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A chloroplastic RNA ligase activity analogous to the bacterial and archaeal 2'–5' RNA ligase

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Abbreviations: ASBVd, *Avocado sunblotch viroid*; PLMVd, *Peach latent mosaic viroid*; CChMVd, *Chrysanthemum chlorotic mottle viroid*; ELVd, *Eggplant latent viroid*; PAGE, polyacrylamide gel electrophoresis

Bacteria and archaea contain a 2′–5′ RNA ligase that seals in vitro 2′,3′-cyclic phosphodiester and 5′-hydroxyl RNA termini, generating a 2′,5′-phosphodiester bond. In our search for an RNA ligase able to circularize the monomeric linear replication intermediates of viroids belonging to the family *Avsunviroidae*, which replicate in the chloroplast, we have identified in spinach (*Spinacea oleracea* L.) chloroplasts a new RNA ligase activity whose properties resemble those of the bacterial and archaeal 2′–5′ RNA ligase. The spinach chloroplastic RNA ligase recognizes the 5′-hydroxyl and 2′,3′-cyclic phosphodiester termini of A*vocado sunblotch viroid* and *Eggplant latent viroid* RNAs produced by hammerhead-mediated self-cleavage, yielding circular products linked through an atypical, most likely 2′,5′-phosphodiester, bond. The enzyme neither requires divalent cations as cofactors, nor NTPs as substrate. The reaction apparently reaches equilibrium at a low ratio between the final circular product and the linear initial substrate. Even if its involvement in viroid replication seems unlikely, the identification of a 2′–5′ RNA ligase activity in higher plant chloroplasts, with properties very similar to an analogous enzyme widely distributed in bacterial and archaeal proteomes, is intriguing and suggests an important biological role so far unknown.

Introduction

RNA ligases constitute a family of enzymes that mediate processing and repair of cellular and viral RNAs. Bacteriophage T4 RNA ligase 1 (T4 Rnl1), the founding member of this family,[1,2] catalyzes the repair of host tRNAs that are cleaved as a result of the antiviral host defense, thereby allowing infection to progress.[3] T4 Rnl1 belongs to the superfamily of covalent nucleotidyltransferases, which in addition to some ATP-dependent RNA ligases includes ATP- and NAD^+-dependent DNA ligases and GTP-dependent capping enzymes. They catalyze nucleotidyl transfer to the 5' termini of polynucleotides via covalent enzyme-(lysyl-N)-NMP intermediates.[4] Other well-characterized RNA ligases include: (1) the yeast and plant tRNA ligase involved in the processing of some nuclear-encoded tRNA precursors;[5-7] (2) a second phage T4 RNA ligase (T4 Rnl2) that forms a branch of the family together with the vibriophage KVP40 Rnl2, the RNA-editing ligases of Trypanosoma and Leishmania, and the RNA ligases encoded by certain eukaryotic viruses and archaea;[8,9] (3) the archaeal and metazoan RNA ligase involved in tRNA maturation[10] and (4) the *Escherichia coli* RtcB component of a repair operon.[11,12]

However, the function of a 2'–5' RNA ligase from bacteria and archaea is not yet known. This RNA ligase, initially detected in extracts from *E. coli* and some other bacterial species, is able to join the two half-molecules of some yeast *Saccharomyces cerevisiae* tRNAs via a 2',5'-phosphodiester linkage.[13] This activity requires a 2',3'-cyclic phosphodiester and a 5'-hydroxyl RNA termini but not an NTP cofactor. It specifically ligates tRNA half-molecules containing nucleoside base modifications and promotes either ligation or the reversible cleavage reaction.[14] The crystal structures of two 2'–5' RNA ligases from the archaea *Thermus thermophilus* HB8[15] and *Pyrococcus horikoshii*[16] resemble that of the cyclic phosphodiesterase (CPDase) from *Arabidopsis thaliana*, despite sharing little sequence similarity. CPDase is involved in the splicing of nuclear-encoded tRNAs by catalyzing the hydrolysis of an ADP-ribose 1",2"-cyclic phosphodiester.[17]

Viroids are small, circular, non-protein-coding RNA pathogens of higher plants.[18-21] They replicate through an RNA-based rolling-circle mechanism in which one or two RNA circularization steps, presumably mediated by RNA ligases, occur.[22-25] Most viroids replicate in the nucleus of infected cells and belong to the family *Pospiviroidae*, but four viroids (*Avocado sunblotch viroid*, ASBVd; *Peach latent mosaic viroid*, PLVMd; *Chrysanthemum chlorotic mottle viroid*, CChMVd and *Eggplant latent viroid*, ELVd), replicate in the chloroplast and belong to the family *Avsunviroidae*.[26,27] Replication in this last family includes two circularization steps, one for the plus (+) and one for the

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minus (–) monomeric linear viroid RNAs,[23] which result from self-cleavage by hammerhead ribozymes and contain 5'-hydroxyl and 2',3'-cyclic phosphodiester termini.[28,29] While there is strong evidence supporting that hammerhead ribozymes mediate self-cleavage in vivo during viroid replication,[26] most likely assisted by host proteins,[30] the role of these ribozymes in the reverse ligation reaction in vivo is less likely. A chloroplastic RNA ligase activity may instead catalyze this reaction.[31]

When searching for an RNA ligase in spinach (*Spinacia oleracea* L.) chloroplasts that could mediate RNA circularization during RNA replication in the family *Avsunviroidae*, we detected an activity able to join in vitro the 5'-hydroxyl and 2',3'-cyclic phosphodiester termini of ASBVd and ELVd RNAs. We aimed at studying the properties of this enzyme and found that it generates an atypical, most likely 2',5'-phosphodiester bond, thus resembling the bacterial and archaeal 2'–5' RNA ligases and suggesting the existence of a similar activity in chloroplasts of higher plants. Despite its involvement in viroid replication seems improbable; such conservation between bacterial and archaeal, and the spinach chloroplastic RNA ligases is remarkable and supports an important role for this activity.

Results and Discussion

Identification of an RNA ligase activity from spinach chloroplasts that is able to circularize RNAs with 2',3'-cyclic phosphodiester and 5'-hydroxyl termini. Searching for a chloroplastic RNA ligase activity that could circularize the monomeric linear RNA intermediates produced during the rolling-circle replication of viroid members of the family *Avsunviroidae*, we prepared intact spinach chloroplasts by centrifugation through a discontinuous Percoll gradient, extracted their proteins under mild conditions (1% Nonidet P40 and 100 mM KCl) and fractioned them by liquid chromatography on a heparin column. Monomeric linear ASBVd RNA of (+) polarity resulting from hammerhead-mediated self-cleavage in vitro (and consequently with 5'-hydroxyl and 2',3'-cyclic phosphodiester termini), was used as substrate to detect potential RNA ligase activities in the chromatographic fractions (**Fig. 1A**). The reaction products were separated by denaturing PAGE and revealed by northern blot hybridization with a complementary ^{32}P-labeled RNA probe. Three of the protein fractions that eluted between 460 and 595 mM KCl contained an activity able to convert the linear substrate into a

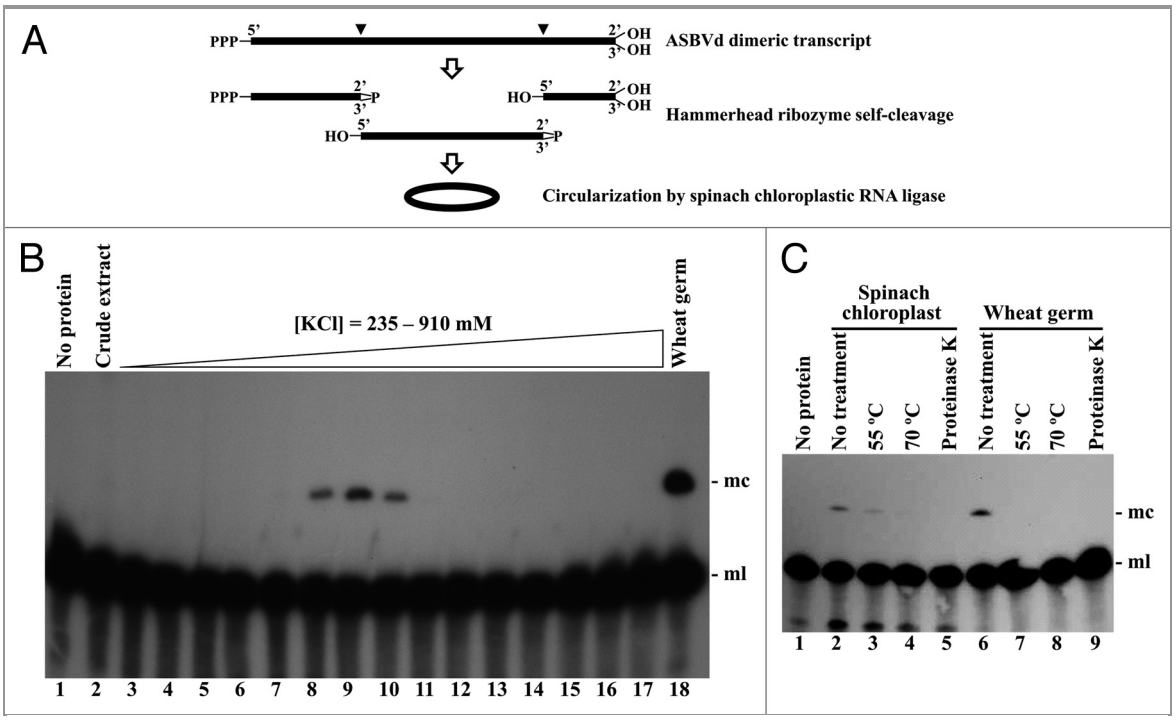

Figure 1. Detection of an RNA ligase activity in spinach chloroplasts. Protein preparations were assayed using the monomeric linear (ml) ASBVd (+) RNA resulting from self-cleavage in vitro. Synthesis of the corresponding monomeric circular (mc) RNA was revealed by denaturing PAGE of the reaction products and northern blot hybridization with a complementary RNA probe. (A) Scheme outlining production of the ml ASBVd (+) RNA from a dimeric transcript by the embedded hammerhead ribozymes (triangles) and subsequent circularization by a chloroplastic spinach activity. (B) RNA ligase activity of the fractions of a spinach chloroplastic extract chromatographed through a heparin column. Lane 1, no protein fraction added (negative control). Lane 2, spinach chloroplastic crude extract (before fractionation). Lanes 3 to 17, column fractions eluting from 235 to 910 mM KCl. Lane 18, ligation with a wheat germ extract containing tRNA ligase (positive control). (C) RNA ligase activity of the spinach chloroplastic fraction with maximum activity (lanes 2 to 5) and of the wheat germ control extract (lanes 6 to 9), without any treatment (lanes 2 and 6), after 30 min incubation at 55°C (lanes 3 and 7), after 30 min incubation at 70°C (lanes 4 and 8), or after 15 min digestion with proteinase K (lanes 5 and 9). Lane 1, control (no protein added). The positions of mc- and ml-ASBVd RNA forms are indicated on the right of both panels.

circular RNA product, which was easily detectable by its low electrophoretic mobility (**Fig. 1B**, lanes 8 to 10). A control reaction with a partially purified preparation of wheat (*Triticum aestivum* L.) germ tRNA ligase[32] produced a species showing the same low mobility (**Fig. 1B**, lane 18).

It has been shown that an isoform of the plant tRNA ligase with an N-terminal transit peptide, possibly arising from alternative translation initiation, localizes to both the chloroplast and the nucleus.[33] Moreover, this enzyme requires 2',3'-cyclic phosphodiester and 5'-hydroxyl RNA termini.[7] Thus, we next asked whether the RNA circularizing activity detected in spinach chloroplasts was attributable to a chloroplastic isoform of the tRNA ligase. However, further characterization of the activity (see below) discarded this possibility.

Properties of the RNA ligase activity from spinach chloroplasts. First, we confirmed that the ligase activity resided in a protein. Aliquots of the fraction with the peak ligase activity were subjected to digestion with proteinase K (37°C for 15 min) or to thermal inactivation treatment (55°C or 70°C for 30 min). As a control, the wheat germ extract containing tRNA ligase was subjected to the same treatments. Next, the ligase activity of each sample was assayed with the monomeric linear ASBVd (+) RNA as substrate. Both the spinach chloroplast and wheat germ RNA ligase activities were affected by the thermal treatments (**Fig. 1C**, compare lane 2 with 3 and 4, and lane 6 with 7 and 8), although the first was partially resistant and retained some activity after treatment at 55°C (**Fig. 1C**, compare lane 2 and 3). However, both activities were completely abolished by proteinase K digestion (**Fig. 1C**, compare lane 2 with 5, and lane 6 with 9). These results support the hypothesis that a protein is responsible for the RNA ligase activity in spinach chloroplasts.

We then proceeded to determine the reaction requirements of the spinach chloroplastic RNA ligase. An aliquot of the fraction with the maximum activity (**Fig. 1B**, lane 9) was dialyzed against a buffer containing 100 mM KCl and the RNA ligase activity was assayed in a reaction buffer with increasing concentrations of KCl. The fraction showed activity between 30 and 250 mM KCl (**Fig. 2A**, lanes 2 to 7) with a peak around 100–150 mM (**Fig. 2A**, lanes 4 and 5). The same fraction was also examined in different buffers from pH 4 to 11. Control ligations in the same conditions were performed with a preparation of recombinant *E. coli* 2'–5' RNA ligase. Both enzymes showed a very similar behavior: they remained active through a wide pH range, but with a maximum between pH 7 and 9 (spinach) and between pH 7 and 10 (*E. coli*) (**Fig. 2B**). The activity was also assayed in the presence of 25 mM EDTA to sequester any remaining divalent cations in the enzyme preparation or with increasing concentrations of Mg^{2+}. Controls with a purified preparation of *A. thaliana* tRNA ligase[25] in the presence of 25 mM EDTA or 4 mM Mg^{2+} were also included in this experiment. In the absence of free divalent cations, the chloroplastic RNA ligase remained active (**Fig. 2C**, lane 2) and increasing amounts of Mg^{2+} did not substantially change the activity (**Fig. 2C**, lanes 3 to 7). In contrast, EDTA treatment abolished activity in *A. thaliana* tRNA ligase (**Fig. 2C**, compare lanes 8 and 9). Resistance to EDTA is quite remarkable because, while most RNA ligases demand a divalent Mg^{2+} cofactor for activity as illustrated by the yeast tRNA ligase,[34] few RNA ligases have been described to operate in the absence of divalent cation cofactors.[35,36] Our own reexamination of some of the properties of the *E. coli* 2'–5' RNA

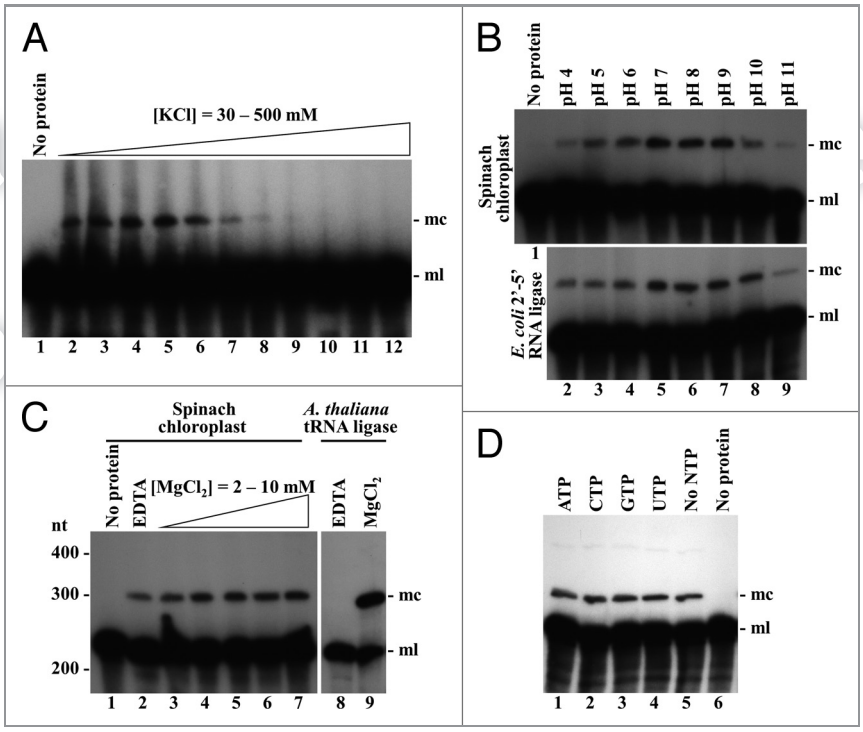

Figure 2. Properties of the spinach chloroplastic RNA ligase. The ligation products of the monomeric linear (ml) ASBVd (+) RNA separated by denaturing PAGE were revealed by northern blot hybridization. (A) RNA ligase activity in the presence of increasing concentrations of KCl. Lane 1, no protein (negative control). Lanes 2 to 12, ligation with a spinach chloroplastic protein fraction in the presence of 30, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mM KCl. (B) RNA ligase activity in buffers with different pH. Lane 1, no protein. Lanes 2 to 7, ligation with a spinach chloroplastic fraction and *E. coli* 2'–5' RNA ligase in reaction buffers containing 100 mM sodium acetate pH 4.0 (lane 2), MES-KOH pH 5.0 (lane 3), MES-KOH pH 6.0 (lane 4), TRIS-HCl pH 7.0 (lane 5), TRIS-HCl pH 8.0 (lane 6), TRIS-HCl pH 9.0 (lane 7), Gly-NaOH pH 10 (lane 8) or sodium bicarbonate pH 11 (lane 9). (C) RNA ligase activity of a spinach chloroplastic fraction (lanes 2 to 7) and *A. thaliana* tRNA ligase (lanes 8 and 9) in the standard ligation buffer (see Materials and Methods) with 25 mM EDTA and no $MgCl_2$ (lanes 2 and 8) or with $MgCl_2$ at 2 (lane 3), 3 (lane 4), 4 (lanes 5 and 9), 5 (lane 6) or 10 mM (lane 7). Lane 1, control (no protein added). (D) RNA ligase activity of a spinach chloroplastic fraction in the presence of 1 mM ATP (lane 1), 1 mM CTP (lane 2), 1 mM GTP (lane 3), 1 mM UTP (lane 4) and no NTP (lane 5). Lane 6, control (no protein added). Positions of mc- and ml-ASBVd forms are indicated on the right of each panel. Positions of RNA standards are indicated to the left of (C).

ligase showed that this enzyme neither needs Mg^{2+} as cofactor for RNA ligation (data not shown).

Finally, we tested the requirement for NTPs substrates in the ligase reaction. The spinach chloroplastic fraction with the maximum RNA ligase activity was assayed in the presence of 1 mM ATP, CTP, GTP or UTP, as well as in the absence of all NTPs. Interestingly, the ligation activity remained unchanged in all five conditions (**Fig. 2D**), strongly suggesting that the enzyme does not need any NTP substrate. To eliminate the possible requirement for more than one NTP species concurrently, we also assayed the activity in the presence of all four NTPs (1 mM each) and found that the amount of ligated product was the same as in the control reaction without NTPs (data not shown). In RNA ligases, this property has only been described for the bacterial and archaeal 2'–5' RNA ligase[13] and for the RNA ligase from *Haloferax volcanii*.[36]

Time course of the RNA ligase reaction. At this point, we had not eliminated the possibility that the spinach chloroplastic RNA ligase was mainly purified as a pre-activated form, for example as a protein-adenylate adduct able to catalyze a single cycle of RNA ligation. For example, DNA ligase 1 from *A. thaliana* is purified, at least in part, in the form of a pre-activated adenylated adduct.[37] To exclude this possibility, we performed a time course assay of the ligation reaction in the presence of 1 mM ATP and without any NTP. The partially purified wheat germ tRNA ligase was assayed in parallel as a control. The time course of ASBVd RNA circularization by spinach chloroplastic RNA ligase was the same in the absence or in the presence of 1 mM ATP (**Fig. 3A**). In contrast, as expected, the wheat germ tRNA ligase only circularized significant amounts of ASBVd RNA in the presence of ATP (**Fig. 3B**). In the presence of 1 mM ATP, the wheat germ tRNA ligase generated circular product at an approximately constant rate throughout the assay, whereas the rate of product formation by the spinach chloroplastic RNA ligase decreased, and the reaction reached equilibrium at a ratio of circular product to initial linear substrate of about 0.02. This behavior very much resembles that of *E. coli* 2'–5' RNA ligase, whose catalyzed reaction quickly reaches an equilibrium with a minor fraction of ligated product.[14]

It could be argued that instead of catalyzing a reaction that reached equilibrium, the spinach chloroplastic RNA ligase could be inactivated during the reaction. To eliminate this possibility, we performed a series of 60 min ligation assays in which either the substrate or the enzyme preparation was replenished after 30 min. While adding more substrate resulted in doubling the amount of ligated product (**Fig. 3C**, compare lanes 2 and 3), replenishing the enzyme preparation had no effect on the final product yield (**Fig. 3C**, compare lanes 2 and 4). These results clearly demonstrate that the spinach chloroplastic RNA ligase was not inactivated during the reaction and, consequently, that overall product formation probably stops as a result of the reaction reaching equilibrium.

Substrate specificity of the spinach chloroplastic RNA ligase. The protein fraction from spinach chloroplasts showing RNA ligase activity on the monomeric linear ASBVd (+) RNA was also assayed for activity on the other monomeric linear (+) RNAs of

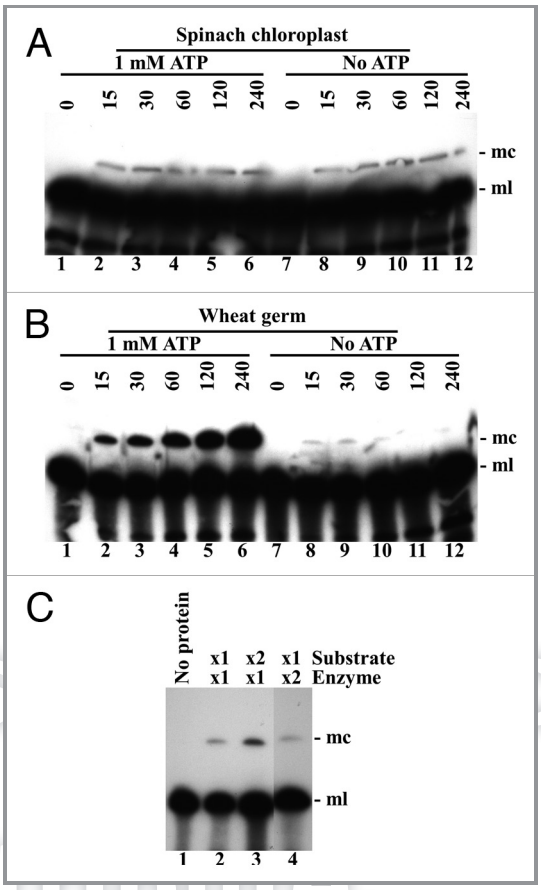

Figure 3. Comparative analysis of the spinach chloroplastic and wheat germ RNA ligases. Products resulting from ligation of the monomeric linear (ml) ASBVd (+) RNA were separated by denaturing PAGE and detected by northern blot hybridization with a complementary RNA probe. Time course assays of the spinach chloroplastic RNA ligase (A) and wheat germ tRNA ligase (B) in the presence of 1 mM ATP (lanes 1 to 6) or without ATP (lanes 7 to 12). Aliquots were taken at 0, 15, 30, 60, 120 and 240 min (lanes 1 to 6 and 7 to 12). (C) Ligation reactions (60 min) with a spinach chloroplastic protein fraction under regular conditions (see Materials and Methods) (lane 2) and after doubling the RNA substrate (lane 3) or the protein preparation (lane 4) at 30 min. Lane 1, control (no protein added). Positions of mc- and ml-ASBVd forms are indicated to the right of each panel.

viroid members of the family *Avsunviroidae*, which contain the same 5'-hydroxyl and 2',3'-cyclic phosphodiester terminal groups. The different substrate RNAs were obtained by in vitro self-cleavage of dimeric transcripts, purified by denaturing electrophoresis, normalized to the level of ASBVd RNA, and subjected to ligation with the spinach chloroplastic fraction. The reaction products were separated by denaturing electrophoresis and analyzed by northern blot hybridization with the corresponding complementary (–) radioactive probes. PLMVd and CChMVd RNAs were not ligated by the spinach chloroplastic activity (**Fig. 4A**, lanes 4 and 6), whereas ELVd RNA was ligated even more efficiently than ASBVd RNA (**Fig. 4A**, compare lanes 2 and 8). These results indicate that the spinach chloroplastic RNA ligase activity shows a strong preference for specific RNA substrates. Interestingly, in the two viroid RNAs (PLMVd and

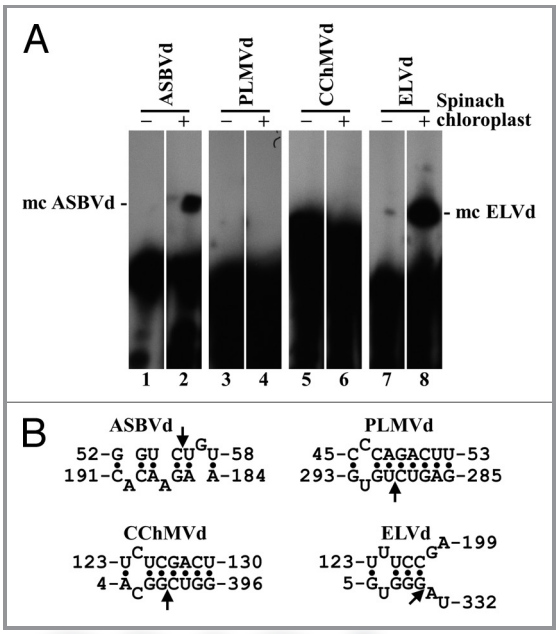

Figure 4. Ligation of self-cleavage products of RNAs from the family *Avsunviroidae* by a spinach chloroplastic protein fraction. (A) Monomeric (+) linear ASBVd (lanes 1 and 2), PLMVd (lanes 3 and 4), CChMVd (lanes 5 and 6) and ELVd (lanes 7 and 8) RNAs were subjected to ligation with no protein added (–, lanes 1, 3, 5 and 7) or with the spinach chloroplastic fraction (+, lanes 2, 4, 6 and 8). Reaction products were separated by denaturing PAGE and revealed by northern blot hybridization. Positions of monomeric circular (mc) ASBVd and ELVd are indicated on the left and right of the panel, respectively. (B) Predicted secondary structures around the termini of the monomeric (+) linear ASBVd, PLMVd, CChMVd and ELVd RNAs. Arrows indicate positions wherein substrates are opened.

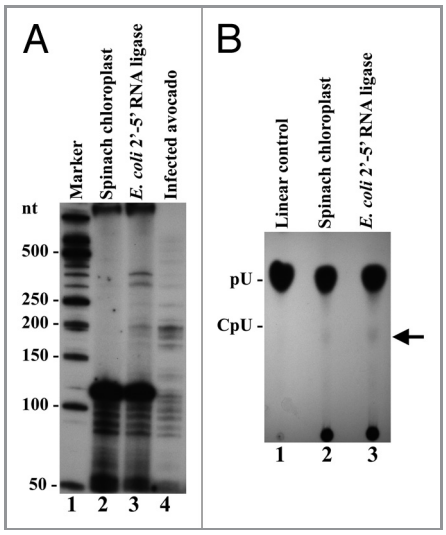

Figure 5. Linkage formed by the spinach chloroplastic RNA ligase. (A) Primer extension analysis of the monomeric circular ASBVd products generated by the spinach chloroplastic RNA ligase (lane 2) and *E. coli* 2′–5′ RNA ligase (lane 3), and of the monomeric ASBVd circular forms purified from infected avocado (lane 4). Products resulting from extension of a ^{32}P-labeled oligodeoxyribonucleotide were separated by denaturing PAGE and revealed by autoradiography. Lane 1, labeled polydeoxyribonucleotide markers with sizes (in nt) on the left. (B) Thin layer chromatography of S1 digestion products of [α-^{32}P]UTP-labeled monomeric circular ASBVd (+) RNA produced by the spinach chloroplastic (lane 2) and *E. coli* RNA ligases (lane 3). Lane 1, control digestion of a linear ASBVd (+) RNA internally labeled during transcription with [α-^{32}P]UTP. Positions of the uridine 5′-monophosphorylated (pU) and of the cytidyl (2′–5′)uridine (CpU) markers are indicated on the left of the panel. The spots likely arising from the pCpU dinucleotide with a 2′,5′-phosphodiester linkage (lanes 2 and 3) are indicated by an arrow.

CChMVd) that were not circularized, the ligation site is predicted to be embedded in a stable double-stranded helix in the minimum free energy secondary structure (**Fig. 4B**).

Linkage produced by the spinach RNA ligase. All previous results suggested that the RNA ligase identified in spinach chloroplasts is similar to the bacterial and archaeal 2'–5' RNA ligase. We next asked whether the linkage produced in the ligation reaction was also similar. First, we performed denaturing electrophoresis and eluted the circular ASBVd RNA product resulting from ligation. Then, we examined the presence of an anomalous nucleotide linkage by primer extension with reverse transcriptase. As controls, we used two monomeric circular ASBVd RNAs forms: one purified from infected avocado (*Persea americana* Mill.) tissue and the other resulting from circularization by *E. coli* 2'–5' RNA ligase. These two controls were prepared as described above and the amounts normalized. The cDNA products obtained by extension of a 5'-[^{32}P]-labeled oligodeoxynucleotide were separated by denaturing PAGE and revealed by autoradiography. When using the RNA circularized in vitro by the spinach chloroplastic activity and the *E. coli* 2'–5' RNA ligase as template, a main product was detected with a migration consistent with the 116-nt long cDNA expected to result from a strong stop of the reverse transcriptase when reaching the viroid C55-A56 phosphodiester bond generated by circularization (**Fig. 5A**, lanes 2 and 3). In contrast, when the ASBVd circular RNA purified from infected tissue was used as a template, the reverse transcriptase went through this bond without stopping (**Fig. 5A**, lane 4). This result indicates that the spinach chloroplastic RNA ligase circularizes ASBVd RNA through an atypical non-3',5'-phosphodiester linkage. Since there is no NTP substrate involved in the reaction, we hypothesize that a 2',5'-phosphodiester bond resulting from a transesterification is the most plausible alternative for this linkage. In line with this interpretation, the intensity of the stop produced during reverse transcription of both circular products generated by the spinach chloroplastic activity and *E. coli* 2'–5' RNA ligase were similar (**Fig. 5A**, compare lanes 2 and 3).

Nonetheless, to further characterize the linkage, ligations with the spinach chloroplastic and the *E. coli* 2'–5' RNA ligases were performed using as substrate monomeric linear ASBVd (+) RNA, internally labeled with [α-^{32}P]UTP, generated by in vitro transcription and self-cleavage. The resulting circular products were purified and treated with S1 nuclease, and the digestion products were separated by thin-layer chromatography. Standards for this experiment were the mononucleotide uridine 5'-monophosphate (pU) and the dinucleotide cytidyl (2'–5')uridine (CpU). A normalized amount of the S1-digested ^{32}P-labeled monomeric linear ASBVd RNA was also included as a control.

Nuclease S1 catalyzes cleavage of RNA yielding 5'-phosphorylated nucleosides and, as a consequence of the internal labeling of substrates, the main spot detected in all cases displayed mobility consistent with that of pU (**Fig. 5B**). But, analysis of the S1-digestion products from the circular ASBVd RNAs generated by the spinach chloroplastic and *E. coli* 2'–5' RNA ligases revealed a faint spot consistent with a pCpU dinucleotide (**Fig. 5B**, compare lane 1 with 2 and 3), further supporting the idea that the spinach chloroplastic RNA ligase activity generates a 2',5'-phosphodiester bond. A similar experiment using the monomeric linear ELVd (+) RNA from self-cleavage as substrate for the spinach chloroplastic and *E. coli* 2'–5' RNA ligases also led to a similar faint spot compatible with a dinucleotide (pApG in the case of ELVd) linked through a 2',5'-phosphodiester bond (data not shown).

The question of RNA circularization in the family *Avsunviroidae*. There are two main reasons to believe that it is unlikely that the RNA ligase activity detected in spinach chloroplasts mediates the ligation step of replication in the family *Avsunviroidae*. First, primer extension analyses reported previously[38,23] and in the present work (**Fig. 5A**), using the natural circular forms of ASBVd as a template, are consistent with ligation of this RNA generating a typical 3',5'-phosphodiester bond. RT-PCR analyses of the natural circular forms of the other members of the family *Avsunviroidae* also support this view (data not shown), with the only exception of PLMVd, the circular form of which accumulating in infected peach [*Prunus persica* (L.) Batsch] has been reported to be closed by a 2'–5' linkage.[39] However, this finding may simply reflect that the monomeric linear RNA resulting from self-cleavage is able to self-ligate in the presence of a divalent cation through a 2',5'-phosphodiester bond.[40] Still, most of the circular PLMVd RNAs that accumulate in vivo are possibly locked by a typical 3',5'-phosphodiester linkage (Delgado and Flores, unpublished data). And second, the spinach chloroplastic RNA ligase does not join the monomeric linear intermediates resulting from self-cleavage of all chloroplastic viroids (**Fig. 4**), and in those cases in which it does (ASBVd and ELVd), the extent of ligation is low under the in vitro conditions assayed here (**Figs. 3C and 4A**).

On the other hand, it is also unlikely that hammerhead ribozymes catalyze both self-cleavage and circularization of the resulting RNAs in the family *Avsunviroidae*. The latter reaction is very inefficient, at least in vitro, and an ELVd RNA with a deletion that does not affect the hammerhead structure (and consequently self-cleaves as efficiently as the wild-type ELVd RNA) is not ligated when expressed in *Chlamydomonas reinhardtii* chloroplasts, thus suggesting that requirements for self-cleavage and ligation in vivo are different for this viroid.[31] A chloroplastic isoform of the tRNA ligase[33] that efficiently circularizes the self-cleaved RNAs of all members of the family *Avsunviroidae* (Nohales, Molina-Serrano, Flores and Daròs, in preparation) appears as a better candidate to mediate the ligation step of replication.

Role of the spinach chloroplastic RNA ligase. The activity of the spinach chloroplastic RNA ligase very closely resembles that of the bacterial and archaeal 2'–5' RNA ligases. However, no genes homologous to those coding for the bacterial and archaeal enzymes have been identified in plants or other eukaryotic genomes. Even if the 2'–5' RNA ligase is not required for *E. coli* survival under normal laboratory growth conditions,[14] its wide distribution in many bacterial and archaeal taxonomic groups suggests an important role. This hypothesis is supported by our finding that an analogous activity is also present in chloroplasts of higher plants. Many bacteria and fungi encode endoribonuclease toxins or ribotoxins—a good example of which is the anticodon nuclease PrrC in *E. coli*[41]—that target RNAs yielding products with 2',3'-cyclic phosphodiester and 5'-hydroxyl termini, like those joined by the 2'–5' RNA ligase. Therefore, 2'–5' RNA ligases may participate in a host defense mechanism against ribotoxins by repairing crucial cellular RNA species that, when sealed through a 2',5'-phosphodiester bond, would become resistant to further ribotoxin cleavage.

Materials and Methods

Plasmids. Plasmids pBdASBVd, pBdPLMVd, pBCChMVd and pBdELVd contain dimeric head-to-tail cDNA inserts of ASBVd (GenBank accession number X52041, from position 154 to 153), PLMVd (AJ005303 with the point mutation C260 U, from 199 to 198), CChMVd (AJ878085, from 295 to 294) and ELVd (AJ536613, from 210 to 209), respectively, cloned in pBluescript II KS(+) (X52327). Plasmids pE25Rl and pERl contain the cDNAs coding for the 2'–5' RNA ligase from *E. coli* and the tRNA ligase from *A. thaliana*, respectively, inserted in pET-23a(+) (Novagen). Expression in *E. coli* strain Rosetta 2(DE3)pLysS (Novagen) transformed with these plasmids produced proteins corresponding to GenBank accession number P37025 plus an N-terminal Met and a C-terminal Leu-Glu-$(His)_6$ (pE25Rl) and GenBank accession number NP_172269.2 plus a C-terminal $(His)_6$ (pERl).

Extraction and fractionation of spinach chloroplastic proteins. Chloroplasts were prepared from spinach (*Spinacia oleracea* L. cv Matador).[42] Leaves (25 g) were ground with an Omni-mixer (Omni International) in 250 ml of chloroplast buffer (50 mM Tricine-NaOH pH 8.0, 0.6% polyvinylpyrrolidone, 5 mM EDTA, 10 mM 2-mercaptoethanol and 0.3 M mannitol) supplemented with 0.1% bovine serum albumin. The homogenate was filtered (20-µm mesh nylon) and centrifuged at 1000 × g for 5 min. The sediments, resuspended in chloroplast buffer, were loaded onto a discontinuous 40–80% Percoll (GE Healthcare) gradient and centrifuged at 10,000 × g for 20 min. Intact chloroplasts were recovered from the interphase between the two Percoll layers, washed with chloroplast buffer and sedimented by centrifugation at 1000 × g for 5 min.

Chloroplasts were then resuspended in 4 ml of extraction buffer (100 mM TRIS-HCl pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1% Nonidet P40 and 10 mM dithiothreitol-DTT-) supplemented with a mix of protease inhibitors (Complete, Roche), and stirred for 10 min to break them and solubilize proteins. The extract was centrifuged twice: first at 10,000 × g for 15 min, and the recovered supernatant spun again at 100,000 × g for 2 h. The final supernatant from the high speed centrifugation

was loaded onto a 1-ml heparin column (Hitrap Heparin HP, GE Healthcare) equilibrated with extraction buffer (except for protease inhibitors) and operated at a flow rate of 0.5 ml/min. After washing with 10 ml of extraction buffer, proteins were eluted with 10 ml of a 0.1 to 1 M KCl linear gradient in the extraction buffer. Twenty fractions of 0.5 ml were collected.

In vitro transcription and purification of viroid RNA. Plasmids pBdASBVd, pBdPLMVd, pBCChMVd and pBdELVd were digested with *Eco*RI or *Xba*I (Fermentas) and subjected to in vitro transcription with bacteriophage T3 or T7 RNA polymerases (Roche) depending on the insert orientation and the viroid strand to be transcribed. Reaction products were separated by denaturing PAGE on 5% polyacrylamide gels containing 8 M urea in TBE buffer (89 mM TRIS, 89 mM boric acid and 2 mM EDTA). Gels were stained with ethidium bromide and the products corresponding to the full-length monomeric linear viroid RNAs (resulting from hammerhead-mediated self-cleavage) were eluted by diffusion. For the synthesis of viroid-specific radioactive probes, 40 µCi of [α-^{32}P]UTP (400 Ci/mmol) were included in the 20-µl transcription reactions. The resulting probes were purified by gel-filtration chromatography using Sephadex G-50 spin columns (Roche).

RNA ligation assays. In general, ligation reactions were performed in 20-µl volume of ligation buffer (50 mM TRIS-HCl pH 7.5, 5 mM DTT, 4 mM $MgCl_2$ and 1 mM ATP), including 100 ng of RNA substrate and 4 µl of the protein preparation to be assayed. In control experiments with the 2'-5' RNA ligase from *E. coli*, reactions were supplemented with 5 mM spermidine and 4 µg of *E. coli* 16S and 23S RNAs (Roche). In other control experiments with the RNA ligase from wheat germ a partially purified preparation was used.[32] Reactions were incubated at 30°C for 30 min and stopped by adding 1 volume of loading solution (98% formamide, 10 mM TRIS-HCl pH 8.0, 1 mM EDTA, containing 4 µg of heterologous *E. coli* 16S and 23S RNAs) and heating at 95°C for 90 sec. Reaction products were separated by denaturing PAGE as described above, electroblotted to positively-charged nylon membranes, cross-linked with UV light and analyzed by hybridization with the appropriate complementary ^{32}P-labeled RNA probes as described.[31]

Primer extension analysis. Oligodeoxyribonucleotide I (5'-TGAAGAGACGAAGTGATCAAG-3'), complementary to positions 151 to 171 of ASBVd (Genebank accession number X52041), was purified by denaturing PAGE and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas). RNA preparations were mixed with the ^{32}P-labeled oligodeoxyribonucleotide I in 6 µl of H_2O, heated at 98°C for 90 sec and snap-cooled on ice. Volume was adjusted to 10 µl with buffer (50 mM TRIS-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 2.5 mM DTT) and 0.5 mM dNTPs, 20 U RNase inhibitor (Fermentas) and 100 U Superscript III reverse transcriptase (Invitrogen). Reactions were incubated at 55°C for 45 min, 60°C for 10 min and 65°C for 5 min, and then stopped by adding a volume of formamide loading solution and heating at 95°C for 90 sec. Reaction products were separated by denaturing PAGE in a 5% gel (8 M urea in TBE buffer) that was dried under vacuum and imaged by autoradiography.

Thin layer chromatography analysis of 2',5'-dinucleotide. RNAs were internally labeled during transcription with [α-^{32}P] UTP, purified by denaturing PAGE followed by elution from the gel, and digested with 100 U of S1 nuclease (Fermentas) for 30 min at 37°C. The nucleotide mixes resulting from digestion were applied to a cellulose chromatography plate (Merck) and separated overnight using a mix of 100 ml 0.1 M sodium phosphate pH 6.8, 60 g ammonium sulfate and 2 ml n-propanol as eluent. The plate was first irradiated with a 254-nm UV lamp to visualize the positions of nucleotide and then revealed by autoradiography.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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