

Processing of RNAs of the Family *Avsunviroidae* in *Chlamydomonas reinhardtii* Chloroplasts[∇]

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The family *Avsunviroidae* comprises four viroid species with the ability to form hammerhead ribozymes that mediate self-cleavage of the multimeric plus and minus strands resulting from replication in the chloroplast through a symmetric rolling-circle mechanism. Research on these RNAs is restricted by their host range, which is limited to the plants wherein they were initially identified and some closely related species. Here we report cleavage and ligation in transplastomic *Chlamydomonas reinhardtii* expressing plus- and minus-strand dimeric transcripts of representative members of the family *Avsunviroidae*. Despite the absence of viroid RNA-RNA transcription, the *C. reinhardtii*-based system can be used to address intriguing questions about viroid RNA processing and, in particular, about the cellular factors involved in cleavage and ligation.

Species of the *Avsunviroidae* family, *Avocado sunblotch viroid* (ASBVd) (19), *Peach latent mosaic viroid* (PLMVd) (18), *Chrysanthemum chlorotic mottle viroid* (CChMVd) (23), and *Eggplant latent viroid* (ELVd) (12), comprise a unique class of plant pathogens because ASBVd and PLMVd, and most likely CChMVd and ELVd, replicate in the chloroplast (13). Like nuclear viroids (family *Pospiviroidae*), they are formed of small, circular RNAs (247 to 401 nucleotides) not encoding any protein but able to autonomously infect their hosts (11, 15, 28). Although ASBVd, PLMVd, CChMVd, and ELVd do not contain the central conserved region characteristic of the family *Pospiviroidae*, they can form hammerhead ribozymes that mediate self-cleavage of multimeric plus and minus strands resulting from symmetric rolling-circle replication (3, 8). The four members of the family *Avsunviroidae* have a restricted host range: they infect only the plants wherein they were discovered and related species (12, 13), a feature limiting the study of these intriguing RNAs. Although no viroid infects *Arabidopsis thaliana*, nuclear transformation of this model plant with dimeric cDNA constructs of members of the family *Pospiviroidae* has revealed its potential in investigation of viroid-host interactions (7). Here, we report that transplastomic *Chlamydomonas reinhardtii* expressing dimeric viroid transcripts can be used to explore viroid-host interactions in the family *Avsunviroidae*.

Transformation of *C. reinhardtii* chloroplasts with dimeric viroid cDNAs. Transplastomic *C. reinhardtii* cell lines expressing different plus-strand viroid transcripts were obtained. These transcripts, which resemble replicative intermediates and are highly infectious when inoculated mechanically into their hosts (5), were from the genera *Avsunviroid* (ASBVd), *Pelamoviroid* (CChMVd), and *Elaviroid* (ELVd) of the family

Avsunviroidae and from *Citrus exocortis viroid* (CEVd) (15) of the family *Pospiviroidae*. A cell line expressing a dimeric minus-strand ASBVd transcript was also included.

Dimeric viroid cDNAs were inserted between the XbaI and EcoRI sites of the chloroplast transformation vector pCrc+157 (27), replacing the β -glucuronidase open reading frame. The recombinant plasmids, in which the insertions were preceded by the promoter, the 5' untranslated region, and the beginning of the open reading frame of the *rbcL* gene (from -70 to +157) and followed by the 3' untranslated region of the *psaB* gene from *C. reinhardtii* (Fig. 1), were particle bombarded (1) into *atpB*-deficient *C. reinhardtii* strain CC-373 (*ac-uc-2-21*). Photoautotrophic transformants were selected, and for each construct, ten lines were grown in high-salt minimal medium at 32°C, with 12/12 h of day/night and bubbling with 2% CO₂-enriched air (27). Slot and Southern blot hybridizations of DNA preparations with ³²P-labeled viroid-specific riboprobes detected the expected sequences and facilitated choosing one highly homoplasmic line per construct.

ASBVd, CChMVd, and ELVd dimeric RNAs, but not CEVd dimeric RNA, are processed in *C. reinhardtii* chloroplasts. To confirm the expression of viroid transcripts, the selected transplastomic lines were analyzed by Northern blot hybridization. Cells from saturated cultures (50 ml) were sedimented, resuspended in buffer (0.1 M Tris-HCl [pH 9.0], 0.1 M NaCl, 10 mM EDTA, and 0.1 M 2-mercaptoethanol), and extracted with phenol-chloroform and sodium dodecyl sulfate. The aqueous phase was adjusted to 35% ethanol, and the RNAs were fractionated on 0.2 g of nonionic cellulose (CF11; Whatman) (8). The eluted RNAs were separated by polyacrylamide gel electrophoresis (PAGE) in 5% gels in Tris-borate-EDTA buffer containing 8 M urea, blotted onto nylon membranes, and hybridized at 70°C in the presence of 50% formamide with strand-specific ³²P-labeled riboprobes (8).

Transcripts from members of the family *Avsunviroidae* were expressed in transplastomic *C. reinhardtii* and correctly processed to monomeric linear (*ml*) and circular (*mc*) RNAs, as revealed by comigration with the forms from ASBVd-infected

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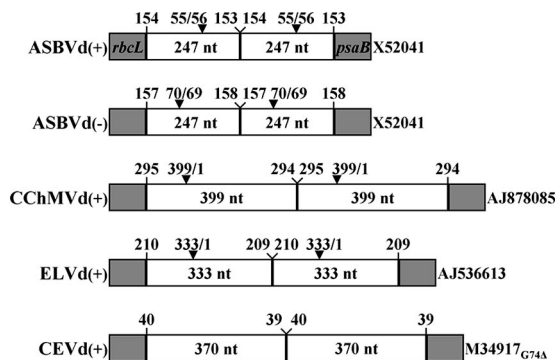


FIG. 1. Bar diagram representing the viroid transcripts expressed in the chloroplasts of transgenic *C. reinhardtii*. The viroid RNAs and their polarities [(+), plus-strand; (-), minus-strand] are indicated on the left, and the database accession numbers of the specific sequence variants are indicated on the right (the subscript in the CEVd sequence refers to a deletion with respect to database entry). Gray bars represent the *C. reinhardtii* *rbcl* 5' and *psaB* 3' terminal regions (not to scale), and open bars represent viroid monomeric units (to scale). Arrowheads indicate the viroid ribozyme self-cleavage sites. The positions at which the viroid sequences start and end in the transcript, the size of each monomeric-viroid unit, and hammerhead self-cleavage sites are also indicated. nt, nucleotides.

avocado (*Persea americana* Mill.), CChMVd-infected chrysanthemum (*Dendranthema grandiflora* Tzvelez), and ELVd-infected eggplant (*Solanum melongena* L.) (Fig. 2A to D). The plus-strand dimeric CEVd transcript was expressed but remained unprocessed, according to the lack of signals comigrating with the *mc* and *ml* RNAs from CEVd-infected gynura (*Gynura aurantiaca* DC.) (Fig. 2E).

To corroborate these results, the RNA preparations from *C. reinhardtii* cell lines expressing dimeric plus-strand RNAs from ASBVd, CChMVd, and ELVd were separated by double PAGE (23) in nondenaturing 5% gels in Tris-acetate-EDTA, with the segment of this gel containing the *mc* and *ml* forms being applied on top of a second denaturing 5% gel (in 0.25× Tris-borate-EDTA and 8 M urea). Under these conditions, the *mc* viroid RNAs comigrating with their *ml* counterparts in the first gel are retarded in the second. Northern blot hybridization of the RNAs from the second gel showed that bands attributed to *mc* and *ml* viroid RNAs (Fig. 2) comigrated again with the *mc* and *ml* forms from their infected plants (Fig. 3). However, because of the higher resolution of double PAGE, the intense bands that migrated below the dimeric plus-strand CChMVd transcript in single-denaturing PAGE (Fig. 2C, lane 3) should be generated by partial self-cleavage products of the dimeric plus-strand CChMVd transcript, because the band corresponding to the *mc* CChMVd form in double PAGE was visible only after prolonged exposure (Fig. 3B, lane 3). Therefore, transplastomic *C. reinhardtii* expressing dimeric viroid RNAs replicating in the chloroplasts of their host plants were cleaved and ligated, but the control viroid replicating in the nuclei was not.

Processing efficiency in *C. reinhardtii* chloroplasts differs among members of the family *Avsunviroidae*. Examination of the hybridizations revealed that processing efficiency was dependent on the particular viroid. Transplastomic *C. reinhardtii* expressing the dimeric plus-strand ASBVd transcript showed a prominent species with the slow mobility expected for the

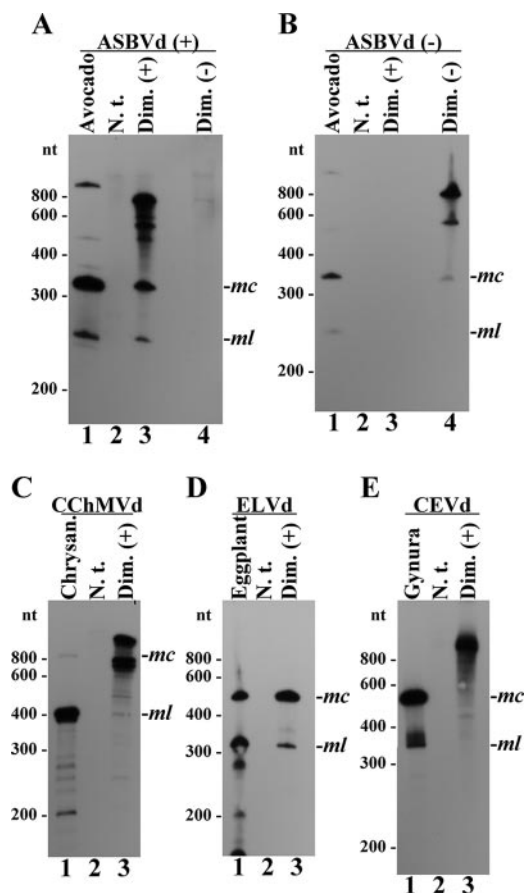


FIG. 2. Northern blot hybridization analysis of transplastomic *C. reinhardtii* expressing dimeric viroid RNAs. Viroid RNA preparations were fractionated by denaturing PAGE and hybridized with riboprobes to detect ASBVd (+) (A), ASBVd (-) (B), CChMVd (+) (C), ELVd (+) (D), and CEVd (+) (E). Lanes 1, controls of ASBVd-infected avocado (A and B), CChMVd-infected chrysanthemum (C), ELVd-infected eggplant (D), and CEVd-infected gynura (E). Lanes 2, controls of nontransformed *C. reinhardtii* (A to E). Lanes 3, transplastomic *C. reinhardtii* expressing dimeric ASBVd (+) (A and B), CChMVd (+) (C), ELVd (+) (D), and CEVd (+) (E). Lanes 4, transplastomic *C. reinhardtii* expressing dimeric ASBVd (-) (A and B). The positions of linear RNA markers, with their sizes in nucleotides, are indicated on the left of each panel, and the positions of the monomeric circular (*mc*) and linear (*ml*) forms of the different viroids are indicated on the right of each panel. (+), plus-strand transcript; (-), minus-strand transcript; N. t., nontransformed *C. reinhardtii* Dim. (+), *C. reinhardtii* expressing dimeric viroid plus-strand transcripts; Dim. (-), *C. reinhardtii* expressing dimeric viroid minus-strand transcripts.

primary transcript, two faster-migrating species corresponding to *mc* and *ml* ASBVd RNAs, and others presumably generated by partial self-cleavage of the primary transcript (Fig. 2A, lane 3). Because *ml* ASBVd RNA results from double self-cleavage of the primary transcript mediated by a double-hammerhead structure (16), self-cleavage at either of the two single sites should generate RNAs with mobilities intermediate between those of the primary transcript and the *mc* form and smaller fragments (Fig. 1). Circularization of the *ml* ASBVd RNA yields the *mc* form that in *C. reinhardtii*, as in ASBVd-infected avocado, accumulates more than its *ml* counterpart (Fig. 2A, lanes 1 and 3). A similar situation was observed with the *C.*

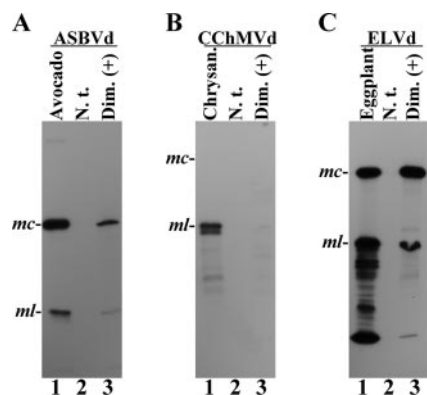


FIG. 3. Northern blot hybridization analysis of transplastomic *C. reinhardtii* expressing different dimeric viroid RNAs after separation by double PAGE. Viroid RNA preparations were separated by two consecutive PAGE steps, first under nondenaturing conditions, with the segment of this gel containing the *mc* and *ml* viroid forms being cut and applied on top of a second denaturing gel. The RNAs from the second gel were blotted and hybridized with riboprobes to detect ASBVd (+) (A), CChMVd (+) (B), and ELVd (+) (C). Lanes 1, controls of ASBVd-infected avocado (A), CChMVd-infected chrysanthemum (B), and ELVd-infected eggplant (C). Lanes 2, controls of nontransformed *C. reinhardtii* (A to C). Lanes 3, transplastomic *C. reinhardtii* expressing dimeric ASBVd (+) transcripts (A), CChMVd (+) transcripts (B), and ELVd (+) transcripts (C). The positions of the monomeric circular (*mc*) and linear (*ml*) forms of the different viroids are indicated on the left of each panel. The bands with faster mobility in panel C, lane 1, most likely result from degradation products of the *ml* form generated during purification. (+), plus-strand transcript; (–), minus-strand transcript; N. t., nontransformed *C. reinhardtii*; Dim. (+), *C. reinhardtii* expressing dimeric viroid plus-strand transcripts.

reinhardtii cell line expressing the dimeric minus-strand ASBVd transcript, although processing appeared less efficient, with minor amounts of *mc* and *ml* minus-strand ASBVd RNAs being detected (Fig. 2B, lane 4), the latter after prolonged exposure (data not shown).

The dimeric plus-strand CChMVd transcript expressed in *C. reinhardtii* was processed to its *ml* and *mc* forms with even lower efficiency. The intense bands observed below that generated by the primary transcript indicated that the latter self-cleaved efficiently at either of its two self-cleavage sites but only to a limited extent at both sites simultaneously, judging from the weak intensity of the band produced by the *ml* form (Fig. 2C and 3B, lanes 3). This result was unexpected because the plus-strand CChMVd single-hammerhead structure is stable (23). Indeed, when the same construct was transcribed *in vitro* under control of the T7 promoter, each hammerhead individually and both concurrently self-cleaved efficiently (data not shown). Possible explanations for this dissimilar behavior *in vivo* and *in vitro* are outlined below. The CChMVd *mc* form from *C. reinhardtii* was visible only after prolonged exposure (data not shown), paralleling the situation of CChMVd-infected chrysanthemum, in which this form accumulates less than its *ml* counterpart (Fig. 2C and 3B, lanes 1) (23).

In contrast, self-cleavage of dimeric plus-strand ELVd RNA in *C. reinhardtii* was efficient at both self-cleavage sites: only two prominent bands corresponding to the processing products of the primary transcript, *mc* and *ml* ELVd RNAs, were observed (Fig. 2D, lane 3). This efficient self-cleavage could result

from either the high catalytic activity of the plus-strand ELVd hammerhead during transcription (12) or from recognition by a cellular factor, particularly an RNA chaperone. The ratio of ELVd *mc* to *ml* forms in *C. reinhardtii* was higher than in ELVd-infected eggplant, indicating efficient circularization (Fig. 2D, compare lanes 1 and 3).

***C. reinhardtii* chloroplasts expressing dimeric ASBVd plus and minus strands do not support RNA-RNA transcription.** To determine whether *C. reinhardtii* chloroplasts support viroid RNA-RNA transcription in addition to correct processing, the presence of viroid cRNA in *C. reinhardtii* expressing dimeric ASBVd plus- and minus-strand transcripts was investigated. Northern blot hybridizations showed no signal when an ASBVd plus-strand-specific probe was used to examine the *C. reinhardtii* cell line expressing dimeric ASBVd minus-strand transcripts (Fig. 2A, lane 4) and when an ASBVd minus-strand-specific probe was used to examine the cell line expressing dimeric ASBVd plus-strand transcripts (Fig. 2B, lane 3). Faint bands in the upper parts of these lanes are most likely nonspecific. *C. reinhardtii* chloroplasts are therefore incompetent for initiating and/or elongating viroid RNAs on a cRNA template, implying that viroid replication could not be completed. Although this may be regarded as a limitation, it presents some advantages (see below).

Potential of transplastomic *C. reinhardtii* for studying *in vivo* RNA processing in the family *Avsunviroidae*. The unicellular green alga *C. reinhardtii* is a model organism with a sequenced genome and a panoply of tools that include transformation of the nuclear, chloroplast, and mitochondrial genomes (17). Despite progress in dissecting the replication of chloroplastic viroids, many aspects remain poorly understood, particularly the host factors involved. This has prompted us to explore the potential for expressing dimeric viroid RNAs in transformed *C. reinhardtii* chloroplasts. Viroids replicate in their hosts through an RNA-RNA rolling-circle mechanism (3). In the symmetric pathway followed by members of the family *Avsunviroidae* (8), *mc* plus- and minus-strand RNAs serve as templates for synthesis of their complementary oligomeric RNAs, which are processed to the *mc* RNAs. Therefore, replication comprises three steps: transcription, cleavage, and ligation. Regarding the first step, available data indicate that a nuclear-encoded chloroplast RNA polymerase (NEP) transcribes ASBVd and PLMVd RNAs, starting at defined regions in each polarity strand (10, 24; M. E. Rodio, S. Delgado, A. De Stradis, M. D. Gómez, R. Flores, and F. Di Serio, unpublished data). Expression of dimeric ASBVd plus- or minus-stranded RNAs in *C. reinhardtii* chloroplasts failed to incite accumulation of their complementary strands, showing that ASBVd, and possibly the other members of its family, are not transcribed. This failure could result from the apparent lack of any NEP-like activity in *C. reinhardtii* (2).

Cleavage in the family *Avsunviroidae* is mediated by hammerhead structures embedded in both polarity strands (14, 19, 26), with available evidence indicating that these ribozymes operate *in vitro* and *in vivo* (8, 10, 23, 24). However, critical aspects of hammerhead ribozymes are unknown, as illustrated by the recent finding of interactions between peripheral regions of natural hammerheads that increase their self-cleavage at the low-magnesium concentrations existing *in vivo* (9, 20). Expression of dimeric ASBVd plus- and minus-strand RNAs and dimeric CChMVd plus- and ELVd plus-strand RNAs in *C.*

reinhardtii chloroplasts showed proper self-cleavage to their *ml* forms. Whereas this is consistent with previous *in vitro* results (14, 19, 26), the extent of the reaction was dependent on the viroid RNA. Moreover, no relationship could be established between the extent of the reaction and self-cleavage mediated by either single- or double-hammerhead structures, indicating the involvement of auxiliary factors from the alga. Because RNA folding occurs during transcription, some viroid RNAs (i.e., CChMVd) could be trapped in conformations catalytically inactive for self-cleavage or display low affinity for cellular RNA chaperones that presumably assist self-cleavage *in vivo*. In this context, a protein binding ASBVd *in vivo* behaves as an RNA chaperone that stimulates *in vitro* ASBVd self-cleavage (6). Alternatively, some of the self-cleavage products could lack elements of a secondary structure that stabilizes transcripts in *C. reinhardtii* chloroplasts (27) and be degraded.

Ligation has been proposed to occur autocatalytically in PLMVd, giving rise *in vivo* to a 2',5'-phosphodiester bond (4). However, the *mc* plus-strand RNA isolated from ASBVd-infected tissue does not appear to have such an atypical bond (D. Molina-Serrano, R. Flores, and J. A. Daròs, unpublished data). Therefore, the involvement of a host RNA ligase producing a 3',5'-phosphodiester bond (21), which might also result from the hammerhead ribozyme catalyzing not only cleavage but also ligation (perhaps assisted by host proteins) (25), must also be considered. Because ligation of viroid *ml* to *mc* RNAs occurs in transformed *C. reinhardtii* chloroplasts and because the ratio between the two forms is similar to that existing in planta, the transplastomic system could serve to identify cellular factors mediating this reaction *in vivo*. Moreover, since there is no viroid RNA-RNA transcription in transgenic *C. reinhardtii*, the effects of mutations in the viroid primary transcript specifically affecting cleavage and/or ligation can be evaluated.

In summary, we believe that the experimental system presented here can facilitate the study of RNA motifs and cellular factors involved in viroid replication in a chloroplastic physiological context. Moreover, expressing foreign genes in the chloroplast is an environmentally-friendly alternative to nuclear transformation (22), and RNAs of the family *Avsunviroidae*, the only ones able to enter and replicate in this organelle, could serve as vectors for this purpose.

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