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Molecular characterisation of a new cassava geminivirus (ACMV-SA) in South Africa

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TLCV	Tobacco Leaf Curl Virus
TYLCV	Tomato Yellow Leaf Curl Virus
TYLCV-Isr	Tomato Yellow Leaf Curl Virus - Israel Isolate
TYLCV-Sar	Tomato Yellow Leaf Curl Virus - Sardinia Isolate
TYLCV-Sic	Tomato Yellow Leaf Curl Virus - Sicily Isolate
TYLCV-Th	Tomato Yellow Leaf Curl Virus - Thailand Isolate
ToLCV	Tomato Leaf Curl Virus
ToLCV-Aus	Tomato Leaf Curl Virus - Australia
Tris	Tris (hydroxy methyl) - aminomethane
U	Units
Vol	Volume
w	watts
w/v	weight to volume
µCi	micro curie
µg	microgram
µl	microlitres(s)
µm	micrometre
%	percentage

h	hour(s)
ICMV	Indian Cassava Mosaic Virus
Kb	Kilobase
KDa	Kilodaltons
l	litre(s)
l	Linearized
LSS	Logarithm of Site Scatter
M	Molar
MSV	Maize Streak Virus
mA	milliamperes
MES	(2(B-Morpholino)-ethane-sulphonic acid)
mg	milligram
min	minute(s)
ml	millilitre
mm	millimetre
MS	Murashige and Shoog
NAA	Naphthalene acetic acid
nm	nanometre
OC	Open circular
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
pmole	picomole
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
Sec	Second(s)
T_4	bacteriophage
TEMED	N, N, N, N, - tetramethyl ethylenediame
TGMV	Tomato Golden Mosaic Virus

LIST OF ABBREVIATIONS

A	ampere
ACMV	African Cassava Mosaic Virus
ACMV-Ken	African Cassava Mosaic Virus - Kenyan Isolate
ACMV-Mal	African Cassava Mosaic Virus - Malawi Isolate
ACMV-Nig	African Cassava Mosaic Virus - Nigeria Isolate
ACMV-SA	African Cassava Mosaic Virus - South Africa Isolate
BAP	Benzylaminopurine
BCTV	Beet Curly Top Virus
BGMV	Bean Golden Mosaic Virus
BGMV-Bra	Bean Golden Mosaic Virus - Brazil Isolate
BS	pBluescript
°C	degree celsius
CCC	Covalently closed circular
2,4-D	2,4 dichlorophenoxyacetic acid
Dicot	Dicotyledon
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
d/ddNTP	deoxy/dideoxyribonucleoside triphosphate
dNTP	deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	Ethylene diaminetetra acetic acid
Fig.	Figure(s)
FS	Forward Scatter
g	force of gravity

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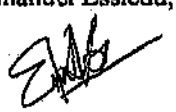
Sincere thanks to Manini Makhema, for painstaking typing this thesis. I would also like to thank Maria, Sarah and Mona at the Microbiology Department for their advice and assistance and my friends and family for their support and encouragement.

Finally I would like to thank the almighty God for his help and provision throughout the duration of this work.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted in partial fulfilment for the degree of Master . of Science in Biotechnology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Emmanuel Essiedu, K.O.



28 day of February, 1995.

In this study, 371bp of the 607bp ACI fragment were sequenced. Sequence comparisons with ACI regions of other geminiviruses indicated that ACI ACMV-SA shared the highest sequence homology with ACMV-Nig (from Nigeria, i.e 80%). However, it also showed a reasonably high sequence homology with Tomato Yellow Leaf Curl Virus (TYLCV)-isolates from Italy (75% with TYLCV from Sardinia and 73% with TYLCV from Sicily) as well as 78% sequence homology with Tomato Leaf Curl Virus (ToLCV) from Australia.

The restriction map of DNA-A of ACMV-SA was established but a convenient restriction site for subcloning the whole of AC1 putative open reading frame (ORF) of the DNA-A genome was not observed. However, using *Pst*1 and *EcoR*1 a large part of it could be subcloned. Cloning of the 607bp ACI fragment of ACMV-SA into plant transformation vectors pBIN19 and pBI121 was not achieved. The transformation control experiments did work, hence the reason for cloning failure may be due to the ligation step.

Healthy plantlets of Nigerian cassava cultivars TMS87100609, TMS63397 and tobacco (Nicotiana tabacum) with developed roots, stems and leaves were successfully propagated from their nodal explants. Calli from immature leaves of thecassava and tobacco cultivars were successfully induced on Murashige and Shoog (MS) medium containing 8mg/l 2,4-D. Cassava calli were creamy in appearance and tobacco calli were greenish in colour. It took 3 weeks to induce the cassava calli while in tobacco 2 weeks was sufficient to obtain callus. Cell suspension cultures of the cassava and tobacco cultivars were initiated using friable calli as inocula. The suspension cells of both cassava and tobacco cultivars were subcultured two weeks after initiation of the primary cultures. Suspension cells counts showed that the viable cell density range of 0.5 - 2.0 x 10^6 cells per ml was attained after 14 days, after which subculturing took place.

Protoplasts were isolated from tobacco and cassava suspension cultures after 5h and 8h of incubation respectively in 2% cellulase Onozuka R10 and 0.1% pectolyase Y23.

ABSTRACT

Cassava (Manihot esculenta Crantz) is a perennial woody shrub grown mainly in Latin America, Africa and Asia for its starchy tuberous roots. The cassava crop provides a lot of calories for both animal and human consumption as well as starch for industrial use. Because of its broad adaptability to a variety of soil and climatic conditions (such as drought tolerance and ability to grow on depleted and marginal soil), the crop is very important to the agroeconomy of several tropical countries. The main threat to the crop's survival is the Cassava Mosaic Disease (CMD) caused by the African Cassava Mosaic Virus (ACMV). This virus is a bipartite geminivirus.

Recently a new strain of ACMV (i.e. ACMV-SA) has been observed to infect cassava crops in Kwazulu-Natal and Eastern Transvaal in South Africa. Initial studies of a 793bp Polymerase chain reaction (PCR) generated fragment sequence of the DNA-A component of the virus reveals that it shares a higher sequence homology with a monopartite TYLCV than with its other bipartite ACMV counterparts. This unique ACMV-strain is a cause for concern which needs to be further investigated so that an effective approach can be taken to combat its devastating influence on cassava in South Africa.

In this project further characterization of DNA-A of ACMV-SA was pursued so that: **(1)** antisense-ACI ORF (putative polymerase) expression could be developed in genetically engineered virus-resistant cassava; **(2)** the new virus could be compared with other similar geminiviruses. The characterization was done by: **(1)** Partially sequencing a 607bp ACI PCR-generated fragment of ACMV-SA cloned into *Pst*1 siteof pBluescript SK(-) and comparing the sequence with other geminiviruses; **(2)** constructing a restriction map of the DNA-A of ACMV-SA cloned into pBluescript KS so as to identify convenient restriction sites for future subcloning experiments. For the Agrobacterium transformation studies, in vitro plantlets, callus and suspension cell cultures for cassava and tobacco were maintained. Protoplast isolation from cell suspension cultures was carried out for purposes of inoculating them with DNA-A of ACMV-SA. An attempt was made to clone the 607bp ACI fragment of ACMV-SA into the plant transformation vectors pBIN19 and pBI121.

MOLECULAR CHARACTERISATION OF A NEW CASSAVA GEMINIVIRUS (ACMV-SA) IN SOUTH AFRICA

EMMANUEL K.O. ESSIEDU

A Thesis Submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in Partial Fulfilment of the Requirements for the Degree of Master of Science.

February 1995.

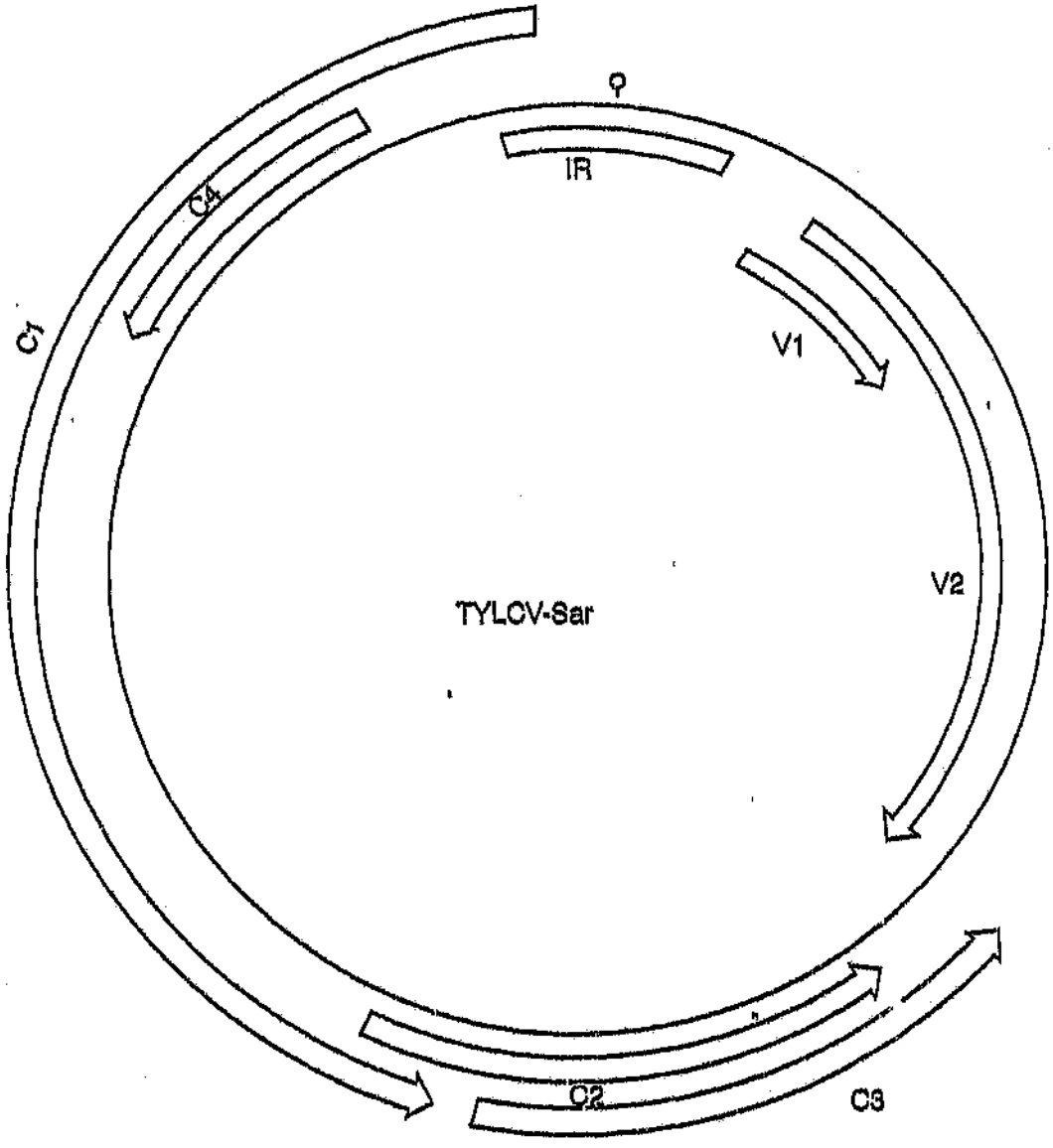


Figure 3: Genome Organization of TYLV ORFS. The virion - sense (+) strand and complementary sense (-) strand are displayed by arrow s . The position of the conserved stem - loop structure (ρ) and intergenic region (IR) are shown (Khey- Pour et al, 1991)

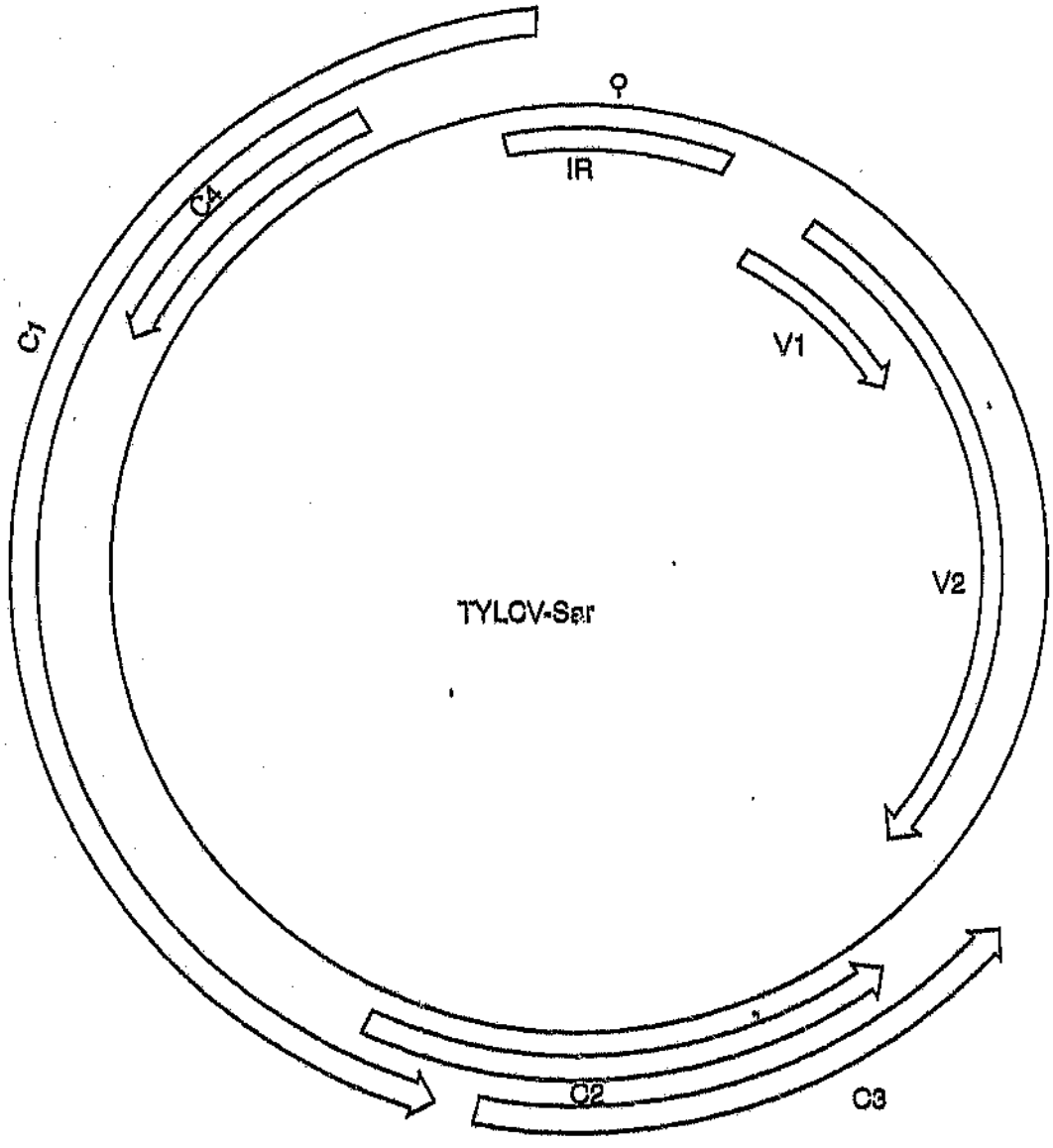


Figure 3: Genome Organization of TYLV ORFS. The virion - sense (+) strand and complementary sense (-) strand are displayed by arrow s . The position of the conserved stem - loop structure (♀) and intergenic region (IR) are shown (Khey- Pour et al, 1991)

leaf abrasion. However, BCTV can be introduced into Nicotiana benthamiana by agroinoculation (Grimsley et al, 1986; Briddon et al, 1989). This technique demonstrates an efficient way of delivering an inoculum directly into tissues in which the virus proliferates and spreads.

Most monopartites have six ORFs that encode polypeptides of over 10 kilodaltons. Two of the ORFs occur on the virion plus strand (including the capsid protein V1 gene) and four on the complementary negative strand (including the C1 gene necessary for replication (Figure 3).

1.3 AFRICAN CASSAVA MOSAIC VIRUS (ACMV)

1.3.1 African Cassava Mosaic Virus in Africa

ACMV is the casual agent of cassava mosaic disease (CMD) in Africa. This is a serious disease of cassava (Manihot esculenta crantz), which has been reported in all cassava growing areas in Africa (Storey and Nicols, 1938) and more recently in India (Menon and Raychandhuri, 1970). It does not occur in the Americas, the recognised origin of the crop (Lazano and Booth, 1974). The disease causes a 60 - 80 percent decrease in tuber yields of plants of susceptible varieties when infected cuttings are used for propagation (Bock, 1983). ACMV is an economically important virus since in some African countries as much as half the human kilojoule intake is derived from cassava. The ACMV was first isolated in East Africa and named Cassava Latent Virus (CLV) (Bock et al, 1978) and only later renamed ACMV (Bock and Wood, 1983) when it was found to cause cassava mosaic disease.

ACMV is a bipartite geminivirus transmitted in a persistent manner by whitefly Bemisia tabaci (Golding, 1936; Storey, 1954). Self (1981) reported that even though the virus is transmitted by a single whitefly, the percentage transmission increases with the number of insects per plant. The virus induces a mosaic pattern on the leaves,and is only found in the yellow and yellow-greenish areas of the mosaic pattern. Viral contents of the leaves increase with increasing symptom intensity, but decreases with age of the leaf and ACMV is usually not detected in mature leaves. Most whiteflies are seen on the young growing cassava leaves and the number of whitefly progressively decrease with the age of the leaf.

Relationships between bipartite geminiviruses can be demonstrated by DNA- hybridizations. Spot hybridizations involving extracts from leaves infected by various geminiviral genomes and probes for ACMV DNA-A or DNA-B revealed that substantial genome homologies exist in the viral DNA-A (i.e. the DNA species which contains the protein gene (Stanley et al, 1985). For example, nucleotide sequence homologies were found between ACMV DNA-A and DNA-A of four other whitefly-transmitted viruses, BGMV, TGMV, Tobacco Leaf Curl Virus (TLCV) and Tomato Yellow Leaf Curl Virus (TYLCV), the last two of which are not sap-transmissible (Roberts et al, 1984). Conversely the nucleotide sequence of DNA-B differed to a greater extent between viruses. It can therefore be concluded that DNA-A probes but not DNA-B probes can be used to identify other bipartite whitefly-transmitted geminiviruses due to a reasonable level of homology between the DNA-A components.

1.2.4 Monopartite Geminiviruses

Monopartite geminiviruses have only one genome component that closely resemble DNA-A of the bipartite geminiviruses (Stanley et al, 1986; Navot et al, 1991; Kheyr-Pour et al, 1991). The single genome is sufficient to start a complete cycle of infection including a systematic spread. Most of these viruses are leaf-hopper transmitted, with the exception of a few like TYLCV which are whitefly transmitted.

Investigative studies have shown that monopartite geminiviruses such as Beet Curly Top Virus (BCTV) and TYLCV can infect a common host such as Nicotiana benthamiana without the presence of the DNA-B component of bipartites. This is due to fundamental differences in the mode of spread. For example, unlike a bipartite ACMV, BCTV needs coat protein (CP) expression for systematic infection of Nicotiana benthamiana (Stanley and Townsend, 1986; Briddon et al, 1989). Also distribution of BCTV appears to be rigidly phloem associated (Esau and Hoefert, 1973), unlike that of ACMV which extends additionally to leaf tissues (Sequeira and Harrison, 1982). This explains why bipartites and not monopartites like BCTV can be transmitted mechanically to Nicotiana benthamiana by

The ORFs occur both in the viral positive DNA strand as well as the complementary (negative) strand of each genome segment. Of the 4-ORFs on DNA-A, one AVI, about 1.0kb in size, occurs in the virion sense ORF and encodes for viral capsid protein of about 30.2 kilodaltons. The other 3 ORFs, ACI, AC2 and AC3 occur on the complementary DNA strand. ACI ORF of about 1.1kb in length encodes the putative DNA polymerase of about 40.3 kilodaltons. BVI and BCI occur in the virion-sense and complementary strand, respectively, of DNA-B. Both ORFs encode proteins responsible for localised cell to cell movement as well as for long distance vascular spread of the virus (Figure 2).

In infected plants DNA-A and B of bipartite viruses usually occur in similar concentrations in their double-stranded (ds) replicative forms (RF) (Ikegami et al, 1981; Stanely and Townsend, 1985; Abouzid et al, 1988; Lazarowitz and Lazdins, 1991). DNA-A can replicate on its own, but DNA-B depends on the presence of A to replicate (Rogers et al, 1986; Elmer et al, 1988). DNA-B is however necessary for systematic spread of the virus (Elmer et al, 1988; Etessami et al, 1988). Disease symptoms appear in plants only when both viral genome components are present at the same time. In an experiment to verify the ability of ACMV DNA-A to spread systematically without DNA-B, it was discovered that agroinoculation of ACMV DNA-A alone into Nicotiana benthamiana resulted in occasional spread of viral DNA throughout the stem, leaves and roots. Although the plants remained asymptomatic, the level of spread was about 5 percent of that observed when both DNA-A and B were present. Viral particles were seen in the upper leaves and this signified that DNA-B was not important for virus assembly (Klinkenberg and Stanley, 1990).

Replication and mutagenesis studies showed that ACMV contains two movement genes on DNA-B (ACMV BCI and BVI) and one on DNA-A (ACMV AC2) (Rogers et al, 1986; Townsend et al, 1986; Brough et al, 1988; Elmer et al, 1988; Etessami et al, 1988, 1991). Both genes on DNA-B were vital for wild type infection, but had no role in viral replication (Sunter et al, 1990). The only viral gene product essential for replication resides on DNA-A.

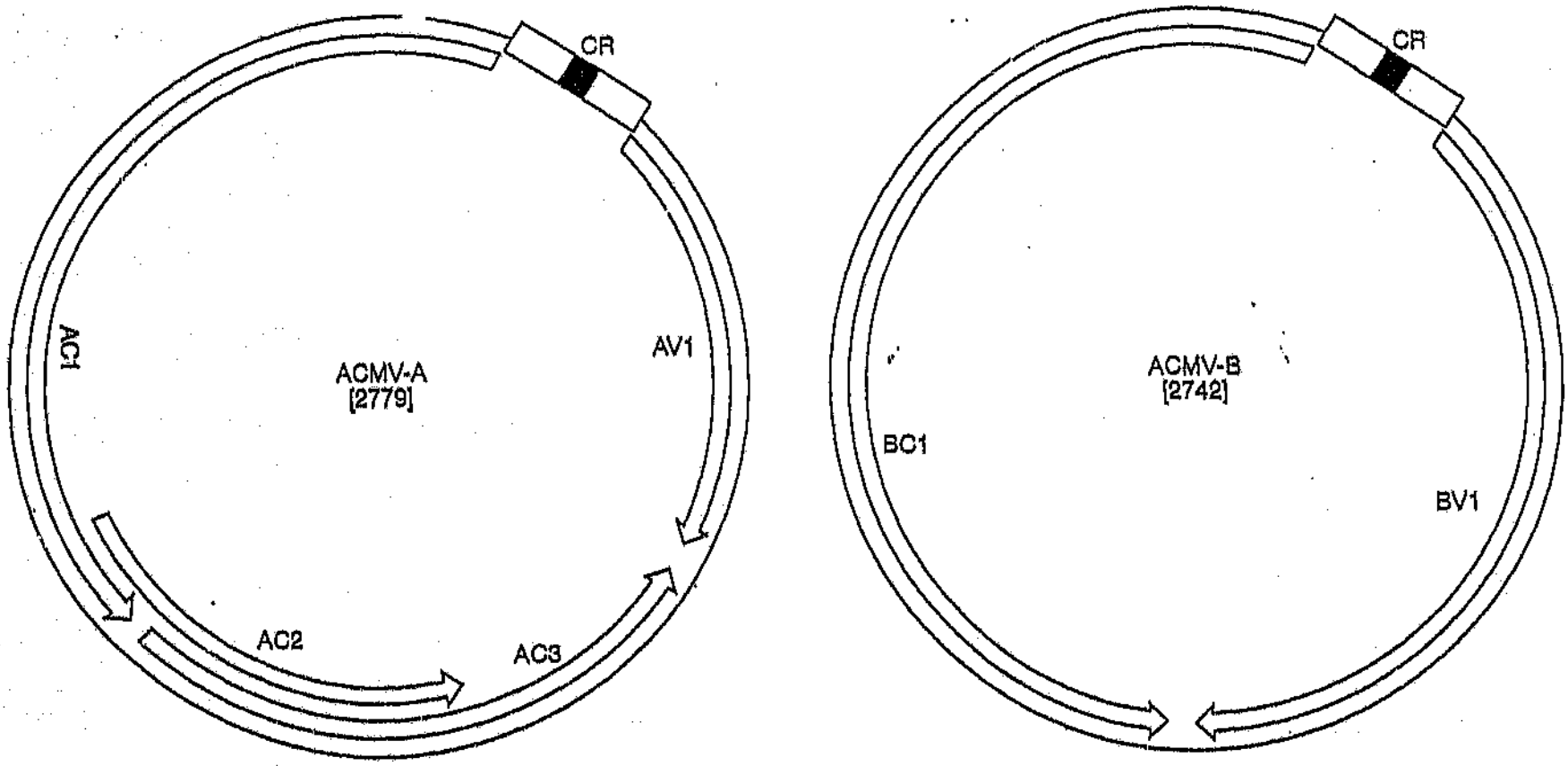


Figure 2: Genetic Map of ACMV-Ken. The nucleotide length of each DNA component is indicated. A ca 200 nucleotide common region (CR) is demonstrated. V- refers to open reading frames encoded directly by Viron (+) DNA and C to those encoded by complementary (-) DNA strand.(Stanley and Gay , 1983).

1.2.3 Bipartite Geminiviruses

Some geminiviruses have a bipartite genome consisting of two circular DNA molecules (designated DNA-A and DNA-B) separately encapsidated in geminate particles (Galvez and Castano, 1976). For instance, the total size of Bean Golden Mosaic Virus (BGMV) DNA fragments formed when the DNA was digested with HhaI was about 5000 nucleotides, which is almost twice the number of nucleotides expected on the premise of the physical size of the DNA. This showed that the BGMV genome is made up of two circular DNA molecules, indistinguishable in size, which may differ only in the nucleotide sequence and thus genetic content (Harber et al, 1981; Bisaro et al, 1982). The bipartites are mainly transmitted by the whitefly (Bemisia tabaci) and they infect only dicots.

The nucleotide sequence of a large number of bipartites geminiviruses are known. The nucleotide sequence of the two genome segments of each virus has been found to be different except for a common sequence (CS) of about 200 nucleotides. At a stretch of 33 to 34 nucleotides, the common sequence of these viruses appears to form a nearby identical stem-loop structure. The stem consists of two AT pairs located at position 4 and 5 from the loop, and nine GC pairs. The loop is composed of 11 or 12 nucleotides and includes a TAATATTAC sequence which is followed by TT, TTT, TA or ATA. The rest of the common sequence of nucleotides varies in these viruses. Restriction maps of the two ssDNA genomes of BGMV and Tomato Golden Mosaic Virus (TGMV) have shown that the two DNA molecules of each bipartite virus are widely different in nucleotide sequence except for a common region (CR) of high homology (Bisaro et al 1982; Harber et al; 1983). The two DNA molecules of each bipartite geminivirus are nearly identical in size and are composed of a slightly different number of nucleotides. For example, DNA-A of ACMV has 2779 nucleotides while DNA-B has 2724 nucleotides (Figure 2).

Putative open reading frames (ORFs), that can encode polypeptides of molecular weight greater than 10 kilodaltons, are present in both DNA-A and B of ACMV. Of these, four ORFs occur in equivalent sites on DNA-A and two on DNA-B.

GEMINIVIRIDAE

MONOGEMINIVIRUSES	INTERGEMINIVIRUSES	BIGEMINIVIRUSES	
		MONOPARTITES	BIPARTITES
♦ Obligatory leaf-hopper transmitted	♦ Obligatory leaf - hopper transmitted	♦ Obligatory whitefly transmitted	♦ Obligatory whitefly transmitted
		Bemisia tabaci	Bemisia tabaci
♦ Have one genome component	♦ Have one genome component		
♦ Have a narrow host range and infect mainly monocots	♦ Have a wide host range and infect mainly dicots	♦Infect mainly dicots	♦Infect mainly dicots
e.g. Maize Streak Virus (MSV)	e.g Beet Curly Top Virus (BCTV)	♦Have one genome component	♦Have two genome components [designated DNA-A and DNA-B]
		e.g.Tomato Yellow Leaf Curl Virus (TYLCV)	e.g. African Cassava Mosaic Virus (ACMV)

Figure 1: Showing Table of Proposed Classification of Geminiviruses [Source: Rybicki, 1994].

GEMINIVIRIDAE

MONOGEMINIVIRUSES	INTERGEMINIVIRUSES	BIGEMINIVIRUSES	
		MONOPARTITES	BIPARTITES
♦ Obligatory leaf-hopper transmitted	♦ Obligatory leaf - hopper transmitted	♦ Obligatory whitefly transmitted	♦ Obligatory whitefly transmitted
♦ Have one genome component	♦ Have one genome component	Bemisia tabaci	Bemisia tabaci
♦ Have a narrow host range and infect mainly monocots	♦ Have a wide host range and infect mainly dicots	♦Infect mainly dicots	♦Infect mainly dicots
e.g. Maize Streak Virus (MSV)	e.g Beet Curly Top Virus (BCTV)	♦Have one genome component	♦Have two genome components [designated DNA-A and DNA-B]
		e.g.Tomato Yellow Leaf Curl Virus (TYLCV)	e.g. African Cassava Mosaic Virus (ACMV)

Figure 1: Showing Table of Proposed Classification of Geminiviruses [Source: Rybicki, 1994].

viral (positive sense) DNA strand and complementary (minus sense) DNA strand. The first stages of the viral replication involves the formation of a complementary DNA strand followed by transcription from the double-stranded DNA molecules. The double-stranded (ds) templates then take part in the synthesis of virus ssDNA molecules by a rolling circle mechanism. The structure that takes part in the synthesis of both the complementary and viral strands is the stem-loop located in the common region (CR) found to be present in genomes of all geminiviruses (Hamilton et al, 1984). The circular DNAs (especially cccDNA) always predominate in all purified viral preparations, although linear DNA forms can be found in small amounts. The linear DNA forms are almost of equal contour length to the circular DNA molecules and are thought to have arisen from circular ones by a single break; moreover, only the cccDNA forms are infectious hence confirming the assumption that native DNA of each geminivirus particle has a circular topology and that the linear DNA is obtained from it by a single nick (Goodman et al, 1980).

1.2.2 Classification of Geminiviruses (Geminiviridae)

Geminiviruses can be classified into three proposed sub-groups (figure 1) depending on their insect vector, host range and number of DNA components (Davis and Stanley, 1989; Rybicki, 1994).

Cassava has the potential to be used as a substrate for producing large amounts of ethanol which can serve as a partial alternative to petroleum. This can help scale down the cost as well as the over reliance on petroleum which normally imposes a drain on an importing nation's foreign exchange reserves.

Despite these several advantages the most worrying set-back to cassava tuber yields in Africa is the Cassava Mosaic Disease (CMD). This debilitating disease is caused by the African Cassava Mosaic Virus (ACMV), a virus that belongs to a family of viruses known as Geminiviruses.

1.2 CHARACTERISTICS OF GEMINIVIRUSES

1.2.1 Structure and Replication

Geminiviruses are a group of small-plant infectious viruses whose nucleic acids are encapsidated in unique geminate (twinned) quasi isometric structure measuring about 30 x 20nm (Harrison et al, 1977; Matthews, 1976). These viruses infect a large number of economically useful plants belonging to the euphorbiaceous, graminaceous, solanaceous and leguminous families (Norris et al, 1994).

Geminiviruses are usually made up of single-stranded covalently closed DNA molecules of approximately 2.7kb in size (Stanley, 1985; Davies and Stanley, 1989). Their single-stranded DNA (ssDNA) content identifies them from the other known group of plant viruses that have a DNA genome, the caulimoviruses, which are made up of double-stranded DNA (Sheperd and Lawson, 1981). The number of nucleotides per DNA molecule of geminiviruses ranges from 2500 to 2800 (Francki et a1980).

The geminiviruses usually accumulate and replicate within the nuclei of infected plant tissue and induce cytopathological effects in the form of distinctive fibrillar rings made up of deoxyribonucleoproteins (Kim and Flores, 1979). Three double-stranded replicative forms of the covalently closed circular (ccc), open circular (oc) and linear molecules of the viral genome can be identified in infected plants. These replicative forms, which are also the transcriptional active forms of the viruses, consist of the

1. INTRODUCTION

1.1 CASSAVA AS A USEFUL CROP

Cassava (Manihot esculenta Crantz) is a very important plant whose carbohydrate rich root tubers have been used over the years as a subsistence crop. The crop was first introduced to East and West Africa from South America in the early 17th century by Portuguese colonizers. From these introduced domains, the crop spread to other tropical areas of Africa. In South Africa, cassava got to the Eastern Transvaal and Northern Natal as a result of the southward migration of the Tonga tribesmen in the 1830's and 1960's. In the rural areas the cassava crop is well known by the older folks and the Zulu tribesman calls it *'ndumbula'*.

Cassava is usually drought resistant, possesses great tenacity to thrive in poor sandy and infertile soils and has a relative resistance to weeds and insect pests. Cassava can grow even under conditions of erratic rainfall and extreme heat. These attributes coupled with the fact that the crop's root tubers can be left in the soil for a long time before harvesting offers a crop a useful security against famine (Lazano and Booth, 1974). Moreover, as it is not season dependent, it can be cultivated all the year round. Cassava is therefore a useful crop that can provide a useful reserve food, and hence provide a healthy defence in the battle against drought and hunger. The introduction and promotion of cassava to the rural populations could go a long way to help to alleviate malnutritional problems in many rural areas in South Africa.

About 60% of all cassava plants produced are eaten, the rest are either processed in industry or fed to animals. The starch content of cassava has a low amylose and high amylopectin content. These features give cassava an unusual viscous characteristic and great dimensional strength, which are vital to the food, textile and paper industries and also as a parent material from which to hydrolyse dextrins for forming adhesives. Cassava is similar to maize in energy value except its protein content is lower. However, if cassava flour is well processed and supplemented mainly with methionine, very high levels of it can be used in rations for pigs, poultry and ruminants (Nestel, 1992).

protoplasts. There are now available several effective enzymes for the degradation of the cell walls of leaf tissues or suspension cells and subsequent release of protoplasts. Some of the commonly used enzymes include pectolyase Y23 and cellulase R.S.

The effective inoculation of protoplasts requires the presence of a concentrated viral DNA, or plasmid with insert e.g AC1gene of high specific infectivity and freshly- prepared suspension of actively metabolising protoplasts. To hasten the DNA infection of protoplasts, the latter are either induced chemically with polycations such as poly-L-ornithine(PLO); polyethyleneglycol(PEG); or electrically by electroporation; to take up the DNA across their cell membranes. The genetic transformation of indica rice cultivar (Datta et al, 1990) has been achieved by DNA transfer to protoplasts. The major limitation to the use of protoplasts , as DNA recipients, are the necessities for cell cultures from which protoplasts can be obtained and the difficulties associated with the regeneration of fertile plants from these transformed protoplast. Delivering foreign DNA into intact plant cells and tissues (e.g. suspension cells) usually eliminates the need for protoplasts. The most successful direct delivery into intact cells invovles the use of DNA-coated particles that are accelerated to velocities permitting their penetration of cell walls and membranes (Klein et al, 1987). Stably transformed tobacco (Klein et al ,1988) and soybean (McCabe et al , 1988) plants have been transformed by delivering DNA directly into intact tissues by particle bombardment.

1.4.2.5 Quantitation of Tissue Culture

The growth of a culture over a time period whether it is a callus, a cell suspension or protoplast culture is characterised by an increase in cell number, volume and cellular complexity (Dodds and Roberts, 1985). There are many parameters that can be used in the analysis of growth rates. These parameters include:

1.4.2.4 Isolation and Inoculation of Protoplasts

The term 'protoplast' refers to the contents of a cell after removal of the cell wall (Cocking, 1972). Protoplasts have been used over the years in fundamental as well as applied studies. The reason for the overwhelming predominance on the use of protoplasts lies in the comparative ease with which they can be infected. The presence of the rigid plant cell wall does not allow efficient infection of plant cells in vitro with plant viruses hence infection of suspension cells, though used to a limited extent, is a less predictable process. Protoplasts can easily be infected in vitro by one of several alternative methods to obtain a population of cells in which a large proportion (about 95%) are infected, and in which the infection if not entirely synchronous is comparatively better than the cells of an infected leaf (Takebe, 1975). Protoplasts can serve as recipients of foreign genes and organelles in genetic modification of plants. In such genetic transformations, the foreign genes are incorporated into the protoplasts in stable forms that allow the genetic informations of the foreign DNAs to be encoded and expressed as new stable characters in transgenic plants.

A large proportion of protoplasts used in virus work have often been isolated from leaf tissue and consists mainly of mesophyll cells. In the isolation of mesophyll protoplasts from leaves, a general rule is to keep the plants under rapid growth conditions with sufficient nutrients, and to collect leaves that have reached or may soon attain their full size. It is usually better to use mature leaves showing no signs of senescence. Reproducible results are obtained only when the above conditions are maintained (Motoyashi et al, 1973; Otsuki et al, 1974). Protoplasts released by overnight digestion are normally not ideal for viral infection as compared to those isolated using shorter isolation times. To by-pass most of the obstacles inherent in the use of leaf tissue, the alternative is to use cell suspension culture as a source for protoplast isolation. A log phase culture of dividing suspension cells, maintained under constant condition and subcultured to a defined regime can provide a reproducible starting material for protoplast preparation. At this growth stage the cell-walls of the suspension cells are moderately thin and cellulosic in nature and will therefore need shorter incubation times with enzyme mixtures in the release of their

After several weeks of culturing on the same medium a callus shows some symptoms of old age, which include: growth retardation, necrosis or browning and dryness. The reasons for such ageing characteristics could be four-fold, namely shortage of important nutrients in the medium, inhibition of diffusion of nutrients, a gradual loss of water from the medium due to evaporation and accumulation of metabolites, most of which could be toxic (Constabel, 1984; Dodds and Roberts, 1985). Hence to maintain a healthy viable callus line, it is very necessary to subculture the callus by transferring a healthy friable callus of a sizeable amount onto a fresh and sterile culture medium. The transferred fragment of callus must be of sufficient size to ensure renewed growth on the fresh medium. Street (1969) recommends that the inoculum must be about 5 - 10 mm in size and 20 - 100mg in weight for a good subculturing. Depending on the extent of growth of callus, subculturing must be done once in every 4 - 6 weeks. It is best to separate callus from explant and break it into inocula for subculturing when the callus tissue has reached a size of 2cm. Ideally, only healthy callus tissue should be subcultured as brown or necrotic tissue may either cease growing completely or exhibit signs of improper growth (Dodds and Roberts, 1985).

1.4.2.3 Initiation, Formation and Maintenance of Cell Suspension Culture

King (1980) define cell suspension culture as a rapidly dividing aggregate of cells suspended in a liquid medium . Cultures of cell suspensions are generally started by transferring fragments of undifferentiated friable callus into a liquid medium that can sustain the growth of the cells (Gamborg et al, 1968). Initially a relatively large amount of callus about 2 - 3g per 100ml suspension medium is needed as an inoculum(Helgeson, 1979). The cell suspension culture is then agitated on a rotating shaker at a speed that swirls the medium in the flask. The swirling action of the liquid medium enables dividing cells to break free from the inoculum. This enables the culture to consist of aggregates of various sizes, residual pieces of the inoculum and remains of dead cells after about 2 - 3 days of culturing (Dodds and Roberts, 1985). Cell suspension cultures, like all growing plant cells always form aggregates which are heterogenous in size as synchrony of cell growth is difficult to achieve. These suspension cultures exhibit three major patterns of growth phases after

and the environmental conditions of culture (Schöpke et al, 1993). As a result of much investigation, a high salt medium developed by Murashige and Skoog (1962) and derivatives from such a medium (Gamborg et al, 1976) have widely been used to culture a large variety of plant species.

In recent years there has been a growing interest in the development of a transfer system of genes which confers desirable traits on the cassava crop. However, before the establishment of any plant transfer methodology, it is necessary to develop an efficient and reproducable plant regeneration system from callus tissue culture or protoplasts. Callus culture systems are usually the material from which suspension cells are derived,and in addition they represent convenient forms for the maintenance and supplies of aseptic cell lines and are often the form of cultures from which plant regeneration is initiated. Cassava leaves and shoots have sometimes been regenerated from callus grown from stem sections (Tiiquin, 1979) or from calli developed from leaf mesophyll protoplast (Shahin and Shepard, 1980).

1.4.2.2 Initiation, Formation and Maintenance of Callus Culture

Callus is mainly a mass of unorganised, loosely arranged thin-walled parenchyma cells that originate from the proliferating cells of the parent plant cell tissue (eg. vascular cambia, storage parenchyma, pericycle of roots, cotyledons, leaf mesophyll and provascular tissue) (Dodds and Roberts 1985). The formation of callus from the explant involves three developmental phases of induction, cell division and differentiation . The induction phase, concerns a stimulation of metabolism to bring the cells to be in a state of readiness for cell division. This stage is followed by a meristematic and active cell division phase. A callus forms after the final stage of cellular cell division takes place (Aichison et al,1977). Generally it takes 2 - 3 weeks to produced a callus growth on the explant. The growth starts at the cut ends of the explants and it occurs either as a protuberance or as a uniform mat across the surface of the explant (Constabel, 1984). The fragile callus growth that can be easily peeled off is known as 'friable' callus culture. A healthy callus may appear wet, yellowish, whitish or greenish in colour.

1.4.2 Plant Tissue Culture

1.4.2.1 General Introduction

The introduction of the concept of totipotency, i.e. a living cell having the usual complement of chromosomes is capable of giving rise to the entire plant, in the early 20th century advanced the idea that, vegetative cells can be used to regenerate artificial embryos. This concept lead to the first successful culturing of excised roots of tomato (White, 1934). Today plant tissue culture or sterile culture of cells, tissues and organs have become a vital tool in basic and applied studies. Tissue culture methods can be used to answer pertinent problems like the 'hypersensitivity' of plants, which is a phenomenon solely associated with intact tissues. The mechanisms of viral replication and pathogenesis can be investigated through tissue culture procedures. Tissue culture technology has also rendered possible the production of virus-free plants through meristem-tip and callus cultures (Hollings, 1965) and has allowed the mass propagation of many cultured plants, in the fields of agriculture, horticulture and forestry.

Laboratory-protocols exist for regenerating over 1000 plantlets from explants and / or callus from ornamental plants, food crops, vegetables,fruits and nut crops, medicinal plants and forest trees (Murashige, 1978; Conger, 1981). The three major ways by which micropropagation has been achieved includes auxillary bud breaking, production of adventitious buds directly or indirectly via callus and somatic embryogenesis directly or indirectly on explants.

Generally, the induction, formation and maintenance of these explants require a suitable culture media. Normally these explants are broadly cultured on a defined media containing five groups of substances, namely macro and micro-nutrients, a carbon/energy source, iron source, some B-vitamins and plant hormones to attain growth (Aitchinson et al, 1977; King and Street, 1977; Phillips, 1980) and organised development (Christianson, 1987). In most cases a comparative study using various mineral salt formulations at various dilutions ensures a selection of an ideal basal medium, that can be used to optimize other factors like plant hormones

to inhibit their replication) there occurred a 4-fold reduction of BCTV (but not ACMV) DNA accumulation compared to TGMV controls (Bejarano and Lichtenstein, 1994). The reason for this result was that although equivalent regions of BCTV and ACMV showed identical overall homology to the TGMV target (i.e. 63 percent for BCTV and 64 percent for ACMV), BCTV displayed a 280 nucleotide region of high homology (i.e.82 percent) within this targeted area. Conversely for ACMV, the homology in the equivalent region was much diffused. This showed that a critical stretch of good complementarity is required to prevent expression of the target mRNA that is effective even along antisense transcript (Bejarano and Lichtenstein, 1994). The antisense strategy has allowed the roles of specific genes to be tested (Smith et al, 1990), has led to the identification of previously unknown genes(Hamilton et al, 1990) and generated new plants with altered characteristics of commercial significance (Van der Krol et al, 1991).

Several procedures have been employed in introducing antisense genes into plant genomes. Some of these methods include **microinjection** ie. direct injection of plasmid DNA carrying antisense gene into cultured cells (Izant et al, 1984) and **electroporation** ie using short electric shocks to make small transient pores in the membranes of target cells, so that they take up DNA from surrounding solution (Ecker et al, 1986). However the most successful way of integrating a gene of interest into dicot plant genomes has been the use of the bacterium, Agrobacterium tumefaciens as a 'vector' (Jones, 1992). This integration procedure commences with an initial cloning of the entire coding region of the gene of interest, eg. AC1 ORF gene, in the antisense orientation into a binary plant transformation vector (eg. pBIN 19) which contains a promoter which would drive the expression of the antisense gene. The clone formed as a result of the cloning is then transferred by triparental mating into Agrobacterium tumefaciens, which is used to transform plant cells in culture. This plant cells are then cultured to generate plantlets that grow into adult plants for propagation through conventional tissue culture methods. The use of Agrobacterium tumefaciens as a vector to mediate the stable introduction of antisense genes into dicots has proved to be a highly versatile and efficient system of studying the effect of antisense RNA on gene expression using reporter gene system with a consequent measurable change in phenotype (Smith et al, 1988; Robert et al, 1989 and Cannon et al, 1990).

helper viruses for their replication and spread in infected plants. Transgenic plants that express these satellite sequences have been found to show reduced symptoms and viral replication (Gerlach et al, 1987). Transgenic tomato plants expressing satellite RNA sequences have proven to be efficient for reduction of symptoms both in green house and field experiments (Tien et al, 1990; Tousch et al, 1990).

(3) The coat protein technique. This consists of the integration of the gene encoding viral coat protein into the genome of the plant. The production of the coat protein serves to protect the plant from invasion by various viruses. This technique has been found to be very effective against RNA containing viruses. As for the DNA viruses, the only cases reported where coat protein mediation had worked is with TYLCV-Th (Thailand Isolate) and ACMV, but in these cases the coat protein levels were extremely low and the protection it offered was limited (Rochester et al, 1990).

(4) The antisense strategy. This involves the blocking of translation of viral RNAs by expressing genes that encode sequences complementary (ie. antisense) to viral genes (Cesareni et al 1991). An antisense gene is that which is inserted into a cell's DNA in the reverse orientation to that in which it is normally found in the cell. In the plant expressing both a sense strand (naturally oriented gene) and its antisense strand (the inserted antisense gene), the intermediate product (messenger RNA) of the antisense gene forms a duplex with that of the sense gene (ie.gene with complementary target nucleic acid sequence). This leads to inhibition or a 'turning down' of normal gene expression to an extent that virtually no gene product is formed. The antisense approach has been used experimentally to regulate gene expression in both plant and animals. The complete antisense sequence of the replicase gene of the Tomato Golden Mosaic Virus (TGMV) was integrated into tobacco plant genome and several lines were reported to exhibit a high level of resistance when challenged with different concentrations of TGMV (Day et al, 1991; Bejarano and Lichtenstein, 1994). When TGMV transgenic tobacco leaves were infected with ACMV and BCTV (to find out if equivalent regions in these viruses carry sufficient sequence homology to allow antisense-specific TGMV-RNA

1.4 RESISTANCE TO VIRAL INFECTION

1.4.1 Transgenic Plants

The presence of one virus in a plant can interfere with infection by another virus or strain, resulting in cross protection (Fulton, 1986). A hypothesis propounded in 1985 (Song and Bertha, 1990) inferred that some main gene products of a pathogen, when present in a plant in an abnormal form in surplus, or at an appropriate stage during the viral replication cycle can interfere with the infection by the invading pathogen (Fitchen and Beachy, 1993). This concept referred to as pathogen-derived resistance has lead to an initiation of stable-gene transfer through genetic engineering techniques for transgenic plants. These transgenic plants containing nucleotide sequences obtained from plant viruses usually show increased resistance to viral disease. Many of such viral sequences give a level of either resistance to/or suppression of disease symptoms, i.e. tolerance. Strategies that are often used in generating such plant viral tolerance include:

(1) The ribozyme strategy. This involves the use of auto-catalytic RNA cleaving molecules or ribozymes to cleave viral RNA molecules. Such cleavages are effective on both the positive and negative strands of the RNAs and are linked with conserved sequence domains. The outcome of in vitro investigations have published cleavage of viral RNAs (Gerlach, 1989 ; Haseloff and Gerlach, 1988), but little or no success have been achieved in in vivo experiments.

(2) The blockage of viral replication using competitor sequences (Morch et al, 19 7), **subgenomic sequences** (Stanley et al, 1990; Stanley, 1983; Stanley and Townsend, 1985), **and satellite** RNAs (Tien and Wu, 1991). The competitor sequences compete with similar sequences that encode the replicase, movement or capsid proteins. Subgenomic molecules are produced by some viruses during infections: for example many geminiviruses produce subgenomic DNA molecules of B-components. It has been demonstrated that the incorporation of a copy of such DNA of ACMV into the genome of tobacco led to decrease disease symptom when the plants were challenged with ACMV (Stanley et al, 1990). Satellite RNAs are also linked with several viruses and are dependent upon

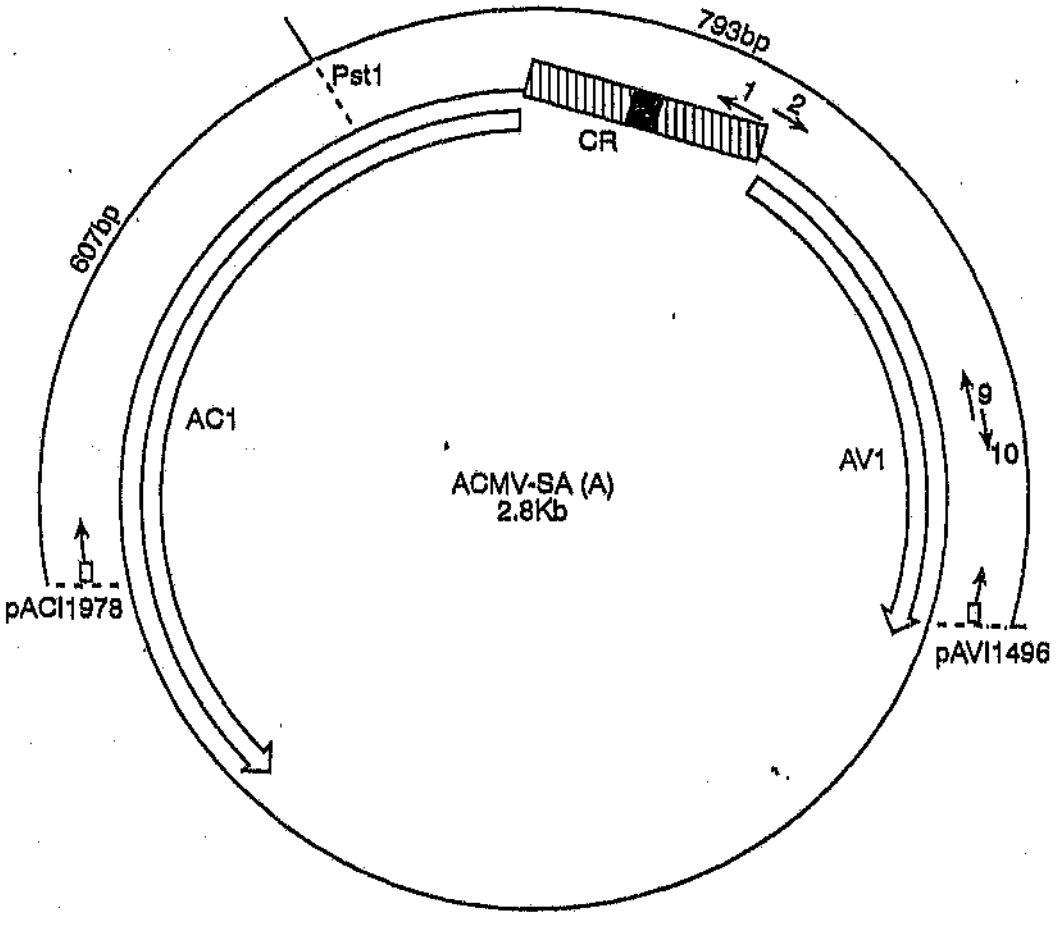


Figure 6: Full Length DNA-A Component of a South Africa Isolate (ACMV-SA of a Cassava- Infecting Geminivirus Cloned into pBluescrpt KS. Abutting primers 9 and 10 were designed from 793bp fragment of the virus to yield a full length DNA component from total nucleic acids from leaf material . V- refers to the putative open reading frame encoded by the virion (+) DNA and C to that encoded by the complementary (-) DNA strand.

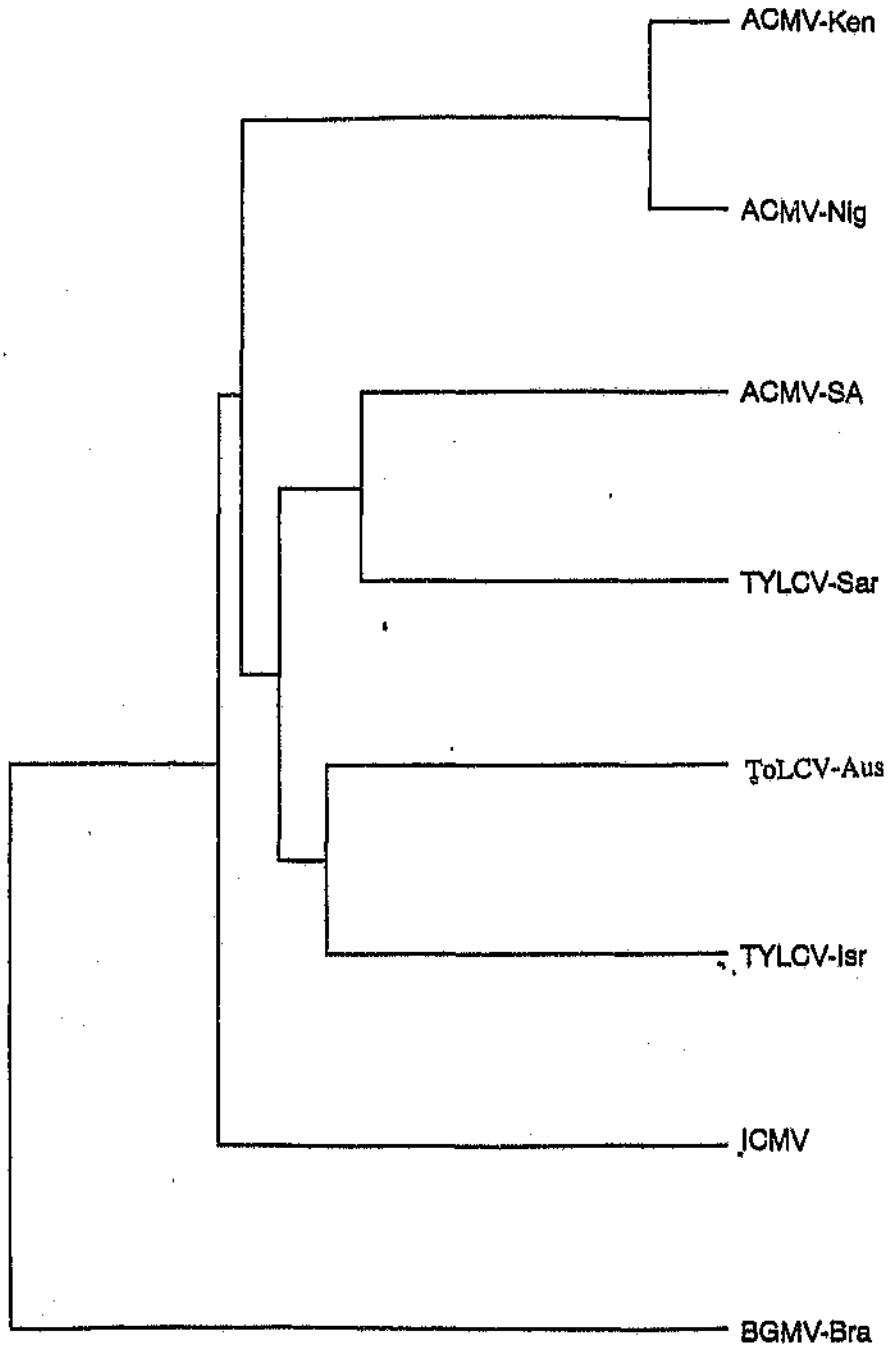


Figure 5: Dendrogram Illustrating the Relationship Between ACMV-SA (793 bp region ; Fig 6) and Nucleotide Sequences of 7 Other Geminiviruses.

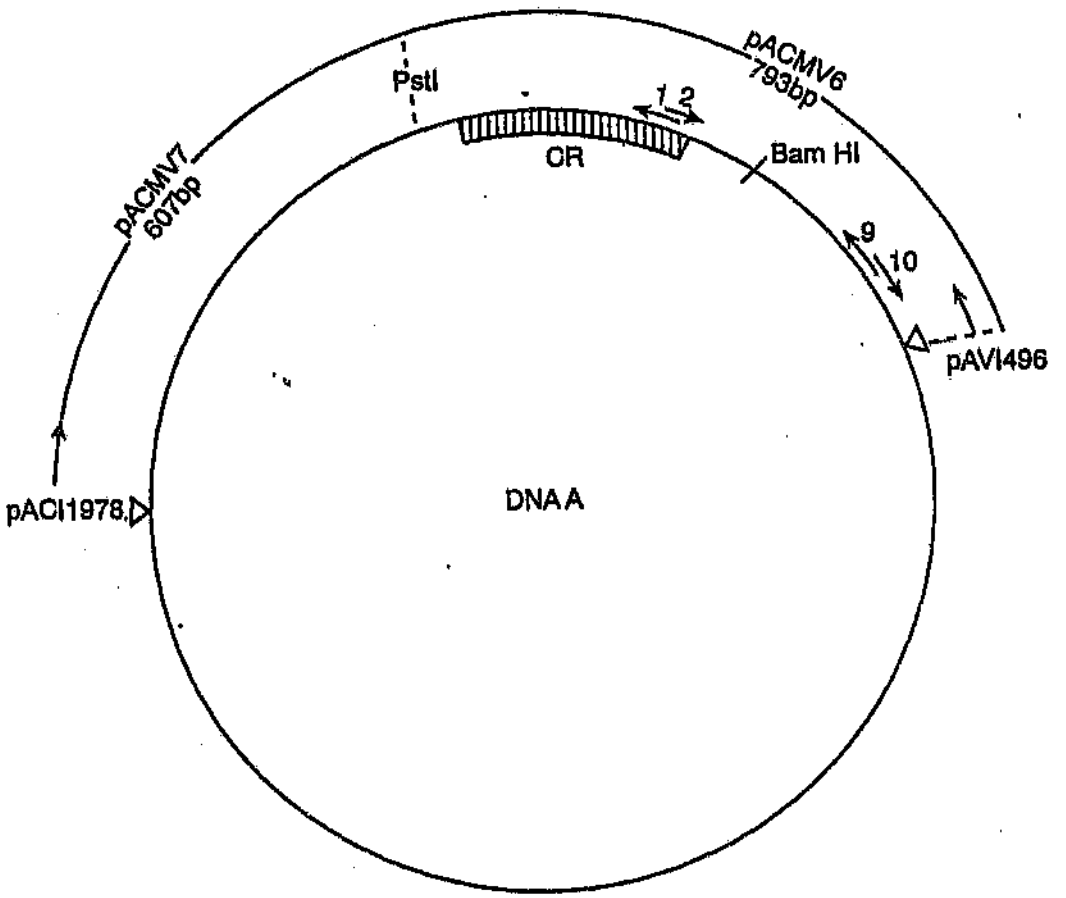


Figure 4: Primer Binding Sites. Primers pAC1 1978 and pAV1 496 were used to generate PCR fragments of approximately 1400bp. An internal *Pst*1 site rendered two fragments - one, a 793bp fragment, was cloned into pBluescript SK(-) yielding pACMV6 and the other a 607bp fragment also cloned into pBluescript SK(-) to form clone pACMV7.

The different strains of ACMV now include the West Kenyan Isolate 844 (Bock et al, 1978), the Nigerian Isolate (Adejare and Coutts, 1982), the Angolan Isolate (Sequeira and Harrison, 1982), the Malawian Isolate and the Indian Isolate (ICMV) (Shanta and Malathi - unpublished results, 1982). The Kenyan Isolate (ACMV-Ken) is the type strain and the nucleotide sequence of its two similar size circular DNA molecules (DNA-A and B) have been(completely) sequenced (Stanley, 1982). The ACMV isolates differ in the severity of symptoms as well as nucleotide sequence, infectivity and several other biological properties.

1.3.2 African Cassava Mosaic Virus (ACMV-SA) in South Africa

Recently a new cassava geminivirus infecting cassava in KwaZulu Natal and Eastern Transvaal in South Africa was reported. A PCR - derived clone named pACMV6 (Doug Maxwell, Dept. Plant Pathology, University of Wisconsin, Madison, pers. comm.), containing a 793bp fragment of the genome (Figure 4), has been sequenced(Menschig, 1993; Kenneth Palmer, Dept. of Microbiology, University of Cape Town, Cape Town, pers. comm.). This 793bp sequence covers the CR and part of the CP sequence. Sequence homology programs have been used to compare the 793bp sequence of ACMV-SA with known nucleotide sequences of some geminiviruses. Dendrograms (Figure 5) demonstrated 79%, 83% and 100% homology in the CP region between ACMV-SA and ACMV-Mal (Malawi Isolate) and TYLCV-Sar (Sardinia Isolate) and TYLCV-Isr (Israel Isolate) respectively.There was 75% and 46% homology in the common region between ACMV-SA and TYLCV-Sar and ACMV-Ken (Kenyan Isolate) respectively (Figure 5). This is quite unique as TYLCV is monopartite and cassava geminiviruses are bipartites. ACMV-SA has putatively been shown to be a bipartite like other cassava geminiviruses(Leigh Berrie, Dept. of Microbiology, University of the Witwatersrand, Johannesburg, pers. comm.), yet the high sequence homology it shares with TYLCV in the common and coat-protein regions makes it special amongst the cassava geminiviruses.

From the sequence of pACMV6, pairs of abutting primers 9 and 10, were designed to amplify a full length DNA-A component of ACMV-SA from total nucleic acids from infected leaf material (Figure 6). This full length ACMV-SA DNA-A was then cloned into *Sma*I site of pBluescript KS and the clone was called pCGV-100 (Kenneth Palmer, pers. comm.).

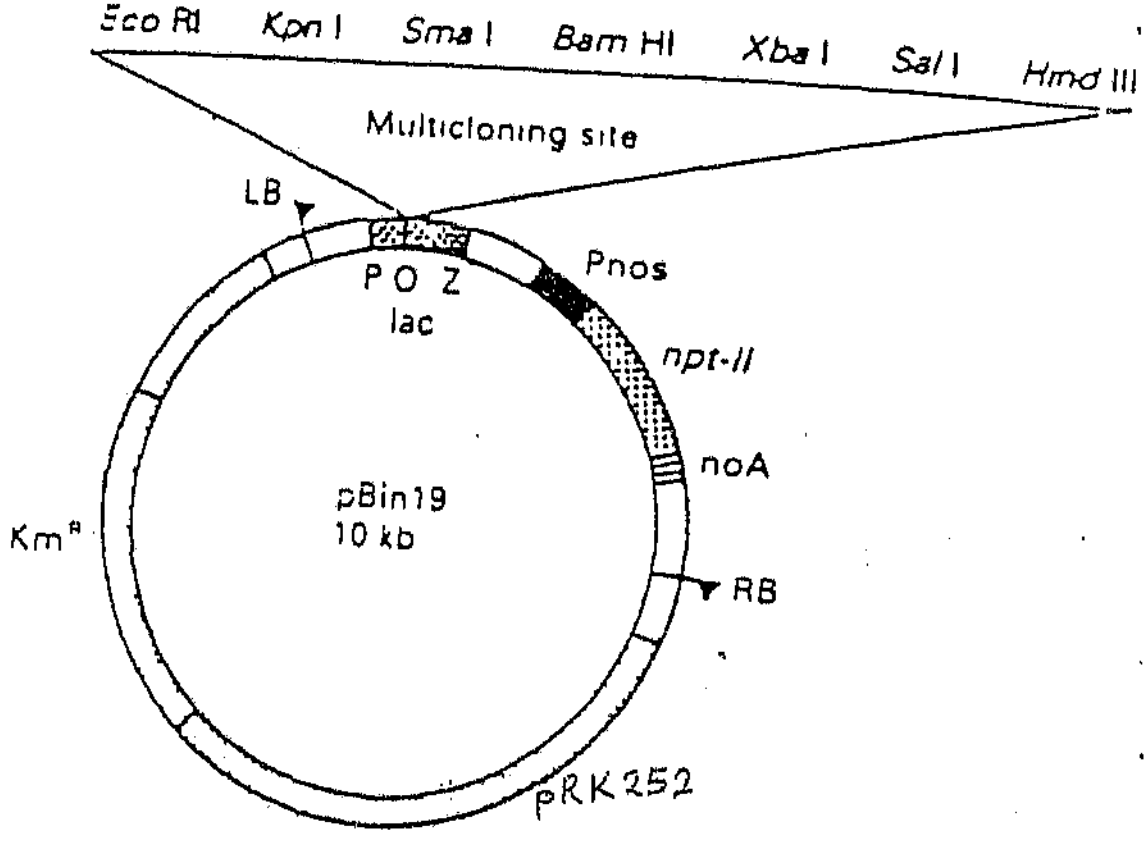


Figure 10: A Map of the Binary Vector pBIN19.LB.LR. = left and right borders: lac = α complimentary region of lac operon; Pnos = nopaline synthase gene promoter; nptII = neomycin phosphotransferase-II coding sequence from Tn5; noA = nopaline synthase gene polyadenylation site; pRK252: derived sequences containing the RK2 wide host range origin of replication. (Bevan,1984)

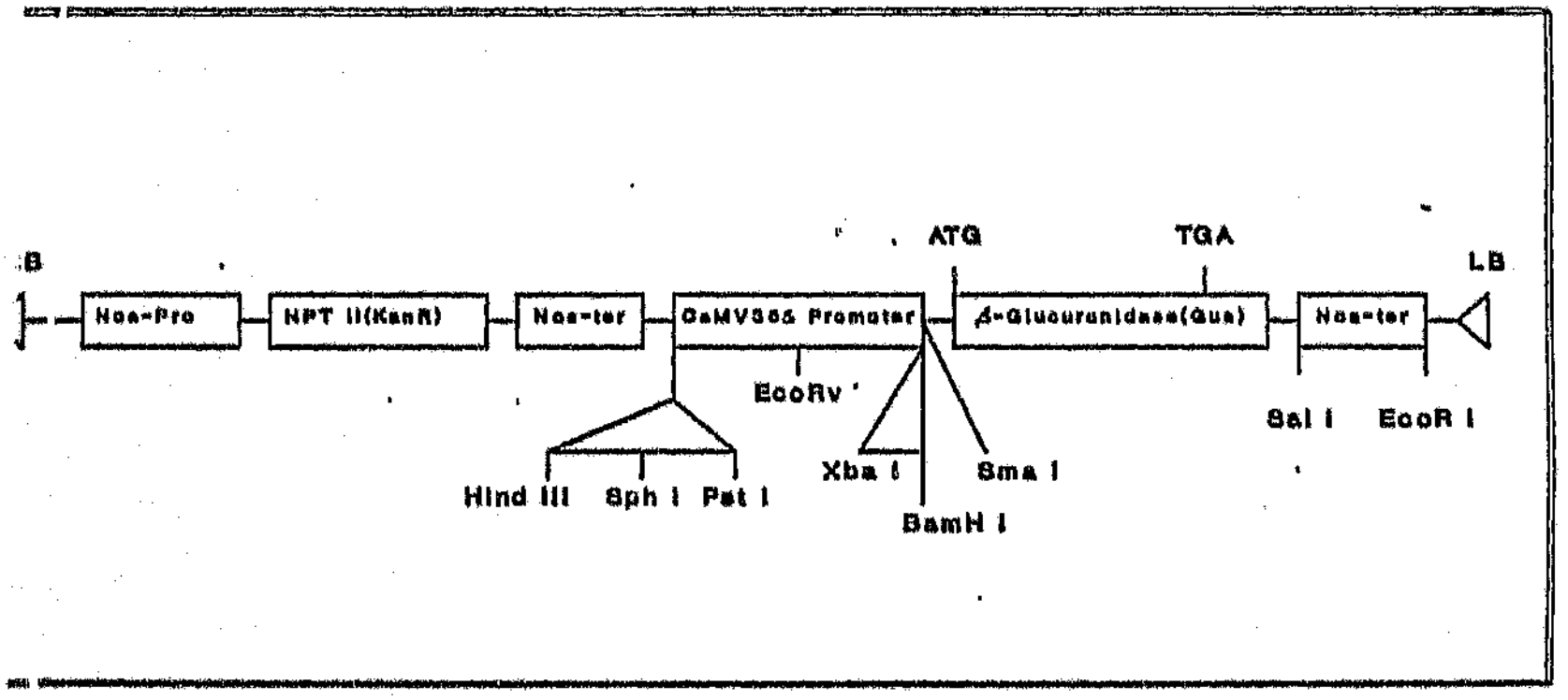


Figure 9: Map of pBI 121 Containing the NPT II and GUS Genes

All restriction enzymes were obtained at a concentration of 1000U/ml from Boehringer Mannheim, Germany, the only exceptions being *Hinc* II (800U/ml) from Promega USA and *Mlu*1 (1000U/ml) Amersham International, U.K.

The total volume of the single and double digest mixture was 10µl. One µl of DNA(3µg pCGV-100), 1µl of 10U restriction enzyme, 1µl of 10 x restriction enzyme buffer and 7µl of sterile distilled water were mixed for single digests whereas the double digest reaction mixture contained 1µl of 10U each of the two enzymes concerned, 1µl of 10 x buffer favourable to both enzymes and 6µl of sterile distilled water. The digests were incubated for 2.5h at 37°C except for *Apa*1 where incubation was at 30°C. Following digestion, the digested products were subjected to electrophoresis in 1% agarose gel with λ-*Pst*I cut markers and 1XTBE (0.IM Tris-base, 0.08M boric acid, 0.001M disodium EDTA, pH8.0) as running buffer. Restriction map of DNA-A of ACMV-SA was then constructed from single and double digest data.

3.3 Cloning the 607bp ACI Fragment of ACMV-SA

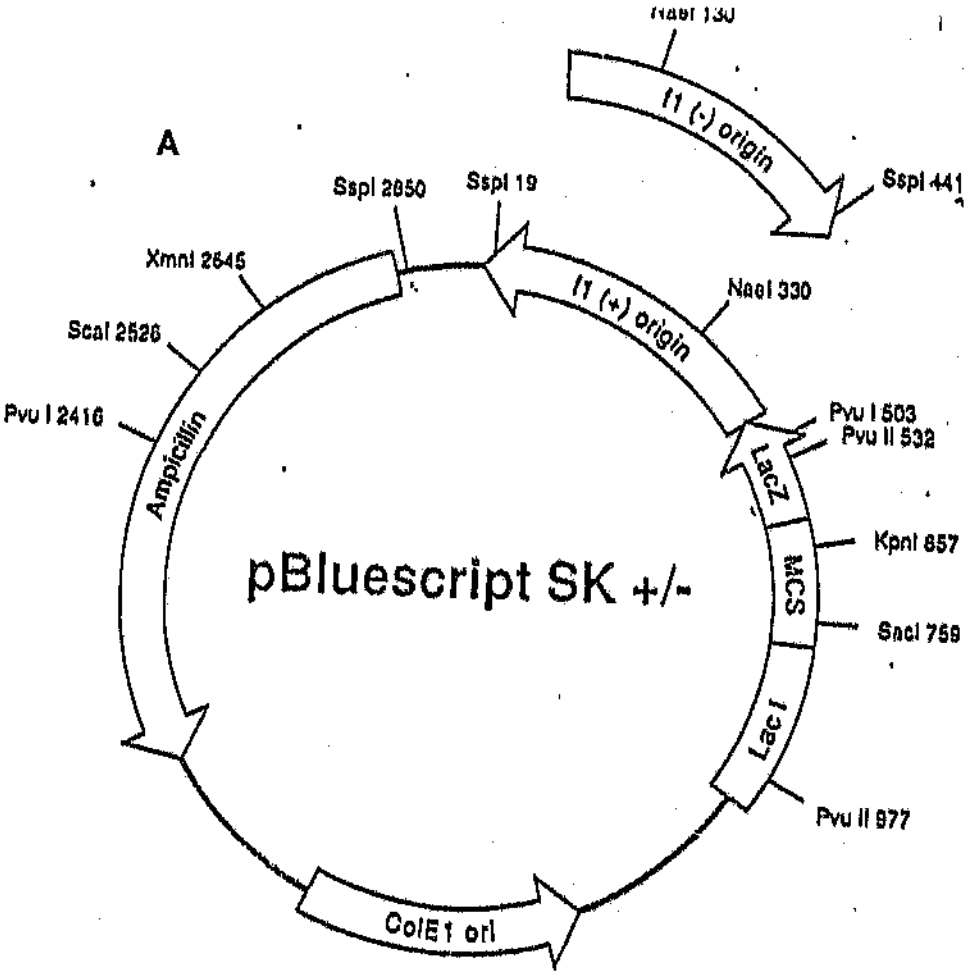
The 607bp ACI fragment of ACMV-SA (Fig 4) is part of the putative DNA polymerase gene cloned into *Pst*1 site of pBluescript SK(-) to obtain clone pACMV7. This 607bp fragment was cut out of pACMV7 with *Pst*1 and attempts were made to clone it into the *Sma*1 site of the plant transformation vectors pBIN19 and pBI121 (Fig 9 and 10).

3.3.1 Reagents and Bacterial Strains

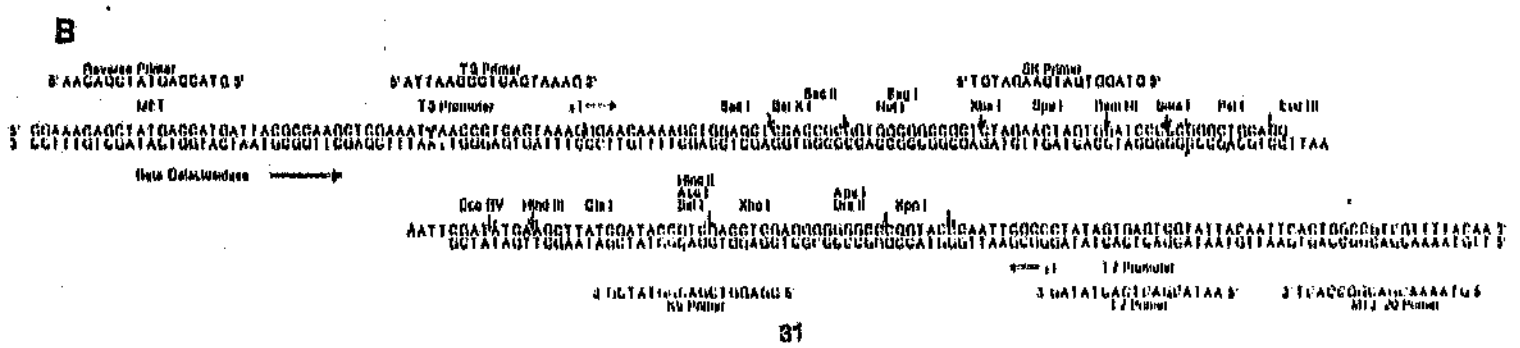
All restrictions enzymes and DNA-modifying enzymes were from Boehringer-Mannheim. The pBIN19 and pBI121 binary vectors were from clonetech laboratories (Palo Alto, CA). Plasmids pBIN19, pBI121 and clone pACMV7 were present in MC1022, JM109 and HB101 E. coli strains respectively. Large scale plasmid preparations of pACMV7, pBIN19 and pBI121 were done using the nucleobond AXpc-Kit 100 and quantified on the spectrophotometer at 260nm. Plasmid pACMV7 was selected on 50µg/ml ampicillin, whilst pBIN19 and pBI121 were selected on 50µg/ml kanamycin during the culturing of E coli cells.

Figure 8 : A. Map of pBluescript SK with the Multiple Cloning Site (mcs) Flanked by the T3 and T7 promoters.

B. Sequence of the mcs; In pBluescript KS the sequence in the mcs is in the reverse orientation.



B



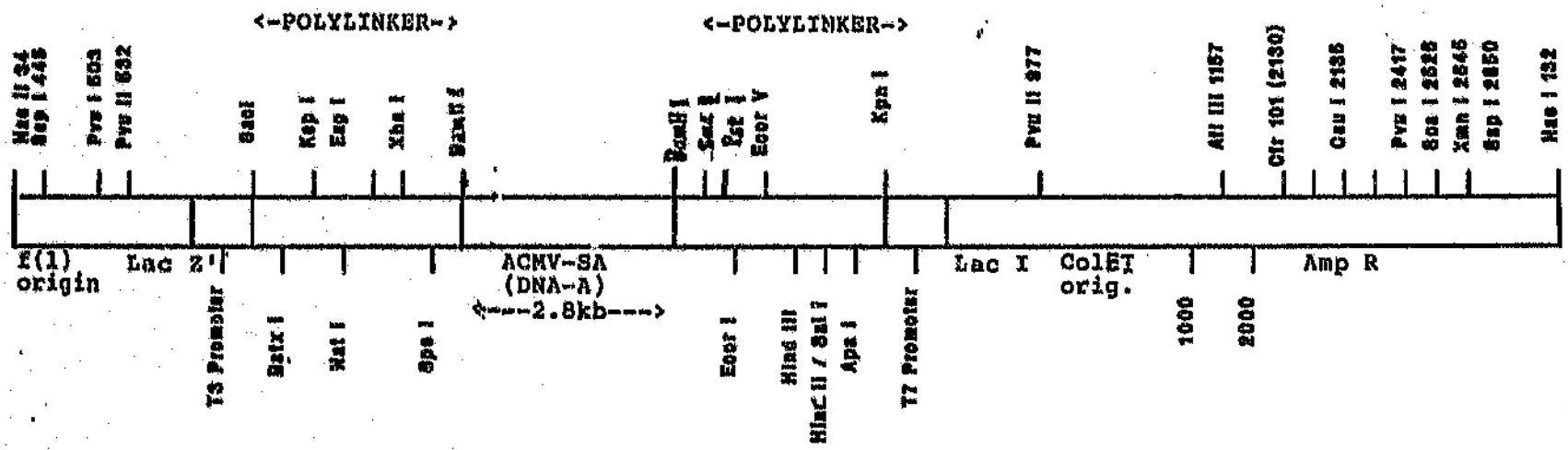


Figure 7: Annotated *Nae* I Linearilised Map of pCGV-100(5.7 kb) (pCGV-100 consist of ACMV-SA DNA-A cloned into *Sma* I site of pBluescript KS).

3.2 Restriction Analysis of DNA-A of ACMV-SA

3.2.1 Restriction Digest Procedure

From the diagram of the map of pCGV-100 plasmid (Figure 7) and pBluescript KS (Fig. 8) various strategies were initiated for performing various single and double digest reactions. For single digests, enzymes were divided into four main groups namely:

1) enzymes that cut in the polylinker to the right of *Sma*I, i.e. *Pst*I, *EcoR*I, *Hind*III, *Sal* I, *Hinc*II and *Apa*I;

2) enzymes that cut in polylinker to the left of *Sma*I, i.e. *Xba*I *Ksp*I and *Sac*I;

3) enzymes that cut in pBluescript KS but not in the polylinker, i.e *PVu*II, *SSp*I, and *Bgl*I; and

4) enzymes which does not cut pBluescript KS at all, i.e. *Mlu*I, *Bgl*II and *Nco*1.

In the double digest two strategies were adopted which included:

1) One enzyme with one or more sites in insert (i.e. ACMV-SA) and another which does not cut in ACMV-SA, but cuts on either sides of *Sma*I site in the polylinker, e.g. *Hinc*II has two sites in ACMV-SA, and *EcoR*V or *Xba*I, which cut only in the polylinker on either the right or left sides of *Sma*I site, respectively.

2) One enzyme which has a unique site in polylinker but no site in ACMV-SA and another which cuts only in ACMV-SA, e.g. *Sac*I (unique site on the left side of *Sma*I in the polylinker) and *Bgl*II (cuts only in ACMV-SA). Thus the following double digest were done, *Sac*I + *Bgl*II, *Hind*III + *Bgl*II, *SSp*1 + *Xba*1, *Mlu*1 + *EcoR*1 and *Mlu*1 + *Xba*1.

1.0ml of ice-cold 70% ethanol and the pellets were dried in a speedy vac concentrator (Lasec - South Africa) and resuspended in 20µlTE (Appendix A). One µl each of resuspended plasmid was run on a 1% agarose gel. The concentration of each suspended plasmid was assessed on a spectrophotometer at 260nm(absorbance of 1=50µg/ml)

3.1.2 Maxi Plasmid Preparation of pCGV-100

In this preparation the alkaline lysis method using the nucleobond Ax pc-kit 100 (Machinery-Nigel, Germany) was used. Five ml LB containing 50µg/ml of ampicillin was inoculated with a single colony of E.Coli containing the plasmid (pCGV-100) and shaken overnight at 300rpm at 37°C. One hundred µl of this cell suspension was subcultured in 100ml LB containing 50µg/ml ampicillin in a 250ml Erlenmeyer flask and shaken overnight at 37°C. The cells were collected in 250ml centrifuge tube at 10,000 x g at 4°C for 20min. The supernatant was poured off and the pellet was resuspended in 4.0ml S1-buffer (50mM Tris-HCl 10mMEDTA, 100µgRNase/ml pH 8.0, stored at 4 °C) containing 2mg/ml lysozyme and incubated at room temperature for 5min. Four ml of buffer S2 (200mM NaOH, 1% SDS) was added, mixed gently and incubated at room temp for 5min. Four ml of buffer S3 (2.8M Potassium acetate pH 5.2, stored at 4°C) was further added, mixed gently by inversion of tube 6 - 8 times till a homogeneous suspension was formed. The suspension was kept on ice for 10min and centrifuged in a Beckman J21 centrifuge at 10,000xg at 4°C for 38min. The supernatant was carefully removed from a white precipitate and loaded on to a nucleobond Ax cartridge pre-equilibrated with 2ml buffer N2 (100mM Tris/H_3PO_4, 15% ethanol, 900mMKCl pH 6.3, kept at room temp). The cartridge was then washed with 2 x 4.0ml buffer N3 (100mM Tris/H_3PO_4, 15% Ethanol, 1150mMKCl pH6.3; kept at room temp). The plasmid DNA was then eluted with 2.0ml buffer N5 (100mM Tris/H_3PO_4; 15% ethanol, 1000mM KCl pH8.5, stored at room temp). The purified plasmid DNA was then precipitated with 0.8 vol of isopropanol (pre-equilibrated to room temp) at 10,000xg at 4°C and washed with 1ml ice-cold 70% ethanol. The pellet was then briefly dried and redissolved in 400µl TE-buffer. One µl was then run on 1% agarose gel with λ-*PstI*-cut markers (Boehringer Mannheim, Germany) (Appendix B). The concentration was measured on a spectrophotometer at 260nm.

3. MATERIALS AND METHODS

3.1 Plasmid Preparation

Both mini and maxi preparations of plasmid (pCGV-100) were carried out. The plasmid was in the DH5 strain of Escherichia coli.(E. Coli)

3.1.1 Mini Plasmid Preparation of pCGV-100

In the mini plasmid preparation, the alkaline lysis method was employed (Sambrook et al, 1989). Five ml of Luria-Bertaini broth(LB) containing 50µg/ml ampicillin (Appendix A) was inoculated with a single colony of E.Coli containing the plasmid(pCGV-100). The culture was incubated at 37°C overnight with shaking at 300rpm on a shaker. Aliquots(1.5ml) of overnight cultures were placed into Eppendorf tubes and centrifugation was carried out at 10,000 x g at 4°C for 1 min to pellet the bacterial cells. The supernatant was decanted and pelleted cells resuspended by vortexing in 100µl ice cold mini preparation buffer (25mM TrisHCl, pH8.0, 10mMEDTA, pH8.0, 50mM Glucose) and incubated at room temperature for 5min. Two hundred µl of freshly prepared solution of buffer II (0.2N NaOH, 1% SDS) was added, mixed by inversion and incubated on ice for 5min. One hundred and fifty µl of ice cold potassium acetate solution pH4.8 (60ml 5M Potassium acetate, 11.5ml Glacial acetic acid and 28.5ml H_2O) was further added, mixed by inversion for 10 sec and incubated on ice for 5min. The resultant solutions were centrifuged at 10,000 x g at 4°C for 5min and the supernatants were transferred into fresh Eppendorf tubes. Dnase-free Rnase (Appendix A) was added to supernatants to a final concentration of 20µg/ml and incubated at 37°C for 20min. One vol of TE-saturated phenol/chloroform/isoamyl alcohol was added, vortexed for a min and centrifuged at 10,000 x g for 2 min. The upper aqueous phase was transferred into fresh tubes and 1 vol of chloroform: isoamylalcohol (24:1) was added, vortexed for a min and centrifuged at 10,000 x g for 2 min. The upper aqueous phase was transferred into fresh tubes and 2.5 vol of ice-cold 100% ethanol was added, mixed and incubated at -20°C overnight. The samples were then centrifuged at 10,000 x g for 5 min and the supernatants decanted. The pellets were then washed with

b) Transgenic plants

1. Propagate sterile cassava and tobacco plantlets in nodal, callus and cell suspension culture as a source of tissue for future inoculation experiments.

2. Protoplast isolation from cell suspension cultures for future inoculation with DNA-A of ACMV-SA.

3. Clone the 607bp ACI fragment of ACMV-SA into vectors pBIN19 and pBI121.

2. GENERAL OBJECTIVES OF THE PROJECT

Cassava is a crop that has a considerable potential, economic and social significance as a source of industrial raw material and food in South Africa. The devastating effect of Cassava Mosaic Disease (CMD) on cassava crops throughout Africa and South Africa, in particular, is of huge economic importance to the cassava industry. A study of causal agent of the disease is therefore vital in providing a remedy for the crop's survival. The new strain ACMV, namely ACMV-SA, which causes the disease in South Africa is unique, in that regions of the nucleotide sequence of its DNA-A, show equal homology with TYLCV (a monopartite) as that of other ACMV isolates such as ACMV-Malawi.

The general aims of this project are to further characterize DNA-A of ACMV-SA with the view to:

1. develop antisense RNA strategies in genetically engineered virus-resistant cassava; and

2. compare ACMV-SA with other geminiviruses.

This project will carry out some of the initial steps required before the ultimate goal of transgenic plants can be achieved.

2.1 Specific Aims

a) Further characterization of ACMV-SA

1. Sequence the 607bp ACI PCR-fragment of ACMV-SA cloned into the *PstI* site of pBluescript SK(-), i.e. clone pACMV7 and compare this fragment with other geminiviruses.

2. Construct a restriction map of DNA-A of ACMV-SA in order to be able to identify convenient restriction sites for future subcloning experiments.

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1) **Fresh weight and Dry weight.** The fresh weight is determined by filtering cells through a pre-weighed nylon fabric filter under slight vacuum, washing the cells in distilled water, drained fully under vacuum, and then re-weighing the cells plus filter. To measure the dry weight of cells, the cells are collected on a pre-weighed dry nylon filter and the cells are dried on the filter in an oven at 60°C until a constant weight has been reached.(Wetter and Constabel, 1982)

2) **Packed cell volume (PCV).** This is obtained by taking a known volume of culture in a graduated plastic or glass centrifuge tube, spinning in a bench centrifuge at 200g for 5 mins and then calculating the volume of the pellet as a function of the volume of the culture and then expressing the value as a percentage of known volume of the culture(Sorokin, 1973)

3) **Cell number counts** are determined by staining the cells and counting them in a haemacytometer slide (Dodds and Roberts, 1985; King and Street, 1977).

4) **Protein and DNA contents.** In total protein estimations, cells are collected on a Whatman glass fibre filter and washed twice with boiling 70% aqueous ethanol and dried with acetone. Dried cells on the filter and transferred into a solution of 1M NaOH and heated at 85°C for 1.5 hr. The product is filtered and the hydrolysed protein in the filtrate is determined by the method of Lowry et al (Dixon, 1985).

5) **Flow cytometry.** This allows the number and types of cell populations present in culture to be analysed(Galbraith, 1984). The instrument allows individual cells or particles to pass in a single file in a fluid stream through a laser beam using several optical parameters for analysis. Each measurement is performed in a few milliseconds and particles microscopically visible (i.e between 0.5μm-40μm) are then assessed.

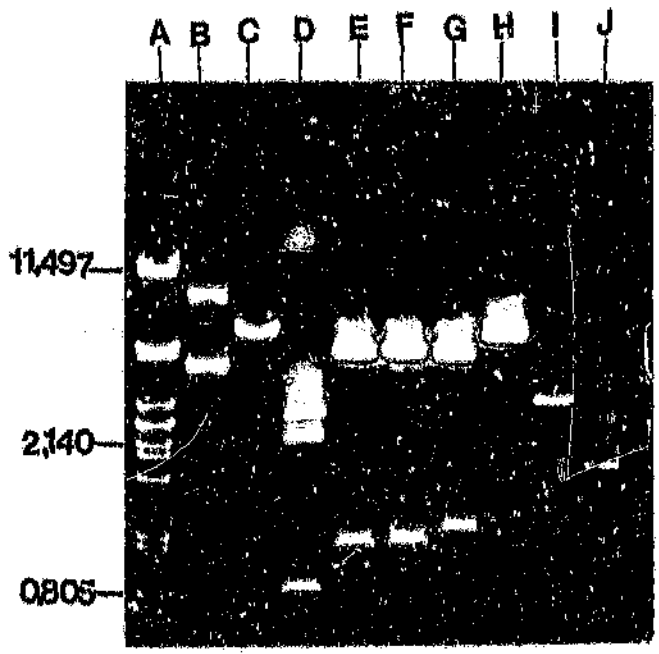


Figure 12: Gel Analysis of pCGV-100. pCGV-100 (3µg) was digested with various single restriction endonucleases and separated by electrophoresis in 1% agarose gel in 1XTBE running buffer at 90V. Lanes A: λ*Pst*1 cut markers whose lengths are given on the left in kilobases. B: uncut pCGV-100 marker. C: *EcoR*V cut pCGV-100 marker. D: pCGV-100 digest with *Ssp*1. E: pCGV-100 digested with *Sal*1. F: pCGV-100 digested with *EcoR*1. G: pCGV-100 digested with *Bgl*I. H: pCGV-100 digested with *Bgl*II. I: pBluescript linearized with *Pst*I. J: uncut pBluescript marker.

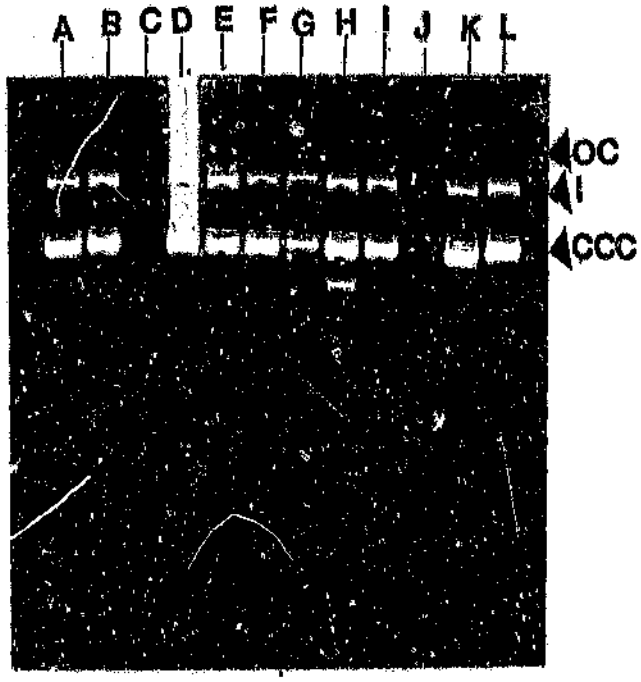


Figure 11: Gel Analysis of Plasmids (pCGV-100) Obtained by Miniplasmid Preparation Procedure. Plasmids were from various 2ml bacterial cultures labelled from A to L. 1µl each of 20µl TE-resuspended clones were run in an 1% agarose gel in 1xTBE running buffer at 90V. oc, 1 ccc (different forms of pCGV-100)

4. RESULTS

4.1 Plasmid Preparation

Figure 11 demonstrates plasmid DNA obtained from mini-preparation of pCGV-100. The average concentration of plasmid per 1.5ml bacterial culture was found to be 0.6µg/µl. The amount of plasmid obtained from lane J culture was little, whilst none was observed in lane C. The plasmid in lane D was contaminated with genomic DNA hence it was not used in the further analysis of pCGV-100. Various plasmid DNA forms of pCGV-100, i.e. open circular [oc], linearized (l) and covalently closed circular (ccc) were observed. In the large scale preparations of the various plasmids, the concentration of plasmid obtained per 100ml bacterial culture was 3µg/µl for pCGV-100 (Fig. 15a, lane I), pACMV7 (Fig. 19, lane 6) and pACMV6 (Fig. 19, lane 2). The yields for the large scale preparations of the plant transformation vectors pBIN19 (Fig. 18, lanes B and D) and pBI121 (Fig. 18, lane E) were 1µg/µl.

The large plasmid preparations produced all migration forms of the plasmid DNA. The plasmid obtained using the nucleobond AX-100 catridge were very clean, hence they were used in the cloning and restriction digest experiments. Using the *Pst*1 λcut markers, the size of the various plasmids were found to be 5.76kb for pCGV-100 (made up of BS of 2.96Kb and DNA-A of ACMV-SA of 2.8Kb) (Fig. 13, lane C), 3.6Kb for pACMV7 (made up of BS of 2.96Kb plus 607bp of ACMV-SA), 10.2Kb for pBIN19 and 11Kb for pBI121 (Fig. 18, Lanes C and F respectively).

3.5.5 Protoplast Isolation from Cell Suspension Cultures of Cassava and Tobacco

In the isolation of protoplast from suspension cells of cassava and tobacco, the method by Nagata and Ishii (1979) was used. The cells for the isolation were from 2-week old in vitro suspension cultures of the Nigerian cassava cultivars TMS63397 and TMS87100609 and tobacco (Nicotiana tabacum L). Ten ml samples of six cassava (three each from the cassava cultivars) and four tobacco suspension cultures were pipetted into 10ml sterile centrifuge tubes and centrifugation was carried out in a Beckman TJ-7 centrifuge at 100xg for 10min. After gently pouring out the supernatant, the pellets were suspended in 10ml sterile filtered enzyme solution (2% cellulase Onozuka R10 (Yakult Biochemicals, Japan) and 0.1% pectolyase Y23 (Kikkoman Shoyu Co. Ltd. Japan) in MES buffer(10mMMES, 2mM$CaCl_2$ 0.4M Mannitol)adjusted to pH5.7 and autoclaved at 15Psi and 121°C for 20min). The digests were incubated at 28°C in a water bath. At intervals of 1hr, 10µl of each digesting sample was collected with a sterile pipette, stained with 10µl of 0.025%w/v Evans blue and observed under the microscope to determine the extent of protoplast formation. Monitoring was carried out for 8h after which the digests were carefully emptied into 10ml sterile centrifuge tubes and centrifuged at 100xg for 5min. The supernatants were decanted and the protoplasts were gently resuspended in 10ml resuspension medium (Appendix B).

These mixtures were then passed through two 212µm and 88µm nylon mesh filters. The filtrates were collected in 10ml sterile centrifuge tubes and centrifugation was carried out again at 100xg for 5min. The pelleted protoplasts were resuspended gently in 5ml of resuspension medium. Ten µl each of resuspended protoplasts and 0.025%w/v Evans blue stain were mixed and counted on a haemacytometer. Only the number of viable protoplasts were counted under the microscope.

through a 212µm nylon mesh filter in order to remove large clumps. The filtrate which were made up of either smaller aggregates or single cells served as inocula for initiating new suspension cultures. Ten ml of the filtrate culture was used to reinoculate 40ml of fresh suspension culture medium. Subculturing was performed once in every 2 weeks.

3.5.4 Determination of the Viability and Enumeration of Cell Suspension Cultures

The Neubauer haemacytometer and the flow cytometer (Coulter Corp, USA) were used in the counting of the number of suspension cells, whilst staining with Evans blue was employed in the assessment of cell viability. Ten µl each of suspension culture and 0.025%w/v Evan's blue stain were mixed and counted on the haemacytometer under the microscope. Both viable cells (that did not take up the stain) and the non viable cells (which took up the stain) were counted separately. The total number of viable and non viable cells per unit volume in four samples each of cassava cultivars TMS63397 and TMS87100609 and six tobacco samples were also estimated during the second and third week of subculturing.

The flow cytometer was also used to count the number of cells in individual cassava and tobacco cultures, and these counts were compared with that from the haemacytometer counts. One ml aliquots from the eight cassava culture samples and the six tobacco samples were collected in Eppendorf tubes through a 212µm nylon mesh filter. One hundred µl each of the fourteen samples was taken through the flow cytometer at a sample flow rate of 50µl/min, sheath pressure of 7.50Psi and a laser power of 15W. The number of cells in each 100µl sample was estimated in a 120sec period. Thereafter the number of cells per ml of culture were determined. The estimation of cell numbers were carried out in the second and third week of culturing.

sterile glass jars and autoclaved at 15Psi and 121°C for 20min.The leaves were excised and collected in distilled water. A 20cm long forcep was then used to place the leaves with cut surface into the callus culture media. All techniques were carried out under sterile conditions in the laminar flow.

Culture Condition: The jars were well sealed and incubated at 25± 2°C in a 12h photoperiod (3000 lux).The callus cultures were examined daily and the approximate size, colour and texture were well noted. The callus was subcultured into fresh media at six weeks intervals. Only healthy friable callus were transferred into new medium; brown, necrotic or dry callus were discarded.

3.5.3 Cell Suspension Culture

Plant Material: Callus obtained from immature leaves of cassava and tobacco were used as inocula for cell suspension culture.

Culture Media: MS liquid medium supplemented with 1% sucrose and 8mgl^{-1} of 2,4D was used. Medium was adjusted to pH5.7, autoclaved at 15Psi and 121°C for 20 min and dispensed in 50ml aliquots into sterile 100ml and 250ml Erlenmeyer flasks.

Processing of Inoculum: Soft and friable callus which easily separates out was used as inoculum. The growing callus was removed from callus culture medium with a long sterile forcep into a large sterile petri dish and chopped into smaller pieces (about 2mm) using sterile forceps. All dead or necrotic callus was discarded and the viable healthy callus was used as inoculum for the liquid suspension culture medium in the Erlenmeyer flask. The flask with their sterile aluminium foil coverings were further sealed with bench parafilm.

Culture Condition: The suspension cultures were agitated continuously on a rotary shaker at 100rpm to reduce the size of cell aggregates and to provide air for the culture. The cultures were incubated at 25°C under diffused light. After 2 weeks the first subculture of the suspension cells were done, by passing the suspension culture

3.5 Propagation of Cassava and Tobacco Plants in Tissue Culture

3.5.1 Nodal Explants

Plant Material: Nodal explants were derived from in vitro shoot cultures of the Nigerian cassava (TMS 63397, TMS 87100609, TMS 87100501) and Tobacco (Nicotiana tabacum) cultivars.

Culture Media: Cassava nodal explants were cultured on Murashige and Skoog (MS) basal medium (Murashige and Shoog, 1962) supplemented with 1% w/v sucrose, 0.011mg/l Naphthalene acetic acid (NAA) and 0.034mg/l benzylaminopurine (BAP) final concentration. Media were adjusted to pH5.7 and 0.8%w/v purified agar was added. Prior to autoclaving at 15Psi and 121°C for 20min, media were dispensed in 20ml and 30ml aliquots into 8.0cm and 12cm sterile glass jars respectively.

Processing Explants: Nodal explants were cut with sterile blades (8mm below node and 3mm above) from shoot cultures, collected in sterile water and using long sterile forceps (20cm long) they were placed with bottom-end of node into the MS basal medium.

Culture Condition: The sterile jars were tightly sealed with parafilm and cultures were incubated at 25± 2°C with 12h photoperiod (3000 lux). The tobacco nodal explants were cultured on half strength basal medium previously described. Subculturing was done once every four weeks.All culturing and subculturing procedures were done under sterile conditions in the laminar flow hood.

3.5.2 Callus Culture

Plant Material: Immature leaves about 3 - 7mm from cassava clones TMS 63397 TMS87100609 and tobacco (Nicotiana tabacum) were used. The leaves were excised from in vitro plantlets and inoculated on MS basal medium supplemented with 1% w/v sucrose and 8mg/l 2,4D. Media were adjusted to pH5.7 and 0.8%w/v purified agar was added. The media was dispensed in 20ml aliquots into 8cm long

3.4.2 Running Sequenced Reactions on 6% Polyacrylamide Gel

Preparation of 6% polyacrylamide gel: 5.7 acrylamide, 0.3g bis acrylamide, 42.0g urea were weighed into a clean graduated beaker. 20ml 5x TBE-buffer (1xTBE: 0.1M Tris-base, 0.08M boric acid, 0.001M disodium EDTA pH 8.0) and 20ml deionised water were added and stirred on a magnetic stirrer at low heat until all solids dissolved. The level of solution was made up to 100ml with deionized water. The solution was filtered through a Whatmann 3MM paper to obtain a clear homogeneous solution. A 750µl aliquot of 10% ammonium persulfate (APS) and 25µl of TEMED were added to the solution and mixed gently, and quickly poured at an angle between sequencing plates to give a gel of size 420 x 330 x 5mm. The comb was then inserted in an inverted position, and the gel allowed to polymerize. After polymerization, the comb was removed and inserted with teeth penetrating gel to an extent to create wells for loading. The plates were mounted in a sequencing tank. The latter was filled with about 1.5*l* of 1 x TBE. The wells were flashed with 1 x TBE to remove polymerized particles. The gel was pre-electrophoresed for 30min at 1800V.

Loading and Running the DNA-Sequencing Gel: The four different reaction samples stored at -20°C were heated at 70°C for 2min immediately before loading on the gel. Two sequencing runs were carried out. In each run 2µl of each sample was loaded per well. The short run samples were loaded after the bromophenol-blue dye from the long run had reached the bottom of the gel. The gel was run at 1800V, 100mA and 60watts. The gel electrophoresis was stopped when the bromophenol-blue dye of the short run reached the bottom of the gel. The plates were split apart and the gel was collected on a wet Whatmann 3MM blotted paper, covered with saran wrap and dried at 70°C on a vacuum operated gel dryer (mode SE1160, Hoefer Scientific Instr., SanFrancisco).

3.4.3 Processing the DNA-Sequencing Gel

The dried gel was exposed to an X-ray film (Agfa) at 70°C in an autoradiograph cassette for 5 - 7 days. The film was developed for 3min then fixed for 5min and washed in water for 2min.

3.4.1 Sequencing Procedure

The Promega traqtrack sequencing method was used with slight modifications (Kenneth Palmer, pers. comm.).

Denaturation : 3.0 µl (4µg) super-coil pACMV7 was placed in an Eppendorf tubeand 15µl of deionised water was added to make up a total volume of 18µl. 2 µl of 2MNaOH, 2mMEDTA was added and incubated at 37 °C for 10 min. The reaction was stopped by adding 3µl of 3M sodium acetate, pH 7.5. The reaction mixture was brought to a final volume of 135µl by adding 112 µl of ice-cold 100% ethanol and incubated at -70 °C for 30 min. The denatured pellet was precipitated by centrifigation at 10,000xg at 4 °C for 10min. The pellet was washed with 500µl of ice-cold 70% ethanol, dried and used for the annealing process.

Annealing of Primer to Denatured pACMV7 Template: 2.0µl of 2pmol designed primer was added to 4.0µg of 2pmol of denatured template, in a molar ratio of 1:1. Five ul of Taq DNA polymerase 5x buffer (250mM Tris-Hcl, pH 9.0 at 25°C, 50mMMgCl$_2$) and 2.0µl of extension-labelling mix (7.5µM each of dGTP, dTTP, dCTP) were added. The total volume was brought to 25µl with sterile water. The reaction was incubated at 37°C for 10min.

Extension/Labelling Reaction: Half a µl of ^{35}S-dATP (10µCi/µl) and 1µl of sequencing grade Taq DNA polymerase (5U/µl) were added to annealed primer/ template mixture, mixed thoroughly and incubated at 37°C for 5min.

Termination Reaction: Six µl each of completed extension/labelling reaction was aliquoted into four tubes containing 1µl of the appropriate d/ddNTP mix and the four tubes were spun down briefly, and incubated at 70°C for 15min. These reactions were finally stopped by the addition of 4µl of stop solution (10mM NaoH, 95% formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) to each tube. The tubes were then stored at -20°C until gel electrophoresis.

3.3.5 Preparation and Transformation of Competent E. coli Cells

Preparation of competent cells and transformation of E. coli was carried out by standard $CaCl_2$ procedure (Cohen et al, 1972).

Five ml of LB was inoculated with a single colony of bacteria (one-shot strain of E coli) and incubated overnight at 37°C on 300rpm shaker. Twenty five µl of overnight culture was used to reinoculated 10ml of LB-broth. Bacterial growth was then monitored until the OD_{600} was 0.4, and then 1.5ml of culture was dispensed in Eppendorf tubes on ice for 5min, then microfuged at 10,000xg at 4°C for 1min. The supernatants were drained off, and pelleted cells were resuspended in 750µl ice-cold 0.1M$CaCl_2$ and placed on ice for 20min, microfuged again at 10,000xg for 1min and pelleted cells resuspended once more in 150µl of ice-cold 0.1M$CaCl_2$ and left on ice until transformation. For transformation, 150µl of competent cells were added to the 10µl ligation mix. The resultant mixture was left on ice for 30min, then heat-shocked at 42°C for 2min, and put on ice immediately and 850µl of LB-broth was added. It was then incubated at 37°C for 1hr. A hundred µl of this reaction mix was then plated on LB-agar containing 50µg/ml anamycin, 45µl of 20mg/ml X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactoside) and 35µl of 100mM IPTG (isopropylthio-B-D-galactoside) and incubated at 37°C overnight. As transformation positive controls 4µl each of uncut pBIN19/pBI121 were used to transform competent cells and TE was used as a negative control.

3.4 Sequencing Strategy

The M13 universal primer had already been used to sequence 196 nucleotides of one of the strands of the ACI fragment cloned into pBluescript SK(-). From the end of this sequence a 16 mer primer 5'ggACAACCAcggAgAc3' was designed to prime further inwards. Sequencing was performed by the dideoxy chain termination method (Sanger et al, 1977) using the Promega taqtrack kit (Q5330, Promega Corp. USA) and ^{35}S-dATP labelling. Sequencing consisted of denaturation of double-stranded pACMV7 template, extension of template by the primer and termination of reaction by the incorporation of the appropriate dideoxynucleotide triphosphates.

3.3.3 Digestion, Desphosphorylation and Extraction of pBIN19 and pBI121

*Sma*1 was used to linearized pBIN19 and pBI121. After digestion for 3hrs, the cut vectors were dephosphorylated in two ways (1) 1µl of 0.5U/µl of calf intestinal alkaline phosphatase (CIP) was used to dephosphorylate the vectors at 37°C for 15min, 56°C for15min and repeated after addition of 1µl of 0.5U of the enzyme. The second method used 0.1U of CIP at 37°C for 30min and 65°C for 45min. Vectors were subjected to electrophoresis in a 1% low melting temperature (LMT) gel and the respective vector bands were cut and extracted as previously described. In an alternative procedure, the extraction of the dephosphorylated vector from the gel was by-passed and rather the dephophorylated mixture was heated at 70°C for 30 min to denature enzymes, and then the vectors were used directly in the subsequent ligation reaction.

3.3.4 Ligation of 607bp ACI Fragment of ACMV-SA into Transformation Vectors

The ligation reactions were performed at different vector:Insert ratios of pmoles of ends: 4 : 1; 3 : 1; 2 : 1; 1. 5 : 1; 1 : 1: 4: 1; 3: 1; 2 and 1: 1. 5 of pBIN19/pBI121 to the ACI insert respectively were attempted]. All ligations were done in a total volume of 10µl. pBIN19/pBI121, ACI fragment and sterile water were added up to 8.5µl in an Eppendorf tube, and heated at 65°C for 10min, then placed on ice for addition of 1µl of 10x T_4 DNA ligase buffer and 0.5µl of 1U/µl T_4 DNA ligase. As a positive ligation control, pBluescript SK (-) was linearlised with *Pst*1, dephosphoryated and ligated to the *Pst*1 cut 607bp AC1 fragment of ACMV-SA. A self-ligation control included *Sma*I cut and dephosphorylated pBIN19 and pBI121. Ligations were carried out at 14°C overnight and ligated products were used to transform competent E coli cells(one-shot, PCR-script kit, stratagene).

3.3.2 Digestion, Extraction and Blunt-Ending 607bp ACI Fragment of ACMV-SA

*Pst*1 was used to cut out the 607bp ACI fragment in pACMV7. The cut fragment was run and extracted from a 1% low melting temperature (LMT) gel. The 607bp band was cut from the gel and placed into an Eppendorf tube and 1vol of TE-buffer was added and heated at 65°C for 10min to melt the agarose. One-vol phenol: Chloroform extraction was done twice. After each extraction the aqueous layer was transferred into a new tube. After the second extraction the aqueous layer was transferred into a tube containing 0.1vol 4M LiCl, the reaction mixture was mixed by inversion and incubated on ice for 2min, then microfuged at 10,000xg for 3min at 4°C and supernatant was collected in fresh tubes and 1 vol of TE-saturated isoamylalcohol (IAA) extraction was done to remove any trace of ethidium bromide. After mixing and centrifuging at 10,000xg the lower aqueous phase was transferred into new Eppendorf tubes containing 1µl of glycogen (20mg/ml). Two and half vol of ice-cold 100% ethanol was added, and incubated at -70°C for 30min, then centrifuged at 10,000xg for 10min. The supernatant was discarded and the pellet washed with ice-cold 70% ethanol, dried and resuspended in 25µl of TE. In blunt ending the *Pst*1 cut fragment, the Klenow fragment of E coli DNA polymerase was used according to Sambrook et al (1989). Twenty four µl of resuspended fragment was added to 1µl of 10U Klenow enzyme; 5µl of 10x nick translation buffer (0.5M Tris-Hcl pH 7.5, 0.1M MgS04, 1mM dithiothreitol, 500µg/ml bovine serum albumin), 1µl of a mixture of all four dNTPs (2mM each) and 19µl of deionised water to make up a final volume of 50µl. The reaction was incubated at room temp for 30min, stopped with 2µl of 0.5M EDTA pH 7.0 and, extracted once with phenol:chloroform to remove the enzymes. The blunt ended 607bp fragment was then separated from the dNTPs by passing the mixture through a millipore filter (ultrafree-MC 30,000 NMVC filter unit, millipore).

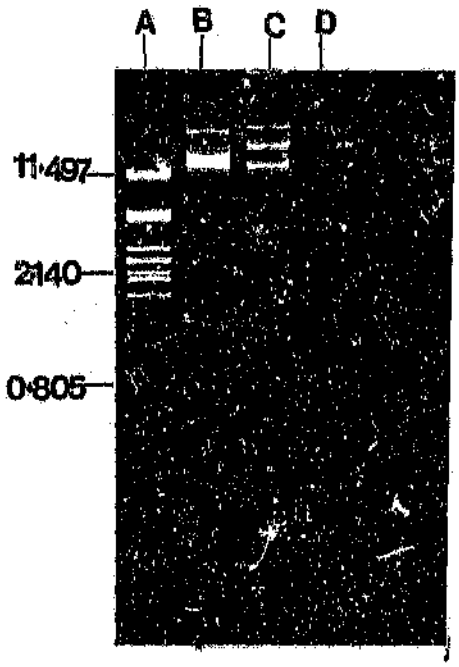


Figure 17: 1% Agarose Gel Analysis of Plasmids Formed from Maxi Preparation of pBIN19 and pBI121. Lanes A: λ*Pst*1 cut markers whose lengths are given in kilobases on the left. B: Uncut pBIN19 plasmid vector. C: pBI121 plasmid vector and D: the blunt-ended 607bp fragment in pACMV7.

4.4 Sequencing of 607 ACI Fragment of ACMV-SA

The M13 forward primer had already been used to sequence 196 nucleotides of the 607ACI bp fragment cloned in pACMV7 (Menschig, 1993). From the end of this sequence, the primer 5'ggACAAccAcggAc-3' was designed and used to further prime inwards. The designed primer which is 16mer was used to obtain a further 175 nucleotides of the ACI fragment. The 196 and 175 nucleotide sequences obtained were that of the complementary (-Ve) sense strand, hence reversing the sequenced primed by both the M13 primer and the designed primer gave the positive virion sense (+) strand sequence. The resulting sequence of 371 nucleotides (26%A, 27%T, 20%C and 26%G) is as shown in Fig. 16(b)

```
5' A G A A C T C C A T  g A A A A T g g g g  A A c c T c A T T T
  g c A T g C C C t C  A T T C A g T T C g  A g g g C A A g T A
  c A A T T g T A C C  A A C C A A c g A T  T C T T c g A C C T
  C A T A T C C C T T  C C A g g T C A A C  A C A T T T C C A T
  C C A A A C A T T C  A g g g A g C T A A  A T C C A g T T c T
  g A C g T C A A g T  C C T A T T T g g A  C A A g g A c g g A
  g A C A c c W W C C  A A T g g g g C g A  g T T T C c g A T c
  g A c g g A c g A c  T g c T c g c g g c  g g A C A A C A A T
  C C g C a a t G A A  A c g T T A c g c c  A A g g C T C T T A
  A c g g C g A g C A  A g T A A A A c A g  A g g C T T c T T A
  A T g T A A T C C g  g g A A C T A g c c  c c c A A g g T T T
  T g T T T T A C A g  T T T A T A A T T T  A A T A g c A T T T
  A g A T A g g g g g  g..... 3'
```

Figure 16b: Nucleotide Sequence of the Virion Sense Strand of 371bp Fragment of ACI ACMV-SA (DNA-A)

4.3 Cloning of the 607bp ACI Fragment into Plant Transformation Vectors

The plant transformation vectors pBIN19 (Fig. 17, lane B), pBI121 (Fig. 17, lane C) and the recombinant plasmid pACMV7 (Fig. 19, lane 6) that harbours the 607bp ACI fragment were successfully isolated from the bacterial cells. Digestion and desphosphorylation of the vectors using *Sma*1 was carried out and a single linear band in each case (Fig. 18, lanes C and F) was observed on the gel . Digestion of pACMV7 with *Pst*1 and extraction of the insert from a LMT- agarose gel yielded a fragment which migrated similarly to λ*Pst*1 cut marker of identical size (Fig. 19, lane 5). Using the Klenow fragment of *E coli* DNA polymerase I, the 3' termini of the extracted 607bp fragment was partially filled in to produce a blunt end insert for cloning into the *Sma*1 cut vectors. A gel of this fragment can be observed in Fig. 17, lane D.

Ligation reactions of several vector to insert ratios were done and the ligated products were transformed in one-shot strain of *E coli*. Unfortunately there was no growth of bacterial cells selected on Kanamycin-LB-agar plates containing X-gal and ITPG in all cases. However, the positive control of transformation of the uncut pBIN19 and pBI121 did exhibit growth of several white colonies on selection media, thereby demonstrating that the transformation step was not the cause of failure to obtain transformation with ACMV-SA ACI fragment. In the transformation of the ligation control reaction between *Pst*1 - cut and dephosphorylated pBluescript and *Pst*1-cut ACI fragment, five white and two blue bacteria colonies grew on ampicillin-LB-agar plate containing X-gal and ITPG. There was no bacteria growth on Kanamycin -LB- agar plates when *Sma*1 cut and desphosphorylated pBIN 19 and pBI121 were self-ligated and transformed.

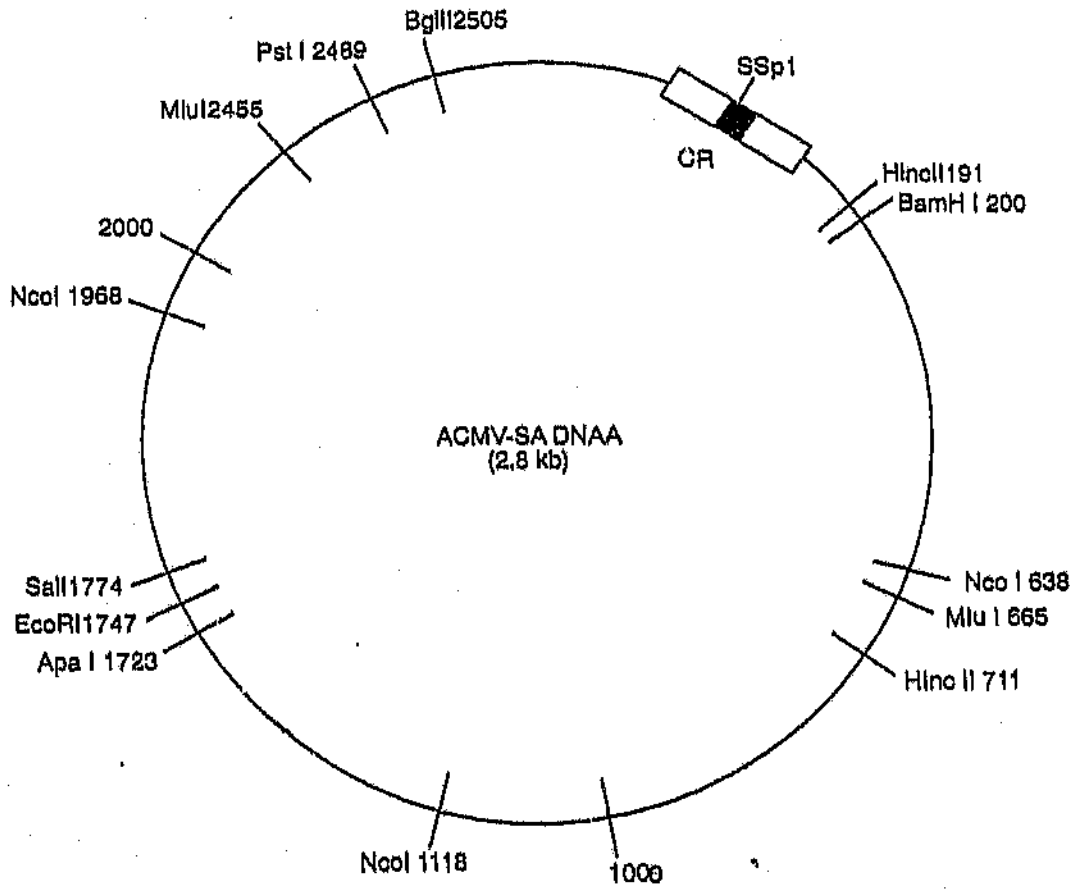


Figure 16a: Restriction Map of ACMV-SA (DNA-A).

The PCR fragment of the genome was approximately 2.8kb, It was excised from an agarose gel and purified. The PCR product was then cloned in pBluescript KS to give clone pCGV-100. CR= Common Region

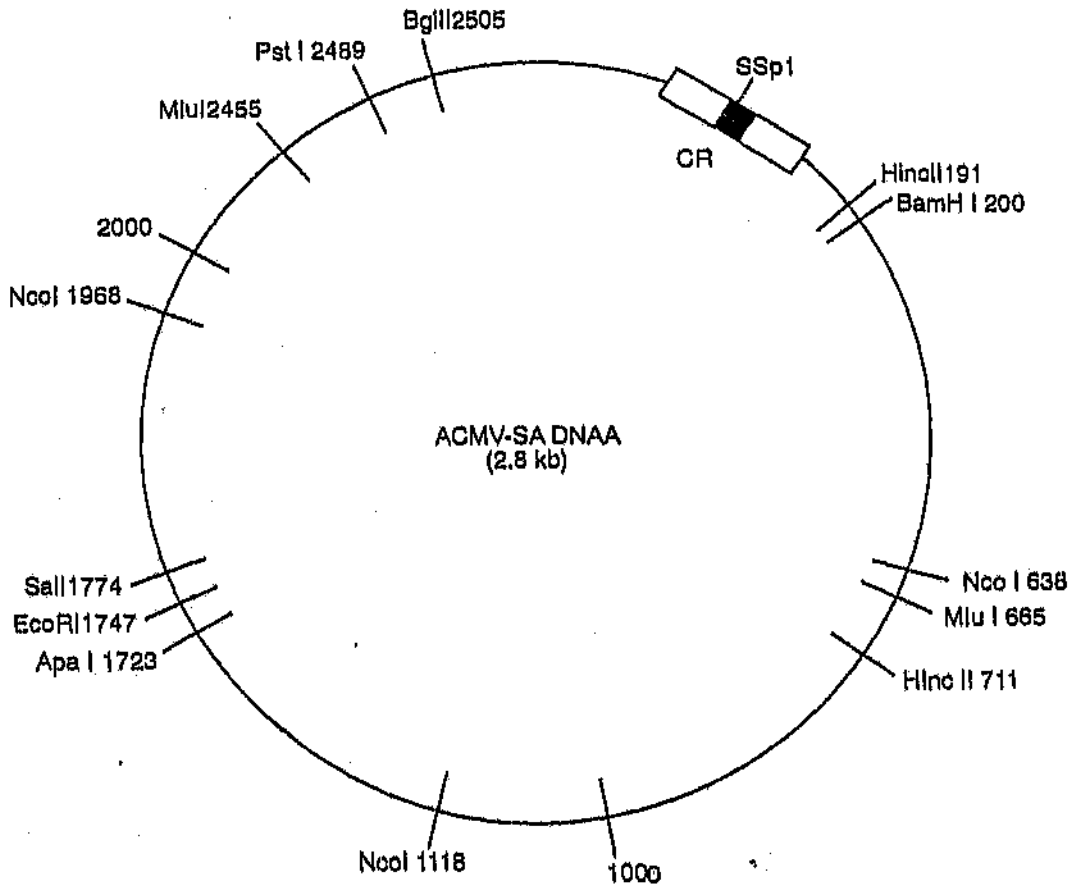


Figure 16a: Restriction Map of ACMV-SA (DNA-A).

The PCR fragment of the genome was approximately 2.8kb. It was excised from an agarose gel and purified. The PCR product was then cloned in pBluescript KS to give clone pCGV-100. CR= Common Region

Table 2: **Restriction Fragment Sizes Produced from Single and Double Digestion of pCGV-100**

ENZYME(S)	FRAGMENT SIZES (bp)
*Ksp*1	5760
*Xba*1	5760
*Bgl*II	5760
*EcoR*V	5760
*Sac*1	5760
*Hind*III	5760
*PVu*II	3349, 2511
*Bgl*I	4467, 1454
*SSp*1	2260, 2100, 850
*Mlu*I	3705, 2055
*Apa*1	4631, 1129
*BamH*1	2985, 2775
*Pst*1	5252, 520
*Hinc*II	4630, 580, 550
*Nco*1	4430, 780, 550
*EcoR*1	4673, 1067
*Sal*I	4673, 1067
*Bgl*II + *Sac*1	3410, 2350
*Bgl*II + *Hind*III	5240, 520
*EcoR*V + *Mlu*I	3372, 2158, 230
*Xba*1 + *Mlu*I	3132, 2158, 470
*EcoR*V + *Hinc*II	4051, 550, 580, 520
*Xba*1 + *Hinc*II	2930, 1700, 550, 580
*EcoR*V + *Nco*1	3540, 850, 780,580
*Xba*1 + *Nco*1	3750, 770, 650, 550
*SSp*1 + *Bgl*II	2180, 2100, 850, 370
*SSp*1 + *Xba*I	2700, 2180, 850, 230

Table 1 :Restriction Analysis of pCGV-100

Enzyme(s) Used	No. of Fragments Produced	No of Sitesin Polylinker Region of BS	No. of Sites in BS but not in Polylinker Area	No. of Sites in DNA-A of ACMV-SA
*Pst*1	2	1	0	1
*Apa*1	2	1	0	1
*EcoR*1	2	1	0	1
*Sal*1	2	1	0	1
*Bgl*1	2	0	2	0
*Bgl*1I	1	0	0	1
*PVu*1	2	0	2	0
*Hind*III	1	1	0	0
EcoRV	1	1	0	0
*Sac*1	1	1	0	0
*Ksp*1	1	1	0	0
*Mlu*1	2	0	0	2
*Hinc*II	3	1	0	2
*Nco*1	3	0	0	3
*Xba*1	1	1	0	0
*SSp*1	3	0	2	1
*BamH*1	2	1	0	1

4.2 RESTRICTION ANALYSIS OF pCGV-100

Seventeen single and ten double digestions were done. The number of sites in pCGV-100 and size fragments produced are shown in Tables 1 and 2 and Figs. 12 to 15. The restriction enzymes *Xba*1, *Hind*III, *Ksp*1 (Fig. 15b, Lane D), *Sac*1 and *EcoR*V have one site each in the polylinker, hence they each gave a linearilized fragment of 5.7kb. *Bgl*II (Fig. 12, lane H) also produce one fragment, but since it has no site in pBluescript, the cut or site should be in the insert (i.e. ACMV-SA). *EcoR*1, *Pst*1, *Apa*1, *Mlu*1, *Sal*1, *PVu*II and *Bgl*I each produced two fragments (Figs. 12, 13 and 14). *EcoR*I, *Pst*1, *Apa*1 and *Sal*1 each has one recognition site in the polylinker hence the second site should be in the insert. *PVu*II and *Bgl*I each has two recognition sites in pBluescript, hence the cuts were made outside of the polylinker and insert. *Mlu*1 has neither a site in the polylinker nor in pBluescript hence the two sites were in the insert.

*SSp*1 (Fig. 12, lane D) and *Nco*1 (Fig. 14, lane F) produced three fragments hence they have 3 recognition site in the plasmid. *SSp*1 has 2 recognition sites in pBluescript hence the third recognition site is located in the insert. *Nco*I has neither a site in the polylinker nor in pBluescript hence all the 3 recognition sites must be in the insert. *Hinc*II (Fig. 14, lane C) produced three fragments. *Hinc*II has one recognition site in the polylinker, hence the two other sites are expected to be situated in the insert.

In the double digest in Figs. 14 and 15a, *Sac*I + *Bgl*II and *Hind*III + *Bgl*II gave 2 fragments (Fig. 15a, lanes 1 and 2). *Mlu*I + *Xba*I and *Mlu*I + *EcoR*V gave 3 fragments (Fig. 15a, lanes 3 and 4), *Xba*I + *Hinc*II (Fig. 15a, lane 7) and *EcoR*V + *Hinc*II (Fig. 14, lane A) produced 4 fragments. *Xba*I + *Nco*I (Fig. 15a, lane 8) and *EcoR*V and *Nco*I (Fig. 14, lane B) produced 4 fragments. *SSp*1 + *Bgl*II (Fig. 15a, lane 5) and *SSp*1 and *Xba*I (Fig. 15a, lane 6) produced 4 fragments each. From these single digest and double digest of pCGV-100 and taking into consideraion the cuts made in the pBluescript vector and length of the fragments observed, a restriction map (Fig. 16a) of ACMV-SA was constructed

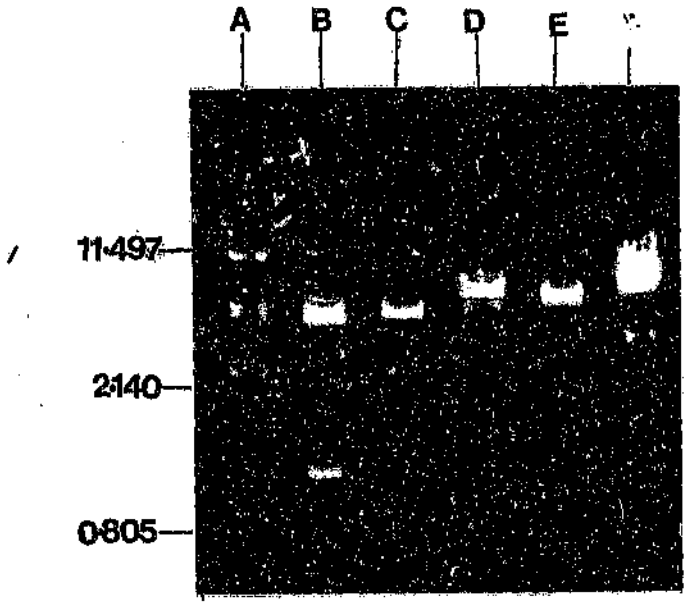


Figure 15b: Gel Analysis of pCGV-100 DNA. pCGV-100 DNA (3μg) was digested with various single restriction enzymes and separated by electrophoresis in 1% agarose gel in 1XTBE running buffer at 90V. Lanes A: λ*Pst*1 cut markers whose lengths are given on the left in kilobases. Lane B: pCGV-100 digested with *Apa*1. C: pCGV-100 digested with *Hinc*II. D: pCGV-100 digested with *Ksp*1. E:pCGV-100 digested with *Pst1*. F: pCGV-100 digested with *Xba*I.

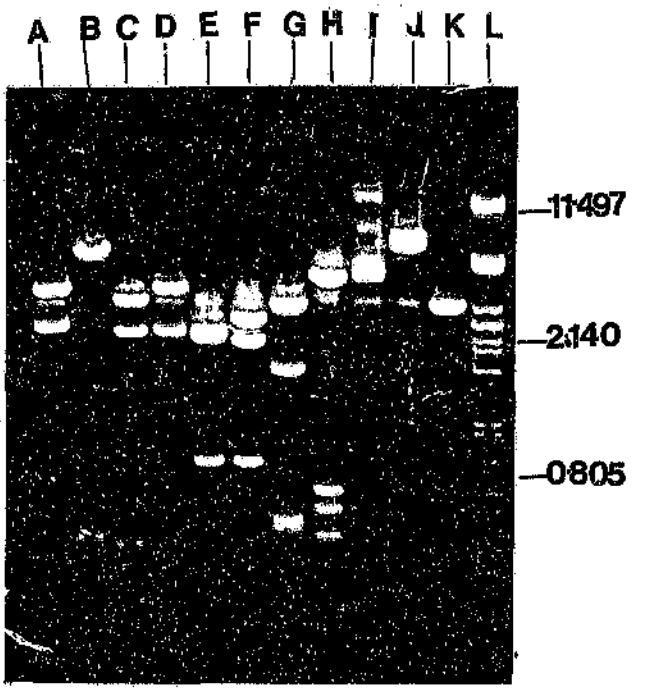


Figure 15a: 1% Gel Analysis of pCGV-100 DNA. pCGV-100 (3μg) was digested with two restriction enzymes and separated by electrophoresis in 1% agarose gel in 1XTBE running buffer at 90V. Lanes A: pCGV-100 digested with *Sac*I and *Bgl*II. B: pCGV-100 digested with *Hind*III and *Bgl*II. C: pCGV-100 digested with *Mlu*I + *Xba*I. D: pCGV-100 digested with *Mlu*I + *EcoRV*. E: pCGV-100 digested with *SSpI* + *Bgl*II. F: pCGV-100 digested with *SSp*I and *Xba*I. G: pCGV-100 digested with *Xba*I and *Hinc*II. H: pCGV-100 digested with *Xba*I + *Nco*I. I: Uncut pCGV-100 marker. J: *EcoRV* cut pCGV-100 marker. K: *Pst*I cut pBluescript marker. L: λ-*Pst*I cut markers whose lengths are given on the right in kilobases.

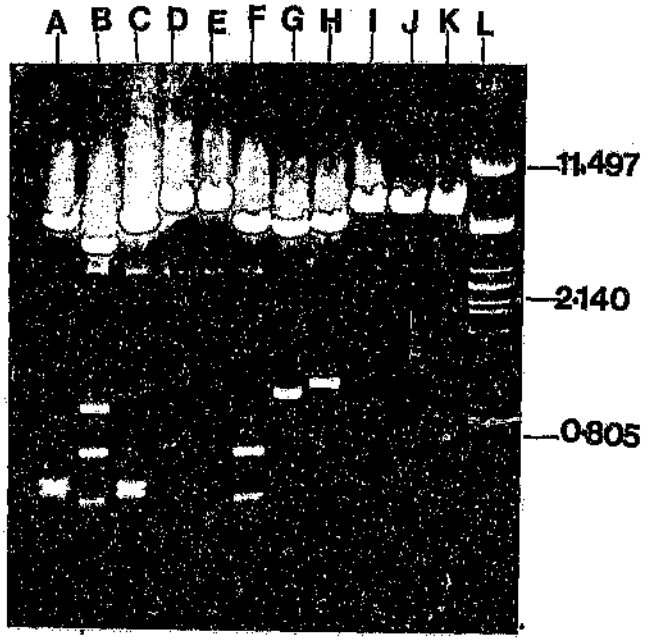


Figure 14 ' Gel Analysis of pCGV-100 DNA. pCGV-100 (3µg) was digested with various restriction endonucleases and separated by electrophoresis in 1% agarose gel in 1 x TBE running buffer at 90V. Lanes A: pCGV-100 digested with *EcoR*V and *Hinc*II. B: pCGV-100 digested with *EcoR*V + *Nco*1. C: pCGV-100 digested with *Hinc*II. D: pCGV-100 digested with *Bgl*II. E: pCGV-100 digested with *EcoR*V. F: pCGV-100 digested with *Nco*1. G: pCGV-100 digested with *EcoR*I. H: pCGV-100 digested with *Apa*1. I: pCGV-100 digested with *Hind*III. J: pCGV-100 digested with *Xba*I. K: pCGV-100 digested with *Sac*1. L: λ*Pst*1 cut markers whose lengths are given on the right in kilobases.

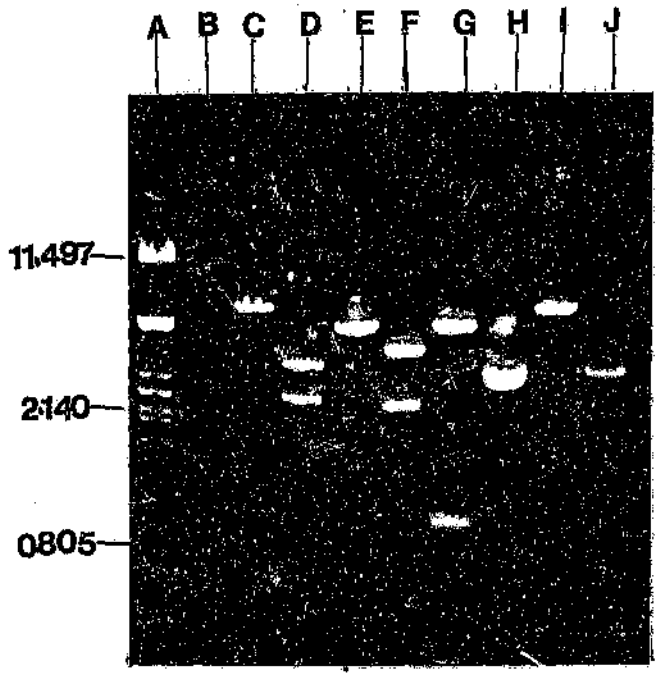


Figure 13: Gel Analysis of pCGV-100 DNA. pCGV-100 (3µg) was digested with various single restriction enzymes and separated by electrophoresis in 1% agarose gel in 1xTBE running buffer at 90V. Lanes A: λPst1 cut markers whose lengths are given on the left in kilobases. B: uncut pCGV-100 marker. C: *EcoR*V cut pCGV-100 marker. D: pCGV-100 digested with *PVu*II. E: pCGV-100 digested with *Hinc*II. F: pCGV-100 digested with *Mlu*I. G: pCGV-100 digested with *Apa*I. H: pCGV-100 digested with *BamH*1. I: pCGV-100 digested with *Pst*1. J: cut pBluescript marker.

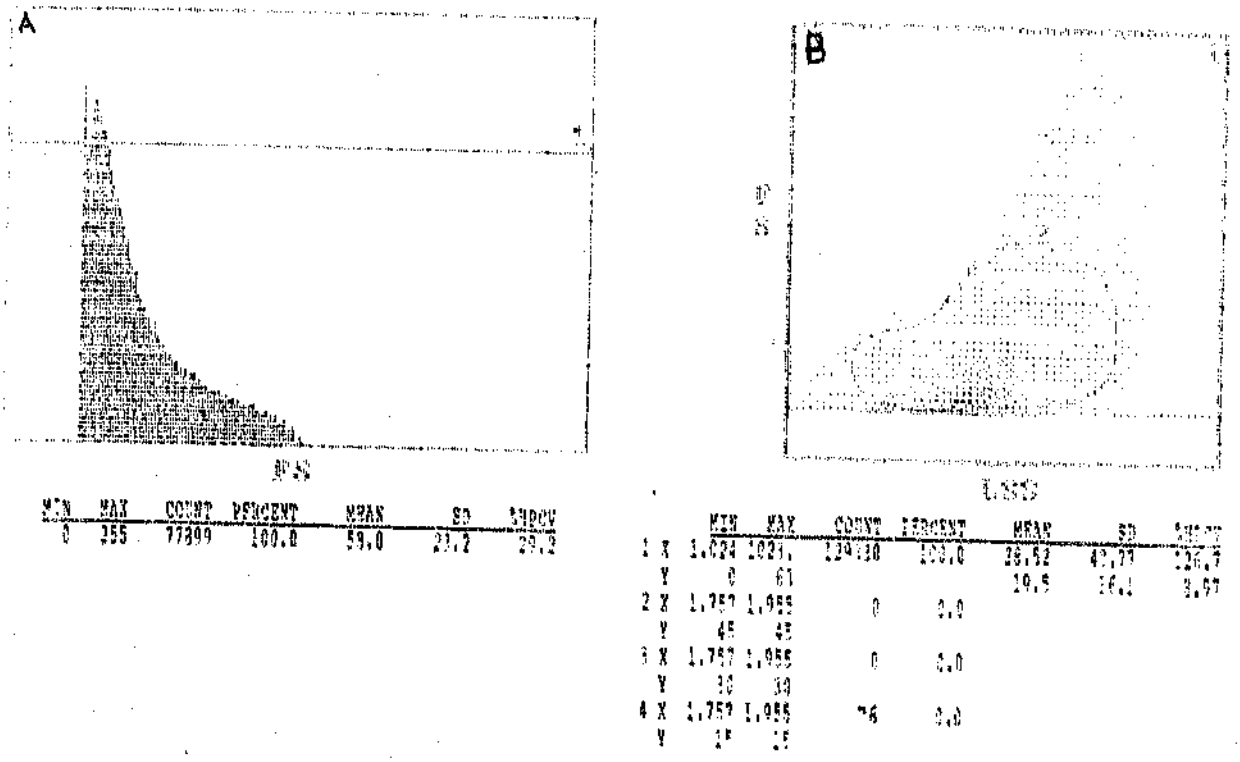


Figure 25: Flow Cytometer Counts of A Population of Cassava (TMS87100609) Cell Suspension Culture. A depicts the appearance of the entire cell population. B depicts the distribution and total counts (129330) of all the cells in the population. The bitmap enclosed by area 2 in B depicts a count (77899) of a selection of cells in the population.

Table 5: Flow Cytometry Counts of Total Number of Suspension Cells Present in Cassava Cultivars TMS87100609, TMS63397 and Tobacco (Nicotiana tabacum)at 14 Days and 21 Days after Initiation of Suspension Culture

Tube Number	Sample	Total number of cells/ ml at 14days	Total number of cells/ml at 21days
1	TMS8710069	2.40×10^6	2.54×10^6
2	TMS8710069	1.96×10^6	3.69×10^6
3	TMS8710069	2.34×10^6	2.36×10^6
4	TMS8710069	1.81×10^6	2.44×10^6
5	TMS63397	1.50×10^6	1.11×10^6
6	TMS63397	1.40×10^6	2.64×10^6
7	TMS63397	0.95×10^6	0.98×10^6
8	TMS63397	1.10×10^6	1.12×10^6
9	Tobacco	1.26×10^6	1.47×10^6
10	Tobacco	0.28×10^6	0.41×10^6
11	Tobacco	0.38×10^6	0.62×10^6
12	Tobacco	0.29×10^6	0.45×10^6
13	Tobacco	1.11×10^5	1.19×10^6
14	Tobacco	0.58×10^5	0.93×10^6

Table 4: Haemacytometer Counts of Viable and Non-Viable Cells at 14 and 21 Days after Subculturing of Suspension Cells of Cassava and Tobacco.

Sample Number	Sample	Viable cells/ml at 14days	Non-viable cells/ml at 14 days	Total #o. of cells/ml at 14 days	Viable cells/ ml at 21 days	Non-viable cells/ml at 21 days	Total No. of cells/ml at 21 days
1	TMS87100609	1.90×10^6	0.50×10^6	2.40×10^6	2.01×10^6	0.53×10^6	2.54×10^6
2	TMS87100609	1.75×10^6	0.20×10^6	1.95×10^6	3.20×10^6	0.45×10^6	3.65×10^6
3	TMS87100609	2.00×10^6	0.30×10^6	2.30×10^6	2.06×10^6	0.30×10^6	2.36×10^6
4	TMS87100609	1.50×10^6	0.25×10^6	1.75×10^6	1.95×10^6	0.45×10^6	2.40×10^6
5	TMS63397	1.25×10^6	0.20×10^6	1.45×10^6	1.65×10^6	0.34×10^6	1.99×10^6
6	TMS63397	1.05×10^6	0.25×10^6	1.30×10^6	1.95×10^6	0.60×10^6	2.55×10^6
7	TMS63397	0.75×10^6	0.15×10^6	0.90×10^6	0.75×10^6	0.20×10^6	0.95×10^6
8	TMS63397	1.00×10^6	0.20×10^6	1.20×10^6	1.02×10^6	0.20×10^6	1.22×10^6
9	Tobacco	0.95×10^6	0.30×10^6	1.25×10^6	1.16×10^6	0.30×10^6	1.46×10^6
10	Tobacco	0.25×10^6	0.03×10^6	0.28×10^6	0.35×10^6	0.10×10^6	0.45×10^6
11	Tobacco	0.30×10^6	0.05×10^6	0.35×10^6	0.56×10^6	0.05×10^6	0.61×10^6
12	Tobacco	0.25×10^6	0.01×10^6	0.26×10^6	0.39×10^6	0.15×10^6	0.54×10^6
13	Tobacco	1.00×10^6	0.05×10^6	1.05×10^6	1.30×10^6	0.25×10^6	1.55×10^6
14	Tobacco	0.50×10^6	0.05×10^6	0.55×10^6	0.80×10^6	0.10×10^6	0.90×10^6



Figure 23: Cell Suspension Culture of Cassava (TMS63397) Cultured in Liquid MS-medium Containing 8mg/l 2,4-D. A shows isodiametric cells in a small aggregate. B - a dividing palisade cell. C, a single oval shape cell. magnification 100X

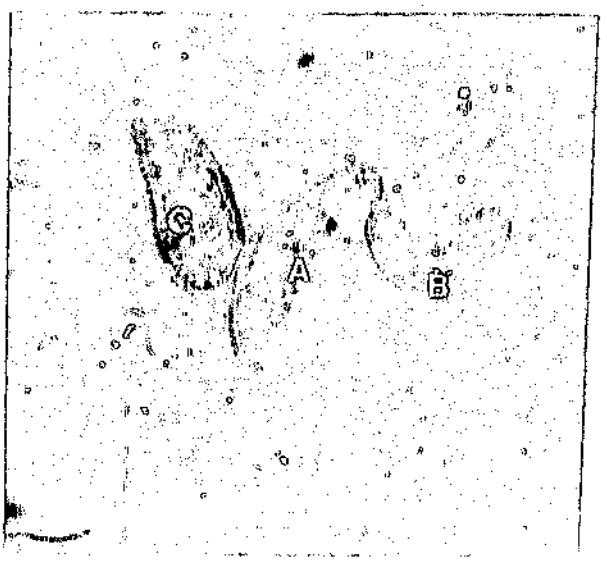


Figure 24: Suspension Cells of Tobacco Culture in Liquid MS-Medium Containing 8mg/l 2,4-D. A & B are banana-shaped cells. C is an oval'-shaped cell. magnification 100x

of non-viable cells per ml for the same cultivar was about 17% of the number of viable cells. In the TMS63397 cultivar, the average number of viable cells per ml was 1.01 x 10^6 whilst the non-viable cells per ml was 20% of the viable cells. For tobacco, the average number of viable cells per ml was 0.54x 10^6, whilst the average count of non-viable cells per ml was around 15% of the viable cells. After 21 days of culture,the average number of viable cells per ml of TMS87100609 cassava cultivar was 2.31 x 10^6, whilst non-viable cells per ml was 25%of the viable cells. In TMS63397,the average number of viable cells per ml was 1.34 x 10^6 whilst non-viable cells per mlwas 25% of the viable cells. For tobacco, the average number of viable cells per ml was 0.76 x 10^6 and non-viable cells was 21%of the viable cells.

There was an increase in the number of viable and non-viable cells in both cassava cultivars as well as in the tobacco cultivar from 14 days to 21 days after culturing. For instance, the viable cells increased by 30% in TMS87100609 , 33% in TMS63397 and 41% in tobacco. The non-viable cells also increased by 45% in TMS87100609, 70% in TMS63397 and 100% in tobacco.The number of non-viable cells on the whole increased greatly in the tobacco and cassava cultivars whilst the viable cells increased moderately in all the cultivars between 14 to 21 days of culture. The number of viable cells counted in each culture of the cultivars indicated that the propagation of the cells in cell suspension culture was higher in the cassava cultivars than tobacco. Amongst the cassava cultivars the number of viable cells was greatest in TMS87100609.



Figure 22: Leaf Explant Showing Cream-Coloured Friable Callus Initiated from Cassava (TMS87100609). A is the remains of the leaf explant left after callus formation. B is the creamy-friable callus.

after culturing. Between 14 - 21days, roots continued to elongate, branch and curl. After 21 days, plantlets assumed heights ranging from 5 - 8cm for cassava cultivars and 4 - 6cm in the case of tobacco. Roots continued branching, and with the cassava cultivar (TMS87100609) more curling activity could be seen under the MS-media.

An adult plantlet with normal growth features were finally observed during twenty-eight days of the nodal explant culture. At this period a developed network of root and shoot system were seen (Figs. 20 and 21). After 28 days plantlets continued to grow but some signs of discolouration of leaves (i.e. yellowing) began to show and subculturing was undertaken.

4.5.2 Callus Culture

Callus formation was achieved with leaves of sizes between 1 - 5mm in length from the cassava cultivars TMS63397, TMS87100609 and tobacco. Leaves longer than 5mm showed little or no callus formation. Callus formation in tobacco was superior to that of the cassava cultivars. Callus was initiated in cassava cultivars 3 weeks after the culturing of the leaf explant whereas in tobacco, callus formation started in the first two weeks. Tobacco callus was greenish in appearance whilst cassava callus was creamy (Fig 22) despite both been grown under light. Callus formation was initiated from the cut ends of the embedded leaves and gradually expanded to form a friable callus. Due to the colour of the tobacco callus, the remains of the leaf explant looked masked, however in the cassava cultivars the remains of the explant was conspicuous. At the end of the fifth week of culture, wet and friable callus had formed to a reasonable size that could be subcultured. After this period the callus became dry and brownish.

4.5.3 Cell Suspension Culture

Table 4 shows haemocytometer counts of cell suspensions 14 and 21 days after initiation of culture from friable callus from cassava cultivars TMS87100609, TMS63397 and the tobacco cultivar. After 14 days of culture the average number of viable cells per ml determined for TMS87100609 was 1.79×10^6 .The average number



Figure 20: Complete Propagated Plantlet of Cassava Cultivar (TMS87100609) fom Nodal Explants. Plantlet was propagated 28 days after culturing explant on MS-medium supplemented with sucrose, NAA and BAP.

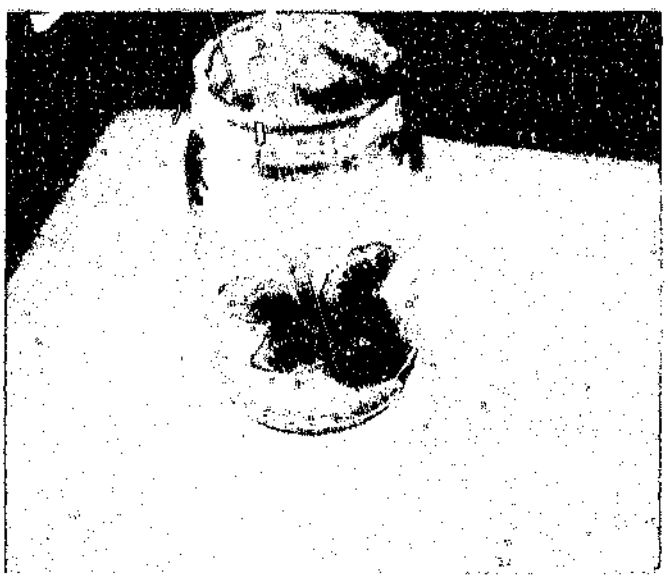


Figure 21: Twenty eight Day old Plantlet of Tobacco (Nicotiana tabacum) Propagated from Nodal Explants Cultured on MS-medium Supplemented with Sucrose, NAA and BAP.

4.5 Propagation of Cassava and Tobacco in Plant Tissue Culture

4.5.1 Nodal Explant Culture

Healthy growth of plantlets were assessed by normal root, stem and leaf development. These attributes were characterized by abundant root formation, increase in height and girth of stem, internodes as well as the maintenance of fleshy green leaves. Poor and reduced growth was noted by poor root, stem and leaf development. Stunted growth and discolouration of leaves were indications of improper growth features. Out of the initial twenty nodal cultures of each cultivar grown in this study, 100% of TMS87100609, 90% of TMS63397 and 50% of TMS87100501 were successfully propagated into plantlets. In the case of tobacco, 75% (15/20) developed into plantlets.

Of the 10 plantlets formed from the cassava cultivar (TMS87100501) nodes, eight were stunted and distorted whilst only two appeared healthy and hence were used for furthersubculturing. Of the two other cassava cultivars, TMS87100609 did demonstrate the best growth characteristics on the culture media. Although the TMS63397 did also exhibit some good growth features too, the rate and extent of growth was greater in TMS87100609.

TMS87100609 nodes were propagated with much ease into plantlets, and their plantlets illustrated better root, stem and leaf development (Figure 20). The aerial growth of these plantlets was only obstructed by the height of the containing vessel.

Tobacco cultivars also showed a high degree of growth, despite the fact that they were cultured on half strength MS-media. Tobacco plantlets demonstrated healthy root, stem and leaf development. Their leaves were large, fleshy and green (Figure 21). Generally, plantlet development commenced in all the cultivars after the first seven days of initiation of culture. Organogenesis commenced with a sprouting out of the nodal explants of young leaves. During the second week, roots appeared as miniature outgrowths from under the nodes.Within the same period, elongation of shoot and development of more nodes and internodes did occur. Complete young plantlets made up of roots, stems and leaves were seen at the end of the fourteen days

4.4.1 Phylogenetic Analysis

The 371bp ACI sequence of ACMV-SA and other whitefly-transmitted geminiviruses were aligned using the PILEUP program (GCG). Geminivirus sequences were obtained from the GENBANK/EMBL databases, that is ACMV-Ken (Kenya isolate), ACMV-Nig (Nigeria isolate), TYLCV-Sar (Sardinia), TYLCV-Sic (Sicily) and Tomato Leaf Curl Virus (ToLCV-Aus) from Australia. The results of the sequence comparisons are summarized in Table 3. The highest ACI homology found was with the ACMV isolate from Nigeria (80% homology), while a 76% homology with the type strain (ACMV- Ken) was noted. However the ACMV-SA also shared a sensibly high homology with TYLCV-isolates from Italy (75% with TYLCV-Sar; and 73% with TYLCV-Sic) and 78% with ToLCV-Aus.

Table 3: Comparison of 371bp of the ACI Fragment of ACMV-SA with ACI Sequences of Other Geminiviruses

	ACI ACMV-Nig	ACI ACMV-Ken	ACI TYLCV-Sar	ACI TYLCV-Sic	ACI ToLCV-Aus
ACI ACMV-SA	80%	76%	75%	73%	76%

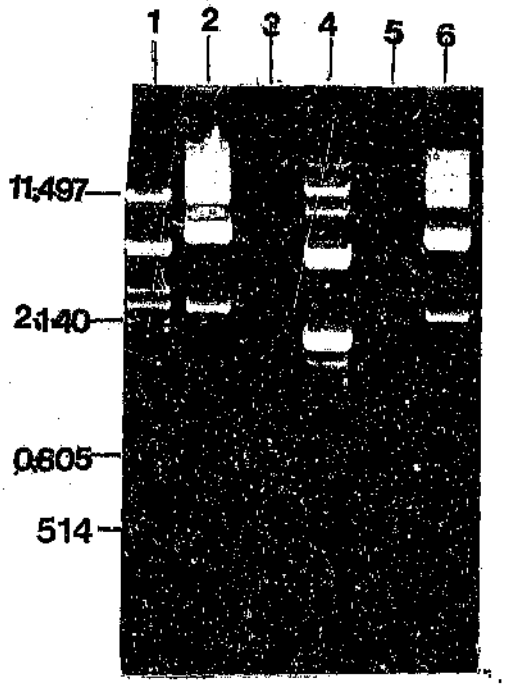


Figure 19: Gel Analysis of Fragments Cloned into pACMV6 and pACMV7. Fragments were cut with *Pst*1, and extracted from 1% LMT agarose gel and identified in 1% agarose gel in 1xTBE running buffer at 90V. Lanes 1: λ*Pst*1 cut markers whose lengths are given on the left in kilobases. 2: uncut pACMV6 plasmid formed from a large scale prep. 3: the 800bp fragment that was cloned into pACMV6. Lane 4: uncut pBluescript marker. 5: the 607bp ACI fragment. Lane 6: uncut pACMV7.

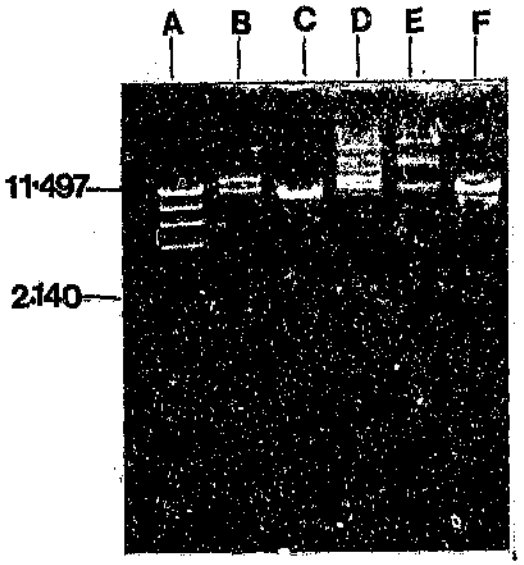


Figure 18: Gel Analysis of Cut Transformation Vectors.

Plant transformation vectors were digested with a *Sma*1, dephosphorylated with calf intestinal alkaline phosphatase(CIP) and separated in 1% LMT agarose gel in 1xTBE running buffer at 90V. Lane A: λ*Pst*1 cut markers whose lengths are given on the left in kilobases. Lanes B and D: uncut pBIN19 vector marker. C: pBIN19 vector cut with *Sma*l and dephosphorylated. E: Uncut pBI121marker. F: pBI121 cut with *Sma*l and dephosphorylated.

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λPst1 Markers in base pairs

11497 5077 4749 4507 2836 2560 2549 2443 2140 1986 1700 1159 1093 805
514 468 448 339 264 247 216 211 200 164 150 94 87 72 15

RESUSPENSION MEDIUM

4.780g/L Murashige Skoog media (MS)
10.0g/L sucrose
10.0g/L glucose
0.034mg/L BAP
0.011mg/L NAA
0.4 mannitol
8mg/L 2,4-D - made up to 900ml with sterile water
pH to 5.7
then addition of sterile water to 990ml, autoclaved at 121°C at 15Psi at 20min then filter sterile 10ml of 100mg/l casein hydrosylate into medium and store at 4°C.

a) **Ampicillin Stock**

500mg ampicillin was dissolved in 10ml sterile water to produce a stock conc. 50mg/ml and stored at -20°C.

b) **RNase-A (DNAase free) Stock**

10mg RNase was dissolved in 10ml of sterile water.Heated at 70°C for 10 min, filter steriled and stored in 1ml aliquots at -20°C.

c) **TE**

10mM Tris-Hcl, pH 7.5; 1mM EDTA

It was shown in this study that cassava and tobacco could be propagated successfully via nodal, callus and cell suspension cultures. These tissue culture systems can subsequently be used in transformation experiments. It was also demonstrated that protoplasts could be readily isolated from cassava and tobacco cell suspension cultures.

6. CONCLUSIONS

Various procedures were employed in the further characterization of the ACMV-SA virus, as well as formulation of preliminary steps that may lead eventually to the establishment of transgenic ACMV-SA cassava plants.

The restriction map of DNA-A of ACMV-SA was constructed, but it showed no convenient sites for subcloning the **entire** region of the ACI open reading frame (ORF). However, the map demonstrated that several restriction sites existed which could be used for other ORFs subcloning. Furthermore, the map revealed that a **large** fragment of the ACI ORF and part of the AC2 ORF could be excised using *Bgl*II and *Apa*I. Such a fragment could be used to produce antisense RNA resistance in transgenic plants.

Cloning of the 607bp ACI fragment of ACMV-SA into plant transformation vectors pBIN19 and pBI121 was not successful. The possible reason for the failure may be attributed to the ligation step in the cloning process. It seems that in order to achieve a breakthrough, every step involved in the cloning process would have to be meticulously reassessed.

Sequence homology studies indicated that ACMV-SA was almost equally related to ACMV-Nig and ACMV-Ken (80% and 76% respectively) as to TYLCV-Sar and TYLCV-Sic (75% and 73% respectively), and ToLCV-Aus (76%). These findings confirm previous results with respect to the common and coat protein regions. In other words, ACMV-SA and TYLCV have been found to share a higher sequence homology than expected along their ACI and AVI ORFs. This result further confirms the earlier assertion that ACMV-SA is indeed a unique cassava geminivirus. This is even more notable since ACMV-SA is a bipartite ,while TYLCV is a monopartite virus. Furthermore since both ACMV-SA and TYLCV show a high sequence similarity within their ACI ORF regions (i.e. which encodes the replication protein), there is a strong probability that both viruses may originate from a common ancestor of ACMV and TYLCV.

cells incubated overnight (i.e.24h), while Cabral et al (1992b) illustrated the enzymatic liberation of protoplasts from cassava leaves digested overnight (16 -18 h). The method used in this study had revealed that protoplasts could be liberated very rapidly from cassava cell suspensions . Protoplasts liberated at shorter isolation times , have been found to be ideal for viral infection compared to those isolated by overnight digestion (Wood,1985).Therefore the procedure adopted here seemed effective and advantageous, for producing easily infectable cassava protoplasts ,as the protoplasts were liberated only 7h - 8h after incubation with enzyme mixtures. Nagata and Ishii (1979) also illustrated the liberation of protoplasts from tobacco leaf tissue within 30min and other leaf tissues like oat and rice in 2h. Using the same concentration of enzyme mixtures as in that experiment , the liberation of protoplasts from suspension cells in this case took a much longer time (5h). Despite the time taken to produce protoplasts from tobacco suspension cells, the method employed in this experiment could be said to be encouraging, as protoplasts derived from culture cells offer some advantage over that obtained from mesophyll leaf cells in that they are uniform and contaminant-free and could be produced in high numbers .

on an MS-medium supplemented with 10mg/l 2,4-D in 2 weeks. It is possible that a higher concentration of 2,4-D could accelerate the initiation of calli from cassava leaves.

Suspension culture cells from calli induced from leaves of the cassava cultivars (TMS87100609 and TMS63397) and tobacco demonstrated that sufficient meristematic and growth activities in the cultures could lead to the production (within the 2 weeks of primary culture initiation) of large amount of cells that can be subcultured or used for other cell manipulation experiments. The viable cell density range observed in all the cultivars at 2 weeks and 3 weeks after initiation of cultures were 0.5 - 2.0 x 10^6 cells/ml and 0.65 - 2.3 x 10^6 cells/ml respectively. Also the number of non-viable cells per ml noted, for the same periods were 0.02 - 0.3 x 10^6 and 0.05 - 0.6 x 10^6 respectively. These results showed that there was no drastic increase in the number of viable cells per ml between the monitoring interval. However, there was about two-fold increase in the amount of non-viable cells within the interval. These findings revealed that subculturing could be done 2 weeks after the initiation of the primary cultures, at which period the viable cell populations are sufficiently high and the amount of non-viable cells are low. These observations seemed to concur with the viewpoint of King (1980), who proposed that subculturing of suspension cells could be done 2- 3 weeks after initiation of primary culture, as cell densities of cultures around this period approach maximum levels. The total cell-counts recorded by the flow cytometer were in most cases higher than that obtained with the haemacytometer, and the possible reason may be that the readings from the flow cytometer may have included cell debris that have sizes and granularities similar to the plant suspension cells. The number of viable suspension cells per ml was less in tobacco than the cassava cultivars. The largest number of viable cells were recorded in the cassava cultivar TMS87100609 which also exhibited the highest growth rate.

In this experiment,some 2-week old cultured tobacco and cassava suspension cells were incubated with enzymes at 28°C; and this resulted in tobacco protoplasts being liberated within 5h, and cassava protoplasts within 7-8h period. In comparisons, Dudits(1984) demonstrated an enzymatic liberation of protoplasts from a 2-day old cultured carrot suspension

ACMV-SA also shares high sequence homology in the coat protein and common region with TYLCVs especially TYLCV-Sar and TYLCV-Isr (Israel isolate). The TYLCVs are monopartites whereas ACMV-SA is bipartite. Following the publication of the first DNA sequence of a geminivirus (ACMV-Ken) (Stanley and Gay, 1983), Kikuno et al (1984) postulated, based upon the sequence comparison between DNA-A and B of ACMV-Ken, that geminivirus genomes may have evolved from monopartite to bipartites through duplication. The close identity between ACMV-SA and TYLCV-isolates may represent actual examples within this process. However, this is merely speculation at present.

Successful tissue culture of cassava and tobacco was achieved in this study. Cassava plantlets were propagated from nodal explants cultured on MS-media supplemented with 0.034mg/l BAP and 0.011mg/l NAA and 1% sucrose. In previous experiments the plantlets were propagated from nodal segments on MS containing 0.1mg/l BAP and NAA and 3% w/v sucrose (Cabral et al, 1992). The procedure used in this project looks more cost effective as less amount of hormones were used. Despite lower hormone additives, the two cassava cultivars TMS87100609 and TMS63397 exhibited healthy growth features. However the third cassava cultivar TMS87100501 was less tolerant of the medium and did not respond favourably to the culture medium used. It could be possible that the growth conditions for the donor plant were unfavourable for subsequent culturing. TMS87100501 may be a genotype that requires higher amounts of hormones to support further development after propagation. It was clear from results that the cultivar TMS87100609 was superior to the other cassava genotypes in plantlet propagated from nodal explants. The tobacco cultivar also responded well on the MS-medium used. Despite being cultured on half medium strength, tobacco nodes developed properly into healthy plantlets with excellent root and shoot systems.

Calli was initiated from the leaves of the cassava cultivars (TMS87100609 and TMS63397) and tobacco on MS-media supplemented with 8mg/l 2,4-D. It took up to 3 weeks to induce calli in the cassava genotypes, whilst tobacco callus was initiated within the second week of culture. Cabral et al (1992a)under the same incubation conditions initiated embryogenic calli from immature cassava leaves

reactions involving blunt-ended vectors such as the examples attempted in this experiment, have been found to inefficient (Sambrook et al, 1989). The reason why the ACI ACMV-SA insert and pBIN19 and pBI121 vectors could not be ligated was because the calculated pmoles of ends for both insert and vector may not have been in correct equimolar concentrations. It is difficult know the exact concentration of each DNA species in a ligation reaction considering the fact that these DNAs are usually purified by gel electrophoresis and are present in such small quantities that only rough estimations of their concentrations are possible. Since the insert and vectors were subjected to various processing protocols prior to the ligation reaction, the likelihood of damage to the termini of some of the DNA molecules cannot be overlooked. The behaviour of the T_4DNA ligase in cloning is very crucial . Although in this experiment, the enzyme seemed funtional as shown by results of the control reactions, it can be fastidious at times. The cloning carried out in this experiment involved blunt-ended DNA fragments, hence the concentration, pH and activity of the enzyme and enzyme buffer cannot be ruled out, as possible contributory factors to the failure of the ligation process. Another possible reason may be that the partial filling of the ACI fragment using the Klenow fragment of E coli DNA polymerase 1, did not work, and self-ligation may have occurred. Other likely reasons may have been the presence of competitor molecules such as some unfiltered remains of the dNTPs left after the partial filling in process. Plant transformation vectors pBIN19 (10.2Kb) and pBI121 (11kb) themselves may be difficult vectors to clone into. They are rather large vectors and their sizes vis-a-vis the size of the insert (607bp ACI ACMV-SA) may also account for the reason why the cloning between insert and vectors did not succeed.

The 371bpACI sequence comparisons of ACMV-SA with other geminiviruses clearly indicates a much closer relatedness of ACMV-SA with the ACMVs from Nigeria than that from Kenya. However, it did also share some unique resemblances with TYLCVs from Italy (i.e. TYLCV-Sar and TYLCV-Sic) and ToLCV from Australia. ACI proteins are the only viral encoded proteins for the replication of the geminivirus genome (Elmer et al, 1988). A high sequence homology in this region depicts some similarities in replicative structures and roles. It has previously been reported that

5. DISCUSSION

The restriction map was constructed with the view of identifying convenient restriction sites for future subcloning, infectivity and sequencing experiments. To produce full length copies of the genomic DNA-A suitable for infectivity investigation it is necessary to use a single cutting restriction enzyme that allows cloning into appropriate vector without damaging the cloning site (Stanley and Gay, 1983). Suitable enzymes for this purpose in ACMV-SA DNA-A are *Apa*1, *EcoR*1, *Bgl*II, *BamH*1 and *Pst*1. The restriction map showed that there are no convenient restriction sites for cloning the entire ACI ORF gene (Fig 6) which is about 1.1kb in length. However, a large portion of it which is about 0.742kb in size could be subcloned as *Pst*1 and *EcoR*1 fragment. *Bgl II* and *Apa I* could be used for subcloning a fragment that will consist of part of AC2-ORF and a large region of AC1-ORF. *Nco*1 could also be used to generate three fragments of approximate sizes 1.37kb, 0.85kb and 0.43kb for subcloning. *Mlu*I is another enzyme that would be suitable for subcloning. It would cut two fragments, one of which would cover about 0.6kb of the AVI - coat protein region (1.0kb), the common region and part of the ACI-ORF. The other fragment would cover a large portion of the ACI-ORF area, the whole of the AC2 and part of AC3-ORFs.

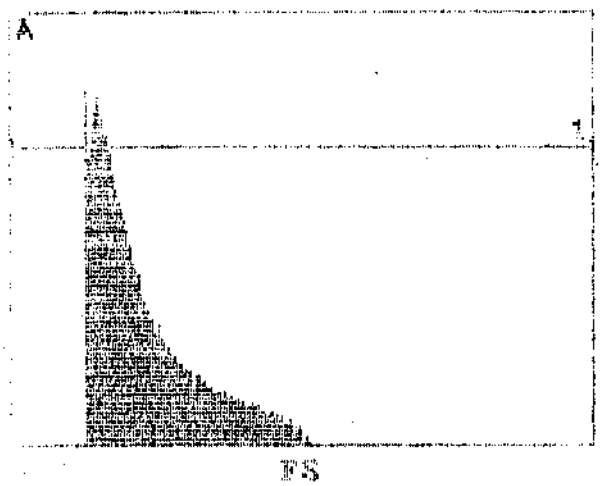
The fact that the transformation control (uncut plasmids) procedure did work implies the possible cause of not obtaining any clones of the 607bp ACI fragment in pBIN19 and pBI 121 could be attributed to the failure of the ligation step. Theoretically, molecular cloning of plasmid vectors is straightforward, however in practice, some procedural steps like ligation are difficult and not easily obtainable. The main factors that affect ligation are ATP concentration, concentration of DNA termini, concentration, pH and activity of ligase, and presence in the ligation mixture of contaminants, e.g. salts, dNTPs, etc. Concentration of ATP alters the ability of T_4DNA ligase to ligate DNA-fragments. A high ATP concentration normally inhibits blunt-ended ligation but favours the ligation of protruding ended fragments (Firman, 1991). Usually the easiest DNA fragments to clone are those that carry complementary protruding termini generated by the digestion of the vectors and/or inserts with the same restriction enzyme. Conversely, ligation

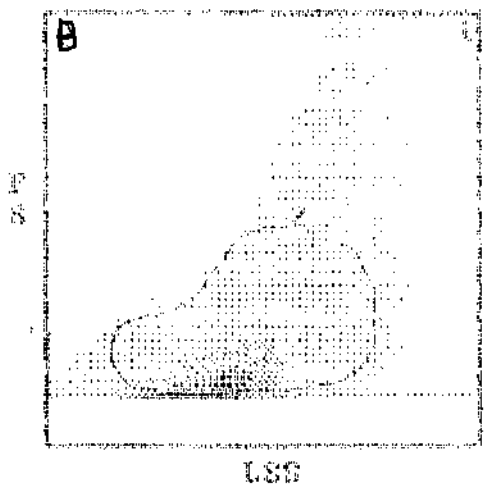
Table 5 demonstrates the flow cytometry counts of the suspension cells in the two cassava cultivars (i.e. TMS87100609 and TMS63397) and tobacco. Fig 25A illustratesthe appearance of populations of TMS87100609 cassava cells in a volume of 100µl cell culture, while Fig 25B, shows the total cell count (129330) of all the cells that is within that volume. The bitmap enclosed by the area marked 2 also in Fig 25B, represents the major size class of cells (77899) in the population.

The total number of suspension cells counted by the flow cytometer f the cassava cultivars and the tobacco after 14 and 21 days of culturing were in most cases not equal to that obtained from the haemocytometer counts. With the exception of two of the TMS63397 cultures (samples 5and 8), a higher flow count was obtained in all the other cultures. The suspension cells varied in sizes and shapes from oval, flat banana-shaped as well as circular shapes. Some of the cells were found clumped together (Figs 23 and 24).

4.5.4 Protoplast Isolation

The enzyme mixture of 0.1% pectolyase Y23 and 2% cellulase Onozuka R10 liberated protoplasts from tobacco suspension cells after 5h of incubation whereas in both cassava cultivars, protoplasts were released after 7- 8h of culturing. Protoplasts yields from cassava cultivars TMS 63397 and TMS87100609 were 2.0×10^5 and 3.5×10^5/ml respectively, while tobacco protoplasts yield was 2.8×10^5/ ml .





MIN	MAX	COUNT	PERCENT	MEAN	SD	%MEAN
0	255	77899	100.0	58.0	33.2	29.7

		MIN	MAX	COUNT	PERCENT	MEAN	SD	%MEAN
1	X	1.024	1023.	129330	100.0	28.52	42.77	126.7
	Y	0	63			19.5	16.1	8.57
2	X	1.757	1.955	0	0.0			
	Y	45	45					
3	X	1.757	1.955	0	0.0			
	Y	30	30					
4	X	1.757	1.955	76	0.0			
	Y	15	15					

Figure 25: Flow Cytometer Counts of A Population of Cassava (TMS87100609) Cell Suspension Culture. A depicts the appearance of the entire cell population. B depicts the distribution and total counts (129330) of all the cells in the population. The bitmap enclosed by area 2 in B depicts a count (77899) of a selection of cells in the population.

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