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Study of the polymerization step of the rolling circle replication of peach latent mosaic viroid

Brief Report

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Summary. We have developed an in vitro transcriptional assay using *Escherichia coli* RNA polymerase to initiate the replication of peach latent mosaic viroid (PLMVd). Regardless of the polarity of the PLMVd strand used as template, initiation in vitro occurred at the same hairpin structure. These initiation sites correspond to the 5′-ends of two small (280 nt) PLMVd-related RNAs found in infected peach leaves. Using a series of truncated PLMVd-derived transcripts, we have demonstrated that the viroid domain composed solely of the self-complementary hammerhead sequences is sufficient to trigger polymerase-driven replication in vitro. These data suggest that the bacterial-like RNA polymerase from peach chloroplasts catalyzes PLMVd replication.

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Viroids are small (∼300 nucleotides, nt), single-stranded, circular RNAs that infect higher plants [6, 17]. These RNA species replicate through a rolling circle mechanism that involves only RNA intermediates, and is considered as being either asymmetric or symmetric depending on the absence or presence, respectively, of the minus circular strand, acting as a template, in the mechanism. Since viroids do not encode any protein, two of the most intriguing questions with regard to their replication are the identification of the host polymerase involved, and the determination of the signal(s) that trigger this enzyme. *Pospiviroidae*, such as the potato spindle tuber viroid, share five typical structural domains, a nuclear localization and an asymmetric replication that requires the host DNA-dependent

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RNA polymerase II (RNAP II) [6, 18]. Conversely, the RNAP II is unable to support the replication of the *Avsunviroidae* such as avocado sunblotch viroid (ASBVd) and peach latent mosaic viroid (PLMVd, Fig. 1A) [8, 9]. These viroids possess self-cleaving hammerhead motifs crucial for their symmetric replication

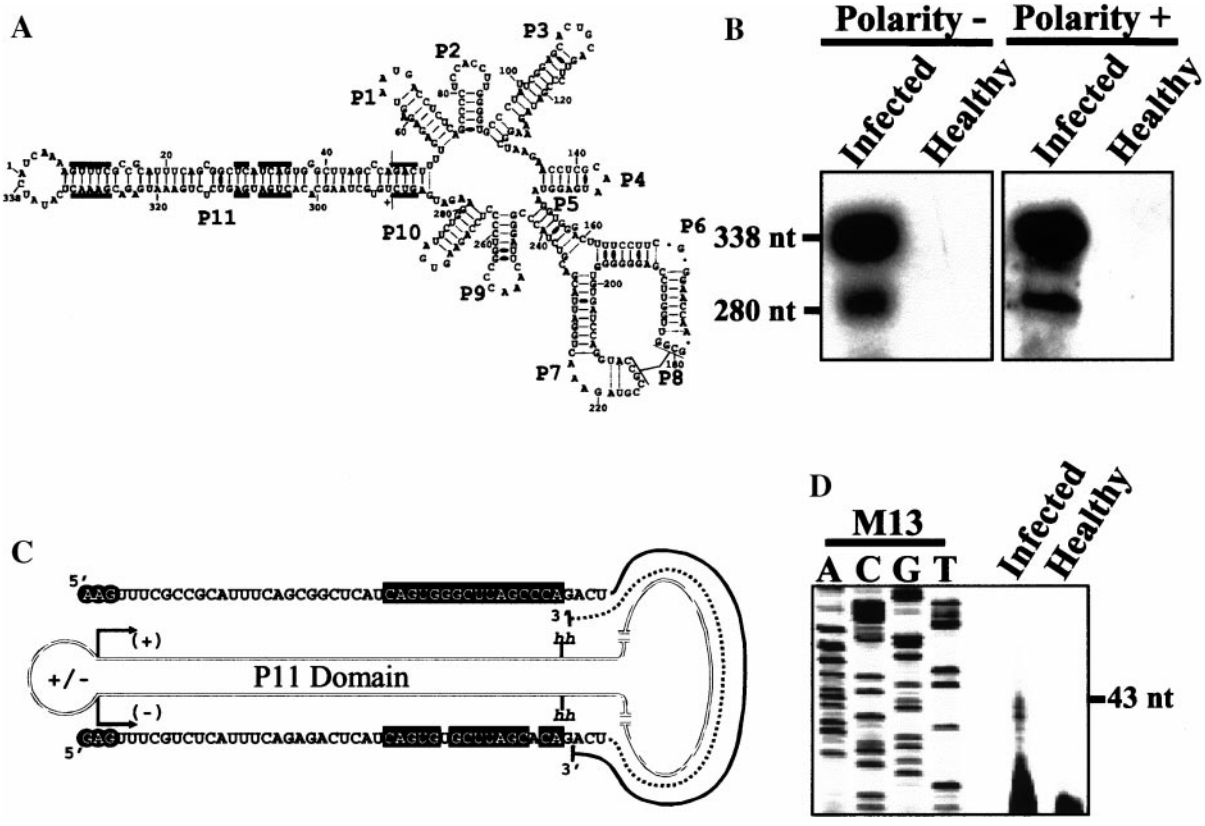


Fig. 1. Structure of the PLMVd intermediates isolated from infected peach leaves. **A** PLMVd sequence and proposed secondary structure. The helix numbering is in accordance with Bussière et al. [4]. The conserved hammerhead sequences of both polarities are indicated by small boxes, and the cleavage sites by arrows. **B** Northern blot hybridization of RNA samples isolated from either infected or healthy peach leaves and probed using PLMVd plus and minus polarity riboprobes, respectively. Adjacent to the gel the length in nucleotides of the PLMVd intermediates are indicated. **C** Schematic representation of the hypothesis formulated for the presence of the 280 nt RNA species. In the middle is the template PLMVd strand. *hh* indicates hammerhead self-cleavage sites. The arrows indicate the positions of the expected 5′ends of both polarities. The sequence above the template is continued by a plain line corresponding to the 280 nt RNA species of plus polarity, while that below is continued by a dotted line corresponding to the 280 nt RNA species of minus polarity. The sequence in the box corresponds to that annealed to by the oligonucleotide in the primer extension assay. The nucleotides illustrated by closed circles are at the 5′ end(s) of the accumulated PLMVd species (see **D**). **D** Primer extension assays using the 280 nt RNA isolated from either PLMVd-infected or healthy peach leaves. The universal primer was used on M13mp19 DNA (M13) to generate a sequence ladder

[6]. Strands of both the plus and minus polarities of ASBVd and PLMVd have been localized in the chloroplasts of infected plants [3, 12], suggesting that the replication of these viroids occurs within this cellular compartment that possesses both a nuclear encoded phage-like RNAP and a plastid encoded bacterial-like RNAP. While the phage-like RNAP was suggested to be responsible for the ASBVd replication based on tagetitoxin resistance [14], nothing was known regarding the polymerization step of PLMVd replication. This brief report presents a model transcription assay using the *Escherichia coli* (*E. coli*) RNAP to study the PLMVd polymerization step, and provides evidence that the peach chloroplastic bacterial-like RNAP may support the replication of this viroid.

All expected PLMVd replication intermediates were detected by Northern blot hybridization [3]. RNA samples were isolated from leaves harvested from both PLMVd infected and healthy peach cultivars using the RNeasy Plant mini kit (Qiagen, Canada). This RNA (5 μg) was then fractionated by electrophoresis through a 5% polyacrylamide gel (PAGE), and Northern blot hybridizations performed by successively using the 338 nt PLMVd linear plus and minus strand-specific riboprobes [3]. These experiments revealed that PLMVd strands of both polarities accumulated predominantly as linear monomers. Circular conformers were found to be present in small amounts, and concatamers were only detected upon over-exposure of the membranes. An enlargement of the section below the linear monomers shows the presence of another abundant RNA species of approximately 280 nt (Fig. 1B, see also Fig. 3 of [3]). Northern blot experiments were rigorously controlled so as to limit cross-hybridization to less than 1% [3]; therefore, these RNA species are of both polarities. They were always detected in RNA samples isolated from infected leaves regardless of the peach cultivar used. As with all of the other PLMVd intermediates, the quantity of accumulated 280 nt species appears to be similar for both polarities, supporting the idea that the rolling circle replication of PLMVd is perfectly symmetric, that is not only the same intermediates present in the cases of both polarities, but they are present in similar quantities.

One likely explanation for the existence of these 280 nt species is that they originate from the first replication cycle, and correspond to the sequence beginning at the initiation site and ending at the self-cleavage site (Fig. 1C). In this case, the 5′ end (i.e. initiation site) should be located in, or near, the left-terminal loop. In order to verify this hypothesis, we undertook the determination of the 5′ end(s) of these RNA species by primer extension. The primer used ($5'$-$_{291}$TGGGCTAAGCCCACTG$_{306}$-$3'$, the numbering corresponds to PLMVd positions) is located in the P11 domain which, according to the hypothesis, would be formed by one strand, consequently it should be easier to anneal the primer to the 280 nt species as compared to complete PLMVd (Fig. 1C). Also, the primer is located at a relatively short distance from the 5′ end(s) which reduces the probability of encountering the problem of premature arrests resulting from the presence of stable secondary structures. Because this primer is perfectly complementary to the product that will be transcribed from the PLMVd strand of plus polarity, as well as those from the minus polarity (with the exception of two residues, 293

and 301), it offers the advantage that it may be used to anneal to PLMVd-derived RNA species of both polarities (Fig. 1C). However, it will not allow us to determine which polarity one strand originates from, nor can it be used to produce a sequence ladder.

The gel-bands corresponding to 280 nt from both infected and heathly peach samples were excised from a non-transfered gel and the RNAs recovered. The RNA was heated for 5 min at 65 °C with 1 pmol of ^{32}P-5′-end-labeled oligonucleotide in 5 μl of annealing buffer (50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 80 mM KCl solution), and then the mixture quickly snap frozen by immersion in −80 °C ethanol/dry ice for 2 min. Synthesis was performed by adding 4 units avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech) in 5 μl of ice cold annealing buffer containing 8 mM DTT and 4 mM dNTP to the primer annealed templates, and then incubating at 42 °C for 30 min. The reaction products were then fractionated by 10% PAGE, and revealed by phosphorimaging (Fig. 1D). A sequence ladder was generated using M13mp19 DNA and the universal primer.

Only three primary products were detected in the RNA of the gel-bands from the PLMVd-infected leaves. Although the polarity of the species serving as templates for the reverse transcriptase cannot be deduced, this assay shows that the 5′ ends of the extension products corresponded to both the AAG and GAG triplets from positions 6 to 8 according to the plus and minus polarity strands, respectively (Fig. 1C). These triplets are at the junction of the left-terminal loop and stem. No longer extension products were detected, suggesting that these signals do not originate from premature arrests; therefore, we considered these residues to be the first nucleotide of the replication products of PLMVd strands of both polarities if the 280 nt species represented such an intermediate. These results do not eliminate the possibility that these 5′-ends may be the result of specific cleavage, nor the existence of additional initiation sites located elsewhere in PLMVd. However, primer extension and rapid amplification of RNA ends by PCR using several oligonucleotides complementary to various regions of PLMVd on total RNA extracted from infected leaves failed to reveal any other potential initiation sites (data not shown).

Having an in vitro transcription assay would be of great value in the study of the PLMVd polymerization step. Prior to initiating this project, we synthesized and purified linear monomeric PLMVd transcripts (i.e. 338 nt) of both polarities that will serve as templates. The use of linear transcripts offers two advantages compared to circular conformers: i. the synthesis is more efficient; and, ii. it provides a template for run-off transcription thereby allowing determination of the initiation site based on the size of the product. Briefly, plasmid pPD1 contains an insert that includes a dimeric end-to-tail copy of PLMVd flanked by the T3 and T7 promoters for the production of plus and minus polarity transcripts, respectively (Fig. 2A). After digestion of the plasmid with the appropriate restriction enzyme, in vitro run-off transcription, DNase (RNase-free) treatment and nucleic acid precipitation, the transcripts were fractionated through a denaturing 5% PAGE gel in 100 mM Tris-borate (pH 8.3), 1 mM EDTA, 7 M urea buffer [1]. Bands

were detected by UV shadowing, excised, and the RNA recovered. During the transcription, the RNA of both polarities self-cleave via their hammerhead motifs, releasing the linear monomeric PLMVd strands.

Initially, we tested various protein extracts from both total cells and isolated chloroplasts from peach leaves as a source of polymerase activity capable of supporting transcription using the synthetic PLMVd strands. If successful, this would have then allowed direct identification of the polymerase involved in PLMVd replication. Unfortunately, these extracts failed to transcribe PLMVd strands. Total wheat germ extract, as well as chloroplastic protein extracts from the leaves of various other plant cells, including those from avocado and spinach, were also prepared and tested to transcribe PLMVd strands. However, none of these extracts supported in vitro polymerization using PLMVd RNAs as templates.

Subsequently, we tested the ability of commercially available RNAP to support the transcription of PLMVd. These assays were performed either in the presence of only one nucleotide (i.e. GTP), or of all four nucleotides (NTP), in order to differentiate between template extension and the synthesis of novel RNA molecules (i.e. transcription), respectively. RNA template (1 μg) was resuspended in 25 μL of 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 mM KCl, 0.2 mM dithiothreitol, 5 μg/ml BSA, 1 mM NTP solution containing 50 μCi of [α-^{32}P]GTP (i.e. for radioactive reactions; Amersham Pharmacia Biotech), the appropriate concentration of RNAP (e.g. 3.8 units of *E. coli* RNAP) added and the reaction incubated at 37 °C for 60 min. The reactions were stopped by adding 1 μL of 1 M EDTA (pH 8.0), extracted with phenol-chloroform, the nucleic acids ethanol precipitated, the pellets resuspended in a mixture of 6 μL DEPC-treated water and 6 μl of formamide dye buffer (i.e. 80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml each of xylene cyanol and bromophenol blue), and the resulting samples fractionated on 12% PAGE gels.

As nuclear encoded phage-like RNAP homologues, we tried the bacteriophage T3 and T7 RNAP (data not shown). Both enzymes catalyzed template extension, but failed to support transcription. As a homologue of a plastid encoded bacterial-like RNAP, we tried the *E. coli* enzyme. A typical gel using the plus polarity RNA strand as template shows that extension products are detected primarily in the upper portion of the gel (Fig. 2B). The same banding pattern is detected under transcription conditions, although with a slightly different intensity resulting from the variation of the total concentration of NTP. More importantly, under the transcription conditions additional specific products ranging from 40–50 nt in size are also observed. The size of these additional RNA molecules suggests that the initiation of synthesis occurred near the left-terminal loop of PLMVd (see Fig. 1A, P11 domain). Using the PLMVd strands of minus polarity as templates also resulted in the detection of transcription products of this size (Fig. 2C). The autoradiograms in Fig. 2 were over-exposed so as to visualize trace bands; however, some bands were not consistently observed during the various experiments and therefore not considered as interesting candidates for the subsequent steps in this study. These results suggest that the signals that trigger the RNAP are

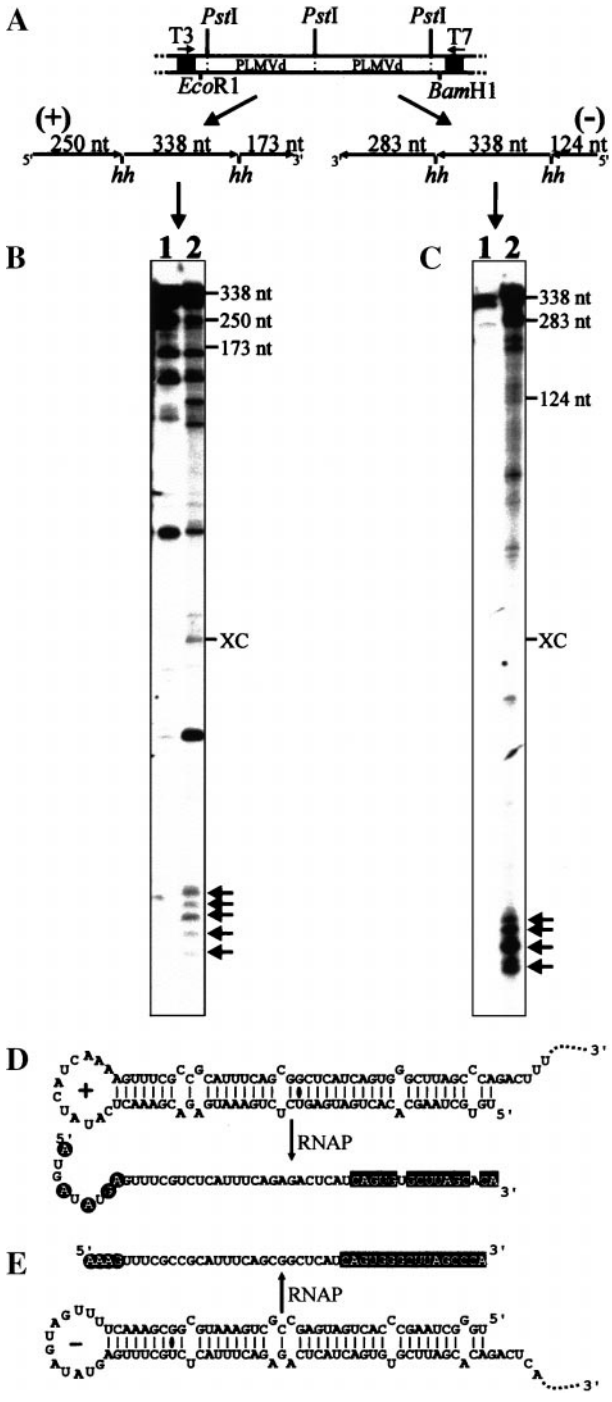


Fig. 2. in vitro transcription of linear monomeric PLMVd RNA templates with *E. coli* RNA polymerase. **A** Schematic representation of the insert in plasmid pPD1 and the run-off transcription products corresponding to the various PLMVd-derived templates. *hh* indicates hammerhead self-cleavage sites. (+) and (−) symbols indicates the polarities of the PLMVd-derived strands. **B**, **C** Autoradiogram of a 12% PAGE gel of transcription assays performed with *E. coli* RNAP using the 338 nt strands of plus and minus polarity,

similar in the strands of both polarities, which is in agreement with the fact that the P11 domains of the plus and minus templates are virtually identical in term of sequence and structure, thereby reinforcing our notion of symmetric replication.

Several experiments were performed to unambigously prove that what we were observing was indeed transcription (data not shown). For example, when the templates were radiolabeled during their synthesis and the assay performed in the presence of non-radioactive NTP, the specific bands (40–50 nt) were not detected. When the assays were performed in the presence of non-radioactive NTP and the resulting RNA molecules 3′-end labeled using T4 RNA ligase and [α-^{32}P]pCp, the specific bands were detected. Thus, these bands are synthesized by transcription catalyzed by the *E. coli* RNAP, rather than being produced either by template extension or specific hydrolysis of the template, respectively. When the incubation was performed in the presence of [γ-^{32}P]ATP, which allows the incorporation of a radioactive phosphate only at the 5′ end of a novel transcript (i.e. no more template extension), only the specific bands were detected. Nevertheless, the incorporation of a single radioactive phosphate required long over-exposition of the gels, and therefore, was not a sensitive enough detection method to warrant modification of the initial assay. Also, if the template was incubated in the presence of DNase either prior to or after the transcripion, the results were similar, demonstrating the absence of DNA contaminants. In contrast, if the template was incubated in the presence of RNase either prior to or after the transcription, no product was detected. Together these results prove that this is a transcription reaction using RNA PLMVd strands of both polarities as template. Finally, all the assays presented in this report so far were performed using solely magnesium as bivalent cation in order to preserve the specificity of the *E. coli* RNAP. In the past, some polymerases were reported to replicate viroids in vitro, but only in the presence of manganese, which significantly reduces the specificity of these enzymes [2, 16]. When 1 mM manganese was added to the reaction mixture, no significant modifications of the results in terms of specificity and efficiency were observed.

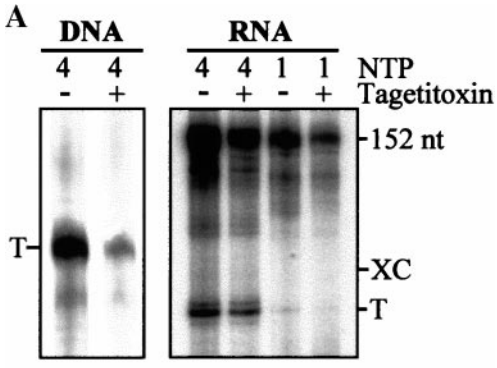
The initiation sites of the *E. coli* RNAP were localized by primer extension as described previously. Because the transcription products are more abundant than their respective templates, only a minimal background due to the templates was observed (data not shown). Using the products derived from the PLMVd strands

Fig. 2 (*caption continued*)
respectively. In each case lane 1 is under template extension conditions (i.e. only GTP is present) and lane 2 is under transcription conditions (i.e. all four NTPs are present). The transcription specific products are indicated by arrows. Xylene cyanol (XC) and position of size markers are indicated on the right side of the gels. **D**, **E** Localization of the initiation sites using the plus and minus PLMVd strands as templates, respectively. These sites were determined by primer extension (not using the above gel). The positions of initiation are illustrated by closed circles on the product. The sequence in the boxes corresponds to the primer

of plus polarity, five extension products were detected possessing 5′ purines (i.e. 4 adenosines and 1 guanosine) that correspond to the presence of either uridine or cytosine on the template, located either in the left-terminal loop (positions C_{333}, U_{335}, U_{337} and U_2) or at the first uridine of the adjacent stem (position U_{332}) (Fig. 2D). Using the products of transcription from the PLMVd strands of minus polarity, four predominant extension products were observed (Fig. 2E). These products have 5′ nucleotides corresponding to an initiation occurring at the consecutive uridine and cytosine residues that form the first two basepairs of the stem adjacent to the left-terminal loop (positions C_{331} and U_{332}), and the first two uridines within the left-terminal loop (positions U_{333}, U_{334}) on the templates. These experiments show that the *E. coli* RNAP initiation sites of transcription on strands of both polarities occur within the left-terminal stem-loop, in the same region as the 5′ end of the natural 280 nt species. More initiation sites are observed in the in vitro assay than in the RNA samples isolated from PLMVd-infected peach-leaves; however, the observation of multiple initiation sites in vitro compared to in vivo is frequently observed (e.g. see [7]). Therefore, we felt that the *E. coli* RNAP might be used as a model enzyme to study the polymerization step of PLMVd replication, and consequently pursued the characterization of this transcription assay.

Based on the fact that a protein extract from avocado chloroplast replicating ASBVd was resistant to tagetitoxin, a phage-like RNAP has been proposed to support the replication of this viroid [14]. Tagetitoxin is known to strongly inhibit both *E. coli* RNAP and the homologous chloroplastic RNAP [10]. For example, a concentration of 12 μM tagetitoxin (Epicentre Technologie) strongly prevented the transcription of a control DNA template (i.e. pBlueScript KS DNA digested by *Sca*I) by the *E. coli* RNAP under our reaction conditions (Fig. 3A). This experiment in the absence of tagetitoxin constitutes a positive control that was performed several times during this research. Considering the number of copies of the plasmid as compared to the number of PLMVd strands, we estimate that the transcription using the DNA as template was at least one order of magnitude more efficient than that using the RNA. Surprisingly, 12 μM tagetitoxin has only a weak effect on the transcription from PLMVd RNA templates by *E. coli* RNAP (Fig. 3A). Only higher concentrations of tagetitoxin (i.e. 60 to 120 μM) led to $\sim$ 50% inhibition with a PLMVd derived RNA template (data not shown). Like actinomycin D [11], the sensitivity to tagetitoxin appears to be affected by the nature of the template (i.e. RNA versus DNA), as a consequence it should be used with caution when utilized as a means of identifying an RNAP involved in RNA pathogen replication. However, these results do not exclude the possibility that ASBVd and PLMVd use different RNAPs for their respective replications, particularily since it is known that these viroids share some features, but differ for several others.

In order to identify which region triggers the RNAP, we tested some PLMVd-derived transcripts. During synthesis of the monomeric PLMVd strand, both the 5′ and 3′ transcripts released by self-cleavage are composed of PLMVd sequences and a small plasmid derived portion (Fig. 2A). All of these RNAs were substrates



B

Template	Length	Polarity	Transcription
	250 nt	+	no
	173 nt	+	yes
	152 nt	+	yes
	124 nt	-	no
	283 nt	-	yes

Fig. 3. Characterization of the PLMVd in vitro transcription assay. **A** Example of autoradiograms of PAGE gels of assays performed using either DNA or RNA as template. The left panel shows the transcriptional controls using pBlueScript KS DNA digested with Sca I (pKS/*Sca* I) as template. The right panel shows the transcription assays performed with PLMVd RNA strands as templates. Template extension and transcription assays are differentiated by the indication that 1 or 4 NTP were presented in the reaction mixture, respectively. Transcription reactions were performed either in the presence (+) or the absence (−) of 12 μM tagetitoxin and analyzed onto a 12% PAGE gel. T indicates synthesis of novel strands (i.e. transcription). Xylene cyanol (XC) and position of size markers are indicated. **B** Compilation of the assays performed using various PLMVd-derived transcripts as templates. The strands supporting the transcription are indicated by the yes in the appropriate row

for the template extension activity, but only the 173 and 283 nt species of plus and minus polarities, respectively, supported the transcription reaction (Fig. 3B). Both of these transcripts contain the intact P11 domain, while the non-template strands of 250 and 124 nt do not. We also show that a 152 nt, plus polarity, PLMVd-derived transcript (i.e. positions 273 to 86; Fig. 3B) that corresponds primarly to the P11 domain supported transcription. Thus, the P11 domain appears to be the essential region of the template for replication. This domain is composed solely of the self-complementary, hammerhead sequences. If this domain serves a similar function in vivo, it would constitute a novel function for the PLMVd hammerhead

sequences. The sequence of the P11 domain within the full-length plus polarity strand was demonstrated to fold predominantly, if not exclusively, into a long stem ending with the large loop [4]. A similar demonstration has not been performed for the strands of minus polarity; however, several biochemical data suggested that it also folds into a long stem [1, 3, 15]. If the presence of this character is indeed a requirement for transcription, this may explain the conservation of the superimposition of the self-catalytic hammerhead sequences of both polarities, i.e. to conserve this long stem. For both ASBVd and the hepatitis *delta* virus it has been reported that the initiation of replication also occurred at long terminal hairpins involving relatively large loops. Apparently, such a hairpin appears to be a common requirement for the initiation of the replication of various RNA pathogens, regardless the RNAP involved [5, 13].

Based on the facts that PLMVd accumulates in chloroplasts [3], that *E. coli* RNAP supports the in vitro transcription of PLMVd strands of both polarities, and that these in vitro initiation sites originated from the same region (i.e. the hairpin) that the 5′-ends of an accumulated PLMVd species, we suggest that the chloroplastic bacterial-like RNAP might be the enzyme responsible for PLMVd replication. However, the data presented here does not completely rule out the possibility that the nuclear-encoded peach chloroplast phage-like RNAP might be the enzyme involved in PLMVd replication. Obviously, a more cogent proof would be the identification of a polymerase from a chloroplast extract. Nevertheless, the model enzymatic assay using the *E. coli* RNAP reported here should be a fundamental building block for the further study of PLMVd polymerization, and for the design of inhibitors preventing the replication of these viroids.

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