Molecular Variability Among Isolates of Prunus Necrotic Ringspot Virus from Different *Prunus* spp.

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ABSTRACT

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Viral sequences amplified by polymerase chain reaction from 25 isolates of Prunus necrotic ringspot virus (PNRSV), varying in the symptomatology they cause in six different *Prunus* spp., were analyzed for restriction fragment polymorphisms. Most of the isolates could be discriminated by using a combination of three different restriction enzymes. The nucleotide sequences of the RNA 4 of 15 of these isolates were determined. Sequence comparisons and phylogenetic analyses of the RNA 4 and coat proteins (CPs) revealed that all of the isolates clustered into three different

groups, represented by three previously sequenced PNRSV isolates: PV32, PE5, and PV96. The PE5-type group was characterized by a 5' untranslated region that was clearly different from that of the other two groups. The PV32-type group was characterized by an extra hexanucleotide consisting of a duplication of the six immediately preceding nucleotides. Although most of the variability was observed in the first third of the CP, the amino acid residues in this region, which were previously thought to be functionally important in the replication cycle of the virus, were strictly conserved. No clear correlation with the type of symptom or host specificity could be observed. The validity of this grouping was confirmed when other isolates recently characterized by other authors were included in these analyses.

Prunus necrotic ringspot virus (PNRSV) is responsible for economically important diseases in most cultivated *Prunus* spp. throughout the world (28). Many variants have been described covering a wide range of biological diversity (3,5,12). Some isolates do not induce symptoms, whereas others produce characteristic necrotic spots and shot holes in young leaves. The existence of clearly different "pathotypes" has been well described in sweet cherry trees, in which isolates could be classified as M or R depending on whether they produced mild mottle or rugose mosaic symptoms, respectively (5,12). Moreover, isolates from this host can be grouped into three different serotypes (CH3, CH9, and CH30) (13). Recently, sequence comparisons among three mild and three rugose isolates belonging to the CH9 serotype revealed that single nucleotide and amino acid differences in both the movement protein (MP) and the coat protein (CP) correlated well with serological relationships and symptom type (9). Unfortunately, this correlation could not be observed when CH30 serotype isolates were compared. On the other hand, Scott et al. (25) recently showed that the CPs and putative MPs of five PNRSV isolates are extensively conserved irrespective of their host species or geographic origins. More importantly, the authors (25) were unable to associate any of the changes observed with the symptoms caused by these isolates in cucumber or with the range of host species that they infect, thus reflecting that the situation could be much more complicated than that in sweet cherry trees.

PNRSV is a positive-sense RNA plant virus with a tripartite genome that belongs to the genus *Ilarvirus* (19). *Ilarvirus* spp. have the same genomic organization, encoding functionally similar translation products, as members of the genera *Bromovirus*, *Cucumovirus*, and *Alfavirus*, all of which belong to the family *Bromoviridae* (4). RNAs 1 and 2 of viruses in the family *Bromoviridae* encode the replicase proteins P1 and P2, respectively. RNA 3 is bicistronic and encodes the MP (or P3) and the CP. CP syn-

thesis occurs via a subgenomic monocistronic mRNA (RNA 4). Studies of evolutionary relationships, considering first RNA 3 (22) and later RNAs 1 and 2 (17,24), led to the proposal that alfalfa mosaic virus (AMV) should be considered a true *Ilarvirus* sp. instead of forming a distinct genus. Accordingly, Sánchez-Navarro et al. (23) showed that the replacement of the MP and CP genes of AMV by the corresponding genes of PNRSV resulted in basic competence for encapsidation and replication in protoplasts and for a low level of cell-to-cell movement in tobacco plants transformed with the replicase proteins.

The relative molecular masses of the CPs of some PNRSV isolates vary within a range of about 27 to 29 kDa (5). Moreover, it has been recently demonstrated that there are differences in the electrophoretic mobility of the RNA 4 transcripts synthesized from the corresponding polymerase chain reaction (PCR) products from six different PNRSV isolates (18). As mentioned above, there are few data about the possible correlation of this heterogeneity and biological properties for PNRSV isolates and for *Ilarvirus* spp. in general. Only in the case of AMV, a putative member of the *Ilarvirus* genus, has the CP gene been shown to play a key role in symptom determination (14). To increase knowledge of the molecular variability among PNRSV isolates and in an attempt to correlate this variability with their biological diversity, we determined the primary structure of the RNA 4 of 15 PNRSV isolates that differ in symptom expression and originate from six different *Prunus* hosts and four different countries.

MATERIALS AND METHODS

Plant material and RNA extraction. Twenty-five PNRSV isolates from different *Prunus* hosts and geographical locations were either obtained from a collection kept at the Istituto Agronomico Mediterraneo (Bari, Italy) or collected from orchards in other countries (Table 1). Total nucleic acid was extracted from 0.5 g of fresh leaf tissue for each isolate as described previously (20). Samples were analyzed for the presence of the virus by nonisotopic molecular hybridization as described (15).

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cDNA synthesis and PCR amplification. First-strand cDNA synthesis was carried out in a 20- μ l reaction containing 50 mM Tris-HCl, pH 8.3; 50 mM KCl; 10 mM MgCl₂; 10 mM dithiothreitol; 0.5 mM spermidine; 1 mM of each dNTP; 40 U of ribonuclease inhibitor (Amersham International, Cleveland, OH); 8 U of AMV-reverse transcriptase (RT) (Promega Corp., Madison, WI); and 100 pmol of antisense primer VP33 (5'-CCGGCTGCAGCT-TCCCTAACGGGC-3'). The 3' 15 nucleotides (nt) of the primer are complementary to the 3' terminus of PNRSV RNA 4 (22) and are preceded by the restriction site *Pst*I (underlined). PCR amplifications were made using primer VP33 and the sense primer VPC (5'-CTTGCCATGGTTTGGCGAATTTGC-3', which contains a sequence identical to nt 1,102 to 1,119 and a *Nco*I site, underlined). For cloning and sequencing purposes, the sense primer used was VP56 (5'-GTTAACGGATCCGTTTCTTTCTTTCTTTCTTCCG-3' containing the first 21 nt of RNA 4, a *Bam*HI site [underlined] and six additional unrelated residues at its 5' end). For the P4090 and C7140 isolates (Table 1), primer VP67 (5'-GTACGGATCCTA-TTTTATTCCCGATTGC-3', identical to nt 730 to 751 of RNA 3, and containing a *Bam*HI site [underlined]) (Fig. 1A) was used. Five microliters of the reverse-transcription products, 10 μ l of 10 $\times$ buffer (166 mM (NH₄)₂SO₄, 670 mM Tris-HCl, pH 8.8, and 0.1% Tween 20), 3 mM MgCl₂, 0.2 mM of each dNTP, and 60 pmol of each primer were mixed in a total volume of 100 μ l for the PCR reactions. Prior to thermal cycling, 1 U of Ecotaq DNA polymerase (Ecogen SRL, London) for restriction fragment length polymorphism (RFLP) analyses or Ecozyme DNA polymerase (Ecogen SRL) for sequencing purposes was added and the 100- μ l samples overlaid with mineral oil (Sigma Chemical Co., St Louis). PCR was carried out in a Perkin-Elmer 2400 thermal cycler (Perkin-Elmer Corp., Norwalk, CT) programmed for one cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 45 s, 51°C for 45 s, and 72°C for 1 min, finishing with an extension at 72°C for 5 min. Amplified products (5 μ l each) were electrophoresed in 1% agarose gels in 40 mM Tris-acetate and 1 mM EDTA, pH 8.0 (TAE), and stained with ethidium bromide to confirm the expected size of the PCR products. A total of 15 μ l of the PCR products generated was directly digested with 10 U of *Eco*RI, *Rsa*I, and *Taq*I (Boeh-

ringer GmbH, Mannheim, Germany) and adjusted to a final volume of 20 μ l with the corresponding buffer. The mixtures were incubated for 4 h at 37°C, except that containing *Taq*I, which was incubated at 65°C. DNA fragments were separated by electrophoresis in 10% polyacrylamide gel for *Eco*RI and *Rsa*I and 15% polyacrylamide gel for *Taq*I, using TAE buffer. The gels were stained in ethidium bromide and photographed using Polaroid film and an orange filter.

The restriction fragments were identified by position in the gel, and the presence or absence (1 or 0, respectively) of each was recorded in a restrictotype matrix to obtain a clustering of the isolates using the RESTML program of the PHYLIP 3.5c package (6).

Cloning, nucleotide sequencing, and sequence comparisons. PCR products obtained with the pairs of primers VP33-VP56 and VP33-VP67 (Fig. 1A) were extracted from 1% agarose gels using USBioclean (Amersham International), cloned into the *Bam*HI-*Pst*I cut pUC18 vector, and transformed into *Escherichia coli* DH5 α cells. For all isolates, positive clones were identified by restriction analysis and sequenced in both orientations by using universal and specific internal primers with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer Corp.). The isolate sequences were named using an eight-letter code. The first three letters correspond to the source of the isolate, followed by two letters indicative of the country, and the last three letters indicating the cultivar (e.g., the first Italian PNRSV isolate obtained from apricot cv. Napoletana would be named AprIt.nap1).

Homology and similarity between sequences were compared using the GAP and OLDDISTANCES programs in the GCG software package (version 9.1; Genetic Computer Group, Inc., Madison, WI). Multiple alignments were obtained using Clustal W (27). Phylogenetic analyses were performed using the following programs of the PHYLIP 3.5c package (J. Felsenstein, University of Washington, Seattle): DNADIST and PROTPARS to calculate genetic distances, NEIGHBOR (unweighted pair group method with arithmetic means) to cluster the variants from the distance data, and SEQBOOT (100 repetitions) and CONSENSE to perform bootstrap analyses. The TREEV 32 program was used to draw the resulting phylogenetic trees, in which apple mosaic virus served as the outgroup.

TABLE 1. Listing and properties of Prunus necrotic ringspot virus (PNRSV) isolates characterized in this study

Isolate	Source ^a	Symptomatology ^b	Restrictotype ^c	Origin	Sequence code ^d	Size RNA 4 nt/aa	Accession no.
A3430	<u>Apricot cv. Cafona</u>	Line pattern, necrosis	EI, RI, TI	Italy	AprIt.caf 1	884/224	AJ133199
A9170	<u>Apricot cv. Napoletana</u>	Asymptomatic	EI, RI, TI	Italy	AprIt.nap 1	883/224	AJ133200
A8360	<u>Apricot cv. Tiryntos</u>	Line pattern, necrosis	EI, RII, TII	Italy	AprIt.try 1	890/226	AJ133201
A6430	Apricot cv. Errani	Severe necrotic line pattern	EI, RII, TII	Italy	...	...	...
A1400	Apricot cv. Ninfa	Line pattern	EI, RII, TII	Italy	...	...	...
A8380	Apricot cv. Boccuccia	Latent	EI, RI, TI	Italy	...	...	...
M3450	Almond cv. Tardy non Pareil	Shot hole	EI, RVI, TI	Italy	...	...	...
M7960	<u>Almond cv. Preani</u>	Shot hole	EI, RI, TI	Italy	AlmIt.pre 1	884/224	AJ133202
M8610	<u>Almond cv. Core</u>	Latent	EI, RI, TII	Italy	AlmIt.cor 1	884/224	AJ133204
M8520	Almond cv. Oliena	Latent	EI, RI, TI	Italy	...	...	...
P1000	Peach cv. Fantasia	Yellowing, rolling of leaves	EI, RI, TI	Italy	...	...	...
P8450	<u>Peach cv. May Crest</u>	Latent	EI, RI, TI	Italy	PchIt.may 1	884/224	AJ133205
P2000	Peach cv. Early Crest	Latent	EI, RI, TI	Italy	...	...	...
Tunez-111	<u>Peach cv. unknown</u>	Few chlorotic spots	EI, RIII, TII	Tunisia	PchTu.unk 1	890/226	AJ133206
P4090	<u>Peach cv. Marilyn</u>	Latent	EII, RV, TV	Italy	PchIt.mry 1	880/224	AJ133207
P4890	Peach cv. Psal5	Latent	EI, RII, TIV	Italy	...	...	...
NcM1	<u>Nectarine (Murcia)</u>	Few chlorotic spots	EI, RI, TIII	Spain	NctSp.mur 1	884/224	AJ133208
C8220	<u>Cherry cv. Lambert</u>	Latent	EI, RI, TI	Italy	ChrIt.lam 1	884/224	AJ133203
C8320	<u>Cherry cv. Marasca</u>	Latent	EI, RII, TIV	Italy	ChrIt.mrs 1	884/224	AJ133209
C7140	<u>Cherry cv. Black Giant</u>	Some years yellowing	EII, RIV, TV	Italy	ChrIt.bla 1	880/224	AJ133210
PL11	<u>Plum cv. unknown</u>	Chlorotic pattern	EI, RII, TII	Albania	PlmAl.unk 1	890/226	AJ133211
S6340	<u>Plum cv. C. Lopez</u>	Asymptomatic	EI, RI, TI	Italy	PlmIt.clf 1	884/224	AJ133212
S5281	Plum cv. unknown	Chlorotic pattern	EI, RI, TI	Italy	...	...	...
S9090	Plum cv. Stanley	Latent	EI, RII, TII	Italy	...	...	...
S9040	<u>Plum cv. Mirabolán</u>	Latent	EI, RII, TII	Italy	PlmIt.mrb 1	890/226	AJ133213

^a The 15 isolates representing the three main clusters of PNRSV isolates obtained by restriction fragment length polymorphism analysis are underlined.

^b Observed in the natural host over several years.

^c Restrictotypes determined after digestion with *Eco*RI, *Taq*I, or *Rsa*I.

^d For the isolates whose RNA sequence was determined, a new nomenclature was established. The isolate sequences were named using an eight-letter code. The first three letters correspond to the source of the isolate, followed by two letters indicative of the country, and the last three letters indicating the cultivar.

RESULTS AND DISCUSSION

RFLP analysis of PNRSV isolates. To obtain preliminary evidence concerning their molecular diversity, 25 PNRSV isolates from six different *Prunus* hosts (apricot, plum, peach, cherry, almond, and nectarine) were chosen for strain discrimination by RT-PCR-RFLP analysis (Table 1). Primers VP56 and VP33, which are located at the beginning and end, respectively, of PNRSV RNA 4, were used in initial attempts to obtain full-length RNA 4 amplicons (Fig. 1A). No PCR products were obtained with isolates P4090 and C7140; therefore, the primer VPC was used instead of VP56 as the sense primer (located at the beginning of the CP cistron), thus providing amplification products consisting of the complete CP cistron and the 3' untranslated region (UTR) for all isolates. RT-PCR products of the expected size (843 to 850 base pairs [bp]) were digested with three different restriction enzymes (*EcoRI*, *RsaI*,

and *TaqI*). Restriction products from representative isolates, and maps based on these data are shown in Figure 1B and C, respectively. *EcoRI* separated the isolates into two groups (EI and EII). Group EII contained isolates P4090 and C7140, each with an identical pattern to the American isolate PE5, whereas group EI contained the rest of the isolates (Fig. 1B and C, Table 1). Group EI could be subdivided into four new groups when digested with *TaqI* (TI, TII, TIII, and TIV), whereas members of the EII group were included in a separate TV group. The TI and TII groups had the same restriction pattern, except that the 139-bp band of the 152/139-bp doublet in TII had a slightly lower electrophoretic mobility than the corresponding band in the isolates of the TI group, leading to the disappearance of this doublet (Fig. 1B, cf. lanes 1 and 2, *TaqI* gel). Sequence determination of some isolates of these two groups revealed a six-nt insertion that could explain this different electrophoretic mobility (described below). The two members of the TV

Fig. 1. A, Schematic diagram of Prunus necrotic ringspot virus (PNRSV) RNA 3 showing the location of primers used for restriction fragment length polymorphism (RFLP) analyses and for cloning and sequencing of the PNRSV isolates. B, RFLPs of reverse-transcriptase polymerase chain reaction products from eight representative PNRSV isolates: lane 1, isolate A3430; lane 2, A8360; lane 3, Tunes-111; lane 4, NcM1; lane 5, M8610; lane 6, C8320; lane 7, C7140; lane 8, P4090; and lane M, molecular weight markers (1-kilobase ladder; Gibco BRL, Bethesda, MD). C, Schematic diagram representing the fragment patterns for each restriction enzyme used. Each pattern is named with its corresponding endonuclease initial followed by a number.

group (or EII) could only be discriminated when the *RsaI* restriction enzyme was used, providing the RIV and RV patterns, respectively. RFLPs RI and RII obtained with this enzyme were very similar to those of the TI and TII groups (Table 1), except that isolate Tunez-

111, belonging to the TII group, possessed a unique *RsaI* restriction type (Fig. 1B, RIII, *RsaI* gel, lane 3). By scoring the presence or absence (1 or 0, respectively) of each restriction fragment in all the isolates, a matrix was generated that allowed calculation of the ge-

	1	110
PV96	GTTTTCTTTTC-TTCTTCCGAATTCT-TTTCACCCAACAATGGTTTGCCGAATTGCAATCATACCCACGCTGGTGGATGCCGTTCTTGCAAGAAGTGCCATGCCGAATG	
PImIt.cif1		
PchIt.may1	TT.....	
AprIt.nap1		
NctSp.mur1	T.....	
AlmIt.cor1		
AlmIt.pre1	T.....	
AprIt.caf1		
ChrIt.mrs1	CT.....	
ChrIt.lam1	C.....A.....	
PV32	AC..C.....TTTG.T.....A.....	
PImAl.abn1	CA..C.....TTTG.T.....	
AprIt.try1	CA..C.....TTTG.T.....T.....	
PImIt.mrb1	CA..C.....T.TG.T.....	
PchTu.tun1	A..C.....TTTG.T.....	
PE5	G.A..T-TC...CT.TCTCA-...AAGCTTT.TC.TT.....	
ChrIt.blal	G.A..T-TC...CT.TTCC-...AA.CTTT.T.TC.....	
PchIt.mry1	G.A..T-TC...CT.TTCC-...AA.CTTT.T.TC.....	
	111	216
PV96	GAGCTCTGGTCCCACTCAGGGCTCAACAGAGGGCTGCGAATAACCCGAATAG-----AAACCCGAATAGGGCTTCGAGTGGTACCGGACCAGCGGTCCGACCACACACCGGT	
PImIt.cif1		T.....
PchIt.may1		T.....
AprIt.nap1		AT.....
NctSp.mur1		T..T..T.....
AlmIt.cor1	G.....G.....TC.....	T.....
AlmIt.pre1		G.....T.....T.....
AprIt.caf1		A.....
ChrIt.mrs1		T.....T.....T.....
ChrIt.lam1		A.....
PV32	A.....G.....GAGTAGG.....T.....C.....GTA.....T.....A.....G.....	
PImAl.abn1	AT.....A.....GAATAGG.....T.....C.....TTA.....T.....AT.....C.....G.....	
AprIt.try1	AT.....A.....GAATAGG.....T.....C.....TTA.....T.....AT.....C.....G.....	
PImIt.mrb1	AT.....A.....GAATAGG.....T.....C.....TTA.....T.....AT.....C.....G.....	
PchTu.tun1	AT.....A.....A.....T.....AGATAGG.....T.....C.....GTA.....T.....AT.....G.....	
PE5	AT.....A.....A.....-----GTG.....T.....A..T.GTA.....T..A.T.....CA...A..	
ChrIt.blal	AT.....A.....A.....-----GTG.....T.....A..T.GTA.....T.TA.T.....TA...A..	
PchIt.mry1	AT.....TA.....A.....-----GTG.....T.....A..T.GTA.....T.TA.T.....TA...A..	
	217	328
PV96	CGTGAAGACCACCTGGACCGTGAGAGGTCCGAATGTGCTCCCCGAATTCCTAAGGGGTTGTAGCACACAAATACCGAGAGGTGACGACGACAGGCGAGTGAAGTACTTGT	
PImIt.cif1		
PchIt.may1		
AprIt.nap1		
NctSp.mur1		
AlmIt.cor1	C.....	
AlmIt.pre1		T.....
AprIt.caf1	TC.....G.....	
ChrIt.mrs1	T.....G.....T.G.....G.....	
ChrIt.lam1	A.....AC.....AT.....G.....A.....	
PV32	T.....G.....T.A.....T.....	
PImAl.abn1	T.....G.....T.A.....T.....	
AprIt.try1	T.....G.....T.A.....T.....C.....	
PImIt.mrb1	C.....T.....G.....TT.A.....T.....	
PchTu.tun1	T.....G.....T.A.....	
PE5	T..T.....T..T.....G.....A..C.....T.....T.....T.G.....T.....C.T..T.....C.T	
ChrIt.blal	T..T.....TA.T.....A.....A..C.....T.....T.....T.G.....T.....T..T.....C.T	
PchIt.mry1	T..T.....TA.T.....A.....A..C.....T.....T.....T.G.....T.....T..T.....A...C.T	
	329	440
PV96	AGCATAGACTTCACGACCACTCTCCCTCAGTTGATGGGTGAGATTGACCCCTATTGACTGTTATAGTCCGAATGAACCTATGAGTTGGAATGGTTGGATGGTGG	
PImIt.cif1		
PchIt.may1		
AprIt.nap1		
NctSp.mur1	T.....	
AlmIt.cor1		
AlmIt.pre1	T.....T.....	
AprIt.caf1		
ChrIt.mrs1	C.....A.....	
ChrIt.lam1	T.....	
PV32	T..T.....T.....A.....G.....	
PImAl.abn1	T..T.....T.....A.....C.....	
AprIt.try1	T..T.....T.....A.....	
PImIt.mrb1	T..T.....C.....T.....A.....	
PchTu.tun1	T..T.....T.....A.....	
PE5	TG.C.....T.....G.....A.....T..T..A..GA...CAA.....C.....A.....C.....	
ChrIt.blal	TG.T.....T.....G.....A.....T..T..A..GA...CAA.....C.....A.....C.....	
PchIt.mry1	TG.T.....T.....G.....A.....T..T..A..GA...CAA.....C.....A.....C.....	

Fig. 2. Nucleotide sequence alignments of the RNA 4 of the 15 *Prunus* necrotic ringspot virus isolates characterized in this study. Isolates whose sequences had been previously determined by other workers (PV96, PV32, and PE5 [7,8,21,22,?]) and that are used here as reference isolates are in bold. Dots indicate identical residues. Dashes denote gaps in the sequence. The extra hexanucleotide present in the PV32 subgroup is marked in bold. The start codon is underlined.

netic distances and the corresponding dendrogram (data not shown). Three main clusters of PNRSV isolates were obtained and 15 isolates representing the three different groups obtained by RFLP analysis were chosen for further characterization (Table 1, underlined).

Sequences and comparative analysis of the RNA 4. Full-length RNA 4 amplified by using primers VP56 and VP33 (Fig. 1A) was obtained from the 15 isolates mentioned above. The corresponding PCR fragments were excised from agarose gels and, after diges-

Fig. 2. (continued from preceding page)

	441		552
VP96	AGGACTATAAGGTGGAACGACCTGATGGTCCGAATGCCCTGTCTAGGAAGGGGTTCTTGAAGGACCAACCGAGAGGTTGGCAGTTCGAACCTCCTTCCGATTTAGATTTCGA		
PlmIt.c1f1			C.....
PchIt.may1	A.....		
AprIt.nap1	A.....C.....		
NctSp.mur1	A.....	A.....	
AlmIt.cor1	A.....		
AlmIt.pre1	A.....		C.....
AprIt.caf1	A.....A.....		
ChrIt.mrs1	A.....A.....		
ChrIt.lam1	A.....T.....		
VP32	T.A.....	T.....C.....	
PlmAl.abn1	T.A.....	C.....C.....	
AprIt.try1	T.A.....	C.....	
PlmIt.mrb1	T.T.A.....	C.....	
PchTu.tun1	T.A.....	C.....	
PE5	G.....A.TAAC.....GAGG.....T.....	A.T.....A.A.....	
ChrIt.blal	G.....ATTAAAC.....C.....G.....	A.T.....G.A.....	
PchIt.mry1	G.....A.TAAC.....C.....G.....	A.T.....G.A.....	
	553		664
VP96	CACCTTTTGCGCGTACGCATCGTGTCTTATCGAATTCAGAACCGAAGTGCCCGCTGGGGCCAAGGTCTTGGTTAGGGATTGTACGTAGTGGTAAGTGATTTACCACGAGTG		
PlmIt.c1f1			
PchIt.may1			
AprIt.nap1			
NctSp.mur1			
AlmIt.cor1		C.....	
AlmIt.pre1		C.....	
AprIt.caf1	C.....		
ChrIt.mrs1		G.....	
ChrIt.lam1			
VP32	C.....	C.....	
PlmAl.abn1		C.....	
AprIt.try1	C.....	C.....	
PlmIt.mrb1	C.....	C.....	
PchTu.tun1	C.....	C.....	
PE5	T.....A.A.....TG.....A.....C.....A		
ChrIt.blal	T.....A.G.....	C.....	
PchIt.mry1	T.....A.G.....A.....	A.....C.....	
	665		776
VP96	CAAATTCGCACTGATGTCTTGCTGGTCGATGAAGACCTGCTTGAGATCTAGAGTGAGATAAGCACACTCGAATTTTCTCCGAATGGAAAGTTCGCACCACCGATAGTGGATA		
PlmIt.c1f1	A.....		
PchIt.may1			
AprIt.nap1			
NctSp.mur1			
AlmIt.cor1			
AlmIt.pre1			
AprIt.caf1	C.....		
ChrIt.mrs1		T.....	
ChrIt.lam1	G.....		
VP32			
PlmAl.abn1			
AprIt.try1			
PlmIt.mrb1			
PchTu.tun1	G.....	T.....	
PE5	G.....C.....	C-.....TA.....T.....T	
ChrIt.blal	G.....C.....	C-.....TA.....T.....T	
PchIt.mry1	G.....C.....	C-.....TA.....T.....T	
	777		884
VP96	TTGCGAATTAGATTTCTGAAGGTCGCTTCCCGACTTTTCATGCTTGGAAATCTTACCTGCGTTAGCAGATGCCCAACGTTGAAGTTGTGGATGCCCCGTTAGGGAAGC		
PlmIt.c1f1			
PchIt.may1			
AprIt.nap1			
NctSp.mur1			
AlmIt.cor1			
AlmIt.pre1			
AprIt.caf1			
ChrIt.mrs1			
ChrIt.lam1	C.....		
VP32	A.....A.....G.....		
PlmAl.abn1	A.....A.....G.....		
AprIt.try1	A.....A.....G.....		
PlmIt.mrb1	A.....A.....G.....		
PchTu.tun1	A.....A.....G.....		
PE5	C.....T.....A.....T.....A.....CTT		
ChrIt.blal	C.....T.....A.....T.....A.....CTT		
PchIt.mry1	C.....T.....A.....T.....A.....CTT		

tion with appropriate restriction enzymes, were cloned into the plasmid vector pUC18. For two isolates, the complete nucleotide sequence of three different clones was established and no heterogeneity was found. It was decided to then select single clones from the rest of the isolates for nucleotide sequencing. RNA 4 sequences were aligned (Fig. 2) and the corresponding phylogenetic tree was generated (Fig. 3A). Three different groups, represented by the first three PNRSV isolates characterized (PV32, PV96, and PE5) (Table 2) (7,8,21,22), were clearly differentiated. The size of the RNA 4 of the different isolates ranged from 880 to 890 nt, with 884 nt being the most frequent size (8 out of 15 isolates), whereas the size of the CP was either 224 or 226 amino acids (aa). Initial visual inspection of the alignment revealed that most of the variability was concentrated in the first third of the molecule (nt 1 to 350). The intragroup percentage of identity was 94.8 to 99.5% for the PV96-type group, 97.9 to 99.2% for the PV32 group, and 96.8 to 99.8% for the PE5 group. The 5' UTR sequences belonging to

the PE5-type were clearly different from those of the other two groups, a fact that is consistent with the inability of primer VP56 to amplify these isolates (described above). Isolates from the PV32-type group were characterized by having an extra hexanucleotide consisting of a direct repeat of the immediately preceding six nt. When the secondary structure around this hexanucleotide was examined in isolates with no duplication (Fig. 4, representative isolate

TABLE 2. Listing of *Prunus necrotic ringspot virus* isolates previously characterized and used as references in this study

Isolate	Origin	References	Accession no.
PE5, <i>Prunus persica</i> L. Batsch	United States	8	L38823
PV96, <i>Prunus mahaleb</i> L.	Germany	7	S78312
PV32, <i>Malus</i> spp.	United States	21, 22	U03857, Y07568
ApMV ^a , <i>Malus</i> spp.	United States	26	U15608

^a Apple mosaic virus.

Fig. 3. Phylogenetic trees of the A, nucleotide and B, amino acid sequences of the *Prunus necrotic ringspot virus* (PNRSV) RNA 4 isolates characterized in this study. Phylogenetic analysis was based on genetic distances (PHYLIP 3.5c package). The numbers near nodes were determined by bootstrap analysis (100 replicates). The PNRSV sequences reported previously are indicated in bold. Amino acid sequences characterized by Scott et al. (25) and Hammond and Crosslin (9) have also been included in B.

Fig. 4. Secondary structure proposed for A, *Prunus necrotic ringspot virus* isolates lacking the hexanucleotide repeat, represented by sequence ChrIt.bla1, and for B, isolates bearing this repeat, represented by PlmAl.unk1. Secondary structures were generated by using the RNA fold program. Models proposed for the generation of these repeats are described in text.

ChrIt.blal), it was noted that the hexanucleotide to be duplicated immediately preceded a very stable stem-loop structure (G-C pairs in three out of six positions in the stem). The duplication could then be explained by assuming an intramolecular rearrangement due to a “jumping” RNA polymerase in discontinuous transcription, following the replicative template switch model previously proposed

in other systems (1,10). The presence of the duplicate hexanucleotide in the PV32-type isolates produced two extra amino acids (NR [Asp-Arg] in all of them except in the case of PV32, in which there was an SR [Ser-Arg] insert) in their CPs (Fig. 5). This duplication has been observed in four different hosts (plum, apricot, and peach in this work and Scott et al. [25]; and in cherry [9,25]), in-

	1	74
PV96	MVCRI C NHTHAGGCR SCK KCHPNALVPLRAQQRAANNPNR--NPNRASSGTGPAVR P QPVVKTWTVRGPNVPPR	
PlmIt.clf1	V.....	
PchIt.may1	V.....	
AprIt.napl	M.....	
NctSp.mur1	LV.....	
AlmIt.cor1	R.....D.....S.....V.....	
AlmIt.pre1	A.....V.....S.....	
AprIt.caf1	R.....	L..
ChrIt.mrs1	V.....I.....V.....	
ChrIt.lam1	R.....N.....L.....S.....	
PV32	S.....D.....S.SR...V...V...I.....	
PlmAl.unk1	D.....NR...V...L...I.....	
AprIt.try1	...F.....D.....NR...V...L...I.....L...	
PlmIt.mrb1	D.....NR...V...L...I.....A.....	
PchTu.unk1	D...I.....V...KNR...V...V...I.....	
PE5	D.....--..V.V.NVV...K.....	
ChrIt.blal	D.....--..V.V.NVV...V...K.....I.....	
PchIt.mry1	D.....--..V.V.NVV...V...K.....I.....	
	75	150
PV96	IPKGFVAHNRHREVTTEAVKYLSIDFTTTLPLQMGQNLTLTIVIVRMNSMSSNGWIGMVEDYKVERPDGPNALSRK	
PlmIt.clf1	Q.....	
PchIt.may1	Q.....	
AprIt.napl	Q.....	
NctSp.mur1	Q.....	
AlmIt.cor1	Q.....	
AlmIt.pre1	F.....Q.....	
AprIt.caf1	Q.....	
ChrIt.mrs1	S.....Q.N.....	
ChrIt.lam1	S.....T.....Q.V.....	
PV32	...Y.....M.....DQ.....	
PlmAl.unk1	...Y.....DQ.....	
AprIt.try1	...Y.S...Q.....DQ.....	
PlmIt.mrb1	...VY.....S.....DQ.....	
PchTu.unk1	...Y.....DQ.....	
PE5	S.....P.....V.....F.....I.N.....I...R.NN.R.....	
ChrIt.blal	S.....V.....F.....I.N.....I...R.IN.E.....	
PchIt.mry1	S.....V.....F.....I.N.....I...R.NN.E.....	
	151	224
PV96	GFLKDQPRGWQFEPPSDDLDFDTFARTHVRVIEFKTEVPAGAKVLVRDLYVVVSDLPVQIPTDVLLVDEDLLEI	
PlmIt.clf1	D.....	
PchIt.may1		
AprIt.napl		
NctSp.mur1		
AlmIt.cor1		
AlmIt.pre1		
AprIt.caf1		
ChrIt.mrs1	R.....	
ChrIt.lam1		
PV32	D.....	
PlmAl.unk1		
AprIt.try1		
PlmIt.mrb1		
PchTu.unk1		
PE5	I.....A.....	
ChrIt.blal	I.....	
PchIt.mry1	I.....	

Fig. 5. Multiple alignment of coat protein sequences of the *Prunus necrotic ringspot virus* (PNRSV) isolates characterized in this study. The cysteine residues involved in the putative zinc-finger domain of PNRSV (21,22) and the arginine residues proposed to be crucial in the RNA binding properties of the coat protein (2) are marked in bold. Note the two extra amino acids present in members of the PV32 group.

dicating that it is not the result of host selection or the adaptation of PNRSV sequence variations.

Sequences and comparative analysis of the CPs. The N-terminus and the C-terminus of the protein were both extremely well conserved with most of the variability at the amino acid level occurring between positions 41 and 58 (Fig. 5). At the N-terminus, the four Cys residues involved in the putative zinc-finger motif previously proposed for PNRSV (16,21) are strictly conserved, as is the arginine residue, which by analogy with AMV, tobacco streak virus, and citrus leaf rugose virus, is involved in the RNA binding properties of the CP (2). Recently, Hammond and Crosslin (9) determined the sequence of the RNA 3 of seven PNRSV isolates that differed in serological relationships and pathology in sweet cherry. They proposed that limited changes at the amino acid level in the CP correlated well with symptom types (pathotypes) in cherry and identified four amino acid substitutions in the isolates causing rugose mosaic. Special attention was paid to the T50-I and F79-Y substitutions. Only one isolate (Chrlt.mrs1) of those sequenced in the current work possessed the T50-I substitution, although it is considered to be a latent isolate (Table 1). More interesting is the fact that the F79-Y substitution was present in all four isolates of the PV32-type group, three of which cause some kind of chlorotic or necrotic syndrome. However, the fact that some isolates that induce a similar symptomatology (e.g., Aprlt.caf1) lack the aforementioned amino acid substitution indicates that the correlation observed for cherry isolates is not necessarily valid for other hosts.

The phylogenetic tree generated from the amino acid sequence data showed essentially the same three groups as described above (Fig. 3B). When the cherry isolates characterized by Hammond and Crosslin (9) were included in this phylogenetic study, isolates of the CH30 serotype (CH30 and CH71) clustered in the PE5-type group, as previously noted. Interestingly, mild isolates of the CH9 serotype (CH61 and CH39) clustered in the PV96-type group, whereas rugose isolates of the CH9 serotype (CH38, CH59, and CH9) clustered in a subgroup within the PV32-type group. On the other hand, isolates 30/4 and CAN, recently described by Scott et al. (25) and which both have the two extra aa, clustered within the PV32-type group, as expected, whereas isolates Prune, Mission, and SW6 fell within the PV96-type group (Fig. 3B), thus validating the universality of the grouping proposed in this work.

It is worth noting that both the N-terminus and the C-terminus of the CP are extremely well conserved and show almost no variability. The CP of PNRSV and of *Iilarvirus* spp. in general has a very different charge distribution at both ends of the molecule, the N-terminus being very basic ($pI > 13$ at pH 7) and the C-terminus extremely acidic ($pI < 4.3$ at pH 7). Taking into consideration this asymmetry and previous experimental evidence, we previously proposed a model for the initiation of infection in which the *Iilarvirus* sp. CP would act as the transactivating domain of many transcription factors (21): the N-terminal region of the CP could bind to the 3' terminal region of the RNA and the acidic C-terminal domain could interact with a replicase complex to facilitate its contact with the genomic RNA. This model was extended by Houser-Scott et al. (11) to accommodate the 3' terminal nucleotide in the correct orientation for the accurate initiation of replication (4). The high degree of conservation of the N- and C-terminus ends shown by the CPs of the isolates sequenced in this work is consistent with this model.

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