CHAPTER 1.11

The Diversity of Cassava Mosaic Begomoviruses in Africa

Peter Markham, Robert Briddon*, Clotilde Roussot*, John Farquhar*, Geoffrey Okao-Okuja** and James Legg****

Introduction

Cassava mosaic disease (CMD) is the most widespread and economically important disease of cassava (*Manihot esculenta* Crantz) in tropical Africa. A disease of cassava was first described in East Africa in the nineteenth century (Warburg, 1894) and cassava mosaic, as it came to be called, was subsequently reported as spreading throughout the cassava-growing areas of Central and West Africa (Calvert and Thresh, 2002). In more recent times, CMD has been reported as occurring at varying levels of incidence throughout the cassava belt of Africa and losses have been estimated at between 12% and 25% of total production (Thresh et al., 1997).

In 1983, the first sequence of a geminivirus to be determined and published was that of *African cassava mosaic virus* (ACMV) (Stanley and Gay, 1983). Later, this virus was included in the genus *Begomovirus* of the family *Geminiviridae* (Briddon and Markham, 1995), consisting of viruses with bipartite genomes transmitted by the whitefly *Bemisia tabaci* (Gennadius). For some time, ACMV was thought to be the only geminivirus species associated with CMD, despite the suggestion by Harrison and Robinson (1988), based upon serological analysis, that CMD was regionally distinct. Some 10 years after the publication of the sequence of ACMV, Hong et al. (1993) characterized a further CMD-associated geminivirus. Although based upon only the sequence of the DNA A genomic component (and therefore lacking any corroborative information on infectivity, since this is determined by the B genome component) this virus was given the name *East African cassava mosaic virus* (EACMV). A further geminivirus species has been found since in southern Africa and named *South African cassava mosaic virus* (SACMV) (Berrie et al., 1998).

During the 1990s, an unusually severe and damaging form of CMD spread rapidly through Uganda and the wider East African region and came to be referred to as the CMD "pandemic" (Otim-Nape et al., 1997; Legg, 1999). A novel recombinant geminivirus was shown to be consistently associated with the pandemic (Zhou et al., 1997), commonly in dual infections with ACMV that gave rise to the severest symptoms (Harrison et al., 1997). The so-called Uganda variant has been shown to be a recombinant between

* John Innes Centre (JIC), Norwich Research Park, Colney, Norwich, UK.
** International Institute of Tropical Agriculture-Eastern and Southern Africa Regional Center (IITA-ESARC), Kampala, Uganda.
*** IITA-ESARC, Kampala, Uganda, and Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent, UK.

EACMV and ACMV, with ACMV providing an approximately 400 bp fragment of the coat protein gene; this virus is therefore best described as EACMV-Uganda (EACMV-Ug). Subsequent studies have identified DNA B components for EACMV-Ug, have confirmed the occurrence in Uganda of EACMV and have demonstrated infectivity through the generation of infectious clones (Pita et al., 2001).

At the beginning of this project it was apparent that the different begomovirus species and strains associated with CMD did not occur necessarily in different regions of Africa. It was therefore a major objective of the work to survey the diversity of cassava mosaic geminiviruses in the main cassava growing areas of Africa and determine their geographical distribution.

Methods and Materials

Cassava is normally propagated by stem cuttings and national collaborators therefore collected stem samples from CMD-infected plants during their project surveys, and shipped these to the John Innes Centre (JIC), UK. The material was planted in compost in containers (7.6 cm diameter) and maintained at about 25 °C (± 5 °C) in glasshouses, with supplementary lighting between October and April.

Samples of nucleic acids for polymerase chain reaction (PCR) analysis were extracted from 0.1 g samples of infected leaf tissue using the Nucleon Phytopure plant DNA extraction kit (Amersham) essentially as described by the manufacturer. The main modification of the procedure was that liquid nitrogen was used in place of dry ice. Samples were stored at –200 °C and used directly in the PCR reactions.

The PCR procedure and the primers utilized have been described previously (Briddon and Markham, 1994). Typically 1 µL, or 1 µL of a 10-fold dilution, of the DNA extract was used in the amplification reactions. Reaction volumes of 100 µL were used and amplification products were cleaned further by phenol extraction and ethanol precipitation before use in the restriction fragment length polymorphism (RFLP) analysis.

Typically 10 µL (of the original 100 µL reaction volume) of the PCR product was used for restriction enzyme digestion in a 100 µL reaction volume following the manufacturers' (Gibco-BRL) instructions. After a 4-hour digestion, RFLP products were resolved on 1% agarose gels in TBE buffer stained with ethidium bromide. For most cases this sufficed to determine the restriction pattern. However, for a few samples the amplification was poor. This necessitated blotting of the gel to Hybond membranes (Amersham) and probing with radioactively labeled probes. As a rule, all gels were blotted to Hybond for subsequent analysis.

Restriction patterns with particular viruses were predicted from published sequences (ACMV: Stanley and Gay, 1983; Morris et al., 1990. EACMV: Hong et al., 1993. EACMV-Ug: Zhou et al., 1997).

For each of the virus species encountered in the restriction mapping procedures, and any viruses with restriction maps that did not conform to known patterns, full length DNA A genomic components were cloned for sequence analysis. Clones were produced by PCR amplification with specific abutting, non-overlapping primers (Briddon et al., 1993). The sequences of these primers were obtained by cloning the PCR products produced with universal primers into pGem T-Easy vectors (Promega). Sequences were

determined by dideoxynucleotide chain termination sequencing using the PCR-based BIG DYE kit (Perkin Elmer Cetus) and specific internal primers (Gibco-BRL). Reaction products were resolved on an ABI 377 automated sequencer. Sequence information was stored, assembled and analysed using Version 7 of the program library of the Genetics Computer Group.

Results

Table 1 summarizes the analysis of the samples. About 13% of samples re-grew initially without symptoms, although 14% of these (2% of the total) did eventually show symptoms after more than 3 months. Less than 1% recovered after initially showing symptoms. Table 2 shows the viruses

Table 1. Summary of analysis of cassava cuttings collected during field surveys. Sampled plants were all showing symptoms when collected.[a]

Country	Total no. of stems	Dead	Re-grown healthy	Re-grown infected	Healthy > infected	Infected > healthy	DNA extract	PCR DNA-A
Tanzania	80	9	0	71	0	0	39	26
Kenya	45	15	0	30	0	2	14	5
Uganda	126	28	11	87	0	0	65	24
Zimbabwe	1	0	0	1	0	0	1	1
Malawi	23	19	2	2	0	0		
Madagascar	193	1	39	153	1	2	30	18
Benin	96	46	7	43	5	2	35	15
Nigeria	68	21	27	20	7	0	25	12
Ghana	4	0	0	4	0	0	4	0
Cameroon	32	24	4	4	0	0	4	0
Total[b]	671	163 (24)	90 (13)	418 (62)	13 (14)	6 (1)	217 (51)	101 (47)

a. "Healthy" indicates plants showing no disease symptoms after about 3 months of re-growth. "Healthy > infected" indicates plants that initially showed no symptoms on re-growth but developed disease later; "Infected > healthy" indicates the converse. Other data: not sampled 39 (9%); yet to be identified 68 (16%).

b. ( ) Percentage of totals; for DNA extract ( ) percentage of successful viral DNA extractions made from cassava mosaic disease samples; for PCR DNA-A ( ) percentage of DNA A products successfully amplified from viral DNA.

Table 2. Summary of virus species isolated following restriction mapping using *Mlu*1, *Dra*1 and *Eco*R1 on polymerase chain reaction products following the use of universal primers to amplify *Begomovirus* genome component A.

Country	Virus species[a]					Total
	ACMV	EACMV	EACMV-Ug	SACMV	Dual	
Tanzania	3	5			1	8
Kenya	1	3			1	4
Uganda	6	2	15		3	23
Zimbabwe				1		1
Madagascar	12	4				16
Benin	12	3				15
Nigeria	8					8
Totals[b]	42 (56)	17 (23)	15 (20)	1	[5]	75

a. ACMV, *African cassava mosaic virus*; EACMV, *East African cassava mosaic virus*; EACMV-Ug, EACMV-Uganda; SACMV, *South African cassava mosaic virus*; Dual, combinations.

b. ( ) percentage of total; [ ] included in total as single infections.

identified from the samples analysed: ACMV comprised 56%, EACMV comprised 43% (of which 53% were EACMV and 47% EACMV-Ug) and 7% were dual infections (of which all except one were combinations of ACMV and EACMV-Ug). Samples from West Africa comprised less than one-third of the total identified (31%) but of these only 13% were EACMV, while this virus accounted for 56% of East Africa samples.

Full-length clones of the DNA A components of CMD-associated begomoviruses were obtained from isolates originating from Uganda and from Zimbabwe. The sequence of the clone originating from Uganda shows this to be highly similar to the sequence of EACMV-Ug reported by Zhou et al. (1997). The DNA A component of this virus is essentially EACMV with an approximately 450 bp insertion of an ACMV sequence in the centre of the coat protein gene. The restriction pattern of the virus originating from Zimbabwe was entirely novel, with no counterpart in the published literature. Sequence analysis of this clone showed it to have most similarity to SACMV (Berrie et al., 1998). The sequence published by Berrie et al. (1998) covers only the coat protein gene of SACMV to which the sequence of the Zimbabwe clone shows 86% similarity. In the absence of the full sequence of SACMV we must provisionally conclude that the Zimbabwe virus is distinct from, but most closely related to, SACMV. Efforts are now under way to obtain the DNA B genomic components of these two viruses for infectivity studies. Attempts also are continuing to produce full-length clones of EACMV and ACMV originating from Madagascar. Although amplification of these viruses has been achieved, it has not yet proven possible to clone them.

Discussion

The results obtained during this screening project indicate that a PCR/RFLP-based approach is able consistently to identify the viruses associated with CMD and identify species or strains that differ significantly from those previously reported. It is unclear whether a protocol based solely upon PCR amplification with diagnostic primers for each virus species/strain could achieve the same.

It is surprising, based on experience of related viruses, to find the genetic stability which is evident from the very low variability detected both in the sequence analysis of clones and from the RFLP analysis. However, this stability makes RFLP analysis a particularly useful diagnostic tool for these viruses. This method also enables plants that are dual-infected to be correctly diagnosed because the overlaid RFLP patterns are evident. Possibly the major drawback to the technique is that high-quality samples of nucleic acids are essential but these may be difficult to extract from cassava. This was most evident in the case of the dried cassava leaf samples sent to JIC from Africa, from which no successful PCR amplification was achieved. The reason for this failure remains unclear. It is possible that sample degradation occurs under tropical (hot and humid) conditions; fungal growth was particularly evident on some of the leaf samples. This problem has been encountered previously where even samples dried under optimal conditions were not suitable for DNA extraction and subsequent begomovirus PCR amplification. However, the cassava leaf samples collected from the glasshouse and dried immediately yielded amplification products more than 1 year later, after storage at room temperature but in a dry atmosphere.

It is clear from the recent reports of new strains/species of begomoviruses identified in association with CMD that the diversity of these agents is greater than initially expected. The procedures utilized for the analysis of the diversity of begomoviruses infecting cassava across Africa were devised to be all encompassing. The use of so-called "universal primers", designed with degeneracy so as to amplify all whitefly-transmitted begomoviruses, ensures that all possible begomoviruses in a particular sample are amplified. The use of specific primers in an analysis like this would risk missing possible new viruses, or mutants of viruses, which have been described previously. The universal primers used (Briddon and Markham, 1994) are well characterized and have been able thus far to amplify all begomoviruses against which they have been tested.

The use of RFLP analysis on PCR-amplified DNA allows several diagnostic restriction enzymes to be used on a single sample to identify a virus. The initial identification of a virus species/strain by a particular restriction pattern was subsequently confirmed by sequence analysis of a full-length clone. Full characterization of a clone requires infectivity studies on cassava (Briddon et al., 1998). Our work has shown that the use of alternate hosts, such as *Nicotiana benthamiana* Domin (*Solanaceae*), can lead to selection of mutants with atypical biological characters (Liu et al., 1997) and thus is not appropriate for diagnostic studies.

Acknowledgements

The authors wish to thank the Danish International Development Agency for providing funds for this project, and all the participants of the Systemwide Program on Integrated Pest Management whose enthusiasm and determination guaranteed its initiation. We also wish to thank all members of the project team who sent material during their regional surveys; without their help and endeavours, the project would not have been possible. We also thank Dr. Richard Gibson and Mr. Ian Robertson for sending extra samples for inclusion. The work was also supported by the John Innes Centre, which is grant-aided by the Biotechnology and Biological Sciences Research Council. The viruses were imported and held under the authority of the Ministry of Agriculture Fisheries and Food, under the Plant Health (Great Britain) Order 1993, Licence Numbers PHL11/2557(4/1998) and PHL/2300(6/1998).

References

Berrie, L. C.; Palmer, K. E.; Rybicki, E. P.; Rey, M. E. C. 1998. Molecular characterization of a distinct South African cassava infecting geminivirus. Arch. Virol. 143: 2253-2260.

Briddon, R. W.; Markham, P. G. 1994. Universal primers for the PCR amplification of dicot-infecting geminiviruses. Mol. Biotechnol. 1:202-205.

Briddon, R. W.; Markham, P. G. 1995. Geminiviridae. *In*: Murphy, F.A.; Fauquet, C. M.; Bishop, D. H. L.; Ghabrial, S. A.; Jarvis, A. W.; Martelli, G. P.; Mayo, M. A.; Summers, M. D. (eds.). Virus taxonomy: Sixth report of the International Committee on Taxonomy of Viruses. Springer, Vienna, AT. p. 158-165.

Briddon, R. W.; Prescott, A. G.; Lunness, P.; Chamberlin, L. C. L.; Markham, P. 1993. Rapid production of full-length, infectious geminivirus clones by abutting primer PCR (AbP-PCR). J. Virol. Methods 43: 7-20.

Briddon, R. W.; Liu, S.; Pinner, M. S.; Markham, P. G. 1998. Infectivity of *African cassava mosaic virus* clones to cassava by biolistic inoculation. Arch. Virol. 143:1487-1492.

Calvert, L. A.; Thresh, J. M. 2002. The viruses and virus diseases of cassava. *In*: Hillocks, R. J.; Thresh, J. M.; Bellotti, A. C. (eds.). Cassava: Biology, production and utilization. CAB International, Wallingford, GB. p. 237-260.

Harrison, B. D.; Robinson, D. J. 1988. Molecular variation in vector-borne plant viruses: Epidemiological significance. Philos. Trans. R. Soc., Lond., B, Biol. Sci. 321:447-462.

Harrison, B. D.; Zhou, X.; Otim-Nape, G. W.; Liu, Y.; Robinson, D. J. 1997. Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. Ann. Appl. Biol. 131:437-448.

Hong, Y. G.; Robinson, D. J.; Harrison, B. D. 1993. Nucleotide sequence evidence for the occurrence of three distinct whitefly-transmitted geminiviruses in cassava. J. Gen. Virol. 74:2437-2443.

Legg, J. P. 1999. Emergence, spread and strategies for controlling the pandemic of cassava mosaic virus disease in east and central Africa. Crop Prot. 18:627-637.

Liu, S.; Bedford, I. D.; Briddon, R. W.; Markham, P. 1997. Efficient whitefly transmission of African cassava mosaic geminivirus requires sequences from both genomic components. J. Gen. Virol. 78:79-94.

Morris, B.; Coates, L.; Lowe, S.; Richardson, K.; Eddy, P. 1990. Nucleotide sequence of the infectious cloned DNA components of *African cassava mosaic virus* (Nigerian strain). Nucleic Acids Res. 18:197-198.

Otim-Nape, G. W.; Bua, A.; Thresh, J. M.; Baguma, Y.; Ogwal, S.; Semakula, G. N.; Acola, G.; Byabakama, B.; Martin, A. 1997. Cassava mosaic virus disease in Uganda: The current pandemic and approaches to control. Natural Resources Institute (NRI), Chatham, GB. 65 p.

Pita, J. S.; Fondong, V. N.; Sangare, A.; Otim-Nape, G. W.; Ogwal, S.; Fauquet, C. 2001. Recombination, pseudorecombination and synergism of geminiviruses are determinant keys to the epidemic of severe cassava mosaic disease in Uganda. J. Gen. Virol. 82:655-665.

Stanley, J.; Gay, M. G. 1983. Nucleotide sequence of cassava latent virus DNA. Nature 301:260-262.

Thresh, J. M.; Otim-Nape, G. W.; Legg, J. P.; Fargette, D. 1997. African cassava mosaic disease: The magnitude of the problem? Afr. J. Root Tuber Crops 2:13-19.

Warburg, O. 1894. Die Kulturpflanzen Usambaras. Mitt. Dtsch. Schutz. 7:131.

Zhou, X.; Liu, Y.; Calvert, L.; Muñoz, C.; Otim-Nape, G. W.; Robinson, D. J.; Harrison, B. D. 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. J. Gen. Virol. 78:2101-2111.