SHORT COMMUNICATION

IDENTIFICATION AND PARTIAL CHARACTERIZATION OF *PRUNUS NECROTIC RINGSPOT VIRUS* ON STONE FRUITS IN JORDAN

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SUMMARY

Prunus necrotic ringspot virus (PNRSV, Genus *Ilarvirus*, Family *Bromoviridae*) was isolated from stone fruit trees showing virus-like symptoms grown in Jordan. Identification of this virus was based on host range, properties in crude sap, transmissibility, and serological tests. PNRSV-J has a limited range of experimental hosts. The dilution end-point of infectivity was 10^{-2}, the thermal inactivation point was 57°C, and purified virus had an *in vitro* longevity of 16 h at 25°C. PNRSV-J was detected by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). PNRSV-J was purified from cucumber leaves harvested 6-8 days after inoculation. The modified purification method gave an adequate virus yield for antibody production. Antiserum produced by immunizing a rabbit had a titer of 1024 in direct antigen coating (DAC)-ELISA, with high specificity to PNRSV. An immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) protocol was useful for the detection of PNRSV in herbaceous and woody plant tissues. Nucleotide sequence and phylogenetic analysis of RT-PCR products derived from RNA3 of PNRSV-J confirmed its identity as an isolate of PNRSV and revealed that it is a member of Group I (PV32) isolates.

Key words: ELISA, host range, IC-RT-PCR, PNRSV, purification.

Stone fruits are affected by several diseases of viral etiology that cause serious reduction in the vigour and production of plants (Németh, 1986). *Prunus necrotic ringspot virus* (PNRSV) is the most widely spread virus and one of the most economically important *Prunus* viruses. It occurs worldwide wherever stone fruits are grown and is a serious pathogen of many woody species, causing various ringspot diseases in peaches, cherries, roses and hops; and mosaic diseases in apples, plums and roses (Németh, 1986).

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Since PNRSV is one of the most important viruses in Jordan (Myrta *et al*., 2003), this research was conducted to achieve the following objectives: (i) identification and characterization of PNRSV, (ii) production of suitable, high titer antiserum, using antigen from the purified PNRSV, and (iii) determination of sensitive, less laborious, quick, and cheap techniques for the detection of PNRSV in stone fruits within the country.

One virus isolate of PNRSV, namely PE-211, isolated from a naturally infected peach tree was selected as a representative of field isolations and used in this work. According to Fulton (1970), a pure culture was obtained for this isolate by successive single lesion transfer from *Cucumis sativus* cv Lahloba and then was increased and maintained by weekly transfers from systemically infected cucumber. For convenience, this isolate will be referred to in this manuscript as PNRSV-J.

In preliminary transmission tests, the transfer of PNRSV-J from infected *Prunus* hosts to herbaceous hosts was found to be unsatisfactory. Sodium sulfite (antioxidant), Na-DIECA (enzyme inhibitor), charcoal (adsorbent), and nicotine were used to facilitate virus transmission and to overcome the inhibitory effects of tannins. The tested buffers were as follows: 0.01 M phosphate buffer pH 7.2, containing 2.5% nicotine; 0.01 M phosphate buffer pH 7.2, containing 0.001 M Na-DIECA and 0.001 M cysteine; 0.01 M phosphate buffer pH 7.8, containing 0.001 M Na-DIECA and 2.5% nicotine mixed with activated charcoal (100 mg ml^{-1}, w/v); and 0.05 M phosphate buffer pH 7.2, containing 2% sodium sulfite. One of the four inoculation buffers tested was effective in facilitating the transmission of PNRSV-J from peach to cucumber cotyledons. This buffer (0.01 M phosphate buffer pH 7.8, containing 0.001 M Na-DIECA, 2.5% nicotine, and 100 mg ml^{-1} charcoal) was, therefore, used for subsequent studies. Substances such as tannins and cell constituents consisting of proteins, polysaccharides, and enzymes may be involved in the inhibition of virus infection (Noordam, 1973; Hill, 1984). Oxidation of phenolic materials is an enzymatic process, depending on the activity of polyphenol oxidase (Fulton, 1966). Copper ion is necessary for the activity of the enzyme, and materials such as Na-DIECA, which chelate the copper ion, will stabilize

PNRSV infectivity in extracts. Also, nicotine reduces the precipitation and inactivation of viruses by tannins (Hill, 1984). The presence of these components in the inoculation buffer contributed to the successful transmission of PNRSV from *Prunus* spp. to *C. sativus*. Nicotine was not required for the subsequent transmission of this virus from cucumber to other herbaceous plants.

PNRSV-J was mechanically inoculated to thirty herbaceous hosts from nine botanical families. Inoculated and systemically infected leaves of cucumber provided infective extracts for host range trials. At least four plants of each of the herbaceous indicators were mechanically inoculated with the sap from the source plants (*C. sativus* cv Lahloba) diluted 1:1 (w/v) with the inoculation buffer (0.01 M phosphate buffer pH 7.2, containing 0.001 M Na-DIECA and 0.001 M cysteine) and mixed with charcoal (100 mg ml^{-1}, w/v).

Results of host range trials showed that PNRSV had a limited host range. Only twelve of the plant species developed symptoms. They proved to be infectious upon back indexing of both inoculated and tip leaves. No symptomless infection was detected by DAS-ELISA in either inoculated or tip leaves. PNRSV-J produced chlorotic ring spots on the inoculated leaves and systemic chlorotic mottle on the tip leaves of *Chenopodium amaranticolor* and *C. quinoa* plants. In *Helianthus annuus*, PNRSV-J incited numerous distinct chlorotic ringspots in inoculated leaves. *Zinnia elegans* reacted to PNRSV-J with chlorosis at the base of the youngest leaf. Chlorotic and necrotic lesions were observed on cotyledons of *C. sativus* cvs Aya, Beit Alpha, Judy, Lahloba, and National Pickling. Systemic symptoms consisted of severe mosaic, compact growth, and top necrosis. These symptoms were typical of PNRSV infection. *Cucurbita moschata* developed a severe chlorosis on inoculated leaves 5-6 days after inoculation. Among Leguminosae plant species, only *Vigna unguiculata* reacted to PNRSV-J by forming red necrotic local lesions in the cotyledons ten days after inoculation. However, Boulila and Marrakchi (2001) showed that *V. unguiculata* reacted to PNRSV by forming a chlorotic line pattern and deformation of the leaf lamina. In *Petunia hybrida*, a solanaceous plant, systemic chlorotic lesions formed that eventually developed into necrotic lesions, and systemic mottling was also observed in plants inoculated with PNRSV-J. *Vinca rosea*, *Gossypium hirsutum*, *Nicotiana benthamiana*, and *N. clevelendii* exhibited systemic symptoms ranging from mild mottle to severe leaf distortion.

Among the different hosts studied, *Gomphrena globosa*, *Cucurbita pepo*, and *Phaseolus vulgaris* were not infected; and the virus could not be recovered from either inoculated leaves or tip leaves, contrary to the results reported by Civerolo and Mircetich (1972) who described isolates systemically invading these hosts. *Vigna unguiculata* reacted to PNRSV by forming red necrotic local lesions. The observed differences in the herbaceous host range were probably due to different virus strains or environmental influences, such as different greenhouse temperatures.

Fifteen peach (*Prunus persica* cv Elberta) seedlings were graft-inoculated from the PNRSV-J source trees in May and June 2001. The inoculum consisted of three buds each per peach seedling. Typical PNRSV symptoms induced on graft-inoculated seedlings of *P. persica* cv Elberta were generally characteristic of those previously reported for PNRSV (Németh, 1986).

Virus properties in plant crude sap, dilution endpoint (DEP), longevity *in vitro* (LIV), and thermal inactivation point (TIP), were determined by procedures described by Hill (1984). The extracted sap from virus-infected cucumbers was infectious by mechanical inoculation to cucumber when diluted to 10^{-2} in inoculation buffer. Infectivity of extracted sap was retained for up to 16 h at room temperature (approximately 25°C). Sap extracted from virus-infected cucumber remained infectious when heated to 57°C for 10 min.

Samples were tested by DAS-ELISA as described by Clark & Adams (1977) following the procedure recommended in the diagnostic kit for PNRSV by the manufacturer (Bioreba AG, Reinach, Switzerland). All of the PNRSV-infected samples reacted positively with antiserum specific for PNRSV using DAS-ELISA. A darker yellow color developed with the positive samples, giving absorbance values (A_{405}) twice or more than those given by healthy samples. In all tests, a visual rating of ELISA plates was in agreement with the ELISA reading.

The optimum harvest time of infected cucumber cotyledons for virus purification was determined by bioassay and confirmed by DAS-ELISA. Cucumber (cv Lahloba) leaves from PNRSV-inoculated plants were harvested 2, 4, 6, 8, 10, and 12 days after inoculation, and tissue was ground in extraction buffer and tested by ELISA. Based on the above assay, PNRSV was purified from cucumber leaves harvested 6-8 days after the inoculation of the cotyledons.

The purification procedure used was similar to that described by Ong and Mink (1989) with some modifications. Stabilization of PNRSV was achieved by including 0.01 M Na-DIECA and 0.01 M sodium thioglycolate in the extraction buffer. It seems that acidification with 36% acetic acid is very efficient in removing host material yet giving high yields of the virus. Furthermore, this procedure requires less time than following previous purification procedures described by Fulton (1957, 1970). The ultraviolet absorption spectrum of the partially purified virus was typical of spherical viruses, with A_{max} at 260 nm. The ratio of $A_{260/280}$ was calculated to be 1.59, which agreed with the reported value for PNRSV (Fulton, 1970).

Antiserum was prepared by intramuscular injection of a nine months old white rabbit with purified virus emulsified in Freund's incomplete adjuvant (Sigma

Chemical Co. Saint Louis, USA). All injections were made twice a week. The unwanted host specific antibodies were removed by cross absorption with healthy components. The antiserum produced was evaluated with direct antigen coating (DAC)-ELISA. The DAC-ELISA was conducted following the procedure of Lommel *et al.* (1982).

Antiserum titer, the reciprocal of the highest dilution of the antiserum, which gave positive reaction with the homologous virus, was 1024 when the antigen was diluted 1:5 and the goat anti-rabbit (GAR) conjugate was used at 1:3000 dilution.

In addition to biological and serological assay, a molecular method was used successfully for the detection of PNRSV. Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) was conducted following the method of Moury *et al.* (2000). The primers used for RT as well as for PCR were designed to amplify products based on the nucleotide sequence of the 3' end of the coat protein gene (RNA-3) of PNRSV. The sequences of PCR primers were the 20-mer Ilar 1 (5'-TTCTAGCAGGTCTTCATCGA-3') and the 17-mer Ilar 2 (5'-CAACCGAGAGGTTGGCA-3') (Operon Technologies Inc., Atlantic Avenue, USA) (Moury *et al.*, 2000; corresponding to nt. 1568-1772 of the PE5 isolate of PNRSV; Hammond and Crosslin, 1995). For nucleotide sequence analysis, primers Mac1 (5'-ACGCG-CAAAAGTGTCGAAATCTAAA-3') and Mac2 (5'-TG-

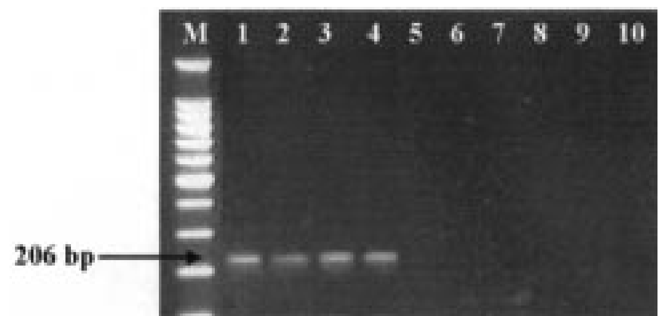

Fig. 1. Gel electrophoresis analysis of amplified *Prunus necrotic ringspot virus* cDNA from immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) using primers Ilar 1 and Ilar 2. Lane M, a ladder of DNA size markers (100-1,500 bp, Promega); lanes 1 and 2 PNRSV-infected cucumber; lanes 3 and 4, PNRSV-infected peach; lanes 5 and 6, healthy cucumber; lane 7 and 8, healthy peach; lanes 9 and 10, water. The arrow shows the position of the amplified fragment (206 bp).

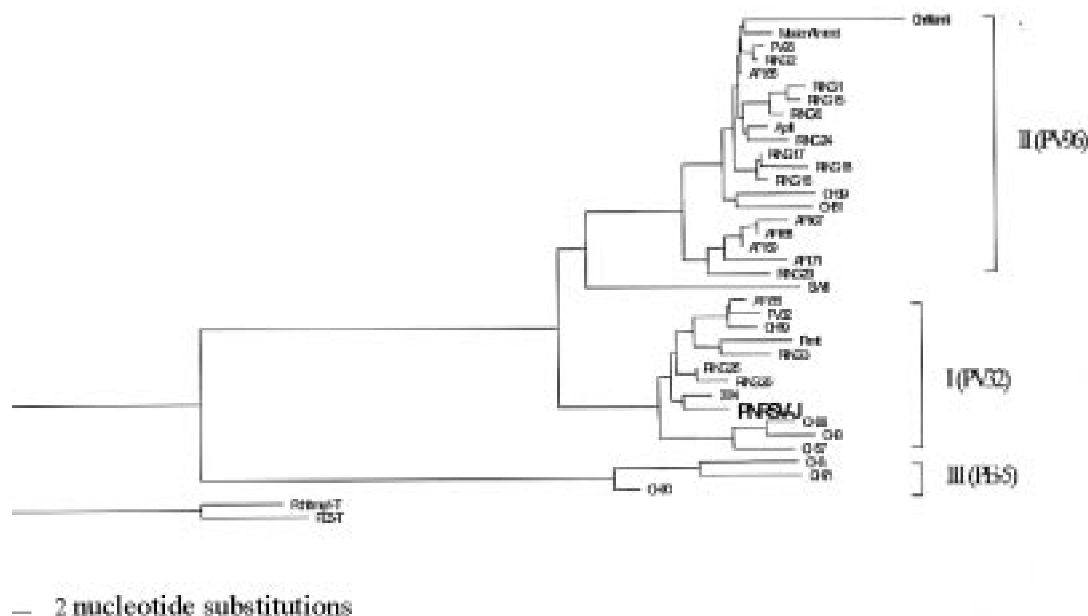

Fig. 2. Phylogenetic relationships as determined by CLUSTAL and PAUP between PNRSV-J and a selection of other known PNRSV isolates. Horizontal distances are proportional to the number of nucleotide changes; the bar indicates two nucleotide substitutions. Vertical branches are arbitrary. Group designations on the right reflect major groupings adopted in two previous reports of PNRSV sequence comparisons (Aparicio *et al.*, 1999; Vaŝková *et al.*, 2000). GenBank accession numbers for the isolates are: ChrItlam1 (AJ133203), Mission Almond (AF013285), PV32 (S78312), RING 2 (AF465215), AF165 (AF170165), RING 1 (AF465214), RING 15 (AF465221), RING 6 (AF465217), aprIt (AJ133200), RING 24 (AF465228), RING 17 (AF465223), RING 18 (AF465224), RING 16 (AF465222), CH39 (AF034990), CH61 (AF034989), AF167 (AF170167), AF168 (AF170168), AF169 (AF170169), AF171 (AF170171), RING 29 (AF465233), SW6 (AF013287), AF156 (AF170156), PV32 (AF465235), CH19 (AF465236), PlmIt (AJ133213), RING 3 (AF465216), RING 25 (AF465229), RING 26 (AF465230), 30/4 (UF57046), CH38 (AF034993), CH9 (AF034992), CH57 (AF034993), CH3 (AF465235), CH71 (AF034995), CH30 (AF034994), Pcht1mry1 (AJ133207), PE5(L38823).

Table 1. Reaction of host plant species to infection with PNRSV-J.

Plant family and species	Symptoms produced [a]		Back indexing [b]
	Inoculated leaves	Non inoculated leaves	
Amaranthaceae			
Gomphrena globosa	NS	NS	-
Apocynaceae			
Vinca rosea	NS	MM	+
Chenopodiaceae			
Chenopodium amaranticolor	CRS	CM	+
Chenopodium quinoa	CRS	CM	+
Compositae			
Helianthus annuus	CRS	NS	+
Lactuca sativa	NS	NS	-
Zinnia elegans	NS	C	+
Cucurbitaceae			
Citrullus vulgaris	NS	NS	-
Cucumis melo	NS	NS	-
Cucumis sativus cvs.			
Aya	CLL	M, TN	+
Beit Alpha	CLL	M, TN	+
Judy	CLL	M, TN	+
Lahloba	CLL	M, TN	+
National Pickling	CLL	M, TN	+
Cucurbita moschata	C	NS	+
Cucurbita pepo cv. Carina	NS	NS	-
Leguminosae			
Lupinus albus	NS	NS	-
Phaseolus vulgaris	NS	NS	-
Pisum sativum	NS	NS	-
Sesbania exaltata	NS	NS	-
Vigna unguiculata	NLL	NS	+
Malvaceae			
Gossypium hirsutum	NS	MM	+
Scrophulariaceae			
Antirrhinum majus	NS	NS	-
Solanaceae			
Datura metel	NS	NS	-
Datura stramonium	NS	NS	-
Lycopersicon esculentum	NS	NS	-
Nicotiana benthamiana	NS	MM	+
Nicotiana clevelandii	NS	MM	+
Nicotiana glutinosa	NS	NS	-
Nicotiana occidentalis	NS	NS	-
Nicotiana rustica	NS	NS	-
Nicotiana tabacum cv. White Burley	NS	NS	-
Nicotiana tabacum cv. Xanthi	NS	NS	-
Petunia hybrida	NS	M, SNL	+
Physalis floridana	NS	NS	-

[a] C = chlorosis, CLL = chlorotic local lesions, CM = chlorotic mottle, CRS = chlorotic ringspots, M = mosaic, MM = mild mottle, NLL = necrotic local lesions, NS = no symptoms, SNL = systemic necrotic lesions, TN = top necrosis.

[b] + = virus detected, - = virus not detected.

GTCCCACTCAGAGCTCAACAAG-3') (MacKenzie *et al*., 1997; corresponding to nt. 1178-1629 of the PE5 isolate of PNRSV; Hammond and Crosslin, 1995) and the Titan One Tube RT-PCR system (Roche Diagnostics Corp., Indianapolis, IN, USA) were used to amplify a portion of the coat protein gene using total nucleic acid extracts of PNRSV-infected peach.

The PCR products were analyzed by 1.5% (w/v) agarose gel electrophoresis in 0.5x Tris-borate-EDTA buffer (Sambrook and Russell, 2001). PCR products synthesized using the Mac1/Mac2 primer pair were excised from gels and cloned into the plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA, USA). Nucleotide sequences were obtained from three cDNA clones using an automated sequencer (Applied Biosystems International Model 377, Perkin-Elmer/Applied Biosystems, Norwalk, CT, USA). Sequence data were analyzed using Lasergene software by DNASTAR for the Apple Macintosh. Multiple sequence alignments with selected PNRSV isolates were performed using Clustal version 5 (Higgins *et al*., 1992) and phylogenetic analysis was performed using PAUP (phylogenetic analysis using parsimony version 4; Swofford, 2001) for phylogenetic relationships and inference of evolutionary trees.

The IC-RT-PCR successfully amplified DNA of the expected size from PNRSV-infected cucumber or infected *Prunus* spp., but not from the healthy control plants. Gel electrophoretic analysis of IC-RT-PCR products obtained from PNRSV-infected cucumber and peach plants using primers Ilar 1 and Ilar 2 showed a fragment with the size expected for amplification (206 bp) (Fig. 1). Sequence analysis of RT-PCR products obtained using the Mac1/Mac2 primer pair reveals a fragment corresponding to nt 1178- nt 1629 (aa22-aa175) of the PE5 isolate of PNRSV (Hammond and Crosslin, 1995). The sequence has been deposited at GenBank with Accession no. AY463362. Phylogenetic comparisons of this nucleotide sequence with the sequence of other known PNRSV isolates revealed that PNRSV-J clusters with the Group I (PV32) isolates (Hammond and Crosslin, 1998; Aparicio *et al*., 1999; Vaŝková *et al.,* 2000; Hammond, 2003) (Fig. 2).

Nucleotide sequence analysis of RT-PCR products obtained from PNRSV-J confirmed its identity as an isolate of PNRSV as shown in the biological and serological assays. PNRSV-J contains a two amino acid insertion (the result of a six nucleotide insertion) in this region of the coat protein gene (near the amino terminus). The significance of this insertion to biological activity of the coat protein is not known, but it does serve as a group-specific feature of the Group I isolates that can be used to discriminate between isolates in Groups I – III (Hammond, 2003). Phylogenetic clustering of PNRSV isolates does not correlate with host species or geographic origion of the isolate, however, there is a general trend for severe isolates to cluster into Group I (Hammond, 2003). Interestingly, PNRSV-J causes chlorosis in *Cucurbita moschata* and top necrosis in *Cucumis sativus* (Table 1).

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Received 3 Spetember 2003
Accepted 14 Juanuary 2004