STRAIN DIFFERENTIATION AND SCREENING FOR RESISTANCE AGAINST ISOLATES OF SOYBEAN MOSAIC VIRUS

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DEDICATION

This dissertation is dedicated to my Uncle Athie Govender who has provided strong foundations and safe havens that allow minds to constantly question and challenge the vistas of the unknown.

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ABSTRACT

Soybean mosaic virus (SMV) is recognised internationally as a limiting factor to soybean *(Glycine max)* production. The disease induces typical mosaic and leaf curl symptoms in infected plants. Yield reductions owing to decreased shoot and root length, wet and dry weights of leaves, oil content, seed size and nitrogen fixing ability of the plant are associated with the disease and are directly linked to host cultivar and virus strain interactions.

There are several biological strains of SMV that exist in the natural population. The results of surveys conducted between the 1985 and 1997 in South African soybean production areas suggested that the G1 strain is most predominant. The only other strain identified during that time was SMV G3. The traditional method for SMV strain differentiation requires the monitoring of symptom expression on a differential cultivar host range, which is time consuming and resource intensive. The first part of this project attempted to develop a method for the specific detection of the SMV G1 strain. The techniques were based on nucleotide sequence differences in that part of the SMV genome that spans the mid-region of the coat protein gene to the end of the non-coding region (NCR), and in the CI protein-coding region of the genome.

Polymerase chain reaction (PCR) cDNA amplification products of the partial coat protein gene and 3'-NCR of seven SMV isolates were cloned and sequenced. The results unfortunately revealed that there were no unique sequences, which could be further manipulated by molecular biology techniques, to specifically detect SMV G1 isolates. Analyses of the CI protein gene involved the use of PCR products that were then subjected to restriction enzyme digestion with *Hae III*. The results produced in the current study did not correspond with previously published information. The method was not found to be useful for the specific detection of the SMV G1 strain.

The second part of the study attempted to identify soybean cultivars that are potential resistance sources to South African isolates of SMV strains, G1 and G3. Twenty soybean cultivars currently in use in South Africa were inoculated with either of the virus isolates using viruliferous aphids. Each inoculated plant was paired with an uninoculated control of the same cultivar to allow pair-wise comparison. The plants were maintained in 10 randomised blocks in an insect-proof screen house until maturity. Symptom development was

monitored closely in the first few weeks, and when necessary Enzyme Linked Immunosorbent Assays (ELISAs) were conducted to determine whether individual plants were infected with SMV when symptoms were ambiguous. Seeds were harvested when the plants reached maturity.

Seed yield was determined according to weight and the difference between the weight of the inoculated and uninoculated plants of each pair was also recorded. These results were subjected to statistical analyses. The cultivars Davis and Ibis were resistant to both the G1 and G3 isolates. Many of the cultivars tested were susceptible to at least one of the virus isolates, as determined by a significant ($P<0.05$) yield reductions. The cultivars PAN 494, Sonop, Dumela, A7119 and Rampage are tolerant of both SMV strains tested since they became systemically infected with the virus but this did not cause significant yield losses. The cultivars Nyala, Bloekom and SCS1 were tolerant to the G1 isolate but susceptible to the G3 isolate. The cultivars Prima and JF91 were completely susceptible to both SMV strains.

Seed transmission rates of the virus isolates were also investigated. In many cultivars it was found that there was reduced seedling emergence. The percentage of seedling transmission varied between 0 and 33% and differed according to virus isolate and cultivar interaction. There was a general trend suggesting a higher rate of seed transmission of the G3 isolate.

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1. INTRODUCTION

1.1 BACKGROUND

The average consumer seldom leaves the supermarket without having purchased at least one product that contains some part of the soybean plant. By virtue of its versatility alone, the humble soybean has become a celebrity in the agricultural markets of the world. In South Africa, about 18% of the beans are processed for extraction of the high quality oil, which is then used in pharmaceutical products. About 10% is consumed by humans as alternatives or supplements to conventional protein and dairy products, and the rest is used in oilcake; a high protein animal feed component. The non-edible products that contain processed forms of this botanical wonder include paints, lubricants and waxes (Anon., 1998).

The concerted effort made by the National Oilseed Producers Organisation, the Protein Research Trust and the Animal Feed Manufacturers Association to increase soybean yield in South Africa, has met with considerable success. The total crop yield increased from 72 000 tonnes in 1994, to 200900 tonnes in 1998. This huge increase in yield of soybean was in part also attributed to the uncertainty of the maize price, which persuaded some farmers to switch to planting soybean on land that was traditionally farmed with maize. However, this was still not enough to meet the requirements of the animal feed industry, and the country had to import an additional 450 000 tons of oilcake in 1998. South Africa usually buys oilcake from the United States of America or Argentina; thus an increase in local production could save the country millions of rands in foreign exchange (Anon., 1998).

The imbalance between supply and demand alone makes soybean a lucrative crop for the farmer; however farming it also offers several good agronomic benefits. An average crop rotation benefit of 40 kg N/ha is generally achieved after planting this nitrogen fixing leguminous crop. Soybean is one of the few crops that, when alternated with wheat ensures two crops per annum. Maize farmers should also consider including soybean into their cropping systems, to reduce risk and guarantee a return on investment. Soybean is more tolerant to acid soils than maize and can therefore pay for the lime, which is required before maize can be cultivated. Above average rainfall and temperature in January and February is less detrimental to a soybean crop than to a maize crop, and since soybean plants flower over

a relatively long period they are consequently more drought tolerant during the flowering stage than maize is during the pollination period (Anon., 1998).

Pest management of a soybean crop usually involves controlling weed, insect and pathogenic microbe populations. Several herbicides are registered for use at different stages of plant growth and care must be taken in choosing herbicides that are not damaging to the cultivar that has been planted. Mechanical weed control is not recommended due to the risk of root damage. Cutworm could pose a problem at the time of seedling emergence, and other insects such as the soybean looper and the green stinkbug, are often problematic during the podding stage. A variety of spraying programs that effectively control these pests are available. In S.A the most important soybean diseases induced by microorganisms include *Sclerotinia* stem rot, downy mildew, and *Soybean mosaic virus* (SMV). In general, good cultural practices such as crop rotation and deep ploughing are recommended to keep disease incidence to a minimum. Fungicides are available for the treatment of stem rot and downy mildew but SMV cannot be controlled, however it may be prevented to some extent by planting resistant cultivars. This virus is prevalent in all soybeans growing regions, and research in this field would greatly enhance crop production (Anon., 1998).

1.2 THE SCOPE OF THE PROJECT

Research on SMV has a long history at the Agricultural Research Council's Plant Protection Research Institute (ARC-PPRI). Under the navigation of Dr Gerhard Pietersen, research on this pathogen has traversed a path that began with surveying the virus status of soybeans in the former Transvaal (Pietersen and Garnett, 1990). Of the twenty-six virus infected plants obtained, eleven tested positive for SMV by ELISA. *Peanut mottle virus (*PeMotV), *Bean yellow mosaic virus* (BYMV) and an unidentified potyvirus were the only other viruses observed during the survey. Pietersen and Garnett (1992) used the differential cultivar host range to determine strain identity of the SMV isolates. All isolates reacted in accordance with that of the type G1 strain. The SMV isolate 86/0020 (collected during the survey) was used to raise antiserum for the production of a $F(ab')_2$ ELISA system. The properties of 86/0020 have been described by Pietersen and Garnett (1992). In a following study thirty-one soybean cultivars were screened for resistance to isolates of the viruses identified in the survey. The cultivars Ibis and PNR 565 were identified as resistant to SMV infection (Pietersen, 1995). That study also noted that cultivars that demonstrated a form of tolerance with mild symptom

expression or insignificant yield loss, but with viral replication in the plant, could serve as reservoirs thus facilitating virus spread to adjoining fields of more susceptible cultivars.

Between 1994 and 1997 the Protein Research Trust funded further surveys to identify viruses in all the South African soybean production regions. The results showed that SMV and a previously unreported rhabdovirus were the only two virological pathogens of consequence (Pietersen *et al.*, 1994, 1995 and 1996). SMV was identified in 134 samples, which represented 19.3% of the total sample size. In most commercial fields the incidence of SMV infection was generally low (below 1% in most cases), there were some exceptions where levels of SMV infection ranged between 10 and 15%. However, in the National Soybean Cultivar Trials and National Germplasm Collection SMV was present in incidences of up to 20% of the whole trial, varying between 0 and 50 % infected plants in single cultivar plots. The SMV isolates collected during the surveys were freeze-dried and stored (Pietersen *et al.*, 2000).

In 1998 the Protein Research Trust commissioned a further study at PPRI, under the leadership of Dr Gerhard Pietersen entitled "Increased soybean yields through control of soybean mosaic virus by identification of resistant sources". The project was to have been conducted over a period of four years with two fundamental aims: (a) the determination of local strains of SMV and (b) the identification of soybean lines and cultivars that are resistant to local and international SMV strains (Pietersen *et al.*, 2000).

The first phase of the project undertook collection of more SMV isolates and the re-establishment of previously stored SMV isolates on susceptible soybean cultivars. The virus was isolated by two serial single aphid (*Myzus persicae*) transfers. A total of 40 SMV isolates, representing the various soybean production areas in South Africa, were subjected to this procedure, following which a total of 29 SMV isolates were obtained. Once the isolated virus had been established in the host, it was stored as a pure isolate and the process of strain determination commenced. A differential soybean cultivar host range (Cho and Goodman, 1979) was used to classify the isolates. Twenty of these isolates were shown to be of the G1 strain, isolate 94/2052 was found to be a G3 strain and the remaining eight isolates produced unusual results and their strain types remain unknown (Pietersen *et al.*, 2000).

The differential cultivar host range as a method of strain determination is a time consuming and resource intensive procedure, which is very limiting to commissioned research that has to follow a strict time-line and budget. It is time consuming in its requirement for the use of isolated virus, a process which adds at a minimum of two weeks to any further research activity, and because the results are based on symptom expression, a characteristic that is notorious for its variation in timing. It is resource intensive in the need for a minimum of 21 healthy seedlings to be inoculated per isolate, thus placing huge demands on growth room space and personnel involved in cultivating, inoculating and maintaining seedlings. These factors involved in SMV strain classification make it difficult to advise plant breeders on regional virus status with respect to strain occurrence, or to utilize agricultural best practice scenarios such as planting cultivars that are resistant to specific virus strains that are endemic to particular cultivation regions.

It was therefore originally envisaged that a key process of the project would be the development of a method of rapid, large-scale detection and identification of all SMV strains. The method was then to be applied to screen up to 300 SMV isolates to fulfill the first aim of the study that was to identify local strains of SMV. However, a year after commencement of the original project, funding for the project was cut from four years to two years. The aim was thus adjusted towards the development of a rapid large-scale method that could specifically detect the SMV G1 strain. From the results represented above it was quite clear that the SMV G1 strain was the most prevalent strain in South Africa, thus if the new method could specifically detect this prevalent strain it would also, by virtue of non-detection, indicate novel strains which could then be studied further. I chose to develop techniques based on nucleotide sequence differences in that part of the SMV genome that spans the mid-region of the coat protein to the end of the non-coding region (Pappu *et al.,* 1993); and in the CI protein region of the genome (Omunyin *et al.*, 1996).

Given the time constraints, the second aim, which was the identification of soybean lines and cultivars that are resistant to local and international SMV strains also had to be adjusted. Thus I proposed to identify soybean cultivars and lines resistant to the only two identified local strains (G1 and G3), by inoculation of plants by aphids, monitoring the infection rate, determining soybean yield in comparison with uninoculated plants, and measuring the rate of seed transmission.

This dissertation will document the research methodology, results and conclusions that have been attained within the stated aims of this study.

2. LITERATURE REVIEW

2.1 THE VIRUS

2.1.1 Taxonomy

In nature, black and white borderlines seem to be non-existent, and viruses may not be exempt from this rule. (Bos,1992)

SMV is a member of the taxonomic family *Potyviridae*, genus *Potyvirus*. The family history dates back to 1959 with the formation of a group to contain *Potato virus Y* (PVY) and 15 other viruses with similar filamentous particles. Twelve years later this was formally named the 'potyvirus group' (Brunt, 1992). Most recently the International Committee on Taxonomy of Viruses established the taxonomic family '*Potyviridae*' (Shukla, 1994). The virus taxonomic family has been defined as "the largest unique group of viruses which could reasonably be assumed to have evolved from a common ancestor, without genome reorganisation or addition of genes or modules from elsewhere" (Rybicki and Shukla, 1992).

The *Potyviridae* are composed of 6 recognised genera: *Potyvirus*, *Rymovirus, Ipomovirus, Macluravirus, Tritimovirus* and *Bymovirus* (Berger *et al.,* 2000). Brandes and Wetter (1959) suggested that plant viruses with anisometric particles could be classified by modal length of their particles. This together with differences in virus vectors, proved to be a good foundation for taxonomic evaluation of familial potyviruses. In general, potyviruses are 650-900 nm in length, 11-15 nm in diameter, have slightly flexuous rod shaped particles and are transmitted by a variety of different organisms (Berger *et al.*, 2000). The rymoviruses and tritimoviruses are transmitted by mites (Berger *et al.,* 2000). Ipomoviruses are transmitted by whiteflies. Bymoviruses, which are bipartite have particles of two modal lengths i.e. 250-300 and 500-600 nm. They are transmitted by the fungus *Polymyxa graminis* (Berger *et al.*, 2000).

The genus P*otyvirus* is by far the largest in this family and has a membership of more than 198 viruses. There are 4 subgroups within this genus and they are differentiated on the basis of coat protein sequence identity, biological and serological properties (Shukla *et al.*, 1994). SMV, together with *Watermelon mosaic virus 2* (WMV2) (which is currently recognised as a strain of SMV - Goodman et. al 1996), *Peanut stripe virus* (PStV), *Zucchini yellow mosaic virus* (ZYMV) and others, belong to the *Bean common mosaic virus* (BCMV) subgroup (Shukla *et al.*, 1994). The PVY subgroup is composed of two distinct viruses viz. PVY and

Pepper mottle virus (PepMoV). The *Bean yellow mosaic virus* (BYMV) subgroup is composed of BYMV, *Pea mosaic virus* (PMV) and *Sweet pea mosaic virus* (SPMV). The *Sugarcane mosaic virus* (SCMV) subgroup is composed of SCMV, *Johnsongrass mosaic virus* (JGMV), *Maize dwarf mosaic virus* (MDMV) and *Sorghum mosaic virus* (SrMV). It seems that the criteria used to classify viruses and the priority given to each are directly correlated to the technology of the time, hence taxonomy is in a constant state of flux.

2.1.2 Impact of Viral Infection

SMV symptoms were first recorded in the USA on soybean (*Glycine max)* in 1915 by Clinton (Brunt *et al.,* 1996). It has subsequently been reported to infect 45 species of in 28 genera of 5 families, including 37 species in 23 genera of the Leguminoseae (Edwardson and Christie, 1986). SMV now occurs world wide, and this is generally attributed to the high rate of transmission through infected seed. The virus induces a variety of symptoms, which are strain and cultivar dependent. Mild strains often produce transient mottling symptoms that can sometimes go undetected in field infections. Virulent strains induce symptoms such as leaf curl, systemic necrosis, bud-blight, male sterility, flower abnormalities, reduced pubescence, seed coat mottling and stunting (Shukla *et al.*, 1994). Most susceptible soybean cultivars, develop transient systemic vein clearing followed by a rolling and distortion mosaic on younger leaves with dark green, later puffed areas along the main veins and chlorosis between the dark coloured areas.

SMV infected plants produce fewer pods, which may sometimes be glabrous, malformed and seedless. According to Edwardson and Christie (1986) other researchers in the field found that yield reductions in susceptible cultivars were due principally to decreased seed weight and decreased pods per plant. Significantly reduced seed size has also been observed in infected plants. This phenomenon may be due to the diversion of cell energy and the reduction of leaf area and photosynthetic capacity associated with symptoms, this probably results in the plants reduced ability to fill out the endosperm in developing seeds.

Ross (1977) studied the effects of naturally transmitted SMV in field plantings of closely related resistant and susceptible soybean cultivars, and found that reductions in seed yields of susceptible lines ranged from 20 to 35%. Yield reductions as high as 100% have been recorded in susceptible cultivars challenged with relatively mild strains of the virus (Tu, 1989). Joshi and Gupta (1976) reported reductions in shoot and root length; and in the wet

and dry weights of leaves, roots and shoots. Oil content, seed size, nodulation and nitrogen-fixing ability of plants are also seriously compromised upon infection with SMV (Shukla *et al.*, 1994). The virus is thus recognised internationally as one of the limiting factors in soybean crop production.

2.2 VIRUS PROPERTIES WITH SPECIAL REFERENCE TO DIAGNOSTIC SIGNIFICANCE

SMV can be detected and identified by a number of techniques based on biological, cytological, antigenic and structural properties.

2.2.1 Morphology and Cytopathology

Particle measurements of SMV range from 650-760 nm (Edwardson and Christie, 1986). These values were derived from the electron microscope observations documented in several reports, and modal length was determined either from purified virus preparations or directly from plant sap. The flexuous rod shaped particles have a diameter of c.11-15 nm (Brunt, 1992).

SMV, like the other potyviruses, possesses the inherent ability to induce cylindrical inclusions (CIs) in the cytoplasm of infected host plants. Though plant viruses in all other 34 recognised groups induce inclusion bodies none induces cylindrical inclusions (Edwardson, *et al.*, 1993). These CIs are proteinaceous striated sheets which are present in various configurations in all cell types, though they have been observed to occur most frequently in epidermal and mesophyll cells (Edwardson and Christie, 1996). Based on CI-type four subdivisions have been created within the *Potyviridae* (Edwardson and Christie, 1996): see table 1.

Table 2.1: Configurations of cylindrical inclusions

Subdivision	CI - Type	Configuration
Subdivision – I	Type – 1	Pinwheels, bundles and scrolls
Subdivision – II	Type – 2	Pinwheels, bundles and laminated aggregates
Subdivision – III	Type – 3	Pinwheels, bundles, scrolls and laminated aggregates
Subdivision – IV	Type – 4	Pinwheels, bundles, scrolls and short usually curved laminated aggregates

Thus far the diagnostic potential of CIs are limited only to establishing that the infectious agent is a member of the *Potyviridae*. Separations of viruses on the basis of CI configuration does not coincide in any discernible way with separations based on vectors, serology or host ranges.

According to Edwardson and Christie, (1986) many reports show type-1 inclusions in SMV infected cells. The same authors in a 1996 publication stated that type-1 and type-3 inclusions were present in the Iizuka and Yunoki (1975) report on SMV infected cells, but type-1 and type-4 inclusions were observed in the Hunst and Tolin, (1982) report. Initially it was thought that such discrepancies were only due to inadequate sampling (Edwardson and Christie (1986). However, subsequent contemplation of the matter has produced a slightly different opinion. When viruses which induce type 3 and type 4 inclusions, are also reported to induce type 1 and type 2 inclusions, it is assumed that the latter are incomplete representations of the former. Hence, if inadequate sampling was the reason behind the original discrepancy then some reports should have contained examples of type 2 inclusions in SMV infected cell, but there are none Edwardson and Christie (1996). The statistical value of this deduction has not been discussed. At present it is generally accepted that some SMV strains induce type 1 CIs, others induce type 3 and others still, induce type 4 inclusions (Edwardson and Christie, 1996). The matter may only be fully resolved when a study that correlates CIs with other strain differentiating characteristics is undertaken by some brave soul.

2.2.2 Physical and Chemical Properties

SMV is a monopartite potyvirus and has a sedimentation coefficient $S_{20,w}$ c.150-160 S, buoyant densities in caesium chloride at 20-25°C of 1.325-1.335g/cm^3, and extinction coefficients of 2.4-2.9 (mg/ml)/cm at 260 nm (Brunt, 1992). The virus remains infective in

plant sap for 2-4 days, has a dilution end-point titre of about 10^{-3} and a thermal inactivation point of less than 60°C. The flexuous rod shaped virus particles contain nucleic acid within a capsid which has helical symmetry and is made up of a single type of coat protein. The molecular weight of the coat protein is 28 300 da (Irwin and Goodman, 1986). The single, positive stranded RNA has a molecular weight of 3.25x 10^6 (Irwin and Goodman, 1986). The complete nucleotide sequence of the G2 and G7 strains has been determined and each was found to consist of 9588 nucleotide residues (Jayaram, Hill and Miller, 1992). The only other protein that is found in a SMV virion is the VPg protein, which is covalently bound to the 5'-end of the genome. Genome organisation and function will be discussed in detail in following sections.

2.2.3 Host Range

A wide variety of plant species have been assessed as potential hosts of SMV. For general diagnostic and other laboratory purposes the specific symptoms induced by SMV on certain plants has proved useful. Local chlorotic lesions develop on *Chenopodium quinoa* and *C. album* plants that have been infected with SMV. This reaction is helpful in establishing virus isolates from field-collected material. The characteristic systemic mosaic and necrotic local lesions that develop on *Macroptilium lathyroides* and *Lablab purpureus* respectively have been exploited as diagnostic tools in host range studies (Goodman *et al.*, 1996). *G. max* cultivars Clark or Rampage must be included in any SMV diagnostic host range, since they do not possess resistance genes to this virus, and always become systemically infected and are therefore also used as maintenance and propagation hosts (Goodman *et al.*, 1996). The diagnostically unsusceptible species like *Cucumis sativa, Vicia faba* and *Petunia hybrida* have also proved useful for diagnostic purposes.

Since SMV is related to the other viruses in the BCMV group, and these viruses seem to share many hosts, care must be taken in the choice of plants to include in a diagnostic host range. The VIDE database (Goodman *et al.*, 1996) suggests that a host range for SMV diagnosis should include *Phaseolus vulgaris*, since all but the cultivars Double white and Princess are unsusceptible to the virus. BCMV and BYMV infect *P. vulgaris* systemically. BCMV does not infect *G. max* systemically and BYMV infects *V. faba* systemically.

Cho and Goodman (1979) recorded that reports of SMV symptoms also varied dramatically according to the virus isolate and soybean cultivar that was used in the experiment. Based on

the results of a host range composed of 8 differential soybean cultivars that were challenged with SMV isolates present in the United State Department of Agriculture (USDA) soybean germplasm collection, they identified 7 SMV "strains". Thus SMV strains G1 to G7 were classified on the basis of virulence in resistant soybean cultivars. Subsequently other SMV isolates were evaluated in this manner. Hunst and Tolin (1982) determined that isolates SMV-VA and SMV-OCM were G1 and G3 strains respectively; however SMV-VA was later shown to be a contaminant (see below).

Several additions have been made to the original 7 type strains reported by Cho and Goodman (1979). The virulent G7a strain was described by Buzzel and Tu (1984). A study conducted with 21 SMV isolates from the Peoples Republic of China showed that 16 of the isolates conformed to the original Cho and Goodman classification. The remaining 4 isolates were more virulent than previously described, thus SMV strains G8, G9, G10 and G11 were added to the strain classification system (Xu *et al.*, 1986). Although this method of classifying strains on pathogenicity and virulence has proved useful, its limitations have also been noted. Xu *et al.* (1986) concluded their paper by stating that; "the situation is obviously more complex than anticipated in an earlier report (Cho and Goodman, 1979) and calls for further analysis of the genetics of SMV resistance in soybeans as well as basic studies on the viruses to understand better the mechanisms controlling resistance." Vance and Beachy (1984) characterised the SMV-N strain as a severe isolate of SMV that was not transmissible by aphids. SMV-N and SMV-VA showed differences in symptom expression, in a differential host range that are typical of different strains of a virus (Shukla and Ward, 1988). However, comparison of the amino acid sequences for the coat protein of SMV-N and SMV-VA showed only a 58% homology (Shukla and Ward, 1988). This suggested that SMV-N and SMV-VA should be considered as distinct potyviruses and not strains of a common virus. The matter was finally resolved when 3' non-coding nucleotide sequence data was compared and it was determined that SMV-N was in fact a strain of WMV 2 (Frenkel, Ward and Shukla, 1989). WMV2 is now considered a strain of SMV (Goodman *et al* 1996).

For practical purposes virus strain determination is most easily accomplished using a host range of *G. max* cultivars, where each cultivar possesses different resistance genes. This was the basis for the paper by Cho and Goodman (1979). Results for the G4 strain published in that paper were later found to be inaccurate and were therefore revised by Cho and Goodman

(1982). Xu *et al* (1986) identified several other strains of SMV in the Peoples Republic of China. The collective results of the studies are summarised in Table 2.2.

Roane *et al.* (1986) applied the gene for gene hypothesis to the soybean - SMV interaction described in table 2.2. By assimilating this information with traditional plant breeding research several genes which confer host resistance to SMV were identified. Resistance to SMV will be discussed in greater detail in section 2.4 and chapter 5.

2.2.4 Transmission

2.2.4.1. Aphid Transmission

SMV is transmitted naturally in a non-persistent manner by several aphid species. There is only one known strain that is not aphid transmissible (Ross, 1968). Edwardson and Christie (1986) cited 33 aphid species that have been reported to transmit the virus. A helper virus is not a requirement of vector transmission. Specific sequence domains on the helper component proteinase (HC-Pro), and the coat protein (CP) are generally accepted as the determinants of aphid transmissibility. Atreya *et al.* (1991) showed that a glycine to glutamic acid mutation in the DAGX motif in the N-terminus of the CP would inhibit aphid transmission of the virus, without affecting virus replication and infectivity. Flasinski and Cassidy (1998), working with PeMoV and PStV, suggested that the coat protein DAGV and DAAA motifs respectively may interact with an HC-Pro domain which is distinct for each virus, to enable aphid transmission. Using mutational analysis of full length clones they have predicted that it is the CCC motif on the HC-Pro that interacts with the DAGX motif for PStV.

Table 2.2: Symptoms of SMV on several soybean cultivars[a]

STRAIN	CULTIVAR										
	Clark	Rampage/ Williams[b]	Davis	York	Marshall	Ogden	Kwanggyo	Buffalo	PI96983	PI483.084	PI486355
G1	-/M[c]	-/M	-/-	-/-	-/-	-/-	-/-	-/-	*	*	*
G2	-/M	-/M	-/-	-/-	N/N	-/-	-/-	-/-	*	*	*
G2a	*	M	*	-	M	-	-	-	-	-	-
G3	-/M	-/M	-/-	-/-	N/N	N/N	-/-	-/-	*	*	*
G4	-/M	-/M	N/N	N/N	-/-	-/-	-/-	-/-	*	*	*
G4a	*	M	*	M	-	-	-	-	-	-	-
G5	-/M	-/M	-/M	-/M	-/-	-/-	N/N	-/-	*	*	*
G6	-/M	-/M	-/M	-/M	N/N	-/-	N/N	-/-	*	*	*
G6a	*	M	*	N	M	-	N	-	-	-	-
G7	-/M	-/M	-/M	-/M	N/N	N/N	N/N	N/N	*	*	*
G7a[d]	*	M	*	*	*	*	*	-	-	*	M
G7b	*	M	*	M	M	M	N	M	M	-	-
G8	*	M	*	M	M	-	N	M	M	-	-
G9	*	M	*	M	M	M	-	M	M	-	-
G10	*	M	*	M	-	-	N	-	-	N	-
G11	*	M	*	M	M	M	M	M	M	N	-

Reactions of soybean cultivars to SMV strains assimilated from Cho and Goodman (1979S), Cho and Goodman (1982), Buzzel and Tu, (1984) and Xu *et al.* (1986). Symbols for symptoms: - = symptomless; M = mosaic symptoms; N = necrosis; * = virus strain not tested on specific cultivar. [b]Cultivars Rampage and Williams are susceptible to all known strains of SMV. [c]Format for symptom symbols : (Reactions on inoculated primary leaves) / (Reactions on noninoculated trifoliate leaves). Where only one symbol is indicated it refers to the reaction on noninoculated trifoliates (systemic symptoms). [d] The cultivar PI 360844 is resistant to the G7a strain (data not represented here).

The aphids' method of feeding is a principal reason as to why they are such a successful group and are important pests in agriculture. The mouthparts are perfectly adapted to piercing plant tissue and extracting sap for food, and they are also the direct means for acquisition and transmission of plant viruses (Harris and Maramorosch, 1977). Both alate and apterous forms of *M. persicae* (Sulzer) are capable of efficient virus transmission (Irwin and Goodman,1981).

Acquisition and inoculation threshold periods are but a few seconds in duration, therefore careful manipulation of the insects is necessary in the laboratory environment to achieve optimal conditions for virus transmission. The proportion of insects that transmit virus is greatly increased following a fasting period as brief as 15 min. (Harris and Maramorosch, 1977). Cho and Goodman (1982b) starved the aphids for 2-6 hours in glass bottles prior to the acquisition feed when they tested the transmissibility of several SMV strains by a variety of aphid species. Aphids cease to be viruliferous only minutes after acquiring virus. Loss of inoculativity follows an exponential curve. Irwin and Goodman (1981) demonstrated optimum transmission from acquisition probes of 30-60 seconds for SMV transmitted by *M. persicae*. Rates of transmission were lower with acquisition access of 15 min. or longer or with acquisition access of 15 sec. or less.

In Asia and parts of Africa, three species of aphids colonise soybean fields: *Aphis glycines*, *Aulacorthum solani* and *Aphis cracivora* they can be agricultural pests as well as vectors of SMV (Irwin and Goodman, 1981). In most other parts of the world aphids are not usually found on soybean crops; they may colonise weeds around the field and the occasional probing of alate aphids on soybean plants during their flight is enough to transmit the virus and account for field spread of SMV. The timing and rate of SMV spread is to a large extent a function of the activity of transient aphids that land on soybean leaves, probe, move to new plants, and probe again.

Soybeans are often grown near or adjacent to maize fields and sometimes have volunteer maize plants growing with them: the vector *Rhopalosiphum maidis* could pose a potential threat to SMV spread in the future (Abney *et al.*, 1976). It would therefore be important to closely monitor the epidemiology of this virus in South Africa as the area of cultivated soybean increases. Ensuring effective farming practices, such as removal of weeds that are

SMV hosts, will go a long way to safeguard soybean crops against the potential threat of a SMV epidemic.

2.2.4.2 Seed Transmission

SMV is transmitted in the seed of soybean plants. The rate of seed transmission generally ranges between 0 and 38%, according to cultivar (Iizuka, 1973), although a transmission rate as high as 68.4% was reported for the cultivar Midwest (Kendrick and Gardner, 1924). Seedborne SMV is the primary inoculum source in epidemics, since the virus is not known to overwinter in any other host plants.

Iizuka (1973) studied the mechanisms involved in seed transmission of the virus. It was suggested that seed transmission was due to the ability of the virus to infect the meristematic cells in infected plants. Soybean plants inoculated just before flowering produced fewer infected seed than those inoculated before that time. Plants inoculated after flowering had started produced still fewer infected seeds. Seed transmissions of SMV through either pollen or ovules of infected plants were obtained by crossing infected and healthy plants. Infected pollen usually transmitted viruses to a fewer number of progeny plants than infected ovules. SMV has been found in immature seed coats and in immature embryos of seed taken from infected plants. Developing embryos were only occasionally infected through direct invasion of SMV from the mother plant.

Bowers and Goodman (1979) investigated the development of seeds on SMV infected plants of two cultivars, one with relatively high incidence of seed transmission, and another in which seed transmission had yet to be demonstrated. Infective SMV was present in the immature seeds of both cultivars. In the transmitting cultivar, the incidence of infective virus in mature embryos was the same as the incidence of seed transmission. During the course of embryo development in the non-transmitting genotype an unexplained form of virus inactivation occurs so that no infective SMV particles could be recovered at embryo maturity. Irwin and Goodman (1981) suggested that some other form of the virus, possibly a replication intermediate, which was possibly associated with cell membranes, may be maintained in embryo cells. Sufficient intact virus would also have to be present since infectivity tests are possible. The results up to that point also suggested that desiccation or other forms of physical inactivation would be unlikely, though they did speculate on the involvement of inhibitors and specific chemicals in the inactivation process.

There are unconfirmed reports that the virus can be transmitted by pollen to the pollinated plant (Goodman, *et al.*, 1996). Since soybean plants are generally self-pollinated it is unlikely that such an occurrence would play an important role in field spread of the virus.

Several cultivars have been screened for their ability to transmit the virus in seed. SMV is not transmitted in the seeds of soybean cultivars Kwanggyo, Hill and Bienville; in addition 19 lines screened by Goodman *et al* (1979) did not transmit virus to their seed. Goodman and Oard (1980) reported that those varieties exhibiting low seed transmission rates also have lower yield reductions in plant weight.

2.2.4.3 Mechanical Transmission

SMV is sap transmissible. Generally, infected material is homogenised in a cold motar and pestle with chilled 50nM sodium phosphate buffer, pH7.0 (as described by Xu *et al*, 1986). A small amount of Celite or Carborundum is added the homogenate and the mixture is then rubbed onto the leaves of a host plant. The abrasives injure the inoculated plant tissue to effect virus penetration. It is best to inoculate the fully expanded primary leaves of young soybean plants (10-14 days old), as the plants are most susceptible to virus infection at this stage. The appearance of symptoms is to a great degree dependent on climatic conditions and may take between 4 and 14 days.

Difficulty in mechanical transmission of certain other (e.g. *Palm mosaic virus*) potyviruses has been attributed to the presence of virus inactivators or inhibitors of infection, in the source plant (Shukla *et. al.*, 1994). Such inactivators or inhibitors are usually O-quinones which are produced by *in vitro* oxidation of host plant polyphenols. The formation of non-infective virus-tannin complexes can sometimes be reduced by preparing inoculum with a cold extractant containing a copper chelating agent such as sodium diethyl dithiocarbamate and/or a reducing agent. The deleterious effects of preformed virus inactivators can usually be reduced by the inclusion of 1-2% polyethylene glycol or polyvynyl pyrrolidone.

Although most members of the *Potyviridae* are considered to be insufficiently infectious to be transmitted by inadvertently by mechanical means there have been some noted exceptions. *Tulip breaking virus* (TBV) and BYMV have been spread by contaminated cutting knives during flower harvesting of tulips and gladioli (Brierly, 1962), and *Passionfruit woodiness*

virus (PWV) has been recorded to be spread by frequent handling (Da Graca, 1976). Epidemiological studies have suggested that *Ryegrass mosaic virus* (RGMV) may be transmitted mechanically by contaminated machinery. There have been no reports as yet of such mechanical transmission of SMV.

2.2.5 Nucleic Acid Composition

SMV is a single stranded positive sense RNA virus. Hill and Brenner (1980) showed that the SMV RNA molecule had a $S_{20,w}$ value of 39.7 before and 25.4 after formaldehyde denaturation. Nucleic acid molecular weight determinations made by linear-log sucrose density gradient centrifugation gave values of 3.46×10^6 and 3.18×10^6, respectively, for native and formaldehyde-treated RNA. Electrophoresis on polyacrylamide gels gave molecular weight values of 3.02 x 106 both before and after formaldehyde denaturation. The nucleic acid melting point (Tm) has been observed to be 49°C in 0.1 M sodium phosphate, pH 7.0, and a hyperchromicity of 30.4%. Nucleotide composition was 29.9% adenylic acid, 24.3% guanylic acid, 14.9% cytidylic acid and 30.9% uridylic acid. The RNA constitutes 5.3% of the virus particle (Hill and Brenner, 1980).

The RNA sequence of the virus has been exhaustively exploited by molecular biological techniques and this will be discussed in greater detail in following sections. Nucleic acid hybridization (NAH) was used to demonstrate the relatedness of WMV2 and SMV in the 3'-noncoding region (NCR). The hybridization of the WMV2 probe of the 3'-NCR probe, with SMV RNA confirmed the earlier report based on amino acid sequences of the CP by Yu *et al.* (1989) that WMV2 and SMV-N were strains of the same potyvirus. The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) has proved to be a very successful diagnostic technique. Several PCR primers have been developed against several regions on the virus genome including the coat protein, NIa, NIb and CI regions (Pappu *et al.*, 1993 and Gibbs *et al.*, 1997). Continuous advances in the technique using degenerate primers, different enzymes, etc. has offered the laboratory worker improvements in speed, reproducibility and fidelity. PCR alone has an important role in diagnostics, however, PCR often is only the first step in a process to determine, either patterns of identity or, the actual RNA sequence. Cloning and sequencing techniques which support sequence determination have also advanced rapidly. Jayaram *et al.* (1992) sequenced the complete genome of the SMV G2 and G7 strains. Regions of other SMV strains have also been sequenced using specific PCR primers to target regions. This will discussed in greater detail in following sections.

2.2.6 Aspects of the Coat Protein and Serology

2.2.6.1 Coat Protein Structure and Assembly

The flexuous rod-shaped particles of potyviruses consist of around 2000 copies of a single species of coat protein, that encapsidates one copy of a single stranded positive sense RNA species (Brunt, 1992). Hill and Brenner (1980) showed the presence of two, or irregularly the presence of three bands on SDS-PAGE gels, when dissociated SMV coat protein was separated by electrophoresis. Amino acid analysis by High Performance Liquid Chromatography (HPLC) of tryptic peptides and protein has added greater value to the diagnostic potential of the coat protein. This technique reflects the extent of sequence identity between proteins and has been shown to clearly distinguish between distinct potyviruses and their strains. Jain *et al.* (1992) confirmed that 14 potyvirus isolates from soybean were strains of one virus, by comparing coat protein peptide profiles. The results of this study also suggested that SMV-N and WMV-2 were closely related, thus implying that WMV-2 could in fact be a pathotype of SMV.

The coat proteins of distinct potyviruses vary considerably in size as elucidated by protein and gene sequencing . The size differences are largely due to heterogeneity in the first quarter of the N-terminal end (Shukla *et. al.*, 1994). It is thought that this variability as been generated by major deletions, frameshift mutations or cleavage site mutations. The next quarter is increasingly conserved and the last half of the coat protein is the most conserved. The conservation is probably due to this region forming the tertiary an quaternary structures necessary for virus assembly (Shukla and Ward, 1989).

The secondary structure of the PVY-D CP as predicted by Shukla and Ward (1989) has ten regions of the α-helices and 4 sections of β-pleated sheets. Although little is known of the 3-D structure of the potyvirus coat protein, it is thought to have some resemblance to that of *Tobacco mosaic virus* (TMV). The N and C terminals are surface located (Shukla *et. al.*, 1988), and the N terminal is immunodominant. The DAG sequence which is involved in aphid transmission is also found on the protein surface. The basic pitch for the potyvirus nucleocapsid helix is 3.3nm with seven to nine CP subunits per ring (Goodman *et al.*, 1976). Approximately six nucleotides are associated with each CP subuinit (Veerisetty, 1979). Since potyviruses are flexuous rods, unlike the rigid TMV rods, and the TMV subassembly units

have been characterised as double discs, but this has not been demonstrated for PVY or other potyviruses, a slightly different subunit arrangement was proposed for the flexuous rod viruses. McDonald and Bancroft (1977) proposed that PVY protein subunits aggregate as stacked rings (almost like a close spiral), rather than stacked discs. The assembly mechanism of virus particles is yet to be adequately elucidated. Jaggdish *et al.* (1991) observed the formation of potyvirus-like particles when the JGMV CP was expressed in *Saccharomyces cerevisiae*. Site directed mutagenesis of highly conserved sequences, viz. WY at positions 130/131 to GP and RQ at 194/195 to DL, had a deleterious effect on the assembly of coat protein subunits.

2.2.6.2 Serology

Serological techniques have provided important tools in the identification, characterisation and epidemiological study of many potyviruses. However, serological interactions with the potyvirus CPs are extremely complex and inconsistent. Variable cross-reactivity of polyclonal antisera, unexpected paired relationships between distinct viruses and lack of cross-reactions between some related strains are some of the major problems associated with potyvirus serology (Shukla *et al.*, 1994).

The various strains of SMV are indistinguishable using polyclonal antisera (Hunst and Tolin, 1982). However, polyclonal antisera have been useful in conducting surveys for the virus (Pietersen and Garnett, 1990), and during routine screening for the virus in commercial farms, seed propagation farms and during cultivar/breeding trials. At the Agricultural Research Council-Plant Protection Research Institute, polyclonal antisera to the SMV G1 isolate 86/0020 (Pietersen and Garnett, 1992) have been raised according Pietersen and Garnett (1990) and is used in dilution of 1/1000 for serologic techniques such as immunoelectron microscopy (IEM), Enzyme-linked immunosorbent assay (ELISA) and Immunocapture reverse transcriptase PCR (IC-RT-PCR).

Refined serological techniques have made use of monoclonal antibodies (Mabs) as tools for strain differentiation. Hill *et al.* (1989) used purified virus, of which the coat protein was digested with trypsin, and then run on a gel which was then blotted and probed with a panel of 12 Mabs. This study showed that the SMV strains could be divided into three serological groups: Group1-G1; Group 2-SMV G2, G3,G4,G6, and G7; Group 3- G5, SMV-O, SMV,12-18, SMV 75-16-1 and SMV-Brazil. However the requirement for purified virus in this

technique means that antigenic properties of the capsid, and especially of the of the surface located epitopes, could be altered, which makes this technique unreliable (Rong *et al.*, 1993). This technique also does not lend itself to rapid analysis of field samples

Hill *et al.* (1994), used antigenic signature analysis to differentiate SMV isolates. This technique is based of the property that Mab binding is influenced both by affinity for and frequency of the epitope it recognises. Antigenic differences among 14 SMV strains were demonstrated with antigenic signature analysis employing a panel of nine well characterised Mabs. The results however did not determine serological relationships amongst the strains used in this study. Thus it would appear that such a technique might identify individual virus isolates without having adequate capability to group the isolates according to strains. However, the method does have scope to be used for identification of unique virus isolates in field trials as it requires minimum processing of plant tissue (Hill *et al.*, 1994).

2.3 GENOME OGANISATION

The genera within the *Potyviridae* with the exception of the bymoviruses, all have monopartite, single stranded, positive sense RNA genomes of approximately 10 000 bases. Most of the information on the genome structure has been generated in studies involving the aphid transmitted potyviruses, of which SMV is a member. The generic and familial potyviruses belong to the picorna-like subgroup of viruses whose genomes have a viral genome-linked viral protein (VPg protein) covalently bonded to the 5'- end, a poly (A) tail at the 3'- end and are expressed as a large polyprotein which is subsequently cleaved by proteases to yield several functional and structural proteins (Shukla *et. al.,* 1994).

The polyprotein has the following order: first protein (P1), helper component (HC-Pro), third protein (P3), 6Kda protein, cylindrical inclusion protein (CI), 6Kda protein, small nuclear inclusion protein (NIa) which includes the VPg protein at its N-terminus, large nuclear inclusion protein (NIb), and the coat protein (CP). In the native state, the RNA genome is preceded by a 5'- non-coding region (5'-NCR) and terminated by the 3'- non-coding region (3'-NCR), see diagram and table 3. It is only the VPg and coat proteins that are detected in purified virus preparations, the other gene products have been identified in infected plant tissue (Dougherty and Carrington, 1988; Rodriguez-Cerezo and Shaw, 1991).

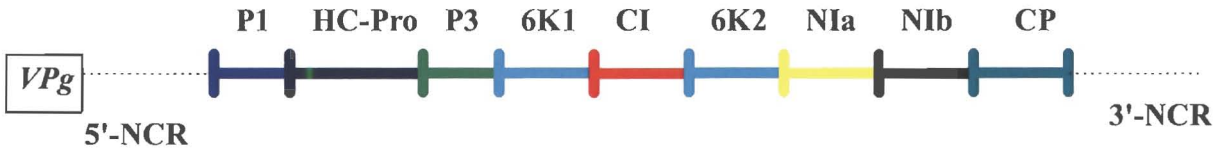


Figure 2.1: Schematic Representation of the SMV Genome

Table 2.3: Genome organisation of SMV

Region	Total Number of Nucleotides	Position of Nucleotides	Total Number of Amino Acids	Position of Amino Acids
5'-NCR	131	1 - 131	-	-
P1	924	132 - 1056	308	1-308
HC-Pro	1368	1057 - 2425	457	309 -765
P3	1040	2426 - 3465	347	766 - 1112
6K1	153	3466 - 3619	52	1113 - 1164
CI	1902	3620 - 5525	634	1165 - 1798
6K2	162	5526 - 5688	54	1799 - 1852
NIa-VPg	567	5689 - 6256	189	1853 -2041
NIa-pro	729	6257 - 6986	243	2042 - 2284
Nib	1151	6987 - 8538	517	2285 - 2801
CP	795	8539 - 9334	265	2802 - 3066
3'-NCR	±259	9335 - poly A tail	-	-
Total RNA without Poly A tail		9588		

2.3.1 The 5'-Non Coding Region

The length of the 5'- NCR varies in length between different species of potyvirus, from 85 nucleotides in *Papaya ringspot virus* (PRSV), to 131 in SMV and 205 in *Tobacco vein mottling virus* (TVMV). Alignment of several potyvirus 5'-NCR sequences (Lain, *et al.*, 1989) has shown that there are two conserved regions, that have come to be known as box a and box b. Box a is part of the Potybox consensus sequence UCAACACAACAU (Shukla, Frenkel and Ward, 1991 and Atreya, 1992). SMV has a total of 15 nucleotides that are identical to the consensus for box a and box b.

Potyviral RNA differs from host mRNA in the absence of a 5'-cap structure. As in most eukaryotic cells the 5'-cap and the 3'-poly(A) tail work together to effect translation, it is thought that some alternative process must exist in potyviruses. Revers *et al.* (1999) suggested that the 5'-NCR could possess an internal ribosome entry site (IRES), and though there was limited evidence (Basso *et al.*, 1994) for this, there is as yet no IRES-like structure that has been identified. The 5'-NCR has also been shown to function as an enhancer in the translation process of *Tobacco etch virus* (TEV) (Carrington and Freed, 1990). Wu and Shaw (1998) studied protoplasts infected with TVMV RNA at timed intervals to understand the process of potyvirus assembly. Their results suggested that the assembly of virus particles begins with the interaction of coat protein subunits with the 5' terminal region of progeny viral RNA molecules.

2.3.2 The P1 Protease (P1)

Much of the work on the P1 protease has been conducted on TEV and TVMV. It is a 35 kDa protein situated at the N-terminal of the polyprotein and has been demonstrated to have autocatalytic protease activity (Verchot *et al.*, 1991). The P1 protease effects the cleavage reaction between itself and the HC-Pro following HC-Pro autocatalytic cleavage from the rest of the polyprotein (Verchot *et al.*, 1991). The cleavage site lies between Tyr (304) and Ser (305) (Mavankal and Rhoads, 1991; Verchot *et al.*, 1992). Alignment of the potyviral P1 protein showed the presence of a Gly-x-Ser-Gly motif which is the consensus of a class of serine proteinases. This motif coincides with residues 254-257 in TEV. Substitution of TEV Ser (256) with most other residues resulted in diminished proteolytic activity (Verchot *et al.*, 1992).

In the rabbit reticulocyte translation system, P1 has failed to demonstrate proteinase activity (Carrington *et al.*, 1990). However in a wheat germ system such activity was observed (Mavankal and Rhoads, 1991). Verchot *et al.* (1992) demonstrated that the processing characteristics of P1 in mixtures of rabbit reticulocyte lysate and wheat germ extract was such that it suggested that a positive acting factor resided within the wheat germ extract. It was further observed that the factor was heat labile.

Beyond the proteinase activity of P1, very little is understood of is other functions in the lifecycle of a potyvirus. P1 protein from distinct potyviruses varies dramatically in size. But, when P1 protein from strains of SMV were compared (Jayaram *et al.*, 1992), it was shown

that there was a sequence identity of 98%. Shukla *et al.* (1994) resolved that the low sequence identity of P1 proteins of biologically distinct potyviruses suggests that P1 particularly in its N-terminal, non-proteinase domain may be involved in some specific virus-host reaction. Cell –to-cell movement may be one such interaction, however the situation is as yet unresolved. Mutant lacking the entire P1 coding region were observed for their abilities in genome amplification and cell-to-cell movement (Verchot and Carrington, 1995). P1 was thus shown to function *in trans* to stimulate genome amplification, however it was not strictly required. The level of cell-to-cell movement and systemic movement of the mutant was also reduced as compared to the parental virus.

2.3.3 The Helper Component - Proteinase (HC-Pro)

The HC-Pro has been associated with several functions in the virus life-cycle viz. proteinase activity, aphid transmission, cell-to-cell movement and most recently in post transcriptional gene silencing (Revers *et al.*, 1999). The proteinase activity has been shown to be effected by the 20 kDa protein located in the carboxyl terminal half of the HC-Pro (Carrington *et al.*, 1989). The cleavage site between HC-Pro and the rest of the polyprotein in TEV has been shown to be Gly (763) – Gly (764) and equivalent sites exist on the polyproteins of other potyviruses (Carrington *et al.*, 1989). The HC-Pro most closely resembles members of the papain-like cysteine proteinases.

Together with the coat protein, the HC-Pro is directly involved in the process of virus transmission by aphids. It has been shown that aphids cannot transmit purified potyvirus preparations unless they have prior or simultaneous access to HCs (Govier and Kassanis, 1974). In PVY and TVMV the apparent molecular weight of HC is 58 and 53 kDa, in its active form HC is thought to be a dimer (Hellman *et al.*, 1983). Immunochemical investigations (Dougherty and Carrington, 1988) suggest that the inclusion body form of amourphous inclusion protein (AI) may be inactive, and HC activity may be associated with a processed form of the inclusion protein. The motif Lys-Ile-Thr-Lys (KITC) has been shown to be essential in aphid transmission (Atreya *et al.*, 1992) and the Pro-Thr-Lys motif has also been shown to be involved (Pirone , 1991). In their review article Revers *et al.* (1999) stated that it has been suggested that HC-Pro acts as a bridge between virion CP and a putative protein receptor in the vector mouthparts, however a direct interaction has as yet not been established. Virion invasion of the immature seed, and therefore seed transmission is thought to be either directly or indirectly mediated by the HC-Pro (Wang and Maule, 1992).

Unlike other plant viruses which have dedicated movement proteins, this function in the *Potyviridae* is the result of the synergistic effects of the CP, CI and HC-Pro proteins (Rodriguez-Cerezo *et al.*, 1997 and Rojas *et al.*, 1997). The *Escherichia coli* expressed HC-Pro of *Lettuce mosaic virus* LMV) and *Bean common mosaic necrosis virus* (BCMNV) have been shown to increase the size exclusion limits of plasmodesmata (Rojas *et al.*, 1997). Long-distance movement of potyvirus particles has been attributed to the HC-Pro, VPg and CP. This phenomenon involves the movement of the infectious agent from the mesophyll via the bundle sheath cells, phloem parenchyma, and companion cells into phloem sieve elements, passive translocation in the phloem and unloading at a remote site to establish further infection foci (Carrington, 1996). Mutational analysis has shown that this function is determined by the central and N-terminal region of the HC-Pro (Klein *et al.*, 1994). Revers *et al.* (1999) cited 3 studies which have suggested that HC-Pro may act as the viral suppressor of the plant defence mechanism effected by post-transcriptional gene silencing (PTGS). The implication is that a complete block of long-distance movement could represent the indirect effect of PTGS in allowing extra time for other resistance mechanisms to come into play. In light of these findings it would be necessary to re-examine information collected thus far, with respect to PTGS in order to fully appreciate the plant/HC-Pro interaction.

2.3.4 The P3 Protein (P3)

The P3 protein has since its first identification as a 42 kDa non-structural protein, remained an enigma (Rodriguez-Cerezo and Shaw, 1991). The TVMV P3 protein is detected in membrane fractions of infected tissue and computer analysis of its deduced amino acid sequence suggests that P3 may be an integral transmembrane protein (Rodriguez-Cerezo and Shaw, 1991). Antiserum against TEV P3 protein was used to determine the subcellular location of the P3 protein in virus infected plant cells. Immunogold labelling with the antiserum showed labels associated with the nucleoli, nuclei and nuclear inclusions. P3 protein in the nucleus and nucleolus could indicate that it too is involved in the early stages of viral replication (Langenberg and Zhang, 1997). The 3' third of the TEV P3 protein has been implicated in the wilting response of Tobasco pepper (Chu *et al.*, 1997). Riechman *et al.* (1995) showed that mutations introduced to the *Plum pox virus* (PPV) in the P3-6K1 cleavage site either cause increased severity or attenuation of symptoms. In SMV it is thought that the pathogens ability to overcome Rsv1 resistance in the host is in some way mediated by the P3 protein and HC-Pro (Hill, 1999; pers. comm.).

2.3.5 The Cylindrical Inclusion Protein (CI)

The CI protein from PPV has been shown to be a RNA helicase (Lain *et al.*, 1990, 1991). The CI protein can unwind RNA duplexes in the presence of NTP and has a nucleic acid stimulated ATPase activity. It functions in the 3' to 5' direction, as is expected since the RNA template is read by the polymerase in the 3' to 5' direction with the newly synthesised complementary chain, primed by the 5'-linked-VPg , growing in the 5'-3' direction (Shukla *et al.*, 1994). Mutational analysis of TEV CI protein (Carrington *et al.*, 1998), identified 2 mutants that were altered in the N-terminal region that replicated to usual levels, but were defective in cell-to-cell movement. Other CI protein mutants were only weakly replicated in infected protoplasts suggesting the role of potyviral CI protein in the replication process.

Some elegant IEM studies by Rodriguez-Cerezo *et al.* (1997) have shown the TVMV CI to be associated with the plasmodesmal connection between mesophyll cells, prior to the appearance of the CP or any other virus-induced features or effects. They also showed the accumulation of CI in the form of conical structures, which appeared to penetrate the host cell walls and connect to similar structures in the adjacent cells. The wall bound deposits of CI contain P3 protein and are associated with complexes containing viral CP and RNA. Their results thus suggested that the formation of specific structures by potyviral CI proteins is required for and plays a direct role in the intercellular passage of viral genetic material in the form of virus particles or complexes containing viral CP and RNA. Roberts *et al.* (1998) showed similar results working with PSBMV, but went further to demonstrate that behind the infection front, CIs no longer associated the host cell-wall or with CP, and collected as the characteristic pinwheel structures in the cytoplasm.

2.3.6 The 6K1 and 6K2 Proteins

The 6K1 and 6K2 proteins appear to be involved in the RNA replication. They have long stretches of hydrophobic amino acids, and have a similar location in the potyviral genome as the 2B and 3A proteins have in the picornaviral genome (Riechmann *et al.*, 1992). However it is important to exercise caution in ascribing function based only on structural evidence. Riechmann *et al.* (1992) went further to suggest that the 6K2 protein could anchor the NIa-VPg to the membranes and that cleavage of the 6K2-NIa junction may be related to some step in the RNA replication process. Mutations in the PPV P3-6K1 cleavage site caused either

attenuated or more severe symptoms (Riechmann *et al.*, 1995). Thus at this stage the functions of the 6K1 and 6K2 proteins arenot quite clear.

2.3.7 The Nuclear Inclusion *a* (NIa)

The NIa or small nuclear inclusion protein plays an important role in the proteolytic processing of the potyvirus polyprotein. It has a 2 domain structure; the N-terminal domain is the genome linked VPg protein (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990) and the C-terminal is the proteinase (Dougherty *et al.*, 1988). In their review, Dougherty and Carrington, (1988), discussed the autocatalytic cleavage by NIa at the CI-NIa junction and at the NIa-NIb junctions. The additional cleavages release the 6K1, 6K2 and and VPg proteins (Garcia *et al.*, 1989; Restrepo-Hartwig and Carrington, 1992)

2.3.7.1 The VPg Protein

The virus coded VPg protein is linked by a phosphoester bond (Shukla *et al.*, 1994) to the 5' end of the virus genome. By analogy of the VPg of other viruses it is believed that the VPg acts as a primer for v RNA synthesis Shahabuddin *et al.*, 1988).

2.3.8 The Nuclear Inclusion *b* (NIb)

The NIb is believed to be a RNA-dependent RNA polymerase of potyviruses (Shukla *et al.*, 1994). It is also the most conserved gene product of potyviruses.

2.3.9 The Coat Protein (CP)

The CP has been associated with functions of encapsidation of viral RNA, vector transmission and cell to cell movement (Atreya *et al.*, 1991 and Rodriguez-Cerezo, 1997). In SMV the CP gene is composed of 795 nucleotides. Jayaram *et al.* (1992) showed that there were 31 nucleotide differences in this region between SMV strains G7 and G2. This represented a 96% homology which translated into only 3 amino acid differences in the region. Sections 2.2.6.1, 2.2.6.2 and chapter 3 provide detailed discussions on the CP.

2.3.10 The 3'-Non Coding Region (3'-NCR)

The 3'-NCR has a dual function. Firstly it interacts with the virus replicase during the initiation of minus-strand RNA synthesis (Bryan *et al.*, 1992). Secondly the presence of the poly-A tail prevents exonucleolytic degradation of the viral nucleic acid (Dolja and Carrington, 1992). Comparison of the 3'-NCR sequences of 14 strains from 7 distinct

potyviruses, showed that the region was approximately the same size in related strains and they shared greater than 80% homology, whilst the length of the 3'-NCR varied considerably between distinct potyviruses and they generally shared less than 50% homology (Frenkel *et al.*, 1989). The authors went further to demonstrate the usefulness of the 3'-NCR to resolve the debate as to the nature of the relationship between WMV2 and SMV-N. The amino acid homology between the coat proteins of WMV2 and SMV-N was 83% (Yu *et al.*, 1989) this lay midway between the ranges of similarity for independent potyviruses (38-71%) and related strains (90-99%). However the authors concluded that the degree of homology and the location of sequence differences between WMV2 and SMV-N is much closer to strains of the same virus than that found between distinct potyviruses. Nucleic acid sequence homology between WMV2 and SMV-N in the CP region was 82% and 78% in the 3'-NCR (Frenkel *et al.*, 1989). Thus based on this evidence Frenkel *et al.* (1989) resolved to support Yu *et al.* (1989) that WMV2 and SMV-N are strains of the same virus. In the same study Frenkel *et al.* (1989) showed that SMV-N had only 60% and 45% homology with SMV-VA in the CP and NC regions respectively. Thus they could not further postulate as to whether WMV2 and SMV-N were isolates of SMV or WMV2. It is important to note at this stage, that "SMV-VA" is no longer recognised as representative of viruses causing soybean mosaic disease and was originally misidentified (Rybicki *et al.*, 1992). Frenkel *et al.* (1992), developed a probe based on the 3'-NCR's of WMV2 and SCMV and expounded further the utility of the region in discriminating between distinct viruses and strains of potyviruses. The WMV2 probe produced positive signals for WMV2 and SMV-N, PCR products and infected plant material but not for isolates of SCMV.

2.4 CONTROL OF SMV

Various strategies based on epidemiological research have been considered for the control of SMV. Irwin and Goodman (1981) discussed methods of vector management including breeding soybean cultivars with resistance to aphids. It is believed that aphids land indiscriminately in plant canopies and determine their natural hosts from others only after probing (Gibson *et al.*, 1977). Wavelength reflectance can attract (yellow spectrum) or repel (ultraviolet spectrum) certain aphid species; thus it was considered theoretically possible to manipulate plant genetics to repel aphid species (Irwin *et al.*, 1981). Similarly reflective mulches were postulated as a method of reducing landing frequencies of aphids in soybean fields (Irwin *et al.*, 1981). However none of these envisaged control measures have ever been found to be practically useful. So too the case with insecticides, they are ineffective at

reducing the spread of non-persistently transmitted viruses as they kill the vector only after initial probing, which is usually adequate to transmit such viruses (Broadbent, 1969). However, Bottenberg *et al.* (1992) reported some success by intercropping cereals of the similar height with soybeans. It is thought that the effectiveness of this practice was related to the reduced aphid landing rates and/or the higher probability of aphids losing their SMV transmission ability after landing and probing non-host plants. The use of cereals of similar height to soybean plants would be to remove the effect of shading and competition.

The greatest success in controlling the disease has come from research into breeding soybean cultivars which incorporate SMV resistance genes. Resistance to SMV has been identified in several soybean cultivars (Cho and Goodman, 1979; 1982 ; Lim, 1985, Ma *et al.*, 1995), however most of them are not resistant to all strains of the virus. The dominant resistance gene in cultivar PI 96983 had been identified and designated as *Rsv* (now *Rsv1*) according to the work of Kihl and Hartwig *et al.* (1979). Alleles of this gene which confer differential reactions to SMV strains G1 to G7 have been identified in the following cultivars Ogden (*Rsv1-t)*, York (*Rsv1-y)*, Marshall (*Rsv1-m*) and Kwanggyo (*Rsv1-k*) (cited by Ma *et al.*, 1995). Wang *et al.* (1998) screened four soybean cultivars from China and identified resistance genes in 3 of them which were not at the *Rsv1* site. Gai *et al.* (1989) found that resistance to each of 4 Chinese strains of SMV was conferred by a separate dominant gene A, C, G or H. All four were in the same linkage group with the order of G-H-A-C. The resistance genes LR1and LR2 were identified in PI486355 and documented by Ma *et al.* (1995).

The primary source of virus inoculum in the field is infected seedlings arising from infected seeds. Many researchers have attempted to develop soybean cultivars with reduced ability to transmit SMV via seed (Irwin *et al.*, 1981). Bowers *et al.*, (1991) found that resistance to SMV seed transmission is strain specific. Tu (1992) reported that higher percentages of seed infection occurred with early infection of plants. It is imperative for soybean breeding programmes to consider disease epidemiology, strain variation and host resistance genes to develop successful strategies.

3. MOLECULAR CHARACTERISATION OF THE PARTIAL COAT PROTEIN GENE AND THE 3'-NONCODING REGION

3.1 INTRODUCTION

Given the difficulties in taxonomic classification, it is no wonder that there has been much deliberation as to the genome fragment that most accurately represents the variation of the entire genome of a given potyvirus isolate. Though the various potyvirus proteins have been characterised, there are comparatively few complete genome sequences available; thus, basing deductions on undisputed empirical evidence is impossible. Shukla *et al.* (1991) compared full genome sequences of four distinct potyviruses and showed that P1, P3 and the N-terminal of the CP were the most variable regions of the polyprotein. The NIb region was shown to be the most conserved region in the same study. Analysis of the sequence diversity of the P1, HC, P3, NIb and CP genomic regions of several Y*am mosaic virus* (YMV) isolates, have shown two levels of diversity: above 90% sequence homology was observed between YMV isolates of the same group (intragroup), regardless of the region concerned, whereas identities between isolates from the different groups (intergroup) were lower and depended on the protein concerned (Aleman-Verdaguer *et al.*,1997).

Comparative analysis of whole CP sequences of the *Potyviridae* have been shown to be quite reliable for differentiation between closely related strains and to show groupings of more distantly related strains (Rybicki and Shukla, 1992). The N-terminal of the coat protein is variable and immunodominant, whilst the C-terminus tends to be conserved with only 18-20 amino acid residues that are surface exposed (Shukla *et al.*, 1994). Comparisons between SMV G2 and G7 have shown that in the 795 nt CP gene, there are 31 differences, representing 96% sequence homology, which translated into only 3 amino acid differences in that region (Jayaram, Hill and Miller, 1992). Analysis of other regions showed that the 5'- and 3'-NTRs showed 90% and 93% homology respectively, whilst other proteins shared nucleotide sequence identities of between 94 and 98%. The coat protein sequences of other virus families has also provided important information on the strain identity of virus isolates. Gillings *et al.* (1993) used restriction analysis of the coat protein gene of the *Citrus tristeza virus* (CTV), to characterise isolates and strains of the virus.

Sequence identity in the 3'-NCR has been touted as a valuable determinant in relatedness of strains. Unlike the CP gene which has extended sequences of homology between distinct potyviruses, similar sequences have not been observed in the 3-NCR (Frenkel *et al.*, 1992). Comparison of the 3'-NCR sequences of 14 strains from 7 distinct potyviruses, showed that the region was approximately the same size in related strains and they shared greater than 80% homology, whilst the length of the 3'-NCR varied considerably between distinct potyviruses and they generally shared less than 50% homology (Frenkel *et al.*, 1989). Section 2.3.10 provides a description on the use of the 3'-NCR sequences as in the characterisation of strains of a distinct virus.

A polymerase chain reaction (PCR) amplification of cDNA which enabled amplification of that region in potyviruses that lies between the mid-region of the CP and the end of the 3'NCR was documented by Pappu and colleagues in 1993. The degenerate primers were based on the conserved WCIEN/WCIDN box of the potyviral CP, and the 3'-poly A tail. The authors had shown that the method combined the advantages of the group-specific PCR detection technique of Langeveld *et al.* (1991) with the strain specific 3'-NCR hybridization / sequencing approach of Frenkel *et al.* (1992). This technique was originally applied to *Dasheen mosaic virus* (DMV), and nucleic acid sequence results of the PCR fragment established it as a distinct potyvirus. The size of the PCR fragment that is generated in this reaction varies according to the potyvirus template that is used. In the case of WMV2 and SMV-N the PCR fragment was found to be approximately 0.7 kb (Pappu *et al.*, 1993).

It was hypothesised the partial coat protein gene sequence together with the 3'-NCR could possess sequences which could be useful in developing a technique for the detection and strain differentiation of SMV isolates. The PCR primers which were developed by Pappu *et al.* (1993) would facilitate amplification of such a target region, and the PCR products would be of an ideal size for further manipulation. In an attempt to prove the hypothesis, PCR products of the target region of several SMV strains were to be generated. The PCR fragments were then to be cloned and sequenced. The sequence information would then be analysed with the intention of determining restriction enzyme sites that would group the isolates according to their respective strains as observed on a differential cultivar host range. Thus, restriction sites that were unique to isolates that are typical of the G1 strain would facilitate specific detection of that strain by restriction enzyme digestion of the original PCR product. The method of RT-PCR and restriction enzyme analysis would then be used to

determine the strain identity of archived SMV infected material plant material. Since the SMV G1 strain has been found to be the most predominant strain in SA (Pietersen *et al*, 2000), the technique would enable rapid positive identification of the G1 strain, and negative results would warrant further research on that isolate and strain determination according to the traditional method of a differential cultivar host range. The method would assist plant breeders in regular determination of SMV strain profiles in soybean growing regions and thereby hopefully provide farmers with more accurate information for determining suitable SMV resistant soybean cultivars.

3.2 MATERIALS AND METHODS

3.2.1 Virus Isolates

The SMV isolates used in following protocols were either purchased from the American Type Culture Collection (ATCC), or collected in South Africa. The following ATCC isolates were utilised: PV716, PV718, PV720, PV721, and PV724. These isolates were contributed to the ATCC by J. Hill and were initially detected by Cho and Goodman (1979) and then utilized in experiments documented in Hill *et al.* (1989 and 1984). These isolates have been confirmed to display strain specific behaviour on a differential cultivar host range in our laboratories (see table below). The South African SMV isolate 86/0020, previously documented by Pietersen and Garnett (1992), is a local SMV G1 strain that was collected in soybean plantations in the Roodeplaat vicinity in 1986. The isolate 94/2052 was collected in soybean fields in the Grobblersdal area in 1994 and has been confirmed on a differential cultivar host to be a G3 strain. The South African viral sources were isolated by two single-aphid transfers (Noordam, 1973; Hollings and Lelliot, 1960). Strain determination (see table 3.1) was conducted as per the differential cultivar host range (Cho and Goodman, 1979).

Table 3.1: Source and description of virus isolates used in this study

Isolate Name	PPRI Accession Number	Strain	Origin
86/0020	86/0020	G1	SA[1]
94/2052	94/2052	G3	SA
PV716	94/2151	G1	ATCC[2]
PV718	94/2153	G3	ATCC[2]
PV720	94/2155	G5	ATCC[2]
PV721	94/2156	G6	ATCC[2]
PV724	94/2158	G7a	ATCC[3]

[1]Pietersen and Garnett, 1992

[2]Hill *et al.*, 1989

[3]Hill *et al.*, 1984

3.2.2 Maintenance Hosts

The virus cultures were usually maintained on the *G. max* cultivar Rampage. On occasion other cultivars such as Marshall, Ogden, Davis, York or Kwanggyo were used, depending on the virus strain and availability of host plants. The seeds were sown in plastic pots containing a potting mix used for all routine propogation. The seedlings were held in an environment controlled disease and insect free glass house until they were approximately 2 weeks old and the primary leaves had emerged.

The seedlings were then transferred to an insect free environment controlled growth room. These disease free seedlings were inoculated with freeze dried or desiccated material which had been stored at 4°C, and rehydrated with inoculation buffer. A pinch of carborundum was added to the inoculum, and this mixture was applied to the primary leaves by gently rubbing it between the thumb and finger tips. No signs of burning or tearing were observed. Virus infection and establishment in the host plants was most effective when the seedlings were inoculated prior to the emergence of the first trifoliates. Symptoms, which were observed between 4 to 9 days after inoculation, included local necrotic lesions, mild to severe mosaics, vein clearing and leaf curl. A Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA)was performed to confirm virus infection if the symptoms were vague. Host plant symptoms were monitored and recorded regularly. Prior to the demise of host

plants, fresh leaf tissue displaying the characteristic symptoms was macerated in approximately 1:10 w/v dilution with buffer and the mixture was inoculated onto healthy seedlings as described above. For the duration of the study, between one and five maintenance hosts were available as virus sources for each of the isolates described above.

3.2.3 The Immuno Capture Reverse Transcriptase Polymerase Chain Reaction

3.2.3.1 Immunocapture

500 µl PCR tubes were coated with SMV 86/0020 polyclonal antiserum in a dilution of 1:1000 and held overnight at 4°C. The tubes were then washed twice with PBS-Tween (0.02M PBS and 0.5ml/l Tween 20). Virus infected plant material was macerated in the ELISA extraction buffer in a 1:100 w/v dilution. The mixture was then transferred to an eppindorf tube and microcentrifuged. 100µl of the light green supernatant was then transferred to the washed PCR tubes. Uninoculated soybean plants were handled similarly and used as controls in parallel assays. The immunocapture process was thus effected, and the tubes were held at 4°C overnight for optimal results. The tubes were washed twice prior to the addition of RT- PCR reagents.

3.2.3.2 Reverse Transcription and Amplification

The single-vial, two stage protocol for reverse transcription PCR was documented by Pappu *et al.*, 1993. Whilst the original authors obtained the nucleic acid templates by crude extraction of the RNA, I modified the procedure to obtain the nucleic acid templates using the immunocapture technique as documented above.

The upstream forward reaction was primed by the degenerate CN48 primer (PPRI accession no. 96/1201) which targeted the WCIDN box and had the sequence TGGTGYATHGANAATGG. The reverse reaction was primed by the oligo d(T) primer (PPRI accession no. 96/1202) which annealed to the polyA tail on virus genome and had the sequence TTTTTTTTTTTTTTTTTTTTN. The PCR reagents were added in the following concentrations the washed PCR tubes: 1µM of each primer, 0.2% Triton X-100, 350 µM of each dNTP, 1.5mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl pH 8.8 at 25°C, 10 mM DTT, 35.6 units HPRI RNAse inhibitor (Amersham), 40 units M-MLV reverse transcriptase (Life Technologies), 1 unit of *Thermus brockianus* DNA Polymerase (DyNAzyme by Finnzymes Oy). The final reaction volume was 100µl, and was overlayed with 50µl of liquid paraffin to prevent evaporation.

A Hybaid Omnigene thermocycler was programmed for reverse transcription at 37°C for 45 min. Template denaturation then took place at 94°C for 1 min (2 min for the first cycle), primer annealing at 42°C for 1 min, and DNA synthesis at 72°C for 2 min. This programme was repeated for 35 cycles and was terminated with a final elongation at 72°C for 10 min. The resulting nucleic acids were separated in a 1% agarose (w/v) gel containing ethidium bromide (0.5µg/ml), in a TAE buffer medium at 100V for 30-40 min. The PCR products were observed in UV light with the UVP Imaging System and results were analysed with UVP Gelworks 1D Advanced (Version 3, 1996) software by Nonlinear Dynamics.

Although PCR products for all isolates were produced, it was decided that for the purpose of developing a method of detection for the G1 strain that it would be necessary to clone and sequence both isolates of the G1 strain. In addition three other isolates viz. 94/2052, PV718 and PV724, were also cloned and sequenced. Previously published SMV sequences which were available with the BLAST search tool included "G2" (PV 723), "G7" (PV722) (Jayaram *et al.*, 1992), SMV-N (Eggenberger *et al.*, 1989) and WMV2 (Frenkel *et al.*, 1989) which is now recognised as a strain of SMV (VIDE database). All these sequences were to be used for comparative analyses that would enable selection of target sequences that would be unique to G1 isolates.

3.2.4 Cloning and Sequencing of PCR Products

Following observation of the PCR products, the 0.7kb fragment was excised from the gel. The Gene Clean™ kit (Bio 101) was then used to purify the DNA fragments from the agarose gel . Standard cloning techniques were utilised (Sambrook *et al.*, 1989). The fragments were treated with PolI to produce blunt ends (Amersham) and T4 polynuceotide kinase (Amersham) enzymes to produce flush ends which were necessary for blunt end cloning. The DNA fragments then had to be removed from the above reaction mixture, as those reagents would inhibit following processes, thus a second Gene Clean™ was performed. The concentration of DNA yielded after each purification step was determined spectrophotometrically.

For blunt end cloning the ratio of pmol 5' ends between insert and vector may vary between 5: 1 and 100: 1 (Dieffenbach *et al.*, 1998). Ratios of between 5:1 and 7:1 were used in this protocol.

Competent cells were obtained either using the Inoue (1990) protocol or from the Stratagene cloning kit. Competent *E. coli* XL1 blue cells were transformed with the ligation product. protocol. The cells were plated out on Luria Bertani agar containing (100µg/µl) ampicillin. IPTG (2.4% w/v in water) and X-gal (2% w/v in N,N-dimethylformadide were used for easy colorimetric determination of transformants (Sambrook *et al.*, 1989). Plates were incubated overnight at 37°C. White colonies indicated the presence of transfected cells which contained plasmid that was ligated to the SMV target sequence, whilst blue colonies indicated the presence of plasmid without the target sequence.

3.2.5 PCR Detection of Full Length Clones

White colonies were picked off the agar plates with sterile toothpicks and transferred to Luria Betani broth containing (100µg/µl) ampicillin. Each colony assumed the name of the SMV strain from which the insert originated and was further given a clone number e.g 86/0020 clone1. The cultures were incubated overnight at 37°C on a shaker.

The M13 reverse primer (PPRI accession number 96/1212) 5'-CAGGAAACAGCTATGAC-3' and the M13 -20 primer (PPRI accession number 96/1211) 5'-GTAAAACGACGGCCAGT which anneal to those regions that flank the multiple cloning site in pBluescript primed these reactions. PCR reagents were used in the following concentrations: 1µM of each primer, 175µM of each dNTP, 0.005% DMSO, 50mM KCl, 10mM Tris-HCl pH 8.8 at 25°C, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 2 units *Thermus aquaticus* DNA Polymerase (BioTaq by BioLine). The reaction mix was generally made up to 100µl and then dispensed as 10µl aliquots into labelled PCR tubes. The plasmid templates contained in the *E.* coli cultures were transferred to the PCR vessel with a sterile pipette tip, which was first immersed in the overnight culture and then in the relevant PCR tube. This was done in the aseptic environment of a laminar flow unit. As above the reaction was effected by a Hybaid Omnigene thermalcycler. Denaturation occurred at 94°C for 2 min in the first cycle and at 93°C for 45 sec in the following 35 cycles. Primer annealing at 43°C for 40 sec, and DNA synthesis at 72°C for 1 min. This programme was repeated for 35 cycles and was terminated with a final elongation at 72°C for 10 min. The resulting nucleic acids were separated in a 1% agarose (w/v) gel containing ethidium bromide, in a TAE buffer medium at 100V for 30-40

min. The PCR products were observed in UV light with the UVP Imaging System and results were analysed with UVP GRAB and UVP Gelworks software.

3.2.6 Automated DNA Sequencing

Nucleic acid sequencing services were rendered by the University of Cape Town, Department of Microbiology, using the "ALF- Express Automated Sequencer".

PCR product from only one reaction (per isolate) was cloned and sequenced, due to time constraints. In retrospect it would have been better to sequence at least two independent clones from independent PCR amplifications, since DNA polymerase is not 100% accurate.

At least three clones of each isolate of SMV were sequenced. In the case of discrepancies the documented sequence was observed 2/3 times. However in the case of isolates 94/2052 and PV 720 results from only two clones were used.

3.2.7 DNAMAN Sequence Analysis Software

Sequence results were analysed with DNAMAN for Windows (Lynon Biosoft, 1996) software package. The multiple sequence editor provided optimally aligned output and is based on the methods of Thompson *et al.* (1994).

3.3 RESULTS AND DISCUSSION

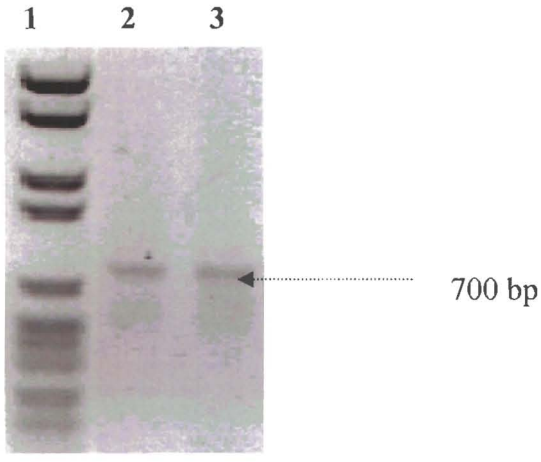


Figure 3.1: A PCR of 86/0020 and PV 716 with the CN48 and oligo d T primers. Lane 1 Boehringer Mannheim Marker VI (2176 bp – 154 bp), Lane 2: 86/0020, Lane 3: PV716.

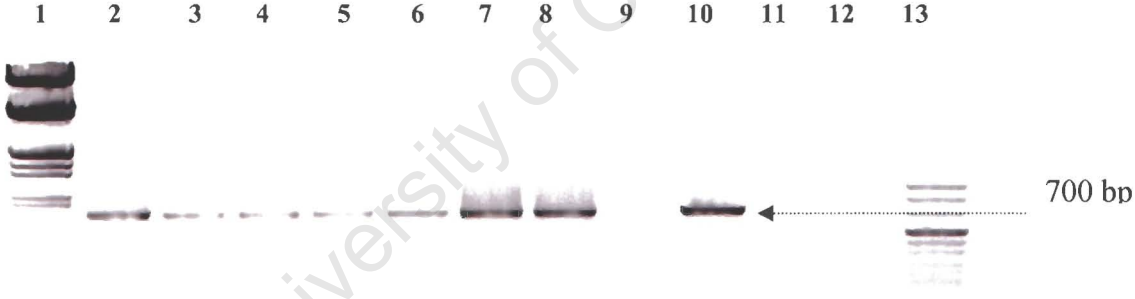


Figure 3.2: Results of PCR of several SMV strains with the CN48 and oligo d T primers. Lane 1: Boehringer Mannheim DNA Marker III (23130 bp-125 bp), Lane 2: PV 718, Lane 3: PV720, Lane 4: PV720 (repeated), Lane 5: PV721, Lane 6: PV721 (repeated), Lane7: PV724, Lane 8: PV724 (repeated), Lane 9: 94/2052, Lane 10: *Ornithogalum mosaic virus* (OrMV) insert in plasmid (positive control), Lane 11: Uninoculated soybean plant sap (negative control), Lane 12: PCR reagents without nucleic acid template, Lane 13: Boehringer Mannheim Marker VIII (1114 bp - 19 bp).

All the isolates tested were amplified under the stated conditions as shown in Figures 3.1 and 3.2. Due to a problem during gel loading, the band for isolate 94/2052 (lane 9) is only vaguely visible in Figure 3.2, adequate PCR product was however obtained for use in following steps. The PCR products were usually approximately 0.7 kb in length with the

smallest fragments being 679 bp. These variations in size appear to be a result of the limitations in the gel screening technique and the software used in size determination. The CN48/oligo dT reaction has previously been documented to produce amplification products of "approximately 0.7 Kb" from WMV2 and SMV-N (Pappu *et al.*, 1993), thus products that fell within a range of 0.7 kb were accepted as specific amplification products of the optimised PCR system.

Although blurred non-specific bands are observed in Figure 3.1, this was not an unusual result since Pappu *et al.* (1993) also observed non-specific amplification with healthy tissue extracts. They attributed this phenomenon to the to the degeneracy of the oligo dT primer which could have primed and amplified polyadenylated mRNA of the host. Since the correct sized bands were excised from the agarose and nucleic acid was then purified with the Gene Clean™ kit, I was able to guard against cloning non-specific bands into vectors.

```
86/0020    CACGTCTCCAGATGCCAATGGTGTGTGGGTAATGATGGATGGAGAGGAACAGATCGAATA   60
PV 720     ---a----------------c--------g----------------------t--g--   60
PV 723     t--a----------t-----c--------g----------------------t-----   60
SMV-N      t--a----------t-----c--------g----------------------t-----   60
PV 724     ---a----------t-----c--------g----------------------t-----   60
PV 716     t--a----------t-----c--------g----------t-----------t-----   60
94/2052    t--a----------t-----c--------g----------------t-----t-----   60
PV 718     ---a----------------c--------g----------------------t-----   60
PV 722     t--a----------t-----c--------g----------------------t-----   60
WMV-2      t--a----------t------a--t-----g--------------a--g--ag-t--g--   60

86/0020    CCCACTGAAGCCCATCGTTGAAAATGCAAAACCAACTTTGAGGCAAATCATGCACCATTT  120
PV 720     t--g----------t--------------------------a----------t-----  120
PV 723     t--g-----a-----t--c----------------------a----------------  120
SMV-N      t--g-----a-----t--c----------------------a----------------  120
PV 724     t--g-----a-----t--c----------------------a----------------  120
PV 716     t--g-----a-----t--c----------------------a----------------  120
94/2052    t--g-----a-----t----------g--------------a----------t-----  120
PV 718     t--g--a--------t--c----------------------a----------t-----  120
PV 722     t--g-----a-----t--c----------------------a----------------  120
WMV-2      t---t-a-----a--t----------------------c-a--a----------------  120

86/0020    TTCAGATGCAGCAGAAGCTTACATTGAGATGAGAAATTCTGAAAGTCCGTATATGCCTAG  180
PV 720     ------------------------------------------------------------  180
PV 723     c-----------------------------------------------------------  180
SMV-N      c-----------------------------------------------------------  180
PV 724     c-----------------------------------------------------------  180
PV 716     c-----------------------------------------------------------  180
94/2052    c-----------------------------------------------------------  180
PV 718     ------------------------------------------------------------  180
PV 722     c--------------------t-----a--------------------------------  180
WMV-2      c-----c----------a--t-----a--------c----------------------  180

86/0020    ATATGGACTACTGAGGAATTTGAGAGATAGAGAGTTAGCCCGTTATGCCTTTGATTTCTA  240
PV 720     ----------------------------c--------g-----c-----t----------  240
PV 723     ------------------------------------c----t--c-----t----------  240
SMV-N      ------------------------------------c----t--c-----t----------  240
PV 724     ------------------------------------c----t--c-----t----------  240
PV 716     ------------------------------------c----t--c-----t----------  240
94/2052    ------------------------------------c----t--c-----t----------  240
PV 718     ---------gt-------------------------------c-----t-----c-----  240
PV 722     ---------g--a--a--------------------------c-----t-----c-----  240
WMV-2      ---c---t----a--a----------c--g--a-----a--c-----t-----c--t--  240

86/0020    TGAGGTCACCTCCAAAACACCGAACAGGGCAAGGGAGGCAATAGCACAAATGAAGGCTGC  300
PV 720     ---------t----------a-------------a--------g-------------  300
PV 723     ------t--t--t--------a-------------a--------g--g----------  300
SMV-N      ------t--t--t--------a-------------a--------g--g----------  300
PV 724     ------t--t--t--------a-------------a--------g--g----------  300
PV 716     ------t--t--t--------a-------------a--------g--g----------  300
94/2052    ------t--t--t--------a-------------a--------g--g----------  300
PV 718     ---------t--t--g-----a-------------a--------g-------------  300
PV 722     ---------t--t--------a-------------a--------g-----a--------  300
WMV-2      ------t--t----------t--t--------a--a--------------------c--  300
```

```
86/0020    AGCTCTCTCGGGAGTTAACAACAAGCTGTTTGGGCTTGATGGAAACATCTCGACCAACTC  360
PV 720     ----------------------------------------------c-----------------  360
PV 723     -------------------------t-------a--------g--------a--------  360
SMV-N      -------------------------t-------a--------g--------a--------  360
PV 724     -------------------------t-------a--------g--------a--------  360
PV 716     -------------------------t-------a--------g--------a--------  360
94/2052    -------------------------t-------a--------g--t-----a--------  360
PV 718     -------------------------t-------a-----------t-----a--------  360
PV 722     -------------------------t-------a-----------t-----a--------  360
WMV-2      -------g-----a------g--g-t-a--------------t--t-----------t--  360

86/0020    CGAAAATACTGAAAGGCACACTGCAAGAGATGTGAATCAAAACATGCATACTCTTCTGGG  420
PV 720     ----------------------------------------g--------c------t----  420
PV 723     --------------------------g--------------------c------t----  420
SMV-N      --------------------------g--------------------c------t----  420
PV 724     --------------------------g--------------------c------t----  420
PV 716     --------------------------g--------------------c------t----  420
94/2052    --------------------------g--------------------c------t----  420
PV 718     --------------------------g--------------------c-----------  420
PV 722     --------------------------g--------------------c-----------  420
WMV-2      ------------g-------------g--c--------g--t---------t-gt----  420

86/0020    CATGGGCCCACAGCAGT...AAAGGCTAGGTAAACTGGCCACAGTTATCATTTCGGGTCG  477
PV 720     -----------------...-------------------------------------  477
PV 723     -----------c-----aat--------a-----t---t--------------------  480
SMV-N      -----------c-----aat--------a-----t---t--------------------  480
PV 724     -----------c-----...---------a-----t---t--------------------  477
PV 716     ---a-------c-----...---------a-----t---t--------------------  477
94/2052    -----------------...---------a-----t---t--------------------  477
PV 718     -----------------aa...----c-a-----t-----------------------  477
PV 722     -----------------aat--------a-----t-----------------------  480
WMV-2      t-----t--g-c---------.-----a-------------t--------g---------  477

86/0020    CTTTATAGTTTGCTATAGTATAGTA.GTTGCACTTCCTTTAAGTATAGTGTGATTGCATC  536
PV 720     -----------t----------t--.---------ttc-----------------a----  536
PV 723     -----------a-----a-------.--------gt------a----------------  539
SMV-N      -----------a-----a-------.--------gt------a----------------  539
PV 724     -----------a-----a-------.--------gt------a----------------  536
PV 716     -----------a-----a-------.--------gt------a----------------  536
94/2052    -----------a-----a-------.--------gt------a----------------  536
PV 718     -----------------a-------.---------t----g------------------  536
PV 722     -----------------a-------.---------t----g------------------  539
WMV-2      t-a--.-----t-----t----a-gt---------tt....--------------tt---  532
```

```
86/0020    ACCTAATAATACTTTTGTTTAGAGTGGTT.TAACCACCTTAGTGTGCTTTATATTATAGT  595
PV 720     ---a------g----------t------.--------------------------g--  595
PV 723     ---a------gt--g-------t------t--------cc-----------g-------  599
SMV-N      ---a------gt----------t------t--------cc-----------g-------  599
PV 724     ---a------gt--c-------t------t--------cc-----------g-------  596
PV 716     ---ag-----gt--c-------t------t--------cc-----------g-------  596
94/2052    ---a------gt--c-------t------t--------cc-----------g-------  596
PV 718     ---a------------------t------c---------c--------------------  596
PV 722     ---a------------------t------t---------c--------------------  599
WMV-2      ----ttat-ctt---atg----t------.-----------------c------------  591

86/0020    TTATGAATGGCAGGGAGAACCATTGCAATGCCGGAGTCCTTTTAAGAGTGATTCTATCAT  655
PV 720     -----------------------------------.----------------t------  654
PV 723     -------------------------tgt--------c-----g----------tc----c  659
SMV-N      -------------------------tgt--------c-----g----------tc----c  659
PV 724     -------------------------tgt--------c-----g----------tc----c  656
PV 716     -------------------------tgt--------c-----g----------tc----c  656
94/2052    -------------------------tgt--------c-----g----------tc----c  656
PV 718     -------------------------tgt-a-t----c-----g----------------  656
PV 722     -------------------------tgt-a-t----c--c--g---------------c  659
WMV-2      -----c--aa-------------a----a-------tg---gt--t------ac----c  651

86/0020    GTATAGTGGCCGAGGTACGGCAATGTTTGTTGTCCCC                        692
PV 720     --..-----------gg                                            669
PV 723     c-c------------g-----------------..t                         695
SMV-N      --c------------g-----------------t                           695
PV 724     --c------------g-----------------.a                          692
PV 716     --c------------g-------------------                          692
94/2052    --c------------g------------------                           691
PV 718     --t--------------------------------.t                        692
PV 722     --t---------------------------------t                        695
WMV-2      -gt-ga-a----------t-------------t                            687
```


Figure 3.3: Nucleotide sequences PCR-amplified cDNA fragments of SMV isolates PV716, PV718, PV720, PV724, 86/0020 and 94/2052. The full sequence of 86/0020 is represented, whilst only the bases that differ from this are represented in the sequences of other isolates. The sequence data of PV723 (G2), PV722 (G7) , SMV-N and WMV-2 were previously published (Jayaram *et al.*, 1992, Eggenberger *et al*, 1989 and Frenkel *et al*, 1989).

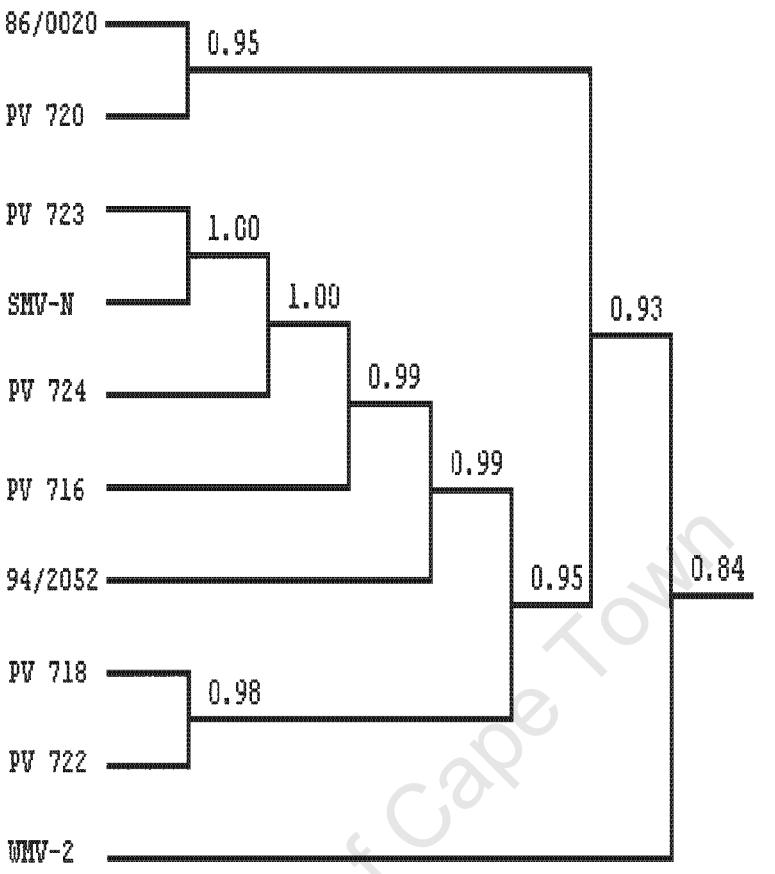


Figure 3.4: Sequence relationship dendrogram (homology tree) of SMV isolates representing that region between the mid CP and the end of the NCR. The tree was generated from the alignment of the nucleotide sequences of the isolates represented in Figure 3.3 (above). The value 1.00 represents 100% homology between sequences linked by a branch. The length of the branches are of no consequence.

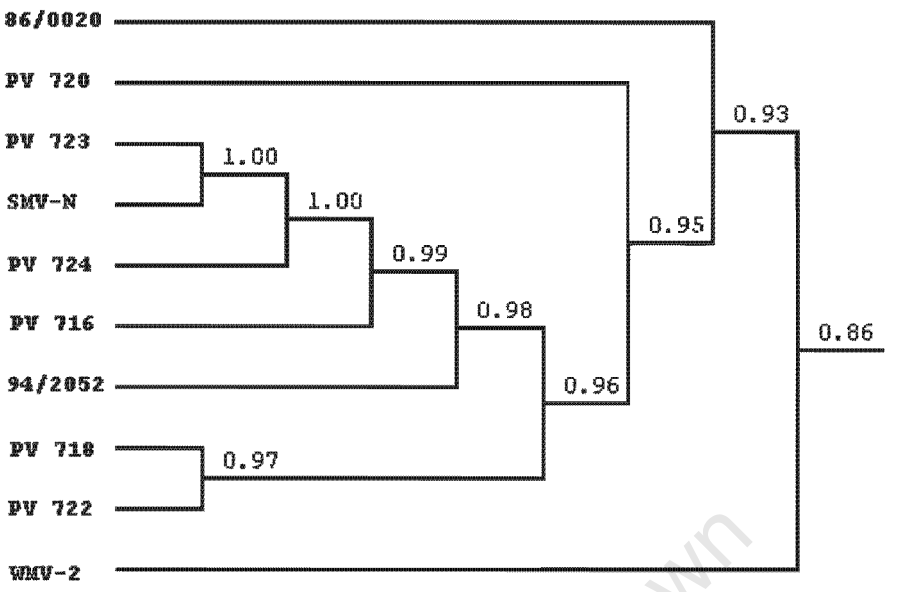


Figure 3.5 : Homology tree generated from the alignment of the nucleotide sequences of the partial coat protein for SMV isolates represented in figure 3.4. The 3'-non-coding region has been excluded in this analysis.

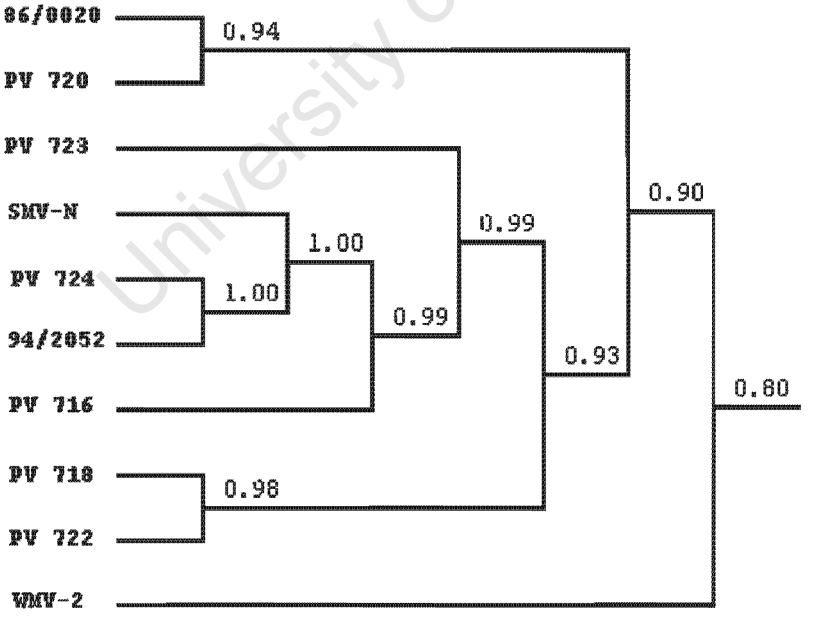


Figure 3.6: Homology tree generated from the alignment of the nucleotide sequences of the 3'-non-coding region of SMV sequences represented in figure 3.4. The nucleotide sequence of the partial coat protein has been excluded in this analysis.

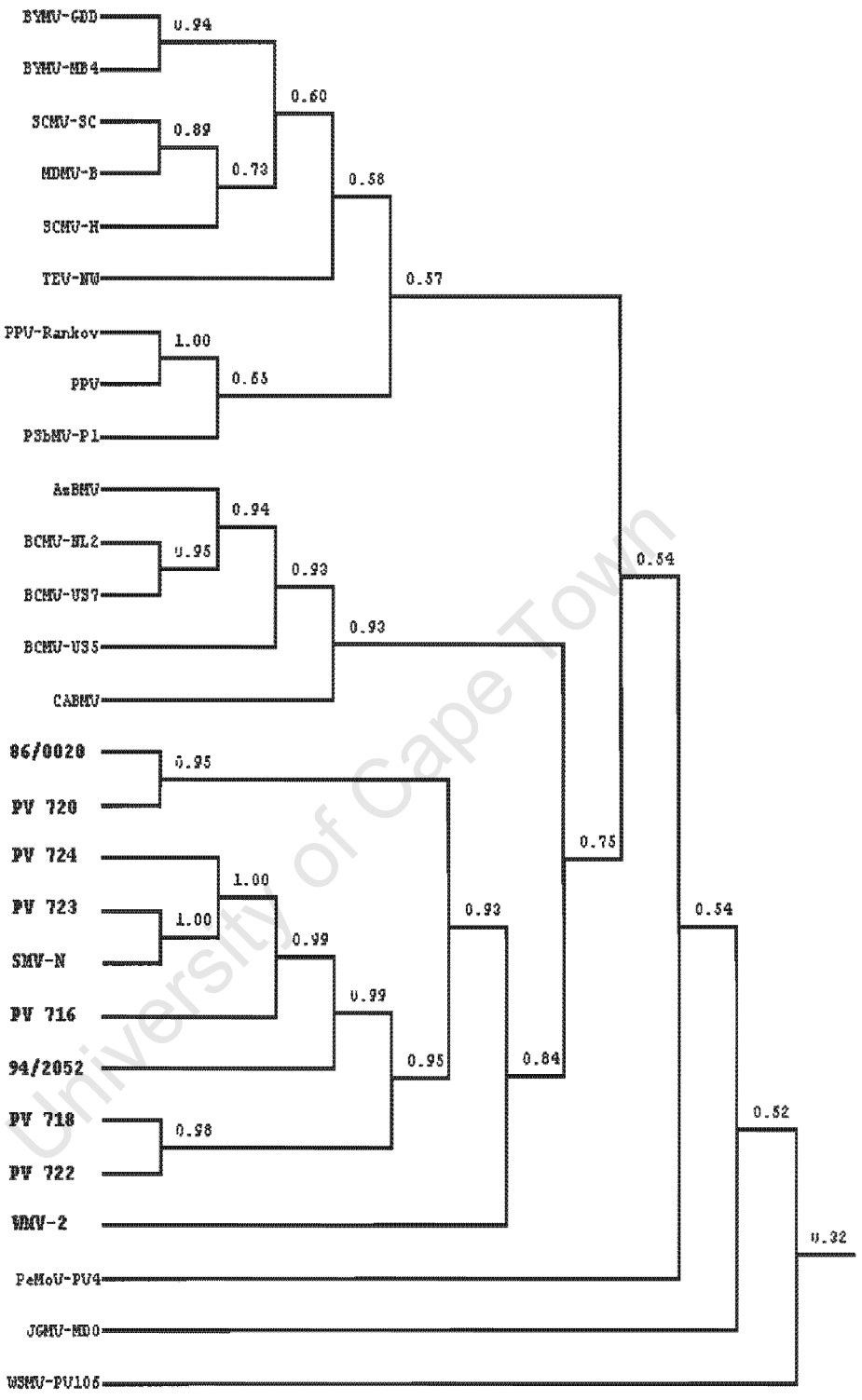


Figure 3.7: Homology tree generated from the comparative alignment of the 10 isolates sequenced in the current study, and other potyvirus genome data that is currently in the EMBL/GENBANK databases. The alignment was done only in that region that spans the mid coat protein gene to the end of the 3'-NCR.

The homology trees (illustrated in Figure 3.4, 3.5 and 3.6) represent the similarity of the sequences that have been analysed by the Multiple Alignment Sequence Editor of DNAMAN (MASED), which is captured in Figure 3.4. The value 1.00 represents 100% homology. There are only minor differences between the homology trees in Figure 3.4, 3.5 and 3.6 which represent the complete sequenced region; only the partial CP and only the 3'-NCR respectively.

The results in Figure 3.4 show that the two isolates which have been classified as SMV G1 strains show only 93% sequence homology in the target region. PV716 and 86/0020 are less related to each other, than they are to the other isolates with respect to the sequenced region. This was not expected, since both isolates display identical symptoms when inoculated on differential cultivar host ranges. According to the results in Figure 3.4, the ATCC G1 strain (PV716) appears to be most closely related to PV724, SMV-N and G2 (PV 723). Isolate PV724 is a G7a strain and has vastly different capabilities in overcoming host resistance markers when compared with the G1 strain. These results suggest that there are no unique sequences that could be further manipulated to by molecular biology techniques to differentiate SMV G1 isolates on the basis of current strain classification.

A similar situation is observed with the G3 isolates. The isolates which represent G3 strains, PV718 and 94/2052, are non-identical in the sequenced region and share greater similarity with other isolates. PV718 appears to be more closely related to PV 722 (G7); and 94/2052 appears to be more closely related to PV724 (G7a). Here again it is demonstrated that in the sequenced region, the degree of homology between isolates does not correspond to strain designation based on symptom expression.

SMV-N and PV723 are shown to have 100% homology. Jain *et al.* (1992) documented SMV-N as having properties of the G2 strain as has been documented of PV723 (Jayaram *et al.*, 1992). This is the only instance where isolates of the same strain showed identical sequence.

Figure 3.7 represents the homology tree generated from the comparative alignment of the 10 isolates sequenced in this study, and other potyviruses. The 10 SMV isolates grouped together. They shared the highest degree of homology with CABMV, BCMV and AzMV isolates. This was expected because Shukla *et al.* (1994) in their discussion of the BCMV subgroup of the *Potyvirus* genus, group these together. WMV-2 shows an 84% homology

with the other SMV isolates which further supports its inclusion as an isolate of SMV and not a distinct *Potyvirus*.

Table 3.2 below shows the projected restriction enzyme (RE) sites on the sequenced region. Certain restriction sites appear to be common (*RleAI, HindIII, PstI* and *HpaI*). There are a few sites that appear to group isolates into two subgroups e.g. *BalI /MscI* and *AhaIII/DraI*. It is however dangerous to make any extrapolation based on this information as there is a considerable amount of sequence variation in the natural SMV population and on the 10 isolates that are represented in this study. Furthermore the subgroupings do not correlate in any sensible manner with current strain classification.

A few RE sites are unique to specific isolates; e.g. 86/0020 has two unique sites (*ApaBI* and *DraI*). There are however no sites that are only common to 86/0020 and PV 716, the two isolates that represent the G1 strain. This observation has meant that it is not possible to develop a technique for the specific detection of the SMV G1 strain based on the methodology discussed in this study.

Table 3.2: The projected restriction enzyme sites of the sequenced regions

Restriction Enzyme	86/0020 (G1)	PV718 (G3)	PV722 (G7)	PV720 (G5)	94/2052 (G3)	PV724 (G7a)	PV723 (G2)	PV716 (G1)	
RleAI	(14)*	(14)	(14)	(14)	(14)	(14)	(14)	(14)	
HindIII	(135)	(135)	(135)	(135)	(135)	(135)	(135)	(135)	
PstI	(301)	(301)	(301)	(301)	(301)	(301)	(301)	(301)	**Common Restriction Sites**
HpaI	(316)	(316)	(316)	(316)	(316)	(316)	(316)	(316)	
ApaI	(428)	(428)	(428)	(428)	(428)	(428)	(428)	(-)	
RleAI	(444)	(444)	(444)	(444)	(444)	(-)	(-)	(-)	
BalI and MscI	(455)	(455)	(458)	(455)	(-)	(-)	(-)	(-)	
AhaIII and DraI	(-)	(-)	(-)	(-)	(516)	(516)	(519)	(516)	
NheI	(-)	(-)	(-)	(-)	(214)	(214)	(214)	(214)	
XcmI	(-)	(623)	(626)	(-)	(-)	(-)	(-)	(-)	
NsiI	(408)	(114)	(-)	(114)	(114)	(-)	(-)	(-)	
Af1II	(-)	(-)	(-)	(514)	(-)	(-)	(-)	(-)	
Eco571	(86)	(-)	(-)	(86)	(-)	(-)	(-)	(-)	**Unique Restriction**
ApaB1	(110)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	
BstEII	(244)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	

The projected restriction enzyme sites in the sequenced regions represented in Fig 3.4, these were determined with DNAMAN using programmes standard database of restriction enzymes which are recorded in Appendix B. * Represents the position of the restriction site on the sequenced regions.

```
86/0020    TSPDANGVWVMMDGEEQIEYPLKPIVENAKPTLRQIMHHFSDAAEAYIEMRNSESPYMPR   60
PV 720     ------------------------------------------------------------   60
PV 723     ------------------------------------------------------------   60
SMV-N      ------------------------------------------------------------   60
PV 722     ------------------------------------------------------------   60
PV 716     ----------v-------------------------------------------------   60
94/2052    ----------d-------------------------------------------------   60
PV 718     ------------------------------------------------------------   60
PV 722     ------------------------------------------------------------   60
WMV-2      ----v----------v-------------------------------------------   60

86/0020    YGLLRNLRDRELARYAFDFYEVTSKTPNRAREAIAQMKAAALSGVNNKLFGLDGNISTNS  120
PV 720     ------------------------------------------------------------  120
PV 723     ------------------------------------------------------------  120
SMV-N      ------------------------------------------------------------  120
PV 722     ------------------------------------------------------------  120
PV 716     ------------------------------------------------------------  120
94/2052    ------------------------------------------------------------  120
PV 718     ------------------------------------------------------------  120
PV 722     ------------------------------------i-----------------------  120
WMV-2      ----------------------------------------a-i-sr--------------  120

86/0020    ENTERHTARDVNQNMHTLLGMGPQQ                                     145
PV 720     -------------------------                                     145
PV 723     ----------------------p-                                      145
SMV-N      ----------------------p-                                      145
PV 722     ----------------------p-                                      145
PV 716     --------------------i--p-                                     145
94/2052    -------------------------                                     145
PV 718     -------------------------                                     145
PV 722     -------------------------                                     145
WMV-2      ----------------------p-                                      145
```

Figure 3.8: Deduced amino acid sequence of the CP fragment of the SMV isolates represented in Figure 3.3. The nucleotide sequences were edited to remove the NCR region prior to projecting translation profiles using the universal genetic code. These sequences represent the partial coat protein, which occurs between residues 2921 and the end of the polyprotein.

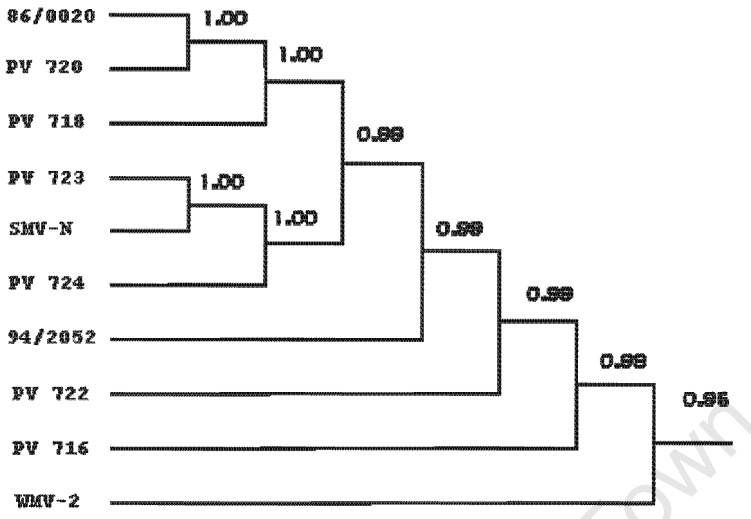


Figure 3.9: Homology tree which represents the relationship between the deduced protein sequences represented in Figure 3.8.

The alignment of the amino acid sequences of the partial coat protein sequence of the given isolates, in most cases, shows greater homology than their corresponding nucleic acid sequences. This phenomenon was expected since Jayaram *et al.* (1992) reported that despite a total of 31 differences in the nucleic acid sequences between the G2 (PV722) and G7 (PV723) strains in the entire CP region, only 3 amino acid differences resulted. Two of the 3 amino acid changes occur in that region of that CP that has been analysed in this study. Jayaram *et al.* (1992) indicated that the change from methionine in isolate PV723 (G2) to isoleucine in strain isolate P722 (G7) at position 97 in Figure 3.8, occurs within the trypsin resistant core and is unusual, since this region tends to be conserved among potyviruses, as documented by Ward and Shukla (1991). It is interesting to note that none of the other isolates represented here share this feature with G7. This methionine to isoleucine change was also not detected in by Jain *et al.* (1992) using HPLC profiles of the coat protein of a G7 isolate known as U670.

3.4 CONCLUSION

Pappu *et al.* (1993) stated that the CN48/oligoT PCR system has 5 advantages over other methods of amplifying the 3' region of potyviruses:

(1) Very little starting material is required;
(2) Crude nucleic acid extracts are required, there is no need for purification;
(3) The reverse transcription and amplification reactions occur in a single tube;
(4) The method can theoretically be applied universally to all potyviruses and;
(5) Both a portion of the CP gene and the 3'-NCR are amplified, which is potentially more useful for taxonomic/probe construction purposes than amplification of part of the CP gene alone.

Certainly in the current study I have confirmed the usefulness of the technique with respect to points 1 and 3. I did not use the method for the crude extraction of nucleic acid (point 2) described by the original authors, but used the immunocapture technique as an alternative and I found that it worked effectively. Though I did not attempt to apply the technique to any other potyvirus (point 4) but SMV, I found that the technique worked effectively for all the isolates and strains that were tested. However regarding point 5, wel find that ourmy results cannot justify this statement.

In the 3'-NCR the minimum degree of homology that was registered in this study of SMV strains was 80%, representing the relationship between WMV2 and the other strains. Frenkel *et al.*, (1989) documented the use of 3'-NCR sequences to resolve that WMV2 and SMV-N were strains of the same virus. They concluded that strains of the same virus would have sequence similarities between 83% and 99% in the 3'-NCR region. Both Frenkel *et al.* (1989) and Pappu *et al* (1993) inferred that 3'-NCR may be useful in developing virus specific probes that may be used to differentiate strains and species of potyviruses. The current study provides evidence to show that neither sequences of the 3'-NCR nor the 3'-region of the CP nor these sequences combined, present unique target sequences (that may be used for probes or other taxonomic techniques) that correspond with strain groupings as determined by the traditional technique of a differential cultivar host range. Our results do however show that all the isolates of SMV that were analysed grouped together with a minimum of 80% homology in the NCR region, when compared with sequences of other related but distinct potyviruses (figure 3.7). Thus I can conclude that the nucleic acid sequence of the 3'NCR can provide reliable taxonomic information as to whether or not an isolate is a strain of SMV, but it cannot be further manipulated towards accurately determining exactly which biological strain it is.

It is noteworthy that isolates of SMV have, since the classical paper by Cho and Goodman (1979), been grouped as strains according to their ability to overcome host resistance genes and the symptoms displayed on a differential cultivar host range. This was an extremely effective method and several successful soybean breeding programmes were developed from that study (Cho and Goodman, 1982 and Bowers *et al.*, 1992). However, there have since been several alternative postulates on the grouping of SMV isolates, none of which have been absorbed as definitive taxonomic principles, but are still worthy of mention. Hill *et al.* (1989) used one-dimensional trypsin peptide maps of the coat protein, immunoblotted with monoclonal antibodies, and found that the SMV isolates that were tested clustered into 3 groups. Cluster A: G1*(* - isolate name not stated); Cluster B: G2*, G3*, G4*, G6*, G7*; and Cluster C: G5*, SMV-O, SMV-12-18, SMV-Ia 75-16-1 and SMV-Brazil. The isolates G2 and SMV Ia-75-16-1 are both classified as G2 strains according to the differential cultivar host range results, yet they fall into different clusters according to this technique. Jain *et al.*, (1992) analysed the high-performance liquid chromatographic (HPLC) peptide profiles of tryptic digests of coat proteins of fourteen strains of SMV and found that whilst no definite clustering pattern was observed, certain isolates had identical peaks. Hill *et al.*, (1994), using antigenic signature analysis, also found that clustering patterns of SMV strains were not observed. The authors stated that their results were not intended to define serological relationships amongst strains since signatures reflected only the relative binding ability of the monoclonal antibodies in the panel for their specific epitopes, and these monoclonal antibodies probably reflected only a portion of the epitopic diversity in the virus isolates.

The study that most closely corresponded with the results of Cho and Goodman (1979) was that of Mansky *et al.* (1993). They studied the variation of cell-free translation profiles of several strains of SMV and found that groupings formed by analysis of products from rabbit reticulocyte lysates correlated with pathogenicity. Strains G1-G7 are as described above according to Hill *et al.* (1989). Cluster A (less virulent strains): G1, G2 and G3; isolate Ia 75-16-1 did not cluster definitively within this group, however it was closely associated. Cluster B (moderately virulent): G4, G5 and G6. The isolates representing G7 and G7a, which are very virulent strains of SMV, were non-clustering. These results were only obtained with the rabbit reticulocyte system, and groupings formed from the analysis of products of a wheat germ extract translation system had no apparent biological significance.

What is possibly only a superficial observation but nonetheless striking, is that those studies which analysed only the coat protein or 3'-NCR show groupings that are somewhat ambiguous and do not make definitive conclusions on the issue of SMV strain determination. However, those studies that conducted analyses on the entire virus, as in the case of Cho and Goodman (1979) using intact infective virions, and Mansky *et al.* (1993) using translation profiles of the entire genome, were much more successful in grouping isolates according to biological relatedness. It is important that future work on the determination of SMV strains guard against making the assumption that there is a direct association between the virus coat, pathogenicity and symptomology. It is vital for future work in this area to determine precisely that/those region/s on the SMV genome that are associated with virulence. Revers *et al.* (1999) stated of potyviruses in general that to some extent our inadequate understanding of symptom expression reflects our lack of knowledge of host proteins that interact with viral proteins, deeper understanding of the host pathogen interaction in future may provide some answers to the questions on isolates of SMV and there groupings as strains.

4. SMV STRAIN DETEREMINATION BASED ON DIVERGENCE IN THE CI GENE SEQUENCE

4.1 INTRODUCTION

The CI and P3 proteins have previously been considered as gene products of the SMV genome that may be involved in symptom expression in the host (Hill, 1999 pers. comm. and Chu *et al*, 1997). Jayaram *et al* (1992) characterised the complete nucleotide sequences of SMV strains G2 (isolate PV 723) and G7 (isolate PV722), which differ in their ability to overcome the host resistance mediated by the *Rsv* genes. The G2 strain (together with strains G1, G3, G4, G5 and G6) is unable to overcome resistance mediated by the host *Rsv* gene (Lim, 1982), whilst the G7 strain is, and therefore able to induce disease symptoms in such plants. The nucleotide sequences revealed a small but useful region of divergence between the two strains that lay within the CI region. Omunyin *et al* (1996) used this region to develop sequence specific oligonucleotide primers for RT-PCR to detect and differentiate SMV stains G2 and G7. Differentiation of strains of the same virus with RT-PCR has also been reported for CTV (Gillings *et al.*, 1993) and *Cucumber mosaic virus* (CMV) (Rizos *et al.*, 1992). The protocols used in those studies were based on an initial RT-PCR with primers derived from variable regions followed with restriction-endonuclease analyses.

Omunyin *et al.*(1996) developed two 20bp downstream primers which were complementary to strains G2 and G7, and identical to each other bar 12 mismatches. The downstream primers for both strains were complementary to sequences at positions 4937 to 4956 in the CI region (see figure 4.1). The upstream primers for the G2 strain G2-5'CI and G2-5'CI-0, were homologous to nucleotides between positions 4518 and 4537 in the former and 4680 and 4699 in the latter. The upstream primer for the G7 strain was homologous to nucleotides between positions 4680 and 4699. The G2-5'CI and G2-3'CI primer set together with the G7-5'CI and G7-3'CI primer set were used in an assay that yielded strain specific RT-PCR fragments from total RNAs of trifoliate soybean leaves infected with either strain or a mixture of both. The authors also demonstrated that G2-5'CI and G2-3'CI primer set would not produce an amplification product from plants infected only with the G7 strain. However the corollary that the G7-5'CI and G7-3'CI primer set would not produce an amplification product from template RNA of the G2 strain was not adequately discussed in that paper.

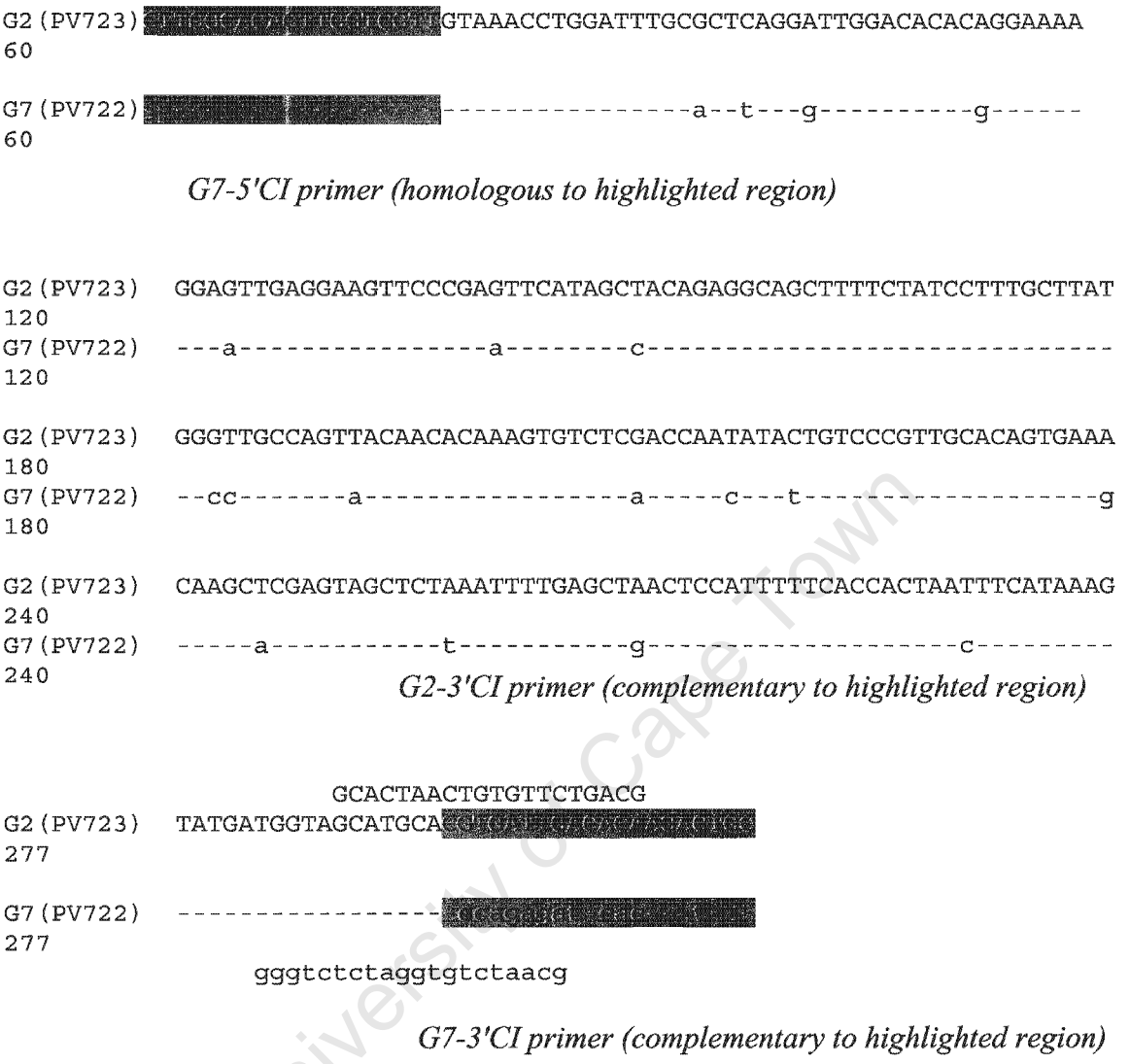


Figure 4.1: The sequence and position of primers in the CI region that provided the foundation for the assay developed by Omunyin *et al.* (1996). Sequence data from Jayaram *et al.* (1992), the sequence of G2 (PV723) is given in full and only bases of G7(PV722) that differ are indicated below.

The G7-5'CI and G7-3'CI primer set also produced RT-PCR products of 277 base pairs, from SMV template RNA of strains G7, G1, G3, G4, G5, and G7a. Though it was not an objective of their initial study to differentiate all the SMV strains, Omunyin *et al.*(1996) found that further treatment of the G7-5'CI and G7-3'CI amplification products with the restriction-endonuclease HaeIII, would further differentiate the G1 strain from all others.

All isolates except that of the G1 strain would produce restriction products of approximately 155 bp and 122 bp.

The following section describes an attempt to test the ability of this assay to differentiate G1 isolates from others. Given the prevalence of this strain in SA soybean growing regions it was thought that specific detection of this strain could provide plant breeders with more information upon which to base their cultivar recommendations.

4.2 MATERIALS AND METHODS

4.2.1 Virus Isolates

Table 4.1: Virus isolates used in these experiments

Isolate Name	PPRI Accession Number	Strain
86/0020	86/0020	G1
94/2052	94/2052	G3
PV 716	94/2151	G1
PV 717	94/2152	G2
PV 718	94/2153	G3
PV 720	94/2155	G5
PV 722	94/2157	G7
PV 724	94/2158	G7a

4.2.2 Maintenance Hosts

The virus isolates were established and maintained on *G. max* hosts as described in section 3.2.2.

4.2.3 IC-RT-PCR

The single-vial, two stage protocol for RT-PCR of Pappu *et al.* (1993) was used.

4.2.3.1 Immunocapture

Whilst the original authors obtained the nucleic acid templates by crude extraction of the RNA, I modified the procedure to obtain the nucleic acid templates using the

immunocapture technique as documented. 500 µl PCR tubes were coated with 50µl of SMV 86/20 polyclonal antiserum at a dilution of 1:1000 and held overnight at 4°C. The tubes were then washed twice with PBS-Tween (0.02M PBS and 0.5ml/l Tween 20). Virus infected plant material was macerated in the ELISA extraction buffer in a 1:100 w/v dilution. The mixture was then transferred to an Eppendorf tube and microcentrifuged. 50µl of the light green supernatant was then transferred to the washed PCR tubes. The tubes were held at 4°C overnight for optimal results. The tubes were washed twice prior to the addition of RT- PCR reagents.

4.2.3.2 Reverse Transcription and Amplification

The upstream forward reaction was primed by the G7-5'CI primer (PPRI accession no. 96/1279) which was homologous to nucleotides 4680-4699 of the G7 genome (Jayaram *et al.*, 1992 and Omunyin *et al.*, 1996) and had the sequence CTTGGCAGAGTTGGTCGTTG. The reverse reaction was primed by the G7-3'CI primer (PPRI accession no. 96/1278) which was complementary to nucleotides 4937-4956 of the G2 and G7 genomes (Jayaram *et al.*, 1992 , Omunyin *et al.*, 1996), and had the sequence; GCAATCTGTGGATCTCTGGG. The PCR reagents were added in the following concentrations to the washed PCR tubes: 1µM of each primer, 0.2% Triton X-100, 350 µM of each dNTP, 1mM $MgCl_2$, 67mM Tris-HCl pH 8.8 at 25°C, 10 mM DTT, 19 units HPRI RNAse inhibitor (Amersham), 10 units M-MLV reverse transcriptase (Life Technologies), 0.5 unit of *Thermus aquaticus* DNA Polymerase (BioTaq by Bioline). The final reaction volume was 50µl, and was overlayed with 50µl of liquid paraffin to prevent evapouration.

A Hybaid Omnigene thermocycler was programmed for reverse transcription at 37°C for 60 min. Template denaturation then took place at 94°C for 1 min (2 min for the first cycle), primer annealing at 55°C for 2 min, and DNA synthesis at 72°C for 3 min. This programme was repeated for 25 cycles and was terminated with a final elongation at 72°C for 7 min. The PCR products were separated in a 1% agarose (w/v) gel containing ethidium bromide, in a TAE buffer medium at 100V for 30-40 min. The gel was observed under UV light with the UVP Grab-It Imaging System and results were analysed with UVP Gelworks 1D Advanced (Version 3, 1996) by Nonlinear Dynamics.

4.2.4 Restriction Digestion

The PCR products were purified from the reaction mixture by phenol chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989). The dry DNA pellet was dissolved in 17.5 µl of sterile distilled water. Restriction digestion with 5 units *HaeIII* in 10 mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, and 1mM dithioerythritol (DTE) at a final reaction volume of 20µl took place at 37°C for 1 hour. The restriction digestion products were separated in a 1% agarose (w/v) gel containing ethidium bromide, in a TAE buffer medium at 100V for 30-40 min. The gel was observed in UV light with the UVP Grab-It Imaging System and results were analysed with UVP Gelworks 1D Advanced (Version 3, 1996) by Nonlinear Dynamics.

4.3 RESULTS AND DISCUSSION

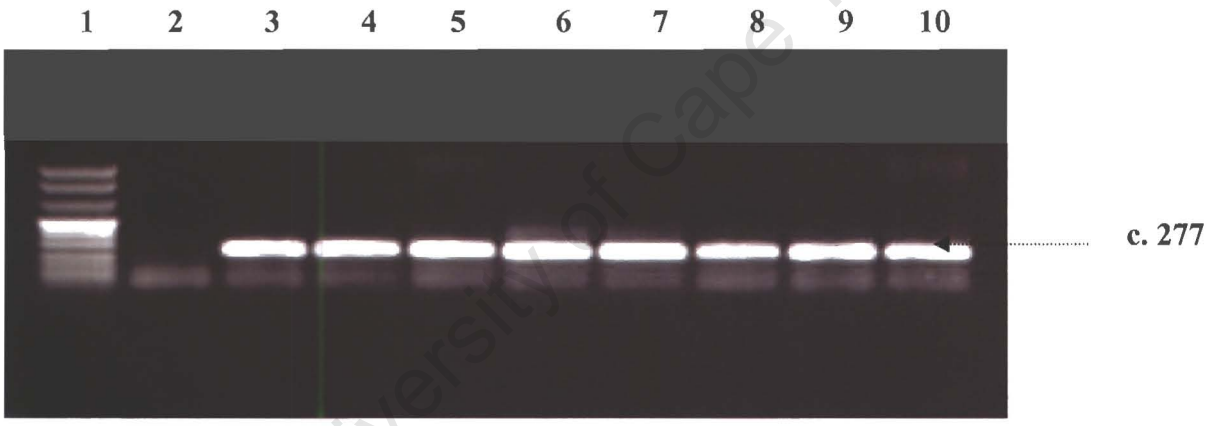


Figure 4.2: RT-PCR of SMV isolates with primers G7-3'CI and G7-5'CI. **Lane 1**: Boehringer Mannhein Marker VIII (1114 bp - 19 bp), **Lane 2**: uninoculated control, **Lane 3**: 86/0020, **Lane 4**: 94/2052, **Lane 5**: PV 716, **Lane 6**: PV 717, **Lane 7**: PV 718, **Lane 8**: PV 720 **Lane 9**: PV 722, **Lane 10**: PV 724.

Figure 4.2 shows that all the isolates tested produced amplification products of approximately 277 bp. The amplification of PV 717 was surprising since the G7 3' CI primer was expected to have a 12 base pair mismatch with the sequence of the G2 strain in the target region. The reaction had been optimized and several levels of stringency were tested with varying $MgCl_2$ concentrations (Figure 4.3), yet PV 717 (G2) repeatedly

produced amplification products of approximately 277 bp. It is also important to note at this juncture that the isolate used as a representative of the G2 strain by Omunyin *et al.*(1996) was PV 723, and different to the isolate used in the current study. Thus, isolate specific sequence variation could account for this result. However, the initial study did not specifically test the ability of the G7-5'CI and G7-3'CI primer pair to produce an amplification product from template RNA of the G2 isolate. The authors also indicated that the G2-5'CI-0 and G2-3'CI primer pair inconsistently amplified a 277 bp fragment of the G2 RNA template and also amplified the characteristic G7 fragment under all conditions. The authors were only able to demonstrate strain differentiating ability of the assay when the G2-3' CI primer was used in conjunction with the G2-5'primer (which anneals to G2 template nucleic acid at a point further upstream than the G2-5' CI-0 primer) and produces a 380 bp product. The evidence presented here could suggest that the 12 base pair mismatch in the target region was not adequate to ensure sequence specific priming, and some amount of non-specific annealing was permitted during the PCR thus compromising the strain differentiating ability of the assay, or that the published sequence was wrong.

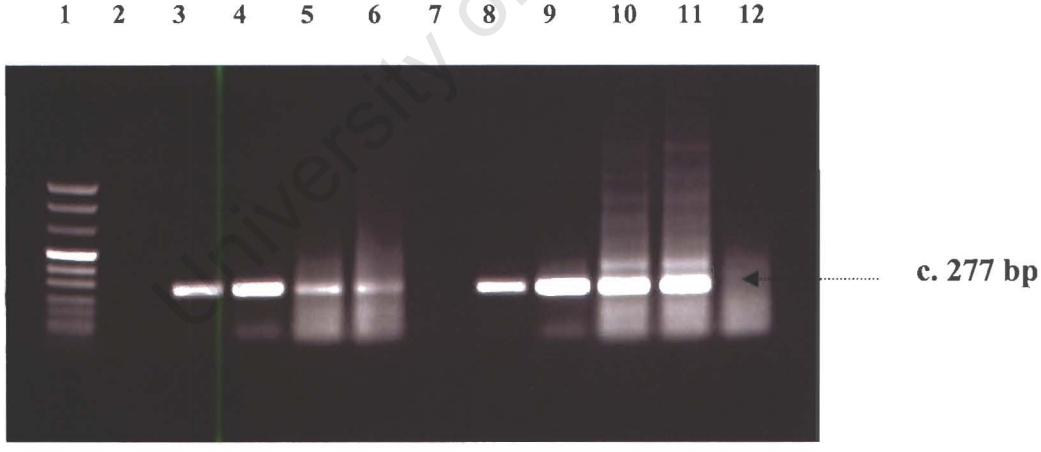


Figure 4.3: Optimising $MgCl_2$ concentration for the PCR. **Lane 1**: Boehringer Mannheim Marker VIII (1114 bp - 19 bp), **Lane 2**: PV 716 with 0mM $MgCl_2$, **Lane 3**: PV 716 with 0.5mM $MgCl_2$, **Lane 4**: PV 716 with 1mM $MgCl_2$, **Lane 5**: PV 716 with 2mM $MgCl_2$, **Lane 6**: PV 716 with 3mM $MgCl_2$, **Lane 7**: PV 717 with 0mM $MgCl_2$, **Lane 8**: PV 717 with 0.5mM $MgCl_2$, **Lane 9**: PV 717 with 1mM $MgCl_2$, **Lane 10**: PV 717 with 2mM $MgCl_2$, **Lane 11**: PV 717 with 3mM $MgCl_2$, **Lane 12**: Uninoculated control.

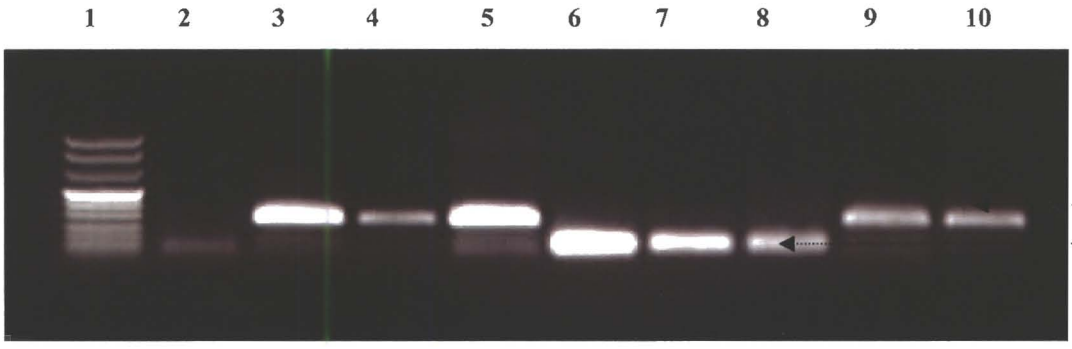


Figure 4.4: Restriction digestion of PCR products with *HaeIII.* **Lane 1**: Boehringer Mannhein Marker VIII (1114 bp - 19 bp), **Lane 2**: Uninoculated control, **Lane 3**: 86/0020, **Lane 4**: 94/2052, **Lane 5**: PV 716 , **Lane 6**: PV 717 , **Lane 7**: PV 718, **Lane 8**: PV 720, **Lane 9**: PV 722, **Lane 10**: PV 724

The results in figure 4.4 show that this study correlated well with those of Omunyin *et al* (1996). with respect to PV 716, PV 718 and PV 720. However even after several repetitions I failed to obtain restriction digestion products from isolates PV 722 and PV 724, whilst the original authors did. The SA G3 isolate 94/2054 cDNA also did not cut. Although both G1 isolates (PV 716 and 86/0020) did not cut as was expected, isolates of other strains also behaved similarly. This makes the assay ineffective as a method for specifically detecting the SMV G1 strain.

Two G3 strains were tested in this assay: I found that they produced different results when treated with *HaeIII.* The SA G3 isolate 94/2052, did not produce restriction products, however PV 718 did, suggesting sequence variation between same strain isolates in the target region.

4.4 CONCLUSION

The assay based on PCR amplification in the CI region of the SMV genome followed by restriction digestion of the PCR products with *HaeIII* has failed in its objective to specifically detect the G1 strain. The method failed possibly because isolate variation in that region makes strain specific detection impossible. Complete genome sequences of all SMV strains would make possible the selection of a more useful target regions for strain differentiation.

The results of the current study have shown that when the G2 isolate PV 717 was tested with the G7 5'CI and G7 3'CI primers, reaction products were always obtained. Omunyin *et al.*(1996) used a different G2 isolate (PV723) to that used in the current study.It is unlikely that the discrepancies are the result of a mix up in viralisolates since host plants were inoculated with stored carefully labeled freeze dried material at the start of the experiment, and then the whole experiment was repeated using fresh inoculum to reconfirm the results. It could be that the sequence variation detected in the CI region between representative isolates of the G7 and G2 strains described by Jayaram *et al.* (1992) is not necessarily also present in all other isolates of those strains, or that the sequence specific oligonucleotide primers described by Omunyin *et al.* (1996) are not in fact able to differentiate between all isolates of the G2 and G7 strains. Omunyin *et al.* (1996) inferred that the ability of the unique sequence upon which G7-5'CI primer is based, allows it to specifically differentiate the G7 strain from the G2 strain. Their closing statement that "This RT-PCR assay has shown that unique RNA sequence specific primers can be employed to detect and discriminate between RNA's of very similar strains" was made without having tested the ability of G7 5' CI and G7 3' CI primer set to amplify G2 template RNA. Omunyin *et al.* (1996) found that the G2-5' CI-0 and G2 3' CI primer pair inconsistently amplified a 277 bp fragment for G2 and also amplified the characteristic G7 fragment under all conditions. This suggests that a PCR was able to proceed even when there was a 12 base mismatch between the primer and template, and is also in contradiction with their closing statement. From extrapolations of their results and those of the current study it would seem that successful PCRs could be achieved even if there is a considerable degree of mismatch between the template and primer in an otherwise optimized PCR system.

5. SCREENING SOYBEAN CULTIVARS FOR RESISTANCE TO TWO SOUTH AFRICAN ISOLATES OF *SOYBEAN MOSAIC VIRUS*

5.1 INTRODUCTION

SMV is considered to be an important factor in limiting soybean yield throughout the world (Quiniones *et al*, 1971; Tu, 1989). Surveys of South African soybean growing regions have shown that although the incidence of SMV was generally below 1% in most commercial fields, some exceptions occurred where the virus was present at levels of 10-15% (Pietersen *et al.*, 1994; 1995 and 1996). The virus was however present in the National Cultivar trials and National Germplasm Collection during the same period at incidences of up to 20% of the whole trial, varying between 0-50% infected plants in a single cultivar. Pietersen and Garnett (1992) found after limited studies involving differential cultivar host ranges, that the SMV G1 strain was most prevalent in South Africa. Subsequent surveys have also detected a few non-G1 SMV strains one of which was confirmed to be a G3 (Pietersen *et al.*, 2000).

Resistance to SMV has been identified in several soybean cultivars (Cho and Goodman, 1979; 1982 ; Lim, 1985, Ma *et al.*, 1995), however most of them are not resistant to all strains of the virus. The dominant resistance gene in cultivar PI 96983 had been identified and designated as *Rsv* (now *Rsv1*) according to the work of Kihl and Hartwig *et al.* (1979). Alleles of this gene which confer differential reactions to SMV strains G1 to G7 have been identified in the following cultivars Ogden (*Rsv1-t*), York (*Rsv1-y*), Marshall (*Rsv1-m*) and Kwanggyo (*Rsv1-k*) (cited by Ma *et al.*, 1995). The resistance genes LR1and LR2 were identified in PI486355 and documented by Ma *et al.* (1995). In South Africa, Pietersen (1995) documented two sources of resistance to the G1 strain viz. the cultivars Ibis and PNR565.

Tu (1989) tested the effect of seven SMV strains on eight soybean cultivars. The effect of the virus on growth; maturity; yield; seed mottling and seed transmission was quantified. It was found that the SMV strain by cultivar interaction was significant for all traits tested. This led the author to conclude that whilst susceptible soybean cultivars remain in use it is necessary to (1) determine their yield loss profile against all major strains, (2) conduct

surveys to determine the virus strains in various districts. This information would enable specific cultivar recommendations for specific areas and thereby promote an increase in yield.

Although SMV epidemiology suggests that plant to plant spread in the field occurs from primary inoculum foci, such as SMV infected seeds that have been planted (Hill *et al.*, 1980), most published experiments on SMV/soybean interactions have involved mechanical inoculation of the host plants. Furthermore Gunasinghe *et al.* (1988) showed that soybean leaf pubescence affects aphid vector transmission and field spread of SMV. Thus the purpose of this study was to determine the yield loss and seed transmission profiles of 20 cultivars when challenge-inoculated with two different South African isolates of SMV. Viruliferous aphids were utilised for the inoculation procedure, so as to mimic the natural infection cycle and thereby incorporate the phenomenon of field resistance. The results of such a study would advise soybean breeders on the SMV resistance traits of currently available germplasm.

5.2 MATERIALS AND METHODS

5.2.1 Virus Isolates

Two South African isolates of SMV were chosen to screen soybean cultivars for resistance to the virus. The isolate 86/0020 (SMV G1 strain) and isolate 94/2052 (SMV G3 strain) were used in this study. The isolate 86/0020 was collected in a soybean plantation in the Roodeplaat vicinity in 1986, whilst 94/2052 was collected in the Grobblersdal area in 1994. The differential cultivar host range method of Cho and Goodman (1979) was used to characterise the collected isolates. Other isolates of the virus utilised in this study were obtained from the American Type Culture Collection.

5.2.2 Maintenance Hosts

The virus isolates were maintained on the *G. max* cultivar Rampage. The seeds were sown in plastic pots containing a potting mix used for all routine propogation. The seedlings were held in an environment-controlled disease and insect free glass house until they were approximately 2 weeks old and the primary leaves had emerged. These disease free seedlings were inoculated with freeze dried or desiccated material which had been stored at 4°C, and rehydrated with inoculation buffer. A pinch of carborundum was added to the

inoculum, and this mixture was applied to the primary leaves by gently rubbing it between the thumb and finger tips. No signs of burning or tearing were observed. Virus infection and establishment in the host plants was most effective when the seedlings were inoculated prior to the emergence of the first trifoliates. Symptoms which were observed between 4 to 9 days after inoculation included local necrotic lesions, mild to severe mosaics, vein clearing and leaf curl. A $F(ab')_2$-ELISA was performed to confirm virus infection. Host plant symptoms were monitored and recorded regularly. Host plants that had been inoculated 4 to 6 weeks previously, and confirmed to be infected with SMV, were used as source plants for the resistance trials.

5.2.3 Aphid Vectors

The green peach aphid *M. persicae*, a known natural vector of SMV, was used in this study. A colony of aviruliferous aphids were maintained on virus free *Capsicum frutescens* plants in an insect proof room.

5.2.4 Aphid Transmission of SMV

Aphids were gently transferred from detached leaves of the *C. frutescens* plants with a small paint brush into an empty Schott bottle. They were starved for 45 - 120 min. prior to the acquisition feed (Harris *et al*, 1977, Cho *et al.*, 1982b). They were then transferred onto SMV inoculum source plants (prepared as described in 5.2.2) for between 2.5 - 5 min. for the acquisition feed (Harris *et al*, 1977, Cho *et al.*, 1982). Ten viruliferous aphids were then transferred onto each of ten labelled healthy soybean plants. After allowing a transmission feed of approximately 1.5 hours, the aphids were exterminated with the systemic insecticide Metasystox. Given the size of the experiment it was necessary to perform aphid inoculations over a period of several days with a team of 5 people. To reduce variability within the experiment, each worker was assigned the task of completing both treatments for a specific cultivar within a specific inoculation session. Aphid transmission was used as a preference to mechanical inoculation in this study, so as to retain the authenticity of an *in vivo* environment where the only means of virus transmission would be via the vector, if a virus free seed source is used.

5.2.5 Resistance Sources

Twenty different cultivars were screened for resistance to SMV in this study. Eighteen of those cultivars were also utilised in the 1998/1999 South African soybean cultivar trials,

which test agronomic properties. The remaining two cultivars Rampage and Davis, which have well established interactions with the virus strains used in this study, were used as positive and negative controls respectively. The cultivars tested in this study together with the labels that were assigned to each cultivar-virus interaction has been documented in Table 5.1.

For each cultivar, 48 plant bags containing a standardised mixture of manure, peat moss, vermiculite and soil were sown with 2 seeds per bag. The potting mixture was also inoculated with *Bradyrhizobium japonicum* bacteria to promote effective nitrogen fixation by the soybean plants and closely simulate farming practices. The seedlings were then tested by ELISA for SMV to confirm the absence of this virus prior to the commencement of the experiment. The plants were then thinned out so that only one plant remained per bag. Only 40 plants per cultivar were retained in the final experiment. They were maintained within the screen house and regularly monitored for insect infestation and fungal diseases.

5.2.6 Experimental Design

Ten replicates of each of the 20 cultivars were inoculated by aphid transmission with either the 86/0020 or 94/2052 virus isolate. To enable pairwise comparison of yield data, each inoculated plant was paired with a non-inoculated control, thus the pair was handled as a unit for the duration of the experiment. Ten randomised blocks were set up within an insect free screen house with dimensions of 15.9m x 5.48m. Each of the 40 treatments was represented once within each of the 10 blocks. Each plant was labelled according to the treatment it represented, the block within which it was present and whether it was the inoculated or non-inoculated component of the pair.

5.2.7 Data Collection and Analysis

Virus infection and disease development was monitored periodically by visual observation of symptoms, ELISA and immunosorbent electron microscopy (ISEM). The percentage of infection was calculated as follows: [The number of infected plants within a treatment/ number of replicates (always 10)] x 100 = %Infection. Just prior to the end of the experiment when the plants still had green leaves, all non-inoculated control plants were again tested by ELISA for SMV infection to establish whether these plants became 'contaminated' during the experiment.

Plants were maintained and monitored regularly. Seed was allowed to mature and was harvested and weighed. The percentage yield loss per treatment was calculated as follows: [(Average yield over 10 replicates of the non-inoculated control) – (Average yield over 10 replicates of the inoculated treatment) ÷ Average yield over 10 replicates of the non-inoculated control] x 100 = Percentage yield loss. Differences in seed yield were calculated between virus infected and uninfected control plants for each replicate of each treatment, and the values were subjected to an analysis of variance. For the purpose of ranking the cultivars according to resistance, tolerance and susceptibility, yield differences were further analysed by Gupta's shortfall test (Gupta, 1965) and cultivars were ranked from 'best' to 'worst' with associated significance indicated.

5.2.8 Seed Transmission

Seed transmission tests were conducted by pooling together the seed of inoculated plants that became infected with the virus, within a specific treatment. Fifty seeds from this pool were then planted and monitored for symptoms of SMV. In those treatments where less than 70% infection was obtained, seeds from all inoculated plants were pooled.

5.2.9 Determination of SMV Resistance Profile of the Cultivar Ibis

The culivar Ibis was challenge inoculated with the following isolates of SMV: PV 716, PV 717, P718, PV720, PV722, PV724, 86/0020 and 94/2052, to determine its resistance profile. Seven Ibis plants and 3 Rampage plants were mechanically inoculated, monitored for symptoms over a three week period. ELISA tests were used to confirm the results of the visual screening in the case of the Ibis plants.

5.3 RESULTS AND DISCUSSION

Table 5.1: Percentage of infection recorded per treatment

CULTIVAR	TREATMENT (inoculated with 86/0020)	%INFECTION	TREATMENT (inoculated with 94/2052)	%INFECTION
SNK 500	T1	100	T21	100
A5409	T2	90	T22	90
PAN494	T3	100	T23	100
SONOP	T4	100	T24	90
NYALA	T5	80	T25	80
HIGHVELD TOP	T6	100	T26	80
KNAP	T7	100	T27	100
CRN 1550	T8	90	T28	100
BLOEKOM	T9	100	T29	60
DUMELA	T10	100	T30	100
A5308	T11	100	T31	80
JF80	T12	100	T32	100
PRIMA	T13	100	T33	90
JF91	T14	100	T34	100
LS555	T15	100	T35	90
SCS1	T16	100	T36	100
IBIS	T17	0	T37	0
A7119	T18	90	T38	90
RAMPAGE	T19	80	T39	90
DAVIS	T20	10	T40	20

Of the 40 treatments that composed the experiment, 21 treatments exhibited 100% infection across the 10 replicates. In the case of the positive control Rampage, I found that only 80% and 90% infection was obtained in treatments 19 and 39 respectively. It is not possible to determine whether this phenomenon is a limitation of the inoculation technique, or whether it is a manifestation of 'field resistance' to aphid transmission of the virus. It is noteworthy that cultivars A7119, Highveld Top, Ibis, Prima and Rampage were previously challenged by 86/0020 by mechanical inoculation (Pietersen,1995). In all cases except Ibis it was found that of the 10 inoculated plants all became infected with the virus. In the current study the results I obtained were slightly different to those of Pietersen *et al.* (1995) in the case of cultivar A7119 where 90% infection was obtained, and in Rampage where only 80% infection was obtained with 86/0020, lending further evidence to the hypothesis of the deficiency of the inoculation technique. The lack of

infection in certain members of the positive control group (Rampage) could alternately be a manifestation of host resistance conferred by trichome density or other physical aspects (Gunasinghe *et al.*, 1988). These could play a role in inhibiting vector transmission of the virus; however, it is beyond the scope of this project to measure such phenomena. Thus, I chose to accept that treatment groups that had less than 100% infection, but still fell within the range of the infection percentage of the positive control group (80% infection), would be considered to be susceptible to infection by that virus. Those plants within that treatment group that did not become infected following inoculation would be considered 'escapes', whether they were induced either by a less than perfect inoculation technique or by resistance phenomena was not considered further and the data set was not altered in anyway to compensate for either of these effects.

The only treatments that had less than 100% infection, but did not fall within the range for escapes were treatments 29 (60 % infection), 17 (10% infection), 37 (0% infection), 20 (0% infection) and 40 (20% infection). Whether the low percentage of infection observed in treatment 29 (which represents cultivar Bloekom inoculated with 94/2052) is a manifestation of resistance or some other phenomenon remains unclear. Treatments 20 and 40 represent the cultivar Davis inoculated with 86/0020 and 94/2052 respectively. These treatments were used as negative controls, since it is supposed to be resistant to both virus strains used in this experiment. The infected plants cannot be explained with any certainty, save to say there are always non-controlled variables in any biological system such as genetic segregation and indeed an ever-present risk of contamination. The complete lack of infection in treatments 17 and 37 is indeed interesting and represents the cultivar Ibis inoculated with 86/0020 and 94/2052 respectively. The result of treatment 17 corresponds well with a previous report of Ibis mechanically inoculated with 86/0020 (Pietersen, 1995). The lack of symptom development and virus detection by ELISA and ISEM led to the conclusion that Ibis was extremely resistant to 86/0020 (a SMV G1 isolate) (Pietersen, 1995), and has been further confirmed by the results of the current study. The current study also indicates that Ibis is extremely resistant to 94/2052, which is a SMV G3 isolate.

The results of the ELISA conducted on the healthy control plants towards the end of the trial showed that although some of the control plants did become contaminated, the

contaminants represented less than 7% of the control population. It is possible that the source of the contamination could have been a few aphids that survived the spray treatment with the aphicide, and then infected the healthy control plants. In future experiments of this nature it may be preferable to use a systemic aphicide that is applied to the soil, which would have more immediate extermination effects on the aphids following the transmission feed. The effect of the contamination was excluded from the experiment by removing contaminated data units prior to statistical analysis. The data units that were removed due to contamination have been captured in table 5.3.

The following data units were also removed from the final data set as they were found to be 'outliers' in the analysis of variance: T11 Block 4, T35 Block 9, T14 Block 2, T20 Block 5, T19 Block 4 and T3 block 5. This brings the total value of missing data to 33 entries of an expected data set of 400 entries, thus representing 8.25% on the initial experiment.

Table 5.2: The subset of results that were removed from the final data set due to contamination of the healthy controls.

CULTIVAR	TREATMENT	BLOCK NUMBER
A7119	T18	B10
HIGHVELD TOP	T6	B10
CRN1550	T28	B10
JF80	T12	B10
LS555	T15	B10
DAVIS	T20	B10
RAMPAGE	T39	B2
A7119	T38	B2
RAMPAGE	T19	B3
DAVIS	T20	B3
SNK500	T1	B3
PRIMA	T33	B3
SONOP	T4	B3
JF80	T32	B4
SCS1	T36	B4
LS555	T15	B4
HIGHVELD TOP	T26	B5
KNAP	T27	B5
CRN1550	T28	B5
A5409	T22	B5
A7119	T38	B8
JF80	T12	B8
SCS1	T36	B8
SONOP	T24	B8
JF91	T14	B8
CRN1550	T8	B9
JF91	T34	B9

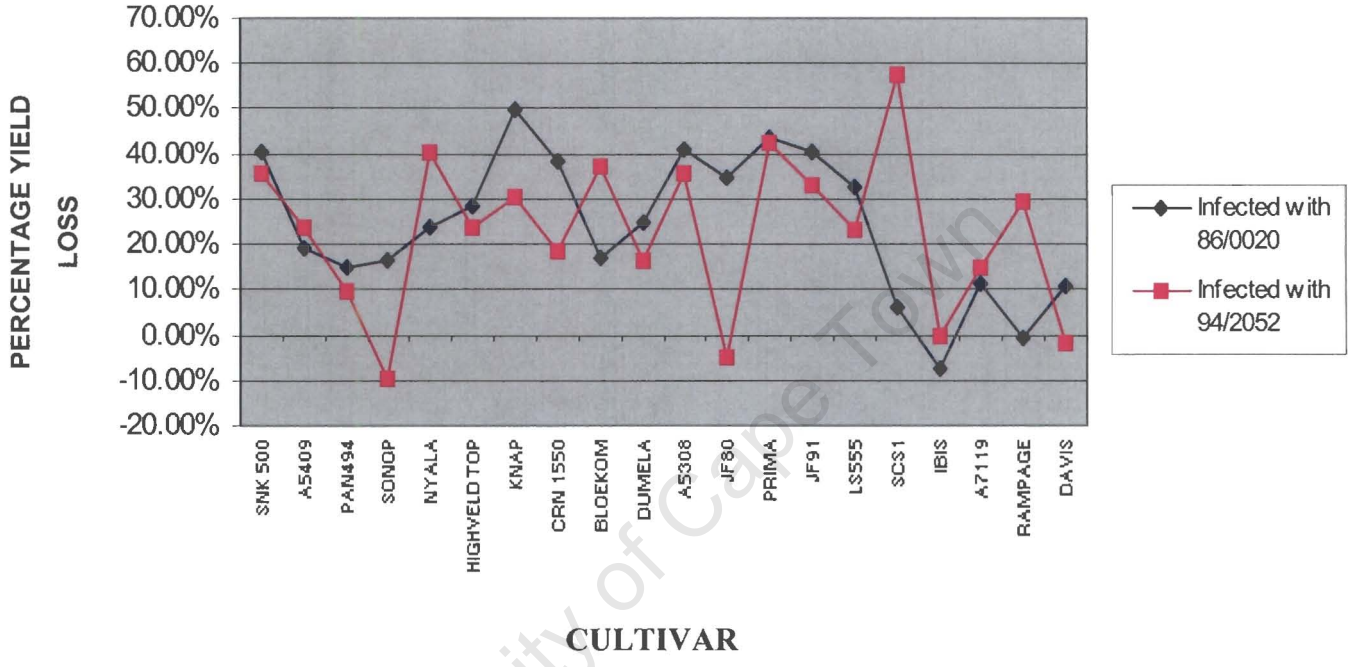
Table 5.3: Yield loss induced by infection with SMV isolate 86/0020

Cultivar	Inoculated With 86/0020	Average Yield of Non-inoculated Plants (g)	Average Yield of Inoculated Plants (g)	Variance of the difference between inoculated and uninoculated plants across 10 replicates	Percentage loss
SNK 500	T1	9.93	5.92	12.14	40.42%
A5409	T2	10.11	8.16	7.28	19.26%
PAN494	T3	7.78	6.63	2.88	14.88%
SONOP	T4	10.38	8.68	19.21	16.31%
NYALA	T5	8.66	6.63	6.95	23.46%
HIGHVELD TOP	T6	13.72	9.82	15.80	28.44%
KNAP	T7	12.45	6.28	13.33	49.61%
CRN 1550	T8	11.64	7.21	11.47	38.01%
BLOEKOM	T9	10.30	8.58	15.86	16.68%
DUMELA	T10	11.04	8.31	15.99	24.72%
A5308	T11	9.97	5.87	3.75	41.12%
JF80	T12	11.31	7.40	23.73	34.58%
PRIMA	T13	11.60	6.53	15.61	43.69%
JF91	T14	19.70	11.77	31.14	40.26%
LS555	T15	16.09	10.81	26.21	32.80%
SCS1	T16	10.42	9.78	12.33	6.14%
IBIS	T17	10.60	11.37	46.22	-7.31%
A7119	T18	14.32	12.71	30.67	11.24%
RAMPAGE	T19	6.39	6.46	5.17	-0.98%
DAVIS	T20	13.71	12.24	9.62	10.73%

Table 5.4: Yield loss induced by infection with SMV isolate 94/2052

Cultivar	Inoculated With 94/2052	Average Yield of Non-inoculated Plants (g)	Average Yield of Inoculated Plants (g)	Variance of the difference between inoculated and uninoculated plants across 10 replicates	Percentage Loss
SNK 500	T21	9.23	5.92	9.59	35.85%
A5409	T22	12.96	9.90	13.20	23.62%
PAN494	T23	9.64	8.70	13.30	9.71%
SONOP	T24	8.35	9.13	24.58	-9.34%
NYALA	T25	10.98	6.56	8.51	40.24%
HIGHVELD TOP	T26	13.91	10.61	9.39	23.76%
KNAP	T27	9.70	6.77	3.56	30.25%
CRN 1550	T28	10.70	8.71	19.98	18.61%
BLOEKOM	T29	11.94	7.51	12.95	37.10%
DUMELA	T30	9.68	8.07	28.12	16.64%
A5308	T31	9.38	6.05	10.96	35.49%
JF80	T32	9.62	10.11	18.23	-5.01%
PRIMA	T33	11.52	6.64	18.15	42.33%
JF91	T34	18.90	12.65	24.65	33.07%
LS555	T35	15.94	12.23	21.22	23.27%
SCS1	T36	11.11	4.72	49.74	57.51%
IBIS	T37	10.64	10.66	12.74	-0.18%
A7119	T38	14.30	12.19	30.20	14.80%
RAMPAGE	T39	6.73	4.75	5.45	29.45%
DAVIS	T40	13.59	13.81	19.31	-1.63%

Figure 5.1: Percentage yield loss induced by infection with SMV isolates 86/0020 and 94/2052



The results in Tables 5.3, 5.4 and Figure 5.1 show the percentage yield loss per cultivar, induced by infection either with 86/0020 or 94/2052. The greatest yield loss was 57.51% which was recorded for the cultivar SCS1, when infected with 94/2052. This was followed by the cultivar Knap which showed 49.61%, yield loss when infected with 86/0020. Six treatments showed a slight net gain in yield following infection with SMV they were; Sonop with 94/2052, JF80 with 94/2052, Ibis with 94/2052, Ibis with 86/0020, Rampage with 86/0020 and Davis with 94/2052. The cause of this phenomenon is unclear and could be the result of experimental error or a complex interaction between the host and pathogen. In general it appeared that cultivars experienced similar yield losses following infection with either of the SMV isolates tested, however there are a few notable exceptions such as in the case of SCS1, which experienced only 6.14% yield loss from 86/0020 infection, but a massive 57.51% loss with 94/2052.

Table 5.5: Analysis of variance (ANOVA) on the difference in the average yield of seed between virus inoculated and non-inoculated plants

Source of Variation	d.f (m.v)	s.s	m.s	v.r	F pr.
Repitition Stratum	9	405.32	45.04	2.81	
Cultivar	19	1225.27	64.49	4.02	<0.001
Virus	1	8.23	8.23	0.51	0.474
Cultivar.Virus	19	531.40	27.97	1.74	0.028
Residual		318(33)	5098.32	16.03	
Total		366(33)	7033.81		

The effect of virus infection was determined per treatment according to the mean difference between the yield of virus inoculated and non-inoculated plants. Table 5.5 shows an ANOVA conducted across the entire experiment to determine the whether the effect of virus infection was significant. The results indicate that there was significant variation between the cultivars used in this experiment. There was also significant variation recorded for the virus and cultivar interaction.

The data was also subjected to a BESTTEST to rank them according to resistance/tolerance (as determined by low yield loss and consequently low shortfall). It was then repeated to rank according to susceptibility (large yield loss and consequently large shortfall). Those cultivars that did not fall clearly into the resistant/tolerant group or the susceptible group were placed into the null group. The results of ranking according to susceptibility are documented in Table 5.6. Results from ranking according to resistance (not shown) were also used to categorize the cultivars according to resistant, tolerant or susceptible as indicated in column 4 of the table.

Table 5.6: Treatments ranked from most resistant cultivar x virus interaction to most susceptible interactions

No.	Treatment No.	Cultivar X Virus	Average Difference (g)	Shortfall	Significance Level of falling into the Susceptible Group
1	17	IBIS X G1	-.7700r[1]	-0.01	0.9754
2	24	SONOP X G3	-.7600t[2]	0.01	0.9746
3	32	JF80 X G3	-.4900t	0.28	0.9604
4	40	DAVIS X G3	-.2200r	0.55	0.9405
5	37	IBIS X G1	-.0200r	0.75	0.9213
6	19	RAMPAGE X G1	-.0200t	0.75	0.9213
7	16	SCS1 X G1	.6400t	1.41	0.8252
8	23	PAN494 X G3	.9400t	1.71	0.7639
9	3	PAN494 X G1	1.0400t	1.81	0.7411
10	20	DAVIS X G1	1.4700r	2.24	0.6322
11	30	DUMELA X G3	1.6100t	2.38	0.5938
12	18	A7119 X G1	1.6800t	2.45	0.5743
13	9	BLOEKOM X G1	1.7200t	2.49	0.563
14	4	SONOP X G1	1.7400t	2.51	0.5574
15	28	CRN 1550 X G3	1.9400t	2.71	0.5006
16	2	A5409 X G1	1.9500t	2.72	0.4977
17	5	NYALA X G1	2.0300t	2.8	0.4749
18	39	RAMPAGE X G3	2.1700t	2.94	0.4354
19	38	A7119 X G3	2.3600t	3.13	0.3829
20	10	DUMELA X G1	2.7300t	3.5	0.2877
21	27	KNAP X G3	2.8200n[3]	3.59	0.2664
22	22	A5409 X G3	2.9400n	3.71	0.2393
23	26	HIGHVELD TOP XG3	3.1900n	3.96	0.188

24	21	SNK 500 X G3	3.3100n	4.08	0.166
25	31	A5308 X G3	3.3300n	4.1	0.163
26	35	LS555 X G3	3.8500n	4.62	0.089
27	6	HIGHVELD TOP XG1	3.9700n	4.74	0.076
28	12	JF80 X G1	4.0200n	4.79	0.071
29	1	SNK 500 X G1	4.0600n	4.83	0.068
30	11	A5308 X G1	4.100n	4.87	0.064
31	25	NYALA X G3	4.4200s[3]	5.19	0.04
32	29	BLOEKOM X G3	4.4300s	5.2	0.04
33	8	CRN 1550 X G1	4.5700s	5.34	0.03
34	33	PRIMA X G3	4.9200s	5.69	0.02
35	13	PRIMA X G1	5.0700s	5.84	0.01
36	15	LS555 X G1	5.3500s	6.12	0.01
37	7	KNAP X G1	6.1800s	6.95	0
38	34	JF91 X G3	6.3900s	7.16	0
39	36	SCS1 X G3	6.4100s	7.18	0
40	14	JF91 X G1	7.7800s	8.55	0

With regard to entry no. 24 (for example) line 2 gives the significance level of its shortfall (as compared to the smallest of the remaining entries) as 97.5%

[1] r = resistant – those treatments in which the test cultivar exhibits a significant level of resistance to the virus strain with which it was inoculated determined by the small loss in yield and, less than 30% of the inoculated plants became infected with the virus.

[2] t = tolerant – those treatments in which the test cultivar exhibits a significant level of resistance to the virus strain with which it was inoculated determined by the relatively small loss in yield, but between 80-100% of the inoculated plants became infected with the virus.

[3] s = susceptible – those treatments in which the test cultivar exhibits a significant level of susceptibility to the virus strain with which it was inoculated due to a large yield loss and 80-100% of the inoculated plants became infected the virus.

[4] n = null – those treatments in which the test cultivar could neither be categorised as resistant nor susceptible.

Standard error of an entry mean = 1.26600

Degrees freedom for error = 318

Number of entries = 40

Table 5.6 indicates the most resistant (r), tolerant (t) and susceptible (s) cultivar/virus strain groups identified with 95% certainty using shortfall methodology. The method is based on the multiple t-distribution test procedure of Gupta and Panchapakesan (1979), programmed by Callitz (1986).

These results show that the cultivars Davis and Ibis are the only two that are resistant to both SMV strains tested. Many of the cultivars tested were susceptible to either of the virus isolates which induced significant ($P<0.05$) yield reductions. Several cultivars were found to be tolerant to both SMV strains viz. PAN 494, Sonop, Dumela, A7119 and Rampage. Certain cultivars were found to be tolerant to 86/0020 but completely susceptible to 94/2052, these included Nyala, Bloekom, and SCS1. The cultivars that were entirely susceptible to both strains were Prima and JF91. The only cultivar that was tolerant of 94/2052 but completely susceptible to 86/0020 was CRN1550. Those treatments that could not be grouped into the resistant, tolerant or susceptible subsets fell into the null group.

Table 5.7: Effect of infection with two different strains of SMV on seed transmission of the viruses in different soybean cultivars

Cultivar	Percentage Of Seedling Emergence From Seed Derived From 86/0020 Infected Parent Plants	Number Of SMV Infected Seedlings Derived From Parent Plants That Were Inoculated With 86/0020	Seed Transmission Of 86/0020 Reflected As A Percentage	Percentage Of Seedling Emergence From Seed Derived From 94/2052 Infected Parent Plants	Number Of SMV Infected Seedlings Derived From Parent Plants That Were Inoculated With 94/2052	Seed Transmission Of 94/2052 Reflected As A Percentage
SNK 500	78%	0/39	0.00%	64%	5/32	15.63%
A5409	82%	0/41	0.00%	42%	2/21	9.52%
PAN494	86%	1/43	2.33%	60%	1/30	3.33%
SONOP	84%	0/42	0.00%	68%	0/34	0.00%
NYALA	64%	0/32	0.00%	56%	2/28	7.14%
HIGHVELD TOP	82%	0/41	0.00%	60%	0/30	0.00%
KNAP	98%	0/49	0.00%	90%	3/45	6.67%
CRN 1550	94%	1/47	2.13%	92%	7/46	15.22%
BLOEKOM	100%	0/50	0.00%	100%	1/50	2.00%
DUMELA	100%	0/50	0.00%	100%	4/50	8.00%
A5308	98%	0/49	0.00%	98%	2/49	4.08%
JF80	98%	0/49	0.00%	100%	3/50	6.00%
PRIMA	94%	0/47	0.00%	94%	0/47	0.00%
JF91	94%	0/47	0.00%	94%	8/47	17.02%
LS555	94%	1/47	2.13%	100%	13/50	26.00%
SCS1	94%	0/47	0.00%	90%	15/45	33.33%
IBIS	100%	0/50	0.00%	82%	0/41	0.00%
A7119	94%	0/47	0.00%	92%	2/46	4.35%
RAMPAGE	80%	0/40	0.00%	100%	0/50	0.00%
DAVIS	76%	0/38	0.00%	98%	0/49	0.00%

Figure 5.2: Percentage of seed transmission of SMV isolates 86/0020 and 94/2052 in soy bean cultivars

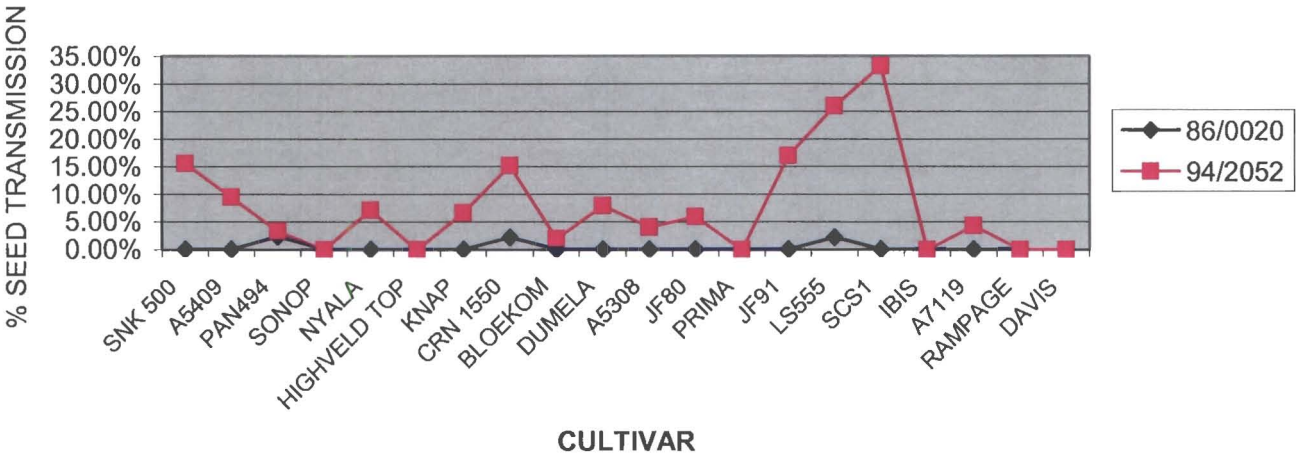


Table 5.7 shows that though there were instances where 100% germination and seedling emergence was achieved, in general there was reduced seedling emergence. Parent plants of cultivar A5409 infected with 94/2052 showed the poorest seedling emergence with only 42% recorded. It is not possible to determine whether this was a result of SMV infection or characteristic of the cultivar itself. I did not observe seedling emergence on the non-inoculated controls as our purpose was to determine the effect of the different virus isolates on seed transmission, though in retrospect it has been realised that the percentage of germination is an important factor to be considered. Bowers and Goodman (1991), found that cultivars Midwest, PI86146 and PI 181549, had between 70 and 79% seedling emergence when infected with SMV strains G1-G7. In the same study they found that the cultivar Merit had between 36.3 and 73.3% seedling emergence. The authors attributed the low seedling emergence to "poor quality seed", although there was no control experiment to ascertain the cause of the poor quality.

The percentage of seed transmission varied between 0 and 33.33% and differed according to cultivar and virus isolate interaction. In general there were lower levels of seed transmission of 86/0020 than of 94/2052. The level of seed transmission and the difference in the interaction between the cultivar and strain was expected and correlates with previous studies. Iizuka (1973) recorded that seed transmission in most varieties was below 38%. Tu (1989) reported a significant cultivar X virus strain interaction for seed transmission. In that study as well there was a general trend showing higher seed transmission in the G3 infected plants than in those infected with the G1 strain. In some

cultivars Tu (1989) reported as high as 70% seed transmission. However, the sampling method used by Tu (1989) could have biased the results, since only mottled seeds were tested for seed transmission. Bowers and Goodman (1991) observed seed transmission rates of between 0 and 32%, which also varied according to the virus strain and host cultivar. However they used randomly selected seeds, as in the current experiment to remove any form of bias. It is interesting to note that the highest level of seed transmission (33.33%) recorded in this study occurred in cultivar SCS1 infected with 94/2052, which also coincides with the treatment that showed the highest level of yield loss.

Table 5.8: Resistance profile of soybean cultivars challenge-inoculated with SMV isolates

ISOLATE	**CULTIVAR**	
	IBIS	**RAMPAGE**
86/0020 (G1)	0/7*	3/3*
94/2052 (G3)	1/7	3/3
PV 716 (G1)	0/7	3/3
PV 717 (G2)	2/7	3/3
PV 718 (G3)	4/7	3/3
PV 720 (G5)	0/7	3/3
PV 722 (G7)	7/7	3/3
PV 724 (G7a)	2/7	3/3

*Reflects the number of plants that became infected over the total number of plants that were inoculated with the specific SMV isolate.

The results shown on Table 5.8 indicate that all plants of the cultivar Rampage, that were inoculated became infected and showed visible symptoms. This suggests that the inoculum was viable and the inoculation technique was effective. In this assay the soybean cultivar Ibis has been shown to be immune to both isolates (86/0020 and PV 716) of the G1 strain. Ibis has also been shown to be immune to the G5 strain, but entirely susceptible to the G7 strain. The result of the interaction between Ibis and the G3 isolates is not consistent with that observed in the earlier resistance trial. The isolate 94/2052 which produced no infection out of 10 inoculated plants in the trial, produced 1 infected

plant out of 7 inoculated plants in the current assay. It is important to remember that aphid vectors were used for inoculation in the trial, but mechanical inoculation was used in the current assay. Furthermore, four out of the seven plants inoculated with the other G3 isolate (PV 718) became infected. These results indicate that the cultivar Ibis is not immune to G3 infection but may have variable levels of resistance to this SMV strain. Although one normally expects an "all or nothing" interaction between SMV isolates and soybean cultivars, in this assay I observed varying levels of infection with the interaction between Ibis and the G2, G3 and G7a strain. Given the limited number of replicates used in this study it is difficult to determine whether this is due genetic out crossing, host-pathogen interaction or merely experimental error.

5.4 CONCLUSION

The results of this study correlate well with previously published work in that significant differences in cultivar response to virus infection were observed (Tu, 1989). The method used to classify cultivars as resistant or tolerant, based on both statistical shortfall methodology and on the percentage of infection across 10 replicates has not been previously documented and was developed specifically for this study. It would be important to refine this methodology in future to include both immune reactions and those with varying symptom expression.

The SMV interaction with the cultivar Ibis was previously documented by Pietersen (1995). He indicated that the cultivar was in use extensively in the Brits Thabazimbi area and found the Ibis cultivar to be resistant to the SMV isolate 86/0020 when mechanically inoculated. The use of aphid inoculations for the purpose of screening for SMV resistance has not been previously documented, but its usefulness has been illustrated in the interaction of the cultivar Ibis with the SMV isolate 94/2052 (G3). When the cultivar Ibis was mechanically inoculated one out of seven replicates became infected with the virus, whilst none of the 10 replicates became infected when inoculations were effected by viruliferous aphids. Furthermore, 4 out of seven Ibis plants became infected when mechanically inoculated with PV 718, also a G3 strain. It is possible that Ibis has resistance genes, which have an inhibitory effect on vector transmission, which would not be recognised if resistance trial methodology were solely based on mechanical inoculations. It would be important to investigate the genetic lineage of Ibis to gain a deeper understanding of the observed reactions.

Seed transmission rate varied between 0 and 33% and was dependant on the virus and cultivar interaction. This correlated well with the results of previous studies (Tu, 1989 and Bowers *et al.*, 1991). This would indicate the need for South African farmers to utilise good quality seed, which where possible has been confirmed to be free from SMV.

This study has provided a starting point in the understanding of the interaction between South African soybean cultivars and SMV isolates. Trends that have been observed in US and Chinese papers have in most cases held true for the local scenario. Further research incorporating geographical information systems, local surveys on the vector and virus would assist in the development of more comprehensive integrated pest management programmes.

APPENDIX A: Standard Methods

1.1 Preparation of Competent Cells (Inoue *et al*, 1990)

SOB Medium *(per litre)*

20g bacto tryptone (2% w/v)

5g yeast extract (0.5% w/v)

0.5g NaCl (10 mM)

Make up to 1 litre. Sterilize in autoclave. Store at room temperature.

Prior to use make up and filter sterilize:

1M $MgCl_2$ and

1M $MgSO_4$.

Add 10ml of each solution ($MgCl_2$ and $MgSO_4$) per litre of the previously sterilized tryptone, yeast medium.

SOC Medium *(per 100ml)*

2ml 20% glucose solution (i.e. 2g in 10ml dH_2O). Filter sterilized.

98ml SOB medium

This must be prepared just prior to use.

TB buffer:

3.024g PIPES {piperazine-N,N- bis (2-ethanesulfonic acid)}(10 mM)

10.88g $MnCl_2$ (55 mM)

2.21g $CaCl_2$ (15 mM)

18.64 g KCl (250 mM)

Add all components EXCEPT the $MnCl_2$. Adjust the pH to 6.7 with KOH. Add $MnCl_2$, make up to 1 litre. Filter sterilize through a 0.45 um filter and store at 4°C.

Thaw stock cells of *E. coli* XL1 Blue, and streak out on Luria Bertani (LB) agar plates. Grow overnight at 37°C. Pick up 10-12 large colonies (2-3 mm diameter) and inoculate 250 ml SOB medium in the sterile 2*l* Erlenmeyer flask. Grow at room temperature with vigorous shaking (200-250 rpm) until OD at 600nm = 0.6. It is necessary to inoculate the medium late in the afternoon on the day before you wish to start the protocol as the culture takes quite

long to reach the correct OD. Leave on ice for 10 min. Transfer culture to sterile 250ml centrifuge bottles. Centrifuge at 4000 rpm in a Beckman JA14 rotor (2500xg) for 10 min at 4°C. Resuspend cells gently in 80 ml of ice cold TB buffer, using a glass pipette. Keep the bottle on ice whilst resuspending. Leave on ice for 10 min. Centrifuge at 4000 rpm in a Beckman JA14 rotor (2500xg) for 10 min at 4°C. Resuspend cells gently in 20 ml of ice cold TB buffer, keep the bottle on ice. Add DMSO with gentle swirling to a final concentration of 7%, (the simplest way is to discard 1.4 ml of the cell suspension and replace it with DMSO). Leave on ice for 10 min. Aliquot and freeze in liquid nitrogen. Cells can be stored in liquid nitrogen for several months. Or store at –70°C.

1.2.Preparation of the Vector

The plasmid chosen for use in this study was pBluescript KS^+. It was treated with *SmaI* according to the method of Sambrook *et al.* (1989). 10-20µg of closed circular plasmid DNA was digested with a twofold excess of *SmaI* (Boehringer Mannheim) for 1.5 hours at 25°C in Buffer A (33mM Tris-acetate, 66mM potassium acetate, 10mM magnesium acetate, 0.5mM DTT, pH 7.9). An aliquot of plasmid was run on a 1% agarose gel to determine whether digestion was complete. A phenol: chloroform extraction was performed and DNA was precipitated with two volumes of absolute ethanol. The DNA was recovered by centrifugation at 12000g for 10min at 4°C. The pellet was then dissolved in 90µl of TE buffer, pH 8.3.

The plasmid was then treated with Calf Intestinal Phosphatase (Promega) according to the suppliers instructions. This process removes the 5'-phosphate groups on the plasmid, thus preventing self-ligation and recircularisation of the plasmid. The phenol:chloroform extraction was repeated to purify the DNA the reaction mixture. The dephosphorylated open circular plasmid DNA was stored in TE buffer, pH 7.6.

1.3 Ligation reaction

For blunt end cloning 30µl of the cleaned PCR product was treated at 37°C with 10 units of DNA polymerase I (Pol I – Amersham International) and 10 units of T4 polynucleotide kinase (Amersham International). The reaction buffer contained 0.5M Tris pH7.5, 0.01M $MgCl_2$, 10mM DTT, 0.5mg/ml BSA, 200µM dNTPs and 0.1M ATP. The final reaction

volume was 100µl. The reaction was stopped after 1 hour by addition of 1µl of 0.5M EDTA. The PCR product was then cleaned again.

The cleaned and polished PCR (7.5µl) product was ligated to the vector with 5 units of T4 DNA ligase (Amersham International), 0.5µg of the pBluescript vector in ligation buffer (0.66M Tris, 0.005M $MgCl_2$, 1mM DTT, 1mM ATP). The final reaction volume was 10µl and was incubated for 16 hours at 22°C.

1.4 Transformation

Pipette 200 µl of the competent cells into a 15ml polypropylene tube (keep these tubes on ice as far as possible). Add 1-5 µl of the plasmid. Leave on ice for 30 min. Heat shock at 42°C for 30 seconds. Place on ice. Add 0.8 ml of SOC medium and incubate at 37°C for 1 hour, shaking vigorously. Transfer 100µl of cells, 40µl X-gal (2% w/v in N,N-dimethylformamaide) and 10µl IPTG (2.4 % w/v in water) onto a LB plate containing antibiotic, spread evenly with a hockey stick. Incubate overnight at 37°C.

2. Storing bacterial cultures

Bacterial cultures were screened for the presence of full length clones. Those cultures that were found to carry the appropriate inserts were cultured in a shaker, overnight at 37°C in L-B broth (10g bactotryptone, 5g yeast extract, 10g NaCL and 950ml H_2O, pH 7.4 adjusted with NaOH made up to one litre and autoclaved) containing 100µg/ml ampicillin. The overnight culture was stored by adding 15% sterile glycerol and freezing at –20°C.

3. F(ab')$_2$ ELISA (Enzyme-linked immunosorbent assay)

Polyclonal antiserum raised to SMV isolate 86/20 was used to prepare F(ab')$_2$ fragments with the pepsin digestion procedure (Campbell and Garvey, 1970). The centre 60 wells of Nunc™ ELISA plates were coated with F(ab')$_2$ diluted in coating buffer (0.05M sodium bicarbonate, pH 9.6) in a 1:1000 ratio. The plates were incubated at 37°C for 3-4 hours. The plates were then washed with PBS-Tween (0.02M PBS and 0.5 ml/l Tween 20). They were then rinsed once and washed 3 times with a 3 minute soaking step between each wash. Plant material was macerated in a 1:10 ratio in extraction buffer (0.02M PBS-Tween, 2% PVP and 0.2% ovalbumin, pH 7.4). The macerate was filtered through

cheese-cloth, and the filtrate was pippetted into the wells of the plate according to a predetermined set up. At least 2 wells were used per sample for the purpose of replication. A positive control, negative control and buffer control were used in every plate. Plates were incubated at 4°C overnight, then rinsed, and washed 5 times as described above. IgG diluted in a 1:1000 ratio with extraction buffer was then added to the plates and incubated for 3-4 hours at 37°C. the plates were then rinsed, and washed 3 times as described above. The conjugate of goat anti rabbit Fc fragment and alkaline phosphate in a 1:2000 dilution with extraction buffer was added to the plates and incubated overnight at 4°C. The plates were then rinsed, and washed 5 times as described above. The substrate ρ-nitrophenyl phosphate (1mg/ml) in substrate buffer (10% diethanolamine, pH 9.8), was used to develop the ELISA at 37°C. Absorbance readings at 405 nm were taken at 15 and 30 minute intervals with the Multiscan MC ELISA reader.

APPENDIX B: List of Restriction Enzymes

Aat I
Acc 65I
Acc I
Acc II
Acc III
Ac II
Acg I
Afl II
Afll II
Age I
Aha III
Alu I
Alw 261
Alw 441
Alw NI
Apa BI
Apa I
ApaL I
Asc I
Asp 7181
Asu I
Asu II
Ava I
Ava II
Avr II
BalI
Bam HI
Ban I
Ban II
Bbel
Bbv I
Bc II
Bg II
Bg III
Bpu 11201
Bsa HI
Bsa OI
Bsc 91I
Bsi I
Bsp 1286I
Bsp 1407 I
Bsp HI
Bsp MI
Bss 1tII
Bst 71I
Bst D102I
Bst EII
Bst NI
Bst Xi
Bsu 361
Cfr OI
CfrI
Csp451I
CspI
Cvn I
Dde I
Dpn I
Dra I
Dra II
Dra III
Drd I
Eag I
Eam 11051
Ecl 136 II
Eco 47 III
Eco 52 I
Eco 56 I
Eco 72 I
Eco t II
Eco I Cr I
Eco NI
Eco RI
Eco RII
Eco Rv
Ene I
Esp I
Fnu 4HI
Fnu DII
Fok I
Fse I
Hae II
Hae III
Hga I
Hgi AI
Hha I
Hind II
Hind III
Hinf I
Hin PiI
Hpa I
Hpa II
Hph I
I-Pol
Kpn I
Mae I
Mae II
Mae III
Mbo I
Mfe I
Mlu 113I
Mlu I
Mn II
Msc I
Mse I
Msp A1I
Msp I
Mst II
Nae I
Nar I
Nco I
Nde I
Nhe I
Nla I
Not I
Nru I
Nsi I
Nsp BII
Nsp I
Pac I
Pfe MI
Pin AI
Ple I
Pma CI
Ppu MI
Pss I
Pst I
Pvu I
Pvu II
Rle AI
Rsa I
Sac I
Sac II
Sal I
Sap I
Sau I
Sci I
Sci RI
SduI
Sfa NI
Sfi I
Sgr AI
Sma I
Sna BI
Spe I
Sph I
Spl I
Spo I
Srf I
Ssp I
Sst I
Sst II
Stu I
Sty I
Sun I
Swa I
Taq I
Tna I
Tth 11 I
Ttth 111 II
Vsp I
Xbu I
Xcm I
Xho II
Xma II
Xmn I
Xov II

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