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# The Molecular Variability Analysis of the RNA 3 of Fifteen Isolates of *Prunus Necrotic Ringspot Virus* Sheds Light on the Minimal Requirements for the Synthesis of its Subgenomic RNA

FREDERIC APARICIO & VICENTE PALLÁS*

*Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Avenida de los Naranjos s/n, 46022 Valencia, Spain*

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**Abstract.** The nucleotide sequences of the RNA 3 of fifteen isolates of *Prunus necrotic ringspot virus* (PNRSV) varying in the symptomatology they cause in six different *Prunus* spp. were determined. Analysis of the molecular variability has allowed, in addition to study the phylogenetic relationships among them, to evaluate the minimal requirements for the synthesis of the subgenomic RNA in Ilarvirus genus and their comparison to other members of the *Bromoviridae* family. Computer assisted comparisons led recently to Jaspars (Virus Genes *17*, 233–242, 1998) to propose that a hairpin structure in viral minus strand RNA is required for subgenomic promoter activity of viruses from at least two, and possibly all five, genera in the family of *Bromoviridae*. For PNRSV and *Apple mosaic virus* two stable hairpins were proposed whereas for the rest of Ilarviruses and the other four genera of the *Bromoviridae* family only one stable hairpin was predicted. Comparative analysis of this region among the fifteen PNRSV isolates characterized in this study revealed that two of them showed a 12-nt deletion that led to the disappearance of the most proximal hairpin to the initiation site. Interestingly, the only hairpin found in these two isolates is very similar in primary and secondary structure to the one previously shown in *Brome mosaic virus* to be required for the synthesis of the subgenomic RNA. In this hairpin, the molecular diversity was concentrated mostly at the loop whereas compensatory mutations were observed at the base of the stem strongly suggesting its functional relevance. The evolutionary implications of these observations are discussed.

**Key words:** *Bromoviridae*, Ilavirus, movement protein, molecular variability, subgenomic promoter

## Introduction

*Prunus necrotic ringspot virus* (PNRSV) is a positive RNA plant virus with a tripartite genome that belongs to the Ilarvirus genus [1]. *Ilarviruses* spp. have the same genome organization, encoding functionally similar translation products to those of members of the genera *Bromovirus*, *Cucumovirus*, *Oleovirus* and *Alfalfa mosaic virus* (AMV), which belong to the family *Bromoviridae*. RNAs 1 and 2 of *Bromoviridae* encode the replicase proteins P1 and P2, respectively. RNA 3 is bicistronic and has two open reading frames (ORFs) encoding the movement protein (MP or P3) and the coat protein (CP). The CP is synthesized via a subgenomic monocistronic mRNA (RNA 4) which is collinear with the 3′-terminal part of the RNA 3. Unlike to bromo-, cucumo- and oleoviruses, initiation of infection by AMV and ilarviruses requires the binding of CP to the inoculum RNAs, a phenomenon denominated genome activation. Moreover, CPs of AMV and ilarviruses are interchangeable in this process [2,3].

PNRSV is a pollen and seed-transmitted [4] worldwide pathogen responsible of economically important diseases in most cultivated *Prunus* sp. [5].

*Author for all correspondence:
E-mail: vpallas@ibmcp.upv.es

It causes a broad range of different symptoms (pathotypes) ranging from none to the characteristic necrotic spots and shot holes depending on the virus isolate. Sequence analysis of the RNA 3 of seven PNRSV isolates from sweet cherry trees collected in USA revealed single nucleotide and aminoacid changes affecting at MP and/or CP level that correlated well with serological relationships and pathotypes [6]. However, similar studies using PNRSV isolates from other hosts and different geographical origins showed not clear correlation between molecular variability and biological properties [7–9] indicating that the results derived from the cherry isolates are not necessarily valid for others hosts. Sequence comparisons and phylogenetic analysis of the RNA 4 and/or the CP of most of the PNRSV isolates characterized so far allowed their division into three main groups, characterized by specific residue substitutions highly conserved in the members of each group [7]. This grouping has remained invariable when other PNRSV isolates collected at Czech Republic were analyzed [9]. To date, most of the molecular variability studies involving the movement protein have been done with PNRSV isolates originating from the USA whereas in Europe only very limited information of the MPs of some Czech PNRSV isolates is available [9].

Most of the positive RNA viruses express their 3′-proximal genes through the synthesis of subgenomic RNAs (sgRNAs) which within the *Bromoviridae* family it is well established that occurs by internal initiation on the negative-strand copy of the genomic RNA (see [10] for a review). Subgenomic promoter (sgp) sequences have been studied for different viruses being some members of the family *Bromoviridae* the ones for which a more detailed description is available. *In vitro* experiments with truncated and deleted transcripts have permitted to identify, on the negative-strand RNA 3 of AMV, BMV and CMV [11–14], short sequences of about 20–30 nt upstream of the sgRNA 4 initiation site which are sufficient for a basal level of transcription. Moreover, mutational analysis of BMV core promoter demonstrated that at least four residues (G −17, A −14, C −13 and G −11, taking the initiation site as +1) are essential for sgp activity probably due to their implication in direct interaction with the replicase [15,16]. By comparing secondary structure predictions of the nucleotide sequence immediately in front of the initiation site for subgenomic RNA 4 synthesis on the RNA 3 minus strand of 26 species of the family *Bromoviridae*, Jaspars [17] has recently proposed the hypothesis that a hairpin structure is required for sgp activity of viruses from at least two, and possibly all five, genera of this family. Supporting this hypothesis, Haasnoot et al. [13] by a mutational strategy have demonstrated that this hairpin structure is required for efficient RNA synthesis *in vitro* in *Alfamovirus* and *Bromovirus*. In this scenario, in addition to the necessary information obtained by site-specific and/or deletion mutations of the hypothetical promoter region, data originating from variability analysis of virus isolates that include 'real mutants' in these specific regions that could reinforce the *in vitro* experimental evidence are welcome. In this paper, the molecular variability analysis of the RNA 3 of fifteen PNRSV isolates has allowed us, in addition to study the phylogenetic relationships among them, to evaluate the minimal structural requirements for the synthesis of the subgenomic RNA in the *Ilarvirus* genus and their comparison to other members of the *Bromoviridae* family.

## Materials and Methods

### *Plant Material and RNA Extraction*

Fifteen 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 preparations were obtained from 0.5 g fresh leaf tissue for each isolate as described previously [18]. Samples were analyzed for the presence of the virus by non-isotopic molecular hybridization as described [19,20].

### *cDNA Synthesis and PCR Amplification*

First strand cDNA synthesis was carried out in a 20 µl reaction containing 50 mM Tris–HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 1 mM of each dNTP, 40 U ribonuclease inhibitor, 8 U AMV-RT and 100 pmol of antisense primer VP90 (5′-ATCAAGCTTATTGCAAATTCGGCA-AAC-3′). The 3′ 18 nt of the primer are complementary to the N-terminus of PNRSV CP cistron and are preceded by the restriction site *Hind* III (underlined).

PCR amplifications were made using primer VP90 and either the sense primer VP91 (5′-TCA-GGATCCGTTTTTACAATCGAAATC-3′, which contains a sequence identical to nt 1–18 of RNA 3 from PV96 isolate) or the sense primer VP92 (5′-TCA-GGATCCGTTTTTACCAAAATGAAATC-3′, with a sequence identical to nt 1–20 of RNA 3 from PV32 isolate), both with a *Bam* HI site (underlined). Thus, isolates previously clustered as PV96-type group and PE5-type group after the phylogenetic analysis made by Aparicio et al. [7] were amplified with the VP90/VP91 primer pair whereas those from the PV32-type group were amplified with VP90/VP92 primers. Five microliters of the reverse transcription products, 10 µl of 10× buffer (166 mM $(NH_4)_2SO_4$, 670 mM, Tris–HCl, pH 8.8, 0.1% Tween 20), 3 mM $MgCl_2$, 0.2 mM of each dNTP, and 60 pmol of each primer were mixed in a total volume of 100 µl for the PCR reactions. Prior to thermal cycling, 2.5 U Ecozyme DNA polymerase was added and the 100 µl samples overlaid with mineral oil. PCR was carried out in a Perkin–Elmer 2400 thermal cycler programmed for one cycle at 94°C for 2 min followed by three cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 90 s, and 27 cycles of 94°C for 45 s, 53°C for 45 s and 72°C for 90 s, finishing with extension at 72°C for 10 min. Amplified products (5 µl each) were electrophoresed in 1% agarose gels in TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide to confirm the expected size of the PCR products.

### *Cloning, Nucleotide Sequencing and Sequence Comparisons*

PCR products obtained with the pairs of primers VP90/VP91 and VP90/VP92 were extracted from 1% agarose gels using USBioclean, cloned into the *Bam* HI–*Hind* III cut pUC18 vector and transformed into *E. coli* DH5α 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). The sequences of the different isolates were named using the the same eight letter code used to describe their RNA 4 sequences (Table 1) [7].

From the GCG software package (Wisconsin Package, version 9.1), GAP and OLDDISTANCES programs were used to compare homology and similarity between sequences, MFOLD and PLOTFOLD programs were used to calculate secondary structures of RNA which, were drawn with RNAviz program. Multiple alignments were obtained using Clustal W [21]. Phylogenetic analyses were performed using the following programs of the PHYLIP 3.5 c package [22]: DNADIST and PROTPARS to calculate genetic distances, NEIGHBOR (UPGMA

*Table 1.* Listing and properties of PNRSV isolates characterized in this study

| Isolate | Source | Symptomatology[a] | Origin | Sequence[b] Code | Size MP (aa) | Accession No. |
|---|---|---|---|---|---|---|
| A3430 | Apricot cv. Cafona | Line pattern, necrosis | Italy | Aprlt.caf 1 | 283 | AJ306810 |
| A9170 | Apricot cv. Napoletana | Asymptomatic | Italy | Aprlt.nap 1 | 283 | AJ306811 |
| A8360 | Apricot cv. Tirynthos | Line pattern, necrosis | Italy | Aprlt.try 1 | 283 | AJ306812 |
| M7960 | Almond cv. Preani | Shot hole | Italy | Almlt.pre 1 | 283 | AJ306813 |
| M8610 | Almond cv. Core | Latent | Italy | Almlt.cor 1 | 283 | AJ306814 |
| P8450 | Peach cv. May Crest | Latent | Italy | Pchlt.may 1 | 283 | AJ306815 |
| Tunez-111 | Peach cv. unknown | Few chlorotic spots | Tunisia | PchTu.unk 1 | 283 | AJ306816 |
| P4090 | Peach cv. Marylin | Latent | Italy | Pchlt.mry 1 | 284 | AJ306817 |
| NcM1 | Nectarine (Murcia) | Few chlorotic spots | Spain | NctSp.mur 1 | 283 | AJ306818 |
| C8220 | Cherry cv. Lambert | Latent | Italy | Chrlt.lam 1 | 283 | AJ306819 |
| C8320 | Cherry cv. Marasca | Latent | Italy | Chrlt.mrs 1 | 283 | AJ306820 |
| C7140 | Cherry cv. Black Giant | Some years yellowing | Italy | Chrlt.bla 1 | 284 | AJ306821 |
| PL11 | Plum cv. unknown | Chlorotic pattern | Albania | PlmAl.unk 1 | 283 | AJ306822 |
| S6340 | Plum cv. C. Lopez | Asymptomatic | Italy | Plmlt.clf 1 | 283 | AJ306823 |
| S9040 | Plum Mirabolan | Latent | Italy | Plmlt.mrb 1 | 283 | AJ306824 |

[a]Observed in the natural host over several years.
[b]Code nomenclature as previously described by Aparicio et al. [7].

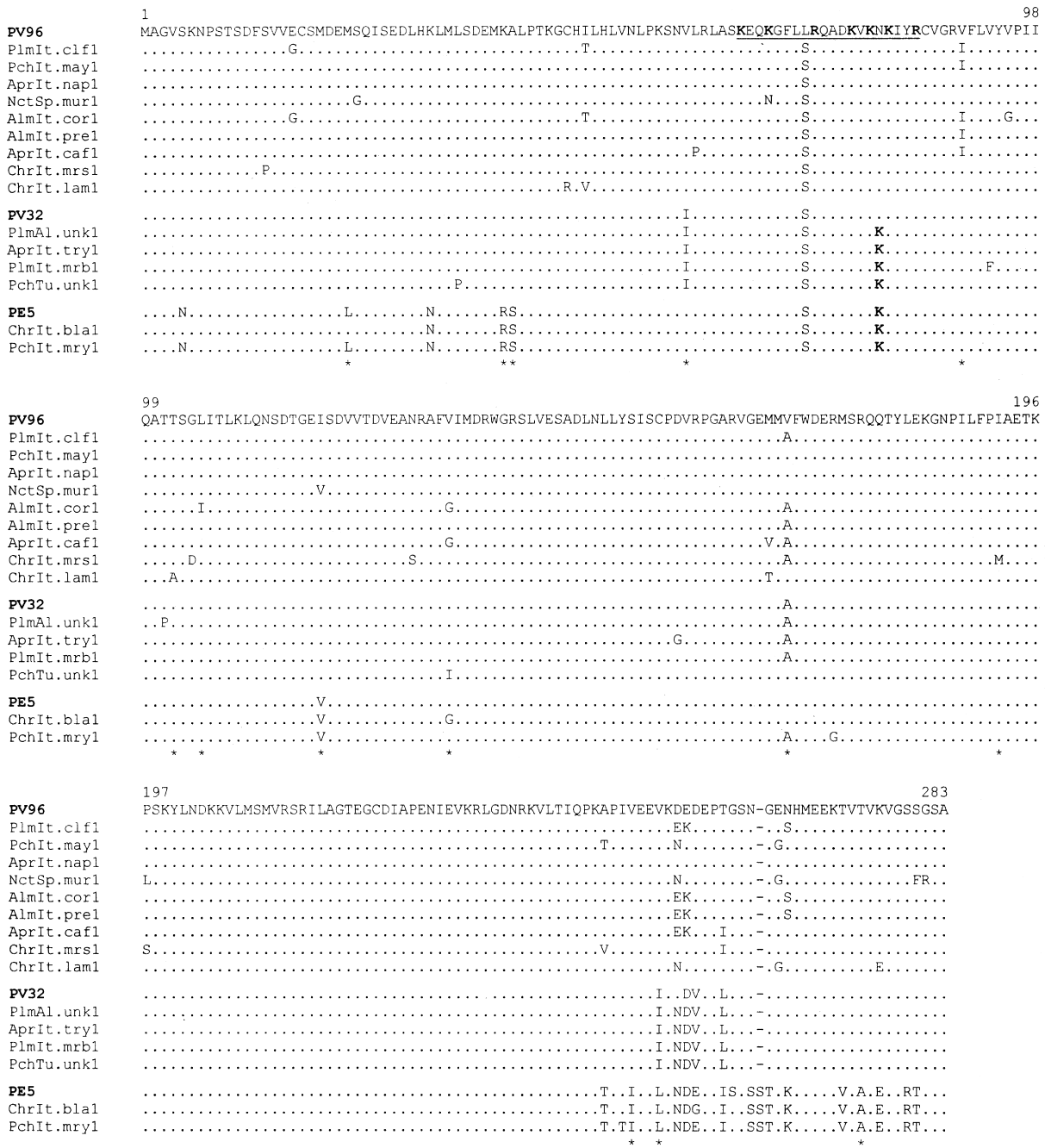


*Fig. 1.* Multiple aa sequence alignment of the movement protein of the 15 PNRSV isolates characterized in this work. In bold, isolates whose sequences had been previously determined (PV96, PV32 and PE5; see Table 2) and used here as reference isolates. Dots indicate identical residues. Dashes denote gaps in the sequence. Conservative aa changes are marked by asterisk at the bottom of the alignment. Note the high variability at the C-terminal fragment of the protein between isolates. The basic region proposed previously as putative RNA binding motif [18] is underlined with the basic residues in bold.

method) 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 ApMV served as the outgroup.

## Results and Discussion

### *Sequence and Comparative Analysis of MPs*

PNRSV RNA 3 encodes the MP and CP which are separated by a non-coding intergenic region. The CP is translated via a subgenomic RNA 4 collinear with the 3′ part terminal of RNA 3, and previously, we have reported the nucleotide sequence of the RNA 4 from fifteen PNRSV isolates [7]. We analyzed here the molecular variability contained within the 5′NTR, the ORF 3A (MP) and the intercistronic region of the same fifteen isolates (Table 1).

Putative translation products deduced from the corresponding MPs ranged between 283 and 284 amino acids (aa) with percentage homology values of 92–99% among all the isolates (data not shown). The alignment of the nucleotide (not shown) and aa sequences (Fig. 1) showed three sequence types represented by the first three PNRSV isolates reported (PV96, PV32 and PE5, Table 2) and characterized by well conserved specific residues within each type. This grouping was similar to the previously obtained after analysis of the RNA 4 sequences [7], indicating that there are three main PNRSV RNA 3 sequence types for the PNRSV isolates. The intragroup homology values at the aa level for the MP sequences was 95–99% among PV96-type isolates, 98–99% among PV32-type and 97–98% for those within the PE5-type. As can be observed in Fig. 1, the first two-thirds of the molecule shows scattered aa changes, most of them being conservatives, whereas the last third shows a first part of a high level of conservation and the C-terminus where most of the variability is concentrated. Most of the plant virus movement proteins have an RNA-binding domain required for the cell-to-cell movement that is characterized by a high accumulation of positively-charged residues [23–27]. Within the family *Bromoviridae*, the RNA binding domain has been mapped at the C-terminus region in BMV and CMV MPs [27,28] whereas in AMV MP was mapped at the N-terminus region [26]. Sequence comparison of the MPs from several Ilarviruses led to the proposal that the highly basic motif located at the N-terminus in the MPs of all Ilarviruses (from aa 63 to 85 in PNRSV MP, underlined in Fig. 1) could be a good candidate for RNA binding [18]. The high degree of conservation of the basic residues found in this region in the fifteen MP sequences characterized here reinforces this suggestion. The observation that a chimeric AMV RNA 3 with MP and/or CP genes replaced by the corresponding genes of PNRSV was functionally equivalent for a limited cell-to-cell movement in tobacco plants transformed with replicase AMV genes P1 and P2 (P12 plants) is consisting with this proposal [29].

Comparative analysis of the aa sequences of the MP of seven PNRSV isolates led to Hammond and Crosslin [6] to identify eight substitutions (four of them at the last 30 aa of the protein) that allowed to differentiate CH9 serotype isolates causing rugose mosaic disease from those causing mild mottle in sweet cherry. As stated above, alignment presented in the Fig. 1 showed extensively sequence conservation with scattered changes in each isolate together with well conserved characteristic substitutions representatives of each group, most of them placed at the C-terminus of the protein. Interestingly, all isolates of the PV32-type characterized here showed, at the C-terminus, the same four substitutions as the CH9 rugose mosaic pathotypes (Fig. 1, I-253, D-256, V-257 and L-260). Three of the four PV32-type isolates cause some kind of chlorotic and/or necrotic syndrome although, some other isolates showing a similar symptomatology (e.g. Aprlt.caf1) lack the aforementioned aa substitutions. In addition, most of the isolates belonging to the PV96-type group were considered to be latent although no discriminatory aa substitutions were found. These results, together with previously analysis [7–9] indicate that the correlation observed by Hammond and Crosslin [6]

*Table 2.* Listing of PNRSV isolates previously characterized and used as reference in this study

| | Isolate | Origin | References | Accession No. |
|---|---|---|---|---|
| PE 5 | *Prunus persica* L. Batsch | USA | [41] | L38823 |
| PV96 | *Prunus mahaleb* L. | Germany | [42] | S78312 |
| PV32 | *Malus* spp. | USA | [18,43] | U03857, Y07568 |
| ApMV | *Malus* spp. | USA | [39] | U15608 |

in cherry isolates is not necessarily valid for other hosts.

Previously, based on RNA 4 sequence analysis, we proposed a phylogenetic PNRSV division into three groups which, since they were represented by the first three PNRSV RNA 3 sequences reported, we named them as PV32-type group, PV96-type group and PE5-type group [7]. Similar grouping was recently obtained by Vašková et al. [9] when it was considered only the last 53 aa sequenced of the MPs of sixteen Czech isolates. In that analysis, phylogenetic groups were named them as groups I, II and III, respectively. Phylogenetic analysis of the MPs including the fifteen PNRSV isolates characterized in this work and the American isolates previously described [6,8] (Fig. 2A) clearly differentiated the three groups mentioned above. Thus, the phylogenetic trees generated from the aa sequence data of the entire MPs show the close relationships between rugose sweet cherry isolates (CH38, CH57 and CH9) and all the isolates bearing four of the eight substitutions identified as characteristic of this pathotype, all of them forming the PV32-type group. A second branch conforming PV96-type group cluster all the American isolates causing mild mottle or no-symptoms in cherry (CH61 and CH39) plus the majority of our asymptomatic and latent isolates. Finally, PE5-type group formed by three cherry isolates and one peach isolate was the more phylogenetically closed to ApMV. When phylogenetic analysis was carried out by taking into account only the last 53 aa of the MPs to include the Czech isolates [9] similar results were observed (Fig. 2B).

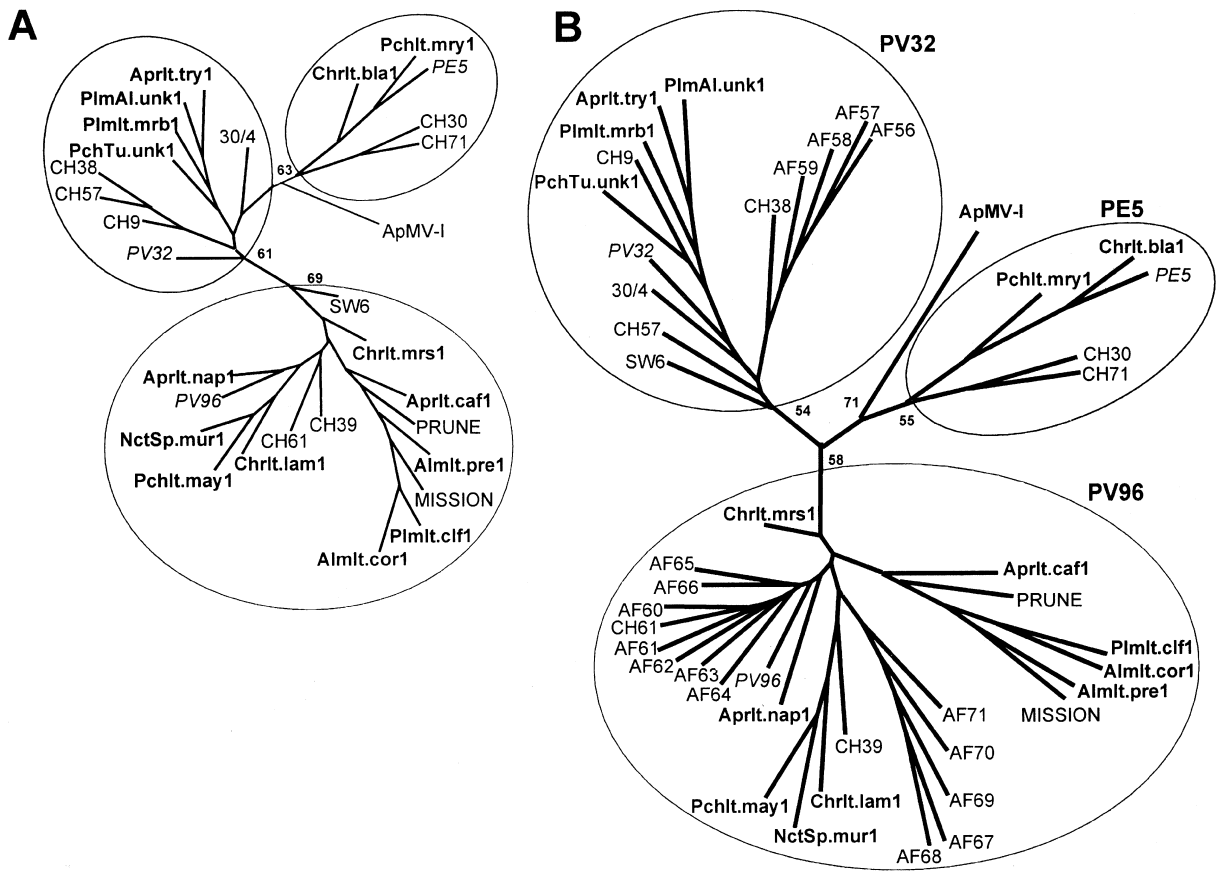


*Fig. 2.* Phylogenetic trees of aa sequences of the movement protein (MP) of PNRSV isolates using (A) the whole sequence of the protein and (B) the last 53 residues of the C-terminus. Phylogenetic analysis was based on genetic distances (PHYLIP 3.5 c package). The numbers near nodes were determined by bootstrap analysis (100 replicates). The PNRSV sequences used as references are in italics and the ones characterized in this study are indicated in bold. Amino acid sequences characterized by Scott et al. [8] and Hammond and Crosslin [6] are included in (A) and those reported by Vašková et al. [9] have been additionally included in (B).

*Sequence and Comparative Analysis of Non-Translated Regions*

The 5′-NTR of the fifteen isolates characterized ranged between 173 and 176 nt length with homology values among 88–99% for all isolates. It is interesting to note that the 5′-NTR of Chrlt.bla1 showed 98% of identity with Chrlt.mrs1 both isolates clustering at the same phylogenetic tree when only this part of the molecule was considered (not shown), whereas they clustered at different phylogenetic groups when coding regions were analyzed (see above). Assuming coordinated evolution of all parts of the genome, all phylogenetic reconstruction should show similar topologies. Differences in the phylogenetic trees obtained when considering different viral genome regions are usually considered as a first criterion for a recombination event [30]. Thus, our results suggest that recombination events are also present in Ilarvirus as a source of molecular diversity. Recombination phenomena have been proposed as one of the causes of phylogenetic radiation in CMV [31] and genetic diversity in BMV [32], both members of the *Bromoviridae* family.

Some members belonging to the *Bromoviridae* family contain in their 5′-NTR sequence elements similar to the ICR2 eukariotic promoters [33]. The ICR2-like motif was found to be required for efficient replication in BMV [34] and such a role was also proposed for CMV [35]. Binding of host-proteins to ICR2 motifs has been reported in BMV [36]. In addition, mutations in the 5′-NTR ICR2 motif of AMV RNA 3 abolished RNA accumulation supporting the idea that this structure is important for plus-strand promoter activity [37,38]. ICR2-like sequences have also been found at the 5′-NTR of ApMV RNA 3 [39]. Figure 3 shows the predicted secondary structure of the 5′-NTR and the molecular variability found

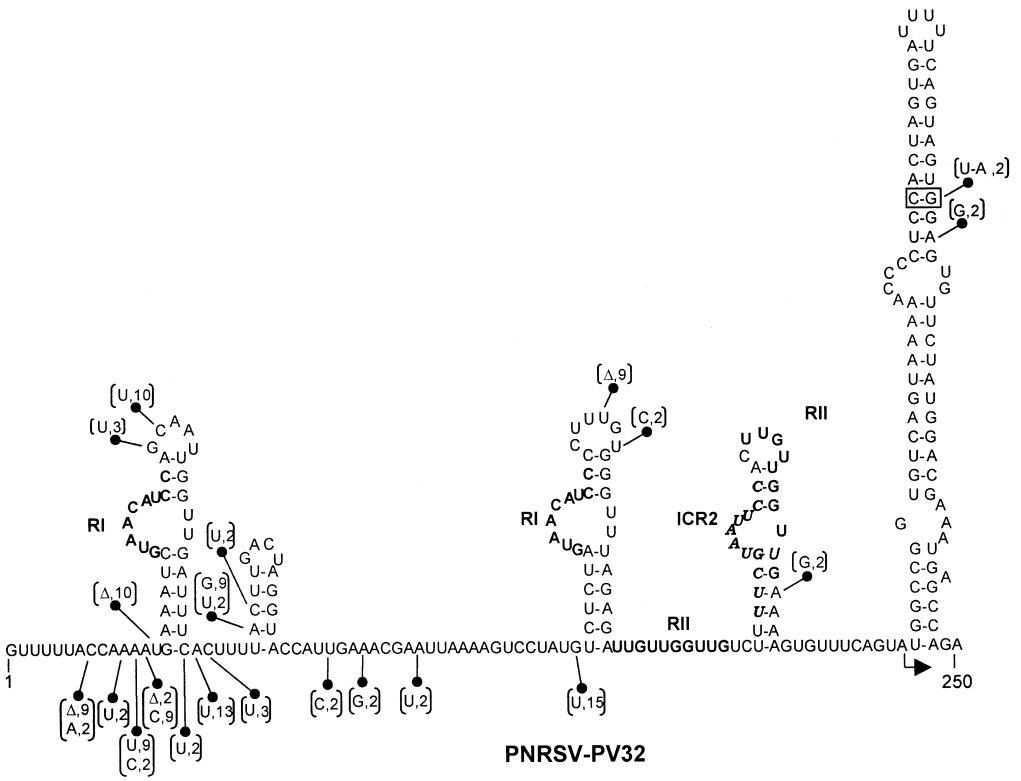


*Fig. 3.* Predicted secondary structure for plus-strand 5′ non-translated region (5′-NTR) of PNRSV-PV32 isolate. The 9- and 11-nt repetitions are indicated in bold letters and denoted as RI and RII, respectively. The ICR 2 motif is in bold italic. Nucleotide changes either substitutions, insertions (+) or deletions (Δ) found in two or more of the fifteen isolates are in parenthesis, including number of affected isolates by the corresponding mutations (e.g. [U,2] indicates that two isolates showed a U residue in this position). The arrow indicates the start site of the MP coding region.

within it. As it can be observed, most of the nucleotides changes were localized in unpaired linear regions or at the loops of the stem-loop structures. Interestingly, the two pairs of direct repetitions (RI and RII in Fig. 3) previously described [18] and the ICR2-like motif were extremely conserved. Finally, the importance of the 5′-NTR in determining host specificity in ilarviruses was recently suggested by using chimera viruses between AMV and PNRSV [40].

Intergenic regions (IR) of the fifteen isolates characterized ranged from 62 to 79 nt length being the most variable region of the RNA 3 showing an

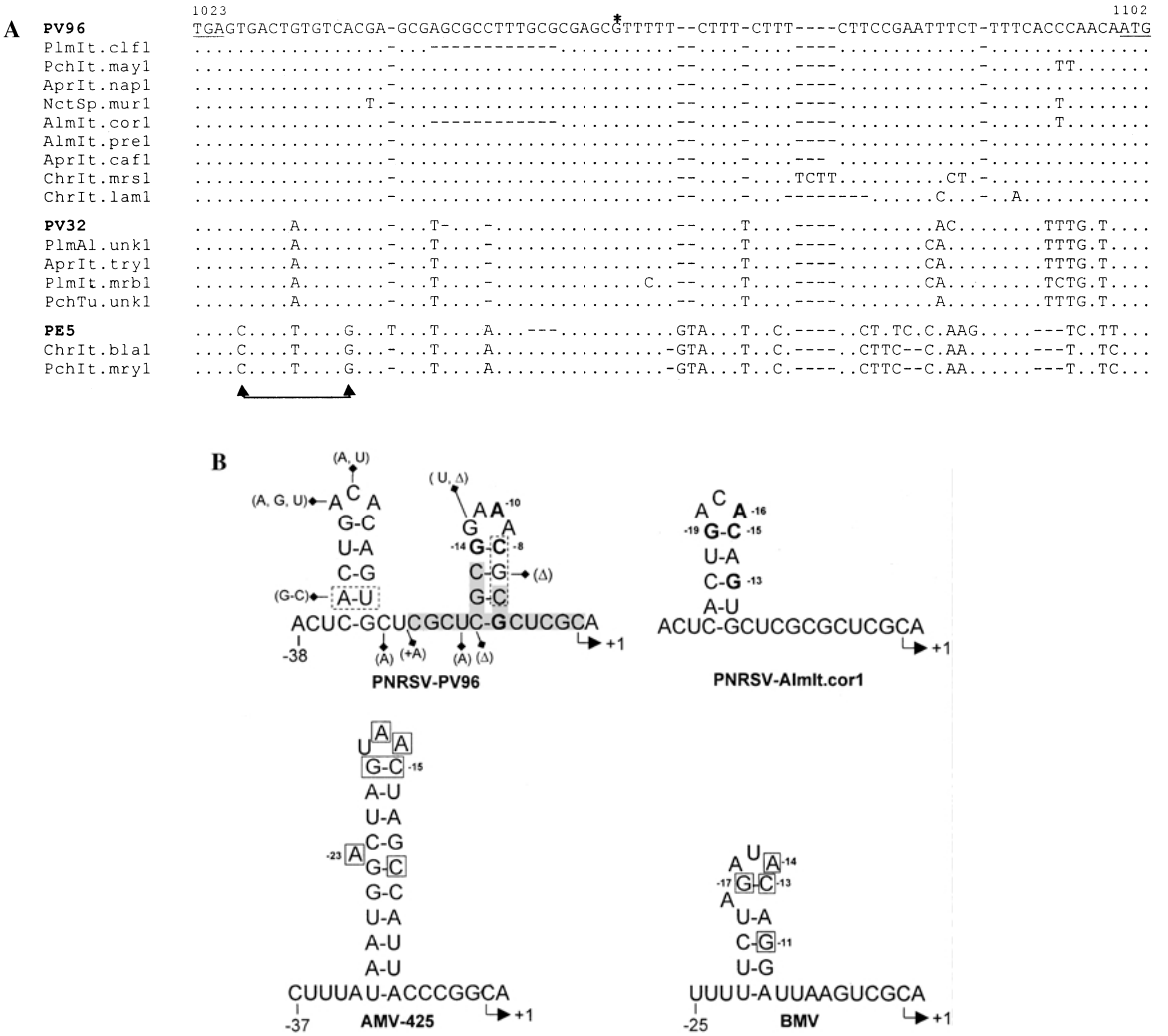


*Fig. 4.* Nucleotide sequence alignment of the non-translated intergenic regions of the fifteen PNRSV isolates characterized in this study. (A) Dots and dashes indicate identical residues and gaps in the sequences, respectively, and stop MP and start CP codons are underlined. Initiation site of the subgenomic RNA 4 is denoted by an asterisk. Double arrowhead indicates a compensatory mutation in isolates of the PE5-type group. (B) Predicted secondary structures of subgenomic RNA 4 core promoter on RNA 3 minus-strand of the PNRSV-PV96 isolate; the one proposed for the two isolates bearing the 12-nt deletion represented by AlmIt.cor 1 sequence, and the sgRNA 4 core promoter structures postulated for AMV and BMV [17]. Nucleotide residues shown to be essential for full sgp activity in AMV [13] and BMV [16] are boxed whereas the corresponding ones in PNRSV isolates are in bold. Nucleotide changes found in the 47 PNRSV isolates, including the fifteen sequences of this study, are indicated on PV96 structure. Sequences run from left (3′end) to right (5′end) and arrow indicate start site of RNA 4. The 7-nt direct repeat at the base of the hairpin structure most proximal to the RNA 4 initiation site is shadow.

overall percentage of identity of 35–75% (data not shown). However, nucleotide sequence alignment also showed three intergenic sequence types characterized by a high sequence homology as well as by the existence of strictly conserved and representative changes (Fig. 4A). The 5′-NTR of the RNA 4 was the region that contributed most to this variability whereas the region upstream the initiation of sgRNA was significantly more conserved as previously noted for PNRSV isolates [7,9] or other members of the *Bromoviridae* family [17].

A core promoter sequence of 20 or 37 nt upstream of the RNA 4 start site (nt +1) was shown to be sufficient for full subgenomic promoter activity in *in vitro* experiments with BMV [11,14] and AMV [13], respectively, whereas for CMV the core subgenomic promoter has recently been mapped between residues −28 and +15 [12]. Computer assisted comparisons led recently to Jaspars [17] to propose that a hairpin structure in viral minus strand RNA is required for sgp activity of viruses from at least two, and possibly all five, genera in the family of *Bromoviridae*. For PNRSV and ApMV two stable hairpins were proposed whereas for the rest of Ilarviruses and the other four genera of the *Bromoviridae* family, only one stable hairpin was predicted [17]. Interestingly, comparative analysis of this region among the fifteen PNRSV isolates characterized in this study revealed that two of them showed a 12-nt deletion that led to the disappearance of the hairpin most proximal to the initiation site (Fig. 4B). The 12-nt deletion was consistently obtained in these two isolates when the RT-PCR reactions were carried out by using other pair of primers that allowed to amplify a shorter region covering the IR (not shown). These results strongly suggest that the most distal hairpin to the subgenomic initiation site would be the minimal structure required for the synthesis of the PNRSV sgRNA as revealed by the following evidences: (i) the distal hairpin is the only one found in the two PNRSV isolates described above; (ii) this distal hairpin contains all the same 4 nt (G −17, A −13, C −13 and G −11) and at the same spatial position than those that were shown to be essential for BMV RNA synthesis and believed responsible for direct interaction with replicase [15,16]; (iii) a 3 nt deletion affecting the stem of the proximal hairpin is present in the PNRSV-PE5 isolate sequenced by Hammond and Crosslin [41] and compensatory mutations were only observed on the stem of the distal hairpin (Fig. 4A and B); (iv) finally, it has been recently shown that base pairing in the top half of the stem of the potential hairpin structure within sgp of AMV RNA 3 is essential for full sgp activity [13] and this top half is very similar to the stem of the distal hairpin proposed for the core promoter of PNRSV sgRNA. In addition, recent evidence indicates that efficient and specific initiation of sgRNA synthesis by CMV replicase *in vitro* requires an upstream RNA stem-loop [12].

It is tentative to speculate that only one stem-loop would be the minimal secondary structure in the *Bromoviridae* family for efficient subgenomic promoter activity and that PNRSV would have acquired a second functional stem-loop through a duplication mechanism conferring to the new variants a selective advantage in the virus life cycle. The existence of one 7-nt direct repeat flanking the proximal hairpin (Fig. 4B) reinforces this proposal. Experiments are in progress to test this hypothesis.

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## References

1. van Regenmortel M.H.V., Fauquet C.M., Bishop D.H.L., Carstens E.B., Estes M.K., Lemon S.M., McGeoch D.J., Maniloff J., Mayo M.A., Pringle C.R., Wickner R.B. (eds), *Virus Taxonomy. Classification and Nomenclature of Viruses.* Wien Seventh ICTV Report. Academic Press, 2000, pp.
2. Bol J.F., J Gen Virol *80*, 1089–1102, 1999.
3. Jaspars E.M.J., Arch Virol *144*, 843–863, 1999.
4. Aparicio F., Sánchez-Pina M.A., Sánchez-Navarro J.A., and Pallás V., Eur J Plant Pathol *105*, 623–627, 1999.
5. Uyemoto J.K. and Scott S.W., Plant Dis *76*, 5–11, 1992.
6. Hammond R.W. and Crosslin J.M., J Gen Virol *79*, 1815–1823, 1998.
7. Aparicio F., Myrta A., Di Terlizzi B., and Pallás V., Phytopathology *89*, 991–999, 1999.
8. Scott S.W., Zimmerman M.T., Ge X., and MacKenzie D.J., Eur J Plant Pathol *104*, 155–161, 1998.
9. Vašková D., Petrzik K., and Karešová R., Arch Virol *145,* 699–709, 2000.
10. Miller W.A. and Koev G., Virology *273*, 1–8, 2000.

11. Adkins S., Siegel R.W., Sun J.-H., and Kao C.C., RNA *3*, 634–647, 1997.
12. Chen M.H., Roossinick M.J., and Kao C.C., J Virol *74*, 11201–11209, 2000.
13. Haasnoot P.C.J., Brederode F.Th., Olsthoorn R.C.L., and Bol J.F., RNA *6*, 708–716, 2000.
14. Marsh L.E., Dreher T.W., and Hall T.C., Nucleic Acids Res *16*, 981–995, 1988.
15. Adkins S. and Kao C.C., Virology *252*, 1–8, 1998.
16. Siegel R.W., Adkins S., and Kao C.C., Proc Natl Acad Sci USA *94*, 11238–11243, 1997.
17. Jaspars E.M.J., Virus Genes *17*, 233–242, 1998.
18. Sánchez-Navarro J.A. and Pallás V., Arch Virol *142*, 749–763, 1997.
19. Pallás V., Más P., and Sánchez-Navarro J.A., in Foster G. and Taylor S. (eds), *Detection of Plant RNA Viruses by Non-Isotopic Dot-Blot Hybridization*. Humana Press, Totowa, 1998.
20. Pallás V., Sánchez-Navarro J.A., and Díez J., Arch Virol *144*, 797–803, 1999.
21. Thompson J.D., Higgins D.G., and Gibson T.J., Nucleic Acids Res *22*, 4673–4680, 1994.
22. Felsenstein J., Cladistic *5*, 164–166, 1989.
23. Citovsky V., Wong M.L., Shaw A.L., Venkataram P.B.V., and Zambryski P., Plant Cell *4*, 397–411, 1992.
24. Jansen K.A.J., Wolfs C.J.A.M., Lohuis H., Goldbach R.W., and Benedictus J.M.V., Virology *242*, 387–394, 1998.
25. Marcos J.F., Vilar M., Pérez-Payá E., and Pallás V., Virology *255*, 354–365, 1999.
26. Schoumacher F., Giovane C., Maira M., Poirson A., Godefroy-Colburn T., and Berna A., J Gen Virol *75*, 3199–3202, 1994.
27. Vaquero C., Liao Y.-C., Nähring J., and Fisher R., J Gen Virol *78*, 2095–2099, 1997.
28. Fujita M., Mise K., Kajiura Y., Dohi K., and Furusawa I., J Gen Virol *79*, 1273–1280, 1998.
29. Sánchez-Navarro J.A., Reusken C.B.E.M., Bol J.F., and Pallás V., J Gen Virol *78*, 3171–3176, 1997.
30. Candresse T., Revers F., Le Gall O., Kofalvi S., Marcos J., and Pallás V., in Tepfer M. and Balázs E. (ed.) *Systematic Search for Recombination Events in Plant Viruses and Viroids*. Springer-Verlag, Berlin, pp. 20–25, 1997.
31. Roossinck M.J., Zhang L., and Hellwald K.-H., J Virol *73*, 6752–6758, 1999.
32. Bujarski J.J. and Kaesberg P., Nucleic Acids Res *15*, 1337–1337, 1987.
33. Marsh L.E., Pogue G.P., and Hall T.C., Virology *172*, 415–427, 1989.
34. Pogue G.P., Marsh L.E., Connell J.P., and Hall T.C., Virology *188*, 741–753, 1992.
35. Boccard F. and Baulcombe D.C., Virology *193*, 563–578, 1993.
36. Dugall R. and Hall T.C., Virology *214*, 638–641, 1995.
37. van der Vossen E.A.G., Neeleman L., and Bol J.F., Nucleic Acids Res *6*, 1361–1367, 1993.
38. van der Vossen E.A.G. and Bol J.F., Virology *220*, 539–543, 1996.
39. Shiel P.J., Alrefai R.H., Domier L.L., Korban S.S., and Berger P.H., Arch Virol *140*, 1247–1256, 1995.
40. Aparicio F., Sánchez-Navarro J.A., Olsthoorn R.C.L., Pallás V., and Bol J.F., J Gen Virol *82*, 9947–9951, 2001.
41. Hammond R.W. and Crosslin J.M., Virology *208*, 349–353, 1995.
42. Guo D., Maiss E., Adam G., and Casper R., J Gen Virol *76*, 1073–1079, 1995.
43. Sánchez-Navarro J.A. and Pallás V., J Gen Virol *75*, 1441–1445, 1994.