DETECTION OF *STRAWBERRY MOTTLE VIRUS* (SMoV) USING RT-PCR – COMPARISON OF TWO RNA EXTRACTION METHODS

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A B S T R A C T

RNA extraction is difficult in plants that contain large amounts of polysaccharides and polyphenolic compounds. Usual protocols for RNA isolation are tedious and result in poor yields with fruit trees and small fruits. Two simple methods were used to extract RNA from leaves of *Fragaria virginiana* UC-11 infected with the *Strawberry mottle virus* (SMoV): the lithium method (homogenization in buffered lithium chloride), and the silica capture method. Sensitivity of virus detection was evaluated using five dilutions (1:50, 1:100, 1:200, 1:400, 1:800) of 1 µl of the total extracted RNA. The lithium method was more efficient in recovering viral RNA than the silica capture method. Following RT and PCR amplification of RNA extracted by the lithium method, specific ethidium bromide stained DNA bands were visible with dilutions up to 1:400. With the silica capture method, RNA of SMoV was detected only in undiluted samples, and no products of RT-PCR were visible on agarose gel.

Key words: *Fragaria virginiana, Strawberry mottle virus,* RNA isolation, RT-PCR

INTRODUCTION

The *Strawberry mottle virus* (SMoV) is one of the most common viruses of cultivated strawberry plants and occurs wherever they are grown. Many strains of SMoV infect strawberry plants asymptomatically, however virulent strains may reduce vigor and yield by up to 30% (Mellor and Krczal, 1987; Martin and Spiegel, 1998). Controlling aphid-borne viruses in strawberries is difficult and currently relies completely on the use of virus-free planting material and protection against the aphid vectors.

Rapid and simple detection methods of these viruses were not available until quite recently. Due to problems with their purification from infected plant

material it had been difficult to develop specific antisera (Adams and Barbara, 1986; Leone et al., 1992). Like other major aphid-borne strawberry viruses, SMoV is currently detected by the time-consuming leaf-graft bioassay on indicator plants. Recent studies on the molecular characterisation of SMoV have made possible new detection methods (Thompson et al., 2002). Polymerase chain reaction (PCR) is rapid and reliable method for detecting plant pathogens (Saiki et al., 1988). Reverse transcription PCR (RT-PCR) has been successfully used to detect SMoV (Thompson and Jelkmann, 2003). RT-PCR can be used by certification and quarantine services to screen strawberry plants on a large scale.

Effective detection of the virus depends on the number of viral particles present in infected plants. Their low concentration can give false negative results. The extraction method used determines the quantity of high-quality viral RNA recovered. The aim of this study was to evaluate two methods for extracting SMoV RNA in terms of yield and quality of the RNA.

MATERIAL AND METHODS

Plant material

SMoV was isolated from three infected strawberry plants: 1248 clone, 'Geneva' and 'Totem'. Isolates were maintained in *Fragaria virginiana* UC-11 indicator plants grown in the greenhouse. In the indicator plants, the isolates induced symptoms characteristic for SMoV. RT-PCR and ELISA confirmed the presence of SMoV and excluded infection by other viruses.

Methods of RNA extraction

Nucleic acids, included viral RNA, were extracted from plant material using either the lithium method or the silica capture method described below.

Lithium method

Solutions

Lithium buffer: 200 mM Tris-Cl, pH 8.5, 1.5% lithium dodecylsulphate, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% tergitol NP-40 and 1% 2-mercaptoethanol (Hughes and Galau, 1988; Spiegel and Martin, 1993).

Procedure

0.1 g frozen plant material was ground in 2.5 volumes of lithium buffer and transferred to a 1.5 ml Eppendorf tube. An equal volume of 6 M potassium acetate, pH 6.5 was added, and the mixture was centrifuged at 14,000 rpm for 10 min. To 0.5 ml of the supernatant, 0.5 ml of isopropanol was added to precipitate nucleic acids, and the mixture was centrifuged. The pellet was washed with 0.5 ml of 70% ethanol, dried, and resuspended in sterile distilled water. The extract was stored at – 20°C until needed.

Silica capture method

Solutions

SEB buffer: 0.14 M NaCl, 2 mM KCl, 2 mM KH_2PO_4, 8 mM $Na_2HPO_4.2H_20$ (pH 7.4), 0.05% v/v

Tween 20, 2% w/v polyvinyl-pyrrolidone 40, 0.2% w/v ovalbumin, 0.5% w/v bovine serum albumin, 0.05% w/v sodium azide (Boom et al., 1990; Thompson and Jelkmann, 2003).

Grinding buffer: 4.0 M guanidine thiocyanate, 0.2 M NaOAc pH 5.2, 25 mM EDTA, 1.0 M KOAc and 2.5% w/v polyvinylpyrrolidone 40.

NaI solution: dissolve 0.75 g Na_2S0_3 in 40 ml water and then add 36 g NaI.

Wash buffer: 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 50 mM NaCl; 50% ethanol

Procedure

0.1 g frozen plant material was ground in 1.0 ml SEB-buffer and transferred to a 1.5 ml Eppendorf tube. 100 µl 10% N-lauryl sarcosyl and 5 µl 2-mercaptoethanol were added. To 100 µl of this mixture, 400 µl grinding buffer was added. The mixture was incubated at 70°C with intermittent shaking for 10 min, placed on ice for 5 min, and centrifuged at 13,000 rpm for 10 min. To 300 µl of the supernatant, 150 µl l EtOH, 300 µl 6 M NaI solution, and 25 µl resuspended silica were added. The mixture was incubated at room temperature and centrifuged. The pellet was resuspended in 500 µl wash buffer. After drying, the pellet was resuspended in sterile distilled water, incubated at 70°C for 5 min, and centrifuged at 13,000 rpm for 3 min. The extract was transferred to a new tube and stored at -20°C until needed.

Effectiveness of the RNA extraction methods

RNA extracted with both methods was measured with a spectrophotometer (GeneQuant *pro*, Amersham). A 260/280 and, in some cases, A 260/230 correlate with the purity of the sample and its suitability for RNA amplification. RNA concentration (ng/µl) was also determined.

RT-PCR

Viral RNA was diluted 1:50, 1:100, 1:200, 1:400, 1:800 and amplified using SuperScript™One-Step RT-PCR with Platinum®Taq (Invitrogen) and specific primer pair Smdetncr4a/Sm2ncr1b (Thompson and Jelkmann, 2003). The expected size of RT-PCR products was 460 bp. RT was conducted at 45°C for 30 min. PCR reactions were carried out for 35 cycles using the following protocol: 60 s denaturation at 94°C, 60 s annealing at 50°C and 60 s primer extension (5 min in final cycle) at 72°C. Amplification products were separated by electrophoresis in a 1% agarose gel. DNA was visualized under UV light after staining with ethidium bromide.

RESULTS AND DISCUSSION

Purity of nucleic acid extracts and concentration of viral RNA were higher with the lithium method than with silica capture. A 260/280 ranged from 1.5 to 1.9 with the lithium method, and from 3.4 to 4.2 with the silica capture method (Tab. 1). Viral RNA concentration ranged from 168

Table 1. Spectrophotometric estimation of purity and concentration of SMoV RNA extracted with the lithium method and the silica capture method from three plants of *Fragaria virginiana* UC-11

Plant	A 260/A 230		A 260/A 280		RNA concentration [ng/µl]	
	lithium method	silica capture	lithium method	silica capture	lithium method	silica capture
1248 clone	0.70	0.012	1.91	3.52	180.0	144.0
'Geneva'	0.59	0.008	1.50	4.23	220.0	124.0
'Totem'	0.42	0.010	1.48	3.41	168.0	100.0

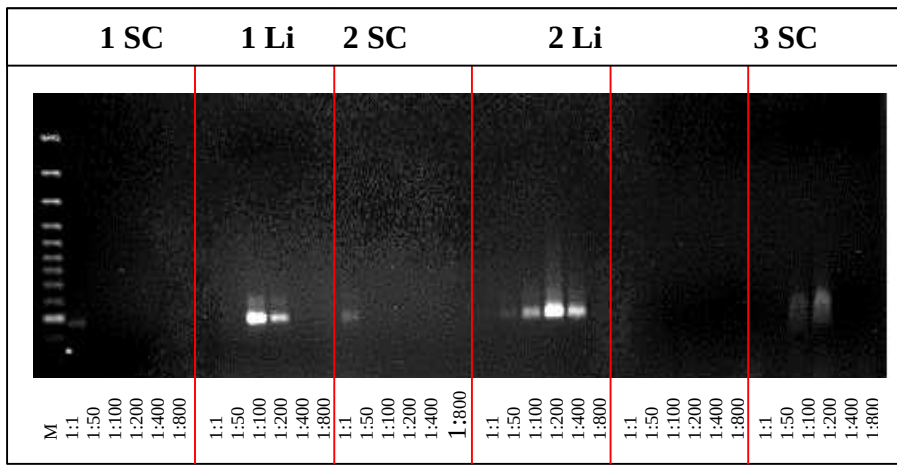

Figure 1. Agarose gel electrophoresis of RT-PCR products from three plants of *Fragaria virginiana* UC-11 infected with SMoV: 1 – 1248 clone; 2 – 'Geneva'; 3 – 'Totem'. Nucleic acids were extracted using the lithium method (Li) or the silica capture (SC) method. Undiluted (1:1) and diluted (1:50, 1:100, 1:200, 1:400, 1:800) extracts were amplified using specific primers Smdetncr4a/Sm2ncr1b. M - 100 bp ladder

to 220 ng/µl with the lithium method, and from 100 to 144 ng/µl with the silica capture method.

With the lithium method, RT-PCR products could be detected from RNA samples diluted up to 1:400. With the silica capture method, RT-PCR products were detected only in undiluted samples from only two of the three isolates tested, 1248 clone and 'Geneva' (Fig. 1). The lithium method provided purer and less contaminated extracts than the silica capture method.

CONCLUSIONS

Two simple methods were used to extract RNA from leaves of *Fragaria virginiana* UC-11 infected with the

Strawberry mottle virus (SMoV): the lithium method and the silica capture method. Both methods do not involve phenol extraction, allow reverse transcription (RT) and PCR amplification of viral sequences, and are less expensive than commercially available kits. RNA concentration and quality were measured by spectrophotometry. Diluting extracts contaminants that may interfere with RT-PCR, restriction analyses, cloning, and sequencing. The extraction method used determines the quantity of high-quality viral RNA recovered, and thus the effectiveness of the detection process. The lithium method was better at extracting SMoV RNA than the silica capture method. In the future, we plan to evaluate other RNA extraction methods, such as the commercial extraction kit RNeasy® Plant Mini Kit (Qiagen).

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WYKRYWANIE WIRUSA CĘTKOWANEJ PLAMISTOŚCI LIŚCI TRUSKAWKI (SMoV) ZA POMOCĄ RT-PCR – PORÓWNANIE DWÓCH METOD IZOLACJI RNA WIRUSA

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S T R E S Z C Z E N I E

W badaniach zastosowano dwie metody izolacji RNA wirusa cętkowanej plamistości liści truskawki (SMoV) – metodę adsorpcji na żelu krzemionkowym (SC) i metodę, w której do buforu ekstrakcyjnego dodawano związki litu (Li). Źródłem izolatów wirusa były rośliny *Fragaria virginiana* UC-11 zakażane SMoV pochodzącym z trzech roślin truskawki (klon hodowlany 1248, 'Geneva' i 'Totem'). Na podstawie pomiarów spektrofotometrycznych oceniano przydatność uzyskanego RNA do testów RT-PCR. Jakość i ilość wyizolowanego RNA wirusa określano na podstawie stosunku A 260/A 230 i A 260/A 280 oraz pomiaru koncentracji RNA wirusa (ng/µl). Po wykonaniu serii rozcieńczeń ekstraktów RNA 1:50, 1:100, 1:200, 1:400, 1:800, przeprowadzono amplifikację RNA w łańcuchowej reakcji polimerazy poprzedzonej odwrotną transkrypcją (RT-PCR) przy użyciu zestawu Super-Script™One-Step RT-PCR with Platinum®Taq (Invitrogen) i starterów specyficznych dla SMoV.

Ekstrakty zawierające kwasy nukleinowe wyizolowane metodą wykorzystującą związki litu były mniej zanieczyszczone, o czym świadczy stosunek absorbancji A 260/A 280, który wahał się w granicach 1,5-1,9. Koncentracja RNA izolowanego metodą wykorzystującą związki litu była wyższa niż w przypadku RNA izolowanego metodą adsorpcji na żelu krzemionkowym. Zależnie od badanej próbki, ilość RNA wynosiła odpowiednio 168-220 ng/µl i 100-144 ng/µl. Po zastosowaniu do izolacji RNA metody wykorzystującej związki litu, w elektroforezie wykrywano produkty amplifikacji w rozcieńczeniu 1:100-1:400. Stosując izolację metodą SC, możliwe było wykrycie nierozcieńczonego produktu reakcji RT-PCR, zaś w przypadku niektórych próbek nie uzyskiwano produktu amplifikacji o oczekiwanej wielkości.

Słowa kluczowe: *Fragaria virginiana,* wirus cętkowanej plamistości liści truskawki, izolacja RNA, RT-PCR