-NAT  LVNTNRDRDV DAGSTGTFTV PRLKAMTSKL SLPKVKGKAI MNLNHLAHYS PAQVDLSNTR APQSCFQTWY EGVKRDYDVT DDEMSIILNG
    PPV-D  LVNTNRDRDV DAGSIGTFTV PRLKAMTSKL SLPKVKGKAI MNLNHLAHYS PAQVDLSNTR APQSCFQTWY EGVKRDYDVT DDEMSIILNG
Consensus  *****kdkDV nagtsGtfsv prlkait*km nlPkvkgkav lnLdHlleYk PqQidlsNtr AtqsqF**Wy eavkdaydvn deqMgii*ng

           181                                                                                                270
      OMV  LMVWCIENGT SPNLNGNWTM MDGDEQIEYP LQPVLENAQP TFRQIMAHFS NAAEAYIEKR NSEQRYMPRY GSQRNLNDYS LARYAFDFYE
  BYMV-GDD  LMVWCIENGT SGDLQGEWTM MDGEEQVTYP LKPILDNAKP TFRQIMSHFS EVAEAYIEKR NATERYMPRY ELQRNLTDYG LARYAFDFYE
    SMV-N  FMVWCIDNGT SPDANGVWVM MDGEEQIEYP LKPIVENAKP TLRQIMHHFS DAAEAYIEMR NSESPYMPRY GLLRNLRDRE LARYAFDFYE
    SMV-V  FMVWCIENGT SPNINGVWTM MDGAEQVEYP LKPMVENADP TLRQIMDHFS DLAEAYIEMR NNEWPYMPRY GLQRNLGDLS LARI.LDFYE
    WMV-2  FMVWCIDNGT SPDVNGVWVM MDGEEQVEYP LKPIVENAKP TLRQIMHHFS DAAEAYIEMR NSESPYMPRY GLLRNLRDRE LARYAFDFYE
    PVY-D  LMVWCIENGT SPNVNGVWVM MDGNEQVEYP LKPIVENAKP TLRQIMAHFS DVAEAYIEMR NKKEPYMPRY GLIRNLRDVG LARYAFDFYE
    PVY-N  LMVWCIENGT SPNVNGVWVM MDGNEQVEYP LKPIVENAKP TLRQIMAHFS DVAEAYIEMR NKKEPYMPRY GLIRNLRDMG LARYAFDFYE
     PeMV  LMVWCIENGT SPNINGVWVM MDGSEQVEYP LKPIVENAKP TLRQIMAHFS DVAEAYIEMR NKKEPYMPRY GLVRNLRDAS LARYAFDFYE
  TEV-NAT  FMVWCIENGT SPNLNGTWVM MDGEEQVSYP LKPMIENAKP TLRQIMTHFS DLAEAYIEMR NRERPYMPRY GLQRNITDMS LSRYAFDFYE
  TEV-HAT  FMVWCIENGT SPNLNGTWVM MDGEDQVSYP LKPMVENAKP TLRQIMTHFS DLAEAYIEMR NRERPYMPRY GLQRNITDMS LSRYAFDFYE
   PWV-TB  FMVWCIENGT SPDINGVWVM MDGDEQVEYP LKPMVENAKP TLRQIMHHFS DAAEAYIEMR CASGPYMPRY GLLRNLRDKN LARYAFDFYE
     TVMV  FMIWCIENGT SPNISGVWTM MDGDEQVEYP IEPMVKHANP SLRQIMKHFS NLAEAYIRMR NSEQVYIPRY GLQRGLVDRN LAPFAFDFFE
   SCMV-JG  LMVWCIENGT SPDINGYWTM VDGNNQSEFP LKPIVENAKP TLRQCMMHFS DAAEAYIEMR NLDEPYMPRY GLLRNLNDKS LARYAFDFYE
   PPV-NAT  LMVWCIENGT SPNINGMWVM MDGETQVEYP IKPLLDHAKP TFRQIMAHFS NVAEAYIEKR NYEKAYMPRY GIQRNLTDYS LARYAFDFYE
    PPV-D  LMVWCIENGT SPNINGMWVM MDGETQVEHP IKPLLDHAKP TFRRIVARFS DVAEACVEKR NYEKAYMPRY GIQRNLTDYS LARYAFDFYE
Consensus  *MvWCIeNGT SpninGvWvM mDGeeQveyP lkPivenAkP tlRqimahFS d*AEAyiemR nseepYmPRY glqRnlrDys LaryafDFyE

           271                                                                                                331
      OMV  MTSRTANRAR EAHIQMKAAA LRNTKTKLFG LDGKVGTEEE DTERHVASDV NRNMHSLLGV .NM.
  BYMV-GDD  LTSRTPVRAR EAHMQMKAAA VRGKSTRLFG LDGNVGTDEE NTERHTAGDV NRDMHTMLGV RI..
    SMV-N  VTSKTPNRAR EAIAQMKAAA LSGVNNKLFG LDGNISTNSE NTERHTARDV NQNMHTLLGM GPPQ
    SMV-V  VTSTTPVRAR EAHNQMKAAA GTQAVPRLFG LDGLVSTQEE NTERHTTDDV NPHMHTLLGV KGM.
    WMV-2  VTSKTPNRAR EAIAQMKAAA LAGINSRLFG LDGNISTNSE NTERHTARDV NQNMHTLLGM GPPQ
    PVY-D  VTSRTPVRAR EAHIQMKAAA LKSAQPRLFG LDGGISTQEE NTERHTTEDV SPSMHTLLGV KNM.
    PVY-N  VTSRTPVRAR EAHIQMKAAA LKSAQPRLFG LDGGISTQEE NTERHTTEDV SPSMHTLLGV KNM.
     PeMV  VTSRTPVRAR EAHIQMKAAA LKSAQSRLFG LDGGVSTQEE NTERHTTEDV SPSMHTLLGV KNM.
  TEV-NAT  LTSKTPVRAR EAHIQMKAAA VRNSGTRLFG LDGNVGTAEE DTERHTAHDV NRNMHTLLGV RQ..
  TEV-HAT  LTSKTPVRAR EAHIQMKAAA VRNSGTRLFG LDGNVGTAEE DTERHTAHDV NRNMHTLLGV RQ..
   PWV-TB  VNAKTSDRAR EAVAQMKAAA LSNVTNKLFG LDGNVATISE DTERHTARDV NQNMHTLLGM GAPQ
     TVMV  VNGATPVRAR EAHAQMKAGR TPQFAAAMFC LDGSVSGQEE NTERHTVDDV NAQMHHLLGV KGV.
   SCMV-JG  INSRTPNRAR EAHAQMKAAA IRGSTNHMFG LDGNVGESSE NTERHTAADV SRNVHSYRGA KI..
   PPV-NAT  MTSTTPVRAR EAHIQMKAAA LRNVQNRLFG LDGNVGTQEE DTERHTAGDV NRNMHNLLGM RGV.
    PPV-D  MTSTTPVRAR EAHIQMKAAA LRNVQNRLFG LDGNVGTQKQ DTERHTDGDV NRNMHTFLGV RGV.
Consensus  vts*TpvRAR EAhiQMKAaa lrnvq*rlFg LDGnv*tqee nTERHta*DV nrnmHtllGv kg**
```

Fig. 5.11. Alignment of the deduced coat protein amino acid sequences of OMV and 14 other potyviruses. Sequences were initially aligned pairwise with the GCG Gap programme, and then finally aligned using the multiple sequence editor LineUp: gaps introduced to optimise alignments are indicated by dots. A consensus sequence was calculated using LineUp: this identifies regions of absolute homology as capital letters, regions of partial homology as lower-case letters, and regions with no discernible homology as asterisks.

A phylogenetic analysis of the OMV CP sequence by the KITSCH programme of the PHYLIP package is shown in Fig. 5.12. The tree generated from this analysis agrees in broad details with that of Shukla & Ward (1989b), in that all of the viruses shown as being even distantly related in their tree are identically grouped in this one. BYMV-GDD, SMV-N, SMV-V, WMV-2, PVY-D, PVY-N, PeMV, TEV-NAT, TEV-HAT, PWV-TB, TVMV, PPV-NAT, PPV-D and SCMV-JG are all shown as distinct viruses in both trees, as is OMV in this tree. The finer details of branching at the base of this tree are not in agreement with the tree of Shukla & Ward (1989b) - however, analyses using PHYLIP'S FITCH and other sequence-based programmes produce branching patterns that are also in broad agreement with this KITSCH tree and the previously published tree (Shukla & Ward, 1989b), but again differ at the base (Rybicki & Burger, unpublished).

5.3.7.2 The NIb region

Directly upstream of the CP gene is a region whose sequence corresponds closely to the NIb gene of TEV-HAT, TVMV, PPV-NAT and PVY-N (Allison *et al.*, 1986, Domier *et al.*, 1986, Maiss *et al.*, 1989, Robaglia *et al.*, 1989). Comparisons of the deduced amino acid sequence of this region of OMV and of the NIb genes of the above-mentioned potyviruses, as well as its genomic position, enabled the prediction of the 518 amino acid protein sequence presented in Fig. 5.9: this is shown as the product of a polyprotein cleavage between the Q and G residues at position -771/-772. Sequence similarity between this and other putative NIb proteins was between 72% and 73% (Table 5.1): this confirms previous findings that the NIb proteins are the most conserved of the mature potyviral proteins (Maiss *et al.*, 1989, Robaglia *et al.*, 1989). An RNA-dependent RNA polymerase function has been proposed for this region: putative RNA polymerases of potyviruses, other plant viruses, and animal viruses contain two short stretches of identical amino acid sequence near their C-termini (Argos *et al.*, 1984, Domier *et al.*, 1987). These two sequences (GxxxTxxxN and GDD) were found 31 residues apart, near the C-terminus of the putative OMV NIb protein (Fig. 5.9).

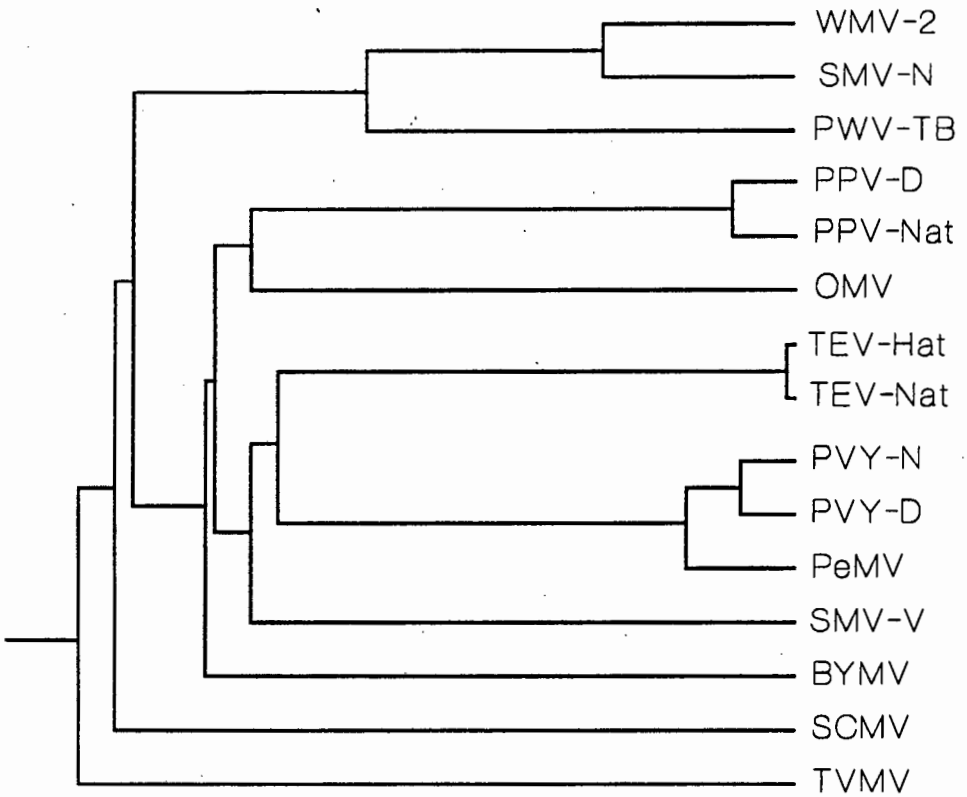

Fig. 5.12. Phylogenetic tree calculated for potyvirus coat proteins. The GCG Distances programme was used to calculate a matrix of pairwise sequence similarities from the sequence alignments used in Fig. 5.11. Values were calculated as the sum of the sequence matches divided by the length of the shorter sequence, excluding gaps. These values were subtracted from 1 to convert them to distance rather than similarity scores and then used as a data matrix for the PHYLIP programme KITSCH. The tree shown is the best of 1234 examined. The horizontal branch lengths are proportional to time of divergence (arbitrary units). Average percent standard deviation was 7.4.

5.3.7.3 The NIa region

The sequence upstream of the NIb gene could encode part of a NIa-equivalent polypeptide of at least 365 amino acids. Amino acid similarity (Table 5.1) as well as size comparisons with other potyvirus NIa genes indicated that the N-terminal cleavage site for this gene was not contained within the pOM16 clone, but would probably be located approximately 60 amino acids upstream on the polyprotein. This suggests that pOM16 contained approximately 85% of the NIa gene. It has been shown that this region

encodes the proteinase responsible for the post-translational cleavage of at least five proteins, all from the C-terminal two-thirds of the polyprotein (Carrington & Dougherty, 1987a,b). A stretch of amino acid sequence near the C-terminus of the NIa protein shows a high degree of homology with proteinases of other plant and animal viruses; it has been suggested that the cysteine and histidine residues, which are conserved in this cluster, are involved at the active site of these proteinases (Argos *et al.*, 1984, Domier *et al.*, 1987). More recently Dougherty *et al.* (1989b) proposed that the conserved residues histidine, aspartic acid and cysteine are important in the catalytic activity of the TEV proteinase. All of these conserved residues were contained within the putative OMV NIa protein (Fig. 5.9), which suggests that this region of pOM16 might be catalytically active.

CHAPTER 6

THE EXPRESSION OF ORNITHOGALUM MOSAIC VIRUS COAT PROTEIN IN *E. COLI*

THE EXPRESSION OF ORNITHOGALUM MOSAIC VIRUS COAT PROTEIN IN *E. COLI*

SUMMARY

A 1243 base pair fragment of a cDNA clone containing the complete OMV coat protein encoding sequence, was subcloned in the pUEX bacterial expression vectors. OMV CP was expressed in *E. coli* as a β-galactosidase fusion protein after an in-frame fusion with the *lacZ* gene of the pUEX2 vector. Expression was temperature-controlled and could be induced by incubation at 42°C for 2 hours. Expressed β-gal::OMV CP fusion protein could be detected in appropriately-transformed *E. coli* colonies by monospecific anti-OMV antibodies in an immuno-colony blot assay. Large quantities of the insoluble fusion protein (M_r 143.7 kDa) was partially purified from sonicated bacterial cells. It was confirmed that the β-gal fusion protein was indeed CP-specific by its positive reaction with a monospecific anti-OMV antibody and a monoclonal anti-potyvirus CP antibody in immunoelectroblot assays. This was taken as proof that the single open reading frame found in the clone does in fact code for OMV coat protein. A specific anti-OMV antiserum was prepared in rabbits using the partially-purified fusion protein as antigen. This antiserum was used to specifically detect OMV in immunoelectroblotting assays.

6.1 INTRODUCTION

Coat protein genes of several plant viruses have been expressed in transgenic plants in order to confer cross-protection against the same virus or strains thereof (Powell-Abel *et al.*, 1986, Loesch-Fries *et al.*, 1987, Van Dun *et al.*, 1987, 1988, Tumer *et al.*, 1987, Cuozzo *et al.*, 1988, Hemenway *et al.*, 1988, Van Dun & Bol, 1988). The expression of potyviral coat proteins in *E. coli* was reported for PRSV (Nagel & Hiebert, 1985) and BYMV (Hammond & Hammond, 1989); the expression of SMV CP in *E. coli*, *Agrobacterium tumefaciens* and tobacco callus was reported by Eggenberger *et al.* (1989). Stark & Beachy (1989) have subsequently reported the broad spectrum protection against other potyviruses in transgenic tobacco plants expressing SMV CP. In order to achieve similar goals with the coat protein of OMV, it was necessary to first express it from cloned cDNA so that it could be compared with the native coat protein.

Stanley and Luzio (1984) have constructed the pEX family of expression vectors from a *cro-lacZ* gene fusion plasmid, which could express large quantities of C-terminal β-galactosidase fusion protein under control of the λ P_R promoter. These vectors contain a polylinker and termination signals in all three translational reading frames near the end of the *lacZ* gene, thus allowing DNA in any reading frame to be expressed as a β-galactosidase (β-gal) fusion protein. The pUEX bacterial expression vectors were constructed from the pEX vectors by including the CI857 gene in the plasmid sequence (Bressan & Stanley, 1987): this allows temperature-controlled over-expression of a β-gal fusion protein in all *E. coli* strains. These proteins are eminently suitable antigens for the raising of specific antisera (Schughart *et al.* 1987).

The production of specific anti-OMV antiserum is hampered by the difficulty in purifying OMV, which in turn can be attributed to the very mucilaginous nature of ornithogalum plant sap, as well as the strictly seasonal availability of ornithogalum plants. Furthermore, antisera raised against biochemically purified plant viruses, invariably contain antibodies against contaminating plant proteins.

The 3'-terminal region of the OMV genome - including the complete CP gene - was cloned and sequenced (Chapter 5). This chapter describes the expression of OMV CP in *E. coli* as a possible first step towards expressing the gene in plants. The production of

anti-OMV antiserum, using partially purified β-gal::OMV CP fusion protein as antigen, is described.

6.2 MATERIALS AND METHODS

6.2.1 Subcloning of OMV coat protein in expression vectors

pOM16Δ18, one of the deletion mutants created for the sequencing of a 3684 bp OMV clone (pOM16; section 5.2.3), contained the putative OMV CP gene of 759 nucleotides, a 187 bp 5' flanking region, the 3' non-coding fragment of 274 bp and a 23 nucleotide poly(A) tail. This 1243 bp fragment was excised from pOM16Δ18 using the restriction enzymes *Hind* III and *Eco* RI. The resulting two 5'-protruding ends were filled in using the Klenow fragment of DNA polymerase I. To approximately 5 µg of *Hind* III/*Eco* RI digested DNA were added 1 µl dNTP mix (0.5 mM of each dNTP) and 8 U of Klenow fragment, and the volume adjusted to 20 µl with 20 mM Tris-Cl pH 8.0; 7 mM $MgCl_2$. After incubation at 30°C for 15 min the reaction was stopped by adding 1 µl 0.5 M EDTA. The blunt-ended insert fragment was separated from plasmid vector by agarose gel electrophoresis (Appendix B.4.5), and gel purified using DEAE cellulose membrane (Appendix B.4.6).

The bacterial expression vectors, pUEX 1, 2 and 3 (Amersham), were linearised with *Sma* I, twice extracted with phenol/chloroform, ethanol precipitated, and treated with calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) to remove 5'-phosphates (Maniatis *et al.*, 1982). Dephosphorylation reactions were carried out at 37°C for 30 min in the presence 50 mM Tris-Cl, pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine. CIP was used at 0.1 U per pmole of vector DNA 5' termini. Reactions were stopped and DNA cleaned up by two phenol/chloroform extractions, ethanol precipitation and a 70% ethanol wash, before final pellets were resuspended in TE buffer (10 mM Tris-Cl pH 8.0; 1 mM EDTA) . Blunt-ended insert DNA was ligated into all three pUEX vectors. For blunt-end ligation reactions approximately 100 ng insert DNA and an equimolar quantity of vector DNA were mixed with 1 U T4 DNA ligase (Boehringer Mannheim), adjusted to a final reaction volume of 20 µl with blunt-end ligation buffer (50 mM Tris-Cl pH 7.6; 10 mM $MgCl_2$; 2 mM DTT; 2 mM ATP), and incubated at 15°C for 16 h. *E. coli* LK-111 competent cells were prepared and transformed as described in Appendix B.4.13.

Presence of the CP-encoding fragment in pUEX was confirmed by agarose gel electrophoresis and colony hybridisation. Quick mini-preparations of plasmid DNA (Appendix B.4.1) were subjected to agarose gel electrophoresis to determine plasmid sizes. The gel-purified *Hind* III/*Eco* RI insert fragment from pOM16Δ18 was [α-^{32}P]dCTP labelled by nick-translation (Appendix B.4.8) and used as probe for colony hybridisations (Appendix B.4.9).

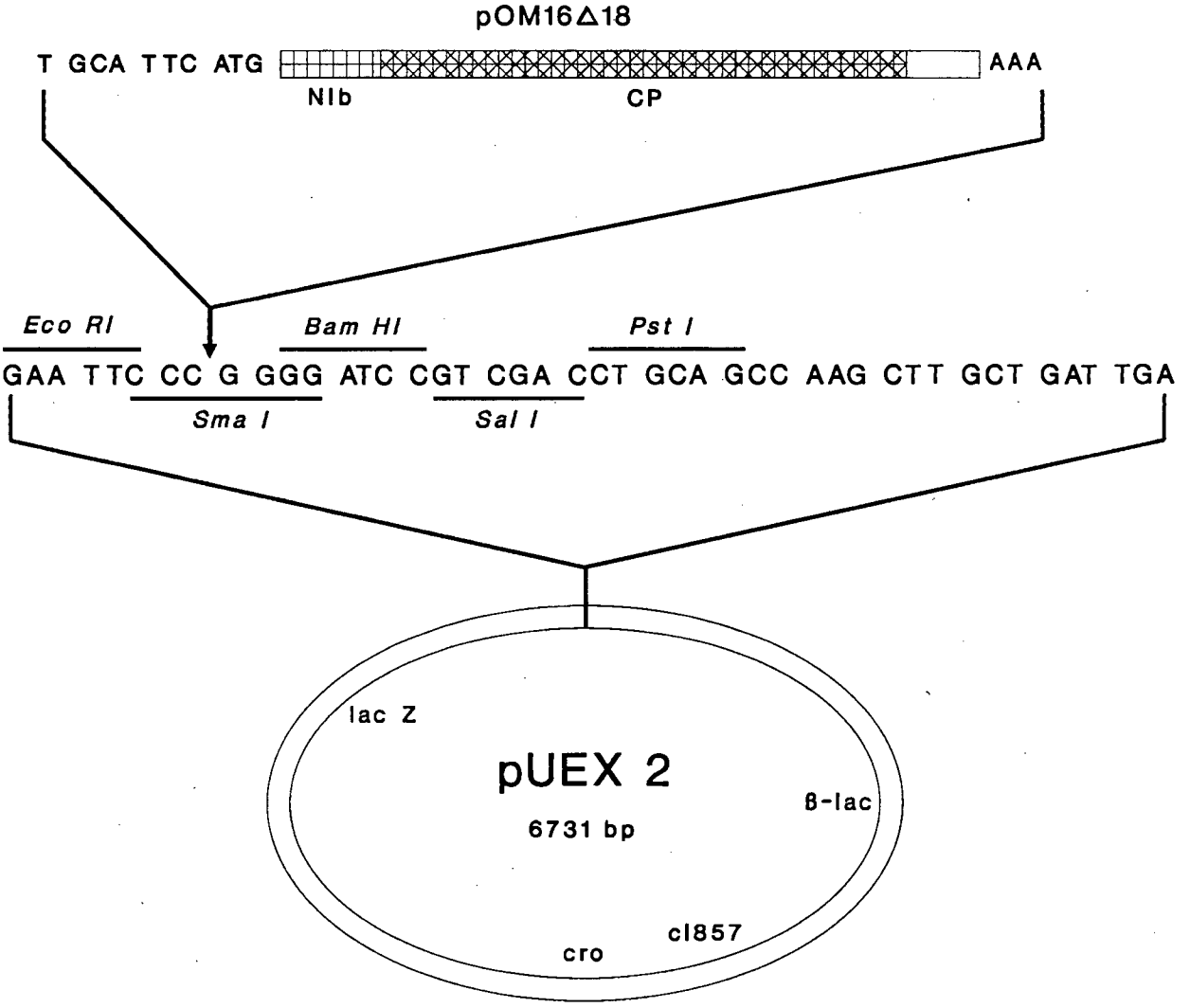

Fig. 6.1. Subcloning of OMV coat protein in the pUEX2 expression vector. The 1243 bp fragment was excised from pOM16Δ18, its ends polished with Klenow (not shown in the diagram), and the blunt-ended fragment inserted in-frame with the *lacZ* gene of pUEX2.

6.2.2 Expression of OMV coat protein in *E. coli*

Transformants which reacted positively in colony hybridisations were screened for the expression of β-gal::OMV CP fusion protein by an immuno-colony blot (ICB) assay adapted from the method of Stanley (1983). Colonies were transferred to nitrocellulose disks (Millipore), placed on filter paper disks soaked in Luria-Bertani medium, containing 100 μg/ml ampicillin (LB-100, Appendix B.4.1) and incubated at 42°C for 2 h. Colonies were then lysed in the presence of 5% SDS for 2 min at 650 W in a microwave oven. Excess SDS was removed by electroelution in a semi-dry carbon electrode electroblot apparatus. Nitrocellulose disks were sandwiched, with colonies towards the cathode, between layers of nappy liners (Johnson & Johnson) and filter paper soaked in 20 mM Tris-base; 160 mM glycine. Electroelution continued for 60 min at 50 V. Filters were incubated for 90 min in blocking buffer (10 mM Tris-Cl; 150 mM NaCl; 0.1% (v/v) Tween 20; 2% (w/v) skimmed milk powder) to saturate protein adsorption sites, then incubated for 90 min in each of diluted monospecific anti-OMV antibodies (see Appendix B.2.2) and goat anti-rabbit alkaline phosphatase conjugate (Bio-Yeda, Israel), then reacted in NBT/BCIP substrate (Appendix B.2.3). Filter disks were washed 3 x 5 min in wash buffer (10 mM Tris-Cl pH 7.4; 150 mM NaCl; 0.1% Tween 20) between every incubation step.

6.2.3 Purification of β-gal::OMV CP fusion protein

Fusion protein was partially purified essentially as described in the pEX technical brochure (Genofit, Switzerland). A 100 ml LB-100 medium culture was grown at 30°C until early log phase (OD_{600} = 0.3) before being transferred to 42°C for 2 h to permit expression of the fusion protein. Cells were harvested by centrifugation, resuspended in 2 ml low salt STE (50 mM Tris-Cl pH 8.0; 50 mM NaCl; 1 mM EDTA) and sonicated for 2 min. After centrifugation at 10 000 *g* for 10 min at 4°C, the insoluble fusion protein pellet was again resuspended in 2 ml low salt STE and used for SDS-PAGE and immunisation of rabbits.

Partially purified fusion proteins were mixed with an equal volume of SDS-PAGE disruption buffer (Appendix B.3.1) and heated to 95°C for 5 min before being subjected to SDS-PAGE in 7% polyacrylamide gels (Appendix B.3.1). Gels were either stained with Coomassie Blue or immunoelectroblotted as described in Appendix B.2.3. Monospecific anti-OMV antibodies as well as a wide-spectrum monoclonal antibody reactive to most potyvirus coat proteins (MAb PTY-1, Jordan & Hammond, 1986) were used to detect β-gal::OMV CP fusion protein.

6.2.4 Production and evaluation of OMV specific antiserum

Partially-purified β-gal::OMV CP fusion protein preparation (250 µl) was diluted 1:1 with low salt STE and emulsified with an equal vol (500 µl) of Freund's incomplete adjuvant for the immunisation of rabbits as described in Appendix B.2.1. Preliminary evaluation of antisera was by immunoelectroblot assays (Appendix B.2.3).

6.3 RESULTS

6.3.1 Subcloning of OMV coat protein in pUEX vectors

The gel-purified, blunt-ended restriction fragment, encoding OMV CP and flanking regions from pOM16Δ18, was inserted into the *Sma* I sites of CIP-treated pUEX vectors (Fig. 6.1); these were used to transform *E. coli*. No religation of the CIP-treated vectors occurred. Forty-eight transformants from each vector were selected for screening by agarose gel electrophoresis and colony hybridisation. Plasmids containing the 1243 bp insert could clearly be distinguished from others in agarose gel electrophoresis (not shown). This result was confirmed by colony hybridisations (Fig. 6.2).

Fig. 6.2. Colony hybridisation of pUEX clones. A gel purified *Hind* III/*Eco* RI insert fragment from pOM16Δ18 was [α-^{32}P]dCTP labelled by nick-translation and used as probe.

6.3.2 Expression of OMV coat protein in *E. coli*

The OMV CP fragment was successfully cloned in all three pUEX vectors. However, only colonies containing the inserts in pUEX2 reacted positively with monospecific anti-OMV antibodies in ICB. Approximately 50% of these insert containing plasmids expressed the β-gal::OMV CP fusion protein in *E. coli* (Fig. 6.3): this was to be expected, as blunt-ended CP-encoding fragments could have been inserted in either sense or anti-sense orientations. The β-gal::OMV CP fusion protein was reliably detected in *E. coli* only by affinity-purified monospecific antibodies to OMV or MAb PTY-1 (Fig. 6.3).

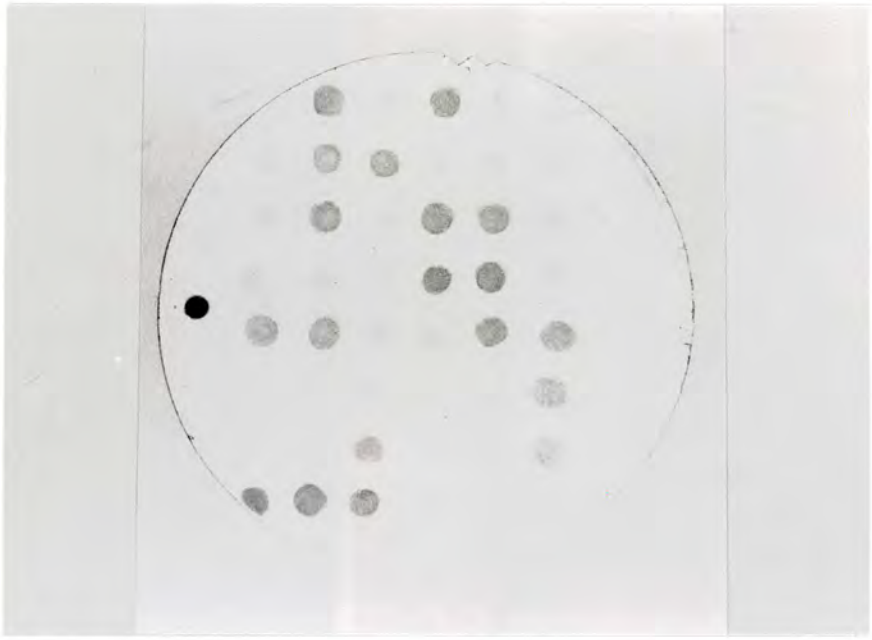

Fig. 6.3. Immuno-colony blots of pUEX2 clones. Colonies were probed with monospecific anti-OMV antiserum.

6.3.3 Purification of fusion protein

Large quantities of β-gal::OMV CP fusion protein were purified from four different positive clones (pOMX37, -39, -41 and -43). An M_r of 143.7 ± 0.8 kDa was determined for the fusion protein in SDS-PAGE, compared to the 114.15 ± 1.12 kDa of β-galactosidase alone (Fig. 6.4). When proteins isolated from a clone containing the OMV CP insert in the antisense orientation (pOMX42) were electrophoresed, no fusion protein band could be detected (Fig. 6.4, lane 9). In immunoelectroblots of the same samples probed with monospecific anti-OMV antibodies, only the fusion protein bands could be detected (Fig. 6.5). MAb PTY-1 also detected β-gal::OMV CP fusion proteins specifically in IEB (Fig. 6.6), which is added proof that the authentic OMV CP was indeed expressed in *E. coli* (Fig. 6.1).

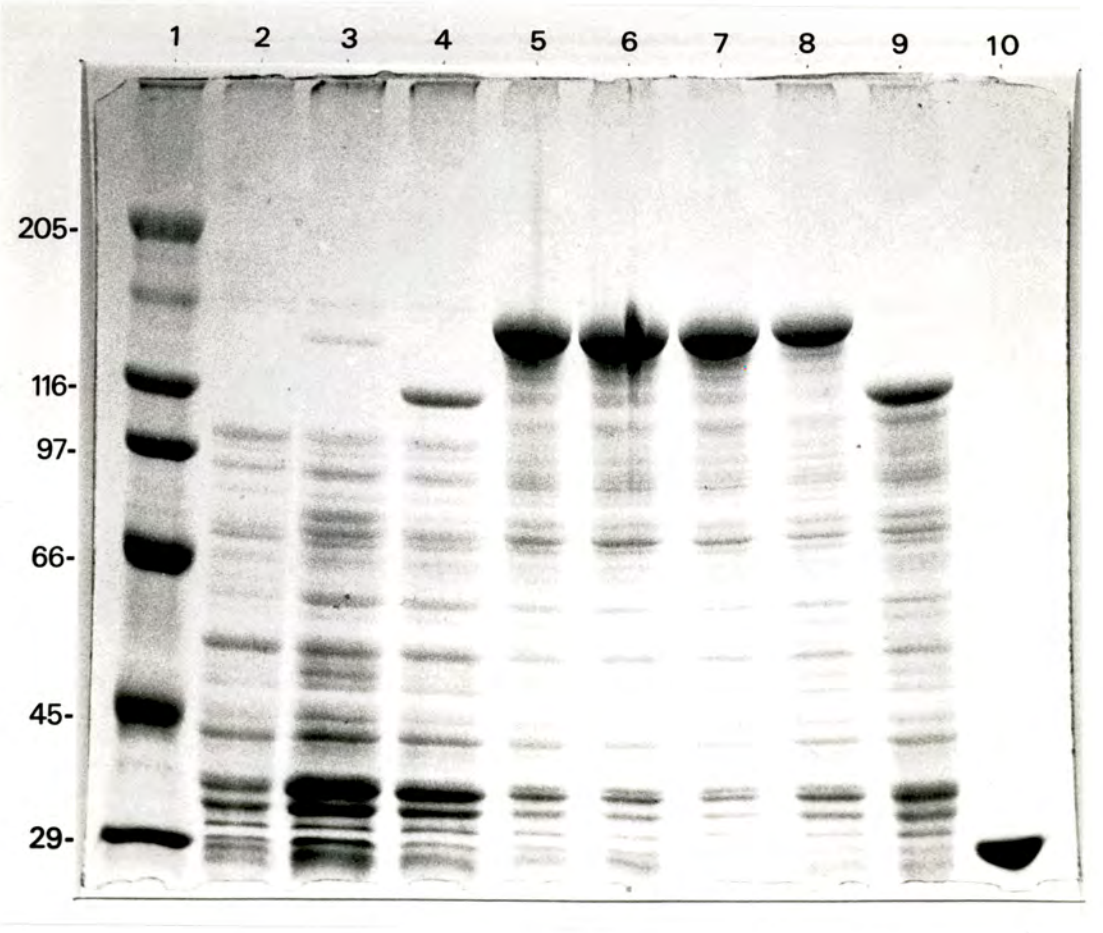

Fig. 6.4. SDS-PAGE of partially-purified protein extracts from *E. coli* containing different plasmids. Lane 1, M_r markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lane 2, *E. coli* LK-111; lane 3, pUC19; lane 4, pUEX2; lane 5, pOMX37; lane 6, pOMX39; lane 7, pOMX41; lane 8, pOMX43; lane 9, pOMX42; lane 10, purified OMV.

6.3.4 Preliminary evaluation of antisera

Antisera obtained from early bleedings of three of the four rabbits immunised with β-gal::OMV CP fusion protein reacted specifically with OMV. No non-specific plant protein bands could be observed in immunoelectroblots of crude OMV infected plant extracts when probed with these antisera (Fig. 6.7).

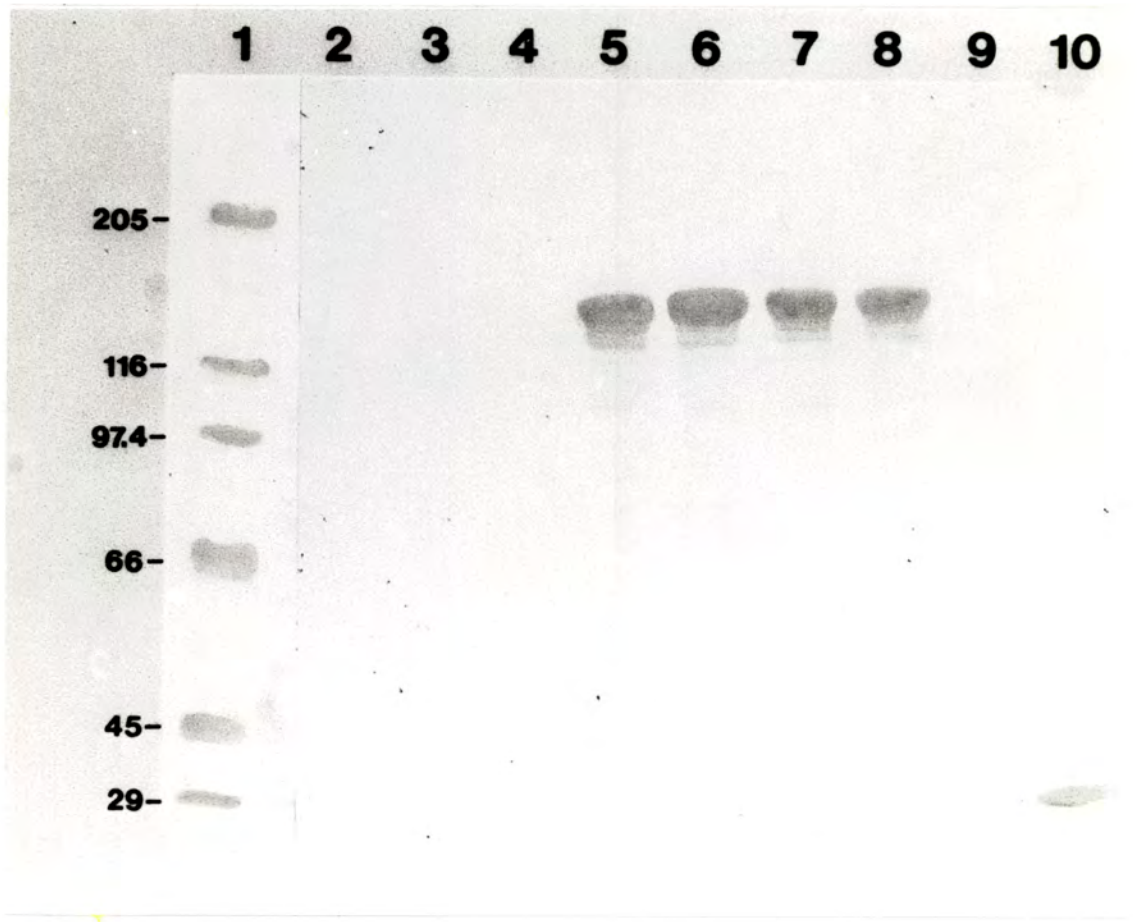

Fig. 6.5. Immunoelectroblot of partially purified *E. coli* protein extracts probed with affinity-purified monospecific anti-OMV antibodies. Lane 1, M_r markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine plasma albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lane 2, *E. coli* LK-111; lane 3, pUC19; lane 4, pUEX2; lane 5, pOMX37; lane 6, pOMX39; lane 7, pOMX41; lane 8, pOMX43; lane 9, pOMX42; lane 10, purified OMV. Marker proteins were stained on the nitrocellulose membrane in a 0.2% solution of ponceau S in 0.3% trichloroacetic acid.

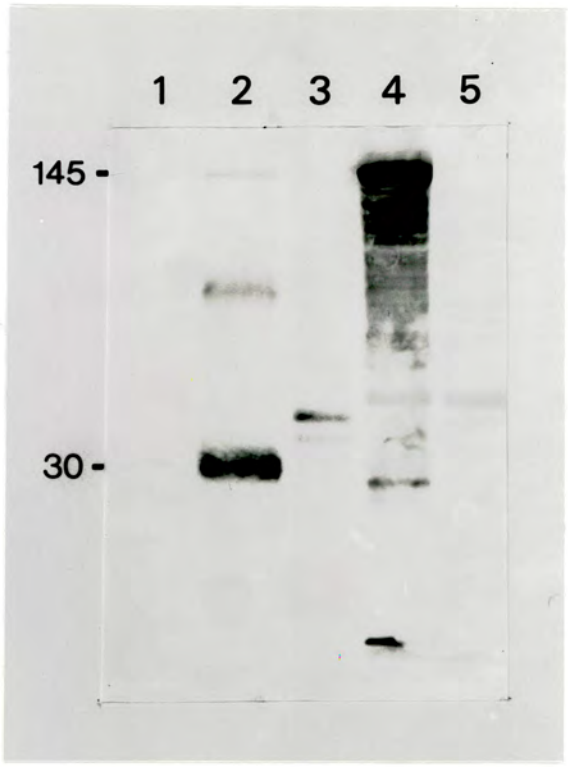

Fig. 6.6. Immunoelectroblot with a potyvirus CP-specific monoclonal antibody (PTY-1). Lane 1, virus-free ornithogalum sap; lane 2, purified OMV; lane 3, purified PWV-SA; lane 4, *E. coli* extract containing pOMX43; lane 5, *E. coli* extract containing pOMX42.

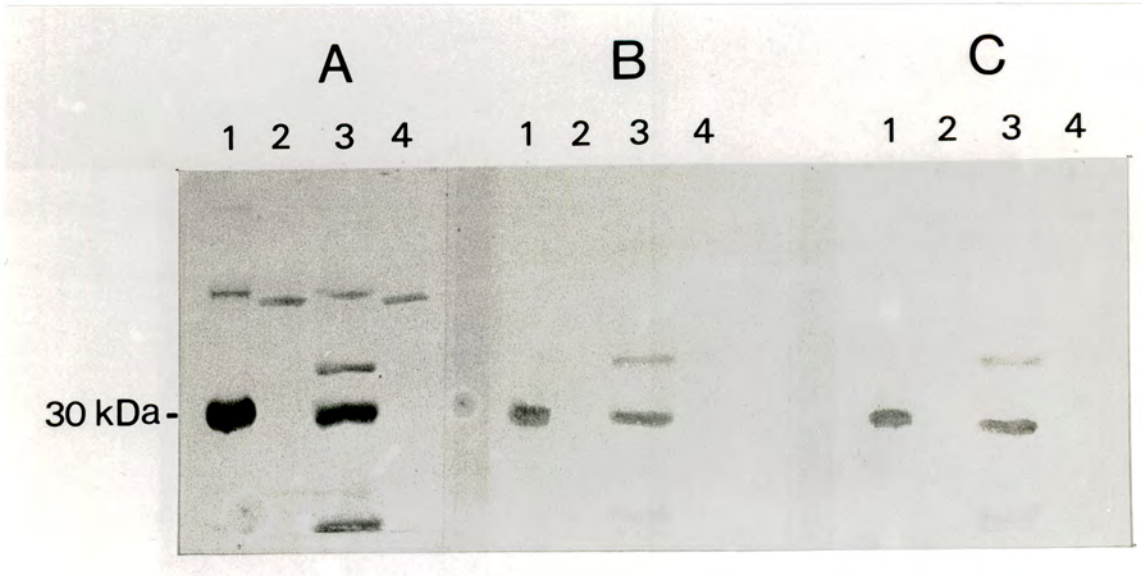

Fig. 6.7. IEB with antisera to: A, purified OMV; B & C, expressed β-gal::OMV CP fusion protein. Lane 1, purified OMV; lane 2, virus-free ornithogalum sap; lane 3, OMV-infected ornithogalum sap; lane 4, virus-free tobacco sap.

6.4 DISCUSSION

The pOMX expression plasmids were created by inserting a 1243 bp fragment, including the OMV CP gene, into the pUEX2 expression vector. These plasmids directed the synthesis of a predicted 145 kDa β-gal::OMV CP fusion protein in transformed *E. coli*.

When screening colonies for fusion protein expression by serological methods, it is essential that the detecting antiserum contains no antibodies to any *E. coli* proteins: absorbing crude anti-OMV antiserum with *E. coli* cell extracts still resulted in false positives in ICB and a background smear in IEB. The use of affinity-purified monospecific antibodies to OMV or a monoclonal antibody to potyvirus CP was required for the reliable detection of the fusion protein in *E. coli* colonies.

Large quantities of the insoluble fusion protein could easily be purified from positive clones. The fusion protein molecular weight of 143.7 kDa determined in SDS-PAGE was very close to the theoretical value of 145 kDa. Since the coding region for the 49 kDa proteinase - which is responsible for cleavage of the CP from the polyprotein - was not present on the expressed cDNA clone, it was not surprising that no post-translational cleavage occurred. Limited post-translational processing by *E. coli* proteases, as was suggested for SMV and BYMV (Eggenberger, *et al.*, 1989, Hammond & Hammond, 1989), did occur, and caused minor bands below the 145 kDa fusion protein band in immunoelectroblots probed with anti-OMV monospecific antibodies and MAb PTY-1.

Antisera prepared against the β-gal::OMV CP fusion protein could be used for specific detection of OMV in ornithogalum and lachenalia sap in immunoelectroblots. However, OMV detection with these antisera was not as sensitive as with conventional anti-OMV antiserum, probably because of the low titre of the early bleeds. It is expected that IgG preparations of later bleeds will be successfully used in the serological detection of OMV in ornithogalum and lachenalia. The possibility of using *E. coli* as a source of viral protein for rabbit immunisation in the long term offers a few distinct advantages: the costly maintenance of virus in laboratory plants would be

eliminated; virus purification, especially from unsuitable host plants such as ornithogalum and lachenalia would not be necessary; and viruses, in this cloned form, could be exchanged between laboratories without the danger of introducing new pathogens into foreign countries.

The expression of OMV CP in transgenic plants as a means of cross-protection against viral infection is beyond the scope of this study, but is an exciting prospect which will be investigated in the future.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Ornithogalum mosaic virus is one of the most serious pathogens of commercially grown *Ornithogalum* and *Lachenalia* spp. in South Africa and elsewhere. However, until recently only the occurrence of the virus, its symptomatology, mode of transmission, and host range have been reported (Smith & Brierley, 1944a, Klesser & Nel, 1976, Derks, 1979, Derks *et al.*, 1983). The reason for this could be that the virus and/or its hosts were considered economically not important enough to warrant a detailed characterisation. A second (and very probable) reason could be the extremely mucilaginous nature of ornithogalum and lachenalia plant extracts, which severely hampered virus purification. The elimination of the polysaccharides in extracts of these plants by hemicellulase digestion, made possible the development of an efficient (albeit somewhat cumbersome) OMV purification protocol, and the subsequent thorough characterisation of the virus.

All the physicochemical properties of OMV were distinctive of the potyvirus group, except that no cytoplasmic inclusions could be found. This could probably also be attributed to the mucilage in the ornithogalum and lachenalia plants which made it impossible to properly dehydrate and embed leaf tissue for ultrathin sectioning. Considering the importance being given to the presence and type of inclusions of a virus in assigning it to, and within the potyvirus group (Edwardson, 1974a, Edwardson *et al.*, 1984), it was decided to confirm OMV's taxonomic assignment by serological and molecular characterisation.

Serological characterisation of the three South African OMV isolates studied (OMV-O, OMV-L and OMV-W), showed that they were all the same strain, and that they are probably also identical to the Dutch isolate of OMV. This is not surprising considering the flower export trade that existed between South Africa and Europe during the 1950s.

Serological relationships with several acknowledged potyviruses were shown in ELISA and IEB, with OMV's closest relative apparently BYMV.

The development of a reliable viral detection method was one of the primary objectives of this study. A single-vial extraction procedure in combination with DAS-ELISA provided a simple but reliable means to detect OMV in ornithogalum and lachenalia plantlets at a very early stage of their development. Virus detection in plant tissue by nucleic acid hybridisation tests was not successful. The highly mucilaginous nature of plant sap caused nucleic acid extracts to block filter membranes when applied to the wells of the multiwell filtration apparatus and little if any adsorption onto membranes occurred.

The role of biological aspects like symptomatology, mode of transmission, host range and epidemiology in plant virus characterisation has always been contentious. The differences in experimental procedures and conditions employed by different laboratories are believed to be largely responsible for the differences (and often contradictions) in results which have been reported in many plant virus groups. Differences in previously reported results on biological aspects of OMV (Smith & Brierley, 1944a, Klesser & Nel, 1976, Derks, 1979, Derks *et al.*, 1983) and those reported here, convinced me to support the notion that these parameters, although useful in preliminary investigations of a virus, cannot be considered conclusive evidence in plant virus characterisation.

Currently, no virus characterisation is complete without also investigating its molecular properties. A portion of the OMV genome was cloned: the nucleotide sequence of this region was determined to confirm OMV's inclusion in the potyvirus taxonomical group; and the coat protein region was expressed in *E. coli* as a first step towards expressing the gene in plants for possible virus resistance.

Nucleotide and deduced amino acid sequence comparisons with several acknowledged potyviruses confirmed that OMV is a potyvirus. Distinct features in potyviral amino acid sequences, which also occurred in the OMV amino acid sequence were: several highly conserved stretches in the 3'-terminal two-thirds of the CP gene; the DAG triplet

- which is seemingly characteristic of aphid-transmitted potyviruses (Harrison & Robinson, 1988) - in the N-terminal region of the CP gene; the two RNA polymerase-specific sequences - GxxxTxxxN and GDD (Argos *et al.*, 1984, Domier et al., 1987) - in the NIb gene; conserved H, D and C residues in the NIa gene, which were reported to be involved in the catalytic activity of the TEV 49 kDa proteinase (Dougherty *et al.*, 1989b); and the consensus recognition sites for the 49 kDa NIa proteinase (Domier *et al.*, 1986, Dougherty *et al.*, 1989a, Robaglia *et al.*, 1989).

The expression of the OMV coat protein in *E. coli* and its subsequent identification with monospecific anti-OMV antiserum, and with a potyvirus CP-specific monoclonal antibody, confirmed that the expressed OMV CP was real. Future work will focus on engineered OMV resistance in ornithogalum and lachenalia. This will entail the insertion of the CP region in a plant expression vector and its subsequent expression in transgenic plants. The expression in plants of other potyviral genes (e.g. the RNA-dependant RNA polymerase) in sense or antisense forms, as possible measures of virus resistance, will also be investigated.

In conclusion; what seemed to be a major stumbling block from the outset of this project, namely the mucilaginous plant sap, indeed turned out to be the villain on several occasions. The mucilage hampered virus purification, made it impossible to characterise cytoplasmic (and possible nuclear) inclusion bodies, and greatly reduced the efficiency of virus detection by nucleic acid hybridisation tests. I believe, however, that the main objectives of this project were met: a virus purification protocol was developed; a reliable virus detection method was devised, and the virus was thoroughly characterised. Physicochemical, serological, biological and molecular characterisation confirmed that OMV was a definite but distinct potyvirus.

Considering all the exciting possibilities that molecular biology holds, especially the expression of viral genes in plants, I believe the work described here has brought us a step closer in "solving the virus problem" in ornithogalum and lachenalia.

APPENDIX A

VIRUS NAMES AND ACRONYMS

AlMV	Alstroemeria mosaic virus
ArMV	Arabis mosaic virus
BaYMV	Barley yellow mosaic
BBWV	Broad bean wilt virus
BCMV	Bean common mosaic virus
BIMV	Bearded iris mosaic virus
BlIMV	Beardless iris mosaic virus
BMV	Brome mosaic virus
BtMV	Beet mosaic virus
BYMV-GDD	Bean yellow mosaic virus (strain GDD)
BYMV-Scott, -G	Bean yellow mosaic virus (Scott and gladiolus isolates)
CERV	Carnation etched ring virus
CLV	Carnation latent virus
ClYVV	Clover yellow vein virus
CMV	Cucumber mosaic virus
DMV	Dahlia mosaic virus
FMV	Freesia mosaic virus
FSV	Freesia streak viruses
GRSV	Gladiolus ringspot virus
HMV	Hyacinth mosaic virus
IFMV	Iris fulva mosaic virus
IMMV	Iris mild mosaic virus
IrV-1	Iris virus 1
IrV-2	Iris virus 2
ISMV	Iris severe mosaic virus
IYMV	Iris yellow mosaic virus

JGMV	Johnsongrass mosaic virus (= SCMV-JG)
KV-1, -2	Kalanchoe latent virus (strains 1 and 2)
LCLV	Lilac chlorotic leafspot virus
LMV	Lettuce mosaic virus
LSSMV	Lilium speciosum streak mottle virus
LSV	Lily symptomless virus
LVX	Lily virus X
LYSV	Leek yellow stripe virus
MaMV	Maclura mosaic virus
MDMV-A, -B	Maize dwarf mosaic virus (strains A and B)
MSV	Maize streak virus
MYFV	Melandrium yellow fleck virus
NaMV	Narcissus mosaic virus
NDV	Narcissus degeneration virus
NeLV	Nerine latent virus (= hippeastrum latent virus)
NeVX	Nerine virus X
NeVY	Nerine virus Y
NeYSV	Nerine yellow stripe virus
NLSYV	Narcissus late season yellows virus (= jonquil mild mosaic virus)
NLV	Narcissus latent virus (= iris mild yellow virus)
NTNV	Narcissus tip necrosis virus
NWSV	Narcissus white streak virus
NYSV	Narcissus yellow stripe virus
OMV-O, -L, -W	Ornithogalum mosaic virus (ornithogalum, lachenalia, and "wild" isolates)
OYDV	Onion yellow dwarf virus
PeMoV	Peanut mottle virus
PeMV	Pepper mottle virus
PLRV	Potato leafroll virus
PokMV	Pokeweed mosaic virus
PPV-D	Plum pox virus (strain D)
PPV-NAT	Plum pox virus (aphid non-transmissible strain)
PRSV	Papaya ringspot virus

PVMV	Pepper veinal mottle virus
PVX	Potato virus X
PVY-1, -2	Potato virus Y (potato and tobacco isolates)
PVY-N, -D	Potato virus Y (strains N and D)
PWV-SA	Passionfruit woodiness virus (South African isolate)
PWV-TB	Passionfruit woodiness virus (strain TB)
RaMV	Ranunculus mottle virus
RRV	Raspberry ringspot virus
RyMV	Ryegrass mosaic virus
SCMV-JG	Sugarcane mosaic virus (Johnsongrass strain; = JGMV)
SLRV	Strawberry latent ringspot virus
SMV-N, -V	Soybean mosaic virus (strains N and V)
SPMMV	Sweet potato mild mottle virus
TBRV	Tomato black ring virus
TBV	Tulip breaking virus
TCBV	Tulip chlorotic blotch virus
TEV-HAT	Tobacco etch virus (highly aphid transmissible strain)
TEV-NAT	Tobacco etch virus (aphid non-transmissible strain)
TGV	Tulip grey virus
TiLV	Tigrida latent virus
TiMV	Tigrida mosaic virus
TMV	Tobacco mosaic virus
ToRSV	Tomato ringspot virus
TRSV	Tobacco ringspot virus
TRV	Tobacco rattle virus
TSV	Tobacco streak virus
TSWV	Tomato spotted wilt virus
TTBV	Tulip tip breaking virus
TuMV	Turnip mosaic virus
TuVX	Tulip virus X
TVMV	Tobacco vein mottling virus
WaTV	"Wa tulip virus"
WMV-1, -2	Watermelon mosaic virus (strains 1 and 2)

WMV-Mor	Watermelon mosaic virus (Moroccan strain)
WSMV	Wheat streak mosaic virus
WSSMV	Wheat spindle streak mosaic virus
ZYMV	Zucchini yellow mosaic virus

APPENDIX B

STANDARD METHODS

STANDARD METHODS

B.1 BIOLOGICAL METHODS

B.1.1 Origin and maintenance of virus infected plants

Ornithogalum thyrsoides Jacq., *O. dubium* Houtt., *O. conicum* Jacq., *O. maculatum* Jacq., *O. pruinosum*, and *Lachenalia aloides* (L.f) Engl., *L. viridiflora* W.F. Baker, *L. mutabilis* Sweet, *L. orchidioides* (L.) Ait., *L. bulbifera* (Cyrillo) Engl., *L. splendida* Diels., and *L. reflexa* Thunb. are the most important species used in the plant improvement programmes at the VOPRI. Except for plants collected from the wild for epidemiological studies, all plant materials were ornithogalum and lachenalia hybrids derived in the above-mentioned programmes. Virus infected bulbs of both genera were planted in heat sterilised soil, containing 25% milled pine bark, and kept in plant growth rooms for 4 to 6 weeks. Day and night temperatures of 22°C and 18°C respectively, a 14 h photoperiod and 70% relative humidity were maintained in plant growth rooms. The plantlets were then transferred to large (1 m x 2 m x 1 m) wooden outdoor cages, which were covered with a fine aphid-proof nylon gauze. Plants were sprayed with a systemic insecticide (oxydemeton-methyl) at two week intervals.

B.1.2 Regeneration and maintenance of virus-free plants

Virus-free callus tissue of ornithogalum and lachenalia hybrid lines, which are used in the plant improvement programmes, was obtained from the VOPRI. Callus tissue was cultured on a basal medium containing the inorganic salts of Murashige and Skoog (1962) with ammendments for ornithogalum propagation and regeneration (Nel, 1981). For lachenalia the auxin indole-3-butyric acid (2 mg/l) was added to the basal medium (Nel, 1983). Explants were kept in glass test tubes at a constant temperature of 22°C and a 16 h photo-period under VHO Gro-lux fluorescent lights. Plantlets were subcultured under sterile conditions onto fresh medium at 8 to 10 week intervals. When roots were adequately developed, plantlets were transplanted into polystyrene trays containing a

heat sterilised, porous soil mixture and kept in plant growth rooms for further development.

B.2 IMMUNOLOGICAL AND SEROLOGICAL METHODS

B.2.1 Rabbit immunisation

Antiserum to OMV was prepared from sucrose gradient-purified virus (0.2 - 0.4 mg virus/injection) and antiserum to β-gal::OMV CP fusion protein from partially-purified fusion protein (250 µl/injection). Antisera were raised in rabbits as described by Rybicki and Von Wechmar, (1981): weekly injections of purified antigen, emulsified in an equal volume of Freund's incomplete adjuvant, were administered for four weeks, and subsequent booster injections were given monthly. Serum was collected three weeks after the initial injections, and weekly thereafter. Approximate antiserum titers were determined by microprecipitin tests (Noordam, 1973).

B.2.2 Monospecific antibody preparation

Monospecific antibodies were prepared by an adaptation of the method of Rybicki (1986). Virus was adsorbed to nitrocellulose filters (BA 85, 0.45 µ pore, Schleicher & Schuell, NH) by soaking filter strips in a dilution of purified virus for 2 h at room temperature. The strips were washed on a shaker with four changes of wash buffer (10 mM Tris-Cl, pH 7.4; 150 mM NaCl; 0.1% (v/v) Tween-20) for 5 min and then incubated overnight in blocking buffer (10 mM Tris-Cl, pH 7.4; 150 mM NaCl; 0.1% (v/v) Tween-20, 2% (w/v) skimmed milk powder) to saturate protein adsorption sites. The strips were then incubated for 2 h in diluted anti-OMV antiserum, which had been host-absorbed with virus-free ornithogalum sap. The filter strips were washed again, and virus-specific antibodies eluted by agitating strips in 10 ml 0.1 M glycine-Cl, pH 2.9 for 10 min. The eluate was poured off, immediately neutralised by the addition of 0.1 M NaOH, dialysed against distilled water, and concentrated by lyophilisation. When monospecific antibodies were prepared for the detection of β-gal::OMV CP fusion protein in *E. coli*, crude anti-OMV antiserum was absorbed with an *E. coli* protein extract prepared by sonication of *E. coli* LK-111 cells.

B.2.3 Immunoelectroblotting

IEB of viral coat proteins was performed as described by Rybicki & Von Wechmar (1982). After electrophoresis the acrylamide gel was laid onto a nitrocellulose filter presoaked in transfer buffer (25 mM Tris-Cl, pH 8.3; 192 mM glycine; 20% (v/v) methanol), and two sheets of presoaked filter paper were placed on either side. This "sandwich" was placed between two 2 cm-thick wads of presoaked nappy (= diaper) liners (Johnson & Johnson) in a custom-made semi-dry carbon electroblot apparatus, orientated with the nitrocellulose filter towards the anode. Blotting continued for 30-60 min at approximately 1 A. Nitrocellulose filters were incubated overnight in blocking buffer to saturate protein adsorption sites, then reacted for 90 min at room temperature with antibodies appropriately diluted in blocking buffer, with gentle shaking. Blots were washed 4 times for 5 min in wash buffer, and incubated for 90 min at room temperature in diluted goat anti-rabbit alkaline phosphatase conjugate (Bio-Yeda, Israel). Blots were washed as before and reacted in substrate buffer (100 mM Tris-Cl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$, containing 0.3 mg/ml nitro blue tetrazolium chloride (NBT) and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Leary *et al.*, 1983).

B.3 PROTEIN METHODS

B.3.1 SDS-PAGE

The discontinuous SDS-PAGE system of Laemmli (1970) was used for protein electrophoresis. Polyacrylamide gels of 7% and 12% were run in vertical slab gel apparati (Hoefer SE 600 or "Mighty-Small", Hoefer Scientific Instruments, San Francisco). Large gels (16 cm long x 14 cm wide x 1.5 mm thick) were run overnight at 10 mA or at 35 mA for approximately 4 h, and small gels (8 cm x 7 cm x 1.5 mm) at 25 mA for 90 min. Samples were dissociated by heating at 95°C for 5 min with an equal volume of disruption buffer (see below). Sigma SDS-6H (Sigma Chemical Company, St Louis, MO) and Pharmacia LMW (Pharmacia Fine Chemicals, Sweden) molecular weight markers were used for 7% and 12% gels respectively.

Gels were stained for 4-16 h in a solution containing 0.2% (w/v) PAGE blue 83 (BDH, UK); 45% (v/v) methanol and 10% acetic acid, and destained in several changes of destain solution (25% (v/v) methanol; 10% (v/v) acetic acid). Gels were either photographed immediately or dried onto filter paper using a Hoefer SE 1160 gel drier.

SDS-PAGE reagents

Resolving gel buffer:	1 M Tris-Cl pH 8.8
Stacking gel buffer:	1 M Tris-Cl pH 6.8
Acrylamide solution:	40% stock solution (acrylamide : bis = 39 : 1) (BDH Electran)
Electrophoresis buffer:	25 mM Tris base; 192 mM glycine; 0.1% (w/v) SDS, pH 8.3
Disruption buffer:	125 mM Tris-Cl pH 6.8; 10% (w/v) SDS; 10% (v/v) 2-mercaptoethanol; 15% (v/v) glycerol; 0.01% (w/v) bromophenol blue.

SDS-PAGE gel preparation table

Stock	Resolving Gel				Stacking Gel	
	7%		12%		4%	
	large	small	large	small	large	small
acrylamide	7.0	1.75	12.0	3.0	1.5	0.5
distilled water	15.6	3.9	10.6	2.65	9.8	3.3
resolving gel buffer	15.0	3.75	15.0	3.75	-	-
stacking gel buffer	-	-	-	-	1.9	0.63
1.5% (w/v) APS	2.0	0.5	2.0	0.5	0.7	0.23
10% (w/v) SDS	0.4	0.1	0.4	0.1	0.15	0.05
80% (v/v) glycerol	-	-	-	-	1.0	0.33
TEMED	0.04	0.02	0.04	0.02	0.04	0.02

B.4 NUCLEIC ACID METHODS

B.4.1 Plasmid DNA preparation

Both small and large scale plasmid DNA preparations were made by the alkaline lysis method of Ish-Horowicz & Burke (1981).

Quick "mini-preparation" of plasmid DNA

Small quantities of plasmid DNA for plasmid size determination could be isolated in under 4 h using this method. Preparations contained chromosomal DNA and were not suitable for further manipulations such as restriction enzyme digestions. Cells from transformed colonies were transferred to microfuge tubes containing 750 µl Luria-Bertani (LB) medium, (1% (w/v) bacto-tryptone; 0.5% (w/v) bacto-yeast extract; 0.5% (w/v) NaCl, pH 7.5), containing 100 µg/ml ampicillin. After shaking violently (with tubes in a horizontal position) at 30°C for 2-3 h, cells were harvested by spinning in a microfuge for 60 sec, and resuspended in 40 µl STE (10 mM Tris-Cl pH 8.0; 100 mM NaCl; 1 mM EDTA). An equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added, the mixture was thoroughly vortexed, and the phases separated by spinning for 3 min in a microfuge. Approximately 5 µl of the aqueous phase was loaded directly onto a 0.8% agarose gel. This technique was used extensively for selection of suitably-sized shortened inserts after Henikoff shortening.

Small scale plasmid DNA preparations

Cell cultures were grown overnight at 37°C in 3 ml LB-100 medium (LB containing 100 µg/ml ampicillin), shaking vigorously. Cells were harvested by centrifugation in a microfuge, the cell pellet drained and resuspended in 300 µl of solution I (25 mM Tris-Cl, pH 8.0; 10 mM EDTA; 50 mM glucose). To this, 600 µl freshly prepared solution II (0.2 M NaOH; 1% SDS) was added, the contents gently mixed by inverting the tube, and then incubated on ice for exactly 5 min. After 450 µl solution III (3 M potassium; 5 M acetate) was added, the tube contents was gently mixed again, and incubated on ice for 5 min. The sample was then centrifuged for 10 min, the supernatant removed to a clean

tube, RNase A added to a final concentration of 50 μg/ml, and incubated at room temperature for 30 min. The sample was extracted with phenol/chloroform and the DNA precipitated with an equal volume of isopropanol. After centrifugation, the DNA pellet was washed with 70% ethanol, lyophilised, and resuspended in TE buffer (10 mM Tris-Cl pH 8.0; 1 mM EDTA).

Large scale plasmid DNA preparation

Cells from a 200 ml overnight LB-100 culture were harvested by centrifugation at 5 000 *g* for 5 min. The cells were resuspended in 4 ml solution I (solutions I, II and III were the same as used in the small scale preparations), 8 ml solution II was added and gently mixed with the resuspended cells. The sample was then incubated on ice for 5 min, 6 ml solution III added, again gently mixed and left on ice for another 5 min. The sample was centrifuged at 10 000 *g* for 10 min and the supernatant transferred to a clean tube. DNA was precipitated with an equal volume of isopropanol and collected by centrifugation. The DNA pellet was resuspended in TE bufffer, caesium chloride and ethidium bromide added to final concentrations of 1 mg/ml and 500 μg/ml respectively, and the refractive index adjusted to 1.390. The samples were sealed in Beckman Quickseal tubes and centrifuged at 55 000 rpm for 12 h in a Beckman Vti 65.2 rotor or at 95 000 rpm in a Beckman TLV-100 rotor for 4 h.

After centrifugation DNA bands were visualised by fluorescence under UV illumination (365 nm). The DNA band of interest was collected through the side of the tube using a sterile 18 gauge hypodermic needle. An equal volume of water was added and DNA precipitated with isopropanol. After the DNA pellet was resuspended in 400 μl TE buffer, RNase A was added to a concentration of 50 μl/ml, and the sample incubated at room temperature for 30 min. Residual ethidium bromide was removed by phenol/chloroform extraction and DNA recovered by ethanol precipitation. Final DNA pellets were resuspended in TE buffer.

B.4.2 Restriction enzyme digests

Both small- and large-scale DNA preparations were used in restriction enzyme digests. Restriction enzymes were obtained from Boehringer Mannheim, Amersham, and Anglian Biotec Limited. The Boehringer Mannheim restriction enzyme incubation buffer set (see below) was used throughout; for digestions with *Kpn* I, BSA was added to the low salt incubation buffer to a concentration of 100 µg/ml. All digestions were done by incubation in a 37°C waterbath for 60 to 120 min, except for *Sma* I, which was incubated at 25°C. Typically 1 to 5 µg DNA was digested using, 2 U restriction enzyme per µg DNA in a 20 µl reaction volume.

Multiple digests were done if required; simultaneously in cases of buffer and temperature compatibility, or sequentially (starting with the enzyme with the lowest salt requirement) if buffers differed. In cases of extreme incompatibility DNA was phenol/chloroform extracted and ethanol precipitated between digestions. A small aliquot of digested DNA was routinely checked for complete digestion by agarose gel electrophoresis in a small "slide" gel (see B.4.5).

Composition of the Boehringer Mannheim restriction enzyme incubation buffer set

Buffer components	Final concentration (mM)				
	A	B	L	M	H
Tris-acetate	33	-	-	-	-
Tris-Cl	-	10	10	10	50
Mg-acetate	10	-	-	-	-
$MgCl_2$	-	5	10	10	10
K-acetate	66	-	-	-	-
NaCl	-	100	-	50	100
DTE	-	-	1	1	1
DTT	0.5	-	-	-	-
2-Mercaptoethanol	-	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

B.4.3 Phenol/chloroform extractions

Nucleic acid preparations were phenol/chloroform extracted to remove contaminating proteins during purification or for the removal of enzymes after enzymatic manipulations of RNA or DNA.

An equal volume of buffered phenol/chloroform/isoamylalcohol (25:24:1) was added to the DNA or RNA preparation, the mixture emulsified by vortexing, and the nucleic acid-containing aqueous phase separated by centrifugation. The extraction was repeated until no precipitate could be seen on the aqueous/organic phase interface (normally three times). Trace amounts of phenol were removed from the preparations by extraction with water-saturated diethyl-ether. Nucleic acids were then precipitated from solution by the addition of NaOAc pH 5.5 to 120 mM and either 2.5 volumes of 96% ethanol or an equal volume of isopropanol, followed by centrifugation. RNA or DNA pellets were washed with ice-cold 70% ethanol, dried, and resuspended in an appropriate buffer.

B.4.4 Sephadex column chromatography for purification of radiolabelled DNA

Sephadex G50 (Pharmacia Fine Chemicals, Sweden) was soaked in TE buffer for a few hours and then autoclaved. A column was poured in a sterile glass pasteur pipette blocked with a glass bead. The sample was loaded and washed through with 150 µl aliquots of TE buffer. Radiolabelled DNA fractions (as determined with a hand-held Geiger counter) were pooled, a 2 µl aliquot was removed and the cpm determined by Cerenkov counting in a Beckman liquid scintillation counter.

B.4.5 Agarose gel electrophoresis

Agarose gel electrophoresis was performed using a horizontal submerged slab gel system. Electrophoresis of large (20 cm long x 15 cm wide x 0.7 cm thick) analytical gels was carried out at 50 V for 16 h, or 100 V for approximately 5 h, in a custom-made perspex apparatus. To obtain quicker results, medium (10 x 6.5 x 0.7 cm) or small (5 x 7.5 x 0.3 cm) gels were run at 100 V in a "Minnie" submarine agarose gel unit

(Model HE 33, Hoefer Scientific Instruments, San Francisco), or a custom-made "slide-gel" apparatus, for 60 or 15 min respectively.

Agarose (Sigma Type 1) or low gelling temperature agarose (SeaPlaque, FMC BioProducts, ME) was used with TBE (89 mM Tris-borate; 89 mM boric acid; 2 mM EDTA pH 8.0) and TAE (40 mM Tris-acetate; 2 mM EDTA pH 8.0) buffers, respectively. Agarose (0.8% to 1.4%, depending on sizes of fragments being analysed) was dissolved in TBE or TAE buffer by melting in a microwave oven, and cooled to approximately 50°C before pouring. Gels were run either with ethidium bromide (0.5 µg/ml) in the running buffer or were stained afterwards. DNA bands were visualised and photographed on a 254 nm UV transilluminator. If DNA was to be recovered from the gels, these were viewed on a 365 nm transilluminator.

B.4.6 DEAE-cellulose membrane purification of DNA fragments

Electrophoretically-separated DNA bands were visualised on a long wavelength transilluminator, and a small strip of DEAE membrane (Schleicher and Schuell, Keene, NH) inserted in an incision made just ahead of the DNA band to be purified. Electrophoresis was resumed until the band was completely bound to the membrane (i.e. no ethidium bromide fluorescence remained visible in the gel). Residual agarose was removed by shaking the membrane for 10 min in a microfuge tube containing NET buffer (150 mM NaCl; 0.1 mM EDTA; 20 mM Tris-Cl pH 8.0). The membrane was shredded with a sterile scalpel blade and transferred to a fresh tube. DNA was eluted in 200 µl high salt NET buffer (1 M NaCl; 0.1 mM EDTA; 20 mM Tris-Cl pH 8.0) by shaking at 65°C for 30 min. The buffer was removed and the elution repeated with 200µl fresh high salt NET buffer. The two buffer fractions were pooled, phenol/chloroform extracted, ethanol precipitated, and final pellets resuspended in TE buffer.

B.4.7 Southern blotting of DNA to nylon membranes

Fractionated DNA bands were transferred to nylon membranes (Hybond N, Amersham, UK) by the method of Smith & Summers (1980).

After electrophoresis the agarose gel was incubated twice for 15 min in two gel volumes of 0.25 M HCl to partially hydrolyse the DNA by acid depurination. The gel was then incubated twice for 15 min in denaturing buffer (0.5 M NaOH; 1.5 M NaCl), and neutralised by two 30 min incubations in 1 M NH_4OAc; 0.02 M NaCl (neutralising buffer). The gel was rinsed in water between all incubation steps, which were done at room temperature with gentle agitation. The gel was then placed on a glass plate and overlaid with a nylon membrane and three layers of filter paper, all which had been pre-wetted in neutralising buffer. A 5 cm-thick wad of dry tissue paper was placed on the gel/membrane/filter paper sandwich, followed by another glass plate and a 5 kg weight. Blotting was continued for 60-90 min. DNA was fixed onto the air-dried membrane by UV irradiation (254 nm) for 5 min. Membranes were used for hybridisation immediately, or stored until used at 4°C in a sealed plastic bag.

B.4.8 Nick-translation labelling of DNA

DNA was radiolabelled with [α-^{32}P]dCTP by nick translation using an Amersham kit (N.5000, Amersham, UK). In a typical 25 µl labelling reaction 1 µg DNA was mixed with 5 µl nucleotide/buffer solution (100 µM each of dATP; dGTP and dTTP in a Tris-Cl pH 7.8 buffer containing $MgCl_2$ and 2-mercaptoethanol), 2.5 µl [α-^{32}P]dCTP (3000 Ci/mmol in aqueous solution at 10 µCi/µl) and 2.5 µl enzyme solution (0.5 U/µl DNA polymerase I; 10 pg/µl DNase I), and incubated at 15°C for 2 h. Labelled DNA was separated from unincorporated dNTPs by chromatography through a pasteur pipette column of Sephadex G50.

B.4.9 Colony hybridisation assays

Colony hybridisation assays were done according to the method of Buluwela *et al.* (1989). Bacterial colonies were transferred to nylon filter membranes (Hybond N, Amersham, UK) by placing dry filters on plates, rubbing them gently, and peeling them off. Filters were placed, colonies facing up, on filter paper disks soaked in 2X SSPE, 5% SDS and microwaved for 3 min at 650 W. The filters were then transferred to filter paper disks soaked in 5X SSPE; 0.1% SDS for 5 min; then sandwiched between clean filter paper disks, and sealed in plastic bags for hybridisation.

B.4.10 Hybridisation assays

After DNA fixing by UV illumination, the nylon filter was placed in a thick plastic bag with pre-hybridisation solution (5X SSPE; 5X Denhardt's solution, 0.5% SDS, 50 $\mu l/cm^2$ filter). Sonicated salmon sperm DNA was denatured by boiling for 5 min, snap-cooled on ice, and added to the pre-hybridisation solution to a final concentration of 100 $\mu g/ml$. The bag was sealed and incubated with shaking at 65°C for 4-16 h.

For the hybridisation reaction the filter was transferred to a new plastic bag, and pre-hybridisation solution and denatured salmon sperm DNA added as before. The probe was denatured by boiling, and added. The bag was sealed and incubated as before for 16 h.

After hybridisation the filter was washed at 65°C for 10 min in low stringency washing buffer (see below). Moderate and high stringency washes were done when necessary (as judged using a hand-held Geiger counter). Finally the filter was wrapped in Saran wrap and subjected to autoradiography.

Buffers for hybridisation assays

1. 20X SSPE

   3.6M NaCl; 200mM sodium phosphate pH 7.7; 20mM EDTA

2. 100X Denhardt's solution

   2% (w/v) BSA; 2% (w/v) Ficoll; 2% (w/v) PVP

3. Pre-hybridisation solution

   5X SSPE; 5X Denhardt's solution; 0.5% SDS

4. Stringency washes

   Low stringency washing buffer 2.0X SSPE; 0.1% SDS

   Moderate stringency washing buffer 0.5X SSPE; 0.1% SDS

   High stringency washing buffer 0.1X SSPE; 0.1% SDS

B.4.11 Autoradiography

Radioactive blots were exposed to Curix RP 1 X-ray film (Agfa, West Germany) at -70°C in X-ray cassettes with intensifying screens. In cases of a weaker signal, as judged using a hand-held Geiger counter, Kodak XAR 5 X-ray film (Eastman Kodak Company, NY) was used.

Dried sequencing gels containing ^{35}S were exposed overnight at room temperature to Curix RP 1 X-ray film in casettes without intensifying screens. Autoradiographs were processed using Agfa chemicals according to the manufacturer's instructions.

B.4.12 cDNA synthesis and cloning

RNA/cDNA hybrid and ds-cDNA yields were calculated after doing duplicate first and second strand synthesis reactions in parallel, and incorporating radioactivity in one of the reactions.

First strand synthesis

Freshly-extracted OMV RNA was resuspended in water to a concentration of 1 µg/µl. In two microfuge tubes the following were mixed:

	Tube A	Tube B
RNA	2.0 µl	2.0 µl
Methyl mercury hydroxide (50 mM)	1.0	1.0
H_2O	2.0	2.0

The tubes were incubated at 60°C for 5 min to denature RNA; 1 µl 700 mM 2-mercaptoethanol was added and tubes left at room temperature for 5 min. The following were then added in order:

	Tube A	Tube B
(Denatured RNA	6.0 µl	6.0 µl)
5X First strand synthesis buffer	4.0	4.0
Oligo d(T) primer	2.0	2.0
dNTP mix	2.0	2.0
RNasin	0.5	0.5
[α-^{32}P]dCTP	2.0	-
H_2O	1.5	3.5
Reverse transcriptase	2.0	2.0
	20.0 µl	20.0 µl

Tubes were incubated at 42°C for 2 h, and reactions stopped on ice. Unincorporated dNTPs were removed by fractionation on Sephadex G50 pasteur pipette columns. A 2 µl aliquot from reaction A was Cerenkov counted and the RNA/cDNA yield calculated (see below).

Second Strand Synthesis

The appropriate fractions from the column chromatography were pooled, RNA/cDNA hybrid molecules ethanol precipitated, and resuspended in 20 µl H_2O. The following were added:

	Tube A	Tube B
(RNA/cDNA	20.0 µl	20.0 µl)
Second strand synthesis buffer	50.0	50.0
dNTP mix	9.0	9.0
[α-^{32}P]dCTP	-	2.0
RNase H	1.0	1.0
DNA polymerase I	3.0	3.0
H_2O	17.0	15.0
	100.0 µl	100.0 µl

Tubes were then sequentially incubated at 12°C for 60 min., 22°C for 60 min., and 70°C for 10 min. One microliter (1 µl) T4 DNA polymerase was added and tubes incubated at 37°C for 10 min. Reactions were stopped by addition of 5 µl 0.5 M EDTA, fractionated again on Sehadex G50 columns and ds-cDNA yield calculated after Cerenkov counting of a 2 µl fraction of reaction B.

Blunt-end Cloning

pUC 19 DNA was digested to completion with *Sma* I, cleaned up by phenol/chloroform extraction and ethanol precipitation, and resuspended in TE buffer.

The following were mixed in a microfuge tube and incubated at 15°C for 16 h:

cDNA (100 ng)	10.0 µl
pUC19 DNA (50 ng)	1.0
10X Blunt-end ligation buffer	2.0
T4 ligase	1.0
water	6.0
	20.0 µl

cDNA cloning buffers and enzymes

1. Methyl mercury hydroxide
   50 mM in water (Alfa Products, West Germany)

2. 5X First strand synthesis buffer
   500 mM Tris-Cl pH 8.3 at 42°C; 140 mM KCl; 100 mM $MgCl_2$

3. 2X Second strand synthesis buffer
   40 mM Tris-Cl pH 7.5; 10 mM $MgCl_2$; 20 mM $(NH_4)_2SO_4$; 200 mM KCl; 100 µg/ml BSA

4. 10X Blunt-end ligation buffer
   500 mM Tris-Cl pH 7.6; 100 mM $MgCl_2$; 20 mM DTT; 20 mM ATP

5. Oligo d(T) primer
   Oligo $d(T)_{12-18}$, 2 µg/µl (Boehringer Mannheim)

6. dNTP mix
   A cocktail containing 2.85 mM dCTP, 5.70 mM dATP, 5.70 mM dGTP and 5.70 mM dTTP (Boehringer Mannheim).

7. RNasin
   RNasin 50 U/µl (Boehringer Mannheim)

8. [α-^{32}P]dCTP
   3000 Ci/mmol, 10 µCi/µl (Amersham)

9. Enzymes
   Reverse transcriptase M-MuLV, 25 U/µl (Boehringer Mannheim)
   RNase H, 20 U/µl (Amersham)
   DNA polymerase I, 8 U/µl (Amersham)
   T4 polymerase, 4 U/µl (Amersham)
   T4 ligase, 10 U/µl (Anglian Biotec Limited)

10. Water
    Glass double distilled water was treated overnight with 0.1% diethyl pyrocarbonate (DEPC) at 37°C, and then autoclaved. All glassware, tips and microfuge tubes were similarly treated.

Calculation of cDNA yield

RNA/cDNA hybrid yield was determined using the equation:

$$X\ \mu g\ RNA/cDNA = \frac{\text{radioactivity incorporated in first strand (dpm)}}{5.54 \times 10^6}$$

This equation was derived as follows:

1. Calculation of the total amount of dCTP used

In the first strand synthesis reaction 6.67×10^{-3} nmol radiolabelled dCTP (20 µCi of a 3000 µCi/nmol stock) and 5.714 nmol unlabelled dCTP (2 µl of a 2.857 mM stock) were used. Therefore a total of 5.72 nmol dCTP was represented by 20 µCi radioactivity.

2. Calculation of dpm per nmol dCTP

1 μCi of ^{32}P represents 2.22×10^6 dpm
therefore 20 μCi = $20 \times 2.22 \times 10^6$ dpm/5.72 nmol dCTP
= 7.76×10^6 dpm/nmol

3. Calculation of dpm per μg cDNA

Assume M_r of dNTP = 350
therefore 1 nmol dNTP = 0.35 μg dNTP
assume dCTP = 25% of the bases
therefore 1 nmol dCTP = 0.35 x 4
= 1.4 μg cDNA

$$\text{The number of dpm/μg cDNA} = \frac{7.76 \times 10^6 \text{ dpm/nmol}}{1.4 \text{ μg cDNA/nmol}}$$
$$= 5.54 \times 10^6 \text{ dpm/μg cDNA}$$

$$\text{therefore cDNA yield} = \frac{\text{total dpm incorporated}}{5.54 \times 10^6}$$

Similarly the second strand cDNA yield was calculated using the equation:

$$\text{X μg ds-cDNA} = \frac{\text{radioactivity incorporated in second strand (dpm)}}{1.23 \times 10^6}$$

B.4.13 Preparation and transformation of competent *E. coli* cells

E. coli LK-111 (*lac I, lac Z*, M15 derivative of *E. coli* K514, Zabeau & Stanley, 1982) competent cells were prepared as described by Chung & Millar (1988).

LK-111 cultures, freshly-grown overnight, were used to inoculate 100 ml LB medium. These were allowed to grow to an early log phase (OD_{600} = 0.5) before being harvested by centrifugation at 1 000 *g* for 10 min at 4°C. The cells were resuspended in 10 ml ice

cold TSB buffer (LB medium, containing 10% (w/v) PEG M_r 4 000; 5% (v/v) DMSO; 10 mM $MgCl_2$ and 10 mM $MgSO_4$) and incubated on ice for at least 10 min. Cells were then either used immediately for transformation or aliquotes thereof stored at -70°C. Frozen cells retained satisfactory competence for two weeks.

For transformation, approximately 50 ng plasmid DNA was mixed with 100 µl competent cells and incubated on ice for 30 min. After the addition of 900 µl of preheated TSB, containing 20 mM glucose, cells were grown at 37°C for 60 min to allow expression of the ampicillin resistance gene. Aliquotes of 100 µl transformed cells were plated on Luria-Bertani agar (LA-100) plates (LB medium, containing 1.5% (w/v) bacto-agar and 100 µg/ml ampicillin). In cases where the inactivation of the β-gal gene of the pUC vector was used as marker, 40 µl X-gal solution (20 mg/ml X-gal in N,N dimethylformamide) was spread on LA-100 plates before transformed cells were plated out.

B.4.14 Exonuclease III/S1 nuclease shortening

Caesium chloride density gradient-purified DNA (20 µg) from pOM16 or pOM21 was digested to completion with *Pst* I and *Bam* HI, cleaned up by phenol/chloroform extraction, and ethanol precipitated. The lyophylised DNA pellet was resuspended in 130 µl exonuclease III buffer and equilibrated at 37°C for 5 min. An 8 µl fraction was removed into another tube (time T=0) containing 25 µl S1 nuclease reaction mix, and placed on ice. Exonuclease III (Boehringer Mannheim 175 U/µl) was added to the remaining DNA to a concentration of 150 U per pmol of DNA 3' termini. Aliquotes of 8µl were removed at 30 sec intervals to tubes (T=1 to T=15) on ice containing 25 µl S1 nuclease reaction mix each. The tubes were incubated at room temperature for 30 min before reactions were stopped by addition of 3.5 µl S1 nuclease stop buffer and incubation at 70°C for 10 min. Fractions of 5 µl were removed from each time interval and run on a 0.8% agarose gel. Four microliters (4 µl) of a Klenow reaction mix was added to each tube, and tubes incubated at room temperature for 3 min; then 1 µl dNTP mix was added and tubes incubated for a further 5 min at room temperature. Ligation was carried out at 15°C for 16 h after 115 µl ligase reaction mix was added to each tube.

Buffers for shortening reactions

1. 10X S1 nuclease buffer
   0.33 M NaOAc pH 4.6; 2.5 M NaCl; 10 mM $ZnSO_4$; 50% (v/v) glycerol

2. Exonuclease III buffer
   66 mM Tris-Cl pH 8.0; 0.66 mM $MgCl_2$

3. S1 nuclease reaction mix (enough for 16 tubes)
   53µl 10X S1 nuclease buffer; 347µl water;
   80U S1 nuclease, 400 U/µl (Boehringer Mannheim)

4. S1 nuclease stop buffer
   300 mM Tris base; 50 mM EDTA

5. Klenow reaction mix
   20 mM Tris-Cl pH 8.0; 7 mM $MgCl_2$;
   16 µl (3-4 U/µg DNA) Klenow fragment, 4 U/µl (Amersham)

6. 10X Ligation buffer
   500 mM Tris-Cl pH 7.6; 100 mM $MgCl_2$; 20 mM ATP (freshly prepared);
   20 mM DTT

7. Ligase reaction mix
   240 µl 10X ligation buffer; 32 µl T4 DNA ligase, 1 U/µl (Boehringer Mannheim);
   1.568 ml water

8. dNTPs
   A cocktail containing 0.125 mM of each dNTP (Boehringer Mannheim)

REFERENCES CITED

ABU SALIH, H. S., MURANT, A. F. & DAFT, M. J. (1968). Comparison of the passive haemaglutination and bentonite flocculation tests for serological work with plant viruses. *J. Gen. Virol.* **2**, 155-166.

ABU-SAMAH, N. & RANDLES, J. W. (1981). A comparison of the nucleotide sequence homologies of three isolates of bean yellow mosaic virus and their relationship to other potyviruses. *Virology* **110**, 436-444.

ABU-SAMAH, N. & RANDLES, J. W. (1983). A comparison of Australian bean yellow mosaic virus isolates using molecular hybridisation analysis. *Ann. Appl. Biol.* **103**, 97-107.

ALBA, A. P. C. & OLIVEIRA, A. R. (1976). Serological studies on viruses of the potato virus Y group occurring in Sao Paulo State. *Summa Phytopathol.* **2**, 178-186.

ALLEN, T. C. (1972). Lily symptomless virus. *CMI/AAB Descriptions of Plant Viruses* **96**, 4 pp.

ALLEN, T. C. & ANDERSON, W. C. (1980). Production of virus-free ornamental plants in tissue culture. *Acta Horticulturae* **110**, 245-251.

ALLISON, R. F., DOUGHERTY, W. G., PARKS, T. D., WILLIS, L., JOHNSTON, R. E., KELLY, M. E. & ARMSTRONG, F. B. (1985a). Biochemical analysis of the capsid protein gene and capsid protein of the tobacco etch virus: N-terminal amino acids are located on the virion's surface. *Virology* **147**, 309-316.

ALLISON, R. F., SORENSON, J. C., KELLY, M. E., ARMSTRONG, F. B. & DOUGHERTY, W. G. (1985b). Sequence determination of the capsid protein gene and flanking regions of tobacco etch virus: evidence for synthesis and processing of a polyprotein in potyvirus genome expression. *Proc. Natl. Acad. Sci. USA* **82**, 3969-3972.

ALLISON, R. F., JOHNSTON, R. E. & DOUGHERTY, W. G. (1986). The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: evidence for the synthesis of a single polyprotein. *Virology* **154**, 9-20.

ARBER, A. (1940). The colouring of sixteenth century herbals. *Nature* **145**, 803-804.

ARGOS, P., KAMER, G., NICKLIN, M. J. H. & WIMMER, E. (1984). Similarity in gene organization and homology between proteins of animal picornaviruses and a plant comovirus suggest common ancestry of these virus families. *Nucleic Acids Research* **12**, 7251-7267.

ASJES, C. J. (1972a). Control of the spread of the brown ring formation virus disease in the lily mid-century hybrid "enchantment" by mineral oil sprays. *Acta Horticulturae* **36**, 85-91.

ASJES, C. J. (1972b). Tulip veinal streak, a disorder probably caused by tobacco ringspot virus. *Neth. J. Plant Path.* **78**, 19-28.

ASJES, C. J. (1976). Virus diseases in gladiolus and their control in the Netherlands. (Abstr.) *Acta Horticulturae* **177**, 240.

ASJES, C. J. (1979). Viruses and virus diseases in Dutch bulbous irises (*Iris hollandica*) in the Netherlands. *Neth. J. Plant Path.* **85**, 269-279.

ASJES, C. J. & SEGERS, L. Ch. (1980). Tulp. *Jversl. Lab. Bloembollenonderz. Lisse* 1979, 50-52.

ASJES, C. J. & SEGERS, L. Ch. (1982). Tulp, Gladiool. *Jversl. Lab. Bloembollenonderz. Lisse* 1981, 55.

ASJES, C. J., DE VOS, N. P. & VAN SLOGTEREN, D. H. M. (1973). Brown ring formation and streak mottle, two distinct syndromes in lilies associated with complex infections of lily symptomless virus and tulip breaking virus. *Neth. J. Plant Path.* **79**, 23-35.

ASJES, C. J., BUNT, M. H. & VAN SLOGTEREN, D. H. M. (1974). Production of hyacinth mosaic virus-free hyacinths and lily symptomless virus-free lilies by meristem tip culture. *Acta Horticulturae* **36**, 223-228.

BALASINGAM, G., MILNE, K. S. & FORSTER, R. L. S. (1988). Sensitive and specific detection of two filamentous viruses from Nerines using cloned cDNA probes. *Acta Horticulturae* **234**, 267-274.

BANTTARI, E. E. & GOODWIN, P. H. (1985). Detection of potato viruses S, X, and Y by enzyme-linked immunosorbent assay on nitrocellulose membranes (dot-ELISA). *Plant Disease* **69**, 202-205.

BARNETT, O. W. & BRUNT, A. A. (1975). Bearded iris mosaic virus. *CMI/AAB Descriptions of Plant Viruses* **147**, 3 pp.

BARNETT, O. W. & ALPER, M. (1977). Characterization of *Iris fulva* mosaic virus. *Phytopathology* **67**, 448-454.

BARNETT, O. W., DE ZOETEN, G. A. & GAARD, G. (1971). Bearded iris mosaic virus: transmission, purification, inclusions, and its differentiation from bulbous iris mosaic virus. *Phytopathology* **61**, 926-932.

BARNETT, O. W., RANDLES, J. W. & BURROWS, P. M. (1987). Relationships among Australian and North American isolates of the bean yellow mosaic potyvirus subgroup. *Phytopathology* **77**, 791-799.

BARON, M. H. & BALTIMORE, D. (1982). Anti-VPg inhibition of the poliovirus replicase reaction of covalent complexes of VPg-related proteins and RNA. *Cell* **30**, 745-752.

BAULCOMBE, D. C., FLAVELL, R. B., BOULTON, R. E. & JELLIS, G. J. (1984). The sensitivity and specificity of a rapid nucleic acid hybridization method for the detection of potato virus X in crude sap samples. *Plant Pathology* **33**, 361-370.

BAULCOMBE, D. C. & FERNANDEZ-NORTHCOTE, E. N. (1988). Detection of strains of potato virus X and of a broad spectrum of potato virus Y isolates by nucleic acid spot hybridization (NASH). *Plant Disease* **72**, 307-309.

BAZAN, J. F. & FLETTERICK, R. J. (1988). Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. *Proc. Natl. Acad. Sci. USA* **85**, 7872-7876.

BEIJERSBERGEN, J. C. M. & VAN DER HULST, C. T. C. (1980). Application of enzymes during bulb tissue extraction for detection of lily symptomless virus by ELISA in *Lilium* spp. *Neth. J. Plant Path.* **86**, 277-283.

BELLARDI, M. G. & MARANI, F. (1985). Nepoviruses isolated from gladiolus in Italy. *Acta Horticulturae* **164**, 297-308.

BELLARDI, M. G., MARANI, F. & BERTACCINI, A. (1988). Narcissus mosaic virus in lily. *Acta Horticulturae* **234**, 457-464.

BERGER, P. H. & PIRONE, T. P. (1986). The effect of helper component on the uptake and localization of potyviruses in *Myzus persicae*. *Virology* **153**, 256-261.

BERGER, P. H., THORNBURY, D. W. & PIRONE, T. P. (1984). Highly sensitive serological detection of potato virus Y. (Abstr.) *Phytopathology* **74**, 847.

BOND, W. P., WHITAM, H. K. & BLACK, L. L. (1983). Indigenous weeds as reservoirs of tomato spotted wilt in Louisiana. (Abstr.) *Phytopathology* **73**, 499.

BOS, L. (1970). The identification of three new viruses isolated from *Wisteria* and *Pisum* in the Netherlands, and the problem of variation within the potato virus Y group. *Neth. J. Plant Path.* **76**, 8-46.

BRAKKE, M. K. & VAN PELT, N. (1970). Properties of infectious ribonucleic acid from wheat streak mosaic virus. *Virology* **42**, 699-706.

BRANDES, J. & WETTER, C. (1959). Classification of elongated plant viruses on the basis of particle morphology. *Virology* **8**, 99-115.

BRESSAN, G. M. & STANLEY, K. K. (1987). pUEX, a bacterial expression vector related to pEX with universal host specificity. *Nucleic Acids Research* **15**, 10056.

BRIERLEY, P. & SMITH, F. F. (1948). Two additional mosaic diseases of iris. (Abstr.) *Phytopathology* **38**, 574-575.

BRUNT, A. A. (1967a). Viruses affecting Iridaceae. *Ann. Rep. Glasshouse Crops Res. Inst.* 1966, 99-100.

BRUNT, A. A. (1967b). Freesia streak virus (FSV). *Ann. Rep. Glasshouse Crops Res. Inst.* 1966, 101.

BRUNT, A. A. (1980). A review of problems and progress in research on viruses and virus diseases of narcissus in Britain. *Acta Horticulturae* **110**, 23-30.

BRUNT, A. A. (1988). New economically-important virus and virus-like diseases of ornamental plants. *Acta Horticulturae* **234**, 505-514.

BRUNT, A. A. & ATKEY, P. T. (1970). A new group of filamentous aphid-borne viruses? *Ann. Rep. Glasshouse Crops Res. Inst.* 1970, 152-153.

BULUWELA, L., FORSTER, A., BOEHM, T. & RABBITS, T. H. (1989). A rapid procedure for colony screening using nylon filters. *Nucleic Acids Research* **17**, 452-452.

BURGER, J. T. & VON WECHMAR, M. B. (1988). Rapid diagnosis of ornithogalum and lachenalia viruses in propagation stock. *Acta Horticulturae* **234**, 31-38.

BURGER, J. T. & VON WECHMAR, M. B. (1989). Purification and some properties of South African isolates of Ornithogalum mosaic virus. *Phytopathology* **79**, 385-391.

BURGER, J. T., BRAND, R. J. & RYBICKI, E. P. (1990). The molecular cloning and nucleotide sequencing of the 3'-terminal region of Ornithogalum mosaic virus. *J. Gen. Virol.*, In Press.

BURGERMEISTER, W. & KOENIG, R. (1984). Electroblot immunoassay - a method for studying serological relationships among plant viruses? *J. Phytopathology* **111**, 15-25.

CARRINGTON, J. C. & DOUGHERTY, W. G. (1987a). Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. *J. Virol.* **61**, 2540-2548.

CARRINGTON, J. C. & DOUGHERTY, W. G. (1987b). Processing of the tobacco etch virus 49K protease requires autoproteolysis. *Virology* **160**, 355-362.

CARRINGTON, J. C., CARY, S. M. & DOUGHERTY, W. G. (1988). Mutational analysis of tobacco etch virus polyprotein processing: *cis* and *trans* proteolytic activities of polyproteins containing the 49-kilodalton proteinase. *J. Virol.* **62**, 2313-2320.

CARRINGTON, J. C., CARY, S. M., PARKS, T. D. & DOUGHERTY, W. G. (1989a). A second proteinase encoded by a plant potyvirus genome. *EMBO Journal* **8**, 365-370.

CARRINGTON, J. C., FREED, D. D. & SANDERS, T. C. (1989b). Autocatalytic processing of the potyvirus helper component proteinase in *E. coli* and *in vitro*. *J. Virol.* **63**, 4459-4463.

CHACONAS, G. & VAN DE SANDE, J. H. (1980). 5'-^{32}P Labeling of RNA and DNA restriction fragments. *Methods In Enzymology* **65**, 75-85.

CHAMBERLAIN, J. A., CATHERALL, P. L. & JELLINGS, A. J. (1977). Symptoms and electron microscopy of ryegrass mosaic virus in different grass species. *J. Gen. Virol.* **36**, 297-306.

CHANG, C. A., PURCIFULL, D. E. & HIEBERT, E. (1985). Purification and partial characterization of nuclear inclusions induced by a pea mosaic isolate of bean yellow mosaic virus. (Abstr.) *Phytopathology* **75**, 499.

CHANG, C. A., HIEBERT, E. & PURCIFULL, D. E. (1988). Analysis of *in vitro* translation of bean yellow mosaic virus RNA: inhibition of proteolytic processing by antiserum to the 49K nuclear inclusion protein. *J. Gen. Virol.* **69**, 1117-1122.

CHIRKOV, S. N., OLOVNIKOV, A. M., SURGUCHYOVA, N. A. & ATABEKOV, J. G. (1984). Immunodiagnosis of plant viruses by a virobacterial agglutination test. *Ann. Appl. Biol.* **104**, 477-483.

CHRISTIE, R. G. & EDWARDSON, J. R. (1977). Light and electron microscopy of plant virus inclusions. *Fla. Agric. Exp. Stn. Monogr. Ser.* **No. 9**, 155 pp.

CHUNG, C. T. & MILLER, R. H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research* **16**, 3580.

CLARK, M. F. & ADAMS, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34**, 475-483.

CLARK, M. F. & BAR-JOSEPH, M. (1984). Enzyme Immunosorbent Assays in Plant Virology. Pages 51-85 in: K. Maramorosh & H. Koprowski, eds. Methods in Virology, Vol. VII, Academic Press, London.

CUOZZO, M., O'CONNELL, K. M., KANIEWSKI, W. K., FANG, R. X., CHUA, N. H. & TUMER, N. E. (1988). Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology* **6**, 549-557.

DAHLGREN, R. M. T. & CLIFFORD, H. T. (1982). The Monocotyledons: A Comparative Study. Academic Press, London, 378 pp.

DE BOKX, J. A. & MAAT, D. Z. (1979). Detection of potato virus Y^N in tubers with the enzyme-linked immunosorbent assay (ELISA). *Med. Fac. Landbouww. Rijksuniv. Gent* **44/2**, 635-644.

DE BOKX, J. A., PIRON, P. G. M. & MAAT, D. Z. (1980). Detection of potato virus X in tubers with the enzyme-linked immunosorbent assay (ELISA). *Potato Research* **23**, 129-131.

DE MEJIA, M. V. G., HIEBERT, E. & PURCIFULL, D. E. (1985a). Isolation and partial characterization of the amorphous cytoplasmic inclusions associated with infections caused by two potyviruses. *Virology* **142**, 24-33.

DE MEJIA, M. V. G., HIEBERT, E., PURCIFULL, D. E., THORNBURY, D. W. & PIRONE, T. P. (1985b). Identification of potyviral amorphous inclusion protein as a nonstructural virus-specific protein related to helper component. *Virology* **142**, 34-43.

DEMSKI, J. W., BAYS, D. C. & KAHN, M. A. (1986). Simple latex agglutination test for detecting rod-shaped viruses in forage legumes. *Plant Disease* **70**, 777-779.

DERKS, A. F. L. M. (1979). Diagnostiek van virusziekten en identificatie van virussen in bloembolgewassen. *Jversl. Lab. Bloembollenonderz. Lisse* 1978, 51.

DERKS, A. F. L. M. & VINK-VAN DEN ABEELE, J. L. (1980). Hyacinth mosaic virus: symptoms in hyacinths, serological detection, and relationships with other potyviruses. *Acta Horticulturae* **109**, 495-502.

DERKS, A. F. L. M. & HOLLINGER, Th. C. (1986). Similarities of and differences between potyviruses from bulbous and rhizomatous irises. *Acta Horticulturae* **177**, 555-561.

DERKS, A. F. L. M., VINK-VAN DEN ABEELE, J. L. & MULLER, P. J. (1980). Bean yellow mosaic virus in some iridaceous plants. *Acta Horticulturae* **110**, 31-37.

DERKS, A. F. L. M., VINK-VAN DEN ABEELE, J. L. & HOLLINGER, Th. C. (1983). Diagnostiek van virusziekten en identificatie van virussen in bloembolgewassen. *Jversl. Lab. Bloembollenonderz. Lisse* 1982, 61.

DERKS, A. F. L. M., HOLLINGER, Th. C. & VINK-VAN DEN ABEELE, J. L. (1985). Identification and symptom expression of four elongated viruses infecting bulbous irises. *Acta Horticulturae* **164**, 309-317.

DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387-395.

DOMIER, L. L., FRANKLIN, K. M., SHAHABUDDIN, M., HELLMANN, G. M., OVERMEYER, J. H., HIREMATH, S. T., SIAW, M. F. E., LOMONOSSOFF, G. P., SHAW, J. G. & RHOADS, R. E. (1986). The nucleotide sequence of tobacco vein mottling virus RNA. *Nucleic Acids Research* **14**, 5417-5430.

DOMIER, L. L., SHAW, J. G. & RHOADS, R. E. (1987). Potyviral proteins share amino acid sequence homology with picorna-, como-, and caulimoviral proteins. *Virology* **158**, 20-27.

DOUGHERTY, W. G. & HIEBERT, E. (1980a). Translation of potyvirus RNA in a rabbit reticulocyte lysate: reaction conditions and identification of capsid protein as one of the products of *in vitro* translation of tobacco etch and pepper mottle viral RNAs. *Virology* **101**, 466-474.

DOUGHERTY, W. G. & HIEBERT, E. (1980b). Translation of potyvirus RNA in a rabbit reticulocyte lysate: identification of nuclear inclusion proteins as products of tobacco etch virus RNA translation and cylindrical inclusion protein as a product of the potyvirus genome. *Virology* **104**, 174-182.

DOUGHERTY, W. G. & HIEBERT, E. (1980c). Translation of potyvirus RNA in a rabbit reticulocyte lysate: cell-free translation strategy and a genetic map of the potyviral genome. *Virology* **104**, 183-194.

DOUGHERTY, W. G. & CARRINGTON, J. C. (1988). Expression and function of potyviral gene products. *Ann. Rev. Phytopath.* **26**, 123-143.

DOUGHERTY, W. G., WILLIS, L. & JOHNSTON, R. E. (1985a). Topographic analysis of tobacco etch virus capsid protein epitopes. *Virology* **144**, 66-72.

DOUGHERTY, W. G., ALLISON, R. F., PARKS, T. D., JOHNSTON, R. E., FEILD, M. J. & ARMSTRONG, F. B. (1985b). Nucleotide sequence at the 3' terminus of pepper mottle virus genomic RNA: evidence for an alternative mode of potyvirus capsid protein gene organization. *Virology* **146**, 282-291.

DOUGHERTY, W. G., CARY, S. M. & PARKS, T. D. (1989a). Molecular genetic analysis of a plant virus polyprotein cleavage site: a model. *Virology* **171**, 356-364.

DOUGHERTY, W. G., PARKS, T. D., CARY, S. M., BAZAN, J. F. & FLETTERICK, R. J. (1989b). Characterization of the catalytic residues of the tobacco etch virus 49-kDa proteinase. *Virology* **172**, 302-310.

DUNCAN, G. D. (1988). The Lachenalia Handbook. National Botanical Gardens, Republic of South Africa, CTP Book Printers, Cape Town, 71 pp.

EDWARDSON, J. R. (1974a). Some properties of the potato virus Y-group. *Fla. Agric. Exp. Stn. Monogr. Ser.* **No. 4** 398 pp.

EDWARDSON, J. R. (1974b). Host ranges of viruses in the PVY-group. *Fla. Agric. Exp. Stn. Monogr. Ser.* **No. 5**, 225 pp.

EDWARDSON, J. R., CHRISTIE, R. G. & KO, N. J. (1984). Potyvirus cylindrical inclusions - subdivision IV. *Phytopathology* **74**, 1111-1114.

EGGENBERGER, A. L., STARK, D. M. & BEACHY, R. N. (1989). The nucleotide sequence of a soybean mosaic virus coat protein-coding region and its expression in *Escherichia coli, Agrobacterium tumefaciens* and tobacco callus. *J. Gen. Virol.* **70**, 1853-1860.

ELLIOT, M. S., ZETTLER, F. W., GALLEGATI, M. & KO, N-J. (1988). A potyvirus infecting *Ranunculus asiaticus*. *Acta Horticulturae* **234**, 39-43.

ELSER, J. E. & ALLEN, T. C. (1969). Intracellular modifications associated with streak mottle virus in *Lilium speciosum*. *Phytopathology* **59**, 11.

FAUQUET, C., DEJARDIN, J. & THOUVENEL, J. C. (1986a). Evidence that the amino acid composition of the particle proteins of plant viruses is characteristic of the virus group. I. Multidimensional classification of plant viruses. *Intervirology* **25**, 1-13.

FAUQUET, C., DEJARDIN, J. & THOUVENEL, J. C. (1986b). Evidence that the amino acid composition of the particle proteins of plant viruses is characteristic of the virus group. II. Discriminant analysis according to structural biological and classification properties of plant viruses. *Intervirology* **25**, 190-200.

FELSENSTEIN, J. (1988). Phylogenies from molecular sequences: interference and reliability. *Annu. Rev. Genet.* **22**, 251-265.

FERREIRA, D. I. & HANCKE, F. L. (1986). Indigenous flower bulbs of South Africa: a source of new genera and species for ornamental bulb cultivation. *Acta Horticulturae* **177**, 405-410.

FOXE, M. J. & WILSON, U. E. (1985). Investigation of virus infection of freesia. *Acta Horticulturae* **164**, 291-295.

FRANCKI, R. I. B. (1980). Limited value of the thermal inactivation point, longevity *in vitro* and dilution end-point as criteria for the characterization, identification and classification of plant viruses. *Intervirology* **13**, 91-98.

FRANCKI, R. I. B. (1983). Current problems in plant virus taxonomy. Pages 63-104 in: R. E. F. Matthews, ed. A Critical Appraisal of Viral Taxonomy, CRC Press, Boca Raton.

FRANCKI, R. I. B., MILNE, R. G. & HATTA, T. (1985). Atlas of Plant Viruses, Vol 2. CRC Press, Boca Raton, 284 pp.

FRENKEL, M. J., WARD, C. W. & SHUKLA, D. D. (1989). The use of 3' non-coding nucleotide sequences in the taxonomy of potyviruses: application to watermelon mosaic virus 2 and soybean mosaic virus-N. *J. Gen. Virol.* **70**, 2775-2783.

FRIBOURG, C. E. & NAKASHIMA, J. (1984). An improved latex agglutination test for routine detection of potato viruses. *Potato Research* **27**, 237-249.

GALLITELLI, D., HULL, R. & KOENIG, R. (1985). Relationships among viruses in the tombusvirus group: nucleic acid hybridization studies. *J. Gen. Virol.* **66**, 1523-1531.

GARCIA, J. A., RIECHMANN, J. L. & LAIN, S. (1989). Proteolytic activity of the plum pox potyvirus NIa-like protein in *Escherichia coli*. *Virology* **170**, 362-369.

GOELET, P., LOMONOSSOFF, G. P., BUTLER, P. J. G., AKAM, M. E., GAIT, M. J. & KARN, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* **79**, 5818-5822.

GOUGH, K. H. & SHUKLA, D. D. (1981). Coat protein of the potyviruses. I. Comparison of the four Australian strains of sugarcane mosaic virus. *Virology* **111**, 455-462.

GOUGH, K. H., AZAD, A. A., HANNA, P. J. & SHUKLA, D. D. (1987). Nucleotide sequence of the capsid and nuclear inclusion protein genes from the johnson grass strain of sugarcane mosaic virus RNA. *J. Gen. Virol.* **68**, 297-304.

GOVIER, D. A. & WOODS, R. D. (1971). Changes induced by magnesium ions in the morphology of some plant viruses with filamentous particles. *J. Gen. Virol.* **13**, 127-132.

GUBLER, U. & HOFFMAN, B. J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263-269.

GUGERLI, P. & FRIES, P. (1983). Characterization of monoclonal antibodies to potato virus Y and their use for virus detection. *J. Gen. Virol.* **64**, 2471-2477.

GUNYUZLU, P. L., TOLIN, S. A. & JOHNSON, J. L. (1987). The nucleotide sequence of the 3' terminus of soybean mosaic virus. (Abstr.) *Phytopathology* **77**, 1766.

HAKKAART, F. A. & VERSLUIJS, J. M. A. (1985). Viruses of alstroemeria and preliminary results of meristem culture. *Acta Horticulturae* **164**, 71-75.

HAKKAART, F. A., MAAT, D. Z. & QUAK, F. (1975). Viruses and meristem culture of Nerine. *Acta Horticulturae* **47**, 51-53.

HAMILTON, R. I., EDWARDSON, J. R., FRANCKI, R. I. B., HSU, H. T., HULL, R., KOENIG, R. & MILNE, R. G. (1981). Guidelines for the identification and characterization of plant viruses. *J. Gen. Virol.* **54**, 223-241.

HAMMOND, J. & CHASTAGNER, G. A. (1988). Natural infections of tulips with turnip mosaic virus and another potyvirus isolate distinct from tulip breaking virus in the U.S.A. *Acta Horticulturae* **234**, 235-242.

HAMMOND, J. & HAMMOND, R. W. (1985). A nucleic acid probe for detection of bean yellow mosaic virus. *Acta Horticulturae* **164**, 373-378.

HAMMOND, J. & HAMMOND, R. W. (1989). Molecular cloning, sequencing and expression in *Escherichia coli* of the bean yellow mosaic virus coat protein gene. *J. Gen. Virol.* **70**, 1961-1974.

HAMMOND, J., BRUNT, A. A., DERKS, A. F. L. M., INOUYE, N., BARNETT, O. W., ALLEN, T. C. & LAWSON, R. H. (1985). Viruses infecting bulbous iris: a clarification of nomenclature. *Acta Horticulturae* **164**, 395-397.

HARI, V. (1981). The RNA of tobacco etch virus: further characterization and detection of protein linked to RNA. *Virology* **112**, 391-399.

HARI, V., SIEGEL, A., ROZEK, C. & TIMBERLAKE, W. E. (1979). The RNA of tobacco etch virus contains poly(A). *Virology* **92**, 568-571.

HARRISON, B. D. & ROBINSON, D. J. (1982). Genome reconstruction and nucleic acid hybridization as methods of identifying particle-deficient isolates of tobacco rattle virus in potato plants with stem mottle disease. *J. Virol. Meth.* **5**, 255-265.

HARRISON, B. D. & ROBINSON, D. J. (1988). Molecular variation in vector-borne plant viruses: epidemiological significance. *Phil. Trans. R. Soc. Lond.* **B 321**, 447-462.

HAY, J. M., FELLOWES, A. P. & TIMMERMAN, G. M. (1989). Nucleotide sequence of the coat protein gene of a necrotic strain of potato virus Y from New Zealand. *Arch. Virol.* **107**, 111-122.

HEARON, S. S. (1985). Detection of kalanchoe latent virus in *Kalanchoe blossfeldiana* by enzyme-linked immunosorbent assay (ELISA). *Acta Horticulturae* **164**, 77-84.

HELLMANN, G. M., SHAW, J. G., LESNAW, J. A., CHU, L., PIRONE, T. P. & RHOADS, R. E. (1980). Cell-free translation of tobacco vein mottling virus RNA. *Virology* **106**, 207-216.

HELLMANN, G. M., THORNBURY, D. W., HIEBERT, E., SHAW, J. G., PIRONE, T. P. & RHOADS, R. E. (1983). Cell-free translation of tobacco vein mottling virus RNA II. Immunoprecipitation of products by antisera to cylindrical inclusion, nuclear inclusion, and helper component proteins. *Virology* **124**, 434-444.

HELLMANN, G. M., SHAW, J. G. & RHOADS, R. E. (1988). *In vitro* analysis of tobacco vein mottling virus NIa cistron: evidence for a virus-encoded protease. *Virology* **163**, 554-562.

HEMENWAY, C., FANG, R. X., KANIEWSKI, W. K., CHUA, N. H. & TUMER, N. E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO Journal* **7**, 1273-1280.

HENIKOFF, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351-359.

HIEBERT, E. & McDONALD, J. G. (1973). Characterization of some proteins associated with viruses in the potato Y group. *Virology* **56**, 349-361.

HIEBERT, E. & McDONALD, J. G. (1976). Capsid protein heterogeneity in turnip mosaic virus. *Virology* **70**, 144-150.

HIEBERT, E., PURCIFULL, D. E., CHRISTIE, R. G. & CHRISTIE, S. R. (1971). Partial purification of inclusions induced by tobacco etch virus and potato virus Y. *Virology* **43**, 638-646.

HIEBERT, E., TREMAINE, J. H. & RONALD, W. P. (1984a). The effect of limited proteolysis on the amino acid composition of five potyviruses and on the serological reaction and peptide map of the tobacco etch virus capsid protein. *Phytopathology* **74**, 411-416.

HIEBERT, E., PURCIFULL, D. E. & CHRISTIE, R. G. (1984b). Purification and immunological analyses of plant viral inclusion bodies. Pages 225-280 in: K. Maramorosh & H. Koprowski, eds. Methods in Virology, Vol. VIII, Academic Press, London.

HILL, J. H. & BENNER, H. I. (1976). Properties of potyvirus RNAs: turnip mosaic, tobacco etch, and maize dwarf mosaic viruses. *Virology* **75**, 419-432.

HILL, E. K., HILL, J. H. & DURAND, D. P. (1984). Production of monoclonal antibodies to viruses in the potyvirus group: use in radioimmunoassay. *J. Gen. Virol.* **65**, 525-532.

HOLLINGS, M. & BRUNT, A. A. (1981a). Potyvirus Group. *CMI/AAB Descriptions of Plant Viruses* **245**, 7 pp.

HOLLINGS, M. & BRUNT, A. A. (1981b). Potyviruses. Pages 731-807 in: E. Kurstak, ed. Handbook of Plant Virus Infections: Comparative Diagnosis. Elsevier/North Holland, Amsterdam.

HOLLINGS, M. & HORVATH, J. (1981). Melandrium yellow fleck virus. *CMI/AAB Descriptions of Plant Viruses* **236**, 4 pp.

HOLLINGS, M., STONE, O. M. & BOCK, K. R. (1976). Purification and properties of sweet potato mild mottle, a white-fly borne virus from sweet potato (*Ipomoea batatas*) in East Africa. *Ann. Appl. Biol.* **82**, 511-528.

HUTTINGA, H. (1975). Properties of viruses of the potyvirus group. III. A comparison of buoyant density, S value, particle morphology, and molecular weight of the coat protein subunit of 10 viruses and virus isolates. *Neth. J. Plant Path.* **81**, 58-63.

ISH-HOROWICZ, D. & BURKE, J. F. (1981). Rapid and efficient cosmid vector cloning. *Nucleic Acids Research* **9**, 2989-2998.

IWAKI, M. & KOMURO, Y. (1972). Viruses isolated from narcissus (*Narcissus* spp.) in Japan. III. Cucumber mosaic virus, tobacco rattle virus and broad bean wilt virus. *Ann. Phytopath. Soc. Japan* **38**, 137-145.

JONES, R. T. & DIACHUN, S. (1977). Serologically and biologically distinct bean yellow mosaic virus strains. *Phytopathology* **67**, 831-838.

JORDAN, R. L. & HAMMOND, J. (1986). Analysis of antigenic specificity of monoclonal antibodies to several potyviruses. (Abstr.) *Phytopathology* **76**, 1091.

KENNEDY, J. S., DAY, M. F. & EASTOP, V. F. (1962). A Conspectus of Aphids as Vectors of Plant Viruses. Commonwealth Institute of Entomology, London.

KITAJIMA, E. W. & LOVISOLO, O. (1972). Mitochondrial aggregates in *Datura* leaf cells infected with henbane mosaic virus. *J. Gen. Virol.* **16**, 265-271.

KITAJIMA, E. W. & COSTA, A. S. (1973). Aggregates of chloroplasts in local lesions induced in *Chenopodium quinoa* Wild. by turnip mosaic virus. *J. Gen. Virol.* **20**, 413-416.

KLESSER, P. J. & NEL, D. D. (1976). Virus diseases and tissue culture of some South African flower bulbs. *Acta Horticulturae* **59**, 71-76.

KNUHTSEN, H., HIEBERT, E. & PURCIFULL, D. E. (1974). Partial purification and some properties of tobacco etch virus induced intranuclear inclusions. *Virology* **61**, 200-209.

KOENIG, R. (1978). ELISA in the study of homologous and heterologous reactions of plant viruses. *J. Gen. Virol.* **40**, 309-318.

KOENIG, R. (1981). Indirect ELISA methods for the broad specificity detection of plant viruses. *J. Gen. Virol.* **55**, 53-62.

KOENIG, R. (1988). Serology and Immunochemistry. Pages 111-158 in: R. G. Milne, ed. The Plant Viruses, Vol. 4. The Filamentous Plant Viruses, Plenum Press, New York.

KOENIG, R. & PAUL, H. L. (1982a). Variants of ELISA in plant virus diagnosis. *J. Virol. Meth.* **5**, 113-125.

KOENIG, R. & PAUL, H. L. (1982b). Detection and differentiation of plant viruses by various ELISA procedures. *Acta Horticulturae* **127**, 147-158.

KOENIG, R. & BURGERMEISTER, W. (1986). Applications of immuno-blotting in plant virus diagnosis. Pages 121-137 in: R. A. C. Jones & L. Torrance, eds. Developments and Applications in Virus Testing, Association of Applied Biologists, Wellesbourne.

KOENIG, R., LESEMANN, D., BRUNT, A. A. & KUNNE, H. (1973). Narcissus mosaic virus found in *Nerine bowdenii*. *Intervirology* **1**, 349-353.

KOZIEL, M. G., HARI, V. & SIEGEL, A. (1980). *In vitro* translation of tobacco etch virus RNA. *Virology* **106**, 177-179.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LANGENBERG, W. G. (1986). Virus protein association with cylindrical inclusions of two viruses that infect wheat. *J. Gen. Virol* **67**, 1161-1168.

LANGENBERG, W. G. & SCHROEDER, H. F. (1973). Endoplasmic reticulum-derived pinwheels in wheat infected with wheat spindle streak mosaic virus. *Virology* **55**, 218-223.

LATORRE, B. A. & FLORES, V. (1985). Strain identification and cross-protection of potato virus Y affecting tobacco in Chile. *Plant Disease* **69**, 930-932.

LAWSON, R. H. (1980). Dissemination of viruses of ornamentals and approaches to reducing spread and improving methods of diagnosis. *Acta Horticulturae* **110**, 143-152.

LAWSON, R. H. (1981). Controlling virus diseases in major international flower and bulb crops. *Plant Disease* **65**, 780-786.

LAWSON, R. H. & HEARON, S. S. (1971). The association of pinwheel inclusions with plasmodesmata. *Virology* **44**, 454-456.

LAWSON, R. H. & CIVEROLO, E. L. (1976). Purification of carnation etched ring virus and comparative properties of CERV and cauliflower mosaic virus nucleic acids. *Acta Horticulturae* **59**, 49-59.

LEARY, J. J., BRIGATI, D. J. & WARD, D. C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. *Proc. Natl. Acad. Sci. USA* **80**, 4045-4049.

LESEMANN, D. E. (1988). Cytopathology. Pages 179-235 in: R. G. Milne, ed. The Plant Viruses, Vol. 4. The Filamentous Plant Viruses, Plenum Press, New York.

LINTHORST, H. J. M. & BOL, J. F. (1986). cDNA as a means of detection of tobacco rattle virus in potato and tulip. Pages 25-39 in: R. A. C. Jones & L. Torrance, eds. Developments and Applications in Virus Testing, The Lavenham Press Ltd, Great Britain.

LISA, V. (1980). Two viruses from rhizomatous iris. *Acta Horticulturae* **110**, 39-49.

LOESCH-FRIES, L. S., MERLO, D., BURHOP, L., ZINNEN, T., HILL, K., KRHAN, K., JARVIS, N., NELSON, S. & HALK, E. (1987). Expression of alfalfa mosaic virus RNA 4 in transgenic plants confers virus resistance. *EMBO Journal* **6**, 1845-1851.

LOGAN, A. E. & ZETTLER, F. W. (1985). Rapid *in vitro* propagation of virus-indexed gladioli. *Acta Horticulturae* **164**, 169-175.

LUBBINGE, J. (1980). Lachenalia breeding. I. Introduction. *Acta Horticulturae* **109**, 289-295.

MAAT, D. Z. (1976). Two potexviruses in nerine. *Acta Horticulturae* **59**, 81-82.

MABUSELA, W. T. & STEPHEN, A. M. (1990). An arabinoglucuronomannoglycan from the leaves of *Ornithogalum thyrsoides*. *Carbohydrate Research*, In Press.

MAISS, E., TIMPE, U., BRISSKE, A., JELKMANN, W., CASPER, R., HIMMLER, G., MATTANOVICH, D. & KATINGER, H. W. D. (1989). The complete nucleotide sequence of plum pox virus RNA. *J. Gen. Virol.* **70**, 513-524.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

MARTELLI, G. P. & RUSSO, M. (1976). Unusual cytoplasmic inclusions induced by watermelon mosaic virus. *Virology* **72**, 352-362.

MATTHEWS, R. E. F. (1981). Plant Virology, 2nd ed. Academic Press, New York, 778 pp.

MATTHEWS, R. E. F. (1982). Classification and nomenclature of viruses. Fourth report of the international committee on taxonomy of viruses. *Intervirology* **17**, 1-199.

MAULE, A. J., HULL, R. C. & DONSON, J. (1983). The application of spot hybridization to the detection of DNA and RNA viruses in plant tissues. *J. Virol. Meth.* **6**, 215-224.

McDONALD, J. G. & HIEBERT, E. (1974). Ultrastructure of cylindrical inclusions induced by viruses of the potato virus Y group as visualized by freeze-etching. *Virology* **58**, 200-208.

MILNE, R. G. (1986). New developments in electron microscope serology and their possible applications. Pages 179-191 in: R. A. C. Jones & L. Torrance, eds. Developments and Applications in Virus Testing, Association of Applied Biologists, Wellesbourne.

MILNE, R. G. (1988). The Plant Viruses, Vol 4. The Filamentous Plant Viruses. Plenum Press, New York, 423 pp.

MILNE, R. G. & LESEMANN, D. E. (1984). Immunosorbent electron microscopy in plant virus studies. Pages 85-101 in: K. Maramorosh & H. Koprowski, eds. Methods in Virology, Vol. VIII, Academic Press, London.

MOGHAL, S. M. & FRANCKI, R. I. B. (1976). Towards a system for the identification and classification of potyviruses. I. Serology and amino acid composition of six distinct viruses. *Virology* **73**, 350-362.

MOGHAL, S. M. & FRANCKI, R. I. B. (1981). Towards a system for the identification and classification of potyviruses. II. Virus particle length, symptomatology and cytopathology of six distinct viruses. *Virology* **112**, 210-216.

MOORE, F. W. (1905). Lachenalia hybrids. *Garden Chronicle* **37**, 210-211.

MOWAT, W. P. (1982). Pathology and properties of tulip virus X, a new potexvirus. *Ann. Appl. Biol.* **101**, 51-63.

MOWAT, W. P. (1985). Tulip chlorotic blotch virus, a second potyvirus causing tulip flower break. *Ann. Appl. Biol.* **106**, 65-73.

MOWAT, W. P., ASJES, C. J. & BRUNT, A. A. (1976). Narcissus tip necrosis virus. *Acta Horticulturae* **59**, 79-82.

MOWAT, W. P., DUNCAN, G. H. & DAWSON, S. (1988). Narcissus late season yellows potyvirus: symptoms, properties and serological detection. *Ann. Appl. Biol.* **113**, 531-544.

MURANT, A. F., RACCAH, B. & PIRONE, T. P. (1988). Transmission by Vectors. Pages 237-273 in: R. G. Milne, ed. The Plant Viruses, Vol. 4. The Filamentous Plant Viruses, Plenum Press, New York.

MURASHIGE, T. & SKOOG, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-497.

NAGEL, J. & HIEBERT, E. (1985). Complementary DNA cloning and expression of the papaya ringspot potyvirus sequences encoding capsid protein and a nuclear inclusion-like protein in *Escherichia coli*. *Virology* **143**, 435-441.

NEL, D. D. (1981). Rapid propagation of *Ornithogalum* hybrid *in vitro*. *Agroplantae* **13**, 83-84.

NEL, D. D. (1983). Rapid propagation of *Lachenalia* hybrids *in vitro*. *S. Afr. J. Bot.* **2**, 245-245.

NEVINS, J. R. (1983). The pathway of eukaryotic mRNA formation. *Ann. Rev. Biochem.* **52**, 441-466.

NOORDAM, D. (1973). Identification of Plant Viruses, Methods and Experiments. Centre for Agricultural Publishing and Documentation Wageningen, Netherlands, 207 pp.

OBERMEYER, A. A. (1978). *Ornithogalum*: a revision of the southern African species. *Bothalia* **12**, 323-376.

O'DONNELL, I. J., SHUKLA, D. D. & GOUGH, K. H. (1982). Electro-blot radioimmunoassay of virus-infected plant sap - a powerful new technique for detecting plant viruses. *J. Virol. Meth.* **4**, 19-26.

OH, C. & CARRINGTON, J. C. (1989). Identification of essential residues in potyvirus proteinase HC-Pro by site-directed mutagenesis. *Virology* **173**, 692-699.

OWENS, R. A. & DIENER, T. O. (1981). Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. *Science* **213**, 670-672.

PALUKAITIS, P., RAKOWSKI, A. G., ALEXANDER, D. Mc E. & SYMONS, R. H. (1981). Rapid indexing of the sunblotch disease of avocados using a complementary DNA probe to avocado sunblotch viroid. *Ann. Appl. Biol.* **98**, 439-449.

PHILLIPS, S. & BRUNT, A. A. (1980). Some hosts and properties of an isolate of nerine virus X from *Agapanthus praecox* subsp. *orientalis*. *Acta Horticulturae* **110**, 65-70.

PHILLIPS, S. & BRUNT, A. A. (1983). Alstroemeria. *Ann. Rep. Glasshouse Crops Res. Inst.* 1981, 142.

PHILLIPS, S. & BRUNT, A. A. (1986). Four viruses in alstroemeria in Britain. *Acta Horticulturae* **177**, 227-233.

PIENAAR, R. D. (1963). Sitogenetiese studies in die genus *Ornithogalum* L.. *S. Afr. J. Bot.* **29**, 111-130.

PIENAAR, R. D. & ROOS, T. J. (1966). Cytogenetic studies in the genus *Ornithogalum* L. II. Karyotype analysis in some biotypes of *O. conicum*, *O. lacteum*, and *O. thyrsoides*. *S. Afr. J. Bot.* **32**, 211-227.

PIENAAR, R. D. & VAN NIEKERK, H. A. (1968). 'n Sitogenetiese ondersoek van 'n aantal hibriede in die genus *Ornithogalum* L.. *Proc. Third Congress S. Afr. Gen. Soc.* 1966, 45-51.

PLESE, N., KOENIG, R., LESEMANN, D. E. & BOZARTH, R. F. (1979). Maclura mosaic virus - An elongated plant virus of uncertain classification. *Phytopathology* **69**, 471-475.

POWELL-ABEL, P., NELSON, R. S., DE, B., HOFFMANN, N., ROGERS, S. G., FRALEY, R. T. & BEACHY, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.

PURCIFULL, D. E. & BATCHELOR, D. L. (1977). Immunodiffusion tests with sodium dodecyl sulfate (SDS) treated plant viruses and plant viral inclusions. *Fla. Agric. Exp. Stn. Tech. Bull.* **788**, 39 pp.

PURCIFULL, D. E., HIEBERT, E. & McDONALD, J. G. (1973). Immunochemical specificity of cytoplasmic inclusions induced by viruses in the potato Y group. *Virology* **55**, 275-279.

PURDY, H. A. (1929). Immunologic reactions with tobacco mosaic virus. *J. Exp. Med.* **49**, 919-935.

RAVELONANDRO, M., VARVERI, C., DELBOS, R. & DUNEZ, J. (1988). Nucleotide sequence of the capsid protein gene of plum pox potyvirus. *J. Gen. Virol.* **69**, 1509-1516.

REDDICK, B. B. & BARNETT, O. W. (1983). A comparison of three potyviruses by direct hybridization analysis. *Phytopathology* **73**, 1506-1510.

REUST, W. & GUGERLI, P. (1984). Oxygen and carbon dioxide treatment to break potato tuber dormancy for reliable detection of potato virus Y (PVY) by enzyme-linked immunosorbent assay (ELISA). *Potato Research* **27**, 435-439.

RIECHMANN, J. L., LAIN, S. & GARCIA, J. A. (1989). The genome-linked protein and 5' end RNA sequence of plum pox potyvirus. *J. Gen. Virol.* **70**, 2785-2789.

ROBAGLIA, C., DURAND-TARDIF, M., TRONCHET, M., BOUDAZIN, G., ASTIER-MANIFACIER, S. & CASSE-DELBART, F. (1989). Nucleotide sequence of potato virus Y (N strain) genomic RNA. *J. Gen. Virol.* **70**, 935-941.

ROCHOW, W. F. & CARMICHAEL, L. E. (1979). Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays. *Virology* **95**, 415-430.

RODONI, B. C. & MORAN, J. R. (1988). The detection of dasheen mosaic virus using the enzyme-linked immunosorbent assay (ELISA). *Acta Horticulturae* **234**, 281-288.

RYBICKI, E. P. (1986). Affinity purification of specific antibodies from plant virus capsid protein immobilised on nitrocellulose. *J. Phytopathology* **116**, 30-38.

RYBICKI, E. P. & VON WECHMAR, M. B. (1981). The serology of the bromoviruses. I. Serological interrelationships of the bromoviruses. *Virology* **109**, 391-402.

RYBICKI, E. P. & VON WECHMAR, M. B. (1982). Enzyme-assisted immune detection of plant virus proteins electroblotted onto nitrocellulose paper. *J. Virol. Meth.* **5**, 267-278.

RYBICKI, E. P. & VON WECHMAR, M. B. (1985). Serology and Immunochemistry. Pages 207-244 in: R. I. B. Francki, ed., The Plant Viruses Vol. 1: Polyhedral Virions with Tripartite Genomes, Plenum Press, New York.

RYBICKI, E. P., VON WECHMAR, M. B. & BURGER, J. T. (1990). Monospecific antibody preparation for use in the detection of viruses. Pages 149-153 in: P. A. Burnett, ed. World Perspectives on Barley Yellow Dwarf. CIMMYT, Mexico. 511 pp.

SANDER, E. & DIETZGEN, R. G. (1984). Monoclonal antibodies against plant viruses. *Adv. Virus Res.* **29**, 131-168.

SANGER, F., NICKLEN, S. & COULSEN, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

SCHUGHART, K., VON WILCKEN-BERGMANN, B. & ESCHE, H. (1987). Expression of adenovirus type 12 E1b 58-kDa protein in *Escherichia coli* and production of antibodies raised against a 58-kDa::β-galactosidase fusion protein. *Gene* **53**, 173-180.

SHAHABUDDIN, M., SHAW, J. G. & RHOADS, R. E. (1988). Mapping of the tobacco vein mottling virus VPg cistron. *Virology* **163**, 635-637.

SHEPARD, J. F. (1972). Gel-diffusion methods for the serological detection of potato viruses X, S, and M. *Mont. Agric. Exp. Stn. Bull.* **662**.

SHEPARD, J. F., SECOR, G. A. & PURCIFULL, D. E. (1974). Immunochemical cross-reactivity between the dissociated capsid proteins of PVY group plant viruses. *Virology* **58**, 464-475.

SHUKLA, D. D. & WARD, C. W. (1988). Amino acid sequence homology of coat proteins as a basis for identification and classification of the potyvirus group. *J. Gen. Virol* **69**, 2703-2710.

SHUKLA, D. D. & WARD, C. W. (1989a). Structure of potyvirus coat proteins and its application in the taxonomy of the potyvirus group. *Adv. Virus Res.* **36**, 273-314.

SHUKLA, D. D. & WARD, C. W. (1989b). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Arch. Virol.* **106**, 171-200.

SHUKLA, D. D., THOMAS, J. E., McKERN, N. M., TRACY, S. L. & WARD, C. W. (1988a). Coat protein of potyviruses. 4. Comparison of biological properties, serological relationships, and coat protein amino acid sequences of four strains of potato virus Y. *Arch. Virol.* **102**, 207-219.

SHUKLA, D. D., McKERN, N. M. & WARD, C. W. (1988b). Coat protein of potyviruses. 5. Symptomatology, serology, and coat protein sequences of three strains of passionfruit woodiness virus. *Arch. Virol.* **102**, 221-232.

SHUKLA, D. D., McKERN, N. M., GOUGH, K. H., TRACY, S. L. & LETHO, S. G. (1988c). Differentiation of potyviruses and their strains by high-performance liquid chromatographic peptide profiling of coat proteins. *J. Gen. Virol.* **69**, 493-502.

SHUKLA, D. D., STRIKE, P. M., TRACY, S. L., GOUGH, K. H. & WARD, C. W. (1988d). The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains the major virus-specific epitopes. *J. Gen. Virol.* **69**, 1497-1508.

SHUKLA, D. D., JILKA, J., TOSIC, M. & FORD, R. E. (1989a). A novel approach to the serology of potyviruses involving affinity purified polyclonal antibodies directed towards virus-specific N-termini of coat proteins. *J. Gen. Virol.* **70**, 13-23.

SHUKLA, D. D., FORD, R. E., TOSIC, M., JILKA, J. & WARD, C. W. (1989b). Possible members of the potyvirus group transmitted by mites or whiteflies share epitopes with aphid-transmitted definitive members of the group. *Arch. Virol.* **105**, 143-151.

SHUKLA, D. D., TOSIC, M., JILKA, J., FORD, R. E., TOLER, R. W. & LANGHAM, M. A. C. (1989c). Taxonomy of potyviruses infecting maize, sorghum and sugarcane in Australia and the United States as determined by reactivities of polyclonal antibodies directed towards virus-specific N-termini of coat proteins. *Phytopathology* **79**, 223-229.

SHUKLA, D. D., TRIBBICK, G., MASON, T. J., HEWISH, D. R., GEYSEN, H. M. & WARD, C. W. (1989d). Localization of virus-specific and group-specific epitopes of plant potyviruses by systematic immunochemical analysis of overlapping peptide fragments. *Proc. Natl. Acad. Sci. USA* **86**, 8192-8196.

SIAW, M. F. E., SHAHABUDDIN, M., BALLARD, S., SHAW, J. G. & RHOADS, R. E. (1985). Identification of a protein covalently linked to the 5' terminus of tobacco vein mottling virus RNA. *Virology* **142**, 134-143.

SMITH, F. F. & BRIERLEY, P. (1944a). Ornithogalum mosaic. *Phytopathology* **34**, 497-503.

SMITH, F. F. & BRIERLEY, P. (1944b). Preliminary report on some mosaic diseases of Iridaceous plants. *Phytopathology* **34**, 593-598.

SMITH, G. E. & SUMMERS, M. D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. *Analytical Biochemistry* **109**, 123-129.

STANLEY, K. K. (1983). Solubilization and immune-detection of β-galactosidase hybrid proteins carrying foreign antigenic determinants. *Nucleic Acids Research* **11**, 4077-4093.

STANLEY, K. K. & LUZIO, J. P. (1984). Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. *EMBO Journal* **3**, 1429-1434.

STARK, D. M. & BEACHY, R. N. (1989). Protection against potyvirus infection in transgenic plants: evidence for broad spectrum resistance. *Bio/Technology* **7**, 1257-1262.

STEIN, A., LEVY, S. & LOEBENSTEIN, G. (1988). Detection of viruses in gladioli corms. *Acta Horticulturae* **234**, 275-280.

STEIN, A., SALOMON, R., COHEN, J. & LOEBENSTEIN, G. (1986). Detection and characterisation of bean yellow mosaic virus in corms of *Gladiolus grandiflorus*. *Ann. Appl. Biol.* **109**, 147-154.

STONE, O. M. (1980). Two new potexviruses from monocotyledons. *Acta Horticulturae* **110**, 59-63.

TABOR, S. & RICHARDSON, C. C. (1987). DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA* **84**, 4767-4771.

TAMADA, T. & HARRISON, B. D. (1980). Factors affecting the detection of potato leafroll virus in potato foliage by enzyme-linked immunosorbent assay. *Ann. Appl. Biol.* **95**, 209-219.

THORNBURY, D. W. & PIRONE, T. P. (1983). Helper components of two potyviruses are serologically distinct. *Virology* **125**, 487-490.

THORNBURY, D. W., HELLMANN, G. M., RHOADS, R. E. & PIRONE, T. P. (1985). Purification and characterization of potyvirus helper component. *Virology* **144**, 260-267.

TOMLINSON, J. A. (1987). Epidemiology and control of virus diseases of vegetables. *Ann. Appl. Biol.* **110**, 661-681.

TORRANCE, L. (1980). Use of protein A to improve sensitisation of latex particles with antibodies to plant viruses. *Ann. Appl. Biol.* **96**, 45-50.

TORRANCE, L. (1981). Use of Clq enzyme-linked immunosorbent assay to detect plant viruses and their serologically different strains. *Ann. Appl. Biol.* **99**, 291-299.

TOYODA, H., NICKLIN, M. J. H., MURRAY, M. G., ANDERSON, C. W., DUNN, J. J., STUDIER, F. W. & WIMMER, E. (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. *Cell* **45**, 761-770.

TUMER, N. E., O'CONNELL, K. M., NELSON, R. N., SANDERS, P. R., BEACHY, R. N., FRALEY, R. T. & SHAH, D. M. (1987). Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *EMBO Journal* **6**, 1181-1188.

VANCE, V. B. & BEACHY, R. N. (1984). Detection of genomic-length soybean mosaic virus RNA on polyribosomes of infected soybean leaves. *Virology* **138**, 26-36.

VAN DER VLUGT, C. I. M., DERKS, A. F. L. M., DIJKSTRA, J. & GOLDBACH, R. (1988). Towards a rapid and reliable detection method for iris severe mosaic virus in iris bulbs. *Acta Horticulturae* **234**, 191-198.

VAN DER VLUGT, R., ALLEFS, S., DE HAAN, P. & GOLDBACH, R. (1989). Nucleotide sequence of the 3'-terminal region of potato virus Y^N RNA. *J. Gen. Virol.* **70**, 229-233.

VAN DORST, H. J. M. (1973). Two new disorders in freesias. *Neth. J. Plant Path.* **79**, 130-137.

VAN DUN, C. M. P. & BOL, J. F. (1988). Transgenic tobacco plants accumulating tobacco rattle virus coat protein resist infection with tobacco rattle virus and pea early browning virus. *Virology* **167**, 649-652.

VAN DUN, C. M. P., BOL, J. F. & VAN VLOTEN-DOTING, L. (1987). Expression of alfalfa mosaic virus and tobacco rattle virus coat protein genes in transgenic tobacco plants. *Virology* **159**, 299-305.

VAN DUN, C. M. P., OVERDUIN, B., VAN VLOTEN-DOTING, L. & BOL, J. F. (1988). Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection. *Virology* **164**, 383-389.

VAN KOOT, Y., VAN SLOGTEREN, D. H. M., CREMER, M. C. & CAMFFERMAN, J. (1954). Virusverschijnselen in freesia's. *Tijdschr. PlZiekt.* **60**, 157-192.

VAN REGENMORTEL, M. H. V. (1966). Plant virus serology. *Adv. Virus Res.* **12**, 207-271.

VAN REGENMORTEL, M. H. V. (1982). Serology and immunochemistry of plant viruses. Academic Press, New York, 302 pp.

VAN REGENMORTEL, M. H. V. (1986). The potential of using monoclonal antibodies in the detection of plant viruses. Pages 89-101 in: R. A. C. Jones & L. Torrance, eds. Developments and Applications in Virus Testing. Association of Applied Biologists, Wellesbourne, United Kingdom.

VAN REGENMORTEL, M. H. V. & BURCKARD, J. (1980). Detection of a wide spectrum of virus strains by indirect enzyme-linked immunosorbent assays (ELISA). *Virology* **106**, 327-334.

VAN SLOGTEREN, D. H. M. (1955). Serological microreactions with plant viruses under paraffin oil. *Proceedings Second Conference on Potato Virus Diseases*, Lisse, Wageningen, The Netherlands.

VAN SLOGTEREN, D. H. M. (1966). Necrosis in the bulb scales of sensitive tulip varieties caused by cucumber mosaic virus. *Med. Fac. Landbouww. Rijksuniv. Gent* **31**, 986.

VAN SLOGTEREN, D. H. M. (1971). Tulip breaking virus. *CMI/AAB Descriptions of Plant Viruses* **71**, 4 pp.

VARMA, A., GIBBS, A. J., WOODS, R. D. & FINCH, J. T. (1968). Some observations on the structure of the filamentous particles of several plant viruses. *J. Gen. Virol.* **2**, 107-114.

VARTAPETIAN, A. B., KOONIN, E. V., AGOL, V. I. & BOGDANOV, A. A. (1984). Encephalomyocarditis virus RNA synthesis *in vitro* is protein-primed. *EMBO Journal* **3**, 2593-2598.

VETTEN, H. J., EHLERS, U. & PAUL, H. L. (1983). Detection of potato viruses Y and A in tubers by enzyme-linked immunosorbent assay after natural and artificial break of dormancy. *Phytopath. Z.* **108**, 41-53.

VIEIRA, J. & MESSING, J. (1982). The pUC plasmids, an M13mp7-derived system for mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259-268.

VON WECHMAR, M. B. (1990). Short and long term maintenance of aphid clones. Pages 321-323 in: P. A. Burnett, ed. World Perspectives on Barley Yellow Dwarf. CIMMYT, Mexico. 511 pp.

WANG, W. C., TRONCHET, M., LARROQUE, N., DORION, N. & ALBOUY, J. (1988). Production of virus-free dahlia by meristem tip culture and virus detection through cDNA probes and ELISA. *Acta Horticulturae* **234**, 421-428.

WATT, J. M. & BREYER-BRANDWIJK, M. G. (1962). Medicinal and Poisonous Plants of Southern and Eastern Africa, 2nd ed. E & S Livingstone Ltd., Edinburgh & London, 1457 pp.

WEINTRAUB, M. & RAGETLI, H. W. J. (1966). Fine structure of inclusions and organelles in *Vicia faba* infected with bean yellow mosaic virus. *Virology* **28**, 290-302.

WEINTRAUB, M., RAGETLI, H. W. J. & LO, E. (1974). Potato virus Y particles in plasmodesmata of tobacco leaf cells. *J. Ultrastruct. Res.* **46**, 131-148.

WEINTRAUB, M., RAGETLI, H. W. J. & LEUNG, E. (1976). Elongated virus particles in plasmodesmata. *J. Ultrastruct. Res.* **56**, 351-364.

WILSON, H. J., GOODMAN, R. M. & ISRAEL, H. W. (1976). Pinwheel inclusions in morphogenesis: a possible alternative to induction by viruses. *Arch. Virol.* **51**, 347-354.

WINSHIP, P. R. (1989). An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Research* **17**, 1266-1266.

YANISH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.

YEH, S. & GONSALVES, D. (1985). Translation of papaya ringspot virus RNA *in vitro*: detection of a possible polyprotein that is processed for capsid protein, cylindrical inclusion protein, and amorphous inclusion protein. *Virology* **143**, 260-271.

ZABEAU, M. & STANLEY, K. K. (1982). Enhanced expression of cro-β-galactosidase fusion proteins under the control of the P_R promoter of bacteriophage λ. *EMBO Journal* **1**, 1217-1224.

ZARET, K. S. & SHERMAN, F. (1982). DNA sequence required for efficient transcription of termination in yeast. *Cell* **28**, 563-573.