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CHAPTER 8

GENERAL DISCUSSION

Cassava (*Manihot esculenta* Crantz) is a basic staple food in the tropics where it provides a cheap source of dietary carbohydrate energy to over 500 million people (FAO, 2003). In Tanzania, cassava is produced mainly by small-scale farmers for food and income generation. Although yield of cassava can be quite high (25-40 metric tonnes/ha) (Plucknett *et al*., 2000), yields are generally low with many famers recording as low as five tonnes per hectare (MAFS, 2003, unpublished data). Cassava mosaic disease (CMD) caused by cassava mosaic geminiviruses constitutes the most limiting factor to cassava production and can be observed in all cassava growing areas (Legg and Raya, 1998) casing yield losses as high as 90% in susceptible cultivars (Thresh *et al*., 1994). Cassava mosaic disease was first observed in Tanzania in 1894 by Warburg (1894) but the disease was poorly studied until the 1990s when two cassava geminivirus species, *East African cassava mosaic virus* (EACMV) (Swanson and Harrison, 1994) and *African cassava mosaic virus* (ACMV) (Ogbe *et al*., 1996) were identified based on serological tests often in mixed infections that resulted in very severe disease. However, subsequent surveys conducted in the country revealed variations in disease severity, incidence and the EACMV Uganda variant (EACMV-UG2), which is associated with severe epidemic of CMD was reported to spread in the Lake Victoria Zone of Tanzania in 1998 (Legg and Raya, 1998; Legg, 1999) from Uganda. These findings emphasized the need for more studies to understand the cassava mosaic geminiviruses (CMGs) using techniques better than serology. The goal of this study was therefore to carry out molecular characterization of CMGs in Tanzania with the following objectives;

1) to identify CMGs and their variability from all the major cassava growing areas using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis and generate a geographical map of CMGs distribution (Chapter 3); 2) determine

molecular characteristics of CMGs in mixed infections (Chapter 4 partly shed light on this); 3) to clone, and sequence representative CMGs to determine the species, strains/isolates and compare the sequences to those available in the GenBank database (Chapter 6) in an effort to provide a detailed description of the CMD syndrome; 4) to uncover molecular factors involved in CMD symptom variability (Chapter 5); and 5) to assess molecular diagnostic methods that can be used for routine diagnosis of CMGs in Tanzania (Chapter 7).

During the current study, a survey was conducted to collect CMD-infected samples from all the major cassava-growing areas in Tanzania (the coastal areas, southern part, and Lake Victoria regions). Restriction and sequence analysis of the PCR-amplified viral DNA products revealed the existence of different CMGs in Tanzania with EACMV being the most widespread throughout the country. The ACMV was not found in any of the samples collected from the coastal areas indicating that only EACMVs are involved in CMD in these areas. Furthermore, there was higher molecular variability in EACMV than in ACMV as reported elsewhere (Fondong *et al*., 2000; Pita *et al*., 2001). A total of 13 EACMV isolated were identified with differing RFLP patterns, with some isolates associated with very severe symptoms, others moderate and only a few mild symptoms. A map was generated to show their geographical distribution in the country presenting the first detailed description of the CMG. This information is useful especially if deployment of cassava resistant material in the country is sought. Existence of different virus isolates/strains can affect the resistance since most of cassava resistant materials are resistant to some but not all virus strains as was observed in Uganda (Sserubombwe *et al*., 2001). Results of the present work also showed the existence of a defective subgenomic DNA viral genome in the CMG mixed infections that appeared to interact with the wild type CMG in the natural infection. Cloning and sequence analysis revealed the DNA genome to be a defect of the EACMV DNA-A genome with some of its genes having been deleted, probably through mutations. However, it was found to retain all the sequences required for replication by the helper virus with which it was found to depend for replication and movement within the plant. Infectivity assays using *Nicotiana benthamiana* test plants and infectious clones, demonstrated the ability of the defective DNA molecules

here referred to as df DNA-A15 to attenuate symptoms only when co-inoculated with EACMV but not ACMV. This was because of sequence compartibility with EACMV but not ACMV. Defective interfering (DI) molecules have been described for geminiviruses with most of them produced through *in vitro* passages of geminiviruses in test plants. For example, a DI described for CMG was found to arise from a deletion of DNA-B component of ACMV genome during a repeated mechanical inoculation of test plants in a greenhouse (Stanley *et al*., 1990). The df molecule reported in this work however, is derived from DNA-A of EACMV in the natural field infection, presenting to our knowledge the first report of df EACMV of that type. The df was not widely spread in the fields suggesting that its formation and occurrence could be a rare event.

Although only EACMV occurred in all the cassava growing areas in the coastal belt strip of Tanzania (Ogbe *et al*., 1996), symptom variability in the infected cassava fields was very high even within the same field and the same cassava cultivars. This variation could partly be caused by different virus strains or other factors (unknown). This observation prompted the investigation of possible involvement of factors other than CMGs in the CMD. Indeed from cassava plants expressing very unique and severe symptoms we identified two novel satellite DNA molecules that interact with CMGs, modulate disease symptoms and break CMD resistance. This discovery represents the first report of DNA satellite molecules to be associated with CMGs. These two molecules, here named satDNA-II and satDNA-III, were found to have no significant sequence similarity to those in the databases qualifying them to be of unknown origin. Satellite molecules described for monopartite geminiviruses such as *cotton leaf curl virus* and *ageratum yellow vein virus* (Mansoor *et al*., 2003) are different from the ones reported here. These findings have changed our way of understanding of CMD etiology and presents a challenge to cassava breeders wishing to breed for CMD resistance as such molecules will have to be considered if stable resistance is to be achieved. Sequence analysis of CMG isolates provided a clear picture of EACMV distribution and highlighted a possible line of their evolutional divergence.

Recently, EACMV species from West Africa, *East African cassava mosaic Cameroon virus* (EACMCV) from Cameroon and Ivory Coast with many DNA sequence recombination features (Fondong *et al*., 2000) were described. In the present study, we found two EACMV strains in southern part of Tanzania, which resembled EACMCV in their DNA sequences, to possess the same type of recombination as the EACMCV. Sequence analysis also showed the two isolates to have originated long ago, excluding the possibility of recent introduction. This suggested that the two EACMCVs (from Cameroon and Tanzania) probably co-existed at an unknown time. A higher number of recombination fragments was identified among EACMV isolates than among ACMV (Chapter 6), partly providing an explanation for the high molecular diversity identified in EACMV isolates during this study and elsewhere (Fondong *et al*., 2000; Pita *et al*., 2001; Saunders *et al*., 2002; Legg and Fauquet, 2004) using both DNA-A and-B components. A total of seven full-length DNA-A genomes of new CMG isolates from Tanzania were deposited to GenBank database as well as one full-length and 13 partial DNA-B component sequences. This has contributed significantly to the geminivirus genetic base available so far in GenBank.

A simple technique of characterizing geminiviruses using Plant DNA stored on FTA cards was evaluated and found to be effective, sensitive and inexpensive for PCR detection of geminiviruses. All downstream analysis of the PCR products such as cloning and sequencing was achieved with a result that was comparable to the conventional method of DNA processing that normally employs the use of the lengthy protocol of phenol-chloroform based extraction. Since samples on FTA cards are stored at room temperature, this tool could be exploited for routine geminivirus diagnosis in the field where laboratory facilities are limited, such as in Africa.

Several issues arising from this work need further investigation in Tanzania:

- The high biodiversity of EACMV observed in Tanzania, probably higher than in any other country where cassava geminiviruses have been characterised so far, may suggest the origin of the EACMV species in Tanzania, which then diverged into other countries. Westward spread of EACMV from East Africa has been

reported recently (Legg and Fauquet, 2004). An investigation of the occurrence of EACMV in a diverse number of weed species present in Tanzania may help to shed light on this observation. The general assumption is that since cassava was introduced into Africa from South America where CMG does not occur, it is obvious that the crop acquired the virus from a weed resident in Africa, possibly Tanzania.

- Factors favoring the formation of defective DNA molecules in infected cassava need further investigation. The fact that df DNA-A 15 was observed in a natural field-infected cassava plant suggests the involvement of a natural factor(s) in the process of defective DNA formation, probably deletion mutations in the plant during DNA replication, and needs further investigation. Furthermore, the exact mechanism of symptom modulation by defective DNA molecules is poorly understood and needs further study.
- How the satellite molecules, discovered during the present study, interact with CMG to enhance CMD symptoms needs to be studied. At the moment it is not clear whether they interact with DNA-A or –B of the geminiviruses and the mechanisms of their replication are not understood. The fact that they significantly enhance symptoms when present with EACMV-UG2, the virus associated with the pandemic of severe CMD, may suggest the involvement of these satellite molecules in the CMD pandemic and needs investigation. It is also possible that other DNA molecules that interact with CMG do exist in nature and await to be discovered.
- There is also a need to further investigate the molecular variability of DNA-B components associated with CMGs in Tanzania. The high symptom variations associated with EACMV, observed during this study, may reflect genetic variation in the DNA-B components that encode the gene for symptoms expression.

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SUMMARY

Cassava (*Manihot esculenta* Crantz) constitutes one of the major staple foods in Tanzania ranking only second to maize. However, its production is limited by cassava mosaic disease (CMD) caused by cassava mosaic geminiviruses (CMGs) that cause significant yield losses of up to 90% and sometimes abandonment of the crop by farmers. Although CMD was recorded long ago in 1894 in Tanzania, information on the nature of the viruses causing CMD was scanty.

Results from the Tanzanian countrywide sample collection survey showed the presence of CMD everywhere cassava is grown with most areas recording high disease incidence and severity. Molecular analysis of collected DNA samples indicated a wider spread of *East African cassava mosaic virus* (EACMV) than *African cassava mosaic virus* (ACMV). Restriction fragment length polymorphism (RFLP) and sequence analysis revealed more molecular variability in the genome of EACMV than in that of ACMV. No ACMV was found in the samples from the east coastal areas or southern part of the country. Cloning and comprehensive nucleotide sequence analysis of different CMGs showed the presence of two strains {EACMCV- [TZ1] and EACMCV- [TZ7]} of *East African cassava mosaic Cameroon virus* (EACMCV) originally described from Cameroon and the Ivory Coast in West Africa. This is the first report to describe and provide a geographical distribution of CMG isolates in Tanzania and to present the presence of EACMCV strains in the eastern part of Africa. A molecular analysis of a naturally occurring truncated form of CMG DNA-A, found coinfected with EACMV in a single plant, was carried out in the laboratory and greenhouse. It was established that the truncated DNA was a defective subgenomic molecule derived from EACMV by sequence deletion. Infectivity assays revealed it to be infectious only when in the presence of the wild-type EACMV from which it was derived and could be replicated by the wild-type virus. Furthermore, it was found to attenuate symptoms of the wild-type virus when coinoculated with EACMV but not with ACMV in *Nicotiana benthamiana* test plants. Cassava mosaic geminiviruses require both DNA-A and –B to establish infection and produce typical CMD symptoms. In addition it was found

that the two novel satellite ssDNA molecules could interact with CMG, enhance CMD symptoms and break resistance. These satellite molecules depend on CMG for replication and movement within the plant and their sequence analysis (nucleotide and amino acid) showed no significant homology to sequences reported in the searchable databases. Since they do not resemble geminiviruses, or other plant DNA viruses reported so far, their origin could not be established. However, their biological role in symptom enhancement and breaking of resistance has added to the CMD complexity and may pose a threat to the development of sustainable control measures for CMD such as development of stable CMD resistant varieties.

In the present study a simple, inexpensive and effective tool of characterizing geminiviruses was evaluated and found to be useful. This tool, which involves characterizing geminiviruses using plant DNA stored on FTA cards (from Whatman Company, USA) at room temperature allowed the recovery of full-length viral DNA-A and –B genomes. Comparison of PCR, clones, and sequences of the virus, derived from the FTA processed DNA, to that obtained using the conventional methods of DNA processing (Dellaporta method), showed very comparable results. In addition, this tool allowed for detection of CMG dual infections commonly occurring in cassava fields and differentiation of different CMG species. This tool proved to be useful particularly for routine virus plant diagnosis in places with limited laboratory resources, such as Africa. From the present study other issues that require further investigation were highlighted.