Journal of Virological Methods 213 (2015) 106–110

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Short communication

A simplified strategy for studying the etiology of viral diseases: *Apple stem grooving virus* as a case study

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Article history:
Received 10 September 2014
Received in revised form 5 November 2014
Accepted 11 November 2014
Available online 5 December 2014

Keywords:
Long distance PCR
Reverse transcription
Infectious transcripts
ACLSV
ASPV
ASGV

A B S T R A C T

A simple method to amplify infective, complete genomes of single stranded RNA viruses by long distance PCR (LD PCR) from woody plant tissues is described in detail. The present protocol eliminates partial purification of viral particles and the amplification is achieved in three steps: (i) easy preparation of template RNA by incorporating a pre processing step before loading onto the column (ii) reverse transcription by AMV or Superscript reverse transcriptase and (iii) amplification of cDNA by LD PCR using LA or Protoscript *Taq* DNA polymerase. Incorporation of a preprocessing step helped to isolate consistent quality RNA from recalcitrant woody tissues such as apple, which was critical for efficient amplification of the complete genomes of *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV). Complete genome of ASGV was cloned under T7 RNA polymerase promoter and was confirmed to be infectious through transcript inoculation producing symptoms similar to the wild type virus. This is the first report for the largest RNA virus genome amplified by PCR from total nucleic acid extracts of woody plant tissues.

Viruses are important pathogens responsible for significant losses to various crop plants grown worldwide. Of them, most viruses have RNA genomes. Plant viruses tend to have single-stranded RNA genomes (Hull, 2008). Amplifying and cloning the complete genomes might be useful to study viral host range, recombination and evolution (Barba et al., 2014). Studies of the molecular evolutionary history of viruses help to provide an understanding of important features of their biology such as changes in virulence and their 'emergence' as epidemics, information which is essential for designing strategies for their control (Duffy et al., 2008). Over the past 25 years, much progress has been made in sequencing the complete genome of plant viruses (Goszczynski, 2014; James et al., 2014), but the ability of the characterized new viral agents to establish a particular disease needs further improvement.

With the versatile *Taq* DNA polymerases, longer cDNAs could be amplified, a step toward amplification of complete genomes of viruses (Barnes, 1994; Cheng et al., 1994; Tellier et al., 1996; Mason et al., 2003; Youssef et al., 2011). However, problems occurred for efficient amplification due to limited amount of viral RNA present in sample, especially for low titer viruses. There are many reports about complete genome amplification of plant viruses (Vives et al., 2008; Youssef et al., 2010). Workers have propagated the virus on herbaceous host and used complete or partial purified virions for cDNA synthesis. For many plant viruses, technical problems in identifying alternative herbaceous hosts, their purification and in experimental transmission have prevented the analysis of their contribution to particular diseases. Beside, in such cases there is a limitation in processing large number of samples.

Infectious cDNA clones of plant viruses are useful tools for the basic study of viral infection, replication and assembly (Vives et al., 2008; Ambrós et al., 2011). Construction of an infectious clone is usually complex and time-consuming. Youssef et al. (2011) used full-length cDNA (FLcDNA) of ACLSV for construction of its infectious clone under T7 promoter and used yeast recombination for cloning of large cDNA's. In several situations, cloning may not be necessary to validate the etiology of a virus, uncloned PCR products can be used directly for transcription (Youssef et al., 2011). LD RT-PCR technique starts with cDNA synthesis from total RNA or from polyA$^+$ RNA (Tellier et al., 1996). The isolation of high-quality RNA from recalcitrant plant tissues is complicated by varying levels of polyphenols, polysaccharides and other compounds that, when

Abbreviation: LD RT-PCR, long distance reverse transcription-polymerase chain reaction.

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http://dx.doi.org/10.1016/j.jviromet.2014.11.017
0166-0934/

Table 1
Virus specific primers used for complete genome amplifications of three viruses and R primers were used for first strand cDNA synthesis.

Primer	Sequence (5′–3′)	Annealing temperature
ASPV F	AGGCAACTTCATAATTTACTCGAAA	55/57
ASPV R	TCTAGTTAAAAAAAAAATAAAAAAAGCATG	
ACLSV F	TGAGTAAACAGATTGACGTAACGC	58/60
ACLSV R	TAAGTCTAAACACTCCAATCAAATACC	
ASGV F	TAACAGGCTTAATTTCCGCGCTTTACGTCAA	65/67
ASGV R	CAAACTCTAGACTCTAGAAAAACCACAC	

oxidized, avidly bind nucleic acids upon cell lysis (Salzman et al., 1999). Once bound, these substances co-precipitate with RNA and make it unusable for downstream enzymatic manipulations.

The results in this study provided the possibility of amplifying the complete viral genome directly from total RNA; avoiding tedious virus purification procedures, using column for quality RNA purification and for handling large number of samples. This technique can be used for amplifying small amount of RNAs even those available from limited starting material. For this purpose, the RNA isolation procedure, RNA concentration for use in cDNA synthesis and LD RT-PCR conditions were optimized using two different RT and long PCR systems which lead to a high level of sensitivity for amplification of complete genomes of *Apple stem pitting virus* (ASPV), *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV). Further, efficient elution and ligation of full length genomes was standardized and infectivity of the transcribed viral RNA from cloned cDNA was also checked.

For complete genome amplification, initially the total RNA was extracted from apple cv. Red Chief, which tested positive for ACLSV, ASGV and ASPV. *Chenopodium amaranticolor* plants inoculated with ASGV were also used for comparative analysis of RNA (yield and quality) and for viral genome amplification. Total RNA was extracted using RNeasy Plant mini kit (Qiagen, Hilden, Germany), but with slight modifications. Frozen leaf tissue (0.5 g) was ground in liquid nitrogen using a mortar and pestle and then homogenized using Tissue Tearer (Biospec, OK, USA) in 5 ml of extraction buffer (4 M Guanidium isothiocyanate, 0.1 M Tris base, 0.1 M sodium acetate, 0.5% PVP-40 and 0.1% β-ME), kept on ice. Supernatant was processed further by adding 0.1% (w/v) N-lauroyl sarcosine and incubating the mixture at 70 °C for 10 min. Mixture was allowed to cool to room temperature and was transferred to Qiagen shredder column and RNA was isolated according to manufacturer's instructions (Qiagen). Quantity of RNA required for reverse transcription was optimized using various concentrations (ranging from 0.2 to 2 μg).

The primers were designed from the available sequences in the NCBI GenBank. Oligocalc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used for the calculation of annealing temperatures of the various primer combinations. Forward primers span the 5′ ends of the viral genomes and 3′ for reverse primers. The primer names, oligonucleotide sequences and their annealing temperature are given in Table 1.

For first strand cDNA synthesis, both AMV (0.2 unit/reaction; Protoscript AMV Long Amplification system, New England Biolabs, Ipswich, MA, USA) and M-MLV reverse transcriptase (200 units/reaction; Superscript III, Invitrogen, Carlsbad, CA, USA) enzymes were used separately in a 20 μl reaction. Aliquots of RNA (varying concentrations) and 1 μl (10 pmol) of gene specific primer or 2 μl of oligo-dT primer were incubated at 70 °C for 5 min. Other reaction components (5× RT buffer, 40 mM dNTP mix and RTase enzyme) were then added according to manufacturer's recommendation and two different temperatures were tried (42 and 50 °C), followed by inactivation of RT enzyme at 75 °C for 10 min. Long PCR reactions were performed in a total volume of 50 μl according to standard protocol (Protoscript AMV Long Amplification system: PS, New England Biolabs and Long Accurate *Taq*: LA, TakaraBio, Otsu, Shiga, Japan). For the optimization of LD PCR, parameters such as annealing temperature, time and amount of cDNA were also evaluated. The annealing time was tested from 10 to 30 s and best results were obtained at 30 s. PCR was performed in two different cycles as follows: (1) a 30 s pre-denaturation step; (2) first 8 cycles of denaturation at 94 °C for 20 s, annealing (−2 °C of A/T used for next 27 cycles) for 30 s and extension at 68 °C for 1 kb/min (as per the size of the amplicon); (3) 27 cycles of denaturation at 94 °C for 20 s, annealing (−2 °C of the temperature obtained for the forward primer in Oligocalc at salt adjusted conditions) for 30 s and extension at 68 °C for 1 kb/min; and (4) a final extension at 72 °C for 10 min. PCR products were analyzed on 1% agarose gel (Ultrapure® agarose gel, Invitrogen) and visualized under UV light after staining with ethidium bromide (0.5 μg/ml). Sizes of the fragments were determined using Gel Doc (Syngene G: Box, Cambridge, UK) by comparison with 1 kb DNA ladder (ThermoScientific, Pittsburgh, USA). The correct size bands were excised and DNA was gel purified using Nucleospin® Gel and PCR purification kit (Macherey Nagel, Düren, Germany) as per manufacturer's instructions, instead that the elution was done twice, *i.e.* using 30 μl MilliQ water once and the same elutant was passed through the column again, to maximize the eluted product concentration. Quality of the gel eluted product was better when isolating from Ultrapure® agarose in comparison to others (data not shown) as depicted by the A_{260}/A_{230} ratio which ranged 1.5–2.0. Ultrapure agarose was an absolute requirement for efficient elution and ligation of large size amplicons.

Ligation reactions were performed after quantifying the eluted product on Nanodrop (Nanodrop 2000, ThermoScientific). Gel eluted products were cloned into pGEM-T easy vector (50 ng; Promega, Wisconsin, USA) before which the calculations were done using Biomath calculator (http://www.promega.com/techserv/tools/biomath/calc06.htm). Molar ratio of 1:3 (vector: insert) was used. Routine transformation was done using *Escherichia coli* JM109 competent cells, prepared according to protocol as described (Inoue et al., 1990). Cells were plated on LB agar-amp plates (Himedia, Mumbai, India) containing 100 g/ml ampicillin and incubated overnight at 37 °C. LB medium was used to grow *E. coli* harboring recombinant DNAs for the plasmid DNA isolation (Holmes and Quigley, 1981). Recombinant clones were end sequenced using T7 and SP6 primers.

For generating infectious transcripts, PCR was carried out using same conditions as mentioned above and a primer pair T7-GV(f)-5′-*TAATACGACTCACTATAGGGCG*AATTAACAGGCTTAATTTCCGCGC-3′ (T7 promoter sequence underlined and italicized) and *Pvu*II-GV (r) 5′-C*CAGCTG*AAACTCTAGACTCTAGAAATTT$(T)_{18}$-3′ with 100 ng of cloned viral DNA to remove the non-viral nucleotides present at the 5′ end of the virus cloned in pGEMT-easy vector. The absence of non-viral nucleotides was confirmed by sequencing. The *in vitro* transcription was carried out in 30 μl reaction consisting of 5 μl (~1.2 μg) of amplified PCR product, 6 μl of Transcription buffer (ThermoScientific), 1.2 mM concentration each of dATP, dCTP, dUTP, 0.05 mM of cap analog (m7G [5′] ppp [5′] G) (Ambion, TX, USA), 1 μl of RNase out (40 U/μl; Promega) and 1.5 μl of T7 RNA polymerase (20 U/μl; ThermoScientific). The transcription reaction was incubated for 15 min at 37 °C, followed by an increase of GTP concentration to 0.5 mM and an another incubation for 2 h at 37 °C.

The transcripts (about 1 μg) were inoculated by gentle rubbing onto carborundum dusted leaves of four week old *C. amaranticolor* plants. Mock (buffer) and wild type virus inoculated *C. amaranticolor* plants were used as negative and positive controls, respectively. The presence of virus was confirmed by RT-PCR using 4F and 4R primer pair (Menzel et al., 2002) and also by northern blot hybridization of the total RNA.