Detection of *Cherry Leaf Roll Virus* and *Strawberry Latent Ring Spot Virus* by One-Step RT-PCR

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Abstract

Kumari S. (2009): **Detection of *Cherry leaf roll virus* and *Strawberry latent ring spot virus* by one-step RT-PCR**. Plant Protect. Sci., 45: 140–143.

A one-step reverse transcription-polymerase chain reaction (RT-PCR) protocol was developed and used for the detection of *Cherry leaf roll virus* (CLRV) and *Strawberry latent ring spot virus* (SLRSV). The protocol was used to test infected screen house plants and also plants from orchards and vineyards where the vector (*Xiphinema diversicaudatum*) of SLRSV was detected from the soil. The one-step RT-PCR protocol is rapid and sensitive and has the potential to be used for the diagnosis of CLRV and SLRSV in routine diagnostic laboratories.

Keywords: CLRV; SLRSV; RT-PCR; *Xiphinema diversicaudatum*

The nepovirus genus belonging to the family comoviridae has a worldwide distribution and principal crops affected are small fruits, fruit trees and grapevine (Brunt *et al*. 1996; Mayo & Robinson 1996). *Cherry leaf roll virus* (CLRV) is an established member of the nepovirus group (Regenmortel *et al*. 2000). It has isometric particles approximately 28 nm in diameter and it occurs mainly in Europe and North America (Bandte & Büttner 2001). It is readily sap-transmissible and has a wide host range. The virus is seed- and pollen-transmitted in several host plants. Unlike many nepoviruses, CLRV appears not to be transmitted by soil-inhabiting nematodes (Jones *et al*. 1981) despite earlier reports (Flegg 1969).

Strawberry latent ringspot virus (SLRSV), a tentative member of the nepovirus group (Regenmortel *et al*. 2000) with a worldwide distribution, has isometric particles 30 nm in diameter (Faggioli *et al*. 2002). The virus can be transmitted with plant sap by mechanical inoculation, through infected seeds, pollen and by soil nematodes *Xiphinema diversicaudatum*. Adults and larvae can transmit SLRSV equally efficiently. It has a broad host range which includes wild and cultivated plants. Perennials are important natural reservoirs.

In the Czech Republic CLRV was detected in plant tissues of cherry, walnut and raspberry (Albrechtová & Chod 1981; Novák & Lanzová 1981; Janečková & Pluhař 1987; Smrčka 1993) and SLRSV was detected in cherry by Novák and Lanzová (1974). The main objective of this study was to detect CLRV and SLRSV in leaves of fruit trees by a polymerase chain reaction for rapid and reliable diagnostics. In this paper, I have also investigated and compared the sensitivity and resilience of two RNA extraction methods.

MATERIALS AND METHODS

Plant material. Apricot and peach plants maintained in a screen house were used as plant mate-

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QG 60123.

rial. Isolates of CLRV and SLRSV were obtained from the Mediterranean Agronomical Institute, Bari-Valenzano, Italy. Leaves were collected from the screen house at one-month interval. Leaves were also collected from orchards where the vector *Xiphinema diversicaudatum* occurs in the Czech Republic. Positive control was used from a plant virus collection of the Department of Virology, Crop Research Institute, Prague-Ruzyně.

RNA extraction by Logemann *et al.* (1987) method

Guanidine hydrochloride extraction. Plant leaves (200 mg) were frozen in liquid nitrogen and homogenised to a fine powder using a mortar and a pestle. To this 2 ml of guanidine buffer (8M guanidine hydrochloride, 20mM of 4-morpholineethane-sulphonic acid, 20mM EDTA, and 50mM mercaptoethanol at pH 7.0) was added and homogenised thoroughly. The extract was then pipetted to 2 ml Eppendorf tubes and the tubes were vortexed for 15 s and then kept in ice for 5 minutes. The tubes were further centrifuged in a pre-cooled (4°C) centrifuge for 10 min at 13 000 rpm.

Phenol/chloroform/isoamyl alcohol extractions. The supernatant is pipetted to new 2 ml Eppendorf tubes and phenol:chloroform:isoamyl alcohol was added at a ratio 25:24:1. Phenol with low pH 4.8 was used. The tubes were further vortexed for 15 s and centrifuged for 10 min at 4°C at 13 000 rpm. The process of removing protein by phenol/chloroform/isoamyl alcohol was repeated twice.

Ethanol precipitation. The RNA-containing aqueous phase was collected and mixed with pre-cooled 700 µl of absolute ethanol and 200 µl of 1M acetic acid for precipitating the RNA and leaving DNA and residual proteins in the supernatant. An overnight incubation at –20°C followed.

RNA-washing step. The precipitated RNA is pelleted at 13 000 rpm for 10 min and washed twice with 500 µl of 3M sodium acetate at pH 5.2 at 4°C. Low-molecular-weight RNAs and contaminating polysaccharides were dissolved, whereas intact RNA remained as a pellet after centrifugation for 5 min at 13000 rpm. The salt was removed by a final wash with 70% ethanol. The RNA pellet is subsequently dissolved in RNase free water.

Nucleic acid isolation by RNeasy plant mini kit – QIAGEN

Plant leaves (200 mg) were frozen in liquid nitrogen and homogenised to a fine powder using a mortar and a pestle, then total RNA was extracted from each sample using the RNeasy plant mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol. RNA was finally eluted with 50 µl of RNase-free water and stored at –20°C until use.

One-step Reverse-Transcriptase Polymerase Chain Reaction. The one-step RT-PCR was performed in 25 µl with the following mastermix: 5.0 µl 5×Qiagen one-step RT-PCR buffer (containing 12.5mM $MgCl_2$), 1.5 µl each primer (10 pmol/µl) (synthesised by Generi Biotech, Prague, Czech Republic) 1.0 µl 10mM dNTP, 1.0 µl of one-step RT-PCR Enzyme mix (Qiagen, Hilden, Germany), and sterile water was adjusted to 24 µl and to this 1.0 µl of RNA was added as a template for RT-PCR.

The following primers were used in the PCR reaction: CLRV 1 (5'-CAT TTC CAT GCG ACC GGT CTT-3'), CLRV 2 (5'-AGT CCG ACA CTC ATA CAA TAA GC-3'), SLRSV 1 (5'-GTT ACT TTT ACC TCC TCA TTG TCC ATG TGT TG-3') and SLRSV 2 (5'-GAC TAT CGT ACG GTC TAC AAG CGT GTG GCG TC-3') (Bertolini *et al.* 2003). Amplification was performed in a MJ research thermal cycler with heated lid. The RNA was subjected to one-step RT-PCR with the following specifications: reverse transcription 30 min at 50°C, initial PCR activation step for 15 min at 95°C, 35 cycles with 45 s at 94°C, 45 s at 55°C, 45 s at 72°C and final extension at 72°C for 10 minutes

An aliquot (5 µl) of each amplification reaction was mixed with 1 µl of 6 × loading dye (Fermentas, MBI) and electrophoresed in high resolution 1.5% agarose gel and run in TAE buffer. The bands were visualized and photographed under UV (312 nm) after SYBER safe binding to the DNA fragments. A 100 base pair and 1 kb marker (Fermentas, MBI) were included on the gel.

RESULTS AND DISCUSSION

Leaves of apricot and peach plants which were infected with CLRV and SLRSV were collected from the screen house for the detection of viruses by the reverse-transcriptase polymerase chain re-

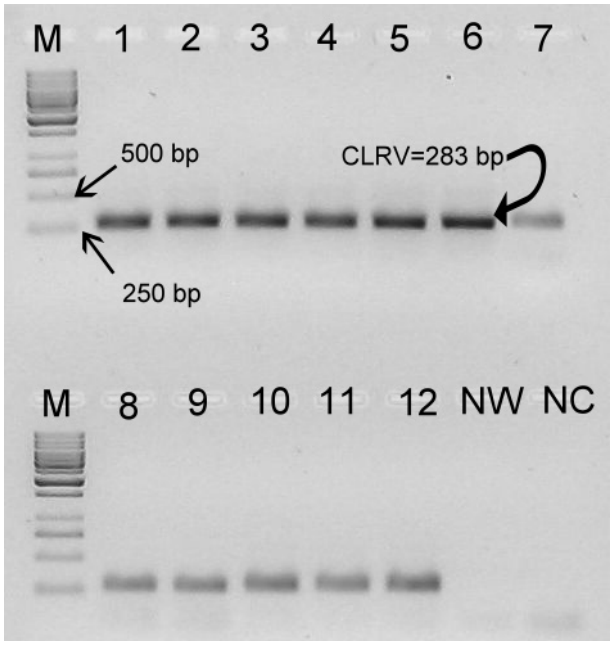

Figure 1. Electrophoresis of the amplified products of *Cherry leaf roll virus*. Lane M: 1 kb DNA ladder (Fermentas); lane 1–6: CLRV (RNA extracted according to Logemann *et al*. 1987); lane 7–12: CLRV (RNA extracted by QIAGEN kit) from leaves of apricot and peach; NW – water negative control; NC – negative control from healthy plant

action. Specific amplification products of 283 bp for CLRV and 181 bp for SLRSV were obtained from all respective positive samples (Figures 1 and 2). No amplification products were obtained from healthy extracts or water control (data not shown for SLRSV). The total RNA of these viruses was extracted using two methods (Qiagen RNeasy plant kit and according to Logemann *et al*. 1987). Amplification products yielded bands of the same intensity for SLRSV using RNA extracted by both methods, while Longeman *et al*. (1987) method produced bands of a little high intensity than the Qiagen kit for CLRV (Figure 1).

Fruit crops belong to the most important crops in the Czech Republic. These crops have a high economic significance in Moravia. Considering the economic importance of orchard viruses in the Czech Republic, I investigated the occurrence of SLRSV in orchards and vineyards where its vector (*X. diversicaudatum*) was recorded. The leaf samples were collected from the locality Hrušky (host – grapevine), Bílé podolí (host – peach, sweet cherry, sour cherry and apple) and Lhenice (host – sweet cherry). From these samples RNA was isolated as described in Material and Methods and one-step RT-PCR was performed using Qiagen one-step RT-PCR kit, but the virus was not detected in any of the studied samples. Although the vector *X. diversicaudatum* was found in these localities, SLRSV was not detected in these orchards and vineyards.

Samples from the screen house which were found positive in this study were previously studied by a serological test by Polak *et al*. (2004). Serological methods are routinely employed for these purposes because they allow the sensitive, specific and simultaneous analysis of many samples in a single microplate (Garnsey & Cambra 1991). However, molecular methods based on the PCR amplification of nucleic acid enable to reach higher sensitivity and facilitate the analysis of sequence data (Bariana *et al*. 1994). Therefore, in this study, one-step RT-PCR method was optimised. This protocol is rapid and sensitive and has the potential to be used for the diagnosis of CLRV and SLRSV in phytosanitary laboratories. As this protocol is verified only on experimentally infected plants, future experiments will be continued to detect the virus in naturally grown trees in geographically different areas and the protocol will be compared between experimentally and naturally infected samples with CLRV and SLRSV.

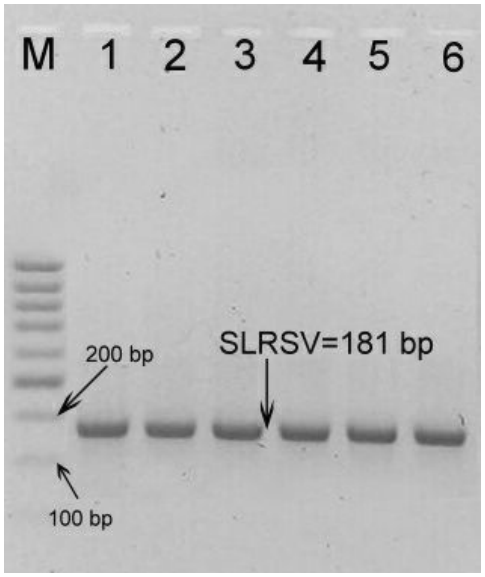

Figure 2. Electrophoresis of the amplified products of *Strawberry latent ringspot virus*. Lane M: 100 bp DNA ladder (Fermentas); lane 1–6 SLRSV (RNA extracted according to Logemann *et al*. 1987) from leaves of peach trees

Acknowledgements. The author is thankful to M. Vítámvásová for technical assistance.

References

Albrechtová L., Chod J. (1981): Identification of viruses in sweet cherry trees (*Prunus avium* L.) with symptoms similar to cherry leaf roll. In: Plant Virology. Proceedings 9th Conference Czechoslovak Plant Virologists, Brno: 17–20.

Bandte M., Büttner C. (2001): Occurrence, transmission and diagnosis of cherry reafroll nepovirus – a literature review. Pflanzenschutzberichte **59**: 1–19.

Bariana H.S., Shannon A.L., Chu P.W.G., Waterhouse P.M. (1994): Detection of five seedborne legume viruses in one sensitive multiplex polymerase chain reaction test. Phytopathology, **84**: 1201–1205.

Bertolini E., Olmos A., López M.M., Cambra M. (2003): Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and Pseudomonas savastanoi pv. savastanoi in olive trees. Phytopathology, **93**: 286–292.

Brunt A.A., Crabtree K., Dallwitz M.J., Gibbs A.J., Watson L. (1996): Viruses of Plants. CAB International, Wallingford.

Faggioli F., Ferretti L., Pasquini G., Barba M. (2002): Detection of *Strawberry latent ring spot virus* in leaves of olive trees in Italy using a one-step RT-PCR. Journal of Phytopathology, **150**: 636–639.

Flegg J.J.M. (1969): Tests with potential nematode vectors of cherry leaf roll virus. In: Report of East Malling Research Station for 1968: 115.

Garnsey S.M., Cambra M. (1991): Enzyme-liked immunosorbent assay (ELISA) for citrus pathogens. In: Graft-transmissible diseases of citrus. In: Roistacher C.N. (ed.): Handbook for Detection and Diagnosis. FAO, Rome: 193–216.

Janečková M., Pluhař Z. (1987): Výsledky testů na přítomnost virů v matečných rostlinách odrůd a novošlechtění maliníku. Zahradnictví, **14**: 267–277.

Jones A.T., McElroy F.D., Brown D.J.F. (1981): Tests for transmission of cherry leaf roll virus using *Longidorus*, *Paralongidorus* and *Xiphinema* nematodes. Annals of Applied Biology, **99**: 143–150.

Logemann J., Schell J., Willmitzer L. (1987): Improved method for the isolation of RNA from plant tissues. Analytical Biochemistry, **163**: 16–20.

Mayo M.A., Robinson D.J. (1996): Nepoviruses: Molecular biology and replication. In: Harrision B.D., Murant A.E. (eds): The Plant Viruses, Polyhedral Virions and Bipartite RNA. Vol 5. Plenum, New York: 139–185.

Novák J.B., Lanzová J. (1974): Sérologická identifikace viru svinutky třešně na některých dřevinách v Československu. In: Sbor. Vysoké školy zemědělské, Praha, Řada A: 205–223.

Novák J.B., Lanzová J. (1981): Virus svinutky třešně z ořešáku vlašského (*Juglans regia*) v Československu. Ochrana Rostlin, **17**: 1–8.

Polák J., ChaloupkovÁ M., Jokeš M. (2004): Biological and serological procedures to detect three nepoviruses in fruit trees. Plant Protection Science, **40**: 121–127.

Regenmortel M.H.V.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., Maniloff J., Mayo M.A., McGeoch D.J., Pringle C.R., Wickner R.B. (2000): Virus Taxonomy. Clsassification and Nomenclature of Viruses. Academic Press, New York.

Smrčka L. (1993): The occurrence of arabis mosaic, tomato black ring and strawberry latent ringspot nepoviruses and cucumber mosaic cucumovirus in raspberry (*Rubus ideaus*) near Prague. Ochrana Rostlin, **29**: 87–92.

Received for publication January 28, 2009
Accepted after corrections October 28, 2009

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