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Replication of Avocado Sunblotch Viroid in the Cyanobacterium *Nostoc* Sp. PCC 7120

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Abstract

Viroids are small infectious RNA molecules that replicate in plants via RNA-RNA replication processes. The molecular mechanism responsible for this replication has attracted great interest, and studies on this topic have yielded interesting biological findings on the processes in which RNA is involved. Viroids belonging to the *Avsunviroidae* family replicate in the chloroplasts of infected hosts. It has by now been established that chloroplasts and cyanobacteria share a common have ancestor. In view of this phylogenetic relationship, we investigated whether a member of the *Avsunviroidae* family could be replicated in a cyanobacterium. The results obtained here show that *Avocado Sunblotch Viroid* (ASBVd) RNA is able to replicate in the filamentous cyanobacterium *Nostoc* PCC 7120. Indeed, Northern blot hybridization showed that linear forms of "minus" polarity were detected in RNA extracted from *Nostoc* cells expressing ASBVd dimers of positive "polarity", and that linear forms of "negative" polarity were detected in RNA extracted from *Nostoc* cells expressing ASBVd dimers of "positive" polarity. ASBVd replication does not impair the growth of *Nostoc*. These results provide the first evidence that a prokaryotic cell possesses all the machinery required to sustain the process of RNA-RNA replication. The data obtained here are of great importance, since they might shed light on the evolution of the cellular factors on which RNA replication processes depend.

Keywords: Avocado Sunblotch viroid; Cyanobacteria; Evolution; Nostoc; RNA-RNA replication

Introduction

Viroids are the smallest pathogens known to exist on Earth. They were found in the early 70s to be the causal agents responsible for the potato tubercle disease, which had been blamed so far on bacteria or viruses [1]. This pioneering study led to the discovery of a highly intriguing non coding RNA, which is able to infect a large panel of crop plants [2,3]. Unlike viruses, viroids are composed of free RNA without any envelope or capsid. They can occur in infected plant cells in two forms: in the positive polarity form, which is the most abundant, and the minus polarity one, which corresponds to a replication intermediate. They are circular, structured RNAs consisting of about 250 to 400 nucleotides that replicate via an RNA-RNA copying process according to a "rolling-circle" mechanism [4]. Two families of viroids are known to exist so far, the *Pospiviroidae,* which include five genera (*pospiviroids*, *hostuviroids*, *cocadviroids*, *apscaviroids* and *coleviroids*) and the *Avsunviroidae,* which include three genera (*avsunviroids*, *pelamoviroids* and *elaviroids*). While the viroids belonging to the *Pospiviroidae* family replicate in the cell nucleus, replication of the members of the *Avsunviroidae* takes place in the chloroplasts of infected plants via a process involving a hammerhead ribozyme (HHR) on which the processing of the transcription products depends. During the replication of *Avsunviroidae*, oligomers are formed and cleaved to monomers, which are then ligated and generate the circular forms. Replication of viroids requires the presence of host factors. The DNA-dependent RNA polymerase II has been found to replicate members of the *Pospiviroidae* family [5], while replication of members of the *Avsunviroidae* family is thought to depend on the nuclear-encoded DNA-dependent RNA polymerase present in the chloroplasts [6].

Several environmental factors are known to affect the replication of viroids in plants. Heat treatment has been found to increase the accumulation of mutations during the replication cycle of the Hop latent viroid, thus increasing the variability of these infectious RNAs [7]. The activity of the HHR of the *Chrysanthemum* chlorotic mottle viroid has been found to resist drastic environmental conditions such as high pressure [8]. In addition, self-cleavage-catalyzed reactions have been reported to occur in the HHR of the *Avocado sunblotch viroid* (ASBVd) exposed to high temperatures (of up to 60°C) [9]. The fact that potato spindle tuber viroid has been detected in potato seeds stored for 21 years shows that this molecule is endowed with remarkable longevity [10].

The structure of viroids, the catalytic activity of some of them, and their long-term persistence have been presented as arguments supporting the idea that they were involved at the beginning of the so-called "RNA world" [11]. We recently established that ASBVd replicates in the yeast *Saccharomyces cerevisiae*, which indicates that the ribozyme is able to sustain the cleavage/ligation reaction during the rolling circle replication process. Since ASBVd replication has continued for 25 generations of the yeast, the viroid replication process can be said to be surprisingly adaptable in this unconventional host [12].

Are *Avsunviroidae* able to replicate in a prokaryotic cell? The aim of the present study was to answer this question. It was therefore proposed to analyze the replication of ASBVd, a typical member of the *Avsunviroidae* family, in the cyanobacterium *Nostoc* PCC 7120. Since ASBVd replicates in chloroplasts, we predicted that cyanobacteria,

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which are share their progenitors with chloroplasts, would be excellent candidates for this study.

Cyanobacteria are among the most diverse and widely distributed phyla of bacteria. In photosynthetic prokaryotes, they have the unique ability to perform oxygenic photosynthesis. They play key roles in the carbon and nitrogen cycles and constitute highly suitable organisms for investigating the evolution of photosynthetic eukaryotes, since the ancestry of the chloroplasts of all photosynthetic organisms can be traced back to cyanobacteria [13]. The authors of taxonomic studies have classified the cyanobacteria in five subsections based on their level of morphological complexity. Organisms in subsections IV (*Nostocales*) and V (*Stigonematales*) are diazotrophic bacteria that can differentiate specific cells called heterocysts, which are involved in nitrogen fixation processes [14]. *Nostoc* PCC 7120 (which will be referred to below as *Nostoc*) belongs to the *Nostocales* subsection. This strain was used here as an experimental model to determine whether ASBVd can replicate in cyanobacteria. This is the first time a viroid RNA replication process has been reported to occur in a prokaryote.

Material and Methods

Strains and growth conditions

Nostoc sp. PCC 7120 (Pasteur Institute collection, France) was grown in BG11 medium at a temperature of 30°C in air under continuous illumination (40 µE $m^{-2}s^{-1}$). Cyanobacterial growth was monitored by measuring the absorbance at 750 nm (OD_{750}). Each experiment was performed in triplicate. *Nostoc* filaments were observed using the Optical microscope Nikon Eclipse E800.

Oligonucleotides

The oligonucleotides used in this study were obtained from Eurogentec (France). Their sequences are listed in Table 1.

Construction of *Nostoc*/ASBVd recombinant strains

The ASBVd dimer was extracted from the pCRII-TOPOdASBVd plasmid expressing the ASBVd DNA dimer [12], using the EcoRI enzyme. The resulting fragment was cloned under the control of the *petE* promoter of the pSK*petE* plasmid [15]. Plasmids expressing dimeric (+) or (-) ASBVd were selected after the sequencing procedure (Millegen, France). The *petEASBVd* fragments were then subcloned into the pRL25 plasmid [15] after performing a linearization step using the NotI and KpnI enzymes. The resulting plasmids were sequenced in order to further confirm the (+) or (-) orientation of the viroid. The recombinant pRLASBVd(+) and pRLASBVd(-) obtained were conjugated into *Nostoc*, and exoconjugants were selected with 50 µg/mL neomycin.

RNA extraction

RNA was extracted using TRizol reagent (Invitrogen, France) in line with the manufacturer's instructions. Chromosomal DNA was removed by treating RNA preparations (50 µL) with 1 µL of DNase (Ambion at 2 U/µL) for 1 hour at 37°C. DNase treatment was checked by performing RT-PCR (see below), omitting the reverse transcription step. Only DNA-free RNAs were used in all our experiments. The concentration of the RNA was determined spectrophotometrically.

Oligonucleotides	Sequence from 5' to 3'
ASBV-O1	GTGAGGATATGATTAAACT
ASBV-O2	TTCTTGTTCTAATAAACAAG
LK ASBV-O1	gatctggagcacgaggacactgc GTGAGGATATGATTAAACT
LK ASBV-O2	gatctggagcacgaggacactgc TTCTTGTTCTAATAAACAAG
LK	gatctggagcacgaggacactgc
rnpB forward	AGGGAGAGAGTAGGCGTTGG
rnpB reverse	GGTTTACCGAGCCAGTACCTCT

Table 1: Sequence of primers used in this study.

Strand-specific RT-PCR

One µg of total RNA was subjected to reverse transcription with ThermoScript reverse transcriptase (Invitrogen) using 2 pmol of LKASBV-O1 and LKASBV-O2 as primers to synthesize (+) and (-) cDNAs respectively. One-tenth of these reactions were used as templates for PCR. Amplifications were performed using the high fidelity Taq polymerase (Jena Biosciences, Germany) in line with the manufacturer's instructions. ASBV-O1 and ASBV-O2 primers (10 pmol) were used in each reaction. The standard program was: 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 45 s at 50°C and 45 s at 72°C, followed by a final 5 min at 72°C. The products (247-nt corresponding to the ASBVd monomers) were separated on a 1.2% agarose gel.

Northern blot hybridization

Total RNA (5 µg) was separated on 6% polyacrylamide-7 M urea gels and electro-transferred (Biorad apparatus, USA) to Hybond N^+ membranes (GE Healthcare, France). Hybridizations were performed in Church buffer [16] at 70°C and filters were washed in 4X SSC, 0.5% SDS at 65°C, before exposure to autoradiographic films.

Riboprobes were generated by performing *in vitro* transcription in the presence of [α-^{32}P]CTP. Briefly, pBdASBVd DNA plasmid was linearized by PdiI enzyme (Thermofisher, France) and then 250 ng (2 µL) were incubated for 2 hours at 37°C in a reaction mixture (20 µL) containing 2 µL reaction buffer 10X (Ambion, France), [α-^{32}P] CTP (5 µL); 10 µCi/mL ; 3000 Ci/mmol ; Perkin Elmer), a nucleotide-triphosphate mixture (NTP: A, U and G; at final concentrations of 0.5 mM each), CTP (10 µM final) and 20 U (2 µL) of T7 (for synthesizing the "-" riboprobe) or T3 (for synthesizing the "+" riboprobe) RNA polymerases (Ambion, France). After adding DNase (1 µL) for 15 min at 37°C, probes were purified using spin G50 columns (GE Healthcare, France) and denatured for 5 min at 100°C before being hybridized with the membranes. The *rnpB* gene encoding the RNaseP subunit B in *Nostoc* was used to perform the internal controls. It was first amplified by PCR using the *rnpB* forward and *rnpB* reverse primers (Table 1) and the PCR product was purified. Five purified picomoles (1 µg ; 10 vL) were then 5' end-labeled by incubating the sample for 10 min at 37°C in a reaction mixture (25 µL) containing an exchange reaction buffer (5X; 5 µL; Promega, France), [γ-^{32}P]ATP (5 µL ; 10 µCi/mL ; 3000 Ci/mmol ; Perkin Elmer), T4 polynucleotide kinase (1 µL; 5 U; Promega, France). The reaction was stopped by adding EDTA 0.5 M (1 µL), and the probe was heated for 10 min at 65°C before hybridization with the previously dehybridized membranes. After the hybridization, the membranes were washed and exposed to autoradiography.

RNA marker (Thermofisher, France) co-migrated with total RNAs samples on the 6% polyacrylamide-urea gels and stained with ethidium bromide before northern blotting.

Results

Replication of negative and positive ASBVd strands in *Nostoc* assessed by strand-specific RT-PCR

Negative (-) and positive (+) dimeric ASBVd DNA were cloned under the control of the copper inducible *petE* promoter of the pRL25 replicative plasmid (Material and Methods). *Nostoc* recombinant

strains bearing either the empty pRL25 plasmid or pRLASBVd(+) or pRLASBVd(-) were grown up to the late exponential phase (OD_{750}: 1). RNAs were extracted and the replication of ASBVd strands was tested using the strand-specific RT-PCR assay, as described in Figure 1. Briefly, the reverse transcription reaction induced using the LKASBV-O1 primer results in the synthesis of the (+) cDNA and that of the LKASBV-O2 primer results in the synthesis of the (-) cDNA. An LK sequence was added to the 5' end of each of these reverse-strand primers. The LK sequence alone was used with the strand-specific primer for the PCR (Figure 1 and Table 1). This procedure ensured that only DNA specifically synthesized during the RT step could be amplified during the PCR [17], thus making it possible after the PCR reaction to detect the RNA-RNA replication of the ASBVd (-) strand. The amplification of the *rnpB* gene encoding the RNase P subunit B was used to check the amount of RNA used in each assay (Figure 2B, Lanes 1 and 2). The data presented in Figure 2A show that both negative and positive strands were replicated in *Nostoc* since DNA species of positive and negative polarities were detected, respectively (Figure 2, Lanes 3 and 5). The transcription, from the *petE* promoter, of ASBVd (of negative and positive polarities) yielded stronger products of negative and positive polarities, respectively (Figure 2, Lanes 2 and 4). All the resulting PCR fragments were about 250 bp long, which is the expected size of the monomeric RNA form (Figure 2, Lane 6). No amplification of the ASBVd strands was observed when the RNAs were extracted from the strain bearing the empty pRL25 plasmid (Figure 2C, Lanes 1 and 2). This result confirms the specificity of the primers used and shows that the amplifications observed actually resulted from the replication of the ASBVd RNA strands.

Replication of negative and positive ASBVd strands in *Nostoc* assessed by Northern blot technique

The replication of the ASBVd in *Nostoc* cells was further investigated using a Northern blot approach. For this purpose, total RNAs were extracted from *Nostoc* cells bearing the pRLASBVd(+) or pRLASBVd(-) plasmids, loaded onto a 6% polyacrylamide gel, and transferred to nitrocellulose membranes. The membranes were probed with riboprobes resulting from *in vitro* transcription of the (+) or (-) ASBVd dimer RNAs extracted from *Nostoc* harboring the empty pRL25 plasmid were used to run negative controls. In the positive controls, we used total RNA extracted from avocado tree leaves infected with ASBVd. The level of *rnpB* transcripts served to monitor the loading control.

When RNAs extracted from *Nostoc*/pRLASBVd (-) were used as a template, linear form were observed when the negative strand was used as a riboprobe (Figure 3A, Lane 5). This confirms that the (-) strands were actually replicated and yielded the complementary (+) strands. Similar results were obtained when the RNAs extracted from *Nostoc*/pRLASBVd (+) were loaded and when the positive strand was used as a riboprobe during Northern hybridization assays (Figure 3B, Lane 8), although the amount of negative strand was much lower than that of positive strand. In both cases, the form detected had the size expected for an ASBVd linear form. This could be assessed thanks to the use of an RNA molecular marker (Figure 3), and positive controls consisting in a linear ASBVd from synthesized *in vitro* and also in RNA extracted from avocado tree leaves infected with ASBVd (Figure 3). This

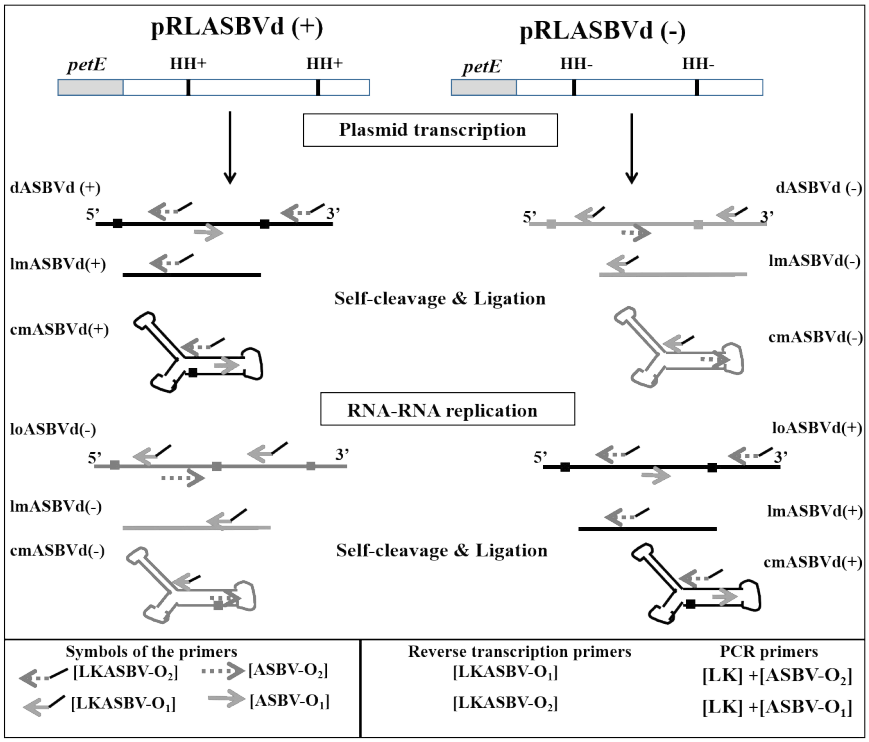

Figure 1: Diagram of strand-specific RT-PCR for analyzing ASBVd replication in *Nostoc*. The pRLASBVd(-) and pRLASBVd(+) plasmids express the ASBVd(-) and ASBVd(+) DNA dimmers, respectively, from the *petE* promoter (grey boxes). HH: hammerhead ribozyme. During the first step, self-cleavage of the ASBVd dimeric form results in the linear monomeric form (lmASBVd) and the circular monomeric form (cmASBVd). During the second step, the RNA-dependent replication process generates linear oligomers (loASBVd), lmASBVd and cmASBVd having the opposite polarity. The primers used during the reverse transcription have a linker sequence at their 5' extremity (LKASBV-O1 and LKASBV-O2). In order to prevent any amplification of DNA from the plasmid, the primers used during the PCR step are LK and ASBV-O2 for the replication of ASBVd(-) and LK and ASBV-O1 for ASBVd(+), respectively, which makes it possible to discriminate between the RNAs resulting from plasmid transcription and those resulting from RNA-RNA replication.

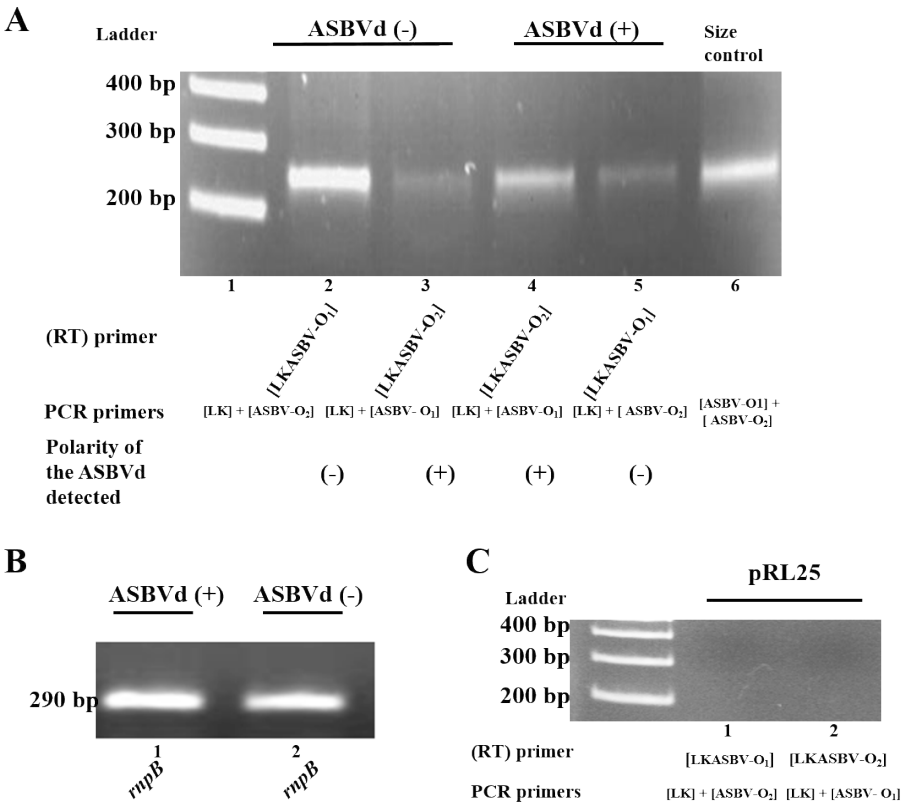

Figure 2: Detection of (-) and (+) forms of ASBVd in *Nostoc* using strand-specific RT-PCR. **A.** Strand-specific RT-PCR performed with RNAs extracted from pRLASBVd(-) or pRLASBVd(+) recombinant strains. For each reaction, the primer used in the reverse transcription step and the polarity of the corresponding amplified cDNAs are indicated at the bottom of the image. The "size control" line corresponds to the result of a PCR amplification step in which the ASBV-O1 and ASBV-O2 primers and the pCRII-topo ASBVd (-) plasmid were used. **B.** Amplification of the *rnpB* gene using RNAs extracted from pRLASBVd(-) versus pRLASBVd(+) recombinant strains. **C.** RT-PCR reactions performed using the LKASBV-O1 and LKASBV-O2 primers and RNAs extracted from a *Nostoc* strain harboring the empty pRL25 plasmid. This experiment was performed with five recombinant *Nostoc* strains obtained by performing five independent conjugations and similar results were obtained.

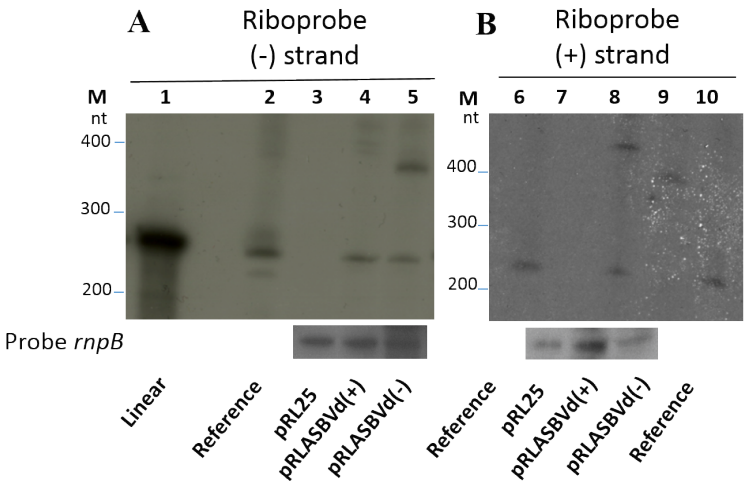

Figure 3: Detection of the (-) and (+) ASBVd forms in *Nostoc* using the Northern blot procedure. RNAs were extracted from a *Nostoc* strain harboring either the empty pRL25 plasmid or pRLASBVd(+) or pRLASBVd(-). They were separated on 6% polyacrylamide-urea gels and analyzed by hybridization using riboprobes corresponding to the ASBVd strands of (-) or (+) polarities. The hybridization using a probe corresponding to the *rnpB* gene served as RNA loading control. Lane 1 corresponds to synthetic linear *in vitro* transcript of ASBVd. Molecular markers (in nucleotide) co-migrated on the 6% polyacrylamide-urea gels are stained by Ethidium bromide before autoradiography. The reference (lanes 2, 6, 10) refers to the total RNA extract from natural ASBVd infected plant. Lane 3 and 7: no product was detected by riboprobe (-) when RNAs extracted from *Nostoc* harboring the pRL25 empty plasmid were loaded. Lane 4: linear transcript form detected by riboprobe (-) when RNAs extracted from Nostoc harboring the pRLASBVd(+) plasmid were loaded. Lane 5: **linear replicative** form detected by riboprobe (-) when RNAs extracted from *Nostoc*/pRLASBVd(-) were loaded. Lane 8: **linear replicative** form detected by riboprobe (+) with RNAs extracted from *Nostoc*/pRLASBVd(+). Lane 9: linear transcript form to be detected by riboprobe (+) with RNAs extracted from Nostoc/pRLASBVd(-) was above the detection limit.

indicates that the cellular machinery of *Nostoc* is able to replicate the negative and negative ASBVd forms; even though the replication and/or stability of the strand of the minus seems more efficient than that of the positive form. Interestingly, "plus" strands were found to be present in significantly higher amounts in infected plant cells than "minus" strands, which suggests that a difference in replication efficiency and/or stability may exist between the two forms of ASBVd having different polarities [18,19].

Impact of the replication of ASBVd on *Nostoc* physiology

The replication of ASBVd in *Nostoc* did not impair the growth of the strain. The generation time of the ASBVd-expressing strains was similar to that of the wild type strain (Table 2). The filaments observed in the wild type strain and the ASBVd-expressing strains were similar (Figure 4). It was therefore concluded that replication of this viroid in *Nostoc* did not have any significant physiological effects on the bacterium, at least under the experimental conditions tested here.

Discussion

Only eukaryotic cells have been found so far to be able to copy viroids via an RNA-RNA replication mechanism. The replication of the ASBVd occurring in the yeast *S. cerevisiae* was an important finding, since it showed that replication of these RNA molecules is not strictly limited to plants and can occur in organisms devoid of chloroplasts [12]. The present study is a further step in this direction, since it shows that a prokaryotic possesses all the machinery required to copy ASBVd; both the (+) and (-) strands were copied in *Nostoc* recombinant cells bearing the dimeric replicative DNA of the ASBVd (Figures 2 and 3).

In a previous study [12], it was reported that ASBVd transcripts with "plus" and "minus" polarities showed differences in electrophoretic mobility under native conditions and in their thermal denaturation profiles. The models obtained by performing RNA-selective 2'-hydroxyl acylation were analyzed using the primer extension (SHAPE) method [20], and the results showed that the two polarities fold into different structures. Structural differences in the regions neighboring the initiation sites of both ASBVd (–) and (+) may influence their respective interactions with the RNA polymerase and/or transcription factors, which might account for the difference observed in the levels of accumulation observed here between the negative and positive forms (Figure 3). In the *Avsunviroidae* family members, the "minus" circular forms have been found to accumulate less extensively than the (+) circular forms at the beginning of the replication process, probably because this form (-) is an intermediate replication form which cannot accumulate in high levels, contrary to the (+) circular form [18]. It was also recently established that in the other viroid family (*Pospiviroidae*), plant cell viroids are subject to decay [19].

***Nostoc* strains**	**Generation time (hours)**
WT	25 ± 3
pRLASBVd(+)	25 ± 5
pRLASBVd(-)	26 ± 4

Table 2: Growth of the *Nostoc* wild type strain in comparison with the ASBVd expressing strains. Generation time corresponds to the population doubling time.

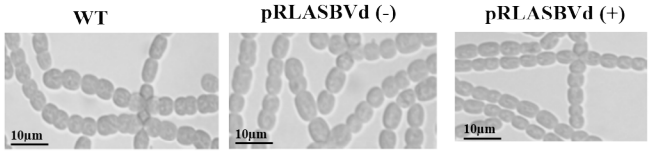

Figure 4: Analysis of the physiological effects of ASBVd replication on *Nostoc*. Light transmission micrographs of the *Nostoc* wild type strain (WT) and either pRLASBVd(-) or pRLASBVd(+) recombinant strains.

The results obtained in this study on the replication of a viroid belonging to the *Avsunviroidae* family in a cyanobacterium were also highly informative from the evolutionary point of view. The nuclear-encoded DNA-dependent RNA polymerase (NEP) was previously found to fulfill this function [6]. It has been also reported that the RNA polymerase of the enterobacterium *Escherichia coli* was able to replicate the Peach latent mosaic viroid, which belongs to the same family as ASBVd [20]. The ability of the *Nostoc* RNA polymerase to replicate ASBVd further supports the idea that bacterial polymerases are able to replicate viroids. The second replication step, which involves the cleavage of the oligomeric RNA intermediates, must be achieved by the HHR, as extensively described in the case of the viroids belonging to the *Avsunviroidae* family in their natural hosts [21-23]. The hammerhead-mediated self-cleavage of the multimeric ASBVd transcripts has been found to be facilitated by the chloroplastic RNA chaperones PARBP33 and PARBP35 [24]. These proteins are RNA-binding proteins involved in the editing and stabilization of transcripts [25,26]. A survey of the *Nostoc* genome has shown the presence of six open reading frames (alr0741, alr2311, alr2087, all2928, alr4683, all277) with approximately 48% amino acid identity with PARBP33. The question as to whether one or several of these proteins interact with the ASBVd transcripts in *Nostoc* and assist the ribozyme in its cleaving function would be well worth investigating.

The self-cleavage of the oligomeric RNAs is followed by the circularization of the resulting monomeric linear viroid RNAs with (+) and (-) polarities, both harboring 5′-hydroxyl and 2′,3′-cyclic phosphodiester termini [27]. This step has been reported to be performed by the chloroplastic isoform of the plant tRNA ligase, which is involved in pre-tRNA splicing and naturally recognizes 5′-hydroxyl and 2′,3′-cyclic phosphodiester termini [28]. A Blast search using several plant tRNA ligases as seeds did not result in the detection of any significant homologues in the *Nostoc* genome or in any of the 127 cyanobacterial genomes sequenced so far [14]. It is therefore possible that RNA ligases showing no homology with plants or other eukaryotic enzymes may exist in cyanobacteria. This would explain the circularization of the RNA occurring during the replication of the ASBVd in *Nostoc*. Discovering these putative enzymes and understanding their natural function is an exciting perspective.

The replication of ASBVd in *Nostoc* had no physiological effects on this bacterium (Table 2 and Figure 4). Replication of this viroid in *S. cerevisiae* has also been reported to be asymptomatic [12]. All in all, these results raise the question as to whether other RNAs may exist in the living kingdoms, where they may replicate via an RNA-RNA process. In this scenario, the viroids could be said to be the submerged part of the iceberg; if they were avirulent against their host plant, as occurs in the case of Eggplant latent viroid [29], their replication would have never been discovered. Do many non-infectious RNAs therefore exist and replicate in living organisms? What might their origin be? Answering these questions constitutes an important challenge for improving our knowledge of the non-coding genome.

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