Journal of Virological Methods 189 (2013) 148–156

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

# A single-tube duplex and multiplex PCR for simultaneous detection of four cassava mosaic begomovirus species in cassava plants

R.C. Aloyce[a,b,*], F. Tairo[a], P. Sseruwagi[a], M.E.C. Rey[b], J. Ndunguru[a]

[a] Mikocheni Agriculture Research Institute, P.O. Box 6226, Dar es Salaam, Tanzania
[b] University of the Witwatersrand, School of Molecular and Cell Biology, P.O. Box Wits 2050, Braamfontein, Johannesburg, South Africa

*Article history:*
Received 4 April 2012
Received in revised form
26 September 2012
Accepted 22 October 2012
Available online 19 November 2012

*Keywords:*
Cassava
Begomoviruses
Detection
Duplex
Multiplex PCR

A B S T R A C T

A single-tube duplex and multiplex PCR was developed for the simultaneous detection of *African cassava mosaic virus* (ACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV) and *East African cassava mosaic Zanzibar virus* (EACMZV), four cassava mosaic begomoviruses (CMBs) affecting cassava in sub-Saharan Africa. Co-occurrence of the CMBs in cassava synergistically enhances disease symptoms and complicates their detection and diagnostics. Four primer pairs were designed to target DNA-A component sequences of cassava begomoviruses in a single tube PCR amplification using DNA extracted from dry-stored cassava leaves. Duplex and multiplex PCR enabled the simultaneous detection and differentiation of the four CMBs, namely ACMV (940 bp), EACMCV (435 bp), EACMMV (504 bp) and EACMZV (260 bp) in single and mixed infections, and sequencing results confirmed virus identities according to the respective published sequences of begomovirus species. In addition, we report here a modified Dellapotra et al. (1983) protocol, which was used to extract DNA from dry and fresh cassava leaves with comparable results. Using the duplex and multiplex techniques, time was saved and amount of reagents used were reduced, which translated into reduced cost of the diagnostics. This tool can be used by cassava breeders screening for disease resistance; scientists doing virus diagnostic studies; phytosanitary officers checking movement of diseased planting materials, and seed certification and multipliers for virus indexing.



## 1. Introduction

Cassava mosaic disease (CMD) is the most limiting biotic factor to cassava (*Manihot esculenta* Crantz) production in sub-Saharan Africa (SSA) (Thresh et al., 1994). Yield losses of 20–95% have been reported in farmers' fields due to CMD (Fargette et al., 1988). The disease is caused by viruses belonging to the genus *Begomovirus*, family *Geminiviridae*, which are transmitted by the whitefly *Bemisia tabaci* (Gennadius) (Dubern, 1994) and spread through planting of infected cassava stakes.

Nine cassava mosaic begomovirus (CMB) species have so far been reported to infect cassava worldwide (Fauquet et al., 2008). In SSA alone, seven of the cassava-infecting CMBs, namely *African cassava mosaic virus* (ACMV), *East African cassava mosaic virus* (EACMV), *East African cassava mosaic Cameroon virus* (EACMCV), *East African cassava mosaic Malawi virus* (EACMMV), *East African cassava mosaic Zanzibar virus* (EACMZV), *East African cassava mosaic Kenya virus* (EACMKV) and *South African cassava mosaic virus* (SACMV) were reported (Fauquet et al., 2008).

Early efforts to detect the causative agents of CMD in SSA first employed enzyme-linked immunosorbant assays (ELISAs) with monoclonal antibodies (Mabs) to distinguish two viruses currently known as ACMV and EACMV (Swanson and Harrison, 1994; Harrison et al., 1997). A key advantage of ELISA is that it is relatively simple to perform and requires fewer resources. However, ELISA requires fresh samples with clear disease symptoms, owing to its failure to detect viruses at very low titer. In addition, ELISA is unable to distinguish other CMBs such as EACMCV, EACMKV, EACMMV, SACMV, EACMZV and the variant East African cassava mosaic virus-Uganda (EACMV-Ug), which has similar epitope profiles in the coat protein as ACMV (Thottappilly et al., 2003).

PCR (polymerase chain reaction), which targets virus nucleic acids, can overcome these difficulties. Degenerate and virus specific primers were developed and used subsequently to detect the CMBs occurring in several African countries, including Uganda (Zhou et al., 1997; Fondong et al., 2000; Pita et al., 2001), South Africa (Berry and Rey, 2001), Rwanda (Legg et al., 2001; Sseruwagi et al., 2005), Senegal (Okao-Okuja et al., 2004), Kenya (Were et al., 2004), Tanzania (Ndunguru et al., 2005) and Nigeria (Ogbe et al., 2006). However, further knowledge of the complexity of the CMBs in SSA,

* Corresponding author at: P.O. Box 6226, Dar es Salaam, Tanzania.
Tel.: +255 754757122.
*E-mail address:* ckitona@yahoo.com (R.C. Aloyce).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.10.007

brought to light through sequencing data obtained from the last decade of studies, indicates even greater genetic diversity among EACMV species and strains than was earlier envisaged (Fauquet et al., 2008).

The development of the multiplex PCR, a technique for detecting multiple viruses by combining multiple primer sets into a single amplification reaction (Deb and Anderson, 2007) enabled the simultaneous detection of ACMV and EACMCV for the first time in cassava (Alabi et al., 2008), and multiple viruses in other plant species (Nie and Singh, 2000; Bertolini et al., 2001; Deb and Anderson, 2007; Roy et al., 2010; Hu et al., 2010). More recently, Abarshi et al. (2012) developed reverse transcriptase (RT) multiplex PCR tool for the simultaneous detection of RNA and DNA viruses co-infecting cassava. The RT multiplex PCR detected reliably the two cassava brown streak associated viruses, *Cassava brown streak virus* (CBSV) and *Uganda cassava brown streak virus* (UCBSV), although it weakly distinguished RNA and DNA cassava viruses.

Currently, there are more CMBs affecting cassava in SSA than were detected by the Alabi et al. (2008) multiplex PCR. Therefore there remains a great need for a more specific, sensitive and reliable diagnostic tool to distinguish the major CMBs in SSA. This study aimed to develop a more specific, sensitive and reliable single duplex and multiplex PCR tool for the simultaneous detection of four major CMB species in east and southern Africa.

## 2. Materials and methods

### 2.1. Collection of CMD virus isolates

A total of 172 cassava leaf samples showing virus and virus-like symptoms of CMD were collected from cassava fields in Kenya (20), Malawi (32), Mozambique (20), Rwanda (20), Tanzania (60) and Zambia (20) between 2010 and 2011. The samples were pressed between papers packed in envelopes and shipped to Mikocheni Agricultural Research Institute (MARI), Tanzania where they were stored in a dry place until DNA extraction and analysis.

### 2.2. DNA extraction

Extraction of DNA was conducted using a modified SDS-based extraction protocol of Dellapotra et al. (1983). In the modified protocol, liquid nitrogen was excluded in DNA extraction process; 50 mg of dry leaf were directly ground in 700 μl of extraction buffer contained 700 mM $NaCl_2$ and 20 mM of β-mercaptoethanol. Other steps remained the same except the final DNA pellets were washed into 700 μl of wash buffer (75% ethanol and 10 mM sodium acetate) instead of 80% ethanol for Dellapotra et al. (1983). The modified extraction protocol was used to extract DNA using both freshly collected and dry leaf samples. Procedures for DNA extraction were similar, except more fresh leaf (100 mg) was used. DNA qualities were checked on 1% agarose gel and the quantity estimated relative to known concentrations of lambda DNA (NEB N3011S, New England Biolabs, Ipswich, MA).

### 2.3. Designing and screening novel primers for CMBs

Published full sequences of ACMV, EACMV, EACMCV, EACMKV, EACMMV and EACMZV species available in the GenBank were used to design novel primers for use in this study (Table 1). The reference sequences were aligned using computer software packages MegAlign of DNAstar and MEGA4 (Tamura et al., 2007) and conserved regions in the DNA-A component specific to virus species

**Table 1**
List of primers used for amplification of cassava mosaic begomoviruses in uniplex, duplex and multiplex PCR.

| Primer name | Sequence (5′ → 3′) | Virus species | Target region | Expected size (nt) | References |
|---|---|---|---|---|---|
| JSP001 | ATGTCGAAGCGACCAGGAGAT | ACMV | AV1/CP | 554 | Fondong et al. (2000) |
| JSP002 | TGTTTATTAATTGCCAATACT | | | | |
| EAB555/F | TACATCGGCCTTTGAGTCGCATGG | CMBs | DNA B | 744 | Fondong et al. (2000) |
| EAB555/R | CTTATTAACGCCTATATAAACACC | | | | |
| ACMV1[a] | GTGGGCCTGGGCTGACACAC | ACMV | DNA A | 948 | This study |
| ACMV2[a] | GCGTAGGAGAGTGGATCTTGTC | | | | |
| EACMKV1[b] | AAGGAGTCAGAGGCTCTTG | EACMKV | DNA A | 669 | This study |
| EACMKV2[b] | CCACGTTTGAATTTCAAATTC | | | | |
| EACMMV1[c] | GTGCCCTGTTCTTCACGGT | EACMMV | DNA A | 503 | This study |
| EACMMV2[c] | ACACACGTCCCAGACGAAA | | | | |
| EACMCV1[d] | AAGTCTGAGGATGTAAACGAG | EACMCV | DNA A | 435 | This study |
| EACMCV2[d] | ACCTAGACGAGGACAAGAATTCC | | | | |
| EACMV1[e] | GTTCGGCTATCACCTTCTAGAACA | EACMV | DNA A | 375 | This study |
| EACMV2[e] | CAAGGCTTACATTGAAAAGGGA | | | | |
| EACMZV1[f] | CCAGGTCGAAGAATCGCTTA | EACMZV | DNA A | 260 | This study |
| EACMZV2[f] | AGGTGTCTCCAATTGCTCTC | | | | |
| EACMMV-F[c] | AACAAGCGACGATCATGGACGTTC | EACMMV | DNA A | 1630 | This study |
| EACMMV-R[c] | ACACACGTCCCAGACGAAA | | | | |
| ACMV-F[a] | GAAGCACCTTGGTATCTGTAAGGTG | ACMV | DNA A | 1106 | This study |
| ACMV-R[a] | CAAGAAGCGCTAAAGGCC | | | | |
| EACMZV-F[f] | GAAACATAAGGAGCTGGT | EACMZV | DNA A | 575 | This study |
| EACMZV-R[f] | AGGTGTCTCCAATTGCTCTC | | | | |
| EACMV-F[e] | CCCCACAACATGCCCGCACT | EACMV | DNA A | 512 | This study |
| EACMV-R[e] | GGCCTTCACAGCCCTTCGGG | | | | |
| EACMCV-F[d] | GGTAATGGGTTTAAGGACTGGT | EACMCV | DNA A | 305 | This study |
| EACMCV-R[d] | CCTGGTTAGACAACTGCATATT C | | | | |
| EACMKV-F[b] | TTGTCCTCCTCGAGCAGATCGTC | EACMKV | DNA A | 238 | This study |
| EACMKV-R[b] | AAGTCCTATATGGACAAGGAC | | | | |
| RBCL-F535 | CTTTCCAAGGCCCGCCTCA | Rubisco L | | 171 | Nassuth et al. (2000) |
| RBCL-R705 | CATCATCTTTGGTAAAATCAAGTCCA | | | | |

[a] Primer designed using GenBank accession nos. AF112352, AF259894, AF366902, AY795982, F126800, FN435276.
[b] Primer designed using GenBank accession nos. AJ717582, AJ717577, AJ717571, AJ717569, AJ717578, AJ717581.
[c] Primer designed using GenBank accession nos. AJ006459 and AJ006460.
[d] Primer designed using GenBank accession nos. EU685323, EU685321, EU685319, EU685326, AF259896, AJ867444.
[e] Primer designed using GenBank accession nos. AJ717546, AJ717553, AJ006458, AY795986, Z83256, AJ717537.
[f] Primer designed using GenBank accession nos. AJ717567, AJ717564, AJ516003, AJ717563, AJ717560, AJ717583.