CHARACTERISATION AND MOLECULAR BIOLOGY OF TWO PLANT VIRUSES

BY

HOWARD KALLENDER

A thesis submitted for the degree of Doctor of Philosophy of the University of London and the Diploma of Membership of Imperial College.

Department of Pure and Applied Biology,
Imperial College,
London SW7 2BB.

1989

"I promised results, not promises!"

ABSTRACT

A fungus-borne disease of potatoes found in Peru, Japan and Western Europe is caused by the furovirus potato mop-top virus (PMTV). The type strain of this virus (PMTV-T) was found to induce symptoms different from those previously reported.

Isolation and purification of PMTV-T using a variety of protocols gave variable and unpredictable results, with generally low yields of virus. Possible reasons for this are discussed. Analysis of purified PMTV-T revealed a capsid protein, consisting of one polypeptide component (M_r 19.1 K), encapsidating three ssRNA species (6.5, 3.2 and 2.5 kb). The smallest of the three was found to be unrelated to either of the two larger species. Double-stranded RNA extracted from PMTV-T-infected leaf material also consisted of three species of similar sizes to the ssRNAs (6.3, 3.2 and 2.3 kb). The PMTV-T genome is compared with those of the other furoviruses.

A whitefly-transmitted disease of tomatoes found in Brazil is caused by the geminivirus tomato golden mosaic virus (TGMV). The genome of TGMV consists of two DNA components, DNA A (2588 nucleotides) and DNA B (2508 nucleotides). Four open reading frames (ORFs) (AL1, AL2, AL3 and AR1) are located on DNA A and two ORFs (BL1 and BR1) are located on DNA B. ORFs BL1 and BR1 were fused to the *Escherichia coli* lacZ (β-galactosidase) gene in a series of recombinant plasmids and expressed in *E. coli* TG2. The cells expressing the β-galactosidase-BL1 fusion protein (M_r 140 K) also expressed a slightly larger protein (M_r 148 K), which is thought to be formed as a result of suppression of the ORF BL1 termination codon.

A β-galactosidase-AL2 fusion protein was purified and used to raise an antiserum in a rabbit. However, this antiserum did not react with any proteins found in samples from infected but not healthy *Nicotiana benthamiana*. An antiserum raised against a β-galactosidase-AR1 fusion protein reacted with purified TGMV capsid protein, confirming that ORF AR1 is the viral capsid protein gene. An antiserum raised against a β-galactosidase-AL1 fusion protein reacted with a protein found in nuclei isolated from TGMV-infected *N.*

benthamiana plants, but not in nuclei from healthy *N. benthamiana*, adding weight to the theory that ORF AL1 protein is involved in TGMV DNA replication.

TABLE OF CONTENTS

Title Page		1
Abstract		3
Table of Contents		5
List of Figures		12
List of Tables		14
Virus Abbreviations		15

POTATO MOP-TOP VIRUS

1.	**INTRODUCTION**	17
1.1	**Introduction to the Furoviruses**	17
1.2	**Occurrence and Biological Properties of Members and Possible Members of the Furovirus Group**	18
1.2i	Beet Necrotic Yellow Vein Virus (BNYVV)	18
1.2ii	Beet Soil-Borne Virus (BSBV)	22
1.2iii	Broad Bean Necrosis Virus (BBNV)	23
1.2iv	Hypochoeris Mosaic Virus (HMV)	23
1.2v	*Nicotiana velutina* Mosaic Virus (NVMV)	24
1.2vi	Oat Golden Stripe Virus (OGSV)	24
1.2vii	Peanut Clump Virus (PCV)	24
1.2viii	Potato Mop-Top Virus (PMTV)	25
1.2ix	Rice Stripe Necrosis Virus (RSNV)	26
1.2x	Soil-Borne Wheat Mosaic Virus (SBWMV)	26
1.2xi	Sorghum Chlorotic Spot Virus (SCSV)	27
1.3	**Virus Transmission**	28
1.3.1	Fungal Vectors	28
1.3.2	Seed Transmission	31
1.4	**Cytopathology of the Furoviruses**	31
1.5	**Virus Purification**	33
1.5.1	Purification of Rod-Shaped Plant Viruses	33
1.5.2	Purification of Furoviruses	34
1.6	**Virus Structure and Composition**	36
1.6.1	Morphology, Size and Structure	36
1.6.2	Composition	40

1.6.2i Capsid Protein 40
1.6.2ii Nucleic Acid 41
1.7 **Genome Organisation and Expression** 41
1.7i Beet Necrotic Yellow Vein Virus 41
1.7ii Soil-Borne Wheat Mosaic Virus 49
1.7iii Sorghum Chlorotic Spot Virus 55
1.7iv Peanut Clump Virus 56
1.8 **Taxonomy** 57
1.9 **Plant Viral Double-Stranded RNA** 58
1.10 **Aims of the PMTV Project** 62

2. MATERIALS AND METHODS 63

2.1 **Abbreviations** 63
2.2 **Safety Precautions** 65
2.3 **Companies and Suppliers of Reagents and Materials** 65
2.4 **Common Buffers Used** 68
2.5 **General Methods** 68
2.5.1 Miscellaneous 68
2.5.2 Agarose Gel Electrophoresis 70
2.5.2i Methyl Mercury Gels 70
2.5.2ii Formaldehyde Gels 70
2.5.2iii Low Melting Point (LMP) Gels 71
2.5.3 Recovery of RNA Fragments from Agarose Gels 71
2.5.4 Polyacrylamide Gel Electrophoresis (PAGE) 71
2.5.4i SDS-PAGE Analysis of Proteins 71
2.5.4ii Molecular Weight Determination of Capsid Protein 72
2.5.4iii PAGE Analysis of dsRNA 73
2.5.5 Gel Staining and Photography 73
2.5.6 Gel Drying 74
2.6 **Virus Propagation and Storage** 74
2.6.1 Origin of Virus 74
2.6.2 Growth of Plants 74
2.6.3 Inoculation of Plants 74
2.6.4 Storage of Virus 75
2.6.4i Dried-Down Leaf Material 75
2.6.4ii Liquid Nitrogen 76

2.7	**Virus Purification**	76
2.7.1	Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a Neutral pH Buffer	76
2.7.2	Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a High pH Buffer	77
2.7.3	Purification by a Method which Involves Clarifying the Sap with Organic Solvents	78
2.7.4	Purification by a Method which Involves Clarifying the Sap by Acidification	79
2.7.5	Purification by a Method which Involves Clarifying the Sap with Carbon Tetrachloride and Concentrating the Virus by PEG Precipitation	79
2.7.6	Purification by a Method which Involves Extracting the Sap in a High pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion	80
2.7.7	Purification by a Method which Involves Extracting the Sap in a Neutral pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion	81
2.7.8	Analysis of Extraction Buffers	82
2.8	**Analysis of Virus Preparations**	85
2.8.1	Electron Microscopy	85
2.8.2	Back-Testing	85
2.8.3	SDS-PAGE	85
2.8.4	Single-Stranded RNA Extraction and Analysis	86
2.8.5	Measurement of Virus Density in Caesium Chloride	86
2.9	**Extraction and Analysis of Viral RNA**	86
2.9.1	Single-Stranded RNA from Purified Virus	86
2.9.2	Preparation of Radioactively-Labelled Probes from Single-Stranded RNA	87
2.9.3	Northern Blot Analysis of RNA	87
2.9.4	Double-Stranded RNA from Infected Plant Material	88

3.	RESULTS AND DISCUSSION	91
3.1	**Virus Symptomatology**	91
3.2	**Storage of PMTV**	99
3.2.1	Short Term Storage	99
3.2.2	Long Term Storage	99
3.3	**Virus Purification**	100
3.3.1	Introduction	100
3.3.2	Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a Neutral pH Buffer	101
3.3.3	Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a High pH Buffer	102
3.3.4	Purification by a Method which Involves Clarifying the Sap with Organic Solvents	104
3.3.5	Purification by a Method which Involves Clarifying the Sap by Acidification	108
3.3.6	Purification by a Method which Involves Clarifying the Sap with Carbon Tetrachloride and Concentrating the Virus by PEG Precipitation	110
3.3.7	Purification by a Method which Involves Extracting the Sap in a High pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion	114
3.3.8	Purification by a Method which Involves Extracting the Sap in a Neutral pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion	116
3.3.9	Analysis of Extraction Buffers	118
3.4	**Capsid Protein Molecular Weight Determination**	124
3.5	**Viral Single-Stranded RNA**	125
3.6	**Viral Double-Stranded RNA**	128
3.7	**Taxonomy of PMTV**	131
3.8	**Future Work on PMTV**	132

TOMATO GOLDEN MOSAIC VIRUS

4.	INTRODUCTION	136
4.1	**Introduction to the Geminiviruses**	136
4.2	**Members of the Geminivirus Group**	137
4.3	**Virus Transmission**	144
4.4	**Host Range**	146
4.5	**Cytology**	147
4.6	**Virion Structure and Composition**	148
4.7	**The Geminivirus Genome**	152
4.8	**Organisation and Expression of the Geminivirus Genome**	155
4.8.1	Whitefly-Transmitted Geminiviruses	155
4.8.2	Leafhopper-Transmitted Geminiviruses	167
4.9	**Geminivirus Replication**	180
4.9.1	Replication of ssDNA Bacteriophages	181
4.9.2	Replication of Geminivirus DNA	185
4.10	**Development of Geminivirus-based Vectors**	189
4.11	**Aims of the TGMV Project**	196
5.	MATERIALS AND METHODS	198
5.1	**Manipulation of DNA**	198
5.1.1	Agarose Gel Electrophoresis	198
5.1.2	Restriction Endonuclease Digestions	198
5.1.3	Ligation	199
5.1.4	Bal-31 Nuclease Reactions	199
5.1.5	End-Filling dsDNA with Klenow Fragment	200
5.1.6	Recovery of DNA Fragments from Agarose Gels	200
5.1.6i	Electroelution onto Whatman 3MM Paper	200
5.1.6ii	Geneclean™	201
5.1.7	Addition of Linkers to dsDNA	201
5.2	**Bacterial Cloning**	202
5.2.1	Growth and Maintenance of Bacterial Strains	202
5.2.2	Vectors used for Cloning	203
5.2.3	Media and Antibiotics	206
5.2.4	Preparation of Competent Cells	207
5.2.4i	Standard Transformation Protocol	207

5.2.4ii	Calcium Chloride Method	208
5.2.5	Transformation of Cells with Plasmid DNA	208
5.2.6	Small Scale Plasmid Preparation ("Miniprep")	209
5.2.7	Large Scale Plasmid Preparation	210
5.2.8	Plasmid Preparation for Sequencing	211
5.2.9	Plasmid Preparation for Bal-31 Digestion	211
5.3	**Dideoxynucleotide Sequencing**	212
5.3.1	Stock Solutions	212
5.3.2	Preparation of Sequencing Gel	215
5.3.3	Sequencing Reactions	215
5.3.4	Sequencing Gel Electrophoresis	216
5.4	**Proteins**	217
5.4.1	Small Scale Preparation of Total Protein from Bacteria	217
5.4.2	Large Scale Preparation of Fusion Proteins	217
5.4.3	Gel Filtration Chromatography	219
5.4.4	Raising Antisera in Rabbits	220
5.4.5	Pre-absorption of Antisera with IPTG-induced *E.coli* TG2: pUR292	221
5.4.6	Pre-absorption of Antisera with *N. benthamiana* Proteins	221
5.4.7	Detection of Proteins with Antisera	222
5.5	**Virus Propagation and Purification**	223
5.5.1	Origin of Virus	223
5.5.2	Growth of Plants	223
5.5.3	Inoculation of Plants	223
5.5.4	Preparation of Virus (High-speed Pellet Material)	224
5.5.5	Isolation of Plant Nuclei	224
5.5.6	Preparation of Total Plant Protein	225

6.	**RESULTS AND DISCUSSION**	
6.1	**Construction of Fusion Proteins**	226
6.1.1	Cloning of ORFs BR1 and BL1	226
6.1.2	Sequencing of Vector/Insert Borders	229
6.1.3	Sub-Cloning into the Expression Vectors	236
6.2	**Identification of Fusion Proteins**	241

6.2.1 Fusion Proteins Derived from TGMV DNA B ORFs 241
6.2.2 Fusion Proteins Derived from TGMV DNA A ORFs 245
6.3 **Characterisation of Fusion Proteins** 245
6.3.1 β-galactosidase-AR1 245
6.3.2. β-galactosidase-AL1 252
6.3.3 β-galactosidase-AL2 254
6.4 **Future Prospects and Applications of Geminivirus Research** 264
6.4.1 Characterisation of Geminivirus Encoded Products 264
6.4.2 Applications of Geminivirus-Based Vectors 272

Acknowledgements 275
References 276

List of Figures

1.	Electron Micrograph of a PMTV-T Particle	37
2.	Genome Organisation of BNYVV	42
3.	Sequence Homologies at the 3' Ends of BNYVV RNAs	50
4.	Genome Organisation of SBWMV	53
5.	Symptoms in *N. debneyi* Leaf Inoculated with PMTV-T	93
6.	Symptoms in *N. benthamiana* Leaves Systemically Infected with PMTV-T	95
7.	Symptoms in *N. benthamiana* Leaf Inoculated with PMTV-T	96
8.	SDS-PAGE of PMTV-T Capsid Protein	112
9.	PMTV-T Single-Stranded RNAs	113
10.	Electron Micrograph of PMTV-T Particles	118
11.	Ferguson Plot Analysis of Polypeptides	126
12.	Molecular Weight Estimation of PMTV-T Capsid Protein	126
13.	Purified PMTV-T RNA 3	129
14.	Total PMTV-T RNA Probed with an RNA 3-Specific Probe	129
15.	PAGE of PMTV-T Double-Stranded RNA	130
16.	Electron Micrograph of Geminivirus Particles	151
17.	Comparison of the Major Stem-Loop Structures	157
18.	Comparison of the Open Reading Frames of the Sequenced Bipartite Geminiviruses	160
19.	Comparison of the 'A-T Box' Region of the Characterised Geminiviruses	165
20.	Major Transcripts of ACMV	166
21.	Comparison of the Open Reading Frames of the Sequenced Monopartite Geminiviruses	169
22.	Major Transcripts of MSV-N	176
23.	Open Reading Frames of the Whitefly-Transmitted Monopartite Geminivirus Beet Curly Top Virus	179
24.	Schematic Representation of ΦX174 DNA Replication; ssDNA → RF DNA	183
25.	Schematic Representation of ΦX174 DNA Replication; RF → RF and RF → ssDNA	184
26.	CaMV Genome	192
27.	CaMV Replication	193

28. Structure of the pEMBL/*Xho*I Vector 204
29. Structure of the lacZ-Fusion Cloning Vectors, pUR290/1/2 205
30. Map of pB/*Cla* 227
31. Bal-31 Time Course Reactions 228
32. Analysis of *Eco*RI Digests of pHC100 and pHC200 Clones 230
33. Restriction Digests of pEMBL/*Xho*I, pHC118 and pHC201 231
34. Bal-31 Deletions in pHC201 232
35. Sequencing of pHC104, 111 and 118 234
36. Bal-31 Deletions in pHC103, 104, 111 and 118 235
37. pHC118 → pURBR1; pHC201 → pURBL1 237
38. *Bam*HI Digests of pUR290, BR1 and BL1 239
39. Maps of pURBR1 and BL1 240
40. SDS-PAGE of IPTG-Induced and Uninduced TG2: pURBR1 and BL1 242
41. Detection of β-gal-AR1 by Western Blotting 250
42. Detection of TGMV Capsid Protein by Western Blotting 251
43. Detection of β-gal-AL1 and its Putative Breakdown Products by Western Blotting 253
44. Detection of the Protein Encoded by TGMV ORF AL1 by Western Blotting 255
45. Growth Curves of TG2 and TG2: pURAL2 258
46. Time Course of IPTG Induction of TG2: pURAL2 Cells 259
47. Ammonium Sulphate Precipitation of Proteins in IPTG-Induced TG2: pURAL2 Extracts 260
48. Purification of β-gal-AL2 by Gel Filtration Chromatography 262
49. Detection of β-gal-AL2 by Western Blotting 263

List of Tables

1. Natural Occurrence of the Furoviruses 19
2. Natural Modes of Spread of Furoviruses 29
3. The Sizes of Furovirus Particles and their Components 39
4. Lengths of RNAs 3 and 4 of 14 BNYVV Isolates, and Associated Symptoms in *Chenopodium quinoa* 47
5. Induction of Symptoms by PMTV-T 92
6. Effects of Organic Solvents on the Purification of PMTV-T 121
7. Effects of Extraction Buffer Content on PMTV-T Purification
   a) Extraction Buffers without Additives 121
   b) 0.5 M Tris-HCl (pH 8.0) plus Additives 121
   c) 0.5 M Tris-HCl (pH 8.0) plus Additives; Pulp Re-extracted 122
   d) 0.1 M Borate Buffer (pH 8.0) plus Additives; Pulp Re-extracted 123
8. Members of the Geminivirus Group 138
9. Potential Proteins Encoded by Conserved ORFs of Whitefly-Transmitted Geminiviruses 158
10. Potential Proteins Encoded by Conserved ORFs of Leafhopper-Transmitted Geminiviruses 171
11. Fusion Proteins Derived from the Six TGMV ORFs 246
12. Percentage of the TGMV ORF-Encoded Amino Acids Present in the Fusion Proteins 247

Virus Abbreviations

AlMV	alfalfa mosaic virus
AbMV	abutilon mosaic virus
ACMV (CLV)	African cassava mosaic virus
BBNV	broad bean necrosis virus
BCTV	beet curly top virus
BDDV	bean distortion dwarf virus
BGMV	bean golden mosaic virus
BNYVV	beet necrotic yellow vein virus
BSBV	beet soil-borne virus
CaMV	cauliflower mosaic virus
CGMV	cowpea golden mosaic virus
CLCV	cotton leaf crumple virus
CSMV	chloris striate mosaic virus
DSV	digitaria streak virus
EMV	euphorbia mosaic virus
EuYVV	eupatorium yellow vein virus
FMV	fern mottle virus
HMV	*Hypochoeris* mosaic virus
HYVMV	honeysuckle yellow vein mosaic virus
JMV	jatropha mosaic virus
MisSV	miscanthus streak virus
MSV	maize streak virus
MVM	minute virus of mouse
MYMV	mungbean yellow mosaic virus
NVMV	*Nicotiana velutina* mosaic virus
OGSV	oat golden stripe virus
PCV	peanut clump virus
PMTV	potato mop-top virus
PSMV	paspalum striate mosaic virus
PYMV	potato yellow mosaic virus
RSNV	rice stripe necrosis virus
SALCV	solanum apical leaf curl virus
SBWMV	soil-borne wheat mosaic virus
SCLV	soybean crinkle leaf virus

SCSV	sorghum chlorotic spot virus
SqLCV	squash leaf curl virus
SV40	simian virus 40
TGMV	tomato golden mosaic virus
TbLCV	tobacco leaf curl virus
TmLCV	tomato leaf curl virus
TYLCV	tomato yellow leaf curl virus
TYMV	tomato yellow mosaic virus
TmPCTV	tomato pseudo curly top virus
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TYDV	tobacco yellow dwarf virus
WDV	wheat dwarf virus
WCMoV	water melon curly mottle virus

POTATO MOP-TOP VIRUS

1. INTRODUCTION

1.1 Introduction to the Furoviruses

There are at least 11 rod-shaped plant viruses (Table 1) which are superficially similar to the tobamoviruses, but with further characterisation have been found to differ in several ways. They occur in low concentrations within plants, are relatively unstable *in vitro*, and have particles of two or more modal lengths, which appear to correspond to an equal number of ssRNA genome components, and which are purified and separated with difficulty. By contrast, tobamoviruses occur within plants in high concentrations, are relatively stable *in vitro*, and have particles of one modal length (similar to the longest particles of the labile tobamo-like viruses), which contain one ssRNA genome component, and are purified with comparative ease. Also, most of the labile tobamo-like viruses are transmitted by plasmodiophorid fungi (Table 2), whereas the tobamoviruses have no known natural vector. Having noted the differences between soil-borne wheat mosaic virus (SBWMV) and the tobamoviruses, Shirako & Brakke (1984a) suggested that SBWMV and similar viruses be classified in a new group, known as the furoviruses (fungus-transmitted, rod-shaped viruses). In 1987 the International Committee on the Taxonomy of Viruses established the furovirus group with soil-borne wheat mosaic virus as the type member of the group. Other members of the group are peanut clump virus (PCV) and potato mop-top virus (PMTV). The group members share the following characteristics: rigid and hollow rod-shaped virions, plasmodiophorid fungus vectors and bipartite RNA genomes. Five other viruses (beet necrotic yellow vein [BNYVV], beet soil-borne [BSBV], broad bean necrosis [BBNV], and rice stripe necrosis viruses [RSNV]) are recognised as possible members of the group (Brunt, 1988; Fauquet *et al*, 1988). Hypochoeris mosaic, *Nicotiana velutina* mosaic and sorghum chlorotic spot viruses all have properties which suggest that they too have affinities with the furoviruses (Brunt, 1988). The

taxonomic status of these 11 viruses will be discussed further in section 1.8

Two further viruses, tobacco stunt (TSV) and lettuce big vein (LBVV), are reported to be transmitted by fungi and to have labile, rod-shaped particles, mostly measuring 18-22 x 330-375 nm (Kuwata & Kubo, 1981, 1984; Kuwata *et al.*, 1983; Masri & Hiruki, 1983). However, unlike the furoviruses, TSV and LBVV are transmitted by zoospores of the chytrid fungus *Olpidium brassicae*, they have a capsid protein with a molecular weight (48,000-52,000) that is almost three times larger, and have a genome of dsRNA instead of ssRNA (Masri & Hiruki, 1983; Dodds & Mirkov, 1983). Because TSV and LBVV have properties that differ markedly from any other labile, rod-shaped viruses, Masri & Hiruki (1983) proposed that they should be grouped separately.

There are several other labile, tobamo-like viruses (barley yellow mosaic, rice necrosis mosaic, wheat yellow mosaic and oat mosaic viruses) which are also transmitted by a plasmodiophorid fungus, *Polymyxa graminis*. However, they differ from the furoviruses in having longer, filamentous particles and inducing cylindrical, cytoplasmic inclusions in infected plants (Inouye & Fujii, 1977; Inouye & Saito, 1975; Saito *et al.*, 1968; Hebert & Panizo, 1975; Hooper & Wiese, 1972).

1.2 Occurrence and Biological Properties of the Members and Possible Members of the Furovirus Group

i) Beet Necrotic Yellow Vein Virus (BNYVV)

This virus induces rhizomania ("root madness"), a disease of sugar beet (*Beta vulgaris* var. *saccarifera*) first found in Italy (Canova, 1959, 1966a) and now known to occur also in Japan (Tamada *et al.*, 1971), France (Putz & Vuittenez, 1974), West Germany (Hamdorf *et al.*, 1977), Greece (Ioanides, 1978), Yugoslavia (Sutic & Milovanovic, 1978), Romania (Codrescu *et al.*, 1981); USA (Al-Musa & Mink, 1981; Duffus *et al.*, 1984), Austria (Koch, 1982), the USSR (Kolomietz, 1983); Czechoslovakia (Novak & Lanzova, 1984); Switzerland (Häni & Bovey, 1983), China (Gao *et al.*, 1983), The Netherlands (Heijbroeck,

Table 1 Natural Occurrence of the Furoviruses (adapted from Brunt & Shikata, 1986)

Virus Name	Abbreviation	Natural Host(s)	Geographical Distribution	References
Beet necrotic yellow vein	BNYVV	Beta vulgaris (sugar beet) Spinacia oleracea (spinach)	Austria	Koch (1982)
			Bulgaria	Jankulova et al. (1984)
			China	Gao et al. (1983)
			Czechoslovakia	Novak & Lanzova (1984)
			England	Hill & Torrance (1989)
			France	Putz & Vuittenez (1974)
			West Germany	Hamdorf et al. (1977)
			Greece	Ioanides (1978)
			Italy	Canova (1959)
				Faccioli & Giunchedi (1974)
				Rana et al. (1978)
			Japan	Tamada & Baba (1973)
				Tamada et al. (1971)
			Netherlands	Heijbroeck (1984)
			Romania	Codrescu et al. (1981)
			Sweden	Lindsten (1986)
			Switzerland	Hani & Bovey (1983)
			United States	Al Musa & Mink (1981)
			USSR	Kolomietz (1983)
			Yugoslavia	Sutic & Milovanovic (1978)
Beet soil-borne	BSBV	Beta vulgaris (sugar beet)	England	Henry et al. (1986)
Broad bean necrosis	BBNV	Vicia faba (broad bean)	Japan	Fukano & Yokoyama (1952) Inouye & Asatani (1968)

Virus Name	Abbreviation	Natural Host(s)	Geographical Distribution	References
Hypochoeris mosaic	HMV	Hypochoeris radicata (cat's ear)	Australia	Greber & Finlay (1981)
		Leontodon autumnalis (hawkbit)	Canada	Brunt & Stace-Smith (1978)
				Singh & McDonald (1980)
			England	Brunt in Brunt & Shikata (1986)
Nicotiana velutina mosaic	NVMV	Nicotiana velutina	Australia	Randles et al. (1976)
Oat golden stripe	OGSV	Avena sativa (oat)	England	Plumb & MacFarlane (1977)
				Adams et al. (1988)
Peanut clump	PCV	Arachis hypogaea (groundnut)	Ivory Coast	Thouvenel et al. (1976)
			India	Reddy et al. (1983)
		Sorghum arundinacearum (great millet)	Senegal	Thouvenel et al. (1974)
			Upper Volta	Bouhot (1967)
				Germani & Dhery (1973)
		Sorghum bicolor (sorghum)	India	Anon. (1987)
		Setaria italica		
Potato mop-top	PMTV	Solanum tuberosum (potato)	Peru	Salazar & Jones (1975)
			Western Europe	Calvert & Harrison (1966)
				Harrison & Jones (1970)
			Japan	Imoto et al. (1981)

Virus Name	Abbreviation	Natural Host(s)	Geographical Distribution	References
Rice stripe necrosis	RSNV	Oryza sativa (rice)	Ivory Coast	Fauquet & Thouvenel (1983)
Soil-borne wheat mosaic	SBWMV	Triticum aestivum (Winter wheat) Hordeum vulgare (barley)	Italy	Canova (1966b)
			Japan	Inouye (1969) Saito et al. (1964)
			United States	McKinney (1923) Brakke et al. (1965) Brandes et al. (1964)
			China	Cai et al. (1983)

1984); Bulgaria (Jankulova *et al.*, 1984); Sweden (Lindsten, 1986) and England (Hill & Torrance, in press). The virus also occurs in Swiss chard (*Beta vulgaris* var. *cycla oleracea*) in southern Italy (di Franco & Gallitelli, 1980).

The most conspicuous symptoms in sugar beet are severe stunting of infected plants and profuse development of lateral roots, the latter giving beets a characteristic bearded appearance from which the name of the disease is derived. In severely affected plants the tap root and some lateral roots are killed, and vascular tissue is discoloured; such plants, especially if infected early in the season, often die. Leaf symptoms occur rarely, but leaf veins sometimes become chlorotic and necrotic. BNYVV can reduce yields of sugar beet crops by about 30 % (Winner, 1984). The virus also causes stunting and conspicuous leaf chlorosis in Swiss chard.

BNYVV is sap-transmissible to 17 of 84 species from 3 of 19 families. In addition to sugar beet it infects at least 14 other species of the Chenopodiaceae, *Tetragonia expansa* (Aizoaceae) and *Gomphrena globosa* (Amaranthaceae). Like similar viruses, however, it infects only the inoculated leaves of most susceptible species, although it usually infects *Beta macrocarpa* systemically. Of susceptible species, *Chenopodium amaranticolor* is a good local lesion assay host and sugar beet seedlings are useful for propagating the virus and as bait plants for testing soils for infectivity (Tamada & Baba, 1973; Putz & Kuszala, 1978; Al-Musa & Mink, 1981).

ii) Beet Soil-Borne Virus (BSBV)

This virus was first detected by growing beet seedlings in *Polymyxa betae*-contaminated soil from Norfolk (Ivanović & MacFarlane, 1982), and has since been found to occur throughout England (Henry *et al.*, 1986). BSBV resembles BNYVV in particle size and shape, and its association with *P. betae*, the vector of BNYVV (Tamada, 1975). However, it differs from BNYVV in inducing different symptoms in test plants (Henry *et al.*, 1986). Also, it is serologically unrelated to BNYVV and fails to induce rhizomania in sugar beet (Ivanović *et al.*, 1983).

The Norfolk isolate of BSBV is sap-transmissible to 12 species of

Chenopodiaceae, but not to 22 species from 9 other families. The virus is restricted to the inoculated leaves of most susceptible species, where it induces chlorotic and/or necrotic lesions. One exception is the Viking cultivar of spinach, in which a symptomless systemic infection is reported to develop (Henry *et al.*, 1986).

iii) Broad Bean Necrosis Virus (BBNV)

This virus induces a severe disease of broad bean (*Vicia faba*), its only known natural host, in the Kyushu of Japan (Fukano & Yokoyama, 1952). Infected plants in early Spring often have young leaves with chlorotic vein-spotting, and older leaves with reddish-brown spots, rings, veinal necrosis and streaks. Severely affected plants are usually also stunted and have leaves that senesce prematurely. However, plants infected later have less obvious symptoms.

BBNV is sap-transmissible to 10 of 35 species in 4 of 9 families (Inouye & Asatani, 1968; Inouye & Nakasone, 1980). At temperatures below about 20 °C it induces systemic infection in *Lathyrus odoratus*, *Pisum sativum* and *Vicia faba*, and all three species are convenient hosts for virus propagation. However, it only infects the inoculated leaves of most other susceptible species, of which *Chenopodium amaranticolor* and *C. quinoa* are useful assay hosts.

iv) Hypochoeris Mosaic Virus (HMV)

This virus is known to occur in *Hypochoeris radicata* ("cats ear") in western Canada, Australia and Great Britain (Brunt & Stace-Smith, 1978a; Greber & Finlay, 1981; Brunt, 1986) and in *Leontodon autumnalis* ("autumn hawkbit") in eastern Canada (Singh & McDonald, 1980). Naturally infected plants of both hosts usually have some leaves that are chlorotic and others that are either symptomless or are inconspicuously chlorotic.

HMV is sap-transmissible to 9 of 51 species from 5 of 11 families, and three species (*Chenopodium amaranticolor*, *C. quinoa* and *Nicotiana clevelandii*) are convenient propagation and assay hosts.

v) Nicotiana velutina Mosaic Virus (NVMV)

This virus occurs naturally in the weed *Nicotiana velutina* in South Australia (Randles *et al.*, 1976). It is sap-transmissible to 22 of 30 species in 4 of 9 families, infecting *Gomphrena globosa* (Amaranthaceae) and 21 species of the Solanaceae and Chenopodiaceae. NVMV induces conspicuous chlorosis in systemically infected leaves of most susceptible hosts, but it only induces lesions in the inoculated leaves of *C. capitatum*, *C. quinoa* and *Spinacia oleracea*.

vi) Oat Golden Stripe Virus (OGSV)

This virus is known to occur in Britain (Plumb & MacFarlane, 1977) and also in France where the synonym 'soil-borne oat stripe virus' is preferred (Hariri & Lapierre, 1985). It was first found infecting autumn sown oats (*Avena sativa*) in south-west England (MacFarlane *et al.*, 1968), but was later found also in Kent (Plumb & MacFarlane, 1977) and in west Wales (Catherall *et al.*, 1977). OGSV, which frequently occurs in naturally-infected plants with oat mosaic virus, alone induces chlorotic striping which is most conspicuous in flag and leaves immediately below them.

OGSV is sap-transmissible from infected to healthy oat cv. Blenda (Plumb & MacFarlane, 1978), and induces chlorotic or necrotic local lesions in *C. amaranticolor*, *N. debneyi* and *N. clevelandii* and occasional systemic infection in the latter.

vii) Peanut Clump Virus (PCV)

This virus occurs in groundnut (*Arachis hypogaea*), sorghum (*Sorghum bicolor*) and *Setaria italica* in India, and in groundnut and great millet (*Sorghum arundinaceum*) in West Africa. Indian PCV was originally thought to be a distinct virus known as Indian peanut clump virus, because it is apparently serologically unrelated to West African PCV (Reddy *et al.*, 1979, 1983). However, cultures from India and West Africa show 21 to 40 % RNA sequence homology, so they are now recognised as isolates of PCV (Mayo & Reddy, 1985; Reddy *et al.*, 1985).

Naturally infected plants in India are severely stunted, have

leaves which are chlorotic or dark green, produce small pods and have darkly coloured roots (Reddy *et al.*, 1979). Similar symptoms often occur in infected plants in West Africa, although severe leaf chlorosis without stunting is characteristically induced by one strain.

The virus has a wide experimental host range. Isolates from West Africa are sap-transmissible to 39 species in 8 families, and those from India to 13 of 20 species in the Chenopodiaceae, Leguminosae and Solanaceae. PCV induces chlorotic or necrotic lesions in inoculated leaves of *Chenopodium quinoa, Canavalia ensiformis, Cyamopsis tetragonoloba, Phaseolus vulgaris* and *Vigna unguiculata*, and chlorosis or necrosis of systemically infected leaves of *Capsicum annuum, Cassia occidentalis, Crotalaria juncea, Nicotiana benthamiana, N. clevelandii* and *N. edwardsonii*. The virus is most conveniently cultured in *N. benthamiana* and assayed in *P. vulgaris, C. quinoa* or *C. amaranticolor*.

viii) Potato Mop-Top Virus (PMTV)

The severity of symptoms induced by PMTV in potato (*Solanum tuberosum*), its only known natural host, is dependent upon a number of factors, including tolerance of the cultivar, virulence of the infecting virus and environmental conditions (Calvert & Harrison, 1966; Harrison & Jones, 1971a, b). Plants of intolerant cultivars are severely stunted and have a characteristic "mop-top" appearance. The leaves of intolerant cultivars usually only develop symptoms in cool conditions (5-15 °C); some may then develop yellow blotches or chlorotic chevron patterns on leaflets. The tubers of some cultivars, such as Arran Pilot, develop so-called "spraing" symptoms in the year of natural infection; such symptoms consist of internal areas of brown necrotic tissue which are sometimes evident as brown rings on tuber surfaces. The necrosis, which occurs at the boundary of healthy and infected tissue, often develops after a sudden marked change in temperature (Harrison & Jones, 1971). Plants of other cultivars, when infected, produce tubers with only raised superficial rings (Calvert, 1968). Tubers produced during the second year of infection may be either symptomless or cracked and distorted, and

stolons may develop internal brown necrotic arcs.

The virus is sap-transmissible to 26 of 37 species in 3 of 14 families (Solanaceae, Chenopodiaceae and Aizoaceae). Symptoms in susceptible species are, like those in potato, often dependent upon environmental conditions (Harrison & Jones, 1970, 1971). Useful indicator plants are *Chenopodium amaranticolor*, which develops lesions in inoculated leaves only, and *Nicotiana debneyi*, *N. benthamiana* and *N. tabacum* cvs Xanthi-nc and Samsun NN, which, in addition to chlorotic lesions in inoculated leaves, develop systemic chlorotic vein-banding. *N. debneyi*, *N. benthamiana* and *N. tabacum* cv Xanthi-nc are convenient assay and virus propagation hosts, and the former is also a useful 'bait' plant for fungal transmission tests.

PMTV is known to occur in Peru, throughout western Europe, in Japan and possibly elsewhere where potatoes are grown (Harrison, 1974; Imoto *et al.*, 1981).

ix) Rice Stripe Necrosis Virus (RSNV)

This virus causes a disease in upland rice (*Oryza sativa*) in several countries of West Africa, although it does not appear to be spreading and occurs at a relatively low frequency (Louvel & Bidaux, 1977; Fauquet & Thouvenel, 1983; Fauquet *et al.*, 1988). Conspicuous chlorotic or necrotic stripes often develop on the leaves of infected plants within three weeks of sowing, and such plants are later severely stunted, have a reduced number of tillers and yield little grain. Although lethal necrosis often follows early infections, plants infected later survive, although their tillering and grain yield are severely reduced (Louvel & Bidaux, 1977).

The virus cannot be mechanically transmitted to rice, its only known natural host. However, it does induce lesions in inoculated leaves of *Chenopodium amaranticolor* and *Nicotiana benthamiana*.

x) Soil-Borne Wheat Mosaic Virus (SBWMV)

This is the best known of the furoviruses and is the type member of the group (Shirako & Brakke, 1984a). It was first recognised in Illinois and Indiana, USA in 1919 (McKinney, 1923), but is now known to occur in many winter wheat growing areas of the USA (e.g. Fellows

et al., 1953; Sill, 1958; Brakke, 1971; Hunger & Sherwood, 1985). It has since also been found in Italy (Canova, 1966b), Japan (Inouye, 1969; Saito *et al.*, 1964), France (Lapierre *et al.*, 1985) and China (Cai *et al.*, 1983). Winter wheat (*Triticum aestivum*) is the major host of the virus, but barley (*Hordeum vulgare*) is also a natural host. In both species the severity of infection varies greatly, being mainly dependent upon a combination of factors, including virus strain, crop cultivar, age of plant when infected and environmental conditions (Brakke, 1971). Thus, some wheat cultivars such as Harvest Queen and its derivatives, Parker 76, TAM 105, Sage, Sturdy and Vona are very susceptible, and tend to become infected early. After infection such plants are severely stunted and have unusually numerous tillers. Symptom development is slower and much less conspicuous in resistant cultivars such as Arkan, Newton, Plainsman V and Homestead (Bockus & Niblett, 1984). In wheat, light green to yellow mosaic leaf symptoms usually first appear in spring, but occasionally develop in autumn, and most frequently develop in plants grown in low-lying areas of fields. Such areas are then apparent from a distance, but symptoms tend to become less conspicuous as plants mature and prevailing temperatures increase (Koehler *et al.*, 1952). In Kansas. it is estimated that SBWMV caused an annual yield loss of 337 x 10^6 kilograms during the period 1975-82 (Sim & Willis, 1982).

The virus is sap-transmissible with difficulty to only a few species of the Graminae and Chenopodiaceae. In addition to wheat and barley, SBWMV also infects rye (*Secale cereale*), *Bromus commutatis*, *B. tectorum* and some *Chenopodium* species (Rao & Brakke, 1969; Paulsen, 1970). SBWMV is best propagated in susceptible wheat cultivars, such as Michigan Amber, and assayed in *C. amaranticolor* or *C. quinoa* (Brakke, 1971).

xi) Sorghum Chlorotic Spot Virus (SCSV)

This virus was originally isolated from sorghum (*Sorghum bicolor*), its only known natural host, in Kansas, USA, and was found to be morphologically and physicochemically similar to SBWMV (Kendall *et al.*, 1988). It induces symptoms which include distinct elongated

chlorotic spots and ring spots, as well as yellowing, on systemic leaves of sorghum. SCSV is not mechanically transmissible to sorghum, although it is readily transmitted to several inbred maize (*Zea mays*) varieties, resulting in systemic symptoms similar to those in naturally infected sorghum. Systemic infection and symptom development are optimal at 25 °C, and infection decreases significantly above 35 °C or below 20 °C. This is in contrast to SBWMV, which causes systemic infection between 15 and 17 °C (Brakke & Rao, 1967). SCSV also infects the inoculated leaves of *C. amaranticolor*, *C. quinoa* and *Nicotiana clevelandii*.

1.3 Virus Transmission

1.3.1 Fungal Vectors

Of the eleven definite or putative members of the furovirus group nine are known to be soil-borne, and of these eight are transmitted by a plasmodiophorid fungus (Table 2). Neither of the viruses that naturally infect weeds - HMV and NVMV - is known to be soil-borne, although adequate transmission tests have not been carried out. Tests on SCSV transmission have found no evidence that the virus is soil-borne. However, the infection pattern of SCSV in the field is consistent with there being a soil-borne vector, and this along with the virus's physicochemical similarity to SBWMV, has led Kendall *et al.* (1988) to predict that SCSV is transmitted in nature by a plasmodiophorid fungus.

The three species of plasmodiophorid fungi that are involved in furovirus transmission - *Polymyxa betae*, *P. graminis* and *Spongospora subterranea* - are obligate parasites that naturally infect relatively few species, but will colonise many more under experimental conditions (Jones & Harrison, 1969, 1972). All three species have comparatively wide geographical distributions; *P. graminis* is known to occur in Africa (Thouvenel & Fauquet, 1981; Fauquet & Thouvenel, 1983), Asia (Inouye & Saito, 1975), Europe (Canova, 1966b; MacFarlane *et al.*, 1968), Australasia (Karling, 1968), North America (Ledingham, 1939; Linford & McKinney, 1954; Karling, 1968; Barr, 1979) and South America (Salazar & Jones, 1975).

P. graminis was the first plasmodiophorid to be identified as a

Table 2 Natural Modes of Spread of Furoviruses

(adapted from Brunt & Shikata, 1986)

Virus	Transmission Through: Seed	Soil	Vector	References
BNYVV	NR	+	*Polymyxa betae*	Tamada *et al.* (1975)
				Vuittenez *et al.* (1977)
BSBV	NR	+	*Polymyxa betae*	Henry *et al.* (1986)
BBNV	-	+	? *Polymyxa* sp.	Fujikawa (1963)
HMV	-	NR	Unknown	Brunt & Stace-Smith (1978)
NVMV	+	-	Unknown	Randles *et al.* (1976)
OGSV	-	+	*Polymyxa graminis*	Plumb & MacFarlane (1977)
PCV	+	+	*Polymyxa graminis*	Thouvenel & Fauquet (1981)
				Thouvenel *et al.* (1976)
				Mayo & Reddy (1985)
PMTV	NR	+	*Spongospora subterranea*	Jones & Harrison (1969)
RSNV	-	+	*Polymyxa graminis*	Fauquet & Thouvenel (1983)
SBWMV	-	+	*Polymyxa graminis*	Estes & Brakke (1966)
				Canova (1966b)
SCSV	NR	-	Unknown	Kendall *et al.* (1988)

Notes: NR - no records
+ - transmission
- - no transmission

virus vector. It was initially found to transmit SBWMV (Brakke *et al.*, 1965; Estes & Brakke, 1966; Rao & Brakke, 1969), but it is now also known to be the vector of OGSV, PCV, RSNV and probably BBNV. Although *P. graminis* is a vector of PCV, it does not usually parasitise one of the two natural hosts - groundnut - in West Africa. It is, however, a common parasite of the other natural host - great millet - which led Thouvenel & Fauquet (1980, 1981) to suggest that *P. graminis* first parasitises great millet from where the virus is probably transmitted to groundnut.

P. betae is the known vector of two furoviruses - BNYVV and BSBV (Tamada *et al.*, 1975; Fujisawa & Sugimoto, 1977; Vuittenez *et al.*, 1977; Ivanović *et al.*, 1983), and *S. subterranea* is the vector of PMTV (Jones & Harrison, 1969).

The relationship between furoviruses and their vectors is described as persistent (Teakle, 1983), because the viruses can survive for many months, or up to 12 years in the case of PMTV, within resting spores in air-dried soil (McKinney, 1937; Rao & Brakke, 1969; Jones & Harrison, 1969, 1972). Unlike viruses that are transmitted non-persistently by *Olpidium brassicae* (Teakle, 1983), furoviruses are acquired by their vectors *in vivo* but not *in vitro*, and transmission is not reduced by treating viruliferous zoospores with specific virus antiserum or resting spores with acid or alkali (Rao & Brakke, 1969; Jones & Harrison, 1969). Moreover, BNYVV-like particles occur within viruliferous *P. betae* zoospores (Tamada, 1975).

The thalli of the fungi acquire virus *in vivo* and then produce viruliferous zoospores, which require moisture for their release and movement (e.g. Jones & Harrison, 1969; Tamada *et al.*, 1975; Fujisawa & Sugimoto, 1977; Ivanović *et al.*, 1983). Virus spread thus often occurs after sustained periods of rainfall or after crop irrigation, and outbreaks of SBWMV are most obvious after extended cool damp Springs (Larsen *et al.*, 1985). Similarly, in Scotland the incidence of PMTV is correlated with total annual rainfall (Cooper & Harrison, 1973).

1.3.2 Seed Transmission

Only two of the furoviruses - NVMV and PCV - are known to be seed-borne. NVMV is seed-borne to a high level (up to 72 %) in *Nicotiana glutinosa, N. clevelandii, N. debneyi* and *N. rustica* but not in *Beta macrocarpa* (Randles *et al.*, 1976). This high level of seed transmission possibly permits NVMV to survive in a semi-arid environment in which vegetative growth and activity of any soil-borne vector is brief and intermittent (Randles *et al.*, 1976).

In West Africa, PCV has been found to be seed-borne in *Arachis hypogaea* but not in *Phaseolus mungo, Sorghum arundinacearum* or *N. benthamiana.* From 6 to 14 % of seeds collected from naturally infected groundnut plants and 19-24 % of those from artificially infected plants are infected (Thouvenel *et al.*, 1978; Thouvenel & Fauquet, 1981). It is interesting to note that the Indian strain of PCV is not seed-borne in groundnut (Reddy *et al.*, 1983).

1.4 Cytopathology of the Furoviruses

The cytopathology of only six of the furoviruses (BBNV, BNYVV, HMV, OGSV, PMTV and SBWMV) has been studied in detail. Each virus was found to occur in the cytoplasm and vacuoles of parenchyma cells, but not within the vascular tissues or organelles.

PMTV particles are sometimes found in parallel array in small aggregates in infected cells of *Nicotiana tabacum* cv. Xanthi-nc, and occasionally numerous particles occur in association with organelles in cytoplasmic masses up to 10 µm wide (White *et al.*, 1972).

BBNV particles have also been found in aggregates of near parallel array, in the cytoplasm of older cells of infected broad bean leaves (Inouye, 1971). Similarly, in oat roots and leaves, OGSV particles usually occur in large cytoplasmic aggregates bounded by a single membrane (Plumb *et al.*, 1977).

BNYVV particles were found in aggregates in a few cells of systemically-infected sugar beet and spinach leaves, and these aggregates were in parallel arrays, layers of which alternated at 45° to form angled layer aggregates (Stocky *et al.*, 1977; Putz & Vuittenez, 1980; Giunchedi *et al.*, 1981; Russo *et al.*, 1981). These aggregates are essentially similar to those induced by strains of TMV

(Herold & Munz, 1967; Warmke, 1968). Similar layered aggregates have also been found in PCV-infected millet cells (M. Dollet, in Thouvenel & Fauquet, 1981).

Particles of HMV were usually found loosely aggregated in the cytoplasm of infected cells, but they were also occasionally found in parallel arrays within the cisternae of endoplasmic reticulum or densely aggregated in paracrystals (Brunt & Stace-Smith, 1978b). Similar crystals have been found occasionally in SBWMV-infected wheat cells (Hibino *et al.*, 1974a, b). Crystalline inclusions are formed in HMV-infected cells and virus particles are commonly found within, or at the periphery of these structures (Brunt & Stace-Smith, 1978b). Most inclusions were enclosed in a membrane which, from its continuity with normal cellular membranes, suggested that the inclusions developed within the cisternae of the endoplasmic reticulum. Crystalline bodies with particles at their peripheries, have also been found in wheat cells infected with SBWMV (Hibino *et al.*, 1974a; Peterson, 1970).

SBWMV, like the other furoviruses, has particles which are either scattered randomly in the cytoplasm or found within aggregates. However, these aggregates differ in form, depending on the isolate. Thus, all SBWMV isolates from Japan formed crystalline arrays, whereas those from the USA did not (Hibino *et al.*, 1974a). However, aggregated particles of all isolates were found in the vacuoles of older cells.

None of the six viruses mentioned causes any apparent damage to organelles. However, most induce the formation of inclusions consisting of interwoven masses of tubules, ribosomes and virus particles (Inouye, 1971; McKinney *et al.*, 1923). These inclusions were found not to be all the same for each of nine SBWMV isolates; those of seven isolates consisted of tubules or endoplasmic reticulum, whereas those of two other isolates consisted of either small or large masses of vesicles and membrane-bound bodies (Hibino *et al.*, 1974a). The sequential development of these inclusions was studied by Hibino *et al.* (1974b), who found that they first appeared in leaves 10-30 mm long, as masses of tangled tubules with virus particles scattered within their interstices. These inclusions enlarged and developed a looser conformation as the leaves grew,

until, in leaves longer than 100 mm, they had mostly disintegrated and virus particles were then found in the cytoplasm and vacuoles.

Similar inclusions consisting of masses of tubules are found in sweet peas infected by BBNV (Inouye, 1971), in oats infected with OGSV (Plumb *et al.*, 1977) and in potatoes infected with PMTV (Fraser, 1976). PMTV inclusions consist of large tufts or clusters of microtubules and occur in the cytoplasm, between cell walls and plasma membranes, and in vacuoles of epidermal, palisade, mesophyll and vascular parenchyma cells and in sieve tubes (Fraser, 1976).

1.5 **Virus Purification**

1.5.1 Purification of Rod-Shaped Plant Viruses

It does not seem unreasonable to expect that virus particles sharing biophysical and morphological characteristics would require similar procedures for their purification. However, among the rod-shaped plant viruses - both rigid and flexuous - the purification protocols reported, vary greatly in their ability to produce intact, infectious virus particles, in reasonable quantities and free from host material.

The tobamoviruses and tobraviruses are easy to purify, because they are relatively stable *in vitro* and are present in high concentrations in the plant (van Regenmortel, 1981; Harrison & Robinson, 1986), but for the other elongated viruses (hordeiviruses [Jackson & Lane, 1981], potexviruses [Purcifull & Edwardson, 1981], carlaviruses [Wetter & Milne, 1981], potyviruses [Hollings & Brunt, 1981], barley yellow mosaic virus (BaYMV) [Huth *et al.*, 1984], closteroviruses [Lister & Bar-Joseph, 1981] and furoviruses [Brunt & Shikata, 1986]) the problems of virus aggregation and/or fragility are frequently encountered during purification.

The rigid hordeiviruses and the flexuous potex- and carlaviruses tend to aggregate during purification and the latter two also tend to fragment. However, this is not particularly important for the purification of these viruses, because of their moderate (carla-) and high (hordei- and potex-) concentrations in plants.

On the other hand, the flexuous poty- and closteroviruses and BaYMV, and the rigid furoviruses are probably present in lower

concentrations in plants, and this, coupled with the instability of the particles and their tendency to aggregate and/or fragment, has caused significant problems in purifying them. In the case of the furoviruses, the problems encountered and the strategies devised to overcome them will be discussed further in the next section.

1.5.2 Purification of Furoviruses

Early attempts to purify SBWMV were very laborious and then often only partially successful, due to the instability of the virus and its tendency to aggregate (Thornberry *et al.*, 1953; Hebert & Coleman, 1955; Hebert, 1963; Brandes *et al.*, 1964; Saito *et al.*, 1964; Rao & Brakke, 1969). The first reliable procedure (Gumpf, 1971), in which up to 150 mg of virus per kilogram of leaf tissue was obtained, involved clarifying leaf extracts with an equal volume of a 1:2 mixture of ethylene dichloride and chloroform followed by differential centrifugation; sedimented virus was further purified by rate zonal centrifugation in sucrose columns containing a detergent. However, Gumpf (1971) noted that longer particles fragmented throughout the procedure, so that shorter particles and/or particle fragments were always present with longer particles in the faster-sedimenting component. A similar procedure in which Triton X-100 was used for clarification, consistently yields 20-30 mg of SBWMV per kilogram of leaf tissue (Powell, 1976; Shirako & Brakke, 1984b). This same method has since also been used successfully to purify SCSV (Kendall *et al.*, 1988), yielding 40-100 mg of virus per kilogram of infected *Zea mays* leaves and 200-500 mg per kilogram of infected *Nicotiana clevelandii* leaves.

BBNV and the Indian strains of PCV have been partially purified from infective leaf extracts clarified with chloroform or diethyl ether by precipitating virus with polyethylene glycol (PEG) and, to further purify the virus, by sedimenting particles through a 20 % sucrose cushion or in a sucrose density gradient column (Nakasone & Inouye, 1978; Reddy *et al.*, 1985).

Up to 12 mg of RSNV per kilogram of rice leaves can be obtained by clarifying leaf extracts with a mixture (1:4) of chloroform and *n*-butanol and, after one cycle of differential centrifugation purifying

the virus further by rate-zonal sucrose density gradient centrifugation (Fauquet & Thouvenel, 1983).

PCV can be purified by two or three cycles of differential centrifugation, after clarification of leaf extracts by acidification to pH 4.2 (Thouvenel *et al.*, 1978; Thouvenel & Fauquet, 1981). The virus can be purified further by isopycnic centrifugation in 45 % caesium chloride, when yields of 20-25 mg of virus per kilogram of leaf tissue are obtainable. HMV can also be purified by differential centrifugation followed by chromatography on controlled pore glass beads, although the virus is so unstable that yields of only 4-8 mg of virus per kilogram of leaf tissue have been obtained (Brunt & Stace-Smith, 1978a).

BNYVV can be purified by precipitating virus from clarified leaf extracts with 4 % PEG and repeating the PEG precipitation after further clarification with fluorocarbon (Tamada & Baba, 1973). A similar procedure, but using 5 % PEG and further purifying virus by centrifugation through a 20 % (w/v) sucrose cushion, yielded 5 to 20 mg of virus per kilogram of leaf tissue (Putz & Kuszala, 1978). Nevertheless, it was noted that considerable losses of virus resulted from aggregation of particles and/or their attachment to larger host components. This procedure was also used for purifying BNYVV by Steven *et al.* (1981), with the addition that zone electrophoresis was used for further purification of PEG-precipitated virus. More recently, silver nitrate and chloroform have been used for clarification before precipitating virus with PEG and then purifying the virus further by sucrose density gradient centrifugation and incubation of the virus preparation with antiserum to normal plant proteins (Koenig *et al.*, 1984). The method used for purifying cauliflower mosaic virus (Hull *et al.*, 1976) has, with slight modifications, also been used to purify BNYVV (Koenig *et al.*, 1984). A non-ionic gradient medium - Nycodenz - has been shown to be useful for isopycnic centrifugation of BNYVV (GÜgerli, 1984).

Initial attempts at purifying PMTV were unsuccessful, due to the instability of the virus particles and their tendency to aggregate with each other and with host components (Harrison & Jones, 1970), which caused the virus to sediment when sap was centrifuged at 8000 g (Harrison & Jones, 1970; Kassanis *et al.*, 1972). PMTV has since been

successfully eluted from this sediment by resuspending in 0.5 M borate buffer (pH 7.5), clarifying the extract with diethyl ether and carbon tetrachloride, and sedimenting the virus by ultracentrifugation. Virus preparations can be decolourised by adding an equal volume of ethylene dichloride, and particles of different lengths then separated by sucrose density gradient centrifugation (Kassanis *et al.*, 1972). A later method for eluting PMTV from the sediment from the first low speed spin (Randles *et al.*, 1976) involved resuspending the pellet in 0.05 M tris-HCl buffer (pH 8.1) containing 0.05 % thioglycerol and, after stirring for two hours at 4 °C, centrifuging the extract through a 55 % (w/v) sucrose cushion twice, and finally subjecting the resuspended pellet to sucrose density gradient centrifugation. This method was also used to purify NVMV, yielding 0.75 to 2.5 mg of NVMV per kilogram of leaf tissue. A modification of this method involving centrifugation through 86.5 % (w/v) caesium chloride, instead of through a sucrose density gradient, has yielded 10 mg of PMTV per kilogram of leaf tissue. (P. Massalski, personal communication).

1.6 Virus Structure and Composition

1.6.1 Morphology, Size and Structure

The furoviruses have particles which, although superficially similar to those of tobamoviruses, differ in some important features. Thus, the particles characteristically occur in low concentrations *in vivo*, are labile *in vitro*, frequently have obvious defects in their protein helices, and have two or more modal lengths less than 300 nm (e.g. Harrison & Jones, 1970; Kassanis *et al.*, 1972; Brunt & Stace-Smith, 1978a; Reddy *et al.*, 1983). The particles of some, such as PMTV and HMV, have helices which frequently uncoil at one end, have fissures throughout their lengths or have parts missing, expanded or partially disrupted (Figure 1). HMV particles frequently fragment where helical discs separate from the particles; such discs can sometimes be seen either attached to particle fragments or, after separation, in end-on view. Fragmentation of HMV particles is not prevented by first fixing leaves in 3 % glutaraldehyde or osmium tetroxide (Brunt & Stace-Smith, 1978a).

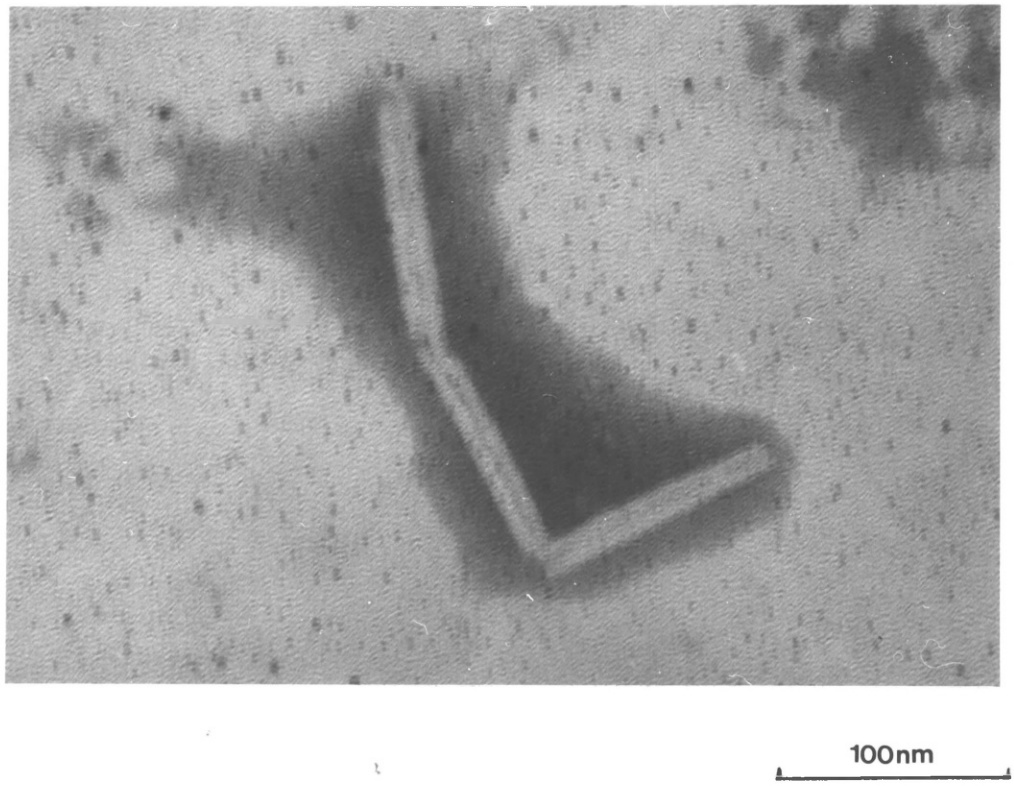


Figure 1 Electron Micrograph of a PMTV-T Particle

The sample was negatively stained with 2 % potassium phosphotungstate (pH 6.8). The final magnification is x 300,000.

Negatively stained furovirus particles have diameters which, although reported to range from 17 to 25 nm, are mostly 18-20 nm wide (Table 3). However, there is not so much uniformity in the number and size of particle modal lengths. Thus, the particles of some (BBNV, HMV, OGSV, PCV, PMTV and SCSV) are of two modal lengths, the particles of three others (BSBV, RSNV and SBWMV) are of three modal lengths, and the particles of BNYVV are of four modal lengths (Table 3).

Most of the viruses have one particle whose modal length falls within the range 100-150 nm and another within the range 250-300 nm. A few, such as BNYVV and RSNV also have one particle 380-390 nm long, while BNYVV, BSBV and SBWMV in addition have one particle 65-110 nm long. The particle length distribution of these viruses is often difficult to establish. For example, although particles of NVMV in extracts during early stages of purification are predominantly 250-300 nm long, those in purified preparations are mostly 100-175 nm long. The increase in the number of short particles was accompanied by a concomitant decrease in the number of longer particles (Randles *et al.*, 1976). This phenomenon has also been observed with several other furoviruses: PMTV particles mostly measure 100-150 and 250-300 nm in clarified sap, but in purified preparations are mostly 40 and 80 nm long (Kassanis *et al.*, 1972); with HMV there was a gradual increase in the number of short particles corresponding to a decrease in the number of long particles during purification (Brunt & Stace-Smith, 1978a); for Indian isolates of PCV purified virus preparations, unlike clarified sap, contain an unusually large number of particle fragments 60-100 nm long (Reddy *et al.*, 1983).

The occurrence of a very wide range of particle lengths, including an abundance of particle fragments in purified preparations, and the apparent fragility of the particles, suggest that the shorter particles, or at least a large proportion of them are fragments of longer fragile particles. However, in the case of SBWMV the occurrence of abnormally short particles in extracts of plants (Tsuchizaki *et al.*, 1973; Brakke, 1977; Hibino *et al.*, 1981) has more recently been attributed to the spontaneous occurrence of deletion mutations in which the deletion of 700 or 1050 bases from RNA in particles 138 nm long results in the production of particles

Table 3 The Sizes of Furovirus Particles and their Components

(Adapted from Brunt, 1988)

Virus	Particle Dimensions (nm)					Coat Protein	Sizes of RNAs (nucleotides)			
	Width	Lengths								
BBNV	25	-	250	150	-	-	-	-	-	-
BNYVV(F)	20	390	265	100	85	20,682	6746	4612	1774	1467
BSBV	19	-	300	150	65	-	-	-	-	-
HMV	21-22,5	-	240	120	-	24,500	-	-	-	-
NVMV	18-19	-	-	100-175	-	21,400	-	-	-	-
OGSV	20	-	305	152	-	-	-	-	-	-
PCV(I)	24	-	249	184	-	24,500	5900	4300		
PMTV	17	-	290	125	-	19,800	-	-	-	-
RSNV	20	380	270	110-160	-	24,000	-	-	-	-
SBWMV	20	-	281	138	92-110	19,700	6500-6900	3500	2450-2800	
SCSV	20	-	260	140	-	20,500	6200	3500		

Notes: (F) - French isolate (F2)

(I) - Indian isolate (L)

References:

BBNV Inouye & Asatani, 1968.

BNYVV(F) Putz, 1977; Bouzoubaa *et al.*, 1985, 1986, 1987.

BSBV Henry *et al.*, 1986.

HMV Brunt & Stace-Smith, 1978a.

NVMV Randles *et al.*, 1976; Randles, 1978.

OGSV Plumb & MacFarlane, 1977.

PCV(I) Reddy *et al.*, 1983, 1985; Mayo & Reddy, 1985.

PMTV Kassanis *et al.*, 1972; Randles *et al.*, 1976; Roberts & Harrison, 1979.

RSNV Fauquet & Thouvenel, 1983; Fauquet, 1985.

SBWMV Gumpf, 1971; Shirako & Brakke, 1984a.

SCSV Kendall *et al.*, 1988.

110 and 92 nm long, respectively (Shirako & Brakke, 1984b).

The detailed structure of only BNYVV particles has been reported. Optical diffraction and image processing techniques have shown that the particles of the French isolate, although of four different lengths, are identical in structure (Steven *et al.*, 1981). The protein helix of each particle type is, like that of tobamoviruses, a single right handed strand, with a pitch of 2.6 nm which, with 49 structural subunits in four complete turns, has 12¼ subunits per helical turn.

1.6.2 Composition

1.6.2i Capsid Protein

Spectrophotometry of purified preparations indicates that furoviruses contain about 95 % protein (Brunt & Stace-Smith, 1978a; Randles *et al.*, 1976; Thouvenel & Fauquet, 1981; Fauquet & Thouvenel, 1983). The protein consists of a single polypeptide in each of the eight viruses that have been studied, with a molecular weight in the range 19,700 to 24,500, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) (Table 3).

The amino acid compositions of the capsid proteins of BNYVV (Putz, 1977), PCV (Indian and West African isolates) and RSNV (Fauquet & Thouvenel, 1987) have been determined, and compared with those of the other rod-shaped viruses - tobamoviruses, tobraviruses and hordeiviruses (Fauquet *et al.*, 1988). Computer analysis revealed that the amino acid compositions of the capsid proteins of RSNV and the West African and Indian isolates of PCV were similar to each other but distinct from those of BNYVV and the other rod-shaped viruses. The amino acid composition of the BNYVV capsid protein was more closely related to that of viruses transmitted by nematodes than to that of fungus-transmitted viruses. These results suggest that PCV and RSNV are members of a distinct group - the furoviruses - and that BNYVV is a member of different group of fungus-transmitted, rod-shaped viruses. However, until more information is obtained on the furovirus group, BNYVV will still be considered as a tentative member.

1.6.2ii Nucleic Acid

Although the nucleic acid of some furoviruses has yet to be isolated and characterised, particles of six (BNYVV, HMV, NVMV, PCV, SBWMV and SCSV) are known to contain approximately 5 % ssRNA (Putz, 1977; Brunt & Stace-Smith, 1978a; Randles *et al.*, 1976; Thouvenel & Fauquet, 1981; Gumpf, 1971; Shirako & Brakke, 1984a; Kendall *et al.*, 1988). Preparations of NVMV were found by PAGE to contain a major RNA species of 2.3×10^6 molecular weight and eight minor components (Randles, 1978). The ssRNAs of BNYVV, PCV, SBWMV and SCSV have been separated and sized by denaturing agarose gel electrophoresis and, in the case of BNYVV, also by sequencing, and for each virus the number of ssRNA species corresponds to the number of particle modal lengths. Moreover, the ratio of ssRNA sizes is roughly equivalent to the ratio of particle sizes (Table 3). This suggests that virus particles of different lengths contain specific ssRNA molecules.

1.7 Genome Organisation and Expression

i) Beet Necrotic Yellow Vein Virus

Each of the four BNYVV particles contains a distinct RNA molecule and all four of these RNA species have been cloned and sequenced (Bouzoubaa *et al.*, 1985, 1986, 1987). They each possess a 5' terminal cap structure, m^7GpppA, and a 3' terminal poly A tail ranging from 65 to 140 residues in length (Putz *et al.*, 1983). RNA 1 is 6746 nucleotides long excluding the poly A tail, and contains a single long open reading frame extending from AUG (154) to UAA (6481) and capable of encoding a polypeptide of M_r 237,389 (237 K). Computer-assisted sequence comparisons have revealed homology between this polypeptide and long non-structural polypeptides from a number of other (+)-strand RNA viruses which are thought to be involved in RNA replication (Bouzoubaa *et al.*, 1987). Two regions of homology exist, designated BN1 and BN2 in Figure 2. BN2 is one of the set of homologous domains typified by the readthrough portion of the tobacco mosaic virus (TMV) p183 protein, the Sindbis virus nsp readthrough protein and the RNA 2-encoded proteins of the tripartite plant viruses (Ahlquist *et al.*, 1985). It contains a candidate 'core

Legend to Figure 2 Genome Organisation of BNYVV

Open reading frames discussed in the text are shown as hollow rectangles, with coding capacity in kilodaltons (K) given within. Translation products which have been detected and mapped are designated by arrows above each RNA. Viral capsid protein is denoted CP. Filled rectangles represent regions of extensive sequence homology among the RNAs. A solid triangle (▲) represents the suppressible RNA 2 amber termination codon, and a solid circle (●) the m^7GpppA 5' terminal cap structure. Regions of sequence homology in RNA 1 with suspected plant virus replicases are shaded. The filled square and diamond indicate the position of the consensus sequence motifs referred to in the text. The extent of deletions detected within RNAs 3 and 4 of isolates G1, G4 and F15 is indicated by the lines beneath each RNA.

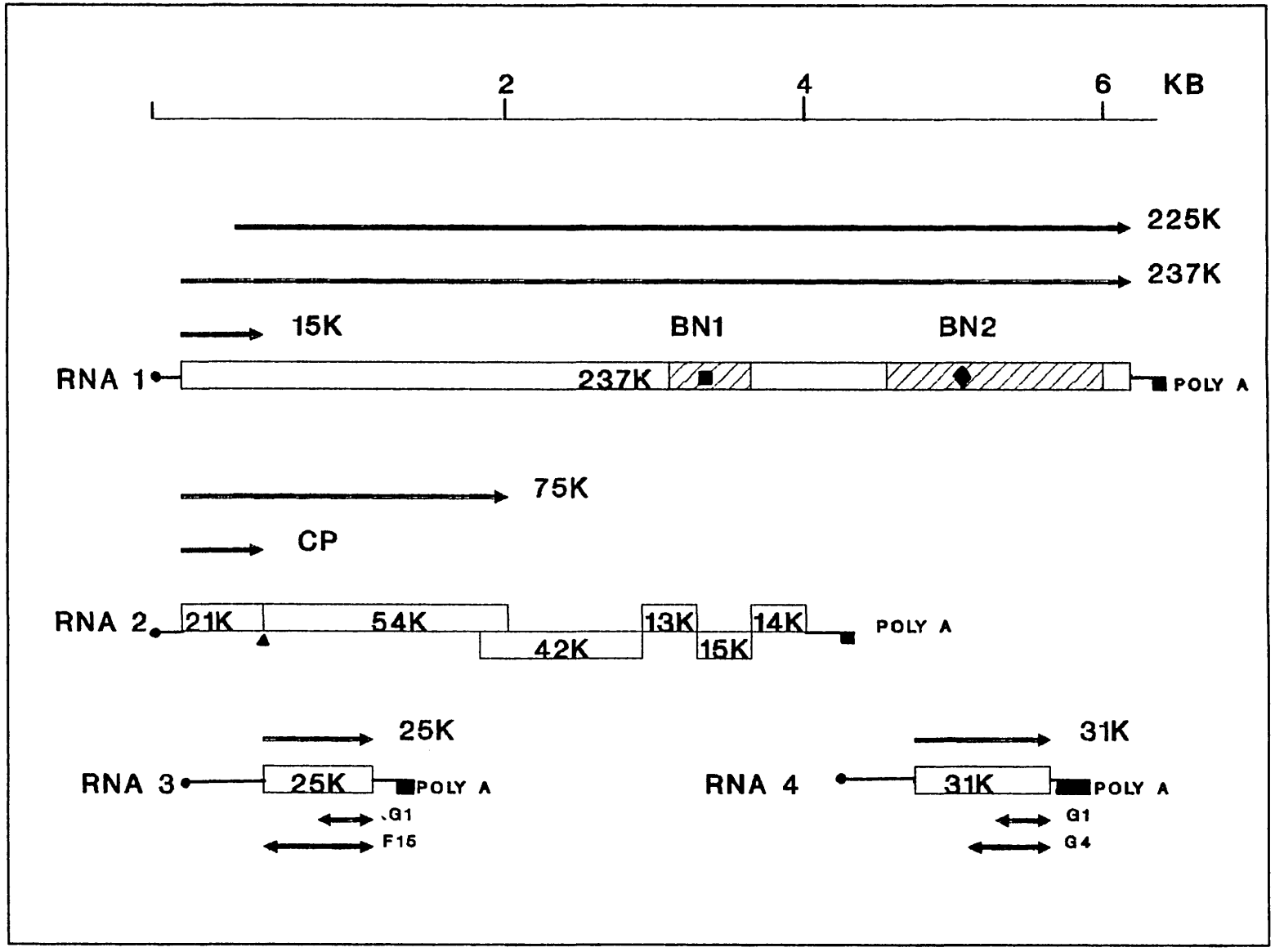


Figure 2 Genome Organisation of BNYVV

polymerase' consensus sequence GXXXTXXXN-X_{20-40}GDD which is found in the putative replicases of all animal and plant (+)-strand RNA viruses characterised to date. Domain BN1 (analogous to the C-terminal portion of TMV p126, Sindbis virus nsP2 and the C-terminal portion of the tripartite virus RNA 1-encoded proteins) contains the sequence motif GXXGXGKT/S, found in many purine nucleoside triphosphate binding proteins and also within the replicases of many but not all (+)-strand RNA viruses (Gorbalenya *et al.*, 1985). Of the four viruses used in the comparison, the BNYVV sequence appears to be most closely but still very distantly related to that of TMV.

The messenger properties of BNYVV RNA 1 are not fully understood. When translated in a wheat germ extract, RNA 1 directs synthesis of roughly equivalent amounts of two long polypeptides: a species of approximately 240 kd, which probably corresponds to translation of the entire open reading frame, and a species approximately 10 kd shorter (Jupin *et al.*, 1988). Comparison of V8 protease digests has shown that these two long polypeptides overlap extensively. *In vitro* translation experiments, using transcripts containing the 5' terminal portion of RNA 1, yield products with lengths consistent with initiation of the larger polypeptide at AUG (154), the first initiation codon in the sequence. Translation of the slightly shorter polypeptide is apparently initiated internally, with the best candidate for initiation being AUG (496) (Jupin *et al.*, 1988). RNA 1 also encodes a 15 kd polypeptide which probably results from premature translation termination (Jupin *et al.*, 1988; Figure 2).

In rabbit reticulocyte lysate, RNA 1 directs synthesis of three polypeptides of approximately 230, 170, and 50 kd (Ziegler *et al.*, 1985). The 230 and 170 kd proteins have been shown to overlap substantially in sequence with one another and with the two long RNA 1-encoded products observed in wheat germ, but the exact relationships among the various species and the nature of the 50 kd translation product are still unknown. Also, it is not known which of the various *in vitro* products are produced *in vivo*. It is interesting to note that (+)-strand RNA viruses usually employ one or more strategies (post-translational processing, suppressible termination codons or separate messengers) to distribute the putative replicase sequence domains, described above, among separate

polypeptides (Zimmern, 1989). The complex *in vitro* translational behaviour of BNYVV RNA 1 may reflect the existence of a novel mechanism to achieve this end.

BNYVV RNA 2 is 4612 nucleotides long, excluding the poly A tail, and contains six closely spaced open reading frames (ORFs) (Figure 2). The 5' proximal ORF begins at AUG (145), the first potential initiation codon in the sequence, and extends to termination codon UAG (709) encoding a polypeptide of M_r 20,682 (Bouzoubaa *et al.*, 1986). This polypeptide has size, amino acid composition and serological properties identical to that of viral capsid protein and, as expected from its 5' proximal position, is the most abundant *in vitro* translation product of RNA 2. Following the capsid protein cistron amber termination codon, the open reading frame continues for an additional 503 codons to UGA (2218). There is convincing evidence that UAG (709) is sometimes suppressed to produce a longer polypeptide (M_r 74,871, but having an apparent molecular weight in denaturing gels of approximately 85 kd) extending to the end of this reading frame and with the capsid protein sequence at its N-terminus (Ziegler *et al.*, 1985). In rabbit reticulocyte lysate readthrough occurs about once out of every ten times that the AUG (709) is encountered. The 75 kd protein and capsid protein are both easily detected in extracts of BNYVV-infected *Chenopodium quinoa* leaves, indicating that suppression of the capsid protein amber termination codon also occurs *in vivo* (G. Jonard, in Brunt & Richards, 1989).

The leaky amber termination codon at nucleotide 709 is flanked by glutamine residues (Bouzoubaa *et al.*, 1986). It is interesting to note that the 126-183 kd leaky amber codon in TMV (Goelet *et al.*, 1982) and a leaky amber codon in turnip yellow mosaic virus genomic RNA (M. D. Morch, in Brunt & Richards, 1989) are also flanked by glutamine residues, suggesting that codon context may be important in suppression.

The 3' terminal half of BNYVV RNA 2 contains four additional ORFs which can encode proteins with M_r 42,024 (ORF 3), 12,819 (ORF 4), 14,762 (ORF 5) and 14,397 (ORF 6) (Figure 2). A subgenomic RNA encoding the 42 kd polypeptide exists in infected cells and in virions (Bouzoubaa *et al.*, 1986). More recently, hybrid selection experiments have provided evidence for a messenger encoding the ORF 4

polypeptide, but the 15 and 14 kd polypeptides encoded by ORFs 5 and 6 have not yet been detected (I. Jupin & V. Ziegler-Graff, in Bouzoubaa *et al.*, 1988).

Other than viral capsid protein, no definitive role has been attributed to any of the other RNA 2 gene products. Extensive sequence homology exists, however, between the BNYVV 42 kd and 13 kd polypeptides and 58 kd and 14 kd polypeptides (also of unknown function) encoded by contiguous regions of RNA β of barley stripe mosaic virus (Bouzoubaa *et al.*, 1987). This sequence homology may reflect a common evolutionary origin or subsequent gene transfer between the two viruses.

BNYVV RNA 3 is 1774 nucleotides long, excluding the poly A tail, and encodes a polypeptide of M_r 24,715 (25 kd), while RNA 4 is 1467 nucleotides long and encodes a polypeptide of M_r 31,854 (31 kd) (Figure 2). There is no detectable sequence homology between the two polypeptides. The 5' non-coding regions of both molecules are unusually long for plant viral RNA, being 445 and 379 nucleotides, respectively. In both cases the non-coding sequence preceding the functional AUG codon contains several apparently non-functional AUG codons, four in the case of RNA 3 and two for RNA 4. Both the 25 kd and the 31 kd polypeptides are produced in cell-free translation systems, although neither RNA is a particularly efficient messenger. This low translation efficiency may be a result of the presence of the relatively long 5' non-coding regions.

The lengths and coding capacities of RNA 3 and RNA 4 mentioned above correspond to the forms characteristic of isolate F3. No RNA 3 and RNA 4 molecules have been found that are longer than this, so these molecules will be referred to as unit length. Examination of other isolates propagated in *C. quinoa* revealed that not all of them contained unit length RNAs 3 and 4 (Richards *et al.*, 1985; Bouzoubaa *et al.*, 1985; Burgermeister *et al.*, 1986; Kuszala *et al.*, 1986; Koenig *et al.*, 1986), but that they may possess deleted versions of one or both RNAs, or lack one or both completely (Table 4).

Two shortened versions of RNA 3 (from isolates F15 and G1) and RNA 4 (from isolates G1 and G4) have been sequenced (Bouzoubaa *et al.*, 1985, 1988), and they were found to be very similar in sequence to the corresponding unit length species, except for the existence of

Table 4 Lengths of RNAs 3 and 4 of 14 BNYVV Isolates, and Associated Symptoms in *Chenopodium quinoa*

(Adapted from Bouzoubaa *et al.*, 1988)

Isolate	Length*		*C. quinoa*
	RNA 3	RNA 4	Lesion Type†
F3	1850	1500	YS
F12	-	-	CS
F13	1850	-	YS
F15	1200	1500	NS
G1	1400	1200	CS
G2	1850	-	YS
G4	-	1100	CS
G5	1850	-	YS
CH1	1600	1500	CS
US1	1850	1500 + 1050	YS
US2	1850	1500	YS
US3	1850	-	YS
Aut6	1300	1200	CS
It1	1850	1500	YS

* Including poly A tail; RNAs 1 and 2 were of uniform length in all isolates.

† YS: yellow spot; CS: chlorotic spot; NS: necrotic spot.

a large internal deletion (Figure 2). In the four examples studied the locus of the deletion right-hand border was relatively constant, while that of the left-hand border varied. It is interesting to note that the deletions were essentially confined to the coding regions of RNAs 3 and 4, indicating that the corresponding polypeptides are not essential for virus multiplication in *C. quinoa*.

The mechanism by which the deletions in RNAs 3 and 4 are generated is not known; a mechanism involving splicing does not seem likely, as no sequences resembling known splicing signals occur at the deletion borders. It is not known whether the deleted forms of RNAs 3 and 4 appear *de novo* during replication or as a result of chance selection and amplification of short RNAs present in small amounts in the original sugar beet inoculum. However, it has been reported that once an isolate has been established on *C. quinoa* its RNA composition usually remains stable, even after as many as forty successive passages (Bouzoubaa *et al.*, 1988).

The RNA content of a BNYVV isolate has been shown to have a dramatic effect on symptom expression. Isolates containing unit length RNA 3 provoke bright yellow local lesions ('yellow spot') in *C. quinoa* or *Tetragonia expansa*, while isolates in which RNA 3 is deleted or lost cause milder symptoms - chlorotic local lesions ('chlorotic spot') - on *C. quinoa* and chlorotic ring or etch ring symptoms on *T. expansa* (Table 4). The only exception to this rule is isolate F15 which contains a shortened RNA 3 of 1200 nucleotides, but causes necrotic local lesions (Table 4). Adding unit length RNA 3 to a mild isolate causes it to express the yellow spot leaf symptoms, thus demonstrating the involvement of RNA 3 in symptom development (Bouzoubaa *et al.*, 1988). It is not known whether RNA 4 has any effect on symptom development.

In contrast to the heterogeneity of RNAs 3 and 4 in isolates of BNYVV maintained on *C. quinoa*, the viral RNA present in the roots of naturally infected sugar beet is uniform in length (Koenig *et al.*, 1986). The only species of RNAs 3 and 4 found were of unit length, suggesting that they are essential parts of the BNYVV genome under the natural conditions of soil transmission and sugar beet root infection, but not under the artificial conditions of mechanical propagation on *C. quinoa* leaves (Lemaire *et al.*, 1988). In the

latter case RNAs 3 and 4 may persist in the isolate in the manner of satellite RNA, undergo deletion or be lost from the inoculum.

Sequence homologies among the four BNYVV RNAs are limited to their extremities. At the 5' terminus the sequence immediately following the cap in all four molecules is a short run of A residues (A3 in RNAs 1, 2 and 4; A4 in RNA 3) and G residues are absent or under-represented. All four RNAs contain long blocks of almost perfect sequence identity over the 3' terminal 70 residues preceding the poly A tails, while RNAs 3 and 4 and RNAs 1 and 2 each possess several additional strongly homologous regions within the last 200 residues (Figure 3). These homologous terminal sequences presumably contain signals governing RNA replication and, possibly encapsidation.

ii) Soil-Borne Wheat Mosaic Virus

The genome of SBWMV consists of two RNA species. The length of SBWMV RNA 1 has been estimated at 6500-6900 nucleotides (Shirako & Brakke, 1984a; Shirako & Ehara, 1986) and is invariant; RNA 2, on the other hand varies in length depending on the growth conditions of the infected plants. Virus isolated from naturally infected wheat plants in the field in early spring contains RNA 2 of about 3500 nucleotides, and is referred to as wild type (WT) virus (Shirako & Brakke, 1984a, b; Hsu & Brakke, 1985b). When naturally infected plants or plants mechanically inoculated with WT virus are maintained at 15-17 °C ("Lab" isolates), deleted forms of RNA 2, of about 2800 and 2450 nucleotides, arise spontaneously and continuously over a period of several months (Shirako & Brakke, 1984a, b; Hsu & Brakke, 1985b). Both RNA 2 deletion mutants were stable and maintainable; however, infections involving the 2450 nucleotide RNA 2 exhibited more severe symptoms than those of the WT virus (Shirako & Brakke, 1984a, b; Shirako & Ehara, 1986). Shirako & Brakke (1984a, b) speculated that the reason the 2450 nucleotide RNA 2 did not predominate in nature was that it could not be transmitted by its natural vector, *Polymyxa graminis*, which would suggest that the region of the genome controlling fungal transmission is within the region of RNA 2 that is deleted.

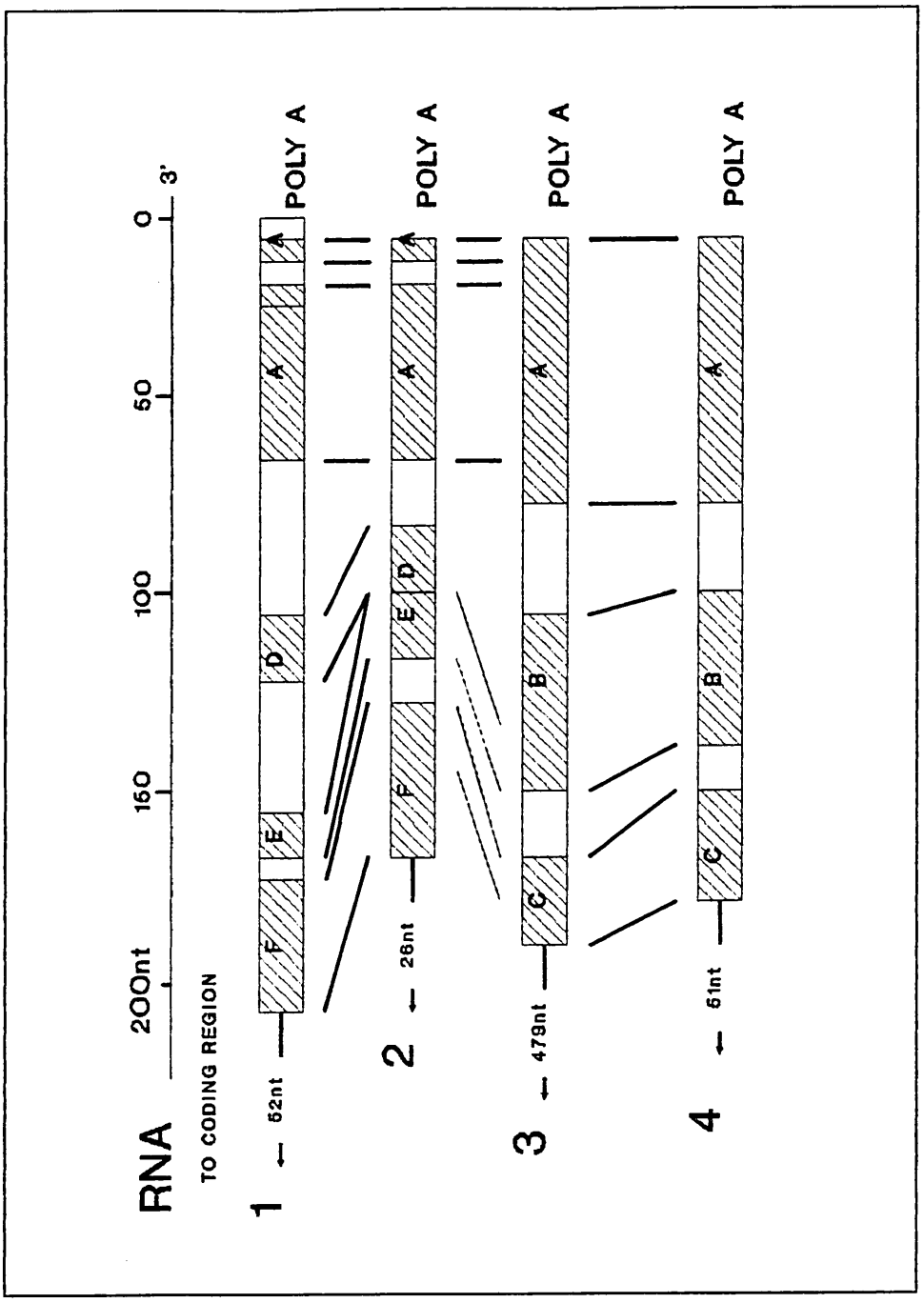


Figure 3 Sequence Homologies at the 3' ends of BNYVV RNAs

Regions of extensive (>90 %) sequence homology are indicated by hatching and joined by heavy lines. Two regions of lesser homology between regions E and F of RNAs 1 and 2, and regions B and C of RNAs 3 and 4 are indicated by fine lines.

It is interesting to note here, a parallel between SBWMV RNA 2 and BNYVV RNAs 3 and/or 4. Both appear to undergo deletions when the virus is propagated under unnatural conditions, such that the *Polymyxa* vector is not required for transmission. Also, SBWMV and BNYVV isolates containing these deleted forms of RNA induce different symptoms in host plants, from those induced by virus containing unit length RNAs. This not only suggests a role for the deleted regions in fungal transmission, but also in symptom development.

It is currently assumed that both RNA 1 plus one of the 3500, 2800 or 2450 nucleotide RNA 2s are required for SBWMV infection, but there are conflicting data in the literature. Gumpf (1971) and Tsuchizaki *et al.* (1975) reported that component 2 particles are not infective whereas component 1 particles are, and others imply that neither component 1 nor its RNA are infectious alone (Shirako & Brakke, 1984a, b).

It is possible that one RNA component encodes a functional replicase, and in this respect is independent of the other component, but is restricted to the initially inoculated cells. This situation exists with several other multicomponent and bipartite genome viruses, such as cowpea mosaic virus (Goldbach *et al.*, 1980), tobacco rattle virus (Lister & Bracker, 1969), red clover necrotic mosaic virus (Osman & Buck, 1987) and nepoviruses (Robinson *et al.*, 1980). The inoculation of the separated SBWMV RNAs or virions to plants would not address this possibility. The separated RNAs need to be inoculated to protoplasts and assayed for the replication of the input RNA. Consequently, it is not clear whether different SBWMV RNA components encode the replicase and the cell-to-cell movement genes or, if so, which.

Unlike the BNYVV RNAs, SBWMV RNA 2 does not bind to oligo dT cellulose or stimulate cDNA synthesis when primed with oligo dT, indicating the absence of a 3' poly A tail (Hsu & Brakke, 1985b). No genome-linked protein was detected at the 5' end of RNA 2 by iodination experiments, nor did the cap analogue 7-methylguanosine phosphate inhibit RNA 2 translation *in vitro* (Hsu & Brakke, 1985b). Thus RNA 2 may not bear a cap structure at its 5' end, although more direct evidence is required to prove this. No information is available about the extremities of the less abundant RNA 1.

In a rabbit reticulocyte lysate, SBWMV RNA 1 directs synthesis of a long polypeptide with a molecular weight estimated at 220 kd, for RNA 1 obtained from a Japanese isolate (Shirako & Ehara, 1986), or 180 kd for an American isolate (Hsu & Brakke, 1985a). In the latter case the longest translation product was accompanied by a number of smaller species, presumably resulting from incomplete translation or internal initiation of translation. When introduced into a wheat germ extract, RNA 1 from the Japanese isolate directed synthesis of 220 kd and 200 kd polypeptides. The 220 kd but not the 200 kd species was also observed when polysomes isolated from SBWMV-infected wheat leaves were added to the wheat germ extract (Shirako & Ehara, 1986). These data thus suggest that the primary translation product of SBWMV RNA 1, like that of BNYVV RNA 1, is a single long polypeptide representing 80-90 % of the coding capacity of the RNA molecule (Figure 4).

Translation of WT RNA 2 (3500 nucleotides) in rabbit reticulocyte lysate produced three species: the 19.7 kd capsid protein in abundant amounts, and lesser amounts of 28 kd and 90 kd polypeptides (Hsu & Brakke, 1985a). The 2800 nucleotide RNA 2 species produced 19.7, 28 and 66 kd polypeptides, while the 2450 nucleotide RNA 2 directed synthesis of 19.7, 28 and 55 kd polypeptides. All of the translation products were precipitated with antiserum against virions, and peptide mapping experiments showed conclusively that the longer polypeptides contained the capsid protein sequence (Hsu & Brakke, 1985a). The time course of appearance of the various polypeptides in the translation mix suggests that the capsid protein gene is situated near the 5' terminus of RNA 2 and that the longer polypeptides arise by readthrough of the capsid protein gene termination codon. The deletions giving rise to the shortened RNA 2 species must occur within the 3' terminal portion of the RNA molecule, encoding the portion of the 90 kd readthrough protein distal to the capsid protein gene (Figure 4).

Comparable experiments have been carried out by Shirako & Ehara (1986) with Japanese SBWMV isolates. When translated in a reticulocyte lysate, WT RNA 2 directed synthesis of 19 kd (capsid protein), 25 kd, 46 kd and 100 kd polypeptides. The 25 and 100 kd species presumably correspond to the 28 and 90 kd polypeptides of the

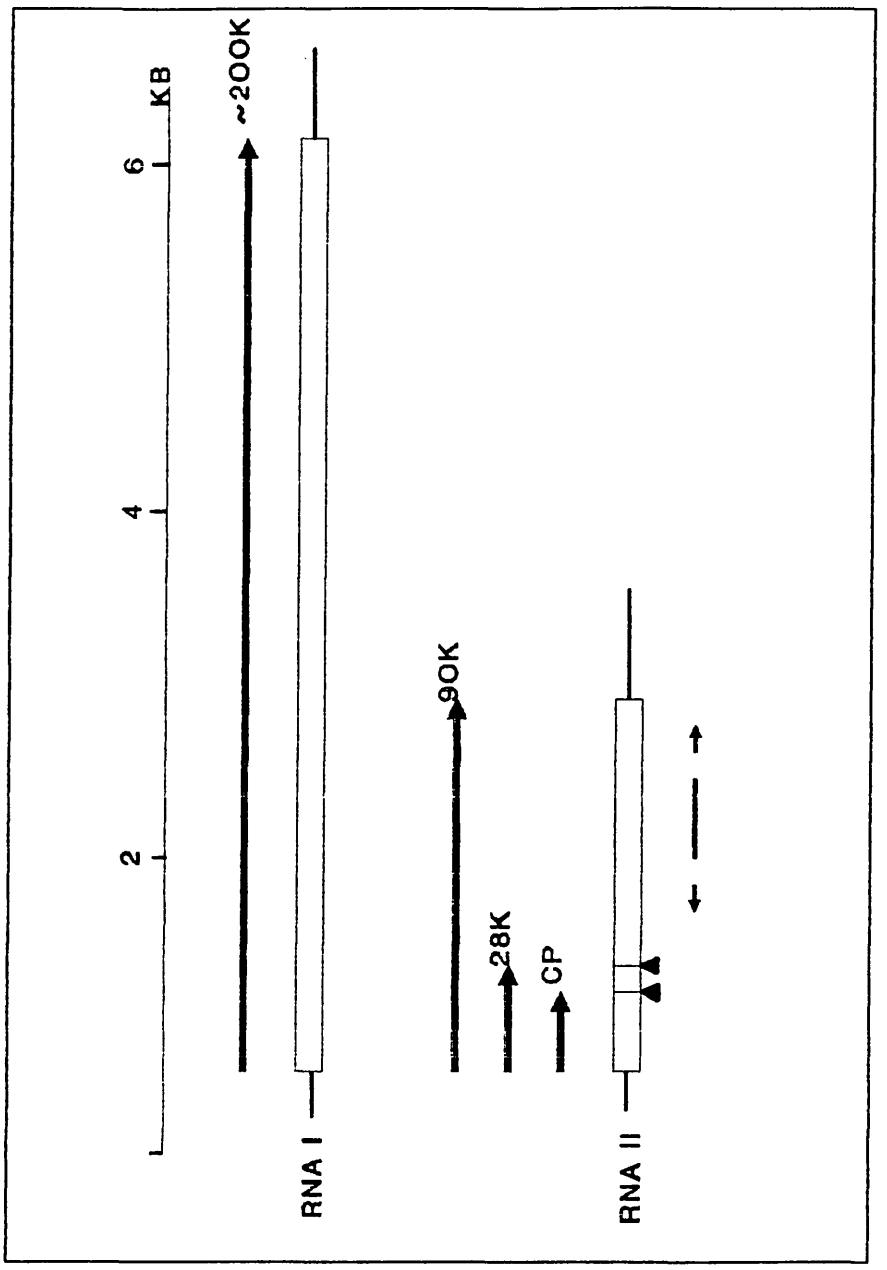


Figure 4 Genome Organisation of SBWMV

Open reading frames are represented by hollow rectangles and the corresponding translational products by arrows above each RNA. CP denotes viral capsid protein. Solid triangles (▲) represent suppresible termination codons in RNA 2. The approximate location and extent of deletions within the 2450 and 2800 nucleotide RNA molecules is indicated by the broken line beneath the RNA.

American isolate, mentioned above (Hsu & Brakke, 1985a). An RNA 2 species containing a 1500 nucleotide deletion produced 19, 25 and 31 kd polypeptides. All of the translation products except the 46 kd polypeptide were immunoprecipitated with virus antiserum. The 46 kd species could arise either from internal initiation of translation at a point beyond the coat protein gene or proteolytic cleavage of the C-terminal portion of the 100 kd polypeptide. It is interesting to note that translation of the two RNAs in a wheat germ extract produced the 19 kd capsid protein and the 25 kd polypeptide but not the longer species (Shirako & Ehara, 1986). This observation is consistent with translational readthrough being responsible for production of the 100 and 31 kd polypeptides from WT and deleted RNA 2, respectively, since wheat germ extract is not thought to contain suppressor tRNAs (Beier *et al.*, 1984a, b). It would appear that the 25 kd species, on the other hand, may be produced by a different mechanism. Alternatively, wheat germ lysate may possess a previously uncharacterised suppressor activity which is responsible for a readthrough event giving rise to the 25 kd polypeptide.

There is evidence that the 90 kd readthrough polypeptide is the SBWMV cytoplasmic inclusion body protein. Firstly, the different types of cytoplasmic inclusion associated with SBWMV infection were found to correspond to the presence of the different lengths of the RNA 2-containing virus component (Peterson, 1970; Tsuchizaki *et al.*, 1973; Hibino *et al.*, 1974a). This led Hsu and Brakke (1985a) to speculate that the deletions within the 90 kd readthrough polypeptide, which produce the shortened 66 and 55 kd polypeptides, are related to these changes in inclusion body morphology. More recently, the inclusion bodies formed during infection by SBWMV isolates containing WT or 2450 nucleotide RNA 2, were both shown to be labelled, in ultrathin sections, by antiserum to SBWMV capsid protein (W.G. Langenberg, in Brakke & Langenberg, 1988), which further implies that the 90 kd polypeptide and the shortened 66 and 55 kd polypeptides, which all contain the capsid protein at their 5' terminals, are the inclusion body proteins in wheat cells infected with the respective SBWMV isolate.

The organisation of SBWMV RNA 2 is similar, in several respects, to that of BNYVV RNA 2 (see previous section). *In vitro* translation

(Ziegler *et al.*, 1985) and sequence analysis (Bouzoubaa *et al.*, 1986) have clearly shown that the 21 kd capsid protein is in the 5' terminal ORF on BNYVV RNA 2, and the 75 kd polypeptide is produced by a partial readthrough of an amber termination codon at the end of the capsid protein ORF (Figure 2). This is more than likely the mechanism employed for the synthesis of the analogous capsid and 90 kd proteins from SBWMV RNA 2. BNYVV RNA 2 possesses several other ORFs. An ORF equivalent to a 42 kd protein was identified that partially overlaps with the 3' end of the ORF encoding the 75 kd product. This protein is expressed from a sub-genomic RNA and has been found in infected plants. It could be analogous to the 46 kd *in vitro* translation product of the Japanese isolate of SBWMV, which appears to be encoded by an ORF distal to the capsid protein ORF, as is the BNYVV 42 kd polypeptide. Near the 3' end of BNYVV RNA 2 there are three additional ORFs which encompass approximately 1.15 kb (Figure 2). Their locations coincide with the deletions in SBWMV RNA 2. As mentioned earlier, there is approximately 1.14 kb of unutilised sequence available at the 3' end of SBWMV WT RNA 2. There may be an uncharacterised association between the deletions in SBWMV RNA 2 and these three small ORFs in BNYVV RNA 2. However, these speculations will only be supported or not when the 3500, 2800 and 2450 nucleotide RNA 2's of SBWMV are sequenced.

iii) Sorghum Chlorotic Spot Virus

The genome of SCSV consists of two non-polyadenylated RNA components with little or no homology. RNA 1 is approximately 6200 nucleotides and RNA 2 is approximately 3500 nucleotides long (Kendall *et al.*, 1988; Table 3). The particle morphology and genome size is essentially identical to SBWMV, although the ratio of virion components and, consequently, RNA components in SCSV is the inverse of that reported for SBWMV. Electron microscopy of purified SCSV virions reveals a greater number of 260 nm particles compared to 140 nm particles, and this is reflected in the relative concentration of RNA 1 compared to RNA 2 (Kendall *et al.*, 1988). The reason for this difference between SBWMV and SCSV is not known.

In vitro translation of unfractionated SCSV RNA using a rabbit

reticulocyte lysate system produced 180 kd, 170 kd, 110 kd, 50 kd, 42 kd, 25 kd and 20.5 kd polypeptides (Kendall *et al.*, 1988). SCSV capsid protein antiserum precipitated three of these proteins, the 20.5 kd capsid protein and polypeptides of 25 and 110 kd. These results are very similar to those for SBWMV, as described in the previous section. SBWMV antiserum precipitates three of the SBWMV *in vitro* translation products, the 19.7 kd capsid protein and polypeptides of 25 and 90 kd (Hsu & Brakke, 1985a). Immunoprecipitations of SCSV translation products with SBWMV antiserum, and vice versa, resulted in the precipitation of minor amounts of the three polypeptides immunoprecipitated by the homologous antiserum. This led Kendall *et al.* (1988) to propose that the SCSV genome organisation is very similar to that of SBWMV. Thus, the SCSV capsid protein gene is located at the 5' end of RNA 2, with the 25 and 110 kd polypeptides resulting from readthrough of the capsid protein gene termination codon.

iv) Peanut Clump Virus

The two genomic RNAs of the Indian strain of PCV are 5900 and 4300 nucleotides long (Reddy *et al.*, 1985; Table 3). Neither RNA is polyadenylated (Mayo & Reddy, 1985) and treatment of the viral RNA with proteinase K has little effect on infectivity, results indicating the absence of a genome-linked protein, or at least one that is important for infectivity (Reddy *et al.*, 1985).

A polypeptide of 195 kd was the longest product formed upon translation of PCV RNA 1 in a rabbit reticulocyte lysate (Mayo & Reddy, 1985). Abundant amounts of a 143 kd polypeptide were also produced. The 195 kd polypeptide takes up most of the coding capacity of RNA 1, so it and the 143 kd species must overlap extensively or be translated in different reading frames. PCV RNA 2 directs synthesis of the 24.5 kd capsid protein and no other product. It is not known whether the capsid protein gene is situated at the 5' end of RNA 2, as is the case for the other three furoviruses characterised so far, BNYVV, SBWMV and SCSV. In most translation experiments a 50 kd polypeptide also appeared, which was encoded by a minor RNA of M_r between 0.5 and 0.9 kd. It is not known whether this

species is a sub-genomic RNA derived from RNA 1 or 2, or a satellite RNA.

1.8 Taxonomy

The better known furoviruses were long considered to have affinities with the tobamoviruses, mainly because their particles are morphologically similar (Fenner, 1976) and their capsid protein molecular weights and amino acid compositions are comparable. Moreover, PMTV and SBWMV are reported to be serologically related to tobacco mosaic virus (TMV) (Kassanis *et al.*, 1972; Powell, 1976), and BBNV to two other tobamoviruses, cucumber green mottle mosaic virus (CGMMV) and odontoglossum ringspot virus (ORSV) (Nakasone & Inouye, 1978). However, the furoviruses clearly differ from the authentic tobamoviruses in their relative instability *in vitro*, their occurrence within infected plants in low concentration, their bipartite genomes and the transmission of most by plasmodiophorid fungi. Moreover, all four viruses (BNYVV, PCV, SBWMV and SCSV) whose RNA has been studied in some detail, have been found to carry the capsid protein gene at the 5' end of RNA 2 and which, unlike those of other viruses, undergoes efficient translational readthrough *in vivo* to produce larger polypeptides. In contrast, the capsid protein gene of the tobamoviruses is situated at the 3' end of the RNA, and is expressed from a sub-genomic RNA (Goelet *et al.*, 1982).

Nevertheless, the exact taxonomic status of BNYVV is uncertain because, unlike PCV, SBWMV and SCSV, its RNAs have a 5' terminal cap structure, m^7GpppA, and a 3' terminal poly A tail (Putz *et al.*, 1983). Also, BNYVV has four genomic RNAs, with RNA 2 being significantly larger than that of the other furoviruses, and the two smallest RNAs having no known counterpart in the other furoviruses (Table 3). On the other hand, the genetic organisation of RNAs 1 and 2 of BNYVV is similar to that of PCV, SBWMV and SCSV: The primary translation product of RNA 1 is a single long polypeptide representing 80-90 % of the coding capacity of the molecule (Ziegler *et al.*, 1985; Mayo & Reddy, 1985; Hsu & Brakke, 1985a; Kendall *et al.*, 1988); and RNA 2 contains the capsid protein gene, which can undergo translational readthrough to produce larger polypeptides

(Figures 2 and 4).

Available evidence, therefore indicates that the labile, fungus-borne, rod-shaped viruses are sufficiently similar to be grouped together as the furoviruses (Shirako & Brakke, 1984a), with the possible exception of BNYVV, which, although more similar to the furoviruses than the other rod-shaped virus groups, is significantly different, and may warrant a taxonomic group of its own. Alternatively, BNYVV may form a sub-group of the furoviruses. However, these speculations will only be supported, or not, when more is known about the genome organisation of the other furoviruses, such as their nucleotide sequences.

1.9 Plant Viral Double-Stranded RNA

Generally, the only homogeneous, high molecular weight dsRNA (i.e. $>0.1 \times 10^6$) found in plants is induced by infection with an RNA virus, either with a ssRNA genome, in which case the dsRNA is the replicative form of the viral RNA, or with a dsRNA genome. However, there have been several reports of dsRNA found in extracts of apparently healthy plants. For example, the cryptic viruses, which do not induce symptoms in host plants and are therefore not easily detected, have segmented dsRNA genomes (reviewed in Boccardo *et al.*, 1987). These viruses have been detected in several plants including sugar beet, spinach, white clover and *Vicia faba*.

The nature of the dsRNAs that do not resemble the genomic dsRNA of the cryptic viruses is less clear. One such dsRNA (M_r 10-13 x 10^6) has been found in *Vicia faba*, and it is thought to be involved in cytoplasmically-inherited male sterility (Grill & Garger, 1981). This dsRNA can be transmitted by the use of dodder (a parasitic angiosperm), with the consequence that male sterility is conferred on the previously fertile, dsRNA-negatve recipient, and the loss of the dsRNA results in the recovery of fertility in previously sterile plants.

Studies of dsRNA in fertile and male-sterile maize (Sisco *et al.*, 1984) or sunflower (Brown *et al.*, 1986) lines did not reveal the degree of association between the presence of dsRNA and male sterility that was reported for *Vicia faba*. Two dsRNAs (M_r 1.9 and

0.5 x 10^6) were detected in some S group cytoplasms of male-sterile maize, and their presence was correlated with an extra degree of male sterility. The dsRNAs detected in sunflower were more variable. Two of the dsRNAs (M_r 1.1 and 0.5 x 10^6) were detected consistently, but were found in fertile as well as male sterile lines and were not considered to be implicated in male sterility. The involvement of dsRNA as a general phenomenon in male sterility is therefore an open question (Hanson & Conde, 1985; Pring & Lonsdale, 1985).

A high molecular weight dsRNA (9.0 x 10^6) has been detected in *Phaseolus vulgaris*, in several cultivars with black seed coats, such as 'Black Turtle Soup' (BTS) (Wakarchuk & Hamilton, 1985; Valverde *et al.*, 1986). This dsRNA hybridised with chloroplast DNA from BTS and other bean cultivars (R.I. Hamilton, in Boccardo *et al.*, 1987). Further dsRNAs have been found in certain cultivars of avocado (Jordan *et al.*, 1983), cucurbit (Nameth & Dodds, 1985), tomato, pepper and mustard (Valverde *et al.*, 1986). For each of these species, there are many cultivars that lack detectable amounts of dsRNA, implying that the presence of dsRNA is not an essential part of the biochemistry of the plant.

Due to the presence of dsRNAs in many healthy plant species, the study of plant viral infections requires proper analysis of healthy controls. However, the absence of dsRNAs in healthy controls grown from the same seed stocks as infected plants is a strong indication that dsRNAs are infection- if not virus-specific.

The value of dsRNA analysis in detection and diagnosis of plant virus infection has been reviewed by Dodds *et al.* (1984, 1988). For most RNA viruses, methods have been developed so that dsRNA can be extracted from small amounts of tissue, even as little as 0.5 g. However, for some viruses large quantities of infected tissue are required to obtain even small amounts of viral dsRNA: for example, 75-100 g of banana plant tissue infected with banana bunchy top disease (BBTD) was needed to yield enough dsRNA to detect when electrophoresed on one lane of a 0.75 mm thick polyacrylamide gel (Dale *et al.*, 1986), and similarly, 50-100g of BSBV-infected *Chenopodium quinoa* was needed to detect viral dsRNA (C.M. Henry, personal communication). No knowledge of the identity of the infectious agent is needed for detection, which is possible even in

the absence of virions, such as in the case of BBTD; this disease was thought to be caused by a virus, because it is persistently transmitted by the banana aphid, *Pentolonia nigronervosa*, and diseased plants have yellowed leaves and damaged phloem tissue (Magee, 1940). However, despite numerous attempts by many different workers to extract and characterise virus particles from BBTD plants, this has not been accomplished. The subsequent isolation of dsRNA from BBTD-infected plants indicates that BBTD is caused by a virus (Dale *et al.*, 1986).

Diagnosis is based on the recognition of a gel-electrophoretic pattern of dsRNA species characteristic of a particular virus or group. In most cases studied so far, the patterns produced are complex but consistent, and illustrate differences between viruses of the different groups (Morris, 1983), similarities between viruses of the same group (Morris, 1983) and small differences between different strains of the same virus (Gildow *et al.*, 1983). A comprehensive catalogue of expected dsRNAs would therefore allow diagnosis of mixed infections as well.

If sufficiently purified from host nucleic acids, dsRNAs are useful as comparatively stable and accessible sources of viral RNA sequences. End-labelling with ^{32}P, as in Jordan & Dodds (1983), provides a source of both (+) and (-) sense probes without the necessity of virus purification, often a more complex and difficult procedure than dsRNA purification. Strategies for the specific molecular cloning of dsRNAs have been reported, although with two of them (Both *et al.*, 1982; Imai *et al.*, 1983) there is the inherent problem of separating the strands long enough for complementary DNA (cDNA) synthesis by reverse transcriptase. The third (Shotniki *et al.*, 1985) employs direct cloning of dsRNAs in plasmid or bacteriophage vectors by addition of DNA linkers. *In vitro* translation of melted dsRNAs again has the problem of re-annealing during incubation, but has been reported for the yeast killer-associated dsRNA (Bostian *et al.*, 1980) and tobacco necrosis virus (TNV) sub-genomic dsRNAs (Condit & Fraenkel-Conrat, 1979), although evidence for the latter was said (in 1979) to be preliminary. An attempt to translate melted TMV dsRNAs was unsuccessful (Palukaitis *et al.*, 1983). Unlike animal picornavirus dsRNAs, plant viral dsRNAs

do not seem to be infectious unless first denatured (Jackson *et al.*, 1971; Shanks *et al.*, 1985), but as such could be useful for infectivity studies when virion ssRNA is not available.

Double-stranded RNA species are often referred to as replicative forms (RF), although there is no evidence that they have this function *in vivo*. Indeed, partially double-stranded RNAs with single-stranded tails (so-called replicative intermediates, RIs) in a complex with membranes and proteins are more likely to be the true replicative structures within the cell, as has been found for turnip yellow mosaic virus (TYMV) (Garnier *et al.*, 1980). RF may be a by-product or end-product or even an artifact of extraction methods. However, the existence of dsRNAs of the same length as genomic and sub-genomic ssRNAs of many viruses implies that analysis of double-stranded forms gives valid information on the genome strategies of the viruses involved. For instance, on extraction of dsRNA from plants infected with cowpea mosaic virus (CPMV), a virus whose gene products arise by polyprotein processing rather than by translation of sub-genomic RNAs (Pelham, 1979; Goldbach & Rezelman, 1983), no double-stranded forms shorter than genomic length are found (Shanks *et al.*, 1985). Tripartite genome viruses (e.g. cucumoviruses) produce dsRNAs for each genomic RNA in addition to a species for sub-genomic RNA 4, and for satellite RNA when present (Kaper & Diaz-Ruiz, 1977). For TMV, a single component virus, four dsRNAs are found, three of which appear to be double-stranded forms of polysome-associated ssRNAs (Palukaitis *et al.*, 1983). Other workers found an additional three (Bar-Joseph *et al.*, 1983) or eight (Dawson & Dodds, 1982) dsRNA species in TMV-infected plants and the latter authors found nine "sub-genomic" ssRNAs, some of which correspond in size to some of their dsRNAs, but these ssRNAs were not found on polysomes and all but the original four ssRNAs (one genomic and three sub-genomic) were shown by Palukaitis *et al.* (1983) to be electrophoretic artefacts. Similar artefactual bands produced on electrophoresis of mixtures of plant RNA and viral RNA have been reported for tobacco etch virus (TEV) (Dougherty, 1983) and tomato bushy stunt virus (TBSV) (Hayes *et al.*, 1984). It has become evident that interpretation of such results is impossible without the appropriate controls. These sorts of effects cannot, however, explain

differences in the number of TMV dsRNAs found by the three groups, since purified preparations of dsRNA should not contain any plant RNA. The disparity may be due to the different resolving capacities of the gel systems employed to visualise the dsRNAs or to anomalous behaviour of some dsRNAs in one or more of the systems.

The only report of dsRNA forms associated with a furovirus is for BSBV (C.M. Henry, personal communication). No successful purification method has been found for this virus, but three viral dsRNA molecules have been extracted from BSBV-infected *C. quinoa* leaves, with molecular weights of approximately 6.4 kb, 2.9 kb and 2.5 kb.

1.10 **Aims of the PMTV Project**

Although PMTV has been designated a member of the furovirus group by the ICTV, on the basis of its rod-shaped particles of two modal lengths and its plasmodiophorid fungal vector, there is no information on the nature of its nucleic acid. The principal aim of this project was to devise a reliable method for purification of PMTV and to further characterise the virus and its nucleic acid.

2. MATERIALS AND METHODS

2.1 Abbreviations

A	absorbance
AMPS	ammonium persulphate
b	base(s)
bp	base pair(s)
BSA	bovine serum albumin
C	centigrade
cccDNA	covalently closed circular DNA
Ci	Curie
CIP	calf intestinal phosphatase
cm	centimetre(s)
cv	cultivar
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide
d	dalton(s)
d-	deoxy, e.g. dNTP, deoxynucleoside triphosphate
dd-	dideoxy
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
deH_2O	deionised water
DIECA	sodium diethyldithiocarbamate
DMSO	dimethyl sulphoxide
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediamine tetracetic acid
EGTA	ethylene glycol-bis(β-aminoethylether)N, N, N', N'-tetra acetic acid
g	gram(s)
IPTG	isopropylthio-ß-D-galactopyranoside
kb	kilobase pair(s)
kd	kilodalton(s)

M	molar
mA	milliamp(s)
MeHgOH	methylmercuric hydroxide
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
M_r	relative molecular mass
µg	microgram(s)
µl	microlitre(s)
ng	nanogram(s)
nm	nanometre(s)
ocDNA	open circular DNA
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PMSF	phenylmethyl sulphonyl fluoride
psi	pounds per square inch
Pu	purine
Py	pyrimidine
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sevag	chloroform: isoamyl alcohol (v/v) 24: 1
ss	single-stranded
TEMED	N, N'-tetramethyl ethylenediamine
tris	tris(hydroxymethyl)aminomethane
TTP	thymidine triphosphate
U	unit(s)
UV	ultraviolet
v/v	volume per volume
V	volt(s)
vol	volume(s)
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

2.2 Safety Precautions

A laboratory coat was worn at all times and disposable gloves were worn when handling hazardous chemicals. Gloves and glasses were worn when using radioisotopes and work was carried out behind a perspex screen. All radioactive waste was disposed of in accordance with Imperial College Safety Regulations. All manipulations involving toxic chemicals were carried out in a fume hood.

An Envair class II microbiological safety cabinet was used for work involving recombinant DNA and all waste materials and media were autoclaved prior to disposal.

The experiments reported here with TGMV were classified by the Department of Pure and Applied Biology Genetic Manipulation Safety Sub-Committee as Good Microbiological Practice and carried out under the Destructive Pests and Diseases of Plants Act with a licence (PHF 29B/75 (106)) issued by the Ministry of Agriculture, Fisheries and Food.

2.3 Companies and Suppliers of Reagents and Materials

Chemicals and equipment were supplied by the following companies:

ALPHA LABORATORIES, 40 Parham Drive, Eastleigh, Hants. SO5 4NU.

AMERICAN CAN COMPANY, Greenwich, Conn. 06930, USA.

AMERSHAM INTERNATIONAL PLC, White Lion Road, Amersham, Bucks. HP7 6LL.

ANGLIAN BIOTECHNOLOGY LTD., Unit 8, Hawkins Road, Colchester, Essex CO2 8JX.

BCL, Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex BN17 1LG.

BDH CHEMICALS LTD., Freshwater Road, Dagenham, Essex RM8 IR2.

BECKMAN RIIC LTD., Progress Road, Sands Industrial Estate, High Wycombe, Bucks. HP12 4JL.

BIO 101, INC.: UK agents, Statatech Scientific Ltd., 50, Newington Green, London N16.

BIO-RAD LABORATORIES LTD., Caxton Way, Watford Business Park, Watford, Herts. WD1 8RP.

BRL: Bethesda Research Laboratory (UK) Ltd., PO Box 145, Science Park, Cambridge CB4 4BE.

CARBORUNDUM COMPANY LTD., Trafford Park, Manchester M17 1HP.

DIFCO LABORATORIES, PO Box 143, Central Avenue, East Molesey, Surrey KT8 08E.

EMscope, Kings North Industrial Estate, Wotton Road, Ashford, Kent.

FISONS SCIENTIFIC EQUIPMENT, Bishop Meadow Road, Loughborough, Leics. LE11 0R6.

GALLENKAMP, Belton Road West, Loughborough, Leics. LE11 0TR.

GILSON MEDICAL ELECTRONICS. Products supplied by Anachem, Anachem House, 20, Charles Street, Luton, Beds. LU2 0EB.

HETTICH. Products supplied by Arnold H. Horwell Ltd., 73, Maygrove Road, West Hampstead, London NW6 2BP.

KODAK LTD., Distribution Southern Region, PO Box 33, Hemel Hempstead, Herts. HP2 7EU.

LANCASTER SYNTHESIS, Eastgate, White Lund, Morecambe, Lancs.

LKB INSTRUMENTS LTD., 232 Addington Road, Selsdon, South Croydon, Surrey CR2 8YD.

MILLIPORE (UK) LTD., Abbey Road, London NW10.

MINI INSTRUMENTS LTD., 8, Station Industrial Estate, Burnham-on-Crouch, Essex CM0 8RN.

MSE SCIENTIFIC INSTRUMENTS, Manor Royal, Crawley, West Sussex RH10 2QQ.

NEW ENGLAND BIOLABS INC., UK Suppliers C.P. Laboratories Ltd., PO Box 22, Bishops Stortford, Herts. CM22 7RQ.

NEW ENGLAND NUCLEAR, DU PONT (UK) LTD., Wedgewood Way, Stevenage, Herts. WD1 8RP.

NBL: Northumbria Biologicals Ltd., South Nelson Industrial Estate, Cramlington, Northumberland NE23 9HL.

OXOID LTD., Wade Road, Basingstoke, Hants. RG24 0PW.

P&S BIOCHEMICALS LTD., 38 Queensland Street, Liverpool L7 3JG.

PHARMACIA LTD., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks. MK9 3HP.

P-L Biochemicals Ltd., PO Box 98, Northampton NN3 1AW.

RAVEN SCIENTIFIC LTD., P.O. Box 2, Haverhill, Suffolk CB9 0ES.

ROSE CHEMICALS LTD., 83 Darenth Road, London N16 6EB.

SIGMA CHEMICAL CO. LTD., Fancy Road, Poole, Dorset BH17 7NH.

STERILIN LTD., 43-45 Broad Street, Teddington, Middx. TW11 8QZ.

UNIVERSAL SCIENTIFIC LTD., The Broadway, Woodford Green, Essex IG8 0HL.

WHATMAN LABSALES, Springfield Mill, Maidstone, Kent ME14 2LE.

WORTHINGTON DIAGNOSTICS SYSTEMS INC., UK Suppliers Flow Laboratories Ltd., PO Box 17, Second Avenue, Industrial Estate, Irvine, Scotland KA12 8NB.

Unless otherwise stated all chemicals were supplied by BDH Chemicals Ltd. or Sigma Chemical Co. Ltd. and were of analytical grade or equivalent.

2.4 Common Buffers Used

BE 40 mM boric acid, 1 mM EDTA, adjusted to pH 7.6 with NaOH and made up as a 10 x stock solution.

SSC 150 mM NaCl, 15 mM trisodium citrate, pH 7.0, made up as a 20 x stock solution.

TAE 40 mM tris base, 1 mM EDTA, adjusted to pH 7.8 with glacial acetic acid and made up as a 50 x stock solution.

TBE 90 mM tris-HCl, 90 mM boric acid, 2 mM EDTA, pH 8.3, made up as a 10 x stock solution.

TE 10 mM tris-HCl, 1 mM EDTA, pH 8.0, made up as a 10 x stock solution.

Column Buffer 20 mM tris-HCl, 150 mM NaCl, 20 mM 2-mercaptoethanol, 1 mM PMSF, pH 7.5.

2.5 General Methods

2.5.1 Miscellaneous

All plasticware (Eppendorf tubes and micropipette tips) and liquid reagents and buffers (except those containing heat-labile or volatile compounds) were sterilised by autoclaving for 20 minutes at 121 °C, 15 psi. Any solutions which could not be autoclaved were filter sterilised through Millipore 0.2 μm filters. Glassware to be used for RNA solutions was sterilised by baking at 180 °C overnight.

All buffers and reagents were made up in glass-distilled water (dH_2O). "Sterile dH_2O" refers to autoclaved glass-distilled water.

Dialysis tubing was cut into pieces and boiled for one hour in 10 mM EDTA (pH 8.0), then for a further hour in fresh EDTA. It was then rinsed ten times in dH_2O, autoclaved in dH_2O and stored at 4 °C.

Solid phenol was melted at 60 °C and redistilled at 180 °C to remove impurities. The phenol was water-saturated by shaking with sterile dH_2O and stored at 4 °C.

Phenol extraction involved adding one volume of water-saturated phenol to a sample, vortexing until an emulsion was formed and then separating the two phases by microcentrifugation for five minutes. The upper, aqueous phase was removed into a clean Eppendorf tube and was then usually re-extracted with a 1:1 (v/v) mixture of phenol and

sevag (24:1 (v/v) mixture of chloroform and isoamyl alcohol) and finally with sevag alone to remove traces of phenol.

Ethanol precipitation involved adding cold, absolute alcohol (2 volumes for DNA, and 2.5 volumes for RNA) to a nucleic acid solution and incubating either on cardice for 20 minutes, at -70 °C for one hour or at -20 °C overnight. Solutions of low ionic strength were made 0.3 M in sodium acetate (pH 5.2) prior to the addition of ethanol. The nucleic acid was collected by microcentrifugation for 10 minutes and the pellet was washed with 70 % ethanol and dried under vacuum.

Formamide was deionised by stirring with 5 g/100 ml Amberlite MB-1 "monobed" resin (BDH), for 30 minutes. The resin was removed by filtration through Whatman No. 1 filter paper and the filtrate was stored in small aliquots at -20 °C.

RNase A was rendered DNase-free by the method of Maniatis *et al.* (1982). RNase A was dissolved to 10 mg/ml in 10 mM tris-HCl (pH 7.5), 15 mM NaCl, heated for 15 minutes at 100 °C and cooled slowly to room temperature. It was stored in aliquots at -20 °C.

DNase I was rendered RNase-free by treatment with proteinase K as described by Tullis & Rubin (1980). DNase I was dissolved to 1 mg/ml in 20 mM tris-HCl (pH 7.5), containing 10 mM $CaCl_2$ and equilibrated at 37 °C for 20 minutes. Proteinase K (Sigma) was added to 1 mg/ml and the solution was incubated at 37 °C for one hour and then stored at -20 °C.

Low speed centrifugations were performed in Beckman J2-21 or MSE HS-18 or HS-21 centrifuges, using the JA-14, JA-20, 8 x 50 or 6 x 300 fixed angle rotors as appropriate. Ultracentrifugations were performed in Beckman L8-55 or L8-70 ultracentrifuges; the rotors used are mentioned at the appropriate places in the text. Small volumes (i.e. less than 1.5 ml) were microcentrifuged in a Hettich microfuge.

UV absorption spectra of nucleic acid and virus preparations were determined using a Beckman Model 35 Spectrophotometer with quartz cells of path length 1 cm. Concentrations of nucleic acids and virus were calculated using the following relationships:

$1\ A_{260} \equiv 40\ \mu g/ml$ ssRNA

$1\ A_{260} \equiv 50\ \mu g/ml$ dsDNA

$1\ A_{260} \equiv 400\ \mu g/ml$ virus

Nucleic acid purities were estimated from the A_{260}:A_{230} and A_{260}:A_{280} ratios, which should both be approximately two.

2.5.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis of nucleic acids was carried out using horizontal "submarine" slab gels, mainly "half-gels (15.5 x 11.5 x 0.5 cm) or "mini-gels" (10.0 x 6.5 x 0.5 cm). Direct current was provided by a Heathkit Model IP-2717 power supply.

2.5.2i Methyl Mercury Gels

RNA samples denatured with methyl mercuric hydroxide (MeHgOH, Lancaster Synthesis) (Bailey & Davidson, 1976) were electrophoresed in agarose gels in the presence or absence of MeHgOH. All manipulations involving MeHgOH were carried out in a fume hood. Gels (1 %) were prepared by briefly boiling the required amount of agarose in dH_2O, cooling to 60 °C and adding 10 x BE buffer and 1 M MeHgOH to final concentrations of 1 x BE and 0, 5 or 10 mM MeHgOH. Samples (5 to 25 µl) contained BE buffer, 10 mM MeHgOH, 10 % (v/v) glycerol and 0.05 % bromophenol blue, and were electrophoresed at 80 V in BE for 1 to 4 hours. Following electrophoresis, gels were treated with 10 mM 2-mercaptoethanol for 30 minutes to sequester the mercury ions, and washed extensively in dH_2O to remove solubilised mercury.

2.5.2ii Formaldehyde Gels

RNA samples denatured with formaldehyde and formamide were electrophoresed in the presence of formaldehyde. Gels (1 %) were prepared by briefly boiling the required amount of agarose in dH_2O, cooling to 60 °C and adding 20 x TAE buffer and 37 % (w/v) formaldehyde (BDH) to final concentrations of 1 x TAE and 6 % formaldehyde. Samples (5-15 µl) contained 1 x TAE, 6 % formaldehyde, 50 % deionised formamide, and were denatured by heating at 60 °C for 5 minutes prior to addition of glycerol and bromophenol blue to 10 % (v/v) and 0.05 % respectively, then electrophoresed in TAE buffer as above. After electrophoresis, gels were treated as in section 2.5.2i.

2.5.2iii Low Melting Point (LMP) Gels

MeHgOH low melting point gels were prepared and run as in section 2.5.2i except that LMP-agarose (Sigma) was used and the gels were allowed to set at 4 °C.

2.5.3 Recovery of RNA Fragments from Agarose Gels

Two aliqouts of the RNA sample of interest were electrophoresed in adjacent wells of a MeHgOH LMP-agarose gel. The gel was cut along a line between the two samples and one half was stained with ethidium bromide as described in section 2.5.5. The RNA bands were visualised on a transilluminator and their distances from the wells measured. This allowed the positions of the RNA bands in the other part of the gel to be estimated, and 3 mm wide strips, corresponding to each RNA band of interest, were excised with a scalpel blade and incubated in a 1.5 ml Eppendorf tube with 0.5 ml 0.1 M DTT each, for 30 minutes at room tewmperature. Then the DTT was discarded, the agarose melted at 70 °C for three minutes and 5 µl of 10 % SDS plus 0.5 ml of a preheated solution of 0.5 M ammonium acetate plus 5 mM EDTA, were added and mixed. The solution was phenol extracted twice and the RNA precipitated with 0.1 volumes of 3 M sodium acetate plus 1.5 volumes of isopropanol overnight at -20 °C. The RNA was collected by microcentrifugation for 15 minutes, washed twice in 70 % ethanol, dried under vacuum, resuspended in the minimum volume of sterile dH_2O and stored at -70 °C. The integrity of the RNA was checked by MeHgOH agarose gel elctrophoresis (section 2.5.2i).

2.5.4 Polyacrylamide Gel Electrophoresis (PAGE)

2.5.4i SDS-PAGE Analysis of Proteins

Proteins were analysed by a discontinuous SDS-PAGE system essentially as described by Laemmli (1970). Slab gels were cast between two glass plates (20.5 x 17.0 x 0.4 cm) separated by 1.5 mm spacers and sealed with paraffin wax. The gels were composed of two layers: a lower, resolving gel (7.5 to 16 % (w/v) acrylamide) and an upper, stacking gel (3 % (w/v) acrylamide) and were made from a 40 % (w/v) stock acrylamide solution (40 g acrylamide and 0.4 g N,N'

methylene bisacrylamide made up to 100 ml with dH_2O, filtered and stored at 4 °C). For the resolving gel, 3.75 ml of 3 M tris-HCl (pH 8.8), 0.3 ml of 10 % (w/v) SDS and 0.25 ml of freshly made 10 % (w/v) ammonium persulphate (AMPS) were mixed with stock acrylamide and dH_2O to give a final acrylamide concentration of between 7.5 and 16 % (w/v) in a 30 ml volume. This solution was degassed for 1 to 2 minutes, 15 µl of TEMED were added and mixed, and the gel immediately cast. The gel was overlaid with 1 ml of dH_2O-saturated butanol to facilitate polymerisation, by excluding oxygen. The gel was allowed to set for 20 to 30 minutes, during which time the stacking gel was prepared by mixing 0.75 ml of stock acrylamide, 2.5 ml of 0.5 M tris-HCl (pH 6.8), 0.1 ml of 10 % (w/v) SDS, 6.6 ml of dH_2O and 75 µl of 10 % (w/v) AMPS. This was degassed for 1 to 2 minutes and then 5 µl of TEMED were added. The butanol overlay was poured from the resolving gel, and the stacking gel was cast and the 12-tooth comb positioned. The gel was allowed to set for 1 hour before use.

Samples (up to 50 µl) were denatured by heating at 100 °C for 5 minutes in 65 mM tris-HCl (pH 6.8), 1 % (w/v) SDS, 2.5 % (v/v) 2-mercaptoethanol, 0.25 % (w/v) bromophenol blue, 7.5 % (v/v) glycerol and cooled briefly on ice. Any insoluble material was removed by microcentrifugation for 5 minutes, before loading the samples onto the gel. Electrophoresis was performed in 0.384 . glycine, 0.05 M tris-HCl (pH 8.3), 0.1 % (w/v) SDS, at 170 V for 2 to 4 hours or at 30 to 70 V overnight.

2.5.4ii Molecular Weight Determination of the Capsid Protein

To determine the relative molecular weight of the PMTV-T capsid protein, the mobility of various molecular weight markers and the capsid protein were compared on different SDS-polyacrylamide gel concentrations using the Ferguson plot (Ferguson, 1964).

Laemmli (1970) gels were used, as described in the previous section, with resolving gel concentrations of 16 %, 12 % and 10 % (w/v) and 5 µg of each marker protein: human γ-globulin (M_r L-55,500; H-23,500), lactate dehydrogenase (M_r 36,500), DNase I (M_r 31,000) and myoglobin (M_r 16,950) were used. Gels were prepared under uniform conditions and PMTV-T and protein marker samples were denatured as in

section 2.5.4ii. Sixteen percent gels were run at 70 v overnight, 12 % gels at 40 V overnight and 10 % gels at 35 V overnight.

After Coomassie blue staining and destaining (section 2.5.5), migration of stained protein bands were measured relative to the migration of myoglobin.

2.5.4iii PAGE Analysis of dsRNA

Double-stranded RNAs were analysed on 5 % polyacrylamide gels made by adding the following in a Buchner flask:

6.25 ml	stock acrylamide (40 % (w/v) acrylamide, 0.4 % (w/v) N,N'-methylene bisacrylamide)
2.5 ml	20 x TAE buffer
0.5 ml	10 % (w/v) AMPS
40.7 ml	dH_2O

After mixing, the solution was degassed for 1 to 2 minutes, 50 M_rl of TEMED were added and then the gel was cast and allowed to set for at least one hour. Samples contained 1 x TAE, 8 % (v/v) glycerol and 0.05 % bromophenol blue and were electrophoresed at 60 V overnight with TAE as the electrophoresis buffer.

2.5.5 Gel Staining and Photography

Nucleic acids were visualised in agarose and polyacrylamide gels on a Chromato-Vue Transilluminator C-63 ultraviolet light box after staining with 0.5 M_rg/ml ethidium bromide for 15 to 30 minutes followed by destaining in dH_2O. Gels containing MeHgOH or formaldehyde were stained as above concurrently with 2-mercaptoethanol treatment (section 2.5.2ii).

Protein gels were stained with 0.25 % Coomassie Brilliant Blue R250 (Bio-Rad) in 45 % (v/v) methanol and 9 % (v/v) glacial acetic acid for 1 hour at room temperature with shaking. Gels were destained in several washes of 45 % (v/v) methanol, 9 % (v/v) glacial acetic acid for several hours. Protein bands were visualised on a light box.

Gels were photographed using a Praktica MTL5 camera fitted with two Y(K2) yellow filters and one UV(O) filter (all Hoya) on 35 mm

Ilford HP5 or Pan F film, which was subsequently developed in Promicrol ultrafine grain developer.

2.5.6 Gel Drying

Agarose and polyacrylamide gels were placed on 2 pieces of wet Whatmann 3MM filter paper, covered with 'Saran Wrap' and dried at 60 °C and 80 °C respectively, under vacuum using a Bio-Rad gel dryer.

2.6 **Virus Propagation and Storage**

2.6.1 Origin of Virus

Three isolates of PMTV were studied: the Todd isolate (PMTV-T, type strain - Harrison & Jones, 1970) was supplied by Dr. P. Massalski from the Scottish Crops Research Institute, Invergowrie, Scotland in six systemically-infected *N. debneyi* leaves. A second isolate was supplied by Dr. P. Mills from the Northern Ireland Ministry of Agriculture in three systemically-infected *N. benthamiana* leaves, and was known as the Northern Ireland isolate (PMTV-NI). The third isolate was supplied by Dr. Alan Brunt from the Institute of Horticultural Research, Littlehampton, West Sussex in a systemically-infected *N. benthamiana* plant, and was known as the IHRL isolate (PMTV-IHRL).

2.6.2 Growth of Plants

Several members of the Solanaceae and Chenopodiaceae plus *Tetragonia expansa* were supplied as young plants at the 4 to 6 leaf stage and were maintained in a glasshouse with an average daytime temperature of 25 °C, falling to 16 °C at night. Natural light was supplemented with mercury vapour lamps on an 18 hour photoperiod. Plants were watered regularly and fed once a week with Baby Bio fertiliser.

2.6.3 Inoculation of Plants

All three PMTV isolates were propagated in *N. benthamiana* and *N. debneyi* and the latter species was also used as a local lesion assay

host. The virus was also inoculated onto the following plants to determine the symptomatology of the virus: *N. clevelandii, N. glutinosa, N. hybrida, N. megalosiphon, N. tabacum* cv Dutch A, Samsun NN, White Burley and Xanthi-nc, *Capsicum annuum, Chenopodium amaranticolor, C. muralae, C. quinoa* and *Tetragonia expansa.*

Plants at the 4 to 6 leaf stage were darkened for 24 hours prior to inoculation, to make them more susceptible to virus infection. The inoculum consisted of either young, infected leaves (approximately 0.5 g) ground up in 1 ml of 10 mM sodium phosphate buffer (pH 7) or 1 ml of partially purified virus (section 2.7). In both cases Celite was added as an abrasive and the plants were inoculated by dipping a gloved finger into the inoculum and rubbing it gently onto one expanding leaf. This procedure was repeated for two other expanding leaves. Twenty minutes after inoculation the leaves were rinsed with water to remove excess Celite.

2.6.4 Storage of Virus

Tests were carried out to determine the stability of PMTV in harvested leaf material stored at various temperatures. Six locally-infected *N. debneyi* leaves were harvested and two leaves were stored at each of the following temperatures: -20 °C, 4 °C and room temperature, for one week. These leaves plus two freshly harvested locally-infected leaves were then ground up in 1 ml of 10 mM sodium phosphate buffer (pH 7) containing Celite and used to inoculate several *N. debneyi* plants (as described in the previous section), which were then observed for symptoms in the subsequent weeks.

The following two methods were used for storing small quantities of PMTV over long periods, such that the virus remained infective.

2.6.4i Dried-Down Leaf Material

Infected leaf material from *N. benthamiana* and *N. debneyi* was cut into very thin strips with a scalpel blade, dried over calcium chloride in a sealed jar and stored at 4 °C. Samples of this leaf material were tested for infective virus content after approximately one year, by back-testing onto *N. debneyi* (section 2.8.2).

2.6.4ii Liquid Nitrogen

Two grams of young, infected *N. benthamiana* or *N. debneyi* leaves were homogenised in 4 ml of 10 mM sodium phosphate buffer (pH 7) using a pestle and mortar, and strained through one layer of muslin. The filtrate was sealed in small, plastic tubes and stored in liquid nitrogen. Aliquots of this filtrate were tested for infective virus content after approximately one year, by back-testing onto *N. debneyi* (section 2.8.2).

2.7 Virus Purification

The purification of PMTV was tried by a number of different methods, based on protocols used for purifying other members of the furovirus group, plus the geminivirus - tomato golden mosaic virus (TGMV).

For each purification either systemically-infected *N. benthamiana* leaves or locally-infected *N. debneyi* leaves were used as a source of virus.

2.7.1 Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a Neutral pH Buffer

This was based on a method used by Kassanis *et al.* (1972) for partially purifying PMTV from *N. tabacum* cv. Xanthi-nc leaves. Approximately 100 g of infected leaf material were ground up using a pestle and mortar and the sap was strained through muslin and the filtrate centrifuged at 10,000 g for 10 minutes. The resulting pellet was resuspended in 0.5 M borate buffer (pH 7.5) using half the original volume of sap, clarified by centrifugation at 10,000 g for 10 minutes and emulsified with an equal volume of ether by gentle shaking. The emulsion was broken by centrifugation and the aqueous phase was similarly treated with an equal volume of carbon tetrachloride, separated from the emulsion and then centrifuged at 30,000 rpm for 75 minutes. The pellets were resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 7.5), incubated

overnight at 4 °C and clarified by centrifugation at 10,000 g for 10 minutes. The final virus preparation was stored at 4 °C.

2.7.2 Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a High pH Buffer

This was based on a method used by Randles *et al.* (1976) for partially purifying NVMV from systemically-infected *N. debneyi* leaves and PMTV from inoculated *N. tabacum* cv. Xanthi-nc or Samsun NN leaves. Approximately 100 g of infected leaf material were homogenised with 200 ml of extraction buffer (0.07 M phosphate buffer, 10 mM DIECA, 0.05 % (v/v) thioglycollic acid, pH 5.6) and strained through muslin. The filtrate was centrifuged at 12,000 g for 20 minutes and the supernatant discarded. The pellet was resuspended in 25 ml of TTG buffer (0.05 M tris-HCl, o.05 % thioglycollic acid, pH 8.1), stirred for two hours at 4 °C, and centrifuged at 12,000 g for 20 minutes. The supernatant was layered over 55 % (w/v) sucrose (in TTG) cushions (1.5 ml per 13 ml tube) and centrifuged at 60,000 rpm for one hour in a Beckman 80Ti rotor. The pellets were resuspended in 1 to 2 ml TTG, incubated overnight at 4 °C and clarified by centrifugation at 10,000 g for 10 minutes. The supernatant was layered over another 55 % sucrose cushion and centrifuged as before. The resulting pellet was treated in one of two ways:

i) it was resuspended in 0.2 to 0.5 ml of TTG, incubated for two hours at 4 °C and layered over a 10-40 % (w/v) sucrose (in 0.05 M tris-HCl, pH 8.1) gradient. This was centrifuged at 30,000 rpm for 25 minutes in a Beckman SW50.1 rotor and fractionated, giving five 1 ml fractions, which were diluted 5 times with 0.05 M tris-HCl (pH 8.1) and pelleted by centrifugation at 30,000 rpm for 75 minutes in a Beckman type 30 rotor. The pellets were resuspended in approximately 0.5 ml of 0.05 M tris-HCl (pH 8.1) and stored at 4 °C;

ii) it was resuspended in 2.5 ml of TTG, mixed with 2.5 ml of 80 % (w/v) caesium chloride (in TTG) and centrifuged at 34,000 rpm overnight in a Beckman SW50.1 rotor. The light-scattering band which contained the virus was collected and diluted 10 to 15 times with

0.05 M tris-HCl (pH 8.1) and pelleted by centrifugation at 30,000 rpm for 75 minutes in a type 30 rotor. The pellet was resuspended in approximately 0.5 ml of 0.05 M tris-HCl (pH 8.1) and stored at 4 °C.

2.7.3 Purification by a Method which Involves Clarifying the Sap with Organic Solvents

Approximately 100 g of infected leaf material were homogenised with 200 ml of 0.03 M phosphate buffer (pH 7.0) containing 0.05 % thioglycollic acid. One of the following organic solvent mixtures was added: i) 1/5 volume of chloroform

ii) an equal volume of a 1:1 mixture of chloroform and butanol

iii) an equal volume of chloroform

iv) ½ volume of carbon tetrachloride

and the mixture was stirred for 10 minutes to form an emulsion. The phases were separated by centrifugation at 10,000 g for 10 minutes and the aqueous phase was collected and centrifuged over a 55 % (w/v) sucrose (in extraction buffer) cushion at 45,000 rpm for 90 minutes in a Beckman 45Ti rotor. The resulting pellets were treated in one of two ways:

i) they were resuspended in approximately 0.5 ml of 0.03 M phosphate buffer (pH 7.0) overnight at 4 °C and the suspension was clarified by centrifugation at 10,000 g for 10 minutes. The supernatant was stored at 4 °C and the pellet was resuspended in approximately 0.5 ml of 0.03 M phosphate buffer (pH 7.0) and stored at 4 °C.

ii) they were resuspended in 1 to 2 ml of 0.03 M phosphate buffer (pH 7.0) overnight at 4 °C and the suspension was clarified by centrifugation at 10,000 g for 10 minutes. The supernatant was layered over another 55 % sucrose cushion and centrifuged as before. The resulting pellet was resuspended in 2.5 ml of TTG, mixed with 2.5 ml of 80 % (w/v) caesium chloride (in TTG) and centrifuged at 34,000 rpm overnight in a Beckman SW50.1 rotor. The light-scattering band which contained the virus was collected and diluted 10 to 15 times with 0.03 M phosphate buffer (pH 7.0) and pelleted by centrifugation at 30,000 rpm for 75 minutes in a type 30 rotor. The pellet was

resuspended in approximately 0.5 ml of 0.03 M phosphate buffer (pH 7.0) and stored at 4 °C.

2.7.4 Purification by a Method which Involves Clarifying the Sap by Acidification

This was based on a method used by Thouvenel *et al.* (1978) for purifying PCV from *C. amaranticolor* leaves. Approximately 100 g of infected leaf material were homogenised with 200 ml of extraction buffer (0.2 M potassium borate buffer (pH 8.2), containing 1 M urea, 10 mM EDTA and 0.5 % (v/v) 2-mercaptoethanol) and strained through muslin. The pH of the filtrate was adjusted to 4.2 with 2 M citric acid, in order to irreversibly precipitate the plant proteins, and this was incubated for 30 minutes on ice. Then the pH was raised to 8.2 with concentrated potassium hydroxide and the solution was centrifuged at 10,000 g for 10 minutes. The supernatant was collected and NaCl added to 4 % (w/v). The pH was adjusted to 6.4 and PEG 20,000 was added to 5 % (w/v), stirred to dissolve and incubated for one hour at 4 °C. This was centrifuged at 16,000 g for 15 minutes and the pellets were resuspended in 20 ml of extraction buffer and centrifuged at 10,000 g for 12 minutes. The resulting supernatant was collected and centrifuged at 30,000 rpm for two hours in a Beckman type 30 rotor. The pellet was resuspended in 2.5 ml of 0.05 M borate buffer (pH 8.2), incubated for one hour at 4 °C and centrifuged at 10,000 g for 10 minutes. The clarified sample was mixed with 2.5 ml of 80 % (w/v) caesium chloride and centrifuged at 34,000 rpm overnight in a Beckman SW50.1 rotor. The virus band was harvested, diluted 10 to 15 times with 0.05 M borate buffer (pH 8.2) and pelleted by centrifugation at 30,000 rpm for 75 minutes in a type 30 rotor. The pellet was resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 8.2) and stored at 4 °C.

2.7.5 Purification by a Method which Involves Clarifying the Sap with Carbon Tetrachloride and Concentrating the Virus by PEG Precipitation

This was based on a method used by Putz & Kuszala (1978) for

purifying BNYVV from *C. quinoa* leaves. Approximately 100 g of infected leaf material were homogenised with 200 ml of 0.1 M tris-HCl (pH 9.0) plus 100 ml of ice-cold carbon tetrachloride, stirred for 15 minutes and centrifuged at 5000 g for 15 minutes. The upper aqueous phase was collected and PEG 6000 and NaCl were added to 5 % (w/v) and 0.2 M respectively. This was stirred for 30 minutes, incubated for 90 minutes at 4 °C and centrifuged at 12,500 g for 20 minutes. The pellets were resuspended in 100 ml of 0.01 M tris-HCl (pH 9.0), incubated overnight at 4 °C and centrifuged at 5000g for 10 minutes. The supernatant was collected and the PEG precipitation was repeated, giving a final volume of approximately 20 ml. This was layered over a 12 ml cushion of 20 % (w/v) sucrose (in 0.01 M tris-HCl, pH 9.0) and centrifuged at 22,500 rpm for 20 minutes in a Beckman SW28 rotor. The supernatant was decanted into a fresh SW28 tube and centrifuged at 28,000 rpm for 75 minutes. The resulting pellet was resuspended in approximately 0.5 ml of 0.01 M tris-HCl (pH 9.0) and stored at 4 °C.

2.7.6 Purification by a Method which Involves Extracting the Sap in a High pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion

This was based on a method used by Shirako & Brakke (1984a) for purifying SBWMV from wheat leaves. Approximately 100 g of infected leaf material were homogenised with 200 ml of extraction buffer (0.5 M sodium borate buffer, pH 9.0, 1 mM EDTA), filtered through muslin and centrifuged at 10,000 rpm for 10 minutes in a Beckman JA-20 rotor. After addition of Triton X-100 to 2 % (v/v) the supernatant was layered onto a cushion of 6 ml of 20 % (w/v) sucrose in extraction buffer and centrifuged at 28,000 rpm for two hours in a Beckman type 30 rotor. The resulting pellet was resuspended in 10 ml of 0.05 M sodium borate (pH 8.0), 1 mM EDTA, incubated for two hours on ice and centrifuged at 10,000 rpm for 10 minutes in a JA-20 rotor. The supernatant was layered onto a 2 ml cushion of 20 % (w/v) sucrose in 0.05 M borate (pH 8.0), 1 mM EDTA and centrifuged at 40,000 rpm

for one hour in a Beckman type 65 rotor. The resulting pellet was resuspended in approximately 0.5 ml of 0.05 M borate (pH 8.0), 1 mM EDTA, the suspension was incubated on ice for two hours and then centrifuged for 10 minutes in a microcentrifuge. The supernatant was stored at 4 °C and the pellet was resuspended in approximately 0.5 ml of 0.05 M borate (pH 8.0), 1 mM EDTA, buffer and stored at 4 °C.

2.7.7 Purification by a Method which Involves Extracting the Sap in a Neutral pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion

This was based on a method used by Stein *et al.* (1983) to purify the geminivirus TGMV from *N. benthamiana* leaves. Approximately 100 g of infected leaf material were homogenised with 200 ml of CEM buffer (0.1 M trisodium citrate. 0.75 % (w/v) sodium sulphite, 5 mM EDTA, 1 % (v/v) 2-mercaptoethanol, 0.325 % (w/v) L-ascorbic acid, adjusted to pH 7.0 with NaOH). The homogenate was made 2.5 % (v/v) with Triton X-100, stirred for 16 hours at 4 °C and filtered through muslin. The filtrate was centrifuged at 10,000 g for 15 minutes and the supernatant was further centrifuged at 30,000 rpm for 75 minutes in a Beckman type 30 rotor. The resulting pellet was resuspended in approximately 10 ml of CEM buffer, incubated for two hours on ice and centrifuged at 10,000 rpm for 10 minutes in a JA-20 rotor. The supernatant was layered onto a 2 ml cushion of 20 % (w/v) sucrose in CEM buffer and centrifuged at 40,000 rpm for one hour in a Beckman type 65 rotor. The resulting pellet was resuspended in approximately 0.5 ml of CEM buffer, the suspension was incubated on ice for two hours and then centrifuged for 10 minutes in a microcentrifuge. The supernatant was stored at 4 °C and the pellet was resuspended in approximately 0.5 ml of CEM buffer and stored at 4 °C.

2.7.8 Analysis of Extraction Buffers

An attempt was made to rationalise the effects of the various components of the extraction buffers used in virus purification. In each experiment a large batch of systemically-infected *N. benthamiana* leaf material was split into 10 g samples, which were then homogenised with different extraction buffers using a Pollähne Press. The homogenate was filtered through muslin and the resulting fractions - filtrate and pulp - were tested further. Each experiment was performed twice under identical conditions.

(i) In the first part of the experiment 16 extraction buffers that differed in molarity, pH and actual buffer were tested:-

1.	0.1 M citrate, pH 6.0	2.3 ml 0.5 M citric acid 88.5 ml trisodium citrate plus dH_2O to 100 ml.
2.	0.1 M phosphate, pH 6.0	6.15 ml 0.1 M $Na_2HPO_4.2H_2O$ 43.8 ml 0.1 M $NaH_2PO_4.2H_2O$ plus dH_2O to 100 ml.
3.	0.02 M phosphate, pH 7.2	36 ml 0.02 M $Na_2HPO_4.2H_2O$ 14 ml 0.02 M $NaH_2PO_4.2H_2O$ plus dH_2O to 100 ml.
4.	0.1 M phosphate, pH 7.2	36 ml 0.1 M $Na_2HPO_4.2H_2O$ 14 ml 0.1 M $NaH_2PO_4.2H_2O$ plus dH_2O to 100 ml.
5.	0.5 M phosphate, pH 7.2	36 ml 0.5 M $Na_2HPO_4.2H_2O$ 14 ml 0.5 M $NaH_2PO_4.2H_2O$ plus dH_2O to 100 ml.
6.	0.1 M tris-HCl, pH 7.2	25 ml 0.4 M tris base 45 ml 0.2 M HCl plus dH_2O to 100 ml.
7.	0.02 M tris-HCl, pH 8.0	25 ml 0.08 M tris base 30 ml 0.04 M HCl plus dH_2O to 100 ml.

8. 0.1 M tris-HCl, pH 8.0	25 ml 0.4 M tris base 30 ml 0.2 M HCl plus dH_2O to 100 ml.
9. 0.5 M tris-HCl, pH 8.0	25 ml 2 M tris base 30 ml 1 M HCl plus dH_2O to 100 ml.
10. 0.1 M borate, pH 8.0	30 ml 0.025 M borax 70 ml 0.1 M boric acid
11. 0.02 M borate, pH 9.0	80 ml 0.005 M borax 20 ml 0.02 M boric acid
12. 0.1 M borate, pH 9.0	80 ml 0.025 M borax 20 ml 0.1 M boric acid
13. 0.5 M borate, pH 9.0	80 ml 0.125 M borax 20 ml 0.5 M boric acid
14. 0.1 M glycine-NaOH, pH 9.0	25 ml 0.4 M glycine 4.4 ml 0.4 M NaOH plus dH_2O to 100 ml.
15. 0.1 M glycine-NaOH, pH 9.4	25 ml 0.4 M glycine 8.4 ml 0.4 M NaOH plus dH_2O to 100 ml.
16. 0.1 M borax-NaOH, pH 9.4	25 ml 0.1 M borax 5.5 ml 0.4 M NaOH

Each 10 g sample of infected leaf material was homogenised with 50 ml of its respective extraction buffer and filtered through muslin. The resulting filtrate was centrifuged at 5000 g for 10 minutes, the supernatant was collected and the pellet resuspended in 50 ml of the same extraction buffer. These two fractions (supernatant and resuspended pellet) were tested for infectious virus content by back-testing onto one *N. debneyi* plant each - 3 leaves per plant - as described in section 2.8.2.

(ii) In the second part of the experiment, the buffer that had extracted the most infectious virus from 10 g of leaf material - 0.5 M tris-HCl (pH 8.0) - was tested further. Ten gram samples of infected leaf material were homogenised with 50 ml of 0.5 M tris-HCl

(pH 8.0) containing one of 8 different additives, each of which is used commonly in virus purification:-

1. 1 M urea
2. 1 mM EDTA
3. 10 mM EDTA
4. 10 mM DIECA
5. 5 mM $MgCl_2$
6. 0.05 % (v/v) thioglycollic acid
7. 0.5 % (v/v) 2-mercaptoethanol
8. 0.25 % (w/v) L-ascorbic acid
9. 1 mM PMSF
10. no additive (control)

The homogenate was filtered through muslin and centrifuged at 5000 g for 10 minutes. The resulting supernatant was collected and the pellet resuspended in 50 ml of the relevant buffer. In addition, in the case of the control (i.e. no additive), the pulp was re-extracted with 50 ml of 0.5 M tris-HCl (pH 8.0) and this along with all of the supernatant and resuspended pellet fractions was back-tested onto *N. debneyi* (section 2.8.2).

(iii) The re-extracted pulp from part (ii) appeared to contain a large proportion of infectious virus. So the third part of the experiment involved a modification of part (ii). Ten gram samples of infected leaf material were homogenised with 25 ml of 0.5 M tris-HCl (pH 8.0) containing the same additives as before, and filtered through muslin. The filtrate was collected and the pulp was re-extracted with another 25 ml of buffer and filtered as before. The resulting filtrate was pooled with the earlier filtrate and centrifuged at 5000 g for 10 minutes. The supernatant was collected and the pellet resuspended in 50 ml of extraction buffer. In the case of the control the pulp was re-extracted a second time and this along with the other fractions was back-tested onto *N. debneyi*.

This part of the experiment was also performed with 0.1 M borate (pH 8.0) - the second best buffer from part (i) - in place of 0.5 M tris-HCl (pH 8.0).

2.8 Analysis of Virus Preparations

Several methods were used routinely for analysing virus preparations: electron microscopy, back-testing onto *N. debneyi*, SDS-PAGE and ssRNA extraction and analysis.

2.8.1 Electron Microscopy

A 20 µl aliquot of purified virus was mixed with 40 µl of 2 % potassium phosphotungstate (pH 6.8) - a negative stain - on a glass slide. One drop of this was then placed on the carbon side of a carbon-collodion-coated grid (400 square mesh) (Athene), the excess liquid sucked off with a piece of tissue and the grid allowed to dry. The grid was viewed in a JEOL 100 S electron microscope. This not only gave an indication of the concentration and purity of the virus, but also of the variation in particle length. The concentration of virus particles was scored as the number of particles per field of vision at magnification x 50,000.

2.8.2 Back-Testing

PMTV-T produces discrete necrotic ring-spots on locally-inoculated *N. debneyi* leaves. Therefore aliquots of the samples prepared in section 2.7.7 were inoculated onto three expanding leaves per plant (section 2.6.3) and after 6 to 10 days the ring-spots were counted, giving a direct measure of the amount of infectious virus in the samples.

2.8.3 SDS-PAGE

Aliquots of purified virus were electrophoresed on 16 % SDS-polyacrylamide gels (section 2.5.3i) alongside molecular weight markers, to determine the amount of viral capsid protein present and the level of host protein contamination.

2.8.4 Single-Stranded RNA Extraction and Analysis

Single-stranded RNA (ssRNA) was extracted from purified virus samples, as described in section 2.9.1, and analysed on MeHgOH (section 2.5.2i) or formaldehyde (section 2.5.2ii) agarose gels to determine the amount and size of viral ssRNA present in the sample, and the level of host nucleic acid contamination.

2.8.5 Measurement of Virus Density in Caesium Chloride

Partially purified virus (sections 2.7.2 & 2.7.4) was suspended in 5 ml of 40 % (w/v) caesium chloride and centrifuged at 34,000 rpm overnight in a Beckman SW50.1 rotor. The light-scattering virus band was collected using a syringe needle and the density was calculated from the refractive index of a 20 µl aliquot, measured with an Abbe '60' refractometer.

2.9 **Extraction and Analysis of Viral RNA**

2.9.1 Single-Stranded RNA from Purified Virus

Purified virus samples were incubated for 30 minutes at 37 °C with 1 % (w/v) SDS, 100 µg/ml proteinase K, 10 mM tris-HCl (pH 8.0) and 1 mM EDTA, to break down the protein coat. The sample was then mixed with an equal volume of a 1:1 solution of sterile dH_2O-saturated phenol and sevag, incubated for 5 minutes on ice with occasional mixing, and microcentrifuged for 5 minutes. The upper, aqueous phase was collected and kept on ice, and the lower, non-aqueous phase was re-extracted twice with an equal volume of 0.1 M tris-HCl (pH 9.0), firstly at room temperature and secondly at 60 °C. The aqueous phases were pooled and re-extracted with an equal volume of a 1:1 mixture of phenol and sevag, and then with an equal volume of sevag. To the final aqueous phase were added 1/15 volume of 3 M sodium acetate (pH 5.2), 1.5 volumes of isopropanol and 1 µl of 10 mg/ml tRNA per 1 ml of RNA in isopropanol, and this was mixed and incubated either on "Cardice" for 20 minutes, at -70 °C for one hour or at -20 °C overnight. The precipitated RNA was pelleted by microcentrifugation for 15 minutes and the pellet was washed twice

with 70 % (v/v) ethanol, dried under vacuum and resuspended in the minimum volume of sterile dH_2O. This final RNA sample was stored at -70 °C.

The quantity and purity of the nucleic acid in the RNA samples was determined by measuring the UV absorbance between 280 and 230 nm (section 2.5.1).

2.9.2 Preparation of Radioactively-Labelled Probes from ssRNA

To prepare a radioactively-labelled probe from ssRNA the following were mixed in a 1.5 ml Eppendorf tube: 5 µCi α^{32}PdCTP, 4 µl 0.1 M DTT, 5 µl 10 x reverse transcriptase buffer (0.5 M tris-HCl, pH 8.3, 0.7 M KCl, 80 mM $MgCl_2$), 25 µl random DNA primers, 2 µl of a mixture of dATP, dGTP and dTTP (12.5 mM each), 1 µg RNA, 1 U reverse transcriptase and sterile dH_2O to a final volume of 50 µl. The reaction was incubated at 42 °C for one hour and then stopped by adding 1 µl 0.5 M EDTA plus 5 µl NaOH and incubating at 65 °C for one hour. The radioactive probe was neutralised with 5 µl 1 M HCl, denatured by heating at 100 °C for ten minutes, cooled on ice for five minutes and used immediately for Northern blot hybridisation, as described in the next section.

2.9.3 Northern Blot Analysis of RNA

RNA electrophoresed on a MeHgOH agarose gel was transferred to a GeneScreen membrane (NEN) by a capillary blot procedure, essentially as described in the manufacturers' instructions.

One or more tracks containing molecular weight markers were cut from the rest of the gel and stained as described in section 2.5.5. The rest of the gel was capillary blotted: a piece of GeneScreen was cut to the size of the gel and soaked in 0.025 M sodium phosphate buffer (pH 6.5) for 15 to 20 minutes. Two pieces of Whatmann 3MM filter paper were wetted in the phosphate buffer and placed on an elevated glass plate so that the ends formed wicks. The gel was then placed on top of the filter paper, and gel spacers placed along each side of the gel. The GeneScreen was carefully placed on the gel, followed by five pieces of filter paper and a 5 to 7 cm stack of absorbent paper towels (all cut to the same size as the gel).

Finally, a weight was placed on top of the paper towels and the transfer was allowed to continue for at least twelve hours. The towels and filter paper were removed and the membrane was carefully lifted from the gel and washed in 0.025 M phosphate buffer (pH 6.5) to remove any residual agarose. The membrane was then placed with the transferred RNA face up on a piece of filter paper and allowed to dry at room temperature. The RNA was baked onto the membrane at 80 °C for two hours.

The membrane was sealed in a plastic bag with 10 ml of prehybridisation solution (50 % deionised formamide, 0.04 & polyvinyl-pyrrolidone (M_r 40,000), 0.04 % bovine serum albumin, 0.04 % ficoll (M_r 400,000), 5 x SSC, 1 % SDS, 100 µg/ml denatured Salmon sperm DNA) and incubated with constant agitation at 42 °C for 16 hours to block any non-specific binding sites. The prehybridisation solution was then replaced with 10 ml of hybridisation solution (50 % deionised formamide, 0.02 % polyvinyl-pyrrolidone (M_r 40,000), 0.02 % bovine serum albumin, 0.02 % ficoll (M_r 400,000), 5 x SSC, 1 % SDS, 100 µg/ml denatured Salmon sperm DNA, denatured cDNA probe) and incubated with constant agitation at 42 °C for 16 hours. The hybridisation solution was removed and the membrane was washed: twice with 100 ml 2 x SSC for five minutes at room temperature, twice with 100 ml of a solution containing 2 x SSC and 1 % SDS for 30 minutes at 65 °C, and twice with 100 ml 0.1 x SSC for 30 minutes at room temperature. Finally, the membrane was dried and autoradiographed in a light-proof cassette, containing an intensifying screen, with Kodak X-Omat S film at -70 °C for an appropriate length of time (1 to 14 days). Films were developed in a Kodak X-Omat automatic processor.

2.9.4 Double-Stranded RNA from Infected Plant Material

Several methods of extracting viral dsRNA from infected plant material were tried, but that of Taylor & Powell (1982) proved to be the most efficient and reproducible. Double-stranded RNA has been successfully extracted from both systemically-infected *N. benthamiana* leaves and locally-infected *N. debneyi* leaves by this method.

2 x Extraction Buffer

2 % (w/v)	cetyltrimethylammonium bromide (CTAB)
0.1 M	tris-HCl (pH 8.0)
20 mM	EDTA
1.4 M	NaCl

"10 % CTAB Solution"

10 % (w/v)	CTAB
0.7 M	NaCl

Precipitation Buffer

1 % (w/v)	CTAB
50 mM	tris-HCl (pH 8.0)
10 mM	EDTA
1 % (v/v)	2-mercaptoethanol

"1.0 M Caesium Chloride Solution"

50 mM	tris-HCl (pH 8.0)
5 mM	EDTA
50 mM	NaCl
1.0 M	caesium chloride

"5.7 M Caesium Chloride Solution"

50 mM	tris-HCl (pH 8.0)
5 mM	EDTA
50 mM	NaCl
5.7 M	caesium chloride

Usually about 100 g of infected leaf material - without any of the larger stalks - were used in each extraction. The leaves were ground to a fine powder in liquid nitrogen using a pestle and mortar, and 2 ml of 2-mercaptoethanol was added. This mixture was slowly stirred into 100 ml of boiling 2 x extraction buffer, ensuring that the temperature never dropped below 15 °C. When the temperature had

risen to 50 °C, 100 ml of sevag was added and the solution mixed until an emulsion was formed. The two phases were separated by centrifugation at 9000 rpm for 10 minutes in a 6 x 300 ml rotor at 20 °C, and the upper, aqueous phase was transferred to fresh 6 x 300 ml bottles. One tenth of a volume of 10 % CTAB solution and 100 ml of sevag were added, mixed to form an emulsion and centrifuged as above. The aqueous phase was transferred to a fresh 6 x 300 ml bottle and an equal volume of precipitation buffer was added. This solution was mixed and incubated at room temperature for at least 30 minutes, to allow a CTAB/nucleic acid precipitate to form. The precipitate was pelleted by centrifugation at 5000 rpm for 5 minutes at 20 °C, resuspended in 10 ml of 1.0 M CsCl solution and layered over 2 ml cushions of 5.7 M CsCl solution in four SW50.1 tubes. These were centrifuged at 32,000 rpm for 15 hours in an SW50.1 rotor at 4 °C. Under these conditions the RNA is pelleted and the DNA is banded at the 1.0 M / 5.7 M CsCl interface. The pellets were resuspended in a total of 2 ml of 10 mM tris-HCl (pH 7.6), 1 mM EDTA, 1 % (v/v) 2-mercaptoethanol. The RNA was re-precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol and incubating on cardice for 20 minutes or at -70 °C for one hour. The RNA was re-pelleted by microcentrifugation for 15 minutes at 4 °C, washed twice with 70 % (v/v) ethanol, dried under vacuum and resuspended in a minimum volume of sterile dH_2O. This final sample of dsRNA was stored at -70 °C.

3. RESULTS AND DISCUSSION

3.1 Virus Symptomatology

The first major aim of this project was to purify PMTV from infected plants, so that it could then be characterised. However, before attempting to purify the virus, it was important to determine which host species was the best source of PMTV for purification. The severity of symptoms induced by a virus is often correlated with the concentration of the virus in the plant, although there are notable exceptions such as Holmes' (1934) masked strain of TMV (now known to be a strain of pepper mild mottle virus). Nevertheless, symptom severity was used as a preliminary indication of which host species could be best used as virus sources for the purification of different PMTV strains.

The type strain of PMTV (PMTV-T) has been reported to induce symptoms in 19 out of 26 Solanaceous species, 8 out of 9 species of Chenopodiaceae and in *Tetragonia expansa*, but not to induce symptoms in 44 species in 13 other plant families (Harrison & Jones, 1970; Randles *et al.*, 1976). In contrast, I found that PMTV-T induced symptoms in only 4 out of 8 Solanaceous species, none out of 3 species of Chenopodiaceae and not in *Tetragonia expansa* (Table 5). Moreover, under my conditions the symptoms induced by PMTV-T in *N. debneyi* and *N. benthamiana* were different from those reported by Harrison & Jones (1970). Symptoms on inoculated leaves first appeared four to eight days after inoculation, and after two to six weeks on non-inoculated leaves, depending on the host plant and the time of year.

PMTV-T is reported to induce spreading necrotic spots on the inoculated leaves and necrotic thistle-leaf patterns on the non-inoculated leaves of *N. debneyi*, although during the summer, systemic infection only occurred in a few plants (Harrison & Jones, 1970). Similarly, I found that PMTV-T induced necrotic spots, usually surrounded by one or more concentric necrotic rings, on the inoculated leaves during winter (Figure 5); but as the weather conditions changed, and the day length and temperature increased, the

Table 5 Induction of Symptoms by PMTV-T

Host Plant	This Report*		Harrison & Jones (1970)		Randles *et al.* (1976)	
	I	N	I	N	I	N
Capsicum annuum	0	0	S	0(-)		
Nicotiana benthamiana	(S)	S	0(+)	(S)	0	S
N. clevelandii	0	0	S	(S)	S	S
N. debneyi	S	(S)	S	S	S	S
N. glutinosa	0	0	S	(S)	S	0(+)
N. hybrida	0	S				
N. megalosiphon	(S)	0	S	(S)		
N. tabacum cv. Dutch A	0	0				
N. tabacum cv. Samsun *NN*	0	0				
N. tabacum cv. White Burley			S	(S)	0	0(+)
N. tabacum cv. Xanthi-nc	0	0	S	(S)	S	S
Chenopodium amaranticolor	0	0	S	0(-)	S	0(-)
C. murale	0	0				
C. quinoa	0	0	S	0(-)	S	0(-)
Tetragonia expansa	0	0	S	0(-)	S	0(-)

I - inoculated leaves
N - non-inoculated leaves
S - symptoms produced
(S) - symptoms only produced in some plants
0 - symptomless
(+) - virus detected in symptomless leaves by back testing onto *N. tabacum* cv. Xanthi-nc, *C. amaranticolour* or *C. quinoa*.
(-) - virus not detected in symptomless leaves by similar tests.
* - no tests for symptomless infection were carried out in this study.

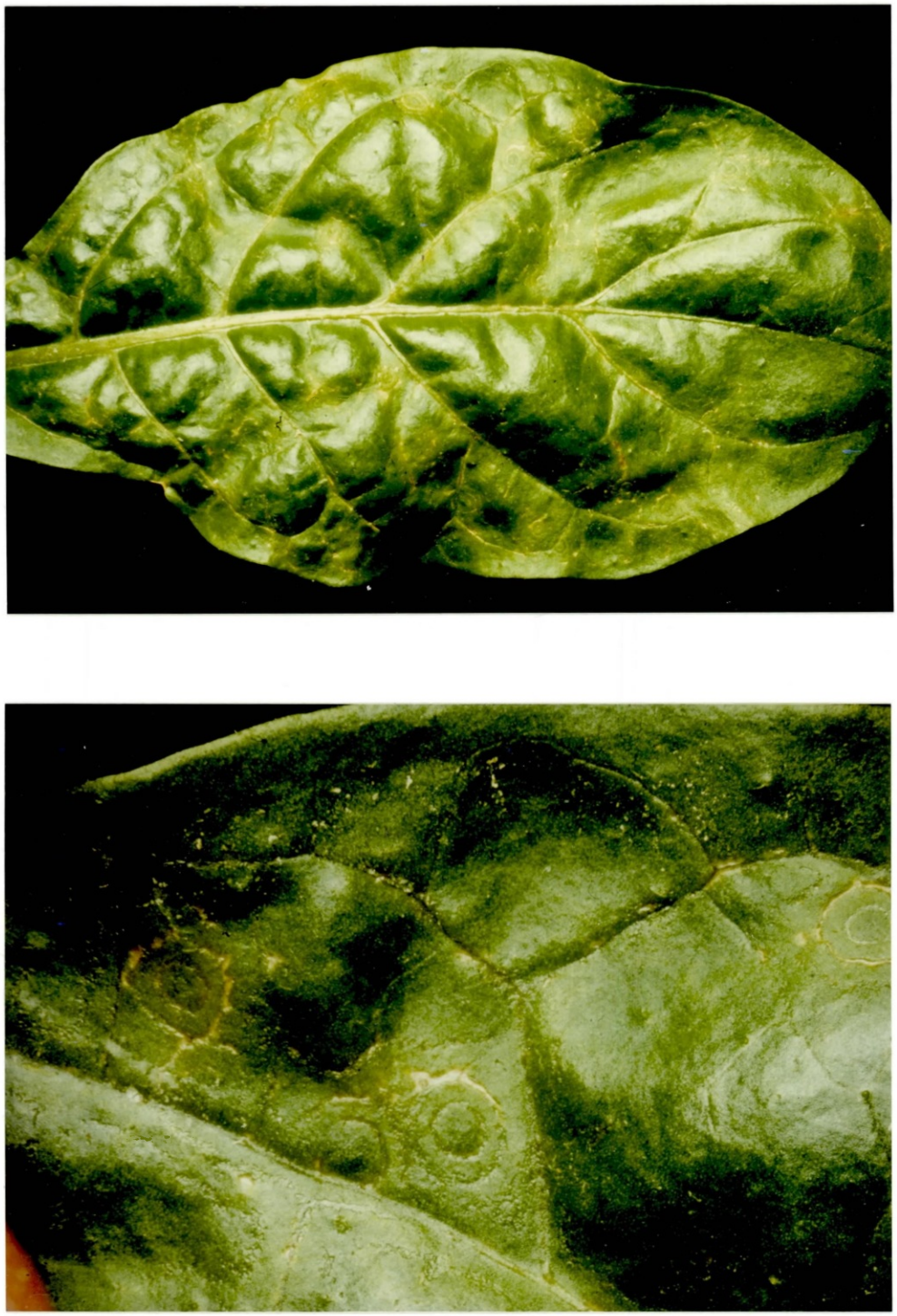


Figure 5 Symptoms in *N. debneyi* Leaf Inoculated with PMTV-T

symptoms changed: chlorotic patches developed on the leaves as a background to the concentric rings. Then the rings gradually appeared less often, so that eventually the symptoms were necrotic spots on a chlorotic background. During late spring the chlorotic patches appeared larger and the necrotic spots often spread within them, forming patches. Symptoms on the inoculated leaves appeared 4 to 8 days after inoculation.

Systemic symptoms were only observed on one batch of *N. debneyi* plants, which were inoculated towards the end of December with sap from a locally-infected leaf. These plants developed concentric necrotic rings on the inoculated leaves and a necrotic thistle-leaf pattern on the non-inoculated leaves. Locally- and systemically-infected leaves from these plants were back-tested onto *N. debneyi*, but these only developed lesions on the inoculated leaves - concentric, necrotic rings. However, those inoculated from locally-infected leaves had more pronounced symptoms than those from systemically-infected leaves.

In the case of *N. benthamiana*, PMTV-T is reported to induce mild symptoms which, as in *N. debneyi*, differ with the time of year. A few chlorotic or necrotic markings have been observed on non-inoculated leaves in winter, but no symptoms appeared on the inoculated leaves (Harrison & Jones, 1970). In contrast, I found PMTV-T to induce more pronounced symptoms in *N. benthamiana* plants: chlorotic patches and distortion of the non-inoculated leaves (Figure 6). During the colder months these symptoms took about two weeks to appear and were more pronounced, but during the summer the symptoms took about three weeks to appear and the non-inoculated leaves were not distorted and the chlorotic patches were mild and sometimes undetectable. One batch of *N. benthamiana* plants, inoculated in late January, developed fine, concentric, chlorotic and necrotic rings on the inoculated leaves (Figure 7), as well as the usual symptoms on the systemic leaves (i.e. chlorotic patches and distortion). However, such symptoms never re-occurred and neither Harrison & Jones (1970) nor Randles *et al.* (1976) reported the induction of local lesions in PMTV-T-infected *N. benthamiana* plants.

N. hybrida plants developed systemic leaf symptoms similar to



Figure 6 Symptoms in *N. benthamiana* Leaves Systemically Infected with PMTV-T

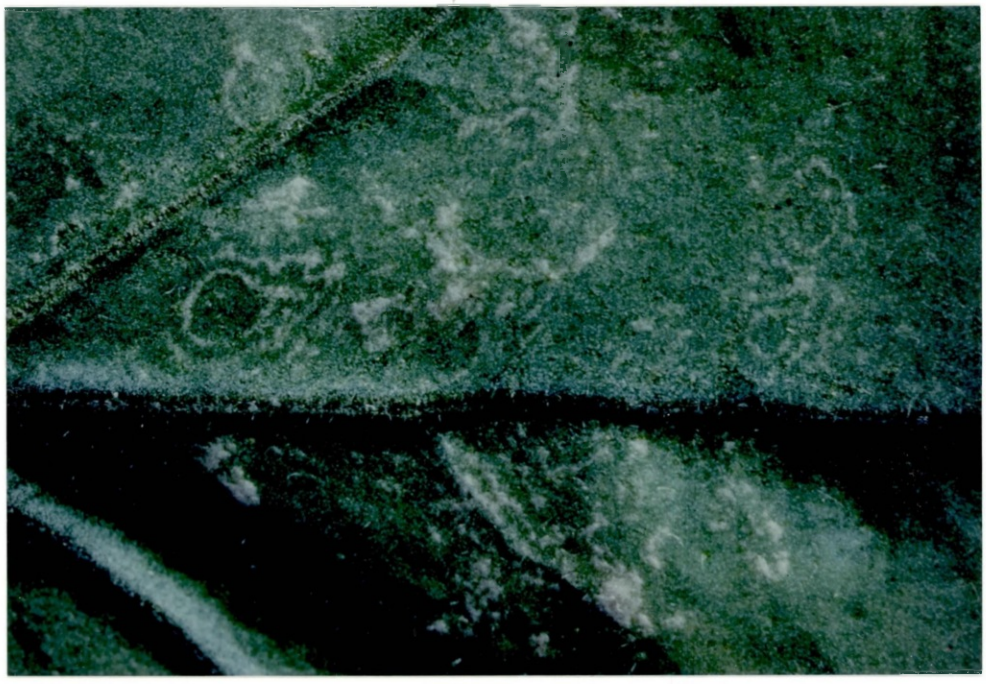


Figure 7 Symptoms in *N. benthamiana* Leaf Inoculated with PMTV-T

those induced in *N. benthamiana* (i.e. chlorotic patches and distortion of leaves), when inoculated with PMTV-T. These symptoms were more pronounced during the winter months and took approximately twice as long (i.e. 4-6 weeks) to appear as those in *N. benthamiana*.

As shown above, there are several differences between the published symptomatology of PMTV-T (Harrison & Jones, 1970; Randles *et al.*, 1976 - both tested at the Scottish Crops Research Institute (SCRI)) and the symptomatology I found at the Institute of Horticultural Research Littlehampton (IHRL), even though the inoculum of PMTV-T had been obtained from SCRI. The conditions in which the plants were grown may have affected the symptoms, if any, that developed. It is possible that the conditions at SCRI and IHRL differ enough to alter the host range. For example, the temperature and amount of light are critical factors in virus symptom development. This was apparent in the way that the symptoms induced in several *Nicotiana* species differed with the time of year. Also, the soil and water used to grow the plants at the two institutes may differ enough to affect the symptoms induced.

Another possible explanation for the differences in symptomatology is virus mutation, which occurs *in vivo* at a low frequency with RNA plant viruses due to the lack of proof-reading activities of the RNA polymerases. For example, in the case of TMV, mutants have been found in preparations obtained after passage of virus through necrotic local lesions (Garcia-Arenal *et al.*, 1984).

Any mutants that occur may become dominant when passaging the virus in a different host, in which the mutant strain may have a selective advantage over the original strain. I obtained PMTV-T from SCRI in systemically-infected *N. debneyi* leaves and propagated it in *N. benthamiana*. This change of host may have given any mutant virus present a selective advantage. The symptoms induced in the non-inoculated leaves of *N. benthamiana* were actually more severe than previously reported (Harrison & Jones, 1970), and when I subsequently inoculated *N. debneyi* plants with the sap from infected *N. benthamiana* leaves, I found that the virus no longer induced symptoms in the non-inoculated leaves. It is possible that a mutant of PMTV-T was present in the non-inoculated *N. debneyi* leaves from SCRI, which had lost the ability to infect *N. debneyi* systemically, and this

mutant became dominant during several passages in *N. benthamiana*, and the original strain was lost. However, detailed characterisation of the genomes of PMTV-T from SCRI and IHRL is required to confirm or deny this suggestion.

Harrison and Jones (1970) compared the symptomatology of PMTV-T with that of nine other isolates of PMTV from different sites in Scotland and Northern Ireland, and found that all ten isolates infected essentially the same species, but induced symptoms of varying severity. All isolates induced concentric necrotic rings in the inoculated leaves of, but no systemic infection in, *Chenopodium amaranticolor*. However, in the inoculated leaves of *N. tabacum* cv. Xanthi-nc, only isolate T induced necrotic lesions; the other isolates caused symptomless infection. There were similar differences in the severity of symptoms induced in *N. debneyi*: isolate T induced obvious necrotic spots on the inoculated leaves and obvious necrotic thistle-leaf patterns on the non-inoculated leaves, whereas the other isolates induced slight necrotic line patterns or no symptoms at all on the inoculated leaves and very slight thistle-leaf patterns on the non-inoculated leaves.

I compared the symptomatology of PMTV-T (Table 5) with that of two other isolates - PMTV-NI and PMTV-IHRL (section 2.6.1) - in the twelve species mentioned above. PMTV-NI and PMTV-IHRL only induced symptoms in two species, *N. benthamiana* and *N. debneyi*, and these symptoms were similar to each other but much milder than those induced by PMTV-T. The tests were carried out during the winter, when the symptoms induced by PMTV-T were most severe. The other two isolates induced chlorotic patches on some of the non-inoculated leaves of *N. benthamiana* and necrotic ring-spots on the inoculated leaves of *N. debneyi*.

Overall, the symptoms induced by the three PMTV isolates tested at IHRL were similar, with PMTV-T the most virulent of the three, and the symptoms induced by the ten PMTV isolates tested at SCRI (Harrison & Jones, 1970) were similar to each other, with PMTV-T the most virulent of the ten. However, there are great differences in symptomatology between the isolates at IHRL and SCRI, even between identical isolates such as PMTV-T. This disparity may be due to some inherent difference in the propagation conditions at the two

Institutes, or a mutation in PMTV-T occurring and becoming dominant, as discussed above.

3.2 Storage of PMTV

3.2.1 Short Term Storage

When PMTV-T-infected leaf material was harvested, it was not always convenient to purify PMTV-T from it immediately. So, tests were carried out to determine the optimum temperature for storing the harvested leaf material over a short period of time (section 2.6.4), such that a minimal amount of virus infectivity was lost. Six locally-infected *N. debneyi* leaves of roughly uniform size and symptom severity were harvested and two leaves were stored at each of the the following temperatures for one week: -20 °C, 4 °C and room temperature. These leaves, plus two freshly harvested locally-infected leaves, were back-tested onto a total of twelve expanding leaves on four *N. debneyi* plants each, and the results are shown in the table below.

The results show that some infectivity is lost when the leaves are stored at 4 °C or room temperature, whereas all infectivity is lost at -20 °C. Therefore, when infected plants were harvested for purification, they were used immediately, if possible, although they were also stored at 4 °C for up to two weeks, before purification.

Storage Temperature	Average Number of Lesions per Leaf
Control	53
Room Temperature	38
4 °C	42
-20 °C	0

3.2.2 Long Term Storage

For long term storage of PMTV from infected leaf material, two methods were tested. Firstly, infected *N. benthamiana* or *N. debneyi* leaves were cut into very thin strips, dried over calcium chloride and stored in a sealed jar at 4 °C (section 2.6.4i). Secondly,

infected *N. benthamiana* or *N. debneyi* leaves were homogenised in 10 mM sodium phosphate buffer (pH 7) (2 ml/g of leaf material) and the homogenate was strained through muslin. The resultant filtrate was sealed in small plastic tubes and stored in liquid nitrogen (section 2.6.4ii).

Samples prepared by each method, for all three isolates of PMTV (PMTV-T, PMTV-NI and PMTV-IHRL), were back-tested onto *N. debneyi* plants after approximately one year's storage; they all induced symptoms on the inoculated leaves. Therefore, samples of all three isolates were stored for long periods by both methods.

3.3 Virus Purification

3.3.1 Introduction

As isolate T was found to be the most virulent of the three PMTV isolates tested, as assessed by symptom severity in infected *N. benthamiana* and *N. debneyi* plants (section 3.1), this isolate was chosen for further study. This initially involved purifying the virus from infected tissue. PMTV-T, as shown in section 3.1, induces conspicuous symptoms in the inoculated leaves of *N. debneyi* and the non-inoculated leaves of *N. benthamiana*, during the colder months. It was difficult to assess in which of these two hosts PMTV-T induced the better symptoms, as the symptoms were different in character (necrotic ring-spots on *N. debneyi* and chlorotic patches on *N. benthamiana*) and were found on different parts of the plants (inoculated leaves of *N. debneyi* and non-inoculated leaves of *N. benthamiana*). So, PMTV-T-infected leaves from both species were initially tested as sources of virus for purification.

Previous attempts to purify PMTV have met with varying results (Harrison & Jones, 1970; Kassanis *et al.*, 1972; Randles *et al.*, 1976). The earliest reported attempt was that of Harrison & Jones (1970) which, although ultimately unsuccessful, did highlight several problems which needed to be overcome for PMTV to be purified successfully. Firstly, virus particles tend to aggregate with each other and some cell components *in vivo* and *in vitro*, so that most of the virus sediments during low-speed centrifugation. Secondly, the particles appear to be fragile, as shown by electron microscopy of

leaf extracts: the ends of many particles seemed to be disintegrating, with the endmost turns of the helix loose. Figure 1 shows a PMTV-T particle breaking up, with the helix unwinding at the breaking point. This indicates that any procedure to break up the virus aggregates must avoid disrupting the particles. Thirdly, PMTV is present in infected cells in low concentration, which makes it even more critical not to disrupt the virus particles when breaking up the virus aggregates, unlike hordeiviruses and potexviruses, whose particles are more stable and are present in high concentrations *in vivo*, such that particle disruption is not a significant problem (Jackson & Lane, 1981; Purcifull & Edwardson, 1981).

As mentioned above, Harrison & Jones (1970) found that most of the virus infectivity of the sap was sedimented during low-speed centrifugation. They obtained the sap by grinding some PMTV-infected *N. tabacum* cv. Xanthi-nc leaves using a pestle and mortar, and this was strained through muslin and centrifuged at 8000 g for 5, 10, 15 or 20 minutes. The infectivities of the supernatant and the resuspended sediments, estimated by counting the number of lesions induced in the inoculated leaves of *N. tabacum* cv. Xanthi-nc or *C. amaranticolor*, indicated that only 40, 17, 5 and 2 % respectively of the infectivity remained in the supernatant. The solubility of the virus was not increased by grinding the leaves in 0.5 M dipotassium hydrogen phosphate. Another attempt to solubilise the virus involved grinding leaves in water-saturated phenol (5 ml/g leaf material) plus 0.02 M phosphate buffer (pH 7.4, 5 ml/g leaf material), but these leaf extracts were only 1 to 3 % as infective as buffer extracts. Harrison & Jones (1970) failed to purify PMTV by several commonly used methods.

3.3.2 Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a Neutral pH Buffer

This was based on a method used by Kassanis *et al.* (1972) for partially purifying PMTV from *N. tabacum* cv. Xanthi-nc leaves. This method relied on sedimenting most of the virus at 10,000 g and eluting it from this sediment using 0.5 M borate buffer (pH 7.5).

The virus was further purified by clarification with ether and carbon tetrachloride, followed by one cycle of differential centrifugation (see section 2.7.1). I have tried this protocol using 100 g of systemically-infected *N. benthamiana* leaves (twice) and 100 g of locally-infected *N. debneyi* leaves (once), but on each occasion the final preparation (0.5 ml) contained very few virus particles, as determined by electron microscopy - less than one particle per field of view at x 50,000 magnification (section 2.8.1) (a good preparation would contain 50 particles per field). Also, significant amounts of host material were still present in the final preparations.

3.3.3 Purification by a Method which Involves Sedimenting the Virus from the Sap by Low-Speed Centrifugation and Resuspending it in a High pH Buffer

This protocol was based on that designed by Randles *et al.* (1976) to purify PMTV and another furovirus, NVMV (section 2.7.2). Again, the method relied on sedimenting as much of the virus as possible during low-speed centrifugation of the leaf extract, followed by elution of the virus in 0.05 M tris-HCl, pH 8.1, containing 0.05 % thioglycollic acid. The virus was further purified by two cycles of pelleting the virus by centrifugation through 55 % (w/v) sucrose cushions, followed by banding on a sucrose density gradient. I tried this protocol on three occasions, with 100 g of systemically-infected *N. benthamiana* leaves. However, there were no light-scattering bands on the sucrose gradients after centrifugation, and when fractionated, there were no well-defined UV-absorbing peaks. This contrasts with the results of Randles *et al.* (1976), who found three closely spaced UV-absorbing and light-scattering zones in the middle of the sucrose gradient. The gradient was fractionated into five 1 ml fractions, each of which was diluted with 0.05 M tris-HCl (pH 8.1) and the virus particles were pelleted by ultracentrifugation, resuspended in 0.5 ml of 0.05 M tris-HCl (pH 8.1), then analysed by electron microscopy, revealing that particles were present in roughly equal, small quantities (less than five particles per field) in all but the top fraction of the gradient, which contained no virus particles. All fractions also contained significant quantities of host material.

Therefore, centrifuging the virus on a sucrose gradient, far from concentrating the virus particles into one or more bands free of host material, has spread the particles and the host material fairly evenly throughout the gradient. This result was not completely unexpected, as PMTV particles have been shown to fragment during partial purification into particles of lengths ranging from about 20 to 300 nm (Kassanis *et al.*, 1972; Randles *et al.*, 1976). As virus particles are separated by size on sucrose density gradient centrifugation, these degraded particles would be spread through most of the gradient and not concentrated in the two bands expected if the virus had remained intact, corresponding to the two particle modal lengths of PMTV (125 and 290 nm, as determined by immunosorbent electron microscopy of PMTV particles from the sap of infected *N. tabacum* cv. Samsun NN; Roberts & Harrison, 1979).

An alternative to the sucrose density gradient as a method of concentrating the virus, was to centrifuge the resuspended pellet from the second sucrose cushion step on a caesium chloride gradient (section 2.7.2ii; P. Massalski, personal communication). The main advantage of using a caesium chloride gradient is that it separates virus particles according to their density, rather than their size. Therefore, all PMTV particles, regardless of length, should band at the same density.

I tried this method, three times, once with 100 g of systemically-infected *N. benthamiana* and twice with 100 g of locally-infected *N. debneyi*, with varying results. The first attempt, with infected *N. debneyi*, was unsuccessful - the resuspended pellet from the second sucrose cushion step contained very few virus particles (less than one per field) and a lot of host material. When this sample was centrifuged on a caesium chloride gradient, it produced no visible band. However, the second attempt with *N. debneyi* was more successful - the resuspended pellet contained 5 to 10 particles per field and a lot of host material; however, there were two light-scattering bands very close to each other in the gradient which, when harvested together, were diluted, ultracentrifuged and the resulting pellet was resuspended in approximately 0.5 ml of 0.05 M tris-HCl (pH 8.1), contained 5 to 10 particles per field and a little host material. It is not known whether both of these bands contained

virus particles and, if so, why there were two bands. The density of the combined bands was measured with an Abbe '60' refractometer (section 2.8.5), and was found to be 1.33 g/ml, which is similar to the densities of PCV (1.32 g/ml) and TMV (1.33 g/ml) in caesium chloride (Thouvenel & Fauquet, 1981).

When this method was tested with systemically-infected *N. benthamiana* there was a lot of host material in the resuspended pellet, but very few virus particles, and one faint band on the caesium chloride gradient which, when harvested, was found to have a density of 1.34 g/ml; when it had been diluted, ultracentrifuged and the resulting pellet had been resuspended in 0.5 ml of 0.05 M tris-HCl (pH 8.1), it was found to contain very few virus particles - less than one per field - plus some host material.

Therefore, the caesium chloride gradient was an improvement on the sucrose density gradient, as it generally banded the virus. Also, the final virus sample appeared to be less contaminated, in that there was less host material present. However, the yields of virus were variable and, at best, quite low.

3.3.4 Purification by a Method which Involves Clarifying the Sap with Organic Solvents

As mentioned earlier, PMTV-T mostly sediments when the leaf extract is centrifuged at low speed, and the two purification protocols tried up to this point both attempted to overcome this problem by maximising the amount of virus sedimented and then eluting it from the pellet. Similar problems were encountered by Putz & Kuszala (1978) when trying to purify another furovirus. They found significant quantities of BNYVV, not only in the pellet from the first low-speed centrifugation, but also in the pulp left over after filtering the leaf extract through muslin. However, Putz & Kuszala (1978) chose a different way of overcoming this problem. Instead of filtering the leaf extract and centrifuging it at low speed, they clarified the leaf extract with an organic solvent - half a volume of carbon tetrachloride - then separated the aqueous and non-aqueous phases by low-speed centrifugation and collected the aqueous phase.

I tried this approach with PMTV-T, testing four organic solvent

mixtures: 1/5 volume chloroform, an equal volume of a 1:1 mixture of chloroform and butanol, an equal volume of chloroform, and ½ volume carbon tetrachloride. The protocol used was as follows: 100 g of infected *N. benthamiana* or *N. debneyi* leaves were homogenised in 200 ml of 0.03 M phosphate buffer (pH 7.0) containing 0.05 % thioglycollic acid. The homogenate was clarified with one of the organic solvent mixtures and then ultracentrifuged over a 55 % (w/v) sucrose (in extraction buffer) cushion. The pellets were resuspended in approximately 0.5 ml of 0.03 M phosphate buffer (pH 7.0) and the suspension was clarified. The supernatant was stored at 4 °C and the pellets were resuspended in approximately 0.5 ml of 0.03 M phosphate buffer and stored at 4 °C. Both samples were tested by electron microscopy. All steps were carried out at 4 °C.

Each of the four organic solvent mixtures was tested at least three times, with a view to testing further the one that gave the best electron microscopy results (i.e. the highest consistent yield of virus particles with the least host material). However, no one organic solvent mixture gave significantly better results than the others (Table 6). In each case, the yields of virus were quite low and preparations contained much host material. Also, there were virus particles present in the resuspended clarification pellets as well as the supernatants, although they were always fewer in number, and the resuspended clarification pellets contained more host material than the supernatants.

Organic solvents, as well as clarifying virus preparations, can also disrupt virus particles, especially when in high concentration. Therefore, although the electron microscopy results were fairly similar for all four organic solvent mixtures, 1/5 volume chloroform was chosen for further study, as it involved the least amount of organic solvent coming into contact with the virus particles.

All virus preparations had so far been analysed only by electron microscopy. I repeated the above protocol, therefore, using 1/5 volume chloroform as the clarifying agent, and analysed the final

Table 6 Effects of Organic Solvents on the Purification of PMTV-T

Clarification Agent	Host Species	Number of Particles per Field†		Host Material
		Supernatant	Pellet	
1/5 vol $CHCl_3$	*N. debneyi*	5-10	1-10	+*
	N. debneyi	5-20	1-10	+
	N. benthamiana	1-10	<1	+
equal vol of	*N. debneyi*	5-10	<1	+
1:1 mixture	*N. debneyi*	0-5	<1	+
of $CHCl_3$ &	*N. debneyi*	0-5	<1	+
butanol	*N. benthamiana*	<1	<1	+
equal vol	*N. debneyi*	0-5	<1	+
$CHCl_3$	*N. debneyi*	1-20	1-5	+
	N. benthamiana	1-10	<1	+
½ vol CCl_4	*N. debneyi*	<1	<1	+
	N. debneyi	1-10	<1	+
	N. benthamiana	5-10	1-10	+

† Number of particles per field, seen with a JEOL 100 S electron microscope at x 50,000 magnification.

* + indicates that quite large quantities of host material were present in the sample.

samples by SDS-PAGE (section 2.7.4iii) as well as electron microscopy, and extracted the ssRNA and analysed it by MeHgOH-denaturing agarose gel electrophoresis (section 2.7.4iv). I repeated the protocol five times, with fairly consistent electron microscope and SDS-PAGE results. The final samples from the five experiments contained 5 to 10, 1 to 20, 1 to 10, 1 to 5 and 0 to 5 particles per field respectively, but also contained much host material. When aliquots of each sample were analysed by SDS-PAGE, they gave patterns very similar to each other and to healthy *N. benthamiana* proteins prepared in the same way. There was no obvious band in any of the virus preparations at the expected position of PMTV-T capsid protein (M_r 19,800; Randles *et al.*, 1976), that was not present in the healthy sample.

Single-stranded RNA extracted from the five virus preparations and analysed by electrophoresis in MeHgOH-denaturing gels varied slightly: the first, third and fifth preparations gave three bands and a blur starting just below the third band and continuing to the bottom of the gel. The three bands were subsequently sized at approximately 6.5, 3.2 and 2.5 kb (Figure 9) and are assumed to be PMTV-T genomic ssRNAs (named RNAs 1, 2 and 3, and discussed further in section 3.5). The second preparation consisted of RNA 1 and a bright blur starting at approximately the position of RNA 2 and continuing to the bottom of the gel. The fourth preparation consisted of just a blur, starting above the expected position of RNA 1 and continuing to the bottom of the gel. The blurs which were found in each of these samples are thought to consist of a mixture of host plant and PMTV-T ssRNAs.

The results of electron microscopy, SDS-PAGE and ssRNA analysis all show that these virus preparations were still heavily contaminated with host plant material. So, I added a second ultracentrifugation step with a 55 % (w/v) sucrose cushion, followed by a caesium chloride gradient step (as described in section 2.7.3), so that after the clarification step this protocol is identical to that described in the previous section. I tried this longer protocol twice with 100 g of systemically-infected *N. benthamiana* leaves, and on each occasion there was a white band in the caesium chloride gradient, which when harvested was found to have a density of 1.32-

1.34 g/ml. However, when analysed by electron microscopy, both preparations contained very few virus particles (less than one per field) plus some host material. SDS-PAGE analysis of both preparations revealed only a few bands and these co-migrated with bands in analogous extracts prepared from healthy *N. benthamiana* leaves. None of these bands migrated at a position expected for PMTV-T capsid protein and they were therefore not thought to be the capsid protein. Single-stranded RNA extracted from these samples gave no bands or even blurs after electrophoresis in a MeHgOH-denaturing agarose gel. It seems, therefore, that the band on the caesium chloride gradients contained mostly host proteins and only a few virus particles. It is unclear why so few particles were found in the band, as it was more intense than the corresponding bands found in CsCl gradients previously obtained (section 3.3.3).

These results suggest that the method involving clarification of the homogenate with organic solvents is no better at purifying PMTV-T than the methods which involve sedimenting the virus from the sap and eluting it from the pellet (sections 3.3.2 & 3.3.1). So other protocols were sought that might produce higher yields of PMTV-T more consistently. It seemed logical to try the protocols that are used routinely to purify other members or possible members of the furovirus group, such as PCV (Thouvenel *et al.*, 1978), BNYVV (Putz & Kuszala, 1978) and SBWMV (Shirako & Brakke, 1984a) (section 2.7.1), as the viruses are biophysically and morphologically similar to PMTV-T.

3.3.5 Purification by a Method which Involves Clarifying the Sap by Acidification

Initially, I tried a protocol based on that used by Thouvenel *et al.* (1978) to purify PCV, as described in section 2.7.4. In this protocol the homogenate (pH 8) was clarified by acidification to pH 4.2 for 30 minutes, in order to irreversibly precipitate any plant proteins. At this pH, the loss of PCV was shown to be negligible. The pH was then raised again to 8, before pelleting the precipitated proteins by low-speed centrifugation. The pH of the supernatant was adjusted to 6.4, and the virus precipitated with PEG 20,000 and NaCl,

and then further purified by differential centrifugation followed by caesium chloride gradient centrifugation.

I tried to purify PMTV-T twice by this method, from 100 g of infected *N. benthamiana*, but with limited success. On the first occasion, no virus particles could be detected by electron microscopy in the resuspended pellet from the high-speed centrifugation (0.5 ml), and consequently, no virus band in the gradient. On the second occasion, the resuspended pellet from the high-speed centrifugation was found to contain less than one virus particle per field, plus a lot of host material; the caesium chloride gradient had a faint band at a density of 1.34 g/ml, contained a similar number of particles, but less host material.

It is interesting to note that between the time of the last purification attempt described in the previous section and the time of the two purification attempts by the protocol described above (approximately three weeks), the systemic symptoms on *N. benthamiana* had become less pronounced (i.e. fainter chlorosis and less distortion of leaves) and the symptoms had taken longer to appear. At the time it was not known whether this was a temporary effect or not, but it was subsequently found that the day length and ambient temperature play an important role in the development of PMTV-T symptoms in *N. benthamiana*, such that, as the days grew longer and warmer, the symptoms became less pronounced (section 3.1). Therefore, when the two attempts at purifying PMTV-T by the method described above were made, the *in vivo* concentration of virus may have been significantly lower than previously and this might explain the very low yields of virus obtained by this method.

During the Summer infection in *N. benthamiana* was almost symptomless (section 3.1) and at this time many attempts were made at purifying PMTV-T, but the yields of virus were negligible. During September the symptoms again became more pronounced, and by the middle of October they were as severe as they had been up to the end of March.

At this point I tried the protocol described above again, with 100 g of PMTV-T-infected *N. benthamiana*. On the first occasion, electron microscopy of the resuspended high-speed pellet revealed 5

to 20 particles per field, plus a lot of host material; and there was a band on the caesium chloride gradient, which had a density of 1.33 g/ml. The band was collected, diluted 10-fold with 0.05 M borate buffer (pH 8.2) and centrifuged. The resulting pellet was resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 8.2) and was found by electron microscopy to contain 1 to 10 particles per field plus a small amount of host material. On the second occasion, the resuspended high-speed pellet contained less than one particle per field plus a lot of host material. When this was centrifuged in a caesium chloride gradient, there was a fairly thick white band which had a density of 1.33 g/ml. The band was collected, diluted 10 times with 0.05 M borate buffer (pH 8.2) and centrifuged. The resulting pellet was resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 8.2) and was found by electron microscopy to contain a lot of host material but no virus particles. When aliquots from each of the final samples were analysed by electrophoresis in an SDS-polyacrylamide gel, they both appeared to contain only host proteins.

From these results, it seems that the earlier assumption that this method was unsuccessful for PMTV-T because of the low *in vivo* virus levels, was not necessarily true. Even when using infected *N. benthamiana* leaves with excellent symptoms, the virus yields were poor. There is one property of PMTV-T that may explain these poor results. As mentioned earlier, PMTV-T can form aggregates with host cell components (Harrison & Jones, 1970). Therefore, if the extraction buffer fails to break up these aggregates, then the virus particles will be co-pelleted with the host proteins. Therefore, for this method to be successful for PMTV-T, the virus particles would have to be dissociated from all plant cell components before lowering the pH to 4.2.

3.3.6 Purification by a Method which Involves Clarifying the Sap with Carbon Tetrachloride and Concentrating the Virus by PEG Precipitation

As the protocol described in the previous section appeared to be no better than any of those already tried, I then tried a protocol

based on that used for purifying BNYVV (Putz & Kuszala, 1978; section 2.7.5). These authors tested many variables for each stage of the purification process, in their attempts to find the optimum method. They found that the extraction buffer which gave the highest yield of virus in the supernatant after a low-speed centrifugation of the leaf extract was 0.1 M tris-HCl (pH 9.0) containing no additives. However, as mentioned in section 3.3.4, the pulp left over after filtering the extract through muslin and the pellet from the low-speed spin were both found to contain significant quantities of virus. To avoid such losses, the leaf extract was clarified with carbon tetrachloride without straining through muslin. In this way, after the two phases were separated, most of the virus was in the aqueous phase. Then the virus was concentrated by two PEG precipitations, because ultracentrifugation, even through a sucrose cushion, formed pellets, from which it was difficult to solubilise the virus.

On the first occasion that I tried this protocol with 100 g of PMTV-T-infected *N. benthamiana* leaves, the final virus preparation (approximately 0.5 ml) contained 20 to 50 virus particles per field and almost no host material. This preparation was shown to be infectious because it induced symptoms on *N. benthamiana* when 50 µl was inoculated onto three expanding leaves. SDS-PAGE analysis of the preparation revealed only one polypeptide species, with an approximate molecular weight of 19,100 (Figure 8a; section 3.4). This is assumed to be PMTV-T capsid protein (M_r 19,800; Randles *et al.*, 1976). When ssRNA was extracted from this sample and analysed by MeHgOH agarose gel electrophoresis, there were three bands, corresponding to the PMTV-T genomic RNAs (6.5, 3.2 and 2.5 kb - Figure 9a), plus a blur starting just below the lowest band and continuing to the bottom of the gel.

Subsequent attempts to purify PMTV-T by this protocol, however, proved to be less successful. On each occasion, 100 g of systemically-infected *N. benthamiana* leaves were used as the starting material. The first such attempt yielded less than one particle per field in electron microscopy and almost no host material, and consequently, there were no bands when an aliquot of this sample was electrophoresed on an SDS-polyacrylamide gel. The next four attempts

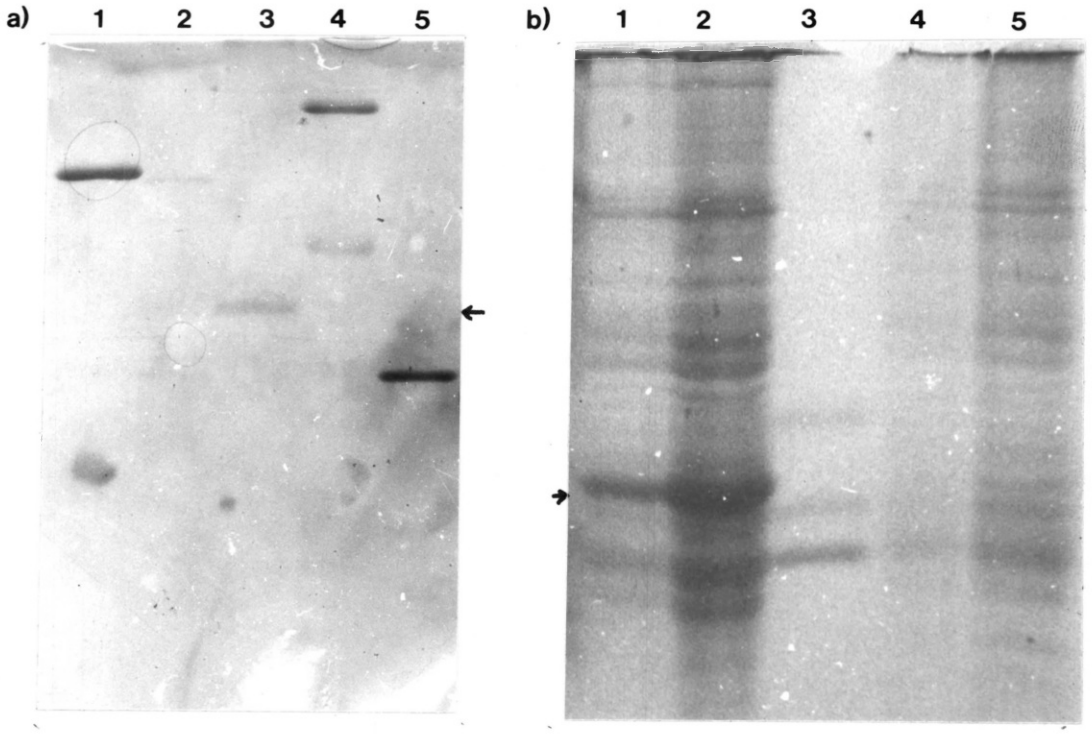


Figure 8 SDS-PAGE of Purified PMTV-T Capsid Protein

Electrophoresis of purified PMTV-T samples in a 15 % SDS-polyacrylamide gel, stained with Coomassie blue. The PMTV-T capsid protein is indicated.

a)

Lane 1 Lactate dehydrogenase (36.5 K).

Lane 2 DNase I (31 K).

Lane 3 PMTV-T purified by the method described in section 3.3.6.

Lane 4 Human γ-globulin (55.5 & 25.5 K).

Lane 5 Myoglobin (16.95 K).

b)

Lanes 1, 2, 4 & 5 PMTV-T purified by the method described in section 3.3.7.

Lanes 1 & 2 5 µl and 20 µl respectively of final supernatant.

Lanes 4 & 5 5 µl and 20 µl respectively of resuspended clarification pellet.

Lane 3 Trypsinogen (24 K); β-lactoglobulin (18.4 K); myoglobin (16.95 K).

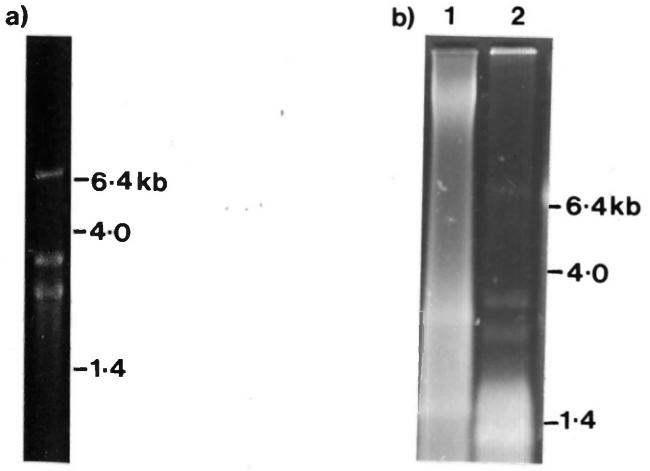


Figure 9 Analysis of PMTV-T Single-Stranded RNAs

Electrophoresis of ssRNA extracted from purified PMTV-T in a MeHgOH-denaturing 1 % agarose gel, stained with 0.5 µg/ml ethidium bromide. The positions of ssRNA size markers are indicated at the right-hand side of the gels: TMV ssRNA (6.395 kb); RCNMV ssRNA (4.0 & 1.4 kb).

a) ssRNA extracted from PMTV-T purified by the method described in section 3.3.6.

b) ssRNA extracted from PMTV-T purified by the method described in section 3.3.7:

Lane 1 ssRNA extracted from the resuspended clarification pellet.

Lane 2 ssRNA extracted from the final supernatant.

yielded similar numbers of particles (1 to 10 per field) and small amounts of host material. When analysed by SDS-PAGE, each of these preparations was found to contain PMTV-T capsid protein as the major protein, plus several other host proteins. Extraction and analysis of the ssRNA from each sample revealed only the two lower ssRNA bands plus the lower blur, as found in the earlier preparation, although the bands were significantly fainter and the blur was brighter.

Therefore, this protocol, as with all of the others tried up to this point, gives variable yields of PMTV-T, although it does seem to give cleaner preparations than the other protocols. On one occasion it gave the best preparation of PMTV-T ever obtained (i.e. the best yield of virus coupled with the lowest level of host material).

3.3.7 Purification by a Method which Involves Extracting the Sap in a High pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion

Due to the variable results gained using the protocol described in the previous section, I tried a protocol based on that used for purifying SBWMV (Shirako & Brakke, 1984a) (section 2.7.6). This protocol involved homogenising 100 g of infected leaf material in 200 ml of 0.5 M borate buffer (pH 9.0) plus 1 mM EDTA, filtering the homogenate through muslin and centrifuging the filtrate at low speed. After the addition of Triton X-100 to 2 % (v/v), the supernatant was treated to two cycles of differential centrifugation involving 20 % (w/v) sucrose cushions and the final pellet was resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 8.0) plus 1 mM EDTA. I tried this protocol six times with PMTV-T-infected *N. benthamiana* leaves, with variable results. The first two attempts yielded very little virus (less than one particle per field in electron microscopy) and a lot of host material. Electrophoresis of RNA extracted from these preparations in a MeHgOH agarose gel revealed a blur from above the position of RNA 1 to the bottom of the gel and this was thought to be mainly plant RNA, thus confirming the electron microscopy results.

The next three attempts were all relatively successful. The first of these yielded 10 to 20 particles per field in electron microscopy, plus some host material in the final clarification supernatant. The final clarification pellet was resuspended in approximately 0.5 ml of 0.05 M borate buffer (pH 8.0) plus 1 mM EDTA and was found to contain 1 to 10 particles per field and a lot of host material. SDS-PAGE analysis of these two fractions revealed many host proteins plus the PMTV-T capsid protein as a relatively major protein (Figure 8b). The resuspended clarification pellet contained significantly more host proteins plus the capsid protein as a minor protein (Figure 8b).

Single-stranded RNA was extracted from each fraction and analysed by electrophoresis in a MeHgOH-denaturing agarose gel (Figure 9b). The clarification supernatant contained PMTV-T RNAs 1, 2 and 3 plus a low molecular weight blur starting below RNA 3. However, the clarification pellet contained just a blur running the whole length of the gel.

It appears from these results that the final high-speed pellet contains aggregated virus, which is difficult to resuspend. A similar result was reported for BNYVV by Putz & Kuszala (1978): when crude BNYVV preparations were ultracentrifuged, the virus was very difficult to resuspend from the resulting pellets.

The next two attempts at purifying PMTV-T by this method resulted in fairly good yields of virus (5 to 10 particles per field in electron microscopy) in the final preparations, and slightly smaller yields (1 to 5 particles per field) in the resuspended clarification pellet. In each case, the virus was present mainly in aggregates and there was also quite a lot of host material present; more so in the resuspended clarification pellets. SDS-PAGE analysis of each sample revealed fewer host proteins than expected from the electron microscopy results. The PMTV-T capsid protein was a fairly minor protein and it was present in roughly equal amounts in each sample.

The next attempt using this protocol yielded very little virus (less than one particle per field in electron microscopy) plus a lot of host material in the final preparation. The resuspended clarification pellet appeared to contain host material only. SDS-PAGE analysis confirmed the presence of large quantities of host

proteins, such that any PMTV-T capsid protein present might not have been observed.

This protocol, when applied to the purification of PMTV-T from systemically-infected *N. benthamiana*, has been shown to give results as variable and unpredictable as any method tried previously. It has, on occasion, produced quite good yields of virus, although with more host material present than when using the method described in the previous section. Furthermore, when the virus was pelleted through a sucrose cushion it was difficult to resuspend completely, probably due to the formation of aggregates. The three viral ssRNAs were extracted intact from the better virus preparations, although a low molecular weight blur was also present, which probably consisted of a mixture of partially degraded plant and viral ssRNA.

3.3.8 Purification by a Method which Involves Extracting the Sap in a Neutral pH Buffer, Clarifying it with Triton X-100 and Concentrating the Virus by Ultracentrifugation through a Sucrose Cushion

As the method described in the previous section produced results as unpredictable as those of all other methods tried previously, a protocol based on that used to purify the geminivirus TGMV was tried (Stein *et al.* 1983; section 5.5.4), for purifying PMTV-T from 100 g of systemically-infected *N. benthamiana*. This protocol involved homogenising infected leaves in neutral citrate buffer containing sodium sulphite, EDTA, 2-mercaptoethanol and ascorbic acid (CEM buffer), stirring with 2.5 % (v/v) Triton X-100 overnight, filtering and spinning at low speed. The resulting supernatant was then subjected to two cycles of differential centrifugation, involving a 20 % (w/v) sucrose cushion for the second high-speed spin. The resulting pellet was resuspended in approximately 0.5 ml of CEM, the suspension was clarified and stored at 4 °C. The clarification pellet was also resuspended in 0.5 ml of CEM buffer and stored at 4 °C.

I tried to purify PMTV-T by this method, on five separate occasions, but with limited success. On each occasion the final

preparation contained large quantities of host material, which tended to obscure the virus particles when preparations were viewed by electron microscopy (an electron micrograph of one of these samples is shown in Figure 10). Therefore, it was difficult to estimate the concentration of virus particles in each preparation. However, the number of particles varied quite considerably from preparation to preparation: the first preparation appeared to contain no virus particles at all by electron microscopy; the second and third preparations appeared to contain more than 10 particles per field; the fourth preparation, less than one particle per field; and the fifth preparation, more than one particle per field. For each preparation, the final clarification pellet was resuspended and analysed by electron microscopy and, as with the virus samples prepared by the method described in the previous section, some of these also contained virus particles i.e. the second and third preparations (less than one particle per field). However, the major constituent of these samples was host material.

SDS-PAGE analysis of each sample obtained by this method confirmed the presence of large quantities of host material and relatively low concentration of virus; there were so many proteins present in each sample that when an aliquot was electrophoresed on an SDS-polyacrylamide gel, there was an almost continuous streak running down the gel.

When ssRNA extracted from each sample was analysed by electrophoresis on a MeHgOH-denaturing agarose gel, in all but two cases there was a blur starting above the expected position of RNA 2 and continuing to the bottom of the gel, but no RNA 1 band was visible. The two exceptions were the final samples from the second and third preparations, which contained RNAs 1 and 2 and a low molecular weight blur starting at the approximate position of RNA 3.

The method described in this section appears to be no better than the other methods tried, and inferior to those methods described in the previous two sections.

3.3.9 Analysis of Extraction Buffers

The protocols tried up to this point all gave inconsistent yields

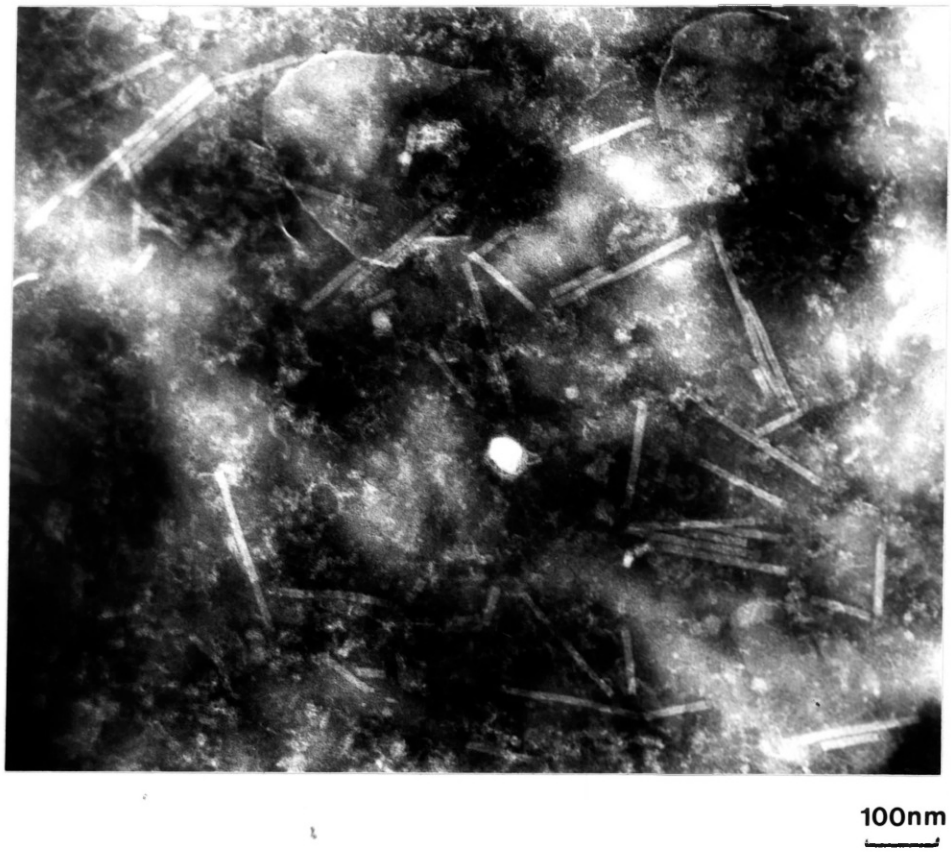


Figure 10 Electron Micrograph of PMTV-T Particles

The PMTV-T particles were purified by the method described in section 3.3.8, and negatively stained with 2 % potassium phosphotungstate (pH 6.8). The final magnification is x 90,000.

of PMTV-T, such that one week they might give a relatively good yield and the next hardly any virus at all. This was despite the fact that when a protocol was repeated, every attempt was made to eliminate any variability in the procedure.

This inconsistency means that the results gained when using each protocol cannot be considered as definitive, even though I tried to eliminate freak results by trying each protocol at least three times. Therefore, no one protocol can definitely be said to be better than any other at purifying PMTV-T, although the protocols described in sections 3.3.6 and 3.3.7 were, on occasion, relatively successful.

As mentioned earlier, Putz & Kuszala (1978) had difficulties purifying BNYVV and these difficulties were similar to those that I have encountered with PMTV-T (e.g. most of the virus content of the leaf extract being pelleted during low-speed centrifugation). Putz & Kuszala (1978) formulated a purification protocol by painstakingly testing many variables for each stage of the purification process, from the type of extraction buffer to the use of density gradients.

Similar tests were made with PMTV-T. Initially, I sought to determine the effects of the various components of the extraction buffers commonly used in virus purification (section 2.7.7). In this way it was hoped to find a buffer or buffers that would release PMTV-T from the aggregates in which the virus is found *in vivo*, without fragmenting the virus particles.

In the first part of the experiment 16 x 10 g samples of systemically-infected *N. benthamiana* leaves were homogenised in 16 extraction buffers, differing in molarity, pH and actual buffer (section 2.7.7i) and filtered through muslin. The filtrate was centrifuged briefly at low speed, the supernatant was collected and the pellet was resuspended in the same extraction buffer. These two fractions (supernatant and pellet) were tested for infectivity by back-testing onto a local lesion assay host - *N. debneyi* (section 2.8.2). At the time of the experiment (Winter/early Spring), the symptoms induced on the inoculated leaves of *N. debneyi* were necrotic spots surrounded by one or more concentric, necrotic rings. The number of necrotic spots on the three inoculated leaves of one *N. debneyi* plant, 6-10 days after inoculation, was taken as a direct measure of the infectious virus content of each sample being tested.

The total number of lesions from the supernatant and pellet fractions of each buffer gives an indication of how stable PMTV-T is in that buffer, and the ratio of the number of lesions in the supernatant and pellet fractions gives an indication of the ability of the extraction buffer to break up the virus aggregates - the more efficient the extraction buffer is at breaking up the aggregates, the more virus should be in the supernatant fraction as opposed to the pellet fraction.

This part of the experiment was repeated once, and the results are shown in Table 7a. There is substantial variation between the two attempts, although it seems that PMTV-T is most stable *in vitro* in 0.5 M tris-HCl (pH 8.0) and 0.1 M borate buffer (pH 8.0). Neither of these buffers appears to have broken up all of the virus aggregates, as the pellet fractions contained substantial amounts of infectious virus.

From these results it was decided to test 0.5 M tris-HCl (pH 8.0) and 0.1 M borate buffer (pH 8.0) further, by using them in conjunction with various additives commonly used in virus purification: urea, which prevents or reverses virus aggregation; EDTA, which chelates Ca^{2+} and Mg^{2+} ions; DIECA, which chelates copper ions; sodium sulphite, thioglycollic acid and 2-mercaptoethanol, which are reducing agents; and PMSF, which is a protease inhibitor.

First of all, 0.5 M tris-HCl (pH 8.0) was tested, alone or with one of the above additives, and the supernatant and pellet fractions from the low speed spin of the filtrate were back-tested onto *N. debneyi*. In the case of 0.5 M tris-HCl (pH 8.0) on its own, one other fraction was also tested - the pulp left over after the homogenate was filtered. This pulp was resuspended in 0.5 M tris-HCl (pH 8.0) and back-tested. The numbers of lesions produced by these various fractions are shown in Table 7b. Again there is a great variability among them, so no definite conclusions can be made. However, it does appear that a large proportion of the virus particles in the *N. benthamiana* leaves are not being extracted, as can be seen by the infectivity present in the pulp fraction.

Thus, a modified version of the above experiment was tried (section 2.7.7iii). Each 10 g sample of infected leaf material was homogenised in half the volume of extraction buffer (25 ml),

Table 7 Effects of Extraction Buffer Content on PMTV-T Purification

a) Extraction Buffers Without Additives

Extraction Buffer	Number of Local Lesions on *N. debneyi* after 6-10 Days			
	Supernatant		Pellet	
0.1 M citrate, pH 6.0	0	3	0	1
0.1 M phosphate, pH6.0	1	0	0	5
0.02 M phosphate, pH 7.2	0	0	1	0
0.1 M phosphate, pH 7.2	1	10	7	0
0.5 M phosphate, pH 7.2	0	12	5	7
0.1 M tris-HCl, pH 7.2	5	15	20	14
0.02 M tris-HCl, pH 8.0	1	3	0	4
0.1 M tris-HCl, pH 8.0	1	11	1	4
0.5 M tris-HCl, pH 8.0	60	32	40	45
0.1 M borate, pH 8.0	45	22	13	20
0.02 M borate, pH 9.0	2	6	3	0
0.1 M borate, pH 9.0	0	14	2	20
0.5 M borate, pH 9.0	2	11	13	1
0.1 M glycine, pH 9.0	0	5	1	5
0.1 M glycine, pH 9.4	0	8	0	0
0.1 M borax, pH 9.4	0	0	1	4

b) 0.5 M Tris-HCl (pH 8.0) plus Additives

Extraction Buffer - 0.5 M tris-HCl, pH 8.0 + :	Number of Local Lesions on *N. debneyi* after 6-10 Days		
	Supernatant	Pellet	Pulp
Control - buffer only	2	35	60
1 M urea	3	6	
1 mM EDTA	6	43	
10 mM EDTA	0	0	
10 mM DIECA	2	10	
0.5 % sodium sulphite	0	0	
0.05 % thioglycollic acid	0	2	
0.5 % 2-mercaptoethanol	0	5	
0.25 % L-ascorbic acid	3	0	
1 mM PMSF	0	10	

c) 0.5 M Tris-HCl (pH 8.0) plus Additives; Pulp Re-extracted

Extraction Buffer - 0.5 M tris-HCl, pH 8.0 + :	Number of Local Lesions on *N. debneyi* after 6-10 Days					
	Supernatant		Pellet		Pulp	
Control - buffer only	70	11	40	25	50	7
1 M urea	30	7	60	8		
1 mM EDTA	110	9	110	3	38	4
10 mM EDTA	23	12	30	3		
10 mM DIECA	18	17	20	12		
0.5 % sodium sulphite	22	0	40	4		
0.05 % thioglycollic acid	5	14	1	5		
0.5 % 2-mercaptoethanol	6	12	10	7		
0.25 % L-ascorbic acid	16	0	12	2		
1 mM PMSF	15	5	35	0		

d) 0.1 M Borate Buffer (pH 8.0) plus Additives; Pulp Re-extracted

Extraction Buffer - 0.1 M borate, pH 8.0 + :	Number of Local Lesions on *N. debneyi* after 6-10 Days					
	Supernatant		Pellet		Pulp	
Control - buffer only	30	12	2	13	10	3
1 M urea	30	30	15	3		
1 mM EDTA	6	12	6	7	6	3
10 mM EDTA	18	12	0	2	0	2
10 mM DIECA	25	14	35	0		
0.5 % sodium sulphite	40	26	40	4		
0.05 % thioglycollic acid	30	10	15	0		
0.5 % 2-mercaptoethanol	32	11	10	0		
0.25 % L-ascorbic acid	0	14	5	5		
1 mM PMSF	7	8	1	20		

containing the same additives as before, and filtered through muslin. The filtrate was collected and the pulp was re-extracted in another 25 ml of extraction buffer and re-filtered. The filtrates were pooled and centrifuged as before. In the case of the 0.5 M tris-HCl (pH 8.0), on its own and with 1 mM EDTA, the pulp was re-extracted again and back-tested along with all of the other fractions.

This part of the experiment was repeated once with 0.5 M tris-HCl (pH 8.0) and twice with 0.1 M borate (pH 8.0); the results are shown in Tables 7c and 7d. The inconsistency of the results between each attempt with 0.5 M tris-HCl (pH 8.0) and between each attempt with 0.1 M borate (pH 8.0) are such that it is hard to say whether or not re-extracting the pulp had released any more virus particles. Moreover, due to the great variability, no additive can be reliably concluded to be better than any other.

This variability, which was consistent throughout, led to the experiment being abandoned at this point, with no clearer view on the effects of the various extraction buffers on PMTV-T purification. However, the inconsistent effect of the various buffers does support the results of the earlier attempts to purify PMTV-T by various methods, as mentioned above.

PMTV-T aggregates *in vivo* with itself and host components (Harrison & Jones, 1970). Therefore, to purify the virus successfully, the aggregates must be dissociated without causing fragmentation of the labile virus particles. All attempts to do so have so far failed to consistently produce significant quantities of pure PMTV-T. Most of the purification protocols have, on occasion, produced reasonable quantities of pure virus, and from these preparations it was possible to partially characterise PMTV-T, as will be described in subsequent sections. However, purifying the virus reliably still remains a major problem if PMTV-T is to be fully characterised.

3.4 Capsid Protein Molecular Weight Determination

The best preparation of PMTV-T, obtained using the purification protocol described in section 3.3.6, contained a high yield of virus almost totally free of host material. This preparation was shown by

SDS-PAGE to contain only one polypeptide species - the viral capsid protein - the molecular weight of which was estimated by comparing its relative mobility when electrophoresed in SDS-polyacrylamide gels (section 2.5.4ii; Figure 8a), with that of known molecular weight markers,

However, in order to determine whether the protein markers and the virus capsid protein were behaving anomalously on SDS-polyacrylamide gels, their electrophoretic mobility was investigated in different gel concentrations. The relative migration (R_f) of each protein, compared to a standard protein (myoglobin), was analysed using the Ferguson Plot (Ferguson, 1964) for each protein (Figure 11). When the lines were extrapolated to zero polyacrylamide concentration (free solution), protein markers and capsid protein extrapolated to the same migration rate, indicating that all the SDS-protein complexes had identical charge densities and none of the proteins had behaved anomalously.

The slope of the Ferguson Plot is a function of the protein size, regardless of its charge, and has been called the retardation coefficient, K_R (Rodbard & Chrambach, 1970). By plotting the K_R values of markers against $\log_{10} M_r$, the K_R value for the capsid protein was used to determine its molecular weight (Figure 12) as 19.1 K.

This value is similar to that (19.8 K) estimated by Randles *et al.* (1976) for PMTV-T capsid protein. Moreover, it is comparable to the capsid protein molecular weights (19.7 to 24.5 K) of the other furoviruses (Table 3).

3.5 Viral Single-Stranded RNA

Single-stranded RNA was isolated from virus preparations (section 2.9.1) and analysed by MeHgOH-denaturing agarose gel electrophoresis (section 2.5.2ii). Those preparations that had little or no host contamination (as judged by electron microscopy and SDS-PAGE analysis) contained three species of ssRNA (Figure 9), which when co-electrophoresed with tobacco mosaic virus and red clover necrotic mosaic virus ssRNAs, were estimated to have sizes of 6.5, 3.2 and 2.5 kb. These three ssRNAs are assumed to be PMTV-T-specific and have

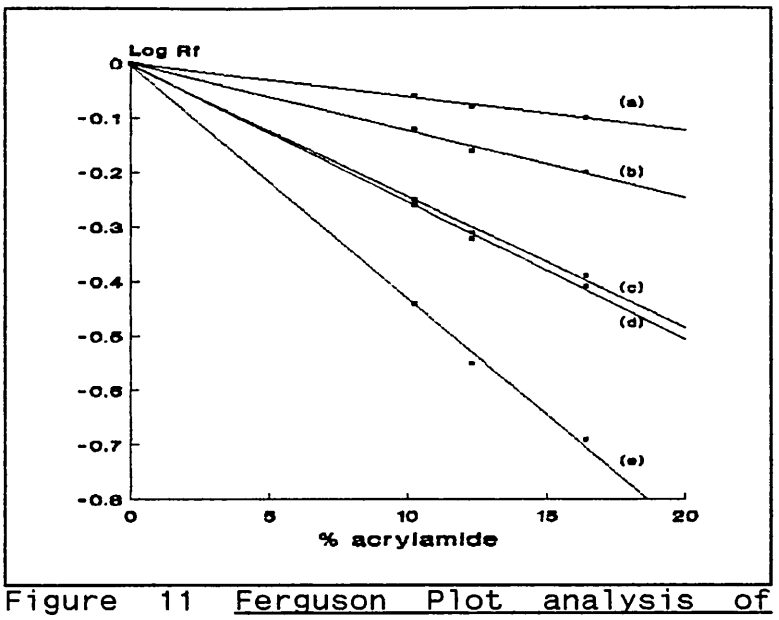


Figure 11 Ferguson Plot analysis of polypeptides

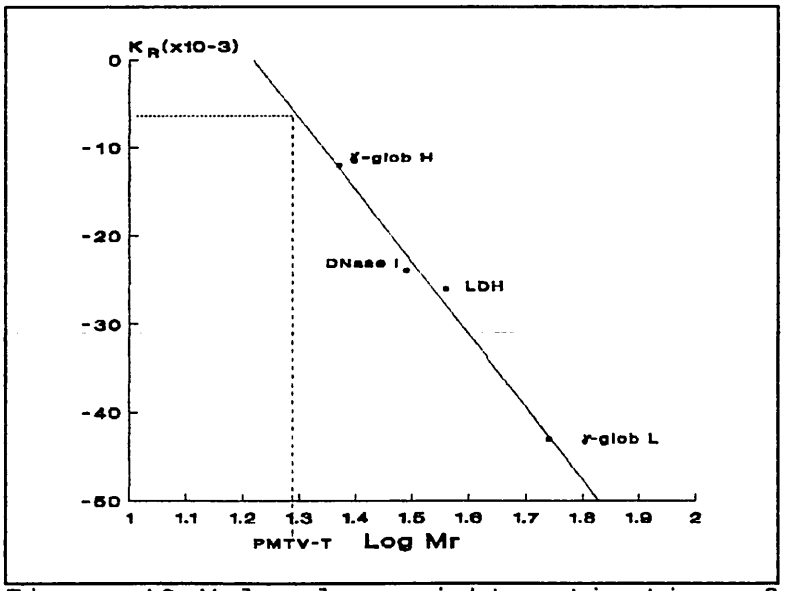


Figure 12 Molecular weight estimation of PMTV-T capsid protein

Notes to Fig. 11 and 12

(a) PMTV-T capsid protein
(b) γ-globulin H (25.5K)
(c) DNase I (31K)
(d) Lactate dehydrogenase (36.5K)
(e) γ-globulin L (55.5K)

been named RNAs 1, 2 and 3, respectively, although it is not known whether they are all genomic RNAs. Interestingly, PMTV-T has only two particle modal lengths (Kassanis *et al.*, 1972; Roberts & Harrison, 1979), which suggests that it has a bipartite, rather than a tripartite, genome. However, the length distribution of particles, as determined by immunosorbent electron microscopy (Roberts & Harrison, 1979), shows a fairly broad peak at 125 nm. The majority of particles in this peak actually range in size from about 105 to 140 nm. So, if the 3.2 and 2.5 kb ssRNAs were encapsidated separately, the corresponding particles would be of very similar size and could not therefore be easily distinguished. Furthermore, there are approximately twice as many particles in the 125 nm peak as there are in the 290 nm peak, adding support to the possibility that the 3.2 and 2.5 kb ssRNAs are encapsidated in particles with very similar modal lengths.

All three of the definitive furoviruses (PCV, SBWMV and SCSV) whose ssRNA genomes have been studied extensively have bipartite genomes (Table 3), with the two RNA components in each case being of comparable molecular weight to PMTV-T RNAs 1 and 2. In the case of SBWMV, however, some isolates also contain a third smaller ssRNA component (2.45 or 2.8 kb), which has been shown to be a deleted form of RNA 2 (Shirako & Brakke, 1984a, b; Hsu & Brakke, 1985b; section 1.7ii). This deleted form of SBWMV RNA 2 only occurs in virus cultures propagated under unnatural conditions (i.e. mechanical propagation), such that the fungal vector is not required for transmission (section 1.7). This has led to the suggestion that SBWMV RNA 2 encodes a protein required for fungal transmission, and thus, when the virus is mechanically propagated this protein is not required, and therefore this RNA can undergo deletion without affecting the viability of the virus.

Interestingly, the three PMTV-T ssRNA components mentioned above were isolated from mechanically-propagated virus; it is possible, therefore, that under natural conditions of fungal transmission of PMTV-T in potato crops, the virus may contain only RNAs 1 and 2, but when mechanically propagated in *N. benthamiana* or *N. debneyi*, RNA 2 may undergo deletion forming RNA 3, without affecting the viability of the virus. PMTV-T RNA 3 is of a similar size to the deleted forms

of SBWMV RNA 2, so it is possible that PMTV-T, like these SBWMV isolates, has a bipartite genome, with RNA 3 being a deleted form of RNA 2.

To investigate this theory, the relationship between PMTV-T RNAs 2 and 3 was studied by Northern blot hybridisation (section 2.9.3). A sample of PMTV-T ssRNA was electrophoresed on a MeHgOH-denaturing LMP-agarose gel (section 2.5.2iii) and the RNA 3 band was excised and the RNA recovered (section 2.5.3). The integrity of the RNA was confirmed by electrophoresis in a MeHgOH-denaturing agarose gel (Figure 13). An aliquot of this purified RNA 3 was used to make a cDNA probe (section 2.9.2), which was then used to probe PMTV-T ssRNA and healthy *N. benthamiana* RNA (section 2.8.3). The RNA 3-specific probe hybridised with RNA 3, as expected, but not with RNAs 1 or 2 (Figure 14), results which show that RNA 3 is not a deleted form of RNA 2, or even RNA 1. Also, the RNA 3-specific probe did not hybridise with any of the healthy *N. benthamiana* RNAs.

This result disproves the theory that the PMTV-T genome is analogous to the SBWMV genome, as discussed above. It is possible that PMTV-T actually has a tripartite genome , thus setting it apart from the other definitive furoviruses, which have bipartite genomes - PCV, SBWMV and SCSV. Another possibility is that the smallest PMTV-T RNA could be a satellite. Although no other examples of satellite RNA have been reported for the furoviruses, there are several other RNA plant viruses with well-known satellites, such as cucumber mosaic virus and tobacco ringspot virus (Baulcombe, 1989). Obviously, further work is required to fully characterise the genome of PMTV-T.

3.6 Viral Double-Stranded RNA

Double-stranded RNA isolated from systemically-infected *N. benthamiana* or locally-infected *N. debneyi* leaves by the method of Taylor and Powell (1982) (section 2.9.4) was analysed by co-electrophoresis in a 5 % polyacrylamide gel with molecular weight markers (*Aspergillus foetidus* and *Penicillium stoloniferum* dsRNAs). Three dsRNA species were found of approximately 6.3, 3.2 and 2.3 kb (Figure 15), and these are probably the replicative forms of the three ssRNA species (6.5, 3.2 and 2.5 kb) isolated from purified

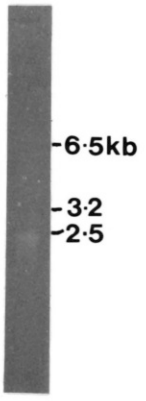


Figure 13 Purified PMTV-T RNA 3

Electrophoresis of purified PMTV-T RNA 3 (section 3.5) in a MeHgOH-denaturing 1 % agarose gel, stained with 0.5 µg/ml ethidium bromide. The positions of ssRNA size markers are shown at the right-hand side of the gel.

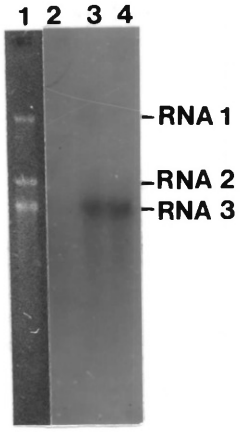


Figure 14 Total PMTV-T RNA Probed with RNA 3-Specific Probe

Electrophoresis in a MeHgOH-denaturing 1 % agarose gel, and either stained with 0.5 µg/ml ethidium bromide (lane 1) or transferred to Genescreen membrane and probed with a RNA 3-specific probe (Lanes 2-4):

Lanes 1 & 3 Total PMTV-T RNA.
Lane 2 Total healthy *N. benthamiana* RNA.
Lane 4 Purified PMTV-T RNA 3.

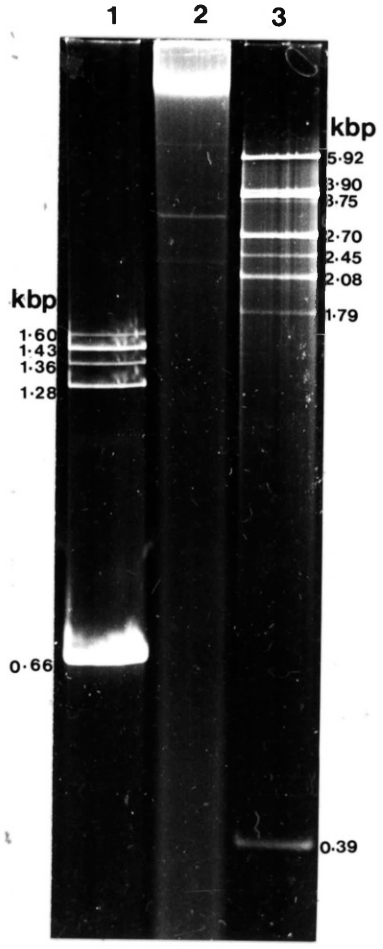


Figure 15 Analysis of PMTV-T Double-Stranded RNA

Electrophoresis of PMTV-T dsRNA in a 5 % polyacrylamide gel, stained with 0.5 µg/ml ethidium bromide.

Lane 1 *Penicillium stoloniferum* virus dsRNA.

Lane 2 PMTV-T dsRNA.

Lane 3 *Aspergillus foetidus* virus dsRNA.

virus preparations (section 3.5). This conforms with the general picture for ssRNA plant viruses; i.e. the dsRNA species isolated from infected tissue correspond in size and number to the genomic and sub-genomic ssRNA species isolated from purified virus particles.

The PMTV-T dsRNAs differ from the ssRNAs with respect to the relative quantities of each RNA component. The three ssRNAs were found in roughly equal proportions (Figure 9), whereas the 3.2 kb dsRNA (RNA 2) was present in much larger quantities than the other two dsRNA components (Figure 15). The reason for this disparity is not known.

No comparative studies have been reported between the ss- and dsRNAs of other furoviruses. However, viral dsRNA has been isolated from BSBV-infected *C. quinoa* plants, due to the difficulty in purifying the virus and, hence the ssRNA. Three dsRNA components have been found, with molecular weights of approximately 6.4, 2.9 and 2.5 kb, which are similar in size to the three PMTV-T dsRNAs (C.M. Henry, personal communication).

3.7 Taxonomy of PMTV

Originally, PMTV was thought to be a possible member of the tobamovirus group, because of its morphological similarity to, and slight serological relationship with TMV (Kassanis *et al.*, 1972), but more recently it has been placed in a new group - the furoviruses (Shirako & Brakke, 1984a; Brown, 1989). The viruses in this group differ from tobamoviruses by their relative instability *in vitro*, their occurrence in infected plants in low concentrations, their particles of two or more modal lengths (Table 3) and their transmission by plasmodiophorid fungi (Table 2). PMTV shares all of these properties with the other furoviruses, and also its capsid protein is of a similar molecular weight to that of the other members (section 3.4; Table 3).

Three of the definitive furoviruses - PCV, SBWMV and SCSV - have been studied in detail at the genome level, and this has confirmed that they should be included in the same group. The main features of the furovirus genome are as follows: a bipartite ssRNA genome (Table 3); RNA 1 (5.9 to 6.9 kb) encoding a single long polypeptide

representing 80-90 % of the coding capacity of the molecule (Mayo & Reddy, 1985; Hsu & Brakke, 1985a; Kendall *et al.*, 1988); and RNA 2 (3.5 to 4.3 kb) containing the capsid protein gene at the 5' end, which can undergo translational readthrough to produce larger polypeptides (Figure 4). Some isolates of PCV and SBWMV were found to contain a third RNA species (Mayo & Reddy, 1985; Shirako & Brakke, 1984a, b), smaller than the other two, which, in the case of SBWMV, has been shown to be a deletion of RNA 2 (Shirako & Brakke, 1984a, b). No such molecule has been found associated with SCSV (Kendall *et al.*, 1988).

One of the possible members of the furovirus group - BNYVV - has been studied extensively at the genome level and this has revealed several significant differences from the genomes of the definitive furoviruses (discussed in section 1.8): The BNYVV genome consists of four ssRNA components with 5' caps and 3' poly A tails (Table 2). The exact taxonomic status of BNYVV is uncertain. It is more similar to the furoviruses than the other rod-shaped virus groups, but it is too different from PCV, SBWMV and SCSV to be considered a definitive furovirus. It could possibly form a sub-group of the furoviruses on its own, or even a new group.

Nothing has been reported previously about the genome of PMTV. In this thesis I have shown that PMTV-T isolated from *N. benthamiana* or *N. debneyi* contains three ssRNA components: RNAs 1 and 2 correspond to the two particle modal lengths of PMTV-T (Roberts & Harrison, 1979), and are of similar sizes to RNAs 1 and 2 of PCV, SBWMV and SCSV (Table 3); and RNA 3 is of similar size to the smaller RNA species found in some isolates of PCV and SBWMV although, unlike SBWMV, PMTV-T RNA 3 is not related to RNA 2.

So, the results presented in this thesis on the genome of PMTV seem to confirm that PMTV should be included with PCV, SBWMV and SCSV in the furovirus group, although this needs to be confirmed by other investigations.

3.8 Future Work on PMTV

The main difficulty with working on PMTV-T was the inability to purify the virus consistently. Consequently, it was not possible to fully characterise the virus. As a pre-requisite for such investigations a reliable purification protocol is required.

The Todd isolate of PMTV (PMTV-T) is not necessarily the easiest isolate of PMTV to purify. It was selected over other isolates solely on the basis of the severity of the symptoms it induced in *N. benthamiana* and *N. debneyi*. However, symptom severity is not always correlated with ease of purification (discussed in section 3.1). So, other isolates of PMTV could be tested to see if any are more readily purified than PMTV-T.

Once ssRNA has been extracted from purified virus, it can be used to make full-length dscDNA, which can be cloned into a suitable plasmid vector and sequenced. The nucleotide sequence will reveal potential open reading frames and will show any homology between the three RNAs. Comparisons could then made with the nucleotide sequence of BNYVV RNAs.

The full-length dscDNA clones of PMTV-T RNA could be transcribed *in vitro* and the resulting transcripts compared with the three PMTV-T ssRNAs. These transcripts could then be translated *in vitro* in wheat germ and rabbit reticulocyte lysates, to see which ORFs are expressed *in vitro*, and to what extent. This would allow direct comparison with the *in vitro* translation products of BNYVV, PCV, SBWMV and SCSV, especially the long polypeptide expressed from RNA 1 and the capsid protein expressed from the 5' end of RNA 2, plus the translational readthrough products of the capsid protein gene.

Infectivity studies could be carried out with various combinations of the three transcripts: i) on whole plants, to ascertain what the infectious unit is for PMTV-T, and ii) on protoplasts, to determine whether RNA 1 can replicate autonomously, as has been suggested for BNYVV and SBWMV, thus implicating one or more of its gene products in replication.

As mentioned in section 3.6, three dsRNAs have been isolated from PMTV-T-infected plants, with sizes relatively similar to the ssRNAs. However, it is not known for sure that these dsRNAs are the

replicative forms of the ssRNAs. So, to confirm or deny this, the dsRNAs could be electrophoresed on a gel, blotted onto GeneScreen and probed with cDNA probes specific to each of the ssRNAs, individually or together.

TOMATO GOLDEN MOSAIC VIRUS

4. INTRODUCTION

4.1 Introduction to the Geminiviruses

The Empress Koken made what was possibly the first reference in the literature to a geminivirus in AD 752, when she described the symptoms elicited by what is now known as Eupatorium yellow vein virus in *Eupatorium chinense*, in a Japanese anthology called "Manyõshũ" (Inouye & Osaki, 1980). Needless to say she didn't know that these were symptoms of a virus infection, and similarly the symptoms of other geminiviruses have been reported right up to the present day without the causal agent being known. It has only been in the last 15 years that the causal agent in each case has been identified as a geminivirus. For example, the disease caused by maize streak virus has been known in tropical Africa since the last century (Fuller, 1901), but it was characterised only recently (Bock *et al.*, 1974).

Since then our knowledge of geminiviruses has increased greatly and some of the major breakthroughs are listed below. In 1974 it was shown that the virus particles had a characteristic geminate morphology (Bock *et al.*, 1974). Goodman (1977a) was the first to show that the geminivirus genome consisted of single-stranded DNA (ssDNA) molecules and Harrison *et al.* (1977) showed that they were circular in form. Bisaro *et al.* (1982) were the first to obtain DNA clones of a geminivirus - TGMV - and a year later Hamilton *et al.* (1983) and Stanley (1983) demonstrated a bipartite genome for TGMV and ACMV respectively, by infection with cloned DNA components. In 1983 Stanley and Gay were the first to publish the complete nucleotide sequence of a geminivirus - that of African cassava mosaic virus - and most recently Hayes *et al.* (1988e) have constructed a vector from the tomato golden mosaic virus genome which is being used to study gene expression in plants. Many other aspects of geminiviruses have been studied and these have been frequently reviewed, with their host range, epidemiology, insect transmission and symptomatology being well documented (Goodman, 1981; Bock, 1982;

Harrison, 1985; Stanley & Davies, 1985; Stanley, 1985; Francki *et al.*, 1985; Lazarowitz, 1987 and Davies *et al.*, 1987).

4.2 Members of the Geminivirus Group

The geminiviruses were first defined as a distinct group by the International Committee on the Taxonomy of Viruses (Matthews, 1979). Definitive members comprise small, quasi-isometric particles found predominantly in pairs and containing circular ssDNA. So far fifteen viruses are known to satisfy these criteria: abutilon mosaic virus (AbMV) (Jeske, 1986); African cassava mosaic virus (ACMV - synonym cassava latent virus CLV) (Harrison *et al.*, 1977); beet curly top virus (BCTV) (Thomas & Mink, 1979); bean golden mosaic virus (BGMV) (Reisman *et al.*, 1979); chloris striate mosaic virus (CSMV) (Francki *et al.*, 1979); digitaria streak virus (DSV); Dollet *et al.*, 1987); euphorbia mosaic virus (EMV) (Jaramillo & Lastra, 1986); miscanthus streak virus (MisSV) (Yamashita *et al.*, 1985); maize streak virus (MSV) (Bock *et al.*, 1977); mungbean yellow mosaic virus (MYMV) (Honda *et al.*, 1983); potato yellow mosaic virus (PYMV) (Roberts *et al.*, 1988); squash leaf curl virus (SqLCV) (Cohen *et al.*, 1983); tobacco yellow dwarf virus (TYDV) (Thomas & Bowyer, 1980) tomato golden mosaic virus (TGMV) (Hamilton *et al.*, 1981) and wheat dwarf virus (WDV) (Lindsten *et al.*, 1980). Of these fifteen only ten satisfy the more stringent criteria of possessing a geminate particle morphology, a genome of one or two circular ssDNA molecules (2.5 - 3.0 kb) and a single capsid polypeptide species (M_r 27,000 - 32,000) as laid down by the International Committee on the Taxonomy of Viruses in 1987 and these are AbMV, ACMV, BCTV, BGMV, CSMV, EMV, MSV, PYMV, TGMV and WDV.

There are several provisional members of the geminivirus group (see Table 8) as determined either by their geminate particles, cytopathology or serological relationship to a definitive geminivirus. It is likely that some of these and some of the definitive members are strains of other viruses, as is the case for DSV, which is thought to be a strain of MSV (Dollet *et al.*, 1986).

Table 8 Members of the Geminivirus Group

Virus Name	Abbreviation	Geminate Morphology	ssDNA Shown	Genome M_r ($\times 10^6$)	Nucs.	Coat Protein	Host Range	Insect Vector	Geographical Distribution
			A) **WHITEFLY-TRANSMITTED GEMINIVIRUSES**						
Abutilon mosaic*	AbMV	+	+	0.8	2660 2640	27,500	Malvaceae	*B. tabaci*	Tropical America
African cassava mosaic*	ACMV	+	+	0.8	2779 2724	32,000	Euphorbiaceae	*B. tabaci*	Africa & India
Bean distortion dwarf	BDDV	spherical particles	+				Leguminosae	*B. tabaci*	China
Bean golden mosaic*	BGMV	+	+	0.8	2646 2587	27,400	Leguminosae	*B. tabaci*	Central & South America
Cotton leaf crumple	CLCV	+					Malvaceae	*B. tabaci*	North America
Cowpea golden mosaic	CGMV						Leguminosae	*B. tabaci*	Nigeria
Eupatorium yellow vein	EuYVV						Compositae	*B. tabaci*	Japan
Euphorbia mosaic*	EMV	+	+	0.96 0.90	2870 2700	32,000	Euphorbiaceae	*B. tabaci*	America
Horsegram yellow mosaic	HYMV	+						*B. tabaci*	India

Virus Name	Abbreviation	Geminate Morphology	ssDNA Shown	Genome M_r (x10^6)	Nucs.	Coat Protein	Host Range	Insect Vector	Geographical Distribution
Honeusuckle yellow vein mosaic	HYVMV						Caprifoliaceae	B. lonicerae	Japan
Jatropha mosaic	JMV	+					Euphorbiaceae	B. tabaci	Central America
Mungbean yellow mosaic	MYMV	+	+				Leguminosae	B. tabaci	South Asia
Potato yellow mosaic*	PYMV	+	+	0.8	2500-2600		Solanaceae	B. tabaci	South America
Solanum apical leaf curling	SALCV	trimeric particles	+				Solanaceae		South America
Soybean crinkle leaf	SCLV	+					Leguminosae	B. tabaci	South Asia
Squash leaf curl	SqLCV	+	+				Cucurbitaceae	B. tabaci	Tropical America
Tobacco leaf curl	TbLCV	+	+				Solanaceae Capricaceae Caprifoliaceae Compositae	B. tabaci	Tropical & Sub-Tropical Far East
Tomato leaf curl & Tomato yellow leaf curl	TmLCV TYLCV	+	+		2800		Solanaceae	B. tabaci	Mediterranean

Virus Name	Abbreviation	Geminate Morphology	ssDNA Shown	Genome M_r ($\times 10^6$)	Nucs.	Coat Protein	Host Range	Insect Vector	Geographical Distribution
Tomato golden mosaic*	TGMV	+	+	0.8	2588 2508	28,500	Solanaceae	B. tabaci	South America
Watermelon curly mottle	WCMoV	+					Cucurbitaceae	B. tabaci	North America
			B) LEAFHOPPER-TRANSMITTED GEMINIVIRUSES						
Beet curly top*	BCTV	+	+	0.93	2993	29,600	Chenopodiaceae Compositae Cruciferae Solanaceae	Circulifer[a] tenellus	Mediterranean
Chloris striate mosaic*	CSMV	+	+	0.71	2750	28,000	Gramineae	Nesoclutha pallida	Australia
Digitaria streak	DSV	+	+	0.71	2701	27,500	Gramineae	Nesoclutha declivata	Australia
Horseradish curly	HCTV						Cruciferae	Circulifer tenellus	USA
Maize streak*	MSV	+	+	0.71[b]	2687[b]	28,000	Gramineae	Cicadulina mbila	Africa & India
Miscanthus streak	MisSV	+	+				Gramineae		Japan
Paspalum striate mosaic	PSMV	+					Gramineae	Nesoclutha pallida	Australia

Virus Name	Abbreviation	Geminate Morphology	ssDNA Genome Shown	M_r ($x10^6$)	Nucs.	Coat Protein	Host Range	Insect Vector	Geographical Distribution
Tobacco yellow dwarf & Bean summer death	TYDV BSDV	+	+			27,500	Solanaceae Leguminosae	Orosius argentatus	Australia
Wheat dwarf*	WDV	+	+	0.9		29,400	Gramineae	Psammotettix alienus	Northern Europe
			C) **TREEHOPPER-TRANSMITTED GEMINIVIRUS**						
Tomato pseudo	TmPCTV						Solanaceae	Micrutallis malleifera	North America
			D) **APHID-TRANSMITTED GEMINIVIRUS**						
Oat chlorotic stripe	OCSV	+					Gramineae	Rhopalosiphon maidis	Canada

Notes to Table 8

* Definitive members.

(+) Geminate morphology/ssDNA genome has been demonstrated.

(-) Geminate morphology/ssDNA genome not yet demonstrated.

[a] Only some host families are shown, host range is much larger.

[b] The figures given for the size of the ssDNA genome are those of the Nigerian isolate (Mullineaux *et al.*, 1984).

Key References for Table 8

AbMV — Jeske & Werz (1980); Abouzid & Jeske (1986); Abouzid *et al.*, *(1988).*

ACMV — Harrison *et al.*, (1977); Bock *et al.*, (1978); Adejare & Coutts (1982); Stanley & Gay (1983).

BCTV — Mumford (1974); Thomas & Mink (1979); Stanley *et al.*, (1986).

BDDV — Zhongxing *et al.*, (1982).

BGMV — Goodman & Bird (1978); Goodman *et al.*, (1980); Howarth *et al.*, (1985).

CGMV — Anno-nyako *et al.*, (1983).

CLCV — Brown & Nelson (1984).

CSMV — Francki *et al.*, (1979, 1980); Andersen *et al.*, (1988).

DSV — Dollet *et al.*, (1986); Donson *et al.*, (1987).

EMV — Kim & Fulton (1984); Jaramillo & Lastra (1986).

EuYVV — Inouye & Osaki (1980).

HYMV — Muniyappa *et al.*, (1987).

HYVMV — Osaki *et al.*, (1979).

JMV — Kim *et al.*, (1986).

MisSV — Yamashita *et al.*, (1985).

MYMV — Honda *et al.*, (1983); Morinaga *et al.*, (1983).

OCSV — Haber & Gill (1984).

PSMV — Grylls (1979); Francki *et al.*, (1985).

PYMV — Debrot (1981); Roberts *et al.*, (1986; 1988).

SALCV — Hooker & Salazar (1983).

SCLV — Iwaki *et al.*, (1983).

SqLCV — Cohen *et al.*, (1983).

TbLCV	Osaki & Inouye (1981).
TYLCV/TmLCV	Russo *et al.*, (1980); Cherif & Russo (1983); Czosnek *et al.*, (1988).
TYMV	Lastra & Gil (1981); deUzcategui & Lastra (1978).
TGMV	Hamilton *et al.*, (1981); Buck & Coutts (1985); Rushing *et al.*, (1987).
TmPCTV	Simons & Coe (1958); Christie *et al.*, (1984; 1986).
TYDV/BSDV	Thomas & Bowyer (1980).
WCMoV	Brown & Nelson (1986).
WDV	Lindsten *et al.*, (1980); MacDowell *et al.*, (1985).

4.3 Virus Transmission

All of the geminiviruses studied so far, with a known vector, are transmitted by insects and these can generally be split into two groups: whiteflies (Aleyrodidae) and leafhoppers (Anchenorrhyncha, Cicavellideae). The only reported exceptions are TmPCTV, which is transmitted by a treehopper (Simons & Coe, 1958) and OCSV, which is transmitted by an aphid (Haber & Gill, 1984).

The whitefly vector is always from the *Bemisia* family and in all but one case it is *B. tabaci*; the exception being *B. lonicerae*, which is the vector of HYVMV (Osaki *et al.*, 1979). The leafhopper vectors vary from virus to virus (see Table 8). Some of these viruses have subsidiary vectors which are closely related to their main vector, such as MSV (*Cicadulina bipunctella zeae, C. parazeae* and *C. storeyi*; Bock, 1974) and BCTV (*Circulifer opacipennis*; Bennett & Tanrisever, 1958).

The virus-vector relationship is similar among geminiviruses. The viruses studied are circulative, require a latent period of at least four hours, persist in the vector for days and sometimes life and do not replicate while in the vector. Leafhoppers and whiteflies do however differ to some extent concerning the viruses they transmit, and this is probably attributable to the different feeding patterns of the two types of insect.

For example, MSV (Storey, 1928; 1938) can be acquired or inoculated by *Cicadulina mbila* in access periods of five minutes, has a minimum latent period of six to twelve hours at 30 °C, and can persist for life. Once the virus has reached the hemolymph it can be transmitted and it can be retained through the molt. BCTV (Bennett & Wallace, 1938; Severin, 1931) and TmPCTV (Simons, 1962) are transmitted in a similar fashion by *Circulifer tenellus* and *Micrutalis malleifera*, respectively. No evidence has been found of maize streak virus or beet curly top virus replicating in their vectors or being passed through the egg into the progeny leafhopper (Storey, 1928; Severin, 1921).

One of the better studied examples of whitefly transmission is that of EMV by *Bemisia tabaci* (Costa & Bennett, 1950). Acquisition and inoculation by *B. tabaci* require minimum access periods of 30 and

ten minutes, respectively, and a minimum latent period of four hours. The virus persists for up to 20 days and passes through the molt from larva to adult but not through the egg to progeny insects. No whitefly-transmitted geminivirus has been found to replicate in its vector.

Geminivirus transmission resembles that of luteoviruses in many ways: virus acquisition and inoculation increase with access period, there is a minimum latent period of several hours, and the virus is able to pass through the moult and persist in the vector for several days or even for life. The route taken by the luteovirus whilst in the aphid vector is from the gut into the hemocoel, then into the accessory salivary glands and finally into the saliva. There is evidence that the virus coat protein is involved in determining vector specificity by controlling the movement of the virus from the hemocoel to the accessory salivary glands (Gildow & Rochow, 1980). Harrison (1985) speculates that this may also be true for the geminiviruses, as those transmitted by whiteflies have antigenically-related coat proteins and are transmitted by the same vector - *B. tabaci* - whereas those transmitted by leafhoppers have antigenically-unrelated or distantly related coat proteins and are transmitted by different vectors (Roberts *et al.*, 1984), except isolates of the same virus, such as MSV (Dekker *et al.*, 1988), which are transmitted by leafhoppers of the genus *Cicadulina* and are serologically related. Moreover, no antigenic relationship has been found between the coat protein of a whitefly-transmitted geminivirus and that of a leafhopper-transmitted geminivirus (Roberts *et al.*, 1984).

Hamilton *et al.* (1984) studied the coat proteins of two whitefly-transmitted geminiviruses - TGMV and ACMV - more closely, using hydrophilicity plots of their amino acid sequences. Hopp and Woods (1981) have found that the most hydrophilic regions of proteins often equate with antigenic determinants, so when Hamilton *et al.* (1984) found that the two major hydrophilic regions of TGMV coat protein had equivalents in the ACMV coat protein they surmised that they might represent common epitopes. Harrison (1985) has suggested that these epitopes may be involved in virus transmission by *B. tabaci*. The use of monoclonal antibodies identified at least two epitopes which were conserved between the whitefly-transmitted viruses ACMV, BGMV, EMV,

MYMV and TGMV but which were not present in the leafhopper-transmitted viruses (Thomas *et al.*, 1986). This supports the view that structural features on the surface of the whitefly-transmitted viruses are required for transmission by *B. tabaci*.

4.4 Host Range

The geminiviruses as a group have a wide host range among mono- and dicotyledonous plants (see Table 8) and this has been one of the reasons why attempts have been made to develop them into vectors with which to study gene expression in plants (Buck & Coutts, 1983; Coutts *et al.*, 1987; Hayes *et al.*, 1988e).

The geminivirus group can be split into three general sub-groups, according to their insect vector (section 4.3) and host range. The first sub-group consists of whitefly-transmitted viruses which only infect dicotyledonous plants, such as AbMV, ACMV, BGMV, EMV, PYMV and TGMV; the second sub-group consists of leafhopper-transmitted viruses which only infect monocotyledonous plants, such as CSMV, MSV and WDV; the viruses in the third sub-group share some properties of each of the other two sub-groups: they are transmitted by leafhoppers, but only infect dicotyledons, and the three known examples are BCTV, BSDV and TYDV, although the last two are probably different strains of the same virus (Thomas & Bowyer, 1980). Two potential members of the geminiviruses fall outside of these three sub-groups: TmPCTV is reported to be transmitted by a treehopper and infects dicotyledons (Simons & Coe, 1958) and OCSV is reported to be aphid-transmitted and infects monocotyledons (Haber & Gill, 1984).

Those viruses that are leafhopper-transmitted generally have a wider host range than those transmitted by whiteflies. For example, BCTV is known to infect over 300 species in 44 families of dicotyledonous plants (Bennett, 1971) mainly within the Chenopodiacae, Compositae, Cruciferae, Leguminosae and Solanaceae and MSV infects all major cereal crops with some 54 grass species as symptomatic hosts (Damsteegt, 1983).

Some examples of narrower host ranges of the whitefly-transmitted viruses are: BGMV, which only infects certain species in the *Phaseolus, Macroptilium, Vigna* and *Calopogonium* genera (Goodman &

Bird, 1978) and MYMV, which only infects seven species in the Leguminosae family (Honda *et al.*, 1983).

Several of the whitefly-transmitted viruses can infect plants in the Solanacae, regardless of their natural host (Bock, 1982), and this has proved an advantage to researchers, allowing the propagation of viruses such as ACMV, TGMV and PYMV in *Nicotiana* species, which are particularly easy to grow and provide a high yield of virus.

4.5 Cytology

For most of the geminiviruses that have been studied, the virus particles have been found by electron microscopy to be confined to the phloem cells in the host plant. Examples are BGMV, MYMV, TbLCV, TYDV and TYLCV (Goodman & Bird, 1978; Thongmeearkom *et al.*, 1981; Osaki & Inouye, 1981; Thomas & Bowyer, 1980; Cherif & Russo, 1983), and this has led to the geminiviruses being often described as phloem-limited (Goodman, 1981; Harrison, 1985; Stanley, 1985). However the particles of several geminiviruses have been found in other tissues as well as the phloem: ACMV particles have been identified in the cortex, mesophyll and epidermis of infected *N. benthamiana* (Sequeira & Harrison, 1982); CSMV particles have been found in every cell type in infected *Chloris gayana*, except epidermal and possibly xylem tracheid cells (Francki *et al.*, 1979); MSV particles have been found in the mesophyll and guard cells of infected *Zea mays* (Markham *et al.*, 1985); and TGMV particles have been found in the mesophyll and possibly the epidermis of infected *N. benthamiana* (Rushing *et al.*, 1987).

In all the cases studied, geminivirus particles accumulate in the nuclei of infected cells, but some viruses have been observed in the cytoplasm, such as CSMV, DSV and MYMV (Francki *et al.*, 1979; Dollet *et al.*, 1986; Thongmeearkom *et al.*, 1981) and the vacuole (CSMV and MYMV). When viruses accumulate in the nucleus they can form randomly arranged aggregates, for example CSMV in *Chloris gayana* (Francki *et al.*, 1985) or crystalline arrays of particles, for example TGMV in *N. benthamiana* (Rushing *et al.*, 1987), or both as in the case of MYMV in mungbean (Thongmeearkom *et al.*, 1981). The virus particles of BCTV, CSMV, TYDV and TbLCV have also been observed in ribbon-like

aggregates, apparently formed from single rows of geminate particles (Esau, 1977; Francki *et al.*, 1985; Osaki & Inouye, 1981).

The cytopathological changes that occur during viral infection are predominantly associated with the nucleus. The sequence of changes in the nuclei of French beans inoculated with BGMV (Kim *et al.*, 1978) is thought to represent the general pattern of changes for geminiviruses that infect dicotyledonous plants. Initially, the nucleoli get larger and then are segregated into granular and fibrillar regions. Then the fibrillar material condenses into various sized rings, sometimes several to a nucleus, and finally virus particles appear in the nucleoplasm. Cytochemical experiments on the granular and fibrillar regions of the segregated nucleoli showed that the granular material consisted mainly of ribonucleoprotein, whereas the fibrillar material was mainly deoxyribonucleoprotein (Kim *et al.*, 1978). As the fibrillar rings are formed before virus particles appear, it was suggested that these structures could be the site of viral DNA replication (Kim *et al.*, 1978).

These nuclear changes have not been detected in monocotyledons infected with geminiviruses. In these plants the only cytopathological change noted was the accumulation of virus particles in the nuclei (Francki *et al.*, 1979; Bock *et al.*, 1974).

As well as the nuclear changes that occur during geminivirus infection, some cytopathological changes have been observed in the cytoplasm. Inclusion bodies occur in JMV-infected *Jatropha gossypifolia* (Kim *et al.*, 1986) and EMV-infected *Damura stramonium* (Kim & Fulton, 1984). However, it is not known whether they are virally-encoded or a host response to viral infection.

4.6 Virion Structure and Composition

One of the most characteristic properties of the geminiviruses is their particle morphology. Each geminate particle measures about 18 x 30 nm with a groove running around the middle at 90° to the long axis. Hatta and Francki (1979) made a detailed study of the CSMV capsid by electron microscopy of virus preparations. They showed that each geminate particle was constructed from two incomplete

icosahedra, each with 12 morphological sub-units or capsomeres, but with one capsomere missing from each icosahedron where they are in contact, making a total of 22 capsomeres per geminate particle. Further work (Francki *et al.*, 1980) suggested that each capsomere consisted of five identical polypeptides, making 110 polypeptides per geminate particle. The M_r of this polypeptide varies among the geminiviruses, from 27,000 to 32,000, as determined by SDS-polyacrylamide gel electrophoresis (see Table 8), 8nd some of the viruses contain more than one polypeptide species. For example, BCTV particles have been found to consist of two major and two minor polypeptides, all M_r 23-27,000 (Harrison, 1985) and BGMV one major polypeptide (M_r 27,400) and two minor ones. It is not known whether these multiple components are related in any way.

The geminivirus genome was first identified as ssDNA for BGMV (Goodman, 1977a, b) by its resistance to RNase A and sensitivity to DNase I and S1 nuclease. Similarly, Harrison *et al.* (1977) demonstrated a ssDNA genome for each of ACMV and MSV, with estimated M_r of 8×10^5 (approximately 2600 nucleotides) and 7.1×10^5 (approximately 2200 nucleotides), respectively. They also observed by electron microscopy that the ssDNA was circular. A genome of circular ssDNA is now considered a prerequisite for membership of the geminivirus group by the International Committee on the Taxonomy of Viruses. The geminiviruses are the only recognised group of plant viruses with a genome of circular ssDNA, although two other viruses have recently been reported to have circular ssDNA genomes which differ from those of the geminiviruses: subterranean clover stunt virus appears to have a divided genome of 3 to 4 circular ssDNA molecules of 850 to 880 nucleotides each (Chu & Helms, 1987), and coconut foliar decay virus appears to have a genome consisting of one circular ssDNA molecule of approximately 1300 nucleotides, which showed no detectable sequence similarity to the geminivirus, DSV, during hybridisation tests (Hanold *et al.*, 1988). The caulimoviruses also have a circular DNA genome, but it is double stranded, and all other plant viruses contain an RNA genome.

It was originally suggested that each monomer of a geminate particle would contain one DNA molecule (Bock *et al.*, 1974; Galvez & Castano, 1975). However, Francki *et al.* (1980) showed that one DNA

molecule filled the whole geminate particle of CSMV. They calculated that a geminate particle containing one circular ssDNA molecule would have about 19 % DNA or about 32 % if two molecules were present per particle. The buoyant density of geminate CSMV particles in CsCl was determined to be 1.35 g cm^{-3} and this, compared with the phage ϕx174 (1.40 g cm^{-3}) which has 25.5 % DNA and fd phage (1.30 g cm^{-3}) which has 12 % DNA, indicated a DNA content of about 19 %, the value predicted for a geminate particle containing one molecule of circular ssDNA. Goodman *et al.* (1980) came to a similar conclusion for BGMV from buoyant density measurements and chemical analysis of DNA and protein content.

Apart from the familiar geminate particles, geminivirus preparations often contain monomeric particles as well, as seen on many electron micrographs (see Figure 16). These are thought to result from breakdown of geminate particles during virus purification or preparation for electron microscopy. To back this up, Larsen and Duffus (1984) showed that geminate BCTV particles were infectious but that the monomers were not. The DNA content of monomeric particles of ACMV (Sequeira, 1982) and MSV (Howell, 1985) has been shown to consist predominantly of degraded viral ssDNA of varying lengths, as opposed to geminate particles which contained intact viral ssDNA. However, sub-genomic ssDNA molecules of almost exactly half genomic size have been isolated from preparations of ACMV (Stanley, 1983; Stanley & Townsend, 1985) and TGMV (Hamilton *et al.*, 1982; MacDowell *et al.*, 1986) containing monomeric and geminate particles and it has been suggested that the monomers may encapsidate these sub-genomic DNAs.

Apart from monomer and dimer particles, trimers and tetramers have been seen in electron micrographs of several geminiviruses: BCTV (Larsen & Duffus, 1984), BGMV (Goodman *et al.*, 1977) and SqLCV (Cohen *et al.*, 1983). They were also present in preparations from transgenic petunia plants containing copies of the TGMV DNA components (Sunter *et al.*, 1987). Two types of virus-like structures were found in the tissue of AbMV-infected plants: geminate particles with a monomer diameter of 16-18 nm and elongated nucleoprotein "chains of pearls" with a diameter of 8-10 nm and a length of 300 nm (Jeske *et al.*, 1977; Jeske & Werz, 1980b; Jeske & Schuchalter-Eicke,

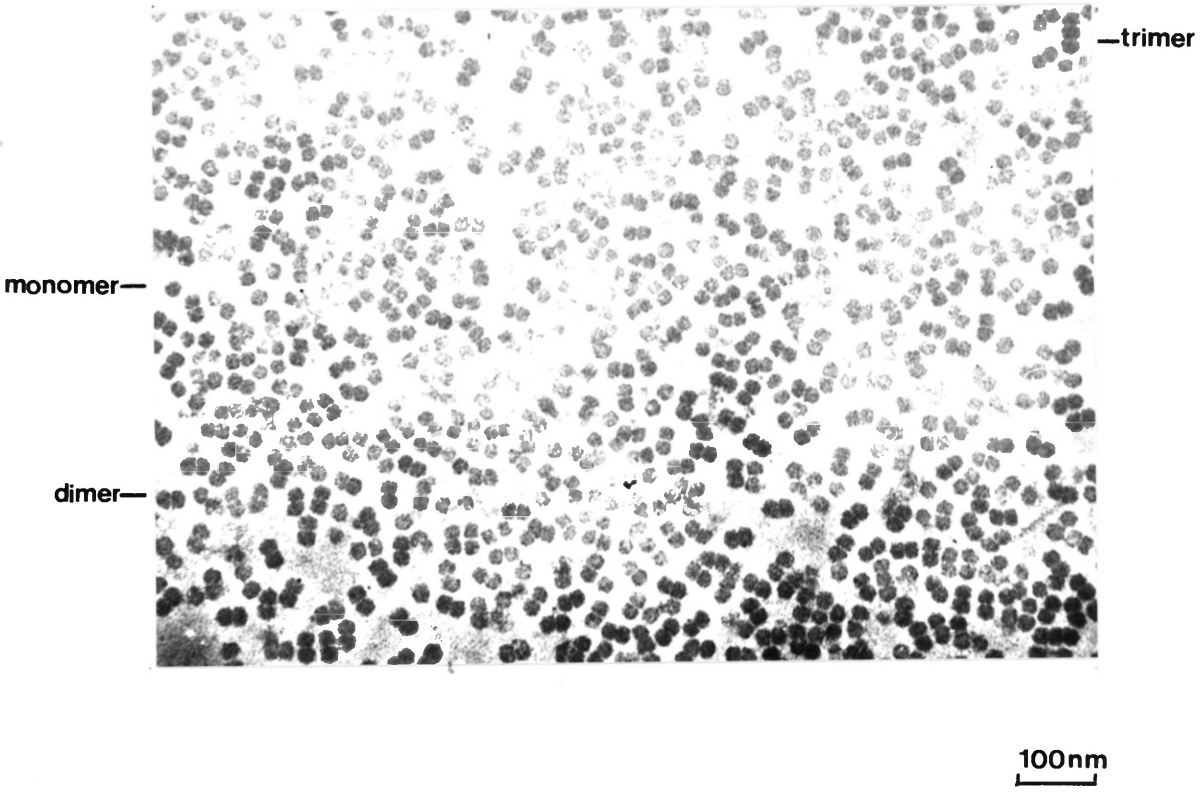


Figure 16 Electron Micrograph of Geminivirus Particles

Electron micrograph of WDV particles; single, paired and trimeric particles are indicated. (Photo courtesy of Dr. Bob Coutts).

1984). The "chains of pearls" were shown to be infectious by Jeske & Werz (1980a). SALCV preparations contain trimeric particles (approximately 52 x 17 nm) (Hooker & Salazar, 1983), but unlike other geminiviruses they are the predominant type. Nucleic acid extracted from SALCV particles contained circular molecules of approximately 3200 nucleotides (Harrison *et al.*, 1985), which is roughly 1½ times the size of geminivirus genomic DNA. Stanley & Townsend (1985) speculated that dimers of ACMV ssDNA could fit in and stabilise tetrameric particles, and extending this argument to SALCV could explain the trimeric particles encapsidating a genome roughly 1½ times larger than other geminiviruses.

4.7 The Geminivirus Genome

Goodman (1977), when commenting on the apparently small ssDNA genome of BGMV (M_r less than 10^6), speculated on the possibility of a multipartite genome of as yet unresolved components for geminiviruses. It wasn't until four years later that restriction endonuclease analysis showed that the BGMV genome was bipartite (Haber *et al.*, 1981). When BGMV ssDNA was cut with *Hha*I it produced fragments with a combined length (5380 nucleotides) that was twice that expected (2510 nucleotides) from electron-microscopic measurements of the DNA (Reisman *et al.*, 1979). A similar result was found with BGMV dsDNA (Ikegami *et al.*, 1981). This implied that BGMV contained a divided genome consisting of two circular ssDNA components of the same physical size but with a different sequence. The presence of two related, but distinct strains of BGMV was ruled out by studies of the kinetics of infection of bean mesophyll protoplasts (Haber *et al.*, 1981).

TGMV has also been shown to have a bipartite genome by restriction endonuclease analysis of virus-specific dsDNA (Hamilton *et al.*, 1982). The calculations from the sizes of the restriction fragments produced, identified two DNA species of M_r 1.7 x 10^6 and 1.65 x 10^6 (approximately 2660 and 2580 nucleotides, respectively) (Bisaro *et al.*, 1982; Hamilton *et al.*, 1982). The bipartite nature of the TGMV genome has subsequently been proven by cloning of two distinct components into pAT153 (Bisaro *et al.*, 1982; Hamilton *et*

al., 1983) and demonstrating their infectious nature by excising them from the vector and mechanically inoculating them onto *N. benthamiana* plants (Hamilton *et al.*, 1983). Both components were required for infection, and symptoms and progeny virus particles were identical to those found during normal viral infection (Hamilton *et al.*, 1983). The two cloned components have subsequently been sequenced: the lengths of DNA components A and B were 2588 and 2508 nucleotides, respectively (Hamilton *et al.*, 1984).

The bipartite nature of the ACMV genome was demonstrated by sequencing the viral ssDNA (Stanley & Gay, 1983). The two components (DNAs 1 and 2) were 2779 and 2724 nucleotides long, respectively. Recombinant DNA clones were constructed that contained full-length copies of ACMV DNAs 1 and 2 inserted into the bacteriophage M13. The inserts, on excision from the vectors, were together shown to be infectious in *N. benthamiana*. The symptoms, cytopathological changes and virus particles produced were indistinguishable from those produced during a native ACMV infection, verifying the bipartite nature of the genome (Stanley, 1983).

Further work on BGMV proved that its genome was bipartite. The two ssDNA species were cloned and shown to be infectious (Morinaga *et al.*, 1983). Sequence analysis of these clones revealed the two ssDNA components to be 2646 and 2587 nucleotides long (Howarth *et al.*, 1985). Also, two components of PYMV have been cloned and shown to be infectious when excised from the vector (Roberts *et al.*, 1988).

Progress on the elucidation of the leafhopper-transmitted geminivirus genome was slower than that of the whitefly-transmitted viruses, mainly due to the difficulties in propagating the former. However, in 1983 Marriott and Symons showed that the CSMV genome contained only one ssDNA component of approximately 2500 nucleotides, by restriction endonuclease analysis. Shortly afterwards, the Nigerian isolate of MSV (MSV-N) was shown to contain one ssDNA component (Mullineaux *et al.*, 1984) which when sequenced was found to be 2687 nucleotides long. At around the same time the sequence of the Kenyan isolate of MSV (MSV-K) was determined showing one ssDNA component of 2681 nucleotides (Howell, 1984). Since then several leafhopper-transmitted geminiviruses have been sequenced and in each case only one ssDNA component is present (WDV: 2749 nucleotides -

MacDowell *et al.*, 1985; BCTV: 2993 nucleotides - Stanley *et al.*, 1986; DSV: 2701 nucleotides - Donson *et al.*, 1987; a South African isolate of MSV (MSV-S): 2690 nucleotides - Lazarowitz, 1988; and CSMV: 2750 nucleotides - Andersen *et al.*, 1988).

It has been difficult to demonstrate the infectious unit as a single ssDNA component for leafhopper-transmitted, monocot-infecting geminiviruses, because neither the virus, the naked DNA nor the cloned DNA is mechanically transmissible, unlike the whitefly-transmitted viruses. However, using a technique known as agroinfection, Grimsley *et al.* (1987) showed the single DNA component of MSV-N to be infectious. This technique, developed with cauliflower mosaic virus (Grimsley *et al.*, 1986), relies on the ability of the bacterium *Agrobacterium tumefaciens* to transfer a portion (T-DNA) of the Ti (tumour-inducing) plasmid it harbours, into plant cells. In the case of dicotyledonous plants, the T-DNA is stably integrated into the nuclear DNA (Chilton *et al.*, 1977, 1980). Several vectors have been developed based on the Ti plasmid, which use the transfer system of *A. tumefaciens* to introduce foreign genes into plant cells (reviewed by Fraley *et al.*, 1986). Grimsley *et al.* (1987) inserted a tandem dimer of MSV-N DNA between the T-DNA border sequences of a broad host range binary vector (capable of replication in both *E. coli* and *A. tumefaciens*). The bacteria were then used to inoculate *Zea mays* seedlings by direct injection into the meristem. Symptoms of infection and viral DNA forms were produced which indicated that the MSV was capable of escaping from the T-DNA (presumably via a recombination or replication mechanism), replicating and spreading through the plant, thereby demonstrating that the single DNA component contained all the necessary viral functions. Lazarowitz (1988) showed that the progeny of agroinfection were transmitted by the vector, *C. mbila*, providing unequivocal proof of the single DNA component genome. This technique has also been used successfully to demonstrate the single DNA component genome of WDV (Hayes *et al.*, 1988b) and DSV (Donson *et al.*, 1988).

BCTV differs from other leafhopper-transmitted geminiviruses in that it infects dicotyledonous and not monocotyledonous plants, and the virus is mechanically transmissible (Mumford, 1972). Recently

Stanley *et al.* (1986) have shown that the cloned DNA component of BCTV is infectious when excised from the vector, by needle-puncture into the crown of *Beta vulgaris* seedlings. Although the efficiency of transmission by this method was low, characteristic symptoms and geminate virus particles were seen in the the plants and the infection was efficiently transmitted to healthy *B. vulgaris* by the natural vector, *C. tenellus* (Stanley *et al.*, 1986). This proves conclusively that BCTV has a single DNA component genome.

4.8 Organisation and Expression of the Geminivirus Genome

4.8.1 Whitefly-Transmitted Geminiviruses

As mentioned in the previous section, the complete nucleotide sequences of three whitefly-transmitted geminiviruses have been determined. TGMV DNAs A and B are 2588 and 2508 nucleotides, respectively (Hamilton *et al.*, 1984); ACMV DNAs 1 and 2 are 2779 and 2724 nucleotides, respectively (Stanley & Gay, 1983) and BGMV DNAs 1 and 2 are 2646 and 2587 nucleotides, respectively (Howarth *et al.*, 1985). To facilitate comparison between these viruses, DNAs 1 and 2 of ACMV and BGMV will be referred to as DNAs A and B, respectively, for the rest of this thesis. Lazarowitz (1987) has reported that two isolates of SqLCV have also been sequenced, although the actual sequence has not yet been published.

Comparison of the sequences of ACMV, BGMV and TGMV indicated a greater degree of homology between each DNA A (60 %) than between each DNA B (40 %) (Stanley, 1985). Furthermore, hybridisation studies between ACMV and other geminiviruses have shown that while homologies were detectable between ACMV DNA A and DNA from BGMV, ToLCV, TGMV, TLCV and TYLCV, no homologies were detected using ACMV DNA B (Roberts *et al.*, 1984). This suggests that the closer relationship between each DNA A than between each DNA B is a general feature of whitefly-transmitted geminiviruses. Hamilton *et al.* (1984) suggested that the reason for the higher degree of sequence homology for DNA A may be that there are more rigid structural constraints on the DNA A-encoded proteins, such as the coat proteins (see section 4.3), which are encoded within the most highly conserved ORFs (approximately 70 % amino acid homology). Hence, the geminate

structure of the virions might impose severe structural constraints on coat protein configuration. However, the role of the coat protein in transmission by the common vector, *B. tabaci* (Roberts *et al.*, 1984), might be responsible for the conservation of the amino acid sequences.

Apart from the sequence relationships between different whitefly-transmitted geminiviruses, there are also homologies between the two DNA components of each virus. The most striking homology is in an intergenic region of approximately 200 nucleotides, which is almost identical within the two components of any virus. This 'common region', as it is known, is, however, completely different among the different viruses, except for one conserved feature of 28-32 nucleotides with the potential to form a hairpin loop structure, consisting of a GC-rich base-paired stem and an AT-rich loop containing the conserved sequence TAATATTAC (see Figure 17). As the 'common region' is in a noncoding sequence and is common to both DNA components of the genome, it is likely that it contains recognition signals essential to one or more common functions, such as DNA replication (see section 4.9.2), transcription or virus assembly.

To investigate the potential coding capacity of the genome, the nucleotide sequences of each virus were screened in all three reading frames and in both DNA senses for open reading frames (ORFs) with the capacity to code for a protein larger than the arbitrary value of 10,000 Daltons when read from the first in-phase initiation codon. Six ORFs were found that were conserved among ACMV, BGMV and TGMV, four on DNA A and two on DNA B, designated AR1, AL1, AL2, AL3, BR1 and BL1, where R and L refer to rightward and leftward orientations with respect to the 'common region' (Stanley & Gay, 1983; Howarth *et al.*, 1985; Hamilton *et al.*, 1984). In addition, ACMV and BGMV have a further six and three ORFs, respectively, which are not related to each other or to any TGMV ORF (Stanley & Gay, 1983; Howarth *et al.*, 1985). Whether they encode virus-specific proteins or have arisen fortuitously is not known.

There is a high degree of sequence homology between each of the analogous ORFs (Howarth & Goodman, 1986), which suggests that they are involved in similar functions. The sizes of the conserved ORFs are shown in Table 9 and their relative positions in the genome are

	Stem	Loop	Stem
Invariant sequence		**TAATATTAC**	
ACMV A and B	GGGGCCAACCG	TA-**TAATATTAC**	CGGTTGGCCCC
BGMV A and B	GCGGCCATCCG	ATA**TAATATTAC**	CGGATGGCCGC
TGMV A	GCGGCCATCCG	TT-**TAATATTAC**	CGGATGGCCGC
TGMV B	GCGGCCATCCG	TTT**TAATATTAC**	CGGATGGCCGC
BCTV	GGGCCATCCG	TTA**TAATATTAC**	CGGATGGCCC
WDV	GTCGGGGGGCCTCCACGCGGG	TTA**TAATATTAC**	CCCGCGTGGTGGCCCCCGAC
MSV-N	GCAGGAAAAGAAGGCGCG	CAC**TAATATTAC**	CGCGCCTTCTTTTCCTGCGA
MSV-K	GAAAAGAAGGCGCG	CAC**TAATATTAC**	CGCGCCTTCTTTTC
MSV-S	GCAGGAAAAGAAGGCGCG	CAC**TAATATTAC**	CGCGCCTTCTTTTCCTGCGA
DSV	GGGGGTGGGGC	GCACCAC**TAATATTAC**A	GCCCCACCCCC
CSMV	CGGGGGGGC	AACCA**TAATATTAC**C	GCCCCCCCG

Figure 17 Comparison of the major putative stem-loop structures

Sequences are aligned to show the invariant 10 base sequence (shown in bold type) and the potential stem structure (underlined).

Table 9 Potential Proteins Encoded by Conserved ORFs of Whitefly-Transmitted Geminiviruses

ORF	M_r (x 10^3)		
	TGMV	ACMV	BGMV
AR1	28.6	30.2	27.7
AL1	40.3	40.3	40.2
AL2	14.9	15.1	19.6
AL3	15.7	15.8	15.6
BR1	29.3	29.3	29.7
BL1	26.5	33.7	33.1

depicted linearly in Figure 18.

Computer-assisted screening of the sequences up- and downstream of the conserved ORFs identified potential promoters with the consensus TATA box sequence TATAA/TAA/T (Breathnach & Chambon, 1981) and polyadenylation signals conforming to the sequence AATAAA (Proudfoot & Brownlee, 1976) for most of the ORFs (Hamilton *et al.*, 1984). The relative positions of these sequences are shown in Figure 18, and their significance in the strategy of transcription is discussed later in this section.

Of the six conserved ORFs, only one has been definitely identified as coding for a virus-specific product, namely the coat protein. A comparison of the experimentally-determined amino acid composition of the ACMV coat protein with those predicted from the nucleotide sequence for the ORFs, indicated that AR1 was the most likely candidate for the coat protein gene (Stanley & Gay, 1983). Townsend *et al.* (1985) provided conclusive proof that AR1 does encode the coat protein: *in vitro* translation of polyadenylated RNA from ACMV-infected *N. benthamiana* produced a protein of approximate M_r 30,000, which was immunoprecipitated by an antiserum raised against ACMV virions. When the polyadenylated RNA was hybridised with a ssDNA fragment of the AR1 ORF, prior to *in vitro* translation, the synthesis of this protein was completely inhibited, and upon melting of the DNA:RNA hybrid, synthesis of the protein was restored.

The coat proteins of TGMV and BGMV can also be assigned to ORF AR1 on the basis of sequence homology with the ACMV ORF AR1 (Hamilton *et al.*, 1984; Howarth *et al.*, 1985).

Although AR1 is the only ORF to have had a gene product definitely assigned to it, quite a lot is known about the putative products of the other five conserved ORFs and their roles in the virus infection. Rogers *et al.* (1986) constructed transgenic petunia plants containing dimeric inserts of TGMV DNA A and showed that the products of this genome component are sufficient to replicate DNA A and encapsidate ssDNA into virus particles (Sunter *et al.*, 1987). Similarly, it has been demonstrated that DNA A of ACMV can replicate independently of DNA B in *N. plumbaginifolia* protoplasts (Townsend *et al.*, 1986). This suggests that DNA A encodes the proteins required for virus DNA replication as well as the coat protein, and DNA B

Legend to Figure 18 Comparison of the Open Reading Frames of the Sequenced Bipartite Geminiviruses

The circular genomes are represented in a linear form. Position 1 is taken as the first T of the invariant sequence of the conserved 'hair-pin' loop. Conserved open reading frames are indicated by large open arrows, non-conserved open reading frames are drawn as thin arrows. White and black triangles indicate the positions of putative promoter and polyadenylation signals, respectively.

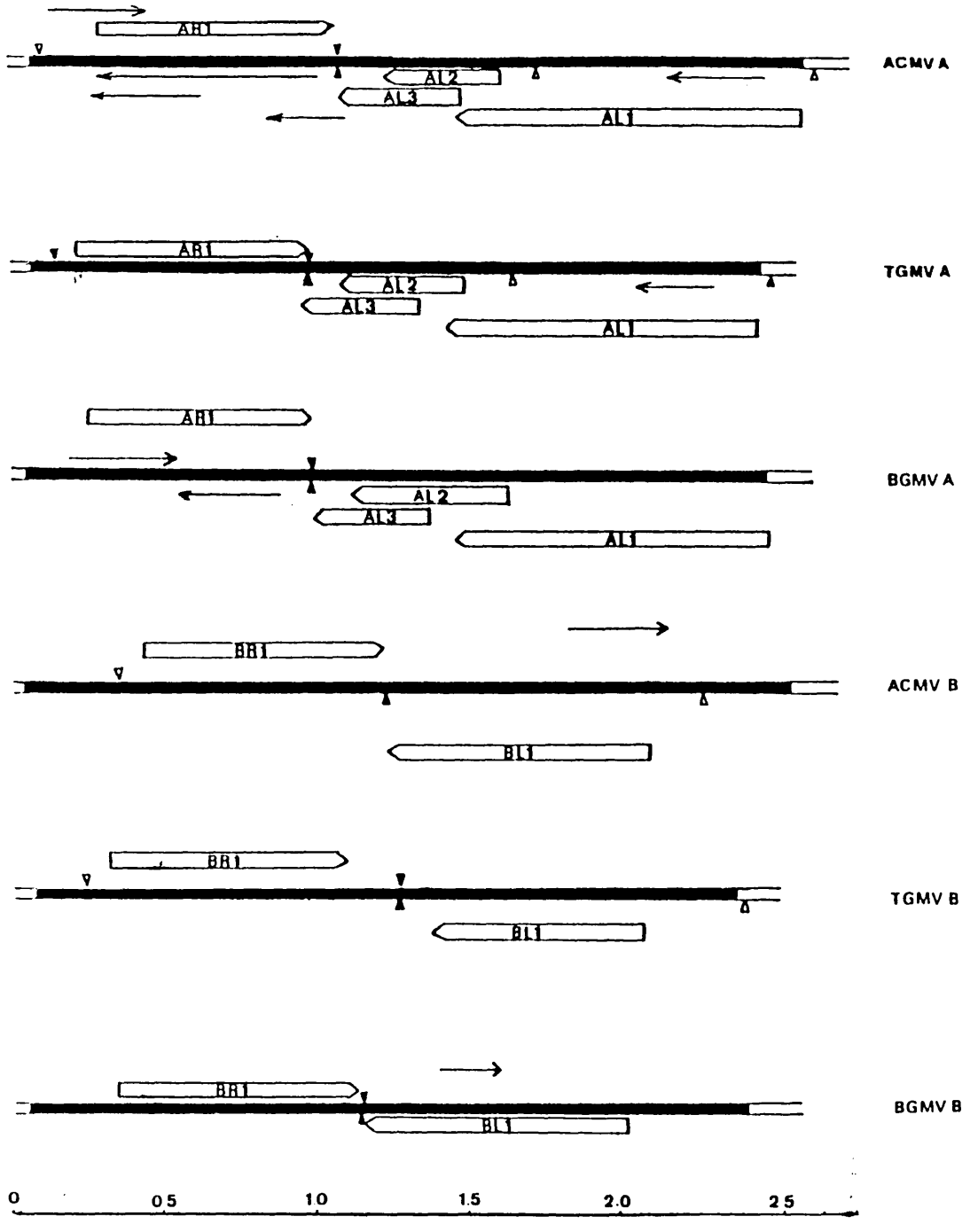


Figure 18 Comparison of the open reading frames of sequenced bipartite geminiviruses

encodes the proteins required for systemic spread and symptom development (Rogers *et al.*, 1986).

Stanley and Townsend (1986) have shown that a functional coat protein is not required for ACMV infectivity and symptom development. They have reported a small deletion in DNA A of ACMV which separates the AR1 ORF into two non-overlapping ORFs that could produce polypeptides with predicted M_r of 8800 and 11,600. This DNA A mutant, in combination with DNA B, was infectious when mechanically inoculated onto *N. benthamiana* plants and produced symptoms indistinguishable from those caused by wild-type ACMV. Similar experiments with mutants of DNA A of TGMV have confirmed that the coat protein coding sequence may be interrupted without loss of infectivity. However, some of these mutants showed delays in the time of symptom appearance and decreased symptom severity in *N. benthamiana* , and some were not infectious at all by mechanical inoculation (Brough *et al.*, 1988; Gardiner *et al.*, 1988). It has been suggested that the lack of infectivity of some of these TGMV coat protein mutants reflects a stringent requirement of the relative sizes of DNAs A and B. If the size of DNA A fell below a critical level it might replicate faster than, and outcompete, DNA B, so that insufficient quantities of the proteins encoded by DNA B would be produced to allow a systemic infection to develop (Brough *et al.*, 1988).

In vitro mutagenesis experiments have also been carried out on the leftward ORFs of the TGMV A component (Brough *et al.*, 1988; Elmer *et al.*, 1988) to determine their roles, if any, during viral infection. Brough *et al.* (1988) introduced mutations which considerably shortened ORFs AL1 and AL2/AL3 and found that none of them were infectious when inoculated onto *N. benthamiana* plants with DNA B, as determined by failure to develop symptoms after 28 days and failure to detect viral DNA by dot hybridisation with ^{32}P-labelled A and B component-specific TGMV probes in DNA prepared from non-inoculated leaves. In similar experiments Elmer *et al.* (1988) also showed that mutations affecting ORF AL1 abolish infectivity. However, they managed to separate the functions of AL2 and AL3 ORFs, showing that mutations in AL2 abolish infectivity for whole plants, but mutations in AL3 just delay and attenuate the symptoms. To

determine if these mutations had a direct effect on viral DNA replication they examined DNA found in petunia leaf discs after the introduction of the TGMV DNA A mutants by agroinfection. They showed that AL2 and AL3 mutants had no detrimental effect on the formation of viral DNAs, but that AL1 mutants totally abolished their formation. Elmer *et al.* (1988) concluded that the AL1, AL2 and AL3 ORFs of TGMV DNA A are genes whose products are required for normal infection and symptom production, and that the product of AL1 ORF is involved in viral DNA replication.

Mutants with alterations in the BR1 and BL1 ORFs of TGMV B have also been constructed and tested for infectivity (Brough *et al.*, 1988), and as with AL1 and AL2/AL3 they were found to be non-infectious when inoculated onto *N. benthamiana* plants with DNA A. The same result was found for the two ORFs of ACMV DNA B (Etessami *et al.*, 1988). This proves that the products of both ORFs are essential for virus proliferation.

Complementation of the non-infectious mutants in the AL1, AL2/AL3, BR1 and BL1 ORFs of TGMV (Brough *et al.*, 1988) and in the BR1 and BL1 ORFs of ACMV (Etessami *et al.*, 1988) with other non-infectious mutants in other ORFs has been demonstrated. An AL1 mutant co-inoculated with an AL2/AL3 mutant plus DNA B, and a BR1 mutant with a BL1 mutant plus DNA A all elicited symptoms on *N. benthamiana* plants. Also, analysis of the viral DNA showed that no detectable recombination or reversion to generate wild-type DNA had occurred (Brough *et al.*, 1988). In a further experiment on the BR1 and BL1 ORFs of ACMV, Etessami *et al.* (1988) cloned DNA fragments containing the two DNA B components into a DNA A coat protein deletion vector, in both orientations, and used them to inoculate *N. benthamiana* plants in the presence of DNA A. They demonstrated that only when the BR1 and BL1 ORFs were cloned in the orientation compatible with expression from the coat protein promoter was there an infection. This shows unequivocally that the products of both the BR1 and BL1 ORFs of ACMV DNA B are essential for virus infection.

It has been proposed that the geminiviruses employ a bidirectional mode of transcription (Stanley & Gay, 1983; Hamilton *et al.*, 1984), because the six conserved ORFs are arranged on both DNA strands converging on a point diametrically opposite the 'common

region', which is similar to the arrangement of genes in SV40 (reviewed by Tooze, 1980). The positions of the potential promoters for all conserved ORFs and polyadenylation signals for all except ACMV ORF BR1 supports this theory. The polyadenylation signals (AATAAA) occur in an AT-rich region (known as the A-T box) situated almost directly opposite the conserved stem-loop structure of the 'common region'. In the case of the AR1 ORF the TAA termination codon overlaps the signal and for all DNA components, except ACMV DNA B, the pairs of polyadenylation signals in opposite orientations form, or are part of, inverted repeats (see Figure 19). The close proximity of these polyadenylation signals suggests that there could be a slight overlap of the transcription termination sites in opposite directions.

Townsend *et al.* (1985) confirmed the bidirectional transcription model for ACMV when they found five major and one minor polyadenylated RNAs from ACMV-infected *N. benthamiana* and mapped them to the viral and complementary senses of both DNAs A and B (Figure 20). Two major transcripts of 1.7, and 0.7 kb were specific to the viral strand of DNA A, one major transcript of 1.0 kb was specific to the complementary strand of DNA A and the other two major transcripts (1.1 and 0.9 kb) were specific to the viral and complementary strands respectively of DNA B. None of the transcripts mapped to both DNA components, implying that they don't encompass the 'common region'. The 1.0 kb transcript, which is the most abundant virus-specific transcript found in ACMV-infected plant tissue, was identified as the coat protein mRNA (see earlier in this section) (Townsend *et al.*, 1985). This mRNA has an untranslated leader sequence of about 160 nucleotides and its 5' terminus maps just downstream of a TATA box. The 3' terminus was mapped to the polyadenylation sequence immediately downstream of the ORF AR1 termination codon. There is a consensus transcription modulation sequence or CAAT box (Benoist *et al.*, 1980) (GGCCAATC) 48 nucleotides upstream of this promoter. The same sequence has been found for the coat protein gene of TGMV, 65 nucleotides upstream of the TATA box (Hamilton *et al.*, 1984). Preliminary analysis of TGMV-infected *N. benthamiana* has similarly identified a DNA A-specific 0.9 kb transcript which mapped to the coat protein gene (Petty *et al.*, 1988). The 5' terminus mapped

```
ACMV A   TTGGCAATTAATAAACATT-GAATTTTATTTCATGAGTCAACT
ACMV B   CTATGTGGGATGATTTAT--T--ATTTATAAATCTTTATGGCT
BGMV A   TAACCAATTAATAAAATTTA-TATTTTATATCATGATTCTCAA
BGMV B   TAACACAAGAATAAATATAC-GTATTTATTTCAAAGACTTTGG
TGMV A   TAACAAATTAATAAAATTT-ATATTTTATTGAATGATTTTCGA
TGMV B   ACATATAATAATAAAT-G-G-ATATTTATTGCAACGTTTTGGG
WDV      GCATTCAGTAATAAAATAA--TATTTTATTTATCTCATGTCAT
MSV-N    CCAGTAATGAATAAAACGC-CGTTTTTATTATATCTGATGAAT
MSV-K    CCAGTAATGAATAAAAACTCCCGTTTTATTATATCTGATGAAT
MSV-S    CCAGTAATGAATAAAAACGCCCGTTTTATTATATCTGATGAAT
DSV      CCAG-AATGAATAAAAAC-TAGTTTTTATTATTGATTGCGAAG
CSMV     TTCATTTTCAATAAACTGTGAGAGTTTGCTTGCCAAACAACAT
BCTV     TAGATGCTATTTTTATTAATAATATTTATTATTAATAATGAAAT
```

Figure 19 Comparison of the 'A-T box' region of the characterised geminiviruses

The nucleotide sequences are aligned to show the relative positions of the putative polyadenylation signals (broken boxes; usually AATAAA). The arrows show the extent of the inverted repeats.

ACMV B and BGMV A contain polyadenylation signals in the virion sense that differ from AATAAA; and no polyadenylation signals have been found in the complementary sense of ACMV B and BCTV, and the virion sense of CSMV.

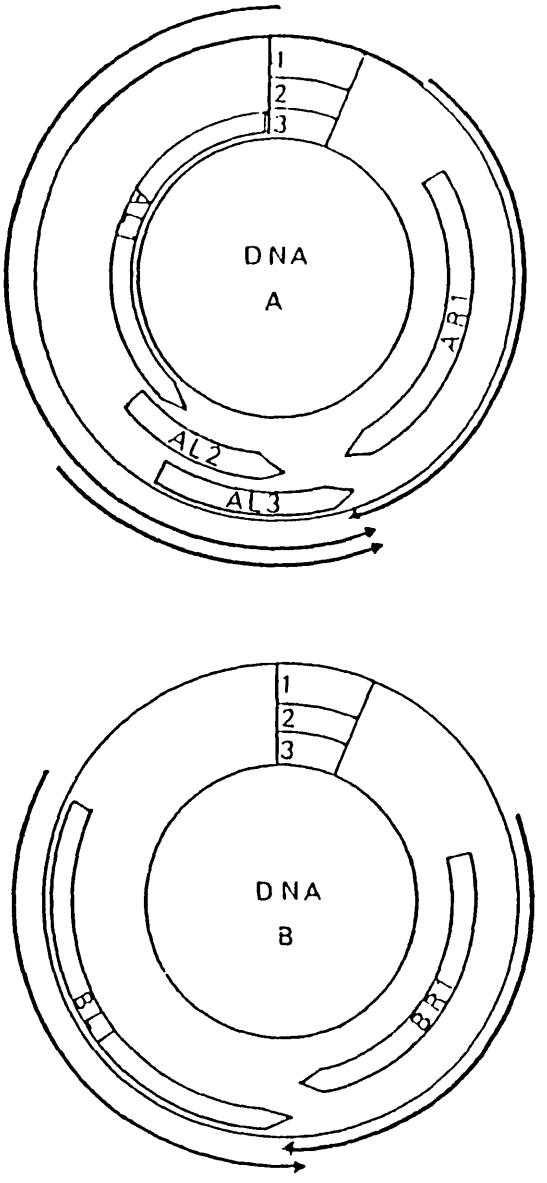


Figure 20 Major transcripts of ACMV

The ORFs conserved in other whitefly-transmitted geminiviruses are shown as open arrows. The 'common region' is shown boxed. The major transcripts referred to in the text are shown as thick arrows on the outside of each map.

downstream of a TATA box resulting in an untranslated leader sequence of 8 or 14 nucleotides and the 3' terminus mapped approximately 30 nucleotides downstream of the AR1 ORF termination codon.

The other four major transcripts of ACMV have not been mapped accurately. However, they are of appropriate sizes and distribution to encode the products of the other five conserved ORFs (Townsend *et al.*, 1985). Hence, the 1.7 kb polyadenylated RNA could encode the product of ORF AL1 and be co-terminal with the 0.7 kb transcript, which could encode the AL2 and/or AL3 overlapping ORFs. Hamilton *et al.* (1984) suggested that the overlapping AL2 and AL3 ORFs of TGMV could be expressed by post-transcriptional processing. However, Townsend *et al.* (1985) found no evidence for transcript splicing in ACMV. The DNA B-specific 0.9 kb and 1.1 kb transcripts were of a size to encode the products of ORFs BR1 and BL1, respectively. All of the transcripts had a suitably positioned TATA box, except the 1.1 kb transcript of the DNA B complementary sense ORF BL1. Townsend *et al.* (1985) have suggested that the minor DNA B-specific 1.35 kb transcript could be a precursor of the 1.1 kb transcript, becoming truncated at its 5' end to produce the smaller final transcript.

By analogy with the papovavirus SV40 (reviewed by Tooze, 1980), in which bidirectional transcription separates the early and late functions onto complementary strands, a similar mode of temporal regulation has been proposed for the geminiviruses (Hamilton *et al.*, 1984; Howarth *et al.*, 1985). Since the coat protein gene , which is probably required late in the cycle, has been located on the viral strand it was proposed that the early genes involved in DNA replication could be expressed from the complementary strand. The position of the promoter for the AL2 and AL3 ORFs within the AL1 ORF would require a coordinated control mechanism to allow expression of these ORFs. A similar temporal regulation of expression of genes on the DNA B component could occur using the identical sequences within the 'common region'. The possibility for intermolecular control between the two DNA components also exists (Stanley, 1985).

4.8.2 Leafhopper-Transmitted Geminiviruses

As mentioned in section 4.7, the complete nucleotide sequences of

several leafhopper-transmitted geminiviruses have been determined, and they range in size from 2681 nucleotides for MSV-K (Howell, 1984) to 2993 nucleotides for BCTV (Stanley *et al.*, 1986).

The sequences of the three isolates of MSV were found to be very similar: MSV-S and MSV-N were identical at 2628 positions, MSV-S and MSV-K at 2624 positions and MSV-N and MSV-K at 2621 positions (Lazarowitz, 1988; Howarth & Goodman, 1986). Comparisons of these sequences with those of DSV, WDV and CSMV, showed that DSV was more related to MSV (64 % homology) than to WDV and CSMV (47 % and 48 % respectively) (Donson *et al.*, 1987; Andersen *et al.*, 1988) and that WDV and CSMV were related to each other and DSV and MSV to roughly the same extent, approximately 50 % sequence homology (MacDowell *et al.*, 1985; Andersen *et al.*, 1988). However, comparisons between all of these viruses and the other leafhopper-transmitted virus BCTV, and also the whitefly-transmitted viruses ACMV, BGMV and TGMV, failed to detect any significant sequence homology (Stanley *et al.*, 1986; MacDowell *et al.*, 1985; Donson *et al.*, 1987; Andersen *et al.*, 1988). In contrast, BCTV has some sequence homologies with the DNA A component of the whitefly-transmitted viruses (Stanley *et al.*, 1986). As BCTV also differs from the other leafhopper-transmitted viruses by virtue of its dicotyledonous hosts, and as such is a sort of hybrid of the leafhopper-transmitted and whitefly-transmitted geminiviruses (Stanley *et al.*, 1986), its genome organisation will be discussed later in this section.

The nucleotide sequences of all of the leafhopper-transmitted viruses were screened in all three reading frames and in both DNA senses for ORFs with the capacity to code for proteins larger than 10,000 Daltons. There are four such ORFs, designated R0, R1, L1a and L1b, where R and L refer to the proposed direction of transcription, that are conserved among all of the viruses, with the exception of MSV-K, which has no ORF R0 and has a truncated ORF L1b, such that it is directly downstream and in-frame with ORF L1a (Howell, 1984). The sizes of the putative proteins encoded by the four ORFs are indicated in Table 10 and their arrangement in the genome is shown in Figure 21.

In the sequences of WDV, DSV and CSMV the ORF L1b is not strictly an open reading frame but an open region (OR), since there is no ATG

Legend to Figure 21 Comparison of the Open Reading Frames of the Sequenced Monopartite Geminiviruses

The circular genomes are represented in a linear form. Position 1 is taken as the first T of the invariant sequence of the conserved 'hair-pin' loop. Conserved open reading frames are indicated by large open arrows, non-conserved open reading frames are drawn as thin arrows. White and black triangles indicate the positions of putative promoter and polyadenylation signals, respectively.

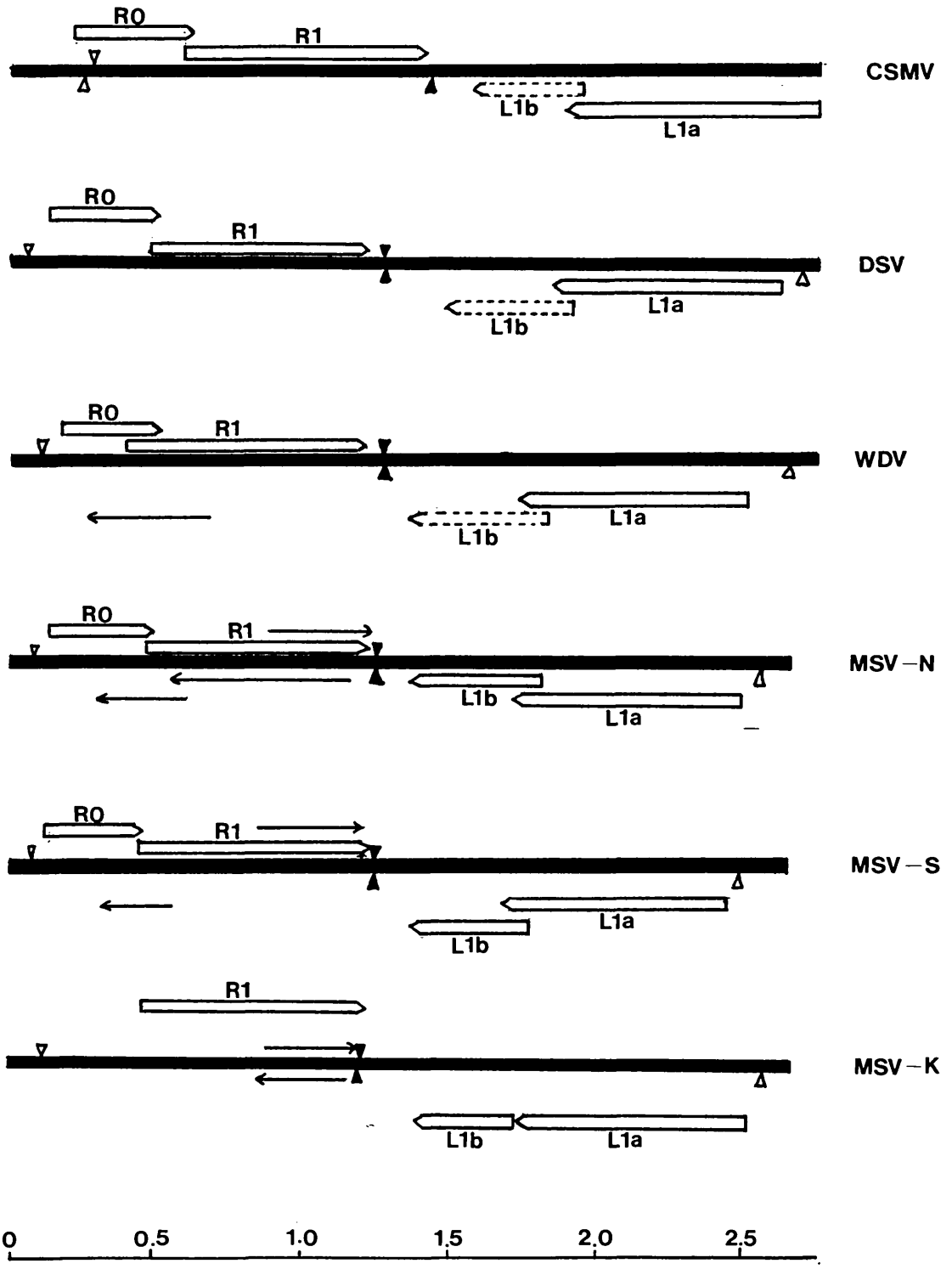


Figure 21 Comparison of the Open Reading Frames of the Sequenced Monopartite Geminiviruses

Table 10 Potential Proteins Encoded by Conserved ORFs of Leafhopper-Transmitted Geminiviruses

a) geminiviruses with monocotyledonous hosts

ORF	M_r (x 10^3)					
	WDV	MSV-N	MSV-K	MSV-S	DSV	CSMV
R0	10.1	10.9	-	10.9	12.1	14.5
R1	29.4	27.0	26.9	26.8	26.6	26.8
L1a	30.2	31.4	31.4	31.3	30.5	33.2
L1b	17.3*	17.8	13.6	17.2	16.9*	15.8*

* L1b of WDV, DSV and CSMV are open regions (OR) assigned on the basis of homology shown with ORF L1b of MSV-N.

b) geminivirus with dicotyledonous hosts

ORF	M_r (x 10^3)
	BCTV
R1	29.6
L1	40.9
L2	20.1
L3	16.1

triplet available. In all three cases there is a high degree of amino acid homology between the putative products of the ORs and the putative products of MSV-N ORF L1b (MacDowell *et al.*, 1985; Donson *et al.*, 1987; Andersen *et al.*, 1988). It was noted that the first three amino acids of the MSV-N L1b ORF are the same as those of the WDV L1b OR, including the methionine start codon (MacDowell *et al.*, 1985). However, the ATG start codon of MSV-N is replaced by GTG in WDV. In procaryotes, GUG codons can function as start codons, but eucaryotes seem to require AUG (Kozak, 1983). Therefore it is unlikely that GTG functions as a start codon for the L1b OR of WDV. MacDowell *et al.* (1985) have suggested that the WDV ORF L1a and OR L1b and the equivalent ORFs in MSV-N could be transcribed on a single mRNA and encode a composite protein, either by translational frameshift or processing of a precursor mRNA. This hypothesis is backed up by the fact that the nearest polyadenylation site for ORF L1a of WDV, DSV and CSMV is downstream of the termination codon of the OR L1b in each case (MacDowell *et al.*, 1985; Donson *et al.*, 1987; Andersen *et al.*, 1988). Further evidence for the formation of a composite protein has come from the comparison of the predicted amino acid sequences of ORF L1a and OR L1b of WDV, DSV and CSMV and the equivalents in MSV-N, with ORF AL1 of ACMV and TGMV (MacDowell *et al.*, 1985; Donson *et al.*, 1987; Andersen *et al.*, 1988). Significant regions of homology were found between ORF L1a of WDV, DSV, CSMV and MSV-N and the N-terminal region of ORF AL1 of ACMV and TGMV, and between OR(F) L1b and the C-terminal region of ORF AL1. However, it has also been suggested that separate functions of the MSV-N ORFs L1a and L1b, found as a single protein in ACMV and TGMV could reflect differences in the control of gene expression in these viruses, possibly as a consequence of infecting mono- and dicotyledonous hosts (Mullineaux *et al.*, 1985).

The ORF encoding the coat protein of leafhopper-transmitted geminiviruses has been identified as the larger virus sense ORF, R1. This was originally identified for MSV-N following a comparison of the experimentally determined amino acid composition with that predicted by the nucleotide sequence and then this was confirmed by hybrid-arrested translation of MSV-specific transcripts (Morris-Krsinich *et al.*, 1985). By analogy, the coat proteins of WDV, DSV and CSMV have been assigned to ORF R1 (MacDowell *et al.*, 1985; Donson

et al., 1987; Andersen *et al.*, 1988).

The ORF R0 of MSV-N has recently been shown to encode a virus-specific protein of M_r 11,000 (Mullineaux *et al.*, 1988). The protein was detected among the products of *in vitro* translation reactions programmed with RNA from infected maize plants and also in total protein extracts from infected leaves, using an antiserum raised against a β-galactosidase-R0 fusion protein in Western blotting analysis. Similarly, the R0 protein was not detected in preparations of virus particles and is therefore proposed to have a non-structural role (Mullineaux *et al.*, 1988). *In vitro* mutagenesis of ORF R0 resulting in the insertion of a translation stop codon, completely abolished the infectivity of the MSV-N DNA, as determined by agroinfection of maize plants: no characteristic symptoms or DNA forms were observed. This may indicate that the R0 protein is essential for MSV-N infection. On the other hand, the abolition of infectivity may have been caused by the slightly larger size of the genome (14 bases), or by the inserted sequence interfering with the expression of the coat protein cistron, perhaps by perturbing the promoter (Mullineaux *et al.*, 1988). Computer-assisted analysis of the predicted amino acid sequences of MSV-N, WDV and DSV ORF R0 proteins revealed a high level of homology, suggesting that the ORF R0 protein of MSV-N has functional counterparts in plant tissue infected with the other two viruses (Mullineaux *et al.*, 1988). However, no amino acid sequence homology was observed between the ORF R0 protein of MSV-N, WDV or DSV and any product encoded by an ORF in the dicotyledon-infecting geminiviruses, suggesting a possible, essential function for the ORF R0 protein in monocotyledon-infecting geminiviruses (Mullineaux *et al.*, 1988).

A large part of each leafhopper-transmitted geminivirus genome has been implicated in protein coding. However, there are two intergenic regions situated almost diametrically opposite each other, known as the starting and terminating intergenic regions (SIR and TIR), reflecting their proposed function in bidirectional transcription (Howell, 1984, 1985). These regions contain inverted repeats capable of forming potential hairpin structures, the most stable of which are in the SIR. It has been suggested that they could be involved in replication or regulation of transcription or be

sites for virus assembly (Mullineaux *et al.*, 1984; MacDowell *et al.*, 1985). The number and position of these hairpin loop structures varies from virus to virus, but the sequence TAATATTAC has been identified in the loop of one hairpin structure in each of MSV-N, MSV-K, MSV-S, DSV, WDV and CSMV, and also the whitefly-transmitted geminiviruses ACMV, BGMV and TGMV (Figure 17, section 4.8.1). This has led to the much vaunted theory that it may function as a recognition sequence for a protein involved in ssDNA replication, analogous to the primosome assembly site of the ssDNA phage ϕx174 (MacDowell *et al.*, 1985; Rogers *et al.*, 1986). In addition, a series of short DNA molecules complementary to part of the TIR have been found for MSV, DSV, WDV and CSMV (Donson *et al.*, 1984, 1987; Howell, 1984, 1985; Hayes *et al.*, 1988c; Andersen *et al.*, 1988) and these are proposed to be involved in priming second strand synthesis. This will be considered in the next section when the possible mechanism of geminivirus replication is discussed.

The nucleotide sequences of MSV, DSV, WDV and CSMV have been screened for potential promoter and polyadenylation signals (Mullineaux *et al.*, 1984; Donson *et al.*, 1987; MacDowell *et al.*, 1985; Andersen *et al.*, 1988). The consensus promoter sequence for animal genes is TATAA/TAA/T (Breathnach & Chambon, 1981), whereas that for plant genes, based mainly on dicotyledonous plant species, is TC/GTATATAA$_{1-3}$C/TA (Messing, 1983). Therefore the sequences were initially screened only for the core 'TATA' sequence and each of the conserved ORFs was found to have at least one TATA box within the region 250 nucleotides upstream of the initiation codon. Consensus polyadenylation signals for plant genes (G/AATAA) (Messing, 1983) were also found within a region 200 nucleotides downstream of the termination codons of most ORFs. As with the whitefly-transmitted viruses the putative polyadenylation signals are situated within AT-rich regions (AT-boxes) in which pairs of polyadenylation signals in opposite directions form, or are part of, inverted repeats (Figure 19). CSMV is the one exception to this: it has only one polyadenylation signal in the AT-box corresponding to ORF L1a and OR L1b (Andersen *et al.*, 1988). However, there is still a short inverted repeat present, capable of forming a hairpin structure.

A bidirectional mode of transcription has been suggested for the

leafhopper-transmitted geminiviruses, due to the arrangement of the four conserved ORFs and their promoter and polyadenylation signals on both DNA strands of the genome, converging on the TIR (Mullineaux *et al.*, 1984). This is similar to the arrangement of genes in the whitefly-transmitted geminiviruses ACMV, TGMV and BGMV (Section 4.8.1) and the papovavirus SV40 (Tooze, 1980). Morris-Krsinich *et al.* (1985) confirmed the bidirectional transcription model for MSV-N when they found one major and two minor polyadenylated RNAs from MSV-N-infected maize leaves and mapped them to the viral and complementary DNA strands (Figure 22). Two of the transcripts, a major 0.9 kb RNA and a minor 1.05 kb RNA, were mapped on the viral sense DNA and the other minor transcript of 1.2 kb was mapped on the complementary sense DNA. The 1.05 kb transcript is potentially dicistronic, expressing both the ORF R0 protein and the coat protein, but it is more likely to express only the ORF R0 protein, a strategy like many (+) strand RNA plant viruses, which have polycistronic genomes but translate only the 5' proximal ORF in the full-length genomic RNA (Davies & Hull, 1982; Dougherty & Hiebert, 1985). The untranslated leader sequence preceding the AUG initiation codon of ORF R0 is only four bases long (AUUCAUGG) (Morris-Krsinich *et al.*, 1985) and it doesn't conform to the consensus sequences flanking the AUGs of genes from animals (CCA/GCCAUGG) (Kozak, 1984) or plants (AACAAUGGC) (Lutcke *et al.*, 1987), although this does not rule out the expression of ORF R0 from this transcript (Kozak, 1986). Furthermore, Morris-Krsinich *et al.* (1985) found a TATA box sequence conforming to the consensus sequence for eucaryotic promoters TATAA/TAA/T (Breathnach & Chambon, 1981), situated 20 bases upstream of the 5' end of the 1.05 kb transcript, but no CAAT box (Benoist *et al.*, 1980).

If the ORF R0 protein is expressed from the 1.05 kb transcript then the coat protein is likely to be expressed from the more abundant 0.9 kb mRNA, whose 5' terminal is situated in the middle of ORF R0. This transcript has the sequence CA/GCCAUGU flanking the initiation codon, which compares favourably with the consensus sequence for animal genes - CCA/GCCAUGG (Kozak, 1984), but there are no TATA or CAAT boxes immediately preceding the 5' terminal (Morris-Krsinich *et al.*, 1985). However, the 0.9 kb transcript could be

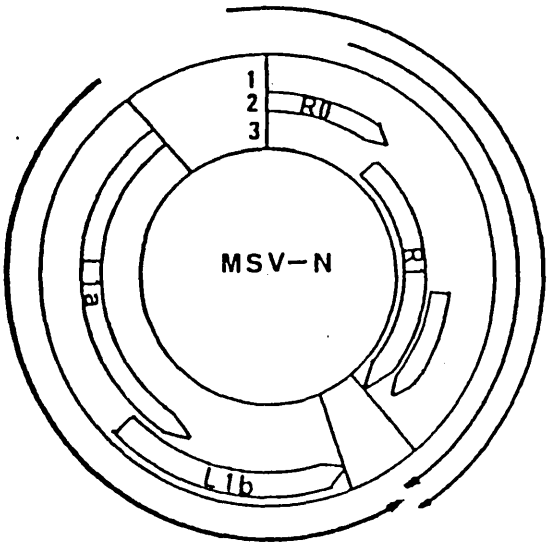


Figure 2 2 Major transcripts of MSV-N

The ORFs conserved in the leafhopper-transmitted geminiviruses are shown as open arrows. The two non-coding regions are shown boxed. The major transcripts referred to in the text are shown as thick arrows on the outside the map.

promoted from one of several T-rich regions 15-60 bases upstream from its 5' end, and Fenoll *et al.* (1988) have demonstrated an upstream activating sequence (UAS), situated in the SIR, which enhances promoter activity in maize protoplasts. The UAS includes the hairpin structure containing the invariant sequence TAATATTAC found in all geminiviruses. However, Fenoll *et al.* (1988) suggest that a GC-rich sequence immediately upstream of the hairpin loop structure may be the essential part of the UAS and not the hairpin loop itself.

The UAS has been tested in various positions and orientations relative to the 5' end of the 0.9 kb transcript, and in single and multiple copies, and it was found that altering its position abolished promoter activity, but reversing its orientation only reduced promoter activity (Fenoll *et al.*, 1988). Multiple tandem copies were more active than a single copy, in both orientations, and the UAS is interchangeable with the analogous distal region (DR) of the CaMV 35S promoter, although the MSV promoter UAS specifically binds proteins in maize nuclear extracts which appear to differ from those bound by the CaMV 35S DR in the same extract (Fenoll *et al.*, 1988).

Morris-Krsinich *et al.* (1985) mapped the 3' terminals of both MSV-N virus-sense transcripts to the same nucleotide, which is 57 bases downstream of a consensus polyadenylation signal (AATAAA) (Breathnach & Chambon, 1981). This 3' end is nine bases from the 3' end of the major species of 'primer-like' DNA molecules found on virion DNA (Donson *et al.*, 1984). The 5' and 3' ends of the 1.2 kb complementary-sense transcript have only been mapped approximately, due to the low abundance of the RNA (Morris-Krsinich *et al.*, 1985). A TATA box has been found approximately 70 bases upstream of the 5' end and an AATAA polyadenylation signal has been found approximately 100 bases upstream of the 3' end. This transcript could encode the putative product of ORF L1b, but its 5' end appears to map slightly downstream of the first ATG of ORF L1a. However, another in-frame ATG 99 bases downstream was covered by this transcript. So, translation from this codon would produce a protein of M_r 27,675 instead of M_r 31,388 from the upstream ATG (Morris-Krsinich *et al.*, 1985).

As mentioned earlier in this section, the genome of BCTV has been

sequenced and consists of one infectious ssDNA component of 2993 nucleotides (Stanley *et al.*, 1986). The sequence was screened in both DNA senses and in all three reading frames for ORFs encoding putative proteins larger than 10,000 Daltons, and six were found: two in the virus sense and four in the complementary sense. The pattern of these ORFs resembles that of DNA A of the whitefly-transmitted viruses, rather than that of the other leafhopper-transmitted viruses (Figure 23). Four of these ORFs have counterparts in the whitefly-transmitted viruses (AR1, AL1, AL2 and AL3) and for the purpose of this thesis they will be known as R1, L1, L2 and L3, respectively, and their sizes are shown in Table 10. The coat protein has been assigned to ORF R1 by analogy with ORF AR1 in ACMV, which has been definitely identified as the coat protein gene (Townsend *et al.*, 1985).

Computer analysis of the known geminivirus nucleotide sequences revealed significant homology between the genome of BCTV and the DNA A components of the whitefly-transmitted viruses (Stanley *et al.*, 1986). The most highly conserved sequences are found within the complementary sense coding regions and the carboxy terminal of the putative coat protein gene, ORF R1. However, the amino acid sequence of the BCTV coat protein showed a higher degree of homology with the coat proteins of MSV and WDV than ACMV (Stanley *et al.*, 1986), which supports the view of a relationship existing between the geminivirus coat protein and the insect vector (Harrison, 1985; section 4.3). Of the remaining BCTV ORFs that have counterparts in the whitefly-transmitted viruses, the largest complementary-sense ORF, L1, is the most highly conserved, reflecting the stringent amino acid sequence conservation within this ORF in all whitefly-transmitted viruses and the analogous OR(F)s L1a and L1b in all leafhopper-transmitted viruses.

The arrangement of the conserved ORFs of BCTV in relation to potential promoter and polyadenylation signals is suggestive of bidirectional transcription, although details of the transcripts are not yet available. In common with the other geminiviruses examined so far, a direct repeat sequence capable of forming a stable stem-loop structure, and containing the sequence TAATATTAC, was identified in the large intergenic region (see Figure 17). Also within this

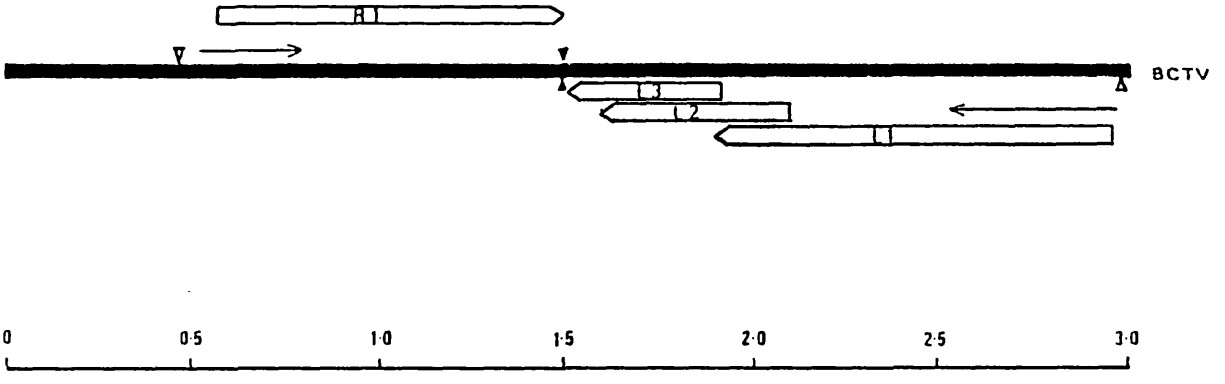


Figure **23** Open reading frames of the whitefly-transmitted monopartite geminivirus beet curly top virus

The circular genome is represented in a linear form. Position 1 is taken as the first T of the invariant sequence. Open reading frames in common with the bipartite geminiviruses are indicated by large open arrows, non conserved open reading frames are drawn as thin arrows. White and black triangles indicate the positions of putative promoter and polyadenylation signals, respectively.

region was identified a 27-nucleotide direct repeat sequence which Stanley *et al.* (1986) likened to the 72-nucleotide transcript enhancer sequence of SV40 (reviewed by Das *et al.*, 1985).

Lazarowitz (1987) described BCTV as the 'missing link' between the monocotyledon-infecting, leafhopper-transmitted geminiviruses and the dicotyledon-infecting, whitefly-transmitted geminiviruses. The molecular characterisation of BCTV has shown that the number of components in the geminiviruses genome is a reflection of the insect vector and not the type of host plants of the virus.

The hybrid nature of the BCTV genome has raised the question of how the members of the different sub-groups of the geminiviruses have evolved. On the basis of the similar organisation and limited amino acid sequence homology, Kikuno *et al.* (1984) suggested that the two components of the whitefly-transmitted geminiviruses had evolved from a common ancestral DNA. Further examination of the nucleotide and amino acid sequences of ACMV, TGMV, BGMV, MSV-N and WDV, and estimations of the divergence of the putative genes (Howarth & Goodman, 1986) supported this view and suggested that geminiviruses with mono- and bipartite genomes were ancestrally related. Stanley *et al.* (1986) have suggested that BCTV could have evolved as a leafhopper-transmitted, single component virus before the development of the bipartite genome of the whitefly-transmitted viruses and that the genome organisation was maintained by the limitations imposed by common hosts. However, it cannot be ruled out that BCTV evolved as a bipartite virus which then lost one component as a result of the change of insect transmission vector.

4.9 Geminivirus Replication

The nucleus of infected plant cells has been implicated as the site of geminivirus DNA replication, from electron microscopy (Kim *et al.*, 1978; Francki *et al.*, 1985) and studies with isolated nuclei (Coutts & Buck, 1985). However, the mechanism of replication is not well understood, although there has been much speculation and comparison with other well characterised systems, for example the ssDNA bacteriophages, SV40, porcine circovirus (PCV) and the minute virus of mouse (MVM) (Stanley & Davies, 1985). The geminiviruses do

not necessarily replicate by any of these strategies, but a comparison of the otherwise unrelated CaMV with the retroviruses and hepatitis B virus (HBV) resulted in the proposal of a CaMV replicative cycle via reverse transcription of an RNA intermediate (Hull & Covey, 1983), so a comparison of the features of the geminivirus genome in common with other viruses could help elucidate their method of replication.

The very nature of the geminivirus genome as circular ssDNA has invited comparison with PCV and ssDNA bacteriophages ΦX174, G4 and fd (closely related to M13). Relatively little is known about the replicative cycle of PCV, but it is the sole representative of the animal viruses shown to have a genome of circular ssDNA. Also, the size of the PCV genome is exceptionally small (1.8 kb) compared with those of other DNA-containing viruses and so it may be multipartite, a further analogy with the geminiviruses. Unlike the geminiviruses, however, PCV DNA is encapsidated within single isometric particles.

The replicative mechanisms of the ssDNA bacteriophages, which have been extensively studied (reviewed by Kornberg, 1980, 1982), will initially be described below and then the common features and information currently available for the mechanism of geminivirus replication will be discussed.

4.9.1 Replication of ssDNA Bacteriophages

Replication of the circular ssDNA bacteriophages ΦX174, G4 and fd is generally considered as three distinct stages: i) conversion of the infecting viral (+)-strand into a double-stranded replicative form (SS→RF); ii) rolling circle replication of the replicative form (RF→RF); iii) production of the encapsidated viral ssDNA (RF→SS) (reviewed by Kornberg, 1980, 1982).

Following infection of *E. coli* cells with ΦX174, fd or G4, the ssDNA ((+)-strand) becomes covered with host encoded ssDNA binding proteins (SSB). In fd and G4 a stem-loop structure remains exposed. The hairpin loop of fd is able to bind *E. coli* RNA polymerase, which then synthesises a short RNA molecule to serve as a primer for DNA polymerase III-catalysed second-strand synthesis. In G4 infection, the host dnaG gene product (primase) binds to the hairpin loop and

synthesises the primer. ΦX174 is closely related to G4, but a deletion in the genome of ΦX174 serves to remove the primase binding site found in G4. Therefore, in ΦX174 (Figure 24), second-strand synthesis is initiated by the binding of a complex of six proteins (the products of dnaB and dnaC genes, the proteins n, n', n'' and i) called the preprimosome, following the recognition of a stem-loop structure by the n' protein. The subsequent binding of primase completes the primosome. The n' and *dnaB* proteins then propel the primosome around the ΦX174 genome in a 5'→3' direction (i.e. anti-elongation direction), displacing SSBs and allowing the primase to synthesise short RNA primers at regular intervals, for extension by *E. coli* DNA polymerase III. When (-)-strand synthesis is complete the primers are removed and replaced with DNA by the nick-translation action of DNA polymerase I, and the RF covalently closed by DNA ligase. The primosome remains attached to the RF which is then probably converted into a negatively supercoiled form by DNA gyrase before being transcribed.

Synthesis of the second-strand of RF DNA, described above, involves only host encoded proteins, but replication of the RF requires a phage-encode protein - the gene A protein of ΦX174 and G4, and gene 2 protein of fd. This protein is an endonuclease-ligase which recognises and nicks the (+)-strand of the RF at a specific site (*ori*) within its own gene. In ΦX174 RF multiplication, the (+)-strands are synthesised continuously by a rolling-circle mechanism (Figure 25). The gene A protein binds to the free 5' end at the nick and the 3'-OH end acts as a primer for DNA polymerase III-catalysed synthesis of a new (+)-strand. The *E. coli rep* protein acts at the replication fork and unwinds the dsDNA helix exposing the (-)-strand to act as a template for synthesis of the new (+)-strand (the original (+)-strand is displaced). The conserved primosome is thought to act as a helicase and assist the *rep* protein in unwinding the dsDNA and may subsequently act to initiate complementary strand synthesis on the displaced (+)-strand template. On completion of the synthesis of the new (+)-strand, the gene A protein cleaves the regenerated origin of replication and ligates and releases the original viral strand which has served as a template for primosome-initiated synthesis of the complementary (-)-strand. The gene A

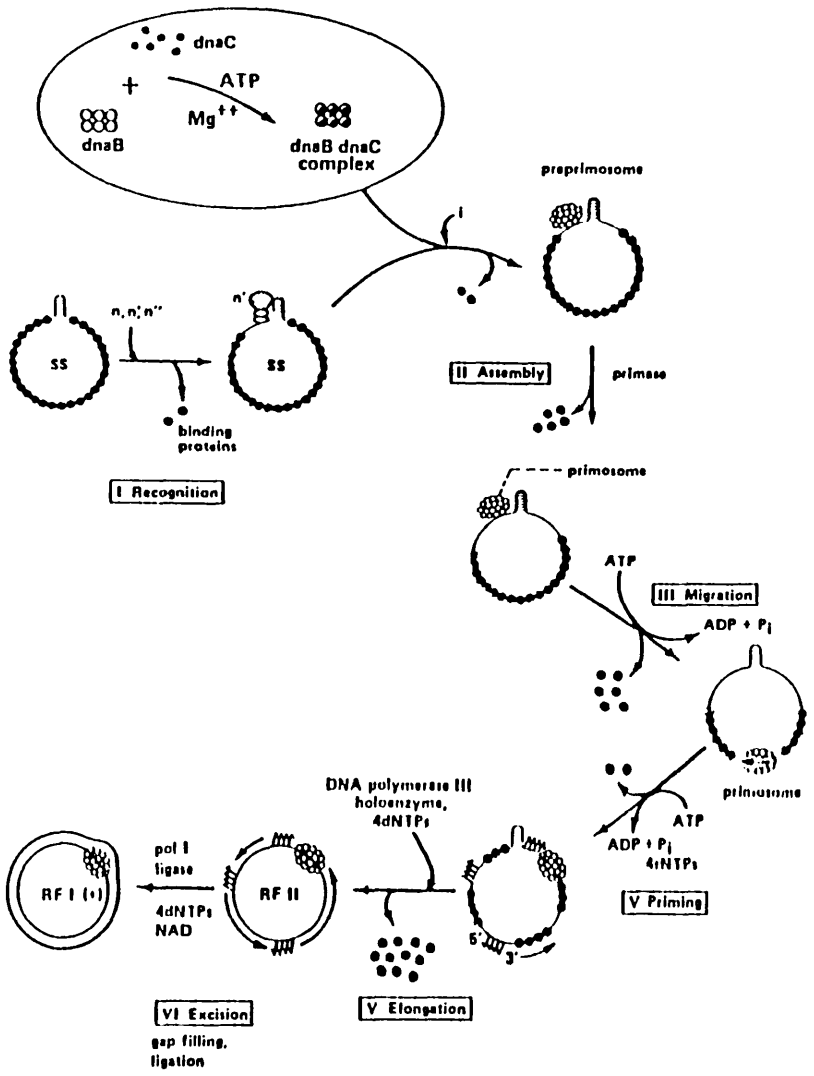


Figure 24 Schematic representation of φX174 DNA replication; ssDNA → RFDNA

(Reproduced from Kornberg (1982)).

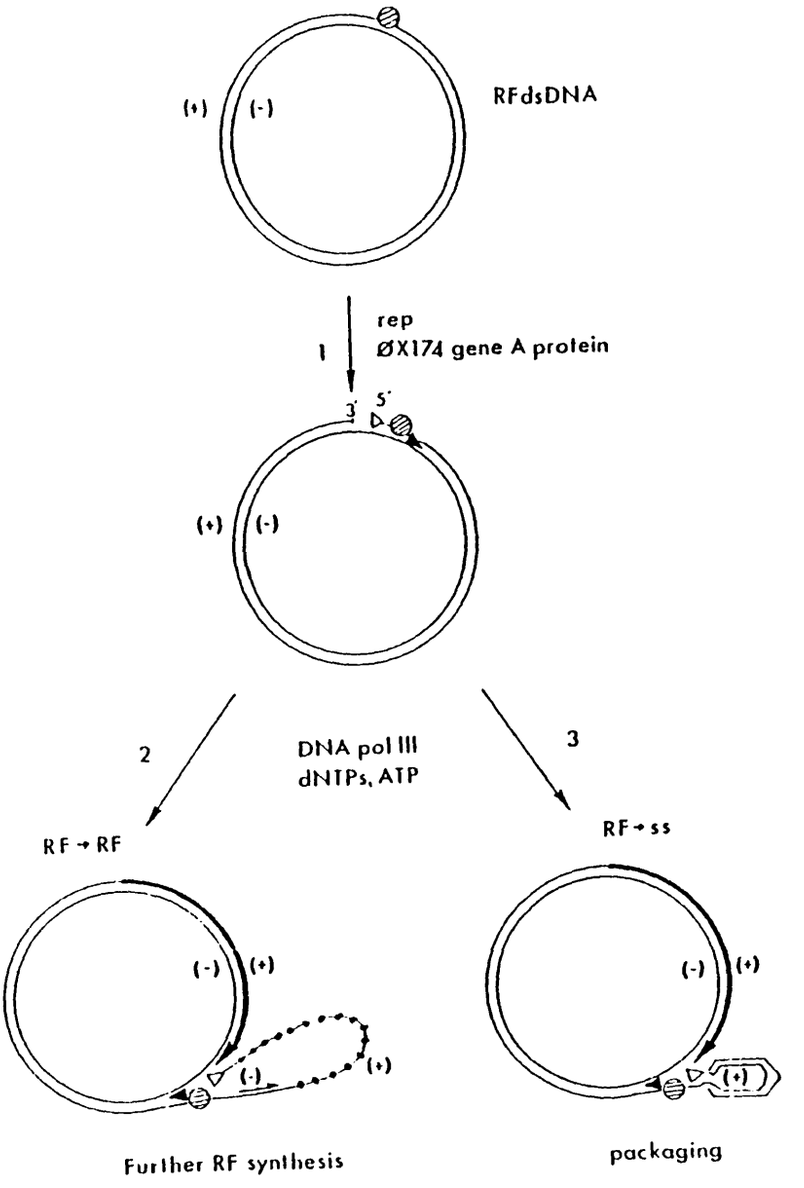


Figure 25 Schematic representation of φX174 DNA replication; RF → RF and RF → ssDNA

1) Nicking and unwinding of RFdsDNA (with conserved primosome; shaded circle) by φX174 protein A (open triangle) and *E.coli* rep protein (black triangle) to allow the initiation of DNA synthesis from the 3' OH terminus. 2) DNA polymerase III synthesises a new (+)-strand on the exposed (-)-strand, while the unwound (+)-strand serves as a template for new (-)-strand synthesis. Progeny RF molecules are formed by this rolling-circle mechanism, newly synthesized strands are shown as heavy lines. 3) Formation of ssDNA: new (+)-strand synthesis proceeds as in (2), but the unwound (+)-strand is cut and joined by protein A and then packaged to form mature φX174 particles (from Kornberg 1980).

protein is linked anew to the 5' end of the parental RF to function in another round of replication. The parental RF with its conserved primosome is thought to be the only RF functional in replication, the other numerous supercoiled RFs serving as transcription templates.

The final stage of the replication cycle is the production of ss (+)-strands. At this stage for ΦX174, seven phage-encoded proteins have accumulated in the *E. coli* cell and function in the assembly of ΦX174 virions. The ssDNA (+)-strands are produced by the same rolling-circle mechanism described above, except this time the (+)-strands are encapsidated in preformed capsid assemblies to form mature virions, and are therefore unavailable as templates for (-)-strand synthesis (Figure 25). In the case of fd and M13, the transition from RF→RF synthesis to RF→SS is determined by the accumulation of the gene 5 protein, which binds to the ssDNA, preventing re-initiation of (-)-strand synthesis.

4.9.2 Replication of Geminivirus DNA

Ikegami *et al.* (1981) were the first to show that geminivirus-infected plants contain virus-specific, circular dsDNA as well as circular ssDNA. This discovery led them to suggest that the dsDNA was a replication intermediate and this has become an integral part of the much vaunted theory of geminivirus replication as a three-stage process: i) the conversion of virion ssDNA to a double-stranded form; ii) the proliferation of the dsDNA, and iii) the production virion ssDNA from this dsDNA intermediate (Stanley, 1985; Davies *et al.*, 1987). This strategy is, in outline at least, the same as that of the ssDNA 'phages mentioned in the previous section. However, little is known about the mechanisms involved in these processes and that which is known will be reviewed in the rest of this section.

The synthesis of the second strand on the virion ssDNA template is thought to require only host factors, as deproteinised virion ssDNA from BGMV (Goodman, 1977a, b), TGMV (Hamilton *et al.*, 1981) and MYMV (Ikegami *et al.*, 1984) has been shown to be infectious. As both DNA components of a bipartite genome are likely to be replicated in the same way, it has been suggested that the host factors recognise similar features on each component. Hence, the 'common region' has

been implicated as containing the origin of replication (Stanley & Gay, 1983). Sequence homologies between the respective 'common regions' of these viruses are limited to the highly conserved hairpin loop structures, containing the invariant sequence, TAATATTAC, in the loop (Figure 17) and these structures have been likened to the primosome assembly site on 'phage ΦX174 ssDNA (Hamilton *et al.*, 1984), at which solely host factors initiate second strand synthesis (Arai & Kornberg, 1981; section 4.9.1).

The leafhopper-transmitted viruses MSV, DSV, WDV and CSMV contain a population of small DNA molecules, approximately 80 bases long and complementary to part of the TIR, associated with virus particles (Donson *et al.*, 1984, 1987; Hayes *et al.*, 1988c; Andersen *et al.*, 1988). These molecules have a variable 3' terminal and several ribonucleotides covalently bound to the 5' terminal of the DNA. They can prime second strand synthesis *in vitro*, producing full-length dsDNA which on subsequent cloning is infectious in plants (Donson *et al.*, 1984, 1987). No evidence has been found of these small DNA molecules in ACMV DNA (Stanley & Townsend, 1985), which has led to the suggestion that they are a characteristic feature of the leafhopper-transmitted, monocotyledon-infecting geminiviruses. It is interesting to note that ACMV and the other dicotyledon-infecting viruses lack the TIR within which the MSV, DSV, WDV and CSMV small DNA molecules bind.

Hayes *et al.* (1988c) have proposed a possible mechanism for the formation of these small DNA molecules which involves the viruses encoding their own primases. Since conversion of ssDNA to dsDNA is a prerequisite for viral transcription and hence formation of virus-encoded proteins in the early stages of an infection cycle, primase would need to be synthesised during a previous infection cycle and priming take place prior to encapsidation. The function of the primase would be to synthesise a short RNA molecule to act as a primer for a DNA polymerase (Kornberg, 1980). Formation of a short double-stranded region could then be a signal for encapsidation. Termination of complementary strand synthesis as a result of encapsidation would be expected to be imprecise and could explain the heterogeneity of the 3' ends of the small DNA molecules. The small DNA molecules would then act as primers for second strand synthesis

during a subsequent infection. The obvious candidate for the virus-encoded primase is the product of ORF R0 in MSV, DSV, WDV and CSMV (Mullineaux *et al.*, 1984; Donson *et al.*, 1987; MacDowell *et al.*, 1985; Andersen *et al.*, 1988), which has no counterpart in the dicotyledon-infecting viruses.

The mechanism involved in the proliferation of the virus dsDNA molecules has not yet been demonstrated, although the possibility of a rolling circle mechanism being used, analogous to that used by ssDNA 'phages, has been suggested (Stanley & Townsend, 1985). Analysis of plants infected with the cloned components of ACMV and TGMV has revealed dimeric and trimeric dsDNA forms of each component, suggesting that they might be intermediates in the replication of viral RF DNA (Hamilton *et al.*, 1982; Stanley & Townsend, 1985; Slomka *et al.*, 1988). However, the possibility that they occur through intermolecular recombination cannot be ruled out.

Subgenomic dsDNA has been isolated from tissue infected with ACMV (Stanley & Townsend, 1985; Coutts & Buck, 1987), TGMV (MacDowell *et al.*, 1986), PYMV (Roberts *et al.*, 1988) and WDV (Macdonald *et al.*, 1988). The subgenomic DNAs are derived by relatively specific deletions of approximately half the DNA B component of ACMV, TGMV and PYMV, and approximately half the single DNA component of WDV. It is interesting to note that the subgenomic DNAs retain the hairpin loop structures containing the conserved TAATATTAC sequence, which is thought to be important for DNA replication. Also, WDV subgenomic DNA retains the 3' end (in the virion DNA sense) of a sequence to which small DNA molecules are bound (Hayes *et al.*, 1988c) and which may be important for the priming of DNA synthesis *in vivo* (Macdonald *et al.*, 1988). Although the subgenomic DNAs can replicate in the presence of genomic DNAs, it is thought that they are non-functional in virus replication, since they are not detected after multiple passage of ACMV from plants originally inoculated with the cloned DNA components (Stanley & Townsend, 1985). However, they are detected after the second passage of TGMV from plants infected with cloned DNA (MacDowell *et al.*, 1986). Infectivity experiments using increasing concentrations of the subgenomic DNA have demonstrated that these molecules delay the formation of symptoms (Townsend *et al.*, 1985; MacDowell *et al.*, 1986) and are probably analogous to defective

interfering nucleic acids of animal viruses (Huang & Baltimore, 1977; Holland, 1985).

The next stage of the infection cycle of ssDNA viruses prior to or concomitant with encapsidation, is the synthesis of viral ssDNA using the complementary strand of the dsDNA replicative form as a template. This usually requires one or more virus-encoded proteins. The replicative origin for this synthesis may overlap with that involved in second strand synthesis, as in M13, or may be distinct, as in G4 and ΦX174 (Meyer *et al.*, 1978; Fiddes *et al.*, 1978). Rogers *et al.* (1986) have suggested that the hairpin loop structure containing the conserved TAATATTAC sequence, which is found in all the geminiviruses characterised to date, could function as the recognition sequence for a virus-encoded protein analogous to the ΦX174 gene A protein.

As well as the concatameric dsDNA forms found in infected plants, variable amounts of concatameric ssDNA have been found in virus preparations of ACMV and MSV (Stanley & Townsend, 1985). These molecules consist predominantly of non-covalently bound dimers of ssDNA as well as the true dimeric ssDNA. The origin of the former is not clear, as there are no major intra- or intermolecular inverted repeats to account for this. However, it was suggested that residual proteins bound to the ssDNA may be responsible for the aggregation. The true dimeric ssDNAs could have occurred either from incomplete strand scission during a rolling replication cycle or by replication of a dimeric dsDNA template (Stanley & Townsend, 1985). As with the dimeric dsDNA, the ssDNA concatamers are routinely observed in infections using cloned ACMV components.

The delimitation of genes essential for geminivirus replication started with the demonstration of the ability of the DNA A components of ACMV and TGMV to replicate autonomously (Townsend *et al.*, 1986; Rogers *et al.*, 1986), thus showing that the DNA A component encodes all of the proteins required for viral DNA replication. Recently, Elmer *et al.* (1988) have shown that TGMV ORF AL1 is the only ORF required for viral DNA replication. They agroinfected petunia leaf discs using an *Agrobacterium tumefaciens* Ti plasmid vector containing DNA A components, with various mutations in ORFs AL1, AL2 and AL3, between the T-DNA borders. The plants that grew from the leaf discs

were subsequently checked for the presence of viral ss- and dsDNAs, and these were found in those agroinfected with the ORF AL2 and AL3 mutants but not with the ORF AL1 mutants, implying that mutations in ORF AL1 totally abolish TGMV DNA replication.

As mentioned in section 4.8.2, there are significant amino acid homologies between the product of TGMV ORF AL1 and the putative products of ACMV ORF AL1 and MSV-N, DSV, WDV and CSMV OR(F)s L1a and L1b, and it has been suggested that OR(F)s L1a and L1b could encode a composite protein, analogous to the ORF AL1 products of the whitefly-transmitted viruses (MacDowell *et al.*, 1985). No evidence is available yet to show that this putative composite protein is required for viral DNA replication. Nonetheless, it is interesting to note that the subgenomic DNA found in WDV-infected wheat tissue contains the whole of ORF L1a and OR L1b (Macdonald *et al.*, 1988), and although the replication of WDV subgenomic DNA may rely on trans-acting factors encoded by genomic DNA, it would not be surprising if the product(s) of ORF L1a and/or OR L1b allowed the subgenomic DNA to replicate autonomously.

4.10 Development of Geminivirus-based Vectors

Considerable progress has been made over the last five years in the development of Ti plasmid based vectors (reviewed by Fraley *et al.*, 1986; Lichtenstein & Fuller, 1987; Schell, 1987) and these are now used routinely for the introduction of foreign genes into plants. Transformed plants are capable of normal growth and the foreign genes are stably inherited. Similarly, foreign genes have been introduced directly without the use of a vector, by treating protoplasts with the polyvalent cation polyethylene glycol (PEG) (Paszkowski *et al.*, 1984; Negrutiu *et al.*, 1987) or by electroporation (Fromm *et al.*, 1985).

These methods of transformation have allowed the analysis of sequences controlling expression (i.e. promoters, enhancers, silencers) of a number of genes, including photosynthetic genes [e.g. the small subunit of ribulose 1,5 bisphosphate carboxylase (SS *rbcS*) (Herrera Estrella *et al.*, 1983; Timko *et al.*, 1985) and the light harvesting chlorophyll a/b binding proteins (cab) (Simpson *et al.*,

1985)], organ-specific and stress-regulated genes [e.g. the major storage proteins of potato tubers (patatin) (Rosahl *et al.*, 1987) and proteinase inhibitor II genes of potato (Sanchez-Serrano *et al.*, 1987; Thornburg *et al.*, 1987)]. In each case the regulatory sequences of the gene being studied were fused to a 'reporter' gene thereby allowing the level of expression to be examined by determining the level of reporter gene activity. Commonly used reporter genes include the chloramphenicol acetyl transferase (CAT) gene from *Tn9* (activity assayed by determining the conversion of ^{14}C-chloramphenicol to its acetate derivative using thin layer chromatography); neomycin phosphotransferase II (*neo*) from *Tn5* (detected by *in situ* phosphorylation of kanamycin (Reiss *et al.*, 1984)). More recently the firefly luciferase gene (catalyses production of light) (Ow *et al.*, 1986) and *E. coli* β-glucuronidase (GUS) genes (assayed fluorometrically) (Jefferson *et al.*, 1987) have been developed as efficient reporter genes.

However, in all studies of foreign genes introduced using the Ti plasmid vectors it takes a number of weeks from the initial transformation event until the progeny plants or tissue are available for analysis. Therefore it would be useful to have more rapid systems and also systems to give gene amplification. In this respect interest has been directed at developing vectors based on plant viruses which would be capable of replicating to a high copy number. The two groups of plant DNA viruses; caulimoviruses and geminiviruses, have attracted the most attention (Hohn *et al.*, 1987; Buck & Coutts, 1983; Coutts *et al.*, 1987) although vectors have also been developed which are based on the RNA viruses TMV and BMV (French *et al.*, 1986; Takamatsu *et al.*, 1987). However, a major disadvantage of using RNA virus-based vectors (and perhaps CaMV-based) is that the lack of proof-reading in the replication mechanism results in a higher error frequency than that of replication via DNA and therefore questions the stability of non-selected sequences in the RNA genome (Van Vloten-Doting *et al.*, 1985).

Attention was initially directed at the caulimovirus CaMV (Hull & Davies, 1983) and this was used successfully to construct a replacement vector which when used to systemically infect turnip plants conferred resistance to methotrexate (Brisson *et al.*, 1984).

However, several features of the CaMV genome (Figure 26) and replicative cycle (Figure 27) have imposed limitations on the development of useful viral vectors: 1) encapsidation is required for systemic spread of CaMV which imposes packaging constraints on any recombinant molecules constructed; 2) *in vitro* mutagenesis studies (Daubert *et al.*, 1983; Dixon *et al.*, 1983) have revealed only two ORFs (II and VII) to be dispensable for systemic virus spread, and hence able to be replaced by a foreign gene; 3) the ORFs are tightly arranged and a 'relay-race' model of translation has been proposed (Sieg & Gronenborn, personal communication, cited in Dixon & Hohn, 1984) whereby the ribosome after completing translation of one ORF remains associated with the mRNA and resumes translation at the next initiation codon. This necessitates that foreign sequences inserted in place of either ORF II or ORF VII have to be tailored exactly so as not to disrupt translation of the following ORF; and 4) the mode of replication via reverse transcription (Figure 27) results in a high mutation/recombination rate (Lebeurier *et al.*, 1982; Choe *et al.*, 1985) probably due to the template switching involved. This has prevented the development of replacement vectors which require complementation of the defective CaMV by a helper virus.

Therefore attention has turned to the development of vectors based on the geminiviruses (Buck & Coutts, 1983; Coutts *et al.*, 1987). These are attractive since they are small and easily manipulated and as a group infect a wide range of mono- and dicotyledonous plants including several agriculturally and horticulturally important plants (section 4.4; Table 8). Recently, gene replacement vectors have been constructed from the genomes of ACMV and TGMV that are capable of replicating and spreading systemically in whole plants (Ward *et al.*, 1988; Hayes *et al.*, 1988e). As the coat protein is not required for DNA replication, and encapsidation is not a prerequisite for systemic spread, ORF AR1 was chosen to be replaced with a foreign gene. In the case of ACMV, the coat protein gene on DNA A was replaced with the CAT gene (Ward *et al.*, 1988), and *N. benthamiana* plants were mechanically inoculated with excised, unit length dsDNA copies of this DNA A construct, in the presence of DNA B. The plants developed severe, systemic symptoms within ten days and the systemically-infected leaves were

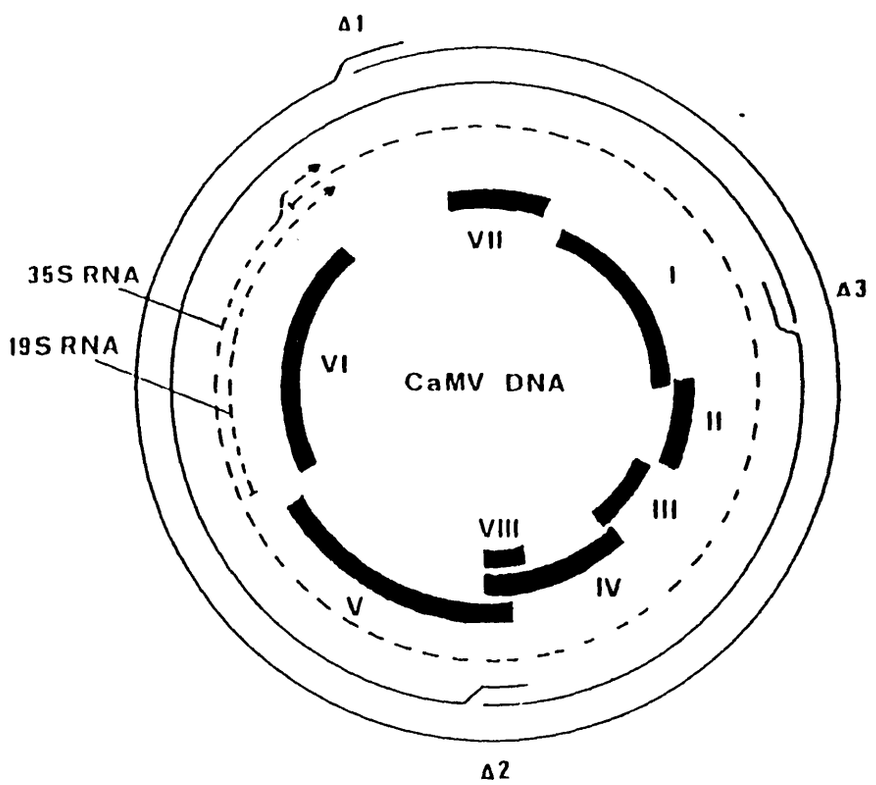


Figure 26 The CaMV Genome

The discontinuities in the double-stranded virion DNA are indicated by open triangles, and the CaMV ORFs as heavy black arrows. The major polyadenylated RNA transcripts are shown as broken lines.

Legend to Figure 27 CaMV DNA Replication

Encapsidated DNA (gapped) is converted to the supercoiled DNA in the nucleus (1) and transcribed to produce the 35S RNA (thin black line) (2). First strand ((-)strand) synthesis by reverse transcriptase is primed by binding of a tRNA at a site (PBS) corresponding to Δ1 near the 5' end of the 35S RNA (3). When the reverse transcriptase reaches the 5' end of the template (4), the extending DNA (-)-strand (heavy black line) switches to the 3' terminal repeat sequence of the same template for elongation to continue (5). A simultaneous RNase H activity digests the RNA template (5, 6, 7). Poly purine tracts (PPT) in viral RNA are believed to serve as primers for synthesis of the (+)- strand DNA, thereby defining Δ2 and Δ3 in the virion DNA (6, 7). DNA synthesis continues (8) and the tRNA is removed to produce the gapped DNA (9). This can either be encapsidated or supercoiled (10) for further transcription.

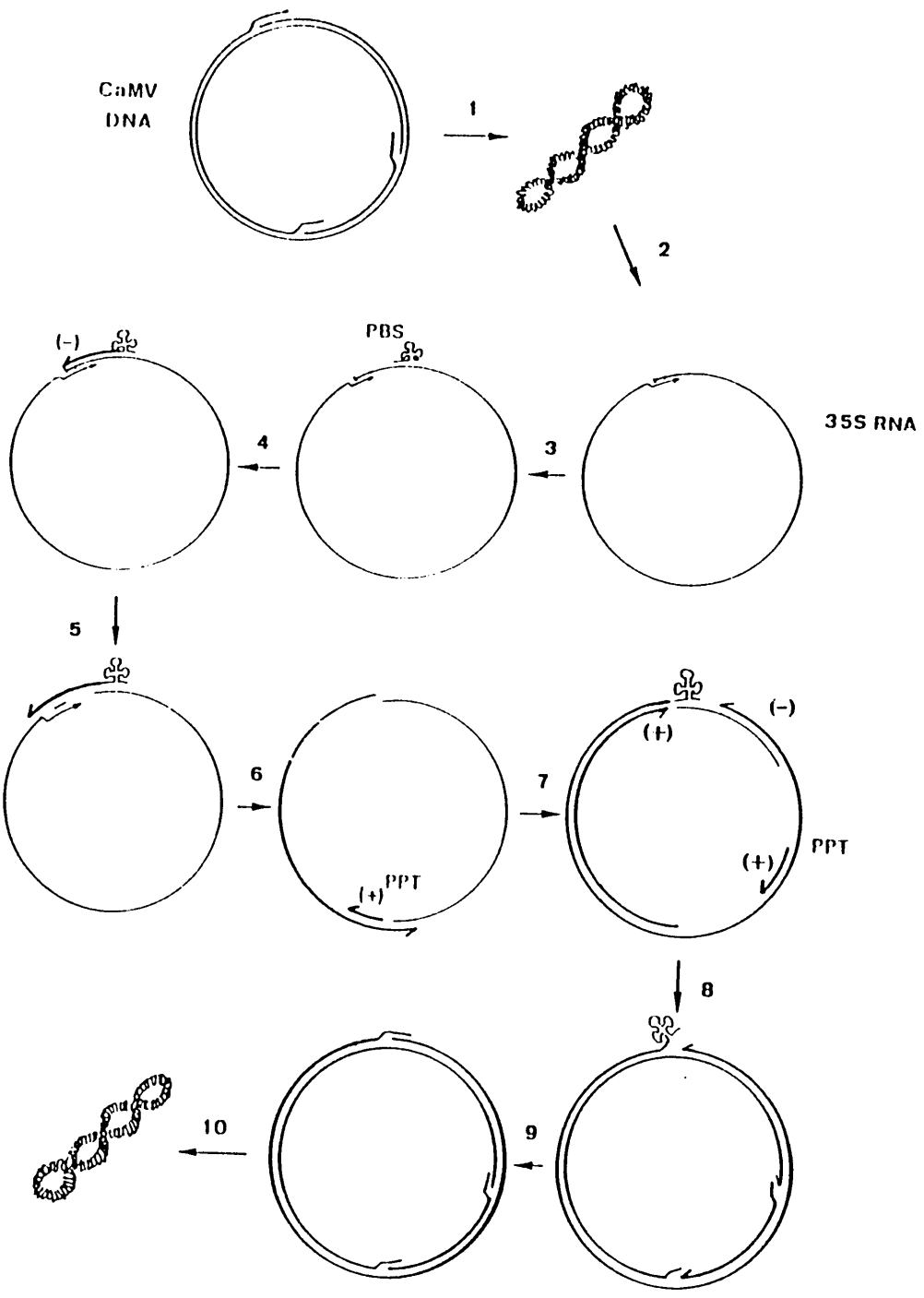


Figure 27 CaMV DNA Replication

shown to contain the DNA A construct in the expected single- and double-stranded forms (by Southern blot analysis) and the CAT enzyme (by Western blot analysis), with an activity of approximately 80 U/mg of soluble protein. Similar levels of activity were found after transmission of the viral DNA by mechanical inoculation of sap expressed from infected leaves, suggesting the construct to be reasonably stable within the plant.

Although the production of CAT provides a sensitive assay for viral DNA replication, the uses of this vector in *N. benthamiana* are limited, because it elicits severe symptoms, and in its present form it is not heritable. However, Hayes *et al.* (1988e) have constructed a gene replacement vector from TGMV, which can be used not only for amplification of gene expression by the systemic infection of plants, but also for heritable gene amplification by the integration of stable master copies of the vector into the plant chromosomal DNA. The vector was constructed as follows: a partial dimer of TGMV DNA A was constructed that contained two copies each of ORF AL1 and the 'common region', both of which are thought to be essential for viral DNA replication (Elmer *et al.*, 1988; Hamilton *et al.*, 1984). The duplications are necessary to ensure formation of monomeric replicating DNA molecules (TA*neo*) in the plant cell. Also, most of the coat protein gene, including the initiation codon, was deleted and replaced with the *neo* gene from *Tn5*. This partial dimer was then cloned between the T-DNA border sequences of the Ti plasmid pBin19 to give pBin19A1.6*neo*, introduced into *A. tumefaciens* and the whole used to agroinfect transgenic B2 *N. tabacum* cv. Samsun plants (plants containing a head-to-tail dimer of DNA B integrated into the chromosomal DNA). None of the plants developed symptoms after 21 days. However, 18 out of 20 plants were shown to be infected when Southern blot analysis of plant DNA revealed the expected viral ss- and dsDNA forms, and an assay of plant extracts revealed a neomycin phosphotransferase (NPT) activity 240 times greater than that found in transgenic pBin19 plants, which correlates well with the average copy number of 490 for double-stranded forms of TA*neo*. From these results Hayes *et al.* (1988e) concluded that TA*neo* is formed from A1.6*neo*, and replicates and spreads systemically through the plant together with DNA B, in an analogous way to DNA A and DNA B during a

normal virus infection.

Transgenic plants containing A1.6*neo* integrated into their chromosomal DNA have also been created, and, as with transgenic B2 plants agroinfected with pBin19A1.6*neo*, single-stranded and double-stranded forms of TA*neo* and functional NPT enzyme were found in plant extracts. This result was also found with the progeny plants, demonstrating that the ability of monomeric DNA molecules to form and replicate was inherited. Thus, Hayes *et al.* (1988e) have shown that a bacterial gene can be amplified and expressed in plants by a replicating TGMV vector and possible applications for such a vector will be discussed in section 6.4.2.

4.11 Aims of the TGMV Project

The DNA genomes of three of the whitefly-transmitted geminiviruses have been sequenced (ACMV, Stanley & Gay, 1983; TGMV, Hamilton *et al.*, 1984; BGMV, Howarth *et al.*, 1985), revealing six conserved ORFs which have the capacity to encode proteins of molecular weight greater than 10 kd. However, when this project was started, there was no knowledge of which of these six ORFs were expressed *in vivo*, or of the function of these putative gene products during the viral infection.

The aim of this project, which was started by I.T.D. Petty (1986) and continued by myself, was to determine which of the six conserved TGMV ORFs are expressed *in vivo* and to identify their gene products, by fusing each of the putative viral genes to the *E. coli* lacZ gene in a series of recombinant plasmids, purifying the β-galactosidase fusion proteins and using them to raise antisera in rabbits. The antisera were then to be used to detect the putative TGMV gene products in various fractions from infected plant material, by Western blot analysis.

Since the start of the project, ORF AR1 of ACMV has been shown to encode the viral capsid protein, and on the basis of its sequence homology with ORF AR1 of BGMV and TGMV, the capsid proteins of the latter two are also assumed to be encoded by ORF AR1. However, this has not been shown unequivocally for either virus.

More recent studies on TGMV have shown that all of the other five

conserved ORFs are genes whose products are required for normal virus infection and that the product of ORF AL1 is required for viral DNA replication (Rogers *et al.*, 1986; Sunter *et al.*, 1987; Brough *et al.*, 1988; Elmer *et al.*, 1988). However, none of these proteins have been identified in infected plants.

5. MATERIALS AND METHODS

5.1 Manipulation of DNA

5.1.1 Agarose Gel Electrophoresis

DNA samples were electrophoresed through 1 % agarose gels (section 2.5.2), prepared by briefly boiling the required amount of agarose (Sigma No. A-6013) in TBE buffer, cooling to 60 °C, adding ethidium bromide (5 mg/ml stock) to a final concentration of 5 μg/ml and pouring into the relevant plates. DNA samples (2 to 10 μl for mini-gels and 20 to 50 μl for half-gels) contained 1 x TBE, 8 % (v/v) glycerol and 0.05 % bromophenol blue and were electrophoresed at 80 V in TBE for 2 to 3 hours for half-gels and for 1 hour for mini-gels.

5.1.2 Restriction Endonuclease Digestions

Restriction endonucleases were purchased from NBL and BRL and stored at -20 °C. Reactions were performed in the buffers provided by NBL and BRL for each enzyme (see below), and these buffers were as 10 x stocks and were stored at 4 °C.

Generally, reactions containing 0.5 to 1 μg DNA in a 10 to 20 μl volume of 1 x restriction buffer were digested with 2 to 10 units of enzyme at 37 °C for 1 to 2 hours. Double digestions were performed sequentially, if the respective restriction buffers differed, with the enzyme requiring the lower ionic strength being used first and then the buffer altered to suit the requirements of the second enzyme. If the respective restriction buffers were the same, the double digestion was performed simultaneously.

Restriction Enzyme	Restriction Buffer
*Bam*HI	150 mM NaCl, 6 mM tris-HCl (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin
*Cla*I	50 mM NaCl, 10 mM tris-HCl (pH 7.9), 10 mM $MgCl_2$, 100 µg/ml bovine serum albumin
*Eco*RI	50 mM NaCl, 100 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 µg/ml bovine serum albumin
*Nae*I	20 mM NaCl, 10 mM tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin
*Pst*I	100 mM NaCl, 10 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 µg/ml bovine serum albumin
*Sal*I	150 mM NaCl, 10 mM tris-HCl (pH 7.9), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin
*Xho*I	150 mM NaCl, 10 mM tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 µg/ml bovine serum albumin

5.1.3 Ligation

For cloning experiments, approximately 50 ng of restriction enzyme-digested plasmid vector was ligated with a 3 to 10 fold molar excess of similarly digested insert DNA. Ligations were performed in a 10 µl reaction containing 66 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 15 mM DTT, 1 mM spermidine, 1 mM ATP and 2 mg/ml BSA, and incubated at 4 to 12 °C overnight or at room temperature for 5-6 hours. For 'sticky end' ligations 2 units of T_4 DNA ligase (Amersham) were added, for 'blunt end' ligations 10 units were added.

5.1.4 Bal-31 Nuclease Reactions

Bal-31 nuclease has both 3'→5' and 5'→3' dsDNA exonuclease activity and an absolute requirement for Ca^{2+}, allowing its rate of activity to be controlled using EGTA. The presence of ribonucleotides and low molecular weight RNA inhibit its activity. Therefore the DNA which was to be digested was first purified by RNase A digestion followed by ultracentrifugation through a 1 M NaCl cushion, under which conditions the DNA is pelleted and the

ribonucleotides remain in the supernatant (see section 5.2.9).

DNA (4 μg) was linearised by restriction endonuclease digestion, phenol extracted, ethanol precipitated and resuspended in 25 μl dH_2O. An equal volume of 2 x Bal-31 buffer (24 mM $CaCl_2$, 24 mM $MgCl_2$, 0.4 mM NaCl, 40 mM Tris-HCl (pH 8.0), 2 mM EDTA) was added and the reaction preincubated for 3 minutes at 30 °C. Then one unit of Bal-31 (Biolabs) was added and the reaction incubated at 30 °C. Samples (10 μl) were taken at 1, 2, 3, 4 and 5 minutes and the reaction in the samples terminated by the addition of EGTA to a final concentration of 20 mM, and cooling on ice. The extent of Bal-31 digestion could be visualised by agarose gel electrophoresis.

Since the Bal-31 exonuclease reaction is not synchronous, further manipulations (i.e. ligations) required the DNA to be repaired using Klenow fragment as described in the next section.

5.1.5 End-Filling dsDNA with Klenow Fragment

Usually 0.5 μg of dsDNA was end-filled in a 10 μl reaction containing 50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 0.1 mM DTT, 50 μg/ml BSA, 0.1 mM dNTPs and 2 units Klenow fragment (NBL). The reaction was incubated at 37 °C for 30 minutes and terminated by the addition of 1 μl 0.5 M EDTA, phenol extracted and the DNA precipitated with 2 volumes of ethanol at -20 °C overnight.

5.1.6 Recovery of DNA Fragments from Agarose Gels

The following two methods for eluting DNA fragments from agarose gels were both found to give good yields of DNA.

5.1.6i Electroelution onto Whatman 3MM paper

The DNA was digested with restriction enzymes and loaded directly onto a 1 % ultra-pure, DNA grade agarose (Bio-rad) gel containing 0.5 μg/ml ethidium bromide. The gel was electrophoresed in TAE buffer and the band of interest was located using a UV transilluminator. The DNA was eluted using a modification of a method of Maniatis *et al.* (1982). An incision was made directly below the DNA band using a scalpel blade, and a piece of Whatman 3MM

paper backed with dialysis tubing, both cut into squares slightly wider and deeper than the band and pre-soaked for 5 minutes in TAE, were carefully inserted using forceps, with the paper nearer the DNA. Electrophoresis was continued until the band had completely entered the paper. The paper and dialysis tubing were then removed and soaked in 3 x 100 µl elution buffer (50 mM Tris-HCl (pH 7.5), 0.1 % SDS, 1 mM EDTA, 0.2 M NaCl) for 10 minutes each. The eluant was pooled, phenol extracted and the DNA precipitated with 2 volumes of ethanol at -20 °C overnight

5.1.6ii Geneclean™

DNA fragments were also recovered from agarose gels using Geneclean™ (Bio 101), essentially as described in the manufacturer's instructions. The DNA was digested, electrophoresed and the band of interest located as described in section 5.1.6i. Then the band (containing no more than 5 µg of DNA) was excised from the gel, placed in a 1.5 ml Eppendorf tube and 2 to 3 volumes of sodium iodide solution were added to it. The resulting mixture was incubated at 45 to 55 °C for 5 minutes with occasional mixing to help solublise the agarose. The Glassmilk™ was vortexed thoroughly so that it was completely in suspension and 5 µl was added to the DNA, which was then mixed, incubated on ice for 5 minutes and microcentrifuged for 5 seconds. The pelleted DNA/Glassmilk™ was washed three times by resuspending in 10 to 50 volumes of NEW wash solution (contains NaCl, tris, EDTA and ethanol - Bio 101) and microcentrifuging for 5 seconds. The final pellet was resuspended in 5 µl TE buffer, incubated at 45 to 55 °C for 2 minutes to elute the DNA from the Glassmilk™, which was then re-pelleted by microcentrifugation for 30 seconds. This was repeated with another 5 µl TE, giving a total DNA eluant of 10 µl.

5.1.7 Addition of Linkers to dsDNA

Non-phosphorylated *Xho*I d(CCTCGAGG) linkers were purchased from Biolabs and were phosphorylated using polynucleotide kinase before ligation to dsDNA.

A 20 µl reaction containing 2 µg of linkers in 10 mM Tris-HCl (pH

7.5), 10 mM $MgCl_2$, 10 mM DTT and 10 µCi [γ^{-32}P]ATP (3000 Ci/mmol, Amersham) was incubated with 10 units of T_4 polynucleotide kinase at 37 °C for 30 minutes. Unlabelled ATP was added to a final concentration of 1 mM along with another 5 units of enzyme, and the reaction incubated for a further 30 minutes. Aliquots of 5 µl were stored at -20 °C.

Blunt-ended DNA (section 5.1.5) was ligated with a 100 fold molar excess of phosphorylated linkers in a 20 µl reaction containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 2 units of T_4 DNA ligase, and incubated at 12 °C overnight. The reaction was terminated by phenol extraction and the DNA ethanol precipitated, resuspended in 20 µl 10 x *Xho*I buffer (NBL) and digested with an excess (40 to 50 units) of *Xho*I at 37 °C for 2 hours. The reaction was terminated by phenol extraction and the DNA ethanol precipitated. The excess linkers were removed by running the resuspended DNA on an agarose gel and excising the band of interest as described in section 5.1.5.

5.2 Bacterial Cloning

5.2.1 Growth and Maintenance of Bacterial Strains

Three strains of *Escherichia coli* were used for cloning experiments:

DH5α — F^-, endA1, hsdR17(r_k^-, m_k^-), supE44, thi-1, λ^-, recA1, gyrA96, relA1, Δ(argF-laczya)U169, ϕ80dlacZΔM15.

TG2 — supE, thi, recA, Srl::Tn10, hsdΔ5(rk$^-$, mk$^-$), Δ(lac-proAB), [F', traD36, proAB, lacI^{q}ZΔM15].

XL1-blue — endA1, hsdR17(rk$^-$, mk$^+$), supE44, thi-1, λ^-, recA1, gyrA96, relA1, lac$^-$, [F' proAB, lacI^{q}ZΔM15, Tn10(tet^r)].

The DH5α strain (Jessee, 1986) was obtained as frozen competent cells from BRL and was used for plasmids with colour selection (section 5.2.2). The TG2 strain was a gift from Dr. I.T.D. Petty and

the XL1-blue strain was obtained as frozen competent cells from Stratagene. These two strains were used for the plasmids pUR290/1/2 (RÜther & Müller-Hill, 1983) and their derivatives, which require the bacterial $lacI^q$ genotype to efficiently suppress the plasmid lacZ gene.

Strains were grown on minimal medium plates (plus supplements), stored sealed with Parafilm at 4 °C and were restreaked every 1 to 2 months. Transformants were maintained on 2xTY plates containing antibiotics. Stocks of host strains and transformants were also stored frozen at -70 °C in 2xTY broth made 15 % (v/v) with glycerol.

5.2.2 Vectors used for Cloning

The vector pEMBL/*Xho*I (Hayes *et al.*, 1988a; Figure 28) is a derivative of pEMBL9 (Dente *et al.*, 1985) with a *Xho*I site in the polylinker, and was a gift from Dr. R.J. Hayes. This vector allows recombinant clones to be identified by colour selection. It contains a non-functional fragment of the *E. coli lacZ* gene (including operator, promoter and N-terminal α-peptide of β-galactosidase), which can be induced by isopropyl-β-D-thiogalactopyranoside (IPTG) and can complement the defective *lacZ* gene of *E. coli* strain DH5α (Gronenborn & Messing, 1978). This activity can be detected using 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal), a colourless lactose analogue, which is hydrolysed by β-galactosidase and results in the colonies produced being blue in colour. When a fragment of foreign DNA is inserted into the polylinker cloning site of the vector this disrupts the α-peptide and destroys its ability to complement the defective β-galactosidase activity. Therefore the X-gal is not hydrolysed and the colonies which result from the transformation of recombinant plasmids are colourless.

The vectors pUR290/1/2 (RÜther & Müller-Hill, 1983; Figure 29) were a gift from Dr. I.T.D. Petty. These plasmids contain the β-lactamase gene which confers ampicillin resistance on *E. coli*, the *lac* promoter/operator region and the *lacZ* structural gene encoding β-galactosidase. There is a polylinker sequence at the C-terminal end of the *lacZ* gene, which is positioned in a different reading frame in each of the three vectors, such that new sequences can be inserted in

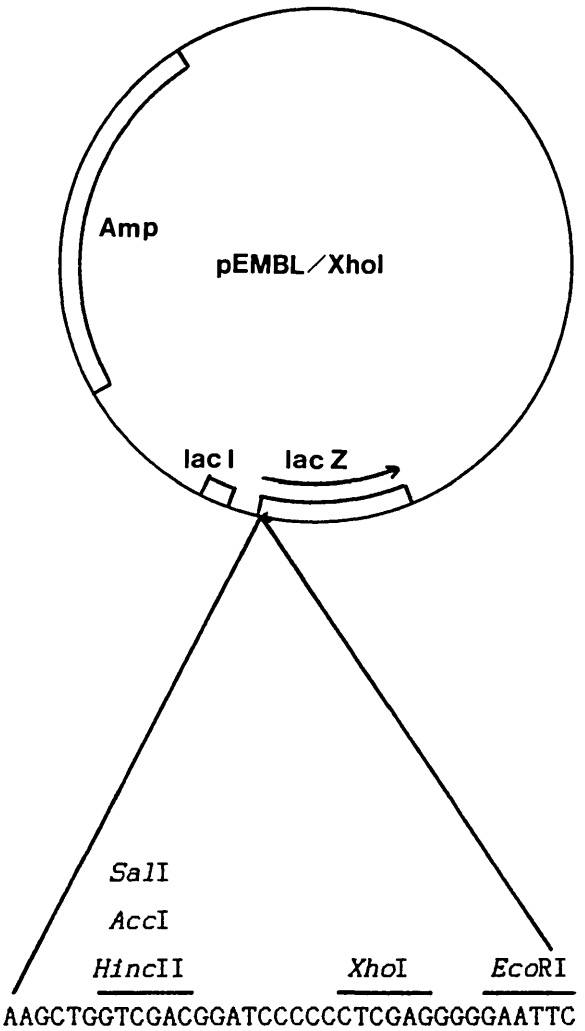


Figure 28 The Structure of the pEMBL/*Xho*I Vector

A schematic diagram of the pEMBL/*Xho*I vector (Hayes *et al.*, 1988a), with the sequence of the polylinker shown below. The β-lactamase gene (Amp) is also shown, which confers ampicillin resistance on host bacteria.

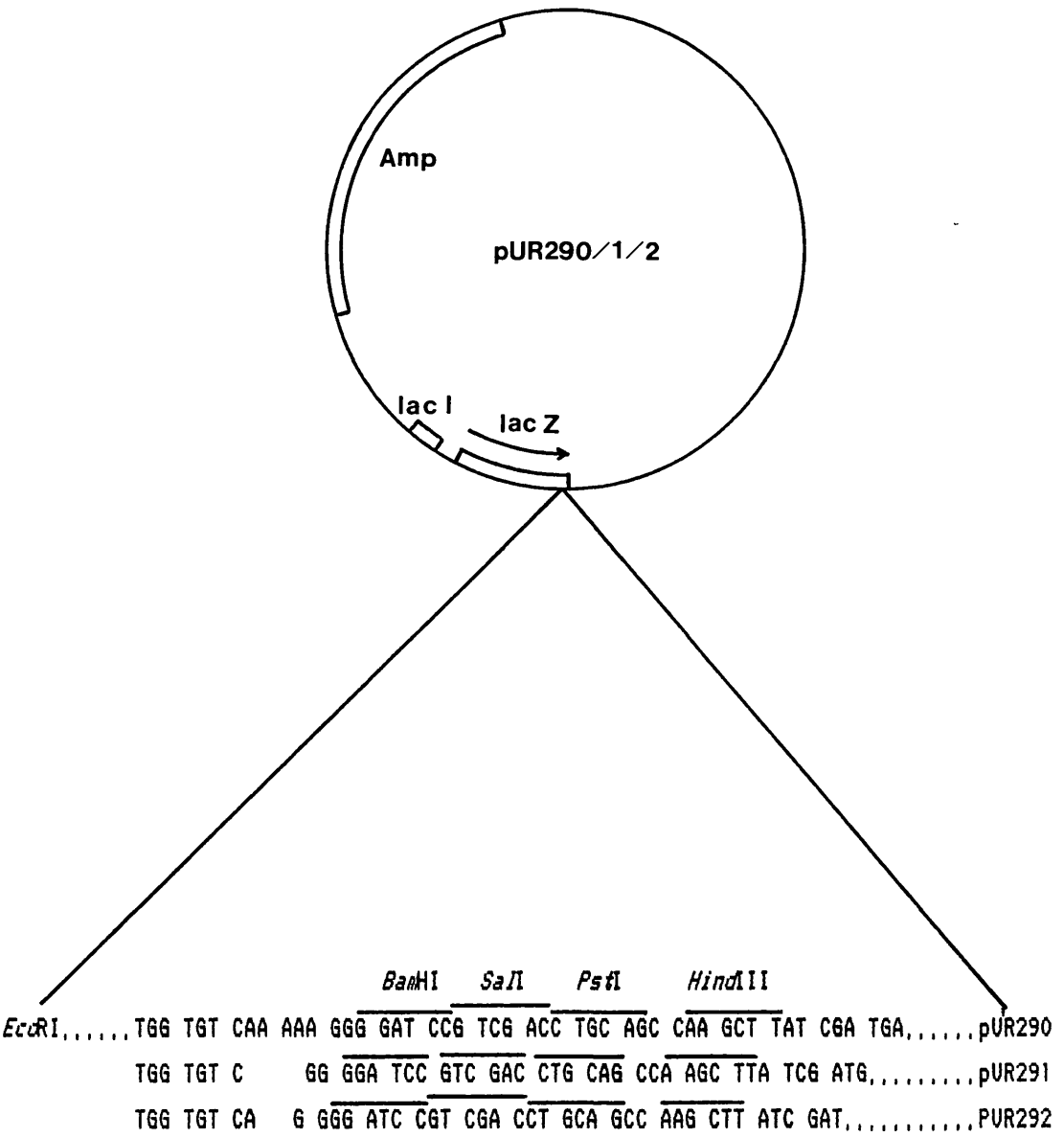


Figure 29 Structure of the lacZ-fusion Cloning Vectors, pUR290/1/2

A schematic diagram of the lacZ-fusion cloning vectors, pUR290/1/2 (RÜther & MÜller-Hill, 1983), showing the sequence of the polylinkers and their reading frames relative to the lacZ gene. They all contain the β-lactamase gene (Amp), which confers ampicillin resistance on host bacteria.

the correct reading frame for expression of a β-galactosidase fusion protein. The vectors must be used in *E. coli* strains with the $lacI^q$ genotype, which suppresses the expression of the lacZ gene. To induce expression of the lacZ gene, the *E. coli* must be grown in the presence of IPTG (sections 5.4.1 and 5.4.2)

5.2.3 Media and Antibiotics

i) Minimal Agar:

500 ml	3 % Bacto Agar (Difco)
500 ml	M9 salts (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, dH_2O to 1 litre)
1 ml	0.1 M $CaCl_2$
1 ml	1 M $MgSO_4$
0.5 ml	1 % (w/v) vitamin B1 (BDH)
1 ml	20 % (w/v) glucose

All components were autoclaved except vitamin B1 which was filter sterilised.

ii) SOB Medium:

2 % (w/v)	Bacto Tryptone (Difco)
0.5 % (w/v)	Bacto Yeast Extract (Difco)
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$

All components were autoclaved except Mg^{2+} which was added from a filter sterilised 2 M stock solution.

iii) SOC Medium:

SOB medium containing 20 mM glucose from a filter sterilised stock solution.

iv) 2 x TY broth:

1.6 % (w/v)	Bacto Tryptone
1 % (w/v)	Bacto Yeast Extract
0.5 % (w/v)	NaCl

v) 2 x TY plates:

2 x TY broth containing 1.5 % (w/v) Bacto Agar (Difco).

Ampicillin (Sigma, sodium salt) was prepared as a 25 mg/ml stock in dH_2O, filter sterilised and stored at -20 °C. It was added to broth and plates to a concentration of 50 to 100 µg/ml.

X-gal (NBL) was prepared as a 20 mg/ml stock in dimethyl formamide and stored at 4 °C.

IPTG (NBL) was prepared as a 24 mg/ml stock in dH_2O, filter sterilised and stored at 4 °C.

5.2.4 Preparation of Competent Cells

5.2.4i Standard Transformation Protocol

The method of Hanahan, (1985) was used to prepare competent cells for most cloning experiments. A single bacterial colony was picked from a minimal medium plate and used to inoculate 5 ml of 2xTY broth in a 10 ml Sterilin tube. This was incubated overnight at 37 °C with shaking. A 150 µl aliquot of this culture was used to inoculate 30 ml of SOB medium in a 250 ml conical flask and this was shaken at 37 °C until the optical density at 550 nm had reached 0.6 (approximately 2.5 to 3 hours). The culture was poured into three Sterilin tubes, chilled on ice for 10 minutes and the cells were harvested by centrifuging at 1000 g for 10 minutes at 4 °C. The pelleted cells in each tube were drained thoroughly, resuspended in 3.3 ml of ice cold FSB and incubated on ice for 10 minutes, before being recentrifuged and resuspended in 0.8 ml ice cold FSB. Dimethyl sulphoxide (DMSO, BDH) was added to 3.5 % (28 µl) and the cells mixed and incubated on ice for 10 minutes. A second 28 µl aliquot of DMSO was added and the cells held on ice for a further 10 minutes before aliquoting 200 µl

samples into chilled Eppendorf tubes, for transformation as in section 5.2.5 or storage at -70 °C.

Frozen Storage Buffer (FSB)

100 mM	potassium chloride
45 mM	manganese chloride
10 mM	calcium chloride
3 mM	hexamine cobalt (III) trichloride (Sigma)
10 mM	potassium acetate (pH 7.5)
10 %	glycerol

filter sterilised and stored at 4 °C.

5.2.4ii Calcium Chloride Method

When a lower transformation efficiency was sufficient, for example, when retransforming an already characterised plasmid, cells were made competent using 50 mM calcium chloride.

A 50 µl aliquot of an overnight culture of *E. coli* was used to inoculate 10 ml of 2xTY broth, which was shaken at 37 °C until the optical density at 550 nm had reached 0.45 to 0.55 (approximately 3 to 4 hours). The cells were chilled on ice for 10 minutes and then harvested by centrifugation for 10 minutes at 1000 g at 4 °C. The pelleted cells were resuspended in 5 ml ice cold 50 mM $CaCl_2$ and incubated on ice for a further 20 minutes before being harvested and resuspended in 1 ml 50 mM $CaCl_2$. The cells were either used immediately or kept for 12 to 24 hours at 4 °C.

5.2.5 Transformation of Cells with Plasmid DNA

Up to 20 ng of DNA in a 10 µl volume was added to 100 to 200 µl of competent cells in a 1.5 ml Eppendorf tube. The mixture was incubated on ice for 20 to 40 minutes, heat shocked at 42 °C for 90 seconds and chilled briefly. Eight hundred microlitres of SOC medium was added and the cells incubated at 37 °C with shaking for 30 minutes. They were then either aliquoted as 200 µl samples into 1.5 ml Eppendorf tubes or else pelleted and resuspended in 200 µl SOC

medium. If TG2 or XL1-blue cells were used with pUR290/1/2 or their derivatives, these samples were immediately spread onto 2xTY plates containing ampicillin. However, if DH5α cells were used, 12 µl of IPTG and 50 µl of X-gal were added to these samples prior to spreading. The plates were allowed to dry before being incubated inverted at 37 °C overnight.

When colour selection was used, recombinants were identified by the formation of white colonies as opposed to the blue colonies formed by the religated vector (section 5.2.2).

5.2.6 Small Scale Plasmid Preparation ("Miniprep")

For "minipreps" of plasmid DNA, a modification of the method of Maniatis *et al.*. (1982) was used. Single colonies of each transformant were toothpicked into 10 ml Sterilin tubes containing 1.5 ml of 2xTY broth plus 50 µg/ml ampicillin, and grown at 37 °C overnight with shaking. The cultures were transferred to 1.5 ml Eppendorf tubes and the cells harvested by microcentrifugation for 1 minute. The supernatants were discarded and the pelleted cells resuspended in 100 µl TE buffer by vortexing . Two hundred microlitres of alkaline SDS (0.2 M NaOH, 1 % SDS) were added to lyse the cells, and the contents mixed gently by inversion, and incubated on ice for 5 minutes to allow complete lysis. Then 150 µl of 3 M potassium acetate (pH 4.8) were added and the mixture was vortexed and held on ice for a further 5 minutes. The cell debris was pelleted by microcentrifugation for 5 minutes and the supernatants transferred to clean Eppendorf tubes and phenol extracted. The aqueous phase was recovered and the plasmid DNA precipitated by adding 1 ml of ethanol and incubating at room temperature for 2 minutes. The DNA was collected by microcentrifugation for 5 minutes. The pellets were washed with 70 % ethanol, dried under vacuum and then resuspended in 50 µl TE containing 20 µg/ml RNase A. The RNA was digested by incubating at 37 °C for 30-60 minutes. The plasmid DNA could then be analysed by restriction enzyme digestion and agarose gel electrophoresis, or stored frozen at -20 °C.

5.2.7 Large Scale Plasmid Preparation

A single bacterial colony containing the appropriate plasmid was toothpicked into 5 ml of 2xTY broth plus ampicillin in a 10 ml Sterilin tube, and grown at 37 °C overnight with shaking. This starter culture was used to inoculate 1 litre of the same broth in a 2 litre conical flask, which was similarly shaken at 37 °C overnight. The plasmid DNA was isolated by an adaptation of the method of Maniatis *et al.*. (1982), in which most of the cellular RNA was precipitated with lithium chloride and the plasmid DNA was banded on a caesium chloride gradient.

The cells were chilled on ice for 15 minutes and then transferred to four 250 ml Beckman bottles and harvested by centrifugation for 10 minutes at 7,000 rpm in a Beckman JA-14 rotor. The supernatants were discarded and the pelleted cells in each bottle resuspended in 5 ml TE buffer, and then pooled into one 250 ml bottle and incubated on ice for 15 minutes. Forty millilitres of alkaline SDS (0.2 M NaOH, 1 % SDS) were added, the contents of the bottle mixed gently by inversion and then incubated on ice for 15 minutes to complete cell lysis. Thirty millilitres of potassium acetate (pH 4.8) were added and the contents of the bottle mixed by sharp inversion and then held on ice for a further 15 minutes. The cell debris was pelleted by centrifugation at 10,000 rpm for 20 minutes and the supernatant transferred to a clean 250 ml bottle. Propan-2-ol (0.6 volumes (54 ml)) was added and the contents mixed and allowed to stand at room temperature for 15 minutes. The nucleic acid was recovered by centrifugation at 10,000 rpm for 25 minutes. The pellet was drained thoroughly and then resuspended in 12 ml TE buffer and transferred to a 50 ml tube. Four millilitres of 8 M lithium chloride were added, the mixture incubated on ice for 30 minutes and then the RNA pelleted by centrifugation at 10,000 rpm for 20 minutes. The supernatant was recovered and the plasmid DNA was precipitated by addition of 32 ml of ethanol and incubation at -20 °C for 1 hour. The DNA was pelleted by centrifugation at 10,000 rpm for 20 minutes, dried and resuspended in TE to give a final volume of 10 ml. Ten grams of caesium chloride and 1 ml of 10 mg/ml ethidium bromide were added and mixed gently until the caesium chloride had dissolved and this solution was

incubated on ice for 30 minutes to precipitate any proteins present. The proteins were pelleted by centrifuging in a bench-top centrifuge (BTL) at 2500 rpm for 10 minutes at 4 °C. The supernatant was collected and the plasmid DNA was banded by ultracentrifuging in a Beckman 80Ti rotor at 50,000 rpm for 24 hours at 20 °C. The lower band of supercoiled plasmid DNA was located using a UV transilluminator and was removed from the tube using a syringe. The ethidium bromide was extracted by washing several times with an equal volume of dH_2O-saturated *n*-butanol until the solution was no longer pink. The caesium chloride was removed by dialysing against 2 litres of TE buffer overnight at 4 °C. The plasmid DNA was ethanol precipitated and resuspended in 0.5 ml sterile dH_2O, before being analysed by restriction enzyme digestion and agarose gel electrophoresis, or stored at 4 °C.

5.2.8 Plasmid Preparation for Sequencing

Plasmid DNA for sequencing was either prepared as in section 5.2.7 or by a modification of the "Miniprep" method (section 5.2.6). In this latter method the modifications were as follows. After the cells had been lysed, treated with potassium acetate and microcentrifuged, the supernatant was incubated at 37 °C for 20 minutes with 20 µg/ml RNase A. This was then phenol extracted, the aqueous phase recovered and the DNA ethanol precipitated. The dried pellet was resuspended in 16 µl of sterile deH_2O, 4 µl of 4 M NaCl and 20 µl of 13 % (w/v) polyethylene glycol (M_r 8000) and incubated for 20 minutes at 0 °C. The DNA was recovered by microcentrifugation for 10 minutes, washed with 1 ml of 70 % ethanol and dried under vacuum. The pellet was resuspended in 20 µl of sterile deH_2O and stored at 4 °C or used immediately. This method usually yielded about 2 µg of plasmid DNA, and there was much less contaminating RNA present than when using the "Miniprep" method.

5.2.9 Plasmid Preparation for Bal-31 Digestion

Plasmid DNA was prepared for subsequent Bal-31 digestion (section 5.1.4) as in section 5.2.7, except for the caesium chloride gradient. Instead of resuspending the final plasmid pellet in TE buffer to give

10 ml, the pellet was resuspended in 0.5 ml of TE buffer and RNase A was added to a final concentration of 10 µg/ml. This was incubated for one hour at room temperature and then layered over a 4 ml cushion of 1 M NaCl, topped up with TE buffer and centrifuged for 7 hours at 35,000 rpm in a Beckman SW50.1 rotor at 20 °C with slow acceleration. The pellet, which contained the plasmid DNA, was washed twice with 70 % ethanol, dried under vacuum and resuspended in 0.5 ml sterile dH_2O and stored at 4 °C.

5.3. Dideoxynucleotide Sequencing

5.3.1 Stock Solutions

i) 10 x TBE (pH 8.9)

162 g	Tris
27.5 g	boric acid
9.5 g	EDTA
deH_2O to 1 litre.	

ii) 8 % (w/v) Acrylamide / 7.5 M Urea

264 g	ultrapure urea
120 ml	40 % (w/v) acrylamide
120 ml	2 % (w/v) N,N'-methylenebisacrylamide
60 ml	10 x TBE
deH_2O to 600 ml.	

The acrylamide and N,N'-methylenebisacrylamide stock solutions were deionised by stirring with 5 g/100 ml Amberlite MB-1 "monobed" resin (BDH) for 30 minutes and then filtered through Whatman No.1 filter paper and stored at 4 °C. The acrylamide/urea stock solution was filtered through Whatman No.1 filter paper and stored at 4 °C in 40 ml aliquots.

iii) 10 x Denaturing Buffer

2 M	NaOH
2 mM	EDTA

autoclaved and stored at 4 °C.

iv) 10 x Annealing Buffer

70 mM	Tris-HCl (pH 7.5)
70 mM	$MgCl_2$
300 mM	NaCl
100 mM	DTT
1 mM	EDTA

autoclaved and stored in 0.5 ml aliquots at -20 °C.

v) Radioactivity

[α^{-35}S] dATP (10 µCi/µl, >600 Ci/mmol) was obtained from Amersham as an aqueous solution and stored in 10 µl aliquots at -70 °C.

vi) Primer

A 17 base forward primer (5'-TGACCGGCAGCAAAATG-3') and a 16 base reverse primer (5'-AACAGCTATGACCATG-3') (New England Biolabs) were used. They were both dissolved in sterile deH_2O to give a concentration of 10 ng/µl and stored in aliquots at -20 °C.

vii) Nucleotide (dNTP/ddNTP) Mixes

The dNTPs and ddNTPs were prepared as 10 mM stocks in sterile deH_2O, and 0.5 mM working solutions of dNTPs and 2 mM working solutions of ddNTPs were prepared by diluting stocks with sterile deH_2O. The dNTP/ddNTP mixes were made up as in the table below.

	A-mix		C-mix		G-mix		T-mix	
	µl	→ µM	µl	→ µM	µl	→ µM	µl	→ µM
0.5 mM dTTP	40	100	40	100	40	100	2	5
0.5 mM dCTP	40	100	4	10	40	100	40	100
0.5 mM dGTP	40	100	40	100	2	5	40	100
2 mM ddGTP	-	-	-	-	50	500	-	-
2 mM ddCTP	-	-	50	500	-	-	-	-
2 mM ddATP	50	500	-	-	-	-	-	-
2 mM ddTTP	-	-	-	-	-	-	50	500
10 x annealing buffer	20		20		20		20	
sterile deH$_2$O	10		46		48		48	

viii) Chase Solution

0.125 mM	dATP
0.125 mM	dCTP
0.125 mM	dGTP
0.125 mM	dTTP

ix) Formamide Dye Mix

98 % (w/w)	deionised formamide
10 mM	EDTA
0.2 % (w/v)	bromophenol blue
0.2 % (w/v)	xylene cyanol

x) 'Sticky' Silane Solution

'Sticky' silane solution was made as below, just prior to use:

10 ml	Absolute ethanol
300 µl	10 % acetic acid
30 µl	silane (BDH)

5.3.2 Preparation of Sequencing Gel

Sequencing gels were cast between two glass plates 38 x 20 x 0.4 cm, one of which had a notch (16 x 2 cm) cut from the top. Spacers and combs were cut from 0.35 mm plasticard (Raven) and were soaked in 50 % Lipsol before being used for the first time, to remove any contaminants which may affect acrylamide polymerisation.

The plates were washed thoroughly with Lipsol and rinsed with dH_2O and industrial methylated spirits. The notched plate was treated with 10 ml 2 % dimethyldichlorosilane in 1,1,1-trichloroethane (BDH, Repelcote) and the un-notched plate treated with 10 ml 'sticky' silane (section 5.3.1x). Both plates were allowed to dry in a fume cupboard and then finally the notched plate was rinsed with dH_2O. The spacers were positioned and the plates sealed along the bottom and sides with electrophoresis tape (Universal Scientific Ltd.).

Freshly prepared 10 % (w/v) ammonium persulphate (320 µl) and 32 µl of TEMED were added to a 40 ml aliquot of acrylamide/urea and mixed gently by inversion. The gel was cast by positioning the plates at an angle of approximately 30° and pouring the acrylamide down one side, so as to form the gel slowly from the bottom and to avoid the formation of air bubbles. The comb was inserted, clamped into position using two bulldog clips and then covered with clingfilm to prevent the gel from drying out. The gel was allowed to set for at least two hours, or more usually overnight.

5.3.3 Sequencing Reactions

Sequencing reactions were performed essentially as described in the Boehringer Mannheim manual - "Guidelines for quick and simple Plasmid Sequencing".

Two micrograms of plasmid (prepared as in section 5.2.8) were denatured by adding 4 µl of 10 x denaturing buffer, making up the volume to 40 µl with sterile deH_2O and incubating for 5 minutes at room temperature. This solution was then neutralised with 4 µl of 2 M ammonium acetate (pH 4.5) and the DNA precipitated by adding 100 µl of ethanol and incubating for 5 minutes at -70 °C. The DNA was

recovered by microcentrifugation for 10 minutes, and the pellet dried and resuspended in 13 µl of sterile deH_2O. The primer was annealed to the template DNA in the following reaction:

13 µl	template DNA
0.5 µl	primer (10 ng/µl)
1.5 µl	10 x annealing buffer

This was incubated at 65 °C for 3 minutes and cooled slowly to 35 °C (approximately 30 minutes). Two microlitres of [α^{-35}S] dATP and 1 µl of Klenow fragment (1 U/µl) were added to the annealing reaction, and this was mixed and aliquoted into four 0.5 ml Eppendorf tubes labelled A, C, G and T (3 µl into each), containing 2 µl of the respective dNTP/ddNTP mix. The solutions were mixed rapidly and incubated at 30 °C for 20 minutes. One and a half microlitres of chase solution was added to each tube, mixed and the whole incubated for a further 15 minutes at 30 °C. The DNA was dried under vacuum, resuspended in 3 µl of formamide dye mix and heated at 100 °C for 5 minutes, before being snap-cooled on ice and loaded onto the sequencing gel.

5.3.4 Sequencing Gel Electrophoresis

The sequencing gel was prepared as in section 5.3.2, and just prior to use, the electrophoresis tape was removed from the bottom of the plates using a scalpel blade. The plates were assembled in an electrophoresis tank and running buffer (1 x sequencing TBE) added to the upper and lower troughs. The comb was removed and the wells of the gel rinsed out, using a syringe and needle, to remove any unpolymerised acrylamide. The gel was electrophoresed for 15 minutes, using an LKB power pack set at 32 W, 1100 V, 30 mA, prior to loading the samples, using a Gilson 'pipetman'. The samples were then electrophoresed until the bromophenol blue dye front was about two thirds down the gel (1.5 to 2 hours). When electrophoresis was complete the gel plates were taken from the tank, the electrophoresis tape removed and the plates gently levered apart. The un-notched plate, to which the gel remained attached, was soaked in a tank of 10

% acetic acid for 60 minutes with occasional shaking. This fixes the gel and washes out the urea. When removed from the tank, the gel was blotted dry with Kimwipe and then dried by baking at 80 °C for at least 1 hour.

The gel was exposed to Kodak X-Omat S film for 1 to 2 days and the film developed using a Kodak X-Omat automatic processor.

5.4 Proteins

5.4.1 Small Scale Preparation of Total Protein from Bacteria

A single bacterial colony containing the appropriate plasmid was toothpicked into a 10 ml Sterilin tube containing 1.5 ml of 2xTY broth, 50 µg/ml ampicillin and 0.5 mM IPTG, and grown at 37 °C overnight with shaking. The cultures were transferred to 1.5 ml Eppendorf tubes and the cells pelleted by microcentrifugation for 1 minute. The supernatants were discarded and the pellets resuspended in 50 µl of TE buffer and 350 µl of SDS-PAGE loading buffer (section 2.5.3i), heated for 5 minutes at 100 °C and cooled briefly on ice. The lysate was cleared by microcentrifugation for 5 minutes and the supernatant stored at -20 °C.

5.4.2 Large Scale Preparation of Fusion Proteins

To prepare each β-galactosidase fusion protein for injection into rabbits to raise antisera (see section 5.4.4) 1 litre of 2xTY broth containing 50 µg/ml ampicillin was inoculated with 5 ml of a stationary phase, overnight culture of TG2 containing the appropriate plasmid. The 1 litre culture was shaken at 37 °C and the bacterial growth monitored by measuring the optical density at 550 nm of 1 ml aliquots removed at 30 minute intervals. After 2 hours the culture had entered exponential growth phase, and IPTG was added to a final concentration of 0.5 mM, to induce expression of the fusion protein, and the incubation continued for 4 hours, with 30 minute sampling as before. To monitor the accumulation of fusion protein, total protein was extracted from the 1 ml samples taken every 30 minutes, as described in section 5.4.1, and 40 µl/OD_{550} aliquots of these extracts were analysed by electrophoresis through a 7.5 % SDS-

polyacrylamide gel (see section 2.5.3i).

Total protein was extracted from the 1 litre culture by the method of Marston (1987). The cells were pelleted by centrifugation at 7500 rpm for 10 minutes in a Beckman JA-14 rotor at 4 °C and resuspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 0.1 M NaCl). Twelve microlitres of 0.1 M phenylmethylsulphonylfluoride (PMSF) in isopropanol and 270 µl of 10 mg/ml lysozyme in lysis buffer were added and the cells were allowed to lyse for 20 minutes on ice, with occasional mixing. Twelve micrograms of deoxycholic acid (Sigma) were added while stirring with a glass rod and the lysate was transferred to a 37 °C water bath and stirred until it became viscous and difficult to stir. Seventy microlitres of DNase I (1 mg/ml in lysis buffer) were added while stirring and the lysate was incubated at room temperature for 30 minutes or until it was no longer viscous. The insoluble bacterial matter, which contained some fusion protein, was pelleted by centrifugation at 10,500 rpm for 10 minutes in a Beckman JA-20 rotor at 4 °C. The supernatant (hereafter known as the "soluble fraction"), which also contained some fusion protein, was collected and stored at 4 °C.

The fusion protein contained in the crude bacterial extracts (soluble and insoluble fractions) was concentrated by ammonium sulphate precipitation prior to purification by gel filtration chromatography.

Contaminating nucleic acids were removed from the crude extracts by adding 0.1 volumes of 30 % (w/v) streptomycin sulphate (in 50 mM sodium phosphate buffer, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM EDTA, 1 mM PMSF), stirring for 15 minutes at 4 °C and centrifuging at 10,500 rpm for 10 minutes in a Beckman JA-20 rotor at 4 °C. The supernatant was collected and saturated ammonium sulphate (pH 8.0) was added dropwise, with stirring, to a final concentration of 25 % (v/v). This solution was then stirred for 30 minutes at 4 °C and centrifuged at 10,500 rpm for 30 minutes. The pellet was resuspended in 5 ml of column buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM 2-mercaptoethanol, 1 mM PMSF) before centrifugation at 10,500 rpm for 10 minutes. The final supernatant was collected and stored at 4 °C.

5.4.3 Gel Filtration Chromatography

The fusion proteins were purified from the crude bacterial extracts by gel filtration chromatography, using a Sephacryl S-300HR column (Sigma) with a packed gel height and diameter of 50 cm and 2.5 cm respectively.

i) Pouring and Packing the Column

The Pharmacia C 26/70 column was used for this separation, with one flow adaptor. All parts of the column were cleaned thoroughly with Lipsol detergent and both the flow adaptor and the bottom piece were wetted by drawing water through them under vacuum, to ensure that no air bubbles were trapped under the nets. The column was assembled and used in a room with a constant temperature of 4 °C. A bottle containing 600 ml of Sephacryl S-300HR was shaken gently until it was an even slurry, and then it was poured into a measuring cylinder and allowed to settle for about 15 minutes. The excess supernatant was decanted and the gel resuspended in 170 ml of column buffer (section 2.4) by shaking gently. The packing reservoir was attached tightly to the column, which was then mounted vertically on a stand. The outlet was closed and the inside of the column wetted with buffer so as to leave a few centimetres of fluid in the bottom. The well-mixed gel suspension was poured in a single movement, down the inside wall of the column, until the reservoir was full. The reservoir cap was then attached to the reservoir, with a piece of tubing leading to a flask containing column buffer. A pump was connected to the column outlet and buffer was pumped through the column at a rate of 2.5 ml per minute for 2 hours, by which time the gel had settled below the top of the column. The reservoir was removed and the flow adaptor inserted. The flow rate was increased to 5 ml per minute and the gel packed for a further 1 hour. Then the adaptor was adjusted to the level of the gel and locked into position.

ii) Sample Application

The column was calibrated, using proteins of known molecular

weight, before crude bacterial extracts were applied to the column. The flow rate was adjusted to 1 ml per minute, and 5 ml of column buffer containing 1 mg each of thyroglobulin (669 kd) and horse apoferritin (443 kd) (both Sigma) were pumped into the column via the inlet tubing. The eluant was monitored at 280 nm using an LKB 8300 Uvicord II Absorptiometer and chart recorder. Any proteins in the eluant appeared as peaks on the chart, and from the elution profile of the marker proteins, the time at which the fusion proteins would be eluted could be estimated.

The crude bacterial extracts (section 5.4.2) were applied to the column in a 5 ml volume, in the same way as the marker proteins, and the OD_{280} of the eluant monitored with the Uvicord. Just before the fusion protein was expected, the collection of eluant in 10 ml aliquots was started and these were analysed by polyacrylamide gel electrophoresis (section 2.5.3i). Those samples containing fusion protein were pooled and the protein concentrated by precipitation with ammonium sulphate, as described in section 5.4.2, to a final concentration of at least 0.2 mg/ml.

5.4.4 Raising Antisera in Rabbits

The rabbit was maintained and injected at the Animal Unit of St. Mary's Hospital, London. It was injected with a total of 300 µg of β-gal-AL2 fusion protein in 100 µg doses. For all 3 injections the protein solution was emulsified with an equal volume of Freund's complete adjuvant (Sigma) prior to injection. The first 2 samples were injected intramuscularly at 0 and 7 days, and the third sample was injected sub-cutaneously at 41 days. A 1 ml blood sample was taken at 0 days and the final bleed was taken at 55 days, yielding about 60 ml.

Serum was prepared from the blood by allowing it to clot overnight at 4 °C and removing the clot by centrifugation for 20 minutes at maximum speed in a Hettich EBA 3S centrifuge. Serum samples were stored at 4 °C after addition of 0.02 % sodium azide (w/v).

5.4.5 Pre-absorption of Antisera with IPTG-induced *E. coli* TG2: pUR292

The antisera were pre-absorbed with IPTG-induced *E. coli* TG2: pUR292 proteins to remove antibodies to β-galactosidase and any contaminating bacterial proteins.

Eight hundred millilitres of 2xTY broth containing 50 µg/ml ampicillin were inoculated with 50 ml of a stationary phase, overnight culture of TG2: pUR292 and incubated at 37 °C for 2 hours. IPTG was added to a concentration of 0.5 mM and the culture incubated for a further 2 hours. The cells were pelleted by centrifugation at 7000 rpm for 10 minutes in a Beckman JA-20 rotor at 4 °C. The pellet was frozen at -70 °C, thawed at 37 °C and resuspended in 30 ml of phosphate-buffered saline (15 mM NaCl, 0.4 mM KH_2PO_4, 1.6 mM $Na_2HPO_4.2H_2O$). This was then sonicated with 5 x 30 second bursts using the large probe (19 mm tip diameter) of an MSE Soniprep 150, with intermittent cooling on ice. The final bacterial extract was stored in 1 ml aliquots at -70 °C or used immediately, as follows: 100 µl of antiserum was added to 1 ml of bacterial extract in an Eppendorf tube and the tube rotated overnight at 4 °C to precipitate any antibodies to either β-galactosidase or any bacterial proteins. This precipitate was pelleted by microcentrifugation for 1 hour at 4 °C and the supernatant containing the cleaned-up antiserum was collected and stored at -20 °C.

5.4.6 Pre-aborption of Antisera with *N. benthamiana* Proteins

Acetone powder was prepared from healthy *N. benthamiana* leaves by the method of Tucker & Fairbrothers (1970) and was used to remove antibodies to *N. benthamiana* proteins from antisera.

The acetone powder was prepared as follows: 1 g of *N. benthamiana* leaf material was ground to a fine powder in liquid nitrogen and washed on Whatman No. 1 filter paper with chilled acetone, until the filtrate was clear. The powder was then soaked in chilled butanol for 20 minutes, pelleted by centrifugation at 4580 g for 20 minutes at -20 °C, rinsed with chilled acetone and dried for 12 hours at -20 °C. The dried powder was stored at -20 °C. One milligram of this powder was added to 1 ml of antiserum in an Eppendorf and the tube rotated overnight at 37 °C to precipitate any antibodies to healthy

N. benthamiana proteins. This precipitate plus the acetone powder were pelleted by microcentrifugation for 15 minutes, and the supernatant containing the cleaned-up antiserum was collected and stored at -20 °C.

5.4.7 Detection of Proteins with Antisera

Proteins were detected with antisera by Western blotting, as described by Towbin *et al.* (1979) and Sherwood (1987).

Electroblot Buffer (EB)

25 mM	Tris-HCl (pH 8.3)
129 mM	glycine
20 % (v/v)	methanol

made as a 10x stock solution without the methanol.

Tris-buffered Saline (TBS)

10 mM	Tris-HCl (pH 7.4)
0.9 % (w/v)	NaCl

made up as a 10x stock solution.

The proteins were separated by electrophoresis through an SDS-polyacrylamide gel (section 2.5.3i) and transferred to a Hybond-C nitrocellulose membrane (0.45 µm pore size, Amersham). Using an E-C Electroblot apparatus (E-C Apparatus), the following were soaked in electroblot buffer and placed between the terminals (-ve to +ve): 2 pieces of Scotch-brite, 1 piece of Whatman 3MM paper, the polyacrylamide gel, 1 piece of nitrocellulose, 1 piece of Whatman 3MM paper and 2 pieces of Scotch-brite, ensuring that there were no air bubbles between the layers. The whole was submerged in a tank containing circulating electroblot buffer and electroblotted at 380 mA for 2 hours or at 150 mA overnight. The apparatus was dismantled and the nitrocellulose cut into 2 pieces - one piece to be probed with an antiserum and the other, containing the marker tracks, to be stained briefly in 1 % amido black (in 25 % isopropanol and 10 % methanol) and destained in several washes of 25 % isopropanol/10 %

methanol.

The first piece of nitrocellulose was soaked in 5 % fat-free dried milk (Waitrose) (in TBS) for 30 minutes, rinsed 3 times in TBS and sealed in a polythene bag with 10 ml of antiserum (diluted in TBS). This bag was rotated for 1 hour and the nitrocellulose was removed and rinsed once in TBS, once in TBS containing 0.05 % (v/v) Tween 20, twice more in TBS and sealed in a fresh bag with 10 ml of 2 µg/ml Protein A peroxidase (diluted in TBS). This was rotated for 1 hour before the nitrocellulose was removed, rinsed three times in TBS and incubated in a solution containing the following: 50 ml of TBS, 10 ml of 3 mg/ml 4-chloro-1-naphthol (in methanol) and 180 µl saturated hydrogen peroxide, for about 5 minutes, by which time any colour reaction would have happened. To stop the reaction the nitrocellulose was placed in TBS and then dried on Whatman 3MM paper.

5.5. **Virus Propagation and Purification**

5.5.1 Origin of Virus

The isolate of Tomato Golden Mosaic Virus was originally provided by Dr. A.S. Costa from the Instituto Agronomico, Estado de Sao Paulo, Brazil and propagated by Dr. W.D.O. Hamilton (Hamilton *et al.*, 1981).

5.5.2 Growth of Plants

Seeds of *N. benthamiana* and *N. tabacum* **cv** Samsun *NN* were supplied by Dr. R.J. Hayes. The seeds were germinated on Fisons compost under a propagation cover. After about one week individual seedlings were transferred to pots of moistened John Innes Potting Compost No. 2 and these kept covered for a further 4 to 5 days to prevent dehydration. Plants were grown in a glasshouse with an average daytime temperature of 25 °C falling to 16 °C at night. Natural light was supplemented with mercury vapour lamps on an 18 hour photoperiod. Plants were watered regularly and fed once a week with a fertiliser (Phostrogen Ltd.).

5.5.3 Inoculation of Plants

The normal propagation host of TGMV was *N. benthamiana*. Plants

were grown to the 4-6 leaf stage, normally 2 to 3 weeks after potting, before inoculation. The inoculum consisted of either young, infected leaves (approximately 0.5 g) ground up with a pestle and mortar, or high-speed pellet material (section 5.5.4). Three expanding leaves per plant were dusted with F320 grade carborundum powder (Carborundum Company) and inoculum (10 to 20 µl) applied by rubbing with a gloved finger. Twenty minutes after inoculation the leaves were rinsed with water to remove excess carborundum.

Symptoms generally became apparent 10 to 16 days after inoculation and plants were harvested after a further 14 days for virus purification.

5.5.4 Preparation of Virus (High-Speed Pellet Material)

High-speed pellet material was prepared according to the procedure of Stein *et al.* (1983). TGMV-infected *N. benthamiana* leaves were homogenised with ice-cold CEM buffer (0.1 M trisodium citrate, 0.75 % (w/v) sodium sulphite, 5 mM EDTA, 1 % (v/v) 2-mercaptoethanol, 0.325 % (w/v) L-ascorbic acid (BDH), adjusted to pH 7.0 with NaOH) at 2 ml/g of leaf tissue, using a Sorvall Omnimixer. The homogenate was made 2.5 % (v/v) with Triton X-100 and stirred for 16 h at 4 °C before filtration through muslin. The filtrate was centrifuged at 10,000 g for 15 minutes and the supernatant was further centrifuged at 40,000 rpm for 2 hours in a Beckman Type 65 or Ti70 rotor. The pellet was gently resuspended in either TE or CEM buffer at 4 °C. At this stage (crude virus) it could be used in Western blotting (section 5.4.6), as inoculum or further purified.

For further purification the material was microcentrifuged for 10 minutes and the supernatant layered onto a 1 ml cushion of 20 % (w/v) sucrose in CEM buffer and centrifuged in a Beckman SW50.1 rotor at 35,000 rpm for 4 hours at 4 °C. The pellet was resuspended in 100 µl CEM buffer. This partially purified virus was used in Western blotting.

5.5.5 Isolation of Plant Nuclei

Nuclei were isolated from the leaves of healthy and TGMV-infected *N. benthamiana* and healthy *N. tabacum* cv Samsun *NN* by a modification of

the method of Coutts and Buck (1985). Plants were kept in the dark for 24 hours prior to harvesting, to reduce starch levels. Leaf material weighing 10 g was harvested, washed three times in cold, deH_2O and chilled to 4 °C. The leaves were then cut into thin strips and homogenised with 20 ml ice-cold buffer A (34 % (w/v) sucrose, 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, 0.07 % (v/v) 2-mercaptoethanol, 0.2 mM PMSF) in a Sorvall Omnimixer with 3 x 20 second pulses at maximum speed. The homogenate was filtered through four layers of muslin (pre-soaked in buffer A) and one layer of 70 µm nylon mesh (Henry Simon) and then centrifuged at 5000 rpm for 15 minutes in a Beckman JA-20 rotor at 4 °C. The resultant pellets were gently resuspended in 20 ml buffer A containing 0.5 % (v/v) Triton X-100 followed by centrifugation at 4000 rpm for 5 minutes. This step was then repeated several times until the pellet was no longer green (*i.e.* almost all contaminating chloroplasts had been removed) prior to the pellet being resuspended in 1 ml buffer A and layered onto a discontinuous Percoll (Sigma) step gradient composed of 35 % (w/v) and 80 % (w/v) Percoll in buffer A (1.7 ml per step). This gradient was centrifuged at 7300 rpm for 40 minutes in a Beckman SW50.1 rotor at 4 °C to separate the nuclei from other cellular components. The nuclei banded at the interface of the two Percoll layers. This band was collected, diluted in 5 volumes buffer A and the nuclei pelleted in a microcentrifuge for 5 minutes. The pellet was resuspended in 0.5 ml buffer A and re-pelleted (5 minutes) to remove the Percoll. Finally the nuclei were resuspended in 0.25 ml storage buffer (8.5 % (w/v) sucrose, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 2 mM DTT, 50 % (v/v) glycerol) and stored at -70 °C or used in Western blotting.

5.5.6 Preparation of Total Plant Protein

Approximately 1 g of leaf material was ground to a fine powder in liquid nitrogen using a pestle and mortar, and resuspended in 2 ml of SDS-PAGE loading buffer (section 2.5.3i), minus the bromophenol blue. This was then heated at 100 °C for 5 minutes and the insoluble material pelleted by microcentrifugation for 5 minutes. The supernatant was collected and stored at -20 °C.

6. RESULTS AND DISCUSSION

6.1 Construction of Fusion Proteins

Fusion proteins were constructed between β-galactosidase and the proteins encoded by the TGMV ORFs BR1 and BL1, using the expression vectors pUR290, pUR291 and pUR292 (RÜther & Müller-Hill, 1983; section 5.2.2). The resulting fusion proteins consisted of β-galactosidase with a C-terminal addition of one of the TGMV-encoded proteins, missing its first few N-terminal amino acids.

Similar fusion proteins derived from each of the four TGMV A component ORFs have been constructed and described by Petty (1986).

6.1.1 Cloning of ORFs BR1 and BL1

The plasmid pB/*Cla* (Hayes *et al.*, 1988a; Figure 30), which contains the TGMV B component cloned into the unique *Cla*I site of pEMBL9 (Dente *et al.*, 1983), was digested with the restriction endonucleases *Cla*I and *Nae*I, so that one fragment (1970 bp) contained both ORFs BR1 and BL1, with *Cla*I cutting 139 bp upstream of the 5' end of ORF BR1 and *Nae*I cutting 87 bp upstream of the 5' end of ORF BL1. Bal-31 exonuclease was used (section 5.1.4) to digest past the 5' ends of each of these ORFs, so that when subsequently cloned into the expression vectors a viable fusion protein would be formed with β-galactosidase. From time-course reactions with Bal-31 (Figure 31) it was concluded that a one minute reaction would digest just past the 5' end of ORF BL1 and a two minute reaction would digest just past the 5' end of ORF BR1 and quite a way past the 5' end of ORF BL1. The resulting shortened DNA fragments were blunt-ended using Klenow fragment (section 5.1.5), ligated to phosphorylated *Xho*I linkers (CCTCGAGG) (section 5.1.7), digested with *Xho*I and separated by agarose gel electrophoresis (section 5.1.1). The fragment containing the ORFs BR1 and BL1 was eluted from the gel using Geneclean™ (section 5.1.6i), ligated with *Xho*I-digested and phosphatase-treated pEMBL/*Xho*I vector (Hayes *et al.*, 1988a) and transformed into *E. coli* DH5α cells (section 5.2.5), which were then plated out onto 2xTY plates containing ampicillin, IPTG and X-gal, and incubated overnight at 37 °C.

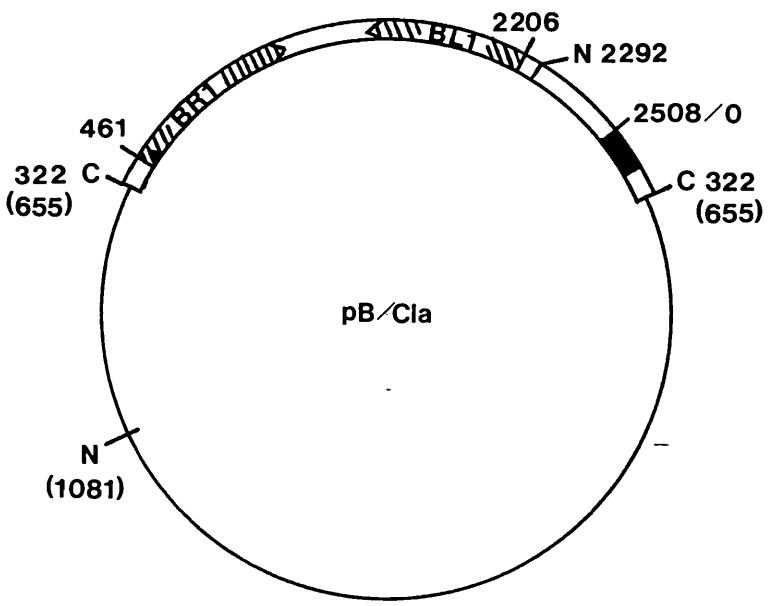


Figure 30 Map of pB/*Cla*

Schematic diagram of pB/*Cla*, showing the TGMV ORFs BR1 and BL1 (hatched boxes) and the 200 bp 'common region' (shaded box). Numbers within brackets represent pEMBL9 nucleotides, and numbers without brackets represent TGMV DNA B nucleotides.

Key: C - *Cla*I; N - *Nae*I.

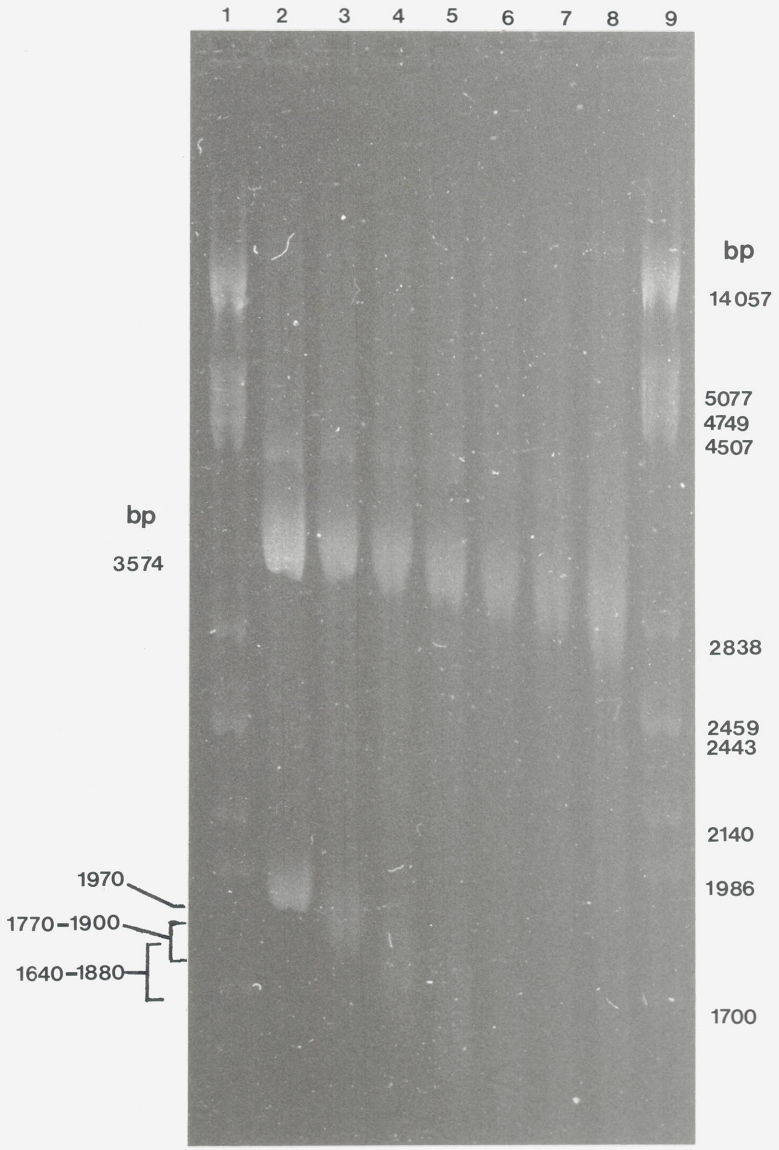


Figure 31 Bal-31 Time Course Reactions

The samples were prepared as follows:- 4 µg of the plasmid pB/*Cla* were digested to completion with *Cla*I and *Nae*I, giving four fragments of 3574, 1970, 551 and 426 bp, and then further digested with Bal-31 exonuclease. Aliquots were taken at one minute intervals and the reaction stopped by adding 10 mM EGTA. Each aliquot was electrophoresed in a 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide.

Lanes 1 & 9 *Pst*I-digested λ DNA.

Lanes 2 - 8 *Cla*I/*Nae*I-digested pB/*Cla* digested with Bal-31 for 0 - 6 minutes, respectively.

A selection of white colonies was picked and the plasmids isolated (section 5.2.6) and analysed by restriction endonuclease digestion and agarose gel electrophoresis. Nineteen clones were identified resulting from the one minute Bal-31 digests and these were designated pHC101-119 (the pHC100 series). Six clones were identified resulting from the two minute Bal-31 digests and these were designated pHC201-206 (the pHC200 series). The orientation of the inserts was determined by *Eco*RI digestion and agarose gel electrophoresis, as there is one *Eco*RI site in the polylinker of pEMBL/*Xho*I and one asymmetric *Eco*RI site in the TGMV-derived insert (Figures 32 and 34)

The sizes of the inserts in the pHC100 and pHC200 series clones were estimated by digesting two representative clones (pHC118 and pHC201) with *Xho*I, electrophoresing them on an agarose gel alongside molecular weight markers (λ DNA digested with *Pst*I) and comparing the distances travelled by the insert bands with the marker bands (Figure 33). From this the *Xho*I inserts of pHC118 and pHC201 were estimated to be 1770 and 1670 bp respectively. Therefore, if both ends had been digested at similar rates, then the 1970 bp *Cla*I/*Nae*I fragment would have been shortened by approximately 100 and 150 bp respectively, at each end. In other words, Bal-31 would have digested past the BL1 5' end after one minute, and past both the BR1 and BL1 5' ends after two minutes.

6.1.2 Sequencing of Vector/Insert Borders

The exact extent of Bal-31 deletion was determined accurately for one of the pHC200 series clones - pHC201 - by dideoxy sequencing the vector/insert border regions using Klenow fragment and the universal M13 forward and reverse sequencing primers (section 5.3). The sequencing ladders contained some ambiguities (bands in all four lanes) and hence are not shown. However, from a consensus sequence derived from four gels, it was found that Bal-31 had deleted 221 bp from one end of the 1970 bp *Cla*I/*Nae*I fragment, but only 96 bp from the other end (shown diagramatically in Figure 34), resulting in an 82 bp deletion at the 5' end of ORF BR1 and a 10 bp deletion at the 5' end of ORF BL1. Therefore Bal-31 had digested the DNA at the BR1

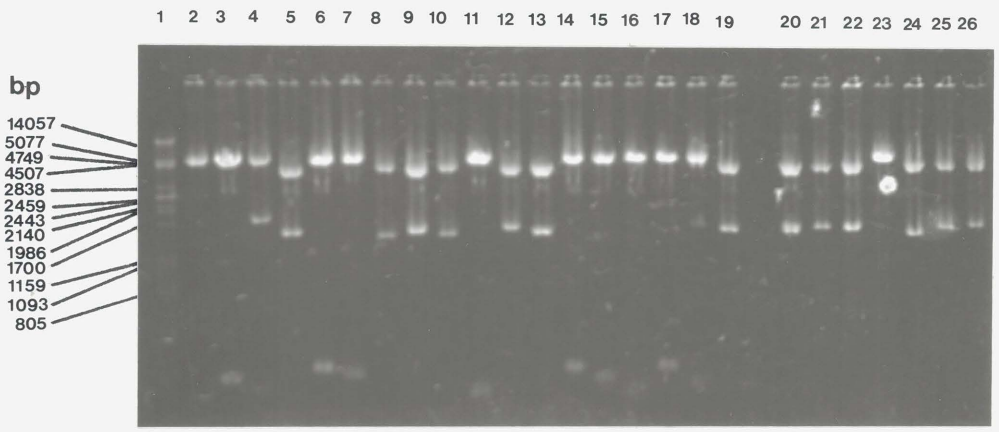


Figure 32 Analysis of *Eco*RI Digests of pHC100 and pHC200 Series Clones

Electrophoresis of *Eco*RI digests in a 1 % agarose gel containing 0.5 μg/ml ethidium bromide.

Lane 1 *Pst*I-digested λ DNA.

Lanes 2-20 *Eco*RI-digested pHC101-119, respectively.

Lanes 21-26 *Eco*RI-digested pHC201-206, respectively.

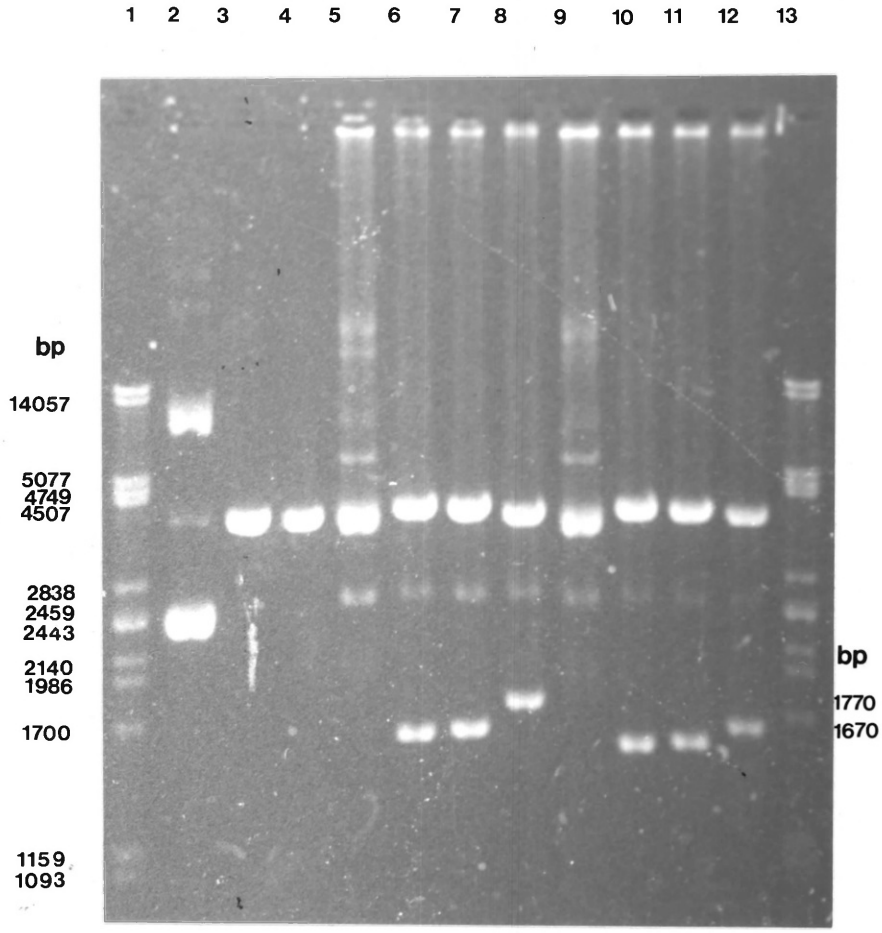
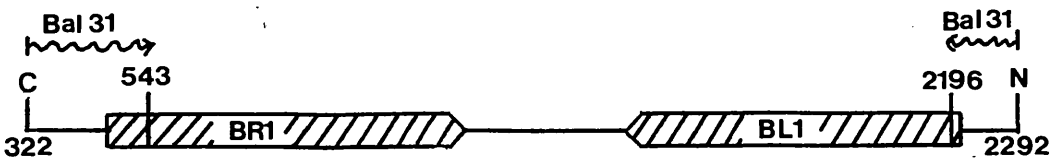


Figure 33 Restriction Digests of pEMBL/*Xho*I, pHC118 & pHC201

Electrophoresis of restriction endonuclease digests in a 1 % agarose gel containing 0.5 μg/ml ethidium bromide. The sizes of the *Xho*I inserts of pHC118 and 201 are indicated.

Lane 1 *Pst*I-digested λ DNA.
Lane 2 pEMBL/*Xho*.
Lane 3 *Eco*RI-digested pEMBL/*Xho*.
Lane 4 *Xho*I-digested pEMBL/*Xho*.
Lane 5 pHC118.
Lane 6 *Hinc*II-digested pHC118.
Lane 7 *Eco*RI-digested pHC118.
Lane 8 *Xho*I-digested pHC118.
Lane 9 pHC201.
Lane 10 *Hinc*II-digested pHC201.
Lane 11 *Eco*RI-digested pHC201.
Lane 12 *Xho*I-digested pHC201.
Lane 13 *Pst*I-digested λ DNA.



1970 bp NaeI/ClaI fragment

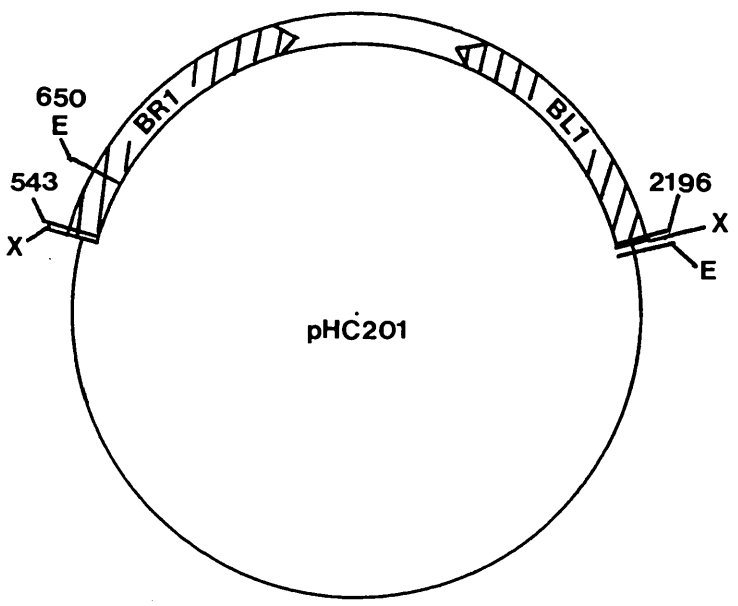


Figure 34 Extent of Bal-31 deletion in pHC201

The figure shows one example of the extent of Bal-31 deletion past the 5' ends of ORFs BR1 and BL1 in the 1970 bp *Cla*I/*Nae*I fragment from pB/*Cla* after a 2 minute digestion, and the subsequent clone - pHC201.

Key: C - *Cla*I; E - *Eco*RI; N - *Nae*I; X - *Xho*I.

end at approximately twice the rate of that at the BL1 end.

The rate of Bal-31 digestion has been shown to depend to a great extent on the DNA sequence and the type of terminus (Guo & Wu, 1983). However, there is no obvious pattern to Bal-31's preference for certain types of terminus. For example, the rate of Bal-31 digestion of *Ava*I ends (C↓PyCGPuG) is faster than that of *Sma*I ends (CCC↓GGG), although their base composition is almost the same. On the other hand, there is a definite pattern of preferences for particular bases, such that AT-rich regions are always digested faster, and G-rich regions slower. Analysis of the sequence of the digested TGMV DNA (Hamilton *et al.*, 1984; MacDowell *et al.*, 1986) revealed that the 221 bp region at the BR1 end of the 1970 bp *Cla*I/*Nae*I fragment was significantly AT-rich, whereas the 96 bp at the BL1 end had an almost equal distribution of the four bases, which would explain the different rates of digestion at each end.

As the 1653 bp *Xho*I fragment of pHC201 contained a copy of ORF BL1 with only a very short deletion at its 5' end, it was ideal for sub-cloning into the expression vectors pUR290, 291 and 292, and subsequent production of a β-galactosidase-BL1 fusion protein containing 230 out of a possible 234 amino acids of putative BL1 protein. This same *Xho*I fragment, if sub-cloned into the expression vectors in the opposite orientation, would produce a β-galactosidase-BR1 fusion protein, which would be missing 28 out of 256 amino acids from the putative BR1 protein. Although this still leaves the majority of the BR1 protein present, it was thought that the pHC100 series clones would contain an even larger proportion of the BR1 coding sequence. Therefore, four of these - pHC103, 104, 111 and 118 - were sequenced in the vector/insert region, at the BR1 end of the insert, using the relevant M13 sequencing primer (a sequencing gel of pHC104, 111 & 118 is shown in Figure 35; and the extent of Bal-31 deletion in all four clones is shown in Figure 36). The clone pHC103 had the shortest Bal-31 deletion at the BR1 end - 132 bp. However, this left the 5' end of ORF BR1 intact. The clone with the smallest deletion of ORF BR1 was pHC118, which was missing the first 5 bp. Therefore, this clone was chosen for sub-cloning into the expression vectors pUR 290, 291 and 292, and subsequent production of a β-

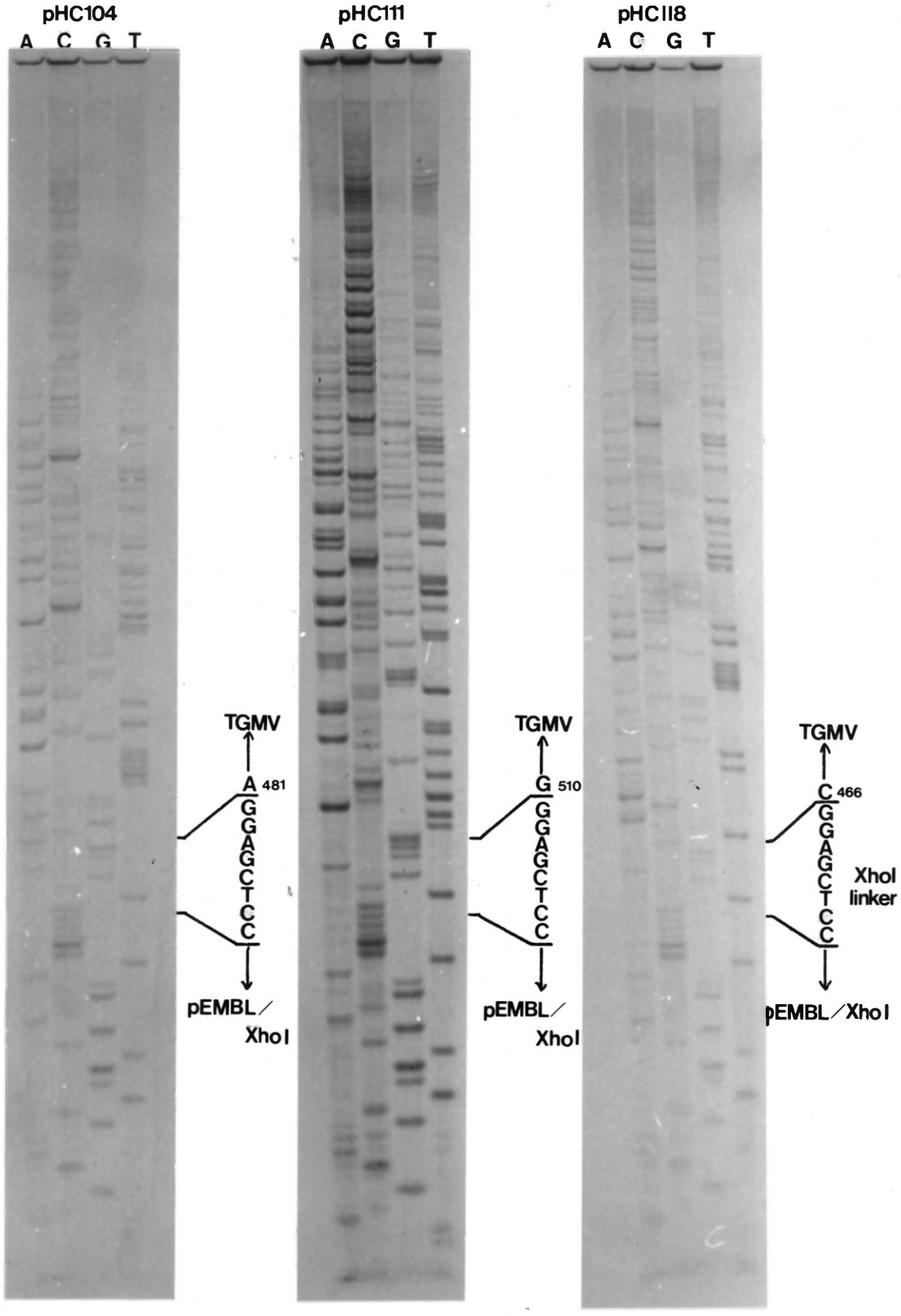


Figure 35 Sequencing Gel of pHC104, 111 & 118

The sequence at and around the vector/insert borders of three pHC100 series clones. The *XhoI* linker and the first nucleotide of the TGMV sequence are indicated. Tracks are labelled with the terminating dideoxynucleotide used in each reaction.

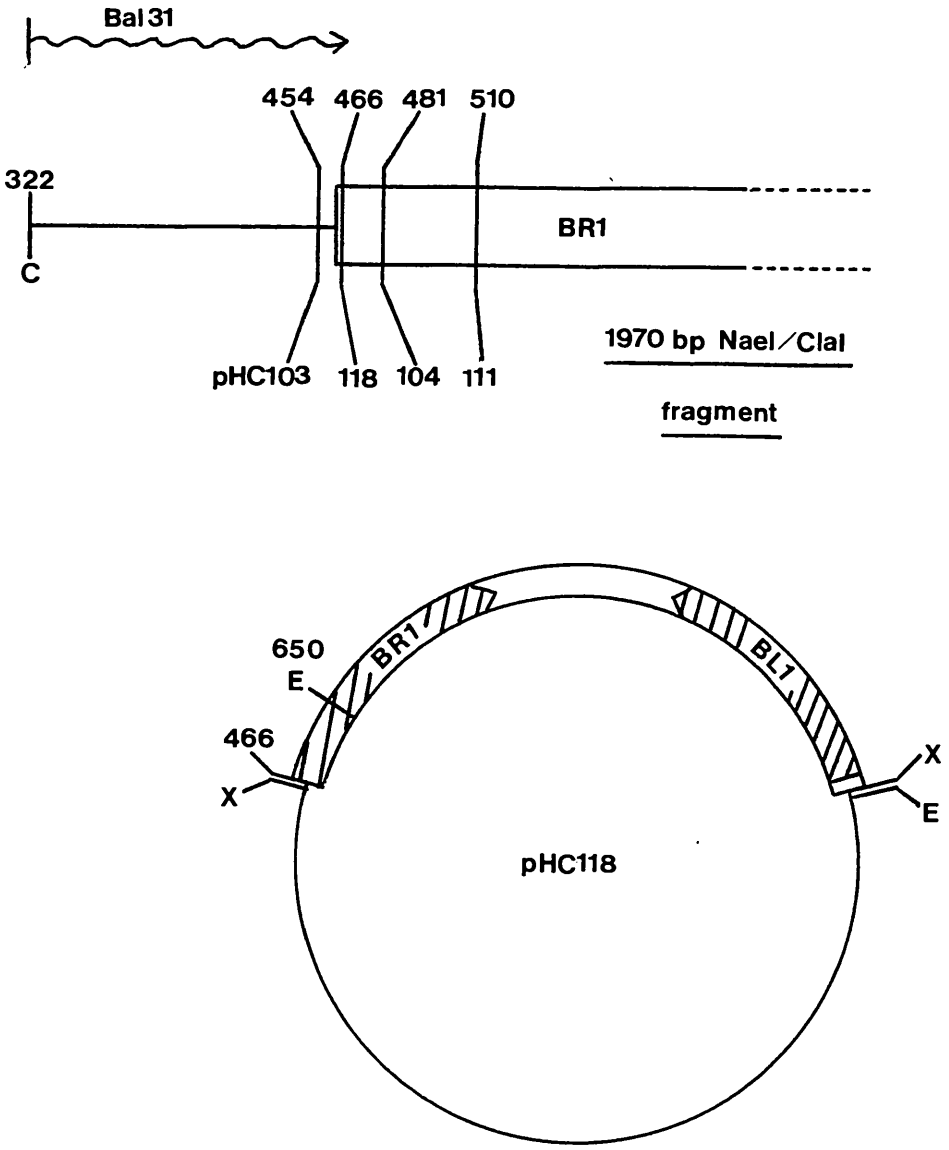


Figure 36 Extent of Bal-31 Deletion at BR1 end of pHC103, 104, 111 & 118

The figure shows four examples of the extent of Bal-31 deletion past the 5' end of ORF BR1 in the 1970 bp *Cla*I/*Nae*I fragment from pB/*Cla* after a 1 minute digestion, and one of the subsequent clones - pHC118.

Key: C - *Cla*I; E - *Eco*RI; N - *Nae*I; X - *Xho*I.

galactosidase-BR1 fusion protein, which would contain 254 out of 256 BR1 amino acids.

6.1.3 Sub-Cloning into the Expression Vectors

From the sequences of the BR1/vector border of pHC118 and of the BL1/vector border of pHC201, it was calculated which of the expression vectors (pUR290, 291 or 292) the TGMV-derived inserts would have to be sub-cloned into, in order that the shortened ORFs BR1 and BL1 would be in the same reading frame as lacZ and thus encode a fusion protein (Figure 37). For expression of a β-galactosidase-BR1 fusion protein, it was calculated that the *Xho*I insert of pHC118 would have to be sub-cloned into pUR292, and for expression of β-galactosidase-BL1 the *Xho*I insert of pHC201 would have to be sub-cloned into pUR291.

Therefore these inserts were excised from pHC118 and pHC201 by digestion with *Xho*I, and separated from the 4 kb *Xho*I fragment by agarose gel electrophoresis. The fragments containing ORFs BR1 and BL1 were eluted from the gel using Geneclean™ (section 5.1.6i), ligated with *Sal*I-digested and phosphatase-treated pUR292 and 291, respectively, and transformed into *E. coli* TG2 cells (section 5.2.5), which were plated out onto 2xTY plates containing ampicillin.

As the two sticky ends of the excised *Xho*I inserts were the same, the inserts could be ligated into the *Sal*I sites of pUR291 and 292 in either orientation. So, in order to identify the clones with the inserts in the correct orientation for subsequent fusion protein production (i.e. the BR1 ORF from the pHC118 *Xho*I insert attached to the lacZ gene of pUR292, and the BL1 ORF from the pHC201 *Xho*I insert attached to the lacZ gene of pUR291), a selection of colonies was picked and the plasmids isolated (section 5.2.6) and analysed by *Bam*HI digestion and agarose gel electrophoresis. Figure 38 shows the plasmids with the inserts in both direction, digested with *Bam*HI and electrophoresed in an agarose gel. The positions of the two *Bam*HI sites in the TGMV-derived insert and one *Bam*HI site in the polylinker allowed the orientation of the insert to be determined (Figure 39). Of the clones derived from pHC118, only those with the insert in the BR1 orientation (i.e. with the 5' end of the shortened ORF BR1

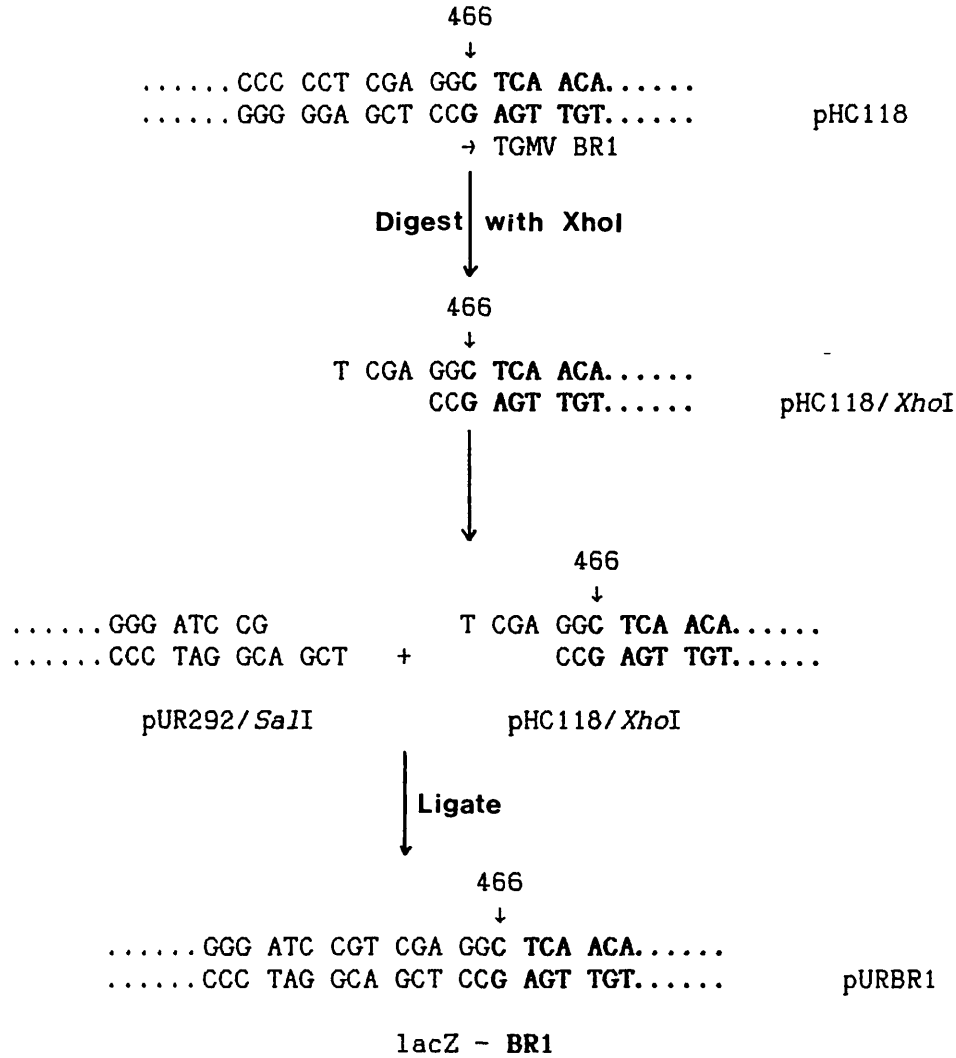


Figure 37a pHC118 → pURBR1

The figure shows how the 1770 bp *Xho*I fragment of pHC118 was subcloned into the *Sal*I site of pUR292 to form pURBR1. The nucleotides in bold type are TGMV sequences, and the nucleotides in ordinary type are vector sequences. The number 466 indicates the first TGMV nucleotide in the sequence, according to the numbering system of Hamilton *et al.* (1984).

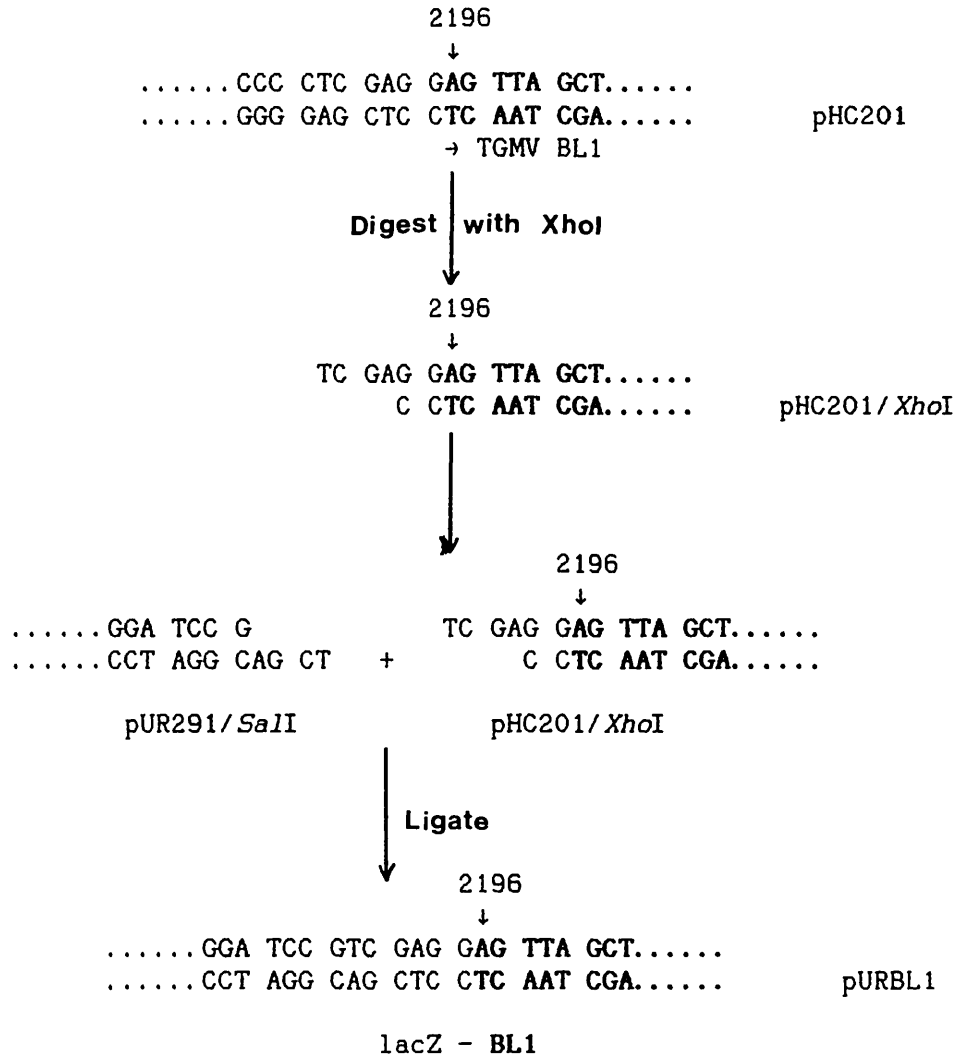


Figure 37b pHC201 → pURBL1

The figure shows how the 1670 bp *Xho*I fragment of pHC201 was subcloned into the *Sal*I site of pUR291 to form pURBL1. The nucleotides in bold type are TGMV sequences, and the nucleotides in ordinary type are vector sequences. The number 2196 indicates the first TGMV nucleotide in the sequence, according to the numbering system of Hamilton *et al.* (1984).

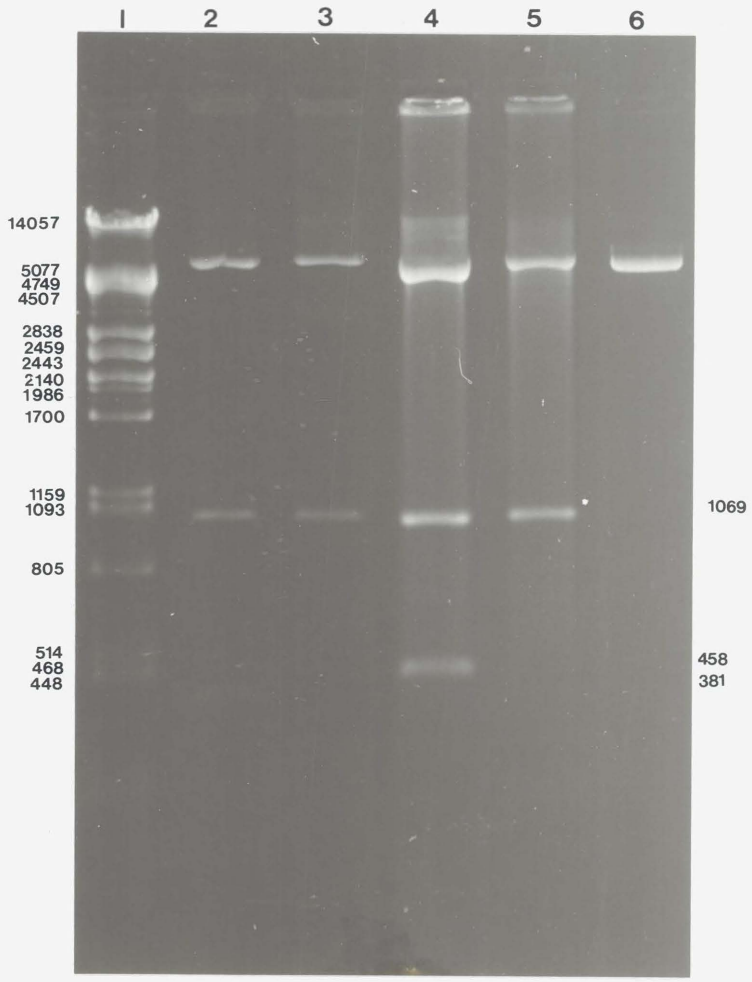


Figure 38 *Bam*HI Digests of pUR290, BR1 & BL1

Electrophoresis of *Bam*HI digests in a 1.2 % agarose gel containing 0.5 µg/ml ethidium bromide.

Lane 1 *Pst*I-digested λ DNA.
Lane 2 pHC201 *Xho*I insert sub-cloned into the *Sal*I site of pUR291 in the wrong (BR1) orientation and digested with *Bam*HI. The lowest band (211 bp) is not visible.
Lane 3 pHC201 *Xho*I insert sub-cloned into the *Sal*I site of pUR291 in the correct (BL1) orientation (pURBL1) and digested with *Bam*HI.
Lane 4 pHC118 *Xho*I insert sub-cloned into the *Sal*I site of pUR292 in the correct (BR1) orientation (pURBR1) and digested with *Bam*HI.
Lane 5 pHC118 *Xho*I insert sub-cloned into the *Sal*I site of pUR291 in the wrong (BL1) orientation and digested with *Bam*HI. The lowest band (<300 bp) is not visible.
Lane 6 *Bam*HI-digested pUR290.

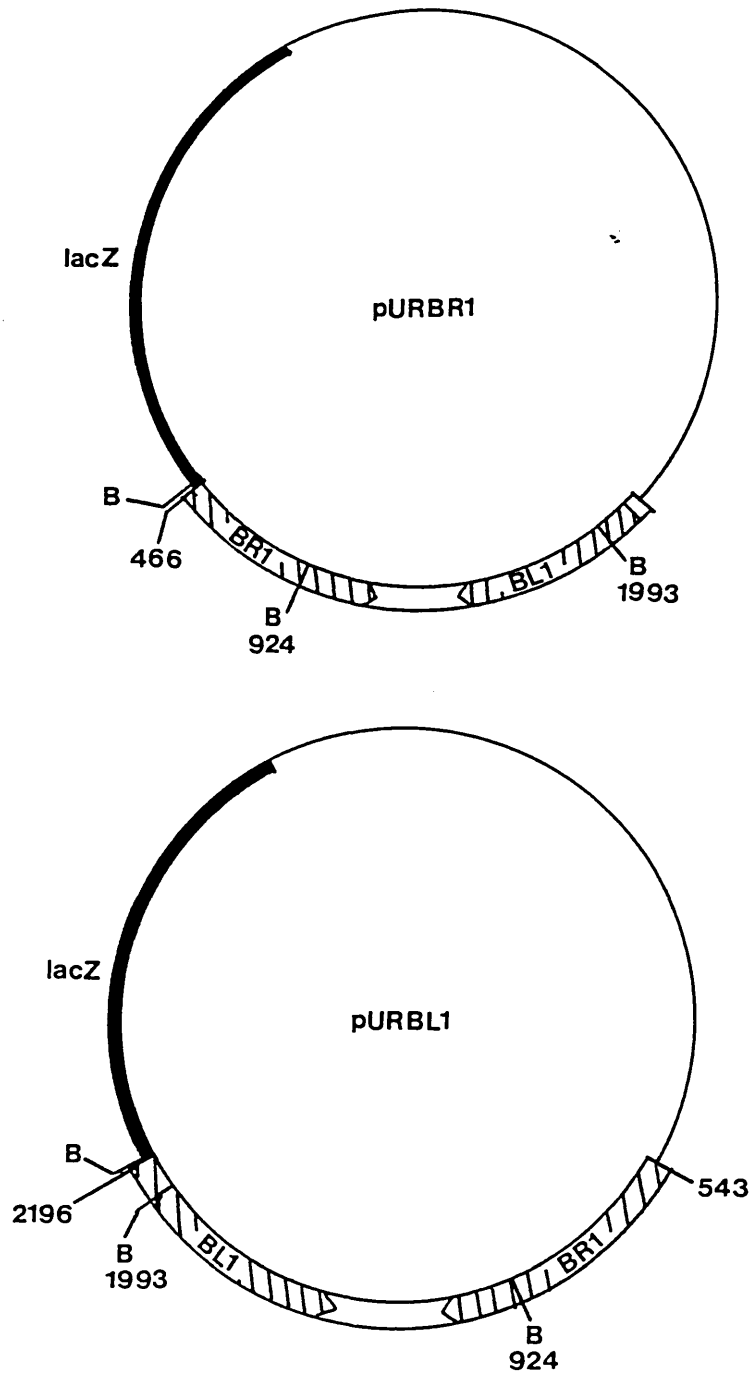


Figure 39 Maps of pURBR1 and pURBL1

Schematic diagrams of pURBR1 and pURBL1, showing the lacZ gene (shaded boxes) and the TGMV ORFs BR1 and BL1 (hatched boxes).

Key: B - *Bam*HI.

attached to the 3' end of lacZ) were selected, and designated pURBR1; and the only pHC201-derived clones selected were those with the insert in the BL1 orientation, and these were designated pURBL1.

6.2 Identification of Fusion Proteins

6.2.1 Fusion Proteins Derived from TGMV DNA B ORFs

E. coli TG2 cells containing the plasmids pURBR1 and pURBL1 were grown up overnight with or without IPTG, so that the IPTG, when present, would induce the expression of the lacZ gene and hence the production of the fusion proteins. Total soluble protein was extracted from these cells (section 5.4.1) and analysed by SDS-PAGE. This revealed that IPTG had induced the production of β-galactosidase plus a larger protein, β-gal-BR1, in the TG2:pURBR1 cells; and β-galactosidase plus three larger proteins in the TG2:pURBL1 cells (Figure 40). However, each of these proteins was a minor cellular protein, which is in sharp contrast to other fusion proteins that have been studied, such as those between β-galactosidase and the proteins encoded by CaMV gene VI or gene I. In these examples, the fusion proteins were the major cellular proteins in induced *E. coli* (Harker *et al.*, 1987).

It is not known why the β-gal-BR1 and β-gal-BL1 fusion proteins are produced in such low quantities in these cells. The plasmid constructs pURBR1 and pURBL1 have been shown by DNA sequence and restriction endonuclease analysis to be authentic, as has the *E. coli* host, TG2, which produced β-gal-AR1 as a major cellular protein when transformed with pURAR1 and grown in the presence of IPTG. Nevertheless, a different *E. coli* host, XL1-blue, was also tried. This strain also contains the $lacI^q$ gene, which encodes a strong repressor of lacZ expression. In the presence of IPTG the repressor is inhibited and hence lacZ expression is induced. When transformed with pURAR1, pURBR1 or pURBL1 and grown in the presence of IPTG, the same proportions of fusion proteins, β-galactosidase and host proteins were present, as when TG2 was the host.

The molecular weights of these fusion proteins were estimated by comparison with protein molecular weight markers, and β-gal-BR1 was found to be approximately 143 K, which compares favourably with the

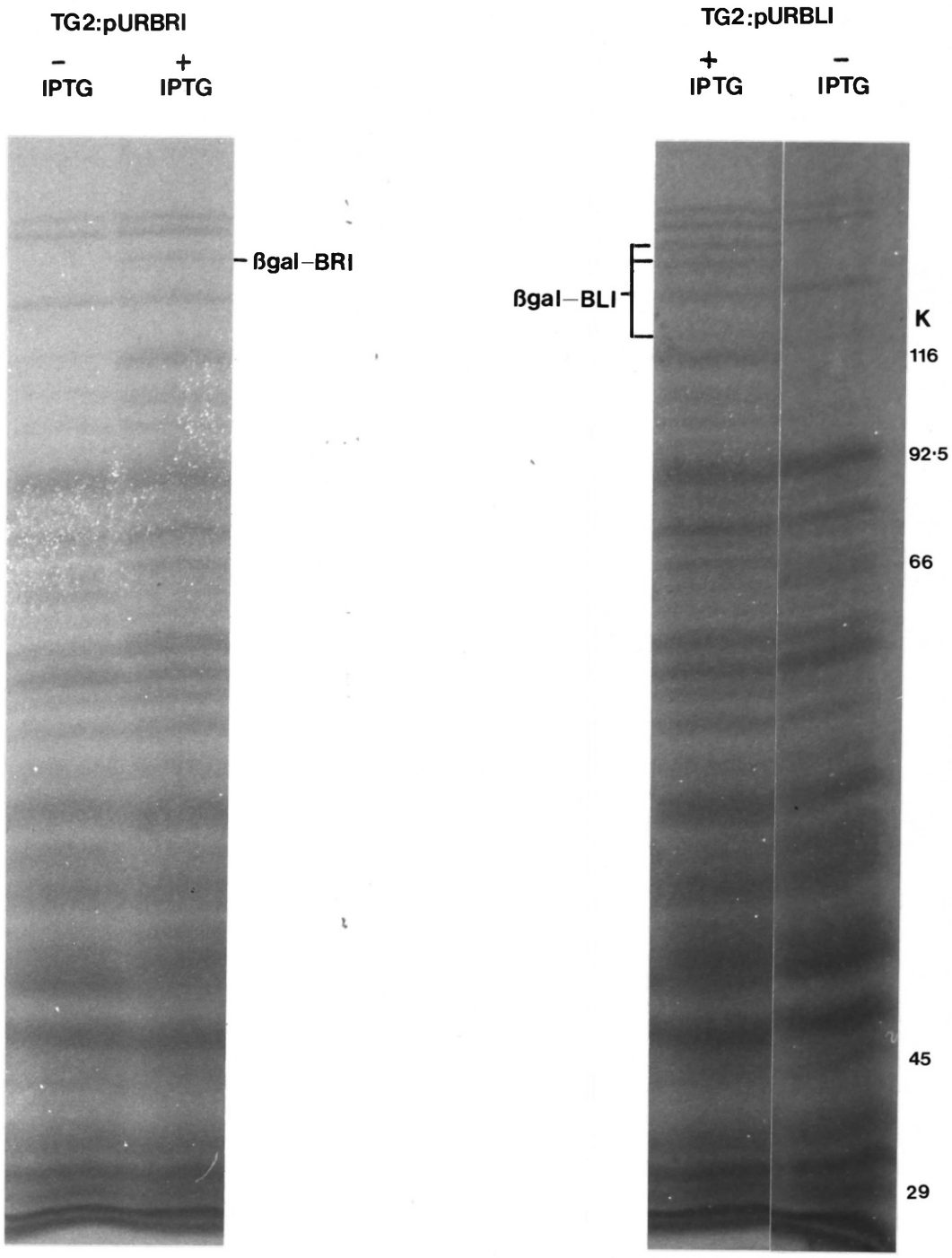


Figure 40 SDS-PAGE of Induced and Uninduced TG2: pURBR1 & BL1

Electrophoresis of IPTG-induced and uninduced TG2: pURBR1 and BL1 cell extracts in a 7.5 % SDS-polyacrylamide gel, stained with Coomassie blue. The induced fusion proteins (β-gal-BR1, 143 K; β-gal-BL1, 148, 140 & 120 K) and β-galactosidase are indicated. The positions of protein size markers are shown at the right-hand side of the gel: β-galactosidase (116 K); phosphorylase b (92.5 K); bovine serum albumin (66 K); ovalbumin (45 K); and carbanic anhydrase (29 K).

figure calculated from the sequence data of 145.1 K. The three proteins found in induced TG2:pURBL1 cells, larger than β-galactosidase, were approximately 148, 140 and 120 K. The intermediate sized protein of 140 K is thought to be the full-length β-gal-BL1 fusion protein, as it is closest to the theoretical molecular weight of 142.0 K. The identity of the other two proteins is not known. However, proteins that are smaller than the full-length fusion protein but larger than β-galactosidase have been noted before. IPTG-induced TG2:pURAL1 cells produced at least two such proteins, in quantities similar to the fusion protein, β-gal-AL1, and it has been suggested that they represent breakdown products of full-length β-gal-AL1 (Petty, 1986). Also, a range of proteins has been observed as breakdown products of β-gal-gene I and β-gal-gene VI fusion proteins from CaMV, although in both cases the full-length fusion protein was still the major band (Harker *et al.*, 1987). Therefore, the 120 K protein found in induced TG2:pURBL1 cells is probably a breakdown product of the β-gal-BL1 fusion protein.

On the other hand, the 148 K protein cannot be a breakdown product, as it is larger than β-gal-BL1. It is possible that it is formed as a result of suppression of the UAA and UAG termination codons at the end of ORF BL1. The next in-phase termination codon - UAA - is 130 bases downstream at nucleotides 1369-1367. If translation continued as far as this, a protein 45 amino acids longer would be produced, and the resulting readthrough fusion protein would have a molecular weight of approximately 147 K, which corresponds well with the 148 K protein found in induced TG2:pURBL1 cells.

The presence of this putative readthrough protein has only been demonstrated in *E. coli* TG2 and XL1-blue cells. Both of these strains contain a mutant glutamine-specific tRNA, which suppresses termination at *amber* (UAG) codons by inserting glutamine. This would explain the readthrough at the UAG codon adjacent to the UAA codon at the end of ORF BL1, but not at the UAA codon itself. It is possible that the sequence in this region, as determined by Hamilton *et al.* (1984), is inaccurate and that there is in fact no UAA codon at nucleotides 1504-1502. Credence is leant to this suggestion by the fact that MacDowell *et al.* (1986) found several minor differences in the sequence of other regions of DNA B. If this is the case, then it

is possible to envisage the production of the 147 K readthrough protein in *E. coli*.

It is interesting to note that there were no putative readthrough fusion proteins found in induced *E. coli* cells containing any of the other five TGMV-derived fusion plasmids, pURAR1, pURAL1, pURAL2, pURAL3 and pURBR1, and of these, only one - pURAL2 - has a UAG termination codon at the end of the TGMV-derived ORF. However, in this case, there is an in-phase UAA termination codon situated four bases downstream. Therefore, UAG suppression would not produce readthrough proteins significantly larger than full-length fusion proteins with any of these plasmids.

Although a β-gal-BL1 readthrough protein appears to be produced in *E. coli*, I can only speculate on whether a BL1 readthrough protein is produced *in vivo*, during normal TGMV infection. Readthrough is known to occur in several plant viruses, as a means to produce more than one protein from one ORF, and the best studied of these is tobacco mosaic virus (TMV). The 5' proximal ORF of TMV RNA can be translated *in vitro* and *in vivo* to yield a major 126 K protein and a minor 183 K readthrough protein (Pelham, 1978; Beier *et al.*, 1980). The latter is produced as a result of suppression of the UAG termination codon of the former and is essential for TMV replication. In tobacco plants this readthrough was found to be caused by the two major cytoplasmic tyrosine-specific tRNAs (Beier *et al.*, 1984a, b). It remains to be seen whether the two suppressor tRNAs will suppress any UAG termination codon or just the one found at the end of the TMV 126 K ORF. There may be some as yet unidentified sequences at or near the UAG codon in TMV RNA, which are involved in $tRNA^{Tyr}$ recognition. As TGMV also infects tobacco plants it is tempting to suggest that these two $tRNA^{Tyr}$s could also suppress the UAG termination codon in TGMV ORF BL1 during infection of tobacco plants.

One of the major incongruities between the six ORFs that are conserved among the three sequenced whitefly-transmitted geminiviruses (ACMV, BGMV and TGMV) is that ORF BL1 of TGMV encodes a protein (26.5 K) that is considerably smaller than the analogous proteins in ACMV (33.7 K) and BGMV (33.1 K). If termination readthrough were to occur *in vivo* for TGMV ORF BL1, the protein produced would have a molecular weight of approximately 34.5 K, which

is more akin to the molecular weights of the BL1 proteins of ACMV and BGMV. This perhaps suggests that the larger 34.5 K protein is the major protein encoded by ORF BL1 of TGMV.

6.2.2 Fusion Proteins Derived from TGMV DNA A ORFs

As mentioned earlier, fusion proteins derived from each of the four TGMV DNA A component ORFs (β-gal-AR1, -AL1, -AL2 and -AL3) have been constructed (Petty, 1986; Tables 11 and 12). *E. coli* TG2 cells containing the fusion plasmids pURAR1, pURAL1, pURAL2 and pURAL3 were grown up, with or without IPTG, and total soluble protein was extracted and analysed by SDS-PAGE (Petty, 1986). The Coomassie blue-stained polyacrylamide gels showed that the IPTG had induced the production of β-galactosidase and β-gal-fusion proteins such that they were the two most abundant cellular proteins. This is in contrast to the β-gal-BR1 and β-gal-BL1 fusion proteins, which were minor cellular proteins. In the case of pURAL1, there were also at least two putative breakdown products of full-length β-gal-AL1, a situation similar to that of β-gal-BL1. All four of the full-length fusion proteins were very similar in size to that estimated from their amino acid sequences.

6.3 Characterisation of Fusion Proteins

6.3.1 β-galactosidase-AR1

An antiserum has previously been raised against the β-gal-AR1 fusion protein by Petty (1986) (described in section 6.2.2), using the following methods:- a large-scale culture of TG2:pURAR1 was grown at 37 °C, with IPTG being added when the OD_{550} had reached 0.2. Aliquots were taken at 30 minute intervals and analysed by OD_{550} measurement and SDS-PAGE. The growth curve was shown to be the same as that for untransformed TG2, thus demonstrating that β-gal-AR1 production had no adverse effects on the growth of the host *E. coli*. SDS-PAGE analysis detected β-gal-AR1 and β-galactosidase one hour after induction and their concentrations reached a peak after two hours. Total protein was extracted from the cells three hours after induction, separated into soluble and insoluble fractions, and

Table 11 Fusion Proteins Derived from the Six TGMV ORFs

fusion plasmid	fusion protein	codons fused*	K^+ fused	M_r of fusion protein (K)
pURAR1	β-gal-AR1	222	25.9	141.9
pURAL1	β-gal-AL1	233	26.5	142.5
pURAL2	β-gal-AL2	86	9.9	125.9
pURAL3	β-gal-AL3	96	11.4	127.4
pURBR1	β-gal-BR1	254	29.1	145.1
pURBL1	β-gal-BL1	230	26.0	142.0

* number of codons from each ORF fused to β-galactosidase.

+ estimated molecular weight of fused amino acids.

Table 12. Percentage of the TGMV ORF-Encoded Amino Acids Present in the Fusion Proteins

ORF	amino acids	fusion protein	amino acids*	percentage†
AR1	247	β-gal-AR1	221	89 %
AL1	352	β-gal-AL1	232	66 %
AL2	129	β-gal-AL2	85	66 %
AL3	132	β-gal-AL3	95	72 %
BR1	256	β-gal-BR1	254	99 %
BL1	234	β-gal-BL1	230	98 %

* number of TGMV ORF-encoded amino acids in the fusion protein.

† percentage of the TGMV ORF-encoded amino acids that are present in the fusion protein.

analysed by SDS-PAGE. Most of the β-gal-AR1 fusion protein was found in the insoluble fraction, which was quite surprising, as β-galactosidase is a soluble protein and the C-terminal AR1 moiety makes up only 18 % of the β-gal-AR1 fusion protein. It is possible that the AR1 moiety is so insoluble that it consequently makes β-gal-AR1 insoluble. On the other hand, Nixon (personal communication, quoted in Petty, 1986) suggested that the culture conditions were important in determining not only the yield of fusion protein, but also its distribution between the soluble and insoluble protein fractions.

The β-gal-AR1 fusion protein was isolated by electroelution from a preparative SDS-polyacrylamide gel, in which the bands were located by precipitation of unbound SDS with sodium acetate (Higgins & Dahmus, 1979), precipitated with ethanol and resuspended in sterile dH_2O. An antiserum was raised against β-gal-AR1 by sub-cutaneous injection of a rabbit with 50 μg of protein emulsified with Freund's complete adjuvant, followed by further injections, each of 50 μg of protein emulsified with Freund's incomplete adjuvant, 14, 29, 49 and 91 days later. The final bleed was taken 105 days after the first injection, and serum prepared from it and from a blood sample taken 42 days after the first injection (section 5.4.4).

Both sera were tested for reactivity against various proteins immobilised on nitrocellulose by Western blotting (section 5.4.7). The 42 day serum (0.5 ml diluted to 10 ml with TBS) was found to contain antibodies which reacted against many proteins in extracts from IPTG-induced TG2:pURAR1 cells (in addition to β-gal-AR1 and β-galactosidase) and in extracts from uninfected as well as TGMV-infected *N. benthamiana* plants. No reaction was obtained against purified TGMV capsid protein. However, the 105 day serum (0.5 ml diluted to 10 ml with TBS) reacted with all of these proteins, plus TGMV capsid protein (Petty, 1986).

These results suggest that antibodies against the AR1 moiety of β-gal-AR1 are reacting against TGMV capsid protein, and hence that ORF AR1 is the capsid protein gene. However, as the serum also reacted against many bacterial and plant proteins, it was not possible to say this unequivocally.

In order to verify that antibodies to the AR1 moiety of β-gal-AR1

react solely against TGMV capsid protein, I removed all antibodies that react against the bacterial and plant proteins from the 105 day serum. This was achieved by pre-absorbing the serum with IPTG-induced TG2:pUR292 proteins (section 5.4.5) and healthy *N. benthamiana* proteins (section 5.4.6). By this method any protein will form an insoluble complex with its antibody, and hence all antibodies against induced TG2:pUR292 proteins and *N. benthamiana* proteins will be precipitated out and can be removed by centrifugation. As the only protein that is present in induced TG2:pURAR1, but not in induced TG2:pUR292, is β-gal-AR1, and the only protein present in TGMV-infected *N. benthamiana*, but not in healthy *N. benthamiana*, is TGMV capsid protein, then the only antibodies left should be anti-β-gal-AR1, if ORF AR1 is the capsid protein gene. However, this cleaned-up antiserum reacted with three of the induced TG2:pURAR1 proteins: β-gal-AR1 (as expected), β-galactosidase and a protein of approximately 60 K (Figure 41a). The continuing presence of antibodies against β-galactosidase implies that there was not enough of the protein present in the induced TG2:pUR292 extract to precipitate all of the antibodies. The identity of the 60 K protein is not known, but it is interesting to note that an antiserum raised against purified TGMV capsid protein (Stein *et al.*, 1983) also reacted with a protein of approximately 60 K in induced TG2:pURAR1 extract (Figure 41b).

The cleaned-up anti-β-gal-AR1 serum was then tested, to ensure that it had retained its reactivity with TGMV capsid protein. Three equal aliquots of partially-purified TGMV capsid protein (section 5.5.4) were immobilised on three separate pieces of nitrocellulose (section 5.4.7). The first piece of nitrocellulose was then stained with amido black, revealing one band at approximately 29 K - the capsid protein. The other two pieces were probed with anti-TGMV capsid protein serum (Stein *et al.*, 1983) and the cleaned-up anti-β-gal-AR1 serum, respectively. Both antisera reacted with the capsid protein band (Figure 42; Kallender *et al.*, 1988). As the anti-capsid protein serum was prepared against this protein, this was to be expected, but as the anti-β-gal-AR1 serum was prepared against β-gal-AR1, then it follows that the AR1 moiety of the fusion protein is

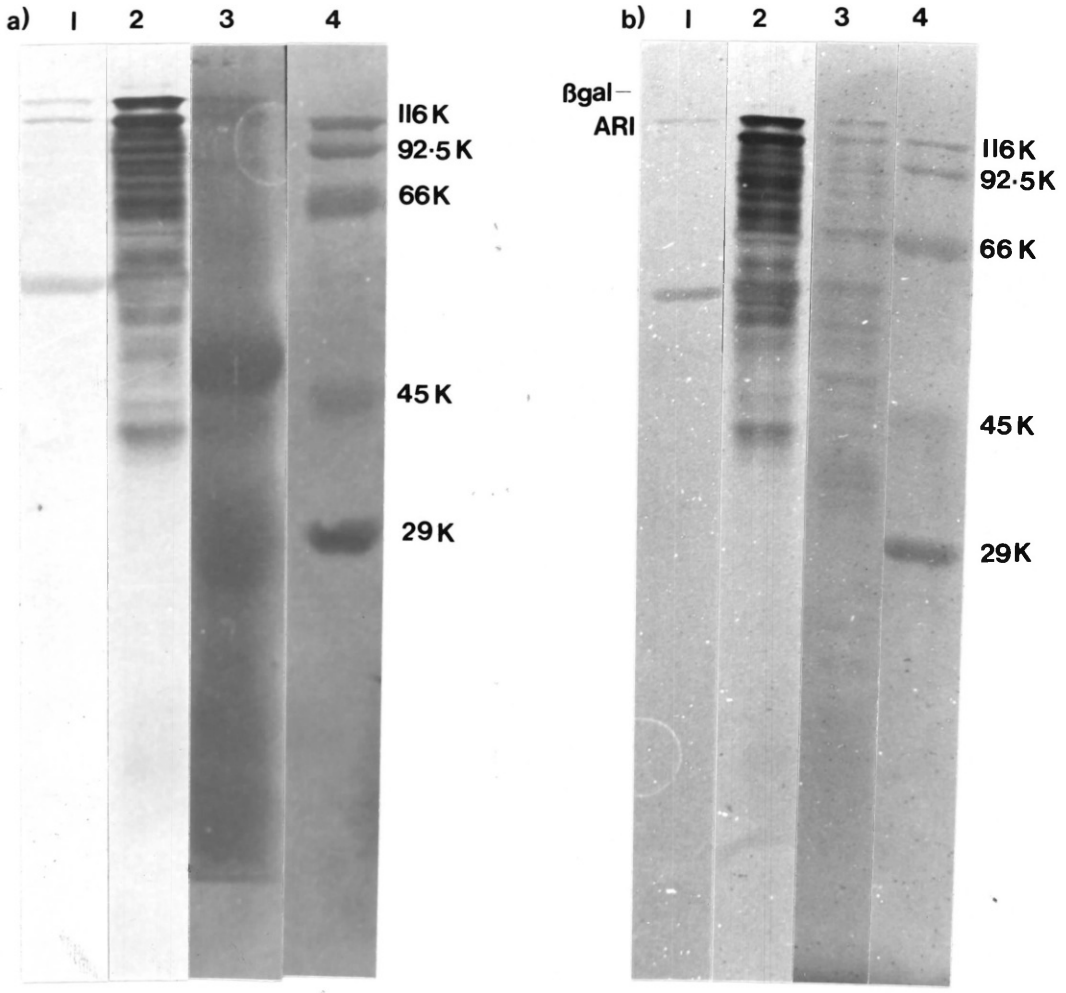


Figure 41 Detection of β-gal-AR1 by Western Blotting

Electrophoresis of IPTG-induced TG2: pURAR1 cell extract in a 7.5 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose.

a) Lanes 1 - 3 were IPTG-induced TG2: pURAR1 cell extract. Lane 1 was probed with anti-β-gal-AR1 serum pre-absorbed with IPTG-induced TG2: pUR292 extract; Lane 2 was probed with crude anti-β-gal-AR1; Lane 3 was stained with amido black. Lane 4 was protein size markers stained with amido black.

b) Lanes 1 - 3 were IPTG-induced TG2: pURAR1 cell extract. Lane 1 was probed with anti-capsid protein serum; Lane 2 was probed with crude anti-β-gal-AR1 serum; Lane 3 was stained with amido black. Lane 4 was protein size markers stained with amido black.

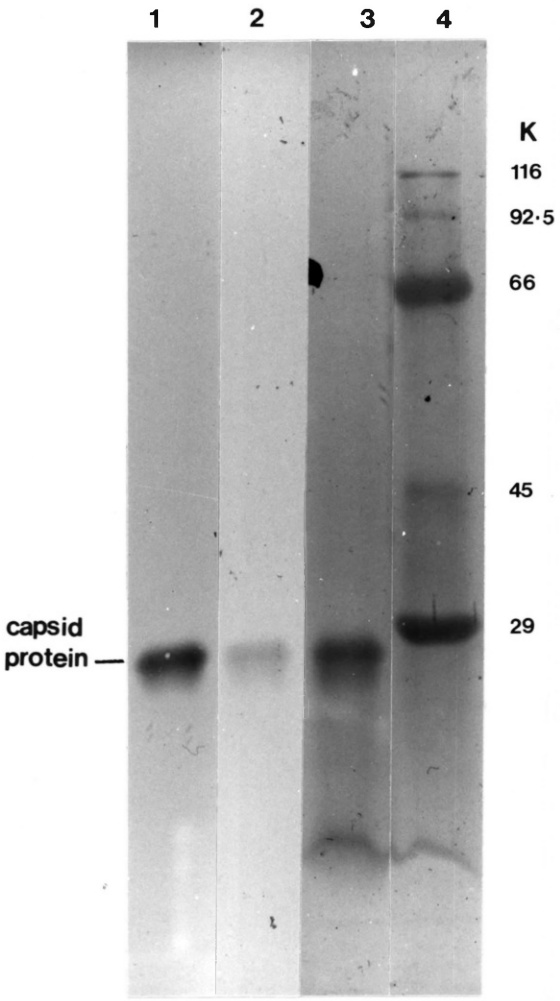


Figure 42 Detection of TGMV Capsid Protein by Western Blotting

Electrophoresis of partially purified TGMV virions in a 15 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose. Lanes 1 - 3 were partially purified TGMV virions. Lane 1 was probed with anti-capsid protein serum; Lane 2 was probed with anti-β-gal-AR1 serum pre-absorbed with IPTG-induced TG2:pUR292 extract and *N. benthamiana* proteins; Lane 3 was stained with amido black. Lane 4 was protein size markers stained with amido black.

probably the C-terminal 25.9 K of the capsid protein, thus implying that ORF AR1 of TGMV is the capsid protein gene. However, it is possible that there were rabbit antibodies present in the anti-β-gal-AR1 serum that fortuitously react with TGMV capsid protein. So, to discount this possibility, a pre-immune serum (Petty, 1986; 0.5 ml diluted to 10 ml with TBS) was tested for reactivity against purified capsid protein (section 5.5.4) by Western blotting, and no reaction was observed. Therefore, the reaction of the anti-β-gal-AR1 serum with capsid protein proves that ORF AR1 is the capsid protein gene of TGMV.

This has also been proved by the analysis of the cyanogen bromide cleavage products of the TGMV capsid protein (Stein, 1985). The cleavage products were separated by high pressure liquid chromatography, and three selected peptides examined by mass spectrometry. The masses of these compared favourably with three of the nine peptides predicted to be formed by cleavage of the product of ORF AR1. Determination of the N-terminal amino acid sequences of two of the peptides by Edman degradation (Edman & Berg, 1967) confirmed their identities and proved conclusively that ORF AR1 encodes the capsid protein.

6.3.2 β-galactosidase-AL1

An antiserum has previously been raised against the β-gal-AL1 fusion protein by Petty (1986) (described in section 6.2.1), using the same methods as those described for the β-gal-AR1 fusion protein in section 6.3.2.

I pre-absorbed the serum prepared from the final bleed with IPTG-induced TG2:pUR292 proteins (section 5.4.5) to remove any antibodies present against these proteins, and then tested its reactivity against total proteins extracted from IPTG-induced TG2:pURAL1 and TG2:pUR292 (section 5.4.1), by Western blotting (section 5.4.7). The cleaned-up serum was found to react with β-gal-AL1 and β-galactosidase, plus the two proteins of intermediate size (Figure 43) that were thought to be partial breakdown products of the β-gal-AL1 fusion protein (Petty, 1986). This result confirms that these two proteins are indeed shorter versions of β-gal-AL1 (i.e shorter at the

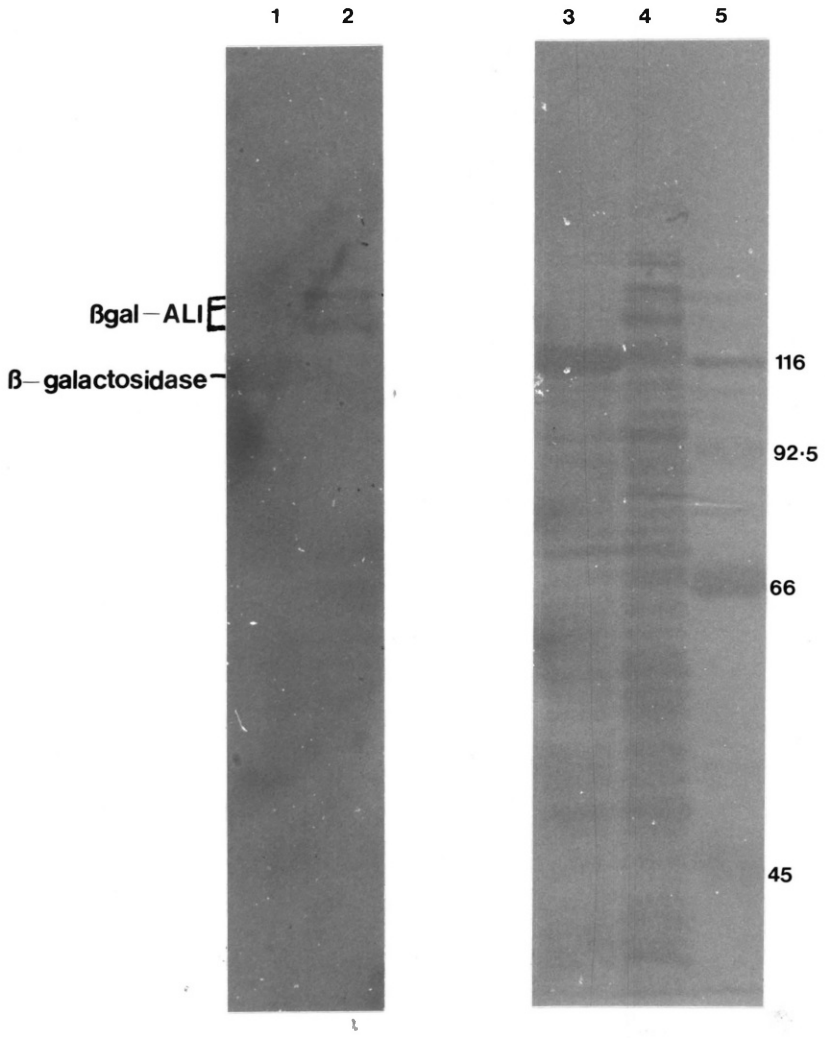


Figure 43 Detection of β-gal-AL1 and its Putative Breakdown Products by Western Blotting

Electrophoresis of IPTG-induced TG2:pURAL1 and TG2:pUR290 cell extracts in a 7.5 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose. Lanes 1 & 3 were induced TG2:pUR290 cell extracts; Lanes 2 & 4 were induced TG2:pURAL1 cell extracts; Lane 5 was protein size markers. Lanes 1 & 2 were probed with anti-β-gal-AL1 serum pre-absorbed with IPTG-induced TG2:pUR292 proteins; Lanes 3 - 5 were stained with amido black.

C-terminal end of the AL1 moiety), and it adds weight to the theory that they are actually partial breakdown products of β-gal-AL1.

Elmer *et al.* (1988) have shown that the product of ORF AL1 is involved in TGMV DNA replication (section 4.8.1) and hence the AL1 protein must be located *in vivo* in the nucleus. Therefore, to identify the protein, the anti-β-gal-AL1 serum was tested for reactivity against proteins from the purified nuclei of healthy and TGMV-infected *N. benthamiana* (section 5.5.5), plus total proteins extracted from healthy and TGMV-infected *N. benthamiana* (section 5.5.6), by Western blotting (section 5.4.7). The cleaned-up serum (0.5 ml plus 9.5 ml TBS) reacted against several proteins found in both healthy and infected samples, which are assumed to be host-specific, but not against any protein found solely in the infected samples.

As the cleaned-up serum was approximately ten times more dilute than the crude serum, it was decided to repeat this experiment using 0.5 ml crude serum (plus 9.5 ml TBS). As a result, the crude anti-β-gal-AL1 serum was found to contain antibodies against several proteins from both healthy and infected *N. benthamiana*, plus one protein found in the nuclei and the total protein extract of TGMV-infected *N. benthamiana* , but not in the healthy samples (Figure 44). This protein has an approximate molecular weight of 39.5 K, which is similar to the expected molecular weight of the ORF AL1 protein, as calculated from the amino acid sequence, 40,285 (Hamilton *et al.*, 1984), and is assumed to be the AL1 protein.

However, further work is required to confirm this. The 39.5 K protein must be identified with a more specific serum i.e. a serum with all antibodies against *N. benthamiana* proteins removed. Also, the 39.5 K protein must be shown not to react with pre-immune serum, to ensure that the reaction of the anti-β-gal-AL1 serum with this protein is not due to the presence of rabbit antibodies.

6.3.3 β-galactosidase-AL2

A large-scale (1 litre) culture of TG2:pURAL2 was grown at 37 °C as described in section 5.4.2, with IPTG being added two hours after inoculation. The bacterial growth was monitored by measuring the

Legend to Figure 44 Detection of the Protein Encoded by TGMV ORF AL1 by Western Blotting

Electrophoresis of various fractions from healthy and TGMV-infected *N. benthamiana* plants in a 12 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose. Lanes 1 & 5 were total protein extracted from TGMV-infected *N. benthamiana*; Lanes 2 & 6 were total protein extracted from healthy *N. benthamiana*; Lanes 3 & 7 were purified nuclei from TGMV-infected *N. benthamiana*; Lanes 4 & 8 were purified nuclei from healthy *N. benthamiana*; Lane 9 was protein size markers. Lanes 1 - 4 were probed with crude anti-β-gal-AL1 serum; Lanes 5 - 9 were stained with amido black.

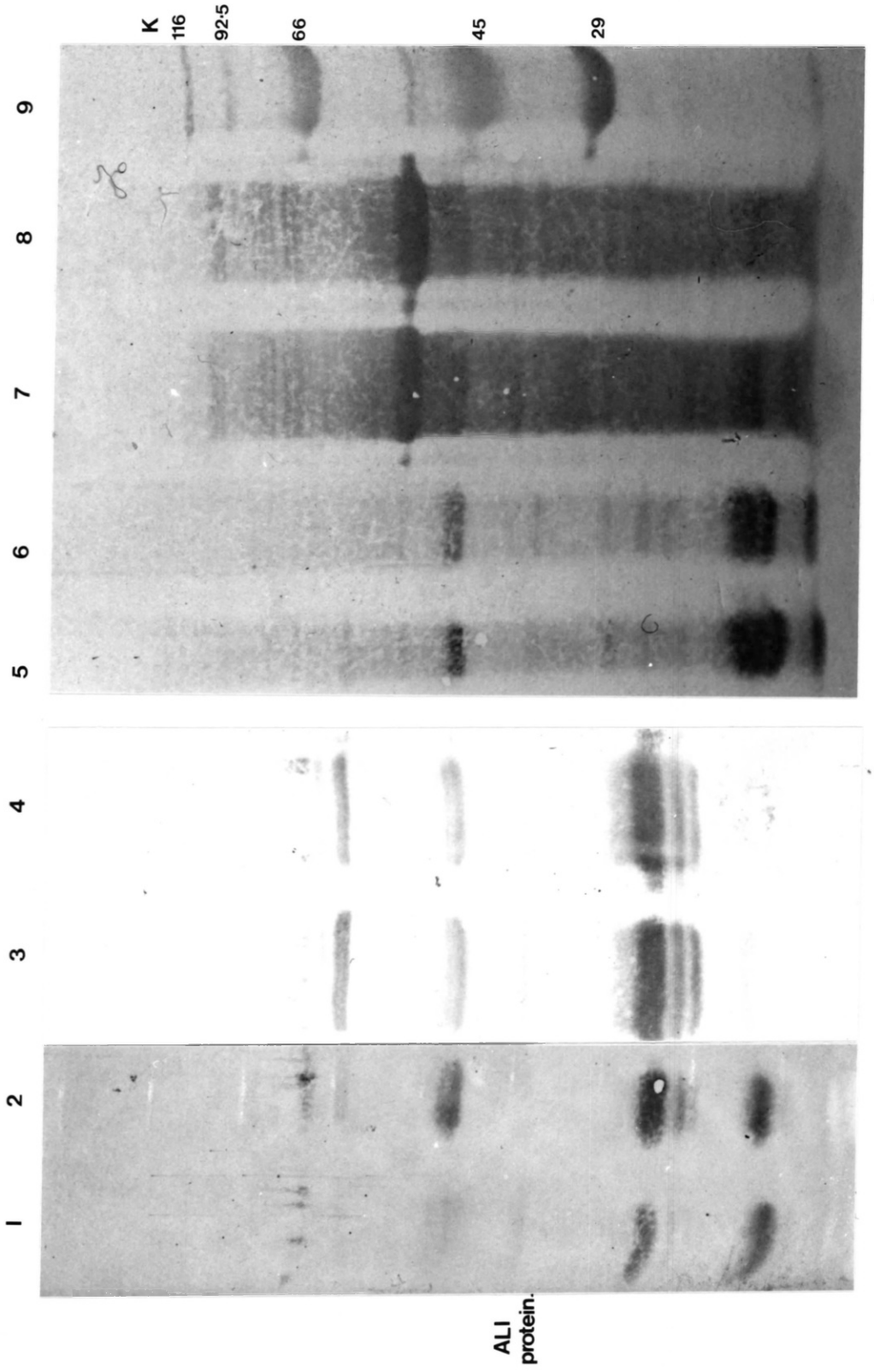


Figure 44 Detection of the Protein Encoded by TGMV ORF AL1 by Western Blotting

OD_{550} of 1 ml samples taken every half-hour and plotting the growth curve. This was found to be the same as the growth curve for untransformed TG2 (Figure 45), which demonstrates that the production of β-gal-AL2 does not affect the growth of the host *E. coli*. The accumulation of β-gal-AL2 in the induced cells was monitored by analysing the total protein from the 1 ml samples by electrophoresis in SDS-polyacrylamide gels. β-gal-AL2 was first detected 30 minutes after induction, and its concentration increased for a further 1½ hours (Figure 46). At this point total protein was extracted from the 1 litre culture by the methods of Marston (1987) and Carroll & Laughon (1987), into soluble and insoluble protein fractions (section 5.4.2). Aliquots of each were analysed by SDS-PAGE, showing that β-gal-AL2 was present in both fractions, with most present in the soluble fraction (Figure 47). The β-gal-AL2 in each fraction was concentrated by precipitation with ammonium sulphate. To find the optimum ammonium sulphate concentration, 1 ml aliquots were treated with 25, 30, 35 or 40 % (w/v) ammonium sulphate and the total protein analysed by SDS-PAGE (Figure 47). For the soluble fraction, similar amounts of β-gal-AL2 were precipitated at each ammonium sulphate concentration, but at the lowest ammonium sulphate concentration (25 %), the fewest host proteins were precipitated. For the insoluble fraction, similar amounts of β-gal-AL2 and host proteins were precipitated at each ammonium sulphate concentration.

The major part of the soluble and insoluble fractions was therefore treated with 25 % (w/v) ammonium sulphate, before purifying the β-gal-AL2 protein further by gel filtration chromatography, on a Sephacryl S-300HR column with a packed gel height and diameter of 50 cm and 2.5 cm, respectively (section 5.4.3). The column was calibrated by running a 5 ml sample containing 1 mg of each of thyroglobulin (669 K) and horse apoferritin (443 K) through it, and monitoring the OD_{280} of the eluant. Thyroglobulin was eluted after 165 minutes and apoferritin after 195 minutes. From this it was calculated that β-gal-AL2, which has a molecular weight of 503.6 K in its native tetrameric form, would elute from the column approximately 185 minutes after loading.

The 5 ml soluble fraction was loaded onto the column and the OD_{280} of the eluant monitored. Five minutes before the β-gal-AL2 was

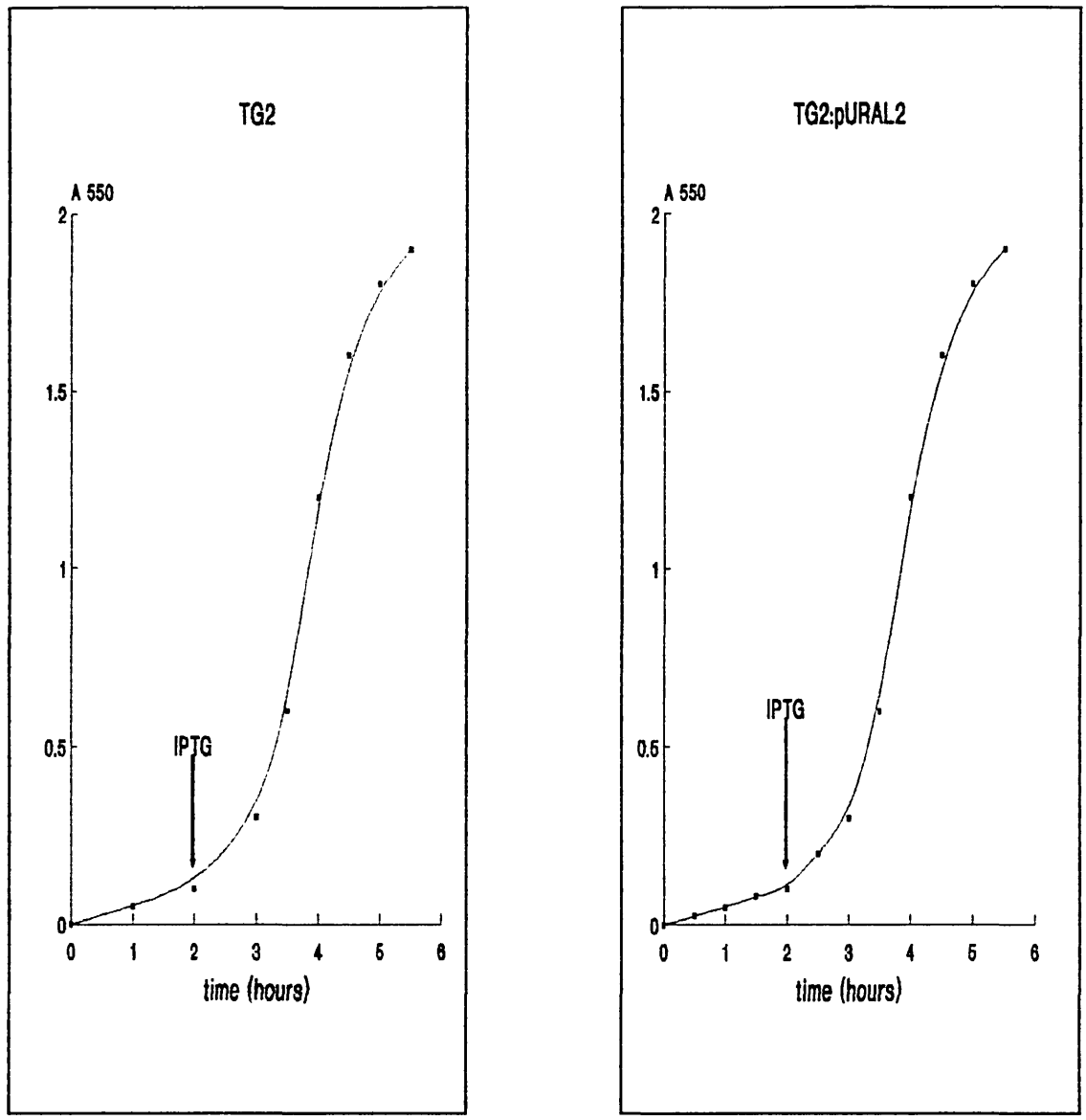


Figure 45 Growth curves of TG2 and IPTG-induced TG2: pURAL2

The growth curves of *E. coli* strain TG2 were determined by measuring the optical density at 550 nm of 1 ml aliquots of a 1 litre culture at various times after inoculation with 5 ml of a saturated overnight culture. The culture of TG2 was in 2xTY broth, and TG2: pURAL2 in 2xTY containing ampicillin. After two hours of growth IPTG was added to the TG2: pURAL2 culture to induce production of the fusion protein.

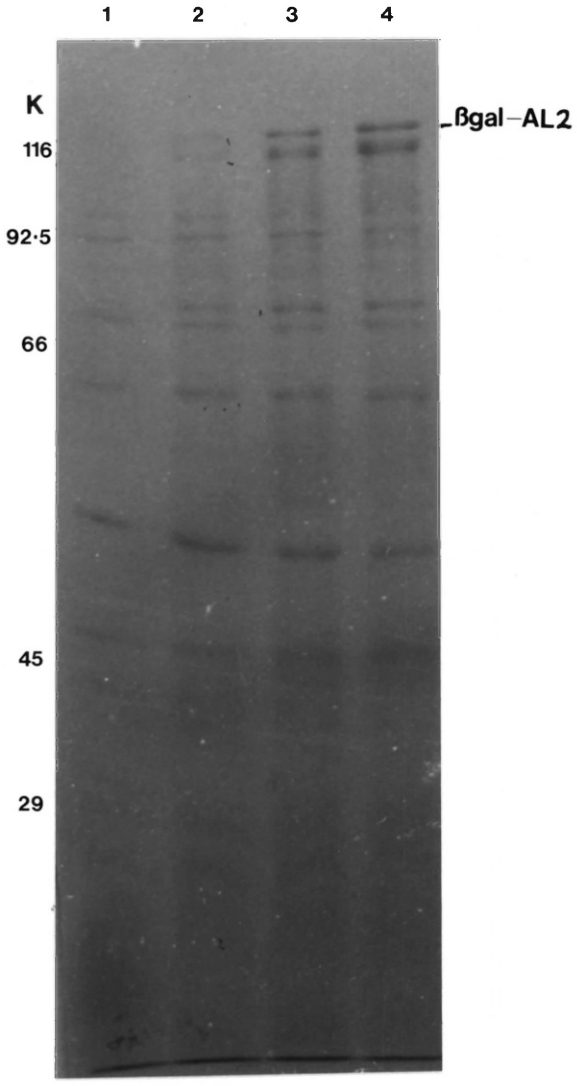


Figure 46 Time Course of IPTG Induction of TG2: pURAL2 Cells

Electrophoresis of IPTG-induced TG2: pURAL2 cell extracts in a 7.5 % SDS-polyacrylamide gel, stained with Coomassie blue. The extracts were taken at the following times after the addition of IPTG: Lane 1 - 0 minutes; Lane 2 - 30 minutes; Lane 3 - 60 minutes; Lane 4 - 90 minutes. The positions of protein size markers are shown on the left-hand side of the gel.

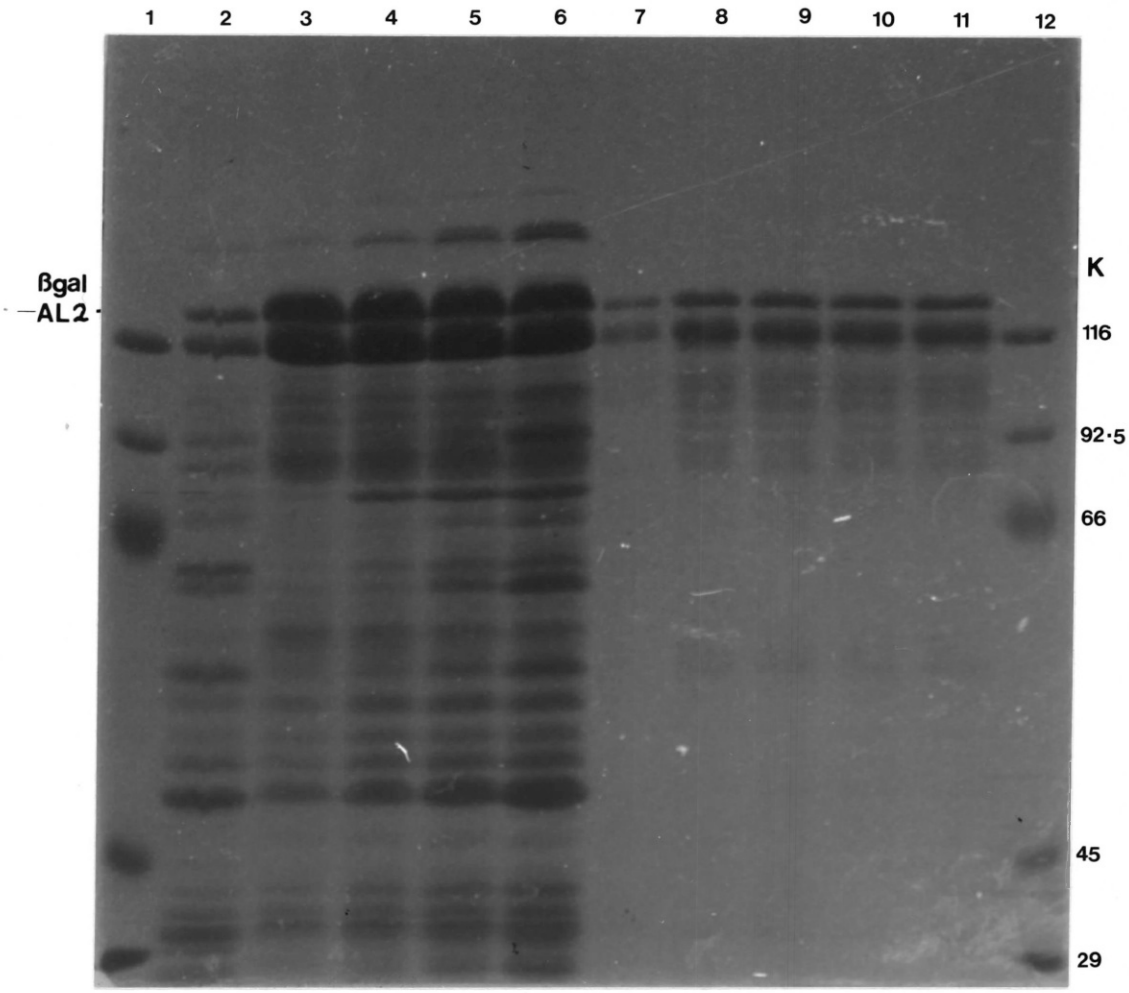


Figure 47 Ammonium Sulphate Precipitation of Proteins in IPTG-induced TG2: pURAL2 Cell Extracts

Electrophoresis of ammonium sulphate-precipitated extracts from IPTG-induced TG2: pURAL2 cells in a 7.5 % SDS-polyacrylamide gel, stained with Coomassie blue.

Lanes 1 & 12	Protein size markers.
Lane 2	10 µl of the soluble fraction.
Lanes 3 - 6	20 µl each of the soluble fraction concentrated with 25, 30, 35 and 40 % (w/v) ammonium sulphate, respectively.
Lane 7	10 µl of the resuspended insoluble fraction.
Lanes 8 - 11	20 µl each of the resuspended insoluble fraction concentrated with 25, 30, 35 and 40 % (w/v) ammonium sulphate, respectively.

expected (180 minutes after loading), the collection of eluant was started, in 10 ml aliquots, and these samples were analysed by SDS-PAGE. The OD_{280} profile showed several peaks, the first and largest of which appeared 185-190 minutes after the sample was loaded. The eluant collected at this time was found to contain the β-gal-AL2 fusion protein and also β-galactosidase (Figure 48, lanes 2 & 3). Therefore, the column had succeeded in separating β-gal-AL2 from the *E. coli* proteins, but not from the β-galactosidase. This was not considered to be a major problem for the subsequent production of an antiserum to β-gal-AL2, because most of the anti-β-galactosidase antibodies could be removed by pre-absorbing the antiserum with IPTG-induced TG2: pUR292 extract (section 5.4.5).

The eluant samples containing the fusion protein were pooled and the β-gal-AL2 and β-galactosidase concentrated by precipitation with 25 % (w/v) ammonium sulphate, to give a final β-gal-AL2 concentration of approximately 0.2 mg/ml, as determined by electrophoresing an aliquot alongside several β-galactosidase samples of varying known concentrations on an SDS-polyacrylamide gel, and comparing the intensity of the bands after Coomassie blue staining.

An antiserum was raised against β-gal-AL2 by injecting a rabbit with three 100 μg doses of purified β-gal-AL2, as described in section 5.4.4. Fifty five days after the first injection, the rabbit was bled to death, and approximately 60 ml of serum was prepared from the blood (section 5.4.4).

Some preliminary tests have been carried out on this serum. Initially, I pre-absorbed the serum with IPTG-induced TG2: pUR292 proteins (section 5.4.5) to remove any antibodies present against these proteins, and then tested its reactivity against total proteins extracted from IPTG-induced TG2: pURAL2 (section 5.4.1) by Western blotting (section 5.4.7). The cleaned-up serum was found to react with β-gal-AL2 and β-galactosidase (Figure 49), thus confirming that there are antibodies present in the serum against the β-gal-AL2 fusion protein.

The cleaned-up serum was then tested for reactivity against total protein extract from healthy and TGMV-infected *N. benthamiana* (equivalent to 1 mg of leaf material; section 5.5.6) and nuclei isolated from healthy and TGMV-infected *N. benthamiana* (equivalent to

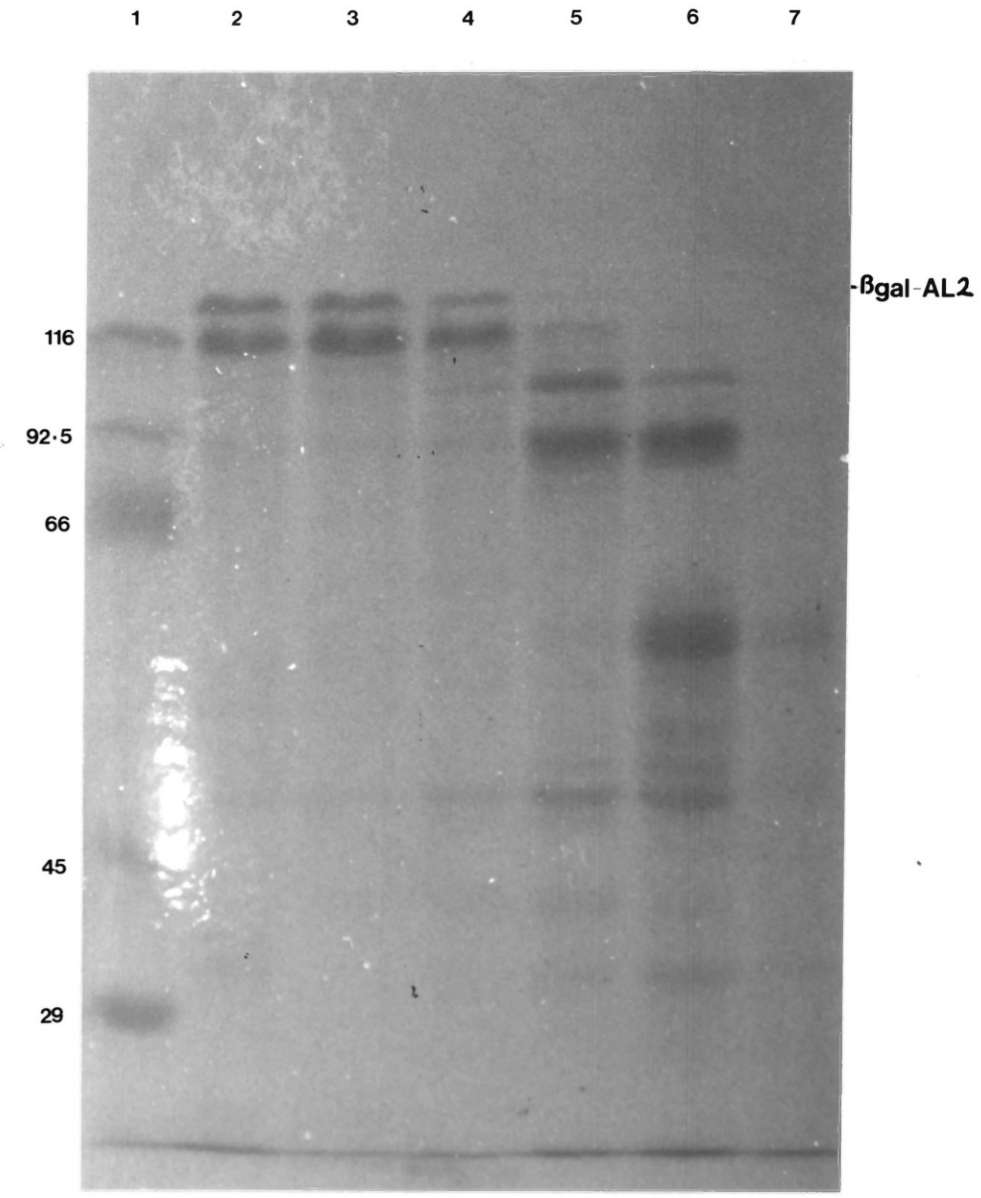


Figure 48 Purification of β-gal-AL2 by Gel Filtration Chromatography

Electrophoresis of aliquots from the 10 ml samples eluted from a Sephacryl column loaded with IPTG-induced TG2: pURAL2 extract in a 7.5 % SDS-polyacrylamide gel, stained with Coomassie blue.

Lane 1 Protein size markers.

Lanes 2 - 7 Samples eluted at 10 minute intervals, starting at 180 minutes (lane 2) and ending at 230 minutes (lane 7).

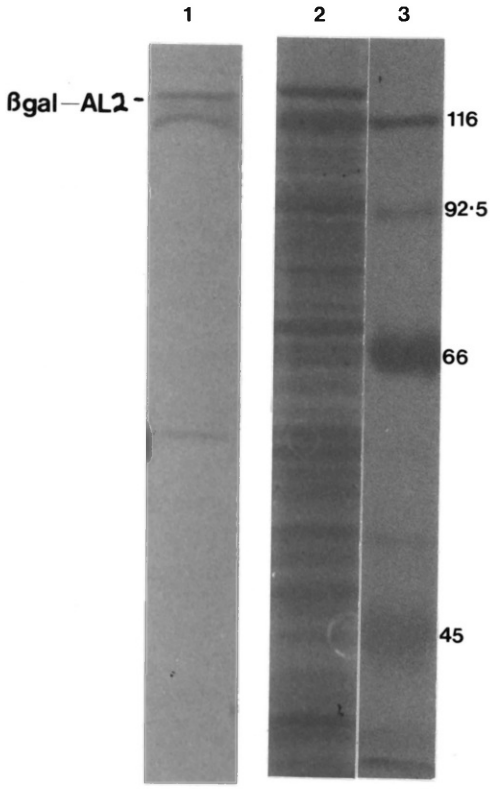


Figure 49 Detection of β-gal-AL2 by Western Blotting

Electrophoresis of IPTG-induced TG2: pURAL2 cell extracts in a 7.5 % SDS-polyacrylamide gel, followed by blotting onto nitrocellulose. Lanes 1 & 2 were induced TG2: pURAL2 cell extracts; Lane 3 was protein size markers. Lane 1 was probed with anti-β-gal-AL2 serum pre-absorbed with IPTG-induced TG2: pUR292 proteins; Lanes 2 & 3 were stained with amido black.

50 mg of leaf material; section 5.5.5), by Western blotting: aliquots of each of the four protein samples were separated by electrophoresis through an 18 % SDS-polyacrylamide gel and electroblotted onto nitrocellulose, which was then probed with 0.5 ml of cleaned-up anti-β-gal-AL2 serum in 9.5 ml TBS (section 5.4.7). The serum was found to react against only one protein (M_r 50-60,000) that was present in all four protein samples and hence is assumed to be a host-specific protein. No reaction was observed against the putative AL2 protein (expected M_r 14,871; Hamilton *et al.*, 1984) in either of the samples from TGMV-infected material. This may imply that the AL2 protein is produced in quantities too low to be detected in total protein extract and nuclei from infected *N. benthamiana*, or that the titre of the antiserum was too low to detect AL2 protein in these samples.

However, further work is required with the anti-β-gal-AL2 serum to try and identify the AL2 protein in extracts from TGMV-infected plants. For example, the crude serum, as opposed to the cleaned-up serum, could be tested for reactivity against total protein extract and nuclei from infected *N. benthamiana*, as it is approximately ten times more concentrated than the cleaned-up serum.

6.4 Future Prospects and Applications of Geminivirus Research

6.4.1 Characterisation of Geminivirus Encoded Products

The only gene product as yet identified and assigned to the TGMV genome, or indeed assigned to any of the characterised bipartite geminiviruses, is the capsid protein demonstrated to be encoded by ORF AR1 (Kallender *et al.*, 1988; section 6.3.2). Future work on TGMV must obviously involve the identification and characterisation of the other viral encoded products and determination of the roles these play in viral gene expression, host range determination, insect transmission and symptom production. The information which is currently available to speculation as to the functions of the gene products of each DNA component will initially be considered below and then possible approaches which could be taken to determine these functions will be discussed.

The experiments of Rogers *et al.* (1986) using transgenic petunia

plants containing tandem inserts of the TGMV DNA components, demonstrated that DNA A is capable of independent replication and encapsidation, and more recently, Elmer *et al.* (1988) have shown that of the four DNA A ORFs only ORF AL1 is required for viral DNA replication. They agroinfected petunia leaf discs using an *Agrobacterium tumefaciens* Ti plasmid vector containing DNA A components, with various mutations in ORFs AL1, AL2 and AL3, between the T-DNA borders. The callus tissue that grew from the leaf discsubsequently checked for the presence of viral ss- and dsDNAs, and these were found in those agroinfected with the ORF AL2 and AL3 mutants but not with the ORF AL1 mutants, implying that mutations in ORF AL1 totally abolish TGMV DNA replication.

As replication occurs in the cell nucleus, it follows that the AL1 protein must be located there. I have confirmed this using an antiserum made against a β-gal-AL1 fusion protein, which reacted with a protein found in infected but not healthy *N. benthamiana*, nuclei with a very similar molecular weight to that of the AL1 protein, as determined from its amino acid sequence (section 6.3.2).

The deproteinised TGMV viral ssDNA has been demonstrated to be infectious (Hamilton *et al.*, 1982) suggesting that the initial step of replication, the conversion of ssDNA to the dsDNA RF, relies entirely on host-encoded enzymes (section 4.9.2). Therefore, it seems likely that the product of ORF AL1 is involved in either the replication of the dsDNA RF and/or production of progeny ssDNA. Elmer *et al.* (1988) have suggested that the AL1 protein is analogous to the gene A and gene II proteins of the ssDNA bacteriophages ΦX174 and fd respectively, which are site-specific nicking endonucleases essential for both replication of dsDNA RF and the conversion of dsDNA RF to ssDNA. Although mutations in ORF AL1 have the same effect on replication as mutations in the ΦX174 gene A or fd gene II, there is no direct evidence that the enzymatic properties of the AL1 protein are similar to those of the bacteriophage enzymes.

The 200 bp 'common region' of TGMV DNAs A and B has been shown to contain sequences required for replication plus transcriptional promoter elements. If replication proteins were expressed from the genome of transgenic plants, *in vitro* mutagenesis of the 'common region' could be carried out to determine its effect on replication.

The product of ORF AL2 has been shown to be essential for systemic infection by TGMV by *in vitro* mutagenesis of ORF AL2 (Elmer *et al.*, 1988).

Characterisation of the single component genome of BCTV (Stanley *et al.*, 1986), a leafhopper-transmitted geminivirus which has a dicotyledonous host range, formally demonstrated that the monopartite genome was a property of the leafhopper-transmitted viruses rather than a 'monocot-dicot' distinction. Therefore the very existence of the DNA B component in the whitefly-transmitted geminiviruses is suggestive of one of its products being associated with insect transmission. However, an 'insect transmissibility factor' cannot be the sole function of either BR1 or BL1 since both products are essential for infection, as demonstrated by Brough *et al.* (1988) and Etessami *et al.* (1988): *in vitro* mutagenesis of either ORF abolished the infectivity of the cloned components when mechanically inoculated.

The products of the DNA B component have also been implicated in cell-to-cell/systemic spread and in symptom production by the studies of Rogers *et al.* (1986). Transgenic petunia plants with tandem copies of DNA A inserted into the chromosome were phenotypically normal but contained a low level of freely replicating viral DNA A. However, when these plants were crossed with plants containing integrated copies of DNA B, one quarter of the progeny seedlings produced symptoms and contained a higher level of freely replicating DNA A and B components. Rogers *et al.* (1986) interpreted these results as indicating that an extrachromosomally replicating DNA B component was necessary for the systemic spread of the virus and for the associated production of symptoms. Alternatively, however the lack of symptoms in the plants with tandem copies of DNA A inserted in the chromosome may have been due to the low copy number of freely replicating DNA A.

The requirement for two DNA B products plus the AL2 product to elicit a systemic infection suggests that they all have a role in cell-to-cell and/or systemic spread. It is possible that the viral DNA is transmitted from cell-to-cell as a DNA-protein complex rather than as virus particles. The product of either ORF AL2, BL1 or BR1 could function by binding to the viral DNA to allow transport through

the plasmodesmata. It is perhaps interesting to note that Kikuno *et al.* (1984) using computer analysis, detected 43 % homology between the amino acid sequences of ACMV ORFs AR1 (capsid protein) and BR1. Similarly, homology was reported between ORFs AR1 and BR1 of TGMV and BGMV (Howell & Goodman, 1986), which may suggest that the product of ORF BR1 could be capable of complexing with the viral DNA. The other DNA B product or the AL2 product may perhaps have a function analogous to that of the 30 K protein of TMV. This protein was originally proposed to be involved in cell-to-cell movement on the basis of work involving temperature-sensitive mutants defective in cell-to-cell spread (Leonard & Zaitlin, 1982; Ohno *et al.*, 1983). Conclusive evidence has been provided using transgenic tobacco plants expressing the 30 K protein, which are capable of complementing a temperature-sensitive mutant defective in cell-to-cell spread (Deom *et al.*, 1987). Although it is not known how such a protein might mediate the cell-to-cell spread of a virus, two general methods have been proposed by Atabekov & Dorokhov (1984). The first is based on the hypothesis that plants do not allow free movement of viruses and therefore a transport protein could modify the host structure directly or indirectly to allow the virus to move through the plasmodesmata. The second is that the protein represses a host resistance response, postulated to be caused by viral infection and to result in the shutting off of intercellular communication, so that the virus can then move from cell to cell. The 30 K protein of TMV, although not precisely located in the transgenic plants, has been reported in the plasmodesmata of infected leaves in wild-type infections (Tomenius *et al.*, 1987). With regard to the transgenic tobacco plants expressing the 30 K protein (Deom *et al.*, 1987), it would be interesting to see if these could complement mutants of either of the TGMV DNA B products, since it has been reported that coinoculation of a TMV bean strain and BGMV allows BGMV, which is usually phloem-limited, to spread systemically throughout the plant (Carr & Kim, 1983).

It is interesting to note that three proteins (the products of AL2, BR1 and BL1) are needed for systemic infection by TGMV, but only one is needed by TMV. The extra two virus-encoded functions during TGMV infection may be the movement in and out of nucleus or between

different cell types of the virus.

With regard to the production of symptoms by TGMV, although it is possible that products from both components are involved, the results of Rogers *et al.* (1986) do not conclusively demonstrate that the major determinant(s) reside on the DNA B component, since symptoms may be induced as a result of the increase in copy number of DNA A. In this respect, agroinfection has recently been used to demonstrate the infectivity of a capsid protein deletion mutant (Hayes *et al.*, 1988d), the symptoms produced by which were milder than those of the wild-type infection, thereby indicating that the TGMV capsid protein has some effect on symptomatology. Products of the DNA A component of ACMV have also been implicated in symptom determination by pseudorecombinant studies using Kenyan and Nigerian isolates which produce different symptoms and have electrophoretically distinguishable capsid proteins (Stanley *et al.*, 1985). The determinant for vein necrosis in *N. tabacum* segregated with DNA A and although this was not directly attributed to the capsid protein, it would be possible to examine further by using conserved restriction endonuclease sites to exchange regions of the genome from each isolate. Similar exchanges of fragments of the genomes of different strains of CaMV (Daubert *et al.*, 1984) indicated that the major determinant of symptomatology is probably the product of ORF VI (Daubert *et al.*, 1984), the major component of the inclusion body (Covey *et al.*, 1981), but that other loci also appear to influence disease expression, probably as a result of their effect on virus replication and systemic spread. Recently, it has been demonstrated that transgenic plants expressing the product of CaMV ORF VI produce viral-like symptoms (Baughman *et al.*, 1988) and in this respect it would be interesting to see what effect the products of the TGMV ORFs have on transgenic plants expressing the individual ORFs.

There is no evidence as yet to give any indication as to host range determinants. It was originally proposed that as host enzymes would need to recognise similar sequences on both components of the bipartite viruses, then the homologous sequences of the 200 bp 'common region' may be involved in the determination of host range (Stanley, 1986). However, analysis of the 'common regions' of ACMV and TGMV which share common hosts in *Nicotiana* species have revealed

no significant homologous sequences. It has also been proposed that the host range of a given virus can be determined by the functioning of the virus-encoded transport protein (Atabekov *et al.*, 1984). Therefore, it may be that one of the products of DNA B is a host range determinant.

The use of Ti plasmid vectors to produce transgenic plants carrying chromosomal insertions of geminivirus genomes (Rogers *et al.*, 1986) and to efficiently infect whole plants via agroinfection (Grimsley *et al.*, 1987) has provided a useful tool with which to study the biology of the geminiviruses. It should now be possible to examine the effects of *in vitro*-generated mutants (such as those described by Brough *et al.* (1988) and Elmer *et al.* (1988)), which are lethal in normal infectivity tests, but perhaps when integrated into every cell of a plant would allow the accumulation of replicative and/or assembly intermediates. Also geminiviruses could be introduced into plants which are not usually susceptible to infection by conventional means, to allow the study of incompatible virus-host interactions.

Future work will obviously include the identification and isolation of the putative geminivirus-encoded products. This thesis describes the work undertaken to try and identify the products of the six ORFs of TGMV. The approach used was to sub-clone each ORF into a translational expression vector (pUR290/1/2), which could subsequently express a fusion protein consisting of β-galactosidase at the 5' end and the TGMV ORF-encoded product at the 3' end. This fusion protein was then purified in large quantities and antibodies were raised to it in a rabbit. These antibodies were then used to detect the ORF-encoded product in TGMV-infected *N. benthamiana* extracts by Western blotting.

This approach has been used successfully to identify the product of ORF AR1 as the capsid protein (section 6.3.1). Also, an antiserum raised against a β-gal-AL1 fusion protein has been shown to react with a protein found in TGMV-infected *N. benthamiana* nuclei but not in healthy *N. benthamiana* nuclei, with a molecular weight (39.5 K) very similar to that expected for AL1 protein (40.3 K, Hamilton *et al.*, 1984) (section 6.3.2). However, this serum also reacted with several other proteins that were present in both healthy and infected

N. benthamiana extracts. But these proteins are thought to be host-specific. In order to prove that the 39.5 K protein is AL1 protein, the serum must be rid of all antibodies except those to β-gal-AL1 and then be shown to still react with the 39.5 K protein by Western blotting. Finally, a pre-immune serum must be shown not to react with the 39.5 K protein, to prove beyond doubt that this protein is encoded by ORF AL1.

The product of ORF AL1 has previously been demonstrated to be essential for TGMV DNA replication (Elmer *et al.*, 1988) and it is therefore assumed to be located in the plant cell nuclei. The 39.5 K protein was found to be present in purified TGMV-infected *N. benthamiana* nuclei, which also points to it being the AL1 protein. If the 39.5 K protein is proved to be the AL1 protein, its exact location in the nucleus at the various stages of virus infection can be demonstrated by immunogold labelling of the nucleus, using the anti-β-gal-AL1 antibodies.

An antiserum has been raised against a β-gal-AL2 fusion protein (section 6.3.3) by the same approach as that used for the β-gal-AR1 and β-gal-AL1 fusion proteins, and this serum, after the removal of antibodies to induced TG2:pUR292 proteins, has been shown to react with the β-gal-AL2 fusion protein, but not to any proteins with a molecular weight similar to that of AL2 protein, as calculated from the DNA sequence (14.9 K, Hamilton *et al.*, 1984). This may be due to the AL2 protein being present in too low a concentration to be detected in total protein extract or isolated nuclei from infected *N. benthamiana*, or the titre of the antibodies directed against the AL2 portion of the fusion protein might be too low. In order to increase the titre of the AL2-specific antibodies, the serum could be fractionated by immunoaffinity chromatography. In priciple this would involve the use of two columns utilising proteins coupled to cyanogen bromide-activated Sepharose. Antibodies specific for the β-galactosidaseportion of a fusion protein would be removed by coupling β-galactosidase to a column and passing the serum over it. The anti-fusion protein antibodies in the effluent would then be recovered by passage over a second column coupled with the purified fusion protein, before subsequent elution and concentration prior to use.

Fusion proteins have been constructed for the other three TGMV

ORFs (β-gal-AL3, Petty, 1986; β-gal-BR1 and -BL1, section 6.1), but no antibodies have been raised against any of them. The β-gal-AL3 fusion protein could be purified from IPTG-induced TG2:pURAL3 cells by the method described for β-gal-AL2 (section 6.3.3) and antibodies raised to it, which could be used to identify the AL3 protein in extracts from TGMV-infected *N. benthamiana* by Western blotting. However, the β-gal-BR1 and -BL1 fusion proteins have been shown to be produced in very low quantities in IPTG-induced TG2:pURBR1 and -BL1 cells respectively, unlike most other fusion proteins constructed using the pUR290/1/2 vectors. Hence, purifying sufficient quantities of these two fusion proteins to raise antisera against them would be very difficult. So, a different approach would be required in order to identify the products of ORFs BR1 and BL1 in extracts from TGMV-infected *N. benthamiana*. One approach would be to sub-clone the ORFs into a different expression vector to see whether more BR1- or BL1-encoded product could be expressed. One possible group of vectors is the pET vectors (Rosenberg *et al.*, 1987), which express gene products in their native form and not fused to a host protein, as with the pUR vectors. The major advantage of this system over the pUR290/1/2 plasmids is that any antibodies raised will be solely against the gene product of interest and not against the host-specific part of a fusion protein.

To examine the function(s) of the ORF AL1 protein in viral replication it would be necessary to isolate it from infected tissue in a biologically active form. Since it is present in infected plants in only very low amounts (Figure 44), transgenic plants could be produced which constitutively express this protein from chromosomal inserts. Alternatively, it could be expressed using a geminivirus-based vector as described in section 4.10. Once isolated the AL1 protein could be examined for its ability to bind to specific sequences of the TGMV genome. It is likely that *in vivo* the geminivirus DNA will be associated with host proteins, possibly in the form of minichromosomes as have been described for AbMV (Jeske & Werz, 1980a) and CaMV (Olszewski *et al.*, 1982), and therefore this DNA form would need to be isolated for the examination of DNA binding. It should also be possible to determine if the AL1 protein has a function analogous to the ΦX174 gene A and fd gene II proteins

in site-specific cleavage of one strand of dsRF. Also, *in vitro* mutagenesis of the 200 bp 'common region' could be performed and its effect on binding and on DNA replication determined. Ultimately, an *in vitro* replication system could be established to identify the proteins and mechanisms involved in geminivirus replication. Perhaps, just as *in vitro* systems of ΦX174 or SV40 and adenovirus have been used to provide information on procaryotic (Kornberg, 1982) and eucaryotic DNA replication (reviewed by Campbell, 1986), studies of the host proteins involved in geminivirus replication could reveal their functions in plant DNA replication, about which little is known.

6.4.2 Applications of Geminivirus-Based Vectors

As described in section 4.10, Hayes *et al.* (1988e) have constructed a gene replacement vector from TGMV, which can be used not only for amplification of gene expression by the systemic infection of plants, but also for heritable gene amplification by the integration of stable master copies of the vector into the plant chromosomal DNA. Such vectors should provide a useful research tool for the study of sequences involved in the regulation of gene expression in plants, including those specifying tissue-specific expression, light inducibility and developmental regulation. The vectors will also have some important commercial applications. An obvious major commercial objective is the development of improved crop plants and in this respect current targets include the engineering of resistance to virus disease, fungal pathogens and to herbicides.

It is possible that these geminivirus-based gene amplification vectors could be used to improve two approaches which have recently been taken to engineer resistance to viral infection. The first of these has been to mimic the poorly understood phenomenon of cross-protection - this is an operational term describing what happens when plants are infected by a mild non-pathogenic strain of a virus and then become resistant to superinfection by more virulent strains. This was accomplished by creating transgenic tobacco and tomato plants expressing the capsid protein gene of TMV (Powell-Abel *et al.*,

1986). These plants exhibited a degree of protection (i.e. delayed and reduced symptoms) when challenged by other strains of TMV, but this was not so apparent when TMV RNA was used, indicating that protection blocks an early stage in the infection cycle, possibly interfering with uncoating of the virion. Similar protection to alfalfa mosaic virus (AlMV) infection has been demonstrated by plants expressing the AlMV coat protein (Tumer *et al.*, 1987). Beachy *et al.* (1987) reported that the level of gene expression of the capsid protein in transgenic plants correlates with the level of protection. Therefore, if the capsid protein was expressed from a viral-based vector capable of replication, a considerably higher level of expression could be obtained, which perhaps in turn would lead to increased protection.

Another approach taken to engineer viral resistance has been to use antisense RNA inhibition (Baulcombe *et al.*, 1987). Antisense RNA (i.e. RNA complementary to the biologically active form) has been used to inhibit RNA-mediated functions in both bacterial (Pines & Inouye, 1986) and eucaryotic systems (Izant & Weintraub, 1984; Kim & Wold, 1985) and therefore could be applicable to control viral infection in plant cells. However, the expression of antisense RNA to viral genes of tobacco rattle virus (TRV) in transgenic tobacco plants afforded no protection to viral infection when the plants were inoculated with TRV (Baulcombe *et al.*, 1987). It is possible that this was due to the antisense RNA not being expressed to a high enough level, in which respect the geminivirus amplification vector could be useful, but it is also likely that the antisense RNA was unable to gain access and hence have any effect on the encapsidated viral RNA. It therefore appears unlikely that antisense inhibition will be a commercially useful antiviral strategy. However, it is still possible that antisense RNA expressed to a very high level using the geminivirus amplification vector, could be used to inactivate/inhibit the expression of selected plant genes, thereby allowing their functions to be studied.

Geminivirus vectors could also provide the high level gene expression which may be required for the development of plants with resistance to fungal pathogens e.g. by the expression of chitinase (Jones *et al.*, 1986); resistance to insect attack, by the expression

of specific insect toxins (Vaeck *et al.*, 1987) or resistance to non-selective herbicides by the over-expression of target enzymes (Shah *et al.*, 1986). They could perhaps also function in a role analogous to that proposed by Hohn *et al.* (1987) for CaMV vectors, in which useful substances could be expressed to a high level in edible plants, as opposed to in microbial cells, thereby bypassing the need to purify these products from contaminating microbial toxins.

ACKNOWLEDGEMENTS

and finally....

I would like to thank my supervisors - Ken Buck and Alan Brunt - for their advice and guidance on all aspects of my PhD; Rob Hayes and Jackie Brown - for their invaluable help, above and beyond the call of duty; Justin Rigden - for a bit of technology transfer here and there; Paul Dodson - for showing me how to "hex" a Sephacryl column and for offerring to lend me his dice; Martin "Roger Irrelevant" Goble - for doing some of the figures in this thesis and for being hat-stand; Bob Coutts - for seeds (and a whole lot more!); Tim Jones and Stuart Miller - for smiling, even when the chips were down; Tony Day - for a variety of things; SERC - for three years' grant; Fred Rolfe and M&R - for three years' beer money; and last but not least Chelsea Football Club - for keeping me out of the lab. on Saturdays.

Adams, M. J., Jones, P. & Smaby, A. G. (1988). Purification and some properties of oat golden stripe virus. Annals of Applied Biology 112, 285-290.

REFERENCES

Abouzid, A. & Jeske, H. (1986). The purification and characterization of gemini particles from *Abutilon* mosaic virus infected Malvaceae. *Journal of Phytopathology* **115**, 344-353.

Abouzid, A.M., Frischmuth, T. & Jeske, H. (1988). A putative replicative form of the abutilon mosaic virus (gemini group) in a chromatin-like structure. *Molecular and General Genetics* **212**, 252-258.

Adejare, G.O. & Coutts, R.H.A. (1982). The isolation and characterization of a virus from Nigerian cassava plants affected by the cassava mosaic disease, and attempted transmission of the disease. *Phytopathologische Zeitschrift* **103**, 193-210.

Ahlquist, P., Strauss, E.G., Rice, C.M., Strauss, J.H., Haseloff, J. & Zimmern, D. (1985). Sindbis virus proteins nsP1 and nsP2 contain homology to non-structural proteins from several RNA plant viruses. *Journal of Virology* **53**, 536-542.

Al Musa, A.M. & Mink, G.I. (1981). Beet necrotic yellow vein in North America. *Phytopathology* **71**, 773.

Andersen, M.T., Richardson, K.A., Harbison, S.-A. & Morris, B.A.M. (1988). Nucleotide sequence of the Geminivirus Chloris Striate Mosaic Virus. *Virology* **164**, 443-449.

Anno-nyako, F.O., Vetten, H.J., Allen, D.J. & Thottappilly, G. (1983). The relationship between cowpea golden mosaic virus and its vector *B. tabaci*. *Annals of Applied Biology* **102**, 319-323.

Arai, K. & Kornberg, A. (1981). Unique primed start of ΦX174 DNA replication and mobility of the primosome in a direction opposite chain synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 69-73.

Atabekov, J.G. & Dorokhov, Yu. (1984). Plant virus specific transport function and resistance of plants to viruses. *Advances in Virus Research* **29**, 313-364.

Bailey, J.M. & Davidson, N. (1976). Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Analytical Biochemistry* **70**, 75.

Bar-Joseph, M., Rosner, A., Muscovitz, M. & Hull, R. (1983). A simple procedure for the extraction of double-stranded RNA from virus-infected plants. *Journal of Virological Methods* **6**, 1-8.

Barr, D.J.S. (1979). Morphology and host range of *Polymyxa graminis, Polymyxa betae* and *Ligniera pilorum* from Ontario and some other areas. *Canadian Journal of Plant Pathology* **1**, 85.

Baughman, G.A., Jacobs, J.D. & Howell, S.H. (1988). Cauliflower mosaic virus gene VI produces a symptomatic phenotype in transgenic tobacco plants. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 733-737.

Baulcombe, D.C., Hamilton, W.D.O., Mayo, M.A. & Harrison, B.D. (1987). Resistance to viral disease through expression of viral genetic material from the plant genome. 1987 Plant resistance to viruses. Wiley, Chichester (Ciba Foundation Symposium 133) p170-184.

Baulcombe, D.C. (1989). Strategies for virus resistance in plants. *Trends in Genetics* **5**, 56-60.

Beachy, R.N., Powell-Abel, P., Nelson, R.S., Register, J., Tumer, N., Fraley, R.T. (1987). Genetic engineering of plant viruses for protection against virus diseases. 1987 Plant resistance to viruses. Wiley, Chichester (Ciba Foundation Symposium 133) p151-169.

Beier, H., Mundry, K.W. & Issinger, O.G. (1980). *In vivo* and *in vitro* translation of the RNAs of four tobamoviruses. *Intervirology* **14**, 292.

Beier, H., Barciszewska, M., Krupp, G., Mitnacht, R. & Gross, H.J. (1984a). UAG readthrough during TMV RNA translation: isolation and sequence of two $tRNAs^{Tyr}$ with suppressor activity from tobacco plants. *The EMBO Journal* **3**, 351-356.

Beier, H., Barciszewska, M. & Sickinger, H.-D. (1984b). The molecular basis for the differential translation of TMV RNA in tobacco protoplasts and wheat germ extracts. *The EMBO Journal* **3**, 1091-1096.

Bennett, C.W. & Wallace, G. (1938). Relation of the curly top virus to the vector, *Eutettix tenellus*. *Journal of Agricultural Research* **56**, 31-51.

Bennett, C.W. & Tanrisever, A. (1958). Curly top disease in Turkey and its relationship to curly top in North America. *J. Am. Soc. Sugar Beet Technol.* **10**, 189-211.

Bennett, C.W. (1971). The curly top disease of sugar beet and other plants. *American Phytopathological Society*. Monograph No. 7, St. Paul, Minnesota.

Benoist, C., O'Hare, K., Breathnach, R. & Chambon, P. (1980). The ovalbumin gene sequence of putative control regions. *Nucleic Acids Research* **8**, 127-142.

Bisaro, D.M., Hamilton, W.D.O., Coutts, R.H.A. & Buck, K.W. (1982). Molecular cloning and characterization of the two DNA components of tomato golden mosaic virus. *Nucleic Acids Research* **10**, 4913-4922.

Boccardo, G., Lisa, V., Luisoni, E. & Milne, R.G. (1987). Cryptic plant viruses. *Advances in Virus Research* **32**, 171-214.

Bock, K.R. (1974). Maize streak virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses, No. 133, Commonwealth Mycological Institute Kew, England.

Bock, K.R., Guthrie, E.J. & Woods, R.D. (1974). Purification of maize streak virus and its relationship to viruses associated with streak diseases of sugar cane and *Panicum maximum*. *Annals of Applied Biology* **77**, 289-296.

Bock, K.R., Guthrie, E.J. & Meredith, G. (1977). RNA and protein components of maize streak and cassava latent virus. *Annals of Applied Biology* **85**, 305-308.

Bock, K.R., Guthrie, E.J. & Meredith, G. (1978). Distribution, host range, properties and purification of cassava latent virus, a geminivirus. *Annals of Applied Biology* **90**, 361-367.

Bock, K.R. (1982). Geminivirus diseases in tropical crops. *Plant Disease* **66**, 266-270.

Bockus, W.W. & Niblett, C. (1984). A procedure to identify resistance to wheat soilborne mosaic in wheat seedlings. *Plant Disease* **68**, 123-124.

Bostian, K.A., Hooper, J.E., Rogers, D.T. & Tipper, D.J. (1980). Translation analysis of the killer-associated virus-like particle dsRNA genome of *S. cerevisiae*: M dsRNA encodes toxin. *Cell* **19**, 403-414.

Both, G.W., Bellamy, A.R., Street, J.E. & Siegman, L.J. (1982). A general strategy for cloning double-stranded RNA: Nucleotide sequence of the Simian-II rotavirus gene 8. *Nucleic Acids Research* **10**, 7075-7087.

Bouhot, D. (1967). Observations sur quelques affections des plantes cultivées au Sénégal. *Agron. Trop. (Maracay, Venez.)* **22**, 888.

Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K. & Putz, C. (1985). Nucleotide sequence analysis of RNA-3 and -4 of Beet Necrotic Yellow Vein Virus isolates F2 and G4. *Journal of General Virology* **66**, 1553-1564.

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K. & Jonard, G. (1986). Nucleotide sequence of Beet Necrotic Yellow Vein Virus RNA-2. *Journal of General Virology* **67**, 1689-1700.

Bouzoubaa, S., Quillet, L., Guilley, H., Jonard, G., & Richards, K. (1987). Nucleotide sequence of Beet Necrotic Yellow Vein Virus RNA-1. *Journal of General Virology* **68**, 615-626.

Bouzoubaa, S., Guilley, H., Jonard, G., Jupin, I., Quillet, I., Richards, K., Scheidecker, D. & Ziegler-Graff, V. (1988). Genome organisation and function of beet necrotic yellow vein virus. In: Developments in Applied Biology 2: Viruses with Fungal Vectors. Eds. Cooper, J.I. & Asher, M.J.C. pp. 99-110. Association of Applied Biologists, Wellesbourne, Warwick.

Brakke, M.K. (1971). Soil-Borne Wheat Mosaic Virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses No. 77, Commonwealth Mycological Institute, Kew, England.

Brakke, M.K. (1977). Sedimentation coefficients of the virions of soil-borne wheat mosaic virus. *Phytopathology* **67**, 1433.

Brakke, M.K., Estes, A.P. & Schuster, M.L. (1965). Transmission of soil-borne wheat mosaic virus. *Phytopathology* **55**, 79.

Brakke, M.K. & Langenberg, W.G. (1988). Experiences with soil-borne wheat mosaic virus in North America and elsewhere. In: Developments in Applied Biology 2: Viruses with Fungal Vectors. Eds. Cooper, J.I. & Asher, M.J.C. pp. 183-202. Association of Applied Biologists, Wellesbourne, Warwick.

Brakke, M.K. & Rao, S.A. (1967). Maintenance of soil-borne wheat mosaic virus cultures by transfer through root washings. *Plant Disease Reporter* **51**, 1005-1008.

Brandes, J., Phillippe, M.R. & Thornberry, H.H. (1964). Electron microscopy of particles associated with soil-borne wheat mosaic virus. *Phytopathologische Zeitschrift* **50**, 181.

Breathnach, R. & Chambon, P. (1981). Organisation and expression of Eukaryotic split genes coding for proteins. *Annual Review of Biochemistry* **50**, 349-383.

Brisson, N., Paszkowski, J., Penswick, J.R., Gronenborn, B., Potrykus, I. & Hohn, T. (1984). Expression of a bacterial gene in plants using a viral vector. *Nature (London)* **310**, 511.

Brough, C.L., Hayes, R.J., Morgan, A.J., Coutts, R.H.A. & Buck, K.W. (1988). Effects of *in vitro* mutagenesis on the ability of cloned Tomato Golden Mosaic Virus DNA to infect *Nicotiana benthamiana* plants. *Journal of General Virology* **69**, 503-514.

Brown, F. (1989). Classification and nomenclature of viruses. Fifth report of the International Committee on Taxonomy of Viruses. *Intervirology* (in press).

Brown, G.G., Bussey, H. & Desrosiers, L.J. (1986). Analysis of mitochondrial DNA, chloroplast DNA and double-stranded RNA in fertile and cytoplasmic male-sterile sunflower, *Helianthus annuus*. *Can. J. Genet. Cytol.* **28**, 121-129.

Brown, J.K. & Nelson, M.R. (1984). Geminate particles associated with cotton leaf crumple disease in Arizona. *Phytopathology* **74**, 987-990.

Brown, J.K. & Nelson, M.R. (1986). Whitefly-borne viruses of melons and lettuce in Arizona. *Phytopathology* **76**, 236-239.

Brunt, A.A. (1988). Labile rod-shaped viruses transmitted by plasmodiophorid fungi - furoviruses. In *Viruses with Fungal Vectors* (Eds. J.I. Cooper and M.J.C. Asher) pp.3-17, Association of Applied Biologists.

Brunt, A.A. & Shikata, E. (1986). Fungus-transmitted and similar labile rod-shaped viruses. In The Plant Viruses, Volume 2, The Rod-Shaped Viruses, pp. 305-335, Eds. M.H.V. Regenmortel & H. Fraenkel-Conrat. Plenum Press, New York.

Brunt, A.A. & Richards, K. (1989). The biology and molecular biology of furoviruses. *Advances in Virus Research* **36**, 1-32. F.A. Murphy & A.J. Shatkin. Orlando, Florida, Academic Press.

Brunt, A.A. & Stace-Smith, R. (1978a). Electron microscopy of a virus from *Hypochoeris radicata* in sap and infected plant tissue. *Journal of General Virology* **41**, 207.

Brunt, A.A. & Stace-Smith, R. (1978b). Some hosts, properties and possible affinities of a labile virus from *Hypochoeris radicata* (Compositae). *Annals of Applied Biology* **90**, 205-214.

Buck, K.W. & Coutts, R.H.A. (1983). The potential of geminiviruses in the construction of plant host vector systems. *Plant Molecular Biology* **2**, 351-354.

Buck, K.W. & Coutts, R.H.A. (1985). Tomato golden mosaic virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses No. 303, Commonwealth Mycological Institute, Kew, England.

Burgermeister, W., Koenig, R., Weich, H., Sebald, W. & Lesemann, D.E. (1986). Diversity of the RNAs in thirteen isolates of Beet Necrotic Yellow Vein Virus in *Chenopodium quinoa* detected by means of cloned cDNAs. *Journal of Phytopathology* **115**, 229-242.

Cai, W., Peng, X. & Mang, K. (1983). Identification of soil-borne wheat mosaic virus causing wheat mosaic in Shandong Province, China. *Acta Phytopathol. Sin.* **13**, 7.

Calvert, E.L. & Harrison, B.D. (1966). Potato Mop-Top, a soil-borne virus. *Plant Pathology* **15**, 134-139.

Calvert, E.L. (1968). The reaction of potato varieties to Potato Mop-Top Virus. *Rec. Agric. Res. Minist. Nth. Ire.* **17**, 31-40.

Campbell, J.L. (1986). Eukaryotic DNA Replication. *Annual Reviews in Biochemistry* **55**, 733-771.

Canova, A. (1959). Appunti di patologica della barbabietola. *Inf. Fitopatol.* **9**, 390.

Canova, A. (1966a). Si studia la rizomania della bietola. *Inf. Fitopatol.* **16**, 235.

Canova, A. (1966b). Ricerche sulle malattie da virus delle frumento transmissible attraverso il terrano. *Phytopathol. Mediterr.* **5**, 53.

Carr, R.J. & Kim, K.S. (1983). Evidence that bean golden mosaic virus invades non-phloem tissue in double-infection with tobacco mosaic virus. *Journal of General Virology* **64**, 2489-2492.

Carroll, S.B. & Laughon, A. (1987). Production and purification of polyclonal antibodies to the foreign segment of β-galactosidase fusion proteins. In : DNA Cloning: A Practical Approach, Volume 3, (Ed. Glover, D.M.) pp. 89-111. IRL Press, Oxford.

Catherall, P.L., Boulton, R.E. & Chamberlain, J.A. (1977). Oat tubular virus. *Rep. Welsh Plant Breeding Stn.* 1976, p. 186.

Cherif, C. & Russo, M. (1983). Cytological evidence of the association of a geminivirus with the tomato leaf curl disease in Tunisia. *Phytopathologische Zeitschrift* **108**, 221-225.

Chilton, M.-D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. & Nester, E.W. (1977). Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**, 263.

Chilton, M.-D., Saiko, R.K., Yadav, N., Gordon, M.P. & Queiter, F. (1980). T-DNA from *Agrobacterium* Ti plasmid is in the nuclear fraction of crown gall tumor cells. *Proceedings of the National Academy of Science U.S.A.* **77**, 4060.

Christie, R.G., Falk, B.W., Kon, J. & Zettler, F.W. (1984). Geminivirus-like nuclear inclusions associated with pseudo-curly top disease in Florida. American Phytopathological Society-Canadian Phytopathological Society Annual Meeting, Guelph, Ontario, Abstract A88.

Christie, R.G., Ko, N-J., Falk, B.W., Heibert, E., Lastra, R., Bird, J. & Kim, K.S. (1986). Light microscopy of geminivirus-induced nuclear inclusion bodies. *Phytopathology* **76**, 124-126.

Chu, P.W.G. & Helms, K. (1987). Purification and characterisation of a novel single-stranded DNA virus associated with subterranean clover stunt disease in Australia. VIIth International Congress of Virology, Ottawa, Ontario, Canada, Abstract R36.1.

Codrescu, A., Ciochia, V. & Vilau, N. (1981). Simptome de rizomania in culturile de sfecla de zahar din Romaina. *Productia vegetale* **10**, 38-40.

Cohen, S., Duffus, J.E., Larsen, R.C., Liu, H.Y. & Flock, R.A. (1983). Purification, serology and vector relationships of squash leaf curl virus, a whitefly-transmitted geminivirus. *Phytopathology* **73**, 1669-1673.

Condit, C. & Fraenkel-Conrat, H. (1979). Isolation of replicative forms of 3' terminal subgenomic RNAs of tobacco necrosis virus. *Virology* **97**, 122-130.

Cooper, J.I. & Harrison, B.D. (1973). Distribution of Potato Mop-Top Virus in Scotland in relation to soil and climate. *Plant Pathology* **22**, 73-78.

Costa, A.S. & Bennet, C.W. (1950). Whitefly-transmitted mosaic of *Euphorbia prunifolia*. *Phytopathology* **40**, 266-283.

Coutts, R.H.A. & Buck, K.W. (1985). DNA and RNA polymerase activities of nuclei and hypotonic extracts of nuclei isolated from tomato golden mosaic virus infected tobacco leaves. *Nucleic Acids Research* **13**, 7881-1897.

Coutts, R.H.A. & Buck, K.W. (1987). Cassava Latent Virus specific DNAs in mosaic diseased Cassava of Nigerian origin. *Netherlands Journal of Plant Pathology* **93**, 241-244.

Coutts, R.H.A., Buck, K.W., Roberts, E.J.F., Brough, C.L., Hayes, R.J., MacDonald, H., MacDowell, S.W., Petty, I.T.D., Slomka, M.J., Hamilton, W.D.O. & Bevan, M.W. (1987). Studies on the replication of tomato golden mosaic virus and the construction of gene vectors. In *Molecular Stategies for Crop Protection*, pp307-318. Edited by C. Arntzen & C. Ryan. New York: Alan Liss, Inc.

Covey, S.N., Lomonossoff, G.P. & Hull, R. (1981). Characterisation of cauliflower mosaic virus DNA sequences which encode major polyadenylated transcripts. *Nucleic Acids Research* **9**, 6735-6747.

Czosnek, H., Ber, R. Antignus, Y., Cohen, S., Navot, N. & Zamir, D. (1988). Isolation of tomato yellow leaf curl virus, a geminivirus. *Phtopathology* **78**, 508-512.

Dale, J.L., Phillips, D.A. & Parry, J.N. (1986). Double-stranded RNA in banana plants with bunchy top disease. *Journal of General Virology* **67**, 371-375.

Damsteegt, V.D. (1983). Maize streak virus : Host range and vulnerability of maize germplasm. *Plant disease* **67**, 734-737

Daubert, S., Shepherd, R.J. Gardner, R.C. (1983). Insertional mutagenesis of the CaMV genome. *Gene* **25**, 201-208.

Daubert, S., Schoelz, J., Debao, L. & Shepherd, R. (1984). Expression of disease symptoms in cauliflower mosaic virus genomic hybrids. *Journal of Molecular and Applied Genetics* **2**, 537-547.

Davies, J.W. & Hull, R. (1982). Genome expression of positive strand RNA viruses. *Journal of General Virology* **61**, 1-14.

Davies, J.W., Stanley, J., Donson, J., Mullineaux, P.M. & Boulton, M.I. (1987). Structure and replication of Geminivirus Genomes. *Journal of Cell Science Supplement* **7**, 95-107.

Dawson, W.O. & Dodds, J.A. (1982). Characterisation of subgenomic double-stranded RNAs from virus-infected plants. *Biochemical and Biophysical Research Communications* **107**, 1230-1235.

Debrot, E.A. (1981). Natural infection of potatoes in Venezuela with the whitefly-transmitted mosaic amarillo tomato virus. International Society of Plant Pathology, Association of Applied Biologists, Keble College, Oxford.

Dekker, E.L., Pinner, M.S., Markham, P.G. & van Regenmortel, M.H.V. (1988). Characterisation of Maize Streak Virus isolates from different plant species by polyclonal and monoclonal antibodies. *Journal of General Virology* **69**, 983-990.

Dente, L., Cesarini, G. & Cortese, R. (1983). pEMBL : a new family of single-stranded plasmids. *Nucleic Acids Research* **11**, 1645-1655.

Dente, L., Sollazzo, M., Baldari, C., Cesarini, G. & Cortese, R. (1985). The pEMBL family of single-stranded vectors. In *DNA cloning : a practical approach* vol. 1. pp. 101-107. Edited by D.M. Glover. Oxford: IRL Press.

Deom, C.M., Oliver, M.J. & Beachy, R.N. (1987). The 30-kilodalton gene product of tobacco mosaic virus potentiates virus movement. *Science* **237**, 389-394.

de Uzcategui, R.C. & Lastra, J.R. (1978). Transmission and physical properties of the causal agent of mosaic amarello de tomato (tomato yellow mosaic). *Phytopathology* **68**, 985-988.

di Franco, A. & Gallitelli, D. (1980). Le virosi delle piante ortensi in Puglia. XXVI. La rizomania della bietola da coste. *Inf. Fitopatol.* **30**, 9.

Dixon, L.K., Koenig, I. & Hohn, T. (1983). Mutagenesis of CaMV. *Gene* **25**, 189-199.

Dixon, L.K. & Hohn, T. (1984). Initiation of translation of the CaMV genome from a polycistronic mRNA: evidence from deletion mutagenesis. *The EMBO Journal* **3**, 2731-2736.

Dodds, J.A. & Mirkov, T.E. (1983). Association of double-stranded RNA with lettuce big vein disease. *VIth International Congress for Plant Pathology, Melbourne, Abstracts*, p. 113.

Dodds, J.A., Morris, T.J. & Jordan, R.L. (1984): Plant viral double-stranded RNA. *Annual Review of Phytopathology* **22**, 151-168.

Dodds, J.A., Valverde, R.A. & Mathews, D.M. (1988). Detection and interpretation of dsRNA. In *Viruses of Fungi and Simple Eukaryotes*, pp 309-326, Eds: Y. Koltin & M.J. Leibowitz, Marcel Dekker Inc.

Dollet, M., Accotto, G.P., Lisa, V., Menissier, J. & Boccardo, G. (1986). A geminivirus serologically related to maize streak virus, from *Digitaria sanguinalis* from Vanuata. *Journal of General Virology* **67**, 933-937.

Donson, J., Morris-Krsnich, B.A.M., Mullineaux, P.M., Boulton, M.I. & Davies, J.W. (1984). A putative primer for second-strand synthesis of maize streak virus is virion-associated. *The EMBO Journal* **3**, 3069-3073.

Donson, J., Accotto, G.P., Boulton, M.I., Mullineaux, P.M., & Davies, J.W. (1987). The nucleotide sequence of a geminivirus from *Digitaria sanguinalis*. *Virology* **161**, 160-169.

Donson, J., Gunn, H.V., Woolston, C.J., Pinner, M.S., Mullineaux, P.M., Boulton, M.I. & Davies, J.W. (1988). *Agrobacterium* mediated infectivity of cloned DSV. *Virology*, **162**, 248-250.

Dougherty, W.G. (1983). Analysis of viral RNA isolated from tobacco leaf tissue infected with tobacco etch virus. *Virology* **131**, 473-481.

Dougherty, W.G. & Hiebert, E. (1985). Genome structure and gene expression of plant RNA viruses. In: Molecular Plant Virology. Volume 2, Replication and Gene Expression, pp23-69. Ed. J.W. Davies. CRC Press, Boca Raton, Florida.

Duffus, J.E., Whitney, E.D., Larson, R.C., Liu, H.Y. & Lewellen, R.T. (1984). First report in the western hemisphere of rhizomania of sugar beet caused by beet necrotic yellow vein virus. *Plant Disease* **68**, 251.

Elmer, J.S., Brand, L., Sunter, G., Gardiner, W.E., Bisaro, D.M. & Rogers, S.G. (1988). Genetic analysis of the Tomato Golden Mosaic Virus II. The product of the AL1 coding sequence is required for replication. *Nucleic Acids Research* **16**, 7043-7060.

Esau, K. (1977). Virus-like particles in nuclei of phloem cells in spinach leaves infected with the curly top virus. *Journal of Ultrastructural Research* **61**, 78-88.

Estes, E.P. & Brakke, M.K. (1966). Correlation of *Polymyxa graminis* with transmission of soil-borne wheat mosaic virus. *Virology* **28**, 772.

Etessami, P., Callis, R., Ellwood, S. & Stanley, J. (1988). Delimitation of essential genes of cassava latent virus DNA 2. *Nucleic Acids Research* **16**, 4811-4829.

Faccioli, G. & Giunchedi, L. (1974). On the viruses involved in rhizomania disease of sugar beet in Italy. *Phytopathol. Mediterr.* **13**, 28.

Fauquet, C. (1985). *Essai de classification des phytovirus par leur protéine capsidaire.* Thèse d'Etat, Volume II, 19/12/85, ULP Strasbourg, 387pp.

Fauquet, C. & Thouvenel, J.-C. (1983). Association d'un nouveau virus en bâttonet avec la maladie necrose à rayures du riz en Côte d'Ivoire. *C.R. Acad. Sci. Ser. D.* **296**, 575.

Fauquet, C. & Thouvenel, J.-C. (1987). Viral diseases of plants in the Ivory Coast. In *Initiation et Documentation Technique* **No. 46**, ORSTOM, Paris, 243 pp.

Fauquet, C., Desbois, D., Fargette, D. & Vidal, G. (1988a). Classification of furoviruses based upon the amino acid composition of their coat proteins. In *Viruses with Fungal Vectors* (Eds. J.I. Cooper and M.J.C. Asher) pp. 19-36, Association of Applied Biologists.

Fauquet, C., Thouvenel, J.-C., Fargette, D. & Fishpool, L.D.C. (1988b). Rice stripe necrosis virus: a soil-borne rod-shaped virus. In *Viruses with Fungal Vectors* (Eds. J.I. Cooper and M.J.C. Asher) pp. 71-82, Association of Applied Biologists.

Fellows, H., Sill, W.H. & King, C.L. (1953). The 1952 epiphytotic of a soil-borne wheat mosaic in Kansas. *Plant Disease Reporter* **37**, 287-289.

Fenner, F. (1976). Classification and nomenclature of viruses: Second Report of the International Committee on the Taxonomy of Viruses. *Intervirology* **7**, 1.

Fenoll, C., Black, D.M. & Howell, S.H. (1988). The intergenic region of maize streak virus contains promoter elements involved in rightward transcription of the viral genome. *The EMBO Journal* **7**, 1589-1596.

Ferguson, K.A. (1964). Starch-gel electrophoresis - application to the classification of pituitary proteins and polypeptides. *Metabolism* **13**, 985-1002.

Fiddes, J.C., Barrell, B.G. & Godson, G.N. (1978). Nucleotide sequences of the separate origins of synthesis of bacteriophage G4 viral and complementary strand synthesis. *Proceedings of the National Academy of Science U.S.A.* **75**, 1081-1085.

Fraley, R.T., Rogers, S.G. & Horsch, R.B. (1986). Genetic transformation in higher plants. *CRC Critical Reviews in Plant Sciences* **4**, 1-46.

Francki, R.I.B., Hatta, T., Grylls, N.E. & Grivell, C.J. (1979). The particle morphology and some other properties of chloris striate mosaic virus. *Annals of Applied Biology* **91**, 51-59.

Francki, R.I.B., Hatta, T., Boccardo, G. & Randles, J.W. (1980). The composition of chloris striate mosaic virus, a geminivirus. *Virology* **101**, 233-241.

Francki, R.I.B., Milne, R.G. & Hatta, T. (1985). *Atlas of Plant Viruses, Volume 1*, pp 33-46. CRC Press.

Fraser, T.W. (1976). Mop-top tubules. The ultrastructure of unusual tubular elements associated with two different leaf symptoms of potato mop-top virus infected potato plants. *Protoplasma* **90**, 15-31.

French, R., Janda, M. & Alquist, P. (1986). Bacterial gene inserted in an engineered RNA virus: efficient expression in monocotyledonous plant cells. *Science* **231**, 1294-1297.

Fromm, M., Taylor, L. & Walbort, V. (1985). Expression of genes electroporated into monocot and dicot plant cells. *Proceedings of the National Academy of Science U.S.A.* **82**, 5824.

Fujikawa, T. (1963). On the broad bean necrosis disease and its control. *Nogyo Oyobi Engei* **38**, 359.

Fujisawa, I. & Sugimoto, T. (1977). Transmission of beet necrotic yellow vein virus by *Polymyxa betae*. *Annals of the Phytopathological Society of Japan* **43**, 583.

Fukano, H. & Yokoyama, S. (1952). On a necrotic virus disease of broadbean, garden pea and spring vetch. *Kyushu Nogyo Kenkyu* **10**, 133.

Fuller, C. (1901). Mealie variegation. 1st Report of the Government Entomologist, Natal, 1899-1900, pp17-19.

Galvez, G.E. & Castano, M. (1975). Stability and purification of bean golden mosaic virus. *Proceedings of the American Phytopathological Society* **2**, 75.

Gao, J.L., Deng, F., Zhai, H.Q., Liang, X.S. & Liu, Y. (1983). The occurrence of sugar beet rhizomania caused by beet necrotic yellow vein virus in China. *Acta Phytopathol. Sin.* **13**, 1.

Garcia-Arenal, F., Palukaitis, P. & Zaitlin, M. (1984). Strains and mutants of tobacco mosaic virus are both found in virus derived from single-lesion-passaged inoculum. *Virology* **132**, 131-137.

Gardiner, W.E., Sunter, G, Brand, L., Elmer, J.S., Rogers, S.G. & Bisaro, D.M. (1988). Genetic analysis of tomato golden mosaic virus: the coat protein is not required for systemic spread or symptom development. *The EMBO Journal* **7**, 899-904.

Garnier, M., Mamoun, R. & Bové, J.M. (1980). TYMV RNA replication *in vivo*: Replicative intermediate is mainly single-stranded. *Virology* **104**, 357-374.

Germani, G. & Dhery, M. (1973). Observations et experimentations concernant le rôle des nématodes dans deux affections de l'arachide en Haute-Volta: la 'chlorose' et le 'clump'. *Oleagineux*, **28**, 235.

Gildow, F.E. & Rochow, W.F. (1980). Role of accessory salivary glands in aphid transmission of barley yellow dwarf virus. *Virology* **104**, 97-108.

Gildow, F.E., Ballinger, M.E. & Rochow, W.F. (1983). Identification of double-stranded RNAs associated with barley yellow dwarf virus infection in oats. *Phytopathology* **73**, 1570-1572.

Giunchedi, L., Langenberg, W.G. & Marani, F. (1981). Appearance of beet necrotic yellow vein virus (BNYVV) in host cells. *Phytopathol. Mediterr.* **20**, 112.

Goelet, P., Lomonossoff, G.P., Butler, P.J.G., Akam, M.E., Gait, M.J. & Karn, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proceedings of the National Academy of Science U.S.A.* **79**, 5818-5822.

Goldbach, R.M., Rezelman, G. & Van Kammen, A. (1980). Independent replication and expression of B-component RNA of cowpea mosaic. *Nature (London)* **286**, 297-300.

Goldbach, R.M. & Rezelman, G. (1983). Orientation of the cleavage map of the 200-kilodalton polypeptide encoded by the bottom-component RNA of CPMV. *Journal of Virology* **46**, 614.

Goodman, R.M. (1977a). Single-stranded DNA genome in a whitefly-transmitted plant virus. *Virology* **83**, 171-179.

Goodman, R.M. (1977b). Infectious DNA from a whitefly-transmitted virus of *Phaseolus vulgaris*. *Nature (London)* **266**, 54-55.

Goodman, R.M., Bird, J. & Thongmeearkom, P. (1977). An unusual viruslike particle associated with golden yellow mosaic of beans. *Phytopathology* **67**, 37-42.

Goodman, R.M. & Bird, J. (1978). Bean golden mosaic virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses No. 192, Commonwealth Mycological Institute, Kew, England.

Goodman, R.M., Shock, T.L., Haber, S., Browning, K.S. & Bowers, G.R. (1980). The composition of bean golden mosaic virus and its single stranded DNA genome. *Virology* **106**, 168-172.

Goodman, R.M. (1981). Geminiviruses. *Journal of General Virology* **54**, 9-21.

Gorbalenya, A.E., Blinov, V.M. & Koonin, E.V. (1985). Prediction of nucleotide binding properties of virus-specific proteins from their primary structure. *Molek. Genetika* **7**, 30-36.

Gould, A.R. & Francki, R.I.B. (1981). Immunochemical detection of dsRNA in healthy and virus-infected plants and specific detection of viral dsRNA by hybridisation to labelled complementary DNA. *Journal of Virological Methods* **2**, 277-286.

Greber, R.S. & Finlay, J.R. (1981). Hypochoeris mosaic virus in Australia. *Aust. Plant Pathol.* **10**, 30.

Grill, L.K. & Garger, S.J. (1981). Identification and characterisation of double-stranded RNA associated with cytoplasmic male sterility in *Vicia faba*. *Proceedings of the National Academy of Science, USA.* **78**, 7043-7046.

Grimsley, N., Hohn, B. & Hohn, T. (1986a). Recombination in a plant virus: template switching in cauliflower mosaic virus. *The EMBO Journal* **5**, 641-646.

Grimsley, N., Hohn, B., Hohn, T. & Walden, R. (1986b). "Agroinfection," an alternative route for viral infection of plants using the Ti plasmid. *Proceedings of the National Acadamy of Sciences, U.S.A.* **83**, 3282-3286.

Grimsley, N., Hohn, T., Davies, J & Hohn, B. (1987). *Agrobacterium*-mediated delivery of infectious maize streak virus into maize plants. *Nature (London)* **325**, 177-179.

Gronenborn, B. & Messing, J. (1978). Methylation of single-stranded DNA *in vitro* introduces new restriction endonuclease cleavage sites. *Nature (London)* **272**, 375-377.

Grylls, N.E. (1979). Leafhopper vectors and plant disease agents they transmit in Australia. In : Leafhopper Vectors and Plant Disease agents (Eds Maramorosch, K. & Harris, K.F.) pp199-214. Academic Press, London

GÜgerli, P. (1984). Isopycnic centrifugation of plant viruses in Nycodenz density gradients. *Journal of Virological Methods* **9**, 249-258.

Gumpf, D.J. (1971). Purification and properties of soil-borne wheat mosaic virus. *Virology* **43**, 588-596.

Guo, L.-H. & Wu, R. (1983). Exonuclease III: use for DNA sequence analysis and in specific deletions of nucleotides. In: Methods in Enzymology, Volume 100, Recombinant DNA, Part B (Eds. Wu, R., Grossman, L. & Moldave, K.) pp93-96. Academic Press, London.

Haber, S., Ikegami, M., Bajet, N.B. & Goodman, R.M. (1981). Evidence for a divided genome in bean golden mosaic virus. *Nature (London)* **289**, 324-326.

Haber, S. & Gill, C.C. (1984) *American Phytopathological Society-Canadian Phytopathological Society Annual Meeting*, Guelph, Ontario, Abstract A87: A geminivirus-like particle from oats.

Hamdorf, G., Lesemann, D.E. & Weidemann, H.L. (1977). Untersuchungen Über die Rizomania-Krankheit an Zucherrüben in der Bundesrepublik Deutschland. *Phytopathologische Zeitschrift* **90**, 97.

Hamilton, W. D. O., Sanders R. C., Coutts, R. H. A. & Buck, K. W. (1981). Characterisation of tomato golden mosaic virus as a geminivirus, *FEMS Microbiological Letters* **11**, 263-267.

Hamilton, W. D. O., Bisaro, D. M. & Buck, K. W. (1982). Identification of novel DNA forms of tomato golden mosaic virus infected tissue. Evidence for a two component viral genome. *Nucleic Acid Research* **10**, 4901-4912.

Hamilton, W. D. O., Bisaro, D. M., Coutts, R. H. A. & Buck, K. W. (1983). Demonstration of the bipartite nature of the genome of a single-stranded DNA plant virus by infection with the cloned DNA components. *Nucleic Acid Research* **11**, 7387-7396.

Hamilton, W. D. O., Stein, V. E., Coutts, R. H. A. & Buck, K. W. (1984). Complete nucleotide sequence of the infectious cloned DNA components of tomato golden mosaic virus: potential coding regions and regulatory sequences. *The EMBO Journal* **3**, 2197-2205.

Hanahan, D. (1985) Techniques for transformation of *E. coli*. In *DNA cloning : a practical approach*, vol. 1, pp 109-135. Edited by D. M. Glover, Oxford : IRL Press.

Häni, A. & Bovey, R. (1983). La Rhizomanie, une virose de la betterave nouvelle pour la Suisse. *Revue Suisse d'Agriculture* **15**, 305-308.

Hanley-Bowdoin, L., Elmer, J. S. & Rogers, S. G. (1988). Transient expression of heterologous RNAs using tomato golden mosaic virus. *Nucleic Acids Research* **16**, 10511-10528.

Hanold, D., Langridge, P. & Randles, J. W. (1988). The use of cloned sequences for the identification of coconut foliar decay disease-associated DNA. *Journal of General Virology* **69**, 1323-1329.

Hanson, M.R. & Conde, M.F. (1985). Functioning and variation of cytoplasmic genomes: lessons from cytoplasmic nuclear interactions affecting male sterility in plants. *Internat. Rev. Cytol.* **94**, 213-267.

Hariri, D. & Lapierre, H. (1985). Purification and serological properties of soil-borne oat stripe virus. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **228**, 72.

Harker, C.L., Mullineaux, P.M., Bryant, J.A. & Maule, A.J. (1987). Detection of CaMV gene I and gene VI protein products *in vivo* using antisera raised to COOH-β galactosidase fusion proteins. *Plant Molecular Biology* **8**, 275-287.

Harrison, B.D. (1974). Potato mop-top virus. *CMI/AAB Descriptions of Plant Viruses* No. 138.

Harrison, B.D. (1985). Advances in geminivirus research. *Annual Review of Phytopathology* **23**, 55-82.

Harrison, B.D., Barker, H., Bock, K.R., Guthrie, E.J., Meredith, G., Atkinson, M. (1977). Plant viruses with circular single-stranded DNA. *Nature* **270**, 760-762.

Harrison, B.D., Duncan, G.H., Roberts, I.M. & Robinson, D.J. (1985). Scottish Crop Research Institute, U.K., Annual Report for 1984, p180.

Harrison, B.D. & Jones, R.A.C. (1970). Host range and some properties of potato mop-top virus. *Annals of Applied Biology* **65**, 393-402.

Harrison, B.D. & Jones, R.A.C. (1971a). Effects of light and temperature on symptom development and virus content of tobacco leaves inoculated with Potato Mop-Top Virus. *Annals of Applied Biology* **67**, 377-387.

Harrison, B.D. & Jones, R.A.C. (1971b). Factors affecting the development of spraing in potato tubers infected with potato mop-top virus. *Annals of Applied Biology* **68**, 281-289.

Harrison, B.D. & Robinson, D.J. (1986). *Tobraviruses*. In The Plant Viruses. Vol 2. The Rod-shaped Plant Viruses (Eds M.H.V. Van Regenmortel and H. Fraenkel Conrat) pp 339-369. Plenum Press, New York & London.

Hatta, T. & Francki, R.I.B. (1979). The fine structure of chloris striate mosaic virus. *Virology* **92**, 428-435.

Hayes, R.J., Buck, K.W. & Brunt, A.A. (1984). Double-stranded and single-stranded subgenomic RNAs from plant tissue infected with tomato bushy stunt virus. *Journal of General Virology* **65**, 1239-1243.

Hayes, R.J., Brough, C.L., Prince, V., Coutts, R.H.A. & Buck, K.W. (1988a). Infection of *Nicotiana benthamiana* with uncut cloned tandem dimers of tomato golden mosaic virus DNA. *Journal of General Virology* **69**, 209-218.

Hayes, R.J., Macdonald, H., Coutts, R.H.A. & Buck, K.W. (1988b). Agroinfection of *Triticum aestivum* with cloned DNA of wheat dwarf virus. *Journal of General Virology* **69**, 891-896.

Hayes, R.J., Macdonald, H., Coutts, R.H.A. & Buck, K.W. (1988c). Priming of complementary DNA synthesis *in vitro* by small DNA molecules tightly bound to virion DNA of wheat dwarf virus. *Journal of General Virology* **69**, 1345-1350.

Hayes, R.J., Coutts, R.H.A. & Buck, K.W. (1988d). Agroinfection of *Nicotiana* spp. with cloned DNA of tomato golden mosaic virus. *Journal of General Virology* **69**, 1487-1496.

Hayes, R.J., Petty, I.T.D., Coutts, R.H.A. & Buck, K.W. (1988e). Gene amplification and expression in plants by a replicating Geminivirus vector. *Nature (London)* **334**, 179-182.

Hebert, T.T. (1963). Precipitation of plant viruses by polyethylene glycol. *Phytopathology* **53**, 362.

Hebert, T.T. & Coleman, N.T. (1955). Rod-shaped particles associated with soil-borne wheat mosaic virus. *Phytopathology* **45**, 348.

Hebert, T.T. & Panizo, C.H. (1975). Oat mosaic virus. *CMI/AAB Descriptions of Plant Viruses No. 145.*

Heijbroek, W. (1984). Distribution of BNYVV in the Netherlands and initiated research programme. *Proceedings of the First International Virology Congress on Rhizomania*, INRA, Colmar, 95-96.

Henry, C.M., Jones, R.A.C. & Coutts, R.H.A. (1986). Occurrence of a soil-borne virus of sugar beet in England. *Plant Pathology* **35**, 585-591.

Herold, F. & Munz, K. (1967). An unusual kind of crystalline inclusion of tobacco mosaic virus. *Journal of General Virology* **1**, 375.

Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J-P., Van Montagu, M. & Schell, J. (1983) Chimaeric genes as dominant selectable markers in plant cells. *The EMBO Journal* **2**, 987-995.

Hibino, H., Tsuchizaki, T. & Saito, Y. (1974a). Comparative electron microscopy of cytoplasmic inclusions induced by nine isolates of soil-borne wheat mosaic virus. *Virology* **57**, 510.

Hibino, H., Tsuchizaki, T. & Saito, Y. (1974b). Electron microscopy of inclusion development of rye leaf cells infected with soil-borne wheat mosaic virus. *Virology* **57**, 522.

Hibino, H., Tsuchizaki, T. & Saito, Y. (1981). Influence of host species and infection stages on the components of soil-borne wheat mosaic virus. *Ann. Phytopathol. Soc. Japan* **47**, 566.

Higgins, R.C. & Dahmus, M.R. (1979). Rapid visualisation of protein bands in preparative SDS-polyacrylamide gels. *Analytical Biochemistry* **93**, 257-260.

Hill, S. & Torrance, L. (1989). *Plant Pathology* 38, 114-122. Rhizomania disease of sugar beet in England.

Hohn, B., Grimsley, N., Pisan, B. & Hohn, T. (1987). Plant DNA viruses as gene vectors. 1987 Plant resistance to viruses. Wiley, Chichester (Ciba Foundation Symposium 133) p185-195.

Holland, J.J. (1985). Generation and replication of defective viral genomes. In: *Virology* (Eds B.N. Fields *et al.*), Chapter 6 pp77-99. Raven Press, New York.

Hollings, M. & Brunt, A.A. (1981). Potyvirus group. *CMI/AAB Descriptions of Plant Viruses* No. 245.

Holmes, F.O. (1934). A masked strain of tobacco mosaic virus. *Phytopathology* **24**, 845-873.

Honda, Y., Iwaki, M., Saito, Y., Throngmeearkom, P., Kittisak, K. & Deema, N. (1983). Mechanical transmission, purification and some properties of the whitefly-borne mung bean yellow mosaic virus in Thailand. *Plant Disease* **67**, 801-804.

Hooker, W.J. & Salazar, L.F. (1983). A new plant virus from the high jungle of the Eastern Andes; solanum apical leaf curling virus (SALCV) *Annals of Applied Biology* **103**, 449-454.

Hooper, G.R. & Wiese, M.V. (1972). Cytoplasmic inclusions in wheat affected by wheat spindle streak mosaic. *Virology* **47**, 664.

Hopp, T.P. & Woods, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Science, U.S.A.* **78**, 3824-3828.

Howarth, A.J., Caton, J., Bossert, M. & Goodman, R.M. (1985). Nucleotide sequence of bean golden mosaic virus and a model for gene regulation in geminiviruses. *Proceedings of the National Acadamy of Sciences, U.S.A* **82**, 3572-3576.

Howarth, A.J. & Goodman, R.M. (1986). Divergence and Evolution of Geminivirus Genomes. *Journal of Molecular Evolution* **23**, 313-319.

Howell, S.H. (1984). Physical structure and genetic organisation of the genome of maize streak virus (Kenyan isolate) *Nucleic Acid Research* **12**, 7359-7375.

Howell, S.H. (1985). Corrigendum: Physical structure and genetic organisation of the genome of maize streak virus (Kenyan isolate) *Nucleic Acid Research* **13**, 3018-3019.

Hsu, Y.H. & Brakke, M.K. (1985a). Cell-free translation of soil-borne wheat mosaic virus RNAs. *Virology* **143**, 272-279.

Hsu, Y.H. & Brakke, M.K. (1985b). Sequence relationships among soil-borne wheat mosaic virus RNA species and terminal structures of RNA-II. *Journal of General Virology* **66**, 915-919.

Huang, A.S. & Baltimore, D. (1977). Defective interfering animal viruses. *Comprehensive Virology* **10**, 73-116.

Hull, R., Shepherd, R.J. & Harvey, J.D. (1976). Cauliflower mosaic virus: an improved purification procedure and some properties of the virus particles. *Journal of General Virology* **31**, 93-100.

Hull, R. & Covey, S.N. (1983). Does cauliflower mosaic virus replicate by reverse transcription. *Trends in Biochemical Science* 8, 119.

Hull, R. & Davies, J.W. (1983). Genetic engineering with plant viruses, and their potential as vectors. *Advances in Virus Research* **28**, 1-33.

Hunger, R.M. & Sherwood, J.L. (1985). Use of symptomatology and virus concentration for evaluating resistance to wheat soilborne mosaic virus. *Plant Disease* **69**, 848-850.

Huth, W., Lesemann, D.-E. & Paul, H.-L. (1984). Barley yellow mosaic virus: purification, electron microscopy, serology, and other properties of two types of the virus. *Phytopathologische Zeitschrift* **111**, 37-54.

Ikegami, M., Haber, S. & Goodman, R.M. (1981). Isolation and characterisation of virus-specific double-stranded DNA from tissues infected by bean golden mosaic virus. *Proceedings of the National Acadamy of Sciences, U.S.A.* **78**, 4102-4106.

Ikegami, M., Morinaga, T. & Miura, K. (1984). Infectivity of virus-specific double-stranded DNA from tissue infected by mung bean yellow mosaic virus. *Virus Research* **1**, 507-512.

Imai, M., Richardson, M.A, Ikegami, M., Shatkin, A.J. & Furuichi, Y. (1983). Molecular cloning of double-stranded RNA virus genomes. *Proceedings of the National Academy of Science U.S.A.* **80**, 373-377.

Imoto, M., Tochihara, H., Iwaki, M. & Nakamura, H. (1981). Occurrence of potato mop-top disease in Japan. *Annals of the Phytopathological Society of Japan* **47**, 409.

Inouye, T. (1969). Filamentous particles as the causal agent of yellow mosaic disease of wheat. *Ber. Ohara Inst. Landwirtsch. Biol. Okayama Univ.* **13**, A7.

Inouye, T. (1971). Electron microscopy of sweetpea cells infected with broad bean necrosis virus. *Ber. Ohara Inst. Landwirtsch. Biol. Okayama Univ.* **15**, 69.

Inouye, T. & Asatani, M. (1968). Broad-bean necrosis virus. *Annals of the Phytopathological Society of Japan* **34**, 317.

Inouye, T. & Fujii, S. (1977). Rice necrosis mosaic virus. *CMI/AAB Descriptions of Plant Viruses No. 172.*

Inouye, T. & Nakasone, W. (1980). Broad bean necrosis virus. *CMI/AAB Descriptions of Plant Viruses No. 223.*

Inouye, T. & Osaki, T. (1980). The first record in the literature of the possible plant virus disease that appeared in "Manyōshū", a Japanese classic anthology, as far back as the time of the 8th century. *Annals of the Phytopathological Society of Japan* **46**, 49-50.

Inouye, T. & Saito, Y. (1975). Barley yellow mosaic virus. *CMI/AAB Descriptions of Plant Viruses No. 143.*

Ioanides, A. (1978). Rhizomania. *To Teutho* **1.10.78**, 13.

Ivanović, M. & MacFarlane, I. (1982). A tubular virus associated with infection of sugar beet by *Polymyxa betae*. *Annual Report of Rothamsted Experimental Station for 1981*, pp. 190-191.

Ivanović, M., MacFarlane, I. & Woods, R.D. (1983). Viruses of sugar beet associated with *Polymyxa betae*. *Annual Report of Rothamsted Experimental Station for 1982*, pp. 189-190.

Iwaki, M., Thongmeearkom, P., Honda, Y. & Deema, N. (1983). Soybean crinkle leaf; A new whitefly-borne disease of soybean. *Plant Disease* **67**, 546-548.

Izant, J.G. & Weintraub, H. (1984). Inhibition of thymidine kinase expression by antisense RNA: a molecular approach to genetic analysis. *Cell* **36**, 1007

Jackson, A.O., Mitchell, D.M. & Siegel, A. (1971). Replication of tobacco mosaic virus . I. Isolation and characterisation of double-stranded forms of ribonucleic acid. *Virology* **45**, 183-191.

Jackson, A.O. & Lane, L.C. (1981). Hordeiviruses. In: *Handbook of Plant Virus Infections and Comparative Diagnosis* (Ed. E. Kurstak), pp. 565-625, Elsevier/North-Holland, Amsterdam.

Jankulova, M., Gueorguieva, P. & Ivanova, A. (1984). Le virus des nervures jaunes et nécrotiques de la betterave, agent de la rhizomanie, détecté en Bulgarie. *Proceedings of the First International Virology Congress on Rhizomania*, INRA, Colmar, 81-87.

Jaramillo, S. & Lastra, R. (1986). Purification and properties of the geminivirus *Euphorbia* mosaic virus. *Journal of Phytopathology* **115**, 193-203.

Jefferson, R.A., Kavanagh, T.A. & Bevan, M. W. (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* **6**, 3901-3907.

Jeske, H., Menzel, D. & Werz, G. (1977). Electron microscopic studies on intranuclear virus-like inclusions in mosaic-diseased *Abutilon sellowianum* Reg. *Phytopathologische Zeitschrift* **89**, 289-295.

Jeske, H. & Werz, G. (1980a). Ultrastructural and biochemical investigations on the whitefly transmitted abutilon mosaic virus (AbMV). *Phytopathologische Zeitschrift* **97**, 43-55.

Jeske, H. & Werz, G. (1980b). Cytochemical characterisation of plastidial inclusions in *Abutilon* mosaic-infected *Malva parriflora* mesophyll cells. *Virology* **106**, 155-158.

Jeske, H. & Schuchalter-Eicke, G. (1984). The abutilon mosaic virus (AbMV) in different leaf tissues of several host species of Malvaceae. *Phytopathologische Zeitschrift* **109** 353-362.

Jeske, H. (1986). The abutilon mosaic virus (AbMV) in different compartments. EMBO Workshop: Molecular Plant Virology. Wageningen, The Netherlands, July 6-10, 1986.

Jessee, J. (1986). New sub-cloning efficiency competent cells : $1x10^6$ transformants/µg. *Focus* **8**, 9-10. Gaithenberg, Bethesda Research Laboratories.

Jones, J.D.G., Grady, K.L., Suslow, T.V. & Bedbrook, J.R. (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *The EMBO Journal* **5**, 467-473.

Jones, R.A.C. & Harrison, B.D. (1969). The behaviour of potato mop-top virus in soil, and evidence for its transmission by *Spongospora subterranea* (Wallr.) Lagerh. *Annals of Applied Biology* **63**, 1-17.

Jones, R.A.C. & Harrison, B.D. (1972). Ecological studies on potato mop-top virus in Scotland. *Annals of Applied Biology* **71**, 47-57.

Jordan, R.L. & Dodds, J.A. (1983). Hybridisation of 5'-end-labelled RNA to plant viral RNA in agarose and acrylamide gels. *Plant Molecular Biology Reporter* **1**, 31-37.

Jordan, R.L., Dodds, J.A. & Ohr, H.D. (1983). Evidence for viruslike agents in avocado. *Phytopathology* **73**, 1130-1135.

Jupin, I., Quillet, L., Ziegler-Graff, V., Guilley, H., Richards, K. & Jonard, G. (1988). *In vitro* translation of natural and synthetic beet necrotic yellow vein virus RNA-1. *Journal of General Virology* **69**, 2359-2367.

Kallender, H., Petty, I.T.D., Stein, V.E., Panico, M., Blench, I.P., Etienne, A.T., Morris, H.R., Coutts, R.H.A & Buck, K.W. (1988). Identification of the coat protein gene of tomato golden mosaic virus. *Journal of General Virology* **69**, 1351-1357.

Kaper, J.M. & Diaz-Riuz, J.R. (1977). Molecular weights of the double-stranded RNAs of cucumber mosaic virus strain S and its associated RNA 5. *Virology* **80**, 214-217.

Karling, J.S. (1968). *The Plasmodiophorales*, Hofner Publishing Co., New York, pp. 256.

Kassanis, B., Woods, R.D. & White, R.F. (1972). Some properties of potato mop-top virus and its serological relationship to tobacco mosaic virus. *Journal of General Virology* **14**, 123-132.

Kendall, T.L., Langenberg, W.G. & Lommel, S.A. (1988). Molecular characterisation of Sorghum chlorotic spot virus, a proposed furovirus. *Journal of General Virology* **69**, 2335-2345.

Kikuno, R., Toh, H., Hyashida, H. & Miyata, T. (1984) Sequence similarity between putative gene products of geminiviral DNAs. *Nature (London)* **308**, 562.

Kim, K.S., Shock, T.L. & Goodman, R.M. (1978). Infection of *Phaseolus vulgaris* by bean golden mosaic virus: ultrastructural aspects. *Virology* **89**, 22-23.

Kim, K.S. & Fulton, R.W. (1984). Ultrastructure of *Datura stromonium* infected with a Euphorbia virus suggestive of a whitefly-transmitted geminivirus. *Phytopathology* **74**, 236-240.

Kim, K.S., Bird, J., Rodriguez, R.L., Martin, E.M. & Escudero, J. (1986). Ultrastructural studies of *Jatropha gossypifolia* with jatropha mosaic virus, a whitefly-transmitted geminivirus. *Phytopathology* **76**, 80-85.

Koch, F. (1982). Die Rizomania der Zuckerrube. *Proc. Int. Inst. Sugar Beet Res., Brussels.* p. 211.

Koehler, B., Bever, W.M. & Bennett, O.T. (1952). Soil-borne wheat mosaic. *University of Illinois Experimental Station Bulletin* **556**, 567.

Koenig, R., Lesemann, D.-E. & Burgermeister, W. (1984). Beet necrotic yellow vein virus: Purification, preparation of antisera and detection by means of ELISA, immunosorbent electronmicroscopy and electro-blot immunoassay. *Phytopathologische Zeitschrift* **111**, 224.

Koenig, R. Burgermeister, W., Weich, H., Sebald, W. & Kothe, C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in sugarbeet roots, but not in leaves from several plant species. *Journalof General Virology* **67**, 2043-2046.

Kornberg, A. (1980). DNA Replication. Freeman, San Francisco.

Kornberg, A. (1982). 1982 Supplement to DNA Replication. Freeman, San Francisco.

Kozak, M. (1983). Comparison of initiation of protein synthesis in prokaryotes, eukaryotes and organelles. *Microbiological Reviews* **47**, 1-45.

Kozak, M. (1984). Compilation and analysis of sequences upstream from the translation start site in eucaryotic mRNAs. *Nucleic Acids Research* **12**, 857-872.

Kozak, M. (1984). Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. *Nucleic Acids Research* **12**, 3873-3893.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eucaryotic ribosomes. *Cell* **44**, 283-292.

Kuszala, M., Ziegler, V., Bouzoubaa, S., Richards, K., Putz, C., Guilley, H. & Jonard, G. (1986). Beet necrotic yellow vein virus: different isolates are serologically similar but differ in RNA composition. *Annals of Applied Biology* **109**, 155-162.

Kuwata, S. & Kubo, S. (1981). Rod-shaped particles found in tobacco plants infected with tobacco stunt agent. *Annals of the Phytopathological Society of Japan* **47**, 264.

Kuwata, S. & Kubo, S. (1984). Properties of two *Olpidium*-transmitted viruses: Tobacco stunt and lettuce big vein. *IVth. International Congress of Virology, Sendai.* Abstract P45-2, p. 331.

Kuwata, S., Kubo, S., Yamashita, S. & Doi, Y. (1983). Rod-shaped particles, a probable entity of lettuce big vein virus. *Annals of the Phytopathological Society of Japan* **49**, 246.

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

Lapierre, H., Hariri, D., Bouchain, F. & Gernaud (1985). Presence of soilborne wheat mosaic virus in France. *Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft* **278**, 73.

Larsen, H.J., Brakke, M.K. & Langenberg, W.G. (1985). Relationships between wheat streak mosaic virus and soilborne wheat mosaic virus infection, disease resistance and early growth of winter wheat. *Plant Disease* **69**, 857-862.

Larsen, R.C. & Duffus, J.E. (1984). A simplified procedure for the purification of curly top virus and the isolation of its monomer and dimer particles. *Phytopathology* **74**, 114-118.

Lastra, R. & Gil, F. (1981). Ultrastructural host cell changes associated with tomato yellow mosaic. *Phytopathology* **71**, 524-528.

Lazarowitz, S.G. (1987). The molecular characterization of geminviruses. *Plant Molecular Biology Reporter* **4**, 177-192.

Lazarowitz, S.G. (1988). Infectivity and complete nucleotide sequence of the genome of the South African isolate of maize streak virus. *Nucleic Acid Research* **16**, 229-249.

Lebeurier, G., Hirth, L., Hohn, B. & Hohn, T. (1982). *In vivo* recombination of cauliflower mosaic virus DNA. *Proceedings of the National Acadamy of Sciences, U.S.A.* **79**, 2932-2936.

Ledingham, G.A. (1939). Studies on *Polymyxa graminis* n.gen., n.sp., a plasmodiophoraceous root parasite of wheat. *Canadian Journal of Research* **17**, 38-51.

Lemaire, O., Merdinoglu, D., Valentin, P., Putz, C., Ziegler-Graff, V., Guilley, H., Jonard, G. & Richards, K. (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa graminis*. *Virology* **162**, 232-235.

Leonard, D.A. & Zaitlin, M. (1982). A temperature sensitive strain of tobacco mosaic virus defective in cell to cell movement generates an altered viral coded protein. *Virology* **117**, 416-424.

Lichtenstein, C.P. & Fuller, S.L. (1987). Vectors for the genetic engineering of plants. In Genetic Engineering 6, pp104-171. Academic Press, London.

Lindsten, K., Lindsten, B., Abdelmoeti, M. & Juntti N. (1980). Purification and some properties of wheat dwarf virus. *Proceedings of the third Conference on Virus Diseases of the Gramineae*, pp27-33, Rothamstead, England.

Lindsten, K. (1986). Rhizomania - a complicated disease in sugar beets which can also occur in Sweden. *Växtskyddsnoticer* **50**, 111-118.

Linford, M.B. & McKinney, H.H. (1954). Occurrence of *Polymyxa graminis* in roots of small grains in the United States. *Plant Disease Reporter* **38**, 711-713.

Lister, R.M. & Bracker, C.E. (1969). Defectiveness and dependence in three related strains of tobacco rattle virus. *Virology* **177**, 416-424.

Lister, R.M. & Bar-Joseph, M. (1981). Closteroviruses. In Handbook of Plant Virus Infections and Comparative Diagnosis, pp809-844. Ed. E. Kurstak. Amsterdam: Elsevier/North Holland Biomedical Press.

Louvel, D. & Bidaux, J.-M. (1977). Observation de nouveaux symptômes pathologiques sur des variétés précoces de riz en Côte d'Ivoire. *Agronomie Tropicale* **XXXII 3**, 257-261.

Lutcke, H.A., Chow, K.C., Mickel, F.S., Moss, K.A., Kern, H.F. & Scheek, G.A. (1987). Selection of AUG initiation codons differ in plants and animals. *The EMBO Journal* **6**, 43-48.

Macdonald, H., Coutts, R.H.A. & Buck, K.W. (1988). Characterisation of a subgenomic DNA isolated from *Triticum aestivum* plants infected with wheat dwarf virus. *Journal of General Virology* **69**, 1339-1344.

MacDowell, S.W., Macdonald, H., Hamilton, W.D.O., Coutts, R.H.A. & Buck, K.W. (1985). The nucleotide sequence of cloned wheat dwarf virus. *The EMBO Journal* **4**, 2173-2180.

MacDowell, S.W., Coutts, R.H.A. & Buck, K.W. (1986). Molecular characterization of subgenomic single-stranded and double-stranded DNA forms isolated from plants infected with tomato golden mosaic virus. *Nucleic Acid Research* **14**, 7967-7984.

MacFarlane, I., Jenkins, J.E.E. & Melville, S. (1968). Viruses infecting barley in S.W. England. *Plant Pathology* **17**, 167.

Magee, C.J.P. (1940). Transmission studies on the banana bunchy-top virus. *Journal of the Australian Institute of Agricultural Science* **6**, 109-110.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbour Laboratory, New York)

Markham, P.G., Pinner, M.S., Boulton, M.I. & Plaskitt, K. (1985). John Innes Institute UK Report for 1983-1984.

Marriott, T.W. & Symons, R.H. (1983). Characterisation of the DNA genomes of Australian geminiviruses. In : Current Communications in Molecular Biology - Plant Infectious Agents - Viruses, Viroids, Virusoids and Satellites, (Eds. Robertson, H.D., Howell, S.H., Zaitlin, M. and Malmberg, R.L.) pp.59-62. Cold Spring Harbor, New York.

Marston, F. (1987). The purification of eukaryotic polypeptides expressed in *Escherichia coli*. In *DNA Cloning: A Practical Approach. Volume 3.* pp.59-87. Ed. D.M.Glover, IRL Press, Oxford.

Masri, S.A. & Hiruki, C. (1983). A new group of elongated plant viruses having a capsid protein of an unusually high molecular weight. *Canadian Journal of Plant Pathology* **5**, 208.

Matthews, R.E.F. (1979). Classification and nomenclature of viruses. Third report of the International Committee on the Taxonomy of Viruses. *Intervirology* **12**, 129-296.

Mayo, M.A. & Reddy, D.V.R. (1985). Translation products of RNA from Indian peanut clump virus. *Journal of General Virology* **66**, 1347-1351.

McKinney, H.H. (1923). Investigation on the rosette disease of wheat and its control. *J. Agric. Res.* **23**, 771.

McKinney, H.H. (1937). Mosaic diseases of wheat and related cereals. *US Department of Agriculture Bulletin* **442**, pp.23.

McKinney, H.H., Eckerson, S.H. & Webb, R.W. (1923). The intracellular bodies associated with the rosette disease and mosaic-like leaf mottling of wheat. *Journal of Agriculture Research* **26**, 605.

Messing, J. (1983). New M13 vectors for cloning. *Methods in Enzymology* **101**, 20-77. Edited by R.Wu, L. Grossman & K Moldave. New York: Academic Press.

Meyer, R.R., Shlomai, J., Kobori, J., Bates, D.L., Rowen, L., McMacken, R., Ueda, K. & Kornberg, A. (1978). Enzymatic conversion of single-stranded ΦX174 and G4 circles to duplex forms: discontinuous replication. *Cold Spring Harbor Symposia on Quantitative Biology* **43**, 289-293.

Morinaga, T., Ikegami, M. & Miura, K. (1983a). Infectivity of the cloned DNAs from multiple genome components of bean golden mosaic virus. *Proceedings of the Japanese Academy* **59**, 363-366.

Morinaga, T., Shimotono, K., Miura, K. & Ikegami, M. (1983b). A two component viral genome of a geminivirus. *Annual Phytopathological Society of Japan* **49**, 424.

Morris, T.J. (1983). Virus-specific double-stranded RNA: functional rôle in RNA virus infection. In *Current Communications in Molecular Biology - Plant Infectious Agents - Viruses, Viroids, Virusoids and Satellites*, pp.80-83. Eds. H.D.Robertson, S.H.Howell, M.Zaitlin and R.L.Malmberg, Cold Spring Harbor, New York.

Morris-Krsinich, B.A.M., Mullineaux, P.M., Donson, J., Boulton, M.I. & Davies, J.W. (1985). Bidirectional transcription of maize streak virus and identification of the coat protein gene. *Nucleic Acid Research* **13**, 7237-7256.

Mullineaux, P.M., Donson, J., Morris-Krsinich, B.A.M., Boulton, M.I. & Davies, J.W. (1984). The nucleotide sequence of maize streak virus DNA. *The EMBO Journal* **3**, 3063-3068.

Mullineaux, P.M., Donson, J., Stanley, J., Boulton, M.I., Morris-Krsinich, B.A.M., Markham, P.J. & Davies, J.W. (1985) Computer analysis identifies sequence homologies between potential gene products of maize streak virus and those of cassava latent virus and tomato golden mosaic virus. *Plant Molecular Biology* **5**, 125-131.

Mullineaux, P.M., Boulton, M.I., Bowyer, P., van der Vlugt, R., Marks, M., Donson, J. & Davies, J.W. (1988). Detection of a non-structural protein of M_r 11,000 encoded by the virion DNA of maize streak virus. *Plant Molecular Biology* **11**, 57-66.

Mumford, D.L. (1974). Purification of curly top virus. *Phytopathology* **64**, 136-139.

Muniyappa, V., Rajeshwari, R., Bharathan, N., Reddy, D.V.R. & Nolt, B.L. (1987). Isolation and characterisation of a gemenivirus causing yellow mosaic disease of horsegram (*Macrotyloma uniflorum* [Lam.] Verdc.) in India. *Journal of Phytopathology* **119**, 81-87.

Nakasone, W. & Inouye, T. (1978). On the serological relationship between broad bean necrosis virus and the tobamovirus group. *Ann. Phytopathol. Soc. Japan* **44**, 97.

Nameth, S.T. & Dodds, J.A. (1985). Double-stranded RNA detected in cucurbit varieties not inoculated with viruses. *Phytopathology* **75**, 1293 (Abstr.).

Negrutiu, I., Shillito, R., Potrykus, I., Biasim, G. & Sala, F. (1987). *Plant Molecular Biology* **8**, 363-373.

Novak, J.B. & Lanzova, J. (1984). Serological proof of beet necrotic yellow vein virus on sugar beet in Czechoslovakia. *Proceedings of the IXth Czechoslovak Plant Protection Conference in Brno* **1983**, 253-254.

Ohno, T., Takamatsu, N., Meshi, T., Okada, Y., Nishiguchi, M. & Kiho, Y. (1983). Single amino acid substitution in the 30K protein of TMV defective in virus transport function. *Virology* **131**, 253-258.

Olszewski, N., Hagen, G. & Guilfoyle, T.J. (1982). A transcriptionally active, covalently closed minichromosome of cauliflower mosaic virus DNA isolated from infected turnip leaves. *Cell*, **29**, 395-402.

Osaki, T., Kobatake, H. & Inouye, T. (1979). Mosaic of honeysuckle (*Lonicera japonica* Thunb.), a disease caused by tobacco leaf curl virus in Japan. *Annals of the Phytopathology Society of Japan* **45**, 62-69.

Osaki, T. & Inouye, T., (1981). Tobacco leaf curl virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses No. 232, Commonwealth Mycological Institute, Kew, England.

Osman, T.A.M. & Buck, K.W. (1987). Replication of red clover necrotic mosaic virus RNA in cowpea protoplasts: RNA 1 replicates independently of RNA 2. *Journal of General Virology* **68**, 289-296.

Ow, D.W., Wood, K.V. De Luca, M., De Wet, J.R., Helinski, D.R. & Howell, S.H. (1986). Transient and stable expression of the fire fly luciferase gene in plant cells and transgenic plants. *Science* **234**, 856-859

Palukaitis, P., Garcia-Arenal, F., Sulzinski, M.A. & Zaitlin, M. (1983). Replication of tobacco mosaic virus. VII. Further characterisation of single-stranded and double-stranded RNAs from tobacco mosaic virus infected plants. *Virology* **131**, 533-545.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak, V., Hohn, T., Hohn, B. & Potrykus, I. (1984). Direct gene transfer to plants. *The EMBO Journal* **3**, 2717-2722.

Paulsen, A.Q. (1970). Reactions of eleven *Chenopodium* species to seven sap-transmissible grass viruses. *Phytopathology* **60**, 1307.

Pelham, H. R. B. (1978). Leaky UAG termination codon in tobacco mosaic virus. *Nature (London)* **272**, 469-471.

Pelham, H. R. B. (1979). Synthesis and proteolytic processing of cowpea mosaic virus proteins in reticulocyte lysates. *Virology* **96**, 463.

Peterson, J. F. (1970). Electron microscopy of soil-borne wheat mosaic virus in host cells. *Plant Disease Reporter* **42**, 304-310.

Petty, I. T. D. (1986). Gene expression of tomato golden mosaic virus. PhD Thesis, University of London.

Petty, I. T. D., Coutts, R. H. A. & Buck, K. W. (1988). Transcriptional mapping of the coat protein gene of tomato golden mosaic virus. *Journal of General Virology* **69**, 1359-1365.

Plumb, R. T., Catherall, P. L., Chamberlain, J. A. & MacFarlane, I. (1977). A new virus of oats in England and Wales. *Annals of Phytopathology* **9**, 365-370.

Plumb, R. T. & MacFarlane, I. (1977). Cereal diseases: a "new" virus of oats. *Annual Report of Rothamsted Experimental Station for 1976.* p. 256.

Plumb, R. T. & MacFarlane, I. (1978). Oat golden stripe virus. *Annual Report of Rothamsted Experimental Station for 1977.* p. 212.

Powell, C. A. (1976). The relationship between soil-borne wheat mosaic virus and tobacco mosaic virus. *Virology* **71**, 453-462.

Powell-Abel, P., Nelson, R. S., De B., Hoffmann, N., Rogers, S. G., Fraley, R. T. & Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science* **232**, 738-743.

Pring, D.R. & Lonsdale, D.M. (1985). Molecular biology of higher plant mitochondrial DNA. *Internat. Rev. Cytol.* **97**, 1-46.

Proudfoot, N.J. & Brownlee, G.G. (1976). 3' non-coding region sequences in eukaryotic messenger RNA. *Nature (London)* **263**, 211-214.

Purcifull, D.E. & Edwardson, J.R. (1981). Potexviruses. In Handbook of Plant Virus Infections and Comparative Diagnosis, pp627-694. Ed. E. Kurstak. Amsterdam: Elsevier/North Holland Biomedical Press.

Putz, C. (1977). Composition and structure of beet necrotic yellow vein virus. *Journal of General Virology* **35**, 397-401.

Putz, C. & Kuszala, M. (1978). La rhizomanie de la betterave sucrière en Alsace. Recherche d'une nouvelle méthode de purification du 'beet necrotic yellow vein virus'. *Annales de Phytopathologie* **10**, 247-262.

Putz, C., Pinck, M., Fritsch, C. & Pinck, L. (1983). Identification of the 3' and 5' ends of beet necrotic yellow vein virus RNAs. *FEBS Letters* **156**, 41-56.

Putz, C. & Vuittenez, A. (1974). Observation de particules virales chez des betteraves présentant en Alsace des symptômes de 'rhizomanie'. *Annales de Phytopathologie* **6**, 129.

Putz, C. & Vuittenez, A. (1980). The intracellular location of beet necrotic yellow vein virus. *Journal of General Virology* **50**, 201.

Rana, R.L., Di Franco, A. & Russo, M. (1978). La rizomania della barbabietola in Italia meridionale. *Inf. Fitopatol.* **28**, 5.

Randles, J.W., Harrison, B.D. & Roberts, I.M. (1976). *Nicotiana velutina* mosaic virus: purification, properties and affinities with other rod-shaped viruses. *Annals of Applied Biology* **84**, 193-204.

Randles, J.W. (1978). *Nicotiana velutina* mosaic virus. *CMI/AAB Descriptions of Plant Viruses No. 189.*

Rao, A.S. & Brakke, M.K. (1969). Relation of soil-borne wheat mosaic virus and its fungal vector, *Polymyxa graminis*. *Phytopathology* **59**, 581.

Reddy, D.V.R., Iizuka, N., Subrahmanyam, P., Rajeshwari, R. & McDonald, D. (1979). A soil-borne disease of peanuts in India. *Proc. Am. Peanut Res. Educ. Soc.* **11**, 49.

Reddy, D.V.R., Rajeshwari, R., Iizuka, N., Lesemann, D.E., Nolt, B.L. & Goto, T. (1983). The occurrence of Indian peanut clump, a soil-borne virus disease of groundnuts (*Arachis hypogaea*) in India. *Annals of Applied Biology* **102**, 305-310.

Reddy, D.V.R., Robinson, D.J., Roberts, I.M. & Harrison, B.D. (1985). Genome properties and relationships of Indian peanut clump virus. *Journal of General Virology* **66**, 2011-2016.

Reisman, D., Ricciardi, R.P. & Goodman, R.M. (1979). Size and topology of single-stranded DNA from bean golden mosaic virus. *Virology* **97**, 388-395.

Reiss, B., Sprengel, R. & Schaller, W.H. (1984). A new sensitive method for qualitative and quantitative analysis of neomycin phosphotransferase in crude cell extracts. *Gene* **50**, 217-223.

Richards, K., Jonard, G., Guilley, H., Ziegler, V. & Putz, C. (1985). *In vitro* translation of beet necrotic yellow vein virus RNA and studies of sequence homology among the RNA species using cloned cDNA probes. *Journal of General Virology* **66**, 345-350.

Roberts, E.J.F., Buck, K.W. & Coutts, R.H.A. (1986). A new geminivirus infecting potatoes. *Plant Disease* **70**, 603.

Roberts, E.J.F, Buck, K.W. & Coutts, R.H.A. (1988). Characterisation of potato yellow mosaic virus as a geminivirus with a bipartite genome. *Intervirology 29, 162-169.*

Roberts, I.M. & Harrison, B.D. (1979). Detection of potato leafroll and potato mop-top viruses by immunosorbent electron microscopy. *Annals of Applied Biology* **93**, 289-297.

Roberts, I.M., Robinson, D.J. & Harrison, B.D. (1984). Serological relationships and genome homologies among geminiviruses. *Journal of General Virology* **65**, 201-223.

Robinson, D.J., Barker, H., Harrison, B.D. & Mayo, M.A. (1980). Replication of RNA 1 of tomato black ring virus independently of RNA 2. *Journal of General Virology* **51**, 317-326.

Rodbard, D. & Chrambach, A. (1970). Unified theory for gel electrophoresis and gel filtration. *Proceedings of the National Academy of Science U.S.A.* **65**, 970-977.

Rogers, S.G., Bisaro, D.M., Horsch, R.B., Fraley, R.T., Hoffmann, N.L., Brand, L., Elmer, J.S. & Lloyd, A.M. (1986). Tomato golden mosaic virus A component DNA replicates autonomously in transgenic plants. *Cell* **45**, 593-600.

Rosahl, S., Schell, J. & Willmitzer, L. (1987). Expression of a tuber specific storage protein in transgenic tobacco plants, demonstration of an esterase activity. *The EMBO Journal* **6**, 1155-1159.

Rosenberg, A.H., Lade, B.N., Chui, D., Lin, S., Dunn, J.J. & Studier, F.W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125-135.

Rushing, A.E., Sunter, G., Gardiner, W.E., Dute, R.R. & Bisaro, D.M. (1987). Ultrastructural aspects of tomato golden mosaic virus infection of tobacco. *Phytopathology* **77**, 1231-1236.

Russo, M., Cohen, S. & Martelli, G.P. (1980). Virus like particles in tomato plants affected by the yellow leaf curl disease. *Journal of General Virology* **49**, 209-213.

Russo, M., Martelli, G.P. & Di Franco, A. (1981). The fine structure of local lesions of beet necrotic yellow vein virus in *Chenopodium amaranticolor*. *Physiol. Plant Pathol.* **19**, 237.

RÜther, U. & MÜller-Hill, B. (1983). Easy identification of cDNA clones. *The EMBO Journal* **2**, 1791-1794.

Saito, Y., Takanashi, K., Iwata, Y. & Okamoto, H. (1964). Studies on the soil-borne virus disease of wheat and barley. I. Several properties of the viruses. *Bull. Natl. Inst. Agric. Sci. Tokyo Ser. C* **1964**, 1711.

Saito, Y, Tsuchizaki, T. & Hibino, H. (1968). Causal virus of wheat yellow mosaic disease. *Annals of the Phytopathological Society of Japan* **34**, 347.

Salazar, L.F. & Jones, R.A.C. (1975). Some studies on the distribution and incidence of potato mop-top virus in Peru. *Am. Potato J.* **52**, 143.

Sanchez-Serrano, J.J., Keil, M., O'Connor, A., Schell, J. & Willmitzer, L. (1987). *The EMBO Journal* **6**, 303.

Schell, J. (1987). Transgenic plants as tools to study the molecular organization of plant genes. *Science* **237**, 1176-1183.

Sequeira, J.C. (1982). Purification, properties and relationships of cassava latent virus. Ph.D. thesis. Dundee University, Scotland.

Sequeira, J.C. & Harrison, B.D. (1982). Serological studies on cassava latent virus. *Annals of Applied Biology* **101**, 33-42.

Severin, H.H.P. (1921). Minimum incubation periods of causative agent of curly leaf in beet leafhopper and sugar beet. *Phytopathology* **11**, 424-429.

Shanks, M., Lomonosoff, G.P. & Evans, D. (1985). Double-stranded replicative form RNA molecules of cowpea mosaic virus are not infectious. *Journal of General Virology* **66**, 925.

Sherwood, J.L. (1987). Comparison of a filter paper immunobinding assay. Western blotting and an enzyme linked immunosorbent assay for the detection of wheat streak mosaic virus. *Journal of Phytopathology* **118**, 68-75.

Shirako, Y. & Brakke, M.K. (1984a). Two purified RNAs of soil-borne wheat mosaic virus are needed for infection. *Journal of General Virology* **65**, 119-127.

Shirako, Y. & Brakke, M.K. (1984b). Spontaneous deletion mutation of soil-borne wheat mosaic virus RNA-II. *Journal of General Virology* **65**, 855-858.

Shirako, Y. & Ehara, Y. (1986). Comparison of the *in vitro* translation products of wild-type and a deletion mutant of soil-borne wheat mosaic virus. *Journal of General Virology* **67**, 1237-1245.

Sill, W.H. (1958). A comparison of some characteristics of soil-borne wheat mosaic viruses in the great plains and elsewhere. *Plant Disease Reporter* **42**, 912-924.

Simons, J.N. (1962). The pseudo-curly top disease in South Florida. *Journal of Economical Entomology* **55**, 358-363.

Simons, J.N. & Coe, D.M. (1958). Transmission of pseudo-curly top virus in Florida by a treehopper. *Virology* **6**, 43-48.

Simpson, J., Timko, M.P., Cashmore, A.R., Schell, J., Van Montagu, M. & Herrera-Estrella, L. (1985). Light inducible and tissue specific expression of a chimaeric gene under control of a pea chlorophyll a/b binding protein gene. *The EMBO Journal* **4**, 2723-2729.

Singh, R.P. & McDonald, J.G. (1980). A chlorotic mosaic of fall hawkbit (*Leontodon autumnalis*). *Can. Plant Dis. Surv.* **60**, 47.

Sisco, P.H., Garcia-Arenal, F., Zaitlin, M., Gracen, V.E. & Earle, E.D. (1984). LBN, a male sterile cytoplasm of maize contains 2 double stranded RNAs. *Plant Science Letters* **34**, 127-134.

Skotniki, A., Skotniki, M.L., Dale, J.L. & Gibbs, A.J. (1985). Direct cloning of double-stranded viral RNA. Abstract from *First International Congress of Plant Molecular Biology*. Ed. G.A. Galan. The University of Georgia Centre for Continuing Education, Georgia, USA.

Slomka, M.J., Buck, K.W. & Coutts, R.H.A. (1988). Characterisation of multimeric DNA forms associated with tomato golden mosaic virus infection. *Archives of Virology* **100**, 99-108. Stanley, J. (1983). Infectivity of the cloned geminivirus genome requires sequences from both DNAs. *Nature (London)* **305**, 643-645.

Stanley, J. (1985). The molecular biology of geminiviruses. *Advances in Virus Research* **30**, 139-177.

Stanley, J. & Davies, J.W. (1985). Structure and function of the DNA genome of geminiviruses. In *Molecular Plant Virology. Vol. II, Replication and Gene Expression*. pp. 191-218. Edited by J.W. Davies. Boca Raton FL: CRC Press.

Stanley, J. & Gay, M.R. (1983). Nucleotide sequence of cassava latent virus DNA. *Nature (London)* **301**, 260-262.

Stanley, J. & Townsend, R. (1985). Characterisation of DNA forms associated with cassava latent virus infection. *Nucleic Acid Research* **13**, 2189-2206.

Stanley, J. & Townsend, R. (1986). Infectious mutants of cassava latent virus generated *in vivo* from intact recombinant DNA clones containing single copies of the genome. *Nucleic Acid Research* **14**, 5981-5998.

Stanley, J., Townsend, R. & Curson, S.J. (1985). Pseudorecombinants between cloned DNAs of two isolates of cassava latent virus. *Journal of General Virology* **66**, 1055-1061

Stanley, J., Markham, P.G., Callis, R.J. & Pinner, M.S. (1986). The nucleotide sequence of an infectious clone of the geminivirus beet curly top virus. *The EMBO Journal* **5**, 1761-1767.

Stein, V.E. (1985). Nucleotide sequence analysis of infectious cloned DNA of tomato golden mosaic virus. Ph.D thesis, University of London.

Stein, V.E., Coutts, R.H.A. & Buck, K.W. (1983). Serological Studies on tomato golden mosaic virus, a geminivirus. *Journal of General Virology* **64**, 2493-2498.

Steven, A.C., Trus, B.L., Putz, C. & Wurtz, M. (1981). The molecular organisation of beet necrotic yellow vein virus. *Virology* **113**, 428-438.

Stocky, G., Vuittenez, A. & Putz, C. (1977). Mise en évidence *in situ* de particules en batonnets, correspondant probablement au virus de la rhizomanie, dans les tissus de diverses chénopodiacées inoculées expérimentalement, ainsi que dans le champignon *Polymyxa betae* Kesk., myxomycète parasitant les racines de plantes malades. *Annales de Phytopathologie* **9**, 536-537.

Storey, H.H. (1928). Transmission studies of maize streak disease *Annals of Applied Biology* **15**, 1-15.

Storey, H.H. (1938). Investigation of the mechanism of the transmission of plant viruses by insect vectors. II. The part played by puncture in transmission. *Proceedings of the Royal Society of London, Series B* **125**, 455-477.

Sunter, G., Gardiner, W.E., Rushing, A.E., Rogers, S.G. & Bisaro, D.M. (1987). Independent encapsidation of tomato golden mosaic virus A component DNA in transgenic plants. *Plant Molecular Biology* **8**, 477-484.

Sutic, D. & Milovanovic, M. (1978). Occurrence and significance of sugar beet root stunting. *Agrochemija* **9**, 363.

Takamatsu, N., Ishikawa, M., Meshi, T. & Okada, Y (1987). Expression of bacterial chloramphenicol acetyl transferase gene in tobacco plants mediated by TMV RNA. *The EMBO Journal* **6**, 307-311.

Tamada, T. (1975). Beet necrotic yellow vein virus. *CMI/AAB Descriptions of Plant Viruses* No. 144.

Tamada, T., Abe, H. & Baba, T. (1971). Relationship between the abnormal growth of sugar beet and a virus isolated from those sugar beet plants. *Bull. Sugar Beet Res.* **13**, 179.

Tamada, T., Abe, H. & Baba, T. (1975). Beet necrotic yellow vein virus and its relation to the fungus *Polymyxa betae. Proc. 1st Int. Congr. Int. Assoc. Microbiol. Soc.* **3**, 313.

Tamada, T. & Baba, T. (1973). Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. *Annals of the Phytopathological Society of Japan* **39**, 325.

Taylor, B. & Powell, A. (1982). Isolation of plant DNA and RNA. *Focus* Vol. 4, No. 3, pp. 4-6.

Teakle, D.S. (1983). Zoosporic fungi and viruses. Double trouble. In *Zoosporic Plant Pathogens*, pp. 233-248. Ed. S.T. Buckazi, Academic Press, London.

Thomas, J.E. and Bowyer, J.W. (1980). Properties of tobacco yellow dwarf and bean summer death viruses. *Phytopathology* **70**, 214-217.

Thomas, J.E., Massalsk, P.R. & Harrison, B.D. (1986). Production of monoclonal antibodies to African cassava mosaic virus and differences in their reactivities with other whitefly-transmitted geminiviruses. *Journal of General Virology* **67**, 2739-2748.

Thomas, P.E. & Mink, G.I. (1979). Beet curly top virus. Commonwealth Mycological Institute/Association of Applied Biologists Descriptions of Plant Viruses No. 192, Commonwealth Mycological Institute, Kew, England.

Thongmeearkom, P., Honda, Y., Saito, Y. & Syamananda, R. (1981). Nuclear ultrastructural changes and aggregates of virus-like particles in mungbean cells affected by mungbean yellow mosaic disease. *Phytopathology* **71**, 41-44.

Thornberry, H.H., Vatter, A.E. & Hickman, D. (1953). Virus-like particles from some plants having symptoms of virus disease. *Phytopathology* **43**, 486.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R. & Ryan, A. (1987). Wound inducible expression of a potato inhibitor II-chloramphenicol acetyl transferase gene fusion in transgenic tobacco plants. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 744-748.

Thouvenel, J.-C., Dollet, M. & Fauquet, C. (1976). Some properties of peanut clump, a newly discovered virus. *Annals of Applied Biology* **84**, 311-320.

Thouvenel, J.-C. & Fauquet, C. (1980). First report of *Polymyxa graminis* in Africa, and its occurrence on new *Sorghum* Moench host species. *Plant Disease* **64**, 957.

Thouvenel, J.-C. & Fauquet, C. (1981). Further properties of peanut clump virus and studies on its natural transmission. *Annals of Applied Biology* **97**, 99-107.

Thouvenel, J.-C., Fauquet, C. & Dollet, M. (1978). Influence du pH sur le virus du clump de l'arachide (peanut clump virus) et nouvelle méthode rapide de purification. *Annales de Phytopathologie* **10**, 93-99.

Thouvenel, J.-C., Fauquet, C. & Lamy, D. (1978). Transmission par la graine du virus du clump de l'arachide. *Oleagineux* **33**, 503.

Timko, M.P., Kausch, A.P., Castesana, C., Frassler, J., Herrera-Estrella, L., Van den Broeck, G., Van Montagu, M., Schell, J. & Cashmore, A.R. (1985). Light regulation of plant gene expression by an upstream enhancer-like element. *Nature* **318**, 579-582.

Tomenius, K., Clapham, D. & Meshi, T. (1987). Localization by immunogold cytochemistry of the virus-coded 30K protein in plasmadesmata of leaves infected with tobacco mosaic virus. *Virology* **160**, 363-371.

Tooze, J. (1980) *Molecular Biology of Tumor Viruses Part 2, DNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, N.Y.

Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science U.S.A.* **76**, 4350-4354.

Townsend, R., Stanley, J., Curson, S.J. & Short, M.N. (1985). Major polyadenylated transcripts of cassava latent virus and location of the gene encoding coat protein. *The EMBO Journal* **4**, 33-37.

Townsend, R., Watts, J. & Stanley, J. (1986). Synthesis of viral DNA forms in *Nicotiana plumbagniofolia* protoplasts inoculated with cassava latent virus (CLV); evidence for the independent replication of one component of the CLV genome. *Nucleic Acid Research* **14**, 1253-1265.

Tsuchizaki, T., Hibino, H. & Saito, Y. (1973). Comparisons of soil-borne wheat mosaic virus isolates from Japan and the United States. *Phytopathology* **63**, 634.

Tsuchizaki, T., Hibino, H. & Saito, Y. (1975). The biological functions of short and long particles of soil-borne wheat mosaic virus. *Phytopathology* **65**, 523-532.

Tucker, A.O. & Fairbrothers, D.E. (1970). Extraction of leaf proteins of *Mentha* (Labiatae) for disc-electrophoresis. *Phytochemistry* **9**, 1399-1403.

Tullis, R.H. & Rubin, H. (1980). Calcium protects DNase I from proteinase K: a new method for the removal of contaminating RNase from DNase I. *Analytical Biochemistry* **107**, 260-264.

Tumer, N.E., O'Connell, K.M., Nelson, R.S., Sanders, P.R., Beachy, R.N., Fraley, R.T. & Shah, D.M. (1987). Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants. *The EMBO Journal* **6**, 1181-1188.

Vaeck, M. Reynaents, A., Hofte, H. Jansens, S., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M. & Leemans, J. (1987). *Nature* **328**, 33.

Valverde, R.A., Dodds, J.A. & Heick, J.A. (1986). Double-stranded ribonucleic acid from plants infected with viruses having elongated particles and undivided genomes. *Phytopathology* **76**, 459-465.

Van Regenmortel, M. H. V. (1981). Tobamoviruses. In: *Handbook of Plant Virus Infections and Comparative Diagnosis* (Ed. E. Kurstak), pp. 541-564, Elsevier/North-Holland, Amsterdam.

Van Vloten-Doting, L., Bol, J.-F. & Cornelissen, B. (1985). *Plant Molecular Biology* **4**, 323-326.

Viuttenez, A., Arnold, J., Spindler, C. & de Larambergue, H. (1977). Test de transmission du virus rhizomanie de la betterave (beet necrotic yellow vein virus) par le champignon *Polymyxa betae* myxomycete associé aux racines. Application a la prospection de la maladie. *Annales de Phytopathologie* **9**, 537.

Wakarchuk, D. A. & Hamilton, R. I. (1985). Cellular double-stranded RNA in *Phaseolus vulgaris*. *Plant Molecular Biology* **5**, 55-64.

Ward, A., Etessami, P. & Stanley, J. (1988). Expression of a bacterial gene in plants mediated by infectious geminivirus DNA. *The EMBO Journal* **7**, 1583-1587.

Warmke, H. E. (1968). Fine structure of inclusions formed by the aucuba strain of tobacco mosaic virus. *Virology*, **34**, 149.

Wetter, C. & Milne, R. G. (1981). Carlaviruses. In Handbook of Plant Virus Infections and Comparative Diagnosis, pp695-730. Ed. E. Kurstak. Amsterdam: Elsevier/North Holland Biomedical Press.

White, R. F., Kassanis, B. & James, M. (1972). Potato mop-top virus in infected cells. *Journal of General Virology* **15**, 175-177.

Winner, C. (1984). Viröse Wurzelbärtigkeit (Rizomania) der Beta-Rübe als Heerausforderung für Forschung und Resistenzzüchtung. *Zuckerindustrie* **109**, 113-120.

Yamashita, S., Nonaka, N., Namba, S., Doi, Y. & Yora, K. (1985). Miscanthus streak virus, a geminivirus in *Miscanthus sacchoriflorus* Benth. *et* Hook. *Annals of the Phytopathological Society of Japan* **51**, 582-590.

Zhongxing, X., Shaohua, X. & Keqiang, M. (1982). A small ssDNA containing virus-bean distortion dwarf virus. *Acta Microbiologica Sinica* **22**, 293-299.

Ziegler, V., Richards, K., Guilley, H., Jonard, G. & Putz, C. (1985). Cell-free translation of beet necrotic yellow vein virus: readthrough of the coat protein cistron. *Journal of General Virology* **66**, 2079-2087.

Zimmern, D. (1989). Evolution of RNA viruses. In *RNA Genetics*. Eds. J. Holland, E. Domingo and P. Ahlquist. CRC Press, Boca Raton, Florida (in press).