A Short Double-Stranded RNA Motif of *Peach Latent Mosaic Viroid* Contains the Initiation and the Self-Cleavage Sites of Both Polarity Strands†

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Viroids represent the lowest step of the biological scale. Their genome is composed exclusively by a small (246 to 401 nucleotides [nt]) circular RNA without any apparent protein-coding capacity (16, 20, 21, 49), a key aspect that sets a demarcating difference with virus genomes, which do code for one or more proteins of their own. Despite their structural simplicity, viroids are able to replicate autonomously in their host plants and to elicit symptoms in most instances. Therefore, viroids offer unique opportunities for studying RNA replication without interference with the concurrent transcription and translation accompanying the replication of viral RNAs.

Viroids replicate through a rolling-circle mechanism, with two alternative pathways mediated by three catalytic activities: RNA polymerase, RNase, and RNA ligase (6). The most abundant viroid circular RNA, arbitrarily considered as having a plus polarity, is transcribed into oligomeric minus strands that, by themselves (asymmetric pathway) or after processing to their monomeric circular counterparts (symmetric pathway), serve as templates for a second RNA-RNA transcription round leading to oligomeric plus strands that are finally cleaved and ligated to the monomeric plus circular forms. There is empirical evidence supporting the asymmetric pathway for *Potato spindle tuber viroid* (14, 25) and for other nuclear viroids of the family *Pospiviroidae* (6, 7, 18, 30, 44) and the symmetric pathway for *Avocado sunblotch viroid* (ASBVd) (48) and other chloroplastic viroids of the family *Avsunviroidae* (8, 11, 28). Remarkably, cleavage in members of the family *Avsunviroidae* is mediated by hammerhead ribozymes (22, 29, 43) embedded in both polarity strands (17, 19).

Viroids do not necessarily require a transcription starting from specific initiation sites, because reiterative copying of their circular genomes would ensure complete transcription. Although this may have represented an advantage in the primitive precellular RNA world—of which viroids are considered “molecular fossils”—by precluding the need of genomic tags (15), the available evidence indicates that in the present cellular habitat, the polymerization of viroid strands starts at defined sites. Specifically, the labeling of the free 5′-triphosphate group of chloroplastic primary transcripts with [α-32P]GTP and guanylyltransferase (in vitro capping), together with RNase protection assays, has mapped the initiation sites for the family *Pospiviroidae*, obtained by in vitro transcription of the *Potato spindle tuber viroid* monomeric plus circular RNA either with a potato nuclear extract or with purified RNA polymerase II from wheat germ and tomato, are restricted to the minus polarity strand and are not coincidental (49). This discrepancy may in part reflect the difficulties in reconstituting in vitro an initiation complex reproducing the in vivo situation.
There are also data on the initiation sites of a second member of the family Avsunviroidae, Peach latent mosaic viroid (PLMVd) (27), derived from primer-extension analysis of the 5’ termini of certain viroid subgenomic RNAs isolated from infected tissue that are presumed to be replication byproducts and from in vitro transcriptions with truncated PLMVd RNAs and the RNA polymerase of Escherichia coli (41). However, the presence of free 5’-triphosphate groups in the subgenomic RNAs was not examined, and the problems inherent in reconstructing a bona fide initiation complex in vitro are enhanced by using a bacterial RNA polymerase that may have a specificity very different from that of its homologous chloroplastic RNA polymerase (PEP, from plastid-encoded polymerase) (47). Moreover, a second RNA polymerase class exists in chloroplasts (NEP, from nuclear-encoded polymerase) (1), with the available evidence suggesting that it is an NEP-like enzyme that is involved in ASBVd replication (39).

Here we report a reassessment of the PLMVd initiation sites based on mapping the 5’-triphosphate termini of PLMVd plus and minus RNAs isolated from infected peach tissue. Our results have identified another initiation site for each PLMVd strand located in the vicinity of the self-cleavage site of its complementary strand. The quasisymmetrical distribution of both initiation sites and, particularly, their close proximity to the self-cleavage sites are intriguing and indicate that the 6- to 7-base pair (bp) RNA motif in which they are embedded is involved in a dual function.

MATERIALS AND METHODS

RNA extraction, partial purification, and Northern blot hybridization. Nucleic acids from young expanding fruits of a PLMVd-infected peach tree (Prunus persica Batsch cv. ‘Springcrest’) were extracted (12) and fractionated on nonionic cellulose (CF11; Whatman) with STE (50 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM EDTA) containing 35% ethanol (38). Control preparations from healthy gynura leaves. The positions of the PLMVd monomeric circular (C) and linear (L) forms and of certain viroid subgenomic (SG) RNAs are indicated on the right. The acid-precipitable counts of riboprobes in panels A and B were the same, and the exposure time for panel B was three times that for panel A.
FIG. 2. (A) Primer extension analysis with primers RF-43 and RF-132 using as templates the natural monomeric linear PLMVd RNAs (lanes L) or the PLMVd plus monomeric linear RNA generated by self-cleavage during in vitro transcription of a linearized recombinant plasmid with a dimeric PLMVd-cDNA insert (lanes SC). (B) Primer extension analysis with primers RF-44 and RF-26 using as templates the natural monomeric linear PLMVd RNAs (lanes L), or the PLMVd minus monomeric linear RNA generated by self-cleavage during in vitro transcription of a linearized recombinant plasmid with a dimeric PLMVd-cDNA insert (lanes SC). In both panels, the sequencing ladders were obtained with the same primers and recombinant plasmids containing a monomeric PLMVd-cDNA insert. For panels A and B, the positions in the 6% sequencing
peach fruits and from gynura leaves (Gynura aurantiaca DC) infected by Citrus exocortis viroid (CEVd) were obtained following the same protocol. RNAs were separated by denaturing polyacrylamide gel electrophoresis (PAGE) in 5% gels containing 1× TBE (Tris-borate-EDTA) and 8 M urea (45), elecroblotted to nylon membranes, and hybridized with strand-specific riboprobes. For preparative purposes, following electrophoresis the gel was stained with ethidium bro- mide, and the monomeric linear and subgenomic RNAs, identified with appro- priate markers, were cut and eluted.

**Analysis by primer extension.** Different PLMVd-specific primers (Table 1) were used to determine the 5′ ends of plus and minus PLMVd RNAs by primer extension assays. Primers (100 ng) were annealed to purified linear PLMVd RNAs (100 ng) in sterile water at 80°C for 3 min and snap-cooled on ice. Extension reactions (in 20-μl final volumes) were carried out in 50 mM Tris-HCl, pH 8.3, containing 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol (DTT), 1 mM each of dATP, dGTP, and dTTP, 0.1 mM of dCTP supplemented with 3 μCi of [α-32P]dCTP (3,000 Ci/mmol), 125 μg/ml actinomycin D, and 200 U of reverse transcriptase (SuperScript II RNase H-; Invitrogen). After incubation at 42°C for 1 h, the reaction mixture was heated at 70°C for 15 min and the cDNAs were analyzed by denaturing PAGE in 6% sequencing gels. The precise sizes of the extension products were determined by running them in parallel sequence ladders obtained with the same primer and a recombinant plasmid with a complete PLMVd-cDNA insert.

**In vitro capping.** The two subunits of the vaccinia virus mRNA-capping en- zyme (guanylyltransferase) were coexpressed in bacteria after proper modification by additions of N-terminal histidine tags for affinity purification by Ni- agarose chromatography (Qiagen) (33). C-terminally-tailed 20 nucleotide preparations (5 μg) from healthy and PLMVd-infected peach tissues and, when indicated, from individual RNAs eluted from denaturing gels were in vitro capped in a reaction mixture (30-μl final volume) containing 50 mM Tris-HCl, pH 7.9, 1.25 mM MgCl2, 6 mM KCl, 2.5 mM DTT, 10 U of human placental RNase inhibitor (Roche Applied Science), 0.3 mM GTP, and 2.5 μl of a purified gua- nyltransferase preparation (which in previous experiments was sufficient to efficiently label with [α-32P]GTP 200 ng of a PLMVd synthetic transcript; data not shown). Following incubation at 37°C for 45 min, the addition of the same amount of fresh enzyme, and incubation for another 45 min at 37°C, the RNAs were extracted with phenol-chloroform, recovered by ethanol precipitation, and resuspended in sterile distilled water.

**RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE).** After in vitro capping, the RNAs were denatured (95°C for 1.5 min) and incubated for 30 min at 50°C with 10 U of calf intestinal phosphatase (CIP) (Roche Applied Science) in a reaction mixture (30-μl final volume) containing 50 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, and 10 U of HPRI. Following denaturation (95°C for 1.5 min), the incubation was repeated with fresh CIP and HPRI, and the RNAs were treated with phenol-chloroform and recovered by ethanol precipitation (this deproteinating treatment was repeated after each subsequent enzymatic step). For decappping, the RNAs (in a final volume of 10 μl) were incubated for 1 h at 37°C with 0.5 U of tobacco acid pyrophosphatase (TAP) (Epicenter Technolo- gies) in 50 mM sodium acetate, pH 6.0, containing 1 mM EDTA, 0.1% β-mer- captoethanol, 0.01% Triton X-100, and 10 U of HPRI. For ligation, the de- capped RNAs were mixed with 250 ng of an RNA adaptor (5′-CGACUGGGAG CACAGGGACACUGGAGCAGGAGGAGGAGAAGGAAA-3′) that was previously denatured at 65°C for 5 min and incubated for 1 h at 37°C with 10 U of T4-RNA ligase (10-μl final volume) in the buffer recommended by the sup- plier (Roche Applied Science) supplemented with 1 mM ATP and 10 U of HPRI. For reverse transcription (RT), the ligated RNAs (500 ng) were mixed with 100 ng of the corresponding complementary primer, heated at 85°C for 5 min, and incubated for 1 h at 42°C in a reaction mixture (20-μl final volume) containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 0.5 mM dNTP, 10 mM DTT, 10 U HPRI, and 300 U of reverse transcriptase. One-tenth of the RT-reaction volume was used for PCR amplification with 0.4 mM each of the dNTPs, 400 ng each of primer, and 1 U of Expand High Fidelity DNA polymer- ase in the buffer recommended by the supplier (Roche Applied Science). After

**RESULTS**

PLMVd plus and minus monomeric linear RNAs from infected tissue have complex populations of 5′ termini. PLMVd RNAs accumulate in vivo at amounts considerably lower than those of ASBVd RNAs (27). Since this could represent a limiting factor in the characterization of the PLMVd initiation sites, we performed some preliminary experiments aimed at identifying the plant organ with the highest content of viroid RNAs. This organ proved to be the young expanding fruits, for which analysis of four different extracts by denaturing PAGE and Northern blot hybridization with strand-specific riboprobes revealed a reproducible series of PLMVd RNAs of both polarities (Fig. 1). The specificities of the signals were shown by the lack of hybridization of the two riboprobes with CEVd, an unrelated nuclear viroid (21) and of each one with the PLMVd in vitro transcript of the same polarity (Fig. 1, lanes 1, 2 and 5). In agreement with previous results (8), the concentrations of the monomeric linear plus and minus forms were significantly higher than those of their circular counter- parts, with the predominant band being generated by the monomeric linear plus RNA; no larger-than-unit PLMVd RNAs could be detected, but several subgenomic RNAs were clearly visible (Fig. 1). The pattern of these viroid-specific RNAs was analogous in PLMVd-infected leaves collected at different develop- mental stages, but their concentration was significantly lower than in young fruits (data not shown). RT-PCR amplification, cloning, and sequencing revealed that variants of the PLMVd isolate here used had sequences similar to that of the reference variant (2, 27).

We anticipated that a fraction of the PLMVd monomeric linear forms should have the characteristic 5′ termini resulting from the hammerhead-mediated self-cleavage of both polarity strands. To confirm this point and to get insight into how complex the population of 5′ termini was, the PLMVd mono- meric linear forms were eluted and reverse transcribed with the minus polarity primers RF-43 and RF-132. Whereas RF-43 led to six cDNAs, RF-132 generated only one well-defined cDNA (Fig. 2A, lanes L). The corresponding 5′ termini mapped at residues U290, C3, C22, C51, C80, and A113 of the PLMVd plus RNA and are referred to as termini 1, 2, 3, 4, 5, and 6, respectively. Only the cDNA with a length consistent with terminus 1 was clearly visible with primer RF-132, most likely because of the relative positions and abundances of the other termini. Extension controls using as a template the linear monomeric PLMVd plus RNA that results from self-cleave-
during in vitro transcription of the linearized pPL30 plasmid containing a dimeric viroid-cDNA insert identified only terminus 1 (Fig. 2A, lanes SC). A functional origin could thus be established exclusively for terminus 1, the most represented in the population of the monomeric plus linear RNAs isolated from PLMVd-infected tissue, with the other 5’ termini probably reflecting sites in the monomeric plus circular RNA particularly sensitive to degradation or the specific site at which the transcription of PLMVd plus RNAs starts.

Parallel extension experiments with the plus polarity primers RF-44 and RF-26 revealed four 5’ termini mapping to residues U66, U48, G3, and U286 of the PLMVd minus RNA (numbers correspond to the plus polarity), referred to as termini I, II, III, and IV, respectively (Fig. 2B, lanes L). Control extensions with the same primers and the linear monomeric PLMVd minus RNA resulting from self-cleavage during in vitro transcription of the linearized pPL30 plasmid identified U48 as the self-cleavage site of PLMVd minus strands (Fig. 2B, lanes SC). Therefore, any of the remaining 5’ termini could correspond to the initiation site of PLMVd minus RNAs.

Setting up a sensitive methodology for mapping the initiation sites of PLMVd RNAs. Because chloroplast primary transcripts possess a 5’-triphosphate group that can be specifically capped in vitro with guanylyltransferase and GTP (3) and because PLMVd RNAs have been localized in chloroplasts (8), we first attempted to label the monomeric PLMVd linear RNAs with a 5’-triphosphate isolated from PLMVd-infected tissue. However, even with RNA preparations from young fruits, the radioactive signal was too faint for applying the RNase protection strategy (38) used previously for identifying the initiation sites of ASBVd RNAs (data not shown). Therefore, we focused our attention on more-sensitive approaches and, particularly, on an RLM-RACE methodology developed for mapping the genuine 5’ termini of eukaryotic messenger RNAs, which is based on a specific property: such termini are capped (35, 46). We reasoned that by introducing an additional step of in vitro capping, this strategy could be adapted for mapping the initiation sites of PLMVd RNAs. Figure 3 summarizes the sequential steps. Only RNAs with a 5’-triphosphate are specifically capped in vitro with guanylyltransferase and GTP; other RNAs, such as those resulting from processing or degradation, have a 5’-monophosphate or a hydroxyl group not susceptible to in vitro capping (13, 36). Subsequent digestion with CIP removes the 5’-monophosphate groups and impedes the ligation of these RNAs to an RNA adaptor. Further treatment with TAP eliminates the cap structure of the genuine 5’ termini and leaves a free 5’-monophosphate suitable for ligation to the RNA adaptor with T4-RNA ligase. The resulting product is then reverse transcribed with RT and a viroid-specific primer, with the cDNA then being PCR amplified (with primers derived from the viroid and the RNA adaptor), cloned, and sequenced. To test its reliability, this methodology was applied to two artificial mixtures formed by 10 ng of an in vitro PLMVd plus or minus transcript, therefore containing 5’-triphosphate termini, combined with an excess of a nucleic acid preparation from healthy peach, which were used to simulate the situation existing in PLMVd-infected tissue. After completing the enzymatic treatments, RT-PCR products with the lengths predicted for both PLMVd transcripts were obtained (Fig. 4A and B, lanes 2), and cloning and sequencing confirmed that they had the expected 5’ termini (data not shown).

In contrast, two controls in which the incubations with guanylyltransferase or the RNA adaptor were omitted failed to generate the same RT-PCR products (Fig. 4A and B, lanes 3 and
found that the experimental approach developed was sensitive and specific.

The initiation site of each PLMVd strand maps in the vicinity of the self-cleavage site of its complementary strand. Application of the RLM-RACE methodology to CF11-fractionated RNA preparations from PLMVd-infected peach tissue led for each viroid polarity strand to one specific RT-PCR product that was absent in parallel controls in which the CF11-fractionated RNA preparation was from healthy peach tissue (Fig. 4A and B, lanes 5 and 6). More specifically, an RT-PCR product of approximately 200 bp was obtained for the plus polarity strand (Fig. 4A, lane 6). Cloning and sequencing revealed that the 5′ terminus of the corresponding PLMVd plus RNA mapped at position 51 (Fig. 4E), which coincides with terminus 4, the major 5′ terminus after that produced by the hammerhead-mediated self-cleavage, in the primer-extension experiment (Fig. 2A). Thus, the results from both experimental approaches (RLM-RACE and primer extension) were mutually consistent. Intriguingly, this position 51 is located only a few nucleotides away from the self-cleavage site of the minus polarity strand (Fig. 4E).

The specific RT-PCR product obtained for the minus polarity was of approximately 150 bp (Fig. 4B, lane 6). The 5′ terminus of the corresponding PLMVd minus RNA mapped at position 286 (Fig. 4E), which coincides with terminus IV of the primer-extension experiment (Fig. 2B). The results from both experimental approaches were again mutually consistent, and position 286 is also located only a few nucleotides away from the self-cleavage site of the plus polarity strand. Therefore, the transcription of each PLMVd RNA starts at almost symmetrical positions in the base of the long hairpin that contains the sequences forming the hammerhead structures, the so-called hammerhead arm (2). Because of their high thermodynamic stability, the branched conformations of minimal free energy predicted for both PLMVd polarity strands contain the long hairpin or a cruciform variation thereof (2, 27). Nuclease probing (9) and natural sequence variability (2, 40) also support the existence of this long hairpin in vitro and in vivo, respectively.

Analysis of PLMVd subgenomic RNAs also confirms the proposed initiation sites. To provide further experimental support for these initiation sites, we explored the existence in vivo of PLMVd subgenomic RNAs with sizes consistent with their initiations at the proposed sites and subsequent self-cleavages at the sites predicted by the corresponding hammerhead structures (before the first replication round was completed). Examination of the patterns resulting from denaturing PAGE and Northern hybridization of RNA preparations from PLMVd-infected fruits showed that, in addition to the prominent bands generated by the viroid linear and circular forms of both polarities, bands generated by PLMVd subgenomic RNAs of the expected size (about 240 nt), also of both polarities, were discernible (Fig. 1). This size is significantly different from that reported previously (about 280 nt) for what seem to be similar or even the same RNAs (41). We ignore the reasons for this discrepancy, but our size estimation appears reliable because the plus subgenomic RNA essentially comigrated with one of the size markers of 239 nt (Fig. 1).

Next, to confirm that the ~240-nt PLMVd subgenomic RNAs indeed had 5′-triphosphate termini, they were eluted from preparative gels and analyzed with the RLM-RACE strategy developed previously (Fig. 3). To increase its sensitivity, a nested instead of a single PCR amplification was applied. For the plus polarity strand, an RT-PCR product of approximately 110 bp was obtained using as templates the eluted PLMVd subgenomic RNAs and also a CF11-fractionated RNA preparation from PLMVd-infected tissue (Fig. 4C, lanes 8 and 6, respectively). This was the expected size for a PLMVd plus subgenomic RNA with the same 5′ terminus determined previously (Fig. 4A), and cloning and sequencing the amplified product showed that this was the case. The reliability of the nested RLM-RACE was verified with an in vitro PLMVd plus transcript (mixed with an RNA preparation from healthy peach), which generated the predicted amplification product of 160 bp (Fig. 4C, lane 2), and by the lack of the same product in two additional controls without guanylyltransferase and without the RNA adaptor (Fig. 4C, lanes 3 and 4, respectively). Similar experiments for the minus polarity strand, using the eluted PLMVd subgenomic RNAs and a CF11-fractionated RNA preparation from PLMVd-infected tissue as templates, led to the RT-PCR product of approximately 200 bp (Fig. 4D, lanes 8 and 6, respectively) expected for a PLMVd minus subgenomic RNA with the same 5′ terminus determined previously (Fig. 4B), and cloning and sequencing of the amplified product confirmed this point. Again, an in vitro PLMVd minus transcript control (mixed with an RNA preparation from healthy peach) produced the predicted amplification product of approximately 250 bp (Fig. 4D, lane 2), which was absent in two parallel controls in which the guanylyltransferase or the RNA adaptor was omitted (Fig. 4D, lanes 3 and 4, respectively). Collectively, the coincidence of the results obtained with variations (single and nested RLM-RACE) of the methodology applied either to a complex RNA mixture or to purified components thereof provides firm support for the functional relevance of the proposed transcription initiation sites.

**DISCUSSION**

How viroids switch the specificities of certain host RNA polymerases to transcribe RNA instead of their physiological DNA templates is one of the most interesting questions that remains to be solved, particularly considering that viroids have evolved this ability with both nuclear and chloroplastic RNA polymerases. A way to begin addressing this issue is to identify where the transcription of viroid RNAs starts, because the adjacent region may provide hints about the nature of the promoters involved. Moreover, since RNA folding occurs during transcription, the initiation sites of nascent viroid strands may determine the adoption of transient metastable structures that are functionally relevant (see below). We previously developed an in vitro capping/RNase protection methodology for mapping the 5′ transcription initiation sites of ASBVd plus and minus RNAs isolated from infected avocado, which, like the 5′ termini of other chloroplastic primary transcripts, are chemically labeled with a triphosphate (38). Here we report an extension of this methodology for other chloroplastic viroids and host transcripts accumulating in vivo at concentrations much lower than that of ASBVd. An example of these viroids is PLMVd, which differs from ASBVd in key aspects, including G+C content, secondary structure, and morphology of the hammerhead ribozymes (27). Moreover, the accumulation in...
FIG. 4. PAGE analysis of the RT-PCR products resulting from the adapted RLM-RACE strategy (A to D) and localization of the 5’ termini of the corresponding RNA templates on the secondary structure predicted for the plus strand of the PLMVd reference variant (E). (A and B) RT-PCR (single) products from plus RNA templates (using primer RF-129 for reverse transcription and primers RF-43 and RF-553 for PCR amplification) and minus RNA templates (using primer RF-26 for reverse transcription and primers RF-446 and RF-553 for PCR amplification). Lanes 1, DNA markers (100 bp and of multiples thereof). Lanes 2, artificial reaction mixtures containing in vitro PLMVd plus or minus transcripts starting at position 1 (the same numbers are used for both polarities) combined with excesses of a CF11-fractionated RNA preparation from healthy peach fruits. Lanes 3, as in lanes 2 but without the guanylytransferase. Lanes 4, as in lanes 2 but without the RNA adaptor. Lanes 5, as
vivo of both polarity strands does not differ very much in PLMVd (reference 8 and this work), whereas the ratio of plus strands to minus strands in ASBVd is considerably higher (11, 28); on the other hand, the PLMVd monomeric linear RNAs are more abundant than their circular counterparts (reference 8 and this work), whereas the opposite occurs with ASBVd (11, 28). Finally, longer-than-unit viroid strands accumulate in ASBVd-infected avocado (11, 28), but they are absent in PLMVd-infected peach (Fig. 1) or accumulate at very low concentrations (8); this difference may result from the higher catalytic efficiency of PLMVd ribozymes, which are stable and operable as single hammerhead structures (4, 27), compared to those of ASBVd, which are unstable and operable as double-hammerhead structures (23). Overall, these significant divergences between ASBVd and PLMVd made unlikely the existence of similar transcription initiation sites and created the stimulus to determine those of the latter.

After checking with proper controls the reliability of the adapted RLM-RACE methodology, its application to RNA preparations from PLMVd-infected peach fruits led to the mapping of the initiation site of each PLMVd strand in the vicinity of the self-cleavage site of its complementary strand. The existence in these preparations of monomeric linear PLMVd RNAs with 5′-termini corresponding to both initiation sites was further confirmed by primer extension analysis. Moreover, the application of a nested modification of the adapted RLM-RACE methodology to two PLMVd subgenomic RNAs of both polarities led to the same transcription initiation sites. We believe that these sites, located at the base of the hammerhead arm (Fig. 4E), reflect the in vivo situation better than those reported previously at terminal loop A (Fig. 4E) of this long hairpin, which were inferred from primer extension analysis of subgenomic PLMVd RNAs isolated from infected tissue and from in vitro transcriptions with a nonphysiological system (41, 42). Moreover, the 12- to 13-nt insertion observed in certain PLMVd variants, inducing peach calico (an extreme chlorosis) (34), introduces an important sequence and structural variability in loop A that argues against this loop

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development, are transcribed by NEP in PEP-deficient tobacco genes, rather than only the housekeeping genes needed early in presumed, because recent results have shown that all plastid has been reported (5). NEP appears more flexible than initially enzyme, because more than one RNA polymerase of this class that proposed to mediate ASBVd replication on the basis of its replication of an NEP-like enzyme, which could be the same as 12942 DELGADO ET AL. J. VIROL. for coexpression of the two subunits of the vaccinia virus mRNA-"ment and the MCyT.

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