Selection of RNA Isolation Method for Molecular Detection of Grapevine Viruses

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Abstract

Grapevines infected with *Grapevine leafroll-associated virus-1* (GLRaV-1) and *Grapevine leafroll-associated virus-3* (GLRaV-3) were selected. Total RNA was isolated from grapevine phloem tissue scrapped from dormant canes by three different methods: extraction with urea buffer followed with phenol-chloroform extraction, method using Concert™ reagent (Invitrogen) followed with chloroform-isopropylalcohol extraction, and procedure using RNeasy Plant Mini Kit (Qiagen). The highest yield of RNA was obtained using Concert™ reagent. If this RNA was used in RT-PCR, GLRaV-1 and GLRaV-3 were easily detected. From RNA isolated by other two methods these viruses were not detected.

Keywords: *Grapevine leafroll-associated virus-1*; *Grapevine leafroll-associated virus-3*; grapevine; ELISA; RT-PCR

INTRODUCTION

Grapevine leafroll is worldwide serious disease of grapevine affecting yield quantity and quality, yield losses up to 40% (WOODHAM *et al*. 1984). The disease is characterised by a downrolling of leaves and reddening or yellowing of blades. It is caused by a group of closteroviruses (BOSCIA *et al*. 1995), from them *Grapevine leafroll-associated virus*-1 (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3) are economically the most important and worldwide most distributed.

Serological detection by ELISA of these two viruses is widely used. More sensitive molecular techniques, mainly different variants of RT-PCR, are being adapted for their detection (ROUTH *et al*. 1998). Despite many publications about detection of these two viruses, some problems still occurs, probably due to erratic distribution and low concentration of viruses in plant tissues (MONIS & BESTWICK 1996). This work was aimed to select optimal method of RNA isolation for establishing RT-PCR detection of grapevine closteroviruses in the Czech Republic.

MATERIALS AND METHODS

Serological detection

Grapevines grown in Faculty of Horticulture at Lednice, Czech Republic, were examined for presence of leafroll symptoms. Dormant canes were sampled and tested by DAS-ELISA with commercial antibodies to identify individual leafroll-associated viruses. Antigens were prepared by grinding of 0.4 g of cortical scrappings in 6 ml of extraction buffer (phosphate buffered saline – PBS pH 7.4 with 2% of PVP K25 and 0.2% of bovine serum albumin). All ELISA were performed in duplicate, and a reaction was considered positive when the mean absorbance at 405 nm was at least three standard deviation units above the negative control.

RNA isolation

Total RNA was isolated from grapevine phloem tissue scrapped from dormant canes using three methods.

1. Procedure using RNeasy Plant Mini Kit (Qiagen). 0.1 g of plant material was grinded in liquid nitrogen.

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450 µl of RLC buffer was added and procedure continued according to instructions of manufacturer.

2. Method using Concert™ reagent (Invitrogen). Sample – about 0.1 g of plant tissue was ground in liquid nitrogen. 0.5 ml of Concert™ reagent was added and mixed. Solution was centrifuged and supernatant collected. 100 µl of 5 M NaCl and 300 µl of chloroform were added. After centrifugation, aqueous phase was transferred to a new tube. Equal volume of isopropyl alcohol was added. Mixture was centrifuged, supernatant discarded and 1 ml of 75% ethanol added to the pellet. After centrifugation, supernatant was decanted and 30 µl of water was added to dissolve the RNA.

3. Extraction with urea buffer. Sample 0.05 g of plant tissue was ground with liquid nitrogen. 1 ml of buffer (6.5M urea, 5M NaCl, 1 M Tris, 0.5M EDTA, 20% sarcosyl, 1% of PVP) was added. 50 µl of 2M sodium acetate, 500 µl of phenol and 100 µl of chloroform were added and mixture was vortexed. Upper phase was taken and 30 µl of 2 M sodium acetate (pH 5.2) and 900 µl of ethanol were added. After precipitation, pellet was resuspended in 500 µl of water and 110 µl of 10 M LiCl was added. After overnight precipitation at 4°C, pellet was collected, resuspended in 300 µl of water and 30 µl of 2M sodium acetate (pH 5.2) and 750 µl of ethanol were added. After precipitation at –20°C for 1.5 hour, liquid was removed and sedimented pure RNA resuspended in 50 µl of water.

RT-PCR

RT-PCR was used to detect the two viruses from total RNA isolated from infected samples by three described methods.

Primers for GLRaV-1 detection were selected according to GOOD and MONIS (2001), forward primer GSP 1 (5'CGA AGA TGG CCG TGT CAA TTA CTG 3') and reverse primer GSP 9 (5'CGC CGC CGA AGT CGT AGA CAA CCA 3').

Primers for GLRaV-3 detection were selected according to MINAFRA and HADIDI (1994), forward primer H 229 (5' ATA AGC ATT CGG GAT GGA CC 3') and reverse primer C 547 (5' ATT AAC TTG ACG GAT GGC ACG C 3').

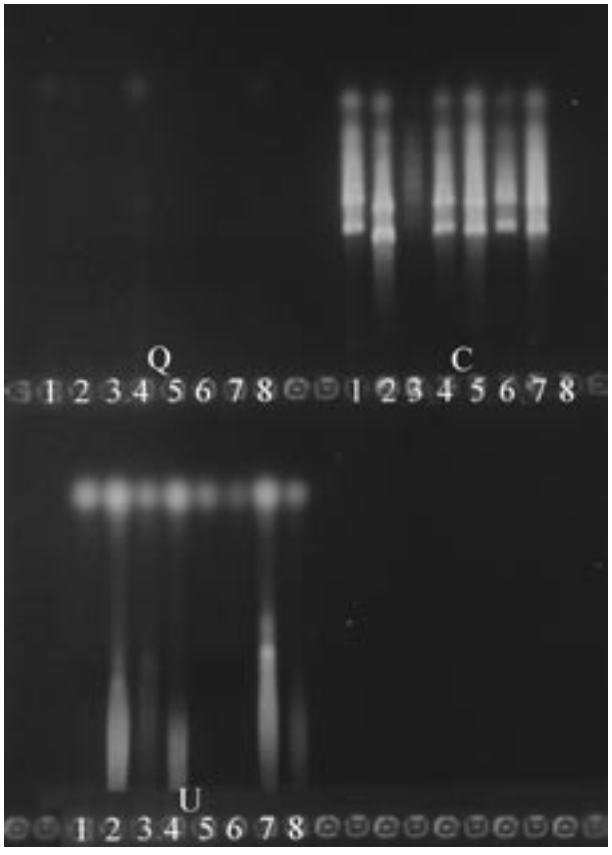
Reverse transcription mixture contained 5 µl of total RNA, 0.4 mM of dNTPs, 0.25 µg of reverse primer, 40 U of RNaseOut (Invitrogen), buffer, 0.01 M DTT, and 100 U of SuperScript™ II reverse transcriptase (Invitrogen). Reaction was performed 1.5 hour at 42°C. RT product (10 µl) was added to the PCR reaction containing PCR buffer, 1 mM of $MgCl_2$, 0.1 mM of dNTPs, 0.25 µg of each primer and 1.5 U of Taq polymerase (Promega).

PCR reactions were run on PTC-200 thermocycler (MJ Research) under following conditions: pre-heating 5 min at 94°C, template denaturation 30 s at 94°C, primer annealing 35 s at 58°C (GLRaV-1) or at 56°C (GLRaV-3), DNA synthesis 1 min at 72°C, 35 cycles. Amplification products were analysed by electrophoresis on 1.5% agarose gel.

RESULTS

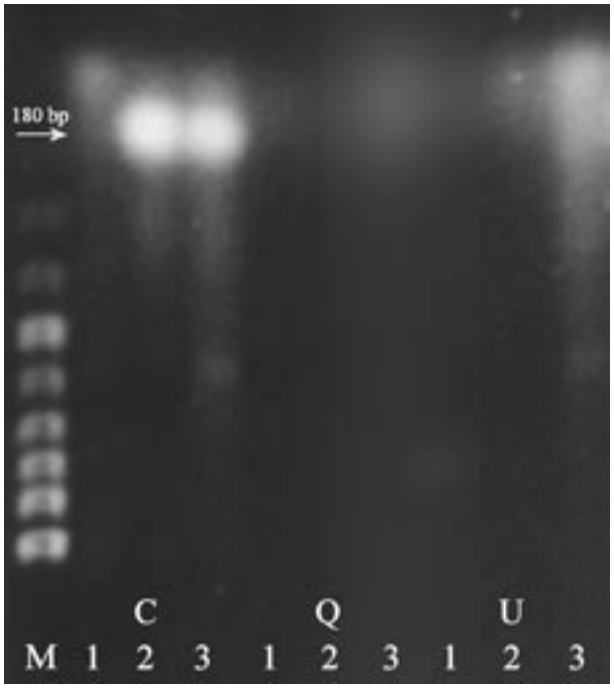
From twenty two grapevines tested serologically, three were found to be infected with GLRaV-1, three with GLRaV-3, three with GLRaV-2, three with GLRaV-6 and two with GLRaV-7. Leafroll- associated viruses were not found in nine grapevines tested.

Three grapevines infected with GLRaV-1 and three with GLRaV-3 were selected. Total RNA was isolated



1–8 – individual samples
Q – method using Qiagen RNeasy Plant Mini Kit
C – method using Concert™ reagent
U – method using urea extraction buffer

Figure 1. 1.5% agarose gel, isolated total RNA by different procedures



1–3 – individual samples
C – method using Concert™ reagent
Q – method using Qiagen RNeasy Plant Mini Kit
U – method using urea extraction buffer

Figure 2. 1.5% agarose gel, RT-PCR detection of GLRaV-1

from grapevine phloem tissue scrappings using three different methods.

Highest yield of RNA was obtained using Concert™ reagent, as visible in Figure 1. Very low amount of RNA was obtained by a method using Qiagen kit. Somewhat more RNA was obtained using urea extraction buffer, but only a few bands are visible on a gel.

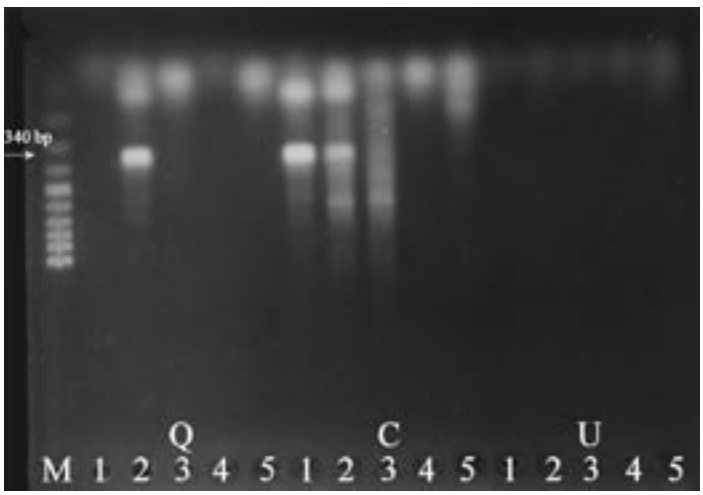
RT-PCR was used to detect GLRaV-1 and GLRaV-3 from total RNA isolated by three different methods from infected samples. RT-PCR for GLRaV-1 detection with primers GSP1 and GSP 9 resulted in infected samples to 180 bp long product (Figure 2). GLRaV-3 RT-PCR detection with primers H 229 and C 547 resulted to 340 bp products (Figure 3).

DISCUSSION

From leafroll-associated viruses tested, all five viruses occurred among tested grapevines. This is first report on occurrence of GLRaV-2, GLRaV-6 and GLRaV-7 in the Czech Republic. Not all grapevines with symptoms of leafroll disease were found to be infected with leafroll-associated viruses. However, they were not tested for presence of GLRaV-4, GLRaV-5 and GLRaV-8 and they may be infected with these not examined viruses.

Different results were obtained when RNA isolated by individual methods was used in RT-PCR. RNA isolated by Concert™ was amplifiable and it was possible to detect GLRaV-1 and GLRaV-3 from it by RT-PCR. In two samples were detected GLRaV-1 and in three samples GLRaV-3 in a good agreement with ELISA results. RT-PCR from RNA isolated by procedure using Qiagen kit was able to detect only one sample infected with GLRaV-3 and did not detect any sample infected with GLRaV-1. No viruses were detected from RNA isolated using urea buffer.

Concert™ reagent is a new product of Invitrogen and was not largely tested and used by plant virologists yet. This work showed its suitability for isolation of total RNA from plants, especially grapevines. Qiagen kit is commonly used by plant virologist. Yield of RNA is known to be low, but is considered to be of high quality and purity. In this work, some samples gave false negative results, probably isolation from



1–5 – individual samples
Q – method using Qiagen RNeasy Plant Mini Kit
C – method using Concert™ reagent
U – method using urea extraction buffer

Figure 3. 1.5% agarose gel, RT-PCR detection of GLRaV-3

grapevine was not completely successful. Some modification of this method was published (MACKENZIE *et al*. 1997) and is probably needed to do so, if we wish to use this method for RNA isolation from grapevine tissue. Method using urea extraction buffer was commonly used in our laboratory for detection of fruit tree viruses. It seems not to be suitable for isolation of RNA from grapevine.

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