# Screening of cassava improved germplasm for potential resistance against cassava mosaic disease

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Master of Science in the School of Molecular and Cell Biology.

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## Research Outputs

## Conferences

Rey, M.E.C., Mvududu, D. Moralo, M. (2015). Improved virus-resistant cassava for small-scale and commercial farmers. Oral presentation at the 8th Annual Agriculture Symposium, 04 June 2015, Pretoria.

Rey, M.E.C., Mvududu, D., Moralo, M. (2016). Screening of CMM6 and AMM2 transgenic cassava lines for resistance to cassava mosaic disease. 19th Biennial SASM conference. Coastlands Umhlanga Durban 17-20 January 2016 (Poster presentation).

Rey, M.E.C., Mvududu, D., Moralo, M. (2016). Screening of cassava expressing stacked non-mismatched or mismatched inverted repeat constructs derived from *African cassava mosaic virus* ORFs. Oral presentation at the 9th Annual Agriculture Symposium, 02 June 2016, Pretoria.

## Abstract

With growing populations and climate change associated drought predicted for the future, cassava can provide one solution for food security and a source of starch for industrial use and biofuels in South Africa, and other countries in the SADC region. One of the severe constraints on cassava production is cassava mosaic disease (CMD) caused by cassava infecting begomoviruse species, including *African cassava mosaic virus* (ACMV), *South African cassava mosaic virus* (SACMV) and *East African cassava mosaic virus* (EACMV). Cassava begomoviruses (CBVs) are responsible for significant yield loss of the starchy tubers. Since no chemical control of virus diseases of plants is possible, one approach to develop virus resistance is via biotechnology, through genetic engineering (GE) of cassava with hairpin RNA (hpRNA) silencing constructs that express small interfering RNAs targeting CBVs and preventing severe disease development. The aim of this project was to subject previously transformed five CMM6 cassava lines (cv. 60444 transformed with a non-mismatched *Africa cassava mosaic virus*-[Nigeria:Ogorocco;1990] (ACMV-[NG:Ogo:90])-derived hpRNA construct, six AMM2 (cv. 60444 transformed with a mismatched ACMV-[NG:Ogo:90]-derived hpRNA construct), six CMM8 cassava lines (cv.60444 transformed with a non-mismatched SACMV BC1-derived hpRNA construct) and seven AMM4 cassava lines (cv.604444 transformed with a mismatched SACMV BC1-derived hpRNA construct) to reproducible trials, and evaluate for response to virus challenge. The ACMV-[NG:Ogo:90] hpRNAi constructs target 4 overlapping virus open reading frames (ORFs) (AC1 replication associated protein/AC4 and AC2 transcriptional/AC3 replication enhancer), while the SACMV hpRNAi constructs target the cell-to cell movement BC1 ORF. Non mismatched constructs consist of a transformation cassette that has an intron separating the sense and antisense arms of the viral transgene whilst mismatched constructs have the sense arm of the viral transgene treated with bisulfite to induce base mutation. This mutated sense arm is then separated from the non mutated antisense arm by a small spacer. Furthermore, a 229 bp inverted repeat hpRNA construct (DM-AES) was designed to target ACMV-[NG:Ogo:90] 117 nt putative promoter region (2714-49 nt), a 91 nt overlapping sequence (1530-1620 nt) between ACMV-[NG:Ogo:90] AC1 3’ end and AC2 5’ end (AC1 3’/AC2 5’-ter) as well as being efficient against SACMV and EACMV due to the inclusion of a 21 nt conserved sequence (1970-1990) of AC1/Rep shared between ACMV, EACMV and SACMV. Cassava landrace T200 friable embryogenic callus (FEC) were transformed with this construct. The

selected transgenic lines were infected with either ACMV-[NG:Ogo:90] (CMM6 and AMM2 transgenic lines) or SACMV (CMM8 and AMM4 transgenic lines) by agro-inoculation and monitored at 14, 36 and 56, 180 and 365 days post infection (dpi) for symptom development, plant growth and viral load. From the ACMV trials 3 lines (CMM6-2, CMM6-6 and line AMM2-52) showed significantly lower symptom scores and lower viral load at 36, 56 and 365 dpi, compared with viral challenged untransgenic cv.60444. This phenotype is described as tolerance, not resistance, as despite ameleriorated symptoms virus replication persists at lower levels. From the SACMV infectivity trials even though all CMM8 and AMM4 transgenic lines had lower symptom severities and viral loads compared with infected untransformed cv.60444, the results were not highly significant ($p > 0.05$). From this study, tolerance or reduction of viral load and symptoms was attributed to the accumulation of transgene-derived siRNAs prior to infection. However there was no observable correlation between levels (semi-qauntitative northern blots) of siRNAs and tolerance or susceptible phenotypes. Tuber yield evaluation of the three tolerant lines (CMM6-2, CMM6-6 and line AMM2-52) showed that the tuber fresh and dry weight at 365 dpi was not affected by the viral presence. These are promising lines for larger greenhouse and field trials. A comparison between the two different constructs showed that the two tolerant CMM6 lines-2 and 6 appeared to perform better (viral load) compared with AMM2 tolerant line-52 with regards to levels of viral amplification. The mismatched construct in AMM4 lines and the non-mismatched construct in CMM8 lines induced the same viral and symptom severity score (sss) reduction. Transformation of T200 FECs with the DM-AES construct was unsuccessful due to the age (more than six months old) of the FECs. FECs are more likely to lose their regeneration and totipotent nature with age. We therefore propose the use of fresh T200 FECs in future transformation studies to test the DM-AES construct.

**Table of contents**

## List of Figures

### Chapter 1

## List of Tables

## List of Appendices

## List of Abbreviations

**AC2, AC4**: Complementary-sense genes found in begomovirus genomes on DNA-A

**ACMV**: *African cassava mosaic virus*

**ACMV-[NG:Ogo:90]**: *[Africa cassava mosaic virus*-[Nigeria:Ogorocco;1990]

**AGO**: Argonaute protein

**ARC**: Agriculture Research Council

**AV1, AV2**: Virion-sense genes found in begomovirus genomes on DNA-A

**BAP**: 6-Benzylaminopurine

**BC1**: Complementary-sense gene found in begomovirus genomes on DNA-B

**CaLCuv**: *Cabbage Leaf curl virus*

**CaMV**: *Cauliflower mosaic virus*

**CBV**: cassava begomovirus

**CEM**: cassava elongation medium

**CIM**: callus induction medium

**CIASA**: Cassava Industry Association of Southern Africa

**BV1**: Virion-sense gene found in begomovirus genomes on DNA-B

**CMD**: Cassava mosaic disease

**CMB**: Cassava mosaic begomovirus

**CMV**: *Cucumber mosaic virus*

**CP**: Coat protein

**CTAB:** Cetyltrimethylammonium bromide

**CR**: Common region shared by DNA-A and DNA-B components of begomovirus genomes

**CTAB**: Cetylmethylammonium bromide

**DCL**: Dicer-like protein

**DNA**: Deoxyribose nucleic acid

**DNA-A**: Bipartite genomic component A

**DNA-B**: Bipartite genomic component B

**dpi**: Days post-inoculation

**ds**: Double stranded

**DTI**: Department of Trade and Industry

**EACMCV**: *East African cassava mosaic Cameroon virus*

**EACMKV**: *East African cassava mosaic Kenya virus*

**EACMMV**: *East African cassava mosaic Malawi virus*

**EACMV**: *East African cassava mosaic virus*

**EACMZV**: *East African cassava mosaic Zanzibar virus*

**EDTA**: ethylene diamine tetra-acetate

**FEC**: Friable Embryonic cells

**GD**: Gresshoff and Doy

**GE**: Genetic engineering

**GFP**: Green fluorescent protein

**GM**: Genetically modified

**GV**: Geminivirus

**hp**: hairpin

**hyg**: hygromycin

**ICMV**: *Indian cassava mosaic Virus*

**IR**: Inverted repeat

**Kb:** kilobase

**LB:** Luria broth

**mRNA**: messenger RNA

**miRNA**: Micro RNA

**MS2**: Murashige and Skoog medium 2

**MSN** Murashige and Skoog Media with 1mg/ml naphthaleneacetic acid

**NAA**: α-naphthaleneacetic acid

**NOP**: Non profit organisation

**NOS** Nopaline synthase

**NSP**: Nuclear shuttle protein

**nt**: nucleotide

**OD**: optical density

**ORF**: Open reading frame

**ori**: Origin of replication

**PCR**: Polymerase chain reaction

**PDR**: Pathogen derived resistance

**PTGS**: Post-transcriptional gene silencing

**qPCR**: Quantitative / Real time PCR

**RdRp**: RNA dependent RNA polymerase

**REn**: Replication enhancement protein

**Rep**: Replication initiation protein

**RISC**: RNA-induced transcriptional silencing

**RNA**: Ribose nucleic acid

**RNAi**: RNA interference

**RT-PCR**: reverse transcriptase PCR

**SA**: South Africa

**SACMV**: *South African cassava mosaic virus*

**siRNA**: Small interfering RNA

**SLCMV**: *Sri Lankan cassava mosaic virus*

**sRNA**: Small RNA

**ss**: Single stranded

**SSE**: Secondary Somatic Embryo

**sss**: Symptom severity score

**SqCLV** *Squash leaf curl virus*

**T-DNA**: Transfer DNA

**TGS**: Transcriptional gene silencing

**TIA**: Technical Innovation Agency

**TMV**: *Tomato mosaic virus*

**TNA**: Total nucleic acid

**ToLCV**: *Tomato leaf curl virus*

**TMV**: *Tobbaco mosaic virus*

**TNA**: Total nucleic acid

**TuMV**: *Turnip mosaic virus*

**TYLCSV** *Tomato yellow leaf curl Sardina virus*

**TrAP**: Transcriptional activator protein

# Chapter 1 Literature Review

## 1.1. Cassava

Cassava (*Manihot esculenta* Crantz) is a tropical, perennial root crop mostly grown in subtropical regions of Africa, Asia and Latin America (Lebot, 2009; Legg *et al*., 2015). It belongs to the Family *Euphorbiaceae* which is most common in the tropics and exists either as shrubs, herbs or trees. The *Euphorbiaceae* family includes rubber, castor beans and cassava (Puonti-Kaelas, 1998). Cassava origins traces back to tropical Southern America and it was introduced in Africa in the 16$^{th}$ century and later Asia by Portuguese traders (Leotard *et al*., 2009; Olsen *et al.*, 1999).

The cassava plant grows to between one and three meters in height. Cassava tuber root is cylindrical in shape and has a circular cross section (Sayre *et al*., 2011). The cross section of the root shows three distinct sections, the bark also called periderm, the peel layer also called phelloderm and the parenchyma which represents the greater percentage of the root and a major hub of starch storage (Lebot, 2004).

**Figure 1. 1** Cassava (*Manihot esculenta* Crantz) plant (left) and high carbohydrate tuberous roots (right) (Stupak *et al*., 2006)

## 1.2. Agricultural Importance of Cassava

Cassava is mainly cultivated for its starchy roots. As the cassava plant grows a few of its roots develop into tubers which act as its major storage unit, and this is the edible portion. Cassava is important because 85% of its tuberous root dry weight is starch. In tropical regions cassava is ranked as the third most important food crop behind maize and rice (FAOSTATS, 2013). Among these top ranked important crops, cassava over the period 1980 to 2013 has had the highest increase in global harvest area, amounting to a 44% expansion. In this same

period 1980-2013 global cassava tuber production has increased from 124 million tonnes to 255 million tonnes (FAOSTATS, 2013). In the wake of climate change, cassava has become a vital crop for food security in Sub-Saharan African. Cassava has an added advantage of being able to grow in poor soils and to survive prolonged dry spells (El Shawkawy, 2004). Mature cassava can be stored 2 to 3 years in the ground making it easy to be drawn upon in times of drought or social challenges. It is these advantages that make it a very reliable subsistent crop for low income farmers (FAOSTATS, 2013; Lebot, 2004).

In most developing countries such as Africa, 90% of the crop harvest is for human consumption, and this is mainly because it is the cheapest source of calories of all the staple crops (Pounti-Kaelas, 2004). Despite its importance as a food source, cassava has growing industrial potential especially as a source of industrial starch and ethanol. The production of ethanol from cassava and utilization of its waste products (peels) is being explored by various cassava-producing countries. According to Adelekan. (2012) the total cassava production from tropical countries could potentially produce 133 million gallons of ethanol per year. Cassava is also being used to produce starch for industrial and food additive purposes. Maize, wheat and rice are major raw materials sources for starch production, but the usage of cassava starch is increasing due to its large starch content, and it has become a major raw material for paper production, detergent manufacturing and bond agents for tablets (Tonukari *et al*., 2015). Nigeria recently embarked on new projects for the production of adhesives from cassava starch and it was found to be equally effective as common chemical adhesives (Ozemoya, 2007).

## 1.3 Constraints of Cassava

Despite several steps being taken to elevate its importance and establishment of a diverse cassava based industry, several issues still challenge such establishment. Cassava production suffers from several biotic (pests and diseases) and abiotic challenges. One post harvest challenge of cassava is the short storage life of the harvest roots (Bull *et al*., 2011). Biotic stresses that affect cassava include pests and diseases. Although a number of crop protection methods can be implemented to reduce the occurrence of diseases, such as crop rotation and using healthy planting material, cassava still remains vulnerable to many diseases. Among the chief menacing diseases is bacterial blight caused by a proteobacterium, *Xanthomonas axonopodis*, which is mainly transmitted from plant to plant (Bart *et al.*, 2012). The other major challenges of cassava are viral diseases, including cassava brown streak disease and cassava mosaic disease (CMD). To date, based on homologous demarcations eleven cassava

mosaic geminiviruses are known to cause CMD (**fig 1.2)**, two being Asian species, namely *Indian cassava mosaic virus* (ICMV) and *Sri Lanka cassava mosaic* (SLCMV). Africa has the highest dynamism of CBVs with nine different species: *African cassava mosaic virus* (ACMV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *South African cassava mosaic virus* (SACMV), *East African cassava Cameroon virus* (EACMCV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Kenya virus* (EACMKV), *African cassava mosaic Burkina Faso virus* (ACMBFV) and *Cassava mosaic Madagascar virus* (CMMGV) (Fauquet *et al*., 2007; Legg *et al*., 2015). Three distinct cassava geminivirus species are more prevalent in Southern Africa (Zimbabwe, South Africa and Mozambique) namely EACMV, ACMV and SACMV (Berry and Rey, 2001).

### 1.3.1 Cassava Mosaic Disease (CMD)

Cassava mosaic disease is currently one of the most endemic viral diseases for cassava in Africa (Legg *et al*., 2015; Patil and Fauquet, 2009). The virus induced disease mainly occurs in India and Africa, but was first reported in Tanzania in 1894 by Warburg. It was only in 1906 that Zimmerman suggested that CMD was a direct result of virus infection. CMD has been reported in India, central, eastern and southern Africa causing loses of over US$1 billion annually (IITA, 2014; SciDev, 2016). Cassava mosaic disease is spread by the use of infected plant material or by an infecting whitefly vector *Bemisia tabaci* (Gennadius) (**fig 1.3**) (Brown *et al*., 1995; Carabali *et al*., 2010; Legg *et al*., 2015; Maruthi *et al.*, 2004). Since cassava is vegetatively propagated transmission by infected plant material is widespread. *B. tabaci* is the only known vector to spread CMD, and is also responsible for the transmission of cassava brown streak viruses (CBSVs) (Maruthi *et al*., 2004). Several *B. tabaci* haplotypes forming distinct sub-Saharan clades have been shown to transmit CBVs in southern and eastern Africa (Berry *et al*., 2004; Esterhuizen *et al.*, 2012; Sseruwagi *et al*., 2004). Whiteflies feed on phloem sap in all growth stages of the plant (Thresh, 1998). Whiteflies can be extensively spread in tropical regions with immigration of close to 7 km downwind (Hirano *et al*., 1993).

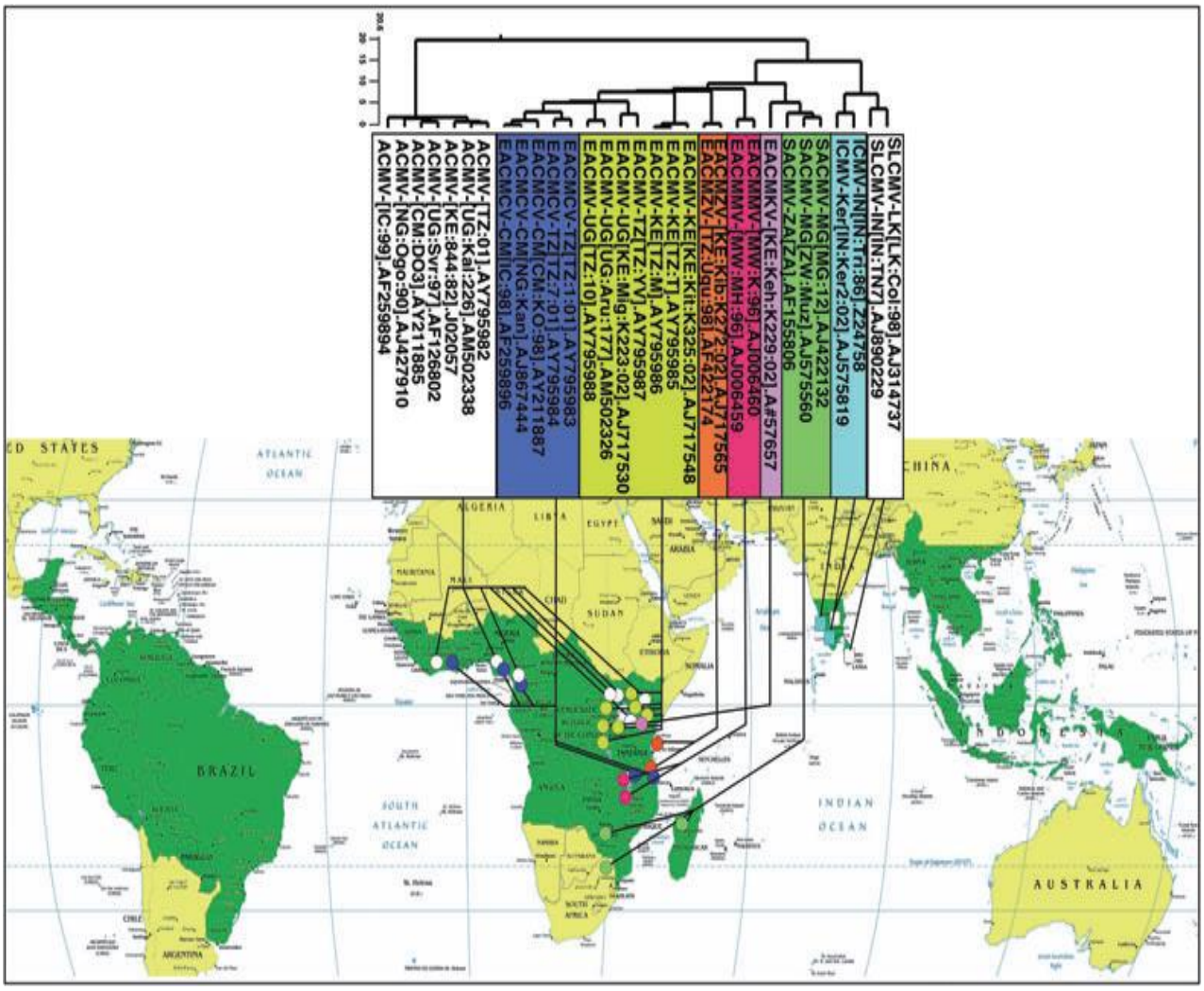


**Figure 1. 2** Map showing Geographical distribution of cassava-infecting begomoviruses together with all the species and strains of cassava mosaic begomoviruses (CB8Vs) in Africa as well as the Indian subcontinent. The green coloured regions indicate areas under cassava cultivation and different coloured dots represent specific specie (Patil and Fauquet, 2009).

**Figure 1. 3** The whitefly *Bemisia tabaci* (Gennadius) (Cuthbertson, 2013)

Cassava mosaic disease symptoms vary between varieties, cultivars and landraces, due to a number of factors such as plant age, temperature and differences in viral strains. Generally, CMD causes chlorotic mosaic on infected leaves. The mosaics are either yellow or green regions on the leaves. Green mosaics usually appear as alternating light and dark green regions on the leaf (Legg *et al*., 2004). Green mosaics are usually mild or moderate. The more severe mosaics appear as yellow and green regions. Yellow regions show areas where the leaves have lost photosynthetic ability this in turn results in reduction of leaf size and plant stunted growth (Vanitharani *et al.,* 2005). ACMV and SACMV symptoms of leaf curling, twisting/malformation and blistering are often also observed (Legg *et al*., 2004; Vanitharani *et al.,* 2005). Some of the symptoms are shown in **fig 1.4**. Infection with CMD greatly reduces yield and tuber size. In severe cases leaves may be distorted, this is because the chlorotic regions seize to grow whilst other surrounding regions grow causing uneven leaf expansions (Thresh *et al*., 1998).

## 1.4 Geminiviruses

Geminiviruses belong to the family Geminiviridae (Brown *et al*., 2015). Geminiviruses affect a range of dicots and monocots causing diseases and deformities. Geminiviruses are characterised by a geminate icosahedral structure, and their genome consist of a single stranded circular DNA (Gutierez, 2000). They replicate by a unique form called rolling circle mechanism in the host cell nucleus, where they rely on host cell replication mechanism (Stanley, 1995; Wang *et al*., 2008). The geminiviridae family is further divided into 7 genera based on genome organisation. The seven generas of geminiviruses are *Begomovirus*,

*Mastrevirus, Curtovirus, Topocuvirus, Becurtovirus, Turncurtovirus and Eragrovirus* (Adams *et al.*, 2013). Of these seven *begomoviruses, mastreviruses, curtoviruses, topocuviruses* have been extensively studied. *Begomoviruses* make up 80% of geminivirus species (Brown *et al*., 2015).

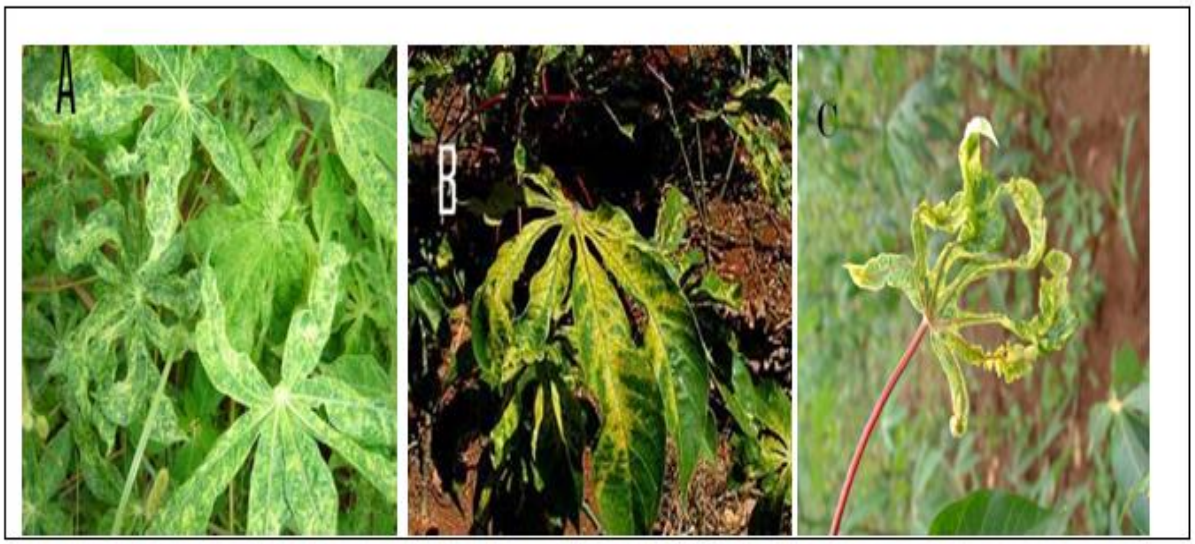


**Figure 1 4** Symptoms of CMD: A green leaf mosaic B, yellow leaf mosaic, C severe leaf deformation (Vanitharani *et al.,* 2005)

### 1.4.1 Begomoviruses

Begomoviruses exist as either mono-or bipartite viruses. Their genome sizes range from 2.5 to 5 kb and usually consist of 6 to 7 genes (Brown *et al*., 2015; Raja *et al*., 2010). Begomoviruses usually have 2 genetic components called DNA-A and DNA-B. DNA-A has six open reading frame coding for four complementary sense genes, namely AC1 to AC4 and two virion sense genes AV1 and AV2 (Brown *et al*., 2012; Vanitharani *et al*, 2005) (**fig 1.5**). AC1 codes for the Rep protein 358 aa in length, and is essential for initiation of replication. AC2 codes for a 135 aa protein (TrAP) which is essential for transcriptional activation, while AC3 codes for REn a 134 aa protein essential for replication enhancement. AC4 is reported in some geminiviruses as a suppressor of RNA silencing (Vanitharani *et al*., 2004). AV1 codes for a coat protein of 258 aa and AV2 encodes a pre-coat protein (Vanitharani *et al*., 2005; Wang *et al*., 2014). AV2 is also known to be a virus suppressor of silencing (VSR) and contribute to symptom severity (Vanitharani *et al*., 2004).

DNA-B has 2 open reading frames coding for a complementary sense gene (BC1) and a virion sense (BV1) gene. BV1 encodes a nuclear shuttle protein (NSP) and BC1 encodes a

cell-to-cell movement protein (MP) (Varma and Malathi, 2003). Geminivirus DNA is replicated in the nucleus so NSPs transport the DNA complex to the cytoplasm where MP is responsible for transportation from one cell to the other. Synonymous to both DNA-A and DNA-B is a 200bp common region within the intergenic region (IR) (Brown *et al*., 2015). In begomoviruse transcription occurs bi-directionally from the IR (Ashraf *et al*., 2014; Eagle and Harnley-Bowdoin, 1997; Stanley, 1995). Opposite oriented promoters exist on either side of the origin of replication site. The common region also contains a small hairpin structure which acts as the initiation site of the rolling circle replication (reviewed by Rizvi *et al*., 2014). The common region also consists of 2 TATA motifs and a Rep-associated protein binding sites called iterons (Ashraf *et al*., 2014;Varma and Malathi, 2003).

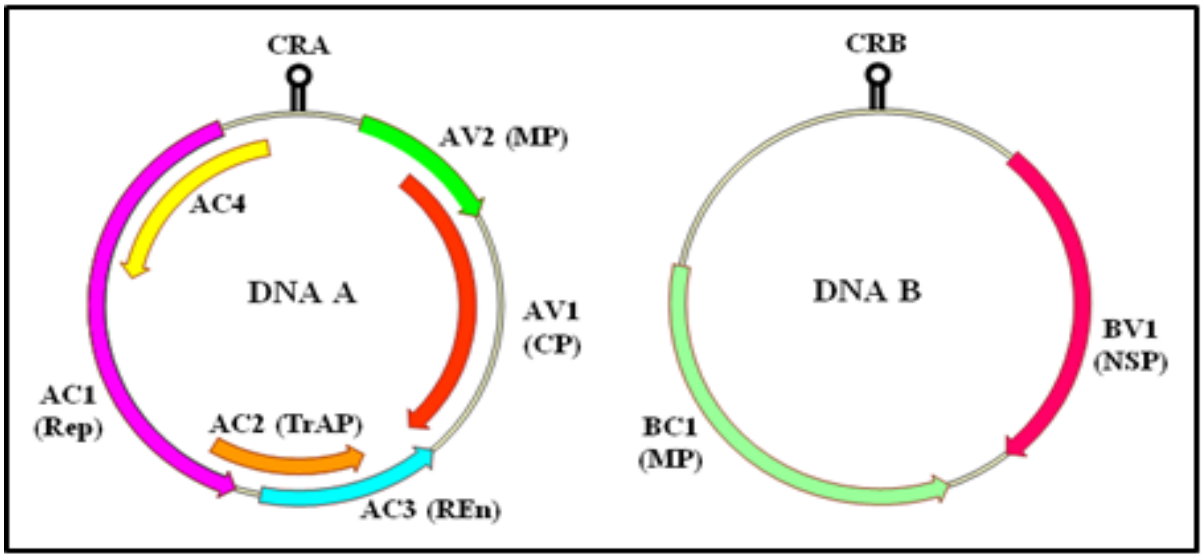


**Figure 1. 5** Bipartite genome of begomoviruses showing their respective open reading frames (Palmer and Rybicki, 1997)

## 1.5 Plants response to viral infection

Plants offer resistance to viruses using various mechanisms. Once a virus has infected a plant, plants are able to counteract the infectivity. Plant resistance to viruses has been described to occur at four different levels by various mechanisms and these are inhibition of replication, inhibition of cell-to-cell movement, inhibition of systemic infection as well as defence response mechanisms (Reviwed by Palukaitis and Carr, 2008). The first line of defence for plants to viral infection is physical and chemical barriers. The second barrier of defence is the plants recognition of virus virulence factors. Once these virulence factors have been recognised the defence response would include the involvement of basal immunity or RNA

silencing (Pallas and Garcia, 2011). Despite these defence mechanisms viruses may overcome them by producing silencing suppressors (Pallas and Garcia, 2011).

## 1.6 RNA silencing

RNA silencing is a gene regulatory mechanism resulting in sequence specific mRNA degradation (Balcombe, 1999; Vantharani *et al.*, 2005). RNA silencing involves two classes of short RNAs, namely small interfering RNAs (siRNA) and microRNAs (miRNA), 21 to 26 nucleotide (nt) in size, which are involved in the regulation of gene expression, regulation of chromatic structure and defence mechanisms against transposons and viruses in eukaryotic organisms (Baulcombe, 1999, 2004; Cerutti *et al*., 2011; Holoch and Moazed, 2015). The same mechanism occurs in fungi and animals where it is called quelling and RNA interference, respectively (Cogoni *et al*., 1997; Napoli *et al*., 1990). Plants main natural defence mechanism against viruses is by RNA silencing. This mechanism of RNA silencing in plants occurs as post transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS).

### 1.6.1 Post transcriptional gene silencing

PTGS occurs by cytoplasmic siRNA or miRNA silencing of endogenous mRNA (Baulcombe, 1999, 2004; Hammond *et al.*, 2000). TGS downregulates gene expressions through siRNA guided methylation of gene promoters or histones resulting in inhibition of transcription. PTGS and TGS have similar mechanisms and both rely on initiation by recognition of double-stranded RNA (dsRNA) from within or outside the cell and their subsequent cleavage by Ribonuclease III like enzymes called Dicers into small nucleotides with size ranging from 21-26 nucleotides (Bernstein *et al.*, 2001). Small interfering RNAs are divided into two classes, short class 21-22 nucleotides and longer class 24-26 nucleotides (Hamilton *et al.*, 2002). These act as guides in sequence specific degradation of homologous target RNA for gene regulation or viral degradation (Hammond *et al.*, 2000; Raja *et al*., 2010). Dicer structure has been reported to have a 10-residual segment consisting of an enzyme site that directly aligns with the dsRNA sequence (MacRae *et al*., 2007). Different Dicer-like enzymes (DCL) have been distinguished based on their different functions, DCL 1 is involved in miRNA biogenesis, DCL2 is involved in viral siRNA production and DCL 3 is involved in retroelements and transposon siRNA production (Garcia-Ruiz *et al*., 2010; Parent *et al*., 2015; Xie *et* al., 2004). Unlike most viruses which have an RNA genome, geminiviruses have a single stranded DNA (ssDNA) genome. RNA silencing is triggered in

viruses with RNA genomes by dsRNA that exist as either viral replication intermediates or folding secondary structures by ssRNA or ssRNA-encoded mRNA (Vanitharani *et al*., 2005).

Geminiviruses are circular single-stranded DNA and replicate through a dsDNA intermediate, and trigger PTGS. Double stranded viral genome induces PTGS in several ways. Firstly, the mRNA of the geminivirus which are a product of bi-directional transcription and overlap at the 3'end forming dsRNA by 3' extension by RNA dependent RNA polymerase (RDRP) 1 or 6 which triggers PTGS. Secondly, early abarrent AC1 transcripts could also serve as templates for dsRNA formation leading to PTGS and lastly the strong tendency of geminiviruses transcripts to fold can easily attract Dicer to cleave the transcripts giving siRNA (Garcia-Luiz *et al*., 2010; Vanitharani *et al*., 2005; Wang *et al*., 2010). Duplex siRNA generated by Dicer act as effectors of the silencing mechanism (Brodersen and Voinnet, 2006). The siRNA duplexes are unwound by helicase activity into two separate strands and either the sense or antisense strand can be loaded into an effector complex called RNA induced silencing complex (RISC), consisting of Agonaute proteins (reviewed in Sherman *et al*., 2015; Vasquez and Hohn, 2013; Voinnet *et al*., 2009). The Agonaute proteins have dicing properties and it guides the siRNA to homologous mRNA where it binds to specific homologous sequences (21-26 nt). The specific mRNA is then subjected to enzymatic cleavage and subsequent degradation (reviewed in Vaucherets, 2008). Short class siRNAs (21-23 nt) are involved in mRNA degradation whilst longer siRNAs (24 nt) have been implicated in DNA methylation (Hamilton *et al*., 2002). The overview of the RNAi pathway is shown in (**fig 1.6)**.

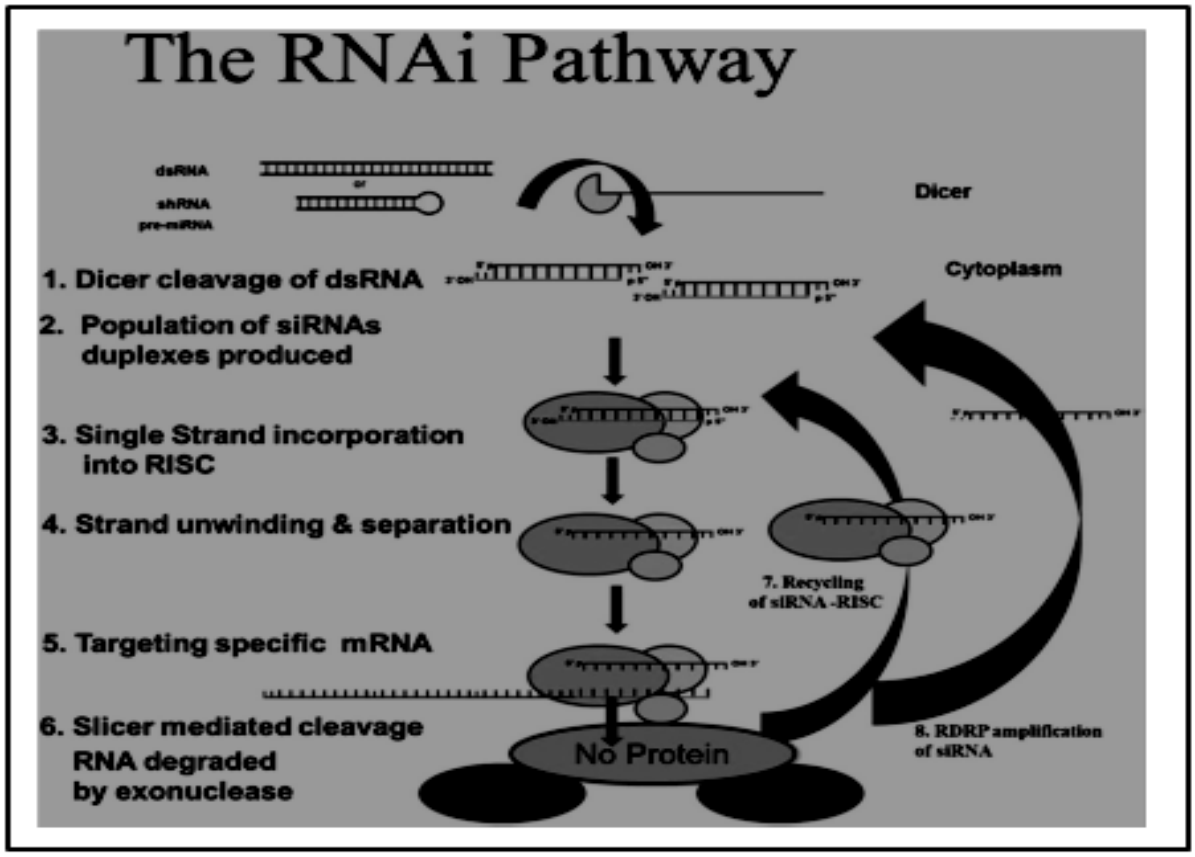


**Figure 1. 6** Overview of steps involved in RNAi pathway involving (**1, 2**) processing of dsRNA intermediates by Dicer into primary siRNA production. (**3, 4**) siRNA unwinding and incorporation into RISC complex (**5**) sequence-specific degradation of complementary mRNA (Sherman *et al*., 2015)

### 1.6.2 Transcriptional gene silencing

Down regulation of transposons, chromosomes as well as protein coding gene is called transcriptional gene silencing (TGS) (Nishumura *et al*., 2012). TGS occurs by three methods (i) DNA methylation (ii) heterochromatic formation and (iii) programmed DNA elimination (Shah *et al*., 2012; Vaucherets *et al*., 2001). Heterochromatic formation occurs when changes are induced on the chromatin by deacetylation of Histone *H3* and methylation at the *lycin 9* of the chromatin (Shah *et al*., 2012). The methylated *lycin* is then bound by heterochromatic binding proteins which cause chromatic condensation and subsequent blocking of transcription (Shah *et al*., 2012). DNA methylation involves the addition of a methyl group to DNA base cytosine by DNA methyltransferase. Methyl binding proteins bind to the transcriptional factor binding site on the promoter and this inevitably stops transcription.

RNA dependent-DNA methylation involves the methylation of DNA regions homologous to dsRNA sequence (Vaucherets *et al*., 2001). RNA dependent-DNA methylation is initiated by dsRNA derived from transgene or subsequent siRNAs from that transgene. The siRNAs are then incorporated into the RNA induced initiation of transcriptional gene silencing (RITS). The RITS/siRNAs complex then directs methylation of target DNA that is homologous to the siRNAs (Wu *et al*., 2010; Xie *et al*., 2004). Plants infected by geminiviruses such as *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Cabbage Leaf curl virus* (CaLCuv) have been shown to trigger TGS by hypermethylation or chromatic methylation of the virus (Paprotka *et al*., 2011; Raja *et al*., 2008). Transgenic plants with geminivirus promoter transgene have been shown to induce hypermethylation thereby reducing accumulation of infecting geminiviruses (Reviewed in Rodriguez-Negrete *et al*., 2013).

### 1.6.3 Role of miRNAs in silencing

Lee *et al.* (1993) reported *Caenorhabditis elegans lin-4* gene produced small RNA instead of coding for a protein. These small RNAs were termed micro RNA (miRNA). Micro RNAs are small endogenous non coding RNAs found in plant animals and viruses. These 21-22 nucleotide are produced from ~70 nucleotide long endogenous precursors called pri-miRNA (Bartel and Bartel, 2003; Voinnet, 2009). DCL1 then processes the pri-miRNA into 21-22 nucleotide miRNA. In plants Dicer DCL1 has been implicated in miRNA precursor processing. Silencing by miRNA can occur in 2 pathways (i) the 3'UTR of the mRNA can base pair with miRNA/RISC complex and inhibit transcription (ii) they can degrade mRNA by sequence specific binding (Finnegan and Matzke, 2003). The complementarities of the miRNA to the target mRNA determines which silencing pathway is taken. In animals translation repression usually occur due to the imperfect base pairing between the miRNA and the target mRNA whilst mRNA degradation occurs in plants due to their high complementarity to target mRNA (Llave *et al*., 2002; Tang *et al*., 2003).

Unlike siRNA, miRNA are not only involved in gene expression regulation but are also response to virus infection. Host miRNA bra-miR158 and bra-miR1885 were upregulated when host *Brassica rapa* was infected with *Turnip mosaic virus* (TuMV) (He *et al*., 2008). Recently EACMV viral miRNA encoded from AC2 and AC4 ORFs targeting the host and itself were identified (Maghuly *et al*., 2014). The identification of premiRNA encoded from the viral ORF could be used to engineer plants against the virus.

## 1.7 Viral RNA silencing suppressors

To counteract plants' viral RNA silencing mechanisms PTGS and TGS, viruses have developed suppressor proteins. The level of suppression exerted by different viruses varies, where some viruses suppress in all infected tissues of the leaves whilst some exert suppression in newly formed leaves (Voinnet *et al*., 1999). Viral suppressors of RNA (VSRs) are capable of stopping the silencing mechanism and generation of siRNA targeting the virus (vsiRNAs) at its most important stages such as RISC assembly, dsRNA recognition and translational inhibition (Burgya and Havelda, 2011). Brigneti *et al*. (1998) showed that *Cucumber mosaic virus* (CMV) produced a protein 2b which suppresses PTGS in *Nicotiana benthamiana*. The model of suppression by the protein has since been implicated in disrupting silencing signal and disruption of the production secondary vsiRNAs (**fig 1.7**) (Diaz-Pandon *et al.*, 2007; Guo and Ding, 2002). Since then several proteins have been shown to suppress silencing. *Cauliflower mosaic virus* (CaMV) P6 protein was shown to disrupt dsRNA-binding protein (Hass *et al*., 2008; Love *et al*., 2007). P14 and P38 proteins from *Pothos latent auresvirus* and *Turnip crinkle virus* have been proven to disrupt production of siRNAs (Merai *et al*., 2005, 2006). Double stranded siRNAs produced against the virus are also a target of VSR. The p19 protein from plant *Tombusviruses* have been proven by crystallography studies to attach with high degree of affinity to double stranded siRNAs thereby preventing siRNA/RISC complex formation (Silhavy *et al*., 2002).

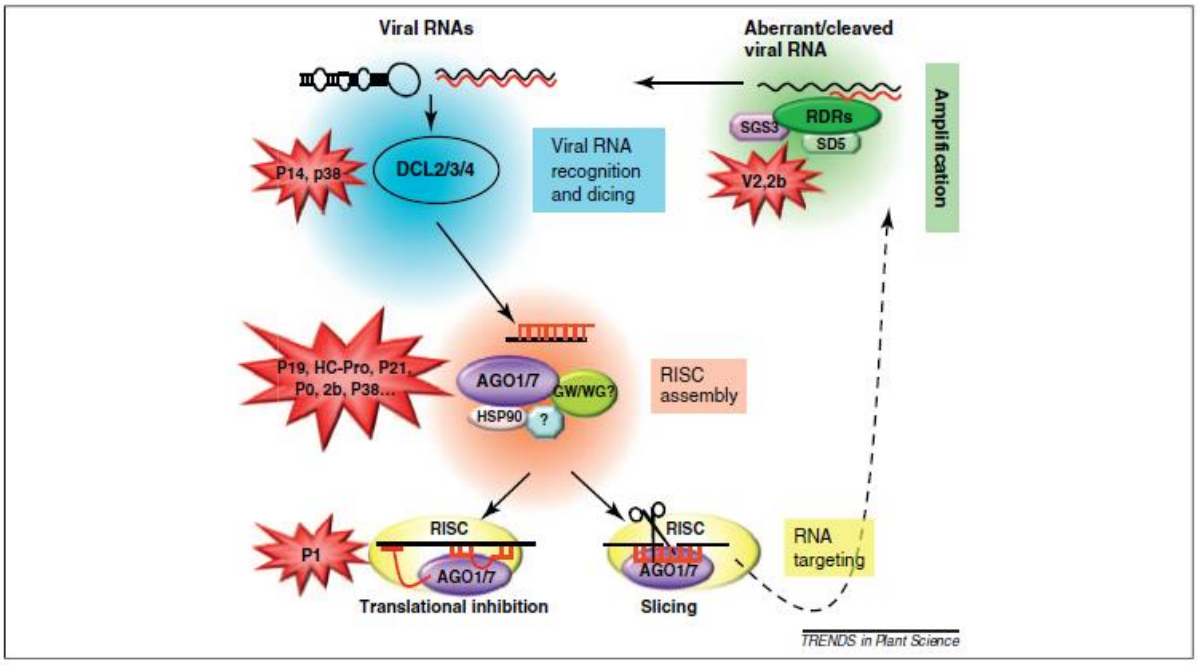


**Figure 1. 7** Model of antiviral RNA silencing mechanism in plants and its suppression by virus-encoded silencing suppressors. The model shows the various target area on the silencing mechanism were certain VSRs (i.e. P14, P38, 2b, P6, P21, P0) interact with the silencing pathways (Burgyan and Havelda, 2011)

### 1.7.1 Cassava infecting geminiviruses as silencing suppressors

Voinnet *et al*. (1999a) reported that ACMV AC2 open reading frame acted as a PTGS suppressor. *Nicotiana benthamiana* was transformed with a strain of *Agrobacterium tumefaciens* expressing a green fluorescent protein (GFP) transgene inserted into its Ti plasmid. A few days after transformation the *N. benthamiana* leaves, which had shown green fluorescence, showed red illumination because of PTGS. *N. benthamiana* was then transformed again with an AC2-PVX expression vector, and days after post infiltration *N. benthamiana* leaves showed green flourescence leading to the conclusion that GFP's PTGS was being suppressed upon infection of *N. benthamiana* with the ACMV AC2 gene construct. The result showed that AC2 was a suppressor of PTGS.

Vanitharani *et al*. (2004) also reported using the similar GFP transgene method that the AC4 gene which lies in the Rep encoding region of ACMV was a suppressor of PTGS. Like previously done by Voinnet *et al*. (1999), *N. benthamiana* with a GFP trangene showed PTGS suppression when infected with an *Agrobacterium tumefaciens* baring an AC4 construct. Vanitharani *et al*. (2004) also showed that the silencing ability of AC4 was virus

specific. AC4 obtained from ACMV-[CM] showed no suppression activity whilst AC4 from ACMV and SLCMV both showed PTGS suppression.

## 1.8 Manipulation of RNA silencing mechanisms to combat plant diseases

Advances in plant biotechnology and understanding of silencing mechanisms in the late 1990 lead to the development of molecular tools for possible introduction of foreign genes. Introduction of foreign genes into plants is termed genetic engineering (GE). Using the siRNA-mediated RNA silencing molecular mechanisms, introduction of a transgene consisting of a partial fragment from the pathogen genome results in production of trangene-derived siRNAs which aid the plants natural defence mechanism PTGS subsequently leading to resistance. This mechanism of introducing pathogen genes in plants is called pathogen derived resistance (PDR) (Peele *et al*., 2001).

### 1.8.1 Pathogen Derived Resistance (PDR)

PDR was first suggested by Sanford and Johnson (1985). Two methods are popular for pathogen derived resistance; the use of a viral gene coding for a protein which offers (protein-mediated resistance) and the use of viral genomic sequences which offer resistance (Peele *et al*., 2001). Powell-Abel *et al*. (1986) first demonstrated the use of *Tobacco mosaic virus* (TMV) coat protein gene for transgenic protein-mediated resistance. Their finding showed that transgenic tobacco with TMV coat protein genes offered resistance to TMV. Since then several viral proteins including movement proteins and replication-associated proteins have been used to induce resistance (Canto *et al*., 1998; Challappan *et al*., 2004; Sjen *et al*., 1996). At first it was believed that expression of the complete viral protein was necessary for silencing to occur but it has since been shown that the transcribed viral sense or antisense only can induce resistance (Fagoaga *et al*., 2006; Harrison *et al*., 1987). It became clear that silencing was being brought about by the transgenic-expressed RNA rather than the protein itself as expression of mutated proteins work too.

### 1.8.2 RNA silencing constructs

The use of sense or antisense transgenes was extensively studied in the early 1990s to compare their efficacy in inducing viral resistance (Fire *et al*., 1991; Smith *et al*., 1990; Takayami *et al*., 1990). Antisense RNA-mediated resistance uses an antisense strand which is specific to a viral sense strand, and complimentary binding of the expressed transgene antisense strand and the viral sense strand results in dsRNA which can be degraded via PTGS. The resulting siRNAs can then act as guide to degradation of homologous infecting

viral molecules. Smith *et al*. (1990) experimented with transforming tomato with *polygalactoronase* (PG) gene with either sense or antisense strand. Both methods resulted in reduction in expression of PG protein. However, the use of sense or antisense transgene has been reported to be unstable due to the low accumulation of siRNAs (Duan *et al.*, 2000).

Waterhouse *et al*. 1999 reported that simultaneous expression of a trangene with antisense and sense viral sequence in a plant induces more efficient virus silencing than antisense or sense alone. They reported that *N. benthamiana* plants transformed with a transgene expressing both arms of the *Nia-Protease* (*Pro*) gene of *potato virus Y* (PVY) had higher resistance compared with transgenes expressing either the sense or antisense polarity. The ability of the transgene to transcribe a RNA that can form a duplex/inverted repeat or hairpin meant it was more plausible for gene degradation to occur at a higher degree. Complete resistance could not be reached because the homologous nature of the sense and antisense strands meant secondary structures in the form of cruciform or Holliday junction like structures could easily form (Eichman *et al*., 2000; Holliday *et al*., 1985).

To circumvent the formation of secondary structures, intron sequences were introduced between the sense and antisense sequence to form inverted repeat (IR) constructs (Smith *et al.*, 2000). Due to the ability of the intron to align the sense and antisense arm in perfect complementarity these IR constructs have been reported to confer higher levels of resistance to viruses (Smith *et al*., 2000; Wesley *et al*., 2001). Using this model several generic vectors for IR/Hairpin constructs have been made. Helliwell and Waterhouse (2003) made pHANNIBAL, pKANNIBAL and pHELLSGATE generic vectors. These generic vectors require the insertion of *de novo* amplified arm of the transgene with suitable flanking sites, into the vectors by using the Gateway directed recombination.

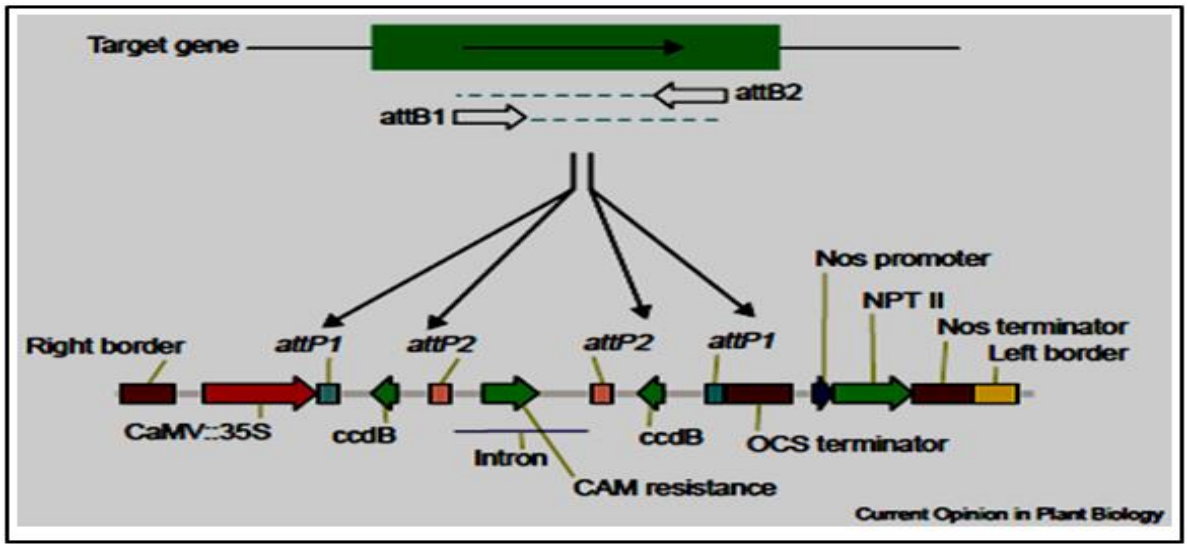


**Figure 1. 8** An overview of the Hellsgate cloning strategy. The target gene is amplified and attB1 sites are added to both flanks of the gene. The target gene is then cloned into the attP1/attP2 or attP2/attP1 in sense or antisense orientation in respect to the intron using Gateway recombination. The recombination sites have a ccdB gene for easy selection. The pHellsgate backbone has a *Cauliflower mosaic virus* (CaMV) 35S promoter that drives the IR cassette, *Agrobacterium* nopaline synthase gene; OCS terminator, the terminator sequence of the *Agrobacterium* octopine synthase gene, Right border and left border sequence from the *Agrobacterium* Ti plasmid (Wang and Waterhouse, 2001)

### 1.8.3 Artificial miRNA constructs for virus resistance

The ability of miRNA to specifically target mRNA transcripts has been adopted to make artificial miRNA (amiRNA) constructs for viral resistance. It was observed that changing several nucleotide bases within a mature mRNA would not affect its functionality hence artificial miRNA could be derived from modification of plant endogenous miRNA precursors. Artificial miRNA have been used to interfere with viral mRNA (Duan *et al*., 2008; Niu *et al*., 2006; Zhang *et al*., 2011). Recently Wabaya *et al*. (2016) recorded resistance ranging from 20-60% against *cassava brown streak virus* when transformed *N.bentamiana* was transformed with an amiRNA targeting a 21 nucleotide conserved sequence of the virus. The advantage of using amiRNA for viral resistance is that they produce short cDNA sequences which reduce the possibility of off targets synonymous with long cDNA sequences (Galun, 2005).

## 1.9 Resistance strategies against cassava mosaic disease

Crop production faces several biotic and abiotic constrains in their growth and management.

Traditional plant breeding has been the major method of enhancing crop physiological and genetic characteristics. Despite its success in other crops, cassava heterozygosity makes traditional breeding methods difficult and time consuming (Bull *et al*., 2011). Since the development of virus resistance strategies such as protein and RNA mediated resistance, both these strategies have been achieved to induce maginal tolerance or in some cases resistance in cassava against cassava begomoviruses (CBVs) via PTGS or TGS (Vanderschuren *et al*., 2007). While introduction of viral coat protein (CP) in tobacco was able to offer resistance against TMV (Abel *et al*., 1986), this strategy was not successful using CP of cassava-infecting ACMV to induce resistance (Frischmuth and Stanley, 1998). It was later determined that using the CP of bipartite geminiviruses such as ACMV would not work because their function in the spread of the virus can be substituted by the BC1 cell-to-cell movement protein (Frischmuth and Stanley, 1998).

Perhaps the best results in developing resistance in cassava has been the use of antisense or IR hairpin constructs consisting of viral coding sequence (Vanderschuren *et al*., 2007). Challappan *et al.* (2004a) observed that the Rep C-terminal region produce high amounts of viral siRNA suggesting that this region could be used to interfere with viral replication if introduced as a transgene (discussed in Chapter 4). Zhang *et al*. (2005) transformed cassava with antisense constructs of AC1, AC2 and AC3 sequences. The infectivity trials of the transgenic cassava with ACMV viral clones showed reduced viral accumulation in the transgenic plants and in addition short sense and antisense RNA homologous to the AC1 transgene were identified and thought to be responsible for triggering PTGS. Inverted repeat constructs consist of a sequence coding for a viral gene, or partial fragment which is followed by a reverse of that same sequence, resulting in dsRNA hairpin when expressed. Transgenic cassava expressing ACMV AC1 homologous hairpin dsRNAs were reported to be resistant to ACMV infection (Vanderschuren *et al*., 2009). The resistance was directly linked to the high accumulation of siRNAs homologous to the AC1 transgene. Previously Vanderschuren *et al*. (2006) showed that IR constructs expressing the common region-containing bidirectional promoter of ACMV-[NG:Ogo:90] DNA-A produced homologous siRNAs that offered resistance to ACMV-[NG:Ogo:90] infection. Taylor *et al*. (2012) noted that IR constructs with a *pdk* intron were not stable resulting in difficulties in cloning. They suggested improving the structure of the IR hp contructs by replacing the intron with a few bases (spacer) and stabilizing the hairpin by introducing mismatches to the sense arm of the IR hairpin construct using bisulfite treatment. Mutated sense arm IR constructs of SACMV BC1

were made by sodium bisulfite treatment and expression of SACMV BC1 mismatched IR constructs in *N.benthamiana* resulted in a reduction in SACMV viral infection (Taylor *et al*., 2012).

### 1.10 Genetic engineering

Knowledge on CBVs viral genome structure, replication and movement of the virus has made it possible to identify possible strategies such as RNA silencing to engineer cassava resistant to CMD (Vanderschuren *et al*., 2009). This is made possible by introduction of new DNA with a desired trait to an organism by a process termed genetic engineering (GE). Plants which express a foreign desired trait due to a gene introduced by GE are called transgenic plants or genetic modified (GM) plants (Bull *et al.*, 2011). Two processes are involved in production of transgenic plants; development of efficient transformation and regeneration procedures. The totipotent nature of plants allows for the generation of new plants from a few cells. Several key steps have been taken to genetically improve cassava for resistance against CMD. The required trait represented by the gene or a section of the gene is introduced into the plant via a disarmed Ti plasmid of *A. tumefaciens* (Gelvin, 2003). Once the construct with the gene of interest has been inserted into the Ti plasmid genome cassava explants are then chosen for the transformation. In cassava, friable embryonic callus (FEC) and somatic cotyledons are the mostly used explants for transformation (Taylor *et al*., 2004).

#### 1.10.1 Transformation of Cassava

Plant transformation involves the stable integration and expression of foreign genes in a plant genome (Gelvin, 2003). Several factors have hampered the improvement of cassava by traditional breeding, among them low fertility and the alloploidy nature of the plant (Munyikwa *et al*., 1999). Plant transformation has been used to genetically improve cassava germplasm for resistance against CMD. The major breakthrough in cassava transformation was originally based on development of friable embryogenic callus (FECs) suspension cultures for transformation and regeneration by Taylor and other researchers in the 1990s (Raemakers *et al*., 1996; Schopke *et al*., 1996; Taylor *et al*., 1996). A more recent improved method using FEC has been developed (Bull *et al*., 2011). Three methods of gene transfer are commonly used to genetically transform cassava, and these are microparticle bombardment, electroporation and *Agrobacterium*-mediated transformation. Electroporation involves mixing the plant cells with the vector DNA in a conductive solution such that when a high electric voltage is passed through the solution temporary pores are formed on the phospholipid bilayer allowing the vector DNA to move into the cell (Neumann *et al.*, 1982;

Sukharev *et al.,* 1992). In microparticle bombardment the vector DNA is coated on small metal particles which are then fired into the host plant tissue (Christon, 1992). The DNA coated particles are propelled into plant cells using high pressure. The third method (discussed below) by *Agrobacteirum tumefaciens* is the most widely used.

### 1.10.2 *Agrobacterium tumefaciens*-mediated transformation

*Agrobacterium tumefaciens* is a soil bacterium that has a Ti plasmid consisting of *vir* genes and T-DNA used by the *Agrobacterium* for infectivity (Gavin, 2003). The T-DNA has repeat sequence called right border and left border which aid in T-DNA integration into the host. Insertion of a foreign gene into the T-DNA segment would result in the foreign gene being transferred to host cells (reviewed by Yuan and Williams, 2012; Pitzschke, 2013). However due to lack of restriction sites on the T-DNA it proves difficult to clone foreign gene into the T-DNA (Gavin, 2003a). Binary vectors systems have been developed (Hoekema *et al*., 1983), where the T-DNA and *vir* gene are separated with one replicon consisting of *vir* (vir helper) and the binary vector consisting of the T-region (reviewed by Murai, 2013).

Many binary vectors have been developed and perhaps the most common are the pCambia vectors (Hajdukiewicz *et al*., 1994). pCambia consist of a multiple cloning site (MCS) flanked by the right and left border. These cassettes also include an antibiotic marker gene and some have GFP (reviewed in Murai, 2013). The gene of interest can be cloned within the multiple cloning sites for transformation into host cells. The transformed pCambia vector can then be transformed into one of the many disarmed *Agrobacterium* strains that only have the (*vir* helper replicon) and the origin of replication. Commonly used disarmed *Agrobacterium* strains include LBA4404 (Ooms *et al*., 1981) and EHA105 (Hood *et al*., 1993).

### 1.10.3 Transformation explant material

Another factor to consider for successful production of transgenic cassava for viral resistance is the type and quality of transformation material. Somatic embryos (SEs) as transformation material have been reported (Ntui *et al.,* 2015; Raemakers *et al.,* 1997; Zhang and Puonti-Kaelas, 2000). However the use of SEs for transgenic integration often leads to development of chimeras and is highly inefficient (Raemakers *et al*., 1997). Cassava embryogenic cell suspensions have also been used as transformation material (Raemakers *et al*., 1996; Schopke *et al*., 1996; Taylor *et al*., 1996). Currently the production of large numbers of independent transgenic plants relies on the use of friable embryogenic callus (FECs) as transformation material. Friable embryogenic callus have a unicellular origin and are totipotent making them

ideal for genetic manipulation by introduction of foreign genes (Taylor *et al*., 1996). Transformation of FECs from model cultivars as well as farmer preferred landraces have been reported successfully (Bull *et al*., 2009; Chetty *et al*., 2013; Nyaboga *et al*., 2015; Taylor *et al*., 2012). *In vitro* cultivation of FECs however requires optimisation for each particular cultivar targeted for genetic manipulation (Nyaboga *et al*., 2015).

## 1.11 Public perception on GM crops and the state of GM technology in Africa

Genetic engineering of crops comes as a solution to the many biotic and abiotic stresses causing food security problems worldwide. Despite a decline in food production in Africa the adaptation of GM crops still faces a lot of challenges. Thus far only South Africa, Egypt, Burkina Faso and Sudan are actively producing GM crops in SSA (James, 2013), whilst countries like Zimbabwe and Zambia have banned GM crop imports. Concerns have been raised about the potential risk of GM crops' transgenes being introduced into the environment through gene flow from GM crops to natural crops, and also potential pest resistance and health issues including toxicity and allegenicity. Significant research has been done over the years to determine if GM crops pose any negative impact on human health or the environment, and thus far results have been inconclusive. There has been contrasting reports on the potential health issues arising from ingestion of GM crops. Jennings *et al*. (2003) proved that glyphosphate resistant genes could not be detected in the gut of pigs fed with glyphosphate tolerant soybeans. In other reports transgene DNA was detected in white blood cells of mice that had been feed with GM crops (Beever and Kemp, 2000). As a result the public perception is still divided as to whether they should accommodate GM crops or not. Countries like Nigeria, Kenya and Uganda are still doing experimental field trials (Okeno *et al*., 2013). However more recent reviews have shown that GM crops are safe for consumption (Jose and Zapeda, 2016; NAS, 2016), and that environmental risk assessments are in place to mitigate the potential risk of gene transfer from GM crops or products containing dsRNA (Heinemann *et al*., 2013).

South Africa has adopted G.M technology, and currently transgenic maize, soybeen and cotton are under cultivation (Bothma *et al*., 2010). The adaptation of GM crops has also been hindered by lack of proper GM legislation in Africa for example development of Bt potato in South Africa by Syngenta and Michigan State University was blocked due to fear of possible trans-boundary movement into its neighbouring countries without proper GM regulation

measures (Eicher *et al*., 2006). Although South Africa is leading in terms of GM technology development and legislation it will not fully develop its potential until its neighbours and the rest of Africa follows suit. There is need by African governments to develop biotechnology frameworks that initiate the development of human resources to properly investigate these concerns as well as draft legislation that implement biosafety measures.

The recent discovery of foreign *Agrobacterium* genes in sweet potato (Kyndt *et al*., 2015) many shift the general perception on GM crops. This discovery means sweet potato is a natural GMO and peoples have been consuming it for decades without consequence. This finding may allay the fears of the public to some extent since it argues for the fact that transfer of genes happens in nature.

## 1.12 Rationale for study

Cassava is one of the four most important staple food sources in the world. Cassava is an excellent staple crop in developing countries due to its high carbohydrate source and its ability to produce sizeable yield in adverse growing conditions. Despite its importance as a security crop in developing countries with limited agricultural resources, commercialisation of cassava in Africa is still limited due to several constraints. In South Africa (SA) cassava is grown as a subsistence crop and most of its production is by small scale farmers with limited resources in areas such as Mpumalanga, Limpopo and Kwazulu- Natal.

SA like most countries in Sub Saharan Africa has recognised cassava as an alternative food source and a potential source of food security, but major intrest is in realising cassava's industrial potential. More recently, commercial cassava cultivation has moved to Swaziland. The Cassava Industry Association of Southern Africa (CIASA) is now registered as an Non profit organisation (NPO) with the Department of Social Welfare. Resources have been allocated by the Technical Innovation Agency (TIA), Department of trade and industry (DTI) and Agriculture Research Council (ARC) to launch cultivation trials of cassava varieties suitable for starch production in SA in the future. The ARC is also looking at mechanization for small scale on-site processing. Starch production here in SA is currently used as raw material for industries such as Monde and Sappi for paper as well as food and textile companies. Cassava is also imported from Thailand to meet local demand. In 2013, South African cabinet approved the National Bio-economy Strategy for SA. One of the pillars of the strategy is addressing commercialization and agro-processing requirements and since cassava

is important for its agro-processing our rationale to improve is yields and subsequent agroprocessing is aligned with the national bio-econony stategy.

Cassava agro-processing potential cannot be fully realised until its major constraint (yield loss due to CMD) is dealt with. One of the focus areas currently is the genetic engineering of cassava for resistance against CBVs. This scientific approach allows for introduction of desired traits into existing cultivars. The Plant Biotechnology Program at The University of the Witwatersrand over the past ten years has been developing transformation and regeneration systems to be able to genetically engineer desired traits into cassava. Currently the laboratory has genetically engineered T200, a high starch landrace variety and cv. 60444 (model laboratory based cultivar) for resistance against EACMV, SACMV and ACMV, but these need to be evaluated. The rationale of this study is to vigorously test these previously transformed lines for potential resistance against CBVs. Once proof of concept is achieved the resistant lines will be used in larger commercial field trials.

The use of sense or antisense constructs has been mostly used for engineering cassava for resistance against CBVS, although this method has resulted in reduced symptoms in several research projects, inverted repeat (IR) transgenes have been proved to be more efficient compared to sense/antisense in inducing PTGS of incoming viruses (Vanderschuren *et al*., 2007). Inverted repeat constructs consist of a sequence coding for a viral gene, or partial fragment which is followed by a reverse of that same sequence, resulting in dsRNA hairpin when expressed. Despite the efficiency of IR their main drawback is the instability of the sequence which causes cruciform structures (Duckett *et al*., 1988). To counter this formation of cruciforms Taylor *et al.* (2012) treated the sense arm sequence with bisulfite to induce base pair mismatch on the sense strand before the assembly of IR or hairpins (hp), in order to stabilize the construct. Several IR constructs have been made in our laboratory in order to produce lines which are CBV resistant using this RNA silencing mechanism. Transgenic cassava from four transformation events lines namely; AMM2, CMM6, AMM4 and CMM8 have shown promise for tolerence in small laboratory trials, and will be targeted for this study. Transformed cv.60444 lines transformed with a partial BC1 (cell to cell movement) transgene of SACMV are coded CMM8. The partial BC1 sense strand was treated with bisulphite to create mismatches. Transformed cv.60444 cultivars transformed with the cognate corresponding mismatched partial BC1 constructs are called AMM4. Transformed cv.60444 lines with a mismatched AC1/4 and AC2/3 IR construct targeting the Rep/VSR and TrAP/Ren ORFs, respectively, are coded AMM2 lines. Transformed cv. 60444 cultivars with

the cognate corresponding non-mismatched AC1/4 and AC2/3 constructs are coded CMM6. This study compares mismatched AMM2 lines and non-mismatched CMM6 lines as well as non-mismatched CMM8 and mismatched AMM2 for resistance or tolerance against ACMV and SACMV, respectively.

The study also includes designing an inverted repeat hpRNA construct (DM-AES) was designed to target ACMV-[NG:Ogo:90] putative promoter region, an overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter) as well as being efficient against SACMV and EACMV due to the inclusion of a 21 nt conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV. The construct was designed with aim of improving the efficiency of RNA silencing and due to the combination of three chosen genomic regions on ACMV-[NG:Ogo:90]. Sharma *et al*. (2014) proved that siRNA have been found to trigger TGS by, methylation of target promoter sequences (hotspots) on the intergenic region of geminiviral DNA thereby leading to reduction of transcription levels. Vanderschuren *et al*. (2007) proved that cassava plants transformed with a viral sequence of the ACMV-[NG:Ogo:90] promoter region showed signs of recovery when infected with ACMV-[NG:Ogo:90] viral strain. The construct was transformed into cassava T200 FECs in preliminary experiments.

## 1.13 Research Objective and Specific aims

The objective of this study is to undertake green house trials to evaluate selected transgenic plants (CMM6, CMM8, AMM2 and AMM4) for response to geminiviruses infection in order to identify resistance/tolerance. Furthermore, a construct targeting ACMV-[NG:Ogo:90] putative promoter region, a 21 nt conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV and the overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter) was made and subsequently transformed into cassava T200 FECs.

## Specific aims

**Aim 1: Evaluation for resistance of selected cassava transgenic plants in green-house trials, and compare transformed lines with mismatched or non-mismatched constructs**

(a) To confirm successful integration of the transgenes in previously transformed CMM6, AMM2, AMM4 and CMM8 cassava plants selected for virus resistance evaluation trials in this study.

Experimental tasks:

- DNA extraction (CTAB method).
- PCR using *GUSPLUS*, *hygromycin* and transgene insert primers.
- Southern blot to determine DNA copy number.
- Total RNA extraction and Northern blot to determine siRNA.

(b) Viral challenging of transformed cassava plants

Experimental tasks

- Macropropagation and multiplication of selected transformed lines.
- Acclimatization and Agroinoculation with ACMV-[NG:Ogo:90] (CMM6 and AMM2) or SACMV (CMM8 and AMM4) infectious clones.
- Agroinoculation of wild-type healthy cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1-RNAi.

(c) Characterisation of ACMV and SACMV challenged transgenic plants for resistance or susceptibility at 5 time points post inoculation (14, 36, 56, and depending on results at later time point 180 and 365dpi)

Experimental task;

- Evaluate leaf symptom severity and (plant height) at all time points.
- Viral load determination using real time q-PCR at 36 and 56 dpi (depending on results at later time points).
- Root yield evaluation after 365dpi.

**AIM 2: Design of a construct targeting ACMV-[NG:Ogo:90], EACMV and SACMV.**

Construction of RNAi IR/hairpin transgenes and transformation into pCambia 1305.1 plant transformation vector

Experimental tasks

- Identifying the sequence of the three target regions and designing an IR hairpin construct.
- Clone the IR constructs into pART7 expression vector.

- Clone the hairpin cassette constructs into plant transformation vector pCambia 1305.1.
- Transform *Agrobacterium* LBA-4404 with pCambia/IR.

(b)Transform and regeneration of cassava T200 FECs

Experimental task

- *Agrobacterium*-mediated transformation of FEC with pCambia/IR construct
- Regeneration of transformed FECs.
- Selection of plants for transgene integration using visual and molecular screening

# Chapter 2

# Screening of cassava expressing stacked non-mismatched or mismatched hairpin RNA constructs derived from *African cassava mosaic virus [NG]* ORFs

## 2.1 Introduction

Cassava is a very important crop in poverty alleviation in sub-Saharan Africa (SSA), and it contributes to 60% of the daily calorie intake. One major constrain to cassava cultivation is cassava mosaic disease (CMD) (Legg *et al*., 2015; Thresh & Cooter, 2005). CMD is caused by bipartite cassava begomoviruses species including *African cassava mosaic virus* (ACMV). African cassava mosaic virus consists of a single-stranded circular bipartite genome comprising of DNA-A and DNA-B. DNA-A has six open reading frame coding for four complementary sense genes that is AC1 to AC4 and two virion sense genes AV1 and AV2 (Vanitharani *et al*, 2005). AC1 codes for the (Rep) 358 amino acids (aa) protein, and Rep is essential for replication initiation. AC2 codes for a 135 aa protein called (TrAP) which is essential for transcriptional activation as well as suppression of post-transcriptional gene silencing. AC3 codes for (REn) a 134 aa protein essential for replication enhancement. AC4 functions as a suppressor of RNA silencing (VSR) and symptom modulator. AV1 codes a coat protein of 258 aa and AV2 encodes a pre-coat protein (Vanitharani *et al*., 2004; Vanitharani *et al.*, 2005). DNA-B has 2 open reading frames coding for a complementary sense gene (BC1) and a virion sense gene (BV1). BV1 encodes a nuclear shuttle protein (NSP) and BC1 encodes a cell to cell movement protein (MP) (Varma and Malathi, 2003).

Plants have a natural defence mechanism called RNA silencing which protects them from virus invasion (Bologna and Vionnet, 2014; Ribeiro *et al.*, 2006; Zvereva and Pooggin, 2012). RNA silencing results in sequence specific inhibition of transcription or translation (Balcombe, 1999). Post transciptional gene silencing (PTGS) plays a major role in plant defence against viral pathogen invasion (Balcombe, 1999; Waterhouse *et al.*, 2001). The process is dependent on the recognition of foreign double stranded RNA (dsRNA). Ribonuclease III like enzymes called Dicer then processes dsRNA to short interfering RNA (siRNA) (Bologna and Vionnet, 2014; Hamilton *et al.*, 2002). Subsequently, one strand of the siRNA then acts as guide strand for RNA induced silencing complex degradation of homologous RNA molecules (Arreger *et al*., 2012; Hammond *et al.*, 2000; Tang *et al.*, 2003).

Several studies in cassava or other related plants which can be affected by geminiviruses have shown that introduction of full-length or truncated segments of the different geminivirus genes induces varying levels of viral tolerance (Hong and Stanley, 1996; Norris *et al.*, 1996; Ntui *et al*., 2015; Vanderschuren *et al*., 2007). Significant levels of resistance to ACMV have been reported in transgenic plants expressing antisense mRNA of Rep, TrAP and REn proteins (Zheng *et al.*, 2005). Increased resistance has also been reported in transgenic plants expressing sense and antisense RNA homologous to Sri Lankan cassava mosaic virus (SLCMV) AV1/AV2 overlapping region (Ntui *et al.*, 2015), and transgenic plants expressing sense and antisense RNA homologous to ACMV-[NG:Ogo:90] AC1 (Vanderschuren *et al.*, 2009). Constructs with self complementary sense and antisense strands forming inverted repeat (IR) are more efficient PTGS inducers as opposed to either sense or antisense strategies (Wang *et al*, 2008; Waterhouse *et al*, 1998). Expression of the IR contruct would result in formation of a hairpin RNA (hpRNA) which is recognised as being double stranded thereby efficiently triggering PTGS (Aregger *et al.*, 2013; Duan *et al*., 2012).

High-throughput gene silencing vectors such as pHELLSGATE and pHANNIBAL which facilitate insertion of the transgene by Gateway recombination in the sense and antisense orientation whilst being separated by a sliceable intron are commonly used in designing IR constructs (Helliwell and Waterhouse, 2003; Wesley *et al.*, 2001). However, large introns in generic vectors like pHellsgate often result in T-DNA deletion and recombination (Nakano *et al.*, 2005). Taylor *et al*. (2012) from our laboratory developed a method to avoid the use of large introns by replacing the intron with a few bases (spacer) and stabilizing the hairpin, by introducing mismatches in the sense arm of the IR hairpin construct using bisulfite treatment. Amplification of the converted fragment would result in uracil being replaced by thymine causing base mismatches. The use of mismatched constructs to induce PTGS in tobacco for virus resistance was successfully reported (Taylor *et al*., 2012).

In this chapter transgenic cassava was evaluated for resistance/tolerance to CMD. The transgenic plants were from two transformation events with either a mismatched (AMM4) or non-mismatched (CMM6) *Africa cassava mosaic virus*-[Nigeria:Ogorocco;1990] (ACMV-[NG:Ogo:90]) IR construct derived from stacking the overlapping AC1/AC4 (2437-2572 nt) and AC2/AC3 (1297-1479 nt) viral sequences, were screened. The ACMV-[NG:Ogo:90] derived (non-mismatched construct) was constructed using the Gateway method where the sense and antisense arms are separated by a 800 bp *pdk* intron; and the mismatched transgene by bisulfite treatment for introduction of mismatches (C to T) in the sense arm of the identical

ACMV-[NG:Ogo:90] IR sequence (Rey *et al*., 2015; Taylor *et al.*, 2012). CMM6 and AMM4 transgenic lines were derived from previous transformation of the model cassava cultivar cv.60444.

The current study evaluates CMM6 and AMM2 lines for viral resistance or tolerance to ACMV-[NG:Ogo:90] by monitoring symptoms, viral replication and plant height in comparison with untransformed cv.60444. Symptoms were evaluated using a 0-3 scale similar to the one described by Ntui *et al*. (2015). The current study evaluates if there is a correlation between the symptoms and the viral load as this helps in defining whether the plants are tolerant or resistant. For resistance/tolerance trials in our laboratory we adopted definitions for virus resistance, tolerance or susceptibility from (Bengyella and Rey, 2014; Lapidot and Friedman, 2002): resistant plants show no symptoms as a result of no virus replication; tolerance is defined as the presence of mild symptoms due to low virus levels; and susceptibility refers to the presence of severe symptoms due to uncontrolled virus replication. Small interfering RNA generation was also evaluated to determine if tolerance/resistance correlated with the siRNAs being produced. The infectivity trials were done in growth facilities and the green-house with controlled environmental conditions. Resistance or tolerance has been shown to differ when conditions vary greatly, such as temperature, viral dosage and light intensity (Chellappan *et al*., 2005; Szittye *et al*., 2003; Vanderschuren *et al*., 2009). The tuber yields of tolerant lines from this trial were evaluated to determine if tolerance resulted in less yield loss of the storage roots, and also if insertion of the transgene would interfere with root formation.

### 2.1.1 Specific Aims

The aim of this research was to subject selected transgenic non-mismatched CMM6 lines 2, 3, 5, 6 and 7, and corresponding mismatched AMM2 lines 30, 41, 44, 52, 53 and 54 lines to reproducible trials and evaluate for response to virus challenge and select potential resistant/tolerant lines for further larger GM trials in future.

**Steps in achieving our aim**

i. To confirm successful integration of transgenes in previously transformed lines
ii. Micro-propagation of CMM6 and AMM2 transgenic cv.60444 lines and acclimatization for (6 weeks)
iii. Agro-infection of transgenic lines with ACMV-[NG:Ogo:90] infectious virus clones
iv. Plant evaluations for virus symptom severity, plant height and viral load at 14, 36, 56 and 365 days post inoculation (dpi)
v. Tuber yield evaluation at 365 dpi
vi. Northern blots to determine siRNA production pre and post infection

## 2.2 Methodology flow chart

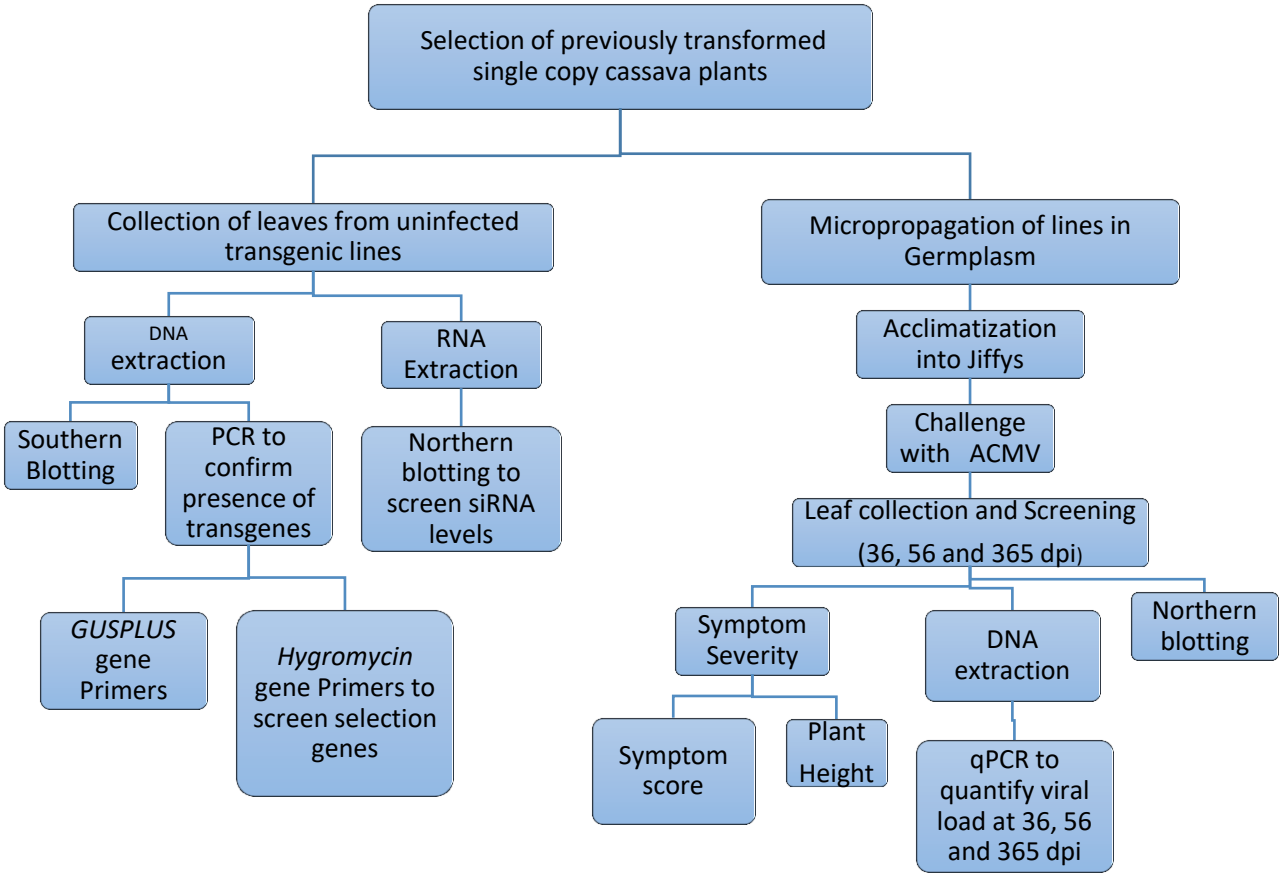


**Figure 2. 1** Work flow diagram for screening of transgenic cassava lines for resistance

## 2.3 Materials and Methods

**Characterisation of transgenic lines**

### 2.3.1 DNA extraction

Leaf tissue approximately (50 mg) was collected from each of the five CMM6, six AMM2 trangenic lines and non-infected healthy cv.60444. The leaf samples were collected in eppendorf tubes, snap-frozen in liquid nitrogen and crushed into a fine powder. The Cetylmethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) was used for total nucleic acid (TNA) extraction. To each eppendorf tube, 500 µl of preheated CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM Ethylene diamine tetra-acetate (EDTA), 100 mM Tris-HCl, pH 8.0) supplemented with 0.2% 2-mercaptoethanol was added, and the sample mixture was vortexed. The sample mixture was then incubated in a 65°C water-bath for 60 min. After 60 min of incubation, 500 µl of chloroform-isoamyl alcohol (24:1) was added followed by mixing and centrifugation at 13,000 rpm for 10 min at 4°C. The resulting aqueous layer after centrifugation containing the TNA was placed into an eppendorf tube and 500 µl of 24:1 mixture of chloroform-isoamyl alcohol was added followed by mixing and centrifugation at 13,000 rpm for 10 min at 4°C. The TNA in the resulting aqueous layer was precipitated using 500 µl isopropanol by mixing then centrifugation for 10 min at 13,000 rpm at 4°C. The supernatant was discarded and the remaining TNA pellet was washed with 500 µl of ice-cold 70% ethanol followed by centrifugation for 10 min at 13,000 rpm at 4°C. The washing step was repeated again. The eppendorf tube was blotted on tissue paper and air dried for 60 min. The pellet was then resuspended in 50 µl TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA) supplemented with 20 µg/ml RNase A (Fermentas). The extracted DNA was quantified and checked for quality on the Nanodrop 1000 spectrophotometer (Nanodrop).

### 2.3.2 Southern blot screening for transgene copy number

To screen for transgene copy number, DNA from the five CMM6 and six AMM2 transgenic lines extracted in **2.3.1** was used for Southern blotting. Transgene copy number analysis was done following the DIG-High Prime DNA labeling and detection starter kit II (Roche Life Sciences) protocol. *Africa cassava mosaic virus*-[Nigeria:Ogorocco;1990] AC1/4:AC2/3 non-mutated strand PCR fragments were produced using the primers described in **Table 2.1**. The fragments were then labeled as probes following manufactures protocol. A total 30 µg of the

extracted DNA was digested with *Hind*III which cuts once within the T-DNA at 37°C overnight. The digested reaction was run on a 1% agarose gel in 1X TAE and transferred by capillary action to a positively charged nylon N+ membrane (Amersham). The membrane was then hybridized with the DIG labeled probe at 42°C overnight. The next day membrane was washed and blocked following the DIG-High Prime DNA labeling and detection starter kit protocol. Biorad Chemi Doc system was used for chemiluminiscent detection by exposing the membrane for 30 min.

### 2.3.3 PCR of *GUSPLUS*, *hygromycin* and virus-derived transgenes

To confirm successful integration of the plant transformation cassette carrying the ACMV-[NG:Ogo:90] IR transgene into the CMM6 and AMM2 trial lines, DNA extracted in **2.3.1** was used as template. Transformed plants were screened for the *GUSPLUS* and *Hygromycin* gene (*hyg*) using *GUSPLUS* forward primer (5'-CAACATCCTCGACGATAGCA-3'), *GUSPLUS* reverse primer (5'-GGTCACAACCGAGATCTCCT-3'), and *hyg* forward primer (5'-TCTCGATGAGCTCATGCTTTGG-3') and *hyg* reverse primer (5'-AGTACTTCTACACAGCCATGGG-3'). Transformed plants were expected to amplify a 181 bp *GUSPLUS* gene fragment using the *GUSPLUS* primers and a 485 bp *hyg* gene fragment using the *hyg* primers. Screening for the CMM6 and AMM2 sense and antisense was done using ACMV-[NG:Ogo:90] AC1/4:AC2/3 primers and AC1/4:AC2/3 modified primers, respectively **Table 1**. The PCR reaction mixture for amplification of these genes contained 1X Taq buffer, 0.2 mM dNTP, 0.2 µM of each primer, 1U Taq polymerase enzyme (Fermentas) and 50 ng template DNA. The reaction was set at 94°C for 2 min and 35 cycles, 94°C for 30 sec, 55°C for 30 sec, primer extension at 72°C 30 sec and final extension step of 72°C for 10 min. The reaction was set up in Thermal cycler (Biorad). The resulting amplified PCR fragment were analysed by electrophoresis on a 1% agarose gel in 1X TAE buffer containing 10 µg/ml ethidium bromide for staining.

**Table 2.1** Primers used to amplify the ACMV-[NG:Ogo:90] DNA-A target region

| Primer | Primer sequence |
|---|---|
| AC1/4 *Xba*I F | 5'-GATCTCTAGAAAGTGAGGTTCCCCATTCTG-3' |
| ACMV AC2/3 *Xba*I F | 5'- GATCTCTAGACCAATCATGGATTTACGCACA-3' |
| AC1/4 *X hoI* F | 5'-GATCCTCGAGAAGTGAGGTTCCCCATTCTG-3' |
| AC2/3 *X hoI* R | 5'-GATCCTCGAGCCAATCATGGATTTACGCACA-3' |
| AC1/4(*X hoI* and *SpelI )*F | 5'-GATCCTCGAGACTAGTAAGTGAGGTTTCCATTTTG-3' |
| AC2/3 *Bgl*II R | 5'-GATCAGATCTCCAATCATAGATTTACACACAGG-3' |

### 2.4.4 RNA extraction

Leaf tissue approximately 50 mg was collected from each of the CMM6, AMM2 transgenic lines and untransformed healthy cv.60444 control. Two leaves per sample were collected in eppendorf tubes, snap-frozen in liquid nitrogen and crushed into a fine powder. Then 500 µl of QIAzol lysis reagent was added to eppendorf tube and the tubes were briefly vortexed. The mixture was left to stand for 5 min at room temperature prior to centrifugation at 12,000 rpm for 5 min. After centrifugation the aqueous layer was poured in 200 µl of chloroform and shaken vigourously. The mixture was left to stand at room temperature for 3 min and then centrifuged at 12,000 rpm for 15 min at 4°C. The resulting aqueous layer was transferred to a new tube with 500 µl isopropanol and mixed by inverting. The mixture was left to stand at room temperature for 10 min and then centrifuged at 12,000 rpm for 10 min at 4°C. After centrifugation the supernatant was discarded and 500 µl of 75% ethanol was added to the tube for pellet cleanup. The mixture was then centrifuged at 75,000 rpm for 5 min at 4°C. The supernatant was discarded and the RNA pellet was dried for one hour before it was dissolved in 30 µl double distilled water. RNA was quantified on a nanodrop and the quality and integrity determined on a 1.2% agarose gel containing 10 ug/µl ethidium bromide run in 0.5X TBE.

### 2.3.5 T-DNA expression

Total RNA extracted from **2.3.4** was used to check for the expression of the T-DNA. For cDNA synthesis 2 µg of RNA from the CMM6, AMM2 transgenic lines and untransformed healthy cv.60444 control was first DNAase treated with 1U DNAase (Thermo Scientific) in 10X reaction buffer. RevertAid reverse transcriptase kit was used to synthesise cDNA strand using OligodT and random primers. The synthesised cDNA stranded was then directly used in PCR to amplify the *GUSPLUS* gene, *hyg* gene and the sense and antisense arms of the

respective transgene. The same primers and primer conditions described **2.3.3** were used. PCR amplicons were visualised on a 1% agarose gel containing 10 µg/µl ethidium bromide run in 1X TAE.

**Sceening trials of transgenic lines for response to ACMV challenge**

### 2.3.6 Bulking up and acclimatisation of selected transgenic plants

The selected five CMM6 lines 2, 3, 5, 6, and 7 and six AMM2 lines 30, 41, 44, 52, 53 and 54 were propagated in tissue culture on Murashige and Skoog 2 (MS2) medium containing 4.4 g/L MS salts, 20 g/L sucrose, solidified with 6.8 g/L plant agar, pH 5.8. For experimental controls, healthy cv.60444 cassava plants, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 (Vanderschuren *et al*., 2009) and pCambia vector only transgenic controls were also propagated. The dsAC1 transgenic plants express an IR construct homologous to ACMV-[NG:Ogo:90] AC1/Replication–associated protein (Rep) sequence (154 bp) from nt position 1690 to 1844 (Vanderschuren *et al*., 2009) transformed in cv.60444. Plants were grown on MS2 media at 28°C under 16 h light/8 h dark photoperiod. After three weeks when roots had developed plantlets were removed from tissue culture and transferred to small jiffie bags (Jiffies International). A total of 25-30 plants were moved to jiffies per each transgenic line, healthy cv.60444 cassava plants, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 and pCambia vector only transgenic controls. The jiffies were then placed in plastic trays and covered with plastic wrap. Trays were placed in a phytotron facility with conditions 28°C, 16 h light (8,000-10,000 lux) and 8 h dark cycles and 60% humidity. After one week holes were poked through the plastic wrap gradually for a few days to help acclimatise the plants.

### 2.3.7 Experimental design

Ten plants per each line were inoculated with ACMV-[NG:Ogo:90] infectious viral clones (n=10). After infection six plants per each line were randomly selected for symptom, viral load, height evaluation and tuber yield evaluations. The second and third upper most apical leaves were scored for symptom evaluation and collected for real time absolute qPCR at 14, 36, 56 and 365 dpi. The two apical leaves collected per plant were pooled into 3 groups of three biological and (2 technical replicates per biological replicate) were used for real time qPCR. Three plants per each of the CMM6 or AMM2 transgenic lines, infected untransformed cv.60444, non-infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 were harvested to determine tuber yield fresh and dry weight. Statistical evaluations were done on the results from each of the characterisation. Student t-

test using (p=0.05) were used to determine the significant differences of the results obtained between transgenic lines and infected untransformed cv.60444. Results were deemed significantly different at $p<0.05$ and insignificant at $p>0.05$. Pearson correlation was used to determine the correlation between the viral load, symptom scores, height, tuber yield evaluation and siRNA band intensities. A positive correlation value signified a strong correlation between the test parameters and a negative correlation value signified no correlation between the test parameters.

### 2.3.8 Agro-inoculation of transgenic plants with ACMV

After 4 weeks the transgenic and control lines were agro-inoculated with ACMV-[NG:Ogo:90] DNA-A and DNA-B infectious clones. Prior to infection Agrodimers A and B mobilized into pCambia 1300 vector (Vanderschuren *et al.*, 2009) and cloned in *Agrobactrerium tumefaciens* LBA4404 were grown separately overnight in Yeast extract peptone (YEP) broth inoculated with antibiotics 100 µg/ml streptomycin, 50 µg/ml kanamycin and 50µg/ml rifampicin until an $OD_{600}$ of between 1.8 and 2.0 was reached. Equal amounts of the appropriate A and B components were mixed. Each plantlet was injected at the nodes with 120µl of *Agrobacterium* culture containing equal volumes of ACMV-[NG:Ogo:90] DNA-A and B dimers. Characterisation of the ACMV-[NG:Ogo:90] challenged transgenic and control lines were done at 14, 36, 56 and 365 dpi. The characterization included height evaluation, symptom scoring using a score scale of 0-3 and real time absolute quantitative PCR for viral load determination.

### 2.3.9 Sampling and symptom monitoring

Africa cassava mosaic virus-[Nigeria:Ogorocco;1990]-infected CMM6, AMM2 lines, infected untransformed cv.60444, and infected ACMV resistant line dsAC1 control plants were monitored and evaluated for viral response at 4 time points post infection (14, 36, 56 and 365 dpi). For symptom severity scoring the second and third upper-most apical fully grown leaves were scored for symptom development using a symptom severity score index of 0-3 (0= no symptoms, 1= faint mosaic/ chlorosis, 2= clear mosaics with or without leaf deformation, 3=severe mosaic, leaf distortion and reduced size). These second and third upper-most fully grown leaves were harvested and frozen in liquid nitrogen and stored at -70°C for DNA and RNA extraction. The height of 6 plants from each of the ACMV-[NG:Ogo:90]-infected CMM6, AMM2 lines, infected untransformed cv.60444, and infected ACMV-[NG:Ogo:90] resistant line dsAC1 control plants were also measured at each time.

**Molecular characterization of challenged transgenic and non-transformed plants**

### 2.3.10 Absolute quantitative PCR of viral load

Viral loads in ACMV-[NG:Ogo:90] infected CMM6, AMM2 transgenic lines, infected untransformed cv.60444, non infected healthy cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 were determined by real time absolute qPCR. Total DNA was extracted from the second and third apical leaves at 36 and 56 dpi from infected transgenic lines, infected untransformed cv.60444, non infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant line dsAC1 following Doyle and Doyle (1987) CTAB based method described in **2.3.1**. Extracted DNA was standardized to 50 ng/µl and DNA from 6 plants (2 apical leaves per plant) was pooled into 3 groups of three biological and (2 technical replicates per biological replicate) were used for real time absolute qPCR. For the qPCR reaction 10 µl of Maxima SYBR green master mix, RepF and R primers to a final concentration of 0.3 µM for each primer, 1 µl of template DNA, plasmid standard or nuclease-free water were added to a total volume of 10 µl. To amplify ACMV-[NG:Ogo:90] virus multiplication primers APA9 forward primer FP2 5'CAATTTCCACCCCAACATTCA3' and reverse primer APA9 RP2 5'GCGTAAGCATCATTCGCTGAT3' were used. The primers were designed to amplify the core coat protein region on ACMV-[NG:Ogo:90] DNA-A (Wyatt and Brown, 1996). The reaction condition were set at initial denaturation 95°C for 10 min for 40 cycles, 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec. The 10 µl sample reaction was setup in Light Cycler 480i (Roche Applied Sciences). The viral loads quantification of the pooled sample was done in triplicate. The ACMV-[NG:Ogo:90] viral loads of the experimental samples were calculated by reference to a standard curve generated by serial dilutions of pCambia 1300- ACMV-[NG:Ogo:90] DNA-A clones ranging from 0.1 pg/µl to 1 ng/µl under the same qPCR reaction condition.

Amount of molecules in pg was generated by the LightCycler software version 4 (Roche Applied Science, Penzberg, Germany) from the pCambia 1300- ACMV-[NG:Ogo:90] DNA-A based standard curve. Using the pg values obtained from LightCycler software version 4 (Roche Applied Science, Penzberg, Germany), the corresponding viral molecules were calculated using Equation 1. Equation 2 was used to calculate the number of viral molecules from the extracted DNA. The viral molecules calculated for each treatment were plotted on a logarithmic graph.

$$viral\ molecules = \frac{G\ DNA\ X\ * 6.022.10^{23}}{Length\ of\ plasmid\ standard\ (bp) * 660} \qquad \text{Equation 1}$$

$$Viral\ molecules\ per\ G\ of\ DNA = \frac{Copy\ number}{Amount\ of\ DNA\ used\ per\ reaction} \qquad \text{Equation 2}$$

### 2.3.11 Northern blot detection of siRNA

Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] IR PCR amplicons were cloned into PTZ57R/T plasmid to facilitate *in vitro* transcription of the IR transgene downstream of the T7 promoter using T7 RNA polymerase. Then 1 µg total plasmid containing ACMV-[NG:Ogo:90] IR PCR amplicon was linearized at the 5'end of the insert with 1U *Eco*RI restriction enzyme. DIG Northern Starter Kit (Roche) was used to produce DIG labeled RNA from the linearised template. For hydrolysis 1X alkaline hydrolysis buffer was used to hydrolyze 2 µg aliquots of the DIG labeled RNA to small sizes by heating the sample at 95°C for 20 min. For siRNA control, an *Arabidopsis* 21 nt miR167 (5-'TAGATCATGCTGGCAGCTTCA-3'), oligonucleotide was 3'end labeled. For size marker a 24 nt primer (5'-TTCAGGAGCCTCCGCCTGTCT-3'), designed not share sequence similarily with the ACMV IR and a 30 nt positive control from the kit were 3' end tail labeled using DIG Oligonucleotide Tailing kit 2$^{nd}$ generation (Roche). Prior to infection, 30 µg of RNA was extracted from CMM6, AMM2 transgenic lines and non-infected healthy cv.60444. Post-infection 30 µg of RNA was also extracted from infected CMM6, AMM2 transgenic lines, non-infected healthy cv.60444 and infected untransformed cv.60444 leaf tissue using QIAzol reagent **(Qiagen)** as described in **2.3.4**. Extracted RNA was separated by electrophoresis using 15% polyacrylamide (19:1) gel cast in 8 M urea and buffered with 20 mM MOPS/NaOH (pH 7). Seperated RNA was blotted to Hybond-N+ membrane (Amersham GE Healthcare) using a semi-dry electro blotter (Sigma Aldrich, SV20-SDB, UK). The electrophoresis buffer was 20 mM MOPS/NaOH (pH 7). Seperated RNA was mobilized onto the Hybond-N+ membrane by 1-ethyl-3[3-dimethylammopropyl] carbodiimide (EDC) (Sigma Aldrich) according to Pall *et al*. (2007). Then 245 µl of 12.5 M 1-methylimidazole (Sigma Aldrich) was added to 9 ml of double distilled water and pH was adjusted to 8.0 prior to adding 0.75g EDC. The volume was then adjusted to 24 ml with double distilled water. The prepared EDC solution was used to soak a Whatman paper and the nylon membrane was placed on top of the membrane wrapped in Saran Wrap and incubated at 60°C for 2 h. Pre-hybridisation of the EDC crosslinked membrane was done for

one hour at 42°C in DIG Easy Hyb buffer. Membrane was then hybridized with the ACMV-[NG:Ogo:90] probe at 42°C overnight. Detection of RNA was done according to the DIG Northern Starter Kit instruction. Biorad Chemi Doc system was used for chemiluminiscent detection by exposing the membrane for 15 min. Band intensity was measured using Quantity tool (Biorad Chemi Doc system). The highest siRNA accumulation was set at 100% reference. Values of the other siRNA accumulations were expressed as percentages of the reference value.

### 2.3.12 Yield evaluation

After 365 dpi three plants from each of the infected CMM6, AMM2 transgenic lines, infected untransformed cv.60444, non infected healthy cv.60444 and infected ACMV-[NG:Ogo:90] resistant line dsAC1 transgenic line were harvested and root yield evaluated to determine the fresh tuber weight. The tubers were dried at 105°C overnight in a dry oven, the next day dry tubers weight were measured to determine the percentage dry weight from the fresh weight. Student t-test statistical evaluations were done on the root fresh and dry weight to determine significant differences between the root yields of infected untransformed type cv.60444 in comparison with infected CMM6, AMM2 transgenic lines, non infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant line dsAC1.

## 2.4 Results

**Characterisation of transgenic lines**

### 2.4.1 Southern blot screening for transgene intergration and copy number

Transgenic lines CMM6 line 2, 3, 5, 6 and 7 as well as AMM2 lines 30, 41, 44, 52, 53 and 54 were screened for transgene copy number using the Southern blot method. The ACMV-[NG:Ogo:90] IR PCR amplicon, *GUSPLUS* PCR amplicon and the *hyg* PCR amplicon probes were successfully labeled (**fig 2.2**). From the results, the DIG-labeled *GUSPLUS* and *hyg* probes (**fig 2.2 lane b and d, respectively**) migrated slower in the gel, as expected, than the 181 bp and 485 bp unlabeled PCR amplicon (**fig 2.2 a lane c, respectively)**. The DIG labeled ACMV IR probes **(fig. 2.2 lane f)** migrated higher than unlabeled 319 bp ACMV-[NG:Ogo:90] IR PCR amplicon (**fig. 2.2 lane e**).

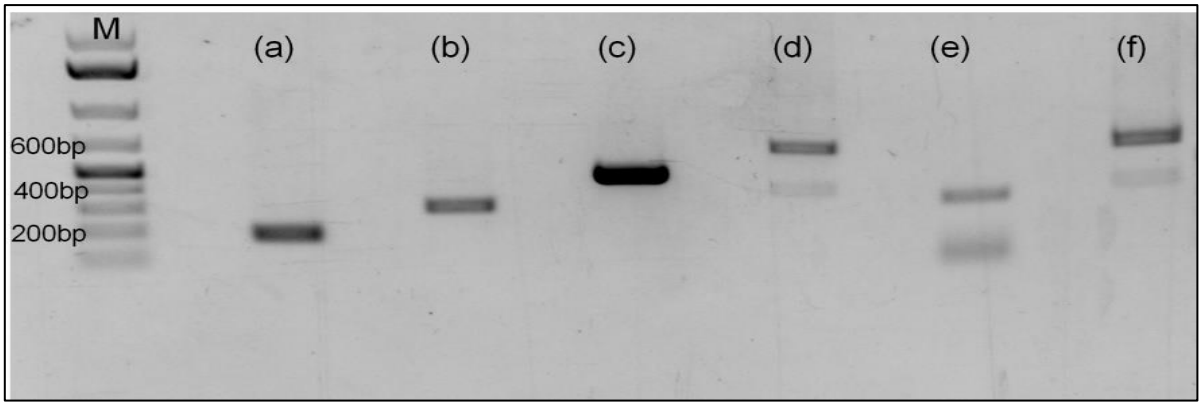


**Figure 2. 2** 1% agarose gel of PCR DIG labeled unmodified ACMV-[NG:Ogo:90] IR, *hyg* and *GUSPLUS* fragment probes. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas). DIG-labeled *GUSPLUS* probe (**lane b**) unlabeled *GUSPLUS* PCR amplicon (**lane a**) DIG-labeled *hyg* probe (**lane d**) unlabeled 485 bp *hyg* fragment (**lane c**) and unmodified ACMV-[NG:Ogo:90] IR DIG-probes (**lane f**) unlabeled 319 bp ACMV-[NG:Ogo:90] IR PCR amplicon (**lane e**)

Southern blot analysis of double digested DNA from five **CMM6** transgenic lines with three DIG labeled probes showed that all five lines had a single transgene copy (**fig 2.3a**). Probing of single digested genomic DNA with DIG labeled-*hyg* showed lines 2, 5 and 6 had a single transgene copy, while lines 3 and 7 had two transgene copies (**fig. 2.3b**). Southern blot results from double digested AMM2 genomic DNA probed with ACMV-[NG:Ogo:90] IR DIG probe showed that all six lines had a single transgene copy (**fig 2.4a**). Probing of single digested genomic DNA with DIG labeled-*hyg* showed that lines 41, 44 and 53 had a single transgene copy while lines 30, 52 and 54 had a double copy (**fig. 2.4b**).

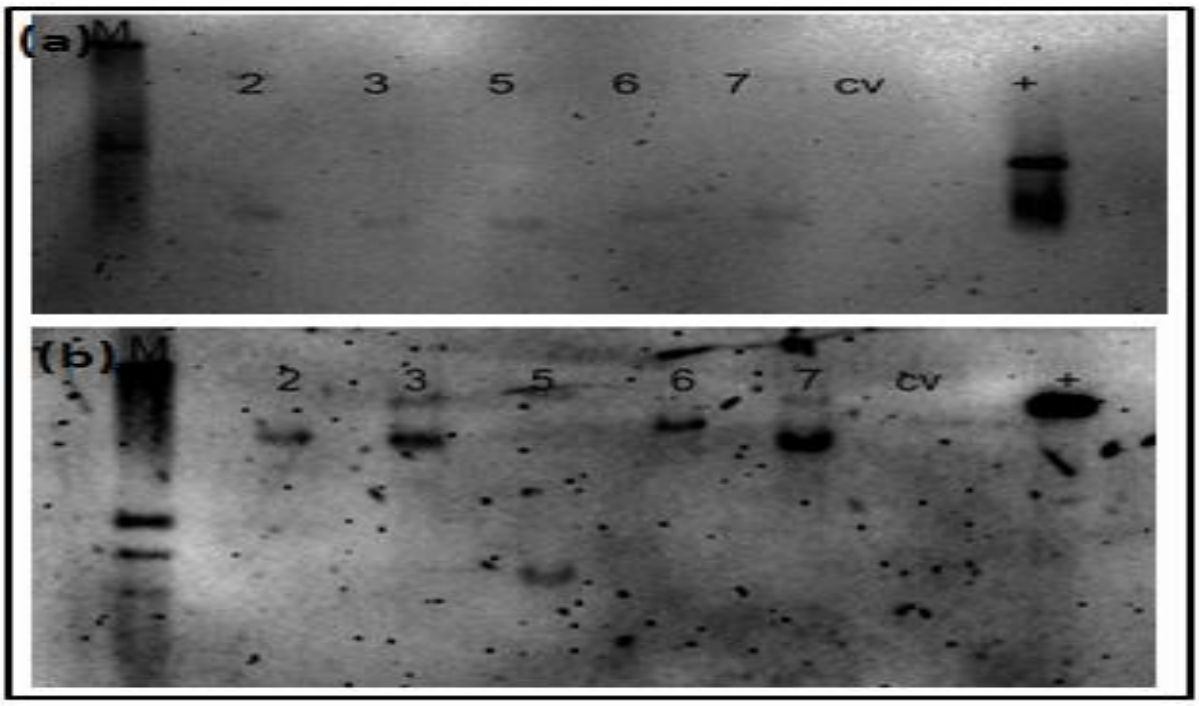


**Figure 2. 3** Southern blot analysis of **CMM6** transgenic lines 2, 3, 5, 6 and 7 for transgene integration using DIG-labeled probes. M = DIG molecular weight marker (Roche), cv. = non-infected healthy cv.60444 and +ve C = pC1305.1/CaMV35S- ACMV-[NG:Ogo:90] IR **(a)** *Hind*III and *EcoR*I-double digested genomic DNA probed with ACMV-[NG:Ogo:90] IR fragment and **(b)** *Hind*III-digested genomic DNA, probed with *hyg* probe

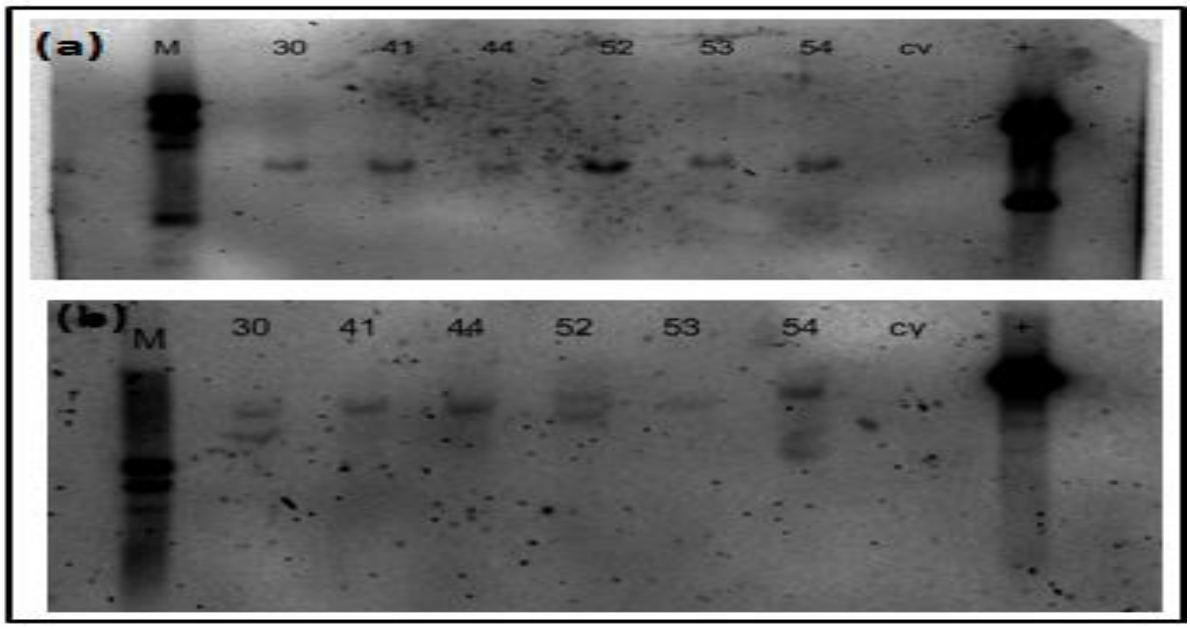


**Figure 2. 4** Southern blot analysis of **AMM2** transgenic lines 30, 41, 44, 52, 53 and 54 for transgene integration using DIG-labeled probes. M = DIG molecular weight marker (Roche), cv. = non-infected healthy cv.60444 and +ve C = *Hind*III and *EcoR*I-double digested pTZR5/T containing MM2hp cassette (a) *Hind*III and *EcoR*I-double digested genomic DNA probed with ACMV-[NG:Ogo:90] IR fragment and (b) *Hind*III-digested genomic DNA, probed with *hyg* probe.

### 2.4.2 PCR of *GUSPLUS*, *hygromycin* and virus-derived transgenes

Positive PCR amplification of *hyg*, *GUSPLUS* and ACMV-[NG:Ogo:90] IR fragment confirmed successful transgene integration in five CMM6 lines. Positive bands for both the 181 bp *GUSPLUS* amplicons and the 485 bp *hyg* fragments were observed (**fig. 2.5b**). All the five CMM6 lines tested positive for PCR amplification of both the 331 bp *Xba*I-arm and the 339 bp *Xho*I-arm of the ACMV-[NG:Ogo:90] IR construct (**fig 2.5a**). Positive amplification was achieved for the positive plasmid controls while no amplicons were obtained for the negative controls, healthy cv.60444 and the non template control (NTC).

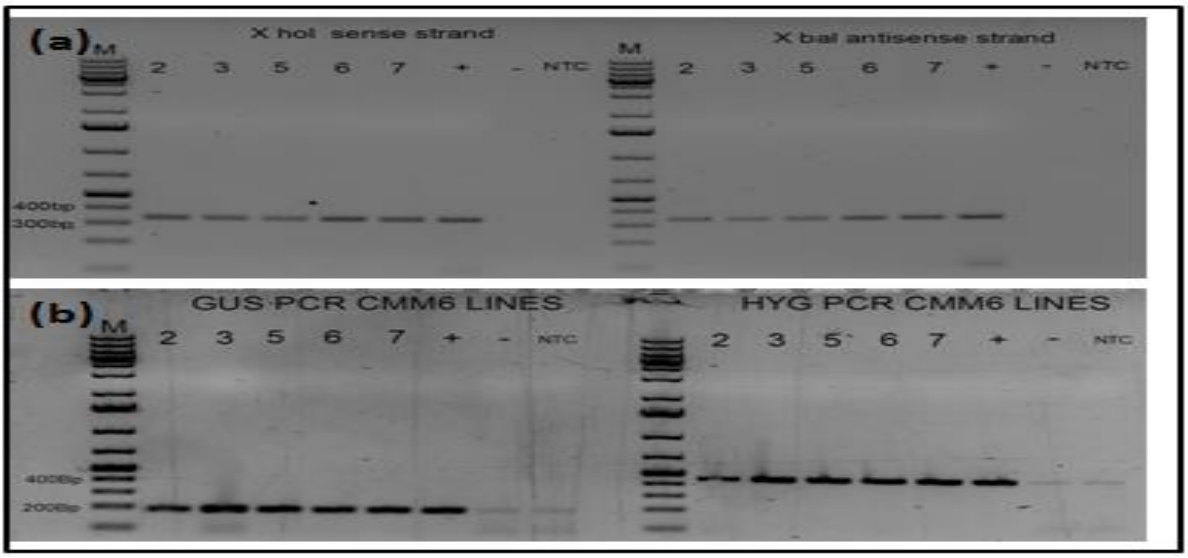


**Figure 2. 5** PCR confirmation of the transgenic status of **CMM6** lines. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas), (-) = untransformed healthy cv.60444 and +ve = positive pC1305.1/CaMV35S- ACMV-[NG:Ogo:90] IR and NTC = non template control. **(a)** PCR amplification of both the 331 bp *Xba*I-arm and the 339 bp *Xho*I-arm of the construct ACMV-[NG:Ogo:90] IR. Lines were screened for **(b)** 181 bp *GUSPLUS* fragment; fragment was successfully amplified in all lines screened and the 485 bp *hyg* fragment was detected in all lines tested.

All the six AMM2 lines were positive for both the 181 bp *GUSPLUS* amplicons and the 485 bp *hyg* fragment **(fig. 2.5b)**. Positive amplification was achieved for the plasmid controls while no amplicons were obtained for the negative control, namely untransformed healthy cv.60444 and the NTC. All the six AMM2 lines tested positive for PCR amplification of the 337 bp mutated sense-arm but the non mutated 331 bp strand could not be detected **(fig 2.5a)**.

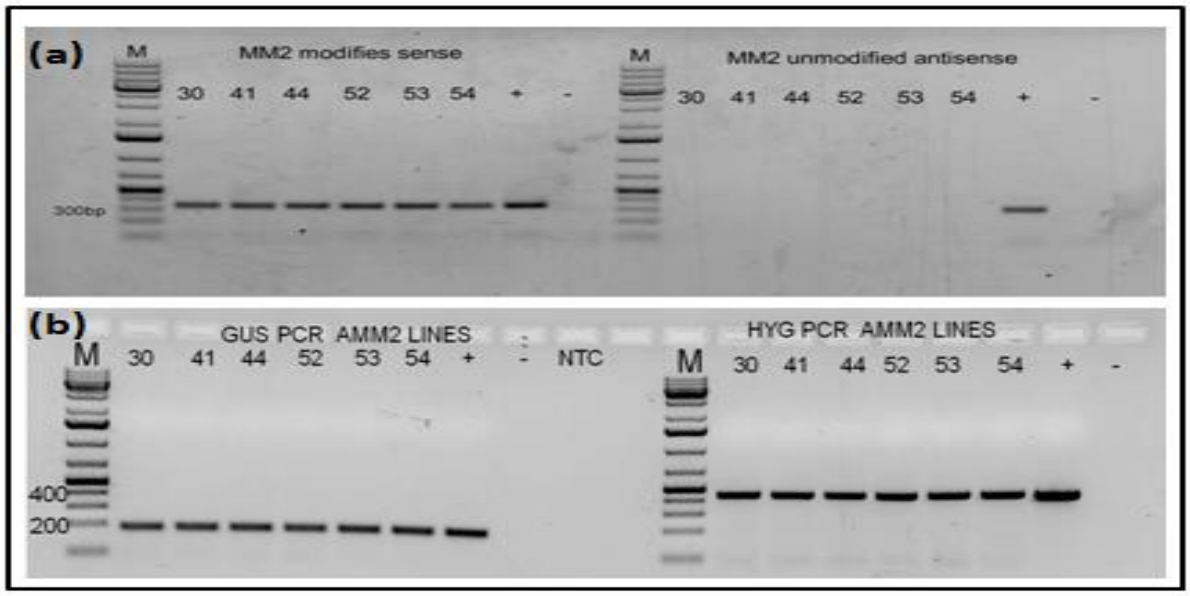


**Figure 2. 6** 1% agarose gel electrophoresis of *hyg*, *GUSPLUS* and ACMV-[NG:Ogo:90] IR construct PCR amplicons of the six AMM2 transgenic lines 30, 41, 44, 52, 53 and 54. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas), (-) = untransformed healthy cv.60444 and + ve = positive pC1305.1/CaMV35S- ACMV-[NG:Ogo:90]-NOg IR. Lines were screened for the **(a)** 337 bp mutated sense-arm amplicon and the 331 bp non-mutated antisense-arm amplicon. **(b)** 181 bp *GUSPLUS* gene amplicon and 485 bp *hyg* gene amplicon

**Screening of transgenic lines for resistance/tolerance or susceptibility at 14, 36, 56 and 365 dpi**

### 2.4.3 Sampling and symptom monitoring

A symptom severity score (sss) index (**fig. 2.7**) was used to evaluate severity based on a scale of 0-3 where 0= healthy leaves, 1= mild chlorosis, 2=clear mosaics with or without slight leaf deformation, 3= severe mosaics and severe leaf deformation. Differences in symptom scores between the infected CMM6, AMM2 transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant line dsAC1 were statistically evaluated at p= 0.05. Values less than this confidence interval were considered significant. P values for the t-tests

are in (**appendix A and B**)

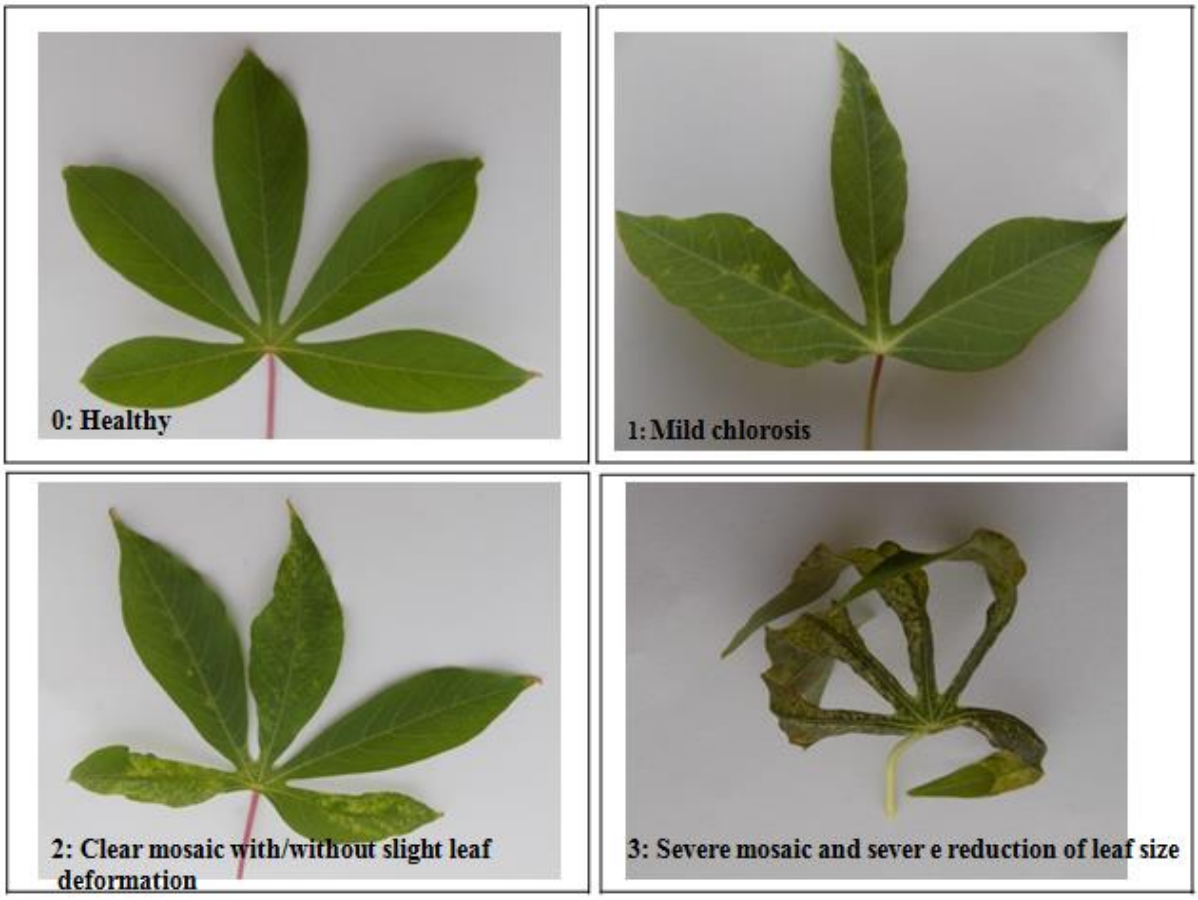


**Figure 2. 7** Cassava mosaic disease symptomatic leaves from trials in this study. A 0-3 scoring scale was used for evaluation. Each image represents each score level (0 being healthy asymptomatic leaves and 3 being fully symptomatic)

Symptom severity score (sss) of CMM6 transgenic lines were noticeably lower than those of infected untransformed cv.60444 plants at all time points (**fig 2.8a**). At 14 dpi CMM6 transgenic lines 2, 3, 6 and 7 had significantly lower ($p<0.05$) (**Appendix A**) symptom scores. The sss of the 4 transgenic lines were (3-fold, 2-fold, 1.7-fold and 1.7-fold) lower compared with infected untransformed cv.60444. CMM6-5 had a 1.5-fold lower sss compared with infected untransformed cv.60444, however there was no significant difference ($p=0.3433$). ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly lower ($p=0.001259$) sss (3-fold) compared with infected untransformed cv.60444. At 36 dpi, transgenic lines CMM6-2 and CMM6-6 had a significantly ($p=0.00002$ and $p=0.0001$, respectively) lower sss, (2.6 and 2.1-fold, respectively) compared with infected untransformed cv.60444. CMM6-5, 7 and 3 also had relatively lower sss (1.2, 1.0 and 1.0-fold, respectively) compared with infected untransformed cv.60444 however, these results

were not significantly different (p>0.05). At 36 dpi, ACMV-[NG:Ogo:90] resistant line dsAC1 had a significantly (p=0.0000183) lower sss (3.2-fold) compared with infected untransformed cv.60444. At 56 dpi, CMM6-2 and CMM6-6 sss remained significantly lower (p=0.00000129 and p=0.0000397, respectively), (2.9 and 2-fold, respectively) compared with infected untransformed cv.60444. CMM6 lines 3, 5 and 7 each had 1.0-fold lower sss but the results were not significantly different (p>0.05). At 56 dpi, ACMV-[NG:Ogo:90] resistant line dsAC1 line had a significantly (p= 0.00000221) lower sss (5-fold) compared with infected untransformed cv.60444.

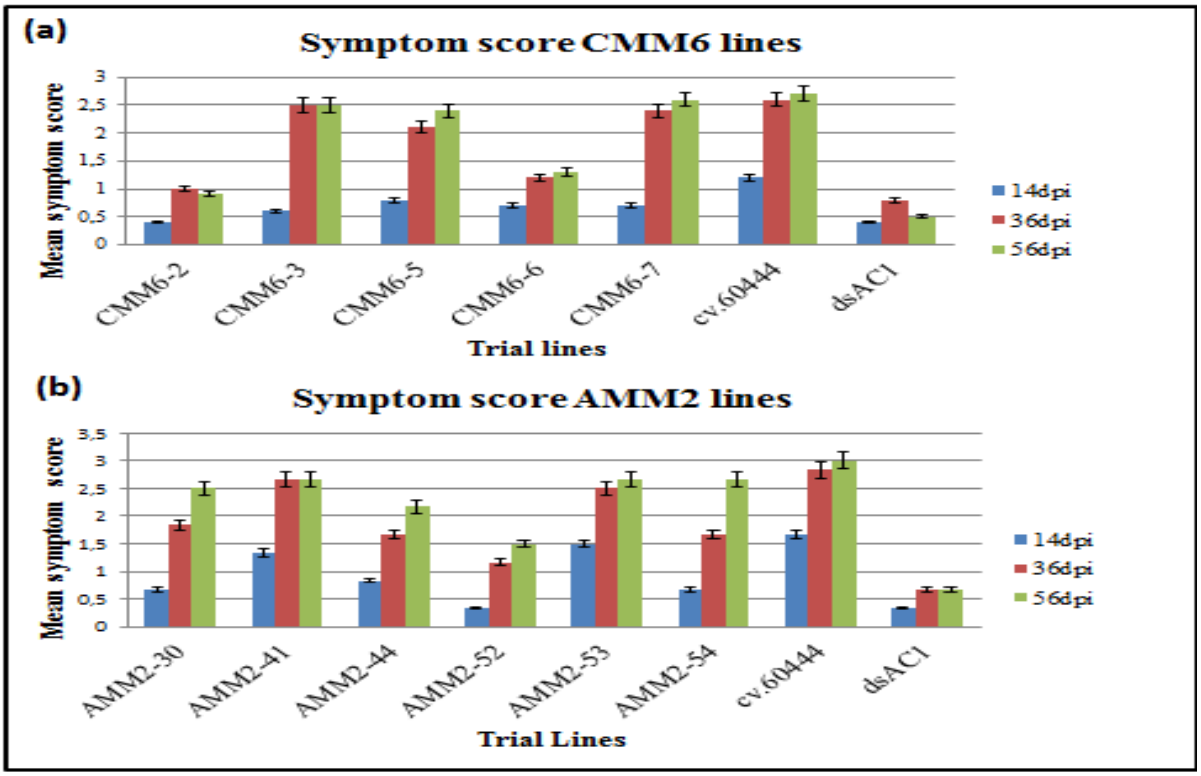


**Figure 2. 8** Symptom severity score of **CMM6** and **AMM2** transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 agro-inoculated with infectious ACMV-[NG:Ogo:90] clones. Plants were evaluated at 14 dpi (blue), 36 dpi (red) and 56 dpi (green) **(a)** CMM6 mean symptom severity scores (**b**) AMM2 mean symptom severity score. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

Symptom severity scores of AMM2 transgenic lines were also observed to be lower than that of infected untransformed cv.60444 **(fig 2.8b)** at all time points. At 14 dpi, AMM2 transgenic lines 30, 44, 52 and 54 had significantly (p<0.05) lower sss (2.5, 1.9, 4.5, and 2.6-fold, respectively) compared with infected untransformed cv.60444 **(Appendix B)**. AMM2-41 and

53 had lower sss, (1.2 and 1.0-fold, respectively) compared with infected untransformed cv.60444, however these results were not significantly different (p=0.2080 and p=0.2997, respectively) compared with infected untransformed cv.60444. ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significant (p=0.0005) lower sss (5-fold) compared with infected untransformed cv.60444.

At 36 dpi, AMM2-52 had significantly (p=0.00001) lower sss (2.4-fold) compared with infected untransformed cv.60444. AMM2-30, 44 and 54 has significantly lower sss ($p < 0.05$), (1.5, 1.75 and 1.75-fold) compared with infected untransformed cv.60444. AMM2-41 and 53 both had 1.0-fold lower sss compared with infected untransformed cv.60444 however, these results were not significant (p=0.2745 and p=0.1297, respectively). At 36 dpi, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly (p=0.000001) lower sss (4.5-fold) compared with infected untransformed cv.60444. At 56 dpi, AMM2-52 and 44 had a significantly (p=0.0006 and p=0.0109, respectively) lower sss, (2-fold and 1.4-fold, respectively) compared with infected untransformed cv.60444. AMM2-53 and 54 both had a 1.0-fold lower sss whilst lines 30 and 41 had a 1.2 and 1.4-fold lower sss, respectively; however, these results were not significantly different ($p > 0.05$).

### 2.4.3 Real time absolute quantitative PCR of viral load

Viral load accumulation in infected **CMM6** and **AMM2** transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 were determined by real time absolute quantitative PCR using DNA from leaves extracted at 36 and 56 dpi.

For **CMM6** lines, at 36 dpi CMM6-2 and CMM6-6 had significantly (p=0.030282 and p=0.0304), lower viral load (918 and 313-fold, respectively) compared with infected untransformed cv.60444 **(fig 2.9a)**. Transgenic lines 3, 5 and 7 had 1.6, 6.8 and 2.6-fold lower viral load, respectively, compared with infected untransformed cv.60444 however these results were not significantly different ($p > 0.05$) **(Appendix A)** from infected untransformed cv.60444. At 36 dpi, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly lower viral load (p=0.0302) which was 4117-fold lower compared with infected untransformed cv.60444. At 56 dpi, CMM6-2 and CMM6-6 viral load (6149 and 2327-fold, respectively), remained significantly lower (p=0.0462 and p=0.0462, respectively), compared with infected untransformed cv.60444. Transgenic lines 3, 5 and 7 had lower viral load (1.0, 0.1 and 1.8-fold, respectively) compared with infected

untransformed cv.60444 however, these results were not significantly different (p>0.05) from infected untransformed cv.60444. ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly lower (p=0.0462) viral load (9633-fold) compared with infected untransformed cv.60444.

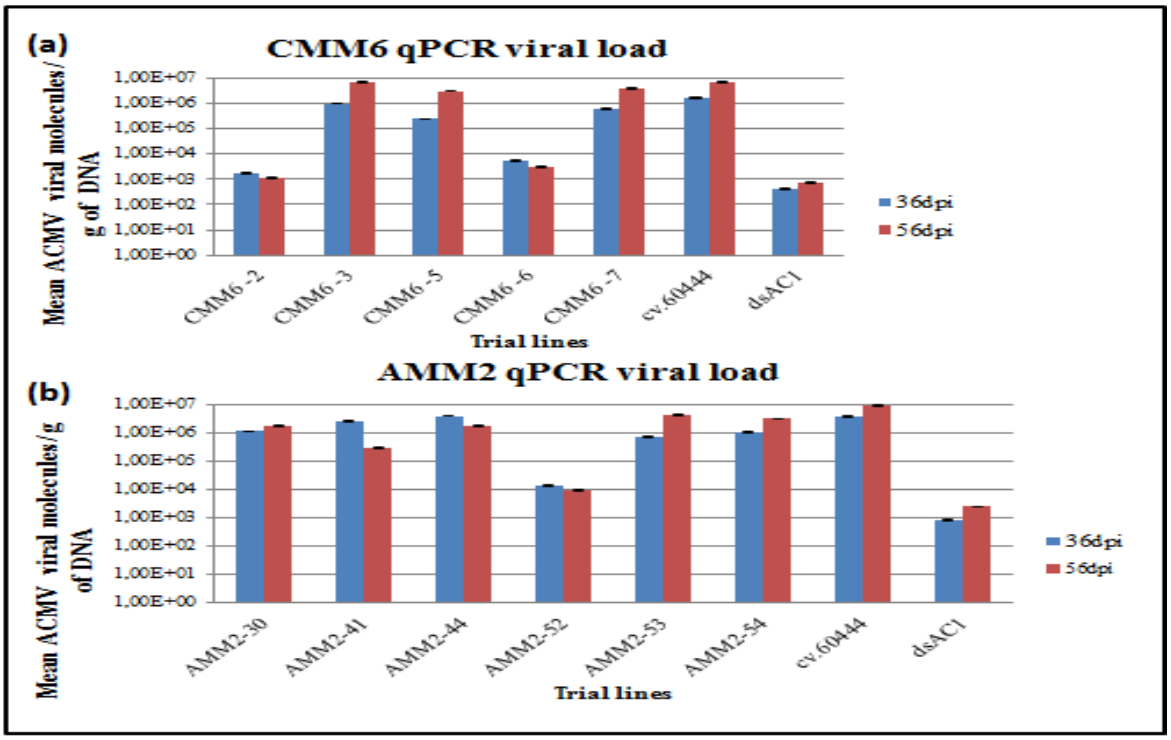


**Figure 2. 9** Viral load of **CMM6** and **AMM2** transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 agroinoculated with infectious ACMV-[NG:Ogo:90] clones. Plants were evaluated at 36 dpi (blue), 56 dpi (red) for **(a)** CMM6 mean absolute viral load quantification (mean viral molecules/g DNA) **(b)** AMM2 mean absolute viral load quantification (mean viral molecules/g DNA). The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

For AMM2 lines, at 36 dpi AMM2-52 has significantly lower (p=0.0338) viral load (278-fold) compared to infected untransformed cv.60444 **(fig 2.9b)**. Transgenic lines AMM2-30, 41, 44, 53 and 54 had 3.4, 1.4, 0.9, 5.8 and 3.6-fold lower viral load, respectively, compared with infected untransformed cv.60444, however these results were not significantly different (p>0.05) **(Appendix B)** from infected untransformed cv.60444. At 36 dpi ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly (p=0.0335) lower viral load (4657-fold) compared with infected untransformed cv.60444. At 56 dpi, AMM2-52 and 41 had significantly lower (p=0.321 and p=0.0349, respectively) viral load (963 and 31.6-fold respectively) compared with infected untransformed cv.60444. Transgenic lines AMM2-30,

44, 53 and 54 had 5.1, 5.2, 2.1 and 2.8-fold lower viral load respectively, compared with infected untransformed cv.60444 however, these results were not significantly different ($p>0.05$) from infected untransformed cv.60444. At 56 dpi ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had significantly ($p=0.0320$) lower viral load (3,575-fold) compared with infected untransformed cv.60444.

### 2.4.4 Plant height evaluation

Plant height evaluation for infected **CMM6** transgenic lines, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 was measured at 14, 36 and 56 dpi. There were no significant differences between the height of CMM6 transgenic lines and that of infected untransformed cv.60444 controls (**fig 2.10a**). At 14 dpi, CMM6-2, 3, 5 and 6 had significantly lower average height ($p< 0.05$) compared with infected untransformed cv.60444. Only CMM6-7 ($p=0.06$) had an average height that was not significant lower compared with infected untransformed cv.60444. At 36 and 56 dpi no significant difference ($p>0.05$) in mean plant height were recorded for all CMM6 transgenic lines and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 compared with infected untransformed cv.60444 (**Appendix A**).

For AMM2 transgenic lines, at 14 dpi AMM2-53 mean plant height was significantly lower ($p=0.0171$) compared with infected untransformed cv.60444 whilst AMM2-30, 41, 44, 52 and 54 had an average height that was not significantly different ($p>0.05$) to infected untransformed cv.60444 (**fig 2.10b)**. At 36 and 56 dpi no significant difference in mean plant height were recorded for all AMM2 transgenic lines and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 ($p>0.05$) compared with infected untransformed cv.60444 **(Appendix B)**.

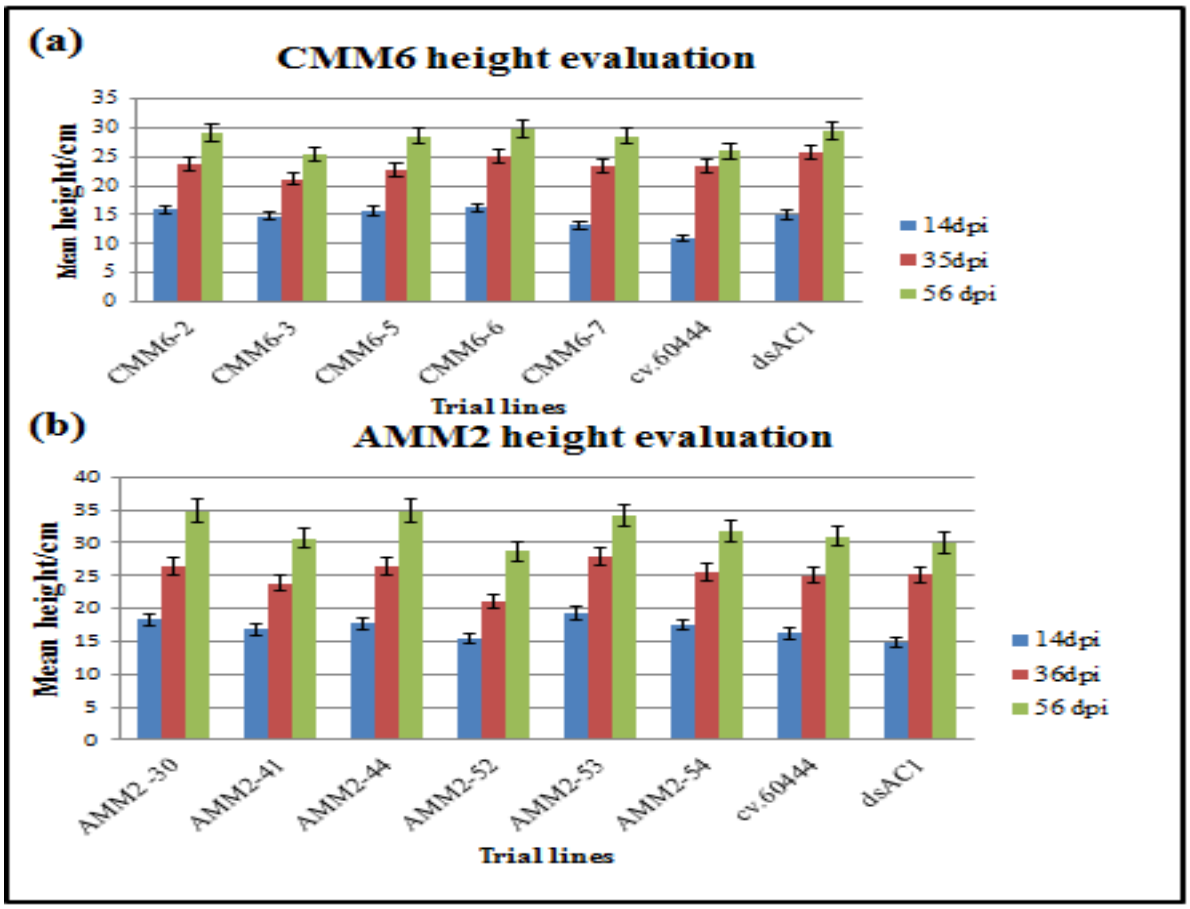


**Figure 2. 10** Height evaluations for **CMM6** and **AMM2** transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant line dsAC1. Plants were evaluated at 14 dpi (blue), 36 dpi (red) and 56 dpi (green) **(a)** CMM6 mean average height **(b)** AMM2 mean average height. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

**Evaluation of CMM6 and AMM2 transgenic lines at 365dpi**

**2.4.5 Symptom score and viral load**

CMM6 transgenic lines were evaluated at 365 dpi. Transgenic lines CMM6-2 and CMM6-6 were asymptomatic. ACMV-[NG:Ogo:90] resistant line dsAC1 was also asymptomatic. CMM6-7 had mild symptoms whilst CMM6-3 and cv.60444 had similar symptoms to those recorded at 56 dpi **(fig 2.8a)**. Viral load of these transgenic lines were also quantified and we observed that the viral loads had decrease from those recorded at 56 dpi. For transgenic lines CMM6-2, 3, 5, 6 and 7 there was a 44, 13.7, 13.2, 116 and 79.3-fold decrease, respectively, in viral load from 56 dpi to 365 dpi **(fig 2.11a)**. Infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic lines dsAC1 had a 4.4 and 20.9 fold decrease, respectively, in viral load from 56 dpi to 365 dpi. At 365 dpi, the viral load of CMM6-2 and

CMM6-6 (60,602 fold) remained significantly lower (p=0.04695 and p=0.04695, respectively) compared with infected untransformed cv.60444. Transgenic lines 3, 5 and 7 had 3.2, 6.5 and 32.7 fold lower viral load compared with infected untransformed wild type cv.60444 however, these results were not significant (p>0.05) **(Appendix F)** from infected untransformed cv.60444. ACMV-[NG:Ogo:90] resistant line dsAC1 had significantly (p=0.046951) lower viral load (44,243 fold) compared with infected untransformed cv.60444.

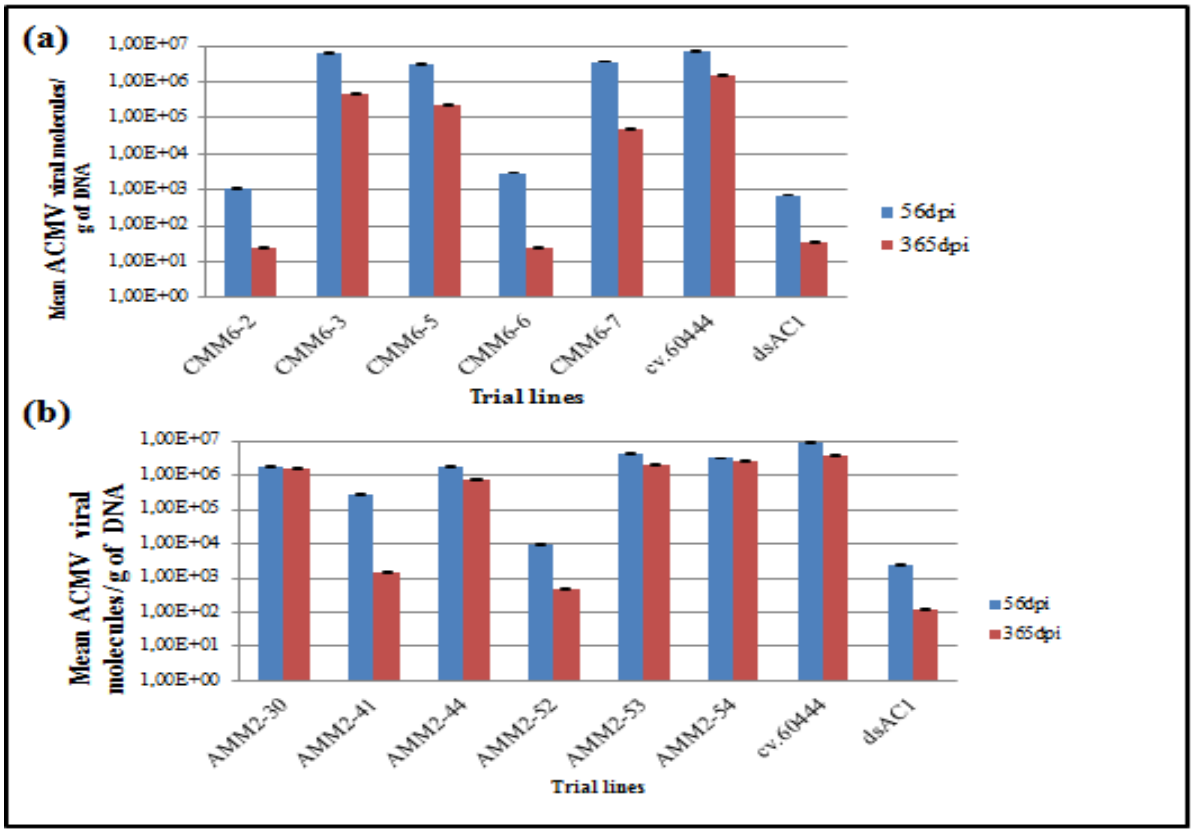


**Figure 2. 11** Mean ACMV viral molecules/g of DNA at 365 dpi compared to mean viral molecules/g of DNA at 56 dpi, quantified using real time absolute qPCR, **(a)** ACMV infected CMM6 transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 **(b)** ACMV infected AMM2 transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant line dsAC1 transgenic line. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

Transgenic lines AMM2-41, AMM2-52 and AMM2-44 were asymptomatic at 365 dpi. Resistant line dsAC1 was also asymptomatic. AMM2-30, 53 and 54 has mild symptoms whilst infected untransformed cv.60444 had similar symptoms to those recorded at 56 dpi (**fig 2.8b**). Viral loads of transgenic lines, infected untransformed cv.60444 and infected ACMV

resistant transgenic line dsAC1 had decreased from those recorded at 56 dpi. For transgenic lines AMM2-30, 41, 44, 52, 53 and 54 there was a 1.1, 197, 2.2, 20, 2 and 1.2-fold decrease, respectively, in viral load from 56 dpi to 365 dpi **(fig 2.11b)**. Controls, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had a 2.4 and 21.5-fold decrease respectively, from 56 dpi to 365 dpi. At 365 dpi, AMM2-41 and AMM2-52 had a significantly ($p=0.0490$ and $p=0.0490$, respectively) lower viral load (2,518 and 7,882-fold, respectively) compared with infected untransformed cv.60444 viral load. Transgenic lines AMM2-30, 44, 53 and 54 had a 2.3, 4.6, 1.8 and 1.4-fold lower viral load compared with infected untransformed cv.60444 however, these results were not significantly different ($p>0.05$) **(Appendix F)** from infected untransformed cv.60444. Viral load of ACMV-[NG:Ogo:90] (31,051-fold) was significantly lower ($p=0.0490$) compared with infected untransformed cv.60444.

### 2.4.6 Yield evaluation

At 365 dpi, three plants per each of the CMM6 or AMM2 transgenic lines, infected untransformed cv.60444, non-infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 were harvested. The tubers yields were weighed to determine fresh weight **(fig 2.12)**. The same tubers were then dried at 105°C overnight in a dry oven and the dry tuber yield was measured. The percentage tuber dry weight was calculated from the tuber fresh weight. Fresh and dry weights (grams) are the average of three tubers per line.


**Figure 2. 12** Pictorial representation of the tuber exhibiting the highest yield from each of the CMM6 trangenic lines 2, 3, 5, 6 and 7, infected untransformed cv.60444, non-infected healthy cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1


**Figure 2. 13** Pictorial representation of the tuber exhibiting the highest yield from each of the AMM2 trangenic lines 30, 41, 44, 52, 53 and 54, infected untransformed cv.60444, non-infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1

From the non mismatched CMM6 yield evaluation, non-infected healthy cv.60444 had the highest average tuber fresh weight of 39.4g **(fig 2.14a)**. For the transgenic CMM6 lines, CMM6-2 and CMM6-6 had an average fresh weight (34.4 and 20.2 g, respectively) that were lower but not significantly different ($p>0.05$) **(Appendix E)** from the fresh weight of non-infected healthy cv.60444. CMM6-3, 5 and 7 had average fresh weights of 5.5, 10.3 and 6.6 g, respectively, and the results were significantly lower ($p<0.05$) from the fresh weight of non-infected healthy cv.60444. Infected untransformed cv.60444 weighed on average 8 g and these results were significantly lower ($p=0.0309$) from the fresh weight of non-infected healthy cv.60444. There was a 3.5 fold difference in average fresh weight between non-infected healthy cv.60444 and its infected counterpart. ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had an average fresh weight of 35.4 g and these results were lower but not significantly different ($p=0.1686$) **(Appendix E)** from the fresh weight of non-infected healthy cv.60444. The average percentage tuber dry weight of transgenic lines CMM6-2, 3, 5, 5 and 7 were 38, 38, 37, 39 and 36%, respectively, **(fig 2.15a)**. Infected untransformed cv.60444 and non-infected healthy cv.60444 had an average of 38 and 39% tuber dry weight, respectively. ACMV-[NG:Ogo:90] resistant dsAC1 transgenic lines had an average tuber dry weight of 39%.

From the mismatched AMM2 trial, non-infected healthy cv.60444 had the highest average fresh weight of 40.5g **(fig 2.14a)**. AMM2-41 and 52 tubers weighed on average 33.4 and 33.6 g, respectively. Fresh weight from these two lines were lower but not significantly different ($p=0.1872$ and $p=0.1893$) **(Appendix E)** from the fresh weight of non-infected healthy cv.60444. Transgenic lines AMM2-30, 44, 53 and 54 had 20.9, 22.5, 15.1 and 16.1 g fresh weights, respectively, and these results were significantly lower ($p<0.05$) compared with the average fresh weight of non-infected healthy cv.60444. Infected untransformed cv.60444 had average fresh weight of 9.3 g which was significantly lower ($p=0.0055$) (4.3-fold) compared with the average fresh weight of non-infected healthy cv.60444. ACMV-[NG:Ogo:90] resistant transgenic lines dsAC1 had an average fresh weight of 37.2 g which was lower but not significantly different ($p=0.3687$) compared with the average fresh weight (40.5 g) of non-infected healthy cv.60444. The average percentage tuber dry weights of transgenic lines AMM2-30, 41, 44, 52, 53 and 54 was 37.4, 38, 37, 37.7, 36.9 and 38 %, respectively **(fig 2.15b)**. Tubers from non-infected healthy cv.60444 and infected untransformed cv.60444 had a 37.9 and 38% tuber dry weight, respectively. ACMV-[NG:Ogo:90] resistant dsAC1 transgenic tubers had a 38.5 % average tuber dry weight.

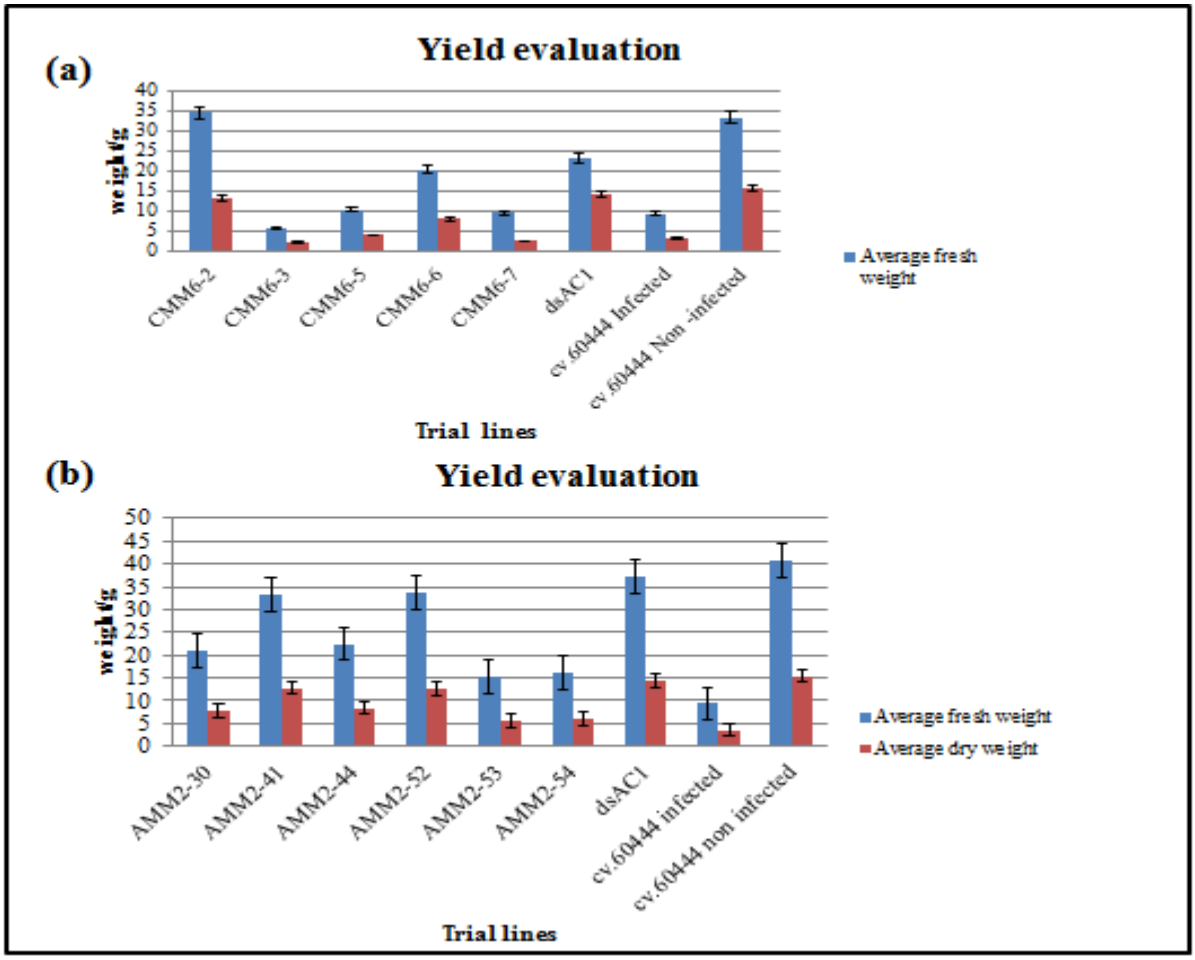


**Figure 2. 14** Mean tuber fresh weight (blue) and mean tuber dry weight (red) of (**a**) CMM6 transgenic lines, infected untransformed cv.60444, non-infected healthy cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 (**b**) AMM2 transgenic lines, infected untransformed cv.60444, non-infected healthy cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

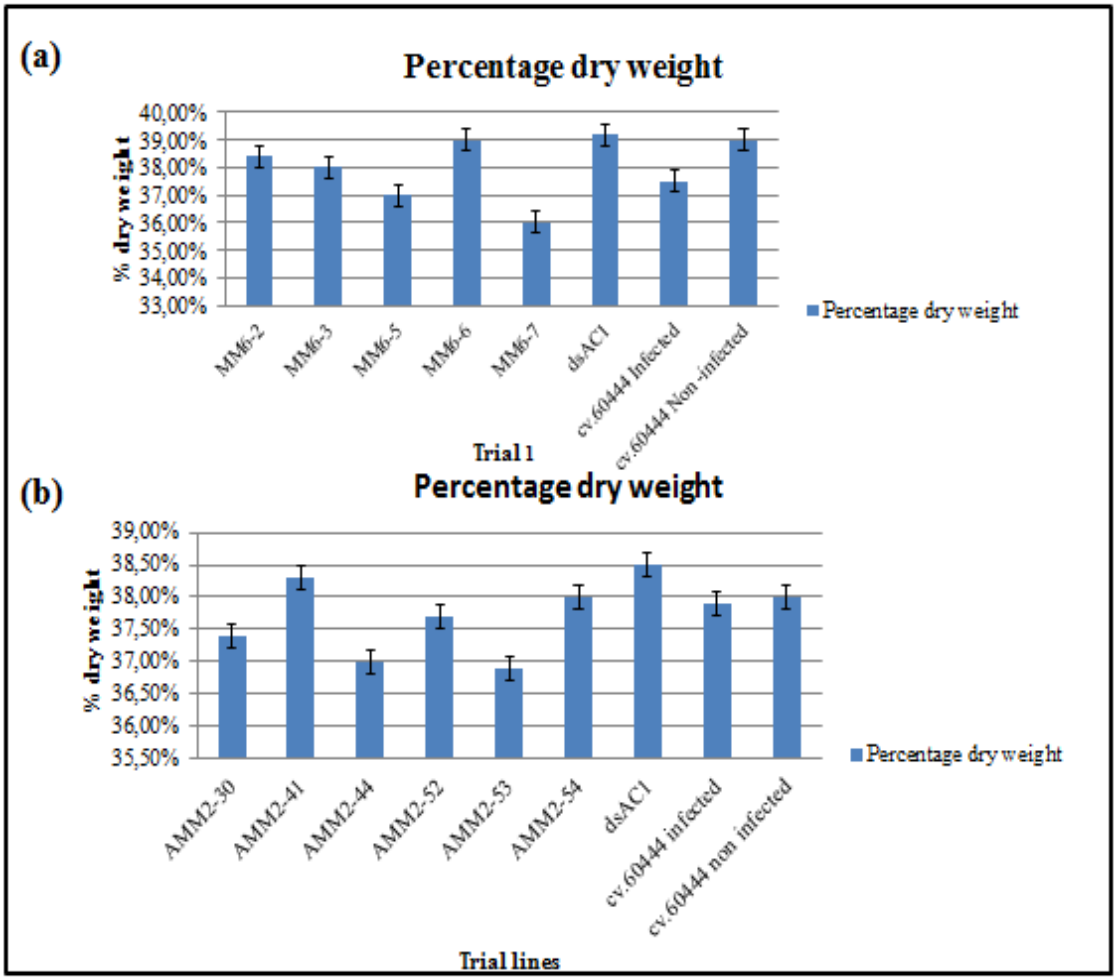


**Figure 2. 15** Mean percentage tuber dry weight of (**a**) CMM6 and (**b**) AMM2 trial lines, infected untransformed cv.60444, non-infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1. The percentage was calculated from the difference between the tuber fresh weight and dry weight. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

## Small interfering RNA evaluation of trial plants

### 2.4.7 RNA extraction

High quality RNA was extracted using the QIAzol method and the integrity of the RNA was checked on a 1.2% agarose gel containing 10 µg/µl ethidium bromide run in 0.5X TBE. The results show that high quality RNA was successfully extracted from pre-inoculated CMM6, AMM2 lines and non-infected healthy cv.60444 (**fig 2.16)**. RNA was also extracted from

infected CMM8, AMM4 transgenic lines, infected untransformed cv.60444 and non-infected untransformed cv.60444.

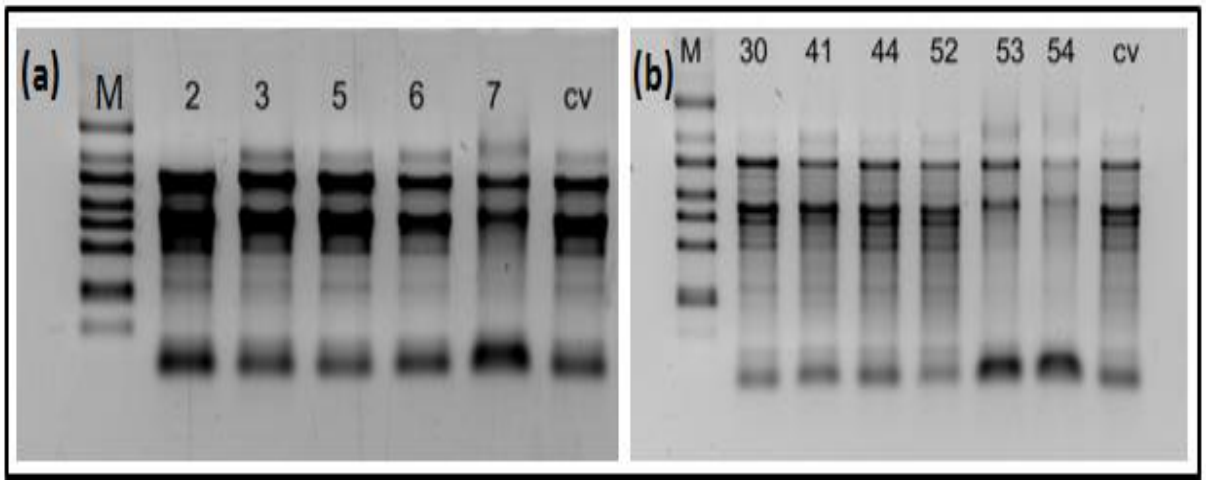


**Figure 2. 16** 1.2% agarose gel electrophoresis of total RNA extracted from (a) CMM6 transgenic lines 2, 3, 5, 6, 7, non-infected healthy cv.60444. (b) AMM2 transgenic lines 30, 41, 44, 52, 53, 54 and non-infected healthy cv.60444

### 2.4.8 T-DNA expression results

Total RNA (**2.4.7)** was used to synthesise cDNA using Random hexamers and OligodT primers. The cDNA was used as template for RT-PCR amplification of *hyg* (485 bp), *GUSPLUS* (181 bp) and ACMV-[NG:Ogo:90] IR to check for expression levels. RT-PCR of the cDNA from transgenic lines resulted in successful amplification of the *GUSPLUS* and h*yg* in both mismatched AMM2 lines **(fig 2.17d)** and non-mismatched CMM6 transgenic lines **(fig 2.17c)**. As expected no amplicons were detected in non-infected healthy cv.60444, and the PCR amplification reaction controls were positive for both *GUSPLUS* and *hyg*. Complementary-DNA from CMM6 and AMM2 lines was successful amplified yielding the 331 bp CMM6 *Xba*I and 339 bp CMM6 *Xho*I **(fig 2.17a)** fragments. The CMM6 arms were strongly amplified as seen by comparison with amplification of endogenous *ubiquitin* gene **(fig 2.17ai)**. AMM2 lines showed low expression of the 337 bp mutated sense arm **(fig 2.17b)** compared to the amplification of endogenous *ubiquitin* gene **(fig 2.17bi)**. The non-mutated antisense arm (331 bp) fragment of the AMM2 hairpin construct could not be amplified from cDNA even though the positive control plasmid was amplified (gel not shown)

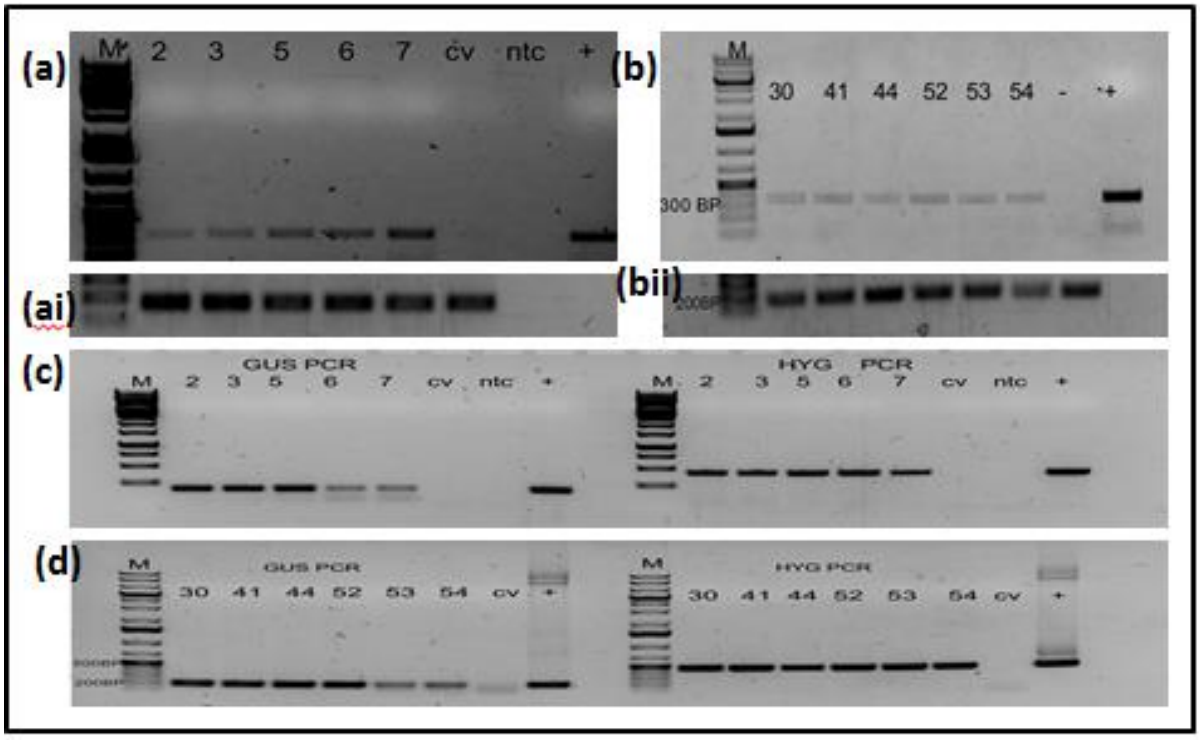


**Figure 2. 17** RT-PCR amplification of transgene sense arm, *GUSPLUS* and *hyg* genes from CMM6, AMM2 and non-infected healthy cv.60444 negative control. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas). cv. = non-infected healthy cv.60444 negative control. + = positive control transgene plasmid (**a**) amplication of CMM6 transgenic lines sense arm (**ai**) amplification of endogenous *ubiquitin* gene from CMM6 cDNA (**b**) amplification of AMM2 transgenic lines mutated sense arm (**bii**) amplification of endogenous *ubiquitin* gene from AMM2 cDNA (**c**) amplification of 181 bp *GUSPLUS* and 485 bp *hyg* from CMM6 transgenic lines cDNA (**d**) amplification of 181 bp *GUSPLUS* and 485 bp *hyg* from AMM2 transgenic lines cDNA

### 2.4.9 Northern blot detection of siRNA

Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] AC1/4:AC2/3 IR RNA was labeled using Northern Starter Kit (Roche). For siRNA control, an *Arabidopsis* 21 nt miR167 (5-'TAGATCATGCTGGCAGCTTCA-3'), oligonucleotide was 3'end labeled. For size marker a 24 nt primer (5'-TTCAGGAGCCTCCGCCTGTCT-3') and a 30 nt positive control from the kit were 3' end tail labeled using DIG Oligonucleotide Tailing Kit 2$^{nd}$ generation (Roche). The labeling results showed successful labeling of the ACMV-[NG:Ogo:90] AC1/4:AC2/3 IR RNA, siRNA control and size marker oligonucleotide **(fig 2.13)**. The labeled RNA and short oligonucleotides were detected at low concentrations of 10 ng/µl and 1 ng/µl.

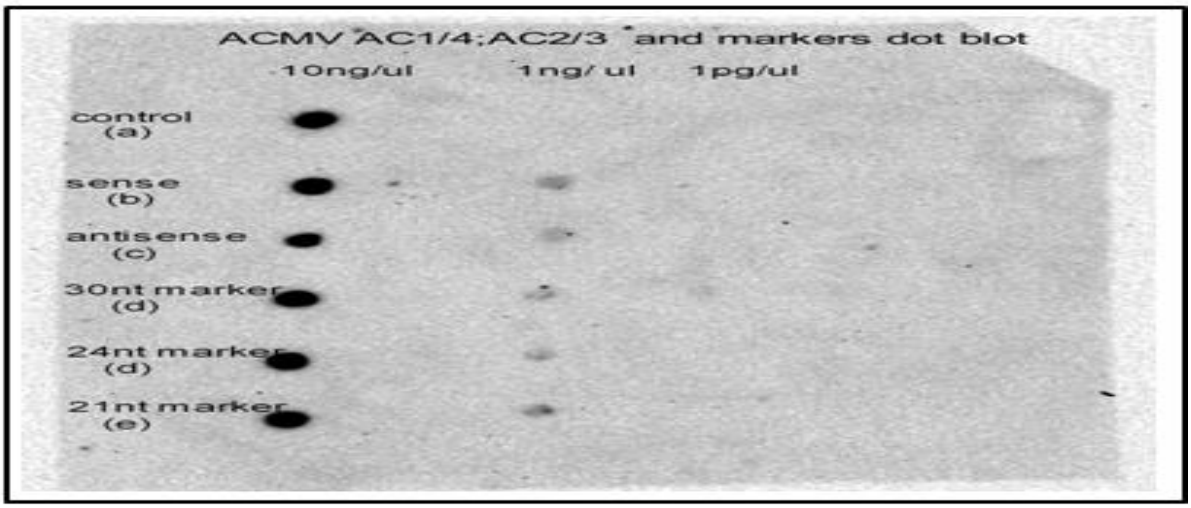


**Figure 2. 18** Determination of the DIG labeling efficiency on (a) ACMV-[NG:Ogo:90] AC1/4:AC2/3 IR sense arm (b) ACMV-[NG:Ogo:90] AC1/4;AC2/3 antisense arm (a) a Northern Starter Kit (Roche) labeled RNA control.(d) labeled DIG oligonucleotide tailing kit 2nd generation (Roche) 30 nt control (d) labeled 24 nt oligonucleotide and (e) labeled 21 nt *Arabidopsis* mi167

Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] AC1/4:AC2/3 IR-derived siRNA molecules accumulation were detected at varying levels (based on semi quantitative relative band intensity comparison) (Biorad Chemi Doc Quantity tool) in CMM6 **(fig 2.14a)** and AMM2 transgenic lines prior to infection (**fig 2.14b)**. Using the siRNAs at the highest concentration being equal to 100%, CMM6-2, 3, 5, 6 and 7 had a 65%, 49%, 87%, 54% and 100% accumulation, respectively. Using the siRNAs at the highest concentration being equal to 100%, AMM2-30, 41, 44, 52, 53 and 54 had a 12%, 89%, 100%, 12%, 58% and 44% accumulation, respectively. Non-infected healthy cv.60444 did not produce siRNAs as expected. Loading control miRNA 167 was detected in CMM6 **(fig 2.14ai)** and AMM2 transgenic lines **(fig 2.14bi)** after stripping of the ACMV-[NG:Ogo:90] AC1/4:AC2/3 IR.

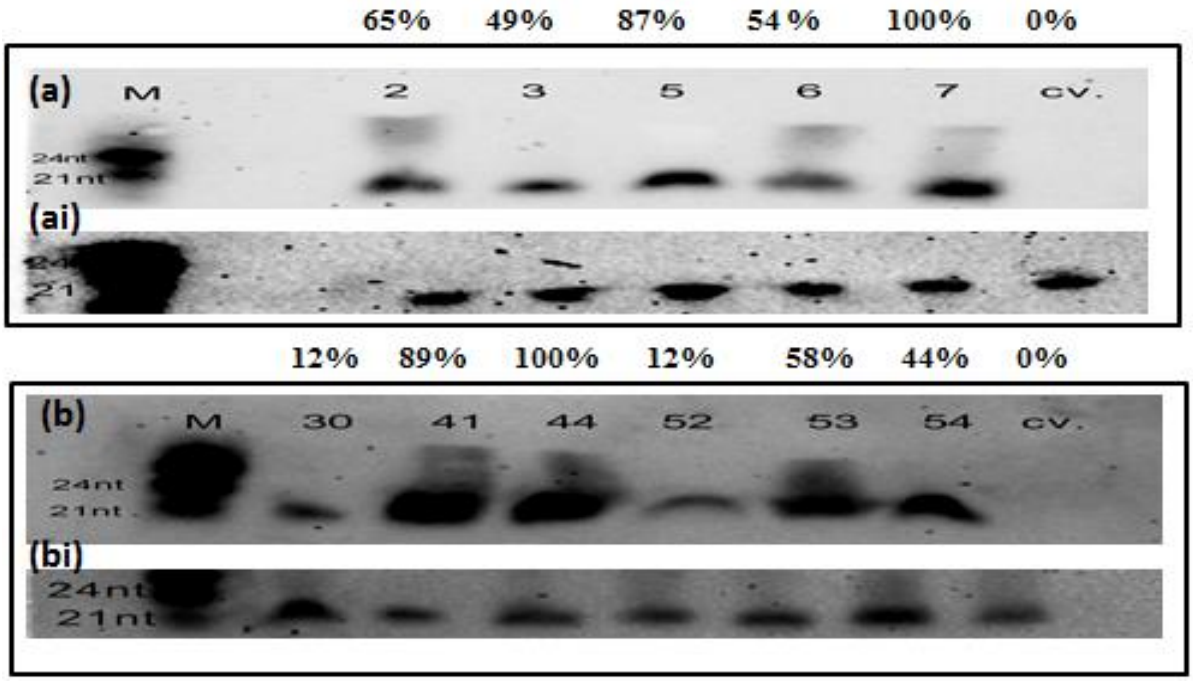


**Figure 2. 19** Northern blot siRNA detection (**a**) CMM6 transgenic lines and healthy cv.60444 probed with DIG-labeled ACMV-[NG:Ogo:90] IR derived probe (**b**) AMM2 transgenic lines and healthy cv.60444 probed with DIG-labeled ACMV-[NG:Ogo:90] IR derived probe. (**ai**) CMM6 and (**bi**) AMM2 transgenic lines and healthy cv.60444 probed with DIG labeled *Arabidopsis* miRNA 167 probe. The percentage numbers above represent relative accumulation levels of the siRNAs

At 365 dpi, ACMV-[NG:Ogo:90] infected CMM6 lines still produced small nucleotides approximately 21 nt bases in size. Using the siRNAs at the highest concentration being equal to 100%, CMM6-2, 3, 5, 6 and 7 had a 51%, 34%, 94%, 24% and 100% accumulation, respectively. Infected untransformed cv.60444 produced siRNAs of approximately 21 nt bases in size (6.4%) (**fig 2.20a**) whilst non-infected healthy cv.60444 did not produce siRNAs. At 365 dpi, ACMV-[NG:Ogo:90] infected AMM2 lines still produced small nucleotides 21 nt bases in size. Using the siRNAs at the highest concentration being equal to 100%, AMM2-30, 41, 44, 52, 53 and 54 had a 46%, 31%, 60%, 50%, 100% and 93% accumulation, respectively. Infected untransformed cv.60444 produced siRNAs 21 nt bases in size (55%) whilst non-infected healthy cv.60444 did not produce siRNAs.

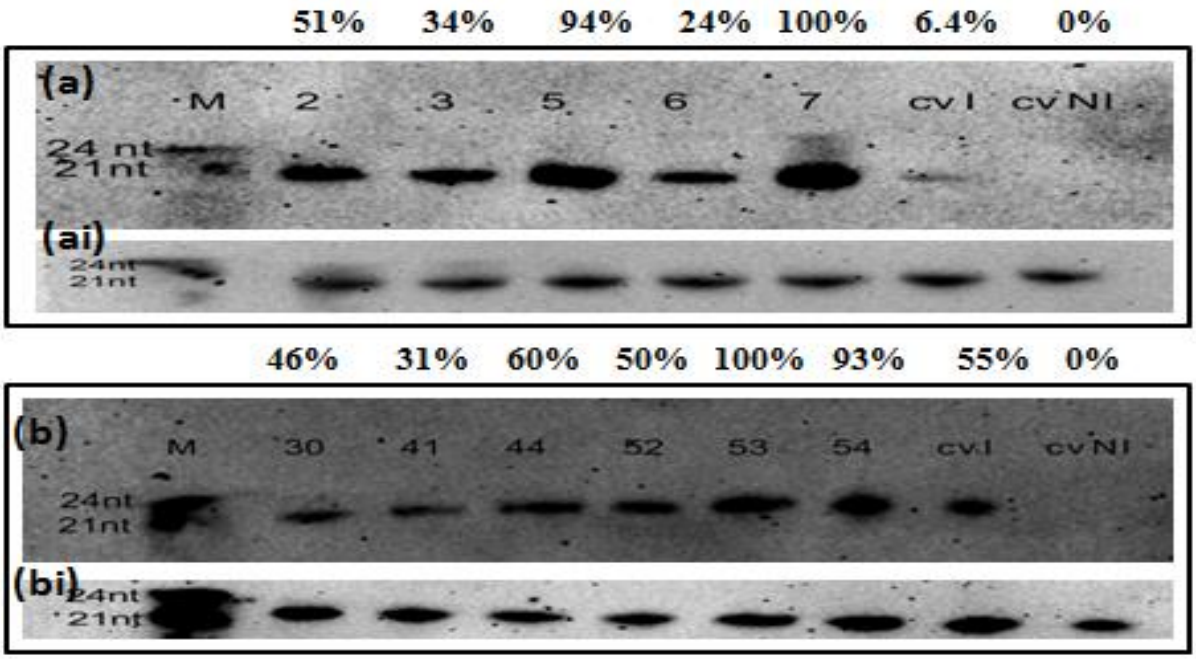


**Figure 2. 20** Northern blot for siRNA detection (a) CMM6 transgenic lines, infected untransformed cv.60444 and non-infected healthy cv.60444 probed with DIG labeled ACMV-[NG:Ogo:90] IR derived probe (b) AMM2 transgenic lines, infected untransformed cv.60444 and non-infected healthy cv.60444 probed with DIG labeled ACMV-[NG:Ogo:90] IR derived probe. (ai) CMM6 and (aii) AMM2 transgenic lines and controls probed with DIG labeled *Arabidopsis* miRNA 167 probe. The percentage numbers above represent relative accumulation levels of the siRNAs

## 2.5 Discussion

The application of RNA silencing to plant disease resistance is well documented (Reviewed in Duan *et al*., 2012; Kumar and Sarin, 2013; Prins *et al*., 2008). Introduction of DNA sequences or segments of viral genes leads to resistance or tolerance by sequence- specific binding of virus transgene-derived siRNA to the homologous infecting viral mRNA, and subsequent degradation. The objective of this study was to evaluate the tolerance, resistance or susceptibility of cassava cv.60444 transformed with a mismatched or non-mismatched inverted repeat (IR) transgene targeting two overlapping ORFs, namely the Rep/VSR (AC1/4) and TrAP/Ren (AC2/3), and to compare the efficiency of both mismatched and non-mismatched. In both our CMM6 and AMM2 trials the transgenics symptom severity was observed to be lower than that of infected untransformed cv.60444 at the symptomatic stage (36d pi) and the recovery stage (56 dpi). A few of these transgenic lines showed mild symptoms and such lines with reduced symptoms in the presence of viral replication are termed tolerant (Bengyella *et al*., 2015; Lapidot and Friedman, 2002). Resistance implies no detection of virus replication and absence of symptoms. Notably, in the two trials we identified tolerant lines CMM6-2, CMM6-6 and AMM2-52 with significantly lower symptom severity scores in comparison with infected infected untransformed cv.60444. However, these three lines, while exhibiting milder symptoms compared with non-transgenic plants, displaced more severe symptoms than the positive ACMV-[NG:Ogo:90] dsAC1 resistant line engineered by Vanderschuren *et al*. (2009). Notably, at 36 and 56 dpi CMM6-2, CMM6-6 and AMM2-52 had significantly lower viral load compared with infected untransformed cv.60444. A positive Pearson correlation was established in CMM6 and AMM2 trials between sss and viral load. CMM6 lines had a positive Pearson coefficient of 0.94 at 36 dpi and positive Pearson coefficient of 0.95 at 56 dpi (**Appendix G**). AMM2 lines had a positive Pearson coefficient of 0.25 at 36 dpi and 0.59 at 56 dpi. It is generally accepted that a positive correlation occurs between sss and viral load. Vanderschuren *et al*. 2009 report attenuated symptoms of dsAC1 transgenic lines correlated with viral titre levels in the leaves. The difference between resistance reported in ACMV-[NG:Ogo:90] dsAC1 transgenic line engineered by Vanderschuren *et al*. (2009) and our study may be contributed to the different viral sequence regions (IR) introduced into cv.60444. Constructs from both studies were hairpin/IR constructs which has been shown to be efficient in RNA virus–model plant systems (Smith *et al*., 2000) and other geminivirus-plant systems (Pooggin *et al*., 2003;

Vanitharani *et al*., 2003). Our ACMV-[NG:Ogo:90] IR targeted 5' AC1/AC4 and AC2/AC3 overlaping sequence whilst the dsAC1 transgenic plants express an IR ACMV-[NG:Ogo:90] AC1/Replication–associated protein (Rep) sequence (154 bp) from nt position 1690 to 1844 (Vanderschuren *et al*., 2009). Geminivirus resistance has been reported in transgenic plants expressing intron dRNA IRs (hairpins) homologous to Rep/AC1 (Ammara *et al*., 2015; Fuentes *et al*., 2006; Ye *et al*., 2014). Rep/AC1 is essential for geminivirus replication (Hanley-Bowdoin *et al*., 2000). The transgene used in this study was a stacked construct against 4 ORFs, namely AC1/Rep, AC4/putative VSR, AC2/TrAP, and AC3/Ren which has not been reported in cassava begomovirus resistance studies to date. Stacking of genes in transgenic plants could be a more viable method to induce broad spectrum durable resistance against diseases (Zhu *et al*., 2012). We expected that the generation of a large number of siRNAs targeting multiple ORFs on the ACMV viral DNA A genome would result in more effective resistance than the dsAC1 line, but this was not the case. Our results were however more consistant with previous attempts to engineer resistance in cassava against ACMV-associated CMD by Vanderschuren *et al*. (2007). Vanderschuren *et al*. (2007) noted attenuated symptoms but not resistance in two out of three cv.60444 lines transformed with ACMV common region viral sequence. Differences in RNA silencing efficiency could be due to other factors such as dosage of challenging virus inoculum or method of virus challenge. In this study we used *Agrobacterium* LB4404-mediated delivery of infectious clones while Vanderschuren *et al*. (2009) attempted biolistics and *Agrobacterium* LB4404 and achieved varying levels of success with different dosages.

Interestingly, at 56 dpi AMM2-41 had a significantly lower viral load compared with infected untransformed cv.60444 when previously at 36 dpi its viral load was not significantly different to that of infected untransformed cv.60444 (**fig 2.9**). This could be a consequence of plant recover from viral infection. Recovery from viral infection and tolerance in non-transgenic geminivirus-infected plants had been demonstrated (Gongora-Castillo *et al*., 2012; Sahu *et al*., 2010). In cassava, tolerance and recovery in TME landrace post-SACMV infection has been demonstrated (Allie *et al*., 2014; Bengyella *et al*., 2015), and since cassava is perennial, transgenic resistance via basal immunity i.e. RNA silencing may be only one of many complex interacting factors throughout the growth stages of the crop. The role of resistance genes in tolerance and recovery has been demonstrated (Bengyella and Rey, 2014), and the interaction between RNA silencing and other immunity related pathways needs further study. This prompted us to evaluate the plants for tolerance after an extended period

of time. CMM6 and AMM2 transgenic lines were evaluated at 365 dpi. Notably, transgenic lines CMM6-2, CMM6-6, AMM2-41, AMM2-52 and AMM2-44 were asymptomatic. ACMV-[NG:Ogo:90] resistant line dsAC1 was also asymptomatic. CMM6-7, AMM2-30, 53 and 54 has mild symptoms whilst CMM6-3 and cv.60444 had similar symptoms to those recorded at 56 dpi. The viral load of these infected transgenic lines CMM6-2, 3, 5, 6 and 7, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant dsAC1 transgenic line had decrease from those recorded at 56dpi (**fig.2.11a)**. For AMM2 transgenic lines AMM2-30, 41, 44, 52, 53 and 54, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant dsAC1 transgenic lines' viral load had decrease from those recorded at 56 dpi (**fig 2.11b)**. The recovery of CMM6-2, CMM6-6, AMM2-41, AMM2-52 and AMM2-44 transgenic lines could be attributed to several factors such as temperature, light intensity, siRNA production or other basal immunity associated genes. Since the plants were moved to the greenhouse with higher temperature and light intensity after 56 dpi these two factors may have contributed to recovery. Light intensity was also shown to play a role in siRNA accumulation. Patil and Fauquet (2014) showed that *N. bethamiana* infected with cassava mosaic virus showed recovery at high light intensity of ≥600 μE/m2/s compared with low light intensity of 150 μE/m2/s. Challappan *et al*. (2005) reported on the effect of temperature on transgene mediated silencing. At 30°C they reported a higher accumulation of viral derived siRNAs compared with lower temperatures of 25°C in transgenic cassava, as a result there were significantly lower symptom severity compared with plants grown at 25°C. It has been reported that low temperature inhibits RNA silencing-mediated defense by the control of siRNA generation (Szittya *et al*., 2003).

Detection of siRNAs is a key component to determining the role of the trangene in inducing PTGS and the resulting resistance. Prior to infection all CMM6 and AMM4 transgenic lines produced transgene derived siRNA in varying levels. Kalantidis *et al*. (2002) showed that presence of siRNAs prior to infection plays an important role in the plants defence against viruses. From our results, surprisingly, transgenic lines CMM6-2, CMM6-6 and AMM2-52 that exhibited tolerance did not have the highest siRNA accumulation levels compared with the other transgenic lines but instead there was high variability in accumulated levels (**Fig 2.9**). Even after infection we could not find a positive relation between siRNAs produced and viral tolerance. This contradicts reports were the presence of virus specific RNA were shown to correlate with viral resistance (Chen *et al*., 2004; Hilly *et al*., 2005; Vanderschuren *et al*., 2009). However it has also been shown that the presence of transgene specific siRNA prior to

infection does not always correlate with virus resistance (Noris *et al*., 2004; Ribeiro *et al*., 2006). This was the case in our transgenic lines. Small interfering RNAs were detected in infected untransformed cv.60444, clearly shows that PTGS was intiated. Ntui *et al*. (2015) suggested that in infected untransformed cassava although siRNAs were being produced the virus was replicating faster and overcoming the plant natural defence mechanism. This was clearly the case for the infected untransformed cv.60444 in this research. While there was a decrease in ACMV-[NG:Ogo:90] infected untransformed cv.60444 viral load at 365 dpi (**fig 2.11a and b**), it was not sufficient to confer resistance, but was likely due to some level of tolerance triggered under high temperatures and light intensity. Certainly the generation of vsiRNAs targeting the virus genome occurs in susceptible plants such as *Arabidopsis* infected with *cabbage leaf curl virus* (Aregger *et al*., 2012) and susceptible cassava landrace T200 infected with SACMV (Rogans *et al*., 2016), but this is not effective in establishing resistance. The movement of the siRNA signal, timing of siRNA induction and other interacting factors play a role. Our findings suggest that the transgene-derived siRNAs detected in CMM6 and AMM2 transgenic lines prior to infection enhanced the decrease in virus replication while the siRNA production post infection in non-transgenic infected cv.60444 was not effective. The variation in CMM6 and AMM2 transgene siRNAs accumulation in relation to the viral tolerance or reduction appears to depend on more than just siRNAs accumulation. It is possible that the quality and specific target of siRNAs produced rather than the quantity could be the key difference between tolerant and non-tolerant lines. While the generation of siRNAs can be predicted by software programs such as pssRNAit (http://plantgrn.noble.org/pssRNAit/) and certain siRNA targets on the viral genome desired, natural siRNA hotspots occur and these are variable and depend on multiple factors (Sharma *et al*., 2014).

The transgenic plants showed normal height in comparison with infected untransformed cv.60444. No significant differences were observed between the height of CMM6, AMM2 transgenic lines and that of untransformed cv.60444 (**fig 2.10**). There have been reports of reduced growth in transgenic plants linked to the insertion of a transgene for example Jorgensen *et al*. (2005) reported reduced growth in cassava engineered to block cytochrome biosynthesis pathway. This however was not the case with our CMM6 and AMM2 transgenic as their height was not affected by insertion of the IR. This is due to successful integration and non-disruption of existing growth related genes by insertion of the transgene. Our four promising tolerant transgenic lines CMM6-2, CMM6-6, AMM2-52 and ACMV-[NG:Ogo:90] resistant line dsAC1 did not show reduction in average fresh tuber yield. The

average tuber yield was not significantly different between these lines and non-infected healthy cv.60444. The tubers from other transgenic lines and infected untransformed cv.60444 were significantly lower compared with non-infected healthy cv.60444. Indeed, CMD has been reported to result in yield loss (Legg *et al*., 2011; Patil and Fauquet, 2009) and these results further confirm their observations. Yields loss would be due to the physiological damage that the virus has on the plant and as expected the tolerant lines would not be affected as severely. Severe CMD symptoms result in reduced leaf size and this would greatly reduce the movement of nutrients from the leaves to the tuberous roots (Cock, 1976; Veltkamp, 1985). Our results demonstrate that our tolerant transgenic lines would be of benefit to farmers in terms of yield output. Perhaps of importance to agro-processors and industrial use is the cassava tuber dry weight. From our tuber dry weight results we observed that the percentage tuber dry weight did not differ significantly between CMM6, AMM2 transgenic lines, infected untransformed cv.60444, non-infected healthy cv.60444 and ACMV-[NG:Ogo:90] resistant line dsAC1. The percentage tuber dry weight these transgenic lines and controls was between 35% and 39% (**fig 2.15**). Our results were similar to these reported by Edah-Djedji *et al.* (2012) of 31.17-39.83%, 37.60-42.99%, 31.54-38.70% and 31.24-39.04% percentage tuber dry weight in non-infected healthy cassava. A good cassava harvest has percentage tuber dry weight that ranges from 30% to 40% (Braima *et al*., 2000; Teye *et al*., 2011). For agro-processing of cassava, the dry starch content is important, and water is removed in the process. Dry matter includes starch content which is important. Therefore both parameters were measured. These results showed that the ACMV-[NG:Ogo:90] IR transgene in CMM6 and AMM2 lines did not interfere with root formation.

Transgene constructs with self complementary sense and antisense strands (IR or hairpins) have been proven to be more efficient PTGS inducers as opposed to either sense or antisense strategies (Wang *et al.*, 2008; Waterhouse *et al.*, 1998). AMM2 and CMM6 lines both express the same IR transgene sequence targeting AC1/AC4:AC2/AC3 of ACMV-[NG:Ogo:90], but CMM6 transgenic lines are a result of genetic engineering of cassava cv.60444 with a transformation cassette that has a 767 base pair intron separating the sense and antisense arms of the IR transgene (non-mismatched construct), while AMM2 lines base mutations were introduced to the sense arm only and instead of an intron a small spacer loop 23 bp was used (mismatched construct) (Moralo, 2015). Previously Smith *et al*. (2000) observed PTGS success when a spacer loop was used to separate two arms of the transgene, and ACMV resistance in cassava was reported with a smaller intron 85 bp (Vanderschuren *et*

*al*., 2009). Exclusion of the intron in mismatched contructs was meant to reduce the size of the construct, and stabilize the IR for cloning and transformation (Rey *et al*., 2015). Taylor *et al*. (2012) went further to introduce mismatches in the sense arm of the SACMV BC1 transgene arms separated by a small spacer. Introduction of this mismatched construct in model plant *Nicotiana benthamiana* resulted in reduced SACMV multiplication upon infection. However, a direct comparison between mismatched and non-mismatched constructs were to date not performed and was one of the objectives of this study. We predicted that both our mismatched and non-mismatched construct would induce resistance to ACMV-[NG:Ogo:90] however we only managed to identify two CMM6 lines and one AMM2 line that had symptom severity and viral loads which were significantly lower in comparison with infected untransformed cv.60444. This was observed in three independent trials, those in this study and previously (Moralo, 2015). However, the two tolerant CMM6 lines-2 and 6 appeared to perform better (viral load) compared with AMM2 tolerant line-52 with regards to levels of viral amplification. It is possible that the observed differences between viral loads of the two tolerant mismatched AMM2 lines and the non-mismatched CMM6 lines may be attributed to the presence of an intron in the non-mismatched CMM6 transgene, providing better stability and dsRNA processing.

Expression of the transgene could also affect siRNA production. CMM6 transgene showed high expression (RT-PCR) of both the sense and anti sense arm, whilst expression of the AMM2 transgene showed weak expression of the mutated sense arm only. Expression of transgene introduced by genetic engineering has been noted to be variable in plants carrying the same transgene (Kohli *et al*., 2010; Longstaff *et al*., 1993; Rooke *et al*., 2003). The variability was suggested to be due to possible chromosomal effects or endogenous transgene silencing due to presence of multiple copies of the transgene (Angell and Balcombe, 1997). However, tolerant transgenic lines CMM6-2, 6 and AMM2-52 had single copy numbers of the transgene. Dalakouran and Tzanopoulous (2011) concluded that high expression of the transgene does not necessarily guarantee resistance after noting that *N. benthmiana* expressing high levels of CMV CP transgene were still susceptible to CMV infection. In terms of recovery at 365 dpi, tolerant transgenic lines CMM6-2, 6 and AMM2-52 were asymptomatic. As earlier discusses recovery observed in these lines may be due to late transgene siRNAs biogenesis and other complex interaction factors (Bengyella and Rey, 2014)

In summary, four potential ACMV-[NG:Ogo:90] tolerant cassava lines were identified from this study and previous trials (Moralo, 2015) and these will be subjected to larger greenhouse trials. There are conflicting reports with regards to the robust endurance of RNA silencing in the field (Beyene *et al*., 2015; Fuentes *et al*., 2016), and GM field trials are also planned in future.

# Chapter 3

# Screening of cassava expressing mismatched or non-mismatched hairpin RNA constructs derived from *South African cassava mosaic virus* BC1/Cell-to-cell movement ORF

## 3.1 Introduction

In South Africa a novel cassava infecting begomovirus *South Africa cassava mosaic virus* (SACMV) was identified (Berrie *et al*., 1998). South African cassava mosaic like most begomoviruses has a DNA A (2800 nt) and DNA B (2760 nt) component and a highly conserved intergenic region (Berrie *et al*., 2001). SACMV DNA B has two genes, BC1 which codes for cell-to-cell movement protein (MP) and BV1 which codes for the nuclear shuttle protein (NSP) (Berrie *et al*., 2001). Begomoviruses utilise movement and cell associated-accessory proteins such as heat shock proteins (Frischmuth *et al*., 2007; Gorovits *et al*., 2013; Moshe *et al*., 2015), for systematic movement of genome DNA-protein complexes through the cytoplasm and via the plasmadesmata into the next cell and into the vascular system for long distance movement (Henley-Bowdoin *et al*., 2013; Krenz *et al*., 2012). Expression of begomovirus movement gene BC1 in transgenic plants has been reported to induce resistance to incoming virus (Taha *et al*., 2016). Silencing of the begomovirus cell-to-cell movement protein induced by BC1 derived siRNAs in transgenic plants is expected to reduce viral transmission from cell-to-cell (Taha *et al*., 2016). Harmse (2007), identified a region on the SACMV BC1 genome from nt position 1532-1753 with several loops on the mRNA transcript which was predicted *in silico* to be an idea region to produce transgene induced siRNAs which would trigger PTGS and subsequently degradation of homologous SACMV MP miRNA.

It is important to expose transgenic cassava lines to green house trials with conditions similar to the field environment. Resistance or tolerance has been shown to differ under variable conditions such as temperature, viral dosage and light intensity. Vanderschuren *et al*. (2009) reported that increasing the viral dosage from 350 to 750 ng had an adverse effect on transgenic resistant lines as they had infectivity percentage similar to wild types. They concluded that the amount of virus derived siRNAs produced in these transgenic lines were only sufficient to impart resistance to a certain dosage of viral inoculum. Chellappan *et al*.

(2005) reported similar findings on the effect of temperature on transgene mediated silencing. At 30 °C they reported a higher accumulation of ACMV-[CM] and SLCMV viral derived siRNAs compared to lower temperatures of 25°C in non-transgenic cassava, as a result there were significantly lower symptom severity compared to plants grown at 25°C.

For resistance trials in our laboratory we adopted definitions for viral resistance, tolerance or susceptibility from Bengyella *et al*. (2015). Resistance plants show no symptom as a result of negligible viral replication, tolerance is defined as the presence of mild symptoms due to replication of low viral levels, and susceptibility refers to the presence of severe symptoms due to uncontrolled viral replication. Well defined symptom scoring scales (sss) have been published to assist with assessing the severity of symptoms. Hahn *et al*., (1980) described a 1-5 sss with 1 representing no symptoms and 5 representing severe symptoms. Fauquet and Fargette (1990) described a 0-5 sss with 0 representing no symptoms and 5 representing severe leaf deformation and symptoms. A symptom severity score scale of 0-3 was reported by Ntui *et al*. (2015). To properly define whether the trial plants are resistant, tolerant or susceptible it is important to monitor the viral multiplication levels in the plants and establish a correlation between the observed symptoms and the viral load. Although a correlation between the observed symptom and viral load varies in different virus host cultivar, several researchers have reported a positive correlation between the observed symptoms and viral multiplication (Chellappan *et al*., 2004; Kaweesi *et al*., 2014). In other cases, a positive correlation cannot be established (Carrillo-Tripp *et al*., 2007; Gomez *et al*., 2009).

Transgene-associated siRNA targeting of geminivirus movement proteins has not been well studied. In this research, a 221 bp SACMV BC1 (1532-1753 nt) mismatched IR construct, with mutated BC1 sense arm was transformed into model cultivar cv.60444 (AMM4 transgenic lines). A non mismatched IR construct of the same selected BC1 sequence was also transformed into model cultivar cv.60444 (CMM8 transgenic lines). The AMM4 and CMM8 transgenic lines were evaluated for resistance, tolerance or susceptibility to SACMV, and a comparison between the two constructs was made

### 3.1.1 Specific Aims

The aim of this research was to subject selected non-mismatched CMM8 transgenic lines 1, 3, 8, 23, 25 and 27 and corresponding mismatched AMM4 lines 11, 33, 34, 46, 59, 68 and 79 to reproducible trials and evaluate for response to virus challenge and select potential resistant/tolerant lines for further GM trials in future.

**Steps in achieving our aim**

i. To confirm successful integration of transgenes in previously transformed lines.
ii. Micro-propagation of CMM8 and AMM4 transgenic cv.60444 lines and acclimatization for (6 weeks)
iii. Agro-infection of transgenic lines with SACMV infectious virus clones
iv. Plant evaluations for virus symptom severity, plant height and viral load at 14, 36, 56 and 180 days post inoculation (dpi)
v. Northern blots for siRNA detection in all lines pre and post infection.

## 3.2 Methodology flow chart

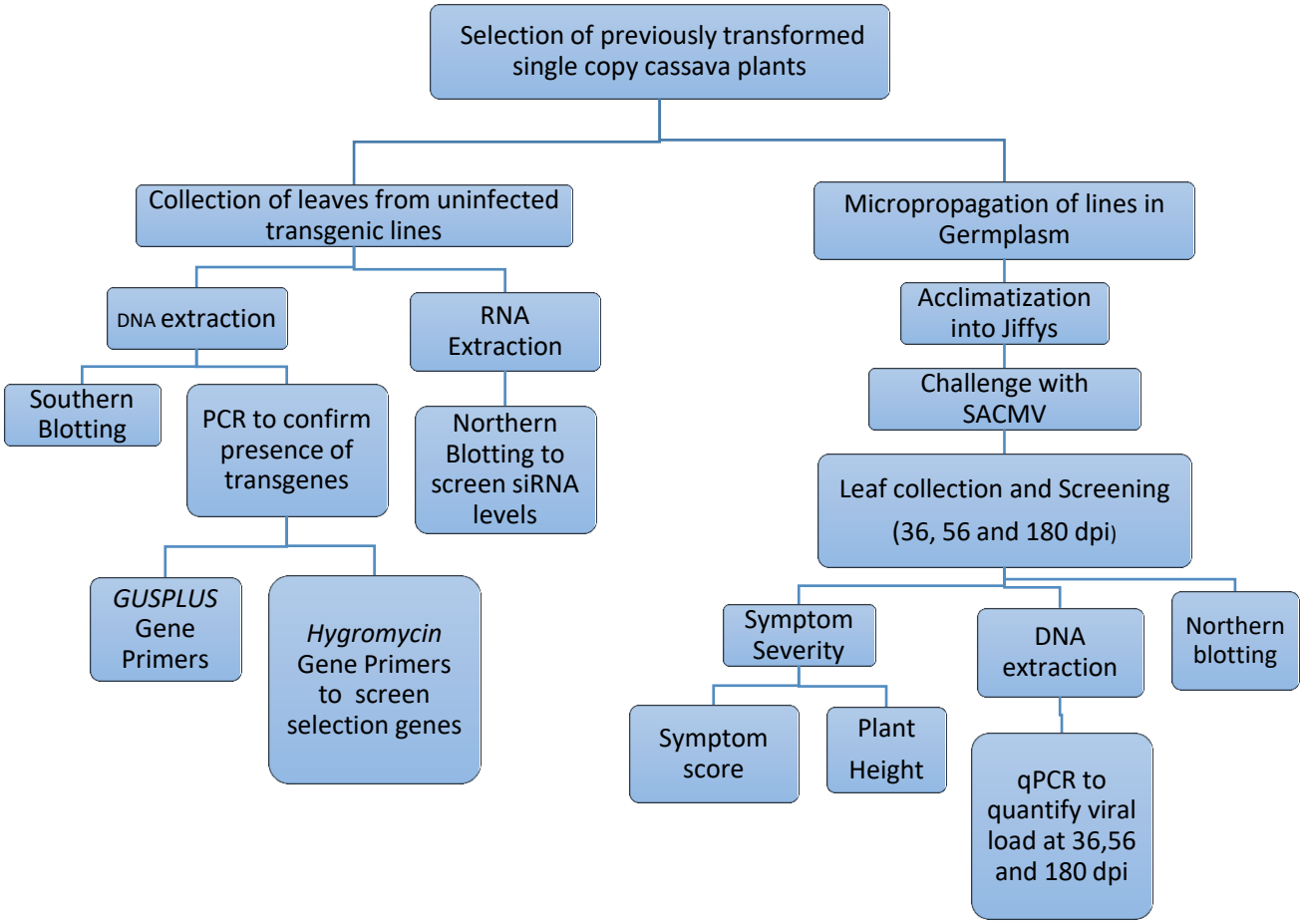


**Figure 3. 1** Work flow diagram for screening of transgenic cassava lines for resistance/tolerance to SACMV

## 3.3 Materials and Methods

### Characterisation of transgenic lines

#### 3.3.1 DNA extraction

Leaf tissue collected from each of the six CMM8 and seven AMM4 trangenic lines chosen for this trial were used for DNA extraction using the CTAB method (Doyle and Doyle, 1987) for total nucleic acid (TNA) extraction. Method used is described previously in **2.3.1**.

#### 3.3.2 Southern blot screening for transgene copy number

Transgene copy number in the extracted DNA from the six CMM8 and seven AMM4 transgenic lines was determined by southern blotting as earlier described in **2.3.2**. BC1 non mutated strand PCR fragments were produced using the primers described in (**Table 3.1**).

#### 3.3.3 PCR of GUSPlus, hygromycin and virus-derived transgenes

Integration of the plant transformation cassette carrying the transgene in the trial lines was confirmed by PCR. Total nucleic acid extracted in **3.3.1** was used as template. Transformed plants were screened for the *GUSPLUS* and (*hyg*) gene using primers listed in **Table 2.1**. Screening for the CMM8 or AMM4 sense and antisense IR was done using BC1 and BC1 modified primers, respectively, (**Table 3.1**) using the same method described in **2.3.3**.

**Table 3. 1** Primers used to amplify BC1 sense and antisense amplicons

| Primer | Primer sequence |
|---|---|
| BC1 F | (5′-TACGATAACCGACCCAGTTGCGTT-3′) |
| BC1 R | (5′-TGCGACTCAAAGGCCGATGTATGA-3′) |
| BC1 (mod+*Xho*I+*Spe*I) F | (5′GATCCTCGAGACTAGTAAATATTCTACGGACATACG-3′) |
| BC1 (mod +*Bgl*II) R | (5′-GATCAGATCTTAGTAGCCCAATCTAAGACCTTGT-3′)] |

#### 3.3.4 RNA extraction

Leaf tissue was collected from each of the six CMM8 and seven AMM4 trangenic lines chosen for this trial. Leaf tissue was also extracted from non-infected healthy cv.60444 plants. Method used is described previously in **2.3.4**.

### 3.3.5 T-DNA expression

Total RNA extracted from **3.3.4** was used to check for the expression of the T-DNA. The synthesised cDNA standed was then directly used in RT-PCR to amplify the *GUSPLUS* gene, *hyg* gene and the sense and antisense arms of the respective transgene using primers in (**Table 3.1)** following methods described in **2.3.5**.

**Sceening trials of transgenic lines for response to ACMV challenge**

### 3.3.6 Bulking up and acclimitisation of selected transgenic plants

Selected six CMM8 lines 1, 3, 8, 23, 25 and 27, seven AMM4 lines 11, 33, 34, 46, 59, 68 and 79, healthy cv.60444 cassava plants, resistant transgenic line dsAC1 (Vanderschuren *et al*., 2009) and vector only transgenic controls were propagated in tissue culture on MS2 medium and acclimatized as described in **2.3.6**.

### 3.3.7 Experimental design

Ten plants per each line were inoculated with SACMV infectious viral clones (n=10). The experiment was designed following specifications described in **2.3.7**.

### 3.3.8 Viral agro-inoculation of transgenic plants

After 4 weeks the six CMM8 lines 1, 3, 8, 23, 25 and 27, seven AMM4 lines 11, 33, 34, 46, 59, 68 and 79, healthy cv.60444 cassava plants, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 and pCambia vector only transgenic controls were agro-inoculated with SACMV DNA-A and DNA-B. Prior to infection *A. tumefaciens* C58C1 SACMV DNA-A and DNA-B infectious dimers were cultured in Yeast extract peptone (YEP) broth as described in **2.3.8** with slight modifications. SACMV Agro-dimers were cultured in YEP inoculated with antibiotics kanamycin and cabenicillin.

### 3.3.9 Sampling and symptom monitoring

The SACMV infected CMM8 and AMM4 trial lines were monitored and evaluated for sss, viral load and height evaluation as described in **2.3.9** response at 3 time points post infection (14, 36, 56 and 180 dpi).

### 3.3.10 Absolute quantitative PCR of viral load

Viral load accumulation in infected CMM8, AMM4 transgenic lines, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 were determined by absolute real time quantitative PCR at 36, 56 and 180 dpi as described in **2.3.10** with slight

modifications. To amplify SACMV viral multiplication primers CCP-F 5'-GCACAAACAAGCGTCGA-3' and reverse primer CCP-R 5'-CTGCCAGTATGCTTAACGTCA-3' were used. The SACMV viral loads of the experimental samples were calculated by reference to a standard curve generated by serial dilutions of pBS-SACMV DNA-A clones ranging from 0.1 pg/µl to 1 ng/µl under the same qPCR reaction condition. The corresponding viral molecules were calculated using Equation 4-3. Equation 4-4 as described previously in **2.3.10**.

### 3.3.11 Northern blot detection of siRNA

BC1 IR PCR amplicons were DIG labeled as previously described in **2.3.11**. For siRNA control, an *Arabidopsis* 21 nt miR167 (5-'TAGATCATGCTGGCAGCTTCA-3'), oligonucleotide was 3'end labeled, and for size marker a 24 nt primer (5'-TTCAGGAGCCTCCGCCTGTCT-3' was 3' end labeled as previously described in **2.3.11**.

## 3.4 Results

### Characterisation of transgenic lines

### 3.4.1 Southern blot screening for transgene copy number

Transgenic CMM8 lines 1, 3, 8, 23, 25, and 27 and seven AMM4 lines 11, 33, 34, 46, 59, 68 and 79 were screened for transgene copy number using southern blot method. The BC1 PCR amplicon, *GUSPLUS* PCR amplicon and the *hyg* PCR amplicon were successfully amplified (**fig 3.2**). From the results, the DIG-labeled *GUSPLUS* and *hyg* **(fig 3.2 lane b and d, respectively)** migrated slower, in the gel, as expected than the 181 bp and 485 bp unlabeled *GUSPLUS* and *hyg* PCR amplicons **(fig 3.2 lane a and c, respectively)**.The unmodified BC1 DIG-probes **(fig. 3.2 lane f)** migrated slower than unlabeled 221 bp BC1 PCR amplicon **(fig. 3.2 lane e)**.

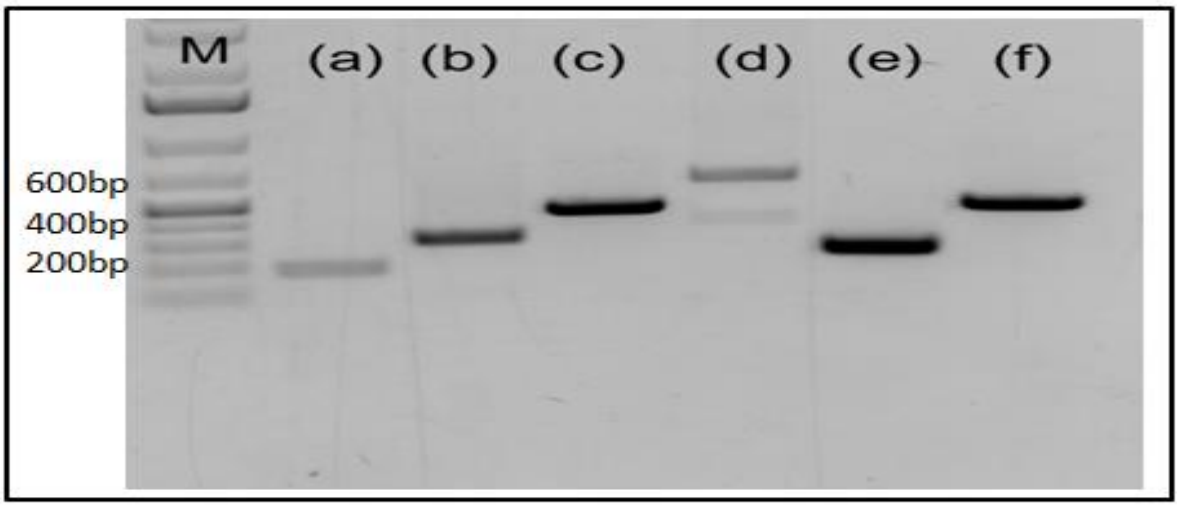


**Figure 3. 2** 1% agarose gel of DIG labeled unmodified BC1 PCR fragments, *hyg* and *GUSPLUS* PCR fragments probes. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas). DIG-labeled *GUSPLUS* PCR amplicon (**lane b**) unlabeled *GUSPLUS* PCR amplicon (**lane a**) DIG-labeled *hyg* probe (**lane d**) unlabeled 485 bp *hyg* PCR fragment (**lane c**) and unmodified BC1 DIG-probes (**lane f**) unlabeled 221 bp BC1 amplicon (**lane e**)

Southern Blot analysis of double digested DNA from CMM8 transgenic lines with the BC1 IR probe showed that all six lines had a single transgene copy (**fig 3.3a**). The same genomic DNA was digested with *Hind*III and probed with DIG labeled-*hyg* probe, and results showed that all lines except CMM8-8 had two transgene copies (**fig. 3.3b**).

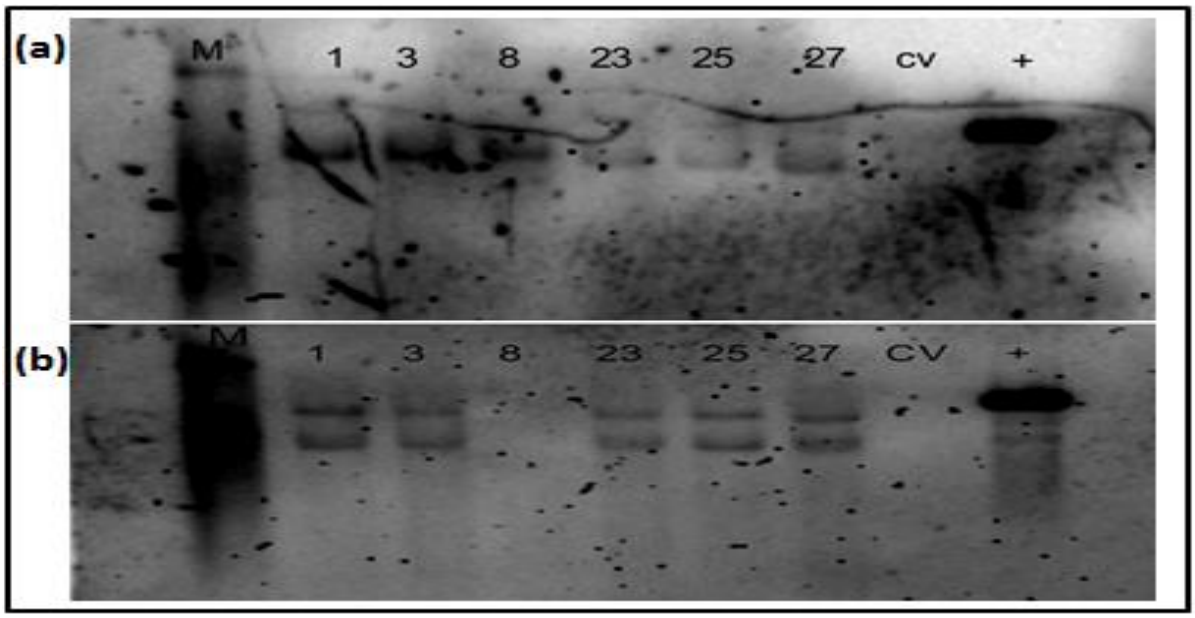


**Figure 3. 3** Southern blot analysis of CMM8 transgenic lines for transgene integration using DIG-labeled probes. M = DIG molecular weight marker (Roche), cv. = untransformed healthy cv.60444 and +veC = pC1305.1/CaMV35S BC1 IR **(a)** *Hind*III and *EcoR*I-double digested genomic DNA probed with BC1 probe and **(b)** *Hind*III-digested genomic DNA, probed with *hyg* probe

Results from southern blot analysis of double digested genomic DNA from the seven AMM4 transgenic lines probed with BC1 IR probe showed that all seven lines had a single transgene copy (**fig 3.4a**). The same genomic DNA was digested with *Hind*III and probed with DIG labeled-*hyg* probe, and results showed single copy of the transgene in all AMM4 lines (**fig. 3.4b**).

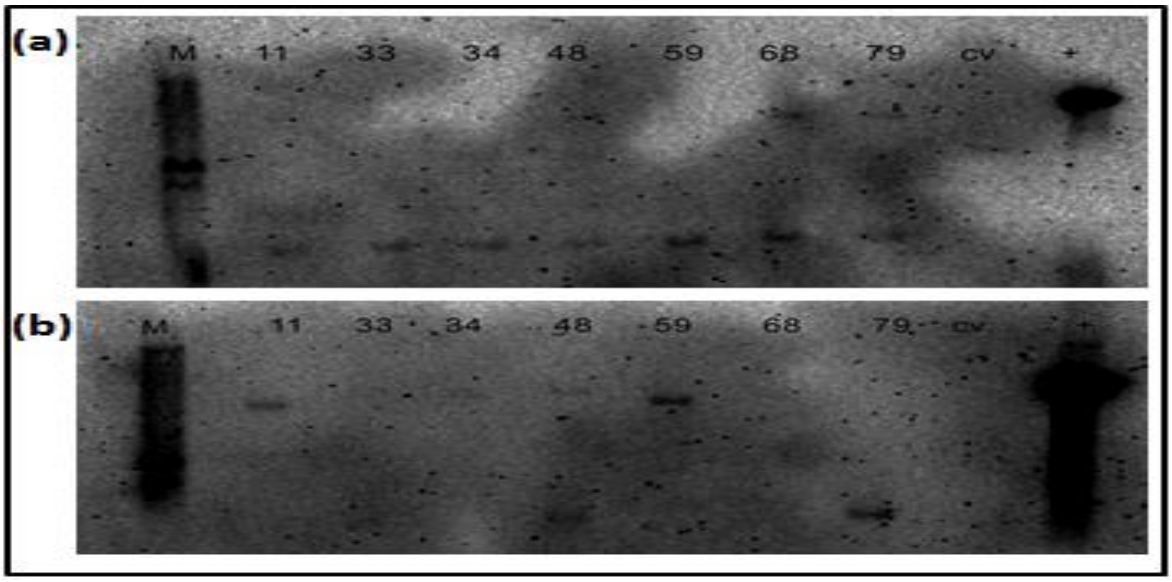


**Figure 3. 4** Southern blot analysis of AMM4 transgenic lines for transgene integration using DIG-labeled probes. M = DIG molecular weight marker (Roche), cv. = untransformed healthy cv.60444 and +ve = *Hind*III and *EcoR*I-double digested pTZR5/T containing MM4hp cassette (a) *Hind*III and *EcoR*I-double digested genomic DNA probed with BC1 IR probe and (b) *Hind*III-digested genomic DNA, probed with *hyg* probe

### 3.4.2 PCR of *GUSPLUS*, *hyg* and virus-derived transgenes

Positive PCR amplification of *hyg* gene, *GUSPLUS* gene confirmed successful transgene integration in the all 6 CMM8 lines **(fig. 3.5b)**. All six CMM8 transgenic lines tested positive for PCR amplification of both the 221 bp sense and antisense arms of the BC1 IR construct **(fig 3.5a)**. Positive amplification was achieved for the positive plasmid controls while no amplicons were obtained for the negative control untransformed healthy cv.60444.

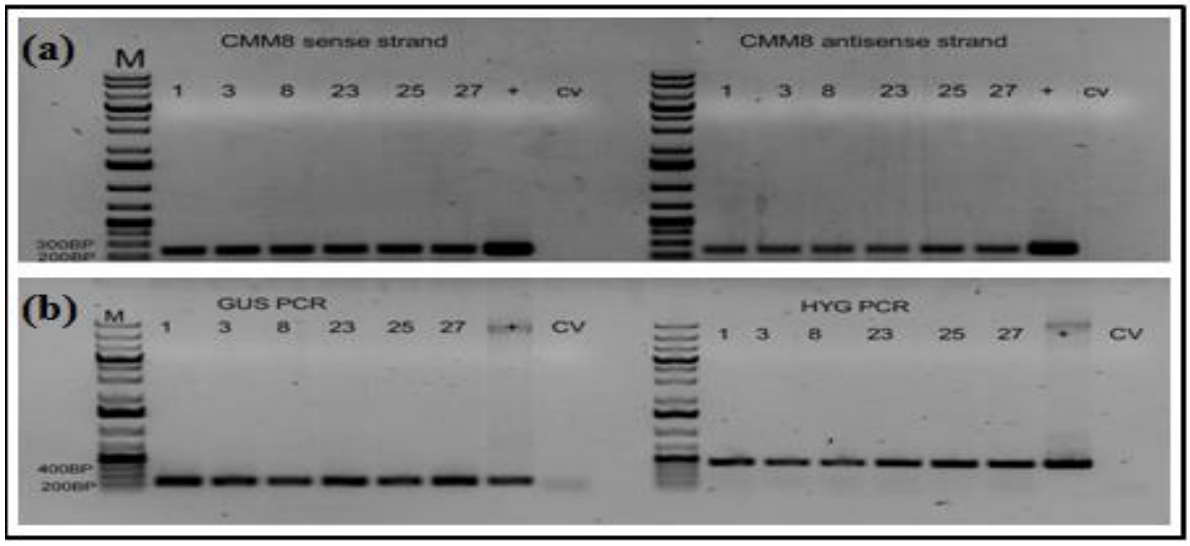


**Figure 3. 5** PCR confirmation of the transformation cassette in CMM8 lines transgenic status. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas), cv. = negative control untransformed cv.60444. Transgenic lines were screened for **(b)** 181 bp *GUSPLUS* fragment was successfully amplified in all lines screened and the 485 bp *hyg* gene fragment was detected in all lines tested.**(b)** PCR amplification of both the 221 bp sense and antisense-arm of the BC1 IR construct in CMM8 transgenic lines

Positive PCR amplification of *hyg* and *GUSPLUS* genes confirmed successful transgene integration in all 7 AMM4 transgenic lines **(fig. 3.6b)**. The 221 bp modified sense arm of the BC1 IR was weakly amplified whilst the antisense arms was not amplified **(fig 3.6a)**. Positive amplification was achieved for the positive plasmid controls while no amplicons were obtained for the negative control untransformed healthy cv.60444.

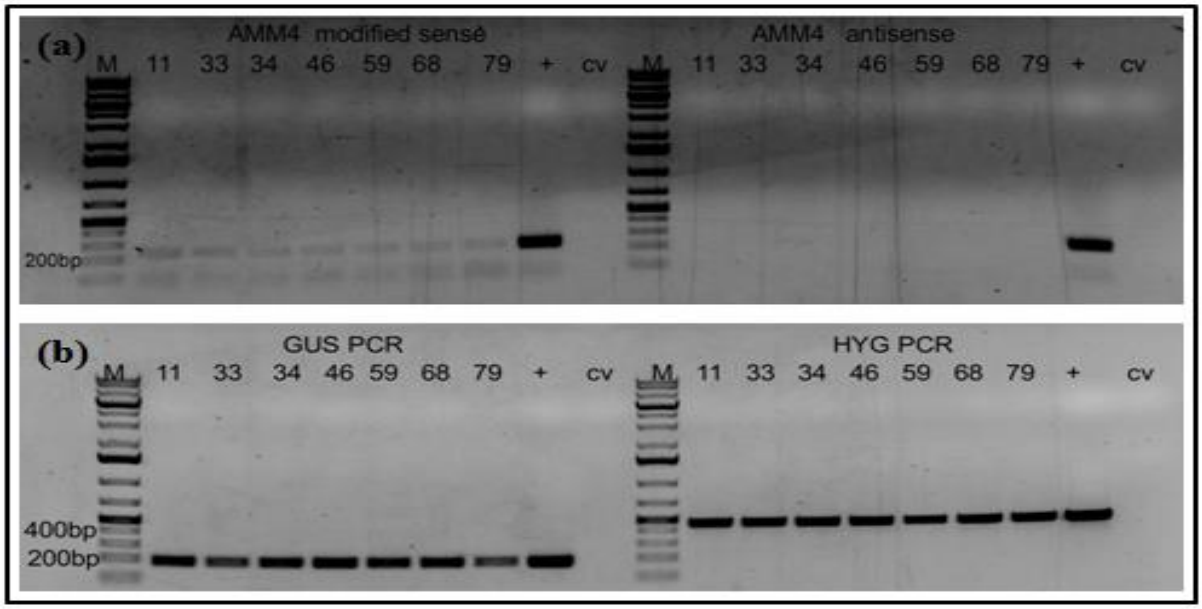


**Figure 3. 6** 1% agarose gel electrophoresis of *hyg*, *GUSPLUS* and the BC1 RNAi construct PCR amplicons of AMM4 transgenic lines. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas), cv. = untransformed cv.60444 and +ve = positive plasmid controls. Transgenic lines were screened for the **(a)** 221 bp mutated sense-arm amplicon and the 221 bp non-mutated antisense-arm. **(b)** 181 bp *GUSPLUS* gene fragment and 485 bp *hyg* gene

**<u>Screening of transgenic lines for resistance/tolerance or susceptibility at 14, 36 and 56 dpi</u>**

### 3.4.3 Symptom severity scoring

A symptom severity score (sss) index (**fig 3.7**) was used to evaluate severity based on a scale of 0-3. Symptom severity score (sss) of **CMM8** transgenic lines were noticeably lower than those of non-infected healthy cv.60444 plants at all time points (**fig 3.7**). At 14 dpi, CMM8-8, 23 and 25 had a lower sss (1-fold each, respectively) compared with infected untransformed cv.60444 however, there was no significant difference ($p>0.05$) between the sss of these lines and infected untransformed cv.60444. CMM8-1 and 27 had a significantly ($p<0.05$) **(Appendix C)** lower sss, (2 and 2.6-fold, respectively) compared with infected untransformed cv.60444. Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] resistant transgenic line dsAC1 was tested for resistance or susceptibility to SACMV, and at 14 dpi, dsAC1 had a lower sss (1-fold) compared with infected untransformed cv.60444 however, these results were not significantly different ($p=0.097$).

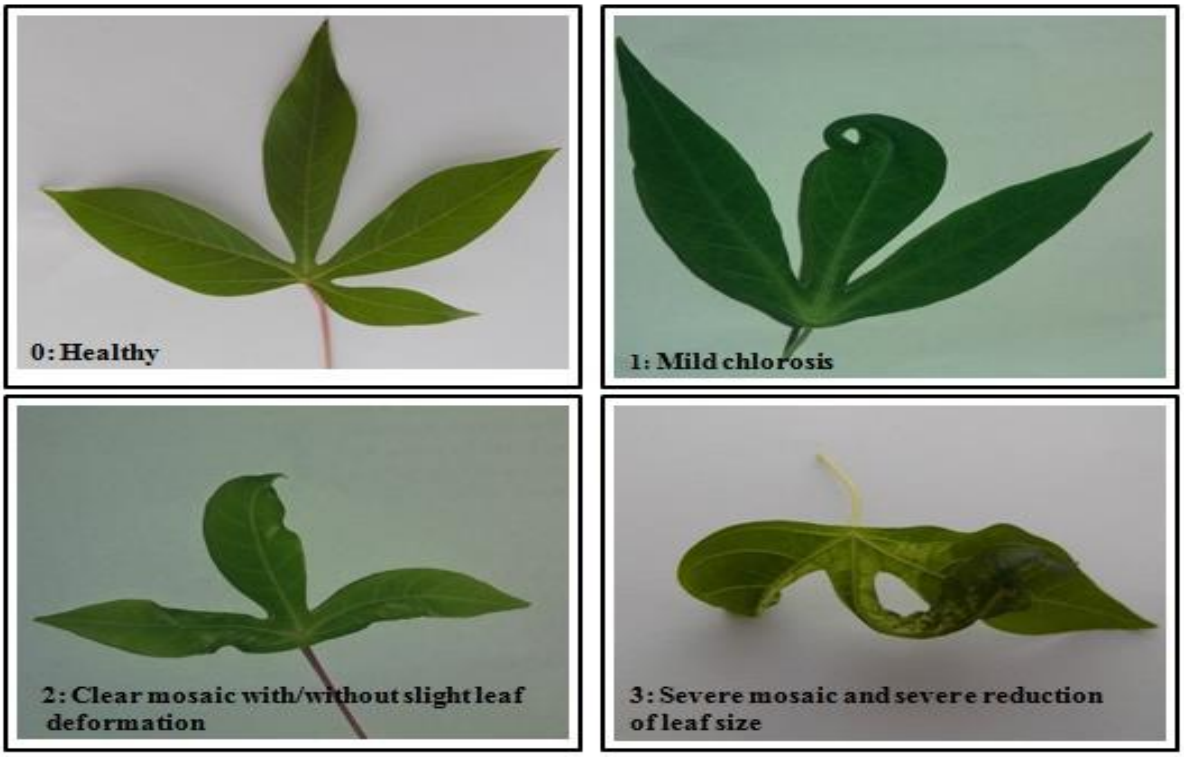


**Figure 3. 7** Cassava mosaic disease symptomatic leaves from trials in this study. 0-3 scoring scale used for evaluation

At 36 dpi, CMM8-1, 8, 23 and 25 had a significantly ($p<0.05$) lower sss (1.5-fold each, respectively) compared with infected untransformed cv.60444. CMM8-3 and 27 both had significantly ($p<0.05$) lower sss (1.0-fold each, respectively) compared with infected untransformed cv.60444. Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] resistant line dsAC1 had significantly ($p<0.05$) lower sss, (1.9-fold) compared with infected untransformed cv.60444. At 56 dpi, CMM8-1, 8, 23 and 25 had significantly ($p<0.05$) lower sss, (1.7, 1.4, 2 and 1.7-fold, respectively) compared with infected untransformed cv.60444. CMM8-3 and 27 had lower sss, (1.2 and 1.0-fold, respectively) compared with infected untransformed cv.60444 however there was no significant difference between the results ($p>0.05$).

Symptom severity scores of **AMM4** transgenic lines were also observed to be lower than that of infected untransformed cv.60444 **(fig 3.8b)** at all time points. At 14 dpi, AMM4-11 and 33 had lower sss, (1.5-fold each, respectively) compared with infected untransformed cv.60444. AMM4-46, 59, 68 and 79 had lower sss (2-fold each, respectively) compared with infected untransformed cv.60444. The sss of all AMM4 transgenic lines at 14 dpi were however not significantly different ($p>0.05$) to the sss of infected untransformed cv.60444 **(Appendix D)**. At 14 dpi, ACMV-[NG:Ogo:90] resistant line dsAC1 had a lower sss, (2-fold) compared with

infected untransformed cv.60444 however, these results were not significantly different (p=0.086). At 36 dpi, AMM4-11, 34, 46, 56, 68 and 79 had lower sss, (1.3, 1.8, 1.6, 1, 1.8 and 1.1-fold, respectively) compared with infected untransformed cv.60444 however, these results were not significantly different (p>0.05). At 56 dpi AMM4-11 and 79 had significantly (p<0.05) lower sss, (1.4 and 1.7-fold, respectively) compared with infected untransformed cv.60444. AMM4-33, 34, 46, 56 and 79 had lower sss, (1, 1.2, 1.2, 1.5 and 1.1-fold, respectively) compared with infected untransformed cv.60444, however these results were not significantly different (p>0.05).

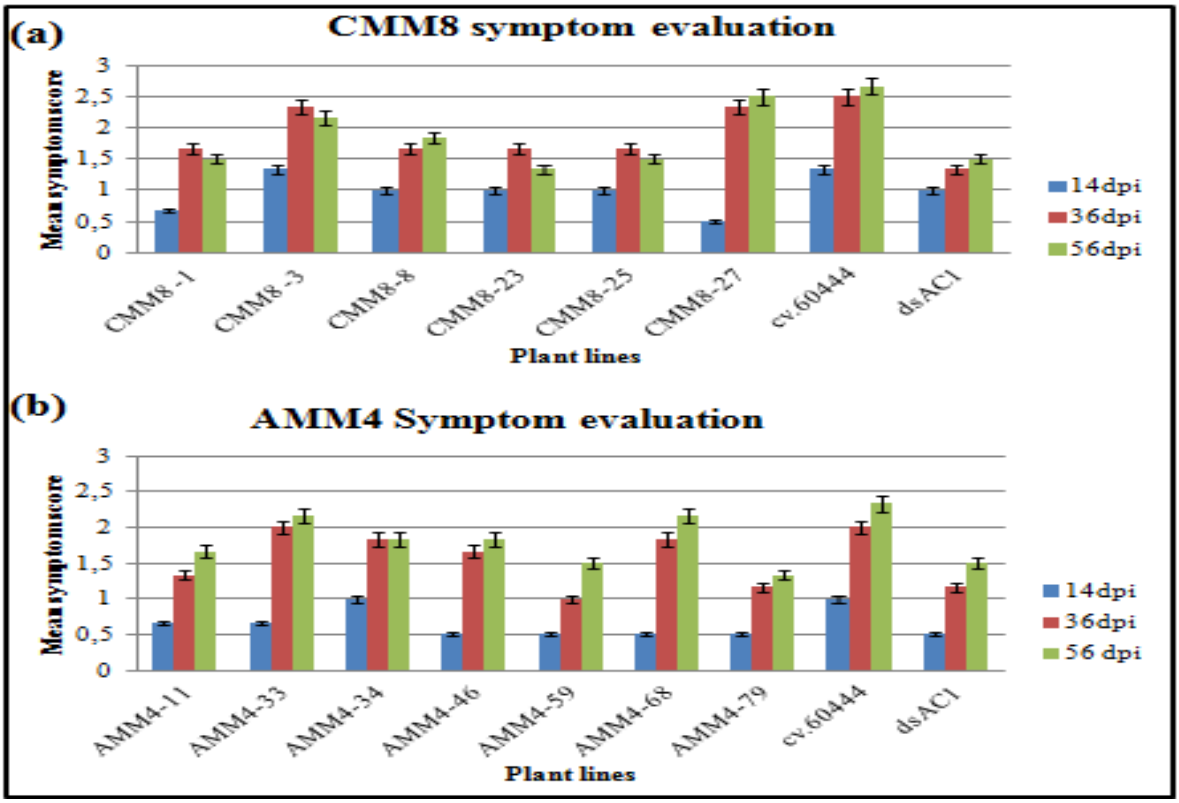


**Figure 3. 8** Symptom severity score of CMM8, AMM4 transgenic lines, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 agro-inoculated with infectious SACMV viral clones. Plants were evaluated at 14 dpi (blue), 36 dpi (red) and 56 dpi (green) **(a)** CMM8 mean symptom severity scores **(b)** AMM4 mean symptom severity score. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

### 3.4.4. Real time absolute quantitative PCR of viral load

At 36 and 56 dpi SACMV viral load accumulation in infected CMM8 and AMM4 transgenic lines, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line

dsAC1 were determined by real time absolute quantitative PCR. At 36 dpi, CMM8 transgenic lines viral loads were not significantly different ($p>0.05$) compared with infected untransformed cv. 60444 (**Appendix C**). CMM8 lines 1, 3, 8, 23, 25 and 27 had a lower viral load, (7.3, 32, 121, 26, 16 and 30-fold, respectively) compared with infected untransformed cv.60444. Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] resistant transgenic line dsAC1 has a lower viral load, (121-fold) compared with infected untransformed cv.60444 however, these results were not significantly different ($p>0.05$). At 56 dpi, CMM8 lines 1, 3, 8, 23, 25 and 27 had a lower viral load, (2.5, 2.3, 6.6, 2, 2 and 18.8-fold, respectively) compared with infected untransformed cv.60444 however, these results were not significantly different ($p>0.05$).

For AMM2 transgenic lines, at 36 dpi AMM4 lines 11, 33, 34, 46, 58, 68 and 79 had lower viral load, (3.2, 4.6, 2.6, 9.9, 3.7, 2.7 and 1.4-fold, respectively) compared with infected untransformed cv.60444. Although all AMM4 transgenic lines had lower viral load compared with infected untransformed cv.60444, statistically these results were not significantly different ($p>0.05$) (**Appendix D**). Africa cassava mosaic virus-[Nigeria:Ogorocco;1990] resistant line dsAC1 has 11.8 fold lower viral load ($p=0.06$) compared with infected untransformed cv.60444. At 56 dpi, AMM4 lines 11, 33, 34, 46, 58, 68 and 79 had lower viral load (82.4, 19.3, 9.1, 388, 147, 85 and 12.6-fold) compared with cv.60444 however, these results were not significantly different from cv.60444 ($p>0.05$). At 56 dpi, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 has a lower viral load, (50-fold) compared with infected untransformed cv.60444 however, these results were not significantly different ($p>0.05$).

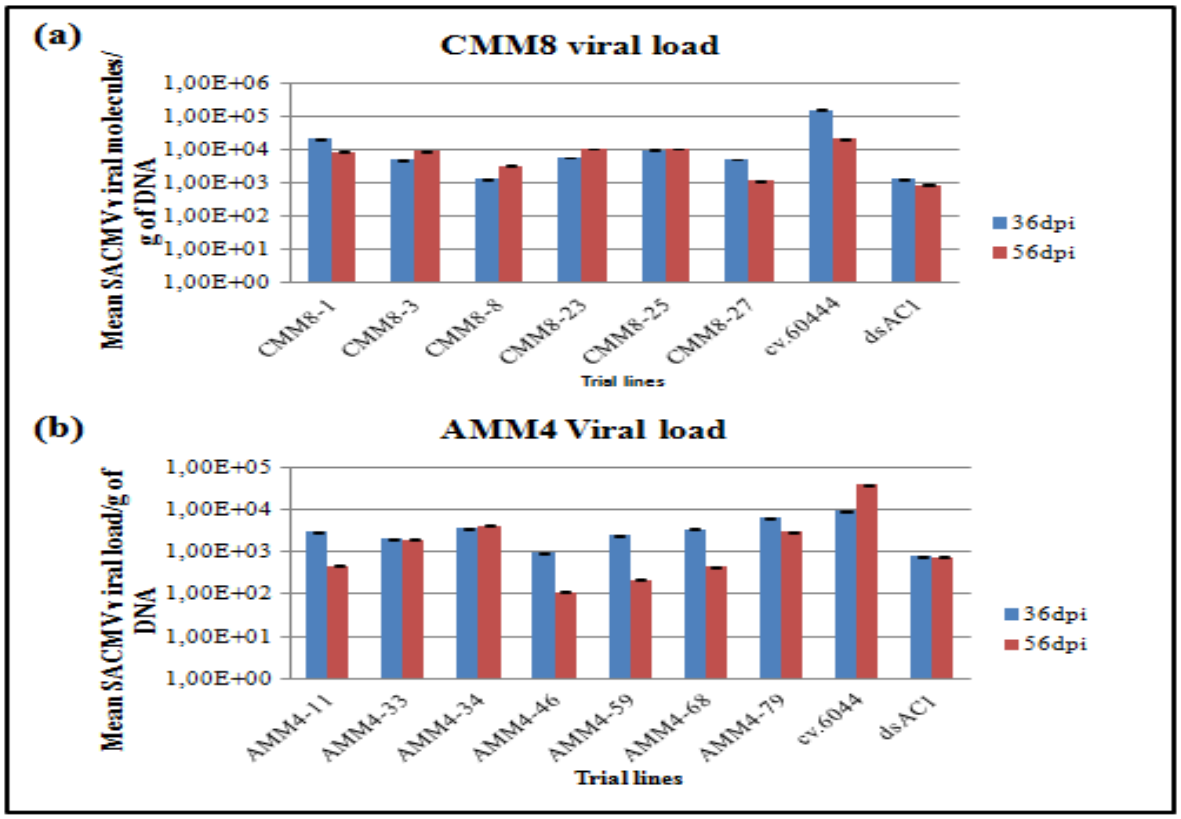


**Figure 3. 9** Viral load evaluation for CMM8, AMM4 transgenic lines, infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 agro-inoculated with infectious SACMV clones. Plants were evaluated at 36 dpi (blue) and 56 dpi (red) **(a)** CMM8 mean absolute viral load quantification (mean viral molecules/g DNA) **(b)** AMM4 mean absolute viral load quantification (mean viral molecules/g DNA). The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

### 3.4.5 Plant height Evaluation

At 14 dpi, CMM8-1, 3, 8, 23, 25 and 27 had average height of 8.8, 8.5, 8.1, 9.1, 8.5 and 8.5 cms, respectively. These results were not significantly different ($p>0.05$) to plant height of untransformed cv.60444 which had an average of 8.1 cms. At 36 and 56 dpi no significant difference ($p>0.05$) in mean plant height was recorded for all CMM8 transgenic lines and the ACMV-[NG:Ogo:90] resistant line dsAC1 compared with infected untransformed cv.60444 **(Appendix C)**.

Plant height evaluations of AMM4 transgenic lines showed that there weren't significant differences between the height of AMM4 transgenic lines and that of infected untransformed cv.60444 **(Fig 3.10b)**. At 14 dpi, AMM4-11, 33, 34, 46, 59, 68 and 79 had average heights of 7.6, 8.6, 7.5, 9, 8.1, 8.1 and 8.1 cms, respectively. These results were not significantly

different (p>0.05) from infected untransformed cv.60444 which had an average height of 7.8 cms. At 36 and 56 dpi no significant difference in mean plant height was recorded for all AMM4 trial lines and ACMV-[NG:Ogo:90] resistant line dsAC1 (p>0.05) compared with untransformed cv.60444 **(Appendix D)**.

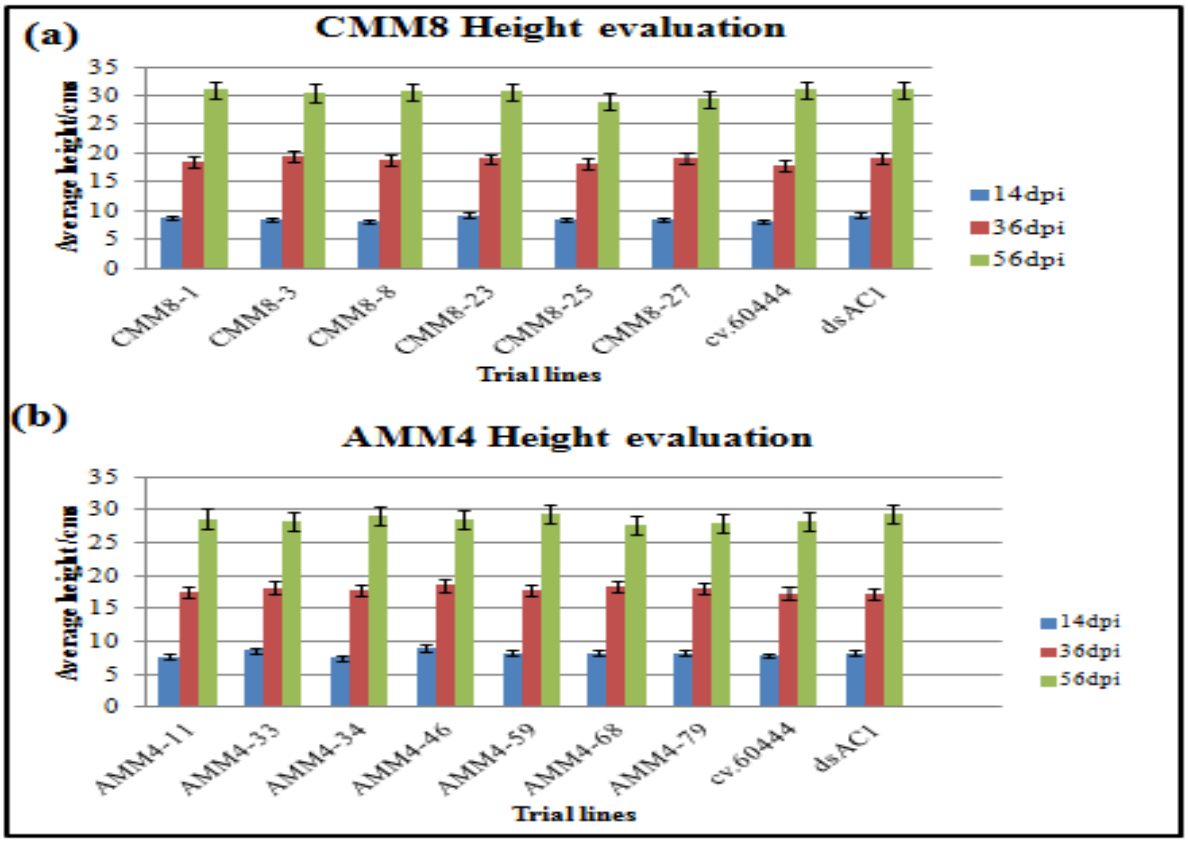


**Figure 3. 10** Plant height evaluation of CMM8, AMM2 transgenic lines, untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1. Plants were evaluated at 14 dpi (blue), 36 dpi (red) and 56 dpi (green) (a) CMM8 mean average height (b) AMM4 mean average height. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

**Evaluation of CMM8 and AMM4 transgenic lines at 180dpi**

### 3.4.6. Symptom scores and viral load

CMM8 transgenic lines were evaluated at 180 dpi for symptom and viral load. At 180 dpi, CMM8-8, 25 and 27 had mild symptoms. CMM8-1, 3, 23, cv.60444 and dsAC1 had similar sss compared with those at 56 dpi. The viral load of CMM8 transgenic lines and controls was also quantified at 180 dpi and there was a decrease in viral loads from those at 56 dpi **(fig**

**3.11a)**. For transgenic lines CMM8-1, 3, 8, 23, 25 and 27 there was a 59, 55, 223, 7, 93 and 9 fold decreases, respectively, in viral load from 56 dpi to 180 dpi. Infected untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had a 2.5 and 2.3 fold decreases in viral load, respectively, in viral load from 56 dpi to 180 dpi. At 180 dpi there was however no significant difference in the viral load between CMM8 transgenic lines, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 and infected untransformed cv.60444 ($p>0.05$) **(Appendix F)**.

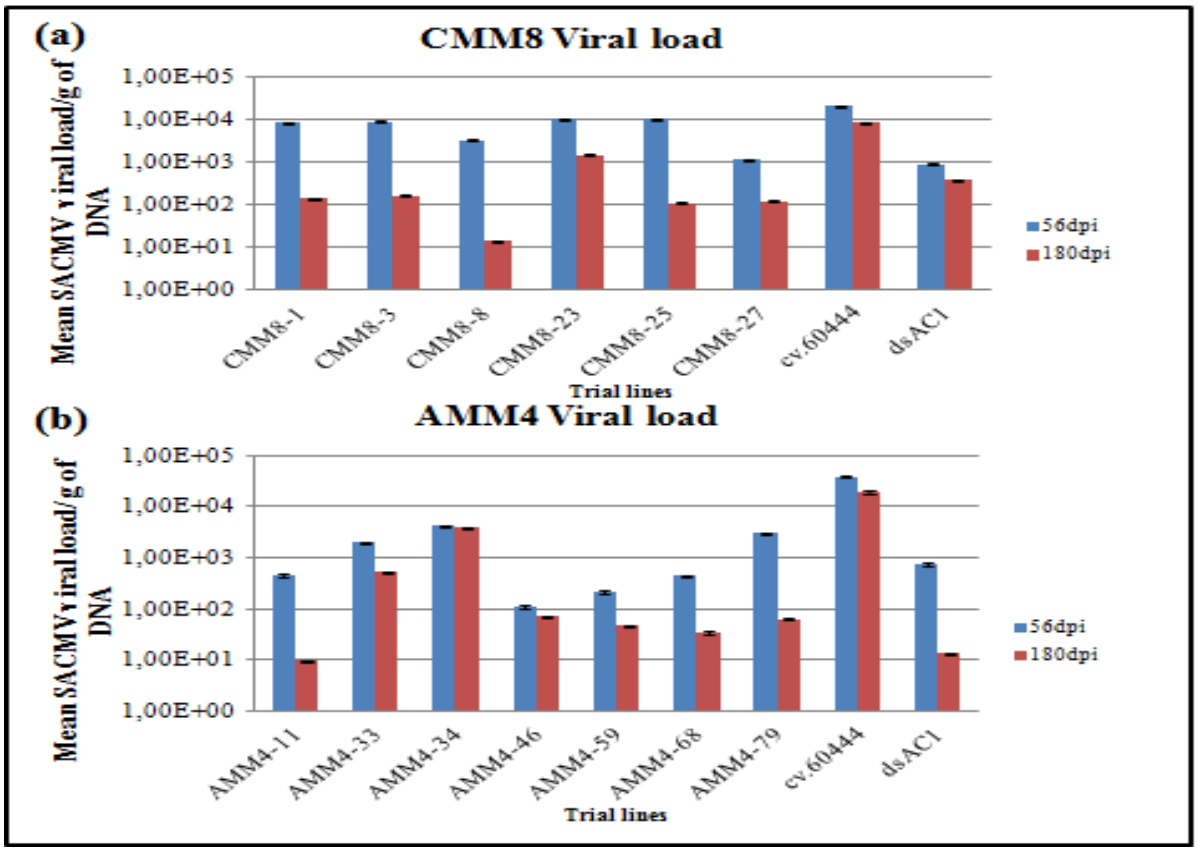


**Figure 3. 11** Mean viral molecules/g of DNA at 180 dpi compared with mean viral molecules/g of DNA at 56 dpi, quantified using absolute real time qPCR, **(a)** Viral load of SACMV infected CMM8 transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 **(b)** SACMV infected AMM4 transgenic lines, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC. The measure of uncertainty is measured by displayed error bars scaled to 95% confidence level

Evaluation of AMM4 transgenic lines at 180 dpi for symptom showed that AMM4-11, 68 and 79 had mild symptoms. AMM4-33, 34, 46 and 59 had similar sss compared with those

recorded at 56 dpi. The viral load of AMM4 transgenic lines, untransformed cv.60444 and ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 was also quantified, and there was a decrease in viral loads from those at 56 dpi **(fig 3.11b)**. For transgenic lines AMM4-11, 33, 34, 46, 59, 68 and 79 there was a 47, 3.7, 1, 1.5, 4.6, 12.9 and 47.4-fold decreases, respectively, in viral load from 56 dpi to 180 dpi. Infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had a 1.9 and 56.8-fold decreases in viral load, respectively, from that viral load at 56dpi. At 180 dpi there was however no significant difference between the viral load of CMM8 transgenic lines, ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 and infected cv.60444 ($p>0.05$) **(Appendix F)**.

**Small interfering RNA evaluation of trial plants**

### 3.4.7 RNA extraction

High quality RNA was extracted from CMM8, AMM4 and non-infected untransformed cv.60444 using the QIAzol method and the integrity of the RNA was checked on a 1.2% agarose gel. The results show that high quality RNA was successfully extracted from CMM8, AMM4 transgenic lines and non-infected healthy cv.60444 (**fig 3.12**). RNA was also extracted from infected CMM8, AMM4 transgenic lines, infected untransformed cv.60444 and non-infected untransformed cv.60444.

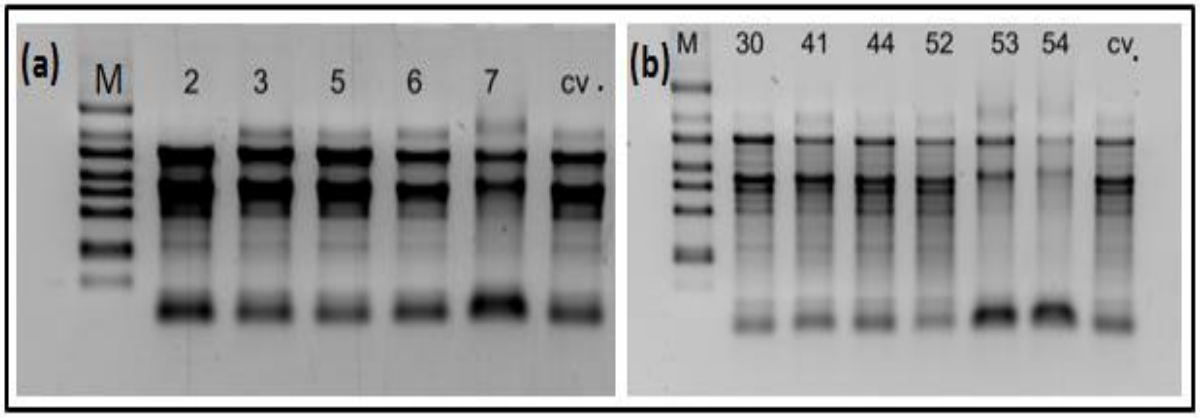


**Figure 3. 12** 1.2% agarose gel electrophoresis (10mg/ml EtBr) of total RNA extracted from (a) CMM8 transgenic lines and non-infected untransformed cv.60444. (b) AMM4 transgenic lines and non-infected untransformed cv.60444

### 3.4.7 T-DNA expression results

Reverse transcriptase-polymerase chain reaction of cDNA from AMM4 and CMM8 transgenic lines resulted in successful amplification of the *GUSPLUS* and *hyg* in both

mismatched AMM4 lines **(fig 3.14c and d)** and non-mismatched CMM8 transgenic lines **(fig 3.13c and d)** and as expected no amplicons were detected in healthy cv.60444. Synthesised cDNA from CMM8 and AMM4 lines was used to amplify the BC1 IR transgene. The results showed successful amplification of the 221 bp CMM8 sense arms in all six selected lines except CMM8-8 **(fig 3.13a)**. The CMM8 antisense arm was successfully amplified in all six selected lines except CMM8-3 **(fig 3.13b)**. Both arms were strongly amplified in comparison with amplification of endogenous *ubiquitin* gene **(fig 3.13ai and bi)**. AMM4 lines showed low expression of the mutated sense arm **(fig 3.14a)** compared with the amplification of endogenous *ubiquitin* gene **(fig 3.14ai)**. The non-mutated antisense arm fragment of the AMM4 hairpin construct was also weakly expressed **(fig 3.14b)** compared with the amplification of endogenous *ubiquitin* gene **(fig 3.14bi)**.

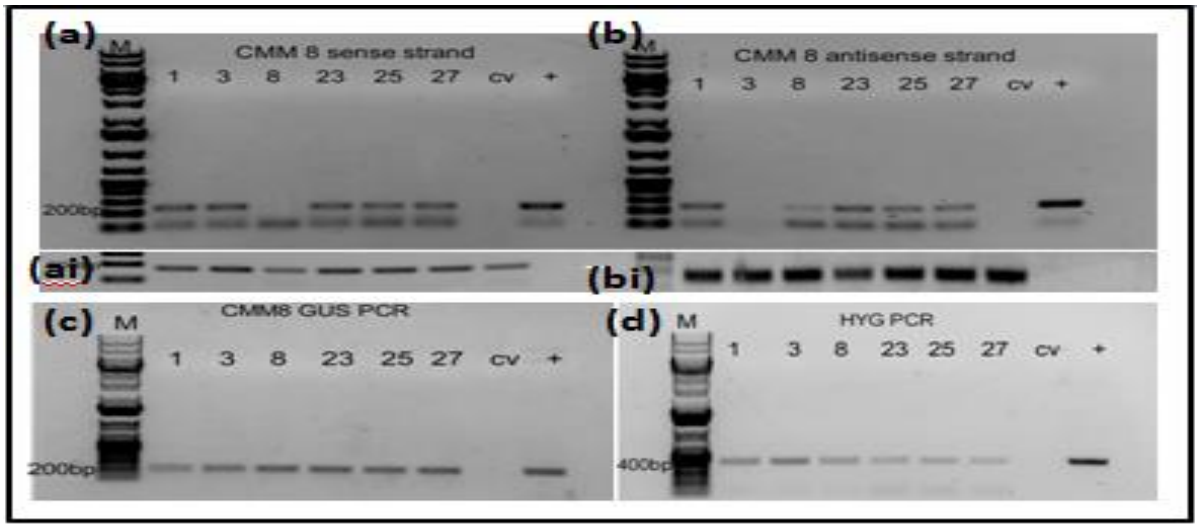


**Figure 3. 13** RT-PCR amplification of the BC1 IR transgene, *GUSPLUS* and *hyg* genes from CMM8, AMM4 transgenic lines and healthy cv.60444 negative control. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas). cv. = healthy cv.60444 negative control. + = positive control transgene plasmid (a) amplication of CMM8 transgenic lines sense arm (aii) amplification of endogenous *ubiquitin* gene from CMM8 cDNA (b) amplication of CMM8 transgenic lines antisense arm (bii) amplification of endogenous *ubiquitin* gene from CMM8 cDNA. (c) amplification of 181 bp *GUSPLUS* from CMM8 transgenic lines cDNA. (d) amplification of 485 bp *hyg* from CMM8 transgenic lines cDNA

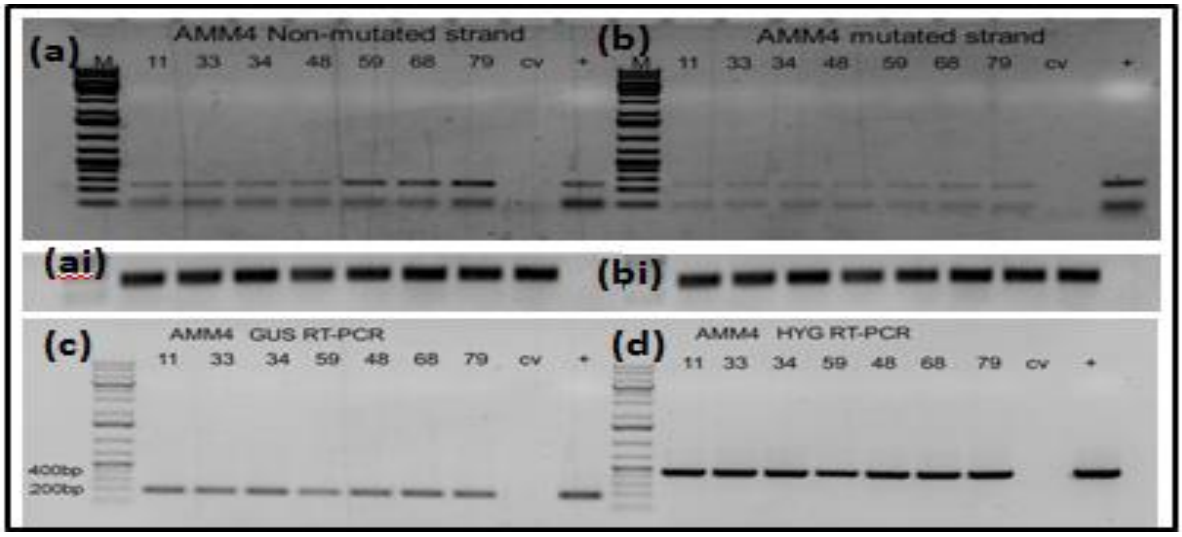


**Figure 3. 14** RT-PCR amplification of BC1 IR transgene, *GUSPLUS* and *hyg* genes from AMM4 and healthy cv.60444 negative control. M= O'GeneRuler 1 kb Plus DNA ladder (Fermentas). cv. = healthy cv.60444. + = positive control transgene plasmid (a) amplication of AMM4 transgenic lines non-mutated antisense arm (aii) amplification of endogenous *ubiquitin* gene from CMM8 cDNA (b) amplication of AMM4 transgenic lines mutated sense arm (bii) amplification of endogenous *ubiquitin* gene from AMM4 cDNA. (c) amplification of 181 bp *GUSPLUS* from AMM4 transgenic lines cDNA. (d) amplification of 485 bp *hyg* from AMM4 transgenic lines cDNA

### 3.4.8 Northern blot detection of siRNA

The labeling efficiency of the Northern Starter Kit (Roche) and DIG oligonucleotide tailing kit 2$^{nd}$ generation (Roche) on SACMV BC1 PCR amplicons, an *Arabidopsis* miR167 21 nucleotide (5'TAGATCATGCTGGCAGCTTCA), a 24 nucleotide (5'TTCAGGAGCCTCCGCCTGTCT) and a 30 nucleotide positive control were tested. The results showed successful labeling of the BC1 RNA as well as the marker nucleotides **(fig 3.15)**. The labeled RNA and short oligonucleotides were detected at low concentrations of 10 ng/µl. At 1 ng/µl the BC1 RNA **(fig 3.15b)** signal was weak compared with the control 1ng/ µl signal.

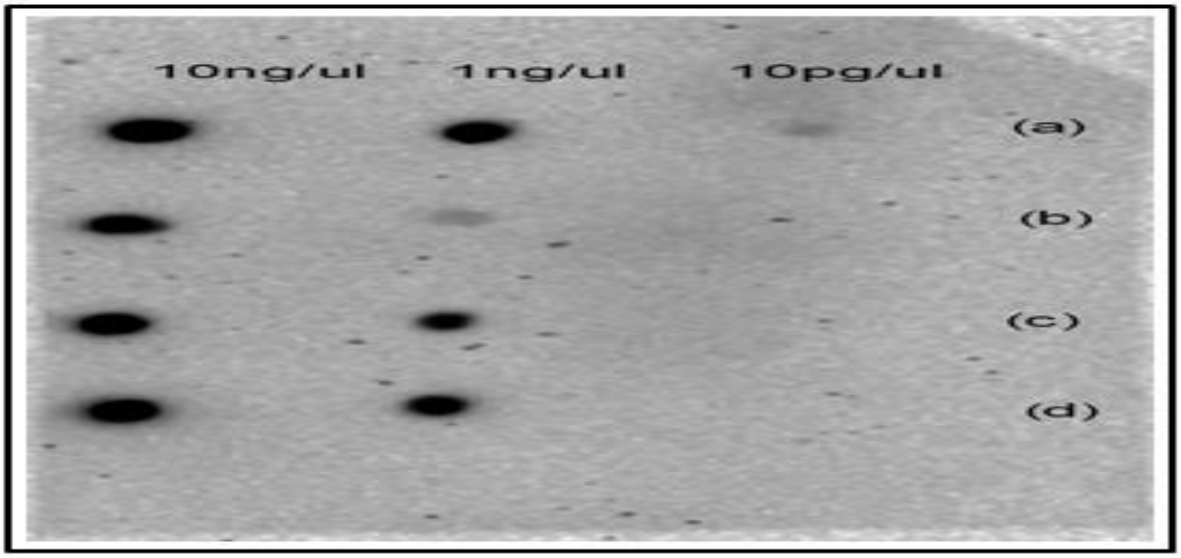


**Figure 3. 15** Determination of the DIG labeling efficiency of the BC1 RNA as well as the marker nucleotides (**a**) a Northern Starter Kit (Roche) positive RNA control. (**b**) BC1 IR sense arm (**c**) labeled 24 nt primer and (**d**) labeled 21 nt *Arabidopsis* mi167

South African cassava mosaic virus BC1 IR-derived siRNA molecules were not detected in CMM8 lines prior to SACMV infection **(fig 3.16a)** however, the probe hybridised to high molecular weight RNA estimated to be around 30 nt base pairs in size. The detected bands were varying in accumulation intensities. Using the siRNAs at the highest concentration being equal to 100% CMM8-1, 3, 8, 23, 25 and 27 had 27%, 49%, 53%, 46%, 77% and 100% accumulation, respectively. Non-infected healthy cv.60444 did not produce siRNAs as expected. AMM4 transgenic lines produced siRNA 21 nt in size (**fig 3.16b)**. The detected bands were varying in accumulation intensities. Using the siRNAs at the highest concentration being equal to 100% AMM4-11, 33, 34, 46, 59, 68 and 27 had 69%, 100%, 53%, 38%, 45%, 57 and 55% accumulation, respectively. Non-infected healthy cv.60444 did not produce siRNAs as expected. Loading control miRNA 167 was detected in CMM8 **(fig 3.16ai)** and AMM4 transgenic lines **(fig 3.16bi)** after stripping the BC1 probe from the membrane.

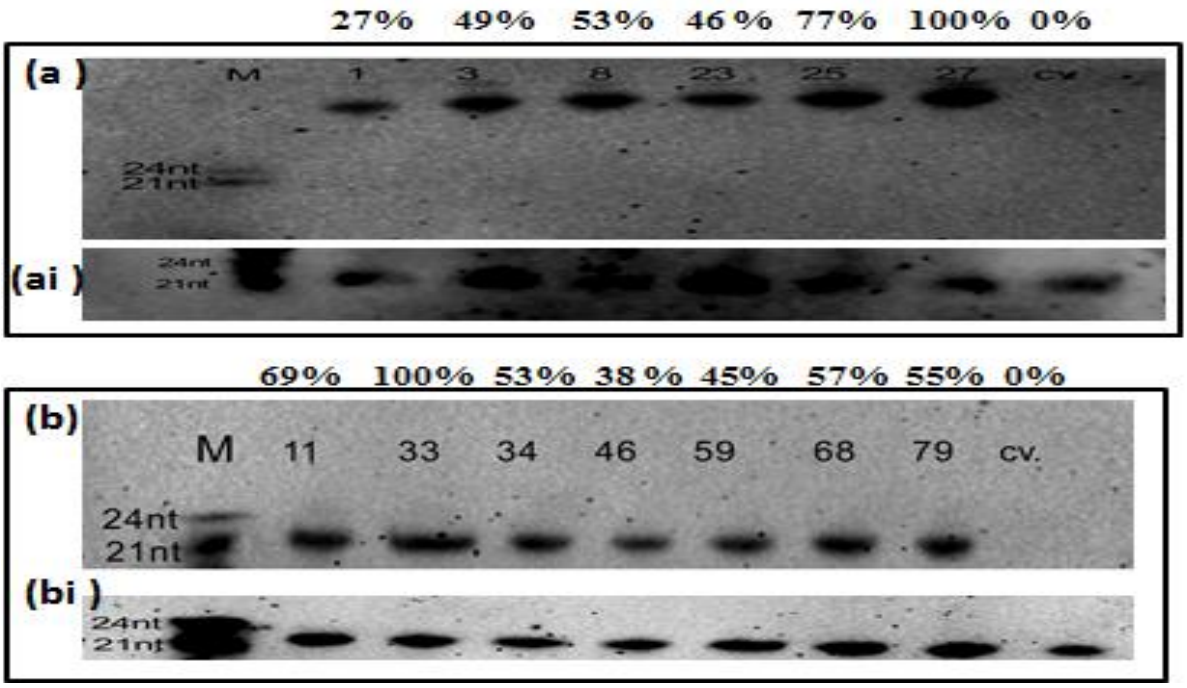


**Figure 3. 16** siRNA detection (a) CMM8 transgenic lines and non-infected healthy cv.60444 probed with DIG labeled BC1 derived probe (b) AMM4 transgenic lines and non-infected healthy cv.60444 probed with DIG labeled BC1 derived probe. (ai) CMM8 and (bi) AMM4 transgenic lines and non-infected healthy cv.60444 probed with DIG labeled *Arabidopsis* miRNA 167 probe. The numbers above represent each trial line

At 180 dpi, SACMV infected CMM8 transgenic lines produced siRNAs approximately 21 nucleotide bases in size. The detected bands were varying in accumulation intensities, and using the siRNAs at the highest concentration being equal to 100% CMM8-1, 3, 8, 23, 25 and 27 had a 4%, 9%, 100%, 42%, 35% and 38% accumulation, respectively. Infected non-transgenic cv.60444 produced siRNAs of approximately 21 nt bases in size with 58% accumulation **(fig 3.17a)**. At 180 dpi AMM4 lines still produced small nucleotides 21 nt in size. The detected bands were varying in accumulation intensities, and using the siRNAs at the highest concentration being equal to 100% AMM4-11, 33, 34, 46, 59, 68 and 27 had an 85%, 70%, 56%, 49%, 67%, 97 and 100% accumulation, respectively. Infected non-transgenic cv.60444 produced siRNAs 21 nucleotide bases in size with 39% accumulation.

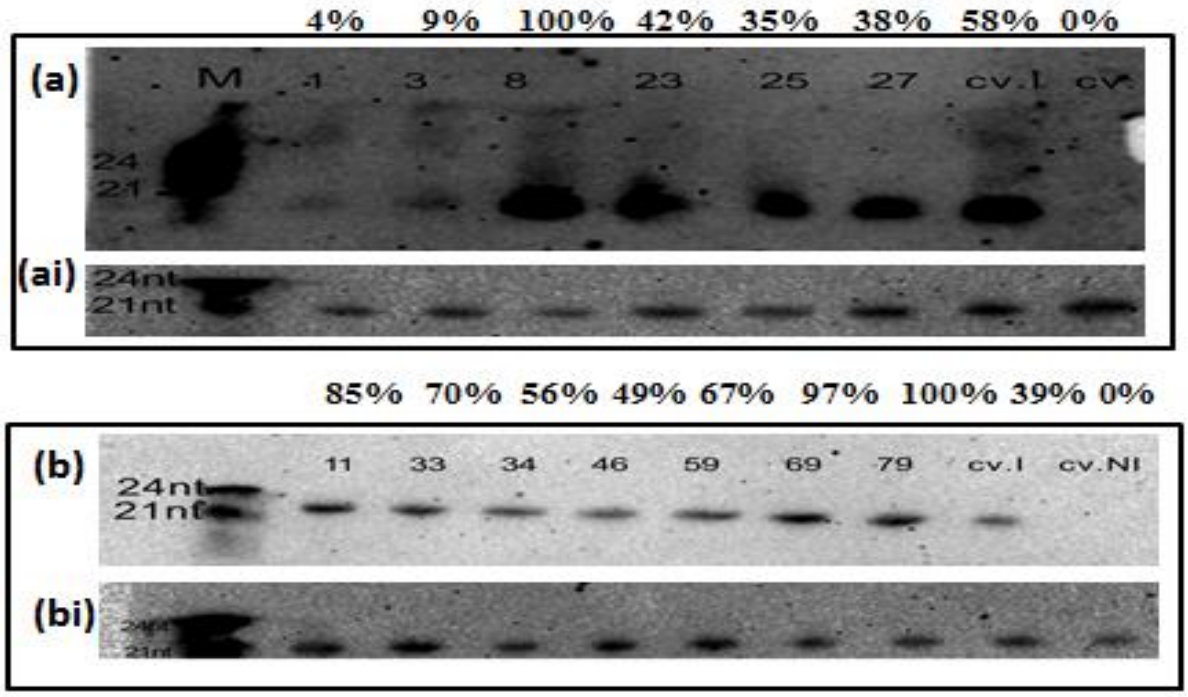


**Figure 3. 17** siRNA detection (a) CMM8 transgenic lines, infected untransformed cv.60444 (cv I) and non-infected healthy cv.60444 (cv NI) probed with DIG labeled BC1 derived probe (b) AMM4 transgenic lines, infected untransformed cv.60444 (cv I) and non-infected healthy cv.60444 (cv NI) probed with DIG labeled BC1 derived probe. (ai) CMM8 and (bi) AMM4 transgenic lines and control line probed with DIG labeled *Arabidopsis* miRNA 167 probe. The numbers above represent each trial line

## 3.5 Discussion

Expression of begomovirus movement gene BC1 in transgenic plants has recently been reported (Taha *et al*., 2016). Silencing of the begomovirus cell-to-cell movement protein (MP) induced by BC1 derived siRNAs in transgenic plants is expected to reduce virus transmission form cell-to-cell in host plants (Taha *et al*., 2016). The objective of this study was to evaluate the tolerance, resistance or susceptibility of cassava cv.60444 transformed with a BC1 mismatched IR construct (AMM4) or non-mismatched BC1 IR construct (CMM8), and to compare the efficiencies of both constructs. Agro-inoculation of the AMM4 and CMM8 lines with SACMV viral cloned resulted in 90 and 95% infectivity, respectively, as evidenced by symptom development on the leaves. CMM8 and AMM4 lines had lower symptom scores compared with infected untransformed cv.60444 (**fig 3.8**). However the difference in symptom severity between CMM8 and AMM4 lines in comparison with infected untransformed cv.60444 were not significantly different at 36 and 56 dpi. In a study by Taylor *et al*. (2012) they used the same mismatched BC1 IR construct (nt position 1532-1753) to agro-transform *N. benthamiana*, and infectivity trials were done with SACMV agrodimers. From their results they identified line L and M with significantly reduced symptoms and viral loads. The difference between their results and ours might be contributed to the geminivirus-plant variety interaction. The extent of PTGS initiation and success is dependent on virus-plant interaction (Chellappan *et al*., 2004). Although *N. benthamiana* is widely used in viral infectivity studies due to its near universal virus susceptibility (Goodin *et al*., 2008), variability in SACMV infection within the *Nicotiana* genus particularly *N. benthamiana* and *N. tubacum* has been reported (Berrie *et al*., 2001). Variation in the significance of symptom reduction between their results and our study might be due to differences in virus-plant interaction between experimental host and natural perennial host cassava.

Viral load quantification by real time absolute qPCR showed that all CMM8 and AMM4 transgenic lines has lower SACMV viral load compared with infected untransformed cv.60444 however the results were not significantly different (**fig 3.9**). Our results were however more consistant with recent attempts to engineer resistance in squash plants against begomovirus *Squash leaf curl virus* (SqLCV) using the full sequence of the virus BC1 gene by Taha *et al*. (2016). They noted virus reduction in transgenic squash plants, however the reduction was not significant, and they concluded that could be due to late expression of the IR construct.

Reduced viral load in transgenic lines could be because of the presence of siRNA prior to SACMV infection. High molecular weight RNA was detected in pre-infected CMM8 lines instead of siRNAs (**fig 3.16**). Absence of siRNA molecule detection could be due to sequestering and trapping of siRNA molecules by the higher molecular weight RNA or late expression of the IR construct (Taha *et al*., 2016). Presence of virus specific RNA has been shown to correlate with viral resistance (Chen *et al*., 2004; Hilly *et al*., 2005; Vanderschuren *et al*., 2009) and the viral reduction could be due to additional boast of transgene derived siRNA to the plants natural PTGS mechanism. All transgenic lines were producing siRNA at varying levels at 180 dpi, we expected siRNAs targeting the movement protein to effectively silence the movement and multiplication of the virus. This suggests that the response of transgenic plants to virus infection does not seem to be determined by the accumulation of siRNAs alone. Bengyella *et al*., 2015 suggested that RNA silencing alone is not sufficient for plant recovery to viral infestation but expression of R genes as a consequence of transcriptome reprogramming also plays a role.

Transgenic line dsAC1 resistant to ACMV-[NG:Ogo:90] (Vanderschuren *et al*., 2009) was used as a transgenic positive control for resistance against SACMV. The ACMV-[NG:Ogo:90] AC1/Replication –associated protein (Rep) sequence (154 bp) from nt position 1690 to 1844 expressed in dsAC1 transgenic line has a 86% sequence similarity with the corresponding sequence on the SACMV AC1 ORF. We assumed that ACMV-[NG:Ogo:90] similarity to SACMV would render ACMV-[NG:Ogo:90] resistant line dsAC1 resistant or tolerant to SACMV. However from our results ACMV-[NG:Ogo:90] resistant transgenic line dsAC1 had lower sss and viral load at 36 and 56 dpi compared to infected untransformed cv.60444 but statistically we could not establish a significant difference between the two. The results suggest that although ACMV and SACMV are related to some extent the ACMV-[NG:Ogo:90] AC1 IR transgene in dsAC1 could not induce broad spectrum resistance to SACMV.

At 180 dpi, viral load and sss of transgenic lines CMM8 AMM4, infected untransformed cv.60444 and infected ACMV-[NG:Ogo:90] resistant line had decreased compared with 56 dpi (**fig 3.11**). The reduction in viral load and attenuation of symptoms can be attributed to factors such as temperature, light intensity, siRNA production or other basal immunity associated genes as a suggested in chapter 2. At 56 dpi plants were moved from the growth facility with average temperature 28°C and light intensity 800 lux to the green house were maximum of 30°C was expected and 1000 lux light intensity. To sum extent, the increase in

light intensity and temperature played a role in plant recovery after viral infection. Patil and Fauquet (2014) who showed that *N. bethamiana* infected with cassava mosaic virus showed recovery at high light intensity of ≥600 μE/m2/s compared with low light intensity of 150 μE/m2/s. While there was a decrease in viral load in SACMV infected untransformed cv.60444 at 180 dpi, it was not sufficient to confer resistance, and the reduction was likely due to some level of tolerance triggered under high temperatures and light intensity. Generation of vsiRNAs targeting the virus genome was reported in susceptible cassava landrace T200 and untransformed cv.60444 cassava infected by SACMV and ACMV, respectively (Chellappan *et al*., 2004; Rogans *et al*., 2016).

At 36 dpi and 56 dpi there was a negative Pearson correlation between the viral load and symptom scores of CMM8 and AMM4 transgenic lines **(Appendix G)**. Several begomovirus resistance trials have failed to establish positive Pearson correlation between viral loads and symptom severity (Cecchini *et al*., 1998; Kaweesi *et al*., 2014). Our results were consistant with those of Challappan *et al*. (2005) were a positive correlation could not be established between the viral load of SLCMV and the symptoms induced by the virus on cassava. Kaweesi *et al*. (2014) reported similar results with *cassava brown streak virus* (CBSV), and they suggested that two separate mechanisms might be responsible for virus multiplication and symptom restriction regardless of high viral loads. This further emphasizes the need to evaluate the two parameters independently when evaluating for viral tolerance or resistance.

In experiments conducted by Hou *et al*. (1999) they observed several negative effects on the depelopment of tomato plants expressing geminivirus *Bean dwarf mosaic virus* movement protein BC1. The anomalies included MP gene deletion whilst surprisingly the adjacent *NPT II* gene was intact, they also observed that BC1 protein could not be expressed in the transgenic line and the transgenic lines had stunted growth. In our research the CMM8 and AMM4 transgenic plants showed normal plant height in comparison with infected untransfomed cv.60444. We observed that there weren't significant differences between the height of CMM8 and AMM4 lines in comparison with infected untransformed cv.60444. This contrasts the observation of Hou *et al*, 1999 were stunted growth of the transgenic lines.

Transgene expression levels in CMM8 and AMM4 lines were highly variable (**fig 3.13 and 3.14**). Expression of transgene introduced by genetic engineering has been noted to be variable in plants carrying the same transgene (Kohli *et al*., 2010; Longstaff *et al*., 1993; Rooke *et al*., 2003). The variability could be due to chromosomal effects or transgene silencing due to presence of multiple copies of the transgene of sections of the transformation

cassette silencing each other (Angell and Balcombe, 1997; Kohli *et al*., 2010). A positive correlation between the expression of the transgene and the level of tolerance induced by the transgenic line after viral infection could not be established. For example AMM4 line 46 had the least viral load at 36 and 56 dpi but its expression was weak compared to lines 68 and 78 which had higher viral loads. CMM8-8 and 27 performed better than all the other CMM8 lines in terms of symptom severity and viral load however expression of the CMM8 sense arm was not detectable whilst CMM8-27 was strongly detected. These results were in strong agreement with Dalakouran and Tzanopoulous (2011) who concluded that high expression of the transgene does not necessarily guarantee resistance after noting that *N. benthmiana* expressing high levels of CMV CP transgene were still susceptible to CMV infection.

From our results the mismatched construct in AMM4 lines and the non-mismatched construct in CMM8 lines induced the same viral and sss reduction. The large size of the transformation cassette has been shown to induce deletion and rearrangement of the insert (Nakano *et al*., 2005). The truncated copy of the transformation cassette in CMM8 lines with the large *pdk* intron as shown by the dual copies of the *hyg* gene detected by southern blot is a clear indication of rearrangement and deletion. Surprisingly the selected AMM4 lines had a single copy of the transgeng.This suggests that replacing the 700bp intron in CMM8 with a small nucleotide loop increased the stability of the transformation cassette. These results were different to those of chapter 2 were the intron in the non-mismatched CMM6 transgene, providing better integration stability and dsRNA processing compared no mismatched construct in AMM2 lines. Taylor *et al*. (2012) showed that introduction of base pair mismatches in the sense arm of in IR sequence as done in the transformation cassette used to transform AMM4 lines can improve the efficiency of the construct to induce PTGS.

In conclusion the BC1 transgene whether as mismatched or non-mismatched construct could not significantly reduce the viral load of SACMV in both AMM4 and CMM8 transgenic lines, respectively, from this study and previous trials (Moralo 2016).

# Chapter 4

# Transformation of cassava landrace T200 with a hairpin RNA silencing construct against African cassava mosaic virus, East African cassava mosaic virus and South African cassava mosaic virus

## 4.1 Introduction

Cassava mosaic disease (CMD) caused by a diverse range of begomovirus species; strains and isolates (Brown *et al*., 2015; Legg *et al*., 2015), including *African cassava mosaic virus* (ACMV), *South African cassava mosaic virus* (SACMV) and *East African cassava mosaic virus* (EACMV) (Brown *et al*., 2015; Legg *et al*., 2015). CBVs are responsible for significant yield loss of the starchy tubers. This diversity makes genetic engineering problematic as these CBVs share only small regions of sequence homology which to exploit for PTGS-induced virus silencing. Geminiviruses like most viruses have been shown to trigger virus induced gene silencing by producing virus specific siRNAs (vsiRNAs) (Aregger *et al*., 2012; Hamilton and Baulcombe, 1999; Pallas and Garcia, 2011). Elevated levels of these vsiRNAs from the transgene have been reported to correlate with viral resistance in *N. bethamiana* and cassava (Chellappan *et al*., 2004a). Many of the CBVs occur in the same geographical location (Rey *et al*., 2012) and as mixed infections on the same field (Fondong *et al*., 2000; Patil and Fauquet, 2009). In southern Africa, ACMV, SACMV and EACMV have been reported (Berry *et al*., 2001; Rey *et al*., 2012). This invariably requires stacking multiple genomic regions from several viruses for construction of the IR repeats against CBVs. Stacking of genes in transgenic plants could be a more viable method to induce broad spectrum durable resistance against diseases (Zhu *et al*., 2012), and targeting of overlapping regions between two genes has been reported to be more effective in silencing (Taha *et al*., 2016)

The laboratory model cassava cultivars have been transformed with genes or segments of genes from the geminiviruses genome (Vanderschuren *et al*., 2009, 2012). Various levels of viral elimination or reduction have been reported in transgenic cassava expressing virus derived transgenes either as antisense RNA, double stranded RNA or inverted repeats (IR) targeting the viral genome or viral promoter region (Ntui *et al*., 2015; Vanderschuren *et al*.,2009,2012; Zhang et *al*., 2005). Selection of which sequence of the viral genome to

introduce as the transgene is very important, thus far the most targeted regions of the viral genome include AC1/Rep ORF and regions of high siRNA production on the viral open reading frame (Chellappan *et al*., 2004a; Vanderschuren *et al*., 2007, 2009). Hot spots on the genome for targeting by vsiRNAs have been reported in many geminivirus studies (Aregger *et al*., 2012; Chellappan *et al*., 2004a; Poogin, 2013). Recently hotspots were reported for cassava T200 and TME3 infected with SACMV (Rogans *et al*., 2016). Hot spots for targeting geminivirus suppressors or the promoter regions have also been identified (Aregger *et al*., 2012; Sharma *et al*., 2014).

The two most common methods for plant transformation are particle bombardment and *Agrobacterium* mediated transformation. Particle bombardment involves the introduction of target DNA into the plant material cells using gold or tungsten metal particles coated with the target DNA (Christon, 1992). The DNA coated particles are propelled into plant cells using high pressure. Particle bombardment has been reported in transformation of cassava even though the transformation numbers were reported to be low (Munyikwa *et al*., 1998; Raemakers *et al*., 1996; Schopke *et al*., 1997). Particle bombardment has a major disadvantage of having high percentages of multiple gene insertion after transformation (Dai *et al*., 2001).

*Agrobacterium*-mediated transformation involves the use of a disarmed Ti plasmid from bacterium *A. tumefaciens w*hich inserts a portion of its T-DNA plasmid into the plant genome (Gelvin, 2003; Yuan and Williams, 2012). In cassava transformation the gene of interest is placed in the 25 bp imperfect repeat sequence which is on either side of the left and right border flanking the T-DNA in a binary vector, such as pCambia. Once the gene of interest has been inserted in this region the gene is then cloned into a disarmed *A. tumefaciens.* The transgene is then transferred using the bacterium natural ability to transfer the T DNA into the plant genome. According to Taylor *et al.* (2004), *Agrobacterium*-mediated transformation is the best method of transformation in cassava with transformation efficiency of 55% possible.

Transformation depends on the source of plant material and the genotype. Several systems incorporating different types of plant sources have been tried for transformation of cassava. Stamp and Henshaw (1987) reported that transformation using germplasm explants was unsuccessful in cassava due to the heterozygous nature. In several studies somatic tissue has been used for *Agrobacterium*-mediated transformation of cassava (Ntui *et al*., 2015;

Raemakers *et al*., 1997; Zhang and Puonti-Kaelas, 2000). Somatic embryos are induced from auxiliary buds (AB) or leaf lobes (ILL) on Murashige and Skoog media. Continuous culture of these somatic embryos results in formation of mature secondary somatic embryos (SSE). The SSE can then be grown in auxin-supplemented Gresshoff and Doy (1974) media to produce friable embryogenic callus (FECs) (Taylor *et al*., 1996). Today the production of large numbers of independent transgenic plants relies on the use of FECs. Transformation of FECs from cassava cultivars has been successfully reported using *Agrobacterium*-mediated transformation (Bull *et al*., 2009; Chetty *et al*., 2013; Nyaboga *et al*., 2015; Taylor *et al*., 1996).

This chapter aims to construct an inverted repeat construct (code named DM-AES) targeting two regions of ACMV-[NG:Ogo:90] as well as being efficient in targeting SACMV and EACMV, due to the inclusion of a third region, a 21 bp sequence conserved sequence between EACMV, ACMV and SACMV, and subsequent transformation of cassava landrace T200 FECs. The construct will be derived from stacking three regions which include (i) sequences from the putatitive leftward promoter region between the 5'end of ACMV-[NG:Ogo:90] AC4 complementary sense ORF to the TATA box plus an additional 49 nt upstream with core elements of the leftward promoter. Trangenic cassava transformed with this region were previously reported to recovery from ACMV infection (Vanderschuren *et al*., 2006) (ii) the overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'). This region was previously shown to produce high levels of vsiRNAs and was targeted by African cassava mosaic virus Cameroon strain ACMV-[CM] for induction of PTGS (iii) a 21 nt conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV. We hypothesise that if this 21 nt conserved sequence could induce PTGS then the transgenic cassava would induce broad spectrum resistance across EACMV, ACMV and SACMV, the most widely spread CBVs. A stacked construct consisting of a combination of these three sequences will be designed into an IR construct where sense and antisense sequence of the stacked construct separated by an 85 bp intron. This IR construct will be used to transform cassava landrace T200 FECs using *Agrobacterium* mediated transformation.

### 4.1.1 Specific Aims

i. Design a construct targeting ACMV-[NG:Ogo:90] putative promoter region, a 21 nt conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV and the overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter).
ii. Clone the IR constructs into pART7 expression vector.
iii. Clone the hairpin cassette constructs into plant transformation vector pCambia 1305.1.
iv. Mobilize pCambia 1305.1/IR into *Agrobacterium* LBA 4404.
Cassava T200 FEC induction.
v. *Agrobacterium*-mediated transformation of FEC with pCambia/IR construct (described in iv)
vi. Regeneration of transformed FECs.
vii. Selection of plants for transgene integration using visual and molecular screening

## 4.2 Methodology flow chart

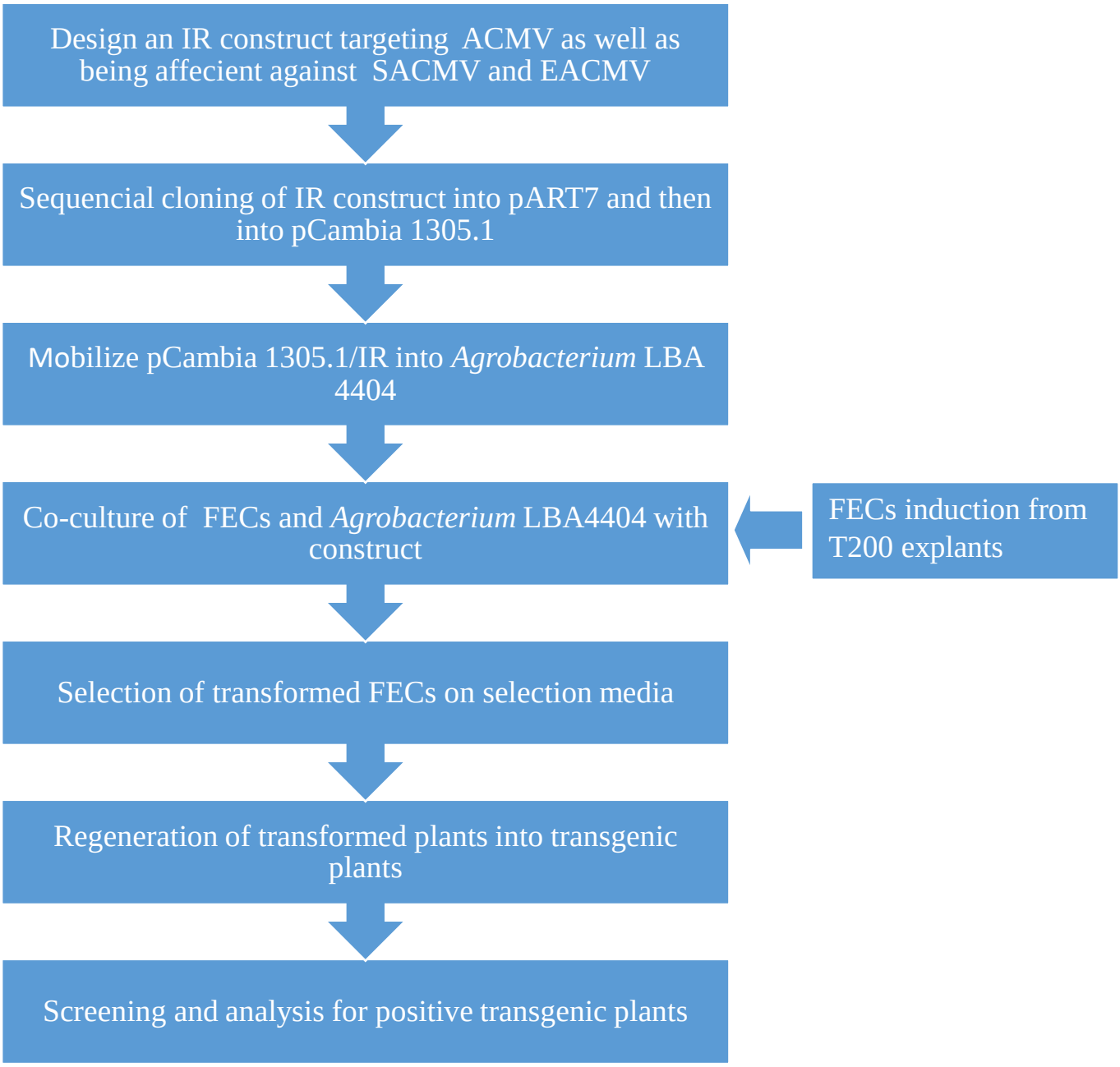


**Figure 4. 1** Flow diagram of the methodology in construct design cloning and transformation into cassava T200 FECs

## 4.3 Materials and Methods

### 4.3.1 Target selection and alignment.

For selection of the construct target regions, the three CBVs ACMV-[NG:Ogo:90], SACMV and EACMV DNA A with accession number obtained from Fauquet *et al.* (2008); Berries *et al*, (2001) and Fauquet *et al*, (2008), respectively, were obtained. Vector NTI Advance suit (version 10.3) software was used to align the three DNA-A sequences. ACMV-[NG:Ogo:90] DNA-A sequence was used to search for the putative leafward promoter region. The second region to be targeted on the ACMV-[NG:Ogo:90] DNA-A was the overlapping region between AC1 3' end and the AC2 5' end. The target region estimated to be between 1200 nt and 1600 nt on ACMV-[CM] ORF was shown by Challappan *et al*, 2004 to be a high target for PTGS. To obtain the conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV, the 3 accession numbers were entered into National Centre for Biotechnological Information site (NCBI). Clusta X Multiple Sequence Alignment Program (Version1.8; 1999) was used to align the 3 CBV isolates AC1 gene to search for a conserved region. The 3 chosen regions, ACMV-[NG:Ogo:90] putative promoter region, a 21 nt conserved sequence of AC1/Rep shared between ACMV, EACMV and SACMV and the overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter) were combined to design our transgene IR sequence. The IR construct was designed in Benchling software (Benchling, Inc) to consist of sense and antisense orientation of our transgene whilst being separated by an 85 bp intron (Vanderschuren *et al*., 2009). The IR construct design was sent to Life Technologies (SA) for synthesis.

### 4.3.2 Digestion and cloning of IR hairpin in pART7

The synthesised IR construct (Life Technologies, SA) was cloned plasmid pMK-RQ. The plasmid was digested with *EcoR*I and *Hind*III enzymes (Fermentas) to cut out the IR construct. The vector pART7 was also subsequently digested with *EcoR*I and *Hind*III enzymes. A 1% agarose gel was used to analyse the digested samples. The 534 bp corresponding to the IR size from pMK-RQ digest was then excised from the gel. The 5013 bp fragment from the *EcoR*I and *Hind*III digested pART7 was also excised. The 2 excised fragments were extracted using GeneJET extraction kit (Thermoscientific), and quantified using NanoDrop 1000 Spectrometer (V3.7). The two fragments with cohesive *EcoR*I and *Hind*III ends were ligated in a 1:1 molar ratio using 1X ligation buffer and 1U T4 DNA ligase (Fermentas) in a 20 µl reaction. The ligation reaction was incubated at 16°C overnight. Then

5 µl of the ligation reaction was used to transform DH5 α chemically competent cells. The transformed DH5 α were spread on LB Agar plates supplemented with 100 mg/ml ampicilin. The plates were incubated at 37°C overnight.

### 4.3.3 Screening of clones

A few colonies were randomly selected from the LBA plate and plasmid DNA was extracted using the GeneJET Miniprep Kit (ThermoScientific). The plasmid extracted was screened for insertion of the 229 bp transgene using Pro1 forward primers and Rev3 reverse primer **Table 1**. The reaction mixture contained 1X Taq buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 1U Taq recombinant enzyme (Fermentas), 20 ng of template DNA and nuclease free water to a final volume of 50 µl. The reactions was set up in a thermal cycler (Bio-Rad) with reaction conditions set at 94°C for 2 min and 35 cycles 94°C for 30 s, 55°C for 30 s, primer extension at 72°C for 30 s and final extension step of 72°C for 10 min. For detection of the sense orientation of the IR construct Pro1 forward primer and intron reverse primers **Table 1** were used for PCR with the same reaction mixture and same reaction conditions as above. For detection of the antisense orientation, the small intron forward primer and Pro1 forward primers were used. The 85 bp intron was also screened using the intron forward and reverse primer **Table 4.1** under annealing temperature of 55°C. To check the orientation of the IR construct in relation to the pART7 *Nopaline synthase* (NOS) terminator and *Cauliflower mosaic virus* (CaMV) promoter pART7 forward and intron reverse primers were used for the sense orientation. For the antisense orientation of the IR construct in regards to the pART7, pART7 reverse primer and Rev 3 primer were used for PCR with the same reaction mixture and reaction conditions as above. A restriction digest of the pART7/IR construct with *EcoR*I and *Hind*III enzymes was performed to check for the presence of the 543 bp IR.

**Table 4. 1** Primers used to screen for the IR construct

| Primer | Primer sequence 5' -3' |
|---|---|
| Pro 1 forward (construt forward) | TTGAACTTTAATTTGAATTAAAAGG |
| Rev 3 reverse (construct reverse) | TGCAATCTTCATCACCCTCACAGA |
| Intron forward | GATATTTAAATTATTTAT |
| Intron reverse | GCGCTCGTACCTGCAGTATA |
| pART7 forward | GTTTGTTATTGTGGCGCTCTATC |
| pART7 reverse | CCCAGCTATCTGTCACTTCATC |

### 4.3.4 Cloning of IR cassette into pCambia 1305.1

A restriction digest of the, pART7/IR construct was done with *NotI* enzyme and blunt ended to separate the CaMV-IR-NOS terminater cassette. Plant transformation vector pCambia 1305.1 was digested with *EcoR*I and *Hind*III (Fermentas). T4 DNA polymerase (Fermentas) was used to blunt-end both the CaMV-IR-NOS cassette and pCambia 1305.1 digests. The blunt polished ends of pCambia 1305.1 digested fragments were then dephosphorylated using FastAP (Fermentas). A 1% agarose gel was used to analyse the digested samples. The 2641 bp and 11,796 bp corresponding to the CaMV-IR-NOS cassette and pCambia 1305.1 digests, respectively, were then excised from the gel. The two excised fragments were extracted using GeneJET extraction kit (Thermoscientific) and quantifies using NanoDrop 1000 Spectrometer (V3.7). The three fragments were ligated in a 1:6 molar ratio using 1X ligation buffer and 1U T4 DNA ligase (Fermentas) in a 20 µl reaction. The ligation reaction was incubated at 16°C overnight. Five µl of the ligation reaction was used to transform DH5 α chemically competent cells. The transformed DH5α were spread on LB Agar plates supplemented with 50 mg/ml kanamycin. The plates were incubated at 37°C overnight.

### 4.3.5 Screening of plant transformation vector for presence of IR cassette.

Plasmid DNA was extracted from the presumptive clones using GeneJET Miniprep Kit (ThermoScientific). The plasmids extracted were screened for insertion of the 314 bp transgene plus intron in the sense and antisense orientation as described in **4.3.3**. The clones were also screened for presence of the *glucuronidase (GUS)* and a portion of the *hyg* genes. Both genes are found on the pCambia 1305.1 vector. To amplify the 181 bp *GUS* gene; *GUSPLUS* F (5'-CAACATCCTCGACGACGATAGCA-3') and *GUSPLUS* R (5'-GGTCACAACCGAGATCTCCT-3') primers were used. For *hyg* screening primer set; *hyg* F (5'-TCTCGATGAGCTCATGCTTTGG-3') and *hyg* R (5'-

AGTACTTCTACACAGCCATGGG-3') were used to amplify a 444 bp portion of the *hyg* resistance gene. The PCR reaction mixture contained 1X Taq buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 1U Taq recombinant enzyme (Fermentas), 20 ng of template DNA and nuclease free water to a final volume of 50 µl. The reactions was set up in a thermal cycler (Bio-Rad) with reaction conditions set at 94°C for 2 min and 35 cycles 94°C for 30s, 55°C for 30 s, primer extension at 72°C for 30s and final extension step of 72°C for 10 min. Fragments were analysed by electrophoresis on a 1% agarose gel containing 10 µg/ml ethidium bromide in a 1X TAE buffer. The positive clones were also digested with *NotI* enzyme to separate the 2641 bp CaMV-IR-NOS cassette. Digests were analysed on 1% agarose gel. A positive clone of the pCambia/IR was sent off for automated sequencing by Inqaba Biotechnical Industries (Pretoria, South Africa). The clones were sequenced using P27-3 and P27-5 primers. Multiple sequence alignment to confirm correct orientation of the IR cassette was performed using Benchling software (Benchling, Inc).

### 4.3.6 Transformation of A. tumefaciens LBA 4404 with IR hairpin cassettes

Freeze-thaw method was used to transform *A. tumefaciens* LBA 4404 with a positive plasmid of pCambia/IR. Five hundred ng of pCambia/IR purified plasmid was added to 100 µl of chemically competent *A. tumefaciens* LBA4404 cells previously stored at -70°C. The mixture was placed on ice to thaw and it was mixed intermittently. This was then snap-frozen in liquid nitrogen for 5 min followed by thawing at room temperature. A 2 ml Yeast extract-phosphate (YEP) broth was then added to transformed cells. This was incubated at 28°C for 3 hrs with gentle shaking (50 rpm). After the incubation period, transformed cells were centrifuged for 5 min at maximum speed and the supernatant discarded. The pellet was resuspended in 50 µl YEP. Transformed cells were then spread on YEP plate containing 50 µg/ml rifampicillin, 50 µg/ml kanamycin and 100 µg/ml streptomycin and incubated at 28°C for 2 days until colonies appeared.

### 4.3.7 Screening of *A. tumefaciens* LBA4404 for the presence RNA silencing hairpin constructs.

Colonies from the *A. tumefaciens* LBA4404 transformation plates were selected and grown overnight at 28°C and 200 rpm in YEP broth containing 50 µg/ml rifampicin, 50 µg/ml kanamycin and 100 µg/ml streptomycin. Plasmid DNA was then extracted from presumptive clones and PCR was done to screen for the presence of the *GUSPLUS, hyg* genes and the IR construct in both orientations as described in **4.3.5**. Amplicons were analysed by

electrophoresis on a 1% agarose gel containing 10 µg/ml ethidium bromide in a 1X TAE buffer.

### 4.3.8 Preparation of *A. tumefaciens* inoculums

The pCambia 1305.1/IR construct transformed into *A. tumefaciens* LBA4044, and *A. tumefaciens* transformed with empty pCambia 1305.1 plant transformation vector, were streaked each on Yeast extract peptone (YEP) medium consisting of 5 g/L Yeast extract, 5g Bacto-peptone and 10 g/L Sodium chloride, solidified with 15 g/L bacterial agar and pH was adjusted to 7.2. Autoclaved YEP medium was supplemented with 50 µg/ml kanamycin, 50 µg/ml rifampicin and 100 µg/ml streptomycin of antibiotics. Plates were streaked with the 2 *Agrobacterium* strains and incubated at 28°C in the dark for 2 days. A single colony obtained from each of the plates was inoculated in YEP supplemented with appropriate antibiotics and 2 mM $MgSO_4$. The inoculated YEP was cultured overnight in the dark at 28°C with shaking at 200 rpm.

Cultures were grown until an optical density (OD) of 0.7-1.0, at λ=600 nm was reached. From this culture 0.5 ml was removed and inoculated in 25 ml of YEP with appropriate antibiotics and 2 mM $MgSO_4$. The culture was grown overnight in the dark at 28°C with shaking at 200 rpm. The OD was measured and when an OD of 0.7-1.0 at λ=600 nm was obtained, the bacterial suspension was centrifuged in a sterile 50 ml centrifuge tube at 4000 g for 10 min at room temperature. The supernatant was removed and the pellet was resuspended in 25 ml of liquid GD medium. The suspension was centrifuged again at 4000 g for 10 min at room temperature. The supernatant was removed and the centrifuge tube was blotted on tissue paper to remove excess liquid. The pellet was resuspended in GD liquid and diluted to an $OD_{600}$ of 0.5. Acetosyringone was added to a final concentration of 200 µM. The cultures were placed on a horizontal shaker (50 rpm) at room temperature for 45 min.

### 4.3.9 *Agrobacterium*-mediated transformation of FEC

The transformed *A. tumefaciens* inoculum prepared in section **4.3.8** were used to transform FECs (10 FEC clumps/plate). For the experiment seven T200 FEC plates were inoculated with *A. tumefaciens* transformed with the pCambia 1305.1/IR plasmid. Then 1 plate was inoculated with *A. tumefaciens* transformed with an empty pCambia plasmid and another plate was inoculated with an empty *A. tumefaciens* culture. One of the plates was left untransformed and was used as a control for regeneration. For the inoculation 100 µl of the respective *A. tumefaciens* was pipette onto each FEC in order to soak the FEC clusters. Plates

were then incubated in the laminar flow uncovered for ~5 min and then sealed with parafilm. The transformed FEC with the bacterial suspensions and the controls were co-cultivated at 24°C for 4 days in 16 h light/8h dark photoperiod.

### 4.3.10 Removal of excess *A. tumefaciens*

After 4 days of co-cultivation period, FECs were gently scraped off the plates using sterile forceps and placed in 50 ml tubes containing 25 ml of GD liquid supplemented with 500 µg/ml carbenicillin. The suspension was vortexed briefly for 5-10 sec and the FEC were allowed to settle. A 25 ml pipette was then used to remove the supernatant, leaving the FECs at the bottom of the tube. Again 25 ml of GD liquid supplemented with 500 µg/ml carbenicillin was added and FECs were washed by gently inverting the tube. The washing of FEC in the GD liquid containing 500 µg/ml carbenicillin was repeated ~5 times until the supernatant became clear. Once the washing GD was clear, the FECs were resuspended in ~10 ml of GD liquid containing 500 µg/ml carbenicillin. The FEC suspensions were pipetted and spread thinly and evenly onto ~10 sterile plastic meshes (pour size of 100 µm). The meshes with the FECs were then blotted each placed on top of 3 layers of sterile filter paper to allow for absorption of excess liquid off the FEC.

### 4.3.11 Recovery of transformed FEC

After *A. tumefaciens* mediated transformation a recovery stage for the FECs is necessary. The mesh/FECs were transferred to GD plates supplemented with 250 µg/ml carbenicillin to facilitate for recovery. The mesh/FEC GD Plates were incubated for 4 days at 28°C for 16 h light/8 h dark photoperiod.

### 4.3.12 Maturation of transformed FECs

For FEC maturation and initiation of antibiotic resistance mesh/FECs were moved to GD containing 250 µg/ml carbenicillin and 5 µg/ml hygromycin for a week at 28°C with 16 h light/8 h dark photoperiod. After a week mesh/FEC were moved to GD containing 250 µg/ml carbenicillin supplemented with 8 µg/ml hygromycin for another week at 28°C with 16 h light/8 h dark photoperiod. The step wise antibiotic selection was rounded off with moving the mesh/FECs to GD containing 250 µg/ml carbenicillin and 15 µg/ml hygromycin for a week at 28°C with 16 h light/8 h dark photoperiod.

### 4.3.13 Determination of transformation success

To determine the transformation success of the FECs a GUSPLUS assay was performed on a small section of putatively transformed material from the mesh/FEC. A small section of FECs

from untransformed T200 plates was tested as the negative control. The isolated FECs were incubated in GUSPLUS assay solution (100 mM Tris/NaCl buffer, 1 mg/ml 5-bromo-4-chloro-3-indolyl-*β*-D-glucuronic acid (X-Gluc) and 1% Triton X-100) at 37°C overnight in the dark .The next day GUSPLUS solution was removed and the material were destained in 70% ethanol, and FECs viewed for staining.

### 4.3.14 Selection and regeneration of transgenic plants

To stimulate the maturation and regeneration of transformed FECs the mesh/FECs were moved to MSN consisting of 4.4 g MS2 medium containing, 20g sucrose, 1 µg/ml 1-Naphthaleneacetic acid (NAA), 15 µg/ml hygromycin and 250 µg/ml carbenicillin. The pH was adjusted to 5.8 and media was solidified with 8 g/L Noble agar. Mesh/FECs were maintained on MSN for 10 days at 28°C with 16 h light/8 h dark photoperiod. The mesh/FECs were subsequently transferred to fresh MSN every 10 days with incubation conditions 28°C with 16 h light/8 h dark photoperiod. The transfer of mesh/FECs was done 8 times. Between cycle 4 and 8 cotyledons started emerging and these were moved to cassava elongation medium (CEM) which consisted of MS2 supplemented with 0.4 µg/ml 6-benzylaminopurine (BAP) a synthetic cytokinin, 20 g/L of sucrose, 2 µM $CuSO_4$ and 100 µg/ml carbenicillin. The media pH was adjusted to 5.8 and solidified with 8 mg/ml gelrite agar. Cotyledons were moved to fresh CEM every 14 days. After 3 cycles juvenile leaves and shoots were supposed to appear but this was note the case and the regeneration experiment was abandoned.

## 4.4 Results

### 4.4.1 Target selection and alignment

ACMV-[Nigeria: Ororoco, 1990] DNA A was aligned to SACMV DNA A and EACMV using Clustal X Multiple Sequence Alignment Program (Version1.8; 1999). For selection of the ACMV-[NG:Ogo:90] DNA-A first target site, the putative promoter region was identified upstream in the common region from the 5'end of ACMV-[NG:Ogo:90] AC4 complementary sense ORF to the TATA box plus an additional 49 nt upstream with core elements of the leftward promoter and ending at the transcriptional start site of AC1. This 117 nt region was identified between 2714 nt and 49 nt. There was a 50.4% sequence similarity between this chosen putative promoter region on ACMV-[NG:Ogo:90] DNA-A and the corresponding region on SACMV DNA A and EACMV DNA A (**fig 4.2**).

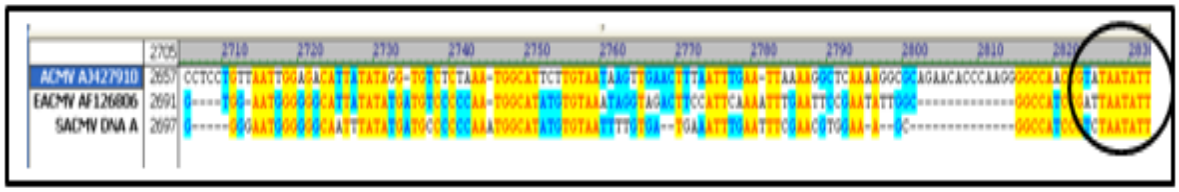


**Figure 4. 2** A segment of the multiple sequence alignment output screen. Alignment was performed on 3 CBV isolates ACMV, SACMV and EACMV to identify the sequence similarity between the chosen putative promoter region on ACMV-[NG:Ogo:90] DNA-A and the corresponding region on SACMV DNA A and EACMV DNA A around the TATA box (circled)

The second region to be targeted was the overlapping sequence between ACMV-[NG:Ogo:90] AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter). A 91 nt region between 1530-1620 nt was chosen on the overlapping region (**fig 4.7**). Clustal X Multiple Sequence Alignment showed that there is a 64% sequence similarity between this 91 nt AC1 3'/AC2 5'-ter overlapping region on ACMV-[NG:Ogo:90] DNA-A and the corresponding region on SACMV DNA A and EACMV DNA A

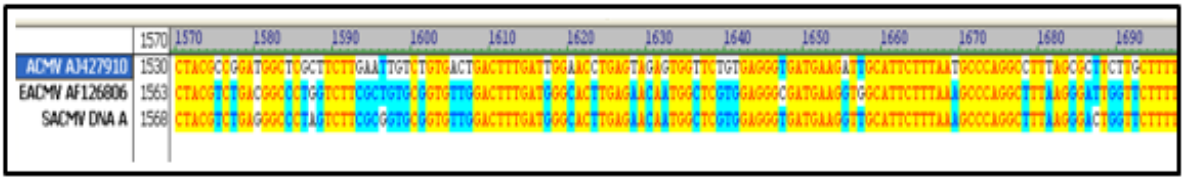


**Figure 4. 3** A segment of the multiple sequence alignment output screen. Showing the second chosen region, a 90 nt region between 1530-1620 nt on the overlapping region of ACMV-[NG:Ogo:90] DNA-A AC1 3' end and AC2 5' end (AC1 3'/AC2 5'-ter). Alignment was performed on 3 CBVs ACMV, SACMV and EACMV to identify the sequence similarity between this 90 nt AC1 3'/AC2 5'-ter overlapping region on ACMV-[NG:Ogo:90] DNA-A and the corresponding region on SACMV DNA A and EACMV DNA A.

Clusta X Multiple Sequence Alignment Program (Version1.8; 1999) was used to align the DNA-A AC1 ORFs of ACMV-[NG:Ogo:90], SACMV and EACMV to search for a conserved region. The conserved 21 bp region was identified between 1970-1990 nt on ACMV-[NG:Ogo:90] DNA-A and the corresponding region on SACMV DNA A and EACMV DNA A.

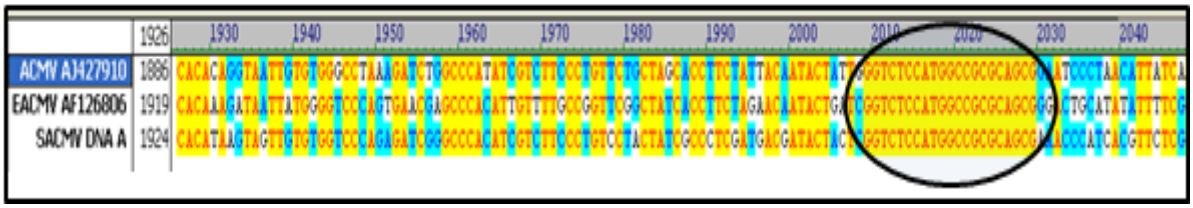


**Figure 4. 4** A segment of the multiple sequence alignment output screen. Alignment was performed on DNA-A AC1 ORFs of ACMV, SACMV and EACMV to identify a 21 nt conserved region along the AC1 genome

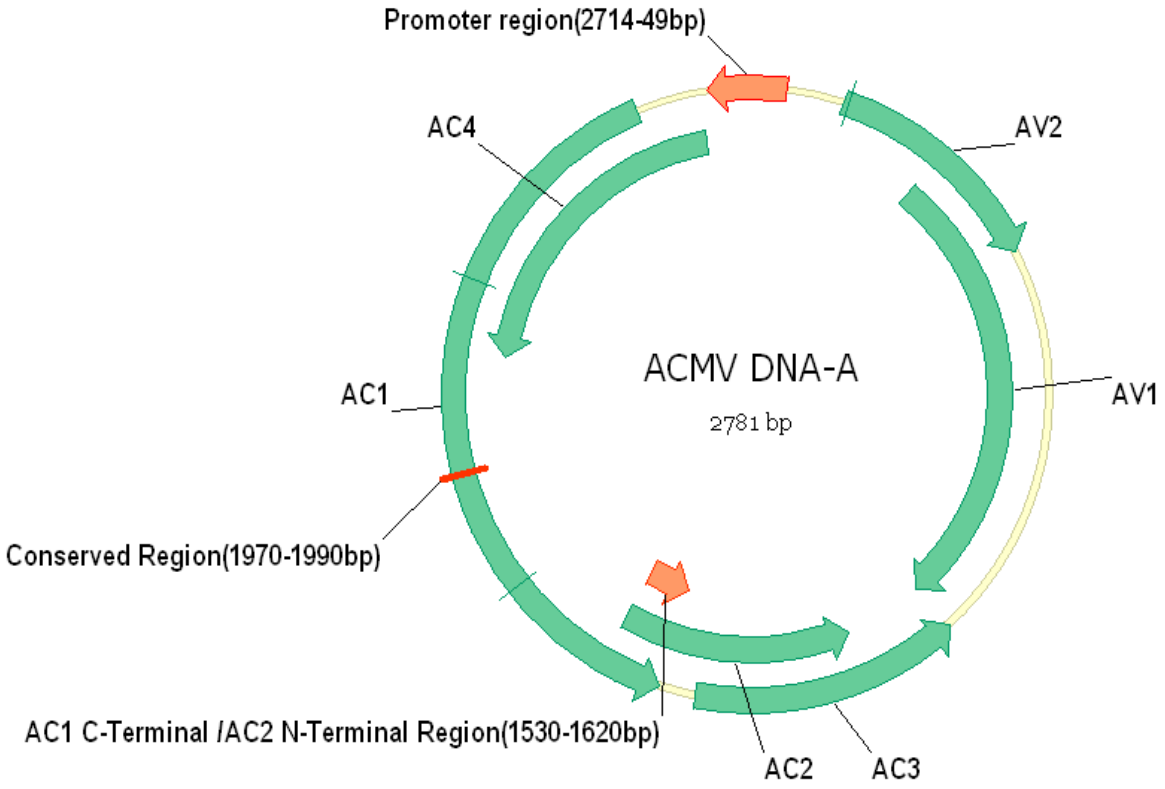


**Figure 4. 5** Diagram of ACMV-[NG:Ogo:90] DNA-A highlighting the three transgene target areas, ACMV-[NG:Ogo:90] 117 nt putative promoter region (2714-49 nt), 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region (1970-1990) and the 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter overlapping region (1530-1620) all shown in orange

The three identified sequences ACMV-[NG:Ogo:90] 117 nt putative promoter region (2714-49 nt), 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region (1970-1990 nt) and the 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter (1530-1620 nt) were combined to design our transgene sequence (229 bp) code named DM-AES (**fig 4.6**).

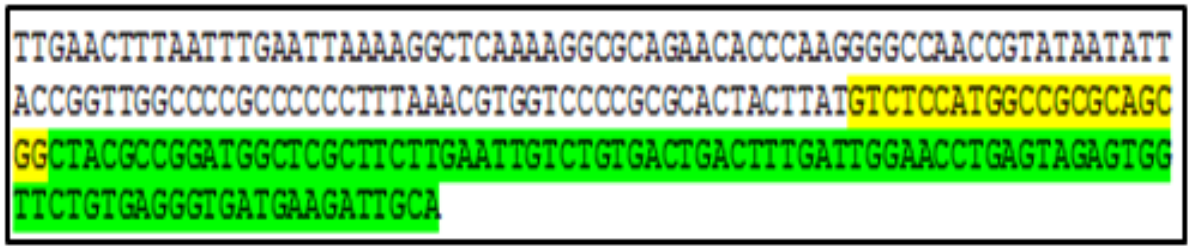


**Figure 4. 6** The combined (DM-AES) transgene sequence consisting of the ACMV-[NG:Ogo:90] 117 nt putative promoter region (white background), the 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region (yellow background) and the 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter

The three proposed potential target areas were entered into pssRNAit (http://plantgrn.noble.org/pssRNAit/) a modified version of siRNA scan program (Xu *et al*., 2006) to predict possible siRNAs target hot spots. **Table 4.2** shows the 8 possible siRNAs from the ACMV-[NG:Ogo:90] 117 nt putative promoter region **Table 4.3** shows some of the 46 possible hit from the 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter overlapping region. The 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region generates only one possible siRNA.

**Table 4. 2** siRNA scan computational results predicting regions along the ACMV-[NG:Ogo:90] 117 nt putative promoter region where effective siRNA could be generated

**8 siRNA Candidates design based upon above parameters for your sequence**

| siRNA (anti-sense) | siRNA * (Sense) | Alignment | | | |
|---|---|---|---|---|---|
| UUAAUUCAAAUUAAAGUUCAA | GAACUUUAAUUUGAAUUAAAA | siRNA 21 | AACUUGAAAUUAAACUUAAUU | | 1 |
| | | User Seq. 1 | UUGAACUUUAAUUUGAAUUAA | | 21 |
| UUUAAUUCAAAUUAAAGUUCA | AACUUUAAUUUGAAUUAAAAG | siRNA 21 | ACUUGAAAUUAAACUUAAUUU | | 1 |
| | | User Seq. 2 | UGAACUUUAAUUUGAAUUAAA | | 22 |
| GAGCCUUUUAAUUCAAAUUAA | AAUUUGAAUUAAAAGGCUCAA | siRNA 21 | AAUUAAACUUAAUUUUCCGAG | | 1 |
| | | User Seq. 8 | UUAAUUUGAAUUAAAAGGCUC | | 28 |
| UGAGCCUUUUAAUUCAAAUUA | AUUUGAAUUAAAAGGCUCAAA | siRNA 21 | AUUAAACUUAAUUUUCCGAGU | | 1 |
| | | User Seq. 9 | UAAUUUGAAUUAAAAGGCUCA | | 29 |
| UUGAGCCUUUUAAUUCAAAUU | UUUGAAUUAAAAGGCUCAAAA | siRNA 21 | UUAAACUUAAUUUUCCGAGUU | | 1 |
| | | User Seq. 10 | AAUUUGAAUUAAAAGGCUCAA | | 30 |
| UUUGAGCCUUUUAAUUCAAAU | UUGAAUUAAAAGGCUCAAAAG | siRNA 21 | UAAACUUAAUUUUCCGAGUUU | | 1 |
| | | User Seq. 11 | AUUUGAAUUAAAAGGCUCAAA | | 31 |
| CUUUUGAGCCUUUUAAUUCAA | GAAUUAAAAGGCUCAAAAGGC | siRNA 21 | AACUUAAUUUUCCGAGUUUUC | | 1 |
| | | User Seq. 13 | UUGAAUUAAAAGGCUCAAAAG | | 33 |
| UGCGCCUUUUGAGCCUUUUAA | AAAAGGCUCAAAAGGCGCAGA | siRNA 21 | AAUUUUCCGAGUUUUCCGCGU | | 1 |
| | | User Seq. 18 | UUAAAAGGCUCAAAAGGCGCA | | 38 |

**Table 4. 3** siRNA scan computational results predicting regions along the 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter overlapping region where effective siRNA could be generated

**46 siRNA Candidates design based upon above parameters for your sequence**

| siRNA (anti-sense) | siRNA * (Sense) | Alignment | | | |
|---|---|---|---|---|---|
| AGAAGCGAGCCAUCCGGCGUA | CGCCGGAUGGCUCGCUUCUUG | siRNA | 21 | AUGCGGCCUACCGAGCGAAGA | 1 |
| | | User Seq. | 2 | UACGCCGGAUGGCUCGCUUCU | 22 |
| AAGAAGCGAGCCAUCCGGCGU | GCCGGAUGGCUCGCUUCUUGA | siRNA | 21 | UGCGGCCUACCGAGCGAAGAA | 1 |
| | | User Seq. | 3 | ACGCCGGAUGGCUCGCUUCUU | 23 |
| CAAUUCAAGAAGCGAGCCAUC | UGGCUCGCUUCUUGAAUUGUC | siRNA | 21 | CUACCGAGCGAAGAACUUAAC | 1 |
| | | User Seq. | 9 | GAUGGCUCGCUUCUUGAAUUG | 29 |
| ACAAUUCAAGAAGCGAGCCAU | GGCUCGCUUCUUGAAUUGUCU | siRNA | 21 | UACCGAGCGAAGAACUUAACA | 1 |
| | | User Seq. | 10 | AUGGCUCGCUUCUUGAAUUGU | 30 |
| GACAAUUCAAGAAGCGAGCCA | GCUCGCUUCUUGAAUUGUCUG | siRNA | 21 | ACCGAGCGAAGAACUUAACAG | 1 |
| | | User Seq. | 11 | UGGCUCGCUUCUUGAAUUGUC | 31 |
| ACAGACAAUUCAAGAAGCGAG | CGCUUCUUGAAUUGUCUGUGA | siRNA | 21 | GAGCGAAGAACUUAACAGACA | 1 |
| | | User Seq. | 14 | CUCGCUUCUUGAAUUGUCUGU | 34 |
| CACAGACAAUUCAAGAAGCGA | GCUUCUUGAAUUGUCUGUGAC | siRNA | 21 | AGCGAAGAACUUAACAGACAC | 1 |
| | | User Seq. | 15 | UCGCUUCUUGAAUUGUCUGUG | 35 |
| AGUCACAGACAAUUCAAGAAG | UCUUGAAUUGUCUGUGACUGA | siRNA | 21 | GAAGAACUUAACAGACACUGA | 1 |
| | | User Seq. | 18 | CUUCUUGAAUUGUCUGUGACU | 38 |

The IR construct was designed to consist of sense and antisense orientation of our transgene (229 bp) sequence whilst being separated by a 85 bp intron sequence. (**fig 4.7).**


**Figure 4. 7** A schematic representation of the IR construct with the intron separating the sense transgene from the antisense transgene

The designed IR construct sequence was entered into Mfold secondary structure prediction software (Zucker, 2003) (http://www.bioinfo.rpi.edu/applications/mfold) to predict the possible RNA structure of the IR construct once expressed. Folding was done under conditions 37°C; ionic condition fixed at $[Na^{+}]$=1 M and $[Mg^{++}]$= 0 M and energy at 1kcal/mol. **fig 4.8** shows the predicted hairpin structure.

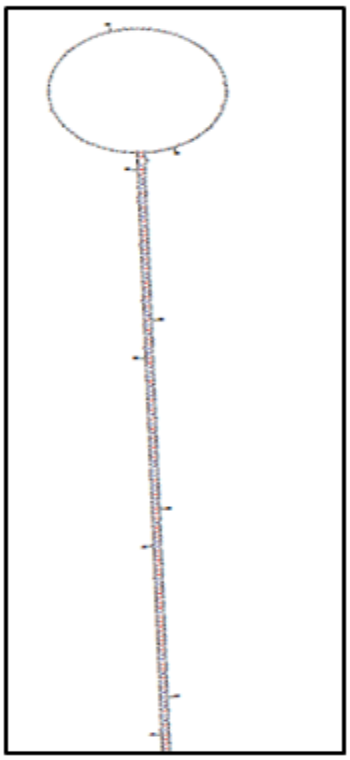

**Figure 4. 8** Computer predicted secondary structure of the IR construct sequence

### 4.4.2 Digestion and cloning of IR hairpin in pART7

The results showed the successful *EcoR*I /*Hind*III restriction of the 543 bp IR construct from pMK-RQ plasmid (**fig 4.9c**), and successful cloning into vector pART7.

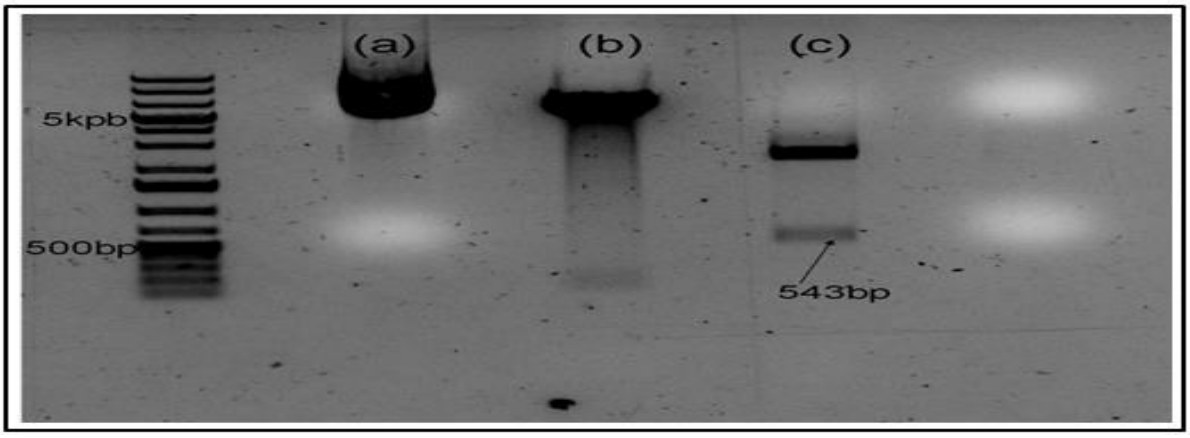


**Figure 4. 9** 1% agarose gel showing pMK-RQ/IR plasmid clones *EcoR*I and *Hind*III digestions. Lane 1= O'GeneRuler 1 kb Plus DNA ladder, Lane (a) Undigested pART7 Lane (b) Digested pART7 (c) Digested pMK-RQ/IR plasmid showing the released IR 543 bp fragment

### 4.4.3 Screening of clones

Positive clones from the IR/ pART7 ligation and cloning were positive for PCR of the construct, intron, sense arm of the transgene and the antisense arm of the transgene (**fig 4.10**), and were in the right orientation (**fig 4.11**). The results showed a colony positive for the transgene in pART7 and the correct orientation

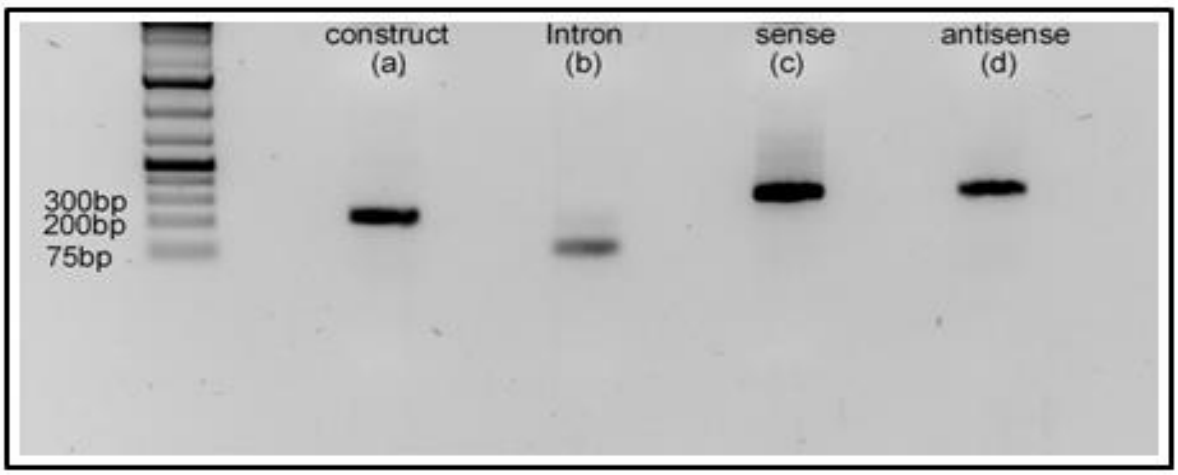


**Figure 4. 10** PCR amplification to screen for presence and orientation of inserts in the completed hairpin constructs. O'GeneRuler 1 kb Plus DNA (Fermentas) (**a**) 229 bp full transgene construct amplicon. (**b**) 85 bp intron amplicon (**c**) 314 bp amplicon to confirm the sense orientation (**d**) 314 bp amplicon to confirm the antisense orientation

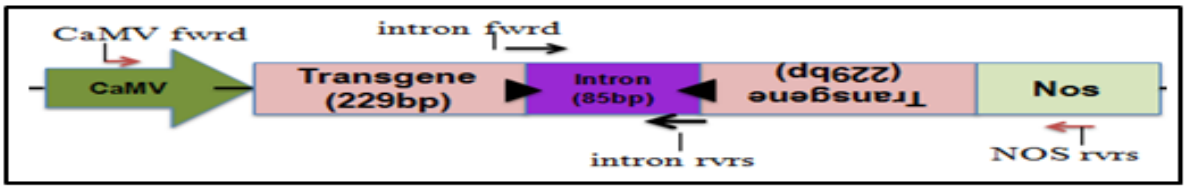


**Figure 4. 11** A schematic representation of the IR cassette between the CaMV promoter and the (NOS) terminator in pART7. The figure also shows the position of the primers used to confirm the orientation of the IR cassette between the CaMV promoter and the NOS terminator

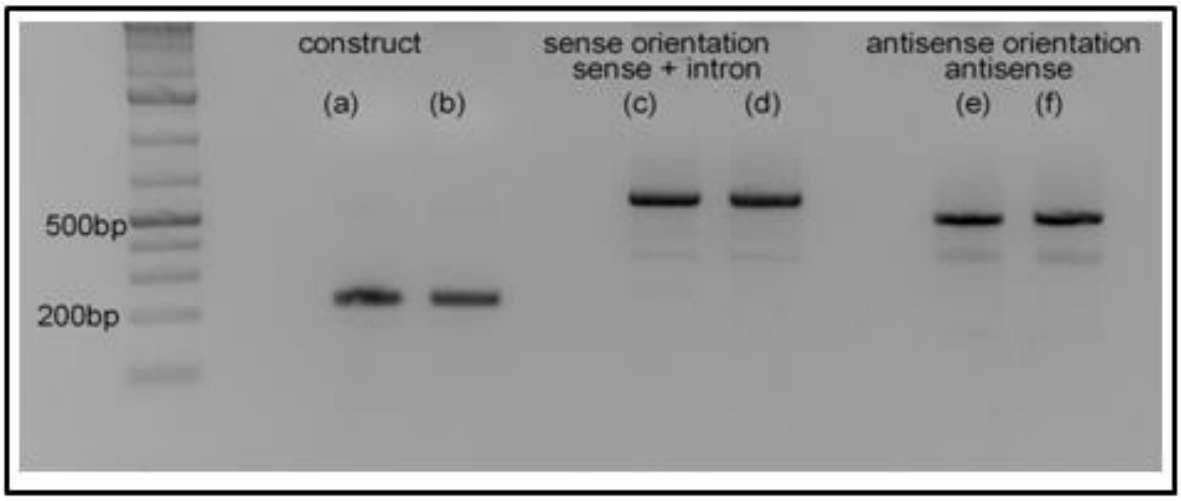


**Figure 4. 12** PCR amplification to screen for presence and orientation of inserts after.ligation. Lane 1 O'GeneRuler 1 kb Plus DNA (Fermentas) (**a**) (**b**) 229 bp transgene amplicon (**c**) (**d**) Sense orientation confirmation amplicons.(**e**) (**f**) Antisense orientation confirmation amplicons

Restriction digestion was also done to confirm the presence of the IR cassette. *Not*I digest of the pART7/IR produced the 2641 bp cassette (CaMV35S promoter, the two 229 bp sense and 229bp antisense hairpin arms and the OCS terminator) (**fig 4.13, lane a**). *EcoR*I and *Hind*III digestion of the pART7/IR positive plasmid produced the expected 543bp IR construct (**fig 4.13**, **lane b**).

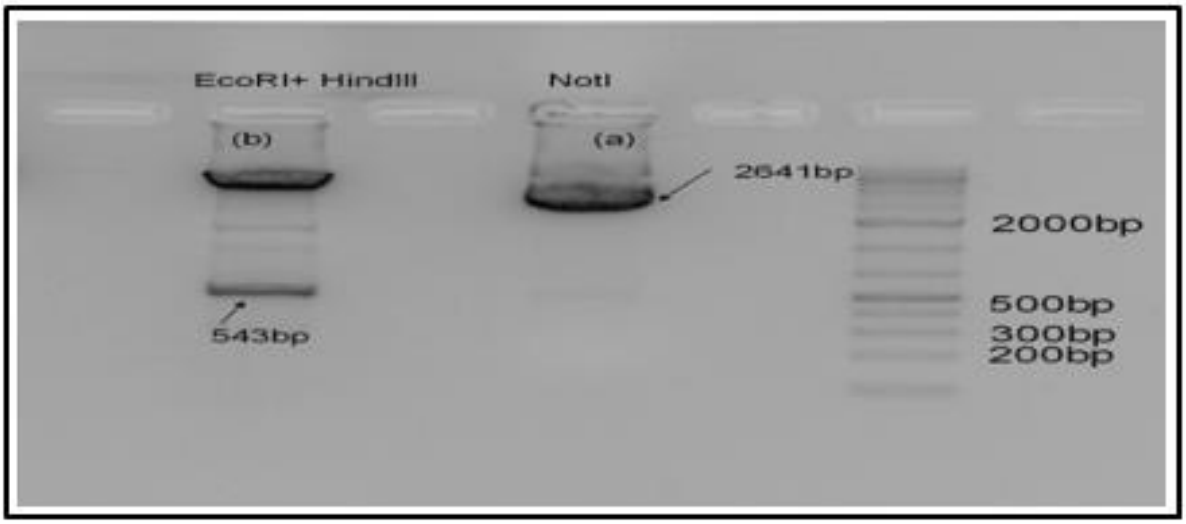


**Figure 4. 13** 1% agarose gel showing pART7/IR plasmid digestion to screen clones for successful ligation of the IR. Lane 3= O'GeneRuler 1 kb Plus DNA ladder, Lane (a) *Not*I digest showing 2641 bp cassette (CaMV35S promoter, the two 229 bp sense and 229 bp antisense hairpin arms and the OCS terminator) Lane (b) *EcoR*I and *Hind*III digest showing 543 bp IR released fragment (lane b)

### 4.4.4 Cloning of IR cassette into pCambia 1305.1.

The expression cassette in pART7 was restricted out using *Not*I enzyme (**fig 4.13**) and cloned into plant transformation vector pCambia 1305.1 restricted with *EcoR*I and *Hind*III. Both restricted fragments were blunt-ended before being ligated. Ligation was successful and this was confirmed by PCR amplification of the sense and antisense arms of the transgene as well as *GUSPLUS* and *hyg* marker genes on the pCambia backbone (**fig 4.14**).

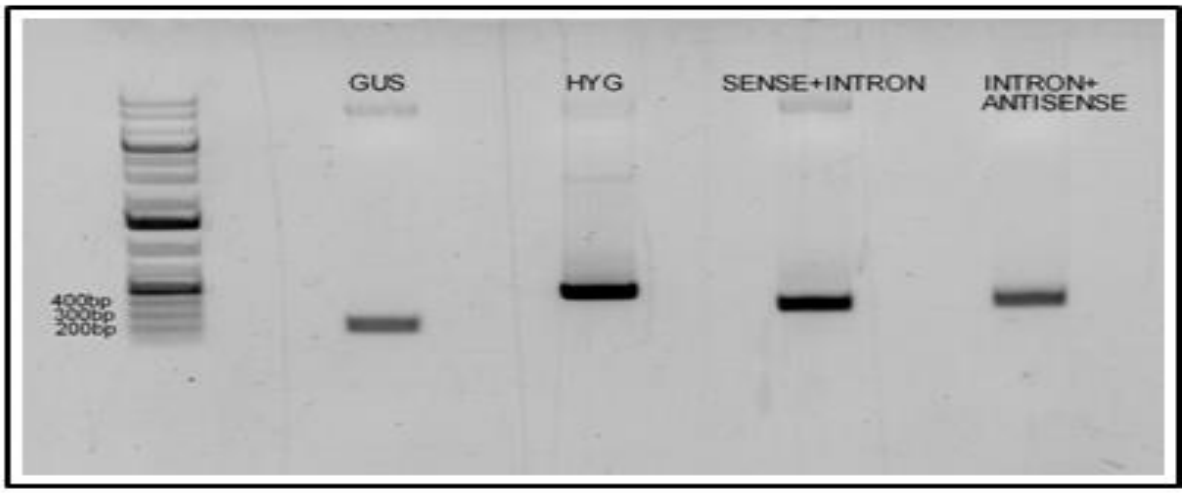


**Figure 4. 14** 1% agarose gel of, PCR amplified products to screen for successful ligation and presence of IR cassette in plant transformation vector p1305.1. Lane 1= O'GeneRuler 1 kb Plus DNA ladder (Fermentas), Lane 2, 181 bp *GUSPLUS* amplicon, Lane 3, 485 bp *hyg* amplicon, Lane 4, transgene sense+ intron 314 bp amplicon, Lane 5, intron+ transgene antisense 314 bp amplicon

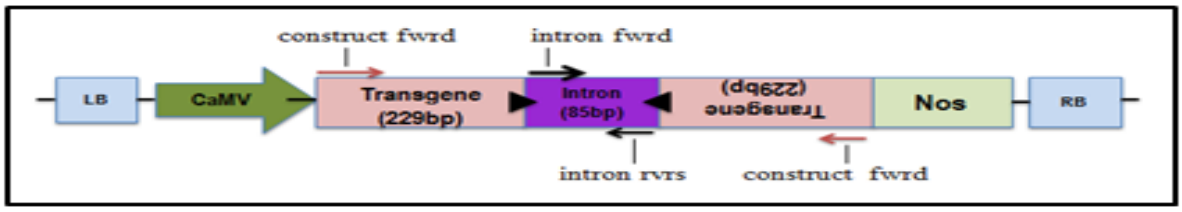


**Figure 4 15** A schematic representation of the transformation cassette with CaMV 35S promoter and NOS terminator as well as the transgene IR within the pCambia left and right border. The figure also shows the location of the primers used to confirm the presents of the transgene

### 4.4.5 Transformation of constructs into Agrobacterium LBA4404

The IR/pCambia plasmid was successfully transformed into *Agrobacterium* LBA4404. The transformation was successfully confirmed as described in **4.4.5** by PCR amplification of the *hyg*, *GUSPLUS* and insert gene as shown in **fig 4.16**.

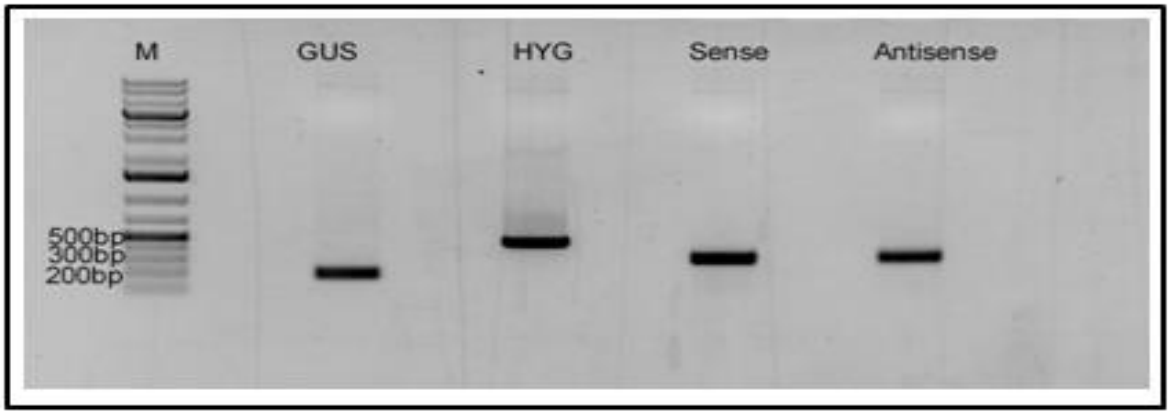


**Figure 4. 16** 1% agarose gel of, PCR amplified products to screen for successful ligation and presence of IR/pCambia in *Agrobacterium*. Lane 1= O'GeneRuler 1 kb Plus DNA ladder (Fermentas); Lane 2, 181 bp *GUSPLUS* amplicon; Lane 3, 485 bp *hyg* amplicon; Lane 4, transgene sense+ intron 314 bp amplicon; Lane 5, intron+ transgene antisense 314 bp amplicon

### 4.4.6 FEC transformation and cotyledon development

Seven T200 FEC plates were inoculated with *A. tumefaciens* transformed with the pCambia 1305.1/IR plasmid vector. One plate was inoculated with *A. tumefaciens* transformed with an empty pCambia 1305.1 plasmid vector and another plate was inoculated with *A. tumefaciens* (no vector) culture. Another plate of T200 FECs was left untransformed. Transformed FECs were positive for transformation by the GUSPLUS assay, as evidenced transformation by the blue stained FEC **(fig. 4.17c).** FECs were moved to Murashige and Skoog Media supplemented with 1mg/ml naphthaleneacetic acid (MSN) media for cotyledon development. Only untransformed T200 FECs produced few embryos after 4 cycles on MSN+H15 media **(fig.4.17d)**. FEC were transferred to fresh MSN+H15 6 times (after every 10 days). The transformed FECs did not develop into cotyledons.

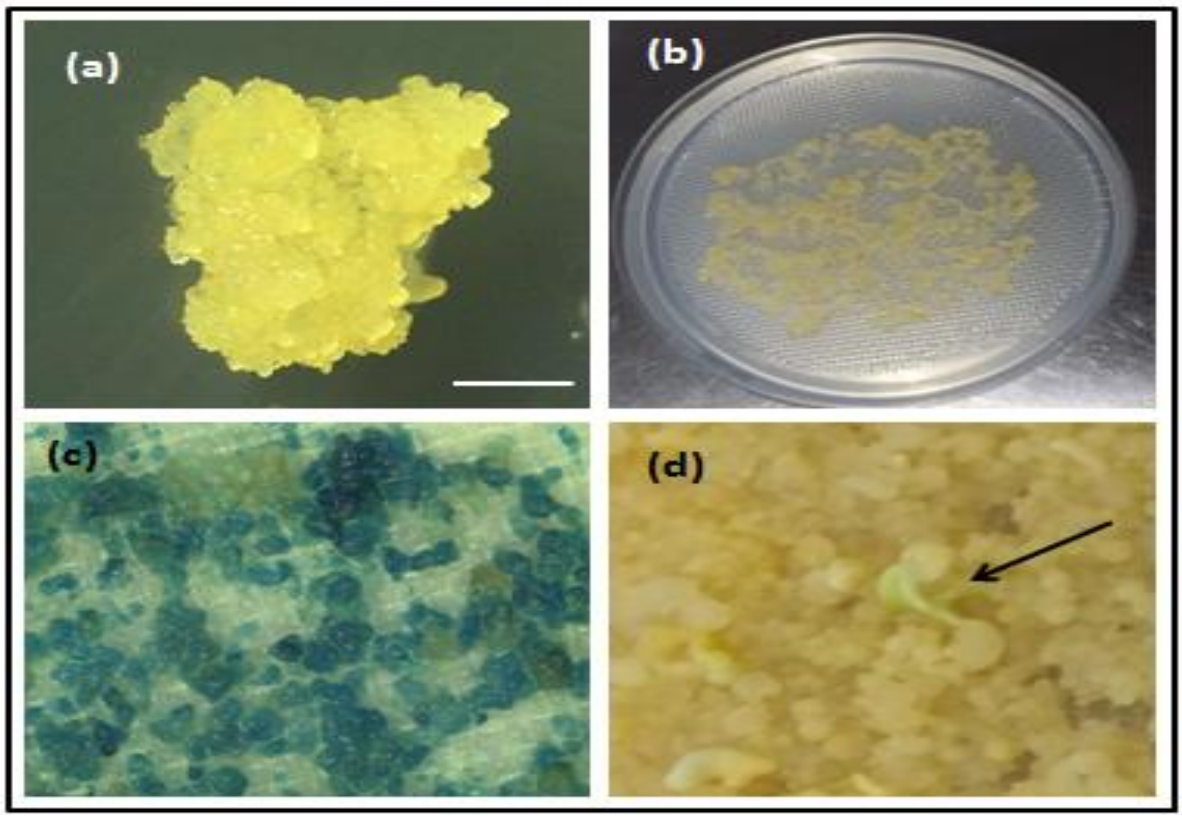


**Figure 4. 17** Steps in producing transgenic plants (a) T200 FECs chosen for transformation (b) FEC clumps following co-cultivation spread onto mesh on GD + C250 (c) Blue stained FEC after *GUSPLUS* assay (d) Small cotyledon (arrow) from untransformed T200 control cotyledon after 4 weeks on MSN+C250+H15

## 4.5. Discussion

The objective of this study was to design an inverted repeat IR construct to target ACMV-[NG:Ogo:90] more efficiently, but concomitantly also be able to target other ACMV genetic variants, EACMV and SACMV, since all these CBVs are widely spread in eastern and southern Africa. Resistance against the ACMV and the other two CBVs was designed through selection of an ACMV-[NG:Ogo:90] 117 nt putative promoter region, 91 nt ACMV-[NG:Ogo:90] AC1 3'/AC2 5'ter high siRNA producing overlapping region and a 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region. The second objective was to transform the construct into South African cassava landrace T200 FECs. T200 was chosen as it is an industry-prefered landrace with high starch content.

**Selection of target region for IR construction**

Sequence similarities between ACMV variants have been reported (Fauquet *et al*., 2007; Pita *et al*., 2001). Full DNA sequence variations have been reported for EACMV and EACMV variants (Fondong *et al*., 2000). Only two closely related variants or strains of SACMV have been reported from Zimbabwe (Briddon *et al*., 2003) and Madagascar (Harimalala *et al*., 2012). For this study it was important to target regions that would confir resistance to ACMV-[NG:Ogo:90]. We chose the CR in the intergenic region of DNA A/B as this is where the leftward promoter of AC1, TATA box as well as iterons resides (Henley-Bowdin *et al*., 1999). By inducing siRNA-mediated TGS of the promoter binding regions on the ACMV-[NG:Ogo:90] putative promoter region , it was hypothesised that transcription of the viruses would be impaired, leading to greater resistance. The unifying feature of geminiviruses, the invariant concensus nucleotide sequence TAATATTAC which makes up the stem loop structure of the common region. (Varma and Malathi, 2005) was identified. We then selected a 117 bp sequence from the 5'end of ACMV-[NG:Ogo:90] AC4 complementary sense ORF to the TATA box plus an additional 49 nt upstream with core elements of the leftward promoter.Vanderschuren *et al*. (2007) reported reduced symptoms and viral load when sequence corresponding to bidirectional promoter region and common region was used to transform cassava, and was also shown to target the promoter region in *Mungbean yellow mosaic virus*-infected mungbean (Pooggin *et al*., 2003). We expected this putative promoter region in our construct to induce the same resistance to CMD through TGS targeting the viral DNA. Transcriptional gene silencing has been shown to occur by methylation between similar regions of the promoter region and the target sequence (Xie *et al*., 2004; Wu *et al*., 2010).

For our second target region we required a highly conserved region (100% match) of ACMV-[NG:Ogo:90], SACMV and EACMV AC1 coding sequence. Geminivirus resistance has been reported in transgenic plants expressing dRNA IRs (hairpins) homologous to Rep/AC1 (Ammara *et al*., 2015; Fuentes *et al*., 2006; Vanderchuren *et al*., 2009; Ye *et al*., 2014). The AC1 gene codes for REP protein essential for replication (Vanitharani *et al*., 2005). Targeting a homologous region of AC1 would inevitably stop viral replication of the three CMGs. In our alignment results we identified a 21 nucleotide sequence with 100% sequence similarity between ACMV-[NG:Ogo:90], SACMV and EACMV. We predict that if this region could produce siRNA it would target the mRNA of the three CMGs offering broad spectrum resistance to the three CBVs. This still need to be tested in future experiments as transformation was not successful in this study.

The most widely used parameter for selection of target regions is the selection of siRNA hotspots on the viral ORF. Chellapan *et al*. (2004) reported that the ACMV-[CM] AC1 3'-ter/AC2 5'-ter overlapping region produces high levels of virus induced siRNAs. For our third target region we selected a 91 nt sequence (1530-1620 nt) of the same region as Chellapan *et al*. (2004) but this time on ACMV-[NG:Ogo:90] strain. We predicted that the chosen region would produce high levels of siRNAs as there was a 97% sequence similarity between ACMV Cameroon [CM] and ACMV-[NG:Ogo:90]. It is therefore most likely that an inverted repeat construct consisting of this AC1 3'/AC2 5' overlapping region would produce efficient siRNAs targeting ACMV-[NG:Ogo:90].

**Efficiency of siRNAs production**

We investigated the probable efficiency of our designed IR construct to produce siRNAs. Based on the siRNA scan (Xu *et al*., 2006) output, eight siRNAs were predicted for the ACMV-[NG:Ogo:90] putative promoter region and forty six siRNAs were predicted from the ACMV-[CM] AC1 3'-ter/AC2 5'-ter overlapping region. Our results clearly show that the ACMV-[CM] AC1 3'-ter/AC2 5'-ter overlapping region is a siRNA hotspot (**Table 4.3**). These result were expected in ACMV-[NG:Ogo:90] as Chellapan *et al*. (2004) had previously shown high levels of virus induced siRNA in ACMV-[CM] which is 97% similarity to ACMV [NG] (Fondong *et al*., 2000). High levels of siRNAs from introduced transgenes in plants have been reported to correlate with viral resistance, and threrefore it is predicted that the designed construct would be in this regard efficient in inducing resistance. Stacking of

three regions has not been reported in the literature to our knowledge, and testing this IR contstruct will prove interesting in further studies.

**Stability of the inverted repeat (IR) construct**

We designed our IR construct to produce a hairpin structure on expression in a host plant. To confirm the secondary structure of the IR, Mfold software (Zucker, 2003) (http://www.bioinfo.rpi.edu/applications/mfold) was used to predict the structure of the expressed IR. From our result a symmetrical haipin loop was predicted (**fig 4.8**). Theoretically this structure would be ideal to induce PTGS. In a plant cell a ribonuclease III like enzyme called Dicer recognises foreign double stranded RNA and cuts the double stranded RNA into small siRNA of between 21-26 nt therefore triggering post-transcriptional gene silencing (Baulcombe, 2004; Hammond *et al.*, 2000; Raja *et al*., 2010). In this study the IR construct was successfully cloned into pART7 expression vector, pCambia binary plant transformation vector as well as transformation into *A. tumefaciens*. In all cases the sense and antisense arms were successfully amplified and sequenced to confirm their presence in these vectors, thus proving that the IR was stable. Inverted repeat contructs are also reported to be more stable if smaller in size (Vanderschuren *et al*., 2009; Taha *et al*. 2016). The IR in this study was 229 nt per arm and contained a small intron (85 bp) (Vanderschuren *et al*., 2009), thereby increasing its predicted stability. The small size of IR constructs also helps to reduce the possibilities of off targets (Jackson *et al*., 2003; Pang *et al*., 1997).

**IR transformation into T200 FEC**

*Agrobacterium tumefaciens* harbouring the designed IR construct was used to transform T200 FECs. *GUSPLUS* assay was performed one week after transformation of the FECs. Our results showed blue colour staining of the FECs confirming successful transformation (**fig 4.7c**). Regeneration of transformed FECs was however unsuccessful. The seven plates of T200 FECs transformed with the IR construct as well as the *A. tumefaciens* only and pCambia (without IR) only controls also did not produce cotyledons. Untransformed T200 FECs produced cotyledons but these did not produce shoots. These results were unusual because other researchers from our laboratory, including the study by Chetty *et al*. (2013) reported transformation success using T200 FECs. The potential of the construct having undesirable negative effects on the regeneration of FECs was ruled out because of the failure by our T200 FECs transformed with the empty pCambia vector control to regenerate.

We hypothesise that the FECs used for the transformation were past their prime in terms of their age (number of transfers onto GD media). FECs had been transferred on 15 cycles (2 weeks per cycle) of GD media were used. (Vanderschuren, personal communication) highlighted that successful regeneration of model cultivar cv.60444 is possible if FECs kept on GD media for less than 20 cycles are used. However Nyaboga *et al*., 2015 recommended that cassava landraces need to be optimised as their regeneration conditions might be different to model cultivar cv.60444. It is possible that after 15 cycles on GD the FECs had lost regeneration potential. FEC quality determines transformation success and older FECs have a minimal potential to regenerate (Raemakers *et al*., 2001; Bull *et al*., 2009). The other reason that might have constributed to failure of our FECs to regenerate could be the observation that after 5 weeks on MSN H15 FECs started browning. Excessive browning of FECs is synonymus with production of phynolics due to stress (He *et al*., 2009) so it is possible that the FECs were stressed.

In conclusion we were able to successfully identify two suitable regions on the ACMV-[NG:Ogo:90] viral genome which we predict to offer resistance to ACMV, as well as identify a third region which could possibly target SACMV and EACMV as well, if transformed into cassava. The designed IR construct was stable, free from undesirable secondary structure and was a predicted siRNA hot spot. The IR construct was successfully transformed in T200 FECs, however the FECs could not regenerate, and we suspect that the FECs used were past their regeneration capacity. We recommend using fresh FECs for transformation of the designed IR into T200 cassava landrace.

# Chapter 5: Conclusions

This study aimed to evaluate selected cassava transgenic lines for resistance, tolerance or susceptibility to SACMV or ACMV. Cassava lines were derived from cassava cv.60444 transformed with a mismatched inverted repeat construct or non mismatched constructs derived from either ACMV-[NG:Ogo:90] AC1/4:AC2/3 transgene (CMM6 lines) or SACMV BC1 movement protein sequence (AMM2 lines). The study objective was also to design an inverted repeat construct consisting of viral sequence that would target ACMV-[NG:Ogo:90] as well as being efficient in targeting SACMV and EACMV, due to the inclusion of a 21 bp sequence conserved sequence between EACMV, ACMV and SACMV and to transform the construct into South African landrace T200. In order to evaluate CMM6 or AMM2 lines (cv.60444 transformed with a non-mismatched or mismatched ACMV-[NG:Ogo:90] AC1/4:AC2/3, respectively) the chosen lines were infected with ACMV infectious clones and evaluated for resistance or tolerance by evaluating the extent of symptom proliferation, viral multiplication and growth patterns. The same evaluation was done for CMM8 (cv.60444 transformed with a non mismatched BC1 sequence) and AMM4 lines (cv.60444 transformed with a mismatched BC1 sequence) but this time after infection with SACMV infectious clones. In summary, all transgenic lines showed reduced symptom scores and viral loads compared with infected untransformed cv.60444. From the ACMV infectivity trials we identified three lines **CMM6-2**, **CMM6-6** and **AMM2-52** which had significantly lower symptom scores and viral loads. These three lines were classified as being tolerant. The term tolerance and resistance have been subject to debate among cassava virologists. For this research we termed these three lines tolerant because they had mild symptoms due to low virus levels. This study, for the first time, showed that stacking of ACMV viral genes in an IR constructs is a viable method to induce tolerance against CBVs. To date in earlier studies, either mismatched or non mismatched IR constructs have been reported to induce CBV tolerance or resistance, but in this study a direct comparison of results was evaluated. A comparison between mismatched inverted repeat constructs or non mismatched constructs showed that both constructs are able to induce tolerance, and that slight differences in the level of induced viral alleviation were not significant. The construct targets were identical but the generation of siRNAs between mismatched and non-mismatched may have been different. It is possible that the quality and specific target of siRNAs produced rather than the quantity could be the key difference between tolerant and non-tolerant lines. Additionally,

differences might have been due to different IR integration stability in the plant genome. In terms of other studies on CBV resistance in cassava, this study is the first to explore the cell to cell movement protein in contrast to previous studies where significant levels of resistance have been reported in transgenic cassava expressing sense and antisense RNA of Rep, TrAP or REn proteins (Vanderschuren *et al*., 2009; Zheng *et al.*, 2005).

The significance of this study to farmers is that the tuber yield fresh and dry weight of these three transgenic lines was not significantly different from the yield of non-infected healthy cv.60444 highlighting that in these lines viral infection did not affect the yield therefore these tolerant lines would be beneficial to farmers. Recent GM food safety endorsements by NAS (2016) are likely to ease farmers and consumer fears on issues of surrounding GM foods. Cassava agro-processors are mostly interested in the tuber yield dry weigh. From this study, percentage dry weight of CMM6 and AMM2 transgenic lines were between 35-49% signifying a good yield according to literature. These results show that transgenic lines **CMM6-2**, **CMM6-6** and **AMM2-52** would be ideal for agro-processing. From the SACMV trials all transgenic lines had lower viral load and symptom severity scores compared with infected untransformed cv.60444 however none of the lines were tolerant (significantly lower sss and viral loads). Reduced viral load reported in the transgenic lines is reported in some cases to be linked to the production of transgene derived siRNAs which were not present in non-infected healthy cv.60444 prior to infection. Although all transgenic lines produced siRNAs only few were tolerant, and this further suggests that reduced viral load and symptoms is in part but not totally dependent on siRNA production. Contradictory results exist in the literature (Ntui *et al*., 2015; Vanderschuren *et al*., 2009) with regard to the correlation between siRNA and resistance, and further work needs to be done to learn more about this meolcular mechanism. Other factors such as the role of basal immunity-associated genes that might contribute to variations in the level of tolerance need to be further examined. The fact that the three tolerant transgenic CMM6 lines were transformed with silencing constructs targeting the ACMV-[NG:Ogo:90] DNA A region, and that none of the CMM8 lines targeting the SACMV cell-to-cell movement protein BC1 were tolerant, suggest that BC1 may not be a suitable target for virus reduction. Perhaps PTGS concommittantly targeting sites on both the AC1/Rep and BC1 ORFs may be more efficient. The siRNA scan *in silico* did predict compatible siRNA production to the target regions on both AC1 and BC1 ORFs, but perhaps this is not sufficient *in vivo*. One possible disadvantage of tolerance over resistance is that tolerant plants still harbour some viruses, albeit at low concentrations, and

these could be picked up by insect vectors. However studies on the correlation between viral load and vector transmission in the field would prove invaluable.

The IR construct designed from ACMV-[NG:Ogo:90] genome in chapter 4 was shown to be stable, free from formation of undesirable secondary structure and the sequence was chosen from a predicted siRNA hotspot. If the predicted siRNA from the 21 nt ACMV-[NG:Ogo:90], SACMV and EACMV AC1 conserved region efficiently bind to the viral mRNA, knockdown of the virus would be achieved resulting in high level of resistance to ACMV, SACMV and EACMV. The IR construct was successfully transformed in T200 FECs however, transformed FECs could not regenerate, and we suspect that the FECs used were past their regeneration capacity. For future work the use of fresh FECs is recommended for transformation of the designed IR construct into T200 cassava variety. Other possible technologies can be explored to improve cassava against CMD such as using endogenous cisgenes to genetically modify cassava. The use of endogenous genes reduces health and environmental concerns brought about by using exogenous genes such as those from viruses. The most promising new technology in crop improvement is the use of gene editing tools. In cassava identifying genes that are involved in resistance and either enhancing them or modifying them using CRISPR (gene editing) is worth exploring further.

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# Appendices

**Appendix A** Student t-test determining the significance of symptom severity, height and viral load between CMM6 lines and cv.60444s at 14, 36, 56 and 180dpi.

| | **Symptom severity score** | | | **Plant height** | | | **ACMV viral load** | |
|---|---|---|---|---|---|---|---|---|
| **Line ID** | **14 dpi** | **36 dpi** | **56 dpi** | **14 dpi** | **36 dpi** | **56 dpi** | **36 dpi** | **56dpi** |
| **CMM6-2** | 0,001259 | 0,00002486 | 0,00000129 | 0,00038 | 0,466606 | 0,090047 | 0.030282 | 0.04622 |
| **CMM6-3** | 0,010441 | 0,20889350 | 0,129786466 | 0,020578 | 0,156219 | 0,422745 | 0.28888 | 0.471875 |
| **CMM6-5** | 0,343372 | 0,055692794 | 0,134998144 | 0,002863 | 0,309185 | 0,141386 | 0.050609 | 0.217455 |
| **CMM6-6** | 0,010936 | 0,000124811 | 0,00000397 | 0,000181 | 0,221183 | 0,061022 | 0.030456 | 0.04625 |
| **CMM6-7** | 0,032308 | 0,299735155 | 0,457532144 | 0,061165 | 0,5 | 0,144466 | 0.137684 | 0.213042 |

**Appendix B** Student t-test determining the significance of symptom severity, height and viral load between AMM2 lines and cv.60444s at 14, 36, 56 and 360dpi.

| | **Symptom severity score** | | | **Plant height** | | | **ACMV viral load** | |
|---|---|---|---|---|---|---|---|---|
| **Line ID** | **14 dpi** | **36 dpi** | **56 dpi** | **14 dpi** | **36 dpi** | **56 dpi** | **36 dpi** | **56dpi** |
| **AMM2-30** | 0,014796 | 0,022093481 | 0,08697419 | 0,099127 | 0,255631 | 0,079753 | 0,088046 | 0,055434 |
| **AMM2-41** | 0,208894 | 0,274507219 | 0,0724638 | 0,300393 | 0,285371 | 0,434198 | 0,347369 | 0,034991 |
| **AMM2-44** | 0,005617 | 0,000731791 | 0,0109361 | 0,089034 | 0,193752 | 0,299735 | 0,487195 | 0,054868 |
| **AMM2-52** | 0,000597 | 0,0000171 | 0,000676392 | 0,033839 | 0,069634 | 0,210197 | 0,033817 | 0,032115 |
| **AMM2-53** | 0,299735 | 0,129786466 | 0,0724638 | 0,017164 | 0,063966 | 0,123417 | 0,055077 | 0,151964 |
| **AMM2-54** | 0,003657 | 0,005340745 | 0,170446566 | 0,138467 | 0,396628 | 0,339588 | 0,098866 | 0,092875 |

**Appendix C** Student t-test determining the significance of symptom severity, height and viral load between CMM8 lines and cv.60444s at 14, 36, 56 and 180 dpi.

| | Symptom severity score | | | Plant height | | | ACMV viral load | |
|---|---|---|---|---|---|---|---|---|
| **Line ID** | **14 dpi** | **36 dpi** | **56 dpi** | **14 dpi** | **36 dpi** | **56 dpi** | **36 dpi** | **56dpi** |
| **CMM8-1** | 0,024666 | 0,010936 | 0,001754 | 0,271625 | 0,321432 | 0,5 | 0,14958671 | 0,251861 |
| **CMM8-3** | 0,5 | 0,299735 | 0,104692 | 0,389807 | 0,171579 | 0,402012 | 0,126146045 | 0,265238 |
| **CMM8-8** | 0,223769 | 0,010936 | 0,024666 | 0,5 | 0,202439 | 0,429851 | 0,121471665 | 0,176172 |
| **CMM8-23** | 0,170447 | 0,010936 | 0,000597 | 0,196561 | 0,085829 | 0,429851 | 0,127349627 | 0,282849 |
| **CMM8-25** | 0,170447 | 0,032308 | 0,001754 | 0,389807 | 0,405817 | 0,151766 | 0,132830793 | 0,281329 |
| **CMM8-27** | 0,010936 | 0,299735 | 0,299735 | 0,394754 | 0,187143 | 0,258422 | 0,126353205 | 0,151745 |

**Appendix D** Student t-test determining the significance of symptom severity, height and viral load between AMM4 lines and cv.60444s at 14, 36, 56 and 180 dpi.

| | Symptom severity score | | | Plant height | | | ACMV viral load | |
|---|---|---|---|---|---|---|---|---|
| **Line ID** | **14 dpi** | **36 dpi** | **56 dpi** | **14 dpi** | **36 dpi** | **56 dpi** | **36 dpi** | **56dpi** |
| **AMM4-11** | 0,170447 | 0,036694 | 0,060925 | 0,390246 | 0,418809 | 0,340773 | 0,129398 | 0,163825 |
| **AMM4-33** | 0,170447 | 0,5 | 0,332126 | 0,154694 | 0,17759 | 0,5 | 0,09382 | 0,172586 |
| **AMM4-34** | 0,5 | 0,343372 | 0,104692 | 0,181609 | 0,267491 | 0,223769 | 0,178042 | 0,186922 |
| **AMM4-46** | 0,086974 | 0,170447 | 0,104692 | 0,085829 | 0,114332 | 0,429157 | 0,071899 | 0,161852 |
| **AMM4-59** | 0,086974 | 0,001547 | 0,010936 | 0,30233 | 0,34821 | 0,147968 | 0,129073 | 0,162443 |
| **AMM4-68** | 0,086974 | 0,3435 | 0,332126 | 0,327239 | 0,156895 | 0,274507 | 0,143557 | 0,163714 |
| **AMM4-79** | 0,086974 | 0,010936 | 0,003657 | 0,285038 | 0,200517 | 0,343372 | 0,355651 | 0,179601 |

**Appendix E** Student t-test determining the significance of the fresh yield weights obtained between CMM6, AMM2 lines and cv.60444s at 365 dpi.

| | **356 dpi** | | **365dpi** |
|---|---|---|---|
| **CMM6-2** | 0,462421 | **AMM2-30** | 0,025118 |
| **CMM6-3** | 0,016642 | **AMM2-41** | 0,187263 |
| **CMM6-5** | 0,029217 | **AMM2-44** | 0,032649 |
| **CMM6-6** | 0,112426 | **AMM2-52** | 0,189356 |
| **CMM6-7** | 0,031146 | **AMM2-53** | 0,011002 |
| **dsAC1** | 0,16862 | **AMM2-54** | 0,012567 |
| **cv.60444 infected** | 0,030934 | **dsAC1** | 0,368798 |
| | | **cv,60444 infected** | 0,00555 |

**Appendix F** Student t-test determining the significance of the viral load of CMM6, AMM2, CMM8 and AMM4 at 180 and 365 dpi against wild type cv.60444.

| | **Viral load 365 dpi** | | **Viral load 180dpi** |
|---|---|---|---|
| **Line ID** | | **Line ID** | |
| **CMM6-2** | 0,04695 | **CMM8-1** | 0,120483 |
| **CMM6-3** | 0,105003 | **CMM8-3** | 0,120995 |
| **CMM6-5** | 0,076487 | **CMM8-8** | 0,117442 |
| **CMM6-6** | 0,04695 | **CMM8-23** | 0,162973 |
| **CMM6-7** | 0,050662 | **CMM8-25** | 0,182527 |
| | | **CMM8-27** | 0,120053 |
| | | | |
| **AMM2-30** | 0,181238 | **AMM4-11** | 0,15842 |
| **AMM2-41** | 0,049044 | **AMM4-33** | 0,164136 |
| **AMM2-44** | 0,099615 | **AMM4-34** | 0,212393 |
| **AMM2-52** | 0,049012 | **AMM4-46** | 0,159105 |
| **AMM2-53** | 0,283499 | **AMM4-59** | 0,158834 |
| **AMM2-54** | 0,493423 | **AMM4-68** | 0,158694 |
| | | **AMM4-79** | 0.159005 |

**Appendix G** Pearson's correlation coefficient measuring the relationships (correlation) between our three test parameters, symptom severity score (sss), plant height, and viral load for A-MM2, A-MM4, C-MM6 and C-MM8, at 14, 36 and 56 dpi.

| **CMM6 lines** | **14 dpi** | **36 dpi** | **56 dpi** |
|---|---|---|---|
| sss and plant height | -0.5378 | -0.7969 | -0.7735 |
| viral load and sss | | 0.94016 | 0.95548 |
| viral load and height | | -0.5038 | -0.9441 |
| **AMM2 lines** | **14 dpi** | **36 dpi** | **56 dpi** |
| sss and plant height | 0.60186 | 0.46499 | 0.49515 |
| viral load and sss | | 0.25096 | 0.5955 |
| viral load and height | | 0.24617 | 0.60967 |
| **CMM8 lines** | **14 dpi** | **36 dpi** | **56 dpi** |
| sss and plant height | -0.10999 | 1.48522 | -0,10999 |
| viral load and sss | | -0.33485 | -0.73075 |
| viral load and height | | -0,55623 | 0.091651 |
| **AMM4 lines** | **14dpi** | **36 dpi** | **56dpi** |
| sss and height | -0.60805 | 0.57307 | -0.36148 |
| viral load and sss | | -0.35572 | -0.11643 |
| viral load and height | | -0.38741 | 0.02578 |