VIRUSES IMPLICATED IN THE WOODINESS DISEASE OF SOUTH AFRICAN PASSIONFRUIT, AND THE MOLECULAR CHARACTERIZATION OF A NEW POTYVIRUS

by

REON J. BRAND

A dissertation submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy in the Faculty of Science, University of Cape Town.

Cape Town, April 1992

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, Reon J. Brand, certify that I approve of the incorporation in this dissertation of material that has already been submitted or accepted for publication.

Associate Professor M. B. von Wechmar
and Dr. E.P. Rybicki
Department of Microbiology
University of Cape Town

THE CHARACTERIZATION OF TWO VIRUSES IMPLICATED IN THE WOODINESS DISEASE OF SOUTH AFRICAN PASSIONFRUIT

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ACKNOWLEDGEMENTS

I wish to express my gratitude towards my supervisors; Associate Professor Barbara von Wechmar for her enthusiasm and supportiveness during the project and for the assistance with aphid transmissions, and Dr. Edward Rybicki for the invaluable discussions and encouragement. I also wish to thank them both for their critical reading of this manuscript. I wish to extend my appreciation to Professor Jennifer Thompson, head of the Microbiology Department, for her support and the use of the facilities.

I wish to thank Dr. Jons Terblanche, Director of the Citrus and Subtropical Fruit Research Institute (CSFRI) for providing the opportunity for me to complete this study on a full-time basis at UCT and for the financial support made available for the project. A word of thanks goes to Dr. Barry Manicom, head of the Plant Pathology section at the CSFRI for his support and cooperation during the project.

I want to express my appreciation to Messrs. G. Kasdorf and M. Jaffer for their expert assistance with the electron microscopy.

I particularly wish to thank Peter Buckton for the technical assistance he provided when required and seldom complaining when I made a nuisance of myself, also for occasionally sharing his lunch with me. I also wish to extend a word of thanks to the UCT laboratory assistants, in particular Mr. D. Solomon for plant maintenance and Messers. D. Roussouw and W. Jacobs for the rabbit injections.

I wish to thank Dr. İ. Roos and my colleagues at Fruitec for their supportiveness during my write-up period, and Messrs. Owen Taylor, Iansley Pool and Jakobus Nel for their assistance with preparing photographs for this manuscript.

A special word of thanks goes to my lab mate and good friend, Dr. Johan Burger with whom I shared most of the excitement and trauma associated with PhD study.

Finally I wish to express my gratitude to my parents and my wife Lizette for supporting me in everything I do.

ABSTRACT

Woodiness disease caused by virus infection is the most serious virus disease of passionfruit and affects economic production of this crop worldwide. A preliminary survey of diseased *Passiflora* material collected from various regions in South Africa revealed the presence of at least three different viruses. A diseased *P. caerulea* rootstock specimen from a woodiness diseased vineyard in Natal was selected as a source for isolation and further characterization of viruses. Two viruses that were present in a mixed infection were isolated and purified from this material: a spherical virus which appeared to be cucumber mosaic virus (CMV) and a filamentous virus which was initially presumed to be an isolate of passionfruit woodiness virus (PWV).

The host range, transmission and prevalence of these viruses were studied by employing techniques such as electron microscopy (negative staining and immunosorbent), electroblot immunoassay, double antibody sandwich enzyme-linked imunoassay and nucleic acid hybridization. In transmission studies, the CMV-isolate and the potyvirus were found to be sap, aphid and graft transmissible. Separation of the two viruses was achieved by passage through a selective host range.

Physicochemical analysis, biological characterization and serology confirmed that the spherical virus was indeed CMV. It has a particle size of approximately 30 nm, a coat M_r of 28 kDa, and ssRNA molecular weights are: 1.29 mDa (RNA 1); 1.15 mDa (RNA 2); 0.81 mDa (RNA 3); 0.35 mDa (RNA 4). Polyclonal anti-CMV antiserum directed to CMV-LupK5, (an isolate from lupins) reacted strongly with this virus. This virus was designated CMV-Pass. The Natal *P. caerulea* potyvirus particles were 670 nm long and 11 nm wide. Physicochemical characterization and serological reaction with anti-PWV and potato virus-Y (PVY) antiserum confirmed that the virus from *P. caerulea* was indeed a potyvirus, but these criteria were inadequate to identify the virus down to the virus/strain level. The Natal *P. caerulea* potyvirus has a CP M_r of 33 kDa, and the ssRNA M_r was estimated to be 2.92 mDa. Biologically this virus differred from previously characterized strains of PWV in that it was able to systemically infect *Nicotiana benthamiana*, but failed to spread systemically in secondary leaves of *Phaseolus vulgaris* cv. Bountiful.

DNA complementary to the 3'-terminal 1436 nucleotides of the Natal *P. caerulea* potyviral genome was cloned into the pUC19 vector and subjected to nucleotide sequence analysis. The sequence contained a single long 5'-ORF which presumably starts upstream of the cloned sequence. Comparative analysis indicated that the clone contained the entire potyviral coat protein (CP) sequence. Phylogenetic analysis of the deduced CP indicated that it was only distantly related to sequenced strains of PWV (71% related to PWV-TB). This

virus thus appeared to be a new and distinct virus causing woodiness symptoms in *Passiflora*, and was subsequently designated South African *Passiflora* virus (SAPV).

The presumptive SAPV CP gene was subcloned in the pUEX2 bacterial expression vector, and expressed as a β-galactosidase::CP fusion protein. This protein reacted with polyclonal antiserum directed against native SAPV virus in electroblot immunoassay (EBIA). Antiserum raised against the expressed fusion protein, reacted with the SAPV CP present in the sap of infected *Passiflora*, thus verifying the authenticity of the gene.

The SAPV CP was thereafter subcloned in the pGSJ280 Ti-plasmid derivative cointegration vector, in frame of an ATG start codon and downstream of the CaMV 35S promotor supplied by the vector. This construct was mobilized into *Agrobacterium tumefaciens* and used for transformation of *Nicotiana tabacum* cv. Petit Havanna SR1. The expression of stable CP in this host was confirmed by EBIA using anti-SAPV antiserum.

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 PASSIFLORA

1.1.1 Passifloraceae

The plant family Passifloraceae comprises 12 genera and about 500 spp. of herbaceous and woody plants, which occur mostly in tropical regions. Of these, about 400 spp. belong to the genus *Passiflora* (Purseglove, 1968). Most are native to the New World, with a few from Asia and Australia, and one sp. in Madagascar. These plants usually appear as climbing perennial woody vines with unbranched axillary tendrils.

Several *Passiflora* spp. produce edible fruit, and for this reason many of these are commonly cultivated in tropical and sub-tropical regions of the world. The fruit (commonly known as passionfruit or granadillas if produced by *P. edulis* Sims or *P. edulis f. flavicarpa*) can be described as a many-seeded berry. The fruits are round to ovoid-shaped, and 4-6 cm in diameter with a tough, waxy rind, and have a single cavity filled with an aromatic orange-coloured pulpy juice with up to 250 small seeds (Morton, 1987).

The most widely cultivated sp. is *P. edulis* Sims. The two most common commercial cultivars derived from this species are *P. edulis f. edulis* (original purple passion fruit) and *P. edulis f. flavicarpa* (yellow passionfruit), which is of unknown origin, but is thought by some to be a hybrid between *P. edulis* Sims and *P. ligularis* q.v. or a naturally occurring mutant of *P. edulis* Sims (Morton, 1987). Fruit size of the latter (5-6 cm diameter) is somewhat larger than that of *P. edulis f. edulis* (4-5 cm diameter), but the pulp is more acidic with generally poorer flavour.

P. edulis is a native of southern Brazil and was introduced into England in 1810. From there it was taken to Australia and South Africa. From Australia, it was introduced into Hawaii in 1880, where it became naturalized. It was introduced into Tanzania from the Natal province of South Africa in 1896 (Purseglove, 1968). Another species widely cultivated in the tropics is *P. quadrangularis* L, a native of South America which produces giant ovate fruit of about 25 x 12 cm. Some other *Passiflora* spp. that produce edible fruits, are: *P. laurifolia* L., known as water-lemon, that grows wild in thickets in the West Indies and north-eastern South America; *P. ligularis* Juss., a native of tropical America now grown extensively for its fruit in the mountainous regions of

Mexico and Central America; *P. mollissima* Baily, the so-called banana passionfruit after its shape, occurring wild in the Andes and cultivated in New Zealand; *P. antioquiensis* Karst., also known as the banana passionfruit, is a native of Colombia.

A number of *Passiflora* spp. are grown as garden ornamentals in the tropics. Of these, *P. caerulea* L. is the most common. This species is also widely used as a rootstock for *P. edulis* locally and abroad (Taylor & Kimble, 1964).

1.1.2 The cultivation of passionfruit in South Africa

Passiflora is produced commercially in several climatologically distinct regions in South Africa. The main areas of production are in the Eastern Transvaal and Natal Midlands: these areas have subtropical climates and are well suited for optimal passionfruit production. The crop is also established in the northern Transvaal (Vaalwater region) as well as the eastern Cape Province. Passionfruit is even cultivated in the western Cape, a region with a typically Mediterranean climate (Fig. 1.1).

The crop is produced mainly for a lucrative local sap/pulp market, but due to disease problems there is an inconsistent fruit supply. The bulk of passionfruit pulp consumed in South Africa is thus imported. The inconsistency of local supply also hampers the effective marketing of the product. The production of passionfruit in SA follows a cyclical trend: every upswing in production is followed by a total collapse of the industry a few years later, mainly due to virus disease problems (Dr. J. Moll, personal communication). The average passionfruit production is thus very low despite healthy consumer interest and the availability of climatologically suitable areas for production.

1.1.3 Diseases and pests of the genus *Passiflora*

Despite the popularity of passionfruit amongst consumers familiar with the fruit, the commercial success of the crop has been limited by diseases and pests. Woodiness, a disease actuated by virus infection, is considered to be the most serious disease of passionfruit world wide.

This disease was shown to be caused by infections of cucumber mosaic virus (CMV) or passionfruit woodiness virus (PWV), or multiple infections of both viruses (Taylor & Kimble, 1964). Woodiness diseased plants have a limited lifespan and produce low yields of deformed fruit with hardened rind and little sap. Several other viruses were also shown to infect *Passiflora* (see 1.3.2).

Serious fungal pathogens of passionfruit are *Alternaria passiflorae*, a fungal disease responsible for brown spotted fruits and defoliation of vines (Simmonds, 1930), and *Fusarium oxysporum f. passiflorae*, causing wilting of plants.

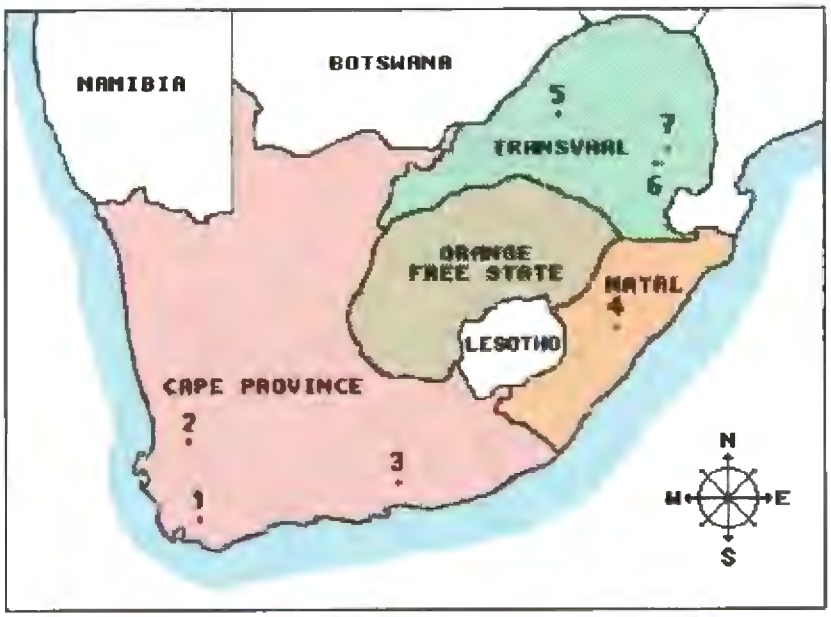


Fig. 1.1 Map of South Africa indicating the geographical regions of passionfruit production.

1 = Franschhoek
2 = Citrusdal
3 = Addo
4 = Nkwalini
5 = Vaalwater
6 = Nelspruit
7 = Burgershall

In 1943, the latter disease caused major problems for the Australian industry, but it was found that the neglected yellow passionfruit *(P. edulis f. flavicarpa)* is both wilt and nematode resistant and it became a very popular choice as rootstock (Morton, 1987). A mycoplasma-like organism eliciting witches' broom symptoms in *P. edulis f. flavicarpa* in Brazil, was reported to have devastating effects on the vines (Kitajima *et al.* 1981).

Several insect pests were reported to physically damage passionfruit crops. They include: the oriental fruit fly, *Dacus dorsalus* Hendel; the melon fly, *D. curbitae* Coquillett; the Mediterranean fruit fly, *Ceratitis capitata* Wied; the red spider, *Brevipalpus papayensis* (Morton, 1987). The stink bug *Nezara viridula L.* was shown to cause damage in South African vineyards (Froneman & Crause, 1989). Although it is known that viruses are transmitted to *Passiflora* by several species of aphids eg. *Myzus persicae* Sultzer (Taylor & Kimble, 1964), *Aphis gossypii* (Greber, 1966) and *Aulacorthum solani* Kaltenbach (this work), only limited data is available on the ability of insects other than aphids to transmit viruses to *Passiflora*.

1.2 OUTLINE OF THE PROJECT

The initial aim of this project was to identify the viruses most often associated with woodiness disease of *Passiflora* in South Africa. Two viruses, cucumber mosaic virus (CMV-Pass) and a potyvirus (initially presumed to be a local strain of PWV), both from the Natal region, were selected for further studies.

These two viruses were to be isolated and subjected to physicochemical, serological and biological characterization. Methods for the sensitive detection of these viruses in *Passiflora* were to be evaluated.

The potyvirus was selected for molecular characterization to facilitate taxonomic assignment of the virus based on coat protein sequence data. A cDNA fragment believed to code for the viral coat protein (CP) was subcloned into plasmid bacterial and plant expression vectors respectively. These respective constructs were used to transform *Escherichia coli* and *Nicotiana tabacum*. The stability and integrity of the expressed CP was evaluated in the resulting transgenic *E. coli* and *N. tabacum*. These results will serve as a foundation for future work to generate transgenic *Passiflora* cultivars expressing the potyviral CP in an attempt to mediate engineered cross protection against potyvirus infection.

1.3 LITERATURE REVIEW

1.3.1 Introduction to plant virus classification

Currently known viruses of eukaryotes are organized in 73 families and groups in a classification system defined by the International Committee on Taxonomy of Viruses (ICTV, Francki *et al.*, 1991). The status of the eukaryotic virus host (vertebrates, invertebrates or plants) is used to define "orders" of virus families. Virus orders are divided into families on the basis of the presence/absence of an envelope, and the type of nucleic acid. Classification on the virus group level (essentially equivalent to a genus) is based primarily on biological, physicochemical and serological parameters, and the replication strategy employed. Some virus families such as Rhabdoviridae, Picornaviridae, Reoviridae, Iridoviridae, Poxviridae, Parvoviridae, Togaviridae and Bunyaviridae can form supergroups crossing the borders of virus 'orders' as defined by Matthews (1982): here the order of viruses infecting plants is divided into five families and 28 virus groups. Plant viruses are of major economic consequence, and are responsible for worldwide annual crop losses estimated to be in the order of US $15 billion.

1.3.2 Viruses infecting *Passiflora*.

Viruses belonging to at least eight different plant virus taxonomic groups have been found to infect *Passiflora* world wide (Brunt *et al.*, 1990). The most serious and prevalent of these are passionfruit woodiness virus (PWV - a potyvirus) and cucumber mosaic virus (CMV - type member of the cucumoviruses). Both of these viruses have been implicated in woodiness disease of *Passiflora* in Australia (Taylor & Kimble, 1964); Brazil (Chagas *et al.*, 1981; Colariccio *et al.*, 1987) and South Africa (Da Graca, 1976). Several strains of PWV with symptom severity ranging from tip blight to mild mosaic in *Passiflora* have been described (Pares & Martin, 1985; Kitajima *et al.*, 1986; Greber, 1966; Shukla *et al.*, 1988b).

Other viruses found to cause disease in *Passiflora* spp., include: passionfruit ringspot (poty) virus (PRV), found in the *Passiflora* spp. of Ivory Coast (De Wijs & Mobach, 1975); passionfruit vein clearing (rhabdo) virus (PVCV) found in Brazilian *P. edulis f. flavicarpa* (Kitajima & Crestani, 1985; Pares *et al.*, 1983); passionfruit yellow mosaic (tymo) virus (PYMV), also found in Brazilian *P. edulis f. flavicarpa* (Crestani *et al.*,1986); tomato ringspot (nepo) virus (TomRV), isolated from Peruvian *P. edulis* (Koenig, 1986); maracuja mosaic (tobamo) virus (MrMV), isolated from Peruvian *P. edulis* (Fribourg *et al.*, 1987); tobacco necrosis (necro) virus (TNV) infecting South African *Passiflora* spp. (Von Wechmar *et al.*, 1991); passiflora latent carlavirus (PLV), (Brunt *et al.*, 1990); and a

spherical virus of unknown group affiliation designated granadilla mosaic virus found in Brazilian *Passiflora* (Chagas *et al.*, 1984).

As this thesis deals with the isolation and characterization of a potyvirus and CMV from South African *Passiflora* with "woodiness" symptoms, only the characteristics of these viruses will be discussed in more depth.

1.3.3 General characteristics of members of the potyvirus group.

The potyvirus group, named after the type member potato virus Y (PVY), was established in 1959 (Brandes & Wetter, 1959) and is currently the largest and most rapidly expanding of the 28 plant virus groups recognized by the ICTV. This group currently has more than 180 definitive and possible members (Hollings & Brunt, 1981; Milne 1988; Ward & Shukla, 1991), comprising about 36% of all known plant viruses. In 1974 potyviruses were reported to infect 1112 species of 369 genera in 53 plant families (Edwardson, 1974). It is thus not surprising that, of all plant virus groups, they collectively pose the greatest economic threat to crops worldwide.

Potyviruses are naturally transmitted through insect vectors and rarely through seed (eg. pea seedborne mosaic virus - Khetarpal & Maury, 1987). Sap transmission through mechanical means is often used under laboratory conditions and also occurs accidentally with crops (such as passionfruit) that require pruning or grafting.

Whilst only aphid vectored potyviruses were up to recently accepted as members of the potyvirus group (Matthews, 1982), the newly proposed taxonomic family of Potyviridae (Barnett, 1991) includes more than 175 members of the aphid transmissible 'genus' Potyvirus, 4 members of the mite transmitted 'genus' Rymovirus, 5 members of the fungus transmitted 'genus' Bymovirus, and sweet potato mild mottle virus, the sole member of the whitefly transmitted 'genus' Ipomovirus.

The rationale for dividing the family Potyviridae into four separate 'genera' on grounds of their vector specificity is in good agreement with molecular phylogenetic analysis based on sequence data (section 1.3.3.3).

In view of the scope of this thesis, the ensuing discussion will focus mainly on the aphid transmissible 'genus' Potyvirus, but limited reference to Potyviridae with other insect vectors will be made in the interests of perspective.

1.3.3.1 Physical characteristics of potyviruses

Aphid transmissible potyviruses are very similar in appearance. The virions are flexuous filamentuous rods with modal length varying from 680-900 nm and 11-12 nm in diameter. Particles are comprised of 5% RNA and 95% capsid protein (CP) and have a density of 1.31 g/cm^3 in CsCl. The ±10 kb long positive-sense single stranded RNA of each virion has a genome linked 5'-

protein, and is encapsidated with approximately 2000 CP subunits, each with a M_r of 30-36 kDa (Hari, 1981; Matthews, 1982). The tertiary structure of stacked CP subunits of potyviruses is such that the N- and C-termini of the CP subunits are surface orientated (Allison *et al.*, 1985; Shukla & Ward, 1989b, Fig. 1.2) Optical diffraction studies showed a helical stack arrangement (with a pitch of 3.3 - 3.5) of CP subunits, when assembled into virions (McDonald *et al.*, 1976; McDonald & Bancroft, 1977).

Particle dimensions of wheat streak mosaic virus (WSMV - 'genus' Rymoviruses) are similar to aphid transmitted potyvirus members, although particles are slightly wider (13 nm). It also differs somewhat in rigidity, and can be confused with carlaviruses or potexviruses in appearance (Shukla *et al.*, 1989a). Barley yellow mosaic virus (BaYMV - 'genus' Bymoviruses) differs from potyviruses transmitted by other vectors in that it has a bimodal particle length of 550nm and 275 nm (Inouye & Saito, 1975). The ssRNA genome of WSMV is about 8.5 kb long (Brakke, 1971), while the genome of BaYMV is divided and the two respective RNA species are 7,6 kb and 3,5 kb in length (Kashiwazaki *et al.*, 1990; Niblett *et al.* 1991). The capsid protein size of WSMV (45 kDa) is conspicuously larger than those of aphid transmitted potyviruses (Lane & Skopp, 1983; Niblett *et al.*, 1991)

1.3.3.2 Genome organization and expression of aphid transmissible potyviruses

For the sake of relevance to potyviruses infecting South African *Passiflora*, which are investigated in this thesis, the genome organization of only the aphid vectored 'genus' Potyvirus will be discussed.

Genome structure

The aphid transmissible potyviral genome is a monopartite ssRNA of 9-10 kb (Dougherty & Carrington, 1988). A small genome-linked viral protein (VPg) is covalently linked to the 5' terminus of the ssRNA through a conserved tyrosine residue in the VPg (Hari, 1981; Murphy *et al.*, 1991), while the 3'-terminus consists of a poly-A stretch of variable length (Hari, 1979).

Genome organization of aphid transmissible potyviruses

The genomes of potyviruses sequenced until now, all have a single open reading frame (ORF), coding for a ca. 350 kDa polyprotein (Dougherty & Carrington, 1988). The gene order in the single ORF was established through *in vitro* cell-free translation studies (Dougherty & Hiebert, 1980; Hellmann *et al.*, 1986).

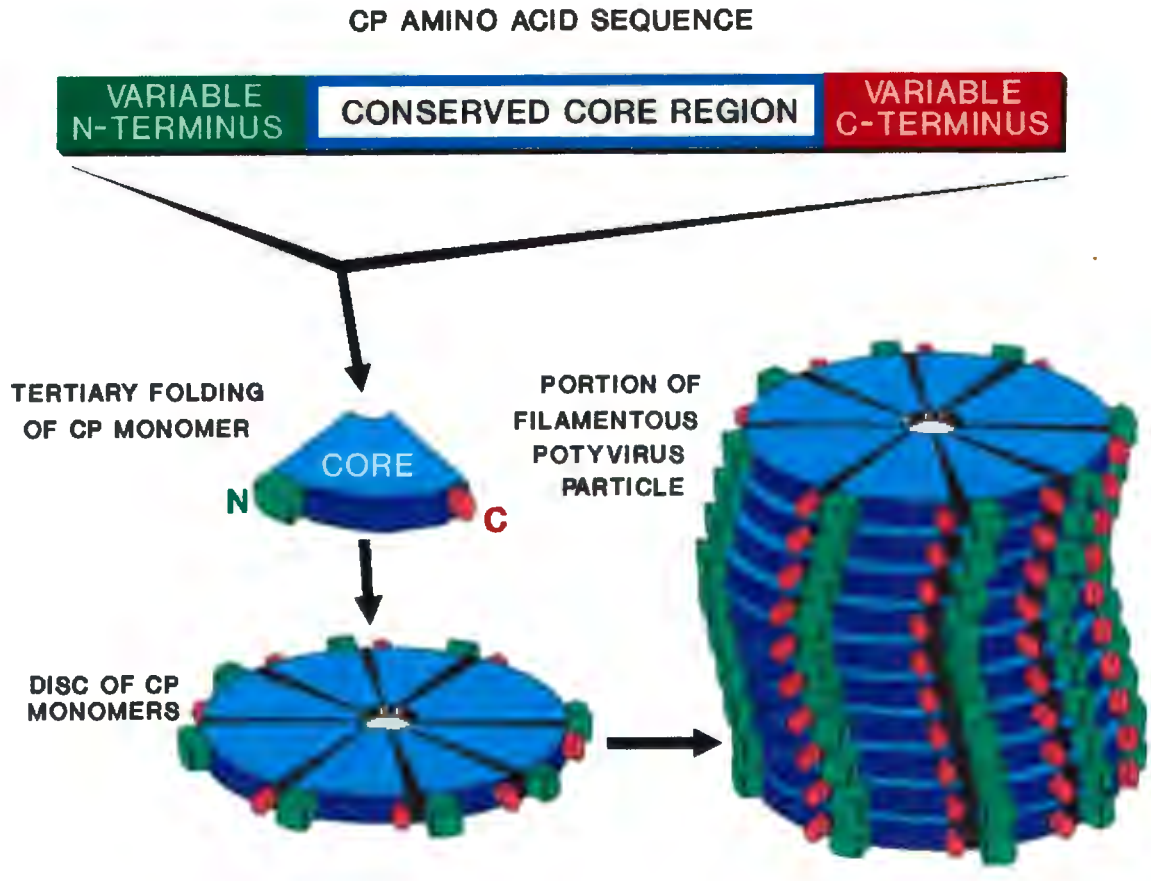


Fig. 1.2 Diagrammatic representation of the structure and assembly of a potyviral coat protein into virions, adapted from Shukla *et al.*, (1989).

Through these studies it also became evident that post translational processing of the polyprotein precursor occurs through an autoproteolytic mechanism encoded by the potyviral genome.

The gene order of all potyviruses sequenced so far is the same, and will henceforth be discussed using the genetic map of tobacco etch virus (TEV) as an illustrative example (Fig 1.3).

The 5'-untranslated leader sequences of potyviruses are approximately 140-200 nucleotides (nt) long, and contain secondary structure motifs thought to act as a binding site for the viral replicase during replication (Allison *et al.*, 1986; Domier *et al.*, 1986). This region is also believed to play a role in the enhancement of translation of the potyviral genome.

Gene products of the first two genes of TEV proximal to the 5'-end are liberated proteolytically from a 87 kDa precursor that appears to be the major translational product in cell-free translational studies with potyviral RNA (Dougherty & Hiebert, 1980; Carrington *et al.*, 1990).

The first gene product of the TEV genome has a size of 31 kDa (Fig. 1.3), and shows limited sequence similarity with the 30 kDa 'movement' protein of tobacco mosaic virus.This is an indication that this protein may also have a role in cell-to-cell movement of potyviruses (Carrington *et al.*, 1990 ; Domier *et al.*, 1987).

The second gene (Fig. 1.3) codes for the 52 kDa helper component protein (HC-Pro) of potyviruses (Carrington *et al.*, 1990; Allison *et al.*, 1986), that is also found within amorphous inclusion bodies in potyvirus infected plant cells (Baunoch *et al.*, 1990).

The HC protein is bifunctional: it is essential for aphid transmissibility of potyviruses (Berger & Pirone, 1986), and the carboxyl terminal half of the HC protein (HC-Pro) constitutes a domain with proteolytic activity, involved in proteolytic processing of the potyviral polyprotein precursor (Carrington *et al.*, 1989).

The third gene from the 5'-terminus of TEV RNA encodes a 50 kDa protein product of unknown function, that has yet to be detected *in vivo* in potyvirus infected plants (Dougherty & Hiebert, 1980).

The gene in the fourth position from the 5'-end of the TEV genome, codes for the 70 kDa cytoplasmic inclusion (CI) protein (Dougherty & Hiebert, 1980). The possible role of this protein in mediating the cell-to-cell translocation of potyviruses through plasmodesmata, is discussed in section 1.3.3.4.

The fifth position in the potyviral genome is occupied by the small nuclear inclusion protein (NIa) gene (Dougherty & Hiebert, 1980). This 49 kDa protein is once again bifunctional. The carboxyl half of this protein contains an amino acid sequence with high homology to thiol-type proteases encoded by several picornaviruses and cowpea mosaic virus (Argos *et al.*, 1984; Allison *et al.*, 1986).

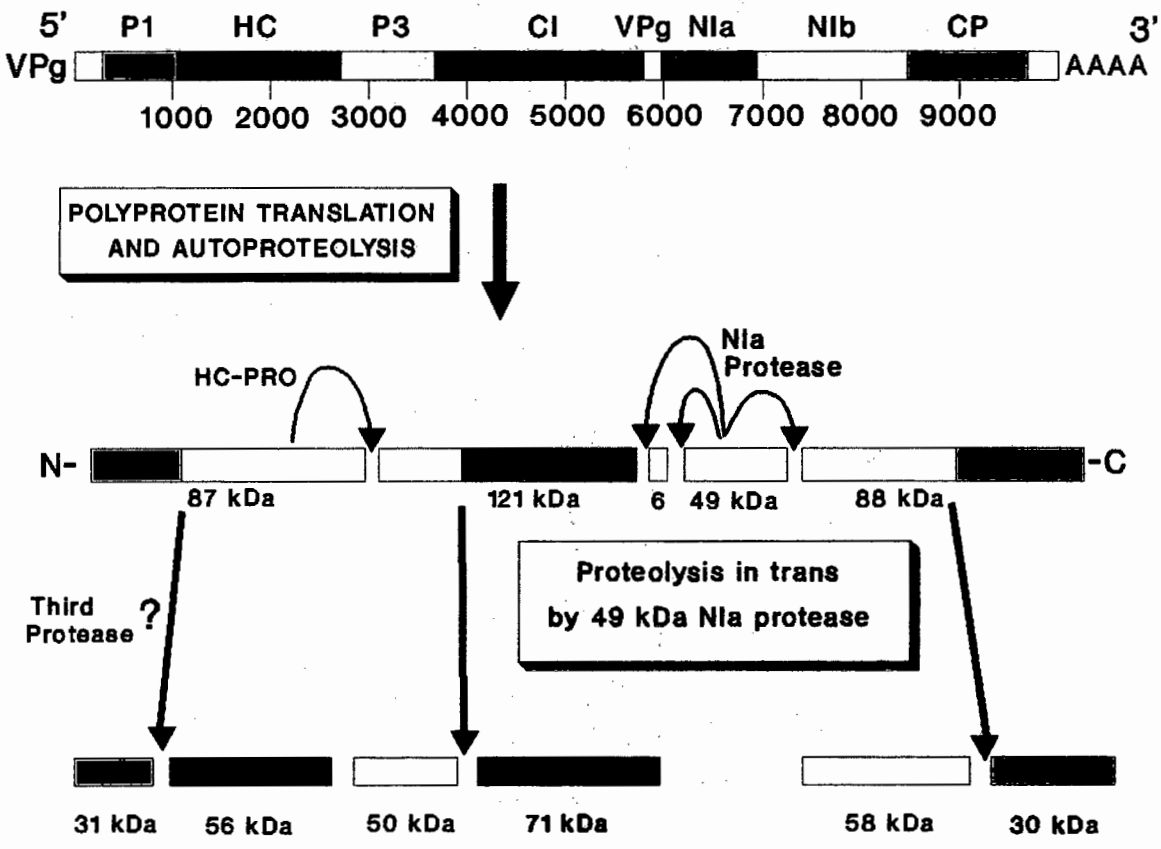


Fig. 1.3 Schematic representation of the general potyviral genome organization, translation and polyprotein processing - adapted from Dougherty & Carrington, (1988).

It was shown that this protease is responsible for at least five post-translational cleavages in the potyviral polyprotein (Carrington & Dougherty 1987a,b; Martin *et al.*, 1990). Very recently it has been established that the 24 kDa N-terminal moiety of the NIa assumes the role of the VPg of potyviruses (Murphy *et al.*,1990).

The sixth potyviral gene encodes the second of the two nuclear inclusion proteins (NIb - M_r 58 kDa). The sequence similarity between this protein and RNA-dependent RNA polymerases of sequenced viruses from other groups suggests a role in the replication of potyviruses (Domier *et al.*,1987).

The CP of potyviruses is encoded by the last gene near the 3'-terminus of the potyviral genome. This has an M_r of 29,6 kDa in the case of TEV (Dougherty & Hiebert, 1980; Allison *et al.*, 1986). Besides its structural function, it was established that a highly conserved "DAG" amino acid motif close to the surface-orientated N-terminus of the potyviral CP is essential for aphid transmissibility (Harrison & Robinson, 1988; Atreya *et al.*, 1990)

The CP gene is followed by a 3'-terminal untranslated region (3'-NTR) varying from 189-475 nucleotides in length, before the potyviral genome terminates in a polyadenylate tract of variable length (Frenkel *et al.*, 1989).

Expression of the potyviral genome

The potyviral polyprotein precursor (ca 350 kDa), is matured into at least eight functional proteins through post-translational proteolytic processing, by at at least three proteases (Carrington *et al.*, 1990, Fig. 1.3).

The NIa protease. This 49 kDa small nuclear inclusion is bifunctional. The C-terminal portion is responsible for at least five post- translational cleavages in the potyviral polyprotein (Carrington & Dougherty 1987a,b), while an N-terminal segment of this gene product is covalently attached to the full length genomic RNA of the virus (Murphy *et al.*,1990). This N-terminal domain of the NIa, designated the VPg (viral protein genome-linked), is proposed to play a role in virus replication.

The NIa protease is structurally related to the class of enzymes known as the trypsin family of serine-type proteases. In this protease, the serine normally found in the active catalytic site of trypsin, is substituted with cysteine. The NIa protease utilizes the free thiol group from a Cys residue (at the conserved catalytic site of the enzyme) as a nucleophile during peptide-bond cleavage, and uses a His residue as a general acid/general base (Dougherty & Carrington, 1988; Parks & Dougherty, 1991).

Through molecular analysis of cleavage sequences recognized by the TEV NIa protease (Dougherty & Carrington, 1988), it was established that these proteases cleave in a specific position in a conserved heptapeptide sequence (Fig. 1.3):

Glu-Xaa-Xaa-Tyr-Xaa-Gln↓Ser/Gly

Analysis of a number of other potyviruses revealed that the Gln on the N-terminal side of the cleavage junction is always conserved, while Ala/Thr can substitute Ser/Gly on the carboxyl site in some potyviruses (Maiss *et al.*, 1989; Lain *et al.*, 1988).

It was established that the determinants for substrate recognition and cleavage reside in the carboxyterminal 150 aa of the protein, and at least three subdomains in this region are involved in accurate recognition and processing of the polyprotein substrate (Parks & Dougherty, 1991).

The HC-Pro protease.

HC-Pro (M_r 52 kDa) is the second potyviral protease shown to participate in the proteolytic maturing of the potyviral polyprotein (Carrington *et al.*, 1989). HC-Pro is a also trypsin-like cysteine protease (Oh & Carrington, 1989). This proteinase appears to catalyse only one proteolytic reaction, that being an autocatalytic cleavage at its carboxyl-terminus (Oh & Carrington, 1989). HC-Pro cleaves selectively between a Gly↓Gly dipeptide (Carrington *et al.*, 1990; Martin *et al.*, 1990) (Fig. 1.3).

A third protease involved in polyprotein processing?

Most of the proteolytic cleavages are accomplished by two virus encoded proteases (NIa and HC-Pro), but it was recently determined that a mysterious third protease of unknown origin is required for processing the junction between the N-terminus of HC-Pro, and the carboxyl end of the N-terminal 35 kDa protein of TEV (Carrington *et al.*, 1990). These reseachers have shown that although the 87 kDa N-terminal portion of the polyprotein is not processed further in *in vitro* translation experiments, complete processing is achieved *in vivo* (to yield a 35 kDa protein and the 52 kDa HC-Pro), when only the sequence coding for the 87 kDa translational product is expressed in transgenic tobacco. Even when the HC-Pro proteolytic activity (located in the carboxyl half of the protein) was inactivated by a point mutation, proteolysis at the N-terminal junction of HC-Pro was unaffected. Translational products from transcripts in transgenic plants with a large deletion in the 35 kDa protein gene resulted in partial processing. If a cryptic protease activity is thus encoded by the potyviral genome, it can only be located in the vicinity of the 35 kDa::HC-Pro junction. The involvement of a host proteinase is not excluded, although unlikely, as no host factor is known to participate in the processing of other picorna-like polyproteins (Carrington *et al.*, 1990).

1.3.3.3 Potyvirus taxonomy

Classical criteria for definition of members of the potyvirus group

The sheer size of the potyvirus group has presented taxonomists with a formidable challenge (Francki, 1983). Classical criteria were inadequate for coping with the growth of this group, and this has led to the gradual decline of the taxonomic orderliness of the potyvirus group into an unsatisfactory state.

The traditionally accepted minimal criteria hitherto required for classification of a virus as a potyvirus include: compliance with the characteristic particle morphology (refer to section 1.3.3.1), the ability to induce the formation of typical cytoplasmic inclusions, and transmissibility by aphids in a non-persistent fashion (Hollings & Brunt, 1981). Other plant viruses with some but not all of these characteristics in common with potyviruses were hitherto assigned to the growing, but ill defined rank of "possible members of the potyvirus group". As mentioned earlier, there are several "poty-like" viruses capable of inducing "pinwheel" cytoplasmic inclusions in infected host cells, but which are vectored by insects other than aphids. These viruses were listed as possible potyviruses in the fourth report of the ICTV (Francki *et al.*, 1985; Milne, 1988; Matthews, 1982).

Classical criteria applied in attempts to establish inter-relationships between potyviruses

Earlier approaches of choice or necessity for resolving the relationships between members of the potyvirus group, or surmised strains of potyviruses, could be described as being subjective rather than impartial. A brief discussion of these relatively crude taxonomic criteria, highlighting their respective merits and shortcomings, follows below.

Symptomatology and host range.

Symptomatology and host range were considered to be important criteria in the definition of potyviruses in the past, probably due to the generally narrow host range of potyviruses and the characteristic symptoms they produce in specific hosts (Edwardson, 1974; Hamilton *et al.*, 1981). There are, however, numerous examples where reliance on these parameters created confusion, resulting in confused taxonomy in the group. This occurred especially in the viruses of plant families *Leguminosae* (Barnett *et al.*, 1987; Bos, 1970; Jones & Diachun, 1977; Tsuchizaki & Omura, 1987) and *Gramineae* (Shukla *et al.*, 1989c), where external factors such as climatic conditions and/or different cultivars/genetic lines of the same plant species can exert a profound influence on susceptibility and symptoms (Hollings & Brunt, 1981). Although some of these problems can be resolved by standardization of the above mentioned

variables, some limitations still remain. For example, different potyviruses infecting legumes had many hosts in common, and displayed very little symptom differentiation (Bos, 1970; Lana *et al.*, 1988). It was also shown recently that a single point mutation in the coat protein gene of tobacco mosaic virus altered the symptom expression from a systemic infection to local lesions. These findings cast serious doubt over the credibility of symptomatology as a marker of genetic relatedness. However, Shukla & Ward (1989a) have suggested that host range and symptoms can potentially serve as an aid to distinguish closely related viruses for which the biochemical relationships have already been established. This approach was used successfully to differentiate four potyviruses infecting *Gramineae* on the basis of their reactions on selected sorghum inbreds (Shukla *et al.*, 1989c).

Cross-protection.

The cross protection phenomenon occurs when infection by one plant virus can prevent a secondary infection by a closely related strain of the particular virus. Distinct viruses generally fail to cross protect against one another. Initially, considerable taxonomic weight was given to cross-protection data, but this criterion failed to consistently accord with other taxonomic data. There are, for instance, examples of unexpected cross-protection between potyviruses hitherto considered as being distinct: cross-protection was observed between bean yellow mosaic virus (BYMV) and soybean mosaic virus (SoyMV), BYMV and bean common mosaic virus (BCMV) and pea mosaic virus and BYMV (Shukla & Ward, 1989a). There are also examples of unexpected failures to cross-protect: the A & B strains of MDMV failed to cross-protect against each other, despite being considered strains of the same virus (Paulsen & Sill, 1970). However recent findings have shown that MDMV-B should rather be considered a strain of sugar cane mosaic virus (SCMV - Shukla *et al.*, 1989c). Much of the cross protection work was done prior to molecular analysis and comparison of the viruses in question. Several of these viruses have subsequently been reclassified on grounds of molecular data. Cross-protection may thus have taxonomic value, provided it is applied after assignment of viruses based on molecular data (Ward & Shukla, 1991).

Particle modal length.

Potyviruses generally have lengths varying from 670nm to 900nm and are 11 to 15 nm wide. The observation by Govier and Woods (1971) that the lengths of some potyviruses were also dependent on the composition of the suspending medium, diminished the status of particle modal length as a taxonomically significant parameter.

Cytoplasmic inclusions.

All members and possible members of the potyvirus group examined thus far, induce characteristic cytoplasmic "pinwheel" inclusions (CIs) in infected plant cells (Edwardson, 1974), rendering this one of the more important classic criteria. This feature is now recognized by the International Committee on Taxonomy of Viruses as a diagnostic feature of the group. Pinwheel inclusions are formed by assembly of single virus encoded CI proteins (liberated from the potyviral polyprotein by autoproteolytic processing) into three dimensional structures. The morphology of these pinwheels have been found to be virus specific, but host independent (Matthews, 1982).

On the basis of inclusion body morphology, the potyvirus group was divided into four subgroups (Edwardson, 1974; Edwardson *et al.*, 1984). Viruses in subgroup I produce tubular and scroll like inclusions; subgroup II induce laminated aggregates; members of subgroup III produce scrolls as well as laminated aggregates. Viruses in subgroup IV induce the formation of scrolls, and short, curved laminated aggregates. It is believed that the morphology of the inclusion protein reflects its primary structure. Although pinwheel formation has indisputable value in assigning a virus to the potyvirus group, Francki *et al.* (1985) observed that care should be taken in interpreting pinwheel characteristics, as potyviruses that were considered to be related strains in fact belonged to different subgroups based on pinwheel morphology, and many distinct viruses produce seemingly identical pinwheel inclusions. Continuing revisions in potyvirus taxonomy based on molecular data may resolve some of these apparently conflicting results, but currently the value of this criterion remains an open question (Shukla & Ward, 1989b; Frenkel *et al.*, 1991).

Nucleic acid hybridization.

Unlike nucleic acid sequence data, which can identify a virus as a member of the potyvirus group, as well as establish its phylogenetic relationships in context of the potyvirus group, cDNA hybridization has little use as a group-specific tool (Ward & Shukla, 1991). This is mainly due to the low homology observed between distinct members of the group. Only seven regions of 15 bases or more of perfect homology could be found through comparison of the entire nucleotide sequences of tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV). Although nucleic acid hybridizations have been used successfully to establish relationships between closely related strains of particular members of the potyvirus group, eg. BYMV (Barnet *et al.*, 1987) and PVY (Baulcombe & Fernandez-Northcote, 1988), it is considered unsuitable for determining hierarchical relationships amongst distinct members of the potyvirus group (Shukla & Ward, 1988).

Classical serology.

Classical serological techniques have been widely applied in the classification of plant viruses due to their simplicity, reliability and low expense (Van Regenmortel, 1966; Van Regenmortel, 1982; Moghal & Francki, 1976). For the purpose of potyvirus taxonomy, the term "classical serology" can be arbitrarily defined as any technique involving the use of polyclonal antibodies raised against a native potyvirus or its entire CP, in intragroup relationship studies.

Probably the most widely applied serological tests in potyviral detection and relationship studies, have been variants of the double antibody sandwich enzyme linked immunosorbent assay (ELISA) (Koenig, 1988; Van Regenmortel & Burckard, 1980). Other more specialized serological approaches to potyvirus characterization include immune specific electron microscopy (ISEM) (Milne, 1986), and *in situ* detection of potyviral proteins by use of immunofluorescent techniques (De Mejia *et al.*, 1985).

Although these techniques were previously considered to be the most suitable for potyvirus classification, it became evident that potyvirus serology was far more complex than anticipated. Hollings & Brunt (1981) noted for instance that bean yellow mosaic virus (BYMV) showed a strong immunological relationship to lettuce mosaic virus (LMV) and bean common mosaic virus (BCMV), but they failed to establish a serological relationship between LMV and BCMV. Perceived serological relationships were often misleading and in conflict with biological properties of potyviruses (Francki, 1983; Hollings & Brunt, 1981). Classical serology did thus, unfortunately, contribute a great deal to the confused state of potyvirus taxonomy.

Better understanding of the tertiary structure of potyviral coat proteins led to the use of N-terminal specific polyclonal antibodies and epitope-specific monoclonal antibodies. Combined with techniques such as electro-blot immunoassays (EBIA) and high performance liquid chromatographic peptide profiling, the status of serology as a very reliable and successful tool in potyvirus taxonomy was re-established (Shukla & Ward, 1989b; Shukla *et al.*, 1988d; Shukla & Ward, 1989a; Kahn *et al.*, 1990; Shukla *et al.*, 1988c; Shukla *et al.*, 1989b).

Improved resolution of potyvirus relationships through molecular structure analysis

Artificial taxonomic barriers implemented by taxonomists for the sake of convenience do not always manifest in nature. It is therefore crucial that any criteria considered in a biological classification system aiming at the establishment of an unbiased order, should most importantly reflect the phylogenetic lineages of such biological entities.

Modern technology employed in potyvirus taxonomy accomplishes this goal by classifying viruses directly or indirectly on the basis of their genetically derived molecular composition.

Modern serological approaches to potyvirus taxonomy.

The discovery that the amino (N)- and carboxy (C)-termini of potyviral CPs are surface-directed and highly variable, while the core portion of the CP is highly conserved (Shukla *et al.*, 1988c), was a breakthrough in potyvirus serology. It was also shown that, depending on the particular potyvirus, 30-67 N-terminal amino acids (aa) and 18-20 C-terminal amino acids can be removed from the CPs, by subjecting the particles to mild trypsin mediated proteolysis.

Such treated core particles were morphologically indistinguishable from untreated virions and retained their infectivity (Allison *et al.*, 1985; Shukla *et al.*, 1988c). Similar degradation of potyviruses is also known to occur during purification and storage (Francki *et al.*, 1985). Due to its high degree of amino acid conservation, the core portion of the potyviral CP lacks virus-specific epitopes. This accounted for the broad specificity of serological cross-reactions observed between distantly related potyviruses, using classical serological techniques.

An affinity adsorption column chromatography method was devised to remove antibodies directed against these virus non-specific core epitopes from polyclonal antisera raised against native virus. This involved the removal of the surface-located virus specific N-terminal region by lysyl- endopeptidase proteolysis. The truncated core CP was then coupled to cyanogen bromide-activated Sepharose gel. Polyclonal antiserum passed through this column was found to retain only highly specific virus antibodies (Shukla *et al.*, 1989b).

The use of such purified N-terminal specific antibodies in EBIA cleared up several inexplicable potyvirus serological relationships. Earlier serological and other attempts to differentiate between the NL1, NL3 and NY15 strains of BCMV and the W strain of blackeye cowpea mosaic virus (BlCMV) had been unsuccessful (Lana *et al.*, 1988). By using N-terminal specific antibodies, (Kahn *et al.*, 1990) showed that BlCMV-W and all the aforementioned BCMV strains, except the BCMV-NL3 strain, were closely related to each other but not to BCMV-NL3. There are however some isolated cases where non-related potyviruses unexpectedly share N-terminal epitopes, as were found for BYMV and clover yellow vein virus (CYVV) (Kahn *et al.*, 1990). Data derived from this method should thus preferably be backed up by other classification criteria, and not used as the sole criterion of relatedness.

As mentioned before, there are at least ten viruses exhibiting "poty-like" morphology that are capable of inducing pinwheel inclusions in plants, but are transmitted by vectors other than aphids (Francki *et al.*, 1985; Hollings & Brunt, 1981). The ICTV listed them as possible members of the potyvirus group

(Matthews, 1982). By means of EBIA, using a broadly group reactive polyclonal antiserum directed to the conserved core portion (aa positions 68-285) of the CP of Johnsongrass mosaic virus (JGMV - a definitive member of the potyvirus group), Shukla *et al.*, (1989a) established a strong serological relationship between the potyvirus group of coat proteins and those of wheat streak mosaic virus (WSMV) (mite transmitted) and sweet potato mild mottle virus (SPMMV) (whitefly transmitted). They concluded their investigation by recommending that the criterion of aphid transmission should be dropped as a prerequisite for membership of the potyvirus group, in the light of common epitopes and other biological properties shared between these and aphid vectored potyviruses.

The use of selected monoclonal antibodies (MAbs) in EBIA or variants of ELISA, has opened new avenues of exploration for the study of potyvirus relationships. Since MAbs react only with a single specific epitope, they can be selected for their ability to react with less or more conserved epitopes. This is extremely useful for epitope mapping (Jordan & Hammond, 1991; Shukla *et al.*, 1989d). The former authors screened a panel of MAbs, and divided them into strain-, virus-, subgroup-, and potyvirus group-specific antibodies. They have also identified an extremely broad spectrum MAb that recognizes a conserved cryptotope on all aphid transmissible potyviruses.

Coat protein amino acid sequence homology.

The potyviral CP is the major genome product in the virion, and accounts for 95% of the virion content, rendering it easily purifyable in moderate amounts. Furthermore it has been established that a highly conserved sequence motif close to the N-terminus of the CP is essential for aphid mediated transmission of potyviruses (Harrison & Robinson, 1988; Atreya *et al.*, 1990). The location of this gene at the 3' end of the virus genome facilitates cDNA cloning and sequence analysis. Not surprisingly, the analysis of this gene made the most significant impact on potyvirus taxonomy in recent times (Ward & Shukla, 1991; Shukla & Ward, 1989a; Shukla & Ward, 1989b; Rybicki & Shukla, in press). The CP sequences of more than 40 strains of 18 definitive potyviruses derived from cDNA- or protein sequencing are now known.

It has been argued that since the CP gene comprises less than 10% of the coding capacity of the potyviral genome, it may therefore have enjoyed a disproportionate measure of attention as a taxonomic criterion (Zettler, in press). However, Rybicki & Shukla (in press) pointed out that computer assisted phylogenetic reconstructions based on sequence data from the entire/partial CP region, gave relationship results very similar to those obtained using the entire viral nucleic acid sequences, and are certainly capable of unequivocally identifying distinct viruses from strains in most instances.

The CP shows no homology with CPs of other virus groups, in contrast with some other potyviral gene products (Domier *et al.*, 1987). The N-terminal

region is highly variable and only shows similarity between strains of the viruses, while the C-terminal two-thirds of the CP is conserved and fulfills a structural role in virion assembly (Shukla & Ward, 1989b). Pairwise comparisons of amino acid homologies between the CPs of 17 strains of eight distinct potyviruses by Shukla & Ward (1989b), revealed a bimodal distribution of sequence homology. Homologies between distinct members of the potyvirus group ranged from 38 to 71% (average 54%) while that between strains of one virus ranged from 90 to 99% (average 95%, refer to Fig. 1.4)

These findings are clearly not consistent with the 'continuum' hypothesis proposed by Hollings & Brunt (1981) in an attempt to explain the unsatisfactory taxonomic state of the potyvirus group.

Although the aforementioned 'rule of the thumb' distinction between members and strains of potyviruses generally applies, it is important to examine sequences in detail, and not only rely on unrefined sequence identity scores to determine whether a virus is a strain or a distinct member, as single deletions or frameshift mutations can sometimes obscure high homologies (Frenkel *et al.*, 1991).

An apparent problem has been observed between pepper mottle virus (PeMV) and PVY. Both of these have structurally very similar CPs, but fail to show a meaningful serological relationship.

Immunochemical studies of overlapping synthetic octapeptides corresponding to the N-terminal peptide regions of PeMV and PVY have shown that this lack of cross reactivity is due to a single sequence substitution at a key contact residue in the major virus specific N-terminal epitope (Ward & Shukla, 1991).

Coat protein sequence data and the genome context of the CP gene in BaYMV (fungus transmitted Bymovirus) and WSMV (mite transmitted Rymovirus) have clarified their indisputable affiliation to the potyvirus group (Kashiwaki *et al.*, 1989; Niblett *et al.*, 1991), as distinct genera in the proposed taxonomic family Potyvirideae (Rybicki & Shukla, in press; Ward & Shukla, 1991). Despite the fact that the CP M_r of WSMV is considerably higher (45 kDa) than reported for aphid transmitted potyviruses (30-36 kDa), the CP sequence homology of WSMV and BaYMV with aphid transmitted potyviruses is low but significant (18 to 24% compared to 38 to 71% observed between different members of aphid transmitted potyviruses). They are far more related to potyviruses than to viruses from any other group. Both of these viruses have 3'-located capsid protein genes, liberated from a polyprotein precursor as occurs with potyviruses. The NIa protease responsible for this cleavage cleaves at Q/S Q/A or Q/G positions (Dougherty *et al.*, 1989, Garcia *et al.*, 1989). In BaYMV cleavage occurs between Q/A (pr 101), and several potential Q/S sites are present in the region of the WSMV polyprotein where cleavage is expected to occur (Niblett *et al.*, 1991).

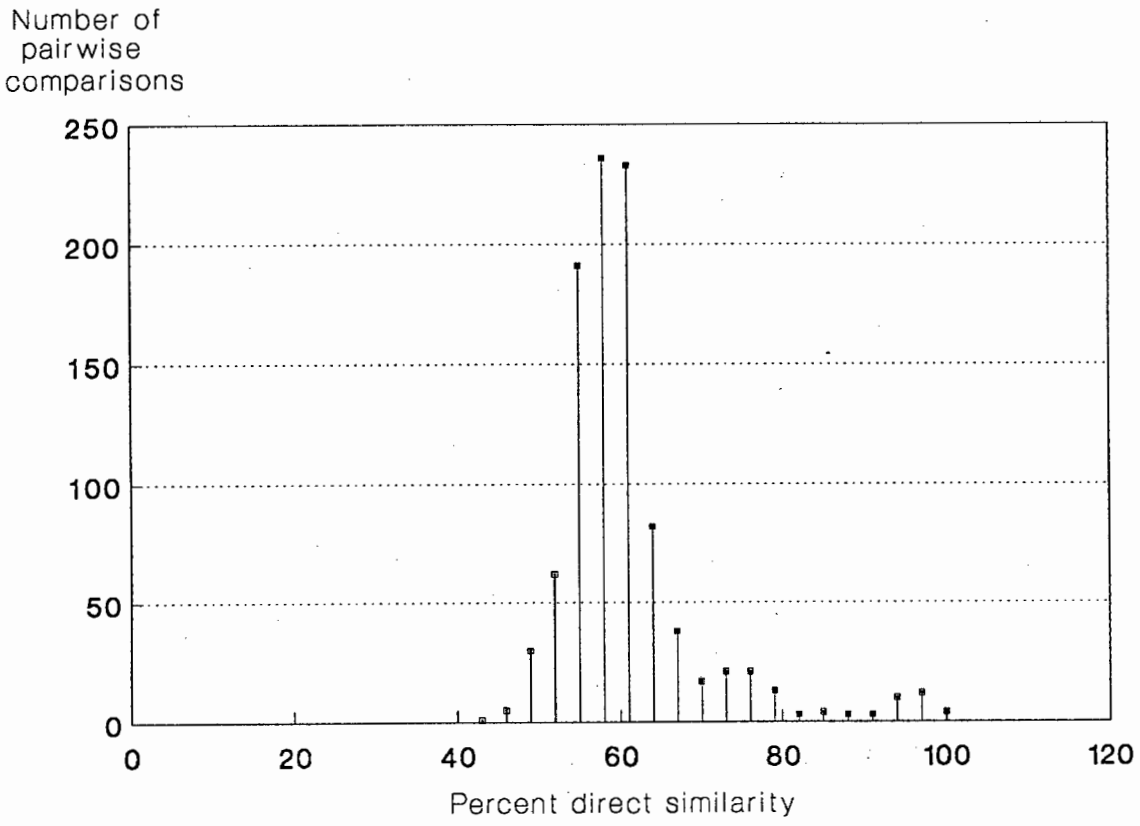


Fig. 1.4 Graphic representation of sequence relationships among 45 potyvirus CPs - 27 distinct members and 18 strains, and 990 pairwise comparisons (Rybicki, unpublished). Most accepted strains appear to fall into the 93-100% sequence identity range, while accepted distinct potyviruses range in similarity from 42-80%, with a major peak from 48-71% (891/990), and a "tail" from 72-83% (58/990).

Furthermore the N- and C-terminal regions of the BaYMV CP are also surface orientated.

It is thus clear that CP sequence data comparisons have immense value as a taxonomic tool in clarifying potyvirus strain relationships. Another reliable, albeit comparatively crude approach used to differentiate between potyviruses and strains, is a "fingerprinting" approach of peptide profiling by high performance liquid chromatographic (HPLC) analysis of trypsin digested CPs (Shukla *et al.*, 1988d; Shukla *et al.*, 1988a; Shukla *et al.*, 1987; Shukla *et al.*, 1988b; McKern *et al.*, 1990). This is, however, a specialized technique requiring expensive hardware, which will probably limit its routine utilization.

Nucleic acid sequence homology.

The nucleic acid sequence is the ultimate factor determining potyvirus identity, as the genotype is the only direct reflection of the phylogenetic status of an organism. Distance relationships amongst potyviruses are, however, more clearly established from aa sequences, partly because polypeptides have twenty "character states" as opposed to four of nucleic acids. This results in lower "noise" levels. Nucleic acids may also vary significantly without affecting protein sequences, because of redundancy of nucleic acid codons, which is not expressed in the encoded aa sequence (Rybicki & Shukla, in press). Sequence comparisons of the entire genomes of TEV-HAT (Allison *et al.*, 1986), TVMV (Domier *et al.*,1986), PPV-NAT (Maiss *et al.*, 1989), PPV-R (Lain *et al.*, 1989a) and PVY-N (Robaglia *et al.*, 1989) revealed that except for the two putative protein coding regions flanking the helper component gene, the sequence identities between corresponding coding regions ranges from 40 to 67%. Of all potyviral genes, the NIb region coding for the putative replicase shows the highest level of conservation (Shukla *et al.*, in press)

Frenkel *et al.*, (1989) observed that sequences of the potyviral 3'-non-coding regions (3'-NCR) are very conserved (83-99%) amongst strains of a potyvirus. There is, however, considerable variation between distinct members of the potyvirus group in this region. This feature can serve as a very quick and useful diagnostic aid for strain assessment of potyviruses (Uyeda *et al.*, 1991).

Sequence data is also a very useful and sensitive measure for revealing distant evolutionary relationships. Despite the bipartite nature of the BaYMV particle, the genome of BaYMV RNA 1 showed a close similarity to aphid transmitted potyviruses in its organization. Despite the low percentage homology between the amino acid sequences of the CP and non-structural genes of BaYMV and aphid transmissible potyviruses, there are several motifs conserved amongst aphid transmissible potyviruses, that are also found in BaYMV (Kashiwaki *et al.*, 1990; Kashiwaki *et al.*, 1989). Two hydrophobic sequences, GXXGXGKS and D(E/D), have been proposed as a consensus motif around phosphate-binding pockets in many NTP-binding proteins. These

sequences are conserved in the CI proteins of potyviruses (Lain *et al.*, 1989b), and they have also been found in BaYMV (Kashiwaki *et al.*, 1990), as well as in the mite transmitted WSMV (Niblett *et al.*, 1991). Two other characteristic stretches, (T/S)GXXXTXXXN(T/S) and GDD are thought to form the core of the RNA-dependent RNA polymerases of positive stranded RNA viruses (Kamer & Argos, 1984). The GDD stretch in BaYMV is also preceded by an N as occurs in the NIb polymerases of four sequenced potyviruses (Kashiwaki *et al.*, 1990). Although sequence analysis of BaYMV RNA 2 revealed an obvious homology to the helper component gene (HC-Pro) of aphid transmitted potyviruses, it disclosed striking differences in genome organization of the 5'-terminal region (Kashiwaki *et al.*, 1991).

New taxonomic considerations for plant viruses.

According to the seventh official guideline for the delineation of viruses promulgated by the ICTV in their fourth report (Matthews, 1982), virus taxonomy has no phylogenetic or evolutionary implications. This overly cautious attitude was probably inherited from an earlier stage when most of the available information, considered useful for the classification of viruses, was based on particle morphology, physicochemical analysis, host range and symptomatology. The more recent revolution in molecular sequence analysis of nucleic acids and proteins, however, has yielded a flood of information revealing clear phylogenetic relationships between viruses. This has completely destroyed the old type of rationale (Goldbach, 1987; Goldbach & Wellink, 1988; Rybicki, 1990; Kingsbury, 1988). At a mid term meeting in 1983, the ICTV executive committee deleted the infamous line 7 from the list of guidelines for the delineation of viruses, although many virologists and even ICTV members are apparently still unaware of this (Kingsbury, 1988).

Virus evolution.

A major classification problem emerging from available molecular data, is the fact that viruses are probably polythetic in origin (Rybicki, 1990). It is thus impossible to construct a tree starting at a single point, but it is possible to draw several evolutionary trees depicting the evolution of viruses from different origins. For the sake of relevance to this chapter, dealing with the potyviruses and cucumoviruses, the possible evolution of the ssRNA viruses will be very briefly discussed.

Sequence data suggest that all plant (+)stranded ssRNA viruses can be grouped into two 'supergroups': (a) the supergroup of 'picorna-like' viruses (related to animal picornaviruses), and (b) the 'sindbis-like' virus supergroup. These viruses exhibit a modular evolution, with a clear phylogenetic lineage of 'cassettes' of gene clusters. Limited new data suggest that a third supergroup

may exist: carmoviruses, tombus viruses and luteoviruses may form part of this supergroup (Goldbach & Wellink, 1988).

The 'supergroup' of 'picorna-like' viruses includes the como, nepo and potyviruses. Although distinctly morphologically diverse, these viruses share the following properties with animal picornaviruses: (a) their RNA genomes have a virus encoded VPg (genome linked virus protein) attached to the 5' end, while the 3' end is polyadenylated; (b) expression of the RNA occurs through the translation of a polyprotein precursor that is matured into functional structural and non-structural proteins by proteolytic processing; (c) a number of the non-structural proteins have more than 20% sequence homology, and these conserved proteins are coded by genes in cassettes of similar gene arrangement (Goldbach, 1987; Goldbach & Wellink, 1988). Most 'picorna-like' viruses generally have a similar capsid structure, with the exception of the potyviruses. Potyviruses have cylindrical filamentuous particles, while the other 'picorna-like' viruses exhibit a spherical, pseudo T=3 capsid morphology. Potyviruses also differ in respect of their -COOH location of the CP in the polyprotein, whereas other picornaviruses have NH- located CPs.

The 'supergroup' of 'sindbis-like' viruses all show varying degrees of relatedness to the alphaviruses of animals. Although they differ widely in morphology, genome structure and translation strategy, all 'sindbis-like' viruses have 5'-cap structures and produce subgenomic RNAs. Furthermore, they all code for nonstructural proteins with homology to three nonstructural proteins of sindbis virus. This group includes the alfalfa mosaic virus group, ilar-, bromo-, cucumo-, tobra-, furo-, hordei-, carmo-, and tombusviruses. Although rod shaped and genomically undivided, tobacco mosaic virus (TMV) is similar to other 'sindbis-like' viruses in coding for four proteins, three of which are related to corresponding 'sindbis-like' viral proteins (Goldbach & Wellink, 1988). The latest addition to the 'sindbis-like' virus supergroup, are the potexviruses, which show sequence affinities with furo- and hordeiviruses.

The overall homology between proteins of the 'picorna-like' and 'sindbis-like' supergroups is very low, but not altogether absent (Goldbach & Wellink, 1988). The replicase gene (RNA dependent RNA polymerase) of some viruses of both supergroups share homologies with each other (Domier *et al.*, 1987). It is thus clear that the viral replicase can serve as an ancient evolutionary marker predating the 'cassette' assembly of viruses.

The features of structural proteins of plant viruses indicate that gene exhange through RNA recombination occurred between the proposed supergroups: there is conspicuous similarity between the coat proteins of TMV (sindbis-like) and potyviral coat proteins (Goldbach & Wellink, 1988; Dolja *et al.*, 1991). There is a conserved 'eight stranded anti-parallel beta barrel' motif common to nearly all isometric viruses regardles of their 'supergroup' affiliation. These features definitively create additional virus classification problems,

especially for the establishment of hierarchies. It has been suggested that, where sequences of different genes display contradictory phylogenetic descent, it is probably wise to regard the core module of genes of the replicational machinery, reflecting the essence of a genome strategy, as the most important criterion of descent (Rybicki, 1990; Bruenn, 1991).

New taxonomic proposals for the potyvirus group.

Surprisingly, molecular data have not altered the group status of any individual potyviruses, except for showing that the potyviral group definition should be broadened to accommodate non-aphid transmissible 'possible' members of the potyvirus group, in a family Potyviridae. However, the molecular data has had a profound impact, on the differentiation and grouping of potyviruses on the virus/strain level.

In order to accommodate the obviously related, yet clearly distinct non-aphid transmissible 'possible members' in the potyvirus group, it has been suggested that the potyvirus group level should be elevated to 'family' status of Potyvirideae comprisin four different 'genera' as discussed in section 1.3.3 (Barnett, 1991).

1.3.3.4 Potyvirus host range and cytopathology

Host range and host defense response

Even though potyviruses are known to infect a wide variety of plants (in 1974 they were reported to infect 1112 species of 369 genera in 53 plant families), individual members have extremely narrow host ranges (Edwardson, 1974). Genes exist in the virus and in the host that determine the susceptibility of the plant host to a particular virus (Van Loon, 1987). In spite of their limited genome size, viruses harbour several pathogenic determinants. The observed cytopathological effect is, however, not neccesarily brought about by a 'viral encoded symptom-inducing protein', but is often due to response of the host defense gene products, or by interference of the virus replication with normal host cytological activity. There are several common host factors involved in pathogenesis and symptom expression, that occur irrespective of the type of pathogen, but it is also known that near-isogenic cultivars of a particular host can sometimes show a completely different reaction to infection by a particular virus strain (Van Loon, 1987).

The defense response of the host cell induced by either fungus, bacterium or virus infection, can include: (a) Induction of enzymes and chemicals involved in cell wall modifications to form a physical barrier restricting spread (cell-to-cell movement) of the pathogen; (b) Induction of enzymes that serve as physical inhibitors of certain kinds of pathogens; (c) Adaption of the host metabolism to stress conditions (Kolattukudy, 1991 *et al.*,; Bol & Linthorst, 1990)

The cytopathological effects associated with potyviral particle components and translational products

Effects of potyvirus infection on chloroplasts

Chlorosis, yellowing and mosaic symptoms result from a fairly general loss of chlorophyll. It has been reported that watermelon mosaic virus-2 (WMV-2) occurs at much higher concentrations in yellow areas than in dark green areas in infected pumpkin leaves showing mosaic symptoms (Suzuki *et al.*, 1989). These authors also detected much higher concentrations of virus encoded proteins (cytoplasmic inclusion-, coat-, and amorphous inclusion proteins) in yellow leaf areas than in green areas.

Gadh & Hari (1986) reported that full length (-)RNA of tobacco etch virus (TEV), was associated exclusively with the chloroplast fraction of tobacco. This (-)RNA was later shown to be in the dsRNA form (Gunasinghe & Berger, 1991). This is a strong indication that chloroplasts are capable of supporting potyviral replication. The same researchers also detected the presence of potyviral coat protein (CP) and helper component (HC) within chloroplasts. It is still unclear whether the potyviral gene products are imported, or whether they occur through translation within the chloroplasts. The possible potyviral replicational activity in chloroplasts may interfere with photosynthesis. Naidu *et al.*, (1984a,b) found that infection by peanut green mosaic virus (PGMV) leads to reduced photosystem II activity in peanuts.

Cytoplasmic 'pinwheel' inclusions

The formation of characteristic cytoplasmic inclusions (CIs) is the most important diagnostic trait of potyvirus infection. All potyviruses encode a single monomer protein (M_r 66-75 kDa), termed the 'cylindrical inclusion protein', that aggregates in infected cells to form inclusions characteristic of the particular potyvirus (Dougherty & Hiebert, 1980; Dougherty & Carrington, 1988). These aggregated CIs can resemble pinwheels, or may appear as scrolls or laminated aggregates (Edwardson 1974; Edwardson *et al.*, 1984). Earlier attempts to classify potyviruses, divided potyviruses into four subgroups, based on the morphologically distinct pinwheel CIs they produce (Edwardson, 1974). Pinwheels have a three dimensional shape: 8-12 tapered arms radiate from a hollow central tube to confer a conical shape to the structure (Calder & Ingerfeld, 1991).

Several functions are associated with the CI proteins of potyviruses: (a) a role in the replication of potyviruses (Domier *et al.*, 1987); (b) a dsRNA template-directed helicase with ATPase activity (Lain *et al.*, 1989b; Lain *et al.*, 1990; Lain *et al.*, 1991); (c) involvement in the cell to cell spread of the virus (Langenberg, 1986; Calder & Ingerfeld, 1991).

(a) The putative role of the CI protein in replication of potyviruses. The CI protein of potyviruses shows significant homology with a corresponding protein of another 'picorna-like' virus, the P2 protein of poliovirus (Domier *et al.*, 1987; Goldbach, 1987). Previous workers have shown that the P2 protein of poliovirus (comprising the 2A, 2B and 2C proteins) plays a role in replication, by inducing the formation of vesicles to which the virus-specific replication complex is bound, and where replication and virus encapsidation occur. Of the three morphological configurations of CIs, only 'pinwheels' were observed to be associated with cellular components (Calder & Ingerfeld, 1991). The pinwheels do not occur randomly throughout the cytoplasm, but instead are found in association with the rough endoplasmic reticulum (rER) or with plasmodesmata (Calder & Ingerfeld, 1991; Lawson & Hearon, 1971). When pinwheels are associated with the rER, it leads to an increased frequency of smooth-surfaced vesicles budding off. These vesicles are of variable size and some have electron-dense contents (Calder & Ingerfeld, 1991; Edwardson, 1974). However, during later stages of infection, the CIs seem to become detached from the cell membranes, which is thought to conflict somewhat with its proposed function as a co-mediator of virus replication (Francki, *et al.*, 1985; Calder & Ingerfeld, 1990).

(b) The helicase and ATPase activity of the CI protein. The recent discovery that the CI protein has nucleotide binding capabilities as well as the ability to unwind dsRNA, provides the strongest evidence yet that this protein is indeed, as previously suspected, involved in the replication of potyviruses. Most positive strand RNA viruses infecting plants and animals, encode proteins containing a so-called nucleotide binding motif (NTBM). Such a conserved motif (GXXGXGKS), occurs in the CI protein of potyviruses, as well as in the 2C protein of poliovirus (Domier *et al.*, 1987). It is also found in other viral and non-viral systems, where it is involved in binding of nucleoside triphosphates, and can be regarded as a general feature of ATP- or GTP-utilizing enzymes (Walker *et al.*, 1982; Hodgman, 1988; Lain *et al.*, 1989b). Lain *et al.*, (1990) demonstrated the helicase activity of the CI protein by showing that it can unwind dsRNA with a 3'-ssRNA poly-A overhang. During this process it also catalyses the conversion of ATP to ADP + Pi which appears a prerequisite for its helicase activity (Lain *et al*, 1991).

(c) The possible involvement of the CI protein in cell-to cell spread of potyviruses. The proteins facilitating cell-to-cell transport of a number of viruses have been analysed and compared (Melcher, 1990). The most well characterized movement protein is the 30 kDa protein of tobacco mosaic virus (TMV) (Deom *et al.*, 1987; Atkins *et al.*, 1991; Deom *et al.*, 1990).

Current data suggest that plant viruses or nucleic acids move from cell-to-cell through plasmodesmata, channels that extend through cell walls and provide cytoplasmic continuity between adjacent cells (Deom *et al.*, 1990). The architecture of plasmodesmata of uninfected cells is, however, restrictive

towards the transportation of molecules of the dimensions of virus particles or viral nucleic acids. Recent studies have shown that TMV encodes a 30 kDa movement protein that is localized within, or on the plasmodesmata, and functions by increasing the permeability limit of plasmodesmata (Wolf *et al.*, 1989; Atkins *et al.*, 1991).

In vivo complementation studies suggested that virus-coded transport proteins are host-specific, rather than virus-specific. Complementation of movement is the ability of a virus to allow the systemic spread of another (even unrelated virus) in a host normally restrictive to the second virus, in double infections. It was for instance shown that TMV (from 'sindbis-like' supergroup) can complement the movement of como- and nepoviruses ('picorna-like' supergroup) in plants that are normally non-hosts for the latter two viruses (Malyshenko *et al.*, 1989). This complementation occurs despite the lack of significant homology between the movement proteins of these viruses. This essentially supports the notion that the cell-to-cell transport of most plant viruses occurs through a physically similar strategy, despite the remarkably low level of conservation of viral movement genes, and the effectiveness of a particular type of transport protein is determined by the host (Malyshenko *et al.*, 1989).

The close association of potyviral CI proteins with plasmodesmata, and their ability to dilate membranes (of the rER), render them the most likely candidates for involvement in cell-to-cell permeation of potyviruses (Calder & Ingerfeld, 1990).

Amorphous cytoplasmic inclusions

Amorphous inclusions (AIs) are non-crystalline masses differing in shape and are produced by several different potyviruses (Edwardson, 1974). Immunological analysis of these structures suggest that they are reservoirs of the helper component (HC) protein (Baunoch *et al.*, 1990). Although HC is essential for aphid transmission of potyviruses, an intriguing observation is the fact that although purified HC has helper activity (Thornbury *et al.*,1985), purified amorphous inclusions do not exhibit any helper activity (Dougherty and Carrington, 1988).

Nuclear inclusions

Although all potyviruses encode two proteins comprising the "nuclear inclusion body", only in some potyviral infections do these proteins aggregate to form stable nuclear inclusion bodies (Dougherty and Carrington, 1988). These structures occur in various shapes and sizes, depending on the potyvirus or strain: they can appear as plates, cubes, bipyramids, etc. (Edwardson, 1974; Edwardson & Christie, 1978). The nuclear inclusion body is composed of two proteins of M_r approx. 49 & 54 kDa respectively, depending on the virus which occur in equimolar amounts (Knuhtsen *et al.*, 1974). Potyviral NIs are encoded

by two distinct potyviral genes (NIa & NIb) and distinct functions have been proposed for them: NIa is a proteinase involved in the autoproteolytic processing of the potyviral polyprotein (Carrington and Dougherty, 1987a,b), while NIb is presumed to be an RNA dependent RNA polymerase on basis of its sequence homology to genes of other viruses (Domier *et al.*,1987).

1.3.4 General features of cucumber mosaic virus (CMV)

Cucumber mosaic virus (CMV) is one of the most widespread viruses, infecting a wide variety of crops and ornamental plants worldwide. This virus exists as a multitude of strains (more than 60 have been reported) that can be genetically and biologically very diverse, despite similar physical, chemical and antigenic properties (Kaper & Waterworth, 1981).

The discussion on CMV will be more brief than the previous section on potyviruses, as a relatively small part of this thesis deals with the limited characterization and detection of a CMV isolate from South African *Passiflora*.

1.3.4.1 Classification

CMV is a single stranded plus-sense RNA plant virus with a functionally divided genome, consisting of three unique RNA species designated RNA 1, 2 and 3 in order of decreasing molecular mass. Each of these RNA species is separately encapsidated in an identical protein capsid, and the presence of all three is required for infection (Kaper & Waterworth, 1981).

As mentioned in section 1.3.3.4, all RNA viruses appear to fall into two supergroups: Sindbis-like viruses and Picorna-like viruses (Goldbach, 1987). While potyviruses, as discussed in section 1.3.3.4, are related to the Picorna-like supergroup, CMV is phylogenetically related to the Sindbis-like supergroup (Goldbach, 1987). Each of these supergroups comprises a number of virus "families" consisting of different but related virus groups.

At present, the ICTV is considering recognition of a "family" of tripartite viruses comprising four separate groups: The Bromovirus group, the Alfalfa Mosaic Virus group, the Ilarvirus group, and the Cucumovirus group (E.P. Rybicki, pers. comm.). All members of these virus groups have in common the fact that they each have genomes consisting of three unique plus-sense single-stranded RNA, separately encapsidated in small polyhedral or bacilliform particles. For the purpose of this thesis, only the Cucumovirus group (particularly CMV) will be further discussed.

CMV is the type member of the Cucumovirus group (='genus'), that also includes tomato aspermy virus (TAV), and peanut stunt virus (PSV). Cowpea ringspot virus CPRSV is included as a possible member in the Cucumovirus group (Matthews, 1982). The physical properties of Cucumoviruses are very similar, and serologically they are distantly interrelated (Francki, 1985).

On the basis of nucleic acid hybridization studies (Gonda & Symonds, 1978; Piazolla *et al.*, 1979; Owen & Palukaitis, 1988), serology (Rybicki & Von Wechmar, 1985), CP peptide mapping (Edwards & Gonsalves, 1983) and nucleotide sequence analysis (Rizzo & Palukaitis, 1989; Quemada *et al.*, 1989; Hayakawa *et al.*, 1989), all CMV strains examined can be subdivided into two subgroups: a major subgroup of 33 strains (Subgroup I) and a minor subgroup of 11 strains (Subgroup II).

1.3.4.2 Particle structure

CMV is a spherical virus, consisting of three native particles with identical protein outer capsid, encapsidating three unique RNA species (1,2 & 3) (Kaper & Waterworth, 1981). The capsid of each particle is about 29 nm in diameter, and consists of 180 identical CP subunits, arranged on a T=3 icosahedrally symmetric surface lattice. Coat protein monomers have a M_r of about 26 kDa, and particle M_r is in the order of 5,3 X 10^6 Da.

RNA contents contribute about 19% of particle mass (Johnson & Argos,1985). Two of the three particles contain RNA 1 (M_r 1.35 X 10^6 Da) and RNA 2 (M_r 1.16 X 10^6 Da) respectively, while the third particle contains RNA3 (M_r 0.75 X 10^6 Da) along with the subgenomic coat protein messenger RNA 4 of M_r 0.35 X 10^6 Da (Johnson & Argos, 1985).

The CMV particle structure is very unstable, especially in the presence of strong salt solutions or even very weak sodium dodecyl sulphate (SDS) solutions (Kaper *et al.*, 1965; Boatman & Kaper, 1976). The particle infectivity is also subject to the presence of RNases. It seems to be more sensitive to RNase-inactivation at pH6 than at pH8 (Ehara & Mink, 1986)

1.3.4.3 Cytopathology

Effects on endogenous plant metabolites

As in the case of potyviruses, infection by CMV has a pronounced effect on the balance of endogenous plant metabolites. There is usually an increased level of ethylene as well as altered levels of other natural plant hormones, that serve as chemical inducers of the general plant stress response (eg. pathogenesis related proteins), leading to the abnormal growth and appearance of the infected host (Bol & Linthorst, 1990; Van Loon, 1987). Plant host response to virus pathogen attack is a general reaction discussed in detail in section 1.3.3.4.

Effect on organelles of the plant host cell

CMV infections are associated with several severe cytological modifications of host tissue. Membranous bodies (MBs) develop in the

cytoplasm due to unusual proliferation of the ER. These MBs are thought to migrate towards the cell vacuoles and fuse with tonoplasts (Giovanni & Russo, 1985). Such tonoplast associated vesicles containing dsRNA have been observed in the host cell, and have been proposed as sites where viral RNA synthesis occurs (Hatta & Francki, 1981).

An association of virions with plasmodesmata (Giovanni & Russo, 1985) has been observed, which is probably the route of cell-to-cell movement of CMV.

Other cytoplasmic organelles, such as chloroplasts and mitochondria, show severe structural and functional impairment in CMV infected cells (Giovanni & Russo, 1985).

Accumulation of virions or viral components in the plant cell

Although assembled virions occur randomly through the cytoplasm of CMV infected cells, virus aggregates are also found in the vacuoles. CMV particles were also observed in up to 70% of the nuclei of infected tobacco cells (Honda & Matsui, 1974).

An association of CMV CP with host chromatin was observed (Van Telgen *et al.*, 1985). Roberts & Wood (1981) correlated symptom severity with CP expression, by showing that a severe strain of CMV (P6) synthesizes considerably more CP than a mild strain (CMV-green).

1.3.4.4 Host range and mode of transmission

CMV has an extremely broad host range and has been reported to infect over 775 species of plants in 365 genera and 85 families. Its host range includes monocots as well as dicots (Kaper & Waterworth, 1981).

More than 60 species of aphids are capable of transmitting CMV in a non-persistent manner (Francki *et al.*, 1979). This type of transmission involves the acquisition of virus during short probes on infected plants by insects which are then able to transmit the virus for a short time. Unlike potyviruses that code for a helper component protein required for non-persistent aphid transmission, cucumovirus transmission is solely dependent on the viral coat protein (Chen & Francki, 1990).

All cucumoviruses are readily transmitted by mechanical inoculation to a wide range of host plants, and are seed borne in many of them (Kaper and Waterworth, 1981). The infection of a wide variety of perennial weeds by CMV, and seed transmission in them, are probably important epidemiological factors in maintaining reservoirs of CMV (Garrett *et al.*, 1985).

1.3.4.5 Genome organization and expression

General features of CMV genomic RNAs

As previously mentioned, the genome of CMV is divided in three RNA molecules (Fig. 1.5), each encoding unique polypeptide products.

All CMV RNAs have in common the fact that they are 5'-capped with m^7Gppp-, and are devoid of a 3'-polyadenylate tail (Symonds, 1975). Instead, the non-translated (approximately 170 nt) 3'-terminal ends of all RNAs of CMV strains contain very similar tertiary structure motifs that resemble tRNAs of plants. The 3'-terminal ends of these structures are in fact tyrosylatable by plant aminoacyl tRNA synthetases (Symons, 1985). Since the 3'-ends of CMV RNAs must be the sites of initiation of viral RNA synthesis, these peculiar features are probably important for recognition by the viral replicase. Although 3'-terminal non-coding regions show a strong conservation of secondary structure between CMV strains of subgroups I & II, the actual sequence conservation in this region is only about 65% (Rizzo & Palukaitis, 1989). However, most of the variation observed between the 3' NCRs of different CMV strains, occurs upstream of the 180 3' terminal nt involved in the conserved structural motif. Changes in the 180 nt 3' terminal region are limited to single nt substitutions that are not expected to alter the secondary structural folding (Owen *et al.*,1990).

There is actually greater sequence conservation (about 80%) in the 5'-terminal non-coding region (of RNAs 1 & 2) between CMVs from subgroups I & II. The sequence conservation in this area is probably important for initiation of translation through ribosome recognition (Rizzo & Palukaitis, 1989).

CMV RNA 1

Sequence analysis of RNA 1 of CMV-Fny (subgroup I) and CMV-Q (subgroup II), has revealed a 3357 nt long RNA molecule with a single ORF of 2979 for CMV-Fny, and a 3389 nt RNA 1 with a 2973 nt ORF for CMV-Q (Fig. 1.5). Sequence homologies for RNA 1 and its translational product in these strains, were calculated as 76% at the nucleic acid level and 85% at the protein level (Rizzo & Palukaitis, 1989).

Comparison of the RNA 1-ORF polypeptide product with sequenced genes from other viruses revealed the presence of nucleotide-binding sequence motifs. This led to suggestions that this ORF encodes a polypeptide product acting as a nucleotide-binding helicase involved in the replication complex of CMV (Rizzo & Palukaitis, 1989; Kamer & Argos, 1984; Hodgeman, 1988). It was shown that only RNA 1 & 2 are required for the replication of CMV (Nitta *et al.*, 1988; Quadt & Jaspars, 1991).

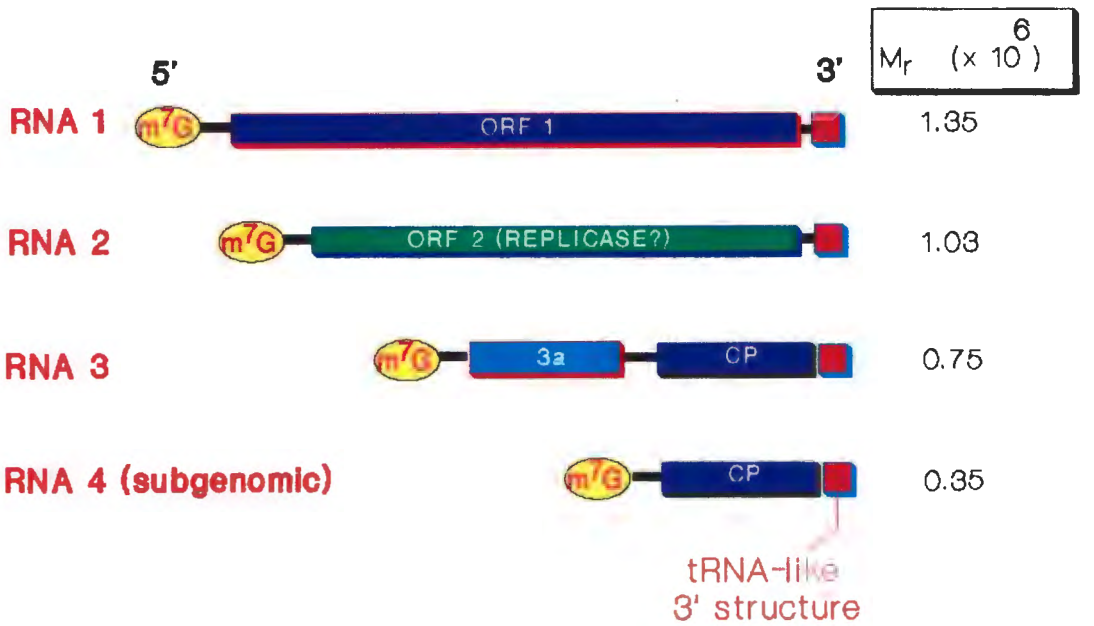


Fig. 1.5 Diagrammatic representation of the genome organization of cucumber mosaic virus (CMV) - adapted from Symons (1985).

CMV RNA 2

Sequencing and analysis of RNA 2 of the same CMV strains mentioned above yielded a 3050 nt RNA 2 containing a single 2571 nt ORF for CMV-Fny, and a 3035 nt RNA 2 molecule with a 2517 nt single ORF for CMV-Q (Rizzo & Palukaitis, 1988). At the nucleotide level, there was 71% homology between the two RNA 2s, while at the protein level, the sequence homology was 73%.

Protein homology was greater (89%) in the central region of the protein than at the N-terminal or C-terminal ends, which were only 64 & 56% homologous, respectively. The level of structural similarity between these proteins is even greater, as out of 38 mismatched amino acids, 20 reflected conservative changes. This high homology between translational products of CMVs from different subgroups suggests a highly conserved functional role for this protein. The "GDD" motif, which is associated with viral replicase proteins (Kamer & Argos, 1984) is present in the central regions of translational products from RNA 2 from both CMVs (Rizzo & Palukaitis, 1988). It is supposed that the CMV RNA 2 product is part of the viral polymerase

CMV RNAs 3 & 4

Sequencing of CMV-Q RNA 3 revealed a 2197 nt RNA molecule with two ORFs. The first ORF (nt 95 - 835) encodes the 279 aa 3a protein, while the second ORF (nt 1220 - 1876) codes for the 218 aa CP (Davies & Symons, 1988). The two genes are separated by an intergenic region of approximately 290 nt long. A sequence conserved in Cucumoviruses and Bromoviruses (GGUUCAA) occurs in the intergenic region of these viruses (Quemada *et al.*, 1989). Analysis of RNA 3 sequences from different CMV strains from subgroups I & II, revealed a remarkably high level of conservation of the RNA 3 sequence amongst members of subgroup I, while RNA 3 sequences between the two subgroups are quite divergent. In spite of the degeneracy of the genetic code, CMV strains in the same subgroup show a strong conservation of both nucleotide sequence and amino acid sequences, suggesting other possible roles for RNA 3 (Owen *et al.*, 1990)

The 3a protein.

In vitro translation of RNA 3 results only in an approximately 35 kDa translation product, encoded by the 3a gene. This means that RNA 3 functions as a monocistronic mRNA, despite the presence of two ORFs (Gould & Symonds, 1982). A putative role in cell-to-cell transport of CMV based on sequence and structural comparisons with the 3a protein of alfalfa mosaic virus (AlMV), has been suggested by Davies & Symons, (1988) and Melcher, (1990), for the 3a protein of CMV . A role of the apparently cognate AlMV 3a protein in cell-to-cell movement of AlMV was proposed on grounds of analysis of AlMV mutants

defective in transport, and on grounds of significant homology to the cauliflower mosaic virus (CaMV) transport protein encoded by CaMV gene I (Huisman *et al.*, 1986; Melcher, 1990).

The CP gene.

The CMV CP is only translated from RNA 4, a subgenomic messenger derived from RNA 3 (Gould & Symons, 1982). The untranslated leader sequences of CMV-O RNAs 3 & 4 contain a sequence ($UGUGUX_{21}UUGAGUCG$), that also is complementary to a sequence in the 18S rRNA of plant ribosomes (Hayakawa *et al.*, 1989).

While the 5'-leader sequences of CMV RNAs 1 & 2 are fairly homologous to each other, these are not homologous to the 5'-leader sequences of RNAs 3 & 4, except for the above mentioned region that is complementary to the 18S rRNA, which is also found in the 5'-leader sequences of RNAs 1 & 2 (Rezian *et al.*, 1985).

CPs sequenced in CMV strains from different subgroups were of the same size order, and although all had an M_r of approximately 25 kDa (Quemada *et al.*, 1989), the actual similarities between CMVs from subgroups I & II were only in the region of 80%, while similarity between members of the same subgroup was distinctly higher (>98%).

1.3.5 The expression of plant virus coat proteins in transgenic plants using *Agrobacterium tumefaciens*

1.3.5.1 Coat protein mediated resistance

Recent technological advances in plant genetic engineering have introduced novel approaches for the transfer of foreign genes into plants. Through such plant transformation, novel plant phenotypes can be produced. The underlying principles and methodology of *Agrobacterium*-mediated plant transformation are briefly discussed in Chapter 5.

A number of plant virus-derived nucleic acid sequences have been expressed in plants, and several of these were found to be useful for the development of virus-resistant plants. Genes expressed in transgenic plants to resist virus disease include virus RNA-specific ribozymes (Gerlach *et al.*, 1987) that cleave specific RNA sequences, viral satellite RNA that attenuate virus symptoms (Harrison *et al.*, 1987), and the virus coat protein gene (Van Den Elzen *et al.*,1989; Abel *et al.*, 1986; Nelson *et al.*, 1987; Beachy, 1990; Grumet, 1990; Kaniewski *et al.*, 1990; Quemada *et al.*, 1991; Loesch-Fries *et al.*, 1987).

The incorporation and expression of the viral capsid protein genes into plants has proved to be the most effective virus resistance strategy yet developed (Beachy *et al.*, 1990). Over the past five years, resistance to viruses in seven different virus groups has been achieved through this approach.

CP-mediated protection has many characteristics in common with the well known phenomenon of viral cross-protection. In viral cross-protection, plants inoculated with a mild strain of a particular virus are usually protected against secondary infections by a severe strain of the same virus. Such plants are usually not protected against inoculation by RNA of another strain.

CP-mediated engineered protection offers the same advantages as conventional cross-protection, and has the same inherent shortcoming in that the protection is easily overcome by RNA inoculation. It has, however, several important advantages over conventional cross-protection. Many viral pathogens have wide host ranges, and can vary in severity from one type of host to another. Thus, mild strain protected plants can act as a reservoir of virus that may be transmitted to other crops. Another danger is that mild strains can mutate to severe strains (Grumet, 1990). While the use of mild strains for protection is restricted by the host range of the mild strain, the expression of a viral CP gene in a non-host can sometimes confer effective broad spectrum protection against distinct viruses (in the same virus group), that are pathogens of the particular host (refer to 1.3.5.2).

1.3.5.2 Possible mechanisms of CP-mediated resistance

The precise mechanism through which CP-mediated protection is manifested is still unresolved. In support of the notion that the CP interferes with the release of RNA from virions, Register & Beachy (1988) have established that CP-mediated protection is attributable to an early event in the infection process. Their results, and the fact that expressed CPs do not protect against RNA inoculation, is consistent with the co-translational dissassembly hypothesis. According to this proposition, host ribosomes bind to the 5'end of the virion and actively uncoat the virus as translation proceeds (Grumet, 1990). A popular hypothesis is that the viral CP blocks the uncoating of virions by re-encapsidation (Register & Beachy, 1988), but other possible mechanisms cannot be ruled out. It was for instance reported that the no CP-mediated cross-protection was observed between the -TCM and -PBL strains of tobacco rattle virus (TRV - 'genus' tobravirus), in spite of the fact that CPs of these strains are able to encapsidate each other (Van Dun *et al.*, 1988).

There are, however, also examples of broad range engineered protection against different viruses in the same group. Expression of the CP gene of SoyMV in transgenic tobacco, is able to confer a high level of protection against PVY and TEV, which are distinct and serologically unrelated potyviral pathogens of tobacco, whereas SoyMV is unable to replicate in tobacco (Stark & Beachy, 1989). The CP of SoyMV shares sequence identities of only 58% and 61% with PVY and TEV respectively.

Thus it appears that at least in some cases, genetically engineered cross-protection does not mimic the classical cross-protection phenomenon observed between related strains (Shukla *et al.*, in press).

1.3.5.3 Manifestation of resistance

CP- mediated resistance is manifested through several measurable criteria. Nelson *et al.*, (1987) reported a 95-98% reduction of necrotic local lesions by TMV on tobacco protected with the TMV-CP gene. Other manifestations of CP-mediated resistance is the delay/prevention of systemic spread of virus in CP-protected plants, and reduced levels of virus accumulation in infected plant tissue (Nelson *et al.*, 1987). The abovementioned CP-mediated resistance can often be overcome by inoculating with higher concentrations of virus, but this is also dependent upon the levels at which the CP is expressed. It was found that homozygous transgenic lines expressing CP were more resistant than heterozygotes (Stark & Beachy, 1989). The use of weaker promotors upstream of expressed genes, also had an adverse effect on levels of resistance (Beachy, 1990).

CHAPTER 2

PRELIMINARY IDENTIFICATION AND BIOLOGICAL SEPARATION OF VIRUSES FROM DISEASED *PASSIFLORA*

SUMMARY

A preliminary survey of diseased *Passiflora* material collected from various regions in South Africa implicated a variable complex of three viruses. A diseased *P. caerulea* rootstock specimen from which the top graft *P. edulis* had died was selected from a woodiness diseased passionfruit vineyard and used as source for virus isolation and characterization. Electron microscopic studies showed that this host was infected with a mixture of three viruses: a filamentous virus, a spherical virus, and an icosahedral virus. By means of preliminary electroblot immunoassay (EBIA) and electron microscopic (EM) studies the spherical virus was identified as cucumber mosaic virus (CMV) and designated the CMV-Pass isolate. The filamentous virus was identified as a potyvirus and temporarily designated the Natal *P. caerulea* potyvirus pending further characterization. The icosahedral virus was identified as tobacco necrosis virus (TNV), but was excluded from further work. Initial attempts to purify these viruses directly from the *P. caerulea* host failed. Preliminary host range studies were undertaken to identify alternative hosts for the propagation and purification of the potyvirus and CMV-Pass. Differences in host reactions of these viruses were utilized to facilitate their biological separation and propagation.

CHAPTER 2

PRELIMINARY IDENTIFICATION AND BIOLOGICAL SEPARATION OF VIRUSES FROM WOODINESS DISEASED *PASSIFLORA*

CONTENTS

CHAPTER 2 PRELIMINARY IDENTIFICATION AND BIOLOGICAL SEPARATION OF VIRUSES FROM WOODINESS DISEASED *PASSIFLORA*

2.1 INTRODUCTION

This chapter describes biological properties and the preliminary identification of viruses from woodiness diseased South African *Passiflora*. The biological properties of the viruses were examined for the following reasons: to assess the symptoms induced by the viruses in single and mixed infections in *Passiflora* species; to identify alternative host plants that were suitable for virus propagation and large scale purification; and to separate the viruses from mixed infections. A strategy for the biological separation of two viruses originating from a single *Passiflora caerulea* host specimen (by differential host range propagation), is presented.

2.1.1 The history of woodiness disease

The woodiness disease of passionfruit was known to occur in New South Wales, Australia, as early as 1891, and was regarded as a serious threat to passionfruit growers by 1897 (Cobb, 1901). The designation of "woodiness" (referring to the economically important criterion of fruit appearance) was proposed by Cobb (1901). In 1928, when the occurrence of woodiness was still rare in Queensland, Australia, R.J. Noble (1928) demonstrated that woodiness disease of passionfruit was due to a sap transmissible virus. The virus subsequently spread rapidly, and by 1932, the commercial lifespan of Australian vines was reduced to two years (Simmonds, 1959).

Since then, the disease has been reported in Kenya (Nattrass, 1944); Nigeria (Martini, 1962); Brazil (Kitajima *et al.*, 1986; Chagas *et al.*, 1981; Colariccio *et al.*, 1987); Japan (Hirata & Kono, 1965); California (Teakle *et al.*, 1963); Italy (Quacquarelli & Martelli, 1969); Sri Lanka (Senanayake, 1972); Malaysia (Ong & Ting, 1973) and South Africa, (Da Graca, 1976).

The woodiness disease of passionfruit is attributed to single or dual infections by two different viruses: passionfruit woodiness virus (PWV) and cucumber mosaic virus (CMV) respectively (Taylor, 1959), although a number of viruses from other virus groups have also been isolated from *Passiflora*

worldwide (refer to Chapter 1 section 1.3.2). The "woody" fruit symptom appears to be a non-specific passionfruit host response to these viruses, and is of no diagnostic value for virus identification (Taylor & Kimble, 1964).

2.1.2 Symptomatology of woodiness diseased *Passiflora*

The symptoms of woodiness of *P. edulis* in South Africa were first described by Malan (1957). A potyvirus presumed to be PWV was isolated from South African woodiness diseased passionfruit (Da Graca, 1976; Da Graca, unpublished). The author reported that he was unable to detect CMV in South African *Passiflora* samples exhibiting woodiness symptoms.

Woodiness-associated symptoms elicited by PWV and CMV in Australian *Passiflora* range from barely discernible symptoms (mild strains) to severe stunting (severe strains). Leaves can show a range of symptoms, including yellow flecking, chlorotic mottle, vein clearing, leaf blistering, puckering or crinkling, and terminal bunchiness, whereas the woody fruit characteristic of the disease occurs throughout all developmental stages. Abnormal thickening of the rind leads to restricted pulp cavity, and bumpy or blistered fruit appearance. The symptoms are also influenced by temperature, and appear more pronounced in colder weather (Fitzell *et al.*,1985; Taylor & Kimble, 1964; Mcknight, 1953). Tip blight and dieback, the most severe symptoms associated with the disease, are associated with dual infections of PWV and CMV, suggesting a synergistic effect (Fitzell *et al.*,1985).

2.2 MATERIALS AND METHODS

2.2.1. Sampling and preservation of diseased *Passiflora* material

Infected Passiflora plants and vines were collected from various climatic regions of production in S.A. as presented in Table 2.1. A map indicating the geographical location of these areas is presented in Chapter 1 Fig. 1.1.

Where plants were not available, cuttings of the original diseased plants were rooted, potted in heat sterilized soil, and kept in plant rooms at day and night temperatures of 24 and 20°C respectively, with a 14-h photoperiod under VHO Gro-lux fluorescent lights and 70% relative humidity. Remaining leaves were desiccated in a vacuum over self-indicating silica gel and stored at 4°C.

Table 2.1 Diseased *Passiflora* specimens collected for virus survey.

Locality	Specimens
Natal Midlands, Nkwalini	*P. edulis*
	P. caerulea
Eastern Transvaal, Burgershall	*P. edulis*
	P. caerulea
	P. flavicarpa
Eastern Transvaal, Nelspruit	*P. edulis*
	P. caerulea
Central Transvaal, Vaalwater	*P. edulis*
	P. caerulea
Western Cape, Citrusdal	*P. edulis*
	P. caerulea
Western Cape, Franschhoek	*P. edulis*
	P. caerulea
	P. flavicarpa
Western Cape, Cape Town Nursery	*P. allardii*
Eastern Cape, Addo	*P. edulis*
	P. caerulea
	P. flavicarpa

2.2.2 Host range studies of viruses

Purification of viruses directly from *Passiflora* leaves (refer to Chapter 3, section 3.1.1), was unsuccessful. It was therefore essential to find alternative hosts suitable for virus propagation and purification. Plant species tested for susceptibility by mechanical sap-inoculation are presented in Table 2.2.

Table 2.2 Plant hosts evaluated for susceptibility to viruses from *Passiflora*

HOST	ORIGIN OF SEED
Nicotiana tabacum (cvs. Xanthi & Soulouk)	Dept. Microbiology, UCT*
N. benthamiana	Dept. Microbiology, UCT*
N. glutinosa	Dept. Microbiology, UCT*
N. clevelandii	Dept. Microbiology, UCT
Phaseolus vulgaris (cv. Bonus)	Virus-free seed donated by Mr. W. Smith
(cv. Bountiful)	G. Pietersen, PPRI
Nicandra physalloides	Seed from healthy plants
Cucurbita pepo (cv Long White Bush)	Commercial supplier (Agricol)
Vigna unguiculata	Grain Crops Research Institute
Cucumis sativus (cv. Rust Resistant)	Commercial supplier (Agricol)
Chenopodium quinoa	Dept. Microbiology, UCT
Zinea elegans	Commercial supplier (Kudu)
Petunia hybrida	Commercial supplier (Kudu)
Capsicum annuum (cv. California Wonder)	Commercial supplier (Kudu)
P. edulis	Seed from healthy (virus tested) plant
P. caerulea	Seed from healthy (virus tested) plant

*Non commercial seed were obtained from Prof. M. B. von Wechmar, Department of Microbiology, UCT.

2.2.3 Transmission by sap inoculation

Leaves of test plants (section 2.2.2) were inoculated with fresh leaf sap that was diluted 1/2 w/v in 0.05M sodium phosphate buffer pH 7, containing 0.02M mercaptoethanol and 0.02M diethyldithiocarbamate. Celite was used as an abrasive. Pure buffer or sap from healthy plants were included as negative controls.

2.2.4 Preliminary diagnosis of viruses

2.2.4.1 Electron microscopic (EM) analysis

Crude sap preparations of virus infected *Passiflora* were adsorbed onto 300 mesh carbon-coated copper grids and negatively stained with 2% uranyl acetate (pH 4.5) or 2% ammonium molybdate (pH 6.5) as described by Milne (1984). The grids were examined in a Philips EM 201C transmission electron microscope (G. Kasdorf, Plant Protection Research Institute, Pretoria (previously at PPRI, Stellenbosch).

2.2.4.2 Electroblot-immunoassay (EBIA)

EBIA of viral coat proteins separated by SDS-PAGE (Rybicki & Von Wechmar, 1982, Appendix B.2.1), was done as described in the Appendix B.1.5. Antisera used for initial identification of viruses were anti-PWV (Taiwan), anti-CMV-LupK5 and anti-PVY antisera respectively (Appendix B.1.6).

2.2.5 Viruses selected for further studies

Virus isolates from an infected *P. caerulea* rootstock from a severely diseased commercial passionfruit vineyard in the Natal midlands of which the *P. edulis* top graft had died (Table 2.1), were selected for further characterization and purification. The choice of material was based upon the fact that the mixed infections of *Passiflora* by CMV and a filamentous virus were detected mainly in diseased *Passiflora* vines from the Natal region, and the high concentration of virus particles observed by preliminary electron microscopic examination in this material. Virus diseased *P. edulis* and *P. caerulea* from this area showed very severe leaf symptoms and dieback of vines.

2.2.6 Separation of viruses from a mixed infection

Susceptibility of alternative hosts (section 2.2.2) to these viruses were evaluated. A strategy based on the host ranges of the different viruses was used for their separation and propagation.

2.2.6.1 Isolation and propagation of the filamentous viral component of woodiness diseased *Passiflora*

A filamentous virus (suspected to be a potyvirus) from infected *P. caerulea* from Natal (section 2.2.5) was propagated by sap inoculation from diseased *P. caerulea* plants onto *P. vulgaris* cv. Bonus seedlings as described in section 2.2.3. Four of the single chlorotic local lesions that appeared on inoculated primary leaves after seven to ten days were cut out using sterile blades. These were used individually as inoculation source for separate *P. vulgaris* cv. Bonus seedlings. Six such serial transfers were performed to obtain single isolates of the filamentous virus.

Dark brown necrotic local lesions were occasionally observed on the inoculated Bonus leaves. These were later shown to be caused by tobacco necrosis virus (TNV - Von Wechmar *et al.*, 1991; Von Wechmar, unpublished results).

2.2.6.2 Isolation and propagation of a spherical virus from woodiness diseased *Passiflora*

A spherical virus (suspected to be cucumber mosaic virus) from infected *P. caerulea* was propagated by sap inoculation onto *C. pepo* cv. Long White Bush and N. tabacum cv. Soulouk (common hosts for CMV, but non-susceptible to infection by the filamentous virus from Natal).

2.3 RESULTS

2.3.1 Symptomatology of infected South African *Passiflora*

Virus diseased *Passiflora* exhibited a variety of symptoms, such as leaf spots, mosaic, leaf blisters with mosaic, severe leaf wrinkle and malformation, overall die-back of vines and decreased yield of malformed "woody" fruit (Fig. 2.1a, e, f & g).

Plants infected with a combination of both the spherical and filamentous viral components showed more severe symptoms than plants only infected with the latter virus, which is in accordance with findings reported for Australian *Passiflora* infected with PWV and CMV (Pares & Martin, 1985; Taylor & Kimble, 1964). The effect of TNV on symptom expression in mixed infections has not been assessed.

2.3.2 Preliminary identification of viruses

Electron microscopic examination of crude sap extracts from woodiness diseased *Passiflora* from the various regions (Table 2.1) clearly showed the presence of filamentous particles. The dimensions of the particles resembled those of potyviruses, but they appeared aggregated in these crude extracts, thus preventing accurate particle size determinations (Fig. 2.2). The four filamentous virus local lesion isolates (section 2.2.6.1) reacted with anti-PWV (Taiwan) and anti-PVY antisera (Appendix B.1.6) in EBIA, and they had identical coat protein (CP) molecular weights (results not shown). One of these isolates was selected for further characterization.

The spherical virus exhibited the typical CMV "doughnut" morphology ie. with a dark staining core. This virus was recognized strongly by the CMV-LupK5 antiserum (Appendix B.1.6) in EBIA (Fig. 2.3a).

LEGENDS:

Fig. 2.1a Commercial vines of *Passiflora edulis* dying of woodiness disease

Fig. 2.1b Symptoms of *Nicotiana benthamiana* that was sap inoculated from a diseased *Passiflora caerulea* (Natal) source that was infected by a mixture of three viruses

Fig. 2.1c *Phaseolus vulgaris* cv Bonus used for serial local lesion transfers of a potyvirus from Natal *P. caerulea*. The holes in the leaf are sites where single local lesions were cut out for inoculation onto *N. benthamiana*.

Fig. 2.1d *N. benthamiana* sap-inoculated from *P. vulgaris* local lesions infected with pure Natal *P. caerulea* potyvirus isolate .

Fig. 2.1e Fruit from *P. edulis*. H = fruit from healthy, virus-free plants; D = fruit from *P. edulis* infected with potyvirus.

Fig. 2.1f Leaf symptoms of *P. edulis*. H = leaf from virus-free plant. D = leaf from plant infected with a mixture of potyvirus, CMV and TNV

Fig. 2.1g Leaf symptoms of *P. caerulea*. H = leaf from virus-free plant. D = leaf from plant infected with a mixture of potyvirus, CMV and TNV

Fig. 2.1h Leaf of *Nicotiana tabacum* cv. Xanthi infected by CMV-Pass.

Fig. 2.1i Leaf of *C. pepo* cv. Rust Resistant infected by CMV-Pass.

Fig. 2.1a

Fig. 2.1b

Fig. 2.1c

Fig. 2.1d

Fig. 2.1e

Fig. 2.1f

Fig. 2.1g

Fig. 2.1h

Fig. 2.1i

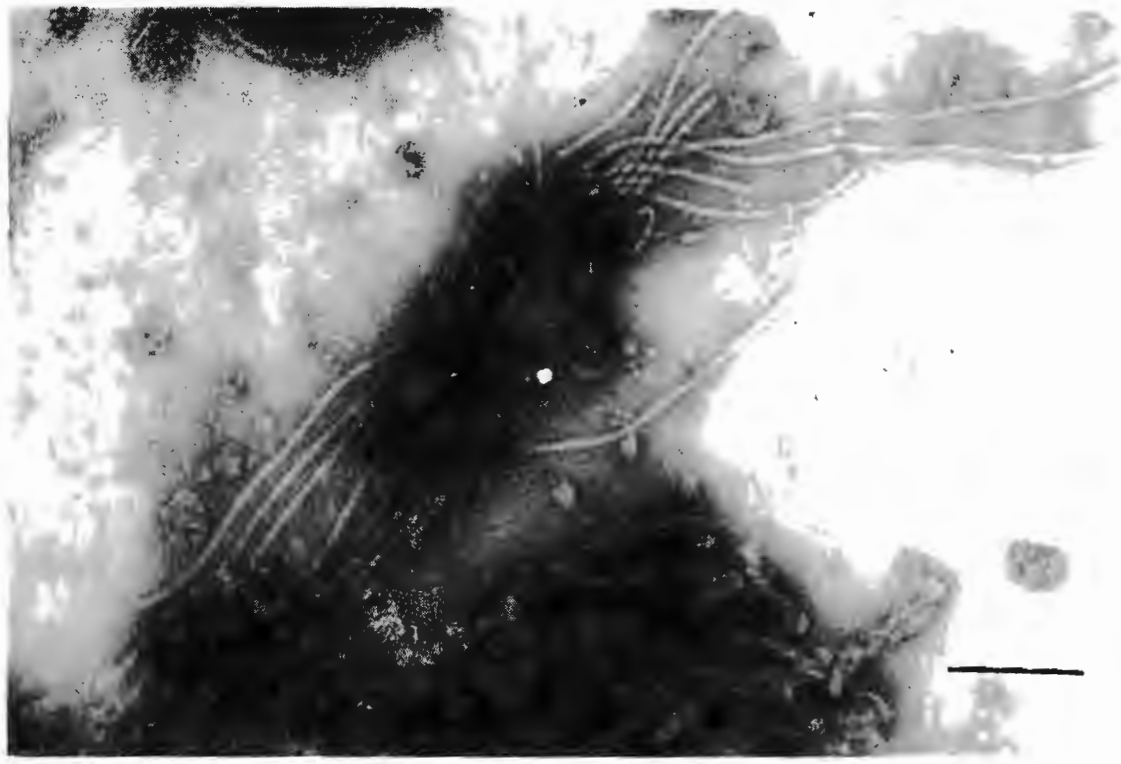


Fig. 2.2 Electron micrograph of aggregated filamentous particles present in crude sap of infected *P. caerulea*. The preparation was stained with 2% (w/v) uranyl acetate. The scale bar represents 100 nm.

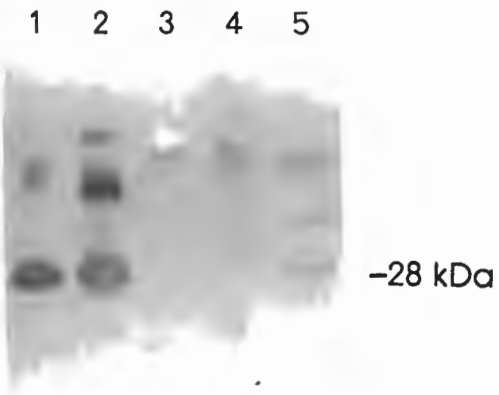


Fig. 2.3a EBIA of infected plant sap using CMV-LupK5 antiserum. **Lane 1,** *N. tabacum* cv. Xanthi inoculated from diseased *P. caerulea*. **Lane 2,** *N. tabacum* cv. Soulouk inoculated as above. **Lane 3,** Healthy *P. edulis* seedling. **Lane 5,** Diseased *P. caerulea* inoculation source

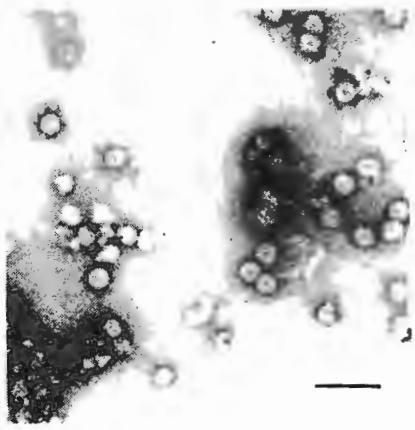


Fig. 2.3b Electron micrograph of spherical particles present in crude sap of *N. tabacum* cv. Soulouk infected by sap inoculation from *P. caerulea*. The preparation was stained with 2% (w/v) uranyl acetate. The scale bar represents 100 nm.

CMV was previously reported to occur at extremely low levels in woodiness diseased *Passiflora* (Taylor & Kimble, 1964). CMV particles were not observed in sap from infected *Passiflora*. However, inoculation onto young healthy herbaceous indicator plants such as *C. pepo* and *N. tabacum* cv. Xanthi or Soulouk, resulted in mosaic and mottle symptoms (Fig. 2.1h & i). Examination of crude sap extracts from these inoculated plants by EM and EBIA, confirmed the presence of CMV (Fig. 2.3a & b).

2.3.3 Sap transmission to alternative hosts

Results of sap transmission tests are presented in table 2.3.

Table 2.3 Results of sap transmission tests

Host	Symptoms	Type Particle	Test
Nicotiana tabacum (cvs. Xanthi & Soulouk)	Mosaic	S	EM
N. benthamiana	Systemic necrosis	S,F,H	EM
N. glutinosa	Mosaic	ND	-
N. clevelandii	Mottle	S	EM
Phaseolus vulgaris (cv. Bonus & Bountiful)	White/yellow local lesions on primary inoculated leaves	F	EM
	Brown local lesions (only sometimes)	H	EM
Nicandra physalloides	Mottle	ND	-
Cucurbita pepo (cv Long White Bush)	Mosaic	S	EM
Vigna unguiculata	Chlorotic lesions & systemic mosaic	F	EM
Cucumis sativus (cv. rust resistant)	Mosaic	ND	-
Chenopodium quinoa	Chlorotic mottle	ND	-
Zinea elegans	Mottle	ND	-
Petunia hybrida	None	-	-
Capsicum annuum (cv. California Wonder)	Mottle	ND	-
P. edulis	Mottle	ND	-
P. caerulea	Mosaic	ND	-

Abbreviations: EM - electron microscopy, S - spherical, F - filamentous, H - hexagonal, ND - not determined at this stage. The results of these sap transmissions were obtained later in the project (as presented in Chapter 3) when specific antisera and nucleic acid probes were available for testing. All other results were also verified in Chapter 3 (refer to Tables 3.2 and 3.3

To find more suitable hosts for separation and propagation of viruses from diseased *Passiflora*, a range of alternative hosts were infected by sap inoculation. The results of these initial transmission tests were scored visually

by observation of host symptom development, and by EM examination of negatively stained preparations.

During the early phase of the project, no specific antisera prepared against the local viruses from *Passiflora* were available. The unavailability of specific antisera prohibited the testing of all the inoculated material. Most of this material was tested only later when antisera and nucleic acid probes specifically against CMV and the filamentous virus were available (refer to Chapter 3).

The following results will be briefly highlighted here in view of their relevance in separation and purification of the viruses:

(a) sap transmission from infected *P. caerulea* directly onto *N. benthamiana*, led to a severe systemic necrotic and mosaic reaction (Fig. 2.1b). EM analysis of *N. benthamiana* sap, demonstrated high levels of CMV as well as potyvirus. A hexagonal virus identified as TNV was also observed in some instances (Von Wechmar *et al.*, 1991; M. B. von Wechmar, unpublished). This indicated that this host supports the replication of three viruses (Fig. 2.4).

(b) sap transmission from infected *P. caerulea* (section 2.2.5) onto *N. tabacum* (cvs. Xanthi & Soulouk) or *C. pepo*, resulted in a systemic mottle in all trials. CMV particles were present in these plants (Fig. 2.1h & i, Fig. 2.4).

(c) sap transmission from infected *P. caerulea* (section 2.2.5) onto *P. vulgaris* (cvs. Bonus and Bountiful) resulted mainly in chlorotic (white/yellow) local lesions on the inoculated primary leaves (Fig. 2.1c, Fig. 2.4). EM-analysis of these chlorotic lesions showed they were associated with the potyvirus. The virus seemed to spread systemically in primary leaves when plants grew older, but did not spread to the secondary growth. Occasionally dark brown local lesions were observed. These were found to be associated with TNV (Von Wechmar *et al.*, 1991; M. B. von Wechmar, unpublished).

2.3.4 Virus separation and propagation

The fact that *P. vulgaris* cv. Bonus was a local lesion host for the Natal *P. caerulea* potyvirus, facilitated the selection of single isolates of the potyvirus by cutting out single local lesions for repetitive serial transfers (Fig. 2.1c, Fig 2.4). Although TNV is also able to infect Bonus (Von Wechmar *et al.*, 1991; M. B. von Wechmar, unpublished), the local lesions induced by this virus were brown and necrotic, and could thus easily be distinguished from the white/yellow chlorotic lesions caused by the potyvirus, with the effect that TNV was eliminated in the first local lesion sap transmission cycle.

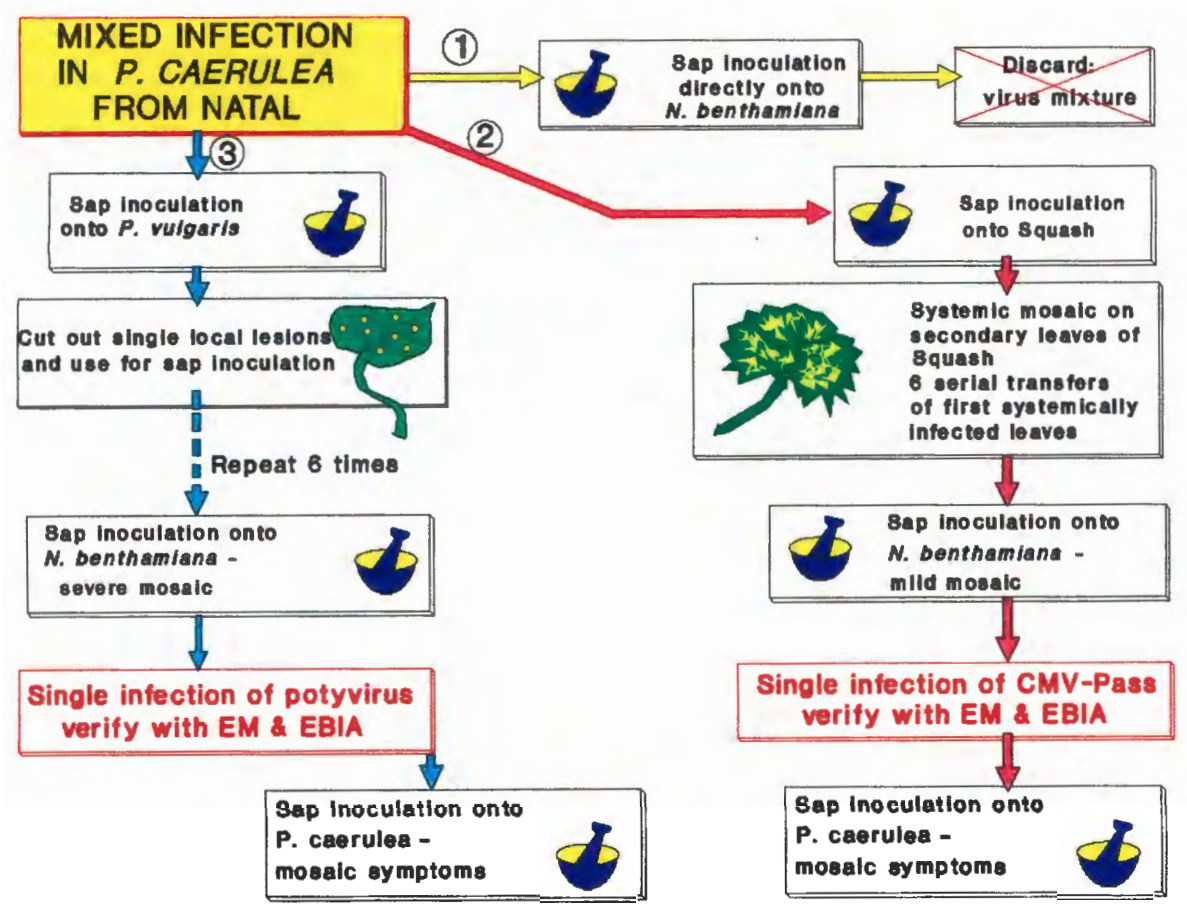


Fig. 2.4 Flow diagram of the use of differential hosts for the separation of viruses infecting passionfruit.

Direct inoculation from the original *P. caerulea* source onto *C. pepo* cv. Long White Bush or *N. tabacum* cv. Xanthi resulted in infection of secondary leaves with CMV-Pass, exhibiting clearly defined mosaic symptoms on the secondary leaves. (Fig. 2.1h & i, Fig 2.4).

N. benthamiana was found to be a very useful and convenient host for the propagation of either of the passionfruit potyvirus or CMV, following inoculation from their respective selective hosts used for separation (Fig. 2.1d, Fig. 2.4). Direct inoculation from *P. caerulea* onto *N. benthamiana*, however, resulted in mixed infections (Fig 2.4).

2.4 DISCUSSION

Symptomatology appeared to be a poor criterion for identifying viruses infecting *Passiflora*. When plants were in a vigorous growth phase, especially in the earlier stages of infection, symptoms could be masked or even be absent. Since the largest proportion of the South African passionfruit industry is still based on unselected seedling and graft material, diversity of host genetic background can also contribute to symptom response. It was observed that individual *P. edulis* and *P. caerulea* seedlings often showed different symptoms when infected from the same *Passiflora* virus source. Speculatively, the ratio of the viruses in the diseased plant could also contribute to altered host response.

Field samples collected from areas of production were in a poor condition, and even younger leaves of *P. edulis* plants appeared leathery and brittle, presumably due to a combination of virus infection and harsh weather conditions. *P. caerulea* samples from the Natal midlands region appeared to be actively growing in spite of very pronounced disease symptoms (the *P. edulis* top grafts of several vines from this region had died, but the *P. caerulea* rootstock continued to survive). From observations it appeared as if virus-infected *P. caerulea* survived for longer when compared with infected *P. edulis* under both field and greenhouse conditions.

The filamentous virus from Natal *P. caerulea* reacted to anti-PWV (Taiwan) polyclonal antiserum in EBIA, indicating that the filamentous virus was a potyvirus, but did not necessarily confirm that it is a strain/isolate of PWV, since it also reacted to anti-PVY antiserum. Potyvirus polyclonal antisera often have wide specificities due to antibodies directed to conserved epitopes in the core region of the potyviral coat protein (Shukla *et al.*, 1988b).

Although the potyvirus under investigation here is implicated in woodiness disease of passionfruit, it is in fact biologically distinct from other strains of PWV, in that it causes chlorotic local lesions on the inoculated primary

leaves of *P. vulgaris* cv. Bountiful and does not spread systemically to secondary leaves in this host. Infection of this host by Australasian PWV, however, is known to result in a systemic infection leading to severe blistering of secondary leaves (Taylor & Kimble, 1964). PWV has furthermore never been reported to infect *N. benthamiana*. Until further characterization studies have been done to verify the identity of this virus, it will be referred to in the subsequent text as the Natal *P. caerulea* potyvirus.

It is not unusual that the CMV particles were undetectable in crude sap extracts of *P. caerulea* by EM examination. This could be due to its renowned instability and low concentration in the woody *Passiflora* host (Taylor & Kimble, 1964). When transferred to herbaceous indicator hosts, however, CMV was readily discernible by EM examination.

The fact that *N. benthamiana* supported the accumulation of high levels of both CMV and the passionfruit potyvirus when sap infected directly from the diseased *P. caerulea* host, prompted the idea that this could be the ideal host for propagation of these viruses for subsequent purification, provided that they were biologically separated prior to propagation in this host.

P. vulgaris cv. Bonus was a useful host for obtaining single isolates of the potyvirus component through serial local lesion transfers. *C. pepo* and *N. tabacum* were susceptible to CMV-Pass infection, but did not allow replication of the poty component, and thus provided the ideal way of separating the two viruses from a mixed infection in *P. caerulea*, before further propagation in *N. benthamiana* for purification. In order to verify that the separated viruses were indeed derived from *Passiflora*, *P. vulgaris* cv. Bonus and *C. pepo* infected with the Natal *P. caerulea* potyvirus and CMV respectively, were used successfully as sap inoculation sources to infect healthy *P. caerulea* seedlings. The properties and possible role of the hexagonal particle identified as TNV (Von Wechmar *et al.*, 1991; M. B. von Wechmar, unpublished) that sometimes occurred, was not further investigated as part of this project.

CHAPTER 3

PURIFICATION, PHYSICOCHEMICAL CHARACTERIZATION, DETECTION AND ETIOLOGY OF VIRUSES FROM PASSIFLORA

SUMMARY

CMV-Pass and the Natal *P. caerulea* potyvirus were purified separately from *N. benthamiana* after their biological separation in selective hosts. These viruses were subjected to physicochemical and serological analysis, and biological studies. The coat protein molecular weights of CMV-Pass and the Natal *P. caerulea* potyvirus were estimated to be 28 kDa and 33 kDa respectively. The ssRNA M_r determined for the Natal *P. caerulea* potyvirus was approximately 2.92 mDa. Molecular weight estimates of CMV ssRNAs were: 1.29 mDa (RNA 1); 1.15 mDa (RNA 2); 0.81 mDa (RNA 3), and 0.35 mDa (RNA 4). Particle sizes were approximately 30 nm for CMV-Pass and 670 (± 42) nm long and 11 nm wide for the Natal *P. caerulea* potyvirus. Physicochemical studies and serology were able to confirm that the filamentous virus from infected *P. caerulea* from Natal was indeed a potyvirus, but these criteria were inadequate to distinguish the virus down to the virus/strain level. This virus isolate did, however, appear to be different from previously characterized strains of PWV in host reactions. The Natal *P. caerulea* potyvirus seemed to be more prevalent than CMV-Pass in commercial passionfruit crops. CMV-Pass appeared to preferentially and perhaps opportunistically infect *Passiflora* that was already infected with a potyvirus. Both CMV-Pass and the Natal *P. caerulea* potyvirus were found to be sap, aphid and graft transmissible, and seemed to act synergystically when present in a mixed infection to produce more severe symptoms.

CHAPTER 3

PURIFICATION, PHYSICOCHEMICAL CHARACTERIZATION, DETECTION AND ETIOLOGY OF VIRUSES FROM *PASSIFLORA*

CONTENTS

CHAPTER 3. PURIFICATION, PHYSICOCHEMICAL CHARACTERIZATION, DETECTION AND ETIOLOGY OF VIRUSES FROM *PASSIFLORA*

3.1. INTRODUCTION

This chapter covers the propagation, purification and physicochemical analysis of CMV and a potyvirus from woodiness diseased South African *Passiflora*. It also describes additional biological properties of these viruses, and strategies for their sensitive detection in crude sap extracts.

In order to understand the etiology and devise strategies for the control of woodiness disease, it was essential to gain information concerning the identity, host range, geographical distribution and transmission of viruses implicated in the disease in different *Passiflora* spp.

3.1.1 Problems with purification of viruses from *Passiflora*

Initial attempts to purify viruses from *Passiflora* spp. were unsuccessful: CMV from *Passiflora* (CMV-Pass) proved to be extremely unstable when purified from this host. Furthermore this virus often occurred at low concentration, and usually occurred as a co-infecting agent with the potyvirus from *Passiflora*. The potyvirus tended to aggregate irreversibly during attempted purification from *Passiflora* spp, and consequently very low yields of purified virus were obtained.

After separating these viruses by passage through selective host ranges (Chapter 2, section 2.3.4), the viruses were propagated separately in *N. benthamiana* for purification.

A strategy entailing the biological separation of the viruses through exploitation of their different host ranges, followed by their separate propagation in *N. benthamiana* for purification, was thus developed (as described in Chapter 2, Fig. 2.4.).

3.1.2 Detection of viruses

Traditional methods of virus indexing were often based on symptomatology, and relied on mechanical inoculations or grafting onto indicator hosts. Traditional serological approaches were variants of immunoprecipitin or gel/liquid agglutinations (Purcifull & Batchelor, 1977; Van

Regenmortel, 1966). All of these techniques have severe limitations, and have been superseded by contemporary, more sensitive detection techniques of controlled specificity, such as variants of amplified immunosorbent assays and nucleic acid hybridizations.

The most widely used immunological techniques used for the detection and diagnosis of virus infection today, include immunosorbent electron microscopy (ISEM), electroblot-immunoassay (EBIA), and several variants of the enzyme-linked immunosorbent assay (ELISA).

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and indirect ELISA are currently the two most widely used immunological methods for virus detection in plants (Koenig & Paul, 1982). The former tends to be much more strain-specific than the latter (Clark & Adams, 1977; Clark & Bar-Joseph, 1984; Rybicki & Von Wechmar, 1985). The outstanding features of these techniques are the high level of sensitivity, and the suitability for large scale use (Koenig, 1988).

A specific advantage of ISEM is that it is capable of detecting low concentrations of virus. It is also capable of revealing strain (antigenic) differences between viruses. Its main limitation is the fact that it requires expensive and sophisticated hardware, and is too laborious and expensive for screening large numbers of samples.

Electroblot immunoassay (EBIA) is a sensitive technique for the detection of viruses, as well as showing up size and immunological differences between proteins (Rybicki & Von Wechmar, 1982). Limitations for the differentiation of potyviral strains include the requirement for affinity-purified N-terminal-specific polyclonal or monoclonal antibodies (Shukla *et al.*, 1991; refer to Chapter 1, section 1.3.3.3). EBIA is also ill-suited for routine screening of large numbers of isolates: the technique entails the separation of denatured viral proteins by SDS-PAGE, followed by the electrophoretic transfer and immobilization onto a nitrocellulose membrane. Specific detection is facilitated by an enzyme-linked immunoassay, visualized indirectly by the precipitation of an insoluble pigmented product on the membrane. The small number of wells per gel, and the amount of labour involved, are the main factors restricting large scale use for routine diagnostic application. The level of conservation in the core domain of the potyviral CPs, increases the potential of EBIA using polyclonal serum (raised against the entire CP) for broad spectrum potyvirus detection. It is thus a more labour-intensive, but a more informative and reliable technique than ELISA, since antigens are compared on grounds of antigenic properties as well as molecular weight.

Nucleic acid hybridization is perhaps the most universally used non-serological method for the detection of plant viruses. Dot-blot hybridization is particularly suitable for sensitive detection of viruses on a large scale. Recent advances in non-radioactive methods for labelling nucleic acid probes made this

a feasible method for routine large scale indexing of plant material (Lo *et al.*, 1988; Hopp *et al.*, 1988; Forster *et al.*, 1985). Due to the degeneracy of the genetic code, genes that are conserved at the amino acid level often show little conservation at the nucleic acid level (refer to Chapter 1, section 1.3.3.3). Nucleic acid hybridizations are therefore often strain-specific and generally unsuitable for broad spectrum detection of potyviruses (Ward & Shukla, 1991).

3.1.3 Control of passionfruit viruses

Whilst CMV is known for its exceptionally wide host range (Chapter 1, section 1.3.4.4), PWV has been reported to infect 44 dicotyledonous plant species in five families. Hosts include 10 species of *Passiflora* and 18 species in *Leguminosae*. Both viruses were shown to be graft, sap and aphid transmissible (Taylor & Kimble, 1964; Mcknight, 1953).

Several different strategies have been implemented in attempts to protect passionfruit crops against woodiness disease. The selection and pre-inoculation of passionfruit with mild strains of PWV (Simmonds, 1959) has yielded short term protection against severe strains, but this tactic failed in the long term, as the mild strain protection was overcome by severe strain infection (Peasley, 1981).

Current strategies for short to medium term relief (in the Australian passionfruit growers industry) are based on the supply of virus-free plant material to the industry. Although the viruses are not seed transmitted, the best Australian cultivar selection for commercial production is an F1 hybrid. Virus elimination via meristem culture has been achieved with *P. caerulea* rootstock, and represents a feasible method of eradication of PWV from infected rootstock (Hakkaart & Versluys, 1981). The costly approach of establishing vineyards of virus-free plants, requires an educated body of growers, that adapt their vineyard practices to avoid possible mechanical transmission of viruses between individual plants, and act quickly to eradicate suspicious looking *Passiflora* specimens from their plantations.

The biological properties of viruses associated with woodiness disease of South African *Passiflora* were investigated to decide on strategies for crop protection. It was decided that the supply of virus-tested plant material to the SA industry was a short term priority, and the creation of genetically engineered crop resistance, a longer term goal. The successful supply of virus-tested plant material to the grower industry is obviously dependent upon reliable detection methods for screening against known viruses.

3.2 MATERIALS AND METHODS

3.2.1 Virus propagation and purification

3.2.1.1 Propagation and purification of the Natal *P. caerulea* potyvirus

Primary leaves with local lesions of *P. vulgaris* cv. Bonus (see Chapter 2) were used as a pure source of potyvirus for the subsequent inoculation of *N. benthamiana*, a host permitting systemic propagation of large quantities of the potyvirus (Fig. 2.4). 200 g of *N. benthamiana* infected with the Natal *P. caerulea* potyvirus was harvested 10 days post inoculation, and homogenized in 600 ml of 0.2 M sodium phosphate buffer pH 7.5 containing 0.1% thioglycollic acid in a Sorvall blender. The homogenate was expressed through a double layer of cheesecloth and centrifuged at 16 000 g for 10 min (low speed cycle) in a Sorvall GSA rotor. Polyethylene glycol MW 6000, NaCl, and Triton-X-100 were added to the supernatant to final concentrations of 6% w/v, 2% w/v, and 1% v/v respectively. The precipitate, collected by low speed centrifugation, was resuspended in 60 ml of 0.05 M Tris-HCl pH 8.8, clarified at low speed, and the supernatant layered on a 25% sucrose cushion for ultracentrifugation at 105 000 g for 90 min in a Beckman type 35 rotor. The resulting pellet was allowed to resuspend for 12 h in 0.05 M Tris-HCl pH 8.8. The virus was further purified by rate zonal centrifugation in a 10-40% (w/v) continuous sucrose density gradient in 0.05 M Tris-HCl pH 8.8 at 115 000 g for 150 min in a Beckman SW28 rotor, followed by fractionation on an ISCO density gradient fractionator with UA-5 UV absorption monitor and 254 nm filter. Fractions were diluted 1:4 with cold 0.05 M Tris-HCl pH 8.8 and precipitated by ultracentrifugation. Final pellets were resuspended in this buffer and kept at 4°C.

3.2.1.2 Propagation and purification of CMV-Pass from *P. caerulea*

CMV-Pass was sap transmitted from *C. pepo* to *N. benthamiana* to propagate the virus for purification (Fig. 2.4). *N. benthamiana* leaves (200g) infected with CMV-Pass were harvested seven days post inoculation, and extracted as described in section 3.2.1.1, with the following modifications: PEG, NaCl, and Triton-X-100 were added to the supernatant to final concentrations of 8.5% w/v, 2.5% w/v, and 2% v/v respectively. After centrifugation the pellet was resuspended in 60 ml of 0.1 M Tris-HCl pH 8.8, clarified, and the supernatant was then centrifuged in a Beckman type 35 rotor at 140 000 g for 90 min. The resulting pellet was resuspended in the same buffer. CMV-Pass was

subsequently further purified by sucrose density gradient centrifugation, as described in section 3.2.1.1.

3.2.2 Physicochemical characterization of viruses

3.2.2.1 Electron microscopic analysis

Transmission electron microscopic analysis was performed as described in section 2.2.4.1., using sap from infected plants or purified virus preparations. Particle size measurements were made from electron micrographs of purified virus preparations on a Summagraphics tablet linked to a Tektronix 4051 microcomputer (Dept. of Biochemistry, UCT). Freshly isolated tobacco mosaic virus was used as a standard for calibration.

3.2.2.2 SDS-PAGE analysis of viral coat proteins

Molecular weight size estimates of the coat proteins from CMV-Pass and the aforementioned potyvirus, were done by discontinuous sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Appendix B.2.1.

3.2.2.3 Purification of single and double stranded RNA

Single stranded RNA

For all ssRNA manipulations, precautionary measures were taken to minimize or avoid degradation by RNases. Glassware was baked for at least 5 hours at 200°C. All Eppendorf tubes and micropipette tips were treated with 0.1% diethylpyrocarbonate (DEPC) for 16 h at 37°C before being autoclaved. Furthermore only analytical grade chemicals and sterile double distilled water that was DEPC treated prior to autoclaving, was used. Gloves were worn throughout all extraction procedures.

Single stranded RNA was extracted from sucrose gradient purified virus preparations by a protocol based on the method reported by Gallitelli *et al.* (1985). Preparations of the Natal *P. caerulea* potyvirus and CMV-Pass were heated at 60°C for 5 min in the presence of 10 mM Tris-Cl pH 8.25; 1% (w/v) SDS; 1mM EDTA. The samples were then extracted with phenol/chloroform and ethanol-precipitated as described in Appendix B.3.3. Finally, samples were resuspended in sterile double distilled water, and immediately used for further manipulations, or stored frozen at -70°C. Before use, the concentration was estimated in a 50 µl micro cuvette by measuring the uv light absorbtion at 260 nm, with an OD of 1 corresponding to a concentration of 25 µg/ml.

Double stranded RNA

The purification of dsRNA (Dodds *et al.*, 1984) was done only for CMV-Pass, as yields obtained with the potyvirus were too low. After purification, CMV-Pass dsRNA was compared to dsRNA profiles of other CMV strains/isolates: CMV-Y and CMV-LupK5 which were available from other projects (refer to Appendix B.3.8.5 for detailed methodology - results not shown).

3.2.2.4 RNA molecular weight estimation

The apparent molecular weights of genomic ssRNA of the potyvirus and CMV-Pass from Natal *P. caerulea* were estimated by electrophoresis under denaturing conditions in agarose gels essentially as described by Maniatis *et al.*, (1982).

Purified ssRNA was denatured by heating at 60°C for 10 min in the presence of electrophoresis buffer (20 mM MOPS; 5 mM NaOAc; 1 mM EDTA), containing 50% (v/v) deionized formamide and 6% (v/v) formaldehyde. Denaturing agarose gels were prepared by melting agarose (1% w/v) in electrophoresis buffer, and adding formaldehyde to 6% (v/w) in a fume hood after cooling to 60°C. The sample loading buffer consisted of 50% (v/v) glycerol; 0.4% (w/v) bromophenol blue; 0.4% (w/v) xylene cyanol; and 1 mM EDTA and 100 µg/ml ethidium bromide. Large size gels (20x15x0.7 cm) were run at 50 V for 16 h. RNA was visualized on a 260 nm uv transilluminator and photographed using monochrome Polaroid film. Sizes of viral ssRNA were estimated graphically by reference to RNA molecular weight markers ("RNA ladder" obtained from Bethesda Research Laboratories). Sizes of RNA molecular weight markers used were 9.5; 7.5; 4.4; 2.4; 1.4; and 0.3 Kb respectively.

3.2.2.5 Binding of the Natal *P. caerulea* potyvirus ssRNA to Hybond-mAP

Purified ssRNA from the Natal *P. caerulea* potyvirus was bound to Hybond-mAP (Amersham, UK) as recommended by the manufacturer: sterile Hybond-mAP was pre-wetted with 0.5 M NaCl and allowed to air dry. Heat denatured ssRNA was chilled on ice and NaCl was added to a concentration of 0.5 M. The RNA was then slowly spotted onto the Hybond-mAP. After two washes with 0.5 M NaCl, the paper was washed with 70% analytical quality ethanol, to wash off any unbound RNA. The paper was subsequently air dried, and poly(A)-RNA was eluted off the paper with water at 70°C for 5 min.

3.2.3 Virus characterization, detection and relationship studies

3.2.3.1 Preparation of plant sap for virus detection and diagnosis.

Sap extracts of plant material (0.5 g/ml) for indexing with serological methods were prepared by homogenizing plant tissue directly in Eppendorf vials with the aid of a glass rod, in the presence of 10 mM phosphate, 150 mM NaCl pH7.4, (PBS buffer), and a small amount of carborundum as an abrasive. Solid material was pelleted by low speed centrifugation in a micro centrifuge, before using the supernatant in immunological tests. The supernatant fluid was used undiluted in DAS-ELISA, and a series of ten-fold dilutions were made for ISEM analysis (section 3.2.3.2). For EBIA analysis, samples were disrupted before SDS-PAGE (Appendix B.2.1).

For hybridization analysis, total plant nucleic acids were extracted by homogenizing approximately 1 g of infected leaf tissue with a mortar and pestle in 3 ml STE buffer (0.1M NaCl, 0.01M Tris-HCl, 1mM EDTA), containing 1% SDS. 600 µl of the homogenate was transferred to an Eppendorf vial, and extracted once with 300 µl of phenol containing 0.8% w/v 8-hydroxyquinoline and 300 µl of a chloroform/isoamylalcohol (24:1 v/v) mixture. After vortexing, the mixture was placed in a 60°C water bath for 5 min and centrifuged in an Eppendorf benchtop centrifuge for 10 min. The aqueous phase was transferred to a fresh Eppendorf tube and precipitated on ice for 2 h by addition of 4% v/v 3 M sodium acetate pH 5.5 and two volumes of ethanol. The total nucleic acids were precipitated by centrifugation, dried under vacuum and resuspended in 100 µl STE buffer.

3.2.3.2 Immunological methods

Antibody preparation

Due to problems with virus aggregation and instability experienced with virus purified from *Passiflora*, all purified virus used as antigens for rabbit immunization were propagated in *N. benthamiana* (refer to section 3.2.1).

Polyclonal antisera were prepared as described by Rybicki & Von Wechmar (1985). Before use in IEB tests or DAS-ELISA, the sera were host absorbed three times with sap from uninfected *N. benthamiana* (Rybicki & Von Wechmar, 1985). The IgG fractions of the sera were purified by two cycles of precipitation with 4 M ammonium sulfate followed by anion exhange chromatography using DE-52 cellulose (Whatman), and subsequently conjugated to calf intestine alkaline phosphatase for use in DAS-ELISA as described by Clark & Bar-Joseph (1984). Methodology of these steps is described in the Appendix (B.1.1-1.3).

Antisera against other viruses were obtained from sources listed in the Appendix B.1.6.

Immunosorbent electron microscopy (ISEM)

Grids for transmission electron microscopy were prepared as described in Chapter 2 section 2.2.4.1. ISEM (Milne & Lesemann, 1984) was performed by adsorbing different dilutions (1/10, 1/100, 1/1000) of specific polyclonal antiserum onto grids for 5 min at room temperature. After washing grids by rinsing with 20 droplets of 0.01 M phosphate buffer pH 7.0, they were floated upside down on droplets of diluted virus or plant sap for 60 min at room temperature. After another wash, virus particles bound to antibodies ("trapped") on the grid were "decorated" by floating grids (for 30 min at room temperature) on droplets of 1/1000 anti-virus antisera. Grids were washed again and negatively stained with uranyl acetate, before being subjected to transmission electron microscopic examination (Chapter 2 section 2.2.4.1).

Enzyme-linked immunosorbent assay (ELISA)

DAS-ELISA (Appendix B.1.4) performed by a modification of the method described by Clark & Bar-Joseph (1984), was employed for indexing plants for the presence of the Natal *P. caerulea* potyvirus. The test was optimized by calibrating as follows: a dilution series of coating anti-virus IgG and anti-virus IgG alkaline phosphatase conjugate to yield the most sensitive level of virus detection whilst minimizing background, was used. Purified virus and virus-free plant sap were included as controls. Dilutions of 1/500 each of anti-virus IgG and anti-virus IgG alkaline phosphatase conjugate produced good results. Anti-virus IgG was prepared from the first weekly bleed after the second booster (Appendix B.1.1).

Electroblot-immunoassay (EBIA)

EBIA of viral coat proteins (Rybicki & Von Wechmar, 1982) separated on the basis of molecular weight by SDS-PAGE (Appendix B.2.1), was done as described in Appendix (B.1.5). This technique was used for detection of virus in plant sap, as well as for limited relationship studies between potyviruses. For relationship studies, disrupted Natal *P. caerulea* potyvirus was loaded into each well of a SDS-polyacrylamide gel. After electrophoresis and electroblotting the nitrocellulose filter was cut into vertical strips, each strip corresponding to a lane on the gel. Individual strips were each incubated in a 1/100 dilution of antiserum directed against a different potyvirus (Refer to Appendix B.1.6 for list of antisera and their respective sources). Strips were then incubated with GAR-IgG alkaline phosphatase conjugate and developed by addition of enzyme substrate as described in Appendix B.1.5.

3.2.3.3 Nucleic acid hybridizations

This was done with ^{32}P-dCTP nick translated (Appendix B.3.8.1) pW9, a 3'-terminal Natal *P. caerulea* potyvirus clone encompassing the entire CP region (refer to Chapter 4, section 4.2.2). CMV-Pass dsRNA (see Appendix B.3.8) was 5'-endlabelled with γ-dATP, using polynucleotide kinase as described in the Appendix B.3.9.3, to serve as a probe for detection of CMV-Pass in plant tissue (Appendix B.3.11 & B.3.12). Total nucleic acid extracts of plant tissue (see section 3.2.3.1), were dot-blotted onto nylon membranes (Hybond-N, Amersham, UK), using a "Minifold" apparatus (Hoefer Scientific) coupled to a water tap vacuum pump. Radioactive isotope labelled probes were hybridized to total denatured nucleic acids bound to these filters as described in Appendix B.3.11. Positive signals on filters were visualized by autoradiography (Appendix B.3.12).

3.2.4 Further transmission studies

3.2.4.1 Origin and maintenance of insect vectors

Aphid transmission studies were performed with aphids of the species *Myzus persicae* (Sulz) and *Aulacorthum solani* (Kalt). *Myzus persicae* was reared from a colony maintained by M.B. von Wechmar, whereas *Aulacorthum solani* was acquired from a citrus tree in the western Cape. Especially in the eastern Transvaal and eastern and western Cape areas, passionfruit is commercially produced in the proximity of citrus plantations. It was hypothesized that aphids found on citrus could be involved in the transmission of passionfruit viruses even if they did not colonize passionfruit vines by choice. Aphids are rarely seen to colonize passionfruit vines by choice, but probably breed on weeds and other horticultural plants in the proximity of plantations. It is possible, however, that they could still act as effective non-persistent vectors of *Passiflora* viruses by a series of quick probings of *Passiflora* hosts, in search of a favored host (Raccah, 1986). Both species were maintained in gauze-covered wooden cages on virus-free, potted *N. benthamiana* kept under VHO Gro-lux fluorescent lights. The cages were kept at 24-26°C on a 12 h day/night cycle.

3.2.4.2 Aphid transmission

Aphids were starved at 4°C for 2-3 h before they were allowed an acquisition period of 5-15 min on *N. benthamiana* and *C. pepo* plants, separately infected with the potyvirus and CMV-Pass respectively. They were subsequently transferred to tested virus-free *P. caerulea* and *P. edulis* seedlings, as well as a range of virus free alternative hosts (Table 2.2), and allowed to feed for 5 h (*Passiflora* species used as receptor hosts were originally grown from seed, screened for viruses by EM and EBIA, and subsequently propagated from rooted

cuttings as tested virus-free clonal material). Aphids were then exterminated by spraying with a systemic insecticide (oxydemeton-methyl). Acquisition feeding on virus-free *P. edulis* was included as a negative control. Plants were then observed for symptom development and indexed by several methods for the presence of virus (Table 3.1 & 3.2).

3.2.4.3 Graft transmission

Cuttings from healthy *P. edulis* plants were wedge-grafted onto infected *P. edulis* and *P. caerulea* rootstocks. Reciprocal grafts of diseased *P. edulis* vines onto healthy *P. caerulea* rootstocks, were also performed. Graft sites were covered with elastic plastic strips, which were removed after three months.

3.2.4.4 Seed transmission studies

Eighty seedlings originating from fruit of virus-infected plants were observed over a period of several months for symptom development. Plantlets exhibiting physiological abnormalities were screened by dot-blot hybridization analysis and EBIA or DAS-ELISA for the presence of CMV-Pass or the Natal *P. caerulea* potyvirus.

3.3 RESULTS

3.3.1 Virus purification

P. vulgaris and *C. pepo* were susceptible to the Natal *P. caerulea* potyvirus and CMV-Pass respectively, but neither of these hosts were optimal for purification of these viruses. These hosts were used for the separation of the Natal *P. caerulea* potyvirus and CMV-Pass and then served as separate source inocula for sap transmission to *N. benthamiana* . *N. benthamiana* was found to be an excellent host for virus purification. Yields of both viruses varied from 4-6 mg per 200g of infected *N. benthamiana* leaf material. CMV, renowned for its instability, retained its stability and infectivity for three weeks when purified from this host. Teakle *et al.*, (1963) reported a higher than usual longevity *in vitro* for CMV isolated from Australian *Passiflora*. The enhanced stability of CMV-Pass was observed only when purified from *N. benthamiana*, and not when purified from *N. tabacum* cv. Xanthi by the same method. Electron microscopic examination of the potyvirus purified from *N. benthamiana* showed very little aggregation and particle breakage.

3.3.2 Physicochemical characterization

3.3.2.1 Electron microscopic analysis

The Natal *P. caerulea* potyvirus appeared as flexuous filamentous particles approximately 11 nm wide and 670 (±42) nm modal length in negatively stained preparations of purified virus (Fig. 3.1a & b). Particle size was similar to the modal length of 670 nm previously reported for Australian isolates of PWV (Taylor & Kimble, 1964; Teakle & Wildermuth, 1967).

Negatively stained preparations of purified CMV-Pass exhibited large numbers of isometric virus particles about 30 nm in diameter with a darker stained central core (Fig. 3.1c). The virus particle size compared well with the size of 30 nm estimated previously for CMV (Kaper & Waterworth, 1981).

3.3.2.2 Size estimation of viral coat proteins and RNA genome size

CMV-Pass

The capsid protein M_r of CMV-Pass was estimated to be approximately 28±0.3 kDa by SDS-PAGE (four determinations, Fig. 3.2). Double stranded RNA extracted from infected *N. benthamiana* as well as ssRNA extracted from the purified virus, showed a 4-band pattern typical for CMV. Sizes of the CMV-Pass-ssRNAs estimated from five separate formaldehyde denaturing gels, were: 1.29 mDa (RNA1); 1.15 mDa (RNA2); 0.816 mDa (RNA3), and 0.356 mDa (RNA4) as shown in Fig. 3.3. The M_r of 28kDa for the capsid protein of CMV-Pass is larger than capsid protein estimations of other strains of CMV (24.5 kDa - Kaper & Waterworth, 1981; Chalam, 1986). RNA sizes, however, correspond well with sizes estimated for other strains of CMV (Francki *et al.*, 1985).

Potyviruses

Potyvirus isolates from most South African *Passiflora* specimens had capsid protein molecular weights of approximately 32-33kDa in SDS-PAGE and EBIA (Figs. 3.2 and 3.4a). This size seems to be similar to previous findings for Australian strains of PWV (Shukla *et al.*, 1988b). Often spurious bands were observed in the 30 - 32 kDa range. This is presumed to be due to degradative proteolysis of the N-terminal portion of the capsid protein, which is not unusual for PWV and some other potyviruses (Shukla *et al.*, 1988b). EBIA results showed that diseased *Passiflora* specimens from the eastern Transvaal region were mainly infected with potyviruses with a slightly smaller capsid protein molecular weight than specimens from either Natal midlands or the western Cape (Fig 3.4a & b).

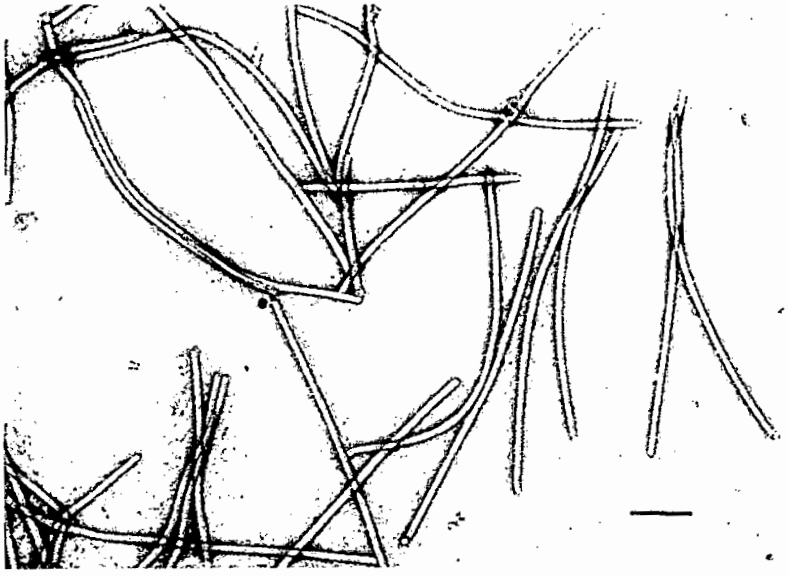


Fig. 3.1a Electron micrograph of a purified preparation of potyvirus from Natal *P. caerulea* stained with 2% (w/v) uranyl acetate. Scale bar represents 100 nm.

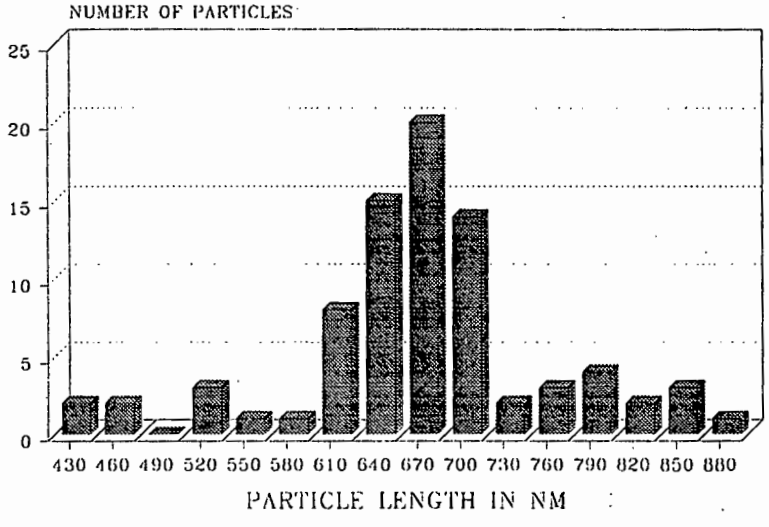


Fig. 3.1b Particle length distribution in a purified preparation of a Natal *P. caerulea* potyvirus stained with 2% (w/v) uranyl acetate. The mean modal length was estimated as 670 ±42 nm.

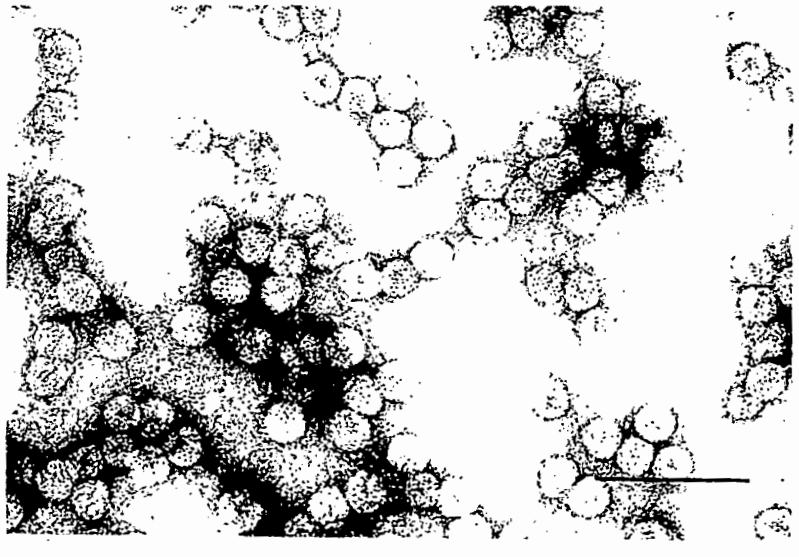


Fig. 3.1c Electron micrograph of a purified preparation of CMV-Pass stained with 2% (w/v) uranyl acetate. Scale bar represents 100 nm.

LEGENDS:

Fig. 3.2 SDS-PAGE of viral capsid proteins. **Lane 1**, CMV-Pass (28 kDa). **Lane 2**, 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), and α-lactalbumin (14.4 kDa). **Lane 3**, Natal *P. caerulea* potyvirus (33 kDa).

Fig. 3.3 Size estimation of ssRNA from CMV-Pass by gel electrophoresis in 1% agarose containing 6% formaldehyde. **Lane 1**, TMV ssRNA. **Lane 2**, "RNA ladder" - ssRNA molecular weight markers obtained from Bethesda Research Laboratories. **Lane 3**, CMV-Pass ssRNA.

Fig. 3.4a & b EBIA for the detection of potyviral CP in infected plant sap using antiserum directed against the Natal *P. caerulea* potyvirus.

Fig. 3.4a **Lane 1**, diseased *P. allardii* from Western Cape Province (W.P.). - infected with a potyvirus. **Lane 2**, potyvirus infected *P. edulis* from Burgershall in the Eastern Transvaal (E.T.). **Lane 3**, potyvirus infected *P. caerulea* specimen from Nelspruit in the E.T. **Lane 4**, potyvirus infected *P. caerulea* specimen from Burgershall (E.T.). **Lane 5**, uninfected *P. edulis* seedling. **Lane 6**, potyvirus infected *P. edulis* from a nursery in the W.P. **Lane 7**, potyvirus infected *P. flavicarpa* from Burgershall (E.T.). **Lane 8**, potyvirus infected *P. caerulea* from Natal midlands. **Lane 9**, purified Natal *P. caerulea* potyvirus .

Fig. 3.4b **Lane 1**, *N. benthamiana*, sap inoculated from *P. edulis* infected with Natal *P. caerulea* potyvirus . **Lane 2**, *P. edulis* seedling, infected by aphid feeding on diseased *P. allardii*. **Lane 3**, *P. edulis* seedling, sap-inoculated from potyvirus infected *P. edulis* from Burgershall (E.T.). **Lane 4**, *P. edulis*, sap inoculated from Natal *P. caerulea* potyvirus infected *N. benthamiana*. **Lane 5**, healthy *P. edulis* seedling. **Lane 6**, *N. benthamiana* infected by aphid feeding on infected Natal *P. caerulea*. **Lane 7**, *P. caerulea* seedling infected by aphid feeding on infected *P. caerulea* from Natal. **Lane 8**, purified CMV-Pass. **Lane 9**, purified Natal *P. caerulea* potyvirus .

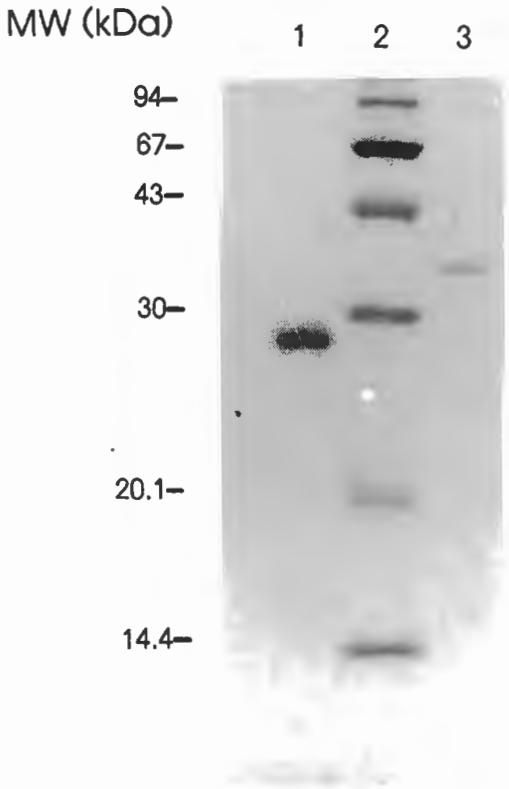


Fig. 3.2

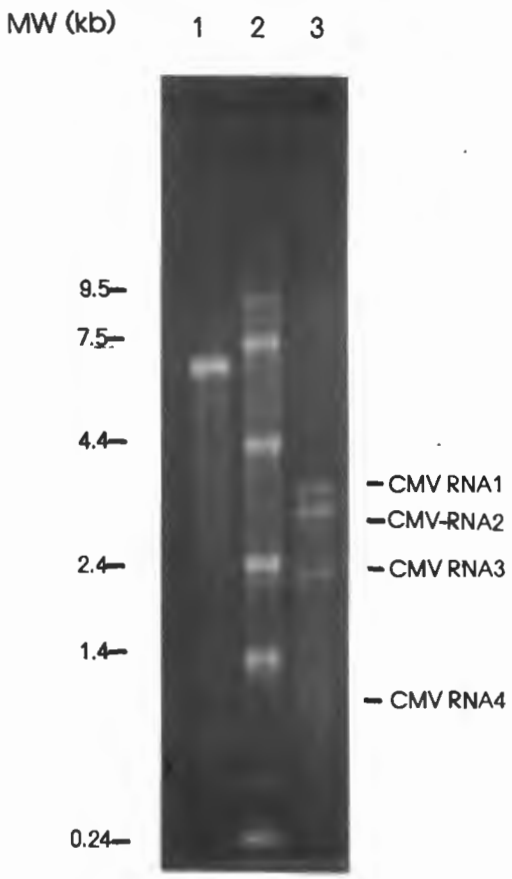


Fig. 3.3

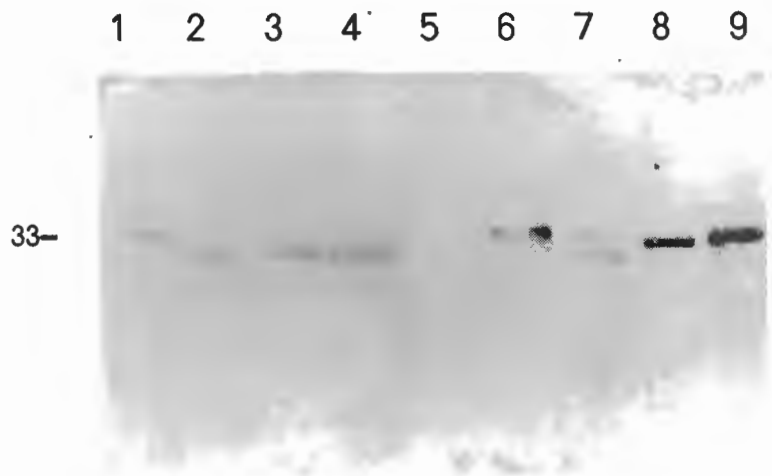


Fig. 3.4a

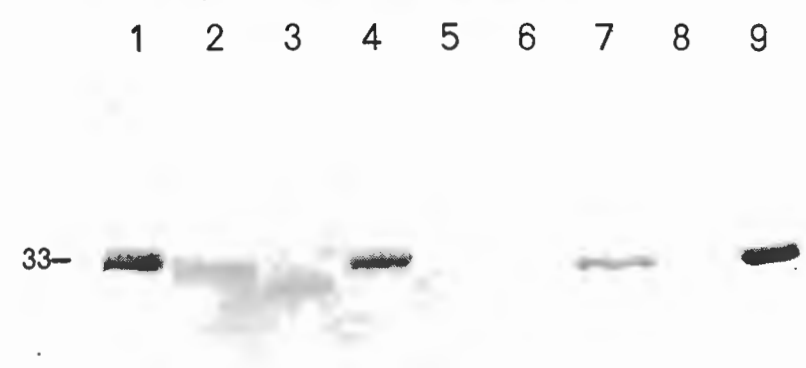


Fig. 3.4b

The M_r of ssRNA from the Natal *P. caerulea* potyvirus was estimated as 2.92±0.08 mDa (8.6 kb) from five separate formaldehyde denaturing agarose gels (Fig. 3.5). The estimated RNA size is in accordance with previous findings for members of the potyvirus group, i.e., 2.9 X 10^6 mDa, 3.07 X 10^6 mDa, 3.04 X 10^6 mDa and 2.90 X 10^6 mDa for OMV (Burger & Von Wechmar, 1989) turnip mosaic virus (TuMV), TEV and MDMV respectively (Hill & Brenner, 1976). The affinity of the ssRNA of this virus for Hybond-mAP indicated that its ssRNA is 3'-polyadenylated, which is a common trait of potyviral RNA, first described by Hari *et al.*, (1979).

3.3.3 Virus diagnosis, detection and serological relationships

3.3.3.1 Electron microscopic detection of viruses in infected material

Flexuous filamentous particles, resembling potyviruses in appearance, were observed in leaf extracts of different diseased appearing *Passiflora* specimens collected from the major areas of commercial production (Table 2.1). Sometimes quite high concentrations of these viruses were present in aggregated bundles in leafdip preparations (Chapter 2, Fig. 2.2).

CMV-Pass, when present, occurred as "doughnut-like" spherical particles in infected plant tissue. As pointed out previously, it was difficult to observe CMV-Pass in sap extracts of *Passiflora* due to the instability and low concentrations of the virus in sap of this host. The presence of CMV-Pass was, however, easily detected in sap of infected herbaceous hosts such as *N. tabacum* or *N. benthamiana*.

A third, isometric virus-like particle was sometimes observed in mixed infections of diseased *Passiflora* (Fig. 3.6). This particle was later identified as tobacco necrosis virus (TNV) by Von Wechmar *et al.* (1991). Further characterization of this virus in *Passiflora* was beyond the scope of this thesis.

EM analysis as a diagnostic tool was more useful for the detection of potyvirus than for CMV in sap extracts of infected *Passiflora*. This technique is generally unsuitable for large scale diagnostic applications, but it is a valuable diagnostic tool for assessing presence of virus particles in samples. It is especially useful in the absence of specific immunological or other diagnostic techniques or when the nature of the infectious viral agent is unknown.

3.3.3.2 Serology

Immunosorbent electron microscopy (ISEM)

In ISEM examinations, potyviruses present in crude sap extracts were efficiently trapped and decorated by an anti-PWV antiserum obtained from Dr. Lin, Taiwan (Fig. 3.7).

ISEM of crude sap of infected *P. flavicarpa* from the Burgershall area, using antiserum specific to the Natal *P. caerulea* potyvirus, showed distinct differences in coating affinity between individual potyviruses viewed simultaneously (Fig. 3.8a). Infected *P. caerulea* material from Natal, however, only showed uniformly coated potyvirus particles (Figs. 3.8b). It was concluded that the specimens from Burgershall may be infected by more than one antigenically different potyvirus.

Serological screening

Serological screening of potyvirus in infected *Passiflora*.

DAS-ELISA was found to be a reliable test for screening plants infected by the Natal *P. caerulea* potyvirus. Results of *Passiflora* leaf specimens screened by DAS-ELISA using a serum directed against the Natal *P. caerulea* potyvirus are shown in Figs 3.9a & b.

In order to standardize the interpretation of DAS-ELISA results, a sample was considered virus-positive when it produced a DAS-ELISA absorbance value (λ=405 nm) which exceeded the mean of the negative controls plus two standard deviations.

Detection of potyviruses by DAS-ELISA and EBIA was most effective in young leaves with virus symptoms. More pronounced symptoms observed in leaves sampled during colder months from vines with little active growth, did not seem to correspond to higher concentrations of virus, as potyviruses could not be readily detected in such tissue. DAS-ELISA was also used to monitor the transmissibility of the Natal *P. caerulea* potyvirus to other hosts (refer to Chapter 2 section 2.2.2). Of all the tests conducted, DAS-ELISA proved to be the most practical, and followed by nucleic acid hybridization, the most sensitive method for detection of Natal *P. caerulea* potyvirus in sap extracts of *Passiflora*. Reliability of the latter technique was, however, subject to using actively growing young leaves from *Passiflora* infected with the Natal *P. caerulea* potyvirus.

The potyvirus was detected by DAS-ELISA in the sap of infected *Passiflora* with higher sensitivity than with EBIA, but EBIA (using anti-Natal *P. caerulea* potyvirus IgG) proved useful for the combined detection of the presence, and possible size (strain) differences in the capsid protein antigen, of potyviruses from different *Passiflora* sources (Fig. 3.4a).

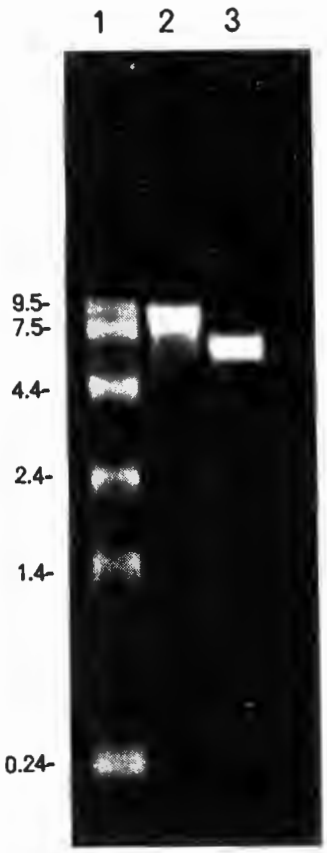


Fig. 3.5 Size estimation of ssRNA from the Natal *P. caerulea* potyvirus by gel electrophoresis in 1% agarose containing 6% formaldehyde. Lane 1, "RNA ladder" - ssRNA molecular weight markers obtained from Bethesda Research Laboratories. Lane 1, Natal *P. caerulea* potyvirus ssRNA. Lane 3, TMV ssRNA.

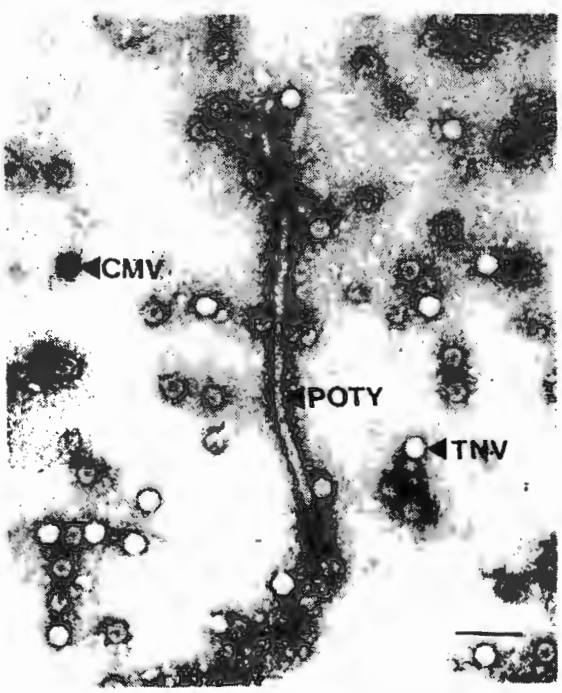


Fig. 3.6 Electron micrograph showing mixture of viruses present in *N. benthamiana* inoculated from naturally infected *P. caerulea*. The scale bar represents 100 nm.

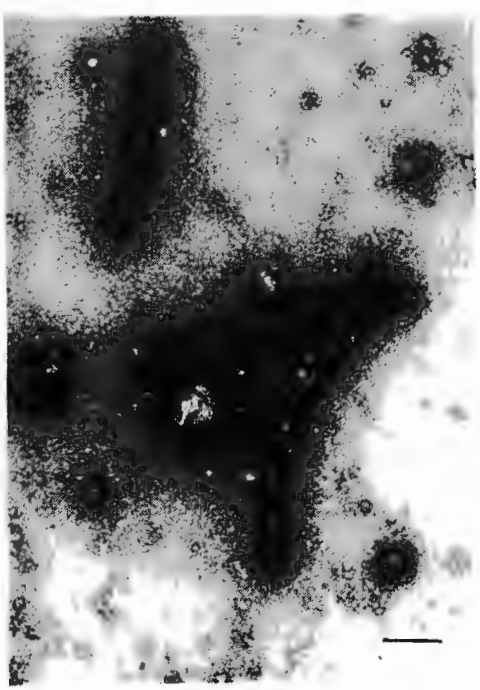


Fig. 3.7 ISEM of infected *P. caerulea* using anti-PWV (Taiwan) antiserum. The scale bar represents 100 nm.

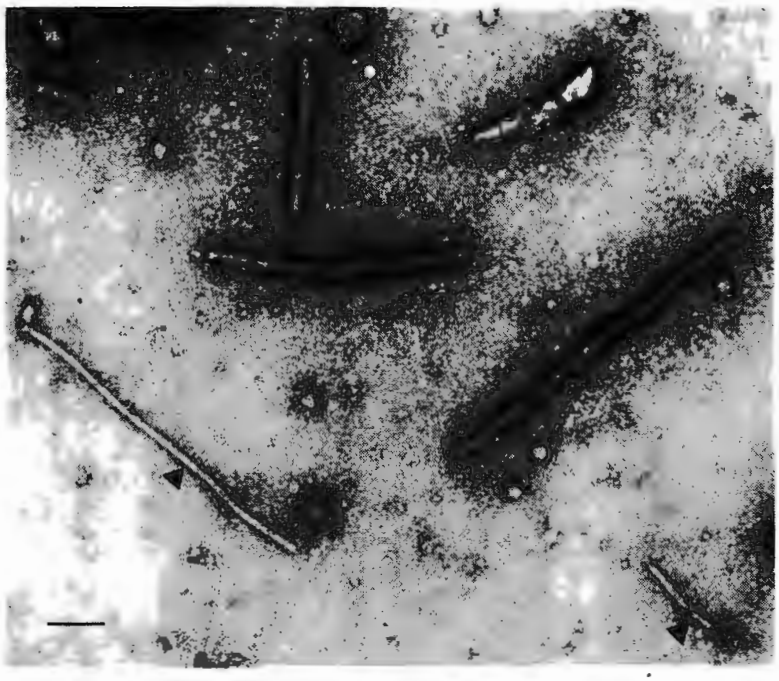


Fig. 3.8a ISEM of infected *P. flavicarpa* from Burgershall, using antiserum prepared against the Natal *P. caerulea* potyvirus. The arrows indicate single particles that are weakly coated by the antiserum. The scale bar represents 100 nm.

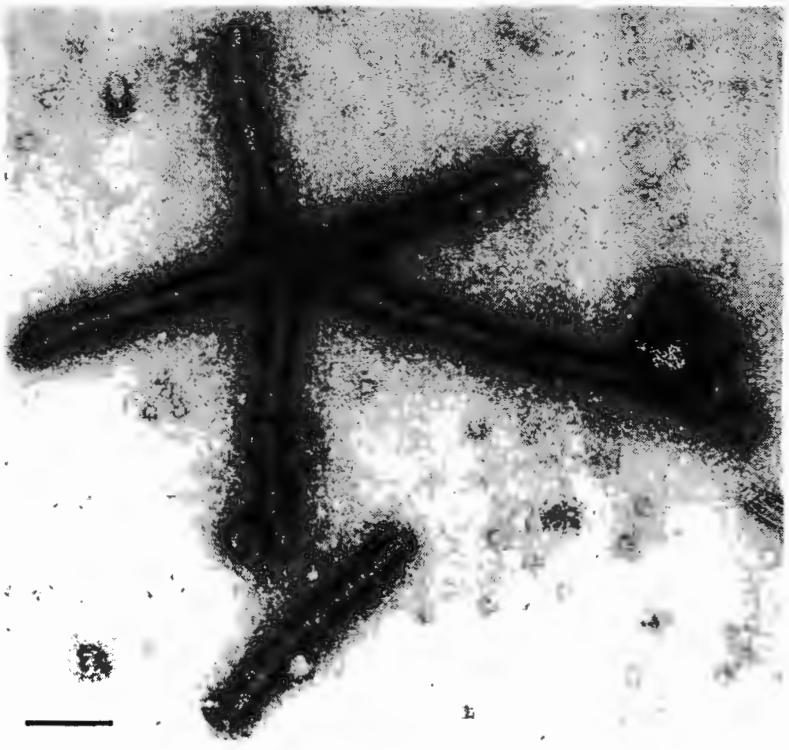


Fig. 3.8b ISEM of infected *P. caerulea* from Natal, using antiserum prepared against the homologous potyvirus. The scale bar represents 100 nm.

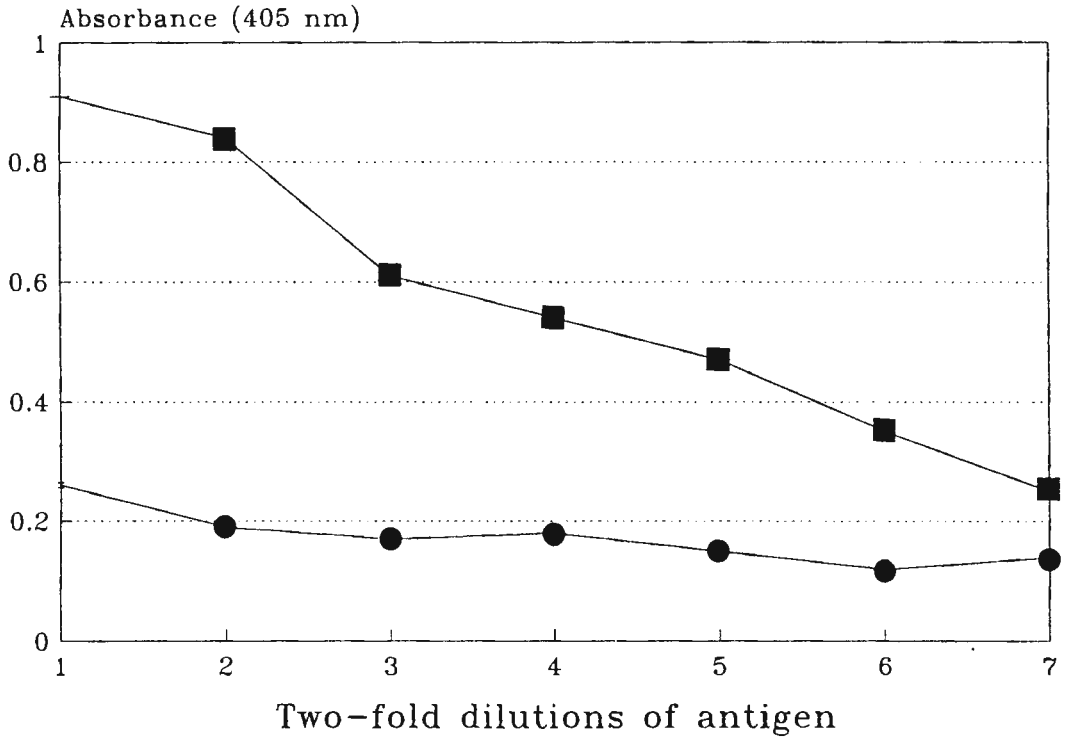


Fig. 3.9a Calibration of DAS-ELISA, using polyclonal IgG directed against purified Natal *P. caerulea* potyvirus. Anti-virus IgG (1 mg/ml) and anti-virus IgG conjugated to alkaline phosphatase were used at a dilutions of 1/500 each. ■ = Dilutions of sap from *P. edulis* infected by the Natal *P. caerulea* potyvirus. ● = Dilutions of sap from virus-free *P. edulis*.

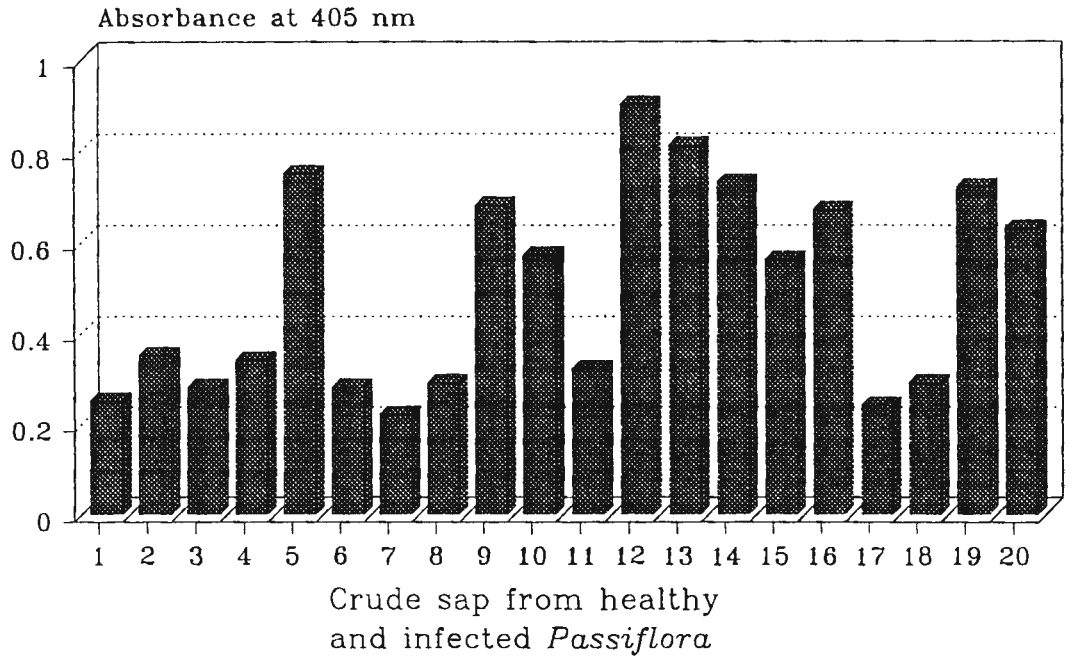


Fig. 3.9b Detection of the Natal *P. caerulea* potyvirus in crude plant sap by DAS-ELISA, using polyclonal IgG directed against purified Natal *P. caerulea* potyvirus. Anti-virus IgG (1 mg/ml) and anti-virus IgG conjugated to alkaline phosphatase were used at a dilutions of 1/500 each. **Sample 1**, sap from virus-free *P. edulis*. **Sample 2**, sap from virus-free *P. caerulea*. **Sample 3**, sap from virus-free *N. benthamiana*. **Sample 4**, sap from virus-free *P. flavicarpa*. **Sample 5**, purified Natal *P. caerulea* potyvirus. **Samples 6 - 10**, sap from *P. edulis* specimens that was infected by aphid transmission from *N. benthamiana* source. **Samples 11 - 15**, sap from *P. edulis* specimens infected by sap inoculation from Natal *P. caerulea* potyvirus-infected *N. benthamiana* source. **Samples 16 - 20**, sap from *P. edulis* specimens that were grafted onto potyvirus-infected *P. caerulea*

DAS-ELISA could detect the presence of the Natal *P. caerulea* potyvirus in as little as 1.5 mg infected Passiflora leaf tissue, which is only about 3 times less sensitive than the dot blot hybridization assay Fig. 3.9a.

The Natal *P. caerulea* potyvirus reacted strongly with antisera against a Taiwanese PWV strain in EBIA (Fig. 3.7), indicating an antigenic relationship between the South African virus and PWV. This reaction could, however, be due to conserved epitopes in the "core" region of the capsid protein, as the serum also reacted strongly with the unrelated OMV coat protein. In the absence of PWV-antigen from Brazil, Taiwan or Australia, it was not possible to examine the serological relatedness between the less conserved N-terminal region of the CP of the Natal *P. caerulea* potyvirus and other isolates of PWV (by the method developed by Shukla *et al.*, (1989b), using N-terminal specific antibodies).

Immunological indexing of *CMV-Pass*.

DAS-ELISA was effective in detecting the presence of CMV-Pass in herbaceous hosts. The high background obtained with sap from *Passiflora*, a woody host, however, often obscured positive reactions. CMV is renowned for its poor antigenicity (Kaper & Waterworth, 1981). Furthermore, this virus was found to occur in very low concentrations in *Passiflora*, particularly in single infections. The sensitivity of DAS-ELISA or EBIA was not adequate to permit reliable routine detection of CMV in Passiflora sap extracts, whereas it was certainly sensitive enough to detect the presence of the CMV-Pass antigen in sap extracts of infected *N. benthamiana*. CMV-Pass was readily detected by EBIA in sap of *Passiflora* only when it occurred as a co-infecting agent with the potyvirus (Fig. 3.10). It was reported previously that CMV occurred in extremely low concentrations in infected *Passiflora*, but can be present in abnormally high concentrations in mixed infections with PWV (Taylor & Kimble, 1964). A similar finding was reported by Poolpol & Inouye, (1986) where co-infection of CMV and zuccini yellow mosaic virus (ZYMV), could result in enhanced replication and higher levels of CMV .

An antiserum directed against a mixture of CMV-Pass and the Natal *P. caerulea* potyvirus, was found to be useful for the detection of either virus or the combined presence of these two viruses in infected *Passiflora* or other hosts. This was once again not reliable for detecting CMV-Pass when occurring in single infections in *Passiflora*, presumably due to low concentrations of the virus (Fig. 3.11).

Interviral serological relationships

Antiserum raised against CMV-Pass, reacted strongly with CMV-LupK5, a CMV isolate from South African lupins (Von Wechmar & Trenor, 1988; M. B. von Wechmar, unpublished) in EBIA (Fig. 3.12). Partial sequence analysis of the CP gene resident on RNA 4 of CMV-LupK5 (S. Dennis, University of Cape Town,

unpublished) indicated that this virus was related to CMV-Q (in CMV Subgroup II). The strong reaction observed of CMV-LupK5 antiserum with CMV-Pass is an indication that the latter virus probably belongs to the subgroup II of CMV strains (Piazolla, Diaz-Ruiz & Kaper, 1979; Rizzo & Palukaitis, 1989; Quemada *et al.*, 1989; refer to Chapter 1, sections 1.3.4.1 & 1.3.4.5). No further relationship studies were performed with CMV-Pass.

Serological relationship studies using potyviral polyclonal antisera (described in Appendix B.1.6) in EBIA against the Natal *P. caerulea* potyvirus coat protein, contributed little to clarifying serological relationships between this virus and other members of the potyvirus group. Shukla *et al.*, (1989b) showed that serological relationship studies based on EBIA can yield sensible results, but only if antibodies directed at the conserved core region of the potyviral CPs are removed from the polyclonal antiserum by affinity column chromatography. The antisera used in this study, were not purified in this manner, and basically all potyviral antisera tested, reacted with the Natal *P. caerulea* potyvirus.
Shukla *et al.*, in press[b] warned that anomalous serological reactions can occur between potyviruses, even if this technique is applied, and that serology remains an imperfect criterion for the identification and classification of potyviruses. They have, for instance, found that antiserum raised against JGMV reacted with WMV-2, but reciprocally, WMV-2-antiserum failed to react with JGMV. Sequence analysis (Chapter 4 section 4.3.5.3) of the Natal *P. caerulea* potyvirus-CP, show a close but distinct relationship with WMV-2, but in EBIA using anti-WMV-2 antiserum, these two viruses ironically show the weakest cross-reaction of all potyviruses tested (Fig. 3.13). A similar phenomenon has been shown to occur between pepper mottle virus (PeMV) and PVY. On the basis of coat protein sequence data, these two viruses appear to be strains of the same virus (Shukla *et al.*, 1988d), yet they only show a very distant serological relationship.

3.3.3.3 Nucleic acid hybridizations

Both the Natal *P. caerulea* potyvirus and CMV-Pass were detected with very high sensitivity in dot blot hybridizations, using radioactive labelled Natal *P. caerulea* potyvirus-cDNA or end-labelled CMV-Pass-dsRNA as probes. The presence of as little as 0.5 ng of viral RNA could be detected, whereas the presence of Natal *P. caerulea* potyvirus-ssRNA in sap of infected leaves could be detected in as little as 0.5 mg leaf tissue (Fig. 3.14a).
For Natal *P. caerulea* potyvirus-specific detection, a probe synthesized by ^{32}P-dCTP nick translation (Appendix B.3.9.1) of pW9, appeared to be very strain specific, predictably so due to the fact that the 1.42 kb 3' terminal clone encompasses the CP gene which is located at the 3' end of the potyviral genome (Hellmann, *et al.*, 1986; Dougherty & Carrington, 1988).

LEGENDS:

Fig. 3.10 EBIA using anti CMV-Pass serum: **Lane 1**, CMV-Pass purified virus. Lane 2, *P. caerulea* from Natal midlands infected with Natal *P. caerulea* potyvirus and CMV-Pass. **Lane 3**, *P. edulis* seedling, sap inoculated from diseased *P. caerulea* used in lane 2. **Lane 4**, *N. tabacum* cv. Xanthi, infected by aphid transmission from diseased *P. caerulea* used in lane 2. **Lane 5**, uninfected *P. caerulea* seedling. **Lane 6**, *P. edulis* seedling infected by aphid transmission from diseased *P. caerulea* used in lane 2. **Lane 7**, *Nicandra physalloides*, a weed found in an infected *Passiflora* orchard. **Lane 8**, *Nicotiana glutinosa*, sap inoculated from infected *N. physalloides* in lane 7. **Lane 9**, CMV-LupK5.

Fig. 3.11 EBIA using antiserum directed against a mixture of purified CMV-Pass and the Natal *P. caerulea* potyvirus. **Lane 1**, purified CMV-Pass. **Lane 2**, purified Natal *P. caerulea* potyvirus. **Lane 3**, diseased *P. caerulea* infected with the Natal *P. caerulea* potyvirus. **Lane 4**, diseased *P. caerulea* infected with a mixture of CMV-Pass and the Natal *P. caerulea* potyvirus

Fig. 3.12 EBIA using anti CMV-Pass serum: **Lane 1**, CMV-Pass, **Lanes 2 & 3**, CMV-LupK5, **Lane 4**, sap from healthy *N. benthamiana*.

Fig. 3.13 EBIA to examine serological cross-reaction of the Natal *P. caerulea* potyvirus CP with antisera directed against the CPs of different potyviruses (Appendix B.1.6). **Lane 1**, Natal *P. caerulea* potyvirus antiserum. **Lane 2**, OMV antiserum. **Lane 3**, PWV (Taiwan) antiserum. **Lane 4**, PVY antiserum. **Lane 5**, WMV II antiserum. **Lane 6**, WMV I antiserum. **Lane 7**, SoyMV antiserum. **Lane 8**, BYMV antiserum. **Lane 9**, MDMV A antiserum. **Lane 10**, MDMV B antiserum.

Fig. 3.10

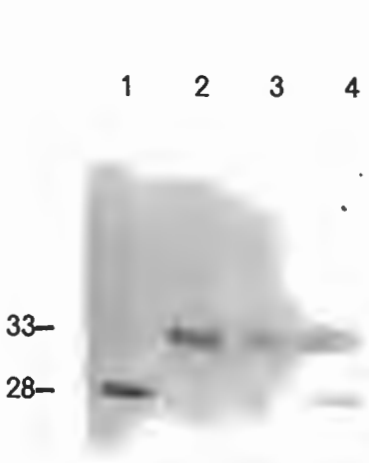


Fig. 3.11

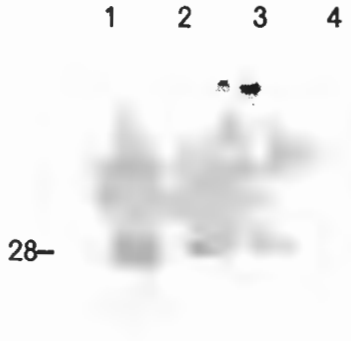


Fig. 3.12

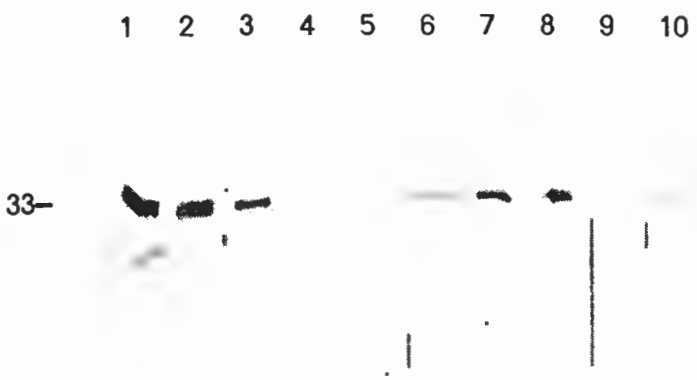


Fig. 3.13

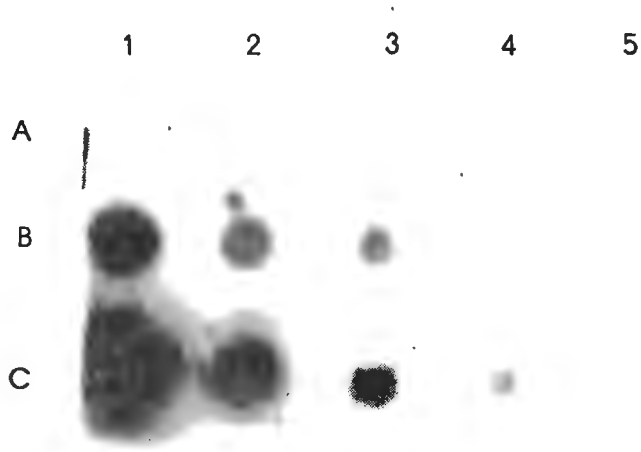


Fig. 3.14a

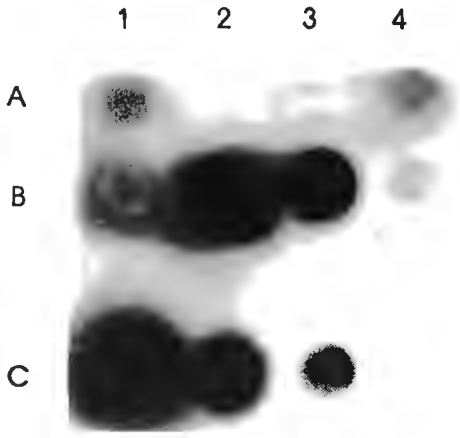


Fig. 3.14b

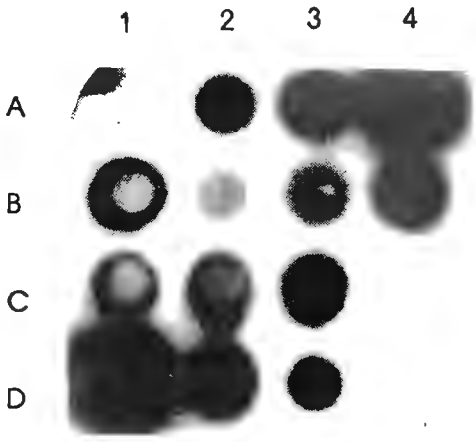


Fig. 3.14c

Fig. 3.14a Investigation of the sensitivity of dot blot nucleic acid hybridization to detect potyviral nucleic acids. Probe: pW9 clone (Chapter 4, section 4.2.4), labelled with ^{32}P-dCTP by nick translation. Lanes A1 to A5, series of ten-fold dilutions of total nucleic acids from sap of virus-free *P. caerulea*, starting with undiluted sap. Lanes B1 to B5, series of ten-fold dilutions of total nucleic acids from crude sap of virus-infected *P. caerulea* from Natal (starting with 50 mg leaf material). Lanes C1 to C5: Series of 10-fold dilutions of purified potyviral ssRNA (starting with 500 ng).

Fig. 3.14b Dot blot nucleic acid hybridization to detect the possible presence of potyvirus in *Passiflora*. Probe: pW9 clone (Chapter 4, section 4.2.4) labelled with ^{32}P-dCTP by nick translation. A1, Diseased *P. edulis* from Franschhoek, western Cape Province (W.P.). A2, *P. edulis* from Burgershall, eastern Transvaal infected with a filamentous (poty-like) virus. A3, *P. allardii* infected with a filamentous (poty-like) virus from W.P. A4, *P. caerulea* from Natal midlands - infected with a mixture of potyvirus and CMV-Pass. B1, *P. edulis* infected by aphid transmission from potyvirus infected *P. caerulea*. B2, *P. edulis* infected with potyvirus by sap inoculation from potyvirus infected *N. benthamiana*. B3, *P. edulis* infected by graft transmission onto potyvirus infected *P. caerulea*. B4, Healthy *P. edulis* seedling. C1, 400 ng Natal *P. caerulea* potyvirus-ssRNA. C2, 40 ng potyvirus-ssRNA. C3, 4 ng potyvirus-ssRNA. C4, 0.4 ng potyvirus-ssRNA.

Fig. 3.14c Dot blot nucleic acid hybridization to detect the possible presence of CMV in *Passiflora*. Probe: CMV-Pass-dsRNA endlabelled with [γ-^{32}P] dATP. A1, virus free *P. caerulea* seedling. A2, CMV-infected *P. edulis* from Burgershall in the eastern Transvaal (B.E.T.). A3, infected *P. allardii* from the western Cape province (W.P.). A4, CMV-Pass infected *P. caerulea*. B1, *P. edulis* infected by aphid transmission from diseased *P. caerulea* source. B2, *P. caerulea* infected by aphid transmission from diseased *P. caerulea* source. B3, *P. caerulea* infected by aphid transmission from CMV-Pass and potyvirus infected *N. benthamiana* source. B4, original source of CMV-Pass from Natal midlands. C1, *P. caerulea* sap-inoculated from *P. edulis* infected with CMV-Pass & potyvirus. C2, *N. tabacum* cv. Xanthi sap-inoculated from CMV-Pass & potyvirus infected *P. edulis*. C3, *P. caerulea* sap-inoculated from CMV-Pass infected *N. benthamiana*. C4, healthy *P. caerulea* seedling. D1, 400 ng CMV-Pass-ssRNA. D2, 40 ng CMV-ssRNA. D3, 4 ng CMV-ssRNA. D4, 0.4 ng CMV-ssRNA

The core portion of potyviral CPs is conserved at the amino acid level (Shukla *et al.*, 1988b,c), but the level of conservation is much less on the nucleic acid level due to the degeneracy of the genetic code, rendering such a probe mainly useful for strain-specific detection. In Fig. 3.14b, it can be seen that the Natal *P. caerulea* potyvirus probe did not hybridize to some potyviral isolates occurring in *Passiflora* species collected at Burgershall in the eastern Transvaal. The presence of potyvirus in these samples was, however, demonstrated by EBIA using anti-Natal *P. caerulea* potyvirus serum (Fig. 3.4a & b). The virus from Burgershall also differed in biological respects from the Natal *P. caerulea* potyvirus in that it was unable to infect *N. benthamiana*.

In the case of CMV-Pass, the use of radioactive labelled probes appeared to be the most reliable and sensitive method for the detection of the presence of CMV in sap of infected *Passiflora* (Fig. 3.14c). By endlabelling CMV-Pass-dsRNA, not only the CP gene of the virus is labelled, but also more conserved non-structural genes e.g. the replicase gene which reduces the strain specificity of the probe, rendering it potentially useful for the detection of a variety of CMV strains in the same group. CMV strains seem to cluster into two groups (I & II) based on nucleic acid homology (Piazolla, Diaz-Ruiz & Kaper, 1979; Rizzo & Palukaitis, 1989; Quemada *et al.*, 1989).

3.3.4 Transmission of passionfruit viruses to alternative hosts

Since the Natal *P. caerulea* potyvirus and CMV-Pass were not readily purified from *Passiflora* spp. (refer to section 3.1.1), various herbaceous hosts were evaluated for their susceptibility and their potential for propagation of these viruses for purification (Chapter 2 section 2.2.2).

3.3.4.1 Transmission studies with CMV-Pass

CMV-Pass was isolated from mixed infections in *Passiflora*. In field-collected specimens examined for the presence of virus, CMV-Pass always co-occurred with a potyvirus.

C. pepo or *N. tabacum* were found to be suitable hosts for separating CMV-Pass from Natal *P. caerulea* potyvirus, as these hosts do not support replication of the latter virus.

When CMV-Pass was separated from the potyvirus in *C. pepo* or *N. tabacum*, it was found to be less readily transmissible to healthy *P. edulis* and *P. caerulea*. The virus was however successfully transmitted by sap inoculation to healthy *P. caerulea* seedlings from CMV-Pass-infected *C. pepo* or *N. benthamiana*, but not to healthy *P. edulis* seedlings (Table 3.1). CMV-Pass-infected *P. caerulea* showed distinct mosaic symptoms two to three weeks after infection, and reacted positively for CMV in a dot-blot hybridization test with end-labelled CMV-Pass-dsRNA as probe (Fig. 3.14c). These results were confirmed by EBIA (Fig. 3.10). CMV-Pass was readily transmitted to *P. caerulea* and *P. edulis* seedlings from a

diseased *P. caerulea* source infected with the Natal *P. caerulea* potyvirus and CMV-Pass.

Table 3.1. Alternative hosts evaluated for susceptibility to CMV-Pass by sap and aphid transmission

	Transmission:			
Recipient Host	Aphids +/Total	Sap +/Total	Tests	Symptom
Nicotiana tabacum (cvs. Xanthi & Soulouk)	6/8	7/8	NA(+), EBIA(+)	Mosaic
N. benthamiana	6/8	8/8	NA(+), EM(+), EBIA(+)	Mottle
N. glutinosa	3/8	4/8	NA(+) EBIA(+)	Mosaic
N. clevelandii	ND	5/8	NA(+)	Mottle
Phaseolus vulgaris (cvs Bonus & Bountiful)	0/8	0/8	EBIA(-)	None
Nicandra physalloides	ND	5/8	NA(+) EBIA(+)	Mottle
Cucurbita pepo (cv Long White Bush)	8/8	8/8	NA(+) EBIA(-/+) EM(+)	Strong Mosaic
Vigna unguiculata	ND	0/8	NA(-)	None
Cucumis sativus (cv. Rust Resistant)	2/8	0/8	NA(+)	Mosaic
Chenopodium quinoa	0/8	0/8	NA(-)	Mottle?
Zinea elegans	ND	2/8	NA(+)	Mottle
Petunia hybrida	ND	0/8	NA(-)	None
Capsicum annuum	ND	3/8	NA(+)	Mottle
P. edulis	3/8	0/8	NA(+)	Mottle
P. caerulea	5/8	4/8	NA(+)	Mosaic

+ = positive infection; NA = Nucleic acid hybridization; EBIA = Electroblot immunoassay; EM = electron microscopic analysis; ND = not determined. All above hosts (except *C. pepo* and *N. tabacum*) were infected using CMV-Pass-infected *N. tabacum* as a source. *C. pepo* and *N. tabacum* were infected from the original *P. caerulea* specimen from Natal that was infected with a combination of CMV-Pass, potyvirus and TNV.

CMV-Pass produced strong systemic mosaic symptoms in secondary leaves of *C. pepo* five days post transmission by aphids, or mechanical sap inoculation from the Natal *P. caerulea* source infected by a mixture of CMV-Pass, potyvirus and TNV. Electron microscopic analysis confirmed the presence of CMV-Pass in *C. pepo*, when infected in either way. In *N. benthamiana*, CMV-Pass produced a yellowing between veins seven days post infection, when inoculated with sap from an infected *C. pepo* source. CMV-Pass was transmitted successfully to seedlings of *N. tabacum* cvs. Xanthi & Soulouk (Fig. 3.10, Table

from a *C. pepo* source by aphids. For results of seed transmission studies see section 3.3.4.3.

3.3.4.2 Transmission studies with the Natal *P. caerulea* potyvirus

Table 3.2. Alternative hosts evaluated for susceptibility to the Natal *P. caerulea* potyvirus

	Transmission:			
Recipient Host	Aphids +/Total	Sap +/Total	Tests	Symptom
Nicotiana tabacum (cvs. Xanthi & Soulouk)	0/8	0/8	ELISA(-)	None
N. benthamiana	8/8	8/8	ELISA(+) EM(+) EBIA(+) NA(+)	Systemic mosaic, & wrinkle
N. glutinosa	0/8	0/8	ELISA(-)	None
N. clevelandii	ND	0/8	ELISA(-)	None
Phaseolus vulgaris (cvs Bonus & Bountiful)	3/8	8/8	EM(+) ELISA(+)	Chlorotic/ necrotic local lesions on primary leaves
Nicandra physalloides	0/8	0/8	ELISA(-)	None
Cucurbita pepo (cv Long White Bush)	0/8	0/8	ELISA(-)	None
Vigna unguiculata	ND	4/8	EM(+) ELISA(+) EBIA(+)	Chlorotic lesions & systemic mosaic
Cucumis sativus ((cv. Rust Resistant)	0/8	0/8	ELISA(-)	None
Chenopodium quinoa	ND	0/8	ELISA(-)	None
Zinea elegans	ND	0/8	ELISA(-)	None
Petunia hybrida	ND	0/8	ELISA(-)	None
Capsicum annuum	ND	0/8	ELISA(-)	None
P. caerulea	5/8	6/8	ELISA(-)	Mottle
P. edulis	4/8	4/8	ELISA(-)	Mottle

+ = positive infection; NA = Nucleic acid hybridization; EBIA = Electroblot immunoassay; EM = electron microscopic analysis; ELISA = DAS-ELISA; ND = not determined. All above hosts (except *N. benthamiana* and *P. vulgaris)* were infected using Natal *P. caerulea* potyvirus infected *N. benthamiana* as a source. *N. benthamiana* was infected using Natal *P. caerulea* potyvirus-infected *P. vulgaris* as a source, and the latter was infected directly from Natal *P. caerulea* potyvirus infected *P. caerulea*.

The Natal *P. caerulea* potyvirus has a narrow host range (see Table 3.2), as is often the case for potyviruses (Hollings & Brunt, 1981).

Infection of *P. vulgaris* by this virus produced small chlorotic (yellow/white) local lesions on the primary leaves 10 days post sap inoculation from the original infected *P. caerulea* host (described in Chapter 2, section 2.3.3).

Local lesion passage through this host separated the Natal *P. caerulea* potyvirus from CMV-Pass when they co-occurred in mixed *Passiflora* infections, (Tables 3.1 & 3.2). *P. vulgaris* could be used as an inoculation source for transmission of the virus to *N. benthamiana*, a host found to be more suitable for virus purification. Symptom expression on *N. benthamiana* (10 days post sap inoculation) produced a systemic leaf wrinkling symptom on new young leaves. *Vigna unguiculata* was susceptible to the Natal *P. caerulea* potyvirus, and often allowed systemic spread of the virus. This seed source was, however, contaminated with other seedborne viruses. The unavailability of virus-free seed prohibited the routine use of this host.

The Natal *P. caerulea* potyvirus was readily aphid- or sap- transmitted from *N. benthamiana*, *P. vulgaris*, *Passiflora* spp. or from purified virus as source, to *P. caerulea* and *P. edulis* seedlings, and vice versa (Fig. 3.4a & b).

3.3.4.3 Indexing for graft- and seed-transmitted virus.

The results of graft transmission studies are summarized in Table 3.3. Progeny seedlings grown from infected parent plants often appeared less vigorous and foliage had a chlorotic appearance. DAS-ELISA, EBIA and nucleic acid hybridization tests conducted to screen for the presence of the Natal *P. caerulea* potyvirus or CMV-Pass in 80 such seedlings all yielded negative results. Both CMV-Pass and the Natal *P. caerulea* potyvirus, were readily graft transmissible.

Table 3.3. Graft transmission studies on CMV-Pass and the Natal *P. caerulea* potyvirus.

Rootstock	Pre-graft Infection Status	Graft	Pre-graft Infection Status	Post-graft Results +/Total
P. edulis (x4)	CMV-Pass Poty	*P. edulis* (x4)	Healthy	CMV-Pass (4/4) Poty (4/4) *(Present in both rootstock and graft)*
P. caerulea (x4)	Healthy	*P. edulis* (x4)	CMV-Pass Poty	CMV-Pass (4/4) Poty (4/4) *(Present in both rootstock and graft)*
P. caerulea (x4)	CMV-Pass Poty	*P. edulis* (x4)	Healthy	CMV-Pass (4/4) Poty (4/4) *(Present in both rootstock and graft)*
P. caerulea (x4)	Poty	*P. edulis* (x4)	Healthy	Poty (4/4) *(Present in both rootstock and graft)*

3.4 DISCUSSION

3.4.1 Identification and purification of viruses from South African passiflora

Physicochemical properties of the Natal *P. caerulea* potyvirus were characteristic of a potyvirus. Immunological reaction with potyvirus antiserum confirmed the group identity of the virus, but data from these studies were inadequate to confirm or deny that the Natal *P. caerulea* potyvirus was a strain of PWV. The former virus was different from PWV in its host reactions (it did not spread systemically to the secondary leaves of *P. vulgaris* cv. Bountiful, and it was capable of systemically infecting *N. benthamiana*). On grounds of the available physicochemical characteristics, serological reactions and biological

criteria, it was thus decided to adopt the temporary designation of "Natal *P. caerulea* potyvirus" for this virus until more conclusive data availed. It was decided to examine the phylogenetic affiliation of this virus by molecular analysis.

The properties of the spherical virus observed in mixed infections corresponded physicochemically and serologically to CMV from subgroup II, and it was designated CMV-Pass isolate.

Biological separation of these viruses on a differential host range proved to be reliable and effective. The use of *N. benthamiana* for purification of these viruses after their biological separation produced good yields of virus. CMV-Pass in particular, was more stable when purified from this host than when purified from *N. tabacum* cv Xanthi or *C. pepo*.

3.4.2 The etiology of viruses implicated in woodiness disease of South African *Passiflora*

Results have shown that a variable complex of viruses is associated with woodiness disease of South African *Passiflora*. At least two different potyviruses with CPs of different M_rs (Figs. 3.8b and 3.4a lane 7), a CMV-Pass isolate and TNV are implicated (Fig. 3.6). EBIA analysis of twelve different CMV-infected *Passiflora* specimens from Natal and western Cape (results not shown) indicated that all CMV isolates tested, had a similar CP molecular weight. In every instance when CMV-Pass was identified in infected field-collected specimens, it occurred in mixed infections with a potyvirus. Such plants exhibited more severe woodiness related symptoms than plants infected with potyvirus alone. However, single infections of potyvirus were sufficient to elicit symptoms in *Passiflora* vines and foliage that were similar to woodiness disease..

Potyviruses were detected in diseased specimens collected from all regions of passionfruit production in South Africa (Chapter 1, Fig. 1.1, Chapter 2, Table 2.1), whereas the presence of CMV-Pass in South African *Passiflora* seemed to be limited mainly to the Natal midlands and western Cape regions. Elsewhere where CMV is a problem in passionfruit, it also tends to be found in regions with moderate climates (Taylor & Kimble, 1964).

Earlier investigations indicated that Australian *Passiflora* subjected to aphid-mediated infection of CMV showed symptoms after four days, but proceeded to an apparent complete recovery, not to show symptoms again (Taylor & Kimble, 1964). These researchers also found that CMV was not readily transmitted by aphids from *Passiflora* (due to very low concentrations), except when it occurred as a co-infecting agent with PWV. Results obtained in this study confirmed these findings. Concentrations of CMV-Pass were generally so low in *P. edulis* infected with only CMV-Pass that serological tests were unreliable. When the Natal *P. caerulea* potyvirus-infected *Passiflora* was subjected

to superinfection by CMV-Pass, more severe symptoms developed. This agreed with observations made by Taylor & Kimble (1964) for CMV and PWV.

CMV-Pass alone was not particularly infectious to *P. edulis*, although *P. caerulea* was readily susceptible to sap inoculation by CMV-Pass (Table 3.1). It thus appears that, under South African conditions, CMV-Pass transmission is far more efficient when superinfecting *Passiflora* that already harbours a potyvirus. Higher concentrations of CMV-Pass occurred in superinfected plants, and the virus was more readily transmitted from such plants.

The Natal *P. caerulea* potyvirus and CMV-Pass were tested only on a limited range of laboratory hosts. Most potyviruses are known to have relatively narrow host ranges, whereas many CMV strains are renowned for their wide host range (Edwardson, 1974; Kaper & Waterworth, 1981). It was thus possible that vegetables and other crops grown near areas of passionfruit production could act as reservoirs of CMV. A specimen of *Nicandra physalloides* (a particularly common weed in Natal passionfruit vineyards) that exhibited mosaic symptoms, tested positive for CMV in EBIA (Fig. 3.10 lanes 7 & 8). It is likely that this weed could act as a field reservoir of CMV-Pass.

The common practice of using untested scionwood is hypothesized to be the most important source of infection that contributed to the virtual collapse of the local passionfruit industry. This could explain the widespread occurence of potyvirus(es) in commercial passionfruit groves in spite of the narrow host range of the Natal *P. caerulea* potyvirus or PWV. CMV has a wide host range. In regions with a temperate climate there is an abundance of this virus in field reservoirs like weeds and other crops which may occur in the proximity of passionfruit plantings from where it could be insect-transmitted to (super)infect *Passiflora*.

CMV infection of *P. caerulea* is particularly difficult to assess visually, since this host goes through phases of pseudo-recovery which can mask the CMV infection. In all cases where CMV-Pass was detected, it was present in *P. edulis* top-grafted onto *P. caerulea*. Another possible explanation for the high incidence of CMV in the Natal midlands could thus be the use of CMV-infected *P. caerulea* rootstock propagated from infected scionwood ot root cuttings.

The role of TNV in South African *Passiflora* is still under investigation, but preliminary findings are that it is more widespread than initially suspected (Von Wechmar *et al.*, 1991; Von Wechmar, unpublished).

ISEM studies (using Natal *P. caerulea* potyvirus-specific polyclonal antiserum) of infected *P. flavicarpa* from Burgershall (see Chapter 2, Table 2.1), indicated the presence of more than one type of potyvirus in a single infected plant specimen. In Fig. 3.8a it is evident that some particles are "decorated" efficiently by the Natal *P. caerulea* potyvirus-specific antiserum, whereas others failed to be "decorated". This finding is supported by EBIA analysis of the same sample. In Fig. 3.4a it is clear that there are two bands, one corresponding to the

higher CP M_r of Natal *P. caerulea* potyvirus, and another corresponding to the lower CP M_r found in other diseased *Passiflora* samples from the eastern Transvaal Burgershall region. This is a further indication that the Natal *P. caerulea* potyvirus can occur as a co-infecting virus with another serologically different potyvirus in *Passiflora*. Failure of the Natal *P. caerulea* potyvirus-cDNA probe to hybridize to potyvirus infected material from the Eastern Transvaal (Fig. 3.14b) is an indication that the "Burgershall potyvirus" is unrelated or very distantly related to the Natal *P. caerulea* potyvirus. The fact that the Burgershall potyvirus was unable to infect *N. benthamiana* suggests that it is also biologically distinct from the Natal *P. caerulea* potyvirus. No further characterization studies were undertaken to identify this potyvirus. It is likely that this mixture of two potyviruses in single plant specimens could arise from top-grafting a potyvirus infected *P. edulis* or *P. flavicarpa* onto a *P. caerulea* rootstock that was infected with another potyvirus since one would expect a degree of cross-protection between strains/isolates of potyviruses with other routes of infection such as insect or sap transmission.

The fact that single or double infections of different potyviruses, or mixed infections of Natal *P. caerulea* potyvirus and CMV-Pass, all result in woodiness symptoms, implies that the observed woodiness disease syndrome is a non-specific host response to infection by one or more different viral pathogens.

The Australian passionfruit industry currently relies on the use of virus-free scionwood to curb the spread of woodiness disease, as the use of mild strains of viruses to cross-protect commercial passionfruit material has failed (Simmonds, 1959; Peasley & Fitzell, 1981). The use of virus-free scionwood is a useful short term strategy to minimize the incidence and spread of virus infection and to permit a fair period of virus-free production.

A series of *P. edulis* vines tested to be virus-free was handed over to plant breeders of the CSFRI for further selection and propagation to supply nurseries with virus-free material. It is recommended that growers propagate plants only from virus-free tested plant stock. Although detection tests failed to implicate seedborne virus in the disease of passionfruit, only seed originating from virus-free material should be used as an added precaution. It is also recommended that the use of the *P. caerulea* rootstock should be suspended due to its high susceptibility for CMV and the potyvirus, and to minimize the risk of virus contamination through nursery practices. Future approaches should investigate the possibility of genetically engineered virus cross protection to offer a long term solution.

CHAPTER 4

MOLECULAR CLONING, PARTIAL NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE NATAL *P. CAERULEA* POTYVIRUS

SUMMARY

DNA complementary to the 3'-terminal 1436 nucleotides of the Natal *P. caerulea* potyviral genome was cloned into the pUC19 vector and subjected to nucleotide sequence analysis. The sequence contained a single 1188 nt long 5'-ORF which presumably starts upstream of the cloned sequence, and is followed by a 3'-terminal 230 nt non-coding region. Comparative analysis indicated that the clone contained the entire potyviral CP sequence. Phylogenetic analysis of the deduced CP indicated that it was only distantly related to hitherto sequenced strains of PWV (71% related to PWV-TB). Comparative analysis of the 3'-terminal non-coding region of this virus, also indicated that it is very divergent (only 50% homology) from PWV-K. This virus thus appeared to be a new and distinct virus causing woodiness symptoms in *Passiflora*, and was subsequently designated South African *Passiflora* virus (SAPV).

CHAPTER 4

MOLECULAR CLONING, PARTIAL NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE NATAL *P. CAERULEA* POTYVIRUS

CONTENTS

CHAPTER 4. MOLECULAR CLONING, PARTIAL NUCLEOTIDE SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE NATAL *P. CAERULEA* POTYVIRUS

4.1 INTRODUCTION

4.1.1 Molecular characterization

Physical and biological data strongly suggest that the Natal *P. caerulea* potyvirus is a member of the potyvirus group. As this group currently comprises 180 definitive and possible members (Hollings & Brunt, 1981; Milne, 1988; Ward & Shukla, 1991), it has therefore become very important to establish reliable criteria for the classification of potyviruses. As pointed out in Chapter 1 section 1.3.3.3, a rational biological classification system should ultimately reflect the phylogenetic lineages of the biological entities in question.

Molecular phylogenies based on CP sequence data and the sequence of the potyviral 3'NCR have made the most significant impact on potyvirus classification in recent times (Rybicki & Shukla, in press; Ward *et al.*, in press). Although the CP gene comprises only 10% of the coding capacity of the potyviral genome, phylogenetic relationship profiles derived from CP sequence data were consistently similar to those obtained using entire potyviral nucleic acid sequences, and are definitively capable of defining viruses down to the strain level, and up to the family level.

As discussed in Chapter 1 section 1.3.3.2, the potyviral genome consists of a single 10 Kb ORF, encoding a ca. 350 kDa polyprotein that is matured through post-translational processing to yield at least eight functional proteins (Carrington *et al.*, 1990). The CP gene is the most distal gene in the potyviral genome, and is succeeded by a 3' NCR that occurs immediately proximal to the 3' terminal poly(A) (Fig. 1.3).

The molecular cloning and nucleotide sequencing of a 1.42 Kb 3' terminal fragment of the Natal *P. caerulea* potyvirus genome, encompassing the entire CP gene, is presented in this chapter. The possible phylogenetic lineage of this virus, based on comparative analysis of potyviral sequence data, is discussed.

4.1.2 Molecular phylogenetic analysis

The use of molecular data provides the ultimate level for studying evolutionary relationships, as it facilitates direct comparisons at or near the

actual level of the gene. The amount of sequence data available for potyvirus comparisons is very large and rapidly accumulating. This necessitates the use of numerical methods, designed according to models of the evolutionary process, for rapid computer-assisted data analysis. Numerical methods for inferring phylogenies from molecular data have existed for over 20 years, and they are basically statistical probalistic estimates of the evolutionary process. The objective of a phylogenetic study is usually to produce an inferred tree, that attempts to reconstruct the most probable genealogical ties between organisms, whereby branch lengths can be analogous to time of divergence between organisms from a common ancestor.

Most of the popularly employed algorithms to calculate such trees are based on two different approaches: distance matrix methods and maximum parsimony methods.

Only the Clustal-V set of programmes (Higgins & Sharp, 1988) was used for phylogenetic analysis of data in this thesis. This computer software package comprises a variety of tools for phylogenetic analysis of sequences, and uses only algorithms based on the distance matrix approach for the construction of inferred phylogenetic trees.

4.1.2.1 Parsimony methods

Algorithms based on the parsimony (or minimum net evolution) approach identify a tree requiring the smallest number nucleotide base substitutions necessary to explain the differences observed in sequence data amongst a set of specimens (Felsenstein, 1988). In practice, however, such algorithms often arrive at more than one tree with the minimum number of changes, and the statistical validity of this approach can be variable, depending on the particular set of data, and the precise analysis used (Felsenstein, 1988).

4.1.2.2 The Distance Matrix approach

Distance methods fit a tree to a matrix of pairwise distances between the species. Numerous methods have been developed for deriving hypothetical phylogenetic trees from similarity/distance matrices (calculated from pairwise sequence comparisons), as reviewed by Nei (1987) and Felsenstein, (1988). The simplest measure of distance is basically the percent sequence divergence: this is the number of different positions divided by the total number of positions considered, expressed as a percentage.

One of the most widely used variants of this approach is the "unweighted pair group method with arithmetic mean" (UPGMA) developed by Sokal & Sneath, (1963). This is a very simple approach of constructing a tree from a distance matrix. It essentially employs a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a step-wise manner. Initially every single specimen

is treated as a separate operational taxonomic unit (OTU), but in the successive round of clustering, the two most similar OTUs, are considered as a single OTU. This cycle is repeated until the tree is complete.

This algorithm, however, only yields reliable results if a molecular clock (the assumption that random evolutionary changes occur stochastically at a constant rate) is assumed (Felsenstein, 1988). Topological errors arising from an invalid assumption of a rate constancy among lineages can be remedied, however, by using a correction algorithm called the "transformed distance method". This method uses an outgroup (eg. an additional OTU, known to have diverged from a common ancestor prior to other OTUs under consideration) to find an approximate position for a root for the tree.

The "neighbour-joining" algorithm developed by Saitou & Nei (1987), estimates an additive tree from a distance matrix derived from multiply aligned sequences under the principle of minimum evolution. Starting with a star-like topology, the algorithm sequentially identifies neighbour pairs that minimize the total length of the tree upon each cycle of the process. The final tree has a parsimonious topology, with branch lengths analogous to relative times of divergence. This tree is also rooted either by assuming a molecular clock, and placing the root along the longest branch, or by including a distantly related "outgroup" that is certain to group outside the rest of the tree.

4.2 MATERIALS AND METHODS

4.2.1 Virus purification and RNA extraction

The Natal *P. caerulea* potyvirus was purified from systemically infected *N. benthamiana* (Chapter 3 section 3.2.1.1) and genomic ssRNA extracted from purified virions as described in Chapter 3 section 3.2.2.3.

4.2.2 cDNA synthesis and molecular cloning

The strategy for cDNA synthesis and molecular cloning is described in detail in Appendix B.3.13, and can be outlined as follows: Polyadenylated ssRNA of the Natal *P. caerulea* potyvirus was utilized as template for annealing of oligo-d$(T)_{12\text{-}18}$ and "random" primed cDNA synthesis, using M-MuLV reverse transcriptase (Boehringer Mannheim). Separate batches of the purified Natal *P. caerulea* potyvirus ssRNA were used for each cloning experiment. Synthesis of the second DNA strand was accomplished by the method of Gubler & Hoffman (1983), using RNase-H, DNA polymerase I and T4 DNA polymerase. Blunt-ended dsDNA was subsequently size-fractionated using Sepharose 4B (Pharmacia) column chromatography and cloned (Appendix B.3.13-14) into the

unique *Sma I* site of the pUC19 polylinker region (Yanisch-Perron *et al.*, 1985, Fig. 4.1)

White colonies, indicative of insertional inactivation of the β-galactosidase gene of the pUC19 vector (Vieira & Messing, 1982), were selected for colony hybridization assays (Buluwela *et al.*, 1989). These were probed with a [γ-^{32}P]-dATP 5' end-labelled Natal *P. caerulea* potyvirus ssRNA probe (Appendix B.3.9.2).

4.2.3 Characterization of clones

Clones which hybridized to the Natal *P. caerulea* potyvirus probe were analysed by restriction enzyme digestion and agarose gel electrophoresis to determine the size and orientation of inserts, and to identify unique restriction sites that were suitable for exonuclease III mediated shortening. The insert of a oligo-d(T)-primed 3' terminal clone was gel-purified and labelled with ^{32}P-dCTP to probe "random primed" clones for the identification of additional 3' terminal clones.

4.2.4 Exonuclease III/S1 shortening

A recombinant plasmid with a Natal *P. caerulea* potyvirus-derived insert of 1.42 kb (pW9) was selected for sequence analysis. This plasmid was subjected to unidirectional shortening from each end of the insert, using exonuclease III and S1 nuclease (Boehringer Mannheim) to produce two sets of overlapping deletion mutants for sequence analysis of each strand, as described in Appendix B.3.15.

4.2.5 Nucleotide sequence determination

Nucleotide sequencing was performed by a modification of the dideoxy chain termination method of Sanger *et al.*, (1977) using *Sequenase 2*™ (a modified T7 DNA polymerase) from a *Sequenase* version 2.0 kit obtained from US Biochemicals (Tabor & Richardson, 1987). For sequencing of the "reverse strand", 5' CAGGAAACAGCTATGAC 3' (obtained from Amersham) was used as a primer, while 5' GTTTTCCCAGTCACGAC 3' (obtained from US Biochemicals) was used as the "forward" sequencing primer. Reactions and gel electrophoresis in denaturing polyacrylamide gels were performed as described in Appendix B.3.16.

4.2.6 Sequence and molecular phylogenetic analysis of the Natal *P. caerulea* potyvirus

Computer analysis and compilation of partial nucleotide sequence data of the Natal *P. caerulea* potyvirus were done using the Genepro version 4.0 software package on an IBM-compatible microcomputer.

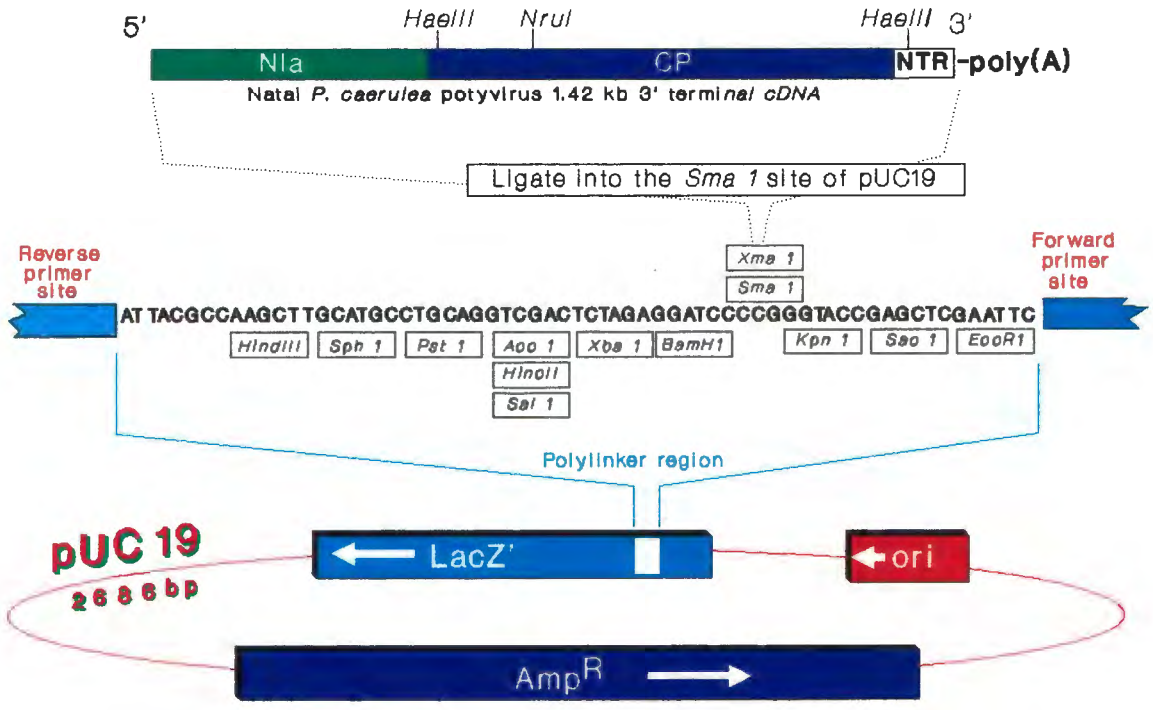


Fig. 4.1 Cloning of cDNA representing the 3'-terminal end of the Natal *P. caerulea* potyvirus genome into the pUC19 vector.

This software was used mainly for retrieving sequences of other potyviral nucleotide and amino acid sequences from the Genbank sequence database. Potyviral sequences not yet listed in GenBank were obtained from recent publications or material submitted for publication. Analysis of the coding context of the Natal *P. caerulea* potyvirus nucleotide sequence data, and prediction of amino acid sequences, were also done by using this programme.

For molecular phylogenetic analysis of the Natal *P. caerulea* potyvirus in relation to other potyviruses, the Clustal-V set of computer programmes was used (Higgins & Sharp, 1988). This package is essentially structured in five separate programmemes that are used sequentially to arrive at a phylogenetic tree. The first three programmemes are used to do multiple alignments of sequences, while the last two programmemes use the alignment output to generate and statistically evaluate a phylogenetic tree. Here follows a brief summary of the processing strategy of the package:

(i) *Pairwise similarity.* Sequences are fed into the programme in a single file and can be in NBRF/PIR, EMBL/SwissProt or FASTA format. The algorithm of Wilbur and Lipman (1984) is then used to do a fast calculation of pairwise similarities between OTUs by comparison of pairwise aligned sequences. The similarity scores are basically calculated as the number of exactly matching residues between two sequences in optimal alignment, minus a fixed penalty for each gap.

(ii) *Cluster analysis.* The similarity matrix obtained from (i) is processed further by the UPGMA algorithm (Sneath & Sokal 1973) to obtain a crude cluster representing the similarity of sequences as a hierarchy.

(iii) *Multiple alignment.* Clustered sequences from (ii) are taken and aligned again using the Wilbur and Lipman (1984) approach, but with two modifications to increase sensitivity. Firstly, the analysis takes into account conservative substitutions in protein alignments, and secondly it allows a small degree of mismatch in the consensus sequences (sequentially updated to a workfile after each alignment).

(iv) *Phylogenetic analysis with "NJTREE".* The multiply aligned sequence data is further processesed by the "NJTREE" programme of Saitou & Nei (1987) to calculate a phylogenetic tree as discussed in 4.1.2.2.

4.3 RESULTS AND DISCUSSION

4.3.1 cDNA cloning

Yields of double stranded cDNA synthesized *in vitro* from the Natal *P. caerulea* potyvirus ssRNA templates varied between 7 and 14%. This seemingly low efficiency is mainly attributed to the first strand synthesis reaction, as second strand synthesis constantly produced an efficiency rate of higher than 90%. Transformation of competent *E. coli* LK111 with ligation mix consisting of 40 ng Natal *P. caerulea* potyvirus-cDNA and 13 ng *Sma I* linearized pUC19 yielded eighty four white transformants in the case of oligo-d(T) primed cDNA and hundred and twenty seven white colonies in the case of "random primed" cDNA. Forty eight of each were selected for colony hybridization assays, of which nine oligo-d(T) primed cDNA clones and twenty three "random primed" clones hybridized to the [γ-^{32}P]-dATP 5' end-labelled Natal *P. caerulea* potyvirus ssRNA probe.

4.2.2 Characterization of clones

In total 32 out of 96 colonies hybridized positively when probed with [γ-^{32}P]-dATP end-labelled Natal *P. caerulea* potyvirus ssRNA (Fig. 4.2a & b. Recombinants with Natal *P. caerulea* potyvirus-derived insert sizes exeeding 500 bp were selected and each was linearized with an appropriate restriction enzyme with preferably one site in the vector, but none in the insert region. These were then run in a 0.8% agarose gel (Fig. 4.2c & d) to estimate insert sizes.

Recombinant plasmids pW1 and pW9 had the largest inserts (approximately 1.4 Kb each) of the oligo-d(T)-primed cDNA clones (pW1 - pW9). Of the "random primed" cDNA clones pW12, pW14, pW24 and pW30 had the largest inserts (approximately 2.2 - 2.4 Kb each).

It was decided to use pW9 for exonuclease III shortening and sequencing, as it seemed very probable that a 1.4 Kb long 3' terminal clone would encompass the entire Natal *P. caerulea* potyvirus CP gene. The aim for characterizing this clone, was mainly to sequence the entire CP-gene, and to subclone this gene into appropriate constructs for expression in *E. coli* and transgenic tobacco.

To establish whether the "random primed" cDNA clones contained inserts overlapping with the 3' terminal pW9 clone, the insert of pW9 was excised from the parent pU19 by *Pst I-EcoR I* double digestion and gel-purified. It was labelled with ^{32}P-dCTP by nick translation (Appendix B.3.9.1) and used to probe DNA of "random primed" clones pW10 - pW31 spotted onto Hybond-N (Amersham). Eight of the "random primed" clones hybridized positively, and thus overlapped with pW9 (Fig. 4.3). The largest "random primed" clone that hybridized to this probe was pW12 (±2.4Kb).

LEGENDS:

Fig. 4.2a & b Colony hybridization using [γ-^{32}P]-dATP end-labelled Natal *P. caerulea* potyvirus ssRNA as probe to identify *E. coli* LK111 colonies containing recombinant plasmids with potyvirus-derived cDNA inserts.

Fig. 4.2c & d Gel electrophoresis of Natal *P. caerulea* potyvirus-derived cDNA cloned into the Sma 1 site of pUC19 (DNA was linearized with appropriate restriction enzymes).

Fig. 4.2c Lane 1, λ (*Sty 1*). Lane 2, pW1 (*EcoR1*). Lane 3, pW2 (*EcoR1*). Lane 4, pW7 (*EcoR1*). Lane 5, pW8 (*EcoR1*). Lane 6, pW9 (*EcoR1*). Lane 7, pW10 (*EcoR1*). Lane 8, pW11 (*BamH1*). Lane 9, pW12 (*BamH1*). Lane 10, pW14 (*EcoR1*). Lane 11, pW15 (*Pst1*). Lane 12, pW16 (*BamH1*). Lane 13, pUC18 (*BamH1*

Fig. 4.2d Lane 1, λ (*Sty 1*). Lane 2, pW17 (*BamH1*). Lane 3 pW20 (*EcoR1*). Lane 4, pW21 (*BamH1*). Lane 5, pW22 (*EcoR1*). Lane 6, pW23 (*BamH1*). Lane 7, pW24 (*BamH1*). Lane 8, pW25 (*BamH1*). Lane 9, pW27 (*EcoR1*). Lane 10, pW29 (*BamH1*). Lane 11, pW30 (*BamH1*). Lane 12, pW31 (*BamH1*). Lane 13, pUC18 (*EcoR1*).

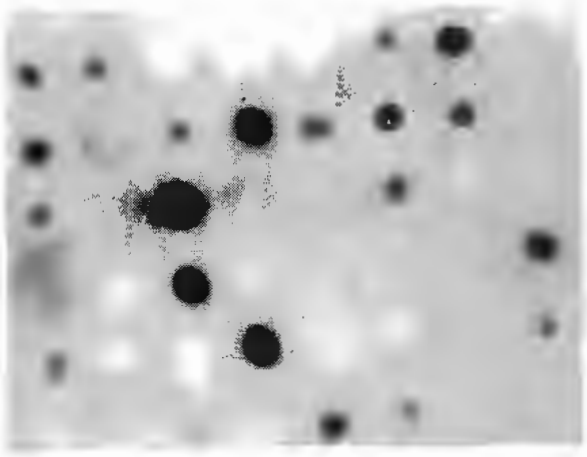


Fig. 4.2a

Fig. 4.2b

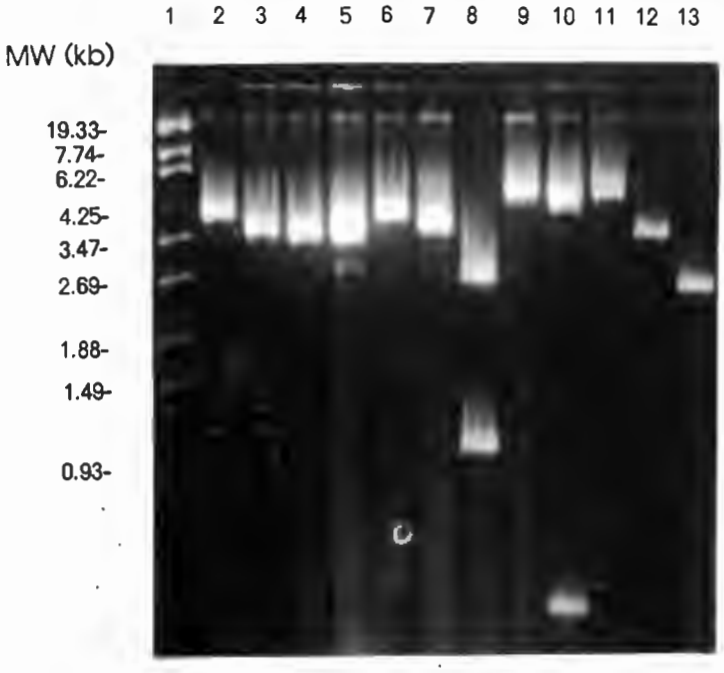


Fig. 4.2c

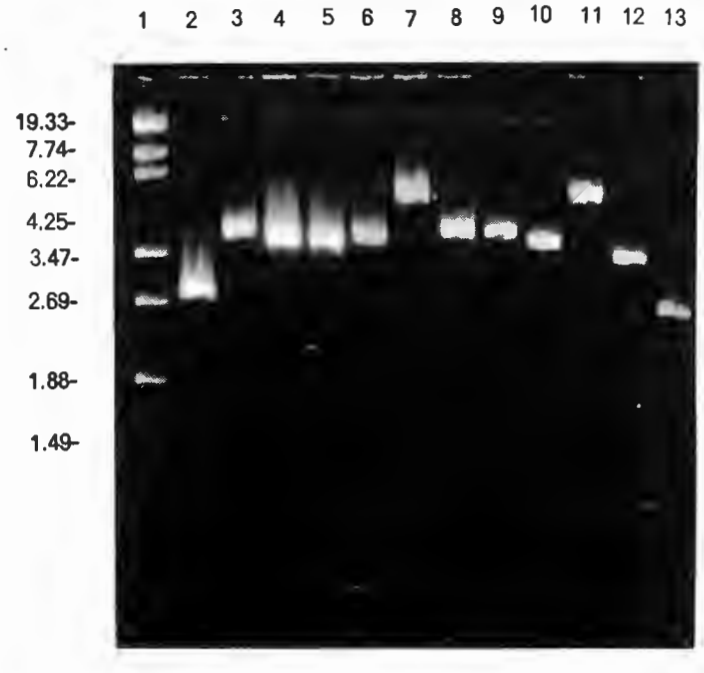


Fig. 4.2d

Plasmids pW9 and pW12 were digested with pUC19 polylinker restriction enzymes, to find unique restriction sites flanking the insert regions that could be used for subcloning or exonuclease III shortening (Figs. 4.4a & b). None of the polylinker restriction enzymes had internal recognition sites in pW9, which facilitated easy exonuclease III shortening.

4.2.3 Exonuclease III/S1 shortening

Progressively deleted constructs were selected so to produce two sets of overlapping deletion mutants, each extending through the entire pW9 insert region (Figs. 4.5 a & b). Deletion increments selected for sequence analysis were in the order of 200 to 400 bp. Seven overlapping deletion mutants were selected for sequencing the "forward primer" DNA strand and eight for sequencing the "reverse primer" strand.

4.3.4 Nucleotide sequencing

The addition of 10% (v/v) DMSO to all steps after the primer annealing reaction greatly enhanced the resolution and contrast of bands, and reduced the background and GC compressions. Typically 200 to 250 bases could be read from a 6% normal sequencing gel that was run for 60 min, while 4.8% gels run for 2-4 h resolved up to 450 bases. One of the deletion templates in the "forward primer" strand produced illegible results, and no other deletion mutant of this particular size could be found. A restriction site just upstream of this illegible stretch of sequence (*Nru I* at base 479 from the "forward priming site") was derived from sequence information obtained from the "reverse primer" strand. pW9 was subsequently cut with *Nru I* and *EcoR I*, and ends were blunted by sequential S1 nuclease and Klenow reactions as described for exonuclease shortening in Appendix B.3.15. The resulting plasmid was self-religated, used to transform *E. coli* LK111 (Figs. 4.6a & b), and sequenced with the forward primer to resolve sequence in this region.

4.3.5 Sequence and molecular phylogenetic analysis of the Natal *P. caerulea* potyvirus in context of the potyvirus group

4.3.5.1 Sequence analysis of the pW9 clone

Sequence analysis of the 1436 nt long pW9 insert confirmed the presence of a 1188 nt ORF starting at position 1, which presumably initiates upstream of the clone as occurs with other partially-sequenced potyviruses (Allison *et al.*, 1986; Domier *et al.*, 1986; Maiss *et al.*, 1989). The ORF terminates in a UAA stop codon at position 1189, followed in frame by two UAG codons in tandem at position 1235, and a short potential ORF of 146 nt with an AUG at position 1288, which terminates in the poly(A) tail.

LEGENDS:

Fig. 4.3 Dot-blot using *EcoR1-BamH1* gel purified insert of pW9 (^{32}P-dCTP-labelled) as a probe to identify Natal *P. caerulea* potyvirus derived clones (pW-) with overlapping sequences. A1, pW10. A2, pW11. A3, pW12. A4, pW13. A5, pW14. A6, pW15. A7, pW16. A8, pW17. B1, pW18. B2,pW19. B3, pW20. B4, pW21. B5, pW22. B6, pW23. B7, pW24. B8, pW25. C1, pW26. C2, pW27. C3, pW28. C4, pW29. C5, pW30. C6, pW31.

Fig. 4.4a Restriction analysis of pW9. Lane 1, λ (*Sty 1*). Lane 2, pW9 *(Pst I)*. Lane 3, pW9 *(Acc I)*. Lane 4, pW9 *(BamHI)*. Lane 5, pW9 *(EcoRI)*. Lane 6, pW9 *(Sac I)*. Lane 7, pW9 *(Hind III)*. Lane 8, pW9 *(Sph I)*. Lane 9, pW9 *(Kpn I)*. Lane 10, pW9 *(Hinc II)*. Lane 11, pW9 *(Sac I + Pst I)*. Lane 12, pW9 *(Pst I + BamHI)*. Lane 13, pW9 *(BamH1 + BstEII)*. Lane 14, pW9 *(BamH1 + Nru I)*. Lane 15, pW9 *(BamHI + EcoRV)*. Lane 16, λ (*Sty 1*)

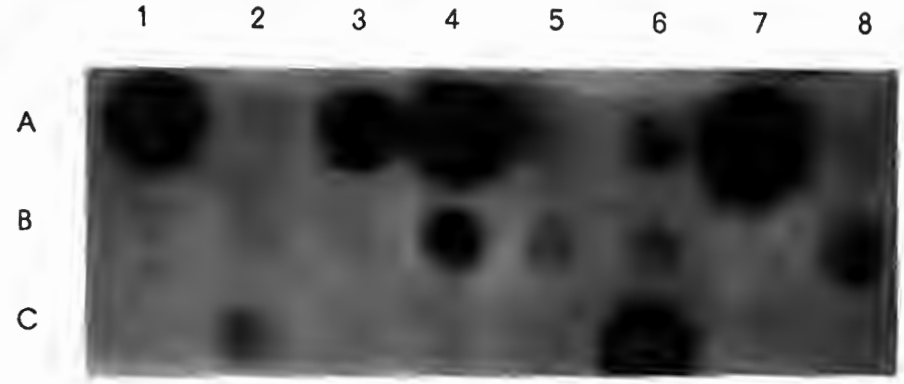


Fig. 4.3

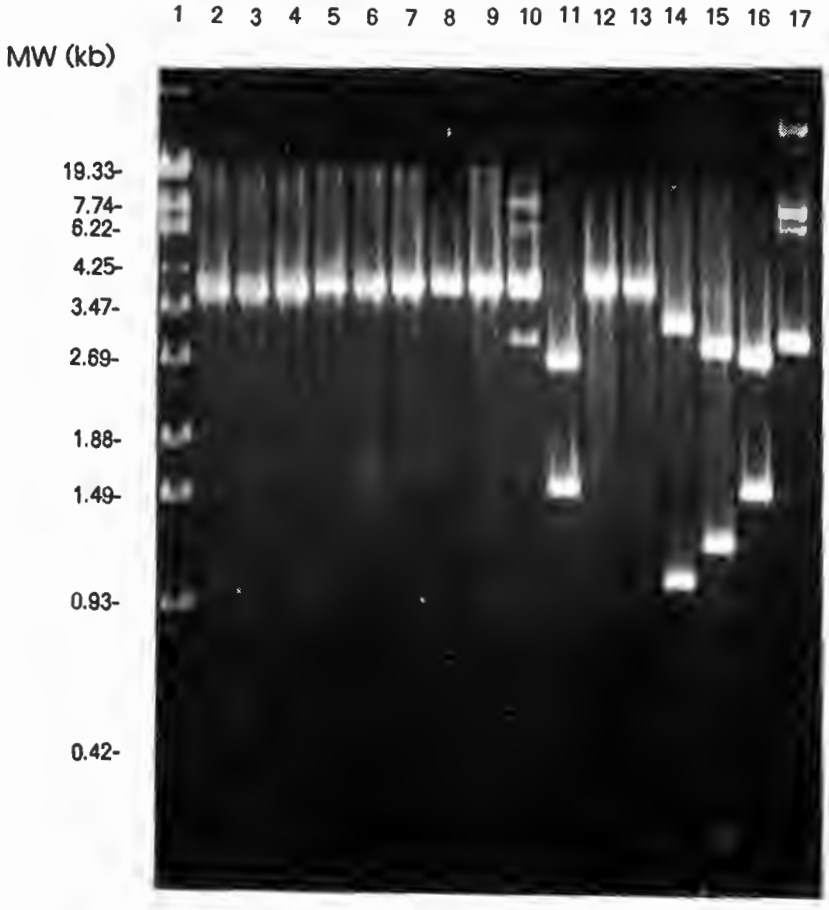


Fig. 4.4a

LEGENDS:

Fig. 4.4b Restriction analysis of pW9. **Lane 1**, pW9 *(Mlu I)*. **Lane 2**, pW9 *(Hpa I)*. **Lane 3**, pW9 *(Nae I)*. **Lane 4**, pW9 *(Sfi I)*. **Lane 5**, pW9 *(Xho I)*. **Lane 6**, pW9 *(Sac II)*. **Lane 7**, pW9 *(BstXI)*. **Lane 8**, pW9 *(Apa I)*. **Lane 9**, pW9 *(BstEII)*. **Lane 10**, pW9 *(Bgl II)*. **Lane 11**, pW9 *(Bcl I)*. **Lane 12**, pW9 *(Nco I)*. **Lane 13**, pW9 *(Nru I)*. **Lane 14**, pW9 *(EcoRV)*. **Lane 15**, pW9 - uncut **Lane 16**, λ (*Sty 1*).

Fig. 4.5a Exonuclease III/S1-nuclease shortening of pW9 for sequence analysis **Lanes 1 & 18**, λ (*Sty 1*), **Lanes 2 to 8**, deletion mutants for sequencing with the pUC19 reverse primer. **Lanes 10 to 16**, deletion mutants for sequencing with the pUC19 forward primer. **Lane 17**, pUC 19 linearized with *Pst I*.

Fig. 4.6a pWΔ71 - an *Nru I - EcoRI* deletion mutant of pW9 for sequencing with the reverse primer. **Lane 1**, λ (*Sty 1*). **Lane 2**, pW9 linearized with *Pst I*. **Lane 3**, pWΔ71.

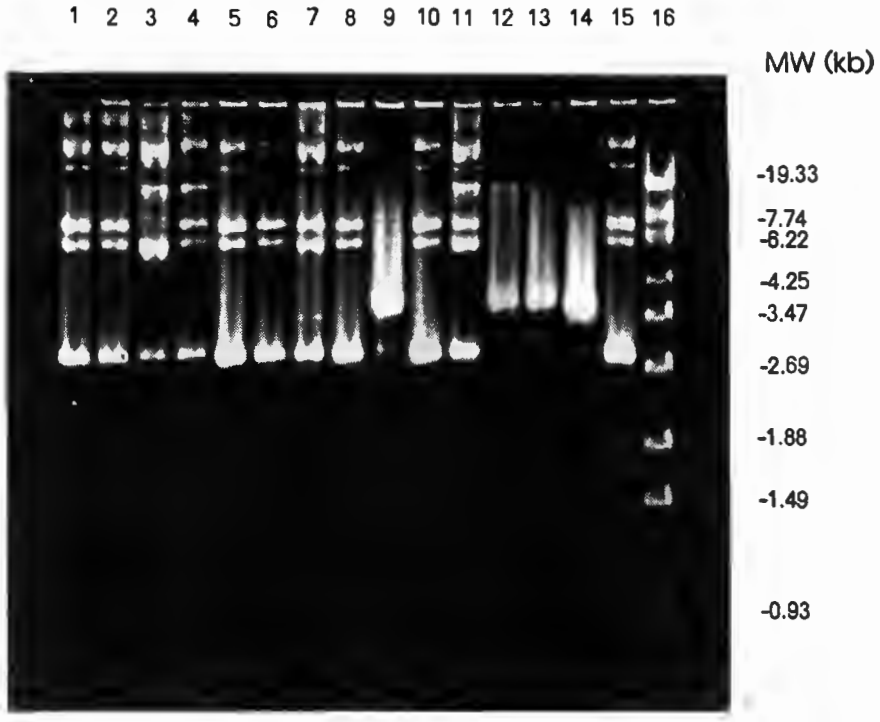


Fig. 4.4b

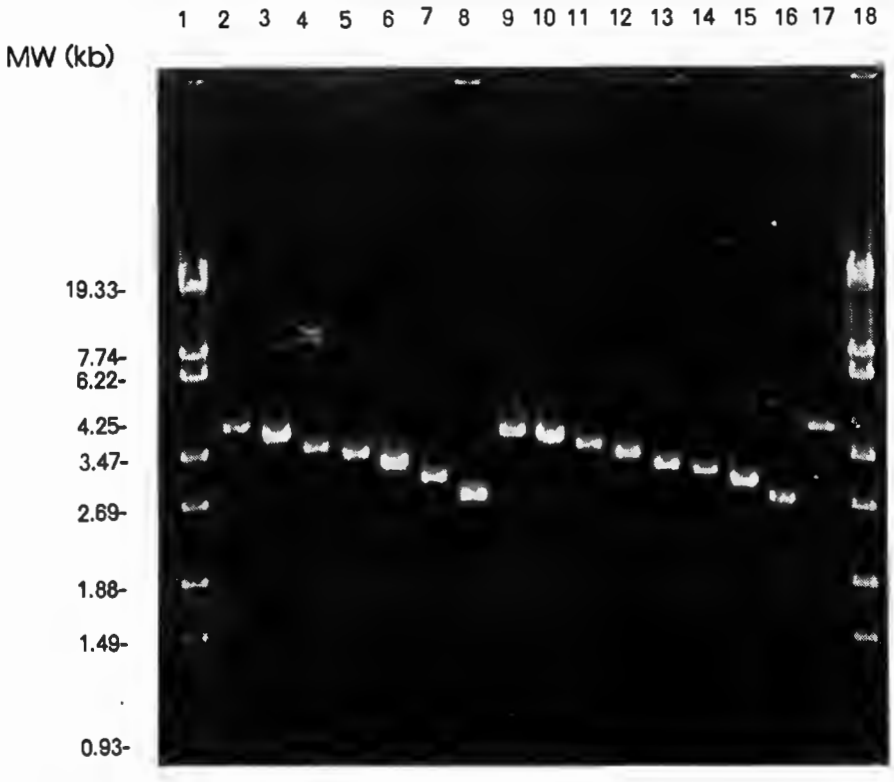


Fig. 4.5a

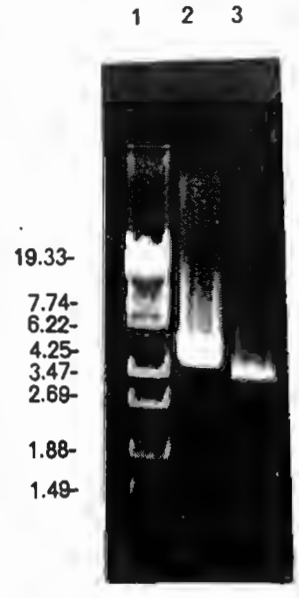


Fig. 4.6a

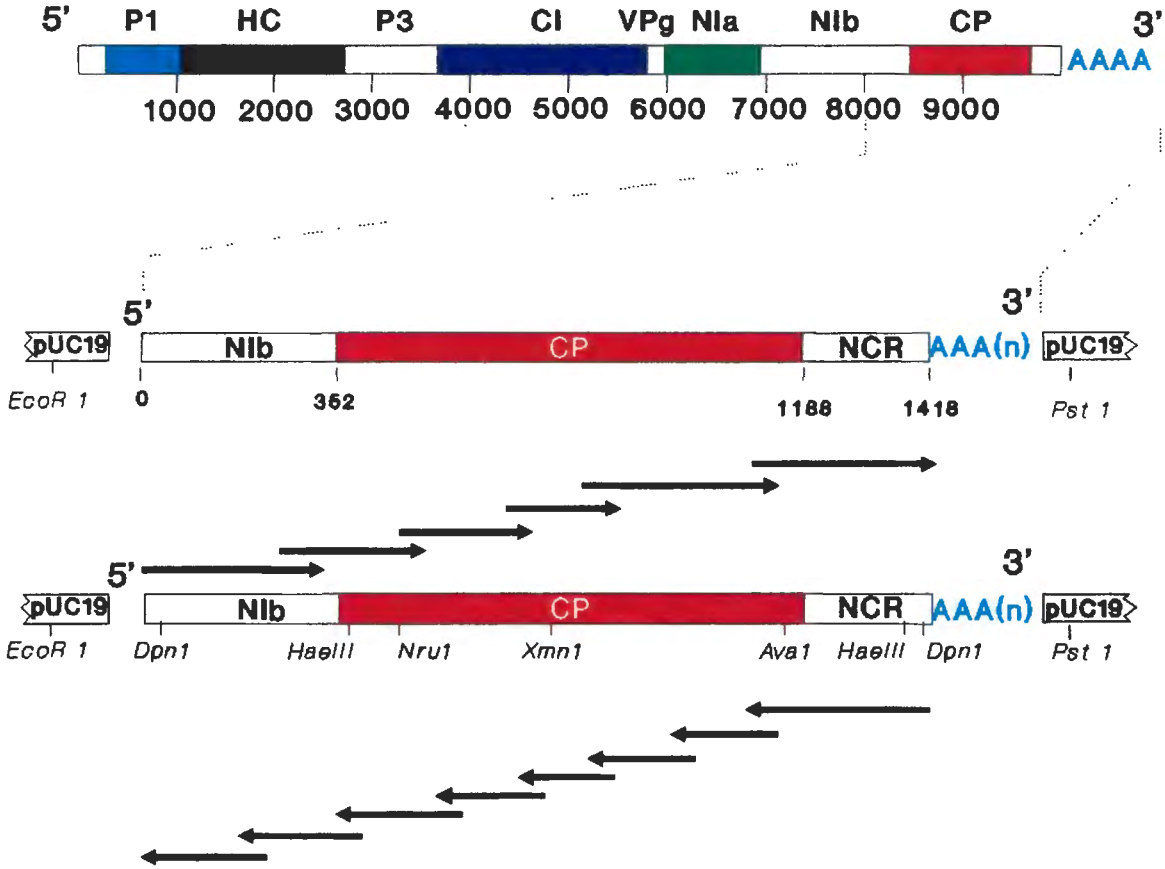
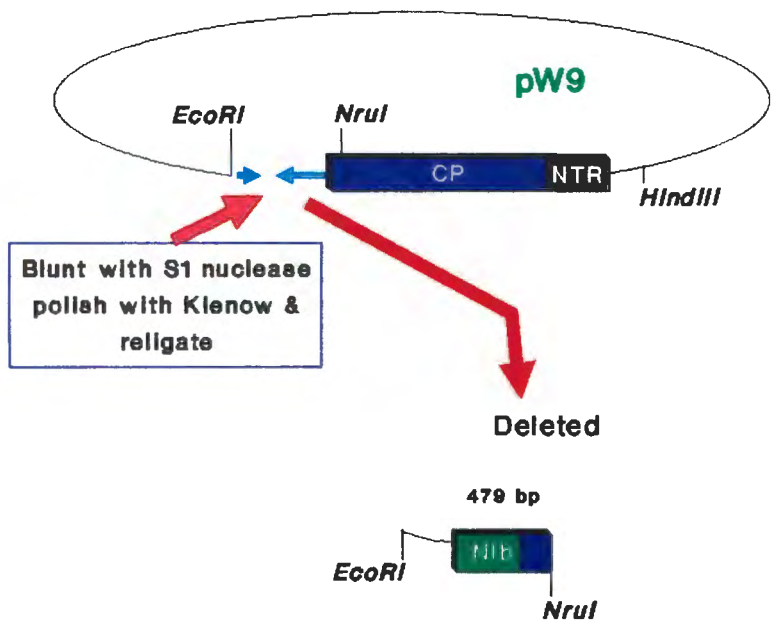


Fig. 4.5b (above) Exonuclease III/S1 shortening of the pW9 clone to create two sets of overlapping deletion mutants for nucleotide sequence analysis of the 3' terminal region of the Natal *P. caerulea* potyvirus.

Fig. 4.6b (below) Deletion of a *Nru I - EcoRI* fragment from pW9 followed by self-religation to create a deletion mutant for sequence analysis.



Both other forward reading frames contained a number of stop codons within the presumptive 3' NCR of 230 bp, which ends in a stretch of 18 adenosine residues (Fig. 4.7). Limited sequence analysis of the pW12 random primed clone showed that the 3' terminal region is identical to the pW9 insert (results not shown). This clone originated from a different batch of purified Natal *P. caerulea* potyvirus, but from the same diseased *P. caerulea* specimen.

4.3.5.2 Sequence analysis of the Natal *P. caerulea* potyvirus CP

The Natal *P. caerulea* potyvirus CP gene was located in the long 5' ORF by comparative analysis and alignment with sequences of other PWV and potyvirus CPs (Fig. 4.8), and by searching for the consensus polyprotein cleavage sites (Q/S, Q/G or Q/A) proposed for other potyviruses (Domier *et al.*, 1986; Robaglia *et al.*, 1989; Carrington & Dougherty, 1987b; Dougherty *et al.*, 1989). Two potential proteolytic processing sites were found (both Q/S), at amino acid residue positions 117/118 and 140/141 respectively (see Fig. 4.7). Cleavage at the former position would result in a CP of 31.7 kDa; processing at the latter a 29.1 kDa polypeptide. The first position (117/118) is proposed as the real cleavage site, as it would produce a CP nearer the size of 33 kDa previously estimated for the Natal *P. caerulea* potyvirus CP by SDS-PAGE (Brand & von Wechmar, unpublished). The cleavage site at aa 140/141 is very unlikely, as it would exclude a CP N-terminal motif (-DAG-), which has been proposed to be essential for aphid transmissibility of potyviruses (Harrison & Robinson, 1988; Atreya *et al.*, 1990; Ward & Shukla, 1991). The "DAG" triplet occurs at aa position 126 in the deduced polypeptide sequence (Fig. 4.7): its presence is in accord with the aphid-vectored transmission previously reported for Natal *P. caerulea* potyvirus (Brand & von Wechmar, unpublished). A block of alternating lysine residues - often found in the N-termini of potyviral coat proteins (-KDKEK-) - occurs immediately downstream of the DAG motif, between positions 128 & 136. Many potyviral coat proteins are subject to partial proteolysis, usually in the surface-directed N-terminal region (Shukla *et al.*, 1988c). The N-terminus of the CP of ornithogalum mosaic potyvirus (OMV), previously sequenced by the same authors (Burger *et al.*, 1990), has a much lower lysine content than the corresponding region of the Natal *P. caerulea* potyvirus. It was also distinctly more stable, and much less subject to degradation. It is therefore fair to speculate that the high lysine content of N-termini of many potyviral coat proteins is perhaps involved in the ubiquitin-targeted protein destruction typical of eukaryotic cells (Ciechanover *et al.*, 1984).

```
CTC GTG GAT GAC TTG TAC ATA CCA AAG CTA GAG CAA GAA AGA ATT GTG TCA ATC TTG GAA      60
L   V   D   D   L   Y   I   P   K   L   E   Q   E   R   I   V   S   I   L   E     -396

TGG GAT CGA AGC AAA GAA CTG CTA CAC AGA ACA GAG GCT GTT TGT GCA GCA ATG ATT GAG     120
W   D   R   S   K   E   L   L   H   R   T   E   A   V   C   A   A   M   I   E     -376

GCA TGG GGA TAT CCT GAA CTG TTG CAG GAA ATC AGA AAG TTT TAC CTT TGG TTG CTG CAC     180
A   W   G   Y   P   E   L   L   Q   E   I   R   K   F   Y   L   W   L   L   H     -356

AGA GAC GAG TTT AAG GAG TTA GCT AGC CTT GGA AAA GCT CCT TAC ATT GCA GAA ACC GCC     240
R   D   E   F   K   E   L   A   S   L   G   K   A   P   Y   I   A   E   T   A     -336

CTT AGG AAA CTT TAC ACT GAC GAA CGA GCC TCG GAG AAG GAA TTA CAG AGG TAT CTT CAA     300
L   R   K   L   Y   T   D   E   R   A   S   E   K   E   L   Q   R   Y   L   Q     -316

GAT ATC CTT TCC TTC TAT GAT GAT TGT GAA TCG GAA GAT GTT GTG CTT CAA TCC GGC CGA     360
D   I   L   S   F   Y   D   D   C   E   S   E   D   V   V   L   Q   S   G   R     -296
                                                            ←Nib]  [CP→

AAG CAA GAA GAG TTG GAC GCG GGC AAG GAT AAA GAG AAA ACG AAG GAA GGC GAA GAG CAA     420
K   Q   E   E   L   D   A   G   K   D   K   E   K   T   K   E   G   E   E   Q     -276

TCA ACG CAG CAG AAG CAA ACA AAG GAC AAA GGA ACC AAG GAA ACA GAG AGG GAC GTC GCG     480
S   T   Q   Q   K   Q   T   K   D   K   G   T   K   E   T   E   R   D   V   A     -256

ACT AGT TCT TCA GGA CAA CTA GTC CCA CGC TTG CAG AAA ATC GGC AAA AAG ATG AAT CTC     540
T   S   S   S   G   Q   L   V   P   R   L   Q   K   I   G   K   K   M   N   L     -236

CCC ATG GTA GCT GGT AAG GTT ATT CTT GAC TTG ACC CAT TTA ATA GAG TAT AAG CCA GCA     600
P   M   V   A   G   K   V   I   L   D   L   T   H   L   I   E   Y   K   P   A     -216

CAG ATT GAC CTG TAC AAC ACC AGA GCA TCA AAG ACA CAG TTC AAC AAG TGG TTT GAA GCC     660
Q   I   D   L   Y   N   T   R   A   S   K   T   Q   F   N   K   W   F   E   A     -196

ATC AAA GAG GAG TAT GAA TTG GAT GAT GAC AAG ATG GGA GTA ATT ATG AAC GGT TTC ATG     720
I   K   E   E   Y   E   L   D   D   D   K   M   G   V   I   M   N   G   F   M     -176

GTT TGG TGC ATC GAA AAT GGA ACT TCA CCT GAT GTT AAT GGA GTG TGG ACT ATG ATG GAT     780
V   W   C   I   E   N   G   T   S   P   D   V   N   G   V   W   T   M   M   D     -156

GGG GAT GAA CAA GTG GAA TTT CCA CTC AAG CCC ATC GTG GAG AAC GCA AAA CCC ACA CTT     840
G   D   E   Q   V   E   F   P   L   K   P   I   V   E   N   A   K   P   T   L     -136

CGA CAG ATT ATG CAC CAT TTC TCA GAC GCA GCT GAA GCG TAC ATA GAG ATG AGA AAT TCC     900
R   Q   I   M   H   H   F   S   D   A   A   E   A   Y   I   E   M   R   N   S     -116

GAA GGG TTC TAC ATG CCC AGG TAC GGA CTG CTT AGG AAT TTG AGG GAT AAG AGC TTG GCA     960
E   G   F   Y   M   P   R   Y   G   L   L   R   N   L   R   D   K   S   L   A     -96

AGG TAT GCT TTC GAC TTC TAT GAG GTT ACA TCC AAA ACT CCT GAC AGA GCA AGG GAA GCA    1020
R   Y   A   F   D   F   Y   E   V   T   S   K   T   P   D   R   A   R   E   A     -76

ATA GCA CAA ATG AAA GCC GCA CGT CTC GCC AAC GTT AAT ACC AGG ATG TTT GGC TTG GAT    1080
I   A   Q   M   K   A   A   R   L   A   N   V   N   T   R   M   F   G   L   D     -56

GGA AAC GTG GCA ACA ACT AGT GAG AAC ACT GAG AGG CAC ACT GCA ACT GAT GTG AAT CAG    1140
G   N   V   A   T   T   S   E   N   T   E   R   H   T   A   T   D   V   N   Q     -36

AAC ATG CAT TCC CTT CTC GGG ATG ACG CAT GGG ATG ACG CAT GGG CAG TAA AGG TTT GGG    1200
N   M   H   S   L   L   G   M   T   H   G   M   T   H   G   Q   *                  -16

TTC CGC CCT CAC CAC AGT TAT CGT CTC ACG AAC TTA GCT AAT GGT TGG TTT TAG TAG ATA    1260

GTA CGC ATA TAT CCA GTT AAA CTA TAT ATG TTA GTG TGG GTC ACC CAC CGG TTG CGT TAT    1320

GTT ACT TTA ATT GAA CTT AGC GGA GAG GCC ATA CCC CGA TCG GAG TGC TCA GAG TGG TTT    1380

TAC CAT GGT AGT CAT CGA GAT TGG TAT GAA AAC TCT CTA AAA AAA AAA AAA AAA AA          1436
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Fig. 4.7 Nucleotide sequence of the 3'-terminal region of the Natal *P. caerulea* potyvirus. The predicted amino acid sequence derived from the long 5'-open reading frame is shown. The predicted polyprotein cleavage site between the coat protein and the Nlb is printed in ***large bold italic*** and indicated by arrows. An alternating lysine rich region occurring in the N-terminus of the CP, is also printed in bold. Repetitive sequences in the 3'-non coding region are underlined.

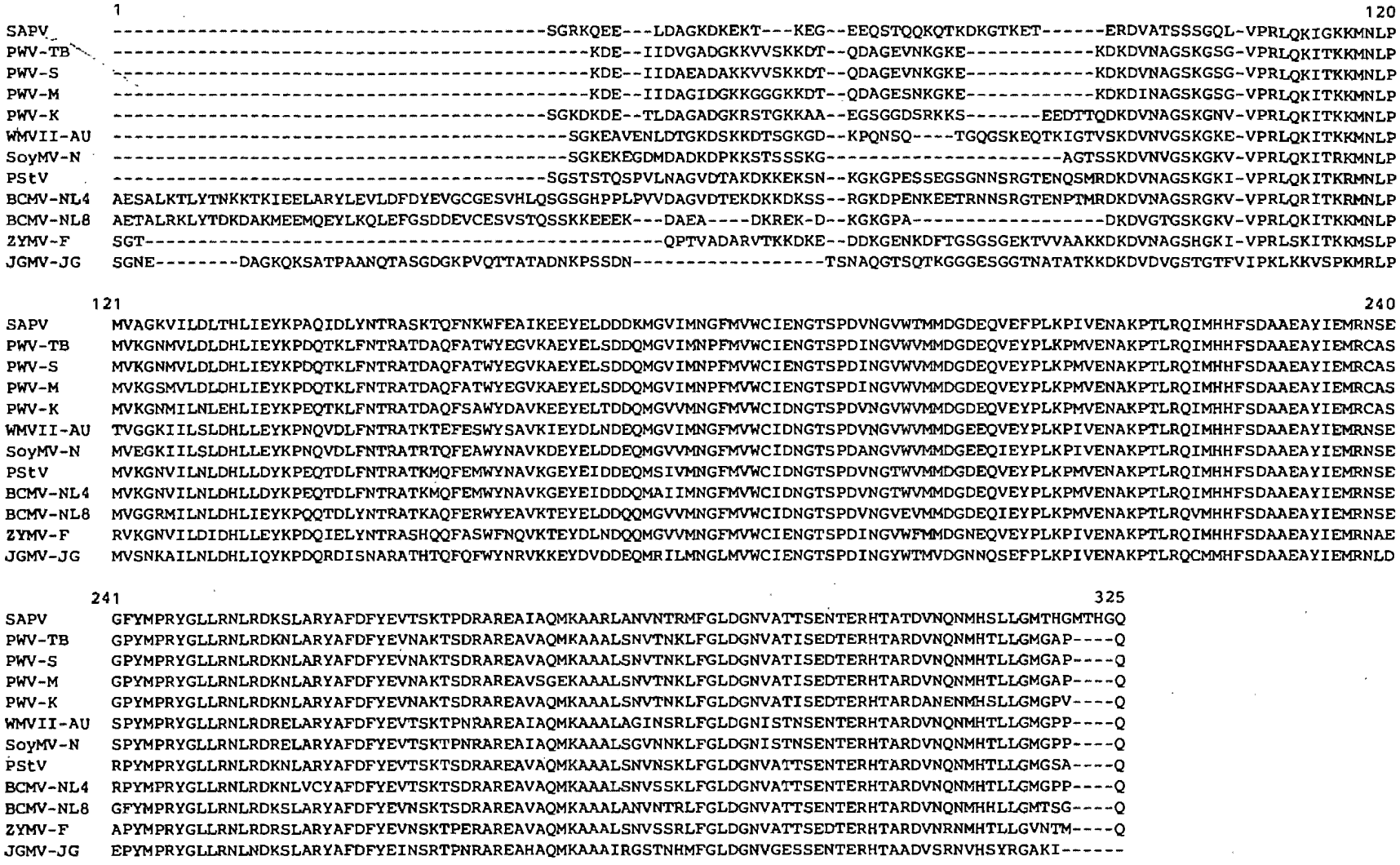

Fig. 3. Alignment of the sequence of the SAPV (=Natal *P. caerulea* potyvirus) coat protein with those of other potyviruses. The deduced coat protein sequence of SAPV was aligned with those of other viruses in the "PWV subgroup", and with JGMV-JG: sequences were obtained from sources detailed in Rybicki and Shukla (1992). BCMV=bean common mosaic virus; JGMV-JG=Johnsongrass mosaic virus strain JG; PStV=peanut stripe virus; PWV=passionfruit woodiness viruses; SoyMV-N=soybean mosaic virus strain N; WMVII-US=watermelon mosaic virus 2, Australian strain; ZYMV=zucchini yellow mosaic virus. Sequences were aligned using CLUSTALV on a VAX 6000/330 mainframe.

4.3.5.3 Phylogenetic analysis of the Natal *P. caerulea* potyvirus based on CP sequence data

It is currently accepted that members of the proposed potyvirus genus have a bimodal distribution of CP amino acid sequence similarities, with distinct members being in the range of 38 to 71% similarity, while strains of given viruses are usually more than 90% similar (Shukla & Ward, 1989b). A recent reappraisal of the relationships among 45 potyvirus CPs - 27 distinct members and 18 strains, and 990 pairwise comparisons (Rybicki, unpublished) - has shown that most accepted strains fall into the 93-100% sequence identity range, while accepted distinct potyviruses range in similarity from 42-80%, with a major peak from 48-71% (891/990), and a "tail" from 72-83% (58/990). This is direct amino acid homology: it should be noted that the calculation of similarity was by the widely-accepted method of dividing the number of direct sequence matches by the length of the shorter sequence, excluding gaps. Sequence similarity between the Natal *P. caerulea* potyvirus CP and CPs of other members of the potyvirus genus varies from 52% to 79% (Rybicki, unpublished). Table 4.1 and Fig. 4.9 shows comparisons between the Natal *P. caerulea* potyvirus and other members of the "PWV subgroup" of the potyviruses: this is a distinct group of viruses forming a related cluster, and includes BCMVs, SoyMV-N, WMV2s, ZYMVs, and PWVs (Ward and Shukla, 1991; Rybicki and Shukla, in press; Ward *et al.*, in press). The Natal *P. caerulea* potyvirus clearly clusters with these viruses (68-79% identity), and is as distinct from JGMV-JG (40% difference) as this is different from all others in the subgroup. Equally clearly it does not cluster specifically with the known PWVs (1-16% difference). At 71% similarity to PWV-TB and 79% to BCMV-NL8, the Natal *P. caerulea* potyvirus is certainly well outside the range of similarity of 90% or higher proposed for strains of a virus, and within the "tail" of 72-83% homology observed amongst distinct viruses. This suggests that the Natal *P. caerulea* potyvirus is a distinct virus within the PWV-related subgroup of generic potyviruses.

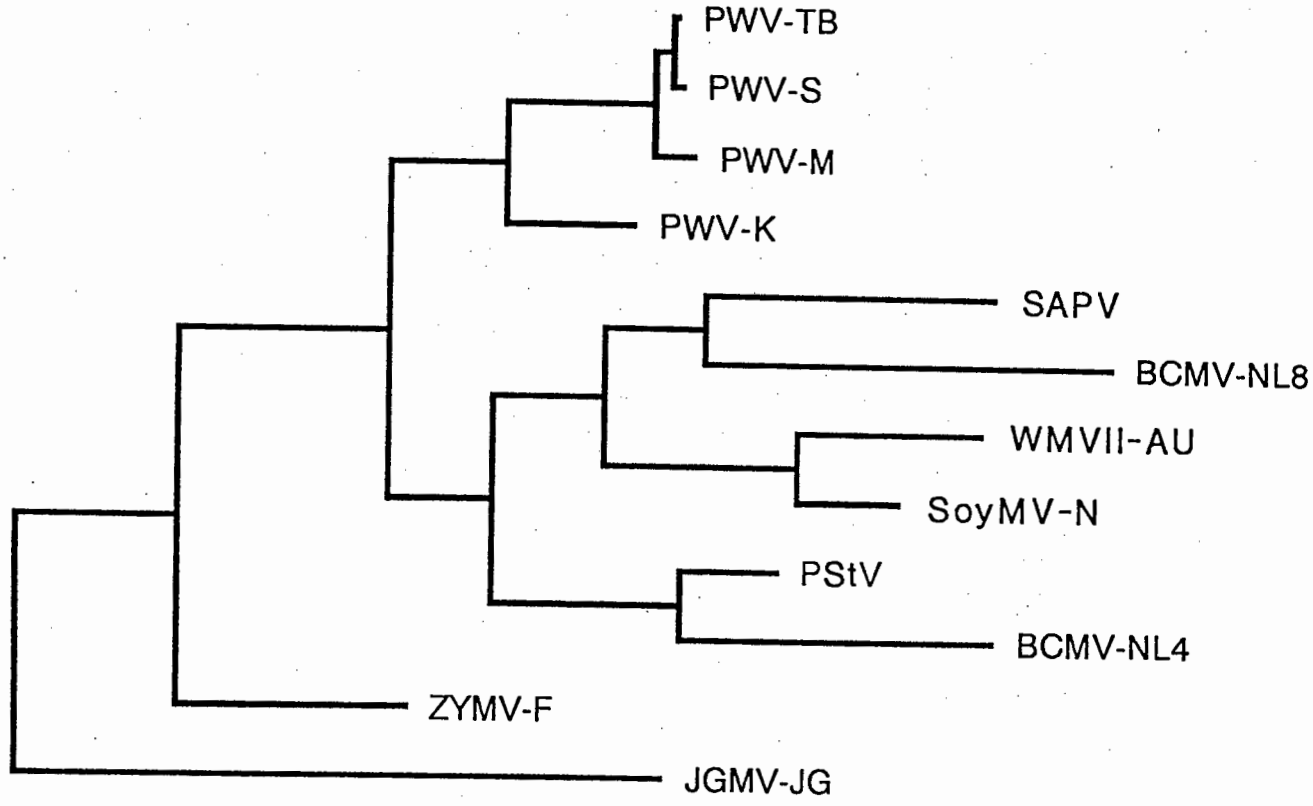

Fig. 4.9 Unrooted phylogenetic tree calculated from potyvirus coat protein distance data using the NJTREE program.

Table 4.1 Pairwise percent sequence difference matrix for SAPV CP and selected other potyvirus CPs

		2	3	4	5	6	7	8	9	10	11	12
1	SAPV	29	30	29	29	26	26	30	30	21	32	40
2	PWV-S		05	15	01	28	24	25	24	21	25	38
3	PWV-M			16	04	29	26	26	26	23	28	39
4	PWV-K				15	29	24	23	25	20	29	40
5	PWV-TB					27	24	25	24	22	25	38
6	WMVII-AU						13	27	28	23	31	39
7	SoyMV-N							23	24	22	27	34
8	PStV								11	18	29	40
9	BCMV-NL4									25	32	49
10	BCMV-NL8										26	43
11	ZYMV-F											37
12	JGMV-JG											

Legend:
Percent pairwise differences calculated using DISTANCES option in PAUP 3.0 on CP sequence alignment produced using CLUSTAL in Figure 3. The distance is the product, expressed as a percentage, of the number of direct sequence matches divided by the length of the shorter sequence, excluding gaps, subtracted from 100.

4.3.5.4 Analysis of the 3' NCR

Comparison of the 3' NCR nucleotide sequence of potyviruses can yield particularly useful information for the distinction of strains, as similarity between strains of a virus usually ranges from 83 to 99%, whereas for distinct viruses it ranges between 39 to 53%, which is effectively non-significant or "random" similarity (Frenkel *et al.*, 1989; Burger *et al.*, 1990). Unfortunately the 3' NCR sequences for PWV-TB and the other closely related strains have not been determined. However, the 3'NCR sequence of a more distantly related Australasian PWV strain, PWV-K, has now been elucidated (Gough & Shukla, in press): although the N-terminus of the CP of this strain is very divergent from other Australasian PWV strains, it clusters consistently with them in all phylogenetic analyses based on CP sequence data (Rybicki and Shukla, in press; Rybicki, unpublished). The 3' NCR of PWV-K shows only 50% homology with the Natal *P. caerulea* potyvirus, however, which is even lower than the 56% similarity between the 3' NCR of the Natal *P. caerulea* potyvirus and WMV2 (Table 4.2).

The 3' NCR of the Natal *P. caerulea* potyvirus contains two direct repeats, GAACTTAGC and TGGTTTTAG respectively (underlined in Fig. 4.7). Repeat motifs in the 3' NCR have also been reported for a number of other potyviruses (Allison *et al.*, 1985; Dougherty *et al.*, 1985; Domier *et al.*, 1986; Van der Vlugt *et al.*, 1989).

TABLE 4.2. Pairwise percent sequence similarities between potyviral genomic 3'-non-coding regions.

Virus (3'-NCR)[1]	Percent sequence identity[2]												
	2	3	4	5	6	7	8	9	10	11	12	13	14
1 PeMV (337)	81	39	39	36	35	28	31	30	29	31	32	23	27
2 PVY-N (331)		40	41	34	32	26	33	33	29	30	32	24	26
3 TEV-HAT (189)			98	50	34	36	28	27	35	31	26	26	24
4 TEV-NAT (189)				51	34	35	29	28	35	32	26	26	24
5 BYMV (169)					33	33	27	26	31	31	26	27	27
6 TVMV (252)						29	28	27	29	28	32	29	28
7 OMV (274)							25	25	29	27	26	26	30
8 PPV-D (218)								97	32	36	31	27	28
9 PPV-NAT (220)									31	34	30	28	28
10 WMV2 (251)										78	54	36	27
11 SMV-N (259)											56	34	25
12 PWV-C (250)												33	32
13 SAPV (234)													31
14 SCMV (475)													

Key:
1 = length of 3'-NCR
2 = calculated using Distances (GCG) on sequences aligned using PileUp: figure is the fraction of division of total sequence matches by length of the shorter sequence, excluding gaps.

4.3.5.5 Concluding notes on the designation and classification of the Natal *P. caerulea* potyvirus

Initially the Natal *P. caerulea* potyvirus was presumed to be a South African strain of PWV. Differences between this virus and PWV were so subtle on physical or biological level, apart from the different host range (Chapter 3 section 3.3.4.2), that molecular data were required to verify that it is indeed a new potyvirus.

Although this virus is able to elicit "woodiness" symptoms in South African *Passiflora*, it is not closely related to Australasian strains of PWV. The Natal *P. caerulea* potyvirus has probably evolved uniquely in response to a particular biological niche, allowing it to interact with its host by interfering on physiological and metabolical levels, to elicit a condition resembling the woodiness syndrome of *Passiflora* observed elsewhere.

Phylogenetic analysis indicated that the Natal *P. caerulea* potyvirus is in fact slightly more closely related to the watermelon mosaic virus-II (WMVII-AU)/soybean mosaic virus-N (SoyMV-N) complex than to the PWV group of strains in the PWV subgroup of potyviruses (Fig. 4.9).

From the CP- and supplementary 3' NCR sequence data it can conclusively be argued that the Natal *P. caerulea* potyvirus should be considered a distinct and new potyvirus infecting *Passiflora*. The designation of South African *Passiflora* virus (SAPV) is proposed for this virus, and it will henceforth be referred to as such.

CHAPTER 5

EXPRESSION OF THE SAPV CP GENE IN *E. COLI* AND TRANSGENIC TOBACCO

SUMMARY

A 992 bp *Hae III* fragment from pW9 encompassing the SAPV CP gene was cloned into the *Sma I* site of the pUEX2 temperature controllable expression vector, in frame with the LacZ gene. The expression of high levels of β-galactosidase::CP fusion protein (MW 146) was induced by incubation at 42°C. The expressed β-galactosidase::CP fusion protein was immunoreactive with anti-SAPV polyclonal antiserum in EBIA. Antibodies prepared against the partially purified fusion protein, reacted with native SAPV in plant sap. After a series of subclonings, the SAPV CP gene was cloned into the the *Bam HI* site of the Ti-derivative pGSJ280 cointegration vector and mobilized into *A. tumefaciens* C58C1 harbouring a resident pGV2260 disarmed Ti plasmid. The resultant *A. tumefaciens* transconjugant was used to transform tobacco leafdisks. Transformants were selected on medium containing kanamycin, and tested for the presence of the CP gene by nucleic acid hybridization. EBIA was used to demonstrate the expression of SAPV CP in the transgenic tobacco.

CHAPTER 5

EXPRESSION OF THE SAPV CP GENE IN *E. COLI* AND TRANSGENIC TOBACCO

CONTENTS

CHAPTER 5. EXPRESSION OF THE SAPV CP GENE IN *E. COLI* AND TRANSGENIC TOBACCO

5.1 INTRODUCTION

The endeavor to express the SAPV CP gene in *E. coli* was undertaken for two reasons: first, it would serve the useful purpose of providing a translation product from the cloned putative SAPV CP gene that could be used for immunological verification (employing polyclonal anti-SAPV serum) of the authenticity of the SAPV CP gene; second, the CP translational product expressed in *E. coli* could be used as an antigen to raise antiserum in rabbits that is free from contaminating plant host-specific antibodies that are invariably present when using virus purified from a plant host.

As reviewed in Chapter 1 section 1.3.5, the expression of a potyviral CP gene in transgenic plants can confer resistance against virus infection. Although tobacco (*N. tabacum* SRI) is not a natural host for SAPV, the expression of the CP gene in this host was undertaken to examine the translational efficiency and stability of this gene in a readily transformable plant host. This served as a preliminary to the eventual expression of the gene in transgenic *Passiflora*, which was beyond the scope of this thesis.

5.1.1 The pUEX series of vectors for the expression of foreign genes in *E. coli*

The pEX1-3 family of expression vectors developed by Stanley & Luzio (1984) directs the expression of large quantities of cro::β-galactosidase (cro-lacZ) fusion protein under control of the λ P_R promotor. The λ P_R promotor is regulated by a temperature sensitive repressor (cIts857) which suppresses transcription at low temperatures but not at elevated temperatures, so that the production of fusion protein can be switched on or off by temperature control.

In the pEX system, the cIts857 repressor gene has to be supplied by a specific *E. coli* host (pop2136), but the pUEX1-3 expression vectors accommodate the repressor gene on the plasmid vectors (Bressan & Stanley, 1987), thus overcoming the requirement for the specific *E. coli* host strain. The pUEX1-3 vectors are identical to the pEX vectors in other respects.

The pEX/pUEX1-3 vectors also provide a multiple cloning site (in all three reading frames) near the 3'end of the lacZ gene, followed by termination signals in all reading frames, thus allowing temperature-controlled expression of a

foreign DNA sequence in any reading frame to yield large quantities of non-soluble β-gal fusion protein.

5.1.2 *A. tumefaciens* mediated transformation of plants.

5.1.2.1 Plant transformation by natural *A. tumefaciens*

A. tumefaciens is a pathogenic soil bacterium that induces plant tumours (crown galls) in dicotyledonous plants. These symptoms are the result of the ability of *A. tumefaciens* to genetically transform plants by inserting a fragment (T-DNA) of its large 180 Kb tumour-inducing (Ti) plasmid into the plant chromosomal DNA (Nester *et al.*, 1991; Schroder *et al.*, 1991; Hooykaas, 1989).

The T-DNA sequence of natural Ti plasmids encodes a variety of genes involved in the differentiation of plant tumors. These oncogenic (onc) genes encode enzymes for the synthesis of natural plant phytohormones, auxin and cytokinin, which incite division of transformed cells. The hormonal imbalance resulting from the expression of T-DNA encoded onc-genes causes the tumorous growth known as crown gall disease.

Besides the onc-genes, the T-DNA also encodes enzymes involved in the production of tumour-specific metabolites called opines, which are either condensates of an amino acid and a sugar (e.g. octopine, nopaline, leucinopine, agropine) or phosphorylated suger derivatives (agropines). These opines are synthesized and excreted by the transformed plant cells, and eventually metabolized as a food source by tumor-inducing *A. tumefaciens* carrying opine catabolic enzymes.

Except for imperfect 25 bp border repeats flanking the T-DNA, none of the T-DNA encoded genes are essential for transfer of the T-DNA region into plants (Leemans *et al.*, 1982). In fact, it has been shown that only the right border is essential for T-DNA transfer. T-DNA derived vectors for the transformation of plants have basically been "disarmed" by removal of the non-essential oncogenic and opine genes. Such vectors usually contain the T-DNA border repeats flanking foreign sequences such as plant-specific promotors, cloning sites, plant polyadenylation signals and marker- and selection genes.

5.1.2.2 Activation of the *vir* genes and T-DNA transfer

The transformation of wounded plants by *A. tumefaciens* is initiated by wound-induced release of plant cell wall components which provide a chemical signal triggering a cascade of interactive events. The production of plant growth hormones by *A. tumefaciens* triggers the plant wound response which amplifies the levels of natural plant phenolic compounds such as acetosyringone (AS) (Pazour & Das, 1990). These plant phenolic substances and a variety of plant sugars are the main components responsible for activating the Ti plasmid-located

vir operons, encoding enzymes involved in the execution of molecular transfer and integration of T-DNA into the plant cell (Nester *et al.*, 1991).

The 22 virulence genes located on the octopine type Ti plasmid are present in seven operons called the *virA* - *virG*, which are co-regulated as a single regulon (Hooykaas, 1989). Transcriptional regulation of the *vir* region is mediated by the *virA* and *virG* gene products. The *virA* gene is constitutively expressed in *A. tumefaciens*, and its polypeptide product spans the bacterial inner membrane. *VirA* acts as a plant phenolic sensor protein, and upon "detection" of acetosyringone, it is autophosphorylated. It then activates (phosphorylates) the *virG* polypeptide, which is located in the bacterial cytosol (Hooykaas *et al.*, 1991; Nester *et al.*, 1991). The phosphorylated *virG* polypeptide acts as a transcriptional activator of all the other *vir* genes.

The other *vir* gene products (B to F) assume active roles in the T-DNA transfer process and can be summarized as follows.

T-DNA is transported into the plant cell in a single stranded form. The "lower" T-DNA strand is liberated from the the parental plasmid by a combination of *virD2* border-specific endonuclease activity and *virD1* topoisomerase activity, after which the *virD2* protein remains attached to the 5' end of the T-DNA strand. This means that a possible role for the *virD2* protein in the integration event cannot be excluded. The *virC* protein binds to a sequence located next to the right border of the T-DNA called "overdrive", and acts as an enhancer of the transfer process (Hooykaas, 1989). Gene products of the *virB* operon are associated with the cytoplasmic membrane, and presumably provide a pore through the bacterial envelope for the exit of the T-DNA (Nester *et al.*, 1991). The *virE2* product of the *virE* operon is a ssDNA-binding product, and present data suggest that it assumes a role in protecting the single stranded T-DNA intermediate.

5.1.2.3 Vectors for *A. tumefaciens* mediated plant transformation

All vectors developed for the *A. tumefaciens* mediated transformation of plants with foreign DNA capitalize on the fact that (a) the DNA transferred to the plant cell is delimited by the 25 bp T-DNA border repeats, (b) the transfer and integration into the plant genome does not require the presence of any other T-DNA sequences, and (c) the *vir* region of *A. tumefaciens* functions *in trans* (Deblaere *et al.*,1987; Walden *et al.*, 1990).

Vectors used with *A. tumefaciens* are of the co-integration or binary type. As only a co-integration vector has been used for this study, only this type of vector will be briefly discussed. Co-integration vectors are derivatives of the Ti plasmid in which the T-DNA has been replaced by a defined sequence (Deblaere *et al.*, 1987). All the *vir* operons required for T-DNA transfer, are, however, still present on the co-integration Ti vector.

Foreign DNA which is to be transferred into the plant genome is cloned between the 25 bp border repeats present on an intermediate vector. This intermediate plasmid also contains selectable marker genes and a sequence (flanking the 25 bp T-DNA border sequences) homologous to the sequence used to replace the T-DNA in the Ti vector. The intermediate DNA construct is introduced into *A. tumefaciens* harbouring a resident co-integration Ti plasmid, by a conjugational process. This is usually achieved by a triparental mating, involving a helper *E. coli* strain capable of providing plasmid encoded mobilization (*mob*) and transfer (*tra*) functions (Walden *et al.*, 1990; Debleare *et al.*, 1987). Due to the absence of appropriate origins of replication, the intermediate vector is not stably maintained in *A. tumefaciens*, but instead, the intermediate plasmid is integrated into the co-integrative Ti plasmid by homologous recombination. The recombinative integration occurs between the homologous sequence shared between the intermediate vector and a sequence substituting the T-DNA in the co-integration vector.

5.2 MATERIALS AND METHODS

5.2.1 Expression of the SAPV CP gene in *E. coli*

5.2.1.1 Subcloning the SAPV CP gene into pUEX2

Useful restriction sites for subcloning the CP gene fragment of pW9 in the pUEX2 *E. coli* expression vector (Bressan & Stanley, 1987; Amersham) were obtained by computer analysis of sequence data. As shown in Chapter 4 Fig. 4.7, the putative SAPV CP gene starts at nucleotide (nt) position 352, and terminates at position 1191 in a TAA stop codon. *Hae* III, a blunt-end cutting restriction enzyme with a four base recognition sequence, cuts twice in pW9: at position 357 (five bases inside the proposed start of the CP gene) and again at position 1349 (well into the 3'non-coding region). It was calculated that insertion of the this fragment into the Sma I site of pUEX2 would result in a β-gal::CP fusion gene synchronized in a single uninterrupted reading frame (Fig. 5.1). Approximately 5 μg of pW9 DNA was cut with *Hae* III, and the 992 bp *Hae* III fragment containing the putative SAPV CP gene was gel-purified for blunt ligation into into the *Sma* I site of the pUEX2 β-gal-fusion expression vector.

Prior to ligation the pUEX2 plasmid was linearized with *Sma* I, extracted twice with phenol-chloroform (Appendix B.3.3) and 5' dephosphorylated with calf intestine alkaline phosphatase (CIP, Boehringer Mannheim). The reaction was performed for 30 min at 37°C in the presence of 50 mM Tris-Cl, pH 9.0, 1mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 1 mM spermidine, using CIP at a concentration of 0.1 U per pmole of vector DNA 5'termini.

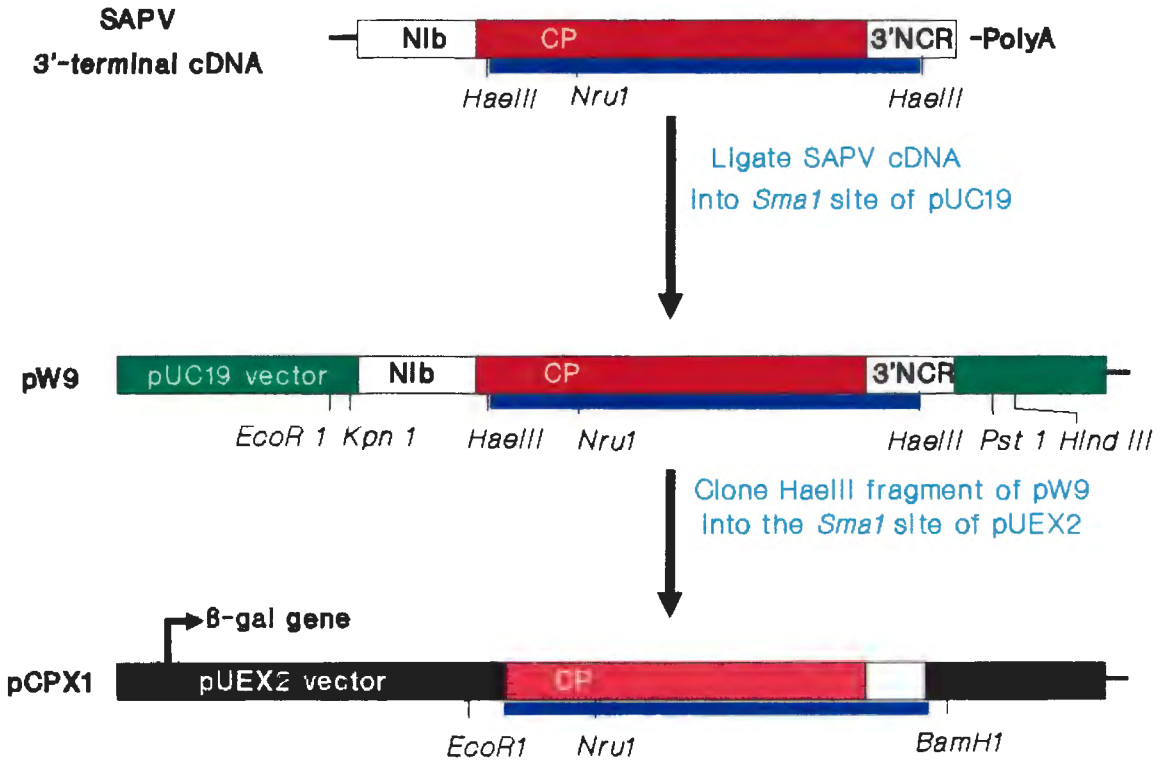

Fig. 5.1 Cloning of the *Hae III - Hae III* fragment of pW9, comprising the CP gene of the Natal *P. caerulea* potyvirus, into the pUEX2 b-gal::fusion protein expression vector.

DNA was purified by repeated phenol-chloroform extraction followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris-Cl, pH 7.5, 1mM EDTA).

The gel-purified *Hae* III CP fragment was then blunt-end cloned into the *Sma* I site of the 5' dephosphorylated pUEX2 vector, by adding 1 U T4 DNA ligase (Boehringer Mannheim) to a 20 µl reaction mix containing 100 ng vector and insert DNA (in a 1:5 molar ratio) in blunt-end ligation buffer (50 mM Tris-Cl, pH 7.6, 10 mM $MgCl_2$, 2mM DTT, and 2mM ATP). The reaction was allowed to proceed for 16 h at 15°C, and used to transform competent *E. coli* as described in Appendix B.3.14.

The presence and orientation of the SAPV CP gene in pUEX2 was verified by restriction analysis (using *BamH*I and *Nru* I) and Southern blotting (Appendix B.3.7), using the *Hae* III DNA fragment described earlier, [α-^{32}P]dCTP labelled by nick translation (Appendix B.3.9.1), as a probe.

5.2.1.2 Expression of the β-gal::SAPV CP fusion protein in *E. coli*

A recombinant (pCPX1) containing the SAPV CP *Hae* III fragment in the "sense" orientation in the *Sma* I site of pUEX2 was selected to evaluate the expression of the β-gal::SAPV CP fusion protein in *E. coli* by SDS-PAGE (Appendix B.2.1) and EBIA (Appendix B.1.5). Fusion protein expressed by pCPX1 in *E. coli*, was partially purified as described in the pEX technical brochure (Genofit, Switzerland). A 100 ml culture (in Luria Bertaini medium containing 100 µg/ml ampicillin - Appendix B.3.1) of *E. coli* harbouring the pCPX1 plasmid was grown at 30°C until early log phase (OD_{600} = 0.3) before incubation at 42°C for 2 h to inactivate the temperature sensitive cIts857 repressor, allowing 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. The insoluble fusion protein was pelleted by centrifugation at 10 000 g for 10 min at 4°C, resuspended in "low salt" STE, and used for SDS-PAGE (7% gels - Appendix B.2.1), electroblot immunoassay (B.1.5) and the immunization of rabbits (B.1.1). Polyclonal antiserum directed against native purified SAPV was host-absorbed three times with plant sap as well as with a sonicated extract of *E. coli*, before being used in EBIA to confirm the expression of the β-gal::SAPV CP fusion protein. Antiserum raised against the partially purified fusion protein was evaluated by EBIA.

5.2.2 Expression of the SAPV CP gene in transgenic tobacco

5.2.2.1 The pGSJ280/pGV2260 intermediate/co-integration vector system

The *A. tumefaciens* strain C58C1-Rif^R harbouring the pGV2260 co-integration Ti plasmid was used for plant transformation. pGV2260 is a derivative of the octopine Ti plasmid pTiB6S3 in which the entire T-region (including T_L and T_R) has been deleted and substituted by pBR322 sequences, including the carbenicillin resistance gene. pGV2260 contains the intact *vir* region encoding the *trans*-acting *vir* gene products necessary for T-DNA transfer and integration (Deblaere *et al.*, 1987).

The pGSJ280 intermediate vector (Fig. 5.2) used in this study (Deblaere *et al.*, 1987), contains a neomycin phosphotransferase (npt-II) selectable marker gene that confers kanamycin resistance to transformed plants under the control of a nopaline synthase promotor. pGSJ280 contains the 35S constitutive promotor from cauliflower mosaic virus (CaMV) upstream of unique *Cla* I (unmethylated) and *BamH* I cloning sites. Foreign coding sequences devoid of an ATG start codon can be fused in frame with an ATG start codon provided by the *BamH* I site to facilitate their expression in transgenic plants, while coding sequences containing their own initiation codon are cloned into the *Cla* I site. The plant expression cassette terminates downstream of the BamH1 cloning site in the polyadenylation sequence derived from T_L DNA gene 7.

Integrative recombination of the pGSJ280 construct into the pGV2260 Ti co-integration vector occurs in the pBR322 derived carbenicillin gene (that replaces the T-DNA in pGV2260, and flanks the T-DNA derived construct in pGSJ280), thus inactivating this gene. The spectinomycin- and streptomycin resistance genes (conferred to the recombinant Ti plasmid by integration of the intermediate pGSJ280 vector), constitute excellent markers for selection in *E. coli* and *A. tumefaciens*.

5.2.2.2 Subcloning the SAPV CP gene for expression in transgenic plants

As discussed in Chapter 1 section 1.3.3.2, the potyviral CP is translated *in vivo* as part of a polyprotein, and is post-translationally liberated through auto-proteolytic cleavage. The SAPV CP gene is thus, like most other potyviral CP genes, devoid of a translation initiation motif (Chapter 4 Fig. 4.7). In order to convert the SAPV CP sequence into a functional gene for expression in transgenic plants, it had to be furnished with a translation initiation codon.

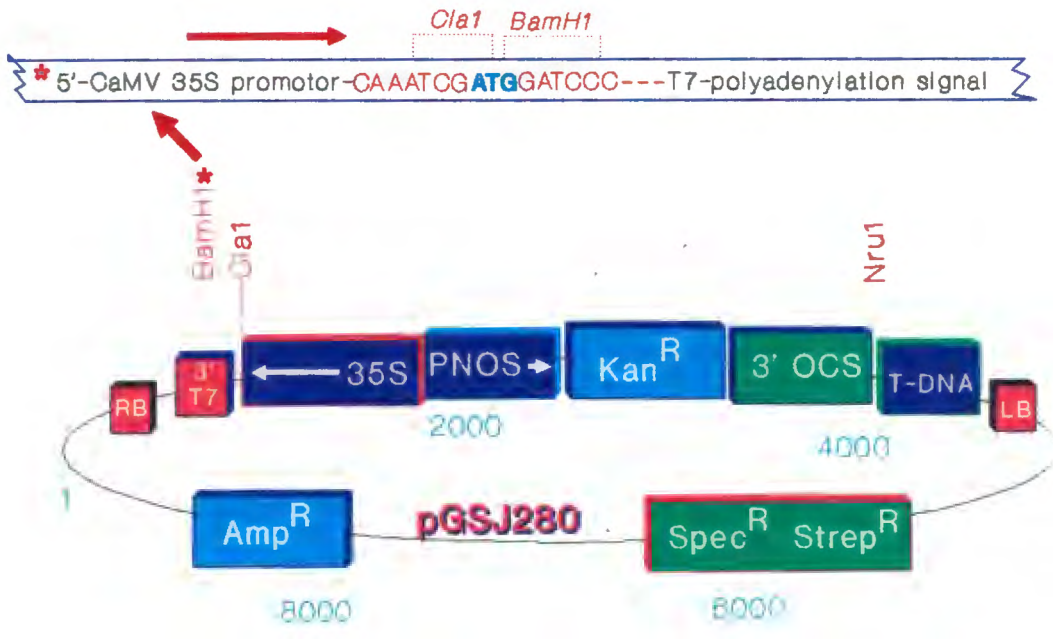

Fig. 5.2 Schematic representation of the pGSJ280 co-integration type vector.

As mentioned before, the T-DNA expression vector, pGSJ280, provides a translational initiation codon (TCGATGGATCCC) downstream of the CaMV 35S promotor sequence, and just upstream of a unique BamH1 cloning site (translation initiation consensus nucleotides in bold, and the *BamH* I site in pGSJ280 underlined). The vector motif is reported to be functional, although its sequence deviates slightly from the preferred dicotyledonous plant ribosome binding sequence (A/GCNAUGGC) (Debleare *et al.*, 1987; Lutcke *et al.*, 1987; Cavener & Ray, 1991). The SAPV CP gene was fused in frame to the ATG-start codon of pGSJ280 through a series of subcloning steps (Fig. 5.3).

Subcloning from the CP gene pCPX1 into pMTL25

The SAPV CP gene was excised from the pCPX1 recombinant vector (see 5.2.1.2) by double digestion with two pUEX2 polylinker restriction sites (*BamH* I and *EcoR* I) flanking the insert. The 5' protruding ends were bluntend by filling in nucleotides with the Klenow fragment of DNA polymerase I as follows: approximately 5 µg of *BamH* I + *EcoR* I digested pCPX1 DNA was incubated at 30 °C for 15 min in the presence of 8 U Klenow, 0.5 mM of each dNTP, 20 mM Tris-Cl pH 8.0 and 7 mM $MgCl_2$ (final reaction volume = 20 µl). The blunt-ended insert fragment was separated from the plasmid vector by agarose gel electrophoresis (Appendix B.3.5) and gel-purified using DEAE cellulose membrane (Appendix B.3.6).

pMTL25, a cloning vector with a palindromic multiple cloning site in the β-galactosidase gene, and bearing the β-lactamase (amp^R) gene as a selectable marker (Chambers *et al.*, 1988; Fig. 5.4), was cut with *Sma* I. DNA was purified by a phenol-chloroform extraction followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris-Cl, pH 7.5, 1mM EDTA). The 32-mer *Sma* I-*Sma* I oligonucleotide was removed from the linearized plasmid by gelfiltration chromatography through a Sephadex G50 pasteur column (B.3.4). The bluntend *BamH* I - *EcoR* I fragment from pCPX1, was then ligated into the *Sma* I cut pMTL25 vector by adding 1 U T4 DNA ligase (Boehringer Mannheim) to a 15 µl reaction mix containing 100 ng vector and insert DNA (in a 1:1 molar ratio) in blunt-end ligation buffer (50 mM Tris-Cl, pH 7.6, 10 mM $MgCl_2$, 2mM DTT, and 2mM ATP). The reaction was allowed to proceed for 16 h at 15°C, and used to transform competent *E. coli* (Appendix B.3.14). Potential recombinant clones with a pCPX1-derived CP insert, were identified as white colonies (insertional inactivation of the β-galactosidase gene of pMTL25) on LB-100 agar plates containing X-gal (Appendix B.3.14). Twelve white colonies were selected, digested with *BamH* I to excise the expected CP gene fragment, and subjected to agarose gel electrophoresis to verify the presence of CP inserts.

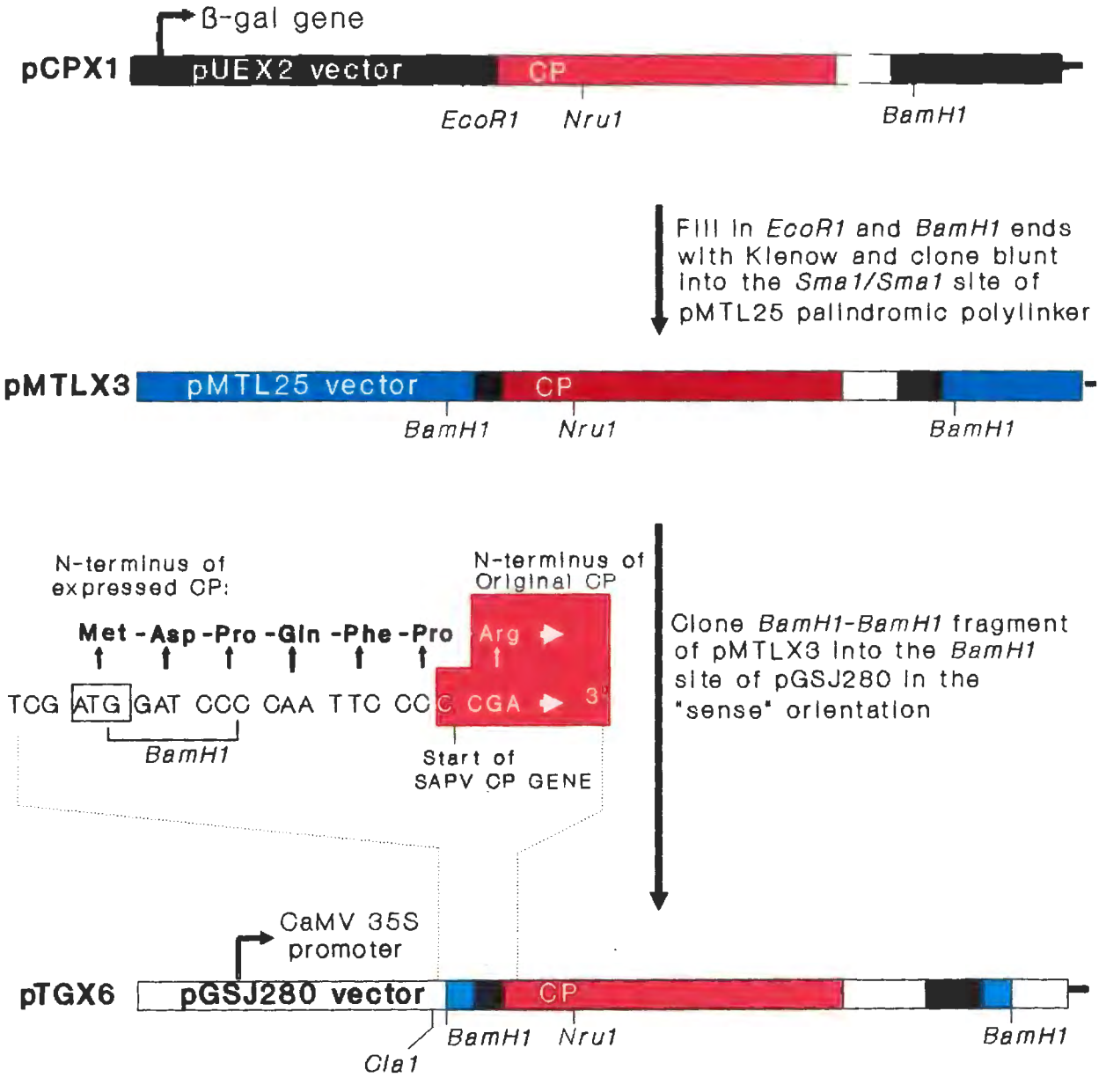

Fig. 5.3 Cloning of the SAPV CP gene into the pGSJ280 co-integration vector in frame with the ATG translation initiation codon provided by the vector.

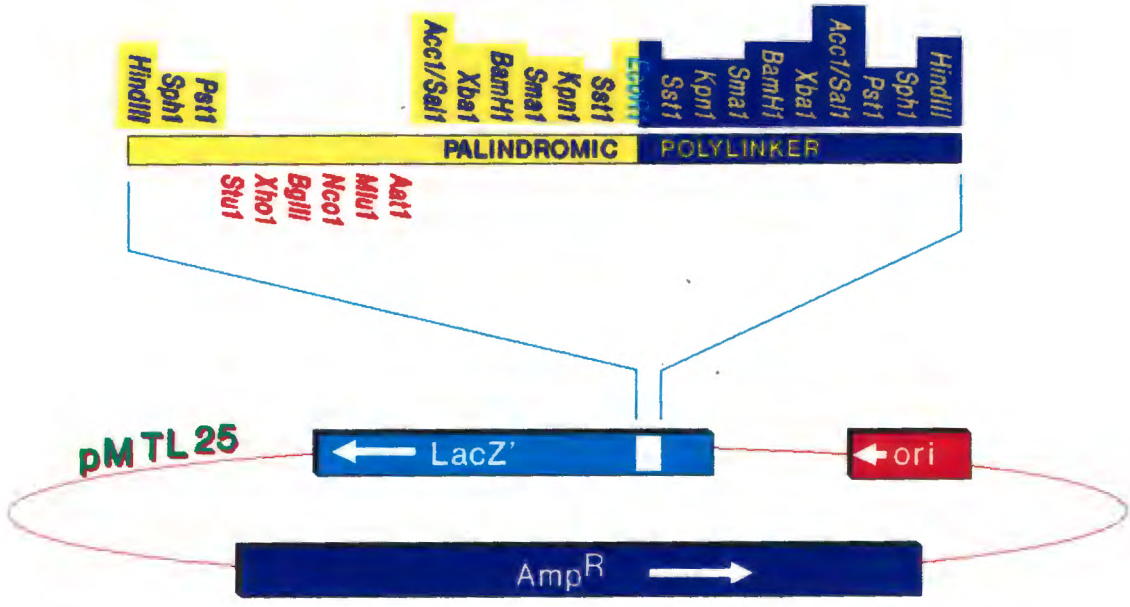

Fig. 5.4 Schematic representation of the pMTL25 palindromic multiple cloning site expression vector

Subcloning from the CP gene from pMTLX3 into pGSJ280

The pMTLX3 vector was digested with *BamH* I, liberating the SAPV CP gene as a *BamH* I - *BamH* I fragment by cutting in the palindromic polylinker on each side of the CP gene insert. The *BamH* I - *BamH* I CP fragment was again separated from the pMTL25 vector DNA by gel electrophoresis (using TAE buffer - Appendix B.3.5) in 1% low melting point (LMP) agarose (Seaplaque). The gel slice containing the CP band was excised from the agarose under UV light (λ = 305 nm), and transferred to a microfuge tube containing an amount of water equalling the volume of the gel slice. The LMP agarose slice was melted at 70°C for 5 min, and equilibrated at 37°C before utilization in subsequent ligation reactions.

Approximately 1 µg pGSJ280 vector DNA was digested to completion with *BamH* I, extracted with phenol/chloroform (Appendix B.3.3) and resuspended in TE buffer following ethanol precipitation. In order to fuse the CP gene in frame with the ATG start codon of pGSJ280, the *BamH* I - *BamH* I CP fragment was mixed in a 5:1 ration (100 µg total DNA) with the pGSJ280 vector (*BamH* I - digested), and ligated for an hour at 20°C in the presence of 1 U T4 ligase, 50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT and 1 mM ATP (final volume of 15 µl). The ligation mixture was used to transform competent *E. coli* LKIII (Appendix B.3.14). Transformation was performed essentially as described in Appendix B.3.14, except the transformation mixture was allowed to express for three hours in LB medium before plating out onto selective medium (Luria Bertaini medium supplemented with 20 µg/ml streptomycin and 50 µg/ml spectinomycin). This was necessary because pGSJ280 is a low copy number plasmid.

Recombinant transformants were identified by colony blotting (Appendix B.3.10), using gel-purified pW9-Hae III CP-insert (^{32}P-labelled by nick translation - Appendix B.3.9.1) as a probe. The presence and orientation of the CP gene recombinants were examined by restriction analysis using *BamH* I to show the presence, and *Nru* I to determine the orientation of the insert.

The integrity of the reading frames of recombinants with the CP gene in the "sense" orientation were verified by sequence analysis (Appendix B.3.16), but using a primer (5' GGAAGTTCATTTCATTTGG 3' - synthesized by the Department of Biochemistry, UCT) homologous to the CaMV 35S promotor region upstream of the CP insert.

5.2.2.3 Conjugation of the intermediate pGSJ280-CP vector construct into *A. tumefaciens*

The pTGX6 T-DNA intermediate pGSJ280-derivative plasmid carrying the SAPV CP gene fused to the CaMV promotor was conjugated into *A. tumefaciens* C58C1 (harbouring the pGV2260 Ti-derivative plasmid) by means of "triparental

mating" as described by Debleare *et al.*, (1987). Bacterial strains and plasmids used for triparental matings were maintained as described in Appendix B.4.3.

E. coli strain HB101 harbouring the pRK2013 plasmid can be used to mobilize pBR-derivative plasmids (like pGSJ280) containing a *bom*-site, because they provide the transfer (*tra*) and mobilization (*mob*) functions in trans (Deblaere *et al.*, 1987). For triparental matings cultures of *E. coli* HB101 (pRK2013) and *E. coli* LKIII (pTGX6) were grown overnight at 37°C in 5 ml LB cultures (with shaking) without antibiotic selection. *A. tumefaciens* C58C1 (pGV2260) was grown up in YEB (Appendix B.4.1.3) medium at 28°C. Of each culture, 100 µl was mixed in a 1:1:1 ratio, and incubated for two to three days on an LB plate. The mobilization mixture was then scraped off the surface of the plate using a sterile spatula, and suspended in 2 ml of 10 mM $MgSO_4$. Serial 1/2 dilutions in 10 mM $MgSO_4$ were plated out on LB supplemented with 100 µg/ml rifampicin, 300 µg/ml streptomycin and 100 µg/ml spectinomycin (LB $Rif_{100}Sp_{300}Sc_{100}$). Plates were incubated for two to three days at 28°C. Transconjugates were selected for their ability to grow on LB $Rif_{100}Sp_{300}Sc_{100}$, streaked out to obtain purified colonies, and verified to be *A. tumefaciens* by the Benedict's test (Appendix B.4.2). The co-integrate structure was examined by Southern blotting (Appendix B.3.7) of total DNA, using ^{32}P-labelled *BamH* I - *BamH* I fragment of pMTLX3 (containing the SAPV CP gene) as a probe.

5.2.2.4 Leaf disk transformation of tobacco

Transformation of tobacco was accomplished by a modification of methods practiced by Draper *et al.*, (1988). Sterile tobacco plants (*N. tabacum* cv. Petit Havanna SR1) were propagated *in vitro* from shoots on half strength Murashige & Skoog (MS) medium solidified with 0.7 % agar as described in Appendix B.4.7.

Leaf discs (± 0.5 cm^2) were cut from 4-8 week old plants, and floated upside down for 5 min in a sterile petridish containing a 1/50 dilution of an overnight culture of recombinant *A. tumefaciens* (carrying a copy of the CP gene between T-DNA borders integrated into the resident Ti plasmid derivative).

The overnight culture of *A. tumefaciens*, was grown for 16 h without selection in Luria Broth (pH 5.6) containing 20 µM acetosyringone (Appendix B.4.4.3), until it reached the late log phase (OD_{600} = 0.2 - 0.4). Acetosyringone was included in the bacterial growth medium for artificial induction of the *vir* genes, resulting in higher transformation efficiency (Sheikholeslam & Weeks, 1987). The bacterial culture was diluted 1/50 with MS medium pH 5.8 (Appendix B.4.5) for co-cultivation with leaf discs.

After co-cultivation, the the leaf discs were blotted on sterile filter paper, and placed with epidermis facing down onto a petri dish containing shoot regeneration medium (SRM - Appendix B.4.5) where they were co-cultivated for two days at low light intensity (12.5 $\mu mol.s^{-1}.m^{-2}$, 16 h photoperiod) at 25°C.

LEGENDS:

Fig. 5.5 Total DNA (uncut) of *E. coli* transformed with pCPX 1 & 2 (the *Hae III - Hae III* fragment of pW9 cloned blunt into the *Sma I* site of the pUEX2 β-gal fusion expression vector). **Lanes 1, 2 & 4**, pUEX2 with no inserts. **Lane 3**, pCPX 1 & 2. **Lane 5**, pCPX 2.

Fig. 5.6a Determination of the orientation of the SAPV-CP gene insert in pCPX 1 & 2. **Lane 1**, pW9 linearized with *Pst I*. **Lane 2**, λ (*Sty 1*). **Lane 3**, pUEX2 linearized with *Sma I*. **Lane 4**, pCPX1 linearized with *Pst I*. **Lane 5**, pCPX2 linearized with *Pst I*. **Lane 6**, pCPX1 digested with *BamHI & Nru I*. **Lane 7**, pCPX2 digested with *BamHI & Nru I*.

Fig. 5.6b Autoradiograph of gel in Fig. 5.6a, using the α^{32}P-dCTP labelled *Hae III - Hae III* fragment of pW9 (section 5.2.1.1) as a probe.

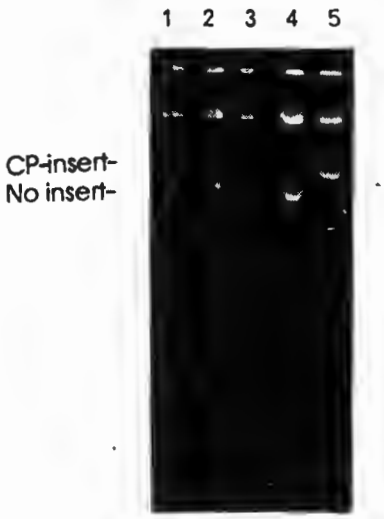

Fig. 5.5

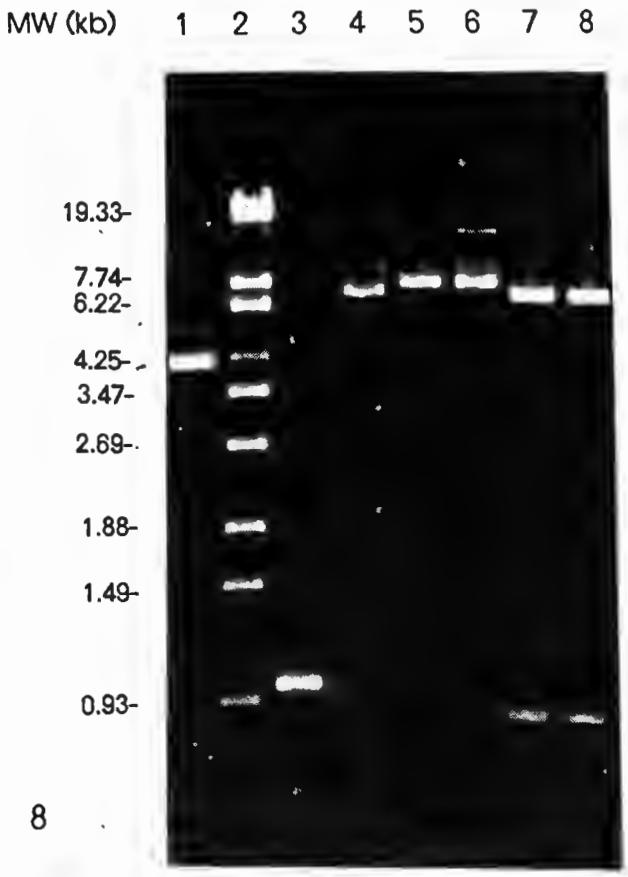

Fig. 5.6a

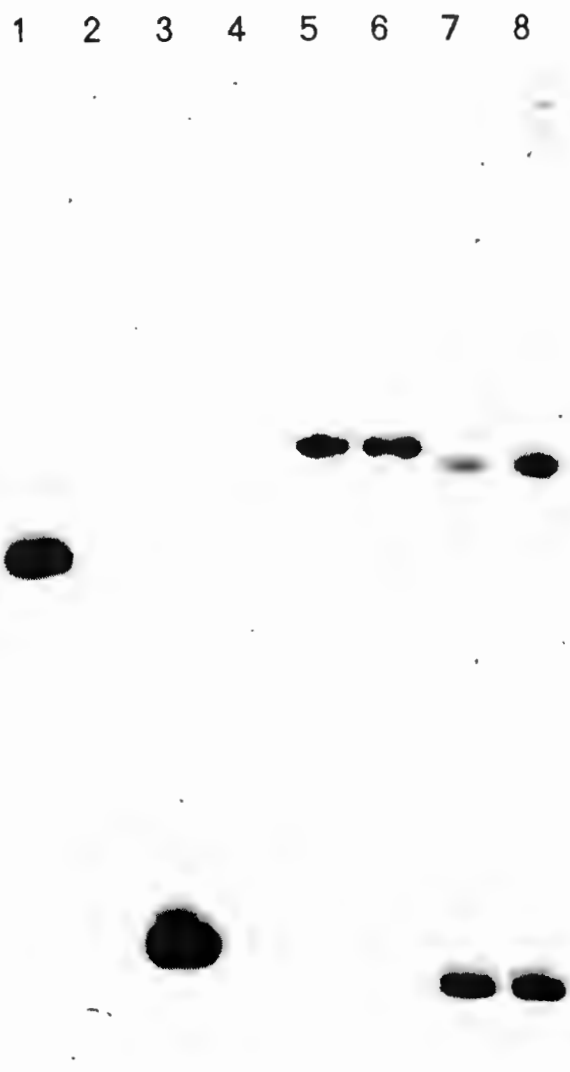

Fig. 5.6b

Discs were then transferred to selective medium, SRM containing 500 µg cefotaxime (cef_{500}; Calbiochem) to eliminate *A. tumefaciens*, as well as 100 µg/ml kanamycin (kan_{100}; Sigma) to select for the growth of transformed tobacco shoots, and incubated at 25°C with 16 h photoperiod (light intensity = 62.5 $\mu mol.s^{-1}.m^{-2}$).

5.2.2.5 Selection and maintenance of transformed tobacco

Shoot forming transformed leaf discs were transferred to fresh selective medium (SRM-$kan_{100}cef_{500}$) and allowed to grow for another three weeks before being transferred to root induction/maintenance medium (RIM-kan_{100} - Appendix B.4.5) and maintained as described in Appendix B.4.7.

5.2.2.6 Detecting the presence of the SAPV CP gene in transformed tobacco

Tobacco plantlets regenerated from transformed leafdisks under antibiotic selection were tested by dot blot hybridization (Appendix B.3.11) using gel-purified pW9-Hae III CP-insert (^{32}P-labelled by nick translation) as a probe. Crude extraction of total plant DNA for dot blotting, was performed as described in Chapter 3, section 3.2.3.1. For hybridization, DNA samples from different plantlets were dot-blotted onto nylon membranes (Hybond-N, Amersham, UK), using a 96-well "minifold" apparatus (Hoefer Scientific) coupled to a water tap vacuum.

5.2.2.7 Analysis of the SAPV CP gene co-integrate structure in transgenic plants

Total DNA from transformed tobacco plants was isolated as described in Appendix B.3.17. Approximately 8 µg DNA was digested for five hours at 37°C using 80 units of *Hae III* in a total volume of 200 µl. DNA was then subjected to phenol/chloroform extraction and ethanol precipitation as described in Appendix B.3.3. The pellet was resuspended in 20 µl TE buffer and subjected to gel electrophoresis (Appendix B.3.5) and Southern blotting (Appendix B.3.11 & 12).

5.2.2.8 Analysis of translational expression of the CP gene in transgenic tobacco

Eight transformant tobacco plants that tested positively for the presence of the CP gene in DNA hybridizations were selected for EBIA (using polyclonal SAPV antiserum; Appendix B.1.5) to examine the translational expression of the CP gene.

5.3 RESULTS AND DISCUSSION

5.3.1 Expression of the SAPV CP gene in *E. coli*

5.3.1.1 Subcloning the SAPV CP gene into pUEX2

Mini extractions of total DNA (Appendix B.3.1) of colonies of *E. coli* transformed with the pUEX2-*Sma* I/pW9-*Hae* III ligation mix were subjected to electrophoresis in agarose gels (Fig. 5.5). Clearly distinguishable plasmids containing inserts were isolated (Appendix B.3.1) and double digested with *BamH*I and *Nru* I.

*BamH*I cleaves 3' of the SAPV CP gene in the pUEX2 polylinker region, while *Nru* I cleaves at insert position 479 from the 5' of the pW9 insert (thus 122 nt inside the 5' end of the *Hae* III-SAPV CP fragment). This meant that a 875 bp fragment would be liberated from recombinants containing the CP gene in the "sense" orientation, and a 109 bp fragment would be excised if the CP gene was in the "antisense" orientation (Fig. 5.6a). Southern blotting confirmed that the recombinants contained the CP gene (Fig. 5.6b). As expected from a blunt end cloning approach, about 50% of recombinants were of the "sense" orientation.

5.3.1.2 SDS-PAGE and EBIA analysis of the β-gal::SAPV CP fusion protein

The recombinant plasmid pCPX1 (containing the CP gene in the "sense" orientation in the *Sma* I site of pUEX2) was selected for expression studies. Large quantities of β-gal::SAPV CP fusion protein were purified from this recombinant clone. An MW of 146 ±0.9 was determined for the fusion protein in SDS-PAGE (Fig. 5.7a) which is in good agreement with the predicted MW of 147.7, and about 30 kDa larger than the native β-galactosidase protein product. In EBIA, the pCPX1 fusion protein reacted with polyclonal SAPV-specific antiserum, which served as additional proof of the authenticity of the SAPV CP gene expressed in *E. coli*. No serological reaction with the SAPV antiserum was evident in lanes expressing only the native β-galactosidase (pUEX2), or a β-gal::SAPV CP fusion with the CP gene inserted in the "antisense" orientation (pCPX3), as exhibited in Fig. 5.7b.

Limited proteolytic degradation of the fusion protein by *E. coli* proteases was evident in EBIA. This was also observed with other potyviral CPs expressed in *E. coli* (SoyMV - Eggenberger *et al.*, 1989; BYMV - Hammond & Hammond, 1989). This effect is probably aggravated due to the activation of heat shock proteases of *E. coli* during the temperature shift step to induce expression of the fusion protein (Maniatis, *et al.*,1989).

LEGENDS:

Fig. 5.7a SDS-PAGE of SAPV CP expressed in *E. coli*. **Lane 1**, Protein MW markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97,4 kDa) bovine serum albumin (66 kDa). **Lane 2**, *E. coli* LK-111. **Lane 3**, *E. coli* LK-111 with pUEX2 vector. **Lane 4**, *E. coli* LK-111 with pCPX1, expressing 147 kDa β-galactosidase::CP fusion protein.

Fig. 5.7b EBIA using polyclonal antiserum directed against SAPV. **Lane 1**, Protein MW markers stained with Ponceau-S: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97,4 kDa) bovine serum albumin (66 kDa), ovalbumin (45 kDa). **Lane 2**, *E. coli* LK-111. **Lane 3**, *E. coli* LK-111 with pUEX2 vector. **Lane 4**, *E. coli* LK-111 with pCPX3 (insert in antisense orientation). **Lane 5**, *E. coli* LK-111 with pCPX1, expressing 147 kDa β-galactosidase::CP fusion protein.

Fig. 5.7c EBIA using antiserum directed against 147 kDa β-galactosidase::CP fusion protein expressed in *E. coli*. **Lane 1**, healthy plant. **Lane 2**, plant infected with SAPV. **Lane 3**, purified SAPV (with some visible degradation of CP)

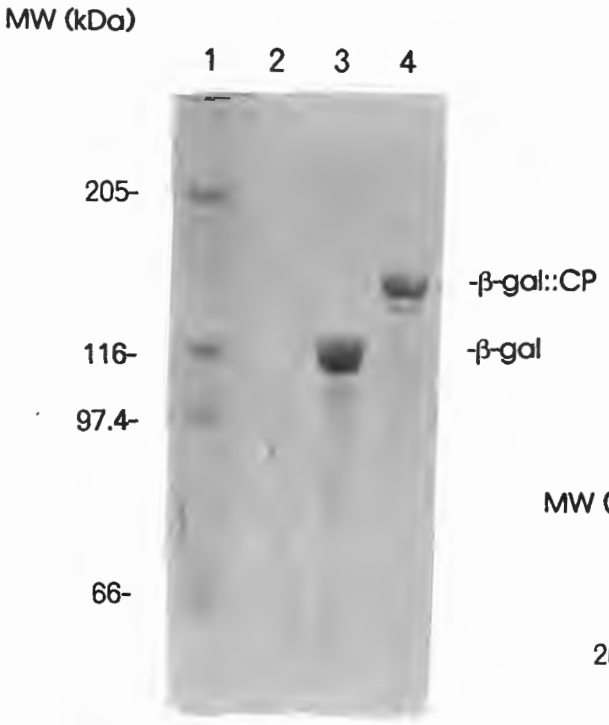

Fig. 5.7a

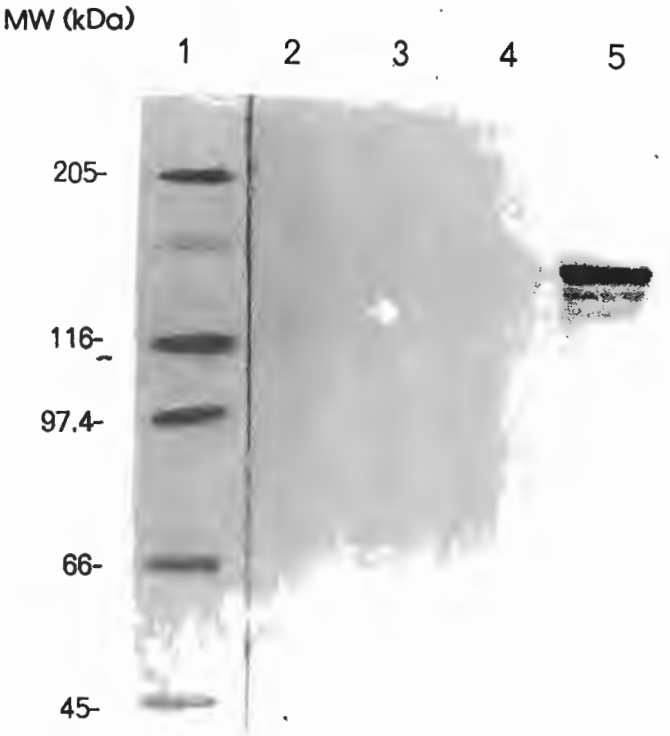

Fig. 5.7b

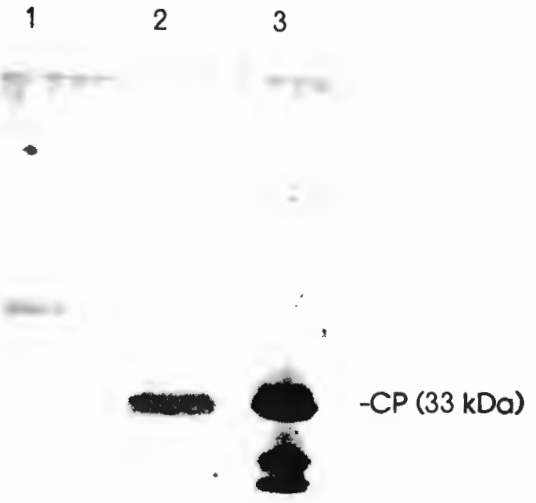

Fig. 5.7c

The expression of foreign genes in expression vectors such as pUEX2, is a convenient approach for verifying the authenticity of a particular gene if a reliable antiserum directed against the gene-product is available.

5.3.1.3 EBIA with antiserum directed against the expressed fusion protein

EBIA showed that antiserum raised against partially purified β-gal::SAPV CP fusion protein detected the presence of the SAPV CP protein in purified virus preparations, as well as in the sap of SAPV-infected plants (Fig. 5.7c). Apart from a single faint non-specific host protein band (consistently occurring in a specific position) in both healthy and diseased plants, the antiserum demonstrated a high level of SAPV CP-specificity.

The fact that antisera directed against the expressed fusion protein appeared less sensitive in EBIA than conventional antiserum raised against native virions could at least in part be attributed to the fact that early bleeds were used, which are normally of lower titre.

The use of *E. coli* to generate large quantities of a particular gene product such as viral capsid protein for rabbit immunization, has distinct advantages over the cumbersome and costly maintenance of a virus strain in laboratory plants, particularly for large scale and long term production of antibodies. It also provides a safe and convenient way of exchanging virus antigens between laboratories.

5.3.2 Expression of the SAPV CP gene in transgenic tobacco

5.3.2.1 Subcloning the SAPV CP gene for expression in plants

Subcloning from the CP gene pCPX1 into pMTL25

Several hundred transformants were obtained from *E. coli* transformed with Klenow-bluntend *BamH* I - *EcoR* I fragment from pCPX1 ligated into the *Sma* I cut pMTL25 vector. Of the 12 white colonies that were selected for restriction analysis, eight had inserts of the expected size (Fig. 5.8). One of these, pMTLX3 was selected for further manipulation.

Subcloning from the CP gene from pMTLX3 into pGSJ280

Since pGSJ280 does not contain a selectable marker for cloning, a large number of transformant colonies were picked for hybridization. Of 135 transformants that were screened, only six hybridized to the pW9-*Hae* III probe (Fig. 5.9). These were selected for identification of the insert orientation by restriction analysis.

Nru I cleaves the SAPV CP gene insert at a site 137 bases downstream of the translation initiation ATG sequence. pGSJ280 also has a *Nru* I site positioned approximately 3500 bases upstream of the aforementioned ATG start codon. Thus, theoretically cleavage of recombinant pTGX clones with *Nru* I should yield two bands of sizes approximately 3500 and 7000 bp respectively if the insert is in the "sense" orientation, and 4500 and 6000 bp if it is in the "inverse" orientation. As shown in Fig. 5.10, two of the six recombinant clones had inserts in the "antisense" orientation, while four had CP inserts in the "sense" orientation. Sequence analysis of two of the recombinants with CP inserts in the positive sense orientation, pTGX1 & 6, confirmed that the reading frames were in synchrony with the pGSJ280 translation initiation motif as theoretically predicted (Fig. 5.11).

5.3.2.2 Conjugation of the intermediate CP vector construct into *A. tumefaciens*, and recombinational insertion into the resident co-integration Ti plasmid

The Ti co-integrate structure (of pTGX-clones integrated into pGV2260) was examined by Southern blotting (Appendix B.3.7) of total DNA, using ^{32}P-labelled *BamH* I - *BamH* I fragment of pMTLX3 (containing the SAPV CP gene) as a probe. Southern blotting of total DNA from the transconjugant *A. tumefaciens* digested with *BamH* I (Fig. 5.12a lanes 3 - 6 and Fig. 5.12b lanes 2 - 5), show bands corresponding with the total size of the CP gene inserted into the *BamH* I site of pGSJ280. Total DNA from the transconjugant *A. tumefaciens* digested with *Cla* I (cuts upstream of the CP insert in pGSJ280) and *BstE* II (cuts close to the 3' terminus of the CP gene insert) showed (as expected) bands slightly smaller than the total CP gene (see Fig. 5.12a, lanes 8-11). This verifies the orientation of the CP insert relative to the CaMV35S promotor. In Fig. 5.12a, lanes 14 to 18 and Fig. 5.12b, lanes 13 to 17 it is clear that *Nru* I-digested total DNA from the transconjugant *A. tumefaciens* differs structurally from *Nru* I-digested total DNA of *A. tumefaciens* harbouring the pTGX6→pGV2260 co-integrate, indicating that recombination had indeed occurred.

5.3.2.3 Leaf disk transformation of tobacco

Eighty four individual tobacco plantlets were regenerated from thirty leaf discs on selective nutrient medium after co-cultivation with *A. tumefaciens* (section 5.2.2.5). The regeneration of plants from tobacco leafdisks is shown in Fig. 5.13. Several plants exhibited physiological abberations, purportedly caused by inactivation or interference of the integrated CP construct with essential plant genes. The majority of plantlets, however, appeared physiologically normal.

LEGENDS:

Fig. 5.8 The pMTLX series of intermediate constructs, containing the SAPV CP gene (liberated from pCPX1 as a *BamH1 - EcoR1* fragment and blunted by S1 nuclease/Klenow) in the *Sma I* site of vector pMTL25. **Lane 1**, pMTL25 linearized with *Sma I*. **Lanes 2, 4 & 5**, pMTLX1, 2 and 3 respectively, digested with *BamH1* which has sites flanking the CP insert in the palindromic multiple cloning site. **Lanes 3, 6 & 7** Transformants with no CP inserts linearized by *BamH1*.

Fig. 5.9 Colony blot to identify six colonies with the SAPV CP sequence cloned into the *BamH1* site of the pGSJ280 co-integration vector, using the α^{32}P-dCTP labelled *Hae III - Hae III* fragment of pW9 (section 5.2.1.1) as a probe (⊕ = positive control pW9).

Fig. 5.10a Gel electrophoresis of pTIX clones (SAPV CP sequence cloned into the *BamH1* site of the pGSJ280 co-integration vector) digested with *BamH1*. **Lane 1**, λ (*Sty 1*). **Lane 2**, pGSJ280 digested with *BamH1*. **Lanes 3 to 8**, pTIX1 to 6 respectively digested with *BamH1*.

Fig. 5.10b Gel electrophoresis of pTIX clones digested with *BamH1*. **Lane 1**, λ (*Sty 1*). **Lane 2**, pGSJ280 digested with *Nru I*. **Lanes 3, 4, 5 & 8**, pTIX1, 2, 3, 6 respectively digested with *Nru I* (all have CP gene in "sense" orientation). **Lanes 6 & 7**, pTIX 4 & 5 respectively digested with *Nru I* (both have CP gene in "antisense" orientation).

Fig. 5.11 Sequence analysis of pTGX6 to verify that the CP gene was correctly fused "in frame" to the ATG-start codon provided by the pGSJ280 parental vector.

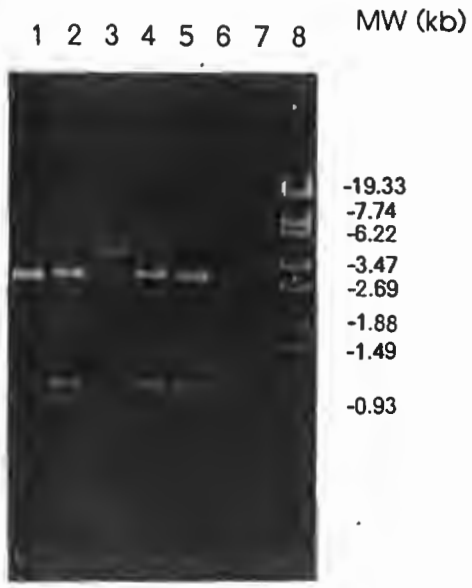

Fig. 5.8

Fig. 5.9

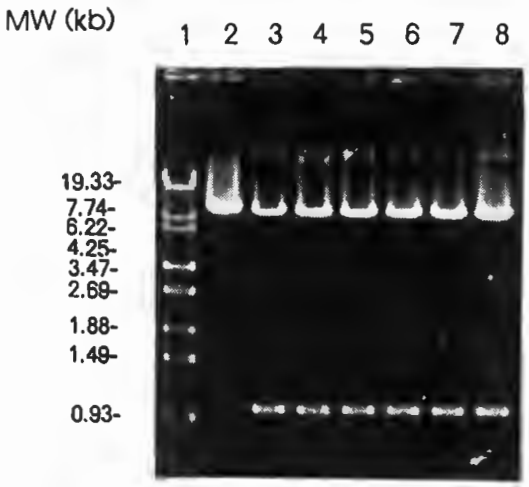

Fig. 5.10a

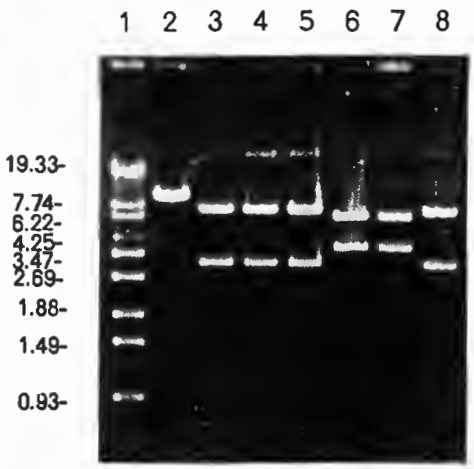

Fig. 5.10b

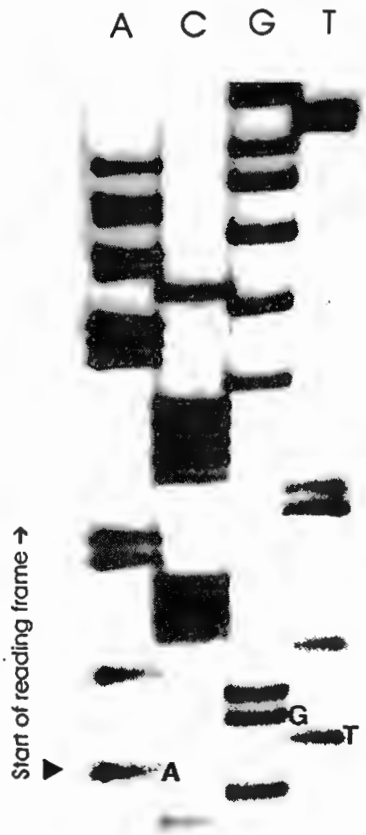

Fig. 5.11

LEGENDS:

Fig. 5.12a

Gel electrophoresis of total DNA from transconjugant *Agrobacterium tumefaciens* digested with different restriction enzymes. **Lane 1**, λ-*Sty* I. **Lane 2**, Total DNA from *A. tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *BamH I*. **Lanes 3 - 6** , Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *BamH I*. **Lane 7**, Total DNA from *Agrobacterium tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *Cla* I and *BstE* II. **Lanes 8 - 11**, Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *Cla* I and *BstE* II. **Lane 12**, pTIX6* purified plasmid DNA digested to completion with *Cla* I and *BstE* II. **Lane 13**, Total DNA from *Agrobacterium tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *Nru* I. **Lanes 14 - 17**, Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *Nru* I. **Lane 18**, pTIX6* purified plasmid DNA digested to completion with *Nru* I. **Lane 19**, λ-*Sty* I.

*All pTIX plasmids consist of the pGSJ280 co-integration vector with the SAPV CP gene inserted in the *BamH* I site downstream of the CaMV 35S promotor.

Fig. 5.12b

Southern blot of total DNA from transconjugant *Agrobacterium tumefaciens* digested with different restriction enzymes, using ^{32}P-labelled *BamH* I - *BamH* I fragment of pMTLX3 (containing the SAPV CP gene) as a probe. **Lane 1**, Total DNA from *Agrobacterium tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *BamH I*. **Lanes 2 - 5** , Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *BamH I*. **Lane 6**, Total DNA from *Agrobacterium tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *Cla* I and *BstE* II. **Lanes 7 - 10**, Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *Cla* I and *BstE* II. **Lane 11**, pTIX6* purified plasmid DNA digested to completion with *Cla* I and *BstE* II. **Lane 12**, Total DNA from *Agrobacterium tumefaciens* C58C1 with the pGV2260 Ti plasmid digested to completion with *Nru* I. **Lanes 13 - 16**, Total DNA from *A. tumefaciens* C58C1 with pTIX6*, pTIX9*, pTIX10* and pTIX14* respectively, integrated into the resident pGV2260 Ti plasmid and digested to completion with *Nru* I. **Lane 17**, pTIX6* purified plasmid DNA digested to completion with *Nru* I.

*All pTIX plasmids consist of the pGSJ280 co-integration vector with the SAPV CP gene inserted in the *BamH* I site downstream of the CaMV 35S promotor.

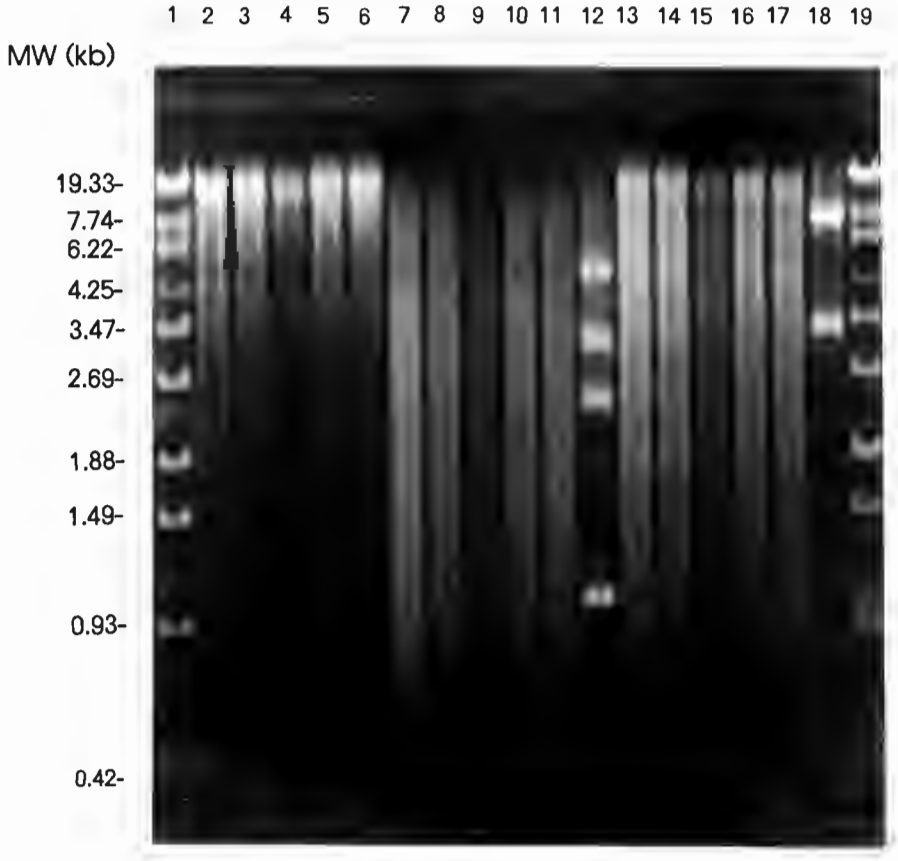

Fig. 5.12a

Fig. 5.12b

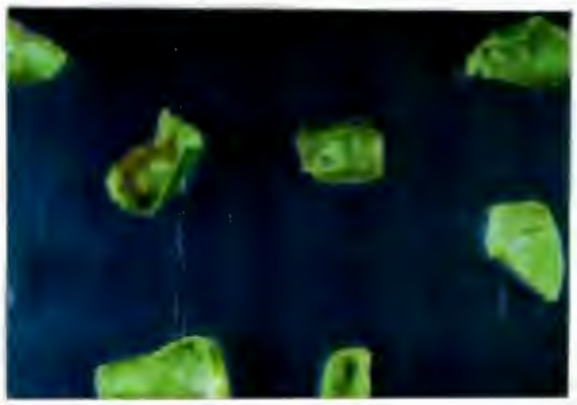

Fig. 5.13a Leaf disks of untransformed *N. tabacum* cv. SRI fail to regenerate into complete plants on medium containing 100 μg/ml kanamycin as a selective antibiotic.

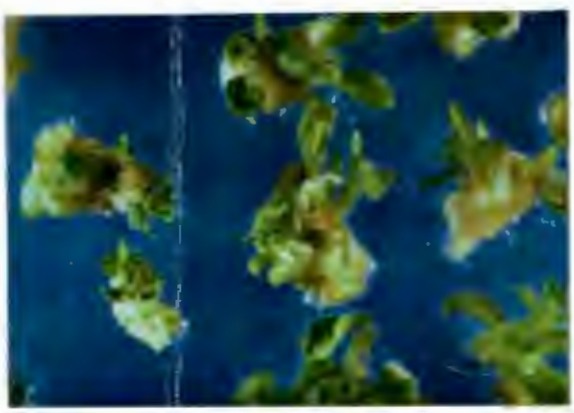

Fig. 5.13b Leaf disks (grown on medium containing 100 μg/ml kanamycin) of *N. tabacum* cv. SRI that was transformed by *A. tumefaciens*, which contained the pTIX6 CP construct integrated into the resident pGV2260 Ti plasmid. The pTIX6 construct also contains the neomycin phosphotransferase gene which confers kanamycin resistance to transformed plants under the control of a nopaline synthase promotor.

5.3.2.4 Expression of the SAPV CP gene in transformed tobacco

Structure of the modified CP gene transferred to transgenic tobacco

In order to fuse the SAPV CP gene in frame to the translational initiation codon in pGSJ280 it was taken through a series of subclonings described earlier (Fig. 5.3). The HaeIII fragment of pW9 starts 4 nt inside the 5' terminus of the CP gene of SAPV and ends in the 3'-NCR (Chapter 4 Fig. 4.7), so that this fragment corresponded to the CP minus two N-terminal amino acids (SG-). In the process of constructing pTGX6 (the CP gene fused in frame with ATG-start codon of pGSJ280), however, additional nucleotides corresponding to six additional amino acids were unavoidably added to the N-terminus of the CP gene (Fig. 5.3). The resultant CP N-terminal aa sequence was thus modified from SGRKQEEL— (original SAPV CP) to MDPQFPRKQEEL—. As the N-terminus of the potyviral does not appear to have a structural role (Shukla *et al.*, 1989b), and is known to be of variable length, these minor modifications were not expected to affect the biological functionality of the modified CP. The risk existed, however, that the N-terminal methionine could be removed by N-terminal methionine specific exopeptidases in plants, as is often the case in eukaryotes. This would result in an expressed CP with an N-terminal Asp residue. While an N-terminal Ser (unmodified CP) or Met (unprocessed modified CP) would exert a stabilizing intracellular influence on the expressed protein, an N-terminal Asp is very likely to have a strongly destabilizing influence on the protein (Bachmair *et al.*, 1986).

Detection of the presence of the SAPV CP gene in transgenic tobacco and analysis of the co-integrate structure

DNA dot blot hybridization analysis of crude extracts of total plant DNA (Fig. 5.14a) showed the presence of the SAPV CP gene in eight out of thirteen tobacco plants (*N. tabacum* SRI-CPX 1 - 8) that were regenerated under kanamycin selection (as described in section 5.2.2.5).

Total DNA from transgenic plants was cut to completion by *Hae III* (Fig. 5.14b). This restriction enzyme has a four base recognition sequence that is not present in the SAPV CP gene, but is present once in the CaMV 35S promotor. The enzyme thus cuts at a specific point upstream of the SAPV CP gene and at a non-specific point in the plant genome downstream of the CP gene when the gene is integrated. Southern blot analysis (using the CP probe described in section 5.2.2.6) showed the presence of the SAPV CP gene and the differences in the co-integrate structure of different tobacco transformants, which is to be expected due to the random nature of the integration process (Fig. 5.14c). The fact that these differences are fairly small can be attributed to the fact that *Hae III*, a frequently cutting enzyme, was used. It appeared that all eight transformed tobacco plants had single copy inserts of the gene. Total DNA of *A. tumefaciens*

harbouring the pTGX6→pGV2260 co-integrate that was also subjected to *Hae III* digestion, showed two bands (Fig. 5.14c), which is probably due to partial digestion of the DNA. It is expected that the lower band represents the CP gene liberated completely from the Ti plasmid.

Expression of the CP gene in tobacco

EBIA confirmed clearly that in each of these eight transformed plantlets, the chimeric CP gene was expressed as a stable, intact protein with mobility in EBIA indistinguishable from the actual SAPV viral CP (Fig. 5.15). This was also an indication that either the N-terminal Met is not post-translationally removed and is thus available to stabilize the protein, or that the Asp does not exert the expected destabilizing effect on the protein *in vivo*.

Unfortunately *N. tabacum* is not a susceptible host for SAPV and this prevented the testing of the cross-protection capabilities of the expressed CP against infection by SAPV. It has been shown before that expression of the potyviral CP gene in transgenic plants can confer protection against a broad spectrum of heterologous potyvirus infections, probably due to the conserved nature of the structural domains of potyviral coat proteins (Stark & Beachy, 1989).

The prospective aim is to express the CP gene in transgenic *Passiflora* in an attempt to protect this commercially important crop against potyvirus infection implicated in the woodiness disease of passionfruit. Recent advances in routines for the *in vitro* culture of *Passiflora*, introduced new possibilities for transformation of this host (Drew, 1991).

LEGENDS:

Fig. 5.14a

Dot blot hybridization of total DNA extracted from tobacco, using ^{32}P-labelled *BamH* I - *BamH* I fragment of pMTLX3 (containing the SAPV CP gene) as a probe. **A1**, Untransformed *N. tabacum* cv. SRI. **A2** to **A7** and **B1** to **B6**, *N. tabacum* cv. SRI transformed by *A. tumefaciens* with the pTIX6 CP construct integrated into the resident pGV2260 Ti plasmid. **B7**, pW9 DNA.

Fig. 5.14b

Gel electrophoresis of total plant and bacterial DNA digested to completion with *Hae* III. **Lane 1**, λ DNA digested with *Sty* I. **Lane 2**, *N. tabacum* SRI-CPX1*. **Lane 3**, *N. tabacum* SRI-CPX2*. **Lane 4**, *N. tabacum* SRI-CPX3*. **Lane 5**, *N. tabacum* SRI-CPX4*. **Lane 6**, *N. tabacum* SRI-CPX5*. **Lane 7**, *N. tabacum* SRI-CPX6*. **Lane 8**, *N. tabacum* SRI-CPX7*. **Lane 9**, *N. tabacum* SRI-CPX8*. **Lane 10**, *N. tabacum* SRI-CPX9*. **Lane 11**, Total DNA from *A. tumefaciens* C58C1 with pTIX6 integrated into the resident pGV2260 Ti plasmid. **Lane 12**, Untransformed *N. tabacum* cv. SRI.

*Different transgenic plants of *N. tabacum* cv. SRI transformed by *A. tumefaciens* with the pTIX6 CP construct integrated into the resident pGV2260 Ti plasmid.

Fig. 5.14c

Southern blot of total DNA from transgenic tobacco plants digested with *Hae* III, using ^{32}P-labelled *BamH* I - *BamH* I fragment of pMTLX3 (containing the SAPV CP gene) as a probe. **Lane 1**, λ DNA digested with *Sty* I. **Lane 2**, *N. tabacum* SRI-CPX1*. **Lane 3**, *N. tabacum* SRI-CPX2*. **Lane 4**, *N. tabacum* SRI-CPX3*. **Lane 5**, *N. tabacum* SRI-CPX4*. **Lane 6**, *N. tabacum* SRI-CPX5*. **Lane 7**, *N. tabacum* SRI-CPX6*. **Lane 8**, *N. tabacum* SRI-CPX7*. **Lane 9**, *N. tabacum* SRI-CPX8*. **Lane 10**, *N. tabacum* SRI-CPX9*. **Lane 11**, Total DNA from *A. tumefaciens* C58C1 with pTIX6 integrated into the resident pGV2260 Ti plasmid - digested with *Hae* III. **Lane 12**, Untransformed *N. tabacum* cv. SRI.

*Different transgenic plants of *N. tabacum* cv. SRI transformed by *A. tumefaciens* with the pTIX6 CP construct integrated into the resident pGV2260 Ti plasmid.

Fig. 5.15

Fig. 7. EBIA using polyclonal antiserum directed against SAPV. **Lane 1**, *N. tabacum* SRI-CPX1*. **Lane 2**, *N. tabacum* SRI-CPX2*. **Lane 3**, *N. tabacum* SRI-CPX3*. **Lane 4**, *N. tabacum* SRI-CPX4*. **Lane 5**, *N. tabacum* SRI-CPX5*. **Lane 6**, *N. tabacum* SRI-CPX6*. **Lane 7**, *N. tabacum* SRI-CPX7*. **Lane 8**, *N. tabacum* SRI-CPX8*. **Lane 9**, Untransformed *N. tabacum* cv. SRI. **Lane 10**, Sap of SAPV-infected *Passiflora edulis*.

*Different transgenic plants of *N. tabacum* cv. SRI transformed by *A. tumefaciens* with the pTIX6 CP construct integrated into the resident pGV2260 Ti plasmid.

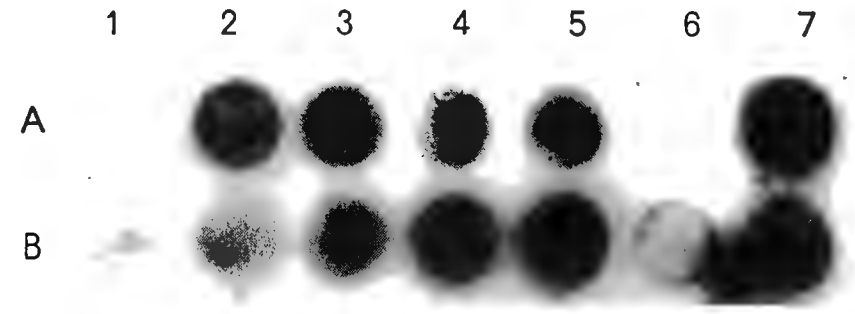

Fig. 5.14a

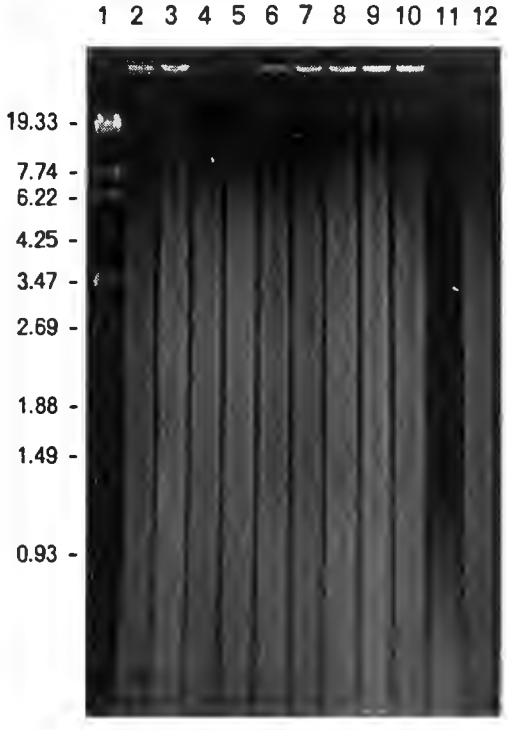

Fig. 5.14b

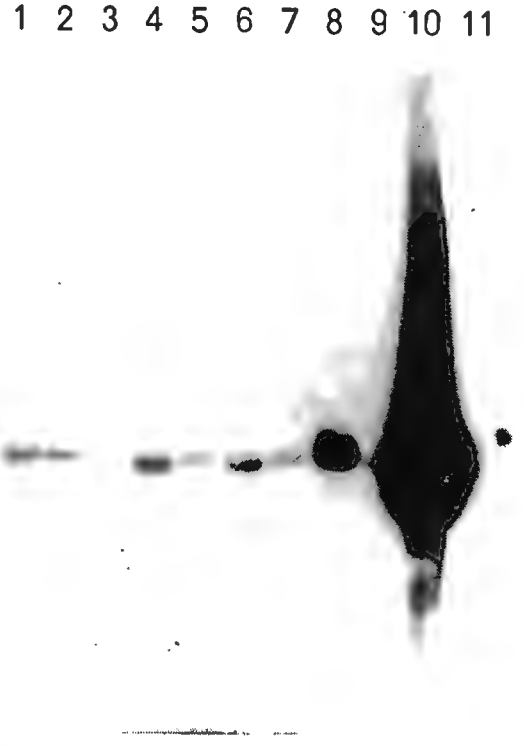

Fig. 5.14c

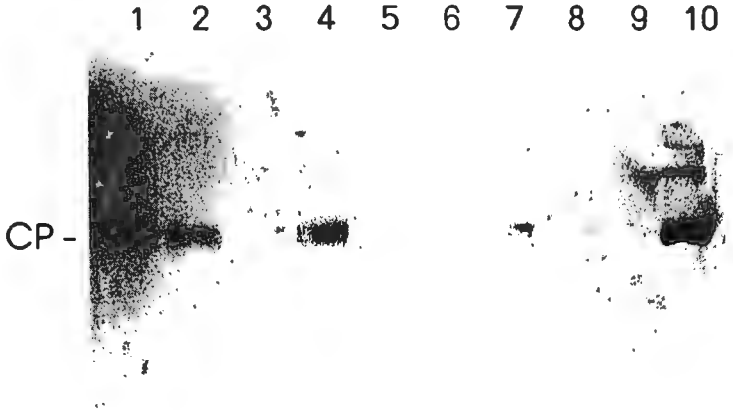

Fig. 5.15

CHAPTER 6. SUMMARY, GENERAL DISCUSSION AND CONCLUSION

6.1 SUMMARY

6.1.1 Survey of viruses in South African woodiness diseased *Passiflora*

Woodiness disease caused by virus infection is considered to be the most serious disease of passionfruit. Although as many as eight distinct viruses from different virus groups have been found to infect *Passiflora* worldwide (Chapter 1, section 1.3.2), the woodiness disorder was hitherto ascribed to infection by PWV, CMV or a mixed infection of both viruses (Taylor & Kimble, 1964).

Passiflora material exhibiting characteristic woodiness symptoms (described by Taylor & Kimble, 1964, and Shukla *et al.* 1988b) was collected from all areas of commercial production in South Africa for a preliminary survey of virus incidence. As it was suspected that the disease complex in this material would be analogous to situations elsewhere, antisera against other strains of CMV and PWV were collected to aid with the initial screening. Filamentous particles were readily discernible in aggregated bundles in EM analysis of crude sap extracts of woody *Passiflora* hosts, but the high levels of background debris observed with these hosts often obscured spherical particles, necessitating the transfer of these particles to a range of alternative hosts for observation by EM. Infected plants were subjected to EBIA after EM examination to aid with the selection of antisera. Filamentous particles resembling potyviruses were found to be the most widespread in samples that were tested. Material from Natal was often found to be infected by a mixture of different viruses. Two of these were identified as CMV and a potyvirus. A third virus that was sometimes present in mixed infections was identified as TNV (Von Wechmar *et al.*, 1991). The role of TNV in infected *Passiflora* was not investigated further in this study.

6.1.2 Biological properties of two viruses implicated in woodiness disease of South African *Passiflora*

A *P. caerulea* rootstock specimen with woodiness symptoms from Natal harbouring a mixed infection of a potyvirus, CMV, and possibly TNV, was selected for further studies. The CMV component and the potyvirus were separated biologically by taking advantage of their different host ranges. Earlier attempts to achieve their separation by physical means failed. Thereafter they

were propagated separately in *N. benthamiana* and purified. The biological properties, host range, and physicochemical properties of CMV (designated the CMV-Pass isolate) were in good agreement with data published previously for CMV from *Passiflora* (Taylor & Kimble).

The potyvirus (temporarily designated the Natal *P. caerulea* potyvirus), whilst sharing many biological and physicochemical properties with strains of PWV (Taylor & Kimble, 1964; Shukla et al., 1988b), differed biologically from strains of PWV hitherto characterized. For instance, it was able to systemically infect *N. benthamiana* (not reported for PWV), and was unable to spread systemically in the secondary leaves of *P. vulgaris* cv. Bountiful as reported by Taylor & Kimble (1964).

As it was uncertain at this stage whether the biological differences between the Natal *P. caerulea* potyvirus and PWV were significant or merely represented subtle strain differences, it was decided to characterize this virus on the molecular level. Even if it did turn out to be a strain of PWV, it would be interesting to obtain the CP nucleotide sequence for the following reasons: (a) up to that point in the project only the CP amino acid sequences of three closely related strains of PWV were known (Shukla *et al.*, 1988b); (b) the CP gene could be used for expression in plants for the future production of virus-resistant *Passiflora* varieties.

6.1.3 Detection of CMV-Pass and SAPV

EBIA and DAS-ELISA were found to be the most useful and reliable methods for the detection of potyviruses in *Passiflora*, whereas dotblot hybridization using 5'-endlabelled dsRNA proved to be more reliable and sensitive for the detection of CMV. The failure of serological techniques to facilitate the reliable detection of CMV in *Passiflora* could be ascribed to the low concentration of this virus in the *Passiflora* host (Taylor & Kimble, 1964) and its notoriously poor immunogenicity (Rybicki & Von Wechmar, 1985). No studies were done to establish whether different strains of CMV occurred in *Passiflora*.

EBIA indicated that the Natal *P. caerulea* potyvirus occurs widespread in most regions of production. However, detection studies using EBIA and ISEM have also indicated that there may be more than one strain of potyvirus involved in woodiness of South African *Passiflora*. An immunogenically distinct potyvirus with a lower CP MW was detected in the Burgershall area, which sometimes appeared to occur in a mixed infection with a potyvirus physicochemically similar to the Natal *P. caerulea* potyvirus. Although the apparent mixture of different potyviruses in a single plant may by due to a lack of cross-protection between these two potyviruses, it is more likely that the situation derived from the practice of unknowingly grafting with infected scionwood.

CMV-Pass was detected mainly in diseased material from the Natal region. In field samples, CMV-Pass always seemed to occur in mixed infections

with the Natal *P. caerulea* potyvirus when present in *Passiflora*. This was interesting because CMV was found to be very infective for *P. edulis* only when this host already harboured a potyvirus. It seems that CMV-Pass acts as an opportunistic pathogen in this instance, preferentially infecting *Passiflora* that already harboured a potyvirus. *Passiflora* material infected by a combination of both viruses showed more dramatic symptoms than either of these viruses cause in a single infection, indicating a synergistic interaction between these pathogens. CMV-Pass has a wide host range and a weed (*N. physalloides*) commonly found in passionfruit vineyards in Natal, was identified as a possible reservoir of virus.

6.1.4 Analysis of the 3'-terminal nucleotide sequence and the deduced CP amino acid sequence of the Natal *P. caerulea* potyvirus

Comparative analysis of nucleotide and deduced amino acid sequences verified that the Natal *P. caerulea* potyvirus should be considered a definitive member of the potyvirus group. The presence of a highly conserved "DAG"-amino acid motif (proposed to be essential for aphid transmission of potyviruses - Harrison & Robinson, 1988; Atreya *et al.*, 1991) positioned close to the N-terminus of the deduced CP of the Natal *P. caerulea* potyvirus, is in accord with the aphid transmissibility of this virus. The 3' NCR of the Natal *P. caerulea* potyvirus contains two direct repeats, GAACTTAGC and TGGTTTTAG respectively (underlined in Fig. 4.7). Similar repeats of unknown function were also reported for a number of other potyviruses (Allison *et al.*, 1985; Dougherty *et al.*, 1985; Domier *et al.*, 1986; Van der Vlugt *et al.*, 1989).

6.1.5 Phylogenetic relationships between the Natal *P. caerulea* and other potyviruses

Molecular phylogenies based on CP and 3'-NCR data have made a significant impact in resolving the chaotic state of potyvirus taxonomy that existed until recently. Such data is adequate for unambiguous designation of viruses down to the strain level, and up to the family level. DNA complementary to the potyviral 3'-terminal region (encompassing the CP and the 3'-NCR) is readily obtainable by oligo-dT primed reverse transcription due to the fact that the ssRNA is polyadenylated, which accounts for the apparent preoccupation of potyvirus taxonomists with this region.

Phylogenetic analysis of CP sequence relationships indicated that the virus is significantly different from other strains of PWV for which the amino acid sequences are known (only 71% amino acid sequence similarity to PWV-TB), while strains of given potyviruses are usually more than 90% similar (Shukla & Ward, 1989b; Rybicki, unpublished). This data suggest that the Natal *P. caerulea* potyvirus is a distinct potyvirus within the PWV-related subgroup of

potyviruses, and not merely a strain of PWV. This new potyvirus was designated South African *Passiflora* potyvirus (SAPV).

6.1.6 Expression of the SAPV CP gene in *E. coli*

Reaction of the SAPV CP gene expressed in *E. coli* with polyclonal anti-SAPV antiserum in EBIA, served to comfirm the authenticity of the cloned gene. Antiserum directed against the β-gal::SAPV CP fusion protein reacted in EBIA with SAPV present in crude sap extracts of infected *Passiflora*. The expression of viral genes in *E. coli* thus provides a feasible approach to obtain polyclonal antibodies free from contaminating anti-plant host antibodies. It could also be a useful method for obtaining antibodies against polypeptide products of poorly expressed genes. Although this antiserum worked in EBIA, it was, however, not suitable for use in ELISA systems. ELISA of native virus probably rely on antibodies directed to external epitopes of potyvirus particles. These epitopes may not be well represented on the exterior of the β-gal::CP fusion protein due to tertiary structural differences, wheras for EBIA, the proteins are blotted onto nitrocellulose in a denatured state and can thus react with antibodies directed to epitopes which may be obscured in native virus preparations.

6.1.7 Expression of the SAPV CP gene in transgenic tobacco

In order to evaluate the translatability and stability of the SAPV CP in transgenic plants the SAPV CP gene was cloned downstream of a CaMV 35S promotor provided by the pGSJ280 cointegration vector, mobilized into *A. tumefaciens*, and used to transform tobacco. The vector construct (pCPX1) used for expression of the SAPV CP gene in *E. coli* also served as an intermediate construct for fusion of the gene in frame to an ATG-initiation of translation motif provided by the plant expression cloning vector. An estimate from EBIA results indicated that the CP gene was expressed at levels which range from a fifth to a tenth of CP levels observed in SAPV-infected *Passiflora*. The apparently satisfactory stability of the expressed SAPV CP observed in transgenic tobacco holds great promise for the eventual expression of the CP gene in *Passiflora*.

6.2 GENERAL DISCUSSION AND CONCLUSION

The two viruses described in this study are important factors in the local woodiness disease complex, but they do not represent the full spectrum of viruses in S.A. *Passiflora*. A second potyvirus/strain that was distinct from

SAPV, as well as TNV (Von Wechmar *et al.* 1991), were also encountered. It is plausible that there may be a number of other undiscovered viruses in local passionfruit, especially in view of the fact that viruses from eight different plant virus taxonomic groups have been reported to infect *Passiflora* world wide (Brunt *et al.* 1990).

It was unexpected that the SAPV seemed to be more geographically widespread than CMV-Pass and yet had a much more restrictive host range than the latter virus. This could, however, be explained by the nursery practice of unknowingly using potyvirus infected scionwood. This practice could contribute to the distribution of the SAPV, while rendering the material more susceptible to CMV present in other crops and plants occurring near passionfruit vineyards. *P. caerulea* appeared to be more susceptible to CMV than *P. edulis*. The limited occurrence of CMV may thus also relate to the relatively recent introduction of *P. caerulea* as a "nematode/*Phytophthora*" resistant rootstock into the South African passionfruit industry. *P. caerulea* is a very vigorous grower, and shoots developing from roots are often observed in vineyards established on this rootstock. Such shoots are usually mechanically mowed at regular intervals, which can result in the mechanical transmission of viruses. Furthermore, such shoots are often surrounded by perennial weeds which may be a source of virus. The apparent ability of this rootstock to mask CMV infection under certain conditions renders it a risk for use in the nursery, and its use should be suspended at least until effective ways of protecting the passionfruit crop against virus infection is established.

Detection techniques employed in this study (including electron microscopy, ISEM, nucleic acid hybridization, DAS-ELISA, SDS-PAGE and EBIA) all suffered from sensitivity restrictions which sometimes prevented the detection of very low amounts of virus in infected tissue.

Recent advances in the detection of potyviruses makes use of the polymerase chain reaction (PCR) to amplify specific virus sequences from reverse transcribed RNA (in crude extracts). This approach holds great promise for diagnostic work (Langeveld *et al.*, 1991; Brand & Rybicki, unpublished results, S. Pappu, personal communication). This technique is extremely sensitive, and can amplify specific sequences from negligible amounts of infected plant tissue. The only prerequisite for this approach is knowledge of the (partial) nucleotide sequence of the virus in question to facilitate the design of primers. For diagnostic purposes these primers are usually targeted at conserved regions in the viral genome, and made degenerate to diminish their strain specificity. DNA amplified in this manner can be subjected to restriction analysis or nucleotide sequencing, which supplements the diagnostic capabilities of this technique to yield phylogenetic data.

This study highlighted the limitations of physicochemical characterization, "classical" serology using polyclonal antiserum, and biological

characterization for the identification of potyviruses on the virus/strain level. The use of 3'-terminal sequence data (including the CP gene and 3'-NCR) to resolve virus/strain relationships in the potyvirus group, has made a tremendous impact in recent years (Rybicki & Shukla, in press; Ward *et al.*, in press; see Chapter 1, section 1.3.3.3). Although SAPV can elicit typical woodiness symptoms in passionfruit and resembles PWV in most other respects, it was shown to be only distantly related to the latter virus by nucleotide sequence analysis of the CP gene and the 3' NCR. The fact that different viruses can elicit a similar disease condition in *Passiflora* indicate that woodiness may be a virus non-specific host response, and can probably be ascribed to different viruses adapting to a particular biological niche in this host, to elicit a physiological disorder resembling woodiness. A similar situation exists with tomato yellow leaf curl gemini virus, where the same apparent disease is caused by distantly related geminiviruses in Africa, Europe, India and Thailand (Keyr-Pour *et al.*, 1991).

The CP of SAPV is subject to degradation which can sometimes be observed in EBIA as multiple bands below the actual CP band in the 33 to 30 kDa position. The CP of OMV (Burger *et al.*, 1990) appears distinctly more stable. A lysine-rich region occurring in the N-terminus, immediately downstream of the "DAG-aphid transmissibility motif" of most potyviral CPs (including SAPV), was found to be absent from OMV, PPV-NAT and PPV-D. It is therefore reasonable to speculate that the high lysine content of N-termini of many potyviral coat proteins is perhaps involved in the ubiquitin-targeted protein destruction known to occur in eukaryotic cells (Ciechanover *et al.*, 1984). This phenomenon may enhance the wide specificity observed with most anti-potyviral polyclonal antisera, by exposing the conserved core region of the CP when used as an antigen for immunization of animals.

The future objective is to express the SAPV CP gene in transgenic *P. edulis* in an attempt to equip the passionfruit crop with CP-mediated cross protection against a broad spectrum of potyviruses (Kaniewski *et al.*, 1990). This was unfortunately beyond the scope of this study, particularly since no reliable methods for the transformation of this crop have been reported. Due to the low infectivity demonstrated by CMV-Pass for the *P. edulis* host in the absence of an endogenous potyvirus infection, it is anticipated that SAPV CP-mediated protection of passionfruit against potyvirus infection, may also offer an indirect inhibition of infection by CMV-Pass.

A viral gene that should be investigated in future as a possible source of wide spectrum protection, is the movement protein such as the 30 kDa protein of TMV (Deom *et al.*, 1987, 1990). *In vivo* complementation studies (Malyshenko *et al.*, 1989) suggested that the virus-coded transport functions operate in a host-specific rather than a virus-specific fashion by increasing the plasmodesmatal exclusion limit (Atkins *et al.*, 1991). This means that when the plasmodesmata of

a host have been modified by one virus, a second virus can spread systemically, even in a host that is usually restrictive to that particular virus.

By expressing a series of "movement protein" deletion mutant clones in transgenic plants, it may be possible to find mutant proteins that would bind to the plasmodesmata, but that would be unable to increase the exclusion limit. The idea is thus to inertly block the plasmodesmatal sites where active movement proteins would normally bind. Such a strategy could be more useful than the CP approach, which only protects amongst some viruses in the same group, as it may be effective against a wide range of unrelated viruses infecting a particular host. The CI protein of potyviruses and the 3A protein of CMV have been identified as possible movement protein genes for these viruses (see Chapter 1, section 1.3.3.4), that can be evaluated in view of protection of passionfruit.

I believe that the work described in this thesis represents significant advancement in contending the woodiness disease of passionfruit, especially in view of recent progress in the *in vitro* culturing of *Passiflora* (Drew, 1991), which may render the transformation of *Passiflora* an achievable short term objective which will certainly be persued in the future.

APPENDIX A:

A.1 GENERAL 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
°C	degrees centigrade
Ci	curie
CI	cytoplasmic inclusion
CIP	calf intestinal alkaline phosphatase
CP	coat protein
cpm	counts per minute
CSFRI	Citrus and Subtropical Fruit Research Institute
cv.	cultivar
Da	dalton
DAS-ELISA	double antibody sandwich enzyme-linked immunoassay
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
ds	double stranded
DTE	dithioerythritol
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EBIA	electroblot immunoassay
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EM	electron microscopy
EtOH	ethanol
g	gravitational acceleration
G	guanine / guanosine
HC	helper component
HPLC	high performance liquid chromatography
ICTV	International Committee on the Taxonomy of Viruses
IgG	gamma-immunoglobulin
ISEM	immunosorbent electron microscopy
Kb	kilobase
M	Molar
Mol	mole
MOPS	morpholinepropanesulphonic acid
M_r	relative molecular weight
NBT	nitro-blue tetrazolium chloride
NI	nuclear inclusion
nt	nucleotide
OD_{600}	optical density at 600 nm wavelength
oligo(dT)	oligodeoxythymidylic acid
ORF	open reading frame
PBS	phosphate-buffered saline

PEG	polyethylene glycol
poly(A)	polyadenylic acid or polyadenylate
PPRI	Plant Protection Research Institute
PVP	polyvinylpyrrolidone
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sp(p)	species (plural)
ss	single stranded
T	thymine / thymidine
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
Tris	tris(hydroxymethyl)aminomethane
U	unit
UCT	University of Cape Town
uv	ultraviolet
v/v	volume per volume (in ml per 100 ml)
w/v	weight per volume (in g per 100 ml)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

A.2 LIST OF VIRUS ACRONYMS

AlMV	alfalfa mosaic virus
BaYMV	barley yellow mosaic virus
BCMV	bean common mosaic virus
BlCMV	blackeye cowpea mosaic virus
BYMV	bean yellow mosaic virus
CaMV	cauliflower mosaic virus
CMV	cucumber mosaic virus
CPRSV	cowpea ringspot virus
CYVV	clover yellow vein virus
JGMV	Johnsongrass mosaic virus
LMV	lettuce mosaic virus
MDMV-A or B	maize dwarf mosaic virus
MrMV	maracuja mosaic virus
OMV	ornithogalum mosaic virus
PeMV	pepper mottle virus
PGMV	peanut green mosaic virus
PLV	passiflora latent virus
PMV	pea mosaic virus
PRV	passionfruit ringspot virus
PSV	peanut stunt virus
PVCV	passionfruit vein clearing virus
PVY	potato virus-Y
PWV	passionfruit woodiness virus
PYMV	passionfruit yellow mosaic virus
SCMV	sugar cane mosaic virus
SoyMV	soybean mosaic virus
SAPV	South African passiflora virus
SPMMV	sweet potato mild mottle virus
TAV	tomato aspermy virus
TEV	tobacco etch virus
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
TomRV	tomato ringspot virus
TRV-TCM or PBL	tobacco rattle virus
WMVII	watermelon mosaic virus-II
WSMV	wheat streak mosaic virus

APPENDIX B

METHODS

CONTENTS

APPENDIX B

METHODS

B.1 SEROLOGICAL PROCEDURES

B.1.1 Rabbit immunisation

Antisera to the Natal *P. caerulea* potyvirus and CMV-Pass respectively were prepared from sucrose gradient-purified virus (0.2 - 0.4 mg virus/injection), and antiserum to β-gal::SAPV-CP fusion protein from partially-purified fusion protein (250 µl/injection). Antisera were raised in rabbits as described by Van Regenmortel (1982): weekly injections of purified antigen, emulsified in an equal volume of Freund's incomplete adjuvant, were administered for four weeks, and four subsequent booster injections were given at monthly intervals. Serum was collected three weeks after the initial injections, and weekly thereafter. Antiserum titers were approximated by microprecipitin tests (Noordam, 1973).

B.1.2 Preparation of virus-specific IgG

Polyclonal anti-Natal *P. caerulea* potyvirus and anti-CMV-Pass antisera were host absorbed three times with sap from virus-free *N. benthamiana*, the host used for propagation and purification of these viruses. For host absorption crude serum was mixed with 25% (v/v) plant host sap and incubated at 37°C for 30 min. The non-specific antibody fraction bound to host components was then removed by centrifugation at 10 000 RPM for 10 min in a Sorvall SS34 rotor. The gamma globulin fractions were purified from these polyclonal antisera by two ammonium sulphate precipitations, followed by DEAE-cellulose (Whatman DE-52) filtration to remove lipids (Clark & Bar-Joseph, 1984). Final IgG concentration was estimated by measuring the absorption at λ=280 nm, and ajusted to 2 mg/ml in half strength PBS (5 mM sodium phosphate; 75 mM NaCl pH 7.4), mixed with an equal volume of glycerol, and stored at -20°C.

B.1.3 Conjugation of alkaline phosphatase to antibodies

Alkaline phosphatase (Seravac, Cape Town, SA, ca 1 u/mg) was conjugated to CMV-Pass-specific and Natal *P. caerulea* potyvirus-specific IgG respectively, by glutaraldehyde-mediated crosslinking (Clark & Bar-Joseph,

1984). Goat anti-rabbit alkaline phosphatase conjugates were also prepared by this method and stored at 4 °C in siliconized glass bottles.

B.1.4 Enzyme-linked immunosorbent assays (ELISA)

DAS-ELISA was performed as reported by Clark & Bar-Joseph, (1984), except that skim milk was used as a blocking agent instead of bovine serum albumin. Microwell plates (Nunc, Denmark), were coated for 90 min at 37°C with virus-specific IgG (2 μg/ml) in 0.05 M sodium carbonate buffer, 9.6. Thereafter plates were coated overnight at room temperature with antigen diluted in blocking buffer (10 mM phosphate, 150 mM NaCl pH7.4, 0.1% (v/v) Tween 20, 0.2% (w/v) skim milk powder), and washed three times (3 min per wash) with washing buffer (10 mM phosphate, 150 mM NaCl pH7.4, 0.1% (v/v) Tween 20). Plates were then incubated for 90 min at 37°C with IgG conjugated to alkaline phosphatase diluted 1/500 in blocking buffer. After another series of three washes, enzyme substrate was added (freshly prepared 1mg/ml p-nitrophenyl phosphate in 10% (v/v) diethanolamine). Light absorbance of the enzyme-substrate reaction was measured and recorded at 405 nm in a Titertek Multiscan ELISA Reader (Flow Laboratories, Sweden).

B.1.5 Electroblot-immunoassay (EBIA)

EBIA of viral coat proteins was performed by a modification of the method 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 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 (section B.1.4) 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).

B.1.6 Antisera used for virus detection and relationship studies.

Antiserum	Origin
Potyvirus:	
Natal *P. caerulea* potyvirus (SAPV)	This study
OMV	J.Burger, UCT collection
PWV (Taiwan)	Prof. N-S. Lin (Acad. Sin. Bot. - Rep. China)
PVY-SA	M.B. Von Wechmar, UCT collection
WMV-2 (Purcifull NY 69)	M.B. Von Wechmar, UCT collection
WMV-1	H. Lecoq, Montfavet, France
SoyMV	R.M. Lister, Purdue University, IN
BYMV-Glad	J.Burger, UCT collection
MDMV-A (SA Johnsongrass isolate)	M.B. Von Wechmar, UCT collection
MDMV-B (SA maize isolate)	M.B. Von Wechmar, UCT collection
CMV:	
CMV-Pass	This study
CMV-LupK5	M.B. Von Wechmar, UCT collection
CMV-Y	M.B. Von Wechmar, UCT collection

B.2 PROTEIN ELECTROPHORESIS

B.2.1 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), β-mercaptoethanol; 15% (v/v) glycerol; 0.01% (w/v), bromophenol blue.

SDS-PAGE gel preparation

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.3 NUCLEIC ACID PROCEDURES

B.3.1 Preparation of bacterial and plasmid DNA

B.3.1.1 Mini-preparation of total bacterial DNA

Small quantities of plasmid DNA for plasmid size determination could be isolated in under four hours 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 micro centrifuge tubes containing 750 µl LB medium (Appendix B.4.1.1) supplemented with 100 µg/ml ampicillin. After shaking vigorously (with tubes in a horizontal position) at 30°C for 2-3 h, cells were harvested by spinning in a micro centrifuge 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 micro centrifuge. 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 (see B.3.15).

B.3.1.2 Plasmid DNA preparation

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

Small scale plasmid DNA preparations

Cell cultures were grown overnight at 37°C in 3 ml LB-Amp_{100} medium (LB containing 100 µg/ml ampicillin), shaking vigorously. Cells were harvested by centrifugation in a micro centrifuge, and 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 (3M potassium acetate, pH 4.8) 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 was added to a final concentration of 50 µg/ml, and the sample 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.3.2 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*, bovine serum albumin (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 buffer 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 (B.3.5)

Composition of the Boehringer Mannheim restriction enzyme incubation buffer set

Buffers	A	B	L	M	H
Buffer components	**Final concentration (mM)**				
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	-	-	-	-
β-Mercaptoethanol	-	1	-	-	-
pH at 37°C	7.9	8.0	7.5	7.5	7.5

B.3.3 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.3.4 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.3.5 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 results more quickly, 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 mini 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.3.6 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 micro centrifuge 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 200ul fresh high salt NET buffer. The two buffer fractions were pooled, phenol/chloroform extracted, ethanol precipitated, and final pellets resuspended in TE buffer.

B.3.7 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.3.8 Extraction and purification of dsRNA

Double stranded replicative intermediate RNA was extracted and affinity-purified essentially as described by Dodds *et al.*, (1984). Seven gram of fresh plant tissue was frozen with liquid nitrogen and ground to a fine powder using a Bosch coffee grinder. The powder was mixed with 14 ml STE buffer (0.1 M NaCl, 0.05 M Tris-Hcl pH 7.0 and 1 mM EDTA), 18 ml water saturated phenol and 16 mg fractionated bentonite. After shaking vigourously for 30 mins, the mixture was centrifuged for 15 min at 8000 g in a Sorvall centrifuge using a Sorvall GSA rotor. Ethanol was added to the supernatant to a final concentration of 16.5% before passing the mixture through a Whatman CF11 column (2.5g dry powder) pre-equilibrated with STE containing 16.5% ethanol. After washing the column with 150 ml STE containing 16.5% ethanol, dsRNA was eluted from the column with three aliquots of 5 ml STE and precipitated. The first aliquot was discarded and the last two aliquots were precipitated by addition of 0.5 ml of 3.0 M sodium acetate pH 5.5 and 12 ml of isopropanol. The resulting pellet was resuspended in 500 µl of 0.2 X STE buffer and incubated with RNase-free DNase

for one hour at 37°C. This was followed by phenol-chloroform extraction and ethanol precipitation as described in Appendix B.3.3.

B.3.8.1 Total nucleic acid extraction for dsRNA isolation

CMV-Pass-infected leaves were frozed in liquid nitrogen, and ground to a fine powder using a Bosch coffee bean grinder. Seven grams of the powder was mixed with 14 ml STE buffer pH 7 (0.1 M Nacl; 0.05 M Tris-Cl; 1 mM EDTA), 2.0 ml of 10% SDS, 18 ml of STE-saturated phenol and 16 mg fractionated bentonite. The suspension was shaken for 30 min at room temperature before the cell debris was pelleted by low speed centrifugation (15min at 15K in a Beckman SS34 rotor). The aqueous phase was collected and made up to 20 ml with STE. Ethanol was then added (4.2 ml of 95% proof commercial ethanol) to obtain a final concentration of 16.5% (v/v). The mixture was left to reach room temperature before fractionation on DEAE cellulose.

B.3.8.2 DEAE cellulose column chromatography of dsRNA

Whatman CF-11 cellulose (2.5 g dry weight) equilibrated in STE-buffered 16.5% ethanol, was poured into the barrel of a 50 ml disposable syringe preplugged with compressed glass wool.

The nucleic acid extract was poured into the column, and excess liquid was dripped out, while dsRNA stayed bound to the affinity column. The column was washed with 120 ml STE-buffered 16.5% ethanol, before the dsRNA was eluted out of the column with 15 ml ethanol-free STE buffer. The eluate was precipitated overnight by addition of 1/20 volumes 3 M NaOAc and 2.5 volumes 95% ethanol. Precipitated dsRNA was pelleted by centrifugation for 30 min at 15K in a Beckman SS34 rotor and resuspended in 0.5 ml TNM buffer (0.1 M Tris-Cl; 1 M NaCl; 0.1 M $MgCl_2$.

B.3.8.3 DNase digestion of dsRNA to remove contaminating DNA

To render DNase free from contaminating RNases, it was incubated in the presence of Proteinase K before being incubated with the dsRNA preparation. Stock solutions of DNase (1 mg/ml) and Proteinase K (5 mg/ml) were made up. To DNase from the stock solution (30 µl) was added 4 µl of 0.45 M $CaCl_2$ and 30 µl of the proteinase K stock solution. This mixture was incubated for 1 h at 37°C.

10 µl of the prepared DNase and 5.1 µl of 0.5 M $MgCl_2$ were then added to 0.5 ml of the dsRNA sample and incubated for a further hour at 37°C before phenol extraction and precipitation as described in Appendix B.3.3.

B.3.8.4 Differential LiCl precipitation

The DNased dsRNA sample was subsequently subjected to differential LiCl precipitation to rid the preparation from contaminating ssRNA. The sample was ajusted to 2 M LiCl by addition of 1/3 volume 8M LiCl. Single stranded RNA was selectively precipitated by overnight incubation on ice, and removed by centrifugation in a micro centrifuge. The supernatant was ajusted to 4 M LiCl by addition of another 1/2 volume 8 M LiCl and incubated overnight on ice. The dsRNA was pelleted by centrifugation, resuspended in 400 µl water, and re-percipitated by ethanol as described in Appendix B.3.3 before finally being resuspended in 50 µl sterile double distilled water. The dsRNA was quantified by uv absorption using an extinction coefficient of 20.

B.3.8.5 PAGE analysis of dsRNA

Best resolution for the analysis of dsRNA was obtained by electrophoresis in 5% polyacrylamide gels.

To prepare 30 ml of a 5% gel for electrophoresis in a vertical Hoefer mighty small apparatus:

40% acrylamide (acrylamide:bisacrylamide 38:1)	3.7 ml
10% ammonium persulphate	0.3 ml
10 x TBE	3 ml
H_2O	22.9 ml
TEMED	20 ml

After polymerization, 3 µl dsRNA sample and 5 µl loading buffer were loaded per well, and run for 4 to 5 h at 50 V. The sample loading buffer consisted of 50% (v/v) glycerol; 0.4% (w/v) bromophenol blue; 0.4% (w/v) xylene cyanol; and 1 mM EDTA and 100 µg/ml ethidium bromide. Fluorescent bands representing dsRNA were visualized on a 260 nm transilluminator.

B.3.9 Labelling of DNA and RNA

B.3.9.1 Nick translation 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 MgCl2 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.3.9.2 5'-Endlabelling of ssRNA

For endlabelling, 1 µg ssRNA was resuspended in 10 µl of 50 mM Tris-HCl pH 9.5, and hydrolysed by heating at 60°C for 25 min. Labelling was performed at 37°C for 1 h by addition of 25 µCi γ-^{32}P-dATP, 10 U polynucleotide kinase (PNK), 5 µl of RNA labelling buffer (50 mM Tris-HCl pH9.5, 45 mM $MgCl_2$, 5 mM dithiothreitol), in a total volume of 20 µl. The reaction was stopped by addition of 20 µl of 0.5 M EDTA, and the unincorporated nucleotides removed by Sephadex G50 pasteur column chromatography (Appendix B.3.4).

B.3.9.3 5'-Endlabelling of dsRNA

Labelling of dsRNA was performed by a modification of the method described by Rosner *et al.*, (1983).

Double stranded RNA (±1 µg) was resuspended in 10 µl hydrolysis buffer (50 mM Tris-HCl pH 9.5; 1 mM EDTA), and hydrolysed by heating at 88°C for 45 min. Labelling was performed at 37°C for 1 h by addition of 25 µCi γ-^{32}P-dATP, 10 U polynucleotide kinase (PNK), 5 µl of RNA labelling buffer (50 mM Tris-HCl pH9.5, 45 mM $MgCl_2$, 5 mM dithiothreitol), in a total volume of 20 µl. The reaction was stopped by addition of 20 µl of 0.5 M EDTA, and the unincorporated nucleotides removed by Sephadex G50 pasteur column chromatography (Appendix B.3.4).

B.3.10 Colony hybridisation assays

Buffers for hybridisation assays

1. 20X SSPE
   3.6M NaCl; 200mM sodium phosphate pH 7.7; 20mM EDTA

2. 100 x 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

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.3.11 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 above). 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.

B.3.12 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 35S 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.3.13 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 Natal *P. caerulea* potyvirus RNA was resuspended in water to a concentration of 1 µg/µl.

In two micro centrifuge 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:

Constituent	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	-
H2O	1.5	3.5
Reverse transcriptase	2.0	2.0
Total volume	20.0 µl	20.0 µl

** In a second cloning experiment, all the above reaction constituents were identical, except that random hexamer primers (0.5 µl) were used in conjunction with oligo-d(T) (1.5 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:

Constituent	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
H2O	17.0	15.0
Total volume	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. cDNA was then precipitated by addition of 2.5 volumes of ETOH and 1/25 volume of 3 M NaOAc pH5.5. The pellets were resuspended in 50 µl TE buffer and size-fractionated by passage through a Sepharose 4B (Pharmacia Fine Chemicals, Sweden) pasteur column, collecting 100 µl fractions. Fractions were counted by Cerenkov counting, and only the first major fraction of the first peak was used for cloning into pUC19 vector.

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 micro centrifuge 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
Total volume	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 Primers
Oligo d(T)12-18, 2 µg/µl (Boehringer Mannheim); Random hexamer primer mix 2 µg/ml (BM)

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

Calculation of cDNA yield

The cDNA yield was estimated as described in the technical manual of the *Riboclone*™ cDNA synthesis system from Promega Corporation. Basically it entails the calculation of the molarity of dNTPs incorporated, from which the MW of cDNA can be estimated. This is achieved by calculation of the percent incorporation radiolabelled dCTP in the first and second strand reactions. The yield can then be estimated if the molarity of labelled and unlabelled dNTPs is known and it is assumed that all dNTP's will be incorporated in approximately equimolar ratios.

B.3.14 Preparation and transformation of competent *E. coli* cells

E. coli LK-111 (Appendix B.4.3.1) competent cells were prepared as described by Chung & Millar (1988).

Freshly-grown overnight LK-111 cultures were used to inoculate 100 ml LB medium. These were allowed to grow to an early log phase (OD600 = 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 Mr 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 aliquotted and 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. Aliquots of 100 µl transformed cells were plated on Luria-Bertani agar (LB-Amp_{100}) plates. 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.3.15 Exonuclease III/S1 nuclease shortening

Shortening of DNA to obtain deletion mutants for sequence analysis was performed according to the method of Henikoff (1984). This entailed the digestion of the recombinant clone DNA with two appropriate unique restriction enzymes to obtain cleavage at two sites in the polylinker region on the same side of the insert DNA. Enzymes are chosen such that the one closest to the insert produces a 5' overhang (from where shortening proceeds), while the site more distal from the insert produces a 3' overhang (protected site resisting exonuclease attack). By exposing such DNA to differential time-intervals of exonuclease attack, a set of nested deletion mutants that can be self-religated is created.

A recombinant plasmid (pW9) with a Natal *P. caerulea* potyvirus derived insert of approximately 1.42 kb was selected for sequence analysis. This plasmid was subjected to unidirectional shortening from each end of the insert, using exonuclease III and S1 nuclease (Boehringer Mannheim) to produce two sets of overlapping deletion mutants for sequence analysis of each strand.

For the reverse primer (5' CAGGAAACAGCTATGAC 3' obtained from Amersham) sequencing reaction, 10 µg caesium chloride density gradient-purified pW9 DNA was digested to completion with *Pst I* (protection site) and *Bam HI* (shortening site), cleaned up by phenol/chloroform extraction, and ethanol precipitated. For sequencing the complementary strand with the "forward" sequencing primer (5' GTTTTCCCAGTCACGAC 3' obtained from US Biochemicals), the same was done using *EcoR I* (protected site) and *Kpn I* (shortening site) instead.

The lyophylised DNA pellet was resuspended in 70 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. Aliquots of 8ul were removed at 30 sec intervals to tubes (T=1 to T=8) 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 (to "polish" ends) 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.

The integrity of the "reverse primer" binding site in each deletion mutant was verified by *Hind III* restriction. The *Hind III* recognition site is located between the *Pst I* site of pUC19 and the "reverse primer" binding site, thus the presence of the *Hind III* site served as an indirect indication that the site was still present. No restriction sites between *EcoR I* and the "forward primer binding site were available, and the presence of these priming sites had to be confirmed by probing deletion mutant transformant colonies with [γ-^{32}P]-dATP endlabelled "forward primer" DNA.

Buffer solutions used for shortening reactions

1. 10 x 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)
   53ul 10X S1 nuclease buffer; 347ul water; 80 U 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. 10 x 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)

B.3.16 DNA nucleotide sequencing

Nucleotide sequencing was performed by a modification of the dideoxy chain termination method of Sanger *et al.*, (1977) using *Sequenase* 2™ (a modified

T7 DNA polymerase) from a *Sequenase* version 2.0 kit obtained from US biochemicals (Tabor & Richardson, 1987).

B.3.16.1 Preparation of DNA templates for sequencing

Double stranded DNA templates used in sequencing reactions were purified by ceasium chloride density gradient centrifugation (Appendix B.3.1) and digested with DNase-free RNase A (1 µl of a 10 mg/ml aqueous stock solution) for 30 minutes at 20°C, followed by one phenol/chloroform extraction and ethanol precipitation (Appendix B.3.3). Five µg of template DNA was then diluted to 18 µl with H_2O, mixed with 2 µl of 2N NaOH, and left at room temperature for 5 min. The alkaline denatured DNA was then precipitated by addition of 120 µl neutralizing/precipitating solution (5% 2M ammonium acetate pH 7.5, 95% ethanol). After 10 min incubation on ice, denatured DNA templates were spun down by centrifugation for 20 min at 4°C. The pellet was washed gently with ice cold 70% ethanol and vacuum-dried.

B.3.16.2 Priming, labelling and termination

For sequencing of the "reverse strand", (5' CAGGAAACAGCTATGAC 3' obtained from Amersham) was used as a primer, while 5' GTTTTCCCAGTCACGAC 3' (obtained from US Biochemicals) was used as the "forward" sequencing primer.

The vacuum-dried DNA templates were resuspended in 7 µl H_2O, 2 µl *Sequenase* buffer (supplied in kit) and 1 µl of appropriate primer (1 pmol/µl). Primers were annealed to the template by heating to 42°C for 5 min followed by slow cooling (15 min) to room temperature.

To each 10 µl primer/template complex were added: 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 (100 Ci/mmol; 10 Ci/µl) and 2 ul *Sequenase* 2™ (1/8 dilution in cold 10 mM Tris-cl pH 7.5; 5 mM DTT; 0.5 µg/µl BSA). Labelling reactions were mixed thoroughly and incubated at 20°C for 3 min. Four aliquots of 4.3 µl each were then transferred, and mixed well with four respective prewarmed termination mixes, each containing a different ddNTP.

Tubes containing termination mix (2.75 µl) were prepared before starting the labelling reactions. Each termination mix consisted of a particular dNTP/ddNTP in a 80µM/8µM ratio, 50 mM NaCl and 10% (v/v) DMSO. For each template there were four tubes: dATP/ddATP, dCTP/ddCTP, dGTP/ddGTP and dTTP/ddTTP. These tubes were pre-warmed at 45 °C, one min before addition of the labelling mix.

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). Reactions were either electrophoresed immediately on a denaturing sequencing gel, or stored at -20°C for up to two weeks.

B.3.16.3 Gel preparation

The gel apparatus used for DNA sequencing was obtained from OMEG Scientific, Cape Town. Glass plates were washed with Contrad (Merck S.A. (Pty) Ltd.) and rinsed thoroughly with deionized water. Denaturing gels of 4.8% polyacrylamide (for extended runs) or 6% for normal runs, each containing 7 M urea (ICN Biomedicals, Inc., Cleveland, OH) were prepared by mixing 60 ml of gel mix with 60 µl of freshly prepared 50% ammonium persulphate (Sigma) and 60 µl TEMED just before pouring. The gel mix was poured smoothly, avoiding bubbles, by using two 25 ml syringes, after which the gel was allowed to polymerize for 30 min before use.

B.3.16.4 Electrophoresis

After assembly of the gel apparatus, the buffer tank was filled with TBE that was pre-heated to 65°C, which eliminated the need for pre-running the gel to heat it up to the running temperature (40 - 45°C). Templates, prepared as described in B.3.16.1-2, were heat denatured in a 80°C water bath for 2 min immediately prior to loading, and snap cooled on ice. The wells were cleared from crystallizing urea by blowing out with a buffer filled syringe, and samples were run at 96 W for approximately 60 min in 6% gels (normal runs) or 92 W for 2,5-4 hrs in 4.8% gels (extended runs). Gel temperature was kept constant at 40 to 45 °C by means of a fan. After running, the top glass plate was lifted and a sheet of Whatmans filter paper was layered over the gel. The gel was "dry-lifted" from the glass plate, and dried onto the filter paper at 80°C for 30 min by means of a vacuum dryer coupled to a water trap and a vacuum pump, before being exposed to Curix RP 1 X-ray film (Appendix B.4.12)

B.3.17 Total DNA extraction from transgenic plants for Southern blot analysis

DNA for Soutern blot analysis was extracted from plant tissue according a modification of the method of Doyle & Doyle, (1990). Approximately 0.15 g young plant leaf material was frozed in liquid nitrogen, and ground to a fine powder with a mortar and pestle. The powder was transferred to a 2.2 ml Eppendorf tube containing 1 ml extraction buffer (100 mM Tris-HCl pH 8.0; 20 mM EDTA pH 8.0; 1.4 M NaCl; 0.2% (v/v) 2-mercaptoethanol; and 2% (v/v) cyltrimethylammonium bromide (CTAB) that was preheated to 60°C. The tube was mixed gently and incubated for 30 min at 60°C with occational gentle mixing to avoid shearing large DNA. The mixture was extracted once with chloroform:isoamyl alcohol (24:1 v/v), and centrifuged for 5' at room temperature. The supernatant was divided and transferred to two fresh 1.5 ml Eppendorf tubes. After precipitation at room temperature by addition of two volumes of isopropanol, DNA was pelleted by centrifugation at 6500 r.p.m. for 1' only. The DNA was then washed for twenty minutes with 75% ethanol containing 10 mM NH_4COOH, dried under vacuum and resuspended in 50 µl TE pH 8.0. DNA was used directly for restriction enzyme digestion and gel electrophoresis. RNA was removed by addition of RNase to the gel loading buffer (10 µl of 10 mg/ml DNase-free RNase per 1 ml loading buffer). The gel was subsequently subjected to Southern blotting.

B.4 MEDIA AND MAINTENANCE OF BACTERIAL STRAINS AND PLANT TISSUE CULTURES

B.4.1 Basic nutrient media used for bacterial cultures

B.4.1.1 Luria Bertani (LB) medium

Bacto tryptone	10 g/l
Bacto yeast extract	5 g/l
NaCl	5 g/l
pH 7.5	

The medium was autoclaved and used in broth form or supplemented with 1.5% agar for solid plates. Antibiotics were added to the medium (after autoclaving and cooling to 50°C) as required.

B.4.1.2 LB plates containing X-gal

40 µl *X-gal (20 mg/ml in dimethylformamide stored at -70°C) and 7 µl **IPTG (23.8 mg/ml in H_2O) was spread onto the surface of LB-Amp_{100} (LB + 100 µg/ml ampicillin) plates, at least 30 min before use.

*X-gal = 5-bromo-4chloro-3-indolyl-β-galactoside

**IPTG = isopropyl-β-D-thiogalactopyranoside

B.4.1.3 Yeast extract/beef (YEB) medium

Bacto beef extract	5 g/l
Bacto yeast extract	1 g/l
Peptone	5 g/l
Sucrose	5 g/l
$MgSO_4$	2 mM
pH 7.2	

The medium was autoclaved and used in broth form or supplemented with 1% agar for solid plates. Antibiotics were added to the medium as required.

B.4.1.4 Lactose agar

Lactose agar was used solely for growing *A. tumefaciens* to perform the Benedict's test.

Lactose	10 g/l
Yeast extract	1 g/l
agar	20 g/l

B.4.2 Benedict's test for *A. tumefaciens*

A. tumefaciens and *A. radiobacter* are the only bacteria studied thus far that are able to metabolize lactose into 3-ketolactose. The presence of 3-ketolactose is scored through its reaction with *Benedict's reagent to form a yellow precipitate of cuprous oxide.

*Benedicts reagent:	Sodium citrate	17.3 g/100 ml
	Na_2CO_3	10.0 g/100 ml
	$CuSO_4.5H_2O$	1.73 g/100 ml

A. tumefaciens is grown for 3 days at 28°C on lactose agar (B.4.1.3). The plates are the flooded with a thin layer of Benedict's reagent, and allowed to stand for two hours for yellow rings to develop.

B.4.3 Maintenance and brief description of bacterial/plasmid strains

B.4.3.1 *E. coli* LK111

The LK111 strain of *E. coli* has the following genotype: F^- ; *thi*1; *thr*1; *leu*B6; *lac*Y1; *ton*A21; *sup*E44; lambda$^-$ rK^- ; mK^+; *lac*I; *lac*ZΔM15; *rec*A^+ (Zabeau & Stanley, 1982). *E. coli* LK111 thus has β-galactosidase activity only when transformed with a plasmid carrying the lac Z'gene, which is able to complement the enzymatically inactive lacZΔM15 gene product. Such plasmids (eg. pUC and pMTL series) are usually constructed so that cloning allows the the inactivation of the lac Z' gene, resulting in blue/white selection of colonies without/with inserts on plates containing X-gal.

This strain was maintained on LB plates/stabs at 4°C, and grown preparatively at 37°C in LB medium.

B.4.3.2 *E. coli* LK111 (pUC19/pMTL25)

The pUC and pMTL series of plasmids belong to the pMB1-replicon series of plasmids (Maniatis *et al.*, 1989) with an inactive *rop* gene, allowing the plasmids to be present at high copy numbers in the *E. coli* host. They carry the β-lactamase gene conferring ampicillin resistance to their *E. coli* host. They also contain the lac Z' gene that can complement the defective *E. coli* LK111 lac ZΔM15 gene. The β-galactosidase complementation result in blue colonies on X-gal plates when the lac Z' gene is not inactivated by insertion of foreign DNA into the multiple cloning site present in this region. *E. coli* LK111 harbouring pUC19 or pMTL25 was grown on LB-Amp_{100} plates/stabs/medium at 37°C, and stored at 4°C on stabs.

B.4.3.3 *E. coli* LK111 (pUEX2)

E. coli LK111 harbouring the pUEX2 plasmid (described in section 5.1.1) was grown on LB-Amp_{100} plates/stabs/medium at 30°C, and stored at 4°C on stabs.

B.4.3.4 *E. coli* LK111 (pGSJ280)

E. coli LK111 harbouring the pGSJ280 plasmid (described in section 5.2.2.1) was grown at 37°C on LB plates/stabs/medium containing 20 μg/ml streptomycin and 50 μg/ml spectinomycin, and stored at 4°C on stabs.

B.4.3.5 *E. coli* HB101 (pRK2013)

The HB101 strain of *E. coli* has the following genotype: *sup*E44; *hsd*S20(r_B^- m_B^-); *rec*A56; *gal*K2; *gal*T22; *met*B1. The pRK2013 low copy number plasmid can be used to mobilize pBR-derivative plasmids (like pGSJ280) containing a bom-site, because they provide the transfer (tra) and mobilization (mob) functions in trans (Deblaere *et al.*, 1987). The plasmed also encodes the neomycin phosphotransferase (nptII) gene conferring kanamycin resistance to the *E. coli* host. *E. coli* HB101 (pRK2013) was grown at 37°C on LB plates/stabs/medium containing 25 μg/ml kanamycin, and stored on stabs at 4°C.

B.4.3.6 *A. tumefaciens* C58C1 (pGV2260)

A. tumefaciens C58C1-Rif^R harbouring the pGV2260 Ti-derivative plasmid (described in section 5.2.2.1) was maintained at room temperature on LB plates/stabs containing 100 μg/ml rifampicin and 100 μg/ml carbenicillin.

B.4.3.7 Long term storage of bacteria

For long term storage bacteria in the late log growth phase in the appropriate medium (containing selective antibiotics if required), was supplemented with 15% (v/v) glycerol, mixed well and stored at -70°C.

B.4.4 Antibiotics, plant hormones and acetosyringone

B.4.4.1 Plant growth hormones

Plant growth hormones were added to tissue culture to stimulate tissue differentiation required for the regeneration of whole plants from leaf discs.

NAA is an auxin-type plant hormone, while 6-BAP and kinetin are cytokinins. Media which drive regeneration from leaf discs generally contain a high ratio of cytokinins to auxins.

GROWTH REGULATOR	[STOCK]	SOLVENT	STERILIZATION
α-Naphtalene-acetic acid (NAA)	10 mg/ml	50% Ethanol	Autoclave in media
6-Benzyl-aminopurine (6-BAP)	5 mg/ml	DMSO	Autoclave in media
6-Furfurylaminopurine (Kinetin)	3 mg/ml	50% Ethanol	Autoclave in media

B.4.4.2 Antibiotics

All antibiotics were filter-sterilized and added to media cooled down to below 50°C.

ANTIBIOTIC	[STOCK]	SOLVENT	STORAGE
Rifampicin (Rif)	50 mg/ml	methanol	3 months @ -20°C
Cefotaxime (Cef)	50 mg/ml	dH_2O	-20°C light sensitive lasts 72 h in medium
Carbenicillin (Cb)	50 mg/ml	dH_2O	1 month @ -20°C
Kanamycin (Km)	12.5 mg/ml	dH_2O	-20°C
Spectinomycin(Sc)	50 mg/ml	dH_2O	-20°C
Streptomycin (Sp)	10/150 mg/ml	dH_2O	-20°C
Ampicillin (Amp)	25 mg/ml	dH_2O	-20°C
Novobiocin (Nv)	50 mg/ml	dH_2O	-20°C
All antibiotics were supplied by Sigma except Novobiocin which was obtained from Boehringer Mannheim			

B.4.4.3 Acetosyringone

A 10 mg/ml stock solution of acetosyringone (obtained from Aldrich), was dissolved in 50% ethanol. The pH was lowered to 5.6 by addition of HCl. The solution was then stored at -20°C.

B.4.5 Plant tissue culture media

Murashige & Skoog medium (MS)

MS salts for plant tissue culture medium (Murashige & Skoog, 1962) was bought premixed from Flow Laboratories.

Shoot regeneration medium (SRM)

SRM consisted of half strength MS medium pH 5.8, containing 0.1 mg/l NAA and 1.0 mg/l 6-BAP solidified with 0.7% plant tissue culture agar. For selection of transformed shoots, SRM was supplemented with antibiotics (SRM-$kan_{100}cef_{500}$).

Root induction and maintenance medium (RIM)

RIM consisted of half strength MS medium pH 5.8, supplemented with 0.2 mg/l kinetin and 0.7% plant tissue culture agar. When used for transformed shoots, RIM-$kan_{100}cef_{500}$ was used for initial rooting. After three weeks on this medium, shoots were transferred to fresh RIM-kan_{100} (no cefotaxime), and subsequent maintenance of regenerated and rooted shoots, was done on this medium.

B.4.6 Sterilization and germination of seeds

Seed from *N. tabacum* cv. Petit Havanna SR1, was surface sterilized by submerging in 70% ethanol containing 0.05% $Tween_{80}$ for 1 min, followed by bathing in sterile NaOCl (0.5-1% available chlorine) containing 0.05% $Tween_{80}$ for 20 min. Thereafter, seeds were transferred to RIM medium (Appendix B.4.5) supplemented 150 µg/ml novobiocin and 50 µg/ml carbendazime (Bavistan - BASF,SA) fungistatic agent for germination.

After germination, sterile seedlings were transferred to bottles containing RIM medium without antibiotic selection and maintained as described in Appendix B.4.7.

B.4.7 Maintenance of plant tissue cultures

Tobacco cultures were incubated at 25°C with 16 h photoperiod of (light intensity = 62.5 $\mu mol.s^{-1}.m^{-2}$). Untransformed tobacco plantlets were kept on RIM without antibiotics, while transformed tobacco shoots were maintained on RIM-kan_{100}. All plants were subcultured every 3-4 weeks by transferring shoots to fresh medium.

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