

**Studies on the Peruvian C1 isolate of
Sweet Potato Feathery Mottle Potyvirus**

by

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Synopsis

Some aspects of the genome of the Peruvian isolate of sweet potato feathery mottle potyvirus (SPFMV C1) and its interaction with host plants were investigated. Cytological studies have confirmed that the cylindrical inclusions (CI) aggregate in the cytoplasm of infected cells which, when probed with antiserum prepared to the ca. 70 kDa protein purified from *N. benthamiana* infected with SPFMV C1, became specifically immunogold labelled. The CI protein of this virus was found to be serologically related to the CI protein of tobacco etch (TEV-HAT), papaya ringspot (PRSV-W), watermelon mosaic (WMV-2) and zucchini yellow mosaic (ZYMV) potyviruses. The helper component-protease (HC-Pro) protein purified from *N. benthamiana* was found to have a M_r of ca. 56 kDa and share common epitopes with potato Y potyvirus (PVY) HC-Pro and PRSV amorphous inclusion (AI) proteins. It is associated with electron dense non-crystalline "AIs", which became immunogold labelled with a polyclonal antiserum to PVY HC-Pro, a monoclonal antibody to PVY/TVMV HC-Pro and, albeit less specifically, to a polyclonal antiserum prepared to SPFMV C1 HC-Pro.

A procedure for the purification of virions (yields of 30-50 mg/100 g) and infective RNA has been developed. Up to 80 % of *N. benthamiana* and *Ipomoea batatas* protoplasts were infected with viral RNA by electroporation. Direct observation of the protoplasts probed by immunostaining showed that protoplasts were infected and supported virus accumulation and production of CI protein for at least for 65 h. Electroporated protoplasts from both "resistant" (S24 and Nemanctc) and "susceptible" (Georgia Red and Jewel) sweet potato genotypes supported similar levels of infection.

The 2490 nucleotide sequences of the 6K2, NIa (VPg-NIa protease) and the 5'-terminus of NIb, and the 1152 bp of the CP coding region and 3'-untranslated (UTR) region were determined. By analogy with other potyviruses, four consensus cleavage sites recognized by the NIa protease were found at the putative junctions of the CI/6K2, 6K2/VPg, VPg/NIa and NIa/NIb proteins. The VPg-NIa and the NIb proteins were shown to contain, respectively, the protease and replicase consensus sequences motifs found in all potyviruses. Comparison of the nucleotide sequence of the UTR and the predicted aminoacid sequence of the CP protein of SPFMV C1 with those previously reported of the C and RC strains and of other potyviruses has confirmed its status as a strain of SPFMV and a distinct potyvirus. The genetic organization of SPFMV C1 RNA is tentatively proposed to be similar to other reported potyviruses. A construct representing a large part of the C-terminal region of the putative CP was expressed in *Escherichia coli* and the product detected by specific antibodies.

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Contents

Chapter 1

General introduction

1.1	Potyviruses	
1.1.1	Taxonomy	1
1.1.2	Structure and organization	2
1.2	Sweet potato feathery mottle virus	5
	Scope	10

Chapter 2

Virus and RNA purification

2.	Summary	13
2.1	Introduction	14
2.2	Materials and methods	
2.2.1	Host plants and virus isolates	16
2.2.2	Virus transmission	16
2.2.3	Virion purification	
2.2.3.1	Purification of SPFMV C1	18
2.2.3.2	Purification of SPFMV C	19
2.2.3.3	Purification of PVX ^C	19
2.2.4	RNA purification	20
2.2.4.1	SPFMV C1 and C	
2.2.4.1.1	Sucrose density gradient	20
2.2.4.1.2	Sodium perchlorate	22
2.2.4.2	PVX ^C	22
2.2.5	Selection of poly(A) ⁺ viral RNA	22
2.2.6	Infectivity	23
2.2.7	Non-denaturing agarose gel electrophoresis	23
2.2.8	Formaldehyde-agarose gel electrophoresis	24
2.2.9	Electron microscopy	24
2.2.10	Immunosorbent electron microscopy	25

2.3	Results and Discussion	
2.3.1	Virion purification	
	2.3.1.1 SPFMV C1	25
	2.3.1.2 SPFMV C	28
2.3.2	RNA purification	
	2.3.2.1 SPFMV C1 and C	29

Chapter 3

Studies on non-structural genome coded proteins

3.	Summary	32
3.1	Introduction	33
3.2	Materials and methods	
3.2.1	Plants and inoculum	44
3.2.2	Cytological studies	
	3.2.2.1 Time course analysis of inclusion development by EM	44
	3.2.2.2 Light microscopy of SPFMV C1 inclusions	45
3.2.3	SPFMV C1 inclusion protein purification	
	3.2.3.1 Helper component	46
	3.2.3.2 Amorphous inclusion	47
	3.2.3.3 Cylindrical inclusion	48
3.2.4	Antisera	49
	3.2.4.1 Polyclonal antisera	49
	3.2.4.2 Monoclonal antibodies	49
	3.2.4.3 Immunoglobulin purification	51
3.2.5	Immunological detection of non-structural proteins.	
	3.2.5.1 Modified indirect ELISA	51
	3.2.5.2 Dot blot ELISA	52
	3.2.5.3 Western blotting	
	3.2.5.3.1 SDS polyacrylamide gel electrophoresis	52
	3.2.5.3.2 Protein transblotting	53
	3.2.5.3.3 Immunological reactions	54
	3.2.5.4 Cytological immunogold labelling	
	3.2.5.4.1 Tissue infiltration and fixation	54
	3.2.5.4.1 Immunogold labelling	54

3.3	Results and Discussion	
3.3.1	Cytological studies	
3.3.1.1	Time course analysis of SPFMV CI inclusion bodies by EM	55
3.3.1.2	Light microscopy of inclusion bodies	58
3.3.2	Helper component	
3.3.2.1	Purification and antisera production	60
3.3.2.2	Immunological detection of HC-Pro protein	61
3.3.2.3	Immunogold labelling of the HC-Pro/AI protein	65
3.3.3	Cylindrical inclusion	
3.3.3.1	CI protein purification and antisera production	70
3.3.3.2	Immunological detection of CI protein	74
3.3.3.3	Immunogold labelling of the CI protein	74

Chapter 4

Expression of SPFMV CI genome transferred into *N. benthamiana* and *I. batatas* protoplasts by electroporation

4.	Summary	77
4.1.	Introduction	78
4.2.	Materials and methods	
4.2.1	Plants and inoculum	
4.2.1.1	Plants	88
4.2.1.2	Inoculum	90
4.2.2	Protoplast isolation and purification	
4.2.2.1	<i>Ipomoea batatas</i>	90
4.2.2.2	<i>Nicotiana benthamiana</i>	92
4.2.3	Protoplast concentration, absence of cell wall and viability assessment	92
4.2.4	Electroporation	92
4.2.5	Immunodetection	
4.2.5.1	F(ab') ₂ indirect ELISA	93
4.2.5.2	Protoplast immunostaining (PIS)	94
4.2.5.2.1	Sample preparation	95
4.2.5.2.2	Immunostaining	95
4.5.	Results and Discussion	
4.3.1	Protoplast isolation	
4.3.1.1	<i>Ipomoea batatas</i>	96
4.3.1.2	<i>Nicotiana benthamiana</i>	100

4.3.2	Electroporation	103
4.3.3	Synthesis of CP and CI proteins in electroporated protoplasts	107
4.3.4	Infection of susceptible and resistant <i>Ipomoea batatas</i> protoplasts	111

Chapter 5

Molecular analysis and sequencing of virus genome

5.	Summary	113
5.1.	Introduction	115
5.2.	Materials and methods	
5.2.1.	SPFMV CI RNA	131
5.2.2.	Antisera	131
5.2.3.	Cloning and transcription vectors	131
5.2.4.	Primers	133
5.2.5.	Bacteria	
5.2.5.1.	Strains	133
5.2.5.2.	Culture and maintenance	134
5.2.5.3.	Competent cells	134
5.2.5.4.	Transformation of competent bacterial cells	134
5.2.6.	Plasmid DNA purification	135
5.2.7.	General techniques for DNA manipulation.	
5.2.7.1	Restriction enzyme digestion	136
5.2.7.2.	Phosphorylation/Dephosphorylation	137
5.2.7.3.	Klenow reaction	137
5.2.7.4.	Ligation and religation	137
5.2.8.	cDNA synthesis	138
5.2.9.	Electrophoresis	
5.2.9.1.	Agarose gel electrophoresis	140
5.2.9.2.	Low melting point agarose gel electrophoresis	140
5.2.9.3.	Polyacrylamide gel electrophoresis	140
5.2.10.	Southern blotting	140
5.2.11.	Identification of recombinant clones	141
5.2.12.	Expression of fused proteins in <i>E. coli</i>	142
5.2.13.	Nucleotide sequencing	
5.2.13.1.	Taq Track sequencing system	143
5.2.13.2.	Cycle sequencing system.	144
5.2.14.	Denaturing sequencing gel	145
5.2.15.	Sequence analysis	145

5.3.	Results and discussion	
5.3.1.	cDNA synthesis and cloning	146
5.3.2.	Sequencing	148
5.3.3.	Sequence analysis	
5.3.3.1.	The 6K2, VPg-NIa protease and flanking regions	149
5.3.3.2.	NIa protease cleavage sites	159
5.3.3.3.	Analysis of the 5'- terminus of NIb gene	161
5.3.3.4.	Analysis of the 3'-end of the CP gene and 3'- UTR	164
5.3.3.5.	Analysis of the CP aminoacid sequence	166
5.3.4	Expression in <i>E. coli</i> of SPFMV CP gene	175
	Abbreviations	188
	Virus names and acronyms	192
	References	194
	Publication	231

List of Figures

Chapter 1

- 1.1 Diagrammatic representation of a poty-, bymo-, como-, nepo- and picornavirus genome organization 6

Chapter 2

- 2.1 Purification protocol for SPFMV C1 virions 21
- 2.2 Effect of homogenization and resuspension buffers used for SPFMV C1 purification 27
- 2.3 Effect of homogenization buffer composition used for SPFMV C1 purification 28
- 2.4 Electron micrograph of purified SPFMV C1 29
- 2.5 Agarose gel electrophoresis of SPFMV C1 RNA 31

Chapter 3

- 3.1 Electron micrographs of *N. benthamiana* mesophyll cells infected with SPFMV C1, time course development of CI bodies 56
- 3.2 Electron micrographs of *N. benthamiana* mesophyll cells infected with SPFMV C1, time course study of inclusion body development 57
- 3.3 Micrographs of *N. benthamiana* tissue infected with SPFMV C1 59
- 3.4. Serological determination of the SPFMV C1 HC-Pro protein purification in dot blot ELISA probed with antisera to PVY and TVMV HC-Pro protein 62
- 3.5. Dot blot ELISA of SPFMV C1 HC-Pro protein developed with homologous antiserum 62
- 3.6 Western blot analysis of SPFMV C1 HC-Pro protein with antiserum to SPFMV C1 HC-Pro (FMV HC2) 64
- 3.7 Western blot analysis of the of SPFMV C1 HC-Pro and AI proteins 64
- 3.8 Analysis of SPFMV HC-Pro protein with MAb 22E8B10 66
- 3.9a. Electronmicrograph of *N. benthamiana* tissue infected with SPFMV C1, probed with antiserum to SPFMV C1 HC-Pro and labelled with GAR-gold 67
- 3.9b. Electronmicrographs of *N. benthamiana* tissue infected with SPFMV C1, probed with antiserum to SPFMV C1 CI protein and labelled with GAR-gold 67
- 3.10 Electronmicrographs of *N. benthamiana* tissue infected with SPFMV C1, probed with antiserum to PVY HC-Pro and labelled with GAR-gold 68
- 3.11. Electronmicrographs of *N. benthamiana* tissue infected with SPFMV C1, probed with MAbs 22E8B10 to HC-Pro protein and labelled with GAR-gold 69

3.11.	Electronmicrographs of <i>N. benthamiana</i> tissue infected with SPFMV C1, probed with MAbs 22F8B10 to HC-Pro protein and labelled with GAR-gold (a to f)	71
3.12	Analysis of the CI protein purification (SDS PAGE)	73
3.13	Western blot analysis of SPFMV C1 HC-Pro protein probed with homologous and heterologous antisera	73
3.14	Electronmicrographs of <i>N. benthamiana</i> tissue infected with SPFMV C1, probed with antiserum to SPFMV C1 CI protein and labelled with GAR-gold (a, b)	75
3.14	Electronmicrographs of <i>N. benthamiana</i> tissue infected with SPFMV C1, probed with antiserum to SPFMV C1 CI protein and labelled with GAR-gold (c)	76

Chapter 4

4.1	Effect of enzyme composition on yield of <i>I. batatas</i> protoplasts	97
4.2.	Effect of several enzyme mixtures on <i>N. benthamiana</i> protoplast concentration	102
4.3.	Effect of number of pulses on the viability of mock-electroporated <i>N. benthamiana</i> protoplasts	104
4.4a	Effect on number of pulses on percentage of viable of <i>N. benthamiana</i> protoplasts electroporated with SPFMV C1 RNA	105
4.4b	Effect on number of pulses on percentage of viable of <i>N. benthamiana</i> protoplasts electroporated with SPFMV C1 RNA	106
4.5	Expression of CP and CI in <i>N. benthamiana</i> protoplasts electroporated with SPFMV C1 RNA	108
4.6.	Protoplast immunostaining (PIS)	110
4.7.	Accumulation of CP in <i>N. benthamiana</i> protoplasts electroporated with viral RNA	111

Chapter 5

5.1.	Diagrammatic representation of the potyviral polyprotein processing	117
5.2	Schematic representation of the TEV VPg-NIa protease	122
5.3	Physical maps of pGEM and pSP64 plasmid vectors	132
5.4.	Product of second strand cDNA synthesis	146
5.5.	Mapping of SPFMV C1 cDNA clones and sequencing strategy	150
5.6.	Nucleotide sequence of SPFMV C1 6K2, VPg-NIa protease and 5'-terminus of NIb and deduced aminoacid sequence	151
5.7.	Comparison of hydrophobicity profiles and alignment of aminoacid sequences of SPFMV C1 6K2 protein and those of other potyviruses	154
5.8.	Aminoacid sequence alignment of SPFMV C1 VPg-NIa protease domain with those of other potyvirus sequences	155

5.9.	Hydrophobicity profiles of SPFMV C1 VPg and VPg-NIa proteins	158
5.10.	Proposed genetic map of SPFMV C1 NIa protease cleavage sites and comparison of several potyvirus hexapeptide consensus sequences recognized by their NIa protease	160
5.11.	Comparison of SPFMV C1 5'- terminus NIb protein with the corresponding region of others potyviruses	162
5.12.	Nucleotide sequences of SPFMV C1 CP and 3'-UTR and deduced CP aminoacid sequence	165
5.13.	Comparison of the aminoacid sequences of SPFMV C1 CP and the ones of the C and RC strains	167
5.14.	Comparison of the aminoacid sequences of SPFMV C1 CP and those of other potyviruses	169
5.15.	Comparison of the hydrophobicity profiles of SPFMV C1, C, RC and PVY ^N CP	174
5.16.	Western blot of SPFMV C1 CP expressed in <i>E. coli</i>	176
5.17.	Dot blot ELISA of SPFMV C1 CP expressed in <i>E. coli</i>	176

List of Tables

Chapter 2

- 2.1. Infectivity of SPFMV C1 RNA in *N. benthamiana* 30

Chapter 3

- 3.1 Reactivity of MAbs to PVY/TVMV HC-Pro with sap extracts of SPFMV isolates infected plants in dot blot ELISA 65

Chapter 4

- 4.1 References on *I. batatas* protoplast isolation 87
- 4.2 *I. batatas* genotypes, showing different responses to SPFMV C1 infection 89
- 4.3 Enzyme mixtures used for isolation of *I. batatas* protoplasts 97
- 4.4 Enzyme mixtures used for isolation of *N. benthamiana* protoplasts 101
- 4.5 Percentage of *N. benthamiana* infected protoplasts reacting with antisera to SPFMV C1 CP and CI 109
- 4.6 Infection of *I. batatas* protoplasts inoculated with SPFMV C1 RNA 112

Chapter 5

- 5.1 Percentage of sequence identity between predicted potyvirus coded proteins 128
- 5.2 Percentage of aminoacid identity between putative SPFMV C1 6K2, NIa and NIb proteins and those of other potyviruses 157
- 5.3. Percentage of aminoacid sequence identity between putative SPFMV C1 CP and those of other potyviruses 172

Chapter 1

General Introduction

1.1 Potyviruses

1.1.1 Taxonomy

Potyviruses are possibly the largest and economically most important of the 34 plant virus groups and families, with at least 180 definitive and possible members, accounting for about 36% of all viruses known to infect plants around the world (Edwardson & Christie, 1991; Agrios, 1990; Milne, 1988; Francki *et al.*, 1985; Hollings & Brunt, 1981). Members usually occur wherever their principal hosts are present, but they are especially prevalent in tropical and subtropical countries. Most potyviruses have narrow, often extremely restricted host ranges, but the different viruses occur in a wide range of monocotyledonous and dicotyledonous plants. The host specialization possibly avoids interspecific competition among potyviruses and has resulted in the unusually large number of specialized variants that are hard to separate on the basis of their biochemical or biophysical properties, or by significant serological differences, but which survive as recognized "strains" or "isolates" (Hollings and Brunt, 1981). As stated by Francki *et al.* (1985), with so many members in this group and the continued description of new candidates, conventional approaches such as host range, symptomatology and serology have failed to successfully resolve the taxonomy of this group. Recent molecular studies are leading to a better understanding of the potyvirus genome structure and expression and appear to provide a rational basis for potyvirus identification and classification. As a result of the latest Potyvirus Taxonomy Workshop, held in Braunschweig, Germany (3-4 September, 1990) a recommendation had been forwarded to the Plant Virus Subcommittee of the International Committee on Taxonomy of Viruses to establish a family of plant viruses called *Potyviridae*, which includes three genera and one possible genus (Barnett, 1992, 1991). The genera are *Potyvirus* [potato virus Y (PVY), type species], the largest genus of non-persistent aphid-borne viruses (73 definitive and 74 possible members), the genus *Bymovirus* [barley yellow mosaic virus (BaYMV), type species] composed of fungal transmitted viruses (5 definitive members), and the genus *Rymovirus* [ryegrass mosaic virus (RgMV), type species] of mite transmitted viruses (9 definitive members). The possible

genus is *Ipomovirus*, with only one definitive member, sweet potato mild mottle virus (SPMMV) and one possible member, sweet potato yellow dwarf virus (SPYDV), transmitted by white-fly (*Bemisia tabaci* Gennadius).

All members in the three genera and one possible genus have flexuous, filamentous rod shaped particles which contain a positive sense ssRNA genome, and produce characteristic "pinwheels" or scroll-shaped inclusions which accumulate in the cytoplasm of infected cells. These are perhaps the main features which have been taken into consideration to justify the incorporation of all of them within the *Potyviridae* family.

1.1.2 Structure and organization

The virions of aphid, mite or whitefly transmitted potyviruses are normally 680 to 900 nm in length by 11 to 15 nm in diameter (Francki *et al.*, 1985), with an M_r of 60 to 70 x 10⁶ daltons. The particles contain approximately 5% nucleic acid and 95% protein and have a sedimentation coefficient of about 150S. Particles have helical symmetry with a pitch of about 3.2 to 3.5 Å (Varma *et al.*, 1968), with 6-8 CP subunits per ring (Veerisetty, 1978) and do not usually show any substructure (Hollings and Brunt, 1981; Huttinga, 1973). The capsid protein (CP) consists of up to 2000 subunits of a single polypeptide of 30 kDa to 37 kDa (Moghal & Francki, 1976). Recent biochemical, serological and sequence analysis of the CP of several potyviruses (reviewed by Ward and Shukla, 1991; Shukla *et al.*, 1991; Shukla and Ward, 1989; Shukla *et al.*, 1989a; Shukla *et al.*, 1988b, Allison *et al.*, 1985) indicates that the N-terminal aminoacids are located on the virion surface, are immunodominant and contain the major virus group specific epitopes, while the C-terminal two thirds of the protein is highly conserved. Mild proteolysis by trypsin of particles revealed that the N- and C-terminal regions of the CP are exposed on the particle surface. This organization is similar to the structural organization of some unrelated plant viruses of the potex and tobamovirus group (Shukla *et al.*, 1988a). In addition to protecting the virus genome from nucleases (Hollings and Brunt, 1981), the biological role of CP in transmission specificity by aphids has been experimentally demonstrated (Gal-On *et al.*, 1992; Atreya *et al.*, 1990; O'Connell-Ziegler *et al.*, 1986).

The potyvirus ssRNA consists of approximately 10,000 nucleotides (10 Kb) (Robaglia *et al.*, 1989; Maiss *et al.*, 1989; Allison *et al.*, 1986; Domier *et al.*, 1986) with a M_r of 3.0 to 3.8 x 10⁶ daltons (Dougherty, 1983; Hari *et al.*, 1979; Makkouk and Gumpf, 1974; Brakke and Van Pelt, 1970). The non-denatured nucleic acid has a sedimentation coefficient of 30S to 40S and 23S to 25S if formaldehyde-denatured (Hill and Benner, 1976; 1980; Makkouk and Gumpf, 1974). Although initially there were conflicting interpretations of the results (Otal and Hari, 1983; Hellmann *et al.*, 1983), it has been established that potyviruses do not synthesize subgenomic mRNA (Dougherty, 1983); only full length mRNA is found associated with polyribosomes (Vance and Beachy, 1984).

The fungus transmitted viruses have bipartite ssRNA genomes, which have a poly (A) tract on the 3'-ends of both of them and a genome-linked protein at both 5'-ends. They are encapsidated in two different rod-shaped virions, approximately 275 and 550 nm long (Kashiwazaki *et al.*, 1991, 1990). The RNA-1 encodes a single large polyprotein, corresponding to a similar ordered gene module conserved in potyviruses, comoviruses, nepoviruses and picornaviruses (Perez Bercoff, 1987; Domier *et al.*, 1987; Goldbach, 1988) and which has been implicated in RNA replication [(Figure 1.1, RNA replication module) (Gorbalenya *et al.*, 1988., Takeda *et al.*, 1986; Goldbach and van Kammen, 1985; Takegami *et al.*, 1983)], but RNA2 shows striking differences in organization when compared with the 5'-terminal region of the potyvirus genome (Kashiwazaki *et al.*, 1991). The mite transmitted viruses, based on wheat streak mosaic virus (WSMV), have a monopartite ssRNA genome of 8.5 Kb, which has 3'-end poly(A) tract, a translation strategy based on polyprotein processing and the CP maps to the 3'-end (Niblett *et al.*, 1991).

The presence of a small protein, VPg, covalently linked to the 5'-terminus and a poly(A) tail linked to the 3'-terminus has been established for all potyvirus RNAs (Ricchmann *et al.*, 1992, 1989). The combination of the VPg at the 5'-terminus and a

poly(A) tail at the 3'-terminus is also known to occur in the plant comoviruses (Argos *et al.*, 1984; Franssen *et al.*, 1984) and nepoviruses (Cornelissen *et al.*, 1986; Mayo *et al.*, 1982) and in the animal picornaviruses (Perez Bercoff, 1987; Kitamura *et al.*, 1981). These features and the distinctive replication gene module (Figure 1.1), place them in the proposed supergroup of picorna-like plant viruses (Goldbach, 1988; Domier *et al.*, 1987).

The VPg of tobacco etch potyvirus (TEV) and pepper mottle potyvirus (PeMV), but not plum pox potyvirus (PPV) RNAs can be removed without any apparent effect on infectivity, when tested by mechanical inoculation (Riechmann *et al.*, 1989; Shahubuddin *et al.*, 1988; Hari, 1981) or cell-free-translation (Siaw *et al.*, 1985). The protein is found in all newly-synthesized viral RNAs (Wimmer *et al.*, 1987; Flanagan *et al.*, 1987., Pincus *et al.*, 1987). The role of poly(A) in translational stability of potyvirus RNAs has not been tested. However, as with cowpea mosaic comovirus (CPMV; Goldbach, 1987) and alfalfa mosaic virus (AIMV; Hucz *et al.*, 1983) RNAs and other eukaryotic mRNAs (Jackson and Standart, 1990; Bernstein and Ross, 1989; Bernstein *et al.*, 1989;) the presence of the poly(A) tail improves the translational stability, probably by protecting the mRNA from indiscriminate destruction by cellular nucleases.

The potyvirus RNA single open reading frame (ORF) codes for a polyprotein precursor (Figure 1.1), which is co- and post- translationally cleaved into at least ten peptides (Riechmann *et al.*, 1992; Dougherty *et al.*, 1990); the order of these products in the polyprotein is: first protein (P1), helper component-protease (HC-Pro), third protein (P3), first small protein (6K1), cylindrical inclusion (CI), second small protein (6K2), VPg-nuclear inclusion protease (VPg-NIa), nuclear inclusion b (NIb) and CP (Figure 1.1). The VPg and the CP are the only gene products detected in virus particles. Six other gene products (P1, HC-Pro, P3, CI, NIa and NIb) have been isolated from infected plants and some of them characterized, while the remaining proteins (6K1 and 6K2) have not been detected *in vivo*, but their formation by proteolytic processing has been deduced from sequencing data or demonstrated *in vitro* (cell-free translation systems) and *in vivo* in

heterologous systems [(e.g. *E. coli*) (Restrepo-Hartwing and Carrington, 1992; Riechmann *et al.*, 1992; Verchot, 1991)].

Determination of the functions of all the potyvirus gene products is incomplete, but it is revealing that some gene products play several roles during the life cycle of the virus (described in Chapters 3, 4 and 5). The recent isolation of full-length cDNA clones of three potyviruses from which infectious transcripts can be synthesized: ZYMV (Gal-On *et al.*, 1991), PPV (Maiss *et al.*, 1992; Riechmann *et al.*, 1990) and TVMV (Domier *et al.*, 1989) opens the possibility for studying the molecular biology of potyvirus gene functions, by applying genetic engineering techniques. Aspects of the potyvirus life cycle such as translation, replication, symptom induction, translocation, transmission and study of gene products functions are now amenable to a new experimental approach.

2. SWEET POTATO FEATHERY MOTTLE VIRUS

The "modified roots" and leafage of sweet potatoes [*Ipomoea batatas* (L.) Lam.] make their major contribution as a crop in countries of the tropical and subtropical regions (International Potato Center, 1992), but their value is also recognized in Australia, Japan, some Mediterranean countries, New Zealand and the United States (Yen, 1982). The crop is vegetatively propagated and this agronomical practice, as well as the abundant presence of insects, has a very important effect in the rapid spreading and persistence of viral diseases.

Among viruses found naturally infecting sweet potatoes, there have been identified at least six filamentous viruses [sweet potato feathery mottle virus, (SPFMV) (Doolittle and Harter, 1945) and sweet potato vein mosaic virus, (SPVMV) (Nome, 1973), transmitted by aphids, sweet potato latent virus (SPLV) (Clark and Moyer, 1988), whose transmission by aphids or other vectors has not been shown, SPMMV transmitted by *B. tabaci*,

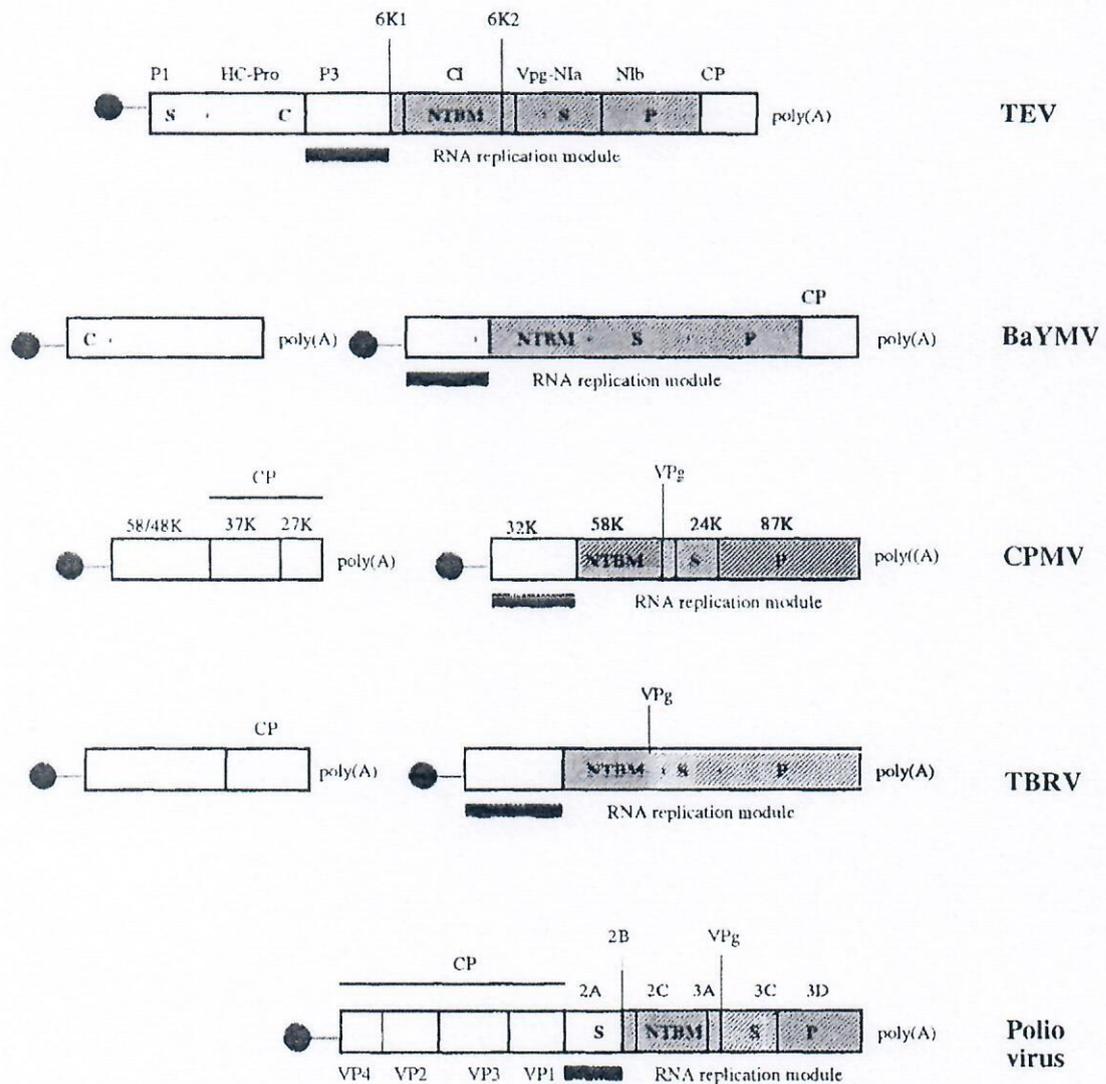


Figure 1.1

Comparison of the genomic RNAs of potyviruses (TEV), bymoviruses (BaYMV), comoviruses (CPMV), nepoviruses (tomato black ring nepovirus, TBRV) and picornaviruses (poliovirus). Shaded areas, in open reading frames, indicate the RNA replication module genes; suggested replication genes are underlined (thick shaded lines); vertical solid lines indicate definitive cleavage sites of the polyproteins and dotted lines sites, whose sequence and exact locations are not known. Characteristic aminoacid sequences features are indicated: **NTBM**, nucleotide-binding motif; **S**, serine-type protease; **C**, cysteine-type protease; **P**, polymerase domain. The name of each of the protein products is indicated above or below the corresponding cistron.

References: TEV; Carrington *et al.*, 1990. BaYMV; Kashiwazaki *et al.*, 1991, 1990. TBRV and CPMV; Goldbach, 1988. Poliovirus; Krausslich and Wimmer, 1988.

(Hollings *et al.*, 1976), sweet potato yellow dwarf virus (SPYDV) (Clark and Moyer, 1988), similar to SPMMV in particle morphology and vector, a closterovirus [sweet potato sunken vein closterovirus (SPSVV) transmitted by *B. tabaci*, (Cohen *et al.*, 1992)], an isometric virus [sweet potato caulimo-like virus (SPCLV) (Atkey and Brunt, 1987)], and an ilarvirus [sweet potato vein clearing virus (SPVCV) (Laakso and Moyer, 1989)]. Other virus-like, whitefly-transmitted agents have been isolated from sweet potato in Nigeria (Rossel and Thottappilly, 1985) and in the United States (Moyer, 1988); however, definitive identification of these agents has not been concluded.

SPFMV is probably the most important and widespread among viruses infecting sweet potatoes, and is one of the causes of the progressive degeneration of the crop (Moyer, 1988). This virus was first found in *I. batatas* (Doolittle and Harter, 1945) and depending on the host genotype, it can cause considerable reduction in yield and loss of market quality in the "modified roots" (Ngeve, 1990; Moyer and Salazar, 1989). Symptoms in sweet potatoes depend more on host genotype and environmental conditions rather than virus strain (Moyer and Salazar, 1989, Clark and Moyer, 1988). Foliar symptoms, including irregular chlorotic patterns (feathering) as well as chlorotic spots sometimes surrounded by purple borders, are the most frequent symptoms of SPFMV.

SPFMV is a definitive member of the *Potyvirus* genus (Barnett, 1991) and has elongated, flexuous particles of 810-865 nm, a value which varies with the strain and method of sample preparation for electron microscopy analysis (Stobbs and Cerkauskas, 1991; Cohen *et al.*, 1988a; Moyer and Cali, 1985). It is mechanically transmitted to several *Ipomoea spp.* (Alconero, 1973) and is non-persistently transmitted by several species of aphids (Kennedy and Moyer, 1982). The probability of SPFMV C transmission through sweet potato seeds is lower than 0.125% (Wolters *et al.*, 1990). The virus reaches a low concentration in infected tissues and most *Ipomoea spp.* exhibit a regular symptomless recovery (Moyer *et al.*, 1980; Hieldebrand, 1958; Daines and Martin, 1964).

The possibility of a protease produced in infected plants being involved in the recovering process has been proposed (Salomon, 1989). For all strains so far tested, infectivity of purified virus preparations has been found to be low and temperature dependent (Cadena-Hinojosa and Campbell, 1981b; Cali and Moyer, 1981).

The genome is an infective, polyadenylated, positive ssRNA, with a molecular weight of 3.65×10^6 daltons, as determined by electrophoresis in methylmercuric hydroxide denaturing agarose gels, and the T_m was calculated to be 51°C (Moyer and Cali, 1985). The CP has a M_r of 3.8×10^4 daltons (Salomon, 1989; Moyer and Cali, 1985). However, as observed with the CP of other potyviruses, the protein is labile (Hiebert *et al.*, 1984; Moghal and Francki, 1976) and polypeptides of 36, 32 and 30 kDa are observed together with the major 38 kDa protein in SDS-PAGE (Salomon, 1989; Moyer and Cali, 1985). The ultraviolet absorption spectra of partially purified virus, corrected for light scattering were reported to have a maximum at 255-262 nm, a minimum at 246-247 nm and a 260/280 ratio of 1.13 ± 0.07 (Cadena-Hinojosa and Campbell, 1981a; Cali and Moyer, 1981). The virus thermal inactivation point is 55 to 60°C (Hollings *et al.*, 1976), dilution end point of 10^{-3} - 10^{-4} (Hollings *et al.*, 1976) and longevity in sap less than 6 h (Cali and Moyer, 1981).

Several isolates have been identified worldwide, based mainly on host range, particle size and serological relationships (Stobbs and Cerkauskas, 1991; Beetham and Mason, 1990; Loebenstein *et al.*, 1960; Olivero and Trujillo, 1989; Green *et al.*, 1988; Usugi *et al.*, 1991; Rossel and Thottappily, 1985; Atcham *et al.*, 1983; Esbenshade & Moyer, 1982; Kennedy & Moyer, 1982; Cali & Moyer, 1981), but only some of them have been described. The common strain (C) which is restricted to Convolvulaceae, induces only foliar symptoms in sweet potato. The russet crack strain (RC) which induces lesions in "modified roots" of cultivar Jersey and foliar symptoms in several other genotypes (Martin and Daines, 1964), is mechanically transmitted to 10 species of *Ipomoea spp.* and

induces local lesions in *Chenopodium quinoa* Willd. and *C. amaranticolor* Coste and Reyn. It induces type 4 CI bodies (Edwardson *et al.*, 1984) in the cytoplasm of infected *I. batatas* and *I. setosa* Kerr., which may vary in size, shape and concentration with time and position in the plant (Lawson *et al.*, 1971). The yellow vein strain (YV) induces severe symptoms in *I. nil* "Scarlet O'Hara" L. Roth (Moyer, 1986). The strain 835, which was isolated in Puerto Rico, induces mosaic, rugosity and leaf deformation in *Nicotiana benthamiana* L. Domin. (Moyer, 1986). The C1 strain was isolated in Peru from a local variety of *I. batatas* (International Potato Center, 1986) and induces mosaic and rugosity on *N. benthamiana* leaves, soft mosaic and rugosity on F1 hybrids of *N. clevelandii* x *N. bigelovii* and local necrotic lesions on *C. quinoa* and *C. amaranticolor* (Lopez, personal communication). SPFMV C, RC, 835 and C1 are all transmitted by several species of aphids in a non-persistent manner and mechanically transmitted with some difficulty to several *Ipomoea spp.* (Moyer, 1986; Moyer and Kennedy, 1978; McLean, 1959; Webb, 1954; Webb and Larson, 1954).

Serological relationships among distinct viruses infecting sweet potatoes or among distinct members of the potyvirus group are extremely complex. Polyclonal antiserum (PABs) to the dissociated core protein of Johnson grass mosaic virus (JGMV) revealed the possibility of epitopes common to SPFMV C (Shukla *et al.*, 1989) whereas PABs to the trypsin-resistant core of the CP of JGMV revealed a possibility of epitopes common to the whitefly-transmitted SPMMV. The results contrast with a study in which several monoclonal antibodies (MAbs) to aphid transmitted (AT) potyviruses reacted with SPFMV and SPLV but not with SPMMV. Thus, SPFMV and SPLV share common epitopes whereas SPMMV appears to be serologically distinct from any of the aphid transmitted potyviruses (Hammond *et al.*, 1992). Strains RC, YV and 835 have been found to be serologically closely related, whereas the C strain is distantly related, by using PABs to C and RC isolates (Abad and Moyer, 1992). However, RC, C, C1 and 835 but not YV have been found to be closely related by using homologous and heterologous PABs to RC,

C and C1 and a MAbs to the Ibadan isolate from Nigeria (Jordan and Hammond, 1991) (Fuentes, 1992; personal communication).

The importance of SPFMV lies in its frequent association with other, mostly unknown, viruses, suggesting that infection with SPFMV increases susceptibility to other viruses (Moyer and Salazar, 1989; Moyer, 1988). There has also been demonstrated a correlation between the presence of combined infections and severity of symptoms (Cohen *et al.*, 1988b; Rossel and Thottappilly, 1985).

1.3 Scope

Viruses are responsible for severe diseases in plants, resulting in major losses in commercially important crops (Agrios, 1990; Milne, 1988). Little is known of how these diseases are induced or why symptoms arise. Different viruses or even isolates that on the basis of their serological relationship are considered strains of a single virus may induce different types of symptoms (Van Loon, 1987). Before the advent of molecular virology, virus classification had been largely based on host range and symptom type (Matthews, 1982). Because both the virus and the host plant contribution to symptom development has a genetic basis, it is necessary to resolve the organization and expression of the viral genes to understand the effect of the viral products on the host and their interactions leading to symptom formation. Expression strategies of several plant viruses have been elucidated (c.g. TMV, Goelet *et al.*, 1982; PVX, Huisman *et al.*, 1988; TEV, Allison *et al.*, 1986; TVMV, Domier *et al.*, 1986; PPV, Lain *et al.*, 1989; PLRV, Mayo *et al.*, 1989; BaYMV, Kashiwazaki *et al.*, 1990, 1991). As more information accumulates on nucleic acid structure and expression, new concepts in virus classification are becoming more important, which in addition to or even instead of the traditional approach may provide a more functional classification probably based on evolutionary relationships. This may allow a better understanding of the host-pathogen interaction and provide the tools that may lead

to more stable genetically engineered resistance to virus induced diseases (Mayo, 1992; Hull and Davies, 1992; Beachy *et al.*, 1990; van den Elzen, 1989; Barrell and Farrell, 1986).

Sweet potato feathery mottle virus (SPFMV) is found nearly everywhere sweet potatoes are grown. Many strains of SPFMV have been identified, and worldwide it has been referred to by many different names. SPFMV has many biological characteristics and cytopathic effects that support its classification as a potyvirus (Barnett, 1991) even though such biochemical properties as capsid protein Mr 38,000 daltons, RNA 3.65×10^6 daltons and virion length 850 nm make it an atypically large potyvirus (Moyer and Cali, 1985). It has a very narrow host range, restricted mainly to *Ipomoea spp.* Several strains or isolates of this virus have been characterized on the basis of their host range and serological differences (Usugi *et al.*, 1991; Vetten, 1990; Olivero-Acuna and Trujillo-Pinto, 1989; Eshenshade and Moyer, 1982; Cadena-Hinojosa and Campbell, 1981a; Cali and Moyer, 1981; Moyer *et al.*, 1980). At present some information is available on the structural characteristics of a common (SPFMV C) and the russet crack (SPFMV RC) strains of this virus (Moyer and Cali, 1985), and nucleotide sequence of the CP gene of both strains had been recently published (Abad *et al.*, 1992). However no information is available on any SPFMV isolate infecting *N. benthamiana* as is the case of Peruvian isolate of SPFMV (C1).

The objective of the work embodied in this thesis was to investigate some aspects of the genome of a Peruvian isolate, C1 and its interaction with host plants. The intention was to first develop a purification method what would allow relatively large quantities of virus and also of full-length infective genomic RNA to be prepared. This would then be used in the development of protocols for infection of both *I. batatas* and *N. benthamiana* protoplasts, establishing systems for investigating virus replication and host interactions at the cellular level.

In order to allow detection and characterisation of one or more of the viral genomic RNA-encoded proteins, and to permit comparisons with other SPFMV isolates and with other potyviruses, it was also proposed to prepare antibodies to virus-coded non structural proteins. These antibodies would also be used to localize the virus coded protein, which aggregates to form specific inclusion bodies in virus infected tissue.

Finally, it was the intention to obtain the nucleotide sequence of as much of the genomic RNA as possible, within the time available, again to allow comparison of this Peruvian isolate of SPFMV with other SPFMV isolates and other potyviruses.

Chapter 2

Virus and RNA Purification

2. Summary

A procedure for the purification of the Peruvian isolate (C1) of sweet potato feathery mottle potyvirus (SPFMV C1) and infective RNA has been developed. The use of Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffer containing urea and Na₂ EDTA (disodium ethylene-diamino-tetraacetic acid) as a base for tissue extraction and virus suspension enabled good yields of virus (35-50 mg/100g) to be obtained from *Nicotiana benthamiana* L. Domin. This protocol can also be successfully applied for the purification of the common (C) isolate (SPFMV C), by using borate-based buffers instead of Hepes-based buffers for tissue extraction and virus suspension. Yields of 10-12 mg/100g were obtained from *Ipomoea nil* L. "Scarlet O'Hara". A short RNA isolation procedure yielded infectious RNA, from which ds cDNA of nearly genome size could be obtained and infection of protoplasts with viral RNA achieved.

2.1. Introduction

Potyvirus particles occur and are assembled in the cytoplasm. Many of the viruses reach a moderate to high concentration in the plant sap; some are easily purified and high yields (50-200 mg/Kg tissue) are readily obtained (e.g. PVY^O and PVY^N, Nakashima, 1987; PeMV, Tolin and Ford, 1983; JGMV, Gough and Shukla, 1981; TEV Dougherty and Hiebert, 1980; TVMV, Hellmann *et al.*, 1980) while others are extremely difficult to purify and low yields (1-5 mg/Kg tissue) are usually obtained (e.g. ZYMV strain FL, Huang and Hseu, 1989; arauja mosaic virus (AjMV), Hiebert and Charudattan, 1984; SPFMV-RC and C, Cali and Moyer, 1981). However most of them lie between these two extremes in the ease with which they can be isolated from their host constituents in reasonably large quantities (Hammond and Lawson, 1988; Hollings and Brunt, 1981, Moghal & Francki, 1976). As observed with other potyviruses (Brunt, 1988), the flexuous virus particles of SPFMV, 810-865 nm in length (Moyer and Cali, 1985), have a strong tendency to aggregate and break during purification (Cali and Moyer, 1981). Several protocols have been developed for the purification of a wide range of potyviruses. However, as reviewed by Brunt (1988), the efficiency of a buffer or additive for the purification of some viruses can be deleterious for others, even strains of the same virus. They can differ greatly in their stability in buffers of different compositions, molarities and pH values.

Homogenization of virus source materials with relatively large volumes of high molarity and high pH buffers has been used in attempts to reduce aggregation of several potyviruses (Gough and Shukla, 1981; Huttinga, 1973; Hiebert and McDonald, 1973). Urea prevents aggregation by weakening hydrophobic and hydrogen bonds, and its use at relatively high concentrations has been shown to be beneficial for the efficient isolation of several filamentous viruses [pea seed-borne mosaic potyvirus (PSbMV), Wang *et al.*, 1992; potato potyvirus A (PVA), Nakashima and Fernandez-Northcote, 1987]. Chelating agents such as Na₂ EDTA (disodium ethylene-diamino-tetraacetic acid) have also been reported to be essential for the purification of several potyviruses [watermelon mosaic potyvirus 2 (WMV 2), Purcifull *et al.*, 1984; cowpea aphid-borne mosaic potyvirus

(CABMV), Taiwo *et al.*, 1982]. However, it has to be used with caution, because it is deleterious for viruses such as AjMV (Hiebert and Charudattan, 1984) which require divalent ions for particle stability. The chelating action of EDTA has the added advantage of assisting in the removal of host ribosomes, polyribosomes and phytoferritin (Brunt, 1988). Several reducing agents [eg. sodium bisulfite, 2-mercaptoethanol, nicotine, ascorbic acid, cysteine, dithiotreitol (DTT), sodium diethyldithiocarbamate (DIECA), thioglycolic acid] have been commonly used to avoid the oxidation of phenolic compounds which will promote the formation of tannin:virus-coat protein complexes. Purification of some unstable viruses (Hiebert and Charundatttan, 1984) has been facilitated by using protease inhibitors to prevent enzymatic degradation of virus coat proteins or by the inclusion of magnesium chloride to stabilize particles and minimize degradation of RNA. Clarification with organic solvents such as chloroform and n-butanol has the added advantage of disrupting ribosomes and phytoferritin. However, their use has had varied degrees of success for the purification of potyviruses. The non-ionic detergent Triton X-100 (alkylphenoxy polyethoxyethanol) disrupts membranes and other lipid-containing tissue, and has proved to be useful in the purification of some flexuous viruses (Hiebert and Charudattan, 1984; Gough and Shukla, 1981; Huttinga, 1973). Concentration by precipitation with PEG (polyethylene glycol) in the presence of NaCl had been reported to induce severe aggregation (Huttinga, 1973); however, this may be attributable to inadequate resuspension of precipitated virus. Further purification by differential centrifugation with the use of a sucrose cushion, is a relatively gentle and effective procedure to eliminate contaminants from virus preparations, and further purification by density gradient centrifugation has been commonly used to separate filamentous viruses from contaminants.

In order to investigate and compare the viral RNA expression, full length infective RNA from SPFMV C1 and C isolates in sufficient quantities for routine infection of protoplasts was required. Many attempts to purify virions of the C1 isolate from *N. benthamiana* by procedures described for the purification of other isolates from *I. nil* or *I.*

batatas (Cali and Moyer, 1981; Cohen *et al.*, 1988a) proved unsuccessful, producing low yields unsuitable for the subsequent preparation of relatively large quantities of infective RNA. The aim of this study was to develop simple and efficient procedures for SPFMV C1 and C infective virion and RNA purification.

2.2. Materials and Methods

Preliminary experimental work was performed at The University of Birmingham, School of Biological Sciences, Birmingham, UK and all final experiments at The International Potato Center (CIP), Lima, Peru.

2.2.1. Host plants and virus isolates

University of Birmingham: glasshouses used for propagating plants were maintained at 20-25 °C during the winter and spring, and 22-28 °C during the summer. Daylight was supplemented with mercury fluorescent (GE,HPMFL/U trulite, 400w, MFB) and high pressure sodium (GE, HPS/U solarcolour 310w) lamps at approximately 100 cm above the plants. They gave a total of 7,000-10,000 lux and 18 h photoperiod. *Ipomoea nil* (L) Roth cv. Scarlet O'Hara, "morning glory": two to three seeds (Nutting and Speed, Longstanton, Cambrige, CB 45DU, UK) were sown into a 7x7x8 cm or a 12x15x20 cm disposable plastic pot in (1:1) commercial soil-less compost (SHL, Lincoln, UK., all purpose compost) and a mixture of loam:peat:gravel (3:2:1). They were germinated under normal glasshouse conditions. Plants were used when the two primary leaves were fully expanded (10-12 days after sowing). *N. benthamiana* seeds (obtained from Dr. Alan Brunt, Institute of Horticultural Research, Littlehampton, West Sussex, UK) were surface sown onto all-purpose compost in a tray covered with "cling film" and grown under normal glasshouse conditions. Seedlings were individually transplanted when 1-2 cm tall into 7x7x8 cm pots and grown in the same glasshouse until they were about 5 cm tall, with 4 primary fully

expanded leaves (usually 15-20 days after transplanting). Infected *I. nil* and *N. benthamiana* plants were grown in glasshouses with environmental conditions similar to those used for propagation. Extra fertilizer (N:P:K, 19:3:4) was supplied every two weeks.

SPFMV C strain, isolated by Dr. J.W. Moyer (North Carolina State University, USA), and SPFMV C1 strain, isolated by Dr. L. F. Salazar (International Potato Center, Lima, Peru), were obtained from Dr. Alan Brunt, Institute of Horticulture Research, Littlehampton, West Sussex, UK. This part of the work was done under Licence No PFH 78A1166 issued by the Ministry of Agriculture and Fisheries and Food, UK.

International Potato Center: *N. benthamiana*, *N. glutinosa* and *C. quinoa* seeds were surface sown on all-purpose peat-sand mixture and transplanted two to three weeks after germination into peatpots. After an additional two weeks, plantlets were transplanted into 10x10x15 cm plastic pots containing a mixture of all purpose compost :coarse sand (2:1). After inoculation, plants were grown under controlled environmental conditions in walk-in growth chambers set to provide 5000 lux light intensity for 18 h at 24°C and 6 h darkness at 20 °C with 85-80% relative humidity; plants were weekly fertilized with 12:12:12, N:P:K mixture (50 ml/pot). *I. nil* "Scarlet O'Hara" seeds were germinated at room temperature (22-24 °C) in humid chambers and individually sown in 10x10x15 cm plastic pots containing a mixture of compost:coarse sand:soil (2:1:1). Virus isolates SPFMV C and C1, potato potexvirus X^C (PVX^C, to be used for RNA marker preparation) and seeds of *N. benthamiana*, *N. glutinosa*, *I. nil* and *C. quinoa* seeds were obtained from CIP, Plant Pathology Department's virus isolates and host plants seed collection.

2.2.2. Virus transmission

Both at UK and in Peru, viruses were transmitted by sap inoculation by rubbing the leaves dusted with Carborundum F500-C6 (The Carborundum Co. Ltd., Manchester, UK) using a cotton pad soaked in inoculum prepared by grinding desiccated or fresh infected tissue in ice-cold 50 mM potassium phosphate buffer (KPB) pH 8.0 containing 500 mM

urea. SPFMV C was inoculated in *I. nil*, SPFMV C1 in *N. benthamiana* and PVXC in *N. glutinosa*, respectively. Systemically infected leaves showing clear symptoms were harvested and cooled for 4-6 h at 4°C before processing for virus purification.

2.2.3. Virus purification

SPFMV C1 and C virions purification were performed at 4 °C unless otherwise indicated. All materials and solutions were sterilized for 20 min at 120 °C and sterile distilled water (SDW) for virions resuspension for RNA purification was treated with 0.1% diethyl pyrocarbonate (DEPC) as explained below (2.2.4).

2.2.3.1. SPFMV C1

For virus purification (Figure 2.1) 50 to 100 g of frozen tissue was immersed in liquid nitrogen for 10 min just before homogenization, powdered and homogenized in a domestic blender for 30 s in 50 mM Hepes [N-(2-hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)] buffer pH 8.0 containing 500 mM urea, 50 mM Na₂ EDTA and 0.5% Na₂SO₃ [1:3, w/v; homogenization Hepes buffer (HHB)]. The mixture was then emulsified for an additional 30 s with (2:1, v/w) ice cold chloroform:carbon tetrachloride (1:1), and centrifuged at 8000 g for 10 min; the supernatant was then filtered through pre-cooled glass wool and mixed with 2% Triton X-100 for 2 h at 4 °C. The homogenate was centrifuged at 8,000 g for 10 min and the supernatant mixed with 6% PEG (MW 6000-8000) containing 200 mM NaCl, stirred for 2 h at 4 °C and centrifuged at 14,250 g for 30 min. The pellets were resuspended with 20 mM Hepes buffer pH 7.2, containing 250 mM urea and 10 mM Na₂ EDTA [1:4, v/w; first resuspension Hepes buffer (FRHB)] for 4-5 h. After clarification at 5000 g for 5 min, the supernatant was layered over 5 ml of 30% sucrose cushion in FRHB and centrifuged at 65,000 g for 2.5 h. Pellets were resuspended in 10 mM Hepes buffer pH 7.2, containing 50 mM urea and 1 mM Na₂ EDTA [1:10, v/w; second resuspension Hepes buffer (SRHB)] for 16-18 h, clarified at 8000 g for 5 min and centrifuged at 103,000 g for 1 h. Pellets were then resuspended in SRHB (1:50, v/w), each 1.5 ml sample mixed with 0.5 g CsCl, 0.5 ml aliquots layered over CsCl density gradients made with 1.2, 1.2, 1.3 ml

of 0.38 g/ml, 0.88 g/ml and 1.35 g/ml (CsCl/SRHB) respectively and the gradients centrifuged at 127,800 g for 3 h in a SW 50.1 rotor. Gradients were fractionated, the virus containing fractions pooled and made up to 10 ml with 10 mM Hepes pH 7.2 and virus sedimented at 103,000 g for 1 h. The final pellet was resuspended in 10 mM Hepes pH 7.2 or, if virus was to be used for RNA preparation, in 150 mM NaCl, 15 mM sodium citrate, pH 7.0 (SSC), or in SDW (30 µl/g fresh tissue). Virus concentration and purity was determined spectrophotometrically ($E_{260} 0.1\% = 2.5$) (Gibbs and Harrison, 1976).

2.2.3.2. SPFMV C

For SPFMV C purification, a protocol based on the one developed for SPFMV C1, with some minor modifications were used. Powdered tissue was homogenized in 250 mM borate buffer pH 8.0 containing 500 mM urea, 10 mM Na₂ EDTA and 0.5% Na₂SO₃ [1:5, w/v; homogenization borate buffer (HBB)], and emulsified with (2:1, v/v) chloroform:carbon tetrachloride (1:1). After precipitation with 6% PEG (MW 6000-8000), pellets were resuspended in 50 mM borate buffer pH 8.0 containing 500 mM urea and 10 mM Na₂ EDTA (FRBB). Suspensions were centrifuged over a 30% sucrose cushion in FRBB, and after pellet resuspension in and clarification, supernatants were layered over a freshly-made 0-40% CsCl gradient in 50 mM borate buffer pH 8.0 containing 50 mM urea and 10 mM Na₂ EDTA (SRBB) and 20% sucrose, and centrifuged at 60,000 g for 3 h at 10°C in a SW28.1 rotor. Fractions were collected and diluted in SRBB. Virions were concentrated and final preparation was resuspended as in SPFMV C1 virion purification.

2.2.3.3. PVX^c

For virus purification, a procedure based on the protocol adopted for SPFMV C1, with some modifications, was used. Frozen tissue was homogenized in 200 mM KPB pH 8.0 containing 0.5% 2-mercaptoethanol (2-ME) and 50 mM Na₂ EDTA (3:1, v/w) for 1 min and emulsified with 20% chloroform. After clarification and treatment with Triton X-100, 4% PEG (MW 6000-8000) and 0.2M NaCl was added, mixed at 4 °C for 1 h and then incubated at room temperature for 1 h. Pellets were resuspended for 4 h with 50 mM

100, 4% PEG (MW 6000-8000) and 0.2M NaCl was added, mixed at 4 °C for 1 h and then incubated at room temperature for 1 h. Pellets were resuspended for 4 h with 50 mM KPB pH 7.2 containing 1% Triton X-100 and after sample clarification, supernatants were loaded over 20% sucrose in 50mM KPB pH 7.2 and 10 mM Na₂ EDTA and centrifuged at 70,000 g for 2.5 h. Sediments were resuspended overnight with the same buffer and after clarification by centrifugation, aliquots of the supernatants were loaded over a 10-40% sucrose density gradient and centrifuged at 60,000 g for 1 h in a SW28 rotor. Fractions were collected and diluted with 4 vols 10 mM KPB pH 7.2 and 10 mM Na₂ EDTA before virion concentration by centrifugation at 110,000 g for 1 h. Pellets were resuspended in DEPC-SDW (4ml/100g tissue).

2.2.4. RNA purification

Purification of viral RNAs was performed at 4 °C unless otherwise indicated. To avoid additional RNAase contamination, all plasticware and solutions (made in MilliQ distilled water) were DEPC (0.1%) treated for 16-18 h at 37 °C before sterilization. Glassware was soaked overnight in nitric acid, rinsed thoroughly with distilled water and DEPC-SDW, and baked at 240 °C for 3 h. Individually wrapped plasticware (pipettes and centrifuge tubes) were used when required. Electrophoresis tanks and accessories used for casting and running agarose gels with RNA samples were soaked in 3% H₂O₂ for 15 min and rinsed thoroughly with DEPC-SDW.

2.2.4.1. SPFMV C1 and C

2.2.4.1.1. Sucrose density gradient centrifugation

Virions in SSC (3.2 ml) containing 40U RNAsin (Stratagene)/mg virus were mixed with 0.8 ml 25% SDS, incubated at 60°C for 5 min, quickly chilled on ice, loaded over a linear 10-40% sucrose (RNAase free, Sigma) density gradient and centrifuged at 150,000 g for 4 h in SW 41 rotor. Fractions were collected from the top of the tube, NaAc pH 5.2 added to 300 mM and the RNA precipitated with 2.5 vols ice-cold EtOH at -70°C. The precipitate was washed with 70% EtOH and resuspended in DEPC-SDW.

100 g tissue		Frozen (-70 °C) or fresh. Immerse in liquid nitrogen for 10 min Powder Homogenize for 30 s in 50 mM Hepes buffer pH 8.0, containing 500 mM urea, 50 mM EDTA, 0.5% Na ₂ SO ₃ (3v:w) (HHB) Emulsify for 30 s with chloroform:carbon tetrachloride (1:1, v:w) Centrifuge at 8000 g x 10 min
Pellets	Supernatant	Filter through glass wool
Debris	Filtrate	Add 2% Triton X-100 Stir for 2 h at 4°C Add 6% PEG 6000 and 0.1M NaCl Stir for 2 h at 4°C Centrifuge at 14,250 g x 30 min
Supernatant	Pellet	Resuspend in 20 mM Hepes buffer pH 7.2, containing 250 mM urea and 10 mM EDTA (FHRB) Centrifuge 5000 g x 5 min
Pellet	Supernatant	Layer over a 5ml 30% sucrose cushion in FHRB Centrifuge 65,000 g x 2.5 h
Supernatant	Pellet	Resuspend in 10 mM Hepes buffer pH 7.2 containing 50 mM urea, 10 mM EDTA (SIRB) Centrifuge 5000 g x 5 min
Pellet	Supernatant	Centrifuge 103,000 g x 1 h
Supernatant	Pellet	Resuspend in SIRB Centrifuge 5000 g x 3 min
Pellet	Supernatant	Mix each 1.5 ml with 0.5 g CsCl Lay 0.5ml over a 1.2,1.2,1.3ml of 0.38g/ml, 0.88g/ml and 1.35g/ml CsCl/SHRB respectively Centrifuge 127,000 g x 3 h (SW50.1 rotor)
	Viral zone	Dilute up to 10 ml with 10 mM Hepes buffer pH 7.2 Centrifuge 103,000 g x 1 hr
Supernatant	Pellet	Resuspend in 10 mM Hepes (30 ml/g tissue) For RNA isolation in water or SSC.

Figure 2.1

Purification protocol for SPFMV C1 virions.

All procedures were carried out at 4 °C unless otherwise indicated

2.2.4.1.2. Sodium perchlorate :

The protocol followed was as described previously by Wilcockson and Hull (1974). Briefly, 800 μ l of virions in DEPC-SDW containing 40U RNAsin/mg virus were mixed with 200 μ l 25% SDS and heated at 60°C for 3 min before adding 3 ml of water-saturated sodium perchlorate (NaClO_4). The mixture was shaken well and centrifuged at 5000 g for 10 min. The lower phase was mixed with 8 ml ice-cold absolute EtOH and incubated overnight at -70°C and nucleic acids recovered as above.

2.2.4.2. PVX^c

For PVX^c RNA isolation, the protocol developed by Vega (1990) was followed. To 1 ml (1mg/ml) virus suspension, 250 μ l 20% SDS, 25 μ l proteinase K (10 mg/ml) and 4 ml water was added. The mixture was incubated at 37 °C for 30 min. The solution was gently mixed with an equal volume of Tris.HCl (pH 7.5)-saturated phenol and centrifuged at 12,000 g for 15 min. Supernatants were collected and mixed with an equal volume of phenol:chloroform (1:1), mixed and centrifuged as above. This step was done twice, and the final aqueous phase re-extracted with an equal volume of chloroform. The final aqueous phase was made to 300 mM NaAc pH 5.2 and RNA precipitated with 2.5 vols ice-cold EtOH, overnight at -70 °C.

2.2.5. Selection of poly (A)⁺ viral RNA

The protocol followed was based on the method described by Aviv and Leder (1972). Oligo(dT) cellulose (0.5 g, Sigma) was packed over sterile glass wool, pre-treated with DEPC, washed several times with DEPC treated water and autoclaved for 15 min at 120 °C. The column was pre-equilibrated with 5 vols binding buffer [BB (500 mM KCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA and 0.2% SDS)]. Purified viral RNA (2-3 mg) was resuspended in 100 ml BB, containing 100U RNasin (Promega), and loaded on top of the column. The filtrate was collected and re-applied twice to the column. The column was then washed with several volumes of BB, until the A_{260} reading was near to zero. Several volumes of elution buffer [EB (10 mM Tris.HCl pH 7.5, 1mM Na_2 EDTA, 0.2% SDS)

were added and all UV absorbing fractions were collected, made 300 mM NaAc and precipitated overnight with 2.5 vols of ice-cold EtOH at -70°C , washed and resuspended for spectrophotometric evaluation.

2.2.6. Infectivity

Purified SPFMV C1 RNAs were diluted in ice-cold water containing 0.05% EDTA treated bentonite (see below), to final concentrations of 1.0, 0.1 and 0.05 $\mu\text{g/ml}$ and inoculated on Carborundum-dusted leaves of 3-4 week-old *N. benthamiana* or *C. quinoa* plants. Purified SPFMV C RNAs were treated as for C1 RNAs and inoculated on *I. nil* Carborundum-dusted cotyledonal leaves. Control plants were mock-inoculated or inoculated with purified virus (0.1 $\mu\text{g/ml}$).

Na_2 EDTA-treated bentonite was prepared according to the method of Fraenkel-Conrat *et al.*, (1961). Bentonite (2g, 200 mesh, Sigma) was mixed thoroughly with 40 ml DEPC-SDW and centrifuged at 3000 g for 15 min. The supernatants were collected and centrifuged again at 10,000 g for 20 min. Pellets were resuspended with 0.1M Na_2 EDTA pH 7.0 and shaken at 25°C for 48 h. Suspensions were then centrifuged at 3,000 g for 15 min. Supernatants were centrifuged again at 10,000 g for 20 min and pellets were resuspended in DEPC-SDW. This step was done twice, and the final pellet was resuspended in 10 mM NaAc pH 6.0. An aliquot was used to determine the dry weight and the remaining pellet resuspended in 10 mM NaAc pH 6.0 to provide a 10% bentonite suspension.

2.2.7. Non-denaturing agarose gel electrophoresis

Non-denaturing agarose gel electrophoresis was performed as described previously by Sambrook *et al.* (1989). Agarose (1-1.5%, Ultrapure, BRL) in TBE was melted, cooled to approximately 60°C and 0.2 $\mu\text{g/ml}$ ethidium bromide added; gels were cast and allowed to solidify at 4°C for 1 h. RNA samples (1-2 mg) in water were mixed with 2 μl 10X loading buffer [(LB) 50% glycerol, 1mM Na_2 EDTA, 0.4% (w/v) bromophenol blue, 0.4% (w/v) xylene cyanol] and loaded into gel wells. The gel was run in ice cold TBE (apparatus

placed on ice) at 75V until bromophenol blue migrated to within 3 cm of the bottom of the gel. An RNA ladder (0.24-9.46 Kb, BRL) and PVY^o or PVX^c RNAs were used for calibration. Migration of the RNAs was monitored by UV (302 nm) transillumination.

2.2.8. Formaldehyde-agarose gel electrophoresis

Denaturing agarose gel electrophoresis was performed as described previously (Rosen and Villa-Komaroff, 1990 and Pappu and Hiruki, 1989). Briefly, 1.5% (w/v) agarose/6.6% (w/v) formaldehyde [(F.W.=30.03), 37% (12.3M)] was prepared as follows: Agarose (0.75 g) was melted in 36 ml of distilled water and allowed to cool to approximately 55°C. 10X running buffer [5 ml, (0.2M MOPS, 10mM Na₂ EDTA, 10mM NaAc, pH 7.0)] was mixed with of deionized 37% (w/v) formaldehyde (9 ml) and added to the cooled agarose. Ethidium bromide was added to a final concentration of 0.2 µg/ml and the gel was cast and allowed to solidify for 1h at room temperature in a fumehood. Purified viral RNA preparations (1-2 µg) were heat-denatured for 10 min at 60 °C in the presence of 0.66 M formaldehyde and 50% (w/v) deionized formamide. After heating, the samples were placed on ice. Following a brief centrifugation, 1 µl of 10X loading buffer [0.5% bromophenol blue (w/v), 0.5% xylene cyanol (w/v), 0.5 mM Na₂ EDTA and 50% glycerol (w/v)] was added. The gel was run at 8V/cm in running buffer with constant recirculation at 4 °C. Monitoring of RNA migration was done as for non-denaturing agarose gel electrophoresis.

2.2.9. Electron microscopy

Assessment of relative virus concentration, particle integrity and aggregation and relative amount of contaminants, was performed under a transmission electron microscope (Jeol 100X or Jeol 1200 EX-3). Virus samples (15-20 µl) at different stages of purification were placed over carbon coated grids, incubated for 2 min, washed with 30 drops SDW, dried briefly and stained for 1 min with 2% PTA (dodecatungstophosphoric acid pH 6.8).

2.2.10. Immunosorbent electron microscopy (ISEM)

To determine whether viral RNA preparations were free from virus coat protein, ISEM was performed according to the method of Hill (1984). In this procedure, support films are precoated with specific antibody to which homologous virus particles become attached and concentrated. The technique gives an effective concentration of virus on the grid from ten-to-a hundred fold. Formvar-coated copper grids were pre-coated with crude antiserum to SPFMV C1 CP (CIP, Plant Pathology Department, Lima, Peru) in phosphate buffered saline (PBS; 137 mM NaCl, 1.5 mM KH_2PO_4 , 8.1 mM $\text{Na}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 2.7 mM KCl, 3.1 mM NaN_3 , pH 7.4) (1:1000) by placing each grid on a 20 μl drop of antiserum for 1.5 h at room temperature. Grids were washed 3 times for 1 min each, by floating them on the surface of several mls of PBS and agitation by sucking and releasing wash buffer using a Pasteur pipette. After draining, antibody coated grids were placed on a 20 μl drop of sample and incubated for 2 h at room temperature. After rinsing with 20 drops of SDW and allowed to dry, grids were stained with 2% PTA.

2.3 Results and Discussion

2.3.1. Virion purification

2.3.1.2. SPFMV C1

Preliminary experiments showed that, in contrast to the experiences of others (Cali and Moyer, 1981; Cohen *et al.*, 1988a) in the purification of other isolates of SPFMV from *I. nil* or *I. batatas*, borate-based buffers proved to be unsuitable for the purification of the C1 isolate from *N. benthamiana*. Based on previous experiences on purification of other potyviruses (Suzuki *et al.*, 1989a; Hammond and Lawson, 1988; Sako *et al.*, 1980), several protocols were tested.

Although tissue homogenization and pellet resuspension with potassium phosphate-based buffers containing urea and Na_2EDTA gave good yields of virus particles, final preparations were contaminated with host components and the majority of the RNA derived from these preparations was non-infective. Contamination with chlorophyll was evident in the clarification solution. Emulsification with chloroform and carbon tetrachloride (1:1, v/w) gave cleaner preparations. Stirring the clarified extract with 2% Triton X-100 for 2-3 h before precipitation with PEG decreased host contaminants substantially. Further purification through a 30% sucrose cushion followed by CsCl density gradient gave preparations with $A_{260/280}$ 1.1-1.2 and yields ranged from 5 to 20 mg/100 g tissue (data from 10 virus purifications). Best results were obtained by homogenization of tissue in a HEPES-based buffer, a finding similar to that of Dougherty and Hiebert (1980) in the purification of TEV and PeMV.

While addition of MgCl_2 to the buffers for virus resuspension was beneficial for purification of C and RC isolates according to Moyer and Cali (1985), replacement of EDTA by MgCl_2 in the HEPES-based buffers led to a significant reduction in yield. The reduction appeared to be due principally to aggregation, a reduction not observed, however when phosphate buffers were used. When EDTA (10 mM) or MgCl_2 (20 mM) were added to the first (20 mM HEPES, pH 7.2, 25 mM urea) and second (10 mM HEPES, pH 7.2, 50 mM urea), HEPES resuspension buffers, average virus yields were 49 and 11 mg per 100 g tissue respectively. However, when added to first (50 mM KPB, pH 7.2, 250 mM urea) and second (10 mM KPB, pH 7.2, 50 mM urea) phosphate resuspension buffers, corresponding yields were 19 and 31 mg per 100 g tissue (Figure 2.2). Variation of HEPES concentration between 20 and 100 mM, of Na_2EDTA between 50 to 200 mM and use of urea at either 1.0 or 0.5 M, in the homogenization buffer, had relatively little effect on virus yield (average of 3 experiments, Figure 2.3), although 50 mM HEPES pH 8.0 containing 500 M urea, 50 mM EDTA with 0.5% Na_2SO_3 as reducing agent was adopted for routine use, because RNAs purified from virions purified using this buffer-system was always less contaminated ($A_{260/280} = 1.9$, average of 4 experiments).

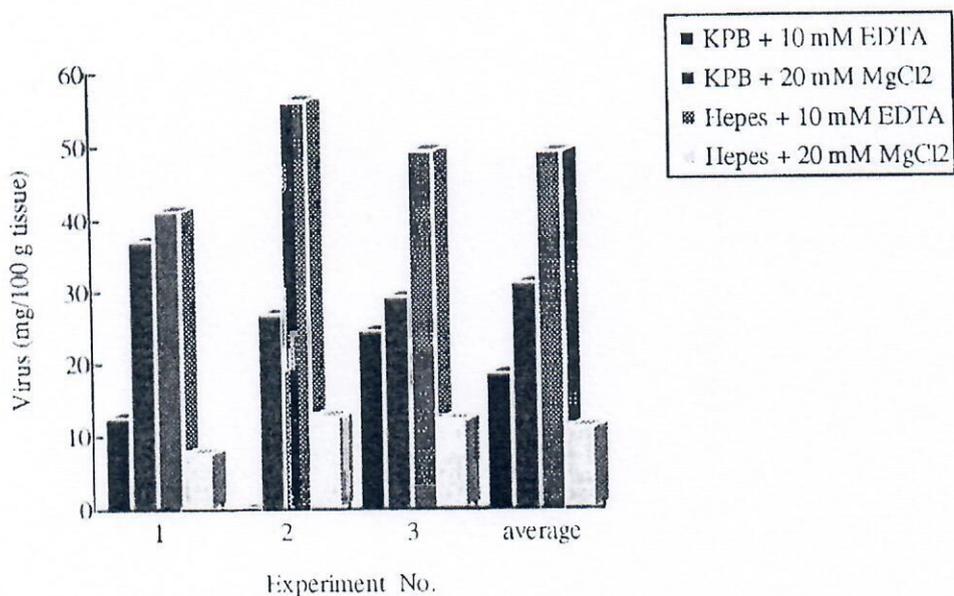


Figure 2.2

Effect of homogenization and resuspension buffers used on SPFMV CI purification on final yield of virions.

Experiment 2: KPb + 10 mM Na₂ EDTA, not performed.

Using information from the various procedures tested above, the procedure described in Materials and Methods was finally adopted for routine virus purification. Virus particles purified by this method are indicated in Figure 2.4. The average yield was 40 to 50 mg/100g issue, with an absorbance ratio ($A_{260/280nm}$) of 1.2-1.3.

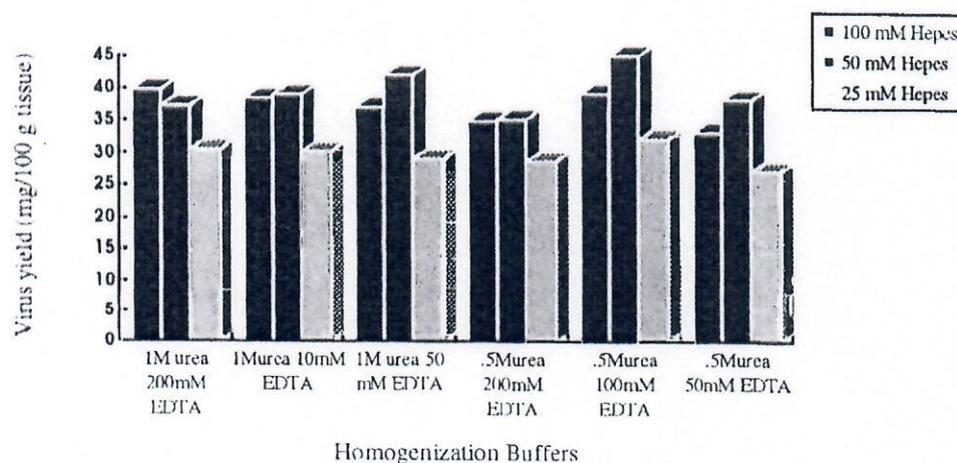


Figure 2.3

Effect of homogenization buffer composition for SPFMV C1 virion yield.

2.3.1.2. SPFMV C

SPFMV C virions, purified by following a protocol described by Cali and Moyer (1981) with a modified CsCl density gradient as described by Cohen *et al.* (1988a), were usually highly aggregated and attached to host contaminants. Insoluble, mucilaginous material was found at the bottom of centrifugation tubes. Preliminary purifications to optimize conditions for extracting virus particles with higher homogenization buffer:tissue ratios (5-10 v:w tissue), decreased particle aggregation but removal of host contaminants was not significantly better. Stirring with 2% Triton X-100 for 2-3 h did, however, decrease host contaminants in final preparations. Sedimentation of virions with PEG in the presence of 100 mM NaCl was efficacious after precipitated virus was adequately resuspended in resuspension buffer containing 1% Triton X-100. Replacement of $MgCl_2$ by Na_2 EDTA in resuspension buffers yielded cleaner preparations of relatively high virus concentration (8-10 mg/100 g tissue), suitable for infective RNA isolation.

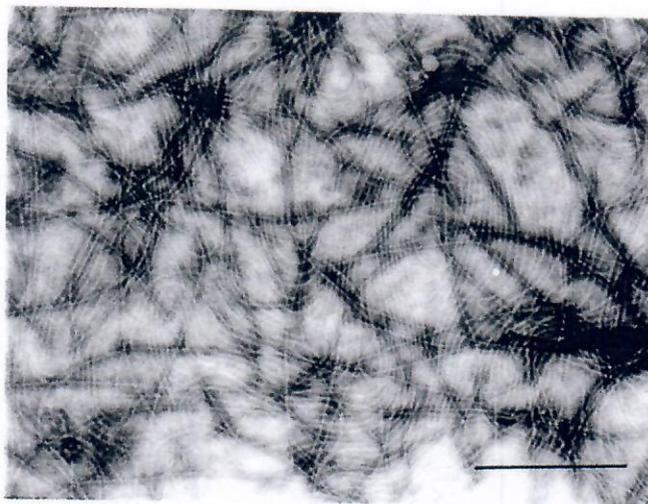


Figure 2.4
Electron micrograph of purified SPFMV C1 virions, stained with 2% PTA
Bar represents 200 nm.

2.3.2. RNA purification

2.3.2.1. SPFMV C1 and C

Incubation of virions in an alkaline dissociation buffer (200 mM ammonium carbonate pH 9.0, 2% SDS, 2 mM EDTA) and proteinase K (10 $\mu\text{g}/\text{mg}$ virus) followed by sucrose density gradients as previously described by Brakke and Van Pelt (1970), had been used previously for the isolation of RNA from other SPFMV isolates (Moyer and Cali, 1985) and for PVY^N (Robaglia *et al.*, 1989). This method was not however, suitable for SPFMV C1. RNA was always non-infective for *N. benthamiana*. Similar results were obtained by using phenol:chloroform:isoamyl alcohol (Brakke and Van Pelt, 1970) treatment of virions dissociated with the same alkaline buffer and proteinase K. Isolation of RNAs by using guanidine isothiocyanate and hydrochloride solutions according to the protocols of Cathala *et al.*, 1983 and Han *et al.*, 1987 have been also tested. Both of them rendered RNAs of less than full-length (2 experiments). By contrast, the procedures outlined in Materials and

Methods, involving SDS treatment followed by sucrose density gradient centrifugation or NaClO_4 treatment, permitted the isolation of full-length infective RNA (Figure 2.5). The yield of RNA was 10-20 $\mu\text{g}/\text{mg}$ virions with an absorbance ratio ($A_{260/280}$) of 1.85-1.93. The NaClO_4 treatment was also suitable for the isolation of infective SPFMV C RNA. The yield of RNA for this isolate was 8-14 $\mu\text{g}/\text{mg}$ virion with an absorbance ratio in the same range as for the C1 isolate.

Solutions of genomic SPFMV C1 and C RNAs purified in this way were infective for *N. benthamiana* and *C. quinoa* (Table 2.1) at 50 ng/ml and for *I. nil* (data not shown, 2 experiments) at 100 ng/ml respectively, and both could be used for infecting protoplasts by electroporation (Chapter 4). SPFMV C1 RNA was also suitable for use in preparation of cDNAs approaching full genome size (Chapter 5). It also hybridized to and could be eluted from oligo(dT) cellulose, suggesting that the genomic RNA of this isolate also has a poly(A) tail. None of the genomic SPFMV C1 RNA prepared by these procedures remained packaged, as verified by ISEM (using purified virions and sap from infective *N. benthamiana* as controls). A band of ca. 20 kD was observed when relatively large amounts of RNA were applied to the agarose gels (not shown). This may be the analogue of VPg-mediated aggregates of TEV, TVMV and PVY potyvirus RNAs reported by Luciano *et al.* (1991) and Rosner *et al.* (1986). This has not, however, been verified.

RNA concentration $\mu\text{g}/\text{ml}$	Number of plants (/4) infected Experiment 1	Number of plants (/4) infected Experiment 2
1.0	4	3
0.1	1	1
0.05	1	0

Table 2.1
Infectivity of SPFMV C1 RNA in *N. benthamiana*.

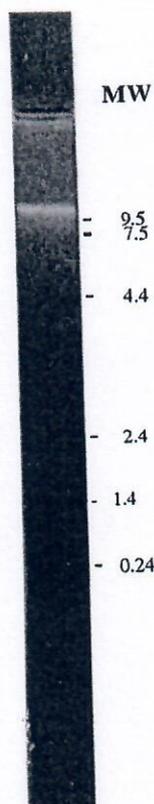


Figure 2.5
Agarose gel electrophoresis of purified genomic SPFMV C1 RN
The 0.2 - 9.5 Kb RNA ladder was used for assessement of the nucleic acid relative size.

The ability of the C1 isolate to infect *N. benthamiana*, coupled with the application of the procedures outlined here, has permitted the isolation of this Peruvian isolate of SPFMV and its genomic RNA in an intact form and in relatively large quantities. This has allowed the performance of further investigations, including electroporation of protoplasts. These would have been difficult, if not impossible, without the availability of such genomic RNA. Also, the application of these protocols with only some minor modifications, for the isolation of relatively large quantities of virions and infective RNA of the C strain, have proved to be feasible.

Chapter 3

Studies on non-structural genome coded proteins

3. Summary

Cytological studies by light and electron microscopy on the localization, development and morphology of sweet potato feathery mottle virus (SPFMV C1) inclusion bodies, have confirmed that the cylindrical inclusion (CI) protein coded by the virus genome aggregates in the cytoplasm of *Nicotiana benthamiana* L. Domin. and *Ipomoea nil* "Scarlet O'Hara" infected cells, in association with the plasmalemma and close to plasmodesmata at early stages of infection, and later as infection develops, in the form of bundles, short laminated aggregates and pinwheels which when probed with antiserum to the ca. 70 kDa protein purified from tissue of *N. benthamiana* infected with SPFMV C1, became specifically immunogold labelled. The CI protein of this virus was found to have a M_r of ca. 70 kDa and to be serologically related to the CI protein of TEV, WMV-2, ZYMV and papaya ringspot (PRSV-W) potyviruses, when probed in Western blots. Nuclear inclusion bodies, in the form of pyramidal crystals, were not observed in cells of *N. benthamiana* or *I. nil*, infected with SPFMV C1, when analyzed either by light or electron microscopy. It appears that the nuclear inclusion (NI) proteins encoded by this potyvirus do not aggregate in the same form as TEV NI proteins. Nucleolar inclusion bodies and crystalline microbodies were occasionally found in the nucleus and cytoplasm, respectively, of *N. benthamiana* infected tissue. The origin of these inclusion bodies has not been determined and requires further study. SPFMV C1 helper component-protease (HC-Pro) protein, purified from *N. benthamiana* infected tissue, was found to have a M_r of ca. 56 kDa, and shares common epitopes with PVY HC-Pro and PRSV-W AI proteins. It is associated with electron dense non-crystalline "amorphous inclusions", usually found near the cell wall and close to the chloroplasts, which became specifically immunogold labelled with a polyclonal antiserum to PVY HC-Pro, a monoclonal antiserum to PVY/TVMV HC-Pro and albeit less specifically with a polyclonal antiserum to SPFMV C1 HC-Pro.

3.1 Introduction

From the biochemical and molecular biology perspective, members of the family *Potyviridae* are unique with respect to the number of non-structural proteins that are synthesized and aggregate into stable forms during infection (Dougherty and Carrington, 1988a). Since these inclusions are products of the viral genome, study of their development, localization within the plant cell and their interaction with virus particles and plant cell constituents, should provide valuable information about the processes involved in viral infections.

Cylindrical inclusions

All members of this group that have been studied cytologically induce unique inclusions referred to as "cylindrical inclusion bodies" (CI), "pinwheels", "bundles", "laminar aggregates" or "scrolls". These inclusions have not been found in tissues infected with viruses of any other of the 34 plant virus groups currently recognized (Edwardson, 1992). Translation and immunological studies have shown that CI bodies are composed of a specific virus encoded protein, unrelated to the CP of the virus, to any other protein produced during infection by the virus or to any host plant protein (Hiebert *et al.*, 1984; Hollings and Brunt, 1981). Studies of TEV and PeMV RNA *in vitro* translations have demonstrated that the capsid and CI proteins are encoded by different portions of the viral genome (Xu *et al.*, 1985; Dougherty and Hiebert, 1980a, b). This information supports the recognition of the formation of CI bodies as a diagnostic feature for the potyvirus group as originally proposed by Edwardson (1974) and approved by the International Committee of Taxonomy of Viruses (Matthews, 1982). Immunological studies have shown that antisera to the CI protein induced by some potyviruses react with the inclusion protein from other potyviruses. Since the CI protein represents a much larger portion of the viral genome than the CP, and antigenic determinants were found to be more conserved than those of CP, at least in some specific cases, it has been suggested that they may be useful

for classification as well as for diagnosis (Mowat *et al.*, 1989; Koenig, 1988; Suzuki *et al.*, 1989; Yeh and Gonsalves, 1984).

Studies on the basic composition and morphology of these inclusions had established that CI are formed by a single protein of 66-75 kDa (Dougherty and Carrington, 1988a; Hiebert and McDonald, 1973) which is assembled into thin striated curved plates (10-20) (Andrews and Shalla, 1974) radiating from a central core and showing a periodicity of ca. 5 nm. These plates, described as "arms", "lamellae" or "septa" (Edwardson *et al.*, 1978) are generally rectangular although in some viruses, at least initially, may be triangular (Andrews and Shalla, 1974). Inclusions having rectangular "arms" are cylindrical in shape and those with triangular "arms" are conical. Inclusions in transverse sections are seen as "pinwheels" and in longitudinal section as "bundles". Sections of uncurled or unfolded "arms" not associated with the "pinwheel" are described as "laminated inclusions" which, when several are closely associated or partially fused, are described as "laminated aggregates". The outermost margin of "arms" may later extend and roll inwards, and in transverse or longitudinal section take the form of a "scroll" or a "tube", respectively (Hollings and Brunt, 1981). Determining the precise shape of the CIs induced by a given virus requires exact cross sections of the structures and studies at different stages of infection to avoid misleading interpretations. The types of CI bodies observed, with a few exceptions, are characteristic for a given virus and are independent of the host plant (Edwardson *et al.*, 1984). This finding led Edwardson (1974) and Edwardson *et al.* (1984) to suggest four subdivisions of the potyvirus group on the basis of the CI structures. Subdivision I contains those viruses inducing tubular scroll-like CI bodies, II contains those forming laminated aggregates, III contains those inducing both scrolls and laminated aggregates and subdivision IV contains viruses inducing scrolls and short curved, laminated aggregates. However, for the CI bodies of some viruses, it had been found difficult to clearly recognize to which subdivision would be the appropriate assignment (Losemann, 1988; Hollings and Brunt, 1981). It is not known which characteristics of the CI protein

determine the shape of the inclusion body, and whether these characteristics are constant for all viruses manifesting a particular inclusion morphology

Temporal studies of CI proteins have shown that the inclusions change in size, shape, number and location with the duration of the infection. Immunogold labelling of TEV CI showed that the protein can be detected the same day as infection, as filamentous threadlike structures, free in the cytoplasm, or closely associated with the plasmalemma, often opposite the plasmodesmata, but not in them. Subsequently, at later stages in the infection, they can be found mostly in the cytoplasm as pinwheel type inclusions (Murphy *et al.*, 1990a; Baunoch *et al.*, 1991, 1988). A similar sequence of events occurring during inclusion development has also been observed in TEV infected tobacco root cells (Andrews and Shalla, 1974) and in SPFMV RC infected *I. setosa* Ker. leaves (Lawson and Hearon, 1971; Lawson *et al.*, 1971). Association of "bundles" with distended endoplasmic reticulum and formation of a large number of smooth surfaced vesicles occurred in older infected leaves (Calder and Ingerfeld, 1990; Lawson *et al.*, 1971).

As mentioned in Chapter 5, the most prominent sequence feature of the CI protein is the presence of a nucleotide-binding motif (NTBM) composed of cassettes of aminoacids that are common to many proteins for which NTP-binding has been demonstrated or postulated. Although still scarce, experimental data indicate that the NTBM-containing proteins are involved in replication (Lain *et al.*, 1991; Domier *et al.*, 1987). Further aminoacid sequence comparisons with several proteins of viral and cellular origin suggested that these NTBM-containing viral proteins possess a helicase activity, related to the unwinding of duplex polynucleotides by using nucleoside triphosphate as an energy source (Company *et al.*, 1991; Lain *et al.*, 1990). As noted by Riechmann *et al.* (1992), only the N-terminal region of the CI protein is related by sequence comparisons with the helicase activity (Lain *et al.*, 1989b). Therefore, the C-terminal region of about 230 amino acids could be involved in some of the other functions which have been proposed for the CI protein, for example the attachment of the replication complex to membranes in a similar way to the NTBM-containing protein 2C of poliovirus (Bienz *et al.*, 1987), the induction

of vesicles in the infected cells or virus transport from cell-to-cell. WSMV (Langenberg, 1986), pea seedborne mosaic potyvirus (PSbMV; Calder and Ingerfeld, 1990) and PPV (Martin *et al.*, 1992) CI bodies were found in close association with both the plasmodesmata and the membrane of the endoplasmic reticulum (ER). ER associated with CI bodies produces large numbers of smooth-surfaced vesicles; some of them, particularly those associated with the arms of the pinwheels, have electron dense contents (Calder and Ingerfeld, 1990). These vesicles are analogous to the vesicles induced by the 2C protein of picornaviruses, which provide a matrix to which the replication complex of poliovirus is bound (Bienz *et al.*, 1987).

Amorphous inclusions

Whereas the appearance of CI bodies is the most conspicuous and universal intracellular effect of all potyvirus infections, a number of other structures have been also reported to be associated with infection by some members of this group. Some strains of WMV-2 and PRSV-W (Suzuki *et al.*, 1989b; Martelli and Russo, 1976), PVY (Christie and Edwardson, 1977; Lesemann, 1988; Baunoch *et al.*, 1990) and PeMV (Edwardson, 1974) but not TEV (Baunoch *et al.*, 1991) induce the formation of irregularly shaped inclusions so-called amorphous inclusions (AI), in the cytoplasm of infected cells (Govier *et al.*, 1977; McDonald and Hiebert, 1975). They appear as a uniform finely granular and electron dense matrix, without any obvious structure (Francki *et al.*, 1985; Edwardson, 1974) or as short rod-shaped cytoplasmic inclusions (Baunoch *et al.*, 1990; Christie and Edwardson, 1977). Apparently, the AI bodies produced by different potyviruses take on different morphological forms (Lesemann, 1988).

The AI of some viruses have been partially purified (Suzuki *et al.*, 1989b; de Mejia *et al.*, 1985a). In the case of PeMV and PRSV-W (watermelon strain 1), these inclusions are not only large in size but also quite stable (Edwardson, 1974). For PeMV, a single polyprotein of 51 kDa is the major component of the AI protein (de Mejia *et al.*, 1985b; Yeh and Gonsalves, 1985). A 112 kDa protein isolated from PRSV-W infected plants

reacted with antisera to the AI protein and was found in *in vitro* studies to function as the precursor for the 51 kDa AI protein (Yeh *et al.*, 1985). Serological cross-reactivity studies have shown that antisera to AI bodies produced by PeMV and PRSV-W immunoprecipitated *in vitro* translation products which are identical to those immunoprecipitated by antisera to the HC-Pro proteins of PVY and TVMV (Hiebert *et al.*, 1984b; de Mejjia *et al.*, 1985b). These findings revealed that the AI protein was related to the HC-Pro protein and suggested indirectly that the AI bodies produced by some potyviruses are pools or reservoirs of HC-Pro protein (de Mejjia *et al.*, 1985b). Reciprocal serological tests done by Western blotting indicated a significant serological relationship between the TVMV HC protein and PRSV-W AI protein. In addition, TVMV HC-Pro protein has a similar size to the PeMV or PRSV-W AI proteins (Hiebert *et al.*, 1984b; Yeh and Gonsalves, 1984). TVMV induces the formation of ring-like cytoplasmic inclusions which appeared to contain HC-Pro protein on the basis of immunofluorescence studies (Hiebert, unpublished). They both co-migrate in SDS-PAGE and were found associated with rod-shaped cytoplasmic inclusions in PVY infected tissues, by immunogold labelling with PVY HC-Pro specific serum (Baunoch *et al.*, 1990). However, antisera against AI protein does not inhibit aphid transmission, as antisera to HC-Pro does, in reconstitution experiments (Hiebert *et al.*, 1984b). It is possible the protein that accumulates in the AI has been modified prior to or during inclusion formation.

Examination of the protein sequence of the putative HC-Pro of TEV, TVMV, PPV and PVY^N (Robaglia *et al.*, 1989) reveals the presence of a cluster of Cys and His residues which is similar to the consensus sequence of metal binding sites in nucleic acid binding proteins (Koonin *et al.*, 1991; Lain *et al.*, 1989a; Sehnke *et al.*, 1989; Evans and Hollenberg, 1988). The cysteine residues in cysteine-rich polypeptides often form intermolecular disulfide bonds in such proteins as human insulin-like growth factor-1 (hIGF-10), which forms dimers (Chaudhuri *et al.*, 1991). It is proposed that hIGF-1 acquires a conformation which exposes at least one cysteine residue in such a way that close proximity between two molecules allows intermolecular disulfide bonds to form a dimer.

Dimerization is prevented by a mutation at residue 27 from Lys to Glu (Chaudhuri *et al.*, 1991). For TVMV HC-Pro protein, a substitution of Lys to Glu change at position 307 might prevent the ionic interaction between the two HC-Pro monomers, as in the case of hIGF-1, and result in the lack of dimerization and the lack of aphid transmission activity of the HC-Pro protein (Atreya *et al.*, 1992). The apparent M_r of the HC-Pro subunit is 53 kDa and 58 kDa for TVMV and PVY proteins, respectively (Hiebert *et al.*, 1984b; Thornbury and Pirone, 1983). However, undissociated, biologically-active glycoprotein (Berger and Pirone, 1986b) was found to be 100 kDa and 150 kDa for the same viruses respectively, suggesting that the active molecule could be a dimer and that the metal-binding sites may be involved in the formation of this structure (Robaglia *et al.*, 1989).

The HC-Pro protein produced by potyviruses in infected cells, has been found to be conclusively related to the transmissibility of the virions by aphids (Pirone and Thornbury, 1983; Sako and Ogata, 1981a; Pirone, 1981; Govier and Kassanis, 1974). Lack of strict HC-Pro-virus specificity has been noted. The proteins of TuMV and PVY differ in size and serological specificity and they can be functionally interchangeable (Pirone, 1981). Purified TEV is aphid transmitted equally efficiently in the presence of PVY HC-Pro and TuMV HC-Pro (Hiebert *et al.*, 1984). BYMV HC-Pro effects the transmission of purified TuMV or PVY, but neither TuMV HC-Pro or PVY HC-Pro effect the transmission of purified BYMV (Pirone, 1983). WMV HC-Pro enabled TuMV transmission, but failed to effect the transmission of PVY. In contrast, TuMV HC-Pro failed to effect transmission of WMV and PVY, but PVY HC-Pro helped to transmit TuMV but not WMV (Sako and Ogata, 1981a). The HC-Pro protein can be extracted from plants infected with either highly, poorly or non transmissible TEV isolates (HAT, PAT or NAT, respectively); however, HC-Pro activity was highest from PAT-infected plants, and aphids given access first to HC-Pro and then to infected plants, were able to transmit the NAT isolate (Pirone and Thornbury, 1983). This probably reflects the presence of common sequences in the HC-Pro proteins of distinct potyviruses.

Potato potyvirus C (PVC), an aphid transmissible-defective strain of PVY (Kassanis and Govier, 1971), produces a HC-Pro protein which is similar in M_r and immunological reactivity to that of PVY but is not functional in aphid transmission (Thornbury *et al.*, 1990). Comparison of the derived amino acid sequence of HC-Pro of PVC, PVY⁰, PVY^N, PPV, TEV and TVMV potyviruses revealed two amino acid changes within areas of conserved sequence in the HC-Pro region that were specific to PVC HC-Pro; one is a change from Lys to Glu and the other is from Ile to Val (Thornbury *et al.*, 1990). As mentioned above, a substitution of Lys to Glu at amino acid 307, of the TVMV HC-Pro protein, completely abolished aphid transmission activity (Atreya *et al.*, 1992). The nucleotide sequence of BaYMV (Kashiwazaki *et al.*, 1990) showed that a region of the polypeptide encoded by its RNA-2 (which corresponds to the 5'-third of the genomes of aphid-transmitted potyviruses) had limited sequence homology with the HC-Pro protein sequence of aphid-transmitted potyviruses. This suggested that a HC-like protein is also produced by BaYMV, although HC-Pro mediated transmission of BaYMV or any other fungus-, mite-, or whitefly-transmitted potyvirus has not yet been reported. Antiserum to TVMV HC-Pro has been found to immunoprecipitate cell-free translated HC-Pro protein of 16 different potyviruses, some of which were either poorly transmitted or nontransmitted by aphids (Hiebert *et al.*, 1984b). This probably reflects the presence of common sequences in the HC-Pro proteins of distinct potyviruses.

The mode of action of the HC-Pro seems to involve binding of the virus particles in some parts of the aphid's alimentary apparatus (Lopez-Abella *et al.*, 1981). This was indicated by the higher amount of ¹²⁵I-labelled HC-Pro protein found in these parts of the insect in a reconstitution experiment (Berger and Pirone, 1986a).

The HC protein (Govier and Kassanis, 1974) has been renamed HC-Pro to encompass its two major activities, aphid transmission (Thornbury *et al.*, 1985) and a

protease (Carrington *et al.*, 1989a,b) responsible for processing at its own C-terminus via an autocatalytic mechanism (Chapter 5). Recently, it has been suggested that the HC-Pro protein may have a third function. It has been suggested that the potential metal binding motifs ("zinc fingers") may form part of the cell-to-cell movement complex (Robaglia *et al.*, 1989). The suggestion that HC-Pro proteins may bind to nucleic acids is strengthened by the observation that they can be purified by absorption to oligo(dT) (Thornbury *et al.*, 1985). Site-directed mutagenesis within the putative 'zinc-finger' motif was found to affect symptomatology and virus accumulation in infected plants (Atreya *et al.*, 1992), however further analysis is needed to elucidate this possible role of the HC-Pro protein in the potyvirus life cycle. It is interesting to note that the three functions of HC-Pro mentioned above may be associated with three different domains of the HC-Pro protein molecule, with the N-terminal domain containing the nucleic acid binding property, the central domain mediating aphid transmission, and the C-terminal domain involved in proteolytic activity.

Nuclear inclusions

A limited number of potyviruses also induce the formation of stable, relatively large, crystalline structures (BYMV and TEV), fibrous inclusions (blackeye cowpea mosaic and gloriosa stripe mosaic potyviruses), fimbriate inclusions (zucchini yellow fleck potyvirus) and lacunose globular inclusions (BeMV and PVA) within the nucleus of infected cells (Edwardson, 1992; Restrepo *et al.*, 1990; Baunoch *et al.*, 1988; Knuhtsen *et al.*, 1974; Matsui and Yamaguchi, 1964). NI bodies are variable in shape and size, depending not only on the virus, but also on the specific isolate (Edwardson and Christie, 1978). The mild strain of TEV induces bipyramidal inclusions, the severe strain induces thin truncated four-sided pyramids and the Madison isolate induces octahedral inclusions (Edwardson, 1992; Matsui and Yamaguchi, 1964), composed of two proteins of 49 kDa (NIa) and 54 kDa (NIb) in equimolar amounts (Dougherty and Hiebert, 1980a). Immunogold labelling of PPV infected tissue indicated that NIa protein was specifically associated with the nuclear

inclusions, which were not like TEV crystalline inclusions, but were of various shapes and also reacted with PPV or TEV NIa antisera and TEV NIB antiserum. Immunogold labelling also indicated that both proteins were also associated with cytoplasmic dense material, similar to the nuclear inclusions and not showing pinwheel shape (Martin *et al.*, 1992). TEV NI protein, not aggregated into inclusion bodies, has been gold labelled at the periphery of the nucleus (Baunoch *et al.*, 1988) and at early stages of infection in both nucleolus and the rest of the nucleus. No labelling of any of the organelles other than nuclei was found, suggesting that both proteins are rapidly transported into the nucleus after synthesis (Baunoch *et al.*, 1991). Spherical nuclear inclusions have been detected in celery infected with celery mosaic potyvirus (Christie and Edwardson, 1977) and large spherical nuclear inclusions have been associated with BeMV infections (Bos, 1970). Similar types of inclusions, found in the cytoplasm and in the nucleus, were reported to be induced by several isolates of BYMV (McWhorter, 1965). Cytochemical tests indicated that the crystals present in the cytoplasm and in the nucleus were of identical constitution. An isolate of BYMV induces both small crystals in the cytoplasm and large angular inclusions in the nucleus (Muller and Koenig, 1965), while another isolate induces large elongate crystals in the cytoplasm and in the nucleus (Edwardson, 1974). PeMV induces flat, angular crystals in both the cytoplasm and the nucleus (Christie and Edwardson, 1977).

Cell-free translation *in vitro* and determination of the sequence of several potyvirus RNAs have shown that both proteins are virus-encoded and their amino acid composition is highly conserved among most potyviruses (e.g. Robaglia *et al.*, 1989; Dougherty and Carrington, 1988a; Domier *et al.*, 1986; Hellmann *et al.*, 1983; Dougherty and Hiebert, 1980b). An antiserum to the NIB of TEV serologically reacts with the protein encoded by 25 potyviruses tested in *in vitro* translation studies, including such potyviruses as PRSV-W that are not known to form NI bodies *in vivo* (Hiebert, unpublished).

The transport of proteins to the nucleus of eukaryotic cells is a highly regulated process. In animal cells, the selective passage through the nuclear membrane depends not

only on the size of the protein but also appears to be governed by amino acid sequences that act as autonomous signals capable of specifying nuclear location (Silver, 1991; Garcia-Bustos *et al.*, 1991; De Robertis, 1983). Entry of proteins into the nucleus appears to involve at least two steps: binding to a nuclear pore receptor or complex and energy-dependent uptake through the pore (Newmeyer and Forbes, 1988). Experimental systems to examine transport sequences involved in nuclear translocation pathways in plant cells have been recently developed (Varagona *et al.*, 1991; Restrepo *et al.*, 1990). It was found that TEV NIa and NIIb proteins that are localized in the nucleus contain a nuclear localization signal (NLS) (Restrepo *et al.*, 1990). Although the majority of NIa molecules are transported to the nucleus, virus particles containing VPg-NIa linked protein are found within the cytoplasm (Murphy *et al.*, 1990b, Lesemann, 1988). The existence of signals at the N terminus of both NIa and NIIb proteins, which direct efficient nuclear transport, has been demonstrated. Two domains within the NIa, one between amino acid residues 1 and 11 (signal domain I) and the other between residues 43 and 72 (signal domain II), were found to function additively for efficient transport of fusion proteins to the nucleus, although either region independently could facilitate a low level of translocation (Carrington *et al.*, 1991; Restrepo *et al.*, 1990). Recently, expression of the genes encoding the NIa or the 6kDa/NIa proteins in transgenic plants, has shown that when the NIa failed to undergo autoproteolysis of its junction with the 6 K2 protein, because of the presence of a Cys-to-Ala substitution in the proteolytic domain of NIa, the fusion protein remained in the cytoplasm. The inhibition of NIa-mediated nuclear transport was found not to be due to changes in the proteolytic domain of NIa, since this alteration had no effect on its translocation in the absence of the 6kDa protein, but to be due to the presence of the 6kDa protein. This result could indicate that subcellular transport of NIa may be regulated by autoproteolysis (Restrepo-Hartwing and Carrington, 1992). Based on the fact that most potyvirus encoded proteins accumulate in the cytoplasm, the proteolytic functions of NIa are presumably carried out prior to nuclear translocation. Cellular or viral factors that influence NIa-mediated autoproteolysis would indirectly affect nuclear translocation of NIa.

It has been proposed that the 6kDa/N1a protein may bind to a cytoplasmic anchor which functions to override the nuclear transport capacity of N1a (Restrepo-Hartwing and Carrington, 1992). The role(s) of N1a and N1b in the nucleus is not known; there is no experimental evidence to suggest that they affect host nuclear functions.

Potyvirus infections also induce other types of inclusions, in addition to the typical CI, AI or NI bodies. Some members induce paracrystalline inclusions, both in the cytoplasm and in the nucleus. These inclusions, since they are needle shaped, may resemble the CI, although they are usually greater in length and they can also be differentiated by their staining reactions by light microscopy (Christie and Edwardson, 1977). Some potyviruses, like BYMV and its various isolates, also induce distinctive cytoplasmic and nuclear crystalline inclusions. Cytochemical tests have indicated that the crystals present in the cytoplasm and the nucleus are of identical constitution (McWhorter, 1965). The presence of such inclusions makes it possible to distinguish between certain strains of BYMV (Muller and Koenig, 1965).

Many of the virus-coded non-structural proteins can be extracted and purified from infected plants and used for the production of specific antisera (Brunt, 1988; Hammond & Lawson, 1988; Hiebert *et al.*, 1984a). The development of sensitive techniques to detect viruses and virus proteins either in solution (Towbin *et al.*, 1979; Clark and Adams, 1977) or *in situ* (Horisberger and Rosset, 1977) have made possible the specific localization of viral products within the plant cell. The immunogold labelling technique offers great potential for the detection of antigen-antibody complexes. Protein A or anti-IgG adsorbed to gold particles specifically binds to such complexes which in turn are easily observed in the EM, because the gold particles are extremely electron dense (Roth *et al.*, 1978), enabling quantification of labelling density. They can be prepared in a range of sizes (5 to 80 nm) and can be used for double or triple labelling in the same specimen (Martin *et al.*, 1992; van Lent, 1988). An essential requirement for the detection of antigens in thin

sections of biological tissue is the retention of antigenic properties of the polypeptides to be detected, and at the same time a satisfying preservation of cellular structures. Recent development of polar embedding resins like Lowicryl K4M (Carlemalm *et al.*, 1982) and London Gold Resin (LGR) and white (Polak and Van Noorden, 1986), have improved preservation of cellular structures and retention of antigenicity.

The aims of the experiments described in this chapter were to initiate a study of SPFMV C1 genome coded non-structural proteins, which either aggregate to form specific inclusion bodies or can be extracted from infected plant tissue. The second aim was the purification of HC-Pro and CI proteins for preparation of antisera, which could allow the characterization of these proteins *in vivo*. The ultimate aim of the investigations was to localize SPFMV C1 CI and AI proteins in infected leaf tissue by using the immunogold labelling technique.

3.2. Materials and Methods

3.2.1. Plants and inoculum

N. benthamiana, *I. nil* and *N. occidentalis* were grown and viruses, SPFMV C1, C, RC, YV and PVY^N were mechanically transmitted using the same conditions and protocols as described in Chapter 2. Plants used for virus inclusion studies by EM and light microscopy (LM) were kept in the dark for 48-72 h before tissue processing.

3.2.2. Cytological studies

3.2.2.1. Time course analysis of inclusion development by EM

The purification of inclusion bodies produced by SPFMV C1 has not been reported and no information was available on host plant localization, development or ultrastructure of these inclusions in *N. benthamiana* or *I. nil* inoculated or systemically infected leaves. A

time course survey based on those parameters was monitored by EM. Samples were collected 6, 15, 22, 30 and 50 days after inoculation. Tissue pieces (1-2 x 5 mm) were fixed in 5% buffered glutaraldehyde [buffer: 9 ml (2.1% NaH_2PO_4 + 0.43% NaOH) + 1 ml (5.4% glucose)] for 18 h and post fixed for 1 h in 2% OsO_4 in cacodylate buffer [0.2M Na cacodylate pH 7.3 (with 1M HCl)]. Samples were thoroughly washed with distilled water, block-stained in 2% UA and dehydrated in an EtOH series (70, 90 and 100%) before embedding in LR White low viscosity epoxy resin at 60 °C for 18 h, followed by 3 changes of resin for 1 h each. Ultrathin sections were cut on a Reichert 4 ultramicrotome with a diamond knife, mounted on formvar-coated copper grids and stained with 2% UA for 10 min. Samples were observed in a JEOL 1200 EX EM.

3.2.2.2 Light microscopy of SPFMV C1 inclusions

Most inclusions are of sufficient size to be readily visualized with the light microscope. This has the advantage of allowing a large field of view to be covered permitting extensive areas of tissue to be searched for the presence of inclusions (Edwardson, 1977). These experiments were performed in order to determine whether crystalline NI and/or AI bodies, similar to the inclusion bodies observed in tissue infected with other potyviruses (Lesemann, 1988; Edwardson, 1977), which were not readily identified in the samples prepared for EM, could be observed by searching a much larger area. Preparation of stains, tissue handling and staining procedures were done according to Christie and Edwardson, (1977) and Dr. U. Jayasinghe (personal communication, CIP, Lima, Peru). Since Azure A and the orange-green (O-G) stains can differentiate between certain types of viral inclusions, each staining procedure was applied to the same piece of tissue. With O-G, which stains most potyviral inclusions, the viral inclusions stain green to olive brown, and are easily distinguished from nuclei and plastids which stain orange and yellow, respectively. 1. Azure: Stain (0.1 g; A-CI 52005, Eastman Kodak) was dissolved in 2-methoxyethanol (100 ml, Eastman Kodak). Just before staining, dye solution was mixed with 0.2M Na_2HPO_4 in distilled water (1:9, v:v). 2. O-G: the orange (102, Pylam Products Co., Inc. 95-10, 218th Street, Queens Village, NY 11428) and the

green (Luxol brilliant green BL, Aldrich Ch. Co.) dyes were prepared and stored separately at room temperature. Each dye (1 g) was dissolved in 2-methoxyethanol (100 ml) and then filtered. The final staining solution was prepared by mixing water, and orange and green dyes. The proportions of orange and green varied depending on the type of tissue under study, but the water content was maintained at 10% of the total volume.

Epidermis samples were taken from non-infected and SPFMV C1 systemically infected *N. benthamiana* and *I. nil* leaves (30-35 days post-infection). Chlorophyll was removed from the plastids by soaking the pieces of tissue in undiluted 2-methoxyethanol for 30 min. After chlorophyll removal, the tissue was transferred to the Azure A solution and stained for 1 h, rinsed with EtOH for 15 min and with 2 methoxyacetate (Sigma). If the same samples were also to be stained with the O-G, they were placed in that stain solution for several hours. After rinsing with 2 methoxyacetate, the stained tissues were mounted in water and observed under a Olympus PM10ADS light microscope.

3.2.3.SPFMV C1 inclusion protein purification

All materials were sterilized and all purification steps were performed at 4 °C, unless otherwise indicated. Specific protein isolation and identification was monitored by spectrophotometry, Western blotting and dot blot ELISA. For the spectrophotometric and immunological analysis of CI and AI proteins, an aliquot of purified sample diluted in final resuspension buffer containing 1-2% SDS was heated at 100 °C for 5 min, cooled at room temperature and centrifuged at 5000 g for 10 min. Supernatants were collected and yields were calculated by using an extinction coefficient $A_{280\text{ nm}} = 1.4$.

3.2.3.1. HC-Pro purification

SPFMV C1 HC-Pro protein was purified by a method similar to that described by Sako and Ogata (1988b). Infected leaves were harvested 3-4 weeks after inoculation (50 g), powdered in liquid nitrogen and homogenized in ice cold 250 mM KPB pH 9.0, containing 20 mM Na₂ EDTA and 20 mM DIECA (1:3, w:v) for 1.5 min. The mixture

was filtered through several layers of cheesecloth and centrifuged at 12,500 g for 20 min. Supernatants were centrifuged at 123,000 g for 90 min, pellets discarded and supernatants mixed with saturated $(\text{NH}_4)_2\text{SO}_4$ (1:3, w:v), incubated for 30 min at room temperature and centrifuged at 15,000 g for 30 min. Pellets were resuspended with 20 mM KPBS pH 7.2 containing 20 mM MgCl_2 , centrifuged at 12,000 g for 10 min and the supernatants layered over a 10 ml DEAE Sephacel column pre-equilibrated with 20 mM KPBS, 20 mM MgCl_2 . The elution profile was scanned at $A_{280\text{nm}}$ and 2 ml fractions were collected and centrifuged at 60,000 g for 40 min. Aliquots of the supernatants (diluted to a protein concentration of 1mg/ml), were analyzed by immunological methods and used for antisera production.

When the protein was to be used as an antigen in serological tests, a semi-purified preparation was prepared; the clarified solution, after ammonium sulfate precipitation was dialyzed against 3 changes of 1/2 PBS pH 7.4, 20 mM MgCl_2 , to remove the traces of ammonium sulfate.

For dot blot ELISA using MAbs to HC-Pro, antigen was prepared according to the protocol of Dr. R. Jordan (personal communication). Infected tissue was homogenized in 100 mM ammonium acetate, 20 mM EDTA, 20 mM DIECA pH 8.8, clarified at 10,000 g for 10 min and diluted in 100 mM Tris, 20 mM MgSO_4 , pH 7.4 (1:10, v:v).

3.2.3.2. AI purification

SPFMV C1 amorphous inclusion proteins were partially purified from systemically infected *N. benthamiana* leaves 3 to 4 weeks after inoculation, according to a protocol described by de Meijia *et al.* (1985a). Pre-cooled tissue (50 g) was homogenized with ice-cold homogenization buffer (HB; 100 mM Tris HCl pH 8.0, 0.5% sodium sulfite) (1:3, w:v). The homogenate was filtered through two layers of cheesecloth and the filtrate layered over a 10 ml 20% sucrose cushion in HB and centrifuged at 5000 g for 10 min. Pellets were resuspended in HB, Triton X-100 added to a final 5% concentration and the mixture stirred for 1 h. The mixture was layered over a 10 ml 40% sucrose cushion in HB and centrifuged at 5000 g for 10 min. Pellets were resuspended as before, and 5% Triton

X-100 was added. Centrifugation and resuspension steps were repeated until almost all the green colour had been eliminated. Final pellets were resuspended in the small volume of HB and mixed with the same volume of 2X- SDS PAGE sample buffer (below: 3.2.5.3.1).

3.2.3.3. CI purification

Systemically infected leaves of *N. benthamiana* plants, 30-40 days after inoculation with SPFMV CI, were harvested and cooled at 4 °C in the dark for 18-20 h. Tissue was homogenized with ice-cold homogenization buffer (HB; 200 mM KPB pH 8.2, 1 M urea, 10mM Na₂EDTA and 0.5% 2-ME) (1:3, w:v) for 1 min and emulsified with chloroform:carbon tetrachloride (1:1, v:w) for 1 min. The homogenate was centrifuged at 5000 g for 5 min. The pellets were re-extracted with HB (1:1, w:v) and re-emulsified with organic solvents (1:0.5, w:v), and the homogenate centrifuged as previously. Supernatants were then mixed and centrifuged at 2,000 g for 5 min. Supernatants were mixed with 5% Triton X-100 and stirred for 2-3 h. PEG 6000-8000 (6%) and 100 mM NaCl was added and stirred for 1 h. The mixture was centrifuged at 12,000 g for 30 min and the pellets were resuspended in resuspension buffer (RB; 50 mM KPB pH 8.0, 0.1% 2-ME and 1% Triton X-100) for 18 h. The suspension was centrifuged at 1000 g for 5 min, the supernatants layered over a 5 ml 40% sucrose cushion in RB, and centrifuged at 40,000 g for 1 h. Pellets were resuspended in 10 mM KPB pH 8.0 and 0.1% 2-ME, by hand. The suspension was then centrifuged at 1000 g for 5 min and the supernatants collected.

For polyclonal antiserum production, the protein suspension was further purified by 10% SDS PAGE. The gel band containing a protein of approximately 70 kDa, previously identified with antiserum to PRSV-W CI protein (see below), was cut with the aid of a razor blade, frozen overnight at - 20 °C, crushed in an ice-cold mortar and washed into a test tube by the addition of 1-2 ml of PBS. For dot blot ELISA or Western blots, partially purified CI protein suspensions were used, after precipitation with PEG and clarification by centrifugation.

3.2.4. Antisera

The following antisera to polyvirus non-structural proteins were received: polyclonal antisera to TEV CI, TEV NIab and PeMV CI from Dr. W. Dougherty, Oregon State University, Nash Hall 220, Corvallis, OR 97331, USA; to TVMV HC-Pro and PVY HC-Pro from Dr. D. W. Thornbury, University of Kentucky, Lexington, Kentucky 40546-0091; to PRSV-W AI, from Dr. D. E. Gonsalves, University of Florida, Gainesville, Florida 32611, USA; to PRSV-W CI, PRSV-W AI, WMV-2 CI and ZYMV CI, from Dr. N. Suzuki, Tohoku University, Sendai 980, Japan. Monoclonal antibody to PRSV-W AI from Dr N. Suzuki, and monoclonal antibodies to a mixture of PVY and TVMV HC-Pro [(Jordan *et al.*, 1990) (HC5G5B11, HC22E8B10, HC1B8B6 and HC5FB8)] from Dr. Ramon Jordan, USDA-ARS, Beltsville, MD 20705, USA. All polyclonal antisera were produced in rabbits and monoclonal antibodies in mice.

Polyclonal antisera and monoclonal antibodies to SPFMV CI CI protein and polyclonal antisera to SPFMV CI HC-Pro protein were prepared according to the protocols described below.

3.2.4.1. Polyclonal antisera

New Zealand rabbits were immunized by multiple intradermal injection (Vaitukaitis, 1981), with purified protein (100-200 $\mu\text{g}/500 \mu\text{l}$ PBS), mixed with Freund's complete adjuvant (1:1, v:v). The titers of the antisera were determined by indirect ELISA four weeks after the first immunization and a booster injection [(antigen 100 $\mu\text{g}/500 \mu\text{l}$ PBS) mixed with Freund's incomplete adjuvant] was given immediately after. From then on, bleeds were done every week for four consecutive weeks.

3.2.4.2. Monoclonal antibodies (MAbs)

Balb/c female mice, 6-8 weeks old, were immunized with 4-5 weekly doses of purified CI protein in phosphate buffer (50-100 $\mu\text{g}/100 \mu\text{l}$) mixed with incomplete Freund adjuvant (1:1, v:v) by intraperitoneal injections, followed by an intravenous (tail) injection

with the same amount of antigen without adjuvant. The titres of the antisera were determined 7 weeks after the first injection by modified indirect ELISA, and a booster injection (100 µg/100 µl) was given 2 to 3 days before fusion.

Cell culture, fusion, cloning by limited dilution and early screening of hybridoma cells was basically done according to the method of Galfre and Milstein, 1981, modified by Dr. John Green (School of Biological Sciences, University of Birmingham, UK). Myeloma cells and mice were kindly supplied by Dr. John Green. NSO/u, Balb/c myeloma cells were grown in complete medium [90% serum free medium (SFM, 1% RPMI 1640, 0.2% Na bicarbonate, 0.06% penicillin, 0.01% streptomycin), 10% foetal calf serum (FCS)] with 5% CO₂ and 95% air at 37°C, until the density was about 5 x 10⁵ cells/ml. For fusion, a hyperimmunized Balb/c mouse was killed by cervical dislocation and the spleen was aseptically removed, teased and washed in SFM (10 ml) by centrifugation at 800 rpm for 5 min. Spleen cells were mixed with myeloma cells (1:10, v:v) and centrifuged for 5 min at 650 rpm. The fusion took place in the presence of 50% PEG 1500 (1g/ml RPMI 1640, 800 µl/10⁸ spleen cells) added over 1-2 min period. The mixture was then further diluted with SFM 1 ml was added over a 1 min period, 8 ml added over 2-3 min period and completed to 40 ml. After centrifugation at 650 rpm for 5 min, the supernatant was completely removed and the cells were resuspended in 40 ml of CMHAT [99% CM (90% SFM, 10% FCS), 1% 100X HAT (hypoxanthine, aminopterin and thymidine)]. Cells were dispensed in 0.2 ml aliquots over four 96 well microtitre plates and placed at 37 °C with 5% CO₂ and 95% air. Supernatants (100 µl) were replaced with the same volume CMHAT at days four, seven and ten and with CMHT (99% CM, 1% HT) on days twelve and fifteen. For selection of cell lines producing antibodies, screening was done by testing the supernatants from wells with colonies of 1-2 mm diameter by indirect ELISA or dot blot ELISA. If interesting antibodies were detected, the hybridomas growing in the positive wells were transferred to 1.5 ml CMHT containing 1.5 x 10⁶/ml feeder cells within a 24 well (2ml/well) Limbro plate. As feeder cells, spleen cells from non immunized mice were prepared as for fusion.

For cloning by limiting dilution, cells growing in the 2 ml Limbro wells were counted in a Neubauer haematocytometer and diluted with CMHT containing 1.5×10^6 /ml feeder cells. Dilutions of hybridoma cells were made to provide 100 cells/ml, 10 cells/ml, 1 cell/ml and 0.5 cell/ml. Each dilution was dispensed into 96 wells microtitre plates (0.2 ml/well). Supernatants were assayed when the clones were well grown (10-12 days). Several clones secreting potentially-useful antibodies were grown up, with CMHT, into 25 ml or 150 ml flasks to be frozen down in liquid nitrogen. Between 5×10^6 and 1×10^7 cells were centrifuged for 5 min at 800 rpm and the pellet resuspended in 1.5 ml. freezing solution (90% FCS, 10% DMSO) at 4 °C and placed within a cryogen tube. The cells were left overnight at - 70 °C, and the vials then immediately transferred to storage in liquid nitrogen. For retrieval cells were thawed quickly at 37 °C and then transferred to CM. The cells were centrifuged at 1000 g for 5 min and the pellet resuspended in 2 ml of CMHT.

3.2.4.3. Immunoglobulin (IgG) purification

Antiserum diluted in 1/2 PBS (1:10) was mixed with 50% saturated $(\text{NH}_4)_2\text{SO}_4$. The mixture was incubated for 30 min at room temperature, centrifuged at 12,000 g for 30 min, the pellets resuspended in 1/2 PBS and dialysed against 3 changes of the same buffer for 18-20 h at 4°C. The solution was clarified at 10,000 g for 15 min.

3.2.5. Immunological detection of non-structural proteins

3.2.5.1. Modified indirect ELISA

The protocol followed was a modified double sandwich ELISA of Clark and Adams (1978). Plates were first coated with 150 µl antigen (10-20 µg/ml) diluted in coating buffer. For CI protein detection, samples mixed with 2% SDS were heated at 100°C for 10 min, cooled at room temperature, centrifuged at 3000 rpm for 5 min and supernatants added into the wells. For polyclonal or monoclonal IgG detection, goat anti rabbit (GAR) or rabbit anti mouse (RAM) IgG respectively, conjugated to alkaline phosphatase (0.25-1.0 µl/ml, Bio-Rad) was used. As substrate, p-nitrophenyl phosphate (0.66 mg/ml, Sigma) was added to each well.

3.2.5.2. Dot blot ELISA

The test was based on a procedure described by Berger *et al.* (1984), modified by Nakashima and Fernandez-Northcote (1987); reactions were performed at room temperature. (i) Samples (30-50 μ l) were blotted on nitrocellulose filters [pre-soaked in tris buffer saline (TBS; 20 mM TrisHCl, 150 mM NaCl pH 7.5) for 5-10 min] and left to dry for at least 1 h (or kept between 3 MM Whatman paper for several weeks). (ii) Filters were soaked in blocking solution [TBS, 5% non-fat dried milk (NFM), 0.25 ml/cm²] for 1 h with gentle shaking, rinsed briefly, immediately transferred to diluted antibodies (1-0.25 μ g/ml) in blocking solution (0.2 ml/cm²), and incubated for 16-18 h with gentle agitation (30 rpm/min). (iii) Filters were washed 3 times, 10 min each with TBS-T (TBS, 0.05% Tween 20). (iv) Filters were then incubated with GAR or MAR IgG coupled to alkaline phosphatase (1-0.2 μ g/ml) in blocking solution (0.2 ml/cm²) with gentle shaking for 1 h. (v) Filters were washed as in (iii), except the last one, which was in alkaline substrate buffer [(ASB), 100 mM NaCl, 5mM MgCl₂, 100 mM Tris HCl pH 9.5]. (vi) Filters were incubated in the substrate solution [0.30 μ g NBT, nitroblue tetrazolium (Bio-Rad)/ml ASB and 0.15 μ g BCIP, 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad)/ml MFM], for 10 to 30 min with gentle agitation. (vii) When reactions reached the appropriate colour intensity (strong purple colour for positive control), filters were rinsed several times with distilled water and dried at room temperature.

3.2.5.3. Western blotting

3.2.5.3.1. SDS polyacrylamide gel electrophoresis (SDS PAGE)

Samples were diluted in 2X sample buffer (SB; 125 mM TrisHCl pH 6.8, 4 % SDS, 30% sucrose, 10% 2-ME, 0.004 % bromophenol blue), boiled for 2 min and centrifuged at 5000 g for 3 min. Supernatants (10 μ l/track) were layered on a 0.5-1.0 cm stacking gel formed on top of a 10 or 12.5 % acrylamide:bisacrylamide, 100 x 80 x 5 mm SDS-PAGE discontinuous buffer system (below) of Laemmli (1970) and run at 100 V until the samples had penetrated the stacking gel and then at 200 V for 45 min using a Mini-Protean II unit

(Bio-Rad). Gels were stained for 2-4 h with 0.1% Brilliant blue (Sigma) in 2.5% methanol and 5% acetic acid and destained for 4-6 h with 30% methanol and 10% acetic acid or silver stained by using the silver staining kit (Bio-Rad), according to the manufacturers instructions.

SDS PAGE:	Gel concentration (%)			
	12.5	10.0	7.5	Stacking gel
Stock solution				
Acrylamide:bisacrylamide (30:0.8)	4.2 ml	3.3 ml	2.5 ml	375 μ l
Water	4.0 ml	4.8 ml	5.7 ml	1.5 ml
Resolving gel buffer (3M TrisHCl pH 8.8)	1.25ml	1.25 ml	1.25 ml	
Stacking gel buffer (0.5M TrisHCl pH 6.8)				750 μ l
10% SDS	100 μ l	100 μ l	100 μ l	30 μ l
10% ammonium persulfate	75 μ l	75 μ l	75 μ l	50 μ l
TEMED	5 μ l	5 μ l	5 μ l	3 μ l

3.2.5.3.2. Protein transblotting

Proteins separated by SDS PAGE were transferred to nitrocellulose filters (0.2 μ m pore) according to the method of Towbin *et al.* (1979), by using a semi-dry electroblotting system (Sambrook *et al.*, 1989). After electrophoresis, transfer buffer (TB; 39 mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol) pre-wetted nitrocellulose filter and gel were placed between two sets of TBS pre-wetted 3MM Whatman paper over the cathode-graphite base of the transblotting system. The sandwich was then covered with the anode-lid and run at 0.65 mA/cm² for 2.0 h. To locate the molecular weight markers, filters with transblotted proteins were stained with Ponceau S, prepared according to Sambrook *et al.* (1989). Ponceau S {0.2g, 3-hydroxy-4[2-sulfo-4-(4-sulfophenylazo)-phenylazo]2,7-naphthalene-disulfonic-acid, Sigma} was mixed with trichloroacetic acid (3g, Cl₃CO₂H) and sulfosalicylic acid [3g, HO₃SC₆H₃-2-(OH)-CO₂H.2H₂O] and the volume made up to 10 ml in distilled water. The filter was incubated with gentle agitation for 5-10 min or until protein bands were visible and was then washed with several changes of deionized water.

3.2.5.3.3. Immunological reactions

Immunological reactions in nitrocellulose filters with electroblotted denatured proteins were performed as described above for dot blot ELISA (steps ii to vi).

3.2.5.4. Cytological immunogold labelling

Tissue infiltration, fixation and immunogold labelling was done as described below according to the procedure of Dr. J. W. van Lent, 1991 (personal communication) and van Lent, 1988.

3.2.5.4.1. Tissue infiltration and fixation

Small pieces of fresh tissue (1x1 mm) from systemically infected leaves of *N. benthamiana* plants, 35 days after inoculation, were cut with a razor blade and fixed under vacuum (55 mbar) in 1% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 1.5 mM CaCl₂ in phosphate/citrate buffer (100 mM Na₂HPO₄ and 2.7 mM citric acid), pH 7.2, for 1 h at room temperature. After infiltration, fixation proceeded for 16-18 h at 4^o. The tissue was then washed with 0.2 M Na cacodylate buffer, pH 7.4 (2 times; 10 min each) and SDW (2 times; 5 min each). Samples were dehydrated at -20 °C with a series of EtOH in water: 50% EtOH (30 min), 50% EtOH (30 min), 70% EtOH (30 min), 90% EtOH (60 min) and 100% EtOH (60 min). Samples were gradually infiltrated with LGR:EtOH (1:1, v:v) for 60 min, LGR 100% for 30 min, LGR 100% for 18 h and finally for 1.5 h. Samples were transferred to gelatin capsules with fresh pure resin and left to polymerise under UV light (375 nm) for 18-24 h at -20°C and at room temperature for 2-3 days. Ultrathin sections were cut with glass knives on a Reichert ultramicrotome and mounted on 150 mesh nickel grids covered with formvar and coated with a 3 nm carbon layer.

3.2.5.4.2. Immunogold labelling

(i) The grids with the ultrathin sections were incubated for 1 h at room temperature on 20 µl drops of PBS-1% BSA. (ii) Grids were then slightly dried and transferred to

a drop of 1-2 µg/ml antibodies in PBS-0.5% BSA and incubated for 2-4 h at room temperature. (iii) Grids were washed with 30 drops of PBS. (iv) Grids were incubated for 1 h in 20µl RAM or GAR-Ag (15 nm) conjugate (Biocell), diluted in PBS-0.5% BSA. (v) After washing as in (iii) and then with 20 drops of distilled water, grids were slightly dried and stained with 2% UA for 5-10 min, washed with distilled water and re-stained with lead citrate (Reynolds, 1963)] for 2-5 min, washed again with distilled water and dried. Specimens were examined with a Jeol 100 or Jeol 1200 EX transmission EM.

3.3. Results and Discussion

3.3.1. Cytological studies

3.3.1.1. Time course analysis of SPFMV C1 inclusion bodies by EM

Studies on the type, development and distribution of virus induced inclusion bodies showed that during early stages of infection (6 days) no pinwheel like inclusion bodies were evident in inoculated or systemic leaves of *N. benthamiana* or *I. nil*. Tubular inclusions, in the form of strands or laminar aggregates, perpendicular to the cell wall and in association with the plasmalemma were found in many cells (Figure 3.1a). Subsequently, at later stages in the infection (15 days) many pinwheel were abundant in parenchyma cells of inoculated leaves, some in association with plasmodesmata and endoplasmic reticulum (Figure 3.1b). Laminar aggregates in association with the plasma membrane and few pinwheel aggregates were present in systemically infected leaves. At later stages of infection (30 days), many bundles, short laminar aggregates and pinwheels were dispersed in the cytoplasm of both inoculated and systemically infected leaves. A larger number of pinwheels in association with laminar aggregates were present in the cytoplasm of systemically infected leaves.

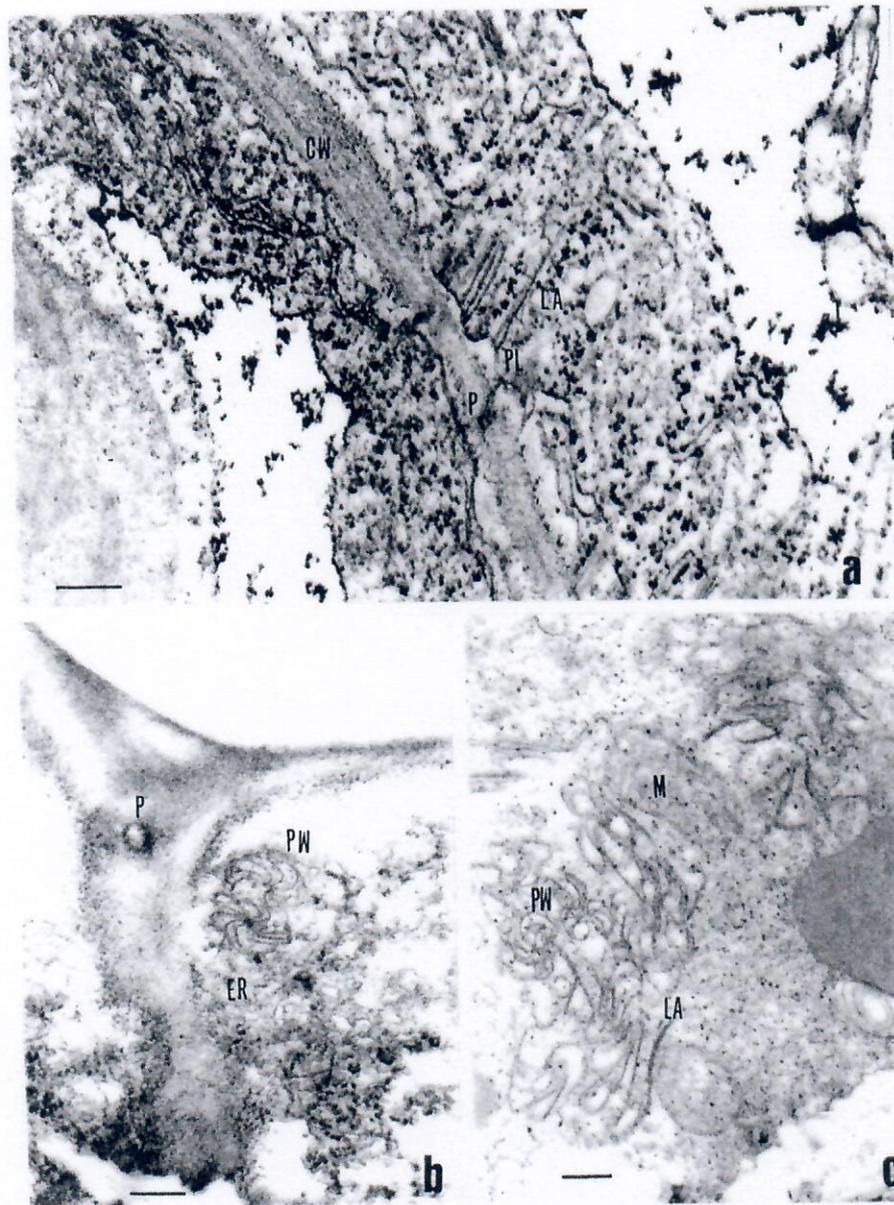


Figure 3.1

Electronmicrographs of *N. benthamiana* mesophyll cells, at various times post-inoculation with SPFMV C1.

Plate (a) 6 days: laminated aggregates (LA) attached to the plasmalemma (PL) in a region adjacent to the plasmodesmata (P), perpendicular to the cell wall (CW).

Plate (b) 15 days: pinwheels (PW) in the cytoplasm, in association with plasmodesmata (P), and with endoplasmic reticulum (ER).

Plate (c) 30 days: abundant CIs: short laminated aggregates (LA), bundles (B) and pinwheels (PW), in association with endoplasmic reticulum (ER), close to mitochondria (M).

Bar represents 250 nm.

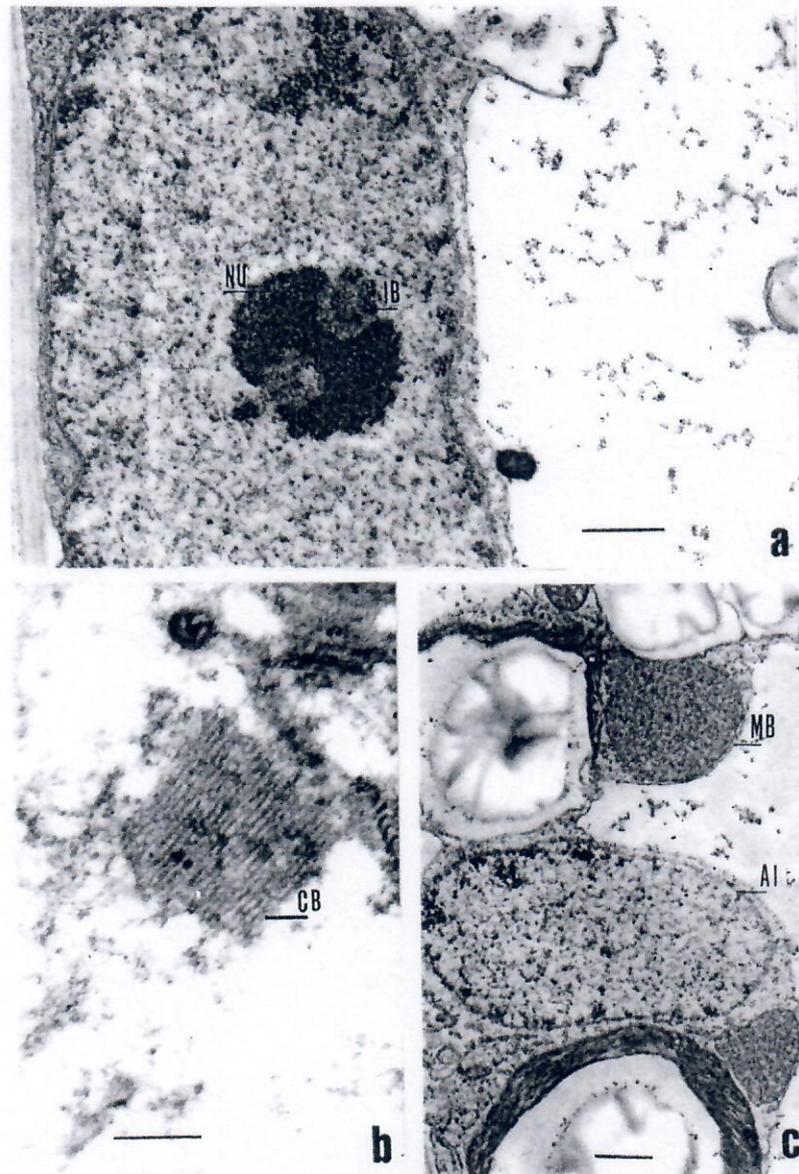


Figure 3.2

Electronmicrographs of *N. benthamiana* mesophyll cells, at 30-40 days post-inoculation with SPFMV C1.

Plate (a), non-crystalline granular inclusion bodies (IB) in the nucleolus (Nu).

Plate (b), Crystal-like inclusion body (CB).

Plate (c), irregular electron dense microbodies (MB) and granular amorphous inclusions (AI).

Bar represents 250 nm.

Abundant finger-like endoplasmic reticulum (not shown) and large vacuoles were found in association with the CI bodies (Figure 3.1c). In addition, some satellite nuclear inclusion bodies were found associated to the nucleolus in systemically infected *N. benthamiana* cells (Figure 3.2a). They are very similar to satellite nuclear inclusions found in cells infected with beet mosaic potyvirus (BeMV) (Martelli and Russo, 1969) and to the nuclear inclusion induced by PVA (Edwardson and Christie, 1983). A few crystalline inclusions (Figure 3.2b) and microbodies (Figure 3.2c) were evident in the cytoplasm of both *N. benthamiana* and *I. nil* parenchyma cells. Tissue from systemic leaves, sampled nearly 2 months after infection showed laminated aggregates, some associated with pinwheels and some loops and circles (apparently formed by dissociation of the pinwheel structure). Cytoplasmic amorphous inclusions, similar to the AI bodies of WMV-1, were also found in some cells (Figure 3.2c). Nuclear inclusion-like structures, similar to the two-, four-, or eight-sided-pyramid crystals, observed in TEV-infected cells were not observed in any of the samples from either *I. nil* or *N. benthamiana* virus infected leaves.

3.3.1.2 Light microscopy of inclusion bodies

SPFMV C1 cytoplasmic CI bodies were very frequently observed in *N. benthamiana* epidermal and guard cells, as large groups of long laminated aggregates and pinwheels, at relatively short distances from the cell wall (Figures 3.3a, 3b and 3c). These types of CI bodies are similar to the cytoplasmic inclusions classified by Edwardson (1974) as subdivision II. At this stage of infection (35 days after inoculation), only a few short laminated inclusions were found associated with the cell wall (3.3a). No other types of inclusion bodies, similar to the ones previously observed by light microscopy of tissue infected with other potyviruses (Christie and Edwardson, 1977; Edwardson, 1974; Bos, 1970; Bos and Rubio-Huertos, 1969; Kassanis, 1939), were identified in the cytoplasm, the nucleus or any other organelle in either infected or non-infected tissue.

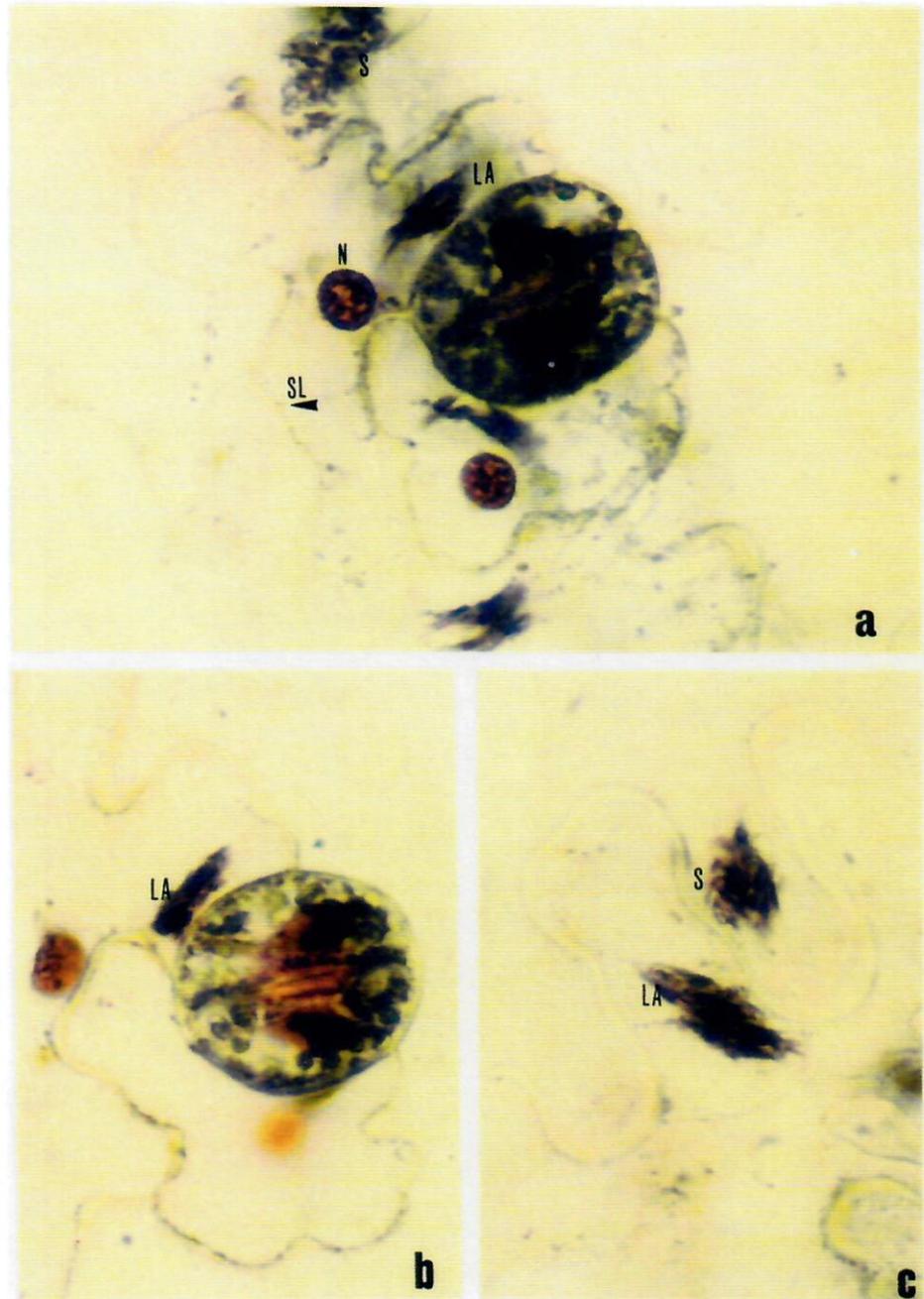


Figure 3.3

Micrographs of *N. benthamiana* tissue, 35 days post-inoculation with SPFMV C1, stained with orange-green (O-G) combination.

Plates (a) and (b), guard and epidermal cells, showing masses of cylindrical inclusion (CI) bodies, in the form of laminated aggregates (LA) and end-on view of scrolls (S). Some short laminated aggregates (SL) are associated with the cell wall (CW). Nucleus stained with the O-G combination. (X 1000).

Plate (c), epidermis cell with a large laminated aggregates (LA) and end-view of scrolls. (X 1000).

These results, from both EM and LM, are in agreement with the observations reported by Lawson *et al.* (1971) on *I. setosa* tissue infected with the RC isolate of SPFMV, in the sense that no crystalline nuclear inclusion bodies, similar to the typical TEV NI pyramid crystals were found in any cells infected with the C1 isolate. It can be suggested, therefore, that NI proteins of this potyvirus apparently do not aggregate in the nucleus of infected cells to form crystalline inclusion bodies. The possibility that other types of inclusions, like the spherical nucleolus inclusion or the microbodies, could be related to the NI proteins requires further study. SPFMV C1 CI bodies were found as laminated aggregates associated with the plasmalemma of both *N. benthamiana* and *I. nil* mesophyll cells at early stages of infection but as large laminated aggregates and pinwheels as the disease progressed; their concentration was relatively high at about 30 days post infection. This stage of infection was, therefore, considered the most appropriate time after inoculation for the purification of the CI protein.

3.3.2. Helper component

3.3.2.1. Purification and antisera production

For the purification of SPFMV C1 HC-Pro protein, saturated ammonium sulphate was used instead of 6% PEG (MW 8000); this procedure yielded a higher concentration of a protein which reacted with PVY HC-Pro antiserum in dot blot ELISA (Figure 3.4). The preparation had a maximum at $A_{280\text{nm}}$ and a minimum at $A_{235\text{nm}}$; when PEG was used preparations with two maximum absorbance peaks at $A_{280\text{nm}}$ and $A_{255\text{nm}}$ and a minimum at $A_{235\text{nm}}$ were obtained (results from 3 purifications, data not shown). Ribosomes or other nucleic acid containing proteins were probably predominantly precipitated in the presence of PEG at the concentration used in this experiment. Sako and Ogata (1981) reported that transmission of TuMV by aphids failed when the HC-Pro protein was purified by PEG but not by ammonium sulphate precipitation. Based on these results, 50% saturated ammonium sulphate was considered more appropriate for SPFMV C1 HC-Pro purification and not PEG MW 6000 as was previously used for PVY HC-Pro and TVMV HC-Pro

purification (Thornbury and Pirone, 1983). In SDS PAGE, a band of ca. 56 kDa was predominant in the preparation; however other bands of lower and higher M_r were observed (not shown). They could be of host origin, or may be products of incomplete denaturation or proteolytic degradation of the HC protein. At the time these experiments were performed, it was not possible to do a more precise identification of these bands by Western blot. In an attempt to prepare an antiserum that could be useful for HC-Pro/AI protein identification by immunogold labelling, aliquots with a high concentration of the ca. 56 kDa protein (assessed by SDS PAGE and spectrophotometry) were used for immunising rabbits (2), according to the protocol described under Materials and Methods. The antisera produced were designated FMV HC-Pro1 and FMV HC-Pro2 respectively.

3.3.2.2. Immunological detection of HC-Pro protein

The specificity of the antisera to SPFMV C1 HC-Pro protein was first evaluated by dot blot ELISA. Serological analysis revealed a similar strong reaction between several dilutions of FMV HC-Pro1 antiserum and sap from both infected and uninfected *N. benthamiana*. The FMV HC-Pro2 produced a strong reaction with sap from infected tissue. A very faint, clear pink coloration was observed with sap from uninfected host tissue (Figure 3.5). No improvement in the specificity of FMV HC-Pro1 antiserum was observed by cross absorption with host proteins (below). This antiserum was considered, therefore not to be appropriate for further work; only FMV HC-Pro2 antiserum was used further.

The size of SPFMV C1 HC-Pro protein and the serological relationship among SPFMV C1, PVY and PRSV-W HC-Pro/AI proteins were assessed in electrophoretically separated, denatured HC-Pro protein from systemically infected *N. benthamiana* with SPFMV C1 (FMV-PPHC). Uninfected host protein was prepared as for FMC-PPHC. Western blotting of the FMV PPHC showed that FMV HC-Pro2 antiserum reacted weakly with a protein of ca. 56 kDa and strongly with a protein of similar size, from a purified PVY^N HC preparation (PVY-PPHC) (Figure 3.6).

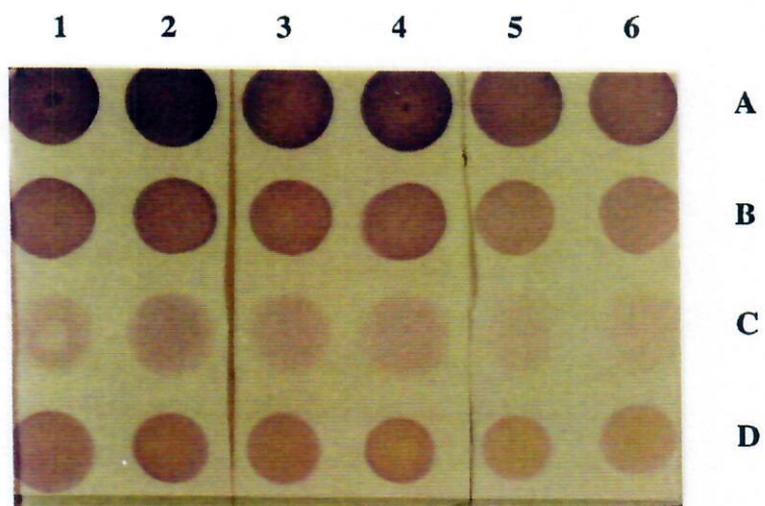
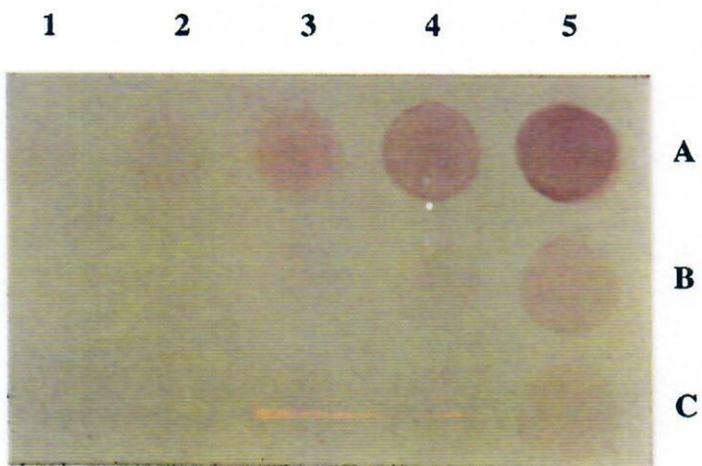
Figure 3.4

Serological determination of SPFMV C1 helper component (HC-Pro) protein purification, in dot blot ELISA. The protein was purified with (A) saturated ammonium sulphate or (B) 6% polyethylene glycol (PEG 8000), 100 mM NaCl. The antigen concentration was adjusted to ca. 10, 20, 50 and 100 ng/ml, (lanes 1 to 5, respectively). Row (C) corresponds to host protein prepared with saturated ammonium sulphate. The protein was probed with a polyclonal antiserum¹ to potato virus Y HC-Pro protein. Conjugate: goat anti rabbit-alkaline phosphatase and substrates: nitroblue tetrazolium and bromochloroindolyl phosphate.

1. Antiserum to PVY HC-Pro from D.W. Thornbury and T.P. Pirone.

Figure 3.5

Dot blot ELISA developed with polyclonal antiserum to SPFMV HC-Pro protein (FMV-HC2). Lanes (1) and (2), antiserum diluted 1:1000; lanes (3) and (4), antiserum diluted 1:2000 and lanes (5) and (6), antiserum diluted 1:4000. The protein samples used to react with the antibodies were as follows. Row (A) SPFMV C1 HC-Pro protein (100 ng/ml). (B) SPFMV C1 systemically infected *N. benthamiana* leaf extract, (C) healthy *N. benthamiana* leaf extract, and (D) SPFMV C1 systemically infected *N. benthamiana* leaf extract (stored at -70 °C). Leaf extract in A, B and C were diluted 1:50 in 20 mM potassium phosphate buffer pH 7.2, 20 mM MgCl₂. Conjugate and substrate, same as in Figure 3.4.



These results could indicate that FMV HC-Pro2 antiserum had more affinity for epitopes conserved in PVY-HC-Pro than in FMV-HC-Pro when denatured for electrophoresis, or alternatively, PVY-HC-Pro protein was present in a higher concentration in the tissue (no previous determination was performed). This antiserum also reacted in Western blots with proteins present in healthy tissue (Figure 3.6, lane 3), and with other proteins present in infected tissue. It was, therefore, not specific for detection of HC-Pro. Reactions of PVY HC-Pro antiserum with a band of ca. 56 kDa present in SPFMV C1 AI protein (FMV-PPAI), in FMV PPHC and in PVY PPHC (very weakly) samples, and the reaction of PRSV- W AI antiserum with a protein band of similar size in FMV PPAI and FMV-PPHC samples (very weak reaction with the first one), confirmed the presence of a protein of ca. 56 kDa related to the HC-Pro/AI protein in SPFMV infected sap (Figure 3.7). Reaction with other protein bands in samples from infected tissue was also evident. The nature of these protein bands was not possible to determine. Cross-absorption of antisera with host protein prepared according to Da Rocha *et al.* (1986) did not reduce significantly the non-specific reactions. However, it is worth mentioning that cross-absorption was done only once; some antisera need to be treated several times for complete removal of non-specific antibodies (Baunoch *et al.*, 1990).

MAbs to PVY/TVMV HC-Pro protein (HC5GB11, HC22E8B10, HC1B8B6, HC5FB8) diluted in TBS (1:1000 and 1:3000) were also used in dot blot ELISA and Western blots to probe SPFMV HC-Pro protein in samples prepared from *N. benthamiana* and *I. nil* infected with SPFMV C1, and from *I. nil* infected with SPFMV C, SPFMV RC and SPFMV YV. Serological analyses (Figure 3.8, Table 3.1) revealed that only the HC-Pro of the C1 isolate of SPFMV reacted strongly with HC 22E8B10 and weakly with HC5G5B11. No detectable reaction was produced between the other two MAbs and SPFMV C1. None of the four MAbs reacted with sap from the RC, C or YV isolates of SPFMV, or with sap from uninfected host tissue. When MAbs were used to probe electrophoretically separated, denatured HC-Pro proteins on nitrocellulose membranes,

Figure 3.6

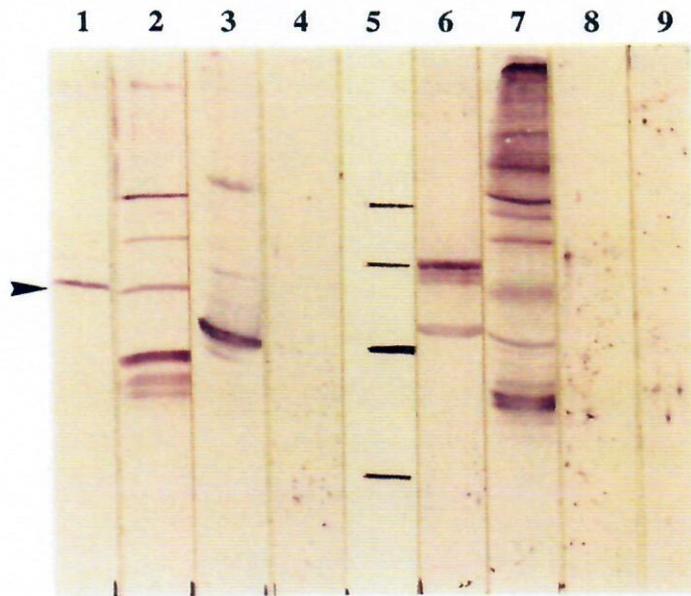
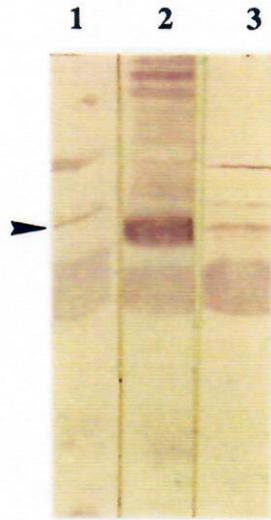
Analysis of SPFMV helper component (HC-Pro) protein reactivity with a polyclonal antiserum to SPFMV C1 HC-Pro protein (FMV HC2). The proteins were probed in Western blots as follows: lane (1), SPFMV C1 HC-Pro, lane (2), PVY HC-Pro and lane (3), healthy host protein. The arrow indicates the ca. 56 kDa protein. Conjugate: goat anti rabbit-alkaline phosphatase and substrates: nitroblue tetrazolium and bromochloroindolyl phosphate.

Figure 3.7

Western blot analysis of the serological relationships of SPFMV C1 helper component (HC-Pro) and amorphous inclusion (AI) proteins to potato virus Y (PVY) HC-Pro and papaya ringspot virus (PRSV-W) AI proteins. SPFMV C1 HC-Pro (lanes 2 and 7), SPFMV AI (lanes 1 and 6) and PVY HC-Pro (lanes 3 and 8) proteins. Proteins from healthy *N. benthamiana* in lanes 4 and 9. The proteins were probed as follows: lanes 1 to 4 with antiserum to PVY HC-Pro¹ (1:1000) and lanes 5 to 9 with antiserum to PRSV-W AI² (1:1000). Lane 5 corresponds to low molecular weight markers. The arrow indicates a ca. 56 kDa protein. Conjugate and substrate as in Figure 3.6.

1 Antiserum to PVY HC-Pro protein from D. W. Thornbury and T.P. Pirone.

2 Antiserum to PRSV-W AI protein from N. Suzuki.



none of the antibodies reacted with SPFMV C1 HC-Pro. The possibility that SDS protein denaturation was affecting the reactivity of the protein with the MAbs was considered. In order to test the effect of SDS denaturation on the reactivity of the protein with the MAbs, different dilutions of SPFMV C1 HC-Pro samples were mixed with 2X SDS-PAGE sample buffer, boiled for 3 min and were probed in dot blot ELISA. A strong reaction was obtained with MAb HC22E8B10 and samples diluted in TSM, but no detectable reaction was obtained with any of the other samples. These results confirmed the hypothesis that HC-Pro protein SDS denaturation affects its reactivity with the MAbs; probably the treatment causes changes in the affinity and/or avidity of the protein or more likely the orientation of the epitopes, and either became non-reactive or the reaction is not detectable.

Virus	Monoclonal antibodies ¹							
	HC 5GB11		HC 5F8		HC 1B8B6		HC 22E8B10	
	1:1000	1:3000	1:1000	1:3000	1:1000	1:3000	1:1000	1:3000
SPFMVC1	+	+/-	-	-	-	-	++++	+++
SPFMV C	-	-	-	-	-	-	-	-
SPFMV RC	-	-	-	-	-	-	-	-
SPYVV	-	-	-	-	-	-	-	-
Control ²	-	-	-	-	-	-	-	-
Control ³	-	-	-	-	-	-	-	-

Table 3.1

Reactivity of MAbs to PVY/TVMV HC-Pro¹ with sap extracts of SPFMV isolate infected plants (1:100 dilution) in dot blot ELISA. - no coloration; +/- faint pinkish; + weak purple; +++ strong purple; ++++ very strong purple.

1. Antiserum to PVY/TVMV HC-Pro protein from Dr. R. Jordan

2. *N. benthamiana*. 3. *I. nil*.

3. 3.2.3. Immunogold labelling of the HC-Pro/AI protein

In situ localization of the HC-Pro/AI protein by immunogold electron microscopy was performed with homologous and heterologous antisera to HC-Pro protein. The

reaction obtained with FMV HC-Pro2 antiserum revealed that labelling was associated with non-crystalline, irregular shaped accumulations of more or less dark-staining material which were found in the cytoplasm, primarily close to the cell wall and near to the chloroplasts. However, labelling was also found in other organelles and in the cytoplasm (Figure 3.9a).

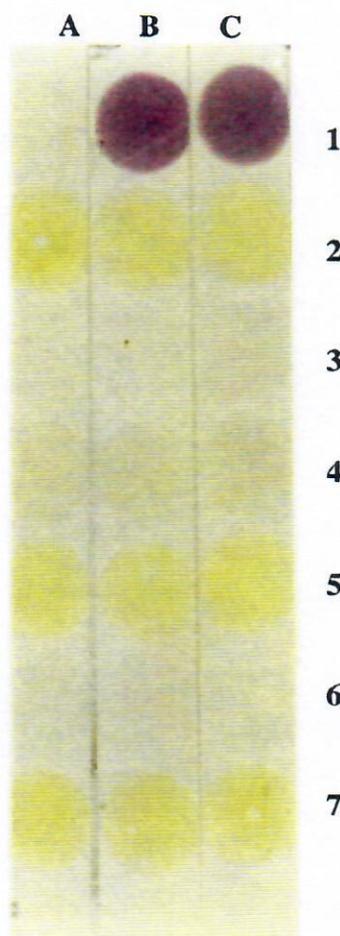
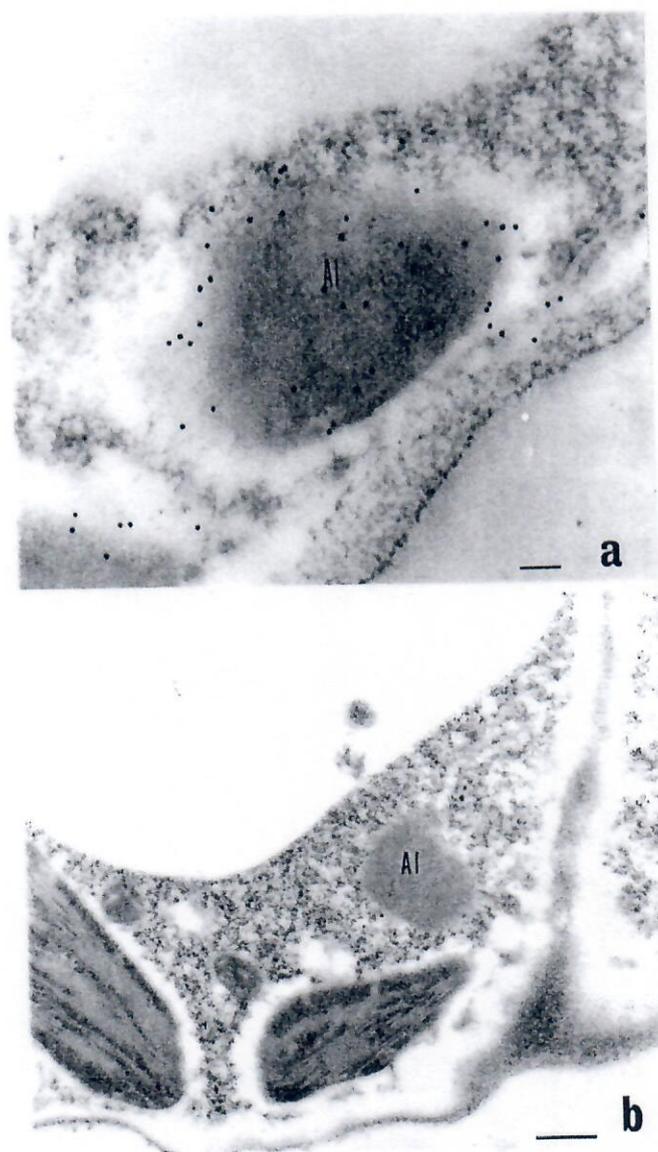


Figure 3.8

Analysis of SPFMV C1, C, RC and SPYVV helper component (HC-Pro), non-denatured and SDS-denatured protein reactivity, probed with a monoclonal antiserum (MAbs HC 22E8B10)¹ to potato virus Y (PVY) and tobacco vein mottle virus (TVMV) HC-Pro protein (1:3000), in dot blot ELISA. The HC-Pro protein in the lanes are as follows. (1) SPFMV C1 from *N. benthamiana*, (2) SPFMV C1, (3) SPFMV C, (4) SPFMV RC and (5) SPYVV from *I. nil*, (6) and (7) proteins of healthy *N. benthamiana* and *I. nil*, respectively. Row (B) and (C) correspond to non-denatured protein samples and row (A) to SDS-denatured protein samples.

¹ Antiserum to PVY/TVMV HC-Pro from R.L, Jordan.

**Figure 3.9a**

Electronmicrograph of *N. benthamiana* infected with SPFMV C1, showing labelling of an electron dense non crystalline "amorphous inclusion" (AI), close to the cell wall. Antiserum to SPFMV C1 HC-Pro (FMV HC2; 1:1000) was used to probe the protein, followed by labelling with goat-anti-rabbit IgG-collodial gold (15 nm). Non-specific labelling of chloroplasts is also evident. Bar represents 250 nm.

Figure 3.9b

Electronmicrograph of *N. benthamiana* infected with SPFMV C1, showing an electron dense non crystalline "amorphous inclusion" (AI). Antiserum to SPFMV C1 CI (1:1000) was used as control to probe the nature of the protein, followed by labelling with goat-anti-rabbit IgG-collodial gold (15 nm). No labelling of the AI was evident. Bar represents 250 nm.

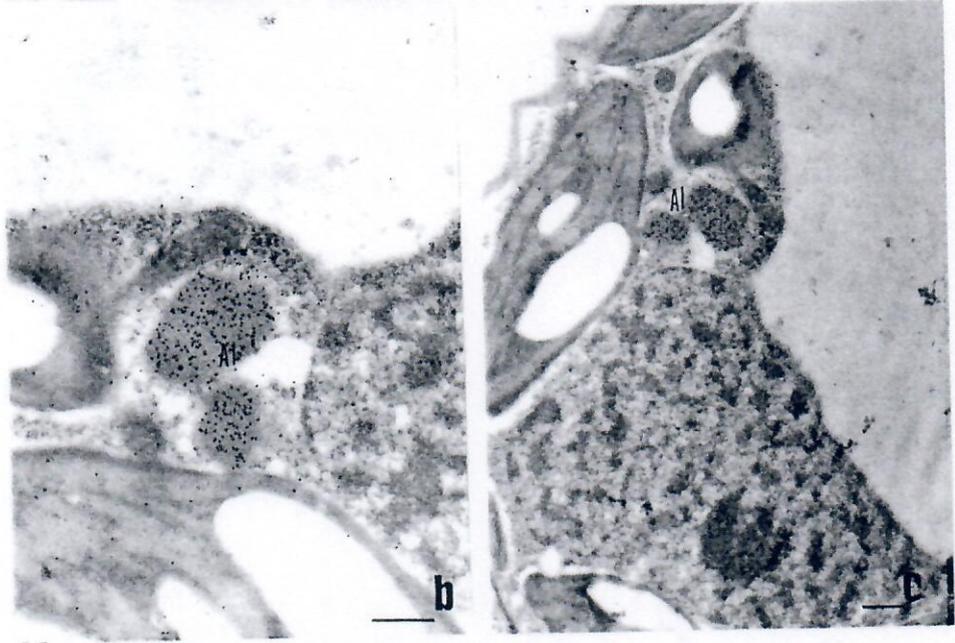
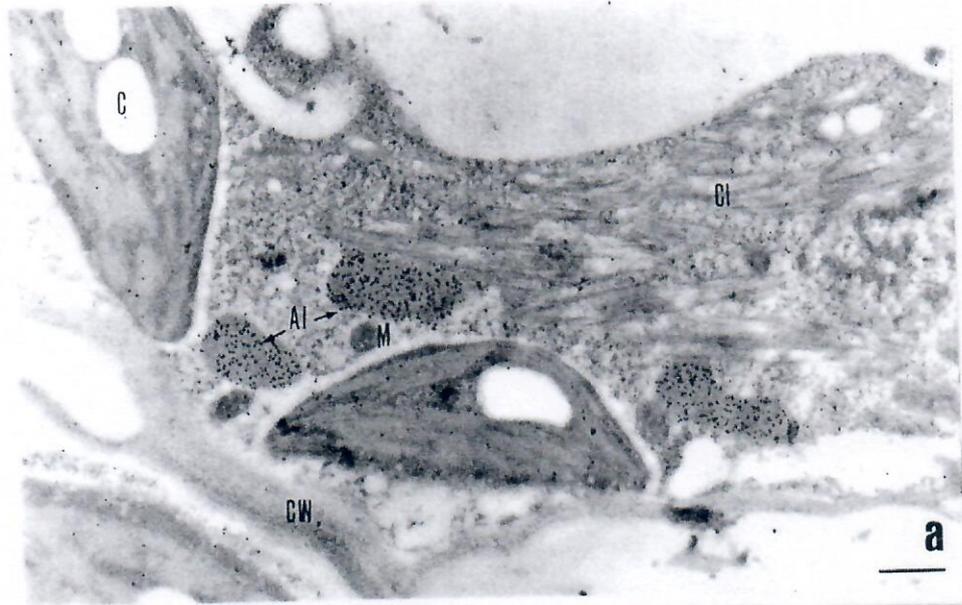


Figure 3.10

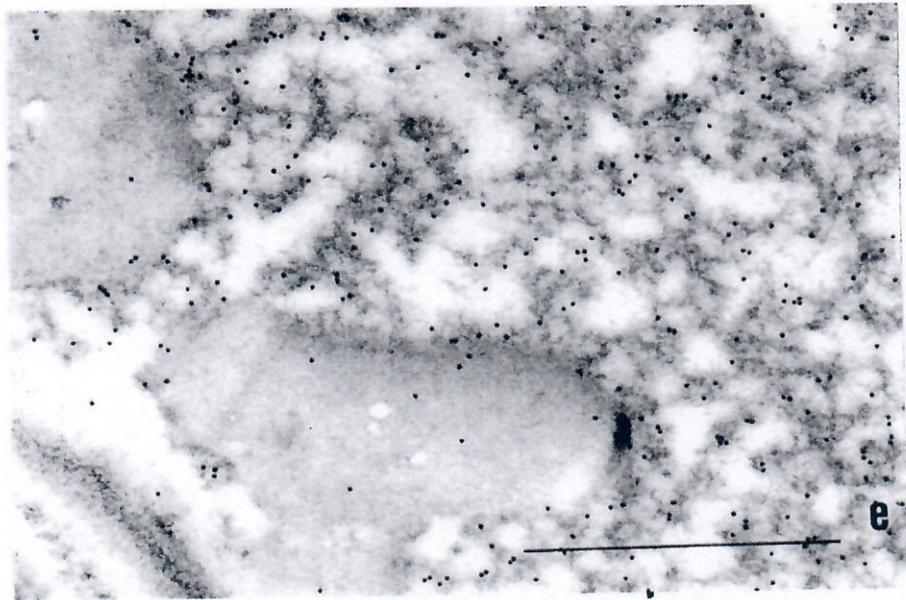
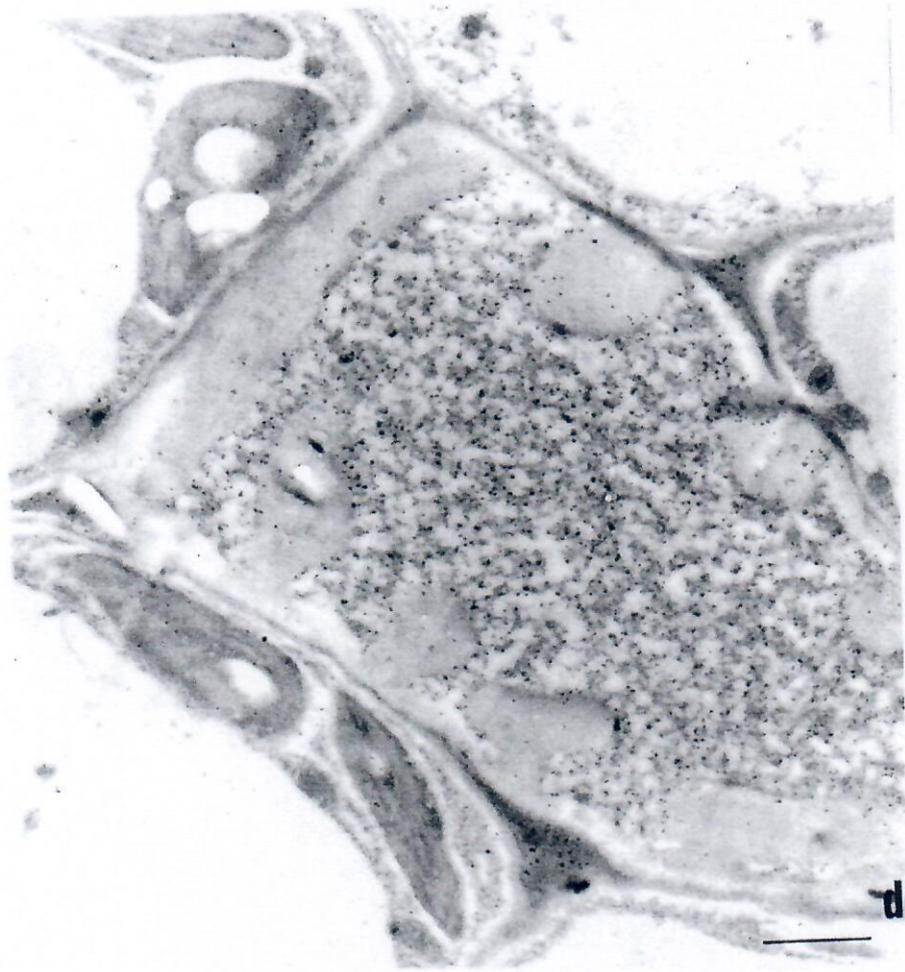
Electronmicrographs of *N. benthamiana* infected with SPFMV C1, showing specific immunogold labelling with antiserum to potato virus Y helper component protein (PVY HC-Pro)¹.

Plate (d) A cell with electron dense material inside an amorphous cytoplasmic irregular inclusion, immunogold labelled with antiserum to PVY HC-Pro¹.

Plate (e) A enlargement of a section in plate (d), showing immunogold labelling of electron dense material with no defined structure.

Bar represents 500 nm.

¹ Antiserum to PVY HC-Pro protein from D. W. Thornbury and T.P. Pirone.



Gold labelling with the PVY HC-Pro polyclonal antiserum was associated only with the dense-non-crystalline bodies (Figures 3.10a,b,c) and in a very few cells with electron-dense irregular cytoplasmic inclusions (Figure 3.10d,e), similar to the inclusion bodies found in *Cucurbita pepo* infected with WMV (Christie and Edwardson, 1977).

Labelling with PVY/TVMV HC-Pro 22E8B10 MAb (Figure 3.11a to e), showed that gold particles were associated with the same type of "amorphous inclusions" labelled with PVY HC-Pro antiserum and the FMV HC-Pro2 antiserum, albeit less specifically in the latter case. Immunogold labelling of the "amorphous inclusions" was not observed in samples treated either with antiserum to SPFMV C1 CI (Figure 3.9b) or to TEV NIab. The morphology of the "amorphous inclusion" bodies observed in this work, which were found to be specifically gold labelled with antisera to HC-Pro protein, is different to most of the amorphous inclusion bodies previously reported for other potyviruses (Christie and Edwardson 1977; Lesemann, 1985). However, it is possible that they could correspond to a different stage in the formation of the irregular cytoplasmic inclusion bodies occasionally found to be gold labelled with PVY HC-Pro antiserum (Figure 3.10d), and to the AI bodies reported for other potyviruses (Martelli and Russo, 1976). Interestingly, similar cytoplasmic inclusions (granular bodies) had been observed in leaf tissue of *N. clevelandii*, *Spinacia oleracea* and *Anthriscus cerefolium*, infected with parnisp yellow fleck virus (Fasseas *et al.*, 1989). This virus has isometric particles and on the basis of its genome structure resembles the picornaviruses (Hemida and Murrant, 1989).

3.3.3.4. Cylindrical inclusions

3.3.3.4.1. Purification and antisera production

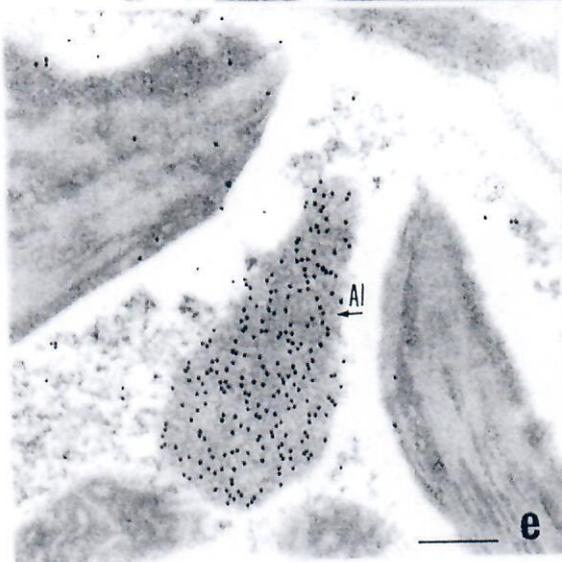
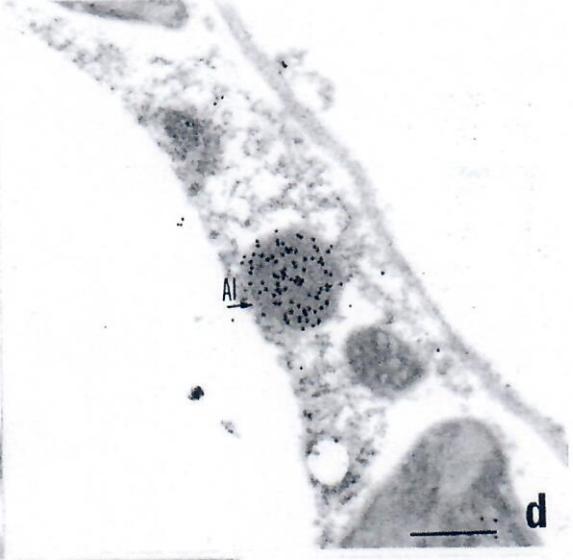
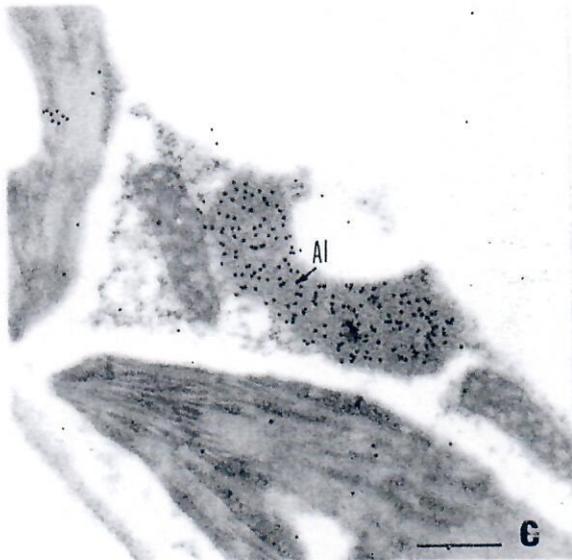
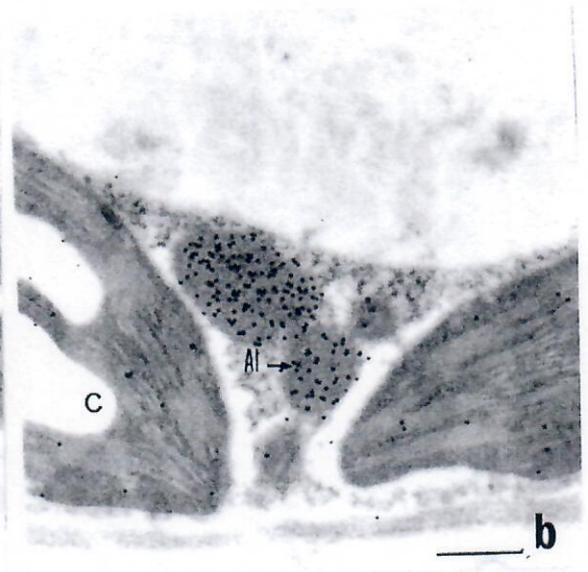
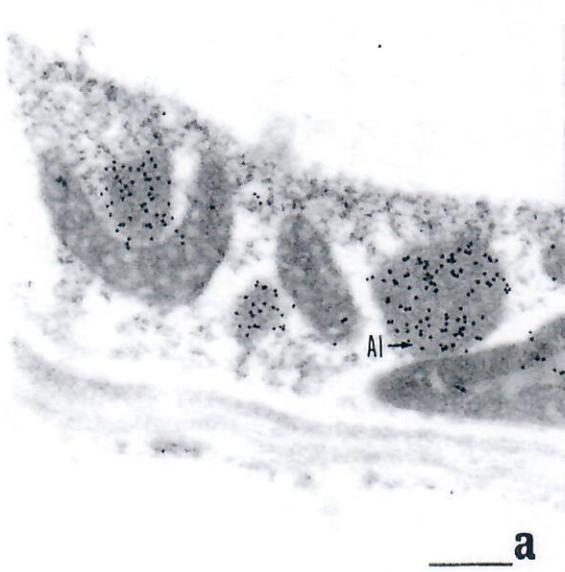
Several attempts to purify CI bodies from *N. benthamiana* or *I. nil* by the protocol described by Hiebert *et al.* (1984a), gave preparations which were highly contaminated with polypeptides of lower M_r than ca. 70 kDa, and also provided a low concentration of CI proteins.

Figure 3.11

Electronmicrographs of *N. benthamiana* infected with SPFMV C1, showing specific immunogold labelling with a monoclonal antiserum to potato virus Y (PVY) and tobacco vein mottle virus (TVMV) helper component (HC-Pro) proteins (MAbs 22E8B10, PVY/TVMV HC-Pro)¹ of several electron dense "amorphous inclusions" (AI). Plates a to f; show several AI bodies close to chloroplast(s) (C) and mitochondria(s) (M) in highly vacuolated cells.

Bar represents 250 nm.

1. MAbs 22E8B10 from R. Jordan.



Purification of CI bodies by the method described under Materials and Methods yielded preparations with a high concentration of the ca. 70 kDa protein, and containing two polypeptides of ca. 55 and 60 kDa. A second cycle of differential centrifugation through a 40% sucrose cushion in the purification protocol improved the quality of the final preparation. Although the relative concentration of the ca. 70 kDa protein was very low, polypeptides of lower M_r were apparently removed (Figure 3.12). The preparations had a maximum absorbance at $A_{275-278 \text{ nm}}$ and a minimum at $A_{250-256 \text{ nm}}$ and a $A_{280/260 \text{ nm}}$ ratio of 1.4-1.43. Electron microscopy of negatively stained CI protein preparations revealed the presence of typical tubular aggregates (not shown), similar to purified CI bodies obtained from other potyvirus-infected plants (Hiebert *et al.*, 1984b; Hiebert and McDonald., 1973). Yields ranged from 1 to 4 mg/100 g tissue (data from 8 preparations).

When polyclonal antiserum produced using aliquots of the CI protein purified as described above as immunogen was analyzed in Western blots, a strong specific reaction with the CI protein was observed; reaction with lower M_r proteins was also evident. However, antiserum produced by immunizing a rabbit with the polyacrylamide gel band containing the ca. 70 kDa protein preparation, as described under Material and Methods, reacted in Western blots with a single protein of ca. 70 kDa (Figure 3.13).

Monoclonal antibody production was not completed due to logistical limitations. However, from 2 fusions, 2% and 10% out of 387 wells into which the products of the fusions were plated contained growing hybridoma colonies. In the initial screening, 26 cell lines producing antibodies which did not show reactivity with host proteins, but reacted weakly with CI protein, were selected. Twenty CI-reactive hybridomas did not remain stable through propagation and cryostorage. Out of the six cell line supernatants, five (FCI 2.1, 2.2, 2.5, 3.9 and 3.32) produced antibodies reactive with CI preparations in dot blot ELISA (data not shown). These cell lines have been frozen down and stored in liquid nitrogen for further work.

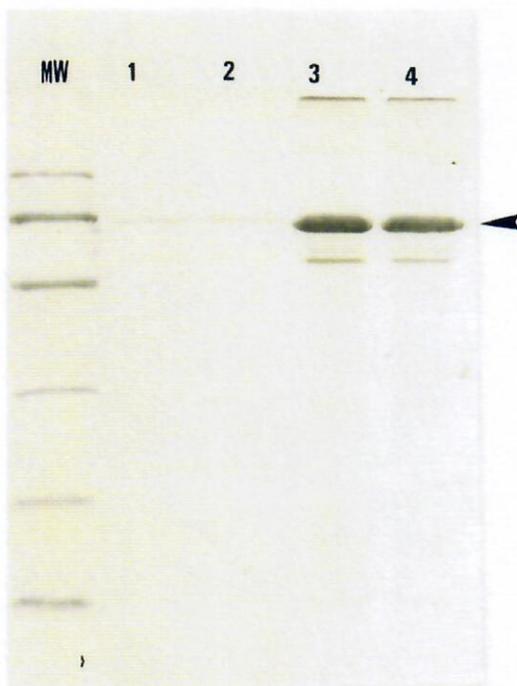
Figure 3.12

Analysis of SPFMV C1 cylindrical inclusion (CI) protein purification by SDS polyacrylamide gel electrophoresis. Lanes 2 and 3 correspond to the CI protein purified with two cycles of differential centrifugation. Lanes 4 and 5, CI protein purified with one cycle of differential centrifugation. Lane 1, low molecular weight markers. The arrow indicates a protein of ca. 70 kDa corresponding to the CI protein.

Figure 3.13

Western blot analysis of the serological relationships of SPFMV C1 cylindrical inclusion (CI) protein. After electrophoretic protein transfer to nitrocellulose membranes, the proteins were probed with antisera to (1) SPFMV C1 CI, (2) TEV CI¹, (3) PRSV-W CI², (4) WMV-2 CI and (5) ZYMV CI proteins. No reaction was detected when the protein was probed with pre-immune antiserum. The arrow indicates the protein of ca. 70 kDa.

1. TEV CI antiserum from W. Dougherty.
2. PRSV-W, WMV-2 and ZYMV CI antisera from N. Suzuki.



3.3.3.4.2. Immunological detection of CI protein

Polyclonal antisera to four virus (TEV, PRSV-W, WMV-2 and ZYMV) CIs were also used to probe denatured SPFMV CI protein in Western blots. Homologous and heterologous reactions were observed with the ca. 70 kDa protein. Slight differences in color intensity was observed with SPFMV CI, WMV-2 and ZYMV antisera, but all them reacted only with the CI protein band. A stronger reaction was observed between the ca. 70 kDa protein and TEV CI and PRSV-W CI antisera than with the other antisera. However, minor reactions with bands migrating slower or faster than the major CI protein band were also evident (Figure 3.13). These results indicated that the CI proteins of all the potyviruses tested were serologically related.

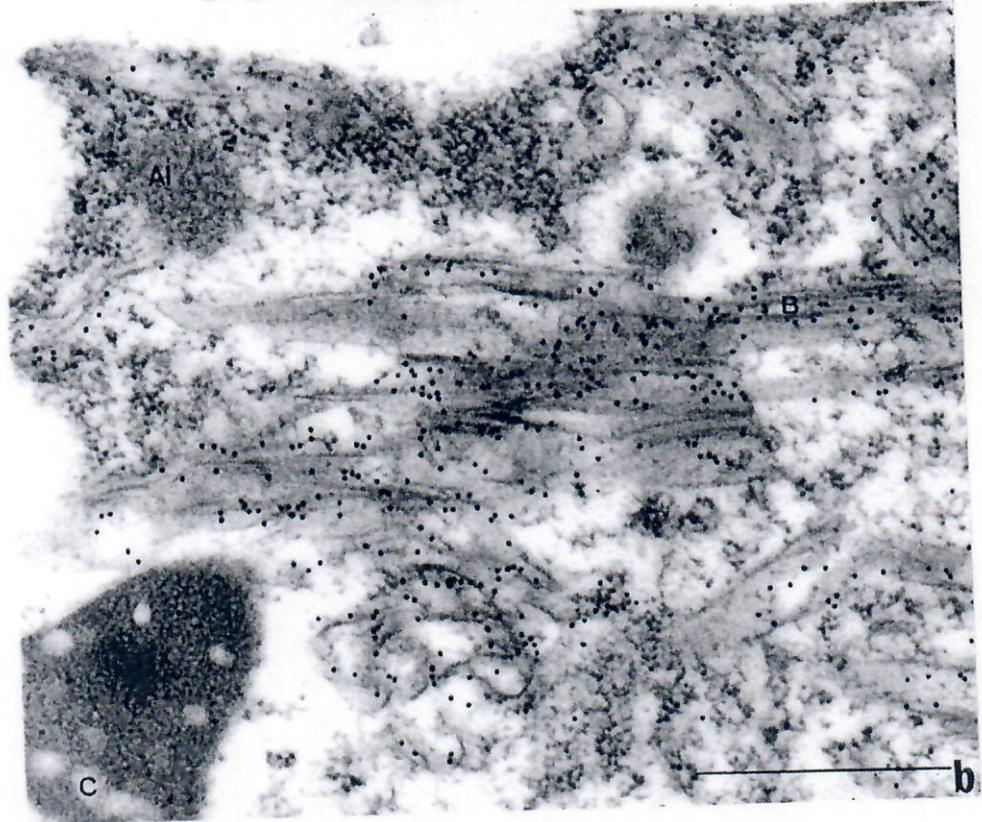
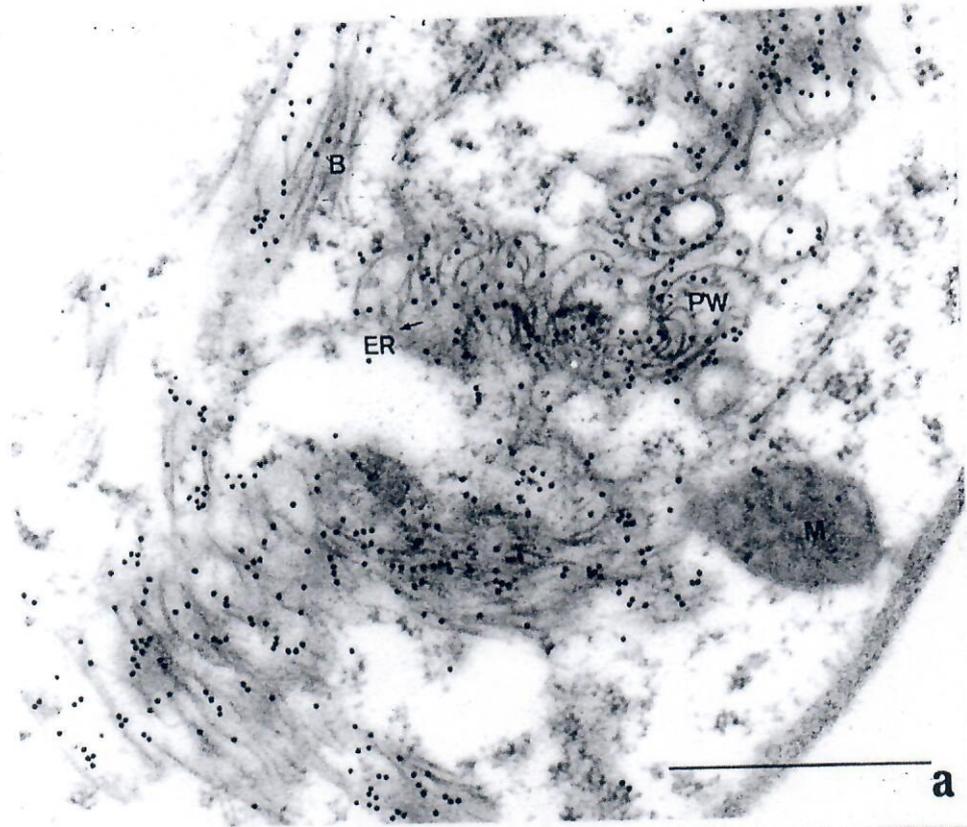
3.3.3.4.3. Immunogold labelling of the CI protein

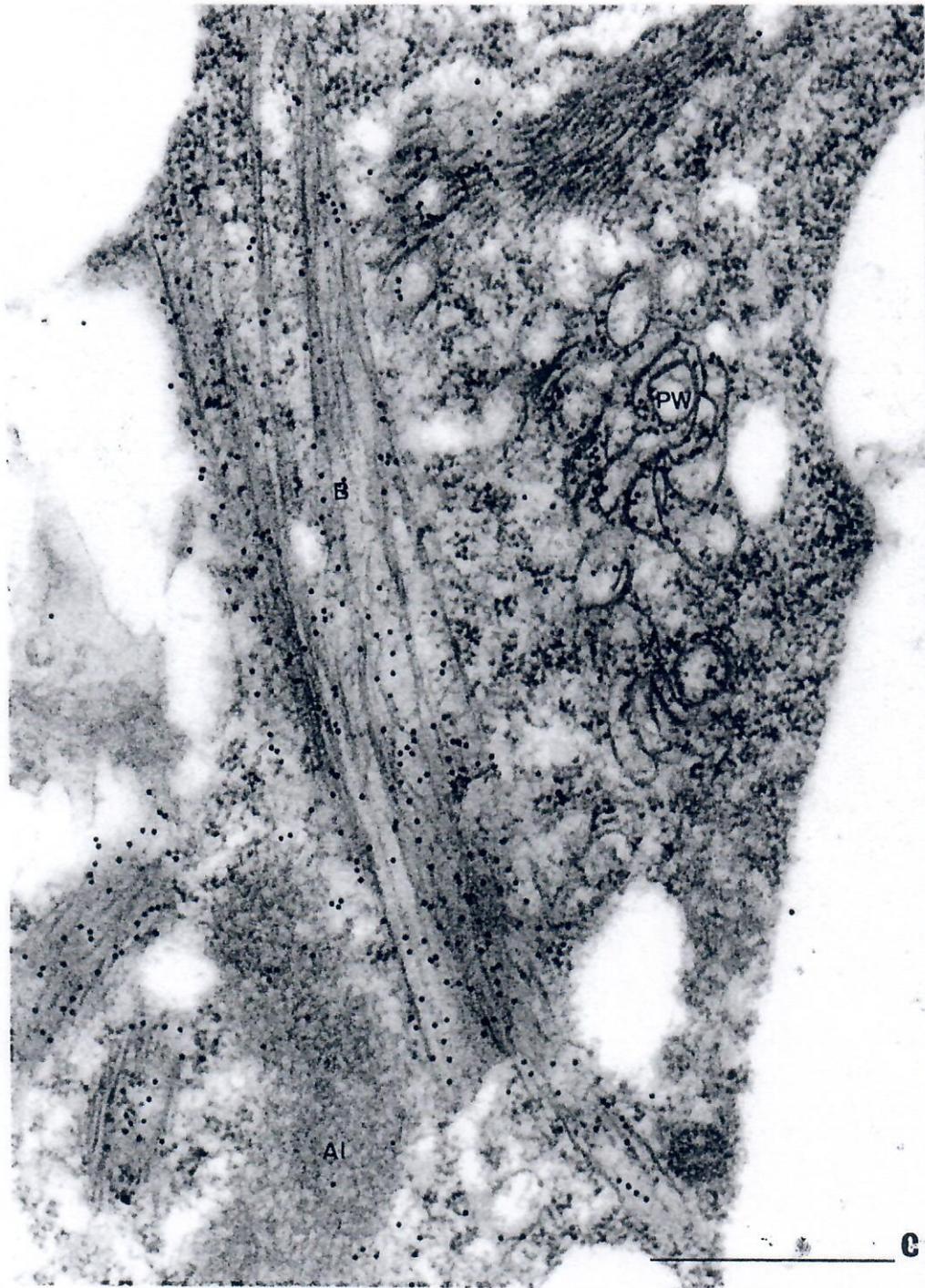
The antiserum produced to the CI protein of SPFMV CI reacts specifically with pinwheels and laminated aggregates inside the cytoplasm of infected *N. benthamiana* cells (Figure 3.14 a,b,c.). None of the organelles contained proteins reacting with this antiserum, confirming that the the CI protein is not transported to other sites of the cell and the antiserum is specific to this virus coded protein. No gold labelling of CI proteins were detected when sections were exposed to PVY HC-Pro antiserum (Figure 3.10c), confirming the nature of the laminated aggregates and pinwheels.

The antiserum to SPFMV CI protein is potentially useful, therefore, both for diagnostic purposes and monitoring CI production in infected cells and tissues.

Figure 3.14

Electronmicrographs of *N. benthamiana* infected with SPFMV CI, showing specific immunogold labelling with antiserum to SPFMV CI CI protein (1:1000). Plates a, b and c, show different views of a large number of CIs: bundles (B) and pinwheels (PW) in the cytoplasm. Some of the arms of the CI bodies are in close association (arrow) with endoplasmic reticulum (ER). A large non labelled "amorphous inclusion" (AI) body is in the cytoplasm, close to the CI bodies. No labelling of chloroplasts (C) or mitochondria (M) over the normal background are noticeable. Bar represents 500 nm.





Chapter 4

**Expression of the SPFMV C1 genome transferred into
N. benthamiana and *I. batatas* protoplasts by electroporation**

4. Summary

Protoplasts from *Ipomoea batatas* L. Lam. mesophyll cells and cell suspension cultures, and from *Nicotiana benthamiana* L. Domin. mesophyll cells, were isolated and infected by electroporation with sweet potato feathery mottle potyvirus (SPFMV) C1 and SPFMV C1 RNA. Up to 80% of protoplasts in mannitol (0.5 M for *I. batatas* and 0.6 M for *N. benthamiana*) containing 7 mM CaCl₂, 5 mM 4-(N-morpholine)ethanesulfonic acid were infected with viral RNA (20 µg/ml) when 2 pulses of 50 µs at 0.8 V/cm field strength were applied. Direct observation of electroporated protoplasts labelled with antisera to the coat protein (CP) and to the non-structural cylindrical inclusion (CI) protein of SPFMV C1 showed that protoplasts were infected and supported virus accumulation and production of CI protein. Analysis by indirect F(ab')₂ enzyme-linked-immunosorbent assay indicated newly synthesized SPFMV C1 CP accumulation after inoculation with viral RNA. Electroporated protoplasts from both "resistant" (S24 and Nemanete) and susceptible (Jewel and Georgia Red) sweet potato genotypes supported similar levels of infection of SPFMV C1 CP, as assessed by immunostaining.

4.1. Introduction

Protoplasts have played an important part in plant virus research since Cocking (1960) first presented a method for their isolation by enzymatic means and Takebe *et al.* (1968) overcame the difficulties in preparing protoplasts from leaf mesophyll and showed substantial virus replication following infection with TMV RNA (Aoki and Takebe, 1969) and TMV (Takebe and Otsuki, 1969). These discoveries allowed for the first time plant virology studies to be performed in separated cells under controlled and synchronized conditions. Protoplast systems have now been established for many viruses, and studies are progressing to elucidate how viruses function in separated cells and how these independent cells respond to virus infection. Actively replicating viral RNA species and translation products from viral genomes in infected protoplasts have been analyzed, and in addition, translation products in protoplasts have been compared to those produced in intact tissue systems [eg. TMV (Blum *et al.*, 1989; Beier *et al.*, 1984a,b; Watanabe *et al.*, 1984); cucumber mosaic cucumovirus (CMV, Wood *et al.*, 1980); TRV (Jobling, 1985); TVMV (Xu *et al.*, 1984a); bromo mosaic virus (BMV, Kiberstis *et al.*, 1981); tobacco rattle virus (TRV, Kubo *et al.*, 1975); barley yellow dwarf luteovirus (BYDV, Young *et al.*, 1989) and tobacco necrotic dwarf luteovirus (TNDV, Kubo and Takagami, 1979)].

Molecular biological analysis of viral RNAs and proteins may help to determine the mechanisms of virus behaviour in living cells and lead us to understand what happens in tissues or whole plants after virus entry (Watanabe *et al.*, 1992; Matsunaga *et al.*, 1992; Angenent *et al.*, 1989; Roenhorst *et al.*, 1988; Watts *et al.*, 1987; Motoyoshi, 1985; Saunder & Mertes, 1984; Leonard and Zaitlin, 1982). The great potential of protoplasts for studies of resistance mechanisms and generation of transgenic plants bearing specific virus genes has been recognized and systems are being developed for that purpose (Smith

et al., 1992; Osbourn *et al.*, 1989; Jones and Jones, 1989; Register and Beachy, 1988). The rapid development of techniques for protoplast micro-manipulation, transformation, fusion and plant regeneration is allowing us to obtain, in a shorter time, information to study virus pathogenesis and to develop genotypes with specific qualities to resist virus entry, multiplication and/or expression.

Introduction of functional RNA molecules into plant cells provides a valuable means for studying the performance of mRNAs *in vivo*. This is particularly true for mRNA with self replicating ability, such as plus-strand virus RNA. Since the first report by Aoki and Takebe (1969), various procedures have been reported for introducing viral RNAs into plant protoplasts. Cationic polymers such as poly-L-ornithine (PLO, e.g. Jarvis and Murakishi, 1980; Maule *et al.*, 1979a; Motoyoshi *et al.*, 1973), fusogenic polymers such as polyethyleneimine (Polymin P, e.g. Kikkawa *et al.*, 1982) or polyethylene glycol (PEG, e.g. Restrepo *et al.*, 1990; Jones and Jackson, 1990; Barker and Harrison, 1984; Maule *et al.*, 1979b), liposomes (e.g. Felgner and Ringold, 1989; Xu *et al.*, 1984b; Watanabe *et al.*, 1984, 1982; Rollo and Hull, 1982; Fukunaga *et al.*, 1981), mild sonication (e.g. Joersbo and Brunstedt, 1990) and, more recently, electroporation have all been used. In the last decade electroporation or electrotransfection, as it is sometimes called when used in reference to plant protoplasts (Hibi *et al.*, 1986), has become a method of choice for specific, rapid and reliable transfer of plant virus nucleic acids [e.g. Watanabe *et al.*, 1992; Matsunaga *et al.*, 1992; Murphy *et al.*, 1990; Uehara *et al.*, 1990; Furfine and Wang, 1990; Saunders *et al.*, 1989; Paje-Manalo and Lommel, 1989; Gibb *et al.*, 1989; Register and Beachy, 1988; Diaz *et al.*, 1987; Luciano *et al.*, 1987; Watts *et al.*, 1987; Hibi *et al.*, 1986; Nishiguchi *et al.*, 1986; Shillito *et al.*, 1985; Beier *et al.*, 1984a,b), virus particles (Register and Beachy, 1988; Okada *et al.*, 1986) or for DNA (e.g. Fromm *et al.*, 1985; Shillito *et al.*, 1985; Smith *et al.*, 1992).

Electroporation is a phenomenon in which the membrane of a cell by exposure to high intensity electric field pulses, can be temporarily destabilized in specific regions. During this period, the membrane is highly permeable to exogenous molecules present in the surrounding media. By late 1970, it was found that an externally applied electric field can cause a dielectric breakdown of the membrane (Zimmermann *et al.*, 1974) and induce "membrane-pore" formation (Kinosita and Tsong, 1977). At the same time, it was found that if the electrical field was applied as a very short duration pulse, the cells could recover from the electrical treatment. This implies that these electric field-mediated "pores" were resealable and could be induced without permanent damage to the cells (Zimmermann *et al.*, 1980). Although the molecular mechanisms by which the electric field interacts with the cell membrane are still under investigation, the basis of the phenomenon is reasonably well known (Chang *et al.*, 1992, Hofmann, 1989; Hofmann and Evans, 1986, Zimmermann *et al.*, 1974). The electric field method has a number of advantages over the conventional methods of cell permeabilization. It is a noninvasive, nonchemical method that does not seem to alter the biological structure or function of the target cells and is highly efficient for delivering mRNAs into protoplasts (Gallie *et al.*, 1989).

An understanding of the underlying mechanisms by which plants resist viruses has become increasingly possible with the development of protoplast systems (e.g. Zheng and Edwards, 1990; Chen *et al.*, 1990; Hirai and Amemiya, 1990; Sulzinski and Zaitlin, 1981). The ability to infect protoplasts with viruses has revealed several situations in which a given virus will multiply in protoplasts from certain plant species that are apparently not susceptible to that virus. Beier *et al.* (1980, 1979) reported a survey of more than 100 cowpea lines for resistance to CPMV. More than 50 of these lines were operationally immune but were sources of protoplasts that supported a CPMV increase equal to that achieved in protoplasts from susceptible cowpeas. Line Arlington was the only source of

resistant protoplasts. This has been taken to indicate that individual cells can be perfectly able to support viral replication but that the virus is unable to move to adjacent cells. Protoplasts from tomatoes with the Tm-2 gene for resistance to TMV support the replication of TMV, suggesting that resistance is manifested at the level of cell-to-cell spread (Motoyoshi and Oshima, 1975). However, tomato protoplasts with the Tm-1 gene do not support TMV replication, indicating that this resistance mechanism operates at an earlier stage (Motoyoshi and Oshima, 1977). Electroporated tobacco protoplasts from cultivars Tennessee 86 (Tn 86) and Kentucky 14 (Ky 14) which are at plant level, resistant and susceptible to TVMV respectively, were infected with viral RNA and supported the accumulation of TVMV, although the final amount of virus accumulated was lower in the Tn 86 protoplasts. These results suggested that resistance in tobacco Tn 86 was primarily due to restricted cell-to-cell virus movement. However, the triggering of "induced immunity" by TVMV in Tn 86, that would stop the movement of the virus from the initially infected cells, has also been postulated (Gibb *et al.*, 1989). Similarly, protoplasts from several potato clones possessing major genes controlling necrotic local lesion formation, and minor genes for resistance to infection with PVY, were infected with PVY⁰ RNA as protoplasts from susceptible potato cultivars, suggesting that resistance is analogous to TMV resistance in tomatoes possessing the Tm-2 gene. However, very few protoplasts from cultivars Corina and Pirola, which possess gene Ry for extreme resistance, became infected. This reaction resembles that of tomato with gene Tm-1 to TMV (Barker and Harrison, 1984). Protoplasts of a wild potato, *Solanum brevidens* Phil. which is resistant to PVY, PVX and potato leaf roll luteovirus, yielding a low virus titre in systemically infected plants, were as susceptible to PVX and PVY as protoplasts from a susceptible *S. tuberosum* genotype. However, preparation of protoplasts from leaves of both *Solanum* spp., inoculated 2 to 5 weeks earlier, showed that the percentage of PVX- and PVY-infected cells of *S. tuberosum* were, respectively 45- to 100-fold and about 1000-fold

greater than the percentage of infected cells of *S. brevidens*. The results suggested that resistance to both PVX and PVY could be associated with slow cell-to-cell spread rather than with slow virus replication (Valkonen *et al.*, 1991).

Even though virus movement is only one aspect of the virus-host interaction that contributes to disease, the significance of this interaction for understanding virus pathogenesis and plant functions involved in cell-to-cell communication is tremendous. A better understanding of plant virus movement proteins and the mechanism by which they function may eventually lead to the development of virus-resistant plants (Fraser, 1990). Mutant movement proteins expressed in transgenic plants may be capable of blocking the function of the wild-type movement protein produced during virus infection.

It is now widely accepted that plant viruses circumvent the cell wall by exploiting plasmodesmata and most of them if not all encode a gene (or genes) for the cell-to-cell movement function (Deom *et al.*, 1992; Melcher, 1990). The rate of transport in the infected tissue appears to be enhanced by the increase in number or size or of structural alterations of plasmodesmata. The small size exclusion limits of plasmodesmata (3 nm; Terry and Robards, 1987) relative to the large size of plant viruses (10-110 nm) or free viral nucleic acid (average of 10 nm for a irregular spheroid; Gibbs, 1976) indicate that plasmodesmata must be modified during virus infection for virus progeny to move from cell-to-cell. In the case of TMV, it has been shown that the 30 kDa protein, the most studied movement-protein, is involved in this function (Meshi *et al.*, 1987; Deom *et al.*, 1987). Although the 30 kDa protein has been reported to accumulate in nuclei of infected protoplasts (Watanabe *et al.*, 1986), most evidence suggests that in intact leaves the protein is associated with the cell wall (Moser *et al.*, 1988) and in substantially higher concentration than that found in protoplasts (Watanabe *et al.*, 1984). The discrepancy between 30 kDa protein production and/or accumulation in protoplasts and intact cells

might be caused by stable deposition of 30 kDa protein found in the cell wall in intact cells. The export of the protein out of the cytoplasm may cause it to be synthesized for longer periods of time in intact cells than in protoplasts.

That viruses cause subliminal infections in some plants yet can infect others systemically would point to specificity in the interaction between movement protein and host factors. The mechanism by which this viral protein operates is, at present, unknown. It has been shown that this protein has the ability to modify the exclusion limit size of the plasmodesmata (Deom, 1991; Wolf *et al.*, 1989) and also has the capacity to bind single stranded nucleic acids (Citovsky *et al.*, 1990). However, the details of the movement protein-plasmodesmata interaction are missing. No information is available about how, or in what form, the movement protein reaches the plasmodesmata, and how this protein increases the molecular size exclusion limit of plasmodesmata. Preliminary analysis by electron microscopy of the movement protein in tobacco plants has failed to identify substructural differences in plasmodesmata, suggesting that TMV 30 kDa protein induces a minor change not readily detectable by electron microscopy (Wolf *et al.*, 1989). Dorokhov (1983, 1980) observed that tobacco cells infected with TMV, contained new types of virus ribonucleoprotein particles (vRNP) differing from mature virus in structure and buoyant density and composed of genomic and subgenomic TMV RNAs and TMV-specific and host-cell proteins. The cytological observations on TMV and AIMV (Stussi-Garaud *et al.*, 1987; Tomenius *et al.*, 1987) supported those observations, since infection is observed in new cells before significant numbers of assembled virus particles can be identified in the first infected cells. Furthermore, particles of these viruses are rarely, if ever, seen in plasmodesmata. This form of movement is also compatible with observations on the temporal expression of the movement protein.

Studies with AIMV have shown that all three of its RNAs and the subgenomic RNA will affect host specificity (Schoelz, 1990). The RNA 3 is a dicistronic messenger coding for the 32-kDa protein, a movement protein (Barker *et al.*, 1983). The 3' proximal ORF of RNA 3, encoding the CP, is expressed via a subgenomic RNA 4 (van Pelt-Heerschap *et al.*, 1987). Using antiserum raised against a C-terminal oligopeptide of the 32 kDa product, this protein was found transiently in a crude membrane fraction from infected tobacco leaves. Subsequently, it accumulated in a cell wall fraction where it remained throughout infection (Godefroy-Colburn *et al.*, 1986). Immunogold labelling of AIMV infected leaves revealed that the 32 kDa protein was in the middle lamella of walls of those cells that had just been reached by the infection front and in which virus multiplication had just begun. The protein was not found in cells (or cell walls) where the virus infection was well established (Godefroy-Colburn *et al.*, 1986). Thus, the 32 kDa protein of AIMV seems to be located only transiently in the cell wall and to be degraded or removed after the virus has passed to adjacent cells (Stussi-Garaud *et al.*, 1987).

Experimental evidence suggests that CPMV employs a mechanism different from that of TMV for movement of virus progeny from cell to cell. By mutational analysis, the 58 and 48 kDa proteins have been found to be essential for movement of virus progeny (Wellink and van Kammen, 1989). The CPMV CPs are also required for cell-to-cell movement. Experimental evidence suggests that intact CPMV particles move along tubular structures that assemble in the cytoplasm and subsequently associate with and extend through plasmodesmata. Immunofluorescence and immunogold labelling localizes the 50 kDa and 48 kDa proteins as well as the CPMV CPs to the tubular structures (van Lent *et al.*, 1988). The requirement of the CPs for movement and the presence of intact virions in the tubular structures suggests that CPMV moves from cell to cell as a virus particle. It is unknown whether the tubules are solely of viral origin or whether host components are also

required. The role of the 58 kDa and 48 kDa proteins in formation of the tubules, and whether they interact directly with the viral genome are yet to be determined. Thus, as far as primary homology is concerned, there are apparently three families of cell-to-cell movement proteins (the TMV, the AIMV and the CPMV) between which there are few or no obvious interrelationships (Hull, 1989).

The P1 protein, which has been recently detected in potyvirus-infected cells (Rodriguez-Cerezo and Shaw, 1991), has been proposed to be involved in the cell-to-cell movement function of virus particles. The 28 kDa (N-terminal) polypeptide of TVMV, but not the analogous proteins of TEV, PPV and PVY, has some primary homology with TMV 30 kDa protein (Domier *et al.*, 1987) and the 29 kDa protein of tobacco rattle tobavirus (TRV) (Lain *et al.*, 1989a; Robaglia *et al.*, 1989). This sequence similarity involves the 30 kDa TMV protein domain that has been implicated in RNA binding (Citovsky *et al.*, 1990). However, the very little sequence similarity found among potyvirus proteins possible involved in cell-to-cell movement, does not exclude attribution of a spread function to P1, since proteins presumably involved in cell-to-cell movement exhibit very little similarity even among members of the same group (Hull, 1989), a fact that could reflect the specificity of the virus-host interactions. In fact, its low conservation and its localization at the 5' end of the polyprotein, as in the case of the proposed movement proteins of the related virus groups, como-(van Lent *et al.*, 1990; Wellink and van Kammen, 1989), nepoviruses and possibly potyviruses (Lain *et al.*, 1989a) support this hypothesis. The CI protein has been found to be associated with the plasma membrane and plasmodesmata early in infection in intact leaves (Chapter 3, this thesis; Langenberg, 1986; Lawson and Hearon, 1971) and with the plasma membrane of protoplasts inoculated with TVMV RNA (Murphy *et al.*, 1990; Xu *et al.*, 1985). Virus particles, were found to be associated with such inclusions (Langenberg, 1988, 1986) an observation which has led

to the suggestion that these structures might align and/or drive the elongate potyviral particles through plasmodesmata (Langenberg, 1986). It is not known whether these particles have exactly the same conformation as mature particles or if the CP is necessary for the interactions with tubular structures and/or the movement protein.

Although a considerable amount of information concerning the organization, structure and nucleotide sequence of some potyvirus genomes is now available (Introduction Chapters 1, 3 and 5), only limited information is available on how the potyviral genome is expressed in infected plant cells. This is due, to a considerable extent, to the difficulties in establishing potyviral infections in protoplasts. Inoculation of protoplasts using PLO or PEG has been limited because of several inherent disadvantages. Severe losses of protoplasts and/or low percentage of infection (< 16%) was obtained by using PLO (Xu *et al.*, 1985, Xu *et al.*, 1984a; Goffinet and Verhoyen, 1979), liposomes (Xu *et al.*, 1984b) and PEG (Valkonen *et al.*, 1991). Electroporation with TVMV and PVY RNAs (Gibb *et al.*, 1989; Luciano *et al.*, 1987) were conveniently used to generate efficient (64-87%) potyvirus infections in tobacco protoplasts which resulted in the synthesis of viral RNA and virus-specific proteins.

Only limited information on *I. batatas* protoplasts isolation is available, and most studies have been aimed toward a potential crop improvement. As shown in Table 4.1, several tissues and enzyme mixtures have been used with varied degrees of success. Recently, the preparation of sweet potato protoplasts from cell suspension cultures and their infection with TMV RNA by electroporation has been reported (Uehara *et al.*, 1990). Electroporation of protoplasts from a cultivar which is resistant to TMV infection at the plant level with virions or RNA yielded up to 10% and 90 % infected protoplasts respectively.

Reference	Source of material	Enzyme mixture and osmotic solution	Yield
Bidney & Shepard (1980)	Green house; Petioles	0.2% Macerozyme R10, 0.4% Cellulase R10, 1/4 MS major elements, 0.6M mannitol, 0.1% casein hydrolysate, 5 mM MES, pH 5.6	non reported
Nishimaki & Nozue (1985)	Callus	3% Cellulase R10, 0.5% Macerozyme R10, 0.3% Pectolyase Y23, 0.7M mannitol, 0.5% CaCl ₂ , 5mM MES, pH 5.5	1.4 x 10 ⁵ /0.6 g
Sihachakr & Ducreux (1986)	Green house; Petioles, stems	1.5% Cellulase R10, 0.5% Macerozyme R10, 0.55M mannitol, pH 5.5	6.5x 10 ⁵ /g (P) 1.3x 10 ⁵ /g (S)
Otani <i>et al.</i> (1987)	<i>In vitro</i> Mesophyll	2% Cellulase RS, 0.05% Macerozyme R10, 0.3% Pectolyase Y23, 0.5% Hemicellulose, 1% Driselase, 0.5M sucrose, 5mM MES	1.8 x 10 ⁶ to 1.2 x 10 ⁷ /g
Sihachakr & Ducreux (1987)	<i>In vitro</i> , Petioles, stems	1.5% Cellulase, 0.5% Macerozyme R10, 19% sucrose, 0.05% MI:S,	1-1.5 x 10 ⁶ /g
Murata <i>et al.</i> (1987)	Green house; Petioles	2% Cellulase, 0.1% Pectolyase Y23, 0.5M mannitol, 5 mM CaCl ₂ , pH 5.8	non reported
Eilers <i>et al.</i> (1988)	Green house; Petioles	2% Cellulase, 0.5% Macerozyme, 0.4M sucrose, 5mM MES, 15 mM arginine, KNO ₃ 4.7 mM, CaCl ₂ 10 mM, MgSO ₄ 0.4 mM, KH ₂ PO ₄ 0.3 mM, pH 5.8	2.5 x 10 ⁵ /g
Kokubo & Sato (1988)	Green house; Petioles	0.4% Cellulase R10, 0.2% Macerozyme R10, 0.6 M mannitol, 0.1% casein hydrolysate, 0.5% CaCl ₂ , 5 mM MES, pH 5.8	3.5 x 10 ⁴ /g
Uchara <i>et al.</i> (1990)	Green house; Petioles	2% Cellulase RS, 0.1% Pectolyase Y23, 0.5M mannitol, 5 mM CaCl ₂ , pH 5.8.	non reported
Liu <i>et al.</i> (1991)	Suspension cell	0.4% Cellulase R10, 0.2% Macerozyme R10, 0.6M mannitol, 0.5% CaCl ₂ , 5 mM MES	non reported

Table 4.1: References on *I. batatas* protoplast isolation.

P = petioles, S = stems

The aims of this work were to develop protocols for the isolation of *N. benthamiana* and *I. batatas* (susceptible and resistant genotypes) protoplasts, and to examine the conditions suitable for their electrotransfection with SPFMV C1 or viral RNA, in an attempt to study virus replication and translation, and the nature of resistance to SPFMV C1 in *I. batatas* genotypes S24 and Nemanete which behave as resistant cultivars at the plant level.

4.2. Materials and Methods

All glassware used for handling protoplasts was siliconized (Sigmacote, Sigma); that used for RNA inoculation was DEPC treated. Enzyme digestion mixtures and incubation media were filter-sterilized, electroporation cuvettes and electrodes were ethanol sterilized and all other solutions and materials sterilized by autoclaving at 120 °C for 20 min.

4.2.1. Plants and inoculum

4.2.1.1. Plants

I. batatas pathogen-free stem cuttings were grown *in vitro* and maintained under controlled environmental conditions (22-24 °C with 5000 lux for 16 h and 8 h darkness at 20 °C). Five cultivars were tested for protoplast isolation and virus inoculation (Table 4.2).

This material was evaluated for SPFMV resistance (International Potato Center, 1992) by the Plant Pathology Department, and provided by the Genetic Resources Department of The International Potato Center, Lima, Peru.

<i>I. batatas</i> cultivars	Plant response to infection			Reaction to virus infection
	Mechanical inoculation	Grafting	Field exposure (high-vector pressure)	
S24	Negative	Negative	Negative	Resistant
Nemanete	Negative	Negative	Negative	Resistant
DLP1914	Negative	Positive	Positive	Susceptible
Jewel	Positive	Positive	Positive	Susceptible
Georgia Red	Positive	Positive	Positive	Susceptible

Table 4.2: *I. batatas* genotypes, showing different responses to SPFMV infection.

Protocols and media recipes for *in vitro* conservation and propagation of sweet potato plants were as routinely used at the International Potato Center, Genetic Resources Department (Tissue Culture Laboratory), Lima, Peru. For long term plant conservation, single node shoot cuttings were grown in 30 ml test tubes containing 5 ml of MS [Murashige and Skoog (Murashige and Skoog, 1962), minimal salts and organics medium, 4.6 g/l (Gibco Lab, 510-3118EA), containing 200 µg/ml putrescine, 20 mg/ml sucrose, 20 mg/ml glucose and 4 mg/ml Phytigel (Sigma). For propagation, 3-5 node shoots were taken from 4-5 old week-old cultures (in conservation media) and subcultured in magentas (GA-7, Sigma) containing 50 ml of sweet potato propagation media [MPB, MS (4.6 g/l), ascorbic acid (200 mg/l), L-arginine HCl (100 mg/l), putrescine (20 mg/l), calcium panthotenate (2 mg/l), giberellic acid (10 mg/l), 3% sucrose; pH 5.8)]. For solid media, 3 mg/ml Phytigel was added.

N. benthamiana plants were grown under conditions similar to those reported by Barker (1980). Seeds were sown and seedlings transplanted under the same conditions used for growing plants for virus purification (Chapter 2). Four to five weeks before

protoplast isolation, seedlings were transplanted into 150 x 150 mm cross-section plastic pots containing a peat:sand mixture (2:1) and grown in a growth chamber, for 16 h at 24 °C with 5000 lux alternating with 8 h at 20 °C in darkness respectively, and 75-80% relative humidity. Plants were fertilized every 4 days with a N:P:K (2:1:1) and 5 days before protoplast isolation with 1/2 MS inorganics.

4.2.1.2 Inoculum

SPFMV C1 and C virus and RNA were purified according to the protocols described in Chapter 2. Viral RNAs were isolated immediately before using for protoplast inoculation. RNase block (40U/ml, Stratagene) was added and the RNA stored on ice.

4.2.2. Protoplast isolation and purification

4.2.2.1. *I. batatas* protoplasts of genotypes Nemanete, Jewel and Georgia Red, were isolated from apical second to third fully expanded leaves of 2-3 week-old *in vitro* grown plants. Leaves were excised, superficially sectioned at several places and soaked in sterile distilled water for 4-5 h in the dark at 22-24 °C with 40 rpm rotatory movement. The water was then discarded and cold pre-conditioning media [PCM; 1/2 strength MPB containing 2% sucrose and 1% coconut water (CW)] added before incubation for 16 h in the dark at 4 °C. PCM was replaced with osmotic solution [OSIB; 0.5M mannitol, 7 mM CaCl₂ and 5 mM MES (Sigma; 4-(N-morpholine)ethanesulfonic acid) pH 5.8], incubated at room temperature for 30 min. Leaves were then transversely cut into 1mm wide strips and placed on enzyme mixture [1 ml/100 mg tissue; 2% Cellulase RS (Yakult Honsha, Co., Ltd.), 1% Driselase (Kyowa Hakko Kogyo, Co., Fluka Biochemika), 0.25% Pectolyase Y23 (Sheishim Pharmaceutical Co.) and 0.05% Macerozyme RS200 (Yakult Honsha, Co., Ltd.) in OSIB, pH adjusted to 5.6]. Plates were then incubated for 18-24 h at 24 °C. Protoplasts were filtered through 120 and 40µ nylon filters, the volume made up to 10 ml

with OSIB solution and the suspension centrifuged at 100 rpm for 90s. The supernatant was discarded, the pellets resuspended in 5 ml OSIB, layered over a 5 ml dextran sulphate (Sigma, MW 60-90K, 0.26% in 0.5M mannitol) cushion, and centrifuged as above. The supernatant was discarded, the pellets resuspended in 10 ml OSIB and centrifuged as before. The final pellets were resuspended in OSIB solution to a final concentration of 2×10^6 cells/ml.

Protoplasts of *I. batatas* S24 were isolated from suspension cell cultures. For cell culture preparation, friable callus was first established from pieces of leaves or "modified roots". The organ was surface sterilized by soaking in 2% calcium hypochlorite, 0.05% Tween 80, for 15 min and rinsing four times with SDW. Tissue was sectioned into large pieces ("modified roots" were peeled, and cut off into 3-5 mm thick pieces) which were laid on petri dishes containing 20 ml of a basic callus inducing medium [CIM, MS salts, 3% sucrose, 0.5 mg/l 2-4, D (2,4-dichlorophenoxyacetic acid), and 3% Phytigel, pH 5.8] and cultured under same environmental conditions as for other *in vitro* culture materials. Every two weeks, calluses were subcultured or used to prepare suspension cells. Callus cells were mechanically dispersed, with the aid of surgical forceps and blades, filtered through 100 μ nylon filters and transferred (1g/50 ml) to 250 ml flasks containing 50 ml CIM (without Phytigel) and supplemented with 1% CW. Suspension cell flasks were placed on a rotatory shaker (80 rpm) in the same environmental conditions as used for callus induction. Cells were subcultured every 5-7 days.

Protoplasts from suspension cells were isolated without any pre-conditioning treatment. Cells from cultures consisting of spherical cells in small clumps were pelleted at 1000 g for 1 min and the pellet resuspended in enzyme solution (100 mg/ml; 2% Cellulase RS, 1% Driselase, 0.25% Pectolyase Y23, OSIB, pH adjusted to 5.6) and incubated for 16-18 h at 22-24 °C. After incubation, protoplasts were purified as described for mesophyll protoplasts and resuspended in OSIB to a concentration of 2×10^6 cells/ml.

4.2.2.2. *N. benthamiana* protoplasts were isolated from the second or third youngest fully expanded leaves of 50-60 day old plants. Leaves were surface sterilised by rinsing in 70% EtOH for 10 s, then in 2.5% calcium hypochlorite containing 0.01% Tween 80 for 30 min. Leaves were rinsed four times with sterile distilled water, cut into transverse (1-2 mm wide) strips and soaked in osmotic solution [OSNB; 0.6M mannitol, 7 mM CaCl₂ and 5 mM MES, pH 5.6] for 30 min at room temperature. Osmotic solution was carefully discarded and replaced with enzyme solution [1ml/100 mg tissue; 1% Cellulase RS, 0.2% Pectolyase Y23, 0.1% BSA (Sigma) in OSNB, pH adjusted to 5.6]. Leaves were incubated overnight at room temperature (22-25 °C), followed by shaking (20 rpm) for 2-3 s and further incubation for 1 h at room temperature. Protoplasts were filtered through two layers of nylon filters (140µ and 50 µ), washed and resuspended in OSNB as described for *I. batatas* protoplast isolation, to 2 x 10⁶ cells/ml.

4.2.3. Protoplast concentration, absence of cell wall and viability assessment

Protoplast concentration was determined by counting cells in a 0.2 mm deep Fuchs-Rosenthal counting chamber (A.H. Thomas, Philadelphia). Complete cell wall digestion was assessed by mixing protoplast suspensions with 1% calcofluor (fluorescent brightener 28, Sigma) in 10% mannitol (1:10/v/v); protoplasts were then observed under a microscope with a UV filter (Villegas, personal communication). Viability was assessed by phase contrast microscopy of protoplasts stained with 0.1% Evans blue in 10% mannitol (Kanai and Edwards, 1973).

4.2.4. Electroporation

In a typical electroporation experiment, a 500 µl aliquot of the protoplast suspension (2 x 10⁵ protoplasts/ml) was placed in an electroporation chamber, a sterile plastic cuvette (Potter *et al.*, 1984), and kept on ice for 10 min. Viral RNA or particles were diluted in

ice cold nuclease free electroporation buffer and added just before the electrical pulse was applied. The cuvette was capped with two stainless flat electrodes, with a 1.9 mm gap between them (BTX model 470, San Diego, California), the contents gently mixed and the electrodes connected to a square wave pulse generator (BTX, Model 200 Cell Manipulator, San Diego, California). The suspension was immediately exposed to 2 by 50 μ s direct current (DC) pulses of 150 volts (0.8 kV/cm). Electrodes were left in the cuvette for 30 s the chamber remaining undisturbed for that time. The cuvette was placed on ice for 10 min and then at room temperature for 30 min. After this time, an aliquot of each sample was taken for viability assessment. Supernatants were discarded and protoplasts resuspended in 1.0 ml incubation medium [Aoki and Takebe (1969); 0.2 mM KH_2PO_4 , 1 mM KNO_3 , 1 mM MgSO_4 , 0.01 mM CaCl_2 , 1 μ M KI, 0.01 μ M CuSO_4 , 1 μ M 2,4-D (2,4-dichloro-phenoxyacetic acid), 0.6 M mannitol, pH 5.8]. The protoplasts were left undisturbed for an additional 20 to 30 min, then the supernatants were discarded and the protoplasts resuspended in incubation medium, with addition of 2.5 μ g/ml Nystatin (Sigma) and 25 μ g/ml Carbenicillin (Sigma). Aliquots (500 μ l, 1×10^4 cells/ml) were dispensed in 1.5 ml microfuge tubes and incubated under 2000 lux light intensity, provided by a cool white fluorescent tube, for 16 h alternating with 8 h darkness, at 22-24 $^\circ\text{C}$ constant temperature.

4.2.5. Immunodetection

4.2.5.1. F(ab')₂ indirect ELISA

The method is based on the protocol described by Barbara and Clark (1982). IgG of SPFMV CI-CP and CI antisera were prepared as described in Chapter 3 .

4.2.5.1.1. F(ab')₂ fragment isolation

F(ab')₂ fragments of IgG were prepared by pepsin digestion (Campbell *et al.*, 1970). IgG (1-2 mg/ml in 70 mM sodium acetate pH 4.0 with 50 mM NaCl_2) was digested by the

addition of pepsin (Sigma, 45 µg/mg IgG) in distilled water and incubation overnight at 37 °C. F(ab')₂ fragments were separated from enzyme and other digestion products by ion exchange chromatography using DEAE Sephacel (Pharmacia).

4.2.5.1.2. Indirect ELISA

(i) Polystyrene plates were coated with virus-specific F(ab')₂ (200 µl, 2 µg/ml) in sodium carbonate buffer, pH 9.6 for 4 h at 37 °C. (ii) Protoplast suspensions diluted in PBS-T containing 2 % PVP (Sigma, 44,000) and 2% NFM were prepared by disrupting the cells by three freezing-thawing cycles (Wood, 1985) and clarification by centrifugation at 3000 g for 2 min. Supernatants (150 µl, 3 wells per sample) were allowed to react with the immobilized F(ab')₂ fragments overnight at 4 °C. Serial dilutions of purified virus in non-infected protoplast suspension were added to the same plates for calculation of virus concentration. (iii) Virus specific IgG (200 µl, 1 µg/ml) in PBS-T and 2% NFM was added to react with the trapped antigen. Plates were then incubated for 3 h at 37 °C. (iv) GAR IgG conjugated to alkaline phosphatase in PBS-T and 1% BSA (200 µl, Bio -Rad, 1:4000) was added and plates incubated for 3 h at 37 °C. (v) Immobilized enzyme-conjugate was assessed by adding enzyme substrate (Sigma, p-nitrophenyl phosphate, 0.75 mg/ml). Reactions took place at room temperature for 60 min and were stopped with 1M HCl (50 µl/well). Absorbance was measured at 405 nm. Between each step, plates were washed with PBS-Tween, three times for 3 min each time.

4.2.5.2. Protoplast immunostaining (PIS)

The procedure for detecting viral antigens in individual protoplasts was based on the protocol described by Luciano *et al.* (1989), with some minor modifications.

4.2.5.2.1. Sample preparation

(i) Protoplasts to be analysed were centrifuged at 100 g for 1.5 min, culture medium removed by aspiration and protoplasts rapidly but gently resuspended in 75% EtOH and incubated for 5 min at room temperature. (ii) Samples were centrifuged as in step 1 and pellets resuspended in 95% EtOH, incubated for 5 min and centrifuged again. This step was repeated one more time, to eliminate most of the chlorophyll. (iii) Final pellets were resuspended in 95% EtOH (1×10^4 protoplasts/ml), stored at -20°C or used immediately for immunostaining.

4.2.5.2.2. Immunostaining

(i) Ethanol-washed protoplasts (50 μl) were fixed on glass microscope slides that had been pre-coated with poly L-lysine (Sigma). To provide positive and negative internal controls on each slide, samples of mock inoculated protoplasts were fixed next to those from infected leaves. (ii) Slides with fixed protoplasts were placed in TBS containing 5% NFM (TBS-B) and shaken gently (30-40 rpm) for 2-4 h at room temperature. (iii) Slides were drained briefly, covered with specific IgG (200 μl , 1-0.5 $\mu\text{g}/\text{ml}$) and incubated for 16-18 h at 4°C . (iv) Slides were washed four times for 15 min each with TBS-T. (v) Slides were drained briefly and covered with GAR IgG conjugated to alkaline phosphatase in TBS-B (200 μl , Bio-Rad, 1:4000), incubated at room temperature for 2 h and washed as in (iv). (vi) Slides were drained briefly and covered with alkaline phosphatase substrate [Bio-Rad, BCIP (0.15 $\mu\text{g}/\text{ml}$) and NBT (0.30 $\mu\text{g}/\text{ml}$)] in APB for 30 to 60 min at room temperature. After developing, slides were rinsed once with distilled water and immediately covered with PBS:glycerol (1:1) and a coverslip.

Antigen presence was assessed by light microscopy. Positive reaction on infective protoplasts exhibited different degrees of purple coloration.

4.3. Results and Discussion

4.3.1. Protoplast isolation

4.3.1.1. *I. batatas*

In preliminary experiments, protoplast isolation from petioles of several *I. batatas* genotypes, performed as described by Murata *et al.* (1987), yielded a reasonable concentration of protoplasts ($1-2 \times 10^5$ protoplasts/g tissue). However, protoplast size varied widely, which was not considered appropriate for the purposes of this research. In a previous work (Otani *et al.*, 1987), it was shown that mesophyll protoplasts of *I. batatas* (Japanese cultivars) could be isolated by using a mixture of several enzymes and pre-treatment with water of leaf tissue prior to enzymatic digestion. Water treatment was reported to not only help to seal the plasmadesmata connecting adjacent cells (Potrykus *et al.*, 1983) but also, probably, to eliminate latex and phenolic compounds present in high concentration in Convolvulaceae, increasing the efficiency of digestion enzymes for removing cell wall components. Treatment of tissue in the dark and at low temperature (4 °C) decreases the rate of accumulation of starch grains and induces predifferentiation, stabilizing the cells (Shepard, 1982). *Solanum melongena* and *N. sanderae* protoplasts, isolated from tissue treated at low temperature in culture medium, were less vacuolated and more stable than protoplasts isolated from untreated tissue (Chung, 1987).

In the present study, several enzyme mixtures and pre-treatments with water and pre-conditioning media were compared for isolation of mesophyll protoplasts of SPFMV resistant (Nemanete and S24) and susceptible (Georgia Red, DLP 1914 and Jewel) *I. batatas* genotypes. Results for Nemanete, Georgia Red and Jewel protoplast isolation were similar and only data for Nemanete are presented.

Mixture	Enzyme mixture			
	Cellulase RS	MacerozymeR200	Driselase	PectolyaseY23
1	1%	0.5%	-	-
2	2%	0.5%	-	-
3	2%	0.5%	1%	-
4	2%	0.5%	2%	-
5	2%	0.5%	2.5%	-
6	2%	-	1%	0.5%
7	2%	0.5%	1%	0.5%
8	2%	0.05%	1%	0.25%

Table 4.3: Enzyme mixtures used for isolation of *I. batatas* protoplasts.

N^o of protoplasts
(x 10⁶ cells/g tissue)

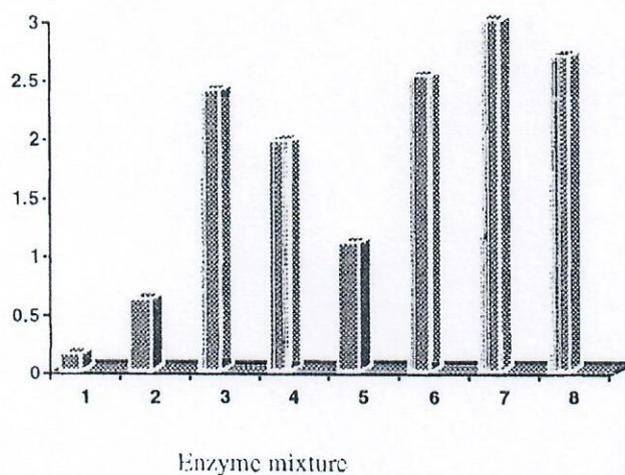


Figure 4.1.

Effect of enzyme composition on yield of *I. batatas* cv. Nemanete protoplasts.

Enzyme mixtures in 0.5M mannitol, 7 mM CaCl₂ and 5 mM MES, pH 5.8.

(1) 1% C + 0.5% M . (2) 2% C + 0.5% M. (3) 2% C + 0.5% M + 1% D. (4) 2% C + 0.5% M + 2% D.
(5) 2% C + 0.5% M + 2.5% D. (6) 2% C + 0.5% P + 1% D. (7) 2% C + 0.5% P + 1% D + 0.5% M. (8)
2% C + 0.25% P + 1% D + 0.05% M.

C= Cellulase RS, M= Macerozyme R200, D= Driselase, P= Pectolyase Y23

Results presented in Figure 4.1 are the average of 3-4 samples (0.25g tissue/sample) from 2 experiments. The best enzyme mixture in terms of protoplast yield was 1% Driselase in combination of 2% Cellulase RS, 0.5% Pectolyase Y23 and 0.5% Macerozyme R200. The effect of 1% Driselase on *I. batatas* protoplasts isolation was evident by comparison with the mixtures without this enzyme. However, increase in Driselase concentration had a negative effect on protoplast yield, probably because enzyme preparations usually contain impurities which, when present in higher concentration, might have a toxic effect on cells metabolism, affecting protoplast stability. From these results, the optimum enzyme mixture for *I. batatas* Nemanete, Jewel and Georgia Red was 2% Cellulase RS, 1% Driselase, 0.5% Pectolyase Y23 and 0.5% Macerozyme R200, but for reasons of economy, 0.25% Pectolyase Y23 and 0.05% Macerozyme R200 were employed in all subsequent protoplast isolations (results with all three genotypes were similar, therefore only data on Nemanete protoplast isolation is shown).

To test the effect of various pre-conditioning regimes, leaves (3 x 0.25g/sample) of genotypes Jewel and S24 were placed in water (5ml/0.1g tissue) and incubated at room temperature with gentle shaking (20 rpm) for 4 to 5 h. Leaves were transferred to pre-conditioning medium [(1ml/0.1g tissue) and incubated in darkness at 4 °C for 16 to 18 h. After cold-treatment, leaves were place in OSIB for 30 min, and protoplasts isolated.

The following pre-conditioning media were used:

1. MS
2. MPB + 2% sucrose
3. MPB + 1% CW + 2% sucrose
4. 1/2 MPB + 2% sucrose + 1% CW
5. Gamborg's B5 + vitamins + 1% CW

Protoplast yield ($2.0\text{-}3.0 \times 10^6$ protoplasts/g tissue) and viability increased when leaf tissue of cv. Jewel was treated with pre-conditioning medium containing 1/2 MPB, 2% sucrose and 1% CW, compared with untreated material ($0.8\text{-}1.2 \times 10^6$); a high percentage of protoplasts (75%) remained viable after incubation for 32 h. With other pre-conditioning media, protoplast yields were slightly lower ($0.8\text{-}1.0 \times 10^6$), but protoplasts were more vacuolated and the percentage of viable protoplasts after incubation for 32 h was lower (50-55%). The experiment was repeated 2 more times, but results were not consistent for all treatments, probably as a result of varying plant-source conditions. However, as was first found, leaves treated with pre-conditioning medium containing 1/2 MPB, 2% sucrose and 1% CW gave similar protoplast yields and about 70% remained viable for more than 48 h after isolation. For the purpose of this work, this was considered the best pre-conditioning media and was used for *I. batatas* mesophyll protoplast isolation.

It is important to note that, even when reasonably high protoplast yields were obtained from Nemanete, Jewel and Georgia Red mesophyll cells under optimal conditions, slight changes in environmental conditions, such as temperature and photoperiod and/or physiological state of the plantlets significantly affected the final yield, size and viability of the protoplasts. In some experiments, only very low concentrations of protoplasts were isolated and it was not possible to make an appropriate analysis of the data. In the case of DLP1914, protoplasts were always extremely variable in size and color, resembling protoplasts from petioles. For this reason, and because other susceptible genotypes were available, DLP 1914 was no longer used in other experiments. For S24, only a very low concentration of protoplasts was isolated, using any of the enzyme mixtures tested, and as explained below, it was only possible to isolate protoplasts of this genotype from suspension cell cultures. Variability between sweet potato genotypes with respect to protoplast isolation has been reported previously (Kokubo & Sato, 1988; Otani *et al.*,

1987; Bidney and Shepard, 1980) and in some cases isolation was impossible or very ineffective, especially if protoplasts were to be isolated from the mesophyll cells. Suspension culture cell-derived protoplasts from several species have been successfully used previously for studies of virus replication, becoming efficiently infected and supporting active virus multiplication [eg. *Lycopersicon peruvianum* PI 126944 with TMV, Matsunaga *et al.*, 1992; *Triticum monococcum* with barley yellow dwarf virus (BYDV), Young *et al.*, 1989; *N. tabacum* with TMV and CMV, Okada *et al.*, 1986; Kikkawa *et al.*, 1982; *Catharanthus roseus* L. with TMV, Fukunaga *et al.*, 1981; *Glycine max* cv. Harosoy 63 with southern bean mosaic virus (SBMV) and CPMV, Jarvis and Murakishi, 1980].

Isolation of protoplasts from *I. batatas* S24 suspension cell cultures was successfully achieved by following the protocol described under Materials and Methods. Yields ranged between 1.6 and 2.0 x 10⁶ protoplasts/g suspension cells and protoplasts appear to be highly stable since 70 to 80% were viable after about 48 h culture. Cell suspensions were maintained without difficulty and were easy to handle. Suspension cell cultures have proved to be a practical source of material for isolation of *I. batatas* protoplasts, especially from genotypes from which it is difficult to prepare a reasonable yield of mesophyll protoplasts.

Although the protocols developed in this work allowed reasonably good yields of protoplasts to be obtained from Nemanete, Jewel and Georgia Red cultivars, maintenance of appropriate source plants was laborious, suspension cells of these genotypes are an alternative source of protoplasts which will be useful for future work.

4.3.1.2. *Nicotiana benthamiana*

Isolation of *N. benthamiana* protoplasts by sequential application of enzymes (two step method) and use of 0.7M mannitol, as described by Barker (1980), yielded a high

concentration of protoplasts; however, protoplast viability dropped significantly during washing and further incubation. It was observed that mannitol brand and lote could affect the osmolarity of the solution. Preliminary experiments showed that the mannitol brand affected the viability of protoplasts isolated using mannitol solutions of theoretically the same osmolarity. Higher concentration of viable protoplasts were isolated with Difco than with Sigma mannitol. The use of 0.6M mannitol (Difco) pH 5.6 proved to yield higher concentrations of viable protoplasts than 0.4, 0.45, 0.5, 0.55, 0.65 and 0.7M mannitol. No significant differences were recorded between 0.55, 0.6 or 0.65 M immediately after isolation but after 5 h incubation, protoplasts in 0.6M mannitol looked healthier and viability was higher than with other treatments (data not shown). All the following *N. benthamiana* protoplast isolations were performed by using 0.6 M mannitol.

Mixture	Enzyme mixture				
	Cellulase R10	Cellulase RS	PectolyaseY23	Driselase	MacerozymeR200
1	2%	–	0.2%	–	–
2	2%	–	0.2%	0.5%	–
3	1.5%	–	0.1%	0.5%	–
4	–	2%	–	–	0.2%
5	–	1%	0.2%	0.25%	–
6	–	1%	0.2%	0.5%	–
7	–	1%	–	–	0.2%
8	–	1%	–	–	0.2%

Table 4.4. Enzyme mixtures used for isolation of *N. benthamiana* protoplasts.

For practical reasons, the one-step enzymatic digestion procedure (Power and Cocking, 1970) for *N. benthamiana* protoplast isolation was tested; the protocol described under Materials and Methods was followed. Two leaves were treated individually with each enzyme mixture. Enzyme mixture numbers 5, 6 and 7 were re-tested two more times; the

the performance of the enzyme mixtures selected was consistent in all the following experiments. Pre-plasmolysed leaves (1g/10 ml) were digested with several enzyme mixtures (Table 4.4) prepared in 0.6M mannitol containing 7 mM CaCl₂, 5 mM MES, pH 5.8. As shown in Figure 4.2, no differences in viable protoplast yield were found by using 1% Cellulase RS, 0.2% Pectolyase Y23 or the same enzymes with the addition of 0.5% Driselase, when viability was assessed immediately after protoplast isolation. However, after incubation for 5 h at room temperature, approximately 25% more protoplasts remained viable when Driselase was not included in the enzyme mixture.

N^o of protoplasts
(x 10⁶ cells /g tissue)

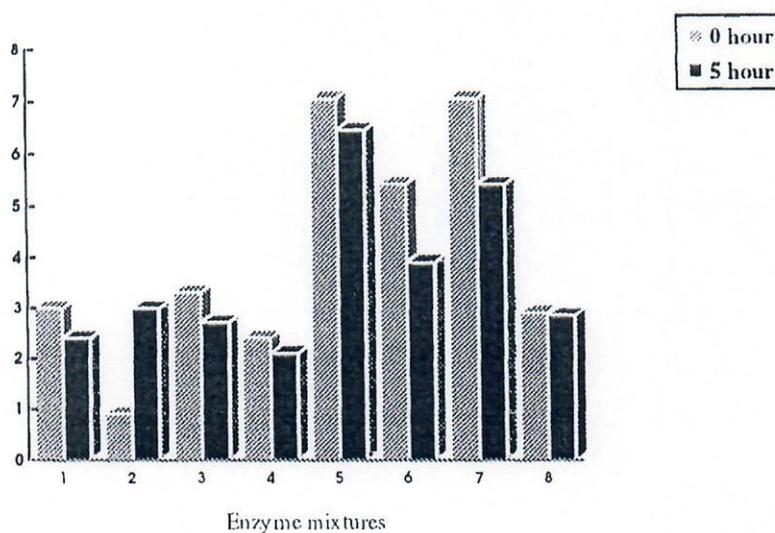


Figure 4.2.

Effect of several digestion enzyme mixtures on *N. benthamiana* protoplast concentration assessed at 0 and 5 h after isolation (enzyme mixtures in 0.6 M mannitol, 7mM CaCl₂, 5 mM MES, pH 5.8).

(1) 2% C + 0.2% P. (2) 2% C + 0.2% P + 0.5% D. (3) 1.5% C + 0.1% P + 0.5% D. (4) 2% C + 0.2% M. (5) 1% C + 0.2% P. (6) 1% C + 0.2% P + 0.25% D. (7) 1% C + 0.2% P + 0.5% D. (8) 1% C + 0.2% M.

C=Cellulase RS, P=Pectolyase Y23, D=Driselase, M=Macerozyme R200.

N. benthamiana protoplasts isolated using enzyme mixture 5 (1% Cellulase RS, 0.2% Pectolyase Y23 with addition of 0.1% BSA in OSNB, pH 5.6) yielded $5 - 7 \times 10^6$ protoplasts/g leaf tissue, 90 to 95% of which were intact and viable. This was thus the best procedure for *N. benthamiana* protoplast isolation.

4.3.2. Electroporation

In order to find suitable conditions for electrotransfection of *N. benthamiana* protoplasts, the effect of several parameters were analyzed. Electroporation medium and the amplitude, length and number of pulses given were first examined by assessing the viability of protoplasts treated without inoculum. These experiments were considered fundamental in view of the fact that many protoplasts may be completely disrupted during the electroporation pulse and measurement of viability after the electrophysical treatment gives an overview of the protoplast behaviour under these conditions. For each parameter, 2-3 samples per treatment were evaluated and the experiments repeated at least twice, unless otherwise stated.

The effect of 1 mM or 7 mM CaCl_2 in the electroporation medium of *N. benthamiana* protoplasts was tested. As is shown in Figure 4.3, viability of protoplasts in medium containing 1 mM CaCl_2 exposed to a field of as low as 0.8 Kv/cm decreased to 90% in relation to the original protoplast population, while in the presence of 7 mM CaCl_2 , nearly 80% of the original population remained viable. The incorporation of CaCl_2 (7 mM) in the electroporation solution was thus found to be essential for *N. benthamiana* protoplast electroporation. Nishiguchi *et al.* (1986) had reported that up to 8 mM Ca^{++} had neither an inhibitory nor a stimulating effect on the percentage of *N. tabacum* cv. Xanthii protoplasts infected with TMV RNA, an observation similar to the findings of Van Beek *et al.* (1985) for infection of cowpea protoplasts with sonchus yellow net virus and of

Hibi *et al.* (1986) for infection of tobacco protoplasts with TMV it was, however, contrary to the report of Watts *et al.* (1987), who found that Ca^{++} ions were apparently inhibitory to *N. tabacum* cv. Xanthi, *N. plumbaginifolia* or *Beta vulgaris* protoplast electroporation. However, it is worth noting that the effect of these ions depends on the quality of the protoplast preparation used. All following experiments were done with the incorporation of 7 mM CaCl_2 in the electroporation medium.

The effect of field strength (kV/cm) on protoplast viability showed that the percentage of viable protoplasts 5 h after being electroporated at 0.75 to 1.0 kV/cm field strength remained being nearly 55% of the original population. However, increasing the field strength to 1.5 kV/cm decreased this percentage to only 15-20% of the original population. When the field strength was less than 0.75 kV/cm (0.5-0.25), 80 to 90% of the protoplasts remained viable after the electric treatment. However when the inoculum was present, a lower percentage of protoplasts were infected at 0.5 kV/cm than at 0.75 kV/cm (data not shown). For practical reasons, 0.8 kV/cm was selected and used in all further experiments.

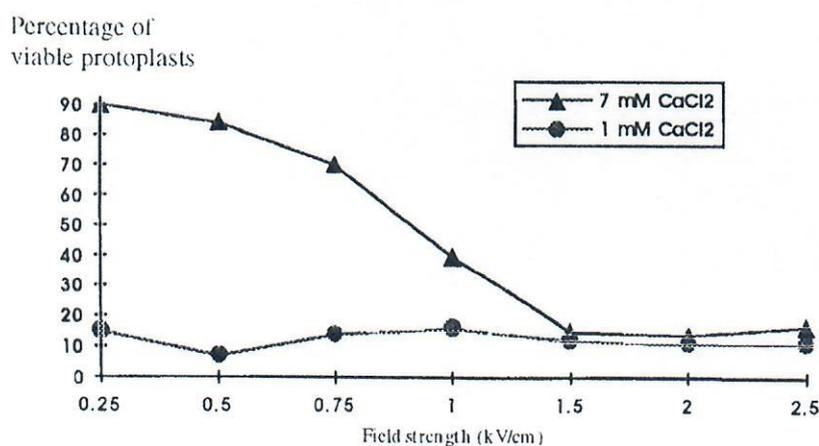
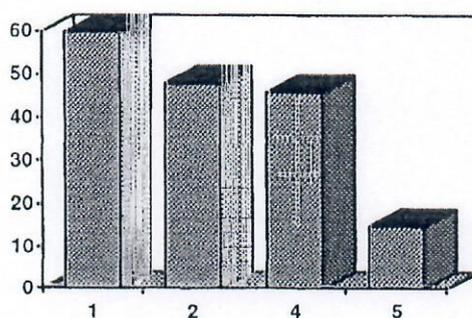


Figure 4.3. Effect of $2 \times 50 \mu\text{s}$ pulses of a square wave direct current, applied at various strengths (kV/cm) and two CaCl_2 concentrations, on the viability of *N. benthamiana* protoplasts electroporated with viral RNA (20 $\mu\text{g}/\text{ml}$), expressed as the percentage of viable protoplasts after 5 h incubation.

The effect of pulse number on viability (Figure 4.4a) and percentage of viable infected protoplasts (Figure 4.4b) was also examined. *N. benthamiana* protoplasts were electroporated according to the protocol described under Material and Methods, except for the number of electrical pulses. When 1, 2 or 4 pulses (50 μ s at 8 kV/cm field strength) were applied to protoplasts without the presence of viral RNA, no differences on protoplast viability were detected and around 50-60% remained viable 24h after the electrical pulse was applied, but when number of pulses applied was increased to 5, a very low percentage of protoplasts (7-15%) remained viable.

Percentage of viable
protoplasts



Number of pulses

Figure 4.4a.

Effect of number of pulses (50 μ s of 0.8 kV/cm) on percentage of viable, mock inoculated *N. benthamiana* protoplasts.

If protoplasts were electroporated with viral RNA (20 μ g/ml), higher percentages (70-80%) of viable infected protoplasts were detected when 2 pulses were applied. When 1 or 4 pulses were applied the percentage was lower (50-55%). This results do not agree

with the findings of Hibi *et al.*, (1989) who had reported no differences on survival or infection of tobacco mesophyll protoplasts with TMV RNA, when 5 to 10 DC pulses (50 μ s and 0.55 to 0.80 kV/cm) were given. However, it is important to consider that the protoplast system, electroporation medium and the type of electroporation chamber used by them were different from the ones used in this work. However, this apparent decrease when only 1 pulse was applied might have resulted from an underestimation of the number of cells infected with 1 pulse, due to a lack of accumulation of CP at detectable levels in all infected cells, and in cells infected with 4 pulses due to a general decline in protoplast viability to support virus replication.

Percentage of viable
infected protoplasts

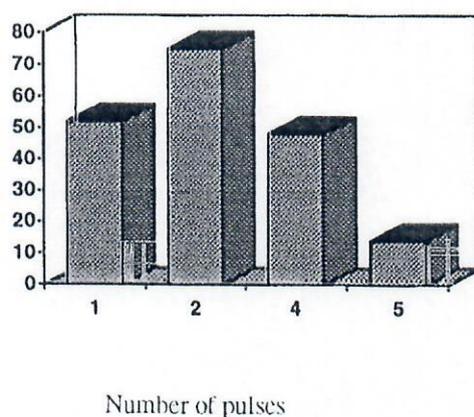


Figure 4.4b.

Effect of number of pulses (50 μ s of 0.80 kV/cm) on percentage of viable infected *N. benthamiana* protoplasts electroporated with SPFMV C1 RNA (20 μ g/ml). Assessment of virus infection by protoplast immunostaining (PIS), with SPFMV C1 CP antiserum.

An optimum SPFMV C1 RNA concentration was not determined. However, 20 µg/ml was used for all the experiments, based on the results of a preliminary experiment (20-60 µg/ml) and from previous findings on the electroporation of sweet potato protoplasts with TMV RNA (10 µg/ml, Uehara *et al.*, 1990), tobacco protoplasts with TMV RNA (10 µg/ml, Hibi *et al.*, 1989) and TVMV and PVY RNA (20-80 µg/ml, Luciano *et al.*, 1989; Gibb *et al.*, 1989).

4.3.3. Synthesis of CP and CI proteins in electroporated protoplasts

Infection of *N. benthamiana* protoplasts with virus particles (20-100 µg/ml) as assessed by PIS with antiserum to SPFMV C1 CP was achieved only when freshly purified inoculum was suspended in 10 mM Hepes buffer pH 7.2 (but not in SDW). Twenty-four h after electroporation, approximately 20% of the protoplasts gave a strong reaction with the antiserum. When higher concentrations of inoculum (150-250 µg/ml) were used, a strong reaction outside the cells was observed, probably because the high concentration of virus particles in the surrounding medium induced attachment to the protoplast wall.

Similar results were obtained with *I. batatas* protoplasts from both susceptible and resistant genotypes when inoculated with virions. At the time these experiments were performed, antisera to CI protein was not available, and an appropriate evaluation of the results was not achieved. However, it is worth noting that these results suggest that pores larger than 11 nm were formed. Infection of *I. batatas* protoplasts with TMV (Uehara *et al.*, 1990) and *Vinca rosea* and tobacco protoplasts with TMV and CMV by electroporation have also been reported, suggesting that in those cases, pores larger than 30 nm were formed (Okada *et al.*, 1986). Infection of protoplasts by electroporation with elongated particles had been considered to be difficult (Benz and Zimmermann, 1981), based on the observation that the diameter of pores produced, in protoplast membranes after an electrical pulse had been applied were calculated to be approximately 3-4 nm.

However, recent studies (Chang, 1992) on red blood cell pore size formation during electroporation has shown the formation of 20-40 nm diameter pores as soon as 3 ms after application of the electric field. After the pores had expanded to 20-100 nm in the first 20 ms, they remained more or less unchanged during the next few seconds. Pore diameters of this magnitude should be sufficient to allow most virus particles to pass through. If these later reports on pore size are found to apply also to plant protoplasts, this will explain the entry of virus particles by electroporation and be especially useful for working with viruses with RNAs which are prone to degradation and antisera to non-structural proteins are available. *N. benthamiana* protoplasts were electroporated in the presence of 20 µg/ml RNA, at an initial field intensity of 0.8 kV/cm (2 x 50 µs pulses) and sampled in triplicate at 15, 25, 35, 45, 55 and 65 h following transfection (Figure 4.5 and Table 4.5). At each time, the percentage of protoplasts reacting in PIS (Figure 4.6) with antisera SPFMV C1 CP and CI proteins (1µg/ml), showed that protoplasts were infected and supported virus accumulation and production of CI protein.

Percentage of viable
infected protoplasts

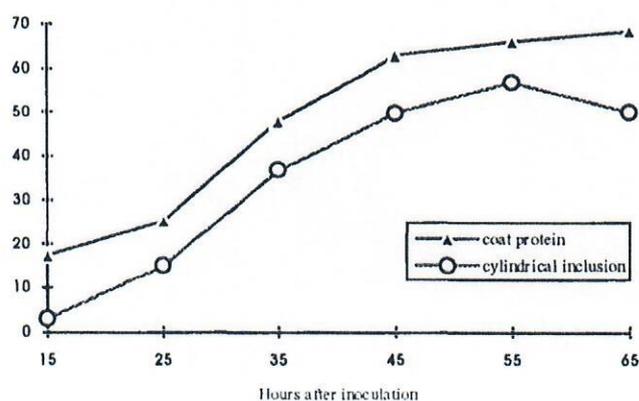


Figure 4.5.

Infection of *N. benthamiana* protoplasts with SPFMV RNA. The percentage of protoplasts reacting in protoplast immunostaining (PIS) with antisera prepared to viral CP and CI proteins (1µg/ml) is indicated. Protoplasts were electroporated in the presence of 20 µg/ml RNA, at an initial field intensity of 0.8 kV/cm (2 x 50 µs pulses) and sampled in triplicate at 10 h intervals after electroporation.

Number Experiment and sample	Coat protein (hours after electroporation)						Number Experiment and sample	Cylindrical inclusion (hours after electroporation)					
	15	25	35	45	55	65		15	25	35	45	55	65
1.1	10	25	39	52	66	69	1.1	6	25	36	45	55	40
1.2	20	30	42	65	68	72	1.2	12	16	33	40	49	48
1.3	22	16	48	80	59	64	1.3	5	18	40	49	56	42
	17.3	23.6	43	65.6	64.3	68.3		7.6	19.6	36.3	44.6	53.3	43
2.1	12	26	46	60	70	72	2.1	10	17	36	50	56	39
2.2	15	26	46	68	68	70	2.2	7	19	35	52	58	55
2.3	20	28	36	53	68	64	2.3	13	25	40	58	60	29
	15.7	26.6	42.6	60.3	68	68.6		10	20.3	37	53.3	58	41
3.1	18	29	40	62	ls	ls	3.1	12	19	40	52	59	45
3.2	20	27	48	68	ls	ls	3.2	10	22	33	56	60	42
3.3	20	26	32	59	ls	ls	3.3	10	17	38	48	60	40
	19.3	27.3	40	63	ls	ls		10.6	19.3	37	52	59.6	42.3
Avr	17.4	25.3	41.6	63.0	66.1	68.5	Avr	9.4	19.7	36.7	49.9	57.0	42.1

Table 4.5.

Percentage of *N. benthamiana* infected viable protoplasts reacting in protoplast immunostaining (PIS) with antisera to SPFMV C1 CP and CI (1 µg/ml) at different times after electroporation with 20 µg/ml viral RNA at initial field intensity of 0.8 kV/cm (2x 50 µs pulses). ls = lost sample.

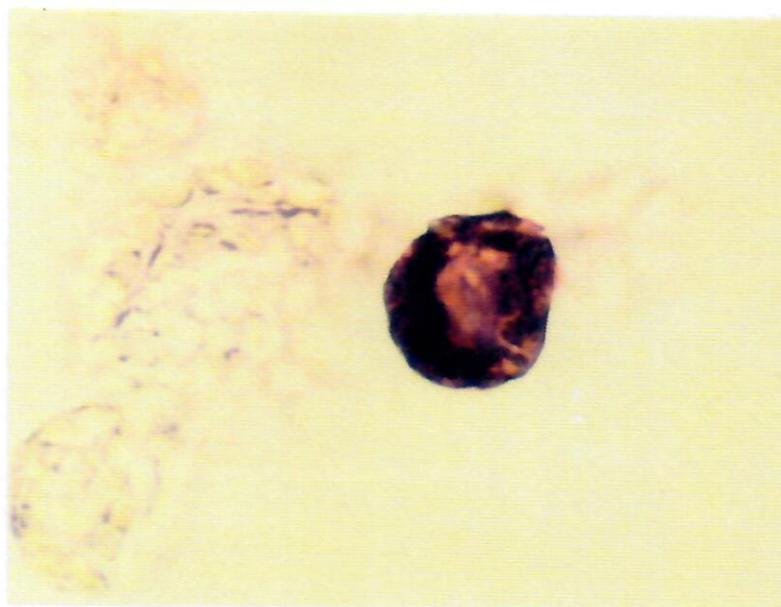


Figure 4.6

Immunostaining of *N. benthamiana* protoplasts (PIS) electroporated with SPFMV C1 RNA (20 $\mu\text{g}/\text{ml}$) at initial field intensity of 0.8 kV/cm (2x50 μs pulses). Protoplasts were collected 45 h after the electrotransfection, treated with SPFMV anti-CI protein (1 $\mu\text{g}/\text{ml}$) and goat-anti-rabbit-alkaline phosphatase conjugated antibodies. Substrates: nitroblue tetrazolium and bromochloroindolyl phosphate.

A time course of CP accumulation of infected protoplasts was determined by indirect F(ab')₂ ELISA on protoplasts electroporated with viral RNA (Figure 4.7). Aliquots in triplicate were taken 5 and 15 h after the electrical treatment. Thereafter, samples (1 x 10⁴ protoplasts/ml) were collected every 10 h up to 65 h. The CP was first detected at 15 h after inoculation, and its amount increased until about 65 h after inoculation. The yield of SPFMV CP accumulated in *N. benthamiana* protoplasts 65 h after inoculation, was estimated to be approximately 1.2 ng/10⁵ infected protoplasts. The appearance and increasing accumulation of SPFMV C1 CP, detected by indirect F(ab')₂ ELISA, provides clear evidence of the successful electrotransfection of viral RNA and its subsequent translation.

Antigen (ng)/10⁵ protoplasts

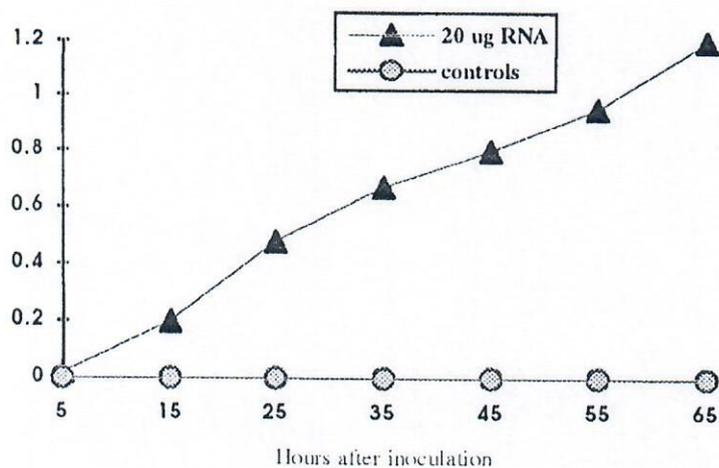


Figure 4.7.

Accumulation of coat protein (CP) in *N. benthamiana* protoplasts electroporated with 20 µg/ml SPFMV C1 RNA at an initial field intensity of 0.8 kV/cm (2 x 50 µs pulses) and sampled in triplicate at 10 h intervals after electroporation. CP accumulation was assessed by indirect F(ab')₂ ELISA with antisera to SPFMV C1 CP (1 µg/ml).

4.3.4. Infection of *I. batatas* protoplasts

I. batatas [S24 (resistant), Nemanete (resistant) Jewel (susceptible) and George Red (susceptible)] protoplasts were infected by electroporation with SPFMV C1 RNA, using the same conditions as described for the *N. benthamiana* protoplast system. Although only a limited number of experiments (2) were performed, the results presented in Table 4.4 indicated that similar numbers of protoplasts from susceptible and resistant genotypes were found to be infected (62-78%). As mentioned before, *I. batatas* S24 and Nemanete have been reported to behave as resistant hosts for SPFMV C1 at greenhouse and field level.

Percentage of viable infected protoplasts

<i>I. batatas</i> genotype	Experiment		
	1	2	Average
S24 (R)	72	80	76
Nemanete (R)	60	63	61.5
Georgia Red (S)	74	82	78
Jewel (S)	77	78	77.5

Table 4.6.

Infection of *I. batatas* protoplasts electroporated with 20 µg/ml SPFMV C1 RNA at initial field intensity of 0.80 kV/cm (2 x 50 µs pulses). Protoplasts were collected 45 h after inoculation, and protoplast infection assessed by protoplast immunostaining (PIS) with antiserum to SPFMV C1 CP (1 µg/ml).

Resistant (R), susceptible (S).

The results obtained here suggest, however, that their protoplasts support virus replication, as those from susceptible genotypes do, although the relative amounts of virus accumulation were not assessed. This might suggest that the inability to detect virus in leaves may be a result of restricted virus spread and not a result of restricted replication. Therefore, it is likely that cell-to-cell movement of virions is prevented or impaired in whole plants, either as a result of a host response to infection, that would stop the movement from the initially infected cells or inability of a transport function to operate. Whatever the mechanism(s) involved, more research needs to be done, to confirm these results and study the factors that might be influencing the transport function performed by virus-coded (or virus-induced) protein(s) in this specific virus-host combination.

Chapter 5

Molecular analysis and Sequencing of SPFMV C1 virus genome

5. Summary

Sweet potato feathery mottle virus C1 (SPFMV C1) RNA was used as a template for the synthesis of cDNA. A partial restriction map covering approximately 95% of the viral genome was constructed. The 2490 nucleotide sequence corresponding to the 6K2, NIa (VPg-NIa protease) and the 5'-terminus of the NIB coding regions, and the 1150 nucleotide sequence of the CP coding region, except for the far 5'-terminus was determined. The VPg-NIa proteinase (21 kDa + 28 kDa) was shown to contain several conserved regions which correspond to consensus protease sequence motifs. Four consensus cleavage sites recognized by the NIa protease were found at the putative junctions between the CI/6K2, 6K2/VPg, VPg/NIa and NIa/NIB proteins. The C-terminus of the NIB protein was shown to have two of the three consensus motifs found in all potyvirus RNA dependent RNA polymerases. The overall sequence of the NIa and 5'-terminus NIB proteins was 62% to 78% identical with the corresponding peptides of PVY^N, TEV, TVMV, PRSV and soybean mosaic potyvirus (SMV). The 6K2 gene protein, as in other potyviruses, is the least conserved peptide (40 to 62% identity with other potyviruses), and has a highly hydrophobic central core, as found in most membrane binding proteins. Comparisons with the reported sequences of other potyviruses indicated that the CP sequence was 94% and 78% identical at the nucleotide level and 98% and 89% at the aminoacid level to the corresponding regions of the C and RC strains respectively, while the aminoacid sequence was 60% to 72% identical to the CP protein sequence of PVY^N, TEV-HAT, PPV-NAT, BYMV, TVMV, WMV and sugar cane mosaic potyvirus (SCMV). The putative sequence motif related to aphid transmission (DAG) was found near the N-terminus of the CP protein. The 3'-terminal non-coding region of 220 nucleotides is followed by a poly(A) tract. A construct representing a large part of the carboxyl terminal region of the putative CP was expressed in *Escherichia coli* and the product was detected by specific antibodies.

5.1. Introduction

Since the first reports in 1986 of the complete genome sequences of TEV and TVMV by Allison *et al.* and Domier *et al.* respectively, the number of reports on potyvirus sequences has increased rapidly, leading to a better understanding of potyviral genome structure and expression. The complete nucleotide sequences have been determined for six more aphid transmitted viruses, PPV-R (Lain *et al.*, 1989), PPV-NAT (Maiss *et al.*, 1989), PPV-D (Teycheney *et al.*, 1989), PRSV (Yeh *et al.*, 1992), PVY^N (Robaglia *et al.*, 1989), PSbMV (Johansen *et al.*, 1991), SMV, (Jayaram *et al.*, 1992) and turnip mosaic potyvirus (TuMV, Nicolas and Laliberte, 1992), and for the fungus transmitted BYMV (Kashiwazaki *et al.*, 1991, 1990), and partial sequences for the 3'-end of the viral RNA of over 40 strains of another 20 viruses (review by Shukla *et al.*, 1991; Shukla *et al.*, 1989), including the mite transmitted virus WSMV (Niblett *et al.*, 1989). The analysis of these data has revealed unsuspected relationships between potyviruses and others groups of plant and animal viruses, allowing functions to be proposed for most of the potyviral gene products. As noted before (Chapter 1), potyviruses are quite similar, in terms of genomic structure and strategy of expression, to the bipartite como- and nepoviruses and to the animal picornaviruses (Goldbach, 1988; Domier *et al.*, 1987). The possible genetic linkage between these viruses is interesting in terms of RNA viruses evolution, because in contrast with como- and nepoviruses, the genetic organization can not easily be inferred from the picornaviral genome (Goldbach *et al.*, 1990; Goldbach and Wellink, 1988; Domier *et al.*, 1987). Among these differences are the location of the CP cistron at the 3' end of the genome, the requirement for at least three proteolytic activities and the sequence similarities between some structural and non-structural potyviral proteins and the equivalent proteins of some otherwise unrelated viruses. From the above, it is possible to suggest that recombination between viral RNA molecules is not a rare event and mixing and joining of distinct gene sets have occurred during virus evolution (Goldbach and Wellink, 1988).

Genome expression

Although the potyviruses are similar to the picorna- and comoviruses in terms of genome organization and strategy of expression, the mechanism of initiation of translation which has been demonstrated or suggested for these two last groups of viruses (Jackson *et al.*, 1990; Thomas *et al.*, 1991) seems not to operate for the potyviruses. *In vitro* and *in vivo* translation of PPV synthetic transcripts have shown that its second AUG codon at position 147 is recognized by ribosomes, probably through a conventional leaky scanning mechanism (Riechmann *et al.*, 1991). *In vitro* competition experiments permitted the proposal of the existence, within the last 63 nt of the TEV 5'-UTR of sequences that might be important for translation (Carrington and Freed, 1990). Taking the results obtained with PPV and TEV together, the specific sequences of the potyvirus 5'-UTR relevant for their translation properties seem to be confined to a very short part of these UTRs. Alternatively, it could be that these properties of the potyvirus leaders are due to the absence of strong secondary structure, as has been described for the enhancing leaders of other plant viruses (Altmann *et al.*, 1990; Jobling and Gehrke, 1987). It has been shown that TEV (Carrington and Freed, 1990) and PSbMV (Nicolaisen *et al.*, 1992) 5'-UTRs can function as enhancers of translation in a cap-independent manner in *in vitro* (RRL) and in protoplast systems and in transgenic plants. In a transient-expression assay in a protoplast system, the TEV 5'-UTR fused to the coding sequence of a reporter gene encoding β -glucuronidase (GUS), was found to induce an 8 to 21 fold enhancement effect over a synthetic 5'-UTR (Carrington and Freed, 1990). The enhancement effect of the 5'-UTRs of PSbMV and TEV were found to be similar in pea and in tobacco protoplasts, indicating a host-independent role of the potyviral 5'-UTR in enhancing gene expression (Nicolaisen *et al.*, 1992).

The single open reading frame (ORF) of the potyviral genome codes for a large polyprotein precursor of 340 to 381 kDa (Yeh *et al.*, 1992; Domier *et al.*, 1986) depending on the potyvirus, which is processed as described below.

Polyprotein processing

Collectively, the nucleotide and aminoacid sequence data, together with a number of biochemical and molecular biological studies of the potyvirus gene products, support a gene order and a mode of polyprotein processing likely to be similar to that presented for PPV in Figure 5.1. Three virus-encoded proteases, NIa protease, HC-Pro and PI have been shown to process the viral precursor polyprotein (Dougherty *et al.*, 1989, 1988; Carrington *et al.*, 1989b; Garcia *et al.*, 1990, 1989a,b; Verchot *et al.*, 1991) co- and post-translational. Soon after the P1-HC-Pro polypeptide is synthesized, it is autocatalytically released from the rest of the growing polyprotein by cleavage at the C-terminus of the HC-Pro peptide (Carrington *et al.*, 1989a). This is the first and obligatory *cis* reaction (Oh and Carrington, 1989). The rest of the growing polyprotein chain continues to elongate, and soon after the synthesis of the NIa protein, this peptide autocatalytically releases from the polyprotein by a cleavage at its N- and C- terminus (Carrington and Dougherty, 1987b). The NIa protease rapidly cleaves the polyprotein at an up stream cleavage site to release the 6K2 protein from the CI protein (Carrington and Dougherty, 1987). In a *trans* reaction, the NIa protease at the 5'-termini of the CP and CI proteins (Dougherty *et al.*, 1989; Garcia *et al.*, 1989; Carrington *et al.*, 1988, 1987), releases the NIb/CP and the P3/6K1/CI proteins, respectively.

Most proteinases can be grouped into one of four classes according to the chemically reactive groups at their active site: Ser, Cys, Asp or Zn²⁺ (Barret, 1986). In most cases, aminoacid sequences surrounding the active-site residues, as well as the spatial relationship of active-site component residues to one another, are highly conserved among members of a given class.

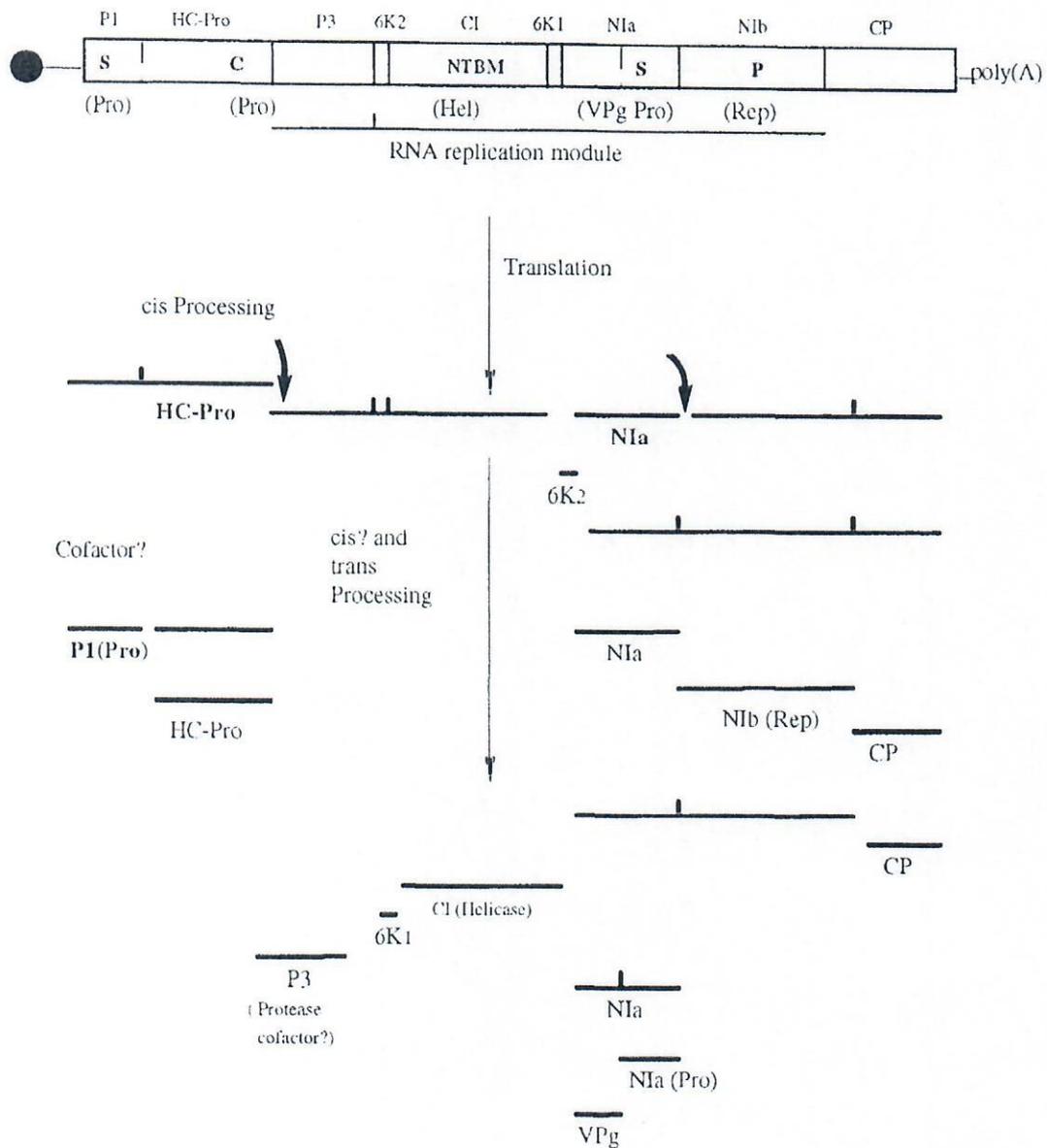


Figure 5.1

Organization of the potyvirus (eg. PPV) genome and a diagrammatic representation of the potyviral polyprotein processing. **S** = serine-type protease domain, **C** = cysteine-type protease domain, **P** = polymerase domain, **NTBM** = nucleotide-binding motif., **Hel** = helicase, **Pro** = protease, **Rep** = replicase. The possible existence of uncleaved NIa-NIb and VPg-NIa (Pro) products is indicated, as is the protease responsible for each cleavage (bold).

Serine proteases would require at least one Ser, His and Asp residue. Aspartic acid proteases would exhibit a requirement for at least two Asp residues (Tang *et al.*, 1973), while zinc-dependent endoproteases would require at least two His residues (Jongeneel *et al.*, 1989). Typical cysteine proteases would show a requirement for at least one Cys and one His residue (Polgar and Halasz, 1982). While, the viral cysteine proteases exhibit a requirement for at least one Cys, His and Asp residue (Bazan and Fletterick, 1988).

Cleavage of the N-terminus of the HC-Pro protein has been demonstrated not to be caused by either the NIa or the HC-Pro proteases (Carrington *et al.*, 1990, 1989b). Processing of the P1/HC-Pro polyprotein in TEV infected plants, transgenic plants and in a wheat germ system resulted in two products, the larger of similar M_r to the native HC-Pro (52 kDa) reacting with anti-HC-Pro serum (Carrington *et al.*, 1990), indicating that proteolysis occurred at or very near the same site in the polyprotein in each system. Point mutation, which abolished the HC-Pro activity (Carrington *et al.*, 1990), did not prevent N-terminal processing of the HC-Pro, indicating that the protease responsible for this cleavage resides in the N-terminal region of the HC-Pro or in the P1 protein. One of the TEV 35 kDa (P1) protein residues (Ser₂₅₆) targeted by mutagenesis was found to be contained in the sequence GSSG, which conforms to the conserved motif GXSG (where X is any residue), found in the active site of cellular serine-type proteases (Barret, 1986). Serine proteases also contains His and Asp residues located toward the N-terminus relative to the Ser as components of the enzymatic active center. One conserved His₂₁₄ residue and one conserved Asp₂₂₃ residue were also found upstream from the conserved Ser₂₅₆ in the TEV 35 kDa (P1) protein. Translation of TEV and TMV RNAs in a wheat germ *in vitro* system, radiochemical degradation analysis of the derived proteins and comparison of their aminoacid sequences with five other potyvirus sequences suggested that the cleavage between the P1 and HC-Pro protein occurs at Ser₂₅₆ or Ser₂₅₇ (Verchot *et al.*, 1991; Mavankal and Rhoads, 1991). Two aminoacids have been recently identified as essential for proteolytic processing (Velchot, 1991). Conserved His and Asp residues are located

upstream from the conserved Ser (HX₈-DX_{30 or 31}-GXSG). It had, therefore, been suggested that the P1 protein catalyzes peptide bond cleavage site by a mechanism similar to that of serine-type proteases (Verchot *et al.*, 1991; Lain *et al.*, 1990; Gorbalenya *et al.*, 1989).

The P1 protein behaves in a similar way to the L polypeptide of the picornavirus foot-and-mouth disease virus, catalysing its own release from the N-terminus of the nascent polyprotein (Strebel and Beck, 1986). The cleavage site for liberating the TVMV P1 and HC-Pro proteins has recently been located between Phe₂₅₆ and Ser₂₅₇ of the polyprotein, 24 aminoacids downstream from the consensus sequence FIVRG (Mavankal and Rhoads, 1991). This cleavage site in PRSV has been also found to be located 24 residues downstream from the FIVRG motif (Yeh *et al.*, 1992).

The cleavage event in *in vitro* systems occurs only to a limited extent, suggesting that it probably requires a co-factor (P3 protein), a host factor, or a specific subcellular environment for activation (Carrington *et al.*, 1990). The involvement of a host factor must be viewed reservedly, since no others members of the "picornavirus-like" superfamily (picornaviruses, comoviruses) recruit host enzymes for polyprotein processing (Krausslich and Wimmer, 1988). The P3 protein encoded by TEV RNA was found to contain a region homologous to the conserved active site sequence of the 2A proteases of picornaviruses and some similarity has been found between the N-terminal part of P3 and the 32 kDa protein of CPMV. The fact that the proteins are located in equivalent positions in their corresponding genetic maps suggests by analogy that P3 could be involved in the regulation of the proteolytic processing of the potyviral polyprotein (Lain, 1990; Vos *et al.*, 1988).

The cleavage of HC-Pro protein at its own C-terminus via an autocatalytic mechanism occurs between a Gly-Gly dipeptide (Carrington *et al.*, 1989a), and although the primary sequence features that define HC-Pro cleavage sites have not been determined, there is a good consensus around the presumed Gly-Gly substrate dipeptides of different potyviruses (TEV, PPV, PVY, TVMV and PSbMV). The HC-Pro autocatalytic processing

event seems to be, at least in RRL *in vitro* system extremely efficient, occurring immediately after ribosomes have traversed the cleavage site coding sequence (Carrington *et al.*, 1989b). The proteolytically active domain of the TEV HC-Pro protein has been localized at its C-terminal half (within a 20 kDa domain), and cleaves between a Gly₇₆₃ and Gly₇₆₄ (Carrington *et al.*, 1989a); the identification of two essential residues in this protease supports the hypothesis that the C-terminal proteolytic domain of HC-Pro most closely resembles members of the cysteine-type protease related to papain (Oh and Carrington, 1989; Barret, 1986). Although a cysteine-type protease comparable to HC-Pro is not encoded by the genomes of the otherwise related como- and picornaviruses, possible analogues have been identified as products of the coronavirus, avian infectious bronchitis virus (Gorbalenya *et al.*, 1989) and the alphavirus, Sindbis virus (Hardy and Strauss, 1989) genomes. Protease p²⁹ that bear a striking resemblance to HC-Pro, and that also autocatalytically cleaves between a Gly-Gly dipeptide, is encoded by a hypovirulence-associated virus-like dsRNA of the chestnut blight fungus, *Cryphonectria parasitica*. In addition there is a significant degree of similarity in the aminoacid sequences flanking the essential Cys and Hys residues of the two proteases and in the spacing of these residues from their respective cleavage sites (Choi *et al.*, 1991). Auto-recognition by HC-Pro involves the interaction of the enzymatic binding site with four aminoacids surrounding the Gly-Gly cleavage site. Substitutions of Tyr, Val, Gly and Gly at positions -4, -2, -1 and +1, respectively, were found to eliminate or nearly eliminate proteolysis (positions relative to the cleavage site; being the cleavage site between positions -1 and +1). Substitutions of Thr, Asn and Met at positions -5, -3 and +2 respectively, on the other hand, were permissive for proteolysis, although the apparent processing rates of some polyproteins containing these alterations were reduced. These results suggested that the auto-recognition by HC-Pro involves the interaction of the enzymatic binding site with four aminoacids surrounding the cleavage site (Carrington and Herndon, 1992).

The N1a protease is responsible for most of the potyviral polyprotein cleavage events, functioning in *trans* at several points in the potyviral polyprotein substrate, in addition to catalysing its own autocatalytic (in *cis*) release from the polyprotein (Himmler *et al.*, 1990; Martin *et al.*, 1990; Garcia *et al.*, 1989a,b; Hellmann *et al.*, 1988; Carrington and Dougherty, 1987a,b). It has been found to be related to the trypsin-like family of the cellular serine proteases, which have a catalytic triad of His, Asp (or Glu), and Cys residues (Argos *et al.*, 1984). The conserved Cys in the C-terminus of many related viral proteases is proposed to be the nucleophile of the trypsin like active site triad (Gorbalenya *et al.*, 1989). The nucleophilic Ser residue of the catalytic triad has been replaced with a Cys residue in the potyvirus protease. A similar replacement was also proposed to take place for picorna-, como- and nepoviruses proteases (Bazan and Fletterick, 1988; Gorbalenya *et al.*, 1989; Demangeat *et al.*, 1991). Replacement of the Cys with a Ser or other aminoacids in the TEV N1a protease results in a protease with no detectable activity (Dougherty *et al.*, 1989). The results of site-directed mutational analysis of the TEV 49 kDa protease gene suggest that the triad of His₂₃₄, Asp₂₆₉ and Cys₃₃₉ (Figure 5.2) is important in the catalytic activity of the protease and that the active His₂₃₄ and Asp₂₆₉ residues are separated by only 34 aminoacids. In this respect the TEV protease seems to be structurally similar to the proposed viral serine proteases of the flavivirus group in possessing a short connecting loop between the Asp and Phe which, respectively, follow and precede the predicted active site His and Asp residues (Dougherty *et al.*, 1989). This is in contrast to the 3C proteases of picornaviruses which typically possess a large linker region (44-51 aminoacids). This protease displays a unique degree of specificity towards its substrate (Rorrer *et al.*, 1992). Analysis of the N1a proteases of PPV and TEV (Garcia *et al.*, 1992, 1991; Dougherty *et al.*, 1989) suggests that the recognition and catalytic sites are closely interlinked and, although residues relevant for the correct interaction with the substrate could be present in other parts of the protein, a main determinant for substrate specificity should lie in a region situated approximately between positions 30 and 90 from the carboxyl end. This region includes the conserved His₃₆₀ of PPV or His₃₅₅ of

TEV, located 15-16 aminoacids downstream the active site Cys, which has been postulated to interact with the Gln at position -1 of the cleavage site (Bazan and Fletterick, 1988). Immunochemical and aminoacid sequence data for several potyviruses have established that the N-terminal part of NIa to be the VPg (Murphy *et al.*, 1990; Riechmann *et al.*, 1989, Shahabuddin *et al.*, 1988).

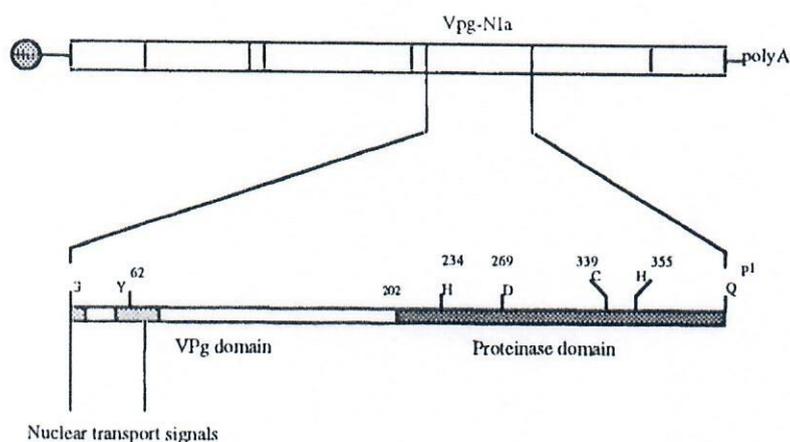


Figure 5.2.

A schematic representation of the TEV 49 kDa VPg-(serine-like protease). The TEV genome is represented at the top of the figure. The VPg has two nuclear localization signals and the Tyr₆₂ (protein-RNA linkage). The NIa has a proteolytic domain localized to the C-terminal 228 aminoacids (shade area). The aminoacid residues proposed to play a key role in the catalytic or substrate binding are indicated along with their positions in the 49 kDa protein. The active site triad is proposed to be His (H₂₃₄), Asp (D₂₆₉) and Cys (C₃₃₉). The conserved H₃₅₅ is proposed to be involved in binding the -1 Gln(Q) of the substrate.

The results of insertion and mutagenesis studies as well as the analysis of 10 potyvirus nucleotide sequences (Ghabrial *et al.*, 1990; Dougherty and Parks, 1989; Carrington and Dougherty, 1988), showed that the NIa protease recognizes a seven aminoacid cleavage sequence, with Gln at position -1 and Gly/Ser/Ala/Thr at position +1, a sequence similar to the cleavage sites reported for picomaviral 3C-like protease cleavage sites (Palmenberg, 1987; Nicklin *et al.*, 1986) and for CPMV (Wellink *et al.*, 1986). A hydrophobic aminoacid generally Val, Leu or Ile, occupies the -4 position. The analysis of the TEV

heptapeptide cleavage site within its naturally occurring polyprotein sequence has revealed that particular aminoacids must occupy certain positions for optimal cleavage (Parks *et al.*, 1992; Dougherty and Parks, 1990; Dougherty *et al.*, 1989). It was shown that Glu at -6, Tyr at -3 and Gln at -1 are required; replacement of these aminoacids at the NIb/CP cleavage site results in no cleavage or cleavage at a very low rate. The +1 position is always occupied by a Gly or Ser in TEV cleavage sites (Dougherty *et al.*, 1989). Although NIa proteases are highly homologous in their overall composition (Garcia *et al.*, 1989b), PPV, TEV and TVMV cleavage sites are efficiently recognized only by their respective proteases (Parks and Dougherty, 1991; Garcia *et al.*, 1989b), indicating that very specific interactions must take place during the proteolytic reaction. The amino acid similarity among the NIa proteins of several potyviruses is lower at their C-terminal end, suggesting that this region of the protease may be important in forming the substrate-binding pocket which allows each of the potyviral NIa proteases to recognize its own unique cleavage sequences (Ghabrial *et al.*, 1990). It has been indicated that cleavage site specificity may be a factor in the successful evolution of potyviruses as pathogens in nature (Dougherty *et al.*, 1989). Viral proteins having such stringent sequence requirements for cleavage sites are unlikely to inactivate host proteins by spurious cleavage events. The probability that they will interfere with host processes is minimized and enables them to most successfully parasitize their hosts.

The NIa protease also appears to catalyze post-translational proteolysis at an internal cleavage site, characterized recently for TEV (Dougherty and Parks, 1991), which separates the VPg and protease domains. The region implicated as the cleavage site lacked homology among different potyviruses, but had nonetheless, some of the features of potyviral sites cleaved by NIa protease. These differences might have the effect of promoting suboptimal cleavage and, in fact, this proteolytic event seems to happen in some, but not in all, the NIa molecules. There is not a consensus cleavage site sequence for the VPg-NIa junction. However, analysis of several potyviral sequences shows that

there is a Glu instead of a Gln at the -1 position and at -4 position, Val or Phe is present (Ghabrial *et al.*, 1992; Johansen *et al.*, 1991). This site is located between the VPg and the protease domains of the NIa protein, and its cleavage might therefore be related to some step of the viral RNA replication process (below). Endoproteolysis of the NIa protein has recently been demonstrated to occur in transformed *E. coli*. The data presented showed endoproteolysis of the 49 kDa NIa protein, with the ensuing release of a 22 kDa protein, which is likely to be the VPg. Fusion of the 6K2 protein with the NIa protein partially inhibited the hydrolytic reaction. Strangely, the C-terminus of the precursor protein, which would have been the NIa protease, was not detected. It was suggested that this domain may be unstable in the *E. coli* cells and was degraded by host proteases or by itself (Laliberte *et al.*, 1992).

Genome replication

The replication process of plant virus RNAs is dependent upon virally encoded proteins as well as cellular structures (Hayes and Buck, 1990), but the functions of these components are largely unknown. Some information on the role of the viral proteins can be obtained by comparison with those of the better known animal viruses. These comparisons have allowed identification of sequences that form the RNA replication module (Figure 5.1), the CI protein, the 6K1 and 6K2, the NIa and the NIb proteins and perhaps the P3 (Riechmann *et al.*, 1992).

Four consensus sequences are conserved with the same linear arrangement in RdRp and in RNA-dependent DNA polymerases encoded by plus-, minus- and double stranded viruses. One of these motifs is the "polymerase site" as defined by a GDD consensus sequence embedded in hydrophobic residues and preceded 21 to 52 aminoacids upstream by the consensus sequence (S/T)GXXXTXXXN(S/T) (Domier *et al.*, 1987). Upstream of this "polymerase site", a strongly conserved region is presented in some plus-strand RNA viruses, which among the potyvirus has the consensus DXXXFDXS sequence (Kamer and Argos, 1984). These motifs are found in the NIb protein which, based on sequence

homology, has been postulated as the potyviral RdRp (Lain *et al.*, 1989a; Robaglia *et al.*, 1989; Poch *et al.*, 1989; Dougherty and Carrington, 1988; Domier *et al.*, 1987) suggesting that this protein may be involved in the replication process.

The two small peptides 6K1 and 6K2 and the VPg-N1a proteins may also play a role in RNA replication. A stretch of hydrophobic aminoacids in the two small peptides and the relative position of the cistrons in the potyviral genetic map suggest that they could be analogous to the picornaviral 2B and 3A peptides respectively (Lain *et al.*, 1989b). Protein 2B seems to be involved in RNA replication (Johnson and Sarnow, 1991), and the function proposed for the 3A is the membrane binding protein of the poliovirus VPg precursor, protein 3AB (Giachetti and Semler, 1991). The 6K2-(VPg-N1a) proteins could be related to binding of the VPg-N1a protein to membranes and the processing of the 6K2-VPg-N1a junctions might be related to some step of the RNA replication process. Some picornavirus VPgs show a significant degree of aminoacid sequence homology, including the Tyr residue at position 62 (Figure 5.2, Argos *et al.*, 1984), with plant como- and potyvirus VPg (Domier *et al.*, 1987). The VPg of CPMV has never been detected in infected cells (Zabel *et al.*, 1984) and by comparisons with the picornavirus VPg, it was suggested that the cleavage of the VPg from its precursor and the formation of the phosphodiester linkage with the RNA, are interconnected events (Jaegle *et al.*, 1987). The involvement of the VPg (or its precursor, peptide 3AB) in RNA replication is borne out by several observations, though the precise role is still unclear (Perez Bercoff, 1987). Sequence analysis of several potyviruses VPg-N1a proteins shows that the N-terminus has a high content of aromatic and basic aminoacids (Lain 1990); this composition is typical of the consensus recognition motifs that interact with RNA (Query *et al.*, 1989). Studies on the function of the VPgs, of other viruses with similar genome organization and expression, suggest that it may be involved in RNA replication, acting as a primer (Andino *et al.*, 1990; Shahabuddin *et al.*, 1988; Takeda *et al.*, 1986), and/or acting as a nuclease during hairpin-primed RNA synthesis, cleaving a replicative form of the RNA (Tobin *et al.*, 1989;

Andrews and Baltimore, 1986), Definitive proof for either of these functions, however has not been obtained for potyviruses VPg protein.

The CI proteins of potyviruses (Lain *et al.*, 1989; Robaglia *et al.*, 1989; Poch *et al.*, 1989; Dougherty and Carrington, 1988; Domier *et al.*, 1987) contain aminoacid sequence motifs common to animal flavi-, hepatitis C- and pestivirus NTBM- containing proteins (Gorbalenya and Koonin, 1989; Lain *et al.*, 1989, Domier *et al.*, 1987). Further aminoacid sequence comparisons with several proteins of viral and cellular origin, such as the eIF-4A factor, suggested that these NTBM-containing viral proteins possess a conserved consensus motif GXXXGXKS and a nucleoside triphosphate-dependent RNA unwinding activity (Lain *et al.*, 1989; Gorbalenya *et al.*, 1988). As suggested from these sequence similarity studies, the PPV CI protein has been shown to interact with RNA and ATP and be able to unwind RNA duplexes. This activity was found to be dependent on the hydrolysis of NTP to NDP and Pi, and thus it can be considered as an RNA helicase activity. In the *in vitro* system used, the CI protein was able to unwind only double stranded RNA with 3' single strand overhangs, showing that the helicase activity of the CI protein functions in the 3' to 5' direction (Lain *et al.*, 1991, 1990). Its possible function may include unwinding of replicative intermediates, genome recombination or unwinding of ssRNA secondary structures, facilitating transcription or translation (Lain *et al.*, 1990). This was the first report of a helicase activity associated with a protein encoded by an RNA virus. Although still scarce, experimental data suggest that the NTBM-containing proteins of positive strand RNA viruses are involved in their genome replication (Li and Baltimore, 1988). In the case of polio- and comovirus, one of the particular functions postulated for these proteins in the replication mechanism is to attach the replication complex to membranes (Bienz *et al.*, 1987; Goldbach and Wellink, 1988). Furthermore, the NTBM-containing 2C protein of poliovirus also seems to be involved in the release of newly synthesized positive sense RNA molecules from the replication complex (Li and Baltimore, 1988).

Potyviral proteins

The greatest similarity between potyviral proteins and those of como- and picornaviruses is between the CI, 6K2, NIa and NIb proteins and the membrane-binding replicase, VPg-protease and RdRp of comoviruses and 2C, 3C and 3D proteins of picornaviruses respectively (Domier, 1987; Allison *et al.*, 1986).

Although the overall sequence identity between distinct viruses is relative low compared with that found between strains, an optimal alignment of the protein sequence of distinct viruses showed that the most conserved regions among them are the CI, NIa, NIb and the CP proteins, while the P1 protein is the most variable gene product (Table 5.1). The only regions in the P1 protein of significant identity between distinct potyviruses are the tetrapeptide MSTI at or near the N- terminus of all but TEV (Shukla *et al.*, 1991) and the FIVRG pentapeptide, which is located in the C-terminal region of all but PSbMV (Johansen *et al.*, 1991). Within the HC-Pro sequence there are three broad regions of strong identity between the different sequences, which span almost the entire HC-Pro protein. The first extends for about 30 residues and starts 50 residues from the FIVRG sequence. The second is about 180 residues from the FIVRG sequence and extends for approximately 100 residues, while the third covers a region of 150 aminoacids at the carboxyl terminus of the HC-Pro. The P3 protein is the second most variable product, showing only restricted regions of sequence identity between the distinct viruses. The most variable region in this protein is a domain which spans 30 residues near the C-terminus of the protein. Upon comparison of the P3 protein (42 kDa) of two strains of SMV (Jayaram *et al.*, 1992) an increase of the local net charge at positions 15 to 29 and a decrease at positions 328 to 340 were reported to be evident. Although their significance is unknown, differences in local net charge have been proposed to affect electrostatic interaction between a host factor and non-structural viral proteins involved in resistance and susceptibility (Meshi *et al.*, 1988).

The exact localization of the N- and C- termini of some potyviral CI proteins has not been established, since they could be generated at one of two protease recognition motifs

(Lain *et al.*, 1989). Although the two predicted small peptides (6K1 and 6K2) delimited by two amino and two carboxyl putative cleavage sites are almost identical in size, significant sequence similarity between the two peptides of three different viruses was not detected, except for the presence of a central hydrophobic region common to both peptides in PPV, TEV and TVMV (Lain *et al.*, 1989). The localization of these two peptides in the potyviral genome resembles that of polypeptides 2B and 3A of poliovirus (Lain *et al.*, 1989). Searching for sequence similarity between these poty- and polioviral proteins, the only common feature found was a stretch of hydrophobic aminoacids proximal to the C-terminus of the 6K2 peptide. The predicted sequences of both peptides of TEV, PPV and TVMV displayed a high percentage of identical aminoacids, as exhibited by the core CI region.

Virus

Protein	SMV ^{G7} /SMV ^{G2}	PRSV/TEV	PRSV/TVMV	PRSV/PVY	SMV ^{G7} /TEV	SMV ^{G7} /PVY
P1	94.0	14.3	10.4	15.0	9.0	13.0
HC-Pro	98.0	48.0	52.0	50.0	43.0	37.0
P3	94.0	32.0	32.0	31.0	25.0	17.0
CI	96.0	54.3	50.1	54.2	51.0	52.0
6K2	100.0	35.1	35.0	35.1	45.0	32.0
VPg	99.0	46.0	54.0	45.3	46.0	45.0
NIa prot.	98.0	47.0	43.4	40.0	34.0	35.0
NIb	99.0	59.0	60.3	58.4	55.0	56.0
CP	99.0	56.2	51.0	57.0	60.0	53.2

Table 5.1

Percentages of sequence identity between predicted virus coded proteins of two strains of SMV (G7 and G2) and between distinct potyviruses: PRSV/TEV, PRSV/TVMV, PRSV/PVY^N, SMV/TEV and SMV/PVY.

References: SMV G7 and G2 : Jayaram *et al.*, 1992; PRSV-HA : Yeh *et al.*, 1992; TEV : Allison *et al.*, 1986; TVMV: Domicr *et al.*, 1986 and PVY^N : Robaglia *et al.*, 1989.

The region of the TuMV polyprotein containing the last 52 aminoacids of P3, all the 6K1 and the first 53 aminoacids of the CI was aligned with that of five potyviruses (PPV, TEV, PVY, TVMV and PSbMV) and the percentage of identity was calculated to be 30%, 83% and 64% for P3, 6K1 and CI respectively. Some stretches of the CI core region showed identity values as high as 80% (Jayaram *et al.*, 1992; Shukla *et al.*, 1991; Lain *et al.*, 1989). The most prominent feature of the alignment is the so-called NTP binding motif (Gorbalenya *et al.*, 1988; Lain *et al.*, 1989) which has been identified in all potyviruses genomes.

For the NIa, the most striking features are five conserved regions containing the two NLSs (Chapter 3), the Tyr residue for VPg attachment to the viral RNA and the protease active sites (above). The NIb is the most conserved gene product. At the N-terminus of the protein, it contains a region, extending about 270 residues, with three consensus sequence motifs in the RdRp domain (above).

The CP is the most characterized gene product of potyviruses and sequence comparisons show that the N-terminus of distinct potyviruses can vary considerably in length (30-95 aminoacids) and sequence, whereas the C-terminal two thirds of the protein are highly conserved. The N-terminus is the only region in the entire CP that is unique to a distinct potyvirus, it is immunodominant and contains virus-specific epitopes (Ward and Shukla, 1991, 1989). The surface-exposed C-terminus is only 18 to 20 aminoacids in length (Shukla and Ward, 1991; Kashiwazaki *et al.*, 1989). Comparisons of the CP aminoacid sequence (Ward and Shukla, 1991, 1989; Shukla and Ward, 1988) and the conserved 3'-UTR (Frenkel *et al.*, 1992, 1989) have led these authors to propose that sequence of this region can be used to identify and differentiate distinct potyviruses and their strains. Analysis of the complete CP sequence of over 30 potyviruses and their strains showed a bimodal distribution of sequence homology. The degree of aminoacid sequence identity between distinct members ranged from 38 % to 71%, while that between

distinct strains of a virus ranged from 90% to 99%. The analysis of these data has allowed confirmation of the status of several strains and distinct potyviruses [eg. WMV-1 and WMV-2 were considered strains of the same virus, but CP sequence data has reinforced the conclusion reached using biological, serological and cDNA hybridization data that WMV-1 is a strain of PRSV (Abu-Samah, 1985)]. However, it is interesting to note that the recent molecular data have not changed the potyvirus group status of individual viruses, apart from confirming the membership of the group of non-aphid transmitted viruses (Shukla *et al.*, 1989b). In contrast, the sequence data have had a dramatic effect on the development of criteria to distinguish distinct potyviruses from strains (Jain *et al.*, 1992; McKern *et al.*, 1991a,b; 1990)

Besides its protective role for the genome, there is a specific involvement of a conserved (I/V)DAG block present at the N-terminus of CP, in virus transmission by aphids (Gal-On *et al.*, 1990; Maiss *et al.*, 1989; Eggenberger *et al.*, 1989; Harrison and Robinson, 1988). This hypothesis has been recently confirmed experimentally through the use of full-length TVMV and zucchini yellow mosaic potyvirus (ZYMV) cDNA clones from which infectious transcripts have been synthesized. For TVMV, a single aminoacid change that alters the DAG block to DAE rendered the virus non-aphid transmissible (Atreya *et al.*, 1990) and for ZYMV, aphid transmissibility was restored by changing a single aminoacid in a corresponding region (Gal-On *et al.*, 1992).

The aims of this work were to determine the nucleotide sequence of the 3'-terminal region of SPFMV C1 (as much as the time allowed). The RNA of the C strain of SPFMV was reported to be longer than that of most potyvirus genomes (Cali and Moyer, 1985) and it was interesting to determine if this apparently longer RNA reflects significant differences at nucleic acid and/or aminoacid level in comparison with other potyviruses. By the end of this work, the sequence of the CP gene of two isolates of this virus which do not infect *N. benthamiana*, as the C1 isolate does, had been published (Abad *et al.*,

1992). It was of interest to compare these CP sequences, because of the differences in host range, serological relationships and symptomatology in *Ipomoea spp.* Determination of the nucleotide sequence should assist in progressing towards the understanding of the behaviour of this important virus in sweet potatoes, and provide the tools for future studies on aspects of its biology and the development of transgenic virus resistant plants.

5.2. Materials and Methods

5.2.1. SPFMV C1 RNA:

Purified viral RNA for cDNA synthesis was prepared according to the method described in Chapter 2, by using sucrose density gradients.

5.2.2. Antisera:

Monoclonal antibody (PTY-1), which reacts with the CP of a wide range of potyviruses (Jordan and Hammond, 1986), was kindly provided by Dr. Ramon Jordan (USDA-BARC, USA).

5.2.3. Cloning and transcription vectors

1. Riboprobe Gemini System series: GEM -4Z plasmid (2746 bp), pGEM -3Z plasmid (3199 bp) and pGEM -3Zf(+) plasmid (3199 bp). They all have a multicloning site, β -lactamase (Amp^R) coding region, the gene encoding the lacZ-a-peptide, which allows recombinants to be selected using blue/white color screening, SP6 and T7 promoters for direct transcription and binding sites of pUC/M13 forward and reverse sequencing primers for dsDNA direct sequencing. The pGEM-3Z and -4Z vectors are identical except for the orientation of the SP6 and T7 promoters. The pGEM -3Zf(+) is similar to the previous ones, but can also be used for the production of circular ssDNA due to the presence of the origin of replication of the filamentous phage f1.

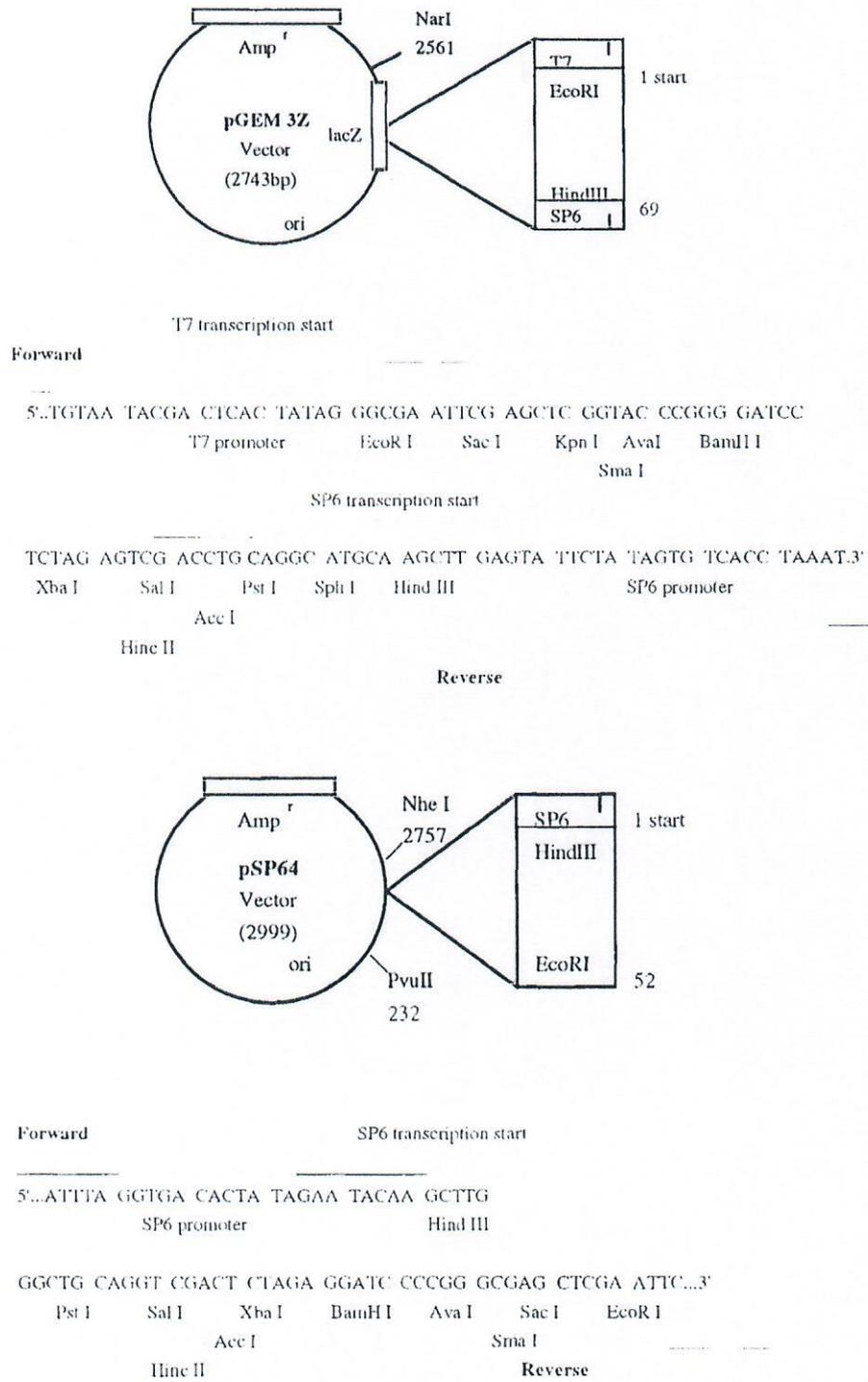


Figure 5.3 : Physical maps of pGEM 3Z and pSP64, showing the multicloning sites, promoters, priming sites, and transcription orientation.

2. pSP series: pSp64 (2999 bp) and pSP65 (3005 bp). They can be used as standard cloning vectors and can also be used for *in vitro* transcription from the SP6 promoter. The pSP64 and pSP65 vectors are nearly identical except for the orientation of their multicloning site regions. They both have the b-lactamase (Amp^R) coding region, and binding sites for pUC/M13 reverse primer.

5.2.4. Primers

Primer	Orientation	Size	Sequence
SP6 primer/promoter:	N/A	19 mer	5'-d(GATTTAGGTGACACTATAG)-3'
T7 primer/promoter:	N/A	20 mer	5'-d(TAATACGACTCACTATAGGG)-3'
pUC/M13 primer:	Forward	24 mer	5'-d(GCCAGGGTTTTCCCAGTCACGAC)-3'
pUC/M13 primer:	Reverse	22 mer	5'-d(TCACACAGGAAACAGCTATGAC)-3'
SP13.7T7	Forward	20 mer	5'-d(CGCTGGGATGGCAATCTTGT)-3'
SP13.7T7Re	Reverse	20 mer	5'-d(ACAAGATTGCCATCCCAGCG)-3'
SP13.7SP6	Forward	24mer	5'-d(GTGGAGCAACCTCAACCGTCGTGG)-3'
SP13.7SP6Re	Reverse	24mer	5'-d(CCACGACGGTTGAGGTTGCTCCAC)-3'
SP14EB4	Forward	20 mer	5'-d(CTAGGTTTCGAGATGCACGC)-3'
SP8SP6Re	Reverse	20 mer	5'-d(CGCAAGCTTGTTTGGGATCC)-3'

5.2.5 Bacteria

5.2.5.1 Strains

Strain	Genotype	Reference
<i>E. coli</i> JM 83	F ⁻ , ara, D (lac-proAB), rpsL, φ 80 d lacZ Δ M15	Yanisch-Peron <i>et al.</i> (1985).
<i>E. coli</i> JM 109	end A1, recA1, gyrA96, thi, hsdR17, (rK ⁻ , mK ⁺), relA1, supE44, Δ(lac-proAB), F ⁺ , traD36, proAB, lacI ^q ZΔM15]	Yanisch-Peron <i>et al.</i> (1985).

5.2.5.2 Culture and maintenance

For small scale DNA purification, a single colony was grown in TY, Amp₅₀ [0.4% Tryptone, 0.5% Yeast, 0.5% NaCl, pH 7.6, Ampicillin, 10 µg/ml (4-5 ml/tube)] with strong agitation (150-200 rpm) at 37 °C for 16-18 h. For medium term conservation, bacterial cells grown in liquid media were plated by the streaking technique (Manniatis *et al.*, 1982) in TY containing 1.5% agar and ampicillin. For long term storage, freshly grown overnight cultures (850 µl) were mixed with 150 µl sterile glycerol and immediately stored at - 70° C.

5.2.5.3 Competent cells

Bacterial cells were made competent for transformation according to the method of Nishimura *et al.* (1990). Medium A [LB broth (1% bacto-tryptone, 0.5% yeast and 1% NaCl, pH 7.5), supplemented with 10 mM MgSO₄ 7H₂O and 0.2% glucose] (50 ml) was inoculated with 0.5 ml of an overnight culture and cells grown with aeration for 3-4 h (mid logarithmic phase). The cells were kept on ice for 10 min, then pelleted at 1500 g for 10 min at 4°C, and the pellets gently resuspended in ice cold medium A (0.5 ml). Then, 2.5 ml of storage solution B [36% glycerin, 12% PEG (MW 7500), 12 mM MgSO₄ 7H₂O added to LB-broth (pH7.0)] was added, and mixed well without vortexing. The competent cells were divided into aliquots (100 µl) and stored at - 70°C. Rapid freezing in dry ice/ethanol was not needed; however cells must be handled gently on ice during all processes before freezing.

5.2.5.4 Transformation of competent bacterial cells

JM83 and JM 109 competent cells were transformed by a procedure based on the method described by Mandel and Higa (1970). An aliquot of competent cells (50-100 µl) was thawed on ice and immediately mixed with DNA (5-10 µl, 20-50 ng) before leaving on ice for 90 min. The cells were then heat-shocked at 42°C for 2 min, chilled on ice for 1-2 min, mixed with prewarmed (37 °C) nutrient broth (400 µl) and incubated at for 1 h

at 37 °C with strong agitation (200 rpm), to allow expression of ampicillin resistance. Cells (50-100 µl) were plated out onto nutrient broth agar plates containing ampicillin (50 µg/ml). For white/blue selection, 100 mM β-D-isopropyl-thiogalactopyranoside¹ (IPTG; 238 µg/100µl water) and 2% 5-bromo-4-chloro-3-indolyl-β galactopyranoside² (X-Gal; 800 µg/40µl dimethyl formamide) were plated immediately before the bacterial cells. Plates were incubated at 37 °C for 18 -20 h.

1. IPTG, functioning as a lac analog, binds and inhibits the lac repressor which serves to induce β-galactosidase activity of vectors containing the lacZ gene.
2. X-gal, chromogenic substrate for β-galactosidase. Bacterial colonies harboring recombinant pGEM-Z vector constructs are white.

5.2.6 Plasmid DNA purification (small scale)

The method is based in a protocol described by Maniatis *et al.* (1982) and modified by M. Querci (CIP, Lima, Peru). An aliquot of an overnight transformed bacterial culture (1.5 ml) was centrifuged for 2 min. The supernatant was removed by aspiration and the pellet was resuspended by vortexing in a freshly prepared ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris HCl, pH 8.0, 4 mg/ml lysozyme (100 µl). After standing at room temperature for 5 min, a freshly prepared alkaline solution (200 mM NaOH, 1% SDS; 200 µl) was added, the contents mixed by inverting the tube, and incubated on ice for 5 min. An ice cold solution of 5M potassium acetate pH 4.8 (150 µl) was added and mixed by vortexing, before incubation on ice for 10 min. The suspension was then clarified by centrifugation for 5 min. To remove remaining proteins, an equal volume of phenol:chloroform (1:1, v:v) was added, mixed by vortexing and the phases separated by centrifugation for 2 min. The supernatant was treated with EtOH (1:2, v:v) and left at room temperature for 10 min before pelleting the nucleic acids by centrifugation for 5 min. The pellet was resuspended within SDW (360 µl) containing RNase (7.5 µg) and incubated at 37 °C for 15 min. Plasmid DNA was precipitated with 5 M KAc (40 µl) and EtOH (1 ml) at - 70° C for 1 h, centrifuged for 10 min and the pellet washed with 70% EtOH. The final pellet was vacuum-dried and resuspended in SDW or T₁₀E₁ (10 mM Tris HCl, 1 mM EDTA, pH 8.0) (30-40 µl).

If larger amounts of same DNA was required, several aliquots were processed, following the same protocol described above. When DNA from a large number of clones was prepared for rapid screening by agarose gel electrophoresis, bacterial cells in 0.5 ml TY media were grown for 5-6 h, and the DNA purified as above, by using proportional amounts of each reagent. The RNase treatment was omitted and the final DNA preparation was resuspended in SDW (10-15 μ l).

5.2.7. General techniques for DNA manipulation

The protocols were mainly according to Maniatis *et al.* (1982) and Sambrook *et al.* (1989).

5.2.7.1. Restriction enzyme digestion

Endonuclease restriction enzymes were obtained from Gibco-BRL, Promega and NBL. For DNA analysis the following reagents were mixed; DNA (1 μ g), restriction enzyme (1-2 u), 10X RE buffer (commercially made buffers, 2 μ l). The volume was made up to 20 μ l with SDW, and the mixture was incubated at 37 °C for 2-5 h.

5.2.7.2. Dephosphorylation/Phosphorylation

Alkaline phosphatase catalyzes the hydrolysis of 5'-phosphate groups from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. Plasmid DNA (1 μ g) was mixed with 10X calf intestinal alkaline phosphatase (CIAP) buffer (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 0.1 mM ZnCl₂, 50 mM KCl and 50% glycerol; 10 μ l) and CIAP (1 μ l, 0.01U/pmol ends) and the volume made up to 100 μ l with SDW. The mixture was then incubated at 37 °C for 30 min. An additional 1 μ l CIAP was added and incubation continued for 30 min. The reaction was stopped with 10 mM TrisHCl, pH 8.0 (70 μ l), 10% SDS (10 μ l) and 100 mM EDTA (20 μ l).

Polynucleotide kinase (PNK) catalyzes the transfer of the γ -phosphate from ATP to the 5'-terminus of polynucleotides or to mononucleotides bearing a 3'-phosphate group.

To plasmid DNA (400 pmol; 3 μ l) was added kinase buffer (10X; 700 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT, 1.5 μ l), ATP (1mM; 1 μ l) and SDW (8.5 μ l). The mixture was incubated at 37^o C for 30 minutes, then at 90^oC for 3 min and chilled on ice.

5.2.7.3. Klenow reaction

The DNA polymerase I large fragment (Klenow) lacks the 5'-3' exonuclease activity of intact DNA polymerase I but retains the 5'-3' polymerase activity, the 3'-5' exonuclease and the strand displacement activities. It is used for filling-in or labelling 3' ends of double stranded DNA, second strand synthesis and DNA sequencing by the dideoxy method. In a typical reaction, plasmid DNA (2 μ g/16 μ l SDW) was mixed with dNTPs (3 μ M; 3.0 μ l), Klenow DNA polymerase (1u; 0.6 μ l) added and the final volume made up to 30 μ l with SDW. The mixture was incubated at room temperature for 30 min and then at 70^oC for 10 min to inactivate the enzyme.

5.2.7.4. Ligation and religations

T4 DNA ligase catalyzes the joining of two strands of DNA between the 5'-phosphate and 3'-hydroxyl groups of adjacent nucleotides in either blunt-ended or cohesi-ended configuration. For ligation of purified cDNA with plasmid vector, the following reagents were mixed in a microfuge tube: insert cDNA (0.5-1 μ g/7.0-8.0 μ l), linearized vector (200 ng/2.0 μ l) and SDW up to 14.2 μ l. The mixture was incubated for 10 min at 65^oC. Samples were chilled on ice for 2 min and 25 mM ATP (0.8 μ l), 5X ligase buffer (4 μ l) and T4 DNA ligase (1u/1 μ l), were added. The reagents were gently mixed, centrifuged and incubated for 16 h at 14-15^oC. For blunt-ended ligations, T4 DNA ligase was increased up to 1.5 μ l. For direct ligation of DNA in low melting point agarose gel (below, 5.2.9.2) (M. Querci, personal communication), the segment of gel containing the DNA was mixed with 20 mM Tris HCl, pH 8.0 and 1 mM EDTA (1:1, v:v), heated at 65^oC for 10 min and an aliquot (8-10 μ l) mixed with linearized vector (100-200 ng; 1-2 μ l). The

mixture was heated at 65°C for additional 10 min, pre-warmed (65°C) 5X ligase buffer (4 µl) added and the mixture cooled down at room temperature for 2-3 min, before adding T4 DNA ligase (1 µl). The mixture was then incubated for 16-18 h at 14-15 °C. Religations were carried out following the same procedure described above, but without addition of vector DNA, and the mixture incubated at room temperature for 3-4 h.

5.2.8. cDNA synthesis

Most of the sequence information was obtained from cDNA synthesized by using a cDNA Plus synthesis system (Amersham) and a Riboclone System (Promega), according to the method introduced by Okayama and Berg (1982) and modified by Gubler and Hoffman (1983).

First strand synthesis: Viral RNA (5 µg) was mixed with either oligo (dT) primer, random primers or SPFMV C1 genome sequence specific primers (5.0 µg), diluted with nuclease-free water up to 15 µl. The mixture was heated at 65 °C for 10 min to denature the RNA and allow primer annealing. The mixture was then quickly cooled down on ice and after centrifugation, the following reagents were added: 5X first strand buffer (250 mM TrisHCl, pH 8.3, 250 mM KCl, 50 mM MgCl₂, 50 mM DTT, 5 mM dNTPs and 2.5 mM spermidine; 7 µl), RNase inhibitor (40U; 1 µl), 100mM DTT (3.5 µl) and 10mM dNTPs (3.5 µl). The reagents were gently mixed, centrifuged and the tube incubated at 37 °C for 2 min. Superscript, [(M-MLV-H-RT) murine moloney leukaemia virus H reverse transcriptase (Gerard *et al.*, 19), (BRL) 25U/µg RNA] and SDW up to 35 µl was added and mixed by gently flicking the tube. To prepare a tracer reaction, 5 µl of the mixture was removed to another tube containing ³²P-dCTP (5 uCi, 400 Ci/mmol). Both reactions were incubated at 37°C for 60 min. To the tracer reaction, 2 mM EDTA (2 µl) and H₂O (93 µl) was added, and both reaction tubes stored on ice until needed.

Second strand cDNA synthesis: For a 100 µl ds cDNA synthesis reaction the following reagents were added, to 30 µl of the first strand synthesis mixture: second strand buffer (10X; 400 mM TrisHCl, pH 7.2, 2.9 M KCl, 30 mM MgCl₂, 0.5 mg/ml BSA and

30 mM DTT) (10.0 μ l), *E. coli* DNA polymerase I (25u), *E. coli* RNase H (1.5U) and nuclease-free water up to final volume of 100 μ l. To prepare a tracer reaction, 5 μ l was removed to another tube containing 32 P-dCTP (5 uCi, 400 Ci/mmol). Both reactions were incubated at 14 $^{\circ}$ C for 2 h and samples heated at 70 $^{\circ}$ C for 10 min. After spinning down, tubes were placed on ice and T4 DNA polymerase (2u/ μ g RNA) was added, the mixture incubated at 37 $^{\circ}$ C for 10 min and the reactions stopped by adding 10 μ l 200 mM EDTA.

Each DNA preparation was made up to a final volume of 200 μ l and mixed with phenol:chloroform (1:1, v:v), centrifuged for 5 min and the supernatant transferred to a fresh tube. This step was repeated twice, and to the final supernatant, tRNA (20 μ g; 1 μ l) was added to improve cDNA recovery. The nucleic acids were precipitated with 7.5M ammonium acetate (1:1, v:v) and EtOH (2.5:1, v:v), incubating at -70 $^{\circ}$ C for 2 hours. After centrifugation for 15 min, the pellets were washed with 70% EtOH and dissolved in 20 μ l of $T_{10}E_1$.

The size distribution of cDNA synthesized in the first and second strand reactions was determined by electrophoresis in a 1% agarose gel. Approximately equivalent numbers of incorporated cpm for each strand and a 32 P-labelled λ DNA markers. [Radiolabelled markers were made by using λ DNA fragments digested with EcoRI/Hind III, in a fill-in reaction with Klenow DNA polymerase. The reaction was performed by mixing EcoRI/HindIII 10x buffer (2.5 μ l), 0.2mM dATP (0.5 μ l), 0.2mM dGTP(0.5 μ l), 32 P-dCTP (2 μ Ci), λ DNA markers/EcoRI/HindIII (1 μ g), Klenow DNA polymerase (1U) and SDW up to a final volume of 25 μ l. After incubation at room temperature for 10 minutes, the reaction was stopped with 200 mM EDTA (2.5 μ l).

For cloning, both cDNA and vector DNA were digested with the appropriate restriction enzyme to generate compatible ends, treated and ligated with the vector, following the protocols described above (5.2.7).

5.2.9 Electrophoresis

5.2.9.1 Agarose gel electrophoresis

The procedure followed was described above (Chapter 2, 2.2.7) with the following modifications. DNA (0.2-1.0 μg) diluted in water or $T_{10}E_1$ was mixed with sample loading buffer SALB [0.15% bromophenol blue, 0.15% xylene cyanole, 0.2% Orange G, 60% sucrose, 50% TBE, (1:10 v:v)].

5.2.9.2 Low melting point agarose gel electrophoresis

Low melting point agarose (1.0-1.2%, BRL-Ultrapur) was dissolved in TAE (40 mM Tris, acetate, 1mM EDTA, pH 7.8). The gel was cast at 4 °C and DNA samples mixed with loading buffer (above). The electrophoresis was carried out with buffer recirculation, at 75 V and 4 °C.

5.2.9.3 Polyacrylamide gel electrophoresis

To a solution containing 1.66 ml acrylamide:hisacrylamide (29:1, w:w), 10% 10X TBE and 0.8% ammonium persulfate (10% stock), TEMED (0.09%), was added and the gel immediately poured between glass plates to form a vertical slab gel (800 mm x 550 mm x 0.4mm). Well formers were inserted and the gel left to polymerise for at least 1 h before assembling the system to be run in TBE. DNA samples (8 μl , 50-100 ng) and a DNA molecular weight marker (PVB6/Hae III, 51-587 bp, 1 μg ; 0.5 μl) mixed with 2 μl SALB were loaded into wells. The gel was run at 100 V until the bromophenol blue dye had migrated to the bottom of the gel. The gel was then removed from between the plates and stained for 5-10 min in 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide in H_2O .

5.2.10. Southern blotting

Transblotting of digested DNA (Southern, 1975) was done according to the protocol described by Sambrook *et al.* (1989). Hybridization of transblotted nucleic acids with a riboprobe was done as described below (5.2.11)

5.2.11. Identification of recombinant clones

Recombinant plasmids selected by white/blue selection [because Close *et al.* (1983) reported that not all insertions into the β -galactosidase fragment of plasmid vector result in white colonies, a portion of light blue colonies were also tested] were screened by nucleic acid hybridization, using either a SPFMV C1 RNA probe (Riboprobe, Promega) or a probe generated by nick translation of a cloned plasmid DNA (Nick translation reagent kit, BRL) on dot or Southern blots. In both cases, the probes were prepared according to the manufacturer instructions. For riboprobe and nick translation radioactive labelling, (^{32}P -UTP; 10 mCi/ml) or (α ^{32}P -dNTP; 800Ci/mmol) were used respectively.

Prehybridization and hybridization conditions were performed according to the protocols in use at CIP, Lima, Peru (Department of Plant Pathology) for routine virus detection (based on Salazar *et al.*, 1988). Prehybridization for the nick translation probe was carried out at 45 °C for 2 h in 5X SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 50 mM NaPO_4 , pH 6.5, 0.1% Denhardt's solution (0.1% Ficoll 40, 0.1% polyvinylpyrrolidone and 0.1% BSA), 2.5 mM EDTA, 0.6% SDS, 5% dextran sulphate and denatured herring sperm DNA (100 $\mu\text{g}/\text{ml}$; 12 μl). Hybridization solution containing the nick translation probe (10⁴ cpm/ml) was added to the prehybridization buffer solution and membranes incubated overnight at 45°C. Membranes were then washed twice for 15 min with 2X SSC and 0.1% SDS at room temperature, once for 30 min with 0.2X SSC and 0.2% SDS at 37°C and once for 30 min with 0.1X SSC and 0.2% SDS at 55°C. Finally, the membrane was rinsed with 0.1X SSC for 5 min at room temperature.

Prehybridization for the riboprobe was carried out at 45 °C for 10 min in 50% formamide, 0.125% SDS and 25 mM sodium cacodylate. Calf-thymus DNA (0.625 mg/ml) was added and incubated for an additional 10 min at 45°C before riboprobe (4 x 10⁵ cpm/ml) was added and the membrane incubated at the same temperature overnight. Membranes were then washed twice for 20 min at room temperature with 360 mM NaCl,

20 mM TrisHCl, pH 7.5 and 0.1% SDS, once for 30 min at 55°C in 0.1X SSC, 0.1% SDS, twice for 10 min at room temperature in 2X SSC, once for 20 min in 2X SSC, RNase (2 µg/ml) and once for 20 min at 50°C in 0.1X SSC, 0.1% SDS. The autoradiographic exposure of the membrane with Kodak X ray film, with intensifier screen was at - 70°C for 24 h.

5.2.12. Expression of fused proteins in *E. coli*

To verify the authenticity of the SPFMV C1 partial CP gene clone, a blunt-ended insert of clone pFMVC1-13, which represents 80% of the viral CP gene, was fused in-frame to the 5' end of the lacZ gene (Guarente *et al.*, 1980) of the pGEM4Z plasmid [based on the T7 expression system developed by Studier (1986)]. The resulting sequence cloned into the pGEM 4Z vector was specifically expressed as a hybrid protein in *E. coli* JM 109. Serological analysis of the expressed proteins was carried out by probing Western blots of the fractionated proteins or dot blots of bacterial extracts with SPFMV C1 CP PAbs and PTY-1 MAbs. The procedure followed was as described by Helfman *et al.* (1984; 1983) except that GAR or RAM antibodies conjugated to alkaline phosphatase were used instead of ¹²⁵I-labelled second antibody. Detection of antibody reactions was followed by development in NBT and BCIP. Overnight cultures (usually in 5 ml LB) were induced by addition of 2mM IPTG, and incubated for an additional 1-4 h at 37 °C; control cultures were grown in the absence of IPTG. The bacterial cells (1 ml) were collected by centrifugation and resuspended in 100 µl of lysis buffer (50 mM TrisHCl, 5mM MgCl₂, 150 mM NaCl, pH 7.5, 3% BSA and 40 µg/ml lysozyme), incubated at room temperature for 30 min, frozen at - 20°C, and then thawed and sonicated for 15 min. Samples were clarified by centrifugation for 10 min at high speed and the supernatants, if used for dot blotting were mixed with same volume of 20 mM Tris, 150 mM NaCl, pH 7.5, or with the same volume of 2X SDS PAGE sample buffer if they were to be dissociated before electrophoresis. Samples were electrophoresed in duplicated lanes of a 10% polyacrylamide

gcl, electroblotted into nitrocellulose filters and analyzed as described in Chapter 2 (3.5.3.3).

5.2.13. Nucleotide sequencing

Sequencing reactions were performed by the dideoxy chain termination method (Sanger *et al.*, 1977). Radiolabelling of the oligonucleotide chain was performed either by the end-labelled primer protocol (Hong *et al.*, 1982) with γ - ^{32}P -ATP (3000 Ci/mmol) or the two-step (extension/labelling) method (Tabor and Richardson, 1987) with α - ^{32}S -dATP (>800ci/mmol) or α - ^{32}P -dATP (1,000Ci/mmol). Sequencing reactions were performed by using the Taq Track sequencing system (Promega) or the Cycle sequencing system (BRL). Both use Taq DNA polymerase isolated from *Thermus aquaticus*, with high processivity (>7600 nucleotides). This polymerase shows thermal stability up to 95°C and gives superior results on double-stranded DNA templates, with direct incorporation of radiolabel (Tabor and Richardson, 1987).

5.2.13.1. Taq Track sequencing system

(i) Alkali denaturation of supercoiled plasmid DNA: approximately 1.6 pmol plasmid DNA was resuspended in SDW up to a final volume of 20 μl 2M NaOH, 2 mM Na_2 EDTA (2 μl) was added and the mixture was incubated for 5 min at room temperature. To neutralize the mixture, 1M Tris HCl pH 4.5 (8 μl) and 3N sodium acetate (3 μl) was added and the DNA was precipitated with 100 % EtOH (75 μl) at 70 °C or 1 h. The mixture was centrifuged for 10 min at top speed, the supernatant decanted and the pellet washed with 70 % EtOH. The dried pellet was then resuspended in 16 μl SDW. (ii) Annealing the template and primer: for each set of reactions, the following reagents were mixed: denatured dsDNA plasmid ((16 μl , approx. 1.6 pmol). Taq DNA polymerase 5x buffer (250 mM Tris-HCl, pH 9.0, 50 mM MgCl_2 ; 5 μl), primer (2 μl , approx. 2-2.5 pmol), extension/labelling mix (2 μl , 7.5 μM) and SDW to a final volume 25 μl . The mixture was then incubated at 37 °C for 10 min. (iii) Extension/labelling reaction:

radiolabel α -³²P-dATP or α -³⁵S-dATP, at approximately 10 mCi/ μ l (0.5-2.0 μ l) and sequencing grade Taq DNA polymerase (5u) were added to the annealed primer/template mixture, mixed by briefly pipetting up and down, and the mixture incubated at 37 °C for 5 min. (iv) Termination reaction: when the extension reaction was complete, aliquots (6 μ l) were added to individual microtubes with each d/ddNTP mix (1 μ l) and mixed briefly by pipetting up and down. The reactions were then incubated at 70 °C for 15 min. To stop the reaction, stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanole; 4 μ l) was added to each tube and the tubes stored on ice or at - 70 °C. Immediately before loading onto the separating gels, the reactions were heated at 90 °C for 5 min. Gels were loaded with 2.0-3.0 μ l of each reaction per track.

5.2.13.2. Cycle sequencing system

(i) End labelling of primer: the following reagents were mixed in a microfuge tube: 5X kinase buffer (1.4 μ l), primer (1 pmol, 0.6 μ l), polynucleotide kinase (1.0 μ l), γ -³²P-ATP (2 pmol, 1.0 μ l); and the volume made up to 5.0 μ l with SDW. The mixture was incubated at 37 °C for 30 min then at 55 °C for 5 min and chilled on ice. If labelled primer was not used immediately, it could be stored at - 20° C for several days. (ii) Pre - reaction mixture: to each 2.5 μ l end-labelled primer the following reagents were added: 10X Taq sequencing buffer (4.5 μ l), template DNA (1 pmol; 2.0 μ l), 0.5 U Taq DNA polymerase (2.5 U; 0.5 μ l), and the volume made up to 36 μ l with SDW. The reaction mixture was mixed by vortexing and placed on ice. (iii) Sequencing reactions: to each microfuge tube containing 1 μ l of the termination mix (ddNTPs), pre-reaction mix (4 μ l) was added, overlaid with 10 μ l of mineral oil, mixed by vortexing and centrifuged for 2 min. (iv) DNA amplification by PCR: tubes were placed in the thermal cycling block (Perkin Elmer) and the following program carried out (i) (95 °C for 30 s, 55 °C for 30 s and 70°C for 1 min) for 20 cycles, (ii) (95 °C for 30 s, 70° C for 1 min) for 10 cycles and (iii) 4 °C (cooling). Tubes were removed from the thermocycler and 5 μ l stop solution

added to each tube, the contents mixed by vortexing and spun down. Samples were heated at 90°C for 5 min just before loading the gel.

5.2.14. Denaturing sequencing gel

Sequencing gels containing 6 or 8% acrylamide in 1.0-1.5M TBE were generally used (Sambrook *et al.*, 1989), with 0.4 mm spacers and sharkstooth combs (Gibco-BRL). Gels were run at constant power (50 watts) at 40-45°C for 2 to 10 h. Gels were fixed (10% methanol, 10% acetic acid for 10 min) dried and autoradiographed using Kodak X-OMAT AR or Amersham Hyperfilm-MP films for 16-20 h at -70 °C, without intensifier screens.

5.2.15. Sequence analysis

Computer analysis of sequence data and compilation of the partial genomic sequence was done using DNASIS (Hitachi Software Engineering) on a microcomputer. This was also used for preliminary comparisons of nucleotide sequences of SPFMV C1 and other potyviruses, obtained from the GenBank sequence database (GenBank, c/o IntelliGenetics, Inc., 700E, CA 94040, USA). Pairwise alignments of the putative 6K2, VPg-N1a, N1b and CP homologous aminoacid sequences of several sequenced potyviruses were done using the Gap program of the University of Wisconsin Genetics Computer Group (UW-GCG) package version 6.1 (Devereux *et al.*, 1984), run on a Vax100 mainframe computer. The programs String/Fetch, Profile, Motifs, Gap, PileUp, Pretty and PlotStructure of the same package were used for sequence exchange, multiple sequence alignments, secondary structure prediction, protein analysis and presentation. Hydrophobicity profiles (Kyte and Doolittle, 1982) were prepared by using the program Hydro (D. Rouch, University of Birmingham, UK., unpublished) run in the Vax100-UW-GCG package.

5.3. Results and Discussion

5.3.1. cDNA synthesis and cloning

SPFMV C1 RNA recovered from sucrose density gradients (Chapter 1) was appropriate for cDNA synthesis under standard conditions. In the reaction shown in Figure 5.4, almost full-length cDNA was synthesized from 5 μ g RNA, by using oligo(dT) priming for first strand synthesis. The successful synthesis of nearly full-length cDNAs of SPFMV C1 by using oligo(dT) priming, confirmed the presence of a poly(A) tract at the 3'-end of the genomic RNA (Chapter 2). cDNAs were prepared, purified and used for cloning according to the protocols described under Materials and Methods. The predicted orientation of interesting clones was confirmed by restriction enzyme analysis of inserts and sequencing of the 3' and/or 5' ends. Some clones, originating from at least 3 different cDNA synthesis experiments, were found to have heterogeneous cDNA inserts corresponding to different regions of the viral genome (not shown).

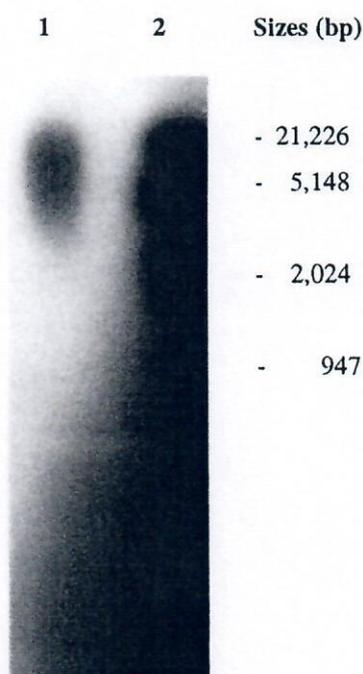


Figure 5.4

Lane 1: SPFMV C1 ds cDNA. Lane 2: EcoRI-HindIII digest of λ DNA, 32 P labelled.

Over 800 clones identified by white/blue screening were screened by colony hybridization and/or agarose gel electrophoresis. Only clones bearing inserts larger than 500 bp (700 to 3500 bp) were selected for further cDNA analysis. Under the experimental conditions used in this work, cDNAs larger than 3500 bp were not successfully cloned into the plasmid vector. Some clones selected by agarose electrophoresis were analyzed by Southern blotting of restriction digests and DNAs detected by hybridization with riboprobes prepared to viral sequences. Clones representing almost the complete genome have been identified and mapped. Their localization in the viral genome was determined by restriction enzyme analysis and partial sequencing of 3'- and/or 5'- ends.

Deduced sequences were used in a search for optimal alignment with the genome sequence of PVY^S (Robaglia *et al.*, 1989). As expected, the 5'-end of the viral genome was not represented in any of the clones, but unexpectedly, the 3' end of the NIb gene and the 5'end of the CP gene (3'-NIb-5'-CP) were also not represented in any of the analyzed clones.

Since the objective of this work was to sequence the 3'-end of the viral genome, no additional work was done on the 5'-end sequences. On the other hand, a series of experiments were conducted in order to obtain the (3'-NIb-5'-CP) region. Strong secondary structure in viral RNA has been reported to interfere with the efficient synthesis of cDNAs (Lorch *et al.*, 1983) and the possibility that this potyvirus RNA could also have similar structures, which would be leading to the results obtained by using standard cDNA synthesis conditions was considered; synthesis of first strand cDNA using two approaches for RNA denaturation prior to first strand cDNA synthesis were conducted. (1) RNA was denatured by using methylmercury hydroxide according to a protocol described by Frankel and Friedmann (1987) and (2) RNAs were denatured by incubation at 75 °C for 10 min. Second strand synthesis, cloning and screening was performed as before. cDNAs were also synthesized in two different experiments by using primers specific to the sequences flanking the (3'-NIb-5'-CP) region. The specific primers were used either for first strand or second

strand synthesis or for the synthesis of both cDNA strands (each one with its corresponding primer). When specific primers were used only for second strand synthesis, the first strand was primed with oligo(dT). Finally, a similar set of experiments, using specific primers as above (5'-N1b-3'-CP) were performed using the polymerase chain reaction (PCR) for amplification of second strand synthesis, according to a protocol described by Wetzel *et al.* (1991a) for PPV cDNA synthesis. cDNAs were separated by agarose gel electrophoresis and sections with DNAs of the approximately expected size (700 to 1500 bp) were extracted and used individually for cloning. However, the results were negative, in the sense that none of the analyzed clones contained the (5'-N1b-3'-CP) region. The factors affecting these results have not been determined and no further work has been done in the limited time available to conclude this work. The possibility that the sequence in this region could translate into a protein which is toxic to the bacterial host cells (Remaut *et al.*, 1981), might be one possible explanation. Another possibility is the presence of defective interfering RNAs (DI RNA) in the RNA preparation used for cDNA synthesis. Hillman *et al.* (1987) reported that tomato bushy stunt virus (TBSV) DI RNAs are a mosaic of virally derived fragments of variable size, but without unique structural features. Further work needs to be done to determine the precise cause of these results.

5.3.2. Sequencing

The sequencing strategy followed to obtain the 3'-end CI, 6 K2, VPg-N1a, partial N1b and CP genes and 3'-UTR nucleotide sequences is shown in Figure 5.5. The clones used for sequencing were as follows: pFMC1-01, pFMC1-02, p-FMC1-07, pFMC1-10, pFMC1-13, pFMC1-18, pFMC1-14, pFMC1-38, pFMC1-60, pFMC1-61, pFMC1-118, pFMC1-151, pFMC1-161 and pFMC1-163. Sequence data were obtained in most cases from more than 3 clones and/or by sequencing both strands.

5.3.3. Sequence analysis

Computer translation of the RNA sequence revealed a coding region of 2490 for the 6K2, NIa genes and flanking regions (3'- end CI and 5'- end NIb) and a coding region of 1150 bp for the CP gene and 3'- UTR. The other two reading frames of the RNA sequence and the three of the complementary strand contained numerous stop codons and few extended ORFs. The CP coding region was followed by a single amber stop codon.

5.3.3.1. The 6K2 , VPg-NIa protease cistrons and flanking regions

The nucleotide sequence and predicted aminoacid sequence of 2490 bp SPFMV C1 genome fragment, containing the 6 K2, VPg- NIa protease and flanking 3'-end of CI and 5'-end of NIb genes sequences, was determined (Figure 5.6). By analogy with the PVY^N genomic nucleotide sequence (Robaglia *et al.*, 1989), and based on the consensus aminoacid sequence recognized by the NIa protease (Dougherty *et al.*, 1989), the N- and C- terminal aminoacids of the 6K2 and VPg-NIa proteins and the cleavage site for the VPg-NIa site, which is characterized by a Glu residue preceding the hydrolysed peptide bond, were tentatively identified. It was postulated that the junction between the CI and 6K2 proteins is at aminoacid positions 7 and 8, the 6K2 and VPg at positions 61 and 62, and the NIa and NIb is at positions 497 and 498, respectively (Figure 5.6). Based on the deduced aminoacid sequences, SPFMV C1 6K2 and VPg-NIa and NIb proteins have been found to be 53, 429 and 309 (5'-terminal region) aminoacids in length, with a calculated M_r of 6 kDa, 49 kDa (VPg 21.5 and NIa 27.6) and 38 kDa respectively.

Comparison of the deduced aminoacid sequences of SPFMV C1 NIa with other published potyviral NIa proteases (Figure 5. 8) suggested that the aminoacids His, Asp and Cys in positions 294, 338, and 399 of SPFMV C1 NIa respectively (boxed in Figure 5.6) were the likely candidates composing the active site of the SPFMV C1 cysteine protease.

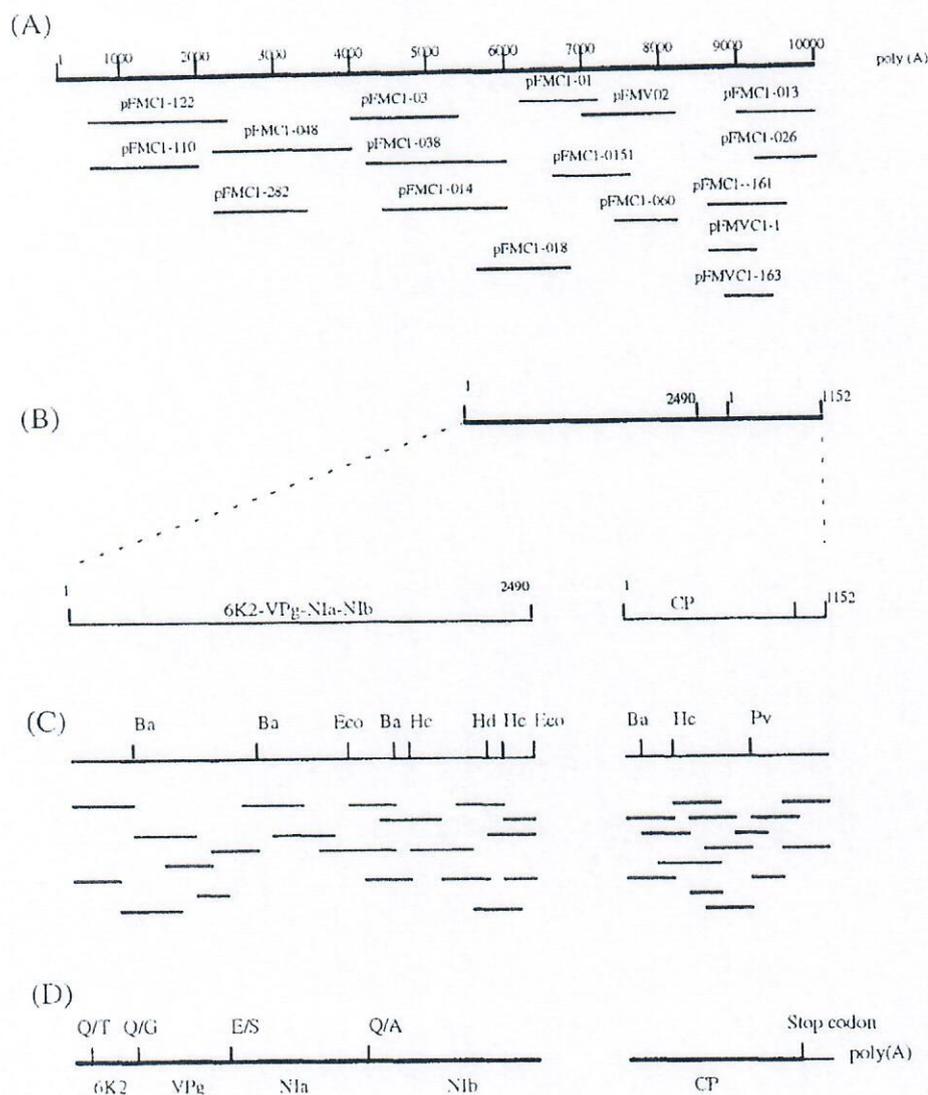


Figure 5.5

(A) SPFMV C1 cDNA inserts cloned into recombinant plasmids (their relative positions were determined by analogy to PVY^N genome sequence).

(B) Genome fragments containing the 6K2, VPg-N1a protease and flanking 3'-end CI and 5'-end coding region (nucleotides 1 to 2490) and genome fragment containing the 3'-end CP coding region and 3'-UTR (nucleotides 1 to 1152).

(C) Restriction sites used for subcloning and sequencing strategy. The sequence was determined directly by sequencing both ends of the insert, using universal forward and reverse primers or specific primers to previously generated sequences. (Av: *Ava*II, Ba: *Bam*HI, Hc: *Eco*RI, Hc: *Hinc*II, Hd: *Hind*III, Pv: *Pvu*II).

(D) The putative 6K2, VPg-N1a protease, N-terminal N1b and the C-terminal CP proteins, and the putative cleavage sites, processed by the SPFMV C1 N1a protease. The thin line between the CP and poly(A) represents the genome's 3'-UTR.

Figure 5.6

Nucleotide sequence of SPFMV C1 6K2, VPg-N1a protease and 5'-terminus of N1b genes, expressed as DNA sequence, and deduced aminoacid sequence. The putative cleavage sites are indicated by arrows. The 6K2 starts at aminoacid position 8, the VPg at aminoacid position 61, the N1a-protease at aminoacid position 249 and N1b at aminoacid position 498, respectively. The boxed residues in the N1a domain correspond to the putative serine-like protease active sites. The Tyr₁₂₂ or ₁₂₉ at the N¹-terminus of the VPg domain is the putative site for covalent linkage protein-viral RNA. The putative RdRp motifs in the N1b domain are underlined.

Comparison of the SPFMV C1 6K2 aminoacid sequence with those of TuMV, PVY and PRSV, indicated 62.3%, 46.0% and 53.0% identity, respectively (Table 5.2). Alignment of this small protein with the corresponding proteins of these other potyviruses for optimal matching showed them to share several consensus basic and aliphatic residues (Lys and Val) in the highly hydrophobic central core (Figure 5.7).

A His residue at position 415 probably corresponds to the His residue usually found 15 to 18 aminoacids downstream of the active site Cys, characteristic of proteases encoded by many positive-stranded RNA viruses that express their genomes as polyproteins (Allison *et al.*, 1986; Argos *et al.*, 1984) and which was proposed to be important in substrate binding, probably interacting with the conserved Gln at the -1 cleavage site position (Bazan and Fletterick, 1988). The tripeptide ARD (residues 327 to 329) is similar to the conserved GRD in TVMV, TEV and PVY (Domier *et al.*, 1986; Allison *et al.*, 1986; Robaglia *et al.*, 1989; Maiss *et al.*, 1989 and), with the Asp residue predicted to be the active site (Parks and Dougherty, 1991). The Gly residue at position 327 is changed to Ala in SPFMV C1. Change in a residue other than the active one has been previously found for the strain G7 (GKD) but not the strain G2 (GRD) of SMV (Jayaram *et al.*, 1992). The strain G7 overcomes resistance conferred by the Rsv gene (Buzzel and Tu, 1984) in the soybean line PI 96983 and has been proposed it could be correlated with the single aminoacid change in the tripeptide GRD (Jayaram *et al.*, 1992). The role (if any) that these differences play in the interaction between virus and putative resistance gene products is unknown.

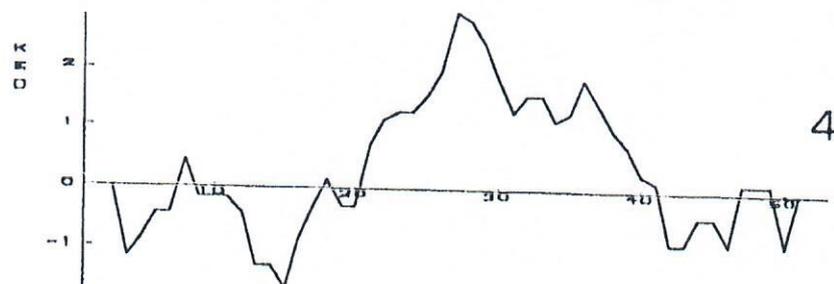
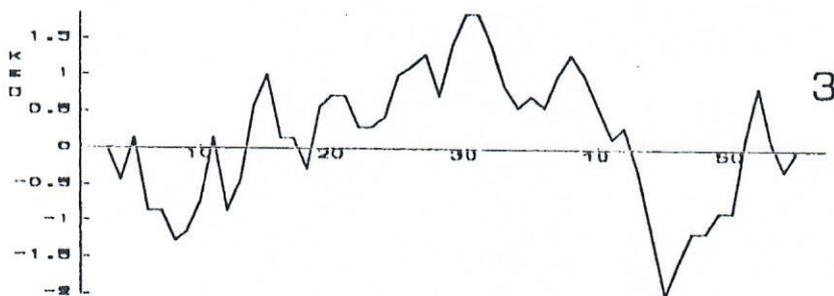
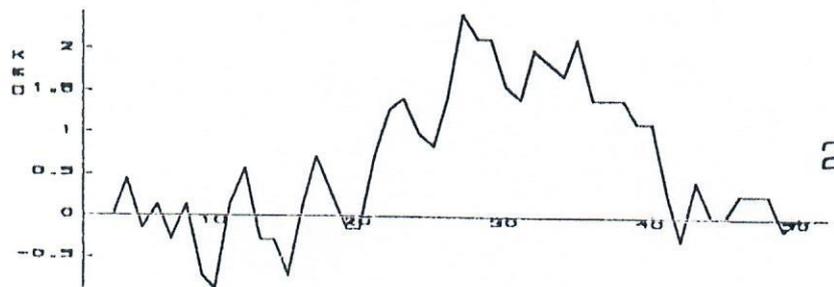
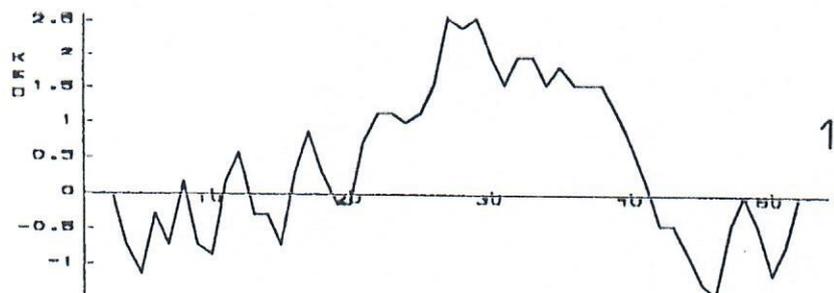
Approximately 25% of the NIa aminoacid residues are identical in all six (PVY^N, PPV, TEV, TVMV, SMV and PRSV) potyviral sequences examined (Figure 5.8), and most of the identity is in the amino terminal three-quarters of the protein. These conserved aminoacids were proposed to be involved in the proper folding of the protein, so that the residues which make up the catalytic triad, or other important features important for enzyme activity, are correctly positioned (Ghabrial *et al.*, 1990; Dougherty *et al.*, 1989).

Figure 5.7

- (A) Comparison of the hydrophobicity profiles (Kyte and Doolite, 1982, window of 7) of the 6K2 protein of SPFMV C1 and PVY^N, TuMV and PRSV.
- (B) Alignments of the aminoacid sequences of the 6K2 protein of SPFMV C1, PVY^N, TuMV and PRSV. Consensus sequence is shown at the bottom of each group, where capital letters represent those aminoacids conserved. Dashes (-) are used where no particular preference is evident.

References:

PVY^N: Robaglia *et al.*, 1989, TuMV: Nicolas and Laliberte, 1992, PRSV: Yeh *et al.*, 1992.



4

	1		50
SPFMV	...tkSDLAn	DLQLKGIWNK	SvITqDLlVl aGVFVGGVWM VFsgakEsfe
TUMV	...stnEMsK	fLQLRGkWNK	tLITrDvlVi cGVLgGGVWM VvqHFRakvs
PVY	...aatSLAK	DLkLkGIWNK	SLVaKDLiIa GaVaIGGIgL IYswFtqsv.
PRSV	svdgifkhir	ELgLRGVWdg	SLmiKDaiVc GftMaGGamL lYqHFRdkft
Consensus	-----	-L-LRG-W--	-----D-----GG-----

	51
SPFMV	qT.VTHQg
TUMV	Ep.VThe.
PVY	ET.VsHQ.
PRSV	nvhVfHQ.
Consensus	---V-H---

Figure 5.8

Alignment of the aminoacid sequences of the NIa (VPg-NIa protease) protein of SPFMV C1 with the corresponding sequences of PVY^N (Robaglia *et al.*, 1989), PPV (Maiss *et al.*, 1989), TEV (Allison *et al.*, 1989), TVMV (Domier *et al.*, 1986), SMV (Ghabrial *et al.*, 1990) and PRSV (Yeh *et al.*, 1992). Capital letters represent those aminoacids positions where two or more of the seven NIa proteinases share identity. Dashes (-) are used where no particular preference is evident. A consensus sequence is shown in the bottom of each block. The consensus sequence motif for the VPg-NIa protease junction is marked with an arrow.

	1					50
PVYGkNKskriQ	aLkFRhARDk
PFV	DAtImLGVLg	GGaWMIFsYL	rDsfkEeVvh	QGEnrrqr.Q	KLkFRqARDn
TEVgKKnQkh	KLkMReARga
TVMV	DlIVvsGVgI	GIGeMLWgFF	kEkmbEPVkf	QGksrr....	rLqFRkARDd
SHV	DAVvaiftLV	GGGWMLWdYF	trvirEPVst	QG..KKRQIQ	KLkFRdAfDr
PRSVgfsaRQRQ	KLrFksAana
SPPMVgkeKRQRQ	KLkFRhARDr
Consensus	-----	-----	-----	-----	-----	-L-----A---
	51					100
PVY	raGFEIdnD	DTIEeFFGSA	YrKKGKqKGT	Tv..GMGksS	PrFINMYGFD	
PFV	rHalEVYgDD	sTmadYFGSA	YsKKGKsKGG	TR..GMGtKL	RKFVNIYGYD	
TEV	rqqYEVaaEp	EalEHYFGSA	YnnKGRtKGT	TR..GMGaKS	RKFINMYGFD	
TVMV	FMGYImhgEg	DTIEHFFGaA	YTKKGGKsKGG	Th..GaGtKa	hKFVNIYGVs	
SHV	KoGrEVYaDD	yTmEHtFGeA	YTKKGGKqKGS	TRtkGMGrKS	RnFtHLYGvE	
PRSV	KLGlEVYgDD	gTIEHYFGeA	YTKKGnkKGG	mh..GMGvKt	RKFVatYGFk	
SPPMV	KLGFVhaDD	qqVE.iFGSA	YTaKGGKqKGG	Tv..GMGaKn	RKFINMYGFD	
Consensus	-----	-----FG-A	Y--KG--KG-	----G-G---	---F---YG--	
	101					150
PVY	PTEYSFJqFV	DPLTGrgtEE	nvyadIrdIQ	ErFsEVRkkM	VenDDiEMQA	
PFV	PTDYnFVRFV	DPLTGHTLDE	nPlmDInLVQ	EHFsqTRndy	IgDDkitMQh	
TEV	PTDFSYLRFV	DPLTGHTiDE	StnapIdLVQ	heFgkVrtrM	LiDDriEpQs	
TVMV	PJIEYSYVRYL	DPvTGA TLDE	SPmTDlniVQ	EHFgEIRrea	IJaDamsqQq	
SHV	FenYSmJRFV	DPLTGHTMDE	hPrvDIrMVQ	qeFeEJRkdM	IgEgEIdrQr	
PRSV	FedYSYVRYL	DPLTGeTLDE	SPqTDIsMVQ	DHFSDIRrky	mDsDsfDrQA	
SPPMV	PTEYSFVRFV	DPLTGlvFEE	SPyTDIqLIQ	EkiGDaRhka	ILegstaIqm	
Consensus	P-----	DP-TG----E	-----Q	-----R---	-----Q-	
	151					200
PVY	LGsHtt.IhA	YFrkdwcDKA	LKIDLmPHHP	LKVCdKtNgI	AkFPEREHEL	
PFV	Imshpg.IvA	YYIkdaTgKA	LKVdLTPHHP	LrVCDKtatI	AGFPPEPEEL	
TEV	LsUht.IhA	YLVnsgTkKv	LKVdLTPHss	LraseKstAI	mGFPEREHEL	
TVMV	.rtdkq.IqA	YFVrnSTmpI	LKVdLTPHiP	LKVCe.sInI	AGFPEREHEL	
SHV	vyhshg.IqA	YFIgknTEeA	LKVdLTPHtP	tllCqnsInAI	AGFPPEEDEL	
PRSV	Lialnt.Ijka	YYVrnSakaA	LeVdLTPHHP	LKVCdNkltI	AGFPDREAEL	
SPPMV	rGhhiilVnA	YYImrIdDaA	LKVdLhPHtP	ysgi.wtNpI	AdFPEREHEL	
Consensus	-----A	Y-----	L--DL-PH--	-----I	--FP-PE--L	
	201					250
PVY	RQTGPavVD	VkdIP....	.agEVEHESK	SLmEGLRDFN	PIAqfVCRlk	
PFV	RQTGhpVfVE	pnaIFkinee	gdEEVDHESK	SLFrGLRDYN	PIASsICqIn	
TEV	RQTGmaVpVa	yDqlPPkn..	..EDltfEge	SLFkCpRDYN	PIsSEICHlt	
TVMV	RiTGpTeTlp	fDalPPEk..	..qEVafESK	aLLkGvRDFN	PIsacVwile	
SHV	RQTGlpqvVs	ksDVPrak..	..ErVEmESK	SvYkGLRDYs	gIstLICqLl	
PRSV	RQTGPprTIq	VDqVFPps..	..ksVhHEgK	SLcGGMraYN	gIASvVChLk	
SPPMV	RQTG.avkVD	IkDVPkEs..	..EVEHESK	iaFrGLRDYN	PIASvICpLs	
Consensus	R-TG-----	----P-----	-----E--	----G-R---	-I-----L-	
	251					300
PVY	vSvEygaSeM	YGfGFGaYIV	aNhHLFRsyN	GsMeVqSMHG	tFrVKNlHsL	
PFV	NSSgarqSvM	FGIGFGgLIv	TNqHLFkRNd	GELTIrShHG	EFVVKdTKTL	
TEV	NeSDGHttsL	YGIGFGpFII	TNkHLFRRN	GtLlVqSLHG	vFkVKNTTTL	
TVMV	NSSDGHserL	FGIGFGpYII	aNgHLFRRN	GELTIKtMHG	EFkVKHsTqL	
SHV	NSSDGHketM	FGVGYGsFII	TNqHLFRRN	GmLTVKtWHG	EFVihNTqL	
PRSV	NES.CkgksL	FGIGYnsFII	TNrHLFkelPI	GELiVKSqHG	kFIVRNTTTL	
SPPMV	erSrwwTSds	YGIGYGVLIi	TNrHLFkRN	GtLTIrSrHG	EFVlvtpprL	
Consensus	-----	-G-G-----I-	--N-HLF----	G-----HG	-F-----L	

PVY	svlPIEGRDI	IIIkMPKDFP	vFPQKLhFRa	PtqnERICLV	GTNFQEKyaS
PFV	klIDekGRDI	VIIIRLPKDFP	PFpkRLqFRt	PttEDRVCLI	GsNFQtkSIS
TEV	QghlIdGRDm	IIIRMPKDFP	PFQKLkFRa	PqREERICLV	tTNFQtKSmS
TMHV	QmkPVeGRDI	IVIkMaKDFP	PFQKLkFRq	PtikDRVCMV	sTNFQqKSVS
SHV	kilfIqGkDV	IIIRMPKDFP	PFgkRnlFRq	PkREERVCMV	GTNFQEKslr
PRSV	QlaPVgktDL	IIIRMPKDFP	PFhsRarFRa	mkagDkVCMl	GvdYQEnhIa
SPEHV	gmkPeadRDI	IIIkMPKDFP	PFpQRlqFRl	PkenERICLV	GsNFQEKSlE
Consensus	-----D-	--I---KDFP	-F-----FR-	-----C--	----Q-----

PVY	SiIUEtSttY	nipgatFWKH	WIEtdnGHCG	LPvVSTaDGc	IVGIHSLaHu
PFV	STmSETSatY	PvdnahFWKH	WIATrKDGHC	LPiVSTrDGs	IIIGHSLaHa
TEV	SmVSDTSetF	PsaDGIFWKH	WIqTKDGqCG	sPLVSTrDGf	IVGIHSLaHf
TMHV	SlVSEsChiv	hkeDtsFWqh	WItTKDGqCG	sPLVSiIDGn	IIIGHSLtHt
SHV	aIVSEsSmIL	PegkGafWih	WItTqDGfCG	LPLVSVnDGh	IVGIHGLtsn
PRSV	SkVSETS.ii	segtGdFgcH	WIATrKDGCG	nPLVSVsDGf	IVGIHSLSts
SPEHV	STISlkqvIL	vmyqItFWKH	WIdTrKDGHC	LaLVSTvDGc	IsGVHSLStt
Consensus	-----	-----F--H	WI-T--G-CG	---VS--DG-	I-G-H-----

PVY	ahT.tHYysA	FdEdFEakYL	rtnEhneWVK	sWvYNpDtVl	WGpLKLkDSt
PFV	THP.QHFYsA	FFDnFETtYL	sNQDnduWIK	qWRYHpdVc	WGSLqLkidi
TEV	THP.nHYFts	vPknF.MeIL	tHQEaqgWVs	gWRlHaDsVl	WGghKvfmSk
TMHV	THg.sHYFve	FPEkFvatYL	. . daadgWcK	nWkFlaDkIs	WGStFlvEda
SHV	dsc.kHFFvp	LtdgFEkeYL	eNaDnlsWdK	HWlWepskIa	WGSLnLvEq
PRSV	Tqd.QHFFak	iPaqFEekvL	rkIDdltwSk	HWSYNinels	WGaLkVwEsr
SPEHV	ihkllaavlh	LilkFaLmsL	drlpgtfWrc	aWgvaagsyg	WG.Yndpvc
Consensus	-----	----F----L	-----W--	-W-----	WG-----

PVY	FkgIFKULKL	VqDLiDhDvV	veQ.....
PFV	FgspFhickL	ItDL.DgEFV	yTQsKtthwL	rDrLEGNLkA	vGacpqrLVT
TEV	PEEpFcpvKe	atqlann.ELV	ysQ.....
TMHV	PEdDFmakKE	VaaImD.DLV	rTQ.....
SHV	PKEeFKIsKL	VsDLfg.ntV	tvQgKkerWv	IdaMEGHLvA	cGqadsalVT
PRSV	PEaIFnaqKe	VnqLnvfEq.
SPEHV	PtqLFItpvh	lqvtvDkDMV	vyaq.....
Consensus	P---F-----	-----	-----	-----	-----

PVY
PFV	KHVVRGKctI	FetYLlthpE	ehe.....
TEV
TMHV
SMV	KHVVRGKcPy	FaqYLVsnqE	aksffepImg	ayqpsrlnkd	afk
PRSV
SPEHV
Consensus	-----	-----	-----	-----	---

Aminoacids 61 to 70 and 102 to 130 (Figure 5.6), share several conserved residues with the recently proposed bipartite NLS sequences, which are located within the VPg domain and which were reported to mediate nuclear translocation of the potyviral NIa protein. These two domains contain a high concentration of basic residues (Arg and Lys) which correspond to the hydrophobic regions (Figure 5.9), as in nuclear translocation signals in animal cells (Roberts, 1989). As in TEV (Carrington *et al.*, 1991), the second NLS overlaps with the Tyr₁₂₂ or ₁₂₉ residue, which is the residue that mediates covalent attachment of the VPg molecule to the 5'-terminus of the viral RNA.

The overall NIa protein (VPg-NIa protease domains) were found to be 63%, 62.3%, 62.0%, 62.0% and 72.0% identical to the corresponding peptides of PVY^N, TEV-HAT, TuMV, PRSV and PPV, respectively (Table 5. 2).

SPFMVCI/

Protein	/PVY ^N	/PRSV	/TEV	/TuMV	/PPV
6K2	46.2 %	53.0%	62.3%	62.3%	54.0%
NIa	63.0%	62.0%	62.3%	62.0%	72.0%
NIb ¹	75.0%	71.3%	78.0%	77.0%	73.0%

Table 5.2

Percentage¹ of aminoacid sequence identity between putative 6K2, NIa and NIb² proteins and those of other distinct potyviruses.

The comparisons were done for optimal alignment of the SPFMV C1 NIb available sequence and the corresponding 5'-terminus residues in the NIb cistron of other potyviruses.

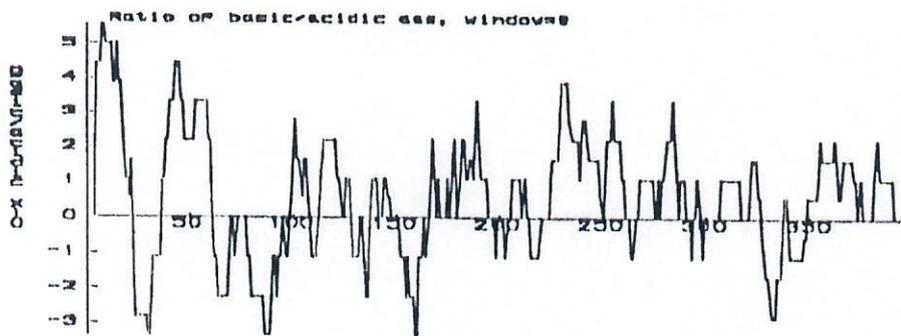
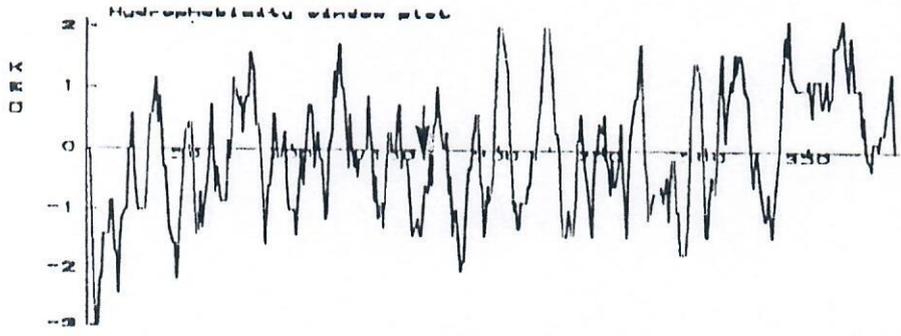
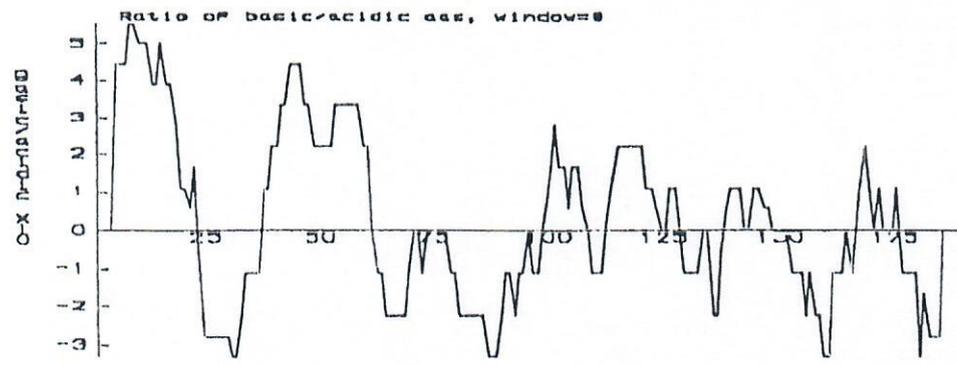
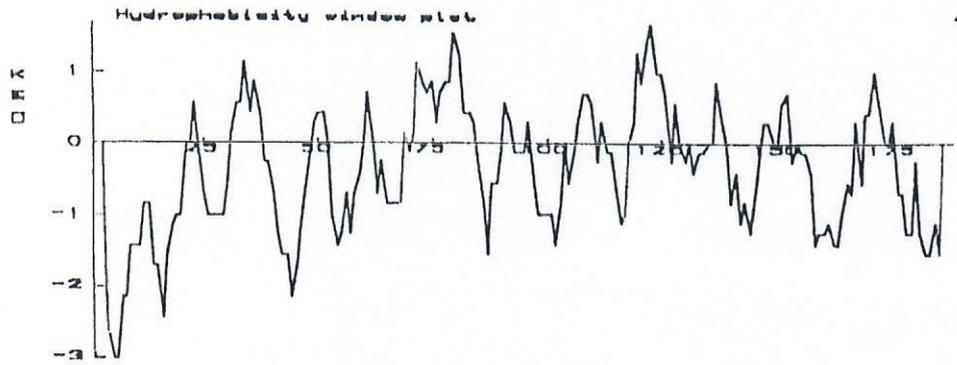
References: PVY^N: Allison *et al.*, 1986, PRSV: Yeh *et al.*, 1992, TEV: Allison *et al.*, 1986, TuMV: Nicolas and Laliberte, 1992, PPV: Maiss *et al.*, 1989.

¹ Percentage of identity was determined by using the program GAP (Needleman and Wunsch, 1970). Gap weight = 3.0 and gap length weight = 0.1.

² N-terminus

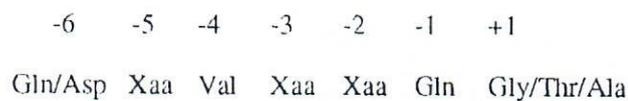
Figure 5.9

- (1) Hydrophobicity and ratio of basic/acidic aminoacids profiles of SPFMV C1 VPg protein. The two regions at the N-terminus are rich in basic residues, characteristic of transmembrane proteins.
- (2) Hydrophobicity and ratio of basic/acid aminoacids profiles of SPFMV C1 VPg-N1a protease proteins. The arrow indicates the junction between the peptides.
(Kyte and Doolittle, 1982; window of 8).

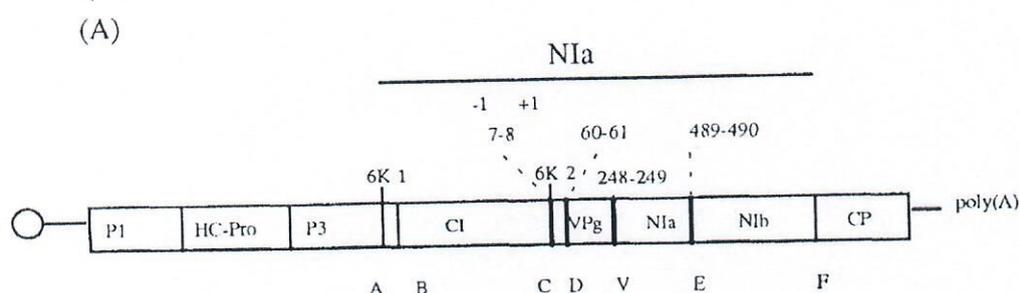


5.3.3.2. NIa protease cleavage sites

Analysis of the deduced aminoacid sequences of the C- terminus CI, 6K2, VPg-NIa protease and N- terminal sequences of NIb proteins, resulted in the tentative identification of three consensus (Gln:Gly/Ala/Thr) cleavage sites processed by the SPFMV C1 NIa protease. Based on the aminoacid sequences surrounding these putative cleavage sites, a consensus SPFMV C1 NIa cleavage site hexapeptide sequence is proposed:



Aminoacids at positions -1 and +1 correspond to the dipeptide cleavage site processed by the NIa protease. The three consensus NIa protease SPFMV C1 cleavage sequences were compared to other potyviral cleavage site sequences (Figure 5.10). The SPFMV C1 NIa protease cleavage site was found always to occur at the peptide bond after a -1 Gln residue, which is in agreement with the consensus potyvirus NIa protease -1 cleavage site aminoacid. This residue appeared to be invariant in the potyvirus group (Ghabrial *et al.*, 1990), while other aminoacids have been predicted for certain -1 positions of picornaviral cleavage sites (Krausslich and Wimmer, 1988). Downstream from the Gln, at position -2, His or Glu were present while at position -3 His, Ser or Val were present. At the position -4, a hydrophobic residue (Val) was found in all three cleavages sites. Presence of Val at this position was found in all of the 5 potyvirus cleavage site sequences examined in this work and in most of the potyvirus NIa cleavage sites reviewed by Shukla *et al.* (1991). Similar aminoacids have also been found at the -4 position of CPMV 24 kDa protease and picornavirus 3C cleavage sites (Wellin and van Kammen, 1988; Hellen *et al.*, 1989). There was not an obvious preference for specific aminoacids at -5 and the -6 position was either a Gln, Glu or Asp, as in most of the NIa potyvirus cleavage sites.



(B)

	SPFMV C1	PVV	TEV	PVY ^N	TVMV
C	QFVHHQ T	ECVHHQ T	ETIYL Q S	QFVHHQ A	EAVRFQ S
D	ETVSHQ G	EEVVHQ G	EPVYFQ G	ETVSHQ G	EPVKFQ G
E	SMVYAQ A	EFVYTQ S	ELVYSQ G	DVVVEQ A	DLVRTQ G
V	SEVEHE S	EEVDHES	EDLTFEG	QEEHE A	QEVAFES

Figure 5.10

(A) A proposed genetic map of SPFMV C1 showing the SPFMV C1 NIa protease cleavage sites. Putative cleavage sites deduced from the amino acid sequence are represented by thick lines. Thin lines represent other NIa protease consensus cleavage sites and cleavage sites for other viral coded proteases. Numbers on top of the thick lines represent the aminoacid position number, with respect to the overall deduced aminoacid sequence for the 6K2, VPg-NIa and flanking regions (Figure 5.6), for the proposed NIa protease cleavage sites. NIa cleavage sites are named A to F, and V.

(B) Comparison of the consensus aminoacid sequences that form the positions -6 to +1 recognized by the NIa protease of SPFMV C1 and four others potyvirus. The peptide bond cleaved between -1 and +1 is indicated by the arrow.

References: TEV, Allison *et al.*, 1986; PPV, Maiss *et al.*, 1989; PVY^N, Robaglia *et al.*, 1989; TVMV, Domier *et al.*, 1986.

Searching the SPFMV C1 NIa protein for the proposed consensus cleavage site sequence, which delimits the VPg and the NIa protease domains, revealed the presence of a potential cleavage site, SEVEHE/S at aminoacids 243 to 249 (Figure 5.6). The most prominent feature of this site, when compared with the respective NIa protease consensus sequences, is the presence of Glu instead of Gln in the position -1 (Figure 5.10). This difference might have the effect of promoting suboptimal cleavage and, in fact, this proteolytic event seems to happen in some but not all NIa molecules (Dougherty and Parks, 1991). Alignment of the aminoacid sequence of SPFMV C1 NIa with those of PVY^N, TVMV, PPV, TEV and SMV revealed similar potential cleavage sites in the corresponding 49 kDa proteins (aminoacids position 228 to 229; Figure 5.8).

5.3.3.3. Analysis of the 5'- end of N1b gene

The N1b gene nucleotide sequence and the aminoacid derived sequence, was not completely determined, as noted above. However, its comparison with the reported PVY^N genome sequence has allowed deduction of the putative N-terminal of the gene, and suggestion of the putative cleavage site between the N1b protein and the NIa protein. The consensus aminoacid sequence (SMVVYAQ/G) for the NIa protease cleavage site was found between aminoacids 498 and 499 (Figure 5.6). Optimal alignment of the SPFMV C1 N-terminus N1b sequence with other potyvirus N1b sequences (Figure 5.11) showed several domains of conserved sequences and conserved features with other known and putative RdRps which are highly conserved among plant, animal and bacteria viruses (Poch *et al.*, 1989; Morch and Haenni, 1987; Domier *et al.*, 1987; Kamer and Argos, 1984). In figure 5.6, aminoacids 741 to 751, **YCDADGSQFDS** (motif A) and 806 to 823, **GNNSGQPSTVVDN(S)LMVV** (motif B) correspond to the consensus motifs found in all potyvirus RdRps, where the (S) represents a Ser which was found in place of the Thr residue in the consensus sequence. An identical substitution was also found in the PVY^N RdRp (Robaglia *et al.*, 1989). The letters in bold represent consensus residues among plus-strand RNA viruses of the polio-like group (Poch *et al.*, 1989).

Figure 5.11

Comparison of the deduced aminoacid sequences of SPFMV C1 5'- terminus N1b and the corresponding region of other potyviruses (350 aminoacids at the 5'-terminus). Capital letters represent positions where two or more aminoacids of the six N1b proteins are identical. Dashes (-) are used where no particular preference is evident. A consensus sequence is shown in the bottom of each block.

The two consensus RdRp motifs (a) and (b) are underlined. Numbers at the beginning and end of each block represent the aminoacid number of the SPFMV C1 5'- terminal N1b protein.

References: PVY^N : Allison *et al.*, 1986, PRSV : Yeh *et al.*, 1992, TEV: Allison *et al.*, 1986, TuMV: Nicolas and Laliberte, 1992, PPV: Maiss *et al.*, 1989.

	1				50
TUMV	tqqnRWMFEq	LTGNLKAIAh	CPSQLVTKHt	VKGKcQmFDL	YLkLh..dEA
PFV	AKtthWlrDk	IeGNLRKAVga	CPgQLVTKHV	VKGKctLFEEt	YLlth..pEe
TEV	gekrrkWvvEa	LsGNLRpVAe	CPSQLVTKHIV	VKGKcPLFEL	YLQLn..pEk
SPFMV	ArsdRWvLDs	cmeiLKAVGq	CPaQLVTKql	VKGKcMLFDL	YLQqD..cDv
PVY	AKhsaWMFEa	LTGNLqAVAt	mkSQLVTKHV	VKGeCrhFte	FLtvDaEaEA
PRSV	.sggRWLFdk	LhGNLkgVas	aPsnLVTKHV	VKGiCPLFrn	YL..EcDeEA
Consensus	-----W----	----L-----	----LVTK--	VKG-C--F--	-L-----
	51				100
TUMV	ReYFqPMLGq	YqKSkLnREa	YaKDLLKYat	PIEaGnIDCD	LFEKt.VeiV
PFV	ReFFqPLMGA	YqKSalNkDA	YVKDLMKYsk	sIvVGaVDCE	qFErA.VdvV
TEV	eAYFkPMMGA	YkpSRLNrEA	FlKDiLKYas	eIEIGnVDCD	LLlElA.IsmI
SPFMV	RsFFkPLMGA	YqKSRLkKEA	YnKDLFKYat	PIInaGdVchk	tFEqAe[ssc
PVY	eAFFrPLMDa	YgKSILNrDA	YIKDiMKYsk	PIDVgVVDrm	hIrkpSIgls
PRSV	kAFFsPLMGh	YmKsvLskEA	YIKDLLKYss	dIvVGeVnhD	vFE.dSVaqV
Consensus	---F-P----	Y--S-L---A	--KD--KY--	-I--G-----	-----
	101				150
TUMV	ISdLrgyGFe	tCNYVTDenD	IFeALNMkSA	VGALYKGGKK	DYFaEFTpEv
PFV	ISMLiskGFs	eCsYVTDPEE	IFsALNMKAA	VGALYsGKKr	DYFkDtsELE
TEV	VtkLkAlGFP	tvNYITDPEE	IFsALNMKAA	mGALYKGGKK	EaLsPI,TDfE
SPFMV	rharAnGfH	kCNYITEPgE	IvkALNMKAA	VGAMYeGKKK	DYFEgMdaED
PVY	.StcnyHGfK	kCaYVTDeGE	IFkALNMKAA	VGAsYgcKKK	DYFEhFTDaD
PRSV	IeLLndHecP	eleYITDsEv	IiqALNMdAA	VGALYtGKKr	kYFEgsTvEh
Consensus	-----	---Y-T----	I--ALNM--A	-GA-Y--KK-	-----
	151				200
TUMV	KEEilkgSce	RLFLGKMGVW	NGSLKAELRP	IEKVEANKTR	TFTAAPIDTL
PFV	KEEFvraSck	RLFMGKkGVW	NGSLKAELRP	kekVEANKTR	sFTAAPiDTL
TEV	qEaMlkaScl	RlytGKLGiw	NGSLKAELRP	iekVEenKTR	TFTAAPiDTL
SPFMV	iEpLfh.sCr	RlyhGKLGiw	NGSFKAELvt	IEKViLnKTR	TFTAAPLDTL
PVY	KEEivmqScl	RlykGllGiw	NGSLKAELPc	KEKILANKTR	TFTAAPIDTL
PRSV	rqaLvrkSce	RLYeGrMGVW	NGSLKAELRP	aEKVIaKkTR	sFTAAPLDTL
Consensus	-----SC-	RL--G--G-W	NGS-KAEL--	-EK----KTR	-FTAAP-DTL
	201				250
TUMV	LGGKVCVDDF	NNQFYdhNlr	aPwDVGMTKF	YcGWDrLLes	LPDGWVYCDa
PFV	LGGKVCVDDF	NNQFYsINLh	CPWsvGMTKF	rgGWdkLLra	LPDGWIYCDa
TEV	LaGKVCVDDF	NNQFYdlNik	aPWIVGMTKF	YqGWneLMea	LPsGWVYCDa
SPFMV	LGGKtCVDDF	NNmFYnnhLk	CPWTVGiTKi	YqGWvrLLts	LPgqWVYCDa
PVY	LGGKVCVDDF	NNQFYskNie	CcWIVGMTKF	YgGWdkLLrr	LPEnWVYCDa
PRSV	LGaKVCVDDF	NNwFYskNme	CPWTVGMTKF	YkGWDeFLkk	FPDGWVYCDa
Consensus	L--K-CVDDF	NN-FY-----	--W-VG-TK-	--GW-----	-P--W-YCDa
	251				300
TUMV	DGSQFDSSLs	PYLINAVLNI	RLgFMEEWDV	GEvMLRNLYT	EIVYTPiSTP
PFV	DGSQFDSSLs	PYLINAVLNI	RLAFMEEWDI	GEQMLsNLYT	EIVYTPiATP
TEV	DGSQFDSSLt	PFLINAVLkV	RLAFMEEWDI	GEQMLRNLYT	EIVYTPiITP
SPFMV	DGSQFDSSLs	PYLINAVLNI	RreFMEEWDI	GgQMLRNLYT	EIVYTPiSTP
PVY	DGSQFDSSLt	PYLINAVLlI	RstYMEDWDV	GlQMLRNLYT	EIVYTPiSTP
PRSV	DGSQFDSSLt	PYLINAVLsI	RLwAMEDWDI	GEQMLkNLYg	EItYTPiITP
Consensus	DGSQFDSSL-	P-L-NAVL--	R--ME-WD-	G--ML-NLY-	EI-YTPI-TP
	301				350
TUMV	DGTIVKkFKG	NNSGQPSTVV	DNtLMVILAv	nYsLkKgGip	sElrDSiirF
PFV	DGTIVKkFKG	NNSGQPSTVV	DNtLMVILAM	tYsLLkLgYh	pDthEcICrY
TEV	DGTIIRkHkG	NNSGQPSTVV	DNtLMVliAM	LYtceKcGin	kEe...iVY
SPFMV	DtTIVtKpiG	NNSGQPSTVV	DNsLMVVLAp	LlflLk....
PVY	DGTIVKkFrG	NNSGQPSTVV	DNsLMVVLAM	hYaLiKecVE	fEeidStcVF
PRSV	DGTIVKkFKG	NNSGQPSTVV	DNtLMVliAM	yYaLrKaGyD	tkTqEdmCVF
Consensus	D-T--K--G	NNSGQPSTVV	DN-LMV----	-----K----	-----

	351				400
TUUV	FVHGDDLLIs	VHPEyEyILD	tMaDnFrELG	LKYtFDGRTR	eKqDLWFMSH
PPV	FVHGDDLvIA	VHPayESJyD	eLqhhFSqLG	LnYtFt tkTe	nKEDLWFMSH
TEV	YVHGDDLLIA	IHPDkaerLs	RFkEsFgELG	LKYEFdctTR	dKtqLWFMSH
SPFMV
PVY	FVHGDDLLIA	VhPEkESILD	RMSqhFSDLG	LnYDFaSRTR	rKEELWFMSH
PRSV	YVHGDDLcIA	IHPDhEhVLD	sFSssFaELG	LKYDFaqRhR	nKquLWFMSH
Consensus	-----	-----	-----	-----	-----
	401				450
TUUV	qGherEGiWI	PKLEPERIVS	ILEWDRSKEP	chRLEAICAA	MIESWGYDkI
PPV	kGiMcEGMYI	PKLEPERIVS	ILEWDRSsEP	iHRLEAICAs	MVEawGYkEL
TEV	RalerUGMYI	PKLEeERIVS	ILEWDRSKEP	sHRLEAICAs	MIEawGYDkI
SPFMV
PVY	RGLIEgMYV	PKLEeERIVS	ILqWDRadlP	EHRLEAICAA	MIESWGYsEL
PRSV	RGiLiDdiYI	PKLEPERIVa	ILEWdkSKlP	EHRLEAtcAA	MIESWGYgDI
Consensus	-----	-----	-----	-----	-----
	451				500
TUUV	THEIRkFYaW	miEQAPFssL	AqEGKAPYIA	ETALRkLYlD	kEpaqEDLttq
PPV	lrEIRkFYsW	VLEQAPYnaL	skDGKAPYIA	ETALkKLYTD	tFaSEtEiEr
TEV	veEIRnFYaW	VLEQAPYsqL	AeEGKAPYIA	ETALkILYTs	qhGtNsEiEe
SPFMV
PVY	THqIRLfySW	lLqqjPFati	AqEGKAPYIA	smALRkLYmD	ravDEEELtA
PRSV	THqIRLfyqW	VLEQAPFneL	AkqGrAPYVs	EvgLRrLYTs	erGSmDELEA
Consensus	-----	-----	-----	-----	-----
	501		522		
TUUV	YLqAiFgDyE	DgvEa.cVYH	Q.		
PPV	YLEAFYsnlt	DEDEshvVvH	Q.		
TEV	YLkvLY.DyD	ipttEHlyFq	..		
SPFMV		
PVY	FtEmmvaldD	EfElDsyevH	hq		
PRSV	YidkYFerer	gDspElIVYH	e.		
Consensus	-----	-----	---		

The highly conserved 'polymerase site' defined by a consensus sequence GDD (motif C) is expected to be located 20 to 25 aminoacids downstream the motif B (Domier *et al.*, 1987). This section was not, however sequenced during the course of this work.

5.3.3.4. Analysis of the 3'-end of the CP gene and 3'-UTR

The nucleotide sequence of 1150 nucleotides at the 3'-terminus of the genomic RNA (excluding the poly (A) sequence), representing most of the CP coding region and the 3'-UTR, has also been determined (Figure 5.12). The poly (A) track, adjacent to the 3'-UTR, varied in length (17-25 nucleotides), with an average of 20 A in most clones. The overall base composition of the CP sequence available is 30% A, 19.5% C, 25% G and 26% U. This composition is around the average for most potyvirus CP gene sequences.

The 3'-UTR of the SPFMV C1 RNA is 220 nucleotides in length. A potential polyadenylation signal (AAGAAA), identical to the sequence found in the RNAs of the RC and C isolates (Abad *et al.*, 1992) and similar to the AAUAAA sequence found in TVMV (Domier *et al.*, 1986), was present 26 nucleotides upstream the poly (A) tail. A putative yeast polyadenylation signal, UAUGU (Zaret and Sherman, 1982) was found 152 nucleotides upstream the poly(A) tail. Similar polyadenylation signals have also been identified in the RNA of TVMV, TEV, PPV and ZYMV, 70 to 90 nucleotides upstream from the poly(A) tail (Grumet and Fang, 1990; Maiss *et al.*, 1989). However, the possibility that this sequence would function as a polyadenylation signal is questionable, since it is further upstream than the usual site for polyadenylation signals. The 3'-UTR of SPFMV C1 RNA is relatively A+U- rich (56%), as are the 3'-UTR of potyviruses.

The overall nucleotide sequence (CP and 3'-UTR) is 93% and 78% identical to the C and RC isolates respectively. The identity between the CP and 3'-UTR of the RC and C isolates was reported to be 77% (Abad *et al.*, 1992). The first third of the C1 CP nucleotide sequence is 91% and 66% identical to the corresponding regions in the C and RC isolates respectively while the 3'-half, excluding the 3'UTR, is 95% and 85% identical,

respectively. This degree of identity is lower than the 100 % reported for the corresponding region in the RC and C isolates.

Comparison of the SPFMV C1 3'-UTR sequence with the corresponding region of the C and RC isolates and other potyviruses CPs indicated that the SPFMV C1 3'-UTR has less than 40% identity with PVY^N, PPV-NAT and TEV-HAT 3'-UTRs, while it is 81% identical to the 3'-UTRs of both the C and RC isolates. This sequence relationship is similar to that found between distinct viruses, but lies at the lower limit for strains of the same virus (Frenkel *et al.*, 1989). It is interesting to note that even when the last 70 nucleotides of the 3'-UTR are 100% identical to the corresponding nucleotides in the C and RC strains, the degree of identity of the overall 3'-UTR is lower than for the CP coding region (93%).

Two repeats of AUUUA pentamers were found in this 3'-UTR region. This motif present singly or in multiple reiterations in a wide variety of oncogen and cytokine mRNA 3'-UTRs (Jackson and Standart, 1990; Malter, 1989), has been demonstrated to be related to mRNA stability. Removal of this region was found to confer significantly greater stability in mRNAs produced from transfected constructs (Greenberg *et al.*, 1986), whereas the addition of a short DNA segment coding for this motif destabilizes previously stable messages (Shaw and Kamen, 1986). A cytoplasmic protein which binds to AUUUA containing RNA in most if not all eukaryotes has been identified (Jackson and Standart). The role (if any) of these motifs in the stability of plant virus RNAs is unknown.

5.3.3.5. Analysis of the CP aminoacid sequence

The CP protein was found to be 930 aminoacids in length with a predicted M_r of 34.5 kDa. The SPFMV C1 CP was aligned (Figures 5.13 and 5.14) with the published CP aminoacid sequences of the C and RC strains and with those of TEV-HAT, TuMV, PVY^N, PPV-NAT, BYMV, PRSV-HA, TMVM, SCMV and WMV. With alignment maximization using gaps, the SPFMV C1 CP sequence shows limited but significant alignment and identity of residues with all potyvirus CPs within the highly conserved central

Figure 5.13

Comparison of the deduced aminoacid sequences of SPFMV C1 CP with the corresponding region of the C and RC strains (Abad *et al.*, 1992). The putative consensus sequence motif (DAG) associated with virus transmission by aphid, found at positions 8 to 10 downstream from the putative cleavage site, is underlined.

Capital letters represent those aminoacids conserved in two or more CP proteins. Dashes (-) are used where no particular preference is evident. A consensus sequence for the aminoacids conserved in the three strains of SPFMV CPs is shown at the bottom of each block.

	1				50
SPFMV C1PEFKD	AGANFPAPKP	KGAFTATrIT	EVTEPgVpKQ	AALReARQKQ
SPFMV C	QSiCdPEFKn	AGANFPAPKP	KGAFTAPeIT	EVTEPEDPKQ	AALRqARQKQ
SPFMV RC	QSSortEFKD	AGAdFPAPKP	KnippPpTIT	EVTDPEDPKQ	AALRAARaKQ
Consensus	-----EFK-	AGA-PPAPKP	K-----IT	EVT-P--PKQ	AALR-AR-KQ
	51				100
SPFMV C1	PAVTPESYGR	DT..GEKSMR	SVStQRVKDK	DVNVGTtGTF	VVPRVKLIHTG
SPFMV C	PAVTPESYGR	DTtKGEKSMR	SVSpQRVKDK	DVNVGTtGTF	VVPRVKLIHTG
SPFMV RC	PATiPESYGR	DTsKekeSiv	gaSskgVrDK	DVNVGTvGTF	VVPRVKMnan
Consensus	PA--PESYGR	DT-----S--	--S---V-DK	DVNVGT-GTF	VVPRVK----
	101				150
SPFMV C1	KMRQPRVNGI	SVVNLQHLAT	YEPEQHNIGN	TRSTQEQFRA	WYEGVKGDYG
SPFMV C	KMRQPRVNGI	SVVNLQHLAT	YEPEQHNIGN	TRSTQEQFRA	WYEGVKGDYG
SPFMV RC	KkRQPmVNGr	aIINFQHLsT	YEPEQfeVaN	TRSTQEQFqA	WYEGVKGDYG
Consensus	K-RQP-VNG-	---N-QHL-T	YEPEQ---N	TRSTQEQF-A	WYEGVKGDYG
	151				200
SPFMV C1	VDDAGMAILL	NGLMVWCIEIEN	GTSPNINGVW	TMMDGDEQVT	YPIKPLLDHA
SPFMV C	VDDAGMAILL	NGLMVWCIEIEN	GTSPNINGVW	TMMDGDEQVT	YPIKPLLDHA
SPFMV RC	VDDtGMgILL	NGLMVWCIEIEN	GTSPNINGVW	TMMDGDEQVT	YPIKPLLDHA
Consensus	VDD-GM-ILL	NGLMVWCIEIEN	GTSPNINGVW	TMMDGDEQVT	YPIKPLLDHA
	201				250
SPFMV C1	VPTFRQIMTH	FSDVAEAYIE	MRNRTKAYMP	RYGLQRNLTD	MSLARYAFDF
SPFMV C	VPTFRQIMTH	FSDVAEAYIE	MRNRTKAYMP	RYGLQRNLTD	MSLARYAFDF
SPFMV RC	VPTFRQIMTH	FSDVAEAYIE	MRNRTKAYMP	RYGLQRNLTD	MSLARYAFDF
Consensus	VPTFRQIMTH	FSDVAEAYIE	MRNRTKAYMP	RYGLQRNLTD	MSLARYAFDF
	251				300
SPFMV C1	YELqSTTPAR	AKEAHMQMKA	AALKNAHNRL	FGLDGNVSTQ	sEDTERHTaT
SPFMV C	YELHSTTPAR	AKEAHMQMKA	AALKNAHNRL	FGLDGNVSTQ	EEDTERHTTT
SPFMV RC	YELHSTTPAR	AKEAHLQMKA	AALKNAKRL	FGLDGNVSTQ	EEDTERHTTT
Consensus	YEL-STTPAR	AKEAH-QMKA	AALKNA-NRL	FGLDGNVSTQ	-EDTERHT-T
	301				
SPFMV C1	DVTRNIHNLL	GMRGVh*			
SPFMV C	DVTRNIHNLL	GMRGVQ*			
SPFMV RC	DVTRNIHNLL	GMRGVQ*			
Consensus	DVTRNIHNLL	GMRGV-*			

and C-terminal domains, while there a lower degree of alignment and identity between the SPFMV strains and others potyviruses at the N¹-terminus.

Substitution in the aminoacid sequence of the C1 strain of Asn for Asp at position 5, Thr and Arg for Pro and Glu at positions 22 and 23, Glu and Asp for Gly and Val at positions 31 and 32, a gap of two aminoacids between residues 59 and 60, and substitution of Phe for Thr at position 67, were the differences within the N- terminus sequence between C1 and C isolates. Substitutions at position 286 of a Gly for a Ser and at position 312 of a His for the Gln, were the only differences found between the C-terminus sequences of both the C and RC isolates. The aminoacid differences between C1 and the C isolate at both the N- and C- terminus are all located in positions where no consensus sequences were found for all the different potyviruses analyzed, whereas the identical aminoacids were at positions previously found to be invariant among potyvirus coat proteins. The gap of two aminoacids at the N- terminus was in the same position as a gap of eleven aminoacids in PVY^S and TEV-HAT, of four aminoacids in BYMV and of fifteen aminoacids in TVMV CP aminoacid sequences, when sequences were analyzed for optimal alignment with the corresponding sequences of SPFMV C1, C and RC (Figure 5.14). To ensure that the gap (6 bp) in the nucleotide sequence was not the result of an error during cDNA synthesis, the sequence of this region was confirmed by sequencing five cDNA clones obtained from two independent cDNA synthesis reactions. The difference in sequence between C1 and the C and RC isolates at positions 286, 312 and 317 at the C-terminus were observed in only two independent cDNA molecules. They might reflect true sequence differences in the starting RNA preparation, or misincorporations during cloning.

As indicated above, most differences in the aminoacid sequence between the C1 and the C isolate of this virus were located in the surface exposed N- and C- terminal domains (Shukla *et al.*, 1989). Since only the core region of the protein has been found to be indispensable for virus assembly, the possible roles of the N- and C-terminals in other biological functions, such as vector- and host- specificity, cross protection, or the initial stages of infection, have been discussed (Shukla *et al.*, 1991).

Figure 5.14

Comparison of the deduced aminoacid sequence of SPFMV C1 CP with the corresponding region of the C and RC strains and others potyviruses.

Capital letters represent those aminoacids conserved in two or more CP proteins. Dashes (-) are used where no particular preference is evident. A consensus sequence for the aminoacids conserved in the ten potyvirus CPs is shown at the bottom of each block.

References: SPFMV C and RC (Abad *et al.*, 1992), PVY^N (Robaglia *et al.*, 1989), TEV (Allison *et al.*, 1986), PPV (Maiss *et al.*, 1989), BYMV (Hammond and Hammond, 1989), TVMV (Domier *et al.*, 1986), SCMV (Frenkel *et al.*, 1991) and WMV (Quemada *et al.*, 1990).

	1				50
SPFMV C1pEFK	DAGAN	PPAPK	PkgaF tAtrITEVTE
SPFMV C	Sic dpEFK	nAGAN	PPAPK	PkgaF tApeITEVTE
SPFMV RC	SsE rtEFK	DAGAd	PPAPK	Pknip ppptITEVTD
TEV	sgTv DAG.....
TUMV	agETL DAd.....
PVY	anDTi DAG.....
PPV	adekeddeee	vdagrplvtt	tqqpivtttt	qqtPitstTL	qAtqamfnpi
BYMV	sdqEqL nAG.....E
PRSV	srgtdD	yqlvcsnnt	vhqsknEav	DAGlnekIkE
TVMV	sDTv DAG.....
SCMV	qsg	nedagkqksa	tpaanqtasg
WMVsg
Consensus	-----	-----	-----	-----	-----
	51				100
SPFMV C1	PgvFKQaaIr	eaRqKqPavt	PESYGRDT..	GeKSMRSVST	qRV.KDKDVN
SPFMV C	PEdPKQaaIr	qaRqKqPavt	PESYGRDTTK	GeKSMRSVSp	qRV.KDKDVN
SPFMV RC	PEdPKQaaIr	aaRaKqPati	PESYGRDTsK	ekeSivgaSs	kgV.rDKDVN
TEV	..adagKkkD	qKdDKvaEga	skdr.....DVN
TUMV	..lteeqkqa	eKekKerEka	ekereRqkql	afKkgkdVaq	eeqKrDKEVN
PVY	...gankkD	aKpEggsiqp	npnkG.....KDKDVN
PPV	ftpatLeptt	rtvphttttt	PpSFGvigne	dtapnaSnav	vRtgrDrDVd
BYMV	ekKdKkEknE	enpDKnsEgg	nsrqiv....p...DrDVH
PRSV	kEKqkEkeKE	kqkEKekDga	sDgndv....StST	ktgerDrDVH
TVMV	...KdKarD	qKladdPtla	iDr.....tKDKDVH
SCMV	dgKpWQtLat	adnkpsDnt	snaqGtsqTK	Gggesggtna	tatKKDKDVd
WMV	kEtvenldag	keskKdasdk	gnkpqnsqvg	qgskeptktg	tvs...KDVH
Consensus	-----	-----	-----	-----	-----V-
	101				150
SPFMV C1	VGTEGTFvVP	RvKlhTgKMR	qPRVnGisVv	NLqHLATYeP	EQhnIgNTRs
SPFMV C	VGTEGTFvVP	RvKlhTgKMR	qPRVnGisVv	NLqHLATYeP	EQhnIgNTRs
SPFMV RC	VGTVGTFvVP	RvKmanKkR	qFmVnGraIi	HPqHLsTYeP	EQfEVANTRs
TEV	aGTSGTFsVP	RinamatKLq	yPRmrGeVv	NLnHLlqYKp	qQIDlSNARA
TUMV	aGTSGTFsVP	RlKslTsKMR	vPRykrVaL	NLDHLllytP	qQIDlSNTRA
PVY	aGTSGTFvVP	RiKaItSKMR	MPTskGatVp	NLEHLLeYaP	qQIDlSNTRA
PPV	aGsiGTFvVP	RlKamTsKLS	LPkVvGKaIM	NLnHLAfySp	aQVDlSNTPA
BYMV	aGTVGTFsVP	klKKIagKLn	iPkIgGKIVL	NLDHLLeYnP	pQDgISNvIA
PRSV	VGTSGTFvVP	RlKsfTdKmv	LPRIkGKtVL	NLnHLLqYnP	qQIDlSNTRA
TVMV	tGTSGTFsIP	RlKKAamnMk	LPkVgGssVv	NLDHLLTYKp	aQefVvNTRA
SCMV	VGstGTFvIP	klKKvspKMR	LPmVsnKaIL	NLDHLlqYKp	DQrDISNARA
WMV	VGskGk.eVP	RlqKiTkKMN	LPtVgGKIIL	sLDHLLeYKp	sQVDlFNTRA
Consensus	-G--G---P	-----	-P-----	---HL--Y-P	-Q----N---
	151				200
SPFMV C1	TQeQFrAWYE	GVKGDYgVDD	agMaIILNGL	MVWCIENGTS	PNINGVWtMM
SPFMV C	TQeQFrAWYE	GVKGDYgVDD	agMaIILNGL	MVWCIENGTS	PNINGVWtMM
SPFMV RC	TQeQFqAWYE	GVKGDYgVDD	tgMGIILNGL	MVWCIENGTS	PNINGVWtMM
TEV	TheQFaWhq	aVmtaYgVnE	EQMkIILNGF	MVWCIENGTS	PNINGtWvMM
TUMV	TrkQFDtWFE	GVmaDYeltE	DkMqIiLNGL	rVWCIENGTS	PNINGmWvMM
PVY	TQsQFDtWYE	aVrmaYDIgE	teMptvMNGl	MVWCIENGTS	PNVNGVWvMM
PPV	pQscPqtWYE	GVrrDYDVtD	DeMsIiLNGL	MVWCIENGTS	PNINGmWvMM
BYMV	TQvQFEAWYs	GVKqaYEVED	sQMGIiLNGL	MVWCIENGTS	gdlqGeWtMM
PRSV	ThsQFEkWYE	GVrnDYglnD	neMqVmLNGL	MVWCIENGTS	PdIsgVWvMM
TVMV	ThsQFEkWhE	nVmaEIElnE	EQMkIvLNGF	MIWCIENGTS	PNIsGVWtMM
SCMV	ThsQFEqLWYn	rVkkEYDVDD	EQMrILMNGl	MVWCIENGTS	PdINGyWtMv
WMV	TktQFEsWYs	aVKvEYDlnD	EQMGVimNGF	MVWCIENGTS	PdVNGVWvMM
Consensus	----F--W--	-V-----	--M----NG-	---WCI-NGTS	----G-W-M-

201 250

SPFMV C1	DGDEQVtYPI	KPLIDHAvPT	FRQIMtHFSD	VAEAYIEMRN	RtkaYMPRYG
SPFMV C	DGDEQVtYPI	KPLIDHAvPT	FRQIMtHFSD	VAEAYIEMRN	RtkaYMPRYG
SPFMV RC	DGDEQVtYPI	KPLIDHAvPT	FRQIMtHFSD	VAEAYIEMRN	RtkaYMPRYG
TEV	DGEDQVsYPI	KPMVEnAqPT	LRQIMtHFSD	lAEAYIEMRN	RErPYMPRYG
TUMV	DGDDQVEFPT	KPLIDHAKPT	FRQIMaHFSD	VAEAYIEkRN	qDrPYMPRYG
PVY	DGnEQVEYPI	KPiVEnAKPT	LRQIMaHFSD	VAEAYIEMRN	kkEpyMPRYG
PPV	DGEtQVEYPI	KPLIDHAKPT	FRQIMaHFSn	VAEAYIEkRN	yEkaYMPRYG
BYMV	DGEEQVtYPI	KPiLDnAKPT	FRQIMsHFSE	VAEAYIEkRN	aterYMPRYG
PRSV	DGEtQVtYPI	KPLIEHAtPs	FRQIMaHFSn	aAEAYIakRN	aterYMPRYG
TVMV	DGDEQVEYPI	eFMVkhAnPs	LRQIMkHFSn	lAEAYIEMRN	sEqvYiPRYG
SCHV	DGnQsEFP	KPiVEnAKPT	LRQcMmHFSD	aAEAYIEMRN	lDePYMPRYG
WMV	DGEEQVEYPI	KPiVEnAKPT	LRQIMhHFSD	aAEAYIEMRN	sEsPYMPRYG
Consensus	DG--Q---P-	-P----A-P-	-RQ-M-HFS-	-AEAYI--RN	----Y-PRYG

251 300

SPFMV C1	LQRNLTDMsL	ARYAFDFYEL	qStTPaRAkE	AHMQMKAAL	kNAhNRLFGL
SPFMV C	LQRNLTDMsL	ARYAFDFYEL	hStTPaRAkE	AHMQMKAAL	kNAhNRLFGL
SPFMV RC	LQRNLTDMsL	ARYAFDFYEL	hStTPaRAkE	AHLQMKAAL	kNAkNRLFGL
TEV	LQRNLTDMsL	sRYAFDFYEL	TskTPVRARE	AHMQMKAAL	RNsgtRLFGL
TUMV	LQRNLTDMsL	ARYAFDFYEM	TsrTPVRARE	AHiQMKAAL	RgAnNnLFGL
PVY	LIRNLTDMgI	ARYAFDFYEv	TsrTPVRARE	AHiQMKAAL	ksAQpRLFGL
PPP	iQRNLTdySL	ARYAFDFYEM	TstTPVRARE	AHiQMKAAL	RNAQNRLFGL
BYMV	LQRNLTdygL	ARYAFDFYkL	TsrTPVRARE	AHMQMKAAL	RgkstRLFGL
PRSV	ikRNLTDisL	ARYAFDFYEv	nSkTPdRARE	AHMQMKAAL	RNtsrRMFGM
TVMV	LQRgLvDmL	ApFAFDFFEv	ngaTPVRARE	AHaQMKAAL	RNsQqRMFcL
SCHV	LIRNLmDkSL	ARYAFDFYei	nSrTPnRARE	AHaQMKAAL	RgstNhmFGL
WMV	LIRNLdDreL	ARYAFDFYEv	TskTPnRARE	AiaQMKAAL	agvnsRLFGL
Consensus	--R---D--L	---AFDF---	---TP-RA-E	A--QMKAAL	-----F--

301 333

SPFMV C1	DGNVSTQsEd	TERHTAtDVt	RNIHNLLGMR	GVh*
SPFMV C	DGNVSTQeEd	TERHTtEdVt	RNIHNLLGMR	GVQ*
SPFMV RC	DGNVSTQeEd	TERHTtEdVt	RNIHNLLGMR	GVQ*
TEV	DGNVgTaeEd	TERHTAhDVN	RNMHtLLGvR	q...
TUMV	DGNVgTtVeN	TERHTtEDVN	RNMHNLLGvq	Gl..
PVY	DGgISTQeEN	TERHTtEDVs	psMHtLLGvk	nm*
PPV	DGNVgTQeEd	TERHTAgDVN	RNMHNLLGMR	GV*..
BYMV	DGNVgTdeEN	TERHTAgDVN	RdMHtMLGvR	i*..
PRSV	DGsVSukEEN	TERHTvEDVN	RdMItLLGMR	n*..
TVMV	DGsVSgQeEN	TERHTvDDVN	aqMHhLLGvk	GV..
SCHV	DGNVgessEN	TERHTAAdVs	RNVHsyrGak	i*..
WMV	DGNISTnsEN	TgrHTArDVN	qNMHtLLGMg	ppQ*
Consensus	DG-----E-	T-RHT--DV-	---H---G--	----

Recently, a comparison between the CP sequences of two strains of SCMV, the SC (infects sugar cane) and MDMV-B (does not infect sugar cane) showed 92% identity in the overall aminoacid sequence, except for a region in the N-terminus, between aminoacids 27 and 70, which was found to be smaller in the SC strain and with relatively low sequence identity (< 30%) with the corresponding region of the MDMB-B strain (Frenkel *et al.*, 1991). It is not known if any of the differences in the aminoacid sequence found between the C1 and the C or RC isolates of SPFMV have any relation to its different host range or indeed to any other biological function.

The presence of a DAG sequence associated with virus transmission by aphids (Atreya *et al.*, 1992, 1991, 1990) was found at the N-terminus aminoacid positions 5 to 8 (Figures 5.13 and 5.14). It is identical to the motif present in the RC isolate, although it differs from the motif in the C isolate, where the Asp was found to be substituted by an Asn (Abad and Moyer, 1992). Sequence analysis of five isolates of PVY, three isolates of PPV, three isolates of JGMV, two isolates of SCMV and two isolates of PWV, indicated that they all have the DAG sequence. However strains of CIYVV and BYMV which are also aphid transmitted all have the NAG sequence (Shukla *et al.*, 1991). The substitution of Asp for a Asn residue is a common mutation in this triplet (Atreya *et al.*, 1991) and could represent an evolutionary recombination event (Goldbach and Wellink, 1988).

The overall aminoacid sequence identity (Table 5.3) between the C1 and the RC (89%) CPs lies in the upper limit of similarity for distinct potyviruses, while the identity between the C and C1 isolates (98%) lies in the range of similarity for related potyviruses (90-99%) (Shukla and Ward, 1989, 1988). The higher percentage of identity at the level of aminoacids than nucleotide indicates that several nucleotide bases are silent, or induce conservative aminoacid replacements. The predicted aminoacid sequence identity between the RC and C isolate CPs was reported to be only 84%. Strains of some viruses have been found to show unexpectedly low identity in this region, but they were considered to be

distantly related strains (Gough and Shukla, 1992; Wetzel *et al.*, 1991b). The high degree of identity with PVY^N, TEV-HAT, TuMV, BYMV and PPV-NAT (71-76%), including the gaps at the N'-terminus, reflects the significant high degree of identity in the core region of these potyviruses.

Reference	Virus	Percentage ¹
1	SPFMV C	98
2	SPFMV RC	89
3	PVY-N	76
4	TEV-HAT	75
5	PPV-NAT	71
6	PRSV	69
7	TuMV	74
8	SbMV	69
9	BYMV	72
10	SCMV	69

Table 5.3

Percentage of aminoacid sequence identity between putative SPFMV C1 CP and those of other potyviruses.

References: 1,2 : Abad *et al.*, 1992. 3: Robaglia *et al.*, 1989. 4: Allison *et al.*, 1986, 5: Teycheny *et al.*, 1989, 6: Yeh *et al.*, 1992, 7: Nicolas and Laliberte, 1992, 8: Jayaram *et al.*, 1992, 9: Hammond & Hammond., 1989, 10: Gough and Shukla, 1987.

¹ Percentage of identity was determined by using the program GAP (Needleman and Wunsch, 1970). Gap weight = 3.0 and gap length weight = 0.1.

The cleavage site between the N1b and CP proteins has been previously predicted to be LEVYHQ/S for the RC and C isolates (Abad and Moyer, 1992), producing a protein of 35 kDa, smaller than the M_r of 38 kDa estimated by for the CP (Moyer and Cali, 1985). For PRSV, the N-terminus of the CP has been predicted to be VFHQ/SKNE (Quemada *et al.*, 1990a) producing a protein of 33 kDa, again smaller than the estimated 36 kDa from the authentic CP (Gonsalves and Ishii, 1980). However, based of the alignment of the N1b of PRSV with those of other potyviruses, it was recently found (Yeh *et al.*, 1992) that

another cleavage site located upstream from the previous one, would generate a protein of 35 kDa. If both sites are used, the N terminus of the CP could be heterogeneous. If a similar situation also applies to SPFMV CP, this may explain why the purified CP of SPFMV is frequently associated with some forms of lower M_r than 38 kDa (Salomon, 1989). So far however, there is not sufficient evidence to draw any conclusions, until the N-terminus of the SPFMV C1 CP sequence is determined and compared with the corresponding chemically determined aminoacid sequence.

Hydrophobicity profiles for the aminoacid sequence of the CP of the C1, C and RC isolates of SPFMV and PVY^N (Figure 5. 15), made with a window of 7 residues (Kyte and Doolittle, 1982) shows that the N-termini of the CP of all three strains SPFMV are similarly strongly hydrophobic, but different from the distinct PVY^N, while the C-terminus is highly conserved and with domains of noticeably lower hydrophobicity. It is important to note that all comparisons and analysis of the nucleotide and derived aminoacid sequences of SPFMV C1 have been done with the sequence determined to date, which lack the N-terminus. Differences in the percentage of identity and M_r of the CP are expected. However, as explained above, these differences might not be highly significant, considering the high percentage of identity founded between the C1 and C isolates in this region. In order to obtain comparable hydrophobicity profiles, the CP sequences of the C and RC isolates started from aminoacid at position 6 down stream from the cleavage site.

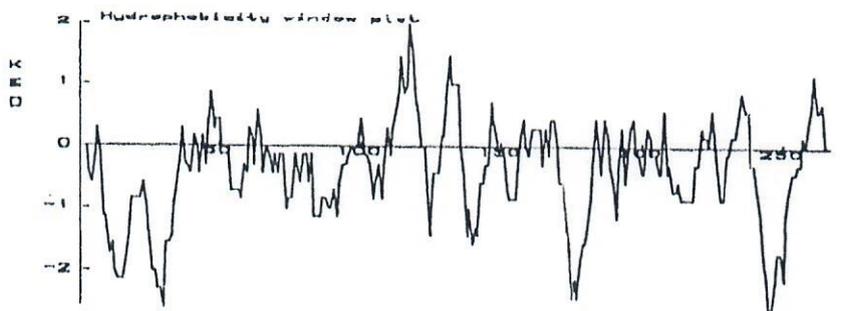
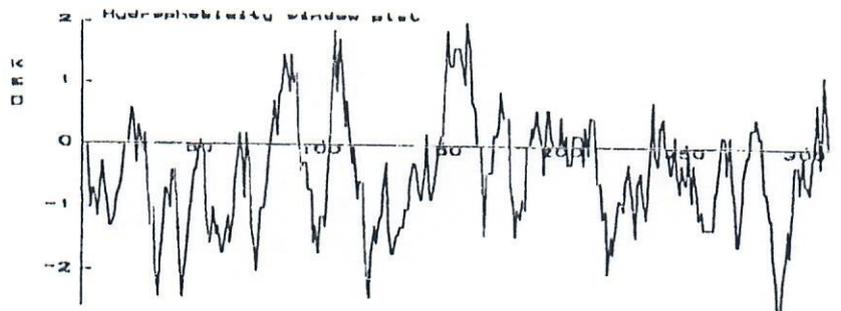
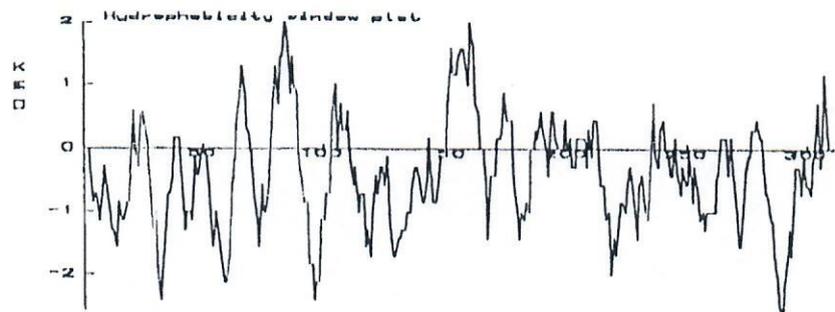
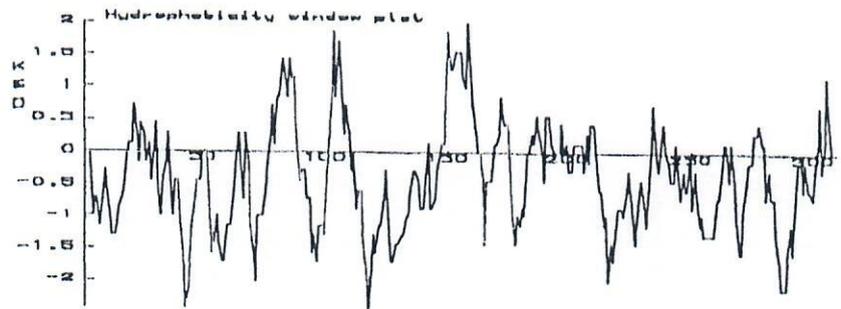
These results confirm previously reported serological relationships (Chapter 1) found between these strains of SPFMV and suggest that although there are biological differences between the C1, C and RC isolates, they are definitely strains of the same virus, with the C1 and C isolates being more closely related to each other than the C1 or C with the RC isolate. The degree of CP aminoacid identity found between SPFMV (C1, C and RC) and PVY^N, TVMV, TEV-HAT, SMV and PRSV (69-73%) confirm their status as distinct potyviruses, but all sharing a common ancestor.

Figure 5.15

Comparison of the hydrophobicity profiles (Kyte and Doolittle, 1982, window of 7) of SPFMV C1 (1) with the C (2) and RC (3) (Abad *et al.*, 1992) and PVY^N (4) (Robaglia *et al.*, 1989).

The numbers refers to the deduced aminoacid sequence in SPFMV C1 CP.

The major differences between profiles of PVY^N and SPFMV C1, C and RC are in the N-terminus, while the central core and C-terminus is highly conserved.



5.3.4. Expression in *E. coli* of SPFMV CP gene

When Western blots of bacterial lysates harbouring clone pFMV C1-13, which contains the C-terminus of the CP coding region, were probed with PTY-1 MAbs, a major band of approximately 30 kDa was detected (Figure 5.16) which was absent in extracts of *E. coli* containing plasmids pFMC1-02, pFMC1-60, pFMC1-60, pFMC1-18 and pFMC1-118. These results suggested that SPFMV C1 CP could be synthesized in *E. coli*. Similar results were also obtained when the bacterial lysates were dot blotted in nitrocellulose membranes (Figure 5.17). The 30 kDa product seems to be a fusion protein composed of a portion of the plasmid β galactosidase gene (15 aa) and part of the SPFMV CP (calculated M_r 26 kDa). The occurrence of two diffuse bands of significantly greater mobility than the genuine CP sequence suggested a proteolytic cleavage within the expressed CP sequence. A bacterial protease may presumably be responsible for these cleavages. Proteolytic degradation of foreign proteins expressed in bacteria has been observed before (Hammond and Hammond, 1989; Van Emmelo *et al.*, 1984).

The pFMC1-13 clone represents the core region and C-terminus of the CP of the SPFMC C1, which makes it a useful clone for the production of an antiserum which could react with a broad spectrum of potyviruses or possibly for engineering coat protein-mediated resistance against potyvirus infection.

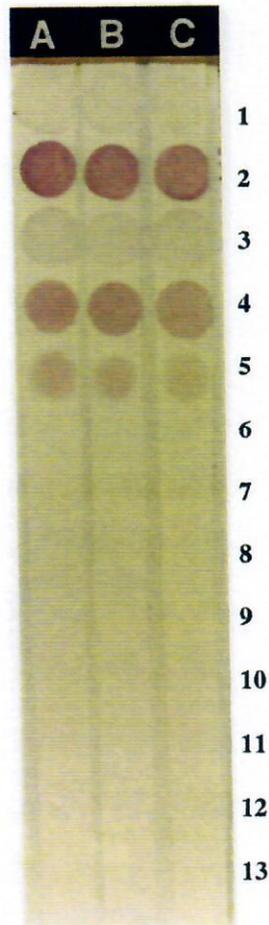
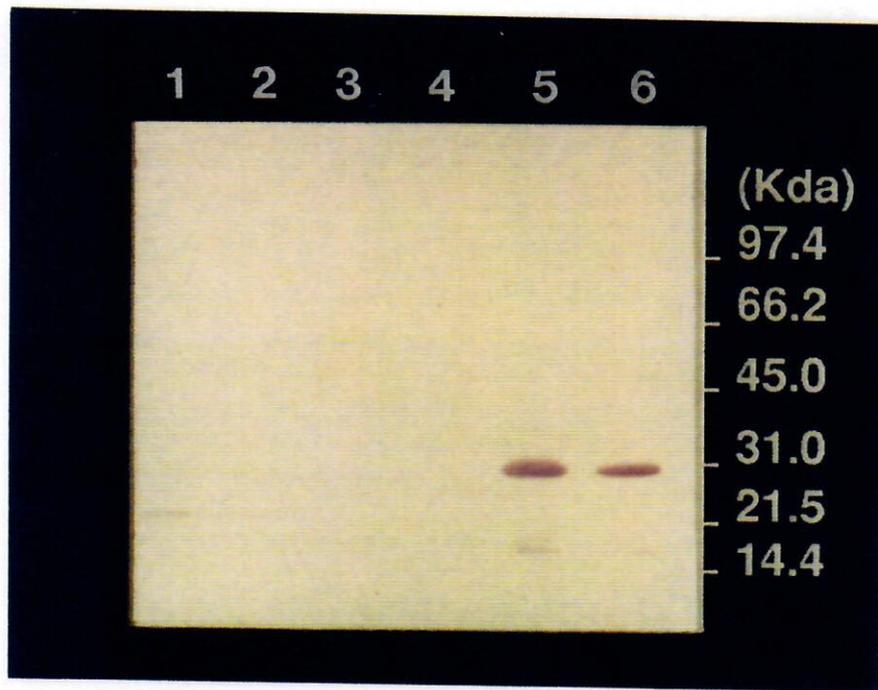
Figure 5.16

Western blot analysis of SPFMV C1 3'-terminus CP expressed in *E. coli* cells harboring pFMC1-13 (lanes 5 and 6). Lanes 1 to 4 correspond to lysates from control *E. coli* cells. After electrophoretic protein transfer to nitrocellulose membranes, the proteins were probed with PTY-1 MAbs to potyvirus CP (Jordan and Hammond, 1991).

Figure 5.17

Dot blot analysis of SPFMV C1 3'-terminus CP expressed in *E. coli* cells harboring pFMC1-13. The proteins were probed with PTY-1 MAbs to potyvirus CP (Jordan and Hammond, 1991). Lanes A, B and C correspond to 1:1000, 1:2000 and 1:4000 dilutions of PTY-1 antiserum, respectively.

Rows 1 to 4 correspond to SPFMV C1, RC, C and YV sap from infected plants (1:20), respectively. Row 5 correspond to lysate of *E. coli* cells harboring pFMC1-13. Lanes 6 to 13 correspond to lysates of control *E. coli* cells.



General Discussion and Conclusions

The need to understand the fundamental aspects of SPFMV structure, replication and expression had been limited by the difficulties in purifying the virions which could then be used for extraction of genomic RNA. In the potyvirus group, yield loss due to virus particle aggregation during purification has been reported to be a major problem. Thus considerable research effort has been directed towards finding suitable buffer systems and clarifying or stabilizing agents for potyvirus purification (reviewed by Brunt, 1988). The results of the work described in this thesis showed that the yield of purified SPFMV C1 and C was greatly affected by the buffer system used and was related to the virus isolate. Both strains of SPFMV have been successfully purified in the presence of EDTA, which had an apparently beneficial effect due to a decrease in aggregation and therefore increase in yield. The structural stability of the virion is essential for the isolation of genomic RNA and for some viruses such as AjMV, depends on the presence of divalent cations (Hiebert and Charudattan, 1984). The incorporation of $MgCl_2$ was recommended for the purification of SPFMV RC and C virions, which were then used for isolation of infective RNAs (Moyer & Cali, 1985). However, it was found that for SPFMV C1 purification using HEPES-based buffer systems, replacement of EDTA by the divalent ion led to a significant reduction in yield, a reduction not observed when phosphate buffers were used. However, the yield of virions purified using phosphate-buffer based systems was always lower than when using HEPES-based buffer systems, and the RNA isolated from those preparations were non-infective in *N. benthamiana*. For the purification of SPFMV C from *I. nil* leaves the use of a borate-based buffer system yielded a higher concentration of purified virus than a HEPES-based buffer system. This result was in agreement with previous findings for the purification of this and other isolates of the virus from *I. nil* or *I. batatas* (Cohen *et al.*, 1988; Moyer & Cali, 1985). It is clear that strains of the same virus can differ greatly in their stability in buffers of different types, molarities, and pH values (Brunt, 1988).

The infectivity of SPFMV C1 RNA was abolished if virions were treated with proteinase K in the presence of SDS. These results suggested that the proteinase K sensitive moiety was associated intimately with the SPFMV C1 genome and probably required for virus replication. Similar results were previously found for PPV (Riechmann *et al.*, 1989) and some other plant viruses (Veerisetty and Sehagal, 1980; Mayo *et al.*, 1979; Harrison and Barker, 1978), but not for SPFMV RC and C (Moyer and Cali, 1985).

The reasons for the differences between the results obtained in this work and the findings of Moyer and Cali (1985) in relation to the effects of Mg^{++} cations on SPFMV C virion purification and proteinase K on virus RNA infectivity have not been totally clarified. Cali and Moyer (1981) reported yields of 5 to 20 μg purified virions/g tissue, by using a procedure similar than Moyer and Cali (1985). In this work, the yield of purified SPFMV C was between 100 and 200 $\mu\text{g/g}$ tissue and the RNA isolated from these preparations was infective in *I. nil* plants at 100 ng/ml, which was considered to be average for a potyvirus (Brunt, 1988; Makkouk and Gumpf, 1974). Other potyviral RNAs which were adequate for protoplast infection have been isolated in the absence of proteinase K and by disruption of virions in the presence of SDS, using a procedure similar to the one used in this work (Gibb *et al.*, 1989; Luciano *et al.*, 1986; Barker and Harrison, 1984). Purification of SPFMV C1 and C by using the protocols developed in this work has allowed relatively high yields of purified virions which were suitable for the isolation of relative large quantities of infective RNA to be obtained, thus making it possible to perform further studies on protoplast infection and the synthesis of cDNAs.

On the basis of the present observations, coupled with the findings reported by Lawson *et al.* (1971), it seems possible to conclude that the CI protein but not the NI protein of SPFMV aggregates in the cytoplasm of infected *N. benthamiana* and *I. nil* cells. The association of the CI bodies with the plasmalemma and endoplasmic reticulum observed by EM in the time course analysis, in this and in a number of other studies (Calder and

Ingerfeld, 1990; Langenberg, 1986) support the possible involvement of the CI with virus cell-to-cell spread and replication. The association of SPFMV virus with the CI bodies has been occasionally observed in some infected cells. However, immunogold labelling with antisera to the viral coat protein should be performed in the future to confirm these observations. According to Edwardson and Christie (1991), SPFMV RC induces type 4-cylindrical inclusions and is a member of subdivision -IV (scrolls and short curved laminated aggregates). However, for the C1 strain, bundles (which are also called laminated aggregates by some researchers) which were initially found in association with the plasmalemma and later as large masses in close association with typical pinwheels were more evident. As stated by Hollings and Brunt (1981) and Lesemann (1988), clear assignation of a virus to a subgroup based on CI morphology is sometimes difficult. Edwardson (1992) indicates that the induction of different types of CI by different strains of the same virus is to be expected, since CIs formation is controlled by the virus genome. Individual viruses may induce their characteristic cylindrical inclusions in different hosts, and different viruses induce their characteristic CIs in the same host.

In a previous report it was shown that a Greek isolate of WMV-2 induces the formation of unusual cytoplasmic amorphous inclusions in *Cucurbita pepo* L. leaves infected with the virus (Martelli and Russo, 1976). The amorphous, electron-dense aggregates found in *N. benthamiana* tissue infected with SPFMV C1, immunogold labelled with monoclonal antiserum to PVY/TVMV HC-Pro, polyclonal antisera to PVY HC-Pro and less specifically with polyclonal antiserum prepared to SPFMV HC-Pro protein, are very similar to the small AI observed in WMV-2 infected cells. However, the large AIs containing flexuous rods which were observed in WMV-2 infected cells were not found in SPFMV C1 infected cells. It is possible that they may represent a different stage of formation or may even not be characteristic of SPFMV C1. The AI bodies occasionally found in some cells infected with SPFMV C1 also resemble the AI bodies reported for PRSV-W1 (Edwardson, 1974), a strain of PRSV [(non-papaya-infecting) (Quemada *et al.*, 1990b; Yeh *et al.*, 1984)]. The CI and AI (HC-Pro) proteins of this virus were also

found to be serologically related to SPFMV CI. The fact that SPFMV CI, WMV-2 and PRSV-W1 CI proteins are serologically related and that their CI and AI proteins aggregate to form similar types of inclusion bodies in the cytoplasm of infected cells may be an indication that their genomes share common sequence in the coding regions for these non-structural proteins. The degree of identity found when the deduced aminoacid sequences of the CP, NIb and NIa of SPFMV were compared with those of PRSV was that normally expressed for distinct potyviruses. However, these sequence comparisons do not mean that the degree of identity at the N-terminus of the polyprotein will be necessarily similar. Future biochemical and genome sequence comparisons are needed to determine more precisely the relationship between these apparently distinct potyviruses. Moreover, the similarity of the SPFMV CI AI bodies with the electron dense AIs found in tissue infected with PsYFV (Fasseas *et al.*, 1989), a virus belonging to a different group and with different particle morphology, but with a similar genome structure to that of the poty- and picorna-viruses, poses new questions on the relatedness of these pathogens.

Evidence presented in this study shows the correlation between the non-structural CI (ca. 70 kDa) and HC-Pro (ca. 56 kDa) virus coded proteins purified from infected *N. benthamiana* plants, with the cytoplasmic CI and AI bodies respectively, induced by SPFMV CI in *N. benthamiana* leaves. The preparative method for SPFMV CI CI protein purification devised in this work and the use of SDS PAGE for isolation of the CI protein to be used as an antigen for antisera production proved successful. The antisera produced to the CI protein, in combination with the immunogold labelling technique has allowed confirmation *in situ* of the identify of the CI protein and the demonstration of its synthesis in protoplasts infected with viral RNA. Therefore, its value in this work and for future studies on the function(s) of the CI protein and the serological relationship of this gene product with similar proteins coded by other potyviruses, and probably also as a means for detecting plants infected with the virus, is evident.

The failure to detect SPFMV NIa and NIb proteins, in either sap extracted from infected tissue or *in situ* by immunological techniques, probing with antiserum to TEV NIa and NIb proteins, does not rule out the possibility that the genome of this virus codes for these proteins but instead, they either do not aggregate in the form of inclusion bodies or they adopt shapes different from the typical crystals observed for TEV NIa and NIb proteins. The observation of the presence of inclusion bodies in the nucleolus of some infected cells lead to the proposal of the second alternative. Further studies using antisera produced to SPFMV NIa and/or NIb proteins purified from either *in vitro* translation products of viral RNA, or proteins produced by expression of cDNAs of the specific viral sequences in *E. coli* are required. Analysis of SPFMV C1 genome sequence data strongly supports the presence of the coding regions for both proteins and comparisons with the aminoacid sequence of TEV NIa and NIb protein sequences show a high degree of identity for both proteins. The two sequence motifs for nuclear transport of these proteins, experimentally determined for TEV NIa protein, were also present in the SPFMV C1 NIa deduced aminoacid sequence. Further studies are needed to determine which residue(s) determine the aggregation into crystals of the NIa and NIb proteins of TEV and BYMV, but not those of most potyvirus (Brunt, 1988; Lesemann, 1988), and if the 6K2-NIa protein junction is efficiently cleaved *in vivo* in cells infected with SPFMV and well as in other potyviruses. It has been recently shown that failure to undergo proteolysis at this junction and the attachment of the 6K2 to the NIa impedes nuclear localization of the TEV NIa (Restrepo-Hartwing and Carrington, 1992). Future work, using SPFMV C1 clones bearing the coding region for the 6K2-NIa proteins and transcription vectors as recently developed by Dolja *et al.* (1992) and Restrepo *et al.* (1990), which allows the *in situ* localization GUS activity to follow the movement of the nuclear inclusion proteins into the nucleus in protoplasts or in transgenic plants, will allow investigation of nuclear transport of proteins of this potyvirus, as well as analysis of the events and interactions that regulate nuclear localization and protein aggregation.

This study has also shown some aspects of SPFMV CI behaviour in isolated *N. benthamiana* and *I. batatas* protoplasts. The one-step isolation protocol developed for *N. benthamiana* protoplast isolation overcomes the technical difficulties of the two-step method previously described by Barker (1980) and allows good yields of viable protoplasts to be obtained in a reasonably short time. This was especially important in these studies where protoplasts were inoculated with freshly isolated viral RNA, and synchronization of times for performing the experiments were critical. The infection of *N. benthamiana* and *I. batatas* protoplasts with viral RNA by electroporation has been successfully achieved. This method, which was previously proved to be more efficient than the inoculation of protoplasts with PLO- or liposome-mediated inoculation methods for TVMV RNA (Luciano *et al.*, 1987), has also been proved to be appropriate for SPFMV RNA inoculation.

The time course of virus infection in *N. benthamiana* protoplasts assessed by direct observation of protoplasts labelled with antisera to virus CP and CI proteins was slightly different from that reported for tobacco protoplasts infected with TVMV RNA (Gibb *et al.*, 1989). The percentage of *N. benthamiana* protoplasts containing detectable virus antigen increased until approximately 45 h after infection, remaining unchanged after that time, while in tobacco protoplasts infected with TVMV a plateau level was reached 25 h after infection. It is not known whether this difference was due to different host protoplast systems, or to differences in virus replication. In both cases, the assessment was performed by direct observation of infection by immunostaining techniques, which is a qualitative rather than quantitative method and differences in the evaluation criteria are expected. The direct observation of approximately 70 % of viable infected protoplasts, labelled with specific antisera to CP and CI proteins, over a period of at least 65 h after inoculation with viral RNA, provides a clear evidence of the successful intracellular delivery of the viral RNA and synthesis of the viral structural and non-structural proteins.

A more detailed study on the viral replication process, by assaying the infectivity extractable from protoplasts at various times after inoculation and the amount of viral RNA synthesized, and determination of the time courses of biosynthesis of other virus coded protein, using appropriate antisera, will provide further information on the virus replication cycle.

The percentage of S24 sweet potato cell suspension culture-derived protoplasts which became infected with viral RNA indicates that suspension cultures provide an alternative to mesophyll protoplasts, with the advantages that uniform and contamination-free protoplasts can be obtained consistently and in large amounts. The fact that SPFMV C1 replicates in isolated protoplasts, but does not accumulate in intact S24 and Nemanete sweet potato plants could reflect of restricted virus transport, based on the block of the virus-specific transport function. However, it is important to note that the resistance in this virus-host system may be also due to the inability of the virus to replicate in host cells organized into leaf tissue (eg. because these cells contain a certain substance preventing virus multiplication). During protoplast isolation, the cell wall is removed and the plasma membrane undergoes fundamental changes, accompanied by partial exchange of the cell contents with the medium and probably loss of some cytoplasmic components. It is possible that all these events alter the composition of the cytoplasm, enabling the virus to replicate in the protoplasts. Rigorous proof that the resistance is due to impaired transport requires demonstration that the virus replicating in protoplasts can also accumulate in primarily infected cells of the intact resistant plant. These experiments have not been performed, partly because of the natural difficulties found in achieving appropriate mechanical virus transmission to sweet potato plants, and also because of limited time to undertake the experiments. An alternative way to overcome this constraint will probably be the inoculation of plants growing *in vitro*, under controlled environmental conditions. It is evident, however, that more experiments need to be done to establish the extent of virus replication in both resistant plants, and in protoplasts derived from them.

The potential of protoplast systems has been underexploited in potyvirus research, due to a considerable extent to the difficulty in establishing viral infections. However, the results reported by other groups (Luciano *et al.*, 1989; Gibb *et al.*, 1989) as well as the findings obtained in this work in the use of electroporation for efficient protoplast infection with viral RNA, and the availability of efficient protoplast systems, will allow protoplasts to be used for further analysis of the potyvirus life cycle, support the studies on the mechanisms of virus resistance, and be used for the introduction of desirable characters, which then can be used for the regeneration of transformed plants.

cDNA clones representing almost the complete SPFMV C1 genome have been obtained. Some have been used for obtaining the nucleotide sequence of several genes at the 3'-end of the viral RNA. Technical cloning problems previously encountered during the cloning of large potyviral RNAs (Turpen, 1989) were also encountered in the course of this work. In particular, the failure to recover cDNA fragments corresponding to the 3'-end of the NIb and the 5'-end of the CP genes was the one that remained unresolved, although various approaches were undertaken to overcome the problem. Analysis of the NIb nucleotide sequence of other potyvirus genomes do not reveal any particular feature that would indicate the presence of strong secondary structure or especially unstable sequences that could be prone to degradation by nucleases during the cDNA synthesis or cloning process. Therefore, the possibility that this sequence may code for a protein toxic to the bacteria can not be ruled out. Further work is needed in order to determine the complete nucleotide sequence of the CP and NIb; direct sequencing of PCR products and direct RNA sequencing will, probably be the next most appropriate approaches.

Molecular criteria are providing valuable insights into the genetic relationships between potyviruses. Evidence is presented, from the analysis of the determined nucleotide sequences data and the deduced aminoacid sequences, that confirms the status of SPFMV as a distinct potyvirus, and it can be suggested that the genome organization and structure

of this virus is similar to other potyvirus genomes. The comparison of the almost complete CP nucleotide sequence, deduced aminoacid sequence and the hydrophobicity profile of the strain C1 with those of the C and RC strains of SPFMV show that despite their biological differences, they are definitively strains of the same virus and the C1 and C are more closely related than either the C1 or C with the RC strain.

The RNA of SPFMV was reported to be approximately 10 Kb (Moyer and Cali, 1985), which would make it an unusually large genome in comparison with other potyvirus genomes, and the possibility of a larger coding region was postulated. However, the nucleotide sequences of the 6K2, VPg-NIa protease and the 5'-terminus NIb genes of SPFMV C1 do not indicate significant differences in the size of these regions, when compared with the corresponding regions of other potyviruses that could support this hypothesis. However, the nucleotide sequence of the CP gene of this strain (C1) and the C and RC strains were found to be longer than the corresponding regions of most potyviruses except for the PPV (Maiss *et al.*, 1989) and PRSV (Yeh *et al.*, 1992) CP genes. The PRSV CP gene codes for a protein of approximately 35 kDa, similar to the CP of SPFMV, and its genome of 10.326 Kb in length was reported to code, among others, for an unusually large P1 protein of 63 kDa in comparison with the 32-35 kDa P1 protein encoded by other potyvirus genomes. The serological relationship between the SPFMV and PRSV CI proteins, the similarity of the inclusion bodies found in infected cells, and the relatively high degree of aminoacid identity found between them (69%, including a deletion of 10 residues in PRSV) could represent a closer relationship between these two distinct viruses or may only be a casual similarity. Additional biochemical data and the sequence of the 5'-terminus of SPFMV are required for further comparisons to be made.

One of the most powerful approaches for identifying gene products encoded by a particular region of cDNA involves the serological detection of the expressed protein in *E. coli* cells (eg. Brand *et al.*, 1993; Oshima *et al.*, 1993; Isomura *et al.*, 1991; Jagadish *et al.*,

1991). The knowledge of the nucleotide sequence and location of coding regions permits the insertion of an appropriate viral restriction fragment into an expression vector. Production of antisera to the protein expressed *in vitro* is also an invaluable tool for the detection of gene products assumed to be encoded by the viral genome, based on sequence analysis, but not previously detected in infected plants (Rodriguez-Cerezo and Shaw, 1991). The expression of a fragment of the coat protein of SPFMV C1 in *E. coli* was considered valuable not only because it allowed the identification of a clone representing part of the CP region of the virus, but also because it could be used for the production of antisera to the potyvirus conserved central core and 3'-terminus of the CP (Shukla and Ward, 1989, 1988).

The potyviruses are one group of plant viruses for which a significant database of amino acid and nucleotide sequences is being developed, especially for the coat protein regions. Additional information on the coat proteins of other potyviruses should allow detailed classification of the viruses in this group. Furthermore, increased nucleotide sequence information is leading to a broader knowledge of the genome structure and organization and is providing the tools for understanding these viruses with a positive sense genome. The *in vitro* synthesis of biologically functional potyvirus RNA transcripts from full-length cDNA clones (Domier *et al.*, 1989; Riechmann *et al.*, 1990; Gal-On *et al.*, 1991), and the development of vectors for introducing into and expressing foreign nucleic acid in plant cells (Maiss *et al.*, 1992) opens the possibility of applying genetic engineering techniques for the study of different aspects of the potyvirus life cycle, including translation, replication, symptom induction, cell-to-cell spread and plant-to-plant propagation under a new experimental approach; the functions that have been proposed for potyviral gene products can now be tested *in vivo*. The availability of cDNA copies of appropriate genes and the understanding of the pathogen and its interactions with the host have led to the development of nonconventional approaches to confer protection against plant virus infections. Transgenic plants expressing potyvirus CP genes have been shown to

be resistant to infection not only by the homologous virus (Lindbo *et al.*, 1992; Regner *et al.*, 1992; Ling *et al.*, 1991; Murphy *et al.*, 1990a; Lawson, 1990), but also by other potyviruses (Stark and Beachy 1989).

Several cDNA clones representing the CP gene of SPFMV have been identified, and its sequence is known. Although none of the clones obtained in this work encode the complete CP, their potential for engineering these sequences for expression in transgenic plants represents a logical start point for the development of nonconventional strategies to confer resistance to this important virus in sweet potatoes.

Abbreviations

A ^o	ampere
Λ	absorbance
AI	amorphous inclusion
ALP	alkaline phosphatase
ASB	alkaline substrate buffer
Amp	ampicillin
AP	ammonium persulphate
AT	aphid transmitted
ATP	adenine triphosphate
BAP	6-benzylaminopurine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
C	cellulase
cDNA	complementary DNA
CI	cylindrical inclusion
CIAP	calf intestinal alkaline phosphate
CM	complete medium
CP	coat protein
CW	coconut water
D	driselase
2-4,D	2,4-dichlorophenoxyacetic acid
DAS	double antibody sandwich
dATP	2'-deoxyadenosine 5'-triphosphate
DC	direct current
dCTP	2'-deoxycytidine 5'-triphosphate
ddNTP	dideoxynucleotide triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate

DEPC	diethyl-pyrocarbonate
DI:CA	sodium diethyldithiocarbamate
DMFM	dimethyl-formamide
DMSO	dimethyl-sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double stranded
DTT	dithiothreitol
EDTA	ethylene-diamino-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	endoplasmic reticulum
F(ab') ₂	antigen specific fragment of IgG molecule
Fc	complement specific fragment of IgG molecule
FCS	foetal calf serum
GAR	goat anti-rabbit
HAT	hypoxanthine, aminopterin and thymide
HIB	homogenization buffer
IIC	helper component
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
ISEM	immunosorbent electron microscopy
Kb	kilobase
kDa	kilodalton
LA	laminated aggregates
LM	light microscopy
LGR	London gold resin
M	macerozyme
MAb	monoclonal antibody
MB	microbodies
ME	2-mercapto-ethanol
MES	4-morpholine ethanesulfonic acid
MI	mitochondria
M MLV RT	Moloney murine leukemia virus reverse transcriptase
MOPS	3-[N-morpholino]propanesulphonic acid
MPB	sweet potato propagation media

MS	Murashige and Skoog
Mr	relative molecular weight
mRNA	messenger RNA
NAT	non -aphid transmitted
NBT	nitroblue tetrazolium
NI	nuclear inclusion
NFM	non-fat milk
NLS	nuclear localization signal
NTBM	nucleotide-binding motif
OA	ovalbumin
O-G	orange-green
oligo(dT)	oligo deoxythymidylic acid
ORF	open reading frame
OS	osmotic solution
P	pectolyase
PAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCM	preconditioning medium
PCR	polymerase chain reaction
PEG	polyethylene glycol
PLO	poly-L-ornithine
Pro	protease
PTA	dodeca tungstophosphoric acid
PVP	polyvinylpyrrolidone
RAM	rabbit anti-mouse
RdRp	RNA dependent RNA polymerase
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNAase	ribonuclease
rpm	revolutions per minute
RRL	rabbit reticulocyte lysate
S	Svedberg units
SALB	sample loading buffer
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SFM	serum free medium
ss	single stranded

SSC	sodium chloride, sodium citrate buffer
TAE	Tris-acetic acid-EDTA buffer
TBE	Tris-borate-EDTA buffer
TBS	Tris buffer saline
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethyl-amino-ethylenediamine
tRNA	transfer RNA
Tris	tris-hydroxymethyl-amino-melthane
Triton X-100	alkylphenoxy polyethoxyethanol
Tween 20	polyoxyethylene sorbitan monolaurate
TY	tryptone-yeast medium
UA	uranyl acetate
UTR	untranslated region
UV	ultraviolet
V	volts
v	volume
VPg	virus protein genome bound
XC	xylene cyanole
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

Virus names and acronyms

Alfalfa mosaic virus	AIMV
Araujia mosaic potyvirus	AjMV
Barley yellow dwarf luteovirus	BaYDV
Barley yellow mosaic bymovirus	BaYMV
Barley mild mosaic bymovirus	BMMV
Bean common mosaic potyvirus	BCMV
Bean yellow mosaic potyvirus	BYMV
Beet mosaic potyvirus	BeMV
Bromo mosaic bromovirus	BMV
Celery mosaic potyvirus	CeMV
Clover yellow vein potexvirus	CYVV
Cowpea aphid-borne mosaic potyvirus	CAMV
Cowpea chlorotic mottle bromovirus	CCMV
Cowpea mosaic comovirus	CPMV
Grapevine chrome mosaic nepovirus	GCMV
Cucumber mosaic cucumovirus	CMV
Johnson grass mosaic potyvirus	JGMV
Lettuce mosaic potyvirus	LMV
Maize dwarf mosaic potyvirus	MDMV
Papaya ringspot potyvirus	PRSV
Parsnip yellow fleck virus	PYFV
Passion-fruit woodiness potyvirus	PWV
Pea mosaic potyvirus	PMV
Pea seed-borne mosaic potyvirus	PSbMV
Pepper mottle potyvirus	PeMV
Plum pox potyvirus	PPV
Potato potyvirus A	PVA
Potato potyvirus C	PVC
Potato potexvirus X	PVX
Potato potyvirus Y	PVY
Ryegrass mosaic ryemovirus	RgMV
Soybean mosaic potyvirus	SMV
Southern bean mosaic virus	SBMV
Sugar cane mosaic potyvirus	SCMV
Sweet potato caulimo-like virus	SPCLV

Sweet potato feathery mottle potyvirus	SFMV
Sweet potato latent virus	SPLV
Sweet potato mild mottle ipomovirus	SPMMV
Sweet potato sunken vein virus	SPSVV
Sweet potato vein clearing virus	SPVCV
Sweet potato vein mosaic virus	SPVMV
Sweet potato yellow dwarf virus	SPYDV
Tobacco etch potyvirus	TEV
Tobacco mosaic tobamovirus	TMV
Tobacco rattle tobnavirus	TRV
Tobacco ringspot nepovirus	TRSV
Tobacco vein mottling potyvirus	TVMV
Turnip mosaic potyvirus	TuMV
Watermelon mosaic potyvirus	WMV
Wheat streak mosaic potyvirus	WSMV
Zucchini yellow fleck potyvirus	ZYFV
Zucchini yellow mosaic potyvirus	ZYMV

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