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MOLECULAR VARIABILITY OF THE COAT PROTEIN GENE OF *Prunus necrotic ringspot virus* ISOLATES

Kinga Sala-Rejczak, Elżbieta Paduch-Cichal
Warsaw University of Life Sciences

Abstract. We have obtained and described the nucleotide sequences corresponding to ilarvirus *Prunus necrotic ringspot virus* (PNRSV] coat protein (CP) gene in eleven Polish isolates, from different *Prunus* and *Rosa* species and four isolates provided in plant material from Australia, Hungary and Italy. Virus identification was possible using specific primers allowed amplifying the 700 bp amplicon of coat protein gene. The product was obtained used IC-RT-PCR. The results indicated no association between the host species or the geographic origin and the PNRSV CP sequence specificity. The CP gene nucleotide sequence of studied isolates allowed for clustering them into the previously reported PV32-I, PV96-II and PE5-III phylogroups.

Key words: PNRSV, CP, IC-RT-PCR, sequencing

INTRODUCTION

Stone fruit trees are affected by a large number of viruses, which cause significant economic losses. Among them the most destructive is ilarvirus *Prunus necrotic ringspot virus* (PNRSV). PNRSV is world-wide distributed and affects many *Prunus* species, including almond, apricot, cherry, nectarine, peach, and plum trees. It is the most common rose virus in Europe [Moury et al. 2000, Paduch-Cichal 2003]. Symptoms caused by PNRSV vary widely from no obvious change to serious damage resulting in significant yield and vigour reduction [Uemoto and Scott 1992].

PNRSV belongs to the genus *Ilarvirus*, family *Bromoviridae.* The PNRSV genome is organized into three plus-stranded RNAs. RNAs 1 and 2 encode for proteins involved in viral replication. RNA 3 encodes for a 5′-proximal movement protein (MP) and for a 3′-proximal coat protein (CP), which is expressed via a sub-genomic RNA 4 [Roossinck et al. 2005].

Recent advances in molecular biology provided new tools for the identification and classification of plant viruses. Strain differentiation by PCR techniques or RLFP analy-

Corresponding author: Kinga Sala-Rejczak, Department of Plant Pathology, Warsaw University of Life Sciences WULS-SGGW, ul. Nowoursynowska 159, 02-776 Warsaw, Poland, phone: (+48) 22 59 320 31, e-mail: kinga_sala_rejczak@sggw.pl

sis have been carried out in several laboratories. Many different isolates of PNRSV have been characterized and phylogenetically grouped into three previously reported groups named PV32-I, PV96-II and PE5-III [Guo et. al. 1995, Hammond and Crosslin 1995, Sanchez-Navarro and Pallas 1997]. Analysis of different isolates revealed a lack of association between specific nucleotide or amino-acids patterns with the symptomatology, host species or geographic origin. Analyses of the coding sequences of CP revealed that protein is highly conserved despite the host and geographical origin of the compared isolates [Scott et al. 1998, Aparicio et al. 1999, Spiegel et al. 1999, Vašková et al. 2000, Moury et al. 2001, Glasa et al. 2002, Yurtmen et al. 2002, Hammond 2003, Spiegel et al. 2004, Salem et al. 2004, Ulubas and Ertunc 2004, Fiore et al. 2008, Oliver et al. 2009]. *Prunus necrotic ringspot virus* is distributed world-wide and cause annually significant losses in stone fruit trees. However, no molecular data have been reported about isolates from Poland until now. The goal of our work was to compare the sequences of cp gene of the Polish isolates with others retrieved from GenBank database and establish the correlation between analyzed sequences and origin of the isolates.

MATERIALS AND METHODS

Eleven PNRSV isolates were obtained in Poland, two were from Italy and two were from Australia and Hungary, respectively. The collected PNRSV isolates are listed in Table 1.

Table 1. *Prunus necrotic ringspot virus* (PNRSV) isolates used in this study

Accesion number	Isolate	Source plant	Variety (where known)	Origin of isolate
EU368735	PNRSV-AL1	almond	'Strout's Papershell'	Australia
EU368736	PNRSV-AL17	almond	'Tardy non Pareil'	Italy
DQ983493	PNRSV-AprI/9	apricot	unknown	Poland
EU368738	PNRSV-Mk	sour cherry	unknown	Hungary
DQ983491	PNRSV-N2	sour cherry	'Łutówka'	Poland
DQ983492	PNRSV-PE56	peach	'Meredith'	Poland
DQ983495	PNRSV-PL1	plum	'Bluefre'	Poland
DQ983499	PNRSV-PL7	plum	'Empress'	Poland
DQ983496	PNRSV-PL9	plum	'Opal'	Poland
DQ983499	PNRSV-PL21	plum	'Empress'	Poland
EU368737	PNRSV-PL38	plum	unknown	Italy
DQ983494	PNRSV-SW2	sweet cherry	'Heidegger'	Poland
DQ003584	PNRSV-R1	rose	'Queen Elizabeth'	Poland
DQ983497	PNRSV-R2	rose	'Ingrid Bergman'	Poland
DQ983498	PNRSV-R3	rose	'Montezuma'	Poland

The PNRSV was isolated from different plant species (almond, apricot, sour- and sweet-cherry, peach, plum and rose). Samples were collected during spring months from buds and leaves. All plant materials were subjected to DAS-ELISA using PNRSV specific commercial polyclonal antiserum (Loewe Biochemica GmbH, Germany) according to Clark and Adams [1977]. PNRSV isolates from different *Prunus* species were transferred to *Prunus aviun* clone F12/1 using chip budding technique. Inoculation of these plants with PNRSV-PL7 and PNRSV-PL21 isolates was not successful [Paduch-Cichal et al. 2007].

Immunocapture – reverse transcription – polymerase chain reaction (IC-RT-PCR) was conducted on the strenght of Wetzel et al. [1992], Nolasco et al. [1993] protocols and Malinowski [2005]. One Tube RT-PCR System (Roche, Germany) was used with reagents from *Tag* PCR Core Kit (Qiagen, USA) according to recommendations of the producer. To perform immunocapture samples were coated with IgG like in DAS--ELISA test (Loewe Biochemica GmbH, Germany). Extracts were prepared from buds and leaves of *P. avium* clone F12/1 infected with PNRSV isolates and plum cv. 'Empress' infected with PNRSV-PL7 and PNRSV-PL21isolates, in 1:20 PBS-TPO (Phosphate buffered saline with Tween, polyvinyl pyrrolidine and ovalbumin), pH 7.4.

The RT-PCR reactions were performed using two primers: NRSCPF3 (5'-ATGGTTTGCCGAATTTGCAATCAT-3') as the sense primer, and NRSCPR4 (5'-GAGTGTGCTTATCTCACTCTAG-3') as the antisense primer [Malinowski and Komorowska 1998]. RT-PCR was performed in a MJ Research PCT-200 thermocycler as follows: 30 min at 50°C and 2 min at 94°C, followed by 35 cycles: 10 s at 94°C, 30 s at 53°C, and 40 s at 68°C. The final incubation was performed at 68°C for 7 min. The RT-PCR products were separated by electrophoresis on 1.2% agarose gel, stained with ethidium bromide.

The PCR products were purified via the Agarose DNA Gel extraction Kit (Roche, Germany) and sequenced (Institute of Biochemistry and Biophysics, Poland) using 3730 DNA Analyzer (Hittachi AB Applied Biosystems, USA) with above mentioned primers using DYEnamic™ ET Terminator Cycle Sequencing Kit. Sequence data were read using Chromas (Technelysium Pty Ltd, Australia) and analysed using DNASTAR programme package (Lasergene, USA) and the internet service NCBI (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments of all fifteen PNRSV isolates with isolates registered in GenBank were performed using algorithm of ClustalW (http://www2.ebi.ac.uk/clustalw) [Thompson et al. 1994].

RESULTS AND DISCUSSION

Virus identification was possible with specific primers (NRSCPF3 and NRSCPR4) [Malinowski and Komorowska 1998], which allowed amplifying the 700 bp amplicon of CP gene of RNA3. The specific 700 bp amplicon was present in all infected with PNRSV plant samples and there was no product in healthy plants. This specific product was obtained in IC-RT-PCR technique.

The CP gene of all fifteen PNRSV isolates was sequenced. There were obtained 675 nucleotides of CP gene sequence for eleven isolates (PNRSV-AL1, PNRSV-AL17,

PNRSV-AprI/9, PNRSV-Mk, PNRSV-PE56, PNRSV-PL1, PNRSV-PL7, PNRSV-PL9, PNRSV-PL21, PNRSV-PL38) and 681 nucleotides for isolates: PNRSV-R1, PNRSV-R2, PNRSV-R3, PNRSV-SW2. The first 24 and the last 4 nucleotides in sequences are the copies of the primers used for RT-PCR. The nearly complete nucleotide sequences of coat protein gene, the new PNRSV isolates reported in this paper were submitted to GenBank (Accession Numbers in tab. 1).

The comparative analysis of the CP gene sequences of studied isolates allowed to classify them into three groups: group 1 – isolates: PNRSV-AL1, PNRSV-AL17, PNRSV-AprI/9, PNRSV-Mk, PNRSV-N2, PNRSV-PE56, PNRSV-PL1, PNRSV-PL9, PNRSV-PL38; group 2 – isolates: PNRSV-R1, PNRSV-R2, PNRSV-R3, PNRSV-SW2; group 3 – isolates: PNRSV-PL7, PNRSV-PL21. The analysis of the nucleotide CP sequences of these PNRSV isolates revealed overall similarities of 97.4% to 99.1% among all isolates in group 1 and group 2. High similarity values were observed between members of these two groups (94.3–96%). The lower percentage of similarity were noted between group 3 and group 2 or group 1 of isolates (88.1–88.5%, 89.4–90.3%, respectively). 100% of similarity was noted between PNRSV-PL7 and PNRSV-PL21 (fig. 1).

Percentage of similarity / isolates															
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	
100	99.1	98.2	98.7	98.7	97.8	98.2	97.8	97.8	95.2	94.7	94.7	94.7	89.4	89.4	**1**
	100	98.7	99.1	99.1	98.2	98.7	98.2	98.2	95.6	95.2	95.2	95.2	89.9	89.9	**2**
		100	98.2	98.2	97.4	97.8	97.4	97.4	96.0	95.6	95.6	94.7	89.4	89.4	**3**
			100	98.7	97.8	98.2	97.8	97.8	95.2	94.7	94.7	94.7	89.9	89.9	**4**
				100	98.7	99.1	98.7	98.7	96.0	95.6	95.6	95.2	89.9	89.9	**5**
					100	99.1	98.7	98.7	95.2	94.7	94.7	94.3	90.3	90.3	**6**
						100	99.1	99.1	95.6	95.2	95.2	94.7	89.9	89.9	**7**
							100	98.7	95.2	94.7	94.7	94.3	89.4	89.4	**8**
								100	95.2	94.7	94.7	94.3	89.4	89.4	**9**
									100	99.1	99.1	97.8	88.5	88.5	**10**
										100	98.7	97.4	88.1	88.1	**11**
											100	97.8	88.1	88.1	**12**
												100	88.5	88.5	**13**
													100	100	**14**
														100	**15**

PNRSV isolates: 1. PNRSV-AL17, 2. PNRSV-N2, 3. PNRSV-Mk, 4. PNRSV-PL38, 5. PNRSV-AL1, 6. PNRSV-PE56, 7. PNRSV-AprI/9, 8. PNRSV-PL1, 9. PNRSV-PL9, 10. PNRSV-R3, 11. PNRSV-R2, 12. PNRSV-R1, 13. PNRSV-SW2, 14. PNRSV-PL7, 15. PNRSV-PL21

Fig. 1. Comparison of the percentage of identity among fifteen nucleotide sequnces of the coat protein gene of *Prunus necrotic ringspot virus* isolates

The relationships among the CP gene sequences our isolates and isolates from GenBank revealed that all fifteen PNRSV isolates were assigned to three previously described model phylogroups: group PV32-I [Acc. No. Y07568, Sanchez-Navarro and Pallas 1997] – PV96-II [Acc. No. S78312, Guo et al. 1995], group PE5-III [Acc. No. L38823, Hammond and Crosslin 1995]. Three PNRSV isolated from rose (PNRSV-R1, PNRSV-R2, PNRSV-R3) and one isolate from sweet-cherry (PNRSV-SW2) were divided into PV32-I group. Isolates: PNRSV-PE56 (peach), PNRSV-AL1 and PNRSV-AL17 (almond), PNRSV-AprI/9 (apricot), PNRSV-PL1, PNRSV-PL9 and PNRSV-PL38 (plum), as well as PNRSV-Mk and PNRSV-N2 (sour-cherry) represented the PV96-II group. However, isolates from plum trees cv. 'Empress' (PNRSV-PL7, PNRSV-PL21) were classified into PE5-III group. The sequence similarity between examined and model isolates indicated over 90% similarities. Isolates PNRSV-PL7 and PNRSV-PL21, showed only 88.1–90.3% similarities to the remaining examined isolates of the PNRSV. Scott et al. [1998] and Aparicio et al. [1999] also received over 90% resemblances in the sequence of CP gene of PNRSV isolates in the ratio to PV32-I, PV96-II and PE5-III model isolates. The sequence of CP gene in rose and sweet-cherry isolates was 6 nucleotides longer than others investigated. Moreover, sequences of two plum isolates (PNRSV-PL7 and PNRSV-PL21) differed in about 40 positions from sequences of isolates belonging to the PV96-II group and in about 50 positions in the ratio to isolates from the PV32-I group. Yurtmen et al. [2002] classified peach isolate PNRSV-M3 and rose isolate PNRSV-Ro into the PV32-I group. Sequences for these isolates were 6 nucleotides longer too. Hammond [2003] classified 68 PNRSV sequences encoding CP, which contained 6 nucleotides additionally, into PV32-I group. Moury et al. [2001] characterized genomes of roses isolates. They stated that isolates didn't probably constitute the independent phylogenetic line and should categorize them to the PV96-II group or PV32-I. Glasa et al. [2002] classified 25 PNRSV isolate of plums and sour-cherries, coming from Slovakia to one group, represented by the PV96-II isolate. Here belongs to this group a lot of isolates of virus coming from USA, central Europe and area of the Mediterranean sea. Oliver et al. [2009] clustered PNRSV isolates from cherry trees in New York in the predominant group PV-96-II too. Ulubas and Ertunc [2004] as a result of phylogenetic analysis and RFLP analysis assigned the majority of examined isolate to the PV96-II group and one from peach to the PV32-I group, none belonged to the PE5-III group. At the presented work only PNRSV-PL7 and PNRSV-PL21 were in this group. Aparicio et al. [1999] divided one peach isolate and one sour-cherry isolate to the PE5-III group.

Amino acids sequences were deduced from the nucleotide sequence of CP gene of all isolates. Putative translation of capsid protein gene of our isolates comprised of 224 (11 isolates) or 226 (4 isolates) amino acids. The coat protein gene of PNRSV-SW2 ('sweet cherry' isolate) and PNRSV-R1, PNRSV-R2 and PNRSV-R3 ('rose' isolates) was 6 nucleotides longer at positions 119–124 than CP gene of the others isolates studied. Addition of 6 nukleotides resulted in two corresponding extra amino acids arginine and asparagine in the putative polypeptyde of virus coat protein.

The alignment of CP amino acid sequences shows a conserved protein, except at its N-terminus, where most of the differences among all isolates are contained. The extra residues of the PV32-I group are inside of the RNA-binding domain described for

PNRSV. Amino acids from 25 to 50 are essential for the protein to bind PNRSV RNA in vitro [Aparicio et al. 2003, Codoñer et al. 2006].

Overall, results of this research and data accessible in literature [Neeleman et al. 1991, Shintaku et al. 1992, Hammond and Crosslin 1998, Scott et al. 1998, Aparicio et al. 1999, Spiegel et al. 1999, Paduch-Cichal 2000, Moury et al. 2001, Glasa et al. 2002, Yurtmen et al. 2002, Hammond 2003, Ulubas and Ertunc 2004] indicate that the membership of PNRSV isolates in one of the several main groups is not correlated with the geographical origin of a given isolate. However, it is not possible to rule out, that changes in the sequence of amino acids of coat protein can affect biological properties of the virus [Neeleman et al. 1991, Hammond and Crosslin 1995 and 1998].

CONCLUSIONS

1. Virus identification was possible using specific primers (NRSCPF3 and NRSCPR4) which allowed amplifying the 700 bp amplicon of coat protein gene of RNA3. The specific product was obtained in IC-RT-PCR technique.

2. The PNRSV isolates coat protein gene nucleotide sequences were classified into the previously reported PV32-I, PV96-II and PE5-III phylogroups.

3. There was no association between the host species or the geographic origin and the PNRSV coat protein sequence specificity.

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ZRÓŻNICOWANIE GENETYCZNE GENU KODUJĄCEGO BIAŁKO PŁASZCZA IZOLATÓW *Prunus necrotic ringspot virus*

Streszczenie. W pracy uzyskano i opisano sekwencje genu kodującego białko płaszcza 11 izolatów wirusa nekrotycznej pierścieniowej plamistości wiśni (*Prunus necrotic ringspot virus*, PNRSV) z różnych gatunków roślin z rodzaju *Prunus* i *Rosa* pochodzących z Polski oraz czterech izolatów wirusa uzyskanych w materiale roślinnym z Australii, Węgier i Włoch. Wykorzystując specyficzne startery, uzyskano produkt 700 par zasad obejmujący gen kodujący białko płaszcza wirusa. Do amplifikacji zastosowano technikę IC-RT-PCR. Wykazano brak korelacji pomiędzy gatunkiem, pochodzeniem izolatu, a sekwencją nukleotydów białka jego płaszcza. Sekwencje genu kodującego CP badanych izolatów przyporządkowano do trzech głównych filogenetycznych grup wzorcowych: PV32-I, PV96-II i PE5-III.

Słowa kluczowe: PNRSV, CP, IC-RT-PCR, sekwencjonowanie

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