Virology **268,** 218–225 (2000)
doi:10.1006/viro.1999.0161, available online at http://www.idealibrary.com on IDEAL®

A Chloroplastic RNA Polymerase Resistant to Tagetitoxin Is Involved in Replication of *Avocado Sunblotch Viroid*

José-Antonio Navarro, Antonio Vera,[1] and Ricardo Flores[2]

Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, Camino de Vera 14, Valencia 46022, Spain

Received November 12, 1999; returned to author for revision December 14, 1999; accepted December 21, 1999

Avocado sunblotch viroid (ASBVd), the type species of the family Avsunviroidae, replicates and accumulates in the chloroplast. Two main chloroplastic RNA polymerases have been described: the plastid-encoded polymerase (PEP) with a multisubunit structure similar to the *Escherichia coli* enzyme and a single-unit nuclear-encoded polymerase (NEP) resembling phage RNA polymerases. On a different basis, sensitivity to tagetitoxin, two major RNA polymerase activities, tagetitoxin sensitive (TS) and resistant (TR), have been found in plastids. The most plausible candidates for the TS and TR RNA polymerases are PEP and NEP, respectively. To gain an insight into the enzymology of the polymerization of ASBVd strands, purified chloroplast preparations from ASBVd-infected leaves were assayed for their *in vitro* ability to transcribe ASBVd RNAs together with some representative genes *(psbA, 16SrDNA, accD,* and *rpoB)* of the three classes of chloroplastic genes according to their promoter structure. High concentrations of α-amanitin had no effect on gene or on viroid transcription, but tagetitoxin (5–10 μM) prevented transcription of all these genes without affecting synthesis of ASBVd strands; only at higher tagetitoxin concentrations (50–100 μM) was a 25% inhibition observed. These results suggest that NEP is the RNA polymerase required in ASBVd replication, although the participation of another TR RNA polymerase from the chloroplast cannot be excluded.

Key Words: avocado sunblotch viroid; catalytic RNAs; ribozymes; hammerhead structures; rolling circle mechanism.

INTRODUCTION

In contrast to virus genomes, viroids, which are small circular RNAs able to replicate autonomously when inoculated in certain plants, do not appear to code for any protein (Davies *et al.,* 1974; Hall *et al.,* 1974). This makes the replication cycle of these molecular parasites extremely dependent on enzymatic host activities. *Potato spindle tuber viroid* (PSTVd) (Diener, 1971b; Gross *et al.,* 1978), the type species of the family Pospiviroidae to which most of the sequenced viroids belong (Flores *et al.,* 1998), accumulates (Diener, 1971a; Harders *et al.,* 1989) and replicates in the nucleus (Schindler and Mühlbach, 1992), as probably do the other members of this family. *Avocado sunblotch viroid* (ASBVd) (Hutchins *et al.,* 1986; Symons, 1981), the type species of the second viroid family, *Avsunviroidae,* accumulates and replicates in the chloroplast (Bonfiglioli *et al.,* 1994; Lima *et al.,* 1994; Navarro *et al.,* 1999), and the same presumably occurs with the other two members of the family, *Peach latent mosaic viroid* (PLMVd) (Hernández and Flores, 1992) and *Chrysanthemum chlorotic mottle viroid* (Navarro and Flores, 1997). Recent data show that PLMVd indeed accumulates predominantly in the chloroplast (Bussière *et al.,* 1999).

Viroids replicate through RNA intermediates (Grill and Semancik, 1978) and follow a rolling circle model (Branch and Robertson, 1984). This model was proposed considering the circular nature of the viroid molecule and the presence in infected tissues of circular and oligomeric viroid RNAs of one or both polarities. The latter were assumed to be the putative replicative intermediates. The model involves two alternative pathways, asymmetric and symmetric, with one and two operating rolling circles, respectively, and three steps catalyzed by (1) an RNA polymerase, which after several RNA–RNA transcription rounds produces oligomeric strands, (2) an RNase for processing the longer-than-unit transcripts to unit-length, and (3) an RNA ligase for circularizing the linear monomeric RNAs. Whether the viroid complementary (−) oligomers resulting from the first RNA–RNA transcription serve directly as the template for the second RNA–RNA transcription, or are previously processed and ligated to the (−) circular monomer, determines whether the pathway is asymmetric or symmetric, respectively. PSTVd follows the asymmetric route (Branch *et al.,* 1988; Feldstein *et al.,* 1998), whereas ASBVd follows the alternative symmetric circuit (Daròs *et al.,* 1994; Hutchins *et al.,* 1985; Navarro *et al.,* 1999). Members of both viroid families are likely to behave in the same way as their type species, and this is for example the case of

[1] Present address: División de Genética, Universidad Miguel Hernández, San Juan, 03550 Alicante, Spain.

[2] To whom reprint requests should be addressed. Fax: 34-96-3877859. E-mail: rflores@ibmcp.upv.es.

0042-6822/00 $35.00

hop stunt viroid (Ishikawa *et al.,* 1984) and PLMVd (Bussière *et al.,* 1999). Interestingly, in the Avsunviroidae family, the second step is mediated by hammerhead ribozymes embedded in both polarity RNAs (Hernández and Flores, 1992; Hutchins *et al.,* 1986; Navarro and Flores, 1997).

There is genetic and biochemical evidence for at least two DNA-dependent RNA polymerase activities with different specificity in plastids (see for a review, Stern *et al.,* 1997). The most clearly characterized has a multisubunit core similar to that of the eubacterial RNA polymerase and is referred to as the plastid-encoded polymerase (PEP) because the homologues of the genes for the α, β, and β' subunits of the *Escherichia coli* RNA polymerase have been identified in the plastid genome (Sugiura, 1992). PEP specificity is provided by σ-like factors encoded in the nucleus (Tanaka *et al.,* 1996) that recognize promoters similar to those of the *E. coli* RNA polymerase with −35 and −10 consensus sequences. Besides PEP, plastids also contain a single-subunit nuclear-encoded polymerase (NEP) similar to phage RNA polymerases (Lerbs-Mache, 1993) that initiates transcription from nonconsensus promoters (Allison *et al.,* 1996; Kapoor *et al.,* 1997; Vera and Sugiura, 1995; Vera *et al.,* 1996). Within this scenario, *Arabidopsis thaliana* has been shown to contain two nuclear genes similar to those encoding the RNA polymerases from T7, T3, and SP6 phages and the mitochondrial RNA polymerase from yeast, whose putative transit peptides are capable of targeting fusion proteins to chloroplasts and mitochondria, respectively (Hedtke *et al.,* 1997). Furthermore, the recent *in vitro* characterization of a tobacco promoter recognized by NEP has revealed a core sequence motif also conserved in plant mitochondrial promoters (Liere and Maliga, 1999).

Considering the existence of more than one RNA polymerase in plastids, the question that now emerges is which is involved in the synthesis of ASBVd strands. Here we report our observations with a system derived from purified chloroplasts of ASBVd-infected avocado leaves that is able to catalyze *in vitro* the transcription of viroid-specific as well as chloroplastic RNAs. By studying the effects on this system of tagetitoxin, which inhibits the bacterial-like but not the phage-like chloroplastic RNA polymerase (Kapoor *et al.,* 1997; Liere and Maliga, 1999; Mathews and Durbin, 1990), we have gained an understanding of the enzymology of the polymerization of ASBVd strands.

RESULTS

Properties of an *in vitro* transcription system from chloroplasts of ASBVd-infected avocado leaves

To improve the quality of the avocado chloroplasts, they were purified from mesophyl leaf protoplasts instead of directly from foliar tissue. Under the assay conditions described, the transcription activity of the chloroplastic preparations, measured as the amount of [α-^{32}P]UMP incorporated into acid-precipitable material, increased over time and reached 4.2×10^7 cpm/mg chlorophyll after 2 h (Fig. 1). The chloroplasts become permeated probably due to the high glycerol concentration of the transcription buffer, because when they were resuspended in an isotonic medium their transcriptional activity was considerably reduced (data not shown); repeat pipetting to favor resuspension of chloroplasts probably also helped to disrupt their membranes. No significant difference in activity was found between preparations from uninfected and ASBVd-infected tissue (data not shown). The transcriptional activity was almost completely abolished when the unlabeled nucleoside triphosphates were not included in the assay (Fig. 1), clearly showing that it was not the result of end-labeling but due to the elongation of RNA strands. Moreover, preincubation of the chloroplastic preparation with RNase-free DNase led to a 75% reduction of the transcriptional activity (Fig. 1), with the remaining activity probably resulting from the partial inaccessibility of chloroplastic DNA to DNase; again, no difference was observed between uninfected and ASBVd-infected tissue. As anticipated from these experiments, essentially no acid-precipitable material was recovered when the product synthesized *in vitro* was treated with RNase (data not shown).

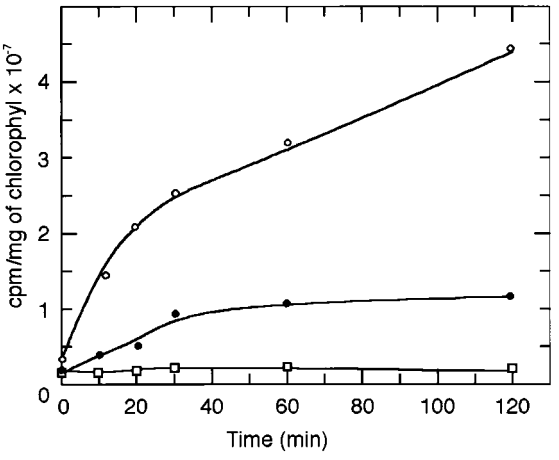

FIG. 1. Progress with time of the *in vitro* RNA synthesis catalyzed by preparations of purified chloroplasts from ASBVd-infected avocado leaves. The chloroplasts were pretreated (●) or untreated (○) with RNase-free DNase before their addition to the transcription mixture, or the transcription mixture was deprived of the unlabeled nucleoside triphosphates (□). Reaction progress with time was estimated by taking one-tenth aliquots of the transcription mixture and determining the acid-precipitable radioactivity.

The avocado chloroplastic system catalyzes the polymerization of ASBVd-specific RNAs

To investigate whether ASBVd-specific sequences were present in the RNAs synthesized *in vitro* with the

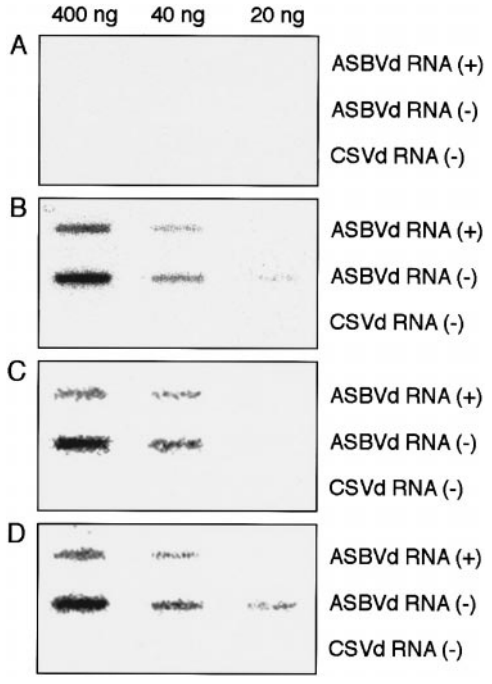

FIG. 2. Slot-blot hybridization signals generated by three different RNAs probed with radioactive transcripts synthesized *in vitro* by purified chloroplast preparations from uninfected (A) and ASBVd-infected (B–D) avocado tissue. (A and B) Reaction mixtures without α-amanitin. (C and D) Reaction mixtures with α-amanitin 1 and 200 μg/ml, respectively. Plus and minus polarities refer to RNAs blotted onto nylon membranes.

chloroplastic preparation from ASBVd-infected leaves, the radioactive transcripts were used to probe membranes to which a set of viroid-specific RNAs were applied in a slot-blot format. To lessen the nonspecific background, hybridizations were made in 50% formamide at 70°C, and because transcription of viroid strands is DNA-independent, the chloroplastic preparations were pretreated with RNase-free DNase to reduce the DNA-dependent RNA synthesis (Marcos and Flores, 1992). Using these experimental conditions, chloroplastic preparations from ASBVd-infected tissue were able to synthesize ASBVd RNAs of both polarities, although as inferred from the intensity of the hybridization signals in the membrane slots, synthesis of ASBVd-plus strands was considerably higher than that of the minus strands (Fig. 2B). As expected, there was a direct relationship between the signal intensity and the amounts of both polarity ASBVd transcripts applied to the membrane. The specificity of these signals was further confirmed by the absence of any trace in the slots containing a minus polarity transcript of chrysanthemum stunt viroid (CSVd), a member of the family *Pospiviroidae* very different in sequence from ASBVd (Fig. 2B). Moreover, no hybridization was observed in transcription mixtures with chloroplastic preparation from uninfected avocado leaves (Fig. 2A).

That synthesis of ASBVd RNAs was plastid-specific was shown by parallel *in vitro* transcription assays with purified nuclei isolated from ASBVd-infected avocado leaf protoplasts. The total incorporation of [α-^{32}P]UMP into acid-precipitable material by equivalent nuclei and chloroplast preparations (on a fresh weight basis) was within the same order of magnitude. However, the ASBVd hybridization signals were 60 times more intense when the membranes were probed with the radioactive transcripts synthesized with the chloroplastic preparation (data not shown).

Effect of α-amanitin on synthesis of total and ASBVd-specific RNAs by the chloroplastic avocado system

The addition of 1 or 200 μg/ml α-amanitin into the transcription assays containing chloroplastic preparations from ASBVd-infected tissue affected their total transcriptional activity in less than 5%, thus underscoring the low level of nuclear contamination. Although it is not possible to prove directly that α-amanitin obtained access to the chloroplast transcriptional machinery because chloroplastic RNA polymerases are resistant to this antibiotic (see Discussion), it can be presumed that this was the case considering the marked inhibitory effects of compounds with a size similar, like tagetitoxin (see later), or considerably higher, like DNase (Fig. 1). The two concentrations of α-amanitin tested were chosen because 1 and 200 μg/ml typically inhibit the nuclear DNA-dependent RNA polymerases II and III, respectively, from different animal and plant systems (Marzluff and Huang, 1984).

Previous *in vitro* studies with a less well characterized cell-free avocado system showed that synthesis of ASBVd-specific RNAs of both polarities was uninfluenced by the presence of α-amanitin in the reaction mixture at the concentrations indicated earlier (Marcos and Flores, 1992). The same results were observed here with the *in vitro* transcription assays containing chloroplastic preparations from ASBVd-infected avocado tissue: no significant differences were observed when the radioactive transcripts synthesized with or without α-amanitin in the reaction mixture were used to probe membranes to which viroid-specific RNAs were applied (Figs. 2B and 2C).

Effects of tagetitoxin on synthesis of specific plastid transcripts and of plus and minus ASBVd RNAs by the chloroplastic avocado system

In contrast to α-amanitin, the incorporation of tagetitoxin in the range of 1–100 μM into the transcription assay led to a pronounced reduction of the overall RNA synthesis (Fig. 3), which is in line with the proposed inhibitory effect of this toxin on the bacterial-like chloroplastic RNA polymerase (Mathews and Durbin, 1990). The residual but significant transcriptional activity observed even in the presence of high concentrations of

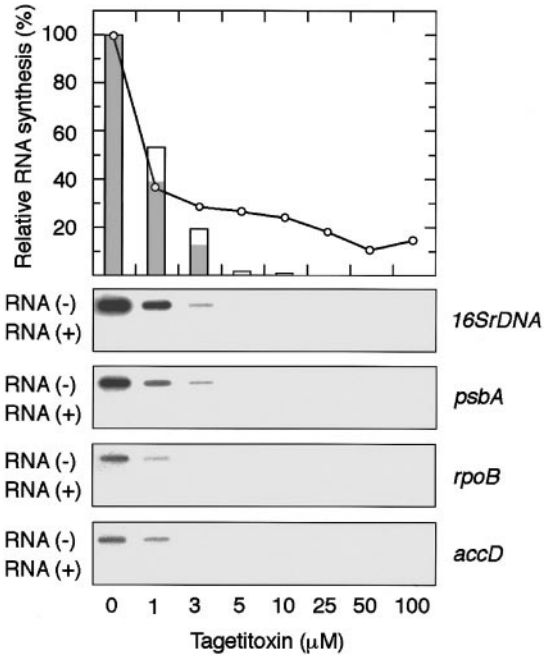

FIG. 3. Effect of tagetitoxin on transcription of individual chloroplastic genes by chloroplast preparations from ASBVd-infected avocado leaves. Radioactively labeled transcripts synthesized *in vitro* in the absence or presence of different concentrations of tagetitoxin were hybridized to immobilized RNAs (400 ng/slot) containing sense (+) and antisense (−) gene-specific sequences. ○, Total transcriptional activity estimated as the acid-precipitable radioactivity. Vertical columns indicate transcription of the *16SrDNA, psbA, rpoB*, and *accD* genes estimated from the intensity of the hybridization signals quantified with a bioimage analyzer; the white background denotes the maximum difference in relative RNA synthesis found between the four genes at each tagetitoxin concentration tested.

the inhibitor (Fig. 3) is consistent with the presence of a tagetitoxin-resistant (TR) chloroplastic RNA polymerase (Liere and Maliga, 1999; Sakai *et al.,* 1998).

For a more precise picture, we also analyzed the effects of tagetitoxin on the transcription of a series of individual plastid genes from avocado. The four genes investigated were selected to include at least one representative of the three classes in which the chloroplastic genes have been classified on the basis of their promoters (Hajdukiewicz *et al.,* 1997): (1) *psbA*, a photosynthetic gene of class I that is transcribed exclusively from PEP promoters, (2) *16SrDNA*, belonging to class II with both PEP and NEP promoters, and (3) *rpoB* and *accD,* two genes of class III with apparently only NEP promoters, encoding a subunit of the PEP core and a subunit of the acetyl-CoA carboxylase, respectively. DNA fragments (approximately 500-1200 nt) of these avocado genes were PCR-amplified using primers derived from regions conserved in the sequences of the homologous genes of tobacco, maize, and rice deposited in the EMBL database (Table 1). After cloning and sequencing the nucleotide identity between the avocado genes and their tobacco, maize and rice homologues were *psbA*, 93–95%; *16SrDNA*, 97–98.5%; and *rpoB* 88–92% and *accD*, 83–89%.

When membranes containing sense and antisense RNA sequences of the four genes were probed with the radioactive transcripts synthesized *in vitro* by a chloroplastic preparation from ASBVd-infected tissue, hybridization signals were observed in the four cases but only in the slots to which the antisense sequences were applied (Fig. 3, lanes 0). Therefore, the four genes were transcribed *in vitro*, although to different levels as indicated by signal intensity. The inhibition profiles induced by increasing concentrations of tagetitoxin were similar in all cases: transcription was reduced by 50–60% and basically disappeared at 1 and 5 μM tagetitoxin, respectively (Fig. 3, lanes 1–5). The overall transcriptional activity took on a slightly different pattern because a residual activity was detected even at the highest tested concentrations of the inhibitor (Fig. 3).

Contrary to the situation observed with the four plastid genes studied, tagetitoxin concentrations in the range of 1–25 μM were essentially ineffective on transcription of plus and minus ASBVd RNAs; only at higher tagetitoxin concentrations (50–100 μM), was a 20–25% inhibition of transcription of ASBVd strands observed (Fig. 4).

DISCUSSION

Polymerization of ASBVd-specific RNAs *in vitro* by a cell-free preparation from ASBVd-infected leaves has been reported before, although at the time no an attempt was made to identify the organelle where viroid replication occurred (Marcos and Flores, 1992). Experiments with that system permitted us to conclude that in contrast to representative members of the family *Pospiviroidae,* whose synthesis is inhibited by the low levels of α-amanitin that typically inhibit the nuclear DNA-dependent RNA

TABLE 1

Primers Used for PCR Amplification of Fragments of Four Avocado Chloroplastic Genes

Gene	Position in tobacco DNA[a]	Sequence
psbA	673 (h)	5′-TCAACTACAGATTGGTTGAAATTGAA-3′
	1393 (c)	5′-GAAACAGGTTCACGAATACCATCAAT-3′
16SrDNA	103258 (h)	5′-TCCGGAATGATTGGGCGTAAAGCGTC-3′
	103996 (c)	5′-GTCCTCAGTTCGGATTGCAGGCTGCA-3′
rpoB	25855 (h)	5′-TTGCATATTAGAAGTCATTAAAGCTC-3′
	27035 (c)	5′-CCTCCCCAATCTGATATTATGGTGCC-3′
accD	61087 (h)	5′-TTGGGGGATATCATTATTGCCGAACC-3′
	60599 (c)	5′-ATTTGAAAATGAGTAGTTCAGATAG-3′

[a] Numbers indicate location of the 5′-termini of primers, and (h) and (c) indicate their homologous or complementary polarity, respectively, with reference to the tobacco plastid DNA sequence (accession number Z00044).

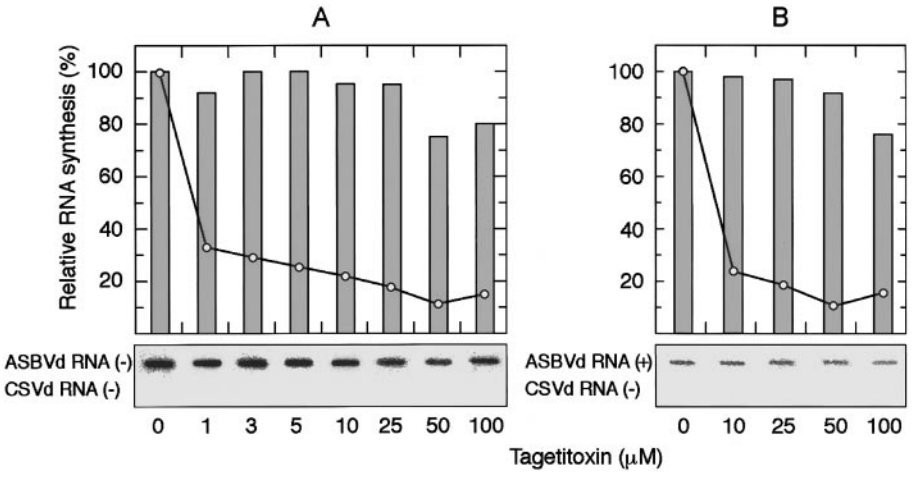

FIG. 4. Effect of tagetitoxin on transcription of plus and minus ASBVd RNAs by chloroplast preparations from ASBVd-infected avocado leaves. Radioactively labeled transcripts synthesized *in vitro* in the absence or presence of different concentrations of tagetitoxin were hybridized to immobilized RNAs (400 ng/slot) containing full-length ASBVd sequences of minus (A) and plus polarities (B), as well as a full-length CSVd transcript of minus polarity used as a negative control. Vertical columns indicate transcription of ASBVd-specific strands estimated from the intensity of the hybridization signals quantified with a bioimage analyzer. The exposure time was 5-fold longer than that of Fig. 3. Others details are as in the legend to Figure 3.

polymerase II (Flores and Semancik, 1982; Mühlbach and Sänger, 1979; Schindler and Mühlbach, 1992), synthesis of ASBVd strands was resistant to α-amanitin, pointing out the involvement of a different RNA polymerase (Marcos and Flores, 1992). Detection by *in situ* hybridization of a weak signal in avocado chloroplasts presumably generated by the ASBVd minus strand (Bonfiglioli *et al.,* 1994) and characterization in purified avocado chloroplasts of two double-stranded RNA complexes containing ASBVd minus RNAs (Navarro *et al.,* 1999) further supported that this organelle is the viroid replication site and therefore suggested the implication of a chloroplastic RNA polymerase. Here we report on the development of an *in vitro* transcription assay from avocado chloroplasts to investigate the nature of this RNA polymerase.

The chloroplastic system showed the general properties of an *in vitro* transcription assay. Run-on systems of this class are assumed to elongate RNA strands whose synthesis has been initiated *in vivo* but not to reinitiate RNA synthesis *de novo*. The system from ASBVd-infected tissue, but not from the uninfected material, was also able to synthesize viroid-specific sequences, being those of the plus polarity predominant as in the *in vivo* situation. The hybridization signals suggesting synthesis of ASBVd minus strands should be regarded with caution because due to the self-complementarity of viroid RNAs, they might also be generated by the great excess of the viroid plus strands. In contrast with the 40–60% reduction of the total RNA polymerase activity caused by α-amanitin in the cell-free system reported previously (Marcos and Flores, 1992), indicating the presence of nuclei, no effect was observed on the transcriptional activity of the chloroplastic system as anticipated from the resistance of chloroplastic RNA polymerases to this antibiotic (Liere and Maliga, 1999; Sakai *et al.,* 1998). Rifampicin (100 μg/ml) also did not affect the transcriptional activity of the chloroplastic system (data not shown), which is also in agreement with previous results (Sakai *et al.,* 1998).

Several studies have revealed that distinct RNA polymerase activities with different sensitivities to tagetitoxin are present in plastids. Taking advantage of the fact that this bacterial phytotoxin inhibits the plastid-encoded RNA polymerase (PEP) (Mathews and Durbin, 1990), but not the nuclear-encoded RNA polymerase (NEP) (Liere and Maliga, 1999), we have used this inhibitor as a tool to explore the activity involved in ASBVd replication. Tagetitoxin strongly inhibited the total transcriptional activity of the chloroplastic system reported here (Fig. 3), indicating a major PEP involvement in this process. The remaining transcriptional activity was likely due to a plastid RNA polymerase resistant to tagetitoxin, presumably the NEP activity reported previously (Liere and Maliga, 1999), or another activity yet to be characterized. When the effect of tagetitoxin on the transcription of four specific chloroplastic genes was analyzed, we observed a similarly strong inhibition in all cases, including, surprisingly, those of *accD* and *rpoB*, which belong to class III and are presumed to be exclusively transcribed from NEP promoters (Hajdukiewicz *et al.,* 1997). We obtained essentially the same inhibition patterns for the aforementioned four genes with a chloroplastic transcription system from tobacco prepared in the same way as that from avocado (data not shown), showing that the unexpected inhibition of *accD* and *rpoB* transcription by tagetitoxin was not peculiar to avocado. Our results are in line with those of a previous report in which transcription of various tobacco plastid genes, including *psbA* and *rpoB,*

was found to be sensitive to tagetitoxin in chloroplast preparations, whereas transcription of the same genes was relatively resistant to this inhibitor in proplastid preparations (Sakai *et al.,* 1998). Moreover, the assumption that *rpoB* and *accD* have only NEP promoters is entirely based on studies with mutant plants that lack PEP and have proplastid-like nonphotosynthetic plastids and substantially increased levels of the NEP-driven transcripts (Allison *et al.,* 1996). Therefore, it is possible that in photosynthetic plastids these two genes may be transcribed by the predominant PEP from alternative promoters or even from the same NEP promoters through a transcription factor able to recruit PEP or NEP depending on their relative abundance. The existence of a modified form of NEP, or even an additional novel RNA polymerase, has been suggested to cope with all aspects of plastid transcriptional regulation (Kapoor and Sugiura, 1999; Sakai *et al.,* 1998).

As opposed to the marked inhibitory effects of tagetitoxin on the overall and gene-specific transcriptional activity of purified avocado chloroplasts, transcription of ASBVd-specific RNAs was essentially resistant to high concentrations of this compound. This indicates that the tagetitoxin-sensitive PEP catalyzing the major fraction of chloroplastic transcription is not involved in the polymerization of viroid strands that is likely mediated by NEP or by another tagetitoxin-resistant chloroplastic RNA polymerase. One pertinent point in this regard is that if the chloroplastic enzyme transcribing ASBVd strands is a DNA-dependent RNA polymerase forced to use a template of RNA instead of DNA as under normal physiological conditions, the remote possibility exists that sensitivity to tagetitoxin might be affected by the nature of the template. However, a parallel argument can be extended to the effects of α-amanitin on the nuclear DNA-dependent RNA polymerase II, which, acting on an RNA template, is the enzyme generally assumed to participate in replication of typical members of the family *Pospiviroidae* (Flores and Semancik, 1982; Mühlbach and Sänger, 1979; Schindler and Mühlbach, 1992), particularly considering that tagetitoxin and α-amanitin present very similar inhibition mechanisms. Tagetitoxin, to which the *E. coli* RNA polymerase and the nuclear DNA-dependent RNA polymerase III are also sensitive, is capable of inhibiting the elongation phase of RNA synthesis by interacting with the ternary complex that contains the RNA polymerase, the template, and the nascent RNA (Mathews and Durbin, 1994).

Yet another argument favoring the involvement of an NEP-like activity in the transcription of ASBVd strands is that the two characterized tobacco promoters recognized by this polymerase are surprisingly short (15–19 nt) and lack regulatory elements outside the promoter (Liere and Maliga, 1999). "Minipromoters" such as these, which are similar in size to those of T3 and T7 phage RNA polymerases thus underscoring the phage-like properties of NEP, appear ideally suited to fit within the small size of the ASBVd genome (247 nt). Our results also suggest that the proposed NEP-like activity is endowed with the ability to transcribe both DNA and RNA templates. Relevant in this context are previous reports showing that DNA-dependent T7 RNA polymerase can catalyze the *in vitro* replication of two small A + U-rich single-stranded RNAs with compact secondary structures that resemble that of ASBVd (Konarska and Sharp, 1990).

MATERIALS AND METHODS

In vitro transcription assay

Young asymptomatic leaves from ASBVd-infected avocado plants (*Persea americana* Miller, cv. Fuerte) and from healthy controls were used to prepare protoplasts, from which chloroplasts and nuclei were purified with a final step of centrifugation through a Percoll gradient (Navarro *et al.,* 1999) and resuspended in 50 mM Tricine-NaOH, pH 8.0, 5 mM $MgCl_2$, 25% glycerol, and 20 mM β-mercaptoethanol by repeat pipetting. The *in vitro* transcription assay was carried out at 30°C for 2 h in a reaction mixture (100 μl) containing 25 mM Tricine-NaOH, pH 8.0, 10 mM $MgCl_2$, 120 mM KCl, 12.5% glycerol, 20 mM β-mercaptoethanol, 0.5 mM each of the unlabeled ATP, CTP, and GTP, and 50 μCi of [α-^{32}P]UTP (Amersham, Arlington Height, IL; specific activity, 400 Ci/mmol). The amount of chloroplasts in the reaction mixture was equivalent to 100 μg of chlorophyll. When indicated, chloroplast preparations were pretreated with 150 U of RNase-free DNase in the presence of human placental RNase inhibitor (Amersham) at 30°C for 15 min. After incubation, transcription mixtures were extracted with phenol–chloroform–isoamyl alcohol (25:25:1), and the nucleic acids were recovered by ethanol precipitation; most of the radioactive precursor that coprecipitated was removed by chromatography through Sephadex G-50 spin columns. One tenth of the reaction product was used to determine the reaction progress estimated as the acid-precipitable radioactivity, and the remainder was used as a probe in the hybridization experiments. When stated, different concentrations of tagetitoxin (Epicenter Technologies, Madison, WI) or α-amanitin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) were added to the reaction mixtures.

Isolation of chloroplastic DNA

Avocado plastid DNA was obtained by extracting intact chloroplasts purified as indicated above with phenol–chloroform–isoamyl alcohol. RNA was digested with RNase A (1 mg/ml) at 37°C for 30 min. The digestion ended with the addition of two volumes of a protease solution (50 μg/ml proteinase K in 12.5 mM Tris–HCl, pH 8.0, containing 0.25% SDS and 5 mM EDTA), incubation

at 37°C for 30 min, and extraction with phenol–chloroform–isoamyl alcohol. The DNA dissolved in the aqueous phase was recovered by ethanol precipitation and resuspended in sterile distilled water.

Plasmid construction

Recombinant plasmids pAS18 and pAS19 containing inserts with the full-length ASBVd sequence in both orientations were the same previously termed p18A and p19A, respectively (Marcos and Flores, 1992). Recombinant plasmid pCS1 was constructed by cloning in the *Eco*RV site of pBluescript II KS$^+$ (Stratagene, La Jolla, CA), a full-length insert of CSVd obtained by RT-PCR using a pair of adjacent primers of opposite polarity derived from the upper strand of the central domain. First-strand cDNA was synthesized on purified circular forms of the viroid with primer RF-74 (5′-GGGATCCCTGAAGGACTTCT-3′) and avian myeloblastosis virus reverse transcriptase. An aliquot (1/20) of this preparation was PCR-amplified with primers RF-74 and RF-75 (5′-GGGGAAACCTGGAGGAAG-3′) and 2.5 U of cloned *Pfu* DNA polymerase using the buffer suggested by the producer (Stratagene). The PCR profile (30 cycles) was 95°C for 40 s, 60°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 15 min.

Partial-length fragments of the coding regions of plastid genes *psbA*, *16SrDNA*, *rpoB*, and *accD* were obtained by PCR amplification of avocado chloroplastic DNA and pairs of primers derived from regions conserved in the corresponding plastid genes of *Nicotiana tabacum, Oryza sativa*, and *Zea mays.* The sequence of the homologous (h) and complementary (c) primers and the location of their 5′ termini within the tobacco plastid DNA are shown in Table 1. The PCR profile was as indicated, and the amplified DNA fragments were cloned into the *Eco*RV site of pBluescript II KS$^+$.

Slot-blot hybridization

Recombinant plasmids linearized with appropriate restriction enzymes were used as template for *in vitro* transcription using T3 or T7 RNA polymerase. The RNAs obtained were applied onto nylon membranes (Hybond N$^+$; Amersham) in a slot-blot format using a bio-dot apparatus (Bio-Rad) and fixed by UV irradiation with a 1800 Stratalinker (Stratagene). The membranes were prehybridized for at least 1 h at 70°C and hybridized overnight at the same temperature in a buffer containing 50% formamide (Marcos and Flores, 1992) and the radioactive transcripts synthesized *in vitro* by the avocado chloroplastic preparations. The membranes were washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature and once in 0.1× SSC and 0.1% SDS for 15 min at 55°C. The hybridization signals were quantified with a bioimage analyzer (Fuji Bas 1500).

ACKNOWLEDGMENTS

We thank A. Ahuir for excellent technical assistance, Drs. C. Hernández and J. A. Daròs for critical reading of the manuscript and suggestions, and Barraclough-Donnellan for the English revision. R.F. was supported by Grants PB95-0139 and PB98-0500 from the Dirección General de Investigación Científica y Técnica de España and by Contract CHRX-CT94-0635 from the European Commission. J.A.N. was the recipient of a predoctoral fellowship from the Generalidad Valenciana, and A.V. was the recipient of a contract from the Ministerio de Educación y Cultura de España.

REFERENCES

Allison, L. A., Simon, L. D., and Maliga, P. (1996). Deletion of *rpoB* reveals a second distinct transcription system in plastid of higher plants. *EMBO J.* **15,** 2802–2809.

Bonfiglioli, R. G., McFadden, G. I., and Symons, R. H. (1994). *In situ* hybridization localizes avocado sunblotch viroid on chloroplast thylakoid membranes and coconut cadang cadang viroid in the nucleus. *Plant J.* **6,** 99–103.

Branch, A. D., Benenfeld, B. J., and Robertson, H. D. (1988). Evidence for a single rolling circle in the replication of potato spindle tuber viroid. *Proc. Natl. Acad. Sci. USA* **85,** 9128–9132.

Branch, A. D., and Robertson, H. D. (1984). A replication cycle for viroids and other small infectious RNAs. *Science* **223,** 450–454.

Bussière, F., Lehoux, J., Thompson, D. A., Skrzeczkowski, L. J., and Perreault, J.-P. (1999). Subcellular localization and rolling circle replication of peach latent mosaic viroid: Hallmarks of group A viroids. *J. Virol.* **73,** 6353–6360.

Daròs, J. A., Marcos, J. F., Hernández, C., and Flores, R. (1994). Replication of avocado sunblotch viroid: Evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. *Proc. Natl. Acad. Sci. USA* **91,** 12813–12817.

Davies, J. W., Kaesberg, P., and Diener, T. O. (1974). Potato spindle tuber viroid. XII. An investigation of viroid RNA as a messenger for protein synthesis. *Virology* **61,** 281–286.

Diener, T. O. (1971a). Potato spindle tuber "virus": A plant virus with properties of a free nucleic acid. III. Subcellular location of PSTV-RNA and the question of whether virions exist in extracts or *in situ. Virology* **43,** 75–89.

Diener, T. O. (1971b). Potato spindle tuber "virus". IV. A replicating low molecular weight RNA. *Virology* **45,** 411–428.

Feldstein, P. A., Hu, Y., and Owens, R. A. (1998). Precisely full length, circularizable, complementary RNA: An infectious form of potato spindle tuber viroid. *Proc. Natl. Acad. Sci. USA* **95,** 6560–6565.

Flores, R., Randles, J. W., Bar-Joseph, M., and Diener, T. O. (1998). A proposed scheme for viroid classification and nomenclature. *Arch. Virol.* **143,** 623–629.

Flores, R., and Semancik, J. S. (1982). Properties of a cell-free system for synthesis of citrus exocortis viroid. *Proc. Natl. Acad. Sci. USA* **79,** 6285–6288.

Grill, L. K., and Semancik, J. S. (1978). RNA sequences complementary to citrus exocortis viroid in nucleic acid preparations from infected *Gynura aurantiaca. Proc. Natl. Acad. Sci. USA* **75,** 896–900.

Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H., and Sänger, H. L. (1978). Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* **273,** 203–208.

Hall, T. C., Wepprich, R. K., Davies, J. W., Weathers, L. G., and Semancik, J. S. (1974). Functional distinctions between the ribonucleic acids from citrus exocortis viroid and plant viruses: cell-free translation and aminoacylation reactions. *Virology* **61,** 486–492.

Hajdukiewicz, P. T. J., Allison, L. A., and Maliga, P. (1997). The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J.* **16,** 4041–4048.

Harders, J., Lukacs, N., Robert-Nicoud, M., Jovin, J. M., and Riesner, D.

(1989). Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy. *EMBO J.* **8,** 3941–3949.

Hernández, C., and Flores, R. (1992). Plus and minus RNAs of peach latent mosaic viroid self cleave *in vitro* via hammerhead structures. *Proc. Natl. Acad. Sci. USA* **89,** 3711–3715.

Hedtke, B., Börner, T., and Weihe, A. (1997). Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. *Science* **277,** 809–811.

Hutchins, C. J., Keese, P., Visvader, J. E., Rathjen, P. D., McInnes, J. L., and Symons, R. H. (1985). Comparison of multimeric plus and minus forms of viroids and virusoids. *Plant Mol. Biol.* **4,** 293–304.

Hutchins, C., Rathjen, P. D., Forster, A. C., and Symons, R. H. (1986). Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Res.* **14,** 3627–3640.

Ishikawa, M., Meshi, T., Ohno, T., Okada, Y., Sano, T., Ueda, I., and Shikata, E. (1984). A revised replication cycle for viroids: The role of longer than unit RNA in viroid replication. *Mol. Gen. Genet.* **196,** 421–428.

Kapoor, S., and Sugiura, M. (1999). Identification of two essential sequence elements in the non-consensus type II *PatpB-290* plastid promoter by using plastid transcription extracts from cultured tobacco BY-2 cells. *Plant Cell* **11,** 1799–1810.

Kapoor, S., Suzuki, J. Y., and Sugiura, M. (1997). Identification and functional significance of a new class of non-consensus-type plastid promoters. *Plant J.* **11,** 327–337.

Konarska, M. M., and Sharp, P. A. (1990). Structure of RNAs replicated by the DNA-dependent T7 RNA polymerase. *Cell* **63,** 609–618.

Lerbs-Mache, S. (1993). The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: Single-subunit enzyme or catalytic core of multimeric enzyme complex? *Proc. Natl. Acad. Sci. USA* **90,** 5509–5513.

Liere, K., and Maliga, P. (1999). *In vitro* characterization of the tobacco *rpo B* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters. *EMBO J.* **18,** 249–257.

Lima, M. I., Fonseca, M. E. N., Flores, R., and Kitajima, E. W. (1994). Detection of avocado sunblotch viroid in chloroplasts of avocado leaves by *in situ* hybridization. *Arch. Virol.* **138,** 385–390.

Marcos, J. F., and Flores, R. (1992). Characterization of RNAs specific to avocado sunblotch viroid synthesized *in vitro* by a cell-free system from infected avocado leaves. *Virology* **186,** 481–488.

Marzluff, W. F., and Huang, R. C. (1984). Transcription of RNA in isolated nuclei. *In* "Transcription and Translation: A Practical Approach" (B. D. Hames, and S. J. Higgins, Eds.), pp. 89–129. IRL Press, Oxford/Washington, DC.

Mathews, D. E., and Durbin, R. D. (1990). Tagetitoxin inhibits RNA synthesis directed by RNA polymerases from chloroplasts and *Escherichia coli*. *J. Biol. Chem.* **265,** 493–498.

Mathews, D. E., and Durbin, R. D. (1994). Mechanistic aspects of tagetitoxin inhibition of RNA polymerase from *Escherichia coli*. *Biochemistry* **33,** 11987–11992.

Mühlbach, H. P., and Sänger, H. L. (1979). Viroid replication is inhibited by α-amanitin. *Nature* **278,** 185–188.

Navarro, B., and Flores, R. (1997). Chrysanthemum chlorotic mottle viroid: Unusual structural properties of a subgroup of viroids with hammerhead ribozymes. *Proc. Natl. Acad. Sci. USA* **94,** 11262–11267.

Navarro, J. A., Daròs, J. A., and Flores, R. (1999). Complexes containing both polarity strands of avocado sunblotch viroid: Identification in chloroplasts and characterization. *Virology* **253,** 77–85.

Sakai, A., Saito, C., Inada, N., and Kuroiwa, T. (1998). Transcriptional activities of the chloroplast-nuclei and proplastid-nuclei isolated from tobacco exhibit different sensitivities to tagetitoxin: Implications of the presence of distinct RNA polymerases. *Plant Cell Physiol.* **39,** 928–934.

Schindler, I. M., and Mühlbach, H. P. (1992). Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: A reevaluation. *Plant Sci.* **84,** 221–229.

Stern, D. B., Higgs, D. C., and Yang, J. (1997). Transcription and translation in chloroplasts. *Trends Plant Sci.* **2,** 308–315.

Sugiura, M. (1992). The chloroplast genome. *Plant Mol. Biol.* **19,** 149–168.

Symons, R. H. (1981). Avocado sunblotch viroid: Primary sequence and proposed secondary structure. *Nucleic Acids Res.* **9,** 6527–6537.

Tanaka, K., Oikawa, K., Ohta, N., Kuroiwa, H., Kuroiwa, T., and Takahashi, H. (1996). Nuclear encoding of a chloroplast RNA polymerase sigma subunit in a red alga. *Science* **272,** 1932–1935.

Vera, A., Hirose, T., and Sugiura, M. (1996). A ribosomal protein gene *(rp132)* from tobacco chloroplast DNA is transcribed from alternative promoters in non-photosynthetic plastids: similar promoter region organization in plastid housekeeping genes. *Mol. Gen. Genet.* **251,** 518–525.

Vera, A., and Sugiura, M. (1995). Chloroplast rRNA transcription from structurally different tandem promoters: An additional novel-type promoter. *Curr. Genet.* **27,** 280–284.