CHAPTER 7

Technical Advance

Use of Plant DNA stored on FTA® cards for recovery and molecular characterization of bipartite and monopartite geminiviruses

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PCR-based geminivirus detection and molecular characterization using FTA® technology was evaluated. Infected plant leaf samples were squashed onto a FTA card that lysed cell membranes, denatured proteins and bound the DNA. Using universal and specific primers against geminivirus DNA-A and DNA-B, PCR products of cassava mosaic geminiviruses (CMGs) and *maize streak virus* (MSV) were obtained. The intensity of PCR bands obtained from FTA-processed DNA was comparable to the conventional phenol-based DNA extraction method. FTA-based technology was sensitive and effective in allowing for detection of *Africa cassava mosaic virus* (ACMV), *East African cassava mosaic virus*

(EACMV) both DNA-A and DNA-B components, in dual or single infection and MSV from samples collected in the field and greenhouse. Cloning and sequencing of PCR products were achieved. PCR products were obtained for DNA templates eluted from FTA cards at a concentration as low as 0.6 x 10^{-4} ug/ul. The results presented here demonstrate FTA technology as an economical, practical, and sensitive tool that allows for collection, shipment, archiving and purification of plant DNA for geminivirus molecular characterization in the field or where laboratory facilities are limited.

7.1 INTRODUCTION

Geminiviruses are small plant viruses with circular single-stranded DNA (ssDNA) genomes encapsidated in twinned (geminate) particles (Harrison, 1985). The *Geminiviridae* family is divided into four genera (*Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus*) according to their genome organizations and biological properties (Fauquet *et al*., 2000, 2003). *Mastreviruses* typified by *maize streak virus* (MSV) are transmitted by leafhoppers in the genus *Cicadulina* and together with the begomoviruses, which are transmitted by the whiteflies (*Bemisia tabaci*) constitute a major constraint to agricultural productivity in all tropical and sub-tropical regions of the world. Maize streak disease caused by MSV is arguably the most significant viral disease of maize in sub-Saharan Africa and the Indian Ocean territories (Rose, 1978; Thottappilly, 1992). *Mastreviruses* infect monocotyledonous hosts and have monopartite single-stranded DNA circular genomes (Lazarowtz, 1992). The begomoviruses constitute the largest genus of the family *Geminiviridae* and the vast majorities of its members infect dicotyledonous plants and have bipartite genomes (with DNAs A and B) (Rybicki *et al*., 2000).

Cassava mosaic disease (CMD) caused by cassava mosaic geminiviruses (CMGs) infects cassava (*Manihot esculenta*), an important food crop in Africa, throughout the African continent and is considered to be the most devastating disease of this crop. Begomoviruses are very diverse and so far six species have been found to infect cassava in Africa namely *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV) *East*

African cassava mosaic Zanzibar virus (EACMZV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV) and *South African cassava mosaic virus* (SACMV) (Fauquet and Stanley, 2003). Furthermore, CMG-associated epidemics are currently threatening cassava production in eastern and central Africa (Legg, 1999; Legg *et al*., 2001).

Usually, geminiviruses are characterized by cloning their genomes by either directly restricting the replicative form, which is isolated electrophoretically from the total nucleic acid (Hamilton *et al*., 1983), or by amplifying the full or partial genomes with geminivirus-specific primers (Wyatt and Brown, 996). It is essential to obtain high quality DNA for these two approaches, but this is difficult for many of the natural hosts such as maize, sugarcane, and cassava due partly to high polyphenol as well as polysaccharide content. Currently, genomic DNA from CMD-infected cassava plants is extracted by a method described by Dellaporta *et al*. (1983) involving a lengthy protocol of DNA extraction, centrifugation, precipitation and purification. In many countries in Africa where cassava and graminaceous crops such as maize are grown, leaf samples have to be collected from fields in remote areas and transported to centralized laboratories for analysis. In this paper we describe a simple approach of isolating viral DNA, PCR, cloning and sequencing of geminiviruses using plant DNA samples squashed onto and stored on FTA cards. FTA cards are commercially available papers that have been impregnated with a patented chemical formula that lyses cell membranes and denatures proteins upon contact. Nucleic acids are physically entrapped, immobilized and stabilized for storage at room temperature (Whatman, 2002). These cards have been used for some time in forensic human biology (Hsiao *et al*., 1999; Vanek *et al*., 2001) but its application for plant virus diagnosis has not been reported. Here the applicability and feasibility of FTA technology for characterization of geminiviruses is described and compared to the conventional DNA extraction method of phenol-chloroform purification described by Dellaporta *et al*. (1983).

7.2 MATERIALS AND METHODS

7.2.1 Sampling of plants. Young symptomatic leaves from CMD or maize streak disease (MSD)-infected plants were squashed on FTA cards (Whatman, USA) within the sample

circle area and allowed to dry under room temperature and stored until use (Fig. 7.1). Briefly, leaf material was placed onto the labeled FTA card and the leaf covered with parafilm. Then a moderate pounding pressure was applied using a blunt object such as a base of test tube or pestle until the extract was drawn through to the back of the FTA card. FTA cards were allowed to dry for at least an hour at room temperature or 1-2 hrs for cassava leaf samples collected from plants growing in the greenhouse. FTA cards containing field samples were archived and transported to the laboratory for analysis. CMD-infected samples were collected from western Kenya. MSD-infected samples were collected from Malawi and Kenya. Samples from the greenhouse or growth chambers were from CMD-infected cuttings collected from Tanzania and Cameroon.

7.2.2 Sample processing from FTA card for PCR analysis. Owing to the mosaic nature of plant virus distribution in the plant leaf tissue, three FTA card discs (2.0 mm) were punched from the applied samples (in a triangular pattern) and transferred to individual 1.5 ml micro-centrifuge tubes. Discs were washed twice with 200 µl of 1X TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and then once with 300 µl FTA® Purification Reagent. The wash solutions were removed and discarded after each wash. The discs were transferred to new 1.5 ml micro-centrifuge tubes to which 8-12 µl of elution buffer (10 mM Tris-HCl, pH 8.5) was added and incubated for 15 minutes. The discs were then removed and discarded and the DNA elute stored and used as template for PCR analysis. A replica of each sample collection was extracted using the method of Dellaporta *et al.* (1983) for comparison.

7.2.3 PCR analysis for cassava mosaic geminiviruses. PCR was carried out in a 25 µl total reaction volume. Sets of universal primers (designed to either amplify near full-length (~ 2.8 kbp) (UNIF and UNIR) or 1020 bp (Universal 1 and Universal 2) geminivirus DNA-A component (Table 7.1) were used. For amplification of MSV, primers MS-F and MS-R (Table 7.1) designed to amplify a 500 bp fragment of the conserved region of the DNA-A genome of MSV. To test the ability of FTA card to produce amplifiable genomic DNA, a transgene (NPTII) was amplified from genetically modified cassava plant (Fig. 7.4b). PCR amplification consisted of 30 cycles of 94^{o}C (denaturation) for 1 minute, 59^{o}C (annealing)

for 1minute 72^{o}C (extension) for 2 minutes. For amplification of coat protein gene sequence, however, the annealing temperature was 45^{o}C. PCR products were analyzed on 1% agarose gel after staining with ethidium.

7.2.4 Detection of dual infection in infected cassava. Dual infections, commonly occurring in a cassava field involving two CMG species, often result in severe infection owing to their synergistic effect. We wanted to find out if DNA stored on FTA card could be used to identify plants singly or dually-infected by CMG species. First, near full-length DNA-A genomes were PCR-amplified using DNA eluted from FTA card as a template. Secondly, the PCR products were recovered from agarose gels, purified and subjected to restriction analysis using the *Eco*RV restriction enzyme. Based on the sequences of all the EACMV and ACMV isolated from the GenBank data base, *Eco*RV has been found consistently to cut ACMV and EACMV giving unique and distinct fragment lengths making it a useful tool for field detection of ACMV/EACMV dual infection (Legg and Fauquet, 2004; Sseruwagi *et al*., 2004).

To find out if this technique could be used to capture different geminivirus species, two primer pairs EAB 555F/ EAB555R (located in the BC1 and the intergenic region) and JSP 001/002 (located at the beginning and the end of the coat protein gene), designed to amplify ~ 555 bp of EACMV DNA-B and ~ 770 bp of ACMV CP, respectively, were used. These primers could also detect dual virus infection.

7.2.5 Cloning, sequencing and sequence analysis. To determine the quality of the viral DNA stored on FTA card in the downstream viral DNA-Analysis and virus characterization, a fragment (556 bp) from DNA-B of EACMCV, a Cameroon isolate of EACMCV was PCR-amplified by primers EAB 555/F and EAB555/R using template DNA eluted from FTA card and from phenol extracts by the method of Dellaporta *et al*. (1983). PCR products were purified and cloned into the pGEM-T Easy vector (Promega) and were completely sequenced in both orientations. Two clones from each (FTA processed and phenol extracted) DNA nucleotide sequences were compared by multiple alignments using the Mega Align option of the DNASTAR computer package. A

corresponding fragment sequence of EACMCV DNA-B from GenBank (AF112355) was used as a reference.

7.2.6 Quantification of DNA and viral DNA eluted from FTA card. The amount of DNA that can be eluted from an FTA card was analyzed using a pre- quantified plasmid DNA carrying a 556 bp geminivirus DNA-B fragment (BC1/IR) from EACMCV. The recombinant plasmid DNA, in quantities (µg/µl) as follows; 0.8, 0.4, 0.2, 0.16, 0.08, 0.05, 0.04, and 0.001, was mixed with 8 µl of sap extracted (by distilled water) from young healthy cassava leaf obtained from the greenhouse and each mix was loaded onto FTA card and left to dry for 1 hr. Four mm punches were then taken from each FTA card and processed normally as described above to elute the DNA. The amount of DNA eluted from the FTA card was determined using a UV spectrophotometer at 260 nm. Then each DNA eluate was used for PCR in a 25 µl total reaction volume to amplify the 556 bp viral DNA fragment. For PCR based analysis of the viral DNA amplification signal, 0.6 µg/µl of the DNA elute was serially diluted down to 10^{-6} -fold and each DNA dilution was used as a template for detection of the 556 bp fragment.

7.3 RESULTS

7.3.1 Polymerase chain reaction for detection of viral DNA

PCR-amplification of near-full length CMG DNA-A genomic component (2.8 Kbp DNA-A) and a 1 Kbp fragment is shown (Fig. 7.2). The intensity of PCR band products obtained from viral DNA stored on an FTA card was comparable with those obtained following phenolic preparation (Dellaporta method). FTA samples collected from the greenhouse and cassava fields gave the expected amplification, demonstrating the sensitivity of the technology in that the 2 µl DNA eluted from an FTA card and used as the PCR template, contained sufficient viral concentration to produce PCR amplification from the leaf sap background kept under ambient conditions.

To test the applicability of FTA technology in the molecular characterization of geminiviruses other than CMGs, MSV-infected plant sap was obtained from plants in Kenya and Malawi. Using primer pairs (MS-F and MS–R) (Table 7.1), designed to amplify *c.* 500 bp DNA fragment from the conserved region, 100% of the samples tested gave a PCR product with strong bands (Fig. 7.4a). These results demonstrated FTA technology to be a sensitive tool for detection of MSV.

7.3.2 Detection of CMG species

For FTA technology to be efficiently and effectively used as a routine tool for PCR-based geminivirus detection, it must allow for CMG species differentiation. To test this, two major cassava–infecting geminiviruses species (ACMV and EACMV) were used. Small viral fragments (556 bp) spanning from the BC1 to the intergenic region (IR) of EACMV DNA-B were successfully amplified using DNA from both extractions (FTA card and phenol purified) (Fig. 7.3). A reproducible PCR product was obtained from all of the samples collected from the greenhouse and between 80–100% of field collected samples. A similar observation was evident when a PCR product (770 bp coat protein gene of ACMV) was used to detect the presence of ACMV in infected cassava leaf samples (Fig. 7.3). In all cases, the PCR product characteristics obtained for the FTA technology were comparable with those from the traditional DNA processing (Dellaporta method). FTA technology not only allowed for the detection of different CMG species present in different samples, but also the detection of mixed infections of EACMV and ACMV. Using a near-full length (2.8 Kbp) DNA-A PCR product obtained using FTA technology, subsequent digestion with *Eco*RV, allowed for the identification of cassava plants infected with either EACMV or ACMV or both (Fig. 7.4a).

7.3.3 Clones and sequence comparisons of viral DNA from phenol and FTA processed DNA

To test the use of FTA technology in the downstream molecular characterization of CMGs, 560 bp PCR fragments of the DNA-B (BC1/1R) of EACMCV obtained using FTA card and Dellaporta methods were cloned and compared. Digestion of the recombinant plasmids PGEM-T Easy carrying the 560 bp fragments with *Eco*RI yielded a restriction pattern shown in Figure 7.5b. Clones from FTA processed DNA were comparable with those from phenol extracted DNA. To test the integrity of the nucleotide sequences of the two sets of clones, duplicate clones from FTA and phenol processed DNA were sequenced and compared. Nucleotide sequence comparison showed 99.8% identity between clones derived from FTA technology and the traditional method of viral DNA processing (Fig. 7.6). This result suggests that viral DNA stored on FTA card maintains its nucleotide sequences throughout processing and amplification by PCR. Therefore FTA technology provides a practical method for analyzing and understanding the molecular and genetical nature of geminiviruses. No significant nucleotide sequence variation was observed when a corresponding EACVCM DNA-B sequence fragment GenBank (acc. no. AF112355) was compared to the clones sequenced in this study.

7.3.4 Quantification of DNA and viral DNA eluted from FTA card

When 0.8 µg/µl of recombinant plasmid DNA was loaded onto an FTA card, up to 78% (0.63 µg/µl) could be eluted. The highest DNA recovery was 100% for 0.2 µg/µl of DNA loaded on an FTA card (Fig. 7.7a). Following PCR analysis of the DNA elute as template, PCR product could be obtained for all but one of the DNA elute samples (Fig. 7.7b). For PCR based analysis of the viral DNA-Amplification signal, 0.6 µg/µl of the DNA elute

was serially diluted down to 10^{-6} -fold and each DNA dilution was used as a template for detection of the 556 bp fragment. Expected PCR products were obtained for DNA concentrations of 0.6 µg/µl with band intensities that decreased progressively to 0.6 x 10^{-4} µg/µl of DNA template (Fig. 7.7c). The PCR amplification signal was lost at DNA template concentrations of 0.6 X 10^{-5} and 0.6x 10^{-6} µg/µl. Real time-PCR analysis produced expected products of 0.66 ± 0.28 ng (± standard error) for DNA template with concentrations as low as 0.04 µg/µl (data not shown). These results suggest high sensitivity of FTA technology in routine virus diagnostics, a tool that is comparable to the conventional/traditional method.

7.4 DISCUSSION

The technology of storage and processing of DNA genomes on fluid storage paper like FTA cards for direct processing by PCR analysis has become accepted (Smith and Burgoyne, 2004), but much less so for plant DNA genomes and PCR. The assay for detection and molecular characterization of geminivirus DNA genomes described here is simple to perform, sensitive and specific. The simplicity of the assay is derived from the use of total plant DNA collected on FTA card that protects the nucleic acids within a sample as soon as it is applied to the coated filtration matrix and lyses cell membranes on contact so that DNA is immediately immobilized and stabilized within the matrix. PCR of all the geminiviruses tested in this study gave amplification products seen in all figures as visible bands. The pre-amplification processing of FTA cards was only a series of washes as compared with the conventional technique for plant DNA processing that usually requires an extensive extraction and purification procedure involving phenol/chloroform/isoamyl alcohol treatment (Dellaporta *et al*., 1983). Thus FTA technology offers advantages over conventional plant DNA processing methods such as the Dellaporta method. They include: the lack of a need for refrigeration, ease of storage, transport and processing, and decreased biohazard risks. It is cost effective in terms of labor, consumables and laboratory instrumentation. Similar advantages of FTA technology have been realized for human DNA processing (Zhong *et al*., 2001) and for wildlife DNA samples (Smith and Burgoyne, 2004). The fact that PCR products of the viral DNA stored on FTA cards was successfully used for down-stream geminivirus analysis such as cloning

and sequencing, detection of CMG species, and mixed infection suggests that FTA technology is a sufficiently sensitive and viable option for routine molecular characterization of germiniviruses.

Transport of plant DNA from collection sites to centralized laboratories is difficult in developing countries because of lack of infrastructure and facilities. FTA technology provides a credible tool with particular application to the problem of transporting infected plant samples to centralized laboratories for analysis. In this study, infected cassava and maize materials collected from Africa (Malawi and Kenya) could be easily transported on the FTA medium and used for virus detection and characterization in St. Louis, USA. PCR analysis showed a successful amplification of full–length viral DNA genome (DNA-A and DNA-B components), as well as specific DNA fragments targeted by the primers indicating that FTA technology can be a useful tool in plant virus diagnosis even at virus species level. Furthermore, the stability of DNA when left on FTA card, and the small area of the paper, that is processed at each time, means that the same samples can be analyzed many times.

The length of time that plant DNA genomes can be stored on FTA card was not tested formally but some material used in other studies containing DNA or RNA genomes were held under ambient conditions for 6 months (RNA) and 11 years (DNA) without noticeable effect on the amplification signal, suggesting that storage time was quite practical for transport from collecting sites to central laboratories (Rogers and Burgoyne, 2000). In this study, however, samples collected from the greenhouse and the fields kept under ambient conditions for 3 months produced PCR amplification with relatively weaker signals observed for field samples for cassava but not for maize. It was unclear whether this slight variation could be attributed to environmental factors, poor sampling techniques in the field as well as the mosaic nature of geminivirus distribution in the infected leaves. Probably, it could be due to difficulties in washing out all the green material that can potentially inhibit PCR amplification of the viral DNA.

In conclusion, this assay, using geminivirus DNA PCR of total plant DNA collected on an FTA card, offers a simple, sensitive and specific tool appropriate for the diagnosis and molecular characterization of geminiviruses. Plasmid and viral DNA can be eluted from the card. DNA integrity is maintained at room temperature and the cards are safe to handle pre and post sample application. Owing to the benefits of FTA technology resulting from the room temperature storage, it is suitable for streamlining DNA purification and the analysis of multiple samples.

7.5 ACKNOWLEDGEMENTS

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Table 7.1. Oligonucleotides used for PCR amplification of CMGs and MSV where K = G+T, R = A + G, S = G + C (Invitrogen, Life Science, USA) (anfl = near-full length)

Primer name	**Sequence** (5′-3′)	**Target virus**
EAB555/F	(5′-TACATCGGCCTTTGAGTCGCATGG-3′)	EACMV DNA-B
EAB555/R	(5′-CTTATTAACGCCTATATAAACACC-3′)	EACMV DNA-B
JSP 001	(5′-ATGTCGAAGCGACCAGGAGAT-3′)	ACMV (AV1/CP)
JSP 002	(5′-TGTTTATTAATTGCCAATACT-3′)	ACMV (AV1/CP)
UNIF	(5' KSGGGTCGACGTCATCAATGACGTTRTAC 3')	CMGs DNA-A nfl[a]
UNIR	(5' AARGAATTCATKGGGGCCCARARRGACTGGC 3')	CMGs DNA-A nfl
Universali 1	(5′-TAATATTACCKGWKGVCCSC-3′)	CMGs DNA-A
Universal 2	(5′-TAATATTACCKGWKGVCCSC-3′)	CMGs DNA-A
MS-F	(5′-ATCCCTCCAAATTCCGACAC-3′)	MSV
MS-R	(5′-TCCATGTACAAAGCTCCTCT-3′)	MSV
N-F	(5′-CCCCTCGGTATCCAATTAGAG-3′)	NPTII
N-R	(5′-CGGGGGGTGGGCGAAGAACTCCAG-3′)	NPTII

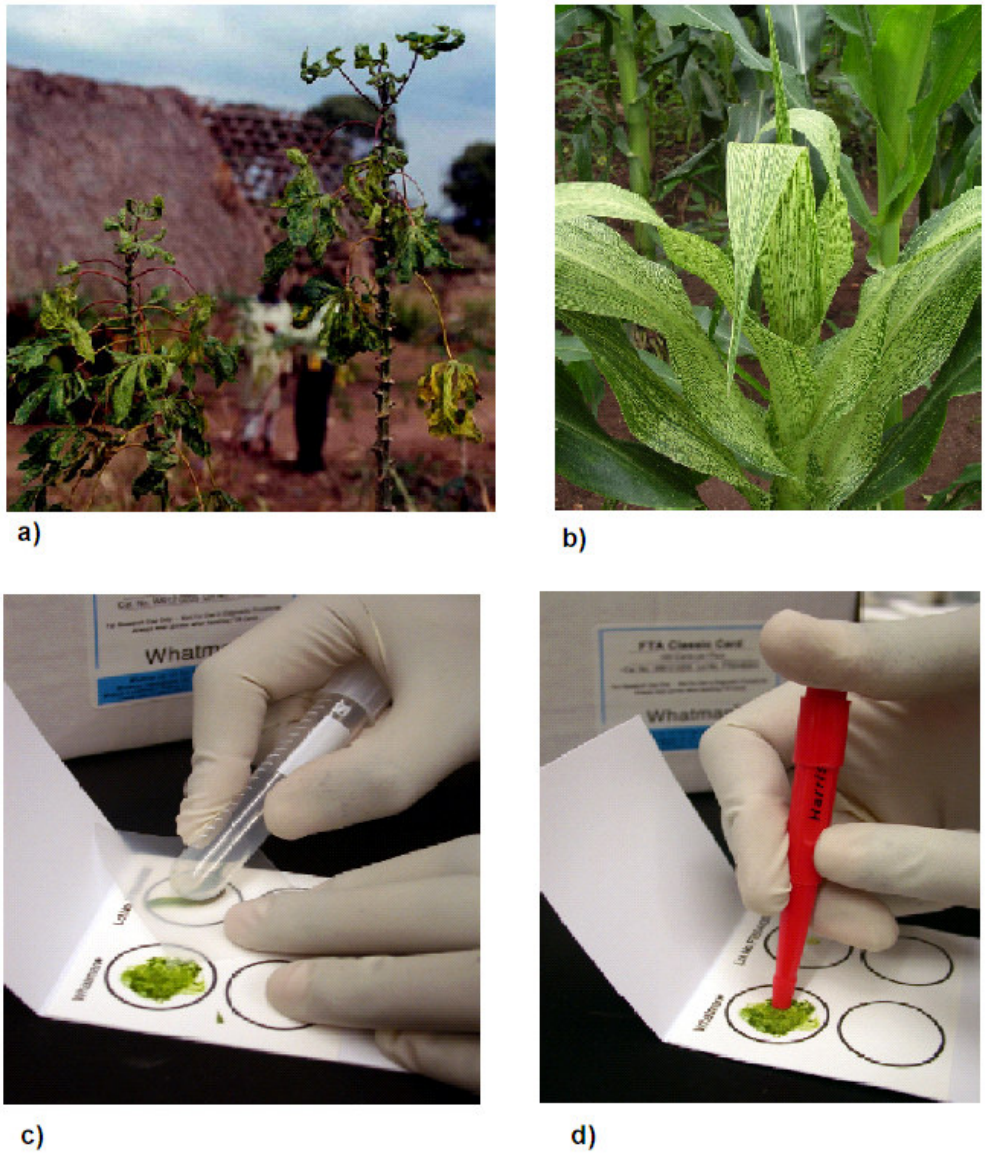

Fig. 7.1. Application of samples collected from infected (a) cassava and (b) maize on FTA card (c). Samples were dried and stored on FTA card at room temperature after which three discs (2 mm) were punched from each sample area of the FTA card (d) pooled and the total plant DNA eluted for use in PCR analysis of cassava mosaic geminiviruses and *maize streak virus* (MSV).

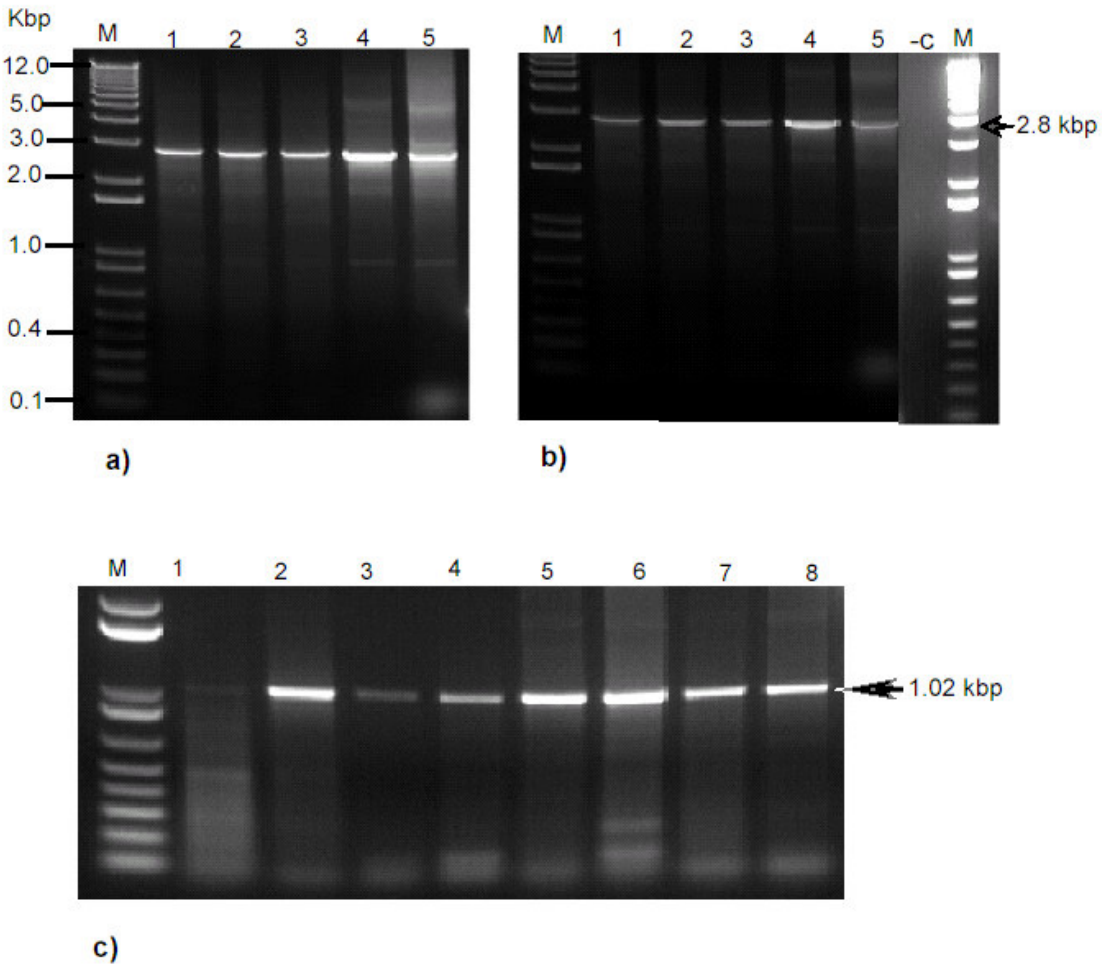

Fig. 7.2. PCR amplification of near-full length DNA-A of CMG using universal primers (UNIF and UNIR) either on DNA processed by the Dellaporta method (a) or eluted from FTA card (b). In addition, primers Universal 1 and Universal 2 (Table 7.1) were used for PCR amplification of ~ 1.0 kbp DNA-A fragment from DNA eluted from FTA card (c). Samples were CMD-infected cassava plants held in the greenhouse.

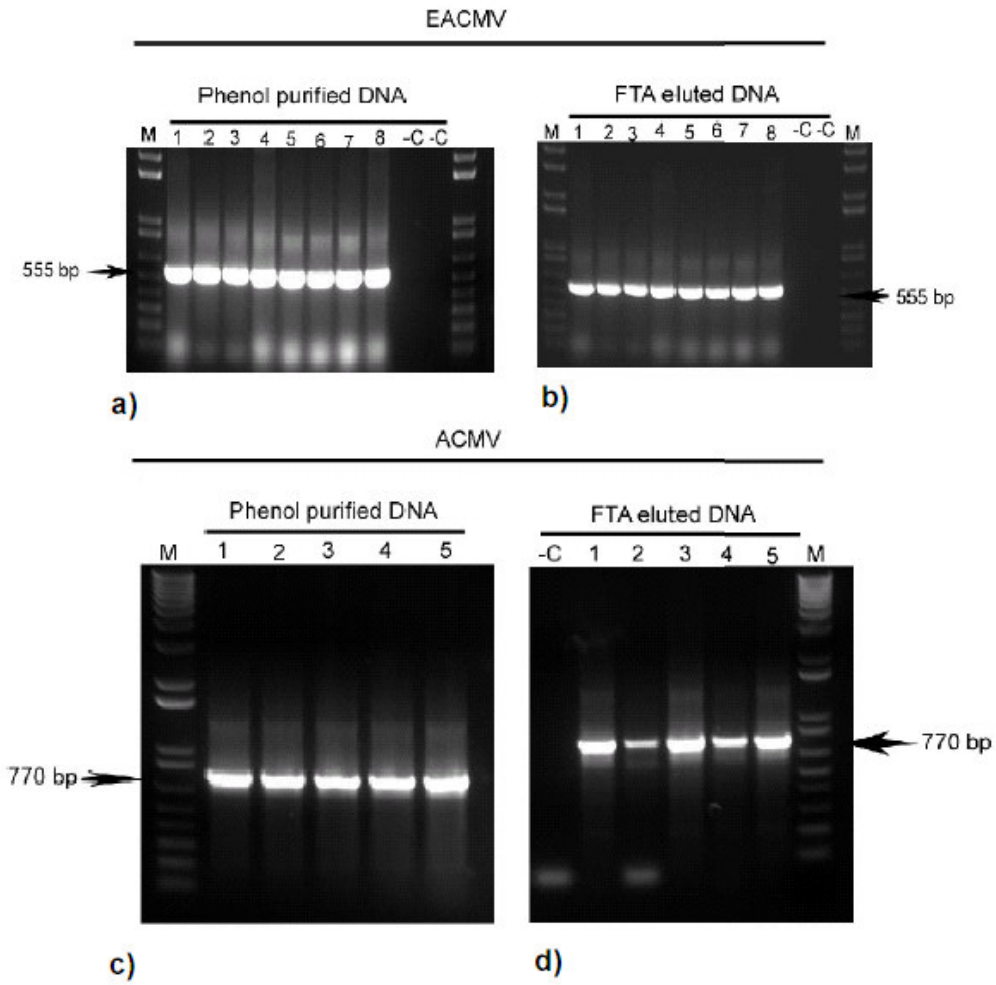

Fig. 7.3. PCR amplification of EACMV and ACMV species on total nucleic acid from cassava leaves processed either by the Dellaporta (phenol purified) method or FTA elution. Amplifications are: 555 bp (IR/BC1) fragment of EACMV DNA-B component (a and b) and 770 bp coat protein gene of ACMV (c and d). Negative controls (-C) contained nucleic acid from healthy cassava leaves. M = 1 Kbp plus DNA ladder (for sizes see Figure 7.2).

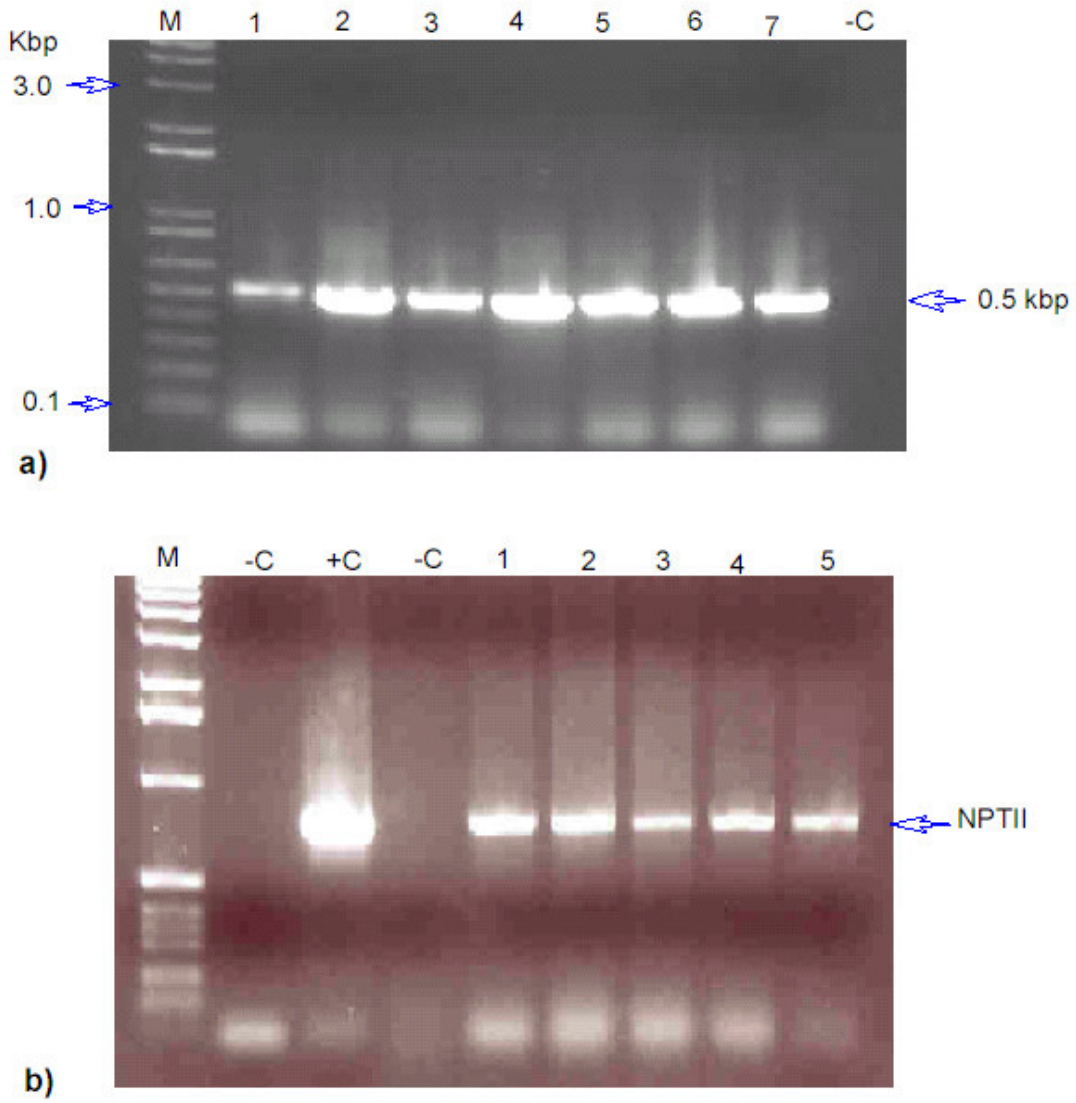

Fig. 7.4. PCR amplification of (a) 0.5 kbp Maize streak virus DNA fragment from infected maize plant leaf samples collected from Kenya and Malawi on FTA cards, (b) NPTII transgene in genetically modified cassava plants held in the greenhouse. Samples (lanes 1-5) were collected on FTA cards and the genomic DNA processed for PCR amplification. Negative control samples (–C) consisted of DNA extracted from healthy cassava plants.

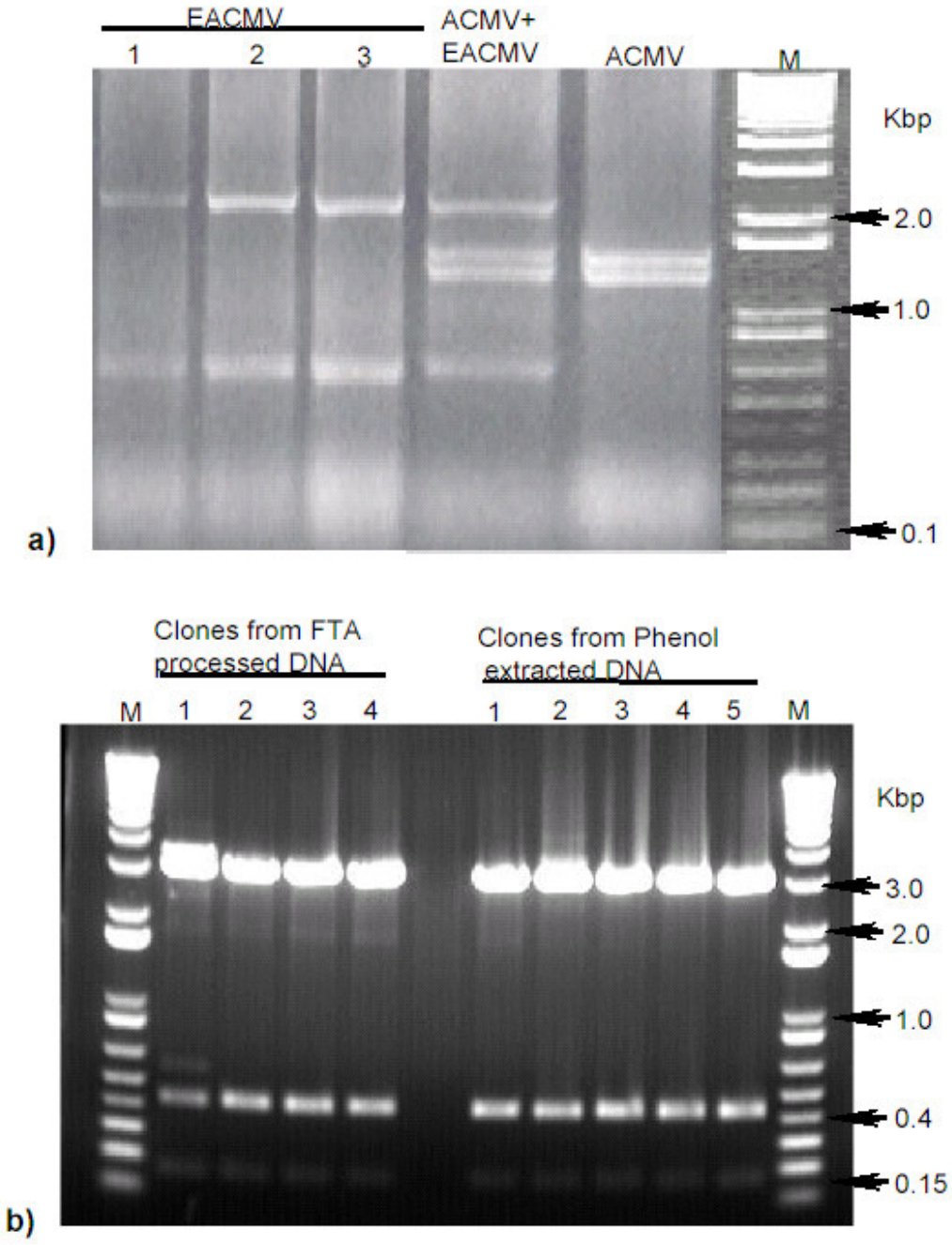

Fig. 7.5. Restriction enzyme digestion of (a) near-full length DNA-A of CMG for detection of ACMV/EACMV dual infection. Samples were collected from CMD-infected plants in the fields, archived on FTA cards, DNA eluted and used for PCR analysis. DNA was recovered from the agarose gel, purified and digested with *Eco*RV for 1.5 hrs at 37^{o}C (b). Recombinant plasmids carrying 556 bp DNA-B viral fragment (IR/BC1) of EACMV.

PCR was done on FTA or phenol processed template DNA, cloned in pGEM-T Easy vector (Promega) and recombinant plasmids recovered by miniprep analysis by digestion with *Eco*RI to release the viral inserts.

Decoration 'Decoration #1': Shade (with solid black) residues that match the Consensus exactly.

Fig. 7.6. Nucleotide sequence comparison of EACMCV-CM clones of viral DNA fragment obtained from FTA or phenol-purified PCR DNA templates. A 556 bp PCR product was cloned and sequenced and sequences of duplicate clones were compared by Multiple Sequence Alignment using MegAlign option of DNASTAR package.

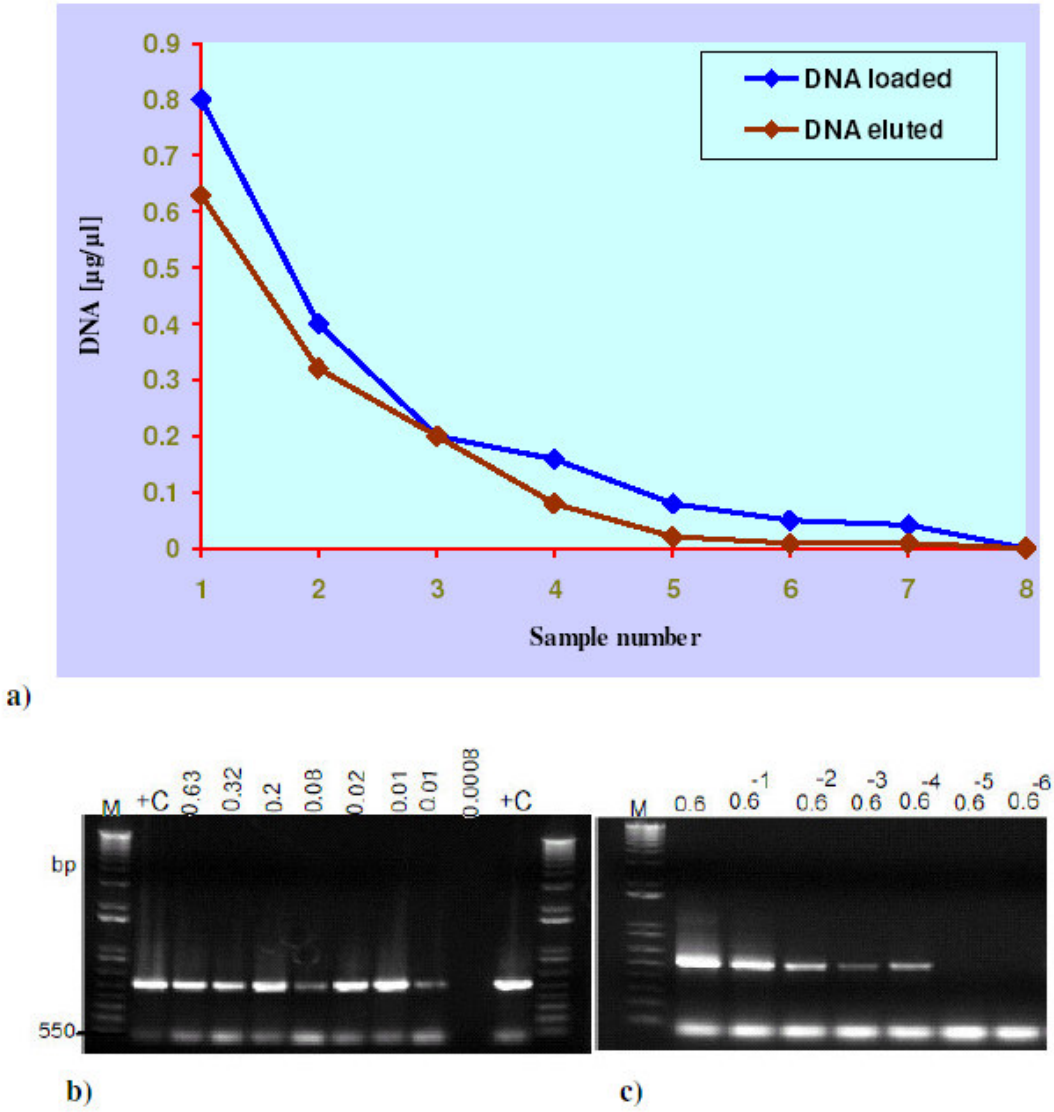

Fig. 7.7. Quantification of recombinant plasmids eluted from FTA cards loaded with known amount of recombinant plasmid DNA + 8 µl of sap from healthy cassava leaf extracted in distilled water (a). The eluted DNA was used for PCR amplification of 555 bp viral DNA fragments insert using primers EAB555F/EAB555R (b). Positive control lanes (+C) contained the 555 bp viral DNA-B fragment PCR-amplified from cassava plants infected with EACMV from the growth chamber. To detect the limit of PCR amplification signal, 0.6 µg/µl was serially diluted to 0.6 X 10^{-6} µg/µl and each dilution used for PCR amplification of the 555 bp viral DNA fragment described above.