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Screening of selected Cassava Cultivars for SACMV Resistance

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CHAPTER 1
INTRODUCTION

1.1 **Overview**

Cassava or manioc (*Manihot esculenta* Crantz) is a 1-3m high woody perennial shrub grown for its starch tuberous roots. Cassava belongs to the *Euphorbiacaeae* family, which also contains other commercially important crops like the castor bean. Cassava is grouped with 160 other species that have shown numerous beneficial qualities, such as dormancy, drought resistance and large yield of crop (Mroso, 2003). It is grown in countries of tropical and sub-tropical Africa, Asia and Latin America between 30°N and 20°S. Cassava is best suited to warm, humid, low-land tropics, but it can also be cultivated in regions of mean annual temperature above 20°C and an annual precipitation between 5000-8000mm. In the tropics cassava is the most important root crop and the fourth most important calorie source after rice, maize and sugar cane.

Cassava is a major source of calories for roughly two of every five Africans and a source of food for approximately 700 million people around the globe (Fauquet & Tohme, 2004) with daily consumption in some countries. In Sub-Saharan Africa over 30 million people get up to 60% of their daily energy intake from cassava (Puonti-Kaerlas, 1998).

The crop is vegetatively propagated by mature woody stem cuttings (or stakes 150-300mm long) planted horizontally, vertically or inclined on flat or ridged soils at densities ranging from 5000 to 20000 cuttings per hectare depending on the cropping system and purpose of production (El-Sharkawy, 2004). Seeds are mostly used in breeding programs depending on the cultivar, though its use in commercial cassava

production is a promising option to eliminate constraints particularly diseases associated with vegetative propagation.

Opportunities for the commercial development however, remain largely undeveloped in contrast to other major zones of cassava cultivation in Asia and South America. Cassava is planted on over 16 million hectares of land with 50% falling within Africa, 30% in Asia and 20% in Latin America. It is also the cheapest energy source in many developing countries and is grown and consumed by the world's poorest and most food insecure households grown by subsistence farmers in poor regions (Were, 2004). Worldwide 65-85% and 90% in Africa of the harvest is used for human consumption. The total world production in 2002 was 185 852 540mt with over 10 mt being produced in Africa alone (Were, 2004). The world's largest cassava producers are Nigeria, Thailand, Indonesia, Brazil and the Democratic Republic of Congo (DRC).

Since cassava leaves are rich in proteins, vitamins and minerals they may be used as a vegetable while the roots are rich in starch. Cassava roots contain up to 85% of their dry weight is starch, Vitamin C, carotenes, calcium and potassium, however their protein content is low. As a raw material, cassava adapts to a wide range of uses: food security crop (consumed in fresh or processed form), cash crop, feed crop and raw materials for industrial uses and can be processed into a wide variety of products for food and industrial uses such as starches, flours, alcohol, glucose and other products. Cassava roots are usually harvested 7-24 months after planting depending on the cultivar, purpose of use and the growing conditions. Since the root perishes at a rapid rate fresh roots need to be eaten on the farm, sold for consumption, processed for starch extraction, dried for flour production, roasted for food products and/or used for animal feed immediately after harvesting (El-Sharkawy, 2004).

In areas with a cool season, e.g., at high altitudes where plant growth and the accumulation of starch in the roots stops, the growing period can be up to three years. Cassava is well adapted to poor soils, allowing acceptable harvests even on marginal and eroded soils which do not support any other crop without costly external inputs and has the unique advantage over, for example, cereal crops in that its harvesting

time is highly flexible. This makes it a good famine reserve since plants can be partially harvested and left growing until the roots are needed. After an initial establishment period cassava is able to survive even prolonged seasonal drought. It produces the highest calorie yields per hectare of all staple crops and has a high efficiency of energy per unit per labor input ratio, as its cultivation requires much lower input than most tropical crops. This crop is produced mainly by resource-limited small farmers who hardly have any funding due to its remarkable properties such as tolerance to adverse environments.

1.2 Cassava in South Africa

Apparently cassava was introduced by southward-migrating tribesmen. The first recorded cassava planting took place with the major tribal movements of the 1830s and 1860s (Woodward *et al*., 1997). Interest in the commercial exploitation of this tuber crop was initially stimulated in 1948 due to its potential source of industrial starch. During 1975/6 the adaptation of this tuber crop was monitored and it was concluded that this crop could successfully be cultivated in the warmer areas of South Africa (Woodward *et al*., 1997). In the 1970s and 1980s there was considerable research done on cassava, particularly the diseases affecting it and some local breeding work was also undertaken. Towards the end of the 1980s, however, all cassava research was terminated due to the worsening of political and economical conditions in South Africa and many of the cassava lines that had been collected in South Africa were lost (Trench & Martin, 1984).

The South Africa Board and Food Manufacturers use about 90000 tonnes of industrial starch a year at a cost of R150 million (Star Business, 1999). Cassava starch is currently being processed in South Africa at CS Manufacturings in Dendron, near Polokwane, in the Limpopo Province. The factory sources 50% of its cassava from CS Manufacturing's own farmland and the balance from commercial farmers who produce on the company's behalf (Nofal, 1999). Commercial cassava farms have recently been established in the Barbeton area in Mpumalanga.

Cassava is cultivated by small scale farmers in a number of regions in South Africa including; Mpumalanga, KwaZulu-Natal and Limpopo Provinces. It is also grown in southern African countries, which include Mozambique, Swaziland, Malawi, Botswana, Zimbabwe and Namibia.

1.3 **Constraints on Cassava Production**

Though cassava is known to be a food security crop in developing countries, cassava production was neglected in breeding programs and often considered as a hardy crop with few problems. Up to 80 tonnes per hectare can be produced under optimal conditions in a twelve month culture period (Puonti-Kaerlas, 1998) but the actual yields can be severely reduced by poor agricultural practices and infestations by insects and various pests. These are estimated to cause 20-50% yield losses world-wide.

1.3.1 Major Pests

Some of the major pests that attack cassava include the cassava green mite, which cause losses of up to 80% (Puonti-Kaerlas, 1998) while leaf consumers like grasshoppers can also cause severe damage. Mealy bugs and green mites are the main pests of cassava in Africa. Nematodes can cause up to 98% losses and the problem is increasing in severity worldwide in areas of intensive cultivation, where crop rotation and fallow periods are either shortened or abandoned altogether. Recently stem borers have been identified as one of the main problems in cassava cultivation in Latin America (Puonti-Kaerlas, 1998).

1.3.2 Major Diseases

Some of the main diseases of cassava include cassava bacterial blight, super-elongation disease, frog skin disease and the cassava mosaic disease (CMD) which can lead up to 100% yield losses. Cassava bacterial blight caused by *Xanthomonas campestris* pv *manihotis* is one of the major pathogens in cassava cultivation world-wide and heavy infestations of bacterial blight can destroy a whole crop. By

traditional breeding some resistant varieties have been produced but so far the resistance obtained appears to be effective only under low infestation pressure (Puonti-Kaerlas, 1998).

1.4 Cassava Mosaic Disease (CMD)

Cassava mosaic disease (CMD) occurs in most cassava producing regions including Africa, India and Sri Lanka. Cassava mosaic disease is caused by several distinct geminiviruses and is responsible for high yield losses in cassava. There is great diversity of these geminiviruses in Uganda, Cameroon, Malawi, Zanzibar, Kenya, South Africa and the Indian subcontinent.

CMD has been recognized in East Africa for more than a century, but for much of this time it has been considered as a minor problem with a limited impact on the regions of cassava production. This disease is caused by begomoviruses, which are transmitted by the whitefly (*Bemisia tabaci*) and also mechanically via infected tools and planting material.

Cassava mosaic disease (CMD) can cause losses of up to 40-50% of the total yields of cassava throughout the African continent. There have been several epidemics of CMD in the past and the occurrence of the disease throughout the continent is very high. The most recent pandemic in Uganda, caused by recombinant EACMV-Ug, resulted in famine related deaths and huge losses in cassava production. The virus is currently spreading from North to South through Uganda and Western Kenya at 15-20km per year. To date no cassava variety that is completely resistant to begomoviruses has been produced through traditional breeding, but in Uganda the use of new tolerant varieties and disease free planting material has shown a positive effect. On the other hand, as the resistance to CMD appears to be recessive and polygenic, new varieties may lead to the loss of local land races and genetic engineering maybe be required to transfer only the desired traits to these cultivars.

An epidemic of unusually severe CMD, initially reported from North-central Uganda has over the course of 1990s, has expanded to cover an area of over 300,000km^2 of East and Central Africa, with devastating effects on cassava

production in the affected zones. Since the acute nature of severe CMD and the observation that it was spreading rapidly was first made in the early 1990s, intensive scientific research was directed at understanding the cause and mode of spread of disease. Studies determined that the epidemic was 'advancing' southwards at the rate of 20-30km and the causal agent was a novel, more virulent form of East African cassava mosaic geminivirus, referred to as the Ugandan variant (EACMV-Ug). Figure 1.1 illustrates the different species of cassava mosaic geminiviruses that are found in different regions of Southern Africa. In South Africa, EACMV, ACMV and SACMV have been found in the KwaZulu-Natal and Mpumalanga provinces. SACMV has also been found in Swaziland and recently an isolate of SACMV has been reported in Zimbabwe (Briddon *et al*., 2004).

The virus vector is the whitefly *B. tabaci* and alsos seems to interact synergistically with virus-diseased cassava host plants, since it reproduces faster on diseased plants than healthy ones. Additionally, new evidence suggests that a novel 'invading' population of *B.tabaci* is associated with the CMD pandemic. *B.tabaci* can also fly up to 7km in its life facilitating the spread of the epidemic over considerable distances each year.

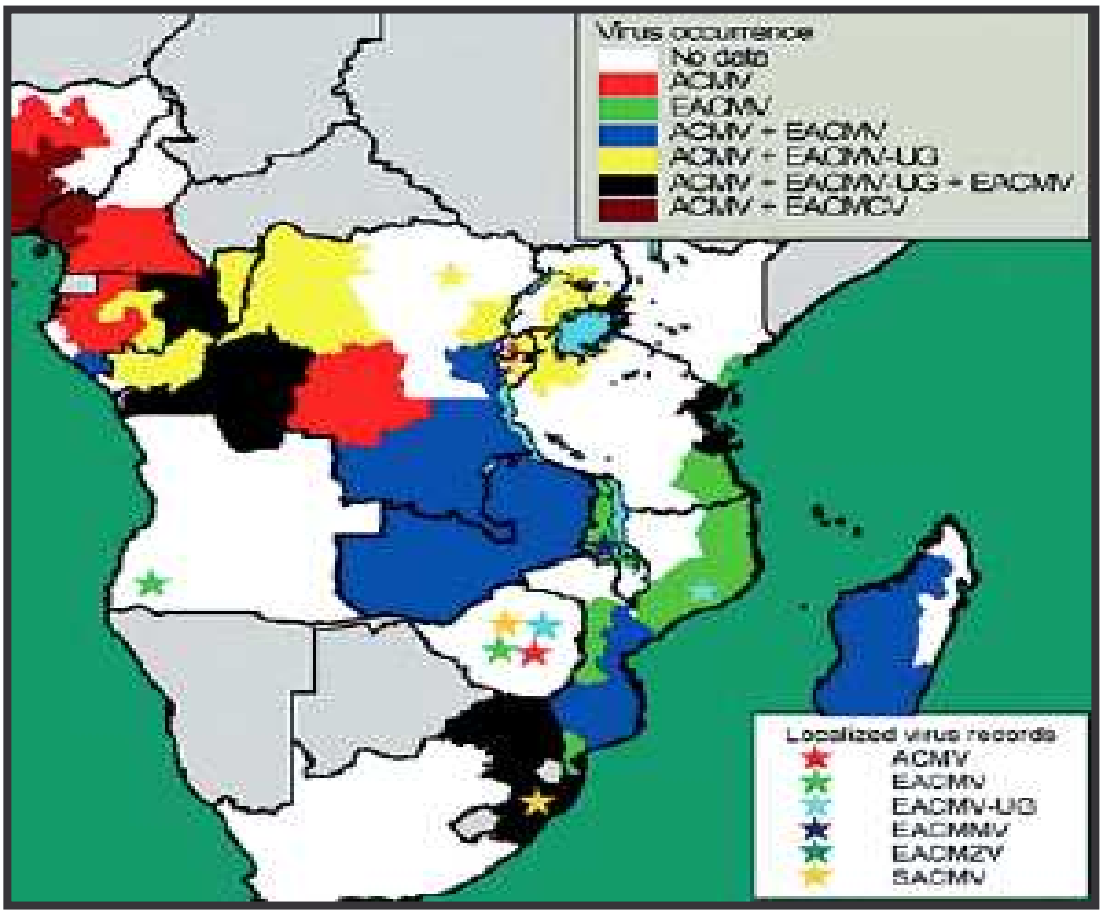

Figure 1.1 – CMV in Different Regions of Southern Africa (Legg, 2004)

1.4.1 Cassava Mosaic Disease in South Africa

The distribution of CMD in South Africa prior to the commercial expansion of cassava can probably be deduced from its present occurrence in subsistence plantings. CMD was initially brought into South Africa most likely by subsistence farmers whilst bringing infected illegal stakes into the country. Large scale movement of planting material by statutory and commercial organizations as well as by individual farmers during the 1970s resulted in some spread of CMD. A severe outbreak of CMD occurred in bulk import of cassava from Mozambique in 1975 (Trench & Martin, 1984).

The distribution of CMD in small-scale and subsistence cassava plantings in the early 1980s in South Africa prior to the commercial expansion of cassava of cassava was undertaken by Trench & Martin (1984). A high incidence of CMD was observed in the Tonga and Mhala Irrigation schemes in Limpopo and Kwa-Zulu Natal as can be seen in the map on Figure 1.2, and low incidence was recorded in subsistence cassava near the Pongola river and immediately south of the Mkuzu river. In 1979 the collection and propagation of the cassava cultivar T400 was a first step for commercial production and was CMD free. However, a CMD epidemic developed on several farms in the Mkuzu area, which was a result of contamination of clean stock. The eradication seemed to have been successful, but several CMD affected areas existed in Swaziland and seem to have spread into subsistance plantings in South Africa.

As cultivars in subsistence plantings in South Africa are very susceptible to CMD, the comparatively limited distribution of the disease in subsistence plantings indicates that CMD-free planting material must have been used initially, while the spread of CMD has probably been restricted by the small size and isolation of peasant plantings. It seemed that there was a buffer zone of CMD-free cassava between the Makatini flats area and the commercial production area. It seems that the areas that were potentially threatened by the disease included Kwazulu-Natal, and areas adjacent to the Tonga and Mhala Irrigation Schemes in the Limpopo Province (Trench & Martin, 1984). The areas affected with CMD are illustrated in the map below (Figure 1.2).

Between 1984 and 1999 no surveys of CMD epidemiology were undertaken. In 1999 a survey of cassava mosaic geminiviruses (CMGs) was undertaken (Berry *et al*., 2001) and CMGs were found to be widespread in all cassava growing regions, except in the newly commercially cultivated plantings in Dendron, Limpopo Province. Currently a survey of CMGs is being undertaken in the Mpumalanga province (USAID-Funded Project).



Figure 1.2 - Distribution of Cassava Mosaic Disease in South Africa (Trench & Martin, 1984)

1.5 Taxonomy of Cassava Mosaic Virus

CMGs belong to the *Geminiviridae* family, which is divided into four genera based on their biological properties and genome organization. These four genus these include:

1 – *Mastrevirus* : these geminiviruses have a monopartite genome and are also known as Subgroup 1 Geminiviridae and are transmitted by leaf hopper vectors to

monocotyledonous plants. *Maize streak virus* is one of the species included in this class.

2 – *Curtovirus:* which have features similar to the *Mastreviruses,* also with a monopartite genome but are transmitted to dicotyledonous plants and are also known as the Subgroup II members. Species which fall within this group include *Beet curly top virus*.

3 – *Topocuvirus*: this genus has only been recognized recently by the International Committee of Taxonomy of Viruses (ICTV) and has one member which is also the type species, *Tomato pseudo-curly top virus* which has a monopartite genome and is transmitted by a treehopper vector to dicotyledonous plants (Fauquet *et al*., 2003).

4 – *Begomovirus:* these viruses are transmitted by the whitefly *Bemisia tabaci* to dicotlyledonous plants. This class of viruses is also known as subgroup III. Most begomoviruses have bipartite genomes (have DNA A and DNA B components) although numerous begomoviruses with a monopartite genome occur. Cassava mosaic viruses fall within this class, upon which this research is be based.

1.5.1 *Begomovirus* –Structure & Open Reading Frames

As mentioned begomoviruses are either monopartite or bipartite, however the majority of begomoviruses have a bipartite genome consisting of DNA A and DNA B each 2.5 – 2.8 kb in size and collectively contain six to seven open reading frames (ORFs) whose molecular weight is larger than 10000 Da. Table 1 below illustrates all the ORFs in begomoviruses.

Table 1.1 – Open Reading Frames on DNA A and DNA B of Bipartite Begomoviruses

DNA A		
	SIZE (Da)	**PRODUCT (Protein)**
AV2 (AR2)	29.7	Coat Protein (CP)
AV1	12.8	Movement
AC1 (AL1)	40.2	Replication Enhancer Protein (Rep)
AC2 (AL2)	19.6	Transcriptional Activator Protein (Trap)
AC3 (AL3)	15.6	Replication Enhancer Protein (Ren)
AC4 (AL4)	12.0	Symptom expression
DNA B		
BV1(BR1)	331.1	Nuclear shuttle
BC1 (BL1)	29.6	Systemic movement

The DNA-A component encodes all viral functions necessary for replication and encapsulation of the single stranded genome. The DNA B component has two ORFS (table 1) both known to provide functions for movement in infected plants (Brown, 1996). In bipartite geminiviruses, the DNA A and DNA B components are both necessary for systemic infection of host plants. Figure 1.3 illustrates the arrangement of all the ORFs found on the bipartite genome.

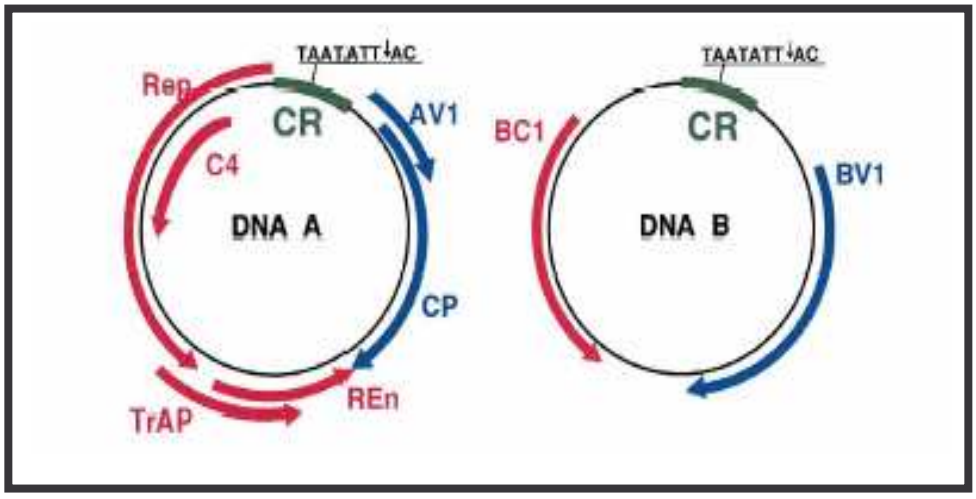

Figure 1.3 – ORFs found on Geminiviruses (Gutierrez, 2004)

1.5.1.1 AV2

The AR1 ORF encodes the coat protein (CP) gene (27-28kDa) which is the most highly conserved gene among the geminiviridae. This gene products main function is to encapsidate the ssDNA genome, one circular molecule per capsid. If the AR1 gene product is absent then this results in a delay or attenuation of disease symptoms (Brown, 1996).

1.5.1.2 AL1 (Rep)

The Replication protein (Rep) is the second most highly conserved gene product of the begomoviruses with a 61-71% similarity at the nucleotide sequence level (Brown, 1996). It is a multifunctional protein and is the only viral protein which is essential for replication which happens via the rolling circle mechanism.

1.5.1.3 AL2 (TrAp)

The gene products of AL2, 3 and 4 have regulatory functions which aid the systemic infection in host plants. The transactivating protein (TrAp) is required for normal accumulation of viral ssDNA. AL2 transactivates the transcription of the

rightward genes, AV2 (CP) and BV1. AL2 functions to not appear to be virus specific.

1.5.1.4 AL3 (REn)

The AL3 gene product or replication enhancer (REn) protein is not essential for replication but has been shown to play an important role in this process (Brown, 1996). It has been shown that this protein functions as a replication enhancer factor.

1.5.1.5 AL4

The function of the AL4 protein has not yet been elucidated. But there is evidence that C4 is involved in either virus movement or functions in symptom expression. There is also evidence that AL4 maybe involved in repression of AL1 gene expression.

1.5.1.6 BR1 and BL1

DNA B components of bipartite begomoviruses are needed for systemic infection. Functional and genetic assays have shown that these proteins are essential for effecting systemic movement for production of disease symptoms in plants. BR1 and BL1 move coordinately in viral movement. The BL1 encodes a movement protein (MP), which localizes to cell walls and has been linked to viral pathogenic properties (Ingham *et al*., 1995).

1.5.1.7 Intergenic Region (IR)

A large intergenic region (IR) which is about 280-350 bases is located between the AR1 and AL1 genes on the A component and between the BR1 and BL1 genes on the B component. Within the IR of bipartite of is a region referred to as the common region (CR) that is identical for the DNA A and DNA B components. The IR/CR contains the origin of replication and promoters responsible for the transcription of leftward and rightward gene sequences that of forming a hairpin

structure and the loop of the structure contains the sequence TAATATTAC which is part of the origin of replication (Brown, 1996).

The cassava geminiviruses are grouped according to their variable nucleotide sequences, the different species include African Cassava Mosaic Virus (ACMV), East African Cassava Mosaic Virus (EACMV), East African cassava mosaic Zanzibar virus (EACMZV), East African cassava mosaic Cameroon virus (EACMCV-CM), East African cassava mosaic Malawi virus (EACMV-MW), the South African cassava mosaic virus (SACMV), Sri Lankan cassava mosaic virus (SLCMV) and also the Indian cassava mosaic virus (ICMV) (Fauquet *et al*., 2003).

1.6 **The Viral Replicative Cycle**

Geminivirus replication takes place in the nuclei of the infected cells where virus particles and virus-induced round structures maybe seen by electron microscopy (Legg, 2004). The replication protein begins viral replication by binding specifically to reiterated motifs (introns) with the IR and introduces a nick into the conserved CR TAATATT/AC sequence. Rep also binds to the plant homologue of retinoblastoma protein (Rb) to regulate cell-cycle progression altering the environment of terminally different cells to give host factors that support viral DNA replication.

TrAp transactivates expression of virion-sense gene expression from both DNA A and DNA B, it also functions in the suppression and post-transcriptional gene silencing. Although the REn protein is not essential it boosts viral DNA replication by several fold. The NSP and MP proteins encoded by the DNA-B component are essential to shuttle viral proteins and DNAs from the cytoplasm to the nuclei and from one cell to the next.

The geminivirus replication cycle can be subdivided into several functionally distinct stages and characterized by specific events. In the early stages of the infection process viral particles are injected by the insect vector presumably coated and the viral genome in this way is transported into the host cell nucleus by mechanisms whose molecular details are unknown. Once within the nucleus the viral genome

commences amplification and this involves an efficient DNA replication process occurs in three stages:

Stage A – Conversion of the genomic circular ssDNA ((c) ss DNA) into supercoiled covalently closed circular dsDNA intermediate or replication form I ((ccc) as DNA (rf1))
Stage B – The amplification of the ds DNA intermediates by a rolling circle mechanism.
Stage C – Production and encapsidation of mature genomic ssDNA into viral particles.

1.7 **Aims & Objectives**

The different begomoviruses in this study were:

1. *South African cassava mosaic virus* (SACMV)
2. *East African cassava mosaic virus* Uganda Variant (EACMV-UG-233-1/2)
3. *African cassava mosaic virus* (ACMV R7-Ug-226-1/2)

In this study, the resistance/susceptibility of different cassava cultivars to SACMV was investigated. EACMV and ACMV were used as comparitive controls in host infectivity studies. The main objectives were:

(i) To propagate the cultivars obtained from The International Institute of Tropical Agriculture (IITA) in Nigeria and test for SACMV resistance/susceptibility. EACMV and ACMV infected plants were used as controls as these cultivars had been tested for EACMV and ACMV resistance by the IITA.

(ii) To test for EACMV and ACMV resistance in the locally grown cultivars T200 and T400 which are grown for commercial starch manufacture SACMV infected plants were used as controls for the T200 and T400 cultivars.

(iii) To infect a model host plant, *Nicotiana benthamiana* as an infection control for begomoviruses ACMV, EACMV and SACMV.

The reason this research is being carried out is so that resistant cultivars can be identified and used in the germplasm pool which will then be used in future breeding programs which is targeted at small-scale farmers who will use the cultivars as a cash crop and also at commercial farmers for starch production.

The project was divided into two sections:

(a) A molecular component which involved isolation and preparation of the Cassava Mosaic Geminiviruses for infectivity studies and

(b) A tissue culture component which was comprised of *in vitro* nodal propagation, transplantation into soil and acclimatization under green house conditions.

CHAPTER 2

Isolation and Purification of DNA Monomers

2.1 Introduction & Overview of Methods

The study was divided into two major sections as can be seen in Figure 2.1, a molecular component where the cassava mosaic virus for infection of cassava plants was prepared which is the main focus of this chapter and a tissue culture component where cassava plantlets were grown under *in vitro* conditions and acclimatized (Chapter 3) to be infected with the virus via agro-inoculation and biolistic bombardment (Chapter 4).

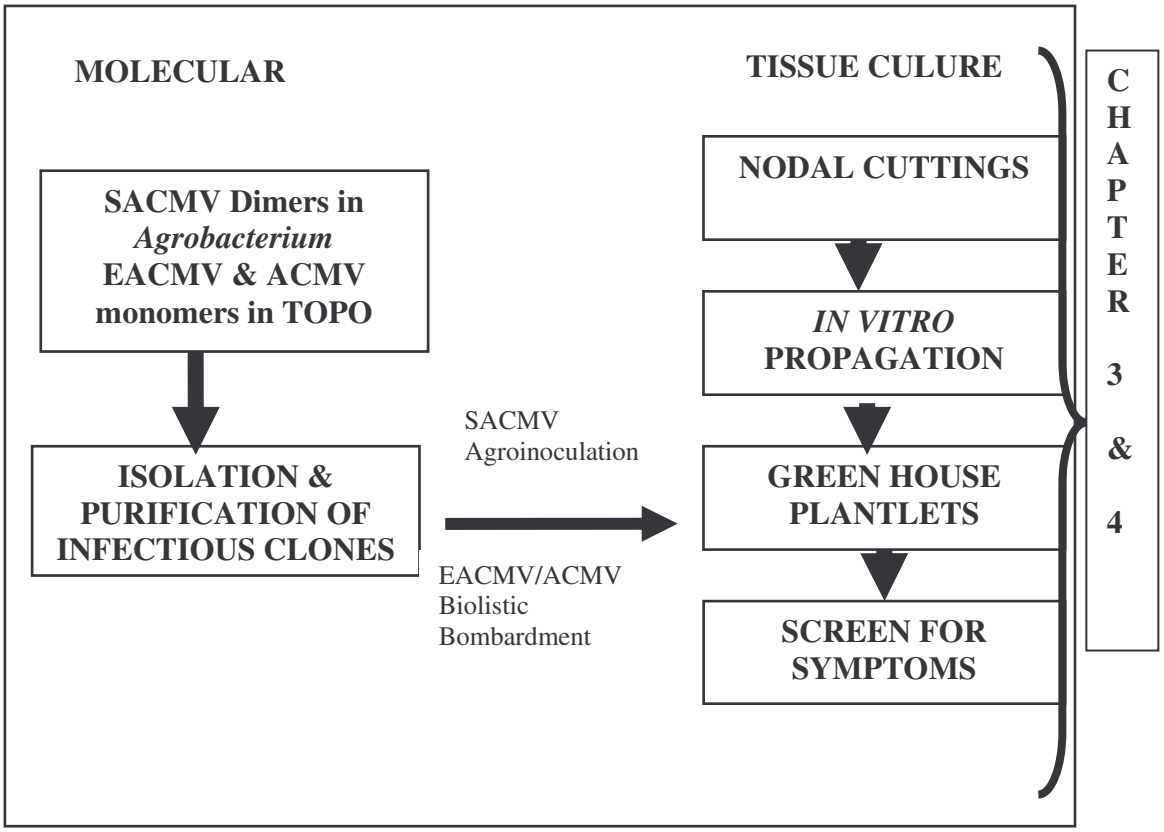

Figure 2.1 Overview of Methods used in Research

2.2 SACMV, EACMV & ACMV Infectious Clones

The infectious *South African cassava mosaic virus* (SACMV) clones used in this study were obtained from the Agro-SACMV constructed by Leigh Berrie (1998). A head-to-tail dimer of SACMV DNA-A and SACMV DNA-B had been subcloned into pBIN19 to form pBINSA-A and pBINSA-B and was mobilized into *Agrobacterium tumifaciens* strain C58C1 creating Agro-SACMV-A and B. *Nicotiana benthamiana* and a variety of cassava cultivars were infected with the virus through agro-inoculation (chapter 4).

The East African cassava mosaic virus (EACMV) and African cassava mosaic virus (ACMV) infectious clones used in this study were obtained from John Stanley at the John Innes Centre, UK in the form of infectious monomers cloned in the TOPO vector (Invitrogen) at the *EcoR1* site (Fig 2.2).

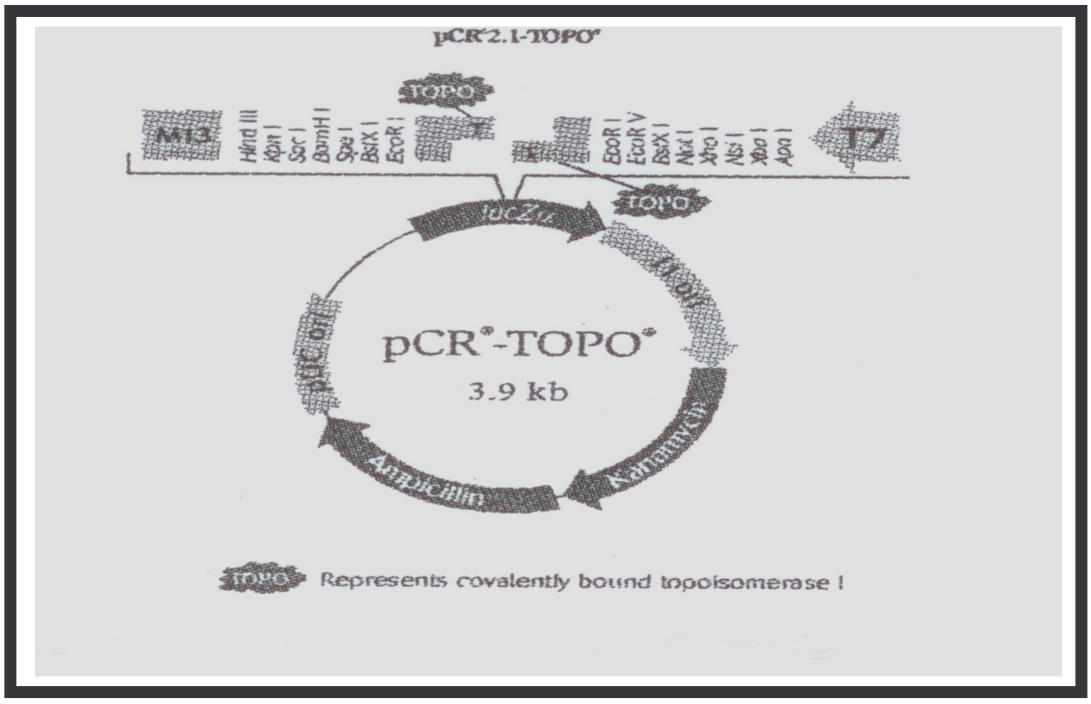

Figure 2.2 Map of TOPO Vector (Invitrogen, 2004)

2.3 **Materials & Methods**

2.3.1 Precipitation of Cloned Viruses

The ACMV and EACMV infectious clones were obtained as DNA-A and DNA-B monomers as mini-prep inserted in the TOPO vector (Fig 2.2). An ethanol precipitation was done, as described in Appendix 1A to precipitate the clones.

2.3.2 Transformation into *E.coli*

The virus recombinant clones were transformed into DH5-α *E.coli* cells using the TSB method which is outlined below, the composition of TSB buffer is described in Appendix 1B. As a positive control pHannibal DNA was used to transform *E.coli* cells and as negative controls LB (Amp/Kan) blank plates were used and untransformed *E.coli* cells.

Transformation (TSB Method)

1. Cultures of *E.coli* DH5- α (~ 1.5 ml) were grown in 30ml Luria Broth (LB – Appendix 1C) and Ampicillin (100µg/ml) and kanamycin (100µg/ml). Overnight (O/N) at 37°C at 200rpm.
2. 1ml O/N culture was added to 30ml fresh LB and grown at 37°C for about 2h until the OD_{600} = 0.3-0.6.
3. 1ml aliquots of culture were spun down at 12000 maximum speed for 2 minutes at room temperature. Supernatant was discarded.
4. The cells were resuspended in 100µl cold TSB by pipetting.
5. They were incubated on ice for 10 minutes.
6. 15µl of DNA (i.e. controls and DNA) was added.
7. The cells were mix thoroughly and incubated on ice for 30 minutes.
8. 900µl TSB and 18ul 5M glucose was added and incubated at 37°C (200rpm) for exactly 1 hour to activate the antibiotic resistance genes.
9. LB agar plates were prepared as described in Appendix 1C (Kanamycin/Ampicillin).

10. Cells were spread onto the prepared plates (450µl and 50µl)
11. The plates were incubated for no longer than 17 hours.
12. The white colonies that were present were harvested as part of the screening process.

2.3.3 Screening for Transformants

Harvesting

1. In Laminar flow aseptically 5ml of LB was poured into 12 tubes and 5µl 100mg/ml Amp and 5µl 100mg/ml Kan was added to each tube, a control tube was used which contained 5ml LB and antibiotics.
2. A colony was picked from the plates using a gilson micropipette and the tip was dropped into the tube. Six duplicate colonies were grown for EACMV/ACMV DNA-A and DNA-B.
3. The tubes were incubated at 37°C O/N at 200rpm.
4. The following day a mini-prep was done to isolate the ACMV/EACMV A and B from the *E.coli* cells.

Following the harvesting of transformants, glycerol stocks were made with 300µl 50% glycerol and 850µl of the O/N stock and stored at -20 ^{o}C and mini-plasmid prep was carried out. The procedure that was carried out is described below.

Mini-Prep Procedure

1. For each of the EACMV and ACMV DNA A and B 1.5ml of the O/N culture was poured into each of the six eppendorf tubes.
2. The cultures were centrifuged at 4°C and thereafter supernatant was removed.
3. The pellet was resuspended in ice cold solution I (100µl) by vortexing vigorously.
4. The tube was left at room temperature for 5 minutes and 200µl freshly prepared solution II was added and mixed by inverting 5 times rapidly.
5. The mixture was incubated on ice for 5-10 minutes.

6. 150µl of ice cold solution III was added and mixed vigorously.
7. The mixture was incubated on ice on for 5-10 minutes then centrifuged at high speed for 10 minutes at 4°C.
8. The supernatant (top layer) was removed into a clean tube.
9. This was centrifuged for a further 3 minutes and the supernatant was removed again into a clean tube.
10. 350µl Isopropanol was added, mixed and left at room temperature for 5 minutes.
11. DNA was precipitated by centrifuging at 4°C at a high speed for a further 10 minutes.
12. Supernatant was removed.
13. The pellet was washed with 1ml of 70% ethanol and Spun at 4°C for 10minutes. Ethanol was removed and a pipette was used to remove.
14. The pellet was allowed to dry (+1hour).
15. The pellet was resuspended in 14ul injection water and 1ul RNASE.
16. The resuspended pellet was left at room temperature for 20-30 minutes.
17. A gel was run with 4ul and the remainder stored at -20°C.

2.3.4 Restriction Enzyme Digests

Restriction enzyme digests were done to excise the ACMV/EACMV A and B from the TOPO vector (Figure 2.2) using *EcoR1* enzyme. This was done by adding 5µl of ACMV/EACMV A and B to 2µl of *EcoR1* buffer, 1µl *EcoR1* enzyme and 7µl of injection grade water and incubating the mixture at 37°C for 3 hours. 2µl of the excised monomers were run on a 0.8% gel. These excised monomers were used to infect cassava cultivars via biolistic bombardment (Chapter 4).

2.4 **Results & Discussion**

2.4.1 Precipitation of ACMV and EACMV Infectious Clones

Successful precipitations were carried out and DNA was present as viewed on gels run.

2.4.2 Transformation

Transformation of EACMV and ACMV monomers into *E.coli* were successfully performed with a high transformation efficiency, over fifty colonies grew on LB plates which were spread with 450ul of cells. Table 4.1 shows the results of the transformation.

Table 2.1 – Results of Transformation with ACMV & EACMV

LB (Amp/Kan) Plates	No. of Transformed Colonies Per Plate (White Colonies)
Blank Plate (Negative Control)	0
E.coli Cells (Positive Control)	0
pHannibal DNA (Negative Control)	>200
ACMV-A (450µl)	224
ACMV-A (50µl)	58
ACMV-B (450µl)	88
ACMV-B (50µl)	15
EACMV-A (450µl)	>75
EACMV-A (50µl)	9
EACMV-B (450µl)	>87
EACMV-B (50µl)	50

2.4.3 Screening for Transformants

Plasmid mini-preps were carried out to screen and to isolate the EACMV and ACMV monomers from the *E.coli* cells. As seen in Figure 2.3 the respective ACMV

monomers are in different confirmations, which are represented by the different bands. The different confirmations of the DNA include circular DNA, linear DNA, supercoiled DNA and closed circular DNA (Edvotek, 1998). Supercoiled DNA has the fastest migration rate of the different forms of plasmid so its seems that the band which has run the longest distance is supercoiled DNA. Closed circular DNA moves the slowest so it is the bands which have run the least that are assumed to be in closed circular DNA form. The rate at which the DNA molecule migrates through the gel depends not only on its size and shape but also on the type of electrophoresis buffer used, the gel concentration and he applied voltage. In this study TAE buffer (Appendix 1F) was used and 0.8% gels.

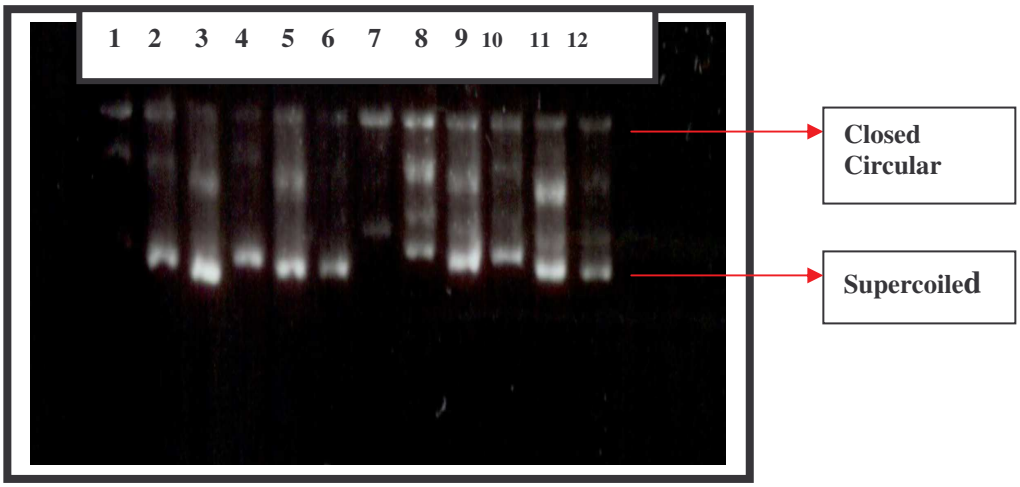

Figure 2.3 ACMV monomers (in TOPO) Plasmid Mini-Prep After Transformation - Lane 1-6 ACMV-A, Lane 7-12 ACMV-B

2.4.4 Restriction Enzyme Digests

Restriction enzyme digests were carried out and the ACMV/EACMV DNA-A and DNA-B monomers were excised from the TOPO vector at the *EcoR1* flanking sites. As can be seen on Figure 2.4a-d the *EcoR1* sites on the ACMV and EACMV DNA-A and DNA-B are shown. The TOPO vector is 3.9kb and ACMV/EACMV