Molecular studies on the interactions between African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV-UG) infecting cassava (*Manihot esculenta* Crantz)

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Saadia Naseem

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Referent:

Prof. Dr. Edgar Maiß

Korreferent:

Prof. Dr. H.-J. Jacobsen

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“In gratitude

to my lovely parents

who have encouraged me;

to my husband ‘Zahid’ & my daughter

‘Bakhtawar’

who have endured me”

Abstract

Cassava mosaic disease (CMD) caused by whitefly transmitted cassava mosaic viruses from the *genus* begomovirus is a most serious impediment to cassava cultivation in Africa and India. While several diverse begomoviruses are implicated in CMD in Africa, *African cassava mosaic virus* (ACMV) widespread in the continent and *East African cassava mosaic virus* Uganda (EACMV-UG) now present in East and central Africa present the principle components of the disease. Both viruses have an overlapping geographic distribution and when present, mixed infections frequently occur. Very severe mosaic disease symptoms in cassava are associated with double infections and synergism on symptoms and virus replication were reported. This study presented here is focusing on the interactions between ACMV and EACMV-UG in double infections of *N. benthamiana* and cassava. This is to attempt to uncover the crucial factors responsible for virus interactions leading to synergism.

Virus concentration was considered to describe synergistic interactions between ACMV and EACMV-UG viruses in cassava. For this purpose the amount of viral genomic components in cassava infections was determined, in absolute and relative quantification experiments, by quantitative PCR. Virus concentration was much higher in symptomatic leaf tissues compared to non-symptomatic leaves and corresponded with the severity of disease symptoms. Much higher virus titres were generally recorded for EACMV-UG compared with ACMV. Mixed infections with both viruses resulted in severe disease symptoms but only a slight increase of EACMV-UG was found. Relative quantification of virus genomes in mixed infections showed higher concentrations of EACMV-UG DNA-A compared to ACMV DNA-A but a drastic reduction of EACMV-UG DNA-B. ACMV concentrations in mixed infections appeared to be unaffected by presence of EACMV-UG with concentrations similar to single infections. The higher concentrations of EACMV-UG DNA-B compared to EACMV DNA-A accumulation in single infections was consistent and irrespective of cassava cultivar and its tolerance status.

One explanation for synergism is that virus movement and spread and/or tissue tropism and invasion is altered in dual compared to single infections. To study this *in vivo*, genome components of infectious ACMV and EACMV-UG virus clones were modified by inserting the gene for green fluorescent protein (GFP) in place of the coat protein (CP) gene and alternatively, by replacing either the BV1 or BV2 genes on the DNA-B genomic components with GFP. All efforts generating infectious virus mutants expressing GFP failed and CP DNA-A mutant viruses did not induce infections in *Nicotiana benthamiana* when inoculated with their cognate DNA-B. Initial GFP fluorescence found in some epidermal cells inoculated with mutated viruses did not develop into systemic signals indicating for GFP expression from replicating virus. Likewise, mixtures of DNA-A and DNA-B with replacements of BC1 or BV1 with GFP did not result in viable virus and GFP expression was only observed in patches of inoculated cells. From this study, it can be assumed that GFP cannot be used as a live monitor to follow replicating cassava mosaic viruses and destructive *in situ* tests are required to resolve virus interactions on a subcellular basis.

Virus-virus interaction in mixed infections and synergism has been postulated as a result from suppression of the host defence mechanism. To study the role of virus genes which are implicated in suppression of silencing mechanisms, ACMV and EACMV-UG AC2 and AC4 genes were isolated and functionally characterized for their activity on the post-transcriptional gene silencing (PTGS) pathway. An *Agrobacterium* based leaf infiltration assay using GFP as a reporter was approached to reveal AC2 and AC4 suppression activity on early stages of post-transcriptional gene silencing. These transient gene expression assays showed that the activity of AC2 to suppress PTGS is weak, while for AC4 an activity on PTGS was not found.

Transient expression of AC2 and AC4 and subsequent infection with ACMV resulted in a sharp increase of ACMV concentration in plants infiltrated with either ACMV or EACMV AC2. This study was extended to assays with AC2 and AC4 transgenic plants. *N.*

benthamiana harbouring AC2 or AC4 genes showed a variety of phenotypic alterations which were most pronounced with ACMV AC2 transgenes. In contrast to transient assays however, challenge inoculations of AC2 or AC4 transgenic plants with ACMV did not result in considerable differences between virus concentrations in transgenic plants compared to infections of wild type *N. benthamiana.*

Key words: African cassava mosaic virus, East African cassava mosaic virus Uganda, mixed infection, synergism, tissue tropisms, AC2, AC4, gene silencing suppression, agroinfiltration, quantitative PCR, GFP fluorescence, transient gene expression.

Zusammenfassung

Die Cassava Mosaikkrankheit (CMD), die durch weiße Fliege übertragene Mosaikviren der Gattung Begomovirus hervorgerufen wird, stellt ein enormes Problem und ernste Bedrohung für die Maniokproduktion in Afrika und Indien dar. Verschiedene Begomoviren können Mosaikkrankheiten auslösen jedoch sind in Afrika das *African cassava mosaic virus* (ACMV), das in allen Cassavagebieten vorkommt, und das *East African cassava mosaic virus* (EACMV-UG) das in Ost- und Zentralafrika verbreitet ist, die bedeutendsten Verursacher der Krankheit. Die geographische Verteilung beider Viren überschneidet sich und so treten häufig Mischinfektionen auf, die mit sehr schweren Symptomen der Mosaikkrankheit einhergehen. Mischinfektionen beider Viren sind mit synergistischen Interaktionen verbunden, die sich in Symptomen und Virusreplikation auswirken. In der hier dargestellten Arbeit sollten Interaktionen zwischen ACMV und EACMV-UG in dualen Infektionen von *N. benthamiana* und Maniok untersucht werden, um die Faktoren zu beschreiben, die zu Synergismus führen.

Zunächst sollte die Konzentration der Viren in Maniok zur Beschreibung der synergistischen Interaktionen zwischen ACMV und EACMV-UG herangezogen werden. Zu diesem Zweck sollten die Genomkomponenten der Viren in absoluten und relativen Werten quantifiziert werden. Hierfür wurde ein quantitatives PCR Verfahren aufgebaut, das es ermöglichte alle viralen Genomkomponenten spezifisch zu quantifizieren. Viruskonzentrationen waren in symptomatischen Blattgeweben viel höher als in nicht-symptomatischen Blättern und mit der Schwere der Symptome korreliert. Im Allgemeinen waren sehr viel höhere Virustiter für EACMV-UG im Vergleich zu ACMV festzustellen. Mischinfektion mit beiden Viren ergaben schwere Krankheitsphänotypen, waren jedoch nur mit geringfügigen Zunahmen von EACMV-UG verbunden. Die relative Quantifizierung der Virusgenome in Mischinfektionen zeigte höhere Konzentrationen von EACMV-UG DNA-A und eine erhebliche Reduktion der EACMV-UG DNA-B Genome,

während die ACMV Replikation von Mischinfektionen mit EACMV-UG unberührt zu sein schien und ähnliche Konzentrationen wie in Einzelinfektionen aufwies. Die in Einzelinfektionen stets höheren Konzentrationen von EACMV-UG DNA-B im Vergleich zu DNA-A, waren ungeachtet des Maniokkultivars und seines Toleranzstatus zu beobachten.

Eine Erklärung für Synergismus ist die in Mischinfektionen veränderte Virusausbreitung und/oder Gewebetropismus und –besiedelung. Um dies *in vivo* zu studieren, wurden Genombestandteile von infektiösen ACMV und EACMV-UG Virusklonen durch Einfügung des „green flourescence protein“ Gens (GFP) anstatt des Hüllproteingens (CP) oder in DNA-B, durch Ersatz der BV1 oder BV2 Gene modifiziert. Alle Bemühungen infektiöse Viren, welche ein funktionales GFP als Reporter exprimieren, herzustellen führten nicht zum Erfolg. DNA-A Hüllproteinmutanten, die mit entsprechender DNA-B gemischt und inokuliert wurden waren in *N. benthamiana* nicht infektiös und die anfänglich sichtbare GFP Fluoreszenz blieb auf wenige epidermalen Zellen beschränkt. Sie entwickelte sich nicht zu systemischen Signalen, die auf GFP Expression durch replizierendes Virus hinwiesen. Ebenso waren Mischungen von GFP an Stelle von BC1 bzw. BV1 in DNA-B mit entsprechender DNA-A nicht infektiös, und auch hier blieb die GFP Expression auf wenige Zellen beschränkt. Es muss davon ausgegangen werden, dass GFP als Vitalmarkierung für ACMV und EACMV-UG nicht verwendet werden kann.

Virus-Virus Interaktion in Mischinfektionen und Synergismus ist ein Resultat von unterdrückten Abwehrmechanismen der Wirtspflanze. Um die Rolle von Virusgenen zu studieren die solche Abwehrmechanismen supprimieren, wurden AC2 und AC4 Gene von ACMV und EACMV-UG isoliert und funktional charakterisiert, um deren Aktivität auf das post-transkriptionale „gene silencing“ (PTGS) zu beschreiben. In Agrobakterium-Infiltrationstests unter Verwendung von GFP als Reporter wurden AC2 und AC4 transient in *N. benthamiana* Blättern exprimiert und auf PTGS Aktivität geprüft.

Transiente Genexpressionstudien zeigten, dass die Aktivität von AC2 nur schwach ist und für AC4 keine Aktivität festzustellen war. Transiente AC2 Genexpression im Virustest führte jedoch zu einem steilen Anstieg der ACMV Konzentration in infiltrierten Pflanzen, die mit ACMV oder auch mit EACMV AC2 behandelt waren. Diese Studien wurden in AC2 und AC4 transgenen *N. benthamiana* Pflanzen weitergeführt. Zunächst zeigten solche transgene Pflanzen eine Vielzahl phänotypischer Veränderungen, die für ACMV AC2 Transgene besonders ausgeprägt waren. Im Gegensatz zu den vorher durchgeführten transienten Expressionsstudien wurden keine wesentlichen Unterschiede zwischen Virusinfektionen in transgenen und Wildtyp Pflanzen in Virusuntersuchungen mit ACMV festgestellt.

Schlagworte: *African cassava mosaic virus, East African cassava mosaic virus* Uganda, Mischinfektion, Synergismus, Gewebeausbreitung, AC2, AC4, post-transkriptionales „Gene silencing“, PTGS, Transiente Genexpression, GFP Fluoreszenz, Agroinfiltration, quantitative PCR.

Contents

Abbreviations

µl	Microlitre
35S	CaMV / CaVMV promoter
A. tumefaciens	*Agrobacterium tumefaciens*
Aa	Amino acid
AC1	ORF 1 on the complementary-sense strand of DNA-A
AC2	ORF 2 on the complementary-sense strand of DNA-A
AC3	ORF 3 on the complementary-sense strand of DNA-A
AC4	ORF 4 on the complementary-sense strand of DNA-A
ADK	adenosine kinase
AV1	ORF 1 on the virion-sense strand of DNA-A
AV2	ORF 2 on the virion-sense strand of DNA-A
BAP	Benzylaminopurin
BCIP	5-bromo-4-chloro-3-indoxylphosphate
bp	Base pair
C58C1	Strain of *A. tumefaciens*
CBB	Cassava bacterial blight
CGM	Cassava green mite
CLSM	Confocal laser-scanning microscopy
CMD	Cassava mosaic disease
CP	Coat protein
CR	Common region

C_T	Threshold cycle
DAS-ELISA	Double-Antibody-Sandwich ELISA
ddH_2O	Double-distilled water
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
dpi	Days post inoculation
DRC6	Democratic Republic of Congo
ds	Double stranded
dsGFP	double stranded, hairpin Green fluorescent protein
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked Immunosorbent assa*y*
EtOH	Ethanol
F	Forward primer
GFP	Green fluorescent protein
HA	Hemagglutinin
HCl	Hydrochloric acid
HC-Pro	Helper component proteinase
hpi	Hours post inoculation
IgG	Immunoglobulin G
IR	Intergenic region

Kb	KilobaseKn	Kanamycin
LB media	Luria-Bertoni medium	
LBA 4404	Strain of *A. tumefaciens*	
LOV	Light, oxygen or voltage sensing	
MES	Morpholinoethansulfonacid	
miRNA	Micro RNA	
MP	Movement protein	
MS media	Murashige and Skoog medium	
N. benthamiana	*Nicotiana benthamiana*	
NAA	Naphthalene acetic acid	
NBT	Nitroblue Tetrazoliumchloride	
NCBI	National Center for Biotechnoly Information	
ND	Not determined	
npt II	Neomycin phosphotransferase II	
NSP	Nuclear shuttle protein	
Nt	Nucleotide	
NTC	Non template water control	
OD	Optical density	
ORF	Open reading frames	
PBST	Phosphate buffer saline-Tween	
PPT	Phosphinothricin	
PTGS	Post-transcriptional gene silencing	

PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RaM-ap	Rabbit anti-mouse alkaline phosphatase conjugate
Ren	Replication enhancer
Rep	Replication associated protein
Rif	Rifampicin
SDS	Sodium dodecyl sulfate
sdm	site directed mutagenesis
siRNA	Short interfering RNA
smRs-GFP	Soluble modified red shift GFP
ss	single stranded
TAS-ELISA	Triple-Antibody-Sandwich ELISA
T-DNA	Transfer DNA
Tm	Melting temperature
TME 117	Tropical *Manihot esculenta* cultivar 117
TMS 30572	Tropical *Manihot* selection 30572
TrAP	Transcriptional activator protein
UNG	Uracil-N-Glycosylase
UV	Ultraviolet
V	Vector
VIGS	Virus induced gene silencing

Virus acronyms

AbMV	*Abutilon mosaic virus*
ACLSV	*Apple chlorotic leaf spot virus*
ACMV	*African cassava mosaic virus*
ACMV-[CM]	*African cassava mosaic virus* (Cameroon virus strain)
BDMV	*Bean dwarf mosaic virus*
BYV	*Beet yellow virus*
CaMV	*Cauliflower mosaic virus*
CBSV	*Cassava brown streak virus*
CMV	*Cucumber mosaic virus*
CsVMV	*Cassava vein mosaic virus*
CTV	*Citrus tristeza virus*
EACMV-UG	*East African Cassava mosaic virus* Uganda (Uganda variant)
EACMV	*East African Cassava mosaic virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
ICMV	*Indian cassava mosaic virus*
JCSMV	*Johnsongrass chlorotic stripe mosaic virus*
MYMV	*Mungbean yellow mosaic virus*
PPV	*Plum pox virus*
PVA	*Potato virus A*
PVX	*Potato virus X*
PVY	*Potato virus Y*

RYMV	*Rice yellow mosaic virus*
SLCMV	*Sri Lankan cassava mosaic virus*
TBSV	*Tomato bushy stunt virus*
TGMV	*Tomato golden mosaic virus*
TGMV	*Tomato golden mosaic virus*
TLCNDV	*Tomato leaf curl New Delhi virus*
TMV	*Tobacco mosaic virus*
ToLCV	*Tomato leaf curl virus*
ToRSV	*Tobacco ringspot virus*

1. General Introduction

1.1 Cassava, a subsistence crop

Cassava, (*Manihot esculenta* Crantz*, Euphorbiaceae*), is universally known by several names: manioca, rumu or yucca (Latin America), mandioca or aipim (Brazil), manioc (Madagascar and French-speaking Africa), tapioca (India, Malaysia), ubi kettella or kaspe (Indonesia), cassava and sometimes cassada (English-speaking regions in Africa, Thailand and Sri Lanka). It is a perennial, woody shrub that grows up to 5 metres tall, and produces enlarged tuberous roots. Its large palmate leaves have five to seven lobes borne on a long slender petiole which grow only toward the end of the branches. Cassava is second to the potato as the most important starchy root crop of the world (FAOSTAT, 2008). More than two hundred species of Cassava are cultivated in regions with tropical and subtropical climates. According to FAO estimates, 238 million tonnes of cassava were produced worldwide in a total area of 18.6 million hectares (FAOSTAT, 2008). The African continent accounts for half of the world production and Nigeria, with 43 million tonnes, making it the world's largest producer.

The value of cassava as a famine relief crop for Africa has long been recognized. Here it is generally grown by smallholder farmers as a single crop or intercropped with maize, legumes and vegetables. The plant withstands droughts and harsh climates and grows in poor soils without significant maintenance and agronomical input (Hahn & Keyser, 1985; Thresh, 2006). Today, on a worldwide scale, cassava cultivation is changing from a subsistence crop to provide daily calories for the poor to a cash crop grown on an industrial scale to produce raw products like cassava flour transformed into starch or alcohol.

Cassava is propagated exclusively by propagation of stem cuttings. Flowering in this plant is a rather complicated process since it does not occur synchronised within a

plantation and it also appears rather scattered. As a consequence cassava breeding is a very difficult, cumbersome and lengthy process. On the other hand, cassava propagation and cultivation is rather easy since cuttings used for propagation are hardy and robust. Cuttings quickly develop roots and grow fast into stable plants which produce tuberous roots ready to harvest after 6-12 month depending on cassava variety and location.

Cassava is cultivated intensively for human consumption in Africa where it presents the major sources of carbohydrates in Sub-Sahara Africa (Dahniya, 1994; Olsen, 2004). Despite its importance, cassava cultivation beyond the expectations and the potential of this crop is by far not exploited. In Africa, socio-economical and biological constraints limit the average yield per hectare to about 10 tonnes, a value that is far below a yield which can lie between 30.8 and 51 tonnes as estimated at research stations (Hahn *et al.*, 1979; Hahn & Keyser, 1985). One constraint to cassava production is represented by diseases and pests. Cassava is affected by serious diseases; Cassava mosaic disease (CMD), by far the most widespread virus disease in Africa (Patil & Fauquet, 2009), cassava brown streak disease (Alicai *et al.*, 2007; Hillocks & Jennings, 2003) occurring in East African countries, vascular bacterial disease caused by *Xanthomonas campestris* pv *manihotis, (*Onyeka *et al., 2008;* Wydra *et al., 2004)* and anthracnose caused by *Colletotrichum gloesporoides* f.sp. *manihotis* Henn. (Onyeka *et al.*, 2008; Owolade *et al.*, 2005). Serious pest problems are the cassava mealybug *Phenacoccus manihoti*, (Bellotti *et al.*, 1999; Steiner, 1991) and the green spider mite *Mononychellus tanajoa* Bondar (Gutierrez, 1987; Raji *et al.*, 2008; Skovgard *et al.*, 1993).

Pests and diseases in concert with poor conditions and/or cultivation practices can cause yield losses that may be as high as 50% in Africa. Cassava breeding for tolerance to abiotic and biotic stresses has resulted in selection of cassava varieties with improved yield, storage and tolerance characters (Ceballos *et al.*, 2004; Dixon *et al.*, 2008; Dixon & Ssemakula, 2008; Lokko *et al.*, 2009; Morante *et al.*, 2010; Raji *et al.*, 2008; Raji *et al.*, 2009).

Management practices to reduce losses due to pests in cassava include site selection, soil improvement practices and selection of appropriate varieties and planting materials. In this respect, major progress has been made in insect control and the widespread use of resistant varieties (Amusa & Ojo, 2002; Bellotti *et al.*, 1999; Hahn *et al.*, 1980a; Raji *et al.*, 2008; Thresh & Cooter, 2005).

Nevertheless, virus diseases and especially those caused by cassava mosaic viruses present the biggest challenge to sustainable cassava production (Legg & Thresh, 2000; Thresh & Cooter, 2005). This is because CMD viruses constantly evolve and hence populations change, new virus genotypes develop to seriously compromise virus tolerance inbred into cassava cultivars since long (Hahn *et al.*, 1980b; Terry & Hahn, 1980). Understanding cassava mosaic virus diseases caused by geminiviruses has reached quite far and extensive knowledge exists on the viruses and their genomes (Legg & Fauquet, 2004; Ndunguru *et al.*, 2005; Patil & Fauquet, 2009; Vanitharani *et al.*, 2005). Much less is known from the plant side and the kinetics of plant infections, the development of disease epidemics and the interactions of virus, insect vector and cassava genotype are far from being understood. These aspects need to be addressed before strategies can be devised to reach sustainable control of these significant diseases.

1.2 The viruses and virus diseases of cassava

Vegetatively propagated crops, like cassava are particularly prone to virus diseases and frequently multiple infections with quite different viruses persist in successive cycles of propagation.

About 20 different viruses of economic importance have been reported in cassava (Calvert & Thresh, 2002). The most significant and by far the most devastating are mosaic diseases caused by geminiviruses (Patil & Fauquet, 2009). Other viruses, like

the *Cassava brown streak viruses* (Alicai *et al.*, 2007; Hillocks *et al.*, 2002; Winter *et al.*, 2010) can reach high significance in certain regions while others are described from cassava but only limited data on their biological or epidemiological significance exist.

Viruses infecting Cassava and their taxonomic affiliations are summarised in table 1.1.

Table 1.1: Viruses infecting cassava

Africa

African cassava mosaic virus (Geminiviridae: Begomovirus)
East African cassava mosaic virus (Geminiviridae: Begomovirus)
East African cassava mosaic Cameroon virus (Geminiviridae: Begomovirus)
East African cassava mosaic Kenia virus (Geminiviridae: Begomovirus)
East African cassava mosaic Malawi virus (Geminiviridae: Begomovirus)
East African cassava mosaic Zanzibar virus (Geminiviridae: Begomovirus)
South African cassava mosaic virus (Geminiviridae: Begomovirus)
Cassava brown streak virus (Potyviridae: Ipomovirus)
Ugandan Cassava brown streak virus (Potyviridae: Ipomovirus)
Cassava Ivorian bacilli form virus (Not assigned)
Cassava kummi viruses
Cassava Q virus

South/ Central America

Cassava common mosaic virus (Potexvirus)
Cassava virus X (Potexvirus)
Cassava vein mosaic virus (Caulimoviridae)
Cassava colombian symptomless virus (Potexvirus)
Cassava American latent virus (Comoviridae: Nepovirus)
Cassava frogskin virus

Asia/ Pacific

Cassava common mosaic virus (Potexvirus)
Indian cassava mosaic virus (Geminiviridae: Begomovirus)
Sri Lankan cassava mosaic virus (Geminiviridae: Begomovirus)
Cassava green mottle virus (Comoviridae: Nepovirus)

Cassava mosaic virus diseases cause production losses reaching almost 90% of harvestable roots (Thresh *et al.*, 1994; Thresh & Otim-Nape, 1994). CMD has been

termed the most damaging plant virus disease in the world, causing famine and the death of thousands of people (Legg *et al.*, 2006).

1.3 Cassava mosaic disease

Cassava mosaic disease is known since 1894 by its first outbreak in part of Eastern Africa now known as Tanzania (Warburg, 1894). It was then recognized later in West Africa and today is present throughout Sub-Sahara Africa, wherever cassava is cultivated and on the Indian continent (Abarshi *et al.*, 2010; Atiri *et al.*, 2004; Hong *et al.*, 1993; Legg & Fauquet, 2004; Mathew & Muniyappa, 1992; Patil *et al.*, 2005; Sserubombwe *et al.*, 2008; Were *et al.*, 2003). Typical symptoms of CMD comprise irregular yellow or yellow–green chlorotic mosaic on leaves, leaf curling and distortion and stunted growth of the plant. Symptoms are usually more severe with younger plant parts and most pronounced in freshly expanding leaves. Plant variety, virus species and strain, cassava genotype, plant age of infection and multiple infections together with environmental factors affect symptom development (Legg & Thresh, 2000).

Several distinct begomovirus species (Table 1.1) and their strains cause mosaic disease in cassava. These viruses are efficiently transmitted by the whitefly *Bemisia tabaci* and widely disseminated by man who use stem cuttings for new plantings. Nine cassava mosaic begomoviruses are recognized out of which seven occur in Africa and two in India. The viruses reported from Africa are *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) and a number of species distinct from *East African cassava mosaic virus* indicating for a high diversity and the probable centre of diversification somewhere in East Africa (Ndunguru *et al.*, 2005; Sserubombwe *et al.*, 2008). Besides ACMV which is present throughout Sub-Sahara Africa, EACMV species have a significance by their regional distribution, the *East African cassava mosaic Cameroon virus* (EACMCV) present in Cameroon, Nigeria and Ivory Coast (Ariyo *et al.*,

2005; Fondong *et al.*, 2000; Pita *et al.*, 2001a) and the *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) are present in certain areas or even more restricted in their distribution to isolated spots.

A severe epidemic form of CMD progressed through much of Uganda in the late 1980s and caused devastating crop losses (Legg *et al.*, 2006; Otim-Nape *et al.*, 1998; Zhou *et al.*, 1997). This epidemic was attributed to a new cassava mosaic virus, a recombinant virus strain, EACMV-UG or Uganda variant (Zhou *et al.*, 1997). This particular virus has dramatically extended its area (Bigirimana *et al.*, 2004; Kumar *et al.*, 2009; Legg *et al.*, 2001; Sseruwagi *et al.*, 2004) and is most likely the most prevalent virus species infecting cassava in Africa.

1.4 Geminiviridae

Geminiviruses belong to a family of plant viruses which have emerged as serious threats to crop production worldwide (Mansoor *et al.*, 2003b; Seal *et al.*, 2006a; Varma & Malathi, 2003). In comparison to plant RNA viruses such as potyviruses or tobamoviruses, viruses in the family *Geminiviridae* were only recently discovered. Known since long as disease causing agents, the nature of the viruses remained unsolved until the end of the 1970s (Harrison, 1985a; Harrison, 1985b). In cassava, the *African cassava mosaic virus*, earlier called *cassava latent virus* was sequence characterised in 1983 (Stanley & Gay, 1983) and the bipartite nature of the virus, requiring 2 DNA genome components for infectivity was also determined only then (Stanley, 1983). Geminiviruses are transmitted by insects and infect monocotyledonous and dicotyledonous plants (Alves-Junior *et al.*, 2009). Their single stranded circular DNA genomes are encapsidated in twin icosahedral particles (Fig. 1.1) of approximately 20 x 30 nm (Fauquet *et al.*, 2008).

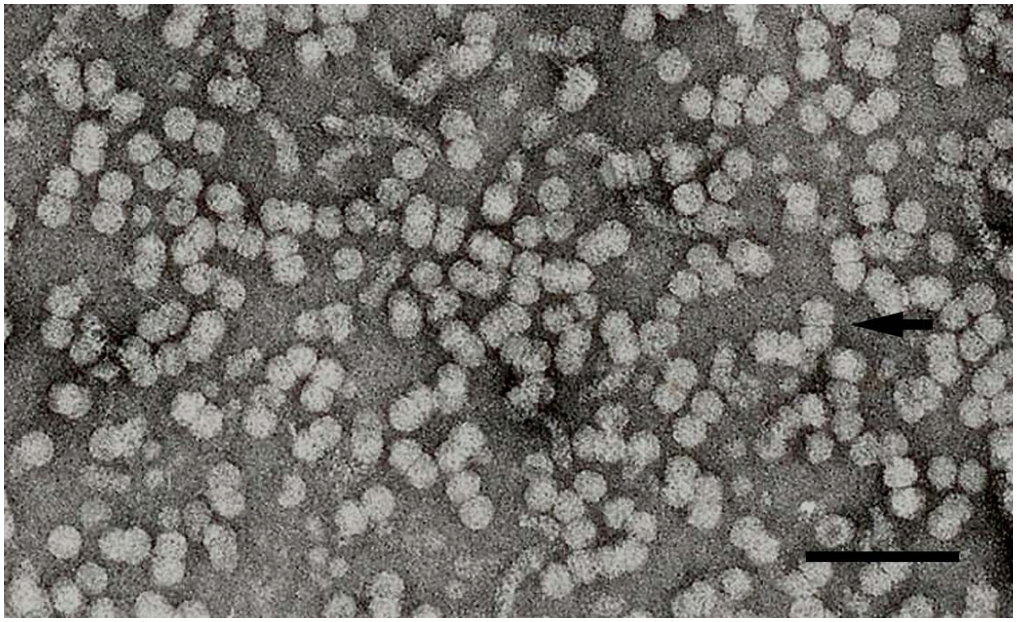

Figure 1.1: Electron micrograph of purified African cassava mosaic virus stained with uranyl acetate. Typical twinned quasi-isometric subunits (arrow). The bar represents 100 nm.

Genome organization and biological properties are key characters to distinguish four genera in the family *Geminiviridae* (Briddon *et al.*, 1996; Rybicki *et al.*, 2000). Mastreviruses and Curtoviruses are transmitted by leafhoppers and topocuviruses by treehoppers. Begomoviruses which are transmitted by whiteflies are the economically most important group (Stanley *et al.*, 2005). Geminiviruses have either one circular genome or, a bipartite genome with two circular, single stranded DNAs, the DNA-A and the DNA-B component of about 2.6 to 2.8 Kb each (Fig. 1.2). The coding regions on the virus genomes are oriented in both, sense and complementary strands. Between the coding ORFs the bipartite begomoviruses have an intergenic region (IR) designated as common region (CR) because it shows identical sequence for both genome components. The CR contains motifs required for the control of replication and transcription along with a putative stem-loop structure which contains the highly conserved nonanucleotide TAATATTAC which is implicated in the initiation of the rolling circle replication (Hanley-Bowdoin *et al.*, 1999). The CR is place of initiation and termination of the replication

through the Rep protein (Arguello-Astorga *et al.*, 1994a; Arguello-Astorga *et al.*, 1994b). Geminivirus diseases can result in yield losses ranging from 12% to 95% (Anderson & Morales, 1994; Briddon *et al.*, 2003; Fauquet & Fargette, 1990; Mansoor *et al.*, 2003b; Monci *et al.*, 2002; Ndunguru *et al.*, 2005). The viruses are present in weeds (e.g. *Ageratum conyzoides*) and in ornamentals (e.g. honeysuckle, *Lonicera japonica* var. *aureoreticulata*) thus are ubiquitous in subtropical and tropical regions. Important world crops like tomato, cotton and cassava but also tobacco and papaya are seriously threatened (Mansoor *et al.*, 2003b). The diseases and the viruses initially endemic in the developing tropics are now disseminated to areas with moderate climate (Sanz *et al.*, 2000). The number of virus species assigned to this family is steadily increasing since new viruses are continuously discovered (Haider *et al.*, 2007). Today, the genus Begomovirus comprises more than 200 species (Fauquet *et al.*, 2008).

1.5 The begomoviruses

During the last three decades, whitefly insects belonging to the genus *Bemisia (Homoptera: Aleyrodidae*), became one of the major pests in world agricultural systems (Jones, 2003; Oliveira *et al.,* 2001). This has ultimately proliferated the problems caused by begomoviruses which are efficiently transmitted by *Bemisia tabaci* (Polston & Anderson, 1997; Varma & Malathi, 2003). Viruses within the genus *Begomovirus* are responsible for serious crop losses in tropical and subtropical regions, particularly in Africa where they cause serious problems in food crops like maize (maize streak viruses) or cassava (cassava mosaic viruses) and South America where tomato production is seriously threatened by viruses of a tomato yellow leaf curl virus complex (Morales & Jones, 2004). Begomoviruses have a distinct geographical separation in their occurrences which is also inherent in their genomes (Harrison *et al.*, 2002; Harrison & Robinson, 1999). Old world viruses are present in Europe, Africa and Asia. They can be monopartite, like the economically most important *tomato yellow leaf curl virus(es)* from

Spain, Italy, Israel and the Mediterranean, or bipartite, like *African cassava mosaic virus* from Africa or, the *tomato leaf curl viruses* from Asia. DNA-A genome components of bipartite viruses are capable of autonomous replication and can produce virus particles but require DNA-B for efficient systemic infection (Stanley, 1983).

Old World virus have an additional ORF, AV2 (bipartite viruses) or V2 (monopartite viruses). Viruses from a specific geography notwithstanding their host are more related than viruses infecting the same hosts in another part of the world (Harrison *et al.*, 2002). Only in the Old World, monopartite geminviruses exist, often in association with satellite DNA. These satellite molecules denoted DNA-ß, are pathogenicity determinants, essential for the induction of the typical disease symptoms and determination of host range (Briddon *et al.*, 2003; Briddon & Stanley, 2006). Satellites are entirely dependent on the helper virus for replication, movement and dissemination, however are somewhat promiscuous since they can be transreplicated by a number of quite different viruses (Briddon *et al.*, 2003; Mansoor *et al.*, 2003a). DNA-ß encode a single protein (ßC1) which has a nuclear localization signal and functions as a suppressor of RNA silencing.

Begomovirus DNA-A genome components encode five or six proteins while the DNA-B genome carries 2 ORFs encoding for two proteins. The genes are positioned in ambisense direction and code for proteins responsible for encapsidation (AV1), viral replication and regulation of gene expression (AC1 and AC3) and for some viruses, for suppression of gene silencing (AC2, AC4) (Vanitharani *et al.*, 2005). The pre-coat protein coded for by the ORF AV2 is a pathogenicity determinant and for some viruses it acts as a silencing suppressor (Chowda-Reddy *et al.*, 2009). On DNA-B, BV1 and BC1 encode two proteins involved in intracellular (nuclear shuttle protein – NSP) and intercellular (movement protein – MP) virus movement (Lazarowitz, 1992). BC1 is found on the complementary strand and mediates cell-to-cell movement while BV1 in virion sense direction controls movement of viral DNA between the nucleus and cytoplasm (Gafni & Epel, 2002).

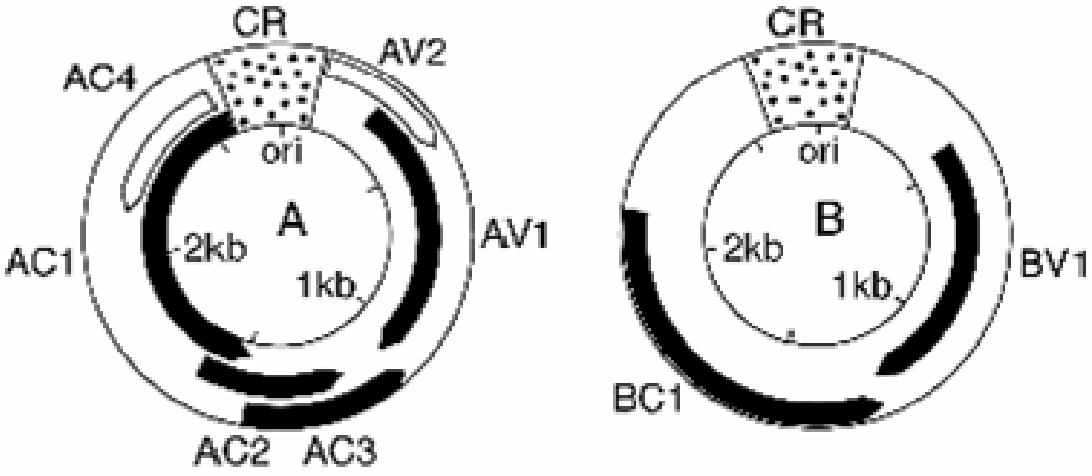

Figure 1.2: Arrangement of open reading frames (ORFs) in bipartite begomovirus genome DNA-A and DNA-B components. All bipartite begomoviruses contain ORFs in black arrows, while AV2 is only present in Old World viruses and AC4 is only present in some viruses. ORFs AV1, AV2 and BV1 are in the virus-sense direction while AC1 to AC4 and BC1 are transcribed from the complementary strand. Arrows indicate the direction of transcription. CR (stippled) represents the 'common region' shared among cognate DNA-A and DNA-B genome components. The number of nucleotides, from the origin of replication (*ori*), is indicated on the inner circle (Harrison & Robinson, 2005).

1.6 Genome expression

In infected cells, begomoviruses are directed to the nucleus where they replicate via a rolling circle mechanism that depends on the host machinery (Jeske *et al.*, 2001; Preiss & Jeske, 2003). Bi-directional transcription also takes place within the nucleus from promoter sequences located in the intergenic region (Hanley-Bowdoin *et al.*, 1999; Sunter & Bisaro, 1991). Host cellular factors and cell machinery both are required for replication, systemic spread, and suppression of antiviral defence mechanisms (Petty *et al.*, 2000). The replication initiation protein (Rep) coded by AC1 functions in virus replication by binding to the stem structure at the origin of replication (ori) (Fontes *et al.*, 1994). It creates a nick in the virion strand (Laufs *et al.*, 1995) and initiates rolling circle replication (RCR) (Hanley-Bowdoin *et al.*, 1999). Rep ligates DNA and represses its own promoter (Eagle *et al.*, 1994; Sunter *et al.*, 1993), plays a role as a DNA helicase (Pant *et al.*, 2001) and can interact with itself (Orozco *et al.*, 1997), with AC3 (Settlage *et al.*,

1996) and with host proteins (Arguello-Astorga *et al.*, 2004; Carrington *et al.*, 1998; Castillo *et al.*, 2003; Castillo *et al.*, 2004; Egelkrout *et al.*, 2002; Kong & Hanley-Bowdoin, 2002; Settlage *et al.*, 2001). AC3 is a supplementary replication enhancing protein (Ren) which increases viral DNA accumulation (Sunter *et al.*, 1990), forms homo-oligomers and interacts with AC1 and host factors (Castillo *et al.*, 2003; Settlage *et al.*, 2005).

The AC2 gene is the transcriptional activator protein (Sunter & Bisaro, 1991) and thus is required for efficient transcription of the late viral sense genes, AV1 and BV1 (Sunter & Bisaro, 1992; Sunter & Bisaro, 1997a). The AC2 consists of three conserved domains: a basic domain with a nuclear localization signal at the N-terminal, a central DNA-binding Zn-finger motif and C-terminal acidic activator domain (Hartitz *et al.*, 1999). It binds to ssDNA in a non specific way and only weakly to dsDNA, suggesting that it is not a canonical transcriptional factor but probably interacts with host plant cellular proteins to trigger transcriptional activation (Hartitz *et al.*, 1999). Furthermore, for a number of begomoviruses, TraP acts as a suppressor of RNA silencing (Selth *et al.*, 2004; Trinks *et al.*, 2005; van Wezel *et al.*, 2002b; van Wezel *et al.*, 2003; Voinnet *et al.*, 1999).

The ORF AC4 is nested within AC1 but is translated from a frame shift (Hanley-Bowdoin *et al.*, 1999). AC4 proteins of bipartite begomoviruses are highly variable and no general role has been ascribed to this gene product. Mutations in the AC4 of ACMV (Etessami *et al.*, 1991) or *Tomato golden mosaic virus* (TGMV) (Elmer *et al.*, 1988) had effects on viral replication or symptom development. Recently, gene silencing suppression activity was assigned to AC4 proteins from ACMV and *Sri Lankan cassava mosaic virus* (SLCMV) (Vanitharani *et al.*, 2004; Vanitharani *et al.*, 2005).

The coat protein (CP) encoded by AV1 is the geminivirus structural protein, a multifunctional protein (Harrison *et al.*, 2002), which forms multimers and encapsidates the viral ssDNA into the characteristic geminate particles (Bottcher *et al.*, 2004). CP is responsible for specific vector transmission by the whitefly *Bemisia tabaci* and it provides

the structural features necessary for virus translocation in the insect (Kheyr-Pour *et al.*, 2000; Noris *et al.*, 1998). *In planta*, a functional CP is not necessary for infectivity and systemic movement of bipartite begomoviruses however it is indispensable for movement of monopartite begomoviruses (Noris *et al.*, 1998) and for efficient accumulation of viral ssDNA (Harrison *et al.*, 2002; Qin *et al.*, 1998). CP interacts with importin and might use this pathway to dock virus particles/nucleoproteins to the nucleus in initially infected cells (Gafni & Epel, 2002; Guerra-Peraza *et al.*, 2005). It was also reported that CP can interact and down regulate AC1 thereby adopting a role in controlling DNA replication (Malik *et al.*, 2005).

The ORF AV2 (pre-coat) is only present in Old World begomoviruses (Harrison & Robinson, 1999). In the monopartite TLCV it has been implicated in ssDNA accumulation (Rigden *et al.*, 1993) and mediates viral DNA export from the nucleus to the plasmodesmata (Rojas *et al.*, 2001). For the bipartite *Tomato leaf curl New Delhi virus*, it was shown to be involved in systemic movement (Padidam *et al.*, 1996).

The genes encoded by the B component of bipartite begomoviruses, BV1 and BC1, provide functions required for virus movement. BV1, the nuclear shuttle protein (NSP) and BC1, the cell-to cell movement protein (MP) coordinate the movement of the viral DNA from the nucleus and across cell boundaries (Gafni & Epel, 2002; Noueiry *et al.*, 1994; Sanderfoot & Lazarowitz, 1995; Sanderfoot & Lazarowitz, 1996).Though it is not precisely known if single stranded or double stranded DNA forms are transported, BV1 packages the viral DNA and interacts with BC1 in the cytoplasm to be transported through the plasmodesmata into the neighbouring cells (Lazarowitz & Beachy, 1999). BC1 and BV1 proteins of several bipartite begomoviruses are virulence determinants in different host plants (Carvalho & Lazarowitz, 2004; Hussain *et al.*, 2005; Ingham *et al.*, 1995; Vonarnim & Stanley, 1992).

1.7 Tissue tropism

Geminivirus replication is exclusively in the nucleus of infected cells (Morilla *et al.*, 2004; Morra & Petty, 2000; Petty *et al.*, 2000; Qin & Petty, 2001; Wege *et al.*, 2001) This is independent from type of infection or plant tissues infected. Begomoviruses can remain limited to the phloem tissues or can be present in cells outside the vascular system. Tissue invasion has no effects on the disease development hence the efficient invasion of the host by a geminivirus is not correlated with symptom severity. Thus tissue specificity is a specific feature of virus and its host and for geminiviruses it is still unclear which host factors are more relevant (Morilla *et al.*, 2004). In fact, different levels of viral replication and transcription in tissues, efficiency of viral proteins to support movement outside the vascular system and differentially acting defence strategies of the host might explain differences in tissue invasion among begomoviruses. *Tomato yellow leaf curl virus* (TYLCV) and AbMV are restricted to phloem cells while ACMV, *Bean dwarf mosaic virus* (BDMV) and *Tomato golden mosaic virus* (TGMV) efficiently invade non phloem tissues and are found in epidermal and palisade cells as well as in spongy parenchyma cells (Wege *et al.*, 2001). In mixed infection with *Tomato yellow leaf curl Sardinia virus* (TYLCSV), tissue specificity is maintained while in mixed infections of begomoviruses with RNA viruses, virus concentrations can be increased in some cases while no effects are seen in others. Thus mixed infection with *Cucumber mosaic virus* (CMV) result in increase of AbMV concentrations and offset of phloem restrictions (Wege & Siegmund, 2007) while mixed infections with TMV did not have this effect.

Host factors might also play role in tissue invasion. This is highlighted by the observation that TGMV promoter elements function in a tissue-specific activation and repression of virion-sense gene expression (Sunter & Bisaro, 1997a). Adaptation to the host might also be significant. *Euphorbia mosaic virus* (EuMV) in its natural host *Euphorbia*

heterophylla L. remains restricted to the phloem but can invade non vascular tissues in the experimental host *Datura stramonium* L. (Kim & Lee, 1992).

1.8 Post-transcriptional gene silencing

Post-transcriptional gene silencing (PTGS) is a natural plant defence mechanism against RNA viruses which involves homology-dependent and specific RNA degradation (Vanitharani *et al.*, 2004). As a counter defence, viruses encode a range of silencing suppressor proteins (Li & Ding, 2006; Voinnet, 2005). These proteins which are functionally and structurally quite diverse interfere with initiation, maintenance and systemic signalling in the RNA-silencing process. More than 30 different types of silencing suppressors have been identified from RNA viruses but also from DNA viruses (Li & Ding, 2006). For example, potyvirus helper component proteinases (HC-Pro) interfere with initiation and maintenance of silencing where short interfering RNAs (siRNAs) are produced. *Cucumber mosaic virus* (CMV) inhibits long-range PTGS-signalling, thereby preventing initiation of PTGS in newly formed tissues. The generation of mobile silencing signals is suppressed by the p25 triple gene block protein of *Potato virus X* (PVX). In geminiviruses, the transcriptional activator protein (TrAp) expressed from AC2 in the monopartite *Tomato yellow leaf curl China virus* has been identified as suppressor of PTGS (van Wezel *et al.*, 2002b). This was confirmed by transient gene expression assays with a PVX vector functionally identifying AC2 genes from several other begomoviruses as suppressors of gene silencing (Dong *et al.*, 2003; Vanitharani *et al.*, 2004; Voinnet *et al.*, 1999). Interestingly, while AC2 was determined as silencing suppressor for EACMV and for *Indian cassava mosaic virus* (ICMV), AC4 was found acting as suppressor of gene silencing for ACMV and for *Sri Lankan cassava mosaic virus* (SLCMV) (Vanitharani *et al.*, 2004). This further highlights the variety of counter–defence strategies adopted by even related viruses acting on different aspects of RNA silencing. Thus synergism phenomena in mixed virus infections might be explained by

the concurrent interaction of unrelated suppressors to suppress the plant defence system.

1.9 Recombination, pseudorecombination and mixed infections

In recent years, ecological and economical damages caused by geminiviruses have increased. This is explained by the expanded geographic distribution of the vector and especially the polyphagous and fecundant *B. tabaci* biotype B whitefly (Anderson *et al.*, 2004; Anderson & Morales, 1994; Morales & Jones, 2004). The rapid invasion of this pest and the displacement, rather elimination, of existing host specialized biotypes has contributed to a rapid increase of begomovirus problems in old crops like tomato since viruses from weeds and ruderal plants once separated were now transmitted to the crop. As a consequence mixed infections caused by multiple begomoviruses exist and often synergism is observed (Ala-Poikela *et al.*, 2005; Ribeiro *et al.*, 2003). Mixed infections create the chances for genetic re-assortment and intra- or interspecific recombination, a phenomenon often reported from geminiviruses (Ala-Poikela *et al.*, 2005; Fondong *et al.*, 2000; Monci *et al.*, 2002; Morilla *et al.*, 2004; Moriones & Navas-Castillo, 2008; Pita *et al.*, 2001b; Saunders & Stanley, 1995). The virus associated with the severe virus epidemic in Uganda, *East African cassava mosaic virus* Uganda (EACMV-UG) is a natural recombinant between ACMV and EACMV where a CP fragment from ACMV was inserted into DNA-A of EACMV (Harrison *et al.*, 1997; Zhou *et al.*, 1997). Thus inter- and intraspecies recombination is a driving factor for geminivirus evolution (Fondong *et al.*, 2000; Lefeuvre *et al.*, 2007; Moriones & Navas-Castillo, 2008; Ndunguru *et al.*, 2005; Padidam *et al.*, 1999; Pita *et al.*, 2001b; Seal *et al.*, 2006b).

Specific aims and objectives

In this work, geminivirus infections of cassava with ACMV and EACMV-UG were studied. These are the most serious viruses in African cassava to date and occur wherever cassava is grown in Sub-Sahara Africa. Various aspects of the virus disease were studied in cassava and in *N. benthamiana*.

ACMV and EACMV-UG once geographically separated now have a largely sympatric distribution. Mixed infections, a landmark of the new cassava virus disease epidemics, occur frequently and are characterised by more severe symptoms and synergistic interactions.

The second chapter of this thesis focuses on replication of ACMV and EACMV-UG in cassava by separately analysing the kinetics of DNA-A and DNA-B genomic components. This was to quantify virus genome components during pathogenesis and to correlate DNA accumulation with incidence and severity of symptoms. In this virus study mixed infections and also susceptible, tolerant and resistant cassava breeding lines were subjected to quantitative assessment of DNA-A and DNA-B genome components. This study analyzed cassava with mixed infections of ACMV and EACMV-UG, to quantify the effects of mixed infections by resolving the concentration and relations of DNA-A and DNA-B genomic components of the viruses involved in these disease complexes. For these studies, real time quantitative PCR was used to determine copy numbers of virus genome components DNA-A and DNA-B in single and mixed infections of cassava genotypes with differential susceptibility/ resistance to ACMV and EACMV.

The objective of the third chapter was to study movement and distribution of ACMV and EACMV-UG in infected *N. benthamiana* plants. For these *in situ* studies DNA-A and DNA-B genomic components were modified to express the green fluorescent protein (GFP) during replication as a reporter of virus movement and tissue invasion. The expression of GFP during replication of the virus should result in emission of

fluorescence signals which could be monitored by confocal laser scanning microscopy. Following the movement of the labelled viruses then would provide visual insights into the infection process of single and mixed virus infections and into synergistic interactions among begomoviruses.

The fourth chapter comprises a functional analysis to elucidate the role of *AC2* and *AC4* genes in ACMV and EACMV infections. In preliminary experiments, the silencing suppression activity of *AC2* (TraP*)* and *AC4* genes of ACMV were examined in transient expression assays as proof of function for AC2 and AC4 as virus gene silencing suppressor proteins. A transgenic model, *N. benthamiana* expressing TraP and AC4 respectively, was established to study in challenge infections with ACMV and EACMV-UG the interactions between constitutively expressed AC2 and AC4 proteins and replicating viruses.

Finally, in the general discussion, a synopsis on the knowledge gains from the studies seeks to bring into perspective the results from the three related but independent studies on cassava begomoviruses with aspects of a broader significance for our understanding of plant virus disease processes.

2. African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV-UG) in single and mixed infected cassava (*Manihot esculenta* Crantz)

Abstract

To assess virus concentrations in susceptible, tolerant and resistant cassava cultivars in infections with *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* Uganda (Uganda variant, EACMV-UG) the amounts of DNA-A and DNA-B of the geminiviruses were analysed by quantitative PCR. The concentrations in absolute and relative quantification experiments of viral DNA-A and DNA-B genome components in single and mixed infections were determined.

Virus concentration was much higher in symptomatic leaf tissues compared to non-symptomatic leaves and corresponded with the severity of disease symptoms. Much higher virus titres were generally recorded for EACMV-UG Ca055 compared with ACMV DRC6. The quantitative assessment also showed that the distribution of both viruses in the moderately resistant cassava cv. TMS 30572 was not different from the highly susceptible cv. TME 117. The highly resistant cassava cv. Albert was immune against ACMV but could be infected with EACMV-UG Ca111 albeit did only support limited virus replication and spread. The differential assessment of DNA genomic components to reveal quantitative relations among the components showed that higher amounts of ACMV DNA-A were present throughout the infection. This was vice versa for EACMV-UG where higher amounts of DNA-B were recorded.

Natural mixed infections with both viruses resulted in severe disease symptoms, however only a slight increase of EACMV-UG. Relative quantification of virus genomes in mixed infections showed higher concentrations of EACMV-UG DNA-A compared to ACMV DNA-A but a drastic reduction of EACMV-UG DNA-B. ACMV concentrations in

natural mixed infections appeared to be unaffected by presence of EACMV-UG with concentrations similar to single infections. The higher concentrations of EACMV-UG DNA-B compared to EACMV DNA-A accumulation in single infections was consistent and irrespective of cassava cultivar and its tolerance status. Since DNA-B is implicated in virus cell-to-cell spread and systemic movement, it can be postulated as a factor driving cassava mosaic disease epidemics.

2.1 Introduction

Begomoviruses causing mosaic diseases (CMD) represent serious problems to cassava cultivation of this important food crop wherever cassava is cultivated in Sub-Sahara Africa and India. In Africa, seven species of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) have been described (Ariyo *et al.*, 2005; Bull *et al.*, 2006; Ndunguru *et al.*, 2005; Were *et al.*, 2003). Genome sequences of ACMV form tight sequence clusters irrespective of their geographical origin. In contrast genomes of EACMV are very different and their genetic diversity is at a level of species and strain diversification. This diversification is most likely driven by frequent recombination between members of this EACMV species complex (Patil & Fauquet, 2009; Pita *et al.*, 2001b).

The geographic distribution of ACMV and EACMV viruses on the African continent today is quite complex. Initially ACMV and EACMV had distinct geographic distributions (Hong & Harrison, 1995) while the dissemination of planting material, human migration over long distances and the efficiency of vector transmission have contributed to a now overlapping occurrence of the viruses albeit with an epicentre in central and eastern Africa (Legg *et al.*, 2006; Ndunguru *et al.*, 2005). Mixed ACMV/EACMV virus infections most probably have caused the emergence of a distinct recombinant strain of EACMV, the so called “Uganda variant virus”, which carries an ACMV type coat protein in an

otherwise EACMV genome (Pita *et al.*, 2001b; Zhou *et al.*, 1997). This particular virus, EACMV-UG is implicated in the serious cassava mosaic disease epidemics first found in Uganda end of the 1980s and moving into neighbouring countries of East and Central Africa (Legg & Fauquet, 2004). The severe disease phenotype of this "new" type of CMD was most often found in mixed infections with ACMV indicating for strong synergisms among both viruses. Mixed infections with severe synergistic disease phenotypes were also found in CMD epidemics in Cameroon and there, ACMV was mixed with *East Africa cassava mosaic Cameroon virus*, EACMCV (Fondong *et al.*, 2000).

The epidemic situation of West and East Sub-Sahara Africa is quite distinct. In West Africa, ACMV is the dominant virus. EACMCV is found in many countries, including Ivory Coast and Nigeria, and mostly in mixed ACMV infections (Ariyo *et al.*, 2005). Outside Cameroon however, the virus has never reached an epidemiological significance comparable to EACMV-UG. This Uganda variant virus has swept from its first sight in the late 1980s throughout eastern and central Africa, from South Sudan (Tadu *et al.*, 2006) to Angola (Kumar *et al.*, 2009) and is the now dominant virus type in major cassava regions of Tanzania, Uganda and the Democratic Republic of Congo. While all other EACMV species and strains more or less remain at their locations of origin, this virus is rapidly spreading and extending its distribution range. Then, in a first phase of virus invasion into a new area, mixed ACMV infection develop, then EACMV-UG is more frequently found and becomes the dominant virus often outcompeting and displacing ACMV (Legg *et al.*, 2006).

This characteristic role and impact of EACMV-UG in the African CMD epidemics provoked a series of key questions to guide the research into elucidating the factors explaining the epidemics. The study presented here focuses on a quantitative assessment of ACMV and EACMV-UG in single and mixed infections in cassava. More severe disease phenotypes of mixed infections can probably be explained by an increase of ACMV and/or EACMV-UG genome DNA-A and DNA-B components. An

increase of virus titre in mixed infections presents more infectious particles thus boosting virus spread by whiteflies. The unilateral increase of EACMV-UG in mixed ACMV/EACMV-UG infected plants may then explain the rapid geographic dispersal of EACMV-UG and why EACMV-UG is then becoming the dominant and later on, the sole virus in CMD.

In this study, ACMV and EACMV-UG were quantified using a quantitative PCR (qPCR) approach, to determine the copy numbers of the genome components DNA-A and DNA-B of the respective viruses in single and mixed infections of cassava. Cassava cultivars with differential CMD susceptibility/resistance characters were used to evaluate the extent of virus replication in susceptible and tolerant cultivars, to quantify each genome DNA-A and DNA-B component during pathogenesis and to correlate it with symptom type, severity and the resistance status of the plant.

2.2 Materials and Methods

2.2.1 **Plants and Viruses**

Virus infections in cassava were induced by inoculations with cloned virus genomes of an *East African cassava mosaic virus* (EACMV–UG, Uganda variant, isolate, Ca055) and an *African cassava mosaic virus* (ACMV, isolate DRC6). Head-to-tail partial multimers of DNA-A genomic components and DNA-B genomic components in the binary vector pGreenII (John Innes Center, UK) were introduced into cassava by biolistic inoculation, essentially following the protocol of Ariyo *et al.* (Ariyo *et al.*, 2006). Subsequently, mixed infections in cassava were introduced by approach grafting of typified ACMV and EACMV-UG infected cassava. Each infection type was established in five plants of each cultivar.

To compare virus replication in cassava, three cassava cultivars (cv.) with differential resistance against cassava begomoviruses were chosen. Viruses were inoculated in a Nigerian landrace, TME 117 (highly susceptible) and an improved cassava cv., TMS

30572 (moderately resistant). A highly resistant cassava cv. Albert, a land race widely grown in Tanzania, was also included in the analysis. This particular cultivar was infected with a wild type isolate of EACMV-UG and expressed, if at all, only very mild symptoms of begomovirus infection. A number of cassava plants, local landraces of unknown genotypes from earlier virus studies, with natural infections of ACMV, EACMV-UG or both viruses in mixed infections, were included in this study.

All cassava plants were kept under greenhouse conditions at 26-28°C and monitored for symptom expression. Biolistically infected plants started with symptom expression about 45 days after inoculation (dpi).

2.2.2 **Sample Preparation**

Tissue samples (approx. 100mg) from newly emerging symptomatic and non symptomatic leaves were taken from each plant, frozen in liquid nitrogen and stored at -80 °C. Total genomic DNA was extracted using a DNAeasy Plant Mini kit (Qiagen Inc., Germany) according to the protocol provided with the kit. DNA was finally dissolved in 100 µl of Tris/EDTA (buffer AE). Prior to qPCR, DNA was quantified using a Nanodrop spectrophotometer ND-1000 (PEQLAB, Germany) and adjusted to a DNA concentration of 2 ng/µl. 10 ng DNA was used as template in each qPCR reaction.

2.2.3 **Primers and Probes design and optimization**

To determine suitable positions for primers and probes, alignments of complete DNA-A and DNA-B genome sequences of isolate Ca055 and DRC6 were carried out using the program ClustalX (Thompson *et al.*, 2002). Consequently forward and reverse primers with the respective fluorogenic probes (TaqMan FAM/minor groove binding [MGB] probes) were designed using the Primer Express software (Applied Biosystems, USA) and/or the Beacon designer 7.0 software (PREMIER Biosoft International, USA). In an

initial step, to eliminate non specific amplification and primer/probe binding, primers and probes selected were subjected to a BLAST search against sequences in Genbank using the integrated BLAST sequence alignment search tool in the software.

TaqMan FAM/MGB assays were developed for specific, differential detection of DNA-A and DNA-B genes of both viruses. For DNA-A genome components of Ca055 and DRC6, primers and probes were designed targeting *AC1* (Rep) genes while primers and probes for amplification of DNA-B were designed in the *BC1* (MP) gene. As an internal control and for quantitation purposes, TaqMan FAM/MGB assays were designed to amplify the *β-actin* gene of cassava.

In qPCR, the primers for EACMV-UG amplified a 138 base pair (bp) fragment while for ACMV DRC6 a 68 bp fragment of the *AC1* gene is amplified. The amplification of DNA-B components resulted in 70 bp and 72 bp fragments of *BC1* genes of Ca055 isolate and DRC6 respectively. The amplicon size generated for *β-actin*, the internal reference control, was 97 bp.

All primer/probe sets were tested using DNA extracted from leaves of cassava plants infected with virus isolate Ca055 or DRC6 and with total DNA from non-infected plants as negative controls. Since the TaqMan FAM assay failed for EACMV-UG *AC1*, a VIC/TAMRA assay was designed and subsequently used. Primer and probe sequences for qPCR detection of DNA-A and DNA-B of EACMV-UG Ca055 and ACMV DRC6 as well as the *ß-actin* gene of cassava are detailed in Table 2.1.

2.2.4 **PCR set up**

Working solutions of primers and probes, DNA samples from plants and plasmid dilutions were stored at -20°C and not thawed more than twice prior to use. Master mixes and water were kept in small aliquots at 4°C. To avoid DNA contamination and carry over, DNA extraction, preparation of master mixes and set up of PCR was done in

separate rooms. All buffers and solutions for qPCR were pipetted using sterile filter tips and composed on ice to avoid errors from evaporation during pipetting. For each sample, triplicate qPCR reactions were composed in 96-well microplate formats and microplates were sealed immediately after loading. Samples, standards, non template controls (NTC) and negative water controls were run on each plate to minimize variations between plates.

The TaqMan Gene Expression Master Mix (Applied Biosystems CO., Foster City, CA) was used which contained AmpliTaq Gold® DNA polymerase (Ultra Pure), Uracil-DNA Glycosylase (UNG), including dNTPs, dUTP, a passive reference dye ROX™, as well as other buffer components.

Standard real time PCR reaction mix (25 µl)

DNA (2 ng/µl)	5 µl
2 x TaqMan Gene Expression Master Mix	12.5 µl
Sense Primer [900 nM]	2.25 µl
Antisense Primer [900 nM]	2.25 µl
TaqMan MGB Probe [200 nM]	0.5 µl
ddH_2O	2.5 µl

Cycling parameters were as follows: 1 cycle at 50°C for 2 min (activation of UNG, Uracil-N-Glycosylase), 1 cycle at 95°C for 10 min (DNA polymerase activation), and 40 cycles, each consisting of 95°C (denaturation) and 1 min at 60°C (annealing and extension).

All real time PCR assays were performed in an Eppendorf realplex^4 ep gradient S Mastercycler (Eppendorf, Germany).

Table 2.1: Primers/TaqMan probes used for the detection and quantitation of East African cassava mosaic virus-UG Ca055, African cassava mosaic virus DRC6 genome components DNA-A and DNA-B and for the endogenous ß-actin gene of cassava

	Accession No.		Oligonucleotide sequence (5'-3')	Amplicon size (bp)	gene	R/Q dyes	Final conc used (nM)
ACMVDRC6 A	FN668378	For ward primer	TGCTAGAGGCGGTCAACAATC	68	Rep	FAM/NFQMGB	225
		Reverse primer	CCGACTTACTGCCGCTGTTAA	…	…	…	225
		TaqMan probe	CTTTGGCGTAAGCATCAT	…	…	…	50
ACMVDRC6 B	FN668379	For primer	TCGAAGGCTCATTCAGAATGAG	72	Mp	FAM/NFQMGB	225
		Reverse primer	TGGACCGTGATGGGCCTAT	…	…	…	225
		TaqMan probe	TAGGCCCAGTGTCATAA	…	…	…	50
EACMV-UG A	FN 668377	For ward primer	CAATGTACCAACCCGAGATTCTT	76	Rep	FAM/NFQMGB	225
		Reverse primer	CTCCCTGAATGTTCGGATGAA	…	…	…	225
		TaqMan probe	TGTGTTGATCGGGATGG	…	…	…	50
EACMV-UG B	FN668380	For ward primer	GGAGAAACAAGACGGAGATTACTCA	70	Mp	FAM/NFQMGB	225
		Reverse primer	GCCTGTGTGTAATGGGTCGTT	…	…	…	225
		TaqMan probe	TGAGCAGTTGGAGTTGG	…	…	…	50
Cassava *ß-actin*	AB158612 (*N.tabacum*)	For ward primer	GCATGCAATCCTTCGTCTTGA	97	*ß-actin*	FAM/NFQMGB	250
	AY179605 (*N.bentha*)	Reverse primer	AGCTGAGGTGGTGAAAGAATATCC	…	…	…	250
		TaqMan probe	TCAGTGAGGATCTTCATCA	…	…	…	50
EACMV-UG A	FN668377	For ward primer	CGAATTGGATGAGAACATGGAGATG	138	Rep	VIC/TAMRA	250
		Reverse primer	CCCGATGCTCATTAACGAAAGAAG	…	…	…	250
		TaqMan probe	TTCCCAATTACAAGCCCTTTCGTACCCG	…	…	…	50

2.2.5 **Preparation of standard curves and DNA quantification**

To determine copy numbers of DNA-A and DNA-B molecules the qPCR was calibrated. Standard curves were prepared with plasmids harbouring a full-length copy of DNA-A or DNA-B component respectively of EACMV-UG Ca055 or ACMV DRC6. The molecular weight of each plasmid was determined from plasmid and vector sizes and plasmid DNA dilutions were prepared to generate standard curves from which copy numbers of the genomic components could be calculated. In this serial tenfold dilution approximately 1.2 ng/µl DNA from non-infected cassava (TMS 30572) was spiked and a target curve starting from 300 molecules to $3x10^7$ genome copies per 5 µl of sample was achieved. The conversion from mass to molecules was done assuming an average molecular weight of a deoxyribonucleotide (330 kDa) and the number of base pairs in the DNA using the formula:

(X g/µl DNA / [plasmid length in basepairs x 660]) x Avogadro's number x 10^{-12}

A similar standard curve was prepared for the cassava *ß-actin* gene by serial dilution of a plasmid harbouring a 1500 bp fragment of the cassava *ß-actin* gene to generate a standard curve starting from 30000 to $3x10^8$ gene copies so that C_T value of unknown sample falls within the range.

The calculation of the standard curves was done using the Eppendorf realplex software (version 2.2) using the C_T values obtained from qPCR as a function of the amount (copies) of nucleic acid target.

The standard curve chart displays the following information:

Slope	Increase in standard curve
Y-Intercept	Point at which the standard curve intersects with the Y-axis (based on amount = 1 [unit])
Efficiency	Efficiency of the PCR , E= $10^{[-1/slope]}$-1
Where a slope (S)	-3.3 (E=1) represents 100% efficiency
Rˆ2	Correlation coefficient of standard curve

Standard curves were verified with GenEX software (BioEPS, Germany) by a linear regression analysis of the threshold cycle C_T value for each replicate of six standard dilutions (for viral components) and five standard dilutions (for cassava *β-actin* gene) over the log of the total DNA amount present in each sample.

For absolute quantitation, the numbers of DNA-A and DNA-B genome molecules of EACMV-UG Ca055 and ACMV DRC6 were determined from the specific standard curves generated for each plasmid containing a molecule of DNA-A or DNA-B genomic component respectively. The standard curves reproduced a linear relationship between the C_T value and the amount of total input DNA. Thus from the fitted line of regression, the target concentrations in the samples (genome copies) were interpolated from the appropriate standard curve. The amount of virus was also calculated by a linear regression analysis of the C_T value of each sample over the log of the total DNA concentration.

For relative quantitation, values obtained in qPCR in amplification of DNA-A and DNA-B genome components were compared with values obtained for amplification of the cassava *ß-actin* gene used as endogenous reference and a DNA from non-infected cassava used as calibrator. The results were expressed as target/reference ratio. For relative quantitation, a mathematical model to calculate the mean normalized target amount was used (Applied Biosystems).

2.2.6 **Statistical analysis**

Results from qPCR were exported in GenEx Professional (version 4.3.8) for verification of standard curves and to quantify target concentrations in samples. Replicates of samples were analyzed to calculate the standard deviation of C_T values. Standard deviation between the samples was evaluated using the log of the amount [copies]. From the standard curves the copy numbers of DNA-A and DNA-B genome components were determined by interpolation. To statistically evaluate all values obtained in separate qPCR reactions, data were presented graphically in box and whisker plots calculated in SigmaPlot 9.0. These plots are based on calculations for the main median from all data; the upper median, for data > median and; for the lower median, from data < median. In a graphical display (Figure 2.4) median values represent the upper (75% of data) and lower limits (25% of data) of the box, with a cross section of the main median. Whiskers extend to the data's smallest and highest values reflecting the extremes. A box plot thus graphically displays the variation of the experimental data and is less influenced by extreme values.

2.3 Results

2.3.1 **Cassava mosaic virus infections in cassava**

Mosaic symptoms on leaves of cassava are typical indications for cassava mosaic disease. Symptoms of CMD are more pronounced on younger leaves of plants infected with cassava begomoviruses. Symptomatic leaves alternating with non-symptomatic are characteristic for the disease (Fig. 2.1).

Figure 2.1: Cassava mosaic disease symptoms on field grown cassava in Yangambi, Democratic Republic of Congo. Single infection of EACMV-UG in a DRC local cassava landrace cultivar "Mbongo".

Cassava cultivars infected with ACMV and EACMV-UG most often show a serious response to virus infections with severe symptoms on almost every leaf consisting of mosaic, leaf malformation and distortion. In the advanced stages of this mixed virus infection, leaves are severely crippled and the entire plant appears stunted (Fig. 2.2).

Figure 2.2: Severe Cassava mosaic disease symptoms of the cassava cultivar "Mbongo" infected with ACMV and EACMV-UG.

For the quantitative assessment of cassava virus infections, cassava cultivars were infected with "reference" cassava mosaic begomoviruses and maintained under glasshouse conditions to monitor for disease progress. Upon biolistic inoculation, cassava plantlets developed leaf symptoms starting on youngest freshly expanding leaves approximately 3-6 weeks after inoculation. Symptoms on cassava differed with viruses and cassava cultivar. ACMV and EACMV-UG infections resulted in leaf symptoms that consisted of mottling, mild to severe mosaic and leaf deformation. In the highly susceptible TME 117, symptoms were most severe but there was no visible difference between symptoms induced by either ACMV or EACMV-UG. However, in ACMV infections, recovery of symptoms with non symptomatic leaves following leaves with pronounced mosaic symptoms was often found (Fig. 2.3A), while in EACMV-UG infections, this phenomenon was less frequently observed (Fig. 2.3.B). In the improved cv. TMS 30572, only mild symptoms were visible with infections of ACMV (Fig. 2.3. C)

while more severe symptoms were recorded for EACMV infections. For ACMV and EACMV this cv. responded with recovery from symptoms. After a symptomatic phase with pronounced mosaic symptoms, apparently healthy leaves developed with only very faint or completely missing symptoms.

Figure 2.3: Cassava mosaic diseases established under glasshouse conditions. Symptoms of ACMV (A) and EACMV-UG (B) in the highly susceptible cassava cv. TME 1117. Single ACMV infections (C) and mixed ACMV/EACMV-UG infections (D) in the improved cassava cv. TMS 30572. Severe symptoms of EACMV-UG in a local land race cv. (E). Faint and transient symptoms of EACMV-UG in the cassava cv. Albert (F).

When mixed infections were introduced, the symptom status of TME 117 already seriously impacted from single infections did not change, while more drastic symptoms

were recorded for the moderately ACMV resistant cv. TMS 30572. In mixed ACMV/EACMV-UG infections, symptoms of severe mosaic and leaf deformation were observed and the recovery phase characteristic for single infections did not happen (Fig. 2.3. D).

Even under optimal conditions for cassava infections established in the laboratory, the cassava cv. Albert was not susceptible to ACMV infection. Upon natural infection with EACMV-UG, only few (1-3) leaves expressed symptoms and infected plants eventually recovered from symptoms (Fig. 2.3 F). However cv. Albert remained infected and recovery was only from symptoms and not from virus infection. Even after a prolonged phase of latent infections with apparently healthy, non symptomatic leaves developing, symptomatic leaves expressing mild symptom were formed after which the plants recovered again. This genotype expressed a high degree of resistance against EACMV-UG. An existing EACMV-UG infection did not change the predisposition to ACMV hence ACMV resistance was maintained.

EACMV-UG causes a serious begomovirus disease in cassava and especially local landraces (Fig. 2.3 E) respond with severe symptoms and a mosaic type with often bright yellow appearance. In advanced stages of the disease, infected plants deteriorate completely.

2.3.2 Development of a real-time PCR assay for quantification of DNA-A and DNA-B genomic components of ACMV DRC6 and EACMV-UG Ca055

Conditions for qPCR were optimized, varying the concentrations of primers and probes for each primer/probe set. Primer concentration between 225 nM and 250 nM and probe concentrations of 100 nM were found optimal with high and reproducible fluorescence signals in qPCR obtained with DNA standards. The amplicon sizes predicted for each primer and probe were confirmed by agarose gel electrophoresis. Primers and probes

designed for DNA-A and DNA-B components of virus isolates Ca055 and DRC6 were specific for their respective targets. Amplification and fluorescence was only recorded when homologous sequences were present in a DNA sample. Occasionally upon qPCR, in samples from non-infected cassava plants and non-DNA template controls, C_T values >35 were recorded, indicating the threshold limits of template detection.

Standard curves for absolute and relative quantitation of DNA-A to DNA-B genomic components were generated for plasmid DNA dilutions which were subjected to 3 replicate qPCR reactions for each dilution (Table *2.*2).

Table 2.2: C_T values obtained in qPCR for DNA- A and DNA-B components of ACMV DRC6 and EACMV Ca055 and amplification of the cassava *ß-actin* gene

Amount of plasmid [copies]	**EACMV-UG Ca055 DNA-A** C_T ± Std. dev.	**EACMV-UG Ca055 DNA-B** C_T ± Std. dev.	**ACMV DRC6 DNA-A** C_T ± Std. dev.	**ACMV DRC6 DNA-B** C_T ± Std. dev.	**β-actin cassava** C_T ± Std. dev.
3.00E+08	-	-	-	-	19.62±0.16
3.00E+07	15.44±0.22	15.79±0.21	14.42±0.15	13.66±0.02	22.87±0.35
3.00E+06	19.06±0.22	19.54±0.18	17.75±0.12	17.80±0.12	27.71±0.41
3.00E+05	22.19±0.25	22.6±0.14	21.23±0.21	21.52±0.37	30.39±0.49
3.00E+04	25.96±0.18	25.57±0.09	24.57±0.14	24.75±0.30	33.78±0.5
3000	28.95±0.31	28.26±0.15	27.9±0.33	28.63±0.22	-
300	32.03±0.39	32.5±0.29	29.91±0.58	31.45±0.40	-

- not determined

The equations of the standard curves for EACMV-UG Ca055 DNA-A and DNA-B obtained by plotting the amount [log of copies] present in each plasmid dilution against C_T (measured) - C_T (predicted) were y = -3.3245x + 40.496; R^2 = 0.9988 and y = -3.2194x + 40.076; R^2 = 0.996 respectively. This indicated an efficiency of 100%. The equations of the standard curves for ACMV DRC6 DNA-A and DNA B obtained by plotting the log of DNA- A or DNA-B copies present in each plasmid against C_T (measured) - C_T (predicted) were y = -3.1783x + 38.458; R^2 = 0.9947, efficiency = 100% and y = -3.613x + 40.999; R^2 = 0.9981, efficiency = 91% respectively. The equation of the standard curve for *β-actin* gene of cassava was y=-3.585x + 50.104; R^2 = 0.9926 reflecting a qPCR efficiency > 90%. Each qPCR system resulted in reliable and reproducible amplification signals with low C_T standard deviations and high correlation coefficients of the standard curves (Table 2.2).

Standard curves generated for plasmid DNA in all qPCR assays revealed highly similar slopes (Fig. 2.4). Since C_T values for plasmid dilutions of the *β-actin* gene resulted in a regression line with a similar slope, the ration between the C_T of the endogenous reference and the C_T of the sample could be calculated.

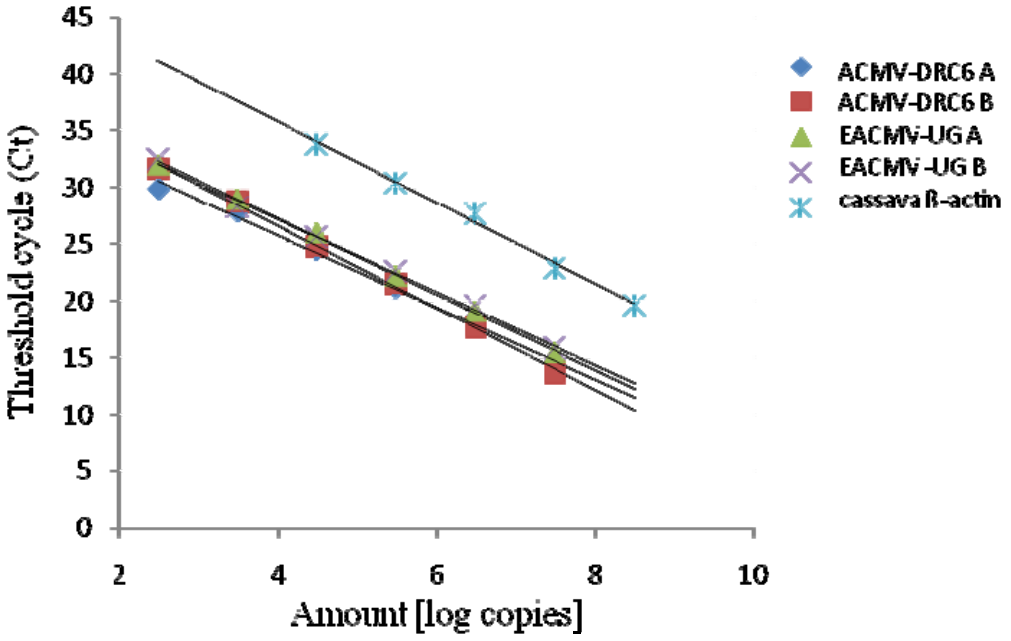

Figure 2.4: Standard curves for plasmid dilutions generated by plotting the log number of plasmid copies of DNA-A and DNA-B components of EACMV-UG Ca055 and ACMV DRC6 and plamids containing *ß-actin*, versus the C_T values obtained. C_T values for each plasmid dilution are means of triplicate qPCR reactions.

For absolute quantification of cassava mosaic virus genome components DNA-A and DNA-B of EACMV-UG Ca055 and ACMV DRC6 the exact copy number of viral components could now be determined from the standard curves generated from qPCR C_T values obtained with defined concentrations of plasmid DNA.

2.3.3 Absolute quantification of EACMV-UG Ca055 and ACMV DRC6 genome components in cassava

Absolute quantification of DNA-A and DNA-B components of EACMV-UG and ACMV showed that virus DNA concentrations were always significantly higher (3 to 4 orders of magnitude) in symptomatic tissues compared to non-symptomatic leaves (Fig. 2.5, 2.6).

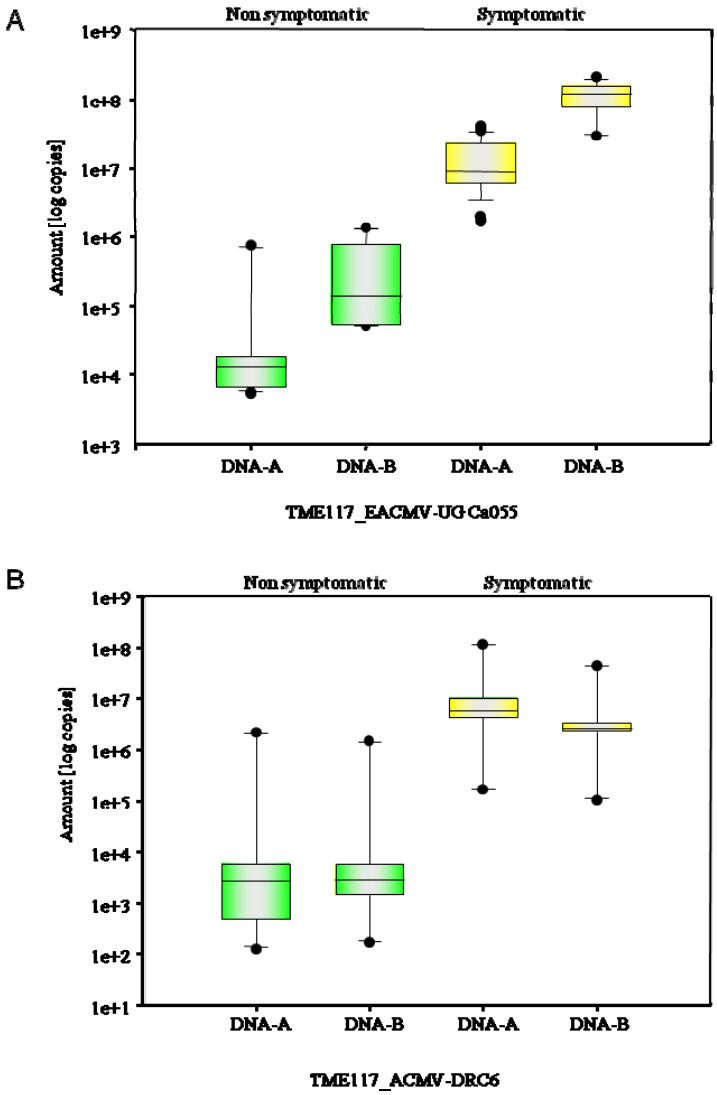

Figure 2.5: Absolute quantitation of EACMV-UG Ca055 DNA-A and DNA-B (A) and ACMV DRC6 (B) in non symptomatic and symptomatic leaf tissues of cassava cv. TME 117.

EACMV-UG Ca055 DNA-B concentrations varied significantly in non symptomatic tissues and also where significantly higher than DNA-A concentrations.

In the highly susceptible cassava cv. TME 117, the molecule numbers, in 10 ng plant DNA, of EACMV-UG Ca055 DNA-A ranged between 5300 for non symptomatic tissues and 3.3 million copies in symptomatic tissues while concentration of DNA-B was between 5200 and 21.2 million copies. The amount of ACMV DRC6 DNA-A ranged between 124 copies and 116 million copies and that of DNA-B between 164 copies and

44.1 million copies in singly infected non-symptomatic and symptomatic cassava leaves of the cassava cv. TME 117.

The study of EACMV-UG Ca055 genome components in the cassava cv. TMS 30572 (Fig. 2.6 A) also showed a large variation of DNA-A and DNA-B genome component concentrations in non symptomatic leaves. Similar to the virus situation in TME 117, the concentration of Ca055 DNA-B exceeded that of DNA-A. In symptomatic cassava tissues, the highest copy numbers recorded for Ca055 DNA-A was 32.6 million molecules while Ca055 DNA-B reached 81.4 million copies.

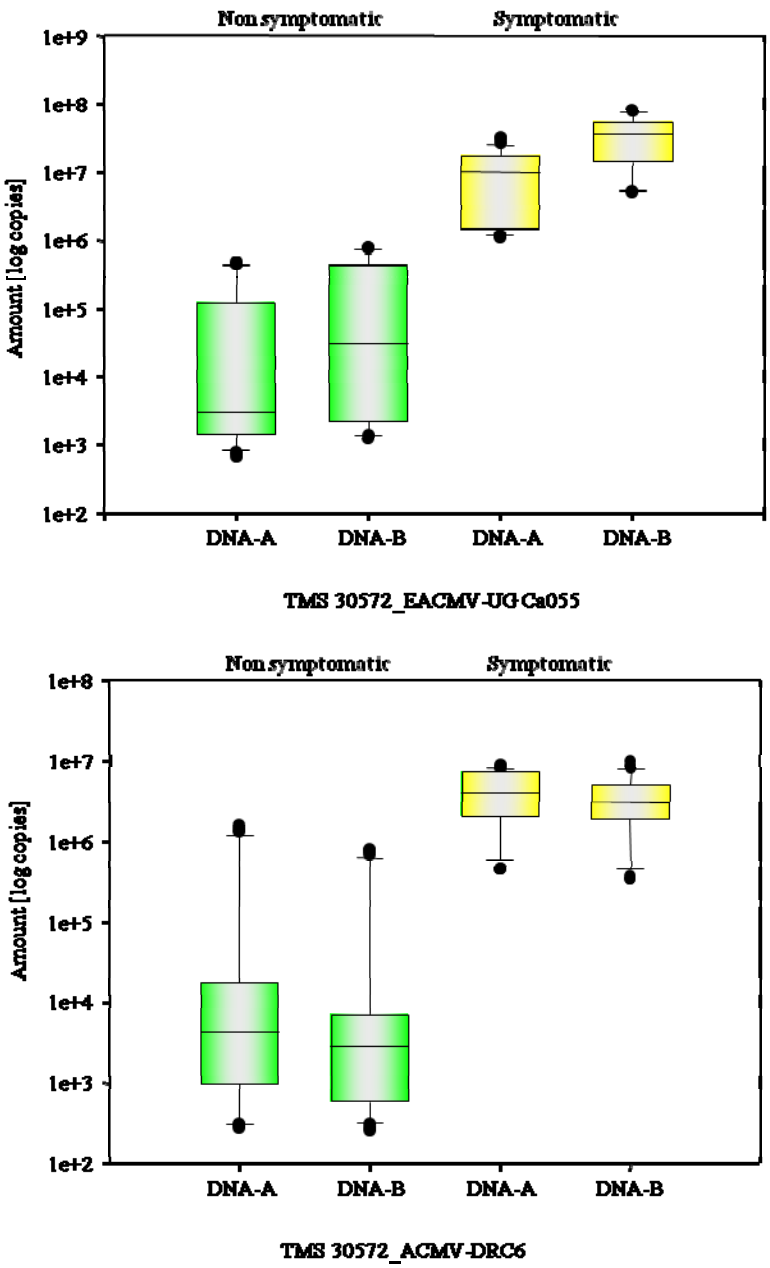

Figure 2.6: Absolute quantitation of DNA-A and DNA-B genomic components of EACMV-UG Ca055 (A) and ACMV DRC6 (B) in non symptomatic and symptomatic leaf tissues of the improved cassava cv. TMS 30572.

In Figure 2.6, the results are taken for the evaluation DNA-A and DNA-B components of ACMV DRC6 in non-symptomatic and symptomatic cassava leaf samples of land race TMS 30572. The ratio of copy numbers of ACMV-DRC6 DNA-A and ACMV-DRC6 DNA-B in non symptomatic and symptomatic cassava leaf tissues was almost 1:1. When

compared with concentrations of EACMV, ACMV concentrations in this cultivar were lower than EACMV ranging between 0.45 million to 8.34 million DNA-A copies in symptomatic leaf tissues compared to more than 32 million DNA-A copies calculated for EACMV-UG in this host.

2.3.4 **Quantification of EACMV-UG genome components in cassava cv. Albert**

In the begomovirus resistant cassava cv. Albert, infected with the wild type isolate EACMV-UG Ca111, low concentrations of DNA-A were detected in qPCR. Upon qPCR amplification of DNA-B, the increase of fluorescence was retarded and non sigmoidal curve shapes were obtained indicating for qPCR failure (Fig. 2.7).

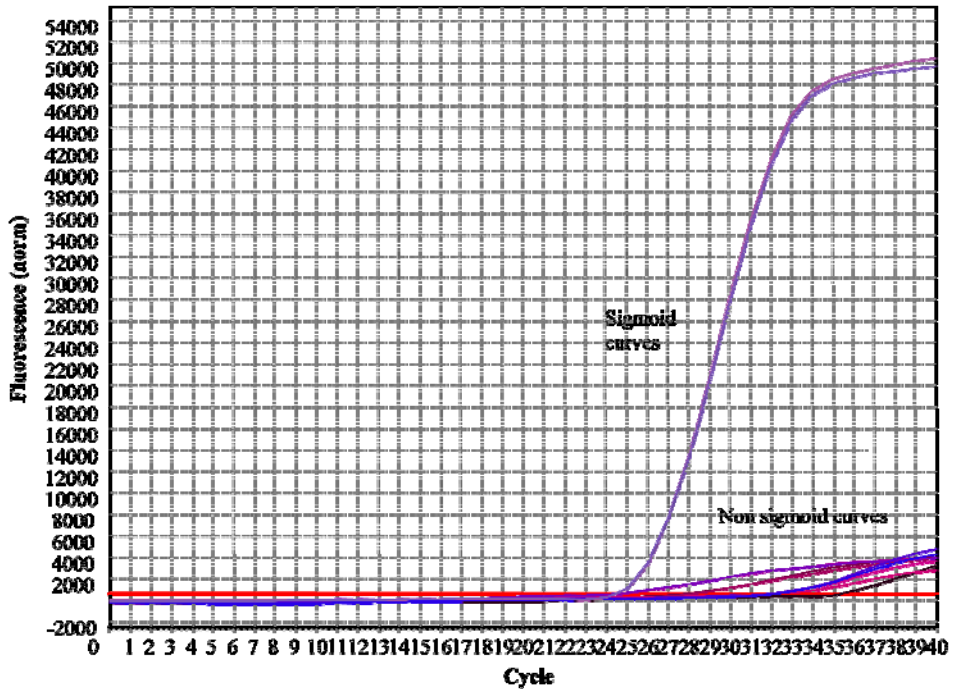

Figure 2.7: Fluorescent signals in qPCR obtained with an EACMV-UG Ca055 DNA B positive control standard sample (sigmoid curve) and signals from qPCR amplification of EACMV-UG Ca111 DNA B (non sigmoid curves). For each sample qPCR was performed in 2 replicates.

qPCR products obtained from these runs were analyzed on 2% agarose gels and an efficient PCR amplification was confirmed. Sequence analysis of the cloned qPCR fragments then revealed sequence deviations in DNA-B of EACMV-UG Ca111 identifying a mismatch of two nucleotides which is localised within the probe.

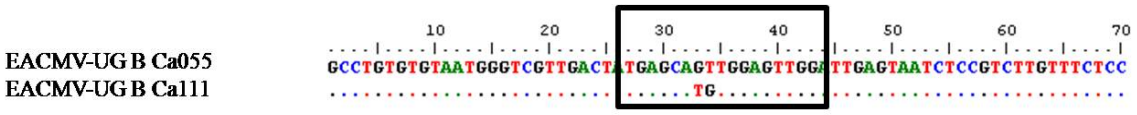

Figure 2.8: Sequence of the EACMV-UG Ca111 DNA-B fragment amplified in qPCR. Box represents probe sequence. Dots are base identities. TG base mismatch is in the core of the probe.

The mismatches of probe-template duplexes in the core region of the probe were most likely responsible for the failure of this probe in qPCR. It shows that mismatches in this region were not tolerated preventing binding of TaqMan probes to the target and development of fluorescence. Since it was not possible to generate infections with EACMV-UG Ca055 in Albert, this cv. was excluded from further experiments.

2.3.5 Relative quantitation of ACMV DRC6 and EACMV Ca055 in cassava cv. TME 117 and cv. TMS 30572

A high variation in copy numbers of DNA-A and DNA-B components was found in cassava plants which was considerably higher in non symptomatic tissues (Figure 2.5) than in leaves with mosaic symptoms. Consequently, for a better assessment of the virus situation relative quantitation, to monitor virus infection in each plant in relation to the endogenous reference *ß-actin*, was pursued (Table 2.3, 2.4 and Appendix).

Table 2.3: Calculation of relative titre of EACMV -UG Ca055 DNA-A and DNA-B in virus infected (non-symptomatic) and non infected, virus-free cassava cv. TME 117. Calculation was according to the relative standard curve method.

		EACMV -UG Ca055			β- actin gene of cassava						
Test sample TME 117_NS	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 30	DNA-A	27.82±0.26	3.81	6.56E+03	24.28±0.1	7.20	1.59E+07	4.14E-04	5.94E+02	0.01	A < B
	DNA-B	22.82±0.13	5.35	2.25E+05	24.28±0.1	7.20	1.59E+07	1.42E-02	5.57E+04		
Ca 32	DNA-A	26.55±0.22	4.19	1.57E+04	24.69±0.06	7.09	1.22E+07	1.29E-03	1.85E+03	0.10	A < B
	DNA-B	24.76±0.04	4.74	5.54E+04	24.69±0.06	7.09	1.22E+07	4.54E-03	1.79E+04		
Ca 34	DNA-A	27.80±0.14	3.82	6.56E+03	24.47±0.03	7.15	1.40E+07	4.68E-04	6.72E+02	0.03	A < B
	DNA-B	24.40±0.06	4.86	7.17E+04	24.47±0.03	7.15	1.40E+07	5.11E-03	2.01E+04		
Ca 30.2	DNA-A	21.97±0.22	5.57	3.74E+05	24.27±0.04	7.21	1.60E+07	2.33E-02	3.35E+04	0.10	A < B
	DNA-B	20.36±0.02	6.12	1.32E+06	24.27±0.04	7.21	1.60E+07	8.23E-02	3.23E+05		
Ca 32.2	DNA-A	21.03±0.05	5.85	7.13E+05	24.4±0.08	7.17	1.47E+07	4.86E-02	6.97E+04	0.35	A < B
	DNA-B	21.16±0.11	5.87	7.43E+05	24.4±0.08	7.17	1.47E+07	5.07E-02	1.99E+05		
Ca 34.2	DNA-A	26.69±0.09	4.15	1.41E+04	24.48±0.02	7.14	1.39E+07	1.02E-03	1.46E+03	0.10	A < B
	DNA-B	24.80±0.04	4.73	5.37E+04	24.48±0.02	7.14	1.39E+07	3.87E-03	1.52E+04		
Ca 91.1	DNA-A	36.56±0.52	0.79	10.74	24.33±0.1	7.19	1.54E+07	6.97E-07	1.00	1.00	A = B
(Calibrator)	DNA-B	38.16±1.89	0.37	3.92	24.33±0.1	7.19	1.54E+07	2.54E-07	1.00		

Table 2.4: Calculation of relative concentrations of EACMV-UG Ca055 DNA-A and DNA-B in symptomatic leaves of virus infected and in leaf tissues of non infected, virus-free cassava cv. TME 117.

		EACMV -UG Ca055			β- actin gene of cassava						
Test sample TME 117_S	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 31	DNA-A	18.09±0.31	6.74	5.57E+06	24.54±0.16	7.13	1.34E+07	4.15E-01	5.96E+05	0.02	A < B
	DNA-B	14.28±0.06	8.02	1.05E+08	24.54±0.16	7.13	1.34E+07	7.80E+00	3.07E+07		
Ca 33	DNA-A	15.77±0.15	7.44	2.74E+07	24.28±0.11	7.20	1.59E+07	1.73E+00	2.48E+06	0.05	A < B
	DNA-B	13.38±0.07	8.30	2.00E+08	24.28±0.11	7.20	1.59E+07	1.26E+01	4.95E+07		
Ca 35	DNA-A	17.79±0.18	6.83	6.75E+06	24.49±0.08	7.14	1.39E+07	4.87E-01	6.99E+05	0.03	A < B
	DNA-B	14.71±0.06	7.89	7.69E+07	24.49±0.08	7.14	1.39E+07	5.54E+00	2.18E+07		
Ca 31.2	DNA-A	19.65±0.13	6.27	1.86E+06	24.48±0.08	7.15	1.40E+07	1.33E-01	1.91E+05	0.02	A < B
	DNA-B	16.00±0.04	7.48	3.04E+07	24.48±0.08	7.15	1.40E+07	2.17E+00	8.54E+06		
Ca 33.2	DNA-A	15.3±0.09	7.58	3.78E+07	24.23±0.09	7.21	1.64E+07	2.31E+00	3.32E+06	0.09	A < B
	DNA-B	13.75±0.18	8.19	1.54E+08	24.23±0.09	7.21	1.64E+07	9.43E+00	3.71E+07		
Ca 35.2	DNA-A	16.6±0.07	7.19	1.54E+07	24.65±0.06	7.10	1.25E+07	1.23E+00	1.76E+06	0.04	A < B
	DNA-B	13.72±0.02	8.20	1.57E+08	24.65±0.06	7.10	1.25E+07	1.25E+01	4.93E+07		
Ca 91.1	DNA-A	36.56±0.52	0.79	10.74	24.33±0.1	7.19	1.54E+07	6.97E-07	1.00	1.00	A = B
(Calibrator)	DNA-B	38.16±1.89	0.37	3.92	24.33±0.1	7.19	1.54E+07	2.54E-07	1.00		

When EACMV-UG Ca055 infections in cv. TME 117 were monitored, the concentrations of DNA-B were always higher compared to DNA-A, whether symptoms were expressed or not (Fig. 2.9). The ratio of DNA-B to DNA-A was similar in all leaf samples (Table 2.3).

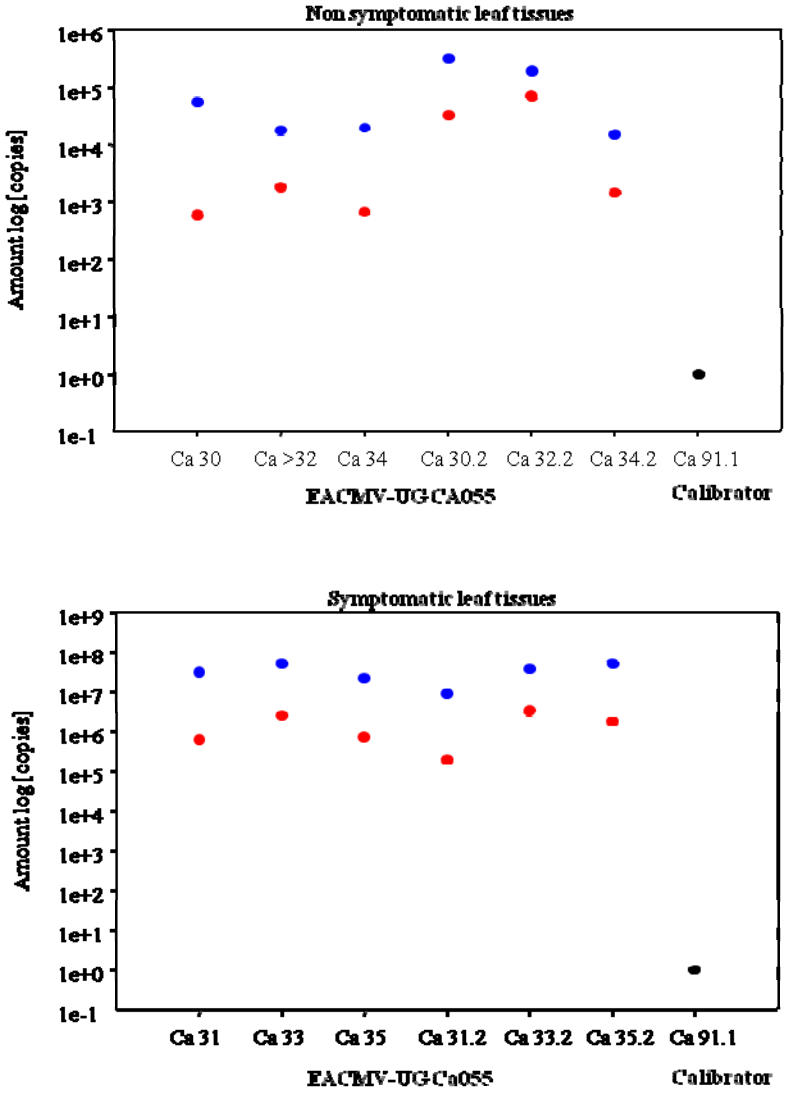

Figure 2.9: Relative amount of DNA-A and DNA-B genome components of EACMV - UG Ca055 in non symptomatic (A) and symptomatic (B) leaf tissues of cassava cv. TME 117. Red dot indicates value for DNA-A concentration, blue dot is value for DNA-B concentration in different samples.

In ACMV DRC6 singly infected cassava cv. TME 117, the proportions of DNA components were slightly reversed. In this highly susceptible cultivar, ACMV DNA-A was always higher than DNA-B although the differences in genome component concentrations were not very pronounced (Fig. 2.10 A). As in EACMV-UG infections, the

concentration of virus in symptomatic leaf tissues was considerably higher than in leaves that did not express symptoms (Fig. 2.10 A & B).

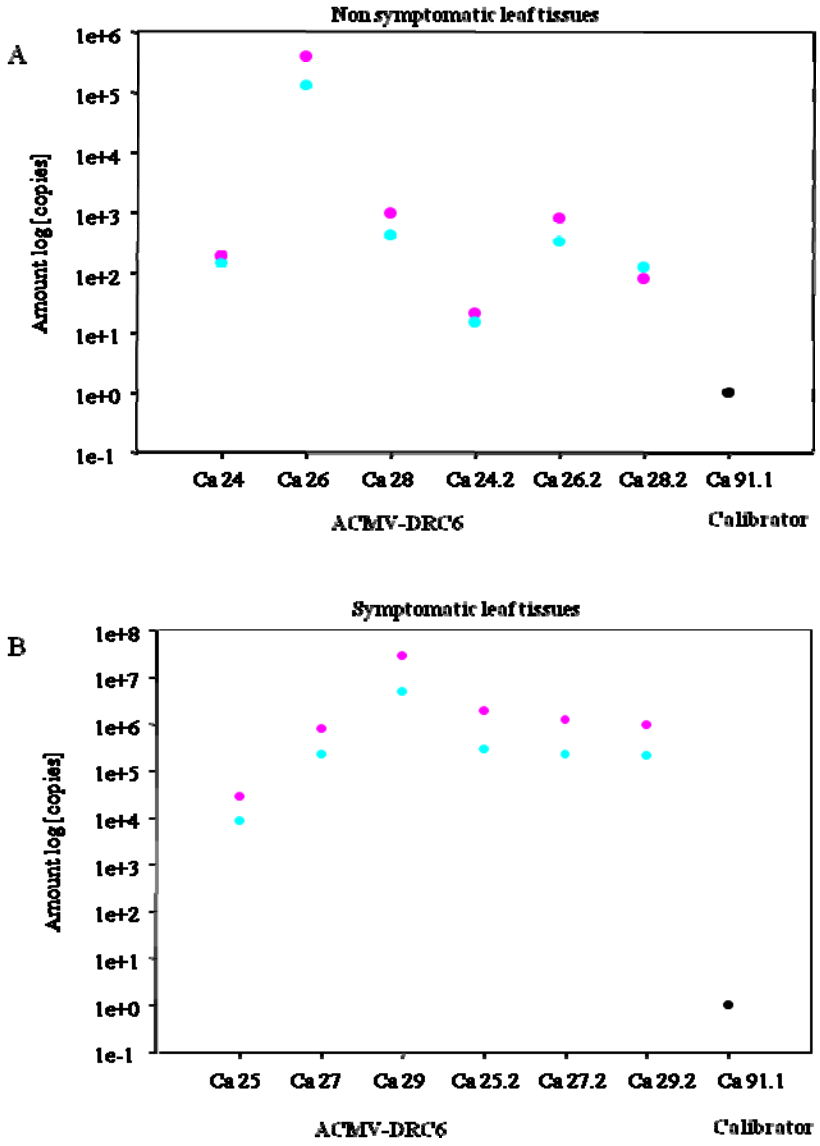

Figure 2.10: Relative amounts of DNA-A and DNA-B genome components of ACMV DRC6 in non symptomatic (A) and symptomatic (B) leaf tissues of cassava cv. TME 117. Red dot indicates value for DNA-A concentration, blue dot is value for DNA-B concentration in different samples.

In the improved cassava cultivar TMS 30572, DNA-B concentrations were higher than amounts of DNA-A. In leaves expressing mosaic symptoms, virus concentrations ranged from approximately 1 million copies of DNA-A versus 5 million copies DNA-B (sample Ca39 Fig. 2.11 B) and approximately 20 million DNA-A copies versus 50 million copies of

DNA-B (Fig. 2.11 B, sample 37). In non symptomatic leaves virus was always present and considerable concentrations of genomic components ranging from 700 to 440000 for DNA- A and from 4000 to 750000 for DNA-B were recorded (Appendix, Table 2.7 & 2.8).

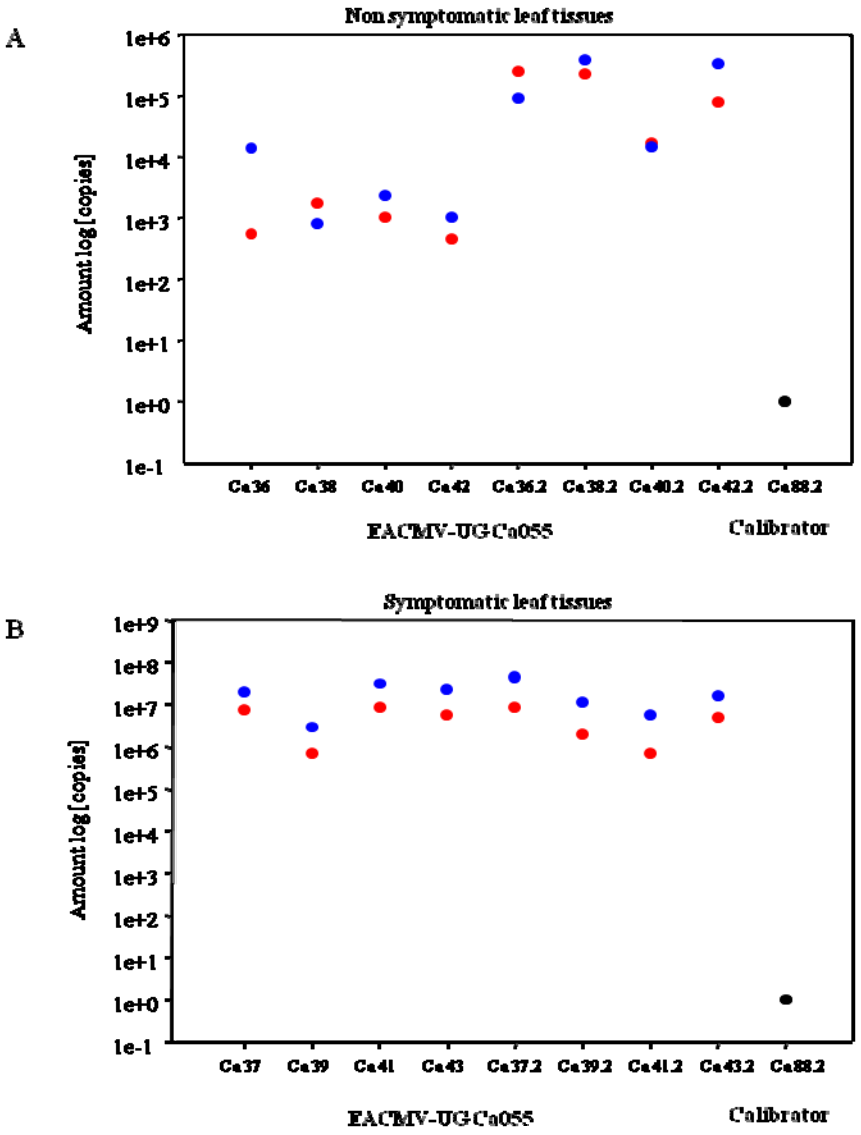

Figure 2.11: Relative amounts of DNA-A and DNA-B genome components of EACMV-UG Ca055 in non symptomatic (A) and symptomatic (B) leaf tissues of cassava plants cv. TMS 30572. Red dot indicates value for DNA-A concentration, blue dot is value for DNA-B concentration in different samples.

In TMS 30572, the ratio between DNA-A and DNA-B components was nearly 1:1 with only slight variations in the proportions of DNA-A and DNA-B in non symptomatic tissues. In non symptomatic tissues of TMS 30572 DNA-A concentrations ranged from a

few hundred copies to 1.5 million molecules (Appendix Table 2.10) while virus concentration in symptomatic tissues was constantly high ranging between 1 and 8 million copies of DNA-A and DNA-B copies respectively.

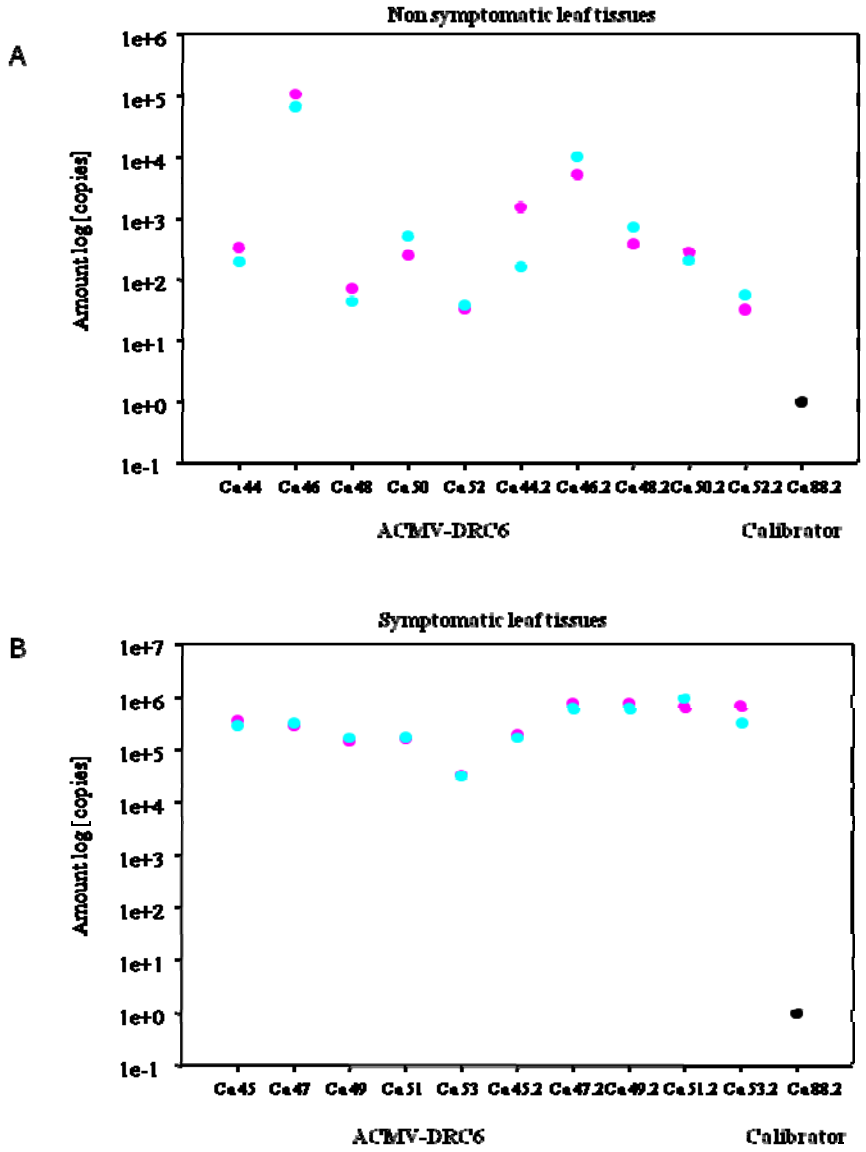

Figure 2.12: Relative amounts of DNA-A and DNA-B genome components of ACMV DRC6 in non symptomatic (A) and symptomatic (B) leaf tissues of cassava cv. TMS 30572. Red dot indicates value for DNA-A concentration, blue dot is value for DNA-B concentration in different samples.

The comparison of virus titres in the susceptible cv. TME 117 and the moderately resistant cv. TMS 30572 showed that EACMV-UG and ACMV were detectable in non symptomatic leaves with considerable variability of the amounts of DNA-A and DNA-B

detected. In symptomatic leaves a fluctuation of virus concentrations was less pronounced and virus titre was increased by several orders of magnitudes. EACMV concentrations in symptomatic tissues were similar in both cultivars but were 10 to 100 fold in excess of ACMV. Similarly ACMV DRC6 concentrations in symptomatic cv. TME 117 and cv. TMS 30572 were not significantly different albeit at much lower values than recorded for EACMV-UG Ca055.

2.3.6 **Relative quantitation of ACMV and EACMV-UG in naturally occurring mixed infections of cassava**

To study “synergism” between ACMV and EACMV in mixed infected plants, natural occurring mixed infections of these viruses in cassava were studied. Mixed infected cassava collected in the Democratic Republic of Congo (DRC6) was subjected to differential virus analysis.

In mixed infections, DNA-A concentrations of both viruses were similar. Virus accumulation was not dramatically different compared with single infections although DNA-A concentrations of EACMV-UG were lower in mixed infections while the amount of ACMV DNA-A was more or less similar. The quantitative assessment of DNA accumulation in mixed infections however showed a dramatic decrease of EACMV-UG DNA-B in mixed infections with DNA-B almost undetectable (Fig. 2.13 A & B).

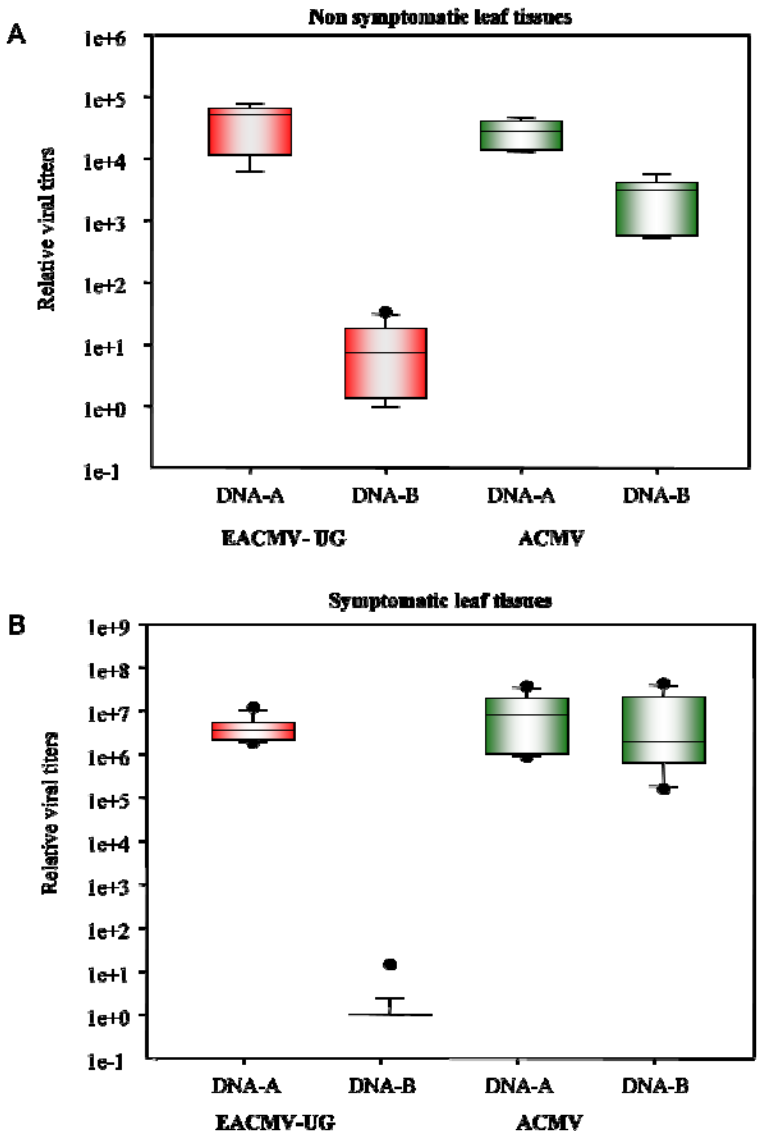

Figure 2.13: Relative quantitation of genome components of EACMV-UG and ACMV in a natural occurring mixed infection of a cassava landrace from the Democratic Republic of Congo (DRC6). Virus DNA-A and DNA-B concentrations in non symptomatic leaves (A) compared to virus titres in symptomatic tissues (B).

The analysis of a further natural mixed infection of ACMV and EACMV-UG (DRC 7) confirmed the virus situation and the quantitative ratios between the DNA genomic components of the two viruses (Fig. 2.14). Compared to single infections, there is no significant change in the level of DNA-A accumulation for both EACMV-UG and ACMV. Several millions of DNA-A copies of both viruses are present in symptomatic tissues while only a few thousand copies of DNA-A can be measured in leaves without virus

symptoms. As with DRC6, the amount of EACMV-UG DNA-B is remarkably low in mixed infected plants and only few DNA-B copies can be traced in leaves from mixed infected plants.

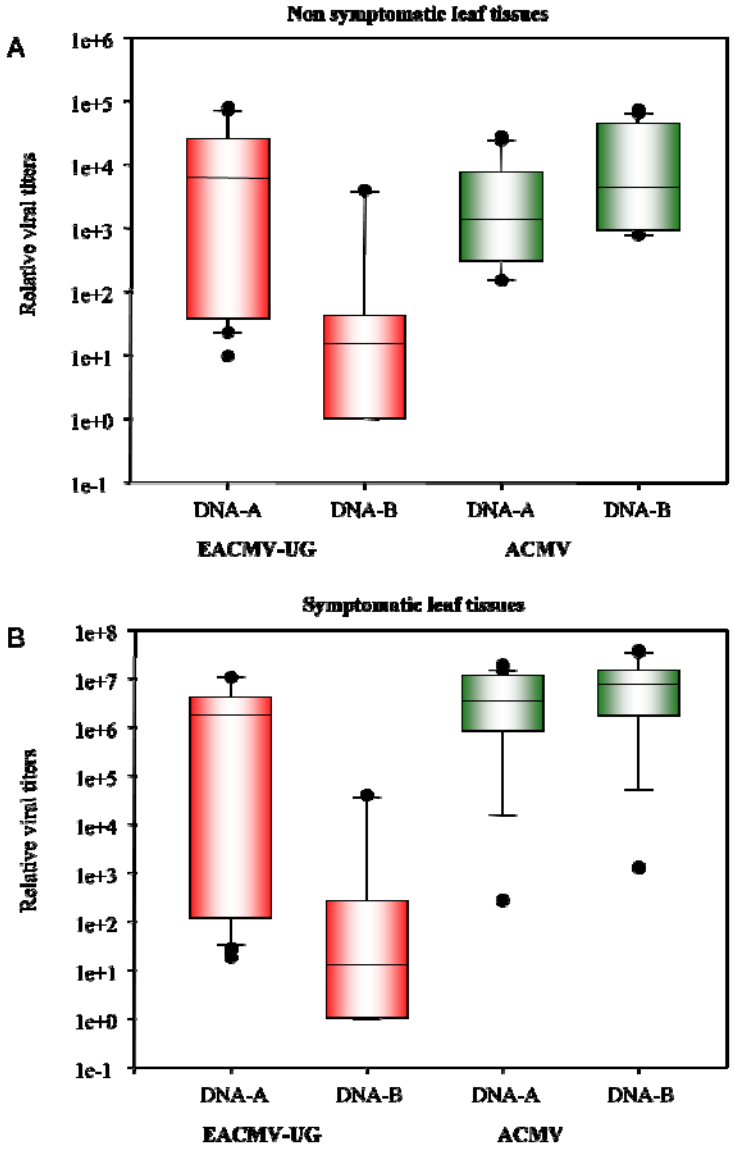

Figure 2.14: Relative quantitation of genome components of EACMV-UG and ACMV in a natural occurring mixed infection of a cassava landrace from the Democratic Republic of Congo (DRC 7). Virus DNA-A and DNA-B concentrations in non symptomatic leaves (A) compared to virus titres in symptomatic tissues (B).

To assess virus concentration in the cassava cv. TME 117 and TMS 30572, natural mixed infections of ACMV and EACMV-UG (DRC 7) were transmitted to respective virus free cassava plants by approach grafting. Newly developing leaves responded with typical virus symptoms approximately 6-10 weeks after grafting.

qPCR assessment of virus amounts in symptomatic leaves of cassava cv. TME 117 and TMS 30572 showed an accumulation of ACMV while C_T values obtained for both EACMV-UG genome components indicated for only trace amounts of EACMV-UG in both cv. (Fig. 2.15 A & B).

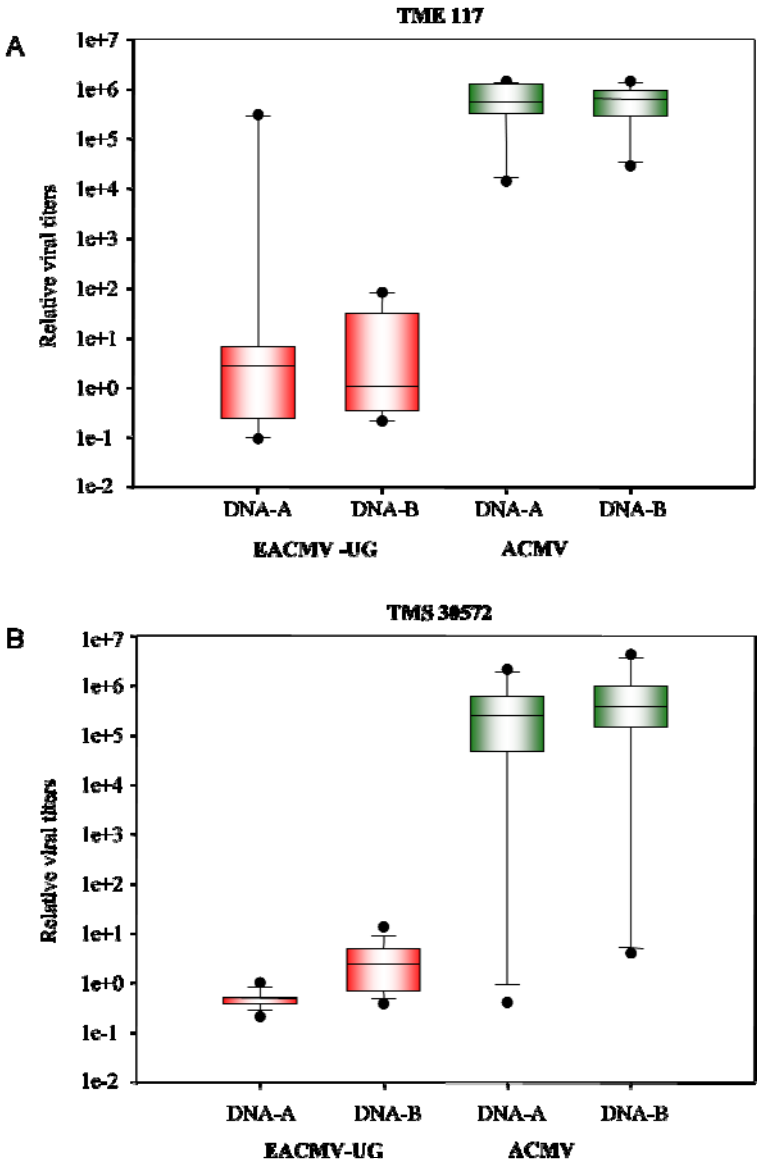

Figure 2.15: Relative quantitation of EACMV-UG and ACMV genome components in mixed infection of cv. TME 117 (A) and cv. TMS 30572 (B). Mixed infections were established by graft transmission from a naturally occurring ACMV/ EACMV-UG mixed infection (DRC 7) onto cassava plants of the respective cultivars.

In a separate grafting experiment, artificial mixed infections were generated by grafting cassava scions from cassava infected with a single virus to cassava plants infected with

the complementary virus. For this experiment, scions of TMS 30572 biolistically inoculated with infectious clones of either ACMV DRC6 or EACMV-UG Ca055 were grafted on a cv. TMS 30572 infected with the corresponding virus to create an ACMV/EACM mixed infection. In all graft combinations evaluated 6-8 weeks after grafting, no evidence was found for movement of the grafted virus into tissues with an established virus infection. And vice versa, the virus from an infected cassava rootstock was never found invading the grafted shoots that established an infection with the corresponding virus.

2.4 Discussion

PCR-based techniques have become standard for detection and identification of viruses. Combined with sequence analysis, species, strains and isolates of RNA and DNA viruses can be differentiated at high resolution and infections with multiple viruses can be determined. By use of real-time PCR it is even possible to quantify virus genomes in plants and insects (Fabre *et al.*, 2003; Korimbocus *et al.*, 2002; Mason *et al.*, 2008) and the level of virus replication in plants infected with viruses and their strains can be compared (Ratti *et al.*, 2004; Ruiz-Ruiz *et al.*, 2007; Vaianopoulos *et al.*, 2009).

Here, infections of cassava with ACMV and EACMV-UG were studied by quantitative real-time PCR to describe replication of both viruses in single and mixed infections. The amount of viral DNA-A and DNA-B components were determined by qPCR and analysed in absolute and relative quantitation experiments, for a differential and separate assessment of both virus genome components during cassava infections. Through absolute quantitation, the exact number of DNA genome components in cassava cultivars was measured whereas by relative quantitation, changes in and between the genome DNA-A and DNA-B components were analysed. This was done by comparing

qPCR measurements for virus genomes in relation to the concentrations calculated for the endogenous cassava *ß-actin* gene.

The *ß-actin* gene of cassava was chosen as endogenous reference because several copies of *ß-actin* are present in the genomes of higher plants and, because concentrations of *ß-actin* measured by qPCR were within the range of virus DNA in infected leaf tissues. Thus, the endogenous *ß-actin* was not only used to validate the analytical procedure, from DNA extraction to qPCR (Korimbocus *et al.*, 2002; Lopez *et al.*, 2006), but also, to normalise the quantity of input DNA. With this, a precise comparison among samples was possible.

Extensive analysis of ACMV and EACMV-UG genome sequences resulted in a number of primer and probe sets suitable for specific amplification of each of the virus DNA-A and DNA-B genome components. With these qPCR systems, an unbiased DNA amplification/quantitation should be achieved which was not disrupted by the presence of non target sequences. Although the sequences of all DNA-A and DNA-B genomes showed substantial differences and regions of high diversity especially in AC1 (Rep, DNA-A) and BC1 (MP, DNA-B) were identified to deduce specific primers and probes, the appropriate system was only found after a series of qPCR experiments with cassava DNA.

The significance of choosing the right primer and probe system became evident when several systems competent in quantifying virus genomic components in single infections failed to specifically detect its target in mixed infections. A specificity problem inherent to TaqMan assays was encountered when a wild type virus infection was studied. Detection of EACMV-UG isolate Ca111, a naturally occurring virus with an almost identical genome sequence to the infectious cloned virus EACMV-UG Ca055, failed using the DNA-B qPCR system and only non sigmoidal curves from artefactual fluorescence at higher Ct (>33) was recorded in qPCR (Fig.2.7). Sequence analysis of the 70bp qPCR amplicon then demonstrated that qPCR failure was because of a 2 base mismatch in the core

region of the FAM/NFQMGB probe preventing accurate probe hybridisation. While PCR amplification efficiently occurred, the probe failed to bind to its target sequence.

Sequence heterogeneity within the probe annealing region does not present problems for SYBR-green based qPCR assays since sequence heterogeneity in regions encompassed by primers do not affect detection (Papin *et al.*, 2004). A SYBR assay also would remove the layer of variation caused by hybridisation efficiency. Nevertheless, the preference for using a TaqMan assay in these experiments was to provide a high level of sensitivity combined with specificity.

The aim of this study was to analyse begomovirus infections in cassava and to compare the concentrations and ratios of 4 virus DNA genome components for which definite sequences were known. Sequence variation, a common feature of field material, was not taken into consideration when qPCR systems for each genome component were developed. The failed qPCR system for EACMV-UG DNA-B illustrates the limitation of qPCR for virus detection in field collected materials. Otherwise negligible deviations in sequence can lead to failure of the assay and cause false negative results. Nevertheless the real-time qPCR developed for the detection and quantitation of EACMV-UG Ca055 and ACMV DRC6 provides a rapid and useful diagnosis tool to identify and differentiate both viruses in field samples. While differential diagnosis for begomovirus species and strains infecting cassava is best done by established, classical PCR methods (Ariyo *et al.*, 2005; Bull *et al.*, 2006) the versatility of this method becomes evident, when extremely low virus concentrations need to be detected e.g. in resistant cassava lines.

The qPCR system developed for each virus genome component resulted in standard curves plotting the C_T values over the logarithm of the amount of input DNA. Highly linear regression lines verified accuracy over the range of dilutions chosen for each system. In qPCR a considerable variability in virus concentration was found among plants. This was more pronounced when virus was quantified in non symptomatic leaves. As shown in the boxplot diagrams which graphically depict results from absolute quantitation experiments

(Fig. *2*.4-2.6), a considerable degree of dispersion of data was recorded. This variability of virus load among plants is most likely because cassava infections cannot be synchronized and biological replications of virus infections in clonally propagated cassava genotypes still display independent infection phases. The architecture of cassava plantlets developing from cuttings can also be quite different. Single shoots (Fig. *2*.3 A-C) and branching types with several shoots (Fig. *2*.3 D, F) make it difficult to choose leaves of similar development and comparable symptoms. Furthermore, a cassava leaf consists of 5 fingers and symptoms within a leaf and between fingers can be quite variable. Hence comparing DNA-A and DNA-B concentrations and virus load is by statistics only which becomes more precise when the number of samples subjected to qPCR is increased. A similar variability in qPCR was found when tomato infections with the begomovirus TYLCSV were quantitatively analysed (Mason *et al.*, 2008). A high variability of virus load was found among leaves of the same plant with significant differences of virus concentrations even between leaves directly following each other. Virus load in the uppermost youngest leaves was less variable and thus these leaves were found most useful for comparative studies. Considering symptoms, recovery from symptoms and leaf stage, the right choice of material for a cassava virus assay was most problematic hence variability was an inevitable parameter.

In spite of a high variability of absolute values, the ratios between virus DNA and *ß-actin* were within comparable range. While with this approach extreme outliers were still recorded, box plots generally showed a more limited dispersion of qPCR data. Even slight variations in quality and quantity of DNA can significantly contribute to variability in qPCR. In relative quantification, DNA preparation affects virus and endogenous control similarly. Hence with a lower standard deviation of the data, this is the method of choice to study viruses in plant infections.

Viruses and cassava cultivars in this study were selected because ACMV and EACMV-UG are major virus species in African cassava cultivation and the viruses were frequently

reported in mixed infections (Harrison *et al.*, 1997) causing severe, synergistic disease phenotypes. The genotypes chosen are typical cassava cultivars; TME 117 a landreace from Nigeria well appreciated for its starch quality however highly susceptible to CMD and TMS 30572, a widely adopted improved cassava cultivar for its resistance characters against ACMV (Raji *et al.*, 2008) and, cassava cv. Albert, popular in Tanzania with high resistance against ACMV and EACMV-UG.

Relative quantitation of the viruses in single infections of cassava showed results similar to those obtained in absolute quantitation assays. Virus load in single infections was much higher in symptomatic tissues than in non symptomatic leaves. The concentration of virus recorded corresponded with the severity of disease symptoms with concentrations of EACMV-UG much higher than ACMV. This finding is consistent with earlier studies on ACMV (Fargette *et al.*, 1996; Ogbe *et al.*, 2003a) where higher virus concentrations correlated with the severity of the disease phenotype. These authors however were unclear whether these findings apply to all categories of cassava genotypes, from highly susceptible to resistant cultivars. Now, from the data of the quantitative assessment it can be deduced that neither ACMV nor EACMV-UG concentrations differed and similar levels were recorded for infections of the moderately resistant cv. TMS 30572 and for the highly susceptible cv. TME 117.

Symptom severity of CMD is used to classify susceptibility and resistance of cassava genotypes (Hahn *et al.*, 1980b). These phenotypic characteristics are field observations that do not take into account the virus status of the plant. Ogbe and co-authors (2003) used TAS-ELISA to quantify ACMV load in cassava cv. among which TME 117 and TMS 30572 were analysed. In both, the moderately resistant TMS 30572 and the susceptible genotype TME 117, ACMV multiplication reached similar levels despite the milder symptoms expressed in TMS 30572. This study extended the ELISA based analysis of Ogbe *et al.* (2003) by differential analysis of ACMV and EACMV-UG infections in these

cultivars. A difference in virus replication between the highly susceptible and the moderately resistant cultivar was not determined.

Resistance evaluation of cassava, done by assessment and scoring of symptom incidence and severity and calculation of disease progress (Egesi *et al.*, 2007) contains a considerable genotype x environment interaction. This is because in natural environments, cassava virus infections rely on whiteflies, are therefore not synchronised and virus load can differ significantly between plants. Under the prevailing experimental conditions of these experiments, virus load and infection were somewhat better synchronised but glasshouse conditions, although identical for all genotypes, are still artificial environments influencing virus replication and plant symptoms. Hence viruses adapted to higher temperatures will replicate to a lower level at ambient glasshouse conditions (23 °C to 26°C) than at 28 to 36°C, which are however temperatures prevailing in cassava locations in Africa. Also, symptoms can be more pronounced in cassava grown at lower temperatures hence observations under glasshouse conditions are not directly comparable to virus and diseases situations in the field. Nevertheless, virus replication in TME 117 and in TMS 30572 was at similar levels and also symptoms were not considerably different. Hence the classification of TMS 30572 as a moderately resistant cultivar is not warranted. In contrast, for cassava cv. Albert, growing conditions had no influence on its virus status. The highly resistant cv. Albert was immune against ACMV and supported only a limited replication, rather maintenance of EACMV-UG, permitting only a limited systemic invasion of virus going along with mild symptoms.

The differential assessment of DNA genomic components by qPCR revealed a striking difference between the viruses. The DNA-A to DNA-B ratios recorded for EACMV-UG were B>A while on ACMV, there was always a higher concentration of DNA-A compared to DNA-B. Symptom type and plant invasion (movement) are predominant features of DNA-B, which was studied intensively with plant infections induced by pseudo-recombinant viruses (Hou & Gilbertson, 1996; Unseld *et al.*, 2000). From these studies,

the increase in symptom severity coincided with accumulation of DNA-B genomic components while plants showing attenuated symptoms had reduced levels of DNA-B. High levels of virus accumulation and consequently more severe symptoms were considered as better adaptability of the virus to the host (Hou *et al.*, 1998). Thus from this quantitative study, EACMV-UG appeared to be the better adapted virus than ACMV reaching far higher virus concentrations in all cultivars than what was recorded for ACMV. The increased DNA-B levels in EACMV-UG infections, indicated for a highly efficient invasion of the “adapted” host. The poor replication of EACMV-UG in cassava cv. Albert and the limited virus movement with mild symptom expression then is a characteristic of a “non adapted” host (Dawson & Hilf, 1992).

In pseudo-recombinant virus studies, symptomless infections always coincided with lower levels of DNA-B accumulation thus mapping symptoms to DNA-B (Schaffer *et al.*, 1995; Vonarnim & Stanley, 1992). In this study, non symptomatic tissues had significantly lower concentrations of both DNA-A and DNA-B genome components. Asymmetric DNA-A and DNA-B regulation in pseudo-recombinant infections (Faria *et al.*, 1994) indicating for virus (Morris *et al.*, 1991) and host encoded factors determining the disease phenotype were not found with ACMV and EACMV-UG infections in cassava. Therefore symptomless phases and recovery can be attributed to the ability of the host to, at least in phases, impair virus replication while a systemic virus invasion in asymptomatic leaves still occurs. In this study a considerable although quite variable level of virus in non symptomatic leaves was found and virus free leaves were only rarely found.

From the mere comparison of virus accumulation, EACMV-UG was found in considerably higher concentrations in infected plants compared to ACMV. Therefore one could conclude that the spread of the CMD pandemics in East Africa which is associated with significant increases of dual ACMV/ EACMV-UG infections is because EACMV-UG acquisition by whiteflies is more efficient due to a higher number of virus particles in

infected plants. Consequently, EACMV-UG would become the predominant virus in areas previously affected only by ACMV. This hypothesis which is based on observations from single virus infections however cannot be substantiated with the observations made with natural and artificial mixed infections in cassava. In cassava mixed infections collected from western Congo (DRC) ACMV concentrations were recorded which were similar to single infections with lower virus load in non symptomatic tissues (Fig. *2.*13 - 2.15). Interestingly, EACMV-UG DNA-A concentrations were also more or less unchanged while the concentration of EACMV-UG DNA-B was drastically reduced. This observation made with highly standardised qPCR systems is in sharp contrast to reports of synergism among ACMV and *East African cassava mosaic Cameroon virus* (EACMCV) where accumulation of EACMCV was very high for both DNA genome components with greatest increase of DNA-B compared to single EACMCV infections (Fondong *et al.*, 2000). The study by Pita and co-workers (Pita *et al.*, 2001b), to describe viral keys determining the CMD epidemic in Uganda also showed a higher accumulation of both EACMV genomes in mixed infections thus indicating for strong synergism between ACMV and EACMV-UG. The higher level of EACMV-UG DNA-B accumulation over DNA-A was consistent with more severe symptoms and was postulated as a factor driving CMD epidemics and spread by whiteflies. The study presented here cannot confirm these earlier reports. ACMV concentrations in mixed infections appear to be unaffected by presence of EACMV-UG while the low accumulation of EACMV-UG DNA-B rather indicates for negative interference. As shown for TYLCV/ TYLCSV mixed infections (Morilla *et al.*, 2004) increased symptoms in dual infections are not necessarily bound to higher DNA accumulation. The TYLCV/TYLCSV infections however are confined to the phloem and hence this might be limiting the accumulation of DNA.

In earlier studies (Fondong *et al.*, 2000; Pita *et al.*, 2001b) tissues reflecting very severe symptoms of synergism among ACMV and EACMCV were compared. Those tissues

reflect ultimate, highly severe stages of infection with severely affected and malformed leaves (see Fondong *et al.*, 2000; Fig. 1a). In fact cassava leaves from mixed infections in this study also showed very severe symptoms but samples for qPCR were not taken from these decline phases of the plant. During plant infections, the kinetics of ACMV and EACMV-UG however might change as was indicated by the graft inoculation experiments (Fig. *2.*15). When natural virus infections in cassava were transmitted to TME 117 or TME 30572, the accumulation of ACMV occurred very fast, while EACMV-UG was barely detected even until 7 weeks after inoculation. This might be because of lower amounts of DNA-B limiting virus movement which probably changes in acute and serious phases of plant infections. This however was not investigated in this study.

Mixed ACMV/ EAMCV -UG infections were studied in the glasshouse under prevailing conditions which were similar for all viruses but do not reflect field situations. Thus EACMV-UG might under higher temperatures reach higher levels of virus accumulation. And furthermore, cassava under field conditions might show more or less pronounced virus symptoms and probably a more pronounced synergism phenotype. This study could not address this aspect. However, using qPCR it could be shown that a moderately resistant cassava genotype still sustains high levels of virus replication and thus might not be the best parent for resistance breeding. Furthermore while only a limited set of plants was subjected to quantitative virus analysis, methods are now developed to further quantify interactions between ACMV and EACMV-UG in natural infections of cassava.

3. Investigations on the distribution and interaction of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) in *Nicotiana benthamiana*

Abstract

To study virus movement and spread in plants infected with geminiviruses, infectious clones of the begomoviruses *African cassava mosaic virus* and *East African cassava mosaic virus* were modified by insertion of the gene for green fluorescent protein (GFP) in place of the coat protein (*CP*) gene and alternatively, by replacing either the *BV1* or *BV2* genes on the DNA-B genomic components with GFP. AV2://GFP fusions and CP DNA-A mutant viruses failed to produce infections in *Nicotiana benthamiana* when inoculated with their cognate DNA-B and infectivity inherent to the parental begomovirus clones was lost. In *N. benthamiana* an initial GFP fluorescence emission was observed in epidermal cells inoculated with mutated viruses only. Weak systemic symptoms from infection with the AV2://GFP mutant virus was found in rare cases, however there was no GFP expression. Complementing mutant DNA-A or DNA-B clones with their respective wild type genomic components restored systemic infections however GFP mutants were not transreplicated to emit fluorescent signals.

Similarly GFP mutants of EACMV-UG DNA-B were also not infectious and mixtures of dysfunctional BC1 or BV1 in DNA-B with wild type DNA-A did not result in viable virus. GFP expression was observed in patches either inoculated with a mutated *BC1* gene or BV1 replaced with GFP. This GFP fluorescence probably arose from an initial replication from a functional DNA-A. From this study, it can be assumed that GFP expression from replicating cassava mosaic begomoviruses is not possible since labelling of DNA-A and DNA-B genome components of EACMV and ACMV with GFP resulted in loss of viability and function.

3.1 Introduction

Cassava mosaic disease is a complex disease caused by nine different species of geminiviruses, within the genus *Begomovirus* of the family *Geminiviridae* (Patil & Fauquet, 2009). Geminiviruses are characterized by small geminate particles containing single stranded circular DNA molecules. Their genome is amplified in the nuclei of host cells by a rolling circle replication mechanism which utilizes dsDNA intermediates as templates for replication and, to generate mature ssDNA genomes. They also serve as templates for transcription during infection (Bisaro, 2006; Hanley-Bowdoin *et al.*, 1999; Saunders *et al.*, 1991). The genomes of geminiviruses are small and encode only few proteins whose open reading frames extend bi-directionally from an intergenic region. Therefore the coding regions are located on both strands, virion sense and complementary, of the ds and transcriptionally active intermediate DNA molecule. The circular DNA genomes of cassava infecting begomoviruses consist of two components, DNA-A and DNA-B, which are essential for infectivity in plants (Stanley & Townsend, 1985; Vanitharani *et al.*, 2005). Both molecules have cognate intergenic regions, also referred to as common region (Fig. 3.1).

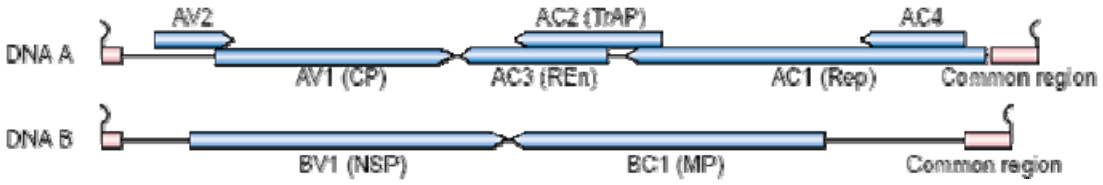

Figure 3.1: Genome organization of a bipartite begomovirus. The common region is highly conserved in the cognate DNA-A and DNA-B components of individual bipartite begomoviruses. It contains elements required for viral DNA replication and gene expression. CP, coat protein; MP, movement protein; NSP, nuclear shuttle protein; Ren, replication enhancer; Rep, replication associated protein; TrAP, transcriptional activator protein. Source: Mansoor *et al.*, 2003

DNA-A has six genes: AC1 encodes a replication–associated protein (Rep) essential for viral DNA replication associated with host DNA polymerase; AC2 encodes a transcription activator protein (TrAP) controlling gene expression and silencing suppression; AC3 encodes a replication enhancer protein (Ren); AV1 and AV2 encode the coat protein (CP) for encapsidation and a pre coat protein, respectively. The small protein AC4 which is found in some geminiviruses is involved in pathogenesis and RNA silencing suppression and together with Rep is implicated in cell cycle progression (Fondong *et al.*, 2007). On DNA-B BV1 and BC1 genes (Fig. 3.1) encode a nuclear-shuttle protein (NSP) and movement protein (MP), respectively (Mansoor *et al.*, 2003b; Vanitharani *et al.*, 2005).

In situ hybridization studies revealed tissue specificity of monopartite (Morilla *et al.*, 2004; Rojas *et al.*, 2001) and bipartite (Morra & Petty, 2000; Qin & Petty, 2001; Wege *et al.*, 2001) geminiviruses with DNA accumulation only found in nuclei of infected cells. Even co-infection with *Cucumber mosaic virus* (CMV) did not change this although an increased number of nuclei with higher amounts of *Abutilon mosaic virus* (AbMV) were found in *Nicotiana benthamiana*, tobacco and tomato (Wege & Siegmund, 2007). Co-infection with CMV however released the phloem restriction of AbMV thus indicating for considerable synergistic effects also existing in co-infections of DNA and RNA viruses. In this, the CMV silencing suppressor gene 2b was implicated. In contrast to CMV, *Tomato mosaic virus* (ToMV) did not change tissue tropism but led to decreased titres of AbMV in co-infected plants, despite severe symptoms indicating for synergistic interactions (Pohl & Wege, 2007). This symptom synergism despite negative interference was explained by a simultaneous action of two viruses on different host pathways resulting in an overall enhanced host response.

Cassava is a vegetatively propagated crop and thus virus diseases are common. Mosaic diseases caused by begomoviruses occur wherever this plant is grown in Sub-Sahara Africa and in India. Frequently multiple virus species are found in cassava and this leads

to more severe symptoms and often decline of the infected plant. One of the fundamental questions in virus synergism is, whether the spatial distribution of viruses, i.e. cell and tissue invasion, differs between single and mixed infections of geminiviruses. If synergism is a consequence of virus to virus and virus to host interaction it is interesting to uncover whether the virus cross talk acts on a cellular basis i.e. both viruses are invading the same cell or, is based on signal transduction between cells or tissues, i.e. when viruses do not enter into cells harbouring other viruses.

This study attempts to visualize movement and spatial distribution of cassava mosaic geminiviruses in *N. benthamiana*. To directly follow cell and tissue invasion of the viruses, direct labelling of the viruses by incorporating the gene for green fluorescence (GFP) into the viral genomes was approached. Movement within the plant and tissue invasion should then be visualised by using confocal laser scanning microscopy monitoring the fluorescence signal (Wang *et al.*, 1997; Wang *et al.*, 1999).

Cell-to-cell movement of geminiviruses in plants is facilitated by CP dependent spread of monopartite geminiviruses and CP independent movement of bipartite begomoviruses. The CP of the bipartite begomovirus ACMV is essential for encapsidation of the DNA components, for vector transmission (Briddon *et al.*, 1990; Liu *et al.*, 1997) and is implicated in movement including nuclear import and export and transport of the viral genome to the cell periphery (Unseld *et al.*, 2001; Unseld *et al.*, 2004). The CP is not essential for systemic infection in *N. benthamiana* (Stanley and Townsend, 1986; Ward *et al.*, 1988). Hence the dispensability of a functional CP for virus movement presents the opportunity to replace sequences of this gene with that of the GFP gene and, to maintain vital viral functions, pathogenicity and movement, *in planta*. In this study the DNA-A of ACMV (isolate DRC6) and EACMV (isolate Ca123) were modified by replacing the CP gene with GFP. In an alternative strategy, the GFP gene was introduced into the DNA-B component of EACMV (isolate Ca055).

3.2 Materials and Methods

3.2.1 **Plants and Viruses**

Virus infections in the *N. benthamiana* were induced by inoculations with cloned virus genomes of *East African cassava mosaic virus* (EACMV isolate Ca123) DNA-A and an *East African cassava mosaic virus* (EACMV–UG, Uganda variant, isolate, Ca055) DNA-B. Initially DNA-A of EACMV isolate 123 and EACMV-UG isolate Ca 055 were manipulated, however site directed mutagenesis (sdm) of Ca123 DNA-A to incorporate restriction sites for cloning was more successful hence EACMV Ca123 DNA-A was used throughout all DNA-A manipulations. Mixtures of genome components Ca123 DNA-A and Ca055 B clones were highly infectious to both cassava and *N. benthamiana* and pseudo-recombinant viruses lead to even more severe symptoms than the original homologous combinations. Manipulations of *African cassava mosaic virus* DNA-A an infectious ACMV clone generated from an isolate collected in the Democratic Republic of Congo (ACMV isolate DRC6) was used. Head-to-tail partial multimers of DNA-A genomic components and DNA-B genomic components in the binary vector pGreenII (John Innes Center, UK) were introduced by biolistic inoculation, essentially following protocols established in the Plant Virus laboratory (Ariyo *et al.*, 2006). Systemic begomovirus infections were verified by PCR using DNA-A specific primers.

3.2.2 **Incorporating GFP into recombinant EACMV and ACMV DNA-A and DNA-B for localization studies**

The basis for generation of EACMV and ACMV chimeric DNA-A components were infectious virus clones comprising head-to-tail partial multimers of genomic components DNA-A and DNA-B used for resistance screening at the DSMZ Plant Virus Department. By site directed mutagenesis, a *Sma* I/*Xma* I site and a *Sph* I site were incorporated into

the *CP* gene for removal of CP sequences and to facilitate directional cloning of foreign gene sequences into the replacement vector (Figure 3.2).

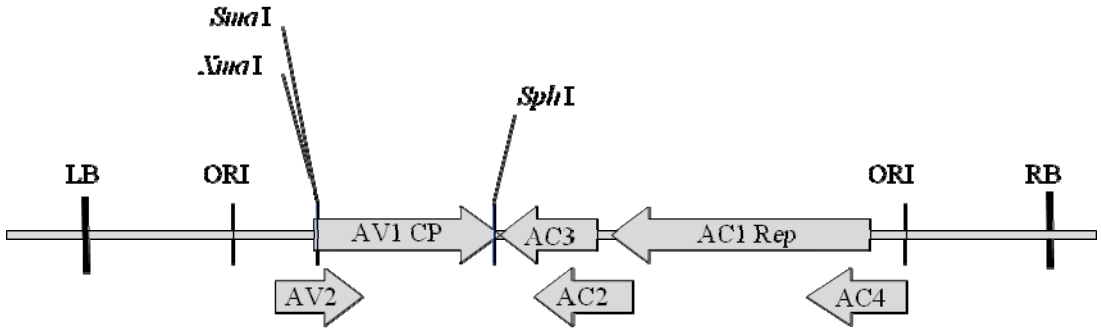

Figure 3.2: EACMV DNA-A vector pSN29_pG EACMV [Ca123]-A_R6. A partial head-to-tail dimer EACMV [123] DNA-A in pGreen II carrying a duplicated, redundant common region fragment (ORI) was modified by site directed mutagenesis to introduce restriction sites for directional cloning of GFP gene sequences.

The constructs were subcloned into the backbone of the binary vector pGreen II (http://www.pgreen.ac.uk) for agro-inoculations of plants. All clones were functionally verified by agro-inoculation to introduce plant infections in *N. benthamiana* and by biolistic delivery in cassava (Winter, unpublished).

Head-to-tail partial multimers of ACMV genomic components DNA-A and DNA-B were similarly developed in pGreen II vectors. Restriction sites were introduced in the CP sequences of ACMV DNA-A by replacing parts of the CP with a synthesized gene fragment carrying *Kpn* I and *Pst* I and *Hin*d III sites at positions appropriate for directional cloning of GFP (Figure.3.6). For transient gene expression and as a control, GFP was cloned under the control of an enhanced Cauliflower mosaic virus (CaMV) 35S promoter in pGreenII. Gene constructions, manipulations and recombinant DNA techniques were performed following established laboratory protocols (Sambrook & Russel, 2001). All restriction endonucleases and DNA-modifying enzymes were used according to the manufacturer's instructions.

A GFP reporter gene, the smRs-GFP gene (Davis & Vierstra, 1998) with improved fluorescence characteristics and protein solubility was found best suited for transient studies. This smRs-GFP was amplified by PCR from a source plasmid carrying this reporter gene in a different context (Dietrich & Maiss, 2003).

3.2.2.1 **GFP labelling of EACMV DNA-A**

To construct all recombinant labelled DNA-A constructs of EACMV-Ca123, template plasmid pSn29_EACMV[Ca123]DNA-A_R6 (Fig. 3.2) was used.

For construction of pSn58_EACMV[Ca123]DNA-A[ΔCPAV2://GFP] (Fig. 3.3), to generate a carboxyterminal GFP fusion to AV2, a *Pst* I restriction site was introduced by sdm upstream the AV2 stop codon. Inverse amplification using Phusion high fidelity DNA polymerase (Finnzymes) with phosphorylated forward R6_dCPAV2://GFP+:

5'-P*-TTACTGCAGtGCATGCATTTCTATGATGCAGTG-3' and reverse primers R6_dCPAV2://GFP-: 5'-P*-GACGTTACTGCAGCCCTTCGGGACATC-3' reconstituted the entire plasmid, excluded the CP and added a CAG codon at a position upstream the AV2 stop codon. After re-ligation of the PCR product the *Pst* I site was reconstituted and the resulting construct was then digested with *Pst* I and *Sph* I to accept GFP. The smRs GFP gene was then inserted to result in pSN58 after amplification with primers R6_GFPAV2dCP+: 5`-TAATCTGCAGTAAAGGAGAAGAACTTTTCACTGG-3`and

R6_GFPAV2dCP-: 5`-TATTAGCATGCTTTTGTATAGTTCATCCATGCC-3` adding *Pst* I and *Sph* I sites respectively.

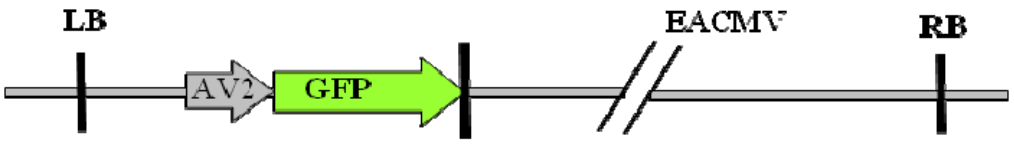

Figure 3.3: pSn58_EACMV [Ca123]-A [ΔCPAV2://GFP] (6686 bp). The smRs GFP is inserted as a translational fusion to the precoat protein AV2 with the CP entirely replaced by GFP

In another strategy smRs GFP was inserted in an AV2 and CP deletion mutant to reach pSn92_EACMV[Ca123]DNA-A[ΔCPΔAV2GFP] (Fig. 3.4). The template plasmid pSn29_EACMV[Ca123]DNA-A_R6 was restricted with *Xma* I and *Sph* I to release the CP fragment which was subsequently replaced by GFP. The resulting intermediate construct was used as template for reverse amplification using phosphorylated forward R6_dCPdAV2+: 5'-P*-ATGTCGAAGCGACCCGGGGAACTTTTC-3' and reverse primer R6_dCPdAV2-: P*-GTTGACGCGCTCTACTACTTCGCGACGAAGTAT resulting in a deleted AV2 and CP, however leaving the AV2 start codon intact.

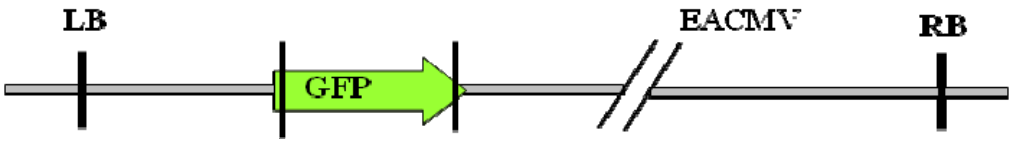

Figure 3.4: pSn92_EACMV[Ca123]DNA-A[ΔCPΔAV2GFP]. The smRs GFP is inserted into the DNA-A genome component replacing AV2 and CP and utilising the AV2 start consensus.

In pSn93_EACMV[Ca123]DNA-A[AV2ΔCPGFP] (Fig. 3.5) the CP was replaced with GFP and keeping the start codon of CP for GFP translation initiation while AV2 was left truncated.

A 734 bp *Sma* I/ *Sph* I fragment of pSN29 was replaced with a 725 bp recombinant GFP construct which was amplified using

SN_R6GFPnew+ : 5'-GGATCCCGGGGTTAGCAAGGGCGA-3' and

SN_R6GFPnew- : 5'-GCATGCGCTTGTACAGCTCGTCCATGC-3', to generate the respective restrictions sites for directional cloning.

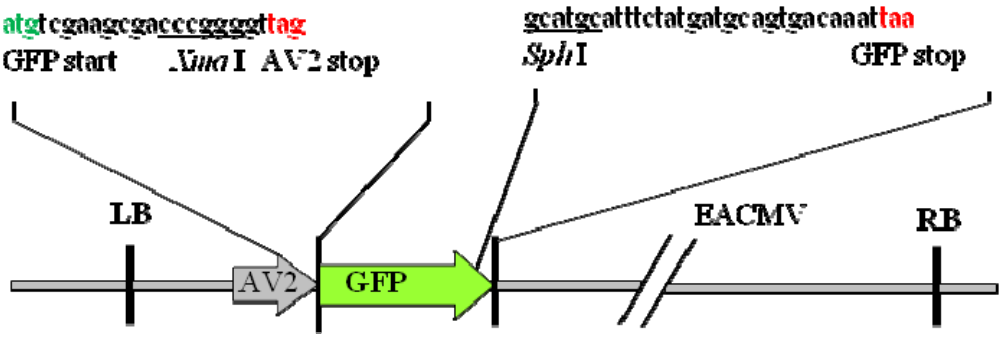

Figure 3.5: pSn93_EACMV[Ca123]DNA-A[AV2ΔCPGFP]. The smRs GFP is inserted into the DNA-A genome component utilising the CP start codon and truncating AV2.

3.2.2.2 GFP labelling of ACMV DNA-A

To construct GFP labelled DNA-A of ACMV, a multimeric cloned DNA-A of an ACMV infectious virus (isolate DRC 6) was used as template. Multiple restriction sites in the ACMV DNA-A genome presented an impediment to follow classical cloning and subcloning strategies. Hence an artificial de novo synthesised gene was inserted that carried restriction recognition sites and partially reconstituted the CP. The resulting plasmid, pSn59_pGACMV[CaDRC6]DNA-A_cloning (Figure 3.6), was used to generate further recombinant ACMV constructs by introducing GFP using the appropriate restriction sites.

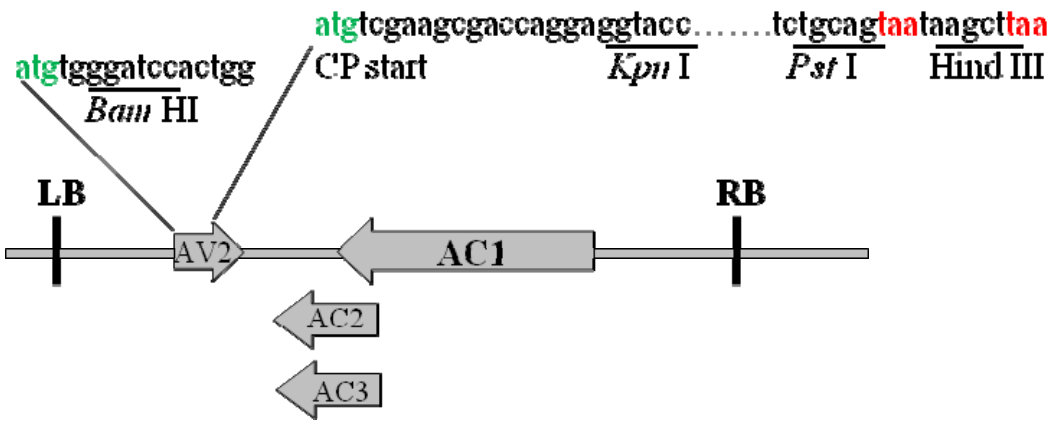

Figure 3.6: pSn59_ pG ACMV [CaDRC6]-A cloning (6116 bp). The artificial gene carries *Kpn* I, *Pst* I and *Hind* III sites to facilitate cloning.

For construction of the recombinant plasmid pSn60_ACMV[CaDRC6]DNA-A[AV2://GFPΔCP], smRs GFP was introduced into the *Pst* I/ Hind III sites of pSN59 to generate a translational fusion with AV2 and truncating CP. To generate pSn61_ACMV[CaDRC6]DNA-A[AV2ΔCPGFP], GFP was introduced via *Kpn* I/ Hind III sites to utilize the start of CP and truncating AV2.

pSn62_ACMV [CaDRC6]DNA-A[ΔAV2ΔCPGFP] then carried a GFP introduced via the *Bam* HI / *Hin*d III restriction sites deleting AV2 and CP but using the AV2 translational context as start.

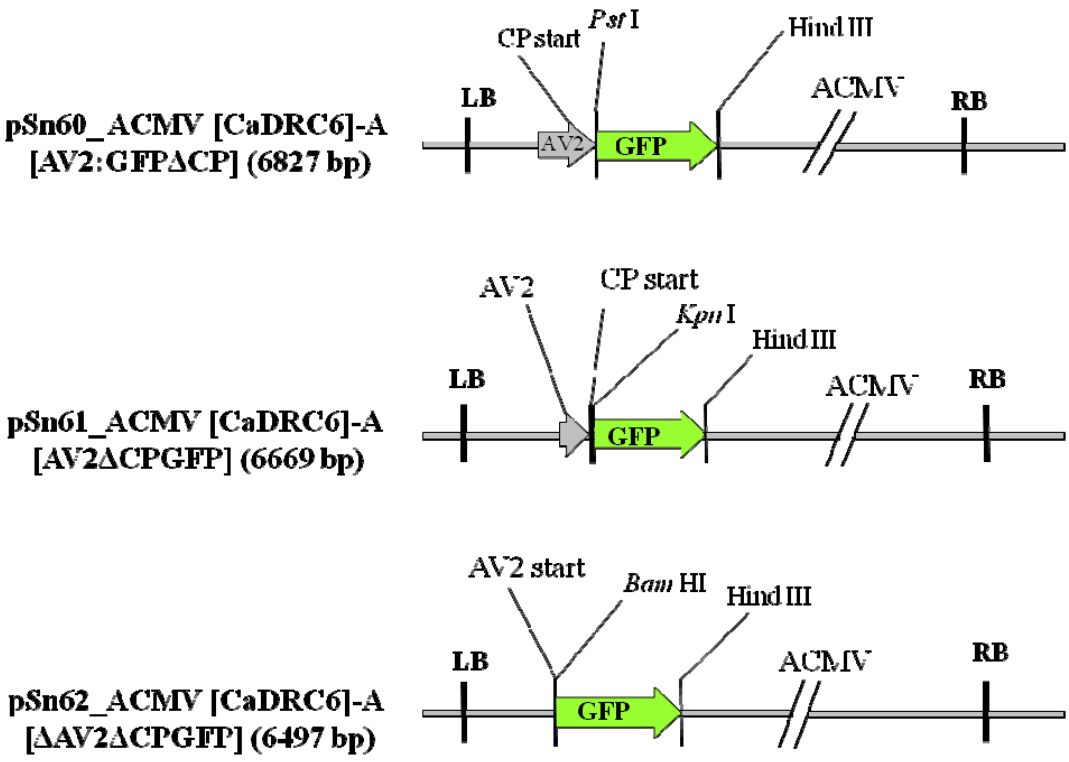

Figure 3.7: GFP constructs in vectors containing ACMV DNA-A genomic components. pSn60_ACMV [CaDRC6]-A [AV2://GFPΔCP], as translational fusion to AV2; pSn61_ACMV [CaDRC6]-A [AV2ΔCPGFP], with GFP translation from the start of CP and, pSn62_ACMV [CaDRC6]-A [ΔAV2ΔCPGFP] utilising the start codon of AV2 and truncating the CP.

3.2.2.3 GFP labelling of EACMV-UG DNA-B

For construction of recombinant labelled DNA-B constructs, a partial multimeric DNA-B of an infectious EACMV-UG (isolate Ca055) clone, pSn001_EACMV[Ca055]DNA-B_1.7 was used as template.

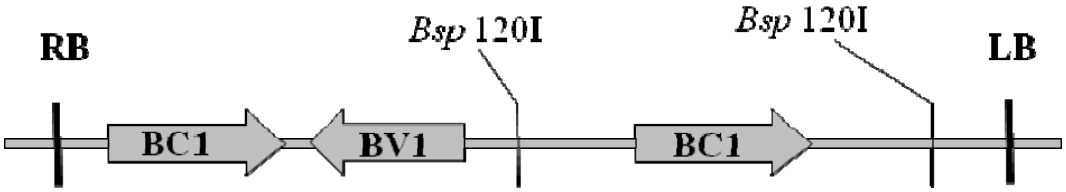

Figure 3.8: Partial head-to-tail dimer pSn001_pG EACMV [Ca055]-B _1.7 carrying a duplicated, BC1 fragment redundant fragment (BC1)

The template plasmid was digested with *Bsp* 120I to eliminate the partial repeat comprising BC1 then treated with Klenow fragment to fill in before ligation to generate an intermediate clone. By sdm, BC1 and BV2 respectively were modified, to introduce the restriction sites *Mlu* I/*Nhe* I in a BC1 construct and the restriction sites *Mlu* I/*Nhe* I in BV1 of another intermediate clone. The smRs-GFP amplified with primers carrying appropriate restriction sites was subsequently introduced replacing BC1 or BV1 respectively. Finally the *Bsp* 120I redundant BC1 fragment was subsequently cloned into the smRs GFP plasmids to reconstruct the partial 1.7 head-to-tail multimer of EACMV DNA-B (Figure 3.9). All the intermediates and end constructs were confirmed by sequencing analysis using the service of MWG.

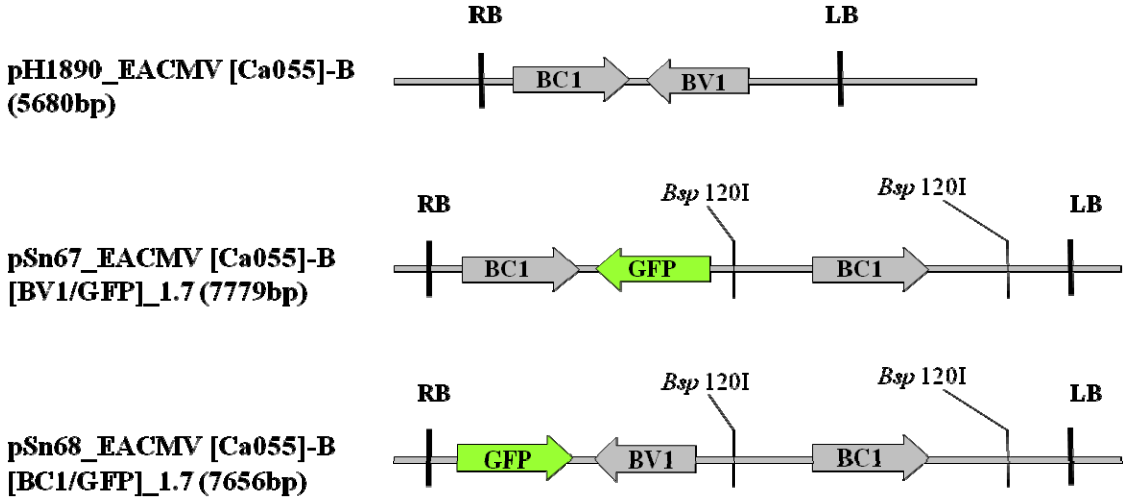

Figure 3.9: GFP constructs in vectors containing EACMV DNA-B genomic components. pH1890_EACMV [Ca055]-B, a monomeric full length clone of EACMV [Ca055] DNA-B an intermediate clone; pSn67_EACMV [Ca055]-B [BV1/GFP], BV1 is replaced by smRs GFP; pSn68_EACMV [Ca055]-B [BC1/GFP], BC1 is replaced with GFP.

3.2.3 Biolistic inoculation of cloned ACMV and EACMV genomes to *N. benthamiana* plants

Wild type and mutated DNA-A components of EACMV were mixed in separate reactions with equal amounts of DNA-B for biolistic inoculation and to initiate systemic virus

infections in *N. benthamiana.* Plants raised under standard greenhouse conditions 16/8 h light/dark photoperiod were inoculated at the two to three leaf stage by particle bombardment (250 psi) with a hand held device, the biolistic PDS-1000/He system, (BioRad, USA) as described by Ariyo *et al.*, 2006. Similarly *N. benthamiana* plants were biolistically inoculated with mixtures of DNA-A and DNA-B of wild type and mutated ACMV isolate DRC6 for inoculation control. For monitoring transient expression, a binary vector construct carrying smRs-GFP under the control of an enhanced 35S promoter (pG-d35S_smRs-GFP) was used as a control and likewise inoculated on leaves of *N. benthamiana*. Wild type Ca123 DNA-A and recombinant GFP constructs of DNA-B were also introduced into *N. benthamiana* to verify viral infectivity and GFP fluorescence.

3.2.4 **Inoculation of cloned ACMV and EACMV genomes to *N. benthamiana* plants by agro-infiltration**

For transient expression, smRs-GFP in the binary expression vector pGreenII was transformed into *A. tumefaciens* strain LBA 4404 (Hoekema *et al.*, 1983) harbouring a pSoup plasmid (Hellens *et al.*, 2000). Similarly all wild type and mutated DNA-A and DNA-B constructs of EACMV and ACMV were also transformed in LBA 4404.

For agroinfiltration patch assay and viral infectious studies, *A. tumefaciens* strains harbouring wild type DNA and the GFP labelled constructs on the cognate DNA-B were infiltrated onto the fully expanded leaves of about 28-35 days old *N. benthamiana* plants. Prior to agroinfiltration, each *A. tumefaciens* strain was grown to an optical density $A_{600\ nm}$ = 1 and subsequently equal volumes of DNA-A and DNA-B harbouring bacteria were mixed. A 1 ml syringe (no needle) was pressed onto the underside of a leaf and arrested by counter-pressure on the other side. Bacterial suspensions were then exerted to infiltrate 3 to 4 leaves. Thus treated plants were kept in the greenhouse at 25 ^{o}C with a 16-h photoperiod and monitored for development of GFP fluorescence.

3.2.5 **GFP Imaging**

GFP fluorescence was monitored using a confocal laser-scanning microscope (CLSM). Leaf samples were prepared from *N. benthamiana* plants inoculated with a recombinant labelled construct and placed in water under glass cover slips. Confocal imaging of GFP labelled viruses in primary inoculated and systemic leaves was performed with the CLSM (Leica, TCS SP2) equipped with a 488 nm argon laser and with fluorescence detected at 500 to 600 nm. Leica confocal software was used to merge the images from the different channels. The specimen were examined using a Leica 10x and 63x dry objectives.

3.2.6 **Verification of virus infections**

Total DNA was extracted from infiltrated and leaves following the treated ones. Using the Qiagen plant DNA extraction kit (Qiagen, Germany). Aliquots of plant DNA were subjected to PCR for amplification and detection of DNA-A and DNA-B genome sequences. A standard PCR profile was followed using a generic Taq polymerase (Invitrogen,USA). Detection of ACMV and EACMV was with series of primers described earlier (Ariyo *et al.*, 2005; Pita *et al.*, 2001a) and using PCR conditions as specified by the authors.

3.3 Results

3.3.1 **Inoculation of GFP labelled EACMV/ACMV DNA-A genome components with cognate DNA-B**

When full length, multimeric infectious clones of wild type DNA-A mixed with DNA-B were introduced by particle bombardment into *N. benthamiana*, typical leaf curling symptoms of begomovirus infections appeared 18-21 days after inoculation. Similarly, multimeric clones of DNA-A and DNA-B of ACMV isolate DRC6 were highly infectious

and caused severe systemic infections that became visible already 12-14 days post inoculation (dpi). Infections were verified by PCR to detect DNA-A or DNA-B of ACMV and EACMV.

DNA-A components of EACMV-GFP mutants were mixed with cognate DNA-B and introduced by particle bombardment or by agroinfiltration into *N. benthamiana* plants. None of the *N. benthamiana* plants inoculated with GFP replacement mutants developed symptoms of EACMV infections. When recombinant GFP constructs were complemented with wild type DNA-A, few plants developed systemic infections but GFP fluorescence was not detected. PCR amplification with DNA-A and DNA-B specific primers confirmed the presence of recombinant virus DNA but only in inoculated leaves (Fig. 3.10).

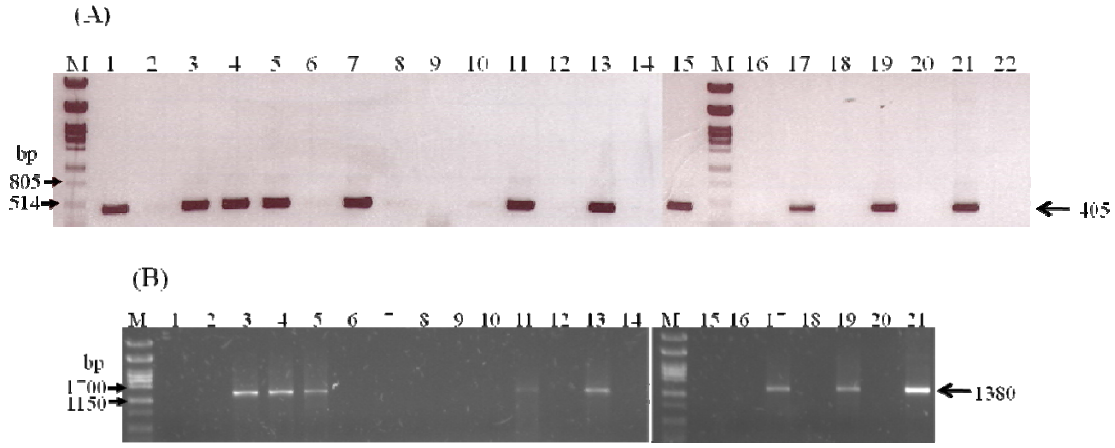

Figure 3.10: PCR amplified fragments from *N. benthamiana* plants agroinoculated with GFP DNA-A replacement mutants and wild type EACMV. DNA-A specific PCR (A); PCR for detection of DNA-B (B).

Lane 1, EA (inf); Lane 2, EA (sys); Lane 3, EA+EB (inf); Lane 4, EA+EB (sys) symptomatic plant; Lane 5, EA+EB (inf); Lane 6, EA+EB (sys) non-symptomatic plant; Lane 7, pSn93 (inf); Lane 8, pSn93 (sys); Lane 9&11, pSn93+EB (inf); Lane 10&12, pSn93+EB (sys); Lane 13, pSn93+EA+EB (inf); Lane 14, pSn93+EA+EB (sys); Lane 15, pSn58 (inf); Lane16, pSn58 (sys); Lane 17, pSn58+EB (inf); Lane 18, pSn58+EB (sys); Lane 19, pSn58+EA+EB (inf); Lane 20, pSn58+EA+EB (sys); Lane 21, positive vector control; Lane 22, mock inoculated plant; M, λ-DNA digested with Pst I. EA, pG_EACMV[Ca123]DNA-A; EB, pG_EACMV[Ca055]DNA-B; pSn93, EACMV[Ca123]DNA-A[AV2ΔCPGFP]; pSn58, EACMV[Ca123]DNA-A[ΔCPAV2://GFP].

inf= infiltrated leaf samples; sys= systemic leaf samples

Similarly, in plants infiltrated with ACMV DNA-A GFP mutants, virus DNA was only detected in inoculated tissues (Figure 3.11, lane 1,7,13) while in leaves following the treated ones, DNA was not detected (Figure 3.11, lane 2, 8,14). Only when mutants were complemented with DNA genomic components reflecting wild type DNA (Figure 3.11, lane 3, 4, 9-12) DNA was found in leaves above the inoculated ones. This was a confirmation however for infections initiated by wild type virus. Since fluorescence was not restored in systemic virus infections, trans-complementation cannot be assumed.

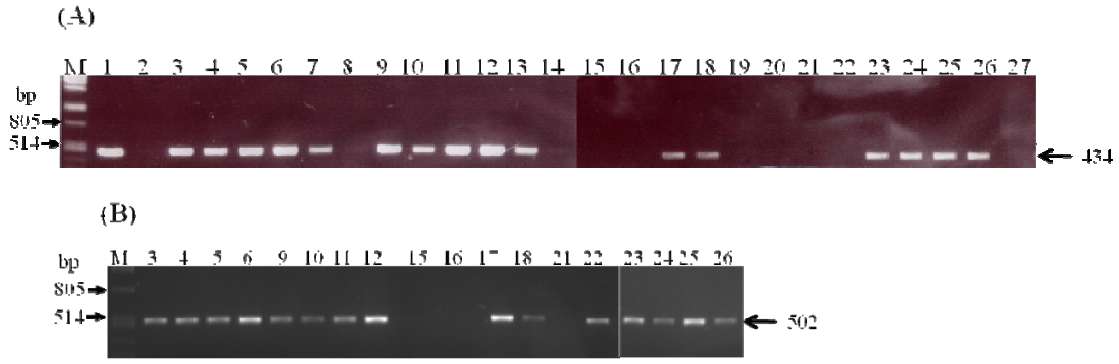

Figure 3.11:PCR amplified fragments from *N. benthamiana* plants agroinoculated with recombinant and wild type ACMV. (A) DNA-A specific PCR (B) DNA-B specific PCR.

Lane 1, pSn59 (inf); Lane 2, pSn59 (sys); Lane 3, pSn59+DB (inf); Lane 4, pSn59+DB (sys); Lane 5, pSn59+DA+DB (inf); Lane 6, pSn59+DA+DB (sys); Lane 7, pSn60 (inf); Lane 8= pSn60 (sys); Lane 9, pSn60+DB (inf); Lane 10, pSn60+DB (sys); Lane 11, pSn60+DA+DB (inf); Lane 12, pSn60+DA+DB (sys); Lane 13, pSn61 (inf); Lane 14, pSN61 (sys); Lane15, pSn61DA+DB (inf); Lane 16, pSn61+DB (sys); Lane 17, pSn61+DA+DB (inf); Lane 18, pSn61+DA+DB (sys); Lane 19, pSn62 (inf); Lane 20, pSn62 (sys); Lane 21, pSn62+DB (inf); Lane 22, pSn62+DB (sys); Lane 23, pSn62+DA+DB (inf leaf); Lane 24, pSn62+DA+DB (sys); Lane 25, DA+DB (inf); Lane 26, DA+DB (sys); Lane 27, mock inoculated plant; M, λ-DNA digested with *Pst* I. DA, pG_ACMV[CaDRC6]DNA-A; DB, pG_ACMV[CaDRC6]DNA-B; pSn59, ACMV[CaDRC6]DNA-A_cloning; pSn60, ACMV[CaDRC6]DNA-A[AV2://GFPΔCP]; pSn61, ACMV[CaDRC6]DNA-A[AV2ΔCPGFP]; pSn62, ACMV[CaDRC6]DNA-A[ΔAV2ΔCPGFP]; inf, infiltrated leaf samples; sys, systemic leaf samples.

Only in case of inoculations with pSN58, a multimeric DNA-A construct carrying a carboxy-terminal AV2://GFP fusion (Figure 3.3), GFP fluorescence was detectable 48 hours post inoculation (hpi) but in inoculated leaves only (Figure 3.14 C&D). GFP mainly

accumulated outside of the nucleus. Discrete GFP spots were regularly detected within the cells and in most cases were associated with the cell periphery indicating a possible plasmodesmata association of the AV2//GFP fusion. But this signal was found in few cells of inoculated leaves only.

Inoculations of ACMV DNA-A GFP replacement mutants in combination with wild type DNA-B did not result in virus infections and only a weak GFP fluorescence was detected in cells of agro-infiltrated tissues. In one case (1/ 30) symptoms developed in a plant infiltrated with pSn60_ACMV[CaDRC6]DNA-A[AV2://GFPΔCP]. The appearance of symptoms was late and weak compared to wild type virus infections (Figure 3.12). In this case, GFP fluorescence was located exclusively in the infiltrated leaves but here it was detected until 19 dpi (Figure 3.14).

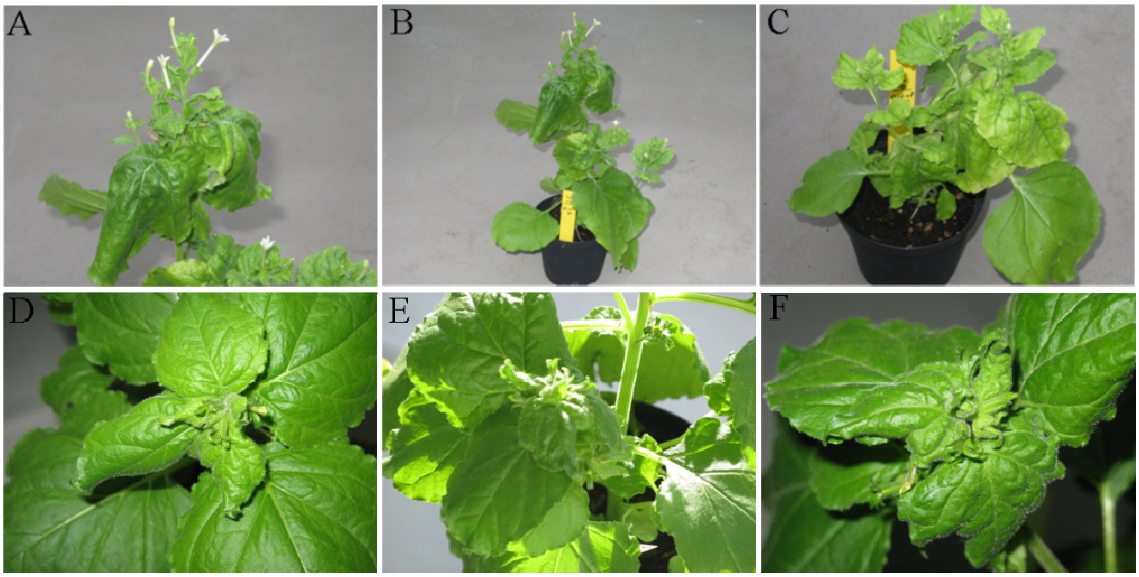

Figure 3.12: Cassava mosaic disease symptoms shown on plants agroinoculated with mutant DNA-A and DNA-B clones of ACMV (A,B) and EACMV (D,E). A, B, pSn60_ACMV[CaDRC6]DNA-A[AV2://GFPΔCP] mixed with cognate wild type DNA-B; C, pSN60 mixed with wild type EACMV DNA-A and DNA-B; D, E, pSn68_EACMV[Ca055]-B[BC1/GFP] mixed with cognate DNA-A; F, infection with wild type EACMV DNA-A and DNA-B.

3.3.2 Inoculation of GFP labelled EACMV/ACMV DNA-B genome components with cognate DNA-A

Since GFP replacement DNA-A mutants of EACMV and ACMV were not infectious in *N. benthamiana* hence did not result in systemic virus infections, GFP was also introduced into DNA-B for infectivity studies. Also in these cases, agroinfiltration of chimeric BV1 DNA-B components mixed with cognate DNA-A, did not result in symptoms of virus infections. However, when DNA-B was complemented with the functional EACMV DNA-B (pSn001), all inoculated plants developed systemic infections. Nevertheless GFP fluorescence remained confined to cells in/ around the infiltrated patches (Fig. 3.14 E).

In agroinfection studies with pSn68_EACMV [Ca055]-B [BC1/GFP], presence of virus was confirmed by PCR in stems, leaves and petioles of a systemically infected plant by EACMV DNA-B specific PCR (Fig. 3.12 and Fig. 3.13, lane 13–16). However again, this was in one plant only, the experiment could not be repeated and furthermore plants failed to show any EACMV symptoms. GFP fluorescence was found only in infiltrated leaves and here until 18 dpi.

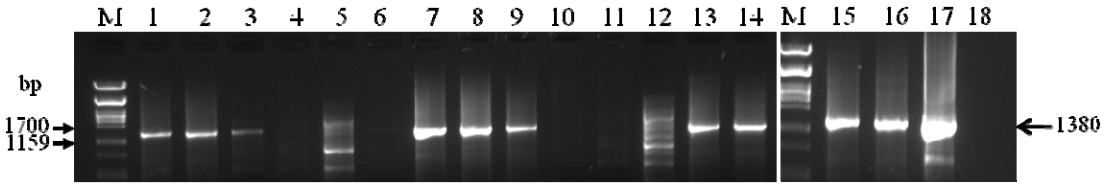

Figure 3.13: Detection of EACMV DNA-B in *N. benthamiana* plants agroinoculated with recombinant GFP replacement DNA-B mutants and cognate DNA-A.

Lane 1,4,7, EA+EB+pSn68 (systemic leaf samples); Lane 2,5,8, EA+EB+pSn68 (petiole samples); Lane 3,6,9, EA+EB+pSn68 (stem samples); Lane 10,13&16, EA+pSn68 (systemic leaf samples); Lane 11&14, EA+pSn68 (petiole samples); Lane 12&15, EA+pSn68 (stem samples); Lane 17, EA + EB; Lane 18, mock inoculated plant; M, λ-DNA digested with *Pst* I. EA, pG_EACMV[Ca123]DNA-A; B, pG_EACMV[Ca055]DNA-B; PSn68, EACMV [Ca055]-B_BC1/GFP.

3.3.3 GFP expression in *N. benthamiana* plants agroinoculated with recombinant GFP DNA-A and DNA-B clones

Transient expression of GFP in *N. benthamiana* plants was studied by inoculation of GPF replacement constructs using as a control a GFP construct, pG-d35S_smRs-GFP under the control of a duplicate CaMV 35S promoter. Fluorescent signals for expression control was observed in CLSM approximately 48-hrs post inoculation. GFP fluorescence was restricted to cells of infiltrated patches only (Fig. 3.14 A).

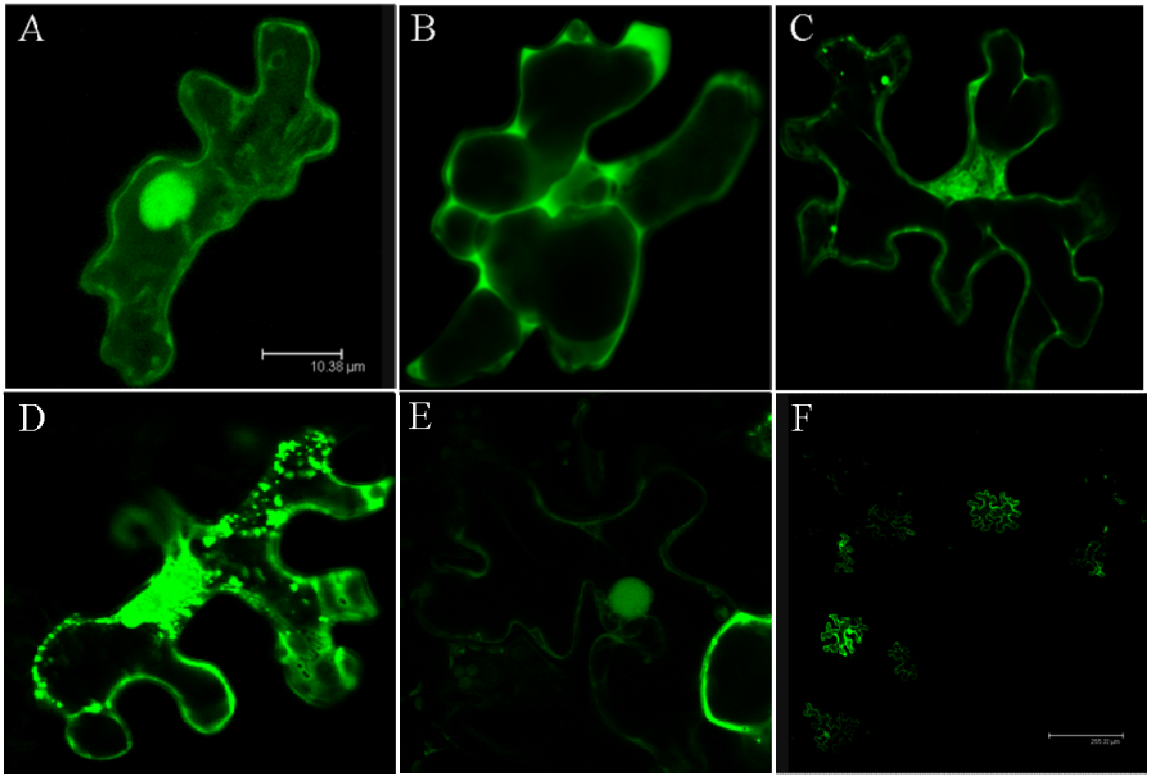

Figure 3.14: Confocal laser scanning microscope images of epidermal cells of *N. benthamiana* bombarded with various African and East African cassava mosaic virus GFP constructs.

A, Transient expression of smRs-GFP in cells bombarded with GFP in a binary vector under the control of CaMV 35S, 48 hours post bombardment (hpb); B, GFP fluorescence in cell bombarded with pSn58_EACMV [Ca123]-A [ΔCPAV2://GFP] 13dpb; C and D, GFP fluorescence 15 dpb showing GFP fluorescence predominantly accumulating outside the nucleus as GFP patches and discrete spots; E, sub cellular localization of transiently expressed pSn68_EACMV [Ca055]-B [BC1/GFP] mixed with wild type EACMV DNA-A and DNA-B 15dpi; F, cellular localization of transiently expressed pSn68_EACMV [Ca055]-B [BC1/GFP] mixed with wild type EACMV DNA-A in epidermal cells of inoculated leaves, 7dpi.

None of the GFP replacement DNA-A or DNA-B mutants when co-inoculated with its cognate DNA resulted in systemic plant infections. However weak symptoms were observed and viral DNA was confirmed by PCR in few plants infiltrated with pSn60_ACMV[CaDRC6]DNA-A[AV2://GFPΔCP] or with pSn68_EACMV [Ca055]-B [BC1/GFP]. In those plants however GFP fluorescence was not detected spreading systemically.

When GFP in pSN 58 and pSN 68 was bombarded onto plant tissues, fluorescence was found scattered and in single cells only (Figure 3.14). For all time points examined GFP remained confined within the single epidermal cells and there was no indication for spread of the GFP signal to neighbouring cells. Nevertheless, this signal persisted for up to 15 dpi (Figure 3.14 D) but during the observation period no further cells started to show fluorescence indicating for cell-to-cell transport and there were no symptoms of virus infection indicating for virus movement.

3.4 Discussion

In this study it was attempted to follow the infection processes of cassava begomoviruses in single and mixed infected *N. benthamiana,* to clarify the tissue tropism for EACMV and ACMV and to determine whether the viruses are capable of invading the same cells. This should shed light on the synergism phenomena observed when the two distinct virus species are invading the same plant (Fondong *et al.*, 2000; Pita *et al.*, 2001b). In earlier studies with cassava viruses, ACMV was found almost in all tissue types (Wege *et al.*, 2001) and hence not restricted to the phloem cells in *N. benthamiana* while Indian cassava mosaic virus was restricted to the phloem (Rothenstein *et al.*, 2007). For EACMV this was not yet determined and it was therefore of great interest to reveal the tissue tropism of EACMV in *N. benthamiana* and furthermore to dissect this in mixed EACMV/ ACMV infections. From such studies one could expect some insights into the level at which the viruses would interact.

For bipartite begomoviruses, cell to cell transport is provided by *NSP* and *MP* genes on DNA-B while a functional CP is not required for systemic infections (Gardiner *et al.*, 1988; Ingham *et al.*, 1995; Padidam *et al.*, 1995). Nevertheless dysfunctional CP result in reduced levels of ssDNA accumulation in infected plants (Briddon *et al.*, 1989; Lazarowitz *et al.*, 1989; Sunter *et al.*, 1990). The dispensability of a functional CP was utilised in earlier studies, to construct infectious *Bean dwarf mosaic virus* (BDMV) clones containing a modified GFP in place of the CP by maintaining the size restrictions to form viable virus (Sudarshana *et al.*, 1998). Using fluorescence analysis, cell-to-cell and long distance movement of BDMV was followed to reveal details of the infection process in *Phaseolus vulgaris*. The expression of the mGFP4 gene from DNA-A was stable through the life cycle of the bean plant hence providing evidence that this GFP gene was efficiently expressed from the CP promoter and stably maintained in the BDMV genome. Hence, this study, using GFP as a non invasive reporter of viral infection in plant tissues, presented the motivation to study cassava begomoviruses in *N. benthamiana.*

As with BDMV the smRs GFP gene introduced into DNA-A would upon complementation with their cognate DNA-B component reconstitutes to an infectious bipartite virus which expresses smRs-GFP during replication and can be followed by emission of a fluorescent signal. In preliminary experiments, essentially following the strategy of Sudarshana *et al.* (1998), the infectivity of EACMV ΔCP mutants was not successfully restored. Hence, in addition to the CP replacement mutants, several alternative routes were pursued.

GFP was introduced into the DNA-A from infectious EACMV or ACMV clones either by utilising the AV2 start and entirely replacing AV2 and CP or, as translational fusion to AV2 replacing CP only. In a study to reveal tissue tropism of ICMV Rothenstein *et al.* (2007) used AV2://GFP fusion constructs to show association of fluorescence with the vascular system as discrete fluorescent foci while GFP expression under the control of

the coat protein promoter resulted in signals evenly distributed in the vascular system of leaf tissues.

All GFP constructs generated during this study failed to induce begomovirus infections in *N. benthamiana*. While in the GFP recombinant BDMV DNA-A genome viability was not compromised and pathogenicity only slightly affected (Sudarshana *et al.*, 1998), viability was completely lost in EACMV and ACMV constructs. The weak systemic symptoms found in rare cases in *N. benthamiana* (pSn60_ACMV[CaDRC6]DNA-A[AV2://GFPΔCP]) were due to presence of virus but GFP expression was not attributed to this. Since this observation was not reproducible it has to be considered artefactual and rather an effect of the inoculation procedure that may have resulted in efficient delivery of virus DNA deep in cells of the vascular system. Virus spread then would be a result of diversion rather than active movement.

The parental infectious begomovirus clones represented EACMV-UG and ACMV viruses that are competent to cause infections in *N. benthamiana* and in cassava similar to wild type infections with no difference in pathogenicity. These infectious virus clones have been very efficiently used to screen for resistance in cassava germplasm (Ariyo *et al.*, 2006). In addition, DNA-A CP replacement vectors were initially designed for virus induced gene silencing (VIGS) to study gene function in cassava (Fofana *et al.*, 2004; Muangsan *et al.*, 2004; Pandey *et al.*, 2009; Robertson, 2004). A number of gene constructs including endogenous genes (Mg-chelatase) and artificial miRNA genes were efficiently expressed using recombinant EACMV (pSN29_pG EACMV [Ca123]-A_DNA-A) or ACMV (pSn59_ pG ACMV [CaDRC6]-A cloning) DNA-A in which CP was partially or entirely replaced by the foreign gene (Winter S, unpublished). Hence loss of function cannot be explained by either size constraints (Etessami *et al.*, 1989; Gilbertson *et al.*, 2003) which would result in restoration of normal genome size or loss of infectivity or loss of pathogenicity.

Attenuated symptoms were found in *N. benthamiana* infected with ICMV GFP recombinant DNA-A constructs (Rothenstein *et al.*, 2007) and in BDMV-GFP infected beans (Sudarshana *et al.*, 1998). It was also evident from VIGS studies on cassava (Winter S, unpublished) where recombinant viruses carrying certain foreign gene sequences caused significantly weaker symptoms compared to symptoms of infections induced by the parent infectious virus clones. This however was less pronounced in *N. benthamiana* than in cassava. Lack of movement of chimeric ACMV and EACMV with truncated and deleted AV2 could also be explained by a function assigned to AV2 to support efficient virus movement in plants (Padidam *et al.*, 1996).

A suggested role of CP in the infection process of bipartite geminiviruses (Pooma *et al.*, 1996), is probably minimal for EACMV and ACMV since VIGS vectors derived from parental clones efficiently replicated and remained stable in cassava and even more so in *N. benthamiana*. The complete loss of infectivity inherent to recombinant GFP clones carrying translational fusions or free GFP translated from AV1 or CP start codons, cannot be explained.

In this study, GFP fluorescence was observed in epidermal cells of *N. benthamiana* leaves inoculated with recombinant EACMV and ACMV DNA (Figure 3.14) but virus infections were not associated with this GFP fluorescence. GFP expression remained localised to few cells only albeit was stable and visible in CLSM for an extended time period (15 dpi). Even when labelled mutant constructs were complemented with infectious DNA-A and DNA-B clones of respective wild type viruses, systemic infections developed. But GFP fluorescence remained unaffected indicating that transreplication of mutant DNA-A did not occur. The GFP fluorescence observed in epidermal cells of *N. benthamiana* bombarded leave tissues observed over time did not change and similar to the GFP transient expression control, there was no spread of the signal to neighbouring, adjacent cells. It therefore can be speculated that the GFP fluorescence observed on bombarded tissues originated from an initial transcription event which was not followed

by even a limited transcription as a consequence of viral replication. Replication of recombinant ICMV expressing GFP and the association of GFP fluorescence with replicating virus was also not convincingly demonstrated by Rothenstein *et al.* (2007). Hence with BDMV representing an exception, it can be assumed that GFP expression from a replicating begomovirus is at most very inefficient hence alternative routes need to be pursued to study virus movement and tissue affiliation.

In addition to GFP recombinant DNA-A clones, DNA-B mutants with GFP replacing either BC 1 or BV1 were generated. It was not assumed that DNA-B constructs would result in infectious viruses when co-inoculated with cognate DNA-A, however it was expected that functional DNA-A will transreplicate DNA-B. Since DNA-A components of certain bipartite geminiviruses are capable of systemically infecting plants when delivered by agroinoculation (Evans & Jeske, 1993; Klinkenberg & Stanley, 1990), dysfunctional BC1 or BV1 in DNA-B when transreplicated from cognate DNA-A would probably still support expression of GFP from either start. However, this was never observed and weak fluorescent signals (Figure 3.14, F) were most likely artefactual and most likely a result of transient expression of GFP in epidermal cells.

Maintenance of genome size is highly significant for replication and movement of geminiviruses (Bisaro, 1994; Gilbertson *et al.*, 2003). Although EACMV and ACMV DNA-A CP replacement mutants were successfully used as VIGS vectors to express foreign gene sequences up to 800 nt in size (Winter S, unpublished), the increased genetic load in plant viruses carrying a fluorescence tag expressed as free or translationally fused protein might severely limit local as well as systemic spread (Toth *et al.*, 2002). A newly developed fluorescent tag reporter, LOV (Light, oxygen or voltage sensing), reporter might overcome the limitations of using GFP for monitoring plant virus infections (Chapman *et al.*, 2008). The coding sequence of iLOV (a domain of LOV, reporter of subcellular protein localization in both plants and mammalian cells) is only approximately

300 bp in size and has been successfully used as a superior reporter to GFP for monitoring local and systemic infections of plant RNA viruses.

In summary, this study has shown that labelling of DNA-A or DNA-B of EACMV and ACMV with GFP inevitably results in loss of function of the constructs. It seems likely that the translatable GFP has a major impact on the infectivity/stability/fitness of the virus constructs. Despite the beauty of using non destructive methods to follow virus infections *in planta*, it also appears that even when mastered, such major interventions have serious impacts on the overall performance of viruses and create artificial systems which counterbalance advantages of real time *in situ* studies.

4. Studies on the role of AC2 and AC4 genes during pathogenesis of African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV-UG) in *Nicotiana benthamiana*

Abstract

To study the role of AC2 and AC4 genes in pathogenesis of ACMV and EACMV, the viral genes were isolated and introduced for gene expression into transgenic *N. benthamiana* plants. Transgenic plants harbouring AC2 or AC4 genes showed a variety of phenotypic alterations which were most pronounced with ACMV AC2 transgenes. Transient expression of AC2 and AC4 genes driven by a Cassava vein mosaic virus 35S promoter and subsequent infection of wild type plants showed a sharp increase of ACMV in plants infiltrated with either ACMV or EACMV AC2. In contrast, challenge inoculations of AC2 transgenic *N. benthamiana* did not resolve any differences to wild type infections. An *Agrobacterium* based leaf infiltration assay was approached to reveal AC2 and AC4 suppression activity on early stages of post-transcriptional gene silencing. In this study, while PTGS effects were confirmed for silencing suppressor genes, HcPro of *Plum pox virus* and P15 of *Johnsongrass chlorotic stripe virus*, in none of the approaches, an activity of AC2 was recorded. Hence it can be assumed that the activity of ACMV or EACMV AC2 to suppress post-transcriptional gene silencing (PTGS) is weak, while for AC4 silencing suppression was not confirmed in this study. Infection experiments with mutant viruses carrying dysfunctional AC2 and AC4 genes showed that AC4 is dispensable for plant infections while AC2 mutations are lethal and AC2 function cannot be complemented.

4.1 Introduction

Plants have adopted a natural defence mechanism against viral pathogens, a pathway called post-transcriptional gene silencing (PTGS) (Baulcombe, 1999; Baulcombe, 1996; English *et al.*, 1996). PTGS or RNA interference (RNAi), or RNA silencing is an active mechanism in genetic regulation of development processes and of gene expression in eukaryotes (Baulcombe, 2005). Likewise it is a protective mechanism for virus defense and in particular effective against RNA viruses, the most important and common pathogens of plants. „RNA silencing "is a process, which consists of 3 key reactions which can differ in mechanism and factors (Voinnet, 2008):

I. Production of double stranded RNA (dsRNA), which represents an ubiquitous initiating cause for RNA silencing;

II. RNAse III (Dicer) catalyzed processing into small single stranded RNA (small interfering RNA, siRNA) consisiting of 21-24 nt sense and anti-sense RNA;

III. siRNA bind to an Argonaute (AGO) protein and is transferred into the RNA induced silencing complex "RNA interference specificity Complex", (RISC) siRNA molecules are responsible for specificity and lead RISC to partial or entirely complementary RNA, which subsequently is specifically degraded.

As a counter defence, viruses encode a range of silencing suppressor proteins which interfere with the silencing pathway and the RNA metabolism at distinct phases (Dunoyer & Voinnet, 2005; Endres *et al.*, 2010; Vance *et al.*, 2009; Voinnet, 2001; Voinnet & Baulcombe, 1997). Thus, systemic silencing is suppressed by P50 of *Apple chlorotic leaf spot virus*; P1 of *Rice yellow mosaic virus* (RYMV) and the coat protein (CP) of *Citrus tristeza virus* (Himber *et al.*, 2003; Lu *et al.*, 2003). These suppressors do not have effects on local gene silencing (Yaegashi *et al.*, 2007). The P21 suppressor of the *Beet*

yellows virus like P19 of *Tomato bushy stunt virus* and HcPro of *Potato virus Y* (PVY) inhibit the RNA-induced silencing complexes (RISC) and target cleavage however, do not affect preassembled RISC. HcPro and P21 also do not prevent siRNA biogenesis from long dsRNA precursors (Chapman *et al.*, 2004; Lakatos *et al.*, 2006). In a different case, the expression of *Hibiscus chlorotic ringspot virus* CP, initiated sense RNA-induced PTGS but has no effects on dsRNA-induced local and systemic PTGS (Meng *et al.*, 2006). This is to illustrate that RNA viruses have developed a great diversity of counter defence strategies aimed at different aspects of gene silencing.

Geminiviruses are DNA viruses replicating in the nucleus and hence have no specific vulnerable dsRNA stage in replication to be targeted by PTGS which is a cytoplasmic process. The geminivirus transcripts are however exposed in the cytoplasm and virus specific siRNAs are also induced as a consequence of transcriptional process in virus infection (Chellappan *et al.*, 2004b; Vanitharani *et al.*, 2005).

PTGS of viral transcripts is led by pathogen derived RNAs (21-24 nt), a phenomenon that is nowadays used to introduce virus resistance in transgenic plants. Overexpression of virus-specific dsRNA induces RNA silencing and induces resistance in virus infected plants. Stable virus resistance already has been generated in a number of economically important plants by transgene expression of viral sequences as so-called “dsRNA hairpins” or ”inverted repeat RNA” (Horser *et al.*, 2002; Wesley *et al.*, 2001).The potential for achieving high level of resistance against geminiviruses using strategies similar to the “hairpin inverted repeat” dsRNA expression was demonstrated (Asad *et al.*, 2003; Chellappan *et al.*, 2004a). For geminiviruses, this silencing does not lead to complete immunity and this is also because of the activity of geminiviral suppressors of gene silencing (Bisaro, 2006; Lucioli *et al.*, 2003).

For begomoviruses, the AC2 (TraP*)* and AC4 have been functionally determined as viral silencing suppressor proteins (Vanitharani *et al.*, 2004). Binding with siRNA and/or miRNA seems a general strategy of viral silencing suppressors (Bisaro, 2006;

Chellappan *et al.*, 2005b; Lakatos *et al.*, 2006; Li *et al.*, 2010; Merai *et al.*, 2006; Ruiz-Ferrer & Voinnet, 2007) and for begomoviruses these genes are also implicated in replication and pathogenesis. Besides the replication initiation protein (Rep) which initiates viral DNA replication, the transcriptional activator protein (TraP) functions as the suppressor of PTGS while the AC4 protein which can interfere with host response to Rep expression might cause similar effects (Vanitharani *et al.*, 2004). TraP is suppressor of gene silencing for a number of begomoviruses (Dong *et al.*, 2003; van Wezel *et al.*, 2002b; Vanitharani *et al.*, 2005; Voinnet *et al.*, 1999) including EACMV. For ACMV, the AC4 protein was shown to interfere with the host response (Vanitharani *et al.*, 2004). In mixed infections of cassava with cassava begomoviruses, the concerted action of both *AC2* and *AC4* genes might lead to synergism with serious implications for disease development. Thus, it is intriguing to speculate that viral synergism is a consequence of two viral suppressors interacting, probably at different sites, with the host silencing machinery. Since begomovirus DNA is translocated to the nuclei, an interaction between diverse DNA viruses in the nucleus can also be assumed (Morilla *et al.*, 2004)

The objective was to study *AC2* (TraP) and *AC4* genes of ACMV and EACMV in transgenic plants and their effects on challenge virus infections with homologous, related and unrelated RNA viruses. Similarly, a transient expression assay was conducted to determine more generally the general mode of silencing suppression of TraP and AC4 expressed from a 35S promoter using GFP as a reporter in a “silencing on the spot assay” as described by Johansen & Carrington (2001).

4.2 Materials and Methods

4.2.1 Gene constructs for transient and stable expression of AC2 and AC4 genes in *Nicotiana benthamiana*

A pGreenII 0229 (John Innes Center, UK) based binary vector was used for *Agrobacterium* mediated transient gene expression and for stable plant transformation. To make use of the *bar* gene as a selective marker, a promoter-terminator cassette was amplified by PCR from a cloning vector carrying a double *Cauliflower mosaic virus* (CaMV) 35S promoter, a CaMV polyA terminator and multiple cloning sites for gene insertion in an optimised transcription/translation context (Dizadji *et al.*, 2008). The primer pair used to amplify the cassette incorporated restriction sites to facilitate subcloning and the amplified product was subsequently inserted into pGreenII to result in pSN40 (Fig. 4.1A). This vector carries *npt*I for Kanamycin selection in bacteria and *bar* as a selective marker *in planta* conferring tolerance to the herbicide phosphinotricin (ppt). pSN40 has a multiple cloning site downstream the enhanced 35S promoter sequence into which the PCR amplified *AC2* and *AC4* genes from EACMV and ACMV were inserted. The putative silencing suppressor genes *AC2* and *AC4* of EACMV and ACMV were amplified from DNA-A genome components of infectious virus clones using primers given in Table 4.1.

Table 4.1: Oligonucleotide primer sequences used for the amplification of AC2 and AC4 genes of specific EACMV and ACMV strains

Primer	Nucleotide sequence (5'-3')	Annealing T_m
Ca-055 AC2_F	GGATCCTAGGAGATATAACAATGCCACCTTCATCAC	69.5
Ca-055 AC2_R	AAGCTTCTAAATACTCTTAAGAAACGACCAGTCTGAGGC	69.5
Ca-123 AC2_F	GGATCCTAGGAGATATAACAATGCCACCTTCGTC	69.5
Ca-123 AC2_R	AAGCTTCTAAATACTCTTAAGAAAAGACCAGTCTGAGGCC	69.5

DRC6 AC2_F	GGATCCTAGGAGATATAACAATGCAATCTTCATCACC	68.4
DRC6 AC2_R	AAGCTTCTAAAGACCCTTAAGAAAAGACCAGTCTGAGG	69.5
DRC6 AC4_F	GGATCCTAGGAGATATAACAATGTATTTCTCACATACCC	68.4
DRC6 AC4_R	AAGCTTTTACATTAAGAGCTCCCGACTTACTGCC	68.3

F: Forward primer, R: reverse primer

Specific restriction sites are shown underlined

Subsequent cloning via *Bam*H 1 and Hind III into pSN40 brought the respective genes in an optimal eukaryotic translation context (Lütcke *et al.*, 1987). All gene constructs used for gene expression assays and plant transformation were confirmed by sequence analysis and expression constructs (Fig. 4.1B) were numbered according to the virus isolate as shown in table 4.1.

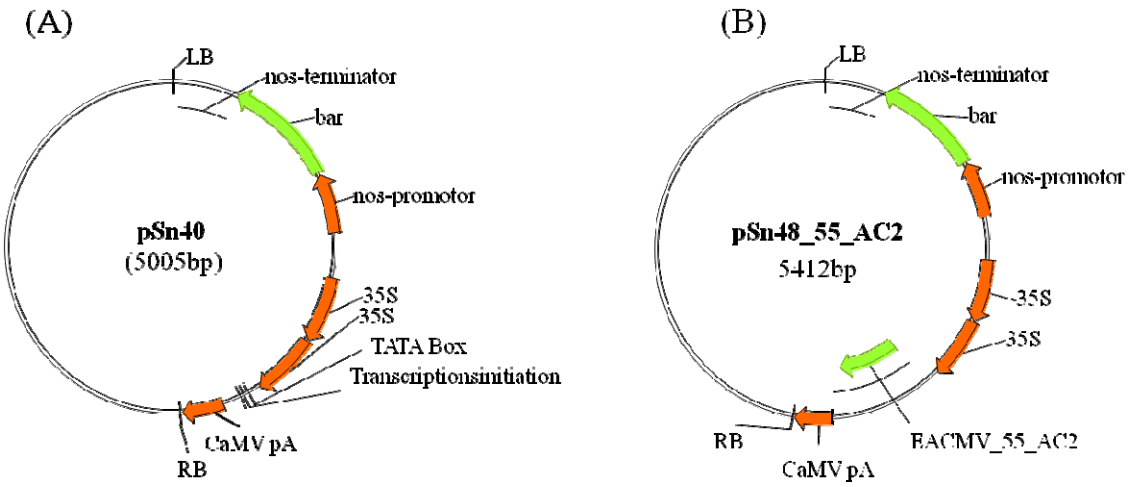

Figure 4.1: (A) Map of pSN40 (pGreenII with enhanced 35S CaMV promoter and bar resistance). (B) Map of transformation construct based on pSN40 containing AC2 gene of EACMV-UG isolate Ca055

4.2.2 **Electroporation of *Agrobacterium tumefaciens***

All gene constructs were transformed into LBA 4404 which already carried the plasmid pSoup, a helper plasmid that provides replicase function for the replication origin of

pGreen which will not replicate in *Agrobacterium* if pSoup is not present (Hellens *et al.*, 2000). 1 µl of the plasmid (conc. 1 µg/µl) was incubated with 40 µl of competent bacterial cells, mixed, transferred to an ice-cold cuvette (Eurogentec) and electroporated in an Easyjet optima Equibio electroporator (Peqlab) using resistance at 335 Ω, voltage at 2.5 kV and capacitance set at 15 µF. Following electroporation (approx. 4.5 s) 1 ml of SOC medium was added and the cells were incubated at 28°C on a rotary shaker for 3 hours. Serial dilutions (10-100 µl) of bacteria were spread on YEB plates containing the corresponding antibiotics for selection. Plates were incubated for 48 hours at 28°C (Sambrook & Russel, 2001).

4.2.3 **Transient gene expression in *N. benthamiana***

Agrobacteria with the respective plasmid constructs were grown overnight at 28 °C to an OD of ~1.0. Following OD measurement, cells were harvested and re-suspended in a medium containing 2% sucrose, ½ MS basal salts (Murashige & Skoog, 1962) to which 10 mM $MgCl_2$, 10 mM MES and 200 µM Acetosyringone (Sigma) was added and left to settle for 3 hr at RT. Bacterial suspensions were infiltrated by gently pressing a 1 ml disposable syringe to the abaxial surface of fully expanded leaves of 3-week-old *N. benthamiana* plants, followed by gentle release of the bacterial suspension in the syringe until the leaves had a water-soaked appearance (Schob *et al.*, 1997). Following agro-infiltration, plants were maintained in a greenhouse at 26 °C with a 16 h photoperiod. Suspensions of *Agrobacterium* carrying the pSN40 and cultures with the silencing suppressors P15 from JCSMV, HcPro from *Plum pox virus* (PPV) and AC2 from SLCMV were also infiltrated as negative and as positive controls, respectively.

4.2.4 **Transgenic plant lines expressing AC2 and AC4 of ACMV and EACMV AC2**

4.2.4.1 **Plant material and transformation**

Leaf disc transformation of *N. benthamiana* was done essentially following standard procedures (Fraley *et al.*, 1985; Horsch *et al.*, 1985). The *N. benthamiana* plants to deliver leaf material for plant transformation were grown in the greenhouse with a 14/10 h light/dark photoperiod at 25 °C. Leaves obtained from 6 week old *N. benthamiana* plants were sterilized for 30 minutes with a 1.2% sodium hypochlorite solution + 1 drop Tween 20. Following sterilisation, leaves were thoroughly washed 5-6 times in sterile water, midribs were removed and leaves were cut into discs of 1.0 cm x 1.0 cm. This was done one day prior to the agro-inoculation. *Agrobacterium* LBA 4404 harbouring the plasmids with respective DNA constructs were grown at 28 °C for 28-36 hrs in YEB medium containing Kanamycin (Kn) and Rifampicin (Rif) while shaking at 180 rpm. Growth of culture was measured by determination of $OD_{600.}$ Bacterial cultures were subsequently adjusted to OD_{600} = 0.6-0.8 and cultures were harvested by centrifugation at 4000g for 10 min at 4 ^{o}C. Pellets were resuspended in one volume of MS-Medium. The suspension was diluted 1:100 (v/v) and leaf discs were immersed for 30 min while slowly shaking on a rotary shaker. The discs were removed from the broth, tapped dry on sterilized filter paper, and carefully transferred onto solid MS medium containing 0.1 mg/l NAA + 1 mg/l BAP (MS-T0). The plates were incubated in dark at 26 °C for 48 hours. Following incubation leaf discs were washed twice with sterile liquid MS-Medium containing Ticarcillin, dried on sterilized filter paper and transferred to MS agar plates containing 0.1 mg/l NAA + 1 mg/l BAP + 500 mg/l Ticarcillin + 6 mg/l PPT (MS-T1). Leaf discs were incubated at 24 °C for 16/8 h light/dark photoperiod, respectively and monitored every day for presence of bacteria or possible contamination. Subculturing was done after every 2 weeks to fresh MS-T1 medium. After approximately 4 weeks, developing calli were subjected to selective MS medium 0.1 mg/l BAP + 300 mg/l Ticarcillin + 6 mg/l PPT (MS-T2). Following shoot development (16th week) the plantlets

were subcultured on root induction medium containing 300 mg/l Ticarcillin + 6 mg/l PPT (MS-T3). Once the plantlets had developed strong roots they were removed from agar, roots were carefully washed with tap water and plants were transferred to pots in the greenhouse and covered to maintain high humidity. After one week, the humidity trays were opened periodically to allow air circulation. After 2 to 3 weeks plants (T0) were hardened and grown under glasshouse conditions at 24°C/18°C day/night temperature conditions.

4.2.4.2 **Selection of transgenic seeds and propagation of T1 and T2 generations**

From each of the constructs including the Sn40 control, 10 putative transgenic plants were grown in the glasshouse for flowering and production of seeds. Seeds obtained from self-fertilized plants were taken, dried and kept at 4 °C prior to use. Surface sterilized seeds (70% ethanol for 2 minutes followed by 5 times rinsing in sterile water) were placed onto MS medium containing 6 mg/l ppt for germination (100 seeds/ line) and kept in a growth chamber at 25/18 °C for 3 to 4 weeks with a 18h daylight period. PPT resistant seeds germinated and comprised T1 generation. Five independent T1 lines were selected for another round of seed germination on ppt containing MS medium and to produce T2 generation seeds. These T2 seeds were sown directly in soil.

4.2.5 **Screening of transgenic plants with RT-PCR**

For evaluation of transgene expression, RNA transcripts of the respective genes were amplified by RT-PCR. For this, total RNA was isolated from putative transgenic plants and treated with DNase to remove all contaminating DNA.

4.2.5.1 **Total RNA extraction from *N. benthamiana***

Total RNA was extracted essentially following the RiboZol™ purification protocol (ribozol-OLS, Omni Life Science, Bremen). Prior to RNA extraction, all materials used for RNA extraction were treated with DEPC before autoclaving. For RNA extraction, 100 mg of plant material was collected in plastic sample bags (BIOREBA) and immersed in liquid nitrogen. RiboZol™ was activated with ß-mercaptoethanol and 2 ml added directly to the thawing leaf tissues which were ground in BIOREBA bags using a manual grinding device (BIOREBA AG, Switzerland). 1 ml of the homogenate was then removed and total RNA was extracted following the protocol. The final nucleic acid pellet was dissolved in 50 µl DEPC-treated water.

4.2.5.2 **RT-PCR**

Aliquots of 8 µl plant RNA were DNAse (Invitrogen) treated, to remove residual DNA which would lead to erroneous PCR amplification. This was achieved by adding 1 µl DNase buffer and 1 µl DNaseI Amp Grade (1U/µl) and incubating at 37 °C for 30 min. After the treatment, DNase was inactivated by heating at 65 °C for 10 min and total RNA was subsequently subjected to a one step RT-PCR using the SuperScript III One-Step RT-PCR system (Invitrogen). RT-PCR was carried out for 30 min at 45 °C for cDNA synthesis followed by an initial denaturing step for 2 min at 94°C and further 30 cycles of 94°C for 15 sec, annealing at 55°C for 30 sec and polymerisation at 72 °C for 30 sec. A final extension at 72 °C for 5 min terminated the reaction cycles. Primers used for amplification/detection of each gene construct are shown in table 4.2.

Table 4.2: Oligonucleotide sequences used as primers for RT-PCR verification of gene expression in transgenes

Primer	Nucleotide sequence (5'-3')
sn_055AC2det_F	CTCCACGAGCCATTGTTCTCTAGT

sn_055AC2det_R	CTTGTGAATCCCCAATGCCTT
sn_DRC6AC2det_F	ATGCAATCTTCATCACCCTCACA
sn_DRC6AC2det_R	TGTGATTGAATCGAACCTGGACT
sn_DRC6AC4det_F	CATTCATTCAAACACTCTCTCTCCC
sn_DRC6AC4det_R	CGTAAGCATCATTCGCTGATTGT

F: Forward primer, R: reverse primer

RT-PCR products were analyzed in a 1% agarose gel electrophoresis. In each RT-PCR detection series, DNA isolated from non transformed *N. benthamiana* and a water control was included.

4.2.6 **Virus infectivity tests, inoculation methods and sample collection**

To study the activity of AC2 genes expressed in transgenic *N. benthamiana* on challenging virus infections, 20 plantlets of each of the transgenic *N. benthamiana* lines expressing AC2 of ACMV and EACMV-UG respectively were inoculated with ACMV DRC6 and with *Potato virus A* serving as control. Transgenic pSN40 vector only and wild type *N. benthamiana* plants were used as controls.

For virus assays, ACMV DRC6 and PVA (DSMZ PV-535) were propagated in *N. benthamiana* to use systemically virus-infected leaves as inoculum for mechanical inoculations. For inoculum preparation, symptomatic leaves of *N. benthamiana* were ground in ice cold Norit buffer (0,05M Na/K phosphate, pH 7.0, 1mM EDTA, 5mM DIECA, 5 mM Thioglycolic acid, pinch of charcoal). Celite 535 was added as an abrasive and transgenic *N. benthamiana* in the 3 to 5 leave stage were infected by gently rubbing leaf homogenates onto 2 to 3 fully expanded leaves. Following inoculation, leaves were washed with tap water to remove debris and to prevent excessive drying out. Inoculated plants were kept at 26 °C and viral symptoms were monitored every other day starting 5 days after inoculation. For virus analysis, samples were collected from leaves showing

symptoms of systemic infection and subjected to ELISA, to quantify virus and to monitor the effect of silencing suppressors on viral replication.

4.2.7 **Enzyme-Linked Immunosorbent Assays (ELISA) for detection and quantification of viruses**

Virus infections were monitored using ELISA tests with antibodies specific for ACMV and PVA. This was done essentially following the ELISA protocols recommended by DSMZ using a TAS-ELISA for detection of ACMV (DSMZ-TAS AS-0421-421/2) and a DAS-ELISA (DSMZ AS-0535) for PVA detection.

4.2.8 **Viral DNA quantification by real time PCR**

To estimate the absolute quantity of virus molecules in transgenic and non-transgenic *N. benthamiana* plants inoculated with ACMV DRC6, a real time quantitative PCR (qPCR) was performed following the qPCR protocol and procedure as described in chapter 2.

4.2.9 **Transient expression of AC2 and AC4 genes in *N. benthamiana* plants**

To evaluate gene silencing activity of AC2 and AC4 genes from ACMV and AC2 from EACMV-UG in transient agro-infiltration assays, AC2 and AC4 genes were amplified by PCR using primers listed in table 4.3.

Table 4.3: Oligonucleotide primer sequences used for amplification of AC2 and AC4 genes, adding a HA Tag (HCA) sequence and *Kpn* I and *Xba* I restriction sites.

Primers	Nucleotide sequence (5'-3')
Sn_055_AC2_HCA_F	ATGGTACCAGGAGATATAACAATGCCACCT
Sn_055_AC2_HCA_R	AAGACTCTAGAACTAGGCATAATCTGGCACATCATAAGGGTAAATACTCTTAA
Sn_055_AC2_pIng_R	AAGACTCTAGAACTAAATACTCTTAAGAAACGACCA

Sn_055_AC4_HCA_F	ATGGTACCAGGAGATATAACAATGGGGTGC
Sn_055_AC4_HCA_R	AAGACTCTAGAACTAGGCATAATCTGGCACATCATAAGGGTAAATGTTGG
Sn_055_AC4_pIng_R	AAGACTCTAGAACTAAATGTTGGCCCTCTCC
Sn_DRC6_AC2_HCA_F	ATGGTACCAGGAGATATAACAATGCAATCTTCAT
Sn_DRC6_AC2_HCA_R	AAGACTCTAGAACTAGGCATAATCTGGCACATCATAAGGGTAAAGACCCTTA
Sn_DRC6_AC2_pIng_R	AAGACTCTAGAACTAAAGACCCTTAAGAAAAGACCA
Sn_DRC6_AC4_HCA_F	ATGGTACCAGGAGATATAACAATGTATTTCTCA
Sn_DRC6_AC4_HCA_R	AAGACTCTAGAACTAGGCATAATCTGGCACATCATAAGGGTACATTAAGAGCT
Sn_DRC6_AC4_pIng_R	AAGACTCTAGAACTACATTAAGAGCTCCCG

F: Forward primer, R: reverse primer; Specific restriction sites are underlined

During PCR, restriction sites *Kpn* I and *Xba* I and further sequences to generate carboxyterminal fusions to an influenca hemagglutinin epitope tag (YPYDVPDYA) were incorporated into the newly synthesized DNA for directional cloning.

PCR amplified genes were cloned into pING71 (kindly provided by Dr. Ivan Ingelbrecht, Biotech unit IITA, Nigeria), a pCAMBIA 2300 derived vector (Hajdukiewicz *et al.*, 1994) in which a Cassava vein mosaic virus (CaVMV) 35S promoter and the 35S Cauliflower mosaic virus terminator (CaMV) cassette was inserted for highly efficient protein expression (Fig. 4.2).

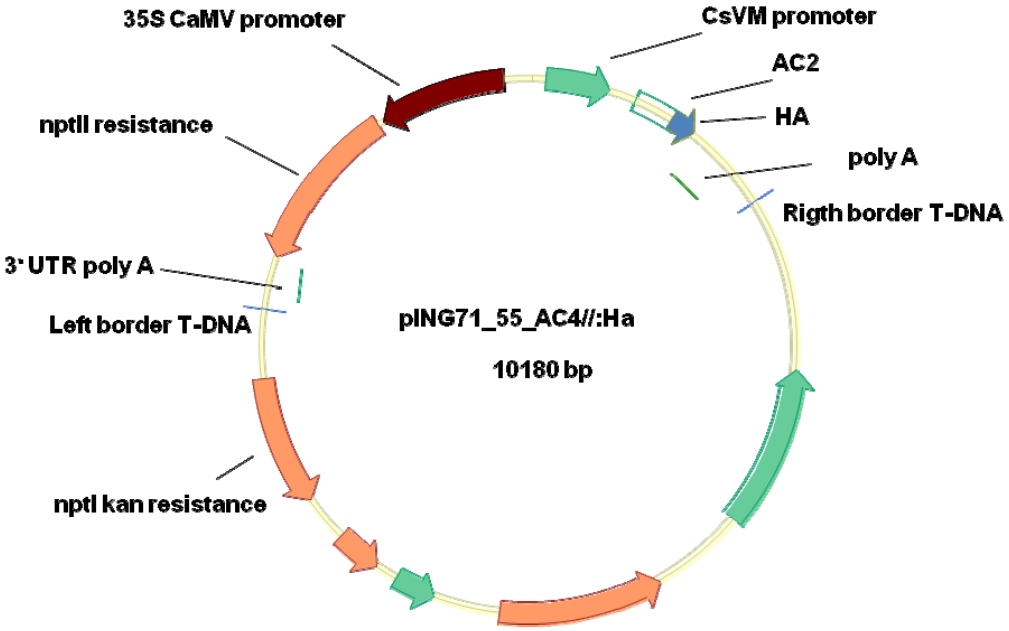

Figure 4.2: Map of pING 71_55AC4Ha (a modified pCambia 2300 vector carrying a 35S Cassava vein mosaic virus promoter for transgene expression. Open and blue arrow indicate gene for expression and the carboxterminal fusion of hemagglutinin.

For expression of GFP a pING71_16cGFP containing the coding region of the 16c GFP while an inverted repeat hairpin dsGFP construct in pBin19 (kindly provided by Prof. M. Varrelmann, University of Göttingen) contained a 16c GFP fragment under the control of a 35S promoter in sense/antisense orientation separated by an intron (Frisch *et al.*, 1995). The constructs in their respective binary vectors were introduced by

electroporation into *A. tumefaciens* C58C1 (Deblaere *et al.*, 1985). To study sense RNA silencing, AC2 and AC4 constructs were mixed with equal concentrations of GFP constructs, left at room temperature for 3 hours in MES medium for activation of *Vir* genes and co-introduced by agroinfiltration (Bendahmane *et al.*, 2000; Johansen & Carrington, 2001; Llave *et al.*, 2000) into *N. benthamiana* (Fig. 4.3).

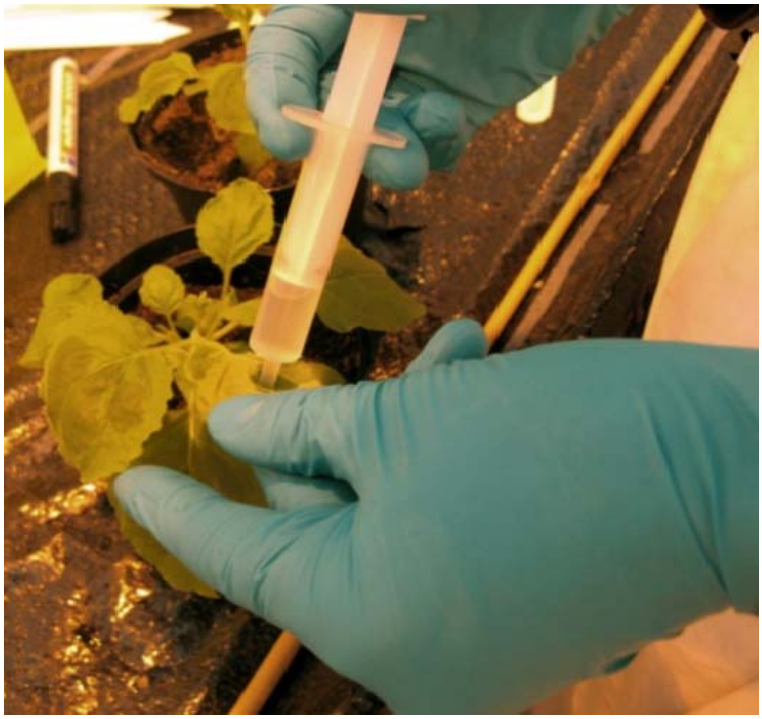

Figure 4.3: Agro- infiltration of *N. benthamiana* plants with needless syringe for transient expression assay. A 1 ml syringe (no needle) is pressed onto the underside of a leaf and arrested by counter-pressure on the other side.

To study silencing suppression activity on dsRNA, the virus constructs were mixed with agrobacteria carrying plasmids with GFP and with dsGFP respectively. Infiltration of the putative viral suppressors with GPF and dsGFP should inactivate the silencing activity from dsGFP expression. For control of function, expression vectors containing HcPro from *Plum pox virus* (kindly provided by M. Varrelmann) and P15 from *Johnsongrass chlorotic stripe mosaic virus* (DSMZ Plant Virus Department) and pSN40 were similarly used for transient expression studies.

Plants were examined daily for GFP fluorescence development under long- wavelength UV light using a high intensity 365nm UV lamp (B-100AP, LTF Germany) and photographed with a digital camera (Nikon D90) mounted with a yellow filter. Images were processed electronically.

4.2.10 **EACMV DNA-A mutants with dysfunctional AC2 and AC4 genes**

To study complementation of gene function, mutations were introduced into *AC2* and *AC4* genes of EACMV-UG isolate Ca123 DNA-A. For construction of AC2 knock out mutants, primer pairs AC2F/R (Table 4.4) introduced stop codons at two positions in AC2. Similarly, primer pair AC4F/R created a G to A transversion, removed the start codon of AC4 and introduced a stop (TGA) without affecting the amino acid sequence of the overlapping ORF AC1.

Table 4.4 Oligonucleotide primer sequences for sdm to generate dysfunctional AC2 and AC4 and for sequencing

Primer	Nucleotide sequence (5'-3')
AC2_F	TAATGAGGCTGCTCGTTCTATCTCCATATCGACTG
AC2_R	GAGGTCTACCCGCCTACGTCTGATGGCCCTG
AC4_F	TCCATGTTCTGATCCAATTCGAAGGCAAGTTCCAATG
AC4_R	GATGAGGCACCCCGTCCTGATGTAGTTCTCTGCAAAC
AC2_seq	ACGGGAAGCCGATTCAAATTAAAGG
AC4_seq	AAGCTTTTTCTTATCCGACGAATATC
EACMV_P1	AAGTACAGTGTTAATGGAAATAACGC

F: Forward primer, R: reverse primer, seq: sequencing primer

For sequence confirmation, AC2_seq and AC4_seq were used for PCR amplification. Agrobacteria harbouring DNA-A mutants were mixed with bacteria containing cognate wild type DNA-B and agroinoculated to establish systemic infections in *N. benthamiana.*

Plants were subsequently maintained in the greenhouse at 25°C with a 16 h photoperiod and monitored for development of symptoms.

4.2.11 **Detection of viral DNA**

Total DNA was extracted from newly emerging and/or symptomatic leaves approximately 21-23 dpi using a DNeasy plant DNA extraction kit following the manufacturer's instructions (Qiagen, Germany). PCR to confirm AC2 and AC4 mutations was carried out with EACMV_P1 and AC4_seq and PCR products of approximately 1375bp were subsequently sequence analysed for presence of the respective mutations using specific sequencing primers (Table 4.4).

4.2.12 **Protein isolation and analysis**

Protein extracts were prepared from *N. benthamiana* leaves 48 h after agroinfiltration using TriFast Gold following the manufacturer's prototol (Peqlab, Erlangen, Germany). The final protein pellet was redissolved in 5 M urea, 50 mM DTT and after centrifugation at 10,000 g for 10 min at 4˚C, a volume containing between 30 and 150 µg of protein was mixed 1:1 (v/v) with loading buffer (0.025M Tris-HCl, 4% SDS and 20% Glycerin) incubated at 95°C for 10 min and loaded onto a 12% discontinuous SDS polyacrylamide gel for electrophoretic separation of proteins. Following electrophoresis, the SDS gel was placed in a 0.5 g/l H_2O Coomassie brilliant blue R-250 (SIGMA) dye solution to which 100 ml glacial acidic acid and 250 ml isopropyl alcohol were added. After a 30 min to 1 h incubation at room temperature, the dye solution was exchanged with H_2O to destain the gel by 2 microwave radiations for 5 min, refreshing the destaining H_2O after each step.

4.3 Results

4.3.1 **Phenotypic aberrations from transgene expression of AC2 and AC4 genes expression in *N. benthamiana***

N. benthamiana was transformed with AC2 from ACMV and EACMV-UG and AC4 from ACMV only. About 100 transgenic events were generated for each of the viral gene constructs. From those regenerating ppt resistant calli, 64 for EACMV-UG AC2 and 52 for ACMV AC4 were positive in RT-PCR indicating AC2 and AC4 mRNA. In ACMV AC2 transformations, most plantlets regenerating from calli did not develop roots and from 5 recovered and regenerating into plants only one remained and proved RT-PCR positive expressing AC2.

Total proteins of certain transgenic lines were analysed to assess whether an additional 15 kDa band in the protein profile would provide an indication for translation of mRNA into AC4 or AC2 (TraP) proteins (Fig. 4.4).

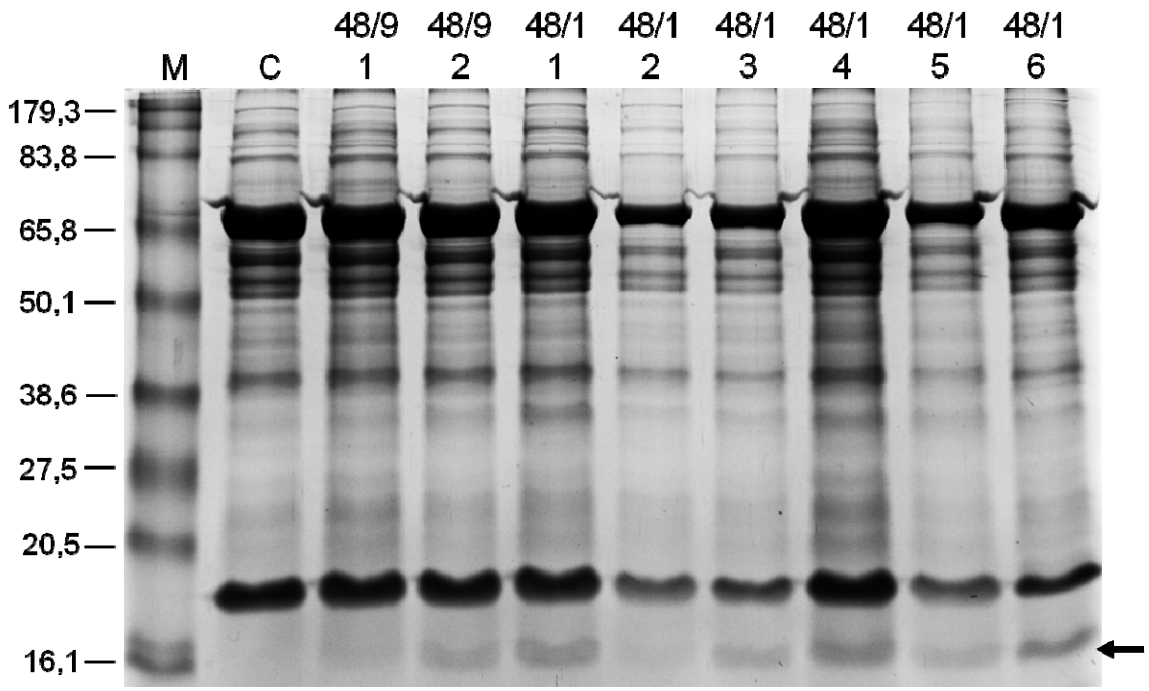

Figure 4.4: SDS PAGE of total leaf extracts from AC2 of EACMV transgenic R0 plants of *N. benthamiana* line SN48 expressing EACMV AC2 (TraP). A 15 kDa protein (arrow) in transgenic R0 plants (line 48/9 1,2; line 48/1, 1-6) was absent in wild type *N. benthamiana* (C). M, Invitrogen-Bench Marker Prestained Protein Ladder.

An additional 15 kDa protein band was only observed in transgenic plants expressing EACMV AC2 while in no other case, a considerable protein expression, indicated by an additional 15 kDa band, was found. Transgenic plants that had germinated on ppt containing medium were transferred to soil and kept in the greenhouse for flowering and production of T1 seeds. During plant development and growth, transgenic *N. benthamiana* plants expressing AC2 and AC4 however showed altered phenotypes. Most striking changes were observed with transgenes expressing ACMV AC2 while phenotypic aberrations associated with AC2 expression of EACMV or ACMV AC4 expression were relatively mild. In ACMV AC2 transgenic plants, leaf deformation and lanceolate spiralling leaves with missing petioles were formed and only three plants developed until flowering. From the limited flower settings only one plant produced seeds while all others were sterile.

Transgenic lines expressing AC2 of EACMV-UG had twisted stems, showed leaf rolling, and developed only few flowers from which seeds were produced. Plants expressing ACMV AC4 were generally stunted and had dark green heavily haired leaves and stems (Fig. 4.5).

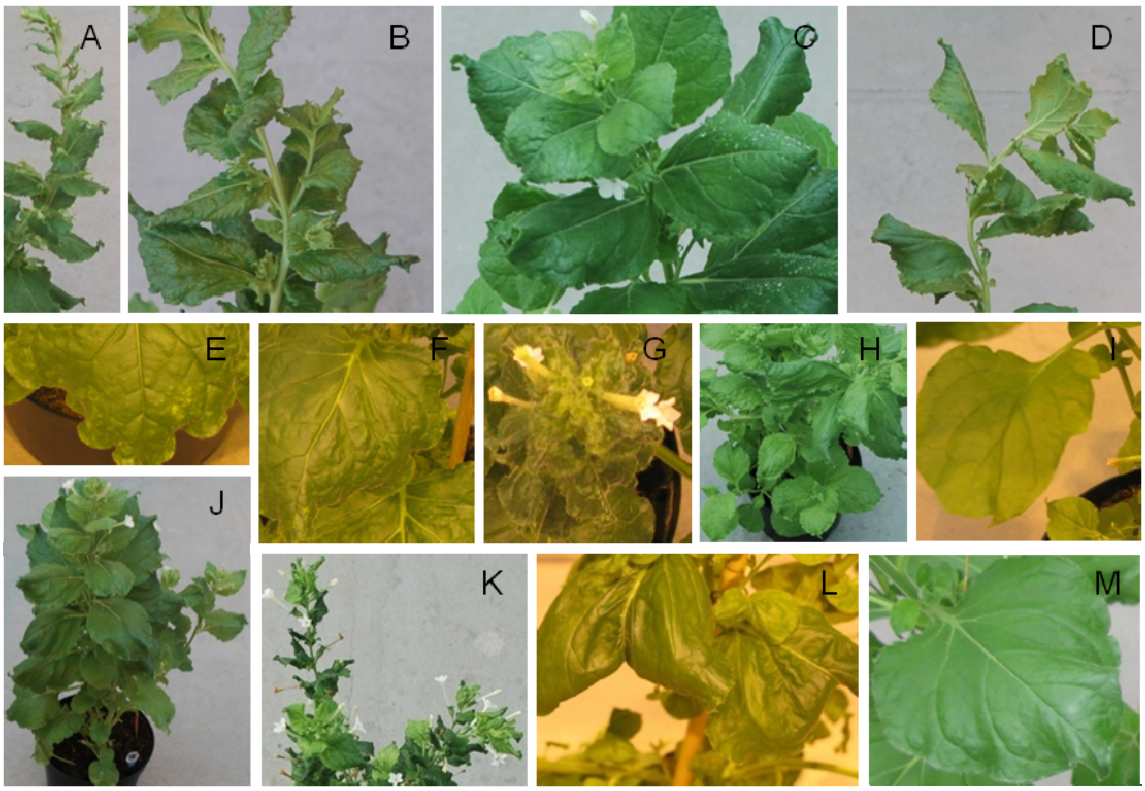

Figure 4.5: Expression of ACMV and EACMV AC2 and AC4 genes in *N. benthamiana*. EACMV AC2 transgenic plants (A-D) with severe leaf curling and rolling, stem bending and reduced or missing petioles; ACMV AC2 transgenic plants (E-I) with chlorotic blistering (E-F), malformed and hairy leaves and stem, sterile flowers (G), stunted growth and cup shaped leaves (H); ACMV AC4 transgenic line (J-L) with dark green and severely malformed leaves; M, leaf of transgenic plant, transformed with pSN40 only.

Three independent R2 lines of EACMV-AC2, ACMV-AC2 and ACMV-AC4, respectively, were subjected to RT-PCR to monitor expression of specific transcripts. mRNA for each of the genes was verified in all plants selected (Fig. 4.6) which subsequently were chosen for challenge infection with ACMV.

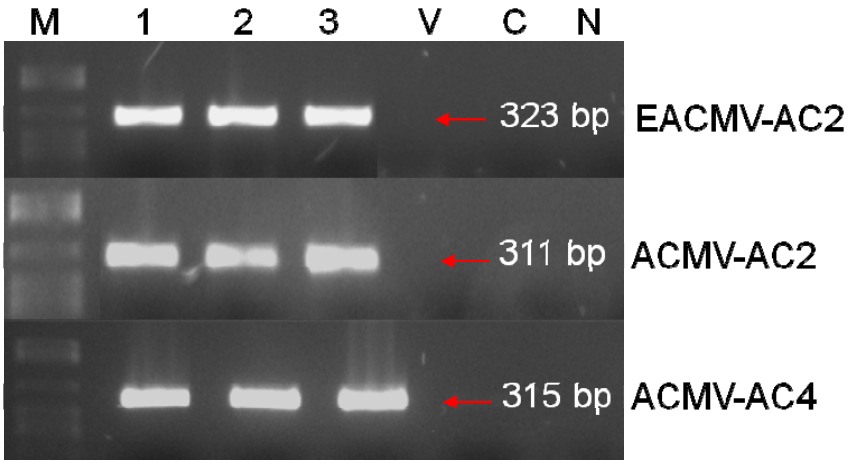

Figure 4.6: RT-PCR detection of AC2 and AC4 transcripts in selected R2 lines of transgenic *N. benthamiana* plants. M= λ DNA digested with Pst I; 1-3 R2 lines, V= vector transformation control, C= Wild type *N. benthamiana*, N= PCR negative control.

4.3.2 Effects of transient expression of AC2 and AC4 genes on replication of ACMV and PVA

Transient gene expression of AC2 and AC4 constructs in wild type *N. benthamiana* was pursued to evaluate whether effects of suppression/ enhancement inherent to a specific gene are already evident and measurable in a transient assay. Gene constructs in their respective binary vector and known viral suppressor genes from different viruses were infiltrated into *N. benthamiana* leaves for transient expression assays. To study interference of the transiently expressed genes with host defence, three days post agroinoculation, infiltrated plants were infected with either ACMV or with the potyvirus PVA, as control. In these experiments only transient expression of EACMV AC2 and ACMV AC2 resulted in sharp increase of ACMV replication (Fig. 4.7), while AC4 expression had no enhancing effects on virus replication.

Transient expression of viral suppressor genes did not have significant effects on PVA replication and pathogenesis. Virus replication was only slightly enhanced compared to wild type infections (Fig. 4.7 PVA). In contrast to infections with ACMV, transient expression of HcPro practically had no effects on plant infections with PVA.

Surprisingly, while expression of AC2 of both African cassava viruses resulted in significant increases of virus replication (Fig. 4.7 ACMV, EACMV), AC2 expression of SLCMV, a virus related to ACMV was not different from ACMV wild type infection as it was also almost negative in inoculated control plants. Expression of HcPro resulted in an increase of ACMV concentrations while ACMV replication was practically unaffected by the expression of the strong silencing suppressor P15 of JCSMV.

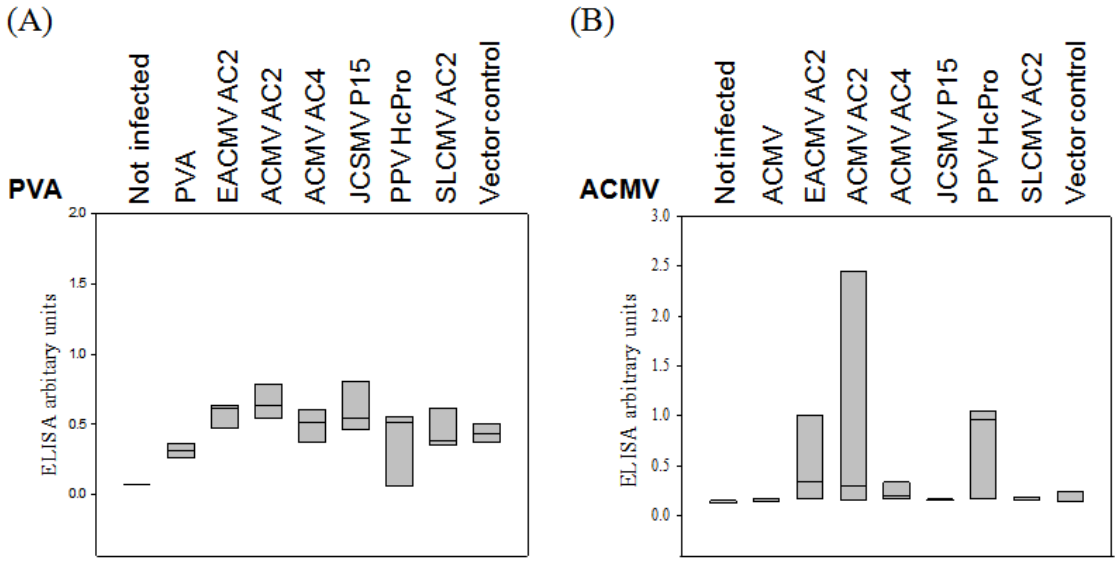

Figure 4.7: Virus replication measured by ELISA of PVA (A, left) and ACMV (B, right) in *N. benthamiana* infiltrated with *Agrobacterium* suspensions transiently expressing AC2 and AC4 constructs or silencing suppressor genes P15 of *Johnsongrass chlorotic stripe mosaic virus* P15, HcPro of *Plum pox virus* or AC2 of *Sri Lankan cassava mosaic virus*. pSn40 vector only construct as control. Box plots reflect mean median from ELISA obtained from each plant within a group.

4.3.3 **Analysis of AC2 and AC4 RNA silencing suppressor activity**

To identify RNA suppression activity, AC2 and AC4 HA tagged proteins were tested in two sets of transient *Agrobacterium* co-infiltration assays in wild type *N. benthamiana* leaves (Llave *et al.*, 2000). In the first assay (Figure 4.8) the effect of the silencer genes on sense PTGS (Wassenegger & Krczal, 2006) initiation were studied by co-infiltration of

agrobacteria containing constructs expressing GFP mixed with pING71 constructs expressing AC2 or AC4. Examination of infiltrated leaves over a period of 15 days dpi revealed that the expression level of GFP was not altered by co-infiltration with *AC2* (Fig. 4.8) or *AC4* genes (not shown) while GFP expression level was highly increased by P15 expression and by co- expression of HcPro used as silencing suppression controls (Fig. 4.8). Thus it can be assumed that the putative silencing suppression of the begomovirus genes AC2 or AC4 does not interfere with early events of sense PTGS.

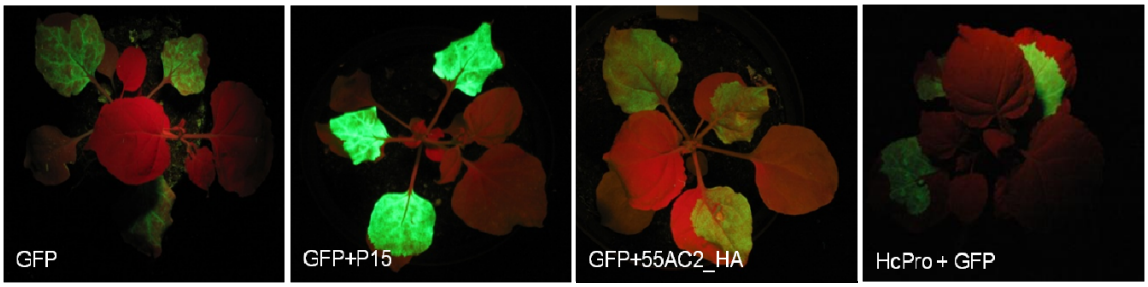

Figure 4.8: Transient expression of GFP co-infiltrated with suppressor constructs in *N. benthamiana* leaves. P15, silencing suppressor of JCSMV; 55AC2_HA, silencing suppressor of EACMV-UG expressed with a HA tag; HcPro, silencing suppressor of PPV. UV light images were taken 7 dpi.

To determine whether AC2 or AC4 could effectively suppress PTGS initiated by dsRNA and thus acting further downstream in the silencing process, plasmids expressing GFP plus plasmids expressing dsGFP were further mixed with silencing suppressor plasmids to reveal whether suppressor proteins interact with dsRNA from GFP inverted repeat expression.

Examination of infiltrated leaves over a period of 15 dpi showed that in patches of dsGFP co-infiltrated with GFP, green fluorescence was completely eliminated. In contrast in patches where P15 was co-infiltrated in a tri-plasmid mix, GFP fluorescence was maintained and intensity was high indicating for binding of P15 to dsRNA (Fig. 4.9). Similarly, but to a much lesser extend HcPro was able to interfere with dsRNA confirming that it also acts as suppressor of hairpin PTGS. In contrast neither EACMV-UG AC2 (Fig. 4.9) nor co-infiltration of ACMV AC2 or AC4 (not shown) interfered with GFP dsRNA

expression and consequently, none of those genes was able to act as suppressor of hp PTGS.

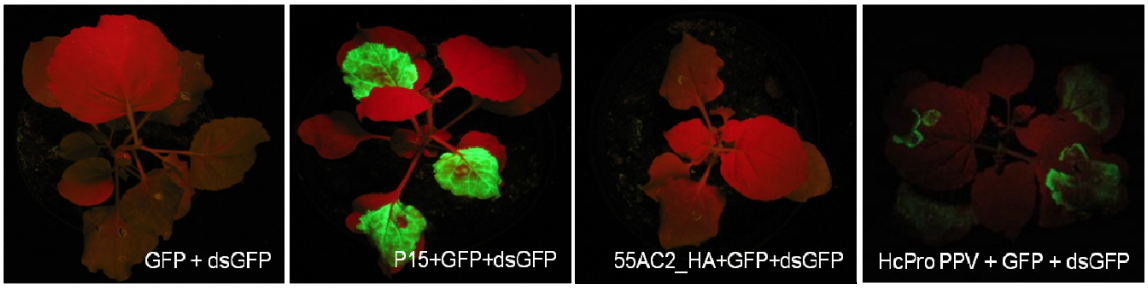

Figure 4.9: Transient expression of GFP and dsGFP mixtures co-infiltrated with suppressor constructs in *N. benthamiana* leaves. P15, silencing suppressor of JCSMV; 55AC2_HA, silencing suppressor of EACMV-UG expressed with a HA tag; HcPro, silencing suppressor of PPV. UV light images were taken 7 dpi.

To determine whether AC2 and AC4 proteins were actually present in patches infiltrated for transient gene expression, total proteins were isolated from treated areas and subjected to protein analysis. In western blot assays using a HA antibody an however weak immunological reaction was found hence confirming expression of functional proteins.

4.3.4 Virus replication in transgenic *N. benthamiana* plants expressing AC2 of ACMV and EACMV

To study interference from expression of homologous ACMV AC2 and heterologous AC2, with virus infection, ACMV infections were introduced into transgenic *N. benthamiana* by mechanical inoculation of leaf sap from systemically infected plants. ACMV infections were readily established and the plants responded with symptoms of systemic infections similar to ACMV infections in wild type non transgenic plants. Severe systemic infections established in a similar time course and neither enhanced virus replication nor spread was recorded compared to vector only transgene controls or wild type *N. benthamiana*. Real time quantitative PCR performed to calculate and compare

the absolute amounts of virus in transgenic and control plants did not show any difference in ACMV loads in the different *N. benthamiana* lines infected with ACMV (Fig. 4.10).

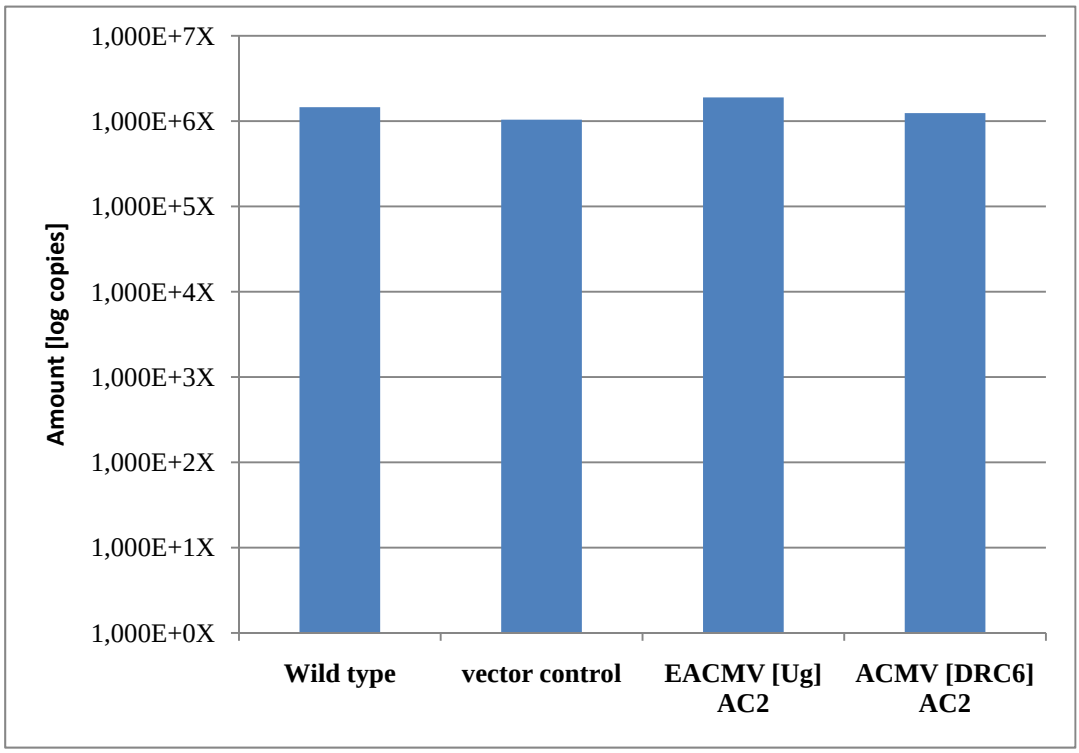

Figure 4.10: Quantification of ACMV [DRC6] in infected transgenic *N. benthamiana* carrying EACMV and ACMV AC2 genes, repectively, assessed 4 weeks dpi.

4.3.5 Trans-complementation of EACMV AC2 and AC4 knock-out mutants in AC2 transgenic *N. benthamiana*

To study whether a dysfunctional gene could be complemented by transgene expression *in planta* of its corresponding wild type gene, multimeric DNA-A clones with AC2 or AC4 mutants were introduced with their cognate DNA-B for agroinoculation to infect *N. benthamiana*. For control of infectivity, wild type EACMV DNA-A and DNA-B were also introduced. The plants inoculated with wild type virus showed mild systemic symptoms which later became obvious 18-21 dpi as mosaic patterns and mild leaf curl on systemically infected leaves. Plants inoculated with a dysfunctional EACMV AC4

developed systemic symptoms in same time course as wild type plants however with symptoms slightly weaker. EACMV AC2 failed to produce any symptoms and PCR from those plants was negative indicating that AC2 mutations resulted in loss of viability. Infectivity of AC2 mutants also was not restored by inoculations onto transgenic plants expressing wild type AC2. Hence it can be assumed that AC2 function cannot be complemented *in trans*.

EACMV mutant AC4 infections in AC2 transgenic *N. benthamiana* were subjected to PCR to analyse to confirm from those infections that a dysfunctional AC4 is stable and not reverted to wild type. Besides, from verification of the mutation in AC4, it could also be concluded that a functional AC4 is not necessarily required for plant infection.

4.4 Discussion

Eukaryotes including plants contain an RNA-directed gene regulatory system, RNA silencing as a wide spread and fundamental component of gene expression. It is activated by dsRNA which subsequently is cleaved into short (21-24 nt) fragments which mediated a number of regulatory and also defense functions in the cells (Brodersen & Voinnet, 2006). RNA silencing also is an inducible defense pathway whereby invading nucleic acids such as viruses are silenced. To invade and replicate in plants, viruses have special silencing suppressor proteins which counteract the plants defense mechanism (Dunoyer & Voinnet, 2005). These virus silencing suppressor proteins are very diverse and unrelated in sequence or structure suggesting separate origins and diverse functional mechanisms. While a large number of studies focused on silencing suppressors of RNA viruses and their interaction with the silencing pathway, suppressors from DNA viruses are much less understood. For the largest group of DNA viruses, the geminiviruses, several proteins have been directly demonstrated to have silencing suppression activity (Raja *et al.*, 2010) and for the bipartite Cassava mosaic viruses,

AC2 for EACMV and AC4 for ACMV, were proven to suppress PTGS (Vanitharani *et al.*, 2004).

The study presented here should provide insights into the mode of action of AC2 and AC4. Transgenic models were generated expressing either of the genes in *N. benthamiana* to study effects on replication of homologous or heterologous or entirely unrelated viruses. The role of *AC2* (TraP) and *AC4* genes in the pathogenesis of ACMV and EACMV-UG infections was further assessed in transient expression assays using GFP to monitor silencing suppression activity.

N. benthamiana transformed with AC2 or AC4 showed various degrees of disturbances from stable expression of the genes. Absent root formation in T0, leaf malformations and reduced flowering and seed setting were frequent with ACMV AC2 plants showing the most striking alterations in phenotype. This was also found in a recent study by Siddiqui *et al.* (2008b) further describing variable effects from expression of silencing suppressor genes in different plant species, *N. benthamiana* and *N. tabacum.* Developmental abnormalities from stable expression of viral suppressor proteins *in planta* were found in a number of studies (Anandalakshmi *et al.*, 2000; Mlotshwa *et al.*, 2005; Pruss *et al.*, 2004) while others apparently did not find phenotypical alterations due to expression of viral suppressor proteins in *N. benthamiana* or tobacco (Mlotshwa *et al.*, 2002; Savenkov & Valkonen, 2002).

The disturbed phenotypes in the transgenic plants are likely from interference of these suppressors with endogenous RNA silencing pathways. This has been shown for virus suppressors interfering with miRNA biosynthesis in *Arabidopsis* (Chapman *et al.*, 2004; Kasschau *et al.*, 2003; Llave *et al.*, 2002; Mallory *et al.*, 2002). This interference at an early step in the silencing pathway would impair the regulation of multiple genes that are regulated by miRNA. It is not clear which steps in the silencing pathway are targeted however, the questions this study tried to address was interference with plant defense

reactions. For these virus infection experiments transgenic plants reflecting a normal phenotype were chosen and infected with ACMV and PVA respectively.

The results of the virus study showed that ACMV and EACMV-UG AC2 expression increased the level of ACMV in plant infections while ACMV *AC2* and *AC4* genes expressed in transgenic plants had no effects. A similar response was reported for expression of AC2 on infection with *Tobacco ring spot virus* and on a *Tobacco mosaic virus* which expresses GFP (Siddiqui *et al.*, 2008a; Siddiqui *et al.*, 2008b). While spread and brightness of GFP was greatly enhanced by AC2 expression, an increased concentration of TRSV was only confirmed for infections of *N. tabacum* while TRSV replication and also the recovery phenomenon in *N. benthamiana* remained unaltered in transgenic plants. A likely explanation is provided from studies to characterise AC2 from MYMV and AC2 from ACMV (Trinks *et al.*, 2005) drawing a functional connection between silencing suppression and transcription activation by AC2. Suppression of silencing by the transcriptional activator protein AC2 required transactivation of host genes which may code for components of an endogenous network that controls silencing. Hence it is a host specific process.

Expression of ACMV AC4 did not interfere with ACMV infections which is somewhat surprising considering its role as gene silencing suppressor gene (Vanitharani *et al.*, 2004) and major determinant of pathogenesis for monopartite begomoviruses carrying a positional homologue C4 (van Wezel *et al.*, 2002a). However, silencing suppression is a feature of viral proteins and in transgenic plants generated in this study, TraP or AC4 protein was not unequivocally confirmed. Thus, a conclusive statement on interaction of expressed protein with replication of viruses cannot be drawn pending the unambiguous confirmation of the proteins expressed.

Virus replication and titre and symptoms severity are probably not adequate to characterise silencing suppression activity. Argument is provided from expression of the AL2 (syn. AC2) gene of TMGMV and virus infection studies (Sunter *et al.*, 2001). Here it

was found that AL1 acting as pathogenicity factor by inhibiting host stress or defence responses acting against DNA and also RNA viruses. Upon infection with viruses, transgenic plants did not show enhanced symptoms or virus replication, however, susceptibility of AL2 transgenes was evident from reduction of latent period and a decrease of inoculum concentration required to infect transgenic plants. These parameters were however not considered in this study.

AC2 and AC4 proteins and silencing suppressor proteins from other viruses were transiently expressed functional assignment of their RNA silencing suppression activity to a respective phase of the process. The results of this experiment proved that RNA silencing suppression is triggered by a variety of inducers like HcPro and P15 acting on sRNA silencing thus reflecting early events and counteracting silencing induced by expression of dsRNA. These results are in sharp contrast to experiments conducted by Vanitharani *et al.* (2004) reporting a reversal of silencing in 16c GFP transgenic plants from expression of either AC2 of EACMCV or AC4 of ACMV. Despite the fact that silencing suppressor genes of different viruses show different behaviour, AC2 suppression was not observed in this study and an effect of SLCMV AC2, reported earlier as a weak silencing suppressor, also was not confirmed. Nevertheless, it has to be stated that the experiments reported here and those by Vanitharani *et al.* (2004) are parallel observations and not directed to a synchronous phase in silencing. While the silencing suppression experiments from this study reflect on early events, the 16c GFP silencing reversal showed that AC2 and AC4 can reverse an already established silencing. This is a later event; hence both observations are independent and consequently not comparable.

The silencing suppression experiments in this study were conducted in analogy to those characterising RNA virus suppressor genes. A direct interaction is presumed, however begomovirus AC2 (AL2) does not bind siRNA or miRNA. Thus silencing suppression by these genes appears to function rather by activation of cellular genes which regulate the

silencing pathway. AL2 alters the host transcriptome, stimulates transcription and activates cellular genes in a transcription-dependent mode (Dong *et al.*, 2003; Trinks *et al.*, 2005; van Wezel *et al.*, 2002b). From studies on pathogenicity functions and interactions with adenosine kinase (ADK), an enzyme required for methyl-cycle maintenance, evidence for transcription-independent silencing suppression is also provided (Hao *et al.*, 2003; Wang *et al.*, 2003; Wang *et al.*, 2005) (Hao *et al.*, 2003; Trinks *et al.*, 2005; Wang *et al.*, 2003; Wang *et al.*, 2005). AL2 reduces ADK activity in geminivirus infections leading to suppression of silencing in a transcription–independent manner. Chromatin methylation is a defence system in plants against geminivirus infections. With AL2 (AC2) inhibiting methylation by interacting with ADK suppression transcriptional gene silencing (TGS) by these genes is likely (Buchmann *et al.*, 2009; Raja *et al.*, 2008). This mechanism or parts thereof however were not studied as direct effects of AC2 expression on viral replication were assumed. Silencing suppression from AC2 transgene expression to dissect TGS and PTGS now is warranted.

PTGS suppression activity of ACMV and EACMCV AC2 and AC4 genes were reported by Vanitharani *et al.* (2004). This study showed that related viruses have different suppressors which act on different silencing pathways. AC4 unlike AC2 can bind single strand siRNA and is able to suppress PTGS by binding small RNA (Chellappan *et al.*, 2005a). This silencing suppressor effect was however not studied in transient assays, as AC4 was not included in the study. Geminiviruses must be able to counteract PTGS and TGS to successfully infect plants (Raja *et al.*, 2010). From evidence provided, including this study, AC2 proteins are at most weak PTGS suppressors and from the body of available evidence, it can be argued that AC2 by opposing methylation are strong suppressors of TGS. Perhaps the role of AC4 then is, to counteract PTGS.

It can be summarized from the results of this study that AC2 of ACMV is a pathogenicity determinant as described by Voinnet *et al.*, (1999) but not a suppressor of PTGS.

Pathogenicity is not related with gene silencing suppression activity (Diaz-Pendon & Ding, 2008).

To study the gene function of ACMV and EACMV mutants with dysfunctional *AC2* and *AC4* genes respectively were generated and tested for their infectivity in *N. benthamiana.* The AC2 knock out mutant of EACMV failed to produce systemic symptoms. This was expected since TraP coded for by AC2 of begomoviruses is essential for efficient transcription of the late viral sense genes, AV1 and BV1 (Sunter & Bisaro, 1992; Sunter & Bisaro, 1997b). Etessami *et al.*, (1991) have shown that disruption of ACMV AC2 prevents plant infections and from this study also cannot functionally be complemented by co-expression of AC2 from DNA-A of a functionally intact virus. In contrast, a dysfunctional AC4 still resulted in plant infection and hence was dispensable for virus infection. AC4 proteins of bipartite begomoviruses are highly variable and somewhat transient in that they exist only in some DNA-A while missing in others. The results of this present study are consistent with finding of other groups that a functional AC4 of bipartite geminiviruses is not essential for infectivity (Bull *et al.*, 2007; Etessami *et al.*, 1991) and has not effects on viral replication or symptom development.

5. General discussion

This study was conducted in the framework of the ongoing cassava virus research at the DSMZ Plant Virus Department. In earlier studies (Ariyo *et al.*, 2005; Were *et al.*, 2003; Were *et al.*, 2004) cassava virus collections from Africa were established and viruses and the diseases they cause in cassava were characterised. Results and knowledge gained from those previous projects provided the impetus for the underlying hypotheses followed in this dissertation.

Virus species in the genus *Begomovirus*, family *Geminiviridae*, are the main and most significant viruses infecting cassava in Africa. The considerable genetic diversity of these viruses (Ndunguru *et al.*, 2005; Sserubombwe *et al.*, 2008) is a result of recombination and intensive genome rearrangements which also include the acquisition of new genome components (Seal *et al.*, 2006b). For recombination to arise, mixed infection is the prerequisite and consequently geminiviruses which replicate in the nuclei of infected cells, have to meet there (Morilla *et al.*, 2004). Mixed begomovirus infections have been reported for many crops (Mansoor *et al.*, 2003a; Mansoor *et al.*, 2003b; Sanz *et al.*, 2000; Xie & Zhou, 2003) and frequently occur in cassava (Ogbe *et al.*, 2003b; Pita *et al.*, 2001b; Tadu *et al.*, 2006). As a consequence very severe symptoms become evident strikingly demonstrating synergistic interactions. Some mixed infections like those reported for ACMV/EACMCV (Fondong *et al.*, 2000) are found at constant frequencies in CMD field situations with ACMV single infections however dominating. In contrast, as is the case of ACMV/EACMV-UG mixed infections, a severe synergistic phenotype (Legg, 1999; Legg & Thresh, 2000; Owor *et al.*, 2004) marks a new virus invasion/combination and this transient phase then is leaving EACMV-UG as the dominant and single virus causing the disease. The epidemiological impact of EACMV-

UG as a key cassava virus in Africa is evident although the parameters for its selective advantage thus driving its dissemination are largely unclear.

One key question addressed in the second chapter was whether virus replication and movement differ in mixed virus infections of ACMV and EACMV-UG compared to single infections with either of the viruses. If in mixed infections the concentrations of EACMV-UG reach high concentrations, asymmetric to ACMV, the epidemiology of EACMV-UG could probably be explained. A further aspect of this study was to assess synergism in relation to virus concentrations and cassava genotype.

In a quantitative assessment, the copy numbers of the genome components DNA-A and DNA-B of the respective viruses in single and mixed infections of cassava were determined. For this a real-time quantitative PCR system based on TaqMan® probes was developed. The cassava *β-actin* gene of which several copies are present in the genome proved an excellent endogenous control gene to validate the analytical procedure, from DNA extraction to qPCR. Thus a precise comparison among virus genome components was possible and as a spin-off, a highly specific real time qPCR for detection and quantitation of EACMV-UG and ACMV is now available for sensitive and rapid differential diagnosis of the viruses in samples with extremely low virus concentrations.

In cassava landraces, generally, virus concentration corresponded with severity of disease symptoms. Considerably lower amounts of virus were recorded when symptoms were absent (recovery phase). This is consistent with earlier studies on ACMV (Fargette *et al.*, 1996; Ogbe *et al.*, 2003a) where higher virus concentrations correlated with the severity of the disease phenotype. However, when the assessment was extended to cassava genotypes with improved resistance/tolerance features, the virus status was evaluated differently. In the moderately resistant cassava cv. TMS 30572 despite the milder symptoms, virus concentration was not too different from that of a highly susceptible cassava cv.TME 117 expressing severe symptoms. From a virological point

of few, considering virus replication and movement, the classification of this cv. TMS 30572 as a moderately resistant cultivar hence is not warranted and the use of this cv. as parent for resistance breeding should probably be revised. Resistance still was confirmed for the cv. Albert which was immune against ACMV but supported a limited replication of EACMV-UG. This was accompanied with virus spread and the expression of mild but transient symptoms.

A differential assessment of DNA genomic components by qPCR revealed striking differences between the viruses. The DNA-A to DNA-B ratios recorded for EACMV-UG were B>A while for ACMV the reverse was true and higher concentrations of DNA-A compared to DNA-B were measured. Symptom type and plant invasion (movement) are predominant features of DNA-B. From studies of with plant infections induced by pseudorecombinant viruses (Hou & Gilbertson, 1996; Unseld *et al.*, 2000) the severity of symptoms coincided with accumulation of DNA-B genomic components while plants showing attenuated symptoms had reduced levels of DNA-B. High levels of virus accumulation and consequently more severe symptoms were considered as better adaptability of the virus to the host (Hou *et al.*, 1998). Thus from this quantitative study, EACMV-UG appeared to be the better adapted virus than ACMV reaching far higher virus concentrations in all cultivars than what was recorded for ACMV. The increased DNA-B levels in EACMV-UG infections, indicated for a highly efficient invasion of the “adapted” host while the limited virus movement of EACMV-UG in cassava cv. Albert was a characteristics of a “non adapted” host. A considerable although quite variable level of virus was recorded in non symptomatic leaves but only rarely virus freedom could be assumed. Notwithstanding, symptomless phases and recovery were attributed to a response of the host to, at least in phases, impair virus replication.

Much higher virus titres were generally recorded for EACMV-UG in infected plants compared with ACMV, however when natural and artificial mixed infections in cassava were studied, ACMV concentrations only reached levels comparable to single infections.

Similarly, EAMCV-UG DNA-A concentrations remained more or less unchanged in mixed infections compared to single infections while EACMV-UG DNA-B was drastically reduced. This observation based on qPCR is in strong contrast to reports of synergism among ACMV and *East African cassava mosaic Cameroon virus* (EACMCV) where accumulation of EACMCV was very high for both DNA genome components with greatest increase of DNA-B compared to single EACMCV infections (Fondong *et al.*, 2000).

In a study by Pita and co-workers (Pita *et al.*, 2001b), a higher accumulation of both EACMV genomes in mixed infections was found indicating for strong synergism between ACMV and EACMV-UG. This was regarded as a driver for EACMV-UG epidemics and preferable spread of this virus by whiteflies. In contrast, the results from this study cannot confirm these earlier reports and ACMV appeared to be unaffected by presence of EACMV-UG. Moreover the low accumulation of EACMV-UG DNA-B rather indicates for negative interference. As shown for TYLCV/TYLCSV mixed infections (Morilla *et al.*, 2004) increased symptoms in dual infections are not necessarily bound to higher DNA accumulation.

The objective dealt with in the third chapter of this dissertation was to study movement and distribution of ACMV and EACMV-UG in infected *N. benthamiana* plants. This study should in parallel follow the quantitative assessment of virus infections and intended to resolve tissue tropism of viruses in mixed and single infected cassava. DNA-A and DNA-B genomic components of infectious clones of ACMV and EACMV-UG (Ariyo *et al.*, 2006) were modified by insertion of the gene for green fluorescent protein (GFP) in place of CP or BV1 or BC1 sequences and to express this reporter gene from transcription during virus infection. The dispensability of a functional CP for infectivity and movement within the plant was utilised to construct infectious bean dwarf mosaic virus (BDMV) clones containing a modified GFP in place of the CP (Sudarshana *et al.*, 1998). By using

fluorescence analysis, cell-to-cell and long distance movement of BDMV was followed to reveal details of the infection process in *Phaseolus vulgaris*.

In preliminary experiments, the strategy of Sudarshana *et al.* (1998) was followed to develop CP mutants of EACMV-UG, however infectivity of EACMV ΔCP mutants was not restored. Consequently, several alternative routes were pursued. GFP was introduced into the DNA-A from infectious EACMV or ACMV clones either by utilising the AV2 start and entirely replacing AV2 and CP or, as translational fusion to AV2 replacing CP only. In a study to reveal tissue tropism of ICMV, Rothenstein *et al.* (2007) used AV2:GFP fusion constructs to show association of fluorescence with the vascular system. None of the GFP constructs generated during this study induced begomovirus infections in *N. benthamiana*. Whereas, in a GFP recombinant BDMV DNA-A genome viability was not compromised and pathogenicity only slightly affected (Sudarshana *et al.*, 1998), viability of recombinant EACMV and ACMV constructs was completely lost. The weak systemic symptoms found in rare cases in *N. benthamiana* with one construct (AV2://GFPΔCP) were due to presence of virus but GFP expression was missing and hence considered artefactual.

The parental Infectious begomovirus clones of EACMV-UG and ACMV cause infections in *N. benthamiana* and in cassava. These infectious virus clones have been very efficiently used to screen for resistance in cassava germplasm (Ariyo *et al.*, 2006). In addition, DNA-A CP replacement vectors were initially designed for virus induced gene silencing (VIGS) to study gene function in cassava (Fofana *et al.*, 2004; Muangsan *et al.*, 2004; Pandey *et al.*, 2009; Robertson, 2004). A number of gene constructs including endogenous genes (Mg-chelatase) and artificial miRNA genes were efficiently expressed using recombinant EACMV-UG DNA-A in which CP was partially or entirely replaced by the foreign gene. Hence loss of function cannot be explained by either size constraints (Etessami *et al.*, 1989; Gilbertson *et al.*, 2003) or loss of pathogenicity. Attenuated symptoms were also found in *N. benthamiana* infected with ICMV GFP recombinant

DNA-A constructs (Rothenstein *et al.*, 2007), in BDMV-GFP infected beans (Sudarshana *et al.*, 1998) and in infections with EACMV VIGS vectors in cassava (Winter S, unpublished) where recombinant viruses carrying foreign gene sequences caused significantly weaker symptoms than the parent virus clones. Lack of movement of ACMV and EACMV chimeras with truncated and deleted AV2 could also be explained by a function of AV2 in efficient virus movement in plants (Padidam *et al.*, 1996). A role of CP in the infection process of bipartite geminiviruses, as suggested by Pooma *et al.*, (1996), is probably minimal for EACMV and ACMV since VIGS vectors derived from parental clones efficiently replicated and remained stable in cassava and even more so in *N. benthamiana*. The complete loss of infectivity inherent to recombinant GFP clones carrying translational fusions or free GFP translated from AV1 or CP start codons can thus not be explained.

Replication of recombinant ICMV expressing GFP and the association of GFP fluorescence with virus replicating virus was not convincingly demonstrated by Rothenstein *et al.* (2007). Hence besides BDMV, it can be assumed that GFP expression from a replicating begomovirus is at most very inefficient hence alternative routes need to be pursued to study virus movement and tissue affiliation.

An alternative to GFP recombinant DNA-A clones, DNA-B mutants with GFP replacing either BC 1 or BV1 were generated. It was not assumed that DNA-B constructs would result in infectious viruses when coinoculated with cognate DNA-A however it was expected that functional DNA-A will transreplicate DNA-B. Since DNA-A components of certain bipartite geminiviruses are capable of systemically infecting plants when delivered by agroinoculation (Evans & Jeske, 1993; Klinkenberg & Stanley, 1990), dysfunctional BC1 or BV1 in DNA-B when transreplicated from cognate DNA-A would probably still support expression of GFP. However, this was never observed.

This study has shown that labelling of EACMV and ACMV genomes with GFP inevitably results in loss of infectivity and it seems likely that the translatable GFP has a major

impact on the infectivity/ stability/ fitness of the virus constructs. Despite the beauty of using non destructive methods to follow virus infections *in planta*, it appears that even when mastered, such major interventions have serious impacts on the overall performance of viruses and create artificial systems which counterbalance advantages of real time *in situ* studies.

Virus-virus interaction in mixed infections and synergism has been postulated as a result from suppression of the host defence mechanism. In the third part of the experiments (4th chapter) *AC2* and *AC4* genes of ACMV and EACMV which are implicated in suppression of gene silencing were characterized for their effects on the post transcriptional gene silencing (PTGS) pathway. A preliminary transient gene expression assay showed that the activity of AC2 to suppress PTGS was weak while expression of AC4 had no effects on PTGS. Silencing suppressors from plant viruses when expressed from transgenes in model hosts can cause serious developmental abnormalities (Anandalakshmi *et al.*, 2000; Mlotshwa *et al.*, 2005; Pruss *et al.*, 2004). *AC2* and *AC4* genes introduced into transgenic *N. benthamiana* resulted in abnormal growth and a series of phenotypic aberrations in this plant. Absent root formation in T0, leaf malformations and reduced flowering and seed setting were frequent with ACMV AC2 plants. Similar effects from expression of silencing suppressor genes in two different tobacco species were described in a recent study by Siddiqui *et al.* (2008b) thus indicating interference of viral suppressors with plant development. Interference at an early step in the silencing pathway would impair the regulation of multiple genes that are regulated by miRNA. It is not clear which steps in the silencing pathway are targeted however, the questions this study tried to address was interference with plant defence reactions. For these virus infection experiments transgenic plants reflecting a normal phenotype were chosen and infected with ACMV and PVA respectively.

The results of the virus tests showed that ACMV and EACMV-UG AC2 expression increased the level of ACMV in plant infections while ACMV *AC2* and *AC4* genes

expressed in transgenic plants had no effects on virus concentration and symptoms. A similar response was reported for expression of AC2 on plant infections with *Tobacco ringspot virus* or *Tobacco mosaic virus* (Siddiqui *et al.*, 2008a; Siddiqui *et al.*, 2008b). From studies to characterize AC2 of MYMV and AC2 from ACMV (Trinks *et al.*, 2005) a functional connection between silencing suppression and transcription activation by AC2 was drawn. Suppression of silencing by the transcriptional activator protein AC2 requires transactivation of host genes which may code for components of an endogenous network that controls silencing.

It was surprising to see no effect of ACMV AC4 expression on ACMV infections considering its postulated role as silencing suppressor gene (Vanitharani *et al.*, 2004) and major determinant of pathogenesis in monopartite begomoviruses [C4 gene] (van Wezel *et al.*, 2002a). In virus infection studies with plants expressing AL2 [syn. AC2] (Sunter *et al.*, 2001) it was found that AL2 acts as a pathogenicity factor by inhibiting host stress or defence responses against DNA and also RNA viruses. This was not evident from enhanced symptoms or increased virus replication but rather from a reduction of latency period and increased sensitivity to inoculation in transgenic plants. The evaluation of virus replication and symptoms severity in this study thus was probably not adequate to characterise silencing suppression activity since latency and inoculum concentration was not considered.

Transient expression of silencing suppressor proteins from several viruses triggers RNA silencing suppression. Inducers like HcPro (potyvirus) or P15 (tombusvirus) act on early events by counteracting silencing induced by expression of dsRNA. Experiments conducted by Vanitharani *et al.* (2004) report a reversal of silencing in 16c GFP transgenic plants by transient expression of either AC2 of EACMCV or AC4 of ACMV. Despite the fact that silencing suppressor genes of different viruses show different behaviour, AC2 suppression was not observed in this study. Furthermore effects of SLCMV AC2 reported earlier as weak silencing suppressor, was also not confirmed.

Nevertheless, it has to be stated that the experiments reported here and those by Vanitharani *et al.* (2004) are parallel observations and not directed to a synchronous phase in silencing. While the silencing suppression experiments from this study reflect on early events, the 16c GFP silencing reversal showed that AC2 and AC4 can reverse an already established silencing. This is a later event; hence both observations are independent and consequently not comparable.

PTGS suppression activity of ACMV *AC4* genes were reported by Vanitharani *et al.* (2004). This study showed that related viruses have different suppressors which act on different silencing pathways. AC4 unlike AC2 can bind single stranded siRNA and is able to suppress PTGS by binding small RNA (Chellappan *et al.*, 2005a). This silencing suppressor effect was however not studied in transient assays since AC4 was not included in the study. Geminiviruses must be able to counteract PTGS and TGS to successfully infect plants (Raja *et al.*, 2010). From evidence provided, including this study, AC2 proteins are at most weak PTGS suppressors and from the body of available evidence, it can be argued that AC2 by opposing methylation are strong suppressors of TGS. Perhaps the role of AC4 then is, to counteract PTGS.

The summary of the experiments of this study is that AC2 of ACMV is a pathogenicity factor as described by Voinnet *et al.*, (1999) but not a suppressor of PTGS and hence pathogenicity is not related to gene silencing suppression activity (Diaz-Pendon & Ding, 2008).

Knock out mutants of ACMV and EACMV with dysfunctional *AC2* and *AC4* genes respectively were generated and tested for their infectivity in *N. benthamiana.* Infection experiments with mutant virus carrying dysfunctional EACMV AC2 failed and loss of viability from mutated AC2. This was expected since TraP coded by AC2 is essential for efficient transcription of the late viral sense genes, *AV1* and *BV1* (Sunter & Bisaro, 1992; Sunter & Bisaro, 1997b). Disruption of ACMV AC2 resulted in loss of infectivity and as

shown in this study cannot functionally be complemented by co-expression of AC2 from DNA-A of a functionally intact virus. In contrast, a virus with dysfunctional AC4 still is infectious and hence AC4 is dispensable for virus infection and has no effects on viral replication or symptom development.

6. References

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7. Figures

8. Tables

9. Appendix

Appendix Table 2.5: Relative concentrations of ACMV [DRC6] DNA-A and DNA-B in virus infected (non-symptomatic) and uninfected cassava plants (genotype TME 117) calculated using the relative standard curve method

		ACMV [DRC6]			β- actin gene of cassava						
Test sample TME 117_NS	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 24	DNA-A	28.77±0.1	3.05	1.11E+03	24.34±0.03	7.18	1.53E+07	7.28E-05	1.89E+02	1.29	A > B
	DNA-B	29.09±0.11	3.26	1.81E+03	24.34±0.03	7.18	1.53E+07	1.19E-04	1.46E+02		
Ca 26	DNA-A	18.38±0.05	6.31	2.06E+06	24.52±0.23	7.13	1.37E+07	1.50E-01	3.90E+05	2.94	A > B
	DNA-B	18.73±0.21	6.17	1.48E+06	24.52±0.23	7.13	1.37E+07	1.08E-01	1.33E+05		
Ca 28	DNA-A	26.47±0.14	3.77	5.91E+03	24.29±0.15	7.20	1.59E+07	3.72E-04	9.66E+02	2.27	A > B
	DNA-B	27.38±0.2	3.74	5.49E+03	24.29±0.15	7.20	1.59E+07	3.46E-04	4.26E+02		
Ca 24.2	DNA-A	31.64±0.15	2.14	1.39E+02	24.18±0.04	7.23	1.70E+07	8.18E-06	2.12E+01	1.41	A > B
	DNA-B	32.49±0.43	2.30	2.07E+02	24.18±0.04	7.23	1.70E+07	1.22E-05	1.50E+01		
Ca 26.2	DNA-A	26.8±0.11	3.66	4.62E+03	24.35±0.03	7.18	1.52E+07	3.05E-04	7.92E+02	2.39	A > B
	DNA-B	27.84±0.13	3.61	4.06E+03	24.35±0.03	7.18	1.52E+07	2.68E-04	3.31E+02		
Ca 28.2	DNA-A	29.9±0.05	2.69	4.89E+02	24.29±0.06	7.20	1.58E+07	3.09E-05	8.03E+01	0.66	A > B
	DNA-B	29.32±0.22	3.19	1.57E+03	24.29±0.06	7.20	1.58E+07	9.92E-05	1.22E+02		
Ca 91.1	DNA-A	36.07±0.63	0.75	5.94	24.33±0.1	7.19	1.54E+07	3.85E-07	1.00E+00	1.00	A = B
(Calibrator)	DNA-B	36.79±0.03	1.10	12.49	24.33±0.1	7.19	1.54E+07	8.10E-07	1.00E+00		

Appendix Table 2.6: Relative concentrations of ACMV [DRC6] DNA-A and DNA-B in virus infected (symptomatic) and uninfected cassava plants (genotype TME 117) calculated using the relative standard curve method

		ACMV [DRC6]			β- actin gene of cassava						
Test sample TME 117_S	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 25	DNA-A	21.84±0.02	5.23	1.68E+05	24.25±0.15	7.21	1.63E+07	1.04E-02	2.69E+04	3.17	A > B
	DNA-B	22.72±0.1	5.05	1.12E+05	24.25±0.15	7.21	1.63E+07	6.87E-03	8.48E+03		
Ca 27	DNA-A	17.3±0.2	6.65	4.53E+06	24.32±0.14	7.19	1.55E+07	2.92E-01	7.58E+05	3.53	A > B
	DNA-B	17.79±0.07	6.43	2.70E+06	24.32±0.14	7.19	1.55E+07	1.74E-01	2.15E+05		
Ca 29	DNA-A	12.82±0.01	8.06	1.15E+08	24.86±0.2	7.04	1.10E+07	1.05E+01	2.73E+07	5.60	A > B
	DNA-B	13.49±0.03	7.64	4.34E+07	24.86±0.2	7.04	1.10E+07	3.95E+00	4.87E+06		
Ca 25.2	DNA-A	16.19±0.06	7.00	1.01E+07	24.43±0.13	7.16	1.44E+07	6.98E-01	1.81E+06	6.36	A > B
	DNA-B	17.47±0.1	6.52	3.34E+06	24.43±0.13	7.16	1.44E+07	2.31E-01	2.85E+05		
Ca 27.2	DNA-A	16.88±0.12	6.78	6.09E+06	24.6±0.09	7.11	1.30E+07	4.70E-01	1.22E+06	5.43	A > B
	DNA-B	18±0.08	6.37	2.36E+06	24.6±0.09	7.11	1.30E+07	1.82E-01	2.25E+05		
Ca 29.2	DNA-A	17.14±0.11	6.71	5.08E+06	24.44±0.06	7.16	1.44E+07	3.54E-01	9.20E+05	4.58	A > B
	DNA-B	18.01±0.1	6.37	2.34E+06	24.44±0.06	7.16	1.44E+07	1.63E-01	2.01E+05		
Ca 91.1	DNA-A	36.07±0.63	0.75	5.94E+00	24.33±0.1	7.19	1.54E+07	3.85E-07	1.00E+00	1.00	A = B
(Calibrator)	DNA-B	36.79±0.03	1.10	1.25E+01	24.33±0.1	7.19	1.54E+07	8.10E-07	1.00E+00		

Appendix Table 2.7: Relative concentrations of EACMV-UG DNA-A and DNA-B B in virus infected (non-symptomatic) and uninfected cassava plants (genotype TMS 30572) calculated using the relative standard curve method

		EACMV [Ug] Ca055			β- actin gene of cassava						
Test sample TMS 30572_NS	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 36	DNA-A	30.15±0.23	3.11	1.30E+03	24.49±0.06	7.14	1.39E+07	9.39E-05	5.40E+02	0.04	A < B
	DNA-B	25.48±0.13	4.52	3.31E+04	24.49±0.06	7.14	1.39E+07	2.39E-03	1.37E+04		
Ca 38	DNA-A	28.89±0.06	3.49	3.09E+03	24.99±0.04	7.00	1.01E+07	3.06E-04	1.76E+03	2.18	A > B
	DNA-B	29.85±0.16	3.15	1.42E+03	24.99±0.04	7.00	1.01E+07	1.41E-04	8.10E+02		
Ca 40	DNA-A	29.69±0.08	3.25	1.77E+03	24.95±0.18	7.00	1.01E+07	1.75E-04	1.01E+03	0.44	A < B
	DNA-B	28.41±0.27	3.60	4.05E+03	24.95±0.18	7.00	1.01E+07	4.00E-04	2.31E+03		
Ca 42	DNA-A	30.97±0.10	2.86	7.30E+02	25.09±0.01	6.97	9.43E+06	7.74E-05	4.46E+02	0.44	A < B
	DNA-B	29.64±0.17	3.22	1.67E+03	25.09±0.01	6.97	9.43E+06	1.77E-04	1.02E+03		
Ca 36.2	DNA-A	21.51±0.36	5.71	5.23E+05	24.69±0.18	7.09	1.22E+07	4.28E-02	2.46E+05	2.66	A > B
	DNA-B	23.00±0.06	5.29	1.97E+05	24.69±0.18	7.09	1.22E+07	1.61E-02	9.27E+04		
Ca 38.2	DNA-A	21.72±0.04	5.65	4.42E+05	24.82±0.19	7.05	1.13E+07	3.91E-02	2.25E+05	0.59	A < B
	DNA-B	21.13±0.04	5.88	7.54E+05	24.82±0.19	7.05	1.13E+07	6.68E-02	3.84E+05		
Ca 40.2	DNA-A	25.41±0.19	4.53	3.44E+04	24.77±0.13	7.06	1.16E+07	2.97E-03	1.71E+04	1.16	A > B
	DNA-B	25.64±0.10	4.47	2.95E+04	24.77±0.13	7.06	1.16E+07	2.55E-03	1.47E+04		
Ca 42.2	DNA-A	23.34±0.34	5.16	1.46E+05	24.92±0.21	7.02	1.06E+07	1.38E-02	7.97E+04	0.24	A < B
	DNA-B	21.45±0.30	5.78	6.12E+05	24.92±0.21	7.02	1.06E+07	5.78E-02	3.33E+05		
Ca 88.2	DNA-A	-	1.00	1.00E+00	25.86±0.14	6.76	5.76E+06	1.74E-07	1.00	1.00	A = B
(Calibrator)	DNA-B	-	1.00	1.00E+00	25.86±0.14	6.76	5.76E+06	1.74E-07	1.00		

Appendix Table 2.8: Relative concentrations of EACMV-UG DNA-A and DNA-B B in virus infected (symptomatic) and uninfected cassava plants (genotype TMS 30572) calculated using the relative standard curve method

		EACMV [Ug] Ca055			β- actin gene of cassava						
Test sample TMS 30572_S	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc. (copies)**	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 37	DNA-A	16.21±0.14	7.30	2.02E+07	24.42±0.66	7.16	1.54E+07	1.31E+00	7.54E+06	0.37	A < B
	DNA-B	15.19±0.06	7.74	5.45E+07	24.42±0.66	7.16	1.54E+07	3.53E+00	2.03E+07		
Ca 39	DNA-A	20.18±0.17	6.11	1.29E+06	24.89±0.07	7.03	1.07E+07	1.20E-01	6.93E+05	0.24	A < B
	DNA-B	18.42 ± 0.09	6.73	5.34E+06	24.89±0.07	7.03	1.07E+07	4.98E-01	2.86E+06		
Ca 41	DNA-A	16.46 ± 0.09	7.23	1.69E+07	24.8±0.1	7.06	1.14E+07	1.49E+00	8.55E+06	0.28	A < B
	DNA-B	15.04 ± 0.24	7.78	6.13E+07	24.8±0.1	7.06	1.14E+07	5.37E+00	3.09E+07		
Ca 43	DNA-A	17.29 ± 0.17	6.98	9.56E+06	25.06±0.09	6.98	9.63E+06	9.93E-01	5.72E+06	0.25	A < B
	DNA-B	15.68 ± 0.02	7.58	3.83E+07	25.06±0.09	6.98	9.63E+06	3.98E+00	2.29E+07		
Ca 37.2	DNA-A	16.59 ± 0.12	7.19	1.55E+07	24.95±0.11	7.01	1.03E+07	1.50E+00	8.63E+06	0.20	A < B
	DNA-B	14.68 ± 0.05	7.89	7.84E+07	24.95±0.11	7.01	1.03E+07	7.60E+00	4.38E+07		
Ca 39.2	DNA-A	18.38 ± 0.02	6.65	4.47E+06	24.55±0.15	7.12	1.34E+07	3.35E-01	1.93E+06	0.17	A < B
	DNA-B	16.22 ± 0.09	7.41	2.60E+07	24.55±0.15	7.12	1.34E+07	1.94E+00	1.12E+07		
Ca 41.2	DNA-A	20.22 ± 0.17	6.10	1.25E+06	24.91±0.09	7.02	1.06E+07	1.18E-01	6.81E+05	0.12	A < B
	DNA-B	17.48 ± 0.00	7.02	1.05E+07	24.91±0.09	7.02	1.06E+07	9.90E-01	5.70E+06		
Ca 43.2	DNA-A	17.27 ± 0.17	6.98	9.70E+06	24.77±0.17	7.06	1.17E+07	8.32E-01	4.79E+06	0.29	A < B
	DNA-B	15.88 ± 0.05	7.52	3.30E+07	24.77±0.17	7.06	1.17E+07	2.84E+00	1.63E+07		
Ca 88.2	DNA-A	-	1.00	1.00E+00	25.86±0.14	6.76	5.76E+06	1.74E-07	1.00	1.00	A = B
(Calibrator)	DNA-B	-	1.00	1.00E+00	25.86±0.14	6.76	5.76E+06	1.74E-07	1.00		

Appendix Table 2.9: Relative concentrations of ACMV [DRC6) DNA-A and DNA-B B in virus infected (non-symptomatic) and uninfected cassava plants (genotype TMS 30572) calculated using the relative standard curve method

		ACMV [DRC6]			β- actin gene of cassava						
Test sample TMS 30572_NS	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc.** (copies)	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc.** (copies)	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 44	DNA-A	26.02±0.2	3.91	8.18E+03	24.15±0.21	7.24	1.74E+07	4.71E-04	3.30E+02	1.70	A > B
	DNA-B	27.86±0.18	3.60	4.04E+03	24.15±0.21	7.24	1.74E+07	2.33E-04	1.94E+02		
Ca 46	DNA-A	18.86±0.13	6.16	1.46E+06	25.06±0.08	6.98	9.60E+06	1.52E-01	1.06E+05	1.63	A > B
	DNA-B	19.77±0.12	5.88	7.53E+05	25.06±0.08	6.98	9.60E+06	7.84E-02	6.53E+04		
Ca 48	DNA-A	28.95±0.12	2.99	9.79E+02	25.05±0.04	6.98	9.66E+06	1.01E-04	7.09E+01	1.61	A > B
	DNA-B	31.06±0.26	2.70	5.11E+02	25.05±0.04	6.98	9.66E+06	5.29E-05	4.41E+01		
Ca 50	DNA-A	27.17±0.15	3.55	3.54E+03	25.04±0.03	6.99	9.76E+06	3.63E-04	2.54E+02	0.49	A < B
	DNA-B	27.21±0.12	3.79	6.12E+03	25.04±0.03	6.99	9.76E+06	6.27E-04	5.22E+02		
Ca 52	DNA-A	30.6±0.08	2.47	2.94E+02	25.72±0.1	6.80	6.32E+06	4.66E-05	3.26E+01	0.87	A < B
	DNA-B	31.95±0.15	2.45	2.86E+02	25.72±0.1	6.80	6.32E+06	4.52E-05	3.77E+01		
Ca 44.2	DNA-A	24.94±0.03	4.25	1.78E+04	25.34±0.3	6.91	8.15E+06	2.19E-03	1.53E+03	9.53	A > B
	DNA-B	29.31±0.07	3.20	1.57E+03	25.34±0.3	6.91	8.15E+06	1.93E-04	1.61E+02		
Ca 46.2	DNA-A	23.39±0.19	4.74	5.52E+04	25.45±0.25	6.87	7.55E+06	7.30E-03	5.11E+03	0.50	A < B
	DNA-B	23.02±0.25	4.96	9.23E+04	25.45±0.25	6.87	7.55E+06	1.22E-02	1.02E+04		
Ca 48.2	DNA-A	26.85±0.05	3.65	4.46E+03	25.3±0.15	6.92	8.27E+06	5.40E-04	3.78E+02	0.53	A < B
	DNA-B	26.99±0.16	3.85	7.07E+03	25.3±0.15	6.92	8.27E+06	8.55E-04	7.12E+02		
Ca 50.2	DNA-A	27.37±0.83	3.49	3.49E+03	25.28±0.22	6.92	8.39E+06	4.16E-04	2.91E+02	1.41	A > B
	DNA-B	28.9±0.34	3.31	2.08E+03	25.28±0.22	6.92	8.39E+06	2.47E-04	2.06E+02		
Ca 52.2	DNA-A	30.21±0.11	2.59	3.91E+02	25.26±0.22	6.93	8.51E+06	4.59E-05	3.21E+01	0.57	A < B
	DNA-B	30.87±0.08	2.76	5.73E+02	25.26±0.22	6.93	8.51E+06	6.74E-05	5.61E+01		
Ca 88.2	DNA-A	35.55±0.19	0.91	8.22E+00	25.86±0.14	6.76	5.76E+06	1.43E-06	1.00	1.00	A = B
(Calibrator)	DNA-B	38.39±1.74	0.65	6.91E+00	25.86±0.14	6.76	5.76E+06	1.20E-06	1.00		

Table 2.10: Relative concentration off ACMV [DRC6] DNA-A and DNA-B in virus infected (symptomatic) and uninfected cassava plants (genotype TMS 30572) as calculated using the relative standard curve method

		ACMV [DRC6]			β- actin gene of cassava						
Test sample TMS 30572_S	**genome component**	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc.** (copies)	**Ct ± Std. Dev.**	**Mean Log Conc.**	**Mean Conc.** (copies)	**Normalized target**	**Fold difference in target with Calibrator**	**A:B**	**A vs. B**
Ca 45	DNA-A	17.23±0.11	6.68	4.75E+06	25.19±0.08	6.95	8.87E+06	5.35E-01	3.75E+05	1.27	A > B
	DNA-B	17.56±0.1	6.50	3.14E+06	25.19±0.08	6.95	8.87E+06	3.54E-01	2.95E+05		
Ca 47	DNA-A	17.48±0.01	6.60	3.96E+06	25.11±0.1	6.97	9.31E+06	4.25E-01	2.97E+05	0.91	A < B
	DNA-B	17.31±0.01	6.56	3.67E+06	25.11±0.1	6.97	9.31E+06	3.94E-01	3.29E+05		
Ca 49	DNA-A	18.39±0.11	6.31	2.06E+06	25.11±0.05	6.97	9.33E+06	2.20E-01	1.54E+05	0.88	A < B
	DNA-B	18.29±0.05	6.29	1.96E+06	25.11±0.05	6.97	9.33E+06	2.10E-01	1.75E+05		
Ca 51	DNA-A	18.22±0.21	6.37	2.34E+06	25.02±0.14	6.99	9.87E+06	2.37E-01	1.66E+05	0.94	A < B
	DNA-B	18.19±0.2	6.32	2.09E+06	25.02±0.14	6.99	9.87E+06	2.12E-01	1.76E+05		
Ca 53	DNA-A	20.47±0.01	5.66	4.56E+05	25.11±0.17	6.97	9.37E+06	4.86E-02	3.40E+04	1.09	A > B
	DNA-B	20.94±0.05	5.55	3.51E+05	25.11±0.17	6.97	9.37E+06	3.75E-02	3.12E+04		
Ca 45.2	DNA-A	18.39±0.02	6.31	2.05E+06	25.5±0.23	6.86	7.33E+06	2.80E-01	1.96E+05	1.12	A > B
	DNA-B	18.59±0.43	6.18	1.53E+06	25.5±0.23	6.86	7.33E+06	2.09E-01	1.74E+05		
Ca 47.2	DNA-A	16.5±0.12	6.90	8.05E+06	25.48±0.03	6.86	7.33E+06	1.10E+00	7.69E+05	1.24	A > B
	DNA-B	16.7±0.06	6.74	5.46E+06	25.48±0.03	6.86	7.33E+06	7.45E-01	6.21E+05		
Ca 49.2	DNA-A	16.47±0.02	6.91	8.22E+06	25.49±0.17	6.86	7.33E+06	1.12E+00	7.86E+05	1.31	A > B
	DNA-B	16.76±0.04	6.72	5.26E+06	25.49±0.17	6.86	7.33E+06	7.18E-01	5.99E+05		
Ca 51.2	DNA-A	16.65±0.08	6.86	7.22E+06	25.41±0.06	6.88	7.67E+06	9.42E-01	6.59E+05	0.68	A < B
	DNA-B	15.95±0.15	6.95	8.91E+06	25.41±0.06	6.88	7.67E+06	1.16E+00	9.68E+05		
Ca 53.2	DNA-A	16.57±0.04	6.88	7.65E+06	25.37±0.16	6.90	7.91E+06	9.67E-01	6.77E+05	2.04	A > B
	DNA-B	17.55±0.04	6.50	3.15E+06	25.37±0.16	6.90	7.91E+06	3.98E-01	3.32E+05		
Ca 88.2	DNA-A	35.55±0.19	0.91	8.22E+00	25.86±0.14	6.76	5.76E+06	1.43E-06	1.00	1.00	A = B
(Calibrator)	DNA-B	38.39±1.74	0.65	6.91E+00	25.86±0.14	6.76	5.76E+06	1.20E-06	1.00		

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Saadia Naseem

Curriculum vitae

Saadia Naseem Ali

Personal details

Date of Birth	31-05-1980
Sex	Female
Marital status	Married
Nationality	Pakistani
E-mail	saadia1@gmail.com
Present address	National Institute for Genomics & Advanced Biotechnology (NIGAB) Park road, NARC-Islamabad, 44000 Pakistan

Educational Qualifications

2004, **M. Phil**.(Biotechnology). National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad /Quaid-i-Azam University-Islamabad, Pakistan

Thesis Title: Molecular studies of genetic instability in *Cicer arietinum* (Chickpea) root nodule isolates.

2001, **M. Sc**. (Biochemistry) University of Agriculture, Faisalabad, Pakistan

Thesis Title: Studies on the fate of Isoproturon applied to control *Phalaris minor* in *Triticum aestivum (wheat)*.

1999, **B.Sc**. (Botony, Zoology, chemistry) University of the Punjab-Lahore, Pakistan

Honors

- DAAD (German academic exchange service) PhD fellowship (2006-to-2010)
- Participant of 55th Meeting of the Nobel Laureates- 2005. Lindau, Germany
- First position in Faculty of Sciences, M.Sc Biochemistry-2001

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