

Characterisation of a subgenomic molecule associated with *South African cassava mosaic virus*

Natasha Abraham

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Declaration

I declare that this research report entitled “Characterization of a subgenomic DNA derived from DNA-B of South African cassava mosaic virus” is my own, unaided work. It is being submitted for the degree of Masters of Science at the University of Witwatersrand, Johannesburg. It has not been submitted for any other degree at any other University.

A handwritten signature in dark ink, reading "Natasha Abraham". The signature is written in a cursive style with a horizontal line underlining the name.

Natasha Abraham

23rd of October 2012

Abstract

Cassava (*Manihot esculenta*) is a major food crop in sub-Saharan Africa, where cassava is mostly used as a subsistence crop. In southern Africa, cassava production is affected by cassava mosaic disease (CMD). The impact of CMD on cassava yield has a devastating impact on the economy in southern Africa as it is also an important industrial crop. *South African cassava mosaic virus* (SACMV) is a distinct geminivirus known to cause CMD in Southern Africa. SACMV is a bipartite virus where the two components DNA-A (2800bp) and DNA-B (2760bp) code for important proteins needed for viral replication, movement and transmission. Defective interfering molecules are a type of subgenomic molecule associated with geminiviruses. DIs are dependent on the helper virus for replication. DIs are known to lead to symptom amelioration in plants infected with its cognate helper virus as a result of interfering with the helper virus replication in the plants. It is believed that they interfere with the replication, by competing for limited host and viral factors needed for replication by the helper virus. They also have a size-advantage to be selected over the helper virus, allowing for increased DI proliferation.

A putative defective interfering (DI) molecule, isolated from a naturally infected field cassava, has been associated with SACMV. This DI molecule is derived from the DNA-B component of SACMV and is 1389bp in size, approximately half the size of DNA-B. In this study, the objectives were to investigate the effect of DI on SACMV replication and symptom development in a model host – *N. benthamiana* and cassava, the natural host of SACMV, in order to determine whether the DI was indeed an interfering molecule. Viral load (DNA-A and DNA-B) were determined using quantitative real-time PCR, and symptoms were scored according to a symptom severity index (1-5). The results from this study show that the DI did influence viral titres in *N. benthamiana* and cassava when infected with SACMV. However, the impact of DI on reducing viral replication and symptom attenuation was different in the two plant systems. In cassava, the symptom attenuation was more pronounced compared with *N. benthamiana*, which correlates to the respective viral titres, thus highlighting the differences in viral-host factor interactions during viral replication between a model host (*Nicotiana benthamiana*) and a natural host (cassava). It was also observed that the DI had an impact on viral replication and symptom attenuation, when it was present with SACMV- from onset of infection, but titres of DNA A and DNA B showed a

cyclic pattern of increases and decreases during the infection process. A surprising observation made from this study was that either the presence of DI or reduced titres of DNA-B, due to interference by the DI, had a direct effect on the ability of the helper virus SACMV to develop a specific chlorotic symptom phenotype in infected leaves, suggesting that DNA B plays an important role in symptom development.

Additionally, transgenic *N. benthamiana* transformed with a DI insert, which only replicates in the presence of the SACMV, was tested as a resistance strategy. Again, quantitative real-time PCR was used to determine viral load and symptoms were scored according to the symptom severity index. Unexpectedly, our results showed that neither decreased viral load nor attenuated symptoms were observed when transgenic *N. benthamiana* was infected with SAMV, deeming the use of DI as a transgenic resistance approach non-viable. This suggests that although DI does decrease viral titres and lead to symptom attenuation during natural systemic infections or experimental inoculations, these DIs don't hold potential in combating SACMV and CMD in a transgenic system.

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Research Output

- Reviews:
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List of Abbreviations

α -DNA	alphasatellites
aa	amino acids
AbMV	<i>Abutilon mosaic virus</i>
ACMV	<i>African cassava mosaic virus</i>
AS	acetosyringone
AYVV	<i>Ageratum yellow vein virus</i>
BAP	Benzylaminopurine
°C	Celsius
CLCuD	cotton leaf curl disease
CMD	cassava mosaic disease
CMV	cassava mosaic virus
CMV	cucumber mosaic virus
CPGMV	<i>Cowpea golden mosaic virus</i>
CPMR	coat-protein mediated resistance
CR	common region
DI	defective interfering molecules
def	defective interfering
dMP	dysfunctional movement proteins
DNA β	betasatellites
dpi	days post inoculation
ds	double stranded
EACMV	<i>East African cassava mosaic virus</i>
EACMCV	<i>East African cassava mosaic Cameroon virus</i>
EACMKV	<i>East African cassava mosaic Kenya virus</i>
EACMMV	<i>East Africa cassava mosaic Malawi virus</i>

FAO	Food and Agricultural Organisation of the United Nations
g	grams
<i>g</i>	Gravitational force
GFP	green fluorescent protein
GUS	beta-glucoronidase
HR	hypersensitive response
ICMV	<i>Indian cassava mosaic virus</i>
IPTG	isopropyl-beta-D-thiogalactopyranoside
IR	intergenic region
KOH	potassium hydroxide
MES	2- (N-morpholino) ethanesulfonic acid
MP	movement protein
NAA	α -Naphthalene acetic acid
NW	New World
NSP	nuclear shuttle protein
OW	Old World
O.D. ₆₀₀	Optical Density measured at 600nm
PCR	polymerase chain reaction
PDR	pathogen derived resistance
PIPES	1, 4-piperazinediethanesulfonic acid
PTGS	post transcriptional gene silencing
PVPP	polyvinylpolypyrrolidone
PYMV	<i>Potato yellow mosaic virus</i>
RCA	rolling circle amplification
RCR	rolling circle replication
Rep	Replication associated protein
RF	replicative form
RNAi	RNA interference

rpm	revolutions per minute
SACMV	<i>South African cassava mosaic virus</i>
SEL	size exclusion limit
siRNA	small interfering RNA molecules
SCR	satellite conserved region
SLCMV	<i>Sri Lankan cassava mosaic virus</i>
SLCV	<i>Squash leaf curl virus</i>
ss	single strand
TbLCZ	<i>N. benthamiana leaf curl Zimbabwe virus</i>
TGMV	<i>Tomato golden mosaic virus</i>
TLCV	<i>N. benthamiana leaf curl virus</i>
TMV	<i>N. benthamiana mosaic virus</i>
TNA	total nucleic acids
ToLCV	<i>Tomato leaf curl virus</i>
TYLCV	<i>Tomato yellow leaf curl virus</i>
wpi	weeks post inoculation

Rationale for study

Cassava (*Manihot esculenta*) is a versatile, drought and insect resistant plant that is grown throughout the world for its starchy tuberous roots. In sub Saharan, such as Swaziland, Mozambique, Angola, Botswana, Zimbabwe and Namibia, cassava is mainly grown as a subsistence crop as it provides food security during difficult droughts and famines. In South Africa, it is mostly grown as a cash crop for industrial purposes in regions of Mpumalanga, Northern Province and Northern KwaZulu Natal. The starch harvested from the cassava is used in different forms in various industries; mainly in the animal feed, paper, textile, food and flour industries. In 2007, Africa contributed to 49% of the world cassava production and cassava was the number one commodity produced on the continent; thus highlighting the enormous impact this crop has on the overall economy of the continent. Cassava provides financial security to small-scale farmers and also creates industrial opportunities. In 2005, the gross income earned from cassava starch production was estimated at R27million in South Africa alone.

Despite its robustness, cassava is susceptible to wide array of diseases caused by bacteria, fungi and viruses. In Africa, the biggest constraint of cassava production is the cassava mosaic disease (CMD) caused by a group of whitefly-transmitted begomoviruses, belonging to the family *Geminiviridae*. Geminiviruses are circular ssDNA viruses of approximately 2.5-2.9 kb, that are either monopartite (a single component DNA-A) or bipartite (components DNA-A and DNA-B). This disease causes the leaves of the plant to distort, blister, shrink and exhibit irregular yellow chlorotic mosaic. There are several different species of cassava mosaic virus affecting cassava production in many sub-Saharan African countries. *South African cassava mosaic virus* (SACMV) is a known CMD causative agent in South Africa. SACMV has a bipartite genome and is transmitted by the whitefly *Bemisia tabaci*. The DNA-A component encodes for several proteins associated with replication, encapsidation and transmission; while the DNA-B component codes for proteins responsible for the systemic movement of the virus through the plant.

One of the contributing factors of the spread of CMD is the epidemic of rising population of its vector, *Bemisia tabaci* and its vegetative propagation. The spread of the disease is further

compounded by the lack of proper phytosanitation by farmers such as the use of infected farm tools and use of infected plant material to plant new fields. Due to limited access and success with chemicals, traditional methods still persists such as crop rotation and use of resistant varieties; but even these seem to be failing.

Genetic engineering is a strategy to combat the problem of crop loss due to pathogens. South Africa is one such country that has implemented genetic engineering of crops and the commercialisation of these genetically-modified crops. Worldwide, research groups have been able to successfully produce transgenic cassava using different strategies against cassava mosaic disease: pathogen-derived resistance using viral movement or coat proteins; and strategies without protein expression such as gene silencing and antisense RNA. Another strategy is 'DNA interference', which involves the use of subgenomic DNA molecules associated with the parent helper virus.

Defective interfering (DIs) DNA molecules are one such type of subgenomic DNA. They are usually derived from the parent helper virus and are generally formed as a result of deletions in the viral genome. In geminiviruses, they are typically derived from the DNA-B component and are approximately half the size of this component. It was discovered that the parent virus replication was substantially decreased in the presence of these defective interfering molecules as a possible result of the DIs competing with the helper virus for essential host factors needed for replication. John Stanley *et al.* (1990) proved that *N. benthamiana* transformed with a tandem repeat of *African cassava mosaic virus* (ACMV) defective interfering DNA showed reduced symptoms compared to wildtype.

A putative defective interfering (DI) molecule has been associated with SACMV. It is half the size of the helper genome (1387bp) and has a 98% sequence homology to the DNA-B component of SACMV. The defective DNA has retained the common region, found on both the DNA-A and DNA-B components of SACMV, containing the origin of replication and the necessary *cis* elements required for replication. The DI also contains part of the BC1 open reading frame found of SACMV DNA-B.

Cassava mosaic disease is endemic in sub-Saharan Africa, including South Africa, and is a threat to cassava commercialisation due to huge yield loss. DIs can potentially be used to reduce virus replication and reduce yield loss. The main objective of the study is to elucidate

the interaction of SACMV with its associated subgenomic DNA molecule; does this subgenomic DNA lead to symptom amelioration by interfering with the replication and movement of its of its helper virus , as demonstrated by many other described Geminivirus DIs?

The specific aims are:

- i. To determine whether the presence of putative defective interfering DNA molecules upon co-infection with SACMV leads to symptom amelioration
- ii. To determine whether the helper virus load decreases if co-infected with the putative defective interfering DNA at different stages of infection in *Nicotiana benthamiana* (model host) and cassava (natural host).
- iii. Whether the presence of DI insert in transgenic *Nicotiana benthamiana* provides resistance to the plant against SACMV infection and leads to symptom amelioration

Chapter 1: Literature Review

1.1. Cassava

Cassava (*Manihot esculenta*) is a perennial woody shrub of the *Euphorbiaceae* family and is considered to be one of the most important crops of the world (Nassar 2001). It is the world's 4th most important basic food source after rice, wheat and maize (Scott *et al.* 2000) and is Africa's second most important food staple in terms of per capita calories consumed (Nweke *et al.*, 2002). It is believed that cassava originated from South America and was introduced in Africa by the Portuguese in the 16th century; and further made its way into Asia by the 18th century (Fauquet and Fargette 1990). Cassava is known by several names around the world such as tapioca, manioc, yucca and kassav (Tonukari 2004) and is used to prepare several different dishes such as Manicuera in the Amazon, Oyek in Indonesia and Lafun in Nigeria (Balagopalan 2002). Cassava is mostly grown for its edible roots but in many parts of the world the leaves are also consumed (Bokanga 1994).

Cassava produces starchy, tuberous roots of which 30-40% are made up of dry matter. Analysis show that 85% of the dry matter consists mainly of starch (amylase and amylopectin) and other soluble carbohydrates (Teles *et al.* 1993). The roots are a poor source of other nutrients but the leaves are a rich source of vitamin A and C; and are also known to contain high levels of proteins (Bokanga 1994) (Figure 1.1).

Uncooked cassava roots and leaves are not fit for human consumption as they contain toxic levels of cyanogenic compounds (Conn 1979). Cassava roots and leaves contain the cyanogenic glycosides, linamarin (95%) and lotaustralin (5%). These compounds naturally protect cassava from herbivory by insects, animals and theft (Nweke *et al.* 2002). It was found that typically in cassava leaves there are approximately 200-1300 mg cyanide (CN) equivalents per kg of dry matter and cassava roots have a lower 10-500mg CN equivalents per kg of dry matter. The recommended allowance by the FAO is 10mg CN per kg of dry matter (Siritunga and Sayre 2004). The concentration of cyanide in cassava depends on various factors such as cultivar, environment and rainfall (Bokanga 1994; Cardoso *et al.* 2005).

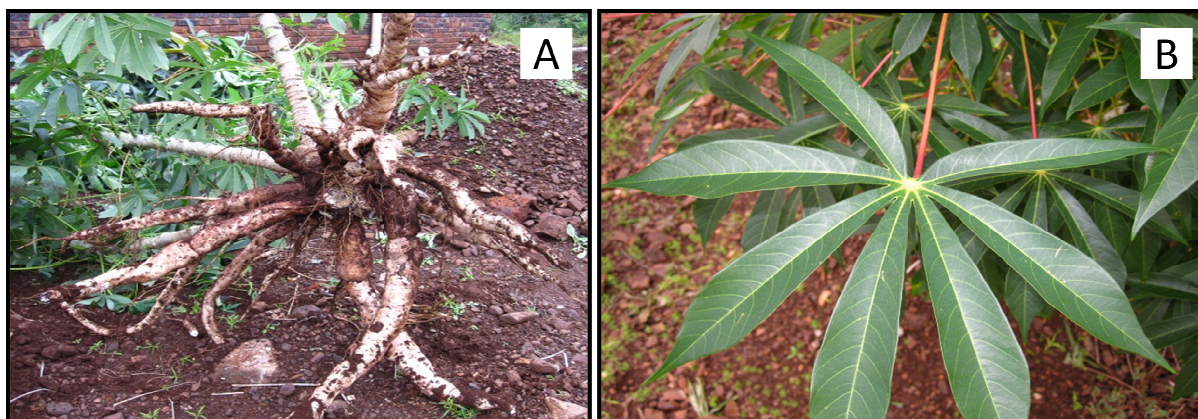


Figure 1:1. Freshly harvested cassava roots (A) and a healthy cassava leaf (B) from a farm in Mpumalanga province.

There have been a number of cyanide-related diseases and deaths, especially in nutritionally compromised individuals (Delange *et al.* 1994). Exposure to low levels of cyanide over a long term from poorly processed cassava has been shown to lead to goiter, tropical ataxic neuropathy (TAN) and cretinism (Osuntokun 1968) whilst acute exposure to high-levels of cyanide has been shown to lead to Konzo or in some cases death (Howlett 1994). This highlights the importance of proper preparation of cassava and its leaves.

Traditionally, in West Africa cassava is prepared by pounding and/or boiling which leads to a significant decrease or complete removal of the cyanogenic compounds (Ngudi *et al.* 2003). Any method that uses grating or crushing is considered to be highly effective as it causes intimate contact between the linamarin and the hydrolyzing enzyme linamarase which causes the breakdown of linamarin to hydrogen cyanide gas, that subsequently escapes into the air (Cardoso *et al.* 2005). Fermentation is also used as a method to remove cyanide in certain parts of the world, for example the cassava is fermented and then fried in Nigeria (Siritunga and Sayre 2004). In Brazil, roasting and frying are the most popular methods (Montagnac *et al.* 2009).

Apart from being used as a food source, its starchy tuberous root is also used for several commercial purposes (Kawano 2003). The cassava plant is used in the animal feed industry as a source of carbohydrates (roots), usually in the form of pellets or chips, and protein (stems and leaves) (Balagopalan 2002). Starch extracted from cassava is used in the production of several food items such as sago, alcohol, dextrose, maltose, fructose, citric acid and lactic acid (Balagopalan 2002). Cassava is a stable ingredient in the paper, textile

and foundry industry (FAO 2010) and is also used for the production of adhesives (Balagopalan 2002).

In South Africa, cassava is mainly grown for commercial purposes in the provinces of Mpumalanga, KwaZulu-Natal and Limpopo; and many neighbouring countries such as Swaziland and Mozambique (Daphne 1980). It is also grown for food security by farmers in these regions as a secondary staple food to maize (Mathews 2000). Recently there has been a renewed interest in cassava starch, not only in South Africa, but also in many tropical and sub-tropical countries, as a source for bioethanol.

The diverse use of cassava in commercial industry makes it an important cash crop for small scale farmers, along with the fact that cassava is such a versatile crop that is resilient to extreme conditions such as drought and poor quality soils with high saline and pH conditions (Asher *et al.* 1980; Okigbo 1980).

Despite the ability of cassava to survive adverse growing conditions and herbivory, world-wide production of cassava is affected by various diseases and pests which lead to great economic and production losses. The most important pests affecting cassava in Africa are cassava mealybug and green spider mites (Hahn *et al.* 1979). In South America, the leading disease of cassava is bacterial blight (CBB). This disease caused by *Xanthomonas campestris* and is considered the most important bacterial disease of cassava. Symptoms include angular leaf spots and cankers along the stem. Bacterial blight causes significant yield loss shadowed only by cassava mosaic disease caused by cassava mosaic viruses (Hillocks and Wydra 2002).

1.2. Cassava Mosaic Disease

Cassava mosaic disease (CMD) caused by geminiviruses is predominant in the Indian subcontinent and Africa. It is the primary biological constraint affecting cassava growth in Africa (Legg and Fauquet 2004). This disease is so devastating that it is termed 'the most damaging plant virus' in the world (Legg *et al.* 2006) and is estimated to have an economic loss of between US \$ 1.9-2.7 billion dollars in recent years (Patil and Fauquet 2009). It has been reported that 17 out of 38 cassava producing countries are severely affected by CMD by Legg and Thresh (2003).

The characteristic symptoms of CMD include irregular yellow or yellow-green chlorotic mosaic of the leaves; stunting of the plant, leaf distortion and crumpling (Figure 1:2.). Plants suffering from severe CMD resemble a 'candlestick' like structure (Legg and Thresh 2003; Legg and Fauquet 2004). Most cultivars are susceptible to this disease which causes a 60 – 80 % decrease in yield (Bock and Woods 1983). CMD is caused by several species and isolates of begomoviruses belonging to the family *Geminiviridae* (Thresh *et al.* 1998). Cassava mosaic geminiviruses (CMGS) are transmitted by an insect vector – the whitefly (*Bemisia tabaci*) (Legg and Fauquet 2004). The current CMD pandemic is attributed to the super abundant population of *B.tabaci*; and the presence of mixed infections, caused by different strains of CMGs acting synergistically. Recombinant strains of CMGs were also found to be present in infected fields (Legg *et al.* 2006).



Figure 1:2. Cassava leaves infected with cassava mosaic disease.

Cassava leaves showing characteristic yellow chlorosis (A) and leaf distortion (B) typical of cassava mosaic disease (Ndunguru *et al* 2005).

1.2.1. Geminivirus classification

The *geminiviridae* family has been classified into 4 genera based upon their genome organisation, host range and insect vectors. They are *Mastrevirus*, *Topocuvirus*, *Curtovirus* and *Begomovirus* (Stanley *et al.* 2005).

Begomoviruses are classified as Old World (OW) or New World (NW) viruses based on geographical distribution, genome organisation and genetic diversity. OW begomoviruses are found in Europe, Asia, Australia and Africa and can be mono or bi-partite; while the NW begomoviruses are restricted to the Americas and are strictly bipartite (Nawaz-ul-rehman and Fauquet 2009). The OW viruses have a highly conserved gene (V2 in monopartites and AV2 in bipartites) that is absent in the NW viruses (Nawaz-ul-rehman *et al.* 2009). No NW monopartite begomovirus existed; that is until global trade recently introduced *tomato yellow leaf curl virus* (TYLCV), a monopartite begomovirus into United States (Nawaz-ul-rehman *et al.* 2009; Briddon *et al.* 2010).

Table 1:1. Different species of classified CMGs (Fauquet *et al.* 2008)

<i>Name of Virus</i>	<i>Assigned Abbreviation</i>	<i>Number of associated variants</i>
<i>African cassava mosaic virus</i>	ACMV	12
<i>East African cassava mosaic Cameroon virus</i>	EACMCV	6
<i>East African cassava mosaic Kenya virus</i>	EACMKV	14
<i>East African cassava mosaic Malawi virus</i>	EACMMV	2
<i>East African cassava mosaic virus</i>	EACMV	56
<i>East African cassava mosaic Zanzibar virus</i>	EACMZV	13
<i>Indian cassava mosaic virus</i>	ICMV	6
<i>South African cassava mosaic virus</i>	SACMV	3
<i>Sri Lankan cassava mosaic virus</i>	SLCMV	11

This table demonstrates 9 different species of CMGs and their associated variants. A mixed infection between different species, such as EACMV-UG and ACMV, has been reported to cause severe symptoms in the field leading to catastrophic damages (Legg *et al.* 2006)

CMGs are classified according to the method recommended by Fauquet et. al (2000). Species are classified according to 89 % pairwise similarity between the DNA-A sequences. Within a species, a pairwise identity of 85 - 94 % corresponds to a strain. A 92 - 100 % pairwise identity between two sequences corresponds to a variant (Fauquet *et al.* 2008). According to this classification system, 7 different species of CMGs has been identified in sub-Saharan Africa (Table 1.1.).

1.2.2. Geminivirus Genome Organisation

Amongst the four groups of *Geminiviridae*, the genus *Begomovirus* has the most complex genome organisation (Yadava *et al.* 2010). The majority of the begomoviruses are bipartite i.e. they comprise of encapsidated geminate particles DNA-A and DNA-B; whilst some are monopartite (DNA-A only) such as TYLCV (Gutierrez 2000). The bipartite components are similar in size, approximately 2.7 - 3.0 kb, but have different sequences except for the common region (CR) (Lazarowitz *et al.* 1992). The interaction between DNA-A and DNA-B of bipartite geminiviruses are highly specific (Y Zafar *et al.* 2006). The CR is about 200 – 250 bp long and is almost identical between two components of a virus, but can differ between viruses (Bisaro 2006). The CR is part of a larger intergenic region (IR), which also contains the origin of replication (*ori*) (a conserved nonanucleotide sequence of TAATATTAC in a hairpin stem loop), promoters for transcription and other regulatory sequences (Hanley-bowdoin *et al.* 1999; Pilartz and Jeske 2003; Yadava *et al.* 2010).

The DNA-A component is 2800 nucleotides in length and has 6 open reading frames (ORF): AV1 and AV2 found on the viral sense strand while AC1, AC2, AC3 and AC4 are found on the complementary strand. These proteins can be categorized into 3 groups (Stanley *et al.* 1986; Hanley-Bowdoin *et al.* 1999; Yadava *et al.* 2010):

- **DNA- replication initiation proteins:**
 - AC1 encoded replication initiator (**Rep**) 354amino acids (aa)
 - AC3 encoded replication enhancer protein (**REn**) 134 aa
- **Host gene regulator and silencing suppressor proteins:**
 - AC2 encoded transcription activator (**TrAP**) 135 aa
 - AC4 (undetermined function) 98 aa
 - AV2 encoded pre-coat protein 116 aa

- **Viral assembly protein :**
 - AV1 encoded coat protein (**CP**) 258 aa

The DNA-B component has 2 ORFs on the sense strand of which BV1 encodes for a nuclear shuttle protein (**NSP**; 258 aa). BC1 on the anti-sense strand codes for a long distance movement protein (**MP**; 307 aa) (Stanley *et al.* 1986; Yadava *et al.* 2010). The NSP and MP are intra and inter-cellular viral movement proteins respectively.

1.2.3. South African Cassava Mosaic Virus

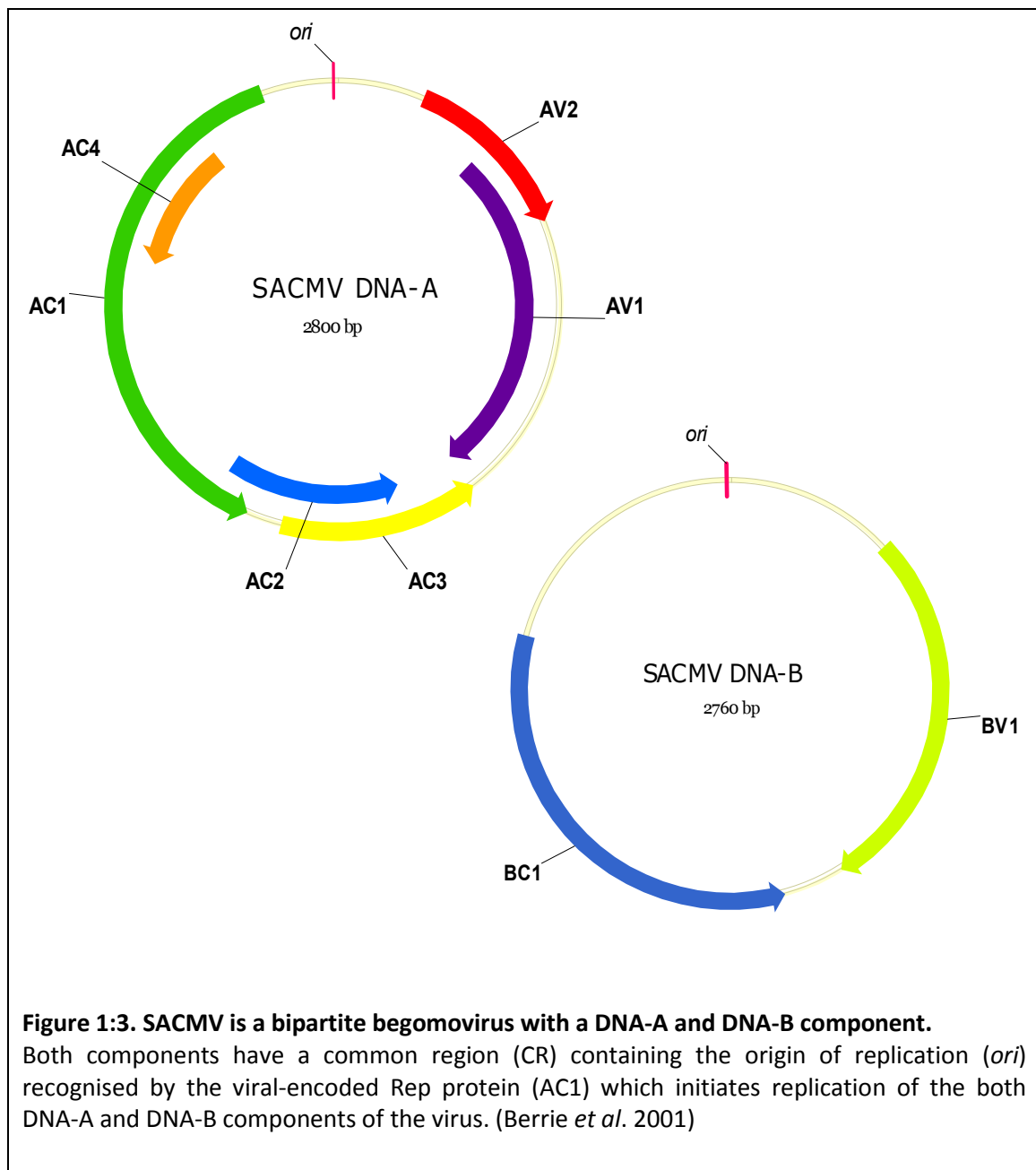
South African cassava mosaic virus (SACMV) was identified as a distinct species in South Africa based on differences in its coat protein (CP) and common region (CR) nucleotide sequences (Berrie *et al.* 1997, 1998). Sequence analysis indicates that the CP and CR of the SACMV are most closely related to the monopartite virus TYLCV. When compared to other bipartite geminiviruses, SACMV is most closely related to EACMV (Berrie *et al.* 2001). The DNA-A component of SACMV is most closely related to EACMV type II isolates from Malawi, while the DNA-B component is most closely related to EACMV isolates from Uganda. The origin of the AC4 ORF in DNA-A has been untraceable hence appearing to be a hotspot for recombination. No recombination events have been determined in DNA-B of SACMV.

SACMV is a typical bipartite begomovirus - composed of DNA-A (6 ORFs) and DNA-B components (2 ORFs) (Figure 1:3) and is transmitted by whiteflies. The DNA-A component of SACMV is 2800 bp and the DNA-B is 2760 bp. The two components have a shared sequence similarity in the 176 bp CR with only 9 nucleotides difference in DNA-A and DNA-B (Berrie *et al.* 2001).

Apart from South Africa, SACMV has been found in infected plants as far as Madagascar, Swaziland and Zimbabwe. Three variants of SACMV exists – *South African cassava mosaic virus-[Madagascar: 12]* (SACMV-[MG: 12]), *South African cassava mosaic virus-[South Africa]* (SACMV-[ZA]) and *South African cassava mosaic virus [Zimbabwe:Muzarabani]* (SACMV-[ZW:Muz]) (Fauquet *et al.* 2008). Infectivity studies indicate that SACMV is able to successfully infect the species *Phaseolus vulgaris*, *Malva parviflora* and *Manihot esculenta* Crantz.

1.2.4. Replication of geminiviruses

Begomoviruses DNA-A and DNA-B both replicate by a rolling circle replication (RCR) mechanism. It is found that only DNA-A is capable of replicating independently, without DNA-B; DNA-B is not able to replicate in the absence of DNA-A. The genome encodes only for a few proteins and depends on essential host factors for their replication (Stanley *et al.* 1986). The replication cycle makes use of double stranded (ds) DNA intermediates and occurs in the nucleus of the infected plant cell (Gutierrez 2002). Once the virus makes its way into the nucleus of the infected cell, the viral ssDNA is converted into intermediate dsDNA, also known as the replicate form (RF), by host cellular enzymes. Once this is achieved, the dsDNA is transcribed and translated to viral enzymes, like the Rep protein (Gutierrez 2000). The Rep protein is crucial for initiating RCR, as it recognises the CR containing the four highly sequence-specific Rep binding sites (iterons) (Yadava *et al.* 2010). More specifically, it recognizes the nonamer nucleotide *ori* and nicks the DNA between the seventh and eighth nucleotide (TAATATT↓AC) (Stanley 1991). The Rep protein is the only viral protein transcribed by the virus that is absolutely vital for replication (Gutierrez 2002). The Rep protein is known to interact with the REn protein; it is thought that Rep/REn oligomers are important, but not essential, for viral replication (Sunter and Bisaro 1992). REn is also thought to play a role in 'enhancing' viral infection and symptom development (Gutierrez 2002). TrAP is responsible for the activation of transcription of the late viral genes CP (AV1) and NSP (BV1) (Sunter and Bisaro 1992). TrAP is also implicated with suppressing plant RNA silencing mechanisms and the hypersensitive response (HR). The AC4 gene product's exact function is still unclear. Studies suggest that it is involved in modulating interactions between Rep and the host factors that can lead to systemic necrosis (van Wezel *et al.* 2002), determining host range, RNA silencing suppression and movement (Seals 2006). The AV2 gene product has been shown to be involved in virus accumulation and symptom development (Padidam *et al.* 1992).



DNA – B has 2 ORF's – BV1, on the sense strand, which codes for a nuclear shuttle protein (NSP). An ORF on the anti sense strand, BC1, which codes for a long distance movement protein (MP) (Yadava *et al.* 2010). The BV1 gene product, NSP, functions as a shuttle protein that transports viral DNA from the nucleus to the cytoplasm. NSP has been found to bind ssDNA, and move the DNA out of the nucleus (Hanley-Bowdoin *et al.* 1999). During early infection, NSP is mainly localized in the nucleus (Gutierrez 2002). The BC1 gene product, MP, is involved with the long distance movement of viral DNA. Thus, MP facilitates the spread of

viral DNA from cell to cell. MP forms endoplasmic reticulum-derived tubules which extend through the cell wall into adjacent cells. When NSP and MP are co-expressed late in the infection cycle, NSP (with bound viral DNA) is relocated to the periphery of the cell, where MP facilitates its movement across the cell wall (Ward *et al.* 1997; Gutierrez 2002). Various studies have shown that the DNA-B of component of bipartite begomoviruses have a role in symptom development (Patil and Dasgupta 2006).

1.3. Resistance to Geminiviruses

In recent years, there has been a significant increase in the number of diseases caused by geminiviruses. This has been linked to the emergence of new virus strains, insecticide resistance, global warming and population increase of insect vectors (Vanderschuren *et al.* 2007). Plant genetic engineering (PGE) has become an important tool in creating resistant crops against these epidemics (Birch 1997).

1.3.1. Pathogen derived resistance

There are several methods being employed to confer geminivirus resistance in crops. Resistance involving the expression of viral proteins, such as coat protein and movement protein in transgenic plants (Powell-Abel *et al.* 1986; Hong and Stanley 1996) is known as pathogen derived resistance (PDR) (Sanford and Johnson 1985; Goldbach 2003).

PDR using CP genes is known as coat-protein mediated resistance (CPMR) and has been successful for certain plants against specific viruses such as transgenic *N. benthamiana* resistant against *N. benthamiana* mosaic virus (TMV) (Powell-Abel *et al.* 1986; Marcel *et al.* 2008). Unfortunately, the CPMR is not a viable strategy for any geminiviruses to date (Shepherd *et al.* 2009). *N. benthamiana* plants transformed with ACMV CP were found to contain low levels of mRNA and CP and the plants remained susceptible to ACMV (Frischmuth and Stanley 1998). Nevertheless, it is believed that CP has the potential to be a good transgenic tool as they control specific interactions with the virus vector, *B. tabaci*. It was determined that a CP-deficient ACMV-KE clone has reduced functional interactions with whiteflies (Liu *et al.* 1997); thus rendering the possibility that the expression of a non-

functional CP could deter the interactions between the virus and its vector reducing the spread of the virus.

Rep, another viral encoded protein, has been shown to be effective against geminiviruses. This strategy uses full, truncated or mutated forms of the Rep gene (Dasgupta *et al.* 2003). Rep protein mediated resistance has been proved successful against CMGs; Hong and Stanley (1996) were able to prove this with transgenic *N. benthamiana* containing the ACMV Rep gene. When infected with ACMV, the plants were able to inhibit ACMV replication. Truncated Rep in transgenic tomato plants showed resistance to homologous virus by repressing the viral Rep promoter, but it also induced resistance against heterologous viruses by forming dysfunctional complexes with its Rep (Lucioli *et al.* 2003). A major drawback of expressing the Rep protein is the possibility of phenotypically abnormal plants due to the fact that the Rep protein interacts with pRBR or other host proteins which may alter the cell cycle and differentiation. The use of a mutated Rep gene reduces the risk of pRBR interaction. A dual strategy of using mutated Rep and REn proteins may confer a far more stable resistance to geminiviruses (Kong *et al.* 2000; Shepherd *et al.* 2009).

Movement proteins have been shown to be effective against a broader range of viruses. Dysfunctional MP (dMP) or mutated MP is generally used as they delay the onset of infection and appearance of symptoms. These MPs compete for NSP interaction or oligomerization. It was found that *Tomato golden mosaic virus* (TGMV) MP was able to confer resistance to an ACMV infection. Although TGMV MP cannot facilitate the movement of ACMV in plants and the MPs of the two viruses only share a 41 % aa sequence identity this suggests that these proteins can work with a wider range of viruses (Marcel *et al.* 2008; Shepherd *et al.* 2009). Overexpression of MP in transgenic plants can be toxic and are known pathogenicity determinants for bipartite begomoviruses, which can lead to undesired traits in the transgenic plants (Hou *et al.* 2000).

One of the important criteria for choosing the right gene for PDR is that it doesn't affect the normal host cell functions. The potential drawback of constitutively expressing a foreign protein in a plant is the formation of a phenotypically abnormal plant due of altered cell cycle or differentiation programs (Kong *et al.* 2000).

A similar strategy for resistance involves the expression of non-viral proteins such as dianthin (a ribosome-inactivating protein), which has antiviral effects against *African cassava mosaic virus* (ACMV) (Hong *et al.*, 1996). Certain studies have attempted to unsuccessfully use transgenic-expressed antibodies targeting geminivirus proteins. These antibodies have been found to only work against CP or RNA-dependent RNA polymerase (RdRP) in RNA viruses (Boonrod *et al.* 2004).

1.3.2. RNA interference

1.3.2.1. RNA silencing

RNA silencing is a process that suppresses the expression of certain genes through sequence specific interactions with RNA at the post transcriptional stage. RNA interference (RNAi) is known to occur naturally in plants, animals and fungi and can occur either through repressing the expression of a gene (usually induced by DNA methylation) or by mRNA degradation. The process of achieving gene silencing via mRNA degradation in plants is known as post transcriptional gene silencing (PTGS) and involves small interfering RNAs (siRNAs) (Hamilton and Baulcombe 1999; Mette *et al.* 2000; Shepherd *et al.* 2009). RNAi has evolved as a defence mechanism against viruses and transposable DNA elements and is also responsible for post-transcriptional and post-translation regulation of gene expression. It also maintains epigenetic regulation of chromatin structure and helps maintain genome integrity by suppressing activity of retrotransposable elements (Matzke and Koote 2001; Agarwal *et al.* 2003).

In transgenic plants, PTGS is triggered by the presence of artificial dsRNA homologous to viral sequences. These artificial dsRNA homologues are constructed by fusing the respective sequences in a sense and antisense orientation with an intron, which is eventually spliced out after post transcription, which creates a dsRNA hairpin structure (Wesley *et al.* 2001; Vanderschuren *et al.* 2007). Alternatively, PTGS can be triggered by the presence of transgenes in the sense/antisense orientation, where the expression of the transgene and the homologous viral gene are silenced simultaneously (Shepherd *et al.* 2009). Ultimately, siRNAs formed will lead to PTGS and often causes the targeted DNA region to be methylated (Vanderschuren *et al.* 2007).

Once PTGS is induced in the cell, it spreads locally and systemically. Short range silencing can be divided into the extensive or non-extensive types. The latter involves the signal spreading to neighbouring 10-15 cells; possibly through the plasmodesmata by siRNAs with no amplification of the signal. The former involves the spread of the signal beyond 10 - 15 cells, to the entire leaf, with signal amplification and secondary siRNA production (Kalantidis *et al.* 2008).

An advantage of PTGS-based resistance is that any coding or non-coding sequence can be targeted without having the need to express any proteins, therefore having fewer effects on plant development. A disadvantage of PTGS is that broad-range resistance is not possible since the sequences need to be homologous (Shepherd *et al.* 2009).

New studies have revealed the potential of microRNAs (miRNAs) in virus resistance. Unlike siRNAs which can be produced from both endogenous and exogenous RNA and are complementary to their sequence, miRNAs are only generated from endogenous mRNA and allow for mismatches with its targets. Qu *et al.* (2007) were able to use artificial miRNAs that targeted sequences encoding the silencing suppressor 2b of Cucumber mosaic virus (CMV) and were successfully able to confer resistance to CMV infection in transgenic *N. benthamiana*.

1.3.2.2. Antisense RNA

Antisense RNA (asRNA) molecules are complementary to a specific mRNA strand and upon binding; it prevents the mRNA from being translated. It may also produce siRNAs and induce PTGS (Asad *et al.* 2003). Suppression of gene expression by asRNA has been used as a resistance strategy to viruses. Vanderschuren *et al.* (2007) were able to successfully use the antisense strategy to produce transgenic cassava plants resistant to ACMV. The genes of Rep, TrAP and REn were inserted in the antisense orientation and upon infection with ACMV it was established that the replication of ACMV was reduced with increased resistance to ACMV. It was shown that resistance is best achieved when the asRNA works against viral mRNAs that code for essential non structural proteins. It has been reported that this method does not work with all viruses. Full-length and truncated antisense Rep failed to provide resistance to SACMV in *N. benthamiana* (Berrie 2000). This approach is only viable for viruses that are closely related to the virus from which the transgene was derived. Broad

range resistance is possible by having multiple sequences targeted using fused antisense RNA sequences (Shepherd *et al.* 2009).

1.4. Subgenomic molecules associated with geminiviruses

Often in plants infected with geminiviruses, one or more smaller DNA, referred to as subgenomic DNAs, are associated with the virus (Patil and Dasgupta 2006). They are found to be present naturally at low levels in geminivirus-infected plants (Stanley *et al.* 1990). These components are related to and dependent on its parents or “helper virus” for replication (Stanley *et al.* 1990). They are usually classified as satellites and defective interfering (DI) molecules (Figure 1:4).

1.4.1. Satellites

Satellites are defined as “viruses or nucleic acids that depend on a helper virus for their replication but lack extensive nucleotide sequence homology to the helper virus and are dispensable for its proliferation” (Mayo *et al.* 2005). Both RNA-associated and DNA virus-associated satellites have been described (Patil and Dasgupta 2006).

The first begomovirus satellite discovered was in tomato plants infected with *Tomato leaf curl virus* (TLCV), a monopartite begomovirus (Dry *et al.* 1997). Some monopartite geminiviruses have been reported to be usually associated with two subgenomic components in their natural host plant, namely beta – or alpha- satellites (R W Briddon *et al.* 2001).

DNA β , or betasatellites, are ssDNA approximately half the size of the helper virus (Nawaz-ul-rehman *et al.* 2009). DNA β s are dependent on the helper virus for their replication, encapsidation, cell-to-cell movement and insect transmission (R W Briddon *et al.* 2004; Nawaz-ul-rehman and Fauquet 2009). They share no sequence similarity to the helper virus except for the *ori* nonanucleotide (TAATATT↓AC) (Dry *et al.* 1997).

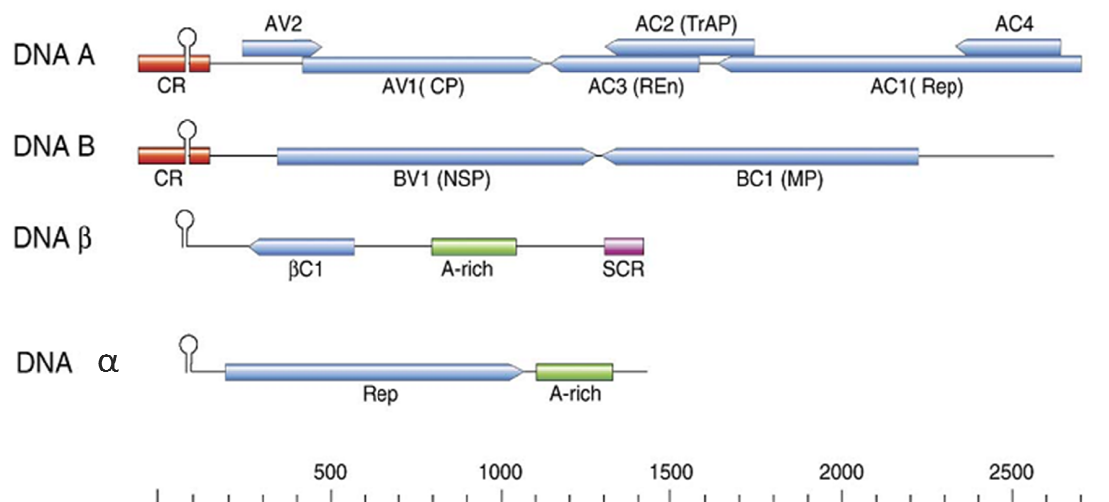


Figure 1:4. A linear map showing the similarities and dissimilarities between alpha- and beta- satellites and their cognate helper virus (R W Briddon and Stanley 2006).

The hairpin loop structure is present in all. The blue regions indicate ORF encoding for specific proteins. The purple region indicates the satellite conserved region (SCR) conserved amongst all DNAβs. The green region represents the adenine-rich region found in all DNAβ and α-DNAs.

DNAβs are classified according to their nucleotide sequence identity – a minimum of 78% pairwise similarity distinguishes a species from an isolate. Currently there are 51 different species of DNA satellites. Additionally, the DNAβ satellites are named according to the system in use for geminiviruses nomenclature (R W Briddon *et al.* 2007).

DNAβ's pathogenicity function was initially discovered associated with *Ageratum yellow vein virus* (AYVV), followed by begomovirus associated with cotton leaf curl disease (CLCuD) (R W Briddon and Stanley 2006). Most monopartite begomovirus seem to require betasatellite for symptom development; in its absence the helper virus are able to infect their natural hosts but induce none to mild or atypical symptoms with low viral titres. It is believed that the discovery of begomovirus associated with DNAβ will increase with time (Briddon *et al.* 2001; Shahid Mansoor *et al.* 2006; Zafar *et al.* 2006)

DNAβ have an adenine-rich sequence and also contain a highly conserved sequence amongst them, known as the satellite conserved region (SCR) of 220 nucleotides. Further analysis has shown that they are grouped according to both host and geographical origin (R W Briddon *et al.* 2003). DNAβs have a highly conserved structure with a single ORF βC1 (that

is also conserved in sequence and position) on the complementary sense for a 13kDa protein BetaC1.

DNA β s are associated with OW begomoviruses but have no associations with NW viruses (R W Briddon and Stanley 2006). Despite this, the origins of DNA β still remain unknown. Unlike DNA-A and DNA-B components of bipartite begomoviruses, the interaction between the helper virus and DNA β is not highly specific (Mansoor *et al.* 2006; Nawaz-ul-rehman and Fauquet 2009). Studies have shown the ability of DNA β to interact with other viruses apart from the parent virus for example AYVV DNA β was shown to interact with DNA-A of SLCMV to cause symptoms typical of yellow vein disease in the host (Saunders *et al.* 2002; Mansoor *et al.* 2006). Trans-replication by a different helper virus is not always possible though; *Cotton leaf curl Multan virus* (CLCuMuV) cannot replicate AYVV DNA β but AYVV can interact with CLCuMuV DNA β (Saunders *et al.* 2008). In an extensive study by Nawaz-ul-rehman *et al.* (2009), involving the trans-replication of two betasatellite species with a NW begomovirus, they were able to hypothesize the ability of betasatellite replication. Since betasatellites do not contain the iteron sequences recognised by Rep of their cognate helper virus and even if they do it is not in the specific arrangement as required for replication; their theory therefore involves the notion of a “universal Rep” or “universal iteron”.

Monopartite begomoviruses associated with DNA β have shown weak suppressor activity against plant RNAi on their own; because the majority of the RNAi suppressor is found on the β C1 (Mansoor *et al.* 2006). Another hypothesized function is that DNA β s may also help with the movement of DNA-A of bipartite begomoviruses in the absence of DNA-B (Saeed *et al.* 2007; Patil and Fauquet 2010). Patil and Fauquet (2010) have demonstrated that CMGs interactions with different betasatellites are diverse, indicating the varied ability of their Reps to bind to them. Phylogenetic analysis of the CMG Reps showed two separate groups of CMGs – ones that could interact with DNA β s and ones that couldn't.

Alphasatellites are “satellite-like molecules”, previously termed as DNA1, associated with betasatellites. As of yet, all begomovirus-DNA β complexes have been associated with an alphasatellite (R W Briddon *et al.* 2004). Like betasatellites, they are dependent on their helper virus for encapsidation, movement and insect transmission but unlike betasatellites, are capable of replication on their own (Nawaz-ul-rehman and Fauquet

2009). Alphasatellites are derived from nanoviruses; and their *ori* contains a conserved nonanucleotide sequence (TAGTATT↓AC) which is conserved amongst all nanoviruses (Briddon and Stanley 2006). The highly conserved alphasatellite genome transcribes a replication-associated protein (36kDa) and similar to DNAβs contain an A-rich region of 200 nucleotides (Briddon *et al.* 2004). The function of alphasatellites has not been elucidated; they are not required by the helper virus for neither the infection of the plant nor development of symptoms (Briddon *et al.* 2004). It has been suggested that perhaps alphasatellites reduce the viral titre in infected plants by competing for limited host factors, so as not to compromise plant-to-plant transmission (Briddon and Stanley 2006). Strong post-transcriptional gene silencing (PTGS) suppressor activity has been found in two alphasatellites (Nawaz-ul-rehman *et al.* 2010). Sequence analysis indicates that alphasatellites tend to loosely group based on geographical origins more than hosts. This also suggests that they are fairly mobile instead of being specifically adapted to a certain helper virus.

Recently, in a study by Paprotka *et al.* (2010), the first two alphasatellites were discovered in the New World – one associated with a Brazilian strain of *Euphorbia* mosaic virus and other with a newly discovered *Begomovirus* species, *Cleome leaf crumple virus*. Sequence analysis indicates that these alphasatellites are different from all other known OW satellites.

1.4.2. Defective Interfering Molecules

Defective interfering (DI) molecules are the third kind of subviral DNA. DIs have been associated with viruses from the families *Geminiviridae*, *Nanoviridae* and *Caulimoviridae* (Patil and Dasgupta 2006).

Stanley and Townsend (1985) first characterized the subgenomic forms associated with ACMV. It was determined that these molecules were derivatives of the DNA-B component and were half the size of DNA-B as a result of a deletion. Consequently, a number of subgenomic forms have been identified with other begomoviruses (Table 1:2). A DI associated with TGMV was also discovered in 1986 by MacDowell *et al.* DIs tend to form more readily in experimental hosts, such as *Nicotiana benthamiana* and *Arabidopsis thaliana*, than natural hosts like cassava. Some well known examples of DIs derived from

DNA-B are associated with *Potato yellow mosaic virus* (PYMV) (Roberts *et al.* 1988) *Cowpea golden mosaic virus* (CPGMV) (Winter *et al.* 1998) and *tomato golden mosaic virus* (TGMV) (MacDowell *et al.* 1986).

Sequence analyses of these DIs indicate that they form due to deletions duplication, inversion or rearrangements of the viral genome and its satellites, or as a result of insertion of foreign DNA (non-viral DNA sequences). They are encapsidated, just like their parent virus and play a role in the movement of the DIs in the plant (Patil and Dasgupta 2006). Some hypotheses suggest that they are formed by specific or non-specific cleavage of ssDNA during breakdown of geminate particles of isometric particles; or they are formed during the conversion of ssDNA to dsDNA during replication as a result of the polymerase “jumping” from one site of the template to another further away in the sequence due to the secondary structure alignment. DIs may also be formed by intra-molecular recombination in a dsDNA form of DNA-B (MacDowell *et al.* 1986; Patil and Dasgupta 2006).

DIs are associated with a delay in the onset and attenuation of symptoms. They have an inhibitory effect on the helper virus as they compete for essential host and viral factors and are thus known as defective interfering molecules (Patil and Dasgupta, 2006).

All known DIs have intergenic regions which contain the *cis*-acting elements and the conserved nonanucleotide sequence required for replication. However, DIs lack the full complement of genes necessary to replicate independently and are always associated with its helper virus. The DIs are maintained at approximately half the size of the cognate helper virus components to facilitate encapsidation and so that they may also be transmission by whiteflies (Patil and Dasgupta 2006).

It has been observed that most DIs are derived from DNA-B compared with DNA-A. Most DIs derived from DNA-A are reportedly only associated with monopartite begomoviruses. These DNA-A DIs contain the intergenic region with all the *cis*-acting sequences required for replication. Also present is the 5' portion of the *Rep* gene and occasionally a small part of the gene responsible for the coat protein (CP) (Patil and Dasgupta 2006). A def DNA-A has been associated with EACMV, a bipartite begomovirus, in an infected field plant. This is the first report of a bipartite begomovirus DNA-A associated DI. Sequence analysis revealed the

presence of the IR and part of the 5' AV1 gene and 80% of the AC1 gene. It has been termed 'df DNA-A 15'' and is most closely related to EACMV-UG2 (Ndunguru *et al.* 2006).

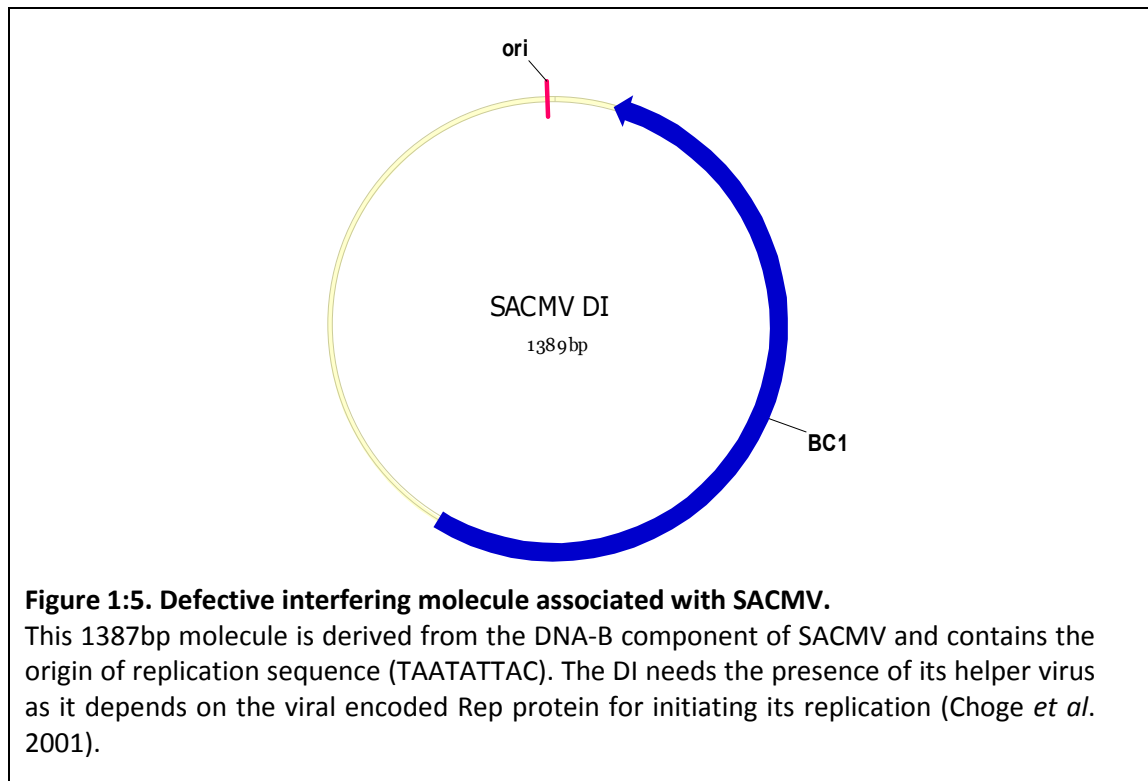
It is believed that the differences in the primary, secondary and tertiary structure between DNA-A and DNA-B may be the reason that there are fewer DIs derived from DNA-A compared to DNA-B (Sunter *et al.* 1985). Another explanation for this phenomenon is that DNA-A derived DIs may severely interfere with the replication of the helper virus hence being lethal and strongly selected against, whereas DIs derived from DNA-B delay the onset of symptoms; hence preventing the virus from killing the plant and consequently being selected for over a period of time (Patil and Dasgupta 2006).

DNA-B derived DIs usually contain part of the ORF coding for MP but the ORF coding for NSP is completely absent. This was the case with the DNA-B DI associated with ACMV characterized by Stanley and Townsend (1985) and SACMV derived DNA-B DI (Choge *et al.* 2001). The SACMV associated DI has a deletion from position 57 to 1429bp in the virion sense of SACMV DNA-B and the ORF BV1 is entirely deleted and the carboxylic terminus of gene BC1 is disrupted with only 82% of the BC1 gene intact (Figure 1:5). Studies have shown that the BC1 protein, and particularly the carboxylic terminus of the BC1 gene, is responsible for the induction of symptoms in bipartite viruses. Studies using transgenic plants expressing these proteins alone have shown to develop early full disease symptoms (Saunders *et al.* 2001). The truncated protein produced by the incomplete MP ORF may also have an effect on symptom amelioration (Patil and Dasgupta 2006).

DI molecules associated with AYVV influence the pattern of leaf chlorosis and leaf curling changing the symptom phenotype and causing a delay in the accumulation of helper virus in *N. benthamiana* (Stanley *et al.* 1997). This ability to interfere with the phenotype of leaf curling indicates that the DI molecules can alter the early stage development of a leaf rather than just interfere with the helper virus replication. This confirms the theory that these AYVV DIs may help to slow down the infection which otherwise would have resulted in the death of the plant, which in turn is lethal to the maintenance of the virus population (Stanley *et al.* 1997; Patil and Dasgupta 2006).

The exact mechanism by which DIs reduce the titre of helper virus in an infected cell is unknown; it is believed that the DI replication interferes with the helper virus replication at

the expense of both DNA-A and DNA-B components in the case of ACMV. The DI competes for the limited host factors essential for virus or DI replication; and also particularly competes for the virus encoded AC1 Rep protein.



1.4.3. Use of defective interfering molecules in engineering resistance

ACMV DNA-B associated DI has been shown to interfere with the replication of the ACMV. ACMV DIs has been efficiently used in a resistance strategy against ACMV infection in transgenic *Nicotiana benthamiana* containing tandem repeats of DNA-B derived DIs. The plants showed reduced symptoms compared to untransformed plants and were found to be specific against ACMV infection only (Stanley *et al.* 1990). The advantage of this method is that it does not rely on transcription or gene expression. The DI will be excised from the integrated template and replicated upon infection in the presence of the helper virus. However, this may only be effective against strains of a particular virus due to specific interactions during replication (Frischmuth and Stanley 1993). Transgenic *N. benthamiana* transformed with tandem repeats of the DI, showed reduced symptoms when infected with ACMV compared to wildtype infected *N. benthamiana* (Stanley *et al.* 1990).

Table 1:2. All known defective interfering molecules associated with begomoviruses (Chgoe *et al.* 2001; Ndunguru *et al.* 2006; Patil and Dasgupta 2006).

Geminivirus family	Virus	Defective DNA Sequence Composition	Size (kb)
Begomovirus (bipartite)	<i>African cassava mosaic virus</i> (ACMV B)	IR and part of BC1 ORF	1.29-1.36
	<i>Tomato golden mosaic virus</i> (TGMV B)	IR and part of BC1 ORF	1.24-1.36
	<i>Potato yellow mosaic virus</i> (PYMV B)	Unknown	Unknown
	<i>Cowpea golden mosaic virus</i> (CPGMV B)	IR and part of BC1 ORF	1.40
	<i>South African cassava mosaic virus</i> (SACMV B)	IR and part of BC1 ORF	1.37
	<i>East African cassava mosaic virus</i> (EACMV A)	IR; part of 5' part of AV1 gene and 80% of AC1 gene	1.52
Begomovirus (monoparite)	<i>Tomato yellow leaf curl virus</i> (TYLCV)	IR found in all DI associated with this virus	0.75-1.37
	<i>Ageratum yellow vein disease</i> (AYVV)	IR and 5' of C1 and V2	1.30-1.34
	Cotton leaf curl disease (CLCuD) - associated viruses	IR and part of AC1	0.25-1.40
	<i>N. benthamiana leaf curl virus</i> (TLCV)	IR and part of AC1 along with additional novel ORFs	1.36-1.42
	<i>N. benthamiana leaf curl Zimbabwe virus</i> (TbLCZ WV)	IR and part of C4, V1 and V2	1.34-1.42

Chapter 2. Effect of DI on SACMV replication in model host plant *Nicotiana benthamiana* and natural host, cassava

Abstract

Defective interfering molecules (DIs) with their associated helper geminivirus, have been shown to interfere with helper virus replication and ameliorate symptoms. In this study, an investigation was performed to determine whether a *South African cassava mosaic virus* (SACMV) DNA-B derived DI interfered with its cognate helper virus replication and/or symptom severity in the model host plant, *Nicotiana benthamiana* and the natural host cassava. A head-to-tail DI dimer was constructed to make an infectious clone, and transformed into competent *Agrobacterium* strain AGL1 cells for agroinfection. The infectious DI dimer was co-agroinfiltrated into *N. benthamiana* with SACMV DNA-A and DNA-B infectious clones (head-to-tail dimers) or SACMV was agroinfiltrated first, and 7 days post SACMV infection, the DI was agroinfiltrated. These were then compared to plants agroinfiltrated with SACMV only. Control plants were mock-inoculated with AGL1 strain containing plasmid with no infectious dimers (*Agrobacterium* control), and another control set of healthy *N. benthamiana* were uninoculated. A similar experiment was performed with cassava, but the SACMV infectious clones were agroinoculated by injection at the 6-8 leaf stage. Symptom severity was scored on a scale of 1-5 over a period of 28 dpi for *N. benthamiana*, and 42 days for cassava. Rolling circle amplification (RCA) and restriction with *PvuII* was performed on extracted TNA to detect DNA-B and DI. Leaf samples were collected at 7, 14, 21 and 28 dpi for *N. benthamiana*, and 14, 21, 28 and 42 dpi for cassava, for each experimental treatment, and total nucleic acids (TNA) were extracted for real time quantitative PCR to determine viral load (SACMV DNA-A and DNA-B).

Rolling circle amplification (RCA) confirmed the presence of replicating SACMV DNA-A and DNA-B. Viral load increased over time post-infection for all treatments, but despite a significant ($p < 0.0001$, 2-way ANOVA) reduction in DNA-A and DNA-B at 7 dpi, and less so at

14 dpi, in *N. benthamiana* co-infected with SACMV DI, compared to SACMV alone, symptoms persisted. At 28 dpi, DNA-A concentrations were significantly higher in the treatment with DI, compared with SACMV alone. SACMV DNA-B increased at 14 and 21 dpi when the DI was present, compared to SACMV alone, but decreased compared to control treatment (SACMV alone) at 28 dpi. In cassava, the viral titres (both DNA-A and DNA-B) were significantly lower at the early time-point 14 dpi and the late-point 42 dpi in plants co-infected with DI. Symptoms were found to ameliorated in plants co-infected with SACMV and DI compared to plants infected with SACMV only and SACMV and 'delayed DI'. It noted that cassava plants co-infected with SACMV and DI showed no chlorosis. This study revealed that SACMV DI did influence virus replication and symptoms, but reductions in virus concentrations (DNA-A and DNA-B) was not sufficiently effective to ameliorate symptoms, and reduce viral load to an acceptable lower level compared to SACMV alone, to be used as a disease control strategy. The study also highlighted the importance of host interactions on viral replication.

2.1. Introduction

South African cassava mosaic virus (SACMV) is a distinct bipartite geminivirus species affecting cassava (*Manihot esculenta* Crantz) in southern Africa (Berrie *et al.* 1998). It is known to cause cassava mosaic disease (CMD), leading to mass yield loss. Subgenomic molecules are often associated with geminiviruses in naturally infected cassava. Defective interfering molecules are a type of subgenomic molecule that have been associated with geminiviruses, and are found to be a result of deletion, insertion, duplication or rearrangement of the parent geminivirus and their satellites (Patil and Dasgupta 2006).

A putative defective interfering molecule has been associated with SACMV in a naturally infected field cassava plant collected from the Mpumalanga Province in South Africa (Choge *et al.* 2001). This DI is derived from SACMV DNA-B, with the ORF BV1 entirely deleted and the carboxyl terminus of gene BC1 is disrupted with only 82% of the BC1 gene intact (Choge *et al.* 2001). Further studies indicated that no subgenomic particles were produced *de novo* when cassava plants were inoculated with total nucleic acid (TNA) from infected cassava (containing SACMV DNA A and B) or agro-biologically inoculated with SACMV DNA-A and DNA-B. Studies of geminivirus associated DNA-B derived DI have reported symptom

amelioration due to their ability to interfere with viral replication by competing with essential host and viral factors needed by the helper virus to replicate (Patil and Dasgupta 2006).

The aim of the study was to investigate whether the SACMV DNA-B derived DI [head-to-tail dimer previously constructed by Kuhn (2003)] interfered with its cognate helper virus replication and/or symptom attenuation in both model, *N. benthamiana* and the natural host cassava.

2.2. Methods and Materials

2.2.1. Infectivity Assay Approach

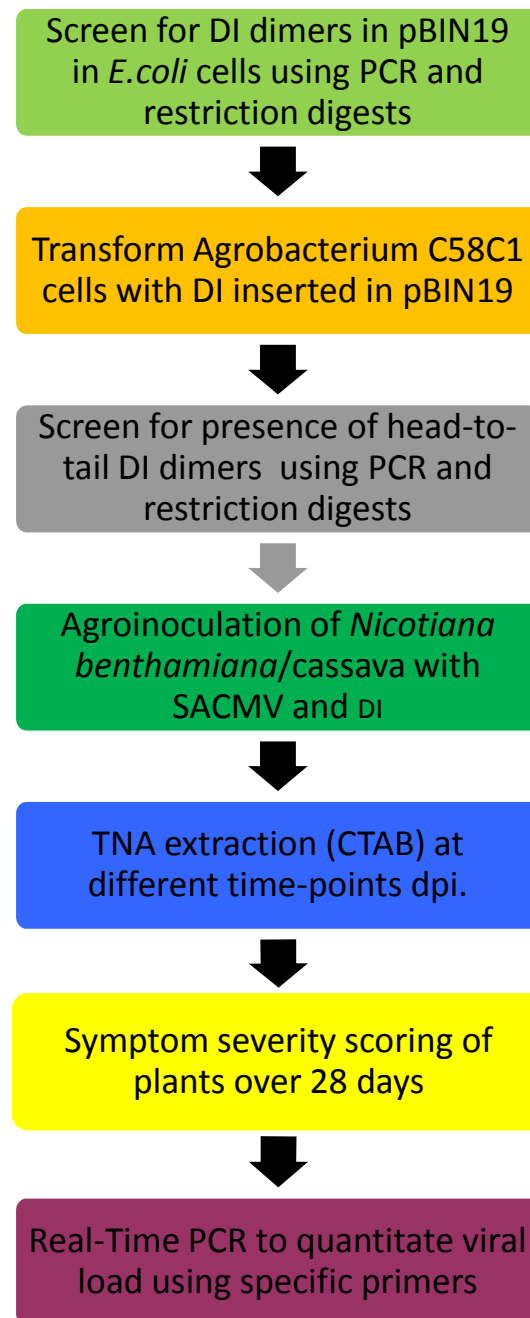


Figure 2:1. An outline of the infectivity assay used in this study.

2.2.2. Construction of DI head-to-tail dimer

A head-to-tail SACMV DNA-B DI dimer previously constructed by Kuhn (2003) was used in this study for agroinfection. The DI was isolated with SACMV in total nucleic acids (TNA) extractions and PCR amplified, using abutting DNA-B primers specific to DNA-B of SACMV, from a naturally infected, field cassava plant from Mpumalanga Province in South Africa was used for the construction of the dimer. Initially, the DI was cloned into the *EcoRI* site of the vector pBluescript (KS) to produce a clone 'pBS SA-DI'. A full restriction digest with the enzyme *ScaI* and a partial restriction digest with the enzyme *EcoRI* was performed on this clone. Selective fragments from this digest were re-ligated to form a head-to-tail dimer in pBS. This dimer was then cloned into a plant binary vector pBIN – 'pBIN19 SA-DI'.

2.2.3. Preparation of competent AGL1 cells

The head-to-tail DI dimer was transformed into competent *Agrobacterium* cells for successful agroinfection. *Agrobacterium tumefaciens* strain AGL1 was chosen as it is one of the more virulent strains used for agroinfection (Jones *et al.* 2005). Competent AGL1 cells were prepared using a method described by Wise *et al.* (2006). An overnight culture of AGL1 was sub-inoculated into YEP broth (10g/L peptone, 10g/L yeast extract, 5g/L sodium chloride, 20mM magnesium chloride, pH 7.2) with 100mg/L carbenicillin. This was placed in a 37°C incubator shaking at 220rpm till an O.D.₆₀₀ between 0.5 and 1.0 was achieved. The culture was placed on ice to chill and centrifuged at 10000g (Beckman J2-21, Beckman Coulter) for 10min at 4°C. The supernatant was discarded and the pellet resuspended with 5mL of ice-cold 20mM CaCl₂. The centrifugation step was repeated as above and the supernatant discarded. The pellet was resuspended in 1mL of cold 20mM CaCl₂ and cells were stored at -70°C in 100µl aliquots.

2.2.4. Transformation of competent AGL1 cells

The competent AGL1 cells were transformed using the freeze/thaw method as described by (Wise *et al.* 2006). Purified plasmid pBIN19 containing the head-to-tail dimer (200ng/µl) was added to 100µl of frozen competent AGL1 cells and thawed on ice, with intermitted mixing. Once the mixture of bacterial cells and plasmid was thawed completely, it was immersed in liquid nitrogen for 5min to snap freeze the cells. The mixture of bacterial cells and plasmid

was thawed at room temperature for 5-10min and transferred into 2mL of YEP broth and incubated with shaking at 28°C for 2-4hours. The cells were centrifuged in a standard benchtop microcentrifuge (MiniSpin Plus, Eppendorf) for 5 min at maximum speed. The supernatant was discarded and the pellet resuspended in 100µl of YEP broth containing 100mg/L carbenicillin and 100mg/L kanamycin. The cells were spread on YEP agar supplemented with antibiotics, 100mg/L carbenicillin and 100mg/L kanamycin, and incubated at 28°C for 2 days.

2.2.5. Selection for putative AGL1 transformants

Several single colonies of AGL1, putatively transformed with the DI dimer, were selected from the YEP agar plates incubated at 28°C for 2 days and used to inoculate 5mL of fresh YEP broth containing 100mg/L carbenicillin and 100mg/L kanamycin and grown to an O.D.₆₀₀ between 0.5 and 1.0. Plasmid isolation was performed using alkaline lysis method based on (J. Sambrook *et al.* 1989). An aliquot of 1.5mL of bacterial culture was centrifuged at maximum speed in a MiniSpin (Eppendorf) for 5min at 4°C. The supernatant was discarded and the bacterial pellet was resuspended by vortexing in 100µl of ice-cold solution I (50mM glucose, 25mM Tris-HCL pH 8.0, 10mM EDTA) and incubated at room temperature for 5min. Thereafter, 200µl of solution II (0.2M NaOH, 1% SDS) was added and mixed by gentle inverting. The suspension was incubated on ice for a maximum of 10min, after which 150µl of ice-cold solution III (3 M potassium acetate, 5 M glacial acetic acid, made up to final volume with sterile distilled water) was added and mixed by inverting the microfuge tubes until a cloudy precipitate was formed. This was then incubated on ice for a maximum of 10min. The suspension was centrifuged at maximum speed for 10min at 4°C. The supernatant containing the plasmid DNA was transferred to a new microfuge tube and the centrifugation step was repeated. The supernatant was transferred to a new tube and 350µl of isopropanol was added, mixed by inversion and incubated at room temperature for 5min. The DNA was precipitated by centrifuging at maximum speed for 10min at 4°C and the supernatant was discarded. The pellet was washed in equal volume, 1mL, of ice-cold molecular grade 70% ethanol (v/v %). The pellet was centrifuged at maximum speed for 10min at 4°C, and the supernatant discarded. The pellet was washed in 70% ethanol for a second time; and the supernatant discarded. The pellet was left to dry and resuspended in

50µl of 1XTE containing 10mg/L RNase A. The extracted pBIN19 plasmid was incubated at 37°C for an hour for RNA degradation by RNase.

A restriction digest was performed on the extracted plasmid using restriction enzymes *Bam*HI and *Hind*III (Fermentas) according to manufacturer's recommendations. The restriction digest was performed at 37°C for 3hours and inactivated at 65°C. This double digest excises the DI dimer from pBIN19.

2.2.6. Agroinfiltration of *Nicotiana benthamiana*

Agroinfiltration was used as the method for inoculation as SACMV is not mechanically transmissible and whitefly inoculation facilities were not available. Eighty *N. benthamiana* plants were grown from seeds in a growth chamber under 16hour photoperiod at 25°C with an acclimatization period of 3 weeks. Sixty plants were used in total for this experiment – 12 plants per treatment.

2.2.6.1. Preparation of infiltration buffer

Agrobacterium tumefaciens AGL1 containing SACMV DNA-A and DNA-B infectious clones [head-to-tail dimers previously constructed by Berrie *et al.* (2001)] and AGL1 containing DI were grown overnight separately, in YEP broth containing 100mg/l carbenicillin and 100mg/L kanamycin, with shaking at 28°C. The following morning, 5mL of the overnight culture was inoculated into 45mL of YEP broth supplemented with 100mg/L carbenicillin, 100mg/L kanamycin, 10mM 2-(N-morpholino) ethanesulfonic acid (MES), 20µM acetosyringone (AS). The culture was allowed to grow overnight with shaking at 28°C. The following morning the cells were centrifuged at 10,000g at 4°C for 10min (Beckham Coulter) and the supernatant discarded. The cells were resuspended in infiltration buffer - made up of 10mM MgCl₂, 10mM MES and 200µM AS to an O.D.₆₀₀ of 1. The cells were incubated at room temperature for 3hours so as to induce the activation of the *Agrobacterium vir* genes that is involved in the T-DNA transfer.

2.2.6.2. The five experimental treatments

Five experimental treatments were performed in this study with 12 plants per treatment. The first treatment was to infect the *N. benthamiana* plants with the helper virus SACMV DNA-A and SACMV DNA-B dimers (collectively referred to as 'SACMV' from now on). After 3

hours of incubation in the infiltration buffer, equal volume of DNA-A and DNA-B were mixed together to make the inoculum used to agroinoculate the plants with SACMV only (helper virus control). The mixture was used to infiltrate the entire adaxial surface of the two lower leaves of the plant, using a needle-less 1ml syringe. The second treatment was to infect the plants with SACMV and DI simultaneously. Therefore, DNA-A: DNA-B: DI was mixed in a ratio of 1:1:2 and used to agroinfiltrate the plants; hereby this treatment being referred to as 'SACMV and DI co-infiltrated' from now. The third experiment was to infect the plants with SACMV only initially and at 7 days post inoculation (dpi) to infect the same set of plants with DI only. Hence, the plants were initially infiltrated with SACMV only and at 7 dpi were infected with DI only; hereby this treatment will be referred to as 'delayed DI' treatment. The fourth set of *N. benthamiana* plants was mock-inoculated with AGL1 strain containing plasmid with no infectious dimers (Agrobacterium control). The last set of plants was the healthy un-inoculated control set of *N. benthamiana*.

2.2.7. Agroinoculation of Cassava

For this study, the susceptible cassava cultivar T200 was used and was infected by agroinoculation via injection, unlike agroinfiltration of *N. benthamiana*. This was principally because the fact that leaves of cassava are waxy and tough, making them fairly impermeable compared to *N. benthamiana*, hence giving poor infiltration results. Agroinoculation was used as the method for inoculation as SACMV is not mechanically transmissible and whitefly inoculation facilities were not available. Agroinoculation involves injecting the bacterial suspensions along the stem of the plant while they are young (6-8 leaves stage) focusing on the nodal part of the stem using a 1mm needle.

The suspensions and the five experimental treatments used for agroinoculation of the cassava plants were the same as described in section 2.2.5.1. and 2.2.5.2..

2.2.8. Total Nucleic Acid Extraction from *N. benthamiana*

Leaf samples were collected at 7, 14, 21 and 28 dpi from each experimental treatment. Total nucleic acids (TNA) were extracted using a modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Fifty mg of leaf tissue were snap frozen in liquid nitrogen and crushed in a microfuge tube using a micropestle in liquid nitrogen. The crushed

tissue was resuspended in 500µl of pre-heated (65°C) extraction buffer containing 2% w/v CTAB, 20mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 and 0.1% v/v β-mercaptoethanol. The suspension was incubated at 65°C for 60min with intermittent inverting. 500µl of chloroform: isoamyl (24:1) was added and mixed by inverting to extract the top aqueous layer from the suspension containing the TNA. This was then centrifuged at 4°C for 10min at the maximum speed using a refrigerated bench top microcentrifuge. The top aqueous layer containing the TNA was transferred to a new microfuge tube and the chloroform: isoamyl step repeated. The nucleic acid present in the aqueous layer was precipitated with equal volume (500µl) of isopropanol (Sigma). The microfuge tube was inverted several times till the TNA precipitated out as visible white stringy material. The TNA was pelleted by centrifuging the suspension in a refrigerated benchtop centrifuge at 4°C for 10min at the maximum speed. The supernatant was discarded and the pellet was washed in 500µl of ice-cold 70% ethanol (molecular grade, Sigma) and centrifuged for 10min at 4°C. This step was repeated and the pellet left to air-dry. Once all the ethanol had evaporated, the pellet was resuspended in 50µl Tris-EDTA(TE) buffer supplemented with 1µl of 10mg/ml RNase A. The pellet was left to resuspend at 37°C overnight to ensure complete RNA degradation. The DNA was stored in -20°C for further use.

2.2.9. Total Nucleic Acid Extraction from cassava

TNA was performed on cassava leaf samples collected at 14, 21, 28 and 42dpi from each experimental group using a modified cetyl trimethylammonium bromide (CTAB) method (Doyle and Doyle 1987) as described in section 2.2.7 with one noted difference. The extraction buffer used in the initial step contained an additional substance, 2% (w/v) polyvinylpyrrolidone (PVPP), which was not used for *N. benthamiana* TNA extractions. This was added as cassava contain a higher concentrations of polyphenols and PVPP is used in the removal of these polyphenols (Sharma *et al.* 2008).

2.2.10. Rolling circle amplification

Rolling circle amplification (RCA) was performed on extracted TNA using the Illustra™ TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences) as per the manufacturer's instructions. Extracted TNA was diluted to a final concentration of 500ng/µl. One µl of the TNA is added to 5µl of sample buffer and sealed in a microcentrifuge tube. This was then

heated at 95°C for 3min and then let to cool to room temperature or 4°C. Whilst the template mixture is cooling, the TempliPhi mastermix was made by adding 5µl of reaction buffer and 0.2µl enzyme mix for each reaction. The mastermix was thoroughly mixed and kept on ice. 5µl of this mastermix was added to each of the cooled down template mix. This was then incubated at 30°C for ±18hrs. Following this, the enzyme was heat-inactivated at 65°C for 10min and the reaction was cooled down at 4°C.

2.2.10.1. Restriction analysis

Restriction analyses on RCA products with the enzyme *PvuII* (Fermentas) as per manufacturer's instructions. Twenty units of enzyme were used to digest 1µl of RCA product at 37°C for 16hrs. DNA-B and DI contain a single *PvuII* restriction site.

2.2.11. Symptom Severity Scoring

Once infected, *N. benthamiana* and cassava plants were routinely observed for symptom development. At the various time points before TNA extraction, the symptom development of the plants were scored according to a symptom severity index (Fauquet and Fargette 1988) (Table 2.1)

Table 2:1. Symptom severity index used to score the plants in the infectivity assays

Symptom Score	Symptom Description
0	No symptoms
1	Mild chlorosis without deformation
2	Clear mosaic with or without slight leaf deformation
3	Strong mosaic all over the leaflets with leaf deformation
4	Same as score 3 except with severe leaf deformation
5	Severe mosaic and severe reduction of leaf size

2.2.12. Real time quantitative PCR to measure viral load

The DNA stored in -20°C was thawed and the suspension homogenized by heating it to 63°C and light vortexing. The samples were spun down using Mini-spin (Eppendorf) and the concentrations of the samples were measured using a NanoDrop® ND-1000 spectrophotometer (NanoDrop technologies). After initial concentration readings, the

samples were diluted down to a final concentration of approximately 100ng/μl in a final volume of 50μl. This diluted TNA samples was used as the template for real-time quantitative PCR. This was done for both *N. benthamiana* and cassava samples.

2.2.12.1. Designing Real-Time PCR primers for SACMV DNA-A and DNA-B

Primers were designed to amplify SACMV DNA-A and DNA-B using PrimerQuest from Integrated DNA Technologies (<http://eu.idtdna.com/Home/Home.aspx>). The real-time CP primers (Forward primer: 5' GCA CAA ACA AGC GTC GAT 3' and Reverse primer: 5' CTG CCA GTA TGC TTA ACG TCA 3') overlap on the AV1 and AV2 ORF's of DNA-A, with an expected amplicon of size of 154bp. The BV1 primer (Forward primer: 5' TAC GCA ATG CCT AGG TTG AAG GAA 3' and Reverse primer: 5' TTC CTT CAA CCT AGG CAT GCC GTA 3') binds to the BV1 ORF present on DNA-B, but is not present on the DI, hence only amplifying DNA-B of SACMV with an expected amplicon of 168bp. These primers were selected from various sets of primers designed (Table 2.2).

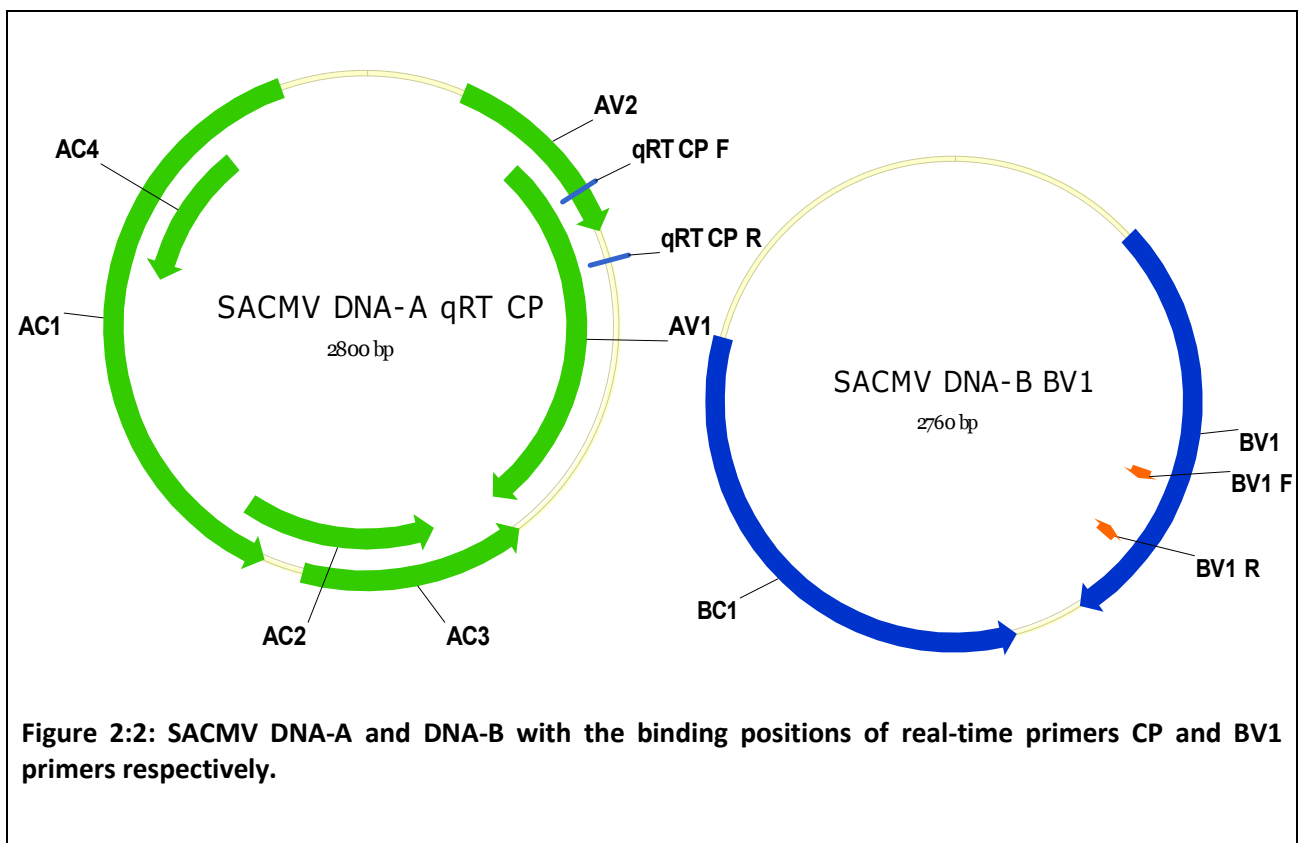


Table 2:2: Primer sets tested for the amplification of SACMV DNA-A from *N. benthamiana* and cassava TNA extracted from infected leaves.

Primer Sets	Primer Sequence (5' → 3')	Amplicon Size(bp)
qRT AV2/AV1 F	TGT GAT CCG AGC CCG TGA TTA TGT	152
qRT AV2/AV1 R	TGA TCG ACG CTT GTT TGT GCC TTG	
qRT AV2 F	GCA CAA ACA AGC GTC GAT CAT GGA	156
qRT AV2 R	ACA TAA TCA CGG GCT CGG ATC ACA	
qRT CP F	GCA CAA ACA AGC GTC GAT	154
qRT CP R	CTG CCA GTA TGC TTA ACG TCA	
Modified qRT CP F	GCA CAA ACA AGC GTC GAT CAT GGA	150
Modified qRT CP R	CTG CCA GTA TGC TTA ACG TCA TCT	

* The highlighted set of primers was eventually selected for both *N. benthamiana* and cassava samples.

2.2.12.2. Construction of standard curve

Standard curves were constructed for each set of real-time primers CP and BV1 using their respective amplicons cloned into in pTZ57RT/T plasmid.

2.2.12.2.1. Cloning amplicons into pTZ57RT/T plasmid

Fifty ng of DNA-A and DNA-B of SACMV in pBS was PCR-amplified with the real-time primers CP and BV1 primers respectively. Each reaction was made up of a Mastermix consisting of 2.5U of DreamTaq (Fermentas), 1X DreamTaq Buffer (Fermentas), 0.2mM dNTPS (Fermentas), and 0.2μM of forward and reverse primer made up to a final volume of 48μl with nuclease free water. The PCR reactions were conducted under the following cycling conditions in MyCycler™ Thermal Cycler (Bio-Rad): 95°C for 3min to activate the polymerase enzyme, with 30cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 30 seconds, primer extension at 72°C for 30 seconds, and a final extension step of 72°C for 5 min. Once successful PCR was confirmed, 4μl of PCR product was ligated into linearised pTZ57RT/T plasmid, using the InstaClone PCR kit (Fermentas), following manufacturer's recommendation.

The ligated product was transformed into the competent *E.coli* DH5 α cells and plated on LB agar plates containing 100mg/L ampicillin, 100 μ M isopropyl-beta-D-thiogalactopyranoside (IPTG) and 20mg/L X-gal. The plates were incubated at 37°C for 16hrs and were screened for white colonies. Several white colonies indicating putative transformation were selected and grown up overnight in LB containing 100mg/L ampicillin at 37°C at 220rpm. An alkaline lysis was performed as described in section 2.2.4 and a PCR performed to confirm successful cloning and transformation as previously described above with their respective primers. Successfully transformed clones were then used in the construction of the standard curves.

2.2.12.2.2. Linearising plasmid for standard curve

For the construction of the standard curves, pTZ57RT/T containing the amplicon was linearised using the restriction enzyme *Eco*RI (Fermentas). 10U of enzyme was used to digest 500ng of pTZ57RT/T plasmid containing the qRT CP and BV1 amplicon respectively for 3hrs at 37°C in Buffer O and was inactivated at 65°C for 20min. Subsequently, the products were purified using a PCR Purification Kit (Roche), using a modified protocol according to manufacturer's recommendation.

2.2.12.2.3. Standard Curve for N. benthamiana samples

Once the plasmids were linearised and purified, an average of 5 concentration readings (NanoDrop® ND-1000spectrophotometer, NanoDrop technologies), was used to create a serial dilution of 10ng, 1ng, 100pg, 10pg, 1pg for each set of primers, with each dilution being set-up with a technical replicate. Each reaction consisted of a Mastermix made up of 2 μ l of LightCycler® DNA Master SYBR Green In (Roche), forward and reverse primers to a final concentration of 0.5 μ M each and a final concentration of 2mM MgCl₂. One μ l of plasmid standard was added to each reaction and made up to a final volume of 20 μ l with nuclease free water. Real-time PCR was run for 40cycles under the following conditions: initial denaturation and enzyme activation at 95°C for 10min, denaturation at 95°C for 15secs, annealing at 60°C for 10secs and elongation at 72°C for 15secs with a single acquisition (fluorescence detection at 520nm) set-up at the end of the elongation phase for each cycle. The above mentioned cycle was set-up for both qRT CP and BV1 primers. The

average crossing points of these standards were used to plot a standard curve on axis of fluorescence against log template concentration.

2.2.12.2.4. Standard Curve for cassava samples

The standard curve for the cassava samples were set-up exactly the same as for the *N. benthamiana* samples as described in section 2.2.10.2.3. with one notable difference - 1µL of 100ng/µL of healthy cassava TNA was used to spike the standard, to minimize the effect of background fluorescence for the calculations of concentrations of the samples. The final volume remained 20µL.

2.2.12.2.5. Standard curve for Ubiquitin

The standard curve for ubiquitin was set-up exactly the same as for the *N. benthamiana* samples as described in section 2.2.10.2.3. with one notable difference – the plasmid was linearized using the restriction enzyme *Bam*HI. The forward primer used for ubiquitin were 18s : 5' GGC AAA TAG GAG CCA ATG AA 3' and the reverse primer used was: 5' GCG AAG ATC AGT CGT TGT TGG 3'. A previously constructed ubiquitin clone was kindly provided by F. Van Schalk.

2.2.12.3. Absolute Quantification

Absolute quantification was performed on *N. benthamiana* and cassava samples for both DNA-A and DNA-B using CP and BV1 primers respectively, at each of the timepoints post infection. Samples selected for real-time qPCR were diluted to a concentration of 100ng/µL and 2µL of this dilution was used for each reaction and each sample was set-up with a technical replicates. Each reaction contained of 2µl of LightCycler® DNA Master SYBR Green In (Roche), forward and reverse primers to a final concentration of 0.5µM each, final concentration of 2mM MgCl₂ and was made up to a final volume of 20µL. The cycling conditions are as mentioned in section 2.2.11.2.3.

2.3. Results

2.3.1. Confirmation of transformed AGL1

The presence of the DI dimer of the expected size of $\pm 3000\text{bp}$ was confirmed by restriction analyses using restriction enzymes *Bam*HI and *Hind*III (Figure 2:3).

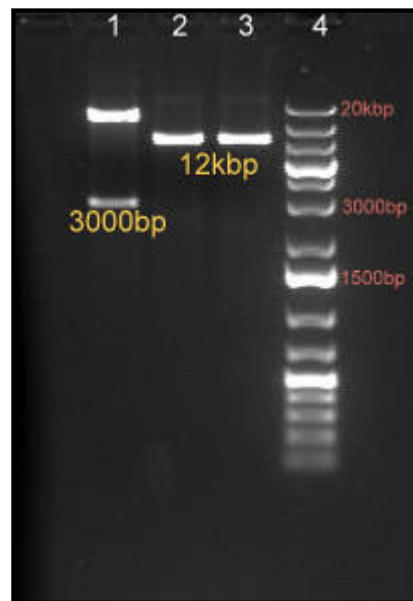


Figure 2:3. 1% agarose gel of restricted pBIN19 containing the DI dimer.

Lane 1: DI-dimer of $\pm 3000\text{bp}$ confirmed by restriction analysis using restriction enzymes *Bam*HI and *Hind*III (Lane 1). Lane 2 and 3 represents pBIN19 SA-DI of $\pm 12\text{kbp}$, cut with one restriction enzyme only *Bam*HI and *Hind*III respectively. Lane 4: O'Gene Ruler 1kb (Fermentas).

2.3.2. Symptom Severity Index

In this study, infectivity studies were performed in order to ascertain whether SACMV DI had any impact on virus replication or symptom phenotype or severity. Severity scores (0 to 5) (Fauquet and Fargette, 1988) are recorded in Table 2.3 and Table 2.4.

2.3.2.1. *N. benthamiana* Infection Assays

From the results (Table 2.3), it was observed that *N. benthamiana* plants co-infiltrated with SACMV and DI exhibited the least severe symptoms (score of 2) at all time-points compared with the other treatments. Plants with the ‘delayed DI’ treatment showed milder symptoms compared with plants infected with SACMV alone, but were more symptomatic compared with plants co-infiltrated with SACMV and DI. The most severe symptoms (4-5) were noted at 28 dpi in plants inoculated with the helper virus SACMV only. Mock-inoculated and healthy plants remained symptom-free across all time-points for all treatments.

Table 2:3: Symptom severity score for *N. benthamiana* at the various time-points (dpi) for the different treatments using the symptom severity index described by Fauquet and Fargette (1988)

Time Points (dpi)/ Treatments	7 dpi	14 dpi	21 dpi	28 dpi
SACMV	0	2	3	4-5
SACMV and DI co-infiltrated	0	2	2	2
SACMV and DI infiltrated at 7 dpi	0	2	2-3	2-3
AGL1 mock-inoculated	0	0	0	0
Healthy	0	0	0	0

*12 plants were used for each treatment, and done in 2 repeats.

These scores correlate to the symptoms exhibited by the plants *N. benthamiana* (Figure 2:4). Plants that were co-inoculated with SACMV and DI or the ‘delayed DI’ treatment showed mild blistering and very little chlorosis at 14 dpi, but at 21 dpi and 28 dpi blistering was intense, and the leaves were curly and reduced in size.

At 42 dpi, plants infected with SACMV, SACMV and DI and 'delayed DI' were stunted compared to wild-type uninfected *N. benthamiana* (Figure 2:5), but stunting was more noticeable in SACMV-only infected plants.

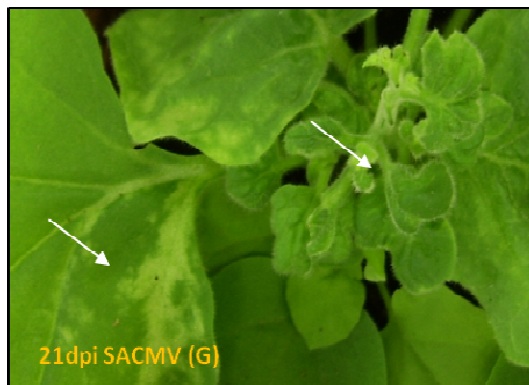
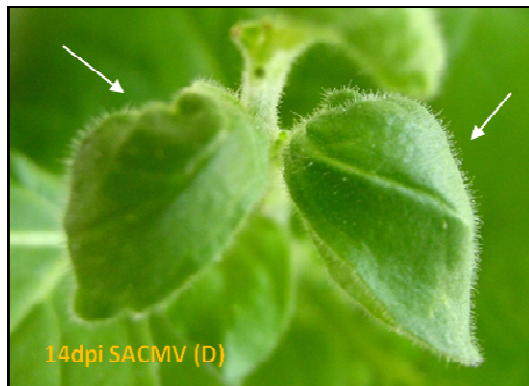




Figure 2:4. *N. benthamiana* plants exhibiting typical SACMV symptoms upon agro-inoculation of different treatments of SACMV and DI at different days post inoculation (dpi).

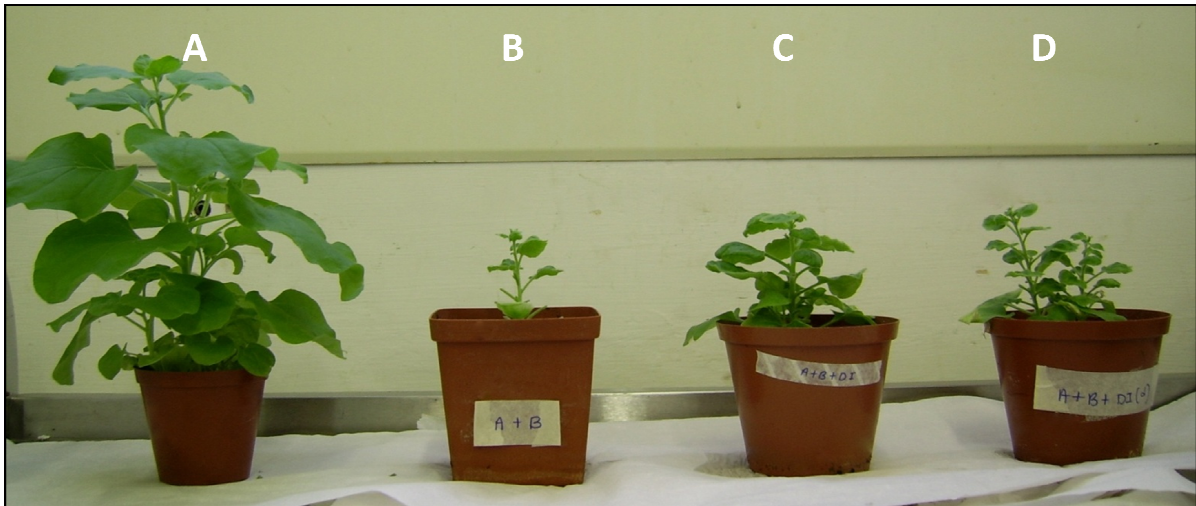
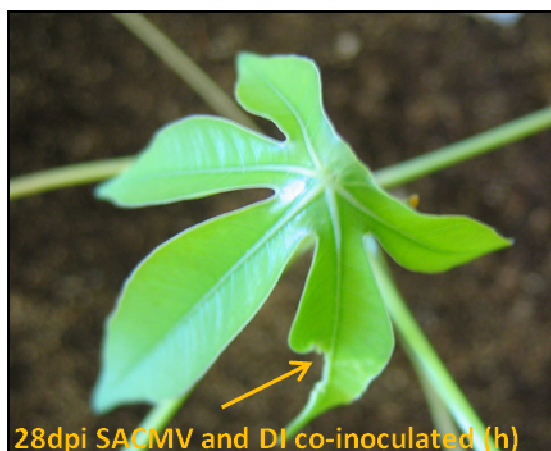
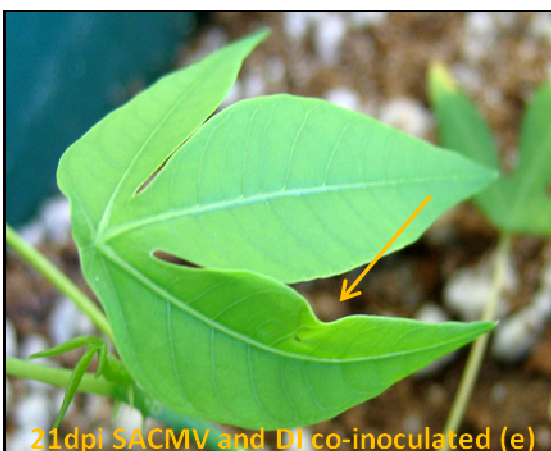
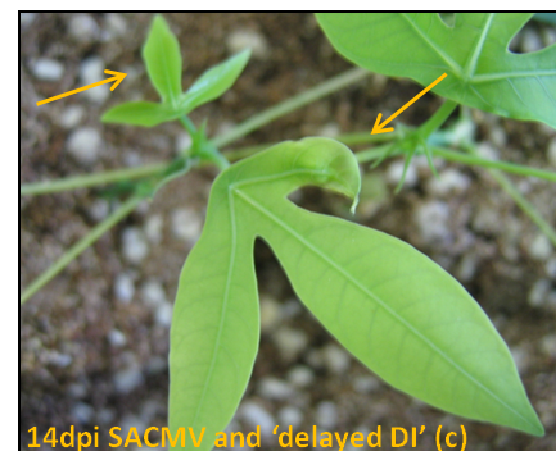
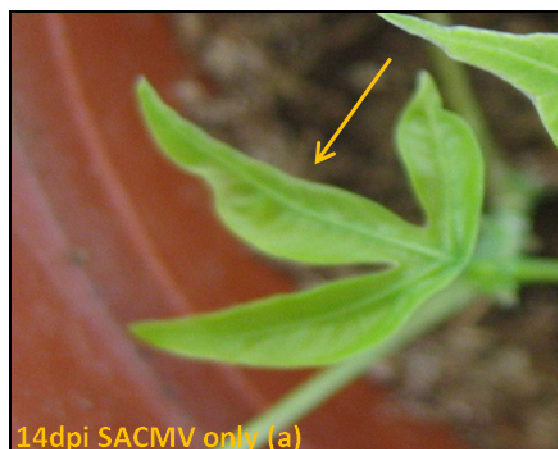


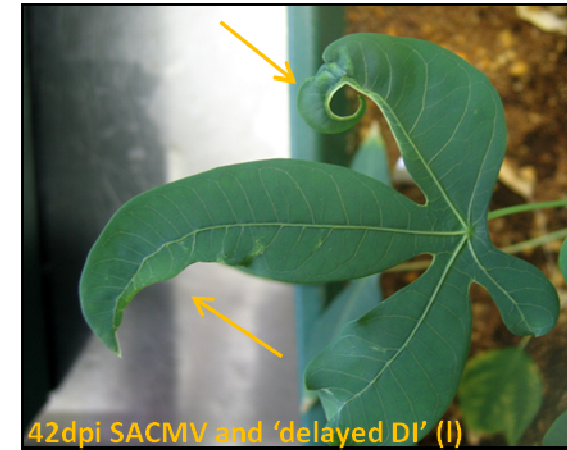
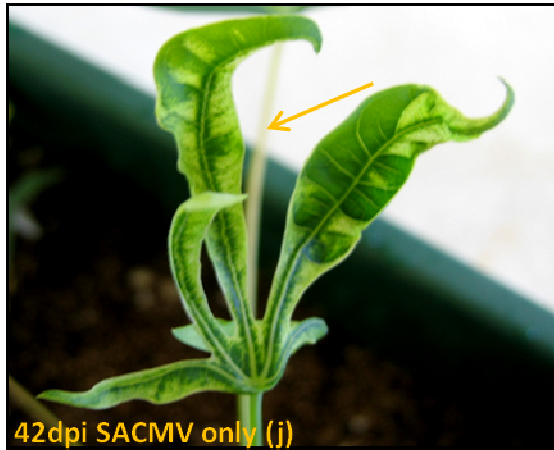
Figure 2:5: *N. benthamiana* plants treated with different treatments displaying different degrees of symptom amelioration at 42 dpi.

Compared to healthy plants (a), plants agro-inoculated with SACMV (B) exhibit severe symptoms with severe stunting, decreased foliage, leaves display chlorosis, leaf curling and blistering. Plants co-infected with SACMV and DI (C) exhibit less severe symptoms compared to (B). Plants are less severely stunted; leaves are bigger and less chlorotic. Plants infected with 'delayed DI' (D) exhibit more symptoms compared to (C) but less than (B).

2.3.2.2. *Cassava Infection Assays*

Similar to the *N. benthamiana* plants, it was observed that cassava plants co-infiltrated with SACMV and DI exhibited the least severe symptoms across all time-points compared with the other treatments (Table 2.4.). Plants with the 'delayed DI' treatment showed milder symptoms compared with plants infected with SACMV, but were more symptomatic compared with plants co-infiltrated with SACMV and DI. Mock-inoculated and healthy plants remained symptom free across all time-points. (Figure 2:6).





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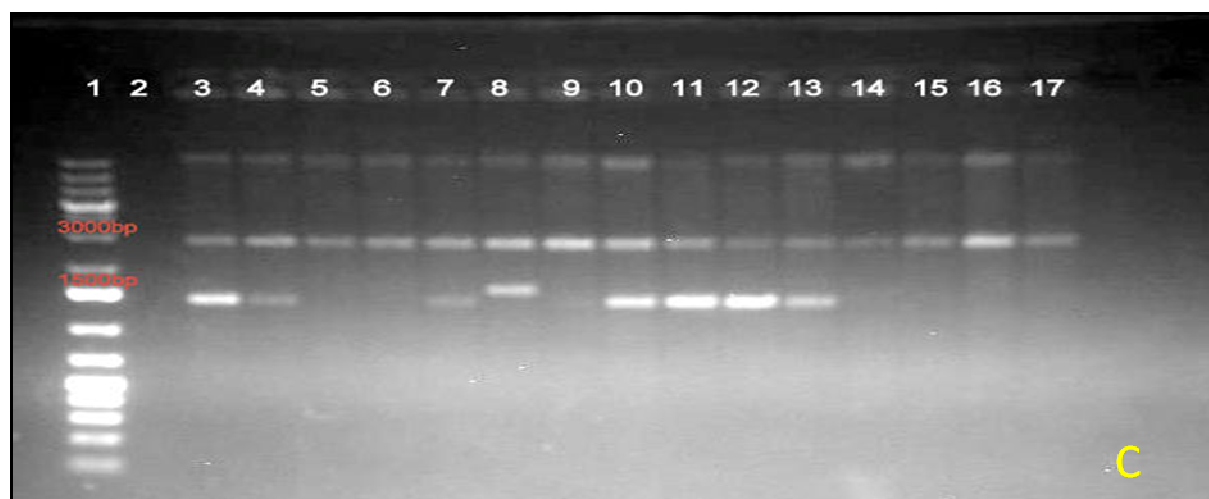
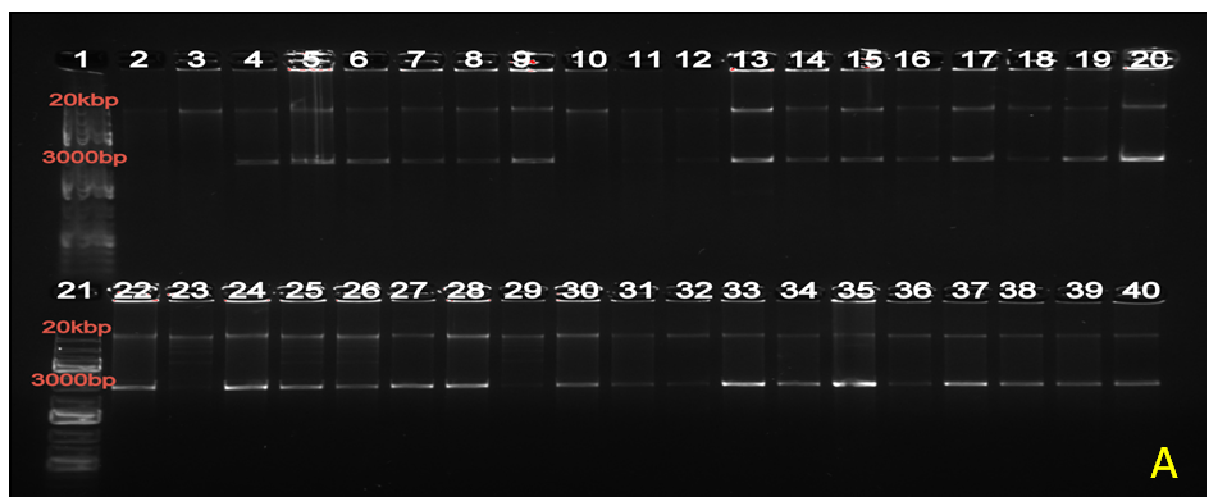
Table 2:4 Symptom severity score for cassava plants at the various time-points (dpi) for the different treatments using the symptom severity index by Fauquet and Fargette (1988).

Time Points (dpi)/ Treatments	14 dpi	21 dpi	28 dpi	42 dpi
SACMV	1	2	3	4-5
SACMV and DI co-infiltrated	0	1	1	1
SACMV and DI infiltrated at 7 dpi	1	1	2-3	2-3
AGL1	0	0	0	0
Healthy	0	0	0	0

At 14dpi, symptom developments in cassava across all treatments were mild. Cassava infected with SACMV only exhibited slightly elevated compared to the other two treatments. By 21 and 28dpi, symptoms are elevated and clearly visible across all treatments. At 42dpi, cassava leaves were severely infected exhibiting symptoms typical of SACMV – leaf curling and deformation, blistering, chlorosis and a decrease in leaf size. Cassava plants co-infected with SACMV and DI showed limited to no symptoms at 42dpi. Plants infected with SACMV and ‘delayed DI’ showed leaf curling and deformation not very little to none chlorosis.

2.3.3. Confirmation of presence of DI

RCA was performed on all samples TNA were extracted from. A restriction digest was performed using *PvuII* which gave a band of approximately 3000 bp (DNA-B) and approximately 1500 bp (DI). No *de novo* production of DI was observed in samples agro-infiltrated with SACMV only. Samples infected with DI and delayed DI retained DI production (but not at 100%) (Figure 2:7 A-E).



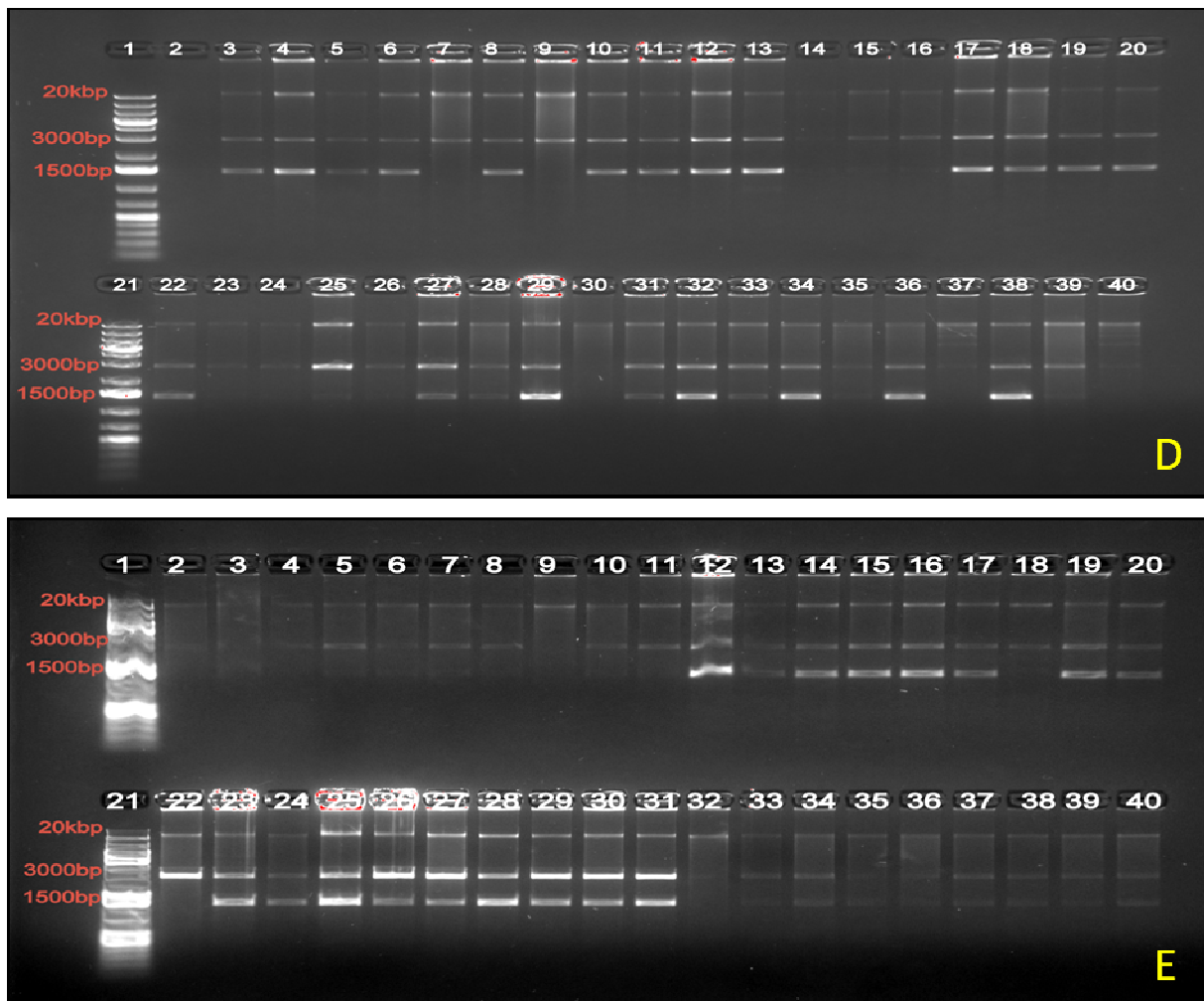


Figure 2:7. 1 % agarose gel showing SACMV DNA-B and DI after restriction analysis using *PvuII* on RCA products.

The bands of approximately 3000 bp in size represent the SACMV DNA-B component, while the bands of approximately 1500 bp represents the DI molecule. The products represented in these gels are from both *N. benthamiana* and cassava infectivity assays from the different treatments at different time-points. In gels A,B,C,D and E – Lane 1: O'Gene Ruler 1 kb (Fermentas).

2.3.4. Virus quantification

2.3.4.1. Confirmation of Cloning

Following design of the primers for real-time qPCR, standard curves were set up. In order to set up standard curves, CP and BV1 primer amplicons were required to be cloned into a plasmid. The presence of the amplicon of each primer set, cloned into plasmid pTZ57RT/T, was confirmed by extracting plasmid from transformed *E.coli* cells and performing PCR analysis (Fig. 2:4) An expected band of 154 bp was amplified using the real-time qPCR CP primers (Lane 5; Fig.2:4). Similarly, the confirmation of successful cloning and transformation of the 168 bp BV1 amplicon (Lane 8; Fig.2:7) was demonstrated. These

clones were used for construction of the standard curve to be used with the CP and BV1 primers.

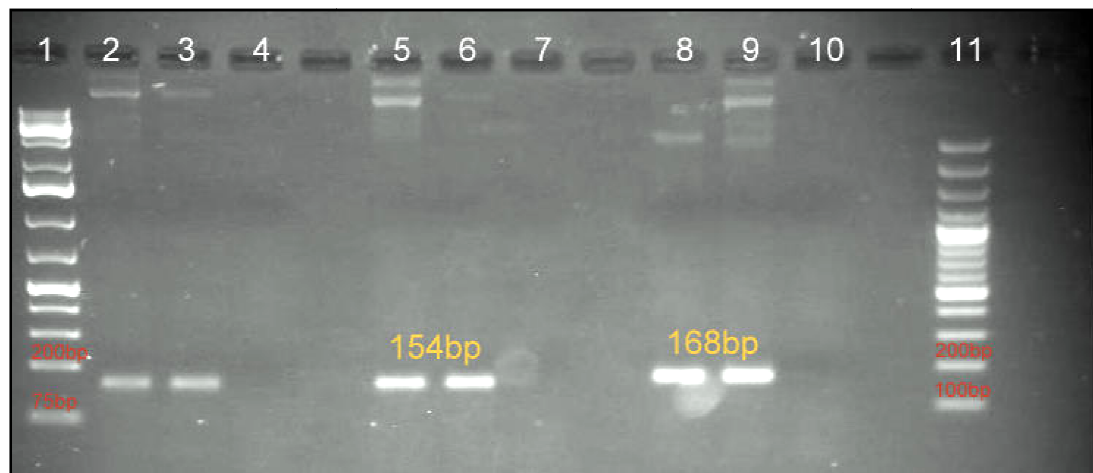


Figure 2:8. 2 % agarose gel showing PCR products of CP and BV1 primers used to construct clones for the standard curves of DNA-A and DNA-B of SACMV for absolute quantification.

Lane 1: O'Gene Ruler 1kb (Fermentas) and Lane 11: GeneRuler™ 100 bp Plus DNA Ladder (Fermentas). Lane 5: amplified expected band of size 154 bp for real-time qPCR CP primers. Lane 6: DNA-A in pBS was used as a positive control and water was used as a non-template control (Lane 7) to eliminate contamination. Lane 8: the expected band size of the 168 bp BV1 amplicon, Lane 9: DNA-B in pBS was used as a positive control and water as non-template control (Lane 10).

2.3.4.2. Construction of standard curves

Standard curves were generated using a serial dilution of linearised plasmid (containing the expected amplicon for primer set) standard from a range of 10 ng/μl to 1 pg for DNA-A and DNA-B with Roche Lightcycler® DNA Master SYBR green (Roche). The graphs were generated using the values of the Crossing points (mean) vs. the logged concentrations of DNA-A or DNA B (ng). Each reaction was conducted in duplicates.

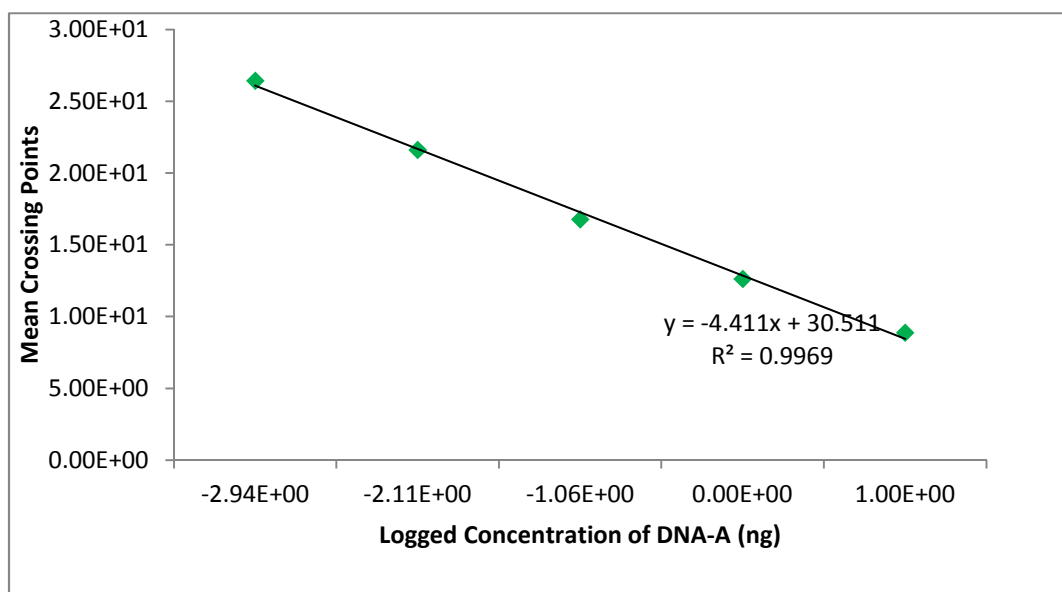


Figure 2:9 Standard curves for real-time CP primers measuring SACMV DNA-A concentrations (ng).

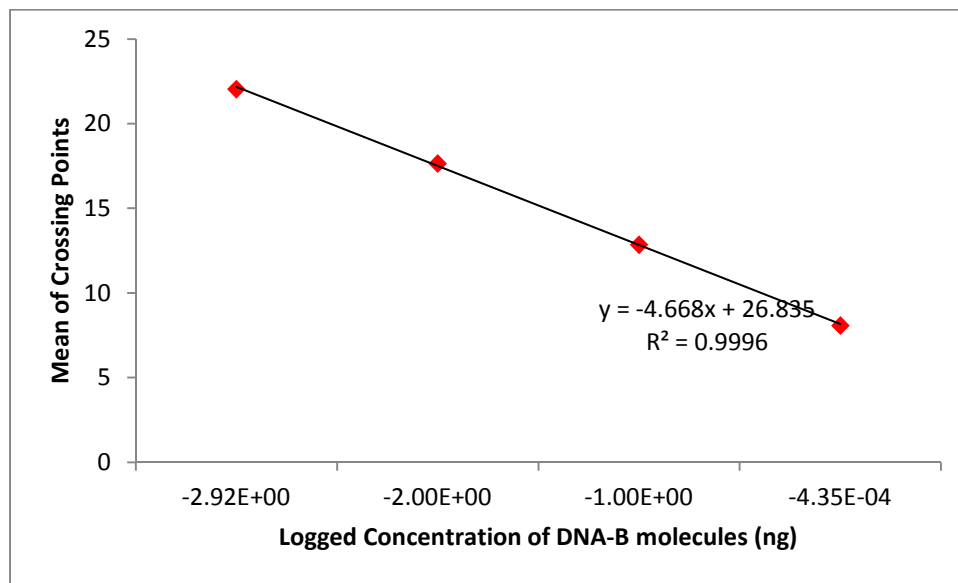


Figure 2:10: Standard curves for BV1 primers measuring SACMV DNA-B concentrations (ng).

Ubiquitin was used as an internal control to normalize the concentrations obtained from cassava. Standard curve for for ubiquitin was created similar to DNA-A and DNA-B standard cuves.

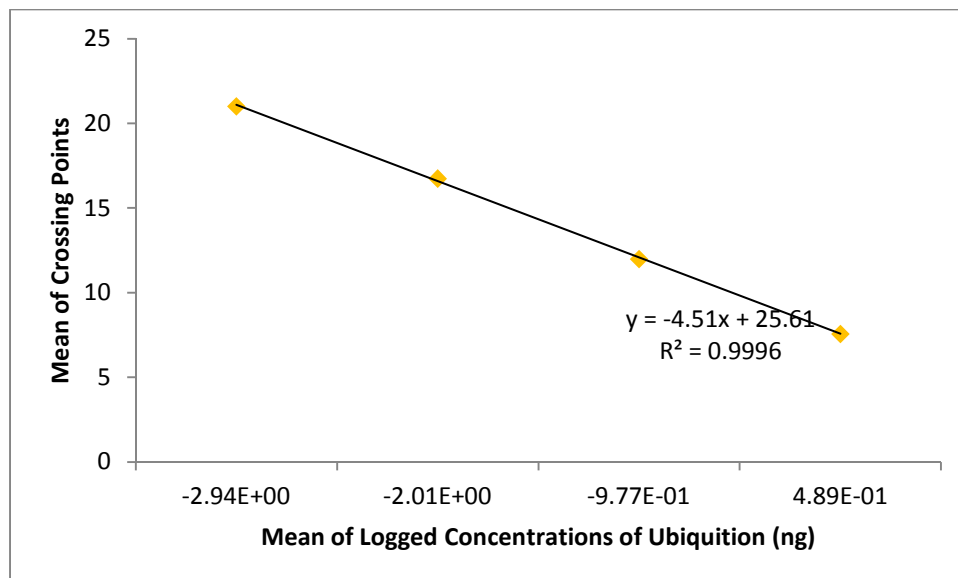


Figure 2:11. Standard curves for ubiquitin primers measuring SACMV DNA-B concentrations (ng).

2.3.4.3. Quantification of viral load of *N. benthamiana*

SACMV DNA-A concentration was quantified using CP primers in *N. benthamiana* samples at various time points for the different treatments. Results from the absolute quantification of the viral loads show that the DNA-A levels were the lowest at 7 dpi and the highest at 28 dpi across the different treatments. At 7 dpi, plants co-infiltrated with SACMV and DI had a lower concentration of DNA-A when compared to plants treated with SACMV and SACMV and delayed DI but by 28 dpi, the levels of DNA-A in plants co-infiltrated with SACMV and DI is higher compared to the other two treatments.

The difference in concentration of DNA-A at the different time points for each treatment, was found to be significant. ($p < 0.0001$, 2-way ANOVA, GraphPad). The difference in concentrations of DNA-A at the various time-points due to treatment was also significant. ($p < 0.0001$, 2-way ANOVA, GraphPad).

Similar to the DNA-A concentrations observed for *N. benthamiana*, it was observed that the DNA-B levels, quantified using BV1 primers - were the lowest at 7 dpi and at 7 dpi, plants co-infiltrated with SACMV and DI had a lower concentration of DNA-A when compared to plants treated with SACMV and SACMV and delayed DI (Figure 2:12).

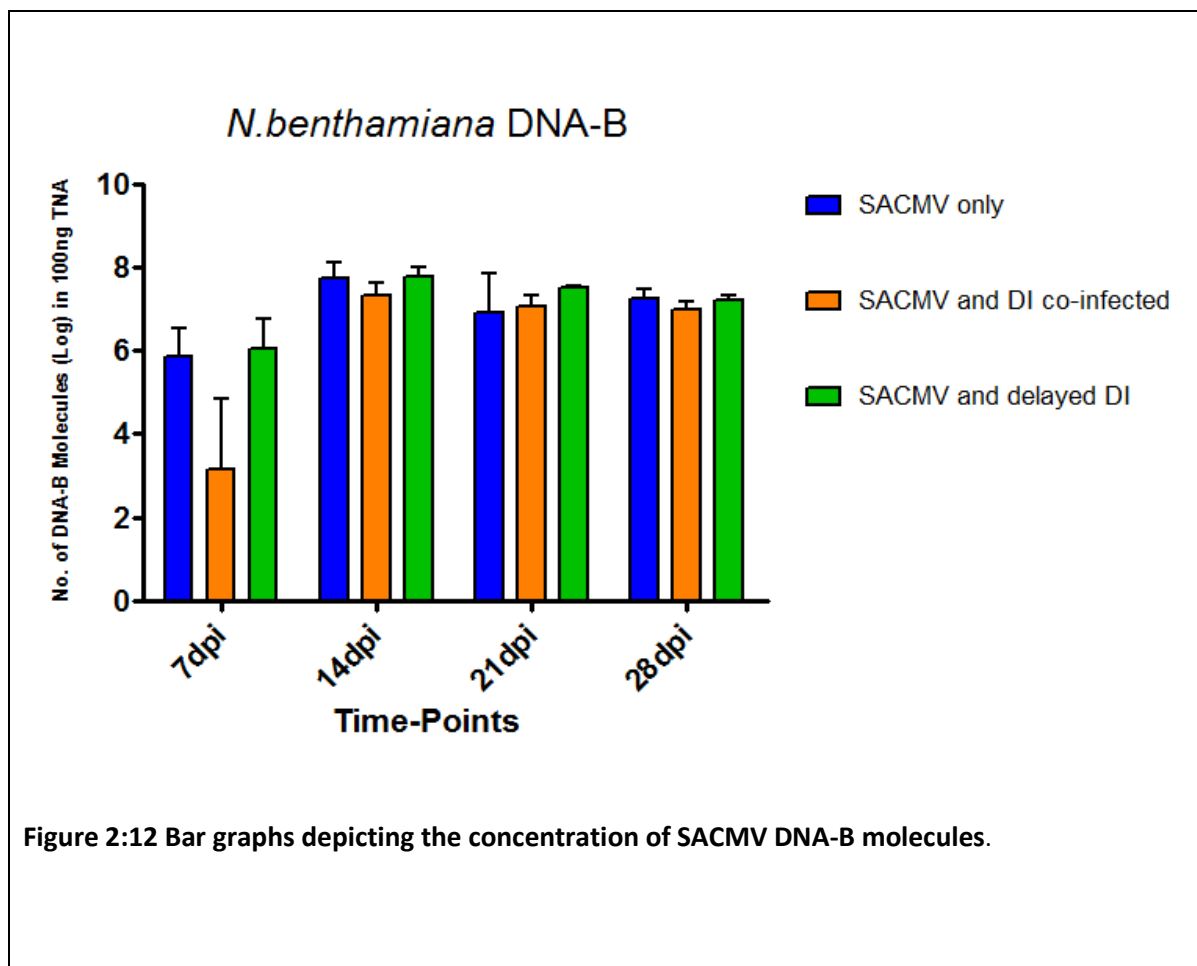
However, unlike the DNA-A concentrations, the highest concentration levels of DNA-B were observed at 14 dpi, not 28 dpi, across the different treatments

The difference in concentration of DNA-B at the different time points for each treatment, was found to be significant ($p < 0.0001$, 2-way ANOVA, GraphPad). The difference in concentrations of DNA-B at the various time-points due to treatment was also significant ($p = 0.0002$, 2-way ANOVA, GraphPad).

2.3.4.4. Quantification of viral load of cassava

Similar to the *N. benthamiana* samples, the concentration of SACMV DNA-A and DNA-B was determined from infected cassava TNA samples from the different treatments. The TNA was extracted from the various experimental treatments at different time-points. This data was normalized using values obtained with the cassava ubiquitin gene.

A significant decrease in DNA-A, at 42dpi, was found in cassava treated with DI, particularly plants co-infected with DI ($p < 0.0001$, 2-way ANOVA) (Figure 2:13).



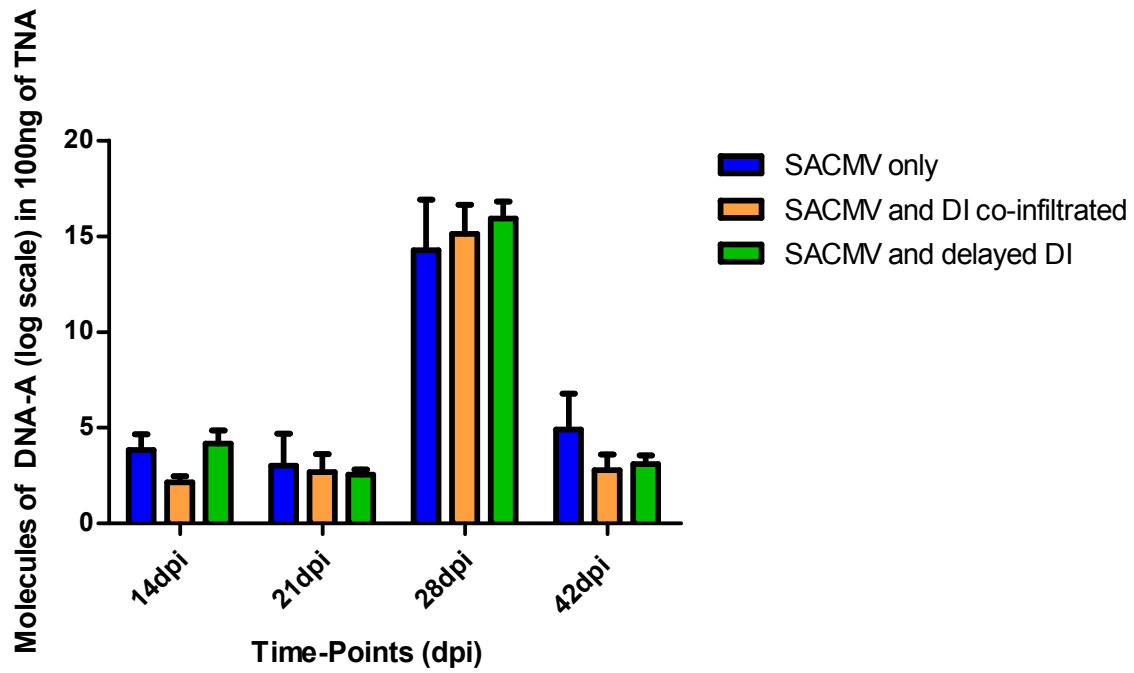


Figure 2:13. Number of molecules of SACMV DNA-A (log scale) found in 100 ng of infected cassava TNA.

A significant decrease in DNA B levels was observed at 14 dpi and 42 dpi in plants co-infected with SACMV and DI ($p < 0.001$, 2-way ANOVA).

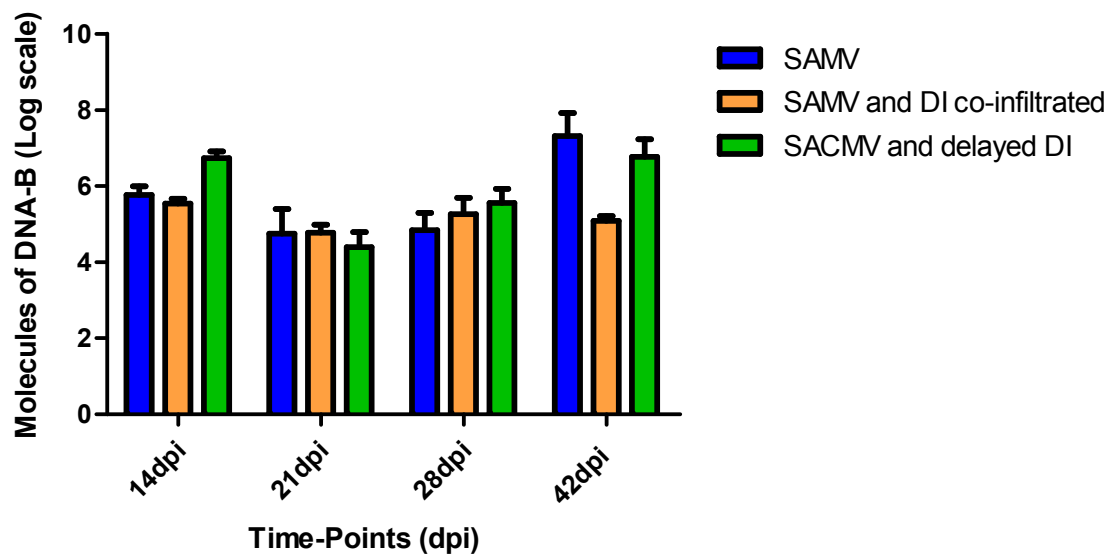


Figure 2:14. Number of molecules of SACMV DNA-B (log-scale) found in 100ng of infected cassava TNA.

The TNA extracted from the various experimental treatments at different time-points. This data has been normalized using values obtained with the cassava ubiquitin gene. A significant decrease in DNA-B levels was observed at 14 dpi and 42 dpi in plants co-infected with SACMV and DI ($P < 0.001$, 2-way ANOVA).

2.4. Discussion

Agroinfection with SACMV DNA-A and DNA-B dimers in *Nicotiana benthamiana* and cassava led to successful infection and symptoms typical of SACMV infection including leaf curling, blistering, chlorosis and severe stunting of the leaves were observed. Results from this study demonstrated that the DI did interfere with its cognate helper virus, SACMV, resulting in modification of symptoms and alterations in virus DNA-A and DNA-B replication (viral loads). Viral titres varied significantly over the time course of infection, showing a modular pattern for both DNA A and B levels. In *N. benthamiana*, while reduced viral loads were noted at some time points post infection, they were never reduced to a level to effectively ameliorate symptoms, which persisted over time. In cassava, however, symptom severity was low (score of 1) throughout the 42 days monitoring, and exhibited significant decreases in DNA A and B titres at 14, 21 and 42 dpi. It would appear that the DI was more effective in its natural host cassava, compared with *N. benthamiana*, in attenuating symptoms.

Nicotiana benthamiana

In the infectivity assay of *N. benthamiana*, the model host plant, virus replication for all treatments (control plants showed no symptoms or virus replication) was detectable at 7 dpi, even though no virus symptoms were visible. By 14 dpi plants showed some signs of infection, and by 28 dpi were fully symptomatic (Table 2.3). There was a 100% (12/12 plants) infection rate for *N. benthamiana* infected with clones of SACMV DNA-A and DNA-B. One of the major reasons *N. benthamiana* has been adopted as a model plant is its susceptibility to a wider range of viruses compared to, for example *Arabidopsis thaliana*, another widely used plant in research particularly for genomic studies. It has been observed that there is variability in the susceptibility of the plant to pathogens within the *Nicotiana* genus and the *N. benthamiana* species. For example, *South African cassava mosaic virus* has been shown to infect *N. benthamiana* but not *N. tabacum* (Berrie *et al.* 2001). It was determined that the “near universal virus susceptibility” of *N. benthamiana* is partly due to the naturally occurring mutation in the RNA-dependent RNA polymerase gene (*NbRdRP1m*) present in its genome (Goodin *et al.* 2008). The 100% infected rate is contributed to the ‘near universal virus susceptibility’ of *N. benthamiana* and agroinfiltration. Agroinfiltration is a procedure used to infect plants, where a suspension of *Agrobacterium*, transformed with

the desired gene, is injected into the intracellular spaces of the leaf through the adaxial side of the leaf (Goodin *et al.* 2008; Vaghchhipawala *et al.* 2011). The suspension of agrobacterium enters the leaf through the stomata and transfers the gene into the plant cells. This method is advantageous for infection and plant transformation as it is convenient and fast. Agroinfiltration is particularly effective in *N. benthamiana* as there are large intercellular space within the leaves and these can be easily infiltrated with a 1ml disposable syringe (Goodin *et al.* 2008; Vaghchhipawala *et al.* 2011).

In *N. benthamiana*, concentrations of SACMV DNA-A and DNA-B, in the young leaves, were the lowest at 7 dpi, for experimental treatments SACMV only and co-infection with SACMV and DI. This is as expected due to limited cell-to-cell viral movement at early stages of virus replication, and is also indicative of the time required for the proliferating virus to move systemically from the initial site of infection, via the phloem, to accumulate in the new young leaves (Carrington *et al.* 1996). By 28 dpi, the plants were fully symptomatic, indicative of virus proliferation and its systemic movement throughout the plant.

In comparison of levels of SACMV DNA-A with DNA B in plants infected with SACMV only, it was found that DNA A was at its highest concentrations at 28 dpi (an increase of 4×10^6 fold from 7 dpi) when the symptoms were the most severe (3-4 score). The genes found on SACMV DNA-A transcribes for proteins mainly associated with replication, encapsidation, transmission and symptom development (Crisanto Gutierrez *et al.* 2004). REn, transcribed by the AC3 ORF on DNA-A, a known DNA replication initiation protein is known to increase symptom severity as a direct result of enhancing viral replication (Levy and Tzfira 2010). Therefore, it could be postulated that the severe symptoms are a result of the high levels of DNA-A accumulating over 28 days, when it results in the most devastating symptom phenotype.

SACMV DNA-B levels in *N. benthamiana* at 28 dpi were only 10 fold higher than at 7 dpi, with a significant increase in titre (a 37 fold increase from 7 dpi) of this component of the helper virus between 7 to 14 dpi, while thereafter it appears to "level off" compared with the exponential increase in DNA A (4×10^6) over the time course of infection. However, despite the highest concentration of DNA-B observed at 14 dpi, the observed symptoms were mild (Table 2.3). SACMV DNA-B ORF's encodes two movement proteins, namely long

distance movement (BC1) and nuclear shuttle protein (BV1). These proteins are responsible for nuclear shuttling and cell-to-cell systemic movement of both DNA components of SACMV and also symptom development (Gutierrez 2000). It can be hypothesized that once DNA A has replicated and transcribed Rep protein, the DNA-B molecules are then preferentially accumulated at high concentrations after the initial replication at the site of entry, to aid the systemic movement of both DNA components of the helper virus. DNA-B is dependent on DNA-A for its replication (Hanley-Bowdoin *et al.* 1999). This is supported by our results where, at 14 dpi, the concentration of DNA-B (7.209×10^7 molecules) was significantly higher than DNA-A (3.951×10^7 molecules).

Defective interfering molecules are known to lead to a decrease in symptom amelioration by a proposed mechanism of interfering with the replication of its helper virus (Patil and Dasgupta 2006). In comparing the three experimental treatments, SACMV alone; SACMV and DI co-infection; and infection with SACMV followed by DI 7 days later, symptom severity evaluations (scoring 1-5) and virus DNA A and B titres (measured by real time qPCR) were measured over time. Symptom amelioration was more prominent in *N.benthamiana* plants co-infected with SACMV and DI, compared to *N. benthamiana* treated with 'delayed DI' (Table 2.3 and Table 2.4).

N. benthamiana plants co-infected with SACMV and DI showed a similar pattern of replication to plants infected with SACMV only (Fig 2:12). The lowest titres of DNA-A were observed at 7 dpi and highest levels of DNA-A were found at 28 dpi (10^5 fold increase from 7 dpi). Similarly, DNA-B levels were lowest at 7 dpi and the highest at 14 dpi (1.5×10^3 fold increase from 7 dpi).

A significant difference in concentrations of DNA-A and DNA-B between the two treatments indicated a strong impact of the presence of DI on the helper virus. At 7 dpi, the plants infected with SACMV and DI had a significant decrease (120 fold) in DNA-A concentration compared to plants infected with SACMV only. DNA-B concentration, at 7 dpi, of plants co-infected with SACMV and DI showed a 100 fold decrease compared to plants infected with SACMV only. This is strong evidence that the DI has significant impact on the early stages of helper virus proliferation.

Studies have shown the importance of the two DNA-B proteins, NSP and MP, for the movement of the virus. NSP is responsible for transport of ssDNA and dsDNA forms from the nucleoplasm to the cytoplasm via the nuclear pore complex (Gafni and Epel 2002). MP is responsible for the cell-to-cell movement of the virus via the plasmodesmata and the long distance movement via the phloem (Noueiry *et al.* 1994). Studies on *Squash leaf curl virus*, a bipartite begomovirus, have shown that its MP has an symptom-inducing capabilities (Pascal *et al.* 1993). SACMV DI contains 82% of the BC1 ORF where the C-terminus is disrupted and the entire BV1 ORF is deleted (Choge *et al.* 2001). It is believed that the decreased size of the DI gives it an advantage during replication to be selected for over full-length SACMV, thus leading to higher levels of DI than DNA-B. Previous studies on *N. benthamiana mosaic virus* (TMV), a positive sense ssRNA virus and *Abutilon mosaic virus* (AbMV), a ssDNA bipartite begomovirus, have signified the importance of the C-terminus of the MP for virus movement (Waigmann *et al.* 2000; Trutnyeva *et al.* 2005; Kleinow *et al.* 2008). It is believed that the C-terminus of the MP is phosphorylated by the cell wall associated kinases which then increases the plasmodesmata size exclusion limit (SEL) and allows the viral to move through the cell wall for cell-to-cell movement. Therefore, the decreased levels of DNA-A and DNA-B could also be contributed to the presence of the truncated BC1 MPs, which cannot be phosphorylated at its C-terminus, hence limiting or interfering with the BC1-DNA A-DNA B complex, which is thought to be the form in which bipartite geminiviruses move through the cytoplasm to the cell wall. Since the DIs also do not transcribe the BV1 nuclear shuttle protein, they would depend on the helper to assist them to move out of the nucleus. This may also contribute to some competition between the cognate helper virus and DI, thereby reducing movement of the helper out of the nucleus. The decreased levels of DNA-B could also be a due to the fact that the smaller DIs use the same limiting host and viral factors that DNA-B use for replication and is selected over full length DNA-B due to their size advantage. The final concentration, at 28 dpi, of DNA-B (1.05×10^7) is substantially lower than the final concentration of DNA-A (8.52×10^{10}). Since DNA A and B of SACMV, the helper virus, also compete for Rep binding in the common region, there is an 'interdependence' between these two components, which is likely to result in a modular pattern of increases and decreases in DNA A in relation to DNA B and vice versa. The presence of an additional defective smaller DNA B component is also likely to influence this process. One could postulate that at 28 dpi, that the higher concentration of DNA A in the SACMV and DI

treatment compared to SACMV alone, could be a consequence of the need to provide Rep for all three component replication. SACMV DNA-B in *N. benthamiana* levels off from 21 dpi, but remains at a high titre (Figure 2:13) would be interesting to be able to accurately quantify the titres of the DI, but since the DI and helper virus DNA B share sequence homology, it was not possible to differentiate with BC1 primers which would amplify both components. However it is not surprising that we are seeing modulation of all three DNA components, as this has also been observed in RNA helper and satellites (Patil and Dasgupta 2006).

Interestingly, the phenotypic effects of the reduced helper virus levels are only observed at the later time point 28 dpi (Figure 2:4 and 2:5). Plants infected with SACMV only exhibit severe stunting, leaf curling, blistering and chlorosis whereas plants co-infected with exhibit limited chlorosis and stunting, leaf curling and blistering (Figure 2:4 and 2:5). DNA-B of bipartite geminiviruses may not always be required for replication and symptom development depending on host and virus. Contrasting results from different studies support this theory. In a study by Patil *et al.* (2007) involving *Sri Lankan cassava mosaic virus* (SLCMV), a bipartite begomovirus – agroinoculation of *N. benthamiana* with only the DNA-A component of SLCMV resulted in upward leaf-rolling and vein-thickening. Thus proving DNA-B not required for symptom development but many of the Old World lacking a DNA-B, require a satellite for natural field infection. However, in a study conducted by Y-ming Hou *et al.* (1998), plants infected with only the DNA-A component of bipartite *Bean dwarf mosaic virus* (BDMV) showed no symptoms, but was positive for systemic infection thus highlighting the importance of DNA-B in symptom development, and often modulation. Similarly, in SACMV, agroinoculation with DNA-A only of *N. benthamiana* lead to positive systemic infection but no symptoms were observed (Kuhn 2003). However it should be noted that this may not reflect the infection in the field of the natural host. Thus one can conclude the importance of DNA-B in symptom development for SACMV. Thus the differences in symptoms between plants infected with SACMV and co-infected with SACMV and DI could be a direct result of the reduced helper virus levels, in particular the DNA-B levels which were significantly lower for plants co-infected with SACMV and DI.

Plants agro-inoculated with the 'delayed DI' treatment, i.e. with SACMV only and then with DI 7 days later, showed contradictory results. Although the concentrations of DNA-A and

DNA-B increased from 7 dpi to 28 dpi for both DNA-A (5×10^6 increase at 28 dpi) and DNA-B (6 fold increase at 28 dpi), no drastic symptom amelioration was observed and the viral load of helper virus was reduced compared to plants infected with SACMV only. This highlights the previously discussed point that the presence of DI interferes with viral replication at early stages of infection (7 dpi or less). However, in *N.benthamiana*, the presence of DI had the most impact at the early stages, where the viral load (both SACMV DNA-A and DNA-B) was lowest at 7 dpi. This suggests that the presence of DI has an effect from the initial infection onwards, interfering with SACMV replication, as shown by the extremely low compared to plants treated with only SACMV and, SACMV and delayed DI. Studies on SLCV have shown that in the presence of a deleted or mutated BV1 of DNA-B, the CP can compensate for the lack of BV 1, thus aiding the movement of the helper virus allowing for systemic spread.

Cassava

N.benthamiana plants were symptomatic by 14 dpi and fully symptomatic by 27 dpi. However, in comparison, the infected cassava plants were only symptomatic 21-24 dpi and were fully symptomatic by 42 dpi (Table 2.4). In cassava plants, 67% of infection rate was achieved overall. Agroinfection of cassava, using clones of SACMV, is far more difficult than *N.benthamiana* owing to the waxy nature of the cassava plants, making it difficult to agroinoculate. In the infectivity assay of cassava, it was viral replication was only detectable by 14 dpi and symptom development was only observed around 21 dpi. By 28 dpi the plants were more symptomatic and by 42 dpi they were fully symptomatic (Table 2.4). Cassava is the natural known host of SACMV and has co-adapted to the cassava host over evolution (R W Briddon *et al.* 2010).

In cassava plants infected with SACMV only, there was a decrease of DNA-A concentration from the first time-point 14 dpi to 21 dpi. The lowest DNA-A concentration was observed at 21 dpi and highest at 28 dpi. This is similar to the pattern observed in *N. benthamiana*, where there was a drop of DNA-A concentration from 14 dpi to 21 dpi and then an increase in DNA-A level from 21 dpi to 28 dpi. By 42 dpi, once again, a decrease in DNA-A concentrations was observed. This could be the indication of a modulating viral proliferation

pattern of DNA A and B components in SACMV's natural host plant, cassava, or the effect of the DI.

In cassava plants, the DNA-B concentrations in plants infected with SACMV only, was highest at 42 dpi and lowest at 21 dpi. There was a 2 fold decrease in DNA-B concentration from 14 dpi to 21 dpi. There was a 63% increase of DNA-B molecules from 21 dpi to 28 dpi. By the final time-point 42 dpi, there was a 250 fold increase of DNA-B from 28 dpi. Similar to DNA-B levels in cassava, the concentration of DNA-B decreased from 14 dpi to 21 dpi. A marginal increase of DNA-B levels from 21 dpi to 28 dpi was observed, similar to the trend in *N. benthamiana*. From a comparison of SACMV levels in both plants systems, there appears to be specific stage of the virus proliferation where the highest accumulation of DNA-A (28 dpi) and DNA-B (14/42 dpi) occurs. This alternating pattern may be a consequence of a cyclic replication pattern typical of a situation with bipartite viruses, such as SACMV, where two DNA components are interdependent on each other for different functions at different times, such as replication (DNA-A) and movement proteins (DNA-B).

Cassava plants infected with SACMV only had the most severe symptoms (4-5) at 42 dpi (Figure 2:6) where the DNA-B levels were the highest, but the DNA- A viral titres were **THE SECOND HIGHEST**. Concentrations of DNA-A and DNA-B were lowest at 21 dpi, when the symptoms were extremely mild (Figure :4). Even though, the concentrations of DNA-A were the highest at 28 dpi, the symptoms were mild. Even though the DNA-A presumably transcribes REn, a known replication enhancer, we assume it is present in high levels before replication of DNA A, it did not contribute to more severe symptoms (Levy and Tzfira 2010). SACMV DNA-B could be playing a greater role in symptom development in cassava its natural host. DNA-B is known as an important symptom modulator in some geminiviruses. A study by Pascal *et al.* (1993) highlighted the importance of this, in particular of the BC1 transcribed MP. Transgenic *N. benthamiana* expressing only the BC1 ORF of SLCV or in conjunction with BV1 ORF developed symptoms typically associated with SLCV. Whilst transgenic *N. benthamiana* expressing only the BV1 ORF of SLCV did not develop any symptoms and the phenotypes were typical to the control healthy *N. benthamiana*, thus showing the importance of the BC1 transcribed MP. This correlates to the high levels of DNA-B at 42 dpi when the most severe symptoms were observed.

The difference in viral titres for DNA-A and DNA-B in *N. benthamiana* and cassava could be a direct result of differences in host factors interacting with the virus (Whitham and Wang 2004).

In cassava, the presence of DI seems to be the most effective in reducing viral load at the later stages of infection, i.e. 42 dpi, particularly affecting SACMV DNA-B (Figure 2 :15). SACMV DNA-A concentrations seem to be lower at the initial stage (14 dpi).

Cassava plants co-infected with SACMV and DI showed symptom amelioration compared to plants infected with SACMV only. Another important observation was that plants co-infected with SACMV and DI developed less chlorosis compared to plants infected with SACMV only, similarly observed in *N. benthamiana*. In a study with SLCMV, DNA-A component only was responsible for upward leaf-rolling and vein-thickening, but when infected with DNA-B component, symptoms observed are downward leaf curling and crinkling (Patil *et al.* 2007). Other studies showed that DNA-B alone can induce ‘typical’ symptoms associated with helper virus, and inoculation with SACMV DNA-A alone produced no symptoms in *N. benthamiana* (Levy and Tzfira 2010). From our results, and other studies, we believe that DNA-B is likely responsible for chlorosis, that is observed with typical symptoms associated with SACMV. Pronounced chlorosis was observed in cassava plants infected with SACMV alone and SACMV and delayed DI, but less so in plants co-infected with SACMV and DI. Therefore, we hypothesize that the attenuation of chlorotic symptoms may be a consequence of the DI modulating DNA-B, leading to lower titres at 14 and 42 dpi, thereby reducing severity of symptoms.

SACMV DNA-A levels in plants co-infected with SACMV and DI were highest at 28 dpi and lowest at 14 dpi. The presence of DI seems to make a significant impact on DNA-A concentration at the early stage of infection i.e. 14 dpi and at the later stages, 42 dpi. There was a 150 fold decrease in DNA-A concentrations in cassava co-infected SACMV and DI, compared to cassava with SACMV only at 14 dpi. This is interesting, as it seems like the DI is having an effect on DNA-A replication at the early stages of the viral replication. A similar trend was observed at the earliest time-point, 7 dpi, in *N. benthamiana* where the DNA-A

levels in plants co-infected with SACMV and DI had lower DNA-A titres at 7 dpi compared to *N. benthamiana* infected with SACMV only (a 120 fold decrease was observed, at 14 dpi a 5 fold decrease was observed in DNA-A levels between the two treatments). The effect of DI could be amplified in the natural host compared with model host, as co-evolution of viral and host factors are important in the outcome of any virus disease. *N. benthamiana* is known as a 'promiscuous' host allowing replication of a wide range of viruses (Goodin *et al.* 2008) therefore, symptoms attenuation is not as pronounced. This could also explain why there was a more significant decrease (150 fold) in DNA-A in cassava compared with *N. benthamiana* (5 fold at 14 dpi.) At 42 dpi, there is a 2000 fold decrease in DNA-A in cassava co-infected with SACMV and DI compared to DI only.

The DNA-B concentrations were the highest at 14 dpi and lowest at 42 dpi. Symptom amelioration was more pronounced in this treatment compared to cassava infected with SACMV only and SACMV and delayed DI. At 42 dpi, the concentration of DNA-B in the treatment with SACMV only infected cassava is 900 fold higher, correlating with the most severe symptoms (score 5), than plants co-infected with SACMV and DI. As previously discussed, the importance of DNA-B, in particular the BC1, has been established in symptom formation. Our study suggests that the low DNA-B concentrations could be the reason for the observed symptom attenuation. The decreased DNA-B could be a result of the smaller DI being favoured over full length DNA-B during replication. The lowered DNA-A levels could be explained by the presence of truncated MP transcribed by DI. As previously discussed, studies have showed the importance of the C-terminus of BC1 in the systemic movement of virus through the plant, Thus, the lack of C-terminus of BC1 could hinder the systemic spread of virus affecting overall viral titre at 42 dpi and symptom development.

Cassava plants agro-inoculated with the 'delayed DI' treatment, i.e. with SACMV only and then with DI 7 days later, once again, showed contradictory results compared to the other 2 treatments. No drastic symptom amelioration was observed although the viral load of helper virus was reduced at certain time-points compared to plants infected with SACMV only. A lack of chlorosis, a direct effect noted in the SACMV and DI treatment, was not noticed in these plants. This highlights a previously discussed point that the presence of DI interferes with viral replication at the critical initial stages of infection (14 dpi), and that any delays in the inoculation of DI, such as in the 7 day delayed treatment, will be less effective

in virus titre modulation. At 14 dpi, concentrations of DNA-A and DNA-B were not lower in the treatment where the DI was only inoculated at 7 days after SACMV, compared with SACMV only and SACMV and DI.

Conclusions

Results from infectivity assays, demonstrating symptom attenuation and decreased viral load in the presence of SACMV-DI along with its cognate virus, SACMV, has now confirmed the 1389bp molecule associated with SACMV DNA-B to be a defective interfering molecule. Infectivity assays conducted on two hosts, *N. benthamiana*, the model host and cassava, the natural host, have exhibited attenuated symptoms in the presence of this molecule as a direct result of decreased viral titre, determined by quantitative real-time PCR of both components DNA-A and DNA-B. It is believed that this is due to the DI molecule competing with the helper virus for limited host and viral factors needed for replication (reflected in lowered viral titres of plants co-infected with SACMV and DI compared to SACMV only). However, it is noted that the interdependence of DI, DNA-B and DNA-A for replication and movement factors, leads to cyclic patterns of replication, reflected in increases and decreases of the two DNA components during the systemic infection process. Additionally, it was observed that the presence of DI affected the ability of the helper virus to form chlorotic mosaic as a symptom in either of the host systems. Very little or no chlorosis was observed in either hosts when co-infected with SACMV and DI, although other typical symptoms such as blistering, leaf curling, stunting and deformed leaves were observed. It was also observed that for DI to successfully interfere with the helper virus replication, the DI needed to be present from the onset of virus infection i.e. DI needed to be present in the cells together with the helper virus at the initial site of infection.

Chapter 3: Transforming *Nicotiana benthamiana* with SACMV derived DI as a resistance strategy

Abstract

Satellites and DIs, associated with cassava-infecting geminiviruses, have been shown to sometimes attenuate infection symptoms. Therefore they are a potential strategy for developing CMD-tolerant cassava as transgenes which are replicated when the cognate helper virus infects the transgenic plants, thereby generating large populations of interfering molecules capable of reducing helper virus replication and movement. The aim of this study was to determine if a SACMV-derived DI transformed as a tandem repeat into *Nicotiana benthamiana*, would lead to symptom attenuation and a decrease in viral titre. The DI dimer insert was cut out of the plant vector pBIN19 with restriction enzymes *Bam*HI and *Hind*III, and transformed into pCAMBIA 1304. *N. benthamiana* leaf discs were agroinfiltrated, in a vacuum chamber at 85kPa for 10 min, with *Agrobacterium* strain AGL1 containing pCAMBIA 1304. Putative *N. benthamiana* transformants were screened by a histochemical assay to detect expression of beta-glucoronidase (GUS). Putative transformants were screened for the DI insert, and hygromycin and GUS reporter genes, using specific primers to each of these, by polymerase chain reaction (PCR), to confirm transformation. Transformants that were positive for all of the molecular tests were acclimatized and bulked up for virus challenge. Six transgenic *N. benthamiana* and wildtype *N. benthamiana* plants were agroinfiltrated with SACMV DNA-A and DNA-B infectious clones. As controls, healthy transgenic and wildtype *N. benthamiana* were not infected. At various time points during the study, plants were scored according to a symptom severity index, and restrictions of rolling circle amplification (RCA) products was performed on

extracted total nucleic acid (TNA) to detect the presence of DI. Viral load was measured by real time quantitative PCR, and compared between transgenic and non-transgenic plants.

Results from this study show that the levels of DNA-A and DNA-B increased in transgenic plants compared to wildtype *N. benthamiana* infected with infectious clones of SACMV DNA-A and DNA-B. Leaf symptom phenotypes of blistering, leaf curling, reduction in leaf size, and some chlorosis, were observed in both wildtype and transgenic plants challenged with SACMV. However, although limited symptom attenuation was observed in transgenic plants, they did appear to exhibit less chlorosis compared to wildtype plants. Stunting was occasionally observed following SACMV inoculation of either wildtype or transgenic plants, but differences between these two were not significant. The DNA-B titres in transgenics were lower at 14 dpi compared with wildtype, and then increased at 21 dpi. Therefore, it would appear that at 14 dpi, there may be some interference by the DI in lowering virus DNA B titres, which resulted in the subsequent delay of onset of symptoms at 21 dpi in transgenic plants. But by 28 dpi, the viral titres were neither lower, nor was symptom attenuation noted, between transgenic and wildtype *N. benthamiana*. The increased viral load and slight symptom attenuation in DI transgenic *N. benthamiana* is not encouraging in terms of using the SACMV DI transgene in cassava as a viable a resistance strategy in cassava.

3.1. Introduction

Satellites and DIs associated with geminiviruses causing CMD are also a potential strategy for CMD-resistant cassava and could also be used as possible transgenes which are replicated when the cognate helper virus infects the transgenic plants, thereby generating large populations of interfering molecules capable of reducing helper virus replication and movement (Patil and Dasgupta 2006). A previous study by Stanley *et al.* (1990) studied the effects of ACMV replication in transgenic *N. benthamiana* plants containing repeats of ACMV derived DI. A single copy of a tandem repeat was used to construct the transgenic plants, and upon agroinfection with both components of ACMV, a decrease in viral loads and symptom attenuation was observed. They observed that the ACMV DI insert episomally replicated in the presence of the helper virus ACMV, but was unable to replicate in the presence of other geminiviruses, *Tomato golden mosaic virus* (TGMV) and *Beet curly top*

virus (BCTV). The presences of the actively replicating DI in the transgenic plants lead to a 20% decrease in the DNA-A component whilst a 70% decrease was observed for the DNA-B component (Stanley *et al.* 1990). The symptoms observed were less severe in the transgenic plants compared to wildtype *N. benthamiana*. However DIs have not always been shown to cause symptom amelioration in other transgenic crops transformed with a DI (Horn *et al.* 2011) therefore for each geminivirus-host system one needs to test the DI.

The purpose of this study was to determine whether transformation of *N. benthamiana* with a SACMV derived DI as a tandem repeat, will lead to symptom attenuation and a decrease in viral titre. The DI episomally replicates only in the presence of the helper virus thereby removing the need of a transgene being constitutively expressed in the transgenic.

3.2. Materials and Methods

All the following steps were performed in aseptic conditions in a laminar flow cabinet, using sterile instruments and equipment.

3.2.1. Cloning of DI dimer insert into pCambia 1304

3.2.1.1. Plasmid extraction of pBIN19 containing the DI dimer

Escherichia coli transformed with pBIN19 containing the DI dimer insert was grown up overnight. Plasmid isolation was performed using alkaline lysis method based on Sambrook *et al* (1989). From the overnight bacterial culture, 1.5 ml was centrifuged at maximum speed in a miniSpin (Eppendorf) for 5 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in 100 µl of ice-cold solution I (50 mM glucose, 25 mM Tris-HCL pH 8.0, 10 mM EDTA) by vortexing and incubated at room temperature for 5 min. 200 µl of solution II (0.2 M NaOH, 1 % SDS) was added and mixed by gentle inversion. The suspension was incubated on ice for a maximum of 10 min. One hundred and fifty microlitres of ice-cold solution III (3 M potassium acetate, 5 M glacial acetic acid, made up to final volume with sterile distilled water) was added and mixed by inverting the microfuge tubes until a cloudy precipitate was formed and incubated on ice for a maximum of 10 min. The suspension was centrifuged at maximum speed for 10 min at 4 °C. The supernatant containing the plasmid DNA was transferred to a new microfuge tube and the centrifugation step was repeated. The supernatant was transferred to a new tube and 350 µl of isopropanol was added, mixed

by inversion and incubated at room temperature for 5 min. The DNA was precipitated by centrifuging at maximum speed for 10 min at 4 °C and the supernatant was discarded. The pellet was washed in equal volume, 1 mL, of ice-cold molecular grade 70 % ethanol (v/v %). The pellet was centrifuged at maximum speed for 10 min at 4 °C, and the supernatant discarded. The pellet was washed in 70 % ethanol for a second time and the supernatant discarded. Finally pellet was left to dry and resuspended in 50 µl of 1 X TE containing 10 mg/L RNase A. The extracted pBIN19 plasmid was incubated at 37 °C for an hour (to allow for RNA degradation by RNase A).

3.2.1.2. Restriction Digest with *Bam*HI and *Hind*III

A restriction digest was performed on the extracted plasmid using restriction enzymes *Bam*HI and *Hind*III (Fermentas) in Buffer R. The restriction digest was performed at 37°C for 3 hours and inactivated at 65°C. This double digest excises the DI dimer from pBIN19.

3.2.1.3. Ligation of the DI dimer in plant transformation vector *pCambia 1304*

Following the double digest, the DI dimer was isolated by gel extraction following the manufacturer's instructions (Qiagen). The extracted DI dimer was then ligated in a *Bam*HI linearised pCAMBIA 1304 vector using a 1:10 ratio (vector:insert) using T4 ligase (Fermentas) according to the manufacturer protocol. Fifteen microliters of the ligation reaction was used to transform competent *E.coli* DHα5 cells which were plated onto LB agar plates containing 100 mg/L kanamycin and incubated overnight at 37 °C. Individual colonies were screened for positive transformation using PCR and restriction digests on extracted plasmid.

3.2.2. Leaf disc Transformation

3.2.2.1. Preparation of leaf discs

Nicotiana benthamiana plants were grown in the greenhouse with a 16 hr light and 8 hr dark cycle. The upper fully expanded young leaves were collected from one month old plants and placed in a large beaker with distilled water for 20 – 30 min. The leaves were then placed in a 10 % bleach solution, containing one drop of Tween 20 for every 50 ml of the 10 % bleach solution, for 8 – 10 min. It was crucial that the leaves were not soaked for

more than 10 min. The leaves were then rinsed five times with distilled water. The leaves were then transferred to sterile filter paper, blotted dry and then stacked in a petri dish. Using a sterile scalpel leaves were cut into 1 cm² squares avoiding the midrib and leaf margins. One hundred leaf disks were placed adaxial side up in pre-culture medium Murashige and Skoog (MS) (without vitamins). Once the media was autoclaved and cooled to a temperature of 55 °C, it was supplemented with a vitamin and growth regulator solution (1000 X stock Gamborg vitamins; 100 µl of Benzylaminopurine (BAP), 10mg/L, 100 µL α-Napthalene acetic acid (NAA) 10mg/L. The leaf discs were limited to approximately 40 leaf disks per plate and incubated at 25 °C for 24 hr with 18 hr in light cycles.

3.2.2.2. *Bacterial Culture Conditions*

Transformed *Agrobacterium* strain AGL1 containing pCambia 1304 and the DI dimer were grown overnight at 30 °C at 220 rpm in YEP media (10 g/L peptone, 10 g/L yeast extract, 5 g/L sodium chloride, 20 mM magnesium chloride, pH 7.2) with the antibiotics 100 mg/L carbenicillin and 100 mg/L kanamycin. An aliquot of this was used to subculture fresh YEP broth containing 100 mg/L carbenicillin, 100 mg/L kanamycin and 0.5 M AS and was inoculated at 25 °C at 220 rpm. The cells were harvested by centrifuging the broth at 5000 g for 20 min at 4 °C (Beckman J2-21 centrifuge, Beckman Coulter). The resulting bacterial pellet was re-suspended in co-cultivation media (MS salts [without vitamins] 30 g sucrose) and 20 mM MES of pH 5.7). This media was supplemented with a vitamin and growth regulator solution containing 1000 X stock Gamborg vitamins; 100 µl BAP (100mg/L), 100 µl NAA (10mg/L) and 200 µM acetosyringone (AS). When the O.D. at 600 nm of the inoculum reached 1.0 the plate was put on ice until further use.

3.2.2.3. *Agroinfiltration of leaf discs*

The prepared *Agrobacterium* co-culture inoculum was transferred to tissue culture jars. Approximately 40 leaf discs were transferred into each jar. The leaf discs were then agroinfiltrated in a vacuum chamber at 85 kPa for 10 min. Following this, the leaf discs were carefully removed and briefly blotted dry on sterile filter paper and placed adaxial side on

solid co-cultivation media (MS salts with no vitamins, 30 g sucrose, 0.8% plant agar, 20 mM MES of pH 5.7 and 200 μ M acetosyringone). This media was supplemented with a vitamin and growth regulator solution containing 1000 X stock Gamborg vitamins; 100 μ l BAP (10 mg/mL), 10 μ l NAA (10 mg/mL). The leaf discs were co-cultivated with the agrobacterium for 3 days at 24 °C with 16 hr light and 8 hr dark cycle. It was ensured that not more than 40 leaf discs were placed on one plate.

3.2.2.4. *Selection of putative transformants*

After three days, the explants were transferred to liquid co-culture media containing 500mg/L cefotaxime and washed three times. The leaf discs were then blotted dry on sterile filter paper and transferred to selection media (MS salts with [no vitamins], 0.8% plant agar and 30 g sucrose using 1M potassium hydroxide (KOH) to bring to a pH of 5.7). Once the media was autoclaved and cooled to a temperature of 55°C, it was supplemented with these filter sterilised vitamin and growth regulator solutions (1000 X stock of Gamborg Vitamin, 10mg/L BAP, 1 mg/L NAA, 500 mg/L carbenicillin and 50 mg/L hygromycin. Approximately, 10 leaf discs were placed per selection media plate at 28 °C under a 16 hr light regime for 2 weeks before they were transferred to fresh selection media. This was repeated till callus clusters were formed along the edges of the leaf discs and multiple shoots were seen to be arising from different single callus clusters along the edges. These were then transferred to rooting media (MS salts [no vitamins] supplemented with filter sterilised vitamin and growth regulator solutions 1000 X stock of Gamborg Vitamin, 1 mg/L NAA , 500 mg/L carbenicillin, 50 mg/L hygromycin). The rooting media was poured into a tissue culture bottle to ensure sufficient plant development and the putatively transformed plants were placed in them with freshly made potassium permanganate (KMnO₄) which acts as an ethylene scrubber to encourage rooting. They were placed at 28 °C with 16 hr light and 8 hr dark cycles. Once the plant had established itself, clones were propagated into fresh rooting media and with each transfer, the antibiotic selection pressure was progressively reduced from 50 mg/L hygromycin to 30 mg/L hygromycin and finally 15 mg/L hygromycin.

3.2.3. Screening for putative transformants

3.2.3.1. *Histochemical assay*

Putative *N. benthamiana* transformants were screened by histochemical assay to detect expression of beta-glucuronidase (GUS). This was performed according a modified protocol by Joseph Sambrook and David William Russell (2001).

3.2.3.2. PCR

Putative transformants were screened using PCR. TNA was extracted as described in section 2.2.8. Several primers were used to detect different targets to confirm transformation. Primers that bind to the DI were used to confirm the presence of the insert (BC1 forward: 5' TAC GAT AAC CGA CCC AGT TGC GTT 3' and BC1 reverse: 5' TGC GAC TCA AAG GCC GAT GTA TGA 3') which yield an amplicon of 100 bp. Fused GUS-GFP primers were used to detect the presence of reporter genes. The mGUS-specific primers (mGUS:GFP forward: 5' TCA AGA CCC GCC ACA ACA T 3' and mGUS:GFP reverse: 5' ATG TTG TGG CGG GTC TTG A 3') were used to amplify a 364 bp region. Primers used to detect the presence of hygromycin the selection marker of the transformation vector were, forward: 5' CCG TCA GGA CAT TGT TGG AC 3' and the reverse primer 5': GCG CCG ATG GTT TCT ACA' amplifying a region of 462 bp.

3.2.3.3. Acclimatization of putative transformants

Putative transformants that were positive for all of the above tests were selected for further studies. The appropriate lines were bulked up and transferred into peat jiffies (Jiffy Products International). Once acclimatised, they were transferred to soil in pots. Plants were acclimatised in 25 °C with 16 hr light and 8 hr dark cycles.

3.2.4. Infectivity Assay

Transgenic plants from one transgenic line, that were positive in all screening methods, and that were phenotypically identical to wildtype *N. benthamiana* plants, were bulked up and selected for SACMV challenge studies. Both wildtype *N. benthamiana* and transgenic *N. benthamiana* were infected to compare the levels of helper virus present. Six transgenic *N. benthamiana* and six wildtype *N. benthamiana* plants were agroinfiltrated with SACMV DNA-A and DNA-B infectious clones. As controls, six transgenic and wildtype *N. benthamiana* were not infected. *Agrobacterium tumefaciens* AGL1 containing infectious clones of SACMV DNA-A and DNA-B were grown overnight separately, in YEP broth containing 100 mg/L carbenicillin and 100 mg/L kanamycin, while shaking at 28 °C. The

following morning, 5 ml of the overnight culture was inoculated into 45 ml of YEP broth supplemented with 100 mg/L carbenicillin, 100 mg/L kanamycin, 10 mM MES, 20 μ M AS. The culture was allowed to grow overnight with shaking at 28 °C. The following morning the cells were centrifuged at 10 000g at 4 °C for 10 min (Beckham Coulter) and the supernatant discarded. The cells were re-suspended in infiltration buffer (10 mM MgCl₂, 10 mM MES and 200 μ M AS with an O.D.₆₀₀ of 1). The cells were incubated at room temperature for 3 hr so as to induce activation of the *Agrobacterium vir* genes that is involved in the T-DNA transfer.

3.2.5. Total Nucleic Acid Extraction

Leaves from both transgenic and wildtype plants were collected at 7 dpi, 14 dpi, 21 dpi and 28 dpi. TNA was extracted using a modified CTAB method (J Doyle and JL Doyle 1987) as described in section 2.2.5.

3.2.6. Symptom Severity Scoring

At various time points during the study, scored according to a symptom severity index (Table 2.1 as shown in 2.2.10).

3.2.7. Confirmation of DI replication

3.2.7.1. Rolling circle amplification

Rolling circle amplification (RCA) was performed on extracted TNA using the Illustra™ TempliPhi 100 Amplification Kit (GE Healthcare Life Sciences) as per the manufacturer's instructions. Extracted TNA was diluted to a final concentration of 500 ng/ μ l. One microlitre of the TNA was added to 5 μ l of sample buffer and sealed in a microcentrifuge tube. This was then heated at 95 °C for 3 min and then allowed to cool to room temperature or 4 °C. While the template mixture cooled, the TempliPhi mastermix was made by adding 5 μ l of reaction buffer and 0.2 μ l enzyme mix for each reaction. The mastermix was thoroughly mixed and kept on ice. Five microlitres of this mastermix was added to each cooled down template mix. This was then incubated at 30 °C for 18 hrs. Following this, the enzyme was heat-inactivated at 65 °C for 10 min and the reaction was cooled at 4 °C.

3.2.7.2. Restriction analyzes

Restriction analysis on RCA products with the enzyme *PvuII* (Fermentas) as per manufacturer's instructions. Twenty units of enzyme were used to digest 1µl of RCA product at 37 °C for 16 hr.

3.2.8. Quantification of viral load

Transgenic *N. benthamiana* plants that were positive for the presence of both SACMV and DI; and wildtype *N.benthamian* plants that were positive for the presence of SACMV were selected to perform real-time PCR to quantify both DNA-A and DNA-B components of SACMV. Absolute quantification was performed on TNA extracted from leaf samples as per described in section 2.2.11., with 18S rDNA used to normalize the values obtained from absolute quantifications of DNA-A and DNA-B. The 18S rDNA primers used were forward primer: 5' GGC AAA TAG GAG CCA ATG AA 3' and reverse primer: 5' GGG GTG AAC CAA AAG CTG TA 3' (accession: AY079155) with an expected amplicon of 139bp. A previously constructed 18s rDNA clone was kindly provided by F. Van Schalk. This was linearized using *EcoRI*. This was used to construct a standard curve as described in 2.2.11.2.3 with the noted exception that the annealing temperature is 57°C. 18S rDNA was used as the endogenous gene to normalize the data obtained for DNA-A and DNA-B.

3.3. Results

3.3.1. Cloning of DI dimer in pCAMBIA 1304

The DI dimer present from the vector pBIN19 was successfully cloned into pCAMBIA 1304 and transformed into AGL1. Plasmid extracted from putative transformant was analyzed by restriction analysis using *BamHI* and *HindIII*, and the ±3000bp DI dimer was detected (Figure 3:1).

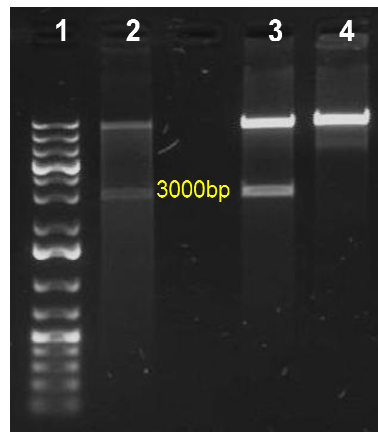


Figure 3:1 0.8% agarose gel showing the presence of the ± 3000 bp DI dimer excised from a successfully cloned pCAMBIA 1304 vector.

Lane 1: O'Gene Ruler 1kb (Fermentas), Lane 2: DI dimer of ± 3000 bp from pCAMBIA 1304 confirmed by restriction analysis using restriction enzymes *Bam*HI and *Hind*III. Lane 3: DI dimer ± 3000 bp from pBIN19 confirmed by restriction analysis using restriction enzymes *Bam*HI and *Hind*III. Lane 4: pCAMBIA 1304(negative control).

3.3.2. *N. benthamiana* leaf-disc transformation

3.3.2.1. Co-cultivation

After the leaves were prepared and cut into discs, they were allowed to acclimatize in pre-culture media adaxial side up for 24 hr (Figure 3:2. A). Once the leaf discs were vacuum infiltrated, they were co-cultivated for 3 days at 24°C to allow for transfer of T-DNA containing the insert in co-cultivation media (Figure 3:2 B).

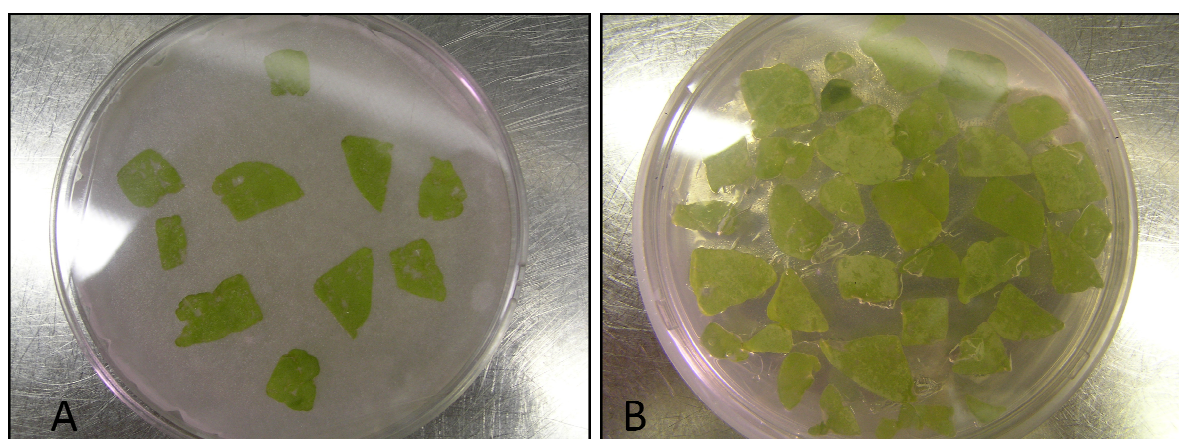


Figure 3:2. Transformation of *N. benthamiana* leaf discs.

Leaf discs placed on sterile filter paper in pre-culture media (A) and upon vacuum-infiltration of the leaf discs with transformed AGL1 containing pCAMBIA 1304 with DI dimer, they were placed on co-cultivation media for 3 days (B).

3.3.2.2. Selection of putative *N. benthamiana* transformants

Once placed on selection media the leaf discs underwent non-specific proliferation and the discs were seen to form callus and increased in size. After approximately 4 weeks, individual shoots were seen arising from the edges of the callus clusters (Figure 3:3.).

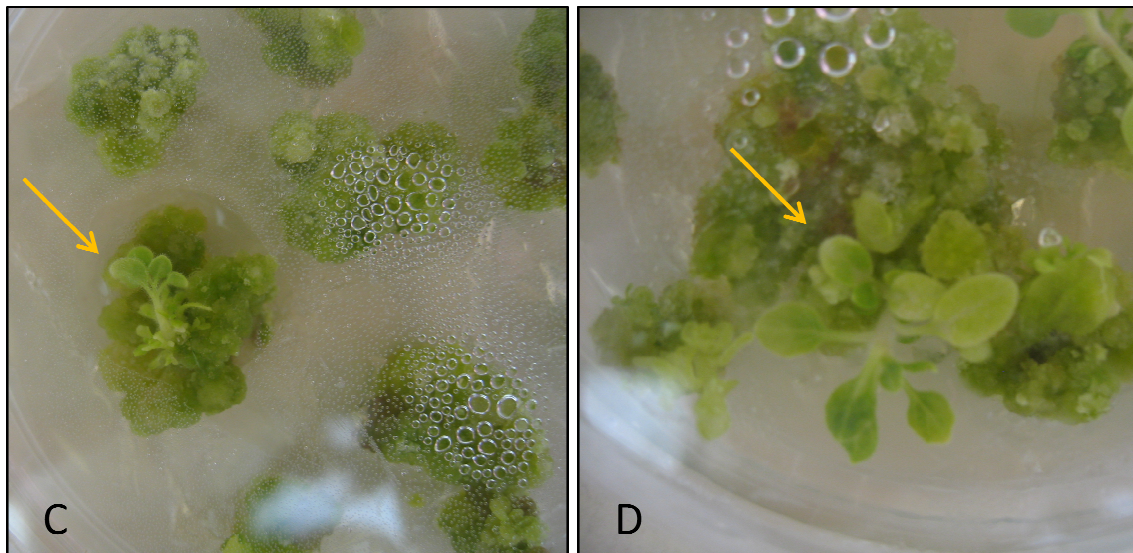


Figure 3:3. Putatively transformed leaf discs on selection media at 10 weeks (c) and 11 weeks (D). The arrow indicates single shoots, representing an individual transgenic line, arising from the edges of the formed callus which were excised and placed in rooting media

The shoots were left to proliferate until about 0.5cm in length and several shoots were then excised and placed in rooting media with hygromycin as the selective pressure. Out of 28 excised shoots, each representing a distinct transgenic line of *N. benthamiana*, only 10 lines survived the move from selection media to rooting media (Figure 3:4).

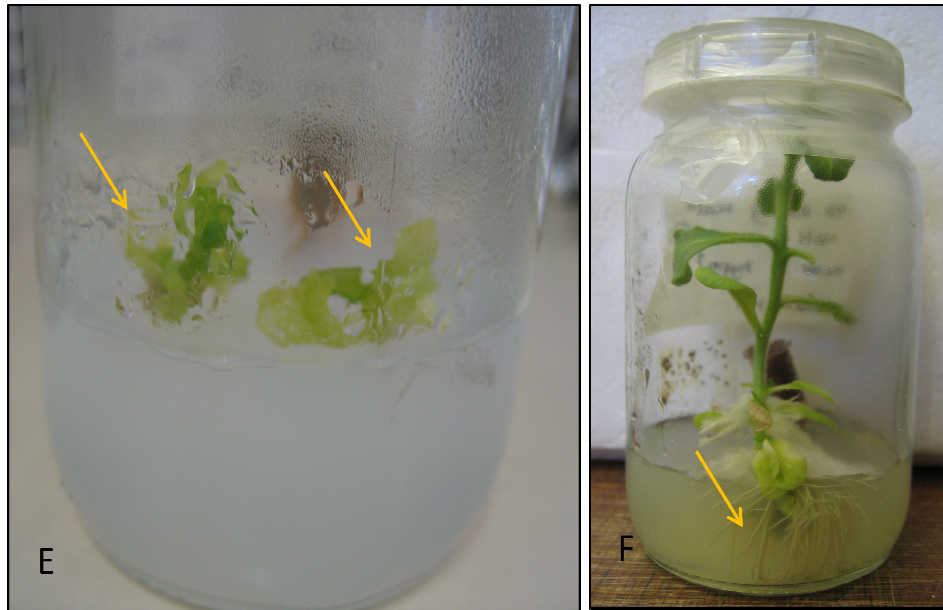


Figure 3:4 Putatively transformed *N. benthamiana* on rooting media.

Freshly excised shoots placed on rooting media (E) as indicated by the arrows. After 3-4 weeks of nodal propagation and transfer into fresh media the shoots have leaves (F) and a well established root system as indicated by the arrow (F) by 15weeks.

Once the transgenic plants were rooted and shooted (Figure 3:5), and tested positive for the DI insert, they were acclimatized and then subjected to challenge with SACMV.



Figure 3:5 Positively transformed *N. benthamiana* successfully acclimatized in growth chambers.

3.3.2.3. Screening putative transformants for insert

Putative *N. benthamiana* transformants were screened by histochemical assay to detect expression of beta-glucuronidase (GUS). Out of the 10 lines, 3 line exhibited uniform strong

GUS expression (Figure 3:6), while other 7 lines either exhibited chimeric or weak GUS expression.

TNA extracted from these putative transformants were used for molecular analysis to determine the presence of markers and the insert. BC1 primers were used to detect the presence of the insert (100 bp) in the extracted TNA (Figure 3:7). The *hptII* primers used to detect the presence of selection marker in the TNA, yielded an expected band of 456 bp (Figure 3:8). The mGFP5 primers were used to detect the presence of the GUS and GFP reporters in the extracted TNA (Figure 3:9). Successful PCR amplification was achieved from the transgenic plants, with all sets of primers, yielding the expected DNA sizes.

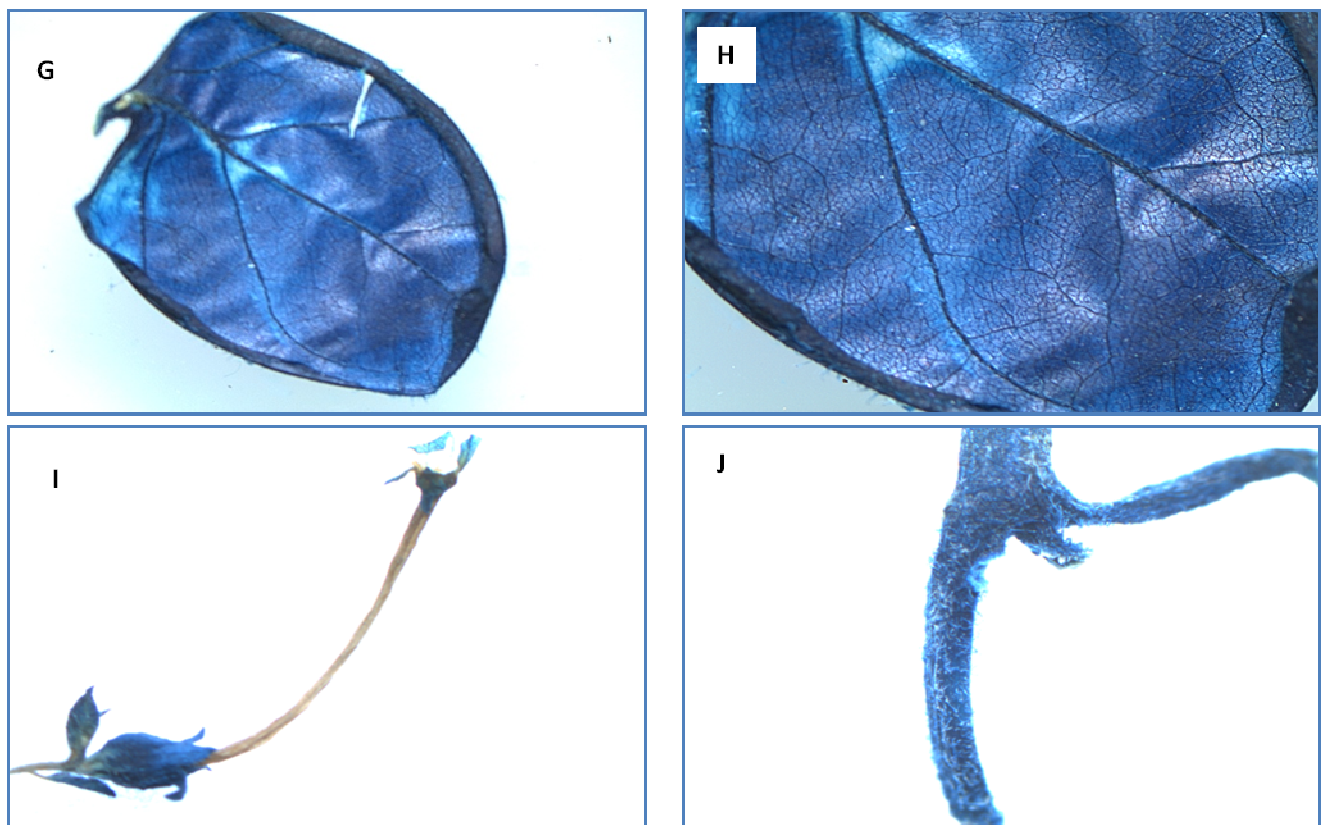


Figure 3:6 Histochemical assay of putative transgenic lines I, IV and X.

Different parts of the plants to test for gus expression – leaves (G, H) , flowers (I) and stem (J) all showed gus expression. Viewed under Olympus SZ2-SZ61 (20X).

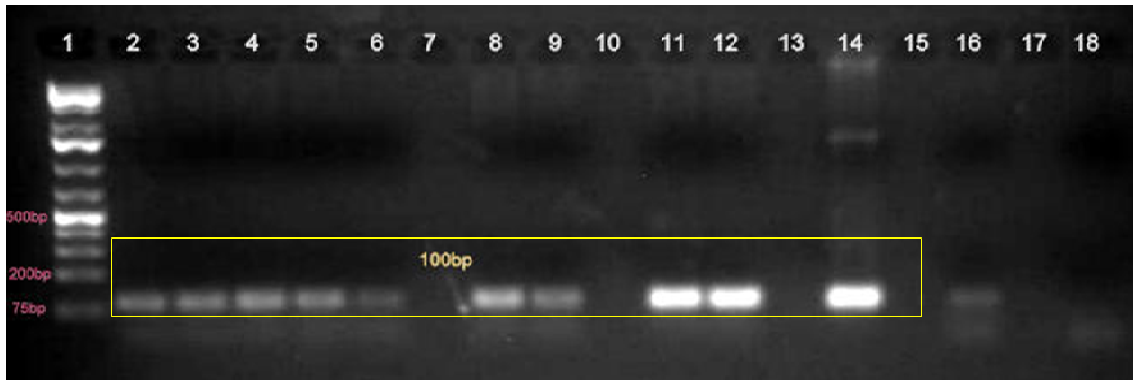


Figure 3:7 A 2% agarose gel showing PCR products (100 bp) of DI.

It is amplified by BC1 primers, (the BC1 primers bind to the BC1 ORD present on the DI) in healthy transgenic *N. benthamiana* TNA. Lane 1: O'gene 1kb ruler (Fermentas), Lane 2 – 6: line Ia, Lane 8-9: line IV, Lane 11-12, Line IIIa, Lane 14: pCAMBIA1304+DI, Lane 16: empty pCAMBIA 1304, lane 18: NTC.

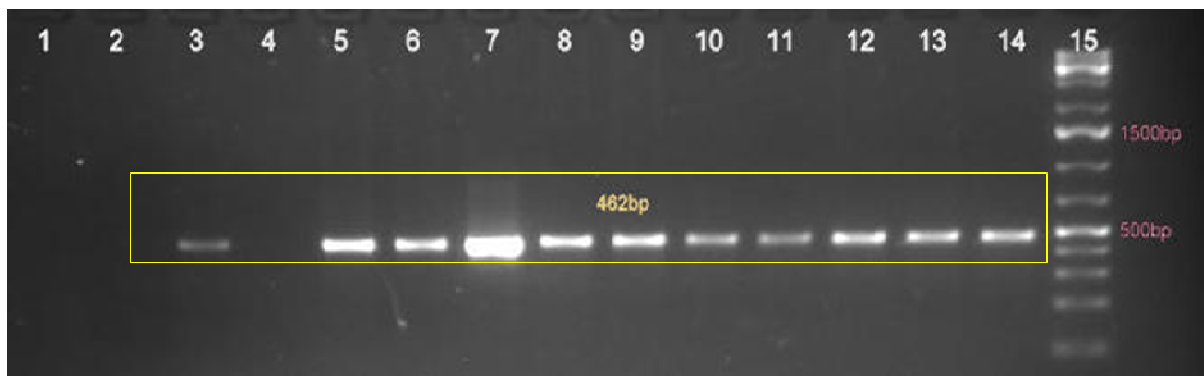


Figure 3:8. 2% agarose gel showing PCR products of *hptII* (462bp) from healthy transgenic *N. benthamiana* TNA.

Lane 1: NTC, Lane 3: pCAMBIA 1304+DI plasmid (positive control), Lane 5-6: *N.ben* Line Iva, Lane 7-8: *N.ben* Line IIIa, Lane 9-14: *N.ben* Line Ia. Lane 15: O'Gene 1kb Ruler (Fermentas).

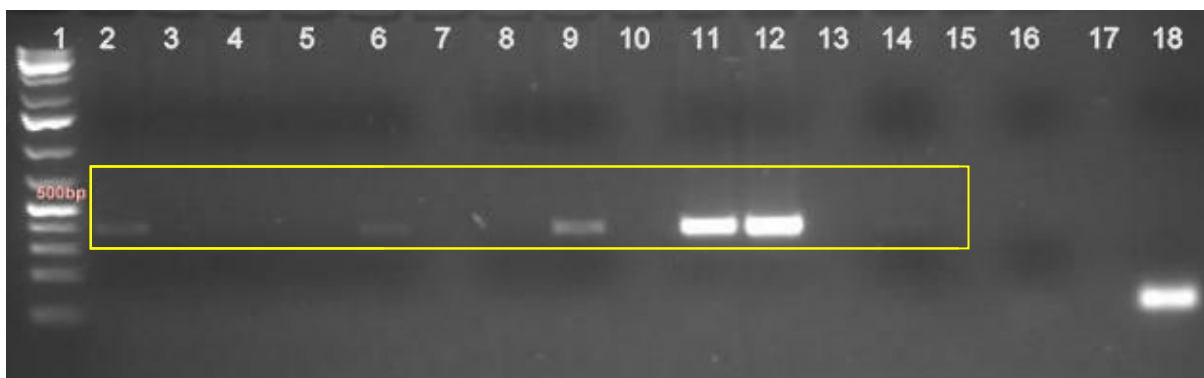


Figure 3:9. 2% agarose gel showing amplified PCR products (364bp) of mGFP5 primers on healthy transgenic *N. benthamiana* TNA.

Lane 1: O'Gene 1kb Ruler (Fermentas), lane 2 – 6: N.ben Line Ia, line 8-9: Line IV, Lane 11-12, Line IIIa, Lane 14: pCAMBIA1304+DI, lane 16: empty pCAMBIA 1304 plasmid, Lane 18: NTC.

3.3.3. Infectivity assay

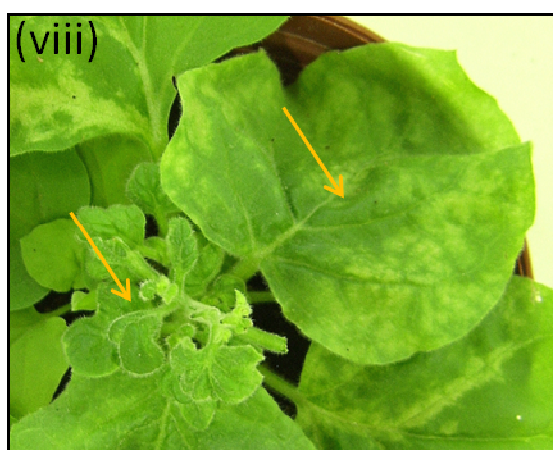
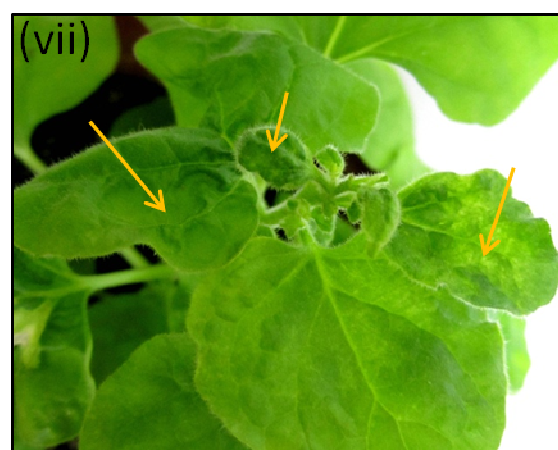
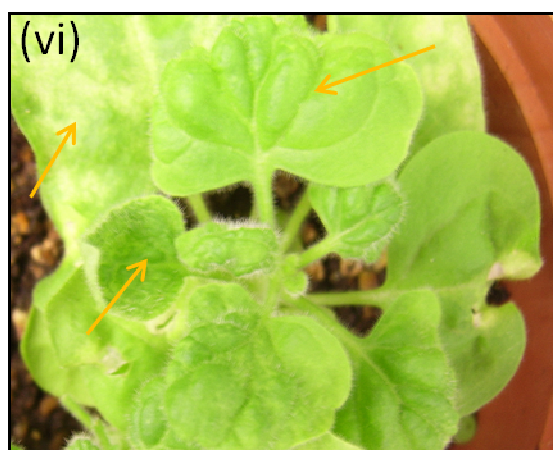
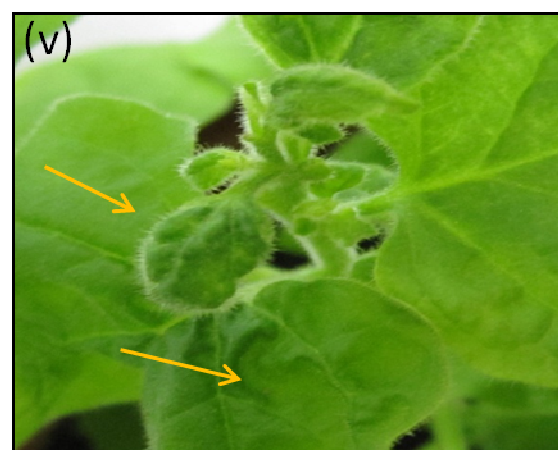
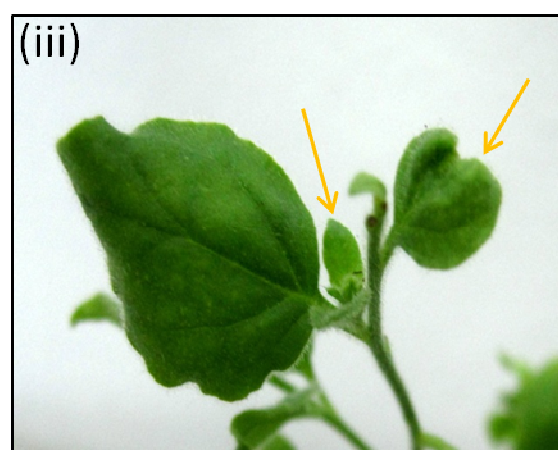
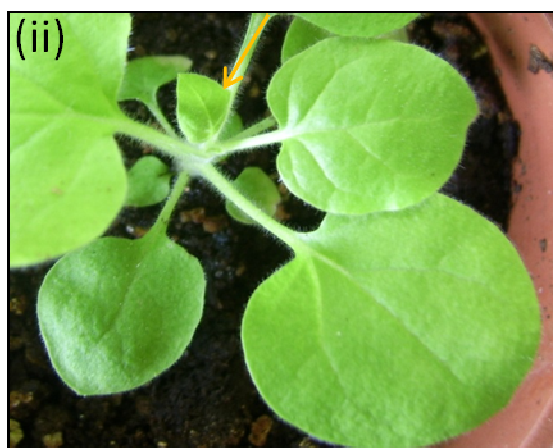
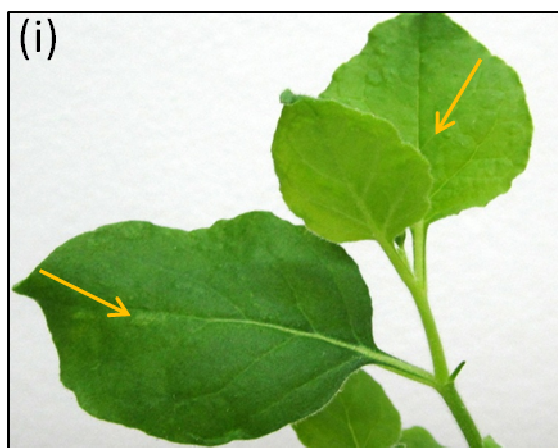
3.3.3.1. Symptom Severity Scoring

Both wildtype and transgenic plants were observed for symptoms at different time points post SACMV challenge. At 28 dpi, the wildtype *N. benthamiana* appeared to be slightly more symptomatic (4 - 5) than transgenic plants. However, no difference in symptom severity was observed, between the two treatments, at earlier time-points (Table 3:1 and Figure 3:10). A delay of onset of symptoms was observed at 21 dpi, but by 28 dpi the symptoms of transgenic plants were similar to that of wildtype.

Table 3:1. Symptom severity scoring of transgenic *N. benthamiana* compared to wildtype *N. benthamiana* at various time points (dpi) using the symptom severity index by Fauquet and Fargette (1988).

Time points/Treatments	7 dpi	14 dpi	21 dpi	28 dpi
Transgenic infected with SACMV	1	2	2	4
Wildtype infected with SACMV	1	2	3	4-5

However, even though there was no observable difference in symptom severity between wild-type and transgenic plants, subtle differences in symptom phenotype were observed in transgenic plants, in that they appeared to be less chlorotic (Figure 3:10 v & vii) compared to wildtype plants (Figure 3:10 vi & viii).



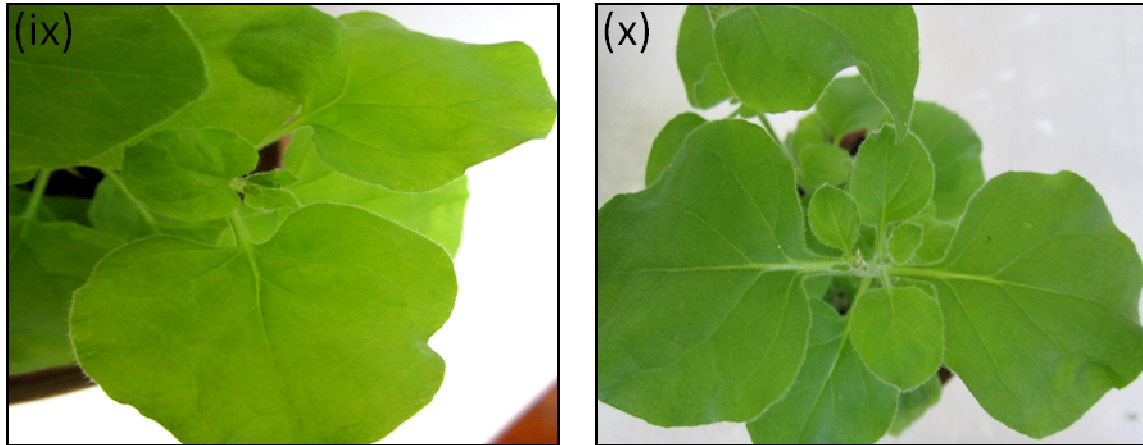


Figure 3:10 Figure (i) to (x) showing symptoms exhibited by both transgenic *N. benthamiana* and wildtype *N. benthamiana* over a 28 dpi time.

Figures (i), (iii), (v) and (vi) show symptoms of transgenic *N. benthamiana* at 7, 14, 21, 28 dpi. Figures (ii), (iv), (vi), (vii) show wild-type SACMV-challenged *N. benthamiana* at 7, 14, 21, 28 dpi and (x) healthy unchallenged plant.

3.3.3.2. Confirmation of replicating DI

The presence of DI replicating in the presence of SACMV helper virus was confirmed using restriction analysis of RCA amplified products. A band of approximately 3000 bp confirmed the presence of the helper virus DNA-B component, whilst the smaller band of approximately 1500 bp confirmed the presence of the DI. Samples that contained both SACMV and DI were further selected for real-time qPCR analysis (Figure 3:11).

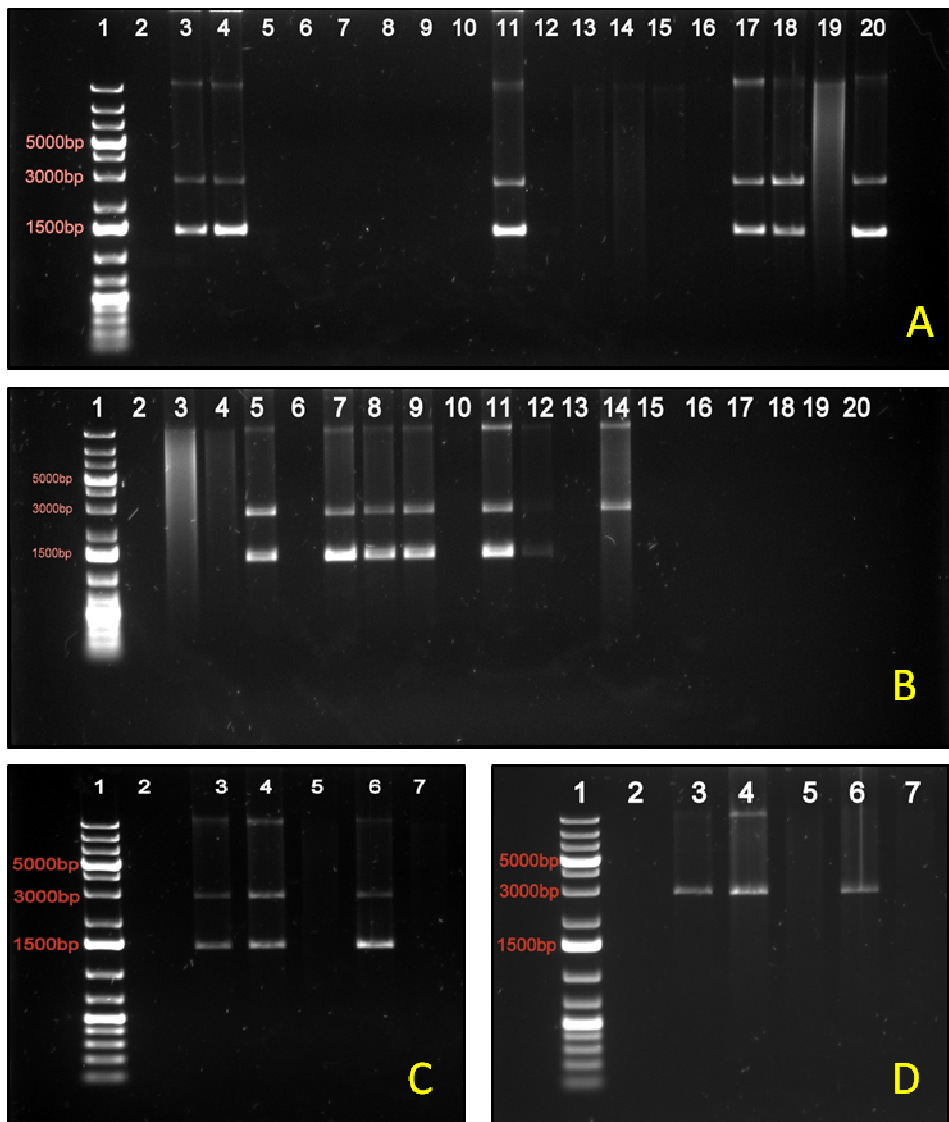


Figure 3:11. A 0.8% agarose gel showing products of *PvuII* restriction analysis of RCA products.

A band of approximately 3000 bp (gels A,B and C) confirmed the presence of the helper virus DNA-B component whilst the smaller band of approximately 1500 bp (A,B,C,and D) confirmed the presence of the DI. Lane 1: O'Gene 1kb Marker (Fermentas) in Figures A,B,C and D.

3.3.4. Virus Quantification

Concentration of DNA-A molecules were found to be higher in transgenic *N. benthamiana* than wildtype *N. benthamiana* at all time points. The lowest concentration of DNA-A molecules observed in the transgenic plants was at the first time point of 7 dpi after which the concentration seems to be stable during the rest of the infection process.

In wildtype *N. benthamiana* plants, SACMV DNA A molecules were detected at 7 dpi, as expected, and increased at 14 dpi (Figure 3:12). Thereafter, the virus titres dropped at 21 and 28 dpi. The lowest concentration of DNA-A molecules observed in the transgenic plants

was also low at the first time point (7 dpi), after which the concentration seemed to be stable during the rest of the infection process. Our qPCR study demonstrated that the concentration of SACMV DNA-A molecules was higher in transgenic *N. benthamiana* compared with wildtype *N. benthamiana* at all time points (Figure 3:12).

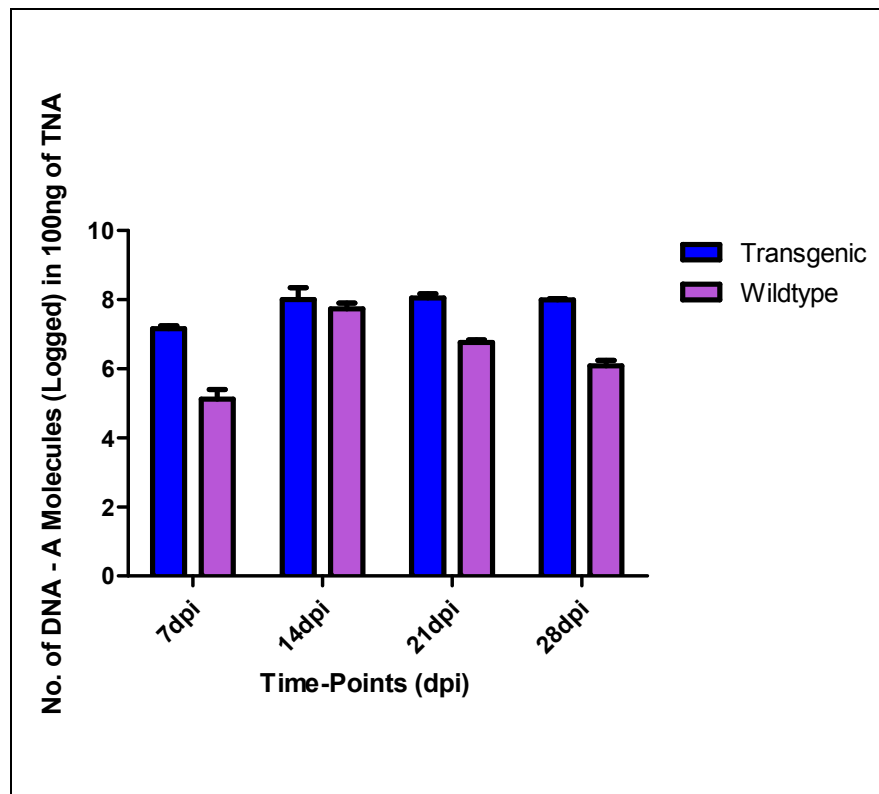


Figure 3:12. Number of molecules of SACMV DNA-A (log scale) found in 100 ng of infected transgenic and wildtype *N. benthamiana* TNA.

The TNA extracted from the various experimental treatments at different time-points. This data has been normalized using values obtained with the *N. ben* 18S rDNA gene. A significant increase of DNA-A concentrations was found in the transgenics *N. benthamiana*.

Concentrations of DNA-B for transgenic *N. benthamiana* was higher at all time-points compared to the concentrations found in wildtype *N. benthamiana* (Figure 3:13). At 7 dpi the lowest concentration of DNA-B was found in both transgenics and wildtype. Analysis by 2-WAY ANOVA found that the difference in viral titre between transgenic and wildtype plants were extremely significant ($P = 0.0009$).

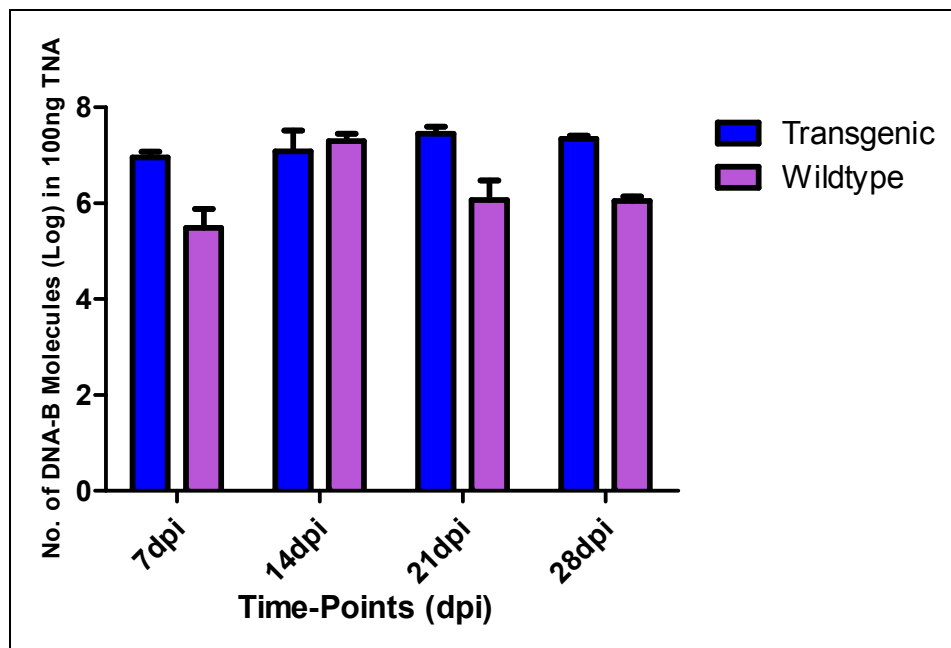


Figure 3:13. No. of DNA-B molecules (logged) in wildtype and transgenic *N. benthamiana* agroinfiltrated with SACMV DNA-A and DNA-B.

The concentration of DNA-B is higher in the transgenic plants compared to the wildtype. All values were normalized using the endogenous gene 18S rDNA gene. A significant increase of DNA-B concentrations was found in the transgenics *N. benthamiana*.

DNA-B levels in transgenic plants were highest at 21 dpi; whilst in wildtype plants they were highest at 14 dpi.

3.4. Discussion

In this study, our objective was to evaluate the potential of a DI molecule in modulating (ameliorating) cassava mosaic disease symptoms in transgenic *N. benthamiana*. If this was the case, one could argue for applying this strategy in SACMV's natural host, cassava. However, *N. benthamiana* transformed with a tandem repeat of DI showed no decrease in viral titre of SACMV. In the presence of the helper virus, the DI is recognised and replicated from the transgene, thereafter undergoing circularisation, episomal replication and amplification. Based on several studies in the literature (Stanley *et al.* 1990, 1997; Patil and Dasgupta 2006), it is postulated that the DI will interfere with the replication of the virus, through some form of competition, leading to decreased viral load and attenuated symptoms. Initially, we postulated an increased viral load representing initial virus proliferation and systemic infection, and a decreased viral load as a result of accumulating DI interfering with the helper virus at later time-points. However, this was not the case in our study, as an increased viral load (both DNA A and B components) was observed for transgenic plants infected with SACMV compared to wildtype. Symptom attenuation was limited, or equally similar, to wildtype *N. benthamiana* infected with SACMV (Figure 3:10). However, a slight delay in onset of symptoms was observed at 21 dpi in transgenic plants, where the symptom severity remained at the score of 2 (scale 1-5) from 14 to 21 dpi (Table 3.1). Subtle differences in symptoms were observed in transgenic plants, they seemed to be less chlorotic compared to wildtype plants (Figure 3:10). It is also notable, that DNA B titres were lower at 14 dpi compared with wildtype, and then increased at 21 dpi. Therefore, it would appear that at 14 dpi, there may be some interference by the DI in lowering virus DNA B titres, causing a slight delay in symptom onset, but this does not last. It may be suggested that 14 dpi is a critical stage in the infection progression of SACMV in *N. benthamiana*, as it represents the stage where the virus is moving systemically and inducing the appearance of detectable symptoms. Since DNA B BC1 ORF codes for the cell-to-cell long distance movement protein, its role at this stage of infection is especially crucial, as it is at this stage that SACMV moves from the initial infection site from cell to cell into the phloem. Any inhibition of this process could be considered significant. The amplified DI is also competing with its cognate helper virus SACMV for movement, since its BC1 ORF is truncated at the 3'end, and it may require SACMV DNA B protein to assist in intracellular

movement from the nucleus to the cell wall. Therefore, based on this study where a slight delay of symptoms, and transient decrease in DNA-B (measured by real-time qPCR) was observed in transgenic plants, compared with wildtype, it is suggested that the DI is interfering at this time-point. This delay is not effective enough to have a longer term impact, and is not sustained as infection progresses. Since there is a lack of experimental studies on transgenic DIs in the literature, and the limited studies do not report consistent results for geminiviruses (Stanley *et al.* 1990; Horn *et al.* 2011) we cannot make any more definite conclusions. Without larger experimental data sets in our SACMV-DI-*N. benthamiana* system, and more examples from other geminivirus-DI-host, it would not be possible to draw any further unequivocal conclusions about the molecular mechanisms at play at this point.

Contradictory to our results, an early study by Stanley *et al.*, (1990), where ACMV DNA-B derived DI was similarly used in a tandem repeat to transform *N. benthamiana*, showed a knockdown of symptoms of the cognate helper virus. Upon infection with ACMV, symptom attenuation was observed in plants over a 3-week period. Using dot- blot analysis on extracted TNA, it was determined that there was approximately a 20% decrease of the DNA-A component in infected transgenic plants compared to control plants, and a 30% decrease of the DNA-B component in transgenic plants. However, in our study, a 60 fold increase in DNA-A levels were observed in transgenics, whilst a 20 fold increase in DNA-B levels were observed in transgenic plants at 28 dpi (Figures 3:11 and 3:12). Although the difference in viral load between transgenic and wildtype are significant (2-way ANOVA, ($P < 0.0001$), the phenotypic effect on symptom amelioration was low. However it must be pointed out that real-time quantitative PCR is in order of magnitude far more sensitive and accurate than dot-blots, and one cannot directly compare viral loads results between the SACMV and ACMV studies. The relevant observation is that even two geminiviruses belonging to the same family, but being distinct species (Patil and Fauquet 2009) may behave differently in the same model host. It would be interesting to see how these DIs behave in the natural host cassava, as no studies on transgenic cassava with a DNA-B DI insert have been reported.

The increased levels of SACMV in transgenic compared to wildtype could be due to several reasons. The location of integration of the DI dimer in the *N. benthamiana* genome could affect SACMV and DI replication in the host.

In a study by Horn *et al.*, (2011), the effects of *Beet curly top virus* (BCTV) associated DI in transgenic *N. benthamiana* on symptom development, upon infection by BCTV, was studied. During the course of the study, they observed ambivalent effects on helper virus levels and symptom development. This was due to several reasons, one of the major ones being the *de novo* production of DI's, not trans-replicated DIs from the transgene. These *de novo* DIs had an attenuating effect on the symptoms of BCTV compared to plants that trans-replicated DI. Additionally, the attenuating effect of these *de novo* DIs were more effective in the absence of the trans-replicated DIs. Horn *et al.* (2011) observed that the presence of the transreplicated DIs exacerbated the symptoms of BCTV in transgenic plants that only contained the transreplicated DI. Although agroinfection of *N. benthamiana* with SACMV DNA-A and DNA-B never appeared to generate spontaneous DIs (during PCR with abutting DNA primers) in our laboratory, and agroinfection of SACMV with DI infectious clones also resulted in detection (by RCA/RFLP) of a single DI population of the correct size, we cannot rule out that we may have the production of *de novo* DIs in our transgenic *N. benthamiana*, and this warrants further interrogation. Since we did not detect for *de novo* DIs, it may be hypothesised that the possible higher viral load in transgenic plants, compared with wildtype, could be due to the presence of transreplicated DIs that induce severe symptoms, similar to the results reported for BCTV (Horn *et al.*, 2011). Another possibility is that for a transgene-derived DI method to be successful, the DI needs to be initially replicated from the transgene by the helper virus when it enters a new cell, followed by episomal transreplication which results in the DI moving systemically with the helper virus. This implies that the DI would need to be replicated in every cell for it to be a successful resistant strategy and that any delays in this, in relation to the helper geminivirus, may result in partial or total ineffective interference by the DI. This hypothesis may be supported by the recent study by Horn *et al.* (2011) which demonstrated the more attenuating effect of *de novo* DIs compared with transgene transreplicated DI molecules. *De novo* DIs generation would always accompany helper geminivirus DNA-A and DNA-B replication, as DIs depend on their cognate helper for replication, and therefore are always present with the helper in

the cell. This may therefore circumvent potential delays in their replication, ensuing rapid interference via competition for viral and host components. Transgene DI transreplication dependence on the helper virus, such as SACMV, may result in a delayed response, allowing the cognate helper virus to establish itself quickly. Furthermore, modulation of helper geminivirus and DI is likely taking place due to the inter-dependence of these two entities (Stanley *et al.* 1990). Dependence of the DI on their cognate geminivirus helper DNA A component for Rep, and possible dependence on DNA B for long distance movement, especially in cases where BC1 is truncated (such as SACMV DI), implies that as the helper geminivirus replication is reduced, so would the dependent DI. This decrease in DI titre would result in an increase in helper geminivirus. Therefore, the balance between DNA A, DNA B and DI is likely to result in a modular pattern of increases and decreases in these DNA components, which may be variable, and which may influence host responses and symptom development. Clearly, more research into the molecular mechanisms of DI interference, and into the inter-relationship between DI, cognate helper geminivirus DNA A, and DNA B, needs to be undertaken.

Conclusion

The increased viral load and slight symptom attenuation in DI transgenic *N. benthamiana* does not encourage the possibility of using the SACMV DI transgene as a resistance strategy in cassava. Although this particular strategy is favoured because the DI dimer is only replicated in the presence of an infection and is not expressed constitutively, it has not proven to be successful in its ability to interfere with the helper virus replication. Another disadvantage of using DIs is that they are highly virus specific, and mixtures of geminivirus isolates often found in the field, and the high recombinant rate of geminiviruses (Legg and Fauquet 2004) does not allow for a broad-based resistance strategy. This specificity has been shown in other studies in transgenic *N. benthamiana* plants with a DI-based BCTV (Logan strain) resistance, which were not resistant to other strains (Stenger *et al.* 1992). No other investigations, to date, of the mechanism of such transgene-derived DI interference, or of any other DNA B DI molecules associated with the different cassava geminivirus species, other than ACMV, and SACMV, have been reported. However, recently a naturally truncated form, 1525bp, of *East African cassava mosaic virus* (EACMV) DNA A was isolated in Tanzania (Ndunguru *et al.* 2006). This defective DNA A resulted in symptom amelioration

and a decrease in EACMV DNA A and DNA B in biolistically inoculated *N. benthamiana*. However, this DI was not tested in transgenic plants. Further studies, such as determining the copy number of the DI tandem present in the transgenic *N. benthamiana* lines may shed some light on the why the SACMV transgene-derived DI was not effective in reducing virus infection to an acceptable level. Further studies such as decreasing the size of the DI, ensuring that the essential *cis* elements required for replication are conserved, and a better understanding of the molecular mechanisms involved in DI interference, may provide a basis for designing better resistance strategies.

Chapter 4: General Conclusion

South African cassava mosaic virus (SACMV) is a known cassava mosaic disease causative agent in South Africa. SACMV is a distinct bipartite geminivirus and a putative defective interfering (DI) molecule has been associated with SACMV. It is half the size of the helper genome (1387 bp) and has a 98 % sequence homology to the DNA-B component of SACMV. The defective DNA has retained the common region, found on both the DNA-A and DNA-B components of SACMV, containing the origin of replication and the necessary *cis* elements required for replication. The DI also contains part of the BC1 open reading frame found of SACMV DNA-B. In this study effect of DI on SACMV replication and/or symptom attenuation in model host, *N. benthamiana* and the natural host cassava, was studied.

Quantitative real-time PCR results from this study show that the DI did influence viral titres in *N. benthamiana* and cassava when infected with SACMV. In both plant systems, at the earliest time-points, there was a significant decrease in SACMV DNA-A and DNA-B viral titres in plants co-infected with SACMV and DI compared to plants infected with SACMV only. This is due to the DI molecule competing with the helper virus for limited host and viral factors needed for replication. Similarly, a significant decrease in viral titres was observed at 42 dpi as well (except for DNA-A in *N. benthamiana*). The lower viral titres correlated with attenuated symptoms observed in *N. benthamiana* and cassava. In *N. benthamiana*, plants co-infected with SACMV and DI exhibited less stunting, chlorosis and leaf curling. In cassava, plants co-infected with SACMV and DI showed less leaf curling, leaf deformation and very limited or no chlorosis. It was interesting that the presence of DI affected the ability of the helper virus to form chlorotic mosaics as a symptom in both host systems. This is similar to the effects observed by DI molecules associated with AYVV. It was found that the AYVV DI influenced the pattern of leaf chlorosis and leaf curling changing the symptom phenotype and caused a delay in the accumulation of helper virus in *N. benthamiana* (Stanley *et al.* 1997). It was also observed that for DI to successfully interfere with the helper virus replication, the DI needed to be present from the onset of virus infection i.e. DI needed to be present in the initial site of infection along with the helper virus.

Satellites and DIs associated with cassava-infecting geminiviruses are a potential strategy for developing CMD-tolerant cassava, and could also be used as possible transgenes which are replicated when the cognate helper virus infects the transgenic plants, thereby generating large populations of interfering molecules capable of reducing helper virus replication and movement. This study aimed to determine if a SACMV-derived DI transformed as a tandem repeat into *N. benthamiana*, would lead to symptom attenuation and a decrease in viral titre. Viral load was measured by real time quantitative PCR, and compared between transgenic and non-transgenic plants. Results from this study show that the levels of DNA-A and DNA-B did increase in transgenic plants compared to wildtype *N. benthamiana* infected with infectious clones of SACMV DNA-A and DNA-B. Symptom attenuation was limited, subtle differences in symptoms were observed in transgenic plants, they seemed to be less chlorotic compared to wildtype plants. The DNA B titres in transgenics were decreased at 14 dpi compared with wildtype, but increased at 21 dpi. Therefore, it would appear that at 14 dpi, there may be some interference by the DI in lowering virus DNA B titres. This seemed to have caused a slight delay in symptom onset, which resulted in the slight delay of onset of symptoms at 21 dpi in transgenic plants. But by 28 dpi, the viral titres were neither lower, nor was any symptom attenuation noted between transgenic and wildtype *N. benthamiana*. A study by Horn *et al.* (2011) on transgenic *N. benthamiana* containing *Beet curly top virus* derived DI showed that DI replicated from the transgenic genome did not attenuate symptoms of infected plants. We believe that for the transgenic strategy to be successful, the DI would have to be episomally replicated in every cell, but may not be happening in this case, thus offering another explanation of why the transgenic study was unsuccessful.

The increased viral load and slight symptom attenuation in DI transgenic *N. benthamiana* does not encourage the possibility of using the SACMV DI transgene as a resistance strategy in cassava. Although this particular strategy is favoured i.e. the DI dimer that is only replicated in the presence of an infection and is not expressed constitutively, it has not proven to be sufficiently effective in transgenics in its ability to interfere with the helper virus replication. Another disadvantage of using DIs is that they

are highly virus specific, and mixtures of geminivirus isolates often found in the field, and the high recombinant rate of geminiviruses (Legg and Fauquet 2004), may prove challenging a broad-based resistance strategy. To our knowledge, the mechanism of such transgene-derived DI interference, or of any other DNA-B DI molecules associated with the different cassava geminivirus species, other than ACMV, BTCV, and now SACMV, have been reported. Our study has contributed further to this understudied field, but many more studies are urgently needed, before this strategy is discarded. Further studies, such as determining the copy number of the DI tandem present in the transgenic *N. benthamiana* lines may shed some light on the why the SACMV transgene-derived DI was not effective in reducing virus infection to an acceptable level. Further studies such as decreasing the size of the DI, ensuring that the essential *cis* elements required for replication are conserved, and a better understanding of the molecular mechanisms involved in DI interference, may provide a basis for designing better resistance strategies.

With current biotechnology techniques available, and new insights into virus-host interactions gleaned through high throughput genomics, proteomics and bioinformatics platforms, the potential of DIs warrants further exploration. We do not have enough transgene-derived DI experiments with different geminiviruses and hosts to conclude that DIs may prove completely ineffective. In our SACMV-*N. benthamiana* system they did not demonstrate a high enough helper virus knockdown, but this DI needs to be tested in cassava, the natural host. Therefore we conclude that although DI does decrease viral titres and lead to symptom attenuation during natural systemic infections or experimental inoculations; much more research is required to understand the molecular mechanisms of DI-induced resistance, and the interdependence of the DI-helper virus-plant host biological system.

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