Long Sequences in the 5′ Noncoding Region of Plum Pox Virus Are Not Necessary for Viral Infectivity but Contribute to Viral Competitiveness and Pathogenesis

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INTRODUCTION

Plum pox virus (PPV) is a member of the Potyvirus genus, the largest group of plant-infecting viruses. The genome of PPV is a messenger-polarity RNA molecule of 9786 nucleotides (nt) in length (Rankovic isolate), with a VPg protein covalently linked to the 5′ end and a poly(A) tail at the 3′ end (Lañá et al., 1989). Upon infection of cells, the genomic RNA is uncoated and its single open reading frame (orf) is translated into a large polyprotein that is co- and posttranslationally processed into the functional proteins (Riechmann et al., 1992). According to current models (Quadt and Jaspers, 1989; Hayes and Buck, 1990; Duggal et al., 1994), the replication of positive-strand RNA viruses is catalyzed by the viral RNA-dependent RNA polymerase (RdRp) complexed to other viral and probably also plant factors. RNA replication is initiated by the synthesis of a full-length negative strand complementary to the genomic positive-strand RNA. A progeny-positive strand is then synthesized using the negative strand as a template. The specificity of the viral RdRp complex for its cognate RNA is achieved through the recognition in cis of signals in the positive and the negative strands.

The tertiary conformation of the promoter located at the 3′ end of the positive-strand RNA, rather than a specific sequence tract, has been proven necessary for its efficient recognition by the RdRp complex and the initiation of the negative strand synthesis of several plant and animal RNA viruses (Dreher and Hall, 1988; Jupin et al., 1990; Kuhn et al., 1990; Pacha et al., 1990; Takamatsu et al., 1990; Van der Kuyl et al., 1991; Cui and Porter, 1995; Rholl et al., 1995). The progeny negative strand seems to remain annealed to the positive-strand template and stem–loop structures in the 5′ NCR of the positive strand. In the case of cucumber mosaic virus (CMV) RNA-3, the nucleotides required for positive-strand synthesis were located between nucleotides 39 and 145 did not alter either the rate of infection or viral accumulation. Nevertheless, these mutants were not able to compete with the wild-type strain in coinoculation experiments. Plants infected with a PPV mutant that lacked nucleotides 127 to 145 showed a very mild symptomatology; the wild-type symptom severity was recovered after spontaneous second-site mutations.

The 5′-terminal 31 nucleotides of the 146-nucleotides-long 5′ noncoding region of plum pox poivirus (PPV) are highly conserved in all the members of the Potyvirus genus. To map the sequences of the 5′ noncoding region that are necessary in vivo for infectivity, we have constructed a nested set of substitution and deletion mutants. While we were not able to infect Nicotiana clevelandii plants with full-length PPV transcripts bearing mutations in the 5′-terminal 35 nucleotides of the viral genome, the deletion of long sequences located between nucleotides 39 and 145 did not alter either the rate of infection or viral accumulation. Nevertheless, these mutants were not able to compete with the wild-type strain in coinoculation experiments. Plants infected with a PPV mutant that lacked nucleotides 127 to 145 showed a very mild symptomatology; the wild-type symptom severity was recovered after spontaneous second-site mutations.

The sequences present in the 5′ terminal loop region of BMV, CMV, and CCMV stem–loops show strong sequence similarity with the internal control regions (ICR) 1 and 2 of the RNA gene promoters (Marsh et al., 1989). In the case of BMV and CCMV RNA-3, the nucleotides required for positive-strand synthesis were located in the 5′-terminal 90 nt of the 5′ NCR (French and Ahlquist, 1987; Pacha et al., 1990), and in also in the intercistronic region of BMV RNA 3 (French and Ahlquist, 1987; Pacha et al., 1990; Traynor et al., 1991). Several ICR-like sequences have also been found in the 5′ NCR of alfalfa mosaic virus (AlMV) RNAs –1 and –3. The 5′-terminal 112 nt of the 314- to 392-nt-long 5′ NCR of the AlMV RNA-3, containing one ICR 2 motif that is also involved in a stem–loop structure, conferred a 10–20% level of RNA-3 accumulation; the next 114 nt, containing two ICR 2 motifs, were necessary to achieve wild-type (wt) levels (Van der Vossen et al., 1993, 1996). It has been postulated that the role of the second and third ICR 2 motifs of AlMV RNAs could be equivalent to that played by the intercistronic region of BMV RNA-3 (Van der Vossen et al., 1993).
A different organization of the positive-strand promoter was shown for the RNA-3 of beet necrotic yellow vein virus (BNYVV), another multicomponent positive-strand RNA virus. The 444-nt-long 5′ NCR of BNYVV does not contain any ICR-like motif but two discrete pairs of complementary sequences that create a secondary structure both in the 5′ end of the positive strand and in the 3′ end of the negative strand. Mutational analysis of the complementary sequences showed that the secondary structure was essential for RNA replication (Gilmer et al., 1992, 1993).

Cis-active sequences necessary for positive-strand RNA synthesis have also been identified in the 5′ NCR of viral monopartite genomes. The 5′-terminal 8 nt of the TMV genome and several downstream redundant elements, which become active when others are deleted, were necessary for viral RNA replication (Takamatsu et al., 1991). In contrast, only the 5′-terminal 8 nt of transcripts were synthesized from these constructs with the TMV genome and several downstream redundant sequences located between nt 39 and 145 are not necessary for genomic RNA replication, but contribute to the competitive fitness of the viral population.

**MATERIALS AND METHODS**

**Plasmids constructs**

Plasmid pGPPV carries a full-length cDNA copy of the PPV genomic RNA cloned downstream of a T7 RNA polymerase promoter (Riechmann et al., 1990).

Plasmid pGG5S6N and substitution and deletion derivatives contain the 5′-terminal 3632 nt of the PPV genome, with different nucleotides substituted and deleted in the 5′ NCR. The substitutions had been previously done by oligonucleotide-directed in vitro mutagenesis and the deletions had been performed using the nuclease BAL-31 (L. Simón-Buela, H. S. Guo, and J. A. García, submitted for publication).

In this study, the fragment PvuII–SalI [nt −50 to 2071; numbering of PPV nt is according to Lain et al. (1989)] of pGPPV was replaced by the corresponding fragment of pGG5S6N and derivatives. The following plasmids were constructed: pGPPVNCol, where the nonfunctional 32AUG codon had been previously changed to 32AAA (Riechmann et al., 1991) and a Ncol site was created between nt 145 and 150 (nt 145AG were changed to 145CC and nt 150U was changed to 150G); pGPPVHpal, where an unique Hpal site was generated, originating an AUG codon in position 31 that leads to a short intraladder off (nt 31U was changed to 31C and nt 32A was changed to 32C); pGPPVBglIII, where an unique BglII site was created between nt 87 and 92 (nt 92A was changed to 92U); pGPPVΔ[1,12] (where nucleotides 1 to 12, included both, were deleted by the BAL-31 treatment); pGPPVΔ[28,35], pGPPVΔ[27,35], pGPPVΔ[22,35], pGPPVΔ[19,35], pGPPVΔ[22,43], pGPPVΔ[39,100], pGPPVΔ[62,87], pGPPVΔ145, pGPPVΔ[126,145], pGPPVΔ[127,145], pGPPVΔ[128,145], and pGPPVΔ[114,145] (Fig. 1).

**In vitro transcription and plant inoculation**

pGPPV and derivatives were cut with PvuII and PstI, restriction enzymes that cut upstream of the T7 promoter and immediately downstream the poly(A) tail at the 3′ end of the PPV sequence, respectively. Capped full-length transcripts were synthesized from these constructs with the T7 Amplicscribe kit (Ambion). The yield and integrity of the transcripts were analyzed by agarose gel electrophoresis.

Three Nicotiana clevelandii primary leaves, a systemic host for PPV, were dusted with carborundum and mechanically inoculated with 2 μl (1 mg/ml of transcript) of the transcription reaction diluted 1:1 in 5 mM phosphate buffer (pH 7.2). Each mutant was tested in at least three separate experiments; eight plants per experiment were inoculated with each transcript. Control plants were inoculated with wt PPV transcripts and mock-inoculated.

**Detection and analysis of progeny viral RNA**

Upper, uninoculated leaves were harvested at different times postinoculation (2 to 8 weeks) and ground in 5 mM phosphate buffer (pH 7.2) (1 g leaves/1 ml buffer). The different proteins in the homogenate were separated by SDS–PAGE in 10% polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblot using an antiserum raised against the coat protein of PPV. Viral infection was also detected by reverse transcriptase-PCR (RT-PCR) on the total RNA isolated from inoculated plants (Riechmann et al., 1991) and by immunocapture-RT-PCR (IC-PCR) of the homogenates (25 μl), using an antiserum raised against the coat protein of PPV to attach the viral particles to the reaction tube and subsequent RT-PCR reaction on the same tube. Two oligonucleotides that amplified the first 248 nt of the 5′ end of the viral genome were employed: 5′-AAAATATAAAAACTCAAC, corresponding to the 5′-first 20 nt, and 5′-GTTCCTCTGGACGGG, complementary to nt 235 to 248. The presence of the mutations in the progeny viral RNA was tested by analyzing the length polymorphism of the amplified fragments electrophoresed in a 2% agarose gel and by sequencing the amplified fragment (nt 1 to 248 of the PPV genome) with the fmol DNA sequencing kit (Promega).

Serial inoculation passages of infected plant homogenates into N. clevelandii plants were performed to determine the rate of infection, the viral accumulation, and the stability of the mutations.

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RESULTS

Sequence analysis of the 5’ NCR of several potyviruses

The genomes of several members of the Potyvirus genus have been sequenced completely. Comparative sequence analysis of the 5’ NCR of 3 potyviruses had revealed the presence of a highly conserved block of 12 nt starting at position 14 of PPV (Laı´n et al., 1989; Turpen et al., 1989). However the multiple alignment of 14 potyviruses indicates that the conserved block includes nt 1 to 31 of the 5’ NCR of PPV (Fig. 2). Further analysis did not reveal the presence of any sequence motif that was repeated several times in the 5’ NCR.

Effects of 5’ NCR mutations on viral infectivity

Plant inoculation analysis revealed that mutations which have introduced AUG codons initiating short intra-

leader orfs (the substitution mutant Hpal and the deletion mutant A[62,87]) and mutations which placed the 345 AUG initiation codon in a poor context for translation initiation, without a purine three nt upstream of the AUG codon (Δ145, Δ[128,145], Δ[126,145], and Δ[114,145]), resulted in noninfectious viruses (Fig. 1). This result is congruent with the repression of translation initiation caused by these mutations in a transient expression system in protoplasts (L. Simóñ-Buela, H. S. Guo, and J. A. García, submitted for publication) (Fig. 1).

We also did not detect virus infection when plants were inoculated with viruses that lacked different sequences in the 5’-terminal 35 nt of the PPV genome (Δ[1,12], Δ[19,35], Δ[22,35], Δ[27,35], Δ[28,35], Δ[22,43]), although in these cases the deletions did not affect RNA translatibility (L. Simóñ-Buela, H. S. Guo, and J. A. García, submitted for publication) (Fig. 1). In contrast, nucleotide substitutions in mutant NcoI and long deletions affecting...
sequences located between nt 37 and 146 ($\Delta[77,91]$, $\Delta[92,130]$, and $\Delta[127,145]$) did not repress viral infectivity and the mutant viruses were detected by symptom observation, Western blots, RT-PCR, and IC-PCR (Fig. 1 and data not shown).

In order to assess the multiplication efficiency of the mutant viruses, N. clevelandii plants were inoculated with homogenates containing the same amount of viral particles. Systemic symptoms (in uninoculated leaves) appeared 8–10 days postinoculation in both wt and mutant-infected plants. The degree of symptom severity was the same for all the viruses with the exception of the one that lacked nt 127 to 145; the chlorotic mottle induced by this mutant in the leaves of N. clevelandii plants was clearly milder than that induced by the wt or the other mutant viruses (data not shown). Although a rigorous kinetic analysis of viral multiplication could not be carried out because of the infection asynchrony, the accumulation of the different mutant viruses was assessed by Western blot with an antiserum raised against the PPV coat protein. No significant difference was observed between the wt and the mutant viruses, even for mutant $\Delta[127,145]$ (Fig. 3), indicating that mutant viruses are able to accumulate to levels similar to those of wild type.

Stability of 5’ NCR mutations through serial passages of plant inoculation

The sequence of the 5’-terminal 248 nt of PPV mutants was determined after serial passages of plant inoculation. All the substitutions (Ncol and BgIII) and the deletions ($\Delta[39,100]$, $\Delta[77,91]$, $\Delta[92,130]$, and $\Delta[127,145]$) were maintained through up to six passages, being the plant material harvested up to 90 days postinoculation. Second-site mutations were detected in two of four independent series of passages of mutant $\Delta[127,145]$. In both cases, the mutation was a point deletion of an uridine or adenosine residue in the sequence stretch $\dddot{\text{G}}$AAAAUUU. Both second-site mutations ($\Delta[127,145]$$\Delta[62$ and $\Delta[127,145]$$\Delta[66]$) restored the severity of wt symptoms, in contrast to the mild symptoms produced by their parental mutant $\Delta[127,145]$ (data not shown). Nevertheless, the viral accumulation was similar with or without the second-site mutation (Fig. 3).

Coinoculations of wt and mutant viruses

The mutational analysis indicated that long sequences located between nt 39 and 145 of the 5’ NCR of PPV were dispensable for viral infectivity. To determine if they contribute somehow to the optimization of the PPV life cycle, we have performed competition experiments between wt and mutant viruses. N. clevelandii plants were inoculated with a mixture of extracts from leaves of infected plants. The presence of the different viruses at different times postinoculation was detected by length polymorphism of the fragments amplified by RT-PCR of total RNA extracted from the infected plants.

N. clevelandii plants were coinoculated with wt and mutant $\Delta[92,130]$ at a proportion (wt:mutant) 1:1, 1:16, or 1:64. Some mutant virus was still detected 18 days postinoculation when it was coinoculated at an excess of 16 or 64 times over the wt virus, but it was not observed at that time when the viruses were coinoculated at equal concentrations. In no case could the mutant virus be detected 42 days postinoculation, regardless of the inoculum composition (Fig. 4A).

No mutant virus $\Delta[127,145]$ was detected 18 and 42 days postinoculation when coinoculated at a concentration equal, 4 times higher or 4 times lower than wt virus (Fig. 4B).

When mutant viruses $\Delta[127,145]$ and $\Delta[92,130]$ were coinoculated at equal concentrations or at a concentration of $\Delta[92,130]$ 4 times higher than $\Delta[127,145]$, only $\Delta[92,130]$ was detected 18 and 42 days postinoculation. When the inoculum contained $\Delta[127,145]$ and $\Delta[92,130]$ at a ratio of 4:1, both viruses were detected 18 days postinoculation, but later on $\Delta[92,130]$ prevailed and was the only virus detected 42 days postinoculation (Fig. 4C).

DISCUSSION

The 5’ NCR of PPV is a 146-nt-long sequence that seems to be devoid of any stable secondary structure due to its very low content of adenosines and guanosines. We have carried out a mutational analysis to determine which nucleotides of this region are necessary for viral infectivity. Full-length PPV transcripts with nt 37 and 38 substituted, or that lacked long sequences located between nt 39 and 145, were able to infect N. clevelandii plants, an herbaceous host of PPV. The presence of redundant sequences, which become active when others are deleted, has been previously postulated for the 5’ NCR of TMV, another monopartite positive-strand plant RNA virus; short deletion analysis of this 5’ NCR showed
that only the 5′-terminal 8 nt of TMV were necessary for viral replication, but the deletion of long downstream sequences (nt 9 to 47 or 25 to 71) also repressed viral infectivity (Takamatsu et al., 1991). In the case of PPV, the sequences that were deleted between nt 39 and 145 without altering the viral infectivity are up to 62 nt long, almost half of the 5′ NCR. This fact, though not conclusive, suggests that there are no redundant sequences in the 5′ NCR of PPV that become active when others are deleted.

Substitution of nt 37 and 38, and deletion of long sequences between nt 39 and 146 did not significantly alter either the rate of plant infection or the viral accumulation. Nevertheless, the conservation of these nt in the 5′ NCR of PPV through evolution suggests that this region plays another role apart from inserting an appropriate translational distance from the 5′ end of the genome to the 141AUG initiation codon. The milder symptomatology produced by mutant Δ[127,145], which has not an apparent correspondence on viral accumulation, might suggest that the deleted sequence of nucleotides plays a role in the interaction with the host. Interestingly, spontaneous second-site mutations, deletion of an adenosine or an uridine in the sequence AAAAAUUUUU, restored the wt symptomatology. It could be speculated that the spontaneous mutations are created to restore the optimum context surrounding the 5′-terminal 31 nt which are necessary for viral infectivity. We cannot discard the presence of second-site mutations in nonsequenced genome regions of other infectious mutant progenies. However, this possibility seems to be unlikely since the time of appearance and the severity of symptoms was the same in plants inoculated with mutant or wild-type transcripts, with the exception of mutant Δ[127,145], where the second-site mutation mentioned above was detected.

It has been previously shown that 144 bases in the 5′ NCR of CCMV RNA 3 were dispensable for systemic infection but important for the relative fitness of the virus to compete with other CCMV genotypes (Pacha and Ahlquist, 1992). In this regard, the infectious PPV mutant viruses Δ[127,145] and Δ[90,130] were not able to compete with the wt virus when coinoculated into N. clevelandii plants. This result indicates that although the deleted sequences are not necessary for viral infectivity, they are required for the optimization of the viral life cycle.

We have recently shown that mutant transcripts Hpal and Δ[62,87], which introduced a short intraleader or, and mutant transcripts Δ145, Δ[126,145], as well as the Δ[128,145] deletion, which left the 141AUG initiation codon in a bad context for translation initiation, caused a significant decrease of the translational levels (between 23 and 52% of the wt ones) in protoplasts transient expression assays (L. Simón-Buela, H. S. Guo, and J. A. García, submitted for publication). This low translation efficiency could explain why full-length PPV transcripts carrying either these mutations or other deletions that also impair the context of the translation initiation codon (Δ145 and Δ[126,145]) were not able to infect plants, while the in vitro transcripts Δ[127,145] (that differs from nonviable mutants in a single base) and Δ[39,100] (with a deletion which embraces that of Δ[62, 87]) were infectious.

Viral infection was also not detected when plants were inoculated with full-length PPV transcripts that lacked different sequences in the 5′-terminal 35 nt of the PPV genomic RNA. Comparative sequence analysis has revealed that the 5′-terminal 31 nt are conserved with little variations in the 5′ end of the potyvirus genomes, thus emphasizing the importance of this region as a target for viral and probably also host factors and suggesting that the entire 31-nt sequence is necessary for these interactions. We have shown in a previous study that neither sequence nor structural elements in the 5′ NCR are necessary for the translation initiation of the PPV genomic RNA (L. Simón-Buela, H. S. Guo, and J. A. García, submitted for publication). Altogether, these results suggest that this 31-nt sequence in the 5′ end of the positive strand, or its complementary in the 3′ end...
of the negative strand, could be the target for the PPV replication complex. If any sequence of the 5′ NCR of PPV participated in the encapsidation of the viral genome, it would also be placed in the 5′-terminal 31 nt of the PPV genome.

Although this 31-nt sequence is conserved in the Potyvirus genus, there is no significant similarity between it and other viral cis-acting regions, as the ICR-like motives of ALMV, BMV, CMV, and CCMV or the sequences in the 5′ NCR of TMV or PVX. Thus, different primary, secondary, or tertiary structures can be involved in providing specificity to the genome replication of different positive-stranded RNA viruses. Further studies are required to determine whether this cis-acting signal heterogeneity is indicating the existence of different mechanisms for the initiation of virus RNA replication. In that case, the requirement of different specific plant factors for each of these mechanisms might contribute to the delimitation of particular virus host ranges.

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