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Transcription of potato spindle tuber viroid by RNA polymerase II starts in the left terminal loop

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Abstract

Viroids are single-stranded, circular RNAs of 250 to 400 bases, that replicate autonomously in their host plants but do not code for a protein. Viroids of the family *Pospiviroidae*, of which potato spindle tuber viroid (PSTVd) is the type strain, are replicated by the host's DNA-dependent RNA polymerase II in the nucleus. To analyze the initiation site of transcription from the (+)-stranded circles into (−)-stranded replication intermediates, we used a nuclear extract from a non-infected cell culture of the host plant *S. tuberosum*. The (−)-strands, which were de novo-synthesized in the extract upon addition of circular (+)-PSTVd, were purified by affinity chromatography. This purification avoided contamination by host nucleic acids that had resulted in a misassignment of the start site in an earlier study. Primer-extension analysis of the de novo-synthesized (−)-strands revealed a single start site located in the hairpin loop of the left terminal region in circular PSTVd's secondary structure. This start site is supported further by analysis of the infectivity and replication behavior of site-directed mutants in planta.

Keywords: PSTVd; Transcription start site; Polymerase II; Enhancer regions; Nuclear extract; Dynabeads

Introduction

Viroids are subviral pathogens consisting of a protein-free, circular RNA molecule containing only 246 to 401 nucleotides. This circular RNA, by definition the (+)-strand, as well as the (−)-strand has no coding capacity. They are not encapsidated. Different biological functions of the viroid RNA, such as recognition by host factors necessary for transcription of strands in both polarities, processing of oligomeric replication intermediates to monomers and ligation to circles, and systemic transport in the plant, require different, partially overlapping structural elements of the RNA (for reviews see Hadidi et al., 2003; Flores et al., 2000, 2004; Tabler and Tsagris, 2004).

Viroids are divided into two families (Flores et al., 1998): *Pospiviroidae* (named after potato spindle tuber viroid, PSTVd) with about 40 different members and *Avsunviroidae* (named after avocado sun blotch viroid, ASBVd) with four members. The RNA of *Avsunviroidae* accumulates in chloroplasts and is replicated by the nuclear-encoded chloroplastic polymerase (NEP) in a symmetric rolling-circle mechanism, in which oligomeric intermediates are cleaved by their hammerhead-ribozyme activity and ligated to circles (for review see Flores et al., 2000). In contrast, the RNA of *Pospiviroidae* accumulates in the nucleolus and is replicated by DNA-dependent RNA polymerase II (pol II) in an asymmetrical rolling-circle mechanism, in which (−)-stranded oligomeric intermediates are transcribed directly into (+)-stranded oligomers. The processing into circles is mediated by host-encoded RNases and a ligase that act on a thermodynamically metastable viroid structure formed by sequential folding (for review see Steger and Riesner, 2003; Flores et al., 2004).

In pospiviroids, well defined structural elements as well as essential nucleotides were found as motifs critical for transcription. Neither the circular (+)-PSTVd nor the (−)-PSTVd replication intermediates do contain a TATA-box motif as promoter element for pol II. But the circular (+)-PSTVd does contain in its native structure GC-rich RNA elements with similarity to house-keeping gene DNA promoters. Further-

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more, site-directed mutagenesis pointed to a thermodynamically metastable structure including a GC-rich hairpin structure (hairpin II, HPII) to be essential for infectivity (Loss et al., 1991). HPII seems to serve as a promoter element for (+)-strand synthesis during the second transcription step (Qu et al., 1993). That is, due to sequential folding during synthesis of (−)-PSTVd, HPII is formed from two distant sequence regions which are part of GC-boxes in the native structure under thermodynamic equilibrium (Repsilber et al., 1999).

Up to now, no transcription factors for replication of pospiviroids have been identified. Therefore, two models for PSTVd transcription are possible: either there are specific start sites for replication, which might be defined by the GC-rich elements in (+)-strands and HPII in (−)-strands, or transcription may start at any position on the viroid RNA, which could be facilitated by the RNA template instead of a natural DNA template.

Specific start sites were found in *Avsunviroidae*, and polymerase recognition appears to involve segments of specific structural elements (Navarro and Flores, 2000; Pelchat and Perreault, 2004). For PSTVd, three start sites have been proposed: two by our group (Fels et al., 2001) and a third one by Tabler and Tsagris (1990). In the following, we show that the proposed start sites at nucleotides A111 and A325 are experimental artifacts due to an endogenous nucleic acid of the nuclear extract used in the experiments rather than representing the 5′-ends of a newly synthesized RNA by the pol II. To avoid any contaminations leading to misassignments, we established a purification system that allows us to isolate de novo-synthesized transcripts from both the endogenous nucleic acids and the template used for in vitro transcription analysis. The true start site was localized to the left terminal loop by using primer-extension analysis of the isolated de novo RNA. Mutagenesis studies on the start site and proposed enhancer regions support the model that the start site is determined by the specific structure. In this respect, PSTVd transcription is similar to the situation found in other viroids such as ASBVd and peach latent mosaic viroid (PLMVd).

Results

Detection of signals after extension of viroid-specific primers in the absence of viroid template

The (−)-strand PSTVd synthesis from exogenously added circular (+)-PSTVd RNA was analyzed in a nuclear extract prepared from non-infected potato (*S. tuberosum*) culture cells. The use of a viroid-free cell culture is essential to avoid a high background of endogenous, constitutively transcribed viroid molecules. In earlier experiments, after transcription, the nucleic acid components were purified by phenol–chloroform extraction and used directly as template in a primer-extension reaction (for details see Fels et al., 2001). Gel-separation of the primer-extension products and autoradiography (see Fig. 1) resulted in a single band corresponding to nucleotide A111. Consequently, A111 was interpreted as a start position of PSTVd (−)-strand synthesis by Fels et al. (2001). The site-directed mutagenesis study (cf. below), showing that an A111 → G mutation reverted during infection in plants, seemed to confirm this interpretation.

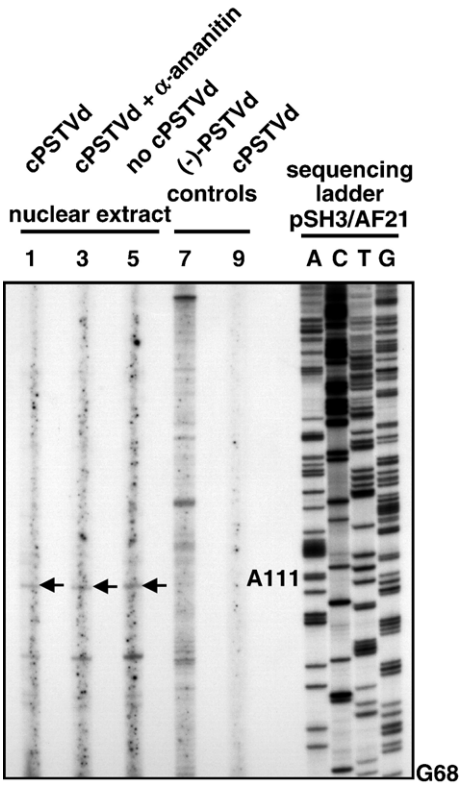

Fig. 1. Primer-extension analysis after transcription reaction in a nuclear extract. The transcription reactions were carried out using 10 µg protein of a nuclear extract from a non-infected potato cell culture followed by purification of the nucleic acids by phenol-chloroform extraction. For primer-extension at 53 °C, the oligonucleotide AF21 (see Fig. 5) was used. Autoradiography of an 8%-PAA sequencing gel loaded with the whole primer-extension sample is shown. Lane 1, in vitro transcription with circular (+)-PSTVd as template (about 100 ng); lane 3, in vitro transcription in the presence of 10^{-6} M α-amanitin; lane 5, in vitro transcription without PSTVd template; lane 7, 100 pg dimeric (−)-PSTVd transcript (pSH3) as positive control for the primer-extension reaction; lane 9, 100 pg circular (+)-PSTVd as control for (−)-strand specificity. All lanes loaded with transcription reactions (lanes 1, 3, 5) show a signal that corresponds to nucleotide A111.

In an additional control, which was not performed, however, in the earlier study, the same signal in primer-extension also appeared in mock-treated controls, that is without addition of circular (+)-PSTVd as template (compare lanes 1 and 5 of Fig. 1). In contrast to a result described in Fels et al. (2001), the signal was not fully suppressed by addition of α-amanitin (see lane 3 of Fig. 1), which indicates non-involvement of pol II. Consequently, the signal is due to unspecific binding of the primer to an endogenous nucleic acid of the nuclear extract and not to a specific binding to newly synthesized (−)-PSTVd RNA. Because these results were reproduced in different, independently prepared nuclear extracts, a contamination during the experimental procedure could be excluded. Evidence supporting the signal as PSTVd-specific by using other PSTVd-specific primers (AF16, AF17; see Fig. 5) could not be obtained (data not shown). Thus, the detected signal is not based on PSTVd RNA but rather on a

cellular nucleic acid species present in the nuclear extract, which binds the 20 nt long PSTVd-specific primer even under stringent conditions. Efforts to sequence the cellular nucleic acid species failed.

Purification of de novo-synthesized (−)-PSTVd RNA and determination of its start-site

Protocol to purify de novo-synthesized (−)-strands

Before reinvestigating the start site(s) of PSTVd transcription, we established a purification protocol to separate the newly synthesized (−)-PSTVd RNA from the endogenous nucleic acids of the nuclear extract. This prevents any unspecific binding of a PSTVd-specific primer to a non-PSTVd nucleic acid, and detection of false signals is excluded. Take note that the circular (+)-PSTVd template in the transcription reaction is present in great excess over the newly synthesized (−)-PSTVd RNA, and that both template and product are nearly self-complementary, which renders specific binding of primers and affinity probes quite difficult.

We used a hybridization-based procedure in which the nucleic acid of interest is bound to biotinylated capture probes of the complementary sequence, followed by immobilization to paramagnetic streptavidin particles. Thus, the target RNA can be separated from the pool of nucleic acids and investigated in subsequent experiments.

A flow-chart of the purification is shown in Fig. 2. In step 1, the circular (+)-PSTVd template was removed from the pool of nucleic acids in the transcription mixture by hybridization to dimeric biotinylated (−)-PSTVd as capture probe and separation by Dynabeads. Because any non-biotinylated (−)-RNA remaining in the sample would lead to artificial and misleading primer-extension signals, a prepurification step was introduced to guarantee that all probe molecules carried at least one biotin label and that any added probe molecule could completely be removed afterwards by binding to Dynabeads. The removal step for circular (+)-PSTVd was repeated to get rid of all (+)-molecules that would otherwise reduce the yield of de novo-synthesized (−)-strands hybridized to (+)-probes due to competition by circular (+)-PSTVd during the following step.

After removal of the template, de novo-synthesized (−)-PSTVd RNA was extracted by hybridization to dimeric biotinylated (+)-RNA and binding of the hybrids to Dynabeads. In step 3, the de novo-synthesized (−)-RNA was released from the hybrids by denaturation at 75 °C in the presence of 8 M urea. During this step, the biotinylated probe molecules remained bound to the streptavidin-coated Dynabeads; a release of probe molecules from Dynabeads was possible at 75 °C only in the presence of 95% formamide.

The purification method was verified by gel electrophoresis and Northern blotting as shown in Fig. 3. As a model for the

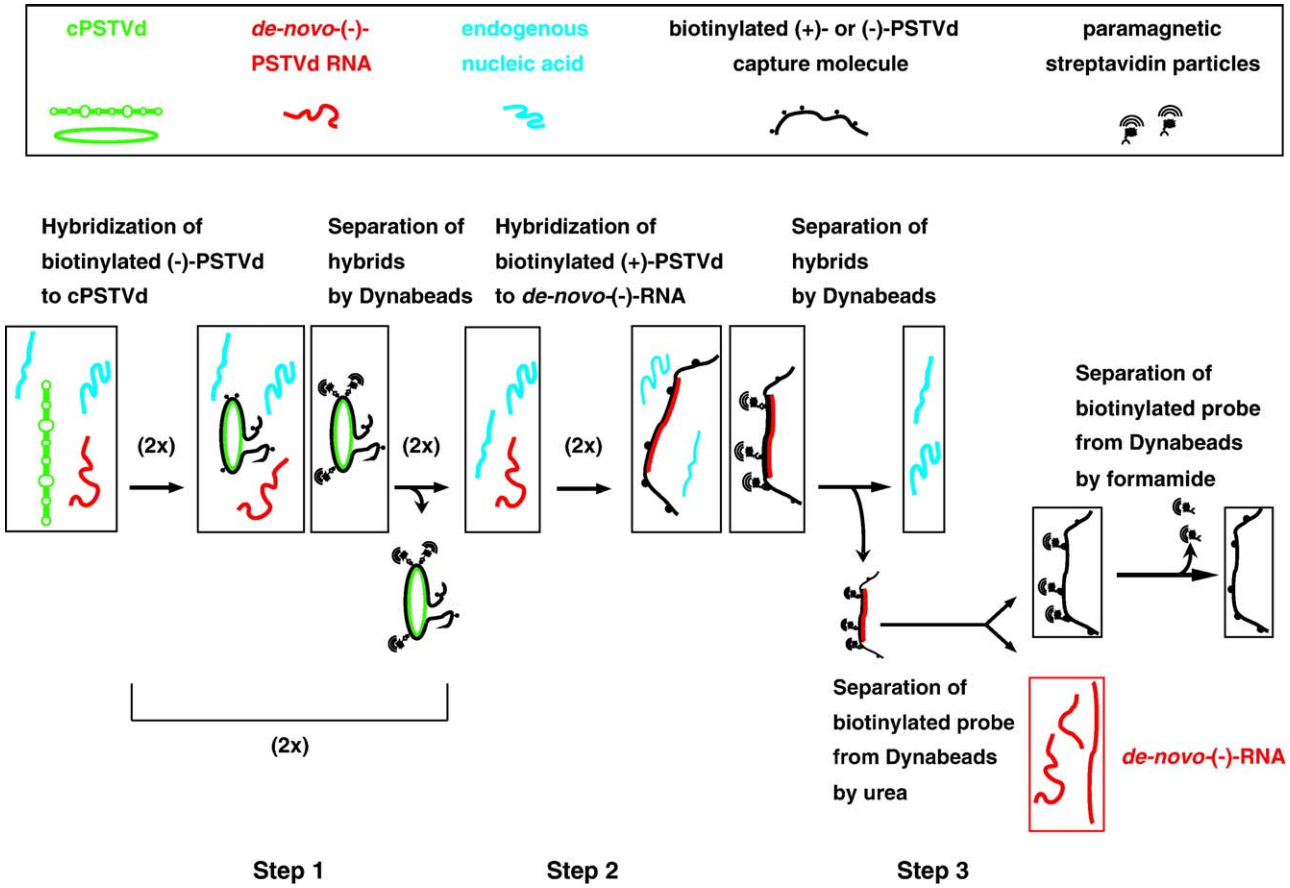

Fig. 2. Scheme for isolation of *de novo*-synthesized (−)-PSTVd RNA after transcription reaction in a nuclear extract. The essential steps of purification are given adjacent to the arrows; the symbols for the different RNAs are explained in the box at top. For details see text.

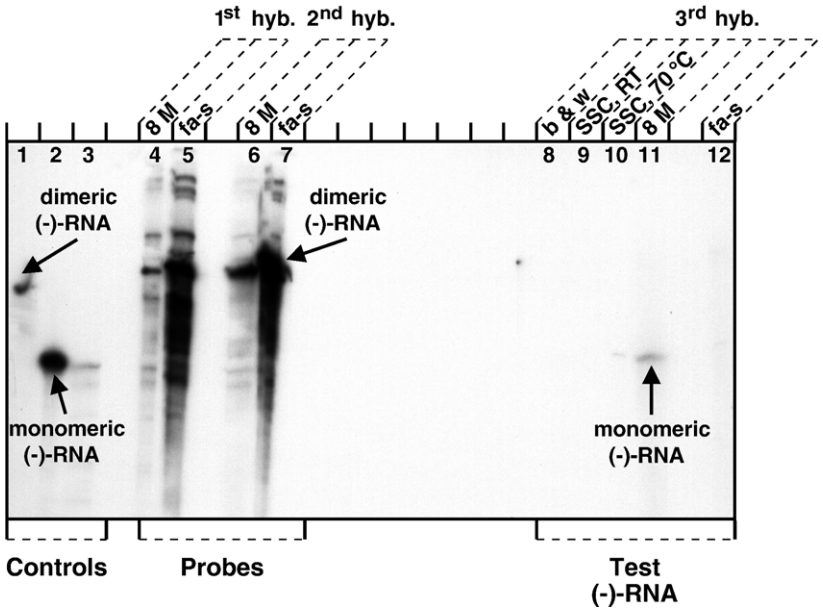

Fig. 3. Effectiveness of the hybrid capture method shown by Northern blot analysis of purified PSTVd (−)-RNA using linear (+)-PSTVd in vitro transcripts as hybridization probes. All three hybridizations were carried out following the protocol shown in Fig. 2 for purification of de novo-synthesized (−)-RNA. At each step, target RNA was isolated by 100 μg Dynabeads. After release from the beads, supernatants of each elution step were ethanol-precipitated and separated by PAGE, followed by nucleic acid transfer onto a nylon membrane. For detection of (−)-PSTVd RNA, radioactively labeled (+)-transcripts were used. Lanes 1–3 served as controls for specificity of the radioactively labeled probes. Lane 1, dimeric (−)-stranded in vitro transcript (pSH3, 3.5 ng); lane 2, monomeric (−)-stranded in vitro transcript (pRH716, 5 ng); lane 3, monomeric (+)-stranded in vitro transcript (pRH715, 100 ng). For step 1 (see Fig. 2), 200 ng monomeric PSTVd in vitro transcript (pRH715) was used as (+)-RNA that was removed from the assay by hybridization to 300 ng biotinylated, dimeric (−)-PSTVd probes (pSH3, lanes 4 and 5). This step was repeated to remove (+)-PSTVd RNA remaining after the first step (lanes 6 and 7). For steps 2 and 3, as (−)-RNA 10 ng monomeric (−)-PSTVd in vitro transcript (pRH716) was used that was isolated from the assay by hybridization to 300 ng biotinylated, dimeric (+)-PSTVd probe (pRH717, lanes 8 to 12). Non-numbered lanes are empty. 8 M, 8 M urea wash; fa-s, formamide wash; b&w, binding and wash (5 mM Tris–HCl, 0.5 mM EDTA, 1 M NaCl); SSC RT/70 °C, wash with 1× SSC buffer at room temperature and 70 °C, respectively. Expected bands are marked by arrows and labels.

transcription assay, we mixed 10 ng of a synthetic monomeric (−)-PSTVd transcript, which should resemble the de novo-synthesized (−)-RNA, with 200 ng of a synthetic monomeric (+)-PSTVd transcript, as a mimic of circular (+)-PSTVd, and endogenous nucleic acids from the nuclear extract used for the actual transcription assay. At each step, the RNA was released from the hybrids bound to Dynabeads by a 8 M urea-denaturation step, the remaining probe RNA was removed from Dynabeads by a formamide denaturation step, and analyzed by Northern blot. In summary, each of the purification steps worked as expected.

We paid special attention to the following, critical steps of the purification procedure: (i) preformed, “wrong” hybrids might result from double-strand formation of (+)-template with newly synthesized (−)-RNA in the transcription assay, which would lead to a decrease in recoverable (−)-RNA; such hybrids were resolved during step 1 by a heat-denaturation step in presence of an excess of (−)-probe molecules; (ii) all nucleic acids could be recovered from the purification procedure without noticeable degradation or contamination with other RNAs; that is, no RNase activity from the transcription assay remained, and no strand breaks were introduced by the repeated hybridization steps. We could also not detect degradation at selected sites in the viroid structure after incubation of circular (+)-PSTVd and of synthetic PSTVd transcripts in the nuclear extract, as already described by Fels et al. (2001).

Purification of de novo-synthesized (−)-PSTVd RNA from a nuclear extract

With the procedure described above, it was possible to purify (−)-PSTVd sequences from a pool of different nucleic acids. If such a purification is successful from a transcription assay, the following questions can be answered: (i) do the nuclear extracts contain a transcriptionally active pol II, and (ii) does the de novo RNA, transcribed by pol II from nuclear extract, have a defined start site?

Two different transcription assays were carried out, the first containing 500 ng of circular (+)-PSTVd as exogenous template, the second was a mock-treated control without any viroid template. Transcription was carried out according to the protocol described earlier (Fels et al., 2001). Subsequent to the transcription assay, the nucleic acids were extracted by phenol–chloroform and precipitated with ethanol. Pellets were redissolved and applied directly to the purification protocol. Eluted fractions were precipitated with ethanol and analyzed by Northern blot hybridization.

As shown in Fig. 4, the new purification protocol was successful in isolating de novo synthesized (−)-PSTVd RNA. Lanes 7 to 10 show a very strong signal representing the biotinylated, dimeric (−)-PSTVd probe used for removal of circular (+)-PSTVd. Due to its (−)-polarity, it is detected by the radioactively labeled (+)-in vitro transcript (compare with lane 1). Lanes 11 to 15 show all fractions of the third hybridization, i.e., isolation of de novo-synthesized (−)-PSTVd RNA. During

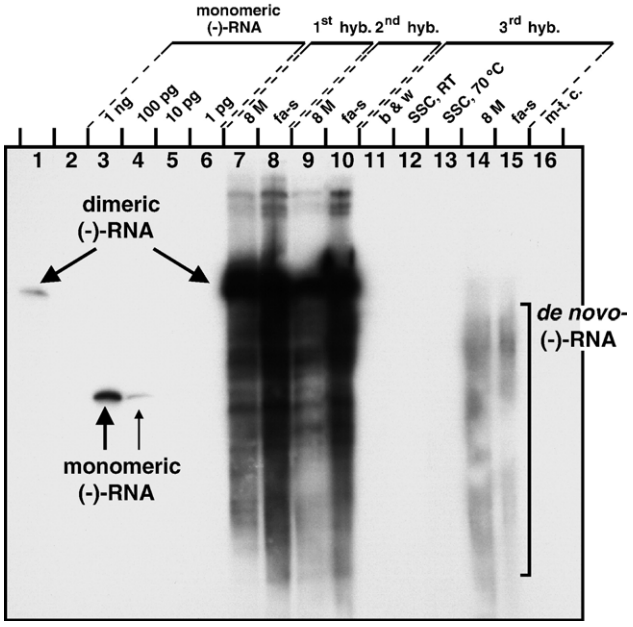

Fig. 4. Detection of de novo-synthesized (−)-PSTVd RNA by Northern blot analysis. From a transcription assay with circular (+)-PSTVd (500 ng) as template in the nuclear extract, the circular (+)-PSTVd was removed by two hybridization steps using 2 × 750 ng of dimeric biotinylated (−)-PSTVd transcripts (pSH3); hybrids were removed by binding to 250 µg Dynabeads at each step; lanes 7 and 8 show fractions of the first hybridization, lanes 9 and 10 of the second hybridization. De novo-synthesized (−)-RNA was isolated by use of 300 ng dimeric biotinylated (+)-PSTVd transcripts (pRH717); hybrids were removed by binding to 100 µg Dynabeads; lanes 11 to 15 show all fractions of this third hybridization. Supernatants of elution steps were ethanol-precipitated and separated by PAGE; afterwards, nucleic acid was transferred to a nylon membrane. Detection of (−)-PSTVd RNA was achieved by hybridization with radioactively labeled monomeric (+)-PSTVd RNA (pRH715). Lane 1, dimeric (−)-PSTVd in vitro transcript (pSH3, 2.5 ng); lane 2, circular (+)-PSTVd as control for binding specificity of the radioactively labeled transcript (200 ng); lanes 3 to 6, monomeric (−)-PSTVd in vitro transcript (pRH716) for determination of detection limit; lane 16, assay without any circular (+)-PSTVd as exogenous template. For labeling see Fig. 3.

the mild wash steps (lanes 11 to 13), no signal is detected; i.e., the captured (−)-PSTVd RNA is tightly bound to its complementary sequence and only detectable after using urea to separate it from its capture probe (lane 14). An additional, but weaker signal is detectable also in lane 15, where formamide was used to disrupt biotin–streptavidin binding and to release the biotinylated capture probe. This is expected because removal of the target RNA from its complementary probe by urea is not always complete. Most importantly, the mock-treated control, where no circular (+)-PSTVd was added to the transcription reaction, revealed no corresponding signal (lane 16). This result emphasizes that signals detected in lanes 14 and 15 are specific and due to newly synthesized (−)-PSTVd transcripts.

The newly synthesized (−)-PSTVd RNA in lanes 14 and 15 does not appear as a distinct band but as a diffuse, heterogeneous length distribution from about 2× unit length down to short fragments. At least three reasons are conceivable for this length distribution: the heterogeneous size could be due to degradation caused by endogenous nuclease activities, it could be due to unspecific initiation of the newly generated transcripts, or the transcripts could be synthesized with specific initiation but unspecific termination. One can summarize, however, that the nuclear extract contains PSTVd transcription activity, de novo-synthesized (−)-PSTVd RNA can be isolated by the new purification protocol, and that this signal is completely missing in the mock-treated control.

Determination of the 5′-ends of the de novo-synthesized transcripts by primer-extension

The 5′-ends of the newly transcribed (−)-PSTVd RNA, which correspond to the start site(s) on the circular (+)-PSTVd template, were determined by primer-extension analysis. Because release of the target RNA from its capture probe is not always complete, supernatants of both elutions (fraction with urea and fraction with formamide) were separately precipitated with ethanol, pellets were redissolved and applied to primer-extension reaction. As primers, either AF9 (position 181–209) or AF6 (position 268–292) was used (see Fig. 5); products were analyzed by gel-electrophoresis and autoradiography (see Fig. 6).

The primer-extension analysis revealed a single specific signal at nucleotide position U359 or C1; that is, there is only a single start site for generation of de novo-synthesized (−)-RNA by pol II in our extract. Discrimination between positions U359 and C1 was not possible due to the limited resolution of the sequencing ladder (Fig. 6, lane 6). None of the other lanes did show a corresponding signal: in the formamide fraction (lane 7), the amount of de novo-synthesized (−)-RNA is probably too low (compare lanes 14 and 15 in Fig. 4), addition of α-amanitin suppressed the (−)-RNA synthesis (lane 8), and no unspecific RNA is generated in the reaction without template

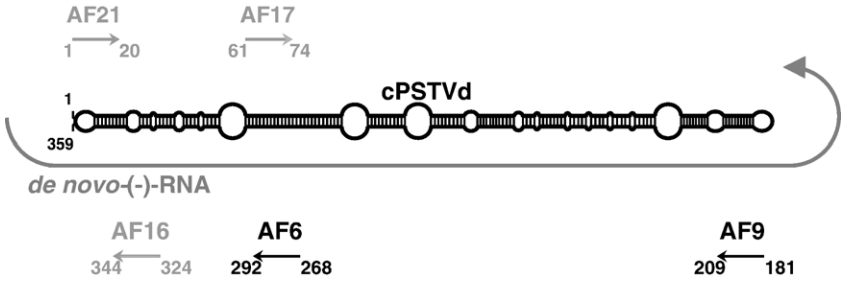

Fig. 5. Localization of primers. Numbering of nucleotides refers to circular (+)-PSTVd. Primers marked in black were used for primer-extension analysis of purified de novo-synthesized (−)-PSTVd RNA (for example see Fig. 6); those in light grey were used for primer-extension analysis in the nuclear extract (for example see Fig. 1).

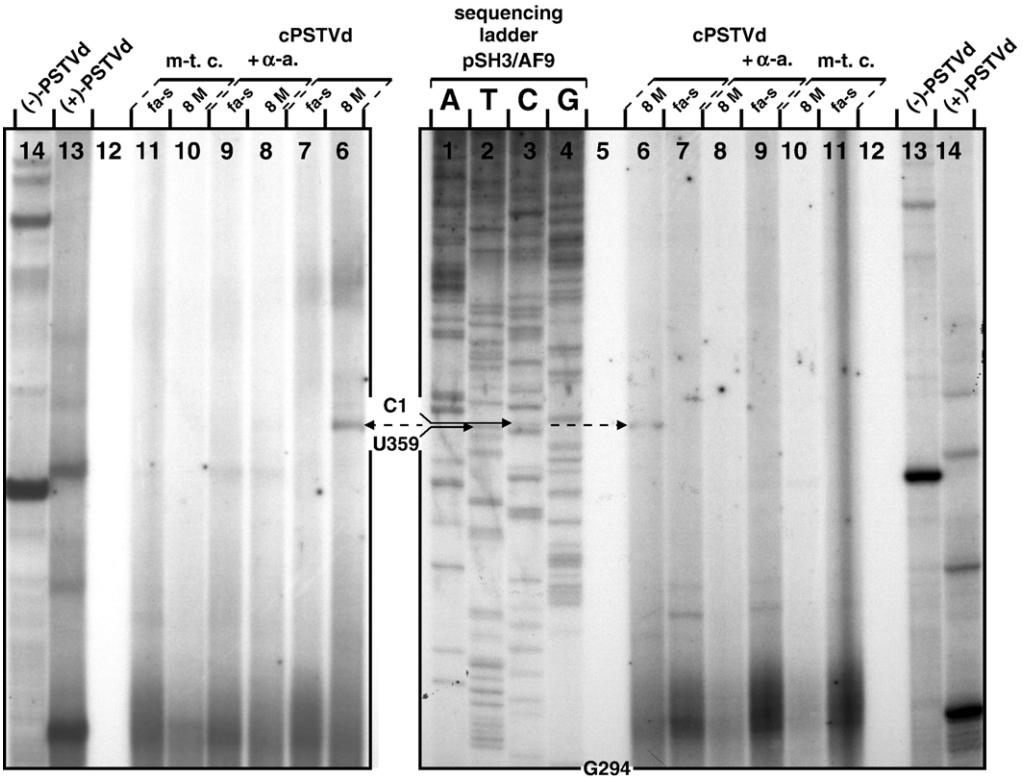

Fig. 6. Determination of the 5′-end of de novo-synthesized (−)-PSTVd RNA by primer-extension analysis of an in vitro transcription reaction in a nuclear extract. Purification of the nucleic acids was achieved by hybridization to biotinylated capture probes, followed by isolation of de novo-synthesized (−)-RNA via Dynabeads. Primer-extension was performed with primer AF9 at 55 °C. Left and right panels show results from two independent analyses with different nuclear extracts. Lanes 1 to 4, sequencing ladder of pSH3; lanes 6 to 11, analyzed samples after transcription in a nuclear extract of non-infected potato-cell culture: fractions applied on the gel are from elutions with 8 M urea (lanes 6, 8, 10) and with formamide (lanes 7, 9, 11), respectively; lanes 6 and 7, in vitro transcription with 5 μg circular (+)-PSTVd as template; lanes 8 and 9, in vitro transcription in the presence of 10^{-6} M α-amanitin; lanes 10 and 11, in vitro transcription without any PSTVd (mock-treated control); lane 13, positive control for primer-extension reaction, 100 pg dimeric (−)-PSTVd transcript (pSH3); lane 14, control for (−)-strand specificity, 1 μg dimeric (+)-PSTVd transcript (pRH717). Only the reaction with circular (+)-PSTVd as exogenous template for transcription revealed a specific signal (lane 6) that could be identified by comparison with the adjacent sequencing ladder as nucleotide U359 or C1. For abbreviations, see Fig. 3.

(lane 10). Furthermore, neither the (−)-probe molecule with its 5′-end at position 343 (lane 13) nor the (+)-strand (lane 14) gives rise to a primer-extension band near positions 359 or 1. Reproductions of the experiment, for example with primer AF6 (see Supplemental Fig. S1) or an independently prepared nuclear extract (Fig. 6), revealed identical results.

Mutagenesis of sites essential for transcription

Mutant viroids with single-nucleotide exchanges in the potential start sites and promoter elements were tested for transcriptional activity in vitro and for infectivity in vivo.

Choice of the mutations

The nucleotides C1 or G359 were determined as start sites. In addition, the two GC-boxes (see Fig. 7) were discussed as important for (−)-strand synthesis (Qu et al., 1993; Fels et al., 2001), whereas HPII is a metastable structural element of (−)-strands and is involved in (+)-strand synthesis (Loss et al., 1991; Qu et al., 1993). Thus, mutations of these positions or in these regions might reveal the importance of these motifs. Either a modified sequence or a change in structure should reduce infectivity or transcriptional activity. Genetic stability of mutations, reversions, or compensatory mutations necessary to regain biological function would point to the importance of the mutated nucleotides. Thus, three kinds of mutations were designed (see Fig. 7):

- The previously proposed start site A111, which could not be confirmed in this study, was altered to G while preserving the predicted secondary structure.
- From primer-extension analysis of this work, the correct start site was determined but it was not possible to differentiate between C1 and U359 as start sites for transcription; therefore, we designed the two mutants C1 → G and U359 → G.
- GC-boxes, which are postulated as promoter elements, can be disrupted with respect to their structure by introducing mismatches. The mutations A343 → C, A344 → C, and C346 → G lead to interior loops in the left GC-box. Therefore, the functionality of this structural element as a promoter is expected to be impaired. Secondary structure calculations predict that eliminating a GC-base pair by changing C346 → G leads to a drastic shift in the viroid structure from the native rod-like end to a “Y”-shaped end (Dingley et al., 2003).

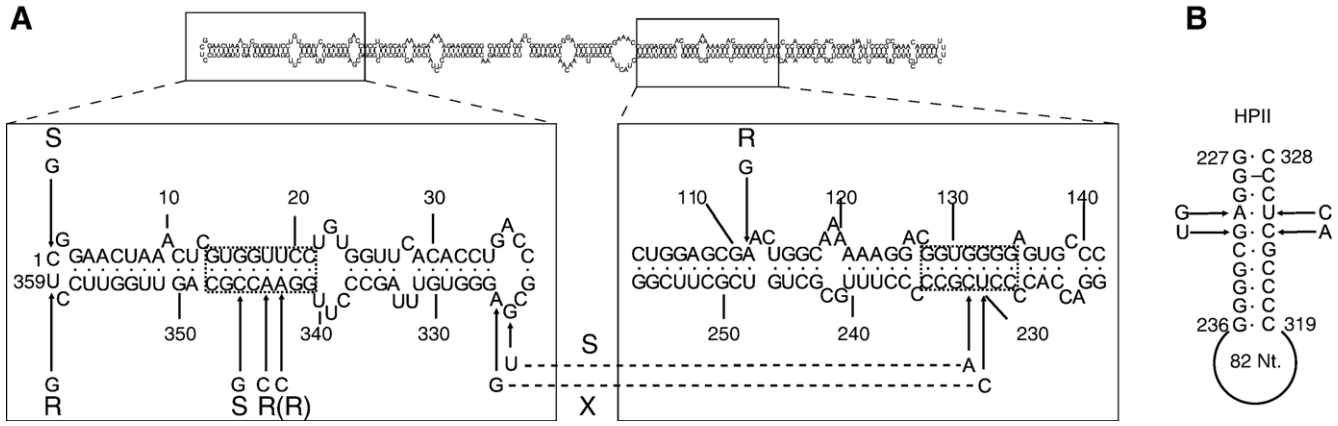

Fig. 7. Positions of single- and double-site mutations in PSTVd. (A) Two sections of the native, rod-like secondary structure of PSTVd. The double-site mutations C231 → A/G324 → U and U230 → C/A325 → G are connected by dashed lines. The left and right GC-boxes are boxed. R and S refer to reverting and stable mutations, respectively; X, in one plant out of eight infected with U230 → C/A325 → G, a single-site reversion G325 → A occurred; for further details on infection, see Table 1. (B) Position of the double-site mutations C231 → A/G324 → U and U230 → C/A325 → G in HPII on the (−)-strand level.

- The double mutations U230 → C/A325 → G and C231 → A/G324 → U are designed to affect the right GC-box. Mutation U230 → C enhances the stability of this GC-box, and C231 → A disrupts the GC-box. A325 → G and G324 → U, respectively, were mutated compensatorily to guarantee the structural integrity of HPII critical for (+)-strand synthesis (see Fig. 7B).

Infectivity tests and genetic stability of the mutants

As described in Materials and methods, mutated circular PSTVd molecules were synthesized and used for inoculation of tomato seedlings *Lycopersicon esculentum* cv. Rutgers. Wild-type PSTVd (strain intermediate DI) served as a positive control. Leaf samples were collected after different time intervals (21, 28, 35, and 42 dpi) and total RNA was extracted. Presence of PSTVd was determined by bidirectional gel electrophoresis (Schumacher et al., 1983).

Infection with wild type was fully established after 3 weeks, whereas the infectivity of the mutants was found to be much lower (see Table 1). Only mutant C346 → G was detectable in three out of four plants, which is a characteristic of a genetically stable mutant. After 4 weeks, however, additional plants exhibited viroid-specific signals. With a delay of 2 weeks, we observed full replicational activity in all plants that were infected with single-site mutated viroids. Only in case of the GC-box mutant A343 → C, three out of four plants contained no viroid after 5 weeks, suggesting that this mutation can be very deleterious for the viroid. The double mutation C231 → A/G324 → U also caused strongly reduced infectivity.

To screen for reversions, we performed RT-PCR reactions from total RNA of those plants which showed a viroid signal in the gel electrophoresis tests, and determined the sequence of the products. With the exception of C346 → G and C1 → G, all single-site mutants had reverted to the wild-type sequence. Even at 42 dpi, the double-mutant sequences were still detectable in all tested plants. Only in one plant infected with U230 → C/A325 → G a reversion G325 → A occurred, which restores the internal loop in the terminal left region and keeps a wobble base pair in (−)-stranded HPII.

Table 1
Time course of infection in individual plants after inoculation with wild-type or mutant PSTVd molecules

Strain	Stability	21 dpi	28 dpi	35 dpi	42 dpi
Intermediate DI	S	4/4	4/4	4/4	n.d.
A111 → G	R	0/4	4/4	4/4	n.d.
A343 → C	R	0/4	1/4	1/4	n.d.
A344 → C	R	0/4	3/4	4/4	n.d.
C346 → G	S	3/4	4/4	4/4	n.d.
U359 → G	R	1/4	4/4	4/4	n.d.
C1 → G	S	1/4	3/4	4/4	n.d.
C231 → A/G324 → U	S	n.d.	7/8	n.d.	8/8
U230 → C/A325 → G	X	n.d.	4/8	n.d.	4/8
Mock inoculation		0/4	0/4	0/4	n.d.

35 or 42 dpi, the PSTVd progeny was sequenced. R and S refer to reverting and stable mutations, respectively; X, in one plant out of four infected with U230 → C/A325 → G, a single-site reversion G325 → A occurred. The presence of PSTVd was analyzed by bidirectional gel electrophoresis. Mock inoculation was performed to exclude cross-contamination. n.d., not determined.

Discussion

Transcription of PSTVd is carried out by DNA-dependent RNA polymerase II (Schindler and Mühlbach, 1992). Transcription from the mature, circular (+)-PSTVd to linear (−)-strands was analyzed here by using an in vitro transcription system consisting of a nuclear extract prepared from a non-infected cell culture of potato, which is a natural host of PSTVd.

Previous investigations on the transcriptional analysis of PSTVd replication, with regard to start sites on the circular (+)-PSTVd, revealed two distinct nucleotides, A111 and A325, as start sites (Fels et al., 2001). Experiments presented here revealed these nucleotides as important for transcription, but not as start sites of the circular (+)-PSTVd molecule. On the contrary, the primer-extension signals, which led to the assignment as start sites, can be attributed to primer binding to an endogenous nucleic acid.

To exclude primer binding to other nucleic acids than the de novo-synthesized (−)-PSTVd transcripts, it was necessary to

eliminate endogenous nucleic acids from the transcription assay. A purification protocol was established that separates newly synthesized (−)-PSTVd RNA from the other nucleic acid components of the transcription assay. Thus, the de novo-synthesized (−)-RNA is the only template remaining in primer-extension analysis. We determined the 5′-end of the (−)-PSTVd transcripts as either nucleotide U359 or C1. Which one of these nucleotides is the true start site could not be decided because of low resolution of the sequencing ladder. In the following, the newly revealed start nucleotide is discussed with respect to further experimental data and literature data on start domains of ASBVd and PLMVd of the family *Avsunviroidae*.

Transcription of circular PSTVd in a nuclear extract

For determination of PSTVd transcription start sites, an analysis of in planta replication intermediates (including unprocessed nascent PSTVd RNAs) would have been extremely difficult: (i) the concentration of intermediates is by far lower than that of the mature circles, (ii) newly synthesized transcripts are immediately processed in subsequent steps of the rolling circle mechanism, and (iii) a differentiation of true replication 5′-ends and ends due to processing and/or degradation would be rather difficult. Thus, we used a nuclear extract from a non-infected potato cell culture that was optimized for transcriptional activity. This cell-free system represents the closest approach possible to the in vivo situation. Following the transcription reaction, it was necessary to isolate sufficient amounts of de novo-synthesized (−)-PSTVd RNA to determine its 5′-end by subsequent primer-extension analysis.

Purification protocol

The amount of de novo-synthesized (−)-PSTVd RNA generated in our nuclear extract is fairly low, which might be either due to degradation during the transcription reaction or because PSTVd as an RNA template is not recognized by pol II as efficiently as the normal DNA. Because the analysis of the start sites of the transcript has been shown to be obscured by the excess of cellular RNA, it was necessary to purify the de novo-synthesized (−)-PSTVd RNA. The purification procedure was optimized to fulfill the following requirements:

1. High specificity of capture probes and of hybridization conditions has to be guaranteed since full-length or longer PSTVd sequences of both polarities tend to hybridize to strands of the same polarity due to the extensive self-complementarity of viroid sequences.
2. All hybridization sequences had to contain biotin labels to be completely removable from solution to avoid cross-contaminations (for details of prepurification, see Materials and methods).
3. The de novo-synthesized (−)-RNA has to be eluted and separated from the capture RNA with high yield.

Both the specific isolation of de novo-synthesized (−)-PSTVd RNA and its elution from the biotinylated complement were successful. Using in vitro transcripts, we could demonstrate that the “target” RNA was the only product detectable in the corresponding elution fraction after purification. Thus, the procedure allowed us purification of newly transcribed (−)-RNA generated by the in vitro transcription system, and the subsequent determination of its 5′-end by primer-extension analysis is unhampered by any additional components.

Left terminal loop as start site for PSTVd replication

With the established purification protocol, we were able to detect the 5′-end at nucleotide U359 or C1. Due to specificity of the purification protocol demonstrated above, the signal cannot originate from endogenous nucleic acids. Furthermore, the signal shows α-amanitin sensitivity which confirms its generation by pol II, because both, pol I as well as pol III, should not be affected by α-amanitin at low concentrations (Weinmann and Roeder, 1974; Wieland and Faulstich, 1978). Final evidence for the specificity of the detected signal attributed to the de novo-synthesized (−)-RNA was its absence in the mock-treated control, with no circular (+)-PSTVd added as transcription template. Both, reproduction of experiments carried out with independently prepared nuclear extracts as well as primer-extension analysis of purified de novo RNA with a different PSTVd primer, led to identical results; i.e., the 5′-end is located at nucleotide position U359 or C1. Additional start sites could not be detected (see Fig. 3 and corresponding text).

One might argue that the detected 5′-end could be a result of a nucleolytic reaction. Because 5′-exonuclease activities have never been described for plants, an artifact of a specific 5′-end resulting from degradation does not seem likely. Furthermore, non-specific degradation of de novo RNA during incubation in the nuclear extract was excluded to the greatest extent possible by addition of RNase inhibitors during the transcription reaction. If (−)-RNA was used as a control (see Fig. 6, lane 13), it did not give rise to a corresponding signal, excluding a specific cleavage at this site.

Elements of the rod-like structure as promoter for pol II transcription

Several promoters for pol II transcription have been described, containing either a TATA-element, an INR(initiator)-sequence, or both. Furthermore, so-termed “mixed promoters” are known, containing either one of these elements. Additionally, promoters are known that initiate pol II transcription independently of a TATA- and/or INR-sequence (Novina and Roy, 1996). Because PSTVd lacks both the TATA-box as well as the INR-element but is nevertheless transcribed by pol II, the rod-like structure itself must contain regions that are recognized and accepted by the enzyme or a transcription factor/enhancer. The rod-like structure of PSTVd corresponds to a stem-loop structure closed by two terminal hairpin loops. In the literature (Beard et al., 1996; Lauber et al., 1997; Carpenter and Simon, 1998; Filipovska and Konarska, 2000; Navarro and Flores, 2000; Pelchat et al., 2001, 2002),

hairpin structures are discussed to be a general motif for recognition by polymerases. Already 1984, binding assays with purified wheat germ pol II showed the enzyme to bind to terminal loops of PSTVd (Goodman et al., 1984). Likewise, recent experiments with PLMVd demonstrated binding of the *Escherichia coli* RNA polymerase – used as a model for the nuclear-encoded chloroplastic RNA polymerase (NEP) – to the ends of the viroid molecule (Pelchat et al., 2001, 2002). Transcriptional experiments with truncated PLMVd transcripts revealed the hairpin structure as sufficient to initiate transcription, which also starts at the terminal loop (see Fig. 8). Furthermore, transcription of both polarities of PLMVd RNA starts at terminal loops, demonstrating that transcription initiation is determined by secondary structure rather than primary sequence of the RNA (Pelchat et al., 2001; Pelchat and Perreault, 2004). The hairpin motif is also known as promoter fragment in ASBVd, where transcription is initiated within a terminal loop (see Fig. 8; Navarro and Flores, 2000). Transcription of ASBVd is also thought to be promoter driven, with binding of the enzyme determined by secondary structure but not sequence.

Beyond viroids, transcription initiation sites within terminal loops are also discussed as essential for promoter activity of viral and subviral RNAs of some (+)-stranded plant viruses (Lauber et al., 1997; Carpenter and Simon, 1998). Another example is the replication of hepatitis delta virus RNA (HDV RNA), a satellite RNA of the Hepatitis B virus. The HDV genome consist – similar to viroids – of a single-stranded circular closed RNA of about 1.7 kb, that forms a viroid-like unbranched structure in the native state. Similar to viroids, its

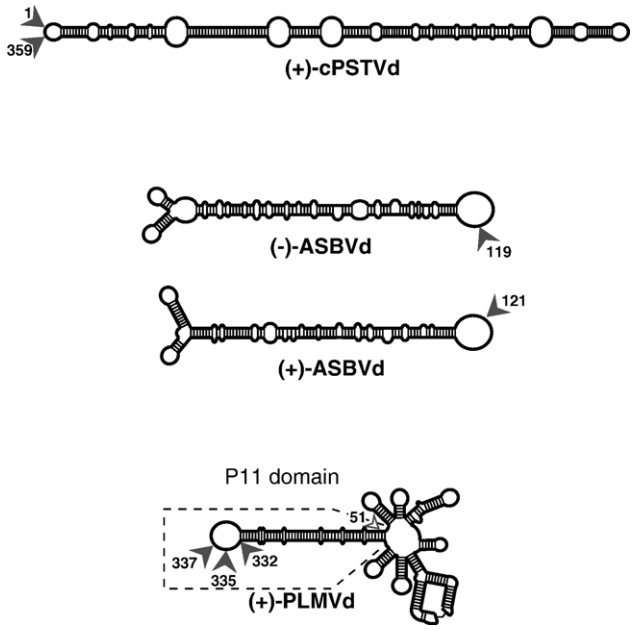

Fig. 8. Schematic representation of viroid secondary structures. (a) potato spindle tuber viroid (PSTVd); (b, c) avocado sun blotch viroid (ASBVd, (+)- and (−)-polarity; Navarro and Flores, 2000); (d) peach latent mosaic viroid (PLMVd; closed arrows point to start sites of *E. coli* polymerase (Pelchat et al., 2002), the open arrow points to in vivo start site (Delgado et al., 2005)). Arrows point to start sites of replication. Note that most start sites are located within a hairpin loop terminating an elongated stem–loop structure.

replication also depends on a host polymerase, most probably pol II (MacNaughton et al., 2002), and the left terminal double-stranded region closed by a loop is discussed as promoter element for pol II with the start site in its close proximity (Beard et al., 1996).

Specificity of transcription start and mechanism of reversion

Mutated viroid sequences are exposed to different degrees of selective pressure in the host plant. Mutation of nucleotides that are essential for the function of the viroid, for example as start sites of transcription, is expected to revert quickly to the wild-type sequence. The fact that mutant G1 turned out to be genetically stable, whereas G359 reverted to the U359, favors U359 as being the start site of PSTVd transcription. It is, however, not obvious from these two mutations if structure or sequence is of greater importance for the start site. The hairpin loop sequence^{357}c(CUCG)g^{3}, with closing pair^{357}C:G^{3}, may belong to either one of the tetraloop families g(CUNG)c (Jucker and Pardi, 1995) or c(YNMG)g (Proctor et al., 2002), which have quite different tertiary structures and structural features for tertiary interactions. In an NMR study on the left terminal region of PSTVd (Dingley et al., 2003), however, resonances for the hairpin loop were found that do not coincide with one of the representative members of the mentioned tetraloop families. So details have to be elucidated by further studies.

Taking together the results from our in vitro and in vivo studies, we gained a more detailed understanding on the mechanistic aspects of reversions. If a mutant sequence is not accepted as a template by pol II, it could be sustained through a low-level transcription by other polymerases (for example pol I or pol III). This could be shown by in vitro studies where some templates exhibited an α-amanitin-independent signal (data not shown). Due to the natural error rate of the polymerases, which might be increased by using the RNA instead of DNA as template, the wild-type sequence can be reestablished, thus leading to full replicational activity, and the maximum titer of viroid is gained within a typical delay of 2 weeks. However, if the basal transcriptional activity is too inefficient, the mutant templates are degraded rather than amplified by the host. We observed this phenomenon in case of mutant C343. Although the mutation does not affect the transcription of the native (+)-stranded circle (data not shown), it appears to have a drastic effect on the (−)-stranded template activity or transport motifs in planta. This mutation turned out to be lethal to the viroid in 3 out of 4 plants (see Table 1).

Mutant viroids with double nucleotide exchanges exhibited very low infectivity. A complete reversion to the wild-type sequence is a very improbable event, because a reversion of a single nucleotide leads to a mismatch in the core of HPII, which is in turn essential for (−)-strand transcription (Loss et al., 1991). Therefore, the replication cycle comes to a halt at this stage. We observed, however, a reversion of G325 → A, which might be interpreted as a first step in a complete reversion to the wild-type sequence. This single reversion is viable because the structural integrity of HPII is left intact; the

G230:C325 base pair is substituted by G230:U325 on the (−)-strand level. Nevertheless, a complete reversion might occur in following passages.

Modular arrangement of structural and functional domains in viroids

The detailed analysis of the transcription process of PSTVd fits to the model of a modular arrangement of functional domains as described earlier (Keese and Symons, 1985; Steger and Riesner, 2003; Tabler and Tsagris, 2004). The left terminal region together with at least one GC-box as the transcription domain could be added to the known list of functional domains, such as the virulence modulating region (Schnölzer et al., 1985; Owens et al., 1996; Schmitz and Riesner, 1998), the processing region (Baumstark et al., 1997; Schrader et al., 2003), and the transport domain (Hammond, 1994; Gozmanova et al., 2003). These domains are not always elements of the native, rod-like structure. Domains for (+)-strand transcription and transport belong to the native structure as expected. The processing domain is definitely part of a metastable structure, which occurs only transiently during the replication cycle, and for the virulence modulating region, the mechanism is not yet known. In a modified version, the modular arrangement holds true also for other viroid families.

Materials and methods

Oligonucleotides

Oligonucleotides used for primer-extension analysis (AF6, AF9, AF16, AF17, AF21) were obtained from Thermo Hybaid, Ulm, Germany. Melting temperatures for each oligonucleotide (given in parenthesis) were determined with POLAND (Steger, 1994) at an ionic strength of 100 mM NaCl with thermodynamic parameters according to Klump (1990), a dissociation constant $\beta = 10^{-3}$ M^{-1}, and a primer concentration $c_0 = 2{\cdot}10^{-8}$ M.

AF6: corresponds to (5′ → 3′) G268–C292 of (+)-PSTVd 5′-GGA AAC AAC TGA AGC TCC CGA GAA C-3′ (58–62 °C)
AF9: corresponds to (5′ → 3′) T181–C209 of (+)-PSTVd 5′-TCA CCC TTC CTT TCT TCG GGT GTC CTT C-3′ (57–65 °C)
AF16: corresponds to (5′ → 3′) G324–A344 of (+)-PSTVd 5′-GAG GGT GTT TAG CCC TTG GAA-3′ (53–60 °C)
AF17: corresponds to (5′ → 3′) G61–A74 of (+)-PSTVd 5′-GAA GGC GGC TCG GA-3′ (57 °C)
AF21: corresponds to (5′ → 3′) C1–C20 of (+)-PSTVd 5′-CGG AAC TAA ACT CGT GGT TC-3′ (56 °C).

Enzymes and enzymatic reactions

Restriction enzymes and T4 polynucleotide kinase for 5′-labeling of synthetic oligonucleotides with [γ-^{32}P]-ATP were used according to standard protocols (Sambrook et al., 1989). T7 RNA polymerase was used for in vitro synthesis of transcripts. Transcripts were internally labeled by addition of [α-^{32}P]-UTP or biotin-16-uridine-5′-triphosphate, respectively, to the transcription mixture. The RNA was eluted from a denaturing gel as described by Krupp (1988), ethanol precipitated, dissolved in 10 µl TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA), and stored at −20 °C.

Synthetic transcripts with PSTVd sequence

Plasmids pRH715, pRH717, pRH716, and pSH3 are derivatives of pRH701 (Hecker et al., 1988). After linearization of plasmids and run-off-transcription with T7-RNA polymerase, the following transcripts were obtained:

pRH715 (368 nts, monomeric (+)): $^{5'}$GG-(147–359/1–146)-GGGAAUU$^{3'}$,
pRH717 (727 nts, dimeric (+)): $^{5'}$GG-(282–359/1–359/1–281)-GGGAAUU$^{3'}$,
pRH716 (368 nts, monomeric (−)): $^{5'}$GG-(146–1/359–147)-GGGAAUU$^{3'}$,
pSH3(731 nts, dimeric (−)): $^{5'}$GG-(341–1/359–1/359–336)-GAAUU$^{3'}$.

The numbering of the PSTVd sequence is according to Gross et al. (1978).

Primer-extension analysis of de novo-synthesized (−)-PSTVd RNA

Purified RNA was annealed with 1×10^{-6} cpm 5′-[γ-^{32}P]-labeled primer (AF6, AF9, AF16, AF17, AF21) in 20 µl reaction buffer (50 mM Tris–HCl, pH 8.3, 100 mM NaCl) at 85 °C for 1 min. After cooling to 35 °C in about 3 h, the samples were ethanol precipitated and redissolved in 20 µl primer-extension buffer (1× first strand buffer, 10 mM DTT, 500 µM of each dNTP, 40 units RNasin (Promega) and 200 units reverse transcriptase (Superscript II; Gibco BRL)). Samples were incubated at a temperature corresponding to the melting temperature of the primer (see figure legends), precipitated with ethanol, redissolved in urea loading solution (4 M urea, 45% formamide, 5 mM EDTA, 0.1% bromophenol blue/xylene cyanol), 0.2× TBE), heat denatured, and analyzed on 8% polyacrylamide denaturing sequencing gels (40%, 19:1, 8 M urea, 1× TBE). Afterwards, gels were exposed to X-ray films (Kodak-X-OMAT, Sigma, St. Louis, USA). To assign primer-extension products, sequencing reactions were carried out on plasmid pSH3 containing a dimeric (−)-PSTVd sequence with primers AF6, AF9, AF16, AF17, or AF21, respectively. All sequencing reactions were performed by using Sequenase II (US Biochemical) following the vendor's protocol.

Preparation of nuclei and nuclear extracts from a non-infected potato-cell culture

Highly purified nuclei from a non-infected potato cell culture (*Solanum tuberosum*, HH258) were prepared according

to Fels et al. (2001). Briefly, a suspension culture is filtered and cells are converted into protoplasts by incubation with cellulase Onozuka R-10 and Macerozyme R-10 (Serva, Heidelberg, Germany). Protoplasts were lyzed mechanically and nuclei purified by centrifugation with Ficoll and Percoll gradients. Ten millimolar NaF was added to all buffers to inhibit endogenous phosphatases. Typical yields of preparations were $1–2{\cdot}10^8$ nuclei per 2 ml nuclear extract, containing protein concentrations varying from 2 to 8 mg/ml. Nuclear extracts were stored at −70 °C for up to 3 months without loss of transcriptional activity.

Transcription of circular (+)-PSTVd as exogenous template with the nuclear extract

Transcription reactions with the nuclear extract were carried out according to Fels et al. (2001). The reaction mix contained 5 to 15 μg protein from the nuclear extract, up to 5 μg circular (+)-PSTVd template, 40 u RNasin, 20 u DNase I (RNase-free), and 20 μg BSA (Molecular Biology Grade; Roche, Germany) in 30 μl transcription buffer (50 mM Tris–HCl, pH 7.9, 60 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$). For a control reaction, the pol II-inhibitor α-amanitin was added at a concentration of 10^{-6} M. Each transcription reaction was preincubated without NTPs at 28 °C for 5 min to form polymerase preinitiation complexes, as well as to digest endogenous DNA. Afterwards, NTPs were added to a final concentration of 600 μM and the solution was incubated at 28 °C for 30 min. Transcription was terminated by addition of 70 μl stop solution (20 mM EDTA, pH 8.0, 200 mM NaCl, 1% SDS). Nucleic acids were extracted by phenol-chloroform, precipitated with ethanol, and redissolved in 20 μl 1× TNE, 8 M urea.

Pretreatment of Dynabeads

Purification of de novo-synthesized PSTVd RNA was achieved by using paramagnetic streptavidin particles (Dynabeads, Deutsche Dynal GmbH, Hamburg). Before use, Dynabeads were pretreated for 2 min at room temperature with solution A (100 mM NaOH, 50 mM NaCl, 0.1% DEPC) or B (100 mM NaCl, 0.1% DEPC), respectively, to remove possible RNase contamination. After each incubation, reaction tubes were put into a rack, containing a magnet at the back wall (Dynal MPC-L, Magnetic Particle Concentrator), Dynabeads were pulled to the tube's wall, and the supernatant was removed. Pretreated, RNase-free Dynabeads were afterwards used to isolate PSTVd RNA.

Synthesis and purification of biotinylated probe molecules

Biotinylated, dimeric PSTVd transcripts of both polarities were synthesized with T7 polymerase in the presence of 2 mM Biotin-16-UTP. During synthesis of biotinylated probes, a low amount of transcripts not containing biotin may be generated. These molecules had to be removed because they prevent optimal purification of de novo-synthesized (−)-PSTVd RNA. According to the vendor's manual, 1 μg Dynabeads will bind to about 1 to 3 ng PSTVd full-length transcript. Two micrograms of 20%-biotinylated transcripts – either pSH3 transcripts as a probe with (−)-polarity or pRH717 transcripts as a probe with (+)-polarity – were purified by use of 1 mg Dynabeads; 15 μg 20%-biotinylated pSH3 transcripts were purified by use of 5 mg Dynabeads. Prepurification was carried out in a volume of 20 ml reaction mix (5 mg Dynabeads (pretreated, RNase-free), 15 μg biotinylated transcript, 5 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, pH 8.0, 1 M NaCl), or 4 ml volume (1 mg Dynabeads (pretreated, RNase-free), 3 μg biotinylated transcript, same buffer). The reaction was incubated at room temperature for 40 min with rotation of tubes head-over-tail to avoid sedimentation of Dynabeads. Afterwards, Dynabeads bound to biotinylated transcripts were magnetically fixed to the tube wall and buffer was removed. Dynabeads were washed by addition of:

+0.1× SSC, 3 min, room temperature;
+0.1× SSC, 4 min, 70 °C;
+formamide solution (95% formamide, 10 mM EDTA, pH 8.2), 4 min, 70 °C.

Incubation with formamide disrupts biotin–streptavidin binding and releases the biotinylated probes. Formamide fractions containing only biotinylated probe molecules were precipitated with ethanol and directly used in hybridization reactions.

Isolation of de novo-synthesized (−)-PSTVd RNA from the transcription assay

Isolation of (−)-PSTVd RNA was achieved by hybridization to biotinylated capture probes of complementary polarity. Circular (+)-PSTVd, used as template in the transcription reaction, is removed from the mixture by hybridization to a high excess of biotinylated (−)-probe molecules. Pellets of transcription reactions as well as corresponding pellets of prepurified (−)-probes were dissolved in a total of 20 μl 1× TNE, heated to 85 °C for 90 s, and cooled slowly to 35 °C. The resulting hybrids were immobilized to Dynabeads, which were washed by addition of the following solutions:

(1) 0.1× SSC, 5 min, room temperature;
(2) 0.1× SSC, 5 min 30 s 70 °C;
(3) formamide solution, 5 min 30 s, 75 °C.

The supernatants from the washing steps 1 and 2, containing de novo-synthesized (−)-PSTVd RNA and other RNAs, were precipitated with ethanol. Pellets containing (−)-PSTVd RNA as well as precipitated prepurified (−)-probes were dissolved in 20 μl 1× TNE. A second hybridization was carried out in the presence of 8 M urea; hybrids were treated as described above. Fractions containing de novo-synthesized (−)-PSTVd RNA (from steps 1 and 2) were precipitated and in the final step hybridized to probe molecules with (+)-polarity. Final hybridization was carried out in the absence of 8 M urea during hybridization. Hybrids were separated as described above and, as a modification, an additional step during the washing

procedure was carried out using 8 M urea at a temperature of 75 °C to disrupt hybrids and to separate de novo-synthesized (−)-PSTVd RNA from its probe molecule:

(1) + (2), see above;
(3) 8 M urea, 4 min, 75 °C;
(4) see (3) above.

Both fractions, the urea fraction 3, containing de novo-synthesized RNA, as well as formamide fraction 4, probably containing de novo-synthesized RNA, were precipitated with ethanol and the pellet was used directly in primer-extension analysis. RNAs from the different elution steps were analyzed by electrophoresis in 5% PAA gels (30:1, 8 M urea, 1× TBE).

Mutant cDNA clones

Plasmid pRZ6-2 carrying a PSTVd intermediate DI sequence flanked by two ribozymes was kindly provided by R.A. Owens (USDA/ARS, Beltsville, MD). For generation of the double-mutant sequences (C231 → A/G324 → U and U230 → C/A325 → G), the wild-type *Eag*I/*Eco*47III fragment was replaced by the corresponding mutant fragment. The GC-box mutants (A343 → C, A344 → C, C346 → G) and mutant A111 → G were obtained by exchanging the *Sty*I/*Sal*I fragment and the *Sac*I/*Eag*I fragment, respectively. The mutations U359 → G and C1 → G were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

Synthesis of mutant PSTVd molecules

Prior to transcription, plasmids were linearized by digestion with *Hin*dIII. Large amounts of linear PSTVd molecules were produced using the Ribomax protocol (Promega). Full-length PSTVd was eluted from a native gel and was circularized in a cell-free wheat germ extract. Twenty micrograms of PSTVd RNA was incubated at 37 °C for 2 h in a total reaction volume of 100 μl containing 50 μl wheat germ extract (Promega), 20 mM Tris–HCl (pH 8.0), 6 mM Mg acetate, 0.2 mM spermidine, 0.4 mM EDTA, and 1 mM ATP (Owens et al., 1996). Total nucleic acid was recovered by phenol-chloroform extraction and precipitation with 15% PEG and 500 mM NaCl. Circular PSTVd molecules were eluted from a bidirectional gel (Schumacher et al., 1983).

Inoculation and RNA isolation

Five nanograms of mutant circular PSTVd RNA was used to inoculate 1 week-old tomato seedlings (*L. esculentum* cv. Rutgers). Leaf samples were collected 21, 28, 35, and 42 dpi. Two hundred milligrams of leaf material was frozen in liquid nitrogen and ground with a mortar in an Eppendorf tube. After addition of extraction buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 2% SDS, 2% β-mercaptoethanol), samples were phenolized and precipitated with 15% PEG and 500 mM NaCl. For detection of viroid, samples were subjected to bidirectional gel electrophoresis (Schumacher et al., 1983).

Sequence analysis of viroid progeny

Total RNA from 10 mg leaf samples of infected plants was used as template in a RT-PCR reaction. Viroid cDNA was obtained using superscript II polymerase (Invitrogen) under hot start conditions (60 °C) and amplified with primers TB1 (5′-171 TTT CGG CGG GAA TTA CTC CTG TCG G147) and QFV6 (5′-209 TCG CGC CCG CAG GAC CAC226) using polymerase Pfx (Invitrogen). The resulting products were purified by a PCR purification kit (Qiagen) and were sequenced in both directions using primer TB1 and QFV6 (Seqlab, Göttingen).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2005.11.039.

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