International Journal of Agriculture Sciences
ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 6, 2019, pp.-8096-8099.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

RAPID AND EFFICIENT HIGH QUALITY VIRAL RNA ISOLATION FROM BANANA BRACT MOSAIC VIRUS INFECTED BANANA CV. GRAND NAINE

EKATPURE S.C.*, SONI K.B., JADHAV P.R., LEKSHMI R.S. AND ALEX S.
Department of Plant Biotechnology, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram, Kerala,695522, India
*Corresponding Author: Email - sachi.ekatpure@gmail.com

Received: March 15, 2019; Revised: March 25, 2019; Accepted: March 26, 2019; Published: March 30, 2019

Abstract: Isolation of high quality viral RNA from a banana plant is quite difficult, due to the presence of high amount of polysaccharides and phenolic compounds in the plant. These compounds not only reduce the yield of RNA but also make it unsuitable for the molecular studies, like cDNA library construction, molecular analysis and RNAseq. In the present study we describe a simple RNA isolation procedure based on Rodriguez-Garcia protocol to isolate high-quality viral RNA from banana plant infected with Banana Bract Mosaic Virus (BBrMV). This modified protocol effectively removes phenols and polysaccharides and gives a yield of 100-200 µg high-quality total RNA per gram of fresh tissue. PCR product of partial BBrMV replicase and movement protein gene from bract sample was sequenced generating 386 nt and 339 nt sequences respectively which were submitted to NCBI database. These sequences showed similarity to replicase (96 %) and movement protein gene (97 %) to that of Trichy isolate, with 100 percent query cover in BLAST result. Isolated RNA showed purity suitable for northern analysis, RT-PCR and cDNA library construction.

Keywords: Banana Bract Mosaic Virus (BBrMV), ssRNA viruses, RNA isolation, Replicase gene, Grand Naine, Polysaccharides and Phenolics

Citation: Ekatpure S.C., *et al*., (2019) Rapid and Efficient High Quality Viral RNA Isolation from Banana Bract Mosaic Virus Infected Banana Cv. Grand Naine. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 6, pp.- 8096-8099.

Introduction

Banana, the most important fruit crop belongs to the family Muscaceae. In India it is widely cultivated due to favourable agro-climatic conditions; however the potential yield cannot be attained due to presence of number of pests and diseases. Among the diseases banana is severely affected by viruses like banana bunchy top virus disease (BBTV) [1], banana streak virus (BSV) caused by a Badnavirus, Banana bract mosaic virus (BBrMV). After BBTV, BBrMV causes higher yield loss up to 40 percent [2]. It was first reported in Philippines [3]. There after its presence has been reported in several Asian countries (India, Sri Lanka, Vietnam, Western Samoa and Thailand) [4-7]. BBrMV spread rapidly through infected suckers and non-persistently transmitted by aphid vectors (*Pentalonia nigronervosa, Rhopalosiphum maidis* and *Aphis gossypi*) [8]. There are no reports specifying resistant cultivars of banana against BBrMV [9]. BBrMV is one of the dreadful viruses causing high economic damage. Hence, its interaction with plant and vector is to be highly monitored to develop control measures. The genome of BBrMV is RNA, so molecular studies essentially requires high quantity and quality of RNA for cDNA library construction of both virus and banana plant. Isolation of good amounts of high-quality viral RNA from the infected plant tissues which are rich in polysaccharides and polyphenols requires methods which can effectively avoid the interference of these compounds [10]. Reverse-transcription polymerase chain reaction (RT-PCR) is the most accurate method for the detection of BBrMV from infected leaf tissues [6,10]. In our experience it is easy to isolate the RNA from leaf and bract samples compared to other plant parts as they are very hardy when immersed in liquid nitrogen. Protocols reported for RNA isolation from banana fruit [11-13], root [14] and greenhouse leaves [15] are lengthy. No method has been standardized specifically for the isolation of large amounts of high-quality viral RNA suitable for cDNA library preparation from virus infected banana leaves and flower bract. Research required the isolation of Banana Bract Mosaic Virus RNA from infected tissues of banana for cDNA preparation. For this we used the protocol described by Rodriguez-Garcia *et al*. [15] with some modification.

Materials and Methods

Plant Tissue source

Leaves and bract of banana plant *var*. Grand Naine with prominent symptoms of BBrMV disease were collected from the field of College of Agriculture, Vellayani (Kerala) and flash frozen in liquid nitrogen and stored at -80ºC until use.

RNA extraction preparation

Sterile distilled water was used to prepare all the solutions and buffers. To dissolve RNA, 0.1 percent (v/v) diethyl pyrocarbonate (DEPC; Sigma) was used. All tips, tubes, and DEPC treated water were autoclaved at 121ºC for 40 min. Mortars, pestles and spatulas were washed with distilled water containing 3% (w/v) Sodium hypochlorite (NaOCl), rinsed with sterile distilled water and autoclaved at 121ºC for 40 min.

Reagents

Extraction buffer contained 150 mM Tris base (hydroxymethyl-hydrochloride), 4% (w/v) SDS, 100 mM EDTA (pH 7.5, adjusted with saturated boric acid), 60 µL β-mercaptoethanol (2%, v/v) and 3% (w/v) PVP-40. Liquid nitrogen used for crushing the plant samples. 5 mM potassium acetate, absolute ethanol, chloroform-isoamyl alcohol (49:1, v/v), Phenol: Chloroform: Isoamyl alcohol (25:24:1 v/v), 12 M LiCl and 70% ethanol were used in subsequent steps

Procedure

BBrMV infected banana leaves and bract samples (0.5 g) were taken in a previously chilled mortar and ground to fine powder using liquid nitrogen. Extraction buffer (3 mL) was added to homogenate and incubated in the hot water bath for 30 minutes at 65ºC. Homogenate (approx. 750 µl) was transferred to sterile 2 ml Eppendorf tube using the cut tip to avoid the RNA damage and was precipitated using 5 mM potassium acetate (66 µL) and absolute ethanol (150 µL).

Tube was vortexed briefly for 1 min. Equal volume of chloroform-isoamyl alcohol (49:1, v/v) was added and vortexed briefly and centrifuged at 16,000 g for 20 min at room temperature. Supernatant was carefully transferred to a fresh tube and this step was repeated to remove any suspended cell debris. Supernatant was taken in a fresh tube, equal volume (850 µL) of phenol: chloroform: isoamyl alcohol (25:24:1) added and vortexed for 10 seconds and centrifuged at 16000 g for 15 minutes at room temperature. Supernatant was collected in a fresh tube and 850 µL of chloroform-isoamyl alcohol (49:1, v/v) was added. Tube was briefly vortexed for 10 seconds and centrifuged at 16000 g for 15 minutes at 4ºC. Supernatant was collected and 3 M LiCl was added to it, mixed by inversion and kept at 4ºC overnight. The content was then centrifuged at 16000 g for 20 minutes at 4ºC. Pellet was recovered at this stage and washed twice with 70 percent ethanol. Pellet was air dried at room temperature under sterile laminar air flow and resuspended in 30 µL of DEPC-treated sterile distilled water. RNA was quantified in a UV-light spectrophotometer and stored at -80ºC until use.

Table-1 *Assessment of absorbance ratios and total yields of RNA obtained from infected banana leaves and flower bract*

Tissue	A260/210	A260/230	A260/280	RNA concentration µg/g fresh weight
Leaf	0.745	1.738	1.961	127.038
Flower bract	0.882	2.31	2.061	202.293

Modification of the Rodriguez-Garcia protocol

In the present study for the isolation of single stranded RNA of BBrMV we used the protocol of Rodriguez-Garcia *et al.* (2010); which was developed for the isolation of fungal RNA from M. fijiensis inoculated banana leaves. Here we tried this protocol for the isolation of ssRNA of BBrMV from the infected banana leaves and flower bract samples. Incubation time was increased from 10 minutes to 30 minutes at 65ºC. Chloroform: Isoamyl treatment was given twice to completely remove the cell debris, protein and phenolic compounds. Samples were kept for precipitation in 3M LiCl overnight at 4ºC instead of -20ºC.

Total RNA analysis

Quantity and quality (purity and integrity) of RNA was determined by measuring the absorbance ratio at A260/210, A260/230 and A260/280 using spectrophotometer, as well as by visual analysis on 1.2 percent agarose gels in 1X TAE buffer.

cDNA preparation

First strand of cDNA was synthesized from RNA (0.5 µg) by using Thermo Scientific Verso cDNA Synthesis Kit (#AB-1453/A) following manufacturer's instructions. The quality of cDNA was analyzed by amplification of γ-actin gene from the banana. For PCR, 100 ng of cDNA was used as a template. Reaction primers were specifically designed based on cDNAs from Musa acuminata: γ-actin (forward primer 5'-CTGGTGATGGTGTGAGCCACACTGTTC-3' and reverse primer 5'-CACTGAGAACGATGTTGCCATACAGGTC-3'). Conditions for PCR were kept at 95ºC for 3 min; 30 cycles of 94ºC for 30 seconds, 59ºC for 30 seconds, 72ºC for 45 seconds, and 72ºC for 5 min. PCR product was run on the 1.2 percent agarose gel with 1X TAE buffer and run at 70 V for 60 min.

BBrMV RNA detection by RT-PCR

Complete genome sequence of BBrMV (Trichy isolate, accession no. HM131454) was retrieved from GenBank [National Centre for Biotechnology Information]. Based on the sequence information, employing NCBI primer blast tool and Primer 3 software, gene specific primers for partial replicase gene (forward- 5'-TATTTCTCAGCGGTCCCACT-3' and reverse- 5' CTGCCACCTGCATCAATAGT-3') and for partial movement protein gene (forward- 5'-GAGGAAGTACGCGCATCAAC-3' and reverse- 5'-ATCTCCATACCACGCCGTAA-3') were designed and synthesized at Sigma Aldrich, Bangalore, India. In a standard PCR mix (20 µl) containing 100 ng of template cDNA, 200 µM dNTPs, 10 pM of each primer, 1 unit of Taq polymerase, 1x Taq polymerase buffer, the cDNA was amplified using gene specific primers in a Thermal cycler (BIO-RAD T100TM). PCR conditions : 95ºC for 3 min; 25 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 45 seconds, and 72ºC for 5 min and final storage at 4ºC. The PCR product was loaded on 1 percent agarose gel with 70 volts for 40 minutes and visualized on the gel documentation unit (Bio-Rad) with ethidium bromide stain.

Results and Discussions

The main aim of our study was to develop a simple, rapid and cost effective protocol for viral RNA isolation from BBrMV infected banana plant for molecular studies. BBrMV, a member of the genus Potyvirus, is non-persistently transmitted by the aphid vectors. Main symptoms of BBrMV are the dark red-brown mosaic pattern on the banana flower bract. Cavendish bananas normally do not show any symptoms of the disease until the beginning of typical mosaic symptoms on bracts. So, for RNA isolation highly infected leaf and flower bract tissues are selected after the flowering [Fig-1].

Fig-1 BBrMV highly infected leaf and flower bract

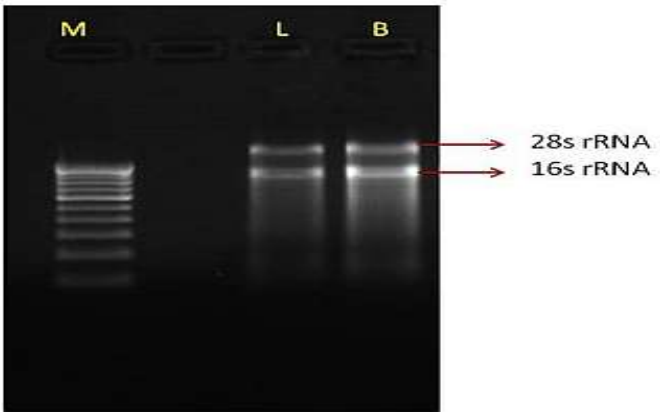

Fig-2 Total RNA extracted from BBrMV infected banana leaves and flower bract samples: Lane M- 100 bp DNA marker, Lane L- RNA from leaf sample, Lane B- RNA from flower bract sample

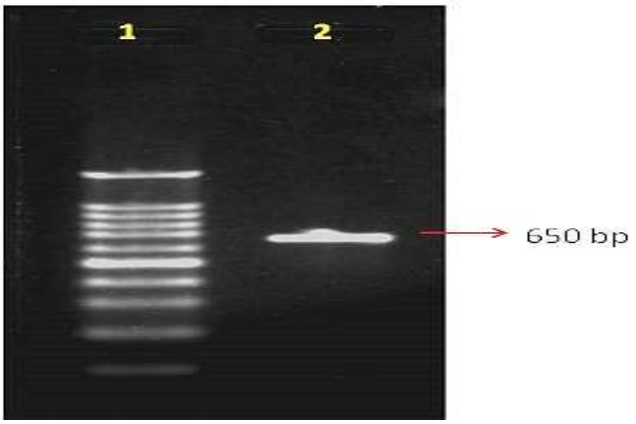

Fig-3 Actin gene amplification: Lane 1- 100 bp DNA marker, Lane 2- Actin gene amplification at 650 bp

Musa species is rich in polysaccharides and phenols and mostly affected by viruses. Several scientific protocols and reagent kits are available for RNA isolation from mucilaginous plant tissues [16-21].

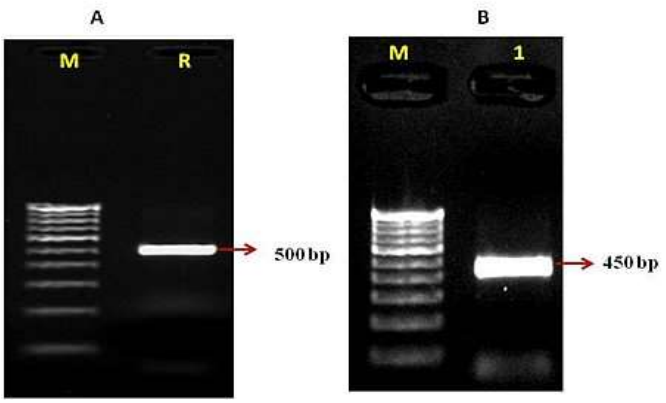

Fig-4 RT PCR of Replicase and movement protein gene:
(A) Replicase gene: Lane M: 100 bp DNA marker; Lane R: Replicase gene amplification at 500 bp
(B) Movement protein gene: Lane M: 100 bp DNA marker; Lane 1: Movement protein gene at 450 bp

Usually clogging in column and column leakage type of problems occurs when using commercial kits for mucilaginous plant samples and makes it unsuitable for RNA extraction [22]. Also, the commercial kits are very expensive. However, a suitable method specifically for recalcitrant plant tissues is yet not available [23]. Standard RNA extraction methods [24-27] are time consuming and laborious; also these methods involves the toxic chemicals, harmful for animals and environment. Also these methods could not be used for virus infected plant tissues as they are rich in polyphenols and polysaccharides components and commercial kits are expensive. Therefore, for isolating nucleic acids from these plants, the protocol was modified so as to get high quality viral RNA. RNA isolated in the modified protocol showed distinct and sharp bands of 28 S and 18 S rRNA on agarose gels without protein, DNA contamination and RNA shearing. The protocol was successful in isolating good-quality RNA from both infected leaf and flower bract tissues of banana [Fig-2]. Yield of isolated RNA measured with spectrophotometry at absorbance ratio A260/210, A260/230 and A260/280. A260/210 (0.7-0.8) and A260/230 (>2) values suggests that weak presence of polyphenols and carbohydrate contaminants in the RNA samples. Whereas absorbance ratio A260/280 of RNA samples was in the range of 1.9 to 2.0 indicating high purity of the samples. The yield of RNA was acceptable and in the range of 127–200 µg/g plant tissue used [Table-1]. The Trizol® reagent [24] and CTAB [25-26] protocol and the method reported by Rodrigues *et al*. [28] tried which gave low yield and degraded RNA (data not shown). Incubation time of tissue homogenate was increased from 10 minutes to 30 minutes at 65ºC to ensure complete breakdown of cells in the isolation buffer. This incubation treatment effectively digested proteins, suspended lipids and celluloid materials and makes nucleic acid free. Twice chloroform–isoamyl alcohol treatment after the phenol–chloroform–isoamyl alcohol step is helpful in the removal of cell debris, protein and phenolic compounds suspended in the supernatant [27]. Precipitation of RNA using lithium chloride was found more effective compared to the ethanol precipitation method [27]. A key advantage of lithium chloride is that it does not efficiently precipitate either protein or DNA and thus helps to reduce contamination by these compounds. Incubation of samples for precipitation in lithium chloride overnight at 4ºC instead of -20ºC effectively increased the RNA quality. Incubation at 4ºC maintains sample in liquid state and it prevents formation of ice crystal and shearing in the precipitated RNA. Saturated phenol and LiCl effectively promote the RNA extraction and gives the RNA free from the DNA contamination as seen in the gel image. Compared to the other protocols reported this protocol is short, simple and cost effective except for the overnight incubation step using lithium chloride. We also found that the protocol can be made still shorter by limiting the precipitation step to 1-3 hours at -20ºC; but, overnight incubation yielded better quality of RNA. On the contrary to leaf sample, flower bract showed the good quality RNA in the spectrometric reading as well as in gel image. cDNA prepared from flower bract RNA sample for amplification of actin (house-keeping gene). The PCR product on separation on 1 percent agarose gel showed a band corresponding to 650 bp which was the expected size of the actin gene [Fig-3]. The result confirmed the successful conversion of mRNA to cDNA. cDNA was also amplified with BBrMV specific primers for replicase and movement protein genes [Fig-4]. The amplicons of virus specific partial genes were gel eluted and sequenced at Agrigenome Cochin (India). Partial replicase gene of BBrMV shows 96 percent and movement protein gene shows 97 percent similarity with the corresponding sequence of Trichy isolate, with 100 percent query cover in BLAST result. Sequences generated were submitted to the NCBI database with accession no. MH253671.1 and MH253670.1 for a partial sequence of replicase and movement protein genes, respectively.

Conclusion

RNA with high yield and quality was isolated from Banana Bract Mosaic Virus infected leaves and flower bract with modifications in the Rodriguez-Garcia protocol. The current protocol is best suited for the isolation of viral RNA from the banana plant cv. Grand Naine. Moreover, this protocol progressively reduced the isolation time and cost. This protocol can be tried for virus isolation in other plants rich in phenols and polysaccharide.

Application of research: High quality viral RNA isolation for molecular studies.

Research Category: Molecular Biology

Abbreviations: BBrMV- Banana Bract Mosaic Virus

Acknowledgement / Funding: Authors are thankful to the Kerala State Council for Science and Technology (KSCSTE), Government of Kerala and College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram, Kerala, 695522, India.

***Research Guide or Chairperson of research: Dr Soni K.B.**
University: Kerala Agricultural University, Thiruvananthapuram, 695522 Kerala
Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Conflict of Interest: None declared

Sample collection: Experimental field, College of Agriculture, Vellayani, 695522

Cultivar/ Variety name: *Musa acuminata var*. Grand Naine

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Ethical Committee Approval Number: Nil

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