MOLECULAR BIOLOGICAL STUDIES OF SOUTHERN AFRICAN ISOLATES OF MAIZE STREAK VIRUS

by

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ABREVIATIONS

A_{260nm}	Optical density (absorbance reading at indicated wavelength)
ACMV	African cassava mosaic virus
BCTV	Beet curly top virus
BGMV	Bean golden mosaic virus
BMV	Brome mosaic virus
BSA	Bovine serum albumin
^oC	Degrees celsius
CsCl	Caesium chloride
CSMV	Chloris striate mosaic virus
D	Dalton
DAS-ELISA	Double antibody sandwich enzyme-linked immunosorbent assay
DEAE	Diethylaminoethyl
DIECA	Diethyldithiocarbamate
EDTA	Ethylene diamine tetra-acetic acid
EtBr	Ethidium bromide
EuMV	Euphorbia mosaic virus
GAR	Goat anti-rabbit antibody
HYVMV	Honeysuckle yellow vein mosaic virus
IgG	Gamma-globulin fraction of serum
kb	Kilobase
M	Molar
MBYMV	Mung bean yellow mosaic virus
mM	Millimolar
M_r	Molecular weight estimated by gel electophoresis
MSV	Maize streak virus
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
S	Svedberg
SDS	Sodium dodecyl sulphate
SLCV	Squash leaf curl virus
TEMED	N, N, N', N',-tetramethylethylenediamine
TGMV	Tomato golden mosaic virus
TLCV	Tobacco leaf curl virus
ToLCV	Tomato leaf curl virus
Tris	Tris (hydroxymethyl)-aminomethane
TYDV	Tobacco yellow dwarf virus
ug	Micrograms
ul	Microlitres
UV	Ultraviolet
v/v	Volume per volume ratio
WDV	Wheat dwarf virus
w/v	Weight per volume ratio

SUMMARY

This thesis deals with an investigation of the nature of variation of maize streak virus (MSV) isolates in Southern Africa. Three isolates from distinct geographical locations, which produced different symptoms, were chosen for this purpose.

Serological tests performed with antisera raised to two of the isolates showed the three isolates to be serologically related but non-identical. This relationship was further verified by cloning and restriction endonuclease mapping of the genomes of the isolates. These maps were then compared to those of published MSV isolates, revealing both similarities and differences. The predicted and experimental restriction patterns were used to construct a phylogenetic tree of all the MSV isolates so far characterised at the genomic level.

A fourth geminivirus (DGV) which infects the grass *Digitaria sanguinalis*, was compared to known isolates of MSV in order to confirm its identification as an MSV strain: DGV was only distantly serologically related to Southern African MSVs, and is probably a distinct virus.

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CHAPTER 1

INTRODUCTION

Maize streak virus (MSV), a geminivirus, is endemic throughout large areas of Africa, and can cause major crop losses. Bock et al. (1974) reported the occurrence of different strains of the virus in Kenya. Since then, however, no further research appears to have been undertaken on strain relationships. It has also been reported that yield losses in maize crops depend to some extent on the virus strain (Bock, 1982). This study was, therefore, initiated to investigate the occurrence and nature of MSV isolates in Southern Africa.

Three isolates were targeted for study, two from South Africa (MSV-CT and MSV-PE) and a third from South West Africa / Namibia (MSV-SWA). The isolates were compared serologically and by restriction endonuclease mapping double-stranded replicative-form or cloned genomic DNA of all three isolates. The restriction enzyme maps of the Southern African isolates were compared with those generated for a Nigerian and a Kenyan isolate (Mullineaux et al., 1984; Howell, 1984).

The different restriction enzyme fragment patterns of the five isolates were used in the construction of a phylogenetic tree. Speculation was also made as to the evolutionary divergence of strains and isolates of MSV.

A geminivirus infecting the grass *Digitaria sanguinalis* (DGV) was similarly compared with MSV-CT, MSV-PE and MSV-SWA, in order to establish whether or not it is a strain of MSV.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The geminiviruses are one of the most recently described taxonomic groups of plant pathogenic viruses, members of which are characterised by their peculiar geminate particles. The group can be further subdivided into two subgroups. The first of these incorporates those geminiviruses that are obligately leafhopper-transmitted and infect monocotyledonous hosts. Members of this subgroup include maize streak virus (MSV), chloris striate mosaic virus (CSMV), and wheat dwarf virus (WDV) (Table 2.1). The second subgroup is composed of those geminiviruses that infect dicotyledonous plant species, and have whitefly vectors. Viruses in this subgroup include tobacco leaf curl virus (TLCV), tomato leaf curl virus (ToLCV), honeysuckle yellow vein mosaic virus (HYVMV), squash leaf curl virus (SLCV), African cassava mosaic virus (ACMV, previously known as cassava latent virus), bean golden mosaic virus (BGMV), mung bean yellow mosaic virus (MBYMV), tomato golden mosaic virus (TGMV), and Abutilon mosaic virus (AbMV) (Table 2.1).

Table 2.1: Members belonging to the geminivirus group of plant pathogens.

Virus	Vector	Natural hosts	Known geographical distribution	Reference
BCTV	L: Circulifer tenellus	Many, especially Chenopodiaceae, Cruciferae, Leguminosae, Solanaceae	USA, Mediterranean	Bennett, 1971
CSMV	L: Nesoclutha pallida	Gramineae	Australia	Francki et al., 1979
MSV	L: Cicadulina mbila	Gramineae, especially maize	Africa	Bock, 1974
TYDV	L: Orosius argentatus	Solanaceae, Leguminosae	Australia	Thomas & Bowyer, 1980
WDV	L: Psammotettix alienus	Gramineae, especially wheat	Sweden	Harrison, 1985
ACMV	W: Bemisia tabaci	Euphorbiaceae, especially cassava	Africa, India	Bock et al., 1978
AbMV	W: B. tabaci	Malvaceae	Many countries	Abouzid & Jeske, 1986
BGMV	W: B. tabaci	Leguminosae	Central and South America	Goodman, 1981a
EuMV	W: B. tabaci	Euphorbiaceae	America	Costa & Bennett, 1950
HYVMV	W: B. lonicerae	Caprifoliaceae	Japan	Harrison, 1985

MBYMV	W: B. tabaci	Leguminosae	Thailand	Honda *et al.*, 1950
TLCV	W: B. tabaci	Solanaceae, especially tobacco	Many tropical countries	Harrison, 1985
SLCV	W: B. tabaci	Cucurbitaceae	USA	Cohen *et al.*, 1983
TGMV	W: B. tabaci	Solanaceae, especially tomato	South America	Hamilton *et al.*, 1981
ToLCV	W: B. tabaci	Solanaceae	Mediterranean	Harrison, 1985

L, leafhopper W, whitefly

The two geminiviruses beet curly top virus (BCTV) and tobacco yellow dwarf virus (TYDV) appear to belong to a third subgroup. Both viruses have specific leafhopper vectors, but like the whitefly-transmitted geminiviruses, they each have a host range which is confined to dicotyledonous plants (Stanley *et al.*, 1986) (Table 2.1).

Geminiviruses are also characterised by the unusual nature of their genomes, which in all cases studied to date are single-stranded circular DNA (Harrison *et al.*, 1977; Reisman *et al.*, 1979; Francki *et al.*, 1980; Hamilton *et al.*, 1984; Stanley *et al.*, 1986).

2.2 History and economic importance of selected geminiviruses

Several early studies on the diseases now known to be caused by, or associated with, geminiviruses have become classical case studies in the literature of plant pathology. For example, BCTV, the cause of major diseases in several crops in the western United States and the Mediterranean, has been the subject of considerable study since the early 1900's (Bennett, 1971). The relationship of the leafhopper vector (*Circulifer tenellus*) to disease spread has been recognised since 1910. Significant progress was already being made before 1950 towards the development of control measures.

These were based upon genetic resistance in crop species, improvements in crop management, and control of vectors with insecticides (Bennett, 1971; Goodman, 1981a).

It has long been recognised that a virus is the causative agent of African cassava mosaic disease in both India and Africa. Warburg described the disease in East Africa in 1894 (as reported by Storey & Nichols, 1938), although it is only recently that the disease agent was identified as a geminivirus. In Kenya inoculations with sap from mosaic-affected cassava transmitted a geminivirus that infected Nicotiana clevelandii and several other solanaceous species (Bock et al., 1978). Initially cassava plants could not be reinfected with the virus and, as it was not found in all mosaic-affected cassava plants (notably those from coastal Kenya) it was named cassava latent virus. Later work showed that plants from the Kenyan coast were infected with a distinct strain of the virus that is transmissible with some difficulty to Nicotiana benthamiana, although rarely to N. clevelandii (Bock et al., 1983). Eventually, both the strains were shown to be transmitted by manual inoculation from Nicotiana spp. to susceptible Brazilian cassava plants which then developed mosaic symptoms (Bock et al., 1983). Thus, cassava "latent" virus was shown to cause mosaic disease and as a result was renamed ACMV.

Fuller (1901) first described a disease affecting maize, which is now thought to be maize streak disease. At that stage maize streak disease had been troublesome for some time in South Africa. Thereafter, Storey (1928) described the transmission of the disease by the leafhopper *Cicadulina mbila* (Naude).

The diseases caused by each of these three viruses has had a detrimental effect on the development of agriculture in the areas where they occur. ACMV typically causes a 70% loss of tuber weight in each infected plant and its incidence in many farming areas in Africa exceeds 80% (Bock & Woods, 1983). BCTV almost caused the collapse of the sugar beet industry in the western USA before the introduction of resistant cultivars (Duffus, 1982). Epidemics of MSV can be very severe, causing most of the crop to be lost. This is especially so when drought or crop maturity causes the host plants of vector leafhoppers to dry out, inducing extensive leafhopper migration (Rose, 1978).

Several breeding programs are devoted to developing and testing maize lines for resistance to MSV. The sources of genes coding for MSV resistance in maize have been known for many years (Rose, 1978), but it was not until the late 1960's that the mode of inheritance was determined (Storey & Howland, 1967). Improved maize cultivars with greater

resistance to or tolerance for infection by MSV are now available from breeding programs in South Africa, Zimbabwe, Kenya and Nigeria (Goodman, 1981c).

2.3 Transmission, symptoms caused by, and host ranges of geminiviruses.

2.3.1 Transmission of geminiviruses

None of the geminiviruses appear to be transmitted via seed to progeny plants, and none seem to spread naturally through contact between infected and healthy plants, by pollen, or through the soil. Field spread of virus from plant to plant is therefore likely to be due only to insect vectors. These vectors are either whiteflies (Aleyrodidae) or leafhoppers (Auchenorrhyncha and Cicadellideae). In all recorded instances the whitefly vector is *Bemisia tabaci*, except for HYVMV which is transmitted by *Bemisia lonicerae*. *B. tabaci* has a wide host range, including at least 300 species found among 63 plant families (Mound, 1983). It has been found that whitefly populations bred on one plant species may be difficult to establish on another, although eventually they may become well adapted to it (Mound, 1983). This indicates that in many cases 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.

This type of restriction does not appear to apply to the transmission of the leafhopper-borne geminiviruses. Amongst the geminiviruses one of the most intensively studied virus-vector relationships is that of MSV and its primary leafhopper vector Cicadulina mbila. Van Rensburg and Kuhn (1977) have reported that there are several subsidiary leafhopper vectors for MSV, but all these species are closely related to C. mbila. They include Cicadulina bipunctella zeae, Cicadulina lateus, Cicadulina parazeae, and Cicadulina storeyi. It is not known whether specific virus strains are preferentially adapted to each vector species. Bock (1982) has shown that MSV can be acquired by the vector in less than 1 hour. Following uptake there is a latent period of 6-12 hours (at 30°C) during which time virus cannot be transmitted. After this period has elapsed, virus can be inoculated within 5 minutes. Storey (1932) found that C. mbila exists in two forms: those able to transmit MSV and those unable to transmit MSV. Inactive individuals were made active for transmission if their gut walls were punctured or if virus was injected into their haemocoele. From these results it was concluded that the latent period represents the time required for the virus to cross the gut wall into the body cavity and thence travel to the salivary glands, where it becomes available for inoculation. It was also found that the passage of MSV from the gut lumen to the hemocoele and subsequent transmission

seems to be under the control of a dominant sex-linked gene. However, individual insects that are able to transmit virus do so with different degrees of efficiency. This is because other genes, which are not sex-linked, can modify the effect of the major gene controlling activity.

A pattern of virus-vector relationship similar to that of MSV and C. mbila is displayed by BCTV and Circulifer tenellus. Acquisition probes of as brief as 1 minute have been shown to result in a low incidence of viruliferous leafhoppers. It has been further demonstrated that after a latent period of 4 hours, insects that had acquired the virus were able to transmit to healthy test plants (Bennett, 1971).

Neither MSV nor BCTV has been shown to replicate in their vector and neither is transmitted to progeny leafhoppers via the eggs (Severin, 1921; Storey, 1928). Furthermore, the frequency of virus transmission and the persistence of virus in the insects was shown to depend on the length of the acquisition access period and on the virus concentration in the source plants (Bennett, 1971). These observations indicate that virus replication in the vector is either very limited, or does not occur at all.

2.3.2 Symptoms caused by geminiviruses

The symptoms induced by the different geminiviruses which infect the Gramineae are similar, and consist mainly of leaf streaking (Bock, 1982). The first sign of maize streak disease is the appearance of pale, circular spots 0.5-2.0mm in diameter, on the lowest exposed portions of the youngest leaves of a maize plant (Rose, 1978). Leaves fully affected characteristically display pronounced chlorosis, which is confined to narrow, broken stripes along the veins (Storey, 1925). The stripes are caused by the failure of the chloroplasts to develop in the tissue surrounding the vascular bundles. Primary veins are less affected than secondary and tertiary veins, so there tend to be pronounced groups of five to seven parallel streaks on the leaves, varying from a few millimeters to several centimeters in length, and from 0.5-1.0mm in width (Fajemisin, 1976). Plants infected at an early stage of growth become stunted and produce undersized and/or misshapen cobs. A reduction in photosynthesis, an increase in respiration, and a reduction in leaf length and plant height occurs (Rose, 1978). Only new tissue develops symptoms and normally there are green symptomless leaves at the base of the diseased plants. This, therefore, enables estimation of the stage of growth of the plant at the time of infection. The rate of decline in maize yield falls logarithmically with increased age at time of infection (Van Rensburg & Kuhn, 1977). Plants infected less

than a week after germination produce no yield, those infected at three weeks produce about half yield, and those infected at eight weeks produce a nearly full yield of maize.

Geminiviruses infecting dicotyledonous plants may be divided into two broad groups on the basis of the symptoms they cause. One group cause leaf curling symptoms such as are caused by TLCV in tobacco, BCTV in several of its many host species (this virus infects more than 300 species found among 44 plant families), and ACMV in Nicotiana clevelandii (Goodman, 1981b). The symptoms include curling and crinkling of the leaf lamina, distortion of leaf shape, reduction of leaf area, and, in some cases, formation of enations. Dwarfing and retardation of root growth are also commonly associated with these symptoms (Bock, 1982).

The second type of symptom associated with geminiviruses is the striking veinal chlorosis, or golden mosaic, typical of dicotyledonous plants infected with the viruses transmitted by whiteflies (Goodman et al., 1977). Bean golden mosaic disease, the mosaic of Euphorbia prunifolia, and tomato golden mosaic disease are caused by viruses that are distinct on the basis of host-range. They are transmitted by the whitefly B. tabaci, and produce similar vein-yellowing symptoms on their various hosts (Goodman, 1981a).

Infected plants initially show chlorosis of veins, that under certain conditions leads to a net-like appearance of the leaf, with strongly contrasting bright yellow veins and solid green interveinal areas (Goodman et al., 1977). As the disease develops, the vein chlorosis broadens into a bright golden mosaic.

2.3.3 Host ranges of geminiviruses

The host range of MSV is extremely wide within the Gramineae. This virus has been found to exist as distinct strains (Bock et al., 1974), several of which are apparently host-adapted to indigenous grass species. These strains differ in host range and pathogenicity but are nevertheless serologically related. For example, a number of strains that induce maize streak disease are unable to infect sugarcane or cause only transient infection and symptoms. In the cases where sugarcane is infected only a few streaks appear, and the virus cannot be recovered from later formed streak-free leaves (Bock, 1982). On the other hand a strain that infects sugarcane is transmissible to maize, where it induces persistent but mild symptoms. A third strain, obtained from guinea grass (*Panicum maximum*), infects neither maize nor sugarcane (Bock et al., 1974).

Wild plants are reservoirs of several geminiviruses and their vectors. In Mauritius, perennial grasses such as Coix lachryma-joli and annual grasses such as Crenchus echinatus and Brachiaria reptans are reservoirs of MSV and C. mbila between maize planting seasons and between maize fields (Autrey & Ricaud, 1983). In the western USA wild species growing near cultivated crops are winter hosts of BCTV and Circulifer tenellus (Bennett, 1967). No wild plant has yet been shown to act as a reservoir for the spread of ACMV to cassava; the virus is, however, maintained and distributed by vegetative propagation. This is due to the planting of infected material by farmers (Bock, 1983).

2.4 Geminivirus particle morphology and properties of virions

Two research groups working independently on curly top disease and a group of scientists working on maize streak disease reported the presence of unusual virus-like particles associated with the diseases (Duffus & Gold, 1973; Mumford, 1980; Bock et al., 1974). Purification of the viruses revealed nucleoprotein virus-like particles occurring predominantly as paired isometric units (Mumford, 1980; Bock et al., 1974; Magyarosy, 1980). Similar paired particles were found in partially purified samples prepared

from beans (*Phaseolus vulgaris*) with symptoms of bean golden mosaic disease (Galvez & Castano, 1976; Goodman *et al.*, 1977b).

Electron micrographs of purified particles of these three viruses (and subsequently other geminiviruses), showed them to be bisegmented structures measuring 20 x 30nm with a groove running around the middle at 90° to their long axis. Each of the two segments appeared to be roughly pentagonal in outline, with the faces in contact being slightly longer than the others. The bisegmented particles typically had a sedimentation of approximately 75S (Bock *et al.*, 1974). Preparations of these bisegmented particles were infective. Virus preparations also contained smaller, approximately isometric structures each resembling one of the segments: these were not infective (Francki & Hatta, 1979; Francki *et al.*, 1980; Larsen & Duffus, 1984; Harrison, 1985).

About 80% of the geminivirus particle is protein, which in all cases so far investigated consists of a single type of subunit. The molecular weight of this subunit ranges from 2.7×10^4 to 3.4×10^4 (Bock *et al.*, 1977; Thomas & Bowyer, 1980; Goodman *et al.*, 1980; Francki *et al.*, 1980; Hamilton *et al.*, 1984) (Table 2.2).

Table 2.2: Properties of geminivirus particles.

Virus	M_r coat protein	Reference	M_r DNA	No. nucleotide residues	Reference
ACMV	3.4×10^4	Harrison *et al*., 1977	8.0×10^5	2779 + 2724	Stanley & Gay, 1983
BGMV	2.7×10^4	Reisman *et al*., 1979	8.0×10^5	2646 + 2587	Howarth *et al*., 1985
BCTV	2.6×10^4	Mumford, 1980	9.3×10^5	2993	Stanley *et al*., 1986
CSMV	2.8×10^4	Francki *et al*., 1980	7.1×10^5	2800	Andersen & Morris-Krsinich, 1987
MSV	2.8×10^4	Harrison *et al*., 1977	7.1×10^5	2687	Mullineaux *et al*., 1984 Howell, 1984
TYDV	2.8×10^4	Thomas & Bowyer, 1980	---	---	---
TGMV	2.7×10^4	Hamilton *et al*., 1981	7.9×10^5	2588 + 2508	Hamilton *et al*., 1984
WDV	2.9×10^4	MacDowell *et al*., 1985	9.1×10^5	2749	MacDowell *et al*., 1985 Commandeur *et al*., 1987

Perhaps the most suprising virion characteristic determined during studies of geminiviruses was the unusually small size and unexpected circular topology of the single-stranded DNA genome. The fact that the DNAs were single-stranded was established by nuclease digestion tests, hyperchromicity in the presence of formaldehyde and thermal denaturation profiles (Goodman, 1981b). The DNAs of BGMV, ACMV, CSMV and MSV have all been shown to consist primarily of closed circular single-stranded DNA by electron microscopy (Harrison _et al._, 1977; Reisman _et al._, 1979; Francki _et al._, 1980). The molecular weight of BGMV and ACMV DNAs is approximately 8 x 10^5 D and that of CSMV and MSV DNAs is approximately 7 x 10^5 D (Table 2.2).

Glutaraldehyde-fixed particles of CSMV have a buoyant density in caesium chloride of 1.35g/cm^3, indicating that the DNA content is about 19%; thus, it can be calculated that each bisegmented particle contains only one circular DNA molecule (Francki _et al._, 1980; Goodman, 1980). Geminivirus DNA preparations also usually contain a certain proportion of linear DNA of the same size as the circular molecules (Harrison _et al._, 1977). These linear molecules can be separated from the circular molecules by gel electrophoresis under denaturing conditions (Goodman, 1981b). Experiments in which _Hha_ I restriction fragments of

linear and circular DNA from BGMV were compared indicated that the two topological forms of the DNA contain the same nucleotide sequences. (Goodman, 1981a).

2.5 Structure and infectivity of geminivirus genomes

If their genomes are monopartite, geminiviruses have the smallest nucleic acids of viruses that replicate autonomously. It was, therefore, important to determine whether isolated DNA molecules comprised a single set of nucleotide sequences. The first evidence that a geminivirus genome might be larger than one circular molecule of about 2500 nucleotides arose from the analysis of BGMV DNA. Single-stranded DNA purified from virus particles and a virus-specific double-stranded replicative form (RF) DNA purified from infected plants (Ikegami et al., 1981) were digested with restriction endonucleases. The sum of the different fragments was about 5000 base pairs, indicating a nucleotide complexity twice that expected on the basis of the physical size of the viral DNA (Haber et al., 1981). The possibility that the virus culture consisted of a mixture of two variants with distinctive genomes could not be excluded. However, the possible existence of a bipartite genome was supported by the shape of the infectivity-dilution curve

obtained from experiments in which bean protoplasts were inoculated with single-stranded virus DNA (Haber et al., 1981).

Work carried out by Stanley and Gay (1983) on ACMV provided conclusive evidence for a bipartite genome of this geminivirus. When the nucleotide sequence of the viral DNA was analysed it was found that the genome was accommodated in two circular molecules of similar length, termed DNA 1 and DNA 2, of 2779 and 2724 nucleotides respectively. In addition, when full-length copies of DNA 1 and DNA 2 were separately cloned into bacteriophage M13, and then excised as the double-stranded form, neither was infective for plants on its own. Mixtures of the two genome parts, however, caused normal infections in Nicotiana benthamiana. Both genome parts are therefore needed for the normal infection of plants and production of virions (Stanley, 1983).

Similar results were obtained in work with TGMV, in which the RF DNA was analysed by restriction endonuclease mapping (Hamilton et al., 1982), and the two genome components were cloned and sequenced. In this case the two genome components were termed DNA A and DNA B (Bisaro et al., 1982; Hamilton et al., 1984). The infectivity of the cloned sequences was determined separately and in mixtures, and results were

similar to those obtained for ACMV (Hamilton *et al.*, 1983). Sequencing and infectivity tests on double-stranded clones of BGMV lead to the same conclusion (Howarth *et al.*, 1985).

Determination of the sequence of MSV gave different results, however. In this case only one circle of DNA, 2687 nucleotides in length, could be detected (Mullineaux *et al.*, 1984; Howell, 1984). Similarly, sequencing of the genomes of BCTV and two strains of WDV (WDV-S and WDV-C) revealed single circular DNAs of 2993 (Stanley *et al.*, 1986), 2749 (MacDowell *et al.*, 1985) and 2750 (Commandeur *et al.*, 1987) nucleotides, respectively. This supports the earlier finding by Marriott and Symons (1982) which indicated that the CSMV genome was monopartite. Andersen and Morris-Krsinich (1987) showed the CSMV genome to be 2800 nucleotides in length.

MSV is not transmissible to plants by mechanical inoculation, and no infection resulted when plants were inoculated with full-length cloned DNA (Mullineaux *et al.*, 1984). Grimsley *et al.* (1987) however, were able to infect maize plants with MSV by inoculating the plants with *Agrobacterium tumefaciens* strains carrying Ti-plasmids into which tandemly repeated dimers of MSV DNA had been cloned. If the T-DNA transfer functions of the *A. tumefaciens* strain were intact, disease symptoms appeared within two weeks of inoculation. No infection occurred with control inoculations using naked MSV DNA, *A. tumefaciens* carrying MSV DNA in a

plasmid lacking T-DNA borders, or in *A. tumefaciens* carrying MSV DNA in a Ti-plasmid containing a mutation in the *vir* A locus. On the basis of these results it was concluded that the cloned viral DNA was biologically active (Grimsley *et al.*, 1987). Furthermore, the successful mechanical inoculation of the cloned single DNA component of BCTV has also been reported, although this occurred with very low efficiency (Stanley *et al.*, 1986). Subsequent insect transmission from mechanically inoculated plants was needed to ensure propagation of curly top disease. These two examples are so far the only published direct demonstrations that the single DNA component of a leafhopper-transmitted geminivirus is sufficient for infectivity.

2.6 Serological relationships among geminiviruses

Roberts *et al.* (1984) reported that strong serological relationships could be detected between five whitefly-transmitted geminiviruses ACMV, BGMV, EuMV, SLCV and TGMV using techniques such as immunosorbent electron microscopy (ISEM). Of five leafhopper-transmitted viruses, only BCTV and TYDV were distantly related. No relationship was detected between CSMV, MSV or WDV nor did these three viruses show any relationship with BCTV or TYDV. No

serological relationship has been reported between the whitefly-transmitted viruses and the leafhopper-transmitted viruses (Roberts et al., 1984).

2.7 Detection of geminiviruses

For some time, the detection of geminiviruses has largely relied on traditional methods such as symptom expression, and transmission of the disease to test plants, often with the help of a specific vector. In general, the concentration of particles of geminiviruses in plants are too low for detection by the older serological tests, but the newer, more sensitive methods are adequate for detection. Immunosorbent electron microscopy (ISEM) is often used, even though difficulties may arise, as the virus particles are unstable in some buffers. Furthermore, most electron-dense staining methods result in only weak contrast (Sequeira & Harrison, 1982; Roberts et al., 1984). The standard "sandwich" enzyme-linked immunosorbent assay (DAS-ELISA) can be applied with more success and has already proved useful in research on ACMV and BCTV (Sequeira & Harrison, 1982; Cohen et al., 1983) and MSV (du Plessis & von Wechmar, unpublished). Spot-hybridization tests using ^{32}P-labelled cloned probes have also been used for detection of ACMV (Roberts et al., 1984; Robinson et al., 1984). Haber et al. (1982) have used both radioisotope-labelled single-stranded

virion DNA of BGMV and restriction fragments of double-stranded BGMV RF-DNA, to probe preparations of low molecular weight total DNA from a number of diseased plant species. As geminivirus particles had only been isolated from a few of these species, the specific binding of the probes to the low molecular weight DNA indicated that geminiviruses similar to BGMV could be the agents of the disease.

2.8 Organisation of and homologies between geminivirus genomes

Sequencing of the genomes of BGMV (Hamilton et al., 1984), TGMV (Stanley & Gay, 1983), ACMV (Howarth et al., 1985), two isolates of MSV (Howell, 1984; Mullineaux et al., 1984), two isolates of WDV (MacDowell et al., 1985; Commandeur et al., 1987), BCTV (Stanley et al., 1986), and CSMV (Andersen & Morris-Krsinich, 1987) has done much to reveal the genomic organisation of geminiviruses. The genomes of ACMV, TGMV and BGMV have a very similar organisation. In each case, the nucleotide sequence of the two genome parts is different, except for one sequence of approximately 200 nucleotides, known as the common region, which is found in a non-coding region of the genome. This region is identical (BGMV) or almost identical (ACMV and TGMV) on each DNA molecule. The common sequence includes a stretch of 33-34 nucleotides, located near the 3'-end of each common region, that appears able to form an almost identical stem and loop structure in

all three cases. The structure consists of a GC-rich base-paired stem and an AT-rich loop containing the conserved sequence TAATATTAC (Fig. 2.1) preceded by TT, TTT or TA. The remainder of the common sequence is different in the three viruses, which has led to the suggestion that it may play a role in determinig host-range, and/or a role in particle assembly (Hamilton et al., 1984; Howarth et al., 1985; Howarth & Goodman, 1986).

A similar sequence has been identified in the single DNA component of MSV, WDV and BCTV (Howell, 1984; Mullineaux et al., 1984; MacDowell et al., 1985; Stanley et al., 1986), and is likewise situated within a non-coding region of the genome. The stem consists of 11 GC pairs plus 8 AT pairs, and the loop, as with the other viruses, includes the sequence TAATATTAC (Harrison, 1985). (Fig. 2.1). These structures are also likely to be involved in host-range determination and particle assembly.

Computer searches of the sequences of BGMV, ACMV, and TGMV revealed six protein coding open reading frames (ORFs) in each virus genome (Stanley & Gay, 1983; Hamilton et al., 1984; Howarth et al., 1985). The most notable feature of the distribution of these ORFs is that they occur both in the virion strand (plus strand) and in the complementary strand (minus strand) of both genome parts (fig. 2.2).

Figure 2.1: A comparison of the conserved sequence of the geminivirus common region.

```
ACMV             GGCCAACCG TATAATATTACCGGTTGGCC
                  ::::: ::: : ::::::::::: :::::
TGMV            CGGCCATCCG TTTAATATTACCGGATGGCCGC
                  ::::: ::: : ::::::::::: :::::
BGMV            CGGCCATCCGATATAATATTACCGGATGGCCGC
                  ::::: ::: : ::::::::::: :::::
BCTV            GGGCCATCCGTTATAATATTACCGGATGGCCC
                          ::   ::::::::::
MSV      GCAGGAAAAGAAGGCGCGCACTAATATTACCGCGCCTTCTTTTCCTGC
                     : ::: :   :::::::::: :::
WDV       GGGGGCCTCCACGCGGGTTATAATATTACCCCGCGTGGTGGCCCCC
```

```
      A T
   A       A
  T         T
  A         T
   C       C
     C   C
      G C
      C G
      C G
      T A
      A T
      C G
      C G
      G C
      G C
      C G
```

References: ACMV (Stanley & Gay, 1983), TGMV (Hamilton *et al.*, 1984), BGMV (Howarth *et al.*, 1985), BCTV (Stanley *et al.*, 1986), MSV (Mullineaux *et al.*, 1984), and WDV (MacDowell *et al.*, 1985). Dots represent the most highly conserved sequence homologies between the various viruses. Gaps have been introduced to maximise sequence homology.

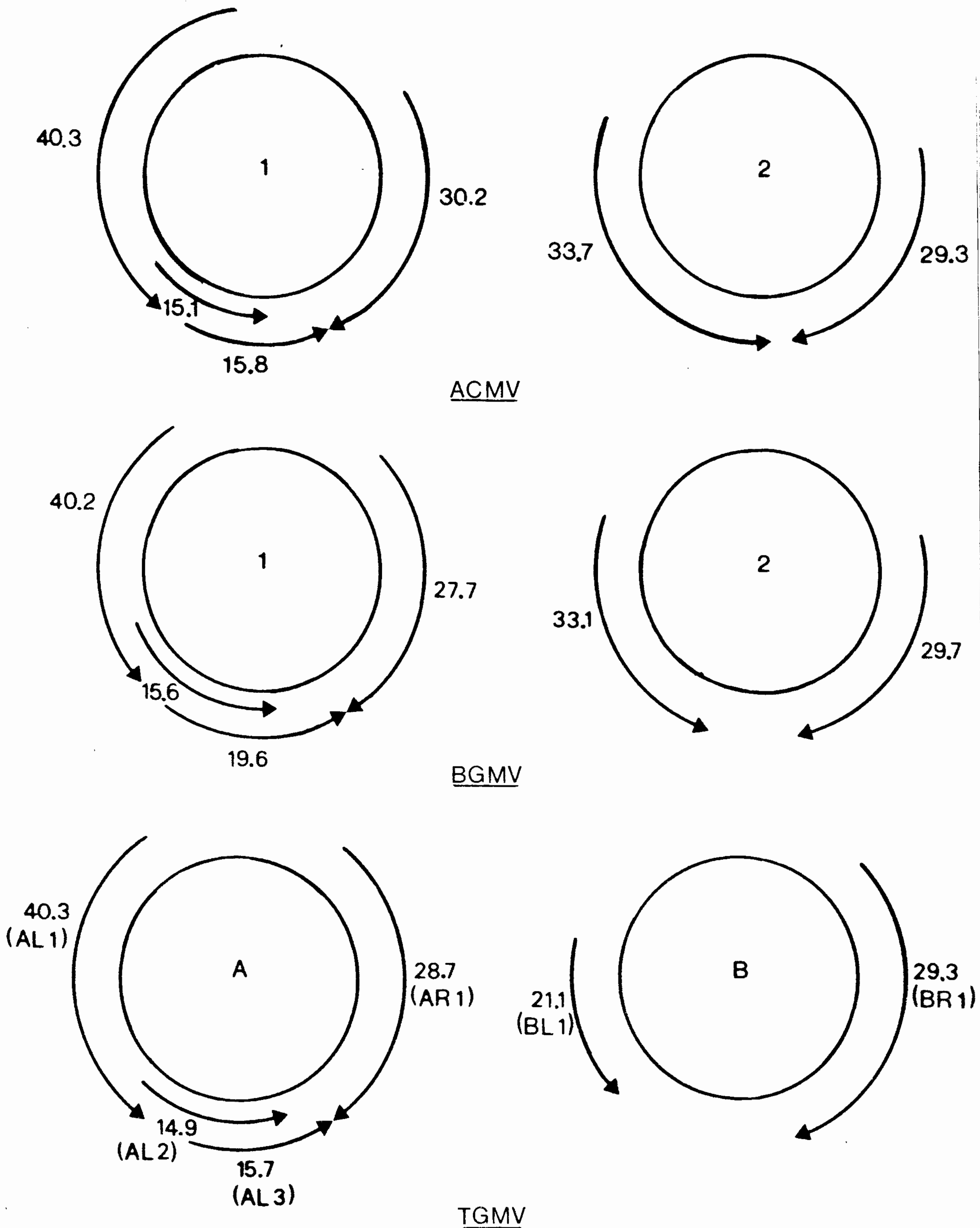
The DNA sequences of a Kenyan (Howell, 1984) and a Nigerian (Mullineaux et al., 1984) strain of MSV are approximately 98% homologous, differing by only 59 nucleotides out of a total of 2680. Only 5 of the 59 differences in sequence are due to insertions or deletions; the remainder are nucleotide substitutions (Howarth & Goodman, 1986). All of the potential ORFs in both MSV isolates begin with an AUG start codon and are associated with a consensus "TATA-like" box and polyadenylation signal sequence of (A/G)ATAA (Howell, 1984; Mullineaux et al., 1984). In WDV all of the ORFs start with AUG codons, with the exception of the ORF that potentially encodes the ~ 17.2kD protein. This ORF begins with GUG (MacDowell et al., 1985). Again, all potential ORFs in WDV have been shown to be associated with TATA-like boxes and polyadenylation signals. One feature which remains unique to MSV is a short DNA molecule of 80 nucleotides in length, found associated with genomic DNA prepared from virions and complementary in sequence to genomic DNA (Donson et al., 1984). This "primer" DNA has a fixed 5' and variable 3' ends and has several ribonucleotides at the 5'-end. It is capable of priming the in vitro synthesis of viral double-stranded DNA. The primer is situated in an intergenic region which is complementary to a region 5' of two inverted repeat sequences capable of forming hairpin structures. It has been suggested that the primer may play a role in the synthesis of viral double-stranded DNA upon infection of the host

(Howell, 1984; Donson et al., 1984). A DNA primer of this type has not been found in any of the other geminiviruses so far investigated (Lazarowitz, 1987).

It was observed that the amino acid sequence of the MSV ~31.3kD and ~17.2kD proteins, predicted from the overlapping ORFs on the viral complementary strand, had homology to N-terminal and C-terminal sections respectively, of the ~40kD protein ORF in AL1 of TGMV (Mullineaux et al., 1985) (Fig. 2.2). In fact, this was true for all the predicted A component ~40kD protein ORFs of the whitefly-transmitted geminiviruses, and the ~31.3kD and ~17.2kD proteins predicted for MSV (Howarth et al., 1985; Mullineaux et al., 1985). Significant homology was found between the ~30.1kD protein of WDV, the ~31.3kD protein of MSV and the NH_2-terminal portion of the ~40.9kD protein of TGMV (MacDowell et al., 1985) (Fig. 2.3). Homology was also found between the ~17.2kD protein of WDV, the ~17.2kD protein of MSV and the COOH-terminal of the ~40.9kD protein of TGMV (MacDowell et al., 1985). The ~40.9kD protein of BCTV has also been shown to have a similar degree of homology with the ~31.3kD and ~17.2kD proteins of MSV and their equivalents in WDV, as was observed for TGMV (Stanley et al., 1986) (Fig. 2.3).

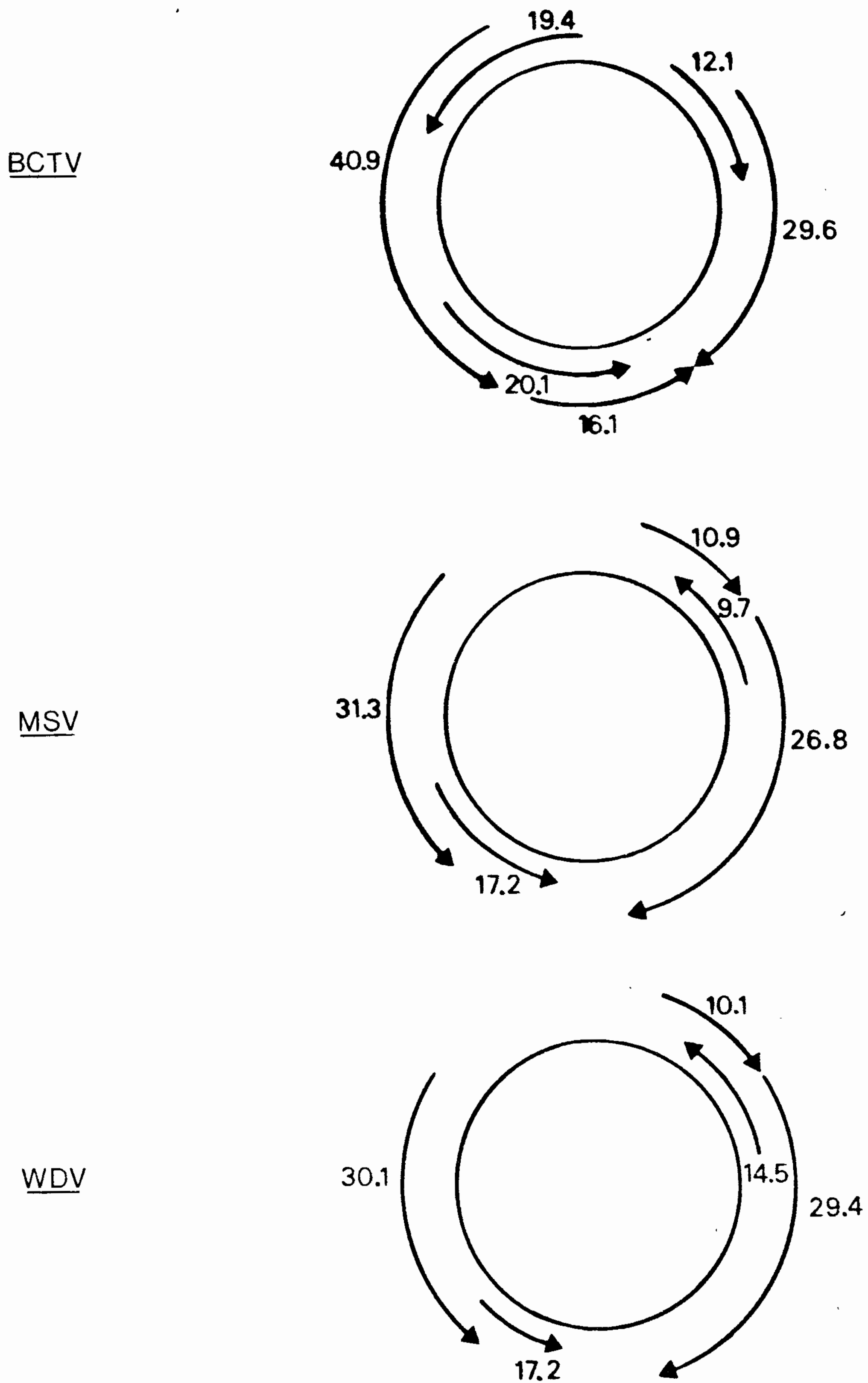
The only published transcription analyses of geminiviruses are those of ACMV (Townsend et al., 1985) and MSV (Morris-Krsinich et al., 1985). In the case of ACMV five virus-

Figure 2.2: Structural organisation of the DNA components of ACMV, BGMV and TGMV.



The maps are based on the published sequences and analyses. The A and B components of TGMV are equivalent to DNA 1 and 2 in BGMV and ACMV. ACMV (Stanley & Gay, 1983), BGMV (Howarth *et al.*, 1985), TGMV (Hamilton *et al.*, 1984).

Figure 2.3: Structural organisation of the DNA components of BCTV, MSV and WDV.



The maps are based on the published sequences and analyses. BCTV (Stanley, et al., 1986), MSV (Mullineaux et al., 1984; Howell, 1984), WDV (MacDowell et al., 1985).

specific transcripts have been identified in virus-infected plants. These transcripts have been mapped to the positive and negative senses of both DNAs, and have been shown to encompass the six ORFs that are most likely to be expressed (Ward et al., 1987). Three are transcribed from DNA 1: a major 1kb transcript (which encodes the coat protein), a 1.7kb and a 0.7kb transcript. Two major transcripts of 1.1kb and 0.9kb hybridise to DNA 2-specific probes. Minor transcripts of 1.35kb, 2.0kb and 2.2kb encoded by DNA 2 have also been detected.

Three MSV-encoded RNA transcripts have been obtained from virus-infected maize leaves, and mapped to the genome (Morris-Krsinich et al., 1985). Two of the transcripts, a major 0.9kb and a minor 1.05kb RNA, were mapped to the virion (+ sense) DNA. These two transcripts each had a 3'terminal at nucleotide 1114 and 5' termini at nucleotides 2682 and 163 respectively. The third minor transcript of 1.2kb was mapped to the complementary (- sense) DNA. A polypeptide of M_r 28 000 D was mapped to the region coding for the two virion-sense transcripts of 1.05kb and 0.9kb. This 28kD protein was immunoprecipitated with antiserum raised to whole virus. The region of DNA covered by the virion-sense transcripts had an ORF with a putative product of 26 969 MW, and has been shown to encode the coat protein (Mullineaux et al., 1987).

In the case of ACMV and MSV the above data is consistent with the bidirectional transcription predicted from sequence analysis of the relevant genomes (Townsend et al., 1985; Morris-Krsinich et al., 1985).

In order to examine the relationship between the geminiviruses, homologies at both the nucleotide and the amino acid sequence level were investigated. The nucleotide sequences of TGMV DNA A and ACMV DNA 1 show 60% gross homology (Stanley & Gay, 1983; Hamilton et al., 1984). No extensive sequence homology was detected between BCTV DNA and either DNA 2 of the whitefly-transmitted geminiviruses or the DNA of the leafhopper-transmitted viruses (Stanley et al., 1986). Experiments with DNA 1 probes, however, revealed imperfect homology between the genomes of all the whitefly-transmitted geminiviruses tested, but no homology was found with the genomes of the leafhopper-transmitted viruses (Roberts et al., 1984; Stanley et al., 1986). Similarly, no extensive sequence homology has been shown between the DNAs of MSV, TGMV and ACMV (MacDowell et al., 1985). A 46% gross homology has been reported between the DNAs of WDV and MSV (MacDowell et al., 1985).

Comparison of ACMV with TGMV and BGMV has indicated that the most highly conserved ORF of these viruses is that encoding the coat protein (71% and 68% direct homology, respectively) (Hamilton et al., 1984; Howarth et al., 1985). This

contrasts markedly with the 15% direct homology between ACMV ORF encoding the coat protein (30.2kD) and the analogous ORF encoding a 26.9kD protein in BCTV (Stanley et al., 1986). Furthermore, when the amino acid sequences were aligned, homology was mainly confined to the carboxy-terminal 20% of the ORFs (40% direct homology), the remaining 80% of the ORFs showing only 8% direct homology (Stanley et al., 1986). Comparison of the BCTV 29.6kD ORF protein with the 27.0kD and 29.4kD coat protein ORFs of MSV and WDV revealed 25% and 22% direct homology, respectively (Morris-Krsinich et al., 1985; MacDowell et al., 1985). These values are lower than the 35% direct homology between the respective MSV and WDV ORFs (MacDowell et al., 1985).

The information above suggests that there are conserved functional domains within the proteins of both the leafhopper-transmitted geminiviruses with monopartite genomes, and the whitefly-transmitted viruses with bipartite genomes. This provides strong evidence for an evolutionary relationship between these two subgroups of viruses (MacDowell et al., 1985; Mullineaux et al., 1985). On the basis of limited amino acid sequence homology between the coat protein gene in ACMV DNA 1 and a gene of similar size in a corresponding position in ACMV DNA 2, Kikuno et al. (1984) have suggested that the bipartite viral genome evolved from a single common ancestral DNA. These workers compared the following: (i) the coat protein genes for WDV,

MSV, TGMV, and ACMV; (ii) two ACMV ORFs capable of encoding proteins of M_r 30 100 (DNA 1) and M_r 29 200 (DNA 2); and (iii) a gene in TGMV DNA 2 which correspondes to that encoding the 29.2kD protein in ACMV DNA 2. Once the predicted amino acid sequences of these six ORFs had been aligned, it was shown that 43% of all the positions compared are occupied by residues that are identical or similar in nature. Furthermore, it was statistically shown that the similarity between the aligned sequences was highly significant, as the probability of this occurring by chance was 4.3×10^{-6} (Kikuno et al., 1986). The presence of such extensive sequence similarity suggests that like the ORF of DNA 1, which is known to encode the coat protein (Stanley & Gay, 1983), the ORF of DNA 2 also encodes a functional protein.

2.9 Geminivirus replication

Cytological evidence indicates that geminivirus DNA replicates, and virions accumulate, in the nucleus of infected cells (Sequeira & Harrison, 1982; Stanley, 1983). Ultrastructural changes in the nucleus characteristic of geminivirus infections include, segregation of the nucleolus and fibrillar bodies (Francki et al., 1979; Kim et al., 1978; Christie et al., 1986); and, aggregation of virus particles in cells of the vascular region (Kim et al., 1978;

Christie et al., 1986). Granular inclusions may occur in the cytoplasm (Esau & Hoefert, 1973; Christie et al., 1986). With many geminiviruses, virus particles and virus particle antigen are found mainly in the phloem, especially in phloem parenchyma cells, and the viruses are considered phloem limited (Harrison, 1985). However, this limitation is not absolute: occasionally cells outside the phloem are infected with BCTV (Thornley & Mumford, 1979), and several types of cell can be infected with ACMV (Sequeira & Harrison, 1982). Furthermore, the detection of AbMV virion ssDNA and RF dsDNA in the plastids of plants infected with AbMV was recently reported by Groning et al. (1987), although they were not able to isolate virus particles from these organelles.

Little detail concerning the replication mechanism of geminiviruses is available. However, there is evidence that TGMV and BGMV generate double-stranded replication intermediates in infected cells (Ikegami et al., 1981; Sunter et al., 1984). Some of these intermediates are greater than unit length (Hamilton et al., 1982). It appears that DNA replication occurs by a mechanism dependent on a circular rather than a linear template (Hamilton et al., 1984). The replication is via DNA complementary strand synthesis; although RNA-dependent DNA synthesis is not ruled out. If a covalently closed circular (supercoiled) double-strand DNA intermediate is involved as suggested by Hamilton et al. (1982), then it appears not to accumulate to the

extent that the relaxed circular forms do (Townsend et al., 1986). Protoplast infection with both single-stranded DNA and double-stranded replication intermediate DNA is apparently possible (Goodman, 1980). This indicates that the initial events of viral DNA synthesis do not require functions provided by coat or other virion proteins (Rogers et al., 1986). Linear single-stranded DNA may be a precursor, although not necessarily one that accumulates: ligation of this molecule would result in the formation of the circular viral DNA, which is the predominant form found in virus particles (Hamilton et al., 1982; Ikegami et al., 1981). DNA preparations purified from BGMV particles usually contain a small amount of linear single-stranded DNA which is similar in length to the circular single-stranded DNA (Reisman et al., 1979; Goodman et al., 1980). Under certain conditions, the amount of linear DNA obtained in extracts is increased with a stoichiometric decrease in the amount of circular DNA (Goodman et al., 1980). All the above data appears consistent with replication via a double-stranded DNA intermediate.

2.10 Possible evolutionary relationships of geminiviruses

BCTV is a monopartite geminivirus, which is leafhopper-transmitted, and has a host range confined to dicotyledonous plants. Based on sequence analysis of the genome of BCTV

(Stanley et al., 1986) it has been suggested that this virus may be viewed as the "missing-link" between whitefly-transmitted geminiviruses and leafhopper-transmitted geminiviruses (Stanley et al., 1986). The organisation of the putative coding sequences of BCTV DNA (see 2.8) is more like that of the DNA 1 component of the whitefly-transmitted bipartite viruses than that of the single component of leafhopper-transmitted viruses. This suggests that this particular genomic organisation is common to geminiviruses that infect dicotyledonous plants. Furthermore, the differences observed in the positions of particular ORFs in MSV and WDV as compared to TGMV, BGMV and ACMV, reflect a monocotyledonous-dicotyledonous difference rather than a monopartite-bipartite distinction.

The hybrid nature of the BCTV genome raises the question of how the members of the subgroups within the geminivirus group have evolved. If the suggestion made by Kikuno et al. (1984) is true, and the two components of the whitefly-transmitted geminiviruses evolved from a common ancestral DNA, then BCTV probably evolved as a leafhopper-transmitted single DNA-component virus before the development of the bipartite genome of the whitefly-transmitted viruses, and an overall simplicity of genome organisation was maintained due to limitations of diversification imposed by common hosts. However, it cannot be ruled out that BCTV evolved as a whitefly-transmitted geminivirus with a bipartite genome and

subsequently lost the requirement for one component as a result of change of insect vector specificity, perhaps mediated by a recombination event involving sequences encoding the coat protein (Stanley _et al_, 1986).

CHAPTER 3

SEROLOGICAL CHARACTERISATION OF DIFFERENT ISOLATES OF MAIZE STREAK VIRUS

3.1 INTRODUCTION

In 1974 Bock et al. described the occurrence of distinct strains of maize streak virus (MSV) in Kenya, and showed that these strains were serologically related but not identical. It was also shown that yield losses as a result of maize streak disease depended to some extent on the virus strain. Since this report, however, very little reference has been made to different strains of MSV. This study was, therefore, initiated to investigate different isolates of MSV obtained from distinct geographical locations in Southern Africa. For this purpose, three isolates were chosen: two from South Africa (MSV-CT and MSV-PE) and a third from South West Africa / Namibia (MSV-SWA). These three isolates were shown to produce different symptoms in infected maize plants in the laboratory (Fig. 3.1). As MSV cannot be transmitted by mechanical inoculation (Storey, 1925; Rose, 1978; Bock et al., 1974; Goodman, 1981a), leafhopper colonies were established to transmit these viruses, so as to maintain stocks of infected material.

Figure 3.1: Maize plants infected with different isolates of MSV. (a) MSV-CT; (b) MSV-PE; (c) MSV-SWA

Figure 3.2: A *Digitaria sanguinalis* plant infected with DGV.

A fourth geminivirus - infecting the grass Digitaria sanguinalis - was obtained from Vanuatu (Formerly New Hebrides). This virus - thought to be a strain of MSV (Dollet et al., 1986) - was maintained, and vegetatively propagated, in Digitaria sanguinalis plants (Fig. 3.2). Digitaria geminivirus (DGV) was compared to known strains of MSV in order to confirm its identification as an MSV strain.

The virus particles were purified from their respective hosts and visualised by electron microscopy. The size of virion coat proteins was determined by polyacrylamide gel electrophoresis. The viruses were compared serologically by means of DAS-ELISA and Western blotting to determine whether the three MSV isolates differed from each other and from DGV.

3.2 MATERIALS AND METHODS

3.2.1 Establishment and maintenance of leafhopper colonies

3.2.1.1 Establishment of leafhopper colonies

Stock colonies of non-viruliferous ("clean") leafhoppers were obtained from Professor M.B. von Wechmar, Department of Microbiology, University of Cape Town.

Clean leafhoppers were allowed to feed on infected dried material that had been rehydrated by soaking in tap water for approximately 30 minutes. Acquisition feeds were from 5-10 hours, whereafter the leafhoppers were transferred to uninfected maize seedlings.

3.2.1.2 Maintenance of leafhopper colonies

All stock colonies were maintained on maize (*Zea mays* L.) cv. PNR 493 and barley (*Hordeum vulgare*) cv. Clipper, which had been previously checked for the presence of seed-transmitted viruses (von Wechmar, personal communication).

MSV-CT viruliferous- and MSV-SWA viruliferous- leafhoppers were quarantined in separate rooms of the building on custom-made trolleys fitted with Gro-Lux fluorescent lighting (Philips) with a 12 hour day / 12 hour night cycle.

MSV-PE viruliferous leafhoppers were accommodated in gauze-lined wooden-frame cages (50x50x50cm) which were placed on custom-made trolleys as described above. These trolleys were housed in a separate part of the building from the other two virus colonies.

Clean leafhoppers were maintained in similar wooden-frame cages on a laboratory windowsill, "spotlighted" with a 60W incandescent globe.

3.2.1.3 Culling of leafhoppers

The systemic insecticides "Dazzel" (containing diazinon) or "Metasystox" (containing oxydementomethyl) were used for routine killing of leafhoppers. To ensure death of all leafhoppers, sprayed plants were left for 12 hours in a non-ventilated room before being transferred to growth rooms.

3.2.2 Maintenance, propagation and source of virus isolates

3.2.2.1 Source of virus isolates

All MSV isolates were obtained by Professor M.B. von Wechmar, Department of Microbiology, University of Cape Town. They were introduced into the laboratory in the form of dried infected maize leaves.

MSV-CT was originally isolated from infected plants obtained from a farm in the Potchefstroom area, in the Transvaal province of South Africa.

MSV-PE was first isolated from infected material obtained from a farm near Port Elizabeth, in the Eastern Cape Province.

MSV-SWA was isolated from infected plants obtained from a farm near Otjiwarongo, in northern South West Africa / Namibia.

DGV was described by Dollet *et al.* (1986) as originating from Vanuatu (formerly New Hebrides). In this laboratory it was isolated from infected *Digitaria sanguinalis* plants originally obtained from Dr. G. Boccardo (Instituto di Fitovirologia Applicata, CNR, Torino, Italy). This infected plant material was introduced into the country with permission from the Directorate of Plant and Seed Control, Department of Agriculture (Permit no. 14/14/A/16/16).

3.2.2.2 Storage of viruses

For short term storage of MSV-CT, MSV-PE and MSV-SWA, infected plant material was sealed in plastic bags and kept at 4°C, for no longer than one week, before extraction of the viruses. In the case of *Digitaria* geminivirus, virus was always extracted from freshly harvested plant material. For long term storage, infected plant material was dried over self-indicating silica gel under vacuum, and the dessicated samples stored in a cryostat (-170°C).

3.2.2.3 Maintenance and propagation of viruses

MSV-CT, MSV-PE and MSV-SWA were propagated in maize cultivar PNR 493. Two-week old maize plants were infected with one of the three viruses by exposure, for several days, to the respective colony of viruliferous leafhoppers. After this inoculation access period, plants were sprayed with a systemic insecticide (see 3.2.1.3), and incubated in a plant growth room under the following conditions: (i) 14 hour day / 10 hour dark cycle, (ii) approximately 70% humidity, and (iii) 24°C day / 21°C night temperature cycle.

DGV was propagated vegetatively from the original infected *Digitaria sanguinalis* specimen, in an insect-free plant growth room, as described above.

3.2.3 Virus purification

3.2.3.1 Maize streak virus strains -CT, -PE and -SWA

MSV was purified from infected maize (cv. PNR 493) plants by a method derived from that of von Wechmar and Milne (1982).

Infected plants were harvested at approximately 3 weeks, after incubation for 12-14 days in a plant growth room (see

3.2.2.3). The freshly harvested plants were homogenised, at room temperature, in a commercial Waring-type blender, with an equal weight/volume (w/v) of 0.1M sodium acetate buffer, pH 4.8 (Miller & Golder, 1950). The homogenate was passed through a single layer of cheesecloth, and the pH immediately adjusted from approximately pH 6.5 to pH 4.8 with 10% (v/v) glacial acetic acid. Host plant debris was removed by low-speed centrifugation (12 000 x g) for 10 minutes, and the virus-containing supernatant was ultracentrifuged (70 000 x g) for 180 minutes. Pellets were resuspended in 0.05M sodium phosphate buffer, pH 7.5 (Miller & Golder, 1950), and the extract was subjected to an additional cycle of differential centrifugation. The final pellet was again resuspended in 0.05M phosphate buffer, pH 7.5. All purified preparations were stored at $4^{o}C$.

3.2.3.2 Digitaria geminivirus

Virus particles were purified according to the method of Dollet *et al.* (1986).

Freshly-harvested plant material was frozen in liquid nitrogen, then ground with an equal weight per volume of 0.1M potassium phosphate buffer, pH 7.0 (Williams & Chase, 1967) containing 10mM EDTA, 10mM DIECA and 20mM Na_2SO_3. After passing the homogenate through a single layer of cheesecloth, host plant debris was removed by low-speed

centrifugation (12 000 x g for 10 minutes). The supernatant was adjusted to 1% (v/v) Triton X-100 and clarified by treatment with 1/10 volume chloroform/butanol (1:1 v/v). Virus particles were concentrated by precipitation with 12% PEG (w/v) and 0.2M NaCl followed by low-speed centrifugation. The pellet was resuspended in 0.1M phosphate buffer, pH 7.0, containing 10mM EDTA, and ultracentrifuged (60 000 x g for 180 minutes). After resuspension in the same buffer and additional low-speed centrifugation, the purified virus preparation was stored at 4°C.

3.2.3.3 Quantitation

Purified virus preparations were quantified spectrophotometrically. Virus suspensions were diluted in the appropriate buffer and ultraviolet absorbance at 260nm and 280nm recorded on a Beckman Model 25 spectrophotometer. A specific extinction coefficient $A_{260/0.1\%}$ (extrapolated UV absorption of a lmg/ml solution in a lcm cuvette) of 7.7 was assumed for all viruses used in this study.

3.2.4 Serological methods

3.2.4.1 Production of antisera

Virus preparations were purified by ultracentrifugation through 10-40% sucrose density gradients (96 000 x g for 150

minutes) in a Beckman SW 28 rotor, before being utilised for antiserum production.

Rabbits were immunized by weekly intramuscular injections with 1ml virus mixed 1:1 (v/v) with Freunds incomplete adujvant, and bled fortnightly after 4-5 injections (Rybicki & von Wechmar, 1981). Antisera were stored at -20°C. Titres were determined by indirect ELISA (see 3.2.5.2).

3.2.4.2 Gamma-globulin purification

The gamma-globulin fraction of rabbit serum (IgG) was purified by an adaptation of the method of Clark and Adams (1977).

Samples of each antiserum used were "host-absorbed" as follows: antisera were diluted with 1/5 volume of freshly-extracted healthy host plant sap and incubated at 37°C for 30 minutes. The aggregates formed by binding of the host plant components to their homospecific antibodies were removed by centrifugation at 6 000 x g for 5 minutes. After the addition of an equal volume of saturated ammonium sulphate, the preparation was left at room temperature for 10 minutes, whereafter the precipitate was collected by centrifugation (6 000 x g for 5 minutes) and resuspended in saline. The preparation was dialysed extensively against half-strength phosphate buffered saline (1/2 PBS) (PBS; 1:1

[v/v] 0.15M NaCl : 0.1M potassium phosphate buffer, pH 7.0), and freeze-dried overnight. The IgG was further purified by passage through a DEAE-cellulose column (Whatman DE-52 anion exchanger) pre-equilibrated in 1/2 PBS. Fractions which had a UV absorbance of 1.4 or higher at 280nm, were collected. These fractions were pooled and adjusted to approximately 1mg/ml (OD_{280nm} = 1.4) and stored at 4°C.

3.2.4.3 Conjugation of gamma globulin with alkaline phosphatase

IgG was conjugated with alkaline phosphatase by a modification of the method of Avrameas (1969).

Alkaline phosphatase (3 000 U/mg, Boehringer-Mannheim) was added to a 1mg/ml solution of purified IgG to a final concentration of 2mg/ml, and the mixture dialysed extensively against 1/2 PBS at 4°C. Glutaraldehyde was added to 0.05% (v/v) final concentration and the mixture incubated at room temperature for 4 hours. Glutaraldehyde was removed by dialysis against several changes of 1/2 PBS and the antibody-enzyme complex stored with approximately 1% (w/v) BSA at 4°C.

3.2.5 Enzyme-linked immunosorbent assay (ELISA)

3.2.5.1 Double-antibody sandwich (DAS)-ELISA

Standard microtitre trays (Nunclon Microwell Plate 96F) were used for all tests. Reaction volumes of 200ul were used for all steps, except for a 300ul substrate reaction volume. The method followed was that of Rybicki and von Wechmar (1982a). Coating antibodies were diluted in PBS (1:1 [v/v] 0.15M NaCl : 0.1M potassium phosphate buffer, pH 7.0). Antigens and conjugate were diluted in PBS-T-BSA (PBS containing 0.05% [v/v] Tween-20 and 0.2% [w/v] BSA). After each step the plates were given three 5 minute washes in PBS-T (PBS containing 0.05% [v/v] Tween-20). A post-coating reaction in PBS-T-BSA, 15 minutes at 37°C, was allowed before the addition of antigen. For purified virus assays, ten-fold dilutions were used with a starting concentration of virus of 0.5mg/ml. Five-fold antigen dilutions were used for assays performed with samples of infected sap. Colour intensity of the reactions was determined at 405nm, using a Titertek Multiskan automatic read-out spectrophotometer (Type 310C, Flow Laboratories).

3.2.5.2 Indirect ELISA

In this test, antigen absorbed onto a plastic support are reacted with rabbit antibodies, the reaction being detected

by the binding of enzyme-conjugated goat anti-IgG globulin (GAR-alkaline phosphatase) antibodies to the immobilised antibodies, and the subsequent colour reaction of the conjugated enzyme with its substrate (Rybicki & von Wechmar, 1981).

The reaction volumes, washes, and temperature and time of incubation remain the same as those for DAS-ELISA (see 3.2.5.1), with the exception of the post-coating step at 37°C with PBS-T-BSA, which was lengthened to 1-1.5 hours.

3.2.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

3.2.6.1 Sample preparation

Samples to be electrophoresed were mixed with an equal volume of protein dissociation mix (10% [w/v] SDS, 10% [v/v] 2-mercaptoethanol, 15% [v/v] glycerol, 0.01% bromophenol blue, 0.125M Tris/HCl pH 6.8), and heated at 100°C for 10 minutes.

3.2.6.2 Electrophoresis

SDS-PAGE was performed by the method of Laemmli (1970), using a Hoefer SE-600 vertical slab gel apparatus (Hoefer Scientific Instruments). Gel sandwiches (1.5mm thick)

consisted of a 12% resolving gel and a 4% stacking gel. Electrophoresis was carried out at 4°C, with a constant current of 35-40mA. When the tracking dye front had reached the bottom of the gels, they were either stained or immunoelectroblotted (see 3.2.7).

Gels were stained by immersion in 0.2% (w/v) Coommassie brilliant blue R-250 (BDH Chemicals) dissolved in a 45:45:10% (v/v) mixture of methanol, water and glacial acetic acid. Destaining of gels was by diffusion in a 25:65:10% (v/v) methanol, water, glacial acetic acid mixture.

3.2.7 Immunoelectroblotting (Western blotting)

3.2.7.1 Electroblotting

The electroblotting procedure described for plant virus proteins by Rybicki and von Wechmar (1982b) was employed.

The transfer cassette of the Hoefer Scientific Instruments Model TE 52 Transphor was prepared as follows : three pieces of Whatman 3MM paper were laid on a foam pad, followed by the polyacrylamide gel, the nitrocellulose filter (Schleicher & Schuell BA 85, 0.45um pore) and a further three pieces of 3MM paper. The cassette was immersed in

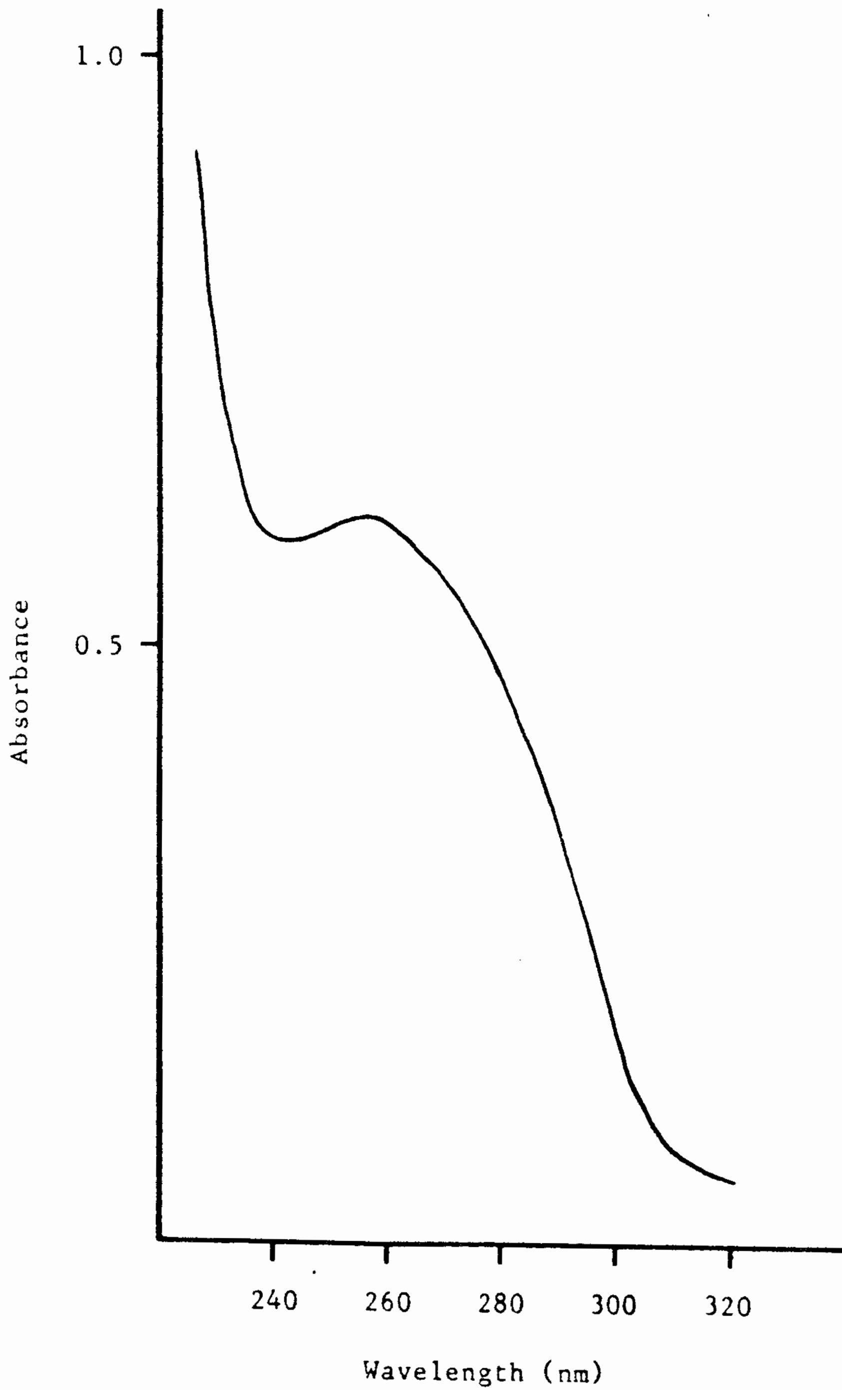
electroblot buffer (0.025M Tris/HCl pH 8.8, 0.192M glycine, 20% [v/v] methanol) in the Transphor tank, with the nitrocellulose towards the anode. Transfer was allowed to proceed for 2-4 hours with a current of approximately 1 ampere (fluctuations in the current occur during the process).

3.2.7.2 <u>Indirect immunoassay</u>

All antisera used in immunoelectroblot tests were host-absorbed (see 3.2.4.2).

After electroblotting (see 3.2.7.1) the nitrocellulose was incubated in blocking buffer (0.01M Tris HCL/saline pH 7.4, 1% [w/v] BSA) at $4^{o}C$ overnight. Rabbit antiserum (1/1000 dilution) was added to the blocking buffer, and the nitrocellulose filter was incubated for 2 hours at room temperature. The filter was washed in at least 4 changes of washing solution (0.15M NaCl, 0.05% Tween-20) before addition of a fresh 1/5000 dilution of GAR-IgG alkaline phosphatase conjugate (Seravac Laboratories, Cape Town) in blocking buffer. After washing, the nitrocellulose was assayed for phosphatase activity, using a freshly prepared substrate solution. The substrate consists of 0.3mg/ml nitro-blue tetrazolium (NBT) (stock solution of 75mg/ml in 70% dimethylformamide) and 0.2mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (stock solution of 30mg/ml in

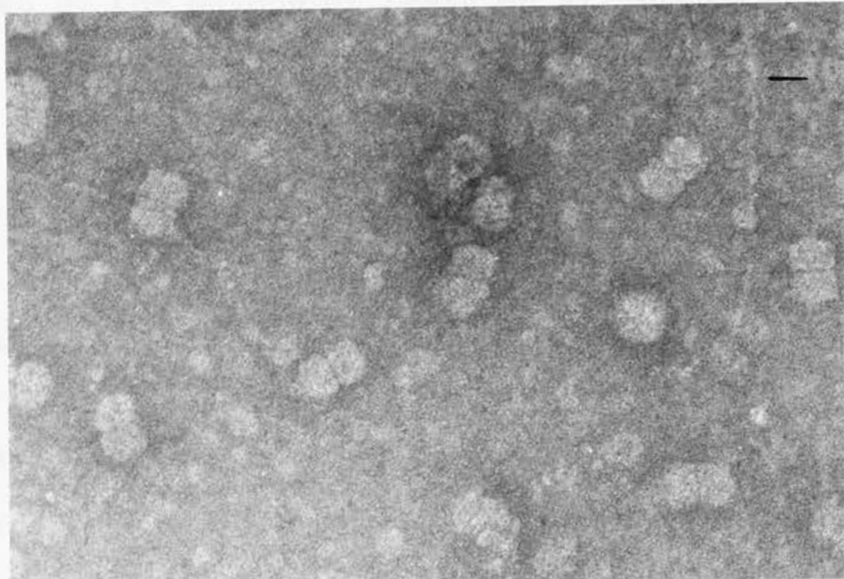
Figure 3.3: An ultraviolet absorption scan of MSV-CT



The virus preparation was diluted 1/100 in sodium phosphate buffer pH 7.5. Purified preparations of MSV-PE and MSV-SWA gave identical spectra.

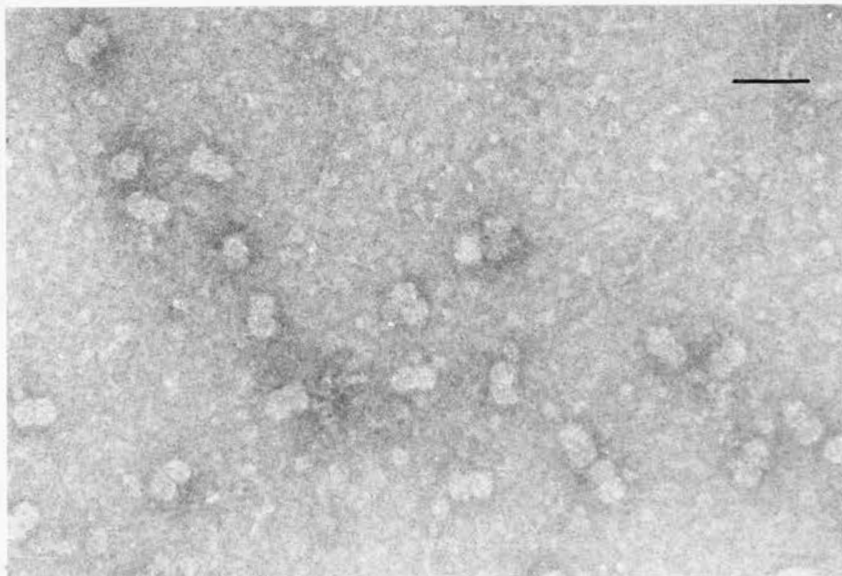
Figure 3.4: Electron micrographs of (a) purified MSV-CT particles, and (b) purified DGV particles.

(a)



The specimen was negatively stained with uranyl acetate and the magnification was 85 000 x. Bar = 10nm.

(b)



The specimen was negatively stained with uranyl acetate and the magnification was 50 000 x. Bar = 50nm.

dimethylformamide) in 100mM Tris-HCl buffer pH 9.5, containing 100mM NaCl and 5mM $MgCl_2$. The substrate solutions were prepared by the method of Leary et al. (1983).

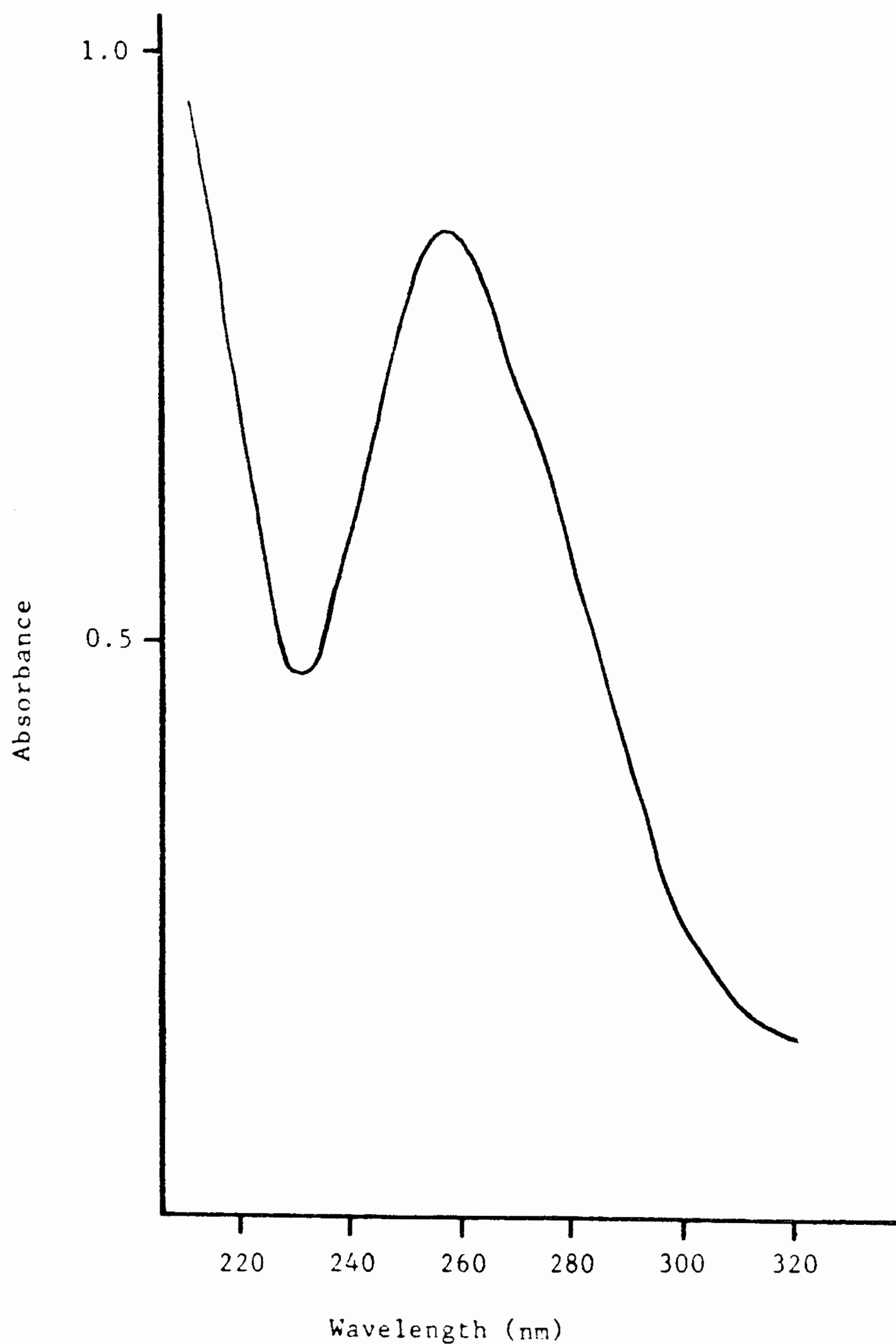
3.3 RESULTS

3.3.1 Purification of viruses

MSV-CT, MSV-PE and MSV-SWA were normally purified from approximately 100g of freshly harvested infected material or from material that had been stored at $4^{o}C$ for no longer than one week. Yields of virus were ordinarily 1-5mg/kg of leaves. Purified preparations of the MSV isolates gave similar UV absorption spectra in the range 220-320nm. The spectrum obtained for MSV-CT is shown in figure 3.3.

Preparations of the three MSV isolates were subjected to electron microscopy to establish whether the characteristic geminate particles were present. Samples were adsorbed to carbon-coated grids and stained with 2% (w/v) aqueous solution of uranyl acetate at pH 5.0 (von Wechmar & Milne, 1982). The grids were viewed in a Zeiss EM109 transmission electron microscope. Preparations of all three isolates contained geminate particles, with no other virus-like particles visible (Fig. 3.4). It was also impossible to distinguish between the geminate particles of the three

Figure 3.5: An ultraviolet absorption scan of DGV



The virus preparation was diluted 1/10 in potassium phosphate buffer pH 7.0

isolates. Furthermore, only paired particles were observed and no singlet particles as had been reported previously by Bock et al. (1974) and Howell (1984). These results are in agreement with published reports that maize streak disease is caused by a single virus and not by a mixed infection (Bennett, 1967; Bock, 1974; Rose, 1978).

DGV could be isolated only at very low yield by the method of von Wechmar and Milne (1982); however, the method devised by Dollet et al. (1986) was more successful. As this virus has no known vector, and is propagated vegetatively, only small amounts of infected material could be processed at a time. This was usually in the region of 10-20g, resulting in a purified virus yield of approximately 0.5-1.5mg/kg leaves. A UV absorption spectrum in the range 220-320nm was determined (Fig. 3.5), for each purification. This spectrum differed from that obtained for the MSV isolates (Fig. 3.3). Electron microscopy of DGV also revealed characteristic geminate particles (Fig. 3.4). Once again these particles could not be distinguished from those of MSV.

3.3.2 Transmission of viruses

Attempts to transmit DGV using the leafhopper *Cicadulina mbila* proved unsuccessful. Transmission experiments were conducted using batches of 20 leafhoppers, both adults and nymphs, that had been cycled over a number of uninfected

maize plants to ensure that they were not harbouring any virus. The leafhoppers were allowed to feed on infected Digitaria sanguinalis plants for a number of different acquisition periods - of 1-2 hours, 2-4 hours and 5-10 hours - before being transferred to healthy maize seedlings. Although the experiments were repeated several times no symptoms appeared on the maize seedlings, and no virus was detected in them in indirect-ELISA tests (results not shown). In order to determine if this lack of transmission was due to the leafhoppers themselves not acquiring the virus, individual insects were allowed different acquisition periods. They were then crushed in potassium phosphate buffer pH 7.0, and tested by means of indirect-ELISA. Positive results were obtained for all samples (results not shown), with no correlation possible between the amount of virus obtained and the different acquisition times. In contrast, the three MSV isolates could be transmitted by C. mbila from infected maize plants, to healthy Digitaria sanguinalis plants. Furthermore, it was possible to transmit these viruses from the infected D. sanguinalis plants back into healthy maize seedlings. As healthy D. sanguinalis plants were grown from seed collected from DGV infected plants, this indicates that like MSV (Storey, 1925; Rose, 1978; Bock et al., 1974, Goodman, 1981a) DGV is not transmitted by seed to progeny plants. Attempts were also

made to transmit DGV mechanically by rubbing purified virus (0.1-0.5mg/ml) onto the leaves of healthy D. sanguinalis plants. This also proved unsuccessful.

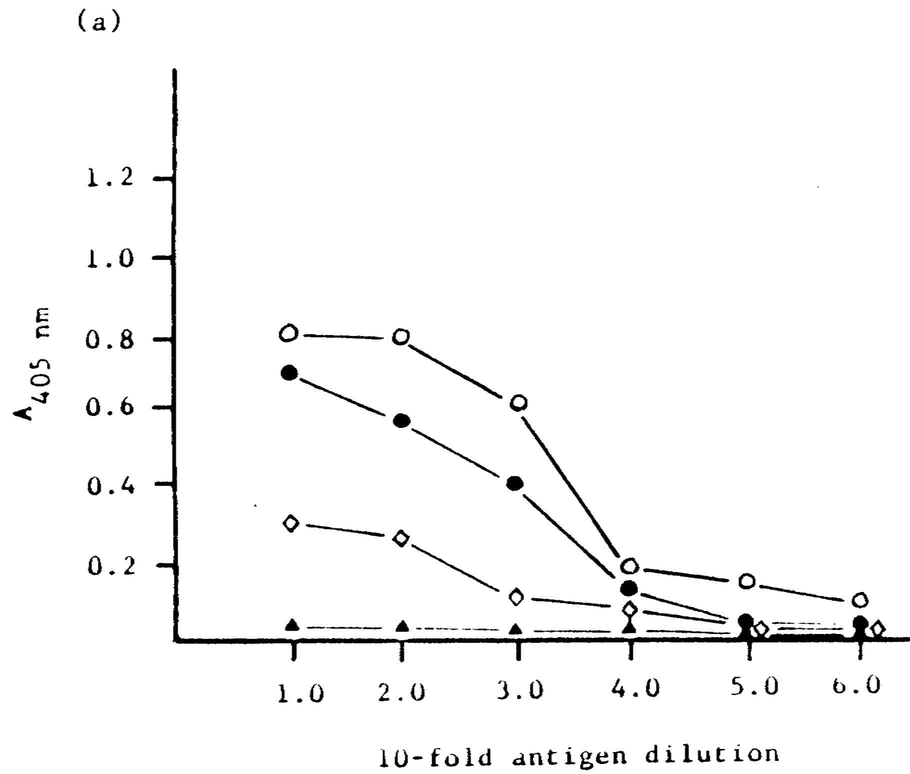
3.3.3 Serological studies

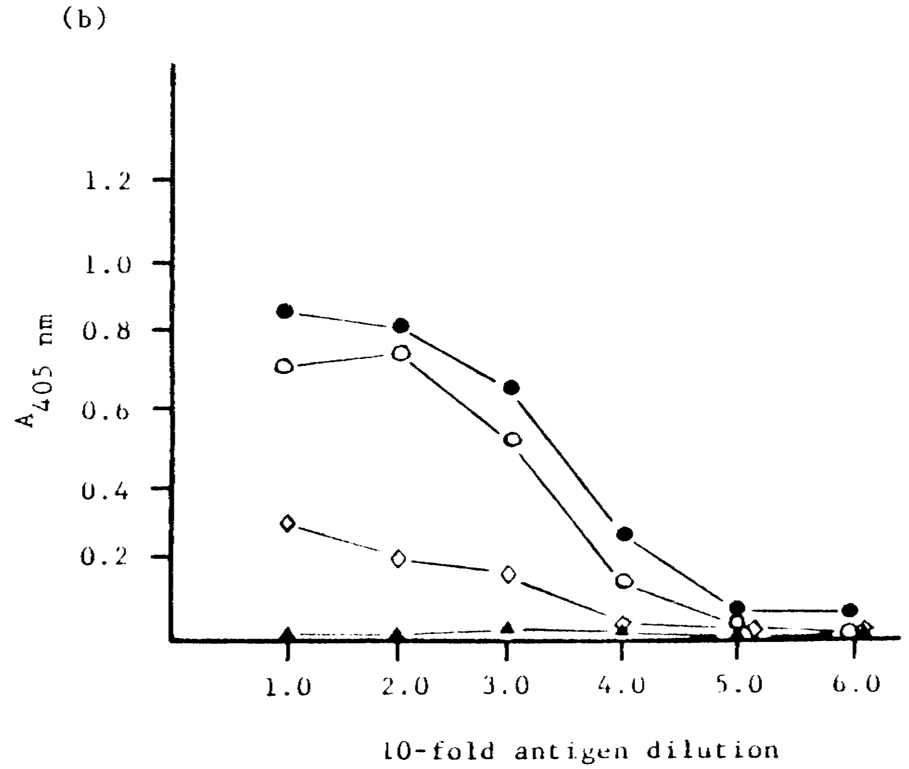
3.3.3.1 Enzyme-linked immunosorbent assays

The titres of antisera raised to MSV-CT and MSV-PE were determined using indirect-ELISA. Those bleedings which showed the highest titres were pooled, and host-absorbed for IgG preparation and conjugation with alkaline phosphatase. Thereafter, the optimal concentrations of the immunoglobulin and conjugate were determined using DAS-ELISA.

DAS-ELISA tests were performed in an attempt to ascertain the serological relatedness between (i) the three Southern African isolates of MSV, and (ii) between MSV-CT and DGV and between MSV-PE and DGV. Two series of tests were performed, one employing the MSV-CT specific immunoglobulin and conjugate at dilutions of 1/500 and 1/700, respectively, and the second using immunoglobulin (1/600 dilution) and conjugate (1/800 dilution) raised against MSV-PE. In both instances, purified preparations of all four virus isolates were used, as well as purified brome mosaic virus (BMV) (obtained from Departmental stocks) as a negative control. Figure 3.6 shows the results obtained. MSV-CT and MSV-PE

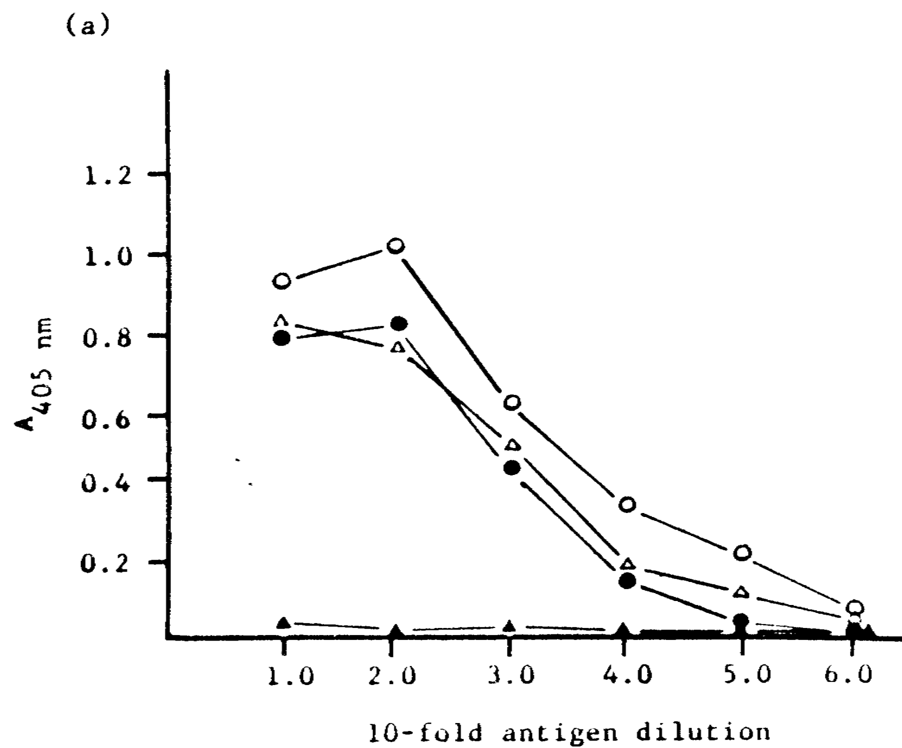
Figure 3.6: Reaction of DGV with (a) MSV-CT antiserum and (b) MSV-PE antiserum

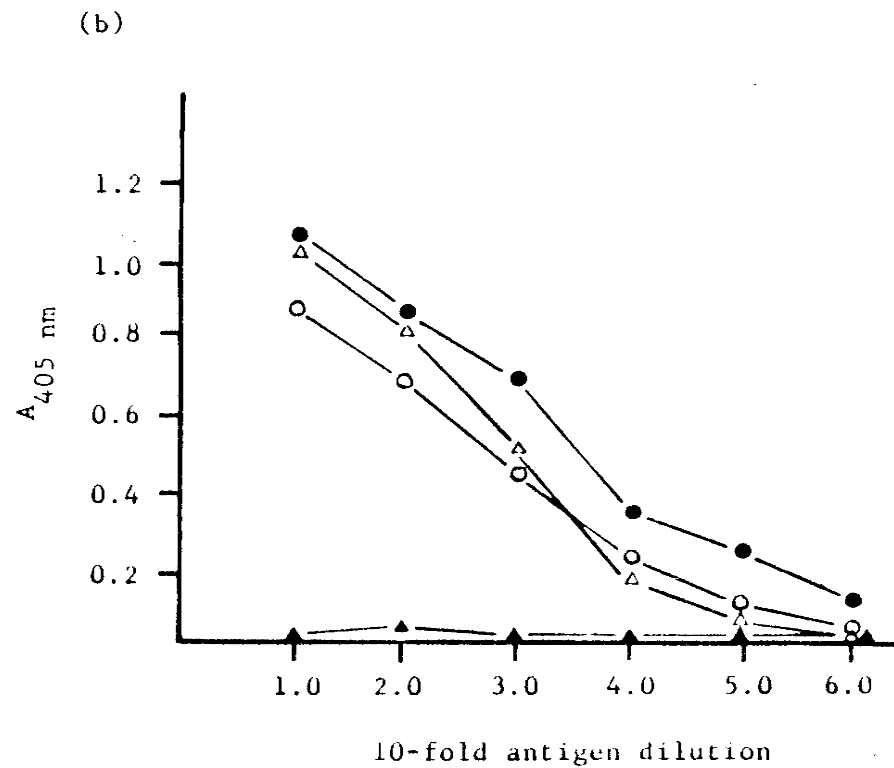




○ MSV-CT
● MSV-PE
◇ DGV
▲ BMV

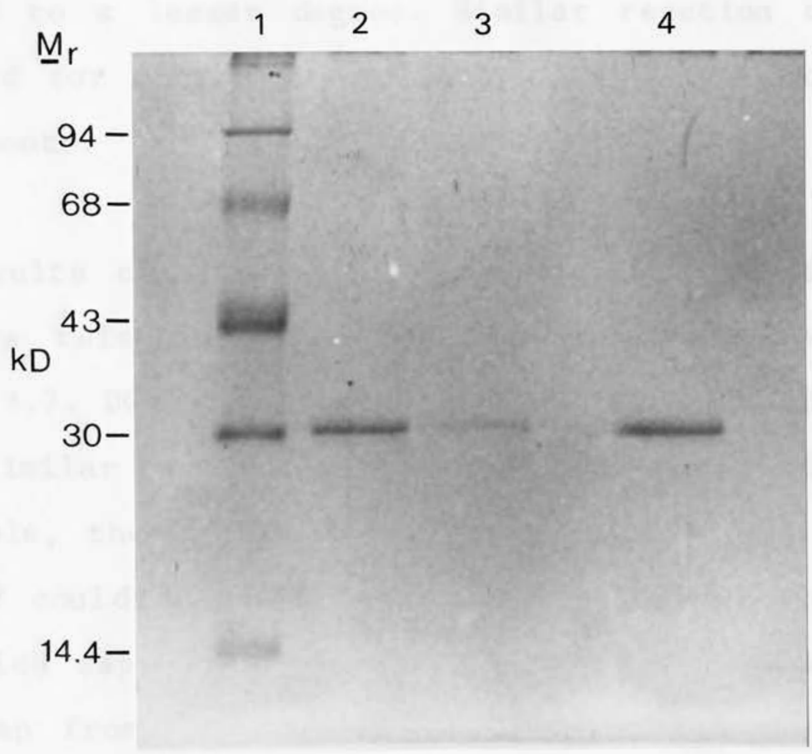
Figure 3.7: Reaction of MSV-CT, MSV-PE and MSV-SWA with (a) MSV-CT antiserum and (b) MSV-PE antiserum





○ MSV-CT
● MSV-PE
△ MSV-SWA
▲ BMV

Figure 3.8: SDS-PAGE of purified preparations of different MSV isolates.



Lane 1: Protein M_r marker (Pharmacia) - phosphorylase B 94kD, bovine serum albumin 67kD, ovalbumin 43kD, carbonic anhydrase 30kD, lactalbumin 14.4kD.

Lane 2: MSV-CT

Lane 3: MSV-PE

Lane 4: MSV-SWA

each showed the strongest reaction with their respective homologous antisera. The other two isolates, in each case, reacted to a lesser degree. Similar reaction curves were obtained for the three isolates in a number of repeated experiments.

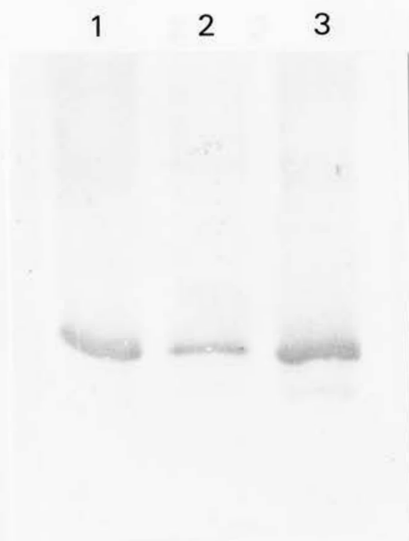
The results obtained for the reaction of purified DGV to antisera raised against MSV-CT and MSV-PE are shown in figure 3.7. DGV appears to react weakly with both antisera, to a similar extent. As antisera against MSV-SWA was not available, the serological relationship between this virus and DGV could not be determined. DAS-ELISA tests were also done with sap containing MSV-CT, MSV-PE, MSV-SWA and DGV, with sap from healthy maize and *Digitaria sanguinalis* as negative controls. The results obtained with both antisera compared favourably with those obtained in the assays with purified virus (results not shown).

3.3.3.2 SDS-PAGE and Western blotting

The capsid proteins of MSV-CT, MSV-PE and MSV-SWA each gave a single band in SDS-polyacrylamide gels, and were determined to have molecular weights in the region of 3.0×10^4 daltons (Fig. 3.8). In immunoelectroblots antisera to both MSV-CT and MSV-PE reacted with the proteins of all

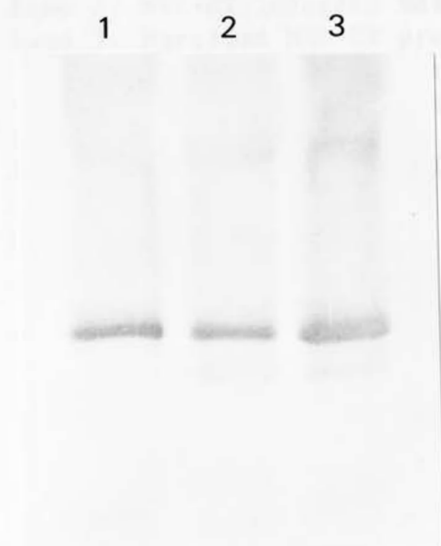
Figure 3.9: Western blot of purified preparations of different MSV isolates. (a) blot probed with MSV-CT antiserum and (b) blot probed with MSV-PE antiserum.

(a)



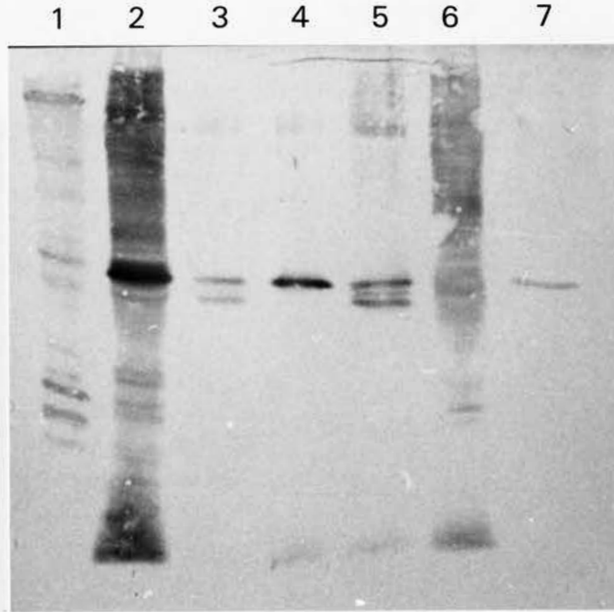
Lane 1: MSV-SWA
Lane 2: MSV-PE
Lane 3: MSV-CT

(b)



Lane 1: MSV-SWA
Lane 2: MSV-PE
Lane 3: MSV-CT

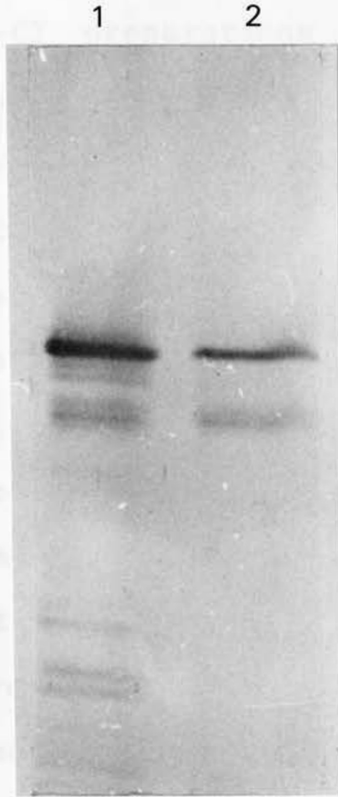
Figure 3.10: Western blot of purified MSV-CT proteolysis products, probed with MSV-CT antiserum.



Lane 1: Viruliferous leafhoppers crushed in protein dissociation mix (see 3.2.6.1)
Lane 2: MSV-CT infected maize sap
Lane 3: Purified MSV-CT preparation stored at $4^{o}C$ for 6 weeks
Lane 4: Freshly purified preparation of MSV-CT
Lane 5: Purified MSV-CT preparation stored at $4^{o}C$ for 4 weeks
Lane 6: Healthy maize sap
Lane 7: Protein M_r marker (Pharmacia) - only the 30kD band is visible

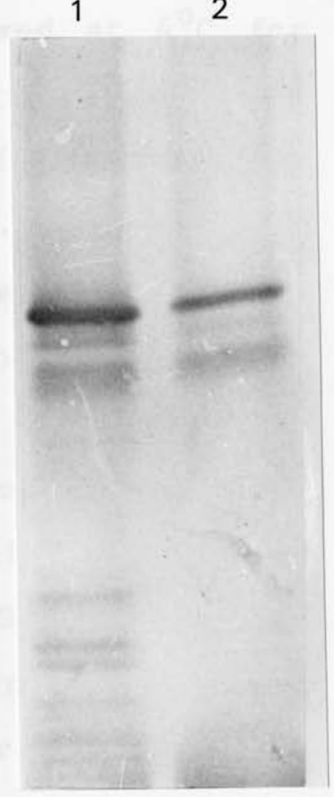
Figure 3.11: Western blot of purified MSV-CT and purified MSV-PE proteolysis products probed with (a) MSV-CT antiserum and (b) MSV-PE antiserum.

(a)



Lane 1: MSV-CT
Lane 2: MSV-PE

(b)



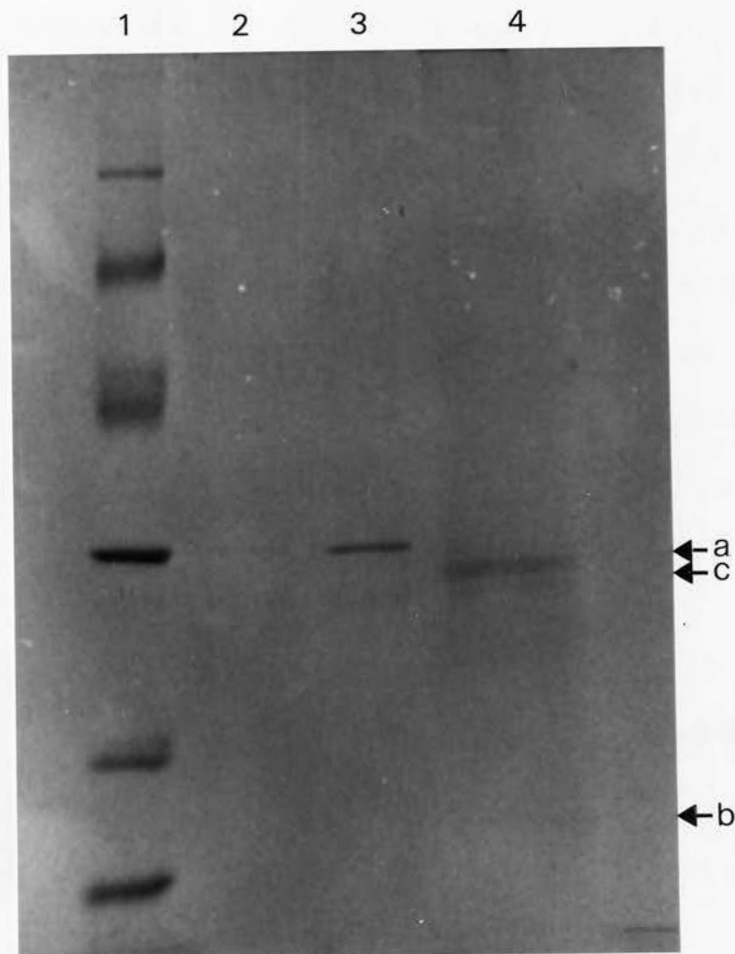
Lane 1: MSV-CT
Lane 2: MSV-PE

three isolates (Fig. 3.9), and no distinction could be made between the isolates on the basis of strength of the reaction.

If MSV-CT preparations that had been stored at $4^{o}C$ for prolonged periods of time were electrophoresed and electroblotted, additional protein bands of lower M_r than the coat protein were often observed. In purified preparations stored for 4 and 6 weeks, two additional bands and one additional band were observed, respectively. The 6 week old preparation was less concentrated, and as a result the second additional protein species was not visible. That these bands were derived from the coat protein, was verified by Western blotting. Antiserum raised to MSV-CT recognised all three species (Fig. 3.10). The final degradation product appeared to have a molecular weight of approximately 2.8 x 10^4 daltons, determined by SDS-PAGE. The incidence and approximate size of these additional bands remained constant in a number of repeated experiments. Degradation products of a similar size and number were also observed for MSV-PE. The apparently proteolysed proteins of both MSV-CT and MSV-PE were recognised by antisera raised to both viruses (Fig. 3.11).

SDS-PAGE of purified DGV protein revealed four bands, with a major doublet of 2.5 x 10^4 and 2.7 x 10^4 daltons, respectively (Fig. 3.12). This doublet, along with a third band of approximately 1.9 x 10^4 daltons, appeared to

Figure 3.12: SDS-PAGE of purified DGV.



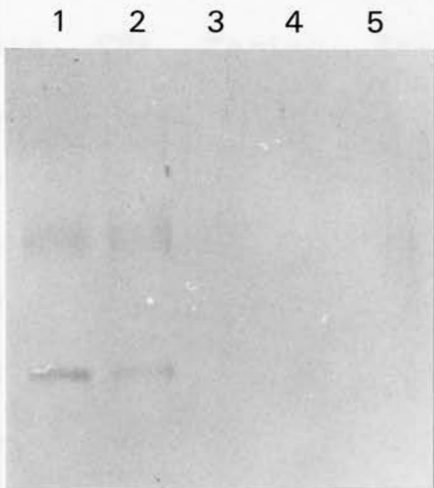
Lane 1: Protein M_r marker (pharmacia) - phosphorylase B 94kD, bovine serum albumin 67kD, ovalbumin 43kD, carbonic anhydrase 30kD, soybean trypsin inhibitor 20.1kD, lactalbumin 14.4kD.

Lane 2: Purified MSV-CT

Lane 3: Purified MSV-PE

Lane 4: DGV - (a) doublet, (b) approximately 19kD protein, and (c) possible host protein

Figure 3.13: Western blot of purified DGV probed with DGV antiserum.



Lanes 1 and 2: Different preparations of DGV
Lane 3: Purified MSV-CT
Lane 4: Purified MSV-PE
Lane 5: Purified MSV-SWA

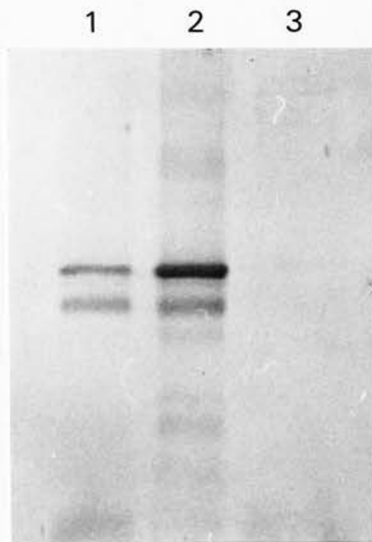
correspond to virion proteins, and the other band to host protein. This was apparent from Western blots with DGV antisera (obtained from Dr G. Boccardo, Instituto di Fitovirologia Applicata, CNR, Torino, Italy), which only recognised these three protein bands (Fig. 3.13). Antiserum against MSV-PE reacted weakly with the DGV doublet protein band, but not with the approximately 1.9kD band (Fig. 3.14). Antisera against MSV-CT showed no reaction with DGV in Western blots (Fig. 3.14), nor did DGV antisera react with MSV-CT, MSV-PE or MSV-SWA in Western blots (Fig. 3.13).

3.4 DISCUSSION

The three isolates of MSV - MSV-CT, MSV-PE and MSV-SWA - were shown both by DAS-ELISA and Western blotting, using antisera raised to MSV-CT and MSV-PE, to be closely related but not identical. In the DAS-ELISA tests MSV-PE and MSV-SWA appeared to react to a similar degree with each antiserum. This suggests that the isolates could be grouped into a tentative serological map (Fig. 3.15), with MSV-PE and MSV-SWA appearing to be more closely related to each other than to MSV-CT. The linear distances (x, y and z in figure 3.15) between the various isolates are rough approximations to "antigenic distances" and not proportional distances. Furthermore, these serological differences agree with the different symptoms produced by each isolate in infected

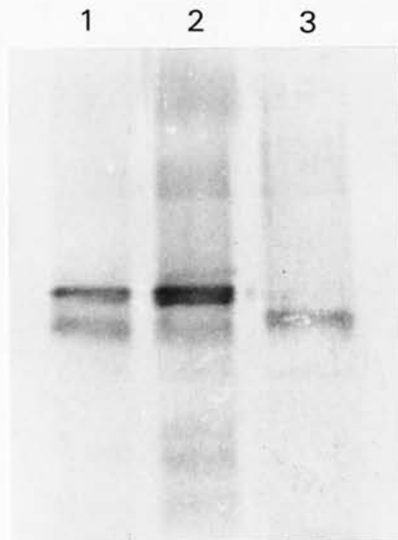
Figure 3.14: Western blot of purified preparations of DGV and different MSV isolates. (a) blot probed with MSV-CT antiserum and (b) blot probed with MSV-PE antiserum.

(a)



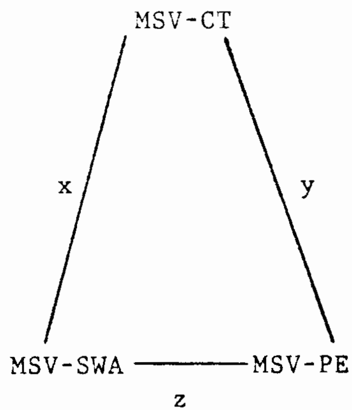
Lane 1: MSV-CT
Lane 2: MSV-PE
Lane 3: DGV

(b)



Lane 1: MSV-CT
Lane 2: MSV-PE
Lane 3: DGV

Figure 3.15: A tentative serological map of the MSV isolates studied in this thesis.



Distances x,y   z; x is not necessarily = y, and is probably   y.

maize plants (Fig 3.1). It therefore seems possible to distinguish between different isolates on the basis of their symptomatology.

DGV reacted weakly with antisera against MSV-CT and MSV-PE in DAS-ELISA tests. This was not the case in immunoelectroblots, however, where no reaction was observed between DGV and MSV-CT antiserum. The slight reaction observed between this virus and MSV-PE antiserum could be explained by these two viruses having an antigenic site in common, which is not present in MSV-CT. The observation that DGV antiserum does not react with MSV-PE could be due to this antiserum having a low titre, and a lesser affinity for MSV-PE. It could also be assumed that *Digitaria sanguinalis* proteins in the DGV preparations were responsible for some of the apparent cross-reaction observed between this virus and MSV-PE and MSV-CT antisera in DAS-ELISA tests.

The size and number of *Digitaria* geminivirus proteins observed in both polyacrylamide gels and immunoelectroblots are not in agreement with results published for this virus by Dollet *et al.* (1986). Comparison of the gel published by them and the gels run in this laboratory revealed a difference in resolution, which may account for the discrepancy in results. They found that DGV protein formed a single band in polyacrylamide gels that corresponded to a molecular weight of approximately 2.7 x 10^4 daltons. In this

laboratory three protein bands were identified for DGV by Western blotting with DGV antiserum. Two of these form a doublet of 2.5×10^4 and 2.7×10^4 daltons, respectively, and the third was determined to be approximately 1.9×10^4 daltons in size. It is thought that this third protein band may be a degradation product of the larger doublet. In addition, Dollet et al. (1986) compared DGV serologically with MSV by means of Ouchterlony gel double-diffusion tests, and did not perform ELISA or immunoelectroblot tests. Consequently they report a higher degree of serological relatedness between the two viruses, than was found in this study.

The apparent proteolysis of the coat protein of MSV-CT and MSV-PE after prolonged storage could be due to the presence of plant proteases in the purified virus preparations. That this was only observed after a period of time could be as a result of the gradual degradation of the virion protein subunits which might expose additional sites for protease digestion.

No attempts were made to transmit any of the MSV isolates mechanically, as it has been reported extensively in the literature that mechanical inoculation of the virus is unsuccessful (Rose, 1978; Goodman, 1981b; Hull & Davies,

1983). Infected maize plants were, therefore, obtained by exposure of healthy plants to viruliferous leafhoppers for specific time intervals.

At present DGV is being regarded as a strain of MSV. This may prove to be incorrect, however, for if the vector (so far unknown) is found to differ from that of MSV, then this virus may need to be classified as a distinct geminivirus. Serological evidence suggests that the literature reports of relatationships between DGV and MSV are questionable, as in this study antiserum to DGV did not react with any of the MSV isolates. Furthermore, only a weak reaction was observed between DGV protein and MSV-PE antiserum in Western blots.

CHAPTER 4

CLONING AND RESTRICTION ENDONUCLEASE MAPPING OF MAIZE STREAK VIRUS ISOLATES

4.1 INTRODUCTION

The serological differences between the MSV isolates MSV-CT, MSV-PE and MSV-SWA (Chapter 3), indicated that the viruses were similar, but distinct from one another. It is postulated that less than 5% of the genomic information of a plant virus is involved in the antigenicity of its coat protein (Hull, 1986). For this reason, it was of interest to see if these serological differences could be correlated with genomic sequence differences.

It was reported by Ikegami *et al.* (1981) that double-stranded replicative form (RF) virion DNA could be isolated from plants infected with BGMV. Furthermore, a low proportion of this DNA was in a covalently closed, circular form. Covalently closed circular DNA can be separated from linear and nicked open circular DNA, as it bands at a higher density in caesium chloride gradients containing an intercalating agent (Maniatis *et al.*, 1982). On the basis of the above, attempts were made to isolate double-stranded RF

DNA from maize infected with MSV-CT, MSV-PE and MSV-SWA and to purify the covalently closed circular forms by centrifugation to equilibrium in caesium chloride-ethidium bromide gradients. This procedure was successful in the case of all three MSV isolates.

After preliminary characterisation, DNA from MSV-CT and MSV-PE was cloned into the *Escherichia coli* positive selection vector plasmid pEcoR251 (M. Zabeau, Plant Genetic Systems, Gent, Belgium) (Fig. 4.1). This vector contains the ampicillin resistance gene and the origin of replication of pBR322, the lambda phage P_r promoter, and the *E. coli* *Eco*RI gene. Construction is such that the *Eco*RI gene is placed under control of the P_r promoter. Transformation to a strain not lysogenic for lambda and methylation minus, results in overproduction of *Eco*RI, which results in cell death. Positive selection is therefore achieved by insertional inactivation of the *Eco*RI gene.

Cloned DNA as well as RF-DNA from MSV-CT and MSV-PE was used for the construction of restriction endonuclease cleavage maps (Maniatis *et al.*, 1982), whereas RF-DNA only from MSV-SWA was used for restriction mapping. These maps were then compared with each other as well as with those generated by computer from the sequenced genomes of MSV-N and MSV-K (Mullineaux *et al.*, 1984; Howell, 1984).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of plasmid

The following methods were used for preparation of pEcoR251 DNA and recombinant plasmid DNA.

4.2.1.1 Small Scale ("miniprep") extraction

Plasmid DNA was extracted from 5ml of overnight culture in Luria broth by the method of Ish-Horowicz and Burke (1981). The cells were harvested by centrifugation and resuspended in 100ul Solution 1 (0.5M glucose, 0.25M Tris/HCl pH 8.0, 0.1M EDTA) followed by incubation at room temperature. After addition of 200ul Solution 2 (0.2N NaOH, 1% [w/v] SDS) and incubation on ice, 150ul pre-cooled Solution 3 (5M potassium acetate pH 4.8) was added. Plasmid DNA was precipitated by addition of an equal volume of isopropanol, and pelleted by centrifugation in an Eppendorf type 4512 microcentrifuge. Purified DNA samples were resuspended in TE buffer pH 8.0 (10mM Tris/HCl, 1mM EDTA) and stored at -20°C.

4.2.1.2 Large scale ("maxiprep") extraction

The method employed for plasmid isolation was that of Ish-Horowicz and Burke (1981) as scaled up for larger volumes.

Plasmid DNA was prepared from a 1 litre overnight culture in Luria broth grown at 37°C in the presence of 100ug/ml ampicillin. The DNA pellet after isopropanol precipitation was resuspended in TE buffer pH 8.0, to which caesium chloride (CsCl) and ethidium bromide (EtBr) were added. The final density of the CsCl solution was $1.55g/cm^3$ ($\underline{n}$ = 1.396) and the concentration of EtBr approximately 600ug/ml. The preparation was centrifuged at 15°C for 16 hours (at 175 000 x g) in a Beckman type VTi65 rotor, and the plasmid DNA was recovered by syringe as described by Maniatis et al. (1982). The EtBr was removed from the DNA preparations by extraction with an equal volume of NaCl-saturated isopropanol. The solution was diluted with 2 volumes of TE buffer pH 8.0 (see 4.2.1.1), and the DNA precipitated by addition of 1 volume of isopropanol. After pelleting by centrifugation for 10 minutes in an Eppendorf microfuge, the DNA was resuspended in TE buffer pH 8.0. The DNA concentration was assayed spectrophotometrically, assuming an absorption coefficient ($A_{260nm/0.1\%}$, see 3.2.3.3) of 20.

4.2.2 Extraction of total DNA from MSV infected maize plants

Infected plant material was ground to a fine powder in liquid nitrogen, suspended in grinding buffer (0.1M Tris/HCl pH 7.0, 0.1M NaCl, 0.1M EDTA, 1% [w/v] SDS) and stirred for 5 minutes. After filtration through a single layer of

cheesecloth, the filtrate was centrifuged for 20 minutes at 12 000 x g. The sample was extracted with a 1:1 mix of phenol:chloroform (8:2 v/v) at least three times, and isopropanol-precipitated at room temperature for 5 minutes. After centrifugation for 15 minutes at 27 000 x g, the pellet was resuspended in TE buffer pH 8.0 (see 4.2.1.1), and isopropanol precipitated a second time. After pelleting, the DNA was stored in TE buffer pH 8.0 at -20°C.

4.2.3 Isolation of the double-stranded replicative form DNA of MSV from infected maize plant tissue

Total DNA was isolated from healthy and infected *Zea mays* (cv. PNR 493) plants by a modification of the method of Ikegami *et al.* (1981).

Infected plant material was frozen in liquid nitrogen, ground to a powder, suspended in grinding buffer (see 4.2.2), and stirred for 5 minutes. The homogenate was filtered through a single layer of cheesecloth, and the filtrate was centrifuged for 20 minutes at 12 000 x g. RNase A (Sigma Chemical Company) (100ug/ml) was added to the supernatant, which was then incubated at 60°C for 15 minutes. This was followed by the addition of Proteinase K (Merck) (100ug/ml) and incubation at 37°C for 10 minutes. The sample was extracted with a 1:1 mix (v/v) of

phenol:chloroform (4:1 v/v) at least three times, and the aqueous phase isopropanol-precipitated (1:1 v/v) at room temperature for 5 minutes. After centrifugation for 15 minutes at 27 000 x g, the pellet was resuspended in TE buffer pH 8.0 (see 4.2.1.1), to which CsCl (final density of solution 1.55g/ml) and EtBr (approximately 200ug/ml) were added. The preparation was ultracentrifuged, and the DNA recovered as described in 4.2.1.2.

4.2.4 Restriction endonuclease digestion

Restriction enzyme digestions of DNA were performed using either high, medium or low salt "restriction buffers" (as described by Maniatis et al., 1982), according to the salt preference of the particular enzyme. Double and triple digests could be carried out simultaneously if the salt and temperature requirements of the enzyme were compatible. If the requirements were dissimilar, digestions were carried out sequentially, using the enzyme with the lowest salt optimum and the highest temperature tolerance first. The salt concentrations and incubation temperatures were then adjusted for the enzymes following in the sequence. Digestion volumes were normally 20ul, with 1 unit of restriction enzyme per lug of DNA. Digestion times were kept to 1-2 hours, to minimize the chance of non-specific degradation. If the digestion products were to be analysed

electrophoretically, the reaction was terminated by the addition of 1/10 volume of DNA sample buffer (0.3M NaOH, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue). If the sample was to be used for subsequent enzyme reactions, the reaction was stopped by extraction with TE-saturated phenol pH 8.0, followed by extraction with water-saturated ether. An equal volume of isopropanol was then added; the DNA was collected by centrifugation in an Eppendorf microfuge, and resuspended in TE buffer pH 8.0 (see 4.2.1.1).

4.2.5 DNA electrophoresis in agarose gels

Electrophoresis of DNA was performed using a horizontal slab gel system with Tris/borate buffer pH 8.3 (TBE: 89mM Tris base, 89mM boric acid, 2mM EDTA), and Sigma Type I agarose. The percentage agarose varied from 0.7% to 1.5% depending on the sizes of the DNA fragments being analysed. Electrophoresis was carried out at 2V/cm overnight or 5V/cm for 4-6 hours. After staining with EtBr (0.5ug/ml buffer) for 15 minutes, the DNA bands were visualised by fluorescence on a 254nm UV transilluminator, and bands were photographed using a Polaroid CU-5 Land Camera and Polaroid Land Pack 667 film. The size of DNA fragments was calculated from the calibration curve obtained from the plot of migration of a series of standard fragments against the log of their molecular weights. DNA standards used were lambda

phage DNA (obtained from Departmental stocks) digested with *EcoRI/Hind* III, *Bgl* I, or *Pst* I. The amount of DNA loaded per lane was in the order of 80-100ng for every fragment expected from digests of lambda or plasmid DNA.

4.2.6 Construction of recombinant plasmids

4.2.6.1 Preparation of vector DNA

The vector pEcoR251 was prepared by digestion of approximately 10ug of the plasmid with 10 units of *Bgl* II, for 1.5 hours at 37°C. The reaction was terminated by phenol extraction and isopropanol precipitation (see 4.2.1.1). The DNA was resuspended in TE buffer pH 8.0 (see 4.2.1.1) at a concentration of approximately 250ng/ul.

4.2.6.2 Preparation of insert DNA

Double-stranded, replicative form DNA (approximately 5ug) isolated from MSV-CT and MSV-PE infected plants (4.2.3) was digested with 5 units of *Bgl* II for 1.5 hours at 37°C. The DNA was then phenol extracted and isopropanol precipitated (see 4.2.1.1) and resuspended in TE buffer pH 8.0 (see 4.2.1.1) at an approximate concentration of 100ng/ul.

4.2.6.3 Ligation of vector and insert

An optimal vector : insert ratio for this DNA combination was found to be 1:1 (mole:mole) (H. Zappe, personal communication), and the optimal concentration of the DNA in the ligation mix was found to be 0.25pM. The ligation reactions were carried out in sterile microfuge tubes to which the DNA was added, followed by 1/10 volume ligation buffer (200mM Tris/HCl pH 7.6, 100mM $MgCl_2$, 100mM DTT, 6mM ATP). The volume of the ligation mix was adjusted to 50ul with sterile distilled water and 0.25 units (approximately 1ul) of T4 DNA ligase was added. Ligation was allowed to proceed at room temperature for 2-3 hours. Thereafter, 10ul phenol was added to the 50ul reaction mix and the volume adjusted to 180ul with sterile distilled water. A futher 20ul of 5M $NaClO_4$ was added, and the contents of the tube mixed by vortexing. An equal volume (200ul) of chloroform was added and after vortexing the mixture was centrifuged in an Eppendorf microfuge for 1 minute. After addition of lug tRNA to the aqueous phase the DNA was isopropanol precipitated and pelleted in an Eppendorf microfuge for 15 minutes. The DNA was resuspended in 50ul of TE buffer pH 8.0 (see 4.2.1.1).

4.2.7 Preparation of Escherichia coli HB101 competent cells

E. coli strain HB101 has the following genotype: F^-, *hsdS20* (r_B^- m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rspL20* (Sm^r), *xyl-5*, *mtl-1*, *supe44*, λ^- (Maniatis *et al.*, 1982).

Competent cells of *E. coli* HB101 were prepared by an adaptation of the method of Mandel and Higa (1970).

A 1/100 dilution of a fresh overnight culture of HB101 in Luria broth was made into fresh Luria broth and the culture incubated at 37°C with constant agitation, until an OD_{600} = 0.2 was reached. This culture was then diluted 1/50 to a 400ml main culture and once more incubated at 37°C with constant agitation until an OD_{600} = 0.2 was obtained. Both broths were prewarmed at 37°C and glucose was added to a final concentration of 0.1% (w/v). After cooling the culture on ice for 5 minutes, the cells were pelleted by centrifugation at 4 000 x *g* for 5 minutes. Cell pellets were resuspended in 1/2 the final growth volume (200ml) ice cold 100mM $CaCl_2$ and the cells kept on ice for 20 minutes. Thereafter, the cells were harvested by centrifugation at 2500 x *g* for 5 minutes and resuspended in 1/100 the final growth volume (4ml) 100mM $CaCl_2$ and 15% glycerol (v/v). These cells were stored on ice, at 4°C, for 16 hours

(overnight) to improve their competency. If the cells were not used immediately they were flash-frozen in liquid nitrogen and stored at -70°C for later use.

4.2.8 Transformation of E.coli HB101 competent cells

Transformation of the competent cells was performed as follows. After ligation of the vector and insert DNAs (see 4.2.6.3), 20 microfuge tubes were set up, each containing, 2ul ligation mix and 100ul competent cells. After 10 minutes on ice the cells were induced to take up DNA by heat-shocking at 42°C for 5 minutes. One ml of Luria broth was added to each of the 20 tubes and the cells kept at 42°C for a further 30 minutes in order for the transformed cells to express the gene for ampicillin resistance. Five such expression mixes were pooled and added to 95ml prewarmed (42°C) Luria broth in a 500ml flask, containing ampicillin at 100ug/ml. These cultures were kept in a waterbath at 42°C for 1 hour with vigorous shaking. The cells were harvested by centrifugation at 4 000 x g for 5 minutes. After resuspending the cells in 4ml Luria broth, the entire suspension was plated (200ul/plate) on Luria agar plates containing 100ug/ml ampicillin. The plates were incubated at 37°C overnight. After checking some colonies for the presence of hybrid-plasmids (see 4.2.1), the transformants

were stored as individual clones in Luria broth and 15% glycerol at -70°C, or as bacterial colonies on Luria agar slants at room temperature.

4.2.9 Membrane hybridisation of DNA

4.2.9.1 "Southern" transfer of DNA from agarose gels to membrane filters

DNA fragments resolved by agarose gel electrophoresis (see 4.2.5) were transferred to nitrocellulose (Schleicher & Schuell, 0.45um pore) or Hybond-N (Amersham International plc) by the method of Southern (1975).

The DNA fragments in the gel were denatured by soaking the gel in an excess volume of 1.5M NaCl and 0.5M NaOH for 1 hour at room temperature with constant agitation. The gel was neutralised by soaking it in an excess volume of 1M Tris/HCl pH 8.0 and 1.5 M NaCl for 1 hour, as above. The gel was placed on a piece of Whatman 3MM filter paper which was in contact with a solution of 10x SSC (1.5M NaCl, 0.15M sodium citrate). A piece of nitrocellulose (or Hybond-N), cut to the exact size of the gel, was soaked in 2x SSC and laid on the gel surface taking care not to trap any air bubbles between the nitocellulose filter and the gel. Two pieces of Whatman 3MM paper were cut to the same size,

soaked in 2x SSC, and placed on top of the nitrocellulose. A 5cm-thick layer of absorbent paper towel was placed on top of the filter paper and the whole stack compressed with a 5kg weight. Transfer was allowed to take place for 16-24 hours.

4.2.9.2 Radioactive labelling of probes

All DNA probes used were labelled to a high specific activity by nick translation (Rigby *et al.*, 1977). The reagents were bought in kit form from Amersham International plc (PB. 5025) and the reaction carried out according to the suppliers instructions, using approximately lug DNA and 50uCi ^{32}P-dCTP (3000 Ci/mM). The reaction mix was incubated at 15°C for 2 hours. When more than 50% of the radioactive nucleotides had been incorporated into the probe DNA (after about 2 hours), the reaction was terminated by addition of 0.5M EDTA to a final concentration of 20mM, and the labelled DNA was eluted in the void volume of a Sephadex G-50 spun column (Maniatis *et al.*, 1982). Specific activities of the order of 5 x 10^7 cpm/ugDNA were routinely obtained. Radioactive probes were stored in lead canisters at -20°C until use. Just prior to use the probe was denatured by heating at 100°C for 10 minutes, followed by flash cooling on ice.

4.2.9.3 Hybridisation

After transfer of the DNA from agarose gels to nitrocellulose (or Hybond-N) filters (4.2.9.1), the filters were baked under vacuum at 80°C for 2 hours. The filters were then wetted in a minimum volume of prehybridisation buffer (5x SSC, 0.5% [w/v] SDS, 10x Denhardt's solution and 200ug/ml denatured salmon sperm DNA), in a heat sealed bag, and prehybridised at 60°C for 4 hours, prior to the addition of denatured labelled probe. This was incubated at 60°C for 16 hours for hybridisation to occur.

Washing of the filters involved use of a series of washes of increasing stringency. The first washes were carried out at 60°C in a solution of 2x SSC (5 minutes), and in a solution of 2x SSC and 0.1% SDS (30 minutes). A subsequent wash was carried out at room temperature in 0.1x SSC and 0.1% SDS for 2 hours with a change of buffer after 30 minutes. The filters were then sealed in plastic bags while still damp, so that they could be washed further after autoradiography if necessary. Plastic bags were taped flat in an X-ray cassette with Fuji X-ray intensifying screens. The autoradiographic film used was Kodak XAR-5. Exposure was usually for 12-16 hours at -70°C. The film was processed using Kodak GBX X-ray developer and fixed according to the manufacturers instructions.

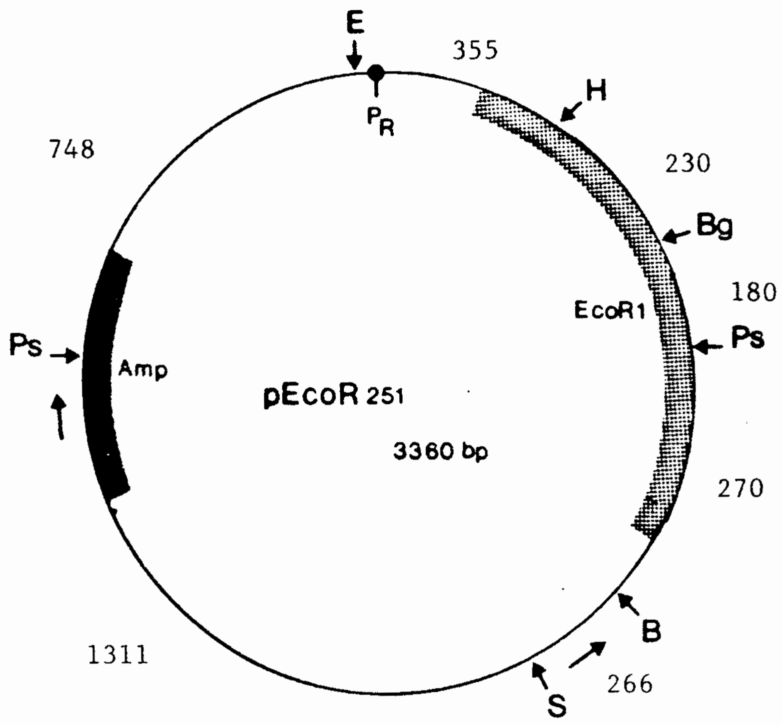
4.3 RESULTS

4.3.1 Construction of recombinant plasmids

Double-stranded RF-DNA was isolated from MSV-CT- and MSV-PE-infected maize plants by passage over CsCl/EtBr gradients, then digested with a number of restriction enzymes in order to determine what unique sites were present for cloning purposes. From published data for MSV-N (Mullineaux *et al.*, 1984) and MSV-K (Howell, 1984), it was expected that there would be a single *Bgl* II site and a single *BamHI* site for each of the MSV isolates. This was confirmed for both isolates by preliminary restriction analyses of RF dsDNA. Furthermore, the MSV DNAs were found to be approximately 2.7kb in size, which is in agreement with published reports (Mullineaux *et al.*, 1984; Howell, 1984). MSV-CT RF-DNA and MSV-PE RF-DNA were restricted with *Bgl* II and cloned into the *Bgl* II site of the positive selection vector pEcoR251, which is approximately 3.36kb in size (Fig. 4.1).

The recombinant plasmids were transformed into *E. coli* HB101 competent cells, and plated on to agar containing 100ug/ml ampicillin. Controls included competent cells with (i) no DNA added, (ii) uncut pBR322 to monitor transformation, and (iii) cut and religated vector to assess ligation efficiencies. All controls were clean, and the

Figure 4.1: **Restriction endonuclease map of the plasmid pEcoR251.**

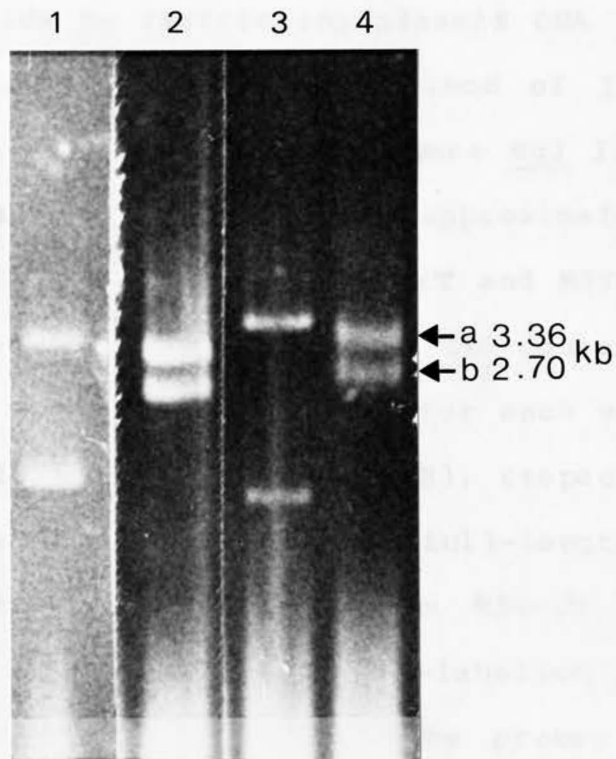


B: BamHI, Bg: Bgl II, E: EcoRI, H: Hind III, S: Sal I.

(H. Zappe, personal communication).

None of the other enzymes used in this study have sites in pEcoR251.

Figure 4.2: Photograph of agarose gel showing restriction fragments of clones pBC100 and pBC200.



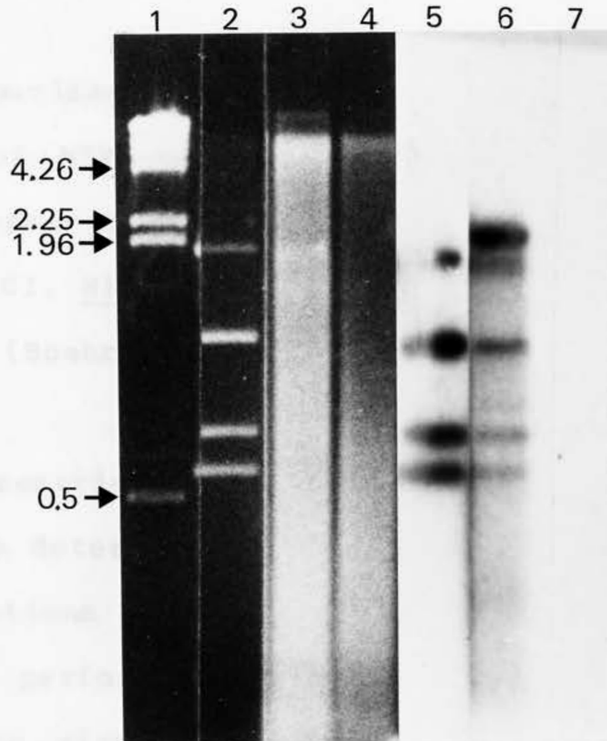
Lane 1: pBC100 BamHI digest
Lane 2: pBC100 Bgl II digest
Lane 3: pBC200 BamHI digest
Lane 4: pBC200 Bgl II digest
(a) Vector pEcoR251 band, (b) insert band, MSV-CT DNA (pBC100) and MSV-PE DNA (pBC200).

transformation frequencies were generally in the order of 5000 transformants per nanogram of uncut vector DNA. A number of colonies were checked for the presence of recombinant plasmids by restricting plasmid DNA isolated by the "miniprep" alkaline hydrolysis method of Ish-Horowicz and Burke (see 4.2.1.1), with the enzymes Bgl II and BamHI (Fig. 4.2). Recombinant plasmids of approximately 6kb in size were identified for both the MSV-CT and MSV-PE cloning experiments. A plasmid that appeared to contain a full-length insert of MSV DNA was selected for each virus: these were named pBC100 (-CT) and pBC200 (-PE), respectively. In order to show that these were in fact full-length clones of the strains, total DNA isolated from MSV-CT and MSV-PE infected plants was probed with ^{32}P-labelled pBC100 and pBC200, respectively. In each case the probes hybridised only with characreristically-discrete RF-DNA fragments and not with the "smeared" plant DNA (result not shown). The success of this experiment led to speculation as to whether it was possible to screen and type field samples thought to be infected with MSV by probing total DNA isolated from these plants with radiolabelled pBC100 or pBC200 DNA. Accordingly, total DNA was extracted from two field-collected samples of maize and restricted with Hind III. The

DNA fragments were separated by electrophoresis on a 1.5% agarose gel, blotted onto nitrocellulose and probed with ^{32}P-labelled pBC200 (Fig. 4.3). From the EtBr-stained gel it was difficult to distinguish the RF-DNA fragments from the smear produced by the plant DNA. On the autoradiograph, however, distinct bands were visible for sample 1 (lane 6) representing hybridisation between the discreet RF-DNA fragments and the pBC200 probe. No hybridisation occurred between the labelled probe and the DNA extracted from sample 2 (lane 7). Although the Hind III restrictions appeared to be partial digestions, in all cases, the fragments produced by digestion of the total DNA of sample 1 match with those produced by digestion of MSV-PE RF-DNA, indicating that the isolates are probably genotypically very similar and that the RF-DNA in the infected plant is probably of one predominant species. The apparent lack of single-stranded DNA on the autoradiograph could be due to the simple DNA extraction procedure used, which does not specifically purify virion DNA. This phenomenon has been noticed by other workers (Grimsley et al., 1987).

RF-DNA isolated from MSV-SWA infected maize plants was not cloned into the vector pEcoR251. It was, however, confirmed

Figure 4.3: Hybridisation of ^{32}P-labelled pBC200 DNA to digests of total DNA extracted from field collected samples thought to be infected with MSV. Lanes 1-4: photograph of agarose gel showing digests of total DNA. Lanes 5-7: autoradiograph of lanes 1-4.



Lane 1: λ DNA cleaved with Hind III generating fragments of 23.72kb, 9.46kb, 6.67kb, 4.26kb, 2.25kb, 1.96kb and 0.5kb.

Lane 2: MSV-PE RF-DNA cleaved with Hind III.

Lane 3: Total DNA of field sample 1 cleaved with Hind III.

Lane 4: Total DNA of field sample 2 cleaved with Hind III.

Lane 5: Autoradiograph of lane 2.

Lane 6: Autoradiograph of lane 3.

Lane 7: Autoradiograph of lane 4.

that, as in the case of MSV-CT and MSV-PE DNAs, MSV-SWA RF-DNA also contained single BamHI and Bgl II restriction endonuclease sites.

4.3.2 Restriction endonuclease mapping of MSV isolates

Restriction endonuclease maps of the MSV inserts in pBC100 and pBC200 and of MSV-SWA RF-DNA were obtained using the following 6 base-pair restriction enzymes: Apa I, Bgl I, Bgl II, Pvu II, Sal GI, Hind III, Sac I, Cla I, Kpn I, Xho I, EcoRI, and Sma I (Boehringer-Mannheim).

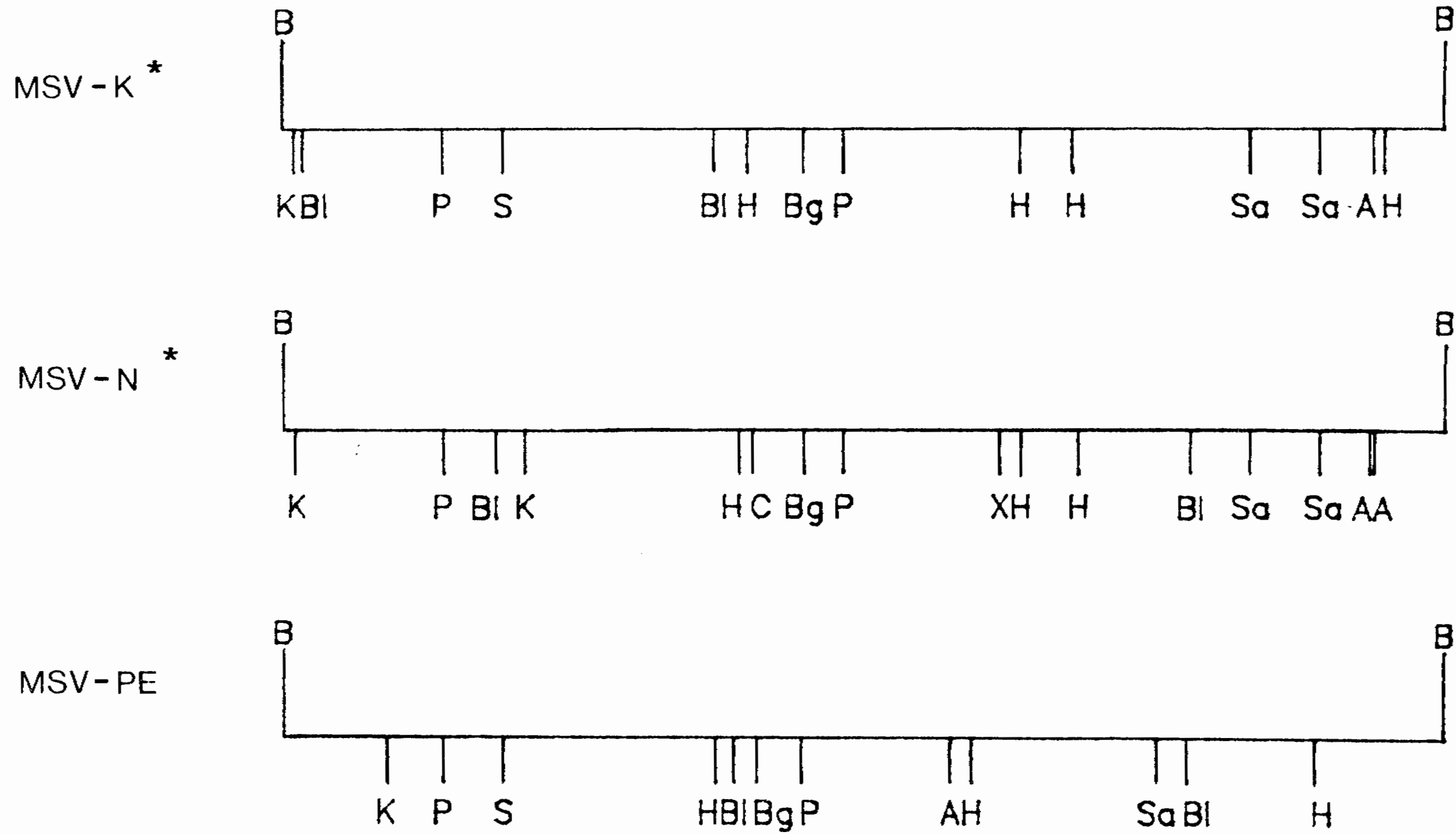
The maps of the restriction enzyme cleavage sites, for each MSV isolate, were determined by digestion of plasmid or RF-DNA with combinations of restriction enzymes. The mapping experiments were performed with enzymes used in groups of three, with three single digestions, all combinations of double digestions, and a triple digestion of a single DNA species performed simultaneously. All digests of a given set were electrophoresed simultaneously with molecular weight markers on a single gel. A calibration curve was constructed for each gel and sizes of each fragment were accurately calculated. If fragments sizes did not add up to the expected value digests were electrophoresed on higher percentage gels with increased amounts of DNA until

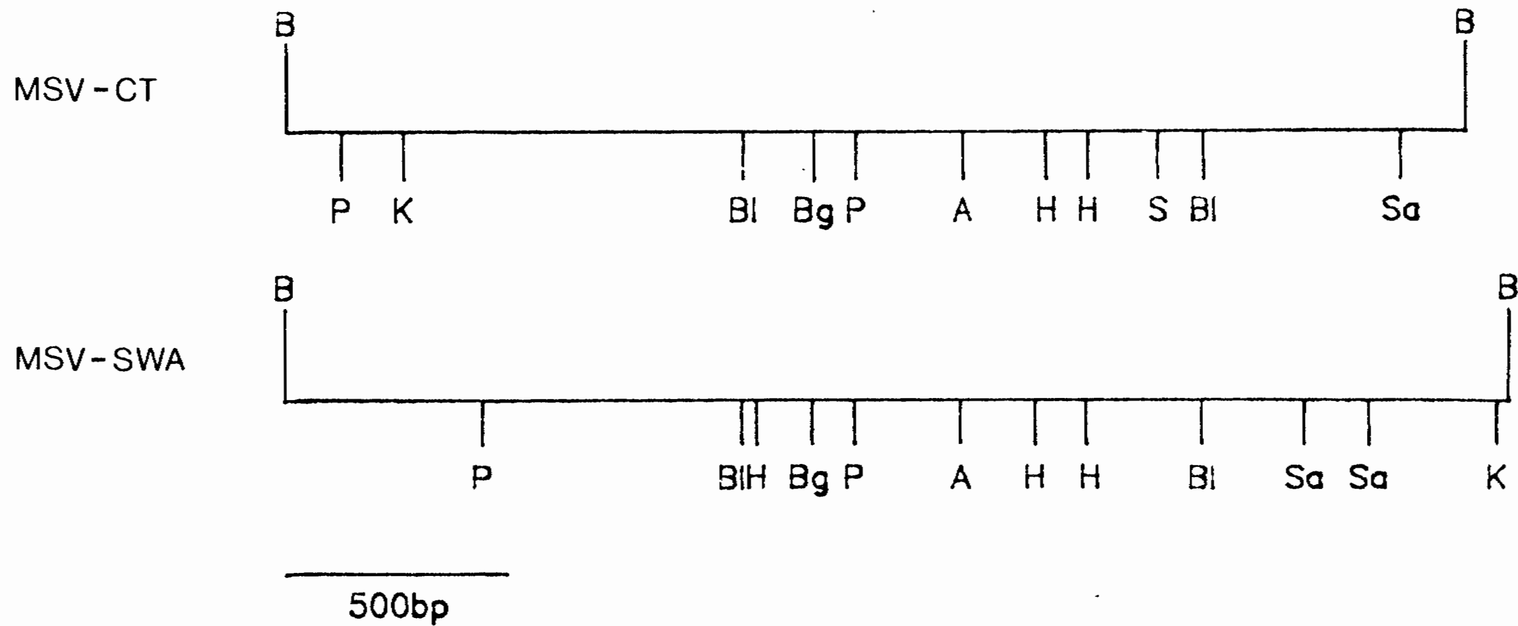
fragments of about 100bp could be visualised. All possible combinations of double digestions of pBC100 and -200 and RF-DNA of all three MSVs were done with all of the enzymes that cleaved the respective DNAs; triple digestions were usually done to clarify ambiguous results. Sample results and an example of the logic used for construction of restriction maps are given in Appendix 1. Maps of the three isolates - linearised at the Bam HI site in conformity with Mullineaux et al. (1984) - are given in figure 4.4.

Single digestions of MSV-CT and MSV-PE RF-DNAs with Apa I, Bgl II, BamHI, Sal GI and Sac I each yielded single restriction fragments of 2700 nucleotides in length. MSV-SWA DNA restricted with Apa I, BamHI, Bgl II and Kpn I consistently yielded a linear molecule of 2800 nucleotides in length; this was true both for digestions of the same batch of DNA with different enzymes, and for digestions of different batches of DNA with the same enzymes (results not shown).

Significant differences - as well as significant similarities - were found between the restriction maps of these three MSV isolates (Fig. 4.4). Differences included both the positions of the restriction sites on the genome, as well as their frequency. Similarities in patterns extended to the predicted patterns of MSV-K and MSV-N (Fig. 4.4): all five isolates have a single BamHI site and a

Figure 4.4: Restriction endonuclease maps of MSV isolates.



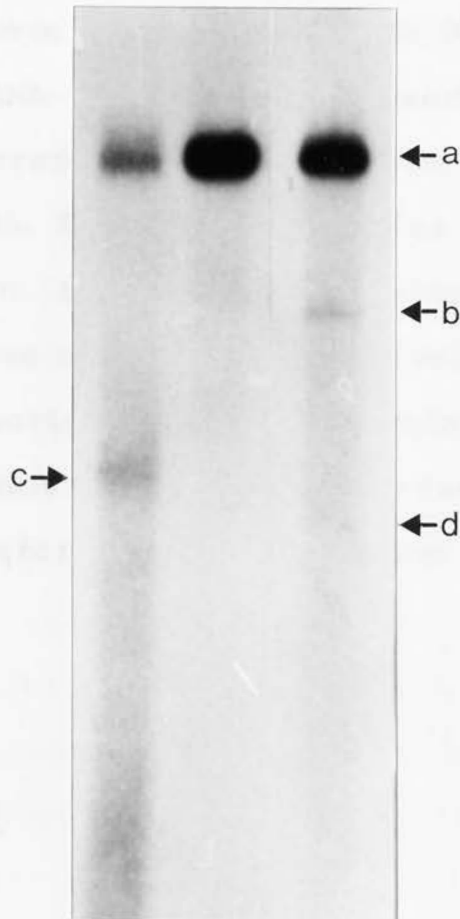


* MSV-N (Mullineaux _et al_., 1984), MSV-K (Howell, 1984). A: _Apa_ I, B: _BamHI_, Bg: _Bgl_ II, Bl: _Bgl_ I, C: _Cla_ I, H: _Hind_ III, K: _Kpn_ I, P: _Pvu_ II, S: _Sal_ I, Sa: _Sac_ I, X: _Xho_ I.

single Bgl II site, and furthermore, the Bgl II site is situated between 1110 and 1250 base pairs in all cases, with the BamHI site designated as position 0. They also all appear to have two Pvu II sites in their DNA which are situated in similar locations on the genomes of all five isolates. MSV-N has two Apa I sites, whereas the other isolates each have a single Apa I site. MSV-K also differs from the other four MSV isolates in having unique Xho I and Cla I sites. Furthermore, MSV-K has four Hind III sites in comparison with the three Hind III sites of MSV-N, MSV-PE and MSV-SWA and the two Hind III sites of MSV-CT. Unlike MSV-CT and MSV-PE, MSV-SWA has no Sal GI site but does have two Sac I sites. MSV-CT and MSV-PE each have a single Sal GI site and Sac I site. In this regard, MSV-SWA appears to more closely resemble MSV-N, which also has two Sac I sites but no Sal GI sites. MSV-K, on the other hand, has both a single Sal GI site and two Sac I sites.

As a cloned DNA probe was available for MSV-CT, attempts were made to identify the polyadenylated RNA transcripts for this virus. Total cellular RNA was isolated from MSV-CT infected maize plants by the method of Maniatis et al. (1982). Poly(A)$^+$ RNA was separated from nonpolyadenylated RNA by chromatography on oligo(dT) cellulose according to the method of Aviv and Leder (1972). Electrophoresis of RNA through formaldehyde gels was performed according to the method of Lehrach et al. (1977), and Northern blotting of

Figure 4.5: Northern blot of total poly(A)$^{+}$ RNA probed with ^{32}P-labelled pBC100.



(a) major transcript; (b), (c), (d) minor transcripts
The sizes of the transcripts were not determined.

separated RNA was done according to the method of Maniatis et al. (1982). RNase A digestion revealed that the mRNA preparations were contaminated with DNA. Three preparations of poly(A)$^+$ RNA were elctrophoresed through formaldehyde gels, transferred to nitrocellulose and probed with ^{32}P-labelled pBC100. In all three samples a major transcript was identified. In two of the samples one and two minor transcripts were observed, respectively (Fig. 4.5). The size of these transcripts was not determined. As this was only a preliminary investigation further research would have to be undertaken before any conclusive evidence could be presented.

4.3 DISCUSSION

The genomes of the two MSV isolates obtained from South Africa and a third obtained from South West Africa / Namibia were found to differ significantly in terms of restriction fragment patterns. This was true for both cloned and plant-purified RF-DNA. Comparison of the restriction patterns with those generated by computer for MSV-K and MSV-N also revealed significant differences. These differences were distributed throughout the genomic DNAs of the five isolates. When examining the restriction maps it became apparent that the "core" section of all the isolates, around the Bgl II site, is very similar. Only minor differences

were observed in the type and position of restriction sites in this area. MSV-N has a Cla I site in this region in place of a Bgl I site, and MSV-CT has no Hind III site in this region. Within the first 1000 nucleotide base-pairs of their genomes, MSV-K and MSV-N are fairly similar, while there appears to have been a rearrangement between the Pvu II site and the Kpn I site in MSV-CT. In this area of the MSV-SWA genome only one Pvu II site is present; the other restriction enzymes used in this study have no sites within this first 1000 base-pair region. This suggests a complete rearrangement of this area in the MSV-SWA DNA. The MSV-SWA genome is also approximately 100 base-pairs longer in length than the other four MSV isolates. These additional nucleotides appear to have been inserted within the last 500 base-pairs of the DNA. Furthermore, MSV-SWA has a Kpn I site at approximately position 2790. This is similar to MSV-N and MSV-K, except that with these two isolates this site is situated at position 10. It is therefore, speculated that with the possible insertion of additional nucleotides into the genome of MSV-SWA, a rearrangement could have occured around the BamHI site resulting in a shift of the Kpn I site. This shift is more apparent on a linear restriction map than it would be on a circular map.

The differences in restriction maps between the various isolates appear to be real, as different selections of MSV-CT clones from the primary transformation event revealed

identical restriction enzyme patterns (results not shown). This was also found to be the case when MSV-CT RF-DNA was recloned into pEcoR251 in a second experiment. Furthermore, restriction fragments obtained from digestion of total DNA isolated from MSV-infected maize plants hybridised to ^{32}P-labelled pBC200 as unique bands and not as a smear. This indicates that those plants infected with MSV are infected with a single strain of the virus, and not with a mixed population of strains. In addition, there appeared to be no variability either within a single population of clones, or between populations of the same clone. Restriction endonuclease mapping, therefore, appears to be an easy means of identifying new isolates. This together with known serological relationships could be used for both detection and characterisation of new strains of the virus.

Morris-Krsinich et al. (1985) reported the detection and mapping of three RNA transcripts for MSV. Two virion-sense transcripts of 1.05kb and 0.9kb and a complementary-sense transcript of 1.2kb (see 2.8). In this study four potential transcripts hybridised to ^{32}P-labelled pBC100. It is not known, however, which of these transcripts map to virion-sense DNA and which to complementary-sense DNA. As these potential transcripts were not sized, and no further research was performed, these results cannot be considered as conclusive.

CHAPTER 5

ANALYSIS OF THE GENETIC VARIATION AMONGST ISOLATES OF MAIZE STREAK VIRUS

5.1 INTRODUCTION

Comparisons of the genetic diversity of strains of MSV have, until recently, been limited to descriptions of serological and host range variants, from which biological and chemical properties and genomic sequence differences may be inferred - but not quantitated. More recently the sequencing of two isolates of MSV (Mullineaux et al., 1984; Howell, 1984) and of the related WDV (MacDowell et al., 1985) has allowed direct sequence comparison and estimation of sequence divergence (Howarth & Goodman, 1982; Kikuno et al., 1984; Mullineaux et al., 1985). Although the ideal method of estimation of genetic relationships between organisms is just such a direct comparison of nucleic acid sequences, it is also possible to reasonably accurately estimate evolutionary distance between related nucleic acids from comparisons of restriction enzyme cleavage patterns (Upholt, 1977). Closely related DNA sequences will have a variable number of restriction cleavage sites in common, depending on the degree of sequence divergence between the DNAs. Analysis

of the fraction of cleavage sites conserved between two DNAs may, therefore, be used to estimate sequence divergence when the sites are mapped (Aoki et al., 1981). Analysis of restriction digests requires that the DNA and the restriction enzyme chosen, be such that the data will provide both a sufficient number of fragments for meaningful comparison, and a sufficiently simple fragment pattern so that fragments may be unequivocally identified (Nei & Li, 1979). It must also be assumed that the cleavage recognition sequence occurs with a distribution and frequency close to that expected in a random sequence of the same base composition (Upholt, 1977). The evolutionary distance is therefore, defined as the number of base substitutions per homologous site that have occurred since the divergence of the two DNA sequences (Aoki et al., 1981).

As previously detailed (Chapter 4), the restriction fragment maps produced for MSV-CT, MSV-PE and MSV-SWA and those generated by computer for MSV-K and MSV-N revealed both similarities and differences between the nucleotide sequences of these isolates. The predicted and experimental restriction patterns were used to calculate evolutionary distances between each MSV isolate and the other four isolates. The values obtained were used in the construction of a phylogenetic tree. Various methods have been proposed for constructing phylogenetic trees from amino acid sequences, nucleotide sequences, and electrophoretic data.

Tateno *et al.* (1982) reviewed a number of methods based on nucleotide sequence analysis, and concluded that the method of Fitch and Margoliash (1967) is the most useful. Where input data are likely to include over-estimates as well as true estimates and under-estimates of the actual distances between taxonomic units, the Fitch and Margoliash (1967) method is the most reasonable for constructing phylogenies from distance matrices. It is an iterative averaging procedure and does not assume a homogeneous rate of evolution among the molecules compared. Since it is an averaging method, each output datum may be equal to, larger than, or smaller than the corresponding input datum. A tree for the three MSV isolates used in this study, and the two published isolates, was therefore constructed according to the method of Fitch and Margoliash (1967). The data obtained from this tree was used for an analysis of the relationships between the various isolates, as well as for speculation on the evolutionary pattern of MSV.

5.2 MATERIALS AND METHODS

Prediction of restriction endonuclease cleavage sites, theoretical restriction patterns, and comparisons of MSV restriction enzyme patterns were performed using Microgenie™ software (Beckman Instruments) on an IBM AT microcomputer.

5.2.1 Estimating evolutionary distances from restriction maps.

The mathematical models of Aoki *et al.* (1981) and Nei and Li (1979) were used for these estimations.

The following equations were applied:

(a) (Aoki *et al.*, 1981)

$$S = \frac{2\sum n_{xy}}{\sum n_x + \sum n_y}$$

(b) (Nei & Li, 1979)

$$\pi = \frac{(-\ln S)}{r}$$

where:

S = the average proportion of shared sites between two strains.

n_x = the total number of restriction sites observed in strain 1.

n_y = the total number of restriction sites observed in strain 2.

n_{xy} = the number of sites shared by both sequences.

π = the nucleotide diversity (average number of nucleotide differences per site between two DNA sequences).

r = the number of base-pairs in the restriction site.

All mutagenic events were assumed to be simple base substitutions equally probable for all nucleotide sites. Furthermore, the substitution rate was assumed to be constant and identical for all directions of base change. Cleavage sites were regarded as common sites if their map positions coincided within the error of 1% of genome length.

5.2.2 Production of an unrooted phylogenetic tree

The π values calculated as shown in 5.2.1 were inserted into the Fitch and Margoliash (1967) algorithm to produce an unrooted phylogenetic tree using Felsenstein's PHYLIP (Phylogeny Inference Package) program (available from J. Felsenstein, Department of Genetics SK-50, University of Washington, Seattle, WA 98195, USA). An unrooted tree was produced as the topological errors for unrooted trees are equal to or smaller than those for rooted trees. This is because in rooted trees an additional error may be generated in the process of rooting (Tateno *et al.*, 1982). Negative branch lengths were avoided in the best tree selected.

5.3 RESULTS

Direct comparison of the nucleotide sequences of MSV-K and MSV-N by computer gave a sequence similarity of 95.7%, which compares favourably with published values (Mullineaux *et al.*, 1984; Howell, 1984). Comparison of computer-predicted restriction fragment patterns for the two isolates using the enzymes listed in Table 5.1 gave a predicted homology of 96.4%, which compares well with the value calculated from sequence analysis.

Table 5.1: Six base-pair restriction endonucleases used in this study.

Apa I, *BamHI*, *Bgl* I, *Bgl* II, *Cla* I, *Hind* III, *Kpn* I, *Pvu* II, *Sac* I, *Sal* GI, *Xho* I

Table 5.2: The number of shared restriction sites between the MSV isolates used in this study.

	MSV-K	MSV-N	MSV-PE	MSV-CT	MSV-SWA
MSV-K	15				
MSV-N	10	15			
MSV-PE	2	2	13		
MSV-CT	3	4	4	13	
MSV-SWA	4	5	4	7	13

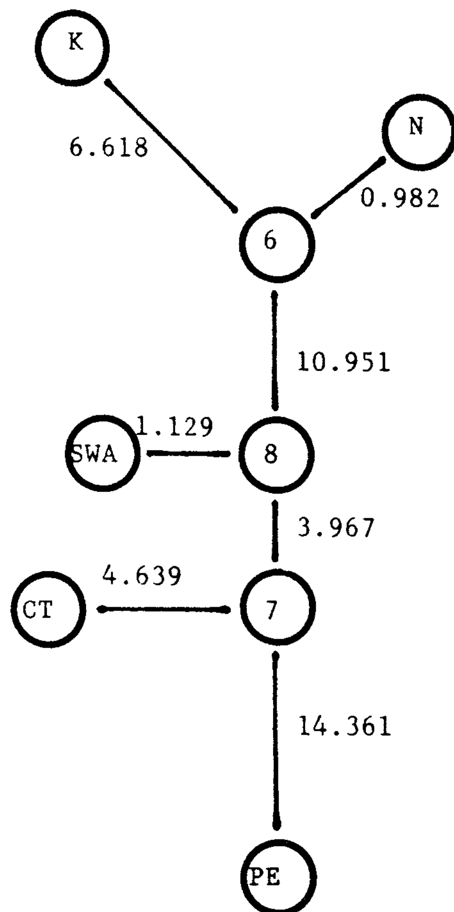
By analysis of the restriction fragment patterns produced for MSV-CT, MSV-PE and MSV-SWA (Fig. 4.4) and those generated by computer for MSV-N and MSV-K (Fig. 4.4), the number of restriction sites shared by each pair of sequences could be calculated (Table 5.2). Using these data the average proportion of shared sites between two isolates (S) and the nucleotide diversity (π) could be determined. Theoretically, $\pi=0$ for identical isolates, and can reach infinity for completely different sequences: however, in the case of the MSV isolates, the maximum value π can reach is 0.45 for 1 common site in 15. The values calculated from the mean of the results obtained for the 6 base-pair restriction enzymes (Table 5.1) for the five MSV isolates are shown in Table 5.3. It appears from Table 5.3 that the relationship between MSV-K and MSV-N is slightly closer than that between MSV-CT and MSV-SWA. MSV-CT is only marginally more closely related to MSV-N than it is to MSV-K, and shows a closer relationship to MSV-PE. MSV-SWA is more closely related to MSV-CT than to any of the other isolates. MSV-PE and MSV-N are the most unrelated, although the relationship between MSV-PE and MSV-K is only marginally closer. MSV-N and MSV-K are the most closely related.

These π values were then used to construct an unrooted phylogenetic tree according to the method of Fitch and

Table 5.3: Calculated genetic distances (π) between the isolates used in this study.

	MSV-K	MSV-N	MSV-PE	MSV-CT	MSV-SWA
MSV-K	-				
MSV-N	0.076	-			
MSV-PE	0.325	0.336	-		
MSV-CT	0.251	0.215	0.190	-	
MSV-SWA	0.209	0.123	0.196	0.097	-

Figure 5.1: Unrooted phylogenetic tree constructed for the isolates used in this study.



The relative genetic distances between the various populations are given on the branches of the tree.

Margoliash (1967), as described above. To evaluate these phylogenies the, "goodness-of-fit" criterion was used. According to this criterion the best tree is that tree whose output distances most closely match the input distances. As it is an indirect means of sequence comparison the values computed are approximate distances and not necessarily minimum estimates of true distances. Fifteen possible trees were examined, the most plausible of which is depicted in figure 5.1. Three additional "populations" (numbers 6, 7, and 8 in the figure) were added to give the shortest possible distances, and hence the best "fit", between the various isolates. The values calculated for the relative genetic distances between the various populations are given on the branches of the tree. The greater these distances, the more diverse the populations (Fitch & Margoliash, 1967).

5.4 DISCUSSION

Maize was first introduced to the continent of Africa at the begining of the 16th century by Portuguese slave traders who established trading posts with the Arabs on the east coast of Africa in an area which is today known as MoÇambique. With the favourable climatic conditions found in the area, and the hardiness of the plant, maize was soon being cultivated by the local inhabitants. This probably led to the spread of this cereal crop to central and north Africa.

There was a separate introduction of maize into southern Africa in 1658, with the establishment of a settlement in the Cape: the Governor, Jan van Riebeeck, imported maize seeds from New Guinea. These two introductions, then, could easily account for the spread of maize throughout the southern half of Africa. Whether the plants introduced to the east coast or those introduced to the Cape were infected with maize streak disease is not known, as the disease was only described in 1901 by Fuller. It is possible that MSV was present in Africa long before the introduction of maize, in "natural" alternate hosts such as different grass species. It has already been reported that both annual and perennial grasses can be reservoirs of MSV (Autrey & Ricaud, 1983). As both MSV and its vector today occur naturally only in Africa, it seems unlikely that either one of them was introduced to the continent via an outside source.

The MSV isolates used in this study as well as the two published isolates do not differ serologically or genetically to any great extent. Furthermore, these isolates all originated from Africa. This implies that MSV isolates have not diverged all that much from their common ancestral form.

The unrooted phylogenetic tree constructed for the MSV isolates (Fig. 5.1) can be explained as follows: a hypothetical single population of MSV (number 8 in the

figure) split to form two hypothetical sub-populations (numbers 6 and 7 in the figure). One of these sub-populations (number 6) gave rise to MSV-N and MSV-K, with MSV-N diverging less than MSV-K. Sub-population number 7 gave rise to MSV-CT and MSV-PE, with MSV-PE more diverged than MSV-CT. As the relative genetic distance between MSV-SWA and the supposed initial population (number 8) is fairly small, it may be speculated that MSV-SWA arose from 8, and thereafter diverged very little from its original form. The divergence of the isolates may have taken place before the introduction of maize, or possibly only after the virus had started to infect maize. From the relative genetic distances calculated between the five MSV isolates in the phylogenetic tree, it can be seen that MSV-N is the most closely related to MSV-K. This relationship is the closest out of all the various combinations calculated for the various isolates. MSV-CT is more closely related to MSV-SWA than it is to MSV-PE, although MSV-PE is more closely related to MSV-CT than to any of the other isolates. MSV-K and MSV-PE are the most unrelated of the isolates, although based on π values the relationship between MSV-K and MSV-PE is marginally closer than that between MSV-PE and MSV-N.

In order to determine fully the relationship between the various isolates, it will be necessary to characterise as many isolates as possible from as many different locations

and hosts as possible. In this way a more accurate picture of the evolutionary divergence of MSV could be constructed.

CHAPTER 6

SUMMARY, GENERAL DISCUSSION AND CONCLUSIONS

6.1 SUMMARY OF RESULTS

Three MSV isolates (MSV-CT, MSV-PE and MSV-SWA) obtained from distinct geographical locations in Southern Africa, were transmitted from infected dried material to healthy maize plants by the leafhopper *Cicadulina mbila*. Extraction of the diseased materials yielded typical geminate particles for each isolate, which could not be distinguished from each other. Antisera were raised to MSV-CT and MSV-PE. DAS-ELISA tests with these antisera revealed that the three isolates were closely serologically related, but non-identical. This correlated with the symptoms produced by each isolate in infected maize plants (Fig. 3.1). Polyacrylamide gel electrophoresis of the virion proteins of the MSV isolates, revealed a single band for each isolate of approximately 3.0×10^4 daltons in size.

A fourth geminivirus (DGV) which infects the grass *Digitaria sanguinalis* reacted weakly with MSV-CT and MSV-PE antisera in DAS-ELISA tests. In immunoelectroblots, however, a weak reaction only developed between DGV virion proteins and MSV-

PE antisera. In SDS-polyacrylamide gels four protein bands were observed for DGV, only three of which reacted with antisera raised to purified DGV particles. Two of these proteins formed a doublet of 2.5×10^4 daltons and 2.7×10^4 daltons, respectively. The third protein of approximately 1.9×10^4 daltons was thought to be a degradation product of the larger doublet. Attempts were made to transmit DGV through seed collected from infected plants, mechanically and using the leafhopper *Cicadulina mbila*, but all proved unsuccessful. DGV was therefore maintained in *Digitaria sanguinalis* plants by vegetative propagation.

As it was possible to transmit MSV-CT, MSV-PE and MSV-SWA from infected maize plants to healthy *Digitaria sanguinalis*, and from the infected *D. sanguinalis* plants back into healthy maize plants, this may prove to be an easy means of maintaining and propagating MSV isolates. In this way it would not be necessary to maintain separate colonies of leafhoppers.

In order to ascertain whether serological differences and symptomatology could be equated with genomic sequence differences, the genomic DNA of each MSV isolate was purified and analysed. The DNAs of MSV-CT and MSV-PE were found to be approximately 2.7kb in size, while that of MSV-SWA was found to be approximately 2.8kb. Double-stranded replicative form DNA of MSV-CT and MSV-PE was cloned into

the positive selection vector pEcoR251, and restriction endonuclease maps were generated. A restriction map of the genome of MSV-SWA was constructed without prior cloning of the DNA. Comparison of these restriction patterns with each other and with those published for MSV-N and MSV-K revealed significant similarities and differences between the five isolates. These included both the positions of the restriction sites on the genome, as well as a difference in the number of some restriction sites present. The most striking discrepancy between the isolates appeared to be in the number, and positions of the Hind III sites within the DNAs. On the basis of these restriction patterns an unrooted phylogenetic tree was constructed. From this tree it could be seen that MSV-N and MSV-K are the most closely related isolates, while MSV-K and MSV-PE are the most unrelated of the isolates.

6.2 DISCUSSION AND CONCLUSIONS

Southern African isolates of MSV were shown to produce different symptoms in the laboratory, to differ serologically and to have different DNA restriction endonuclease patterns from each other and foreign isolates. The differences between the various isolates appeared to be constant within a given population of virus. Data obtained from serological tests suggested that the MSV isolates could

be grouped into a serological map (Fig. 3.15). From this map it appeared that MSV-PE and MSV-SWA were more closely related to each other than to MSV-CT. Conversely, a phylogenetic tree based on the similarities and differences observed between the restriction enzyme maps of the various isolates, showed MSV-CT to be more closely related to MSV-SWA than to MSV-PE. This apparent discrepancy in results can be understood if one bears in mind that less than 5% of the genomic information of a plant virus is involved in the antigenicity of its coat protein (Hull, 1986). For this reason it would be better to base identification and characterisation of MSV strains and isolates on genomic sequence differences than on serological differences. As MSV-CT, MSV-PE and MSV-SWA as well as MSV-N and MSV-K originated from distinct geographical locations, it is, therefore, suggested that further isolates could be typed relative to "standard strains". This could be performed without recourse to sequencing, and perhaps without recourse to cloning. Strains could be mapped by probing Southern-blotted, endonuclease-digested, total DNA extracts of infected maize plants with radioisotope-labelled MSV-DNA. Information on strain diversity generated in this way could perhaps be correlated with differences in symptom expression and virulence, host range, vector transmissibility and/or preference, and other factors important to the epidemiology

of the virus. This data could be of great importance to maize breeders, as very little is currently known about the genetic variation of this virus.

Genome similarities between geminiviruses in general and the genetic variation of maize streak in particular have been discussed in some detail (see 2.10, 5.4); this section will discuss further aspects of the evolution of geminiviruses. Kikuno et al. (1984) detected homology between genes I (encoded on DNA 1) and V (encoded on DNA 2) of ACMV, and Howarth and Goodman (1986) observed the same relationship in BGMV and TGMV (see 2.8). This result suggested that the bipartite genome evolved from a monopartite ancestor, and furthermore, that the geminiviruses with mono- and bipartite genomes are ancestrally related (Stanley et al., 1986) (see 2.10). Howarth and Goodman (1986) constructed an evolutionary tree, based on an estimate of divergence in gene II, the most highly conserved geminivirus gene (see 2.8). They found that, assuming a linear rate of change over time, the monopartite-bipartite divergence is estimated to have taken place twice as long ago as the divergence of the bipartite geminiviruses from their common ancestor. The bipartite geminiviruses were found to be less diverged than the monopartite viruses, which may reflect the several features they have in common. In addition to the postulated divergence to a bipartite genome, these viruses have the same insect vector and are found in plants grown in similar

subtropical environments. The greater phylogenetic distance observed between the monopartite viruses may be consistent with the greater biological differences between these viruses: for instance, their vectors belong to different genera, whereas all the bipartite geminiviruses are transmitted by Bemisia species. MSV infects maize and other grasses in Africa, WDV infects wheat and is found in northern Europe, CSMV infects a number of species in the Gramineae in Australia, while BCTV infects a number of different dicotyledonous plant species in the USA and Mediterranean. These four viruses also differ from each other on a molecular level. The cereal geminiviruses have, therefore, diverged considerably since the original bipartite-monopartite split, and are now located in distinct geographical regions. In Africa, however, MSV appears to have diverged more slowly - or perhaps more recently - to give distinct but closely-related isolates. There may be a wide variety of cereal geminiviruses in Africa, only one or a few of which infect maize; thus, we may be getting a false idea of the natural variation by only studying maize isolates. It is feasible to screen on a large scale - using radioisotope-labelled MSV DNA - for relatives of MSV in all kinds of Gramineae. Such a project could yield a fund of valuable information for virologists, plant pathologists and breeders alike.

Grimsley *et al.* (1987) recently developed a procedure, involving *Agrobacterium*, of making MSV DNA infectious. As the MSV genome was cloned as tandem head-to-tail repeat units, this technique could be used to assay for homologous recombination between different isolates. This could lead to the formation of a number of chimaeras, the serological and biological properties of which would be very interesting to study. Furthermore, crossover-points in different sites in the genome could result in the creation of novel viruses with new symptomatology (B. Hohn, personal communication). Thus, the determinants for symptom expression, virulence and host range could fairly quickly (and relatively easily) be characterised at the level of DNA sequence. Such information could lead to measures for the control of maize streak and other cereal geminivirus-caused diseases at the molecular level.

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Restriction endonuclease fragments of recombinant plasmid pBC100 produced by cleavage with the enzymes *BamH1*, *Bgl11* and *Pvu11*.

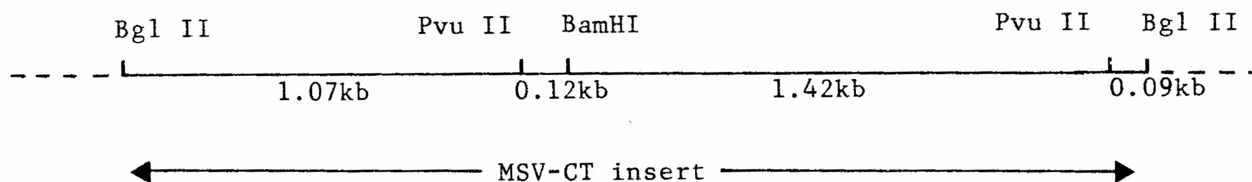
Digest	Fragments					
BamH1	4.1kb	1.96kb				
Bgl11	3.36kb	2.7kb				
Pvu11	4.52kb	1.54kb				
BamH1 + *Bgl11*	2.91kb	1.51kb	1.19kb	0.45kb		
Bgl11 + *Pvu11*	3.36kb	1.54kb	1.07kb	0.09kb		
BamH1 + *Pvu11*	3.98kb	1.42kb	0.54kb	0.12kb		
BamH1 + *Pvu11* + *Bgl11*	2.91kb	1.42kb	1.07kb	0.45kb	0.12kb	0.09kb

APPENDIX I

Mapping Strategy

An example of a mapping experiment on pBC100 with the enzymes BamHI, Bgl II and Pvu II is given in the table opposite. The recombinant plasmid pBC100 was cleaved by Bgl II to release the MSV-CT insert of 2.7kb. As pEcoR251 has no other Bgl II site the remaining 3.36kb fragment represents the vector. The Bgl II site was therefore taken as the left and right end points of the linearised MSV-CT DNA. The other enzyme restriction sites were mapped relative to these two end points. BamHI restriction of pBC100 produced two fragments of 4.1kb and 1.96kb. Furthermore, a BamHI/Bgl II double digest produced four fragments of 2.91kb, 1.51kb, 1.19kb and 0.45kb. As the BamHI site in pEcoR251 is 0.45kb downstream of the Bgl II site, the BamHI site in the insert must be either 1.51kb or 1.19kb from the lefthand Bgl II end point. A Pvu II digestion of pBC100 gave two fragments of 4.52kb and 1.54kb, and a Pvu II/Bgl II double digest produced four fragments of 3.36kb, 1.54kb, 1.07kb and 0.09kb. A BamHI/Pvu II double digest likewise gave four fragments of 3.98kb, 1.42kb, 0.54kb and 0.12kb. As there is no Pvu II site in pEcoR251 the small 0.12kb BamHI/Pvu II fragment must occur within the insert; furthermore, the two

Pvu II sites must be 1.54kb apart, and one of them must be 0.09kb from either the left or right Bgl II insert end point. This Pvu II site is situated 0.09kb from the righthand end point resulting in one of the Pvu II/BamHI fragments being 0.54kb in size. If the Pvu II site was situated 0.09kb from the lefthand end point Pvu II/BamHI cleavage would have resulted in one of the fragments being 3.0kb in size, which is not the case. Likewise, the BamHI site within the insert must be 1.19kb from the left Bgl II end point. If it was 1.51kb from this point a BamHI/Pvu II double digest would not result in one of the fragments being 0.12kb in size, but 0.44kb in size. That these conclusions were correct was verified by a BamHI/Bgl II/Pvu II triple digest producing six fragments of 2.91kb, 1.42kb, 1.07kb, 0.45kb, 0.12kb and 0.09kb. The linear map of the sites for these three enzymes in the linearised MSV-CT insert would therefore be as follows:



If the BamHI site is taken as the left- and righthand end points of the linear insert, this figure is the same as that shown for MSV-CT in figure 4.4. In this same way the sites in MSV-CT for the enzymes Kpn I, Bgl I, Apa I, Hind III, Sal

GI and *Sac* I were determined. In order to double-check that the map was in fact correct MSV-CT RF-DNA was isolated and mapped in the same way, with the same results. The restriction map of MSV-PE RF-DNA was also found to be the same as that for pBC200 DNA. For this reason MSV-SWA was mapped on RF-DNA only.