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ORIGINAL PAPER

Biological and molecular characterization of *Prunus necrotic ringspot virus* isolates from three rose cultivars

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Abstract *Prunus necrotic ringspot virus* (PNRSV) is a rose and stone fruit tree pathogen. Three different PNRSV isolates, originating from three rose cultivars were studied. These PNRSV isolates were characterized using molecular techniques. Nearly the complete nucleotide sequence (1,630 nucleotides) of RNA3 of the isolate PNRSV-R1 has been determined (GenBank Acc. No. DQ003584). The sequence of the MP gene of the PNRSV-R1 isolate was determined, the first such results for a rose-derived PNRSV isolate. The reaction of PNRSV infection on test plants was also investigated. *Cucumis sativus* cv. Wisconsin, *Cucurbita maxima* cv. Buttercup and *Cucurbita pepo* cv. Melonowa Żółta appeared to be the most useful test plants for the differentiation of isolate-specific pathogenicity.

Keywords *Prunus necrotic ringspot virus* (PNRSV) · Rose virus isolates · IC-RT-PCR · Movement protein (MP) gene · Coat protein (CP) gene

Introduction

Prunus necrotic ringspot virus (PNRSV) belongs to the genus *Ilarvirus* and the family *Bromoviridae*. PNRSV is the causal agent of many economically important diseases in most cultivated *Prunus* species worldwide. Apart from almond, apricot, peach, plum, sour- and sweet-cherry trees, roses are also attacked by this virus (Németh 1986). PNRSV is the most commonly found rose virus in the United Kingdom, France and Poland (Thomas 1981, 1984a, b; Moury et al. 2000, 2001; Paduch-Cichal 2003). Some field-grown rose cultivars show no symptoms of PNRSV infection, whereas others develop line patterns, ringspots or yellow nets on leaves (Thomas 1981, 1984a; Curtis and Moran 1986; Wong and Horst 1988; Paduch-Cichal 2003). PNRSV infected roses flower earlier than healthy plants, but produce deformed flowers. The virus infection influences many flower traits, e.g., a significant reduction in number and diameter of the flowers, their fresh and dry weight, as well as reduction in number, diameter, and length of the shoots or in the number of flower petals was observed (Thomas 1982; Moran et al. 1988; Paduch-Cichal and Sala-Rejczak 2007).

PNRSV has a tripartite genome, where RNA1 and RNA2 encode proteins involved in viral replication and RNA3 encodes the 3a (putative movement protein, MP) and the 3b protein (coat protein, CP) (Fauquet et al. 2005).

There are different opinions on the correlation between the amino acid sequences in MP and/or CP of *Prunus* isolates of PNRSV and the host specificity, symptoms or serological properties of the virus (Hammond and Crosslin 1998; Aparicio et al. 1999; Hammond 2003; Vašková et al. 2000; Glasa et al. 2002; Spiegel et al. 2004). Only Moury et al. (2001) studied and discussed this subject for rose isolates of the virus. Therefore, we decided to compare the biological and molecular characteristics of three rose PNRSV isolates from Poland.

Materials and methods

The following PNRSV isolates were studied: PNRSV-R1, obtained from rose cv. Queen Elizabeth (Department of

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Fig. 1 Local numerous chlorotic spots on cotyledons of *Cucumis sativus* cv. Wisconsin inoculated with PNRSV-R3

Fig. 2 Local necrotic rings on cotyledons of *Cucurbita maxima* cv. Buttercup inoculated with isolates PNRSV-R2 (*right*) and PNRSV-R3 (*left*)

Ornamental Plants, Warsaw University of Life Sciences WULS-SGGW, Poland); PNRSV-R2, obtained from rose cv. Ingrid Bergman (Botanical Garden, Warsaw University, Poland); PNRSV-R3, obtained from rose cv. Montezuma (Botanical Garden, Warsaw University, Poland).

The determination of host range for the three virus isolates described above was conducted using the following species of test plants: *Chenopodium amaranticolor, Chenopodium murale*, *Chenopodium quinoa, Cucumis sativus* cv. Wisconsin, *Cucurbita maxima* cv. Ambar and cv. Buttercup, *Cucurbita pepo* cv. Astra, cv. Gagat, cv. Melonowa Żółta and cv. Soraya, *Phaseolus aureus*, *Nicotiana rustica*, and *Nicotiana tabacum* cv. Samsun. Infected rose plants served as the sources of PNRSV for the inoculation of test plants. Leaves from infected source plants were ground in mortar with HEPES buffer (pH 8.0) with the addition of 4% PEG 6000. Five test plants of each species or cultivar were used per each PNRSV isolate. Local disease symptoms were recorded 4 or 5 days after the inoculation and the systemic symptoms were recorded 14–21 days after inoculation.

Immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) was performed on the basis of procedures described by Wetzel et al. (1992), Nolasco et al. (1993), and Candresse et al. (1994), later adapted by Malinowski (2005). The following RT-PCR primer pairs were used: as the sense primer 5′-atggtttgccgaatttgcaatcat-3′ and as the antisense primer 5′-gagtgtgcttatctcactctag-3′ (Malinowski and Komorowska 1998). This pair of primers allowed to obtain a 700 bp amplicon, which is a fragment of the RNA3 CP coding sequence. Another pair of primers was designed to amplify a 900 bp fragment of the RNA3 putative movement protein (MP) gene. The reactions were performed using two primers: the sense primer: 5′-gttggttgaatagtgtttcagtatggcc-3′ and the antisense primer: 5′-agcgtgggtatgattgcaaattcgg-3′ (Scott et al. 1998).

PCR for the CP gene was performed as follows: 30 min at 50°C, 2 min at 94°C. There were 35 cycles performed for 10 s at 94°C, 30 s at 53°C, 40 s at 68°C followed by additional extension for 7 min at 68°C. PCR for the MP gene was done as follows: 30 min at 50°C, 2 min at 94°C, 25 cycles: 10 s at 94°C, 30 s at 62°C, 40 s at 68°C, 10 cycles: 10 s at 94°C, 30 s at 62°C, 175 s at 68°C and finally 7 min at 68°C.

Electrophoresis of the RT-PCR products was performed on 1.2% agarose gels stained with ethidium bromide. For the IC-RT-PCR test, samples were coated with IgG from Loewe Biochemica GmbH (Germany). Amplicons were sequenced in the Institute of Biochemistry and Biophysics Polish Academy of Science (Poland). The sequences were analyzed using the NCBI site: http://www.ncbi.nlm.nih.gov./blast/ and Lasergene: version 5 software (DNASTAR, USA). Nucleotide sequences of tested PNRSV isolates were compared both among each other and with the sequences registered in GenBank.

Results

Cucumis sativus cv. Wisconsin (Fig. 1), *Cucurbita maxima* cv. Buttercup (Fig. 2) and *C. pepo* cv. Melonowa Żółta (Fig. 3) exhibited differing symptoms after the inoculation with three PNRSV isolates (Table 1). *Chenopodium amaranticolor* and *C. murale* as well as *Phaseolus aureus* were infected with the isolate PNRSV-R3 and PNRSV-R2, respectively. The reaction of cucurbita plants (*C. maxima* cv. Ambar, *C. pepo* cv. Astra, Gagat, Soraya) to all isolates was similar. The PNRSV-R1 isolate seemed to be the most pathogenic. It produced more severe symptoms on *Cucurbitaceae* plants than PNRSV-R2 or PNRSV-R3. Abundant systemic chlorotic spots and leaf deformation were found on *Chenopodium quinoa* inoculated with the PNRSV-R1 isolate (Fig. 4), whereas PNRSV-R3 caused local symptomless infection, and PNRSV-R2 did not infect this plant.

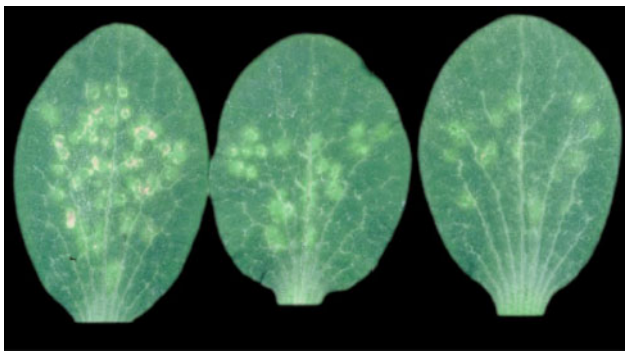

Fig. 3 Local chlorotic rings and necrosis on cotyledons of *Cucurbita pepo* cv. Melonowa Żółta inoculated with isolates PNRSV-R1 (*left*—two cotyledons) and PNRSV-R2 isolate (*right*—one cotyledon)

Fig. 4 Systemic numerous chlorotic rings on the leaves of *Chenopopodium quinoa* inoculated with PNRSV-R1 isolate

Table 1 Symptoms produced on test plants by three PNRSV isolates from roses

Test plant species	PNRSV isolates		
	R1	R2	R3
Chenopodium amaranticolor	−/−	−/−	L/−
Chenopodium murale	−/−	l/s	−/−
Chenopodium quinoa	l/S	−/−	l/−
Cucumis sativus cv. Wisconsin	L/−	L/−	L/−
Cucurbita maxima cv. Ambar	L/S	L/−	L/−
Cucurbita maxima cv. Buttercup	L/S	L/−	L/−
Cucurbita pepo cv. Astra	L/s	l/S	l/S
Cucurbita pepo cv. Gagat	L/S	l/−	l/−
Cucurbita pepo cv. Melonowa Żółta	L/s	L/−	L/−
Cucurbita pepo cv. Soraya	L/s	l/s	l/s
Phaseolus aureus	−/−	l/s	−/−
Nicotiana rustica	l/S	l/S	l/S
Nicotiana tabacum cv. Samsun	−/−	−/−	−/−

−/−, no infection (checked by back inoculation to susceptible host); l/−, latent infection on inoculated leaf, no systemic infection; L/−, visible symptoms on inoculated leaf, no systemic infection; l/s, latent infection; l/S, latent local infection, visible systemic infection; L/s, visible symptoms on inoculated leaf, latent systemic infection; L/S, visible symptoms on inoculated leaf, visible systemic infection

Amplification of the CP gene of RNA3 produced specific PCR products of the expected size of 700 bp for plants infected with each of the three PNRSV isolates studied. Nucleotide sequence of the CP gene was read (681 bp) (Fig. 5). Such a product was not obtained when healthy plants were analyzed. After amplification of the RNA3 MP gene fragment, PCR products of the expected size of 900 bp were found in plants infected with each of the three PNRSV isolates. No product was observed in samples from healthy plants.

Analysis of nucleotide sequence of the CP gene proved that the sequences determined for particular isolates are very similar. Sequence similarity among all the three PNRSV isolates was 98.7–99.1%.

The amino acid sequences of the CP of the three PNRSV isolates studied were deduced and shown to be 226 amino acids (Fig. 6).

Nucleotide sequence of the MP gene (852 bp) was read for the PNRSV-R1 isolate. The amino acid sequence of the MP gene of the PNRSV-R1 isolate was deduced (283 amino acids).

The nearly complete nucleotide sequence of RNA3 of the PNRSV-R1 isolate has been determined. The RNA sequence is 1,630 nucleotides and contains two open-reading frames of 681 nucleotides (ORF1) and 852 nucleotides (ORF2), separated by an intergenic region of 76 nucleotides. The sequence has been deposited at GenBank with Acc. No. DQ003584.

Discussion

The different host susceptibility to various PNRSV isolates is known (Hobbs 1951; Cropley et al. 1964; Waterworth and Fulton 1964; Tuzovic and Digiaro 1992; Paduch-Cichal 2000, 2003; Salem et al. 2004). However, we did not find any information in the literature about differences in PNRSV pathogenicity among isolates derived from roses. Such differences in symptoms produced by three rose PNRSV isolates inoculated on nine selected test plants are reported here. *Chenopodium amaranticolor* and *C. murale* plants reacted with symptoms and in *Phaseolus aureus* systemic symptomless infection was noted to a single PNRSV isolate only. The reaction of test plants belonging to *Cucurbitaceae* differed to the extent that can be used for virus pathogenicity differentiation. Regarding our present results, we may recommend *Cucumis sativus* cv. Wisconsin, *Cucurbita maxima* cv. Buttercup, *C. pepo* cv. Melonowa Żółta, *Chenopodium amaranticolor* and *C. quinoa* as

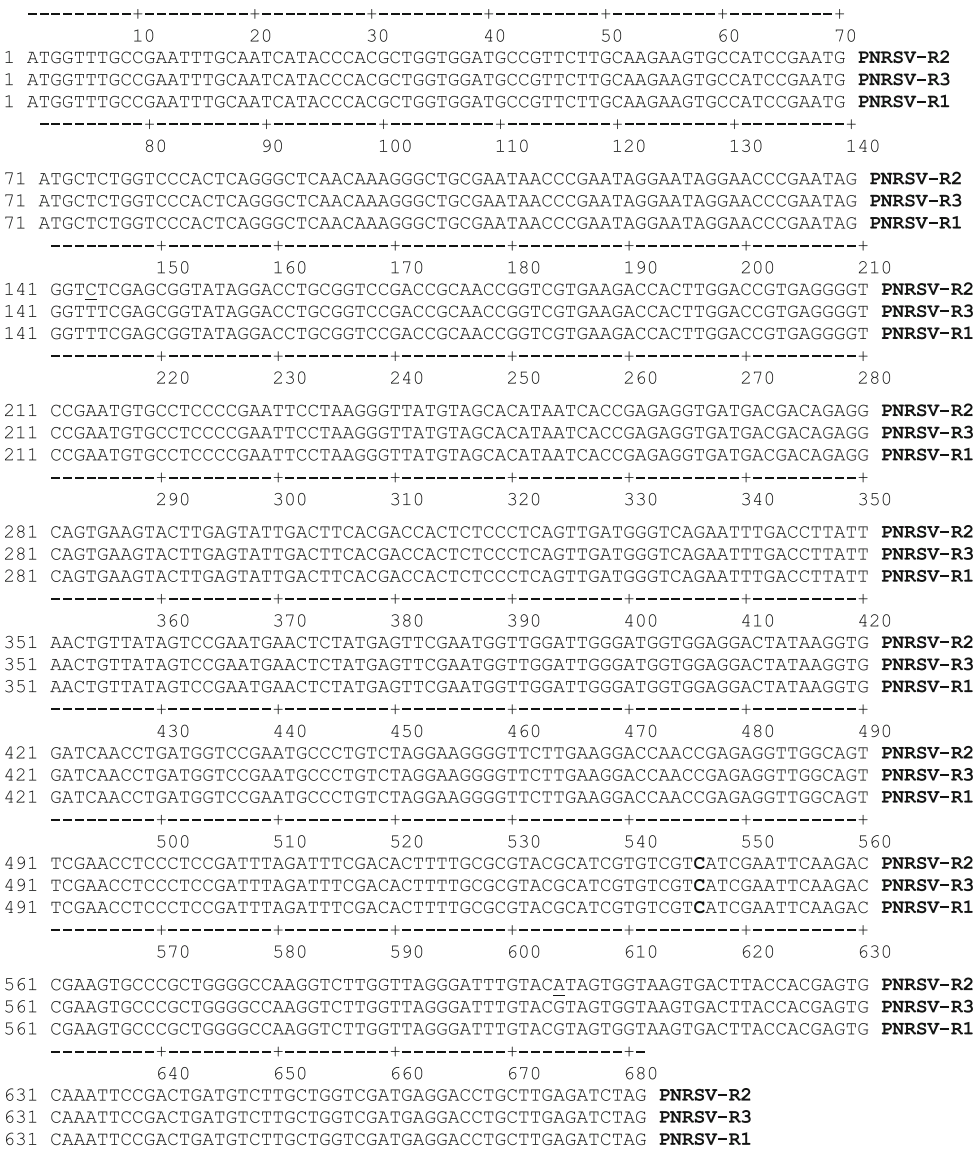

Fig. 5 Nucleotide sequence of the CP gene of three PNRSV isolates

specially useful for revealing the differences among rose PNRSV isolates. *Cucurbita pepo* cv. Astra and Gagat can be used as test plants for the differentiation of PNRSV isolates too. No significant differences in pathogenicity among the PNRSV isolates studied here and virus isolates previously reported are observed (Hobbs 1951; Cropley et al. 1964; Waterworth and Fulton 1964; Tuzovic and Digiaro 1992; Paduch-Cichal and Skrzeczkowska 1994; Paduch-Cichal 2000, 2003; Salem et al. 2004; Sala-Rejczak 2007). We found *Nicotiana rustica* as a new useful biological difference indicator of PNRSV isolates obtained from *Prunus* (Paduch-Cichal 2000; Sala-Rejczak 2007) and rose.

No significant differences among the three studied PNRSV isolates were noted in the crude sap properties. Thermal inactivation point (TIP)—59°C was higher (2–14°C) than that reported for almond, apricot, plum or sour–sweet cherries PNRSV isolates. Dilution end point (DIP) (from 1/32 to 1/64 for PNRSV-R3 and for PNRSV-R1;1/128–1/256 to 1/256–1/512 for PNRSV-R2) observed in this study was similar to the previously reported ones (Paduch-Cichal and Skrzeczkowska 1994; Paduch-Cichal 2000, 2003; Sala-Rejczak 2007). Rose PNRSV isolates were less stable in plant sap at room temperature (longevity in vitro; LIV; 6 h for PNRSV-R1;10 h for PNRSV-R2 and PNRSV-R3) than *Prunus* isolates (16–35 h) studied by Paduch-Cichal and Skrzeczkowska (1994), Paduch-Cichal (2000, 2003) and Sala-Rejczak (2007).

Methods based on the IC-RT-PCR technique using specific primers (Malinowski and Komorowska 1998) allowed to obtain amplicons of 700 bp of the RNA3 CP gene for all the three PNRSV isolates studied. After sequencing, these RNA fragments showed a high degree of similarity (98.7–99.1%).

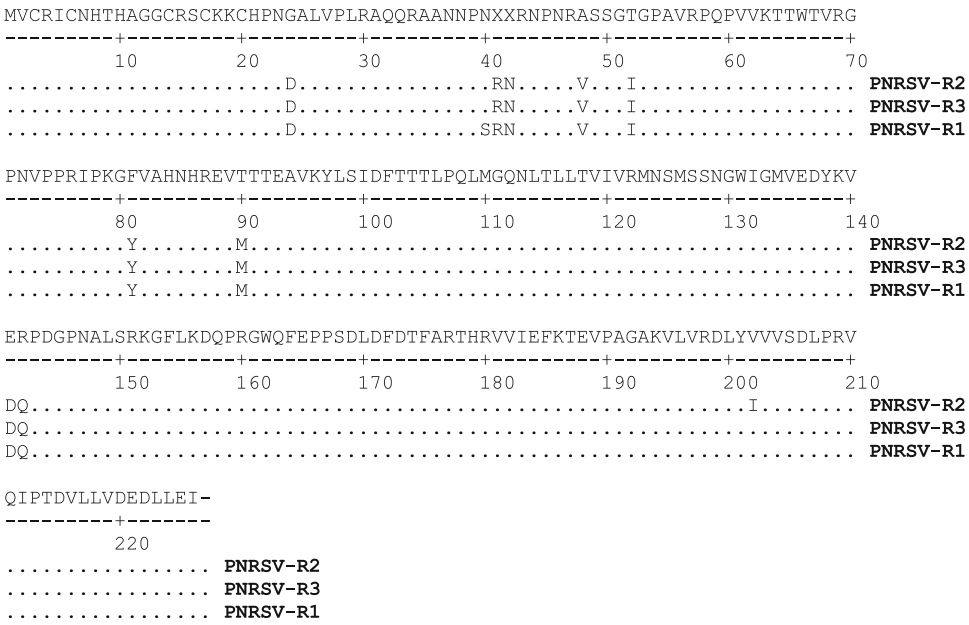

Fig. 6 The sequences of amino acids of the CP of rose PNRSV isolates with consensus sequence (GenBank Acc. No. S78312, Guo et al. 1995)

Comparison of the PNRSV-R1, PNRSV-R2, and PNRSV-R3 CP genes with the sequences of the PNRSV isolates collected in GenBank showed a high degree of similarity and allowed to classify all the isolates studied into the PNRSV-PV32 standard group (Sanchez-Navarro and Pallas 1997). Hammond (2003) reported that the sequence analysis of the RNA3 CP gene of rose PNRSV isolates RING 13, RING 25 and RING 26 isolated in France allowed to classify them into the PNRSV-PV32 standard group. Similarly, the sequences of the CP gene of RNA3 of French rose PNRSV isolates MAJHO, MATI and NOIR also represented the PNRSV-PV32 standard group (Moury et al. 2001). Similar results were obtained by Yurtmen et al. (2002) using PNRSV-R0 isolate from Italian rose cv. Mercedes. In the presented work, classification in the PNRSV-PV96 standard group for sequences of the MP gene of RNA3 of the PNRSV-R1 isolate was also established. To our knowledge, no information on the sequences of the MP gene in other rose isolates of PNRSV is available in the literature. In addition, the nearly complete nucleotide sequences (1,630 bp) of RNA3 of PNRSV-R1 isolates were obtained. The highest similarity (98%) was found between the PNRSV-R1 isolate and a rose PNRSV isolate from India (GenBank Acc. No. AJ969095), isolate from *Malus* sp.(GenBank Acc. No. Y07568) and isolate from peach (GenBank Acc. No. U57046).

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