Small-circular satellite RNAs (scRNAs) and viroids are small, replicating, circular subviral RNAs (194–450 bases) and are mostly plant pathogens, with human hepatitis delta virus satellite RNA being the only known exception (Flores et al., 2005; Taylor, 2003). scRNAs, unlike viroids, depend on helper RNA viruses for replication and encapsidation. scRNAs do not encode functional open reading frames and have no sequence similarity to their helper virus genome, but do possess a self-splicing ribozyme activity. The ribozyme activity is involved in autocatalytic processing during a rolling circle mode of replication (Branch & Robertson, 1984; Bruening et al., 1991; Flores et al., 2005). The RNA-dependent RNA polymerase of the helper virus, with host factors, forms a replicase complex that replicates the satellite RNA of lucerne transient streak virus (scLTSV) through the symmetrical rolling circle model (Collins et al., 1998; Daro’s, 1994; Gellatly, 1994, 1995). Studies in vitro and in vivo have proved that scLTSV can self-cleave in both polarities (Sheldon & Symons, 1993).

Three isolates of scLTSV are known. The two Australasian isolates (324 nt) (Keese et al., 1983) and one Canadian isolate (322 nt) share only 80% sequence similarity (AbouHaidar & Paliwal, 1988). RNA folding of all three isolates of scLTSV shows that up to 70% of residues are base-paired (Fig. 1a) (Zuker & Stiegler, 1981; Zuker, 2003). This structure is similar to that of viroids (Gross & Riesner, 1980) and other scRNAs associated with the sobemovirus group (Haseloff & Symons, 1982). The relationship between scLTSV and its helper virus is also host-dependent. Turnip rosette virus (TRosV) was reported to support replication of scLTSV effectively in Brassica rapa, Raphanus raphanistrum and Sinapis arvensis, but not in Thlaspi arvense or Nicotiana bigelovii (Sehgal et al., 1993a). Specific host factors involved with the RNA-dependent RNA polymerase (RDRP) of the helper virus may be a reason for host specificity (Sehgal et al., 1993a, b). The presence of scLTSV in LTSV virions has a significant influence on symptoms (Jones et al., 1983).

We report here that insertion and/or deletion mutants that cause perturbations to the overall scLTSV molecule are lethal to its infectivity. Foreign sequences introduced into scLTSV will only be tolerated if they maintain the overall cross-like structure. However, the inserted foreign nucleotides are removed in successive generations to reproduce the wild-type scLTSV.

Full-length monomeric and head-to-tail dimeric and trimeric cDNA clones of scLTSV were constructed in a modified pBS+ plasmid at the Ncol (position 122) restriction site (Maniatis et al., 1989). RNA transcripts from clones of both polarities were generated in vitro using T3 or T7 polymerases. Each transcript was co-inoculated with satellite-free TRosV (as a helper virus) to B. rapa. T7 RNA polymerase transcripts contained 21 non-scLTSV
Role of scLTSV sequence and structure in replication

(e) 322I-9

\[ dG = -123 \]

(f) 280D-4

\[ dG = -105 \]

(g) 280D-50

\[ dG = -106 \]

(h) 280D-108

\[ dG = -79 \]
sequences (from pBS (hammerhead ribozymes in plus and minus sense). Their polarities were infectious, presumably following self-splicing trimeric (not monomeric) scLTSV RNA transcripts of both RNA transcript alone or buffer alone. Only dimeric and infected with LTSV (non-host) alone, scLTSV monomeric TRosV alone. No virions could be recovered from scLTSV RNA progeny is observed in plants infected with trimeric scLTSV positive- or negative-sense transcripts. No progeny in plants infected with TRosV plus dimeric and/or virions and total RNA showed the presence of scLTSV. The RT-PCR product of RNA extracted from purified 303 of scLTSV were used for most RT-PCRs to detect specific DNA primers located at positions 11–30 and 322–303 of scLTSV were used for most RT-PCRs to detect positive-sense scLTSV RNA.

The RT-PCR product of RNA extracted from purified virions and total RNA showed the presence of scLTSV progeny in plants infected with TRosV plus dimeric and/or trimeric scLTSV positive- or negative-sense transcripts. No scLTSV RNA progeny is observed in plants infected with TRosV alone. No virions could be recovered from B. rapa infected with LTVS (non-host) alone, scLTSV monomeric RNA transcript alone or buffer alone. Only dimeric and trimeric (not monomeric) scLTSV RNA transcripts of both polarities were infectious, presumably following self-splicing (hammerhead ribozymes in plus and minus sense). Their progeny RNA was identical to the native scLTSV. Foreign sequences (from pBS+) were discarded in vivo during the self-splicing or replication processes. scLTSV replicates via a symmetrical rolling-circle pathway (Hutchins et al., 1985). Positive- and negative-sense dimeric and trimeric RNAs of scLTSV were equally infectious in the current study, indicating the highly infectious nature of this RNA pathogen (Symons, 1992).

To investigate the structure–function relationship of scLTSV and to determine specific sequences and/or structures that may play a role in its biological function, a library of mutants was constructed (Fig. 1a) and each of them was cloned as head-to-tail trimers in pBS (position 280) followed by a partial end-filling to generate a 3 nt insertion at three different positions throughout the scLTSV sequence were disrupted (Fig. 1). A series of smaller insertion and deletion mutants at various positions throughout the scLTSV sequence were also generated (Fig. 1). Three small insertion mutants, with a 3 nt insertion at three different Ddel sites of scLTSV (at positions 17, 167 and 284), were created by a partial Ddel digest followed by end-filling. One Ddel site (at position 17) was located near the end of the rod, opposite the hammerhead domain. This insertion was close to a GAGAUUUU sequence that is conserved in all the scRNAs associated with sobemoviruses. The other two Ddel sites (positions 284 and 167) were found near the BglII site and the hammerhead (+) sites, respectively. All three mutants were non-infectious. A BglII digestion (position 256) was followed by a partial end-filling to generate a +2 nt insertion, and a BglII digestion (position 280) followed by a +2 insertion and a –1 base deletion at the unique NciI site located at position 242 (–1 base deletion generated by NciI restriction followed by mung bean nuclease digestion) were synthesized (Fig. 1a). Only the –1 base deletion mutant at the NciI site was infectious. This mutation is in the region known to show large sequence variability between all scLTSV isolates. The effect of the small insertion/deletion clones on the wild-type secondary structure varied with each clone.

Another mutant, 322I-9, with a 9 nt insertion at the left end (position 1) of the molecule (Fig. 1e), was also infectious. This clone was generated by insertion of a 9 nt palindrome (NolI site; GCGGCCGC). scLTSV cDNA was digested with AluI (positions 4 and 319) and then ligated to the NolI synthetic linker with compatible AluI sticky ends. The resulting hairpin extended the loop at the left end without disturbing the overall secondary structure of the molecule.

Total plant RNA (or RNA from purified virus) was extracted 5, 12 and 21 days post-infection (p.i.) from systemically infected (uninoculated) leaves. Full-length RT-PCR products of 322I-9 clones were synthesized and sequenced. Results showed that the NolI sequence (9 nt) nucleotides at the 5’ end and 37 nucleotides at the 3’ end. Similarly, T3 RNA polymerase transcribes included 45 and 12 nucleotides, respectively, at the 5’ and 3’ ends.

Infectivity assays were conducted to determine the capacity of each clone to replicate, move from cell-to-cell and package its RNA into TRosV capsid. Only total plant RNA (or purified virus) extracted from systemically infected uninoculated leaves was used in this study. Mutations that might theoretically affect cell-to-cell movement or minus RNA synthesis specifically were not investigated. Two specific DNA primers located at positions 11–30 and 322–303 of scLTSV were used for most RT-PCRs to detect positive-sense scLTSV RNA.

The RT-PCR product of RNA extracted from purified virions and total RNA showed the presence of scLTSV progeny in plants infected with TRosV plus dimeric and/or trimeric scLTSV positive- or negative-sense transcripts. No scLTSV RNA progeny is observed in plants infected with TRosV alone. No virions could be recovered from B. rapa infected with LTVS (non-host) alone, scLTSV monomeric RNA transcript alone or buffer alone. Only dimeric and trimeric (not monomeric) scLTSV RNA transcripts of both polarities were infectious, presumably following self-splicing (hammerhead ribozymes in plus and minus sense). Their progeny RNA was identical to the native scLTSV. Foreign sequences (from pBS+) were discarded in vivo during the self-splicing or replication processes. scLTSV replicates via a symmetrical rolling-circle pathway (Hutchins et al., 1985). Positive- and negative-sense dimeric and trimeric RNAs of scLTSV were equally infectious in the current study, indicating the highly infectious nature of this RNA pathogen (Symons, 1992).

To investigate the structure–function relationship of scLTSV and to determine specific sequences and/or structures that may play a role in its biological function, a library of mutants was constructed (Fig. 1a) and each of them was cloned as head-to-tail trimers in pBS (position 280) followed by a partial end-filling to generate a 3 nt insertion at three different positions throughout the scLTSV sequence were disrupted (Fig. 1). A series of smaller insertion and deletion mutants at various positions throughout the scLTSV sequence were also generated (Fig. 1). Three small insertion mutants, with a 3 nt insertion at three different Ddel sites of scLTSV (at positions 17, 167 and 284), were created by a partial Ddel digest followed by end-filling. One Ddel site (at position 17) was located near the end of the rod, opposite the hammerhead domain. This insertion was close to a GAGAUUUU sequence that is conserved in all the scRNAs associated with sobemoviruses. The other two Ddel sites (positions 284 and 167) were found near the BglII site and the hammerhead (+) sites, respectively. All three mutants were non-infectious. A BglII digestion (position 256) was followed by a partial end-filling to generate a +2 nt insertion, and a BglII digestion (position 280) followed by a +2 insertion and a –1 base deletion at the unique NciI site located at position 242 (–1 base deletion generated by NciI restriction followed by mung bean nuclease digestion) were synthesized (Fig. 1a). Only the –1 base deletion mutant at the NciI site was infectious. This mutation is in the region known to show large sequence variability between all scLTSV isolates. The effect of the small insertion/deletion clones on the wild-type secondary structure varied with each clone.

Another mutant, 322I-9, with a 9 nt insertion at the left end (position 1) of the molecule (Fig. 1e), was also infectious. This clone was generated by insertion of a 9 nt palindrome (NolI site; GCGGCCGC). scLTSV cDNA was digested with AluI (positions 4 and 319) and then ligated to the NolI synthetic linker with compatible AluI sticky ends. The resulting hairpin extended the loop at the left end without disturbing the overall secondary structure of the molecule.

Total plant RNA (or RNA from purified virus) was extracted 5, 12 and 21 days post-infection (p.i.) from systemically infected (uninoculated) leaves. Full-length RT-PCR products of 322I-9 clones were synthesized and sequenced. Results showed that the NolI sequence (9 nt)
Fig. 2. Sequence variations (shown on the native folding of 322I-9) of progeny RNAs for 322I-9 mutants recovered from infected plants at 5–21 days p.i. Only the left side of the original 322I-9 (with sequence variations) is shown. (a–c) RNA progeny sequences of 322I-9 clones (I9T-1–9) recovered from infected plants after 5 days p.i. Sequences of I9T-1–6 and I9T-9 (a) are identical to that of the original 322I-9 mutant. (d–g) Sequences of clones (I9T-11–20) recovered after 12 days p.i. Sequences of all clones in (g) and those recovered after 21 days p.i. (not shown) were identical to that of wild-type scLTSV. ‘Compensatory’ mutations are shown outside boxed nucleotides.

http://vir.sgmjournals.org

http://vir.sgmjournals.org
was replicated and packaged by TRosV capsid protein. Sequencing of 10 clones from 5 days p.i. showed that the *Not*I restriction site had been maintained (Fig. 2a–c; clones I9T-1–6, I9T-9), and the sequences were identical to that of the original 322I-9 mutant except for two clones (I9T-7 and I9T-8), which possessed additional point mutations adjacent to the *Not*I restriction site. However, another 10 clones, selected from 12 days p.i. (Fig. 2d–f), showed several mutations. Data indicated that only three of the 10 clones retained the *Not*I site. In each clone that retained the *Not*I sequence, two or three point mutations were present in close proximity to the insertion site, while the rest of the sequence was unchanged (Fig. 2d–f; clones I9T-11, I9T-14 and I9T-15). Presumably, these mutations have a role in stabilizing the RNA from local structural stresses created by the insertion of a foreign sequence. It must be noted that all the 'stabilizing' mutations occur at nucleotides that are conserved among the various strains of scLTSV. Clones generated from TRosV and native scLTSV-infected plants did not show any such mutations. It was concluded the cloning, amplification and other experimental processes alone could not account for the observed mutations in the *Not*I-retaining progeny RNAs. RNA folding of those mutants revealed that the secondary structures are preserved and very similar to that of 322I-9 (not shown), presumably because the terminal hairpin is quite stable and it tolerates a small change in the primary sequence of those clones. Intriguingly, in about 70% of the clones from day 12 p.i. (Fig. 2g; I9T-12, -13 and -16–20) examined, the inserted *Not*I sequence was missing entirely, suggesting a complete reversion to wild-type. In contrast to the day 5 p.i. samples, in which the 9 nt insertion was conserved, it appears that the *Not*I site is eliminated abruptly thereafter, rather than transitionally. All progeny of clones obtained from 21 days p.i. had lost the *Not*I site and reverted to wild-type scLTSV.

From the data presented here, it is clear that scLTSV is not tolerant of any modifications to its overall sequence and/or structure. The only viable mutants were the −1 base deletion at the *Nco*I site and the insertion of 9 nt at the left end (position 1) of the scLTSV molecule. Identical results were obtained whether total cellular RNA or RNA extracted from purified virus of systemically (uninoculated) infected plants was used. This result confirms that the mutations affected replication and/or cell-to-cell movement and not packaging of scLTSV in the TRosV capsid. Comparison of scLTSV (Canadian and Australasian) isolates revealed that some sequences (positions 62–75, 85–100, 245–270 and 298–315) are not conserved. However, the folding into a cross shape (Fig. 1a) of the isolates is quite preserved (Supplementary Fig. S1, available in JGV Online). Some of our insertion and deletion mutations targeted those non-conserved areas. The sequence covering positions 100–220 is quite conserved and involves the ribozymes (positive and negative sense), and was not targeted by the mutations.

scLTSV has been shown to replicate in association with four sobemoviruses (Francki *et al.*, 1983; Jones & Mayo, 1984; Paliwal, 1984; Sehgal *et al.*, 1993a, b), including TRosV, that are normally devoid of satellite RNAs. Mutation studies on other satellite RNAs have yielded varying results. Masuta & Takanami (1989) found that, although a cauliflower mosaic virus satellite was tolerant of small insertions (four bases), deletion mutations were detrimental to the viability of the satellite RNA. On the other hand, Dalmay & Rubino (1995) reported that transcripts from mutant clones of cymbidium ringspot tombusvirus satellite (sCyRSV) that lacked up to eight nucleotides at the 3′ end were biologically active and yielded RNA progeny that had the 3′ end restored, as in wild-type RNA. However, deletions and substitutions at the 5′ end produced non-viable molecules. Any mutation in the internal regions affected the replication of the satellite RNA.

It is clear that our deletion/insertion mutants modified the native sequence and/or structure that is essential for the helper virus (and host) replicase to replicate the scLTSV RNA. They may also contribute to the packaging and/or transport of scLTSV within the cell. Since scLTSV has a very small genome (322 nt), it is presumed that every nucleotide has a function. Most of our mutants were lethal. This is not due to the lack of packaging (clones generated from total cellular RNA) by the capsid protein, but probably arises because their sequence/secondary structure was destabilized or changed to result in poor replication or no replication or no virus movement (see Fig. 1). Similar results have been described for the replication of viroids (Zhong *et al.*, 2008; Baumstark *et al.*, 1997; Bussière *et al.*, 1996). The (−1) deletion mutant at the *Nco*I site was not lethal. Similar results for deletion mutants in the (−) hammerhead region of the molecule were infectious (Sheldon & Symons, 1993).

Because mutant 322I-9 was capable of replication and packaging, and the insertion was maintained in progeny RNA, the notion that overall structural integrity is important to scLTSV replication is supported. Although the extra nucleotides are maintained for the first few rounds of replication, they do not have any 'biological function' and are soon eliminated. In conclusion, some rare single nucleotides (such as the −1 deletion at the *Nco*I site) are dispensable for the replication of scLTSV; others could be added and tolerated (322I-9). However, there exists no tolerance for sequence/structural perturbations. scLTSV, unlike other scRNAs, has an optimal size and sequence that cannot be modified.

The (−) and (+) strands of scLTSV were equally infectious. However, since only the scLTSV (+) strand is encapsidated, the origin of assembly sequence (OAS)/secondary structure should be present only in the (+) strand. Searches for common secondary structures at the 3′ ends of the (+) and (−) strands of TRosV (helper) that encompass the origin of genomic RNA replication revealed no obvious similar primary or secondary structures. The hammerhead ribozyme in scLTSV probably forms only transiently, as described by Song & Miller (2004) for the satRPV RNA.
Satellite RNAs are so compact that any given sequence may participate in more than one function, such as self-cleavage/ligation, origins of replication, cell-to-cell movement and assembly, which are achieved by different conformations of the same sequence. They become important model systems for understanding the minimum molecular requirements for replication, assembly and interactions with helper virus–host factors. Future experiments may shed some light on the role of structural variations of scLTSV that seem to interact with the host component of RDRP to allow the replication of scLTSV in *B. rapa* but not in *N. bigelovii* or *T. arvense* (Sehgal et al., 1993a, b). In conclusion, in this group of satellite RNAs, the entire sequence seems to be important for replication. This result is different from that described for tomato bushy stunt virus satellite RNAs (Rubino & Russo, 2010) or defective-interfering RNAs (Wu et al., 2009), where small sequences and/or structures were identified as sufficient for replication.

### References


