Assessing the role of DNA methylation in response of cassava to *South African cassava mosaic virus*

by

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Dissertation

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DECLARATION

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ABSTRACT

Manihot esculenta Crantz (cassava) is a root crop vegetatively propagated by stem cuttings and serves as a dietary staple for poverty alleviation in tropical America, Asia and Africa. The tuber crop feeds hundreds of millions worldwide and has been positioned as the third most staple carbohydrate source in Africa. As with all crops, cassava is faced with challenges such as the geminivirus South African cassava mosaic virus (SACMV) which leads to cassava mosaic disease (CMD) that results in substantial losses to production and consequently having a negative impact on the economy. Cassava landraces T200 and TME3 display susceptibility and tolerance to SACMV, respectively. Furthermore, TME3 displays a recovery phenotype. Plant hosts have developed strategies to defend themselves against invading geminiviruses by employing epigenetic modifications via DNA methylation. The first aim of this study was to compare the SACMV viral load and global methylation status of T200 and TME3 during symptomatic (32 dpi) and recovery (in TME3; 67 dpi) phases of SACMV infection using relative qPCR, ELISA and Liquid Chromatography Mass Spectrometry (LC-MS/MS), respectively. Infectivity results revealed that T200 plants had higher viral load than TME3 plants at 32 and 67 dpi. Global DNA methylation was higher in AGL1 control T200 plants at 32 and 67 dpi compared to TME3, suggesting that hypermethylation may be a hallmark of this landrace. This correlates with the global transcriptome results where a significantly higher number of genes are downregulated at 32 and 67 dpi in T200 compared to TME3. Global plant DNA methylation was significantly decreased in SACMV-inoculated T200 but marginally inTME3 at 32 and 67 dpi compared to AGL1-inoculated controls. This result suggests that SACMV targets/supresses methylation related genes/pathways to induce genes required for systemic spread and DNA replication. It is therefore concluded that hypomethylation is related to susceptibility. The second objective of the study was aimed at quantifying the expression levels of *MET1*, *CMT3*, *AGO4*, *SAM* and *AGO5* in T200 and TME3 at 32 and 67 dpi. Relative qPCR revealed that SACMV infection led to decreased expression of *MET1*, *CMT3*, *AGO4* and *SAM* in T200 and TME3 plants at 32 and 67 dpi compared to AGL-1, which correlated to hypomethylation observed in infected cassava. Interesting, the expression levels of *AGO5* only increased at 67 dpi in TME3 plants at 67 dpi, suggesting a role in transcriptional gene silencing (TGS) known to be associated with recovery to geminiviruses in several plant hosts. In summary, our results demonstrate that

SACMV causes a decrease in global DNA methylation in T200 and TME3 plants by decreasing *MET1*, *CMT3*, *AGO4*, and *SAM* expression leading to a decrease in *de novo* methylation. In conclusion, further analysis on the gene-specific methylation profile between T200 and TME3 landraces requires further study.

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ABBREVIATIONS

ACMV	*African cassava mosaic virus*
ADK	Adenosine kinase
AGO	Argonaute
BCTV	*Beet curly top virus*
CaMV	*Cauliflower mosaic virus*
CaLCuV	*Cabbage leaf curl virus*
CBSV	Cassava brown streak virus
CMBs	Cassava mosaic begomoviruses
CMD	Cassava mosaic disease
CMGs	Cassava mosaic geminiviruses
CCP	Coat core protein
CP	Coat protein
CR	Common region
DAFF	Department of Agriculture, Forestry &Fisheries
dC	Deocycytidine
DCL3	DICER-LIKE 3
DCL4	DICER-LIKE 4
DRDM	Domain rearranged DNA methyltransferase
DML	Demeter-like
Dpi	Days post inoculation
CMT 3	Chromomethylase 3
dsDNA	Double stranded DNA
EACMCV	*East African cassava mosaic Cameroon virus*

EACMCV-C	*East African cassava mosaic Cameroon virus-C*
EACMKV	*East African cassava mosaic Kenya virus*
EACMMV	*East African cassava mosaic Malawi virus*
EACMV	*East African cassava mosaic virus*
EACMV-U	*East African cassava mosaic virus-Uganda*
EACMZV	*East African cassava mosaic Zanzibar virus*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ETI	Effector triggered immunity
ET	Ethylene
FAO	Food and Agriculture Organization
GVs	Geminiviruses
HEN-1	Hua enhancer
hmC	Hydroxymethylcytosine
HRP	Horseradish peroxidase
ICMV	*Indian cassava mosaic virus*
ICMV	*Indian cassava mosaic virus*
ICTV	International Committee on Taxonomy of Viruses
JA	Jasmonic acid
MAMP	Microbe associated molecular pattern
MANE	Matched Annotation from NCBI and EMBL-EBI
mC	Methylcytosine
MET1	Methyltransferase 1
miRNA	Micro RNA
min	Minutes

mM	Millimolar
N/A	Not Applicable
nt	Nucleotide
NTC	Non-template control
nm	Nanometer
OD	Optical density
ORF	Opening reading frame
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PRR	Plant recognition receptors
PTGS	Post-transcriptional gene silencing
PTI	PAMP triggered response
qPCR	quantitative polymerase chain reaction
QqQ	Triple Quadrupole
R	Resistance
RCR	Rolling circle replication
RdDM	RNA-directed DNA methylation
RDR	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA-interference
SA	Salicylic acid
SACMV	*South Africa cassava mosaic virus*
SAM	S-adenosylmethionine
siRNA	Small interfering RNA
SLCMV	*Sri Lankan cassava mosaic virus*
SLCMV-I	*Sri Lankan cassava mosaic virus-India*

sRNA	small RNA
ssDNA	Single-stranded DNA
ssDNA	single-stranded DNA
TAE	Tris, acetate and EDTA
TBE	Tris, borate and EDTA
TE	Transposable elements
TGS	Transcriptional gene silencing
TME	Tropical *Manihot esculenta*
TYLCV	*Tomato yellow leaf curl virus*
UBQ10	Ubiquitin 10
U/V	Ultraviolet light
v/v	Volume per volume
vsRNA	Viral small RNA
w/v	Weight per volume

OUTPUTS FROM THIS STUDY

1. Rorisang Seutane, Chrissie Rey and Vanessa Meyer (2021) Assessing the role of DNA methylation in response of cassava to *South African cassava mosaic virus.* Oral presentation presented at SASM 2021 virtual conference, 4-6 May 2021

CHAPTER 1: LITERATURE REVIEW

1.1 *Manihot esculenta* and its uses

Manihot esculenta Crantz, native to South America and commonly known as cassava, is one of the ancient root and tuber crops utilized as dietary staple in tropical America, Africa, and Asia (Parmar *et al*., 2017). It is an important food security crop that can be cultivated in poor soil and low rainfall conditions and yield high production. It is regarded as the third most vital food crop; serving as a carbohydrate source for people living in the subtropics and tropics areas (Deepika *et al*., 2015). Cassava forms the basis of various products such as alcohol, flour, animal feed (Motielal *et al.*, 2017), within the food processing and chemical industries, starches in textiles, products for cosmetics and pharmaceuticals. This crop is useful in that both the tuberous root and the leaves (**Fig. 1.1**) can be consumed as a vegetable, while the stem can be used for propagation (Latif & Joachim, 2015.

Figure 1.1: Healthy leaves of cassava. Cassava leaves that were not inoculated.

Cultivation of cassava is favoured in tropical equatorial areas from 23,5°N to 23,5°S (Parmar et *al*., 2017; Fig. 1.2). To date, approximately 103 countries worldwide produce cassava. Approximately 50% of the global cassava cultivation takes places in Africa (Alabi *et al.*, 2011), as reported by the Food and Agriculture Organization (FAO) of the United Nations. The most prominent production of cassava in Africa takes place in Nigeria, with 31-60 million tons produced annually.

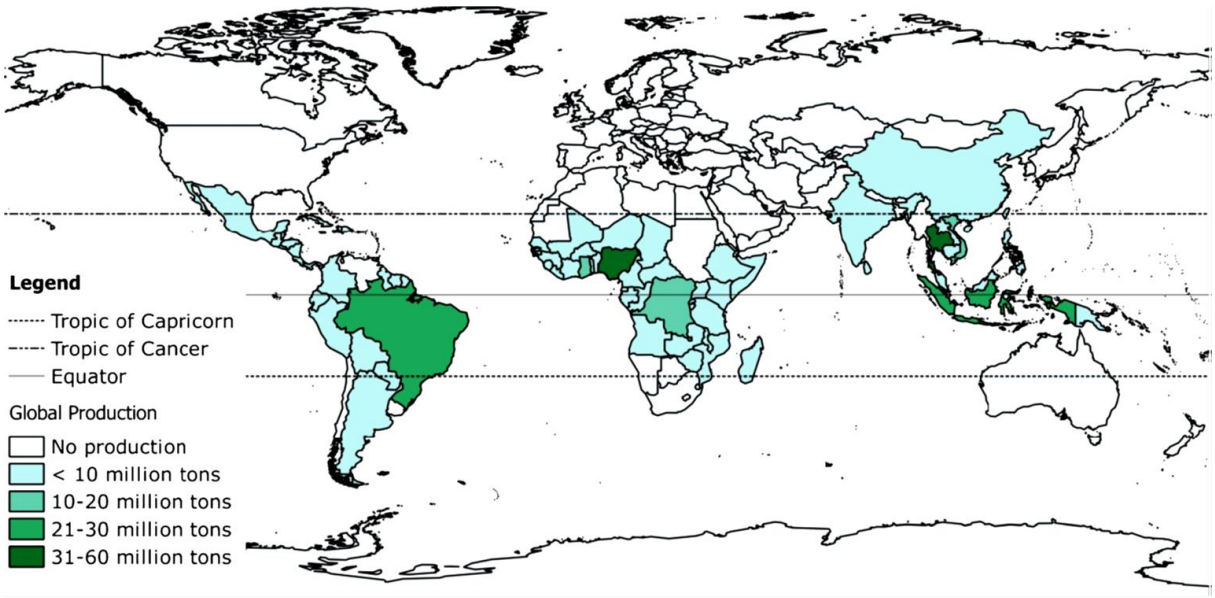

Figure 1.2: Global production of cassava by country. Casava production peaks in tropical equitorial regions (Taken from Parmar *et al.*, 2017).

1.2 Limitations of Cassava

Despite the fact that cassava is able to grow under limited conditions (such as poor soil and drought), the biggest challenge cassava is faced with is abiotic (cold, drought, salinity, UV radiation and heat) and biotic (pathogen infection and herbivores) stressors *(*Arikan *et al.,* 2018). Successful cultivation of cassava is influenced by the magnitude of insect infestations, parasitic nematodes, bacterial and viral attacks experienced by the plant. Various diseases are responsible for affecting cassava in the field. The two major viral diseases that pose the biggest threat in production is Cassava brown streak-mosaic disease (CBSD) (CMD). CMD is caused by whitefly-vector transmitted geminiviruses including the African cassava mosaic virus (ACMV), South African cassava mosaic virus (SACMV;Fig 1.2) and East African cassava mosaic virus (EACMV) (Berrie *et al.*, 1998; Rey & Vanderschuren, 2017), of which ACMV accounts for a third of the total crop loss in Africa.

Figure 1.3: Cassava leaves infected with South African cassava mosaic virus. Symptoms of yellow and pale green chloratic mosaic,size reduction and distortion can be observed as indicated by the arrows.

1.3 Cassava mosaic geminiviruses (CMG)

Plants viruses mainly have RNA genomes, however nanoviruses and geminiviruses have DNA genomes. Plant infecting geminiviruses are the largest and important DNA viruses and they have single-stranded DNA (ssDNA) which are made up of either monopartite (DNA A) or bipartite (DNA A and DNA B) genomes (Borah *et al*., 2016). The ssDNA structure is stabilized by protein-DNA interactions, wherein each genome is encapsulated into a twin icosahedral particle. Geminiviruses are responsible for causing disease in many crops including monocotyledonous and dicotyledonous plants.

1.3.1 Taxonomy

Currently there are nine genera that make up the family *Geminiviridae*, which are constructed according to nucleotide sequence similarities and genome organization. These include *Begomovirus, Grablovirus, Capulovirus, Eragovirus, Curtovirus, Becurtovirus, Topucovirus, Mastrevirus* and *Turncurtovirus* (Varsani *et al*., 2017). Of these nine genera, *Begomovirus* is the most prevalent. Begomoviruses are transmitted by whitefly (*Bemisia tabaci)* tand are detrimental to cassava as well as other vegetable and ornamental plants (Czosnek *et al.*, 2017). There are seven begomovirus species that causeCMD in Africa and two CMD species from India including Sri Lankan cassava mosaic virus and Indian cassava mosaic virusv(*S*LCMV and ICMV) (**Fig. 1.4**). Additionally there are several distinct variants or strains that have been recognised (Brown *et al*., 2015). Despite the widespread cultivation of cassava, CMD has only been evident in Africa and on the Indian subcontinent (Fargette *et*

al., 2006). The most common feature among viruses that belong in the nine genera is that these pathogens mostly contribute to the economic loss of herbaceous plants and weed species in the world (Rwahnih *et al*., 2018).

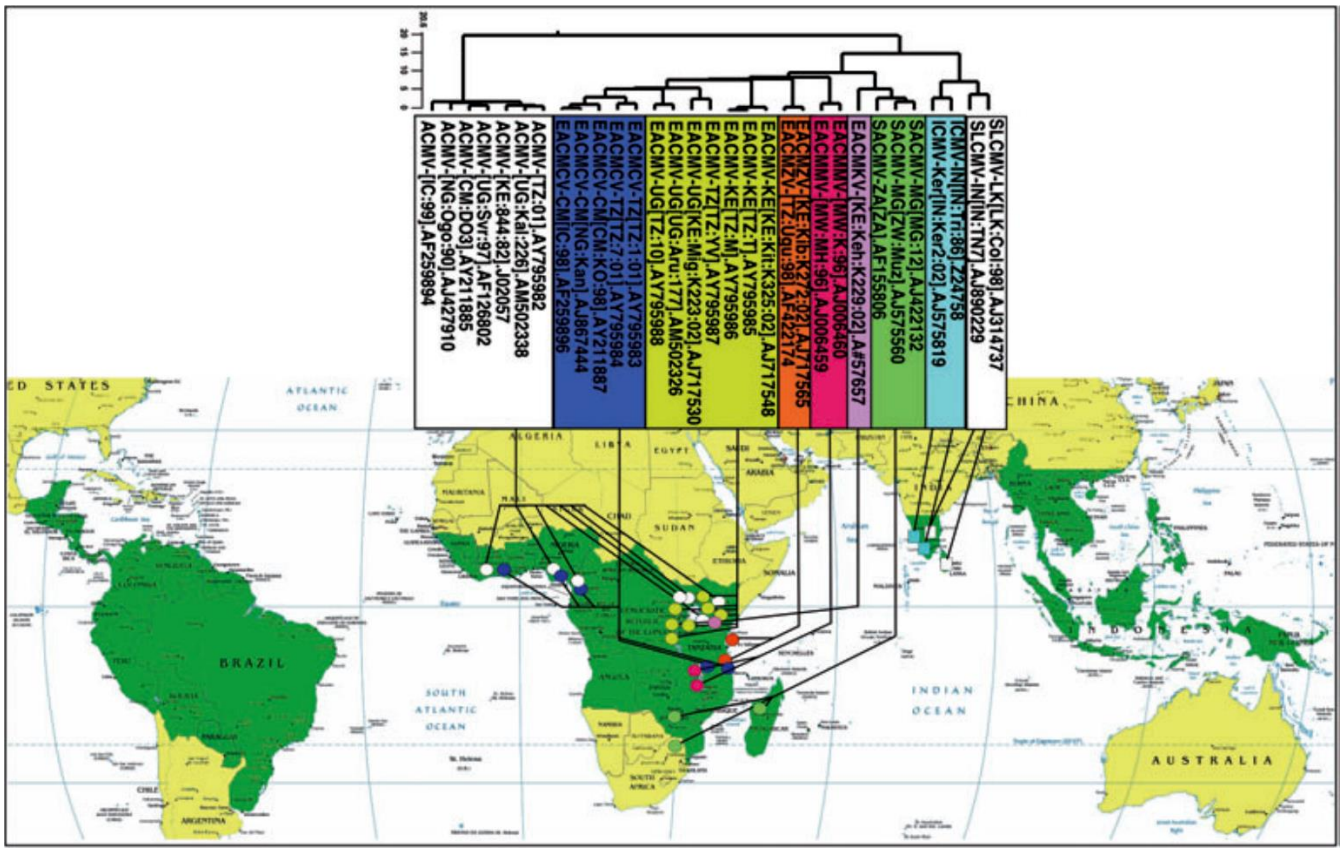

Figure 1.4: Geographical distribution of cassava mosaic geminiviruses (CMG). The map shows the distribution of CMG on the Indian subcontinent and Africa (Patil *et al*., 2009).

1.3.2 Genome structure and organisation

CMGs have a germinate particle (30 x 20 nm) and are bipartite. The size of each component (DNA-A and DNA-B) is about 2.8 kb. The DNA-A component is able to replicate independently and yield virions while the DNA-B component is vital for systemic infection (Saeed *et al.*, 2007). Both DNA components are required for systemic infection. The two components share a mutual area which encompasses a sequence of ~200 nt. Within the shared region there is a well-preserved stem-loop structure, governing elements such as the nonanucleotide TAATATTA↓AC sequence that denotes the origin of virion-strand DNA replication (Lazarowitz, 1992), the TATA box, as well as the iterons which act as binding sites (Alabi *et al.,* 2011). There are two overlapping virion-sense open reading frames (ORFs)

namely coat protein (AV1) and (pre-coat protein) AV2 that are encoded by DNA-A, as well as four overlapping complementary-sense ORFs namely, replicationassociated initiator protein (Rep) (AC1), transcriptional activator protein (TrAP) (AC2), replication enhancer protein (REn) (AC3) and RNA-silencing suppressor protein (AC4) (**Fig. 1.5**). DNA-B encodes two proteins including one virion sense called nuclear shuttle protein (NSP) (BV1) and one complementary sense cell-to-cell movement protein (MP) (BC1) which are necessary for virus translocation (Raghavan *et al.*, 2004).

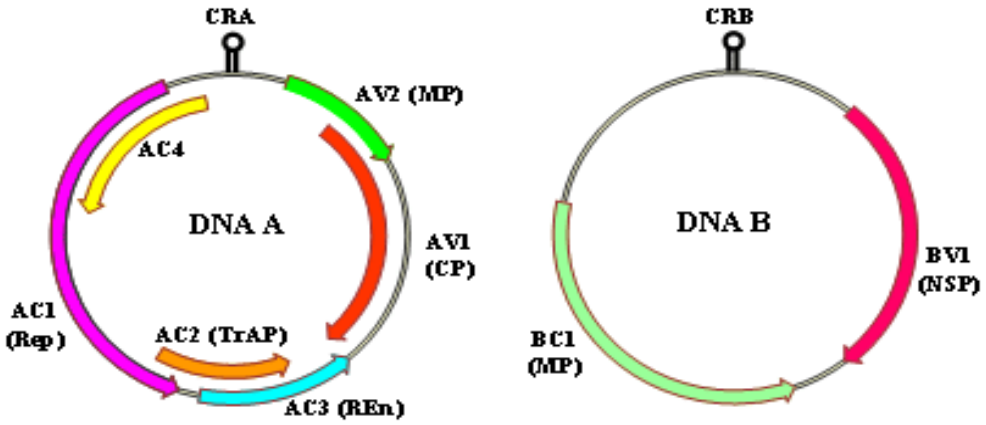

Figure 1.5: Genome organisation of bipartite DNA-A and DNA-B of begomovirus. Open reading frames (ORFs) and the directions in which transcription takes place. DNA-A is made up of six ORFs, four are in the complementary sense which are the AC1, AC2, AC3 and AC4 and two which are in the virion sense, namely AV1 and AV2. DNA-B is made up of two ORFs which is the complementary sense BC1 and the virion-sense BV1 (Taken from Alabi *et al.*, 2011).

1.3.3 Infection cycle in the host

The infection cycle of begomovirus begins in the phloem of cells of a host plant after transmission by *Bemisia tabaci* Gennadius. Transmission can also occur vegetatively through stem cuttings or grafting of infected budwood to healthy cassava plants. Once the viral genome enters the cell, it reaches the nucleus, and the replication cycle begins. The replication cycle can be separated into three phases. The first phase is denoted by the conversion of the genomic ssDNA into dsDNA, which is a supercoiled and covalently-closed circular intermediate (Hanley-Bowdoin *et al.*, 2013) forming mini-chromosomes by associating with histones. This is then followed by amplification of the viral ssDNA by a rolling circle replication (RCR) mechanism where the initiator protein, Rep, is necessary for the insertions of an endonucleolytic nick (↓) that is within the nonanucleotide sequence

TAATATTA↓AC of the intergenic regions of DNA-A or -B (Gutierrez, 2000). Then genomic ssDNA or dsDNA is transported to neighbouring cells through plasmodesmata where it travels to the nucleus. Geminiviruses can also thereafter be encapsulated to form mature viral particles but do not need assembled virions to move. The replication mechanism of begomoviruses is solely dependent on the DNA replication mechanism of the host (**Fig. 1.6**).

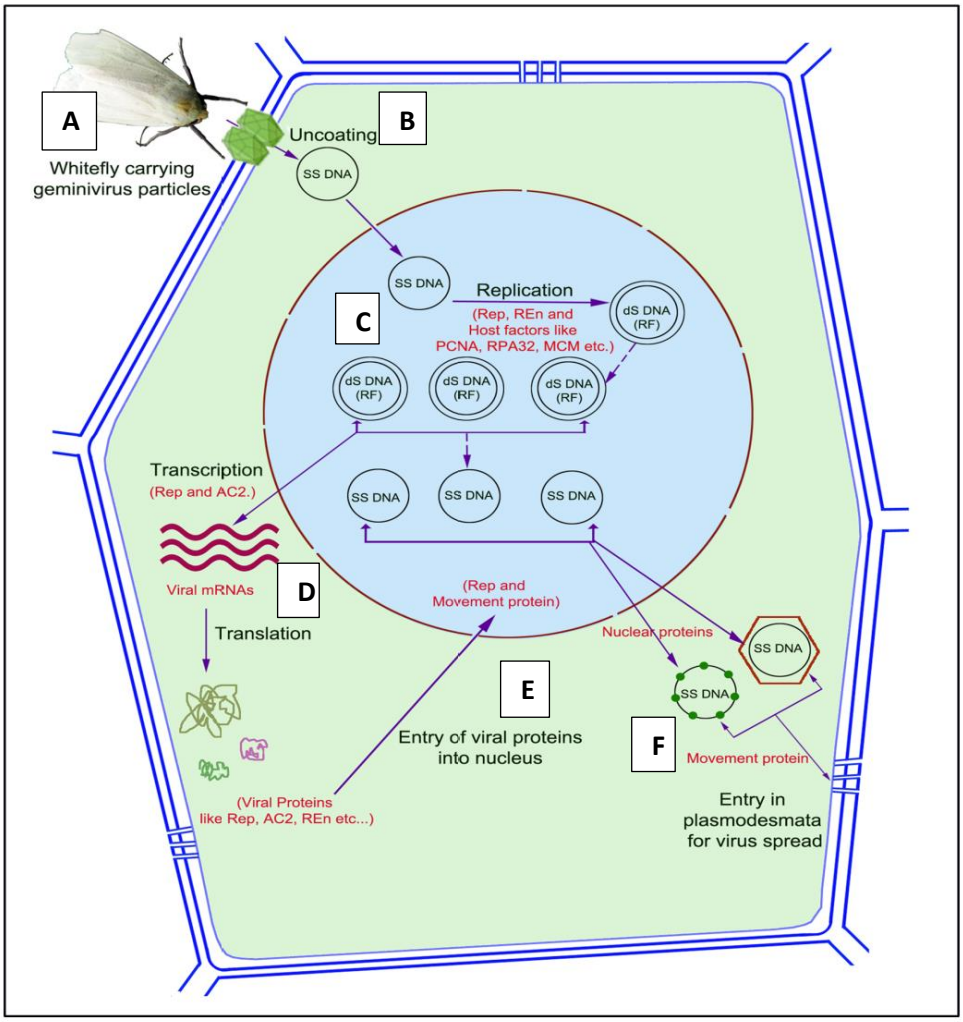

Figure 1.6: Overview of steps involved in the lifecycle of geminivirus. (a) Whitefly transmitting vector carrying geminivirus particles. **(b)** Firstly, uncoating takes place, the viral single stranded DNA (ssDNA) is released to the cells of the infected cytoplasm thereafter the viral DNA is carried to the nucleus to carry out amplification. **(c)** The ssDNA is converted subsequently to double stranded (dsDNA) by making use of the host cell system. **(d)** Transcription occurs in the nucleus as a result of the association of viral DNA with the nuclear proteins and thereafter translation takes place in the cytoplasm resulting to various viral proteins. **(e)** This is followed by the movement of the viral proteins into the nucleus that is needed to carry out transcription and replication. **(f)** Nuclear

proteins and viral DNA enter into neighbouring cells via the plasmodesmata with the assistance of movement proteins causing a progressive virus spread (Taken from Pradhan *et al*., 2017).

1.3.4 South African cassava mosaic virus (SACMV)

South African cassava mosaic virus is present in South Africa, Madagascar, Zimbabwe and Swaziland (Briddon *et al*., 2004; Rey *et al*., 2012). Originally it was recognised as an ACMV isolate in the eastern region of South Africa (Berrie *et al*., 1997; Rey *et al*., 2001). Its bipartite genome comprises of 2 800 nt and 2 760 nt making up DNA-A and DNA-B, respectively (Berrie *et al*., 2001). There is a close relationship between South African cassava mosaic virus (SACMV) and East African cassava mosaic virus (EACMV) in genomic components, with the DNA-A component displaying 85% similarity with EACMV-MH and MK, and the DNA-B component displaying 90% similarity with EACMV-UG2-Mld and EACMVUG3. Nonetheless, SACMV is considered a distinct virus species due to the sequence similarity in DNA-A component being lessthan 90% (Brown *et al*., 2015).

1.4 Plant immunity or defense to viruses

Plants are challenged by various pathogens, and they are constantly defending themselves against attacks. To defend themselves, plants have developed intricate mechanisms to recognize and distinguish pathogens. The first line of defence in plants which initiates immunity signalling is the specific microbe and pathogen-associated molecular pattern (MAMPs and PAMPs) collectively known as the PAMP-triggered immunity (Zipfel, 2014). This defense mechanism has however not been identified for virus infections. In the past years substantial progress in gathering information on the mechanisms of virus resistance in plants has been made, which include understanding the role resistance (R) protein-associated effector triggered immunity(ETI) and RNA silencing play in defence (Rey & Fondong, 2018). RNA silencing or interference (RNAi) is an innate immune mechanism which is triggered by dsRNA derived from viruses, by multiple ways. RNAi can target the virus either by targeting its mRNA , known as post transcriptional gene silencing (PTGS), or in the event of DNA viruses by siRNA-mediated DNA silencing of the DNA genome, known as transcriptional gene silencing (TGS) (Matzke and Mosher,2014). Geminiviruses, as ssDNA

viruses are targeted by both PTGS and TGS (Ramesh *et al*., 2017)**.** However, these viruses counteract these defense mechanisms to carry out infection.

1.4.1 Pattern-effector triggered immunity

The first line of defence in plants is associated with recognition receptors (PRRs) such as receptor-like kinases and receptor-like proteins that recognises pathogen and/or microbial associated molecular patterns (PAMPs/MAMPs) on the plant cell exterior (Deleris *et al.*, 2016, Bart *et al*., 2012). Pattern-triggered immunity (PTI) is involved in defense against non-viral pathogens such as fungi and bacteria. Upon infection, the pathogens primary objective is to compromise the immunity of the plant by employing effector proteins with the intention to suppress PTI thus allowing pathogen proliferation. This state is termed effector-triggered susceptibility (ETS) (Hatsugai *et al*., 2017), which is recognised by resistance (R) gene proteins. Effector-triggered immunity (ETI) is then triggered by the response of R proteins. As a consequence of ETI the signalling cascade leads to hypersensitive response (HR) and programmed cell death (PCD). The HR leads to activation of a prolonged memory-dependant Systemic Acquired Resistance (SAR) in distal healthy tissues (Bart *et al*., 2012). The balance between salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) is important because they are involved in regulating particular pathogen-related genes involved in triggering SAR.

The common aspect in PTI and ETI is that calcium signalling is required. Once the pathogens have been recognized, calcium ions are the earliest responses thus activating downstream responses. (Gao *et al*.,2014; Stael *et al*., 2015; Kruijt *et al*., 2005).

PTI has been studied to be triggered by Arabidposis LRR protein (Pruitt *et al*., 2021)tThe link between the interaction of between R proteins which contain nucleotide-binding site domain and a leucine-rich repeat domain (NBS-LRR) and other immunity related genes (IRG) as a possibility in understanding the mechanism of tolerance and susceptibility (Louis & Rey, 2015) (.Louis & Rey (2015) identified upregulation of several NBS domain-LRR genes in response to SACMV, and their potential influence on plant tolerance.

1.4.2 Post transcriptional gene silencing (PTGS)

Observations of gene silencing were first discovered in transgenenic petunia plants expressing a flavonoid gene which is responsible the color of the flowers called the chalcone synthase (CHS) gene (Napoli *et al*., 1990). PTGS is a natural RNA-mediated defense mechanism that is triggered by invading viral RNAs. RNA transgenes are inserted into what? to produce double stranded RNA (dsRNA). PTGS takes place in the cytosol and degrades sequence-specific mRNA. This silencing mechanism is observed in animals and fungi and commonly referred to as RNA interference(RNAi) and quelling, respectively. The process of PTGS takes places in two different steps, the initial step involves the formation of dsRNA and the effector step leads to mRNA degradation (Ashfaq *et al*., 2020). PTGS is triggered by dsRNAs which are cleaved into smaller RNA classes, siRNA and miRNA. miRNAs are initiated internally by non-coding RNAs, they form imperfect fold-backs, and they play a vital role post transcription in controlling gene expression via mRNA degradation or translation repression(Cai *et al*., 2009). siRNAs are products of intermediates of viral replication or through or from the action of RNA-dependent RNA polymerase (RDR) on ssRNAs (Ossowski *et al*., 2008). The process of exogenous RNA degradation to produce small RNAs (sRNAs) relies on specific RNA nucleases in plants. Firstly, RNAse III-like protein, called Dicer, cleaves dsDNA and pre-microRNA into short dsRNA fragments and then loaded into an RNA-induced silencing complex (RISC) (Amarzguioui *et al*., 2003). siRNAs are the most diverse and abundant classes of small RNAs species in plants (Pooggin, 2013). Small RNA silencing products produced by cytoplasmic RNA nucleases can range from 21-22 nucleotides (nt) to 24-26 nt depending on the mechanism of inactivation. For instance, siRNAs are classes of 21-22 nt sequences for transcripts inactivation, while 24-26 nt sequences are used during TGS (Ossowski *et al*., 2008). In addition, siRNAs produced *in vitro* are more specific to viral target transcripts and induce efficient silencing as compared to those produced in *vivo* which can be nonspecific (Amarzguioni *et al*., 2005). microRNAs are involved in the regulation of plant growth, development and environmental challenges (abiotic and biotic stressors)(Wang *et al*., 2019).

1.4.3 Transcriptional gene silencing (TGS)

Transcriptional gene silencing (TGS) is another defence mechanism against DNA viruses (Ramirez-Prado *et al.,*2018). TGS depends on methylation of viral DNA sequences to impede transcription suggesting that this innate immunity is exclusive to DNA viruses (Veluthambi *et al.,* 2003). Biogenesis of small RNAs is conditional, however both miRNA and siRNA are produced by cleavage of long dsRNAs with Dicer RNAse III-like protein into 21-24 nt molecules of siRNAs and miRNAs followed by loading onto RNA-induced silencing (RISC)/Argonaute (AGO) complex for cleavage of targeted mRNA transcripts (Amarzguioui *et al*., 2003; Brosseau & Moffett, 2015). Interestingly, the produced small RNAs are characterised by their enclosed 2 nt 3' overhangs. Nevertheless, both small classes of small RNAs undergo similar processing mechanism of dicing and there a vast difference in their biogenesis. It was previously reported by Maghuly *et al*., (2014) that EACMV may produce miRNAs that target the genome of plants however the mechanism is still to be proven. Additionally, silencing transitivity mechanism is another way in which RDR can amplify siRNAs, where the cleaved products of AGO/RISC complex are converted into dsRNAs and processed to form secondary siRNAs that can travel systemically throughout the plant (Duan *et al*., 2012).The key point to take into consideration is while siRNA require perfect complementarity to stimulate transcript cleavage, incompatibilities of up to five nucleotides can still stimulate transcript cleavage with miRNA to prevent RNA synthesis (Sharma *et al*., 2015).

1.4.3 Viral defence mechanisms

Viruses counteract host RNA silencing defence response. To do thist, viruses have evolved antiviral RNA silencing suppressor genes, whose products interfere with the host RNA silencing mechanism (Sharma *et al*., 2015). These suppressors operate at various levels, by either directly interfering with Dicer, RISC and AGO proteins or indirectly by binding to siRNA duplexes (Duan *et al*., 2012). One of the targeted pathways by viruses is DNA methylation because it is an epigenetic defence mechanisms in which plants use to defend themselves against invading DNAs such as geminiviruses. RNA-directed DNA-methylation (RdDM) is a process by which transcriptional gene silencing (TGS) occurs. This process includes the activity of DCL3 that results to 24-nucleotide (nt) siRNAs, which are

subsequently methylated at the 3' end by Hua enhancer (HEN1). A silencing effector complex forms when the methylated siRNA is loaded onto AGO4 which then targets promoters or gene bodies, preventing transcription (Fondong, 2017). TGS is also critical in defence against DNA viruses by targeting the histones that form part of the DNA geminiviral minichromosomes which form in the nucleus (Ramirez-Prado *et al*., 2018; Saze *et al*., 2012). Replication of geminiviruses takes place in plant cell nuclei, and because geminiviruses have ssDNA they use an intermediate dsDNA template to replicate via the rolling circle mechanism (RCA) in the plant host (Paprotka *et al.,* 2011). Minichromosomes form because of the association of dsDNA intermediated with cellular histone proteins. Geminiviruses encodes for suppressor protein AL2 and L2 that negate the defence of RNA silencing by inhibiting adenosine kinase (ADK) (Rodríguez-Negrete *et al*., 2013). The methylation-based defence of geminiviruses is therefore negated by protein AL2 and L2, as the methyl group donor S-adenosyl methionine (SAM) requires ADK (Raja *et al.,* 2008). Thus the possible host defence mechanism to counter balance the action by AL2 and L2 protein is histone methylation of the viral minichromosomes (Deuschle *et al.*, 2016). An example of methylation of geminiviruses was demonstrated by Raja *et al.,* (2008) where *Arabidopsis* plant mutants were hypersusceptible after infection with geminiviruses *Cabbage leaf curl virus* (CaLCuV) and *Beet curly top virus* (BCTV) because they lacked methylation pathway components such as met 1, cmt3, drm2, drm1, kyp2, nrpd2A, ddm1, ago4, adk2 and adk1 (Raja *et al.*, 2008).

Epigenetic defence mechanisms such as RdDM of cytosine residues and histone H3 lysine 9 dimethylation (H3K9me2) of chromatin are important characteristics that plants have evolved to protect themselves against invading transposons and DNA including geminiviruses. The outcome of infection and symptom remission is determined by the balance between active and repressed viral chromatin (Ojolo *et al*., 2018; Veluthambi and Sunitha, 2021). Host recovery is closely linked to the equilibrium favouring repressed state (Ceniceros-Ojeda *et al*., 2016; Coursey *et al*., 2018).

1.4.4 Recovery

Recovery is regarded as the initial severe symptomatic infection of the host that gradually becomes less on newly developing leaves (Jovel *et al*., 2007). The phenotypic observation of the host appears almost symptomless and there was a correlation found between decreased virus titers and SACMV DNA A and B (Ghoshal & Sanfaçon, 2015). In geminiviruses, TGS has also been associated with recovery. Pepper golden mosaic virus (PepGMV)-infected pepper plants show a recovery phenotype associated with the presence of virus-derived small RNAs, The results suggesting that PepGMV is targeted by both posttranscriptional and transcriptional gene silencing mechanisms (Rodriguez-Negrete *et al*., 2009 This is an advantage in crops because the host becomes tolerant to diseases such as CMD. Tolerance to SACMV has been demonstrated in the West African cassava landrace TME3 (Allie *et al*., 2014), and also several other geminivirus infections.

1.5 Epigenetics

In the early 1940's the term epigenetics was defined by Conrad Waddington as "causal interactions between genes and their products, which bring the phenotype into being", where its molecular basis was poorly understood (Waddington, 1942). Presently, epigenetics is referred to as a change in gene expression, independent of DNA sequence, but associated with DNA methylation, histone modifications and small RNA-mediated methylation (Yadav *et al.,* 2018).

1.5.1 DNA methylation

DNA methylation plays a significant role in gene expression of physiological processes involved in growing and developing plants. DNA methylation is the establishment of 5-methylcytosine (5mC) by addition of a methyl group on the C_5 position (Bartels *et al*., 2018). The occurrence of heritable C-methylation patterns in evolutionary divergent species indicates the importance of cytosine methylation in the evolution of plant species (Hafiz *et al*., 2001). As a mechanism for epigenetics, DNA methylation regulates gene expression, genome stability, transposable element (TE) silencing, and genomic imprinting (Zhu *et al*., 2009; Bartels *et al*., 2018). DNA methylation is essential for many biological developments, with aberrant DNA methylation patterns related with plant failure in fruit ripening and root nodulation in tomato plants (Zhang *et al*., 2018). It also regulates the plant response to

environmental factors such as abiotic and biotic stress (Zhang *et al*., 2018). For example, Xue *et al*., (2017) disclosed that locus-specific DNA methylation and demethylation events were induced by cold stress in cassava South China cultivar 8. DNA methylation in plants ensues on all cytosine residues in the sequence context CHG, CHH and CG (H=A,C or T), unlike in mammals where it mostly takes place in symmetric CG context however non-CG methylation does take place in embryonic stem cells and neuronal cells (Lee *et al*.,2017). Although all three forms have been observed, CHH is definitely rare across most species, but CHG methylation is fairly common in some gymnosperms. For angiosperms, CG methylation is the most common (Takuno *et al*., 2016). Sequence-specific methylation is established by different enzyme classes of DNA methyltransferase; however the catalysis of DNA methylation is done DNA by employing S-adenosyl-1-methionine which is the methyl donor (Zhang *et al.,* 2021). Methyltransferase 1 (MET-1) mediates CG methylation, while CHH methylation is mediated by domain rearranged DNA methyltransferase 1 (DRDM1), and CHG methylation is facilitated by plant-specific chromomethylase 3 (CMT3) (Rabinowicz *et al*., 2003). Therefore single genomic regions depicts DNA methylation patterns of co-operative or competing interactions of the three methylatranferases, as well as the silencing pathway they function in (Meyer, 2011). In *Arabidopsis thaliana*, enzymes such as chromomethylase 3 (CMT3) and methyltransferase 1 (MET1) are involved in maintaining DNA methylation. Another enzyme that is involved is domains rearranged methyltransferase 2 (DRM2) which is accountable for the methylation of CHH (Zhong *et al*., 2014). However, methylation in all contexts is mediated via RNA-directed RNA methylation using DRM2. This pathway has two stages which involve biogenesis of siRNAs and a methylation targeting stage. Plant development also requires active demethylation. This process is facilitated by repressor of silencing 1 (ROS1), Demeter (DME), Demeter-like 3 (DML3) and Demeter-like 2 (DML2) (Li *et al*., 2018). To complete this process, the family of glycolases is accompanied by base excision repair (BER)-dependent mechanism (Penterman *et al*., 2007; Zhu 2009).The response to biotic stress in plants has been observed with regards to dynamic DNA methylation and demethylation (Dowen *et al*., 2012; Deng *et al*., 2018; Chinnusamy *et al*., 2014). For example, the rate of infection by bacterial pathogens has been allied with a decline in global DNA methylation levels (Pavet *et al.*, 2006). Immune response regulation in plants is associated with RNA and DNA methylation, histone post-transcriptional

modifications and chromatin remodelling (Tariq and Paszkowski, 2004; Castellano *et al*.,2016).

1.5.2 Gene regulation

Gene-associated methylation takes place in the promoter region and this DNA methylation normally inhibits gene transcription, while in other cases it promotes gene transcription. Gene expression is a consequence of recruited proteins that are involved in gene expression or the inhibition of binding of transcription factors to DNA; this is regulated by DNA methylation (Zhang *et al*., 2018; Li *et al.*, 2021).

1.5.2.1 RNA-directed DNA methylation

TGS is an outcome of steady suppression of transcription and mainly affects chromosomal repeats, transposons, and transgenic inserts. Studies have also found the interaction between RNAi and the formation of heterochromatin as a trigger for TGS (Martienssen and Moazed, 2015; Nishimura *et al*., 2012). The *cis* conformation of TGS has been believed to be the reason behind the development of secondary DNA structures that draw methylation and heterochromatin components (Vaucheret and Fagard, 2001). Protein binding is induced by DNA methylation as this organizes the methylated cytosine thus remodelling the chromatin (Alberts *et al.,* 2002). In TGS the virus genome replication or transcription of genes associated with methylated promoters are inhibited by promoter methylation (Sharma and Ikegami, 2008), suggesting that the virus is required to retain unmethylated DNA in infected cells. The plant host uses siRNA-directed *de novo* methylation as a defence mechanism to silence the genes of DNA viruses (**Fig. 1.7**).

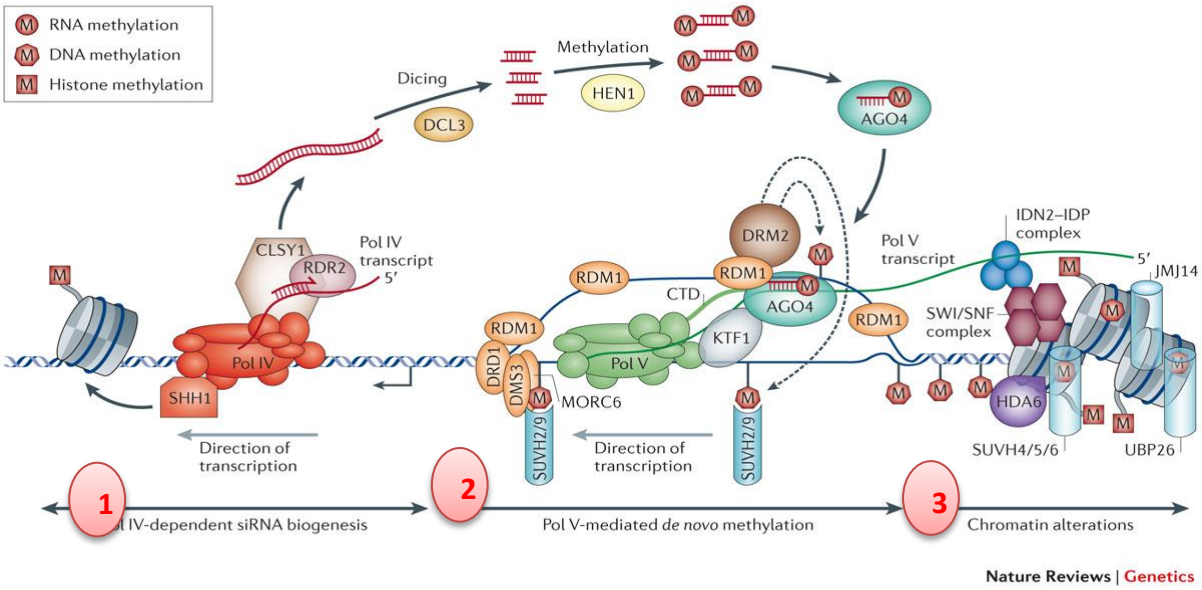

Figure 1.7: The order of RdDM pathway. The figure depicts the typical pathway for RNA-directed DNA methylation**. 1.** The step is the RNA polymerase (Pol IV)-dependent biosynthesis of siRNA 2. The second stage involves Pol V *de novo* methylation **3.** Lastly chromatin alterations are made by adjusting the SWI/SNF complex (Taken from Matzke and Mosher, 2014).

1.5.2.2 siRNA-directed methylation

Small RNAs are non-coding RNA molecules that are roughly 20-30 nt long and are involved in RNA silencing. In *Arabidopsis*, rice and moss, miRNAs direct DNA methylation (Jia *et al*., 2011). DCL3 generates these miRNAs (20-22 nt) that associates with AGO4 protein. DNA methylation can be guided at their individual gene region in *cis* and in *trans* by 23 - 27 MIR-derived species which may sometimes result in TGS. There are two distinct classes of sRNAs in plants that vary in size and functionality. The first being a class of 21-22 nt that is involved in mRNA degradation, and the second a 24-26 nt class that is required in directing DNA methylation and silencing in the plant system (Finnegan, 2003).

1.5.3 Methylation of histones and transposable elements

Histone methylation is important in maintaining the stability of the genome which it achieves by silencing the repetitive sequences. Thus far, histone methylation in plants has been studied in *Arabidopsis* and rice, and high levels of H3K4 di-methylation (H3K4me2) were detected whereas lesser levels of H3K9me2 and H3K9me3 levels were detected in the basal state (Jackson *et al*., 2004). The silenced regions are involved with histone H3K9 and H3K27 tri-methylation, whereas histone3-Lysine 4/36 methylation is linked with actively

expressed genes (Liu *et al.,* 2010). Furthermore, H3K9me2 is often allied with transposons and repetitive elements where it functions to compact the chromatin preventing expression of these sequences. A study done by Cui *et al*., 2013 revealed that histone H3K4 demethylase controls transposon activity in rice, they found that active histone removal modification is involved in silencing transposable elements (TEs). TEs are transportable DNA sections that are able to change their position in the genome thus causing mutations or reversing them. TEs are capable of altering the genome size and genetic identity of the cell (Bennetzen and Wang, 2014; Ikeda and Nishimura, 2015). Transposons have two main groups; these groups are established on their mode of mobility. Class I TEs (which are the most common in plant genomes) use the copy and paste mechanism. These TEs can replicate with the aid of reverse transcription of mRNA and integrate into a new location. Class II TEs, also known as DNA transposons, use the cut and paste approach. These TEs can be removed from the chromosome and incorporated into a new position (Ikeda and Nishimura, 2015). Repression of the activity of TE by DNA methylation was demonstrated by Zakrzewski (2017) in sugar beet. Furthermore DNA methylation also maintains genome integrity through transposon refining and it is a preserved form of genetic regulation involved in TGS. TEs are capable of altering the genome size and genetic identity of the cell (Ikeda and Nishimura, 2015). TEs are also involved in plant immunity (Deleris *et al.,* 2016; Alvarez *et al*., 2020).

1.6 Rationale and motivation for the study

It has been estimated that about 500 million people in Africa (Fessenden, 2014) rely on cassava as their staple food. Cassava's tubers are carbohydrate-rich but protein-poor (Fessenden, 2014). With the price of maize increasing in the food industry, cassava can potentially replace maize as an energy or starch source. The total production yield of cassava in the world has been reported to be 11 853 133 hg/ha (FAO, 2018) and about 800 million of people across the world relies on cassava as an essential food (Parmer *et al*., 2017). Cassava production is threatened by CMD, which is the biggest constraint in the cultivation of cassava (Legg *et al.,* 2015; Rey & Vanderschuren, 2017). Cassava is a versatile crop with agro-processing uses such as animal feed in the form of cassava chips, or as a

source of starch, flour, or tapioca for the food and beverage industry (Richardson, 2011). Bioenergy is another potential application. The industrial potential of cassava starch can potentially improve the economic empowerment as well as the livelihoods of many people in South Africa.

1.6.1 Problem Identification

Due to crop losses in cassava from CMD, natural resistance or tolerance to CMD needs to be identified, and the molecular basis of these natural mechanisms elucidated. Through several cassava breeding programs (reviewed in Legg *et al.,*2015) and genome wide association studies (GWA) (Wolfe *et al.,*2016), a QTL locus *CMD2* on chromosome 12 of cassava was associated with qualitative dominant resistance to CMD in several cassava germplasm banks. Several other minor loci on other chromosomes are also thought to contribute to quantitative resistance. The *CMD2* locus was found to be dominant in TME3 Nigerian landrace (Akano *et al*., 2002) but, the molecular mechanisms of resistance/tolerance to CMD are not known. In a transcriptome study by Allie *et al.,* (2014), TME3 was found to be tolerant to SACMV and recovered at 67 days post infection, while a southern African landrace T200 was shown to be susceptible. There are a number of different varieties of African cassava that exhibit the recovery phenotype namely, TME3, TME6, TME7, and TME279 (Gedil *et al*., 2012). These varieties are more resistant to some CMGs. Louis and Rey (2015) described cassava landraces as tolerant, susceptible or resistant as a result of virus (a)virulence and virus accumulation.. DNA methylation patterns can change when plants are infected with pathogens (Hewezi *et al.,*2018). A change on the levels of methylation in plant genomic DNA was noted by Peng and Zhang (2009) where hypermethylation at the genome-wide level and hypomethylation of resistance-related genes are seen after pathogenic infection.

1.6.2 Hypothesis

As a result of the effects of geminiviruses, it was hypothesized that there is an interaction between CMG and the epigenome of plants as a way to regulate gene expression. Here, we hypothesize that SACMV will increase global DNA methylation in cassava as a defence mechanism.

1.7 Objectives and aim

The main aim of this study was to elucidate the role of DNA methylation in the CMD-tolerant and CMD-susceptible cassava landraces TME3 and T200, respectively, in response to infection with SACMV.

Specific objectives

(i) To agroinoculate T200 and TME3 cassava plantlets with infectious SACMV DNA-A and DNA-B clones and sample at 32- and 67-days post infection.

(ii) To determine the viral load and symptom scoring of T200 and TME3 at 32 and 67dpi and perform statistical analysis on the effect of SACMV in cassava.

(iii) To extract high quality DNA and compare the global methylation status using ELISA of T200 and TME3 symptomatic (32 dpi) and recovery (in TME3; 67 dpi) phases of SACMV infection.

(iv) To validate global methylation using an analytical chemistry technique Liquid chromatography-mass spectrometry.

(v) To extract high quality and intact RNA from cassava and quantify the effect of SACMV on the expression of genes pertaining to proteins associated with methylation.

CHAPTER 2: MATERIALS AND METHODS

2.1 Methodology flow chart

The following diagram outlines the methods that were followed to achieve each objective (Fig. 2.1)

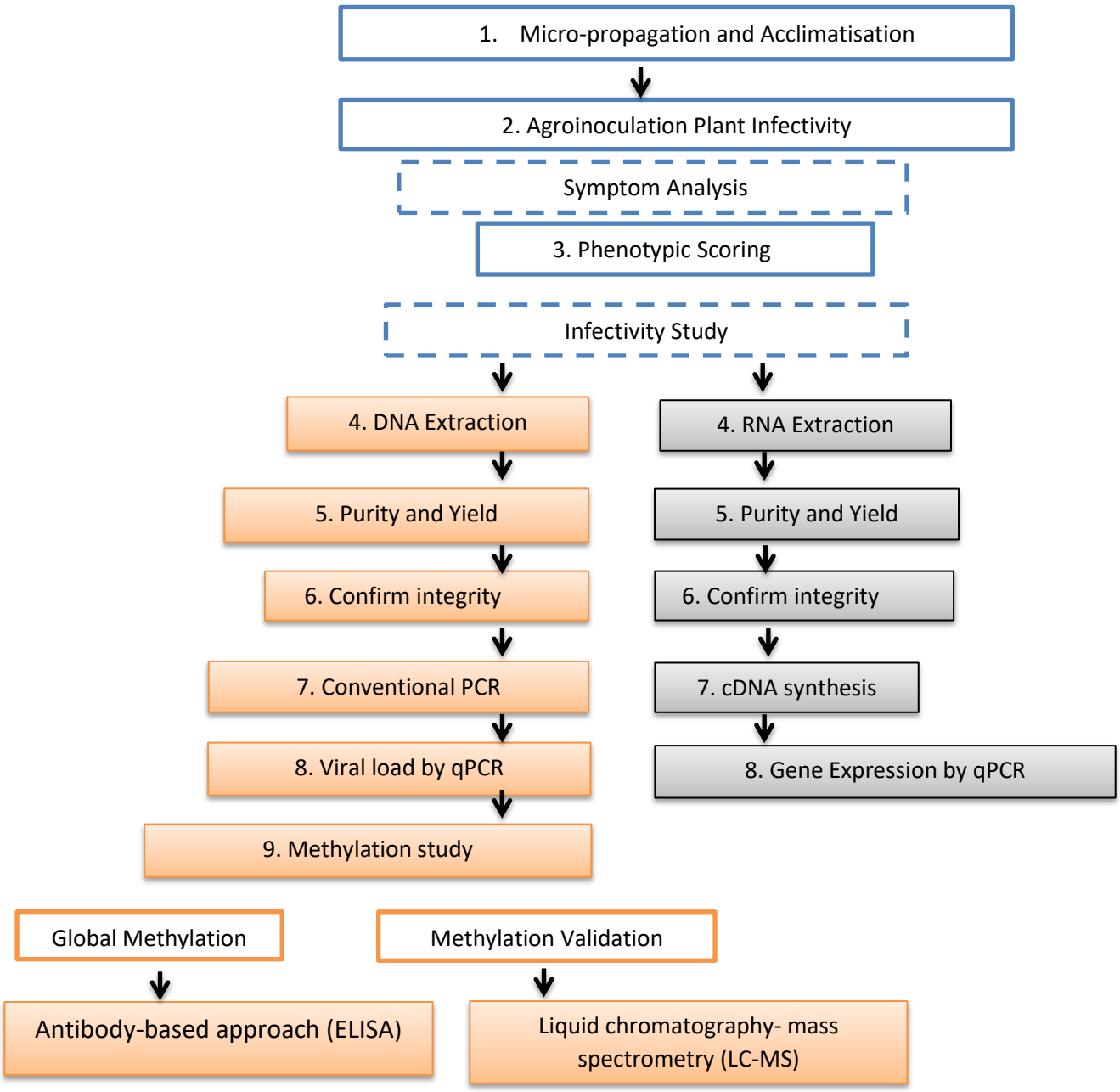

Figure 2.1: Work flow diagram depicting the overall approach to the methodology. The following diagram depicts an overall outline of the methods followed to achieve the objectives of this study. The chapter also describe the methodological steps followed in the thesis.

2.2 Plant material and micro-propagation

Cassava T200 (tolerant) and TME3 (resistant) landraces were used. The germplasm was maintained in sterile tissue culture and propagated in soil. Cassava landraces were micro-propagated by nodal cuttings on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and appended with 20 g/L sucrose and 7.8 g/L plant agar (Sigma Aldrich, St Louis), pH 5.8. The growing conditions for the cassava explants were allowed to grow in were 25 °C under a 16-h photoperiod at a light intensity of 150 µEm−2 sec−1. After approximately 10 days, when the plantlets showed roots, they were transferred into Jiffy® pellets (Jiffy Products International). The Jiffy® pellets were placed on a tray, covered with plastic film and placed in a controlled growth chamber at 28 °C for a 16 h dark, and 8 h light photoperiod. Acclimatized plantlets grown until the4-6 leaf stage was reached.

2.3 Agroinoculation of plants

Once the cassava plantlets were successfully acclimatized, plantlets were inoculated (**Fig. 2.2**) with *Agrobacterium tumefaciens* (*Agl)* strain AGL1 containing head to tail infectious SACMV DNA-A and DNA-B dimers previously cloned into the binary vector pBIN19 (Berrie *et al*., 2001). The two transformed cultures of DNA-A and DNA-B were separately cultured in Luria Bertani (LB) Broth, supplemented with 100 µg.ml−1 carbenicillin and 100 µg.ml−1 kanamycin. An additional culture that contained *Agrobacterium tumefaciens* Agl1 only was used as a negative control. The cultures were grown on a shaking incubator overnight at 30 °C until they reached optical densities (OD600) of 1.8-2. For each culture, 5 ml was sub-inoculated into 30 ml fresh LB broth supplemented with antibiotics. The cultures were again grown in a shaking incubator overnight at 30 °C until they reached optical densities (OD600) of 1.8-2. Approximately 25 ml of each of the cultures were aliquoted and centrifuged at 13 000 x *g* for 20 min until a pellet formed. The supernatant was discarded and the pellet carefully washed with distilled water by centrifugation at maximum speed for 15 min. Finally, the pellet was re-suspended in 5 ml of fresh LB broth after discarding the supernatant. The Agl-SACMV DNA-A and Agl-SACMV DNA-B cultures were then combined and the AGL1-only culture was used as a mock. Individual TME3 and T200 plantlets were inoculated with 100 µl of AGLl1 DNA-A/DNA-B along the stem using a hypodermic needle and a 1 ml Hamilton syringe.

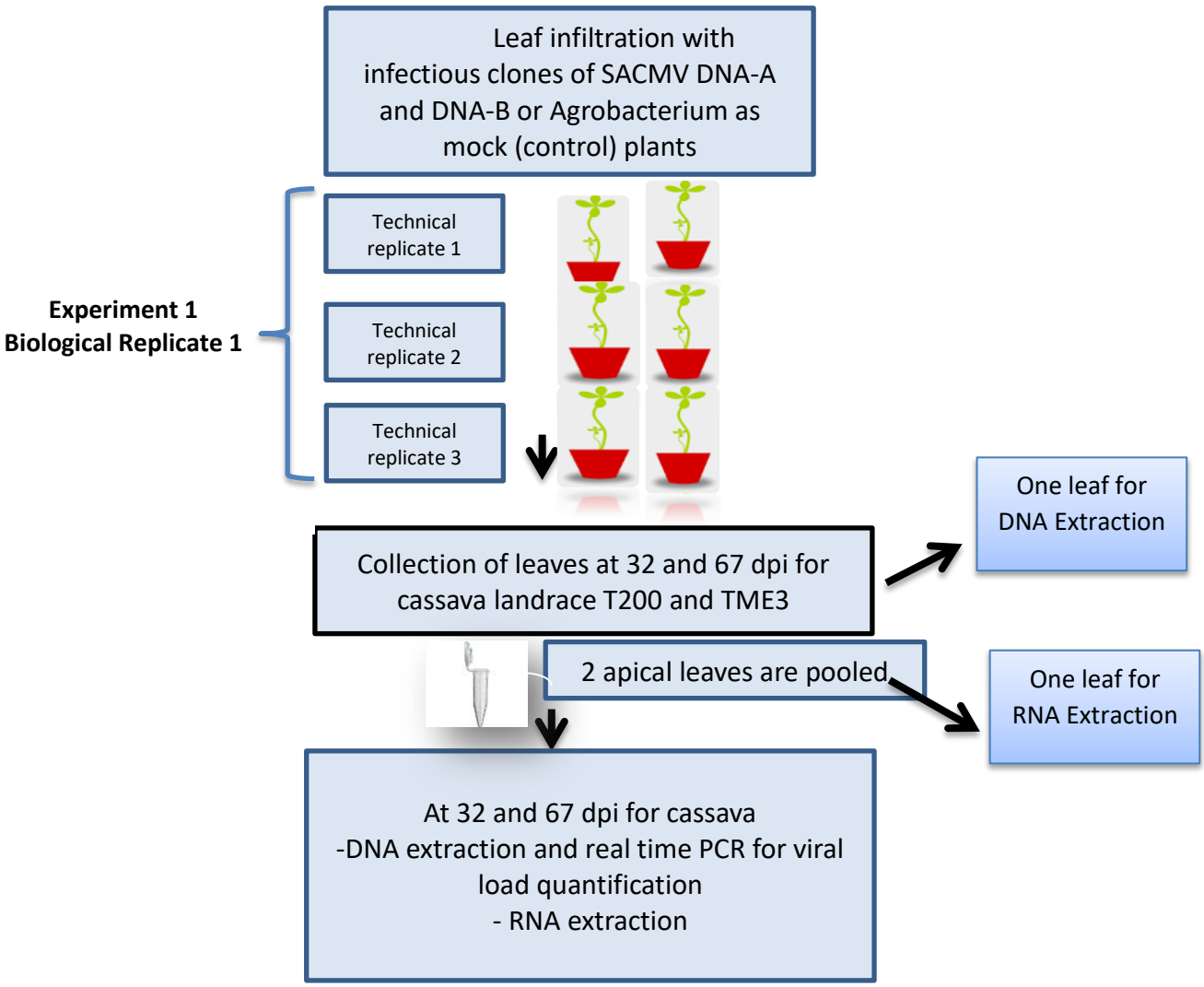

Figure 2.2: Experimental design outline. Both TME3 and T200 was agroinoculated with infectious clones of SACMV DNA-A and DNA-B, in each biological replicate 6 T200/TME3 plants were inoculated with SACMV and 6 T200/TME3 plants were AGL1 mock inoculated at 32 and 67 dpi, a total of 48 plants were used. The experiment was repeated 3 times. In each biological replicate 2 leaves were pooled together.

2.4 Symptom scoring

South African cassava mosaic virus infected and agroinoculated (control) TME3 and T200 plants were monitored and assessed for viral response at 2 time points (32 and 67dpi). The second and third upper-most apical fully grown leaves were scored for symptom severity using a symptom severity score index of 0-3 (Table 2.1). For all plants, plant height was measured at each time point.

Table 2.1 Symptom severity scoring index

Symptom score	Symptom description
0	No symptoms
1	Faint mosaic/chlorosis
2	Distinct mosaic with or without leaf deformation
3	Severe mosaic, leaf distortion and reduced size

2.5 Sample collection

Leaf tissue material was harvested at 32 and 67 dpi. The two apical leaves were picked and collected in 2 ml Eppindorf tubes and snap-frozen in liquid nitrogen. The leaves were stored at -80 °C. The leaves were then crushed using liquid nitrogen and were not allowed to thaw until proceeding with nucleic acid extractions.

2.6 DNA extraction

Total genomic DNA was extracted using the Cetylmethylammonium bromide (CTAB) method developed by Doyle and Doyle (1987). To each Eeppendorf tube containing of 50 mg of fine crushed plant tissue, 500 µl of preheated CTAB (2% (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM Ethylene diamine tetra-acetate (EDTA) 100 mM Tris-HCl, pH 8.0) (Sigma Aldrich) was added. Each mixture was then supplemented with 1 µl 0.2% (v/v) β-mercaptoethanol (Sigma Aldrich) and briefly vortexed. The mixture was then incubated at 65 °C for 30-60 min. After incubation 500 µl of chloroform: isoamylalcohol (24:1) (Sigma

Aldrich) was added to each extract and spun down at a 13 000 x g for 10 min at 4 °C. The top aqueous layer containing Total nucleic acid was extracted (approximately 250-500 µl) and placed into a new Eppendorf tube. To the TNA, equal volume of chloroform: isoamylalcohol (24:1) was added followed by mixing and centrifugation at 13 000 x g for 10 min at 4 °C. The TNA in the resulting spin was extracted and placed into a new eppindorf tube. This was followed by a precipitation step where equal volume (250-500 µl) of isopropanol (Sigma Aldrich) was added, and the tube was gently inverted. The samples were incubated at 4 °C for 60 min, followed by centrifugation at 13 000 x g for 10 min at 4 °C. The supernatant was discarded, and the TNA pellets were washed in 500 µl of ice-cold 70% (v/v) ethanol. The pellets were centrifuged at 13 000 x g for 10 min at 4 °C. The supernatant was discarded and the pellets were washed again in 500 µl of ice-cold 70% (v/v) molecular grade ethanol (Sigma Aldrich). The supernatant was discarded and the DNA pellets were allowed to air-dry for approximately 30-60 min. The pellets were re-suspended in 100 µl of nuclease free water (Sigma Aldrich) supplemented with 1 µl of RNase A (ThermoFisher Scientific). The quantity and quality of extracted DNA was assessed using Nanodrop 1000 spectrophotometer and integrity was assessed on a 1.2% (w/v) agarose gel containing 10 ug/µl ethidium bromide run in 1X TBE (Bio-Rad).

2.8 Nucleic acid purity and integrity

The extracted DNA and RNA samples were quantified at 230, 260 and 280 nm on a NanoDrop® Spectrophotometer. The 260/280 nm ratio was used to assess the purity of the nucleic acids. The 260/230 nm ratio was a secondary measure of nucleic acid purity. To assess the integrity of the extracted nucleic acids, samples were electrophoresed through an agarose gel. DNA fragments were analysed by electrophoresis on a 1% (w/v) agarose gel containing 10 µg/ml ethidium bromide in a 1X TAE (Tris, acetate and EDTA) buffer. 1.5 g of agarose (Lonza) was dissolved in 150 ml of 1X TAE buffer by heating the solution. The buffer was cooled under running water; thereafter 3 µl of ethidium bromide was added. For RNA fragments a 1X (Tris, borate and EDTA) TBE buffer solution was used to run the agarose gel. To make a 1% (w/v) agarose gel for the RNA samples, 1.0 g of agarose (Lonza, Basel, Switzerland) was dissolved in 100 ml of 1X TBE buffer by heating the solution. After cooling the solution, 3 µl of ethidium bromide (Sigma) was added. One µl of the ThermoFisher

Scientific 6X DNA/RNA loading dye was mixed with 5 µl of DNA/RNA sample and the sample loaded into the wells of the set gel. The gels were run at 90 mV for 30 min. The image was viewed and captured on a Gel Doc™ XR system (Bio-Rad).

2.9 Confirmation of viral infection by conventional PCR

To confirm successful viral infection of SACMV in TME3 and T200 plants conventional PCR was used. The core coat protein on DNA-A was amplified from the extracted DNA (Table 2.2). PCR reactions were prepared (Table 2.3) with approximately 500 ng of genomic DNA. To activate Taq DNA polymerase the 20 µl reaction was heated at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, primer extension 72 °C for 45 sec, and a final extension step of 72 °C for 5 min. The primers used targeted the coat protein (CP) and UBQ10 (internal reference) (Table2.2) Genomic DNA extracted from mock-inoculated leaf tissue was used as negative control to confirm no infection and an additional no template control (NTC) was used as a negative control which serves as a general control for unnecessary nucleic acid contamination (Table 2.3). DNA-A (25ng) cloned into pBluescript was used as a positive control. Amplicon products were assessed by electrophoresis on 1.2% (w/v) agarose gel containing 10 µg/ml ethidium bromide in a 1X TAE buffer.

Table 2.2 Target and reference primers used for amplification

	Gene Name	Primer sequence (5'- 3')	Product size(bp)	Accession Number	MANE ID[a]
Target Gene	*CP*	FP: GCACAAACAAGCGTCGARP: CTGCCAGTATGCTTAACGTCA	550	AF155806	N/A
Reference Gene	*UBQ10*	FP:TGCATCTCGTTCTCCGATTG RP:GCGAAGATCAGTCGTTGTTGG	166	DV441403	Manes.07G019300

[a]Not applicable- No manes ID for the virus.

Table 2.3 Components of the PCR master mix reaction

Components	Volume (µl)	Final concentration
10X DreamTaq Buffer	2.0	1X
dNTPs (10Mm)	0.4	0.2 mM
10µM Forward Primer	0.4	0.1 µM
10µM Reverse Primer	0.4	0.1 µM
Template DNA (25 ng/µl)	2.0	-
1XDreamTaq Polymerase (1.25U)	0.1	-
Nuclease-free Water	14.7	-
Total Volume	**20**	**-**

2.10 Determining viral load by qPCR

Viral load was quantified in the samples that tested positive for infection with SACMV using quantitative PCR. In each biological trial (3 trials), 6 plants were used for each landrace. For each replicate, two apical leaves from two plants were pooled resulting in 3 replicate samples. Total genomic DNA was extracted from 50mg of each of the pooled replicate samples as described in section 2.6. The concentration used for the extracted DNA was 25 ng/µl. A master mix was prepared (Table 2.5) with the primers in Table 2.4. To each well the master mix (9 µl) was added first followed by 1 ul of template DNA. For the NTC, nuclease free was used to replace the template DNA. A two-step cycling program (Table 2.5) on a CFX-96 Thermal Cycler (Bio-Rad) was used for analysis.

Table 2.4 Target and reference primers used for amplification

	Gene Name	Primer sequence (5'- 3')	Product size(bp)	Accession No.	MANE ID[a]
Target gene	*CP*	FP: ACGTCCGTCGCAAGTACGAT RP:ATTGTCATGTCGAATAGTAG	95	AF155807	N/A
Internal control	*UBQ10*	FP: TGCATCTCGTTCTCCGATTG RP:GCGAAGATCAGTCGTTGTTGG	166	DV441403	Manes.07G019300

[a]Not applicable- No manes ID for the virus.

Table 2.5 Master mix components for qPCR reaction

Components	Volume (µl)	Final concentration
2X Maxima SYBR GREEN/ROX Master Mix	5	1X
10µM Forward Primer	0.3	750 nM
10µM Reverse Primer	0.3	750 nM
Water, nuclease-free	2.4	-
Total Volume	**9**	**-**

Table 2.6 Two-step PCR cycling conditions

Cycle	Temperature(°C)	Duration	Reaction
1	50	2 min	UDG pre-treatment
1	95	5 min	Initial denaturation
40	95	15 sec	Denaturation
	60	30 sec	Annealing
	72	60 sec	Extension
			Melt curve

2.11 Global methylation quantification by enzyme-linked immunosorbent assay

To quantify changes in global DNA methylation level in response to SACMV in cassava plants, an enzyme-linked immunosorbent assay (5-mC DNA ELISA Kit; Zymo Research, Irvine, CA) was used. A standard curve was prepared from methylated (positive) and unmethylated (negative) DNA standards (Table 2.7). The extracted DNA samples (100 ng) together with the standard curve samples (controls) were pipetted in PCR tubes and taken up to a volume of 100 µl with 5-mC coating buffer and denatured for 5 min at 98 °C. After the DNA samples and controls were denatured, the tubes were immediately incubated on ice for 10 min. The preceding steps are light sensitive and were done under low light to avoid interference of the conjugates (Vancells, 2017). The denatured DNA samples of 100 µl were added to the wells, covered in foil and incubated at 37 °C for 1 h. The 5-mC coating buffer was discarded and the wells were washed three times with 200 µl 5-mC ELISA buffer. To each well 200 µl 5-mC ELISA buffer was added, and the plate was covered in foil and incubated at 37 °C for 30 min. An antibody mix was prepared while the plate was incubating (Table 2.8). After incubation, the 5-mC ELISA buffer was discarded, 100 µl of the antibody mix was added to the well and the plate was covered in foil and incubated at 37 °C for 1 h. The antibody mix was discarded and the wells were washed three times with 200 µl 5-mC ELISA buffer. Lastly, to each well 100 µl of the HRP developer solution was added, and the wells were left to stand at room temperature for 1 h to allow the colour to develop. Absorbance was measured at 405nm on a Multiskan GO® Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A standard curve was constructed, to quantify the methylation

level with the absorbance on the y-axis and % 5-mC on the x-axis. Two technical replicates were done for the standard curve and 3 technical replicates were done for the sample DNA with at least 3 independent biological replicates.

Table 2.7 Reaction components for negative and positive DNA controls

%5-mC	0% methylated DNA (µl)	100% methylated DNA (µl)
0	10	0
5	9.5	0.5
10	9.0	1.0
25	7.5	2.5
50	5.0	5.0
75	2.5	7.5
100	**0**	**10.0**

Table 2.8 An antibody mix made up of Anti-5-Methylcytosine and Secondary Antibody in ELISA Buffer.

	Dilution	Volume(µl)	Example (18 wells)
5-mC ELISA Buffer	N/A	(#wells +2) x 100	2000 µl
Anti-5-Methylcytosine	1:2 000	Buffer Vol./ 2 000	1 µl
Secondary Antibody	1:1 000	Buffer Vol./ 1 000	2 µl
Total Volume			x^a

[a]The total volume was dependent on the number of wells.

2.12 Methylation validation by liquid chromatography tandem mass spectrometry (LC-MS/MS)

To validate global methylation levels quantified by ELISA, LC-MS/MS was performed as it is a highly sensitive and robust method (Liu & Hesson, 2013;Le *et al*., 2011). The levels of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) in genomic DNA were measured.

2.12.1 Sample preparation

To assess the global methylation status of DNA using LC-MS/MS, DNA was efficiently degraded to its individual nucleoside components prior to injection onto the mass spectrometry device. To obtain 5 ng/µl of hydrolysed DNA, 500 ng/µl of the genomic DNA sample was prepared (Table 2.9) and hydrolysed (DNA Degradase Plus ™ Zymo, South Africa). The reaction mixture was incubated at 37 °C for 2 hs and inactivated with 175 µl of 0.1% (v/v) formic acid (Sigma Aldrich, St Louis, MO).

Table 2.9 Components for DNA samples

Components	Volume(µl)
DNA Samples	*2*
10X Buffer	2.5
DNA Degradase	1.0
dH_20	19.5
Total	**25**

2.12.2 DNA calibration

The following three standards, 2'-Deoxycytidine (dC), 5-Methyl-2'- deoxycytidine(5mC) and 5-(hydroxymethyl)-2'- deoxycytidine(5hmC) were prepared for the construction of the calibration curve to calculate the actual percentage of 5mC and 5hmC in every test sample. A scale of 0-10% (Table 2.11) was used to measure the varying amounts of either 5mC or 5hmC in constant amounts of cytosine. The three standards were prepared (Table 2.10) and hydrolysed as described in 2.14.1. Calibration standards were prepared by mixing increasing amounts of hydrolysed 5mC/5hmC in the presence of the same amount of hydrolysed cytosine (Table 2.11).

Table 2.10 Reaction components for the various DNA standards

Components	Volume(µl)	Final Concentration
DNA Standards	*20*	-
10X Buffer	2.5	1X
DNA Degradase	1.0	-
dH_20	1.5	-
Total	**25**	**-**

Table 2.11 Standard Curve Dilutions

Percentage (%)	5mC/5hmC (µl)	Cytosine (µl)	Methanol (µl)
10.0	*0.5*	4.5	100
5.0	2.5	2.5	100
2.0	2.0	3.0	100
1.0	2.5	2.5	100
0.5	2.5	2.5	100
0.1	1.0	4.0	100
0	5.0	0	100

2.12.3 Analysis on Mass Spectrometry

In this step the hydrolysed DNA of both the test samples and standards were analysed by LC-MS/MS-MRM. The 5mC and 5hmC were identified and quantified using UPLC 1290 Infinity Series equipped with a triple quadrupole (QqQ) mass spectrometer. Optimization was done prior to obtain the optimal MS parameters required to detect both the 5mC and 5hmC. This was done by connecting the column directly to the Jet Stream. 5 ul of the hydrolysed DNA was injected onto a UPLC-C18 column using two mobile phases that consisted of water with 0.1% (v/v) formic acid (A) and methanol with 0.1% (v/v) formic acid (B), for chromatographic separation. For 10 min an isocratic flow of 95% (A) and 5% (B) was maintained. The column used operated at 30 °C with the flow of 0.3 ml/min. The positive ion polarity was selected for the optimization of the QqQ parameters. The operating conditions were set as follows: gas temperature of 300_C, drying nitrogen gas of 9 L/min, nebulizer pressure of 40 psig, sheath gas temperature of 350_C, sheath gas flow of 10 L/min, capillary voltage of 3500 V, nozzle voltage of 1000 V. The conditions for nucleotide analysis were as follows: the fragmentor voltage of 90V, collision energy 5 eV and cell accelerator of 7 V, with a scan time of 1000 ms which gave a value of 3.2 cycles/sec. Three transitions were monitored for multiple reaction monitoring (MRM): that of 5hmC was monitored by transitions of m/z 258.0 to 141.90, that of 5mC was monitored by m/z 242.20 to 242.20 and that of dC was monitored by the transitions' of m/z 243.25 to 126.95. To get

the total amount of standards in test samples a calculation was done in the following manner: MRM peak areas divided by the sum of the standard peak areas (5mC/5mC + 5hmC + C). The calibration curves were plotted in that the known percentages are on the y-axis and the peak ratios are on the x-axis. The actual percentage for both 5mC and 5hmC in test samples was obtained by interpolation from the respective linear calibration curve.

2.13 RNA Extractions

High quality RNA was extracted using the QIAzol method (Rio *et al*., 2010). For each sample, two leaves were pooled and crushed into fine powder. In each sample of 50 mg plant tissue, 500 µl of QIAzol lysis reagent (ThermoFisher Scientific) was added and the mixture was briefly vortexed. The mixture was incubated at room temperature for 5 min before adding 200 µl of chloroform (ThermoFisher Scientific) and mixing for 15 sec on a vortex. The QIAzol-chloroform mixture was incubated for 3 min at room temperature and centrifuged at 12 000 rpm for 15 min at 4 °C. The upper aqueous phase was then transferred into a new eppindorf tube and 500 µl of isopropanol (ThermoFisher Scientific) was added and gently mixed. To precipitate the RNA, the extract was incubated at at room temperature for 10 min before centrifugation at 12 000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet resuspended in 500 µl of 75% (v/v) molecular grade ethanol (ThermoFisher Scientific,) was added to the pellet and the mixture was centrifuged at 75,000 rpm for 5 min at 4 °C. The supernatant was discarded and the RNA pellet was allowed to air dry for up to one h before it was dissolved in 50 µl of nuclease free water (Sigma Aldrich). The quantity and quality of extracted RNA was assessed using a Nanodrop 1000 spectrophotometer (Nanodrop) and the integrity was assessed on a 1.2% (w/v) agarose gel containing 10 µg/µl ethidium bromide run in 1X TBE (Bio-Rad).

2.14 DNase clean-up

Prior to cDNA synthesis extracted RNA was treated with DNase I to remove DNA contaminants (Table 2.12) using the RevertAid First Strand cDNA synthesis kit (ThermoFisher Scientific, South Africa). A master-mix of 10X Reaction Buffer with $MgCl_2$ and DNase I was made in a Nuclease-free tube. To each reaction tube, 1 µl of RNA was added and incubated

at 37 °C for 60 min. The reaction was terminated by adding the 1 µL of 50 mM EDTA to the samples and incubated at 65 °C for 10 min of 50 mM EDTA was added and the samples were incubated at 65 °C for 10 min. The purity and concentration of each sample was assessed using spectrophotometry on a Nanodrop Spectrophotometer.

Table 2.12 Reaction component to remove genomic DNA from RNA

Components	Volume (µl)	Final Concentration
RNA	Volume required for 1µg [a]	500 ng/µl
10X Reaction Buffer with $MgCl_2$	1	1X
DNase I, RNase-free	1	1 U/µl
Water, nuclease-free	to 10	-
Total	**10**	-

[a] The amount of RNA added is dependent on the concentration.

2.15 cDNA synthesis

Complementary DNA (cDNA) was synthesised from 500 ng RNA using the RevertAid First Strand cDNA synthesis kit (ThermoFisher Scientific). The priming pre-mixing components were first prepared (Table 2.13) and incubated at 65 °C for 5 min. The mixture was cooled on ice prior to adding part 2 mixture (Table 2.13), Finally, the reaction was terminated by incubating at 70 °C for 5 min.

Table 2.13 Reaction components for cDNA

Components	Volume (µl)	Final Concentration
1. Priming pre-mixture		
Extracted RNA	Volume required for 500ng[a]	
Oligo(dT)$_{18}$ Primer	1	-
Nuclease- free water	Up to 12	-
Sub-Total	**12**	**-**
2. cDNA synthesis reaction mixture		
5X Reaction Buffer	4	1X
RiboLock RNase Inhibitor	1	20 U/µl
10 mM dNTP Mix	2	0.2mM
RevertAid M-MuLV RT	1	200 U/µl
Sub-Total	**8**	**-**
Total	**20**	**-**

[a] The amount of RNA added is dependent on the concentration.

2.16 Gene expression

To quantify the expression of SAM*, DMT, AGO4, AGO5* and *CMT3* in SACMV-infected T200 and TME3, RT-qPCR was performed. The primers (Table 2.14) for SAM*, DMT, AGO4, AGO5* and *CMT3* were designed using the PRIMER3 software (version 4.1.0; http://primer3.ut.ee/). A master mix was prepared (Table 2.16) on ice. A master mix of 9 µl was added first to a 96-well clear PCR plate (Bio-Rad)and 1 µl of the prepared cDNA was added to master mix. The plates were sealed with a clear seal and cDNA was allowed to amplify according to a two-step cyclic program (Table 2.16) on a CFX-96 Thermal Cycler (Bio-Rad, Johannesburg, South

Africa). For each gene, the experiment was carried out in three independent biological replicates with three technical replicates.

Table 2.14 Target and reference primers used for amplification

	Gene Name	Gene description	Primer sequence[a] (5'- 3')	Product size(bp)	Accession number	Manes ID
Target genes	*SAM*	S-adensosylmethionine synthase	FP:TTGAAGACAGCTGCATACGG RP: CTTGAGGCTTCTCCCACTTG	91	N/A[b]	Manes.01G233200
	DMT	DNA(cytosine-5)-methyltransferase 1	FP: TTGGCTTCACTCCTCGAGAT RP: CTCTGGGGCAGCAAATACAT	105	N/A	Manes.13G155300
	CMT3	Chromomethylase 3	FP: TGTCGGTGATCCTTGCCTGAT RP:GCGTCGAGCTGGTAAGATCTCT	139	N/A	Manes.03G089100
	AGO4	Agonaute 4	FP: TCCCTGGTGGAGAAATCAAG RP: ACACGGCCTTCAATATCAGC	149	N/A	Manes.18G121900
	AGO5	Agonaute 5	FP: TGCCTCGTGACTTTTGTCAG RP: TTGTGAACATCAGCCAGAGC	133	N/A	Manes.04G011400
Internal control	*UBQ10*	Ubiquitin 10	FP: TGCATCTCGTTCTCCGATTG RP: GCGAAGATCAGTCGTTGTTGG	166	DV441403	Manes.07G019300

[a] The forward primer (FP) and reverse primer (RP) are given in the 5'- 3'direction.
[b] Not applicable- primers were designed using Primer3.

Table 2.15 Two-step PCR cycling conditions

Cycle	Temperature(°C)	Duration	Reaction
1	50	2 min	UDG pre-treatment
1	95	5 min	Initial denaturation
40	95	15 sec	Denaturation
	60	30 sec	Annealing
	72	60 sec	Extension
			Melt curve

Table 2.16 Components of the qPCR master mix reaction

Components	Volume (µl)	Final concentration
2X Maxima SYBR GREEN/ROX Master Mix	5	1X
10µM Forward Primer	0.3	750 nM
10µM Reverse Primer	0.3	750 nM
Water, nuclease-free	2.4	-
Total Volume	**9**	-

2.17 Statistical Analyses

To statistically analyse the data on symptoms severity score, plant height, viral load and methylation status, a student's T-test was performed to determine if the was a statistical difference between SACMV-inoculated, AGL1- inoculated and healthy plants in TME3 and T200. DNA Methylation was assessed using Kruskal-Wailis test. Pearson's correlation coefficient was used to establish if there was any correlation between DNA methylation and viral load. A two-tailed p-value < 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 Quality Control

3.1.1 Nucleic Extractions

To assess the quality of the extracted DNA and RNA, nucleic acids were electrophoresed on a 1% agarose gel. The results showed high molecular weight and intact DNA (Fig. 3.1). For RNA, the results illustrated three intact bands representing the 28S, 18S and 5S rRNA subunits of the ribosome (Fig. 3.2). Additionally, extracted DNA was accepted as pure and free from RNA and protein contamination with a 260/230 ratio of 1.8-2.0. Extracted RNA was considered pure with no phenol and DNA contamination with a 260/230 ratio of 2.0-2.0.

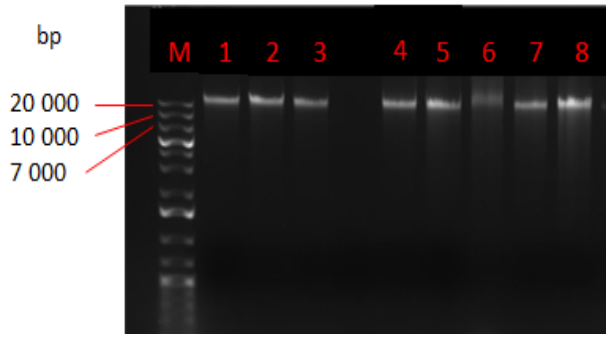

Figure 3.1 The agarose gel-electrophoresis (1%) shows intact, high molecular weight DNA. M= Thermoscientific O'GeneRuler 1kb plus. Lanes 1-2: 32 dpi T200, lanes 3-4: 67 dpi T200, lanes 5-6: 32 dpi TME3 and lanes 7-8: 67 dpi TME3.

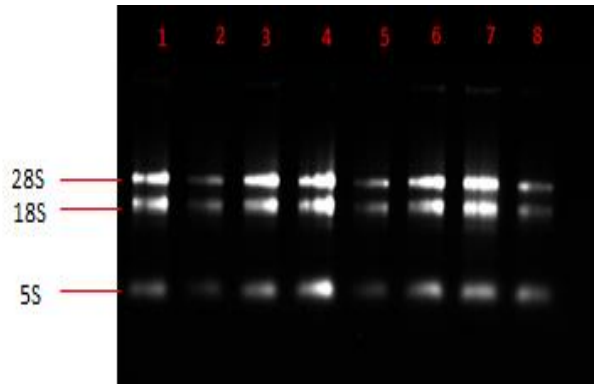

Figure 3.2 The agarose gel-electrophoresis (1%) depicting intact total RNA. Lanes 1-2 depict T200 SACMV-inoculated, lanes 3-4 TME3 SACMV-inoculated, lanes 5-6 T200 *Agrobacterium* AGL1-inoculated, and lanes 7-8 TME3 AGL1-inoculated (control).

3.1.2 Primers for cDNA

Gel electrophoresis was run to assess whether the correct cDNA product was amplified during RT-qPCR of *AGO4, AGO5, DMT, SAM* and *CMT3* (Fig. 3.3). The observed product sizes for each gene corresponded with the Biomart data mining tool hosted in Phytozome, where a 149 bp product for *AGO4*, a 133 bp product for *AGO5,* a 129 bp product *MET1,* a 105 bp product for *SAM* and a 139 bp product for *CMT3* was present. A no template control (NTC) was assayed in parallel, and no products were amplified. Furthermore, a melt curve analysis was done for each gene to verify a single product amplification using the Bio-Rad CFX Maestro software. Each gene showed one peak on the melt curve above the threshold line, which correlates with one product being, amplified (Fig. 3.4).

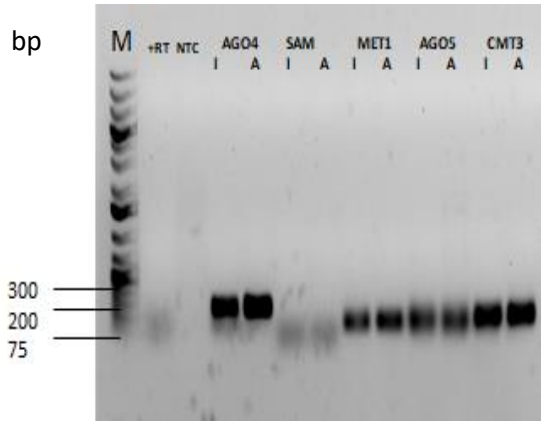

Figure 3.3: Amplifying primers for genes of interest after cDNA synthesis. 1.2% agarose gel shows RT-qPCR products for each primer. Each gene had a single band of the estimated base pair (bp). Each primer pair (*AGO4*, *SAM*, *MET1*, *AGO5* and *CMT3*) shows RT-qPCR products for SACMV and Agl-infected plants. For each gene, a single band is observed at the expected base pair (bp) size (M: molecular weight marker, NTC: no template control, I: inoculated-SACMV, A: AGL1, +RT: reverse transcriptase.

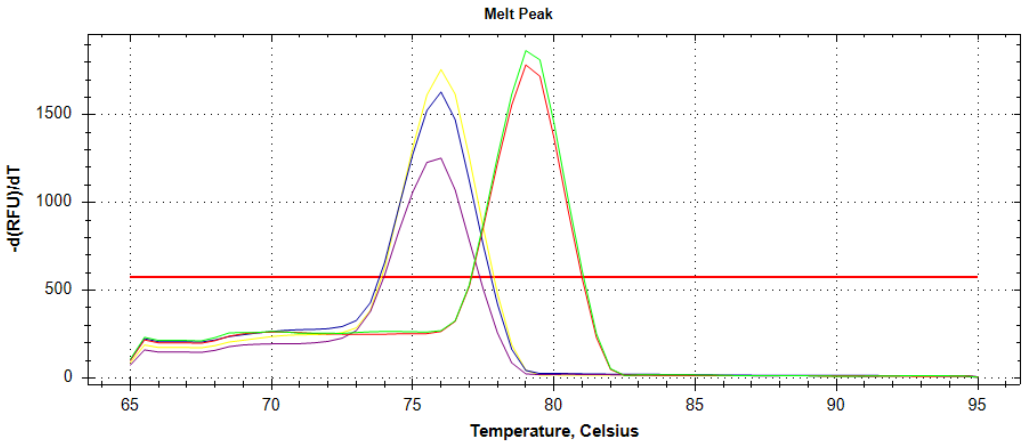

Figure 3.4: The melt peaks depict the specificity of oligonucleotide sequences used. The melt curves show single peaks which are above the threshold for each of the primer pairs (yellow= *AGO4*, blue=AGO5, purple= *SAM*, green= *MET1* and red=*CMT3*).

3.2 Infectivity study

3.2.1 Symptom development and scoring

To assess the difference in severity of the symptoms between T200 and TME3, plants infected with SACMV-[ZA: 99] were observed for symptom development over a period of 67 days. At 32 dpi T200 (Fig. 3.5 A) and TME3 (Fig. 3.5 C) plants were symptomatic with the apical leaves exhibiting leaf curling, size reduction and mild mosaic. By 67 dpi T200 (Fig. 3.5 B) and TME3 (Fig. 3.5 D) leaves were fully symptomatic with severe mosaics and severe leaf deformation. To confirm infection at an early infection (12dpi), a PCR was done and leaf tissue tested positive for SACMV that targets a 95 bp region of CCP of SACMV-[ZA: 99] (Fig. 3.6). At 32 dpi, T200 had a symptom severity score (sss) of 1.8, while TME3 had a sss of 2, however the sss difference between the two plants was not statistically significant. At 67 dpi T200 had a significantly higher sss of 2.8 compared to TME3 with a sss of 1.9 ($p < 0.050$) (Fig. 3.7).

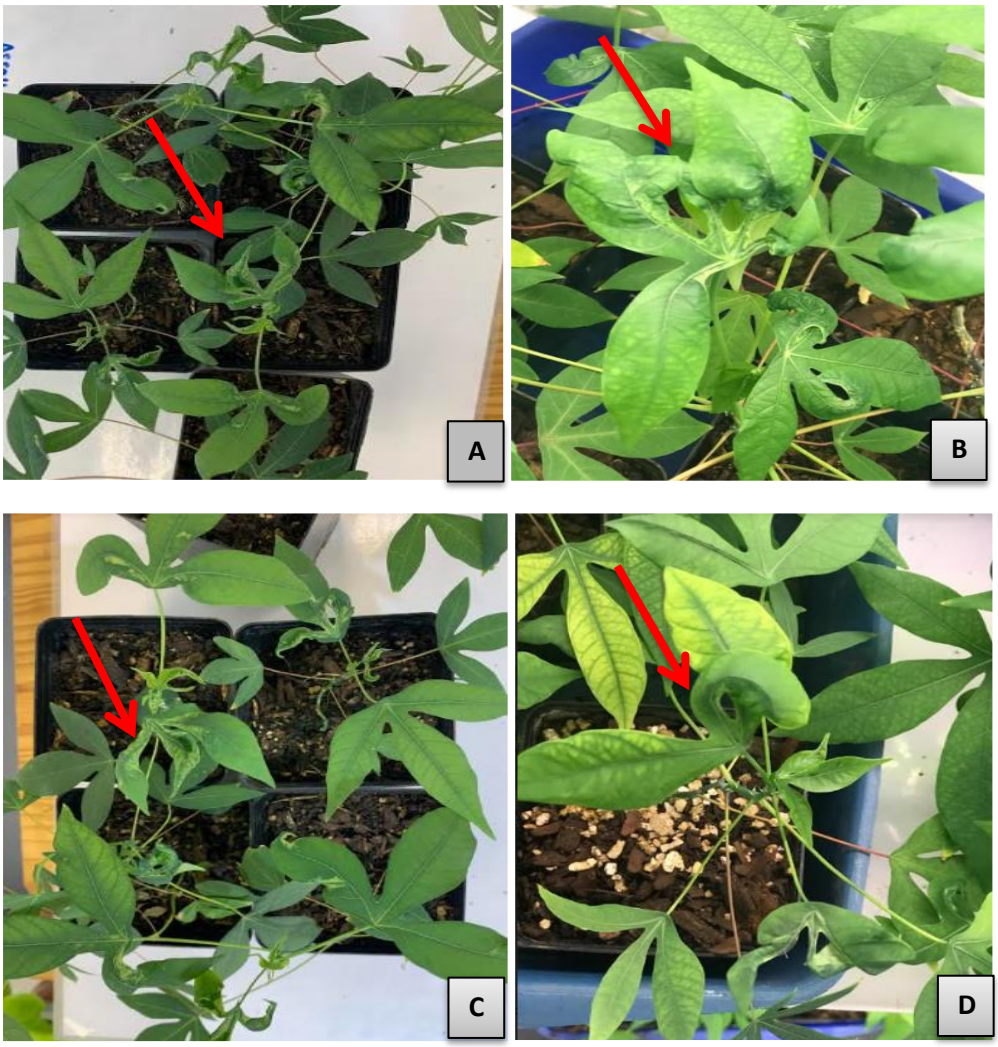

Figure 3.5: Symptom development of SACMV infected T200 and TME3 at 32 and 67 dpi. (A) At 32 dpi in T200 and TME3(C) apical leaves showed symptoms of leaf curly and size reduction. (B) At 67 dpi in T200 and TME3 (D) leaves have fully developed symptoms of severe mosaic and reduction in size.

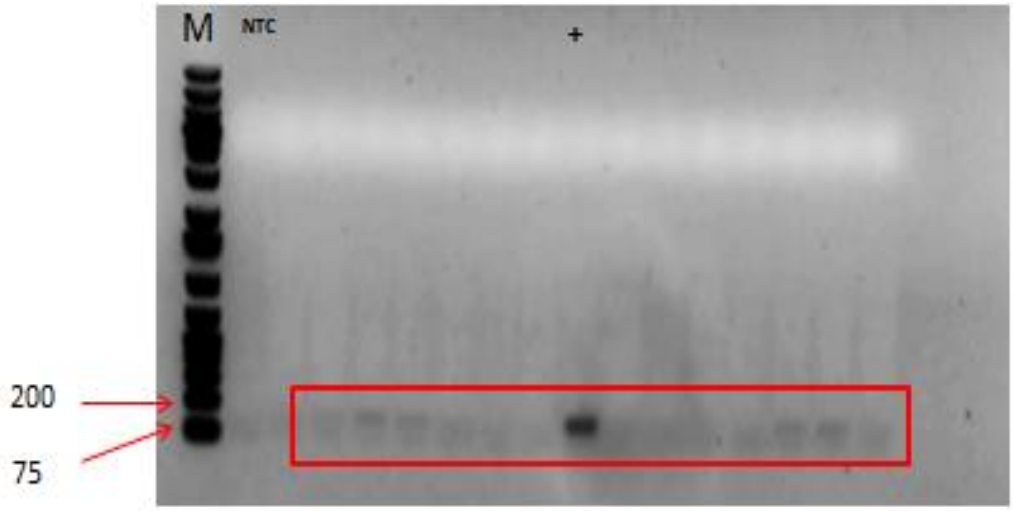

Figure 3.6: SACVM-viral confirmations by conventional PCR. PCR was done to confirm the infection of plants at 12 dpi. The target was a 95 bp amplicon from the coat protein of SACMV. M= Thermoscietific O'GeneRuler 1kb plus, "NTC"= No template control, and "+"= PCR that was positive containing DNA-A cloned into a pBluescript plasmid.

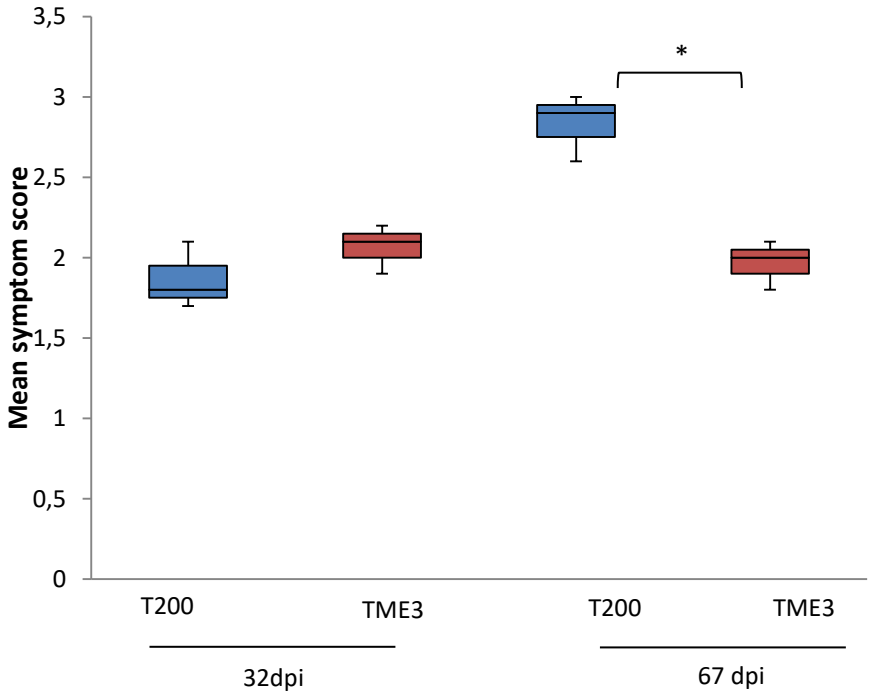

Figure 3.7: Symptom scoring on SACMV infected T200 and TME3 plants at 32 and 67 dpi. Plant symptoms were evaluated at 32 and 67dpi. The boxplot depicts the mean symptom score in response to SACMV infection. The median is represented by the horizontal line within each boxplot. The lower and upper ends of boxes represent the first and third quartile respectively. Whiskers show the minimum and maximum values. A t-test was done to compare significant differences between plants, where T200 was statistically higher than TME3 at 67 dpi (*P < 0.050). Errors bars represent standard deviations of the mean (n=3).

3.2.2 Plant height evaluation

To evaluate the difference in height between T200 and TME3, plant height was measured in SACMV-[ZA: 99] infected plants at 32 and 67 dpi. At 32 dpi T200 plants had a significantly higher average height of 10 cm while TME3 had an average height of 7.4 cm ($P < 0.050$) (Fig. 3.8). At 67 dpi T200 plants measured an average height of 14 cm, while TME3 plants measured at an average height of 12.8 cm; although this difference was not statistically significant.

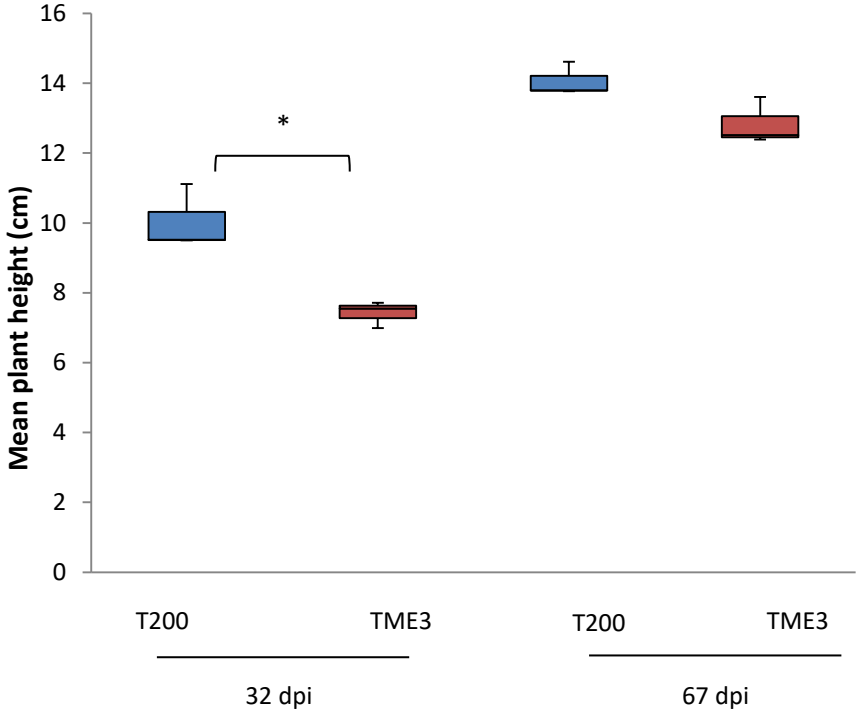

Figure 3.8: Height evaluations of SACMV-inoculated T200 and TME3 plants. The box-plot depicts the mean height in TME3 and T200 plants inoculated with SACMV at 32 and 67 dpi. The median is represented by the horizontal line within each boxplot. The lower and upper ends of boxes represent the first and third quartile respectively. Whiskers show the minimum and maximum values. A t-test was done to compare significant differences between plants; pairwise comparisons confirmed that T200 plants were significantly higher than TME3 plants at 32 dpi (* $P < 0.050$). Errors bars represent standard deviations of the mean (n=3).

3.2.3 Viral load

To quantify the relative accumulation of viral load in T200 and TME3 at 32 and 67 dpi, extracted DNA was amplified using RT-qPCR. At 32 dpi, T200 showed significantly higher viral load of 3.3 while TME3 showed a viral load of 1.4 ($P < 0.010$). At 67 dpi, T200 showed

significantly higher viral load of 6 while TME3 measured a viral load of 2.3 ($P < 0.001$) (Fig. 3.5).

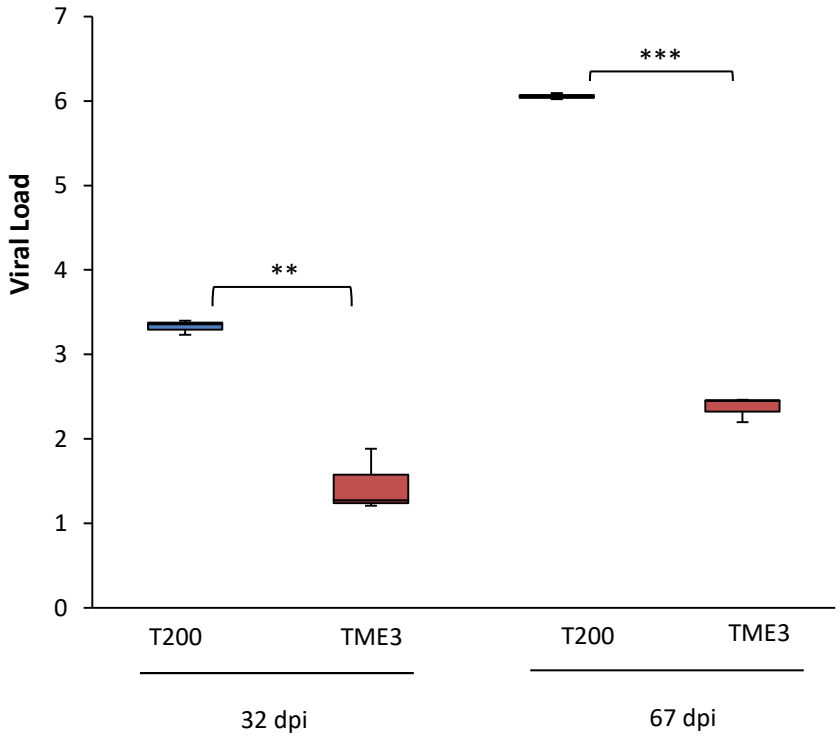

Figure 3.9: Relative quantification of viral load in T200 and TME3 infected with SACMV. Relative quantification was done on plants that tested positive for the virus with conventional PCR. The samples were amplified using a CFX-96 Thermal Cycler (Bio-Rad) in a real time system. The median is represented by the horizontal line within each boxplot. The lower and upper ends of boxes represent the first and third quartile respectively. Whiskers show the minimum and maximum values. A t-test was done to compare values, statistical findings were indicated as **$P < 0.010$ and ***$P < 0.01$. Errors bars represent standard deviations of the mean (n=3).

3.3 DNA methylation

3.3.1 DNA methylation by ELISA

To assess the effect of SACMV on host DNA methylation, quantification of global methylation level was evaluated in TME3 (shown in maroon in Figures below) and T200 plants (shown in blue in Figures below) at 32 and 67 dpi. Prior methylation quantification, a standard curve was done to quantify the percentage of 5-mC in the DNA assay (Fig. 3.10). At 32 dpi, in T200 plants SACMV significantly decreased methylation compared to AGL1 ($P < 0.050$; Fig. 3.11A). In TME3 plants there was no significant change in global methylation at 32 and 67 dpi. Interestingly, T200 plants inoculated with AGL1 showed significantly higher global methylation with a percentage of 31.36 compared to TME3 plants (12.28%; Fig. 3.12). T200 plants inoculated with SACMV were also significantly higher compared to TME3 at 32 dpi. A similar trend was noticed at 67 dpi; where T200 plants that were AGL1-inoculated had significantly higher methylation compared to TME3 plants.

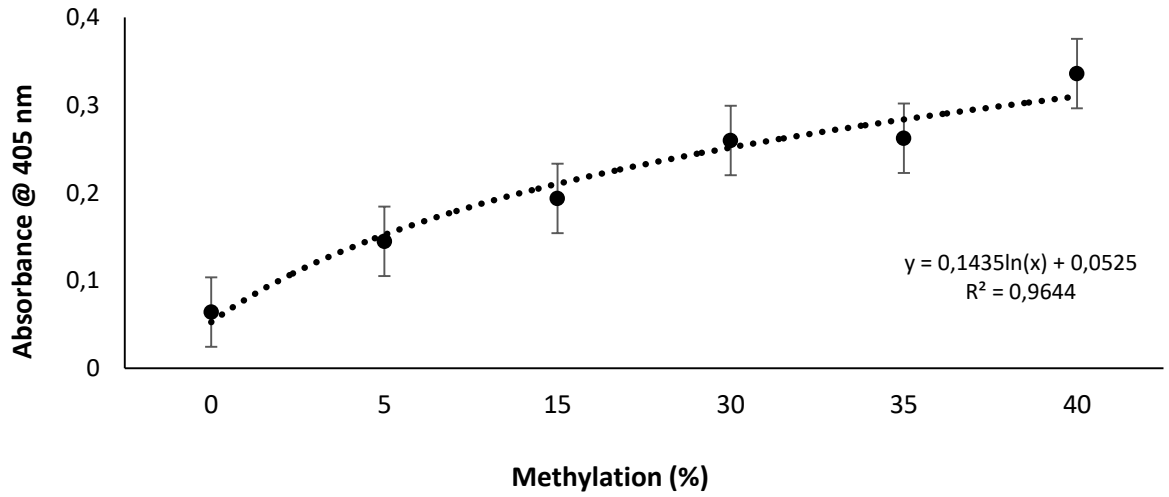

Figure 3.10: An illustrative standard curve of the DNA methylation standards. The standard curve shows the typical (n = 3) of increasing concentration of DNA on a logarithmic scale. The r^2-value of 0.9644 shows a solid logarithmic relationship amongst 5-mC in a DNA sample and absorbance. Error bars signify ±1 SD below the mean.

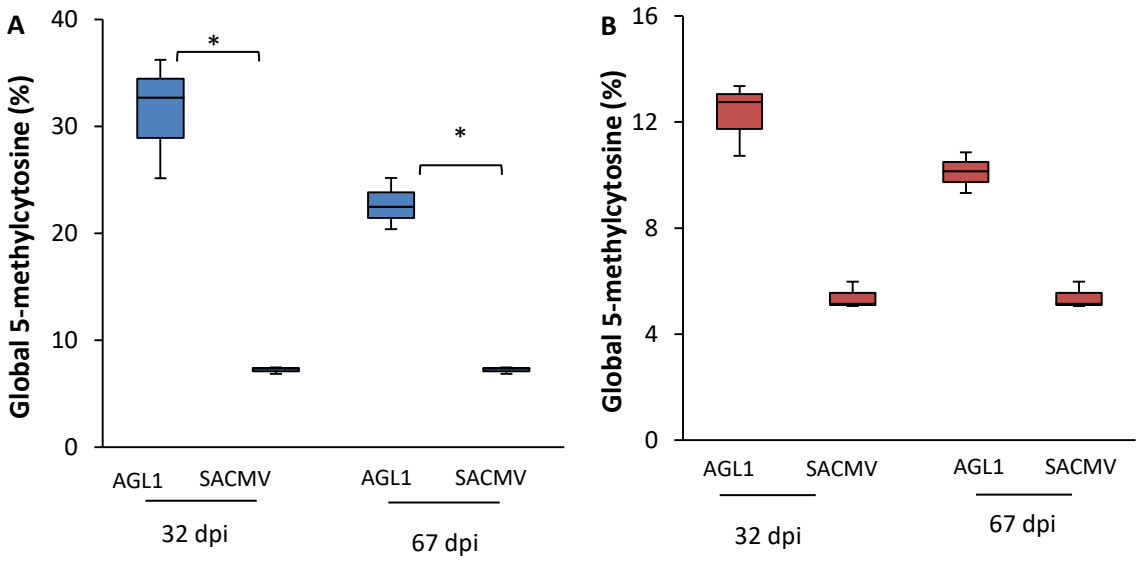

Figure 3.11: Boxplots showing the comparison of global DNA methylation between SACMV-infected and AGL1 controls in T200 (A) and TME3 (B) plants at 32 and 67 dpi. Methylation was assessed using ELISA. Pairwise comparison revealed that at 32 and 67 dpi SACMV significantly decreased global methylation in T200 plants compared to AGL1. However, there was no significant change in global methylation in TME3 plants at either 32 or 67 dpi. Significant differences are indicated as *P < 0.050. Errors bars represent standard deviations of the mean (n=3).

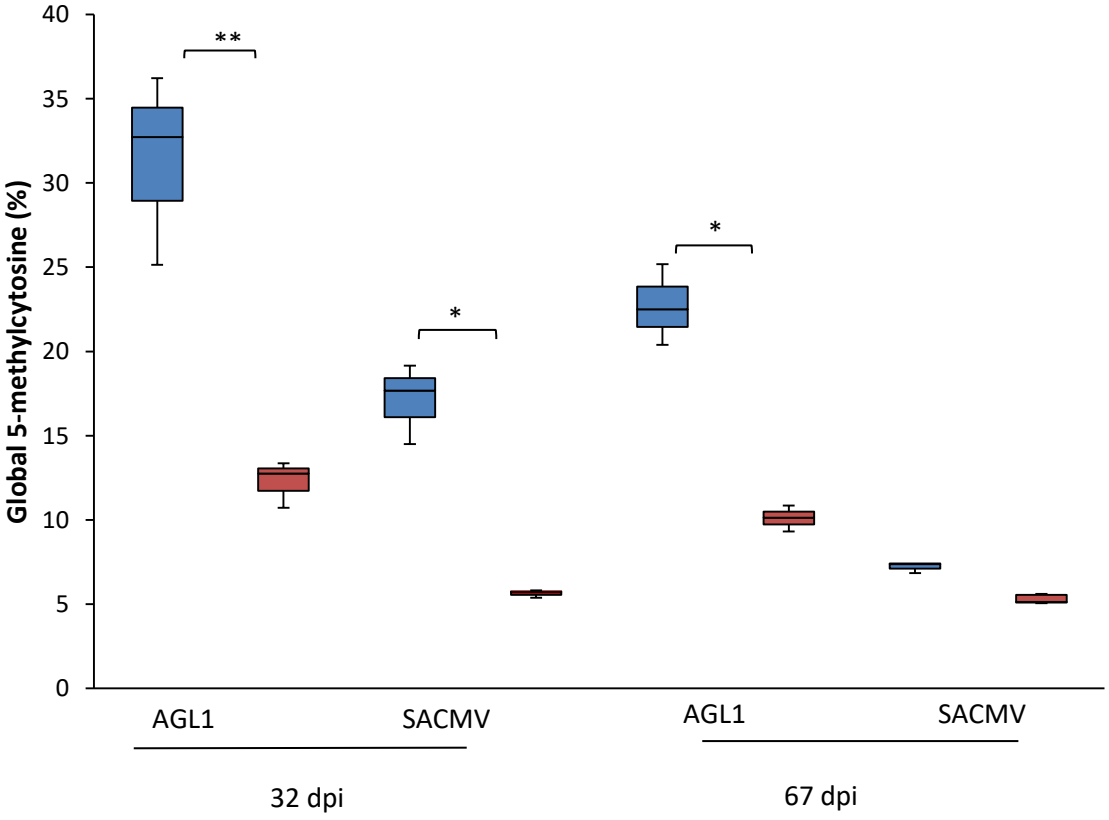

Figure 3.12: Comparison of global methylation between T200 and TME3 in AGL1 and SACMV inoculated plants.. This data was derived from ELISAs. Boxplots show that at 32 dpi T200 (blue) has significantly higher global methylation levels compared with TME3 (red). At 67 dpi, global methylation was only significantly higher in AGL1 inoculated T200 compared to TME3. While methylation was slightly lower in SACMV-infected TME3, this was not significant. The median is represented by the horizontal line within each boxplot. The lower and upper ends of boxes represent the first and third quartile respectively. Whiskers show the minimum and maximum values. Significant differences are indicated as *P < 0.050 and **P <0.010. Errors bars represent standard deviations of the mean (n=3).

3.3.2 DNA methylation by LC-MS

To verify whether a decrease in global DNA methylation corresponds with SACMV infection in T200 and TME3 plants, DNA methylation (5mC) and hydroxymethylation (5hmC) was quantified by LC-MS/MS. LC-MS/MS was used to further quantify the methylation differences between T200 and TME3 plants. To quantify the percentages of 5mC and 5hmC, standard curves were constructed from the measured 5mC or 5hmC peak/ total cytosine plotted against the ratio of either 5mC (Fig. 3.13A and B) the samples. At 32 dpi, in 5mC and 5hmC a similar trend in methylation pattern was noticed, where T200 plants had a higher methylation than TME3 plants (Fig. 3.14 A and B). In agreement with the ELISA method, a decrease in DNA methylation was noticed in SACMV-infected plants compared to AGL1 infected plants. The methylation (5mC) difference between AGL1 and SACMV was statistically significant in T200 plants (P < 0.010) and not significant in TME3 plants (Fig. 3.14). A difference in 5hmC between AGL1 and SACMV plants was picked up; however this difference is not statistically significant.

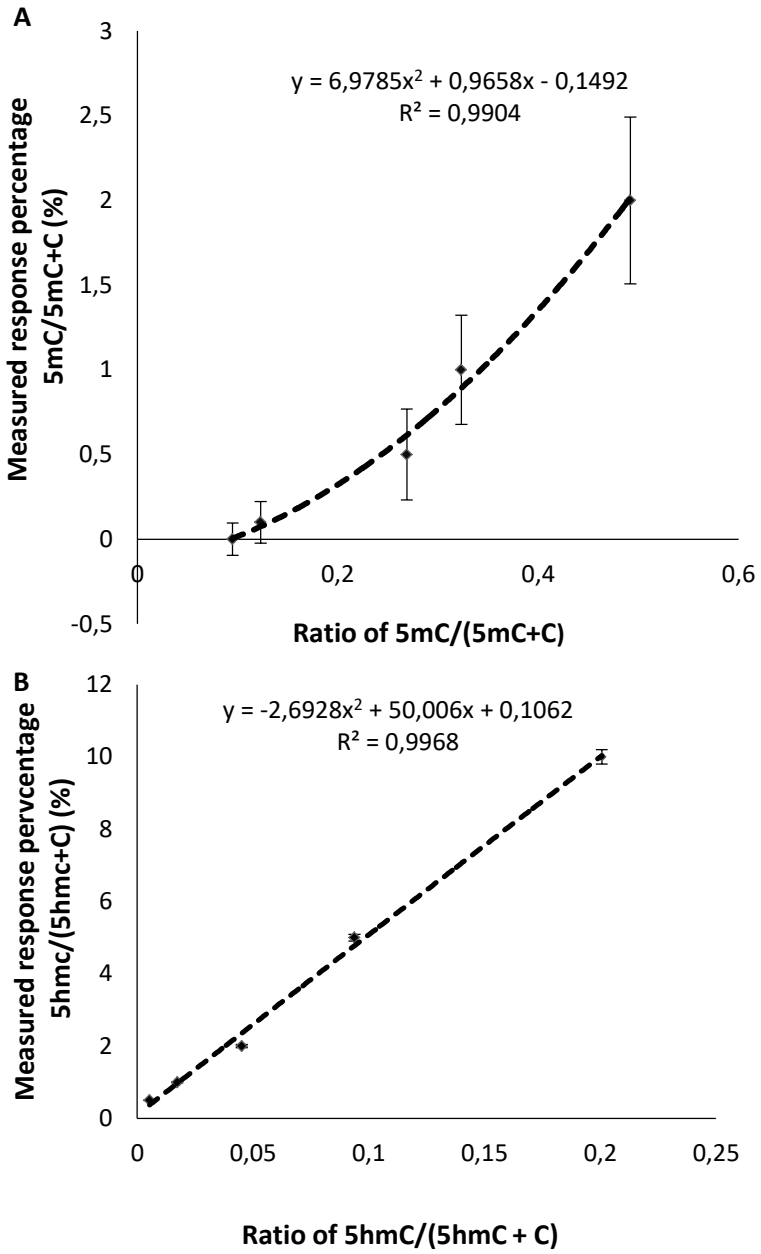

Figure 3.13: A representative standard curve for quantification of 5'-methylcytosine and 5'-hydroxymethylcytosine. The standard curve plots the average (n = 3) mean relative methylation area of DNA methylation in percentage and increasing ratio of methylated DNA in the total pool of cytosine. The r^2-value was 0.9904(A) and 0.9968(B). Error bars represent the standard deviations of the mean.

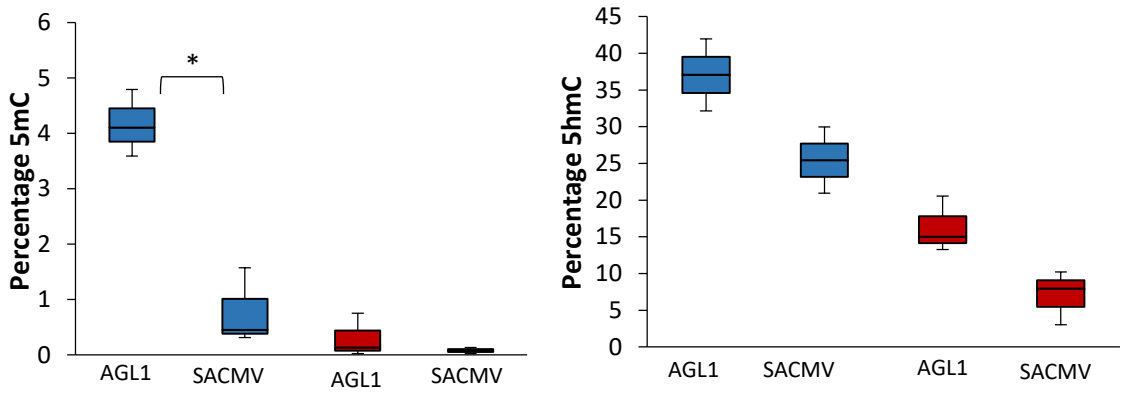

Figure 3.14: A Comparison in percentage DNA methylation (5mC) and hydroxymethylation (5hmC) between T200 and TME3 at 32 dpi. As assessed using LC-MS the boxplots show DNA methylation (5mC) and hydroxymethylation (5hmC) in T200 (blue) and TME3 (maroon) at 32 dpi. The methylation percentages shown by the boxplots represent the sample percentage which was extrapolated from the standard curve. There was a significant change in 5mC between AGL1 and SACMV in T200 plants. Statistical findings were indicated as *P < 0.050. Errors bars represent standard deviations of the mean (n=3).

3.4 Gene expression

3.4.1 SACMV down-regulates *de novo MET1* and *CMT3* expression In T200 and TME3 plants.

It has previously been reported that plant viral infections such as geminiviruses are associated with host DNA methylation and thus they interfere with transcriptional mechanisms regulated by epigenetic modification of host DNA. To assess whether the changes in DNA methylation in response to SACMV is associated with a change in the expression of mRNA levels of maintenance genes (*MET1 and CMT3*), expression of these genes was quantified by RT-qPCR. In T200 plants, SACMV significantly down-regulated the expression of *MET1* at 32 and 67 dpi relative to AGL1-inoculated plants (P < 0.010) (Fig. 3.15A). In TME3 plants, SACMV down-regulated the expression of *MET1* and it was significant at 32 dpi (P < 0.010) and not 67 dpi relative to AGL1-inoculated plants (Fig. 3.15B). A similar trend was noticed with *CMT3*, in T200 plants, where SACMV significantly down-regulated the expression of *CMT3* at 32 (P < 0.050) and 67 dpi (P < 0.010) relative to AGL1-inoculated plants (Fig. 3.15C). In TME3 plants, SACMV down-regulated the expression of CMT3 at 32 and 67 dpi, this was significant at 67 dpi (P < 0.050) (Fig. 3.15D).

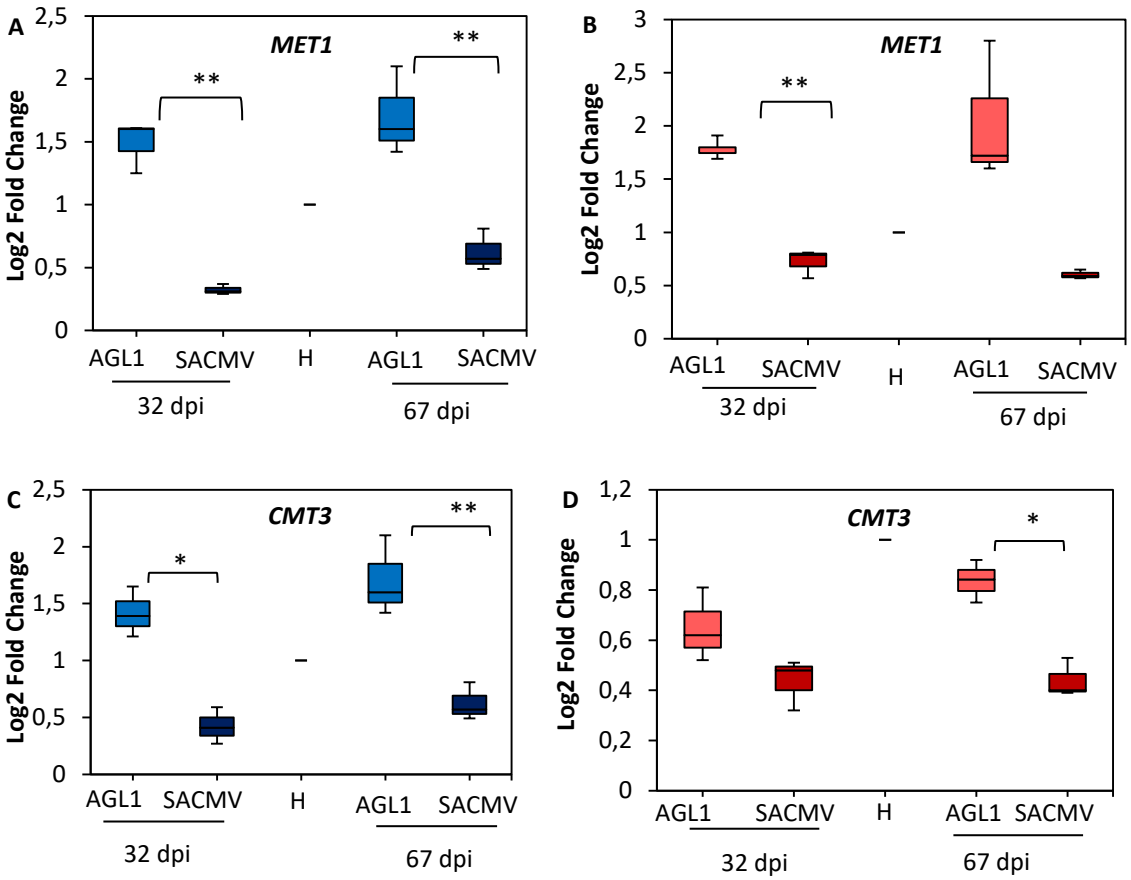

Figure 3.15: SACMV down-regulates the expression of CMT3 in TME3 and T200 at 32 and 67 dpi. Boxplots show differential gene expression of (A and C) T200, (B and D) TME3, fold change in response to SACMV, AGL1l, and healthy (H) which is used as baseline, quantified by RT-qPCR. Significances are indicated by *P < 0.050 and **P < 0.010. Errors bars represent standard deviations of the mean (n=3).

3.4.2 SACMV down-regulates *AG04* expression In T200 and TME3 plants.

To assess whether the changes in DNA methylation in response to SACMV relates to a change in the expression of *AGO4* (*de novo* methylation gene involved in the RdRM pathway), mRNA levels were quantified. SACMV down-regulated the expression of *AGO4* in T200 and TME3 at 32 and 67 dpi, however the result was only significant at 67 dpi in T200 plants and in TME3 plants it was significant at 32 dpi (P < 0.050) (Fig. 3.16 A and B).

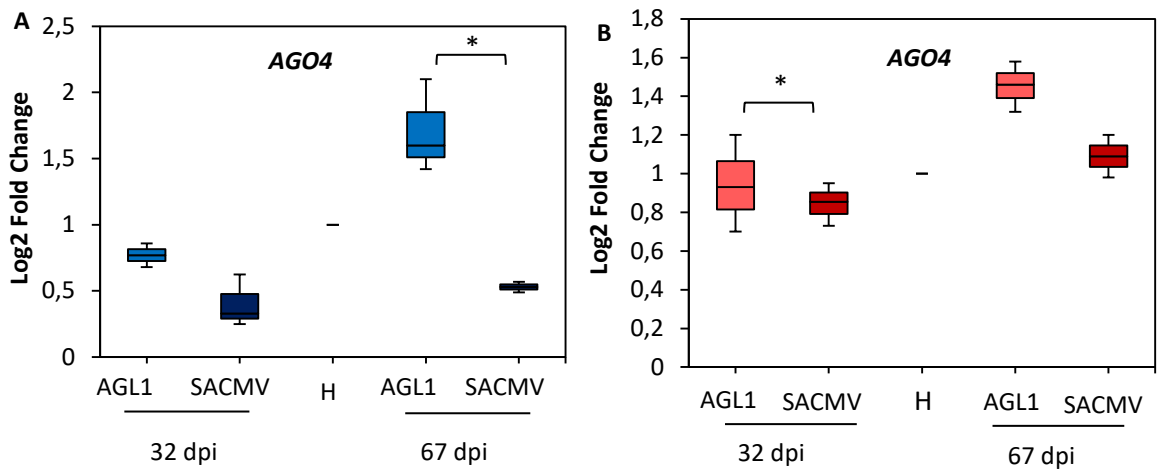

Figure 3.16: SACMV down-regulates the expression of *AGO4* in T200 and TME3.Boxplots show differential gene expression of (A) T200, (B) TME3, fold change in response to SACMV, Agl, and healthy (H) which is used as baseline, quantified by RT-qPCR. The median is represented by the horizontal line within each boxplot. The lower and upper ends of boxes represent the first and third quartile respectively. Whiskers show the minimum and maximum values. Significant differences are shown by *P < 0.050. Errors bars represent standard deviations of the mean (n=3).

3.4.3 SACMV down-regulates *SAM* expression in T200 and TME3 plants.

To assess whether the changes in DNA methylation in T200 and TME3 plants, in response to SACMV-inoculation relates to a change in the expression of the gene regulating the availability of methyl groups, *SAM*, the fold change was quantified. SACMV down-regulated the expression of *SAM* in T200 plants, at both 32 and 67dpi, relative to AGL1 (Fig.3.17A). Interesting, in TME3 plants, SACMV significantly down-regulated the expression of *SAM* at 32 and 67 dpi relative to AGL1 ($P < 0.010$) (Fig. 3.17B).

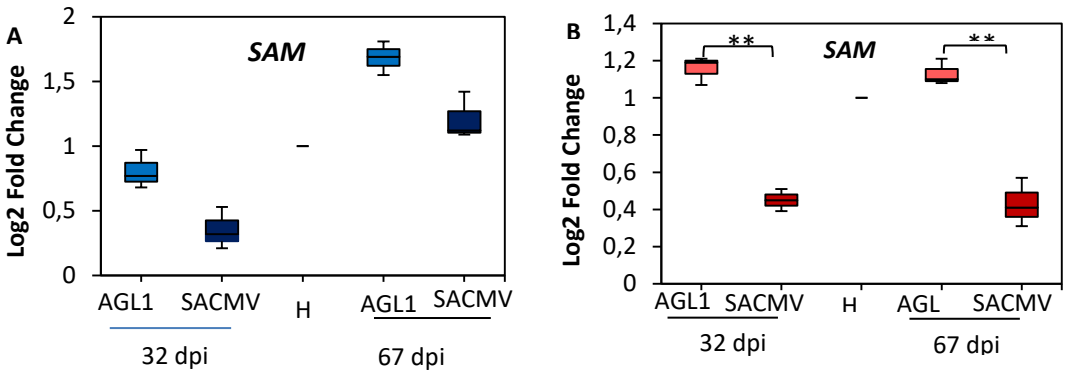

Figure 3.17: SACMV significantly down-regulates the expression of SAM in TME3 at 32 and 67 dpi. Boxplots show differential gene expression of (A) T200, (B) TME3, fold change in response to SACMV, Agl, and healthy (H) which is used as baseline, quantified by RT-qPCR. Significances are indicated are shown by *P < 0.050 and **P < 0.010. Errors bars represent standard deviations of the mean (n=3).

3.4.4 SACMV down-regulates *AGO5* expression in TME3 at 32 dpi, while inducing expression at 67 dpi

To assess whether the changes in DNA methylation in response to SACMV relates to a change in the expression of mRNA levels of a gene involved in the miRNA pathway, *AGO5* was quantified by RT-qPCR. SACMV had no significant change in T200 plants at 32 and 67 dpi, but significantly down-regulated *AGO5* in TME3 at 32 (P < 0.010) while significantly inducing it at 67 dpi (P < 0.010) relative to AGL1 (Fig. 3.18B).

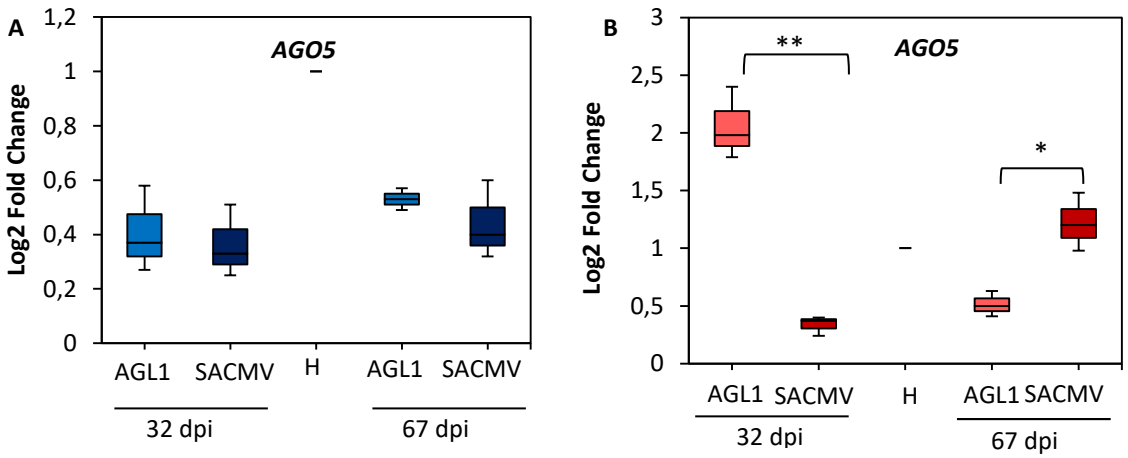

Figure 3.18: SACMV up-regulates the expression of AGO5 in TME3 at 67 dpi. Boxplots show differential gene expression of (A) T200, (B) TME3, fold change in response to SACMV, Agl, and healthy (H) which is used as baseline, quantified by RT-qPCR. Significances are indicated by *P < 0.050 and **P < 0.010. Errors bars represent standard deviations of the mean (n=3).

CHAPTER 4: DISCUSSION

Cassava is vulnerable to a number of begomoviruses that lead to CMD. Much like other plants, cassava has developed mechanisms such as employing cytosine DNA methylation as a defense against these geminiviruses. This study was aimed at investigating the global methylation response in susceptible (T200) and tolerant (TME3) cassava landraces upon infection with SACMV. A further comparison was done between the methylation changes between TME3 and T200. The results showed that SACMV decreased global methylation in cassava by down-regulating the expression of *MET1*, *CMT3*, *AGO4* and *SAM*, while up-regulating the expression of *AGO5* at 67 dpi in TME3 plants. This suggested that geminiviruses are successful at altering the host's methylation profile.

4.1 Quality Control

4.1.1 Nucleic Acids

The extracted nucleic acids which were obtained from leaf tissue material were intact. In this study, on average, the extracted DNA had a 260/280 nm ratio of 1.88 and a 260/230 nm ratio of 1.67. The quality of the overall extracted DNA was satisfactory; however it contained a bit of amounts of protein and phenol. According to Desjardins and Conkin, (2010) DNA absorbs light strongly at 260 nm. Thus a 260/280 nm ratio of 1.8 indicated pure DNA. Obtaining high quality and quantity DNA of good integrity was important as DNA methylation depends on the integrity of DNA. For RNA extractions, average extracted RNA had a 260/280 nm ratio of 2.0 and 260/230 nm ratio of 2.2, suggesting that RNA free from DNA and protein contamination was extracted. It was important to obtain RNA with an OD ratio of 2.0 or more because it is characterised as pure (Lucena-Aguilar *et al*., 2015)). Thus the slightest deviation from the 260/280 OD ratio of 2.0 may contain trace amounts of phenol (Bustin *et al*., 2009), thus interfering with downstream applications such as qPCR.

4.1.2 Primers for cDNA

The synthesized cDNA products were tested for primer specificity on a 2% agarose gel for all the genes. Firstly, the single band denoted a single product, where water was used as template. Additionally a single melt peak above the threshold on the qPCR melt curve was

detected. Smaller peaks that are below the threshold peak are not recommended as these can be a result of primer dimers.

4.2 Agroinoculation of cassava with SACMV infectious clones

Severity symptom scoring and plant height were the phenotypic analyses that were done to assess the viral progression effects. At 67 dpi, T200 plants inoculated with SACMV exhibited significantly higher symptom score than TME3 plants inoculated with SACMV. At 32 dpi, T200 plants inoculated with SACMV exhibited a significantly higher plant height than TME3 plants inoculated with SACMV even though both showed virus symptoms. This was expected as it has been reported that SACMV does not affect plant height (Allie *et al*., 2014). In this study Agrobacterium mock-inoculated plants did not develop any disease symptoms, which was expected as this bacterium does not cause disease symptoms in cassava. Agrobacterium is known as a common micro-organism used in research and biotechnology to generate transgenic plants successfully (Lacroix and Citovsky, 2016). The symptom severity score index (Fauquet *et al*., 1987) was used to measure the progression of symptom development over time in plants. In T200 plants the mean symptom severity score at 32 and 67 dpi revealed increasing development of symptoms overtime. The West African landrace TME3 with a marker-linked CMD2 resistant gene (Akano *et al*., 2002) had previously been reported to show resistance to CMD, however in this study we observed that TME3 gets infected by SACMV when agroinoculating with infectious clones of SACMV. The mean symptom severity score peaked at 32 dpi and leaves were fully symptomatic in TME3. At 67 dpi, the mean symptom severity score was lower than 32 dpi, interesting, in TME3 plants new emerging leaves were displaying milder symptoms (recovery phenotype) compared to T200 plants suggesting that TME3 is tolerant. T200 plants being a susceptible host showed a higher symptom score especially at 32 dpi, which was expected due to its high susceptibility to SACMV (Allie *et al*., 2014). In contrast, TME3 makes provision for the virus throughout its entire life cycle, and thus is able to recover after 67 dpi. Recovery is a phenomenon in certain perennial plants such as cassava that may prompt virus tolerance over the long duration of this perennial crop growth. Tolerant hosts are said to be able accommodate virus replication but at at lower levels by modulating the host transcriptome response (Allie *et al*., 2014) and eliciting a recovery from viral-symptoms (Bengyella *et al.,* 2015). New emerging T200 leaves failed to recover from severe symptoms at 67 dpi. In contrast , TME3

new leaves showed no to reduced symptoms. In cassava, recovery was observed in another study (Chellappan *et al.,* 2004), where infection with African cassava mosaic virus (ACMV) and Sri Lankan cassava mosaic virus (SLCMV) displayed symptom reemission over time. Phenotypic recovery is a well-studied phenomenon across several plant species infected with geminiviruses (Hagen *et al.*, 2008; Rodriguez-Negrete *et al.*, 2009; Sahu *et al.*, 2010; Ghoshal & Sanfacon, 2015).

Virus load of SACMV DNA A generally correlated with virus symptom severity. At 32 and 67 dpi, T200 plants had significantly higher viral fold change than TME3 plants. For both susceptible T200 and tolerant TME3, viral load peaked at full systemic infection (32 dpi). However, at 32 and 67 dpi, T200 plant had significantly higher viral fold than TME3 plants. Significantly, T200 plants had the highest viral fold change at 32 and 67 dpi, and this correlated with the average severity symptom score. The progression in infection in T200 may be attributed to suppression of transcription at 32 and 67 dpi. The expression of distinctive resistance gene analogs (RGAs) is associated with tolerance and recovery observed in TME3. Non-recovery to SACMV in T200 could possibly be associated with the number of RGAs downregulated (Allie *et al*., 2014; Bengyella *et al*., 2015). Pierce and Rey (2013) demonstrated a similar viral trend like that in cassava of virus suppression using an *Arabidopsis*-SACMV pathogen model system. Furthermore, higher infection in T200 may also be due to suppression of basal immunity and RNA silencing (Kumar *et al*., 2015; Zhang *et al*., 2018; Zhan *et al*., 2019). Chellappan *et al.,* (2004) went on to report that the positive phenotype recovery in cassava from African cassava mosaic virus (ACMV) and Sri Lankan cassava mosaic virus (SLCMV) is a consequence of the production of virus-derived small interfering RNAs (siRNAs) via PTGS. Profiling of siRNAs and miRNAs in SACMV-infected cassava T200 plants support the role of small RNAs in suppression of RNA silencing (Rogans & Rey, 2016; Bizabani *et al*., 2021). Tolerance has been associated with a lower accumulation of viral RNAs (Paudel & Sanfaçon, 2018). Tolerance and susceptibility in TME3 and T200, respectively, have also been linked to resistance genes (Louis and Rey, 2015). Additionally, it is possible that transcriptional silencing of the SACMV ssDNA genomes A and B could be silenced, reducing virus load in TME3. In 2015, Ghoshal and Sanfacon discussed that DNA viruses such as geminiviruses can achieve recovery via 24 nt siRNA-mediated transcriptional gene silencing (TGS) of viral mini-chromosomes. In T200 and TME3, 24 nt

siRNA populations were identified as the largest class for virus-derived small RNAs (vsRNAs) mapping to SACMV DNA A and DNA B (Rogans *et al.,* 2016). The aforementioned study further went on to show an increased number of 24 nt vsRNAs at recovery stage (67 dpi) in TME3 which was suggested to be as a result of TGS of genomic SACMV DNA. Our study has confirmed that T200 displayed severe symptoms compared and TME3 displayed symptom recovery at 67 dpi. Furthermore, T200 showed a higher viral load than TME3.

4.3 Global DNA methylation

4.3.1 Global methylation decrease in SACMV-inoculated plants compared to AGL1-inoculated plants

Global methylation of the cassava genome decreased in SACMV-inoculated T200 and TME3 plants compared to mock AGL1-inoculated plants at 32 and 67 dpi. The decrease in global methylation levels in SACMV-inoculated plants shows that geminiviral pathogens such as SACMV target methylation related pathways by repressing genes involved in methylation This is in agreement with Li and Wang (2019), who reported that viral suppressors interact with methyl cycle enzymes to regulate host methylation status. Previously studies have shown that viral infections can induce epigenetic modifications in plants (Duan *et al*., 2012; Yang *et al*., 2011, 2016; Ye *et al*., 2016; Zhang *et al*., 2011; Zhao *et al*., 2016; Diezma-Navas *et al*., 2019). One way in which plants establish global DNA methylation is through 24nt siRNAs that can direct RNA-directed DNA methylation (RdDM) and transcriptional gene silencing (TGS). Plants use DNA methylation as a mechanism to protect against invading viral pathogens, so as to retain their genome stability and gene regulation, and therefore it is not surprising that viruses would suppress TGS in order to establish a successful infection, especially since they replicate in the nucleus (Rajeevkumar *et al*.,2015). It had been hypothesized that SACMV-inoculated plants would increase methylation, however results herein indicate that host methylation is targeted by geminiviruses.This is the first in vivo study indicating that hypomethylation may be a defence strategy of cassava whitefly-transmitted begomoviruses causing cassava mosaic disease (CMD).

Previous studies have demonstrated that geminivirus evolved to counteract repressive host methylation as this would avoid methylation of their DNA genomes. For example, Buchmann *et al*., (2009) reported the suppression of TGS by geminiviral proteins AC2 and C2

which interfere with *Nicotiana benthamiana* host methylation. In their study they found a 14% decrease of the total methylated cytosine residues of *Nicotiana benthamiana* plant genome that *Potato virus X* expressing the AC2 protein. The AC2 is a known suppressor of host gene silencing, allowing geminiviruses to establish infection (Vanitharani *et al*., 2004; Yang *et al*., 2007; Guerro *et al*., 2020; Soitamo *et al*., 2012). They also further proved for the first time that geminiviral proteins can silence TGS. In a study done by Castillo-Gonazlez *et al*., (2015) they found that the geminivirus encoded transcriptional activation protein (Trap/AC2/C2) reduces CHH methylation in the host and possibly in the viral genome too by inhibiting the activity of kryptonite (KYP). Kryptonite plays a crucial role in TGS in the host and thus reprogramming of this component interferes with DNA and histone methylation. The decreased methylation in SACMV-inoculated T200 and TME3 appears to be a common response to viral infection of plants. For example, a study by Butterbach *et al.,* (2014) showed that upon mixed infection in tomato with the monopartite begomovirus *Tomato yellow leaf curl virus* (TYLCV) and *Cucumber mosaic virus* (CMV), a tripartite ssRNA cucumovirus, genome hypermethylation is compromised because the *Ty*-1-encoded RNA-dependent RNA polymerase encoding gene that confers resistance is suppressed during infection. In summary, we propose that SACMV infection induces an epigenetic modification (hypomethylation) in susceptible cassava T200 and TME3 plants, in order to avoid de novo methylation of DNA A and B that may be targeted by via cytosine and histone methyltransferases during replication of DNA minichromosomes in the nucleus. Geminiviral DNA is known to form minichromosomes with histone proteins in the nucleus (Piedra-Aguilera *et al*., 2019; Luna and Lozano-Durán, 2020).The reported hypomethylation in our study is suggested to be correlated to a SACMV-mediated decrease in methyltransferase expression. Methyltransferase1 (*MET1*) is regulated by DNA methylation because geminivirus counteract silencing pathways via viral suppressors of RNA silencing by interacting with host siRNAs. Geminiviruses thus trigger silencing of defense genes, including methylation related genes.

4.3.2 Higher global DNA methylation in T200 (susceptible) host compared to TME3 (tolerant) landraces

Global methylation is significantly higher in T200 compared to TME3 in both AGL1 control and SACMV infected plants at 32 dpi (Figure 3.12), but only in AGL1, and not SACMV-

infected, inoculated plants at 67 dpi. Notably there is no significant difference at 67 dpi in methylation levels between T200 and TME3 in SACMV infected plants, suggesting that host DNA methylation levels are not greatly altered at this later stage of infection compared to systemic infection (32 dpi) when the virus is replicating and spreading quickly. Methylation also seems to fluctuate more in susceptible T200 between control and infected plants compared to TME3 which appears to be more stable and whose levels are always lower than T200 under all treatments. This stability in TME3 may also be a hallmark of its tolerance and recovery phenotype. . The transcriptional control of defence genes by DNA methylation and demethylation is a central regulatory process in plants. Lower global methylation in TME3 plants suggests that R genes may be less highly downregulated than in T200 plants, and this may contribute to tolerance and recovery at 67 dpi observed in TME3 (Allie *et al*., 2014). Recovery is an important phenomenon because it has been associated with PTGS (Chellapan *et al.,* 2004) and TGS (Rodriguez-Negrete *et al.,* 2009; Butterbach *et al.,* 2014). It was demonstrated by Raja *et al.,* 2008 that plant hosts employ methylation as an epigenetic defence by methylating viral DNA. While PTGS and TGS of geminiviral DNA has been demonstrated in other studies, the association of viral genome methylation in recovery in SACMV-infected tolerant TME3 is not proven. However, the association with methylation of geminiviruses with recovery is widely established, for example in Beet curly top virus (BCTV) (Akbergenov et al, 2006), Mungbean yellow mosaic India virus (Yadav & Chattopadhyay, 2011), and ACMV in cassava (Ermak *et al*., 1993; Chellappan *et al*., 2004 Akbergenov *et al.*, 2006). RNA silencing pathways contribute to recovery as a consequence of virus-derived small RNAs (vsRNAs) targeting the invading virus. This recovery is mainly attributed to methylation (accumulation of 24 nt siRNA-mediated TGS). Additionally, PTGS (21-23 nt siRNA mediated) is also involved in resistance, tolerance and recovery to geminiviruses. For example, recovery was reported in cassava and *Nicotiana benthamiana* infected with ACMV-[CM] (Chellappan *et al*., 2009) and in *Capsicum annuum* L. infected with Pepper golden mosaic virus (PepGMV) (Rodriguez-Negrete *et al.,* 2009), leading to a lower virus titre was observed. In a study conducted by Bengyella *et al.*, (2015) they found that the number of resistance (R) genes downregulated in T200 are much higher than those in TME3 plants. Therefore, it appears that virus infections cause global changes in the host transcriptome. Many of these changes are mediated through methylation-associated regulation. T200 plants also display massive disturbances in growth and development as a

result of downregulated genes (Allie *et al.,* 2014). Higher global genomic methylation in T200 correlates to the transcriptome data (Allie *et al*., 2014; Bengyella *et al*., 2015) where the number of downregulated genes at 32 dpi (1000) and 67 dpi (800) were higher than in TME3 (150 and 200 genes at 32 and 67 dpi, respectively). It is hypothesised that this could be, in addition to other regulatory factors, attributed to hypermethylation in the susceptible T200 landrace compared to SACMV-tolerant TME3.

4.3.3 LC-MS method for validation of ELISA results

The LC-MC/MS method measured the global 5-methylcytosine (5mC) and 5-hydromethylcytosine (5hmC) patterns. The results revealed that global 5mC and 5hmC in SACMV-inoculated plants was lower than AGL1-inoculated plants at 32 dpi. However, the 5-hmC modification in cassava T200 and TME3 plants was below detectable limits at 67 dpi. Several studies have reported that the 5hmC in plants is a DNA modification that is biologically low in abundance in the plant genome (Uchida *et al*., 2013, Liu & Hesson, 2013; Jang *et al*., 2014; Erdmann *et al*., 2015; Mahmood & Dunwel, 2019). Despite different methylation levels between the two techniques, both methods that were used in our study reveal a similar trend, in that T200 displayed a higher level of global methylation compared to TME3. The differences between the two methods may be attributed to the fact that LC-MS/MS has high specificity compared to the ELISA. Using ELISA to quantify global DNA methylation a much faster and cost effective way, in addition to that this method requires a minimum of 10 ng of extracted DNA thus allowing investigations with minimal DNA concentrations which is more advantageous compared to LC-MS/MS that required 500 ng of extracted DNA per sample (Wei *et al*., 2010). However, ELISA based methods are prone to high inconsistency and thus they can only be used to get an approximation of global methylation (Kurdyukov & Bullock, 2016). In light of this study result, LC-MS/MS should be forerunner method for studying methylation where specificity and sensitivity are concerned.

4.4 Expression of genes involved in DNA methylation

4.4.1 SACMV decreases the expression of MET1 and *CMT3* in both T200 and TME3

In T200 plants, SACMV significantly downregulated the expression of *MET1* and *CMT3* at 32 and 67 dpi compared to AGL1 plants. This downregulation correlated with the decrease in

global methylation. In TME3 plants, SACMV also downregulated the expression of *MET1* and *CMT3* at 32 dpi compared to AGL1, however *MET1* and *CMT3* expression was not significantly downregulated at 67 dpi. *MET1* and *CMT3* are known as methyltransferase genes that are responsible for symmetric (CG and CHG) cytosine methylation maintenance in plants (He *et a*l., 2011; Ashapkin *et al*., 2016; Yang *et al.*, 2016). Cytosine methylation plays a vital role in defending the genome against invading viral DNA, and it also directs which genes are to be switched on or off thus promoting TGS. Results herein therefore propose that MET1 and CMT3 may play a role in methylation of SACMV DNA at the recovery phase of TME3 at 67 dpi, leading to a lower virus load observed (Allie *et al*. 2014). However geminiviruses inhibit methylation-mediated TGS as a counter defense against the host. For example, *Tomato yellow leaf curl Sardinia virus (TYLCSV)* reduced the expression levels of maintenance methyltransferase genes in *Nicotiana benthamiana,* a similar trend observed in T200 at 32 dpi and at 67 dpi, which may have prevented recovery in T200. It was also established that the expression of *TYLCSV* replication associated protein (Rep) in *Arabidopsis* downregulated the expression of the *MET1* and *CMT3* genes thus inferring that the geminiviral protein Rep which is responsible for the repression of *MET1* and *CMT3* (Rodriguez-Negrete *et al.,* 2013). This correlates with the findings of this study, suggesting that SACMV Rep is involved in suppression of *MET1* and *CMT3* expression in both TME3 and T200 at systemic infection stage (32 dpi), leading to a global methylation decrease in SACMV-inoculated plants compared to AGL1-inoculated control plants. It is additionally proposed that higher SACMV replication and higher expression levels of Rep in T200 compared to TME3 may contribute to the repression of *MET1* and *CMT3* and higher global methylation observed in SACMV-infected T200 compared to TME3. Interestingly, in SACMV-infected TME3 plants, expression of *MET1* and *CMT3* was not significantly downregulated at 67 dpi compared to AGL1, strongly supporting the hypothesis that recovery in TME3 at 67 dpi is associated with the number of SACMV-responsive plant resistance gene analogs (RGAs) in TME3 compared to T200. It is concluded that SACMV interferes with host plant defence mechanisms to favour its replication, which successfully leads to a decrease in the expression of methylation genes, and consequently reducing global methylation.

4.4.2 SACMV decreased expression of *AGO4* in T200 and TME3 plants

A decrease in the expression of *AGO4* was observed in both SACMV-infected T200 and TME3. *AGO4* in plants is involved in the transcriptional gene silencing pathway (TGS), and recruits 24-nt viral siRNA as a means to methylate invading viral nucleic acids (Vinutha *et al.*, 2018). Plants control gene expression through methylating cytosine residues, and this can be done by *de novo* methylation during TGS via RNA-directed DNA Methylation (RdDM). Thus, host gene silencing system intersects with the activity of RNA-directed DNA Methylation (RdDM) to regulate gene expression and protect against viruses, transgenes, transposons and retransposons. Various studies have proven that geminiviruses counteract this defence by interfering with the plants methylation mechanism (Yang *et al.,* 2011; Rodríguez-Negrete *et al*., 2013). This counter defence could suggest that SACMV interrupts the TGS pathway thus down regulating the expression of *AGO4* to favour its accumulation. Vinutha *et al*., 2018 showed that geminiviruses encoded viral suppressor of RNA silencing (VSR) such as AC4 interacts with *AGO4* in tomato by halting the formation of the RISC assembly, and alters the host methyl cycle to favour the replication of *Tomato leaf curl New Dehli virus* (*ToCLDNV*).

4.4.3 *SAM* expression is decreased in SACMV infected plants

In T200 and TME3 plants, SACMV downregulated the expression of *SAM* at 32 and 67 dpi compared to AGL1 plants. *SAM* is a methyltransferase that is known as the universal cofactor and plays a vital role as a signal molecule (Struck *et al*., 2012; Mäkinen and De, 2019). In a study done by Yang *et al*., (2011) and, it was demonstrated that *Tomato yellow leaf curl China virus* (TYLCCV) with its beta-satellite (βC1) can reverse TGS by targeting the methyl cycle so as to limit the production of *SAM* by blocking the activity of S-adenosyl homocysteine hydrolase thus reducing host genome cytosine methylation (Buchmann *et al*., 2009; Zhang *et al.*, 2011). South African cassava mosaic virus possess the βC1 protein and thus the down regulation of *SAM* during infection of T200 and TME3 plants could be attributed to the fact that βC1 inhibits the activity of SAM during viral infection.

4.4.4 SACMV increases expression of *AGO5* in TME3 but not in T200 plants

In TME3 plants, SACMV up-regulated the expression of *AGO5*, but downregulated the expression of *AGO5* in T200 plants at 67 dpi compared to AGL1 plants. Concurrent with the

increase in global methylation in T200 plants, we observed an increase in the expression of *AGO5* in T200 at 67 dpi. *AGO5* is involved in miRNA (microRNA)-directed target cleavage in Arabidopsis (Thieme *et al*., 2012), where *AGO5* specifically targets microRNA156 (miRNA156). miRNA156 (miR156) functions by suppressing the transcripts of SQUAMOSA-PROMOTER BINDING LIKE (SPL) transcription factors. SPL proteins form a major family of plant-specific transcription factors that play important roles in plant growth and development. Recently it was established by Bizabani *et al*., 2021 that members of the mes-miR156 family target the SPL family in both TME3 and T200 however mesi-miRNA156 was downregulated in TME3 and the SPL was up-regulated. So it is suggested that the up-regulation of *AGO5* in TME3 plants at 67 dpi would result to the downregulation of mesi-miRNA156 and up-regulation of SPL, and thus SPL could be involved in growth recovery in TME3 plants.

Based on this study hypomethylation was observed and the expression of MET1, CMT3, AGO4 and SAM was down-regulated in T200 and TME3 plants but interestingly AGO5 expression was up-regulated in TME3 plants. Therefore, showing that methylation is one of the targeted pathways during viral infections and that epigenetic function can be mediated by various transcriptional pathways.

CHAPTER 5: CONCLUSIONS

5.1 Rationale and findings of the study

This study examined the effects of SACMV-infection on epigenetic mechanisms governing the change in global DNA methylation in T200 and TME3 plants, and to observe its effects on mock-inoculated plants in comparison to SACMV-inoculated plants. SACMV decreased global methylation in T200 and TME3 plants by downregulating the expression of *CMT3, MET1, SAM* and *AGO4*, demonstrating that SACMV interferes with host methylation.. This study is the first proposing the association of epigenetic and transcriptional regulatory mechanisms of SACMV-infected cassava plants. However, SACMV infection resulted in a significant decrease in global methylation (hypomethylation) in T200 compared to AGL1 control plants at both time points, while no significant decrease in methylation in TME3, compared to AGL1-inoculated controls, was observed at both 32 and 67 dpi. From this we can conclude that hypomethylation is associated with susceptibility in T200 and not in TME3.

5.2 Implications of the study

We conclude that SACMV infection leads to downregulation of the expression of CMT3, MET1, AGO4 and SAM in cassava plants, which effects methylation of host genes and possible suppression of host defence. This implies a necessity to develop biotechnology techniques that will aid cassava plants to prevent the geminiviral infection from counter host defence, and maintain a strong epigenome.

5.3 Limitations of the study

A limitation in this study was that we did not analyse the role of demethylation genes, or the protein level of the genes involved in methylation. The enzyme activity of the methylation genes was also not measured. However, it can be suggested that there is an increase in enzyme activity because of the changes in the global methylation status. We also know that methylation status is not only limited to methylation related genes but also to the host response genes.

5.4 Future work

Future work could include, looking at the response to SACMV and measuring the expression levels of demethylation genes, as well as look at how demethylation genes affect global DNA methylation. Moreover, future research should also include the observation of the interaction between histone and DNA modifications at the same time. Additionally a metabolome study can be done to understand the model interaction between geminivirus infection in host plants.

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