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BIOTECHNOLOGICAL APPROACHES TOWARDS THE GENETIC AND PHYTOSANITARY IMPROVEMENT OF *EUPHORBIACEAE*

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ABSTRACT

The family *Euphorbiaceae* is of significant importance as it comprises economically valuable crop species such as: cassava, which is mainly used as a staple food and serves for food security of millions in Asia and Africa, and *Jatropha* which serves mainly as a bio-fuel and pharmaceutical crop, with possibility of reclaiming marginal soils, reducing risks of erosion and desertification. *Jatropha* and cassava face challenges of both biotic and abiotic stresses. Furthermore *Jatropha* plant requires more genetic improvement to increase yields and reduce the level of toxins to make it more suitable for biodiesel production and allow for use as a feed stock. Biotechnological approaches using different molecular tools are employed to face these challenges and have opened new avenues for research to study different strategies on their improvement towards achieving better adaptation and quality. In this work, different strategies which may contribute towards the improvement of *Euphorbiaceae* were employed: 1) The investigation of genetic variation in *Jatropha* through ISSR and Ecotilling shed more light on the nucleotide polymorphism and provide clues to adaptive processes and populations history 2) The use of different molecular detection techniques led to the identification of viruses infecting *Jatropha* and cassava. The approaches used included ELISA, rolling circle amplification, PCR, and sequencing. *Jatropha* samples analyzed using ELISA did not contain any of the three RNA viruses tested: *Cassava brown streak virus*, *Cucumber mosaic virus* (CMV) and *Cassava common mosaic virus* (CsCMV). Also the cassava samples were negative for CMV and CsCMV. Only three cassava samples had CBSV. Improved diagnostic approaches allowed the detection of pathogens in *Jatropha* and sequencing of the entire DNA A molecules of 40 Kenyan isolates belonging to *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus-Uganda* (EACMV-UG). This is the first report of the occurrence of ACMV and EACMV-UG on *Jatropha*. New PCR primers were designed of which those amplifying longer sequences led to a phylogenetic tree of isolates used to predict the evolutionary aspects of begomoviruses in *Jatropha*. Primers amplifying shorter sequences were a reliable diagnostic tool that allowed for *Geminivirus* detection from symptomatic and asymptomatic samples including *Jatropha* samples from Ethiopia. In addition, for the first time sequencing and characterization of a naturally occurring defective form of ACMV DNA-A, which at 1420 bp is around half the expected full size, was detected in *Jatropha*. 3) The understanding of the complex plant-virus interaction at the molecular level opens new avenues to better understanding of the mechanisms of plant defense or virus pathogenesis. The *in silico* approach was applied to identify miRNA encoded by ACMV and EACMV-UG. Furthermore, plant miRNA with ability to bind ACMV and EACMV-UG DNA-A genomes were predicted. The method led to the identification of virus miRNA encoded in ORFs including the AC2 and AC4 which are suppressors of RNA silencing and pathogenesis related proteins. Mapping of their targets on the *Jatropha* and cassava ESTs showed that the target pathways have molecular functions in binding, catalytic activity, nucleic acid binding transcription factor activity, electron carrier activity, enzyme regulatory activity, structural molecule activity, transporter activity and nutrient reservoir activity. Some of these targets were found to be pathways involved in plant defense. In addition, several plant miRNAs were identified that could target the ORFs of both ACMV and EACMV-UG viruses. These miRNAs represent potential plant miRNA mediating antiviral defense. Multiple different miRNAs could target single ORFs showing that the plant employs a cooperative regulation mode which could enhance defense. The outcomes of this research may lead to the improvement of *Euphorbiaceae* health to a reduction of losses caused by viruses.

CHAPTER I: Introduction

1.1 *Euphorbiaceae*

Euphorbiaceae is one of the largest and genetically diverse plant family with plants ranging from large woody trees to simple weeds (Mwine and Van Damme, 2011). The family has nearly 322 genera and 8910 species (Rajesh et al., 2009). Among the species are plants having economic impacts on world economies, including *Ricinus communis* (castor bean), *Hevea brasiliensis* (rubber tree), *Manihot esculentum* (cassava) and *Jatropha curcas* (Rajesh et al., 2009; Mwine and Van Damme, 2011).

Jatropha or physic nut is a drought resistant shrub, found abundantly in many tropical and subtropical regions throughout Africa and Asia (Openshaw, 2009). The center of origin of *Jatropha* is in tropical South America contrary to Central America, as is generally suggested (Carels, 2009). *Jatropha* is grown for harvesting the unique oil contained in its seeds, which can be used for the production of biofuel (Vollmann and Laimer, 2013; Maghuly et al., 2013). The crop has the ability to grow under rainfall regimes ranging 200 mm to over 1,500 mm in semi-arid, arid, and tropical humid conditions (Makkar et al., 2009). It has the capacity of reclaiming marginal soils by exploring the soil with an adequate root system which results in recycling nutrients from deep soils, providing shadow to the soil and thereby reducing risks of erosion and desertification (Openshaw, 2000; Dagar et al., 2006). It grows up to a height of 3 to 5 meters and its average life with effective yield is about 50 years (Singh et al., 2007; Makkar et al., 2009).

Jatropha was exported in the 18 th century to Cabo Verde and Guinea Bissau in West Africa, from where it was dispersed to other countries of Africa and Asia (Valdes-Rodriguez et al. 2013). All the provenances exported were reported to be toxic while the non toxic provenances are only found in Mexico (Valdes-Rodriguez et al. 2013). In Kenya the crop was introduced by Portuguese but there are no records on the source of origin of the material (Machua et al. 2011). However, it was suggested that the germplasm in Kenya originates from different countries of Africa and Asia with entry routes from Sudan, Ethiopia, Egypt and India (Machua et al., 2011). Close to 3,860 acres of Kenyan land has been covered by *Jatropha* plantations (Muok and Källbäck 2008). The plant also became naturalized in bush lands and along rivers in Central, Western, and Coastal regions of the country (Tomomatsu and Swallow 2007). The introduction of *Jatropha* in Kenya was meant to alleviate poverty and to offer the farmers a new and sustainable cash crop in order to increase household income. In addition, a substantial part of the energy in Kenya is generator based and *Jatropha* oil

represents a suitable bio-fuel. *Jatropha* oil is also a valid substitute for kerosene for cooking and lighting. There are thus uses from the level of households to the national power supply (Muok and Källbäck 2008). The crop is also intended to create employment and ensure energy security in the long run (Mogaka et al. 2010). Kenya aims at using Straight *Jatropha* oil (SJO) to produce biodiesel as a substitute for conventional diesel and other fossil fuel sources by the year 2020 (Mogaka et al. 2010).

Due to the toxicity of its leaves, *Jatropha* is not browsed and therefore used traditionally in protecting hedges. The seeds contain semi dry oil, an efficient substitute for diesel fuel. The oil yield from kernel is estimated between 46 and 58% derived from semi-dry oil (Kalimuthu et al., 2007).The predominant fatty acids in *Jatropha* oil consist of monounsaturated (44.9%), polyunsaturated (33.4%) and saturated fatty acids (21.6%) (Devappa et al., 2010). The major fatty acids in *Jatropha* oil are oleic (44%), linoleic (33.3%), palmitic (14.7%) and stearic (6.7%) acids (Devappa et al., 2010).

Jatropha is one of the most valuable crude drugs of primitive times and is still widely used in medicine (Soomro and Memon, 2007). Medically it is used as a remedy for alopecia, burns, eczema, inflammation, paralysis and yellow fever. In addition, the oil is also used for burning and spinning in the manufacture of hard soaps, candles, paints and lubricants (Roy, 1990) The seed cake is nutrient rich and therefore suitable as fertilizer. The byproducts of *J. curcas,* such as fruit coats, seed hulls and fruit pulp can be used for the production of bio- gas by anaerobic fermentation (Jongschaap et al., 2007). In addition, the seed cake remaining after oil extraction can serve as a highly nutritious and economic protein supplement in animal feed, if toxins are removed (Becker and Makkar, 1998). Various antinutritional factors are present in the kernel meal of *Jatropha* which include: trypsin inhibitors, phytate, saponins, lectins and phorbol esters (Martinez-Herrera et al., 2010). Makkar and Becker (2011) obtained a patent (Pub No. US2011/0311710 A1) for detoxification of *Jatropha* meal for feeding farm animals, inventing a method resulting in the removal of phorbol esters to a undetectable level by using alkali (sodium hydroxide) during liquid extraction and a further wash using short chain alcohols (methanol). Other attempts to detoxify phorbol esters from *Jatropha* meal showed that phorbol esters could be reduced to a tolerable level of 0.09 mg/g, when the meal was heated at 121°C for 30 min (with 66% moisture) and washed 4 times with 92% methanol (Aregheore et al., 2003). The meal derived from the treatment had a crude protein content of 68% which was far higher than the CP content of most oil seed meals e.g soybean (45.7%) (Aregheore et al., 2003). Trypsin inhibitors were successfully inactivated when defatted meal was extracted with 90% ethanol for 2 hours at room temperature (Martinez-Herrera et al.,

2006). A double solvent extraction (hexane/ethanol system) coupled with moist-heat treatment have been evaluated to detoxify phorbol esters, lectins and tyrpsin inhibitors (Chivandi et al., 2004). In the procedure, minced kernels were soaked in hexane for 8 h. This was followed by a subsequent soaking in 95% ethanol for 6 h and 3 cycles of ethanol extraction at 35°C each of 45 min. After ethanol extraction a heat treatment at 90°C was performed (Chivandi et al., 2004). The results showed that lectins and trypsin inhibitors were inactivated completely while a high concentration of residual phorbol esters (1.90 mg g^{-1}) was observed in the resultant meal (Chivandi et al., 2004)

Cassava (*Manihot esculenta*) is the only member of the *Euphorbiaceae* that is cultivated for food as tropical crop (Fauquet and Fargette, 1990). Although the exact location of the first domestication of cassava is not known, current consensus based on botanical, genetic and archeological evidence supports the South American origin of cassava, pointing to the southern border of the Amazon region as the center of cassava domestication (Gibbons, 1990; Olsen and Schaal, 1999; Olsen and Schaal, 2001). It was first brought to West Africa in the form of flour by Portuguese traders and first cultivated in 1558 in the Congo basin (Carter et al., 1995). Multiple introductions of cassava took place through the Portuguese trading stations in Guinea, Sierra Leone, Angolan Coast and Congo River. It is not clear how the crop moved into East Africa, although it is speculated to have been through Portuguese trading stations at Mozambique, Sofala, Zanzibar, Pemba and Mombasa in Kenya (Carter et al., 1995).

Cassava is suited to warm humid lowland tropics and can be cultivated in most areas where the mean annual temperature exceeds 20 °C with annual rainfall that varies between 500mm and 800mm (Puonti-Kaerlas, 1998). It is a major factor in food security across Sub-Saharan Africa and is consumed both in fresh or processed form. It is the fourth most important source of carbohydrates for human consumption in the tropics, after rice, sugar, and maize (Bellotti et al., 1999). Furthermore, the crops ability to thrive on degraded soils, tolerate drought and produce acceptable yield makes it of economic importance in tropical agriculture where it is popularly cultivated by small scale farmers for subsistence (Thresh and Cooter, 2005). In addition, since cassava has indeterminate and perennial growth patterns, tubers could be left in the soil for several months until needed for utilization (Thresh and Cooter, 2005). Cassava is also a cash crop, feed crop and can be used as raw material for industrial uses such as starch and alcohol (Wright, 1996; Were et al., 2004).

1.2 Genetic diversity in *Euphorbiaceae*

Genetic diversity determines the ability of individuals within a species to adapt to different environments and provides a populations history in a species evolution (Gilchrist et al., 2006; Esfahani et al., 2009).

The level of genetic diversity and differentiation of *Jatropha* is partly attributed to the mode of introduction of *Jatropha* in many countries as an exotic species (Achten et al., 2010). Due to the ability of *Jatropha* to successfuly grow and occupy large areas in a short period, it is possible that African and Asian populations result from a narrow germplasm origin. This results in a complex genetic history and might contribute to potential genetic bottlenecks (Achten et al., 2010). From the time cassava was introduced to Africa from its center of origin, selection for adaptation to African conditions has contributed to considerable diversity within the African cassava accessions (Beeching et al., 1993).

Genetic diversity is the basis for crop improvement, making it important to identify genetically distinct plants for breeding purposes (Brummer et al., 1995). Identification of genetic diversity based on morphological characteristics requires much time and is limited by the fact that morphological differences may be epigenetic or genetic based characters (Astarini et al., 2004; Keivani et al., 2010). With time, the approaches for detecting and assessing genetic diversity have extended from analysis of discrete morphological traits to biochemical and molecular traits (Muthusamy et al., 2008). The development of molecular markers (Jinek and Doudna, 2009) provides new dimensions, accuracy and perfection to the screening of germplasm (Tar'an et al., 2005; Keivani et al., 2010). Molecular markers are useful for the assessment of genetic diversity and relatedness between or within populations and species (Weising et al., 2005). Molecular markers have advantages over conventional approaches based on phenotype as they are stable and detectable in all tissues under all growth conditions, differentiation, development, or defense status. Additionally, they are not confounded by environmental, pleiotropic and epistatic effects (Mondini et al., 2009). The discovery of the polymerase chain reaction (PCR) (Mullis, 1990) favored the development of different DNA marker systems such as random amplification of polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), expressed sequence tag (EST)-SSR (EST-SSR), Sequence-characterized amplified region (SCAR) and single nucleotide polymorphism (SNP) (Vos et al., 1995; Basha and Sujatha, 2007; Arif et al., 2010; Ikbal et al., 2010; Gupta et al., 2012).

1.2.1 Genetic diversity in castor bean

Knowing the sequence of an entire plant genome allows to develop markers across the genome which will facilitate the characterization of genotypes (Prochnik et al., 2012). The current published castor bean genome is based on a 4.6x genome coverage from Solexa sequencing. The estimation of the whole genome size is approximately 320 megabases, organized in 10 chromosomes (Chan et al., 2010).

Genes involved in the biosynthesis of fatty acids and triacylglycerols, corresponding mainly to ricinoleic acid and triricinolein, were identified in the draft genome sequences of castor bean (Chan et al., 2010). In addition, 121 potential disease-resistance related proteins were identified which will contribute to understanding and improving biotic stress resistance in members of the *Euphorbiaceae* (Chan et al., 2010). This genome sequencing allowed evaluating the genetic diversity of castor bean accessions using SNP markers. In the study, a total of 676 samples were analysed using SNPs, which revealed a low level of genetic diversity and minimal geographic structuring of populations worldwide due to mixing of genotypes (Foster et al., 2010).

Furthermore, the chloroplast genome has been sequenced, from which sequence data of genes that encode for structural and functional components of the organelle were assembled (Rivarola et al., 2011). This data was used to generate SNP markers to analyze the genetic diversity of castor bean from 5 geographical locations (Ethiopia, India, U.S. Virgin Islands, Puerto Rico, El Salvador, Greece, and Mexico) which revealed low levels of genetic diversity (Rivarola et al., 2011).

Breeding work has attempted to develop castor bean lines with reduced levels of the toxin ricin, *Ricinus communis* agglutinin (RCA_{120}) toxins and dwarf-internode growth habit (Pinkerton et al., 1999; Auld et al., 2001; Auld et al., 2003). Texas Tech University developed and released in 2002 an open-pollinated germplasm population of castor bean, TTU-LRC (Reg. no. GP-3, PI 631156) (Auld et al., 2003). The eight F6 parental lines of TTU-LRC have reduced levels ricin and RCA_{120}, as well as dwarf-internode growth habit. necessary for mechanized harvest (Auld et al., 2003). In addition, through induced mutations and subsequent selection and identification of pistillate variants, the longevity in maturity time of castor bean has been reduced from being a perennial to a high yielding annual domesticated crop (Singh, 1976; Sujatha et al., 2008; Divakara et al., 2009).

Allan et al. (2008) used AFLP markers to assess the genetic diversity of 200 samples from 41 castor bean accessions collected from 35 countries.in five continents. The results showed a low level of genetic diversity (H_E = 0.126). Castor bean EST sequences exhibit high

frequency of SSR sites (Qiu et al., 2010). Qiu et al. (2010) used castor bean EST sequences to design EST-SSR markers and used them to characterize 24 castor bean samples from different countries, which showed a moderate level of genetic diversity (HE = 0.41).

1.2.2 Genetic diversity in *Hevea brasiliensis*

A draft genome of the rubber tree exists and comparison of it with other 17 sequenced plant genomes using 144 single copy orthologous shows that rubber tree shares the closest ancestry with the other *Euphorbiaceous* species, cassava (Rahman et al., 2013). Although the current genome assembly covers half the estimated genome size of the rubber tree (1.1 GB assembled from a 2.15 GB genome), it captures majority of the rubber genome gene space. Approximately 70,000 putative genes have been annotated based on protein alignments from closely related species, RNA-seq, and *de novo* gene prediction software packages. The genome of rubber tree is made up of 18 chromosomes (Rahman et al., 2013).

Furthermore, a chloroplast genome sequence of the rubber tree exists and it is about 161,191 bp in length. (Tangphatsornruang et al., 2011). In addition, a total, 22,756 unigenes with an average length of 485 bp have been obtained from the transcriptome with 39,257 EST-SSRs identified as potential marker sites to be used in accelerating research progress in molecular biology of rubber tree (Li et al., 2012).

Wind damage is a problem in rubber tree growing countries which results in losses of a high number of rubber trees in plantations (Venkatachalam et al., 2007). The incorporation of a dwarf trait into high yielding rubber clones would be useful for generating a high yielding tree with a desirable architecture (dwarf stature) that will further allow for high density planting (Venkatachalam et al., 2007). Venkatachalam et al. (2004) identified a dwarf genome specific RAPD marker which can be used in rubber improvement. The primer OPB-12 generated a DNA marker of 1.4 kb from both natural and controlled F1 hybrid progenies of dwarf stature, originating from a cross of a dwarf parent and a normal cultivated clone.

Gouvea et al. (2010) analysed the genetic diversity of 60 rubber tree genotypes from Asia, Amazon, Africa and IAC clones (Agronomic Institute (IAC), Brazil). From 80 characterized SSRs, 68 were polymorphic and informative. A high diversity ($H_{T'} = 0.58$) and high gene differentiation ($G_{st'} = 0.61$) were observed among the 60 genotypes, indicateing a high genetic variation which may be useful for breeding purposes (Gouvea et al., 2010).

1.2.3 Genetic diversity in cassava

RAPD is based on amplification of genomic DNA with single primers of arbitrary nucleotide sequence to detect polymorphisms in the absence of specific nucleotide sequence information (Williams et al., 1990). RAPD has been applied to distingusih bitter cassava varieties from

sweet ones. Cassava, varieties with high hydrogen cyanide (HCN) concentrations are referred to as bitter and are mainly consumed as flour, starch or glucose. Accessions with low HCN levels (less than 100 ppm in fresh roots), known as sweet cassava, can be consumed cooked or processed (Vieira et al., 2011). Colombo et al. (1998) used RAPD markers to investigate the genetic diversity of 31 Brazilian cassava accessions from 4 genotypes collected from different locations. Varieties cultivated in large scale for flour production proved to be related as they grouped together and separated from those destined to" *in natura*" consumption

AFLP is a fingerprinting technique based on selective PCR amplification of restriction fragments from a total digest of genomic DNA (Weising et al., 2005; Mondini et al., 2009). AFLP based markers are reproducible and exhibit high levels of polymorphisms (Vos et al., 1995). Presence or absence of polymorphisms between two or more genotypes results from: (1) gaining or losing a site of restriction (2) insertion, deletions or reversions within an amplified fragment and (3) mutations of sequences flanking the restriction site (Weising et al., 2005; Mondini et al., 2009).

Elias et al. (2000) used AFLP to estimate the genetic variability of cassava accessions grown by farmers in Guyana and wild accessions, which revealed a high level of variability. These local varieties are an important source of genetic diversity and the interaction between human and ecological factors could have facilitated the dynamics of this diversity (Elias et al., 2000). Second et al. (1997) analyzed accessions from South America and observed introgression into cassava from *M. glaziovii*. The diversity of cassava itself was high, but the diversity was narrow in a single Amazonian field (Second et al., 1997). Although domestication appeared to have evolved primarily from *M. esculenta* ssp. *flabellifolia* and *peruviana*, it seemed that some other species also contributed (Second et al., 1997).

SSR or microsatellites are polymorphic loci present in DNA that consist of repeated units of two to six base pairs in length (Arif et al., 2010). Microsatellites developed for particular species can often be applied to closely related species, but the percentage of loci that will be successfully amplified may decrease with increasing genetic distance (Jarne and Lagoda, 1996).

In cassava, SSR markers have been useful in illustrating the role played by farming practice in maintaining and/or adding to cassava genetic diversity. Montero-Rojas et al. (2011) developed 33 SSR markers to evaluate genetic diversity of 23 accessions of Puerto Rico and 162 samples with unknown genetic background collected from different townships of the Island. The genetic diversity (H_E) of the unknown cassava samples (0.7174) was higher than in cassava samples originating from Puerto Rico (0.6996). Farmers traditional practices like

intercropping and incorporation of volunteer seedlings contributes in recombination of genotypes to the cultivated stocks. Furthermore it allows for selection and adaptation in cassava which result to high levels of genetic diversity (Montero-Rojas et al., 2011).

Fregene et al. (2003) in their study of 283 cassava accessions from different countries using SSRs, attributed the high genetic diversity found to agricultural practices of 'slash and burn' by Amerindians farmers. Due to the preferential out-crossing nature of cassava a large number of volunteer seedlings surviving the 'slash and burn' practice, can germinate in the field (Fregene et al., 2003). Natural and artificial selection, acts on these seedlings leading to new accessions of cassava in the field (Fregene et al., 2003; Montero-Rojas et al., 2011). Furthermore it is possible that whilst selecting, utilizing and distributing landraces with their preferred agronomic and quality traits, farmers have inadvertently added useful cassava mosaic disease (CMD) resistant accessions to the germplasm available to them (Lokko et al., 2006). Using 18 SSR primers, Lokko et al. (2006) detected the genetic difference of CMD resistant and susceptible landraces that had prior been accessed based on diseases scores. The CMD resistant accessions clustered into distinct groups, suggesting the presence of alternative sources of resistance to CMD other than from *Manihot glaziovii* (Lokko et al., 2006).

EST-SSRs are SSR markers developed from EST sequences and are transferable across closely related species and their potential as functional anchor markers in defining genes that affect traits of interest (Zou et al., 2011; Ukoskit et al., 2012). Consequently, they are useful as markers for identifying conserved genomic regions among species and genera, evolutionary studies and comparative genomics (Zou et al., 2011; Ukoskit et al., 2012).

In cassava, the existing ESTs reveal a high degree of genetic diversity between cassava genotypes which is partly attributed to the allotetraploid nature of cassava and because the domestication of this crop has not been quite intense (Anderson et al., 2004). Generating additional cassava ESTs may give more insight to the distribution and divergence of orthologous genes and their allelic diversity, knowledge which will serves as a bench mark for mapping and breeding based on the SNP diversity that already exists (Anderson et al., 2004; Zou et al. 2011).

The mapping of two CMD resistance genes *CMD1* (recessive) and *CMD2* (dominant) on the cassava genetic linkage map (Fregene et al., 2001; Akano et al., 2002) contributes to marker assisted breeding in cassava (Bi et al., 2010). In addition, the identification of a SCAR marker (RME1) and SSR markers (SSRY28 and NS158) associated with *CMD2* has helped the fast tracking of CMD resistant germplasm (Bi et al., 2010). Bi et al. (2010) agro-inoculated 18 cassava cultivars collected from China, Thailand and other Asean countries with infectious

clones of ACMV-N*Og* and used the markers RME1, SSRY28 and/or NS158 to evaluate their levels of resistance. Only 3 cultivars showed resistance-associated bands. This led to a conclusion that African cassava germplasms that harbor CMD resistance genes such as *CMD2* can assist in breeding for CMD resistance (Bi et al., 2010).

SNPs or insertions / deletions (INDELS) manifest genetic diversity present in a plant species (Gilchrist et al., 2006). This variation in the nucleotide sequence is a determinant of heritable phenotypic difference that can be exploited for crop improvement (Till et al., 2007; Simsek and Kacar, 2010). The roots of commercial cassava cultivars contain a limited amount of provitamin A carotenoids. However conventional breeding and genetic modification to increase the level of provitamin A carotenoids in cassava have been attempted (Rojas et al., 2009; Welsch et al., 2010). For conventional breeding, the heterozygous nature of the crop renders varietal recovery difficult, and long breeding cycles slows down the progress of this endeavor (Rojas et al., 2009). Advancement has shown that an SNP present only in yellow-rooted cultivars co segregates with colored roots in a breeding pedigree. This newly characterized phytoene synthase allele will further provide means to improve cassava provitamin A content (Welsch et al., 2010).

At present, a draft genome sequence of cassava has been generated using a 454 based whole genome shotgun strategy (Prochnik et al., 2012). A total of 22.4 billion bp of raw sequence data was generated which were assembled into 12,977 scaffolds spanning a total of 532.5 Mb. The genome sequence covers 69% of the predicted whole genome size (770 Mb) and 96% of protein-coding gene space (Prochnik et al., 2012). However, known SSR and SNP markers are sparsely distributed across the cassava genome which may make it not ideal for fine-mapping (Prochnik et al., 2012).

1.2.4 Genetic diversity in *Jatropha*

Ikbal Boora and Dhillon (2010) used RAPD markers to detect for genetic diversity in 40 *Jatropha* accessions from different eco-geographical regions of India. Out of 50 primers used 44 polymorphic primers yielded 328 bands of which 308 (93.90%) were polymorphic showing a broad genetic base. In contrast Bash and Sujatha (2007) showed that the level of genetic diversity is low in *Jatropha* from India when analyzing 42 accessions using 400 RAPD primers and finding molecular polymorphism was 42.0%. Accessions from different locations in China and Malaysia showed high level of genetic diversity when characterized by RAPD (Chen et al., 2011, Rafii et al., 2012). A narrow level of genetic diversity was reported in 192 accessions from different regions of Brazil characterized by RAPD and SSR (Rosado et al., 2010). Characterization of accessions from Africa using RAPD showed a narrow

genetic base in 40 accessions from Ghana with 10 primers showing a polymorphism of 24.9% (Owusu Danquahl et al., 2012). However a broad genetic diversity was obtained in 160 accessions collected from 8 locations in Kenya, which will be useful for breeding and genetic improvement programmes (Machua et al., 2011). This level of genetic diversity may be explained by the modes of germplasm introductions from different genetic backgrounds, which suggest that *Jatropha* in Kenya could have originated from countries of Asia and Africa (Machua et al., 2011).

ISSR are valuable for effectively detecting low levels of genetic variation (Sica et al., 2005). It involves the amplification of DNA segments present in between two identical microsatellite repeat regions oriented in opposite direction (Pradeep Reddy et al., 2002; Gradzielewska et al., 2012).

The presence of toxic phorbol esters in *Jatropha* is a major concern and the identification of varieties which are non-toxic is important. During mechanical oil extraction from seeds, a high percentage (70-75%) of phorbol esters are extracted along with the oil, while the rest are still retained in the pressed cake, making both cake and oil non edible (Makkar et al., 2009; Devappa et al., 2012). High concentrations of phorbol esters (4.05 mg/g) has been reported in toxic genotypes from Chiapa de Corzo, while phorbol esters were absent in seven samples from non toxic genotypes from Veracruz, Puebla and Morelos (Martinez-Herrera et al., 2010). ISSR markers have been used to distinguish non-toxic *Jatropha* accessions of Mexican origin from those which are toxic (Basha and Sujatha, 2007). Analysis of 42 toxic accessions from India and one non-toxic accession from Mexico showed a moderate polymorphism of 33.5%. Furthermore 12 ISSR were specific to the non-toxic Mexican genotype and distinguished it from the Indian accessions (Basha and Sujatha, 2007). ISSR analysis of 224 accessions from different regions of South China and Mayanmar showed high levels of genetic diversity, suggesting that *Jatropha* in these regions could have been introduced from different places (Cai et al., 2010). In addition, Maghuly et al. (2011) analysed *Jatropha* accessions from different countries using ISSR markers and the results showed variations not only between individuals but also between different regions. This is part of the ongoing research at the Plant Biothechnology unit of BOKU University, which involves the analysis of *Jatropha* accession grown and maintained both *in vivo* and *in vitro*.

A major aim in the genetic improvement of *Jatropha* is the development of high yielding varieties both in terms of seed yield and oil content. To contribute to this process of yield improvement, identification of plants with vegetative or floral traits linked to productivity is vital. AFLP markers showed that *Jatropha* accessions from Chiapas, Mexico, have a high

level of polymorphism with the analysis of the frequency and distribution of polymorphic fragments showing the highest number of rare fragments in one single accession. This accession was found to exhibit traits of agronomic importance (such as the presence of 100% pistillated flowers). In addition divergent collections were detected from regions where high average oil content and other characters associated with productivity have been found (Ovando-Medina et al., 2011; Pecina-Quintero et al., 2011). Furthermore, AFLP has been used to show that the MesoAmerican region could be the center of origin of *Jatropha* (Ovando-Medina et al., 2011). The species in this region have a higher diversity than in other parts of the world, with polymorphic rates at an average of 81.1%.

Analysis of 48 *Jatropha* accessions from 6 different states of India (Uttar Pradesh, Gujarat, Rajasthan, Madhyapradesh, Chhattisgahr and Andhra Pradesh) using AFLP showed a broad genetic base (Tatikonda et al., 2009). With seven primers 770 fragments were generated, of which 680 (88%) were polymorphic, 59 were unique (accession specific) and 108 were rare and present in less than 10% of the accessions. The majority of the accessions from the regions have been shown to have a high oil content (Tatikonda et al., 2009).

Tanya et al. (2011) developed 49 microsatellites markers for characterizing 26 Mexican, 3 Chinese, 3 Thai and 4 Vietnamese *Jatropha* accessions. Eight of the primers amplified bands which were polymorphic while 5 markers were able to distinguish between 26 non-toxic Mexican from 10 toxic Asian accessions (Tanya et al., 2011). Through sequencing the *Jatropha* genome, Sato et al. (2011) discovered 100 microsatellites and used them to determine the genetic diversity of 12 *Jatropha* lines obtained from Thailand, Indonesia, Madagascar, Mexico, Guatemala, Tanzania, Cape Verde and Uganda. The lines from Guatemala and Mexico were found to be genetically distinct from the other lines (Sato et al., 2010).

The total length of the currently published *Jatropha* genome obtained by sequencing using a combination of Sanger method and new-generation multiplex methods is 285 858 490 bp. It is made up of 120 586 contigs (276 710 623 bp total) and 29 831 singlets (9 147 867 bp total) covering 70% of the estimated whole genome size of approximately 410 megabases (Sato et al., 2010). A total of 40 929 complete and partial structures of protein encoding genes have been annotated and their comparison with genes of other plant species show that 4% of the genes are specific to the *Euphorbiaceae* family (Sato et al., 2010).

EST-SSRs have been applied to investigate genetic diversity of *Jatropha* and have helped to identify collections that can be used for *Jatropha* improvement (Wen et al., 2010; Xu et al., 2012). Wen et al. (2010) designed 36 EST-SSRs and 20 genomic SSRs based on cassava

sequences and applied them to investigate genetic diversity in 45 *Jatropha* accessions. A total of 183 polymorphic alleles were detected, indicating a broad genetic background and led to a conclusion that collections from Yunnan (China) could be used to enrich the genetic background of *Jatropha* for breeding (Wen et al., 2010).

SCAR markers have become useful for distinguishing toxic varieties of *Jatropha* from non-toxic in a mixed population. These markers could be applied in quality control for selective cultivation of non-toxic varieties (Mastan et al., 2012). Basha et al. (2009) developed 10 SCAR markers of which 3 from RAPD were specific to toxic genotypes and 5 RAPD and 2 ISSR were specific to non-toxic genotypes. Three of the markers (RSPJ-1, RSPJ-2 and ISPJ-3) distinguished toxic genotypes from other parts of the world from non-toxic Mexican genotypes. Further biochemical characterization supported the association of the developed SCAR markers with non-toxic traits (Basha et al., 2009.). Bash and Sujatha (2007) converted two polymorphic ISSR markers (ISPJ1 and ISPJ2) to SCAR markers which could differentiate the Indian accessions from the Mexican genotype.

TILLING (targeting induced local lesions in genomes) is a reverse genetic approach that identifies SNPs in a gene of interest from a mutagenized population through treatment with mutagens (Till et al., 2007). Application of TILLING technique to identify natural variation in genes is refered to as Ecotilling. Maghuly et al. (2011, 2013) applied Ecotilling to investigate the genetic diversity of 1300 *Jatropha* accessions. The accessions were collected from 14 different countries from America (Bolivia, Brazil, Mexico and Paraguay), Africa (Cape Verde, Ethiopia, Guinea-Bissau, Kenya, Madagascar, Mali and Senegal) and Asia (China, India and Indonesia). A total of 23 rare SNPs were identified, showing that polymorphism was less frequent between the accessions (Maghuly et al., 2013).

Genotype-by-sequencing technologies might further provide wider genome coverage and thus assay a larger number of sequence polymorphisms (Silva-Junior et al., 2011). Through sequencing of whole-transcriptome, Silva-Junior et al. (2011) discovered 768 SNPs and found low level of sequence polymorphism and highlighted the need to widen the current germplasm base for successful breeding. Gupta et al. (2012) discovered 2,482 SNPs among 148 *Jatropha* collections from India, Africa and America which revealed that the level of diversity was narrow among the Indian genotypes as compared to genotypes from America and Africa.

1.3 Viral diseases of *Jatropha* and cassava

Jatropha curcas and cassava are susceptible to infections by several viruses (Table 1), including: cassava mosaic geminiviruses (CMG) leading to CMD (cassava mosaic disease), *Cassava brown streak virus* (CBSV) leading to CBSD (Cassava brown streak disease), *Cassava common mosaic virus* (CsCMV) causing CsCMD (Cassava common mosaic disease), and *Cucumber mosaic virus* (CMV).

Table 1: Viruses infecting cassava and *Jatropha*

Table 1: Viruses infecting cassava and *Jatropha*						
Host plant	**Virus**	**Genus/Family**	**Symptoms**	**Vector**	**Distribution**	**References**
cassava, *Jatropha*	*African cassava mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	Africa	Stanley and Gay, 1983 Thottappilly et al., 2003 Ramkat et al., 2011
cassava	*East African cassava mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	East Africa	Pita et al., 2001 Thottappilly et al., 2003
cassava	*East African cassava mosaic Cameroon virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	West Africa, Tanzania	Fondong et al., 2000
cassava	*East African cassava mosaic Kenya virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	East Africa	Bull et al., 2006
cassava	*East African cassava mosaic Malawi virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	Malawi	Zhou et al., 1997
cassava	*East African mosaic Zanzibar virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	Zanzibar, Madagascar	Maruthi et al., 2004
cassava, *Jatropha*	*East African cassava mosaic virus-Uganda*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	Africa	Pita et al., 2001 Ramkat et al., 2011
Jatropha	*Jatropha mosaic Nigeria*	*Begomovirus/ Geminiviridae*	Mosaic, leaf blistering and mottling	whitefly	Nigeria	Kashina et al., 2013

	virus					
cassava	*Indian cassava mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	Indian, Sri Lanka	Hong et al., 1993
Jatropha	*Jatropha Mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, blistering on leaf surface	whitefly	Puerto Rico, Jamaica, India	Aswatha Narayana et al., 2007 Gao et al., 2010
cassava	*South African cassava mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	South Africa, Zanzibar, Madagascar, Zimbabwe	Berrie et al., 2001
cassava	*Cassava brown streak virus*	*Ipomovirus/ Potyviridae*	Brown, elongate necrotic stem lesions, secondary and tertiary vein chlorosis, corky brown necrosis in tuberous roots	whitefly	Africa	Monger et al., 2001
cassava	*Uganda Cassava brown streak virus*	*Ipomovirus/ Potyviridae*	Brown, elongate necrotic stem lesions on, secondary and tertiary vein chlorosis, corky brown necrosis in tuberous roots	whitefly	Africa	Winter et al., 2010
cassava	*Cassava Ivorian bacilliform virus*	Unassigned/ *Ourmiavirus*	symptomless	unknown	Cote d´ Ivoire	Thottappilly et al., 2003
cassava	*Cassava virus C*	*Ourmiavirus/ unassigned*	pronounced leaf fleck	unknown	Cote d´ Ivoire	Thottappilly et al., 2003
Jatropha	*Cucumber mosaic virus*	*Cucumovirus/ Bromoviridae*	Mosaic	aphids	India	Raj et al., 2008
cassava	*Sri Lankan cassava mosaic virus*	*Begomovirus/ Geminiviridae*	Mosaic, leaf distortion and stunting	whitefly	India, Sri Lanka and India	Rothenstein et al., 2006
cassava	*Cassava American latent virus*	*Nepovirus/ Comoviridae*	Symptomless	unknown	Brazil and Guyana	Thottappilly et al., 2003
cassava	*Cassava vein mosaic virus*	*Cavemovirus/ Caulmoviridae*	vein mosaic	unknown	Brazil	Thottappilly et al., 2003
cassava	*Cassava*	Tentative	Symptomless	unknown	Columbia	Thottappilly et al., 2003

	Colombian symptomless virus	*Potevirus/ Flexiviridae*				
cassava	*Cassava virus X*	*Potexvirus/ Flexiviridae*	Symptomless	unknown	Columbia	Thottappilly et al., 2003
cassava	*Cassava common mosaic virus*	*Potevirus/ Flexiviridae*	Mild mosaic	unknown	South and North America, Africa and Asia	Kitajima et al., 1965
cassava	*Cassava frogskin-associated virus*	Tentative *Oryzavirus*	Frog skin symptoms in tubers	unknown	South America	Thottappilly et al., 2003
cassava	*Cassava green mottle virus*	*Nepovirus/ Comoviridae*	Local and systemic mottle	unknown	Australasia and Pacific islands, Solomon islands	Thottappilly et al., 2003
cassava	*Cassava symptomless virus*	Unassigned *Nucleorbhabdo virus/Rhabdoviridae*	symptomless	unknown	unknown	Thottappilly et al., 2003

1.3.1 *Cassava mosaic Geminivirus*

Geminiviruses are a group of plant viruses that contain circular single stranded (ss) DNA genomes. They infect a wide range of plant species and are responsible for considerable crop losses (Fauquet et al., 2008). Identified alternative hosts of CMG include a wild relative of cassava *Manihot glaziovii* Müll) (Sserubombwe et al., 2008), *Senna occidentalis, Combretum confertum, Centrosema pubescens, Pueraria javanica* and *Leucana leucocephala* (Alabi et al., 2008; Monde et al., 2010). Based on organization of the genome, insect vector and host range, the family *Geminiviridae* is classified into four genera: (1) *Mastrevirus*, (2) *Curtovirus*, (3) *Topocuvirus* and (4) *Begomovirus*.

Begomoviruses including CMG as causal agents of CMD are transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Harrison, 1985; Varma and Malathi, 2003; Fauquet et al., 2008). CMGs are encapsidated in characteristic twinned (geminate) isometric particles approximately 15-20nm by 25-35nm in size together (Harrison, 1985; Fauquet et al., 2008). The viruses contain protein subunits arranged in an icosahedral array of 22 pentamers of of approximately 30kDa (Bock and Guthrie, 1978). The genome of CMG begomoviruses consists of two components, termed DNA A and DNA B (Lazarowitz et al., 1992; Hanley-Bowdoin et al., 2004). The virus DNA A has six overlapping open reading frames (ORFs) which include the replication associated-protein (Rep/AC1), transcriptional activator protein (TrAP/AC2), replication enhancer protein (REn/AC3), pathogenicity enhancer protein (AC4), coat protein (CP/AV1) and precoat protein (AV2) (Sunter et al., 1990; Paszkowski et al., 1993; Höfer et al., 1997; Voinnet et al., 1999). AC1, AC2, AC3 and AC4 are located on the antisense strand

while the AV1 and AV2 are located on the sense strand. AC1 is required for replication (Paszkowski et al., 1993) while AC2 is required for the transcription – activation of plus strand gene transcription, and suppression of post – transcriptional gene silencing (PTGS) (Voinnet et al., 1999). AC3 is not essential for infection but enhances viral DNA accumulation by several-fold (Sunter et al., 1990). AC4 plays a role in pathogenicity and PTGS (Vanitharani et al., 2004). The AV1 is essential for viral transmission by whiteflies (*Bemisia tabaci*) and is highly conserved amongst the begomoviruses originating from the same geographical region (McGrath and Harrison, 1995; Maruthi et al., 2005). AV2 is involved in virus movement (Hofer et al., 1997). The DNA B encodes 2 ORFs: movement protein (MP, BC1) and nuclear shuttle protein (NSP, BV1) that act co-operatively to move the virus both within and between cells in host plants (Hanley-Bowdoin et al., 2004).

The genomic components DNA A and DNA B of begomoviruses share a high sequence nucleotide (nt) identity of more than 90% in the intergenic region (IR) of approximately 200 nt called the common region (CR) (Harrison and Robinson, 1999; Pita et al., 2001). The CR contains promoter and sequence elements required for DNA replication and transcription, including the invariant TAATAT/AC where the initiation of rolling circle DNA replication takes place (Lazarowitz et al., 1992; Eagle et al., 1994; Chatterji et al., 1999).

In addition to genomic components, smaller sized DNAs referred to as defective DNA (def DNA) often occur naturally in *Geminivirus* infected plants (Patil and Dasgupta, 2006; Ndunguru et al., 2006). Def DNAs are usually half the size of the full length genomic component and may be formed by sequence deletion, duplication, inversion or rearrangement of viral DNA. Insertion of foreign sequences has been reported in some cases (Patil et al., 2007; Stanley et al., 1997). Some of the def DNAs have been shown to interfere with virus proliferation as they are associated with a delay and attenuation of symptoms by competing with the genomic components for cellular resources (Frischmuth and Stanley, 1991; Stanley et al., 1990; Patil and Dasgupta, 2006; Ndunguru et al., 2006).

Members of seven species of *Begomovirus* have been identified in association with CMD in Africa: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) (Berrie et al., 1998; Ndunguru et al., 2005; Bull et al., 2006; Alabi et al., 2008).

These viruses are believed to have evolved from African viruses that existed in alternative hosts and adapted to cassava upon its introduction to Africa (Bull et al., 2006). Initially the

viruses existed in distinct geographical regions (Hong and Harrison, 1995), but more recently their distribution has become more complex (Bull et al., 2006). This has been attributed to cassava being vegetatively propagated, a process that perpetuates the virus and further leads to dissemination of the virus (Chellappan et al., 2004). Trade and human migration caused by drought and conflict can result in the spread of infected planting material over great distances (Alabi et al., 2008). In addition, CMGs have the inherent capacity to recombine between each other, and are thereby constantly evolving to generate new biodiversity (Padidam et al., 1999; Fondong et al., 2000; Pita et al., 2001). This, combined with their ability to act in a synergistic manner makes them highly opportunistic and capable of generating dramatic new epidemics (Zhou et al., 1997). Furthermore, recombination and pseudorecombination occurs during mixed infections in the field giving rise to members of novel virus species with increased virulence and adaptation to new host species (Bull et al., 2007; Patil and Fauquet, 2009). An example is presented by the recombinant virus strain *East African cassava mosaic virus – Uganda* (EACMV-UG), which caused a severe form of CMD in Uganda with a serious reduction in cassava yields (Zhou et al., 1997). EACMV-UG is an aggressive, rapidly spreading virus and has since then been identified from East and West African countries (Ariyo et al., 2005; Bull et al., 2006; Monde et al., 2010; Ramkat et al., 2011). EACMV-UG occurred as a recombination of DNA A between ACMV and EACMV (Zhou et al., 1997). EACMV-UG consists of most of the CP gene of ACMV inserted in an EACMV like-A component (Zhou et al., 1997).

Plants infected with EACMV-UG express more severe symptoms than those infected with ACMV, but plants infected with the two virus together (mixed infection) are even more severely diseased than both of the single infection conditions (Harrison et al., 1997). This suggests the occurrence of a synergistic interaction between the two viruses, which leads to 10-50 fold increase in viral DNA accumulation, substantially increasing the potential for a higher efficiency of vector transmission (Legg and Fauquet, 2004).

Cassava plants infected with CMGs express a range of symptoms which depend on the virus species/strain virulence and abundance, environmental conditions and sensitivity of the host genotype (Fargette and Thresh, 1994). Symptoms consist of yellow or pale green chlorotic mosaic leaves which show distortion and crumpling. The symptoms are readily distinguished from those of mineral deficiency and cassava green mite damage as the virus induced chlorosis and malformation of leaflets is asymmetric about the midrib (Legg and Thresh, 2003). Severely affected plants have stunted growth and greatly diminished tuber root yield (Were et al., 2004). Yield reduction of tuberous roots due to CMGs range from 20 to 95%

depending on variety and stage of growth which infection occurs (Thresh et al., 1994). African continental yield losses are estimated to be in the range of 19 - 27 million metric tonnes annually, corresponding to US $1.5 billion (Pita et al., 2001). This high impact on the economy indicates that CMD is among the most damaging plant virus disease in the world (Legg and Fauquet, 2004).

Symptoms of CMGs on *Jatropha* infected plants include: leaf curling, leaf distortion, blistering on leaf surfaces and reduction of leaf size (Ramkat et al., 2011). ACMV spread in *Jatropha* is facilitated by high population density of whiteflies with a disease incidence value of up to 45% being observed in field plants after 16 weeks of planting (Appiah et al., 2012). The size of the whitefly populations has also been positively correlated with virus spread about one month after invasion, which corresponds with the time required for symptom development to occur (Fauquet and Fargette, 1990).

1.3.2 *Cassava brown streak* disease (CBSD)

CBSV and *Uganda cassava brown streak virus* **(**UCBSV) are two distinct virus species known to cause CBSD (Ogwok et al., 2010; Winter et al., 2010). The brown necrotic streaks that occur on the green portions of the stems of CBSD sensitive varieties give the disease its name (Legg and Thresh, 2003). Both virus species belong to the family *Potyviridae*, genus *Ipomovirus* (Monger et al., 2001; Mbanzibwa et al., 2009a, 2009b; Winter et al., 2010; Mohammed et al., 2012). The CBSV virus particles are sub-microscopic flexuous rods, approximately 650nm in length and show the existence of pinwheel inclusions in the cells of diseased plants (Were et al., 2004; Monger et al., 2001). The genome structure of CBSV and UCBSV are similar but they differ from other ipomoviruses (Monger et al., 2010). The genome is monopartite comprising a positive single stranded RNA (ssRNA), which consists of 9069 nucleotides and predicted to produce a polyprotein of 2902 amino acid (Mbanzibwa et al., 2009b). It lacks a helper component proteinase but contains a single P1 serine proteinase that strongly suppresses RNA silencing and encodes a putative nucleoside triphosphate pyrophosphatase (Maf/HAM1h) protein situated between the viral replicase and the CP (Mbanzibwa et al., 2011). HAM1h is implicated in reduction of mutations of viral RNA (Mbanzibwa et al., 2009a). CBSV and UCBSV have been referred to as coastal and highland endemic viruses respectively (Mohammed et al., 2012). CBSV is present predominantly in the coastal lowland areas up to 1000 meters above sea level of Tanzania and Mozambique, while UCBSV is predominant in highland areas (Lake Victoria Basin, Democratic Republic of Congo, Uganda, Kenya and Malawi) of 1200-1500 meters above sea

level in East Africa (Bock, 1994; Monger et al., 2001; Mahungu et al., 2003; Alicai et al., 2007; Mbanzibwa et al., 2009a).

Symptoms of CBSD vary with growing conditions (temperature, rainfall, and altitude), type of variety, age of the crop and the virus isolate involved in causing the symptoms (Hillocks, 2000). The diseases cause symptoms that are more prominent on lower leaves, appearing as yellow chlorosis associated with the secondary and tertiary veins, or a general blotchy chlorotic mottle. Unlike CMD, there is no leaf distortion and size reduction (Legg and Thresh, 2003). Foliar symptoms are less conspicuous and farmers often are unaware of them, until the crop is harvested and corky, yellow brown necrosis on roots becomes evident (Were et al., 2004). However, some cultivars show marked foliar symptoms but without or delayed root symptoms and vice versa (Mohammed et al., 2012). In addition, some isolates of CBSV may cause more severe symptoms than UCBSV in some varieties of cassava (Winter et al., 2010).

Currently, the whitefly *Bemisia tabaci* is the only experimentally proven vector for CBSV (Maruthi et al., 2005). It is still to be demonstrated if it also transmits UCBSV (Mohammed et al., 2012). Whitefly *Aleurodicus disperses,* whose population density has been directly correlated with CBSD incidence is also a potential vector of these viruses (Mware et al., 2009). The disease is also spread by propagation of infected cuttings (Legg and Thresh, 2003).

1.3.3 *Cassava common mosaic virus*

CsCMV is classified as Potexvirus on the basis of its particle morphology, serology and inclusion bodies. CsCMV virions are 15 nm * 495 nm semiflexous rod (Kitajima et al., 1965). The genome consists of ssRNA of 2 * 10^6 Da and a single coat protein of a molecular mass 2 of 1 kDa (Nolt et al., 1991; Calvert et al., 1996).

The virus infects species belonging to several families of dicotyledonous plants (Kitajima et al., 1965; Zettler and Elliott, 1986). Other than cassava, alternative hosts include *Chenopodium album*, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Datura stramonium*, *Euphorbia heterophylla*, *Euphorbia Lathyrus*, *Gossypium hirsutum*, *Jatropha gossypifolia*, *Jatropha podagrica*, *Nicotiana benthamiana*, and *Ricinus communis* (Zettler and Elliot, 1986). Plants infected with CsCMV present mosaic symptoms and chlorotic areas that are often limited by veins. CsCMD can cause losses in yield of more than 30% (Zettler and Elliot, 1986; Calvert et al., 1996). There are no known vectors of CsCMV and the primary source of inoculum is through infected planting material.

1.3.4 *Cucumber mosaic virus*

CMV is a member of the family *Bromoviridae*, genus *Cucumovirus*. *Bromoviridae* have either isometric particles, 26-35 nm in diameter or bacilliform particles whose symmetry is based upon the icosahedron. The CMV strains are divided in three subgroups (IA, IB and II) based on serology and nucleotide sequence identity (Mochizuki and Ohki, 2012). Cucumoviruses have tripartite genomes that consist of three positive senses ssRNA; RNA1 (3.3 kb), RNA 2 (3.0 kb), RNA 3 (2.2 kb) and the sub-genomic RNAs 4 (1.0 kb) and 4a (0.7 kb) designated in decreasing order of molecular mass (Suzuki et al., 2003; Mochizuki and Ohki, 2012).

CMV infects more than 1200 species of 100 plant families including both monocot and dicot (Mochizuki and Ohki, 2012). These include vegetables, ornamentals, legumes and other important crops (Roossinck et al., 1999). Symptoms caused by CMV infection vary with the host species or strain of CMV, and include stunt, mosaic, chlorosis, dwarfing, leaf malformation and systemic necrosis (Roossinck et al., 1999). CMV is spread by aphid transmission in a non-persistent manner (Mochizuki and Ohki, 2012). CMV is commonly detected by ELISA.

1.4 Host pathogen interactions

Host pathogen interactions are often described as an arms race between measures employed by plants to limit the extent of pathogen infection and disease and counter measures by pathogens to suppress host defense (Eckardt, 2011). Different interactions are generated between the plant and the virus during each stage of the viral cycle. If the viral particle is not recognized by a host plant, compatible interaction between the plant and virus will occur (Hammond-Kosack and Jones, 2000). This interaction may be favourable for the virus (Hammond-Kosack and Jones, 2000; Stange, 2006). However, if the plant recognizes the viral particle, an incompatible interaction that is unfavourable for the virus is established (Stange, 2006).

Plants are capable of counteracting the harmful effects of viruses due to the absence of essential host susceptibility factors (passive resistance) or to the existence of several defense layers that the virus has to overcome (Pallas and Garcia, 2011). As an initial step of defense, the plants use several existing physical and chemical barriers. However if a pathogenic virus succeeds in overcoming this, it would have to face the non-specific defensive reactions which the plant responds to some molecular patterns that are common to different pathogens (Pallas and García, 2011). Such measures include the host plant´s ability to activate gene silencing pathways directed against the pathogen (Eckardt, 2011). On the other hand, a virus is in a

position to trigger infection if it has evolved to acquire virulence factors to counteract basal defense and suppress gene silencing by the host by producing silencing suppressors (Bisaro, 2006; Jones and Dangl, 2006; Pallas and García, 2011; Eckardt, 2011). Silencing suppressors not only affect antiviral defense but also interfere with the plant physiological processes that depend on RNA silencing, an interference that may contribute to the pathogenesis of different viruses (Pallas and García, 2011). In CMG infected plants the AC2 and AC4 genes are known to be silencing suppressors (Voinnet et al., 1999; (Vanitharani et al., 2004; Bisaro, 2006).

As the virus spreads through leaf tissues, the infection of new cells will starts from zero hence the amount of suppressor necessary to efficiently suppress RNA silencing in a given cell is reached with a certain delay (Amari et al., 2012). Due to this, an efficient RNA silencing suppression is limited to cells behind the infection front and vsRNAs that remain non-sequestered in leading front cells may trigger the silencing of host genes (Amari et al., 2012). Virus-host interactions change from early to later stages of infection in that the virus first subverts RNA silencing at the leading front of infection and subsequently controls this activity as soon as the silencing suppressor reaches the critical level (Amari et al., 2012). The host virus-interaction involved in RNA silencing are complex and the outcome of infection likely depends on the balance between host recognition features specific to a particular virus/virus gene and the efficiency of viral silencing suppressors (Bisaro, 2006).

1.4.1 Utilization of viral sequences to generate virus resistant *Euphorbiaceae* crops

The breeding of CMD resistant cassava has been attempted through classical breeding. However this has been quite difficult due to high heterozygosity of cassava and strong inbreeding depression of elite varieties (Zhang et al., 2005). A further limitation to the process is the rapid evolution of CMGs in the field leading to new aggressive strains (Zhou et al., 1997; Akano et al., 2002; Legg and Thresh, 2003). However, the advent of biotechnological approaches has led to the possibility to manipulate the plant´s defence system against viruses to generate virus resistant plants (Zhang et al., 2005; Vanderschuren et al., 2007). Further more, it can used to introduce new resistant traits or genes which are currently absent within the cassava germplasm (Zhang et al., 2005; Prins et al., 2008). One such approach is the utilization of hairpins homologous to the viral sequences, which has been shown to be effective in generating CMD cassava resistant plants. Vanderschuren et al. (2007) used sequences from almost the entire common region of DNA A and B including the bidirectional promoter of the ACMV-Kenya isolate (Genbank NC 001467) to design constructs, which were mobilized into *Agrobacterium tumefaciens* and used for transformation of cassava plants. The transgenic plants generated expressed small interfering RNAs (siRNA) which led

to the attenuation of CMD symptoms following inoculation with ACMV-N*Og* infectious clones (Vanderschuren et al., 2007). Other attempts involved improved antisense RNA technology by targeting the ACMV viral mRNAs of AC1, AC2 and AC3 (Zhang et al., 2005). The full coding sequences of these ORFs were inserted separately in antisense orientation to the 3´UTR of the hygromycin phospho-transferase gene under the control of the *cauliflower mosaic virus* (CaMV) 35S promoter and used for transformation. Further analysis performed on leaf discs showed that viral DNA accumulation was strongly decreased and the resistant cassava plants showed delayed and attenuated CMD symptoms depending on the viral titre (Zhang et al., 2005). The ability to resist ACMV infection was concluded to be via PTGS. Similarly, broad spectrum resistant cassava plants towards ACMV, EACMV, SLCMV have been generated by using constructs from the entire AC1 gene of a Kenyan isolate of ACMV (Chellappan et al., 2004). The levels of AC1 mRNA were suppressed upon challenging with the geminiviruses and the viral DNA accumulation was reduced to 98% when compared to the controls. AC1 integration was found to initiate cross protection against several geminiviruses via PTGS. This robust cross protection has an implication towards field deployment of transgenic plants as an alternative to control CMGs (Chellappan et al., 2004).

The transgenic approach has further been applied in the development of CBSV resistant plants. The CBSV (GenBank JN091565) coat protein region conserved for both CBSV and UCBSV (positions 538 to 1063) was placed into a binary expression vector, electroporated into *Agrobacterium tumefaciens* and used to transform cassava plants co-infected with CBSV and EACMV (Vanderschuren et al., 2012). All transgenic lines did not support virus replication, even under high viral pressure. Furthermore, these transgenic lines were consistently resistant to UCBSV (Vanderschuren et al., 2012).

1.4.2 Virus induced gene silencing (VIGS)

RNA silencing is a nucleotide sequence-specific process that induces messenger RNA (mRNA) degradation or translation inhibition at the post-transcriptional level or epigenetic modification at the transcriptional level, depending on RNA-directed DNA methylation (Duan et al., 2012). It includes also quelling in fungi and RNA interference in animals (Cogoni and Macino, 1997; Fire et al., 1998; Baulcombe, 2004). RNA silencing is triggered when double stranded RNAs (dsRNAs) or hairpins are processed into microRNA (miRNA) or small interfering RNA (siRNA) duplexes (Meister and Tuschl, 2004; Qu et al., 2007).

Besides the regulatory roles in plant development, RNA silencing also functions as a natural antiviral defense mechanism, a process also known as virus induced gene silencing (VIGS) (Dunoyer and Voinnet, 2005). Host RNA silencing machinery targets and processes the virus-

derived dsRNA, which results from pathogen replication or by a host-encoded RNA polymerase using a viral RNA template into virus-derived small RNAs (vsRNAs) (Lu et al., 2003; Duan et al., 2012). Although the vsRNA are generally considered to be siRNAs, many of these molecules may be miRNAs because their hairpins have great similarity to miRNA precursors (Dunoyer and Voinnet, 2005). The vsRNAs are then recruited to host RNA-induced silencing complexes (RISC) complexes, to target and inhibit gene expression and protein translation in the viral genome (Baulcombe, 2004; Dunoyer and Voinnet, 2005). The virus RNA in this process is thus both an initiator and a target of the silencing mechanism (Lu et al., 2003).

RNA silencing can be induced locally and spread throughout the plant. This aspect of the process likely reflects its role in viral defense (Vance and Vaucheret, 2001). As a counter defense strategy, many plant viruses have evolved viral suppressors of RNA silencing to counteract antiviral silencing providing strong evidence for the antiviral nature of RNA silencing (Voinnet at al., 1999; Bisaro, 2006). Furthermore, viruses can also exploit RNA silencing to modify host gene expression directly because of homologies between vsRNAs and host transcripts, which may lead to development of viral disease symptoms (Dunoyer and Voinnet, 2005; Pallas and García, 2011; Amari et al., 2012).

1.4.3 microRNA

Many levels of gene regulation in plants and viruses are influenced by different classes of small non-coding RNAs (Bartel, 2004; Zamore and Haley, 2005). Granted that miRNA mediated gene silencing serves as a general defense mechanism against plant viruses, it would not be surprising that viruses also employ miRNAs to circumvent the defense systems (Baulcombe, 2004; Grundhoff, 2011). The involvement of both host and virus miRNA shows a plant-virus interaction that will either lead to a virus winning the race and plants being more susceptible or plants winning by being more resistant.

miRNAs are small non-coding RNA molecules, of approximately 21-24 nt in length that are key regulators of gene expression (Bartel and Bartel, 2003; Baulcombe, 2004; Anselmo et al., 2011). After the discovery of the first members of the miRNA family, lin-4 and let-7 from *Caenorhabditis elegans* (Lee et al., 1993; Reinhart et al., 2000), several miRNAs have been identified in plants, animals, viruses and green algae (*Chlamydomonas reinhardtii*) by molecular cloning, next generation sequencing and *in silico* approaches (Lagos-Quintana et al., 2001; Lau et al., 2001; Bartel, 2004; Jones-Rhoades and Bartel, 2004; Wang et al., 2005; Berezikov et al., 2006; Ruby et al., 2006; Molnár et al., 2007; Stark et al., 2007; Anselmo et al., 2011; Grundhoff, 2011; Grundhoff and Sullivan, 2011).

miRNAs are encoded in various loci in the genome, ranging from introns of protein-coding genes to non coding regions and exons (Rodriguez et al., 2004). miRNAs in introns of protein coding host genes are not transcribed separately, but processed from the introns and thus they have the same regulatory elements and primary transcript as their host genes (Lee et al., 2004). The biogenesis of mature miRNAs involves several endonuclease steps depending on where they are located in the genome. RNA polymerase II (Pol II) is responsible for transcribing most plant and animal miRNA from introns (Lee et al., 2004). However there are suggestions that RNA polymerase III is required for the transcription of miRNA from exonic regions that contain Alu repeats (Borchert et al., 2006). Alu repeats are mobile elements in the genome (transposons) of about 300 nt in full length and contain a recognition site for the restriction enzyme *Alu*I (Gu et al., 2009). The biogenesis of viral miRNAs is usually the same as for cellular miRNAs (Bogerd et al., 2010).

In plants, miRNA biogenesis is confined to the nucleus and only mature miRNA are transported to the cytoplasma (Kurihara and Watanabe, 2004). During biogenesis, plant miRNA genes are transcribed by Pol II to generate a stem loop containing primary miRNA (pri-miRNA), which can range in size from several hundred bps to tens of kbs (Cai et al., 2004; Lee et al., 2004). Pri-miRNAs are subsequently capped, spliced and poly-adenylated (Kurihara and Watanabe, 2004). Pri-miRNAs are processed to mature miRNAs in two steps by the dicer like enzyme1 (DCL1), a double-stranded RNA binding protein the hyponastic leaves1 (HYL1) and a C2H2 zinc-finger protein serrate (SE) (Vaucheret et al., 2004; Dong et al., 2008; Zhu, 2008). The DCL1 with the help of HYL1 and SE makes a first cut on the pri-miRNA to produce precursor-miRNA (pre-miRNA). To produce a mature miRNA duplex which has 2 nt overhangs at the 3´ends on both strands it takes a second cut. The last nucleotide at the 3´end is then methylated by hau enhancer1 (HEN1) a small RNA specific methyl transferase to protect them from uridylation and associated destabilization (Yu et al., 2005). The mature miRNA duplexes are transported to the cytoplasm by hasty (Xu et al.) an Exportin-5 homologue in plants (Bollman et al., 2003; Park et al., 2005). Based on the thermodynamic stability of each end of this duplex, one of the strands is preferentially incorporated into the argonaute (AGO) protein which is the catalytic center of plant RISC producing a biologically active miRNA and an inactive miRNA* (passage strand) (Vaucheret et al., 2004; O'Toole et al., 2006). miRNAs show perfect complementarity to their target mRNA sequences and guide their degradation or block their translation to regulate plant development, biotic and abiotic responses (Mallory and Vaucheret, 2006; Brodersen et al., 2008; Zhu, 2008).

The miRNA loaded RISC binds to the target mRNA in a sequence specific manner that cleaves the target or prevents the translation inhibition. miRNA target sites can be found throughout mRNA transcripts (5' untranslated region (UTR), ORFs and 3'UTR) (Nielsen et al., 2007; Selbach et al., 2008; Bartel, 2009). Furthermore an mRNA can contain multiple sites for the same or different miRNAs, while several different miRNAs can act together to repress the same gene (Grimson et al., 2007; Baek et al., 2008; Selbach et al., 2008). The mechanism of inhibition is based on the degree of complementarity of the miRNA to the target site. Perfect or near perfect complementarity results in cleavage, whereas imperfect complementarity results in impaired translation (Hutvagner and Zamore, 2002).

In directing target cleavage, small RNAs direct the argonaute 1 component of RISC to a specific complementary molecule to slice a single phosphodiester bond. The cleavage site is precisely between the nucleotides pairing to residues 10 and 11 of the miRNA (Hutvagner and Zamore, 2002). The cut fragments are then released and the RISC will subsequently recognize and cleave additional transcripts (Jones-Rhoades et al., 2006).

The mechanism of how miRNAs translationally inhibit a particular mRNA is still unclear (Gu and Kay, 2010). It occurs when the decrease in protein product is greater than the observed decrease in mRNA due to blockage in translation. Translation can be blocked during ribosome initiation, elongation or termination (Gu and Kay, 2010). Most research findings suggested that most animal miRNAs function by translational repression, and that these mechanism occurr in a few rare exceptions in plants (Aukerman and Sakai, 2003; Chen, 2004; Arteaga-Vázquez et al., 2006; Axtell, 2008; Mallory and Bouché, 2008). However at present there are genetic and biochemical evidence supporting that translational inhibition by miRNAs is widespread in plants. Interestingly, the same miRNAs can induce translational repression on their target mRNA, irrespective of the near-perfect complementarity between the miRNA and target sequences that causes cleavage (Eckardt, 2009; Lanet et al., 2009). This makes it difficult to distinguish the relative importance of miRNA-mediated translational repression in plants and suggest that it could be less important for miRNA activity than transcript cleavage (Yang et al., 2012).

1.4.3.1 Virus miRNA

While there is limited information on miRNA encoded by plant viruses, some human viruses encode miRNAs which they utilize to modulate both their own gene expression and that of their host cells (Pfeffer et al., 2004; Qi et al., 2006). miRNAs have several features that make them useful to viruses. The evolution of a miRNA complementary to a new target gene can occur more easily than the evolution of a novel regulatory protein (Umbach and Cullen,

2009). Furthermore, miRNAs are not antigenic and the down regulation of specific genes allows the virus to establish a favorable environment for its own replication while attenuating or avoiding the host immune response (Umbach and Cullen, 2009). Viruses can also exploit RNA silencing to modify host gene expression directly because of homologies between virus-derived small RNAs and host transcripts. They can further exploit the host nucleic acids as part of their infection strategy (Dunoyer and Voinnet, 2005).

In viruses infecting humans, many viral miRNAs were found to target cellular mRNAs of important proteins involved in apoptosis, a mechanism of the host's immune system that avoids viral spread through programmed cell death. The translation of anti-apoptotic proteins inhibits induction of cell death and favors viral survival (Choy et al., 2008; Seto et al., 2010). Furthermore viral miRNA interfere with crucial steps important in reducing the level of virus infection by; preventing cell recognition through inhibiting recognition from natural killer cell and cytotoxic T cell by inhibiting viral T cell antigen synthesis (Sullivan et al., 2005; Stern-Ginossar et al., 2007). In addition viral miRNAs can have the same seed sequence as cellular miRNA and can in turn mimic the biological function of these cellular miRNA, hence ultimately interfering with many host pathways, benefiting its replication (Gottwein and Cullen, 2007; Skalsky et al., 2007).

On the other hand, viruses have suppressors of gene silencing that can interact with the plant miRNA pathways. An example is the AC4 protein encoded by *African cassava mosaic virus* which can bind to single-stranded miRNA and inhibit miRNA-mediated negative regulation of gene expression in plants leading to developmental defects (Chellappan et al., 2005).

1.4.3.2 Plant miRNA

miRNAs in plants have been found to regulate genes involved in plant growth and development, biotic and abiotic responses (Chellappan et al., 2005; Jones-Rhoades et al., 2006; Bazzini et al., 2007). The majority of the early identified miRNAs are abundantly expressed in plants and approximately 50% of their validated targets are transcription factors involved in leaf, shoot and root development, vascular development, floral identity, flower development, flowering time and hormone signaling (Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Mallory et al., 2004; Nikovics et al., 2006).

The miR156 and miR157 are grouped in one miRNA family due to their high degree of sequence similarity and conserved target. They both target the squamosa-promoter binding like proteins (SPL), which are transcription factors involved in regulating plant growth and development (Schwab et al., 2005). A balance of miR156/157 and miR172 regulates juvenile-to-adult vegetative phase transition which leads to flowering (Chuck et al., 2007a; Chuck et

al., 2007b). Overexpression of miR156/157 causes a decrease in the level of miR172 and leads to an extended juvenile stage and prevents early flowering through translational inhibition of SPL3 (Wu and Poethig, 2006; Gandikota et al., 2007). Furthermore, miR172 determines flowering time and sex in maize through an interaction with photoreceptors. Targets of miR172 include Apetala2 gene (AP2) and AP2-like target of eat1 (TOE1) and TOE2 genes belonging to the class A floral homeotic genes. miRNA resistant forms of AP2 causes an abnormal floral structure (Aukerman and Sakai, 2003; Chen, 2004).

miR159 and miR319/JAW sequences differ by three nucleotides and have different targets which are myeloblastosis (MYB) and toxin-coregulated pilus (TCP) transcription factor genes respectively (Reinhart et al., 2002; Schwab et al., 2005). MYB transcription factor genes are involved in flowering and male fertility and bind to the promoter regions of a number of genes including the floral meristem identity gene LEAFY. It has been observed that overexpression of miR159 decreases the accumulation of MYB mRNAs and result in male sterility, while plants expressing miR159 resistant mutant MYB33 showed upward leaf curl and short petioles (Millar and Gubler, 2005; Schwab et al., 2005). The TCP transcription factor genes are responsible in controlling leaf shape and overexpression of miR319 downregulates TCP mRNA and plants exhibit uneven leaf shape and delayed flowering (Schwab et al., 2005).

miR164 targets mRNAs encoding cup shaped cotyledon-like (CUC) NAC domain transcription factors. CUC1, CUC2, and CUC3, function in initiating the shoot apical meristem and establishing organ boundaries (Laufs et al., 2004; Mallory et al., 2004). In CUC1/CUC2 double-mutants, seedlings exhibit fused lateral organs, fused cotyledons as they fail to separate, and a failure in apical meristem formation as seedling meristem gets arrested (Aida et al., 1999). miR164 mediated regulation is therefore necessary for proper formation and separation of adjacent organs. In addition, expression of a wild-type CUC1 gene resulted in phenotype in which ectopic meristems developed from the adaxial sites of both cotyledons and rosette leaves and with a reduced root branching (Takada et al., 2001; Mallory et al., 2004).

miRNAs have been found to target hormonal signal transduction pathways in plants. The miR160, miR167, and miR390 target Auxin Response Factors (ARFs) directly or indirectly (Jones-Rhoades et al., 2006; Xie et al., 2010). miR160 has binding sites on ARF10, ARF16 and ARF17 that are important in seed germination, root, leaf, and flower organ development (Mallory et al., 2005). miR167 guided cleavage of ARF6 and ARF8 which regulate ovule and anther development (Rhoades et al., 2002) and miR390 guides the targeting of ARF2, ARF3 and ARF4 (Allen et al., 2005; Jones-Rhoades et al., 2006; Xie et al., 2010).

Plant miRNAs play important roles in response to abiotic stresses. miR398 targets two closely related copper-zinc superoxide dismutases (CDS); a cytosolic CSD1, and chloroplastic CSD2 that are involved in antioxidant responses. It also targets COX5b-1, a subunit of the mitochondrial Cytochrome C Oxidase (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004). Under low copper conditions, miR398 down regulates its target gene expression an important mechanism for plants to mobilize copper from non-essential to essential copper proteins, including plastocyanin which is mandetory for photosynthesis (Yamasaki et al., 2007; Xie et al., 2010). In addition, plants overexpressing a miR398-resistant form of CSD2 accumulate more CSD2 mRNA and are consequently more tolerant to oxidative stresses, high light and heavy metals than plants overexpressing a regular CSD2 (Sunkar et al., 2006).

Plant miRNAs have a role in nutrient sensing. The miR395 is 100-fold induced upon low-sulfate levels. It targets a low-affinity sulfate transporter AST68 and three ATP sulfurylases (APS1, APS3 and APS4) involved in sulfate assimilation pathways (Jones-Rhoades and Bartel, 2004). Another miRNA, miR399 is involved in the regulation of plant responses to phosphate (Pi) nutrient deficiency (Fujii et al., 2005). It targets a phosphate transporter (Jones-Rhoades and Bartel, 2004) and further has multiple sites at the 5´untranslated region of a gene encoding a putative ubiquitin conjugating enzyme-E2 (UBC24, PHO2) (Sunkar and Zhu, 2004). Under low Pi stress condition, miR399 is highly induced, whereas the target PHO2 mRNA is reduced. A downregulation of PHO2 mRNA levels under low phosphate conditions is important for primary root elongation (Fujii et al., 2005). However it has been observed that transgenic *Arabidopsis* plants overexpressing miR399 accumulate more phosphate than wild type plants (Sunkar et al., 2007).

Although siRNA are known to play a direct role in antiviral defense in plants (Mahmood-ur-Rahman et al., 2008; Pérez-Quintero et al., 2010) there is no proof of naturally occurring plant microRNAs with antiviral activity (Pérez-Quintero et al., 2010). However, research using genetically modified viruses and plants, has been able to show that complementarity between a plant miRNA and the virus genome is enough for antiviral activity (Pérez-Quintero et al., 2010). Previous findings have shown that the alteration of several nucleotides within the miRNA sequence does not affect its biogenesis (Niu et al., 2006; Duan et al., 2008; Khraiwesh et al., 2008). This has made it possible to modify miRNA sequences to create artificial miRNAs (amiRNA) directed against any gene of interest resulting in its post transcriptional silencing (Niu et al., 2006; Khraiwesh et al., 2008). It is important to select target sites of amiRNA that have optimal accessibility in order to obtain high resistance (Duan et al., 2008). Furthermore, it is possible to optimize sequences of amiRNA to knock down the

expression of a single gene or several highly conserved genes without affecting the expression of other genes (Khraiwesh et al., 2008). Artificial pre-miR171a cleaves the 2b (silencing suppressor of CMV) and inhibits its gene expression conferring resistance to CMV (Qu et al., 2007). Multiple virus resistance by introducing amiRNA targeting more than one virus family is also possible. These was shown through the modification of an *Arabidopsis thaliana* miR159 precursor to express amiRNA targeting viral mRNA sequences encoding two gene silencing suppressors, P69 of *Turnip yellow mosaic virus* and Hc-Pro of *Turnip mosaic virus* conferred resistance to both viruses (Niu et al., 2006).

Cross-talk between the pathways of abiotic and biotic stress response might be a reality (Sanan-Mishra et al., 2009). Bioinformatics predictions show that miR156, miR159, miR166, miR160 and miR395 have potential targets in the virus genomes (Pérez-Quintero et al., 2010). *In silico* analysis could also reveal that several tomato miR/miR* sequences exhibit propensity to bind to *Tomato leaf curl New Delhi virus* (ToLCNDV) associated (Naqvi et al., 2011).

Plant miRNAs can accumulate to a higher percentage in virus-infected plants (Tagami et al., 2007). Begomoviruses generally increase the accumulation of miRNA, which leads to a decreased translation of genes involved in the development of plants (Amin et al., 2011). *Nicotiana benthamiana* plants infected by begomoviruses ACMV, *Cabbage leaf curl virus, Tomato yellow leaf curl virus* and *Cotton leaf curl Multan virus* showed an increase in the level of miR159, miR164, miR165/166, miR167, miR168 (Amin et al., 2011). Similarly, In *Lycopersicon esculentum* infected by ToLCNDV, miR159/319 and miR172 was observed to increase and might be associated with leaf curl symptoms (Naqvi et al., 2010). miR163, miR164 and miR167 increased 2-3 times in *Arabidopsis thaliana* plants infected with *Tobacco mosaic virus* (Tagami et al., 2007). This was attributed to a high binding activity of the virus replication protein to the miRNAs or that transcription of some miRNAs is also activated (Tagami et al., 2007).

1.4.4. Prediction of miRNAs

Since it is difficult, expensive and requires a large amount of time to systemically detect miRNAs from a host or pathogen genome by available experimental techniques, computational approaches have been applied to identify pre-miRNA in diverse plant species, animals and a few viruses (Berezikov et al., 2006; Zhou et al., 2009; Wang et al., 2011).

An important generic features shared by all miRNA genes is the secondary structure (pre-miRNAs), in which a mature miRNAs appears to reside on one arm (5' or 3') of the stem loop (Ambros et al., 2003). These secondary structures are vital features used in the computational

identification of miRNAs (Bartel, 2004; Xue et al., 2005; Ng and Mishra, 2007; Zhou et al., 2009; Xuan et al., 2011). Other important characteristics considered are high evolutionary conservation (Li et al., 2010) and thermodynamic stability of the secondary structure features based on minimum free energy (MFE) (Bonnet et al., 2004). A stable secondary structure is needed to avoid early degradation during miRNA biogenesis (Bonnet et al., 2004). MFE is estimated by considering the minimum energy values obtained by complementary base pairs decreased by the stacking energy of successive base pairs or increased by the destabilizing energy associated with non-complementary bases (Zuker and Stiegler, 1981; Bonnet et al., 2004; Jin et al., 2008).

At present, miRNA computational approaches can be divided into three types: (1) comparative genomic approach based on evolutionary conservation, (2) homology based approach and (3) *ab initio* prediction based on sequence and structure features (Berezikov et al., 2006; Zhou et al., 2009). In comparative genomics, the conservation of pre-miRNAs in their primary sequence and/or their secondary structure is the basis of prediction. This approach has shown that miRNA are highly evolutionary conserved from species to species in the plant kingdom (Zhang et al., 2006) and animal kingdom (Pasquinelli et al., 2000). Softwares used in this approach consider multiple alignments of sequences across related species where conserved pre-miRNAs are searched (Stark et al., 2007). Examples of these softwares are miRscan (Lim et al., 2003), MirFinder (Bonnet et al., 2004) and miRseeker (Lai et al., 2003). MirFinder has been used to predict potential miRNA in *Arabidopsis thaliana* genome, based on the conservation of short sequences between the genomes of *Arabidopsis* and *Oryza sativa* (Bonnet et al., 2004).

The availability of known miRNA, their pre-miRNA and structures in miRBase (www.mirbase.org) provides a basis for their utilization in homology based approaches (Tempel and Tahi, 2012). Homologous or near matches of a know miRNA are predicted by identifying genomic regions with sequence similarity to the know miRNA and are capable of forming miRNA hairpin precursors. In addition to this, searching regions close to known miRNA genes for other stem loop can lead to the identification of miRNA genes belonging to a cluster (Bartel, 2004). This approach has been used to search for new miRNAs by analyzing EST data (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Zhang et al., 2006).

ESTs are useful for the prediction of miRNA since: (1) they can be used in identification of miRNA from plants species where no genomes have been published and (2) they provide a direct evidence for miRNA expression that cannot be inferred from genomic sequence surveys since they are derived from transcribed sequences (Altschul et al., 1997; Matukumalli et al.,

2004; Zhang et al., 2008). Examples of softwares used in this approach are ERPIN (Legendre et al., 2005) and miRAlign (Wang et al., 2005).

Although comparative genomics and homology-based approaches provide important techniques to predict miRNAs, they are limited in identifying novel miRNAs in situations where no close homologues are known due to data limitation or miRNAs evolution (Berezikov et al., 2006). These approaches are for instance not suitable for prediction of miRNA from viruses since for many viruses only very distant evolutionary orthologs are known (Grundhoff et al., 2006). To overcome this limitation, *ab initio* approaches to miRNA prediction have been developed (Sullivan et al., 2005; Jiang et al., 2007). *Ab initio* methods rely on intrinsic characteristics of known miRNAs to define sets of features that can accurately describe the structure and sequence of these transcripts (Wilbert and Yeo, 2011). For the majority of *ab initio* methods, an early step secondary structure predictor like RNAFold, Mfold, or UNAFold are applied before further filtering steps are performed to predict pre-miRNAs. These methods can be classified into three categories (Tempel and Tahi, 2012) of which category 1 and 3 were used in the current study: 1) Category 1 classifies a pre-miRNA input sequence as true or false pre-miRNA for instance miPred (Jiang et al., 2007). MiPred are algorithms that classify real and pseudo pre-miRNAs using a random forest prediction model. For any hairpin resembling a pre-miRNA, the random forest-based classifier predict it as a real pre-miRNA which are considered to be stable if it has a MFE < -20 kcal/mol, *P*-value < 0.05 and continuously paired nucleotides at high frequencies. For pseudo pre-miRNA they have MFE > -20 kcal/mol, *P*-value > 0.05 and continuously unpaired nucleotides at high frequencies (Jiang et al., 2007; Chen et al., 2009). 2) Category 2 takes the genomic sequence as input and use other information to predicted pre-MiRNA from the input sequence. They require that a known pre-miRNA is supplied and based on this it will predict new pre-miRNA from the input genomic sequence. An example is miR-abela that has been used to search for pre-miRNA clusters in human, mouse and rat genomes (Sewer et al., 2005). 3) Category 3 are purely *ab initio* and they take the genomic sequences as input and search for all possible pre-miRNAs without the need of any other information (Tempel and Tahi, 2012). An example is VMir that predict pre-miRNA in viruses (Sullivan and Grundhoff, 2007). At the start, the method uses RNAFold to identifying robust stem–loop hairpins which retain the typical folding structure regardless of the precise location of the start/end of the folded transcript (Sullivan and Grundhoff, 2007). The folded hairpins are then scored based on size, number of copies and sliding windows where a similar hairpin is detected (Sullivan and Grundhoff 2007; Tempel and Tahi, 2012).

1.4.5. Prediction of miRNA targets

An important initial step of analyzing miRNA to perform the regulatory task is to recognize its target genes (Yue et al., 2009). One microRNA can have binding sites in multiple targets and alternatively one target can be repressed by multiple microRNAs (multiplicity and co-operativity) (John et al., 2004). Prediction of miRNA targets provides an alternative approach to assign biological functions (Brennecke et al., 2005). Research has shown that most known plant miRNAs bind to the protein-coding region of their mRNA targets with perfect or nearly perfect sequence complementarily, which highly facilitates computational predictions (Wang et al., 2004). However the possibility that a larger numbers of mRNAs could be targeted for translational inhibition through imperfect sites is likely to occur since small RNA-mediated translational inhibition appears to be widespread in plants (Brodersen et al., 2008). The psRNATarget web based server is a prediction tool that takes into account translational inhibition potential, when finding miRNA targets in plants (Dai and Zhao, 2011).

Although a detailed target recognition mechanism is still elusive, majority of research done suggests that the Watson base pairing of miRNA with its target´s mRNAs is the key (Yue et al., 2009). At the 5'end of the miRNA there is a seed region which is located on nucleotides 2-8 (Grimson et al., 2007) and Watson-Crick paring of the mRNA target site to this seed region is an important factor for miRNA target prediction (Lewis et al., 2003; Bartel, 2004). Biochemical and structural findings have supported the relevance of this region in target recognition (Jinek and Doudna, 2009). The seed region is the most evolutionarily conserved region of miRNAs and is further commonly complementary to target sites (Rhoades et al., 2002; Lewis et al., 2003; Bartel, 2004). It has also been suggested that pairing to the 3' end of miRNAs also plays a role in target recognition particularly if a perfect seed match cannot be detected (Bartel, 2009). The 3' end can supplement and compensate seed pairing and consequently improves binding specificity and affinity (Grimson et al., 2007; Bartel, 2009; Sturm et al., 2010). Supplemental and compensatory base-pairing by an additional extended pairing in 3'end is centered around nt 13-16 of the miRNA, and can complement perfect seed matches or ameliorate targeting when the seed match is not perfect (Doench and Sharp, 2004; Brennecke et al., 2005; Grimson et al., 2007; Wilbert and Yeo, 2011). In addition, functional "centered sites" based on a continuous base pairing of position 4 or 5 to position 14 or 15, lacking substantial 5′ or the 3' ends pairing have been observed (Shin et al., 2010). Such relaxed seed approaches are important in the case of viral miRNA which are used to regulate their own or host genes (Ghosh et al., 2009). Furthermore, conserved seeds are not applicable

to viruses since they evolve fast and are highly adapted to specific hosts (Gottwein and Cullen, 2008).

While not all miRNA target sites adhere to seed complementarity, alternative approaches based on the use of free energy of mRNA:miRNA hybridization as an alternative feature have been applied (Lekprasert et al., 2011). A RNA duplex is in a thermodynamically more stable state when the free energy is low (more negative), which means the binding of the miRNA to the mRNA is stronger (Lekprasert et al., 2011). The RNAhybrid (Rehmsmeier et al., 2004) is one of the methods based on algorithms that are rooted in thermodynamics and uses it as the initial indicator of potential miRNA binding site. The method takes candidate target sequences and a set of miRNAs and searches for most favorable energetically binding sites consistent to user defined structural constraints (Rehmsmeier et al., 2004; Alves et al., 2009).

CHAPTER II: Aim of the work

The introduction of non-edible biodiesel from *Jatropha* would provide a suitable alternative and further solve interrelated environmental problems including deforestation, soil erosion and desertification. The crop can alleviate poverty by offering the farmers a new and sustainable cash crop in order to increase household income. This therefore calls for the need of promising *Jatropha* varieties that are high yielding, adaptable to environmental conditions and resistant to diseases and pests. Unfortunately, *Jatropha* has not been domesticated which makes accurate prediction of yields difficult. Furthermore there are no improved varieties with desirable traits for specific growing conditions and limited information is available about genetic diversity (Vollmann and Laimer, 2013; Maghuly et al., 2013). Evaluation of available *Jatropha* genetic stock to access the genetic variation for economically important characteristics will serve as a benchmark for selection of parents that will be important for breeding programs.

Furthermore, plant viruses cause important diseases responsible for massive losses in crop production and quality. *Jatropha* and cassava both in the family *Euphorbiaceae* succumb to similar viral diseases. Increase of virus infection in these crop species threatens their cultivation and availability in the market. Viruses are intracellular plant pathogens and are spread either by biological vectors, mechanical inoculation or vegetative propagation. As a strategy to control viruses, it is a prerequisite to limit their spread, which calls for a need of a detection tool. Plant virus detection has mainly been by ELISA and nucleic acid-based assays, however with improvement in biotechnology, other strategies like rolling cycle amplification can allow the detection of viruses in plants with low virus titers. Further utilization of a combination of these technologies allows the coverage of more pathogens to be detected and provide a clear picture of disease-causing infections.

Consequently, the achievement of high quality and quantity of yields needs the employment of an integrated approach that starts with the selection of plant genotypes that are promising and a need of preventive measures to avoid planting of infected material by developing accurate diagnosis. Therefore the milestones of the thesis contributed to:

1. Investigations of genetic variations in a large collection of *Jatropha* accessions. The first topic intends to shed more light on the nucleotide polymorphisms of *Jatropha* germplasm collected from different parts of the world through ISSR and Ecotilling.
2. Identification of viruses infecting *Jatropha* and cassava through improved biotechnological approaches.

The second topic involves the study of the virus status of *Jatropha* and cassava, due to the crucial epidemiological importance as both crop plants belong to the same family. This study added to the development of improved strategies in virus detection which may contribute to the control of their spread.

3. Evaluation of plant-virus interaction in *Jatropha* and cassava as mediated by miRNA. The third topic intends to explore through an *in silico* approach the identification of miRNA encoded by cassava mosaic geminiviruses and those from the host plant that can bind to this viruses. It further shows a mapping of their targets in *Jatropha* and cassava. Mapping disease resistance genes in *Jatropha* and cassava populations and identifying miRNAs both of pathogens and hosts will improve the understanding of host pathogen interaction and may in turn benefit *Euphorbiaceae* health.

CHAPTER III:

Investigation of genetic variation in *Jatropha curcas* by Ecotilling and ISSR

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Background

The ability of species to adapt to different environments resides in their genetic diversity. This diversity, most commonly manifested as Single Nucleotide Polymorphisms (SNPs), can provide clues to the adaptive processes and population histories that have played a role in the species' evolution. A number of different techniques for identifying SNPs have been developed, all having their limitations.

Reverse genetics approaches rely on the detection of sequence alterations in target genes to identify allelic variations in natural or mutant populations. Ecotilling, a variant of TILLING (Targeting Induced Local Lesions IN Genomes) technique, allows high-throughput analyses of natural genetic diversity in plants (Comai et al., 2004), particularly in species with limited genetic diversity.

Jatropha curcas L. is a perennial, monoecious shrub of the *Euphorbiaceae* family, native to America but distributed widely in the tropical and subtropical areas (Cano-Asseleih et al., 1989). Wild or semi-cultivated types of *J. curcas* can grow well under unfavourable climatic and soil conditions (Katwal and Soni, 2003). *J. curcas* has attracted a great deal of attention worldwide, regarding its potential as a new energy plant. The seeds of *J. curcas* contain 30-45% oil (Openshaw, 2000) with a high percentage of monounsaturated oleic and polyunsaturated linoleic acid (Akintayo, 2004). For genomic analyses, *J. curcas* is an interesting model species, since it has a relatively small genome (2C DNA content of 0.850 ± 0.006 pg or C DNA content of 0.416 × 109 bp) (Carvalho et al., 2008).

However, to achieve specific breeding goals in *Jatropha* for wider ecological adaptation, disease resistance and novel seed quality, the use of germplasm from different group and regions is necessary. Understanding the population structure of the alternative bioenergy plant *Jatropha curcas* is challenging due to limited genetic variability and information on phylogenetic relationships between accessions and related species. The development of cultivars of *Jatropha curcas* by conventional breeding will profit largely from biotechnological support (pathogen-free accessions with specific traits, non-toxic, high yielding varieties).

The knowledge about *J. curcas* remains limited and little genomic research has been done so far (Wen et al., 2010). In fact, the genetic map of *J. curcas* is not well-developed and only few molecular markers exist that could be used to clearly distinguish world wide accessions. Therefore, a resource database of SNPs in *J. curcas* would provide researchers with a tool for answering questions concerning population structure or adaptation and allow comparison of this species with related species.

Methods

The identification of novel SNPs that account for natural variation was used to study genetic diversity and the relationships between and within *Jatropha* species. ISSRs (Inter Simple Sequence Repeats) also were considered as a tool in selecting germplasm for breeding purposes.

An *in vitro* germplasm collection of 1200 accessions from 12 countries was established. This collection will serve different purposes: a) conserve valuable genetic resources, b) survey genetic variation, and c) serve as starting material for genetic improvement with different breeding goals.

Ecotilling was applied to 12 different genes of interest related to stress tolerance, toxin and oil metabolism. 50 ISSR primers were used to assess the genetic diversity of *Jatropha curcas* and related species. Four different pooling strategies were used to identify homozygous and heterozygous SNP variations. In fact, variation was analyzed both within a single tree (heterozygous) as well as between individual trees and a reference samples. Due to the reported low variations between *Jatropha* accessions (Achten et al., 2010; Vollmann and Laimer, 2013) and large size of our collection, the 8 x 8 pooling strategy was chosen to estimate the level of variations among 12 selected genes.

Results and conclusions

To elucidate genetic relationship among *Jatropha* accessions from different regions and related species, a dendrogram was produced using NJ analysis of NeiÂ´s genetic distance for 5 ISSR markers. The dendrogram is divided into two groups, one containing all *Jatropha* accessions and the other containing the related species. The main *Jatropha curcas* cluster is divided into two subclusters, one containing samples from Kenya and the other containing the remaining *Jatropha* accessions. The data showed clear variations not only among individuals but also between different regions.

Ecotilling was found to be more efficient for large-scale studies of genetic variation in *Jatropha*, compared to RAPD, SSR and AFLP. Ecotilling is a low cost, high-throughput reverse genetic method for haplotyping and SNPs discovery.The level of differentiation observed was based on the geographic distribution pattern, i.e. it was higher in the centre of origin. ISSR analysis yielded highly reproducible patterns with 5/50 primers.

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CHAPTER IV:

Biotechnological approaches to determine the impact of viruses in the energy crop plant *Jatropha curcas*.

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Key words: *Euphorbiaceae*, virus detection, ELISA, PCR, RCA, RT-PCR, ACMV, CBSV, CMV, EACMV-UG and Def DNA

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Abstract

Background

Geminiviruses infect a wide range of plant species including *Jatropha* and cassava both belonging to family *Euphorbiaceae*. Cassava is traditionally an important food crop in Sub – Saharan countries, while *Jatropha* is considered as valuable biofuel plant with great perspectives in the future.

Results

A total of 127 *Jatropha* samples from Ethiopia and Kenya and 124 cassava samples from Kenya were tested by Enzyme-Linked Immunosorbent Assay (ELISA) for RNA virus and polymerase chain reaction for geminivirus. *Jatropha* samples from 4 different districts in Kenya and Ethiopia (analyzed by ELISA) were negative for all three RNA viruses tested: *Cassava brown streak virus* (CBSV), *Cassava common mosaic virus*, *Cucumber mosaic virus*, Three cassava samples from Busia district (Kenya) contained CBSV. To develop diagnostic approaches allowing reliable pathogen detection in *Jatropha*, involved the amplification and sequencing of the entire DNA A molecules of 40 Kenyan isolates belonging to *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus – Uganda*. This information enabled the design of novel primers to address different questions: a) primers amplifying longer sequences led to a phylogenetic tree of isolates, allowing some predictions on the evolutionary aspects of Begomoviruses in *Jatropha*; b) primers amplifying shorter sequences represent a reliable diagnostic tool. This is the first report of the two Begomoviruses in *J. curcas*. Two cassava samples were co – infected with cassava mosaic geminivirus and CBSV. A Defective DNA A of ACMV was found for the first time in *Jatropha.*

Conclusion

Cassava geminiviruses occurring in *Jatropha* might be spread wider than anticipated. If not taken care of, this virus infection might negatively impact large scale plantations for biofuel production. Being hosts for similar pathogens, the planting vicinity of the two crop plants needs to be handled carefully.

Introduction

Geminiviruses are a group of plant viruses that contain circular single stranded (ss) DNA genomes encapsidated in small twinned icosahedral capsids (Stanley et al., 2005). They infect a wide range of plant species and are responsible for considerable crop losses (Varma and Malathi,

2003). Members of this virus family have been classified into four genera: *Begomovirus*, *Curtovirus, Mastrevirus, and Topocuvirus*, depending on genome organization, host range and type of insect vectors (Fauquet and Stanley, 2003). The genome of cassava mosaic geminivirus (CMG) belonging to the genus *Begomovirus* consist of two components termed DNA A and DNA B each of ~ 2.7 – 3.0 kb (Stanley et al., 2005; Yadava et al., 2010). The virus DNA A plus strand encodes the coat protein (CP/AV1) essential for viral transmission by whiteflies (*Bemisia tabaci*) (Höfer et al., 1997; Stanley et al., 2005). There are four overlapping open reading frames (ORFs) on the complementary strand, of which the replication associated – protein (Rep/AC1) is absolutely required for the replication of both genomic components (Etessami et al., 1991; Paszkowski et al., 1993). The replication enhancer protein (REn/AC3) is not essential for infection but enhances viral DNA accumulation (Etessami et al., 1991). The transcriptional activator protein (TrAP/AC2) is required for the transcription – activation of plus strand gene transcription**,** and is also involved in suppression of post – transcriptional gene silencing (PTGS) (Voinnet et al., 1999; Vanitharani et al., 2005). The functions of two other DNA A encoded proteins AV2 and AC4 remains unclear although possible roles in movement (AV2), pathogenicity and PTGS (AC4) have been demonstrated (Vanitharani et al., 2004; Chellappan et al., 2005). DNA B encodes the movement protein (BC1/MP) and a nuclear shuttle protein (BV1/NSP) required for cell – to – cell and long distance spread of virus in host plant (Stanley et al., 2005). Both DNAs contain a 200–250 bp region of high sequence homology known as the common region which is a part of a large intergenic region (IR) that contains the origin of replication (Yadava et al., 2010).

Seven species of *Begomovirus* have been identified so far in association with cassava mosaic disease (CMD) in Africa: *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Kenya virus* (EACMKV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV) and *South African cassava mosaic virus* (SACMV) (Zhou et al., 1997; Ndunguru et al., 2005; Bull et al., 2006; Fauquet et al., 2008). The distribution of the viruses has become more complex, since they invade new geographical regions and host plants (Harrison et al., 1997; Bull et al., 2006). This was attributed to their evolution which was more rapid than anticipated through mutational changes, recombination of double stranded (ds) DNA intermediates and re – assortment of gene

components (pseudo – recombination) (Harrison et al., 1997; Zhou et al., 1997; Fondong et al., 2000; Pita et al., 2001; Ndunguru et al., 2005; Bull et al., 2006). In fact, recombination played a role in the emergence of a new geminivirus that resulted in severe epidemics almost eliminating cassava (*Manihot esculenta*) in Uganda and Central Africa (Zhou et al., 1997; Legg and Fauquet, 2004). The symptom severity (due to synergism) was linked to the occurrence of *East African cassava mosaic virus* – Uganda (EACMV – UG) arising from recombination of EACMV and ACMV) (Ndunguru et al., 2005). Synergism refers to a situation where one virus affects a co – infecting virus by allowing its increased accumulation in the host plant by facilitating its replication, its movement to tissues that otherwise would not be invaded, resulting in more severe symptoms than caused by each single infection (Untiveros et al., 2007). Synergism between EACMV and ACMV is due to a selective advantage conferred by each partner linked to post transcriptional gene silencing (PTGS). In plants, PTGS operates as an adaptive immune system targeted against viruses (Voinnet, 2001). To counteract this defence system, viruses have developed suppressor proteins (Bisaro, 2006). ACMV and EACMV – UG possess two PTGS suppressors AC4 and AC2 respectively, with differential roles that target different steps in RNA silencing in a temporal and spatial manner (Voinnet, 2001; Vanitharani et al., 2005; Fondong et al., 2007). Therefore using more than one type of PTGS suppressor provides an advantage to viruses synergistically interacting in mixed infections, leading to more severe symptoms (Vanitharani et al., 2004; Vanitharani et al., 2005). Furthermore, geminiviruses may be associated with small sub genomic DNA molecules termed as Defective (Def) DNAs, which are the result of partial deletion to approximately half the genome, even disrupting genes (Stanley et al., 1990; Ndunguru et al., 2006). They may also result from sequence duplication, inversion or rearrangement of viral DNA, and recombination between DNA A and DNA B components (Patil et al., 2007). Additionally, Cassava brown streak disease (CBSD), caused by *Ipomoviruses* (family *Potyviridae),* has been reported to lead to severe yield losses in cassava plantations in Africa (Mbanzibwa et al., 2009; Winter et al., 2010).

Both host plants under study, cassava and *Jatropha,* belong to the family *Euphorbiaceae. Jatropha* is a drought resistant shrub native in tropical America, but is now widely grown in many tropical and subtropical regions for biodiesel production (Openshaw, 2000; Gao et al., 2010). Based on the genetic relationship of cassava and *Jatropha* and the detrimental impact of Begomoviruses in cassava, the question arose, whether *Jatropha* would be threatened by

comparable epidemics, if planted on larger extensions, or in spatial neighbourhood. Therefore it was necessary to develop diagnostic approaches allowing reliable pathogen detection in *Jatropha*, which involved the amplification and sequencing of the entire DNA A molecules of 40 Kenyan isolates belonging to ACMV and EACMV – UG. This information enabled the design of novel primers to address different questions: a) primers amplifying longer sequences led to a phylogenetic tree of isolates, allowing some predictions on the evolutionary aspects of Begomoviruses in *Jatropha*; b) primers amplifying shorter sequences represent a reliable diagnostic tool, given that so far only limited serological tests are available.

Methods

Sample collection

A total of 127 *Jatropha* samples from Ethiopia and Kenya and 124 cassava samples from Kenya were used in this study. The Kenyan samples were collected during a survey conducted in September 2009 and November 2010 covering four districts: Kakamega, Siaya, Busia (Western region) and Nakuru (Rift valley region) growing *Jatropha* and cassava together (Figure 1). Ten plants of *Jatropha* and cassava showing typical virus symptoms and ten symptomless plants were sampled from five fields in each district. Young leaves were picked from the plants and placed in sample collection tubes over silica gel for further detection of viruses. Two cuttings of approximately 30 cm long were also taken from 5 symptomatic and 5 asymptomatic plants and planted in glasshouse for future use.

Enzyme-Linked Immunosorbent Assay

Double Antibody Sandwich ELISA (DAS – ELISA) was performed on all Jatropha and cassava plant accessions to detect the presence of RNA viruses such as: CMV and CsCMV using commercially available kits (DSMZ GmbH, Germany and AC Diagnostics). Triple – Antibody Sandwich – ELISA (TAS – ELISA) was performed to determine the presence of CBSV (DSMZ GmbH, Germany). An ELISA sample was taken as positive, when its OD value was at least three times higher than the negative control. All determinations were run in duplicate.

DNA extraction and rolling cycle amplification

Total genomic DNA was extracted from leaves using the DNeasy plant Mini Kit (QIAGEN, Hilden, Germany) according to the supplier's instructions. RCA was performed using the TempliPhiTM Kit (Amersham Biosciences) according to the supplier's instructions.

Polymerase chain reaction

Six different primers were designed (Table 1) based on multiple alignments of full length DNA A sequence of geminivirus from *Jatropha* and cassava available in the NCBI Genbank to amplify the variable regions to yield longer (2800 bp) and shorter sequences (380 – 1085 bp) (Table 2). PCR was conducted in a total volume of 25 µl using 2.5 µl 10x PCR buffer (QIAGEN), 1µl $MgCl_2$ (25mM), 0.5 µl of each primer (10pmol), 0.5 µl dNTP, 0.15 µl HotStarTaq Polymerase (QIAGEN HotStar Plus TM PCR), 1 µl of total genomic DNA or RCA (1:30) product. Total genomic DNA was used with all the primer sets that amplify shorter sequences while the RCA was used for primer sets that amplify longer sequences (Table 2). For RCA to amplifying longer sequences, the PCR cycling conditions consisted of an initial denaturation step of 95°C for 5 min followed by 35 cycles of 1 min at 94°C, 1 min annealing temperature (Table 2) and 2 min at 72°C. A final step of 10 min at 72°C ended the cycle. For primers pairs amplifying shorter sequences, the difference in PCR conditions was in 30 cycles of 40 s at 94°C and 40 sec annealing temperature (Table 2). The PCR products were analyzed by electrophoresis in a 1% agarose gel. A subsequent purification of full length PCR products was done using QIAquick PCR purification kit (QIAGEN).

Extraction of RNA and RT–PCR

RT–PCR was performed to detect CBSV and CMV in *Jatropha* and cassava. Total RNA was extracted from 100 mg of *Jatropha* and cassava leaves using Spectrum ^{TM} plant total RNA kit (SIGMA – ALDRICH) according to the supplier's instructions. cDNA was synthesized from 3 µg of genomic RNA using SuperScript II ^{TM} reverse transcriptase primed with oligo$(dT)_{12-18}$ (Invitrogen). The CBSV specific primers CBSV 10F: 5′ATCAGAATAGTGACTGCTGG 3′ and CBSV 11R: 5′ CCACATTATTATCGTCACCAGG 3′ (Monger et al., 2001) amplifying 230 bp were used for PCR amplification of the cDNA template. The reaction mix and PCR cycling conditions were as performed previous (Monger et al., 2001). For CMV detection, Cucumoviruses universal primers CPTALL – 3: 5′ GACTGACCATTTTAGCCG 3′ and CPTALL – 5: 5′ YASYTTTDRGGTTCAATTCC 3′ (Choi et al., 1999) amplifying 940 bp were used for PCR with the reaction mixture and cycling conditions as described previously (Choi et al., 1999).

Sequence analyses

Multiple sequence alignments of geminivirus full length DNA A sequences was carried out using the Clustal program (MegAlign, DNAStar). A phylogenetic tree was constructed from multiple alignments by performing a heuristic search. Multiple alignments were analyzed by maximum parsimony with full-length DNA A using Phylogenetic Analysis Using Parsimony (PAUP) and a bootstrap analysis with 1000 replicates was performed.

Results

Symptomatology

Jatropha plants growing in the field showed symptoms ranging from reduced leaf size, malformation and severe dwarfing of 1 – 3 year old plants. Symptoms on *Jatropha* plants growing in the glasshouse were registered after 3 weeks as severe leaf yellowing coupled with browning of newly formed leaves, leaf malformation, reduced leaf size, mild to severe chlorotic specks and chlorosis in some plants. Drying (like burning) and rolling of leaves from tips was observed on 3 months old cuttings. In cassava the symptoms observed in plants grown in the field or maintained in the glasshouse were similar: mosaic, severe reduction and distortion of leaves, and stunted growth of some plants.

Virus detection by Enzyme-Linked Immunosorbent Assay (ELISA)

The *Jatropha* samples did not contain any of the three RNA viruses tested: Cassava brown *streak virus* (CBSV), *Cucumber mosaic virus* (CMV) and *Cassava common mosaic virus* (CsCMV) when analyzed by ELISA (see Additional file 1, Table S1). Also the cassava samples were negative for CMV and CsCMV. In fact, only three cassava samples from Busia district contained CBSV, as detected by ELISA (see Additional file 2, Table S2).

Virus detection by Polymerase chain reaction (PCR)

All *Jatropha* and cassava samples were tested by PCR for the presence of geminiviruses. The primer pair JC3F and JC4R amplified longer sequences of DNA A which were used the construct a phylogenetic tree. Primer pair JC6F and JC2R amplified a shorter sequence of 380 bp from AC1, AC2 and AC3 and distinguished reliably positive from negative samples.

When symptomatic *Jatropha* samples from Kenya were tested with primer pair JC3F and JC4R, 69% were positive. The primers were able to further detect virus in 67% of asymptomatic samples. For symptomatic *Jatropha* samples collected from Ethiopia, 61% tested positive with the primers JC6F and JC2R (see Additional file 1, Table S1). With the same primers, 75% of

symptomatic samples from Kenya tested positive while 20% of asymptomatic samples were detected positive.

For cassava, when symptomatic samples were amplified with primer pair JC3F and JC4R, 63% were positive while all asymptomatic samples tested negative. The primer pair JC6R and JC2R tested all (100%) symptomatic samples positive while only 6 % of asymptomatic plants were positive (see Additional file 2, Table S2).

Samples testing positive with primer JC6R and JC2R yielded bands with different intensity on gel electrophoresis, which were classified as weak positive (+), moderate positive (++) and strong positive (+++) (see Additional file 1, Table S1 and Additional file 2, Table S2). Only 2 cassava samples were co – infected with CBSV and CMG (see Additional file 2, Table S2).

Reverse transcription – polymerase chain reaction (RT–PCR)

All *Jatropha* samples tested negative for CMV and CBSV. The cassava samples were negative for CMV and only one sample tested positive for CBSV.

Sequences and phylogenetic analysis of DNA A

Complete nucleotide sequences of forty DNA A components typical of Begomoviruses in the Kenyan samples were determined, of which 34 sequences were from *Jatropha* and 6 from cassava (see Additional file 3, Table S3).

Figure 2 shows a phylogenetic comparison of the complete DNA A sequences of the Begomoviruses isolates obtained from *Jatropha* and cassava in this study and other Begomoviruses associated with the two host plants publicly available in the Genbank (Table 3) (Bock et al., 1981; Stanley and Gay, 1983; Morinaga et al., 1987; Hong et al., 1993; Zhou et al., 1997; Fondong et al., 2000; Berrie et al., 2001; Saunders et al., 2002; Maruthi et al., 2004; Ndunguru et al., 2005; Ndunguru et al., 2005; Bull et al., 2006; Rothenstein et al., 2006; Alabi et al., 2008; Sserubombwe et al., 2008; Monde et al., 2010). Phylogenetic analyses clearly indicated that they belong to CMG involved in CMD as they had close identities with sequences already deposited in public databases. All viruses characterised in this study could be grouped with two previously identified Begomoviruses found in cassava in Western Kenya, namely EACMV – UG and ACMV, but not with the species EACMKV [GenBank: NC011583.1], EACMZV [GenBank: NC004655.1] and strain EACMV – KE [GenBank: AJ717552.1], which were found in Kenya previously (Bull et al., 2006). The viruses also did not group with EACMV – TZ [GenBank: AY795987.1], EACMCV – TZ [GenBank: AY795983.1], EACMCV – CM [GenBank:

NC004625.1], EACMMV [GenBank: AJ006459.1], SACMV [GenBank: NC003803.1], ICMV – IN [GenBank: NC001932.1], ICMV – ker [GenBank: AJ575819.1], SLCMV – IN [GenBank: AJ890224.1] and SLCMV – LK [GenBank: AJ314737.1] found elsewhere in Africa and Asia (Hong et al., 1993; Harrison et al., 1997; Zhuo et al., 1997; Saunders et al., 2002; Maruthi et al., 2004). No close relationship was shown between the viruses and *Jatropha* begomoviruses from Asia and South America; JCMV [GenBank: GQ924760.1], CYVMV [GenBank: EU727086.2], JYMIV [GenBank: NC011309.1] and JLCV [GenBank: NC011268.1].

The first group of viruses comprises ACMV, showing nucleotide (nt) identity from 95% (isolate JN053426) to 97.3 % (isolates JN053431 and JN053430) with the ACMV reference sequence [GenBank: NC001467.1] (see Additional file 3, Table S3). The second group is closely related to, but distinct from the strains EACMV – KE and EACMV – TZ. The sequences showed approximately 90.7% nt identity (JN053450) to 92.3% nt identity (JN053440 and JN053444) with the EACMV – KE [GenBank: AJ717552.1] and only 90% nt identity (JN053451, JN053441, JN053452 and JN053453) to 90.5% nt identity (JN053433, JN053440, JN053442, JN053444 and JN053446) with EACMV – TZ [GenBank: A1795987.1]. To indicate that they are clearly isolates of the strain EACMV – UG, they had high nt identity ranging from approximately 94.1% (isolate JN053439) to 98.7% (isolates JN053440, JN053442 and JN05344) with the EACMV – UG [GenBank: NC004674.1] reference sequence (see Additional file 3, Table S3).

The viruses infecting *Jatropha* in Western Kenya occur on overlapping territories, since ACMV and EACMV – UG were both found in all the districts analysed (see Additional file 3, Table S3). EACMV – UG occurred with a higher prevalence than ACMV. Generally speaking, out of 34 viral sequences found in *Jatropha* 24 (71%) were EACMV – UG, while 10 (29%) were ACMV. In Busia, a district neighbouring Uganda, EACMV – UG was most prevalent with 15/24 (63%) compared to 7/24 (29%) and only 2/24 (8%) from Kakamega and Siaya respectively. ACMV was found more frequently with 6/10 (60%) on samples from Kakamega district, compared to 2/10 (20%) from Siaya and Busia respectively.

ACMV Def DNA A

A Def DNA A was present in a *Jatropha* leaf samples collected from Western Kenya. PCR analyses with primer JC3F and JC4R of sample K1J5 amplified the expected 2.8 kb of a near full length DNA A component of the Begomovirus sequence and an additional shorter fragment

(Figure 3). Sequencing of the smaller fragment revealed a size of 1420 bp, which was named Def K1J5. This Def (Genbank JN101951) showed 96.6% nt identity with the ACMV reference sequence [GenBank NC001467.1] and a low nt similarity (69.7%) with the EACMV – UG reference sequence [Genbank NC004674.1] (see Additional file 3, Table S3). The complete sequences of DNA A components of ACMV reference sequence [GenBank NC001467.1] was used for size comparison with the ORFs of Def K1J5. On the virion sense strand, AV1 and AV2 were entirely missing. In the complementary sense strand, AC4 was the only intact gene at 422 bp while AC1 was 1070 bp long. Two ORFs, AC2 and AC3, which are found in DNA A of CMGs, had a size of 326 bp and 178 bp respectively (Figure 4). The IR contained the first 11 bp of the replication site of geminiviruses.

Discussion

Cultivated cassava is believed to be the principle reservoir for CMD associated begomoviruses because of its perennial growth and scale of production. However, alternative hosts have been identified, including *Manihot glaziovii* Müll, a wild relative of cassava native to Brazil (Sserubombwe et al., 2008), *Senna occidentalis, Leucana leucocephala, Combretum confertum, Centrosema pubescens* and *Pueraria javanica* (Alabi et al., 2008; Monde et al., 2010). A strain of *Cassava latent geminivirus* (CLV – V) was previously isolated from naturally infected *Jatropha multifida* growing in the Coastal districts of Kenya (Bock et al., 1981). First reports on geminivirus infections on *J. curcas* indicated the occurrence of *Jatropha curcas virus* closely related to *Cassava mosaic virus* in India, reaching a disease incidence from 25 to 47% (Aswatha Narayana et al., 2007; Raj et al., 2008b; Gao et al., 2010) A phylogenetic analysis of the virus genome showed that *Jatropha curcas* mosaic disease (JCMD) is caused by *Jatropha curcas virus,* a new strain of *Indian cassava mosaic virus* (ICMV) (Gao et al., 2010). *Jatropha* is further host of CMV (family *Bromoviridae Cucumovirus*) (Raj et al., 2008a). In this study, we report for the first time the detection of *Begomovirus*: ACMV and EACMV – UG in *Jatropha*. Furthermore, a defective molecule derived from DNA A of the bipartite *Begomovirus* ACMV was detected in *J. curcas.* Also the presence of a co – infection with CMG and CBSV was found in cassava plants.

The phylogenetic tree of the complete DNA A sequences indicates that the ACMV and EACMV – UG isolates were closely related to those isolated previously in Western Kenya (Bull et al., 2006; Stanley et al., 1983). From an evolutionary perspective, it is an indication that the

geminiviruses infecting *Jatropha* from the sampled areas are as a result of spread of viruses from an inoculum source occurring where the plants are growing. This is further supported by the idea that the EACMKV, EACMV – KE and EACMZV previously identified (Bull et al., 2006) from Eastern and Coastal parts of Kenya were not found to be present in *Jatropha*, since the viruses have a distinct geographical distribution (Bull et al., 2006). Geminivirus dissemination occurs through cuttings or whiteflies. The viruses identified in this study were not closely related with those infecting cassava and *Jatropha* in other parts of Africa, Asia and South America suggests that there has been no movement of infected *Jatropha* cuttings and viruliferous whiteflies from other areas to Western Kenya. In fact, recombination results in severe epidemiological consequences such as the emergence of isolates with increased virulence capable of overcoming host resistance or with a host range wider than the original one (Lozano et al., 2009). Recombination and synergism that have long occurred in cassava (Harrison et al., 1997; Zhou et al., 1997; Fondong et al., 2000; Pita et al., 2001; Legg and Fauquet, 2004; Ndunguru et al., 2005; Bull et al., 2006) could have led to the current spread of the virus in the field to infect *Jatropha* plants. The recombinant EACMV – UG was the most prevalent strain virus found whilst the other strains of EACMV were not identified. In line with this are previous claims, that EACMV in Western Kenya has been largely displaced by EACMV – UG, which is considered a more virulent strain (Bull et al., 2006). In the current study the presence of EACMV – UG and ACMV on different *Jatropha* plants in the same field indicates the opportunity for mixed infections. For example plants K4J1 (EACMV – UG isolate JN053453) and K4J2 (ACMV isolate JN053425) (Figure 2) stand close to each other in the same field, hence offering good opportunities for more recombination to occur. EACMV – UG and ACMV are associated with severe synergistic epidemics on cassava that swept through Uganda and continues to affect surrounding countries including Kenya (Zhuo et al., 1997; Legg and Fauquet, 2004; Were et al., 2004; Bull et al., 2006; Fondong et al., 2007). Specifically the two viruses have differentially acting suppressors of PTGS overcoming the hosts defence mechanisms (Vionnet et al., 1999; Vionnet, 2001; Vanitharani et al., 2004, Vanitharani et al., 2005; Bisaro, 2006). ACMV (recovery – type) has a strong AC4 suppressor and EACMV – UG (non – recovery – type) has a strong AC2 suppressor causing unusually severe symptoms. As a result, ACMV will leave only EACMV – UG to be spread and become the predominant virus in the area (Were et al., 2003; Vanitharani et al., 2005; Bisaro, 2006). In the absence of a synergistic interaction, only one virus in a co – infected plant

will become predominant and persist (Vanitharani et al., 2004, Vanitharani et al., 2005; Bull et al., 2006). Synergism may lead to a 10 – 50 fold increase in viral DNA accumulation which substantially increases the potential for a higher efficiency of vector transmission to even infect non cassava host plants (Legg and Fauquet, 2004; Monde et al., 2010). This might further explain, why the EACMV – UG appears as predominant virus in *Jatropha*. Co – infection of CMG and CBSV threatens cassava production in Busia distict of Kenya. Recent studies have shown how evolution is shaping the populations of CBSV and *Uganda cassava brown streak virus* (UCBSV) in cassava causing significant problems (Mbanzibwa et al., 2009; Winter et al., 2010; Mbanzibwa et al., 2011). Mixed infection results in increase in the titer of one or both viruses and elicits disease symptoms that are more sever than the sum of those induced in single infection (Vanitharani et al., 2004; Untiveros et al., 2007).

In addition to genomic components, smaller sized Def DNA often occurs naturally in geminivirus infected plants (Patil et al., 2007). The plant, from which the defective DNA molecule was isolated in the current study, did not display particular symptoms differing from the neighbouring plants growing in the same field, meaning that it could not have been picked up on purpose due to a previous selective decision. The Def DNA molecule found in the plant K1J5 had lost the entire AV1 and AV2 genes and large portions of other genes. Sub genomic Def DNA molecules associated with a number of Begomovirus seem to be fairly uniform in structure and retain their IR and a large portion of AC1 (Ndunguru et al., 2006) as observed also in this study. These deletions might affect the replication of the molecule and it might depend entirely on its helper virus for replication. Geminivirus Def DNA invariably rely on the respective viruses for replication as observed for *Tomato leaf curl virus* that lacked an ORF required for replication and encapsidation and were not expected to be capable of autonomous replication, however they were replicating in the presence of the viral DNA (Behjatnia et al., 2007). In cassava, Def DNA has been previously reported occurring in DNA A of EACMV and DNA B of ACMV, and were found to be associated with a delay in symptom development and amelioration (Stanley et al., 1990; Ndunguru et al., 2006). However, no naturally occurring Def DNA A of ACMV has been found previously in *Jatropha curcas* and we report it for the first time. The role of this small Def DNA molecule in the biology of ACMV in *Jatropha* in nature is still unclear.

Conclusion

We have shown for the first time cassava Begomoviruses and their associated sub – genomic Def DNA molecules to be naturally occurring in field growing *Jatropha* plants. The occurrence of the Begomoviruses further poses a challenge in the elimination strategy of CMG in field grown cassava as a result of increase in inoculum from different hosts, and calls for an elimination strategy of the viruses in *J. curcas* in order to save the crop which is an important biofuel and pharmaceutical crop. Molecular detection techniques showed the presence of geminivirus even in asymptomatic plants. The new natural host (*J. curcas*) of the two viruses opens new avenues for further recombination of the viruses to occur which indeed becomes a threat both to cassava an important food crop to Sub Saharan countries and *Jatropha*. There is a possibility of *Cassava mosaic virus* in *Jatropha* being more widespread than anticipated, since we have detected it also in *Jatropha* samples from Ethiopia. This has led to hypothesize that other neighboring countries growing *Jatropha* could be facing similar challenges with this plant. The primer pair JC6F and JC2R amplifying a sequence of 380 bp allows the detection of Begomoviruses in symptomatic and asymptomatic cassava and *Jatropha* plants and can therefore be recommended for a large scale screening of field samples.

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Table 1**.** List of the oligonucleotides used in this study

Primer designation*	**Primer sequence (5′ to 3′)**
JC1F	GGAAGATAGTGGGAATGCCNCCTTTAATTTGAA
JC2R	AARGAATTCATGGGGGCCCARAGRGACTGGC
JC3F	RTCGACGTCATCAATGACGTTGTACCAKGCG
JC4R	GTHGAYCCSCACTAYCTMAARCACTTCAARG
JC5R	GGCCATCCGGTAATATTAWWCGGATGG
JC6F	CCATTCATTGCTTGAGGAGCAGTG

*Primer designation: F denotes forward R means reverse.
Primer sequence: R represents A or G; K represents G or T; H represents A, C or T; Y represents C or T; S represents G or C; M represents A or C; W represents A or T.

Table 2. Primer combinations and annealing temperatures used to detect geminiviruses in *Jatropha curcas* and *Manihot esculenta* in PCR and RCA

Forward primer	**Reverse primer**	**Length**	**Annealing temperature**	**Part of genome amplified by PCR and RCA**
JC1F	JC5R	1085 bp	60°C	PCR: amplifies part of AC1 and entire AC4
JC3F	JC5R	971 bp	63°C	PCR : amplifies part of AC1 and entire AC4
JC3F	JC4R	2800 bp	64°C	RCA : amplifies the entire DNA A
JC3F	JC2R	2800 bp	64°C	RCA : amplifies the entire DNA A
JC6F	JC4R	410 bp	55°C	PCR : amplifies part of AC2 and AC3
JC6F	JC2R	380 bp	55°C	PCR : amplifies part of AC1, AC2 and AC3

Table 3. Geminiviruses used for comparisons, assigned abbreviations and their genomic sequence accession numbers

Virus	Abbreviation	Genbank accession number	Reference
African cassava mosaic virus	ACMV	FN435271.1	(Monde et al., 2010)
	ACMV	EU685320.1	(Alabi et al., 2008)
	ACMV	AF259894.1	(Pita et al., 2001)
	ACMV	AJ427910.1	(unpublished; Briddon)
	ACMV	AY211884.1	(Fondong et al., 2000)
	ACMV	NC001467.1	(Stanley and Gay, 1983)
	ACMV	AY795982.1	(Ndunguru et al., 2005)
Bean golden yellow mosaic virus	BGYMV	NC001439.1	(Morinaga et al., 1987)
Croton yellow vein mosaic virus	CYVMV	EU727086.2	(unpublished; Raj et al.)
East African cassava mosaic Cameroon virus – Cameroon	EACMCV – CM	NC004625.1	(Fondong et al., 2000)
East African cassava mosaic Cameroon virus – Tanzania	EACMCV – TZ	AY795983.1	(Ndunguru et al., 2005)
East African cassava mosaic virus – Kenya	EACMV – KE	AJ717552.1	(Bull et al., 2006)
East African cassava mosaic virus – Tanzania	EACMV – TZ	AY795987.1	(Ndunguru et al., 2005)
East African cassava mosaic virus – Uganda	EACMV – UG	AJ618959.1	(Sserubombwe et al., 2008)
	EACMV – UG	FN435279.1	(Monde et al., 2010)
	EACMV – UG	NC004674.1	(Pita et al., 2001)
	EACMV – UG	AJ717524.1	(Bull et al., 2006)
	EACMV – UG	AJ717532.1	(Bull et al., 2006)
	EACMV – UG	AJ717533.1	(Bull et al., 2006)
	EACMV – UG	AJ717534.1	(Bull et al., 2006)
	EACMV – UG	AJ717535.1	(Bull et al., 2006)
	EACMV – UG	AM502329.1	(Sserubombwe et al., 2008)
	EACMV – UG	AM502331.1	(Sserubombwe et al., 2008)
	EACMV – UG	AF126806.1	(Pita et al., 2001)
	EACMV – UG	FN435280.1	(Monde et al., 2010)
	EACMV – UG	AY795988.1	(Ndunguru et al., 2005)
East African cassava mosaic Kenya virus	EACMKV	NC011583.1	(Bull et al., 2006)
East African cassava mosaic Malawi virus	EACMMV	AJ006459.1	(Zhou et al., 1997)
East African cassava mosaic Zanzibar virus	EACMZV	NC004655.1	(Maruthi et al., 2004)
Indian cassava mosaic virus – India	ICMV – IN	NC001932.1	(Hong et al., 1993)
Indian cassava mosaic virus – Kerela	ICMV – Ker	AJ575819.1	(Saunders et al., 2002)
Jatropha curcas mosaic virus	JCMV	GQ924760.1	(Gao et al., 2010)
Jatropha leaf curl virus	JLCV	NC011268.1	(Unpublished; Pal and Mukherjee)
Jatropha yellow mosaic India virus	JYMIV	NC011309.1	(Unpublished; Raj et al.)
South African cassava mosaic virus	SACMV	NC003803.1	(Berrie et al., 2001)
Sri Lankan cassava mosaic virus – India	SLCMV – IN	AJ890224.1	(Rothenstein et al., 2006)
Sri Lankan cassava mosaic virus – Sri Lanka	SLCMV – LK	AJ314737.1	(Saunders et al., 2002)

Figure 1. Map of Kenya showing the sites of cassava and *Jatropha* plant material sampling (black triangles). A survey was conducted in Western Kenya and Rift valley where the two plants are being grown together.

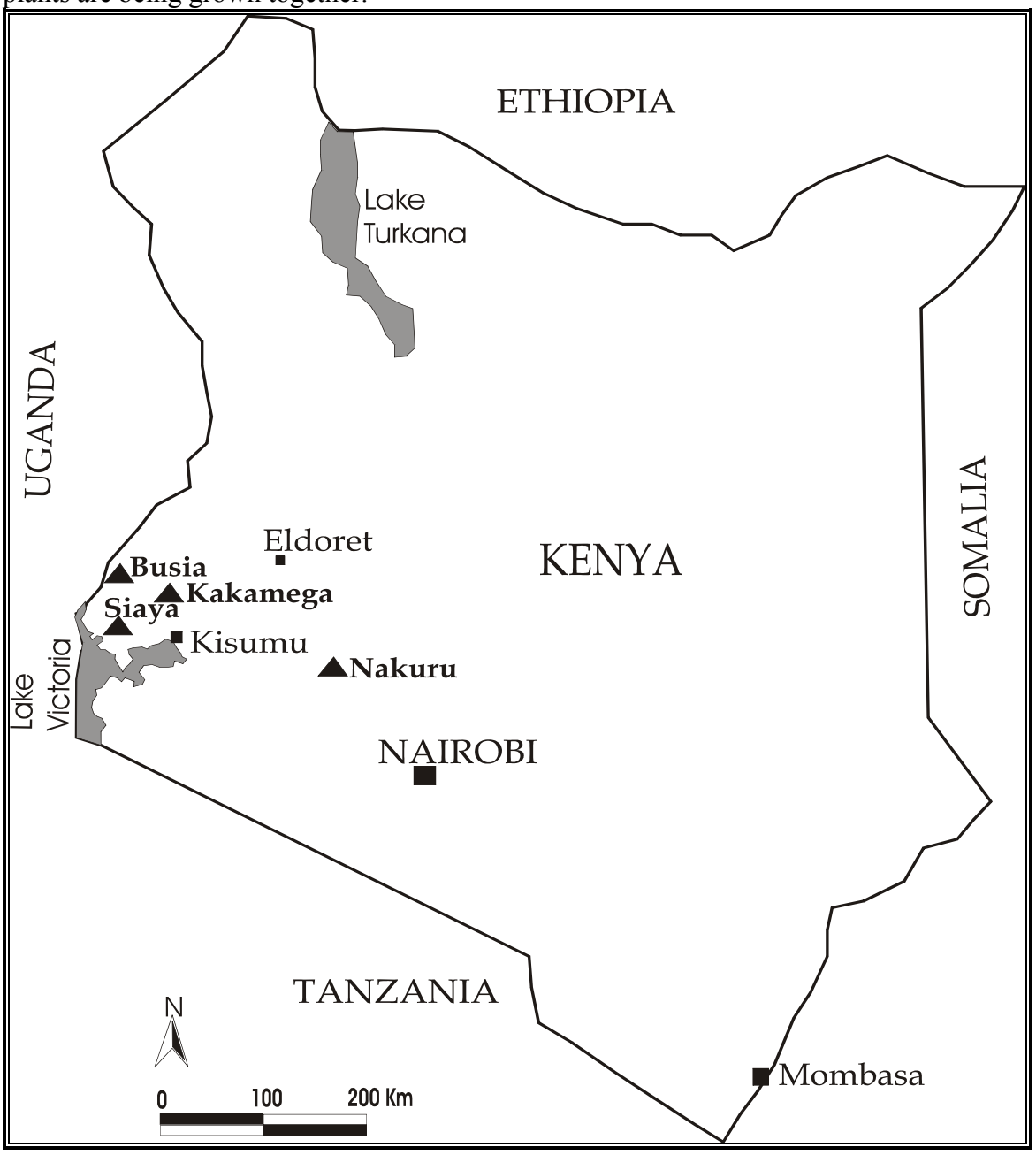


Figure 2. Phylogenetic alignment of nucleotide sequences of cassava mosaic geminivirus isolates obtained from *Jatropha* and cassava in the study and other related Begomoviruses from the Genbank. Viruses obtained from the study have been written in bold. Geminivirus type group species BGYMV was used as an out group. Abbreviations and Genbank Accession numbers are given in Table 3.

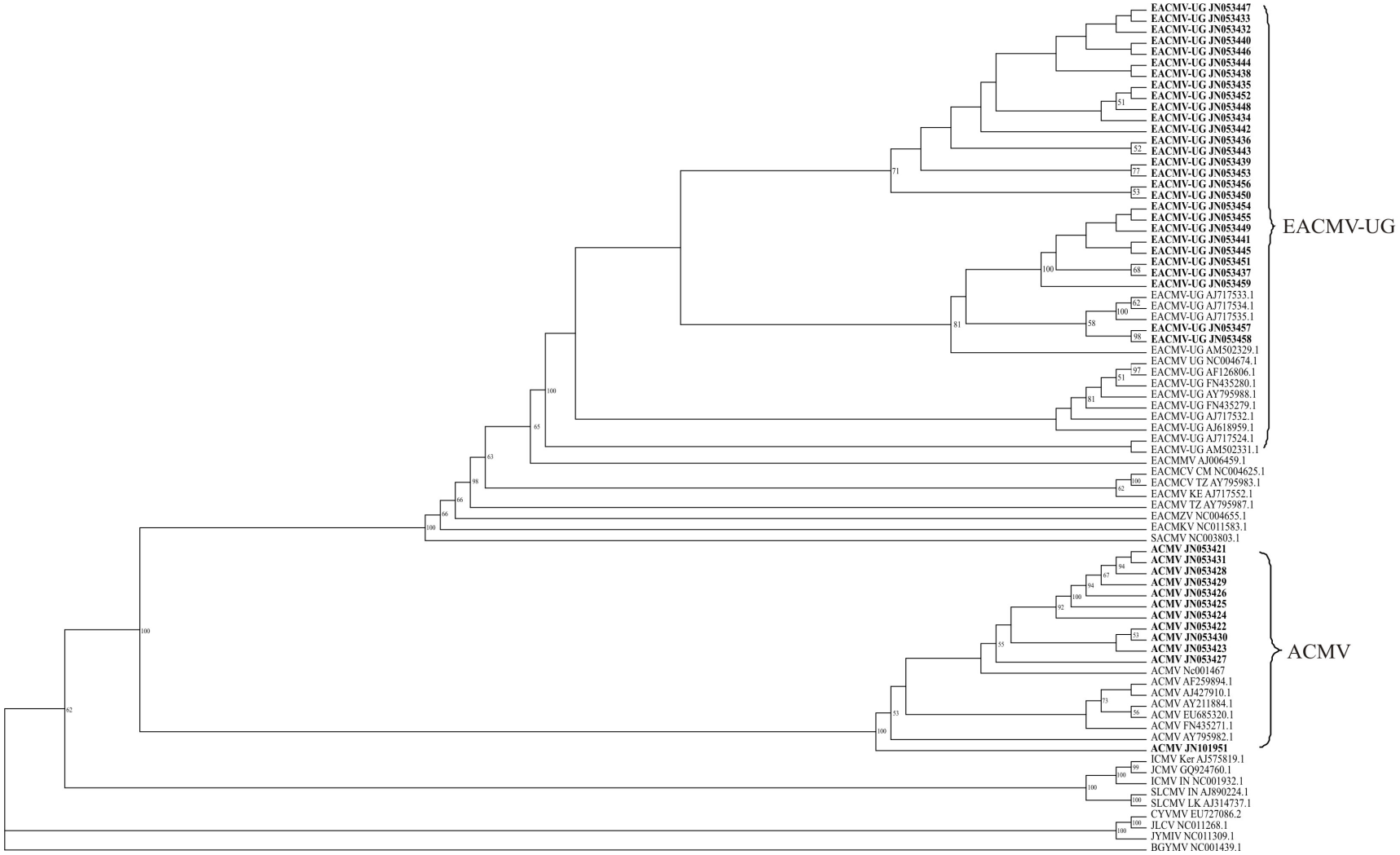


Figure 3. PCR amplification of defective DNA A of ACMV from *Jatropha*. PCR amplification was performed using primer JC3F and JC4R on DNA extracted from field – grown *Jatropha* plants. Lane 1 shows the defective DNA A, lane C+ and C- positive and negative controls. Lane M = marker VIII (Roche Applied Science)

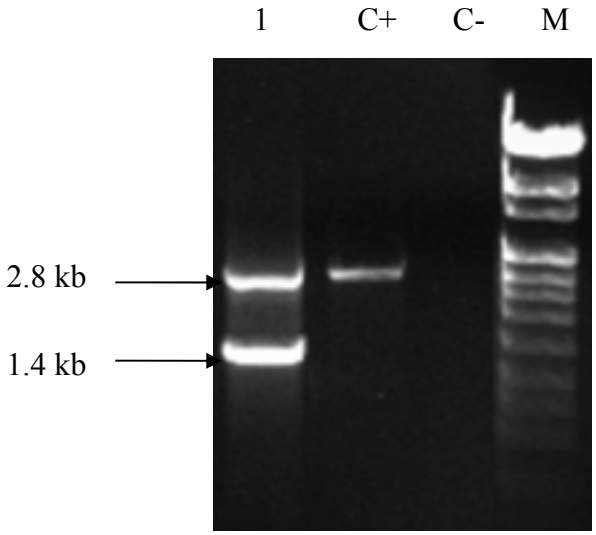
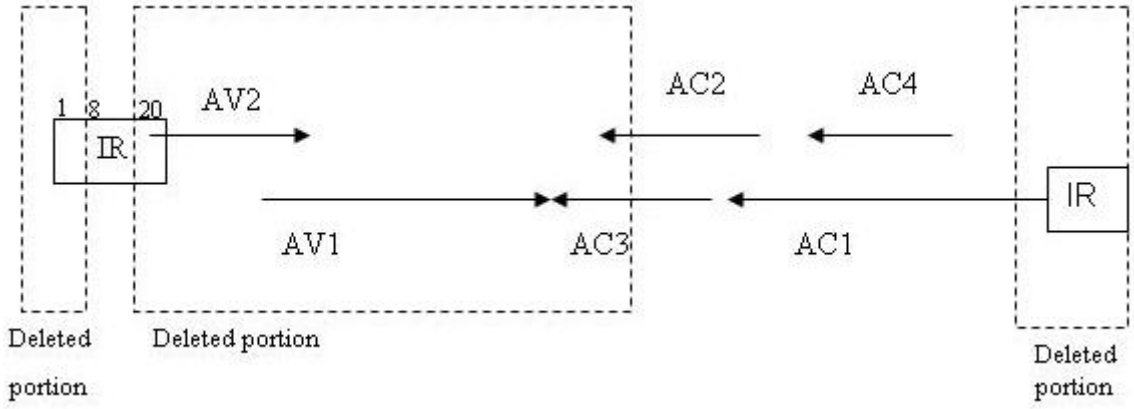


Figure 4. Schematic genome organization of subgenomic Def K1J5. Predicted ORFs in both directions: AC1 – 4 are complementary sense strands; AV1 and AV2 are virion sense strands. The deleted part of the genome based on the known genome of a full length DNA A component of ACMV [GenBank NC001467] is shown (dashed). Nucleotide positions 1, 8 and 20 are indicated.



CHAPTER V:

Virus (pathogen) versus plant (host) miRNAs: who determines the outcome of the battle?

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Key words: Pathogen defense, *African cassava mosaic virus*, *East African cassava mosaic virus-Uganda*, *Jatropha*, Cassava, biofuel, miRNA prediction, virus miRNA, target prediction

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Abstract

Considering the importance of miRNAs in the regulation of essential processes in plants including defense mechanisms, it is not surprising to find that some plant viruses encode miRNA able to interfere with viral or host cell gene expression. In the current study, computational approaches were performed to a) predict virus encoded miRNA in 39 complete DNA-A sequences of 11 African cassava mosaic virus (ACMV) and 28 East African cassava mosaic virus-Uganda (EACMV-UG) isolates, b) investigate whether virus encoded miR/miR* sequences have the capacity to bind to genomic sequences of the host plants Jatropha or cassava and c) investigate whether plant encoded miR/miR* sequences have any potential to bind to the DNA-A genome of the pathogens ACMV and EACMV-UG.

14 different viral pre-miRNA hairpin sequences and 111 viral miR/miR* occurring as isomiRs were identified (84 miR/miR* correspond to ACMV and 27 miR/miR* to EACMV-UG). These virus miRNA were located in ORFs AC1, AC2, AC3 and in the intergenic region. In addition, target genes for virus miRNA were predicted in the genomes of Jatropha and cassava, indicating that they are involved in biotic response, metabolic pathways, binding and transcriptional factors.

In addition, 84 plant miR/miR* were identified that could target the ORFs of both ACMV and EACMV-UG, representing potential plant miRNA mediating antiviral defense. 39 miR/miR* were found to target the AC1 gene, indicating a cooperative regulation.

This is the first collection of predicted viral miR/miR* of ACMV and EACMV-UG and host plant miRNAs, providing a reference point for further studies of miRNA identification in pathogens and their host plant. These findings will also improve the understanding of host-pathogen interaction pathways and the function of viral miRNAs in plants.

Introduction

Jatropha curcas (*Euphorbiaceae*) is a drought resistant plant, native to tropical America, now widely cultivated in tropical and subtropical regions for harvesting a unique oil contained in its seeds, which can be a source of raw material for the production of biofuel (Vollmann and Laimer, 2013; Maghuly et al., 2013). On the other hand, cassava (*Manihot esculenta* Crantz) is a key staple food in sub-Saharan Africa and a potential source of biomass for bioethanol production. However, these economically important crops can be infected by several geminiviruses (Aswatha Narayana et al., 2007), which reduce their productivity. The genomes of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus–Uganda* (EACMV-UG), members of the genus *Begomovirus*, consist of DNA-A and DNA-B, each of 2.7 – 3 kb (Ramkat et al., 2011). The DNA-A component encodes six overlapping open

reading frames (ORFs) (Naqvi et al., 2011). The virion ORFs AV1 and AV2 are located on the sense strand and the ORFs AC1, AC2, AC3 and AC4 on the complementary strand. AV1 (coat protein/CP) is essential for viral transmission by whiteflies, *Bemisia tabaci,* while AV2 (precoat protein) is involved in virus movement (Höfer et al., 1997). AC1 (replication associated-protein/Rep) is required for replication (Paszkowski et al., 1993), while AC2 (transcriptional activator protein /TrAP), required for the transcription – activation of plus strand genes**,** is also involved in suppression of post – transcriptional gene silencing (PTGS) (Voinnet et al., 1999). AC3 (replication enhancer protein/REn) enhances viral DNA accumulation (Etessami et al., 1991). AC4 (pathogenicity enhancer protein) plays a role in pathogenicity and PTGS (Vanitharani et al., 2004). The intergenic region (IR) contains the invariant TAATAT/AC motif responsible for the initiation of rolling circle DNA replication (Fontes et al., 1994).

RNA silencing is a conserved defense mechanism that plants and other eukaryotes use to protect their genomes against aberrant nucleic acids. This process uses short RNAs (20-30 nt) to recognize and manipulate complementary nucleic acid (Baulcombe, 2004). Any pathogen able to establish a successful infection must evade this line of defense (Naqvi et al., 2011). As a result, several viruses encode ORFs termed suppressors of PTGS that compromise the RNA silencing pathways of the host plant, including both microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Dunoyer and Voinnet, 2005). In this way viruses can control viral and host gene expression (Sullivan and Ganem, 2005; Gao et al., 2012).

miRNAs constitute a class of small RNAs of 21-24 nucleotides that regulate gene expression at the post-transcriptional level by targeting specific messenger RNAs (mRNAs) for cleavage or translational repression (Jones-Rhoades et al., 2006). They are expressed in all plants and animals, as well as in several viruses (Jones-Rhoades et al., 2006; Anselmo et al., 2011; Grundhoff, 2011; Grundhoff and Sullivan, 2011; Gao et al., 2012). Primary miRNA (pri-miRNA) transcripts are first cleaved by the nuclear based Dicer like enzyme, resulting in the release of short stem loop precursor miRNA (pre-miRNA) (Bartel, 2004). miRNA genes are transcribed from long pre-miRNAs ranging 70 to 300 nucleotides in which mature miRNA resides either in the 5′or 3′ arm (Ambros et al., 2003; Bartel and Bartel, 2003). miRNAs are then processed from stem-loop regions by a cytoplasm based Dicer like enzyme and are loaded into the RNA-induced silencing complex (RISC) where they directly cleave mRNAs (Bartel, 2004).

There are different methods for identifying miRNA including cloning, NextGen sequencing and computational approaches. Due to the difficulty to systematically detect miRNAs from a

host or pathogen genome by available experimental techniques, especially for those with low expression (Shen et al., 2012) the computational approach has been applied to identify miRNAs (Berezikov et al., 2006; Zhou et al., 2009; Wang et al., 2011). At present, computational approaches can be divided into three types: a) the integrated approach based on algorithms, b) comparative genomic approach based on evolutionary conservation and c) *ab initio* prediction based on sequence and structure features (Berezikov et al., 2006). An integrated approach uses two or more computational approaches to improve the sensitivity or specificity of predictions (Xiao et al., 2009). The comparative genomic approach is not suitable for virus miRNAs prediction, since for many viruses, only very distant evolutionary orthologs are known (Grundhoff et al., 2006). Thus *ab initio* prediction methods appear the method of choice (Jiang et al., 2007). In the current study we performed a combination of different *ab initio* computational approaches to investigate if a) virus miRNAs have the capacity to interact with host ORFs or b) host miRNAs have any possibility to suppress virus ORFs.

Due to the devastating impact by begomoviruses on Jatropha and cassava, the situation calls for the provision of stable virus resistant plants to offer a long term solution. Based on the knowledge that members of the *Euphorbiaceae* family share the same pathogens (Ramkat et al., 2011), identifying miRNAs both of pathogens and hosts will improve the understanding of host pathogen interaction and in turn benefit *Euphorbiaceae* health. The obtained results reveal important implications for miRNAs encoded by begomoviruses, which are located in genes acting as suppressor of PTGS, and their targets in plant pathogenesis related pathways. They also show how plants can utilize cooperative regulation by employing multiple miRNAs to target the ORFs of ACMV and EACMV-UG to strengthen their defense mechanisms against viruses.

Methods

Datasets Thirty three complete DNA-A sequences (9 from ACMV and 24 from EACMV-UG) isolated from infected *Jatropha curcas* and six complete DNA A sequences (2 from ACMV and 4 from EACMV-UG) isolated from infected cassava (4) were used to predict virus miRNAs (Table 1). *Jatropha* and cassava expressed sequence tags (ESTs) from the GenBank (http://www.ncbi.nlm.nih.gov/genbank) were used to predict virus miRNA targets.

Potential ACMV and EACMV-UG miRNA hairpins

The VMir Analyzer (Grundhoff et al., 2006; Sullivan and Grundhoff, 2007) was used to identify novel miRNA hairpins encoded by ACMV and EACMV-UG. Each virus sequence (Table 1) was processed individually. Since both viruses have a circular genome and to

display all hairpins in direct or reverse orientation, for conformation and orientation the options "circular" and "any" were chosen. To obtain the main hairpins, the results from each sequence were further filtered using VMir Viewer (Sullivan and Grundhoff, 2007). The filter values for "minimal score" and "window counts" were set to the most stringent parameters of 115 and 35 respectively (Sullivan and Grundhoff, 2007). After the completion of the prediction, the recorded hairpins were compared to one another and categorized to retain only the largest, i.e. the main hairpin (Grundhoff et al., 2006). Also the web interface Vir-Mir db (http://alk.ibms.sinica.edu.tw (Li et al., 2008) was searched for predicted viral hairpins.

Classification of hairpins and prediction of secondary structures

The viral hairpins obtained after filtering, were further screened with MiPred (http://www.bioinf.seu.edu.cn/miRNA/) to distinguish real from pseudo pre-miRNA, using a hybrid feature, including local contiguous structure sequence composition, minimum of free energy (MFE) of the secondary structure and *P*-value of randomization test (Jiang et al., 2007). For any pre-miRNA like hairpin the random forest-based classifier predicts whether it is a real pre-miRNA (MFE < -20 kcal/mol, *P*-value < 0.05 and continuously paired nucleotides at high frequencies) or pseudo pre-miRNA (MFE > -20 kcal/mol, *P*-value > 0.05 and continuously unpaired nucleotides at high frequencies (Jiang et al., 2007; Chen et al., 2009).

The secondary structures of hairpins grouped as real pre-miRNA were predicted using the RNAshapes (Steffen et al., 2006).

Prediction of virus miR/miR* with capacity to bind Jatropha and cassava ESTs as targets

The ACMV and EACMV-UG real pre-miRNAs sequences with MFE< -20 kcal/mol, were compared to ESTs of *Jatropha* and cassava using BlastN with a sensitive setting of word length 7 (Wang et al., 2005). Sequences of 17-24 bp with < 5 mismatches located directly on the 5´and 3´arms of the hairpin structures were retained and used in RNAhybrid (**http://bibiserv.techfak.unibielefeld.de/rnahybrid/submission_.html** (Rehmsmeier et al., 2004) and psRNATarget (**http://plantgrn.noble.org/psRNATarget** (Dai and Zhao, 2011) to identify complementary regions of predicted virus miRNAs in *Jatropha* and cassava. The selection of targets of miRNAs by RNAhybrid considered the following parameters : a) at least 17 of 21 nucleotides should exhibit complementarity with their target sequence, b) the seed region should exhibit high sequence complementarity (only 1 mismatch allowed), c) any mismatch in the seed region should be compensated by strong binding beyond the seed region (Naqvi et al., 2011), d) end overhangs should not be more than 2 nucleotides, e) G:U base

pairs should not be treated as mismatches (Alves et al., 2009), f) the miRNA:target pair should have a low free energy of binding (maximum - 20 kcal/mol). The latter criterion was used for miRNA target prediction in various plants (Zhang et al., 2006; Pérez-Quintero et al., 2010). To score the complementarity between miRNA and their target transcript, the default cut-off threshold 0-2.0 for lower false positive and 4.0-5.0 for higher prediction coverage were used in psRNATarget analyses. miRNA sequences from the 5´and 3´arm were represented as miR and miR*, respectively.

Plant miR/miR* with capacity to bind the ACMV and EACMV-UG genome as a target

The possible existence of plant miRNAs having the potential of binding to ACMV (Acc. no. JN053423; JN053421) and EACMV-UG ORFs (Acc. no. JN053454; JN053447) was investigated (Table 1). All currently known plant mature miRNAs were obtained from the miRBase, release 18 (http://www.mirbase.org). To avoid any redundant or overlapping miRNAs, the non-redundant miRNA sequences were extracted as query sequences for the Blast search. A total of 1552 miR/miR* plant sequences were used to predict viral targets on ACMV and EACMV-UG by employing RNAhybrid and the psRNATarget (Rehmsmeier et al., 2004). The stringency parameters for RNAhybrid were set as follows: 3 hits per target, -25 kcal/mol energy cut-off and maximum 1 bulge or loop size per side. The same parameters as described above were used to select putative miR/miR* with the best hybridization sites on the genome for both viruses. To score the complementarity between miRNA and their target transcript, for psRNATarget, the default parameters were used as described above.

psRNATarget was further used to predict targets for the plant miR/miR* found to target the ACMV and EACMV-UG genome on the *Jatropha* and cassava ESTs.

Results and discussions

Prediction of miRNAs from ACMV and EACMV-UG genomes

Computational approaches were used for the first time to scan and filter the DNA-A genomes of 11 ACMV and 28 EACMV-UG isolates to identify novel pre-miRNA hairpins encoding miRNAs which could target the *Jatropha* and cassava genome (Table 1). Figure 1 gives an overview of the strategies adopted for the search and prediction of novel virus (ACMV and EACMV-UG) miRNA addressing targets in their host plants *Jatropha* and cassava.

As a result, a total of 14 different predicted pre-miRNA hairpin sequences (9 from ACMV and 5 from EACMV-UG) were obtained (Table 2). A summary of the hairpin sequences, the number of sequences encoding the hairpin and their location in the virus genome is shown in Table 2.

One of the important features that distinguish miRNAs from other endogenous small RNAs is the ability of the pre-miRNA sequence to form a stem loop hairpin structure (Ambros et al., 2003; Berezikov et al., 2006). It is also known that the secondary structure of pre-miRNA is an essential feature for the computational identification of miRNAs (Xue et al., 2005; Xuan et al., 2011). Furthermore, the presence of many pseudo hairpins made the filtering of hairpins in the computational analysis necessary. For this purpose, the MiPred was used to show that out of the 14 different predicted pre-miRNA, 10 were real (ACMV 1-8, EACMV-UG 1-2) and 4 were pseudo miRNA precursors (ACMV 9, EACMV-UG 3-5) (Table 2).

The length of the real pre-miRNA hairpin sequences ranged from 62 bp (ACMV 8) to 100 bp for the longest hairpin (ACMV 6). Pseudo pre-miRNA harpins varied from 65 bp (EACMV-UG 5) to 84 bp (EACMV-UG 3) (Table S1). The MFE ranged from -19 kcal/mol (ACMV 8) to -38.7 kcal/mol (ACMV 6) for real pre-miRNA hairpins, and from -20.5 kcal/mol (ACMV 9) to -34.4 kcal/mol (hairpin EACMV-UG 3) in the pseudo pre-miRNA harpins. Prediction confidence values ranged from 52.10% (ACMV 1) to 75.40% (ACMV 4) for real and from 50% (EACMV-UG 3) to 77% (ACMV 9) for pseudo pre-miRNA hairpins (Table S1). Although the real and pseudo pre-miRNAs had similar ranges of MFE, prediction confidence and sequence length, the results show clearly that most *P*-values are very low, all real pre-miRNA hairpins having *P*-value < 0.03. Within the pseudo pre-miRNAs only EACMV-UG 4 had a low *P*-value of < 0.02. Comparing each of the basic MiPred structure units between real and pseudo pre-miRNA hairpins showed that the real pre-miRNA structures have continuously paired nucleotides [“)))” or “(((“], appearing at higher frequencies than in the pseudo pre-miRNAs where unpaired structures like [“...”] appear more often (Table S1), as described by (Xue et al., 2005) and (Jiang et al., 2007). Since the real hairpin ACMV 8 had a MFE > -20 kcal/mol, only 9/10 real pre-miRNA hairpin sequences (7 from ACMV and 2 from EACMV-UG) were retained for further analyses,

A stable secondary structure is a prerequisite functionally critical for early stages of the mature miRNA biogenesis to avoid early degradation (Bartel, 2004). Nucleotides G and C contributes to the stabilization of the secondary structure of a stem-loop hairpins and the more GC content a sequence contains, the more stable will be the secondary structure (Wang et al., 2012), it means the GC content of pre-miRNA should be between 24 and 71% (Jin et al., 2008). The nucleotide composition of the real viral pre-miRNA hairpins showed that the hairpins ACMV 7 and EACMV-UG 1 had the highest content of A+T (59.26 and 51.76 %, respectively) and ACMV 5 and EACMV-UG 2 had the highest C+G content (45.95 and 49.25%, respectively) (Table 3).

The localization of pre-miRNA hairpins in the ACMV ORFs showed that AC1, AC2, AC4 and IR encoded 2 pre-miRNA hairpins each, while AV2 encoded 1 pre-miRNA hairpin (Table 2). The pre-miRNA hairpins ACMV 3-5 were encoded by the highest number of ACMV sequences (8 out of 11, 72%, Table 2). In EACMV-UG, 1 pre-miRNA hairpin was encoded in by AC1, while 2 were encoded by AC2 and the IR (Table 2). The pre-miRNA EACMV-UG 1 was predicted by the highest number of EACMV-UG sequences (25 out of 28, 89%, Table 2). For both viruses no pre-miRNA hairpin could be located in AC3 and AV1 (Table 2). Interestingly, a comparison revealed no similarity between ACMV and EACMV-UG hairpins. The secondary structures of 9 real pre-miRNA hairpin sequences predicted by RNAShapes (Figure. 2) confirmed the data obtained by MiPred (Table S1).

When searching the Vir-Mir db (Li et al., 2008) for virus hairpins, only 3 deposited EACMV hairpins were retrieved. One of the EACMV sequences (ID 18042) predicted from NC-004674.1 (Li et al., 2008) was similar to the hairpin EACMV-UG 3 found in this study (Table 2). This hairpin was encoded by 23/24 (95%) EACMV-UG sequences. No virus hairpin sequence has been previously deposited in the Vir-Mir web for ACMV.

Virus miR/miR* isomer sequences and location on secondary structures

Due to lack of information on miRNA encoded by plant viruses and incomplete genome information from *Jatropha* and cassava, it was a challenge to locate candidate miRNAs on the hairpin sequences. Nine real pre-miRNA hairpin sequences were compared with the *Jatropha* and cassava ESTs using BlastN. A total of 980 *Jatropha* and 1240 cassava sequence hits between 12-36 bp were obtained and carefully allocated on the secondary structures of predicted hairpins using RNAShapes. Excluding those that fell directly on the stem loops, 111 *Jatropha* and cassava sequences of 18-24 bp located on the 5´or 3´arms of the secondary pre-miRNA harpins were classified as putative virus miRNA candidates (Table 4). 49/111 predicted mature miRNA sequences were located on the 5´arm, and 62 on the 3´ arm of pre-miRNA hairpins (Table 4). 84/111 miR/miR* corresponded to ACMV and 27 to EACMV-UG (Table 4).

Some miRNA sequences exhibited variations in a few nucleotides at the 3´or 5´end, leading to the production of multiple mature variants, which therefore were referred to as isomiRs (Table 4). Cloonan et al. (2011) reported that isomiRs are biologically relevant and target pathways of functionally related genes. In some case, the predicted isomiRs even shared a common seed region and could bind to the same targets, for instance ACMV-mir-3-17* to 3-19*, located on AC4, all targeted the ring finger protein family (Acc.no. GW876074). ACMV-mir-5-1 to 5-2, located on AC2, targeted an AT-rich interactive domain (GW878601). ACMV-mir-5-5 and 5-

6 targeted phospholipase D (GW613466). ACMV-mir-5-14 to 5-15 cysteine protease inhibitor (GT976828). ECMV-UG-mir 1-2 and 1-3, located on AC2 targeted serine/threonine protein kinase (Table 4 and Table S4). All isomiRs groups with the same seed region shared the same targets, only for ACMV mir-1-1 and 1-2, ACMV mir-3-10* and 3-11* and EACMV-UG 1-14* and 1-15* no common target was found.

Virus miR/miR* with putative targets in the *Jatropha* genome

Putative target genes of *Jatropha* were predicted for the 111 miR/miR* by RNAhybrid and psRNATarget. Using the latter was important as it provide scores for target selection (Dai and Zhao, 2011). Scores ranging between 0-2.0 provide lower false positive prediction rate, while scores of 4.0-5.0 provide higher prediction coverage, which can be subjected to further experimental validation (Dai and Zhao, 2011). However it could not predict targets for all the virus miRNA compared to RNAhybrid. The RNAhybrid provides as an alternative feature the MFE, based on the use of free energy of mRNA:miRNA hybridization, which can be used for target selection (Alves et al., 2009). These is important for virus target prediction, as viruses evolve too fast and are typically highly adapted to a specific host (Gottwein and Cullen, 2008) and not all miRNA target sites adhere to seed complementarity (Lekprasert et al., 2011).

In addition to gain better understanding of the functional role of predicted miRNA, the obtained targets were annotated by BlastX and UniProt (www.uniprot.org) Gene Ontology (GO) molecular function (Table S2 and S3).

Based on the RNAhybrid analyses, a total of 234 targets were predicted for 78 ACMV- and 27 EACMV-UG miR/miR*. For ACMV-miR-1-11*, 3-17*, 5-7*, 4-2, 5-9*, 6-4 and 6-15* no target was predicted. The different miRNA targets were grouped into 9 molecular functions, where 83 (35.5%) of targets possess binding functions, 68 (29%) showed catalytic activity, while 43 (18.4%) were proteins with unknown molecular functions. In addition, 9 (3.9%) predicted targets were involved in enzyme regulator activity, 13 (5.6%) had structural molecule activity, 5 (2.1%) electron carrier activity, 5 (2.1%) transport activity, 5 (2.1%) nucleic acid binding transcription factor activity, 2 (0.85%) nutrient reservoir activity and 1 (0.43%) molecular trunsducer activity (Table S2).

Using the psRNATarget, 621 targets were predicted for 79 ACMV- and 26 EACMV-UG-miR/miR* (Table S3). For ACMV-miR-2-3, 42, 5-7*, 6-12*, 6-18* and EACMV-UG miR 6-3 no targets were predicted. 260 targets (41.9%) showed binding activity and 163 (26.2%) catalytic activity, while 94 (15.1%) were proteins with unknown molecular functions. 17 targets (2.7%) possess nucleic acid binding transcription factor activity and 9 (1.5%) showed electron carrier activity. 21 targets (3.4%) showed enzyme regulator activity, 27 (4.4%)

structural molecule activity, 20 (3.2%) transporter activity, 2 (0.03%) molecular trunsducer activity and 8 (1.3%) nutrient reservoir activity (Table S3).

Interestingly, ACMV-mir-1-7, 7-3 and EACMV-UG-mir-2-4* and 2-5* targeted a protein similar to heat shock proteins (Acc. no. GT972247). Production of heat shock proteins can be triggered by exposure to different stress conditions, such as pathogen infection, inflammation, exposure of the cell to toxins, nitrogen deficiency in plants, or water deprivation (Santoro, 2000). ACMV-mir-1-4 and ACMV-mir-6-9* targeted an ADK (Adenosine kinase) protein, predicted from hairpins that located on AC2 and AC1, respectively, while ACMV-mir-3-15* targeted a leucine-rich repeat family protein (Table S2 and S3). Both proteins are typical representatives of proteins involved in biotic response. The leucine rich repeat is present in the majority of immune receptors that form the innate immune system in plants (Padmanabhan et al., 2009). Wang et al. (2003) reported an increase in ADK activity as factor of the host response to virus challenge, therefore the AC2 of geminivirus is a premeditated counter response for inhibition of ADK activity. Furthermore, ACMV-mir-5-12*, 6-9*, 7-3* and EACMV-UG-mir-2-4*, 2-5*, 2-8* -all miR*- targeted a zinc finger protein (Table S3). It was found that mutations in the zinc-finger correlated with the loss of biological function in inducing necrosis and suppressing PTGS in plants (Van Wezel et al., 2003). Further ACMV-miR 5-12* and ACMV-miR 6-9* located on AC1, which is a replication associated protein. Although the predicted miRNA were not validated experimentally, there is a high probability of host miR* to bind virus or plant ORFs as displayed by sequence complementarity. miR* sequences have been demonstrated to accumulate in response to pathogen invasion and thus their role in basal defense is hypothesized (Chapman et al., 2004). It can be speculated that in order to avoid successful establishment of pathogen, a host might activate silencing of some transcripts by use of miR* sequences (Naqvi et al., 2011).

The serine/threonine protein kinase was targeted by EACMV-UG-mir-1-4 and EACMV-UG-mir-1-5, both located on AC2, known to be involved in suppression of PTGS. In eukaryotic cells, regulation of signal transduction pathways through enzymatic protein phosphorylation by serine/ threonine kinase is a widely distributed mechanism (Hanks et al., 1988), suggesting that a serine/threonine protein kinase plays either a direct role in AC2 mediated pathogenesis or in PTGS suppression (Van Wezel et al., 2003).

In addition of the 621 predicted targets, 4 targets (2 binding and two with unknown molecular functions) had a score of 1 (0.64%). A score of 1.5 was obtained for 2 targets (one enzyme regulatory and one binding function) (0.32%). Further, a score of 2 for 10 targets (6 binding, 3 catalytic activity and one unknown function (1.6%), a score of 2.5 for 41 targets (6.6%), while

a score of 3 was predicted for 56 targets (9.01%) and a score of 3.5 was obtained in 102 targets (16.42%). The highest number of 162 targets (26.1%) had a score of 4. Further a score of 4.5 was obtained for 129 targets (20.8%) and 115 (18.5%) with a score of 5 were obtained (Table S3).

Virus miR/miR* with putative targets in the cassava genome

Also putative target genes for the 111 miR/miR* were predicted and annotated in the cassava ESTs from the GenBank as described for *Jatropha*.

Based on the RNAhybrid analyses, 84 ACMV- and 27 EACMV-UG-miR/miR* targeted 370 cassava ESTs. The different miRNA targets were assigned to 8 groups of molecular functions, of which 172 targets (46.5%) with molecular functions as binding, 67 (18.1%) catalytic activity, and 35 targets (9.4%) possess structural molecule activity proteins, while 60 targets (16.2%) were proteins with unknown molecular functions. Further, 5 targets (1.3%) showed nucleic acid binding/transcription factor activity, 4 targets (1.08%) belong to enzyme regulator activity, 15 (4.05%) transporter activity and 11 (2.97%) electron carrier activity. The nutrient reservoir activity protein was targeted only once (0.27%) by ACMV-mir-6-3 (Table S4).

Analyses with psRNATarget revealed that 81 ACMV- and 26 EACMV-UG-miR/miR*, targeted 688 cassava ESTs (Table S5). For ACMV-mir-1-10*, 3-10*, 6-12* and 6-18* no target was found. The highest number of 361 targets (52.5%) showed molecular functions as binding, 125 (18.2%) catalytic activity, 54 targets (7.9%) had structural molecule activity and 89 (12.9%) were proteins with unknown molecular functions. Two proteins (0.29%) with nutrient reservoir activity were targeted by ACMV-mir-3-16* and ACMV-mir-6-3, while ACMV-mir-4-1 targeted a molecular transducer activity protein. In addition, 7 targets (1.01%) were predicted with enzyme regulator activity, 27 (3.9%) transporter activity, 12 (1.7%) electron carrier activity and 10 (1.45%) nucleic acid binding/ transcription factor activity (Table S5).

In addition, data showed that reticulum-3 protein was targeted by ACMV-mir-5-14* and 5-15*) with a score of 1 (0.29%), while 7 targets with binding molecular function (1.02%) had a score of 1.5. A score of 2 was obtained by 12 targets (1.7%), a score of 2.5 by 36 targets (5.2%), and a score of 3 by 84 targets (12.2%). For 128 targets (18.63%) a score of 3.5 was obtained. The highest number of 158 targets (22.99%) reached a score of 4, while 143 had a score of 4.5 (20.8%) followed by 118 (17.17%) with a score of 5 (Table S5).

Plant miR/miR* with putative targets on DNA-A of ACMV and EACMV-UG

The current study revealed a number of plant miR/miR* potentially targeting viral genomic regions of ACMV and EACMV-UG. Host-encoded miRNAs are involved in modulating plant viral diseases symptoms observed as developmental abnormalities by binding to virus – encoded PTGS suppressor proteins (Chellappan et al., 2005). On the other hand, AC4 of ACMV and AC2 of EACMV-UG are unique virus encoded PTGS proteins that bind to target mRNAs and presumably inactivate mature host miRNAs (Chellappan et al., 2005). Therefore any host miRNA sequences targeting ORFs AC4 and AC2 encoded by these viruses could be a potential molecule to develop a resistance strategy and possible achieve immunity against viral diseases.

The approach used to search plant miRNA targets in DNA A of ACMV and EACMV-UG is shown in Figure. 3.

Binding of plant miR/miR* to DNA-A of ACMV

RNAhybrid analyses of all plant miRNAs from the miRBase Database (release18) revealed 24 miR/miR* sequences of 20 miR/miR* families, having putative targets in the DNA-A of ACMV (Table S6). 18 of the miR/miR* sequences (14 miR and 4 miR*) had binding sites within AC1. The family miR164 (a, c, d) was found to be targeting the same position, differing only in one nucleotide. This also occurred in miR169aa and miR169* as well as miR1107 and miR1117, which were located in an overlapping region of AC1 and AC4. Furthermore, miR2094-3p and miR2668 shared binding sites on AC1 and AC4. AC3 was targeted by 3 miR sequences (miR397a, miR397b and miR841c), AV1 by 2 miR sequences (miR160a and miR1864), and AV2 only by miR2640a, while no miR/miR* sequence was found to have a significant complementarity with AC2 and IR (Table S6).

psRNATarget analyses showed a total of 14 miR families containing 15 miR sequences, which had targets in the DNA-A of ACMV (Table S7). Both miR159a and miR159b shared overlapping binding sites on AC1 and AC4 at position 2242. Results from *in vitro* binding assays (Chellappan et al., 2005) revealed the ability of AC4 of ACMV to bind single stranded forms of miR159 and presumably inactivate the mature miRNAs, thus blocking the normal miRNA-mediated regulation of target mRNAs and thus resulting in developmental disturbances. Our data also showed that AC1 was targeted by miR395b, miR868 and miR4243 at positions 1786, 1687 and 2008, respectively. The overlapping ORFs AC2 and AC3 were targeted by miR397b at position 1095. The miR4246 also targeted the AC3 at position 1065. AV1 was targeted by 6 miRs and AV2 by two miRs. However, no miR sequence was found to target the IR (Table S7). Our analyses also revealed that each program

detected different targets at different positions; however miR397b was located on similar targets by both programs.

Binding of plant miR/miR* to DNA-A of EACMV-UG

RNAhybrid identified 23 different miR/miR* families containing 27 miR/miR* sequences, with putative targets in DNA-A of EACMV-UG. 22 miR/miR* sequences were miR and 6 miR* (Table S6). AC1 was targeted by 11 miR/miR* sequences (3 conserved and 8 non-conserved miRNAs), while AC4 only by miR1118. The miR171 family was represented by 4 members (miR171a, b, d, and f) with differences in nucleotide composition, all targeting AC2 and AC3, and overlapping at either position 1364 or 1366. In addition, AC2 and AC3 were targeted by miR859, miR1111, miR1520j and miR2104. AV1, AV2 and IR were targeted by 3 (miR160f*, miR1082b, miR1446a), 2 (miR399c*, miR2927) and 4 (miR478a, miR482, miR2119, miR3633b) miR/miR* sequences, respectively (Table S6). The miR2119 targeted two different positions (1791 and 65) in AC1 and IR respectively, indicating that multiplicity of miRNA targets within the viral genome is possible. It is known that IR is indispensible for viral replication and such a miRNA interaction could be effectively utilized by the host plant to attenuate viral replication at an early stage (Naqvi et al., 2011). The predicted data also shows that plants employ a cooperative regulation mode (Sunkar and Zhu, 2004; Naqvi et al., 2011) by using multiple different miRNAs to target ACMV and EACMV-UG at AC1 to disrupt viral replication. However such strategies are weakened by the infecting virus carrying AC2 and AC4, known to be efficient suppressors of gene silencing.

psRNATarget showed that 25 different miRs containing 22 miR families targeted DNA-A of EACMV-UG (Table S7). AC1 and AC2 were targeted by miR2588a and b at position 1580. AC1 was further targeted by 7 miRs at different positions. AC2 and AC3 were targeted by 4 sequences of family miR171 (a, b, d, f) and miR859. AC4 was targeted by miR2668 at position 236. AV1 was targeted by 6 miRs, while AV2 and IR were each targeted by 2 non-conserved miRs (Table S7).

Results obtained with both programs were confirmed for miR171 (a, b, d and f) and miR859, which targeted both AC2 and AC3, and for two miRs (miR472, miR4390) and miR1446a that targeted AC1 and AV1 respectively (Table S6 and S7).

Predicted putative target location of plant miR/miR* in Jatropha and cassava ESTs

In the current study, plant miRNAs from conserved and highly expressed families (e.g miR156, miR160, miR164, miR166, miR169 and miR171) were shown to have potential targets in the genomes of 2 begomoviruses. This could suggest that highly expressed plant miRNAs have multiple functions as well as multiple targets (Pérez-Quintero et al., 2010).

Furthermore, these miRNA families have multiple targets within the genomes of the host plants, and some of them have been shown to be differentially expressed in response to viral infections, playing a role in pathogen defense activities (Chellappan et al., 2005; Pérez-Quintero et al., 2010; Naqvi et al., 2011).

Analyses by psRNATarget revealed the location of 72 plant miR/miR* sequences in Jatropha ESTs (Table S8). miR164a, miR164c and miR164d (Table S8) with a score of 1.5, all located in the NAC domain (Acc. no. GT978826), a validated target for miR164 (Jones-Rhoades and Bartel, 2004). The four miR 171 (a, b, d and f) all had different targets (Table S8). However, miR 159 (a, b, c), with a score of 2.5 and miR 319c located on CSD (Copper/Zinc Superoxide Dismutase 2) at similar position. miRNAs 159, 164 and 319 have been identified to regulate leaf deformations linked to geminivirus infections (Naqvi et al., 2010), while the induction of disease symptoms after infection of plants with ACMV has been attributed to the accumulation of miR159 and miR164 (Amin et al., 2011). In addition, the miR159a, b, c and miR319c targeted the CSD2 responsible for abiotic stress. miR164a, c and d located in *Jatropha* ESTs targeted the NAC domain, which control leaf development and determine the patterns of the leaves (Nikovics et al., 2006). Zeng et al. (2010) and Patanun et al. (2012) predicted and detected miR156, miR159, miR160, miR164, miR166, miR169, miR170, miR171, miR319, miR395, miR397, miR399, miR477, miR482 and miR1446 in *Jatropha*, cassava, *Ricinus communis* and *Hevea brasiliensis* in response to abiotic stress, development, transcription factors and metabolism.

69 different plant miR/miR* sequences were successfully localized in cassava ESTs (Table S9). miR156g located in the squamosa promoter-binding protein (DV456109) while miR164d targeted the WRKY transcription factor (DR085222). Both miR169aa and miR169* targeted a nuclear transcription factor Y subunit A-1 (DV443290, DV455197, DV445967) while miR397a and miR397b targeted laccase (DR087678) (Table S9).

Conclusions

Granted that miRNA mediated gene silencing serves as a general defense mechanism against viruses, it would not be surprising that viruses also employ miRNA to circumvent the host plant´s defense system (Grundhoff and Sullivan, 2011). Interestingly, viral miRNAs are derived not only from non-coding and intronic regions, but also from protein-coding mRNA (Gottwein and Cullen, 2008; Grundhoff and Sullivan, 2011; Gao et al., 2012). The discovery of virus encoded miRNAs playing a crucial roles in pathogenesis, throws a new light on host-pathogen interactions (Scaria et al., 2006). In addition to regulate the endogenous expression of genes, a host can use miRNA pathways as defense against viruses (Pérez-Quintero et al.,

2010). Furthermore, miRNA are produced by both begomoviruses and their two hosts and can benefit either the virus or the host depending on particular interactions. Such interactions are likely to occur in viral pathogenesis determining the degree to which host restrict viral infection (Gottwein and Cullen, 2008).

Our data support the hypothesis that virus encoded miRNAs can target critical proteins associated with biotic responses in *Jatropha* and cassava. The predicted viral miR/miR* showed complementarity to several regions in the *Jatropha* and cassava genome including proteins with molecular functions such as binding, catalytic activity, enzyme regulator activity, electron carrier activity, nucleic acid/ transcription factor activity, nutrient reservoir activity, structural molecule activity, transporter activity and signal transducer activity (Table S2- S5).

Despite host encoded miR/miR* having the capacity to bind crucial ORFs of ACMV and EACMV-UG, as revealed in this study, these viruses undergo rapid mutations and recombination events, which could in turn lead to a loss of target for plant miRNAs. Since miR/miR* are ~ 21 nt in length, minute changes in the viral genome provide them with opportunity to escape miRNA related defense pathways (Naqvi et al., 2011). However, in the current study, the regions in the virus genome targeted by the host miRNAs were rather conserved, which renders the probability to escape from plant miRNA attack low (Table S7-S8).

Using computational approaches showed for the first time that ACMV and EACMV-UG encode possible pre-miRNA hairpins. So far, this study is the first target computational prediction for viral miR/miR* (both ACMV and EACMV-UG) in *Jatropha* and cassava ESTs. Also, these findings will be useful for the further identification of miRNAs in viruses and plants, and will speed up progress in *Eurphorbiaceae* genome research. They can be further used to engineer virus resistance via RNA based strategies, which could offer a long term solution by providing resistance to the important begomoviruses; ACMV and EACMV-UG (Gottwein and Cullen, 2008).

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Table 1. The complete DNA A sequences of 11 ACMV and 28 EACMV-UG isolated from *Jatropha curcas* and cassava **(in bold)** used in this study (Ramkat et al., 2011).

ACMV	EACMV-UG
JN053421	JN053432
JN053422	JN053433
JN053423	JN053434
JN053424	JN053435
JN053425	JN053436
JN053426	JN053437
JN053427	JN053438
JN053428	JN053439
JN053429	JN053440
JN053430	JN053441
JN053431	JN053442
	JN053443
	JN053444
	JN053445
	JN053446
	JN053447
	JN053448
	JN053449
	JN053450
	JN053451
	JN053452
	JN053453
	JN053454
	JN053455
	JN053456
	JN053457
	JN053458
	JN053459

Table Fourteen viral pre-miRNA hairpins from 11 ACMV and 28 EACMV-UG isolates from *Jatropha* and cassava were classified as real or pseudo. The number of sequences compared, their location in the virus genome and their hairpin sequences are shown.

Name of pre-miRNA	Number of virus sequences used for pre-miRNA predictions	Location of pre-miRNA in ORFs of viral DNA-A	Position*	Real / pseudo pre- miRNA	pre-miRNA hairpin sequence
ACMV 1	7 / 11	AC2	1350-1433	Real	AGCAATGAATGGCGTGTATACCTGGGAAATAAACAATCCCCTGTATTTC ACAATCACCAGGCACCAACAACGACCATTCCTGCT
ACMV 2	4 / 11	AC4	2549-2614	Real	TTTGGGTATGTGAGAAAGACATTCTTGGCTTGAATTCAAAACGAGGAGTT CTCATGTTGACCAAG
ACMV 3	8 / 11	AC4	2442-2521	Real	GTTCTCCATTCTGATGCAGCTCTCTACAGATTTTAATGAACTTAGGGTTTG ATGGGAGAGAGAGTGTTTGAAGGAAGGAC
ACMV 4	8 / 11	AV2	160-246	Real	TCCAGACTCGGTACATGGGCTTAGGTGTATGCTTGCAATTAAATATTTGC AGGCCTTAGAGGATACATACGAGCCCAGTACTTTGGG
ACMV 5	8 / 11	AC1	1645-1718	Real	TTCTTGCTTTTCCTCGTCTAGGAACTCTTTATAGGACGAGGTAGGTCCTGG ATTGCAGAGGAAGATAGTGGGAA
ACMV 6	6 / 11	AC1	1839-1941	Real	ACCAGGCAGCAATATGAGACCTTTGGACTAGGTCCAGGTGTCCACATAGG TAATTGTGTGGGCCTAAAGATCTGGCCCATATCGTCTTCCCTGTTCTGCT
ACMV 7	4 / 11	IR	2622-2704	Real	AGAATGCCATTTAGAGACACCTATATAATGTCTCCAATTAACAGGAGGA CTTGCTCAAGAGTGTCTCTAGTTGAGTGTCTC
ACMV 8	3 / 11	IR	44-105	Real	GTATGTCGGCCAATCATGTTGTAGCTGTAAAAGTTATGTATTAGTGGTGG GCCACTATATAC
ACMV 9	5 / 11	AC2	1234-1310	Pseudo	CAGTCTGAGGCTGTAAGGTCGTCCAGATCTCGAAGTTGAGAAAACATTTG TGAATCCCCAGCGCCTTCCTCAGGTTG
EACMV-UG 1	25 / 28	AC2	1416-1500	Real	TTTCGAAATAGAGGGGATTTGTTATGTCCCAGGTAAAAACGCCATTCCTT GCTTGAGGCGCAGTGATGAGTTCCCCTGTGCGAGA
EACMV-UG 2	10 / 28	AC1	2208-2268	Real	GGAGGGCCAGCATTTAGCTCAGGTATATGCAGACGCGTTAAATGCTTCGT CTAAATCGAGGCTCTTC
EACMV-UG 3	24 / 28	AC2	1535-1618	Pseudo	CGAGCAGCCGCATTCGAGGTCGACCCGCCTACGTCGGACGGCCCTGGTCTTC GCTGTGCGGTGTTGGACTTTGATGGGCACTTG
EACMV-UG 4	9 / 28	IR	2675-2751	Pseudo	AAGTCTATAGCAATCGGTGGAATGGGGGGCAATATATATGATGTCCCCCA ATGGCATATGTGTAAATAGGTAGACTT
EACMV-UG 5	9 / 28	IR	2628-2692	Pseudo	ACCGGCTCTTGGCATATTGGCTGTCGTTTTGGATCGGGGGACACTCAAAAC TCCAGGGGAACGGT

*position based on JN053428 and JN053454

Table 3. Nucleotide content of 7 real ACMV and 2 real EACMV-UG miRNA hairpins

Virus	Pre-miRNA	A %	C %	G %	T %	A+T %	C+G %
ACMV	ACMV 1	33.33	28.57	16.67	21.43	54.76	45.24
	ACMV 2	29.23	13.85	26.15	30.77	60	40
	ACMV 3	26.25	13.75	28.75	31.25	57.5	42.5
	ACMV 4	25.29	19.54	26.44	28.74	47	40
	ACMV 5	22.97	16.22	29.73	31.08	54.05	45.95
	ACMV 6	22	25	25	28	50	50
	ACMV 7	29.63	19.75	20.99	29.63	59.26	40.74
EACMV-UG	EACMV-UG 1	23.53	20	28.24	28.24	51.76	48.24
	EACMV-UG 2	23.88	22.39	26.87	26.87	50.75	49.25

Table 4. The novel predicted virus miR/miR* sequences from ACMV and EACMV-UG real pre-miRNA hairpins using *Jatropha* and cassava sequence hits from BlastN, showing length, location on 5′or 3′ arms of hairpins and sequence of the seed region using RNAShape. Identical seed regions are highlighted in bold.

Pre-miRNA hairpins	miR/miR*	miR/miR*sequence	miR/miR* length	Location of in 5′or 3′ arm	Seed region
ACMV 1	ACMV-mir-1-1	AGCAAUGAAUGGCGUGUAUACCUG	24	5′	**GCAAUGA**
	ACMV-mir-1-2	AGCAAUGAAUGGCGUGUAUA	20	5′	**GCAAUGA**
	ACMV-mir-1-3	CAAUGAAUGGCGUGUAUACCUG	22	5′	AAUGAAU
	ACMV-mir-1-4	AUGAAUGGCGUGUAUACCUGGG	22	5′	UGAAUGG
	ACMV-mir-1-5	UGAAUGGCGUGUAUACCUGGGAA	23	5′	GAAUGGC
	ACMV-mir-1-6	AUGGCGUGUAUACCUGGGAAAUA	23	5′	UGGCGUG
	ACMV-mir-1-7	UGUAUACCUGGGAAAUAAACA	21	5′	**GUAUACC**
	ACMV-mir-1-8	UGUAUACCUGGGAAAUAAAC	20	5′	**GUAUACC**
	ACMV-mir-1-9*	CCAGGCACCAACAACGACCAU	21	3′	**CAGGCAC**
	ACMV-mir-1-10*	CCAGGCACCAACAACGACCAUUC	23	3′	**CAGGCAC**
	ACMV-mir-1-11*	CCAGGCACCAACAACGACCAUUCC	24	3′	**CAGGCAC**
	ACMV-mir-1-12*	CAGGCACCAACAACGACCAUUCCU	24	3′	AGGCACC
	ACMV-mir-1-13*	GGCACCAACAACGACCAUUCCUGC	24	3′	GCACCAA
	ACMV-mir-1-14*	CCAACAACGACCAUUCCUGC	20	3′	CAACAAC
ACMV 2	ACMV-mir-2-1	UUUGGGUAUGUGAGAAAGAC	20	5′	UUGGGUA
	ACMV-mir-2-2	UUGGGUAUGUGAGAAAGACAUU	22	5′	UGGGUAU
	ACMV-mir-2-3	UGGGUAUGUGAGAAAGACAUUCUU	24	5′	GGGUAUG
	ACMV-mir-2-4	GGUAUGUGAGAAAGACAUUCUUGG	24	5′	GUAUGUG
	ACMV-mir-2-5	GUAUGUGAGAAAGACAUUCUUGG	23	5′	UAUGUGA
	ACMV-mir-2-6	AUGUGAGAAAGACAUUCUUGGCUU	24	5′	UGUGAGA
	ACMV-mir-2-7	UGUGAGAAAGACAUUCUUGGCUUG	24	5′	GUGAGAA
	ACMV-mir-2-8*	CAAAACGAGGAGUUCUCAUUUGA	23	3′	AAAACGA
ACMV 3	ACMV-mir-3-1	GAUGCAGCUCUCUACAGAUUU	21	5′	AUGCAGC
	ACMV-mir-3-2	UUCUCCAUUCUGAUGCAGCUCU	22	5′	UCUCCAU
	ACMV-mir-3-3	UCUCCAUUCUGAUGCAGCUCUA	22	5′	CUCCAUU
	ACMV-mir-3-4*	UUAGGGUUUGAUGGGAGAGAGAG	23	3′	UAGGGUU
	ACMV-mir-3-5*	UAGGGUUUGAUGGGAGAGAGAGUG	24	3′	AGGGUUU
	ACMV-mir-3-6*	AGGGUUUGAUGGGAGAGAGAGUGU	24	3′	GGGUUUG

	ACMV-mir-3-7*	GGGUUUGAUGGGAGAGAGAGUGUU	24	3′	GGUUUGA
	ACMV-mir-3-8*	GGUUUGAUGGGAGAGAGAGUGUUU	24	3′	GUUUGAU
	ACMV-mir-3-9*	GUUUGAUGGGAGAGAGAGUGUUUG	24	3′	UUUGAUG
	ACMV-mir-3-10*	UUUGAUGGGAGAGAGAGUGUUUG	23	3′	**UUGAUGG**
	ACMV-mir-3-11*	UUUGAUGGGAGAGAGAGUGUUUGA	24	3′	**UUGAUGG**
	ACMV-mir-3-12*	UUGAUGGGAGAGAGAGUGUUUGAA	24	3′	UGAUGGG
	ACMV-mir-3-13*	UGAUGGGAGAGAGAGUGUUUGAAG	24	3′	GAUGGGA
	ACMV-mir-3-14*	GAUGGGAGAGAGAGUGUUUGA	21	3′	AUGGGAG
	ACMV-mir-3-15*	UGGGAGAGAGAGUGUUUGAAGGAA	24	3′	GGGAGAG
	ACMV-mir-3-16*	GGGAGAGAGAGUGUUUGAAGGAAG	24	3′	GGAGAGA
	ACMV-mir-3-17*	GGAGAGAGAGUGUUUGAAGGAAGG	24	3′	**GAGAGAG**
	ACMV-mir-3-18*	AGAGAGAGUGUUUGAAGGAAGGA	23	3′	**GAGAGAG**
	ACMV-mir-3-19*	AGAGAGAGUGUUUGAAGGAAGGAC	24	3′	**GAGAGAG**
ACMV 4					
	ACMV-mir-4-1	GGUACAUGGGCUUAGGUGUAUGCU	24	5′	GUACAUG
	ACMV-mir-4-2	ACAUGGGCUUAGGUGUAUGCUUGC	24	5′	CAUGGGC
	ACMV-mir-4-3	CAUGGGCUUAGGUGUAUGCUUGCA	24	5′	AUGGGCU
	ACMV-mir-4-4	UGGGCUUAGGUGUAUGCUUGCAA	23	5′	GGGCUUA
	ACMV-mir-4-5	GCUUAGGUGUAUGCUUGCAA	20	5′	CUUAGGU
	ACMV-mir-4-6*	UACAUACGAGCCCAGUACUUUGG	23	3′	ACAUACG
	ACMV-mir-4-7*	AUACGAGCCCAGUACUUUGG	20	3′	UACGAGC
ACMV 5					
	ACMV-mir-5-1	UCUUGCUUUUCCUCGUCUAGGAA	23	5′	**CUUGCUU**
	ACMV-mir-5-2	UCUUGCUUUUCCUCGUCUAGGAAC	24	5′	**CUUGCUU**
	ACMV-mir-5-3	CUUGCUUUUCCUCGUCUAGGAACU	24	5′	UUGCUUU
	ACMV-mir-5-4	UUGCUUUUCCUCGUCUAGGAACUC	24	5′	UGCUUUU
	ACMV-mir-5-5	CUUUUCCUCGUCUAGGAACUCU	22	5′	**UUUUCCU**
	ACMV-mir-5-6	CUUUUCCUCGUCUAGGAACUCUU	23	5′	**UUUUCCU**
	ACMV-mir-5-7*	GAGGUAGGUCCUGGAUUGCAGAGG	24	3′	AGGUAGG
	ACMV-mir-5-8*	GUAGGUCCUGGAUUGCAGAGGAA	23	3′	UAGGUCC
	ACMV-mir-5-9*	AGGUCCUGGAUUGCAGAGGAAGA	23	3′	GGUCCUG
	ACMV-mir-5-10*	GGUCCUGGAUUGCAGAGGAAGAU	23	3′	GUCCUGG
	ACMV-mir-5-11*	CUGGAUUGCAGAGGAAGAUAGUG	23	3′	UGGAUUG
	ACMV-mir-5-12*	UGGAUUGCAGAGGAAGAUAGUGGG	24	3′	GGAUUGC
	ACMV-mir-5-13*	GGAUUGCAGAGGAAGAUAGUGGGA	24	3′	GAUUGCA
	ACMV-mir-5-14*	GAUUGCAGAGGAAGAUAGUGGGA	23	3′	**AUUGCAG**
	ACMV-mir-5-15*	GAUUGCAGAGGAAGAUAGUGGGAA	24	3′	**AUUGCAG**
ACMV 6					
	ACMV-mir-6-1	AGGCAGCAAUAUGAGACCUUU	21	5′	GGCAGCA
	ACMV-mir-6-2	GGCAGCAAUAUGAGACCUUUGGAC	24	5′	GCAGCAA
	ACMV-mir-6-3	AGCAAUAUGAGACCUUUGGACUAG	24	5′	GCAAUAU
	ACMV-mir-6-4	AUGAGACCUUUGGACUAGGUCCA	23	5′	UGAGACC
	ACMV-mir-6-5	CUUUGGACUAGGUCCAGGUGUCCA	24	5′	UUUGGAC
	ACMV-mir-6-6	GACUAGGUCCAGGUGUCCACAUAG	24	5′	ACUAGGU
	ACMV-mir-6-7*	UUGUGUGGGCCUAAAGAUCU	20	3′	UGUGUGG
	ACMV-mir-6-8*	UGUGGGCCUAAAGAUCUGGCCCAU	24	3′	GUGGGCC
	ACMV-mir-6-9*	CCUAAAGAUCUGGCCCAUAUCGUC	24	3′	CUAAAGA
	ACMV-mir- 6-10*	AAGAUCUGGCCCAUAUCGUCUUCC	24	3′	AGAUCUG
	ACMV-mir-6-11*	AGAUCUGGCCCAUAUCGUCUUC	22	3′	GAUCUGG
	ACMV-mir-6-12*	GAUCUGGCCCAUAUCGUCU	19	3′	AUCUGGC
	ACMV-mir-6-13*	UCUGGCCCAUAUCGUCUUCCCU	22	3′	CUGGCCC
	ACMV-mir-6-14*	UGGCCCAUAUCGUCUUCCCUG	21	3′	GGCCCAU
	ACMV-mir-6-15*	GCCCAUAUCGUCUUCCCUGUUCUG	24	3′	CCCAUAU
	ACMV-mir-6-16*	CCAUAUCGUCUUCCCUGUUCUGCU	24	3′	CAUAUCG
	ACMV-mir-6-17*	CAUAUCGUCUUCCCUGUUCUG	21	3′	AUAUCGU

	ACMV-mir-6-18*	UCGUCUUCCCUGUUCUGCU	19	3′	CGUCUUC
ACMV 7	ACMV-mir-7-1	AGAAUGCCAUUUAGAGACACCU	22	5′	GAAUGCC
	ACMV-mir-7-2*	AGAGUGUCUCUAGUUGAGUGUCU	23	3′	GAGUGUC
	ACMV-mir-7-3*	AGUGUCUCUAGUUGAGUGUCU	21	3′	GUGUCUC
EACMV-UG 1	EACMV-UG-mir-1-1	UUUCGAAAUAGAGGGGAUUUGUUA	24	5′	UUCGAAA
	EACMV-UG-mir-1-2	UCGAAAUAGAGGGGAUUUGUUAUG	24	5′	**CGAAAUA**
	EACMV-UG-mir-1-3	UCGAAAUAGAGGGGAUUUGUUAU	23	5′	**CGAAAUA**
	EACMV-UG-mir-1-4	CGAAAUAGAGGGGAUUUGUUAU	22	5′	GAAAUAG
	EACMV-UG-mir-1-5	GAAAUAGAGGGGAUUUGUUAUGU	23	5′	AAAUAGA
	EACMV-UG-mir-1-6	AAAUAGAGGGGAUUUGUUAUGUC	23	5′	AAUAGAG
	EACMV-UG-mir-1-7	AAUAGAGGGGAUUUGUUAUGUC	22	5′	AUAGAGG
	EACMV-UG-mir-1-8	AUAGAGGGGAUUUGUUAUGUCCCA	24	5′	UAGAGGG
	EACMV-UG-mir-1-9	AGAGGGGAUUUGUUAUGUCC	20	5′	GAGGGGA
	EACMV-UG-mir-1-10	GGGAUUUGUUAUGUCCCAGGUAA	23	5′	GGAUUUG
	EACMV-UG-mir-1-11	AUUUGUUAUGUCCCAGGUAA	20	5′	UUUGUUA
	EACMV-UG-mir-1-l2*	UUGCUUGAGGCGCAGUGAUGAGUU	24	3′	UGCUUGA
	EACMV-UG-mir-1-13*	UGCUUGAGGCGCAGUGAUGAGUUC	24	3′	GCUUGAG
	EACMV-UG-mir-1-14*	GCUUGAGGCGCAGUGAUGAGUUCC	24	3′	**CUUGAGG**
	EACMV-UG-mir-1-15*	GCUUGAGGCGCAGUGAUGAG	20	3′	**CUUGAGG**
	EACMV-UG-mir-1-16*	CUUGAGGCGCAGUGAUGAGUUCCC	24	3′	UUGAGGC
	EACMV-UG-mir-1-17*	GAGGCGCAGUGAUGAGUUCCCCUG	24	3′	AGGCGCA
	EACMV-UG-mir-1-18*	AGGCGCAGUGAUGAGUUCCCCU	22	3′	GGCGCAG
	EACMV-UG-mir-1-19*	AGUGAUGAGUUCCCCUGUGCGAGA	24	3′	GUGAUGA
EACMV-UG 2	EACMV-UG-mir-2-1	CAGCAUUUAGCUCAGGUAUAU	21	5′	AGCAUUU
	EACMV-UG-mir-2-2	AGGGCCAGCAUUUAGCUCAGGU	22	5′	GGGCCAG
	EACMV-UG-mir-2-3*	UAAUGCUUCGUCUAAAUCGAGG	22	3′	GAGCUAA
	EACMV-UG-mir-2-4*	UGCUUCGUCUAAAUCGAGGCU	21	3′	GCUUCGU
	EACMV-UG-mir-2-5*	GCUUCGUCUAAAUCGAGGCUC	21	3′	CUUCGUC
	EACMV-UG-mir-2-6*	UUCGUCUAAAUCGAGGCUCUUC	22	3′	UCGUCUA
	EACMV-UG-mir-2-7*	UCGGAGCUAAAUCUGCUUCGU	20	3′	CGGAGCU
	EACMV-UG-mir-2-8*	GCGUUAAUGCUUCGUCUA	18	3′	CGUUAAU

Figure 1. An overview of the search and prediction of novel virus (ACMV and EACMV-UG) miRNAs addressing targets in their host plants *Jatropha* and cassava.

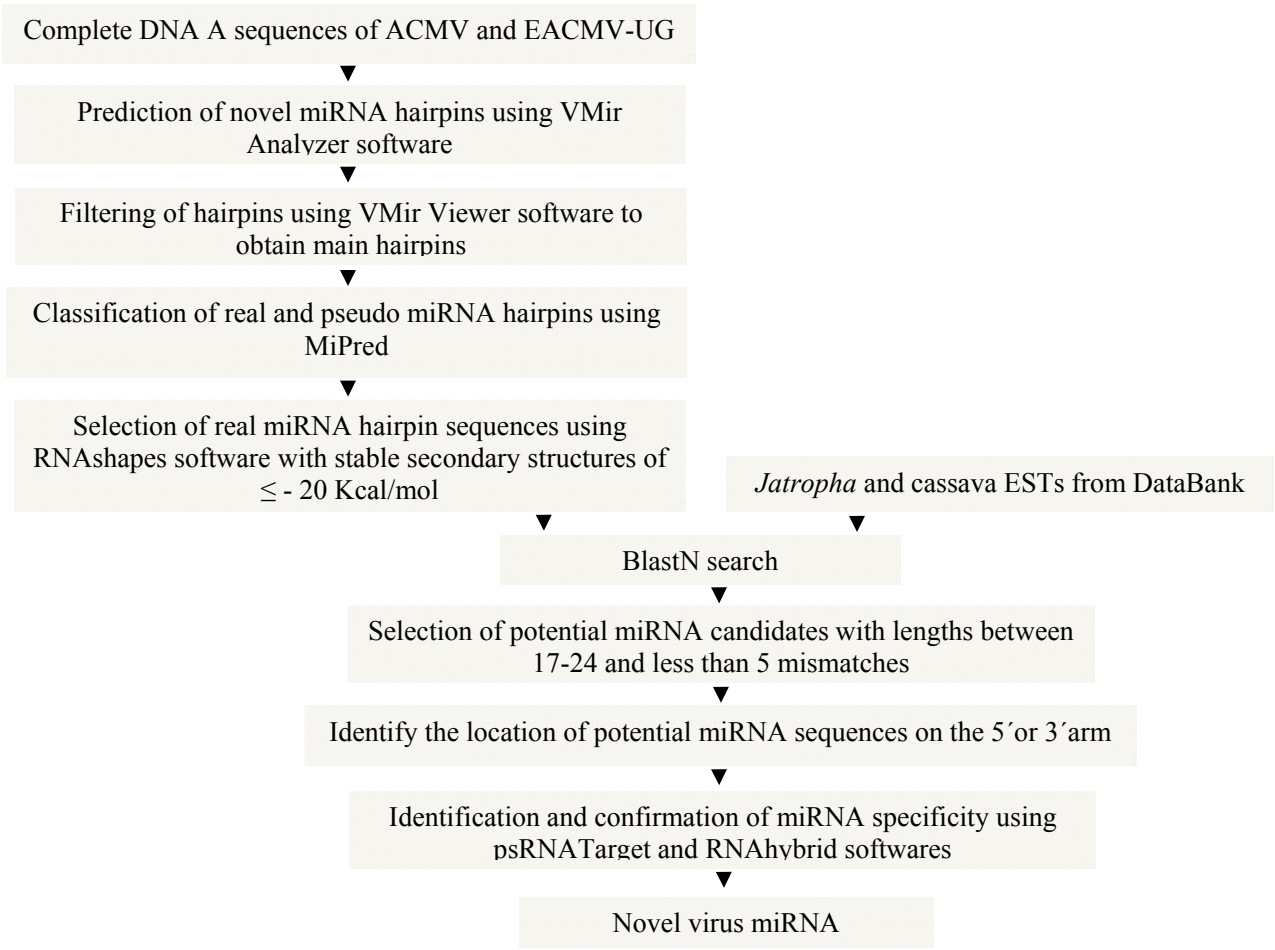
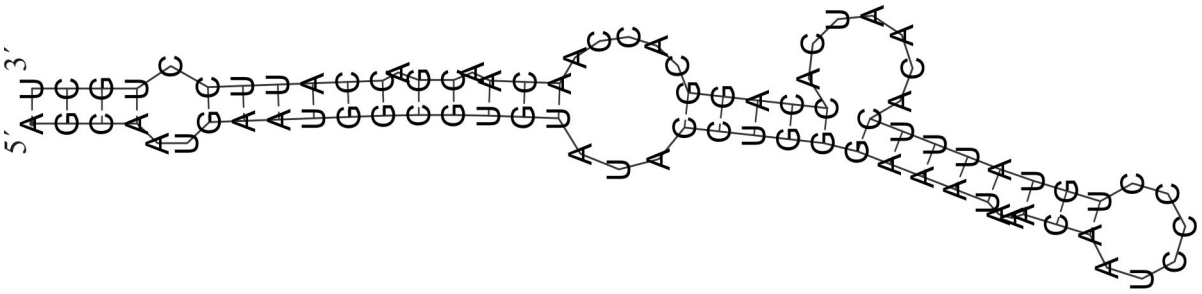
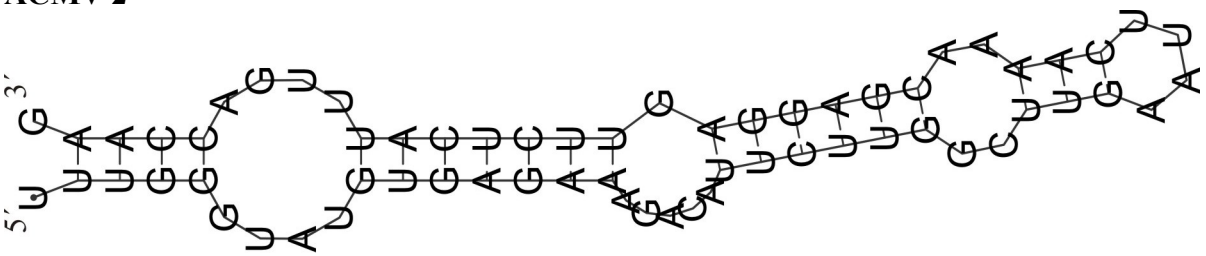


Figure 2. Secondary structures of 9 real predicted viral pre-miRNA hairpins using RNAshapes.

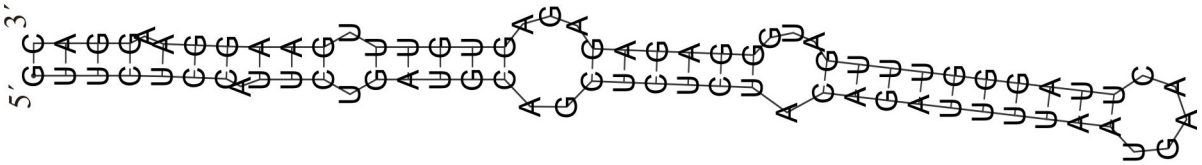
ACMV 1 MFE: -21.71



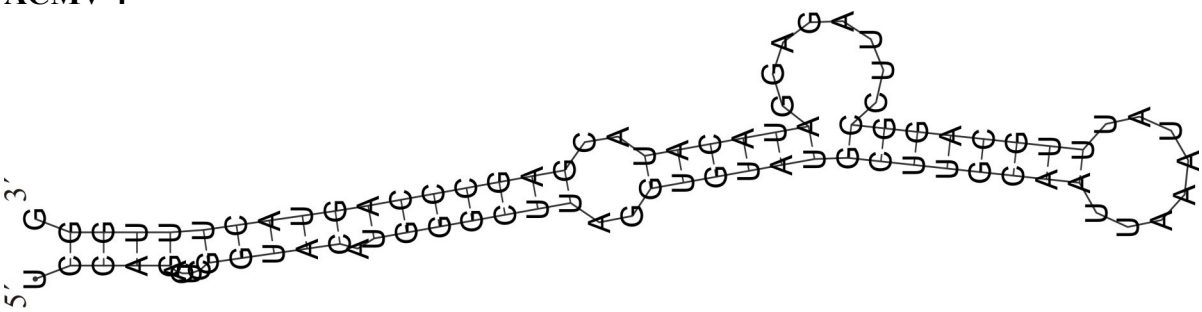
ACMV 2 MFE:-20.8



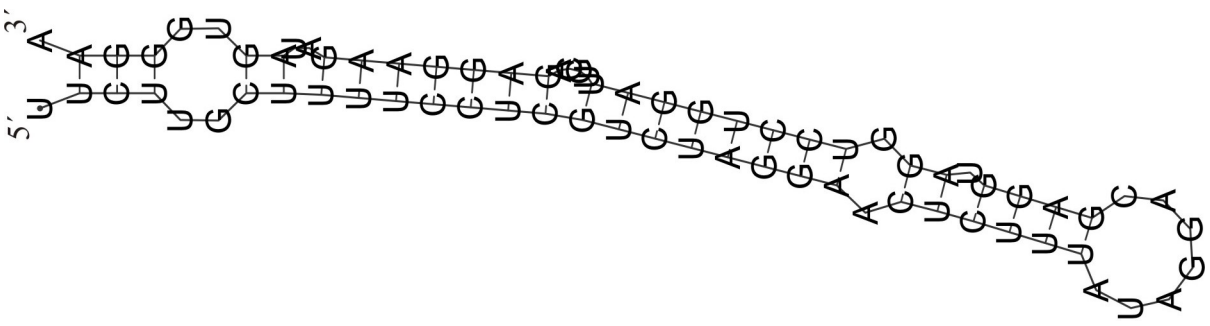
ACMV 3 MFE: -25.9



ACMV 4 MFE: -35.7



ACMV 5 MFE: -27.5



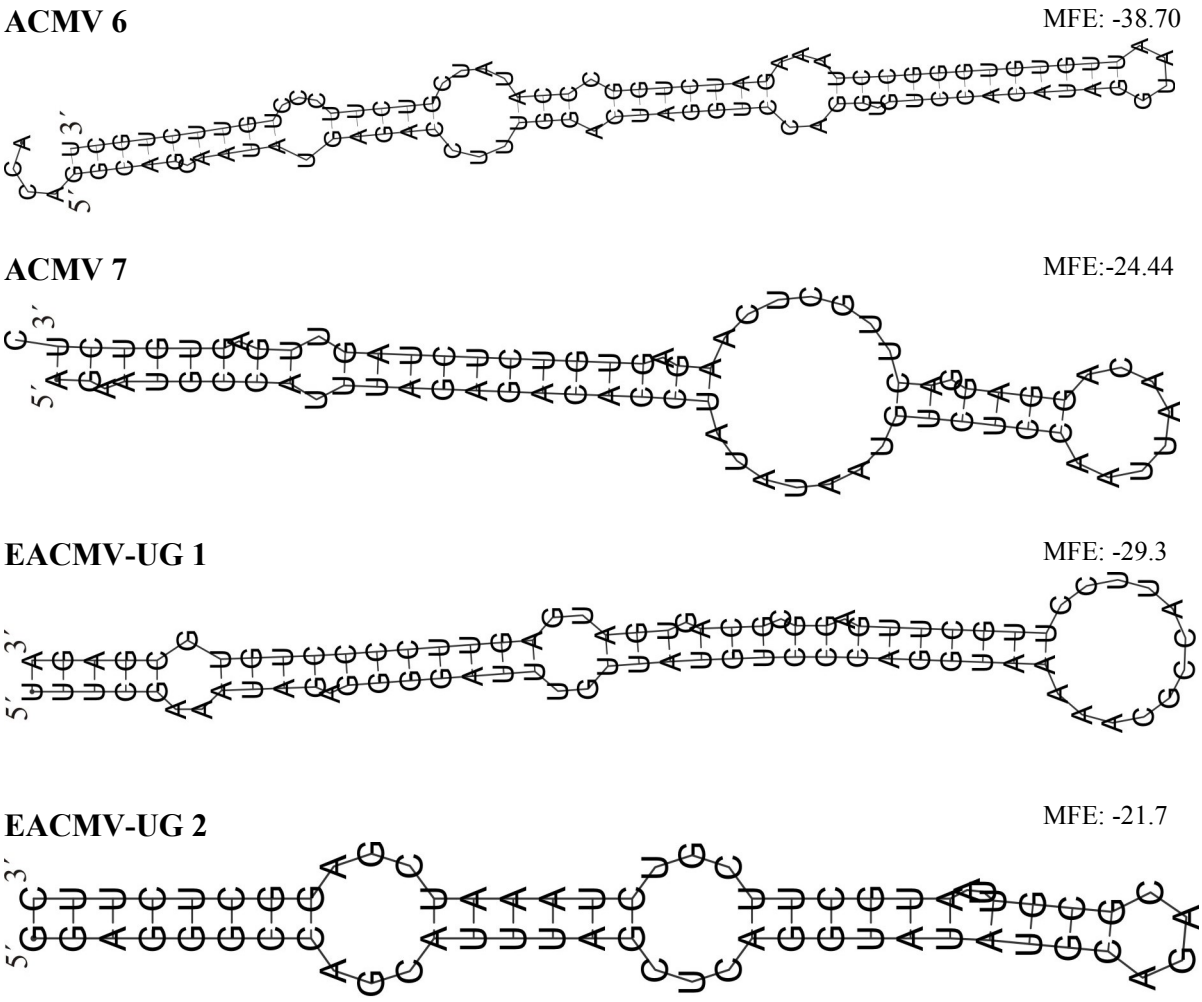
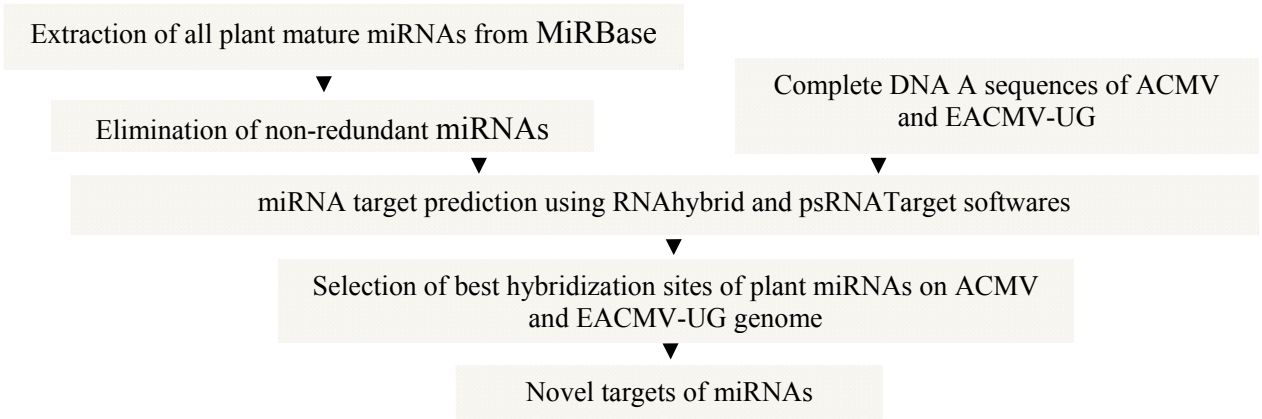


Figure 3. An overview of the approach used to search plant miRNA targets in DNA A of ACMV and EACMV-UG.



CHAPTER VI: General discussion and conclusion

The family *Euphorbiaceae* is of significant importance as it comprises economic valuable crop species such as: a) *Hevea brasiliensis*, which is cultivated commercially for latex biosynthesis (natural rubber production) (Venkatachalam et al., 2007), b) *Ricinus communis* is a biofuel plant and has other uses in medicines, cosmetics, pharmaceutical and industries (Sujatha et al., 2008), c) Manihot esculenta is mainly used as a staple food and serves for food security of millions in Asia and Africa, who strongly depend on it (Calvert and Thresh, 2002), d) *Jatropha curcas* serves mainly as a bio-fuel crop and pharmaceutical crop, with possibility of reclaiming marginal soils, reducing risks of erosion and desertification. Furthermore it can alleviate poverty by offering the farmers a new and sustainable cash crop (Vollmann and Laimer, 2013; Maghuly et al., 2013). Owing to the relevance of these plants there is growing need to focus on their improvement towards achieving better adaptation to biotic responses, abiotic responses, high quantity and quality of yields.

Plant improvement is a combination of principles and methods of changing the genetic constitution of a plant to make it more suitable for human need. The key for success of any genetic improvement programme involves the identification of differences in the traits of economic importance among plants and to improve these traits with available scientific knowledge (Farooq and Azam, 2002). The development of molecular marker provides new dimensions which are more accurate, efficient and quick for the analysis of the genetic diversity of plants to speed up the process of varietal evaluation (Tar'an et al., 2005). Furthermore the information generated on genetic variation within the populations is important for conservation and exploitation of genetic resources for crop improvement programs (Varshney et al., 2005).

Markers that are conserved are more likely to be transferable in economic species in the *Euphorbiaceae* family, such as the *Hevea brasiliensis*, *Ricinus communis* (castor bean), cassava and *Jatropha* (Zou et al., 2011). EST sequences are used to develop markers which are valuable resources for the study of genetic diversity, biotic and abiotc responses, growth and development of members of the *Euphorbiaceae* family (Anderson et al., 2004). ESTs already exist for *Ricinus*, *Hevea*, cassava and *Jatropha*. In addition there exists a detailed genetic map of the rubber tree (Lespinasse et al., 2000) and many ESTs from cassava cross-hybridizing with *Hevea* DNA can be used for further mapping purposes (Anderson et al., 2004). Such an approach should help in establishing syntenic regions between the different genomes and facilitate positional cloning of genes of interest in these species. Additional high

throughput gene discovery and sequencing in *Euphorbiaceae* members can further provide the groundwork for unlocking genetic diversity within this family (Anderson et al., 2004). Of equal importance, it will enhance the ability to control growth and productivity of various members of *Euphorbiaceae* and increase the possibility for map-based cloning (Anderson et al., 2004).

In view of this, Divakara et al. (2009) and Sujatha et al. (2008) suggested that the genetic enhancement and domestication of *Jatropha* should follow the same course as that of castor bean since the wild castor bean once considered as a minor oilseed crop has become a major industrial crop (Sujatha et al., 2008; Divakara et al., 2009). Castor bean has been improved from a perennial wild species to a high yielding annual domesticated crop having short internodes with varying flower sexuality ratios from completely pistillate to predominant male types (Singh, 1976; Sujatha et al., 2008; Divakara et al., 2009). The success is mainly attributed to the use of induced mutation, screening and selection of germplasm and identification of pistillate variants (Sujatha et al., 2008; Divakara et al., 2009).

In surveying the genetic variation in *Jatropha curcas* and related species, a total of 1200 accessions from different countries were analyzed by ISSR, AFLP and Ecotilling (Maghuly et al., 2011, 2013). The ISSR markers divided the accessions in two groups, one containing all *Jatropha* accessions and the other containing the related species (Chapter III, Maghuly et al., 2011). The *Jatropha* accessions from Kenya were clustered in their own group while the remaining accessions were placed in another cluster. Ecotilling was successful in detecting SNPs between *Jatropha* and related species which revealed that polymorphism was less frequent between *Jatropha* accessions (Maghuly et al., 2013). The low variability found in *Jatropha* candidate genes analyzed suggest that genetic diversity may be limited for traditional breeding approaches and developing novel diversity through mutagenesis should be applied (Maghuly et al., 2013).

Plant viral diseases cause major production and economic losses in *Euphorbiaceae* (Berrie et al., 1998; Ndunguru et al., 2005; Bull et al., 2006; Alabi et al., 2008; Gao et al., 2010; Ramkat et al., 2011). There is need for effective control strategies to be put in place to minimize this situation and ensure food security and sustainable agriculture. Since different viruses affect crops from the same family and even result to mixed infections, this brings a unique set of challenge related to the biology of each of the virus causing infection. One of the major strategies towards fight against plant viruses is the need of their rapid detection and identification.

To address the question of viruses infecting *Jatropha* and cassava, molecular identification methods using PCR and RCA were applied for detection (Chapter IV, Ramkat et al., 2011). Since there are limited commercial ELISA tests available for viruses infecting *Jatropha*, a PCR procedure using newly developed primers (JC6F and JC2R) was established. These primers yield short products of 380 bp and allow the detection of CMGs in symptomatic and asymptomatic cassava and *Jatropha* plants (Chapter IV, Ramkat et al., 2011). These primers are recommended for the use as an efficient early screening tool for geminiviruses to reduce the risk of spreading of the viral diseases. Techniques for the single-step amplification of whole genomes have been developed into powerful tools for phylogenetic analyses. The bacteriophage phi29 DNA polymerase has been used for the efficient amplification of circular DNA viral genomes by RCA mechanism (Johne et al., 2009). Since the viral titer in *Jatropha* infected plants was low, the utilization of RCA was of great importance for virus amplification. PCR was performed on the RCA products using primers designed to amplify the complete *Geminivirus* DNA A genome. Sequencing of the products revealed for the first time the presence of ACMV and EACMV-UG in *Jatropha* plants from the same fields, indicating possibilities of mixed infections. Begomoviruses possess some characteristics that enable them to propagate so successful in their host plants such as synergism and recombination (Fondong et al., 2000; Pita et al., 2001). The synergism between ACMV and EACMV-UG is due to a selective advantage conferred by each virus providing differentially acting suppressors of PTGS that are required to overcome host defenses (Vanitharani et al., 2004; Bisaro, 2006; Bull et al., 2006). Synergism has been shown to be a key factor in the genesis of CMG pandemics in East and Central Africa (Harrison et al., 1997). Synergism increases the viral titer in the plant thus enhancing whitefly transmission capacity (Patil and Fauquet, 2009). Recombination and synergism that have long occurred in cassava (Fondong et al., 2000; Pita et al., 2001) could have led to the current spread of the virus in the field to infect *Jatropha* plants.

Furthermore, the presence of mixed infections with CMG and CBSV was detected in cassava (Chapter IV, Ramkat et al., 2011). These diseases can be cross exchanged among the plant species of the *Euphorbiaceae*, especially between cassava and *Jatropha*. The presence of a mixed infection will change the management of disease control in cassava and *Jatropha* crops, because most focus has been on the presence of begomoviruses. This knowledge of mixed infection will further help to set adequate measures as to maintain the production of these crops.

In addition to genomic components, a def DNA (Def K1J5) was detected from DNA A of one isolate of ACMV in *Jatropha*. Smaller sized DNAs often occur naturally in *Geminivirus* infected plants, usually are half the size of the full length genomic component and may be formed by sequence deletion, duplication, inversion or rearrangement of viral DNA (Patil and Dasgupta, 2006; Ndunguru et al., 2006; Patil and Fauquet, 2009). The role of this small Def DNA molecule in the biology of ACMV in *Jatropha* in nature is still unclear. However, Def DNAs have been shown to interfere with virus proliferation as they are associated with delay and attenuation of symptoms (Patil and Fauquet, 2009).

The devastating impact by begomoviruses on *Jatropha* and cassava production calls for the provision of a long term solution that can eradicate the viruses. In general, viral diseases can be prevented by using virus free planting materials, virus resistant cultivars and appropriate cultural controls. Planting resistant cultivars is the most effective and economical way to control plant virus diseases (Legg and Fauquet, 2004). Virus resistant cultivars can be generated through conventional breeding by utilizing natural sources of resistance. However with the advent of modern biotechnology, non-conventional methods have also been used to confer virus resistance by transferring primarily virus-derived genes or non-viral genes (Reddy et al., 2009). An example utilizing virus derived genes is from the research by Vanderschuren et al. (2007), where sequences from the CR of ACMV-Kenya isolate were used to design constructs that were mobilized into *Agrobacterium tumefaciens* and used for transformation of cassava plants. Subsequently, the transgenic plants generated expressed siRNA which led to the attenuation of CMD symptoms following inoculation with ACMV-N*Og* infectious clones (Vanderschuren et al., 2007).

For developing resistant cultivars, it is important to understand the molecular biology of the virus and its interaction with the particular host (Germundsson, 2005). In recent years, small RNAs have been demonstrated to play an important role in plant development and to be implicated in host pathogen interactions (Bazzini et al., 2007). Several classes of small RNAs have been described which include miRNAs. The discovery of miRNA opened up avenues for understanding gene expressions and plant pathogenesis investigations (Lu et al., 2008). Given that miRNA mediated gene silencing act as a general defense mechanism against plant viruses, it is also possible that viruses can employ miRNA to circumvent the host plant´s defense system (Lu et al., 2008).

Considering the relevance of miRNA in defense, computational approaches were used to: a) predict virus encoded miRNA in DNA A sequences of ACMV and EACMV-UG, b) investigate whether virus encoded miR/miR* sequences have the capacity to bind to genomic

sequences of *Jatropha* or cassava and c) investigate whether plant encoded miR/miR* sequences have any potential to bind to the DNA A genome of ACMV and EACMV-UG (Chapter V). Data obtained show for the first time that ACMV and EACMV-UG encode possible pre-miRNA hairpins located in AC1, AC2, AC4, AV2 and IR. This is quite relevant, since the AC1 is required for virus replication, while AC2 and AC4 are involved in suppression of PTGS and pathogenicity (Paszkowski et al., 1993; Voinnet et al., 1999; Vanitharani et al., 2004; Bisaro, 2006). RNA silencing is a natural defense response of plants against invading viruses. Viruses too encode certain proteins that can block the RNAi pathway and are referred to as suppressor of gene silencing (Voinnet, 2001, 2005). The AC4 of ACMV interact/interfere with the host miRNA pathway resulting in plant development defects which are exhibited as disease symptoms (Chellappan et al., 2005).

Furthermore, the predicted virus miR/miR* had complementarity to several regions in the *Jatropha* and cassava genomes with most miR/miR* having multiple targets. Viruses can exploit RNA silencing to modify host gene expression directly because of homologies between virus miRNA and host transcripts, which lead to cleavage or translation of several classes of mRNAs favoring the infection process (Dunoyer and Voinnet, 2005). At the same time, a viral genome can be targeted by a multitude of host miRNA (Simón-Mateo and García, 2006). Several plant miRNAs have potential targets in the ACMV and EACMV- UG DNA A genomes. This suggests a way in which plant miRNAs could have a role in pathogen defense (Pérez-Quintero et al., 2010). Bioinformatic predictions have been used to show that several miRNA including those from conserved families like miR156, miR159, miR166, miR160 and miR395 have potential targets in the virus genomes (Pérez-Quintero et al., 2010; Naqvi et al., 2011). Furthermore, these miRNAs have been shown to be differentially expressed in response to *Begomovirus* infections suggesting their probable role in defense activity and disease symptom development (Simón-Mateo and García, 2006; Naqvi et al., 2010; Amin et al., 2011).

In conclusion, the information generated by molecular markers on *Jatropha* contributes towards the understanding about its population structure, adaptation and phylogenetic relationship with related species. The identified novel SNPs in *Jatropha* can be used as markers for marker assisted selection.

Molecular techniques allowed the identification of viruses infecting *Jatropha*, giving evidence that the crop acts as alternative and/or reservoir hosts for CMGs and could contribute to continued virus evolution and future disease epidemics. However many additional virus species infecting *Jatropha* remain to be identified since comprehensive sampling and

characterization work has only been done from material collected from a fraction of geographical region affected by CMD. A sensitive, reliable and rapid diagnostic tools for the detection of the viruses need to be incorporated in phytosanitary programs for routine monitoring of the diseases. In this respect, the primers JC6F and JC2R designed in this study can be suggested for *Geminivirus* screening. Since cassava and *Jatropha* are vegetatively propagated, distribution of infected vegetative cuttings largely contributes to long distance dissemination of the disease. Provision of virus free planting material will be vital in minimising the spread of the diseases. A prerequisite to this is the availability of an elimination stratagey for the viruses.

The identification of miRNAs both of pathogens and hosts will further improve the understanding of host pathogen interaction. These miRNAs can further be used to engineer virus resistance via RNA based strategies which could offer a long term solution of resistance to ACMV and EACMV-UG and may in turn benefit *Euphorbiaceae* health.

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CHAPTER VII: Additional papers

1. Occurrence of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus-Uganda* (EACMV-UG) in *Jatropha curcas*
2. Detection of phytoplasma associated with *Rubus idaeus* and *Rubus fruticosus*

VII.1. Occurrence of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus – Uganda* (EACMV-UG) in *Jatropha curcas*

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Background

Jatropha curcas is a drought resistant shrub native in tropical America, now widely grown in many tropical and subtropical regions for biodiesel production (Openshaw, 2000). First reports on virus infections in *Jatropha* indicated the occurrence of viruses closely related to *Cassava mosaic virus* in India, reaching a disease incidence from 25 to 47%. This might represent a major constrain to the production of *Jatropha* in large scale (Raj et al., 2008b). The genome of *Cassava mosaic geminiviruses* (CMG) consist of two components termed DNA A and DNA B (~ 2.7 – 3.0 kb) (Yadava et al., 2010). Furthermore, *Jatropha* has been described as host of *Cucumber mosaic virus* (CMV) (Raj et al., 2008a).

Methods

In this study we attempted to detect and molecularly characterize viruses infecting *Jatropha* in Eastern Africa (Kenya and Ethiopia). Detection methods will be valuable tools for early screening of plant viruses in order to make appropriate decisions and selection of planting material.

A total of 127 *Jatropha* samples from Ethiopia and Kenya (districts: Kakamega, Siaya, Busia and Nakuru showing typical virus symptoms and symptomless plants were used in this study. ELISA was performed to detect the presence of three RNA viruses: CMV, *Cassava common mosaic virus* (CsCMV) and *Cassava brown streak virus* (CBSV). PCR was performed using newly designed primers based on multiple alignments of full length DNA A sequences of geminiviruses available in the NCBI Genbank, reported to infect either *Jatropha* or cassava. This allowed amplifying the variable regions of full length (2800 bp) and shorter sequences (380-1085 bp). PCR products were sequenced. A phylogenetic tree was constructed from multiple alignments by performing a heuristic search. Multiple alignments were analyzed by maximum parsimony with full-length DNA A using Phylogenetic Analysis Using Parsimony (PAUP) and a bootstrap analysis with 1000 replicates.

Results and Conclusions

None of the Jatropha samples analysed was infected with the RNA viruses CBSV, CMV and CsCMV. PCR primers amplifying a 380 bp fragment of AC1, AC2 and AC3 yielded positive results with 75% of the symptomatic samples from Kenya and further detected 20% of asymptomatic samples as positive. Furthermore, 61% of symptomatic *Jatropha* samples from Ethiopia were positive. Full length primers were able to detect 69% symptomatic *Jatropha* samples from Kenya, and also in 67% of asymptomatic samples. PCR analyses of sample K1J5 amplified the expected 2.8 kb of a near full length DNA A component of the *Begomovirus* sequence and an additional shorter fragment.

Complete nucleotide sequences of 34 DNA A components typical of Begomoviruses were determined in the Kenyan samples. Thirty three sequences ranged from 2770 bp to 2816 bp while one (K1J5) consisted only of 1416 bp and termed as a defective (Def) DNA. Phylogenetic analyses indicated that the defective molecule belongs to geminiviruses involved in CMG, representing a Def from DNA A of the bipartite *Begomovirus* ACMV. All viruses characterized in this study grouped with two previously identified Begomoviruses found in cassava in Western Kenya, namely EACMV – UG and ACMV. The Def DNA showed 96.6% sequence identity with the ACMV reference sequence [GenBank NC001467.1].

In this study, we report for the first time the detection of Begomovirus: ACMV and EACMV – UG in *Jatropha* from Kenya. From an evolutionary perspective, the phylogenetic data indicate that the virus isolates from the study were closely related to those isolated previously in Western Kenya from cassava (Stanley and Gay, 1983; Yadava et al., 2010). Recombination and synergism that have long occurred in cassava (Harrison et al., 1997; Zhou et al., 1997) could have led to the recent spread of the virus in the field to infect *Jatropha*. Presence of EACMV – UG and ACMV on different *Jatropha* plants in the same field indicates the opportunity for mixed infections, hence offering good opportunities for more recombination to occur. EACMV – UG and ACMV are associated with severe synergistic epidemics on cassava. Synergism lead to a 10 - 50 fold increase in viral DNA accumulation which substantially increases the potential for a higher efficiency of vector transmission to even infect non cassava host plants (Harrison et al., 1997; Zhou et al., 1997; Legg and Fauquet, 2004; Monde et al., 2010). This explain why EACMV - UG is the predominant virus in *Jatropha*. The deletions occurring in the Def DNA found in the study might affect the replication of the molecule and it might depend entirely on its helper virus for replication.

There is a possibility of Cassava mosaic virus in *Jatropha* being more wide spread than anticipated, since we have detected it also in *Jatropha* samples from Ethiopia.

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VII.2. Integration of PCR, RFLP, cloning and sequencing in identification of phytoplasma associated with *Rubus idaeus* and *Rubus fruticosus*

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Keywords: *Rubus spp.*, phytoplasmas, X disease (16SrIII), *Rubus* stunt (16SrV), PCR/RFLP, cloning and sequencing, reference sequence

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Abstract

Phytoplasmas infecting *Rubus* fruit species cause considerable harvest losses. The small sizes and poor taste of infected fruits makes these disaeses a major economic threat. Early detection of phytoplasma is of prime importance to minimize spread of disease to larger areas. An efficient detection and elimination method would therefore be highly desirable for handling the threat. A high number of plants showing symptoms attributed to phytoplasma infection were observed in *Rubus spp.* in the field. In order to obtain a reference sequence from *Rubus* plants a 1852 bp fragment amplified from the 16S rRNA-23S rRNA region was cloned and sequenced, and finally determined as a representative of group 16SrV phytoplasmas. Nested PCR using the universal primer pair R16mF2/R16mR1 followed by R16F2nM/R16R2 allowed the detection of phytoplasma in 35/133 (26.3%) samples, corresponding to 28/39 plants (71.8%). Nested PCR using the group V specific primer pair R16(V)F1/R16(V)R1 on *Rubus* plants showed that 21/34 samples were associated with a phytoplasma belonging to the group 16SrV. This was confirmed by RFLP analysis using the restriction enzyme *Mse*I. The presence of a phytoplasma belonging to 16SrIII was confirmed in one *Rubus* cultivar.

Introduction

Phytoplasmas are obligate intracellular plant-pathogenic bacteria, restricted to the phloem and they belong to the class Mollicutes (Weisburg et al., 1989). They infect many economically important plants worldwide, including small fruit species like *Rubus ideaus* (red raspberry) and *Rubus fruticosus* (blackberry) in the family *Rosaceae* (Lee et al., 1995; Mäurer and Seemüller, 1995; Marcone et al., 1997; Davies, 2000; Vindimian et al., 2004; Borroto-Fernández et al., 2007; Valiunas et al., 2007; Cieslinska, 2011). Phytoplasma infections cause symptoms on host plants which are often non specific to a particular group of phytoplasma (Tolu et al., 2006). Typical symptoms induced by phytoplasmas in infected plants include: stunting, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferations and fruit malformations (Valiunas et al., 2007; Bertaccini and Duduk, 2009; Cieslinska, 2011). Phytoplasma infected fruits are of small sizes and have poor taste (Heinrich et al., 2001; Laimer, 2009) which can interfere with traits and quality desired by consumers. These symptoms in *Rubus* are associated with infection by phytoplasmas belonging to the following groups: elm yellows (Lee et al., 1995), X disease (Davies, 2000), aster yellows and stolbur (Borroto-Fernández et al., 2007). Phytoplasmas and are transmitted from plant to plant by grafting and other vegetative propagation techniques and by specific phloem feeding insects, particularly the leafhoppers, planthoppers, and psyllids (Bertaccini and Duduk, 2009).

In *Rubus spp.* infections with phytoplasmas of groups 16SrI, 16SrIII, 16SrV and 16SrXII have been reported previously (Bertaccini et al., 1995; Lee et al., 1995; Mäurer and Seemüller, 1995; Marcone et al., 1997; Davies, 2000; Jarausch et al., 2001; Ermacora et al., 2003; Sertkaya et al., 2004; Vindimian et al., 2004; Borroto-Fernández et al., 2007; Valiunas et al., 2007; Cieslinska, 2011; Malembic-Maher et al., 2011; Oberhänsli et al., 2011). Infact, due to its distinct biological niche and genomic differentiation, it was proposed that *Rubus* stunt phytoplasma (16SrV) represent a novel distinct candidate taxon: `*Candidatus* Phytoplasma rubi´ (Malembic-Maher et al., 2011). It is therefore of importance to avoid spreading of phytoplasma diseases in this field crops. Furthermore, for plant breeders it is important to avoid infected plant material for grafting and to have healthy parental material for breeding work. To achieve this, an early detection of infected plants and an efficient sanitation procedure are prerequisites (Oberhänsli et al., 2011). Polymerase chain reaction (PCR) based methodologies are mainly used for phytoplasma detection (Gundersen and Lee, 1996). Primers design on phytoplasma specific DNA-probes or 16S rRNA gene sequences and random fragment length polymorphism (RFLP) of the same gene are used for identification and classification of different phytoplasma groups (Lee et al., 1998).

A high number of field growing *Rubus spp.* plants showing symptoms attributed to phytoplasma infection were observed in Italy and Germany. For this reason PCR, RFLP and molecular cloning were employed to detect and identify phytoplasma infecting this plant species.

Material and methods

Plant samples and DNA extraction

From a high number of field growing *Rubus spp.* plants showing symptoms attributed to phytoplasma infection a representative sample of 39 plants (32 *Rubus idaeus* and 7 *Rubus fruticosus*), originating from Italy and Germany, were used in this study. Total genomic DNA was extracted from 133 samples of leaf veins, bark scrapings, flowers, flower buds, fruits, sepals, stipules, roots and petioles with DNeasy Plant Mini Kit (QIAGEN, Germany) according to the supplier's instructions.

Partial cloning and sequencing of a reference strain

For the cloning and sequencing of a reference strain, the universal primer pair P1/P7 was used to amplify a 1852 bp fragment covering the entire 16S rRNA gene, 16S-23S intergenic spacer region and 5'end of 23S rRNA gene. Amplification was performed in 25 ul final reaction volume containing 2.5 µl of 10x *Ex Taq* buffer, 2 µl of dNTPs mixture (2.5mM each), 0.125 µl *TakaRa Ex Taq*™ HotStart, 1 µl of each primer (10 pmol), and 2 µl of total genomic DNA.

PCR was performed for 35 cycles under the following conditions: 30s denaturation at 94°C (10s at 98°C for the first cycle), 30s annealing at 55°C and 1min extension at 72°C. A final step of 10 min at 72°C ended the cycle. A subsequent nested PCR was performed using different primer pairs (P1/RUBR, PA2F/PA2R, and RUBF/P7) (Table 1) to obtain a set of overlapping PCR products covering the whole region amplified by primers P1 and P7. Products of P1/P7 primed PCR were diluted 1:40 with sterile distilled water and used as template in the nested PCR. The PCR mix and cycle were similar to above except in annealing temperature of 60°C for primer pairs P1/RUBR and RUBF/P7. The PCR products were electrophoresed on 1.5% agarose gel stained with ethidium bromide.

The P1/RUBR, PA2F/PA2R, and RUBF/P7 primed PCR products from samples R.i. 29 were purified using the QIAquick gel extraction kit (QIAGEN) following the supplier's instructions, and eluted in sterile distilled water. The products were ligated into pGEM®-T Easy Vector System (Promega) and transformed to competent cells of *E. coli* JM109 following the instructions given by supplier. Transformants were selected on LB agar plates containing ampicillin (100 µg ml^{-1}), 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (80 mg ml^{-1}) and isopropyl β-D-1-thiogalactopyranoside (0.5 mM). The transformed bacterial colonies were isolated using the alkaline-lysis method, digested with NotI-HF (New England BioLabs), visualized on agarose gels to confirm the presence of an inserted fragment and send for sequencing. The obtained sequences were analyzed using the DNASTAR Lasergene software and compared with phytoplasma sequences from GenBank using BLASTn (http://ncbi.nlm.nih.gov/BLAST/).

PCR

Nested PCR was performed using different primer sets to allow for general or group specific diagnosis (Table 1). For general diagnostic purposes, nested PCR was carried out by using first the universal primers R16mF2/R16mR1 amplifying approximately 1500 bp, followed by the universal primers R16F2nM/R16R2 or 16SrV specific primers R16(V)F1/R16(V)R1 amplifying 1250 bp and 1100 bp respectively . The first PCR was carried out according to Gundersen and Lee. (1996) except for an annealing temperature of 55°. The amplicons was diluted in 1:40 and used as a template in nested PCR. PCR mix and cycle conditions were similar to those used in the first round PCR except for an annealing temperature of 64°C and 56°C for R16F2nM/R16R2 and R16(V)F1/R16(V)R1 respectively.

RFLP analysis of PCR products

Nested PCR products from primer R16F2nM/R16R2 were excised from the gel, eluted using QIAquick gel extraction kit (QIAGEN) and subjected to digestion with restriction enzyme

MseI (New England Biolabs) following manufacturer's instructions. Digested products were separated by electrophoresed on 2% agarose gel stained with ethidium bromide and resulting patterns compared with profiles of the restriction patterns of reference strains of phytoplasmas (Lee et al., 1998).

Results

Sequencing

The sequences obtained from clones of PCR products from P1/RUBR, PA2F/PA2R, and RUBF/P7 showed the presence of phytoplasma in sample (R.i. 29). The sequences from the 3 PCR products could be aligned giving 1852 bp. Blasting this sequence to other phytoplasma sequences reported in Genbank revealed that it belonged to phytoplasma 16SrV with 99% sequence similarity to *Rubus* stunt isolate (AC: Y16395) and FD isolate (AC: X76560).

Nested PCRs for general or group specific diagnosis

Positive samples produced the expected 1250 bp fragment of phytoplasma 16S rDNA after nested PCR using general primers R16F2nM/R16R2. From a total of 39 different plants, 28 (71.8%) were positive. In general, from the 133 samples collected from different plant parts, 35 (26.3%) were positive of which 14 out of 56 (25%) were leaf veins, 10 out of 49 (20.4%) bark scrapings, 4 out of 9 (44.4%) sepals and 4 out 7 flowers (57.1%) and 1 out of 3 (33%) fruits. In addition, 1 out of 2 samples (50%) analyzed from the roots and petioles were positive while no products were amplified from flower buds and stipules (Supplementary Table 1). Furthermore, nested PCR with primer pair R16(V)F1/R16(V)R1 specific for group 16SrV phytoplasma gave positive results in 21 out of 34 (61.8%) samples (Table 2).

RFLP

Comparison of RFLP patterns of 16S amplicons amplified by nested PCR with universal primer R16F2nM/R16R2 with those previously published for 16S rDNA (Lee et al., 1998) revealed the presence of phytoplasma belonging to 16Sr III and 16SrV groups on *Rubus* samples analyzed. *MseI* digest yielded fragment profiles similar to that of 16SrV group phytoplasma in 20 samples (Table 2). A second profile was generated from one sample (R.i. 8) and it belonged to 16SrIII group phytoplasma (Table 2).

Discussion

Phytoplasmas are apparently confined to the phloem tissues in infected plants, can be present in low titers and are often unevenly distributed among the plant host organs (Lee et al., 1995), making their detection difficult (Nejat et al., 2009). The most reliable diagnostic methods encompass the collection of samples from different parts of an individual plant to be tested. This was quite relevant in the current study as detection showed that not all samples collected

from an infected plant yielded positive signals. Furthermore, utilization of sensitive techniques such as PCR and nested PCR are important in phytoplasma identification, with the RFLP analysis of PCR products providing additional evidence of differentiation into group species (Bertaccini, 2007). In the present study, cloning and sequencing of a partial reference sequence representative of group 16SrV was achieved in sample R.i. 29. Nested PCR using the group V specific primer pair R16(V)F1/R16(V)R1 on *Rubus* plants showed that 61.8% of samples were associated with a phytoplasma belonging to the group 16SrV(*Rubus* stunt phytoplasma subgroup). RFLP analysis using the restriction enzyme *Mse*I, verified the presences of these phytoplasmas. Phytoplasma belonging to 16SrIII (X disease phytoplasma) was confirmed in one *Rubus* cultivar. The obtained results are in agreement with previous findings that showed presence of group 16SrIII and 16SrV in *Rubus* spp. (Davies, 2000; Cieslinska, 2011; Malembic-Maher et al., 2011).

In conclusion, presence of phytoplasmas infecting *Rubus spp.* is a seriuos threat to its cultivation especially that they are economically important fruit species. The presence of these phytoplasmas is connected to their transmission with infected plant material during vegetative propagation. This therefore calls for a rapid elimination strategy to be utilized in the production of propagation and breeding plant material.

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Table 1 List of primers used in this study

Primer	Primer sequence 5´ to 3´	Source
R16mF2	CATGCAAGTCGAACGGA	(Gundersen and Lee, 1996)
R16mR1	CTTAACCCCAATCATCGAC	(Gundersen and Lee, 1996)
R16F2nM	GAAACGGTTGCTAAGACTGG	this study
R16R2	TGACGGGCGGTGTGTACAAACCCCG	(Gundersen and Lee, 1996)
R16VF1	TTAAAAGACCTTCTTCGG	(Lee et al., 1994)
R16VR1	TTCAATCCGTACTGAGACTACC	(Lee et al., 1994)
P1	AAGAGTTTGATCCTGGCTCAGGATT	(Schneider et al., 1995)
P7	AAGAGCCGATGAAGGACG	(Schneider et al., 1995)
RubF	GTGGTGCATGGTTGTCGTCAG	this study
RubR	CTAACATCTCACGACACGAACTGA	this study
PA2F	GCCCCGGCTAACTATGTGC	(Heinrich et al., 2001)
PA2R	TTGGTGGGCCTAAATGGACTC	(Heinrich et al., 2001)

Table 2. Results obtained in infected *Rubus idaeus* (R.i.) and *Rubus fruticosus* (R.f.) from nested PCR using general primers 16F2nM/R16R2, group five specific primers (R16(V)F1/R16(V)R1) and random fragment length polymorphism (RFLP) with restriction enzyme *MseI* on 16F2nM/R16R2 product. + (very weak positive), ++ (weak positive), +++ (strong positive), ++++ (very strong positive), (-) negative sample

Sample	Tissue tested	R16F2nM/ R16R2	R16(V)F1/R1 6(V)R1	RFLP	Sample county of origin
R.i. 1	bark scrapings	+	-	not tested	Italy
R.i. 2	sepals	+	-	16SrV	Italy
R.i. 3	sepals	+	+	16SrV	Italy
R.i. 4	leaf veins	+	+	16SrV	Italy
R.i. 5	leaf veins	+	+++	16SrV	Italy
R.i. 6	bark scrapings	+	-	not tested	Italy
R.i. 7	leaf veins	+	-	16SrV	Germany
R.i. 8	bark scrapings	+	-	16SrIII	Italy
R.i. 9	petiole	+	++++	not tested	Italy
R.i. 10	roots	+	++++	16SrV	Italy
R.i. 11	sepals	+	+++	not tested	Germany
R.i. 12	flowers	+	++	16SrV	Germany
R.i. 13	flowers	+	++++	not tested	Germany
R.i. 14	flowers	+	+++	not tested	Germany
R.i. 15	flowers	+	+++	not tested	Germany
R.i. 16	leaf veins	+	++++	16SrV	Germany
R.i. 17	bark scrapings	+	+++	not tested	Germany
R.i. 18	leaf veins	+	++	16SrV	Italy
R.i. 19	bark scrapings	+	-	not tested	Italy
R.i. 20	sepals	+	-	uncertain	Italy
R.i. 21	bark scrapings	+	-	not tested	Italy
R.i. 22	bark scrapings	+	-	16SrV	Italy
R.i. 23	leaf veins	+	+	16SrV	Italy
R.i. 24	leaf veins	+	-	not tested	Germany
R.i. 25	leaf veins	+	-	16SrV	Germany
R.i. 26	leaf veins	+	-	not tested	Germany
R.i. 27	leaf veins	+	+++	16SrV	Germany
R.i. 28	leaf veins	+	+++	16SrV	Germany
R.i. 29	leaf veins	+	+++	16SrV	Germany
R.i. 30	leaf veins	+	not tested	16SrV	Germany
R.i. 31	bark scrapings	+	-	not tested	Italy
R.i. 32	bark scrapings	+	+	16SrV	Italy
R.i. 33	bark scrapings	+	+	16SrV	Italy
R.f. 1	leaf veins	+	+	16SrV	Germany
R.f. 10	fruit	+	++	16SrV	Germany

ANNEXES

Annex 1: List of abbreviations

AC4	Pathogenicity enhancer protein
ACMV	*African cassava mosaic virus*
AFLP	Amplified fragment length polymorphism
amiRNA	Artificial miRNAs
AP2	Apetala2 gene
ARFs	Auxin Response Factors
AV2	Precoat protein
bp	Base pair
CBSD	Cassava brown streak disease
CBSV	*Cassava brown streak virus*
CDS	Cu-Zn superoxide dismutases
CMD	Cassava mosaic disease
CMG	Cassava mosaic geminiviruses
CP/AV1	Coat protein
CR	Common region
CsCMD	Cassava common mosaic disease.
CUC	Cup shaped cotyledon-like
Da	Dalton
DCL1	Dicer like enzyme1
def DNA	Defective DNA
DNA	Deoxyribonucleic acid
dsRNAs	Double stranded RNAs
EACMCV	*East African cassava mosaic Cameroon virus*
EACMKV	*East African cassava mosaic Kenya virus*
EACMMV	*East African cassava mosaic Malawi virus*
EACMV	*East African cassava mosaic virus*
EACMZV	*East African cassava mosaic Zanzibar virus*
ELISA	Enzyme Linked Immunosorbent Assay
EST	Expressed sequence tag
HCN	Hydrogen cyanide
HEN1	hau enhancer1
HST	Hasty
HYL1	Hyponastic leaves1
IR	intergenic region
ISSR	Inter simple sequence repeats

kDa	kilodaltons
Maf/HAM1h	Putative nucleoside triphosphate pyrophosphatase
MFE	Minimum free energy
miRNA	microRNA
MP/BC1	Movement protein
mRNA	Messenger RNA
MYB	Myeloblastosis
Nm	Nanometer
NSP/BV1	Nuclear shuttle protein
nt	Nucleotide
ORFs	Open reading frames
PCR	Polymerase chain reaction
Pol II	RNA polymerase II
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PTGS	Post-transcriptional gene silencing
RAPD	Random amplification of polymorphic DNA
RCA	Rolling circle amplification
REn/AC3	Replication enhancer protein
Rep/AC1	Replication associated-protein
RISC	RNA-induced silencing complexes
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SACMV	*South African cassava mosaic virus*
SCAR	Sequence-characterized amplified region
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SPL	Squamosa-promoter binding protein
SSR	Simple sequence repeats
ssRNA	Single stranded RNA
TCP	Toxin-coregulated pilus
TILLING	Targeting induced local lesions in genomes
TOE	Target of eat
ToLCNDV	*Tomato leaf curl New Delhi virus*
TrAP/AC2	Transcriptional activator protein
UCBSV	*Uganda cassava brown streak virus*
UTR	Untranslated region
VIGS	virus induced gene silencing
vsRNAs	virus-derived small RNAs

Annex 2. Description of plant material used in the study

In this study, plant material from *Jatropha* and cassava were used. For the investigations of genetic variation in *Jatropha*, a total of 1300 plants growing in the glasshouse and tissue culture were used. The plants were composed of *Jatropha curcas* and four related species *Jatropha multifida*, *Jatropha hieronymi*, *Jatropha podagrica* and *Jatropha macrocarpa*. The plants were collected from 14 different countries on three continents. From America plants were collected from Bolivia, Brazil, Mexico and Paraguay. In Africa, collections were obtained from Cape Verde, Ethiopia, Guinea-Bissau, Kenya, Madagascar, Mali and Senegal. The collections from Asia came from China, India and Indonesia.

Plant material used for identification of viruses infecting *Jatropha* and cassava were collected from Ethiopia and Kenya. The plant material from Ethiopia was from Amhara district. In collecting plant material from Kenya, a survey was conducted covering 4 *Jatropha* and cassava growing districts in the Western part of the country: Busia, Kakamega, Nakuru and Siaya. These districts are situated in a warm and wet medium altitude region with rainfall ranges from 1000 – 2500 mm/year that occurs in 2 seasons. The survey was conducted in two years by selecting fields with known virus problems and plants with symptomic appearance and asymptomatic. In the first year (August, 2009) the sites sampled included Busia, Kakamega and Siaya while in the second year (October, 2010) only *Jatropha* fields from Nakuru were sampled.

During the survey plant material was collected from 5 *Jatropha* and 5 cassava fields in each district. For each of the fields, ten symptomic and ten asymptomatic plants from *Jatropha* and Cassava were sampled. Young leafy shoot samples were picked from plants and placed in sample collection tubes over silica gel and labelled. In addition, two cuttings of bud sticks approximately thirty centimetres long and four centimetres in diameter were taken from five of the sampled plants. For preservation purpose, the ends of the cuttings were dipped in melted bee wax.

The symptoms observed for during sampling on plants actively growing included: (i) Leaf curl and malformation, (ii) chlorosis, (iii) mosaic patterns and blisters on leaves, (iv) severe mosaic accompanied by yellow spots and (iv) chlorotic specks on leaves. On plants undergoing dormancy, symptoms observed for include: (i) Stunted growth of the whole plant (ii) plants with short internodes (iii) brown necrotic spots or streaks on the stems.

Annex 3. Poster presentations

1. Maghuly, F., Ramkat, R., Taassob-Shirazi F., Jankowicz –Cieslak, J., Laimer, M. 2012. Analysis of genetic variation among and within *Jatropha* species using dominant and codominant markers. In: International conference on Molecular Ecology, February 4-8, Vienna, Austria.
2. Maghuly, F., Jankowicz-Cieslak, J., Ramkat, R., Till, B., Laimer, M. 2012. Reverse genetics and dominant markers to determine the genetic variation in *Jatropha curcas*. Plant and Animal Genome XX Conference, January 14-18, San Diego, CA, USA.
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5. Maghuly, F., Ramkat, R., and Laimer, M. 2013. Prediction of microRNAs encoded by Begomovirus infecting *Euphorbiacea*. Plant Genetics and Breeding Technologies, February 18-20, Vienna, Austria. In: International conference on Plant Genetics and Breeding Technologies, February 18-20, Vienna, Austria
6. Ramkat, R., Maghuly, F., and Laimer, M. 2013. Detection of phytoplasma in *Rubus* and *Fragaria* species. In: International conference on Plant Diseases and Resistance Mechanisms, February 20-22, Vienna, Austria.
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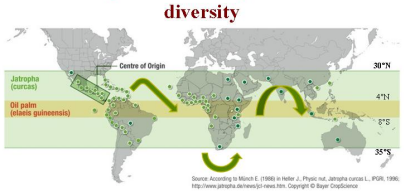
Analysis of genetic variation among and within Jatropha species using dominant and codominat markers

Maghuly F.[1], Ramkat R.[1], Taassob-Shirazi F.[1,2], Jankowicz-Cieslak J.[2], Till B.[2] and Laimer M.[1]

1 Plant Biotechnology Unit (PBU), Dept. Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria,
2 Plant Breeding Unit, FAO/IAEA Agricultural and Biotechnology Laboratory, International Atomic Energy Agency, P.O. Box 100, Vienna, Austria

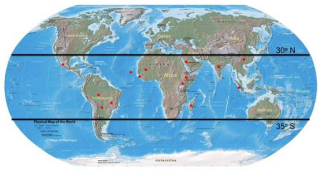
This species of the family *Euphorbiaceae* has gained attention as alternative bioenergy plant worldwide in tropical and subtropical areas for the production of biodiesel. *Jatropha* can be planted on poor, contaminated soils which are not suitable for food production. *Jatropha* is also used in plantations of hedges and barriers to soil erosion. Furthermore *Jatropha* is used as source of fuel wood, for the production lamp oil, soap, colors and smear oils and for some medicinal applications.

Worldwide migration of *Jatropha curcas* affects its diversity



J. curcas is thought to be native to the Americas, and to have been transported via the Cape Verde and Guinea Bissau to Asia and Africa by the Portuguese ships 400 years ago. The export of material from the Americas could have been a single event.

PBUs *Jatropha* collection …



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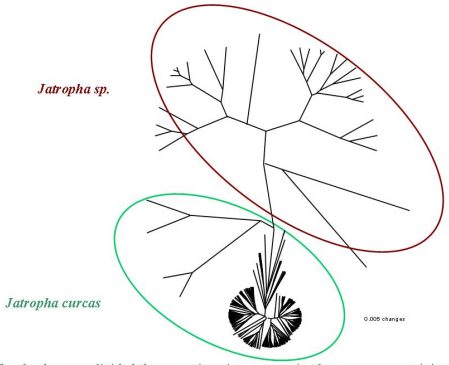
Genetic relationship among Jatropha accessions and related species, using 5 polymorphic ISSRs



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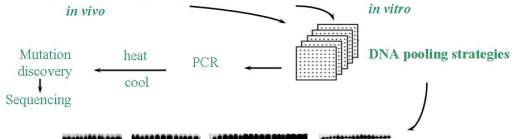

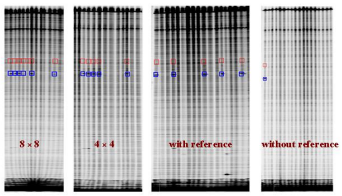

Preliminary allele frequency estimations

Target Gene	Size (bp)	Major alleles	Minor alleles
AF	1267	15	5
EU06	2000	NA	NA
EF03	1286	1	2
DQ98	2500	3	0
EU10	2800	2	2
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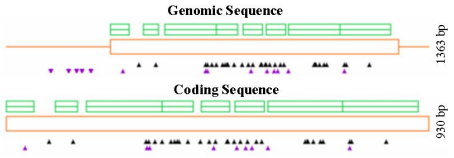
EU39	950	0	0
EI23	2800	3	0
EU21	1500	11	0
DQ15	1119	0	14
DQ66	1700	0	0
SUSYII	1500	4	0

SNPs were detected in 11/12 genes tested. The least polymorphic locus, EU39 has no SNP and the most polymorphic locus AF has 20 SNPs. However the most minor alleles were found in DQ15. 4/12 genes showed a higher SNP variation.

Distribution of SNPs in a AF target gene



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- **Due to the high number of samples, our analyses reveal a higher variation in *Jatropha* accessions compared to previous reports, particularly in the center of origin.**
- **5/45 ISSR primers analysed showed polymorphism and 4/36 AFLP primer showed higher polymorphism (data not shown).**

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Reverse genetics and dominant markers to determine the genetic variation in *Jatropha curcas*

Maghuly F.[1], Jankowicz-Cieslak J.[2], Ramkat R.[1], Till B.[2] and Laimer M.[1]

1 Plant Biotechnology Unit (PBU), Dept. Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria,
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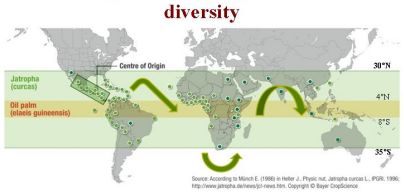

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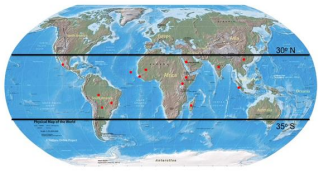

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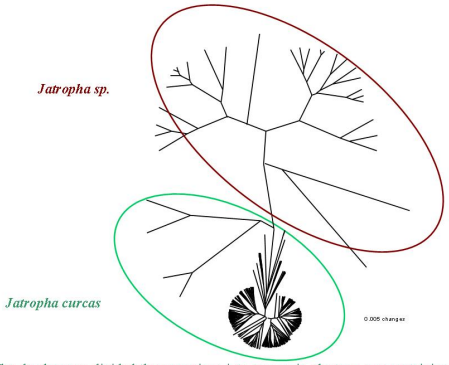

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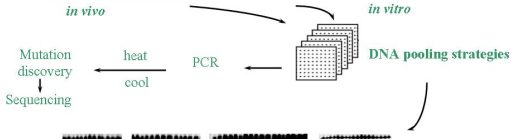

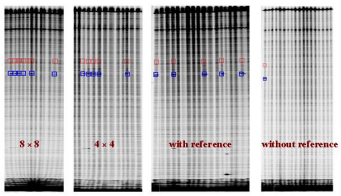

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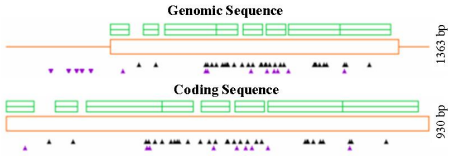

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Occurrence of *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* – Uganda (EACMV-UG) in *Jatropha curcas*

Rose Ramkat, Alberto Calari, Fatemeh Maghuly and Margit Laimer

Plant Biotechnology Unit, Department of Biotechnology, BOKU University, Muthgasse 18, 1190 Vienna, Austria

Jatropha a biofuel plant, belong to the family *Euphorbiaceae*. So far, little information is available about viruses infecting *Jatropha*. In this study 112 Jatropha samples from Kenya and Ethiopia were analysed. Two species of Cassava mosaic geminivirus were detected, showing a high sequence similarity to EACMV-UG (98.7%) and ACMV (97.3%). A naturally occurring defective (Def) of DNA-A of ACMV was also found . Cassava begomovirus occurring in *Jatropha* might be more widespread than anticipated. If not taken care of, this might negatively impact large scale plantations for biofuel production.

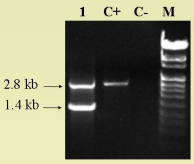
Jatropha plant showing symptoms of reduced leaf size, malformation and mosaic.

Cassava plant showing symptoms of mosaic, leaf distortion and chlorosis.

Country	District	Jatropha	
		Symptomatic	Asymptomatic
Kenya	Kakamega	80.0%	50.0%
	Busia	62.5%	22.0%
	Siaya	37.5%	16.6%
	Nakuru	11.0%	0%
Ethiopia	Amhara	61.5%	-

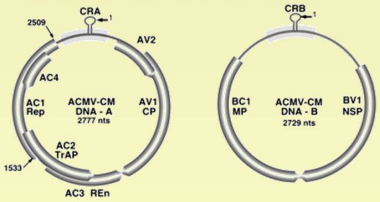
DAS-ELISA and TAS ELISA was used to determine the presence of *Cucumber mosaic virus* (CMV), *Cassava common mosaic virus* (CsCMV) and *Cassava brown streak virus* (CBSV). All *Jatropha* samples were negative. PCR primers designed to amplify 380 bp of part of AC1, AC2 and AC3 of geminiviruses yielded positive results in symptomatic and asymptomatic plants to variable degrees depending on the region of origin.

Bemisia tabaci the vector transmitting cassava mosaic geminiviruses

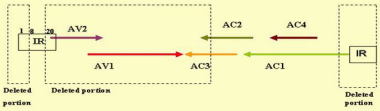


PCR of defective DNA A of ACMV from *Jatropha*. PCR was performed on Rolling Circle Amplification products from DNA extracted from field – grown *Jatropha* plants. Lane 1 shows the defective DNA A, lane C+ and C- positive and negative controls. Lane M= marker VIII (Roche Applied Science).

The genomes of majority of the begomoviruses consists of two components termed DNA – A and DNA – B



Genome organization of DNA-A and DNA-B of *African cassava mosaic virus* – Cameroon. DNA-A contains six ORFs: AC1–AC4, AV1, and AV2. DNA-B contains ORFs BV1 and BC1. AC1 encodes replication-associated protein (Rep). CP, coat protein; TrAP, transcriptional activator protein; REn, replication enhancer protein; MP, movement protein; NSP, nuclear-shuttle protein.



Naturally occurring Def DNA A of ACMV was found in Jatropha which at 1416 bp is around half the expected genome. The deleted part of the genome based on the full length DNA A component of ACMV [GenBank NC_001467] is shown in dashed boxes.

Phylogenetic tree of the alignment of virus sequences obtained from *Jatropha* from the study and other related virus sequences from the Genbank. Viruses obtained from the study have been written in bold. Full length sequences (2.8 bp) of 40 geminivirus isolates from Kenya were detected showing a high sequence similarity to **98.7% of *East African cassava mosaic virus*–Uganda (EACMV–UG) and 97.3% of *African cassava mosaic virus* (ACMV).**

Conclusion Cassava begomoviruses occuring in Jatropha might be more widespread than anticipated since we have detected it from samples from Ethiopia. If not taken care of, this might negatively impact on large scale plantations for biofuel production. The additional natural host of ACMV opens new avenues for further recombination of the viruses to occur which indeed becomes a threat both to *Jatropha* and other important host crops.

References:

- Stanley, J. et al., 2004. Family Geminiviridae. In Virus Taxonomy: Eight Report of the International Committee on Taxonom of viruses. London, Elsevier/Academic Press, 301 – 326.
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Rolling circle amplification for genetic and functional studies of plant viruses infecting African *Euphorbiaceae*

Alberto Calari, Rose Ramkat, Fatemeh Maghuly and Margit Laimer

Plant Biotechnology Unit, Department of Biotechnology, BOKU University, Muthgasse 18, 1190 Vienna, Austria

Jatropha, a biofuel plant, and cassava, an important food crop in Sub- Saharan countries, belong to the family *Euphorbiaceae*. While several viruses infecting cassava were described, so far, little information is available about viruses infecting *Jatropha*. In this study 112 Jatropha and 124 cassava samples from Kenya and Ethiopia were analysed. Two species of Cassava mosaic geminivirus were detected, showing a high sequence similarity to EACMV-UG (98.7%) and ACMV (97.3%). A naturally occurring defective (Def) of DNA-A of ACMV was found in *Jatropha*. Cassava begomovirus occurring in *Jatropha* might be more widespread than anticipated. If not taken care of, this might negatively impact large scale plantations for biofuel production.

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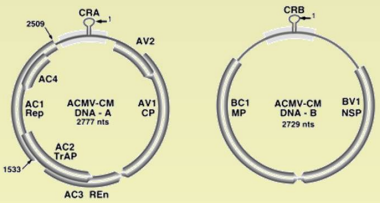

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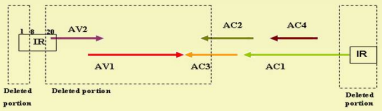

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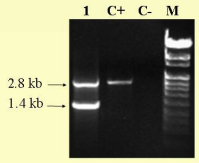

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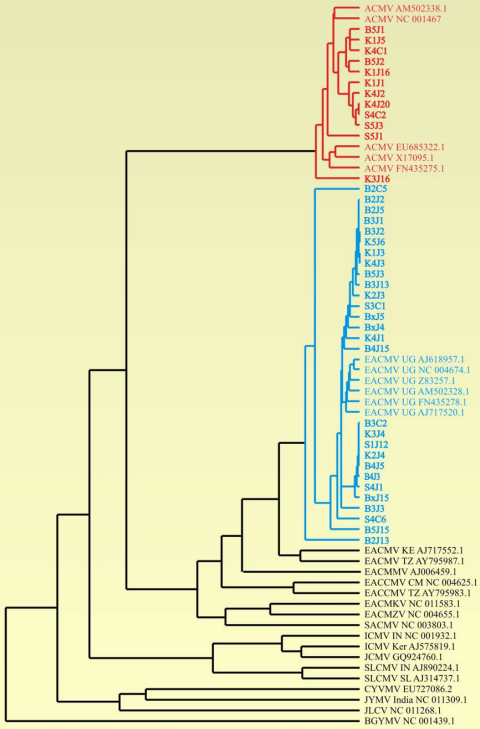

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Conclusion Cassava begomoviruses occuring in Jatropha might be more widespread than anticipated. If not taken care of, this might negatively impact on large scale plantations for biofuel production. It further poses a challenge to the elimination strategy of *Cassava mosaic virus* in field grown cassava as a result of increase in inoculum from different hosts. The additional natural host of ACMV opens new avenues for further recombination of the viruses to occur which indeed becomes a threat both to cassava an important food crop to Sub Saharan countries and *Jatropha*.

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Prediction of microRNAs encoded by *Begomovirus* infecting *Euphorbiaceae*

Fatemeh Maghuly, Rose Ramkat and Margit Laimer

Plant Biotechnology Unit, Department of Biotechnology, BOKU University, Muthgasse 18, 1190 Vienna, Austria

African cassava mosaic virus (ACMV) and *East African cassava mosaic virus-Uganda* (EACMV-UG) are members of the genus *Begomovirus*, family *Geminiviridae*. They cause severe losses in many economically important crops like cassava and *Jatropha*. miRNAs constitute a class of small, non-coding RNAs of approximately 21-24 nucleotides in length that regulate gene expression at the post-transcriptional level by targeting specific messenger RNAs for cleavage or translational repression (Jones-Rhoades et al., 2004). They are present in all plants, animals and in some viruses. There are different methods for identifying miRNA including cloning, NextGen sequencing and computational approaches. In this study, the computational approach was used to identify miRNAs encoded by ACMV and EACMV-UG. Prediction of virus miRNA and their targets on *Jatropha* and cassava will improve the understanding of host pathogen interaction and may in turn benefit *Euphorbiaceae* health.

Fig 1a: *Jatropha* plant showing *Geminivirus* symptoms of reduced leaf size, malformation and mosaic.

Fig 1b: Cassava plant showing *Geminivirus* symptoms of mosaic, leaf distortion and chlorosis.

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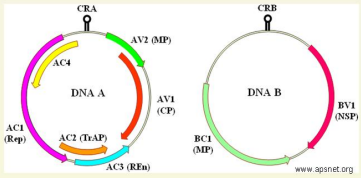

Fig 2: Genome organization of DNA-A and DNA-B of cassava-infecting begomoviruses. ORFs are denoted as either being encoded in the virion-sense (V) or complementary-sense (C) strand. CRA, common region A; CRB, common region B; CP, coat protein; MP, movement protein; Rep, replication-associated protein; TrAP, transcriptional activator protein; REn, replication enhancer protein.

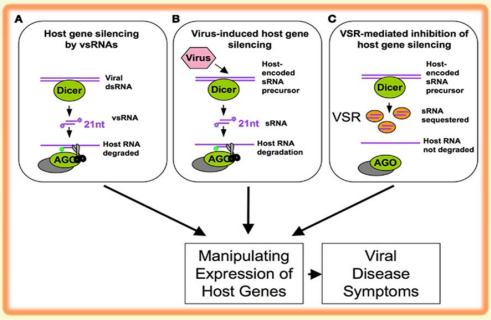

Fig 3: Viral infection triggers production of virus derived small RNAs (vsRNAs) which include miRNA and siRNA by the host gene-silencing machinery. RNA silencing functions as a natural antiviral defence mechanism. vsRNAs associate with Argonaute (AGO) family proteins and guide the resulting RNA-induced silencing complexes to complementary RNA or DNA targets. Plant viruses encode RNA silencing suppressors (VSR) to counteract the antiviral RNA silencing response (Amari et al., 2012). Viruses can also exploit RNA silencing to modify host gene expression directly because of homologies between vsRNAs and host transcripts.

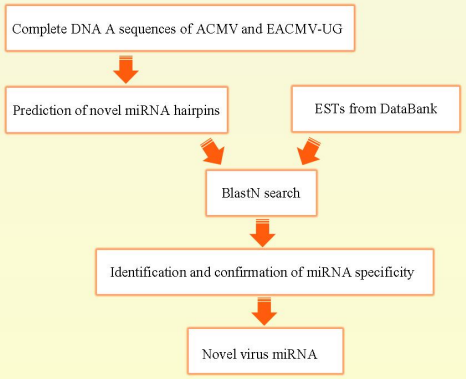

Fig 4: Outline of steps followed to search a total of 40 ACMV and EACMV-UG DNA A sequences isolated from infected *Jatropha* and cassava (Ramkat et al., 2011) used to predict virus miRNAs. This led to the identification of 9 real precursor miRNA (Pre-miRNA) hairpins, 23 novel virus miR/miR* and their targets in *Jatropha* and cassava.

Virus	Pre-miRNA	A %	C %	G %	T %	A+T %	C+G %
ACMV	ACMV 1	33.3	28.57	16.67	21.43	54.76	45.24
	ACMV 2	29.2	13.85	26.15	30.77	60	40
	ACMV 3	26.3	13.75	28.75	31.25	57.5	42.5
	ACMV 4	25.3	19.54	26.44	28.74	47	40
	ACMV 5	23	16.22	29.73	31.08	54.05	45.95
	ACMV 6	22	25	25	28	50	50
	ACMV 7	29.6	19.75	20.99	29.63	59.26	40.74
EACMV-UG	EACMV-UG 1	23.5	20	28.24	28.24	51.76	48.24
	EACMV-UG 2	23.9	22.39	26.87	26.87	50.75	49.25

Table 1: Nucleotide content of 7 ACMV and 2 EACMV-UG real miRNA hairpins. Nucleotides G and C contribute to the stabilization of the secondary structure of stem-loop hairpins and the higher the GC content of a sequence, the more stable the secondary structure will be. The GC content should range between 24-71%. In the current study the GC nucleotide content of the predicted hairpins was between 40-50%.

	Jatropha		Cassava	
Molecular function	**ACMV**	**EACMV-UG**	**ACMV**	**EACMV-UG**
Binding	38%	34%	47%	44%
Catalytic activity	34%	19%	14%	30%
Structural molecule activity	4%	8%	13%	1%
Transporter activity	2%	3%	4%	3%
Enzyme regulator activity	5%	-	1%	2%
Transcription factor activity	1%	4%	2%	-
Molecular transducer activity	-	1%	4%	-
Nutrient reservoir activity	1%	-	-	-
Unknown	15%	27%	15%	20%

Table 2: The different miRNA targets on *Jatropha* and cassava ESTs based on the RNAhybrid grouped into 8 molecular functions. The largest group of targets possesses binding functions, while the smallest group comprizes targets involved in nutrient reservoir activity.

Conclusion: This is the first collection of predicted viral miR/miR* of ACMV and EACMV-UG, providing a reference point for further studies of miRNA identification in pathogens. The obtained results reveal important implications for miRNAs encoded by begomoviruses, which are located in genes acting as suppressor of PTGS, and their targets in plant pathogenesis related pathways.

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Detection of Phytoplasmas in *Rubus and Fragaria* species

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Phytoplasma diseases affect small fruit species in the family *Rosaceae* including *Rubus idaeus*, *Rubus fruticosus* and *Fragaria ananassa*. Infected plants may show a variety of symptoms such as stunting, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation and fruit malformations. These diseases in *Rubus* are associated with infection by phytoplasmas belonging to the following groups: elm yellows (Lee et al., 1995), X disease (Davies, 2000), aster yellows and stolbur (Borroto Fernández et al. 2007). In strawberry, phytoplasma belonging to groups 16SrI, 16SrIII and 16SrVI, 16SrXII and 16SrXIII have been reported (Jomantiene et al. 1998; 2001). In this study, a total of 47 plants (32 *R. idaeus*, 7 *R. fruticosus* and 8 *F. ananassa*) were analyzed. DNA was extracted from a total of 142 samples. PCR, RFLP and molecular cloning were used to detect the phytoplasmas.

Fig 1a: *Rubus idaeus* plant showing symptoms of extended sepals

Fig 1b: *Rubus idaeus* showing proliferation of flower buds from a young fruit

Fig 1c: *Fragaria ananassa* showing leafy structures growing from the fruits

Fig 1d: *Rubus idaeus* showing growth of young fruits from mature fruits

Primer	Primer sequence 5´ to 3´	Source
R16mF2	CATGCAAGTCGAACGGA	Gundersen & Lee 1996
R16mR1	CTTAACCCCAATCATCGAC	Gundersen & Lee 1996
R16F2nM	GAAACGGTTGCTAAGACTGG	this study
R16R2	TGACGGGCGGTGTGTACAAACCCCG	Gundersen & Lee 1996
R16VF1	TTAAAAGACCTTCTTCGG	Lee et al. 1994
R16VR1	TTCAATCCGTACTGAGACTACC	Lee et al. 1994
P1	AAGAGTTTGATCCTGGCTCAGGATT	Schneider et al. 1995
P7	AAGAGCCGATGAAGGACG	Schneider et al. 1995
RubF	GTGGTGCATGGTTGTCGTCAG	this study
RubR	CTAACATCTCACGACACGAACTGA	this study
PA2F	GCCCCGGCTAACTATGTGC	Heinrich et al. 2001
PA2R	TTGGTGGGCCTAAATGGACTC	Heinrich et al. 2001

Table 1: Oligonucleotides used for detection in this study. The primers RubF, RubR and R16F2nM were designed in this study. Primers R16mF2/R16mR1, R16F2nM/R16R2, R16VF1/ R16VR1 were used for the diagnosis of phytoplasmas. Primers P1/P7, P1/RubR, PA2F/PA2R, RubF/P7 were used for cloning purposes.

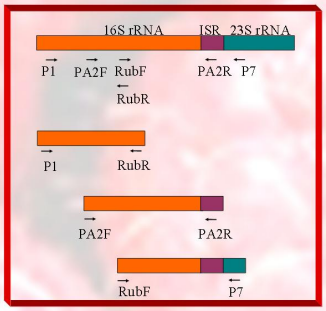

Fig 2: Amplification strategy used for the cloning of 16SrRNA sequences of phytoplasmas from *R. idaeus*. Three different fragments with overlapping regions were amplified, cloned, and sequenced to obtain the full length fragment. A 1852 bp fragment corresponds with 99% sequence similarity to *Rubus* stunt isolate AC: Y16395 and FD isolate AC: X76560.

Sample	Tissue tested	R16mF2/R16mR1	R16VF1/R16VR1	RFLP
R.i. 1	leaf veins	+	+	16SrV
R.i. 2	sepals	+	+	16SrV
R.i. 3	sepals	+	-	16SrV
R.i. 4	leaf veins	+	+	16SrV
R.i. 5	bark scrapings	+	-	not tested
R.i. 6	leaf veins	+	-	16SrV
R.i. 7	bark scrapings	+	-	16SrIII
R.i. 8	petioles	+	+	16SrV
R.i. 9	roots	+	+	16SrV
R.i. 10	sepals	+	-	uncertain
R.i. 11	bark scrapings	+	+	16SrV
R.i. 12	leaf veins	+	+	16SrV
R.i. 13	bark scrapings	+	-	not tested
R.i. 14	leaf veins	+	+	16SrV
R.i. 15	bark scrapings	+	-	16SrV
R.i. 16	bark scrapings	+	-	16SrV
R.i. 17	leaf veins	+	+	16SrV
R.i. 18	leaf veins	+	+	16SrV
R.i. 19	leaf veins	+	-	not tested
R.i. 20	leaf veins	+	-	16SrV
R.i. 21	leaf veins	+	+	16SrV
R.i. 22	leaf veins	+	+	16SrV
R.i. 23	leaf veins	+	-	not tested
R.i. 24	bark scrapings	+	-	not tested
R.i. 25	bark scrapings	+	+	16SrV
R.i. 26	bark scrapings	+	+	16SrV
R.i. 27	flowers	+	+	16SrV
R.i. 28	sepals	+	+	not tested
R.i. 29	flowers	+	+	not tested
R.i. 30	flowers	+	+	not tested
R.i. 31	flowers	+	+	not tested
R.f. 1	flowers	+	+	16SrV
R.f. 2	fruit	+	+	16SrV
F.a. 1	fruit	+	not tested	not tested
F.a. 2	fruit	+	not tested	16SrIII
F.a. 3	sepals	+	not tested	16SrVI

Table 2: Summary of samples showing the respective tissues tested using PCR with universal primers R16mF2/R16mR1, group 16SrV specific primers (R16VF1/R16VR1) and RFLP of R16F2nM/R16R2 products using *MseI*.

Conclusion *Rubus spp.* plants were found to be infected with phytoplasmas belonging to groups 16SrIII and 16SrV, while *Fragaria spp.* were infected with phytoplasmas from group 16SrIII and 16SrVI. In fact, the presence of the group 16SrV member *Candidatus* Phytoplasma rubi has been reported by Malembic-Maher et al. 2011. This is a serious threat for *Rubus* cultivation in the light of their economical importance, which calls for the design of a rapid elimination strategy to be applied in the production of propagation material.

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Expression profiles of *CaM* and *Ltp* in response to different stresses in *Prunus sp.*

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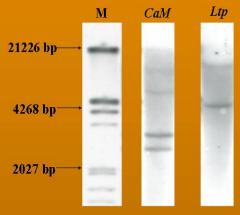
Introduction

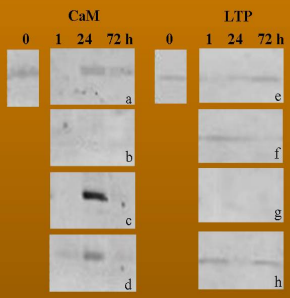
Changes in environmental conditions play a key role in altering gene expression. Therefore, transcription factors and their target genes are promising candidate for crop improvement. Calcium binding proteins and pathogenesis-related (PR) proteins are crucial components of the inducible repertoire of plant stress and defence. Considering the important role played by calmodulin (CaM) and lipid transfer protein (LTP) in mediating plant signal transduction, the current study investigated the expression of *Ltp* and *CaM* genes in *Prunus incisa x serrula* (PIS) under various abiotic stress conditions. In order to find out, whether expression of these proteins is regulated in parallel or independently, the expression profiles of *CaM* and allergenic proteins like *Ltp* were compared under different stress conditions.

Methods

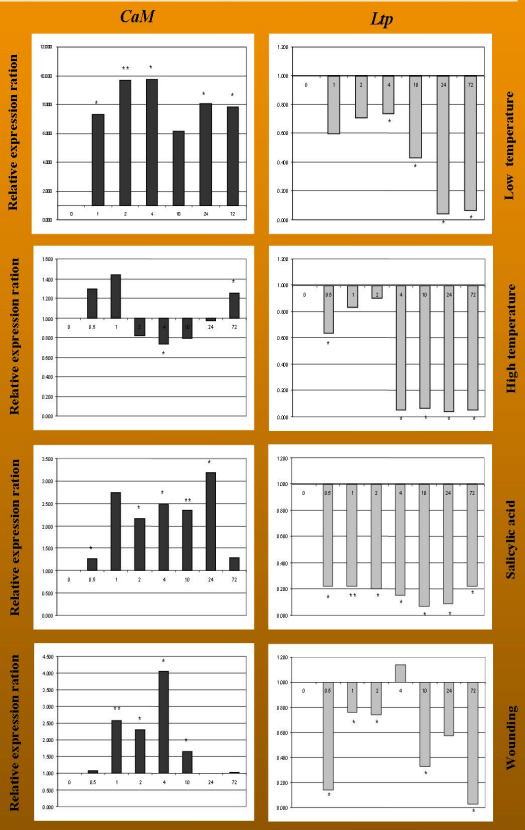
- Transcription and translation levels of both genes were analysed by Real Time qPCR and Western Blot in leaves and roots of micropropagated plantlets under low and high temperatures, salicylic acid and wounding stress, harvested after 0, 0.5, 1, 2, 4, 10, 24 and 72 h.
- Western blots were performed using commercial mAbs against calmodulin or a rabbit pAb against apple LTP as primary and AP-conjugated mouse anti-rabbit-IgG as secondary antibodies.
- Quantitative PCR was analyzed with the software tool REST.



Southern blot was performed to investigate the number of genes corresponding to *CaM* and *Ltp* in the genome. Genomic DNA from PIS plants was digested with EcoRV and the resulting fragments were hybridized with the *CaM* and *Ltp* probes. *CaM* hybridized to two or four fragments and *Ltp* to two fragments. These results suggest that each of genes is present in several copies in the PIS genome. M) DIG-labelled DNA Molecular weight Marker III.



Western blot indicated that the amount of CaM protein increased during 24 h and decreased after 72h under low temperature, salicylic acid and wounding stresses (c-d) compared to unstressed plantlets (0). However, high temperatures induced a very low expression (b). The amount of LTP decreased during 24h under low- and high temperature and wounding stresses (f, h), while after salicylic acid treatment the expression was very low (g). After 72h the amount of LTP increased upon low temperature and wounding (e, g) and decreased under high temperature stress (f).



Expression profiles of both genes altered under all stress conditions analyzed. Relative gene expression showed a lower *Ltp* expression, when compared to unstressed plantlets after different treatment times. *CaM* mRNA was significantly up-regulated in leaves, whereas *Ltp* mRNA was down-regulated under all stresses and at all time intervals. In roots both genes were down regulated (data not shown). All expression values are normalized to the expression value of *Actin*. Relative expression is calibrated base on the corresponding gene expression at "0" time. Significance is indicated as *p = 0.001 and **p = 0.03. PCR efficiency for *Actin* amplification was 90% with correlation coefficients (R^2) = 0.99, for Ltp 89% with R^2 = 0.997 and for CaM 100% with R^2 = 0.996.

Discussion

- Southern blot results indicated the presence of isoforms and confirmed the expected variability.
- Real Time qPCR data showed that both genes respond differently to various stresses. Indeed, expression of *Ltp* is regulated by different factors than *CaM* under stress response conditions.
- Furthermore, a high variation of both genes was observed in leaf, compared to root tissues. This tissue-specific expression hints to a potential role of different isoforms in PIS.
- Western blot analyses suggested that CaM and LTP are differentially regulated in response to different stresses in PIS plants. Competition among CaM isoforms for target proteins may occur, and therefore different transcriptional regulation can significantly affect the ratio of CaM isoforms.
- The different expression levels of CaM and *Ltp* genes observed suggest that these two genes may play an important but independent role in the adaptation of plants to environmental stresses.
- However, further studies will be performed in order to assess the role of CaM and LTP, as well as to understand the function of individual isoforms.

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Annex 4. Curriculum vitae

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- **2011** Bioinformatical analysis of phytoplasma sequences. Ttraining held in Vilnius, Lithuania (4 days)
- **2009** Pesticides and food safety in integrated pest management, Wageningen University, Netherlands (2 weeks)
- **2009** Integrated pest management and food safety policy and institutional innovations, Wageningen University, Netherlands (2 weeks)
- **2002-2006** Masters of Science studies in Agronomy; crop protection at Egerton University, Kenya
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- **1996-2001** Bachelor of science degree course in Agriculture education and extension, Egerton University, Kenya.
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- **2006-2009** Divisional Agricultural extension officer, Ministry of Agriculture
- **2002-2009** Collaborative research with Egerton University
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TEACHING EXPERIENCE

- **2011-2012** Teaching Molecular phytopathology course (954.309) at BOKU University under the supervision of Ao.Univ.Prof.Dr.phil. Margit Laimer.
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PUBLICATIONS AND PRESENTATIONS

Peer reviewed papers

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- Maghuly**, F.,** Jankowicz-Cieslak, J.**,** Calari A.**, Ramkat, R.,** Till**,** B., Laimer, M. 2011. Investigation of genetic variation in *Jatropha curcas* by Ecotilling and ISSR. BMC Proceedings 5 (Suppl 7), O50.
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