

Molecular Evidence for the Association of a Strain of Uganda Variant of East African Cassava Mosaic Virus to Symptom Severity in Cassava (*Manihot esculenta* Crantz) Fields in Togo

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Abstract: Problem statement: This study was carried out to demonstrate that the severity of Cassava Mosaic Disease (CMD) in Togo, is not only influenced by synergism between cassava *Begomoviruses* in presence, but essentially by recombination between the different *Begomoviruses* infecting cassava. **Approach:** Foliar samples presenting typical biological features of *Begomoviruses* infection were collected from cassava and wild infected plants from different regions of Togo and analysed by PCR targeting the Coat Protein (CP). The PCR products obtained from different isolates of two major *Begomoviruses* species infecting cassava in Togo were then sequenced and compared with the sequenced of the African cassava mosaic *Begomoviruses* identified to date and available in NCBI GenBank database by phylogenetic analysis. **Results:** The results indicate that not only the two major *Begomoviruses* could be in synergistic interaction in infected cassava in Togo as it has been shown between African Cassava Mosaic Virus (ACMV) and East African Cassava Mosaic Virus (EACMV) elsewhere, but could also create recombinants which would be highly interfering in the development of symptom severity in the country. **Conclusion/Recommendations:** The study confirmed the assumption that the symptom severity in cassava fields in Togo is rather caused by recombination between different *Begomoviruses* in presence than by synergistic interaction. More investigations should be done to give insight to this founding.

Key words: Cassava mosaic disease, phylogenetic analysis, recombinant strain, sequences, synergistic interaction, Uganda variant

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a staple crop grown throughout Africa and it is the most important source of dietary carbohydrate in sub-Saharan Africa^[1,2]. The crop is able to produce acceptable yields even on very marginal soils and in drought conditions, but is susceptible to a number of pathogens and losses to cassava production occur in all areas due to Cassava Mosaic Disease (CMD), caused by various *Begomoviruses* (family Geminiviridae) transmitted whiteflies^[3-6]. The severity of the pandemic was attributed to a synergistic interaction between African Cassava Mosaic Virus (ACMV) and a recombinant strain of East African Cassava Mosaic

Virus (EACMV), known as the Uganda variant (EACMV-UG)^[7]. It was shown in Cameroon that EACMV-CM was a double recombinant capable of synergism with ACMV^[8]. The association of these *Begomoviruses* recombinants with the severe cassava mosaic disease epidemic was obvious although no explanations for the advantage of the heterogenous combination at the level of their CP genes, over the pre existing homologous association, could be provided.

The aim of this study is to describe the complexity of symptom severity in different cassava production regions of Togo caused by cassava mosaic *Begomovirus* disease by presenting some biological features of their infection which could be created by intermolecular recombination events.

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MATERIAL AND METHODS

Sample collection and virus isolates: Foliar samples were collected from cassava and wild infected plants (*Cassia hirsuta*, *C. obtusifolia*, *C. occidentalis*, *Manihot glaziovii*, *Pupalia lappacea*, *Strophantus hispidus* and *Tetrapleura tetraptera*). All these infected plants displayed mosaic, leaf deformation, yellowing and stunting symptoms and were collected from five economic regions of Togo between 2004 and 2005 for suitable molecular analysis.

Extraction of the total DNA: The total viral DNA was extracted from the various foliar samples collected according to plant DNA mini preparation method^[9]. Fifty mg of cassava infected leaf tissue was ground in 500 μ L of Dellaporta extraction buffer (100 mM Tris pH 8, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol) with pestle in 1.5 mL microtube and 33 μ L of 20% (w/v) of SDS were added, the mix was vortexed and incubated at 65°C for 10 min. Then 160 μ L of 5 M potassium acetate were added to the extract and vortexed and finally centrifuged 10 min at 14,000 g. The supernatant (580 μ L) was transferred to new 1.5 mL microtube and 350 μ L of ice cold isopropanol was added, vortexed and centrifuged for 10 min at 14,000 g and the supernatant was removed and discarded; the pellet was dried for 5 min in a Speed-Vac dryer and resuspended in 50 μ L of RNase A (10 μ g mg⁻¹ in 10 mM Tris-HCl pH 8, 1 mM EDTA) and left at room temperature for 30 min. DNA was finally precipitated for 30 min at -80°C in 2.5 volumes of absolute ethanol in the presence of 0.3 M sodium acetate. The pellet was washed twice in 1 mL of ethanol 70%, dried for 5 min in a Speed-Vac dryer and resuspended in 500 μ L distilled water.

Diagnosis by PCR: Identification of the *Begomoviruses* in the samples was done by Polymerase Chain Reaction (PCR) amplification using specific degenerate primers targeting the Coat Protein (CP), namely: JSP001/JSP002, JSP001/JSP003 and JSP012/JSP013 which identify respectively ACMV (African Cassava Mosaic Virus), EACMV (East African Cassava Mosaic virus) and ICMV (Indian cassava Mosaic Virus)^[8]. These primers are shown in Table 1: JSP001/JSP002 and JSP001/JSP003 give fragments of 770 base pairs; primer pair JSP012/JSP013, specific to ICMV gives fragments of 702 base pairs.

The negative and positive controls (Table 2) were provided by Dr. Max Schoenfelder of DSMZ (Germany). One of the positive controls for ACMV was provided by Dr. Peterschmitt of CIRAD (France) and was isolated in Côte d'Ivoire.

Table 1: Specific primers used for the identification of ACMV, EACMV and ICMV

Primer	Sequence (5'→3')	Target region
JSP001	ATG TCG AAG CGA CCA GGA GAT	5' ACMV/ EACMV CP
JSP002	TGT TTA TTA ATT GCC AAT ACT	3' ACMV CP
JSP003	CCT TTA TTA ATT TGT CAC TGC	3' EACMV CP
JSP012	GTC CAT ATA GGT AAR GTN ATG	5' ICMV CP
JSP013	CCT GCT CCT TGC TNG CYT ART	3' ICMV CP

Source: Fondong *et al.*^[8]

Table 2: List of used positive controls

Reference No.	DSMZ	Origin	Description
PV-0421		Nigeria	ACMV-NG: Cassava infected leaves
PV-0423		Kenya	EACMV: Cassava infected leaves
PV-0424		Sri Lanka	ICMV: Cassava infected leaves
PV-0872/ Ca-161		Cameroun	EACMV: Cassava infected leaves

The PCR reaction was performed in a volume of 25 μ L containing 2 μ L of extracted DNA, 1 unit of Taq Polymerase Extrapol 1 (Euro bio), 1.5 mM MgCl₂, 100 μ M of each dNTP and 0.8 μ M of each primer the reaction buffer provided by the manufacturer. The PCR conditions are as followed: a 94°C denaturation step of 2 min followed by 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature (50°C for ACMV and EACMV and 52°C for ICMV) and 1 min at 72°C and then a final elongation step of 10 min at 72°C.

Virus isolates sequenced: PCR products obtained from Coat Protein (CP) gene (~770 bp) were sent for sequencing at Grenoble in the Laboratory of Bioanalysis, in the unit of Genome express, Cogenics and submitted to GenBank to be numbered. The CP gene sequences obtained from *Begomoviruses* isolates occurring in Togo were compared to the GenBank accession numbers of the published cassava *Begomovirus* CP gene sequences^[10]. The GenBank sequences used in this study were:

- for ACMV: AY211462, AY211466, AY562421 and AY 562429
- for EACMV: AF259896 from Ivory Coast/Cameroon, AJ717534 from Kenya; AJ006461 from Malawi, AY795985 from Tanzania and AF126804, AF423178 and AY562424 from Uganda
- and for ICMV: AJ314739

Sequence analysis: All sequences for the selected viruses were aligned using the ClustalV method of aligning multiple sequences of BioEdit^[11]. Phylogenetic analyses were conducted on matrices of aligned sequences by using the neighbour-joining and bootstrap options of DARWin5. The phylogenetic tree was estimated using the neighbor-joining method^[12] with the unweighted pair-group method with arithmetic average (UPGMA) distance matrix (MegAlign

program). One-thousand bootstrap replications were performed to place confidence estimates on major groups resolved in the tree. The aligned sequences were subjected to the same multiple alignments approaches to detect intermolecular recombination events. Dendrograms were viewed, manipulated and printed by using DARWin5^[13].

RESULTS

Cassava mosaic *Begomovirus* disease and symptoms severity in the fields: Symptom severity caused by cassava mosaic *Begomovirus* disease in the different investigated regions is various and complex through the expression of the infections (Fig. 1). The percentages of the diseased cassava plants in the prospected regions are as follows: 67.3% for the Central Region; 55.01% for the Maritime Region; 56% for the Plateaux Region; 78% for the Kara Region and 85% for the Savannas Region (Table 3). Concerning the severity of the symptoms (note of 4-5) in these regions, the rates are: 13.02% for the Central Region; 4.9% for the Maritime Region; 7.58% for the Plateaux Region; 35.14% for the Kara Region and 26.66% for the Savannas Region (Table 4).

Six hundred ninety seven collected foliar samples were analyzed by PCR with degenerate primers specific to ACMV, EACMV and ICMV. Results obtained from this analysis revealed the presence of the three targeted *Begomoviruses*. The results also revealed that the three *Begomoviruses* are often in mixed infection with the following rates: 39.45% in the case of ACMV and EACMV, 1.72% in the case of ACMV and ICMV, 1.29% in the case of ACMV, EACMV and ICMV. 22% of the samples were infected by non identified viruses (in fact all the samples collected in this study present typical symptoms of virus) (Table 4).

Sequence analysis and comparison: Hundred and fourteen sequences of CP gene were obtained from isolates of Togo and two of them were submitted to EMBL Database and numbered. These numbered sequences were: Accession EU155148 for EACMV and Accession EU152125 for ICMV. Sequence analysis and comparison of ACMV, EACMV and ICMV from Togo with all the available nucleotide sequences of Gen Bank EACMV is presented in Table 5.

Phylogenetic analysis: CP gene sequences were considered for this analysis due to their importance for *Begomoviruses* replication gene expression^[14]. The phylogenetic tree was estimated using the neighbor-joining method^[13] with the Unweighted Pair-Group Method with Arithmetic average (UPGMA) distance matrix (MegAlign program).



Fig. 1: Various symptoms caused by cassava mosaic *Begomovirus* disease in the different cassava production areas in Togo: Leaf curling symptom with yellowing caused by ACMV on a local cultivar Yovovi; Leaf curling symptom caused by EACMV on a selected cultivar “KH₁04” and leaf curling symptom small leaves caused by ACMV in mixed infection with EACMV on a selected cultivar KH₁04

Table 3: Percentages of diseased cassava plants and symptom severity rates

Regions	Diseased plants Percentages	Symptom severity Rates
Central	66.69 (±10.75)	13.03 (±6.25)
Kara	78.17 (±8.53)	35.14 (±10.37)
Maritime	55.25 (±17.23)	4.91 (±0.77)
Plateaux	56.21 (±10.56)	7.58 (±6.00)
Savanna	85.10 (±4.10)	26.66 (±12.81)

Note: In bracket, the standard deviation

One-thousand bootstrap replications were performed to place confidence estimates on major groups resolved in the tree (Fig. 2 and 3). From the information we had on the trees, sequence identity was calculated; the criteria utilised to distinguish between different viruses were; 90-100% for isolates, 80-90% for strains and less than 80% for species demarcation^[10], due to the number of genetic recombination now known to occur among cassava *Begomoviruses*. The CP gene sequences of all the isolates found in our collection were similar to the published sequence of the recombinant EACMV-UG (96% of identity)^[7].

DISCUSSION

The results from our field surveys reveal that these viral diseases are present in all the zones of cassava production in Togo. Percentages of cassava plants severely attacked (evaluation note from 4-5) and which could reflect the severity of the disease in the prospected fields vary from one locality to another with the rate of 4.5-36%. This high rate of symptom severity on the plants in the different regions (Table 3) could be explained by a possible co-infection by ACMV and EACMV or a recombination between both viruses

which unusually induces severe symptoms under field conditions^[8,14] or by the susceptibility of some cultivars in the prospected fields. This assumption of possible co-infection by ACMV and EACMV which was suspected during our surveys in the different cassava production zones through the severity of symptoms observed was confirmed by the results we obtained by PCR by using degenerate primers specific to the target *Begomoviruses* infecting cassava. Though ICMV which is known to be present in India was also identified in several cassava production zones; the hypothesis of possible co-infection was also

established. The results obtained also reveal that the different *Begomoviruses* identified are often in mixed infections with the incidence rates showed in Table 4. This founding suggested the possibility that these viruses can act in synergism as it was the case in Uganda^[14] and these mixed infections could also incite recombination within the populations of *Begomoviruses* which infect cassava in this country and could explain the high rate of symptom severity on the plants in the different regions as it was observed during the surveys (Table 3 and Fig. 1).

Table 4: Distribution of ACMV, EACMCV and ICMV in cassava production zones of Togo

Regions	Isolates analyzed	Percentages of <i>Begomoviruses</i> identified in the different regions					
		ACMV alone	EACMV alone	ICMV alone	ACMV+EACMV	ACMV+ICMV	ACMV+EACMV+ ICMV
Maritime	145	21.38	12.41	1.38	36.55	0.00	0.00
Plateaux	307	29.32	0.33	0.00	54.07	1.63	0.98
Central	165	38.79	1.21	2.42	25.45	3.64	3.64
Kara	71	36.62	7.04	1.41	16.90	1.41	0.00
Savanna	9	66.67	11.11	11.11	22.22	0.00	0.00
Total	697	31.13	3.87	1.15	39.45	1.72	1.29

Table 5: CP sequences identity (%) of selected cassava *Begomoviruses*

Isolats Du Togo	References GenBank								
	ACMV AY 211461	ACMV AY 526421	EACMV AF 259896	EACMV AY 795985	EACMV AJ 717534 UG2M	EACMV AF126804	EACMV AF423178 UGNa	EACMV AY562424 UG2S	EACMV AY562424 UG2S
AC49005M (12-147)	98	97	79	77	93	93	94	93	93
AC3805K (16-73)	98	97	79	78	93	93	94	93	93
AC22905P (28-331)	99	97	79	77	93	93	94	93	93
EAC05-06C (25-149)	78	77	96	95	81	80	80	80	80
EAC05-30M (30-114)	78	77	96	94	81	81	81	80	80
EAC05-38K (16-73)	78	77	97	95	80	81	80	80	80
EU155148	94	93	79	77	96	96	96	96	96

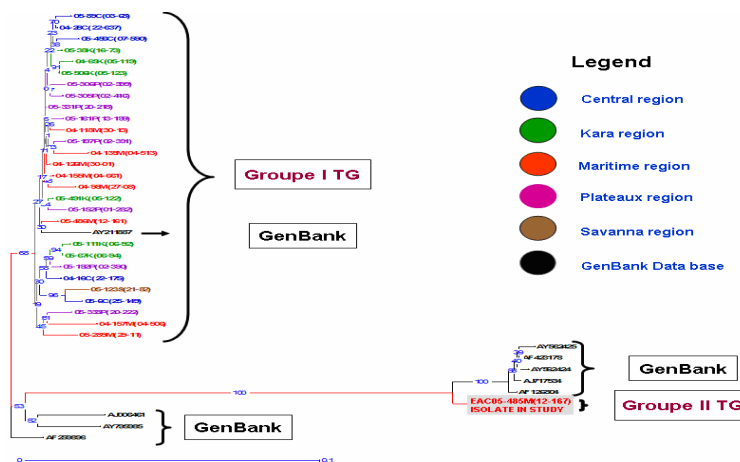


Fig. 2: Consensus phylogenetic tree (10000 bootstrap replications) obtained from comparison of the CP sequences of selected EACMV identified in Togo (black and red bullet) and the GenBank accession number of the published CP *Begomoviruses* sequences from various part of Africa

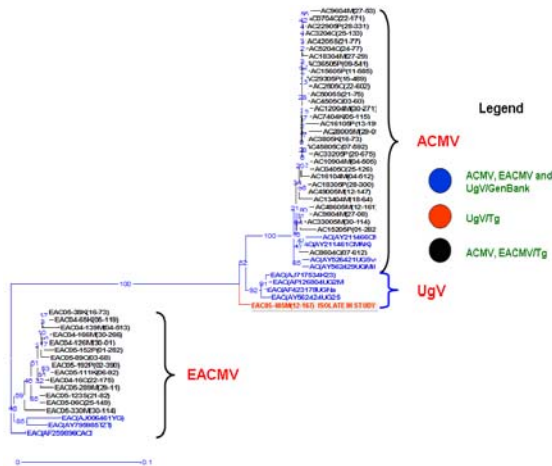


Fig. 3: Consensus phylogenetic tree (10000 bootstrap replications) obtained from comparison of the CP sequences of selected cassava *Begomoviruses* occurring in Togo (black and red bullet) and the Gen Bank accession number of the published CP *Begomovirus* sequences from various part of Africa

Phylogenetic analysis and sequence comparison reveal the evidence of the existence of a recombinant strain between the EACMV and the ACMV in Togo confirming also observations on symptom severity in cassava production areas in Togo and the mixed infections revealed by results obtained from PCR analysis. The CP gene sequence of the isolate found in our collection was similar to the published sequence of the recombinant EACMV-UG^[6]. These results allowed a better understanding of the severity of cassava mosaic disease nowadays in Togo. It is possible that emergence of new *Begomoviruses* by recombination may occur in Togo^[15]. For some other authors, this type of recombination is known for *Begomoviruses*^[16]. The sequence in study was submitted to EMBL Database and numbered (Accession EU155148). Maximum of 96% sequence identity was obtained with the Uganda variant of EACMV (UgV) isolates in NCBI GenBank. Lower (77-93%) sequence identity was obtained with other isolates of EACMV which are different from the sequence in study. It is very important to notice that the high degree of molecular variation found among isolate sequences and distinct grouping of isolate sequences was unexpected (Fig. 2 and 3).

The criteria followed to distinguish between different viruses were; 90-100% for isolates, 80-90% for strains and less than 80% for species demarcation^[10]. Cluster alignments revealed a degree of nucleotide (nt) sequence identity with 96% identical nt sequences between an accession from Togo, EU155148

and the Uganda variant of EACMV (UgV) isolates in NCBI GenBank. The same accession from Togo, EU155148 compared to other EACMV isolates in NCBI GenBank from various part of Africa like Kenya and Malawi, the sequence identity is 77-79%. But if compared to ACMV isolates in NCBI GenBank, the sequence identity is 93-94%. It is obvious that the accession EU155148 from Togo, is a strain of the Uganda variant of EACMV (UgV) named in our study, the Uganda variant of EACMV from Togo (UgV/Tg).

CONCLUSION

In this study, we demonstrated that the symptom severity caused by cassava mosaic *Begomovirus* disease on cassava plants in the different regions could not be explained only by a co-infection between ACMV and EACMV but also by a recombination between both viruses which unusually induces severe symptoms under field conditions. This finding is very important due to the new development of the disease in sub-Saharan region.

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