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Analysis of *cis*-Acting Sequences Involved in Plus-Strand Synthesis of a Turnip Crinkle Virus-Associated Satellite RNA Identifies a New Carmovirus Replication Element

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Satellite RNA C (satC) is a 356-base subviral RNA associated with turnip crinkle virus (TCV). A 3′-proximal element (3′-UCCCAAAGUAU) located 11 bases from the 3′ terminus of satC minus strands can function as an independent promoter in an *in vitro* RNA-dependent RNA polymerase (RdRp) transcription system. Furthermore, in the absence of a 5′-proximal element, the 3′-proximal element is required for complementary strand synthesis *in vitro.* Site-directed mutagenesis was conducted to investigate the functional significance of this element and the 3′ minus-strand terminal sequence "3′-OH-CCCUAU," which contains the minus-strand 3′-end sequence "3′-OH-CC_{1-2}(A/U)(A/U)(A/U)" found in all carmovirus RNAs. Single mutations in the 3′-terminal sequence, which we have named the carmovirus consensus sequence (CCS), suppressed satC plus-strand synthesis to undetectable levels in protoplasts while still permitting some minus-strand synthesis. However, single and multiple mutations introduced into the 3′-proximal element had little or no effect on satC accumulation in protoplasts. *In vivo* genetic selection (SELEX) of the minus-strand 3′-terminal 21 bases revealed that all satC species accumulating in plants contained the 3′ CCS. In addition, the 3′-proximal element preferentially contained a sequence similar to the CCS and/or polypurines, suggesting that this element may also contribute to accumulation of satC *in vivo.*

INTRODUCTION

Replication is a fundamental activity of an RNA virus. Replication of positive-sense RNA viruses involves the synthesis of complementary minus-strand intermediates from the parental RNA template followed by synthesis of nascent plus strands. Initiation of replication requires the specific recognition of *cis*-acting RNA elements by the viral RNA-dependent RNA polymerase (RdRp) and/or associated host factors, and many sequences and/or structures required for minus-strand synthesis have been characterized for a number of RNA viruses (Buck, 1996).

Most viral RdRp extracts cannot use minus-strand RNA as template; thus promoters for plus-strand synthesis have been less extensively characterized. Reverse genetic approaches using cell cultures to monitor viral RNA accumulation indicate that the 5′-terminal region of plus-strand RNA or the complementary 3′-end region of minus strands contains *cis*-acting signals for plus-strand synthesis (Duggal *et al.,* 1994; Buck, 1996). For example, a cloverleaf structure at the 5′ ends of poliovirus and brome mosaic virus (BMV) plus-strand RNAs, but not the similar structure on minus strands, is necessary for plus-strand synthesis, possibly through interactions with host factors that keep the 3′ end of nascent minus strands single-stranded and accessible to the viral RdRp (Pogue and Hall, 1992; Andino *et al.,* 1990, 1993; Duggal and Hall, 1995). On the other hand, some 5′ elements delineated on plus strands *in vivo* likely function in their minus-strand orientation for plus-strand synthesis. For example, minus-strand sequences in BMV RNAs that are essential for plus-strand synthesis are similar to the internal control region of tRNA gene promoters (Pogue *et al.,* 1990, 1992). The 3′-terminal regions of Sindbis virus and potato virus X minus strands are also thought to be involved in plus-strand synthesis (Niesters and Strauss, 1990; Pardigon and Strauss, 1992, 1996; Kim and Hemenway, 1996; Miller *et al.,* 1998).

Turnip crinkle virus (TCV), a member of the genus *Carmovirus,* is a single-stranded positive-sense RNA virus with a genome of 4054 bases (Carrington *et al.,* 1989; Oh *et al.,* 1995). TCV is naturally associated with a number of subviral RNAs including 194-base satellite RNA D (satD; previously known as sat-RNA D) and satellite RNA C (satC; previously known as sat-RNA C; Simon and Howell, 1986). SatC is a 356-base recombinant RNA that is composed of sequences similar to satD at its 5′ end and two regions derived from TCV genomic RNA at its 3′ end (Fig. 1A).

Both plus and minus strands of TCV subviral RNAs are templates for synthesis of complementary strands when

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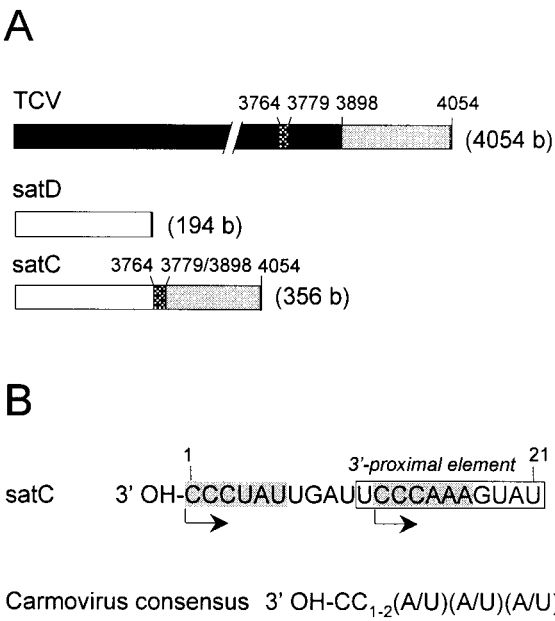

FIG. 1. (A) Schematic representations of TCV, satD, and satC. Similar regions are shaded alike. Numbers indicate positions with respect to locations in TCV genomic RNA. (B) The 3′-end 21 bases of minus-strand satC. The 3′-proximal element is boxed. The CCS in the 3′-terminal and the 3′-proximal element region is shaded. Arrows denote the putative transcription initiation sites for complementary strand synthesis *in vitro*.

added to an *in vitro* transcription assay system that uses RdRp-active extracts prepared from infected turnip plants (Song and Simon, 1994). This assay system led to the identification of a stem-loop structure at the 3′ end of satC plus strands as the promoter for complementary minus-strand synthesis (Song and Simon, 1995). Mutagenesis and genetic selection studies conducted *in vivo* confirmed the importance of this hairpin structure for satC accumulation (Stupina and Simon, 1997; Carpenter and Simon, 1998). Two short elements on satC minus strands, one located 11 bases from the 3′ end (3′-proximal) and the other located 41 bases from the 5′ end (5′-proximal) were also identified using the *in vitro* transcription system as important for plus-strand synthesis (Guan *et al.,* 1997). These two elements appeared to have redundant functions since only one was required for complementary strand synthesis *in vitro.* Both RNA elements are composed of multiple consecutive C residues followed by multiple consecutive purines and have no obvious internal secondary structures. In addition to these two elements, all carmovirus RNAs share a similar sequence at their 5′ ends (called the carmovirus consensus sequence or CCS) of unknown function. Here we report that the 5′-terminal CCS of satC is important for satC plus-strand accumulation in protoplasts. In addition, sequence in the 3′-proximal element region in satC minus strands is not random. For efficient accumulation of satC *in vivo,* the sequence may comprise an extension of the 3′-terminal CCS, a second CCS and/or multiple purines.

RESULTS AND DISCUSSION

The 3′-terminal CCS of satC minus strands is required for satC plus-strand accumulation *in vivo*

The 3′-proximal element (3′-UCCCAAAGUAU) is contained within positions 11 to 21 of satC minus strands (Fig. 1B; all numbering is from the 3′ end of the minus strand). When the 3′ 21 bases of satC minus strands are joined to the 3′ end of a Qβ bacteriophage-associated midivariant (MDV) RNA, which alone is not a template for the TCV RdRp, complementary strands are synthesized by the TCV RdRp *in vitro* (Guan *et al.,* 1997). Addition of nonviral bases to the 3′ end of satC minus strands resulted in transcription initiating exclusively internally in the vicinity of the three consecutive C residues within the 3′-proximal element. Like the 3′ terminus of satC minus strands (3′-OH-CCCUAU), the 3′-proximal element contains a CCS "3′-CC_{1-2}(A/U)(A/U)(A/U)" whose complement is found at the 5′ ends of all carmovirus plus-strand genomic, subgenomic, and subviral RNAs (Guan *et al.,* 1997; see Fig. 1B).

These observations suggested that both the 3′ CCS and the 3′-proximal element of satC minus strands have a role in satC replication. However, deletion of the 3′-terminal 10 bases including the entire 3′ CCS had no effect on promoter activity *in vitro,* suggesting that the 3′-terminal CCS might not be important for complementary strand synthesis (Guan *et al.,* 1997). To investigate the importance of the 3′-terminal CCS in satC accumulation *in vivo* (Fig. 2A), satC plus-strand transcripts containing mutations in the CCS were inoculated together with TCV genomic RNA onto protoplasts of *Arabidopsis thaliana.* Total RNA extracted from the infected protoplasts at 24 h postinoculation (hpi) was used for RNA gel-blot analysis of both plus- and minus-strand satC. As shown in Fig. 2B, a single-base mutation of C to A at position 2 (construct R3), which disrupted the 3′-terminal CCS, eliminated detection of satC plus strands. A single-base mutation of C to G at position 3 (construct R1) also generated no detectable plus-strand satC. Alteration of the same base to A (construct R2) maintained the CCS, and satC accumulated to detectable levels that were 15% of wild-type. These results suggest that in the context of the remaining satC promoter sequence, three consecutive C residues are preferred. When the U residue at position 4 was changed to G (construct R4), a low level of plus-strand RNA (7% of wild-type) was detected. Replacement of U with G at position 6 (construct R5) reduced accumulation of satC plus strands to 2% of wild-type. Similar results were obtained when double-base mutations were introduced into positions 5 and 8 (construct R7). No satC plus strands were detectable when triple-base mutations were introduced into positions 5 to

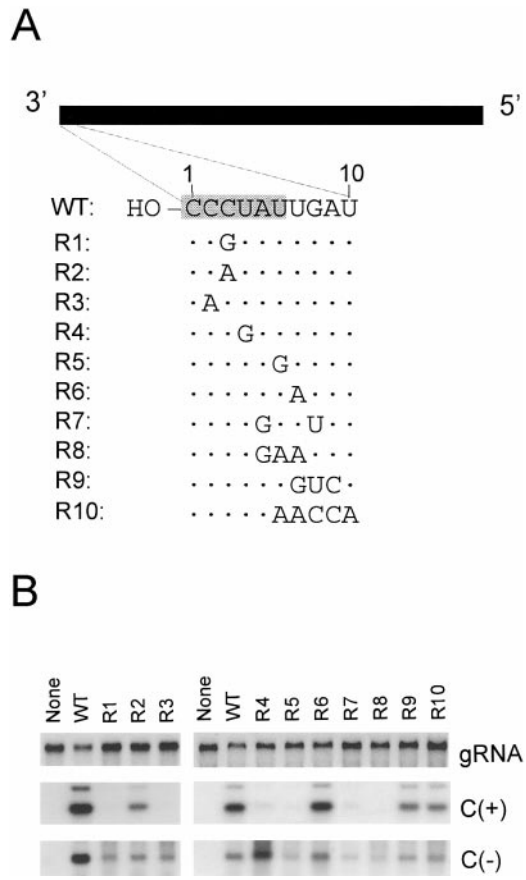

FIG. 2. Mutagenesis studies of the minus-strand 3′-terminal sequences of satC in protoplasts. (A) Mutations introduced into the 3′-terminal region. SatC minus strand is represented by a black bar. The 3′-terminal 10 bases are shown under the bar and positions with respect to location in the minus strand are indicated. The CCS is shaded. Dots represent no change of bases. Names of the constructs containing the mutations are shown to the left. (B) RNA gel-blot analysis of TCV genomic RNA (gRNA), satC plus strand [C(+)], and minus strand [C(−)]. For probing gRNA and C(+), equal amounts of total RNA isolated from 2×10^5 protoplasts at 24 hpi were used. Double-stranded RNA prepared from 8×10^5 protoplasts were used to detect satC minus strands. None, no sat-RNA added.

7 (construct R8). In contrast, changing the U at position 7 to A (construct R6) did not affect satC accumulation. Triple-base mutations in positions 7 to 9 (construct R9) or five-base mutations in positions 6 to 10 (R10) reduced satC plus-strand synthesis by only 50%, suggesting that mutations beyond the 3′-terminal CCS and/or U to A change at position 6, which maintained the CCS, have less severe effects on satC accumulation.

Although plus-strand RNA was undetectable or nearly undetectable for constructs R1, R3, R5, or R8, minus-strand RNA of these mutants was still detectable in protoplasts and accumulated from 25 to 40% of wild-type (Fig. 2B). Accumulation of minus strands of other mutants was also less affected than that of plus strands. Altogether, these results suggest that the 3′-terminal CCS of satC minus strand is important for accumulation of satC in protoplasts and that the sequence likely functions during plus-strand synthesis.

Since the requirement of the 3′-terminal CCS in satC minus strands *in vivo* differs from prior *in vitro* studies where the 3′ CCS was deleted without negative effect on complementary strand synthesis (Guan *et al.,* 1997), the effect of site-specific mutations in the 3′-terminal CCS on transcription was assayed *in vitro.* A chimeric RNA template [MC3′(−)] was constructed by joining the 3′-terminal sequence (positions 1 to 40) of satC minus strands to the 3′ end of the Qβ bacteriophage-associated MDV RNA (Fig. 3A). *In vitro* transcription of MC3′(−) RNA using TCV RdRp extract produced two products: one template-sized whose synthesis apparently initiates at

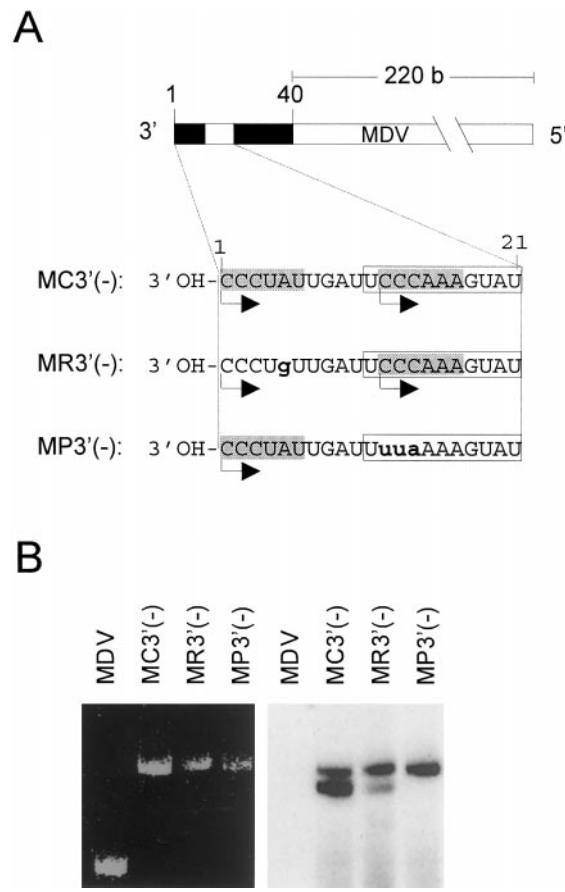

FIG. 3. *In vitro* transcription using partially purified RdRp and chimeric RNA templates containing mutations in the 3′-terminal or 3′-proximal regions of satC minus strands. (A) Schematic representation of the chimeric RNAs derived from MDV RNA (220 bases) and the 3′-end 40 bases of minus-strand satC (black bar). Small open box represents the 3′-proximal element. The 3′-terminal 21 bases (positions 1 to 21) of satC minus strands are shown. The 3′-terminal CCS and the 3′-proximal element CCS are shaded. The 3′-proximal element is boxed. Mutations introduced into the 3′-terminal region or the 3′-proximal element are denoted by bold lowercase letters. Construct names are shown to the left. Arrows denote the putative transcription start sites *in vitro.* (B) Denaturing gel analysis of ^{32}P-labeled products synthesized *in vitro.* The ethidium bromide-stained gel showing the migration positions and the relative levels of the templates is shown to the left of the autoradiogram.

the 3′ terminus of satC minus strands, and the other smaller than template-sized corresponding to initiation in the vicinity of the internal C residues in the 3′-proximal element (Fig. 3B). Changing the A residue in position 5 in the 3′-terminal CCS to G generated construct MR3′(−) (Fig. 3A). *In vitro* transcription of MR3′(−) revealed that the mutation had little or no effect on full-length product synthesis (Fig. 3B). This is in contrast with the *in vivo* results where alteration of this A to G reduced satC accumulation dramatically (construct R7, Fig. 2B). However, levels of the internally initiated transcripts were reduced by 70%, suggesting that the mutation interferes with RNA-RdRp internal initiation in the 3′-proximal element region.

The difference between the *in vivo* and *in vitro* results on the necessity of the 3′-terminal CCS suggests that factors required to initiate plus-strand strand synthesis on isolated minus strands differ from factors necessary for replication beginning with the infecting plus strands. One difference between *in vitro* transcription and *in vivo* replication is that minus-strand templates are directly added to the RdRp extract for *in vitro* transcription, while *in vivo,* the minus strands used for plus-strand synthesis must first be synthesized from plus-strand templates. During synthesis of minus strands *in vivo,* the elongating minus strand likely separates from the plus-strand template with the exception of a short region where active elongation is occurring. Although the length of this transient duplex region is not known for transcription by RdRp, studies using *Escherichia coli* DNA-dependent RNA polymerase (DdRp) indicate that the heteroduplex region in the elongation "bubble" is composed of 9 to 12 bp (Kainz and Roberts, 1992; Wilson *et al.,* 1999). When transcription is completed, the short remaining duplex between the 3′ end of the nascent RNA and the template likely need to dissociate for further RNA synthesis. One possible reason for an *in vivo* but not *in vitro* need for the 3′-terminal CCS is that the multiple consecutive A or U bases facilitate this dissociation. Sivakumaran and Kao (1999) recently reported that substitutions of A or U with G at the the BMV minus-strand 3′ terminus greatly reduced plus-strand RNA synthesis *in vitro,* suggesting that the 3′-terminal region is involved in initiation of RNA synthesis. Although our results indicate that an A to G change in the 3′-terminal CCS of satC minus strands had little or no effect on transcription initiation at the 3′ terminus *in vitro* (MR3′(−), Fig.3), the 3′-terminal CCS, particularly the 3′-end three C residues, likely also play an important role in initiation of plus-strand synthesis *in vivo.* Many other plant RNA viruses such as tobamoviruses, cucumoviruses, tymoviruses, alfalfa mosaic virus, and potexviruses also contain multiple A or U bases following the 3′-end C residue in minus-strand RNA (Ohno *et al.,* 1984; Owen *et al.,* 1990; Chng *et al.,* 1996; Hellendoorn *et al.,* 1996; Van Rossum *et al.,* 1997; Kim and Hemenway, 1999).

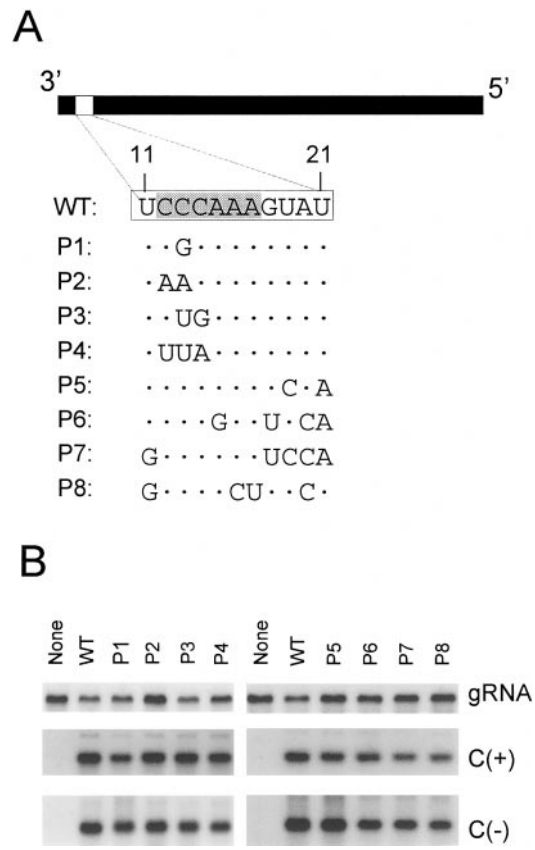

FIG. 4. Mutational analysis of the 3′-proximal element in protoplasts. (A) Mutations introduced into the 3′-proximal element. The 3′-proximal element is denoted by a small open box. Sequence of the 3′-proximal element is shown under the bar and positions with respect to location in the minus strand are indicated. The CCS is shaded. Construct names are shown to the left. (B) RNA gel blot of TCV genomic RNA, and plus- and minus-strand satC containing mutations in the 3′-proximal element. See legend to Fig. 2.

Mutations in the 3′-proximal element have little or no effect on RNA replication in protoplasts

The 3′-proximal element (contained within positions 11 to 21 of satC minus strand) is essential for transcription *in vitro* in the absence of the 5′-proximal element (Guan *et al.,* 1997). To test if the 3′-proximal element is required for satC accumulation in protoplasts, mutational analysis was conducted as described above for the 3′-terminal CCS. Mutagenesis was concentrated on the three consecutive C residues since all TCV promoters contain three consecutive C residues (Simon, 1999). None of the single-, double-, or triple-base mutations in the three consecutive C residues had a significant effect on satC accumulation (P1 to P4, Fig. 4B). Similar results were obtained when 2- to 5-base mutations were introduced into regions other than the three consecutive C residues (P5 to P8, Fig. 4B). These results suggest that the 3′-proximal element, while important for transcription *in vitro* in the absence of the 5′-proximal element, is not required *in vivo.*

To investigate if the multiple consecutive C residues in the 3′-proximal element are required for promoter activity of the element *in vitro,* the three C residues in the element in construct MC3′ (−) were replaced by "UUA" (3′ to 5′ orientation) to generate construct MP3′(−) (Fig. 3A). *In vitro* transcription of MP3′(−) had no effect on full-length complementary strand synthesis but produced no detectable internal initiation product (Fig. 3B). This result is consistent with the finding that the same mutations had little or no effect on RNA accumulation in protoplasts (P4, Fig. 4B). These results suggest that the three C residues in the 3′-proximal element are only required by the TCV RdRp for internal transcription initiation *in vitro* or when the element is serving as a promoter *in vitro* in the absence of the 3′ CCS (Guan *et al.,* 1997).

In vivo genetic selection of the 3′-terminal CCS and the 3′-proximal element of satC minus strands

To help resolve the sequence requirements for satC accumulation in the 3′-terminal 21 bases of minus strands, *in vivo* genetic selection or SELEX (Systematic Evolution of Ligands by EXponential enrichment) was conducted. This method has advantages over site-directed mutagenesis by allowing side-by-side selection from a large number of random sequences as well as sequence evolution (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Carpenter and Simon, 1998). A population of satC transcripts containing 19 randomized bases in positions 3 to 21 was coinoculated with TCV helper virus onto 59 turnip seedlings (the complement of the C residues in positions 1 and 2 is required for efficient transcription by T7 RNA polymerase *in vitro* and was not altered). The large number of plants inoculated was to establish a high sequence complexity for genetic selection. Total RNA was extracted at 21 days postinoculation (dpi) from the upper uninoculated leaves and assayed by agarose gel electrophoresis followed by staining with ethidium bromide. Forty-six of the 59 plants accumulated satC-like species (data not shown). None of the 32 sat-RNA molecules cloned from eight randomly selected plants was wild-type and different satC species were found in each plant (Table 1). Two RNA molecules (S1-16 and S1-18) obtained from plant 33 contained extra bases in the randomized region probably inserted by the TCV RdRp during replication. While several RNA molecules (e.g., S1-2, S1-8, S1-9) had a C residue at position 3 similar to wild-type minus-strand satC, most RNA molecules (13/20 species) contained an A or U residue at this position. However, all the recovered sat-RNA species contained at least three A or U bases following the 3′-terminal two or three C residues, similar to the sequence found at the 3′ ends of minus strands of other carmovirus RNAs and establishing the requirements for a CCS. Some RNA molecules (e.g., S1-2, S1-9, and S1-13) had A or U residues that extended to positions 9 or 10. These results strongly indicate that the CCS at the 3′ terminus of minus strands is required for satC accumulation in plants, which is consistent with the results obtained from the site-directed mutagenesis studies using protoplasts (Fig. 2). Altogether, these results indicate that the 3′-terminal CCS in satC minus strands is required for replication and/or stability of the RNA *in vivo.*

TABLE 1

First-Round *in Vivo* Selection

Plant	Name	Sequence[a]	No. of clones sequenced
	WT	3′HO-*cc*CUAUUGAUUCCCAAAGUAU	
Plant 8	S1-1	*cc*AAUACCAGUC**AGGUGAAGA**	5
	S1-2	*cc*CAAUAAACAUUCU**AAG**UAG	2
	S1-3	*cc*UUUAUACCGAUUU**AAUAAA**	1
Plant 22	S1-4	*cc*AUUUCAAUGUAC**AAAAG**UU	1
	S1-5	*cc*uUUUCAAUGUAC**AAAAG**UU	2
Plant 26	S1-6	*cc*AUUUAACGAUC**AAAGGAA**C	3
	S1-7	*cc*AUUCAAGGGUGCCAACUAA	2
	S1-8	*cc*CAUUGCCAGCUUAUCAUAU	1
Plant 30	S1-9	*cc*CAAUAAUCGAGUGCUCAGU	2
	S1-10	*cc*AAAGAAGGUCUAC**AAAG**UG	1
	S1-11	*cc*AAUAACAGCGUCCCCU**GAG**	1
Plant 31	S1-12a	*cc*CAUUGAACUUUC**AAAAAGA**	2
	S1-12b	*cc*auUUGAACUUUC**AAAAAGA**	1
Plant 32	S1-13	*cc*CUAAAAUUGAAUAC**AGAGA**	1
	S1-14	*cc*CAUUUAACCGCG**CAAAG**CU	1
	S1-15	*cc*CAUUUCGUUAAUGUCCGAC	1
Plant 33	S1-16	*cc*UUUUAAGUCGAGC**AGUAAAGG**	2
	S1-17	*cc*UUAAGUCGAGC**AGUAAAGG**	1
	S1-18	*cc*UUUUAACGGCUGUGCUC**GAA**	1
Plant 36	S1-19	*cc*AAAACGACCU**AGGAAAGG**?	1
		Total clones sequenced	32

[a] Only the 3′-end 21 bases of sat-RNA C minus strands are shown. The two unchanged C residues at the 3′ terminus are shown by lowercase letters in italics. Sat-RNA molecules obtained from different plants are separated by dividers. The wt 3′-proximal element is boxed. Polypurines within this element are in bold. The 3′-terminal CCS and the 3′-proximal element CCS are shaded. Sequences similar to the CCS are boxed in broken lines. Regular lowercase letters denote differences from an arbitrarily selected "parental" molecule. The question mark represents an ambiguous base.

Compared with the 3′-terminus of minus strands, the *in vitro* identified 3′-proximal element was much less conserved. All the recovered sat-RNA species contained different sequences in the three consecutive C region of the 3′-proximal element. Unlike wild-type satC, none of the cloned RNA molecules contained a CCS in the 3′-proximal element region. However, sequences similar to the CCS composed of at least three A or U bases following a single C residue were present in the 3′-proximal element region of 10/20 recovered RNA species. In addition, most of the cloned sat-RNAs (16/20 species), like the wild-type sat-RNA, contained consecutive purines in the 3′-proximal element region.

TABLE 2

Second-Round *in Vivo* Selection

Name	Sequence[a]	Plant[b] 1	2	3	4	5	6	7	8
WT	HO- *cc*CUAUUGAUUCCC**AAA**GUAU								
S2-1a	*cc*CAUUACCGAACC**GGGG**UGA	2		2					
S2-1b	*cc*CAUUACCGAACC**GGGG**cGA		5						
S2-2	*cc*CAUUCAUCAGUGC**GAA**CUA							1	4
S2-3	*cc*CAAUACUCAAACCUU**AAA**C							1	
S2-4	*cc*CAAUAUCAACCAU**GAAG**UA						1		
S2-5	*cc*CUUUGAACUUUC**AAAAAGA**					1			
S2-6	*cc*AUUAGACCAAUAACCUAGU				8		1	2	1
S2-7	*cc*AUUAUCGUCCGC**AGAA**CCU	1							
S2-8	*cc*AUUUAUAAGGUCAUUA**GGU**					1	3		
S2-9	*cc*AUUUGUUGCUACCAGUCGU			3					
S2-10	*cc*AUUUAUUGUCC**GAGAGG**CC					1			
S2-11	*cc*AUAAGCUGCUUCC**AAGAAA**	1				5			
	Total clones sequenced	44							

[a] See the legend to Table 1. Sequence groups are separated by dividers. Two consecutive C residues following an A or U residue in or near the wt three consecutive C region in the 3′-proximal element are underlined.

[b] Number of clones of the sequences found in different plants is indicated.

To identify sat-RNA molecules that are more fit to accumulate, total RNA from the 46 first-round plants was pooled and reinoculated onto 30 turnip seedlings. Three weeks later, satC-sized molecules accumulating in 8 randomly selected plants were cloned and sequenced (Table 2). None of the RNA molecules previously identified in the first round were recovered (although sequence S2-5 differed from S1-12a or S1-12b by a single base), indicating that the RNA molecules identified in the first round were outcompeted for accumulation in the second round by molecules that were likely present but not cloned from the first round. Like the sequences found in the first round, all RNA species obtained in the second round contained the CCS at the 3′ terminus of minus strands, and most RNA molecules (10/12 species) had consecutive purines in the 3′-proximal element region. Nine of 12 RNA species contained two consecutive C residues following an A or U residue near the wild-type three consecutive C region in the 3′-proximal element region. The consecutive C residues were followed by polypurines in some RNA molecules such as S2-1a, S2-1b, S2-10, and S2-11. In RNA species S2-3 and S2-6, the consecutive C residues were followed by at least three A or U bases to form a CCS. S2-6 was the only species found in more than 3 plants. In addition, sequences containing a single C residue followed by three or more A or U bases were found in the 3′-proximal element region of RNA molecules S2-3, S2-5, and S2-8.

Total RNAs from second-round plants were combined and inoculated onto 12 turnip seedlings for a third and final round of selection. Cloning of the progeny sat-RNA accumulating in eight of the infected plants at 21 dpi indicated that the wild-type minus-strand TCV genomic RNA 3′-terminal CCS (3′-CCAUU) was present in all clones (Table 3). Two main species (S2-6 and S2-8), previously found in the second round, were recovered in multiple plants. Like wild-type satC, S2-6 contained the CCS at the 3′ terminus as well as in the 3′-proximal element region. This suggests that the carmovirus consensus sequence in the 3′-proximal element contributes to RNA accumulation *in vivo.* S2-8 was found in six plants and contained eight A or U residues following the 3′-end C residues, suggesting that a long stretch of A or U bases at the 3′ terminus may also enhance RNA accumulation. Other TCV-associated RNAs, including sat-RNA D, defective interfering RNA G, and the two subgenomic RNAs (1.45 and 1.7 kb), do not contain a second CCS near their terminal CCS and also contain seven to nine A or U bases following consecutive G residues at the 5′ ends of their plus strands. The major SELEX species S2-8 also contained a sequence (3′-CAUUA) similar to the carmovirus consensus sequence in the 3′-proximal element region. Four previously unsequenced sat-RNA molecules (S3-1, S3-2, S3-3, and S3-4) were also cloned, but each was a minor species detected only in single plants.

Competition assays were carried out to determine how well species S2-6 and S2-8 competed for accumulation with wild-type satC. Equal amounts of plus-sense

TABLE 3

Third-Round *in Vivo* Selection

Name	Sequence[a]	Plant[b] 1	2	3	4	5	6	7	8
WT	HO- *cc*CUAUUGAUUCCC**AAA**GUAU								
S2-6	*cc*AUUAGACCAAUAACCUAGU	1		5	4	2			1
S2-8	*cc*AUUUAUAAGGUCAUUA**GGU**	3	3			1	4	3	2
S2-8a	*cc*AUUUAUAAGGUCgUU**AGGU**					1			
S3-1	*cc*AUUUGCUUGCAAAAAGACG	1						1	
S3-2	*cc*AUUAACUGUCCGU**AAGAA**U					1			
S3-3	*cc*CAUUUAACCGCGCAAA**G**CU								1
S3-4	*cc*CAUUUAUCAGUGC**GAA**CUA		1						
	Total clones sequenced	35							

[a] See the legend to Table 1. Sequence groups are separated by dividers.

[b] The number of clones of each sequence found in the plants is indicated.

transcripts of wild-type satC and sequence S2-6 or S2-8 were coinoculated with TCV helper virus onto six plants. Sat-RNA molecules accumulating in the six plants were cloned 3 weeks later. Only two out of 19 clones were S2-6 in plants coinoculated with wild-type satC, while only wild-type satC was recovered in plants coinoculated with sequence S2-8. These results indicate that wild-type satC is more competitive for accumulation than either of the two RNA species identified by *in vivo* SELEX.

Mutations introduced into the 3′-proximal element had little or no effect on satC accumulation in protoplasts (Fig. 4B). However, *in vivo* SELEX results suggest that the sequence in this region is not random and thus this element may be involved in enhancing RNA accumulation in plants. Like wild-type satC, the two third-round winners S2-6 and S2-8 contained a CCS or a sequence similar to the CCS in the 3′-proximal element region and many of the other selected sequences contained polypurines within this region. It is noteworthy that in the site-directed mutagenesis study, mutations were introduced only into the region of the three C residues or the polypurines but not both. In addition, these mutants were not subjected to direct competition with wild-type satC.

Although wild-type satC was not recovered in the *in vivo* SELEX, the wild-type sequence was recovered when the 5′-proximal element (14 bases) was subjected to selection (Guan *et al.,* 2000). It is likely that a combination of the shorter randomized sequence and the necessity for specific nucleotides at many of the 14 positions of the 5′-proximal element contributed to recovery of the wild-type sequence in that study.

In addition to the requirement of specific sequences, *cis*-acting structures and/or RNA-RNA interactions between the 3′ terminus of minus strands (or the 5′ terminus of plus strands) and internal regions of viral RNA can be important for plus-strand synthesis. For example, *cis*-acting sequences and stem-loop structures in the 5′ region of PVX genomic RNA are required for plus-strand synthesis (Kim and Hemenway, 1996; Miller *et al.,* 1998). Furthermore, long-distance interactions between the 3′ terminus of PVX minus-strand RNA (or the 5′ terminus of the genomic RNA) and an internal site are required for plus-strand RNA accumulation (Kim and Hemenway, 1999). In addition to the minus-strand 3′-terminal CCS, 3′-proximal element, and 5′-proximal element (Guan *et al.,* 2000), a hairpin structure called the motif1-hairpin is required for plus-strand synthesis *in vivo* and may serve as a replication enhancer (Nagy *et al.,* 1999). Further studies are currently underway to understand how these elements function together during satC plus-strand synthesis.

MATERIALS AND METHODS

Site-directed mutagenesis

Site-specific mutations were introduced into the 3′-terminal region of satC minus strands using polymerase chain reaction (PCR). Oligonucleotides T7C5′GNN, T7C5′N3, and T7C5′N4 (Table 4) were used separately with oligo 7 as primers for the PCRs. The template was plasmid pT7C(+), which contains a full-length satC cDNA immediately downstream from a T7 RNA polymerase promoter (Song and Simon, 1995). The PCR product was treated with T4 DNA polymerase and cloned into the *Sma*I site of pUC 19. All the clones were sequenced and contained a T7 RNA polymerase promoter upstream of full-length plus-sense satC containing site-specific mutations in the 3′-terminal region.

To introduce mutations into the 3′-proximal element, oligonucleotide T7C5′N1 or T7C5′N2 was used with oligo 7 as the primers. All PCR products were cloned into pUC 19 as described above.

In vitro transcription and inoculation of *Arabidopsis* protoplasts

*Sma*I-digested plasmids were subjected to *in vitro* transcription using T7 RNA polymerase (Carpenter *et al.,* 1995). The synthesized RNA contained the wild-type 3′ end. Protoplasts (5×10^6) prepared from Col-0 callus cultures were inoculated with 20 μg of TCV genomic RNA transcripts and 2.0 μg of either wild-type or mutant satC transcripts as described previously (Kong *et al.,* 1995).

RNA gel-blot analysis

Equal amounts of total RNA isolated from 2×10^5 protoplasts at 24 h postinoculation were used for RNA gel-blot analysis of TCV genomic RNA and plus-strand satC (Kong *et al.,* 1995). An oligonucleotide complementary to positions 269 to 288 of TCV and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase was used to detect the genomic RNA. The same blot was reprobed with an oligonucleotide complementary to positions 175 to 199 of satC to detect satC plus strand. For probing minus-strand satC, total RNA extracted from 8×10^5 infected protoplasts was annealed with 1.0 μg of wild-type or mutant satC plus-strand transcripts in 30 μl of annealing buffer (300 mM KCl, 50 mM Tris-HCl, 1 mM EDTA at pH 8.0) and treated with RNase A (final concentration 1.0 μg/ml) as described by Ishikawa *et al.* (1991). Annealed RNAs were then treated with 0.1 mg/ml of Pronase and 0.4% SDS at 30°C for 30 min and recovered by extraction with phenol-chloroform and precipitation with ethanol. The recovered RNAs were then subjected to gel-blot analysis as described by Kong *et al.* (1995) using an oligonucleotide complementary to positions 57 to 78 of satC minus strand. Autoradiograms were scanned with a GS-700 densitometer (Bio-Rad).

Construction of RNA chimeras

RNA chimeras were constructed by joining positions 1 to 40 of satC minus strands to the 3′ end of MDV RNA, a

TABLE 4

List of Oligonucleotides Used for PCR in This Study

Application/ construct	Name	Sequence[a]	Position in satC-RNA	Polarity[b]
Site-directed mutagenesis	T7C5′N1	5′-T7-GGGATAACTAA**NNN**TTTCATAC	1–22	+
	T7C5′N2	5′-T7-GGGATAACTA(A/**C**)GGG(T/C)(T/**G**)(T/**A**)(C/**A**)-(A/**G**)(T/**G**)(A/**T**)CAATACTACGCAACTAA	1–38	+
	T7C5′GNN	5′-T7-G**NN**ATAACTAAGGG	1–14	+
	T7C5′N3	5′-T7-GGG(A/**C**)(T/**C**)(A/**G**/**C**)ACTAAGGGTTTCATAC	1–22	+
	T7C5′N4	5′-T7-GGGA(T/**C**)(A/**T**)(A/**C**/**T**)(C/**G**/**A**)(T/**G**)(A/**T**)AGGGTTTCATA-CAA	1–24	+
Construction of chimeric RNA	C5′	5′-GGGATAACTAAGGGTTTCA	1–19	+
	C5′m	5′-GGGATAACTAA**AAT**TTTCATAC	1–22	+
	C5′mc	5′-GGGA**C**AACTAAGGGTTTCATA	1–21	+
	oligo C(−)40	5′-CATTAGTTGCGTAGTATTG	22–40	−
SELEX	T7C5′SEL	5′-T7-GG**NNNNNNNNNNNNNNNNNNN**CAATACTACGCAAC-TAATGC	1–41	+
	C22-41	5′-CAATACTACGCAACTAATGC	22–41	+
5′-RACE PCR	5′-RACE-G10	5′-GGCCACGCGTCGACTAGTACGGGGGGGGGG		
pT7S2-8	T7(−)3′sw1	5′-T7-GGTAAATATTCCAGTAATC	1–19 (in S2-8)	+
pT7S2-6	T7(−)3′sw2	5′-T7-GGTAATCTGGTTATTGG	1–17 (in S2-6)	+
3′-primer	oligo 7	5′-GGGCAGGCCCCCCGTCCGA	338–356	−

[a] "T7" indicates T7 promoter sequence (5′-GTAATACGACTCACTATA). "N" represents randomized bases. Mutations are denoted by bold letters. Alternative mutations at a particular position are included in parentheses.

[b] "+" and "−" polarities refer to homology and complementarity with sat-RNA C plus strand, respectively.

small RNA associated with Qβ bacteriophage. cDNA fragments containing positions 1 to 40 were amplified by PCR from template pT7C(+). For construction of chimeras MC3′(−), MP3′(−), and MR3′(−), oligonucleotides C5′, C5′m, and C5′mc were used respectively with oligonucleotide C(−)40 as primers for the PCR. The PCR products were treated with T4 DNA polymerase and cloned into the *Sma*I site of pT7MDV (Axelrod *et al.,* 1991). Plasmids containing positions 1 to 40 of satC in minus-strand orientation were selected and linearized with *Sma*I for *in vitro* transcription using T7 RNA polymerase.

In vitro transcription using the TCV RdRp

In vitro transcription using partially purified TCV RdRp was carried out as described previously (Guan *et al.,* 1997).

In vivo genetic selection

In vivo genetic selection was performed as described previously (Carpenter and Simon, 1998). To generate full-length satC templates containing randomized bases in positions 3 to 21, a cDNA product containing positions 22 to 356 of satC was amplified by PCR from pT7C(+) using primers C22-41 and oligo 7. The PCR fragment was purified through an agarose gel to eliminate contamination of full-length satC sequence in pT7C(+). The purified product was then used as template in a second PCR with primers T7C5′SEL and oligo 7 to generate full-length plus-sense satC cDNA containing randomized bases in positions 3 to 21.

For plant infection, transcripts (~5 μg/plant) synthesized directly from the second PCR product were coinoculated with TCV helper virus (total RNA extracted from plants infected with TCV genomic RNA transcripts, ~5 μg/plant) onto 59 two-week-old turnip seedlings (Li *et al.,* 1989). Total RNA was prepared from uninoculated leaves at 21 days postinoculation and subjected to agarose gel electrophoresis to determine which plants were accumulating satC-like species. SatC-sized species accumulating in 8 randomly selected plants were amplified by 5′-RACE (rapid amplification of cDNA ends) PCR as described below and cloned into the *Sma*I site of pUC 19. All clones were sequenced using a primer complementary to positions 60 to 79 of satC plus strand.

For the second-round inoculations, total RNA from first-round 46 plants (the other 13 plants did not contain satC species) containing relatively equal amounts of sat-RNA species as visualized by ethidium bromide-stained agarose gels was pooled and reinoculated onto 30 turnip seedlings (~5 μg/plant). SatC-sized species extracted from 8 plants at 21 dpi were assayed as described above. For the third-round selection, total RNA from the 30 plants of the second round were pooled and reinoculated onto 12 plants (~5 μg/plant).

5′-RACE PCR of satC-like species

5′-RACE PCR was carried out according to Gibco BRL's 5′-RACE PCR protocol with modifications. Briefly, satC-sized species were purified from agarose gel and subjected to reverse transcription in the presence of oligo 7. After being treated with RNase A (0.1 μg/μl) and RNase H (1 U/μl) mix for 30 min at 37°C, the first-strand cDNA was purified from agarose gels to eliminate the unused primer and dNTPs. The purified cDNA was then treated with terminal deoxynucleotidyltransferase in the presence of 2 mM dCTP. Oligonucleotide 5′-RACE-G10 was used with oligo 7 in a PCR to amplify the dCTP-tailed cDNA.

Competition assays between wild-type satC and sequence S2-6 or S2-8

Since S2-6 and S2-8 clones obtained from *in vivo* SELEX contained additional bases added during cloning, it was necessary to remove the additional bases to generate biologically active RNA. To construct full-length cDNA of sequences S2-6 and S2-8, oligonucleotides T7(−)3′sw1 and T7(−)3′sw2 were used, respectively, with oligo 7 as primers in the PCR. PCR products were treated with T4 DNA polymerase and then cloned into the *Sma*I site of pUC 19 to generate pT7S2-6 and pT7S2-8, which contained full-length cDNA of sequences S2-6 and S2-8 (in plus-sense orientation) immediately downstream from a T7 RNA polymerase promoter, respectively. To obtain RNA transcripts, plasmids pT7S2-6 and pT7S2-8 were linearized with *Sma*I and then subjected to *in vitro* transcription using T7 RNA polymerase.

To conduct competition assays, equal amounts of transcripts (0.2 μg/plant) of wild-type satC and sequence S2-6 or S2-8 were coinoculated onto six turnip seedlings together with TCV genomic RNA transcripts (2 μg/plant). Total RNA was isolated at 21 dpi and satC-like molecules were cloned as described above.

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