



Protocols

Extraction of Geminiviral DNA from a Highly Mucilaginous Plant (*Abelmoschus esculentus*)

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Abstract. A protocol is described for the extraction of geminiviral DNA from bhendi yellow vein mosaic virus-infected *Abelmoschus esculentus* (known as bhendi or okra) containing high amounts of mucilage and other phenolic compounds. This method involves extraction with a buffer containing sodium citrate at pH 6 and PEG precipitation of the virus followed by alkali lysis. The extraction buffer eliminates the mucilage and other polyphenols, PEG precipitates the viral particles and DNA and the alkali lysis enriches the replicative forms of the viral DNA. The extracted DNA could be digested with restriction enzymes and cloned without any interference from chromosomal DNA. The quality of the DNA extracted by this method was compared to three other common plant DNA extraction protocols and was found superior. This method was used for PCR amplification and cloning of the 2.7 kbp DNA-A of BYVMV.

Introduction

A large number of crop plants are susceptible to infection by geminiviruses, a family of viruses named after their unique geminate morphology. The viral genome consists of one or two small circular single stranded DNA (2.6-3.0 kbp), which replicates via the rolling circle mechanism in the plant nucleus (reviewed by Bowdoin et al., 1999). Usually, geminivirus genomes are cloned by either (a) directly restricting the replicative form which is isolated electrophoretically from the total infected nucleic acid (Hamilton et al., 1982), or by (b) amplifying the full or partial genomes with geminivirus-specific primers (Wu et al., 1996). It is essential to obtain high quality DNA for these two approaches, but this is difficult for many of the natural host plants due to the high polysaccharide and polyphenol content. Polysaccharides have viscous, glue-like texture and make the DNA unmanageable in pipetting and unsuitable for PCR since they inhibit *Taq* polymerase activity (Fang et al., 1992). Ikegami et al. (1981) and Hamilton et al. (1982) have overcome this problem by extracting the viral DNA from virus-infected non-mucilaginous permissive hosts like tobacco. However for the characterization of all the viral genomic components, including defective and satellite DNA

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responsible for infection of the natural host, the viral DNA has to be extracted from the natural hosts (Saunders et al., 2000).

Bhendi yellow vein mosaic virus (BYVMV) (Brunt et al., 1996) is a member of the *Begomovirus* genus of the family *Geminiviridae*, infecting bhendi (okra) plants by whitefly transmission (Zhou et al., 1998). High mucilage content in the plants affects the purification of the viral DNA. The protocol described here is simple, inexpensive and gives pure viral DNA without ultra-purification.

Materials and Methods

DNA extraction

Total DNA extraction was done according to: 1) Dellaporta et al. (1983), 2) a CTAB method (Doyle and Doyle, 1987; Porebski et al., 1997), and 3) the citrate buffer extraction with alkali lysis (present work). The alkali lysis step is the same as that described by Birnboim and Dolly (1979) for plasmid DNA isolation. DNA obtained by the CTAB method was also subjected to alkali lysis omitting the phenol extraction and RNase treatment steps. This allowed comparison to the citrate method.

Citrate method

Solutions required:

1. Extraction buffer: 100 mM sodium citrate pH 6, containing β -mercaptoethanol (50 μ L /10 mL)
2. Chloroform:isoamyl alcohol 24:1(v/v)
3. PEG 6000
4. 2 M NaCl

Protocol

Extraction

- Collect 1 g of bhendi leaves and grind with liquid nitrogen using a mortar and pestle. Transfer the finely ground powder to a flask containing 6 mL of extraction buffer.
- Add 2 mL of chloroform:isoamyl alcohol (24:1) and mix it thoroughly to form an emulsion.
- Transfer it to a centrifuge tube and spin at 15,000 g for 15 min at 4°C.
- Transfer the top aqueous phase to a beaker containing NaCl and PEG 6000 (to a final concentration of 0.2 M and 7% respectively). Stir it for 3 h at 4°C using a magnetic stirrer.
- Spool out the undissolved mucilage and transfer the solution to polypropylene tubes and spin at 18,000 g for 25 min at 4°C.
- Drain off the supernatant and dry the pellet to avoid any trace of PEG. Take the pellet and dissolve it in 600 μ L of 0.1 M citrate buffer pH 6 without any β -mercaptoethanol. Give a quick spin at 1,100 g to remove the insoluble materials¹.

Cloning

DNA extracted by the citrate method was digested with *Hind* III and was cloned into the *Hind* III site of pBSIISK+ vector (Stratagene). Various clones containing inserts of sizes ranging from 0.5 to 2.7 kbp were selected and digested with restriction enzymes *Eco*R V, *Bam*H I, and *Pst* I and used for Southern hybridization.

Results and Discussion

We were able to successfully amplify small viral fragments (0.77 kbp coat protein gene) using DNA from all the extractions. However, full-length amplification was possible and reproducible only with the modified citrate method. There was a faint 2.7 kbp amplification in the modified CTAB method with alkali lysis. Thus, this extraction protocol could be considered as the second best for PCR (Figure 1).

The presence of virus in the extract was checked on SDS PAGE. It was also found to give a positive reaction with an antiserum against African cassava mosaic virus in both ELISA and Western blot (data not shown).

The amount of total nucleic acids obtained from 1 g of leaf by various extractions was determined spectrophotometrically. The concentrations of the nucleic acids were: Dellaporta 5.5 µg, CTAB 15.2 µg, CTAB with alkali lysis 5.8 µg and citrate with alkali lysis 1.12 µg. For the Southern blots, the total nucleic acids obtained from 1 g of infected leaf was loaded on the gel. The probe used was a full-length DNA-A clone of BYVMV, derived from the PCR amplification using abutting primers on the nucleic acids extracted by the modified citrate method described here. The DNA-A was cloned into the pGEM-T vector (Promega) and was completely sequenced (GenBank acc. no. AF241479) and its phylogenetic relationship with other begomoviruses, in particular, its high degree of identity with the Pakistani isolate of okra yellow vein mosaic virus (Zhou et al., 1998) was determined (Jose and Usha, unpublished results). In the denaturing Southern blot, both single- and double-stranded viral DNA hybridized with the probe. It showed the presence of virus in all extractions except in the Dellaporta method (Figure 2). In the non-denaturing Southern, single stranded DNA from CTAB, both with and without alkali lysis and citrate methods showed the presence of viral DNA. Among the extractions, the citrate method with alkali lysis was found best, as the full length genome remained intact (Figure 3). This observation is in accordance with the findings of Muniyappa et al. (1991), that tomato leaf curl geminivirus particles were more stable in citrate buffer than in Tris buffer.

Although the concentration of total DNA in the CTAB and Dellaporta extractions was higher, the quantity of viral DNA, both single- and double-stranded, was greater in the citrate method and CTAB with alkali lysis. Also, the presence of the chromosomal DNA in the other extraction protocols makes the citrate method reliable for cloning the viral DNA.

When the total DNA extracted by the citrate method was cloned after restriction digestions, all the recombinant plasmids obtained were either full or partial viral DNA-A clones. This confirms the presence of more than one type of DNA-A forms in BYVMV which vary in restriction patterns (Figure 4).

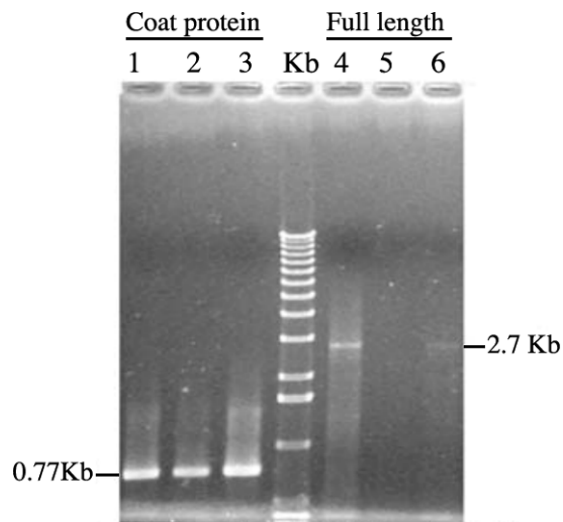


Figure 1. PCR on 250 ng of total nucleic acids extracted by different methods. Amplifications are: 0.77 Kb coat protein gene and 2.7 Kb full length DNA-A of BYVMV. Lanes: (1) and (4) Citrate, (2) and (5) Dellaporta, (3) and (6) CTAB with alkali lysis.

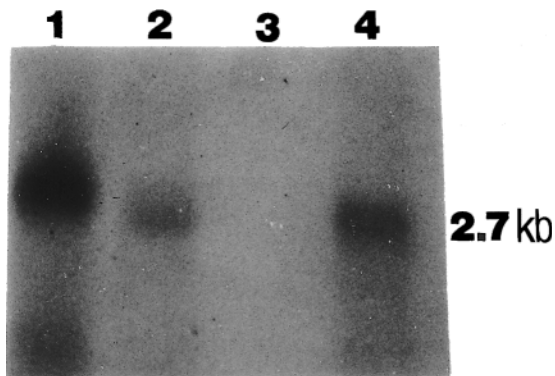


Figure 2. Southern hybridization of BYVMV DNA extracted from 1 g of infected leaf by various methods. The blot was probed with BYVMV DNA-A probe. Lanes: (1) CTAB with alkali lysis, (2) CTAB, (3) Dellaporta, (4) Citrate.

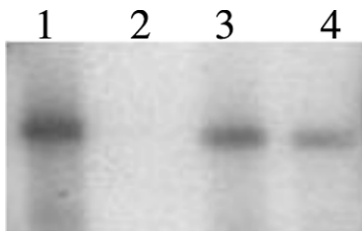


Figure 3. Non-denaturing Southern analysis of the single stranded DNA of BYVMV extracted from 1 g of infected leaf by different methods. The blot was probed with labeled DNA-A probe of BYVMV. Lanes: (1) Citrate, (2) Dellaporta, (3) CTAB with alkali lysis, (4) CTAB.

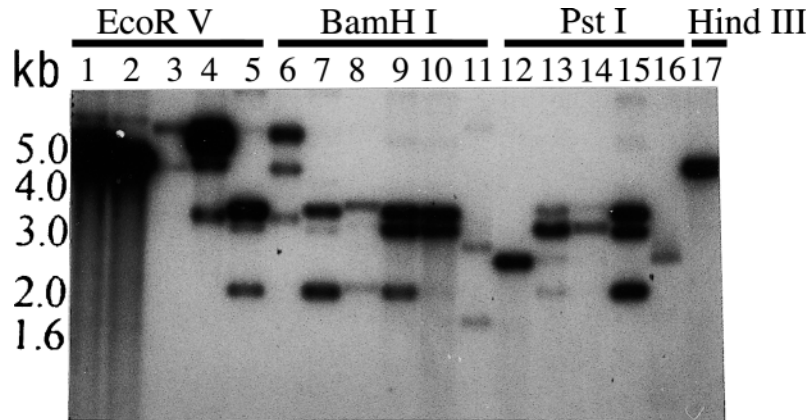


Figure 4. Southern analysis of the pBSIISK+ clones obtained by *Hind* III digestion and subsequent cloning of the BYVMV DNA extracted by citrate method. The blot was probed with labeled DNA-A probe of BYVMV. Lanes: (1-16) 16 different clones, (17) positive control (pGEM clone of BYVMV DNA-A). Clones were digested with *EcoR* V, *BamH* I, *Pst* I and *Hind* III.

The protocol described here is thus useful for obtaining good quality viral DNA, from highly mucilaginous plants such as bhendi (okra).

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