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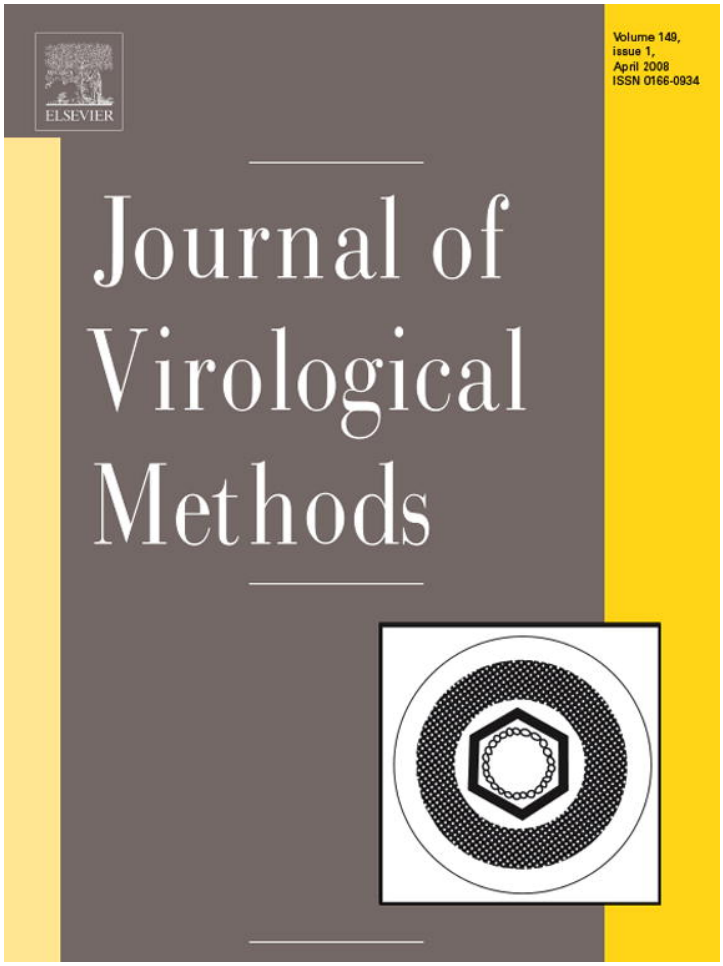

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Short communication

A new method for extraction of double-stranded RNA from plants

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Abstract

The occurrence of high molecular weight double-stranded RNA (dsRNA) in plants is associated with the presence of RNA viruses. DsRNA is stable, can be extracted easily from the majority of plant species and provides an excellent tool for characterization of novel viruses that are recalcitrant to purification. Several protocols have been developed for dsRNA purification, the majority of which are based on extraction with phenol and chloroform. We have developed a protocol for dsRNA extraction based on a lithium salts buffer that does not require organic solvents other than alcohols. The method yields comparable amount of dsRNA to protocols described previously and yields consistently dsRNA from *Vaccinium* hosts that have been recalcitrant to dsRNA purification using traditional protocols. The quality of the dsRNA purified is such that it can be used for downstream enzymatic reactions including reverse transcription-polymerase chain reaction and cloning.
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Double-stranded RNA (dsRNA) forms during replication of positive- and ambisense-RNA viruses. The thermodynamic stability of the dsRNA molecule and its resistance to RNAse degradation makes it an excellent matrix for virus characterization when virus purification is not feasible due to low titer, instability of particles or presence of inhibitors. DsRNA is an excellent alternative to purified particles for virus characterization for most plant species. However, attempts to extract dsRNA from blueberry plants infected with *Blueberry scorch virus* (BlScV; genus *Carlavirus*) or *Blueberry shock virus* (BlShV; genus *Ilarvirus*) were rarely successful, although we could extract significant amounts of dsRNA from other hosts infected with carlaviruses and ilarviruses (Kraus et al., 2008; Tzanetakis et al., 2004b; Tzanetakis and Martin, 2005a). This led to the hypothesis that host factors in blueberry interfere with dsRNA extraction, since over the last 5 years we have cloned more than 30 virus species from dsRNA templates purified from several hosts including *Rubus*, *Fragaria*, *Pisum*, *Mentha*, *Solanum*, *Rosa*, etc. without significant problems.

Since the development of the first protocol for purification of dsRNA from plants by Morris and Dodds (1979), several other methods have been developed to optimize yield or accommodate the needs of a particular virus/host combination (Bar-Joseph et al., 1983; Choi and Randles, 1997; Valverde et al., 1990; Yoshikawa and Converse, 1990). Multiple modifications of some of these protocols to improve dsRNA extraction from blueberry were unsuccessful. Another protocol (Benthack et al., 2005), that does not use organic solvents in the initial steps of extraction that may interfere with the extraction process from blueberry was unsuitable for dsRNA extraction from small fruit crops.

A modification of the Hughes and Galau (1988) total nucleic acids extraction protocol was used for molecular detection of blueberry viruses (Martin et al., 2006). This protocol was further modified to facilitate isolation of dsRNA from blueberry and several other hosts (Fig. 1). The new method and the method of Yoshikawa and Converse (1990) were compared by extracting dsRNA from plants infected with the following viruses: (1) *Pea enation mosaic virus-1* and *Pea enation mosaic virus-2* from *Pisum sativum* 'Early Freezer 680' (Fig. 2A); (2) Mint virus

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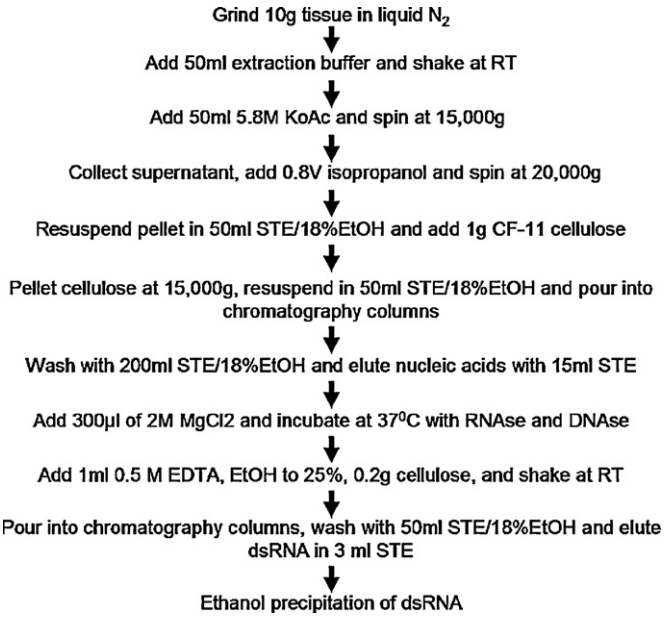

Fig. 1. Outline of the novel protocol for double-stranded RNA extraction from plants.

X, Mint vein banding associated virus and *Strawberry latent ringspot virus* from *Mentha* × *gracilis* (Fig. 2B); (3) *Fragaria chiloensis latent virus* and Fragaria chiloensis cryptic virus from *Fragaria chiloensis* (Fig. 2C); (4) Blueberry fruit drop associated virus (BFDaV) and BlShV from *Vaccinium corymbosum* (Fig. 2D); (5) *Beet pseudo-yellows virus*, *Strawberry mild yellow edge virus*, Strawberry necrotic shock virus and Strawberry pallidosis associated virus from *Fragaria* × *anannasa* (data not shown).

For the novel dsRNA extraction protocol 10 g of leaf tissue was pulverized to fine powder with liquid N_2 using a mortar and pestle. The tissue was transferred to a GSA bottle and 50 ml of extraction buffer (200 mM Tris–Cl, pH 8.5, 300 mM lithium chloride, 10 mM EDTA, 2% polyvinyl pyrrolidone MW 40,000, 1.5% lithium dodecyl sulfate, 1% deoxycholic acid, 1% NP 40 "Tergitol" and 1% β-mercaptoethanol) were added. The slurry was agitated on a horizontal shaker at 400 rpm for 20 min. Fifty milliliters of 5.8 M potassium acetate (4 M potassium, 5.8 M acetate) were added to the homogenate and centrifuged in a Sorvall RC5 Plus® centrifuge using the SLC-1500 rotor at 15,000 × *g* for 10 min. The supernatant was collected after filtration through miracloth and 0.8 vol of isopropanol was added. Centrifugation followed at 20,000 × *g* for 20 min. The supernatant was discarded and the pellet was resuspended in 50 ml of STE/18%EtOH (18% ethanol, vol/vol, in 50 mM Tris–Cl, 100 mM NaCl, 1 mM EDTA, pH 7.1). After the addition of 1 g of CF-11 cellulose (Whatman) to bind the nucleic acids, the homogenate was agitated on a horizontal shaker at 400 rpm for 30 min. The cellulose was pelleted by centrifugation at 15,000 × *g* for 5 min and resuspended in 50 ml STE/18%EtOH. The previous step was repeated and the resuspended pellet was poured into a 2.5-cm diameter column fitted with a fritted glass filter, where it was allowed to drain then washed twice with 100 ml of STE/18%EtOH. After the final wash the column was allowed to drain completely before adding 15 ml of STE to elute the nucleic acids. DNA and ssRNA digestion was accomplished by addition of 30 U of DNAse I (Sigma), 1000 U RNAse T1 (Sigma) and 300 µl of 2 M $MgCl_2$. The digestion was incubated for 1 h at 37 °C. The reaction was terminated by addition of 1 ml 0.5 M EDTA, pH 8.0, and 5 ml of 100% EtOH. After the addition

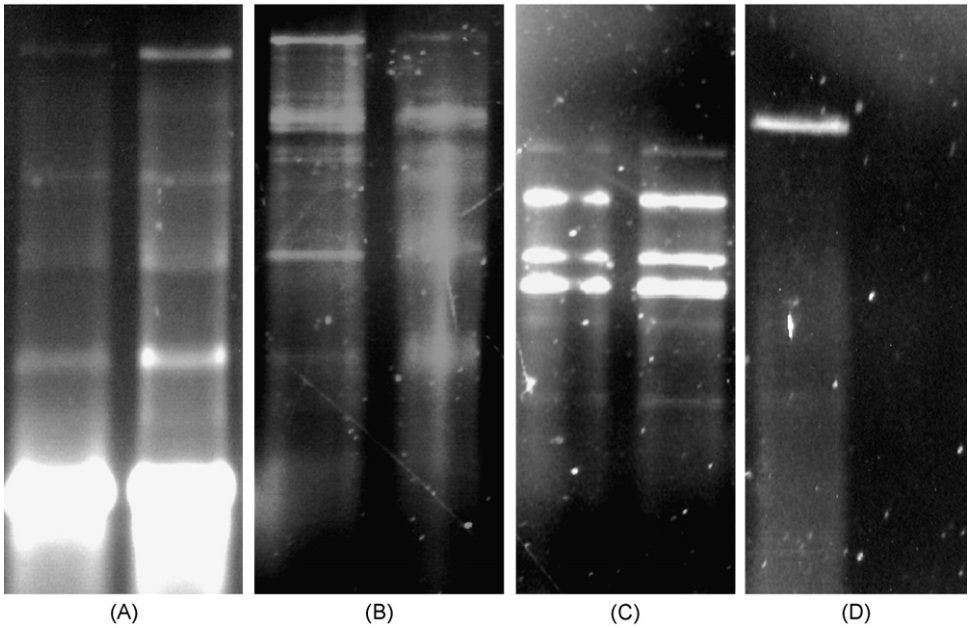

Fig. 2. Double-stranded RNA purified using the novel (left) or the Yoshikawa and Converse (1990) (right) extraction protocols. Amounts have been normalized to represent dsRNA extracted from the same amount of tissue. (A) *Pisum sativum* infected with *Pea enation mosaic virus-1* and *Pea enation mosaic virus-2*; (B) *Mentha* × *gracilis* infected with Mint virus X, Mint vein banding associated virus and *Strawberry latent ringspot virus*; (C) *Fragaria chiloensis* infected with *Fragaria chiloensis latent virus* and Fragaria chiloensis cryptic virus; (D) *Vaccinium corymbosum* infected with Blueberry fruit drop associated virus.

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Table 1
Primer sequence used to evaluate quality of dsRNA and presence of inhibitors in RT-PCR

Virus[a]	Primer	Nucleotide sequence (5′–3′)	Length (b)	Reference
BFDaV	BFDaVF	CTGAGGCGGTGAAGCATTATAG	1265	Martin et al. (2006)
	BFDaVR	GCCCGTCTGTATGCTCCTAACAA		
BlShV	BlShVF	TTCCGATAATGGAAACTMAACC	416	Keller K.E. (unpublished)
	BlShVR	ATTACAGWACTTGCARACCATG		
FClLV	FClLVF	ACCACTTCACCACCAGATCG	350	Tzanetakis and Martin (2005a)
	FClLVR	CAAGCCAACTCACCATGACC		
MVX	MVXF	GGCAATGGCACTGTCA	332	Tzanetakis et al. (2006)
	MVXR	GGCGTTCAGATA GTAGCG		
PEMV-2	PEMV-2F	GTHGCYAAGGGRTTYAAYGC	385	Keller K.E. (unpublished)
	PEMV-2R	ACRATGCARTCRTCWCCRTTGT		
SPaV	SPaVF	GTGTCCAGTTATGCTAGTC	517	Tzanetakis et al. (2004a)
	SPaVR	TAGCTGACTCATCAATAGTG		

[a] Virus acronyms: BFDaV = Blueberry fruit drop associated virus; BlShV = *Blueberry shock virus*; FClLV = *Fragaria chiloensis latent virus*; MVX = Mint virus X; PEMV-2 = *Pea enation mosaic virus-2*; SPaV: Strawberry pallidosis associated virus. Bases: H = A/T/C, M = A/C, R = A/G, W = A/T, Y = C/T.

of 0.2 g of CF-11 cellulose to bind the dsRNA, the homogenate was placed on a horizontal shaker at 400 rpm for 30 min and was then poured into a 1-cm diameter column. After draining, the samples were washed twice with 25 ml STE/18%EtOH. Three milliliters of STE were added to the drained column to elute the dsRNA. The collected dsRNA was precipitated with 2.5 volumes of EtOH and separated on a 1% agarose gel stained with 100 ng/ml ethidium bromide, then visualized using UV light.

The protocols of Benthack et al. (2005), Choi and Randles (1997) and Yoshikawa and Converse (1990) were performed as originally described to evaluate their efficiency to isolate dsRNA from blueberry. The protocol of Yoshikawa and Converse (1990) was modified sequentially by: (1) increasing the molarity of the extraction buffer two or fivefold of that of the original protocol; (2) elevating the pH of the extraction buffer to 9.5; (3) increasing the EtOH content used to bind nucleic acids to the cellulose matrix from 18% to 25% or 50%; (4) eliminating the nuclease digestions; (5) eliminating several of the STE/18%EtOH washes to determine if any of these would reverse the interference observed when extracting dsRNA from blueberry. This protocol in its original form was used in parallel experiments with the novel protocol to compare dsRNA yields (Fig. 2).

Cloning of dsRNA templates extracted from blueberry or strawberry was performed using the reverse transcriptase-based protocol described by Tzanetakis et al. (2005b) or DOP-PCR (Telenius et al., 1992). cDNA obtained in the first step of the Tzanetakis et al. (2005b) protocol was used in the RT-PCR experiments. One microliter of a 100 μl RT reaction that had dsRNA extracted from the equivalent of 1 g of tissue as template, was added to a 25 μl PCR reaction using Taq polymerase (GenScript, NJ) according to manufacturer's instructions, and primers at 0.4 μM each (Table 1). The reaction, performed on a Stratagene Robocycler, started with 3 min denaturation at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30–90 s at 72 °C depending on the amplicon size, followed by a final incubation at 72 °C for 10 min. The amplicons were visualized after separation on a 2% agarose gel stained with 100 ng/ml ethidium bromide.

The use of dsRNA for identification of new blueberry viruses has been very inconsistent. More than 20 extractions were performed before the isolation of a small amount of dsRNA from BlScV-infected blueberry plants (Martin, personal observation) and more than 50 trials were carried out before isolating dsRNA from BFDaV-infected plants (Martin et al., 2006). DsRNA yield was not improved employing the protocols of Benthack et al. (2005), Choi and Randles (1997), Yoshikawa and Converse (1990) or several variations on the latter. The Benthack et al. (2005) and Choi and Randles (1997) protocols were found inappropriate for *Rubus* and *Fragaria* as well, since the final dsRNA products were quite viscous and difficult to pipette. Using blueberry tissue, there was no tRNA or DNA observed in the agarose gels with all published protocols tested, even when the RNAse and DNAse digestions were excluded. This suggests that blueberry contains products that prevent binding of nucleic acids to the cellulose powder or that once bound they are not eluted. A similar phenomenon was observed when trying to use glass milk to purify nucleic acids from blueberry.

This inconsistency of isolation of dsRNA from blueberry can be overcome by the new protocol. More than 15 extractions have been carried out using tissue infected with BFDaV with 100% success in isolation of viral dsRNA. The dsRNA yield of the

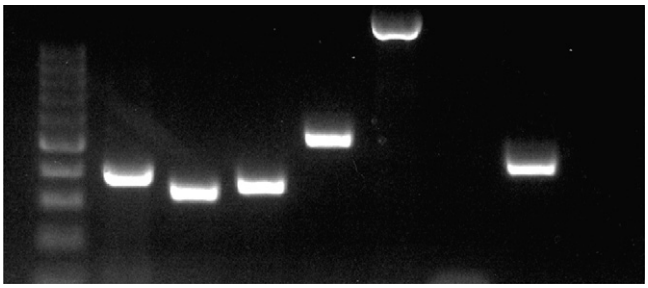

Fig. 3. RT-PCR amplification of double-stranded RNA templates purified with the novel protocol to confirm utility in enzymatic reactions. Lane 1: 100 bp ladder (NEB); lane 2: *Pea enation mosaic virus-2* isolated from *Pisum sativum*; lane 3: Mint virus X isolated from *Mentha* × *gracilis*; lane 4: *Fragaria chiloensis latent virus* isolated from *Fragaria chiloensis*; lane 5: Strawberry pallidosis associated virus isolated from *Fragaria* × *anannasa*; lanes 6–7: Blueberry fruit drop associated virus isolated from *V. corymbosum* using either the novel (lane 6) or the Yoshikawa and Converse (1990) protocols (lane 7); lanes 8–9: *Blueberry shock virus* isolated from *V. corymbosum* using either the novel (lane 8) or the Yoshikawa and Converse protocols (lane 9).

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new protocol is comparable to that of the Yoshikawa and Converse method on the other hosts tested (Fig. 2). The new protocol eliminates the problems with polysaccharides encountered with several hosts and some of the protocols previously developed. The quality of dsRNA, a major concern for enzymatic reactions when extracting nucleic acids from small fruit crops, was evaluated by using it as templates for shotgun cloning using two cloning protocols and RT-PCR. The quality was such that it did not inhibit enzymatic reactions involved in cloning as verified by shotgun cloning from *Fragaria* and *Vaccinium* and amplification of six different virus species obtained from five different plant species (Fig. 3). Furthermore, this protocol, unlike the majority of the dsRNA extraction protocols described previously does not use any of the corrosive organic solvents such as phenol and chloroform that can be harmful in the laboratory and the environment.

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