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The strands of both polarities of a small circular RNA from carnation self-cleave *in vitro* through alternative double- and single-hammerhead structures

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ABSTRACT

The sequence of a circular RNA from carnation has been determined and found to consist of 275 nucleotide residues adopting a branched secondary structure of minimum free energy. Both plus and minus strands of this RNA can form the hammerhead structures proposed to mediate the *in vitro* self-cleavage of a number of small infectious plant RNAs and the transcript of satellite 2 DNA from the newt. Minus full- and partial-length transcripts of the carnation circular RNA including the hammerhead structure showed self-cleavage during transcription and after purification, indicating the involvement of a single-hammerhead structure in the self-cleavage reaction. In the case of the plus transcripts only a dimeric RNA, but not a monomeric one, self-cleaved efficiently during transcription and after purification, strongly supporting the implication in this process of a double-hammerhead structure theoretically more stable than the corresponding single cleavage domain. However, a plus monomeric transcript self-cleaved after purification at a slow rate in a concentration-independent reaction which most probably occurs through an intramolecular mechanism. Comparative sequence analysis has revealed that the circular RNA from carnation shares similarities with some representative members of the viroid and viroid-like satellites RNAs from plants, suggesting that it is a new member of either these two groups of small pathogenic RNAs.

INTRODUCTION

A small circular RNA, which could be a viroid or a satellite RNA, associated with a stunting syndrome in carnations has been identified in the USA (1). Analysis by polyacrylamide gel electrophoresis established the circular structure of this RNA and an estimated molecular weight of 80,000–85,000 for it (1). On the other hand, a disease with symptoms of stunting, abnormal shoot proliferation and poor setting and malformation of the flowers has been reported in Italy in association with a viroid-like RNA (2). Subsequent investigations with Italian samples revealed that such a small RNA was present in two circular forms designated carnation stunt associated viroid slow and fast (CarSAV-slow and CarSAV-fast respectively), based on their electrophoretic mobilities (3). CarSAV-slow has an estimated size of 280 nucleotide residues and partial sequencing of it has shown that it can fold into a hammerhead structure (4), as those proposed to act in the *in vitro* self-cleavage of two viroids, some plant satellite RNAs and the transcript of the newt satellite 2 DNA (5–12).

We have reported previously (13) the existence in Spanish carnation plants of a circular RNA, which on the basis of its estimated size of 275 nucleotide residues appears very similar to those identified in the USA and Italy. Here we present the complete sequence of an Spanish isolate of this RNA together with data indicating that single- and double-hammerhead structures most probably mediate the *in vitro* self-cleavage of the minus and plus strands respectively, although in the latter case a less efficient single cleavage domain also appears operative. Since the published partial sequence (76 nucleotide residues) of the Italian CarSAV-slow (4) is highly homologous with a portion of the RNA here described, we have kept the name CarSAV for it, although both its viroid nature and causal relationship with the stunting syndrome in carnation remain to be fully demonstrated.

MATERIALS AND METHODS

Purification of CarSAV

Nucleic acid preparations enriched in CarSAV were generated by extracting leaves from carnation plants (*Dianthus caryophyllus* L.) with buffer-saturated phenol and fractionating the nucleic acids dissolved in the aqueous phase by cellulose chromatography (14). Purified circular forms of CarSAV were obtained by two

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consecutive electrophoreses under non-denaturing and denaturing conditions (15, 16).

Sequencing of RNA and DNA

CarSAV fragments were produced by limited digestions of the circular form catalyzed by RNases T1 and U2, and their sequences were determined by partial enzymic hydrolysis and electrophoresis on polyacrylamide gels containing urea (17) after 5'-end labelling with (γ-^{32}P)ATP and polynucleotide kinase (18). CarSAV cDNA inserts in pUC 18 were sequenced with chain-terminating inhibitors (19) using T7 DNA polymerase. In some cases, to improve the resolution, Taq DNA polymerase together with 7-deaza GTP were used and the gels contained 40% formamide in addition to 7 M urea.

Preparation and cloning of CarSAV cDNA

First strand cDNA was synthesized on circular CarSAV as described (20) using AMV reverse transcriptase and the complementary 34-mer oligodeoxyribonucleotide primer 5'TCCAGAACACCCGAACCAACTCAACCCTTCATCC3' derived from direct RNA sequencing. Synthesis of the second cDNA strand was performed by the replacement method (21) and the final double-stranded cDNA was cloned in the *Sma*I site of pUC18.

In vitro self-cleavage of CarSAV RNAs during transcription and after purification

The *Eco*RI−*Bam*HI fragment of a pUC18 recombinant plasmid containing the full-length sequence of CarSAV plus a repetition of two residues was subcloned in pBluescript II KS+ (Stratagene) and transcripts of plus and minus polarities were obtained with T7 and T3 RNA polymerases respectively. A second pUC18 recombinant plasmid containing the complete sequence of CarSAV plus a repetition of 145 residues was digested with Asp700 (GAANN/NNTTC) in order to generate a monomeric cDNA which was purified and subjected to ligation. The dimeric product of this reaction was subcloned in the *Sma*I site of pBluescript II KS+ and a recombinant plasmid containing a tandem dimeric insert was selected. Transcripts of plus and minus polarities were obtained as indicated above.

Non-radioactive transcription reactions contained: 50 ng/μl of template DNA linearized with the appropriate restriction enzyme (as indicated in the Figures), 0.5 U/μl of T7 or T3 RNA polymerase, 40 mM Tris−HCl pH 7.2, 6 mM $MgCl_2$, 10 mM DTT, 4 mM spermidine, 0.5 U/μl of human placental ribonuclease inhibitor and 0.5 mM each of the four ribonucleoside triphosphates. Radioactive transcription reactions contained the same ingredients except that they were supplemented with 1 μCi/μl of [α-^{32}P]UTP and the concentration of the unlabelled UTP was reduced to 50 μM. In both cases, the reactions were incubated at 37°C for 1 h. Transcription products were separated in polyacrylamide gels (5%) containing urea which were stained with ethidium bromide and/or autoradiographed. Complete transcripts, and in some cases the self-cleavage fragments thereof, were excised from the gel and eluted as described (22).

Self-cleavage of the purified transcription products was performed by incubating them in 50 mM Tris−HCl (pH 8), 5 mM $MgCl_2$, 0.5 mM EDTA (self-cleavage buffer) at 40°C for 1 h (22), except in some indicated experiments where the incubation time was increased. Prior to incubation the samples were heated in 1 mM EDTA (pH 6) at 100°C for 1 min and snap cooled on ice. Products from the self-cleavage reactions were analyzed by electrophoresis in urea polyacrylamide gels (5%) and autoradiography.

Secondary structure of CarSAV and sequence comparisons

The secondary structures of lowest free energy were determined by means of a program developed for this purpose (23). The fragments of highest similarity scores between CarSAV and some representative viroid and satellite RNAs (see Table 1) were obtained with the BESTFIT program (gap weight 5, length weight 0.3) of the GCG package (24). The statistical significance of the alignments was tested by a procedure (25) which estimates the probability as a function of the sequence length that a given alignment, including mismatches or not, is due to randomness.

RESULTS

Sequence and proposed secondary structure of CarSAV

Figure 1 shows the behaviour in denaturing polyacrylamide gels of the CarSAV RNA present in nucleic acid preparations from some carnation plants. A circular structure of approximately 275 residues was deduced for CarSAV from the comparison in this type of gel of its mobility with those of citrus exocortis viroid (CEVd) and avocado sunblotch viroid (ASBVd). In other carnation extracts some additional weaker bands with mobilities in the range of those of CarSAV, ASBVd and even higher, were also observed (data not shown).

Eight cDNAs clones of CarSAV were sequenced in both directions. One longer-than-unit clone made it possible to check the sequence corresponding to the primer and to rectify two incorrect assignments. Figure 2 (inset) shows the primary structure of CarSAV, which is a circular RNA of 275 residues consisting of 84 G (30.5%), 57 C (20.7%), 62 A (22.5%) and 72 U (26.2%) having, therefore, a G+C content of 51.2%. Sequence heterogeneity was found in the two positions indicated in Figure 2 (inset).

The CarSAV secondary structure of lowest free energy corresponds to a branched conformation (Figure 2) where 70.5% of the residues are paired with contents of GC, AU and GU base pairs of 47.4%, 36.1% and 16.5%, respectively. The search for

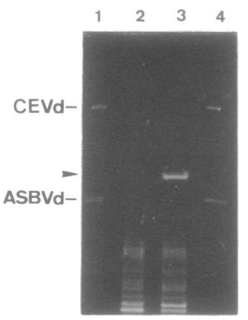

Figure 1. Analysis by double polyacrylamide gel electrophoresis of nucleic acid preparations from carnation plants. The first non-denaturing gel was stained with ethidium bromide and the segment between the purified viroid standards CEVd and ASBVd was cut and applied on top of a second denaturing gel whose electrophoretic pattern is presented. Lanes 1 and 4, circular forms of CEVd and ASBVd. Lanes 2 and 3, extracts from two carnation plants, one of them containing a circular RNA (CarSAV) indicated by an arrowhead.

other secondary structures within the 10% of the minimum free energy value also led to branched conformations (data not shown).

Plus and minus RNAs of CarSAV can form hammerhead structures

Figure 3 shows that plus and minus RNAs of CarSAV have the 13 conserved residues flanked by the other typical elements of the hammerhead structures proposed around the self-cleavage sites of ASBVd (5), peach latent mosaic viroid (PLMVd) (6), some satellite RNAs of the sobemo-, nepo- and luteoviruses (7–11) and the transcript of the newt satellite 2 DNA (12).

The hammerhead structure of the minus CarSAV RNA (Figure 3A) has a stable helix III (we will follow hereafter the nomenclature proposed in reference 26) and short loops 1 and 2, and in these two aspects is similar to most other hammerhead structures with the exception of those of ASBVd (plus and minus), of satellite of barley yellow dwarf virus, sBYDV (plus) and of the transcript of the newt satellite 2 DNA. The minus CarSAV hammerhead structure has the peculiarity that the residues A 11 and C 10 (numbers 10 and 30 respectively in minus CarSAV RNA, the same numbers are used in the minus as in the plus polarity) preceding and following the conserved sequences GAAAC and GA respectively, do not base pair, a situation previously reported in the plus hammerhead structure of sBYDV (11).

The hammerhead structure of the plus CarSAV RNA has a helix III of two base pairs closed by a loop 3 of only two C and G residues (Figure 3B) and in this respect is identical to the hammerhead structure of the transcript of the newt satellite 2 DNA (12). These two hammerhead structures are also similar in having the helix II, but not the helix I, closed by a short loop 2. Since the stability of the hammerhead structures of plus and minus ASBVd and newt RNAs has been questioned (5, 27), more stable double-hammerhead structures have been proposed for these RNAs which may only be active as dimers (28). Figure 3C shows the double-hammerhead structure proposed for a dimeric plus CarSAV RNA.

A residue C 17 (numbers 275 and 51 in plus and minus CarSAV RNAs respectively) precedes the predicted self-cleavage sites of the CarSAV hammerhead structures, as it occurs in most of the other known hammerhead structures with the exceptions of those of satellite of lucerne transient streak, sLTSV, (minus) (8) and sBYDV (plus) (11), where the residue found in this position is a A. The residue 7 (numbers 231 and 33 in plus and minus CarSAV RNAs respectively) between the conserved sequences CUGA and GA is a U and a A in the plus and minus CarSAV hammerhead structures respectively, in agreement with the residue found in this same position (U, A or C, but never G) in the other naturally occurring hammerhead structures.

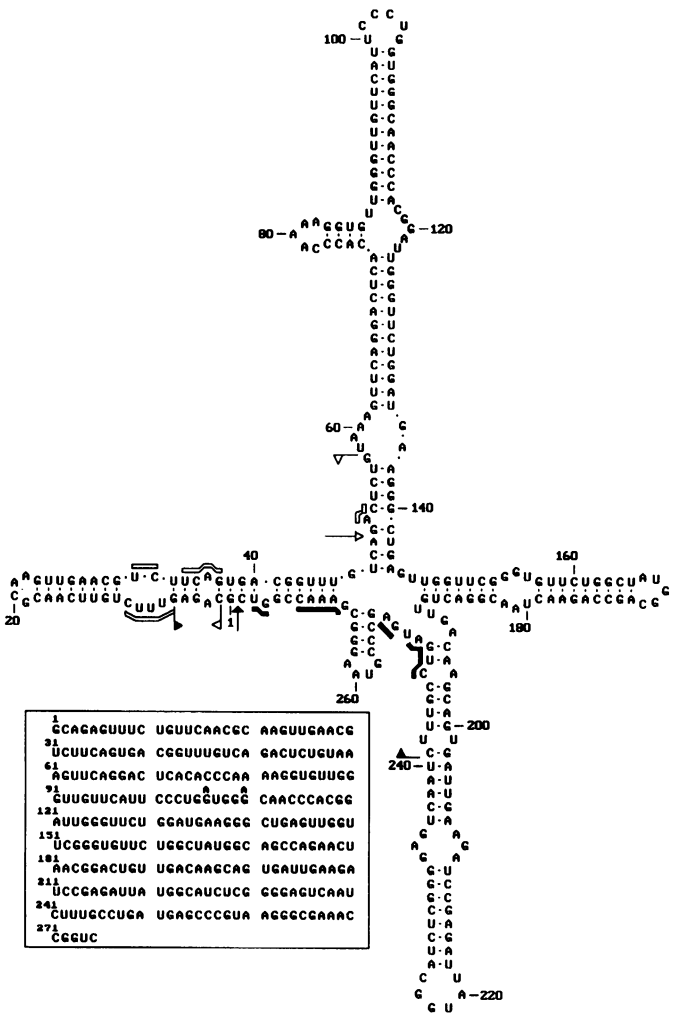

Figure 2. Secondary structure of lowest free energy obtained for CarSAV. Plus and minus self-cleavage domains are delimited by flags, the 13 conserved residues characteristic of the hammerhead structures are indicated by bars, and the predicted self-cleavage sites by arrows. Solid and open symbols refer to the plus and minus polarities, respectively. (Inset) Sequence of CarSAV with the substitutions found at two different positions indicated above the reference sequence (these substitutions do not alter the proposed secondary structure). The Asp700 site is located between residues 26 and 35.

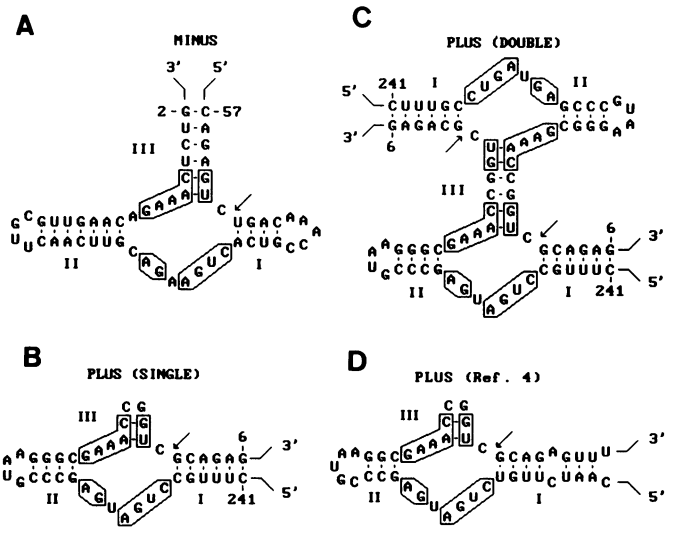

Figure 3. Hammerhead structure of minus CarSAV RNA (A), single- and double-hammerhead structures of plus CarSAV RNA (B and C respectively), and hammerhead structure of CarSAV-slow (D) as depicted in reference (4). The structures are represented using the adopted convention (8, 26) where arrows denote the predicted self-cleavage sites, helices are labelled I to III, and the 13 conserved residues present in all hammerhead structures (with the exception of that of plus sBYDV RNA where only 11 residues are conserved) are boxed. The same numbers are used in the minus as in the plus polarity.

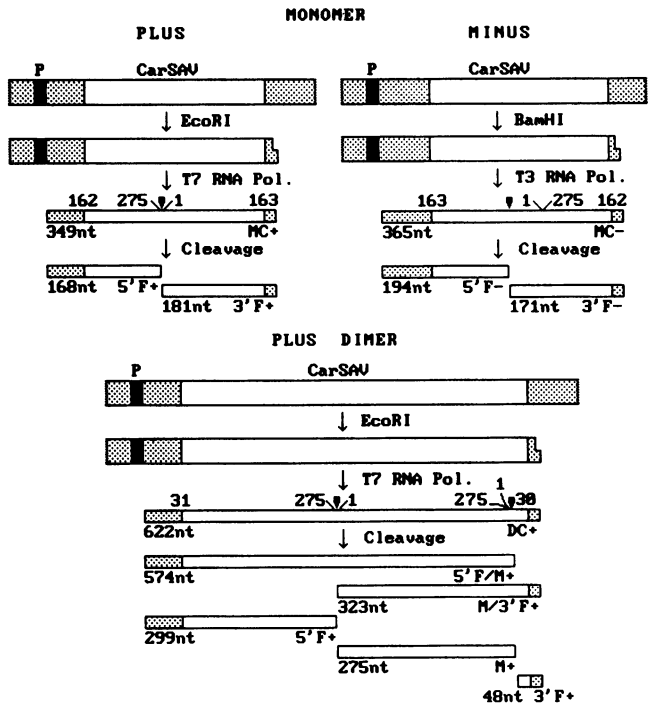

Figure 4. Diagrams of plus and minus DNA templates and of the products generated by transcription with either T7 or T3 RNA polymerases of templates linearized with appropriate restriction enzymes. Hatched boxes, vector sequences; solid boxes, RNA polymerase promoters; open boxes, CarSAV sequences. The complete transcripts are C+ and C− (with a preceding M or D to indicate monomeric or dimeric length, respectively) and the cleavage fragments 5′F+, 3′F+, 5′F− and 3′F−. The cleavage fragments of longer-than-unit length resulting when self-cleavage of the dimeric plus transcript occurred at only one of the two possible sites are 5′F/M+ and M/3′F+. Nucleotide (nt) residue numbers in CarSAV RNAs are given above the products and their expected size below. The sites of self-cleavage are indicated by arrowheads.

The monomeric plus CarSAV RNA has a secondary structure of lowest free energy (at 37°C) of −367 kJ/mol, whereas the value for that containing the hammerhead structure is −327 kJ/mol. The corresponding values for monomeric minus CarSAV RNA are −285 kJ/mol (most stable) and −272 kJ/mol (with hammerhead), and for dimeric plus CarSAV RNA −801 kJ/mol (most stable) and 667 kJ/mol (with hammerhead).

Full- and partial-length minus CarSAV RNAs self-cleave both during transcription and after purification

Monomeric minus CarSAV RNA synthesized *in vitro* as indicated (Figure 4), self-cleaved efficiently during transcription (Figure 5A, lane 4) since in addition to the complete transcript MC−, discrete product 5′F− and 3′F− were observed of the size expected for self-cleavage taking place as in the other hammerhead structures (9, 10). That cleavage occurred at the predicted site was confirmed by reverse transcription of the 3′F− fragment, using as a primer a 26-mer oligonucleotide (positions 270−20), and subsequent determination of the 3′-end of this cDNA by comparing its electrophoretic mobility with the ladders obtained by sequencing with dideoxynucleotides and the same primer a monomeric CarSAV clone (data not shown).

Monomeric minus CarSAV RNA purified from preparative gels also self-cleaved efficiently when it was incubated under standard self-cleavage conditions (Figure 5A, lane 5). Minus RNA transcripts from partial-length CarSAV cDNA clones containing the hammerhead structure, also displayed self-cleavage at the expected site both during transcription and after purification (data not shown).

Dimeric plus but not monomeric plus CarSAV RNA self-cleave efficiently during transcription and after purification

In vitro synthesis of plus CarSAV RNA from linearized monomeric clones of CarSAV cDNAs (Figure 4) led to the complete transcript MC+ but not to self-cleavage fragments

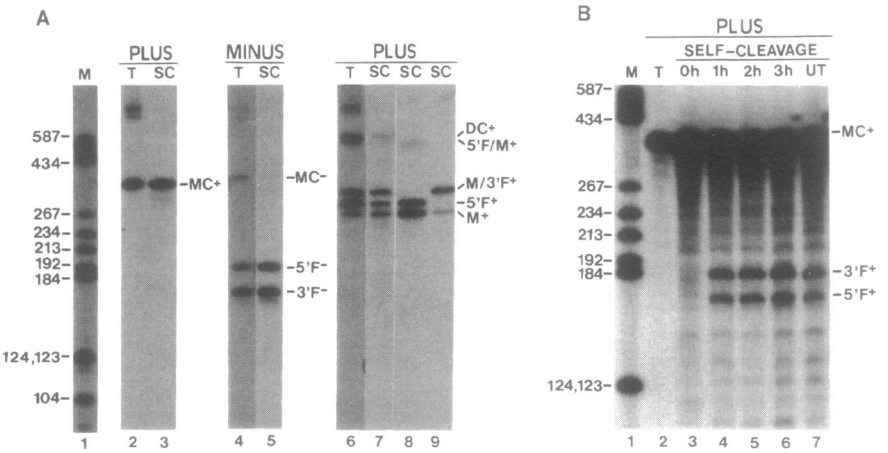

Figure 5. *In vitro* synthesis and self-cleavage of monomeric and dimeric CarSAV RNAs. The positions of the products (see Figure 4) are indicated on the right side. (A) Analysis of the transcription (T) reactions and self-cleavage (SC) reactions of purified products by PAGE and autoradiography. Lane 1, radioactive DNA markers (prepared by digesting pBR322 with *Hae*III and end-labelling) with their sizes in residues on the left. Lanes 2 and 4, plus and minus transcription products from the monomeric clone. Lanes 3 and 5, self-cleavage of purified MC+ and MC− after heating at 100°C for 1 min in 1 mM EDTA (pH 6), snap cooling on ice for 5 min, and incubation at 40°C for 1 h in the self-cleavage buffer. Lane 6, plus transcription products from the dimeric clone. Lane 7, 8 and 9, self-cleavage of purified DC+, 5′F/M+ and M/3′F+ respectively, after the same treatment as in lanes 3 and 5; the 3′F+ has migrated out of the gel. (B) Analysis of the transcription (T) reaction and of self-cleavage (SC) reaction of plus monomeric CarSAV RNA by PAGE and autoradiography. Lane 1, as in (A). Lane 2, plus transcription products from the monomeric clone. Lanes 3, 4, 5 and 6, self-cleavage of purified MC+ after the same treatment as in lanes 3 and 5 of (A) but with different incubation times. Lane 7, self-cleavage of purified MC+ after the same treatment as in lanes 3 and 5 of (A) but in the presence of a 500-fold excess of unlabelled transcript (UT). The intensity of the bands and the background is higher in (B) because the autoradiography was exposed for 96 h instead of for 24 h as in (A).

Table 1. Observed and minimum expected significant lengths between the fragments of highest similarity of CarSAV and some representative viroid and viroid-like satellite RNAs

Sequence	Aligned Fragments	Percent Similarity	Length Observed	Length Expected
CarSAV	146 UUGGUUCGGGUGUAUCUGGCUAUGGCAGCCAGAACUAAC 183	63.16	38*	32
PSTVd	190 UUUCUUCGGGUGUCCUUCCUCGCGCCCGCAGGACCACC 227			
CarSAV	79 AAAAGGUGUUGGGUUGU 95	76.47	17	19
CCCVd	223 AAAAGGUGUCCCUUUGU 239			
CarSAV	71 UCACACCCAAAAGGUGUU 88	77.78	18*	17
ASSVd	309 UAAAACACAAUAGGUGUU 326			
CarSAV	143 GAGUUGGUUCGGGU 156	85.71	14*	14
Cb1Vd	118 GAGUUGCUUCGGCU 131			
CarSAV	193 ACAAGCAGUGA 203	90.91	11	12
ASBVd	10 ACAAGAAGUGA 20			
CarSAV	125 GGUUCUGGAUGAAGGG 140	87.50	16*	13
PLMVd	272 GAUUCUGGAUGAAAGAG 287			
CarSAV	249 GAUGAGCCCGUAAGGGCGAAAC 270	77.27	22*	18
sLTSV	192 GAUGGCCCGGUAGGGCCGAAAC 213			
CarSAV	80 AAAGGUGUUGGGUUGUU 96	82.35	17*	15
sSCMoV	256 AAAAGUGUUGGAAUGUU 272			
CarSAV	203 AUUGAAGAUCCGAGAUUAUGGCAUCUCGGGAGUCAAUC 241	61.54	39*	33
sBYDV	86 AGUAAAGAUCUCCAAUUGUGGCACCACCAGGUGCCACC 124			
CarSAV	245 GCCUGAUGAGCCCGUAAGGGCGAAACCGGUC 275	80.65	31*	20
sTRSV	18 GUCUGAUGAGUCCGUGAGGACGAAACAGGAC 48			

* Significant at the 0.05 level.
CCCVd: coconut cadang-cadang viroid. ASSVd: apple scar skin viroid. Other abbreviations are indicated in the text.

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thereof (Figure 5A, lane 2). Incubation of this complete transcript under standard self-cleavage conditions also did not produce visible discrete fragments attributable to a self-cleavage reaction (Figure 5A, lane 3). These results indicated that an efficient single cleavage domain could not be formed by the monomeric plus CarSAV RNA under the aforementioned conditions, in accordance with the theoretically instable helix III of its hammerhead structure (Figure 3B) which only has two base pairs and is closed by a loop 3 of only two residues. When the RNAs resulting from the *in vitro* transcription of linearized dimeric clones of CarSAV cDNAs (Figure 4) were analyzed, in addition to the complete transcript DC+, three main products were also detected with the expected sizes for the CarSAV linear monomer, M+, and for the two other fragmentb 5′F+ and 3′F+ arising by self-cleavage at the two possible sites present in transcript DC+ (Figure 5A, lane 6); other products 5′F/M+ and M/3′F+ were considered to result from self-cleavage at only one of the two possible sites of transcript DC+ (Figure 5A, lane 6). It was concluded, therefore, that self-cleavage during transcription of a dimeric plus CarSAV RNA was most probably mediated by a double-hammerhead structure (Figure 3C), which should also be active when the purified DC+ transcript was incubated under standard self-cleavage conditions, since the same band pattern was observed (Figure 5A, lane 7).

Monomeric plus CarSAV RNA self-cleaves at a slow rate after purification

In an attempt to detect minor amounts of self-cleavage products which could arise during the incubation of purified monomeric plus CarSAV RNA, the autoradiographs were overexposed. Under these conditions, two discrete fragments 5′F+ and 3′F+ with the expected sizes for the products of the self-cleavage were observed (Figure 5B, lane 4). That cleavage occurred at the predicted site was confirmed by reverse transcription of the 3′F+ fragment using as a primer the 34-mer oligonucleotide employed for obtaining the CarSAV clones (complementary to positions 131−164) and subsequent determination of the 3′-end of this cDNA as indicated above (data not shown). The self-cleavage was stimulated by extending the incubation time (Figure 5B, lanes 5 and 6) but not by adding high amounts of the unlabelled transcript to the reaction mixtures (Figure 5B, lane 7).

Relationships of CarSAV with other infectious small RNAs from plants

Table 1 shows the fragments of highest similarity between CarSAV and some representative viroid and viroid-like satellite RNAs. It can be observed that the percentage of similarity between fragments is more than 80% in the cases of satellite of tobacco ringspot virus (sTRSV), satellite of subterranean clover mottle virus (sSCMoV), *Coleus blumei* 1 viroid (Cb1Vd), PLMVd and ASBVd, although in this latter viroid the fragment length is slightly shorter than the minimum one expected to be significant. On the other hand, the fragments corresponding to sTRSV, sBYDV and potato spindle tuber viroid (PSTVd) are particularly long, with observed lengths of more than 30 residues, which in these three cases are higher than the minimum lengths expected to be significant. From these observations it appears, therefore, that CarSAV is related with viroid and viroid-like satellite RNAs, which according to a previous study (30), seem to have a monophyletic origin.

DISCUSSION

Determination of the complete CarSAV sequence has shown that it is a circular RNA of 275 residues competent to fold into the hammerhead structure reported previously in the self-cleaving domains of some small pathogenic RNAs from plants (5, 11) but so far in only one RNA from animal origin, the transcript of the newt satellite 2 DNA (12). The comparative analysis of the hammerhead structures of CarSAV and the other naturally occurring structures of this class reveals some interesting features. First, CarSAV can form hammerhead structures in the strands of both polarities, as it also occurs in the cases of ASBVd (5), PLMVd (6), sLTSV (8) and sBYDV (11). The conformations of lowest free energy of plus and minus monomeric, and plus dimeric CarSAV RNAs do not contain the hammerhead structures and, therefore, the corresponding purified transcripts are not expected to self-cleave unless they are subjected to a thermic pretreatment to promote the appearence of the alternative conformations containing the hammerhead structures. Second, monomeric minus and dimeric plus CarSAV RNAs self-cleave during transcription and after purification most probably through single- and double-hammerhead structures respectively, resembling in this regard ASBVd RNAs (5, 29), although in the case of dimeric minus ASBVd RNA self-cleavage is mediated by a double-hammerhead structure during transcription but by a single-hammerhead structure after purification (29). Third, the hammerhead structure of plus CarSAV RNA has the helix III and loop 3 identical to those of the newt RNA (12). Consistent with the theoretical instability of this hammerhead structure, we have not been able to detect self-cleavage of monomeric plus CarSAV RNA during transcription, whereas as mentioned above, dimeric plus CarSAV RNA self-cleaves efficiently during transcription (and after purification), strongly indicating the involvement of a double-hammerhead structure in this reaction. However, we have observed that monomeric plus CarSAV RNA self-cleaves after purification by the predicted site in a concentration-independent reaction of slow rate. Our results are

in very good agreement with those obtained with the monomeric newt RNA (31) and suggest that the reaction very probably occurs through an intramolecular mechanism. Since the most similar regions of the single-hammerhead structures of the plus CarSAV and newt RNAs are those encompassing helix III and loop 3, it is possible that although they appear to be thermodynamically unlikely, these structures, or variants thereof, may form transiently or may be stabilized by different factors including divalent cations, non-Watson–Crick base pairs and tertiary interactions such as base triplets, as it was pointed out previously for the single-hammerhead structures of ASBVd RNAs (5), which also appear (specially that of the plus polarity) theoretically instable. Alternatively, the self-cleavage of monomeric plus CarSAV RNA through a single-hammerhead structure may be dependent on sequences external to the conserved self-cleavage domain. This possibility has been advanced and supported experimentally for the self-cleavage of the monomeric newt RNA (32), where an internally looped extension to helix I of the hammerhead structure is important for activity. If this turns out also to be the case with the single cleavage domain of plus CarSAV RNA, more than one type of active extension to helix I favoring self-cleavage should exist since these two regions are different in the newt and CarSAV RNAs. In this context it should also be pointed out that in the CarSAV RNA most residues of the upper strand of the extension to helix I are involved in forming the minus hammerhead structure. Fourth, in the minus CarSAV hammerhead structure the central loop is more open in its left part close to the base of helix II since residues A 11 and C 10 (numbers 10 and 30 respectively in minus CarSAV RNA), flanking the conserved sequences GAAAC and GA, are not involved in a Watson–Crick base pair; in spite of that this hammerhead structure self-cleaves very efficiently (Figure 5A). The same two residues exist in identical positions in the hammerhead structure of the plus sBYDV RNA, which also exhibits two other atypical features: an unusual base pair in helix III and a pseudoknot-like element (33). And fifth, the plus and minus CarSAV hammerhead structures are contained in a segment of 92 residues, indicating that as in the cases of sLTSV (8) and PLMVd (6), approximately one-third of the CarSAV sequence is compromised in the formation of both self-cleaving domains which, like those of sLTSV, are partially overlapping (Figure 2). However, conversely to what has been observed in sLTSV and PLMVd, the plus and minus CarSAV hammerhead structures and their corresponding self-cleavage sites (separated by 50 residues) do not occur in opposite positions in the proposed secondary structure (Figure 2).

As indicated in the Introduction, the RNA whose complete sequence is reported here is very probably the same as the partially sequenced CarSAV-slow from Italy (4). However, by comparing the published 76 residue long sequence of CarSAV-slow with the corresponding homologous region of the RNA here characterized (termed CarSAV in the present work) we have found five substitutions, three of them located at positions 2.1, 2.5 and 2.6 of helix I of the hammerhead structure, and two extra residues in CarSAV, one of them a G located at position 11.4 (residue 62) which base pairs with the C 10.4 (residue 57) and makes helix II of the corresponding hammerhead structure of four instead of three base pairs (Figure 3B and 3D). Unpublished results quoted in (4) indicate that RNA transcripts from a cDNA clone of CarSAV-slow undergo a self-cleavage reaction which is associated with the hammerhead structure depicted in Figure 3D. We have observed that monomeric plus CarSAV RNA self-cleaves after purification through a single-hammerhead structure (Figure 3B) or a variant thereof, although this reaction occurs at a much slower rate than that displayed by dimeric plus CarSAV RNA whose efficient self-cleavage is very probably mediated by a double-hammerhead structure (Figure 3C). Moreover, the elucidation of the complete CarSAV sequence has revealed that monomeric minus CarSAV RNA can also form a theoretically stable hammerhead structure and that *in vitro* self-cleavage occurs as predicted by this structure.

We have not been able to associate the presence of CarSAV with stunting symptoms in carnation plants. The relatively low correlation between the presence of CarSAV RNAs in Italian samples and the development of the stunting syndrome, also casts some doubt on the role played by these RNAs in causing the disease (3), although the possibility exists that a combination of environmental factors as yet untested might be critical to elicit the symptoms. On the other hand, infectivity assays reported previously (3) using carnation seedlings and purified CarSAV-slow and -fast RNAs appear to indicate that they replicate autonomously, although there is virtually no sequence similarity between the partially sequenced CarSAV-slow and any known viroid (or circular satellite RNA) with the exception of a few remnants of the central region from PSTVd (4). However, we have failed to reproduce these infectivity assays in our preliminary attempts with purified CarSAV. Furthermore, a more extensive comparative analysis (Table 1), has revealed that CarSAV shares similarities not only with viroids but also with viroid-like satellite RNAs, indicating that this approach does not discriminate to which of the two groups belongs CarSAV. Therefore, additional experiments are needed to prove convincingly that CarSAV is a viroid and that it is the causal agent of the stunting syndrome of carnation.

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