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REGULAR ARTICLE

Extraction and molecular detection of viral dsRNA from different infected plants

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Abstract

Extraction of viral double stranded RNA (dsRNA) from infected plants is helpful in identification of the viruses involved in infection. To date, there have been several methods developed to isolate dsRNA; however, type of the plant and virus is determinative in extraction efficiency. In this study we extracted dsRNA from different woody and herbaceous plants through a modified method which reduces the costs and time of extraction procedure. This method is based on different affinity of nucleic acids for the cellulose CF-11 in1X STE (Sodium chloride Tris EDTA) buffer containing 16 % ethanol. There is no phenol treatment or mini columns used in the isolation procedure. Extracted dsRNAs were identified by ribonuclease treatment and RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction). We have applied the procedure on five different hosts representing *Amaranthaceae*, *Vitaceae*, *Fabaceae* and *Rosaceae* infected with four different viruses representing *Secoviridae* and *Bromoviridae*.

Key words: Double stranded RNA, plant viruses, polymerase chain reaction, viral infection

Introduction

Plant viruses cause significant losses in crops. It is estimated that the global loss of over 1 billion US dollars is due to viral infection in only vineyards (Mckenzie and Pathirana, 2007). Despite the conducted studies relating to grapevine, the necessity to identify and control viruses is still main issue (Bashir et al., 2009; Sokhandan-Bashir et al., 2012). The first step to control the viral diseases is to identify the virus. Considering the stable structure of dsRNA in comparison to RNA molecules, dsRNA extraction is a promising tool to elucidate the viral genome sequence (Al Rwahnih et al., 2011; Espach et al., 2012; Deker and Parker, 2014). Majority of the RNA viruses are producing dsRNA molecules which are the copies of virus genome; these high molecular weight segments can be used in virus identification. To date, several attempts have been done to extract dsRNA from many different plants such as barley (Morris and Dodds, 1979), grapevine (Azzam et al., 1991), sour cherry (Zhang et al., 1998), rice (Okada et al., 2015), potato (Blouin et al., 2016), however the procedure is either time consuming or relatively costly; which sometimes is not affordable for some laboratories or student works without financial support.

There have been reported several methods to extract viral dsRNA since the past fifty years, but the extraction time, tissue amount, plant

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and infected virus and extraction costs have been challenging during the time. In most of the established methods, dsRNA is extracted through a cellulose column using buffers containing different percentage of ethanol (Barber, 1966) and phenol extracted from infected tissues (Morris and Dodds, 1979).

The present study was conducted with an aim to extract *Secoviridae* and *Bromoviridae* dsRNA from different woody and herbaceous plants with emphasis on extraction costs. We have modified Rezaian et al. (1991) method to extract dsRNA. Different virus and infected plants extends the versatility of the isolation procedure. Grapevine (*Vitis vinifera*), globe amaranth (*Gomphrena globosa*), common bean (*Phaseolus vulgaris*), quinoa (*Chenopodium quinoa*) and rose (*Rosa kordessi*) plants were collected from different regions of Iran. To confirm the isolated dsRNA, molecular detection was carried out.

Material and methods

Plant materials

Based on the viral symptoms, infected grapevine, quinoa and rose leaves were collected from different regions of the northwestern provinces of Iran (Table 1). *Cucumber mosaic virus* (CMV)-infected Globe amaranth and *Peanut Stunt virus* (PSV)-infected common bean leaves were obtained through mechanical inoculation in insect-proof greenhouse at 24 ± 2°C without any supplemental light (Table 1). After 14 d the inoculated plants were harvested and stored in a plastic bag at 4°C until use.

Healthy grapevine leaf tissue was used as a negative control. Besides, gradually frozen infected grapevine leaf tissue (not frozen in liquid nitrogen) was applied to investigate the effect of tissue freshness on extracted dsRNA.

DsRNA extraction

DsRNA extraction was carried out by homogenization of 0.5 - 2 g fresh leaf tissue (0.5 g of common bean and quinoa and 2 g of grapevine, rose and globe amaranth) in 2-4 ml lysis buffer (200 mM Tris, 500 mM NaCl, 10 mM $MgCl_2$, 3 % SDS, 10% Ethanol, 1% 2-Mercapto ethanol) and divided in 2 to 4 tubes (1 ml per each 2.0 ml micro centrifuge tube). After a 10 second vigorous vortex, tubes were incubated at 37°C water bath for 10 min. Thereafter, chloroform was added in 1:1 ratio and mixed. Centrifugation was done at 11300 rpm for 20 min. at 4°C. The upper layer was transferred into a new tube and chloroform added in 1:1 ratio and followed by a 7 min centrifugation at 11300 rpm (4°C) (this step could be repeated to remove leftover tissue particles). Upper layer was placed into a new tube; 0.2 ml absolute ethanol was added per each 1 ml of the supernatant, inverted for several times and followed by a 5 min. centrifugation (11300 rpm at 4°C). In order to enmesh the dsRNA molecules, 15 mg CF-11 cellulose (Sigma-Aldrich Corp., Schnelldorf, Germany) was added in each tube and mixed vigorously, and maintained at room temperature for 5 min. After a 7 min. centrifugation at 11300 rpm (4°C), the upper phase was removed and 1 ml washing buffer (1X STE [100 mM NaCl, 10 mM Tris-HCL, 1 mM EDTA, pH 8.0]/16% ethanol) was added and mixed vigorously. Centrifugation for 5 min. separated the pellet and aqueous phases. Transparent phase was removed and washing step repeated. To elute the pellet after removing upper phase, 150 µl 1X STE was added and incubated at room temperature for 5 min. After 11300 rpm centrifugation for 5 min., upper phase was transferred into a fresh tube. Divided samples of the same type were collected in one tube. Absolute ethanol was added in twice the volume of the solution and kept at -20°C for an hour. To precipitate the expected dsRNA, tubes were centrifuged at 11300 rpm for 20 min. at 4°C. Pellet was exposed to dry for 15 min., then dissolved in 30 µl ddH_2O and maintained at -20°C (Fig. 1).

Table 1. Infected plants used as the sources of dsRNAs.

Plant material	Infecting virus	Collected region
Grapevine (*Vitis vinifera*)	*Grapevine fanleaf virus* (GFLV)	Khelejan, East Azerbaijan province, Iran
Globe amaranth (*Gomphrena globosa*)	*Cucumber mosaic virus* (CMV)	inoculated in greenhouse
Common Bean (*Phaseolus vulgaris*)	*Peanut stunt virus* (PSV)	inoculated in greenhouse
Quinoa (*Chenopodium quinoa*)	*Grapevine fanleaf virus* (GFLV)	Mayan, East Azerbaijan province, Iran
Rosa (*Rosa kordessi*)	*Prunus necrotic ring spot virus* (PNRSV)	Khoy, West Azerbaijan province, Iran

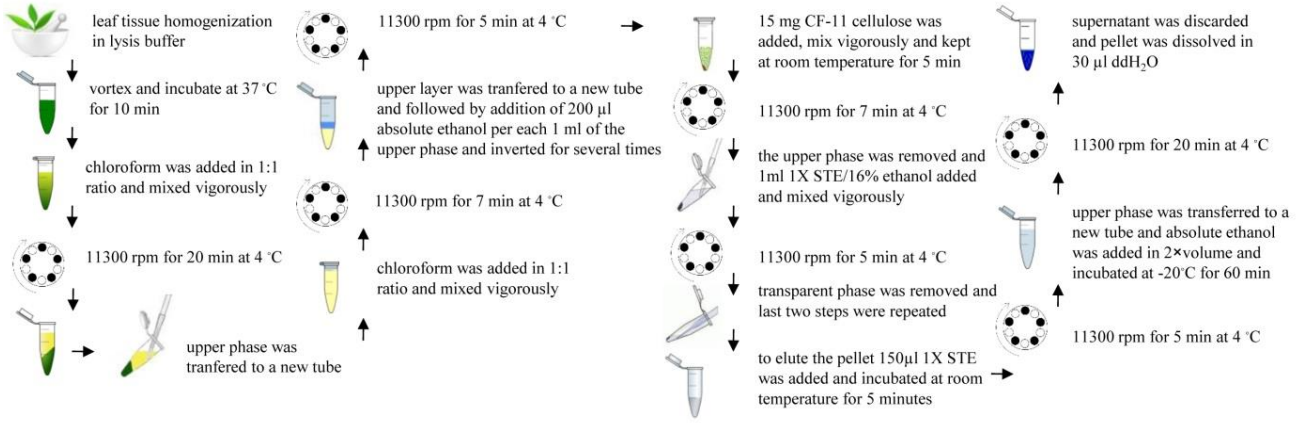

Fig. 1. The schematic diagram showing the procedure of viral dsRNA extraction from infected leaves of grapevine, globe amaranth, common bean, quinoa and rose plants.

DNaseI and RNaseA treatment

To confirm the extracted dsRNA and eliminate the possible host DNA and ssRNA (single stranded RNA) contamination, samples were subjected to DNaseI (Sigma-Aldrich Corp., St. Louis, USA) treatment in presence of 30 mM $MgCl_2$, and RNaseA (Thermo Fisher Scientific Inc., Waltham, USA) treatment in presence of 30 mM NaCl. To perform the reaction, 1µl DNaseI and 1µl RNaseA were added separately to 10 µl of dsRNA samples and incubated for 30 min at 30°C and terminated by incubation at 75°C for 10 min.; control reactions without any enzyme treatment were set as well. Ten microliter of the enzyme treated dsRNA was electrophoresed on 1.6 % agarose gel (1X TBE buffer) stained with ethidium bromide and visualized by ultraviolet (UV) light. Lambda DNA-*Eco*RI plus *Hind*III size marker (Thermo Fisher Scientific Inc., Waltham, USA) was used to identify the desired bands.

Virus detection through RT-PCR

RT-PCR was performed by using coat protein specific primers to confirm the identity of extracted viral dsRNA (Table 2). To perform cDNA synthesis, 5 µl dsRNA sample plus 10 pmol reverse primer was adjusted to the total volume of 12.5 µl and preheated at 80°C for 5 min and then chilled on ice and briefly centrifuged. It was added to the prepared solution comprised of 200 unit Revert Aid Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, USA), accompanied with 1X RT buffer, 20 unit RNase inhibitor and 1 mM dNTPs; then incubated at 42°C for 60 min and terminated by 10 min incubation at 70°C.

PCR amplification of first strand cDNA was performed by using one unit of *Taq* DNA Polymerase (SinaClon Co., Tehran, Iran) in a total reaction mixture of 20 µl containing 1X reaction buffer, 1.5 mM $MgCl_2$, 1 mM dNTPs and 10 pmol of each primer. The optimized reaction conditions were as follows: initial denaturation at 94°C for 1 minute followed by 30 cycles of denaturation at 94°C for 40 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 40 seconds and a final elongation step at 72°C for 7 minutes.

Table 2. Coat protein specific primers used in amplification reactions.

Infected virus	Coat protein specific primer	Expected product length (bp)
Grapevine fanleafvirus[1]	F: 5′- GAACTGGCAAGCTGTCGTAGAA-3′ R: 5′- GCTCATGTCTCTCTGACTTTGACC-3′	350
Cucumber mosaic virus[2]	F: 5′- GCTTCTCCGCGAG-3′ R: 5′- GCCGTAAGCTGGATGGAC-3′	867
Peanut stunt virus[3]	F: 5′-CGGATCCGATGGCATCTAGATCTGGTAACGG-3′ R: 5′- CAGTCGACGACCGGGAGCTTGGAAGC-3′	670
Prunus necrotic ring spot virus[4]	F: 5′- GCTCTAGACTAGATCTCAAGCAGGTC-3′ R: 5′- AACTGCAGATGGTTTGCCGAATTTGCAA-3′	670

1. Izadpanah et al., 2003; 2. Rizos et al., 1992; 3&4. our unpublished study

Results

Using the modified method, we successfully extracted viral dsRNA from *Chenopodium quinoa* (Fig. 2. a4), *Rosa kordessi* (Fig. 2. a5), *Vitis vinifera* (Fig. 2. b7), *Gomphrena globosa* (Fig. 2. c11, 12) and *Phaseolus vulgaris* (Fig. 2. c13). To confirm the double stranded RNA structure of the extracted molecules they were subjected to DNaseI treatment; according to the results dsRNA bands still existed after DNase I treatment which confirmed the RNA structure of the isolated molecules (Fig. 2. b8). However, RNaseA treatment in 30 mM NaCl led to total degradation which confirmed the extraction as well (Fig. 2. b9). There was no any dsRNA observed at negative control (healthy grapevine) extraction (Fig. 2. a2). There was no dsRNA extracted from frozen leaves of grapevine (Fig. 2. a3). These leaves were directly stored at -20 °C after collecting without deep freeze in liquid nitrogen. The virus (GFLV) was not stable in leaf tissue during couple of days maintenance at -20 °C while it was not deep frozen prior to stock.

According to the RT-PCR results, dsRNA extracted from PNRSV infected-rose (Fig. 3. a2), PSV infected-common bean (Fig. 3. a3), GFLV infected-quinoa (Fig. 3. b5), GFLV infected-grapevine (Fig. 3. b6), CMV infected-globe amaranth (Fig. 3. b7) were confirmed through virus coat protein sequence amplification which verified the accuracy of extraction procedure.

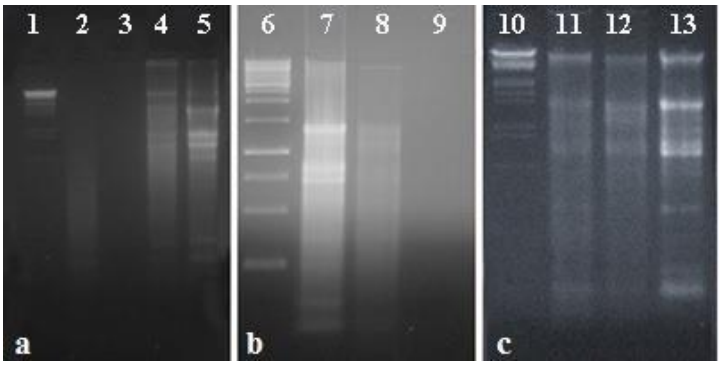

Fig. 2. Agarose gel electrophoresis (1.6%) of extracted viral dsRNA from: frozen leaf of GFLV-infected grapevine (a2) and fresh leaf of healthy grapevine(a3), GFLV-infected *Chenopodium quinoa*(a4), PNRSV-infected *Rosa kordessi*(a5), GFLV-infected *Vitis vinifera*(b7), CMV-infected *Gomphrena globosa*(c11 &c12), PSV-infected *Phasolus vulgaris*(c13 Extracted dsRNA molecules were subjected to DNase I (b8) and RNase A in presence of 30 mM NaCl (b9) to confirm the RNA structure of the extracted molecules. Lambda DNA-*Eco*RI plus *Hind*III size marker (a1 & c10), Gene Ruler 1kb DNA Ladder size marker (b6) (Thermo Fisher Scientific Inc., Waltham, USA) were used to trace the desired bands.

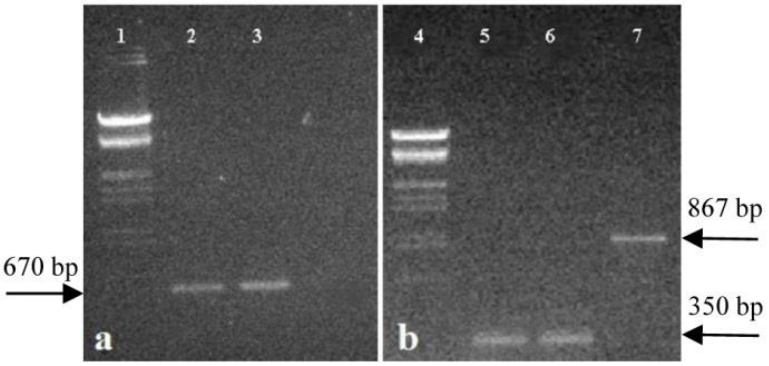

Fig. 3. Agarose gel (1.2%) electrophoresis of RT-PCR products; PNRSV infected-rose (a2), PSV infected-common bean (a3), GFLV infected-quinoa (b5), GFLV infected-grapevine (b6), CMV infected-globe amaranth (b7). DNA-*Eco*RI plus *Hind*III size marker was used to trace the desired bands (a1, b4).

Discussion

Majority of the RNA viruses produce dsRNA molecules which are the copies of virus genome; therefore, dsRNA extraction analysis can be considered as a promising tool in virus identification. There have been several studies since the first reports of dsRNA extraction to improve the procedure (Morris and Dodds, 1979; Morris et al., 1983; Rezaian et al., 1991). Developing more effective and practical extraction method is necessary to enmesh the low quantity dsRNA molecules. Most of the researches were almost to reduce the complexity, time and costs imposed by extraction procedure. There have been significant improvements during the time (Balijja et al., 2008; Okada et al., 2015; Blouin et al., 2016; Khankhum et al., 2017); In order to use the isolated dsRNA in downstream reactions, it should be in a sufficient quantity. According to the studies, plant hosts could be one of the main factors interfering with dsRNA extraction (Tzanetakis et al., 2008). High quantity of tannins, phenolic compounds and polysaccharides in some plants is problematic as interferes with nucleic acid extraction (Loomis, 1974). To facilitate the extraction in such recalcitrant plants improved methods have been established (Rezaian e al., 1991); however some of the protocols have been time consuming and labor-intensive (Speiegel, 1987; Li et al., 2007).

In a primary study, relatively large amount of plant tissue was used to extract dsRNA (Speiegel, 1987). In that study, 50 g of fresh leaves were used to extract dsRNA; this amount reduced by five times (10g) in another study (Tzanetakis and Martin, 2008). Large amounts of plant tissue could be restrictive when the access to infected tissue is limited. In a recent study, Blouin et al., (2016) decreased down the amount to 0.1 g but the related costs through their method may not be affordable for some laboratories with limited financial resources. To enrich dsRNA from infected plant extracts, they used anti-dsRNA monoclonal antibodies. Available dsRNA extraction kits (ABC Scientific, Inc., CA, USA) have facilitated the extraction procedure. By using little amounts of tissue (0.05-0.3 g) dsRNA could be extracted in a short time however the extra charge is imposed.

Okada et al., (2015) used self-prepared cellulose powder based micro-spin columns to purify dsRNA molecules that effectively reduced the expenses and extraction time. They performed the extraction on a limited type of plants which could be challengeable. Host extract compounds could mal-effect the dsRNA extraction procedure. In woody plants such as grapevine virus purification could be hampered by phenolic compounds which inhibits the dsRNA extraction (Loomis, 1974). Unlike most of the protocols in which fresh or frozen leaf tissues were employed, Khankhum et al., (2017) used desiccated foliar tissues and seeds to extract dsRNA.

Unlike majority of the methods which require relatively large amount of plant tissue, we isolated dsRNA from 0.5 - 2 g fresh leaf tissue which makes it possible to do the extraction from less amounts of tissue, especially it is important when our access to infected plant is restricted. Total procedure completes in less than 3 hours. Moreover, to avoid extra costs extraction kits and some reagents utilized in already established methods, such as liquid nitrogen, phenol, PVPP and bentonite are not used during the extraction procedure. In this study we did not use any chromatographic columns or ultracentrifuge which may considered as a limitation in access for some laboratories with limited financial resources.

In this study, we modified the Rezaian et al., (1991) method to accommodate the need for dsRNA extraction from some challenging virus/plant combination. Since plant host and virus are one of the main factors interfering with ribonucleic acid extraction, the presented combination of virus/host will be helpful in future dsRNA studies. The combination is representing *Amaranthaceae*, *Vitaceae*, *Fabaceae* and *Rosaceae* plants infected with *Secoviridae* and *Bromoviridae* viruses.

Conclusions

We applied the mentioned modified method with the aim of reduction in extraction time and plant material with emphasis on extraction costs. Appling the method on different infected woody/herbal plants extends the versatility of the procedure for future studies in related fields.

Authors' Contributions

A.D. Khabbazi conducted the study and prepared the manuscript. N.S. Bashir supervised all the research activities and

criticized the manuscript. S.D. Khabbazi contributed to molecular assays and manuscript preparation. H. Ighani contributed to molecular assays.

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