MOLECULAR CHARACTERISATION OF POTATO YELLOW MOSAIC VIRUS.

BY

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ABSTRACT

A whitefly transmitted virus from Venezuela, potato yellow mosaic virus (PYMV), has been propagated, isolated and characterised. The virus was mechanically transmissible to tobacco and petunia, but only graft transmissible to potato and tomato plants. All plants developed characteristic symptoms. PYMV was purified by two methods and electron microscopic examination showed it to have a geminate morphology. Serological tests with an antiserum raised to another South American geminivirus, tomato golden mosaic virus (TGMV), and PYMV showed no homologous reactions between the two.

Nucleic acid analysis from PYMV infected tissue, by agarose gel electrophoresis, Southern transfer and radioactive probing, illustrated the presence of at least ten different virus specific forms. Nuclease digestion tests revealed that these species consisted of both single and double-stranded (ds) DNA forms. A covalently-closed-circular dsDNA form (infectious for tobacco plants) was purified, digested with restriction endonucleases and cloned into plasmid and bacteriophage vectors. Two distinct clone types were obtained, both *ca.* 2500 base pairs in length but with different restriction patterns. The bicomponent nature and geminiviral status of PYMV was confirmed when it was demonstrated that a combination of both clone types was required to cause systemic infection of mechanically inoculated *Nicotiana benthamiana* plants after excision from their cloning vectors. Strand-specific, radioactively labelled DNA synthesised *in vitro* from these clones was used to probe Southern transfers of nucleic acids isolated from infected plants. The results confirmed that the genome of PYMV contained two distinct components, termed A and B. Sub-clones from the infectious clones were constructed in bacteriophage M13 and sequenced by the chain-termination method. Comparative computer analysis of the incomplete sequence data indicated that PYMV is a distinct member of the geminivirus group.

Potato tissue cultures have been established and used for protoplast isolation and inoculation with TGMV. Using a number of inoculation and culture techniques, no viral DNA replication was demonstrated.

This thesis is dedicated to my parents and Judith, without their support and encouragement it would never have come to fruition.

"Forget about faith!", Chaing said, *"You didn't need faith to fly, you needed to understand flying. This is just the same."*

From *Jonathan Livingstone Seagull* by Richard Bach.

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Abbreviations of virus names

Abutilon mosaic	AbMV
Bean golden mosaic	BGMV
Bean summer death	BSDV
Beet curly top	BCTV
Cassava latent virus	CLV
Cauliflower mosaic	CaMV
Chloris striate mosaic	CSMV
Cotton leaf crumple	CLCV
Euphorbia mosaic	EMV
Honeysuckle yellow vein mosaic	HYVMV
Horseradish curly top	HSCTV
Jatropha mosaic	JMV
Maize streak	MSV
Mungbean yellow mosaic	MYMV
Oat chlorotic stripe	OCSV
Potato yellow mosaic	PYMV
Solanum apical leaf curling	SALCV
Squash leaf curl	SLCV
Tobacco leaf curl	TobLCV
Tobacco yellow dwarf	TYDV
Tomato golden mosaic	TGMV
Tomato leaf curl	TLCV
Tomato yellow mosaic	ToYMV
Tomato yellow leaf curl	TYLCV
Wheat dwarf	WDV
Watermelon curly mottle	WCMoV

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1. INTRODUCTION

1.1 Members of the geminivirus group

Geminiviral infections are responsible for a number of major crop diseases in tropical and sub-tropical regions. Notable among these are the diseases caused by cassava latent virus (CLV) and maize streak virus (MSV) throughout Africa, bean golden mosaic virus (BGMV) and tomato golden mosaic virus (TGMV) in Central and South America, and beet curly top virus (BCTV) in the Mediterranean basin from where it is thought to have spread to western parts of North America. The relationship of geminiviruses with certain insect vectors (table 1) and their economic importance was well established long before the disease agents themselves were isolated; a viral etiology for the diseases was assumed rather than proven. *Eupatorium* yellow vein virus, a geminivirus, may well have been the earliest described plant virus in a classic Japanese anthology by Empress Koken in the year 752 (Inouye and Osaki, 1981).

The name of this virus group was derived from the capsid morphology of its members, being twinned, incomplete isometric particles. Harrison *et al.* (1977) proposed the formation of this new virus group and in 1978 The International Committee on the Taxonomy of Viruses recognised the geminiviruses as a distinct plant virus group (Matthews, 1979) on the basis of their capsid morphology and genomes of circular single-stranded (ss) DNA. A comprehensive review published in 1981 (Goodman, 1981) listed ten members of the geminivirus group, since then the number of definitive and provisional members has more than doubled (table 1). Currently only eight viruses have been demonstrated as definitive members on this basis, namely, abutilon mosaic virus (AbMV) (Jeske, 1986), BCTV (Stanley *et al.*, 1986), BGMV (Reisman *et al.*, 1979), CLV (Harrison *et al.*, 1977), chloris striate mosaic virus (CSMV) (Francki *et al.*, 1979), MSV (Bock *et al.*, 1977), TGMV (Hamilton *et al.*, 1981) and wheat dwarf virus (WDV) (MacDowell *et al.*, 1985). Potential members of the group have been identified on the basis of their particle morphology, relationships with cells and tissues, and serological relatedness to definitive members. Most members, whether definitive or potential, can be subdivided on the

Table 1. MEMBERS OF THE GEMINIVIRUS GROUP

VIRUS[a]	HOST RANGE	INSECT VECTOR	COAT PROTEIN MOL. WT.	DNA MOL. WT.[b]	REF
		WHITEFLY TRANSMITTED			
Abutilon mosaic	Malvaceae	*Bemisia tabaci*	27,500	$8,9 \times 10^5$	Costa (1976), Jeske & Werz (1976) Abouzid & Jeske (1986) Jeske (1986)
Bean distortion dwarf	Leguminosae Solanaceae	*Bemisia tabaci*	-	-	Zhongxing *et al*, (1982)
Bean golden mosaic	Leguminosae	*Bemisia tabaci*	27,400	$8,6 \times 10^5$	Goodman (1977a) Goodman *et al*, (1980) Reisman *et al*, (1979)
Bean golden yellow mosaic	Leguminosae	*Bemisia tabaci*	-	-	Galvez & Castano (1976)
Cassava latent	Euphorbiaceae	*Bemisia tabaci*	34,000	$9,0 \times 10^5$	Harrison *et al*, (1977) Bock *et al*, (1977,1978)
Cotton leaf crumple	Malvaceae	*Bemisia tabaci*	-	-	Brown & Nelson (1984)
Cowpea golden mosaic	Leguminosae	*Bemisia tabaci*	-	-	Annonyako *et al*, (1982)
Eupatorium yellow vein	Compositae	-	-	-	Inouye & Osaki (1980)
Euphorbia mosaic	Euphorbiaceae	*Bemisia tabaci*	-	-	Costa & Bennett (1950) Christie *et al*, (1986)
Honeysuckle yellow vein mosaic	Caprifoliaceae	*Bemisia lonicerae*	-	-	Osaki *et al*, (1979)
Jatropha mosaic	Euphorbiaceae	*Bemisia tabaci*	-	-	Bird *et al*, (1984)
Mungbean yellow mosaic	Leguminosae	*Bemisia tabaci*	-	-	Thongmeearkom *et al*, (1981);Honda *et al*, (1983)
Solanum apical leaf curling	Solanaceae	-	-	-	Hooker & Salazar (1983)
Soybean crinkle leaf	Leguminosae	*Bemisia tabaci*	-	-	Iwaki *et al*, (1983)
Squash leaf curl	Cucurbitaceae	*Bemisia tabaci*	-	-	Cohen *et al*, (1983)
Tobacco leaf curl	Solanaceae Caricaceae Caprifoliaceae Compositae	*Bemisia tabaci*	-	-	Osaki & Inouye (1981)
Tomato golden mosaic	Solanaceae	*Bemisia tabaci*	27,900	$8,4 \times 10^5$	Buck and Coutts (1985) de Uzcategui & Lastra (1978)

Tomato leaf curl & tomato yellow leaf curl	Solanaceae	*Bemisia tabaci*	-	-	Cherif & Russo (1983) Cohen & Nitzany (1966)
Watermelon curly mottle[c]	Cucurbitaceae Chenopodiaceae Compositae Cruciferae Leguminosae	*Bemisia tabaci*	-	-	Brown & Nelson (1986)
		LEAFHOPPER TRANSMITTED			
Bean summer death	Leguminosae	*Orosius argentatus*	-	-	Thomas & Bowyer (1980)
Beet curly top[c]	Chenopodiaceae Compositae Cruciferae Leguminosae Solanaceae	*Circulifer tenellus*	29,600	9.8×10^5	Mumford (1974) Thomas & Mink (1979) Stanley *et al*, (1986)
Chloris striate mosaic	Gramineae	*Nesoclutha pallida*	28,000	-	Francki *et al*, (1979,1980)
Horseradish curly top	Cruciferae	*Circulifer tenellus*	-	-	Duffus *et al*, (1982)
Maize streak	Gramineae	*Cicadulina mbila*	28,000	8.7×10^5	Harrison *et al*, (1977) Bock *et al*, (1977)
Miscanthus streak	Gramineae	-	-	-	Yamashita (1983)
Tobacco yellow dwarf	Solanaceae	*Orosius argentatus*	27,500	-	Thomas & Bowyer (1980)
Wheat dwarf	Gramineae	*Psammotettix alienus*	29,400	9.0×10^5	Lindsten (1980) MacDowell *et al*, (1985)
		TREEHOPPER TRANSMITTED			
Tomato pseudo curly top	Solanaceae	*Micrutalis malleifera*	-	-	Christie *et al*, (1984) Simons & Coe (1958)
		APHID TRANSMITTED			
Oat chlorotic stripe	Gramineae	*Rhopalosiphon maidis*	-	-	Haber & Gill (1984)

[a] Definitely recognised group members (Goodman, 1981), are shown underlined.

[b] DNA molecular weights determined from nucleotide sequences (averaged for bicomponent viruses).

[c] Only example host families are shown, host ranges are much wider.

basis of either their insect transmission vector or whether they have a mono- or dicotyledonous host range.

1.1a Whitefly-transmitted geminiviruses.

Debrot *et al.* (1963) first reported an important disease of tomatoes in South America, tomato yellow mosaic. The disease agent shares many of the physical properties associated with geminiviruses (de Uzcategui and Lastra, 1978), it is sap transmissible and *Bemisia tabaci* is the disease vector (Lastra and de Uzcategui, 1975). A similar disease, tomato yellow mosaic (ToyMV), was first reported in Brazil (Matyis *et al.*, 1975) and the physical properties of the virus are somewhat similar to TGMV (Costa *et al.*, 1977). It is thus possible that TGMV and ToYMV may be related to one another. However, initial serological studies did not support this view (Lastra, pers. comm.). Hamilton *et al.* (1981), working with a Brazilian isolate of TGMV, provided evidence its for the classification as a geminivirus by demonstrating that its genome consisted of predominantly circular ssDNA.

Bean golden mosaic disease, first reported from Brazil (Costa, 1965) occurs in most areas of the tropical and subtropical Americas where beans are grown (Bird and Maramorosch, 1975) and in tropical Africa and Asia (Goodman and Bird, 1978). A similar disease, bean golden yellow mosaic disease was reported from Puerto Rico (Bird *et al.*, 1973). The causal agent of both diseases is transmitted by the whitefly *Bemisia tabaci* and infectious geminate virus particles have been isolated from infected beans (Galvez and Castano, 1975; Goodman , 1977b); BGMV from El Salvador and Brazil and BGYMV from Puerto Rico are assumed to be the same virus (Goodman, 1977b). The genome of BGMV has been cloned, sequenced and shown to consist of two ccc ssDNA molecules (Howarth *et al.*, 1985), which in combination are infectious for plants (Morinaga *et al.*, 1983).

Storey and Nichols (1938) first described a disease of cassava, African cassava mosaic disease (A-CMD), which was vectored by *B. tabaci* and was also sap transmissible (Bock and Guthrie, 1978). CLV which has been isolated from A-CMD-infected cassava by sap transmission onto *Nicotiana clevelandii* (Bock *et al.*, 1978) is

characterised by possessing geminate particles (Bock *et al.*, 1977). CLV is now recognised as the causal agent of A-CMD as purified preparations cause characteristic symptoms on a Brazilian cultivar of cassava (Bock and Woods, 1983). CLV has been reported from East Africa (inland and costal districts of Kenya)(Bock *et al.*, 1978; 1981), West Africa (Walter, 1980), Nigeria (Adejare and Coutts, 1982), Angola (Sequeira and Harrison, 1982) and India (Bock and Harrison, 1985). The genome of CLV has also been shown to consist of ssDNA (Harrison *et al.*, 1977).

Abutilon mosaic virus, responsible for the green and yellow variegations of *Abutilon* species, has been purified by Jeske and Werz (1980b) and its similarities to other geminiviruses has been demonstrated (Jeske and Schuchalter-Eicke, 1984). Two apparent forms of the virus were isolated; particles 16-18nm in diameter, occurring in pairs and aggregates, and "chains of pearls" composed of subunits 8-10nm in diameter. The nucleic acid purified from the latter gave a positive diphenylamine test. It has now been shown by immunological techniques that virus coat protein accumulates in the nuclei of the vascular bundle area and to a lesser extent in nuclei of the mesophyll (Jeske, 1986).

Mungbean yellow mosaic virus which causes an important disease of mungbean and soybean in Southern Asia, India (Nariani, 1960; Nene, 1972) and Thailand (Thongmeearkom *et al.*, 1981) was originally classified as a geminivirus on the basis of ultrastructural changes induced in infected tissue. Infectious geminate particles have since been purified and the virus is transmitted by *B. tabaci* (Honda *et al.*, 1983).

Yellow leaf curl, a major disease of tomato in the South East Mediterranean basin and neighbouring countries has been reported from the Sudan (Yassin and Nour, 1965), India (Varma *et al.*, 1975), Nigeria (Lana and Wilson, 1976), Israel (Cohen and Nitzany, 1966; Russo *et al.*, 1980) and Tunisia (Cherif and Russo, 1983). The ultrastructural changes accompanying yellow leaf curl infections suggest that TYLCV is also a geminivirus (Cherif and Russo, 1983).

Tobacco leaf curl occurs widely in tropical and sub-tropical countries but has also been reported from temperate regions including

Japan, Europe and the USA. The virus responsible is transmitted by *B. tabaci* and geminate particles have been visualised by electron microscopy of purified preparations (Osaki and Inouye, 1978; 1981). Similar virus particles have been isolated from tomatoes with yellow dwarf disease (Osaki and Inouye, 1978) and it is believed the same virus causes both diseases (Osaki *et al.*, 1979).

Similarly the causal agent of yellow vein mosaic of honeysuckle is a virus serologically identical to or closely related to TLCV (Osaki *et al.*, 1979). Although this virus can be transmitted by *B. tabaci* it has also been suggested that *B. lonicerea*, a common Japanese whitefly species found on honeysuckle, may be the natural vector (Osaki *et al.*, 1979).

A mosaic disease of *Euphorbia prunifolia* has been reported from Brazil (Costa and Bennett, 1950), Puerto Rico (Bird *et al.*, 1975; Bird and Maramorosch, 1978) and of *E. heterophylla* from South Florida (Kim and Fulton, 1984). The virus responsible is transmitted by *B. tabaci* and concentrated preparations of virus from *E. prunifolia* containing isometric particles, 30nm in diameter, have been isolated (Costa, 1976). Geminate particles have been observed in infected *Datura stramonium* leaf dip preparations (Kim and Fulton, 1984). A recent report has shown EMV possesses a ssDNA genome (Jaramillo and Lastra, 1986).

A whitefly-borne disease of squash, squash leaf curl, was reported in America by Flock and Mayhew (1981). The disease-causing agent has been purified and infectivity associated with single, dimer, trimer and tetramer isometric particles (Cohen *et al.*, 1983).

Another suspected geminivirus SALCV has been found in cultivated and wild solanaceous plants near Peru (Hooker and Salazar, 1983). The virus is transmissible by grafting but not by aphids, whiteflies or by mechanical means. Triplet particles, 17 x 52nm, have been purified along with monomers and dimers, which are thought to be breakdown products of the triplets. This predominance of trimers may be necessary to encapsidate the larger genome of SALCV first described by Harrison *et al.* (1985) which by measurements of molecules in electron micrographs, appeared to be *ca.* 3200 nucleotides long. Harrison *et al.* (1985) also showed there was no detectable serological relationship

between SALCV and several other geminiviruses. Thus SALCV has properties typical of geminiviruses but seems not to be closely related to other well characterised members of the group.

1.1b Leafhopper-transmitted geminiviruses.

Beet curly top disease causes widespread loss of sugar beet crops in western North America and the Mediterranean. A virus was first purified from beet curly top-infected shepherds purse (*Capsella bursa-pastoris*) by Duffus and Gold (1973). Infectious purified material contained small spherical particles 19-20nm in diameter, in clusters. Similar isometric particles, 18-22nm in diameter, occurring mostly in pairs were isolated from beet curly top infected *Nicotiana tabacum* (Mumford, 1974), and trimers have been observed in preparations from shepherds purse (Larsen and Duffus, 1984). BCTV was shown to contain DNA by the diphenylamine test (Magyarosy *et al.*, 1977). The genome of BCTV has been cloned and sequenced (Stanley *et al.*, 1986) and shown to consist of one covalently-closed-circular (ccc) ssDNA molecule.

A curly top virus-like entity transmitted by *Circulifer tenellus* has also been isolated from horseradish plants affected by brittle root disease in Illinois. Geminate particles have been isolated from these plants and the agent was neutralised by antiserum to BCTV. In plant infectivity bioassays, using non-viruliferous beet leafhoppers, neutralised material failed to cause infection. However, the relationship between this curly-top entity and BCTV remains unclear as the host ranges for the viruses differ (Duffus *et al.*, 1982).

Maize streak disease causes severe losses in irrigated maize crops in much of Africa, and the disease has also been reported in India (Rose, 1978). Isometric particles have been seen in thin sections of streak-infected maize (Sylvester *et al.*, 1973) and purified particles, consisting of a mixture of single particles 20nm in diameter, and paired particles 30 x 20nm, were shown to be infectious for plants (Bock *et al.*,1974). Similar particles, which are serologically related to MSV, have been isolated from streak-infected sugar cane (South Uganda) and guinea grass (Central Kenya) (Bock *et al.*, 1974). The viral nucleic acid was originally identified as RNA when the

precipitin bands formed between the virus and its antiserum in agar gels failed to stain with acridine orange after treatment with RNase (Bock *et al.*, 1974). Extracted virion nucleic acid was later shown to be resistant to RNase and sensitive to DNase and electron microscopy illustrated the nucleic acid to be predominantly circular ssDNA (Harrison *et al.*, 1977).

A stunting disease of wheat which is transmitted by the leafhopper *Psammotettix alienus* was first reported in Czechoslavakia (Vacke, 1961) and later in Sweden (Lindsten *et al.*, 1970). Purification of WDV and probable identification as a geminivirus was first achieved by Lindsten *et al.* (1980). Here it was shown that the infectious purified material contained geminate particles and the viral nucleic acid was sensitive to DNase and gave a positive diphenylamine test. MacDowell *et al.* (1985) have cloned and sequenced the DNA of the Swedish isolate of WDV.

A striate disease of grasses in Australia was first described by Grylls (1963) and was later referred to as chloris striate mosaic disease (Grylls and Waterford, 1976). Infectious particles mostly geminate in form of dimensions 18 x 30nm, with some single particles, 18nm in diameter, have been isolated from infected *Chloris gayana* (Francki *et al.*, 1979). The virus is transmitted by the leafhopper *Nesoclutha pallida* and later Francki *et al.* (1980) demonstrated CSMV possessed a ssDNA genome.

Tobacco yellow dwarf and bean summer death diseases which are transmitted by the leafhopper *Orosius argentatus*, have been recognised in Australia for many years (Hill, 1941; Bowyer and Atherton, 1971). Infectious virus-like particles occurring in pairs (20 x 35nm in size) have been isolated from plants with tobacco yellow dwarf and bean summer death diseases and BSDV is considered to be a strain of TYDV (Thomas and Bowyer, 1980). TYDV DNA has been partially characterised, verifying the classification of TYDV as a geminivirus (Marriott and Symons, 1963).

Most of the leafhopper-transmitted geminiviruses have not been successfully sap transmitted despite the fact that MSV and CSMV appear to infect all the cells in the leaf except the epidermis (Goodman, 1981). The infectivity of these viruses has therefore been

demonstrated using non-viruliferous leafhoppers in plant infectivity bioassays. Groups of caged insects were allowed to feed on purified virus preparations and were then transferred to healthy plants. The appearance of typical symptoms in these plants indicated infectious virus was present in the preparation fed to the leafhoppers. BCTV has been successfully transmitted mechanically by the use of a high pressure injector to ensure that the virus reached the required internal tissue responsible for, and capable of, initiating virus replication (Mumford, 1972).

The majority of well characterised whitefly-transmitted geminiviruses are readily sap transmissible even though the viruses are mainly confined to phloem tissue (Goodman, 1981). This demonstration utilised investigations where infectious purified virus was successfully transmitted by mechanical means rather than by whitefly feeding experiments. Geminiviruses seem to be present at low concentrations in diseased tissues (Costa, 1976). Therefore any failures to achieve infection may be a feature of the virus not being deposited within the required tissues at an adequate concentration to initiate infection.

1.2 Host range and vector transmission

Information on the host range of many geminiviruses is incomplete. However, amongst the leafhopper-transmitted viruses, CSMV was found to infect eight cultivars of wheat, three of oats, two of barley and three species of grasses; *Chloris gayana*, *Ixopherus unisetus* and *Dactylis glomerata*. Seven other grass species were not susceptible to the virus. *Zea mays* L. developed symptoms of CSMV but *Sorghum almum* and *S. vulgare* did not (Grylls, 1963). The infectivity of CSMV in dicotyledonous plants has not been investigated.

MSV was found to have a large host range within monocotyledonous species, 54 grass species being found to be symptomatic hosts (Damsteegt, 1983). Out of 529 maize hybrids, inbreds, exotic lines and sweet corn cultivars, only four were non-susceptible. Several *Sorghum*, *Tripsacum* and *Zea* species were susceptible and the major cereal crops barley, rye wheat and rice were also susceptible (Damsteegt, 1983). Serologically related strains of MSV adapted to indigenous grass species exist, which differ in host range and pathogenicity (Bock *et al.*, 1974). The strain that causes maize streak inflicts only a transient infection on sugar cane, whereas the sugar cane strain can give a mild, persistent infection in maize. A virus causing a striate mosaic disease of *Digitaria sanguinalis*, serologically related to MSV, has been described in Vanuatu (formerly New Hebrides) and may comprise another strain of the virus (Dollet *et al.*, 1986).

The susceptibility of various hosts to infection by CSMV and MSV was investigated using leafhopper feeding experiments under controlled conditions, however the incubation period of different corn hosts to MSV infections was found to vary (Damsteegt, 1983). One experimental corn hybrid was not palatable to *C. mbila* therefore most of the plants escaped infection. Grylls (1963) found that there was a difference in the ability of leafhoppers to acquire virus from different host plant species, thus *C. gayana* was a better source than *I. unisetus*. Damsteegt (1983) also proposed that the low MSV infection levels could also indicate heterogeneity in the seed samples rather than true resistance. Despite these possible sources of error, MSV and CSMV appear to have wide host ranges amongst monocots.

Similarly BCTV has a wide host range amongst the dicots extending from members of the *Urticaceae* to the *Compositae*, including 44 plant families and more than 300 species. Although the vector, *C. tenellus*, feeds readily and can breed on some on grasses, no monocots have been found susceptible to BCTV (Bennett, 1971). The related virus TYDV infects 32 species in 7 families amongst the dicots (Bock, 1982), suggesting therefore that two sub-groups exist within the leafhopper-transmitted geminiviruses, those which infect monocots and those which infect dicots.

The whitefly-transmitted geminiviruses appear to have a more limited host range. BGMV was found to infect only certain species in the legume genera, *Phaseolus, Macroptilium, Vigna* and *Caloplgonium*, but not 21 species in 8 other families (Goodman, 1981a).

ToYMV has been successfully transmitted mechanically to several species of the Solanaceae family including, *Datura stramonium* and *Nicotiana tabacum*. However several species of the following families were immune to mechanical inoculation and virus transmission by *B. tabaci* : *Solanaceae, Leguminosae, Malvaceae, Chenopodiaceae, Cruciferae, Euphorbiaceae* and *Cucurbitaceae* (de Uzcategui and Lastra, 1978). Interestingly the primary host of TGMV (tomato) is susceptible to mechanical inoculation only with extreme difficulty; 1.6% of plants infected as compared with a 100% infection of *N. glutinosa* plants (Bird and Maramorosch, 1978).

Bock *et al.* (1981) compared four CLV strains, CLV-M (Mt. Kenya), CLV-N (Nigeria), CLV-T (Uganda) and CLV-C (coastal strain), and found them to have distinct and narrow host ranges. Investigations were carried out using *B. tabaci* feeding experiments and two strains of CLV were distinguished using *Nicotiana* species. All strains infected *N. benthamiana*, indeed this proved to be the only susceptible host for CLV-C. The diagnostic host for the type strain CLV-T was *N. debneyi*, it being the only strain capable of infecting this species. ClV-C was easily distinguished because it was the only strain which did not infect *N. clevelandii*.

Similarly MYMV exhibited a narrow host range in mechanical inoculation tests. Among 26 plant species belonging to 6 families, only 7 species in the *Leguminosae* family were susceptible to infection

(Honda *et al.*, 1983). In transmission studies with TLCV using its whitefly vector only, a narrow host range comprising species from *Solanaceae*, *Compositae* and *Caprifoliaceae* was demonstrated (Osaki and Inouye, 1981).

It appears, despite the lack of extensive studies, that the host ranges of the whitefly-transmitted geminiviruses is fairly limited. Sap inoculation tests have shown inhibitory factors in the plant sap may be interfering with infection, for example, *D. stramonium* is considered a poor virus source of TGMV for exactly this reason (Bird and Maramorosch, 1978).

The results available imply that the monocot-infecting geminiviruses have only leafhopper vectors whereas the dicot-infecting geminiviruses can have either a leafhopper or a whitefly vector.

Geminiviruses are transmitted by their leafhopper or whitefly insect vectors in a persistent or circulative manner and no geminivirus is known to be transmitted through seed to progeny plants. It is therefore believed that the field spread of virus from plant to plant is entirely reliant on insect vector transmission (table 1).

In all instances the whitefly vectors are species of *Bemisia* and in all but one instance, *B. tabaci*, which has wide host range including at least 300 species in 63 plant families. There are indications that particular insect populations may differ (Mound, 1983), for example in the Sudan Gezira cotton was heavily infested but nearby cassava was only lightly infested, whereas in southern Nigeria the situation was reversed. Whether these observations reflect non-genetically determined host preferences or relate to genetically determined biotypes with limited host ranges is not clear. It is however common experience that insect populations raised on one species may be hard to establish on another. Whatever the underlying reason for such observations, such findings indicate that in many instances a virus is more likely to be spread from one plant to another of the same species than to a plant of a different species. Such restraints seem not to apply equally to the transmission of leafhopper-borne geminiviruses.

Although the vector relations of different geminiviruses are not identical, there is a general similarity. There viruses studied in detail are circulative, requiring a latent period in the vector of four

hours or more, and they persist in the vector for days and in some instances for life. Storey (1928, 1938) found that MSV can be acquired or inoculated by *C. mbila* in acquisition periods of 5 minutes, possesses a minimum latent period in the vector of 6 to 12 hours at 30°C, and can be retained for life. Transmission does not begin before the virus has reached the haemolymph and virus can be retained through ecdysis. Similar results have been obtained for the transmission of BCTV by *C. tenellus* (Bennett and Wallace, 1938) and for that of tomato pseudo-curly top virus by *Micrutalis malleifera* (Simons, 1962). Neither MSV nor BCTV have been shown to replicate in their respective vectors and neither is transmitted *per os* (Severin, 1921; Storey, 1928). In addition, the frequency of transmission by, and the persistence of virus in individual insects depends on the length of the acquisition period and on the virus concentration in the source plant (Bennett, 1971).

The vector relationships of whitefly-transmitted geminiviruses (Bird and Maramorosch, 1978) are different from those of BCTV and MSV, the virus not being acquired or inoculated as rapidly, possibly reflecting a difference in the feeding behaviour of the vectors. In one of the better studied examples, the transmission of EMV by *B. tabaci* requires a minimum acquisition and inoculation times of 30min and 10min respectively, and a latent period of 4h (Costa and Bennett, 1950). The virus is retained for up to 20 days and passes through the molt from larva to adult but not through the egg to progeny insects. As with leafhopper-transmitted geminiviruses there is no evidence for geminivirus replication within the whitefly vectors.

Several parallels have been noted with the insect transmission of geminiviruses and that of luteoviruses by aphids (Harrison, 1985). In luteovirus transmission virus particles pass from the gut into the haemocoel and from there into the cells of the accessory salivary glands and then into the saliva. Recent evidence suggests that the hind gut is the site where luteoviruses pass from the gut lumen into the haemocoel (Gildow, 1984), and there is evidence to implicate that luteovirus capsid protein determines vector specificity by controlling the passage of virus from the haemocoel into the accessory salivary glands (Rochow, 1970; Gildow and Rochow, 1980). The same may be true

for geminiviruses, because several viruses transmitted by *B. tabaci* have antigenically closely related particles, whereas the antigenic relationship between the leafhopper-transmitted geminiviruses, each of which has a specific and different vector, is distant or undetectable (Roberts *et al.*, 1984). The capsid proteins of CLV and TGMV have been compared in detail at the amino acid and sequence level. From this data hydrophilicity plots have been drawn which show both the main hydrophilic regions in the coat protein of TGMV are also present in that of CLV, but that CLV also has three additional unshared hydrophilic regions (Hamilton *et al.*, 1984). Similarly, serological evidence suggests that CLV has all the epitopes found on TGMV (Stein *et al.*, 1983), along with one or more which are not shared (Sequeira and Harrison, 1982). It may be that the shared hydrophilic regions have a role to play in the transmission of the viruses by *B. tabaci*.

1.3 Geminiviral symptomology and cytopathology

The leafhopper-transmitted geminiviruses CSMV, MSV and WDV cause characteristic symptoms in their respective monocotyledonous hosts. CSMV infections result in a characteristic leaf mosaic pattern consisting of fine parallel chlorotic streaks (Slykhuis, 1963). The symptoms of MSV infections vary depending on the host species (as discussed in section 1.2), from a diffuse leaf mosaic to severe plant stunting, leaf curling and streaking (Damsteegt, 1983). WDV infection results in plant dwarfing, increased tillering and suppressed heading, indeed infected wheat heads never leave their sheaths (Tomenius and Oxelfelt, 1980).

The leafhopper-transmitted geminiviruses BCTV and TYDV (BSDV) and the whitefly-transmitted geminiviruses which infect dicotyledonous plants have names indicative of the three most consistent symptoms observed on infected plants (Bock, 1982) namely leaf curling, yellowing and stunting (hence the names shown in table 1). However, under certain conditions, and on particular hosts symptoms may vary, for example, BCTV on tomatoes causes general yellowing, and a strain of TLCV in honeysuckle causes golden mosaic symptoms (Osaki *et al.*, 1979; Goodman, 1981a).

In all cases studied, geminivirus particles accumulate in the nuclei of infected cells (Francki *et al.*, 1985). However, there are some instances where particles have also been observed in the cytoplasm. It has been suggested that these particles were extruded from the nuclei through breaks in the nuclear envelope (Lastra and Gil, 1981), but whether such breaks are a direct result of virus infection is unknown.

The particles of most geminiviruses are confined to phloem cells, but some also appear to invade other tissues. Extensive electron microscopic and immunochemical studies on several species of BCTV-infected plants indicate that the virus is confined to the phloem and the virus-induced hyperplastic phloem cells (Esau and Hoefert, 1973; Esau, 1977; Esau and Magyarosy, 1979, 1980; Mumford and Thornley, 1977). Immunochemical studies on CLV-infected plants detected numerous nuclei containing virus, but some cells of other tissues were also infected (Sequeira and Harrison, 1982). In contrast, CSMV particles

were detected by electron microscopy in all cell types of infected *Chloris gayana* leaves except the epidermal and possibly the xylem tracheid cells (Francki *et al.*, 1979). MSV probably also invades other tissues besides the phloem, as it is acquired by the vector, like CSMV, after brief probes in the mesophyll (Storey, 1938; Grylls, 1963).

Kim *et al.* (1978) studied, by electron microscopy, the sequence of structural changes in the nuclei of French bean leaves inoculated with BGMV. The nucleoli increased in size and then segregated into granular and fibrillar regions. The fibrillar material then condensed into rings of various sizes, often several to a nucleus, and finally virus particles appeared in the nucleoplasm. Cytochemical investigations indicated that, in segregated nucleoli, the granular material was composed of mainly ribonucleoprotein, whereas the fibrillar material was mainly deoxyribonucleoprotein (Kim *et al.*, 1978). Light microscopy has also been used to study geminivirus-induced nuclear inclusions (Christie *et al.*, 1986). Here fibrillar rings were readily detected as large blue-violet inclusions in infected phloem cells by staining tissues with the stain azure-A.

Fibrillar bodies are the most common intranuclear structures induced by geminivirus infections. Since their appearance precedes the appearance of virus particles (Kim and Fulton, 1984; Kim *et al.*, 1978), and they contain DNA (Kim *et al.*, 1978), and the fact that they are associated with virus particles once they appear, it has been suggested that these structures are the sites of viral DNA synthesis and virus assembly (Kim *et al.*, 1978; Lastra and Gil, 1981). Ultrastructural studies of *Jatropha gossypifolia* infected with jatropha mosaic virus (Kim *et al.*, 1986) have demonstrated that many fibrillar bodies were ring-shaped and consisted of orderly aligned spherical "beads" of high electron density embedded in the less electron dense fibrillar matrix. Presently, the nature and role of these structures are not known.

Certain general conclusions can be made concerning the cytopathology of geminivirus infections. The whitefly-transmitted virus infections of dicots cause characteristic changes in cell nuclei, namely hypertrophy of the nucleus and segregation into granular and fibrillar regions. Such features are absent from leafhopper-transmitted virus infections, including those of BCTV which

apparently only infects the phloem cells of dicots. The presence or absence of such features may thus distinguish between the two groups of geminiviruses. Ring-like structures have also been observed in BCTV infected spinach and sugarbeet plants, but these structures were thought to be the remains of nucleoli (Esau and Hoefert, 1973).

1.4 Relationships among the geminiviruses

The causal agents of geminivirus diseases were originally studied with respect to symptomatology and vector specificity. For example, AbMV and EMV were classified as distinct viruses on the basis of host range and symptomology (Costa and Bennett, 1950; Costa and Carvelho, 1960). Their serological relationship to one another, if any, is unknown. Similarities in disease symptoms however, can be misleading. Chloris striate mosaic disease was originally referred to as Australian wheat striate mosaic disease because of its similarity to North American wheat striate disease, which is in fact caused by a Rhabdovirus (Slykhuis, 1963; Sinha and Behki, 1972). In addition, as *B. tabaci* is the vector for over 25 different plant diseases (Costa, 1976) such a classification could cause further confusion.

Geminivirus relationships have been successfully examined using gel immunodiffusion tests and immunosorbent electron microscopy (ISEM) to detect serological relationships. Spot-hybridisation experiments (Roberts *et al.*, 1984) have also been used to detect homology between geminiviral DNA preparations.

Gel immunodiffusion tests are the method most often employed for serological comparison, but this method is not the most sensitive one and requires virus to be at a concentration of a least 50µg/ml in the extracts. ISEM is a more sensitive technique and is suitable for examining virus present in infected tissue at much lower concentrations. ISEM is claimed to be as sensitive as enzyme-linked immunosorbent assay (ELISA) which can detect purified virus at a concentration of 5ng/ml or less (Roberts and Harrison, 1979; Sequeira and Harrison, 1982). ELISA has been used to investigate virus relationships (Cohen *et al.*, 1983) but is more successfully employed in screening plants for infection with particular viruses (Sequeira and Harrison, 1982). However, the high background results obtained using

this technique can often be misleading when examining viral relationships (S.E. Adams, pers. comm.).

For some geminiviruses, insufficient virus is isolatable to successfully raise an antiserum, or for the virus to be tested in heterologous antiserum reactions (eg. BSDV, Thomas and Bowyer, 1980). Yields of virus can however be improved by altering the virus extraction procedure or by changing the host plant. For instance, CLV antigen in infected cassava tissue was found to be at a concentration of 1-7% of that purified from comparable extracts produced from *N. benthamiana* plants (Sequeira and Harrison, 1982).

The use of radioactively-labelled probes to detect nucleotide sequence homology in spot-hybridisation assays is a more sensitive assay method still. Southern blotting can detect sequences that occur at the single copy level (Maniatis *et al.*, 1982). However, great care must be taken when interpreting any results from spot-hybridisation tests. Homologies between viral genomes are best studied in detail when the complete sequence of the viral genome is known. This aspect will be dealt with later in the thesis.

The known serological relationships between geminiviruses are illustrated in table 2. The ISEM results were based on the number of virus particles trapped on antiserum coated grids, divided by the number found on uncoated grids, and as some antisera were found to be especially reactive to ISEM, only large increases in the numbers of particles trapped were counted as positive (Roberts *et al.*, 1984). The strain of CLV referred to in table 2 is the East African strain. Several other strains of CLV have been characterised and in immunodiffusion serological tests spur reactions developed when CLV-C (coastal strain) or CLV-M (Mt. Kenya strain) were tested against CLV-T (East African strain) or CLV-N (Nigerian strain), indicating that the viruses were closely related but not serologically identical (Bock *et al.*, 1981). Sequeira and Harrison (1982) have shown that the East African, Nigerian and Angolan strains do not produce spurs in gel diffusion tests, indicating that they may be serologically identical.

The results shown in table 2 indicate that strong serological relationships exist between the whitefly-transmitted viruses tested. The leafhopper-transmitted geminiviruses, CSMV, MSV and WDV seem

Table 2. SEROLOGICAL RELATIONSHIPS AMONG GEMINIVIRUSES

VIRUS	ANTISERUM: Whitefly-transmitted					Leafhopper-transmitted						
	BGMV	CLV	HYMV	SLCV	TLCV	BCTV	BSDV	CSMV	TYDV	MSV	WDV	REFS.
Whitefly-transmitted												
BGMV	1/256,•	1/32,*•		•		×		×	×	×	×	8,9
CLV	1/256,*•	1/512,•		•	−	1/1,×		−,×	−,×	−,×	×	2,8,9
EMV	•	•		•		×		×	×	×	×	8
SLCV	1	1		1/128		⊗		⊗				3,4
TLCV	−	−	+		1/32					−		7
TGMV	•	•		•			×	×	×	×	×	8
Leafhopper-transmitted												
BCTV	×	×		×		1/256,•		×	−,∘	×	×	8,10
CSMV		−						1/128		−	−	5
TYDV		−				1/16		−	1/128	−		10
MSV	×	−,×		×		×		−,×	×	1/256,•	−,×	1,2,8
WDV	×	×		×		×		−,×	×	−,×	1/128,•	6,8
BSDV									1/8			8

Notes to table 2.

Heterologous and homologous antibody titres determined by gel diffusion tests are given.

(+), positive gel diffusion test but titre not determined

(-), negative result by gel diffusion

(*), spur reaction given by these two viruses

(•), strongly positive relationship as determined by ISEM

(◦), weak relationship as determined by ISEM

(×), no relationship as determined by ISEM

(⊙), positive reaction determined by ELISA

(⊗), negative reaction determined by ELISA

( ), blank space indicates not determined

References

1. Bock *et al.* (1974)
2. Bock *et al.* (1978)
3. Cohen *et al.* (1983)
4. Dodds *et al.* (1984)
5. Francki *et al.* (1979)
6. Lindsten *et al.* (1980)
7. Osaki and Inouye (1981)
8. Roberts *et al.* (1984)
9. Sequeira and Harrison (1982)
10. Thomas and Bowyer (1980)

unrelated to any other viruses. However, ISEM showed BCTV to be distantly related to TYDV.

Spot hybridisation experiments using radioactively-labelled probes prepared from cloned CLV DNA components 1 and 2 showed that BGMV, TGMV, TLCV and TYLCV all gave a positive but weak reaction with the DNA-1 probe and no reaction with the DNA-2 probe, whilst BCTV, MSV and WDV gave no reaction with either probe (Roberts *et al.*, 1984). These results indicate that not only are the whitefly- and leafhopper-transmitted geminiviruses serologically unrelated but they lack any DNA sequence homology, as determined by this method. As DNA-1 contains the CLV coat protein gene (Townsend *et al.*, 1985), sequence homology within this gene would explain any serological relationship.

1.5 Virion structure

Geminate virus particles consist of a single coat protein species encapsidating a genome of ssDNA. The virion nucleic acid has been shown to be ssDNA by various methods: S1-nuclease and DNase I digestion, hyperchromicity in the presence of formaldehyde and thermal denturation profiles (Harrison *et al.*, 1977; Goodman, 1977a; Francki *et al.*, 1980; Hamilton *et al.*, 1981). Molecular weight estimates for the coat proteins of definitive and probable members by SDS-polyacrylamide gel electrophoresis give similar values (table 1.). For viruses of known nucleotide sequence the experimentally determined values compare favourably with those deduced from the theoretical coding capacity of the coat protein open reading frames (ORFs).

Intact geminate particles measure approximately 20 x 30nm with some slight variation between different geminiviruses and the stains used to visualise them. Using high resolution electron microscopic analysis of CSMV, Hatta and Francki (1979) concluded that geminate particles are composed of two incomplete icosahedra with a T=1 surface lattice containing a total of 22 capsomeres, each consisting of 5 polypeptides. The icosahedra are considered to be joined at the discontinuities created by the missing capsomeres to give the characteristic flattened appearance at the interface. For CSMV, based on this model and size estimates of 2.8×10^4 and 7.1×10^5 for the molecular weights of the coat protein and ssDNA, respectively, Francki *et al.* (1980) predicted that each geminate particle would contain either 19 or 32% DNA depending on whether one or two DNA molecules were encapsidated within it. Bouyant density centrifugation demonstrated the genome of CSMV to be 19% DNA (Francki *et al.*, 1980), strongly suggesting that only a single DNA molecule is encapsidated within each geminate particle. A similar conclusion was reached by Goodman *et al.* (1980) who used bouyant density measurements and chemical analysis to determine that BGMV has a DNA content of 20%.

It is generally considered that the majority of single particles are derived from dissociated geminate particles caused by damage to the virions during isolation and/or preparation for electron microscopy. Intact viral ssDNA has been shown to be encapsidated in geminate particles of CLV and MSV, while the single particles contain

predominantly degraded linear ssDNA of non-uniform length (Sequeira, 1982; Howell, 1985). However, purified preparations of CLV single particles are enriched in a minor population of circular ssDNA molecules of a defined length approximately half that of the genomic DNAs (Sequeira, 1982; Stanley. 1983). Such genomic species are known to be present in several different geminivirus preparations (Hamilton *et al.*, 1982, 1983; Stanley and Townsend, 1985)(see section 1.7). These circular molecules may be encapsidated in complete icosahedra. Structures consisting of three or more single particles have been observed in preparations of BGMV (Goodman *et al.*, 1977), trimeric and tetrameric structures have also been found associated with SLCV (Cohen *et al.*, 1983), and trimeric structures in BCTV (Magyarosy, 1980; Larsen and Duffus, 1984). These structures clearly exhibit the characteristic flattening at each interface showing them to be intact units and not aggregates of single and/or geminate particles.

Because there appears to be a stringent size selection process for the encapsidation of the small circular ssDNAs of CLV within single particles and genomic ssDNAs within geminate particles, Stanley and Townsend (1985) argued that encapsidated dimers of genomic ssDNA could be accommodated in, and serve to stabilise tetrameric particles. SALCV particles are predominantly trimeric in appearance composed of three in-line quasi-isometric units each comparable in size to those of the geminiviruses (Hooker and Salazar, 1983). Using a similar argument, it might be expected that these structures have the potential to encapsidate ssDNA molecules half as large again as the geminivirus genomic ssDNAs. Consistent with this idea was the report by Harrison *et al.* (1985) that the estimated size of the SALCV genome, by measurement of contour lengths on electron micrographs, was 3200 nucleotides, significantly larger than the values ranging between 2265 to 2510 nucleotides obtained for other geminiviruses using a similar technique (Harrison *et al.*, 1977; Reisman *et al.*, 1979; Francki *et al.*, 1980; Hamilton *et al.*, 1981).

1.6 Genome structure

To date the genomes of six geminiviruses have been sequenced in their entirety: CLV (West Kenyan isolate 844, Stanley and Gay, 1983; infectious clones, Stanley and Davies, 1985); TGMV (Hamilton *et al.*, 1984); MSV (Kenyan isolate, Howell, 1984, 1985; Nigerian isolate, Mullineaux *et al.*, 1984); BGMV (Howarth *et al.*, 1985); WDV (MacDowell *et al.*, 1985) and BCTV (Stanley *et al.*, 1986).

Although size estimates of the circular ssDNAs, ranging from 7.1 to 8.0 x 10^5 Daltons corresponding to 2265-2510 nucleotides, have proven slight underestimates, the relatively small size of the genome in comparison with other eukaryotic viruses led Goodman (1977) to suggest that geminivirus genomes might be multipartite. While there are many examples of multipartite RNA viruses, Haber *et al.* (1981) described the first evidence for any DNA virus having a divided genome. Size estimates of BGMV DNA restriction enzyme digests resulted in a cumulative fragment size of twice that expected if a single DNA species was present. Similarly restriction endonuclease digestion of ds-circular TGMV DNA (extracted from agarose gels loaded with DNA preparations from infected *N. benthamiana* plants) gave rise to fragments whose combined size was twice that expected for unit length viral DNA (Hamilton *et al.*, 1982). Restriction enzyme digestion of cloned ds TGMV DNA by 12 enzymes enabled the molecular weight of each component to be calculated. The molecular weight of component A, calculated as the average sum of the fragments for each enzyme was found to be 1.70 x 10^6 (*ca.* 2660bp), while component B had a molecular weight of 1.65 x 10^6 (*ca.* 2580) (Bisaro *et al.*, 1982).

Sequencing of the Kenyan type strain of CLV DNA, revealed two DNA components (Stanley and Gay, 1983). The larger CLV DNA 1 component was found to have 2779 nucleotides and the second, smaller, component, DNA 2, to have 2724 nucleotides. The viral genome was restricted with the enzymes *Hae*III and *Hha*I and the digestion products were sequenced using the method of Maxam and Gilbert (1977). DNA polymerase-directed second strand synthesis was then performed on the viral DNA template using a primer complementary to a known CLV sequence, GGAATTCC. This method generated ds forms of both DNA 1 and 2, despite the lack of an exact complementary sequence to the primer in DNA 2. It was

assumed that the sequence GGAATTCA in CLV DNA 2 was sufficiently homologous to hybridise to the primer (Stanley and Gay, 1983). The dsDNA was restricted with various enzymes and cloned into several different M13 vectors (Messing *et al.*, 1981). Dideoxynucleotide sequencing (Sanger *et al.*, 1977) was used to determine the majority of the CLV DNA sequence (Stanley and Gay, 1983). Although bipartite geminiviral DNAs are apparently separately encapsidated, they are of sufficiently similar length and G + C content that separation when within virus particles or as isolated DNAs has proven difficult and has only been achieved by extended gel electrophoresis of the DNA (Haber *et al.*, 1983).

The divided genome hypothesis has now been proven following infectivity studies using full-length copies of both components of the CLV genome (Stanley, 1983), the TGMV genome (Hamilton *et al.*, 1983) and the BGMV genome (Morinaga *et al.*, 1983). From the knowledge of the nucleotide sequence, full-length copies of both components of the CLV genome were cloned into M13 vector DNA (Stanley , 1983). However, not all types of potential full-length clones were stably maintained in M13 vectors. CLV DNA 1 failed to be cloned at its unique *Bcl*I site and *Cla*I site in pKC7 (Rao and Rogers, 1979) and pAT153 (Twigg and Sherratt, 1980), possibly because the intact viral DNA was expressed within the vector and the products were detremental to the host (Stanley, 1983). A hypothesis considered more probable than at the time since the report of Petty *et al.* (1986) that the TGMV coat protein gene promoter sequences can function in *E. coli*. In order to clone CLV DNA 1 at its unique *Mlu*I site, M13mp8 (Messing and Vieira, 1982) was adapted by Stanley (1983) to include an *Mlu*I site. When clones pJS092 (DNA 1 cloned at its *Mlu*I site) and pJS094 (DNA 2 cloned at its *Pst*I site) were excised from their bacteriophage replicative forms as linear dsDNAs, purified by sucrose density gradient centrifugation and *N. benthamiana* plants inoculated with 1μg of each cloned DNA component either separately or as a mixture, it was found that both components were required to initiate infection. Infected plants displayed characteristic virus-induced symptoms, nuclear cytopathological structures normally associated with infection and geminate particles

were isolated from this tissue which were serologically indistinguishable from the native virus.

TGMV dsDNA was purified from infected plant DNA by agarose or polyacrylamide gel electrophoresis (Hamilton *et al.*, 1982; Bisaro *et al.*, 1982) and extracted from gels according to the method of Dretzen *et al.* (1981) or Schuerch *et al.* (1975). TGMV dsDNA A was cloned into pAT153 at its unique *Eco*RI in both orientations (pBH401 and pBH404) and ds TGMV B DNA cloned at its unique *Cla*I site in both orientations (pBH602 and pBH604) (Bisaro *et al.*, 1982; Hamilton *et al.*, 1983). Plants were inoculated with *Eco*RI (pBH404) and *Cla*I (pBH604) excised TGMV inserts (50μg of insert DNA per component was applied either separately or as a mixture).

Ds BGMV DNAs were synthesised on a viral DNA template using AMV reverse transcriptase and random primers. Both DNA components were cloned into pBR322 at single restriction enzyme sites, *Hin*dIII for DNA I and *Cla*I for DNA II. Excised cloned linear dsDNA of both components were used in infectivity tests on *P. vulgaris* plants and infection was only achieved when both components in a mixture were inoculated to plants (Morinaga *et al.*, 1983).

For infectivity, excision of the cloned viral genome from the recombinant plasmids was apparently required (Hamilton *et al.*, 1983). Similarly full-length cloned CaMV was infectious for plants only after excision from its cloning vector (Howell *et al.*, 1980). The reason for these results may be related to the fact that in both cases the viral genome is interrupted within an essential region by its cloning site and after excision from its cloning vector recircularisation may be a limiting step prior to initiating infection. However, uncut CaMV genomes flanked by homologous arms of viral DNA can infect plants by intragenomic recombination (Walden and Howell, 1983) and tandem full-length CaMV dimers in a recombinant plasmid are also infectious without excision (Lebeurier *et al.*, 1982). Stanley and Townsend (1986) have since shown that intact recombinant DNAs containing single copies of either component of the CLV genome can elicit infection when mechanically inoculated to host plants, albeit at a reduced efficiency, in the presence of the other component which has been excised from its plasmid.

The results of these infectivity studies using the cloned components of CLV, TGMV and BGMV illustrated that both components of the genome were required to produce infective progeny. Therefore the two DNA molecules of these geminiviruses represent the infectious entity of the viruses for whole plants and confirms the bipartite strategy of their replication in whole plants.

More recent studies have shown CLV DNA 1 to be capable of independent replication in transfected protoplasts (Townsend *et al.*, 1986). Totipotent leaf mesophyll protoplasts of *Nicotiana plumbaginifolia* were inoculated with 10μg of each gradient purified excised insert from full-length copies of the CLV cloned genomes (pJS092 and pJS094). Following transfection, virus specific DNAs began to appear 48-72h after inoculation with either the cloned DNAs or purified virus, coincident with the onset of host cell division. Infected cells accumulated supercoiled forms of both DNA components as well as virion ssDNA. When protoplasts were inoculated with the two cloned DNA components separately both supercoiled and ss molecules were synthesised by cells inoculated with DNA 1 alone but DNA 2 failed to replicate independently (Townsend *et al.*, 1986). It has also been demonstrated (Rogers *et al.*, (1986) that TGMV A DNA replicates independently of B DNA in transgenic plants. Here phenotypically normal petunia plants carrying chromosomal inserts of tandem direct-repeats of TGMV DNA A or TGMV DNA B were produced using an *Agrobacterium tumefaciens* Ti plasmid transformation system (Fraley *et al.*, 1985). Hybridisation analysis techniques revealed that the tandem, direct-repeat A transformed plants contained free ss and ds A component DNAs. No free B component DNA was detected in plants carrying tandem repeats of the TGMV B DNA (Rogers *et al.*, 1986).

Thus it appears component A of TGMV and component 1 of CLV encode all the gene products required to direct their replication in susceptible host tissue.

Southern blot analysis of encapsidated and intracellular forms of TGMV (Bisaro *et al.*, 1982) and CLV (Stanley 1983; Stanley and Townsend, 1985) using radioactively labelled probes from each DNA component have shown that DNA components are present in approximately equimolar amounts in systemically infected tissues. However all attempts to find

two genomic components for the leafhopper-transmitted geminiviruses that infect monocotyledonous plants have failed thus far. Analysis of cccDNA isolated from MSV-infected *Zea mays* suggests the presence of a single DNA (Mullineaux *et al.*, 1984). Likewise exhaustive sequence analysis of both WDV and MSV virion DNAs could only accommodate the data within a single circular molecule of 2687 nucleotides (MSV) and 2749 nucleotides (WDV) (Howell, 1984, 1985; Mullineaux *et al.*, 1984; MacDowell *et al.*, 1985). However, as neither of these viruses have been successfully mechanically transmitted back to their hosts the infective unit of MSV and WDV remains to be defined. It is believed that this inability to mechanically transmit these viruses, or cause infection with viral or cloned viral DNA, may be a problem of delivery of the DNA into the region where infection is initiated (Mullineaux *et al.*, 1984). Evidence therefore suggests that the leafhopper-transmitted geminiviruses that infect monocotyledonous plants have a single DNA component. This notion is further supported by the positive demonstration of the infectious nature of clones of BCTV, a leafhopper-transmitted geminivirus which possesses an extensive dicotyledonous host range. Here cloned BCTV DNA has been unambiguously demonstrated to consist of a single DNA component which is infectious for plants (Stanley *et al.*, 1986).

1.7 Genome organisation

1.7a Whitefly transmitted geminiviruses

Comparisons of the nucleotide sequences of BGMV, CLV and TGMV indicate that there is a greater degree of homology between each larger DNA (60%) than between each smaller DNA (40%). This conforms with the hybridisation data of Roberts *et al.* (1984) where homologies were detectable between CLV 1 DNA and DNA from BGMV, TobLCV, TGMV, TLCV and TYLCV while none were detected by probing with CLV 2 DNA. Comparisons of the two sequenced genomic components of these geminiviruses revealed that while over 90% of the sequences are unique, there exists regions of approximately 200 nucleotides that are almost identical. In the case of BGMV this common region comprises a perfect 205 nucleotide match. The common region of all three viruses is situated for the most part within an intergenomic region, except for the amino-terminus of an apparently common ORF in TGMV and BGMV which overlaps the *ca.* 200 nucleotide common regions in the larger DNA component by 13 and 6 nucleotides respectively. The common regions between different viruses exhibit little homology except for a highly conserved inverted repeat sequence that can form a stable hairpin structure consisting of a GC-rich stem and AT-rich loop (figure 1). The fact that this common region contained by far the greatest degree of sequence heterogeneity amongst these viruses prompted the suggestion that it conferred host specificity (Howarth *et al.*, 1985). However, using this hypothesis it is difficult to explain why TGMV and CLV DNA show a similar lack of homology between their common regions, but share common hosts in for example *Nicotiana* spp. It may transpire that this non-coding sequence, common to both DNA components, may contain recognition signals for the origin of DNA replication, possible promoter sequences for transcription and may be involved in virus assembly.

Consistent with the notion that the common region is important for viral DNA replication has been the identification of a small subgenomic DNA in CLV and TGMV infected tissue and purified virus preparations, which retains this region in its entirety (Stanley and Townsend, 1985; MacDowell *et al.*, 1986). An examination of the encapsidated forms of

Invariant sequence **TAATATTACC**

BCTV GGGCCATCCGTTA**TAATATTAC**CGGATGGCCC
BGMV1 GCGGCCATCCGATA**TAATATTAC**CGGATGGCCGC
BGMV2 GCGGCCATCCGATA**TAATATTAC**CGGATGGCCGC
CLV1 GGGGCCAACCGTA-**TAATATTAC**CGGTTGGCCCC
CLV2 GGGGCCAACCGTA-**TAATATTAC**CGGTTGGCCCC
TGMVA GCGGCCATCCGTT-**TAATATTAC**CGGATGGCCGC
TGMVB GCGGCCATCCGTTT**TAATATTAC**CGGATGGCCGC
MSV-K GAAAAGAAGGCGCGCAC**TAATATTAC**CGCGCCTTCTTTTC
MSV-N GCAGGAAAAGAAGGCGCGCAC**TAATATTAC**CGCGCCTTCTTTTCCTGC
WDV GTCGGGGGCCTCCACGCGGGTTA**TAATATTAC**CCCGCGTGGTGGCCCCCGAC

Figure 1. Comparisons of the major putative stem-loop structures
This diagram shows the potential hairpin structures containing the invariant 10 base sequence (the invariant loop of which is shown in bolt type). The stem of the potential hairpin loop structures are underlined.

CLV DNA illustrated the presence of a circular ssDNA half the size of the genomic ssDNA. This molecule was approximately 1200 nucleotides in size (Sequeira, 1982; Stanley, 1983). As discussed earlier, a large proportion of these molecules are encapsidated in single isometric particles. Southern blot analysis of the intracellular and purified DNAs from both CLV and TGMV has demonstrated that their sub-genomic DNAs originate from the smaller genomic component (Stanley, 1983; Stanley and Townsend, 1985; Hamilton *et al.*, 1983). Our knowledge of the infectious nature of the cloned components of TGMV and CLV DNA demonstrates that the sub-genomic DNA species is not required for infection and it has been concluded that this DNA represents a nonessential helper-dependent defective molecule. Double-stranded DNA of a sub-genomic species of similar size and component specificity has been isolated from both CLV and TGMV extracts of infected *N. benthamiana* tissue (Stanley and Townsend, 1985; MacDowell *et al.*, 1986). Full-length clones from these ds forms of both CLV and TGMV sub-genomic DNAs have been constructed and sequenced to ensure that no major deletions or insertions had occurred, as was noted for defective DNAs associated with animal DNA virus infections (Huang and Baltimore, 1977). With CLV, in all of the clones examined deletions occurred within 150 nucleotides downstream of the common region of DNA 2 and deleted approximately half the molecule, completely removing the 29,300 Dalton ORF and the carboxy-terminus of the 33,700 ORF (Stanley and Townsend, 1985). For this DNA to be maintained within the virus population, the sub-genomic molecules must presummably retain the origins of replication and assembly but still be of a suitable size to be encapsidated. Since the deletions do not encroach downstream of nucleotide 1720 in CLV DNA 2, Stanley and Townsend (1985) suggested that this region might be important for at least one of the above functions.

The coding capacity of the sequenced geminiviral genomes has been investigated by screening all three reading frames in both DNA senses for open regions with coding capacities greater than the arbitrarily selected value of 10,000 Daltons when read from the first in-phase potential initiation codon (figure 2). Six analogous ORFs were found when CLV (Stanley and Gay, 1983), TGMV (Hamilton *et al.*, 1984) and BGMV

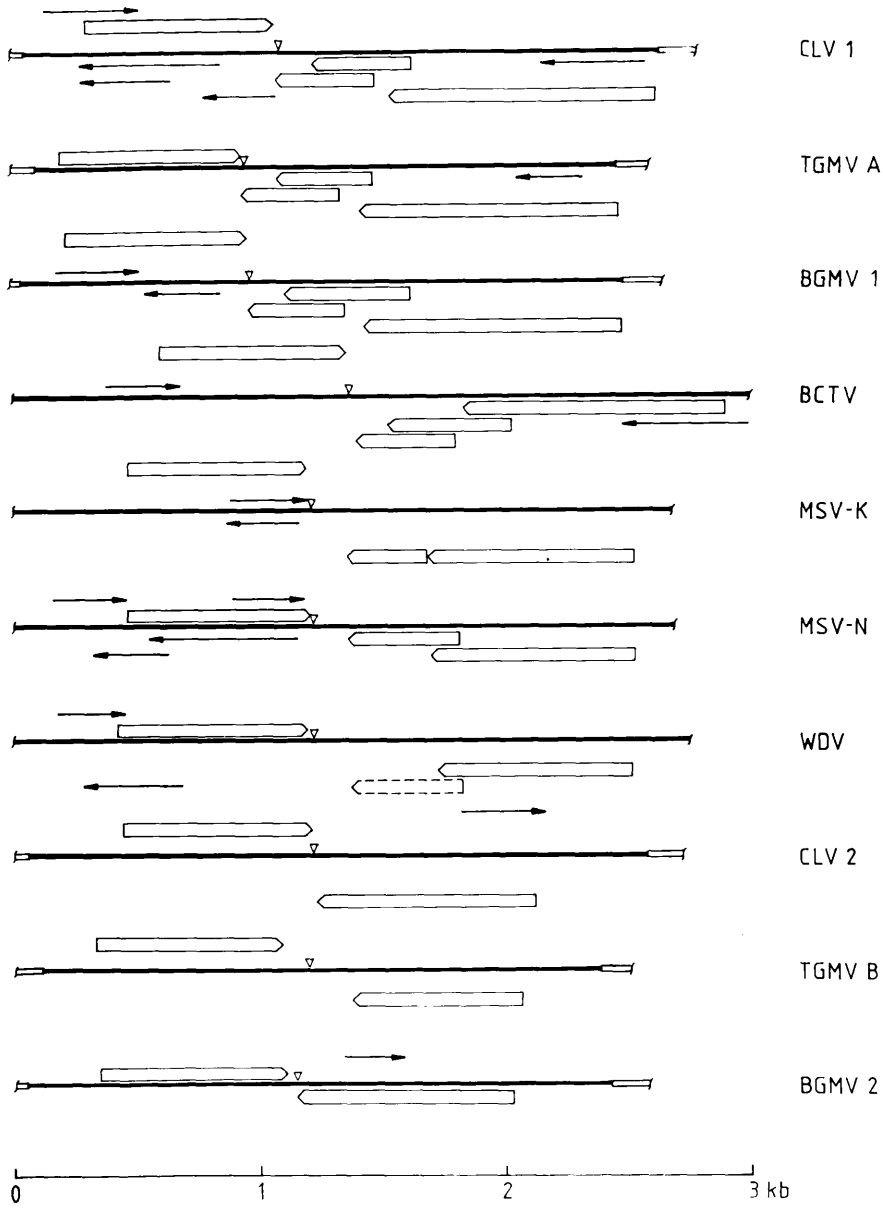


Figure 2. Open reading frame comparison of the sequenced geminiviruses. The circular genomes are represented in a linear form. Position 1 has been taken as the first T of the invariant 10 base sequence. White boxes within the thick black line define the limits of the 200 base common region between the bipartite viruses. Conserved ORFs are indicated by the large open arrows and non-conserved ORFs by the thin solid arrows. The region of the transcription termination signal is indicated by an open triangle. (Diagram courtesy of I.T.D. Petty).

(Howarth *et al.*, 1985) were compared. The high degree of sequence homology observed between these six ORFs suggests they may be involved in producing similar proteins possessing similar functions. Other ORFs found in individual viral genomes may have arisen fortuitously and may not actually be involved in protein synthesis, however this is unproven. For all three viruses only one virus-specific product, coat protein, has been identified and ascribed to its ORF. The size estimate of 29,500 Daltons for the coat protein of the Kenyan isolate of CLV (Stanley *et al.*, 1985) compares well with the theoretical potential coding capacities of the 30,200 and 29,300 Dalton ORFs located on CLV DNA 1 and 2, respectively. On the basis of amino acid composition and partial protein sequencing the 30,200 ORF of CLV and the 28,651 ORF of TGMV it has been postitulated that they encode coat protein (Stanley, 1985; Stein, 1985). For CLV this was definitely proven following analysis of RNA transcripts from extracts of CLV infected *N. benthamiana* plants (Townsend *et al.*, 1985). A 1.0 kb transcript, which upon translation in rabbit reticulocyte lysate produced a product immunoprecipitable with an antiserum raised to purified CLV virions, was demonstrated to be the coat protein mRNA by hybrid arrest of capsid synthesis using ssDNA complementary to the 30,200 Dalton ORF. The coat protein ORF is the most highly conserved of all the potential coding regions between the three viruses, reflecting the strong immunogenic cross-reaction observed between TGMV, CLV and BGMV (Roberts *et al.*, 1984).

From the distribution of ORFs in relation to the positions of potential promoters conforming to the consensus TATA box sequence TATAA/TAA/T (Breathnach and Chambon, 1981) and polyadenylation signals AATAAA (Proudfoot and Brownlee, 1976) bidirectional transcription, from both strands of each DNA component has been suggested (Hamilton *et al.*, 1984; Townsend *et al.*, 1885; Coutts and Buck, 1986). The ORFs on both DNA components of TGMV, CLV and BGMV converge to a point diammetrically opposite to the common region and are separated by only a few nucleotides (Stanley and Davies, 1985). Using extracts from CLV infected tobacco plants Townsend *et al.* (1985) detected three major transcripts of 1.7, 1.0 and 0.7 kb specific to DNA 1. Two transcripts, namely the 1.7 and 0.7 kb, were transcribed from the viral strand while

the 1.0 kb transcript came from the complementary strand. Two major transcripts of 1.1 and 0.9 kb were specific to DNA 2. The larger transcript came from the viral strand and the smaller one from the complementary strand. The most abundant transcript in plant tissue showing advanced systemic symptoms was the 1.0 kb transcript encoding the virus coat protein. Now that a protoplast system, synchronised for DNA synthesis, is available for studying geminivirus replication *in vitro* (Townsend *et al.*, 1986; as discussed in section 1.6) it may be possible to study in detail the relative abundance of each transcript at earlier stages in infection and so gain an insight in to the virus replication strategy.

1.7b Leafhopper-transmitted geminiviruses

Exhaustive sequence analysis of virion DNA of a Nigerian isolate of MSV (Mullineaux *et al.*, 1984) and cloned dsDNA of WDV (MacDowell *et al.*, 1985) demonstrated the presence of only a single circle of DNA consisting of 2687 and 2749 nucleotides, respectively. The sequence of the Kenyan isolate of MSV has been determined (Howell, 1985) and found to be of similar size to the Nigerian isolate. Both MSV DNA sequences were derived from native isolates of the virus and it was not unexpected that a number of nucleotides substitutions were encountered. Most heterogeneities which occurred within ORFs on the DNAs were conservative third base substitutions. No significant DNA sequence homologies were clearly apparent when MSV DNA and the other sequenced whitefly-transmitted geminiviral DNAs were compared. MacDowell *et al.* (1985) however detected significant amino acid sequence homologies when the predicted proteins from ORFs on WDV, MSV, TGMV and CLV DNAs were compared. These results suggest a clear relationship between, and common ancestral origin, for the single and bicomponent geminiviruses. Both MSV and WDV DNAs contain inverted repeat sequences homologous to those found in TGMV, CLV and BGMV DNA (figure 1), emphasising the possible universal role for the putative hairpin structure in geminiviral replication.

Applying the same analysis procedures used previously to investigate the sequences of other geminiviral DNAs, MSV and WDV DNA were found respectively to encode possible seven and five proteins.

The only known MSV-specific protein is the coat protein of estimated size 28,000 Daltons (Bock *et al.*, 1977). Three RNA transcripts encoded by MSV DNA were detected in polyadenylated RNA from virus-infected maize tissue (Morris-Krsinich *et al.*, 1985). Two of the transcripts, a major 0.9 kb and a minor 1.05 kb RNA, were mapped onto the virion sense DNA and the third 1.2 kb transcript was mapped onto the complementary sense DNA, demonstrating that transcription of MSV is similar to the other geminiviral DNAs investigated at the molecular level in that it is also bidirectional. A virus-specific polyadenylated RNA translated *in vitro* produced a 28,000 Dalton polypeptide which was specifically immunoprecipitable with an antiserum raised against purified MSV. Hybrid-arrested translation mapped this transcript onto the long ORF on the virion sense MSV DNA (Morris-Krsinich *et al.*, 1985). This ORF had a potential amino acid composition, calculated from the nucleotide sequence data, which closely agreed with that determined for coat protein (Bock *et al.*, 1977).

The amino acid sequence homologies between the 31,400 and 17,800 Dalton ORFs of the Nigerian isolate of MSV and WDV with the 40,200 Dalton ORF of both TGMV and CLV (Mullineaux *et al.*, 1985; MacDowell *et al.*, 1985) suggests a conservation of functional domains of the proteins. In the case of WDV the smaller ORF has no ATG initiation codon (MacDowell *et al.*, 1985). The AUG initiation codon of MSV is replaced by a GTG codon which is functionally opperational and can initiate translation in prokaryotes but is not known to do so in eukaryotes where AUG codons initiate translation (Kozak, 1983). MacDowell *et al.* (1985) suggested that the WDV ORFs of 30,156 and 17,292 Daltons may be transcribed on a single mRNA which encodes a composite protein.

Nucleotide sequence analysis of both isolates of MSV was made possible by the synthesis *in vitro* of dsDNA by DNA polymerase-directed second strand synthesis on a template of virion DNA. It was found, however that second strand synthesis could be initiated without the need to supply exogenous primer (Donson *et al.*, 1984; Howell, 1985). A similar result has been obtained for WDV (H. Macdonald pers. comm.). If the MSV DNA was heat denatured and snap-cooled the self-priming activity was lost, strongly suggesting the presence of a fragment

complementary to, and associated with, the viral ssDNA. Donson *et al.* (1984) have isolated from MSV preparations a population of 'nested' DNA molecules. These molecules had ribonucleotides covalently linked to the DNA species' discrete 5' deoxyribonucleotide terminus. The major species of DNA had a sequence of 80 nucleotides which was complementary to a region 5' , almost exclusively in an intergenic region, to two hairpin structures on the MSV genome. These molecules with associated primer were capable of initiating second strand synthesis *in vitro*.

Stanley *et al.* (1986) have constructed a number of clones of a Californian isolate of the leafhopper-transmitted geminiviruses BCTV. Several putative full-length clones were isolated, which when excised from their bacteriophage vectors and inoculated by needle-puncture into the crown of seedlings proved infectious. This result demonstrated unambiguously the single component nature of the BCTV genome. The nucleotide sequence of one infectious clone (pBCT028) was determined and found to consist of 2993bp. Comparison with other geminivirus has shown that the genome organisation of BCTV closely resembles that of the larger DNA of the whitefly-transmitted members. The four conserved ORFs in the whitefly-transmitted geminiviruses have homologous counterparts in BCTV (figure 2) with the exception of the putative coat protein which is more closely related to those of the leafhopper-transmitted members. This result suggests a strong interrelationship between coat protein and the insect transmission vector. In the region analogous to the common regions of the whitefly-transmitted geminiviruses, BCTV DNA was found to possess inverted repeat sequences similar to those described earlier (figure 1).

While CLV DNA 1 has been found to encode the virus DNA replication functions (Townsend *et al.*, 1986) in addition to coat protein, the role of DNA 2 in the geminivirus infection cycle remains obscure. This is emphasised by the observation that the host range of BCTV (Bennett, 1971) overlaps those of BGMV, TGMV and CLV. It is possible that the number of geminiviral DNA components present in any particular virus correlates with the insect vector and implies that at least one function of the smaller genomic DNA species is involved with whitefly transmission. This further suggests that the BCTV coat protein is the

sole virus-encoded protein responsible for the interaction of BCTV with its leafhopper vector. However. as demonstrated for CLV, DNA 2 is a prerequisite for successful mechanical transmission, thus it must encode at least one function in addition to a putative insect transmission factor. Townsend *et al.* (1986) recently proposed that the spread of CLV from peripheral leaf cells to phloem cells where it proliferates may be mediated by a DNA 2 gene product. The fact that BCTV is apparently phloem-limited (Esau and Hoefert, 1973) while CLV has been observed in the cortex, mesophyll and epidermis of *N. benthamiana* (Sequeira and Harrison, 1982) is consistent with this notion.

The possible hybrid nature of the BCTV genome raises the question of how the members of the potential subgroups within the geminivirus group have evolved. Kikuno *et al.* (1984) suggested, on the basis of similar organisation and limited amino acid sequence homology, that the two components of the whitefly-transmitted geminiviruses evolved from a common ancestral DNA.

1.8 Geminiviral replication strategy

The events concerned with DNA replication can be divided into three stages, the conversion of virion ssDNA to a double-stranded (ds) form, the proliferation of the dsDNA, and finally the production of virion ssDNA from this dsDNA intermediate. Very little is known about the mechanisms by which these processes occur in geminivirus infections.

During the virus infection cycle different viral gene products will be required at different stages and in different quantities. Therefore for the most advantageous use of the host cell synthetic machinery a method of switching genes on and off is essential. There are two possible solutions to this problem, the viral genes might be clustered either by physical separation as a multicomponent genome or on complementary strands of the same genomic component. The whitefly-transmitted geminiviruses appear to have features of both of these systems. The organisation of the genome shows obvious similarities to that of the papovaviruses in which bidirectional transcription occurs from an intergenic region to a position diammetrically opposite on the circular genome, segregating early and late genes on complementary strands. One early gene product of SV40, the large T antigen, is known to be autoregulatory at the level of transcription (Tegtmeyer *et al.*, 1975; Alwine *et al.*, 1977; Khoury and May, 1977) and the control of transcription and DNA replication are strongly interdependent (Wasylyk *et al.*,1983). By analogy, genes on opposite strands of both genomic DNAs of the whitefly-transmitted geminiviruses are possibly expressed at different times during infection. As the coat protein, a gene product required late in the infection cycle, is located on the viral strand of the larger DNA, the products of the complementary strand ORFs might be required at an earlier stage, possibly in DNA replication. Similarly, the two putative gene products of the smaller DNA might be expressed at different times. It is possible that transcripts on both components are controlled by identical sequences within the common region.

Deproteinised virion ssDNA from BGMV (Goodman , 1977a,b), TGMV (Hamilton *et al.*, 1983) and MYMV (Ikegami *et al.*,1984) is infectious. This results suggests that host factors alone are necessary for the synthesis of a second strand on the virion ssDNA template and neither

capsid protein nor any other virion associated protein is involved. Since it would be expected that host factors would recognise similar features on both components of a bicomponent genome Stanley and Gay (1983) suggested that the sequences common to both components might contain the origins of replication. An analogy has been drawn (Hamilton *et al.*, 1984) between these sequences and the primosome assembly site on bacteriophage øX174 ssDNA where host factors alone initiate second strand synthesis (Arai and Kornberg, 1981). The DNA primer for second strand synthesis which is found bound to encapsidated MSV DNA possesses 5' terminal ribonucleotides (Donson *et al.*, 1984). These residues presumably result from RNA polymerase-directed *de novo* synthesis on a short stretch of DNA, somewhat analogous to the initiation of DNA synthesis in eukaryotes (Hay and DePamphilis, 1982). Limitation of the primer size to 80 nucleotides may be a consequence of either packaging constraints or sequence-specific termination of a DNA polymerase (Donson *et al.*,1984). No such primer was found bound to CLV ssDNA under similar conditions in which the MSV primer was detected (Stanley and Townsend, 1985).

Virus-specific cccDNA, ocDNA and linear DNA forms have been identified in crude extracts of *N. benthamiana* tissue infected with TGMV (Hamilton *et al.*, 1982), genome length cccDNA has been isolated from *Z. mays* infected with MSV (Mullineaux *et al.*, 1984) and CLV infected *N. benthamiana* (Stanley and Townsend, 1985). Only ocDNA has been isolated from BGMV and MYMV infected *P.vulgaris* (Ikegami *et al.*, 1981, 1984). This inability to isolate cccDNA from these two virus infections may reflect a host-specific variation of a rate-limiting step of the replication process resulting in the accumulation of the oc intermediate. The suggestion that these dsDNAs are true intermediates is strengthened by the demonstration of their infectivity when mechanically inoculated onto whole plants (Hamilton *et al.*, 1983; Ikegami *et al.*, 1984). It is likely that the cccDNA is confined to the nucleus where it may adopt a transcriptionally active minichromosome structure, similar to that described for CaMV (Olszewski *et al.*, 1982). Coutts and Buck (1985) detected TGMV specific cccDNA in nuclei and hypotonically leached extracts of nuclei from infected *N. benthamiana* leaves. These preparations were found to have *in vitro* DNA and RNA

polymerising activities, producing virus specific products. The demonstration that negatively supercoiled TGMV cccDNA contains specific S1-nuclease sensitive sites (Sunter *et al.*, 1984) has prompted suggestions that stable cruciform structures within the cccDNA might act as determinants in either the regulation of transcription or the origin of DNA replication (Sunter, 1985). The conserved inverted repeat sequences located within the region common to both DNA components may produce the necessary localized destabilizing effect to form such structures.

At least two high-molecular-weight dsDNAs thought to represent concatamers comprising tandemly arranged viral genomes have been observed in extracts of both TGMV- and CLV-infected *N. benthamiana* tissue (Hamilton *et al.*, 1982; Stanley and Gay, 1985). Whether or not these dimeric dsDNAs represent true intermediates in the replication cycle of the viruses remains to be elucidated but they are routinely observed in infected plant material.

A late event in the virus infection cycle, which may occur simultaneously with encapsidation, is the synthesis of virion DNA from the circular dsDNA template. This process may be under the control of one or more virus encoded proteins acting in an analogous manner to the gene A product of øX174 (Brown *et al.*, 1982). This protein is required for site-specific nicking of the double-stranded replicative form, from which viral ssDNA is displaced by a rolling circle mechanism. The same gene product is also responsible for cleavage of the nascent ssDNA from the replicative intermediate and rejoining the termini to produce the circular form that is encapsidated. Analysis of virion DNA isolated from CLV-infected *N. benthamiana* tissue has demonstrated the occurrence of variable amounts of concatameric ssDNA (Stanley, 1983; Stanley and Townsend, 1985). Dimeric ssDNA with a circular conformation has been isolated from purified CLV and MSV virions (Stanley and Townsend, 1985). The dimers are presumably derived from either incomplete strand scission of the nascent ssDNA during excision from the replicative intermediate or synthesis on a dimeric dsDNA template. The possibility that these dimeric ssDNAs are encapsidated within tetrameric virus particles has been discussed earlier (section 1.5).

The exact determination of the functions and temporal control of geminiviral gene product synthesis may well be more easily elucidated by utalising recent advances in; synchronously infectable protoplasts (Townsend *et al.*, 1986); *Agrobacterium* transformation technology (Rogers *et al.*, 1986); site-directed mutagenisis, or a combination of all three systems.

1.9 Scope of this thesis

The work in this thesis forms part of a larger programme at Imperial College on the molecular biology of geminiviruses aimed at understanding the viral replication strategy and using this information to develop plant gene vectors from geminivirus replicons. This type of study has at least three possible benefits, an improved understanding of the molecular basis of geminivirus pathogenicity, an insight into DNA replication in plants and the production of a geminivirus based vehicle for introducing useful traits into plants. Other work in the group has concentrated on the Brazilian isolate of TGMV and the Swedish isolate of WDV. When a preliminary report was published describing a potentially new geminivirus infecting potatoes (Debrot, 1981) it was decided to obtain an isolate of this virus with a view to completely characterising it as a member of the geminivirus group and using it as the basis of a potato transfection system.

Initial attempts to passage the virus from freeze-dried infected material proved unsuccessful. Eventually I was able to transmit the virus from fresh infected material and passage it to other hosts. Detailed analysis of the DNA forms present in infected tissue allowed the isolation of a double-stranded form suitable for molecular cloning and the demonstration that the genome of potato yellow mosaic virus, PYMV, (the name given to this virus) consisted of single-stranded DNA.

The relationship of PYMV to other geminiviruses was examined by various means, all of which suggested that this virus was a distinct member of the geminivirus group.

Concurrent with this work I established several different potato cell cultures for the production of protoplasts to be used in transformation experiments, with the aim of studying geminivirus replication *in vitro*. Much effort was concentrated on attempting to establish a system where cultured potato protoplasts readily underwent division, believing this, correctly, to be a prerequisite for the detection of geminivirus replication.

2. MATERIALS AND METHODS

2.1 Abbreviations

AMPS	ammonium persulphate
Ap	ampicillin
b	base(s)
bp	base pair(s)
BSA	bovine serum albumin
cccDNA	covalently closed circular DNA
CIP	calf intestinal phosphotase
Cm	chloramphenicol
Cr	carbenicillin
cv.	cultivar
d-	deoxy eg. dNTP; A°, G°, T°, C°, -dNTP mixes
dATP	deoxyadenosine triphosphate
dCTP	deoxycytodine triphosphate
dGTP	deoxyguanosine triphosphate
dd-	dideoxy
dH_2O	distilled water
deH_2O	deionised water
DHFR	dihydrofolate reductase
dicot	dicotyledonous plant
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid (disodium salt)
h	hour(s)
km	kanamycin
kb	kilobase or kilobase pairs
kd	kilodalton
kV	kilovolt
lx	lux
MDal	megadaltons
min	minute(s)
mol. wt.	molecular weight
M_r	relative molecular weight
monocot	monocotyledonous plant

Nm	neomycin
ocDNA	open circular DNA
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PLO	poly-L-ornithine
psi	pounds per square inch
Pu	purine
PVP	polyvinylpyrolidine
Py	pyrimidine
RF	replicative form
RF I	replicative form I (supercoil) DNA
RF II	replicative form II (open circular) DNA
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
s	second(s)
Sevag	chloroform : isoamyl alcohol (v/v), 24 : 1
ss	single stranded
SSC	a buffer
TAE	a buffer
TBE	a buffer
Tc	tetracycline
TCA	trichloroacetic acid
TEMED	N,N'-tetramethylethylene diamine
TGA	thioglycollic acid
TNE	a buffer
tris	trishydroxymethylaminoethane
TTP	thymidine triphosphate
uv	ultraviolet

Amino acid nomenclature

Both the three-letter and one-letter codes are used in the text. These codes are as follows:

Amino acid	Three-letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

2.2 Safety considerations

A laboratory overall was worn at all times. Disposable gloves were worn when handling radioactively labelled compounds, hazardous chemicals and during recombinant DNA work.

Radioactive isotopes were handled behind a perspex screen and all radioactive waste was disposed of in accordance with Imperial College Safety regulations.

Work with recombinant DNA was registered with the Genetic Manipulation Advisory Group (GMAG) and classified by the Department of Pure and Applied Biology under the category of Good Microbiological

Practices. All waste media and materials that contact bacteria containing recombinant DNA, were autoclaved prior to disposal.

Work with potato yellow mosaic virus was carried out under the Destructive Pests and Diseases of Plants Act with a licence (PHF 29A/136) issued by the Ministry of Agriculture, Food and Fisheries (MAFF), with a similar licence (HH 12034 A/73) for work concerning tomato golden mosaic virus.

2.3 Chemical companies and addresses

All reagents, unless otherwise stated, were obtained from BDH or Sigma and were of analytical grade. Other chemicals and equipment were supplied from the following companies:

1. American Can Co. : American Can Company, Greenwich, Connecticut 06830, USA.
2. Amersham : Amersham International plc, White Lion Road, Amersham, Buckinghamshire. HP7 6LL
3. Anglian : Anglian Biotechnology Ltd., Unit 8, Hawkins Road, Colchester, Essex, CO2 8JX
4. BCL : Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex. BN17 1LG
5. BCL Cellophane : BCL Cellophane, Bath Road, Bridgewater, Somerset, TA6 4PA
6. BDH : BDH Chemicals Ltd., Freshwater Road, Dagenham, Essex, RM8 1R2
7. Becton Dickinson : Becton Dickinson Labware, 1950 Williams Drive, Oxnard, California 93030, USA.
8. Beechams : Beechams Pharmaceuticals, Research Division, Clarendon Road, Worthing, West Sussex, BN14 8QH
9. Beckman : Beckman Instruments, Inc., 2500 Harbor Blvd., Fullerton, CA, USA.
10. Bio-Rad : Bio-Rad Laboratories Ltd., Caxton Way, Watford Business Park, Watford, Herts. WD1 8RP
11. BRL : Bethesda Research Laboratory (UK) Ltd., P.O. Box 145, Science Park, Cambridge, CB4 4BE
12. Buchler Instruments : Haeke Buchler Instruments, 244 Saddle River Road, Saddle Brook, New Jersey 07662, USA.

13. Carborundum Company : Carborundum Company Ltd.,Trafford Park, Manchester, England. M17 1HP
14. Difco : Difco Laboratories, P.O. Box 143, Central Avenue, East Molsey, Surrey. KT8 08E
15. Fisons : Fisons Scientific Equipment, Bishop Meadow Road., Loughborough, Leics., LE11 0RG.
16. Flow Laboratories : Flow Laboratories Ltd., P.O. Box 17, Second Ave., Industrial Estate, Irvine, Ayrshire. KA12 8NB.
17. Gallenkamp : Gallenkamp, Belton Road West, Loughborough, LE11 0TR
18. Gilson : Gilson Company Inc., P.O. Box 677, Worthington, Ohio 43085, USA.
19. Hawksley : Hawksley & Sons Ltd., 12 Peter Road, Lansing, West Sussex. BN15 8TH
20. Henry Simon : Henry Simon Ltd., P.O. Box 31, Stockport, Cheshire, SK3 0RT
21. Hewlett-Packard : Hewlett-Packard GmbH, Hewlett-Packard Strasse, Postfach 1180, 7517 Waldbronn 2, West Germany.
22. Kodak : Kodak Ltd., Distribution Southern Region, P.O. Box 33, Hemel Hempstead, Herts. HP2 7EU
23. LKB : LKB Instruments Ltd., 232 Addington Road, Selsdon, South Croydon, Surrey. CR2 8YD
24. Marine Colloids Division : Marine Colloids Division, FMC Corporation, Rockland, ME 04841, USA.
25. Medicell International : Medicell International, 239 Liverpool Road, London. N1 1LX
26. Meiji Seika Kaisha : U.K. suppliers ; Genetic Research Instrumentation, Gene House, Station Road, Tukeley, Bishops Stortford, Herts. CN22 6SG
27. Microflow : Microflow Ltd., South Way, Walworth Industrial Estate, Andover, Hants. SP10 5AG
28. Millipore : Millipore Corp., 80 Ashby Road, Bedford, MA, USA
29. MSE : MSE Scientific Instruments, Manor Royal, Crawley, West Sussex. RH10 2QQ.
30. New England : New England Biolabs Inc., U.K. suppliers C.P.Laboratories Ltd., P.O. Box 22, Bishops Stortford, Herts. CM22 7RQ

31. New England Nuclear : NEN Products, Du Pont Company, 549, Albany Street, Boston, Massachusetts 02118, USA.
32. P&S : P&S Biochemicals Ltd., 38, Queensland Street, Liverpool L7 3JG
33. Pharmacia : P-L Biochemicals Ltd., P.O. Box 98, Northampton, NN3 1AW
34. Raven : Raven Scientific Ltd., P.O. Box 2, Haverhill, Suffolk CB9 0ES
35. Rohm & Hass : Rohm & Hass Co. Ltd., Independence Mall West, Philadelphia, PA 19105, USA.
36. Rose : Rose Chemicals Ltd., 83 Darenth Road, London N16 6EB
37. Seishin Pharmaceuticals : Seishin Pharmaceutical Co. Ltd. , 9-500-1, Nagareyama, Nagareyama-shi, Chiba-ken, Japan.
38. Sigma : Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset BH17 7NH
39. Sterilin : Sterilin Ltd., 43-45 Broad Street, Teddington, Middlesex. TW11 8QZ.
40. Squibb & Sons : E. R. Squibb & Sons , Hounslow, Middlesex, England.
41. Universal : Universal Scientific Ltd., The Broadway, Woodford Green, Essex. IG8 0HL
42. Wacker Chemicals : Wacker Chemicals Ltd., The Clock Tower, Mount Felix, Bridge Street, Walton-on-Thames, Surrey. KT12 1AS
43. Whatman : Whatman Lab. Sales, Springfield Mill, Maidstone, Kent. ME14 2LE
44. Worthington Diagnostics Systems Inc., U.K. suppliers Flow Laboratories Ltd., P.O. Box 17, Second Avenue, Industrial Estate Irvine, Scotland. KA12 8NB
45. Yakult Honsha Co. Ltd. : Yakult Honsha Co. Ltd., 1-1-19 Higashi-Shinbashi, Minato-Ku, Tokyo, 105 Japan.

2.4 Commonly used buffers and media

SSC : 150 mM NaCl, 15 mM tri-sodium citrate (pH 7.0), made up as 20 x stock solution.

TAE : 40 mM tris-HCl (pH8.0), 20 mM acetic acid, 1 mM EDTA, made up as either a 25 x, or a 10 x stock solution.

TBE : 90 mM tris-HCl (pH 8.2), 90 mM boric acid, 2 mM EDTA, made up as a 10 x stock solution.

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

TNE : 100 mM tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, made up as a 10 x stock solution.

5 x loading buffer (agarose gels) : 0.25% bromophenol blue, 30% glycerol, 1 x TAE

The following media were used for tissue culture and protoplast experiments:

Murashige and Skoog medium (MS)

MS medium was supplied as a powder by Flow Labs Ltd. and made up as a 2 x stock and stored at -20°C. Final concentrations of the constituents are as follows, with Iron being added as FeNaEDTA rather than the salt specified in the original formulation (Murashige and Skoog, 1962).

		mg/l
calcium chloride	$CaCl_2.2H_2O$	440.0
cobalt chloride	$CoCl_2.6H_2O$	0.025
copper sulphate	$CuSo_4.5H_2O$	0.025
iron sodium EDTA	FeNaEDTA	36.70
boric acid	H_3BO_3	6.20
potassium dihydrogen phosphate	KH_2PO_4	170.0
potassium iodide	KI	0.83
potassium nitrate	KNO_3	1900.0
magnesium sulphate	$MgSO_4.7H_2O$	370.0
manganese sulphate	$MnSO_4.4H_2O$	22.30
sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
ammonium nitrate	NH_4NO_3	1650.0
zinc sulphate	$ZnSO_4.7H_2O$	8.60
myo-inositol		100.0
nicotinic acid		0.50
thiamine HCl		0.10
pyroxidine HCl		0.50
glycine		2.00

Medium A

Medium A (Shephard and Totten, 1977) was prepared as described by Nelson *et al.* (1983) and contained the following constituents:

	mg/l
KNO_3	190.0*
$CaCl_2.2H_2O$	44.0*
$MgSO_4.7H_2O$	37.0*
KH_2PO_4	17.0*
FeNaEDTA	3.67
H_3BO_4	0.60
$MnCl_2.4H_2O$	2.00
$ZnSO_4.7H_2O$	0.90
KI	0.08
$NaMoO_4.2H_2O$	0.03
$CuSO_4.5H_2O$	0.003
$CoSO_4.7H_2O$	0.003
myo-inositol	10.0
casein hydrolysate (Difco)	100.0
thiamine HCl	0.05
nicotinic acid	0.50
pyroxidine HCl	0.05
folic acid	0.05
biotin	0.005
glycine	0.20
1-naphthaleneacetic acid (NAA)	1.00
6-benzylaminopurine (BAP)	0.50

The salts of Medium A were prepared as a 10 x stock solution. The vitamins were made as a stock solution and contained 0.05mg/ml thiamine HCl, 0.5mg/ml nicotinic acid, 0.05mg/ml pyroxidine HCl, 0.05mg/ml folic acid and 0.005mg/ml biotin (stored at -20°C). Glycine was prepared as a 2mg/ml stock solution, NAA as a 1mg/ml stock solution in 30% ethanol and BAP as a 0.5mg/ml stock solution (each stored at -20°C). The medium was prepared by combining these stock solutions and the solid constituents as described above and the pH was

adjusted to pH 5.8 prior to autoclaving and storage at 4°C. Those salts marked (*) are the major salts of Medium A used in the preparation of the enzyme medium for protoplast release.

Rm Medium

R medium (Shephard 1980), as modified by Nelson *et al.* (1983), was prepared as follows:

	mg/ml
KNO_3	4700.0
$CaCl_2.2H_2O$	1100.0
$MgSO_4.7H_2O$	950.0
KH_2PO_4	430.0
FeNaEDTA	36.7
H_3BO_4	3.1
$MnCl_2.4H_2O$	9.9
$ZnSO_4.7H_2O$	4.6
KI	0.42
$Na_2MoO_4.2H_2O$	0.13
$CuSO_4.5H_2O$	0.013
$CoSO_4.7H_2O$	0.015
glycine	2.0
nicotinic acid	5.0
thiamine HCl	0.5
pyroxidine HCl	0.5
folic acid	0.5
biotin	0.05
glutamine	146.0
casein hydrolysate	100.0
sucrose	3423.0
D-mannitol	1812.0
NAA	1.0
BAP	0.4

This medium was adjusted to pH 5.8, filter sterilised and stored in aliquots at -20°C.

2.5 General Methods

2.5a Miscellaneous

Centrifuges

An MSE HS 18 or HS 21 centrifuge was used for high speed centrifugation, and Beckman L8-55 or L1 centrifuges for ultracentrifugation. Where quoted, 'xg' values are based on the average rotor radius. All microcentrifugation was carried out using a Hettich Mikroliter centrifuge which operates at a constant relative centrifugal force of 11,300g.

Preparation of Dialysis tubing

Tubing (visking size 2-18/23mm ; Medicell International) was boiled in a weak solution of sodium bicarbonate followed by 10 mM EDTA for 30 min each. After washing 10 times in dH_2O the tubing was autoclaved and stored at 4°C in dH_2O.

Sterilisation

All plastic, glassware and solutions were sterilised by autoclaving at 121°C, 15 psi, for 20 min or by baking at 200°C overnight (glassware only), before use. If a particular solution was heat labile then it was sterilised by filtration through a sterile Millipore filter apparatus containing a membrane of 0.2μm pore size.

Siliconisation

All glassware used in tissue culture was siliconised by rinsing with 5%(v/v) dichlorodimethylsilane in chloroform followed by at least ten washes in deH_2O prior to baking overnight at 200°C.

Determination of virus and DNA concentrations

The concentrations of purified virus or plasmid DNA were estimated from uv absorption spectra measured on a Beckman Model 35 spectrophotometer using silica cells with a path length of 1cm.

The concentration of a pure virus solution was calculated according to the relationship :

$$7\ A_{260}nm \equiv 1mg/ml$$

reported for CLV (Sequeira and Harrison,1982). DNA concentrations were calculated according to the relationship:

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

Pure DNA preparation should have an $A_{260}nm$ / $A_{280}nm$ >1.8 (Maniatis *et al.*, 1982).

Laboratory Reagents

All chemicals were obtained from either BDH or Sigma. Concentrations given in the form of percentages (%) were weight per volume (w/v) unless otherwise stated.

2.5b Agarose gel electrophoresis

Submerged horizontal slab gels were used of various sizes but most commonly they were either 15 x 11 x 0.5 cm or "mini" gels, 10 x 6.5 x 0.5 cm (Bio-Rad, 'mini-sub' DNA electrophoresis system). 1% agarose gels were made by boiling the appropriate weight of agarose (Sigma No. A-6013) in TAE or TBE. Ethidium bromide (Sharp *et al.* 1973) was added to a final concentration of 1µg/ml from a 10mg/ml stock solution stored at 4°C in the dark. Alternatively gels were stained after electrophoresis by immersion in buffer containing 1µg/ml ethidium bromide. LKB 2103 power-supplies were used as direct current power sources for electrophoresis. The conditions of electrophoresis varied greatly depending on the buffer system employed and results required but typically electrophoresis was carried out in either TAE or TBE at 60V-150V for 3-5 h for the larger gels and 15-60 min for "mini" gels. Sample volumes varied from 5µl to 20µl and were made 1 x (v/v) in loading buffer (see section 2.4). After electrophoresis DNA bands were visualised using a short-wave ultraviolet transilluminator. Photographs of gels were taken on 35mm Ilford PanF film using a Praktica MTL5 camera fitted with two Y(K2) yellow filters (Hoya) and one UV(0) filter (Hoya).

2.5c Analytical polyacrylamide gel electrophoresis

The Laemmli (1970) method of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 16.5 x 20.6 x 0.2 cm slab gels were used. The gels were composed of resolving or separating gel, the concentrations of which varied from 10 to 20% depending on the experiment, overlaid with a 3% stacking gel. For a 10% resolving gel, 10ml of acrylamide stock solution (30g acrylamide and 0.8g N,N'-methylenebisacrylamide made up to 100ml final volume with dH_2O) was mixed with 3.75ml of 3M tris-HCl(pH 8.9), 0.3ml of 5% (v/v) TEMED, 0.3ml of 10% SDS and dH_2O to a final volume of 30ml. 0.15ml of freshly

prepared 10% ammonium persulphate (AMPS) was then added and approximately 28ml of the gel poured into the glass plates, which had been previously sealed with electrophoresis tape (Universal Scientific Ltd.) and baked for 4h at 80°C. Resolving gels were overlaid with ethanol, to keep the interface between resolving and stacking gel straight. Whilst the resolving gel polymerised the stacking gel was prepared as follows: A mixture of 1ml 30.8% acrylamide stock solution, 1.25ml 0.47M tris-HCl (pH 6.7), 0.1ml 5% TEMED , 7.45ml dH_2O, 0.1ml 10% SDS, and finally 0.1ml 10% AMPS was added. The ethanol was removed from the resolving gel and the stacking gel poured over it. The comb was put in position and the gel allowed to set for at least 2h. Well sizes were approximately 0.85 x 1.5 cm, enabling the use of a maximum sample volume of 100µl.

Before loading, samples were heated at 100°C for 3 min in 1% SDS, 1% 2-mercaptoethanol, 0.002% bromophenol blue and 10% sucrose. Samples were then placed on ice prior to loading. Electrophoresis was in 0.38M glycine, 0.05M tris (pH 8.3), 0.1% SDS, at 40V-90V overnight, depending on the gel concentration, or 150V during the day. After electrophoresis gels were briefly rinsed in dH_2O to remove SDS, and stained for 1h in Coomassie blue (0.025% Coomassie Brilliant Blue R, 45% (v/v) methanol, 9% (v/v) acetic acid) and destained in 45% (v/v) methanol and 9% (v/v) acetic acid, and visualised over a cold light transilluminator.

Analysis of DNA

Partially denaturing urea-polyacrylamide slab gels 16.5 x 20.6 x 0.2 cm were used. The gels were 4% polyacrylamide containing 8M urea and 0.1% SDS (Buck and Ratti, 1977). For 50ml of gel, 5ml of 40.4% acrylamide stock solution (40g acrylamide : 0.4g N,N'-methylenebisacrylamide made up to 100ml with water), 40ml of 10M urea containing 0.25% SDS, 2ml of 25 x TAE, 0.5ml of 5% (v/v) TEMED and 2.5ml of 1.5% AMPS were added together and mixed before pouring.

Samples were heated in 1% SDS, 0.002% bromophenol blue and 10% sucrose, at 60°C for 10 min before loading. Electrophoresis was at 90V

overnight in 0.1% SDS in TAE (top reservoir), 0.1% SDS, 8M urea in TAE (bottom reservoir).

After electrophoresis gels were washed repeatedly in dH_2O to remove SDS and urea and then stained with 1µg/ml ethidium bromide for approximately 30 min. Bands were visualised using a short-wave ultraviolet transilluminator.

2.6 Virus propagation and purification

2.6a Origin of virus

Both tomato golden mosaic virus and potato golden mosaic virus were obtained in systemically infected leaves of tobacco. These viruses were gifts, TGMV from Dr A.S. Costa of the Instituto Agronomico, Estado de Sao Paulo, Brazil, and PYMV from Dr J.R. Lastra then at the Instituto Venezolano de Investigacions Cientifica, Caracas, Venezuela.

2.6b Plant propagation

Several members of the tobacco genus were used as hosts for both viruses. The most common propagation host was *Nicotiana benthamiana* but two other *Nicotiana tabacum* cultivars, Xanthi and Petit Havana (clone SR1) have been used. Other plants propagated were, potato (*Solanum tuberosum* L. *ssp tuberosum*), tomato (*Lycopersicon esculentum*), bean (*Phaseolus vulgaris*) and petunia (*Petunia hybrida*).

Tobacco plants were grown from seed germinated on Fisons compost under a propagation cover. Seeds took approximately 5 to 8 days to germinate and were then individually potted, using John Innes Potting Compost Number 2, into 10 cm square pots. These seedlings were covered with a polythene sheet to prevent dehydration for the first 2 to 3 days after potting.

Tomato and petunia plants were germinated and planted out as described for tobacco plants. At 4 to 6 weeks post-germination these plants were ready for inoculation; at this stage *N. benthamiana* plants were between 5 to 8 cm in height and supported 4 to 6 leaves. Plants were kept in a greenhouse maintained at an average daytime temperature of 25°C, which fell to *ca.*16°C at night. Relative humidity was greater than 60% and natural lighting was supplemented by mercury vapour lights with a photoperiod of 18h.

Bean plants, *Phaseolus vulgaris cv.* The Prince, were kindly provided by the Botanical Supply Unit of University of London, as germinated seedlings in Fisons compost. These seedlings were grown under the environmental conditions described above to the fully expanded, primary leaf, stage prior to inoculation (section 2.6c).

Potato plants were grown from virus seed tuber certificate (VSTC) grade tubers under different environmental conditions.

Greenhouse conditions were, an average daytime temperature of 20°C, which fell to 10°C at night. Relative humidity was greater than 50% and natural lighting was supplemented by mercury vapour lights with a photoperiod of 16h. Seed tubers were allowed to shoot in the greenhouse for 10 to 14 days before shoots were cut-out and individually planted into 15cm round pots.

All plants were regularly watered, as required, and supplemented, once a week, with an NPK fertiliser (Phostrogen®).

2.6c Plant inoculation

Several sources of inoculum were used for mechanical inoculation;

1. Partially purified virus prepared from infected plants.
2. Purified virus.
3. Cloned virus DNA, excised from plasmids.

Plant derived inocula were prepared by methods that will be described in section 2.6e, inoculation with cloned virus DNA used an aqueous solution of both viral DNA components which had been excised from their cloning vehicles. For TGMV approximately 50μg of each DNA component was applied per plant, whereas PYMV only required approximately 10μg of each DNA component to obtain a high efficiency of infection. All inocula required the same method for mechanical transmission regardless of inoculum type. *N. benthamiana* plants at the 4 to 6 leaf stage were lightly dusted with F320 grade carborundum powder (Carborundum Company). The inoculum was then rubbed into the three uppermost leaves with a glove covered finger. Inoculated plants were rinsed with tap water to remove residual carborundum and aid in the sealing of lesions caused by abrading the leaf surface.

Plants displaying symptoms 12 to 16 days post-inoculation and were harvested 7 to 14 days later.

2.6d Detached scion grafting

Detached scion grafting is the most common method of grafting between potato, tomato and tobacco plants (Smith, 1935). Grafts were performed by cutting the stem of the plants at a node and then making 'W' shaped cuts in them. The plants to receive these apicies had complimentary, reciprocal incisions made in them. The graft union was

then sealed with Parafilm® (American Can Co.) and covered with a propagator cover to prevent wilting. Grafts performed between potato and tobacco plants took approximately 2 to 3 weeks to form a union and start growing vigorously to produce new shoots. Several types of graft were undertaken, including controls;

1. Potato apicies onto infected tobacco scions
2. Infected tobacco apicies onto healthy potato scions
3. Healthy potato apicies onto healthy potato scions
4. Healthy tobacco apicies onto healthy tobacco scions
5. Healthy tobacco apicies onto infected tobacco scions.

2.6e Virus purification and storage

Two methods were used to obtain purified virus from systemically infected plant tissue, all operations in both methods were carried out at 4°C. The method of Stein *et al.* (1983) was used to purify both TGMV and PYMV. Infected leaf material was homogenised in 0.1M trisodium citrate, 0.75% sodium sulphite, 5mM EDTA, 1% (v/v) 2-mercaptoethanol, 0.325% L-ascorbic acid, adjusted to pH 7.0 with NaOH (CEM buffer), (2mg/ml of tissue) using a Polytron homogeniser (Kinematica GMBH, Luzern, Schweiz). The homogenate was made 2.5% (by volume) in Triton X-100 (Rose Chemicals) and stirred overnight at 4°C and filtered through 2 layers of muslin. The filtrate was centrifuged at 10,000 xg for 15min and the supernatant decanted and centrifuged at 125,000g for 2h. The pellets were resuspended in CEM buffer (1/20 the original volume) using a rubber-policeman, and left overnight at 4°C (this material is the partially purified virus referred to in section 2.6b and commonly used as a plant inoculum and a source of viral specific DNA forms). The material was centrifuged at 8,000 xg for 20min and the supernatant was layered over a 1ml cushion of 20% sucrose in CEM buffer and centrifuged at 150,000 xg for 3h. The repelleted virus was resuspended in (1/30 original volume) CEM buffer and the suspension centrifuged at 8,000 xg for 20min. Approximately 1.5ml of the supernatant was then layered onto 38ml gradients of 10% to 50% sucrose in CEM buffer and centrifuged at 53,000 xg for 16h. The gradients were

either preformed using a gradient maker (Buchler Instruments) or step gradients, which were allowed to diffuse overnight at 4°C.

After centrifugation the gradients were viewed under fibre optic illumination and fractionated from either the top or the bottom. The material was passed through either a Uvicord (LKB) monitor, at 260nm, attached to a chart recorder (LKB) or a spectrophotometer fitted with a flow-cell, monitoring at 260nm. Fractions (2ml) were collected using a microcol TDC80 fractionator (Gilson) and those containing virus were dialysed against 2 x 4 litres of 0.1M trisodium citrate , 1mM EDTA (CE buffer), adjusted to pH 7.0 with NaOH, and stored at 4°C (this is the material referred to as purified virus in section 2.6e) or purified further by Cs_2SO_4 density centrifugation.

Solid Cs_2SO_4 was added to the dialysed virus until a density of approximately $1.3gcm^{-3}$ was obtained. The refractive index of the solution was determined using a refractometer, and converted to ρ^{25} (density at 25°C) using the equation:

$$n_D^{25} = 0.0730\ \rho^{25} + 1,2646$$ (Hearst and Vinograd, 1965)

The solution was then centrifuged for 24h at 11,5000 xg at 4°C. Gradients were viewed under fibre optic illumination and manually fractionated from the top. The virus fraction was dialysed against 2 x 4 litres of CE buffer and stored at 4°C. Virus from which coat protein was to be extracted was stored at -20°C.

The second method of virus purification was based on that of Adejare and Coutts (1982). Infected plant tissue was homogenised in 1.5ml/g 100mM sodium citrate (pH 7.0), 1mM EDTA, 1% (v/v) thioglycolic acid (TGA). The homogenate was filtered through two layers of muslin, stirred with an equal volume of chloroform : butanol (1:1, v/v, Mumford, 1974 and Bock *et al.*, 1978) for 15min at 4°C and then centrifuged at 10,000 xg for 15min. The aqueous layer was removed by asperation and the solvent interface layer re-extracted 4 times with a total of 0.5ml/g of the homogenisation buffer. The aqueous phases were combined, to give a final extraction rate of 2ml/g and centrifuged at 10,000 xg for 15min. The supernatant was decanted and centrifuged for 2h at 125,000 xg. The virus pellet was resuspended with a rubber-policeman overnight at 4°C in 50mM sodium citrate (pH 7.0), 5mM EDTA. This partially purified virus was clarified by

microcentrifugation for 10min, the pellets re-extracted with fresh buffer and the supernatants pooled prior to centrifugation at 125,000 xg for 2h. The pellets were resuspended over 4h in a total of 1ml 50mM sodium citrate (pH 7.0), 5mM EDTA and clarified by microcentrifugation for 10min at 4°C. The pure virus supernatant was transferred to clean tubes and stored in 100µl aliquots at -20°C.

2.6f Electron microscopy

20µl of pure virus (from either of the above purification methods) was added to a formvar coated electron microscope grid (kindly supplied by Mr Ken Laing). The buffer was drawn off through the arms of the forceps holding the grid with a filter paper wedge. The grid was washed with 3 to 5 drops of 50mM sodium citrate (pH 7.0), 5mM EDTA before staining with 3 to 5 drops of uranyl formate, sodium hydroxide (pH 4.5) (Roberts *et al.*, 1984) or 3% phosphotungstic acid (PTA) for 10min. The grid was blotted dry on filter paper and then stored on dry filter paper prior to examination on a Phillips 301 B electron microscope .

Uranyl formate stain was prepared by adding 1M NaOH, dropwise, to a saturated aqueous solution of uranyl formate (a kind gift from Dr I.M. Roberts) until the precipitate formed no longer re-dissolved upon agitation. Litmus was used to check that the pH was 4.5 and the stain filtered prior to storage at 4°C. Just prior to use the stain was diluted 1:5 with sterile dH_2O (Dr I.M. Roberts pers. comm.).

2.6g.i DNA extraction

DNA to be extracted from any form of plant tissue underwent the same extraction method. The sample was made 0.5% SDS, 0.5mg/ml proteinase k and incubated at 65°C for 10min before being extracted with an equal volume of TAE saturated phenol and microcentrifuged for 5min. The upper aqueous phase was transferred to a clean tube and then extracted repeatedly with water saturated diethyl ether. To the aqueous layer was then added 1:10 (v/v) 3M sodium acetate (pH 5.5) and 2 volumes of cold (-20°C) absolute ethanol. The DNA was precipitated at -20°C overnight prior to microcentrifugation at 4°C for 10min. The ethanol was discarded and the pellet resuspended in a minimum

volume of TAE. Samples of this DNA were electrophoresed on 1% agarose gels to determine the best working volume for resuspension.

2.6g.ii Preparation of purified virus supercoiled DNA

Supercoiled geminiviral DNA was purified by the method of Sunter *et al.* (1984). Partially purified virus, as referred to in sections 2.6c and 2.6e, was resuspended in TNE and 2 volumes 0.2M NaOH, 1% SDS, gently mixed and incubated for 5min on ice. To this mixture was added 1.5 volumes 3M potassium acetate (pH 4.8) and after vortexing it was incubated for 60min on ice. The precipitate formed was pelleted by centrifugation at 10,000 xg for 10min and the supernatant pipetted through a layer of muslin into a clean tube. Nucleic acid was precipitated by the addition of 2.5 volumes of cold (-20°C) absolute ethanol and incubation at -20°C overnight. The nucleic acid precipitate was centrifuged at 10,000 xg for 15min, the supernatant discarded and the pellet vacuum dried before resuspending in TNE. The density of this solution was then adjusted to 1.55g/ml with CsCl and ethidium bromide added to a final concentration of 600mg/ml. The CsCl solution was centrifuged to equilibrium at 35,000rpm for 65h at 20°C in a Beckman SW50.1 rotor, after which a band of ultraviolet fluorescent DNA could be seen when viewed by ultraviolet illumination. This band was removed with a sterile needle and syringe and transferred to an Eppendorf tubes. Ethidium bromide was removed by exhaustive extraction with CsCl saturated propan-2-ol and the supercoiled DNA diluted with 2 volumes dH_2O before precipitating with 6 volumes of cold (-20°C) absolute ethanol at -20°C overnight. The DNA was microcentrifuged for 10min at 4°C and the pellet washed with cold (4°C) 70% ethanol before vacuum drying and resuspending in a minimum volume of TE. The supercoiled DNA product was of a sufficient purity to be directly digested with restriction endonucleases without further purification.

2.6h Nuclease digestions

Several different nuclease digests have been performed on different types of viral nucleic acids.

Gross deoxyribonuclease I digestion of purified virus

Pure virus has associated with it co-purified viral specific double-stranded DNA species, these species were digested with bovine pancreatic DNase I. This enzyme is an endonuclease that hydrolyses dsDNA or ssDNA to a complex mixture of mono- to oligonucleotides with a 5'-phosphate termini (Melgar and Goldthwaite, 1968). Pure virus was made 100mM $MgCl_2$, 5µg/ml DNase I (from a 1mg/ml stock stored frozen at -20°C) and incubated at 25°C for 15min. Mg^{2+} ions were then chelated by the addition of 500mM EDTA (pH 8.0) to a final concentration of 50mM.

S1 nuclease digestion

S1 nuclease (*Aspergilus oryzae*) degrades single-stranded DNA (Vogt, 1973) to yield 5'-phosphoryl mono- or oligonucleotides. Moderate amounts of the enzyme cleave duplex nucleic acids at nicks or small gaps. Ethanol precipitated DNA was pelleted, dried and resuspended in S1 buffer (100mM sodium acetate, 0.28M NaCl, 9mM $ZnSO_4$ (pH 4.6), divided into aliquots, and digested with increasing concentrations of S1 nuclease (BRL), typically 0, 0.5, 1.5, 3.0 and 10 units/µl. Reactions proceeded at 37°C for 30min and were stopped by the addition of an equal volume of S1 termination buffer (2.5M ammonium acetate, 50mM EDTA). Samples were extracted with phenol, Sevag and ethanol precipitated prior to resuspension in TAE and agarose gel electrophoresis.

Ribonuclease A digestion

Bovine pancreatic RNase A is an endoribonuclease that specifically attacks RNA pyrimidine nucleotides at the 3'-phosphate group and cleaves the 5'-phosphate linkage to the adjacent nucleotide. The end products are pyrimidine 3'-phosphates and oligonucleotides with terminal pyrimidine 3'-phosphates (Davidson, 1972). RNase A (2mg/ml stock stored frozen at -20°C) was added to the digestion mixture to a concentration of 1µg/µl and incubated at 37°C for 30min. Samples were extracted with phenol, Sevag and ethanol precipitated prior to resuspension in TAE and agarose gel electrophoresis.

Exonuclease III digestion

E. coli Exo III catalyses the stepwise 3' to 5' removal of 5' mononucleotides from dsDNA carrying a 3'-OH end (Weiss, 1976). The enzyme also carries three other activities; an endonuclease specific for apurinic DNA, an RNase H activity (Rogers and Weiss, 1980) and a 3'-phosphotase activity. DNA to be digested was resuspended in Exo III buffer (66mM tris-HCl (pH 8.0), 0.66mM $MgCl_2$, 1mM 2-mercaptoethanol), Exo III (Pharmacia)added to 16 units/µl and incubated at 37°C for 2h prior to extraction with phenol, Sevag and ethanol precipitation. Samples were resuspended in TAE and electrophoresed through agarose.

2.7 Serological methods

The antiserum used in all serological studies presented here was that raised by Dr V.E. Stein (Stein *et al.*, 1983). TGMV gave a single precipitin line with this antiserum to an end-point titre of 1/256.

2.7a Gel immunodiffusion plates

Serological tests by gel immunodiffusion were by double-diffusion (Ouchterlony, 1962) in 1% agarose gels containing 70mM sodium phosphate (pH 7.0), 0.02% sodium azide. Molten agarose (10ml, of a 2% solution) in dH_2O was diluted 1:1 in the above buffer, poured into a petri dish (Sterilin) and allowed to set. These gels were stored at 4°C in a sealed box containing moist tissue paper to prevent dessication. Wells were cut (5mm in diameter and 3mm apart) in the gel using a cork borer. Two-fold serial dilutions, in 70mM sodium phosphate, of purified virus and/or antiserum were used to obtain optimum conditions for interaction and precipitin line formation. Gels were incubated at RT in a moist box for 48h and examined using dark ground illumination. Gels were then washed thoroughly in 0.07M sodium phosphate (pH 7.0), 0.15M NaCl to remove unbound proteins, stained with Coommasie Blue and destained (see section 2.5c) before being viewed over a cold white light transilluminator. A control sample of healthy *N. benthamiana* plant material was always included in each plate. The healthy sample was prepared by extracting tissue as in section 2.6e by the Triton extraction method, as far as the first high-speed pelleting stage.

2.7b Western dot hybridisation

Viral protein samples were bound to a nitrocellulose sheet (Bio Rad), marked out in 2 x 2 cm squares, by application in an aqueous volume of 20µl and allowed to dry. The nitrocellulose was incubated in blot-saline (0.3M NaCl, 10mM tris-HCl (pH 7.4)), containing 3% BSA, at 40°C for 60min with constant agitation. After washing 5 times for 5min each, with agitation, in blot-saline, the nitrocellulose was incubated with constant agitation for 3h at RT in a 1:16 dilution of TGMV antiserum in 3% BSA in blot-saline. The nitrocellulose was washed 5 times for 5min each in blot-saline and then incubated with 0.75% (v/v) fluorescein isothiocyanate-conjugated sheep anti-rabbit antiserum (a kind gift from Dr S.E. Adams) in 3% BSA in blot-saline for 60min at RT. After washing 5 times for 5min each in blot-saline the nitrocellulose sheet was visualised on a uv transilluminator and photographed. Healthy plant extracts, produced as described in section 2.7a, were always included as controls.

2.7c Immunosorbent electron microscopy

Immunosorbent electron microscopy (ISEM, Katz and Kohn, 1984) was used in an attempt to concentrate virus particles on the electron microscope grid. Formvar coated grids were incubated on drops of TGMV antiserum placed on wax covered paper for 30min at RT. Grids were washed with 5 drops of 50mM sodium phosphate buffer (pH 7.0) and incubated on drops of purified virus placed on wax paper for 30min at RT. After washing in 5 drops of 50mM sodium phosphate (pH 7.0) the grids were incubated for a further 30min on antiserum to "decorate" trapped virus particles and make them more electron dense. Grids were washed, stained and visualised as described in section 2.6f.

2.8 Tissue culture

All tissue culture manipulations were carried out aseptically in a laminar air flow cabinet (Microflow).

2.8a Establishment and maintenance of plant cultures

Plant tissue was cultured in three different forms:

1. Shoot cultures
2. Callus cultures
3. Suspension cultures

2.8a.i Shoot cultures

Originally shoot cultures of potato initiated by Dr S.E. Adams were the source material for protoplast experiments. However, later cultures were initiated as described below.

The establishment and maintenance of shoot cultures was based on the methods of Thomas (1981) and Nelson *et al.* (1983). Axillary buds were excised from plants grown as described in section 2.6b and surface-sterilised by rinsing in absolute ethanol followed by soaking for 20min in 10% (v/v) aqueous solution of household bleach (Parazone). After rinsing thoroughly in six washes of sterile dH_2O the sterile buds were transferred to plastic capped jars (Gallenkamp) containing 50ml of basal medium consisting of the inorganic salts and vitamins of Murashige and Skoog (1962) with 20g/l sucrose, 10g/l 'Noble' agar (Difco) and 0.25mg/l BAP. This level of BAP is five times that in normal basal medium used for sub-culturing plants and was used to enhance shoot proliferation. To prepare 1l of basal medium 20g sucrose was dissolved in 500ml 2 x MS (Flow Labs., see section 2.4) followed by the addition of 0.5ml 0.5mg/ml BAP and *ca.* 400ml deH_2O. The media was adjusted to pH 5.8, made up to 1l with deH_2O and 50ml was added to each jar containing 0.5g 'Noble' agar. The media was the autoclaved and allowed to set before use. The buds were incubated at 25°C in 2,500lx with the plastic lids of the jars slightly loosened to avoid the accumulation of ethylene which can cause abnormal growth of shoot cultures (Hussey and Stacey, 1981). After initiation of the first shoots the cultures were propagated 4 to 6 weeks by cutting the growing shoots into segments , each possessing a leaf and an axillary

bud, and transferring them to fresh basal medium in which the concentration of BAP was reduced to 0.05mg/ml. After the shoot cultures were established , 20 to 30 stem segments were grown per jar. Any cultures which became contaminated with bacteria or fungi were discarded.

2.8a.ii Callus cultures

Potato callus was initiated from leaf or stem tissue of plants grown as described in section 2.6b. Excised tissue was surface sterilised, as described for shoot culture initiation, and placed on a callus inducing medium, CM18 (Dr M.G.K. Jones, pers. comm.), containing the inorganic salts and vitamins of MS with 20g/l sucrose, 10g/l 'Noble' agar, 2mg/ml BAP and 1mg/ml NAA. The medium was adjusted to pH 5.6 prior to autoclaving and poured into sterile petri dishes (Sterilin) after autoclaving. Plates were incubated at 25°C in the dark. The resulting undifferentiated callus tissue which grew was sub-cultured every 2 weeks and was used as the source material for establishing potato suspension cultures.

2.8a.iii Suspension cultures

A suspension culture cell line of *Solanum tuberosum* L. *ssp tuberosum cv.* Maris Bard was originally obtained as a gift from Dr M.G.K. Jones of Rothamsted Experimental Station. The cell lines currently in use are however independently derived from potato stocks grown at Imperial College. Suspension cultures were initiated from the callus material described above by placing callus into a liquid medium and shaking them at 120rpm in a rotary shaker maintained at 25°C in the dark. The liquid medium was based, once again, on the inorganic salts and vitamins of MS with 30g/l sucrose, 2g/l casein hydrolysate, 9.9mg/l thiamine HCl, 9.5mg/ml pyroxidine HCl, 4.5mg/l nicotinic acid, 2mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), and 0.25mg/l kinetin. The callus material breaks up with agitation but continues to grow and divide to produce a fine suspension of cell clusters. Suspensions were sub-cultured every week by transferring 10ml of culture to 40ml fresh medium in a 250ml flask using a sterile wide-mouthed pipette (Sterilin).

2.8b Protoplast isolation

2.8b.i Protoplast isolation from shoot cultures

The procedure for the isolation of protoplasts from shoot cultures was that of Adams *et al.* (1985), which was based on the method of Nelson *et al.* (1983). Protoplast isolations were made from shoot cultures which had been growing for *ca.* four weeks after sub-culturing. Shoots were cut into 1 cm pieces using a sterile scalpel on a baked tile. Samples of approximately 2g were soaked overnight at 4°C in 50ml medium A (Shephard and Totten, 1977) containing 1mg/ml NAA, 0.5mg/ml BAP but no sucrose or agar (see section 2.4) in a sterile and siliconised 150ml Erlenmeyer flask. Medium A was then replaced with 50ml 0.45M D-mannitol (pH 5.6) and the samples were incubated at 30°C in a shaking water bath at 50 strokes/min to plasmolyse the cells. After 30 min the mannitol was replaced with 50ml 1.5% cellulase R10 and 0.3% macerozyme R10 (Yakult Honsha) in 0.4M D-mannitol and the major salts of Medium A at pH 5.6 (see section 2.4). This enzyme medium was filter-sterilised prior to use (see section 2.5a). The tissue was incubated for 3 to 4h at 30°C in a shaking water bath (50 strokes/min) in the dark. The released protoplasts were filtered through a sterile nylon cloth with a mesh size of 77μm (a gift from Henry Simon Ltd.) by gently transferring the protoplast suspension with a sterile wide bore plastic pipette (Alpha Laboratories) through the mesh into two sterile plastic Falcon tubes (Becton Dickinson). The protoplasts were sedimented by centrifugation at 28 xg for 40s in a BTL bench centrifuge and washed by decanting the enzyme solution and gently resuspending the protoplasts in 20ml 0.4M D-mannitol. The resuspended protoplasts were washed twice more by centrifugation and resuspension in 10ml 0.4M D-mannitol. Finally, the protoplasts from each tube were combined and the yield was determined by counting samples in a modified Fuchs-Rosenthal haemocytometer (Hawksley).

Protoplast viability was determined using the exclusion dye phenosafranine (Widholm,1972). The phenosafranine was made 0.4M in D-mannitol to give a final concentration of 0.1%. Samples of the protoplast suspensions were mixed with an equal volume of the stain and examined microscopically. Protoplasts which remained green were

regarded as intact and viable whereas those damaged protoplasts stained red. Those protoplasts which took up stain were not counted.

2.8b.ii Protoplast isolation from suspension cultures

Protoplasts were isolated from approximately 25ml (packed cell volume) of Maris Bard suspension culture cells, which had been passaged three days previously. The cells were harvested by allowing them to settle in a sterile Falcon tube and pipetting off the supernatant. These cells were resuspended in 40ml of an enzyme 'cocktail', to release protoplasts. Several different enzyme mixtures were used, but the two most common mixtures are listed below:

2%	Cellulase R10 (Yakult Honsha)
0.3%	Macerozyme R10 (Yakult Honsha)
0.3%	Pectolyase Y23 (Seishin Pharmaceuticals)

or

4%	Meicelase (Meiji Seika Kaisha)
2%	Rhozyme HP150 (Rohm & Hass)
0.03%	Macerozyme R10 (Yakult Honsha)

Both mixtures were dissolved in 0.4M D-mannitol containing the major salts of medium A (see section 2.4). These solutions were then centrifuged at 1,800 xg for 10min in a BTL bench centrifuge, the supernatant decanted and adjusted to pH 5.8 before being filter sterilised (see section 2.5a). Enzymatic digestion was carried out at 25°C for 4 to 5h in an orbital incubator at 80rpm. The protoplasts released were gently pipetted through sterile nylon cloth (Henry Simon Ltd.) of decreasing pore size (550μm,180μm and 77μm), washed and counted as previously described for for shoot culture derived protoplasts and then inoculated by one of the following methods.

2.8c Inoculation of protoplasts

Potato protoplasts were inoculated by several techniques, using either polyethylene glycol (PEG) or poly-L-ornithine (PLO) as agents to stimulate uptake of the inoculum. PEG inoculation was by the method of Maule *et al.* (1980). 100μg cloned TGMV DNA (50μg A component DNA and 50μg B component DNA) was added to 2ml filter sterilised 40% PEG

(Mr. 6000), 3mM calcium chloride, which had been pre-chilled on ice in a sterile Falcon tube. Freshly prepared protoplasts (1 x 10^5 to 1.5 x 10^5) were sedimented by centrifugation at 50 xg for 40s, resuspended in 200µl 0.4M D-mannitol and added to the PEG/calcium chloride/TGMV DNA mixture. After shaking gently for 20s the suspension was diluted with 20ml 0.4M D-mannitol, 0.1mM calcium chloride with gentle shaking and incubated at RT for 30min. The protoplasts were then washed two times with 10ml 0.4M D-mannitol, 0.1mM calcium chloride by centrifugation and gentle resuspension. The cells were then resuspended in 10ml 0.4M D-mannitol, 5mM magnesium chloride, containing 100µg DNase I and incubated at 25°C for 15min (Lurquin and Kado, 1977). This post-inoculation DNase treatment removes residual DNA not taken up by the protoplasts. After this treatment the protoplasts were washed twice in 0.4M D-mannitol by centrifugation and gentle resuspension before finally being taken up in an incubation medium (see section 2.8d).

PLO inoculation was based on the method of Suzuki and Takebe (1978). 100µg cloned TGMV DNA (50µg A component DNA and 50µg B component DNA) or 20µg pure TGMV (DNase I treated as in section 2.6h) was added to 1ml 0.4M D-mannitol containing 10µl 1mg/ml PLO and this mixture was pre-incubated for 10min at 25°C. This pre-incubation mixture was added to 1 x 10^5 to 1.5 x 10^5 freshly prepared and sedimented protoplasts which had been resuspended in 1ml 0.4M D-mannitol, 5mM zinc sulphate and 1mM calcium chloride and the mixture incubated for 60min at 25°C. After inoculation the protoplasts were washed in exactly the same manner as above.

2.8d Protoplast culture and sampling

Freshly inoculated, protoplasts were finally resuspended in Rm medium (see section 2.4) at a plating density of 100,000 cells/ml and supplemented with 200mg/ml carbenicillin, (Beechams) and 100U/ml Nystatin (Squibb and Sons)(see appendix 5.2) to control microbial contamination. Cells were cultured at 20°C in the dark for 24h before transferring to continuous Cool White fluorescent illumination of 1,500lx at 20°C. Antimicrobial agents were supplemented every 48h after isolation with further additions of 100mg/ml carbenicillin and

50U/ml Nystatin. Samples of 100,000 protoplasts were taken over a time course. Samples were microcentrifuged for 30s, the supernatant discarded and the cells resuspended in 400µl TNE before the DNA was extracted as described in section 2.6g. Alternative plating techniques were used when this method did not produce regenerated calli.

2.8d.i Radioactive precursor incorporation

The state of protoplast nucleic acid metabolism was monitored in liquid culture by addition to the medium of the radioactive nucleic acid precursors, ^{3}H-UTP ([5,6-^{3}H]-uridine-5'-triphosphate, Amersham, 43Ci/mmol., Francki *et al.*, 1971, Coutts *et al.*, 1975) and ^{3}H-thymidine ([methyl-^{3}H]-thymidine, Amersham, 49Ci/mmol., Zelcer and Galun, 1976). Incorporation was assayed in one of two ways, either continuously by the addition of precursor (2µCi/ml of medium) to the whole culture and by taking 1ml extracts over a time course or pulsed by the addition of precursor (2µCi) to a 1ml aliquot of the protoplasts taken from the main culture and incubated for 2h in a sterile, siliconised, glass scintillation vial. In both cases samples were analysed similarly. Labelled protoplasts were microcentrifuged for 30s, the supernatant discarded and the cell pellet resuspended in 100µl TE. The cells were collected on 2.5 cm discs of Whatman 3MM (Whatman) paper and washed with 50ml 5% trichloroacetic acid (TCA), 10mM sodium pyrophosphate, followed by 30ml ethanol. The discs were dried, placed in 8ml of aqueous scintillant (see Appendix 5.3) and the amount of radioactivity incorporated into TCA insoluble material determined by counting in a Tri-carb C2425 scintillation counter (Hewlett Packard).

2.8d.ii Feeder layer plating

Feeder layer plates (Shneyour *et al.*, 1984) were prepared by autoclaving 0.4% low gelling temperature agarose (Sea Plaque, Marine Colloids) dissolved in 2 x CM18 medium (see section 2.4) and allowing it to cool to 37°C. To this was added 4ml of three day Maris Bard suspension culture cells and the mixture poured immediately into a sterile petri dish. After allowing the agarose to gel a circular sheet of sterile 525Pu cellophane (a gift from BCL Cellophane) was overlaid onto it. 2ml protoplasts (10,000/ml), three days post-inoculation, were

pipetted onto the cellophane, as a thin monolayer. Petri dish-sized cellophane discs were prepared by boiling in 5mM EDTA, washing in deH$_2$O and autoclaving in layers between filter paper soaked in CM18 medium. Plates were incubated at 20°C in continuous Cool White fluorescent illumination of 1,500lx.

2.8d.iii Agarose bead culture

Bead culture, based on the method of Shillito *et al.* (1983), was another method used for protoplast culture. In this method *ca.* 50,000 three day post-inoculation protoplasts were centrifuged at 28 xg for 40s and the supernatant removed before resuspending them in 5ml 2 x Rm medium (see section 2.4). These cells were immediately added to 5ml 0.8% low gelling temperature agarose (at 37°C) which had been dissolved and autoclaved in deH$_2$O. The mixture was poured into a 5cm diameter, sterile, petri dish and allowed to set for at least 2h. The gel was then cut into four equal parts and two added to 40ml sterile CM18 medium in a 250ml wide mouth jar (Gallenkamp). This culture was then shaken on a rotary shaker at 120rpm maintained in the dark at 25°C.

2.9 Preparation of radioactive DNA and hybridisation methods

2.9a Nick-translation of plasmid DNA

Plasmid DNA was radioactively labelled with [α^{32}P] dCTP by the method of Rigby *et al.* (1977).

Buffers and reagents :

5 x nick translation buffer : 500mM tris-HCl (pH7.4)
100mM 2-mercaptoethanol
50mM $MgCl_2$
stored in aliquots at -20°C.

Nucleotides :

300µM dATP
300µM dTTP
300µM dGTP
in sterile dH_2O and stored at -20°C.

The following reagents were added to an Eppendorf containing 20µCi [α^{32}P] dCTP (Amersham) which had been dried *in vacuo* :

	Nick translation mix (µl)
5 x nick translation buffer	20
300µM dATP	5
300µM dTTP	5
300µM dGTP	5
3mg/ml nuclease free BSA (BRL)	1
1µg plasmid DNA template	1 to 10
sterile dH_2O (to make final volume 100µl)	49 to 51

After gentle vortexing, two 1µl aliquots were removed and dried onto two 2.5cm diameter discs of Whatman 3MM paper, to be used later as controls for background and input radiation. DNase I, from a frozen stock at 1mg/ml, was added to, the reaction mix to a final concentration of 4µg/ml followed by 2 units DNA polymerase I (Amersham). The reaction was incubated at 16°C for 90min and terminated by the addition of 10µl of 1M NaOH. 1µl aliquots were removed at 30min intervals and dried onto 2.5cm diameter discs of Whatman 3MM paper. One of the two original discs was placed directly into 8ml liquid scintillant (appendix 5.3), but all others were washed in 50ml 5% TCA, 10mM sodium pyrophosphate followed by 30ml ethanol and drying. Individual discs were scintillated in 8ml of a liquid

scintillant and counted in a Tri-carb C2425 scintillation counter (Hewlett Packard). After subtracting background radiation from the figures obtained, the percentage incorporation, as a fraction of the input radiation, was determined. Figures for incorporation varied between 50 and 80%.

Labelled DNA was fractionated away from unincorporated [α^{32}P] dCTP by the 'spun-column' procedure (Maniatis *et al.*, 1982). This method is very useful when several preparations of DNA are labelled simultaneously or when it is necessary to change the buffer in which the DNA is dissolved. A sterile 2ml syringe barrel was plugged with a disc of Whatman 3MM paper and filled with a slurry of sephadex G50 or G75 (Pharmacia) which had been previously prepared by boiling in TE and autoclaving. The syringe was placed, so that it hung, in a 15ml corex centrifuge tube which was centrifuged at 167 xg for 2min. The packed column was then loaded with 75µl of TE and centrifuged at 167 xg for 1min with a 1.5ml, capless, Eppendorf placed under the barrel of the syringe. If the volume eluted was not *ca.* 75µl then the column was discarded and a new one prepared. The nick translation reaction-mix was added to the centre of the column and, with a fresh Eppendorf in place, it was centrifuged for 1min at 167 xg. The column was then loaded with successive amounts of TE (75µl) and centrifuged after each addition at 167 xg for 1min, with a clean tube collecting each eluate. Tubes were analysed for radioactivity either with a hand-held Geiger counter or in the scintillation counter. It was noted that the amount of label eluted possessed two peaks, one within the first 5 Eppendorfs and the second later in the 15th or more Eppendorf. The first peak contained the ^{32}P-labelled DNA and was used for hybridisation. Just prior use, the probe was denatured by boiling for 5min and 'snap-cooled' in an ethanol ice bath at -20°C.

2.9b Preparation of strand-specific DNA probes

Strand-specific probes were prepared from M13 ssDNA templates isolated as described in section 2.10j. Reactions were based on the method of Hu and Messing (1982) using the M13 hybridisation probe primer (Biolabs):

$^{5'}$ CACAATTCCACACAAC $^{3'}$

The following reaction mix was prepared :

	Reaction mix (µl)
10ng/µl boiled primer	1
10 x annealing buffer (section 2.11b.i)	1
0.1M DTT	1
1 to 2µg M13 ssDNA template	7 to 1
sterile dH_2O	6 to 0

After gently vortexing and briefly microcentrifuging the mixture was incubated at 65°C for 15min and then allowed to cool to RT for 30min. To the annealed template and primer was added 1µl of nucleotide mix (dATP, dTTP and dGTP each at 500µM). This mixture was then microcentrifuged and transferred to a Eppendorf tube containing 10µCi [α^{32}P] dCTP which had been dried *in vacuo*, and 4 units of large subunit DNA polymerase I (see section 2.11b) added. The reaction was incubated at 16°C for 60min before termination by the addition of 1µl 500mM EDTA. Incorporation was not monitored because of the small reaction volume but it has been estimated that it was in excess of 60%. The labelled DNA was fractionated by the 'spun column' procedure (section 2.9a). The probe was <u>not</u> denatured by boiling before use.

2.9c <u>Southern transfer</u>

Transfer of DNA from 1% agarose gels was as first reported by Southern (1975) and according to the methods described for Gene Screen™ and Gene Screen *Plus*™ hybridisation membranes in the New England Nuclear catalogue. The two types of membrane used during this study used slightly different methods for transfer. All incubation steps in the following protocols were accompanied by constant agitation.

<u>Transfer to Gene Screen™ (GS)</u>

DNA samples were electrophoresed as described in section 2.5b, photographed and the wells cut away. The DNA in the gel was depurinated in 0.25M HCl for 15min and denatured for 30min in 0.2M NaOH, 0.6M NaCl. The gel was washed in 3 changes of 1 x SSC (section 2.4) for 20min each. During this time the Gene Screen™ membrane was cut to size and soaked in 1 x SSC for 15 to 20min. An elevated glass plate, covered with a sheet of Whatman 3MM, in a

glass baking dish was soaked in 1 x SSC. The gel was laid in the middle of this paper and surrounded with Parafilm® strips to prevent buffer passing around the gel. The transfer membrane was positioned on the gel, carefully excluding all air bubbles, and 5 sheets of gel sized Whatman 3MM placed on top of it. A 3 to 6 cm stack of absorbent paper towels was laid over the 3MM sheets and approximately 500ml 1 x SSC added to the dish. The whole structure was covered with Cling-Film®, a large weight (approximately 800g) was placed on top of the paper and capillary transfer of the DNA allowed to take place overnight. The structure was dismantled, the membrane washed in 1 x SSC, before being dried and baked for 2 to 4h at 80 to 100°C.

Transfer to Gene Screen *Plus*™ (GS+)

Transfer to GS+ was as described for GS except for a few details. After depurination and denaturation the gel was incubated in 1.5M NaCl, 0.5M tris-HCl (pH 7.5) for 30min. A sheet of GS+ was cut to size, rinsed in dH_2O and soaked in 10 x SSC for 15min. The transfer structure was set up as above using 10 x SSC as the buffer and placing the concave side (side B) face down onto the gel. After overnight transfer the membrane was soaked in 0.4M NaOH for 60s, followed by 60s in 0.2M tris-HCl (pH 7.5), 2 x SSC and drying. GS+ did not require baking to bind the transferred DNA.

Gels were re-stained with ethidium bromide after blotting to assess the amount of DNA which had transferred to either membrane type. Occasionally bi-directional blots were produced using GS+ placed either side of the gel

2.9d Solid-phase hybridisation

Several hybridisation methods were used during the study and these are detailed bellow. Hybridisation was performed in two layers of heat sealed plastic bags. Membranes were prehybridised in the hybridisation solution, lacking the radioactive DNA to block any sites of non-specific binding, for a minimum of 4h at the hybridisation temperature. Approximately 10ml of solution was used per $50cm^2$ of membrane.

SSPE/formamide hybridisation

Buffers

20 x SSPE : 2.40M NaCl
0.30M sodium citrate
0.26M NaH_2PO_4
0.02M EDTA

50 x Denhardt's sol. 5% ficoll
(Denhardt, 1966) 5% polyvinylpyrrolidine
5% BSA

20ml hybridisation mix was prepared by mixing the following :

10ml deionised 99% formamide (Rose)
5ml 20 x SSPE
2ml 10% SDS
0.4ml 50 x Denhardt's solution
0.2ml 10mg/ml sheared/denatured Salmon sperm DNA
2.4ml dH_2O

Hybridisation was for 18 to 24h at 42°C in the presence of 100 counts per second of labelled DNA per ml of buffer. The membrane was washed twice in 100ml of each of the following; 2 x SSC for 5min; 2 x SSC, 1% SDS at 65°C for 30min and 0.1 x SSC for 30min. After briefly drying the membrane was wrapped in Saran Wrap™.

Heparin/dextran hybridisation (Singh and Jones, 1984)

Buffers

20 x SET : 2M NaCl
0.6M tris-HCl (pH 7.4)
40mM EDTA

20ml hybridisation mix was prepared by mixing the following :

4ml 20 x SET
4ml 50% dextran sulphate
0.4ml 10% SDS
0.4ml 5% sodium pyrophosphate
0.2ml 50 mg/ml heparin (sodium salt in 4 x SET)
5.5ml dH_2O

Hybridisation was for 18 to 24h at 65°C in the presence of 100 counts per second of labelled DNA per ml of buffer. The membrane

was washed twice in 100ml of each of the following; 2 x SET for 5min; 2 x SET, 0.2% SDS at 65°C for 30min; 1 x SET, 0.2% SDS at 65°C and 1 x SET for 5min. After briefly drying the membrane was wrapped in Saran Wrap™.

Formamide hybridisation

Buffers

5 x P : 1% polyvinylpyrrolidine
1% BSA
1% ficoll
0.25M tris-HCl (pH 7.5)
0.5% sodium pyrophosphate
0.5% SDS

20ml hybridisation mix was prepared by mixing the following :

10ml deionised 99% formamide (Rose)
2ml 5 x P
2ml 10mg/ml sheared/denatured Salmon sperm DNA
6ml dH_2O

Hybridisation was for 18 to 24h at 42°C in the presence of 100 counts per second of labelled DNA per ml of buffer. The membrane was washed twice in 100ml of each of the following; 0.3M sodium chloride, 0.06M tris-HCl (pH 8.0), 2mM EDTA for 5min; 0.3M sodium chloride, 0.06M tris-HCl (pH 8.0), 2mM EDTA, 0.5% SDS at 60°C for 30min; 3mM tris-HCl. After briefly drying the membrane was wrapped in Saran Wrap™.

Autoradiography

Membranes were autoradiographed in a light proof cassette with Kodak X-Omat S film. The length of time of autoradiography depended on the degree of label bound to the membrane (intensifying screens were used if necessary, at -70°C). Films were processed in a Kodak X-Omat automatic processor.

2.9e Solution hybridisation

Because the positive strand is always contained in the bacteriophage viral particle, two recombinant phage with a DNA fragment cloned in opposite orientations into the RF DNA will hybridise only via the insert region (Messing, 1983). Therefore, to test whether two recombinant phages have DNA complimentary to each other they were hybridised together, in solution, and electrophoresed in agarose. 1µl of each M13 ssDNA clone (prepared as in section 2.10j) to be tested was added to 8µl of TNE and heated to 65°C for 60min. After annealing, each sample was made 1 x in agarose gel loading buffer (section 2.4) and electrophoresed in 1% agarose, containing ethidium bromide, as described in section 2.5b. Samples which were complimentary, or had regions of sequence in common, hybridised via their insert region, and formed a figure of eight-like structure which migrated more slowly in the gel. This method has been used to examine all geminiviral full-length clones which were available (TGMV, kindly supplied by Dr W.D.O. Hamilton; CLV, kindly supplied by Dr J. Stanley and PYMV, as isolated during this work).

2.10 Bacterial cloning

2.10a Growth and maintenance of bacterial strains

Escherichia coli bacterial strains were subcultured monthly on minimal medium plates containing supplements specific for the particular auxotrophic requirements of each strain (see appendix 5.1) and stored ,sealed, at 4°C.

Minimal agar :

500ml	3% Bactoagar (Difco)
500ml	2 x M9 salts
1ml	0.1M $CaCl_2$
1ml	1M $MgSO_4$
1ml	20% glucose

These components were autoclaved separately and combined prior to pouring the plates.

Supplements :

0.5ml 1% Thiamine-HCl (Vitamin B_1) for JM 101 and TG2 strains.

50mg/l L-proline for strain HB 101

Supplements were filter sterilised (section 2.5a)

M9 salts :

6g	Na_2HPO_4
3g	KH_2PO_4
0.5g	NaCl
1g	NH_4Cl
	dH_2O to 500ml

Frozen stocks of cells were stored in minimal broth containing 20% (v/v) glycerol at -70°C, to be replated when required.

Plasmid clones containing recombinant DNA were stored in sealed plates at 4°C on L-agar plates containing the appropriate antibiotic and subcultured on a monthly basis. Overnight culture stocks were stored at -70°C in L-broth or 2TY-broth containing the antibiotic(s) and 20% (v/v) glycerol.

L-broth

1.0% Bacto Tryptone (Difco)
0.5% Yeast extract (Difco)
0.5% NaCl
0.1% glucose
pH 7.0-8.0 (≈3ml 1M NaOH/l broth)

2TY-broth

1.6% Bacto Tryptone
1.0% Bacto Yeast Extract
0.5% NaCl

2.10b Small scale restriction enzyme digestions

Various restriction enzyme digests have been used in this work. The enzymes were used with various restriction buffers depending on their requirements for NaCl (Maniatis *et al.*, 1982). The conditions of digestion were as recommended for each particular enzyme (summarised in the Biolabs catalogue 1986), at their individual optimum reaction temperatures. The buffers used were:

10 x buffer	NaCl	tris-HCl(pH 7.5)	$MgCl_2$	DTT	Spermidine
Low	0mM	10mM	10mM	1mM	10mM
Medium	50mM	10mM	10mM	1mM	10mM
High	100mM	50mM	10mM	1mM	10mM

These small scale digests were carried out in volumes of 10 to 20µl in the appropriate buffer, the enzyme being at a concentration of 2-5 units/µg DNA and the DNA concentration not exceeding 150µg/ml. If sufficient material was available digestions were checked by 1% agarose gel electrophoresis. Plasmid vector digests were commonly phosphatase treated to prevent self-religation. Calf-intestinal phosphatase, CIP, (BCL) was added to the restriction enzyme digest (0.1 unit/µl volume of digestion) and the mixture incubated at 37°C for 1h. The mixture was then extracted with 1 volume of TE saturated phenol and the aqueous layer transferred into a clean Eppendorf tube. Extraction with Sevag and transfer of the aqueous layer to a clean Eppendorf tube was followed by precipitation with 2 volumes of ethanol, pelleting, drying and resuspension in a minimum volume of TE.

2.10c Electroelution of DNA

General methods have been developed (Wu *et al.*, 1976) to recover DNA from agarose gels, none of them being entirely satisfactory. The efficiency with which DNA is extracted from agarose gels is a function of its molecular weight. DNA fragments less than 1kb in length can be recovered in a virtually quantitative yield, but as the molecular weight of the DNA increases, the yield drops. The most successful method for electroelution in this work is based on the method of Mc Donnell *et al.* (1977) and is described below.

Restriction enzyme fragments were subjected to agarose gel electrophoresis in TBE and bands of interest visualised using a long-wave (300-360nm) uv transilluminator. A slice of agarose containing the band was excised with a scalpel, placed in a dialysis bag containing a minimum volume of TBE and closed with 'mediclips' (Medicell), avoiding trapping any air bubbles. The bag was immersed in a shallow layer of TBE in an electrophoresis tank at right-angles to the electrodes. The DNA was electroeluted out of the agarose slice at 150V for 45min, with the current polarity being reversed 100V for the last 20s. The progress of the eluting DNA was monitored by placing the bag on the uv transilluminator. The dialysis bag was opened carefully and the buffer removed with a pasteur pipette. This buffer was then microcentrifuged for 5min to remove any agarose fragments and the supernatant extracted with an equal volumes of phenol followed by Sevag and precipitated with 2 volumes ethanol at -20°C overnight. The DNA precipitate was pelleted by microcentrifugation, vacuum dried and resuspended in TAE. Electroeluted DNA was always analysed by electrophoresing an aliquot in 1% agarose before any further manipulation to check the integrity and concentration of the eluted DNA.

2.10d Ligation

In ligation reactions insert DNA was present in 2 to 3-fold molar excess with respect to the vector DNA. Two important controls were always performed in parallel with the actual cloning ligation reaction. These controls were, unligated vector DNA (to indicate the extent of vector cleavage by the cloning restriction enzyme) and the digested vector DNA alone (to monitor the efficiency of the ligation reaction). Occasionally, digested vector DNA which had been phosphatase treated was used for ligation in the absence of foreign DNA, to assess the efficiency of the phosphatase reaction.

Buffers and reagents

10 x ligation buffer : 0.5M tris-HCl (pH 7.7)
0.1M $MgCl_2$
50mM spermidine
10mM DTT

10mM rATP was made up in sterile dH_2O and 40% PEG (Mr. 6000) was sterilised by autoclaving. T_4 DNA ligase (Anglian) was diluted prior to use in 20mM sodium phosphate (pH 7.6), 1mM EDTA, 5mM DTT, 60mM KCl and 50% (v/v) glycerol to 2 units/µl (except for 'sticky-end' ligations, where ligase was at 10 units/µl). All reagents were stored at -20°C.

Ligation reactions were set up as follows and incubated at 4°C overnight :

	Ligation mix (µl)
Insert DNA (*)	6 to 1
Cleaved vector DNA 10ng/µl	1
10 x ligation buffer	1
10mM rATP	1
T_4 DNA ligase (†)	1
dH_2O	5 to 0

(*) : absent in control

(†) : 2 units/µl or 10 units/µl, for 'sticky' or 'blunt-end' ligations respectively. 2.5µl 40% PEG was also added to 'blunt-end' ligations and the amount of dH_2O reduced accordingly.

Ligation reactions were also performed in agarose at concentration of less than 0.1%. Plasmid full-length clones of TGMV digested with the

cloning restriction enzyme were electrophoresed in 1% low gelling temperature agarose (Marine Colloids) containing ethidium bromide (1µg/ml). The viral specific insert was excised from the gel by visualising on a uv transilluminator and the slices heated to 68°C for 10min, to dissolve the agarose. The molten agarose containing the DNA was diluted 1:10 with the appropriate ligase buffers and ligated at 16°C overnight. After extraction with phenol, Sevag and ethanol precipitation the DNA was resuspended in TAE and analysed by 1% agarose gel electrophoresis in the presence of ethidium bromide. Re-ligated TGMV DNA was visualised with a similar mobility to ocDNA and cccDNA found in virus infected tissue extracts.

2.10e Preparation of competent cells

Transformation competent bacterial cells of *E. coli* strains JM101 or TG2 were prepared by the method of Maniatis *et al.* (1982). A single bacterial colony was taken from a minimal agar plate and grown overnight with, shaking at 37°C in 1.5ml of 2TY-broth in a Sterilin tube. 25µl of this culture was used to inoculate 7.5ml 2TY-broth which was shaken vigorously at 37°C until OD_{550} = 0.3 was achieved (*ca.* 4h.). It was important that cells were in the log phase growth before $CaCl_2$ treatment. The cultures were then chilled on ice for 5min prior to centrifuging for 5min at 3,500 xg at 4°C. The supernatant was discarded and the cells resuspended in 10ml of cold sterile 50mM $CaCl_2$ with gentle mixing. These cells were stored on ice for 20min and then recentrifuged as above. The supernatant was again discarded and the cells resuspended in 1ml cold 50mM $CaCl_2$ and used the same day for transformation. In all experiments two control transformations were always performed, one using undigested vector DNA the other lacking any vector DNA. Thus it was possible to monitor both the efficiency of transformation and whether the antibiotic selection was adequate.

2.10f Plasmid transformation

2 to 20ng of DNA, in a minimum volume (less than 10µl) was added to 200µl of competent cells and incubated on ice for 40min. Cells were then heat-shocked at 42°C for 3min and 800µl 2TY-broth added to them. After incubation at 37°C, without shaking, for 30min 200µl aliquots of

the transformation mixture were spread onto the plates of 2TY-agar containing the relevant antibiotics for selection. If the plasmid being used contained the β-galactosidase expression system then agar plates were spread with 30μl 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal ; Anglian), made up in dimethylformamide, and 10μl 24mg/ml isopropyl-β-D-thio-galactopyranoside (IPTG ; BRL) prior to spreading the transformed bacteria. Plates were then inverted and incubated at 37°C overnight.

2.10g Plasmid 'miniprep' protocol

Single colonies of interest, from the transformation plates, were replated and toothpicked into 1ml L-broth containing the relevant antibiotic in a Sterilin tube and incubated with vigorous shaking overnight at 37°C. Cells were pelleted by microcentrifuging for 5min in 1.5ml Eppendorf tubes and the supernatant removed. The cells were resuspended in 100μl 50mM glucose, 25mM tris-HCl (pH 8.0), 10mM EDTA, 4mg/ml lysozyme and incubated at RT for 5min. 200μl 0.2M NaOH, 1% SDS was then added and mixed gently before incubating for 5min on ice. 150μl 3M potassium acetate (pH 4.8) was added,the tube vortexed and incubated for a further 5min on ice. Following microcentrifugation for 5min the supernatant was removed and extracted with an equal volume of 1:1 TE saturated phenol:Sevag (24:1, chloroform:isoamyl alcohol (v/v)) and microcentrifuged for 5min. The upper aqueous layer was removed and extracted with an equal volume of Sevag, microcentrifuged and the aqueous upper phase added to 900μl RT absolute ethanol. After a 2min ethanol precipitation, at RT, the plasmid was pelleted by microcentrifugation for 5min. The supernatant was discarded and the DNA pellet vacuum dried prior to resuspension in 50μl TE. This solution was treated with 1μl 2mg/ml RNase A before use in restriction enzyme digests.

2.10h Large scale plasmid isolation protocol

A single colony containing recombinant plasmid was grown up overnight in 3ml L-broth containing the appropriate antibiotic(s) in a sterile 10ml Sterilin tube, shaking at 37°C and used to inoculate 250ml main culture of the same broth. The culture was shaken overnight at 37°C. Recombinant plasmid was isolated by an adaptation to the method of Ish-Horowicz and Burke (1981) in which most of the cellular RNA was precipitated with lithium chloride.

The cells were chilled on ice for 20-30 min and centrifuged at 4,500 xg for 10 min. The 250ml bottles used were previously sterilised with industrial methylated spirits and rinsed with sterile dH_2O. The pellet was resuspended with a rubber policeman,in 5ml of 10mM EDTA, 25mM tris-HCl (pH 8.0),followed by a 5ml wash, and transferred to a 50ml polypropylene tube. The suspension was recentrifuged at 4,000 xg for 10 min, the pellet being resuspended in 3ml of a solution containing 50mM glucose, 10mM EDTA, 25mM tris-HCl (pH 8.0). Lysozyme (12mg/ml) was added and the mixture was incubated at 0°C for 30 min. The mixture was then gently shaken with 6ml of a freshly prepared solution of 0.2M NaOH, 0.5% SDS and stored at 0°C for 30 min. 4.5ml of 3 M potassium acetate (pH 4.8) was then added, shaken gently, and the mixture was incubated on ice for 30 min. After centrifugation at 14,000 xg for 10 min, the supernatant was transferred to another centrifuge tube, 30ml cold (-20°C) absolute ethanol added and the mixture was left at -20°C for 30 min. The precipitate was harvested by centrifugation at 8,000 xg for 10 min and resuspended by gentle agitation in 3ml of 100mM NaCl, 50mM tris-HCl (pH 7.8). At this stage, if ultrapure plasmid DNA was required, it was loaded onto a CsCl/ethidium bromide gradient and centrifuged to equillibrium and treated as described for the purification of viral supercoiled DNA (section 2.6g.ii). Otherwise, 1ml of 8M LiCl was then added and the mixture incubated at 0°C for 15-30 min. After centrifugation at 8,000 xg for 10 min the supernatant was collected, 2 volumes of cold absolute ethanol were added, and the mixture was incubated for 30 min at -20°C. The DNA precipitate was collected by centrifugation at 8,000 xg for 10 min and resuspended in 0.5ml TNE . The solution was transferred to a 1.5ml Eppendorf tube and 25µl of RNase A

(stock stored at -20°C in sterile dH_2O at 10mg/ml) was added and the solution incubated at 37°C for 1h. The DNA was extracted twice with an equal volume of TNE saturated phenol and the aqueous phase re-extracted with an equal volume of Sevag.

After addition of 2 volumes of cold absolute ethanol to the aqueous phase and storing for 1h at -70°C or overnight at -20°C the DNA precipitate was recovered by microcentrifugation for 10 min at 4°C and washed in 70% ethanol, before being vacuum dried and resuspended in 50µl TE .

This method was commonly 'scaled-up' to purify plasmid from main cultures of 1 litre.

To prepare M13 RF DNA the same method was used but cultures were initiated differently. To 10ml 2TY-broth was added 1ml TG2 or JM101 overnight culture and to 1ml 2TY-broth was added 100µl of the same culture plus 20µl frozen, M13 infected, bacterial cells (section 2.10j). Both cultures were then incubated at 37°C for 6h whilst shaking vigorously before being added together into 1 litre of 2TY-broth which was then further incubated overnight at 37°C with shaking. Cells were then harvested and treated exactly as described for the preparation of plasmid DNA.

2.10i M13 bacteriophage transformation

Two to 20ng of DNA, in a minimum volume (less than 10µl) was added to 200µl of competent cells and incubated on ice for 40min. During this incubation 3ml molten top agar (2TY broth without yeast extract but containing 0.6% Bacto agar) was prepared and allowed to cool to 42°C. To the top agar, held at 42°C, was added 20µl 24mg/ml IPTG, 30µl 20mg/ml X-gal, 200µl of the bacterial overnight culture and 50µl of the transformation mixture. The cells were then allowed to heat-shock for 3min. The top agar was then shaken and immediately poured onto prewarmed (37°C) bottom agar plates (2TY broth containing 1.5% Bacto agar). The top agar was allowed to set before the plates were inverted and incubated at 37°C overnight.

2.10j Template preparation of M13 clones

It was convenient to prepare 12 (or multiples of 12) templates simultaneously, due to the capacity of the microcentrifuge.

Aliquots of 1ml 2TY-broth were dispensed into 12 x 10ml Sterilin disposable tubes and 100µl of an overnight bacterial culture added to each. Individual white plaques, seen on the bottom agar plates after incubation, were toothpicked into the tubes (one plaque per tube) and shaken vigorously at 37°C for 6h. The cultures were then poured into 1.5ml Eppendorf tubes and microcentrifuged for 5min. 800µl of the supernatant was then transferred into clean Eppendorf tubes and 200µl of 2.5M NaCl, 20% PEG (Mr. 6,000) added. The remaining cells were either taken up in TAE, 15% (v/v) glycerol and stored at -70°C, or 'minipreped' for RF DNA as in section 2.10f. After mixing , the supernatants were incubated at RT for 15min, microcentrifuged for 5min and the supernatants discarded. All traces of the PEG supernatant were removed after a second brief microcentrifugation of the tubes. 100µl of TE and 100µl TE saturated phenol:Sevag (1:1) was added and each tube extensively vortexed to resuspend the PEG/bacteriophage pellet. After microcentrifugation for 5min the aqueous upper layer was transferred to a clean tube, extracted with 100µl Sevag and microcentrifuged for 2min. The aqueous layer was transferred to a clean tube, being careful to avoid any solid material at the interface. 10µl of 3M sodium acetate and 250µl of cold (-20°C) absolute ethanol was added to each tube and the tubes stored overnight at -20°C. The DNA precipitate was pelleted for 10min by microcentrifugation, the supernatant discarded and the pellet vacuum dried. The M13 ss DNA template was taken up in 30µl TE and stored at -20°C.

2.11 Dideoxynucleotide Sequencing

2.11.a The sequencing gel

2.11a.i Preparation of electrophoresis plates

Electrophoresis plates were 200 x 380 x 4 mm and each pair had one notched plate with a 160 x 20 mm notch cut out from the top. Prior to use new plates were soaked in concentrated HCl for 30min, followed by a further 30min in Decon 90 (BDH). Immediately before use plates were washed with Decon 90, rinsed thoroughly with water, absolute ethanol and finally acetone. The notched plate was then coated with 2% dimethyl-dichlorosilane in 1,1,1-trichloroethane (BDH Replicote) and the other with gamma-methacryloxypropyl-trimethoxysilane (Wacker Chemicals) or 'sticky' silane, according to the method of Garoff and Ansorge (1981). 75µl of 'sticky' silane and 750µl of 10% (v/v) acetic acid were added to 25ml absolute alcohol immediately before required, shaken well and poured over the 2 back plates. Both plates were allowed to dry in a fume cupboard and were rinsed thoroughly with absolute ethanol and dried. Once a particular plate had been treated with either silane or 'sticky' silane it was never treated with the other chemical mixture.

Plasticard (Raven Scientific), 0.3mm thick was used for spacers and combs. Spacers were 1cm wide and comb wells approximately 5mm wide, 1mm apart or, 2.5mm wide spaced 1mm apart. Before use the plasticard was soaked in 50% (v/v) Lypsol to remove agents which prevent polymerisation of acrylamide. 'Wedge' gels were commonly used, here a wedge of plasticard (1cm x 5mm) was placed on top of the normal spacers at the bottom of the plates.

The gel plates were sealed along the sides and bottom with electrophoresis tape (Universal Scientific Ltd.).

2.11a.ii Preparation of polyacrylamide gels

Stock solutions

10 x sequencing TBE consisted of tris-HCl (162g/l), boric acid (27.5g/l) and EDTA (9.5g/l), pH 8.7. 6% acrylamide, 7M urea was made up by dissolving 288g urea (Rose), 34.2g acrylamide, 1.8g N,N'-methylene bisacrylamide, 60ml 10 x sequencing TBE, to a final volume of 600ml.

The acrylamide solution was then filtered and stored at 4°C in individual aliquots of 35ml (one required for each gel).

Just prior to use an acrylamide aliquot was taken and to it was added 210µl 10% AMPS, 28µl TEMED. This solution was placed into a 50ml beaker and the gel poured continuously down one side of the plates, which were held at an angle of approximately 30° from the horizontal. The plates were filled slowly being careful to avoid any air bubbles. When full the plates were lowered gently and rested at an angle between 10° and 20° from the horizontal and the comb inserted. After attaching bulldog clips to the top (and the bottom of wedge gels) the gel was allowed to polymerase for at least 2h. Commonly the gels were allowed to set overnight without any loss of resolution, however if they were left overnight the gel plates were sealed with 'cling-film', to prevent evaporation.

Before use , the electrophoresis tape at the bottom of the plates was slit open with a scalpel and the comb removed with the aid of dH_2O to dissolve any solidified urea. The plates were assembled in place on the electrophoresis tank (Raven Scientific) with 3mm thick sheets of aluminium fixed to them, these ensure the even dissipation of heat during electrophoresis. Running buffer, 1 x sequencing TBE, was added to each trough and the wells of the gel rinsed using a syringe and needle to remove any unpolymerised DNA in them.

2.11a.iii Gel and plate treatments after electrophoresis

After the required electrophoresis time, gel plates were removed from the tanks and the electrophoresis tape cut open with a scalpel. The plates were then levered apart and the gel remained attached to the plate treated with 'sticky' silane. The gel was then fixed and the urea washed out for 30min in 2 litres of 10% (v/v) acetic acid with occasional agitation. After blotting dry the gel was baked dry in an oven for at least 2h at 80°C.

The plate was allowed to cool to RT, placed in a light proof cassette and a piece of X-ray film (Kodak X-Omat S film, 18 x 43cm) placed directly onto the gel in a dark-room. Exposure time varied from 1 to 2 days. Films were developed using a Kodak X-Omat automatic processor and read over a cold light transilluminator. Sequencing gels

were removed from plates by an overnight soak in 100mM KOH or 10 to 20% Decon 90.

2.11b The sequencing reaction (Sanger *et al.* 1977)

2.11b.i Buffers and reagents

10 x annealing buffer : 600mM NaCl
(*Hinc* II buffer) 70mM tris-HCl, pH 7.5
70mM 2-mercaptoethanol
70mM $MgCl_2$
stored at -20°C.

Formamide dye mix : 0.03% xylene cyanol FF
0.03% bromophenol blue
20mM EDTA
made up in deionizied formamide (pH 7.0)

M13 primers

One of two M13 sequencing primers were used at all times:

a. Pentadecamer universal primer (Biolabs) :

5' GTAAAACGACGGCCAGT 3'

b. 15 base universal primer (BRL) :

5' ATGTTGCAGCACTGA 3'

Primer was diluted with dH_2O and stored at the working concentration of 2ng/µl in aliquots at -20°C.

Large fragment DNA polymerase

Large fragment DNA polymerase I (Klenow fragment, Klenow and Henningsen, 1970) was obtained from Amersham and diluted to 1 unit/µl in 50mM potassium phosphate , 0.25mM DTT and 50% (v/v) glycerol, pH 7.0, just prior to use. The enzyme dilution buffer was stored at -20°C.

Radiolabel

[α-^{35}S] dATP (>600 Ci/mmol., Amersham) in aqueous solution was used directly in the sequencing reaction.

Nucleotide stock solutions

Deoxynucleoside triphosphate (dNTP) stocks (BCL) were made up as described by Maniatis (1982) in dH_2O to a concentration of 10mM and 0.5mM and they were stored at -20°C.

dNTP mixes	A°	T°	C°	G°
0.5mM dTTP	20µl	1µl	20µl	20µl
0.5mM dCTP	20µl	20µl	1µl	20µl
0.5mM dGTP	20µl	20µl	20µl	1µl
50mM tris-HCl, pH 8.0, 1mM EDTA	5µl	5µl	5µl	5µl

A°, G°, T°, C° were stored at -20°C.

Dideoxynucleotide triphosphate, ddNTPs

10mM stocks were kept frozen in 5mM tris-HCl, pH 8.0, 1mM EDTA. Working solutions were diluted in dH_2O and stored at -20°C. Initially dideoxy concentrations were :

0.04mM ddATP
0.40mM ddTTP
0.20mM ddCTP
0.30mM ddGTP

These concentrations were adjusted to the required levels by assay in sequencing reactions. Added to the each dNTP mix was the appropriate ddNTP in an equal volume just prior to use. For each sequencing reaction (A,T,C,G) 2µl of this chain termination mix was required.

dATP chase

0.5mM dATP was prepared by dilution of the 10mM stock in 5mM tris-HCl, pH 8.0, 0.1mM EDTA and stored at -20°C.

2.11b.ii Annealing of primer to template

For each clone :

M13 ssDNA template	6µl
10 x annealing buffer	1µl
2ng/ml M13 primer	1µl
dH_2O	2µl

M13 template DNA was prepared as described in section 2.10j and approximately 0.5µg DNA was used in each sequencing reaction. The annealing mixture was vortexed, microcentrifuged briefly, incubated at 65°C for 5min and then allowed to cool to RT for 30min.

2.11b.iii Dideoxy sequencing

After the 30min hybridisation reaction, 1μl [α-^{35}S] dATP and 1μl (1 unit) of Klenow fragment was added to each clone. After vortexing and a brief microcentrifugation, 2.5μl aliquots of this mix were dispensed into each of 4 Eppendorf tubes labelled A, T, C and G and the DNA was microcentrifuged to the bottom of the tube. 2μl of the previously prepared chain termination mixes, stored on ice until required, were then pipetted onto the rim of the appropriate tube and the reactions started by a brief microcentrifugation and incubated at RT. After 20min, 2μl of the 'chase-solution' was added to the rim of each tube and they were re-centrifuged. At 35min, 4μl of the formamide dye mix was added to the rim of each tube and they were again microcentrifuged to terminate the reaction. Reactions were commonly stored at -20°C overnight before electrophoresis.

Before loading onto the gel, samples were boiled for 5min with their lids open to denature and concentrate the samples. A drawn out melting point tube, attached to a screw-barreled syringe was used for loading. The gel slots were flushed out immediately before loading with a syringe and needle which contained running buffer. 2μl of ^{35}S-labelled sample was loaded per track for one or two gels, as quickly as possible after boiling. After the 4 samples of a particular clone had been loaded they were electrophoresed into the gel until a clear separation could be seen between the two dyes present in the termination mix.

Electrophoresis was carried out with an LKB model 2103 power supply set at 40W, 1,600V and 35mA. Usually short run gels were terminated when the bromophenol blue dye reached the end of the gel. Long runs were allowed to run for 2h after the bromophenol blue had electrophoresed from the gel.

2.11c T-tracking

Newly isolated unknown clones were usually screened by T-tracking. For 20 clones 45μl of annealing solution was prepared consisting of 18μl primer, 9μl 10 x annealing buffer and 18μl dH_2O. 2μl of this was added to 2μl M13 ssDNA template and heated to 65°C for 5min, before being allowed to hybridise at RT for 30min. A reaction solution was

prepared containing 16µl T°, 16µl ddTTP, 4µl (4units) Klenow fragment, 6µl [α^{35}-S] dATP, 4µl dH_2O, and 2µl of this was added to each of the annealing reactions. After incubation at RT for 20min, 2µl of chase and then 15min later 4µl of formamide dye mix were added to each reaction. Samples were all boiled and loaded (without running in each clone), as described above. Electrophoresis, treatment of the gel and autoradiography was the same as before. Sequence patterns were compared to determine which clones were dissimilar.

2.11d Sequencing strategy

Two methods were implemented to obtain subclones from the full-length PYMV clones. The initial method involved excising one of the full-length clones at the restriction site used to clone it and then electroeluting the viral DNA as described in section 2.10c. This linear DNA was religated (see section 2.10d) and the DNA obtained was subcloned into M13mp18 or 19 (Yanisch-Perron *et al.*, 1985) as full length genomes or restriction fragments, prior to sequencing.

A more sophisticated method of generating subclones for sequencing involved the sequential digestion of full length clones with exonuclease III (exo III). The method used was based on that of Henikoff (1984) but replaced the S1 nuclease digestion step with mungbean nuclease. The method was based on two useful features of exo III: (1) progressive digestion at a very uniform rate and (2) failure to initiate digestion at DNA ends with four-base 3'-protrusions. CsCl purified ds RF DNA was prepared (see section 2.10h) and 2.5µg subjected to restriction enzyme digestion with two enzymes known only to cut in the polylinker of M13mp18 or 19 and not within the cloned insert (digest were performed as described in section 2.10b.). The enzymes were chosen such that the one which cut closest to the sequencing primer binding site left a four-base 3'-protrusion which protected it from subsequent exo III digestion, whilst the other enzyme produced a 5'-protrusion susceptible to exo III digestion. After digestion the reactions were extracted with an equal volume of TAE saturated phenol, followed by an equal volume of Sevag (microcentrifuging for 5min between each and removing the upper aqueous phase) before the DNA was precipitated with 2 volumes of cold (-20°C) ethanol at -20°C overnight. The DNA was

pelleted by microcentrifugation for 15min at 4°C and the supernatant removed before being vacuum dried. The DNA was dissolved in 66mM tris-HCl (pH 8.0), 0.66mM $MgCl_2$ to a concentration of approximately 100μg/ml (25μl). To this was added 25 units exo III (Biolabs, 16U/μl) which gave an excess of enzyme over DNA ends calculated to be approximately 10:1 (Wu *et al.*, 1976a). The reaction was incubated at 23°C and samples (8μl) were taken at 60, 105 and 150min. Each sample was then treated as follows. The exo III reaction was stopped with 24μl 0.2M NaCl, 5mM EDTA (pH8.0) and the enzyme heat inactivated at 80°C for 10min. DNA was directly precipitated with 2 volumes of ethanol and incubated at -70°C for 2h. After pelleting and drying the DNA as described before it was resuspended in 5μl 100mM sodium acetate (pH 5.0), 60mM NaCl, 1mM $ZnSO_4$ and 1 unit of mungbean nuclease added (Biolabs). The final reaction volume was made upto 10μl with sterile distilled water and incubated at 30°C for 30min. The reaction was stopped by the addition of 10μl 0.4M NaCl and extracted with 20μl 1:1 mixture of TAE saturated phenol and sevag prior to two ether extractions. After ethanol precipitation the DNA was pelleted and washed with 500μl 70% ethanol, dried *in vacuo*, and dissolved in 30μl 20mM tris-HCl (pH 8.0), 7mM $MgCl_2$. To 20μl of this was added 2μl 0.1U/μl Klenow (Amersham) and after incubation for 3min at 37°C, 2μl of a deoxynucleotide mix containing dATP, dTTP, dCTP and dGTP (each at 125μM) was also added. This reaction was further incubated for 5min at 37°C. This procedure efficiently removes both 5'- and 3'-protrusions, leaving ligatable blunt ends (Henikoff, 1983). To each reaction was then added 10μl 0.5M tris-HCl (pH 7.7), 0.1M $MgCl_2$, 10μl 100mM DTT, 20μl 0.5mg/ml BSA, 20μl 25% PEG (Mr 6000), 10μl 10mM ATP, 5μl dH_2O and 1μl T_4 DNA ligase (Anglian, 2.5U/μl). The ligation reactions were incubated at RT for 4h and either frozen or used directly for transformation. Transformation was exactly as described in section 2.10i with 5μl of each ligation reaction being diluted in 15μl TE prior to addition to the competent cells.

Plaques obtained were screened by preparing M13 template DNA and sizing the clones by electrophoresis in various concentrations of agarose gels against full-length clones and M13mp18.

Suitably different sized clones were then sequenced as described previously.

2.11e Computer analysis

Sequence data was compiled and stored in the Edinburgh Regional Computer Centre VAX/VMS service and accessed via a telephone/modem link through the Imperial College computer system (Cyber 174) on an IBM Personal Computer. All sequence analysis was performed using the program library of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984).

3. RESULTS AND DISCUSSION

Note: All agarose gels shown in this section were electrophoresed in the presence of ethidium bromide and the direction of electrophoresis is from top to bottom. In sequencing gels the DNA sequence is read from the 5' to the 3' end.

3.1 Infection of plants with PYMV

3.1a Propagation

The first stage of the project was to transmit the Venezuelan isolate of *Mosaico Amarillo de la Papa* to susceptible plants. Infected tissue samples were initially provided as freeze-dried *N. benthamiana* leaves (kindly supplied by Dr Ramon Lastra). This tissue was resuspended and homogenised in 100mM phosphate buffer (pH 7.0) and inoculated to *N. benthamiana* plants. These plants did not develop symptoms of infection up to four weeks after inoculation. A sample of fresh infected *N. benthamiana* tissue was used for further experimentation. Following homogenisation of this tissue in either 100mM phosphate buffer (pH 7.0) or TAE buffer and inoculation to *N. benthamiana* plants, characteristic symptoms of infection developed 10-14 days post-inoculation. From these observations it would appear that PYMV is unstable to freeze-drying and thus infected tissue was never stored in this fashion. Two cultivars of *Nicotiana tabacum* were also shown to be hosts for PYMV, *viz. N. tabacum cv.* Xanthi and *N. tabacum cv.* Petit Havana, clone SR1. PYMV was also successfully mechanically transmitted to *Petunia hybrida* F1 hybrid cultivars but french bean (*Phaseolus vulgaris cv.* The Prince) was not susceptible.

All attempts to mechanically transmit PYMV to either potato plants (*Solanum tuberosum* ssp. *tuberosum*) or tomato plants (*Lycopersicon esculentum*) of several cultivars proved unsuccessful. However, in both instances these plants were successfully infected by detached scion grafting with high efficiency. Symptoms appeared on the non-infected tissue within 3 to 4 weeks in potatoes and 14 to 21 days in tomatoes. Differences in the time taken for infection to become established may be a reflection of the ease with which the different tissues formed a stable graft unions in these host plants.

It was decided however to use *N. benthamiana* plants as the propagation host for PYMV for a number of reasons; 1. this plant species is a proven host for TGMV (a virus to which PYMV may be closely related) for which a purification protocol has already been devised (Stein *et al.*, 1983); 2. plants can be easily raised under the greenhouse conditions available and 3. unlike other *Nicotiana* species, *viz. N. tabacum cv.* Xanthi infected with TGMV (M.J. Slomka, pers. comm.), there appears to be no occurrence of symptomless infection in these plants.

3.1b Symptomatology of PYMV infection

The effects of PYMV infection on each of the plant species that were successfully mechanically inoculated with the virus are shown in figures 3-7. All plants displayed similar symptoms, characteristic of geminivirus infection (Matthews, 1979). In PYMV infected plants inoculated leaves often produced small chlorotic spots 2 to 5mm in diameter (10-14 days post-inoculation). This feature was most clearly apparent on the inoculated leaves of petunia plants (figure 7). These chlorotic spots never developed into necroses and no local lesion host for PYMV has been discovered. Systemic symptoms were most obvious on those leaves that developed after inoculation. The symptoms consisted of golden-yellow mottling of leaves and a gross deformation and stunting of the plant. The appearance of deformed apical leaves normally occurred 9 to 21 days post-inoculation. These symptoms were usually followed by a deformation and mosaicing of the axial shoot leaves first above and subsequently below the area of inoculation. This pattern of symptom development possibly reflects the normal growth sequence for these plants and is consistent with the rapid translocation of infectious agents through the normal solute transport channels. It is interesting to note that symptom appearance seemed to closely follow the positioning of metabolically active meristematic tissue. This may reflect the viruses requirement to infect those cells actively involved in DNA replication, as suggested for the successful transfection of protoplasts with CLV (Townsend *et al.*, 1986).



Figure 3. PYMV infected *N. benthamiana*.
Crown of a mechanically inoculated plant 21 days post-infection, showing characteristic symptoms of virus infection *i.e.* stunting, leaf distortion and mosaic.



Figure 4. PYMV infected *N. tabacum* cv. Xanthi.
Crown of a mechanically inoculated plant 18 days post-infection, showing the relatively mild symptoms of PYMV infection in this host.



Figure 5. i) PYMV infected *N. tabacum* cv. Petit Havana, clone SR1. Mechanically inoculated plant 25 days post-infection, showing clear symptoms of virus infection manifested as severe leaf distortion.

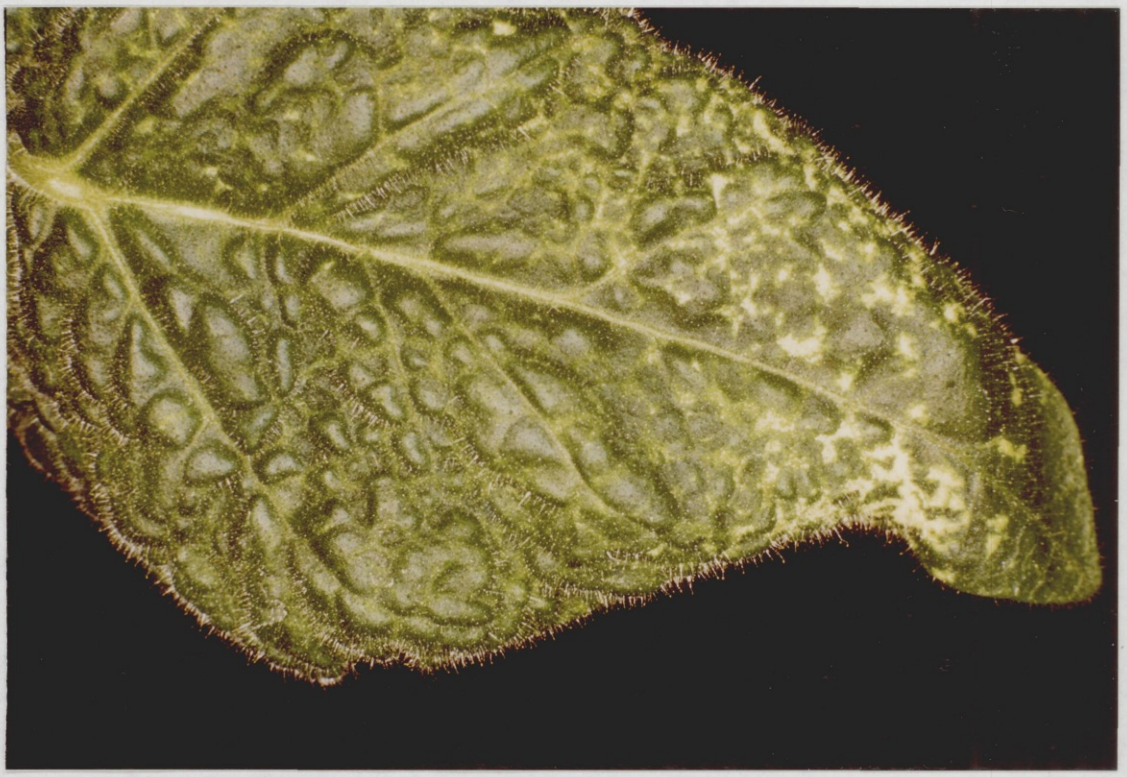


Figure 6. ii) PYMV infected *N. tabacum cv.* Petit Havana, clone SR1. Single leaf from the plant shown in figure 5, displaying the golden-yellow mosaic and leaf distortion characteristic of geminivirus infected plants.



Figure 7. PYMV infected *Petunia hybrida* F1 hybrid cultivar.
Petunia plants displayed the most characteristic symptoms of geminiviral infection of the plants tested, following PYMV infection. Clearly shown above are chlorotic spots on the inoculated leaves.

However, this hypothesis is not proven because there is no direct evidence to link the position of symptom occurrence with active geminiviral replication.

Generally, the susceptibility of plants to infection with PYMV decreased with their increased age and older plants which did become infected often displayed milder symptoms and a much reduced degree of stunting following symptom appearance. Also some variation in symptomatology was observed when plants grown and inoculated in the summer months were compared to those grown and inoculated in the winter months, the latter producing reduced chlorosis. These observations clearly reflect the condition of the plant material used for inoculation as the same inoculum source was used in both sets of experiments.

Bare stems that remained on plants from which leaves had been harvested produced further systemically infected tissue. However, in the studies reported here only fresh, young systemically infected tissue was used.

Graft infected potato and tomato plants produced very similar symptoms to mechanically inoculated tobacco and petunia plants (figures 8-11). One exception being that potato plants did not display any stunting effects as was initially described in PYMV infected plants (Debrot, 1981). This result may have be a reflection of environmental effects, particularly as it is unlikely that the greenhouse conditions used in these studies would simulate the field conditions originally utilised in Venezuela. Detached scion grafts of healthy tobacco plants on infected tobacco stocks produced identical symptoms to those observed on mechanically infected plants, while no symptoms were observed on control grafts of healthy tissue to healthy plants.



Figure 8. Detached scion graft of healthy *Solanum tuberosum* ssp. *tuberosum* cv. Maris Bard on a PYMV infected stock plant of *N. benthamiana*.

The graft union can be clearly seen between the upper healthy potato plant and the lower, PYMV infected tobacco plant. The graft union took *ca.* 2 weeks to become established.



Figure 9. Leaf of graft infected *Solanum tuberosum* ssp. *tuberosum* cv. Maris Bard.

A leaf from a potato plant systemically infected with PYMV by detached scion grafting, showing symptoms of golden-yellow mosaic and leaf distortion.



Figure 10. Detached scion graft infected *Lycopersicon esculentum* cv. Money Maker.

PYMV infected tomato plant showing bright yellow mosaicing, leaf distortion and stunting. Detached scion grafting using infected *N. benthamiana* plants was used to elicit the infection.



Figure 11. Leaf of graft infected *Lycopersicon esculentum* cv. Money Maker.

Leaf of PYMV graft infected tomato plant, showing clear symptoms of bright yellow mosaic and leaf distortion.

3.1c Virus purification

Both methods of virus purification described earlier (section 2.6e) produced highly infectious virus preparations. Indeed the preferred inoculum for plant inoculation was that produced from extracts of pellets obtained after the first ultracentrifugation step during the Triton extraction procedure (Stein *et al.*, 1983). Hereafter such inocula will be referred to as a high-speed pellet inocula.

Initial attempts to purify the virus in a sodium citrate buffer following extraction with chloroform:butanol (1:1) mixture and differential centrifugation, yielded an excellent preparation of virus specific ssDNA (see section 3.2a, figure 15) but electron microscopy failed to detect any virus particles. The final preparation of virus produced by this method was infectious for *N. benthamiana* plants, but a low yield, in a small volume, made such preparations impractical for routine use. In contrast this extraction method has proven successful for the purification of CLV from infected *N. benthamiana* plants, producing virus yields of 3mg/100g of the extracted tissue (Adejare and Coutts,1982). In this study a single DNase I and S1-nuclease sensitive nucleic acid species was isolated from the purified virus which was infectious for plants. While PYMV was not purified with the same efficiency as CLV was previously by this method it was possible to isolate similar ssDNA nucleic acid species from the final extracts (figure 15).

As freeze drying appeared to disrupt the virus, or at least reduce its infectivity (section 3.1a), purification involving freezing infected tissue in liquid nitrogen was avoided.

Hull *et al* (1976) initially utilised the non-ionic detergent Triton X-100 to improve the purification of CaMV by presumably disrupting inclusion bodies where the virus accumulated. The analysis of subcellular fractions of TGMV infected leaf material indicated that a large proportion of the viral infectivity remained associated with nuclear debris (Hamilton, 1983). Therefore it was believed that the use of a detergent during extraction may disrupt and solubilise nuclear membranes, thus releasing more virus. This method proved successful for TGMV (Stein *et al.*, 1983), with yields being increased five-fold to give an estimated 5mg of virus/kg fresh weight infected tissue, using

the extinction coefficient determined for BGMV for spectrophotometric analysis of the final preparation (Goodman and Bird, 1978). The Triton extraction method was used to purify PYMV but was only moderately successful. Whilst high-speed pellet inocula were highly infectious and contained all the expected intracellular virus specific DNA forms (see section 3.2b), further purification steps did not yield large amounts of virus and less than 100μg of virus/kg fresh weight infected tissue was produced. It may be useful, in any future work, to increase the time of incubation, or Triton concentration, in the extraction protocol, in an attempt to improve virus yields.

3.1d Electron microscopy

As discussed in the previous section no geminate particles were observed in virus preparations produced following chloroform:butanol extraction of citrate buffered extracts. Neither of the two negative staining agents, uranyl formate or PTA, gave any positive results. However, a geminate morphology for PYMV was clearly demonstrated when virus purified using the Triton extraction procedure was negatively stained with 3% PTA and examined in the electron microscope (figure 12). Grids prepared from this virus preparation displayed a low density of particles (ca. 10-15 particles per square). Attempts to negatively stain PYMV using uranyl formate were unsuccessful, despite the report by Roberts *et al.* (1984) that this stain improved the visualisation of other geminiviruses. It appears that PYMV may be unstable in this particular stain.

PYMV possessed the characteristic paired particle morphology of geminiviruses, with a flattened edge at the ajoining interface. Very few unpaired particles were seen in the preparations examined suggesting that the extraction protocol used did not readily disrupt the virus. If unpaired particles encapsidate a sub-genomic DNA species (discussed in section 1.5) this may be the reason why few single particles were seen in the preparation shown, as the virus used was purified from plants infected with a first passage high-speed pellet inoculum from plants infected with cloned DNA, which contained very little sub-genomic DNA.

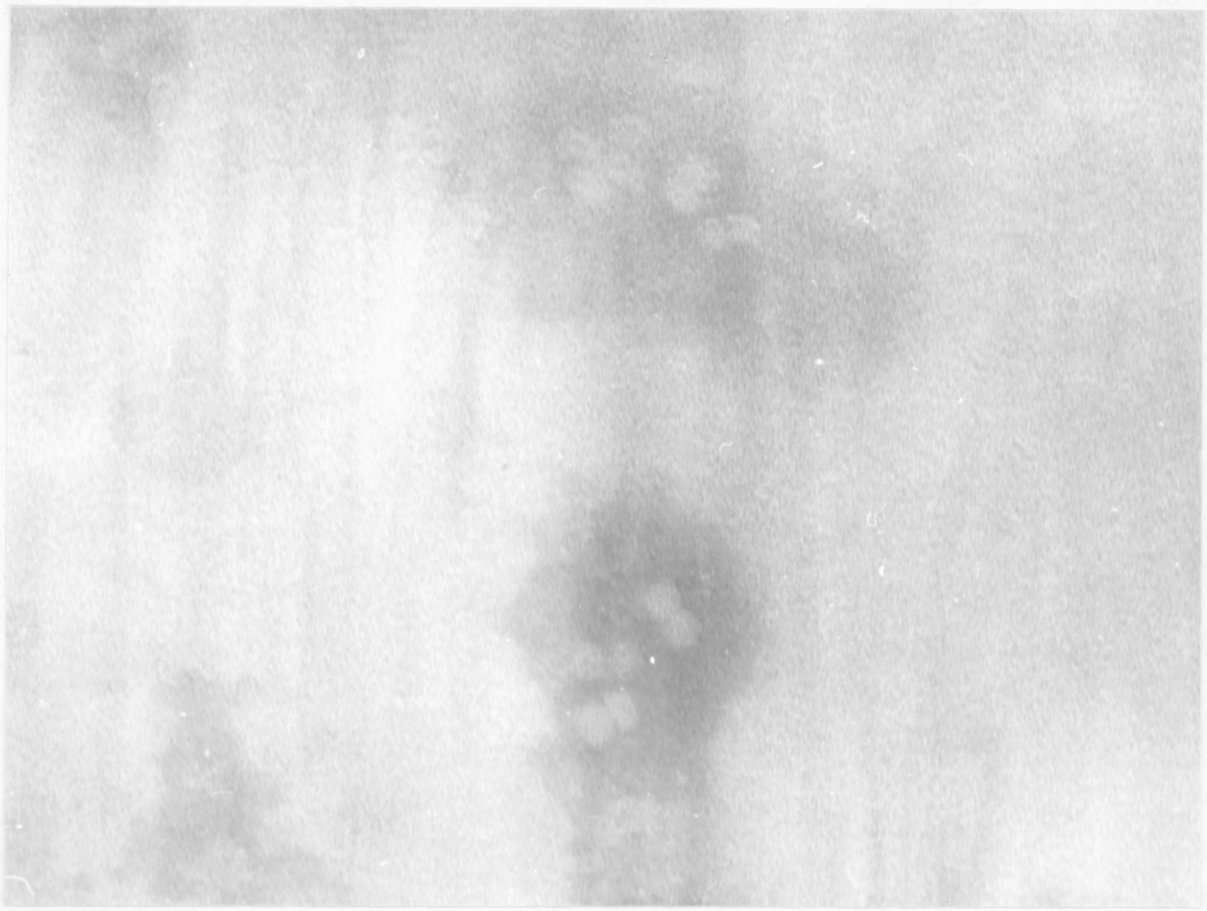


Figure 12. Electron micrograph of PYMV stained with 3% PTA
PYMV particles were purified by the Triton extraction procedure, note the apparent flattened interface at the ajoining surface of the geminate particles.

3.1e Capsid protein and serological analysis

In gel immunodiffusion tests purified TGMV gave a single precipitin line with its homologous antiserum (titre 1/256) but the antiserum gave no reaction with purified PYMV (figure 13). Similarly, ISEM using an antiserum to TGMV failed to 'trap' any PYMV particles on negatively stained grids. In both cases these tests were performed with purified virus which was infectious for *N. benthamiana* plants and contained encapsidated viral DNA species. It was thus assumed that preparations contained intact virus particles. However, thus far it has not been possible to clearly visualise PYMV capsid protein on denaturing protein gels (figure 14). Using the same purification and extraction procedure TGMV capsid protein was identified as a protein of *ca.* 30,000 molecular weight (figure 14), which bound TGMV antiserum on Western blotting (I.T.D. Petty, pers. comm.).

Therefore preliminary serological tests suggested that PYMV may not be serologically related to TGMV. However, until PYMV capsid protein can be purified in sufficiently large enough quantities for it to be visualised on a polyacrylamide gels, this statement is equivocal. Gel immunodiffusion assays are rather insensitive and require *ca.* 50μg of virus to produce a visible precipitin line.

Further attempts to investigate any serological relationships between TGMV and PYMV by Western dot-blotting proved unsuccessful, due to the high background fluorescence observed between the antiserum to TGMV and extracts of healthy *N. benthamiana* sap. Such contamination of the antiserum with host-specific antibodies may not have been detected in gel immunodiffusion tests because these antibodies may not form the precipitable complexes required to visualise a precipitin line. However, in the dot-blot system a precipitating antigen/antibody complex is not required to produce a positive result as the test is carried out on a nitrocellulose solid-support membrane.

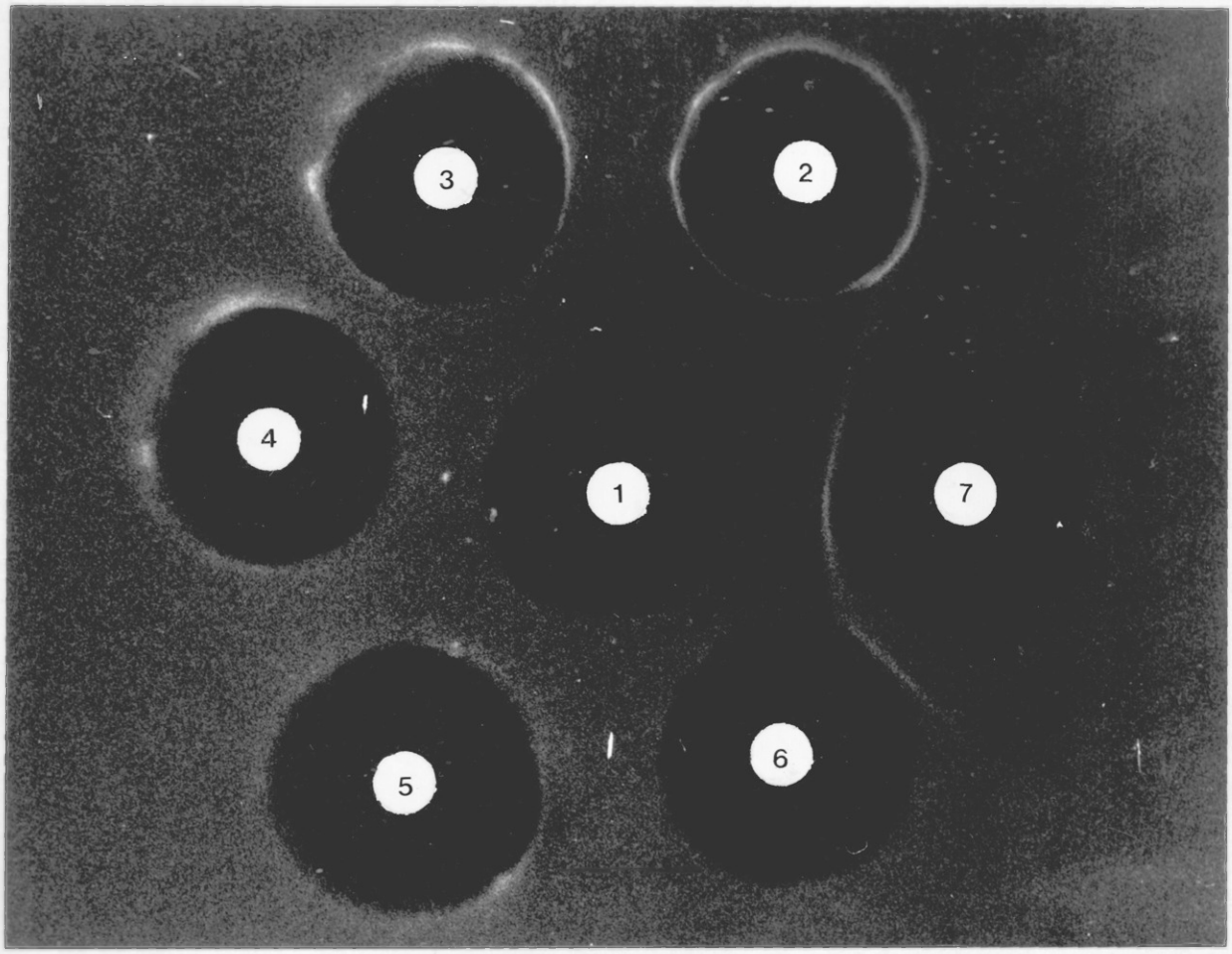


Figure 13. Analysis and serological comparison of TGMV and PYMV by gel immunodiffusion.

Wells contained:

1. TGMV antiserum
2. PYMV, purified by the Triton extraction procedure (in all cases)
3. 1:1 dilution of PYMV
4. 1:2 dilution of PYMV
5. 1:4 dilution of PYMV
6. Healthy sap
7. TGMV, purified by the Triton extraction procedure

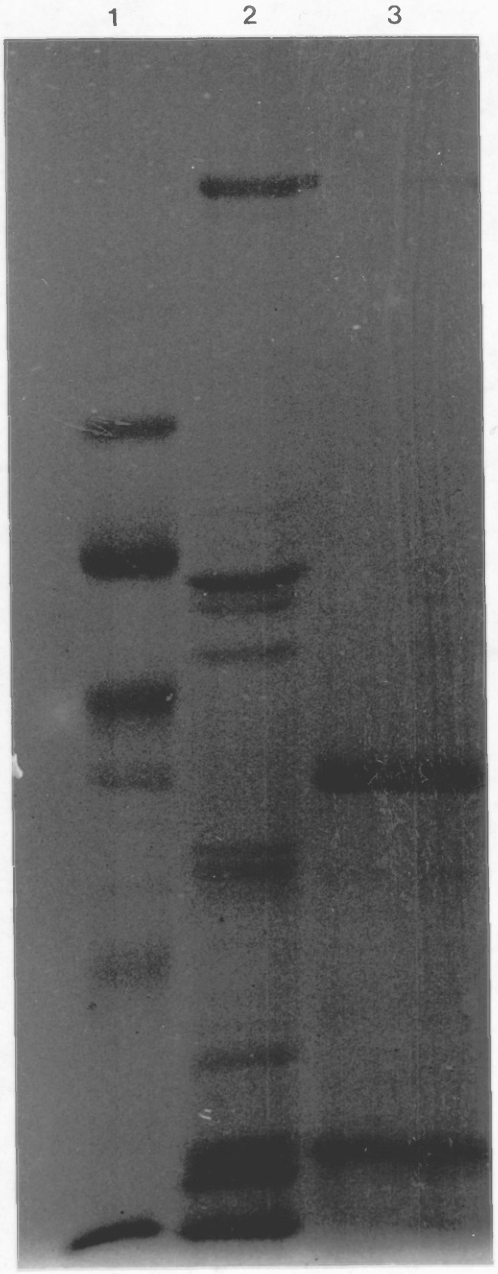


Figure 14. 10% polyacrylamide-SDS gel electrophoresis of purified PYMV and TGMV following sucrose density centrifugation.

1. Molecular weight markers; BSA (M_r 66,000); γ-globulin (M_r 55,000 and 23,500); ovalbumin (M_r 47,000); DNase 1 (M_r 30,000); cytochrome C (M_r 14,500)
2. 50µl PYMV, purified by the Triton protocol
3. 50µl TGMV, purified by the Triton protocol

3.2 Characterisation of viral specific DNA forms

3.2a Encapsidated DNA species

Samples of infectious PYMV purified by the chloroform:butanol extraction procedure were treated with DNase I (section 2.6h) and nucleic acids then extracted and electrophoresed in agarose (figure 15). Two protected species were observed which represent the encapsidated DNA forms of PYMV. Both DNA bands had equivalents of similar mobility in nucleic acid extracts produced from infected TGMV tissue. A radioactively labelled cDNA was prepared from an aliquot of the DNA sample loaded in Lane 2, using DNA polymerase I (large, Klenow fragment), and random primers prepared from sheared and denatured salmon sperm DNA (this method is identical to that described for producing nick-translated DNA (section 2.9a) except for the omission of DNase I and the addition of the two constituents mentioned above). The high degree of incorporation obtained indicated that the DNA species present were at least partially single stranded, because the Klenow fragment of DNA polymerase I has no nick-translating activity (Klenow and Henningsen, 1970) . When the denatured radioactive DNA produced was used to probe a Southern blot of the gel shown in figure 15, the DNA species observed earlier both hybridised (figure 16). It is interesting to note that not all virus-specific TGMV DNA forms seen in Lane 1 (figure 15) hybridised to the PYMV probe, suggesting a limited degree of homology between the two. The degree of cross-hybridisation observed in this type of experiment was however dependant on the hybridisation temperature and the stringency with which the membrane was washed following hybridisation. In this instance the hybridisation temperature was 65°C and the blot was probed and washed as described in those experiments that used heparin/dextran (Singh and Jones, 1984; section 2.9d). When a similar blot was probed and washed by another procedure using formamide (section 2.9d) a greater degree of cross-hybridisation between the PYMV cDNA probe and TGMV DNA forms was observed (figure 17).

The two encapsidated DNA species found in the PYMV preparations appear to correspond to TGMV ssDNA genomic and sub-genomic forms (Hamilton *et al.*, 1982).

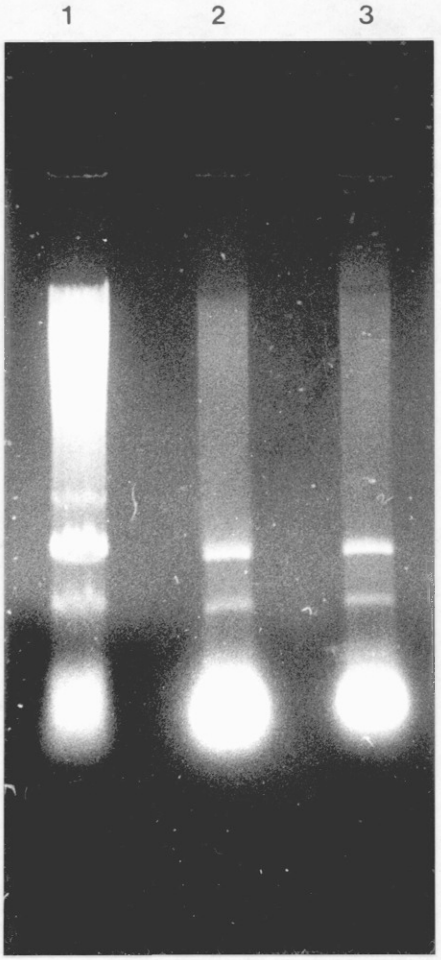


Figure 15. Agarose gel electrophoresis of encapsidated PYMV DNA.

1. A crude preparation of TGMV DNA, prepared from a high-speed pellet inoculum
2. PYMV DNA extracted from virus particles prepared by the chloroform:butanol extraction procedure
3. PYMV DNA from virus particles prepared by the chloroform:butanol extraction procedure, which had been treated with DNase I prior to extraction.

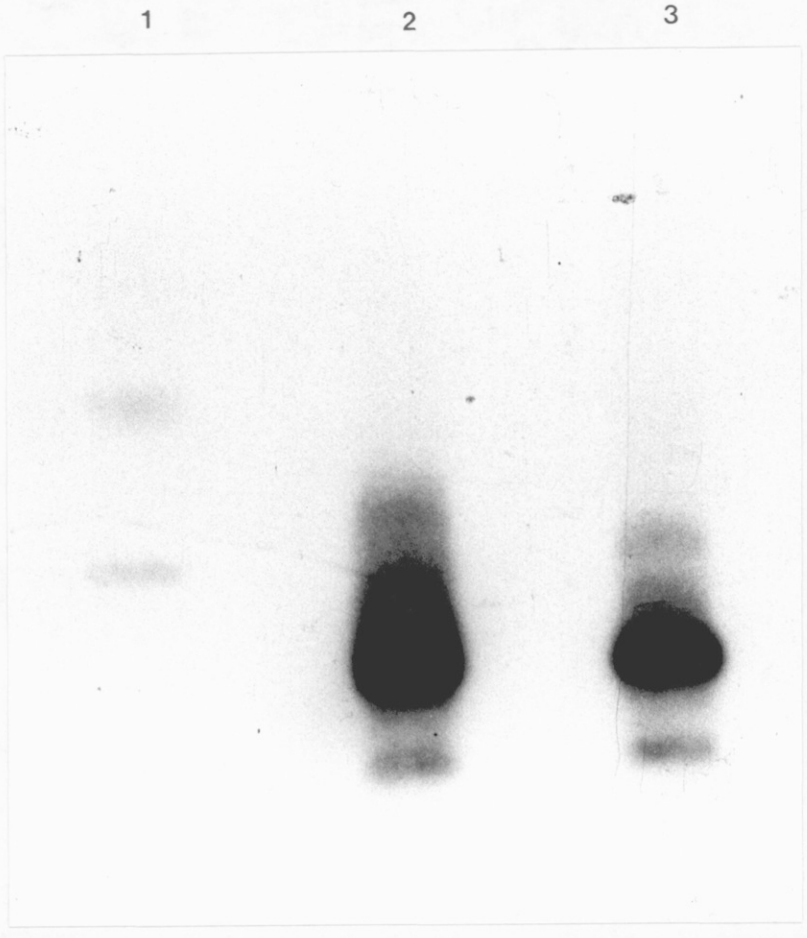


Figure 16. Autoradiogram of Southern blot of encapsidated PYMV DNA probed with [α-^{32}P] labelled PYMV cDNA.

The nucleic acids were transferred onto Gene Screen™, probed and washed as described for the heparin/dextran procedure (section 2.9d).

1. A crude preparation of TGMV DNA, prepared from a high-speed pellet inoculum
2. PYMV DNA extracted from virus prepared by the chloroform:butanol extraction procedure
3. PYMV DNA from virus prepared by the chloroform:butanol extraction procedure, which had been treated with DNase I prior to extraction.

3.2b Intracellular forms of PYMV DNA

The intracellular DNA forms produced during PYMV infection were examined by extracting nucleic acids from high-speed pellet inocula, electrophoresing them in agarose gels, transferring them to a solid support membranes and probing the transfers with radioactively labelled, virus-specific DNA probe. The patterns which emerged from this type of analysis were somewhat complex (figures 17 and 19).

Nuclease sensitivity tests identified six different nucleic acid forms in extracts of PYMV infected *N. benthamiana* tissue (figure 17), all six species were sensitive to DNase but resistant to RNase treatment. The major DNA species corresponding to the encapsidated genomic DNA was extremely sensitive to S1-nuclease treatment, confirming it to be single-stranded in nature. A second highly S1-nuclease sensitive species corresponded to the encapsidated sub-genomic DNA, indicating that it too was composed of single-stranded DNA. Further, double-stranded DNA forms were apparently part sensitive to S1-nuclease treatment. The most sensitive of these DNA species was suspected to be negatively supercoiled DNA (scDNA), a possible dsDNA replicative form of the viral ssDNA which may be a transcriptionally active viral complex (Stanley, 1985). Sunter *et al.* (1984) reported that TGMV scDNA possessed S1-nuclease hypersensitive sites and it would appear that PYMV DNA extracts may also contain such species. By increasing the concentration of S1-nuclease used during the treatments of the extracts all scDNA was converted to ocDNA which in turn (at higher S1 concentrations) was converted into linear dsDNA. A double-stranded ocDNA form of the sub-genomic DNA species was also sensitive to high concentrations of S1-nuclease. As discussed in the previous section the formamide method of probing and washing blots appeared to be less stringent than the heparin/dextran method because it resulted in a much greater degree of cross-hybridisation between PYMV and TGMV (figure 17). This result suggests that the two viruses may be more closely related than figure 16 initially implied.

When a Southern blot of intracellular PYMV DNA forms produced from all of the known hosts for the virus was hybridised with

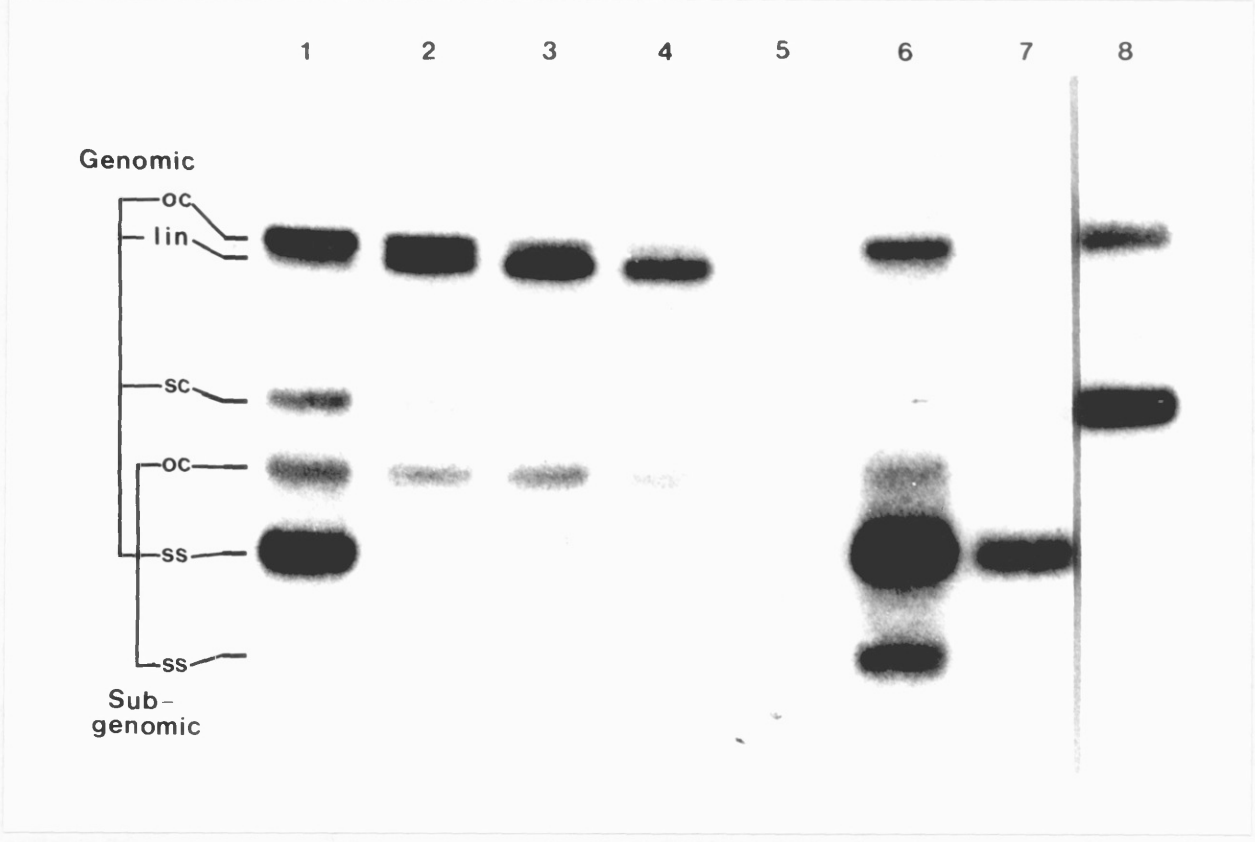


Figure 17. Nuclease sensitivity of PYMV intracellular DNA forms.
Autoradiogram of an agarose gel transfer onto Gene Screen™ probed and washed using the formamide procedure (section 2.9d), with a [α-^{32}P] labelled PYMV cDNA.

1. A crude preparation of PYMV DNA, isolated from a high-speed inoculum
2. as 1, digested for 30min at 37°C with 5units S1-nuclease
3. as 1, digested for 30min at 37°C with 15units S1-nuclease
4. as 1, digested for 30min at 37°C with 30units S1-nuclease
5. as 1, digested with 2µg DNase I
6. as 1, digested with 2µg RNase A
7. gel purified TGMV ssDNA
8. gel purified TGMV scDNA

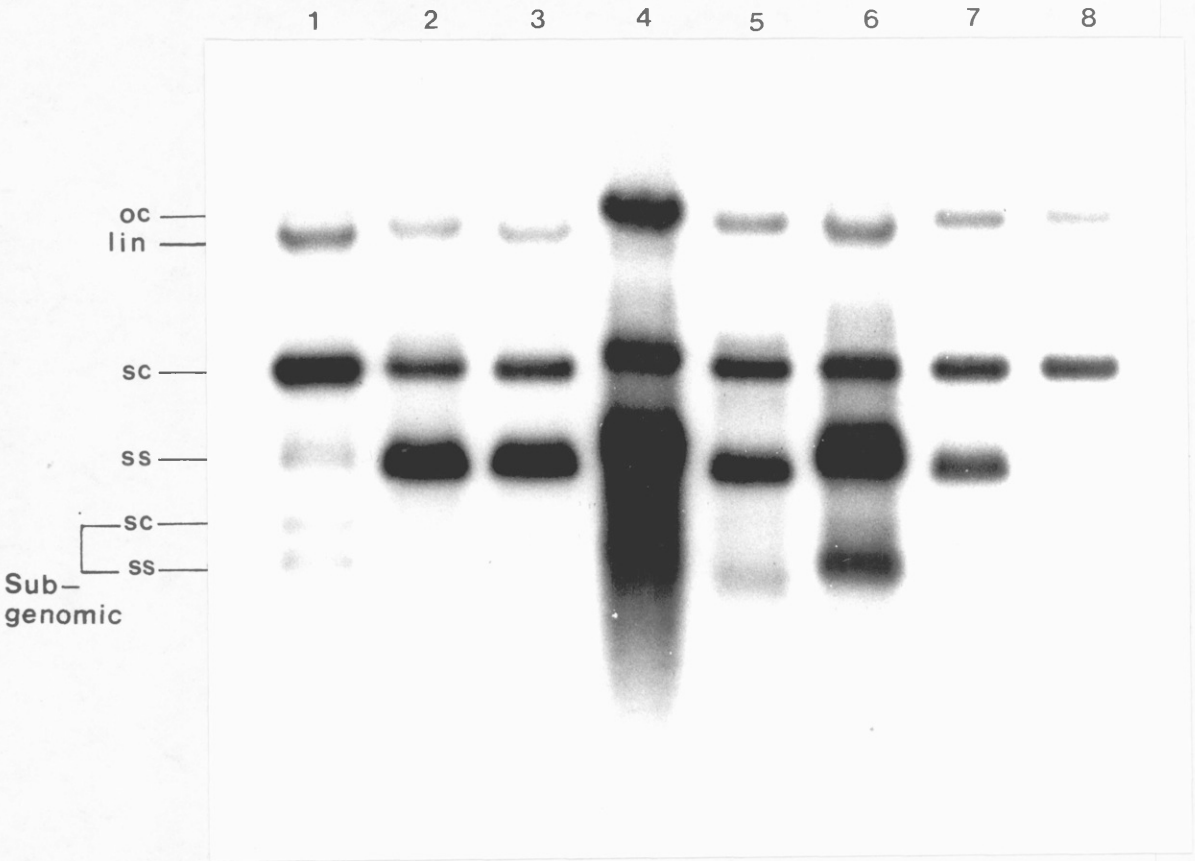


Figure 18. Intracellular DNA forms present in extracts of tissues derived from all known hosts of PYMV

Autoradiogram of an agarose gel transfer onto Gene Screen (+)™ of crude extracts of nucleic acids produced from high-speed pellet inocula prepared from the different known host plants of PYMV, described below. The blot was probed with strand-specific [α-^{32}P] labelled DNA clones mMAH(-) and mMBE(-) (section 3.4b). The DNA samples illustrated were produced from extracts of;

1. *N. benthamiana* plants infected with wild-type (multiple passage) inoculum
2. *N. benthamiana* plants infected with cloned PYMV DNAs excised from M13 cloning vectors
3. *N. benthamiana* plants infected with cloned PYMV DNAs excised from the plasmid cloning vectors
4. *Solanum tuberosum* ssp. *tuberosum cv.* Maris Bard plants infected following graft inoculation
5. *N. benthamiana* plants infected with extracts produced from infected potato plants
6. as above following one passage through tobacco plants
7. *Petunia hybrida* F1 hybrid plants infected with wild-type (multiple passage) inoculum form extracts of tobacco
8. *Lycopersicon esculentum cv.* Money Maker plants infected following graft inoculation

strand-specific probes made to DNA clones of the virus complementary sense DNAs (see section 3.4b), some subtle differences in hybridisation were noted (figure 18). The most obvious variation was in the amounts of sub-genomic DNA isolated from extracts produced from each of the host plants. N. benthamiana plants inoculated with both cloned components of the PYMV genome, excised from the cloning vectors (section 3.4b), did not contain any detectable sub-genomic DNA. This observation is similar to reports that plants infected with the cloned components of either TGMV or CLV did not generate detectable levels of sub-genomic DNA (Hamilton *et al.*, 1983; Stanley, 1983). However, dissimilar from these observations with TGMV and CLV was the fact that sub-genomic DNA was detectable in extracts of plants inoculated with cloned PYMV DNA when the plants were either grown for extended periods following the appearance of symptoms (up to 4 weeks) or when the inoculum from plants infected with cloned PYMV was passaged three or more times through *N. benthamiana*. It has also been demonstrated that when an extract known to contain sub-genomic DNA species was used as an inoculum source for a different plant host to that from which it was derived, the level of sub-genomic DNA in the latter decreased. For instance, following passage of PYMV from a multiple passaged tobacco derived inoculum to *Petunia hybrida* F1 hybrid cultivars no sub-genomic DNA species were detected in extracts of these plants. Since the multiple passage of petunia derived inocula through petunia plants has not been investigated in this study it is not possible to state whether sub-genomic DNA replication is supported in this host. However, if host alterations to virus-specific DNAs are analogous to those found in potato following transfer from tobacco then one would be expect to detect increasing levels of sub-genomic DNA in further passages of PYMV through petunia.

The virus specific DNA forms in extracts prepared from graft-infected potato plants were much more susceptible to nuclease degradation during isolation than those derived from any other PYMV host. This presumably reflects higher levels of nuclease activity in potato tissue. Concommitant with this observation was the slightly retarded mobility in agarose gels of DNA species isolated from potato tissues, suggesting increased amounts of host contaminating materials

in these preparations. This problem was not resolved even after exhaustive extraction of the nucleic acid preparations with 70% ethanol. However, figure 18 does indicate that sub-genomic DNA was present in graft-infected potato tissue. In contrast the intracellular DNA forms isolated from graft-infected tomatoes showed a complete lack of any detectable ssDNA, either genomic or sub-genomic. This surprising result suggests that PYMV may infect and replicate in tomato plants without producing ssDNA and presumably virus. One conclusion from this observation was that the systemic spread of infection throughout the plant may occur in the absence of encapsidation, an important consideration when considering the construction of a plant transformation vector based on the geminiviral replication origins (section 4.3). This observation awaits a more detailed analysis of the replication and expression of PYMV in tomatoes and its other hosts. The possible role of the different virus-specific DNA forms in geminivirus replication has been discussed previously (section 1.8) and thus PYMV may be similar in this respect. However, the absence of any detectable ssDNA forms in PYMV infected tomatoes was an exception but it may transpire that if PYMV is allowed to infect tomatoes for very extended periods of time (*i.e.* greater than 4 months) ssDNA may be detected.

The complexity of the geminiviral intracellular DNA forms found in extracts of infected plants was more clearly demonstrated when strand-specific probes for each strand of both cloned components of the viral genome were used to probe four identical Southern blots of various types of PYMV DNA (figure 19). The exact methods employed for the construction of the clones used to synthesize these probes is described in section 3.4. Two single-stranded DNA preparations from both PYMV components cloned in M13 phage vectors, corresponding to either the virion or complementary sense DNA, were each hybridised to one of four identical Southern transfers. Figure 19 shows that only probes made to the B component of PYMV hybridised to the sub-genomic DNA. This is most clearly illustrated in lanes 2 and 3 (figure 19) hybridised with B+ (virion sense) or B- (complementary sense) specific probes. Lane 2 contained gel purified sub-genomic scDNA isolated from a CsCl gradient of a crude extract of PYMV DNA, hence the DNA is in a

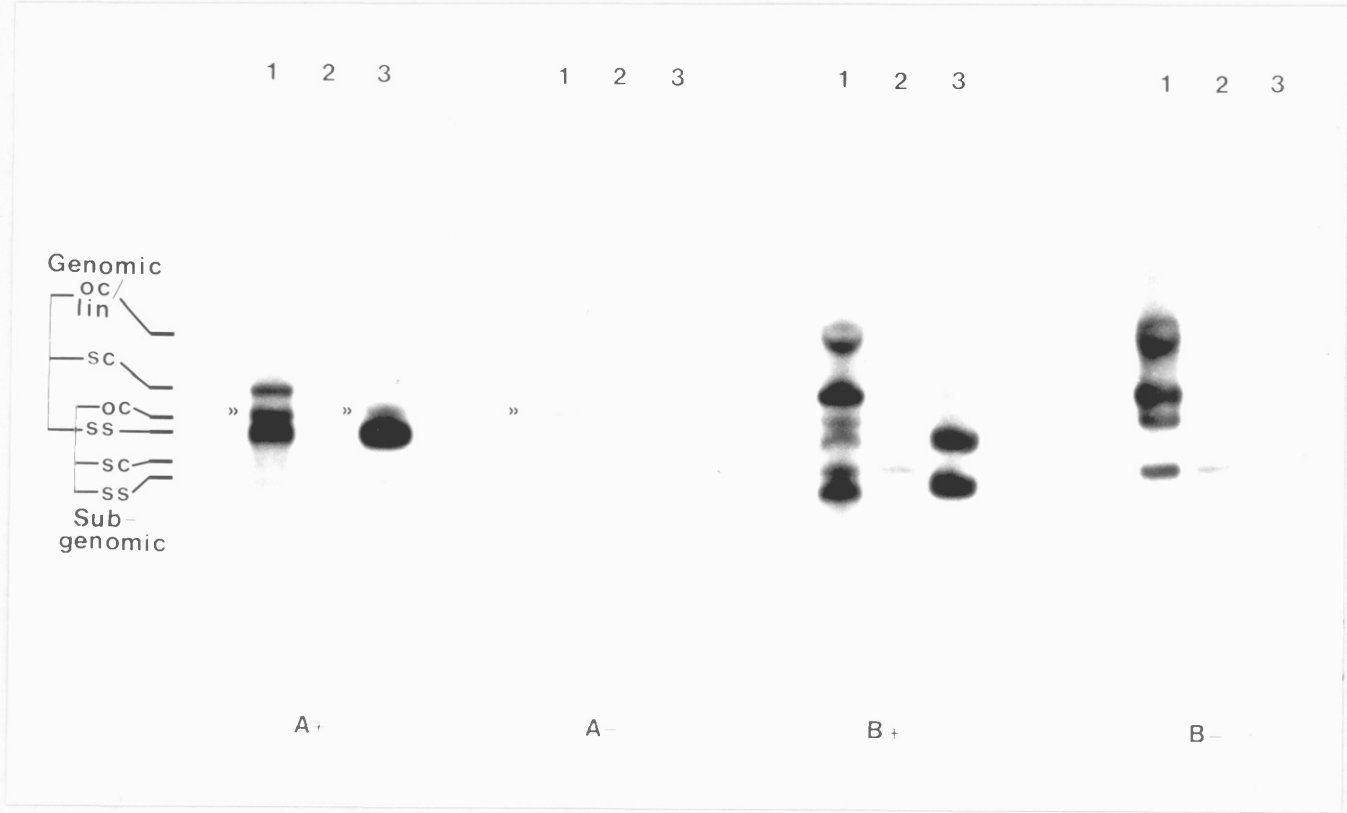


Figure 19. Strand-specific probing of PYMV intracellular DNA forms.
Four identical transfers of agarose gel electrophoresed nucleic acids onto Gene Screen (+)™ were probed and washed by the SSPE/formamide procedure (section 2.9d) and hybridised with strand specific [α-^{32}P] labelled probes of each strand sense of both DNA components of PYMV. The component is indicated as A or B and the sense each probe detected is shown as +, for virion sense and −, for the complementary sense (section 3.4b). Lanes contain;

1. A crude PYMV DNA extract prepared from a high-speed pellet inoculum
2. Gel purified sub-genomic scDNA from CsCl gradiented PYMV DNA (section 2.6g.ii)
3. Encapsidated PYMV ssDNAs, purified by the chloroform:butanol procedure.

double-stranded form that hybridised with equal intensity to radioactive probes of both senses. However, lane 3 which contained genomic and sub-genomic ssDNAs isolated from chloroform:butanol purified virus, hybridised to the complementary sense clone probe, which only detected virion strand DNA. PYMV A component specific DNA only cross-hybridised weakly with B specific, sub-genomic DNA, as illustrated in lane 3 (panel A+), which was probed with an A component specific, complementary stranded DNA probe. This observation may be a result of homology between common regions on the two components of the genome analogous to those found previously with the genomic components of CLV, TGMV and BGMV (Stanley and Gay, 1983; Hamilton *et al.*, 1984; Howarth *et al.*, 1985). Using this method of analysis one previously undescribed DNA species was detectable (indicated by (>>) in figure 19). This species (which does not occur reproducibly in all extracts) appears to be a double-stranded A component-specific DNA form with a similar mobility in the gel system employed to sub-genomic ocDNA. Thus this DNA may be a defective, sub-genomic DNA from PYMV A DNA which has no detectable single-stranded DNA counterpart form. Further analysis of the molecular rearrangements and/or deletions that have occurred to produce this DNA species may give an insight into the mechanism of ssDNA production during geminiviral infection. It may also prove possible to clone this species from crude PYMV DNA preparations using a restriction enzyme map generated from nucleotide sequence analysis of the full-length PYMV A DNA clone.

3.3 Tissue culture

3.3a Protoplast isolation

Two types of sterile source material of potato (*cv.* Maris Bard) were maintained for potato protoplast isolation, *viz.* shoot cultures and suspension cultures (section 2.8a). Protoplasts were successfully isolated from both tissue sources, with an average yield of 10^6 cells/g for shoot cultures and 10^6 cells/10ml packed cell volume of suspension culture cells. Protoplast viability was assessed by the exclusion of the vital stain phenosafranine and then counting unstained cells on a haemocytometer under a light microscope (Widholm, 1972). Immediately after isolation cell viability was greater than 90% from both cell lines of Maris Bard, which always decreased during culture at a rate dependant on the culture medium employed to resuspend the freshly isolated protoplasts. The most suitable culture medium for the protoplasts isolated in this study was Rm medium (Shephard, 1980 as modified by Nelson *et al.*, 1983), where cell viability remained greater than 75% for up to 120h post-isolation. As tissue culture techniques are by their very nature labour intensive, it was decided to concentrate on preparing protoplasts from the suspension cultures because of; 1. the ability to produce very large numbers of protoplasts at regular weekly intervals; 2. the fact that protoplasts isolated from one batch to another were identical, owing to the strict control of environmental conditions and regular sub-culturing into fresh medium.

Suspension cultures were initiated from friable callus of potato which itself was initiated from surface sterilised leaf tissue (figure 20). Callus was added to a liquid medium based on the inorganic salts and vitamins of MS medium (Murashige and Skoog, 1962), supplemented with sucrose, casein hydrolysate, thiamine HCl, pyroxidine HCl, nicotinic acid, 2,4-D and kinetin, at levels described earlier (section 2.8a.iii). Within 2 to 3 weeks of incubation (at 25°C, with shaking at 120rpm) a suspension culture of finely dividing cells developed that was suitable for protoplast isolation. Stocks of callus on CM18 medium were maintained and sub-cultured monthly.

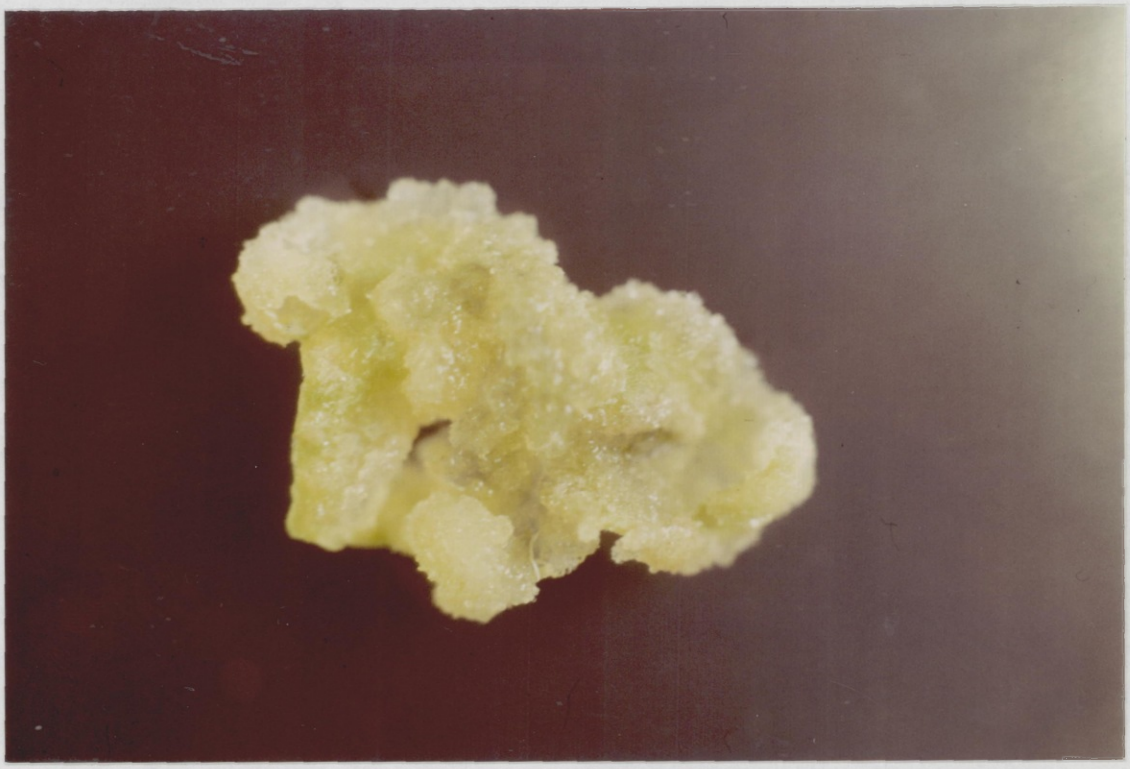


Figure 20. Freshly initiated Maris Bard callus.
Callus of *Solanum tuberosum* ssp. *tuberosum cv.* Maris Bard, initiated on callus initiation medium, CM18 (Dr M.G.K. Jones, pers. comm.)

In the event of microbial contamination of the suspension cultures, such calli were used to reinitiate new suspension cultures.

Initial attempts to isolate protoplasts by the method described in section 2.8b.ii, using cellulase R10, Macerozyme R10 and Pectolyase Y23, from one week old suspension culture cells resulted in poor yields (less than 200,000 protoplasts/10ml packed cell volume of suspension culture cells). These protoplasts were highly vacuolated and regenerated cell walls at very low frequency (as judged by staining with the cellulose specific stain Calcofluor White and examination by uv-light microscopy). Tewes *et al* (1984) reported similar difficulties with the isolation of protoplasts from suspension cultures of *Lycopersicon esculentum* but the problem was circumvented by isolating protoplasts from logarithmically dividing cell suspensions. A similar approach was attempted in this study, by isolating protoplasts from cell suspension sub-cultures produced three days earlier. When suspension culture cells were examined by light microscopy, at different times after sub-culture, it was noted that one week old material consisted of small clusters of highly vacuolated cells (figure 21). By contrast, cells from logarithmically dividing lines, three days after sub-culture, consisted of small chains of plasma rich, partially vacuolated cells (figure 22). When protoplasts were isolated from cell suspensions of this type, yields improved and the protoplasts readily synthesised new cell walls within 24h of culture. It would appear that one week old suspension culture cells may be in a semi-differentiated state unsuitable for the isolation of protoplasts.

Freshly isolated protoplasts of this type (figures 23 and 24) were thus used for further studies on either protoplast metabolism in culture or for inoculation with TGMV.

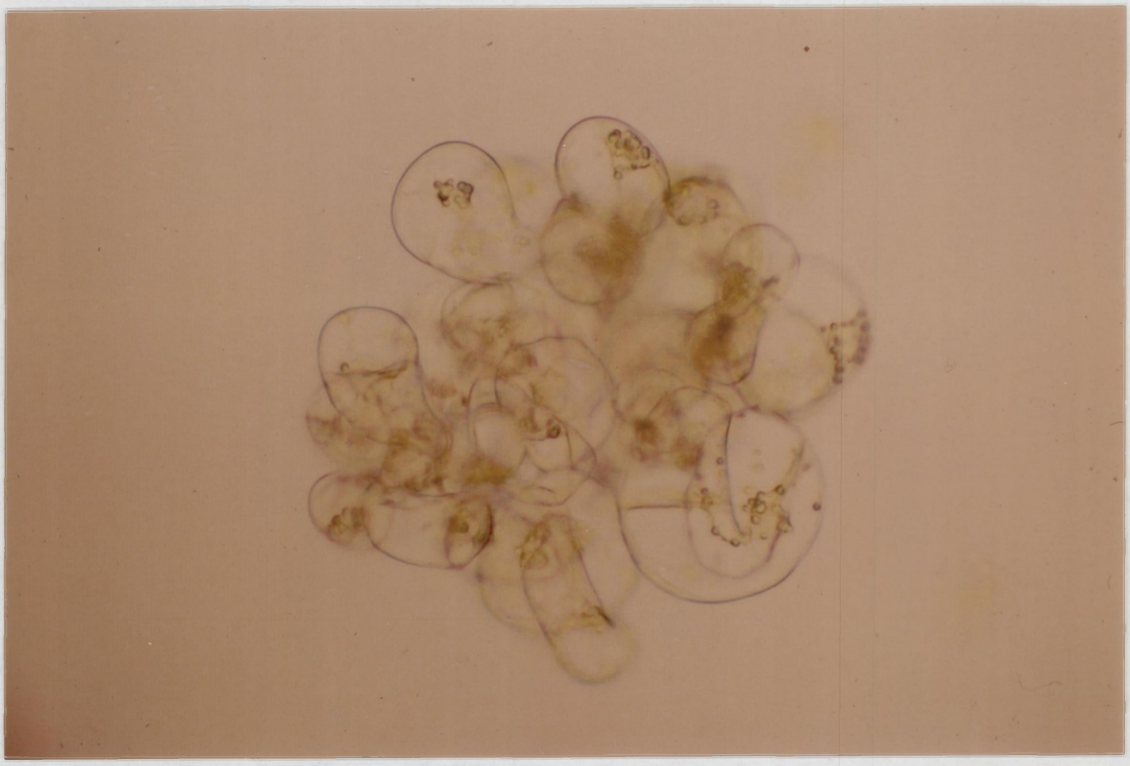


Figure 21. i) Suspension culture cells of Maris Bard.
Photomicrograph of *Solanum tuberosum* ssp. *tuberosum* cv. Maris Bard suspension culture cells, one week after sub-culture.



Figure 22. ii) Suspension culture cells of Maris Bard.
Photomicrograph of *Solanum tuberosum* ssp. *tuberosum* cv. Maris Bard suspension culture cells, three days after sub-culture. Note the partial vacuolation of cells.

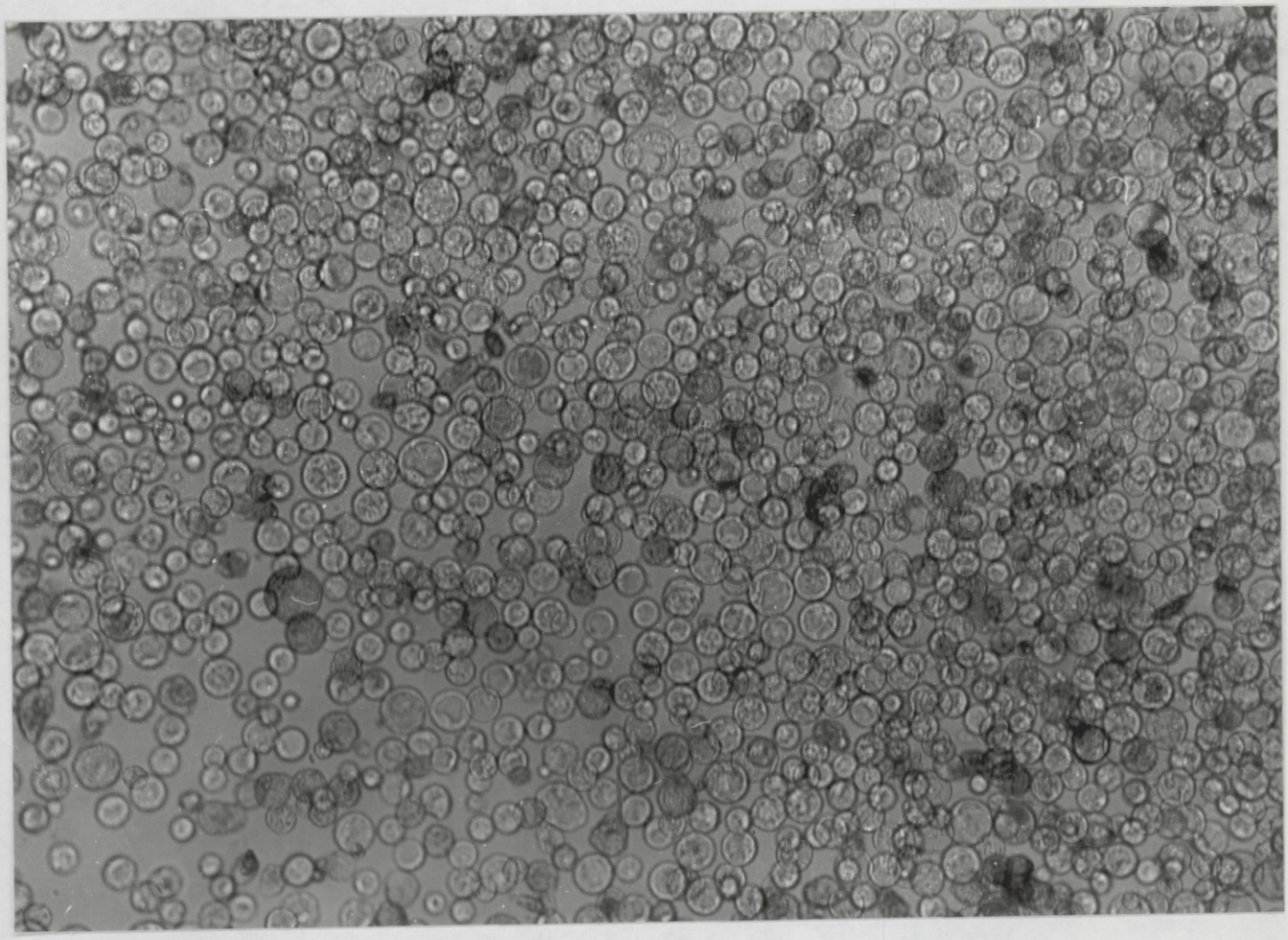


Figure 23. Suspension culture derived protoplasts of Maris Bard.
Photomicrograph of suspension culture derived protoplasts of *Solanum tuberosum* ssp. *tuberosum* cv. Maris Bard.

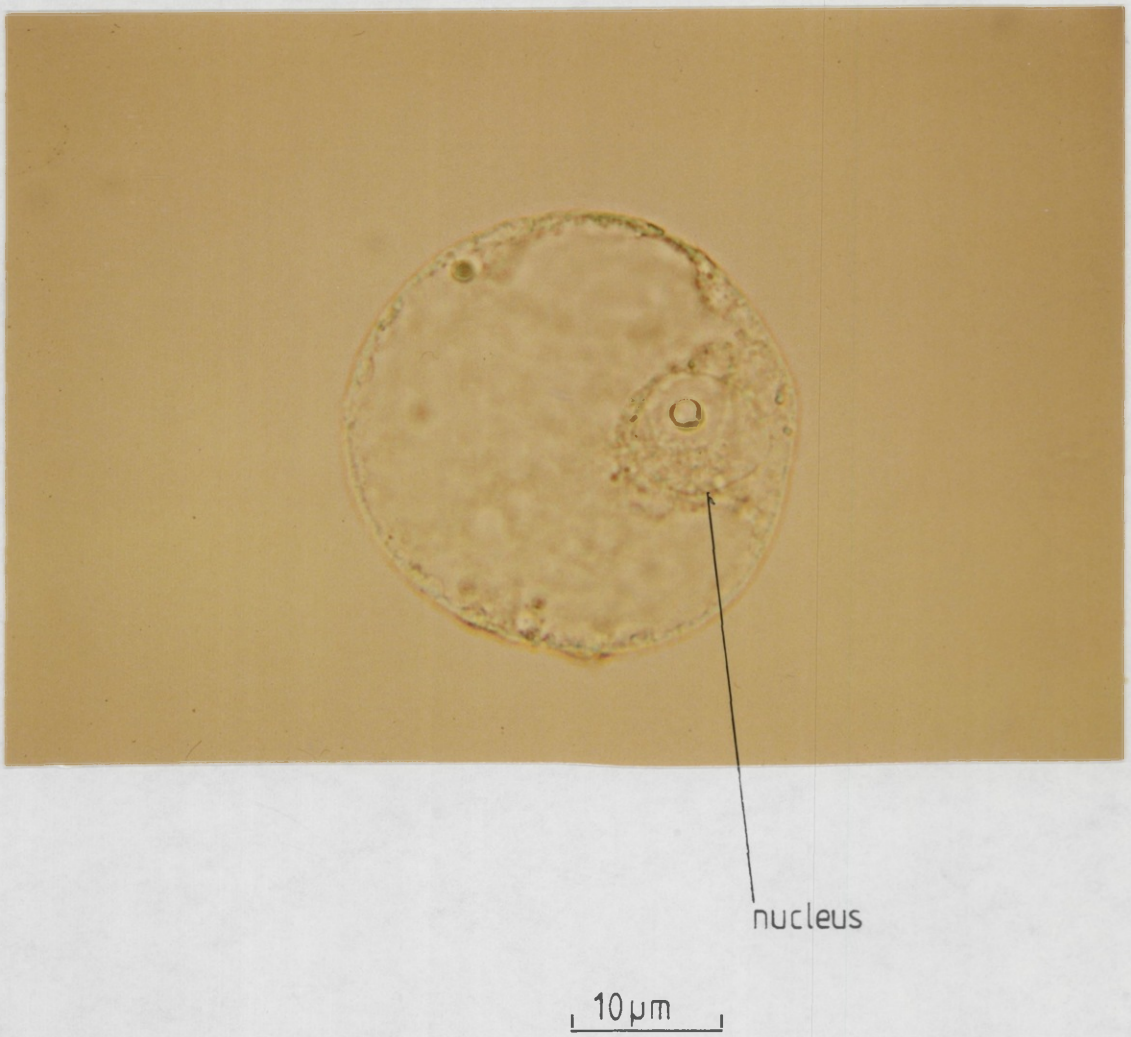


Figure 24. Suspension culture derived protoplast of Maris Bard. Photomicrograph of an individual suspension culture derived protoplast of Solanum tuberosum ssp. *tuberosum cv.* Maris Bard, with a clearly distinguishable nucleus and nucleolus.

3.3b Inoculation with TGMV

The *in vitro* infection of protoplasts by plant viruses depends on two basic processes: the attachment of sufficient amounts of the inoculum to the outer plasma membrane, and their subsequent entry into the protoplasts. Infection can only be achieved under experimental conditions which stimulate both processes without causing excessive damage to the protoplasts. The initial procedure for inoculation (from which most other methods have developed) was that of Takebe and Otsuki (1969). In this work a 90% infection rate for tobacco protoplasts with tobacco mosaic virus was achieved by treating mesophyll protoplasts with a mixture of the virus and the polycation poly-L-ornithine (PLO).

In this study two methods were used to introduce TGMV into freshly isolated potato protoplasts, involving the polyvalent cations polyethylene glycol (PEG) or PLO. Although the actual mechanisms by which polycations stimulate the uptake of virus have not been elucidated, their presence is essential for successful infection to result in several virus-host-protoplast systems (Takebe, 1977). Polycations may form complexes with negatively charged virus particles and nucleic acids. Since the surface of isolated protoplasts is negatively charged (Grout and Coutts, 1974) one might envisage a situation where neutralised polycation/inoculum complexes become adsorbed at the cell surface. The entry of virus into the protoplasts might also be explained by a stimulated endocytosis-like process at the plasmalemma surface or by the actual formation of micro-lesions in the plasmalemma and evidence has been put forward supporting both mechanisms (Cocking and Pojnar, 1969; Burgess *et al.*, 1973).

An inoculation procedure that utilised PEG in the presence of calcium chloride, similar to that described by Maule *et al.* (1980) was used to introduce 50μg of both cloned DNA components of the TGMV genome, excised from their cloning vectors, into suspension culture derived protoplasts (section 2.8c). Additionally isolated protoplasts were inoculated with PLO, by the method of Suzuki and Takebe (1978), in combination with 20μg purified TGMV (DNase I treated to remove any unencapsidated DNA species which co-purified with the virus). Following inoculation the protoplasts were treated with DNase I to remove any DNA which had not been taken up by the cells and then

cultured in Rm medium. Samples of *ca.* 100,000 viable protoplasts were taken over an extended time course, their nucleic acids extracted and subjected to agarose gel electrophoresis (figure 25). After Southern blotting of such gels the transfers were probed with mixtures of nick-translated plasmid DNAs specific for both components of TGMV (pBH401 and pBH602). The resulting autoradiograms are shown in figures 26 and 27. Both autoradiographs show that while inoculum had been introduced into the cells no *de novo* synthesis of viral DNA had occurred. If replication had occurred then the production of ds viral specific DNA forms in the case of virus inoculated protoplasts and the production of ss viral specific DNA forms in protoplasts inoculated with cloned components of the TGMV DNA genome would have been apparent. The viral DNA which was introduced into the protoplasts was not appreciably degraded until the later stages of cell culture *ca.* 7 to 10 days post-inoculation when cells were dying. No regenerated calli were recovered from the inoculated protoplasts, indicating that cell division, if any, was limited. Protoplast viability during inoculation and culture was high and only 10 to 20% of the protoplasts were lost during inoculation with cell death occurring at the rate of 5 to 10% per day during subsequent culture.

An inability to detect viral replication in the cell systems described was probably due to the lack of host DNA replication within the protoplasts (section 3.3d). Townsend *et al.* (1986) have shown that CLV replication in *N. plumbaginifolia* protoplasts is apparently coincident with the onset of host cell division, *i.e.* 48-72h after inoculation. The infection of protoplasts with plant RNA viruses is independent of host cell DNA metabolism and explains the ease with which such systems have been investigated previously. This result, and others like it, prompted a study of host cell DNA metabolism in suspension culture derived potato protoplasts and the use of different culture techniques to obtain an efficient means of regenerating these cells at least to the stage of callus production.

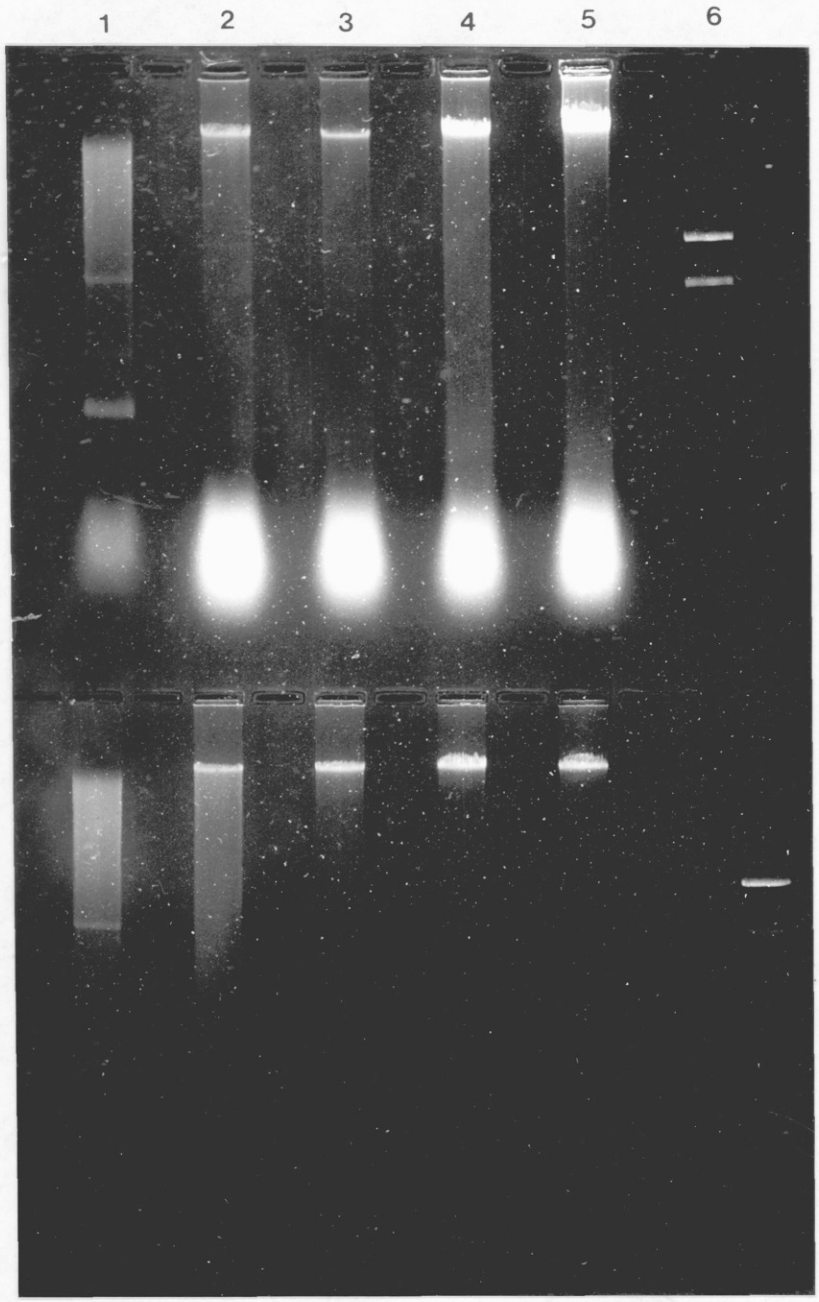


Figure 25. Agarose gel electrophoresis of nucleic acids extracted from TGMV inoculated suspension culture derived protoplasts at various times post-inoculation.

Panel A: Samples from protoplasts inoculated in the presence of PEG with 50μg of each of the cloned components of TGMV excised from their cloning vectors.

Panel B: Samples from protoplasts inoculated with 20μg purified TGMV in the presence of PLO.

Lane 1. A crude extract of TGMV DNA isolated from a high speed pellet inoculum

Lane 2. Protoplast nucleic acid sample 0h post-inoculation

Lane 3. Protoplast nucleic acid sample 48h post-inoculation

Lane 4. Protoplast nucleic acid sample 72h post-inoculation

Lane 5. Protoplast nucleic acid sample 120h post-inoculation

Lane 6. Cloned TGMV DNA components and cloning vector DNA (lower band corresponds to linear TGMV DNA from extracts of crude DNA from infected plants, while the upper band corresponds to linear plasmid DNA)

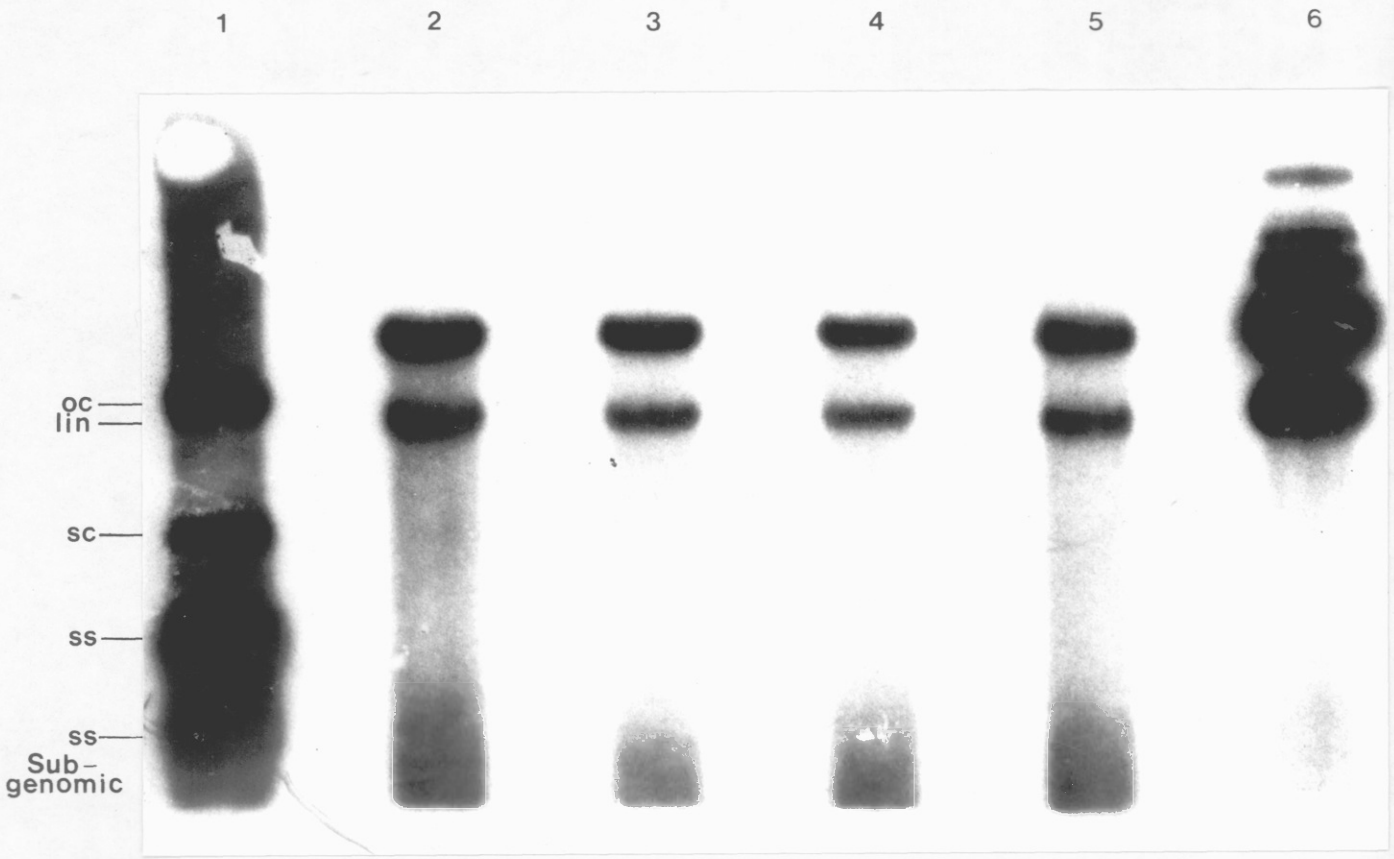


Figure 26. Autoradiogram of Southern transfer of nucleic acids extracted from TGMV clone inoculated suspension culture derived protoplasts at various times post-inoculation.

Samples from protoplasts inoculated in the presence of PEG with 50µg of each of the cloned components of TGMV excised from their cloning vectors, probed with nick-translated cloned TGMV DNA.

Lane 1. A crude extract of TGMV DNA isolated from a high speed pellet inoculum
Lane 2. Protoplast nucleic acid sample 0h post-inoculation
Lane 3. Protoplast nucleic acid sample 48h post-inoculation
Lane 4. Protoplast nucleic acid sample 72h post-inoculation
Lane 5. Protoplast nucleic acid sample 120h post-inoculation
Lane 6. Cloned TGMV DNA components and cloning vector DNA (lower band corresponds to linear TGMV DNA from extracts of crude DNA from infected plants, while the upper band corresponds to linear plasmid DNA)

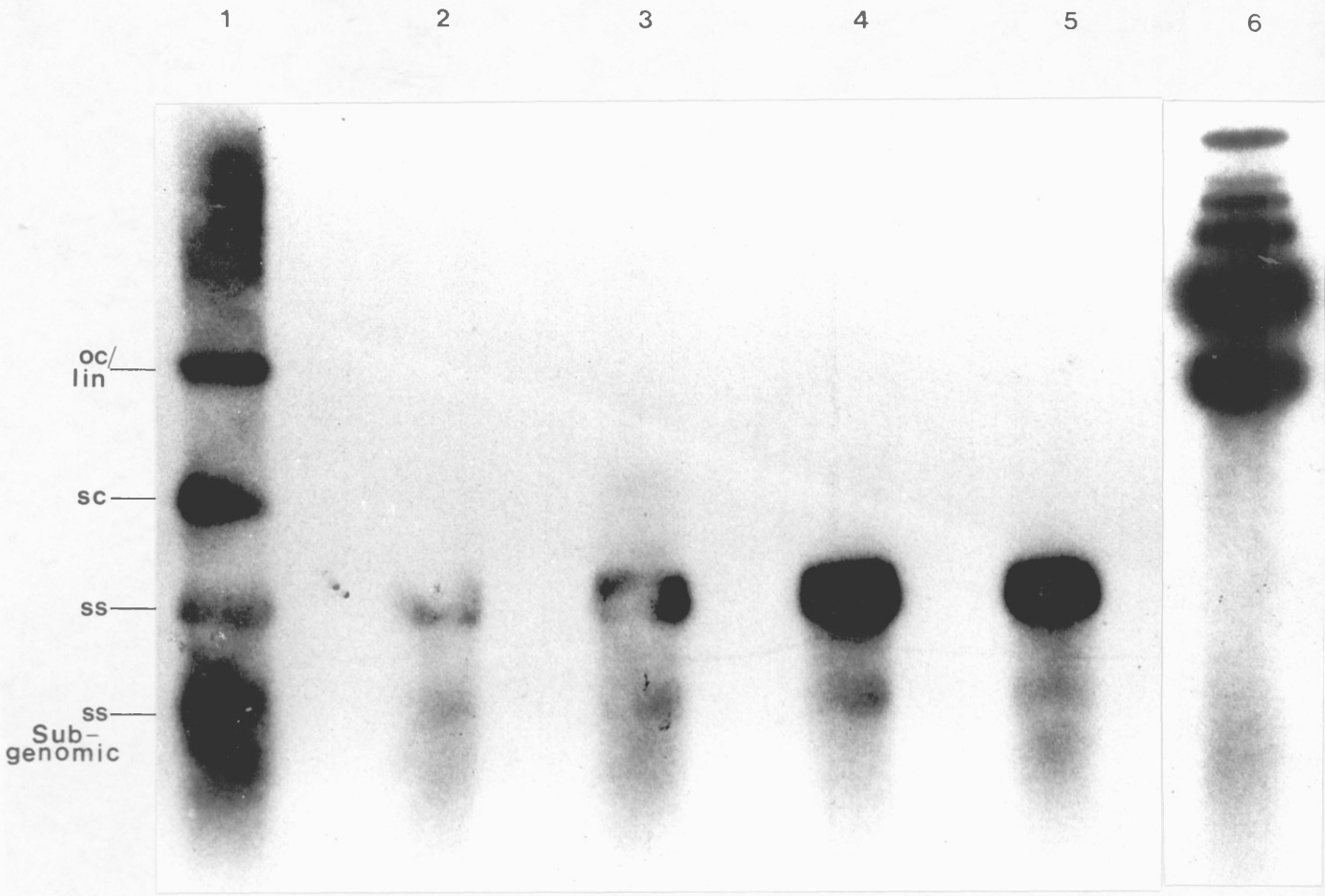


Figure 27. Autoradiogram of Southern transfer of nucleic acids extracted from purified TGMV inoculated suspension culture derived protoplasts at various times post-inoculation.

Samples from protoplasts inoculated with 20μg purified TGMV in the presence of PLO, probed with nick-translated cloned TGMV DNA.

Lane 1. A crude extract of TGMV DNA isolated from a high speed pellet inoculum
Lane 2. Protoplast nucleic acid sample 0h post-inoculation
Lane 3. Protoplast nucleic acid sample 48h post-inoculation
Lane 4. Protoplast nucleic acid sample 72h post-inoculation
Lane 5. Protoplast nucleic acid sample 120h post-inoculation
Lane 6. Cloned TGMV DNA components and cloning vector DNA (lower band corresponds to linear TGMV DNA from extracts of crude DNA from infected plants, while the upper band corresponds to linear plasmid DNA)

3.3c Protoplast culture techniques

In an attempt to regenerate calli from potato protoplasts several different culture techniques were investigated. Shneyour *et al.* (1984) described a procedure for the efficient plating of protoplasts at very low densities (as few as 200 cells/ml). Here, feeder cells from exponentially growing *Petunia* suspension cultures were embedded in low-gelling temperature agarose and overlaid with a cellophane membrane on which protoplasts to be nurse-cultured were plated. This method resulted in a plating efficiency of greater than 50% for *Petunia* protoplasts at densities of less than 10^{3} cells/ml. This procedure was adapted for use with potato suspension cultures and protoplasts derived from this material (section 2.8d.ii). While the feeder layer of suspension culture cells grew rapidly, no protoplast regeneration to form microcalli was observed over a four week incubation period.

Agarose plating and bead culture was first described by Shillito *et al.* (1983) as a means of stimulating the development of protoplast-derived colonies from a number of plant species. Here, protoplasts were embedded in low-gelling temperature agarose and cultured for varying periods of time, depending on the source material being investigated, prior to the agarose being sliced, placed into a liquid medium and incubated on a gyratory shaker. This method proved successful for culturing protoplasts derived from *N. tabacum*, *L. esculentum*, *F. hybrida*, *Brassica rapa*, *Crepis capillaris* and *Hysocyamus muticus*. However, when this method was adapted for suspension culture derived protoplasts of potato (section 2.8d.iii) no callus colonies were observed. However, bead culture should still be persued as a means of culturing potato protoplasts and if combined with the new culture media reported by Fish and Karp (1986) it may prove more successful. It is a powerful method for culturing protoplasts in the type of selective media used in gene transfer experiments. It allows the selective medium to be replaced easily and repeatedly without disturbing the development of the cell colonies in the agarose beads, thus generating a continuous and controlled selection pressure during the important early stages of protoplast culture (Paszkowski *et al.*, 1984).

3.3d Radioactive precursor incorporation

The incorporation of radioactive precursors into TCA precipitable products was used as a means of following the metabolism of cultured protoplast cells. Two different incubation methods were used in labelling experiments, protoplast samples were either taken from the main culture, the label added and the sample incubated for two hours before harvesting or the label was added to the main culture and continuous incorporation assessed by removing aliquots at various times after incubation.

Continuous labelling of suspension culture derived protoplasts of potato with [^{3}H]-UTP revealed that RNA metabolism recovered almost immediately after initiating culture and indeed may never have been interrupted. This finding agrees with a similar study which analysed [^{3}H]-uridine incorporation in two different cultivars of cucumber (*Cucumis sativus cv.* China and Ashley) (Coutts *et al.*, 1975).

Pulse labelling of suspension culture derived protoplasts of potato with [^{3}H]-thymidine were unsuccessful in demonstrating any significant degree of incorporation. Continuous labelling experiments demonstrated a ten-fold increase in the amount of TCA precipitable radioactive products over a 96h protoplast incubation period. However, the level of incorporation declined after this time, a result hardly expected if cells were truly undergoing DNA synthesis. This decrease in incorporation was coincident with the onset of a rapid fall in cell viability. Thus it is possible that the incorporation of [^{3}H]-thymidine may have been the result of host cell DNA repair mechanisms while the decline in TCA precipitable radioactivity may have been due to nuclease digestion as degraded protoplasts lysed releasing their contents.

Zelcer and Galun (1976) have shown that [^{3}H]-thymidine incorporation into protoplasts of *N. tabacum cv.* Xanthi reaches a maximum 48h after culture, with a nearly 40-fold increase in the level of TCA precipitable radioactive products being demonstrable. This peak in incorporation preceded nuclear division by *ca.* 18h indicating that freshly isolated tobacco protoplasts undergo a premitotic stage during which DNA replication occurs. The degree of incorporation of [^{3}H]-thymidine into potato protoplasts in this study never approached these levels.

There have been numerous reports concerning the plating efficiency of shoot culture derived potato protoplasts, *i.e.* the number of calli recovered as a percentage of the number of protoplasts plated. Initial studies (Thomas, 1981) reported a plating efficiency of less than 0.001% but in a recent report (Fish and Karp, 1986) indicated that a plating efficiency of 95% was possible, using the same cultivar of potato, *viz.* Maris Bard.

The obvious conclusion from these studies is that much work remains to be done to optimise the culture conditions of potato suspension culture derived protoplasts so as to obtain a high plating efficiency, a prerequisite for the study of geminivirus replication *in vitro* in potatoes. The importance of tissue culture in studying the replication strategy of geminiviruses will be discussed in section 4.

3.4 Bacterial cloning

3.4a Purification and isolation of PYMV scDNA

Negatively supercoiled DNA from *N. benthamiana* plants infected with PYMV was purified by the method of *Sunter et al.* (1984) (section 2.6g.ii), using cellular extracts from infected plants prepared by the Triton extraction procedure (Hamilton *et al.*, 1982). These preparations were treated initially with NaOH/SDS and subsequently with potassium acetate, a procedure analogous to the alkaline lysis protocol for the production of plasmid 'miniprep' DNA (section 2.10g). Following phenol extraction and ethanol precipitation the DNA was centrifuged to equilibrium in CsCl/ethidium bromide density gradients. Occasionally, uv fluorescent bands were visible in these gradients and if present they were removed with a sterile syringe and needle. However, in most instances no fluorescent bands were observed and the only indication of the presence of a band was a discrete change in optical density approximately ¾ of the way down the tube. When this region was removed and extracted it was found to contain PYMV scDNA. Agarose gel electrophoresis of fractions isolated from such a gradient is shown in figure 28. These results show that several types of supercoiled molecules were present in the bands which were extracted, including those corresponding to sc genomic DNA, sc sub-genomic DNA and possibly sc dimers of the genomic DNA. During the extraction procedure after centrifugation a considerable amount of 'nicking' of the supercoiled molecules occurred, this was unavoidable but did not affect subsequent analysis.

Further analysis consisted of small scale restriction enzyme digestions (section 2.10b) of the scDNA with enzymes potentially suitable for cloning the genome of PYMV into the M13mp18 or 19 series of bacteriophage cloning vectors (Yanisch-Perron *et al.*, 1985). The products of these reactions were subjected to agarose gel electrophoresis and bidirectional Southern transfer. The products of the restriction enzyme digests contained a complex mixture of products, illustrated in figure 29. Very little linear genomic length PYMV DNA was present in the undigested scDNA (lanes 1 and 8, both loaded with unrestricted PYMV DNA in combination with *Hin*dIII digested λ DNA).

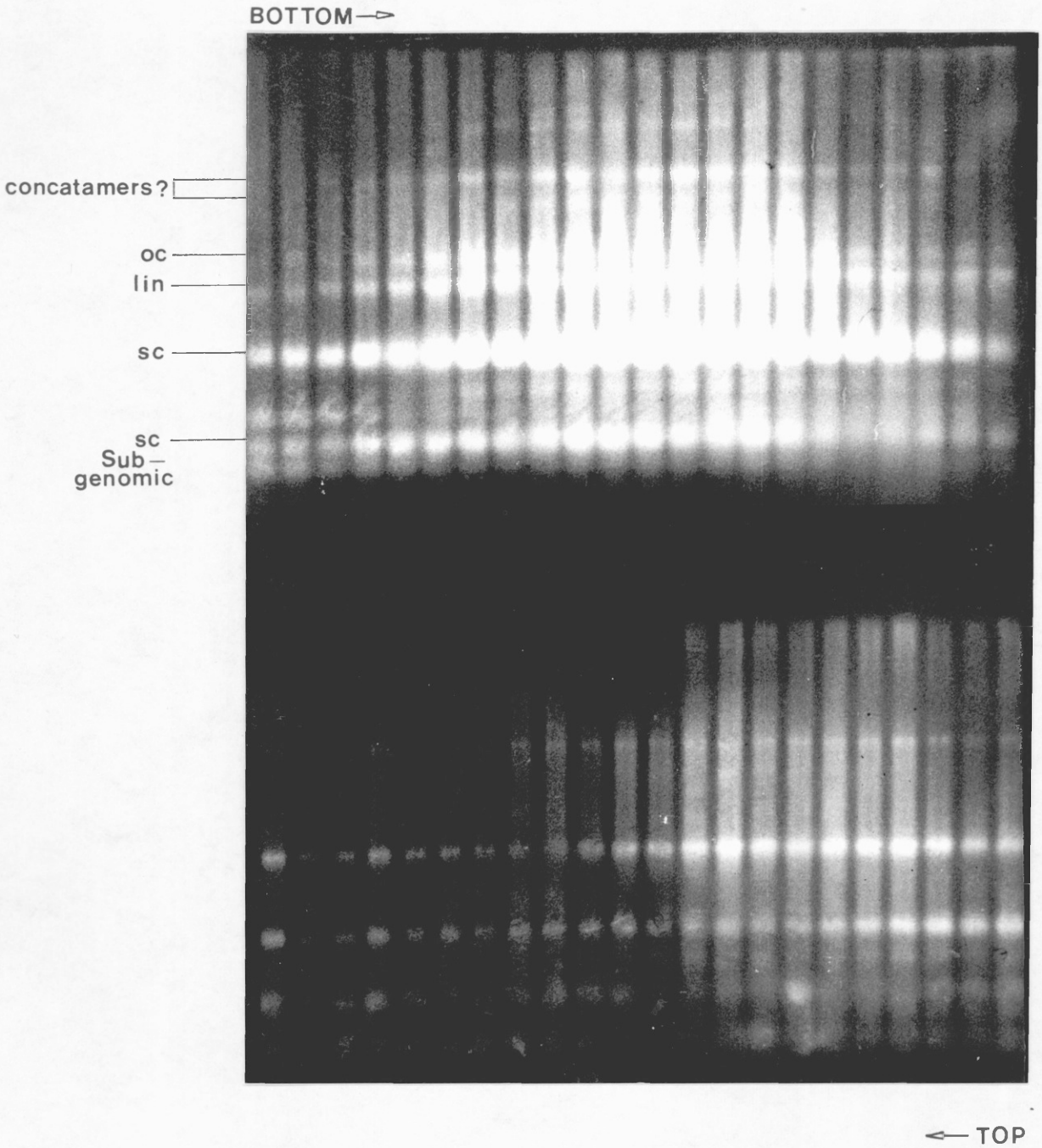


Figure 28. Agarose gel electrophoresis of fractions from a CsCl/ethidium bromide density gradient of PYMV intracellular DNA.

Gradient fractions run from left to right and from the bottom of the gradient to the top, as indicated. DNA at the top of the gradient may well have been trapped by the proteinacious material which collected in this region.

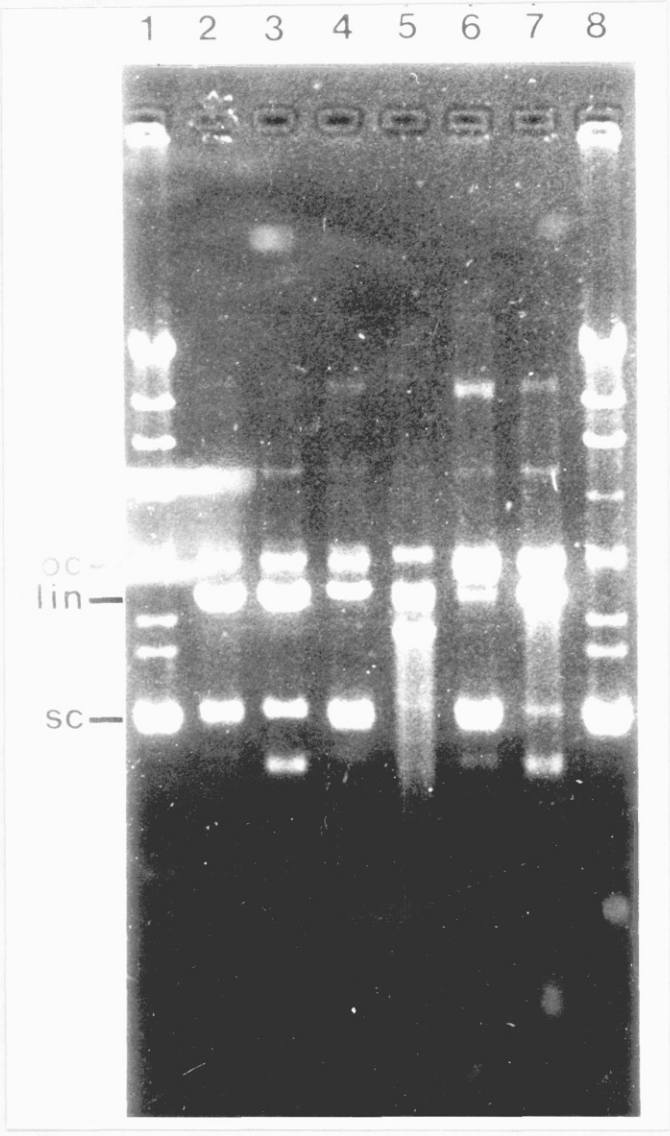


Figure 29. Restriction enzyme digests of purified PYMV scDNA.

1% agarose of small scale restriction enzyme digests of CsCl/ethidium bromide purified PYMV scDNA.

Lane 1. undigested PYMV scDNA loaded with *Hin*dIII digested λ DNA (appendix 5.4)
Lane 2. PYMV scDNA digested with *Bam*HI
Lane 3. PYMV scDNA digested with *Eco*RI
Lane 4. PYMV scDNA digested with *Hin*dIII
Lane 5. PYMV scDNA digested with *Kpn*I
Lane 6. PYMV scDNA digested with *Mlu*I
Lane 7. PYMV scDNA digested with *Pst*I
Lane 8. undigested PYMV scDNA loaded with *Hin*dIII digested λ DNA (appendix 5.4)

By contrast, all restriction enzyme digested PYMV scDNA contained a proportion of linear DNA, which might in some cases have been the result of incomplete digestion. Enzymes which appeared to be single-site cutters within the PYMV scDNA were subsequently utilised in cloning experiments.

3.4b Molecular cloning and restriction mapping of PYMV

Based on the results of the restriction enzyme analysis (figure 29) it was decided to attempt to clone PYMV scDNA at its putative *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I sites, into the reciprocally digested bacteriophage vector M13mp18 and to transform these recombinant DNAs into *E. coli* strain TG2. All four transformations yielded white bacteriophage plaques in the lawn of bacteria, indicating that the clones possibly contained virus-specific inserts. These clones were initially screened by preparing ssDNA templates from the respective plaques (section 2.10j) and electrophoresing them in agarose gels to determine whether they contained a genuine inserted DNA fragment or not (figure 30). These experiments confirmed that all four clones types did contain inserted fragments. Preparations of dsDNA from these clones were then analysed by restriction enzyme digestion. In nearly all cases each clone type, when digested with the enzyme used for the original cloning, excised a linear DNA insert of *ca.* 2500-2600bp (figure 31). These results suggest that these clones may well represent full-length cloned copies of the PYMV genome as their mobility coincided closely with that of full-length clones of other geminiviruses (section 1.6). Exceptional to this observation were those clones constructed at *Pst*I sites. These *Pst*I clones were difficult to excise from the cloning vector and were not amenable to further analysis (see figure 46 which illustrates the results of the nucleotide sequencing of the *Pst*I clones). Clone nomenclature was as follows; m or p indicated whether clones were produced in M13 or plasmid vectors; M indicated that the clones contained the genome of the causal agent of *Mosaic Amarillo de la Papa* (now known as PYMV); the next letter indicated the first letter of the restriction enzyme used to construct the clone; and finally the clones were enumerated.

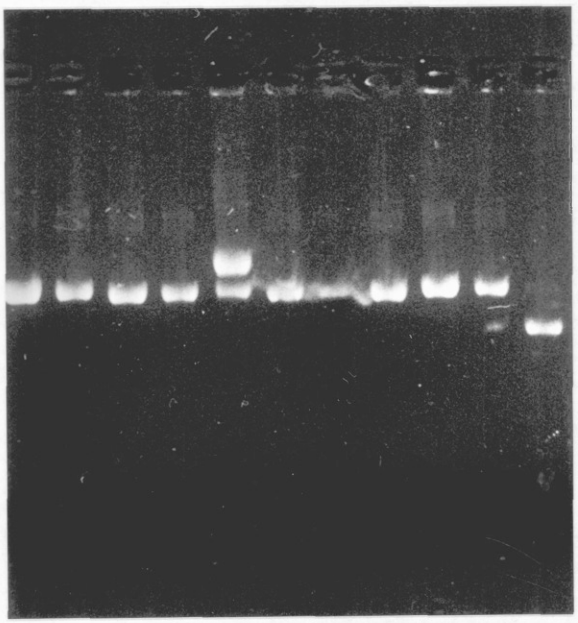


Figure 30. Analysis of PYMV cloned DNA in M13 ssDNA preparations. Electrophoresis through a 1% agarose gel of M13 ssDNA preparations from white plaques obtained in PYMV cloning experiments. The far left-hand lane contains wild-type M13mp18 ssDNA, suggesting that all the other DNA preparations investigated contained cloned inserts.

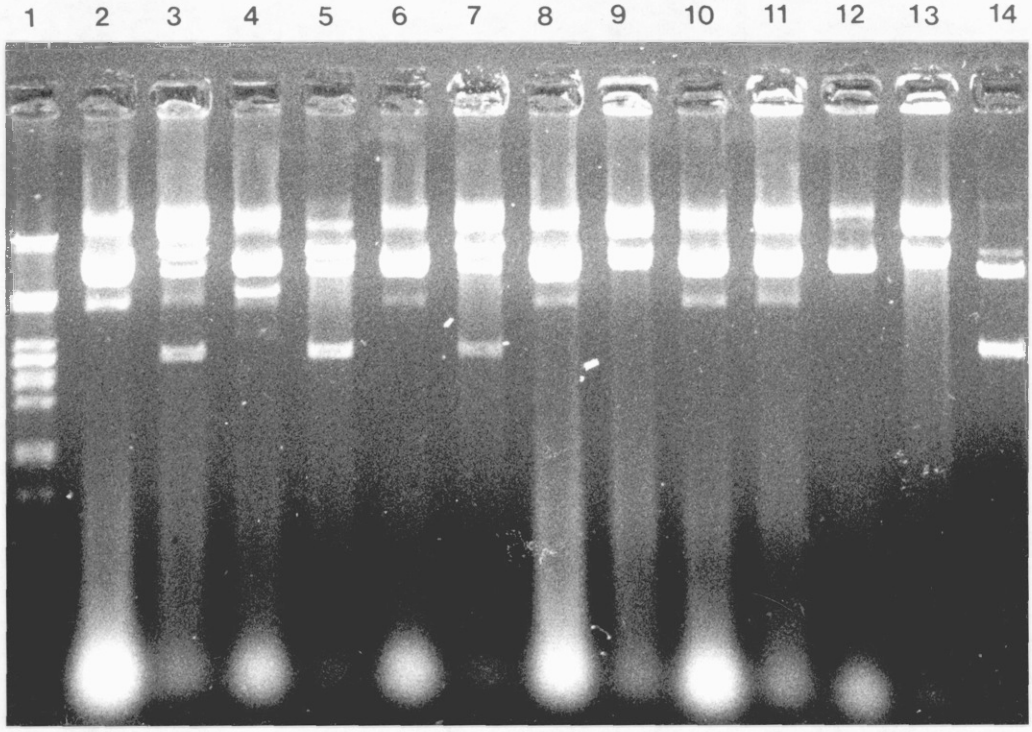


Figure 31. Agarose gel of PYMV clones digested with various restriction enzymes.

Lane 1. *Pst*I digested λ DNA (see appendix 5.4)
Lane 2. mMH1
Lame 3. mMH1 digested with *Hin*dIII
Lane 4. mME1
Lane 5. mME1 digested with *Eco*RI
Lane 6. mMB1
Lane 7. mMB1 digested with *Bam*HI
Lane 8. mMH1 digested with *Eco*RI
Lane 9. mMH1 digested with *Bam*HI
Lane 10. mME1 digested with *Hin*dIII
Lane 11. mME1 digested with *Hin*dIII
Lane 12. mMP1
Lane 13. mMP1 digested with *Pst*I
Lane 14. pBH401 digested with *Eco*RI (full-length TGMV clone)

Thus, clone mMH1 is an M13 clone of PYMV at its *Hin*dIII site, and this particular clone was the first plaque picked from the plate.

Figure 31 illustrates that mMH1 was not digested by either *Eco*RI or *Bam*HI and that neither mME1 nor mMB1 were digested by *Hin*dIII. This result was the first indication that the recombinants contained two different populations of cloned DNA suggesting that PYMV might possess a bicomponent genome. This theory was tested further by hybridising bidirectional blots of the gel shown in figure 29 with nick-translated probes prepared from mMH1 and mME1 clones (figure 32). The two autoradiographs differ in hybridisation pattern and indicate differences in the products of the restriction enzyme digestion reactions not apparent in agarose gels. For example, lanes 3 and 4 appeared very similar when they were compared in the gel, except for a smaller DNA fragment which was found in lane 3. However, when the two autoradiographs of Southern transfers were examined, completely different hybridisation patterns were noted between lanes 3 and 4. After probing lane 4 with mMH1 a mainly linear PYMV DNA was noted, consistent with the notion that apparently full-length clones were obtained following *Hin*dIII cloning. However, after probing lane 4 DNA with mME1 no linear PYMV DNA was apparently present. The obvious conclusion from this result was that PYMV indeed possessed a bicomponent genome, with one component containing a unique *Hin*dIII site and the other a unique *Eco*RI site. This conclusion is supported by the reverse analysis, where when lane 3 was probed with mMH1 mainly scDNA was found, while the same lane apparently contained only linear DNA after probing with mME1. A further smaller DNA species identified in lane 3 after probing with mME1 may be representitive of a sub-genomic scDNA which, by analogy with TGMV and CLV, was derived from the smaller genomic component. Thus the clone nomenclature was amended to include an A or B notation to indicate which component the clones corresponded to similar to the nomenclature adopted previously for TGMV (Bisaro *et al.*, 1982). Thus mMH1 became mMAH1, and mME1 became mMBE1. Two unexplained observations in these experiments were apparent. Firstly, if the smaller DNA molecule was the supercoiled form of the sub-genomic DNA why was its occurrence so variable given

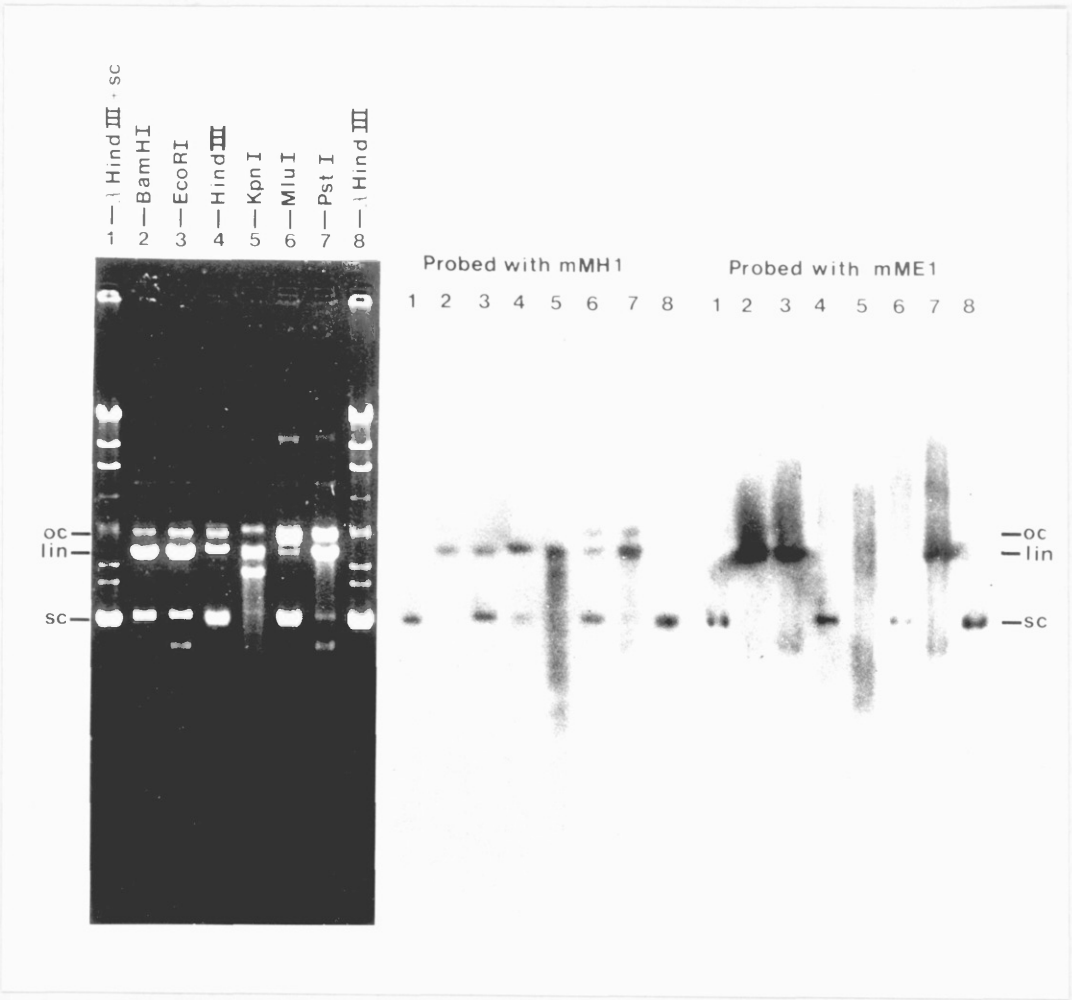


Figure 32. Agarose gel of restriction enzyme digested PYMV scDNA and the corresponding autoradiographs from bidirectional transfers of the same gel hybridised with different nick-translated PYMV clones.

The agarose gel is the same as that shown in figure 29, hence the lane numbers correspond to the same DNA samples. Bidirectional Southern transfers on gene screen™ membranes were probed with [α-^{32}P] labelled PYMV clones as indicated, using the formamide hybridisation and washing procedure (section 2.9d).

that all the samples run on the gel were from the same preparation of supercoiled DNA. Secondly, lane 2 contains a sample of *Bam*HI digested scDNA which when used for cloning gave rise to the same component clones as those obtained with *Eco*RI. Therefore it is difficult to explain why when probed with mMH1, lane 2 did not hybridise to an undigested scDNA species. Both of these points remain unresolved although it is possible that these observations are the result of a failure of the DNA in these regions of the gel to transfer to the membrane. However, as will be shown later, all the tentative suggestions put forward from the analysis of these results have proven correct, in that they are both consistent and compare favourably with studies on other geminiviruses which contain a bicomponent genome.

Other full-length PYMV clones were constructed in the plasmid vector pBR328 (Soberón *et al.*, 1980). Unique *Nco*I sites were found in both components of the PYMV genome (M.J. Slomka, pers. comm). These sites were used to clone PYMV scDNA, digested with *Nco*I, into the unique *Nco*I site in the chloramphenicol resistance gene of pBR328. The plasmid was digested and treated with phosphatase prior to ligation and transformation into *E. coli* strain JM101. Bacteria containing plasmids were then selected on plates containing both ampicillin and tetracycline (appendix 5.2). Colonies which grew were then toothpicked onto two replica plates one containing the antibiotics ampicillin and tetracycline the other containing ampicillin, tetracycline and chloramphenicol. Bacteria which contained recombinant DNA (*i.e.* plasmid DNA with an insert DNA) failed to grow on the chloramphenicol plate due to the insert in the *Nco*I site which inactivated the chloramphenicol antibiotic resistance gene. The DNA from the corresponding colonies of interest (which grew on the replica plate) was then isolated by the method described in section 2.10g. This DNA was analysed by restriction enzyme digestion and agarose gel electrophoresis (figure 33). Two distinct clone types were obtained, one with an insert size of *ca.* 2500-2600bp and the other of *ca.* 1400bp. This second clone type (*e.g.* pMBN5) had an insert of the same size as the sub-genomic DNA. In subsequent restriction enzyme analysis (figure 34) this clone was shown to have a *Bam*HI site but the *Eco*RI site was deleted, no further detailed analysis has yet been performed

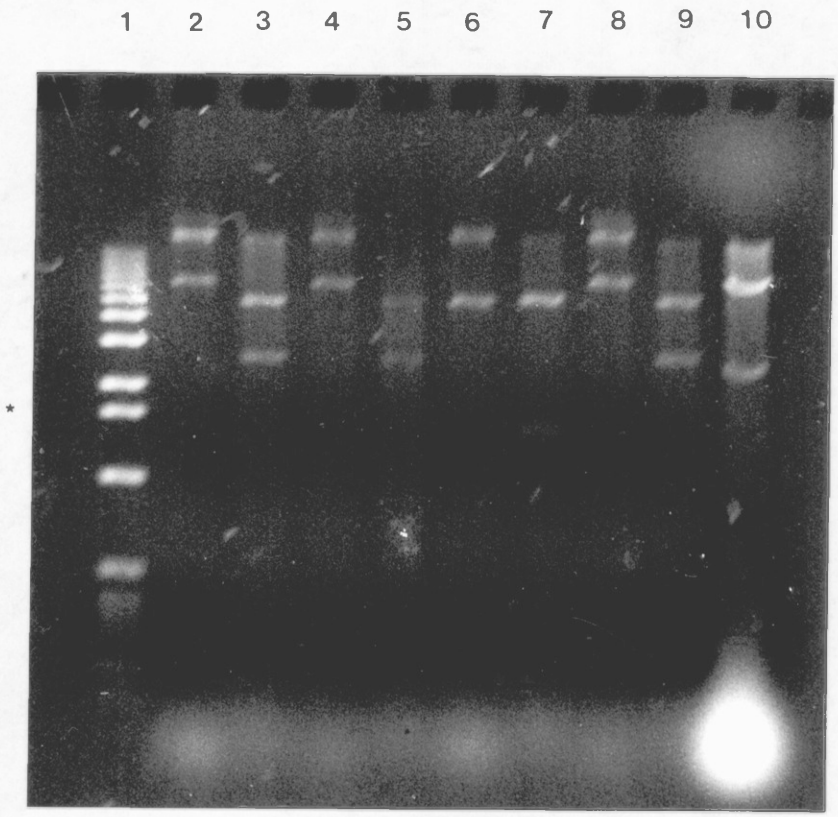


Figure 33. Agarose gel electrophoresis of restriction enzyme digests of PYMV DNA clones produced at the *Nco*I site in the pBR328 plasmid vector.

(*) indicates the 1.6Kb fragment in the kilobase ladder (appendix 5.4).

Lane 1. kilobase ladder marker DNA
Lane 2. pMN2
Lane 3. pMN2, *Nco*I digested
Lane 4. pMN4
Lane 5. pMN4, *Nco*I digested
Lane 6. pMN5
Lane 7. pMN5, *Nco*I digested
Lane 8. pMN25
Lane 9. pMN25, *Nco*I digested
Lane 10. mMAH1, *Hin*dIII digested

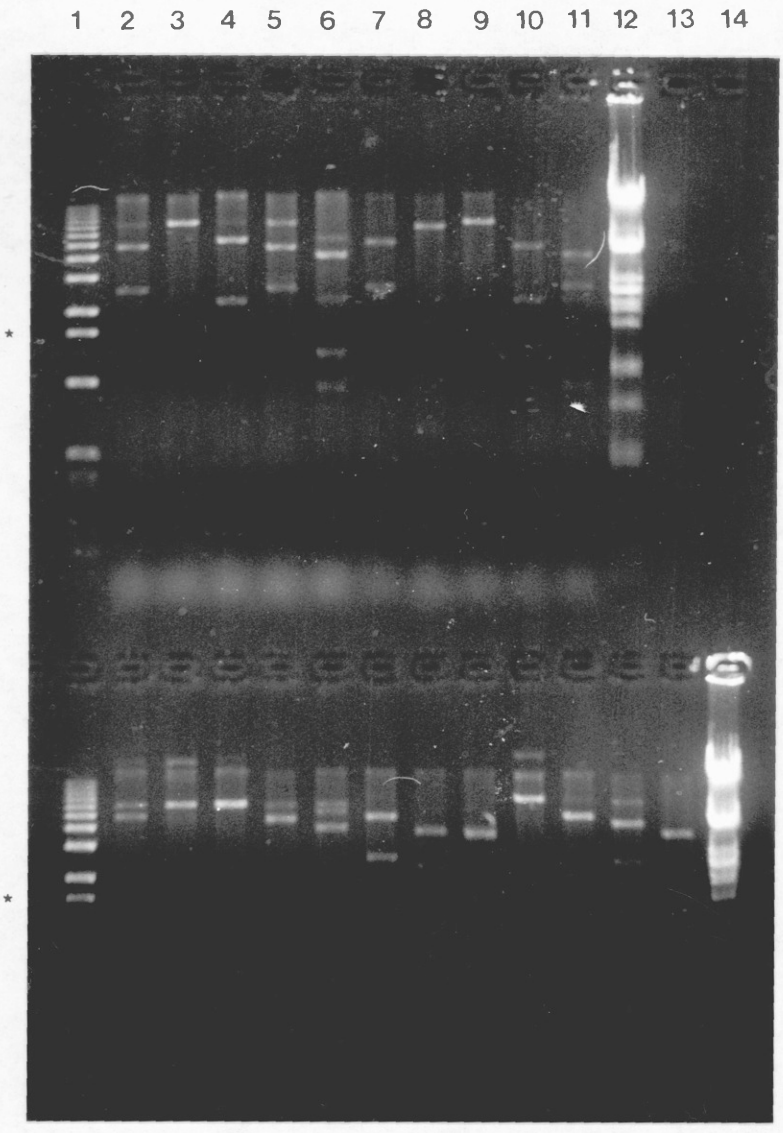


Figure 34. Agarose gel electrophoresis of restriction enzyme digested pMN clones.

(*) indicates the 1.6Kb fragment in the kilobase ladder (appendix 5.4).

Panel A (upper tier)	Panel B (lower tier)
Lane 1. kilobase ladder marker DNA	kilobase ladder marker DNA
Lane 2. pMN2, *Nco*I digested	pMN5, *Nco*I digested
Lane 3. pMN2, *Eco*RI digested	pMN5, *Eco*RI digested
Lane 4. pMN2, *Hin*dIII digested	pMN5, *Hin*dIII digested
Lane 5. pMN2, *Nco*I, *Eco*RI digested	pMN5, *Nco*I, *Eco*RI digested
Lane 6. pMN2, *Nco*I, *Hin*dIII digested	pMN5, *Nco*I, *Hin*dIII digested
Lane 7. pMN4, *Nco*I digested	pMN5, *Bam*HI digested
Lane 8. pMN4, *Eco*RI digested	pMN5, *Nco*I, *Bam*HI digested
Lane 9. pMN4, *Hin*dIII digested	pMN25, *Nco*I digested
Lane 10. pMN4, *Nco*I, *Eco*RI digested	pMN25, *Eco*RI digested
Lane 11. pMN4, *Nco*I, *Hin*dIII digested	pMN25, *Hin*dIII digested
Lane 12. *Fst*I digested λ DNA	pMN25, *Nco*I, *Eco*RI digested
Lane 13.	pMN25, *Nco*I, *Hin*dIII digested
Lane 14.	*Fst*I digested λ DNA

on this clone. Clones pMN2 and pMN25 were digested with *Hin*dIII, while pMN4 was digested with both *Bam*HI and *Eco*RI (figure 34). Thus these clones were ascribed the names pMAN2, pMAN25 and pMBN4, in line with the nomenclature used for the full-length clones obtained in M13 vectors. These clones produced a relatively poor yield of plasmid possibly due to the active tetracycline resistance gene carried on the plasmid. Indeed Lee and Edlin (1985) reported that expression of tetracycline resistance in pBR derivatives reduced the reproductive fitness of plasmid-containing *E. coli*.

Restriction enzyme analysis of the PYMV DNA genome was confined to mapping sites present in the apparently full-length clones. This procedure was performed by digesting clones with restriction enzymes in both single and double digests and determining the sizes of fragments on agarose gels by comparison to standards consisting of dsDNA markers (appendix 5.4). Examples of such gels are shown in figures 35-37, and from this data crude restriction maps of the two components of PYMV DNA were constructed (figure 38).

The relative strandedness of the PYMV M13 clones was analysed using solution hybridisation tests (Messing, 1983). It was demonstrated that because M13 bacteriophage always encapsidate the positive ssDNA strand, two recombinant phage with a DNA fragment cloned in opposite orientations into the RF DNA will hybridise only via the inserted DNA. Thus it is possible to determine the polarity of clones relative to one another. The result of solution hybridisation tests between mMAH and mMBE clones is shown in figure 39 following hybridisation and agarose gel electrophoresis. The presence of slow migrating bands (slower than those of the ssDNA alone) indicates clones of opposite strands of DNA. However, this test was sensitive enough to detect cross-homology between the different components of PYMV DNA, presumably via their common region on the genome (as discussed previously). Homology between clones of opposite orientations was clearly demonstrated as all unit length ssDNA hybridised to form several slower migrating species upon electrophoresis. Strand-specific probes were synthesised to each strand of both components of the PYMV clones mMAH1, mMAH2, mMBE1 and mMBE2 (section 2.9b). These probes were used for hybridisation with

four identical Southern transfers illustrated in figure 19. From these results the strandedness of the M13 clones with respect to the viral encapsidated species was determined. Thus the nomenclature for the PYMV M13 clones was extended to incorporate the strandedness of the ssDNA produced from each clone, by replacing the clone number with either (+) or (-). In all further studies the following clone nomenclature was utilised;

mMAH+, formerly mMAH2
mMAH-, formerly mMAH1
mMBE+, formerly mMBE1
mMBE-, formerly mMBE2

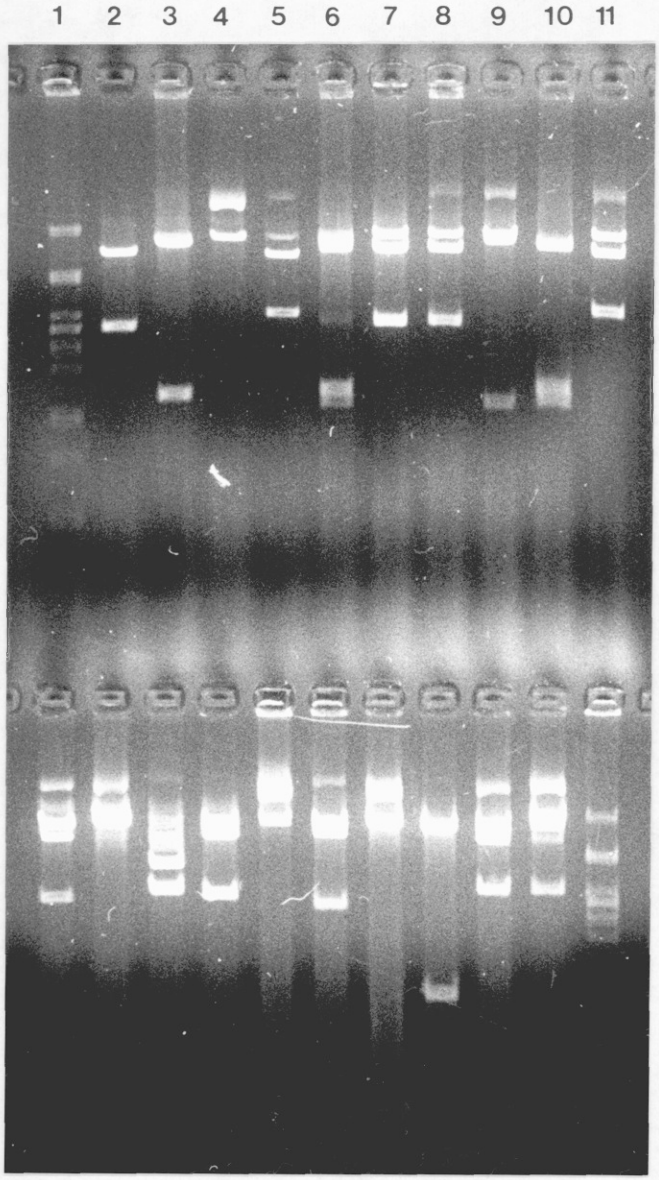


Figure 35. i) Agarose gel electrophoresis of restriction enzyme digested PYMV clones.

Panel A (upper tier)	Panel B (lower tier)
Lane 1. *Pst*I digested λ DNA	mMH1, *Hin*dIII digested
Lane 2. mMB1, *Bam*HI digested	mMH1, *Bam*HI digested
Lane 3. mMB1, *Eco*RI digested	mMH1, *Cla*I digested
Lane 4. mMB1, *Hin*dIII digested	mMH1, *Bam*HI, *Hin*dIII digested
Lane 5. mMB1, *Cla*I digested	mMP1, *Pst*I digested
Lane 6. mMB1, *Eco*RI, *Hin*dIII digested	mMP1, *Eco*RI digested
Lane 7. mMB1, *Hin*dIII, *Bam*HI digested	mMP1, *Hin*dIII digested
Lane 8. mMB1, *Eco*RI digested	mMP1, *Bam*HI digested
Lane 9. mMB1, *Bam*HI digested	mMP1, *Cla*I digested
Lane 10. mMB1, *Bam*HI, *Eco*RI digested	mMB4, *Cla*I digested
Lane 11. mMB1, *Cla*I digested	*Pst*I digested λ DNA

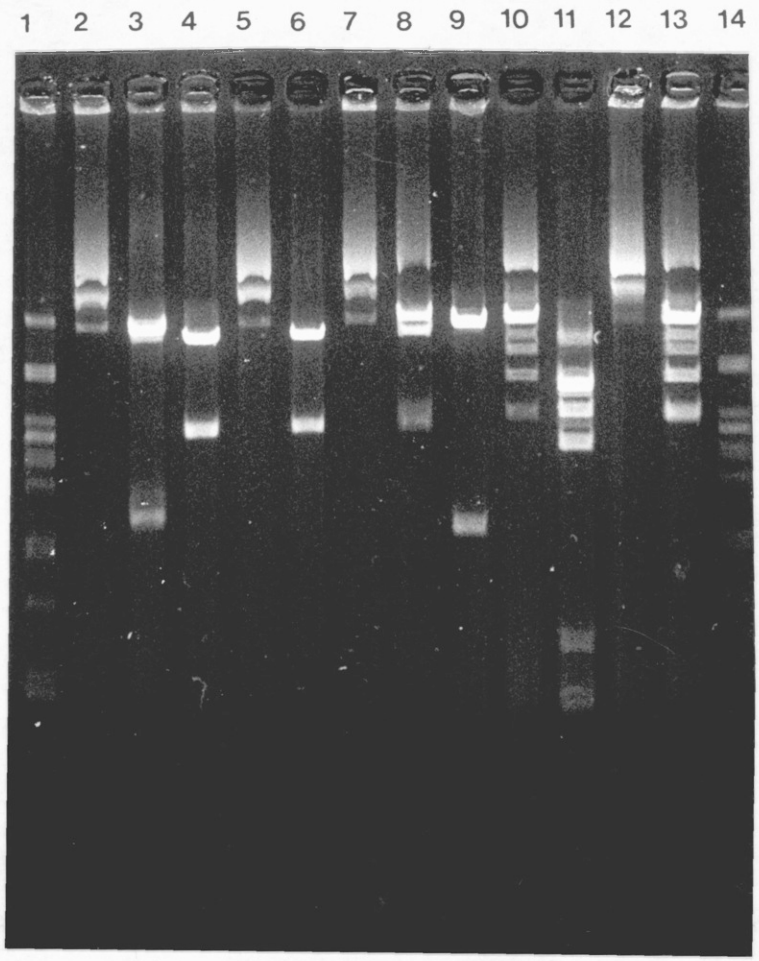


Figure 36. ii) Agarose gel electrophoresis of restriction enzyme digested PYMV clones.

Lane 1. *Pst*I digested λ DNA
Lane 2. mMB1, *Pst*I digested
Lane 3. mMB1, *Pst*I, *Eco*RI digested
Lane 4. mMB1, *Pst*I, *Bam*HI digested
Lane 5. mMB1, *Mlu*I digested
Lane 6. mMB1, *Mlu*I, *Pst*I digested
Lane 7. mMB1, *Pst*I digested
8. mME1, *Pst*I, *Eco*RI digested
9. mME1, *Pst*I *Bam*HI digested
10. mMH1, *Cla*I digested
11. mMH1, *Cla*I, *Hin*dIII digested
12. mMH1, *Pst*I digested
13. mMH1, *Cla*I, *Pst*I digested
14. *Pst*I digested λ DNA

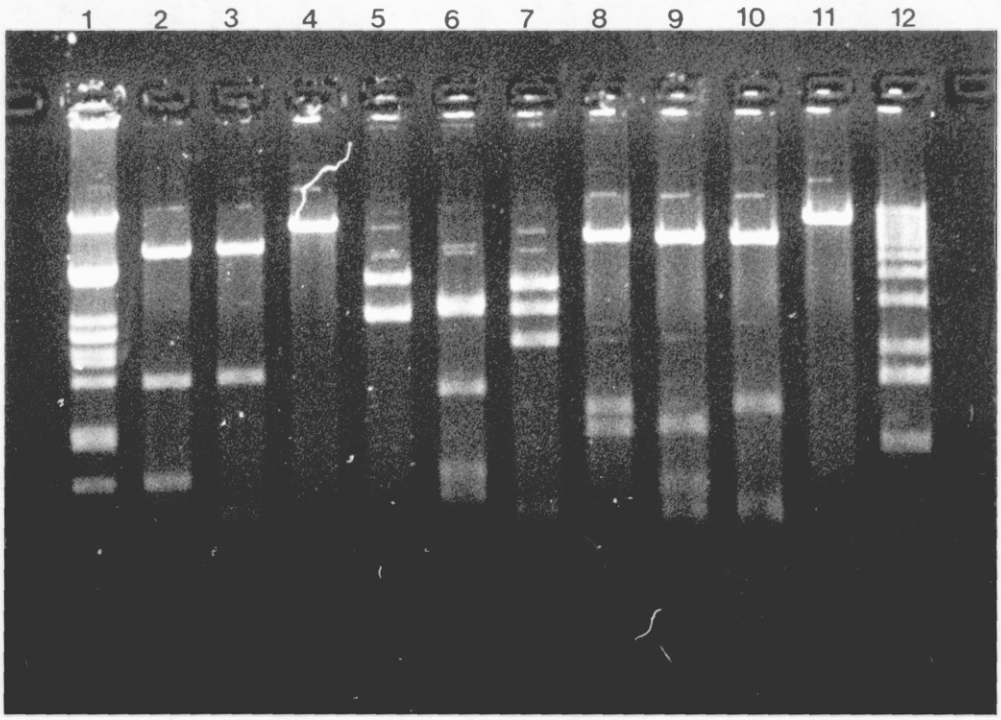


Figure 37. iii) Agarose gel electrophoresis of restriction enzyme digested PYMV clones.

(*) indicates the 1.6Kb fragment in the kilobase ladder (appendix 5.4).

Lane 1. *Pst*I digested λ DNA
Lane 2. mMH1, *Ban*II digested
Lane 3. mMH1, *Ban*II, *Hind*III cut
Lane 4. mMH1, *Pst*I, digested
Lane 5. mMH1, *Cla*I digested
Lane 6. mMH1, *Ban*II, *Cla*I digested
7. mMH1, *Hind*III, *Cla*I digested
8. mME1, *Ban*II digested
9. mME1, *Ban*II, *Eco*RI digested
10. mME1, *Ban*II, *Bam*HI digested
11. mME1, *Pst*I digested
12. kilobase ladder marker DNA

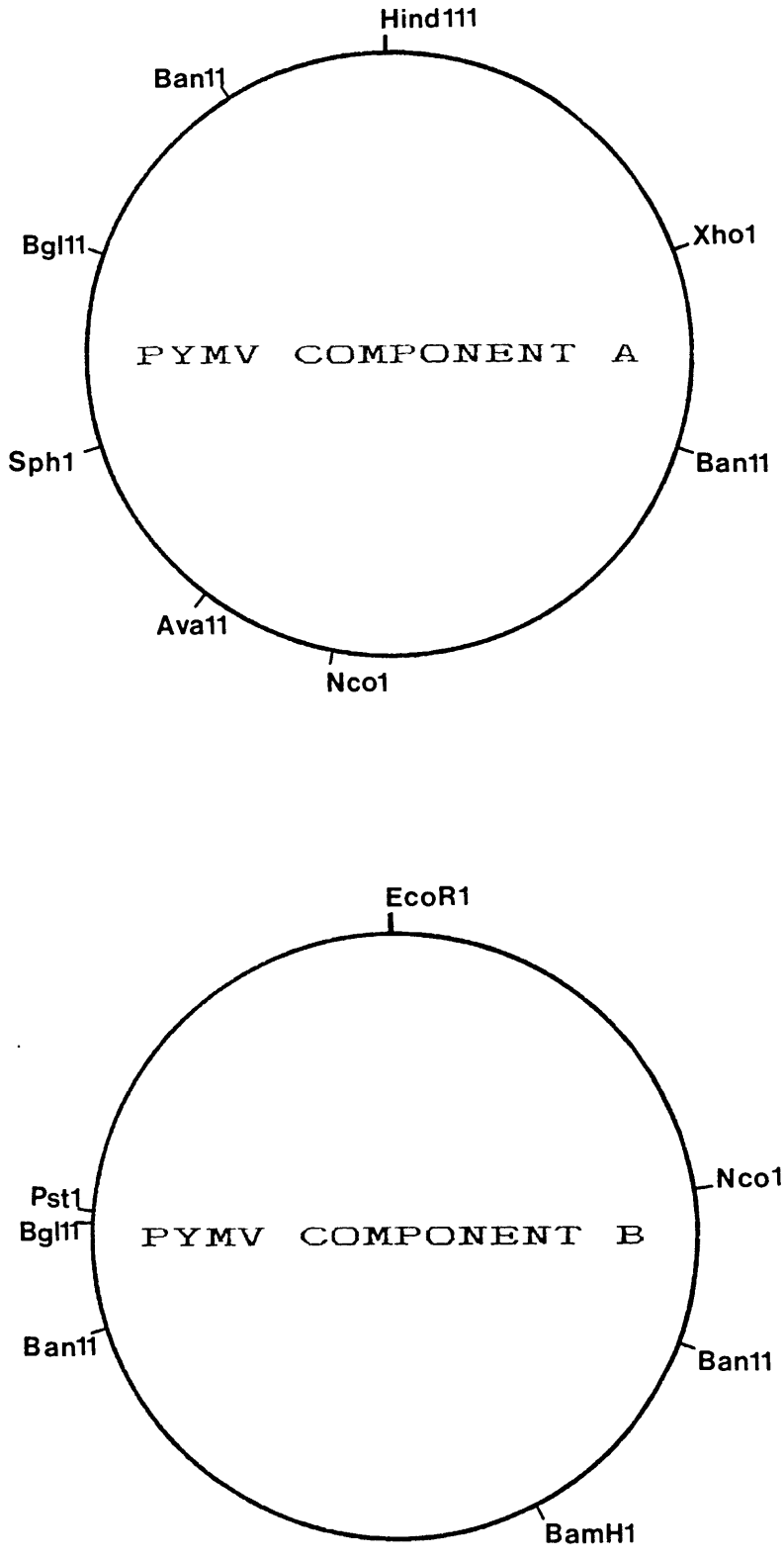


Figure 38. Restriction map of the two genomic components of PYMV.

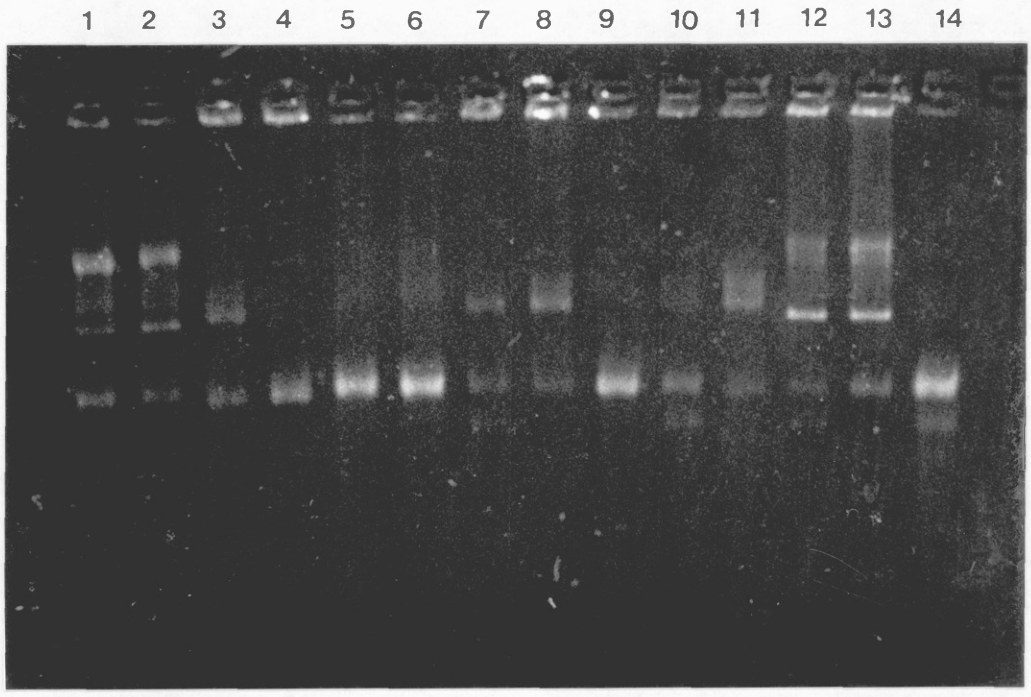


Figure 39. Agarose gel electrophoresis of solution hybridisation products of various clones of PYMV DNA in M13 vector DNA.

Lane 1. mME1+mME2
Lane 2. mME1+mME3
Lane 3. mME1+mMH1
Lane 4. mME1+mMH4
Lane 5. mME2+mME3
Lane 6. mME2+mMH1
Lane 7. mME3+mMH2
Lane 8. mME2+mMH4
Lane 9. mME3+mMH1
Lane 10. mME3+mMH2
Lane 11. mME3+mMH4
Lane 12. mMH1+mMH2
Lane 13. mMH1+mMH4
Lane 14. mMH2+mMH4

3.4c Homology between different geminiviral clones

Following the discovery that solution hybridisation tests could detect homologies between the two components of PYMV it was decided to analyse the degree of homology between different geminiviruses using this method. M13 clones of TGMV and CLV DNA (kindly provided by Drs W.D.O. Hamilton and J. Stanley, respectively) were used for studies of hybridisation between themselves and with PYMV DNA in all possible combinations. The resulting agarose gels, and a diagram interpreting these results (figures 40 and 41), show a complex pattern of hybridisation. In all cases opposite strands of the same clone hybridised strongly, thus to simplify the diagram lines indicating this result were omitted. Levels of the degree of hybridisation were attributed to different hybridisation patterns observed following electrophoresis. Strong homology was indicated by the complete hybridisation of all ssDNA into slower migrating forms. Intermediate homology was indicated by the appearance of two or more slower migrating bands, with some unhybridised ssDNA remaining. Weak homology was attributed to those combinations which showed only one slower migrating DNA form. No homology was indicated by the absence of any slower migrating DNA forms. The data presented here agrees with the findings of Hamilton *et al.* (1984), who analysed the homologies between TGMV and CLV at the nucleotide sequence level, in that it illustrates TGMV A DNA to be more closely related to CLV 1 DNA, than their respective second, smaller, components are to one other. With reference to PYMV DNA it appears that both genomic components are more closely related to their TGMV equivalents than to those of CLV. The relationship between CLV 2 DNA and PYMV is somewhat complex, both components of PYMV hybridised with either sense of CLV 2 DNA. In the absence of complete sequence data for PYMV 2 DNA the only interpretation that can be put on this complex hybridisation pattern is that CLV 2 DNA may contain at least two regions of DNA which have inverted homology to sequences lying outside the putative common regions of the PYMV DNA components. The interpretation of this type of data will be greatly assisted by a knowledge of the complete nucleotide sequence of PYMV DNA.

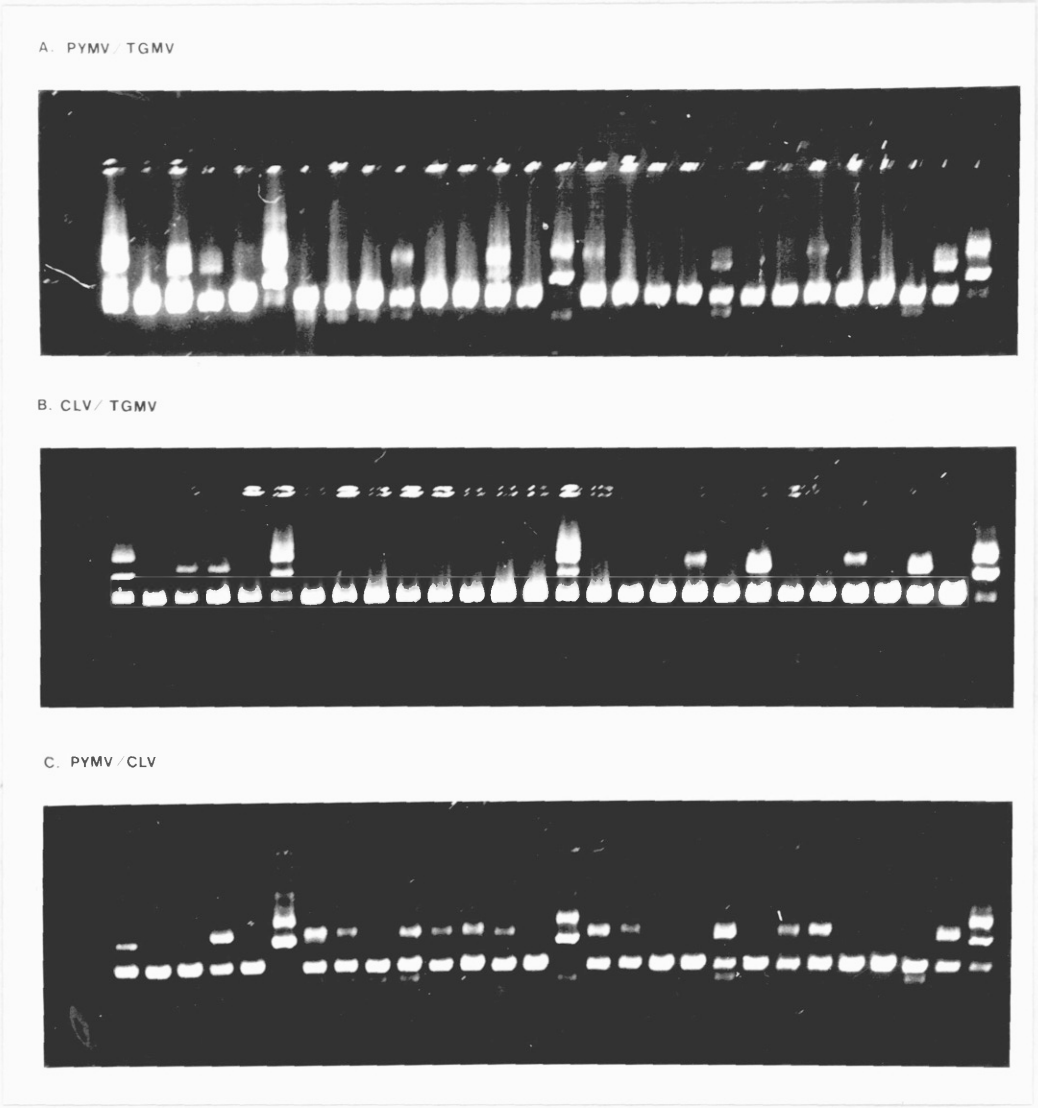


Figure 40. Agarose gel electrophoresis of the solution hybridisation of different geminiviral clones in M13.

A description of the samples loaded in each lane is shown overleaf.

Legend to figure 40, showing the samples loaded in each lane.
The clone names are abbreviated to the first letter of the virus name, followed by the component (A=1 and B=2) and then a + to indicate the viral strand or a - to indicate the complementary strand. Lanes are numbered from left to right.

A. PYMV/TGMV

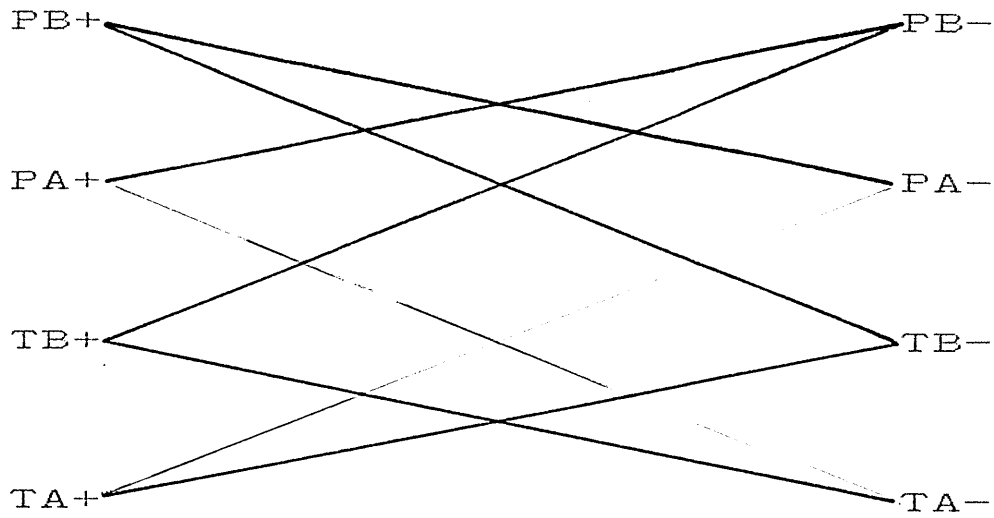
1. TB+ & TB-
2. TA- & TB-
3. TA- & TB+
4. TA+ & TB-
5. TA+ & TB+
6. TA+ & TA-
7. PA+ & TB-
8. PA+ & TB+
9. PA+ & TA-
10. PA+ & TA+
11. PA- & TB-
12. PA- & TB+
13. PA- & TA-
14. PA- & TA+
15. PA- & PA+
16. PB- & TB-
17. PB- & TB+
18. PB- & TA-
19. PB- & TA+
20. PB- & PA+
21. PB- & PA-
22. PB+ & TB-
23. PB+ & TB+
24. PB+ & TA-
25. PB+ & TA+
26. PB+ & PA+
27. PB+ & PA-
28. PB+ & PB-

B. CLV/TGMV

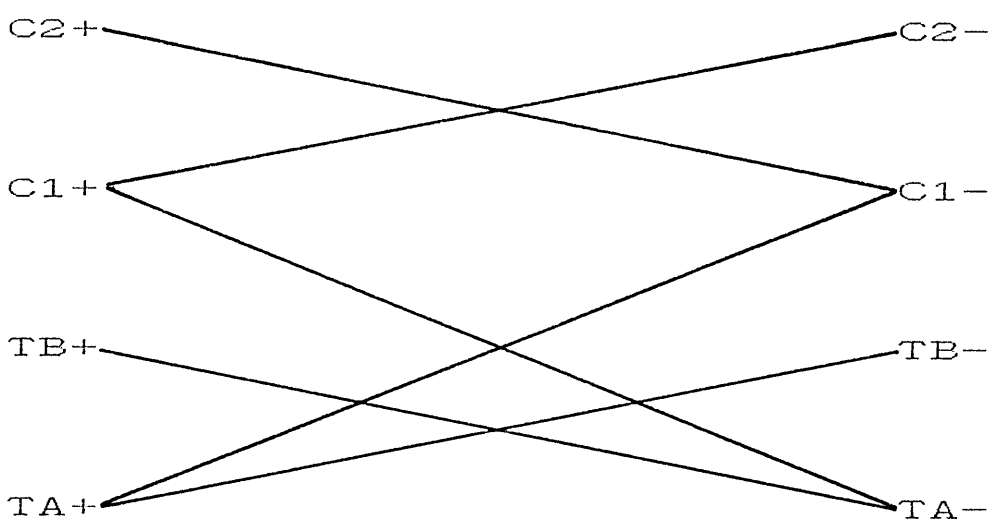
1. C2+ & C2-
2. C1- & C2-
3. C1- & C2+
4. C1+ & C2-
5. C1+ & C2+
6. C1+ & C1-
7. TB- & C2-
8. TB- & C2+
9. TB- & C1-
10. TB- & C1+
11. TB+ & C2-
12. TB+ & C2+
13. TB+ & C1-
14. TB+ & C1+
15. TB+ & TB-
16. TA- & C2-
17. TA- & C2+
18. TA- & C1-
19. TA- & C1+
20. TA- & TB-
21. TA- & TB+
22. TA+ & C2-
23. TA+ & C2+
24. TA+ & C1-
25. TA+ & C1+
26. TA+ & TB-
27. TA+ & TB+
28. TA+ & TA-

C. PYMV/CLV

1. C2+ & C2-
2. C1- & C2-
3. C1- & C2+
4. C1+ & C2-
5. C1+ & C2+
6. C1+ & C1-
7. PA+ & C2-
8. PA+ & C2+
9. PA+ & C1-
10. PA+ & C1+
11. PA- & C2-
12. PA- & C2+
13. PA- & C1-
14. PA- & C1+
15. PA- & PA+
16. PB- & C2-
17. PB- & C2+
18. PB- & C1-
19. PB- & C1+
20. PB- & PA+
21. PB- & PA-
22. PB+ & C2+
23. PB+ & C2-
24. PB+ & C1-
25. PB+ & C1+
26. PB+ & PA+
27. PB+ & PA-
28. PB+ & PB-



CLV/TGMV



PYMV/CLV

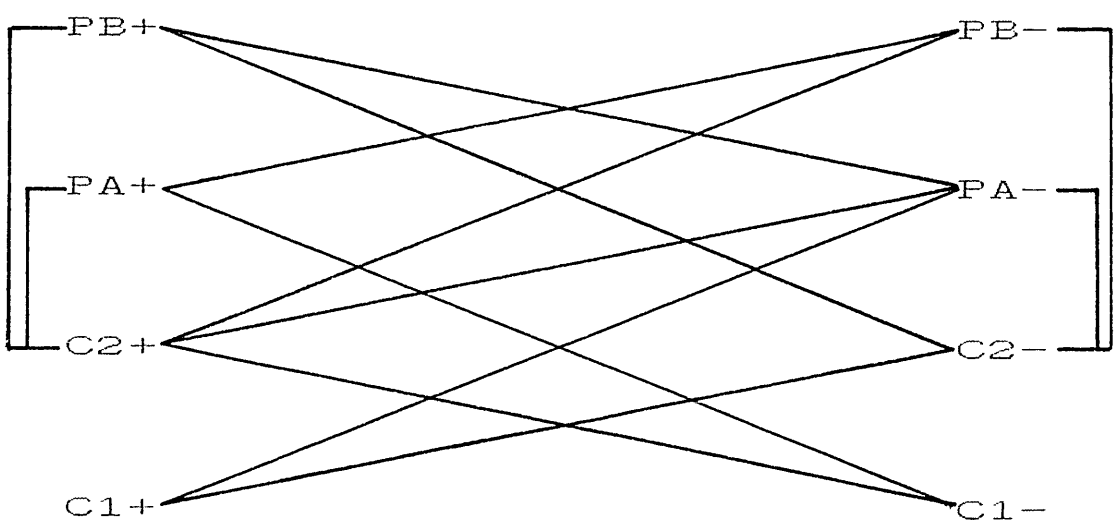


Figure 41. (see next page for legend)

Figure 41. Diagramatic representation of the results obtained from the solution hybridisation data using different geminiviral M13 clones.

This diagram is based on the hybridisation results illustrated in figure 40. Viral components which hybridise together are joined by lines, and the relative degree of hybridisation is colour-coded:

red = strong
black = intermediate
blue = weak

The clone names are abbreviated to the first letter of the virus name, followed by the component (A=1 and B=2) and then a + to indicate the viral strand or a - to indicate the complementary strand.

In all cases opposite strands of the same clone hybridised strongly, thus to simplify the diagram lines indicating this result were omitted. All lines shown in the diagram indicate homology between different viral DNAs.

3.4d Infectivity studies with cloned PYMV DNA

The clones of PYMV DNA have been tested for their infectivity on whole plants in a number of ways. *N. benthamiana* plants were mechanically inoculated with apparently full-length clones of both PYMV components together, in a mixture, and separately. It was found that these clones were indeed infectious, producing symptoms characteristic of PYMV infection (figure 3). Successful infection was only obtained when plants were inoculated with both components of PYMV DNA after excision from their respective cloning vectors by the restriction enzymes used for cloning. This finding was in agreement with similar studies that used the cloned DNA components of TGMV (Hamilton *et al.*, 1983), CLV (Stanley, 1983) and BGMV (Morinaga *et al.*, 1983). Attempts to infect *N. benthamiana* plants with unexcised clones of both DNA components failed, as did attempts to infect plants with excised clones of only one DNA component alone. The intracellular DNA forms present in all inoculated plants were analysed by nucleic acid extraction, gel electrophoresis and hybridisation to nick-translated probes produced form clones mMAH1 and mMBE1 (figure 42). This figure shows that those plants which developed symptoms contained the characteristic intracellular DNA forms known to be associated with PYMV infection. One exception to this result was the lack of any detectable sub-genomic DNA forms in plants infected with cloned DNA which were not allowed to grow for extended periods of time after symptom occurrence (discussed in section 3.2b).

Stanley and Townsend (1986) reported that it is possible to elicit infection with clones of CLV DNA without to excising both viral DNA components from their cloning vectors. It was reported that plants inoculated with full-length clones where only one component was excised from its cloning vector was sufficient to obtain successful infection, albeit at a reduced frequency. This feature has not been investigated for the PYMV clones.

For cloned PYMV DNA the frequency of infection (the number of plants that developed symptoms within 21 days after inoculation as a percentage of the total number of plants inoculated) was *ca.* 80-90% when *ca.* 10μg of excised insert DNA from each component was applied to *N. benthamiana* plants. This figure is ten-fold greater then that

required to successfully infect plants with the cloned CLV DNAs (Stanley, 1983), but five-fold less than that reported for the cloned components of TGMV DNA (Hamilton *et al.*, 1983). These figures may reflect the infectious nature of each individual virus on *N. benthamiana* plants.

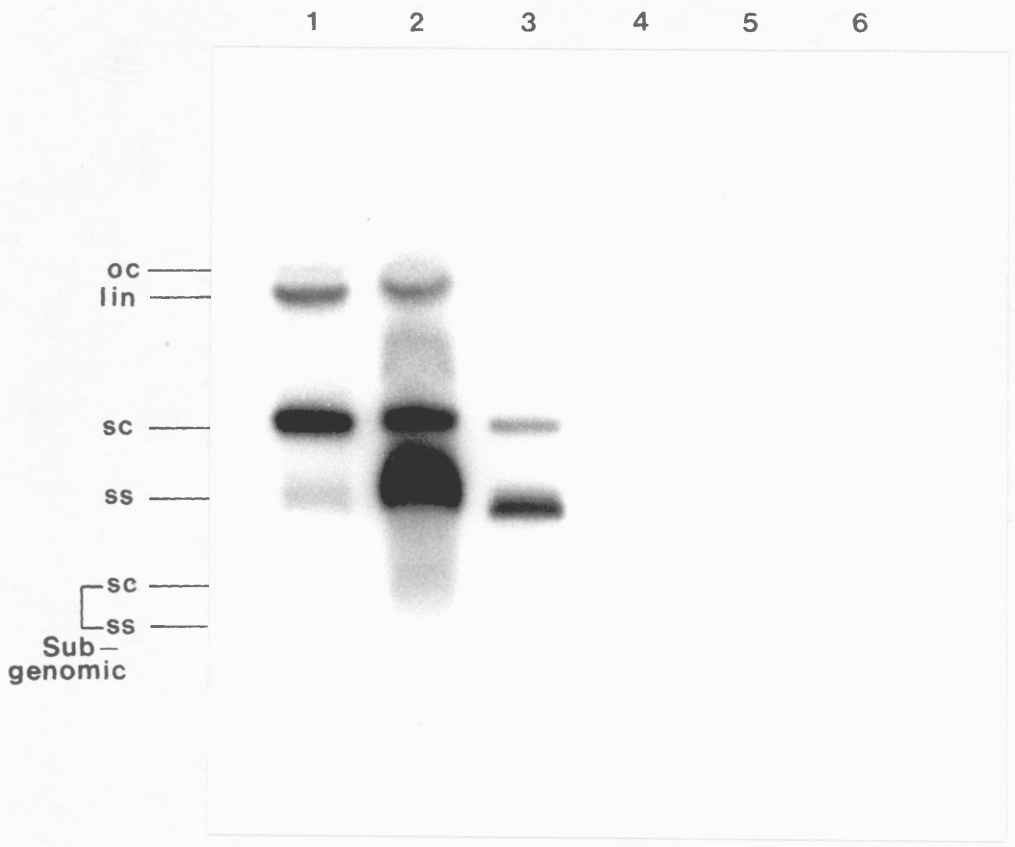


Figure 42. Intracellular DNA forms in extracts of plants inoculated with PYMV cloned DNA.

Autoradiogram of a Southern transfer onto Gene Screen (+)™ from an agarose gel of virus-specific DNA forms isolated from high-speed pellet inocula. The samples are from *N. benthamiana* plants inoculated with the following;

1. wild-type (multiple passage) inoculum
2. excised PYMV DNA clones, mMAH1 and mMBE1 (*ca.* 10μg of each insert)
3. excised PYMV DNA clones, pMAN2 and pMBN4 (*ca.* 10μg of each insert)
4. excised PYMV DNA clone, mMAH1 (*ca.* 20μg of insert)
5. excised PYMV DNA clone, mMBE1 (*ca.* 20μg of insert)
6. unexcised PYMV DNA clones, pMAN2 and pMBN4 (*ca.* 60μg of each clone)

3.5 Dideoxynucleotide sequencing

3.5a The sequencing system

Two rapid methods of sequence analysis have been developed over the last eight years (Sanger *et al.*, 1977; Maxam & Gilbert, 1977). The Maxam and Gilbert method relies on base-specific chemical cleavage of DNA fragments, and while double-stranded DNA can be used in this procedure it requires strand separation or an equivalent fractionation of each restriction enzyme fragment to be studied. In addition, end labelling and sequence analysis from a 5' or 3' end necessitates the use of relatively large amounts of purified fragments, a radioisotope of high specific activity and a relatively long exposure time for sequence readings (Poncz *et al.*, 1982).

The dideoxy technique of Sanger *et al.* (1977) relies on synthesising a complementary strand to a single stranded template using DNA polymerase I (Klenow sub-fragment) and selectively terminating the reaction using dideoxynucleoside triphosphates. Applying this techniques to DNA cloned in the male specific single stranded bacteriophage M13 makes a highly attractive, rapid sequencing system (Sanger *et al.*, 1980).

DNA to be sequenced is digested with restriction enzymes, random endonucleases, or sonicated and the fragments cloned into genetically engineered versions of M13, *e.g.* M13mp18 (Yanisch-Perron *et al.*, 1985). The RF is used to transform a male *E.coli* host, *e.g.* JM101, and the transformed cells are mixed with fresh cells and spread on agar plates. Transformed cells excrete phage which serve as foci for phage infection which are seen as plaques (areas of retarded growth against a background lawn of *E. coli*).

A complementation assay is used to distinguish recombinant phage from the original. Many of the M13 vectors contain part of the *E. coli lac* gene including the operator, promoter and N-terminal 145 amino acid residues of β-galactosidase, the α-peptide (Gronenborn & Messing, 1978). This part of the β-galactosidase gene can be induced by isopropyl-βD-thiogalactopyranoside (IPTG) but is non-functional. *E. coli* JM101 cells have their own β-galactosidase activity removed from the chromosome but a defective β-galactosidase, missing residues 11-14, is present on the JM101 F' episome. Thus in the presence of IPTG, a functionally

active β-galactosidase is formed from the two defective polypeptides. The presence of this activity can be identified by including 5-bromo-4-chloro-3-indoyl-β-galactoside (X-gal), a colourless lactose analogue, which is hydrolysed to colour the plaques blue. Foreign DNA can be cloned into M13 mp18 at a series of unique sites within the α-peptide (Messing *et al.*, 1981). This is possible due to the insertion of a synthetic linker containing these sites in the α-peptide coding region. The α-peptide is interrupted when DNA is cloned into these sites thus destroying the complementation with the episomal defective β-galactosidase. The original phage produces blue plaques whereas any recombinant phage produces white (colourless) ones which can be isolated and grown-up.

Once the DNA from a recombinant phage has been purified, dideoxy sequencing can proceed using a 'universal primer'. This is a short synthetic ssDNA fragment complementary to the M13 DNA immediately 3' to the unique insertion sites. Any M13 clone can therefore be sequenced using this primer.

Large fragments have been reported to become deleted when cloned into M13 (Messing *et al.*, 1981), but no difficulty has been found in cloning fragments greater than 3Kb in this laboratory.

JM101 is derived from the *E. coli* K12 strain 71-18 and has a *lac pro* deletion on its chromosome. *E. coli* strain TG2 has a similar genetic background but has been crossed to incorporate a *Rec*A mutation, reducing recombination frequency, and therefore reducing the frequency with which foreign DNA present in the bacterial cells is deleted (see appendix 5.1 for the precise bacterial genotypes). Both bacterial strains have a *tra*D36 mutation which reduces the efficiency of conjugation, complying with the GMAG and NIH guidelines. The proline marker on the F' episome can be used to select against losing the episome by growing the bacteria on minimal media containing glucose.

3.5b Sequence data obtained

The sequencing methods employed in this study have been described in detail (section 2.11). This methodology was closely adhered to but problems were still encountered, particularly in obtaining ssDNA template of a suitable quality. It appears that great care and

diligence must be taken at all stages during the preparation of ssDNA. An example of the quality of the sequence gels finally obtained is shown in figure 43.

The sequence data obtained to date was derived from two types of clones. Initially full-length clones of PYMV in M13mp18 were sequenced in both orientations. Clones obtained by the ExoIII/mungbean nuclease method (section 2.11d) (a kind gift from M.J. Slomka) were also sequenced, and the two sources of data combined to generate two large regions of PYMV sequence. Sequence data was analysed using the program library of the University of Wisconsin Genetics Computer Group (Devereux *et al.*, 1984), to find regions of overlap and combine them into larger pieces of sequence.

Clones constructed for use with the ExoIII/mungbean nuclease sequencing strategy consisted of full-length copies of the PYMV A component DNA cloned at its *Bgl*II site into the *Bam*HI site of M13mp19. This cloning step was possible because these restriction enzymes produce DNA molecules with complementary over-hanging extensions, which greatly increases the efficiency of ligation. The cloning junction therefore consisted of a hybrid *Bgl/Bam* site which could no longer be successfully digested with either restriction enzyme. Although the M13mp series of cloning vehicles lack a polylinker site for *Nco*I it is possible to construct such a site by digesting M13mp8 or 9 with *Hin*dIII, removing the 5' extensions with mungbean nuclease and then using T_4 DNA ligase to recircularise the molecules. Thus the sequence in the polylinker will have been altered from CCAAGCTTGG (which contains a *Hin*dIII site), to CCATGG, which is the recognition sequence for the restriction enzyme *Nco*I. Such a construct could be utilised to clone both components of the PYMV genome at their unique *Nco*I sites into the same site now present in such a vector. This would enable sequencing data to be obtained from these regions for both components of the PYMV genome. Full-length clones of PYMV DNA A were been constructed at the following sites *Hin*dIII, *Sph*I, and *Bgl*II. It would also be possible to clone full-length PYMV DNA A molecules at its *Xho*I site into the *Sal*I site present in the polylinker region.

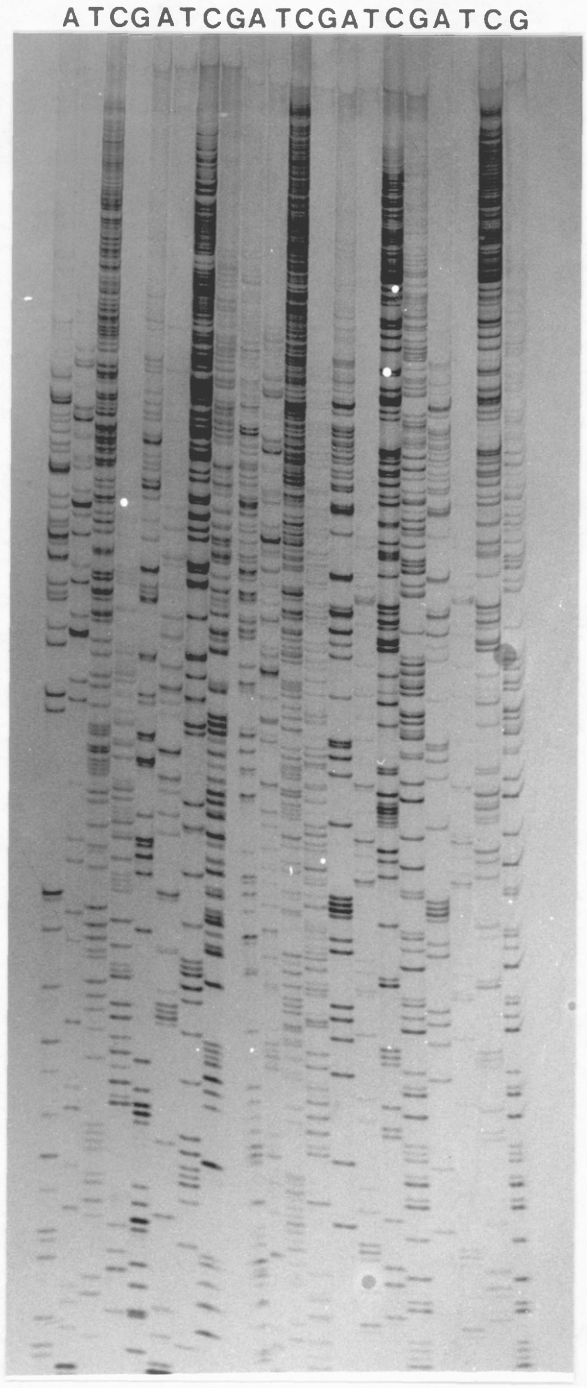


Figure 43. Example of an autoradiographed sequencing gel

Sequencing gel of PYMV A component sub-clones generated by the ExoIII/Mungbean nuclease method. Tracks are labelled with the terminating dideoxynucleotide used in each reaction.

3.5c Comparison with other geminiviruses

Alignments of the two major regions of PYMV DNA sequence obtained to date, with the sequence of TGMV DNA A are shown in figures 44 and 45. The majority of this sequence data was obtained from clones of the viral strand of PYMV. The sequence in the region of the *Hin*dIII site of PYMV (figure 44) aligns to the end of the ORF AL1 in TGMV DNA (Hamilton *et al.*, 1984) and was shown to be 77% homologous at the nucleotide sequence level. The levels of homology between PYMV DNA A and BGMV DNA 1 were of the same order but those between PYMV DNA A and CLV DNA 1 were less (65%). This concurs with the finding that BGMV and TGMV DNAs are more closely related to one another than either is to CLV DNA (Harrison, 1985). This greater degree of homology between the two South American viruses may reflect a closer common ancestry between them rather than African CLV. It is therefore not surprising that PYMV (another geminivirus of South American origin) should show a greater degree of nucleotide sequence homology with the viruses of similar geographical origin.

PYMV DNA sequence in the region of the *Bgl*II site was shown to be 83% homologous to TGMV DNA A and 80% homologous to BGMV DNA 1, in the region diammetrically opposite the common regions (figure 45). This area contains the consensus polyadenylation signals for leftward and rightward transcription, known as the A-T box. PYMV DNA A was similar in sequence to the other geminiviral DNAs with regard to its A-T box (figure 46). When the A-T boxes of PYMV and TGMV DNAs were compared, they were shown to be over 90% homologous with only four mis-matched bases (out of 41 bases) at the periphery of the region illustrated in figure 46.

Clone p1304, sub-cloned by the ExoIII/mungbean nuclease method, was found to contain the potential hairpin region found in all other geminiviruses (figure 1.). Therefore figure 1 has been redrawn to include the A component putative hairpin structure of PYMV DNA (figure 47). To obtain readable sequence data from this clone it was necessary to perform the sequencing reactions at temperatures well in excess of the normal room temperature. Sequencing reactions involving this clone were incubated at 65°C to denature the secondary structure of the ssDNA possibly formed by the putative hairpin loop sequences.

Nucleotide sequence analysis of clone mMP1 has demonstrated it to be a B component clone of PYMV DNA (figure 48). This apparently full-length clone of PYMV DNA B was shown to be 83% homologous to TGMV DNA B in the region of ORF BL1 (Hamilton *et al.*, 1984). The high degree of homology between PYMV DNA B and TGMV DNA B in this region was unexpected because other geminiviruses differ more significantly from one another in the sequences of their smaller components rather than their large components (Hamilton *et al.*, 1984; Howarth *et al.*, 1985). It may be that this relatively small 125 base sequence is not representative of the whole of the PYMV B component DNA, since further sequence data for PYMV DNA B is not available this question remains unanswered. The sequence of clone mMBP1 (figure 48) illustrates the presence of an unaltered *Pst*I site at its cloning junction. Therefore, the difficulty observed in excising the insert from this clone (discussed in section 3.4b) is not due to molecular rearrangements at this site, as was believed initially.

No attempts to search the PYMV DNA sequences for ORFs was undertaken as the sequence data obtained to date is not complete, in that the sequence data cannot be verified until clones in both orientations have been investigated.

```
          101                                                       150
pymv.gap  .......... .......... .......... .......... ...TTtGCTt
tgmv.gap  gccacagttc aggtcaattc gtcgccttct aatagctctt cgtTTaGCTg

          151                                                       200
pymv.gap  gcCTGTGTgc cttcTTGATA GAGGGGGGct gTGAtGgtGA tGAAgacCGC
tgmv.gap  ctCTGTGTtg agctTTGATA GAGGGGGGag tTGAgGaaGA cGAAtttCGC

          201                                                       250
pymv.gap  ATTcTttAgA GTCCAGTTtT TgAGaGacGc aTTTTCgTCT TTGTCGAGGA
tgmv.gap  ATTaTggAaA GTCCAGTTcT TtAGtGgaGt gTTTTCcTCT TTGTCGAGGA

          251                                                       300
pymv.gap  AAgCTTTATA GCTgGaACCC TCaCCAGGAT TGCAgAGCAg CGATTGAtGG
tgmv.gap  AAaCTTTATA GCTaGcACCC TCtCCAGGAT TGCAcAGCA. CGATTGAcGG

          301                                                       350
pymv.gap  GATACCgCCT TTAATTTGcA VCaGGCTTTC CGTAcTTACA VTTtGatTGC
tgmv.gap  GATACCtCCT TTAATTTG.A ACtGGCTTTC CGTAtTTACA GTTaGtcTGC

          351                                                       400
pymv.gap  CAATCcCTTT GGcCCCCAAg cAGTTCTTTC CAgTGcTTtA gCTTTAGATA
tgmv.gap  CAATCtCTTT GGgCCCCAAt gAGTTCTTTC CAaTGtTTcA aCTTTAGATA

          401                                                       450
pymv.gap  TTGCGGTGcG ACgTCATCaA TGACGTTATA CTCcACtTca TTcGAaTAgA
tgmv.gap  TTGCGGTGtG ACaTCATCgA TGACGTTATA CTCaACcTtg TTtGAgTAaA

          451                                                       500
pymv.gap  CCCTgGgATY GAaRTCaAgA TGaCCGCTCA AgTAATTATG TGGGCCTAag
tgmv.gap  CCCTaGaATT GAgATCcAaA TGcCCGCTCA AaTAATTATG TGGGCCTAgt

          501                                                       550
pymv.gap  actCGcGCCC ACATcGTCTT gCCtGTtCGA gaATCaCCtT CaATtATAAT
tgmv.gap  gaaCGaGCCC ACATaGTCTT tCCcGTcCGA ctATCgCCcT CgATgATAAT

          551                                                       600
pymv.gap  ACTAATAG.. .TCTgtctSC GCagccgGcA gCctcTTCCg AAATAAccaT
tgmv.gap  ACTAATAGgt cTCTccggCC GCgcagcGgA aCtctTTCCa AAATAAtttT

          601                                                       650
pymv.gap  CtGCCCAcTc GTtgCATCTC GTCgGGaACG TTcAGTgAAa GAgGAGAgtg
tgmv.gap  CaGCCCA.Tt GTctCATCTC GTCtGGcACG TT.AGTaAAt GAtGAGAcgt

          651                                                       700
pymv.gap  GaAACGGAGG AgcCCA.... .......... .......... ..........
tgmv.gap  GgAACGGAGG AagCCAtggt tcaggagtct tatcaaatat cctatctaaa
```

Figure 44. i) Computer generated sequence alignment.

Alignment of the known nucleotide sequence of the TGMV A component DNA with an assembled sequence of PYMV A component DNA in the region of the *Hin*dIII site (boxed) shown in figure 38. Ambiguous bases were coded as described in section 5.5.

```
          51                                                      100
pymv.zip  .......... .......... .......... ....GCAtGC TaTKGTrAGG
tgmv.zip  ccaaggttac tggtggtcaa tatgccagca acgaGCAgGC TcTGGTtAGG

          101                                                     150
pymv.zip  cGctTTCTGG AAGGTCAAsA cCA...cgtg tTtacACCAC CAGGAaGCtG
tgmv.zip  aG.aTTCTGG AAGGTCAAtA aCAatgtcgt cTacaACCAC CAGGAgGCaG

          151                                                     200
pymv.zip  GcAAATAcGA GAATCATACT GAGAACGCCC TaTTATTGTA cATGGCATgG
tgmv.zip  GgAAATAtGA GAATCATACT GAGAACGCCC TgTTATTGTA tATGGCAT.G

          201                                                     250
pymv.zip  TACTCATGCC TCaAAtCCTG TaTATGCaAC acTTaAgAcT TCGgATCTAT
tgmv.zip  TACTCATGCC TCtAAcCCTG TgTATGCgAC .gTTgAaAaT TCGaATCTAT

          251                                                     300
pymv.zip  TTTTATGATT CGATcttAAA TTAATAAAAT TTATATTTTA TTGAATGATT
tgmv.zip  TTTTATGATT CGATaacAAA TTAATAAAAT TTATATTTTA TTGAATGATT

          301                                                     350
pymv.zip  TTCcAGTACA TaatTTAcAT ATGATCTaTC TGTTGCaAAA CGAACAGaTC
tgmv.zip  TTCgAGTACA TgcgTTAtAT ATGATCTgTC TGTTGCgAAA CGAACAGcTC

          351                                                     400
pymv.zip  TAAatTAcaA TTGTTAAatT gaAaATgACa CCTAACcGgT CtAaGTAC.A
tgmv.zip  TAA..TAacA TTGTTAA..T acAtATaACg CCTAACtGtT CaAgGTACaA

          401                                                     450
pymv.zip  gtDaACTAAD TgTcTAAAWC TAgcTtAAat AaWTCTgtaC CCAGAAGCTG
tgmv.zip  caTcACTAAG TaTtTAAATC TAttTaAAta AgTTCT...C CCAGAAGCTG

          451                                                     500
pymv.zip  TCaTCGAWGT CGTCCADACT TGGAARTTcA GgtAWGCtTT GTGGAGAWCC
tgmv.zip  TCgTCGATGT CGTCCATACT TGGAAGTTgA GaaATGCcTT GTGGAGATCC

          501                                                     550
pymv.zip  AAWGCTCTCC TgARGTgTGT GGTTGAACCg GATTTGgAcG ctGTATATCC
tgmv.zip  AATGCTCTCC TcAGGT.TGT GGTTGAACCt GATTTGtAaG tgGTATATCC

          551                                                     600
pymv.zip  TGGTccTGGT GTAttGcatg TCCTCgACtt gGtTTATCTT gGAAATAGAG
tgmv.zip  TGGTgtTGGT GTAgaGggga TCCTCtACgc tGaTTATCTT .GAAATAGAG

          601                                                     650
pymv.zip  GGGATTTtcT ATCTCCCAGA TAaAcACGCC ATTCTCTGCc TGAcG.....
tgmv.zip  GGGATTTgtT ATCTCCCAGA TAtAgACGCC ATTCTCTGCt TGAgGcacag
```

Figure 45. ii) Computer generated sequence alignment.

Alignment of the known nucleotide sequence of the TGMV A DNA component with an assembled sequence of PYMV A DNA component in the region of the *Bgl*II site (boxed) shown in figure 38. Ambiguous bases were coded as described in section 5.5.

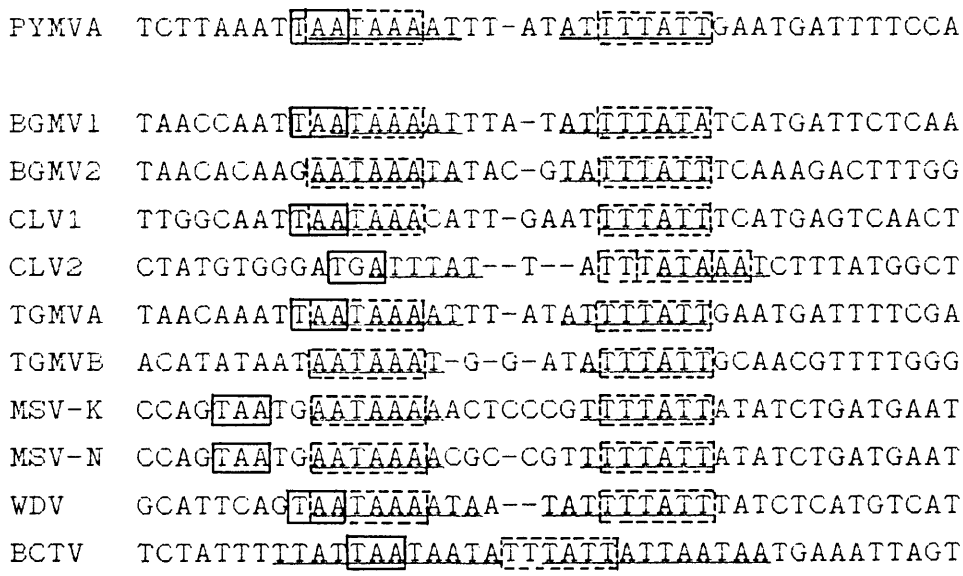


Figure 46. Transcriptional consensus polyadenylation signals. Sequences are aligned to show the relative positions of the polyadenylation signals (broken boxes). Termination codons are boxed and the underlinings show the extent of the inverted repeats.

Invariant sequence **TAATATTACC**

PYMVA GGGGCCATCCGTTA**TAATATTAC**CGGATGGCCCC

BCTV GGGCCATCCGTTA**TAATATTAC**CGGATGGCCC
BGMV1 GCGGCCATCCGATA**TAATATTAC**CGGATGGCCGC
BGMV2 GCGGCCATCCGATA**TAATATTAC**CGGATGGCCGC
CLV1 GGGGCCAACCGTA-**TAATATTAC**CGGTTGGCCCC
CLV2 GGGGCCAACCGTA-**TAATATTAC**CGGTTGGCCCC
TGMVA GCGGCCATCCGTT-**TAATATTAC**CGGATGGCCGC
TGMVB GCGGCCATCCGTTT**TAATATTAC**CGGATGGCCGC
MSV-K GAAAAGAAGGCGCGCAC**TAATATTAC**CGCGCCTTCTTTTC
MSV-N GCAGGAAAAGAAGGCGCGCAC**TAATATTAC**CGCGCCTTCTTTTCCTGC
WDV GTCGGGGGCCTCCACGCGGGTTA**TAATATTAC**CCCGCGTGGTGGCCCCCGAC

Figure 47. Comparisons of the major putative stem-loop structures in geminiviral DNAs.

This diagram shows the potential hairpin structures containing the invariant 10 base sequence (the invariant loop of which is shown in bold type). The stem of the potential hairpin loop structures are underlined.

```
          1                                                        50
pymv.b    .......... .......... .....gTTcG tGTCGgAAAC TcTGTAATAg
tgmv.b    tcgcaaaatg agtcctctgg tgaacaTTaG aGTCGcAAAC TtTGTAATAt

         51                                                       100
pymv.b    AGTTTCCATG GGATaGGaaT CTTTtAGgGA RAAaAAgGAa GcTGAGAAAT
tgmv.b    AGTTTCCATG GGATtGG.gT CTTTgAGcGA GAAgAAcGAc GaTGAGAAAT

        101                                                       150
pymv.b    AGTGGAGATC TATgTTGCAT CtTaACgGGA AcAGTCCATG ACGCcTGcAg
tgmv.b    AGTGGAGATC TATaTTGCAT C.TcACcGGA AaAGTCCATG ACGCtTGtAa

        151                                                       200
pymv.b    .......... .......... .......... .......... ..........
tgmv.b    ggattcattg tcagtcattc ttttgtcatg aatctccacg accacggatc
```

Figure 48. iii) Computer generated sequence alignment.

Alignment of the known nucleotide sequence of the TGMV B DNA component with the determined sequence of clone mMP1 (*Pst*I site boxed), an apparently B component specific PYMV DNA clone.

3.5d Future plans

It is hoped that the sequencing of both components of PYMV DNA will be completed at Imperial College, and a more comprehensive comparison with the other sequenced geminiviruses undertaken. A knowledge of the complete sequence of PYMV DNA will facilitate explanations for the solution hybridisation data and may indicate regions of interest within the DNA that might be concerned with the host range determinants of PYMV.

4. SUMMARY

4.1 Characterisation of PYMV as a geminivirus

The work presented in this thesis has characterised PYMV as a *bona fide* member of the geminivirus group (Matthews, 1979). This conclusion is based on three main observations; 1) The visualisation of a geminate morphology for the virus particles; 2) The demonstration that the genome of the virus contains single-stranded DNA and 3) The production of characteristic geminivirus disease symptoms in host plants after inoculation. This new geminivirus has a unique host range within the *Solanaceae*, infecting potatoes, tomatoes, tobacco and petunia. Attempts to mechanically transmit PYMV to members of the *Leguminosae* however proved unsuccessful. To determine the complete host range of PYMV it would be necessary to perform whitefly transmission experiments, for which the required insect containment facilities are elaborate and beyond the scope of this work.

The genome of PYMV was demonstrated to be bicomponent, analogous to the genomes of the other whitefly-transmitted geminiviruses characterised to date, both components being to required to elicit infection when mechanically inoculated to *N. benthamiana* plants.

The intracellular virus specific DNA forms isolated from tissue infected with PYMV are analogous to those isolated and described previously for extracts from TGMV and CLV infected plants (Hamilton *et al.*, 1982; Stanley and Townsend, 1985; MacDowell *et al.*,1986). The use of Triton X-100 in the virus extraction buffer improved the isolation of the intracellular PYMV DNA forms. It may be that this detergent solubilises membranes with which these PYMV dsDNA forms might be associated. Viral nucleic acids may have been centrifuged down in the absence of detergent since they appear to be associated with membranous components. The involvement of membranes in DNA replication has been suggested for a number of microorganisms. There is evidence that the bacterial chromosome, particularly the replication origin, terminus and replication fork, become membrane associated (Kornberg, 1980; 1982). In *Bacillus subtilis* the binding of the origin for DNA replication to membranes is regulated by mutations that control the initiation of DNA replication (Winston and Sueoka,

1980). In this system one mutation within this area of nucleic acid inhibits both chromosomal and plasmid replication and membrane binding, whereas another mutation inhibits replication and binding of chromosomal DNA but does not affect the plasmid. Membranes may also be involved in the replication of eukaryotic DNA (Kornberg, 1980) and chromatin , particularly the nascent DNA, is bound to a proteinacious lamina which is associated with the nuclear membrane (Dijkwel *et al.*, 1979). Geminivirus DNA replication may thus also be associated with membranous structures.

Supercoiling is thought to be essential for DNA replication, transcription and recombination in both prokaryotic and eukaryotic systems (Kornberg, 1980) and a definite correlation has been shown between supercoiling and transcriptional activity in plasmids (Kano *et al.*, 1981). As PYMV scDNA is infectious when mechanically inoculated to *N. benthamiana* plants, transcriptional promoters and initiators present in the geminivirus genome must be recognised by the host RNA polymerase system. Subsequent translation of the transcripts produced may produce gemini-related regulatory proteins, in addition to the viral structural proteins, which possibly further direct DNA replication, or control transcription. The elucidation of the exact structures and functions of the geminiviral origin/promoter sequences is the subject of immediate research interest in a number of laboratories.

4.2 *In vitro* geminivirus replication

The inability to obtain totipotent protoplasts capable of division, from the cell lines available, under the conditions of culture utilised, is probably the reason why attempts to study geminivirus replication *in vitro* in potato protoplasts were unsuccessful. Another method of analysing geminivirus DNA replication *in vitro* was to establish PYMV infected potato cell lines. However, no detectable amounts of PYMV specific DNA, were found in calli initiated from surface sterilised infected potato tissue. This result is in contrast to similar experiments with TGMV infected tobacco tissue culture derived calli (M.J. Slomka, Pers. Comm.).

There is also the possibility of directly infecting suspension cultured cells with virus. Suspension cultured cells of *Trifolium* ssp. and soybean have both been directly infected in this fashion (Jones *et al.*, 1981; Wu and Murakishi, 1978). Previous experiments utilised a direct mixing of virus with the suspension cultures in order to elicit infection. This may be a worthwhile proposition for the geminiviruses, possibly in conjunction with the synchronisation of cell division. Several chemicals have been described which interfere with plant cell division, *e.g.* aphidicolin and fluorodeoxyuridine, and can be used to inhibit cell division at particular phases in the cell cycle (Sala *et al.*, 1980; Cress *et al.*, 1978). Aphidicolin has been shown to inhibit α-like polymerase activity in tobacco and subsequently block cell development at the S-phase (Sala *et al.*, 1982). This process is reversible and when the inhibitor is removed all cells move into the same phase of replication (Cress *et al.*, 1978). This may be important since, as with mammalian cell cultures (Wigler *et al.*, 1979), plant cell cultures may contain a sub-population of transformation competent cells. Such competence is not a stably inheritable trait but is rather a 'transient property' of the cells. Previous work has demonstrated that the cell cycle phase of plant protoplasts has effects on their capacity to replicate tobacco mosaic virus (Gould *et al.*, 1981). This phenomenom has been tentatively explained in terms of a cyclic variation of the charge on plant cell membranes. Gould and Ashmore (1982) describe results suggesting that PLO-mediated binding of DNA to plant protoplasts is more efficient in certain cell cycle phases than others. Such variation in DNA binding is likely to be one of the key components that defines the transformation competence of cultured plant cells. The ability to inoculate cells at a specific time in their divisional cycle, when transformation capacity is highest, would enhance the possibility of eliciting virus replication. Another inhibitor, 2,6-dichlorobenzonitrile (DB), has been used in combination with aphidicolin to synchronise tobacco protoplast division (Meyer *et al.*, 1985). DB inhibits cell wall formation without affecting nuclear nuclear division (Meyer and Herth, 1978). Prolonged incubation of protoplasts with DB therefore results in the production multinuclear cells as cytokinesis was inhibited. In combination, these two

inhibitors synchronise cell division at the S-phase and prevent cell wall formation during incubation. Meyer *et al.* (1985) demonstrated that tobacco protoplasts synchronised in this fashion were transformed with increased efficiency (*ca.* two orders of magnitude higher than in unsynchronised protoplasts), provided that the transformation was performed at S- or M-phase in the cell cycle. After completion of the M-phase transformation efficiencies decreased to the level of unsynchronised cells. Nagata *et al.* (1980) proposed that the use of M-phase protoplasts may increase transformation efficiencies because these cells do not possess a nuclear membrane.

However, a prerequisite for all these procedures is a regenerable protoplasts system where cultured protoplasts undergo division at a relatively high frequency.

4.3 The potential of geminiviruses as vectors

Early attempts to introduce 'foreign' genetic material into plant cells showed that while the DNA enters cells it is not replicated or expressed (Lurquin and Kado, 1979). Subsequent experiments indicated that for successful replication and expression, defined sequences of DNA are required which are recognised by the host enzymes involved in replication. These sequences can be provided by a vector that may also contain sequences that allow the successfully transformed cells to be identified and selected. Selection markers are an advantage but not a necessity. Plant viruses can be considered as foreign nucleic acids that successfully replicate in and are expressed in plant cells. Thus, they must contain the nucleic acid sequences that can be recognised by plant cells to perform these functions. For this reason plant viruses have attracted much attention as possible vectors for introducing genes into plant cells.

Of the three hundred or so well characterised plant viruses, *ca.* 78% possess a genome comprising ssRNA in the plus or messenger sense. Approximately 13% contain either ssRNA, in the minus sense, or dsRNA, while only 3% contain either dsDNA or ssDNA. As far as genetic manipulation is concerned it is this 3% of plant viruses containing a DNA genome that have attracted the greatest attention.

As a group the geminiviruses infect a wide range of monocots and dicots (section 1.2) including both agriculturally and horticulturally important crop plants and therefore offer much potential for development as useful plant vectors. To overcome any possible packaging constraints and the pathogenic effects associated with viral infection, a geminivirus-derived plasmid vector system could be constructed enabling episomal replication of the vector. It has been demonstrated that encapsidation is not a prerequisite for the systemic spread of virus infection throughout the plant (Stanley and Townsend, 1986), thus these episomal vectors would not only be confined to use in protoplast systems. Such vectors would be analogous to the plasmid shuttle (able to replicate in both eukaryotic and prokaryotic cells) vectors derived from the animal papillomaviruses and papovaviruses (reviewed by Rigby, 1983). Derivatives of pBR322 lacking the 'poison sequences' which inhibit SV40 DNA replication in eukaryotic cells (Lusky and Botchan, 1981), and containing the SV40 origin of replication, replicate efficiently at high copy number following transfection into COS cells (Gluzman, 1981). This system has been utilised to study the transcription of human globin genes and identified a sequence 55 to 87bp upstream from the mRNA capping sequence required for efficient transcription of the α-globulin gene (Mellon *et al.*, 1981). A geminivirus-derived plasmid vector could similarly be used to investigate the expression of plant genes in protoplasts. These vectors could be constructed from a geminivirus origin of replication and the virus gene(s) required for dsDNA replication, an origin of replication from a bacterial plasmid and an antibiotic resistance marker to enable selection for the vector in plants and bacteria (Buck and Coutts, 1983). One main advantage of using such a vector is that a potentially high copy number could be obtained, leading to a high level of expression of cloned genes from their own, or artificially added plant promoters.

An alternative to the episomal maintenance of a geminivirus vector would involve integration of the vector sequences into the plant chromosome. This situation could be brought about in one of two ways. The most obvious system to use is based on the natural transforming ability of Ti plasmid DNA (Herrera-Estrella *et al.*,1985; Fraley

et al., 1986). The main problem with such a system may be the limited host range of the Ti plasmid, confining experiments to use with vectors for dicots (excluding the most economically important crop plants, the monocots). An alternative to this is that the inclusion of a plant gene into a geminivirus plasmid vector may lead to integration into the plant genome by homologous recombination (Buck and Coutts, 1983). This strategy has proven successful for yeast plasmid vectors (Orr-Weaver *et al.*, 1981). Circular hybrid yeast plasmid vector molecules which contain a copy of a yeast gene will integrate into the yeast chromosome by a single reciprocal crossover event resulting in a yeast chromosome containing two copies of the yeast gene separated by vector sequences. However, it may not be necessary to incorporate a complete homologous plant gene into a geminivirus vector. When thymidine kinase$^-$ (tk^-) mouse cells are transformed to tk^+ phenotype by the herpes simplex virus-1 tk gene, in the presence of carrier DNA (either SV40 DNA or pBR322 DNA), defined sequences form the carrier DNA can become linked to the selectable marker gene possibly as a result of homologous recombination. This region of homology was identified as being between 16-19bp (Anderson *et al.*, 1982). Thus a geminivirus based vector may only require a very short plant sequence homology to promote integration.

A particular gene system of interest with respect to crop improvement in potatoes are those involved in the pathway concerned with the production of the aphid alarm pheromone, (E)-β-farnesene. This plant pheromone simulates the production of the aphid alarm pheromone, and is released from the leaf hairs of *Solanum berthaultii* in response to aphid attack. Dawson *et al.* (1982) have shown that the resistance to aphid transmission of potato virus Y is associated with the release of (E)-β-farnesene. The ability to introduce this trait into commercial varieties of potato could greatly reduce the incidence of aphid transmitted plant viruses in the field.

The manipulation of eukaryotic viral genomes requires a detailed knowledge of their primary nucleotide sequence structure. This information is available for several of the members of the geminivirus group (section 1.6). Initially the construction of geminivirus based vectors could serve as a means of gaining an insight

into the replication strategy of geminiviruses. This in turn might make the construction of more elegant vector systems possible.

5. APPENDICES

5.1 Genotypes of *Escherichia coli* bacterial strains

HB 101 : F⁻, *hsd* S20 (r^-_B, m^-_B), *rec A13*, *ara*-14, *pro* A2, *lac* Y1, *gal* K_2, *rps* L20 (Sm^r), *xyl*-5, *mtl*-1, *Sup* E 44, λ^-

JM 101 : Δ(*lac-pro*), *Sup* E, *thi*, F' (*tra* D 36, *pro* AB, *lac* I^q, ΔM15) (Yanisch-Perron *et al.*, 1985, and references therein)

TG2 : Δ(*lac-pro*), *Sup* E, *thi*, F' (*tra* D 36, *pro* AB, *lac* I^q, *lac* Z, ΔM15), *hsd* Δ5 (r^-, m^-), *rec* A, *Srl*::*Tn10*$^{Tet^r}$ (Dr T.J. Gibson, pers. comm.)

5.2 Antibiotic stocks

The following levels of antibiotics were used where ever they are mentioned in the text and these levels were obtained from filter sterilised stocks stored as aliquots at -20°C.

Ampicillin (Am), sodium salt, 50μg/ml from a 25mg/ml stock made up in dH_2O.

Carbenicillin (a gift from Beechams Pharmaceuticals)(Cr), 200μg/ml from a 400mg/ml stock made up in sterile deH_2O.

Chloramphenicol (Cm), 10μg/ml from a 10mg/ml stock made up in absolute ethanol.

Kanamycin sulphate (Km), 50μg/ml from a stock made up in dH_2O.

Nystatin (Squibb & Sons), 100U/ml from a 1000U/ml stock suspension made up in sterile deH_2O.

Tetracycline hydrochloride (Tc), 15μg/ml from a 15mg/ml stock made up in 50% ethanol.

5.3 Liquid scintillant

Liquid scintillant consisted of the following:

triton X-100 (Rose)	500ml
2,5-diphenyloxazole (PPO)	6g
1,4-bis[2-(5-phenyloxazole)]benzene (POPOP)	0.15g

Made up to 1000ml with toluene.

5.4 Marker DNA sizes in restriction enzyme digests

Fragment sizes, in base pairs, of HindIII digested λ DNA

23,100
9,400
6,600
4,400
2,300
2,000
560 (†)
125 (†)

Fragment sizes, in base pairs, of *Pst*I digested λ DNA

12,300
5,077
4,749
4,507
2,838
2,554
2,459
2,140
1,986
1,748
1,159
1,095
805
514 (†)
498 (†)
339 (†)

Those fragments labelled (†) are not usually clearly seen when electrophoresed in 1% agarose gels.

Fragment sizes, in base pairs, of the kilobase ladder (Biolabs).

6,000
5,000
4,000
3,000
2,000
1,600
1,000
500
400

Each gel where these markers were used has (*) to indicate the 1.6Kb fragment.

5.5. Nucleotide ambiguity codes

When a particular nucleotide in a sequencing gel was ambiguous, the following code was used to allow meaningful computer analysis to be performed on the data.

Ambiguity	Code
A or C	M
A or G	R
A or T	W
C or G	S
C or T	Y
G or T	K
A or C or G	V
A or C or T	H
A or G or T	D
C or G or T	B
G or A or T or C	X

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