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Successful application of FTA® Classic Card technology and use of bacteriophage ϕ29 DNA polymerase for large-scale field sampling and cloning of complete maize streak virus genomes

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Abstract

Leaf samples from 155 maize streak virus (MSV)-infected maize plants were collected from 155 farmers' fields in 23 districts in Uganda in May/June 2005 by leaf-pressing infected samples onto FTA® Classic Cards. Viral DNA was successfully extracted from cards stored at room temperature for 9 months. The diversity of 127 MSV isolates was analysed by PCR-generated RFLPs. Six representative isolates having different RFLP patterns and causing either severe, moderate or mild disease symptoms, were chosen for amplification from FTA cards by bacteriophage ϕ29 DNA polymerase using the TempliPhi™ system. Full-length genomes were inserted into a cloning vector using a unique restriction enzyme site, and sequenced. The 1.3-kb PCR product amplified directly from FTA-eluted DNA and used for RFLP analysis was also cloned and sequenced. Comparison of cloned whole genome sequences with those of the original PCR products indicated that the correct virus genome had been cloned and that no errors were introduced by the ϕ29 polymerase. This is the first successful large-scale application of FTA card technology to the field, and illustrates the ease with which large numbers of infected samples can be collected and stored for downstream molecular applications such as diversity analysis and cloning of potentially new virus genomes.

Keywords: Maize streak virus (MSV); Geminivirus; FTA cards; Rolling circle amplification; Phi29 DNA polymerase; MSV diversity

1. Introduction

Geminivirus-caused diseases are a major constraint to the production of economically important crops such as tomato, maize, cotton, bean and cassava (Moffat, 1999; Bosque-Pérez, 2000; Mansoor et al., 2003; Legg and Fauquet, 2004; Vanderschuren et al., in press). In sub-Saharan Africa, substantial economic and subsistence losses occur in the important staple food crop, maize, due to maize streak disease (MSD) caused by the geminivirus maize streak virus (MSV). Increased knowledge of the virus, such as epidemiology, sequence diversity and evolution, and identification of non-maize over-wintering hosts, is vital in order to implement preventative strategies. For this reason, there is a need for rapid and easy collection and storage of MSV-infected plant material for downstream virus purification and molecular-based studies. Using Whatman FTA® Classic Cards (www.whatman.com), viruses can be sampled by simply pressing infected leaves onto this paper-based technology. Use of FTA cards has the potential to reduce cost, sampling time and sample storage space. By enabling the preservation of samples at room temperature these cards also allow sampling of viruses from remote areas.

Initial investigations into the use of FTA cards for sampling geminivirus-diseased plants were carried out by Ndunguru et al. (2005) using greenhouse-sampled plants and a small

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number of cassava and maize plants from the field (6 and 7, respectively). In the present study, the large-scale application of the method to the field is reported, where 155 maize samples were collected from three regions in Uganda, and stored on FTA cards for downstream applications in the laboratory. Rather than using a PCR-based method, as in Ndunguru et al. (2005), to isolate geminivirus DNA from plant samples stored on FTA cards, a method employing rolling circle amplification by the bacteriophage ϕ29 DNA polymerase (TempliPhi™, Amersham Biosciences) was used. Geminivirus genomes are circular and can therefore be used as templates for the ϕ29 DNA polymerase amplification method (Inoue-Nagata et al., 2004). This has the advantage that viral DNA can be cloned from samples containing very low concentrations of viral genomes without the need for specific primers (as are required for PCR), thus enabling the amplification and cloning of previously unknown genomes (Haible et al., 2006).

This paper describes a simple and rapid method of collection and storage of large numbers of MSV-infected maize samples in the field. It demonstrates that viral DNA sampled using FTA cards is suitable as a template for both PCR and TempliPhi™ amplification, which enables the cloning of full-length virus genomes from these cards. The protocol presented should drastically reduce the effort required to collect, store, analyse and clone geminivirus genomes sampled from natural environments.

2. Methods and materials

2.1. Collection of samples

One hundred and fifty-five maize plants displaying symptoms characteristic of maize streak disease were sampled from 155 different fields in 23 districts in the eastern, western and north-central regions of Uganda in May–June 2005. One young symptomatic leaf removed from a potentially MSV-infected plant was placed onto a single FTA® Classic Card under parafilm (American National Can™, Chicago, II). Each leaf was then gently pressed onto the card through the parafilm until plant sap penetrated the matrix of the paper. This process, referred to hereafter as "leaf-pressing", is illustrated in Ndunguru et al. (2005). After leaf-pressing, the cards were left to air dry for approximately 5 min before being placed into plastic bags for storage at room temperature. Additionally, 305 leaf samples were picked, stored in a cooler box and taken back to the laboratory in batches of 100, for DNA extraction using a conventional method (CTAB) and other downstream applications. The severity of disease symptoms in each sampled plant was scored using a five point scale as illustrated in Shepherd et al. (2007).

2.2. Isolation of genomic DNA and RFLP analysis on PCR-amplified fragments

DNA was recovered from diseased plant samples pressed on FTA cards, which had been stored at room temperature for 9 months, as described by Ndunguru et al. (2005) with minor modifications. Three discs were punched from each card and washed initially with 300 μl of Tris-EDTA (TE) buffer (pH 8) in an eppendorf tube, by sucking the buffer up and down vigorously for 5 min using a pipette. This was followed by washes with (1) 300 μl of 70% ethanol, (2) TE buffer and (3) 300 μl of FTA® Purification Reagent, in that order. Discs were then transferred to fresh eppendorf tubes and air-dried for 20 min. Viral DNA was eluted by soaking the discs in 12 μl of Tris–HCl buffer (10 mM Tris–HCl pH 8) overnight. During this step most plant genomic DNA should remain on the disc, whereas smaller nucleic acid molecules such as that of MSV genomes should be preferentially eluted (Ndunguru et al., 2005). In addition, as a comparison to FTA card extraction, DNA was extracted from leaf samples stored at −80 °C using CTAB as described by Kiprop et al. (2002).

Two previously described degenerate primers (Willment et al., 2001): 215–234 and 1770–1792 (which bind at nucleotide co-ordinates 361–343 and 1746–1764, respectively; numbering starts from the last A of the conserved TAATATTAC sequence of the MSV-Kom isolate, in the 5′ → 3′ direction of the primer) were used to amplify a 1.3-kb product from each sample. PCRs were carried out in 50 μl reactions using 2 μl of DNA preparation as template. Amplifications were performed as described in Willment et al. (2001). For RFLP analysis, 5 μl of PCR products were digested with each of the seven restriction enzymes recommended by Willment et al. (2001) for distinguishing different virus isolates: these are *Rsa*I, *Hpa*II, *Hae*III, *Cfo*I, *Hind*III, *Bam*HI and *Sau*3A. A code letter was assigned to each different restriction pattern observed with each of the seven enzymes, such that each sample was represented by a string of seven letters representing a restriction profile (see Supplementary Table 1 and Supplementary Figs. 1 and 2).

2.3. Amplification of virus DNA using the TempliPhi™ amplification kit

After RFLP analysis, six representative samples originating from plants that had mild [1.5 on the symptom severity scale in Shepherd et al. (2007)], moderate (severity of 2.5) and severe (severity of 4) MSD symptoms were selected for further molecular analysis to determine the efficacy of the FTA card storage system. The selected isolates were MSV-UMba27 (from Mbarara, western Uganda), MSV-UKas42 and MSV-UKas43 (both from Kasese, western Uganda), MSV-UKab48 (from Kabarole, western Uganda), MSV-UMub49 (from Mubende, western Uganda) and MSV-UWak56 (from Wakiso, central Uganda). DNA eluted from FTA cards was used as a template for rolling circle amplification by bacteriophage ϕ29 DNA polymerase using the Templiphi™ amplification kit (Amersham Biosciences) according to the kit instructions. In brief, 2.5 μl of eluant potentially containing circular MSV genomes was added to 2.5 μl of the kit's sample buffer, and the mixture was heated to 95 °C for 3 min. After cooling to room temperature, 5 μl of reaction buffer and 0.2 μl of enzyme mix (provided in the kit) were added, followed by incubation at 30 °C for 20 h.

2.4. Cloning of virus genomes and sequencing

The TempliPhi™ amplification product (presumably a concatemer of linear viral genomes) was first digested with a series

of restriction enzymes that are known to cut once in most MSV genomes, in order to select a unique restriction enzyme site for cloning the full-length genomes. In all cases, 1 μl of the TempliPhi™ product was sufficient for the digestion and cloning steps. *Bam*HI was chosen for all six samples as it was in all cases found to cut the amplification products into ~2.7-kb fragments, the approximate size of complete MSV genomes. The ~2.7-kb *Bam*HI-fragments were gel-purified using the Invisorb® spin DNA extraction kit (Invitek, Germany) and the purified product inserted into *Bam*HI-digested pGEMZf+ (Promega Biotech). The resulting insert DNA was sequenced using M13 forward and reverse primers and additional internal sequencing primers. (MSV F1: 5′-GAC CTC ATC AAY ACM TAT GCC C-3′, MSV F2: 5′-GTT GGG AGT GAG AAC GCA GTG G-3′, MSV F3: 5′-CAT TTC CTT CAT CCA RTC TTC ATC CG-3′, MSV F4: 5′-GGT GAG GAK KGY GGA TGA GGA TYT GRT G-3′). Sequencing was outsourced to Macrogen Inc., Korea, who use ABI3730XL and ABI3700 automated sequencers. Additionally, a 1.3-kb product was amplified from the 6 FTA-eluted samples representing mild, moderate and severe isolates, using proof-reading DNA polymerase (Accuzyme, Bioline, UK) and degenerate primers (Willment et al., 2001) as described above. The purified product was cloned into the pGENE-JET (Fermentas) vector and sequenced using pGENE-JET primers supplied with the kit. This enabled a comparison between the sequences of whole MSV genomes amplified from FTA cards using Templiphi™ and the 1.3-kb product amplified from FTA cards by PCR.

3. Results

3.1. PCR and RFLP analysis on MSV-infected field samples collected and stored on FTA cards

Of the 155 samples processed from FTA cards, 91% (141) gave the expected amplicon of 1.3 kb and 9% (14) were PCR-negative. Similarly, of the CTAB-extracted samples, 92% (281) gave a positive PCR product and 8% (24) were PCR-negative. The strength of PCR signal for both FTA and CTAB-extracted samples was comparable (Fig. 1). An analysis of the recovered samples in relation to symptoms observed on sampled plants showed that most (137/155) sampled plants had moderate to severe symptoms, and these had a higher recovery of virus in comparison with mildly diseased plants (72% of the sampled plants with mild symptoms gave a positive PCR product, compared with 93% of sampled plants with moderate/severe symptoms). The positive PCRs indicated that viral DNA was sampled, stored at room temperature and extracted from FTA cards 9 months after field sampling at a success rate comparable to more laborious methods such as CTAB, which requires freezing of leaf samples for storage, grinding of leaf material in liquid nitrogen, the use of centrifuges and several incubation steps. Whereas it is possible that viral DNA in the PCR-negative samples had not survived storage on, and extraction from, the FTA cards, it is also possible that these samples were from plants either mistakenly identified as having MSV symptoms, or plants infected with MSV variants that were not amplifiable with the PCR primers we used.

In the vast majority of cases restriction analysis of PCR products yielded fragment sizes reported in previous studies employing the same PCR-RFLP analysis (Willment et al., 2001; Martin et al., 2001). We did, however, observe one or more unique restriction fragment patterns that had never been reported previously for each of the enzymes *Rsa*I, *Hpa*II, *Hae*III and *Hind*III (Supplementary Fig. 1). Based on RFLPs observed with each of the 7 enzymes, samples were grouped into 28 variants, each represented by a different restriction profile (Supplementary Table 1 and Supplementary Fig. 2). Sixty percent of samples were grouped into one category while the remaining isolate groups made up the other 40%.

To estimate the degree of MSV diversity present within individual fields, we compared the RFLP profiles of samples from the 96 fields for which we achieved two successful CTAB and one successful FTA card derived PCR amplification products. Despite 60% of samples collected countrywide sharing the same RFLP profile, in only 20.8% of fields did all three samples have the same RFLP profile. Whereas two different RFLP profiles were detected in 44.8% of fields, three different RFLP profiles were detected in 34.4% of fields.

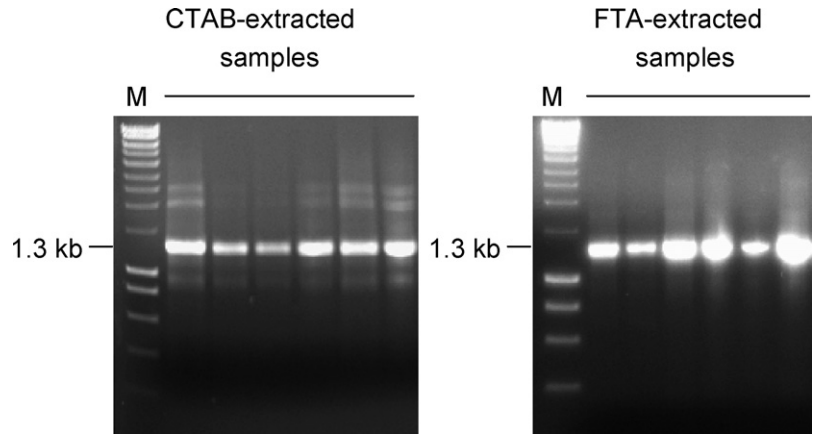

Fig. 1. PCR products from FTA® Classic Card-extracted DNA compared with CTAB-extracted DNA. A 1.3-kb product was amplified using a MSV replicative form-specific degenerate primer pair, 215–234 and 1770–1792 (Willment et al., 2001). M = HyperLadder I DNA molecular weight marker (Bioline, Germany).

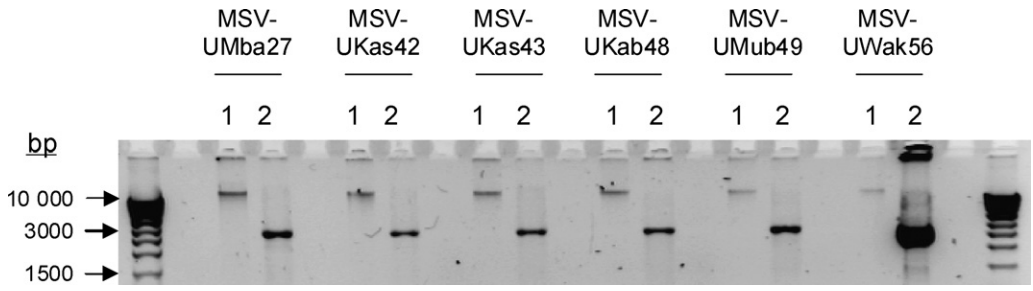

Fig. 2. TempliPhi™ amplification products of MSV-UMba27, MSV-UKas42, MSV-UKas43, MSV-UKab48, MSV-UMub49 and MSV-UWak56 followed by digestion with *Bam*HI. The ~2.7-kb circular MSV genome is amplified as a concatemer (1); digestion with *Bam*HI releases linear full-length genomes (2) that are then inserted into a *Bam*HI-digested cloning vector, pGEMZf+. Note that the apparent faster migration of the *Bam*HI-digested MSV-UWak56 sample was due to its being overloaded and not due to a size discrepancy in comparison with the other samples.

3.2. Cloning and sequencing of full-length virus genomes using TempliPhi™

The identity of six full-length virus genome clones (MSV-UMba27, MSV-UKas42, MSV-UKas43, MSV-UKab48, MSV-UMub49 and MSV-UWak56), amplified using TempliPhi™ (Fig. 2) and representing mild, moderate and severe isolates, was verified by sequencing the genomes in pGEMZf+. Full sequences can be accessed at the following genbank accession numbers: MSV-UMba27, EF015781; MSV-UKas42, EF015780; MSV-UKas43, EF015779; MSV-UKab48, EF015782; MSV-UMub49, EF015783; MSV-UWak56, EF015778.

In addition, each 1.3-kb product amplified by PCR from the same FTA card-eluted samples was sequenced. That the 1300 nucleotides of sequence shared by the PCR and TempliPhi™ amplicons were identical for all six samples indicates both that the two amplification methods have comparable fidelity, and that neither is especially error prone. Sequences of the 1.3-kb products from the degenerate primer PCRs can be accessed at the following genbank accession numbers: MSV-UMba27_degen, EF015772; MSV-UKas42_degen, EF015777, MSV-UKas43_degen, EF015775; MS V-UKab48_degen, EF015773; MSV-UMub49_degen, EF015774; MSV-UWak56_degen, EF015776.

Phylogenetic analysis (maximum likelihood with HKY model using inference of model parameters from the data and 100 bootstrap replicates; Guindon and Gascuel, 2003) of the sequences determined in this study with other publicly available MSV sequences (all aligned using the POA method of Lee et al., 2002, with default settings) indicated that they are all clearly from viruses belonging to the "maize type" or A strain of MSV (Fig. 3; Martin et al., 2001). Following the naming convention proposed by Martin et al. (2001), the sequences are all of the A1 (MSV-UMba27, MSV-UKas42, MSV-UKab48 and MSV-UMub49) and A_5 (MSV-UKas43 and MSV-UWak56) subtypes previously identified in a study of Kenyan MSV diversity as being common in East Africa (Martin et al., 2001).

4. Discussion

Ndunguru et al. (2005) evaluated the efficacy of FTA (r) Classic Card technology for the sampling, retrieval and PCR-based analyses of DNA viruses, and demonstrated that, due to the lack of bulk offered by paper-based collection, the potential number of samples that could be collected within a given time and location should significantly increase compared with other currently used sampling methods. The present report goes beyond a demonstration of potential utility to being the first practical application of the technology to large-scale sampling in the field, in which leaf samples from 155 MSV-infected maize plants were collected from 155 fields in Uganda. Following collection the samples were stored at room temperature for between 8 and 9 months before being processed. Over 90% (141/155) of the samples contained MSV DNA that was amplifiable by degenerate primer PCR. Of these, 127 (90%) yielded more than 1 μg of DNA, suitable for a diagnostic RFLP analysis using seven

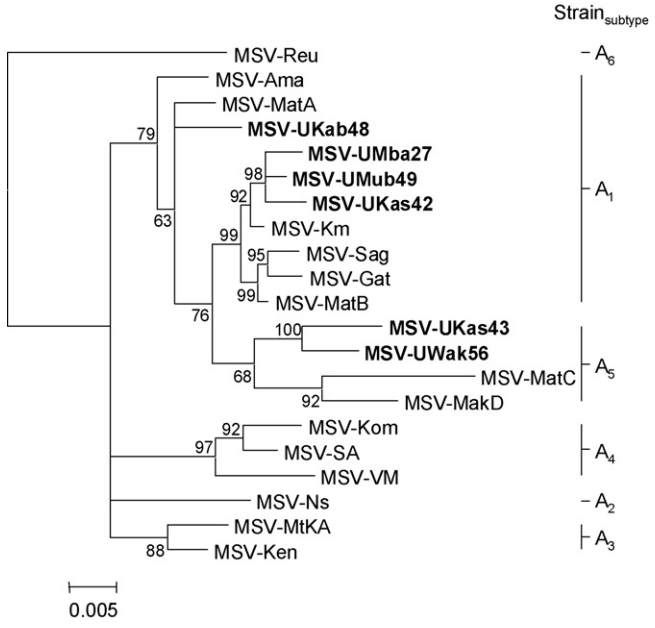

Fig. 3. Classification of Ugandan MSV full genome sequences using the maximum likelihood method of phylogenetic reconstruction (HKY model with all parameters estimated from the data; Guindon and Gascuel, 2003). The strain/subtype classification scheme used is that proposed by Martin et al. (2001). The tree was constructed using 100 full maximum likelihood bootstrap replicates and numbers associated with branches represent the degree of bootstrap support for each branch. Branches with less than 60% support have been collapsed. All six sequences determined in this study (in bold) fall clearly within the subtype A_1 and A_5 groups.

restriction enzymes previously described for MSV (Willment et al., 2001; Martin et al., 2001). This compares very well to established sampling methods for which 92% (281/305) of samples yielded PCR-amplifiable MSV DNA, 91% (257 samples) of which were suitable for diagnostic RFLP analysis.

Compared with sampling and storage of intact MSV-infected plant material followed by CTAB extraction of viral DNA, there was a valuable saving in terms of time and resources using FTA cards. To extract DNA from, for example, 20 samples using CTAB would take at least 4–5 h, compared with ~1 h from FTA cards. While FTA card extraction requires few reagents and no expensive laboratory equipment, CTAB extraction requires liquid nitrogen, numerous chemicals (including toxic reagents such as chloroform, isoamyl and β-mercaptoethanol), a bench top centrifuge and either a water bath or heating block (Kiprop et al., 2002). This makes FTA cards particularly useful for resource poor African institutions, even to the extent that electricity is not required.

PCR-RFLP analyses, and PCR-cloning of virus genomes are heavily dependent on *a priori* knowledge of at least the approximate sequence of the genomes being analysed. We therefore investigated the use of a non sequence-specific circular DNA amplification technique, called TempliPhi™ for the cloning of complete MSV genomes from FTA cards. As viral genomic DNA concentrations on the cards could potentially influence the success of their amplification we attempted to clone full genomes from samples obtained from plants displaying severe (disease rating = 4), moderate (disease rating = 2.5) and mild (disease rating = 1.5) MSD symptoms (two plants in each symptom category). As MSV symptom severity correlates well with viral DNA concentration (Shepherd et al., 2005), the severely infected samples should contain the most and the mildly infected samples the least viral DNA. As virus DNA was efficiently amplifiable by the ϕ29 DNA polymerase from all six samples, it is clear that the quality and quantity of DNA stored on, and elutable from, the cards is sufficient for virtually any analytical purpose. With our method, only 2.5 μl of FTA card-eluted DNA was used in the TempliPhi™ reaction in a total reaction mix of 10.2 μl. Of this reaction mix, just 1 μl of amplified product was used for the cloning of full MSV genomes. Demonstration that the cloned and sequenced genomes had sequences that differed from one another and other sequenced MSVs, and that the sequence of each genome was identical to the homologous region amplified from FTA card-eluted DNA by degenerate primer PCR, meant that we had not inadvertently cross-contaminated the samples. This also demonstrated that neither the TempliPhi™ nor the PCR amplifications were inherently error prone, with both inducing false polymorphisms in less than one in every 7.8×10^3 nucleotides sequenced. We have since used the TempliPhi™ technique to clone and sequence 68 full-length virus genomes, the entire process, once optimised, taking just 3 days (Owor et al., unpublished).

There are several downstream applications for FTA card archived samples. According to the manufacturers, samples have been stored for up to 14 years with no change in the integrity of the attached DNA (Ndunguru et al., 2005). With improved vascular puncture inoculation (VPI; Louie, 1995) techniques (Redinbaugh et al., 2001; Redinbaugh, 2003), it may be possible to directly inoculate plants with viral DNA eluted from FTA cards using VPI. Alternatively, MSV genome concatemers generated during ϕ29 DNA polymerase amplification may also be infectious using these techniques.

In conclusion, our results have demonstrated that use of FTA® Classic Cards facilitates large-scale and geographically wide field sampling of viruses such as MSV, and yields DNA of sufficient stability, quantity and quality for a wide range of potential downstream analyses. The techniques we have investigated should be applicable to other plant viruses with DNA genomes. Additionally, non-specific ϕ29 DNA polymerase amplification of circular viral genomic DNA sequences from plant material sampled from FTA cards should vastly simplify the discovery of novel viruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2006.11.004.

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