The conserved 5′ apical hairpin stem loops of bamboo mosaic virus and its satellite RNA contribute to replication competence

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ABSTRACT

Satellite RNAs associated with Bamboo mosaic virus (satBaMVs) depend on BaMV for replication and encapsidation. Certain satBaMVs isolated from natural fields significantly interfere with BaMV replication. The 5′ apical hairpin stem loop (AHSL) of satBaMV is the major determinant in interference with BaMV replication. In this study, by in vivo competition assay, we revealed that the sequence and structure of AHSL, along with specific nucleotides (C60 and C83) required for interference with BaMV replication, are also involved in replication competition among satBaMV variants. Moreover, all of the 5′ ends of natural BaMV isolates contain the similar AHSLs having conserved nucleotides (C64 and C86) with those of interfering satBaMVs, suggesting their co-evolution. Mutational analyses revealed that C86 was essential for BaMV replication, and that replacement of C64 with U reduced replication efficiency. The non-interfering satBaMV interfered with BaMV replication with the BaMV-C64U mutant as helper. These findings suggest that two cytosines at the equivalent positions in the AHSLs of BaMV and satBaMV play a crucial role in replication competence. The downregulation level, which is dependent upon the molar ratio of interfering satBaMV to BaMV, implies that there is competition for limited replication machinery.

INTRODUCTION

The genomes of positive-strand RNA viruses contain multiple functional RNA elements (1) that are responsible for performing specific tasks in a variety of fundamental viral processes including the synthesis of viral RNA (2,3), translation of the viral gene (4), cell-to-cell movement (5) and formation of the viral particle (6–8). Satellite RNAs (satRNAs) are subviral agents that depend on their cognate helper viruses for replication, movement and encapsidation. Since there are little or no significant sequence similarities between satRNAs and helper viruses (9–11), the RNA elements required for satRNA replication are distinct from those of the helper. However, increasing evidence has shown that satRNAs contain specific cis-sequences and/or structure adaptations that recognize the RNA-dependent RNA polymerase (RdRp) of the helper virus, thus enabling replication (12,13). For example, the conserved 5′ terminal T-shaped domain and downstream domain found in different tombusvirus genomes and satRNAs are involved in viral RNA replication (14,15); the 3′ terminus of Cucumber mosaic virus (CMV) satellite RNA shares considerable sequence and structural similarity with the tRNA-like structure of CMV genomic RNAs (16); and mimicry of the 5′- and 3′-termini was found in Bamboo mosaic virus (BaMV) and its associated satellite RNA (satBaMV) (17–19). These and other satRNAs have evolved various elements or structures to mimic their helper viruses in order to use the resources provided by these helpers (12).

Similar to RNA viruses, satRNAs contain great genetic heterogeneity as a result of error-prone replication of RNA genomes (20). The replication of satRNA could enhance, attenuate or not affect the pathogenicity of the helper virus depending on the propagation host, the helper virus and the satRNA variant (21). Some domains in satRNAs have also been found to be responsible for pathogenic phenotypes. For example, the region near the 5′ end of the satRNA variant NM3c involved in this variant’s replication is also required for downregulation of...
Groundnut rosette virus (GRV) replication and attenuation of GRV-induced symptoms (22); the necrogenicity domain of CMV satRNAs is a hairpin structure containing an octanucleotide loop and an adjacent stem within the 5' half of the minus-strand that induces necrosis in tomato plants (23). Moreover, domains of satRNA variants involved in the pathogenic phenotypes might also be involved in their replication competition. For example, tomato plants co-inoculated with CMV-77.2 and mixtures of two satRNA variants, Tfn-satRNA (a benign variant) and 77-satRNA (a necrogenic variant), showed necrosis symptoms and the selective accumulation of 77-satRNA (24); satRNA B10, which attenuates the symptoms induced by TBSV, was the dominant species when co-inoculated with non-interfering satRNA B1 under the help of TBSV-Ch (25). Whether the necrosis domain of CMV satRNA is involved in the replication competition among satRNA variants is still unknown. The domain of satRNA B10 required for attenuating TBSV-induced symptoms also remains to be determined.

BaMV belongs to the potexvirus genus and has a single-stranded, positive-sense RNA genome of ~6400 nt. The genomic RNA of BaMV contains five conserved open reading frames (ORFs) flanked by 5'- and 3'-untranslated regions (UTRs) of 94 and 142 nt, respectively (Figure 1A) (26,27). ORF 1 encodes a 155-kDa viral replicase containing capping enzyme (28–30), helicase (31) and RdRp domains (32). ORFs 2–4 encode triple block proteins of 28, 13 and 6 kDa, respectively, which are involved in cell-to-cell movement of the virus (33,34). ORF 5 encodes the 25-kDa capsid protein, which is required for viral RNA encapsidation, movement and symptom expression (35). Some BaMV isolates also contain satBaMV, which is a linear RNA molecule of 836 nt with a single ORF for a 20-kDa protein flanked by a 5'-UTR of 159 nt and a 3'-UTR of 125 nt (36). Recent studies revealed several common functional RNA elements and structures in the 5'- and 3'-UTRs of BaMV and satBaMV (12,19). In the 5'-UTR, the GAAA(A) repeat is conserved in the very termini and conserved secondary structure of the apical hairpin stem loop (AHSL) of BaMV and satBaMV play crucial roles in BaMV and satBaMV replication (17). The RNA structure and nucleotide sequence required for this attenuation of satBaMV to mediate downregulation of BaMV replication (47). The maintenance of both the secondary structure and the specific nucleotides of the AHSL are required for satBaMV-mediated interference of BaMV replication (17).

In this study, we performed in vivo competition assay by examining the progeny population in Nicotiana benthamiana co-infected with a mixture of interfering and non-interfering satBaMVs. We found that the interfering satBaMV was the dominant species. The RNA structure and nucleotide sequence required for this dominance were determined to be within the AHSL region, and were also conserved in all natural BaMV isolates. Our results show that the conserved 5' AHSLs of BaMV and satBaMV play a crucial role in replication competence.

MATERIALS AND METHODS

Plasmid construction

Plasmid pBSF4 and pBSL6 are full-length cDNA clones of satBaMVs with a T7 RNA promoter at the 5' end (46,49). SatBaMV mutants pBSF4-1 and pBSF4-5 derived from pBSF4 and mutants pBSL6-1 and pBSL6-11 derived from pBSL6 were described previously (17,47). Plasmid pBaMV is a full-length cDNA clone of BaMV-S with a T7 RNA promoter at the 5' end (50). Plasmid pCB is a full-length cDNA clone of BaMV-S with a double 35S promoter from Cauliflower mosaic virus at the 5' terminus in the pCass2 vector (51); plasmid pCBSF4 is a full-length cDNA clone of satBaMV BSF4 in the same vector (51). Plasmid pBaORF1 with a full-length cDNA of BaMV-S ORF1 and plasmid pBaORF1dGDD with the GDD motif deleted in BaMV-S ORF1 are also cloned in the pCass2 vector (52). To generate pCBSL6, a PCR fragment was synthesized from a pBSL6 template with the primers BS104 (5'-GAAAAACTCACCGCAACGA) and BS99 (5'-CGGAAATTCTTATG). The PCR product was further cleaved with EcoRI and phosphorylated by T4 polynucleotide kinase, then ligated into a StuI/EcoRI-digested pCass2 vector. For construction of pCBSF4-1,
a PCR fragment was synthesized from a pBSF4-1 template with the primers Sat-F (5'-GGTACCGAAAAC TCACCGCAACGAAAC) and SatF4-1-R (GAATTCATCTTTAGCGTCTTTATTCGG). The PCR product was further cleaved with KpnI and EcoRI, and ligated into a KpnI/EcoRI-digested pCass2 vector. Plasmid pCBSL6-1 was constructed in the same way except that the template and reverse primer used in the PCR were pBSL6-1 and SatL6-1-R (GAATTCATCTTTAACGTCTTTATTCGG), respectively. The mutant pBaMV-C86U was constructed by site-directed mutagenesis using a double-PCR method (48) in which an ~100-bp fragment of BaMV 5' end was amplified from the pBaMV template by the primer pair B162 [5'-GCTCTAGAGTAATACGACTCACTATAGAAAAG(C)CAT(C)TCCAAAACA(G)-3'] and C86U-R (5'-ATTGGTTGCCACACGCGAGG-3').

Figure 1. Genetic maps and 5' and 3'-UTR secondary structures of BaMV and satBaMVs. (A) Genome organization and ORFs of BaMV and satBaMV. ORFs and the molecular weight of encoded proteins are shown in boxes. Two subgenomic RNAs of BaMV are presented by lines. (B) The secondary structures of the 5'-UTRs of BaMV and satBaMVs. The AHSL structures of BaMV and satBaMV are boxed, and contain an apical loop and two internal loops (IL-1 and IL-2). Black circles indicate the start codon of BaMV ORF1. The common GAAA(A) repeats in the 5'-UTRs of BaMV and satBaMV are underlined. (C) The secondary structure of the BaMV 3'-UTR contains a cloverleaf-like ABC domain, a stem loop D (D domain) and a pseudoknot (E domain) (40,41). The secondary structure of the satBaMV 3'-UTR is similar to that of BaMV except for the lack of A and E domains (19).
using *Pfu* DNA polymerase (Promega, WI, USA). This fragment was purified and mixed with a reverse primer, B80 (5'-AGCTTTGCCACACATC-3'), to amplify an ~1-kb fragment of the BaMV-C86U mutant. The PCR product was further cleaved with *BamHI* and *XbaI* and ligated into a *BamHI/XbaI*-digested pBaMV vector. The mutant pBaMV-C64U was constructed by the same way except that the reverse primer used in the first PCR was C64U-R (5'-CGATTGTAGGAGACAAATTGG-3'). For construction of pCB-C64U, pBaMV-C64U was cut with *MfeI* and *BamHI* and the fragment was ligated into a *BamHI/MfeI* digested pCB vector.

**Construction of the 5' end cDNA clones of natural BaMV isolates**

For analysis of the 5' sequences and secondary structures of natural BaMV variants, an ~500-bp cDNA fragments were amplified from the BaMV RNAs by the primer pair B82 [5'-GAAGGCTGAAAAG(C)CAT(C)TCCAAC A(G)-3'] and B165 (5'-GGGAGGCGGGGGTAG ATAG-3'), using a RT-PCR kit (GE Healthcare, Buckinghamshire, UK). The amplified cDNA fragments were further cloned in a pGEM T-easy vector (Promega, WI, USA). All of the BaMV constructs were verified by DNA sequencing.

**Synthesis of RNA transcripts in vitro**

Plasmids were linearized by restriction enzyme. RNA transcripts with a 5' cap structure (m7GpppG) were synthesized in vitro with T7 RNA polymerase as previously described (49).

**Virus purification and viral RNA extraction**

One-month-old *N. benthamiana* plants were inoculated with pCB, an infectious cDNA clone of BaMV-S (33). Nearly 7–10 days after infection, infected leaves were ground using a mortar and pestle, and crude sap was used for a second round of inoculation. Virions were purified from BaMV-S-infected leaves and viral RNA was extracted as described previously (53,54).

**Protoplast inoculation and northern blot analyses**

Preparation and RNA inoculation of protoplasts from *N. benthamiana* were as previously described (53). For each inoculation, protoplasts of 2 × 10^5^ cells were inoculated with 1 μg of BaMV-S RNA alone or co-inoculated with different doses of satBaMV transcripts by electroporation (53). Total RNA extraction, glyoxalation, and northern blot analyses were carried out as previously described (49). For northern blot analyses, BaMV RNA and satBaMV RNA were probed with 32P-labeled RNA probes specific for BaMV 3' end (55) and full-length satBaMV, respectively (49). The hybridized membranes were washed by buffer and exposed on X-ray film (Kodak). Isotope intensity was processed and quantified by ImageQuant program (GE Healthcare, Buckinghamshire, UK).

**Protoplast isolation and transfection**

For direct DNA uptake experiments, protoplasts were isolated from 30-day-old *N. benthamiana* leaves as previously described (27). For each inoculation, different micrograms of plasmids were co-inoculated into 4 × 10^5^ protoplasts by mixing with polyethylene glycol solution and incubated at 25°C. Thirty-six hours after transfection, protoplasts were harvested for northern blot analyses (49).

**Plant inoculation**

For mix-infection analyses, five leaves of 2-month-old *Chenopodium quinoa* plants were inoculated with 0.3 μg of BaMV-S RNA alone or co-inoculated with 0.3 μg of satBaMV transcripts. Local lesions generated on *C. quinoa* were counted at 7-day post-inoculation (dpi) and leaves were harvested for photography and Northern blot analysis. For mutational analyses, *C. quinoa* plants were inoculated with 0.5 μg of pCB or pCB-C64U alone or co-inoculated with 0.5 μg of pCBSF4 and pCBLSL6. Leaves were harvested for photography and Northern blot analysis at 10 dpi.

**RT-PCR and sequencing**

The full-length satBaMV progenies were amplified by reverse transcription followed by PCR reaction. The conserved primer BS-43 [5'-GGCCTCTAGAT(15)-3'] (*XbaI* site is underlined) hybridized to the 3'-UTR of satBaMV was used for reverse transcription and primer BS-19 (5'-TGCTTGCAGTAATAAGACTACTATAGAAACTCACCCAAACGA-3') (*PstI* is underlined and the italic sequence represents the T7 promoter) hybridized to the 5'-UTR of satBaMV was added for PCR reaction. The resulting RT-PCR products were column-purified (Viogene, Taipei, Taiwan) and the amplified cDNA fragments were cloned in pGEM T-easy vector and used for DNA sequencing.

**RESULTS**

**Interfering satBaMV is dominant in the mix-infection with non-interfering in *N. benthamiana* protoplasts**

Satellite RNAs associated with BaMV in the natural field include interfering and non-interfering isolates, and several interfering satBaMV have been identified, e.g. BSL6, BB23, DL11, DL16 and DL19 (17,46,47). To mimic the potentially mixed infection of satBaMV, full-length cDNA clones of natural satBaMVs were amplified by reverse transcription and primer BS-19 (5'-TGCTTGCAGTAATAAGACTACTATAGAAACTCACCCAAACGA-3') (*PstI* is underlined and the italic sequence represents the T7 promoter) hybridized to the 5'-UTR of satBaMV was added for PCR reaction. The resulting RT-PCR products were column-purified (Viogene, Taipei, Taiwan) and the amplified cDNA fragments were cloned in pGEM T-easy vector and used for DNA sequencing.
29 of 29 cDNAs derived from progenies of mixed infection were BSL6 (Figure 2B), indicating the dominance of BSL6 in the progeny population. The BSL6 is also the dominant species of the satBaMV progeny population when mix-infected with another non-interfering isolate satBaMV-BV17 (data not shown). These data suggest that the replication competence of BSL6 is higher than that of BaMV and non-interfering satBaMVs.

To test whether this event is host-dependent, we also inoculated both of the satRNAs with BaMV RNA onto C. quinoa and observed the formation of local lesions. As expected, three repeated experiments showed that mix-infection of BSF4 and BSL6 markedly reduced the lesion formation on C. quinoa to ~15% compared to BaMV inoculation alone (data not shown). These results demonstrate that BSL6 is the dominant species of the
satBaMV progeny population in mix-infected protoplasts and plants.

A single nucleotide within the AHSL of BSL6 is crucial for dominance in mixed infection

We previously showed that the interchange of the AHSL between BSF4 and BSL6 alters the ability of satBaMV to interfere BaMV RNA accumulation (47). To further examine whether the modified interfering satBaMV (BSF4-1) is more competent than the non-interfering satBaMV mutant (BSL6-1) and BaMV during replication, BSF4-1 and BSL6-1 were used to perform an in vivo competition assay. BSF4-1 was derived from BSF4 with the AHSL replaced by that of BSL6 with the AHSL replaced by that of BSF4 (Figure 2A). As shown in Figure 3B, BSF4-1 diminished the accumulation of helper RNA to ~2% of that when inoculated with BaMV alone. Inoculation with a mixture of equal amounts of BSF4-1 and BSL6-1 greatly reduced BaMV RNA accumulation to ~10% of that when inoculated with the helper virus alone. Full-length cDNA of RT-PCR followed by sequence analysis of satBaMV further revealed that 24 of 25 cDNAs derived from progenies of BSF4-1 and BSL6-1 mixed infection were BSF4-1, indicating the stable propagation and dominance of interfering BSF4-1 in the progeny population (Figure 2B).

A single-nucleotide change in the internal loop I (IL-I) of AHSL is sufficient to alter the biological activities of satBaMV (17). To further examine whether this change could compete for the RdRp complex, we investigated the replication competence of BSF4-5 and BSL6-11. BSF4-5 was derived from BSF4 with U82 replaced by C82 in the IL-I, and BSL6-11 was modified from BSL6 with C83 replaced by U83 (17). We found that BSF4-5 and mixed-infection of BSF4-5 and BSL6-11 greatly diminished the accumulation of helper RNAs to ~2% and 7%, respectively, of that with BaMV alone. Of 28 cDNAs derived from progenies of BSF4-5 and BSL6-11 mixed infection, 26 were BSF4-5, indicating the dominance of BSF4-5 in the progeny population (Figure 2B).

These data suggest that the modified interfering satBaMVs, even those with a single-nucleotide-substitution, compete for the replication machinery better than BaMV and non-interfering satBaMVs.

Replication efficiency of BSL6 is higher than that of BSF4

Recently, it was shown that over-expression of BaMV ORF1 was able to support satBaMV replication in N. benthamiana protoplasts, whereas the GDD motif (amino acids crucial for RdRp activity) deletion mutant of ORF1 did not (52). Thus, we set up an ORF1-dependent system to support satBaMV replication. As shown in Figure 3A, under the support of BaMV replicase (pBaORF1), BSL6 is the dominant species of the satBaMV progeny population in pCBSF4 and pCBSL6 mix-infected protoplasts (Figure 3B). Similarly, 15 of 20 satBaMV progeny was pCBSF4-1 when mix-infected with pCBSL6-1, suggesting that the AHSLs of satBaMV play a crucial role in competition for replicase in a BaMV-free system (Figure 3B). It also ruled out the possibility of RNA-RNA interaction between BaMV and satBaMV involved in BSL6 satBaMV-mediated interference of BaMV replication.

Since BSL6 is the dominant species of the satBaMV among progeny population of mix-infected protoplasts and plants, we further analyzed the replication efficiency of BSF4 and BSL6 by BaMV replicase in N. benthamiana protoplasts. As shown in Figure 3C and D, the accumulation levels of both BSF4 and BSL6 RNAs were
progressively elevated by increasing doses of BaMV replicase. Apparently, the increasing folds of BSL6 RNA were higher than those of BSF4 (Figure 3D). These results indicated that replication efficiency of BSL6 is higher than that of BSF4.

All natural BaMV isolates contain the conserved AHSL structures

Since the 5' end of BaMV-S also contains an AHSL structure similar to that of satBaMV (Figure 4A) (17,18), we further analyzed the 5' secondary structures of other natural BaMV isolates. As shown in Figure 4B, all 33 natural BaMV isolates sequenced contained the conserved AHSL structures regardless of whether they harbored satellite RNA or not. These results imply a co-evolution of the 5' conserved AHSL structures in BaMV and satBaMV. Interestingly, all of the BaMV variants also contained the same internal loops 54UGC66 in the IL-1 and C64 in the IL-II as was found in interfering BSL6. The nucleotides at the equivalent positions of non-interfering satBaMV BSF4 and BSL4 were 50UGU52 (BSF4) and U60 (BSL4), respectively (Figure 4A).

C64 and C86 in the AHSL contributes to BaMV replication efficiently

Similar to satBaMV, the AHSL also plays a crucial role in BaMV replication. Disrupting the structure or changing the loop sequences of AHSL affects the accumulation of BaMV-S RNAs (18). Since C60 and C83 in the internal loops of AHSL were crucial for downregulation of BaMV replication by BSL6 satBaMV (17), we wondered about the role of the conserved C64 and C86 of AHSL in BaMV replication. When N. benthamiana protoplasts were inoculated with the BaMV-C86U mutant, caused the accumulation of viral RNA was undetectable by Northern blot analysis at 24 hpi (Figure 5). Similarly, no symptoms or viral RNA could be found at 10dpi when C. quinoa plants were inoculated with the BaMV-C86U mutant (data not shown), indicating that the nucleotide C86 of BaMV was essential for replication. However, replacing C64 with U reduced the replication efficiency of BaMV in both N. benthamiana protoplasts and C. quinoa plants to 20 and 46%, respectively (Figures 5 and 6). These results suggested that, similar to findings in satBaMV, C64 and C86 in the AHSL are also crucial to BaMV replication.
BSF4 satBaMV interferes with the replication of BaMV-C64U

The 5' end of the BaMV-C64U mutant contains the AHSL with sequence and structure similar to that of non-interfering satBaMV. To evaluate the replication competence of BaMV-C64U and non-interfering satBaMV, C. quinoa plants were inoculated with pCB, the infectious clone of BaMV-S (33), or pCB-C64U alone or combined with the infectious clones of satBaMV (pCBSF4 or pCBSL6). Table 1 summarizes the local lesion production from three independent experiments. The pCB alone induced production with an average of about 173 lesions per leaf, while the lesions induced by pCB-C64U were at a level of 70% of those produced by pCB. The presence of BSL6 satBaMV resulted in great reduction of local lesion formation to a level of 7.5 and 8.3% of those produced by pCB and pCB-C64U, respectively (Table 1). Co-inoculation with BSF4 satBaMV slightly reduced the lesion number to 75% of that by pCB alone. However, the presence of BSF4 satBaMV significantly reduced the formation of local lesions to a level of 18.2% of those produced by pCB-C64U. Similar results were also found in another non-interfering satBaMV (satBaMV-BV17) (data not shown), indicating that non-interfering satBaMV could downregulate BaMV-C64U replication. Northern blot analyses revealed that BSL6, but not BSF4, greatly suppressed the accumulation of both BaMV-S genomic RNA and 1.0-kb subgenomic RNA in inoculated leaves at 10 dpi, whereas both BSF4 and BSL6 significantly decreased the accumulation level of BaMV-C64U RNAs (Figure 6B). Moreover, the accumulation level of BSF4 RNAs was significantly higher than that of BSL6 with the help of BaMV-C64U (Figure 6). This may be due to a greater reduction of BaMV by BSL6 compared to that by BSF4, and thus the replication of BSL6 was sequentially affected.

SatBaMV-mediated downregulation of BaMV replication is dose-dependent

We have routinely shown that BSL6 interferes with BaMV replication when the same weight (1 μg) of BaMV and satBaMV RNAs is used for co-inoculation in N. benthamiana protoplasts (46). Since the molar ratio of satBaMV/BaMV is 8:1 on an equal weight basis, we examined whether BSL6 could interfere with BaMV replication at low molar ratios. Nicotiana benthamiana
protoplasts were co-inoculated with a combination of 1 μg of BaMV-S RNA and different doses (1, 0.5, 0.25, 0.125 and 0.0625 μg) of BSL6 satBaMV transcripts. This converts the input molar ratios of satBaMV/BaMV to 8, 4, 2, 1 and 0.5, respectively. As shown in Figure 7, the accumulation level of BaMV RNAs was increased when the input molar ratio of satBaMV/BaMV was decreased (Figure 7, lanes 3–7). When the input molar ratio of satBaMV/BaMV was progressively reduced to 0.5, the level of BaMV RNAs was progressively increased to 41% of that of BaMV alone (Figure 7, lane 7). Interestingly, the accumulation level of BSL6 RNA was increased when the input molar ratio of satBaMV/BaMV was decreased (Figure 7A).

In summary, satBaMV-mediated interference with BaMV replication is dose-dependent, suggesting that this downregulation event may be due to competition with the helper virus for access to the replication machinery.

**DISCUSSION**

We previously showed that the sequence and structure of satBaMV 5’ AHSL are crucial for interference of BaMV replication (17,47,48). In this study, we demonstrate that both BaMV and satBaMV contain the conserved 5’ AHSL structures and sequences that are involved in replication competence, but that the particular nucleotides evolved in the AHSL for the efficient replication of BaMV are more constrained than those in satBaMV. Moreover, the interference of satBaMV in the downregulation of BaMV replication in the infected *N. benthamiana* protoplasts is dose-dependent, suggesting that satBaMV interference with BaMV replication may be due to their competition for access to limited replication machinery.

Interfering satBaMV with a conserved AHSL and specific C83 is more competent than the non-interfering variant during replication

The error-prone replication of RNA replicons results in the quasispecies nature of RNA viruses and satellite RNAs in the natural field. Although satBaMVs have evolved into interfering and non-interfering species in the natural field (17,46,47), BaMV supported both interfering (BSL6) and non-interfering satBaMV (BSF4) replication equally in *N. benthamiana* protoplasts. In our competition assay in *N. benthamiana* protoplasts, a mixed population of BSF4 and BSL6 interfered with BaMV replication, and BSL6 was the sole dominant among progeny satBaMVs (Figure 2). Similar results were also found in BaMV-free satBaMV replication system (Figure 3B). Moreover, the replication rate of BSL6 was higher than that of BSF4 by the help of BaMV replicase alone (Figure 3C and D). These are strong lines of evidence suggesting that BSL6 outcompetes with BaMV and other satBaMVs for the replication machinery. Further analysis showed that the structure and specific nucleotides (C64 and C83) of the AHSL, which are involved in interference with BaMV replication, are also required for replication competition among satBaMV variants (Figure 2B). Since a single nucleotide substitution
Conserved AHSLs in the 5′ ends of BaMV and satBaMV

As a parasite of BaMV, satBaMV has evolved a 5′ AHSL structure similar to that of BaMV but with a relaxed degree of constrain for replication. Two major AHSL structures, conserved and less conserved, are present in the 5′-UTRs of natural satBaMV isolates (45). Most of satBaMV isolates maintain the conserved AHSL structure, a result of the co-variation of nucleotide substitutions in the HV region (45). In contrast, the nucleotide sequences of the AHSL in our BaMV isolates were much conserved and no co-variation was found in the stems of the AHSL, suggesting a purified selection (Figure 4). It is possible that the formation of the bottom stem of the BaMV 5′ AHSL requires 2 nt in the ORF1 start codon (Figure 1B), which is essential for the translation of RdRp.

A comparison of the sequences of the AHSLs of BaMV and satBaMV shows that the greatest divergence is located at the apical loop and in the base pairs within the bottom stem. It is worth noting that the structure and the nucleotides of the AHSLs in BaMV and satBaMV required for their replication were not totally the same. SatBaMV can tolerate sequence variations on the apical loop of the AHSL, whereas BaMV cannot (17,18). The change of cytosine (C) to U in the IL-I of the AHSL causes a loss in interfering ability but does not affect the replication ability in satBaMV (17). However, mutation at the equivalent position (C 86→U) of the BaMV 5′ AHSL diminishes replication (Figure 5). Moreover, replacing the upper cytosine (C 83) with U in the IL-II of the AHSL reduces the replication efficiency of BaMV-C64U (Figures 5 and 6), whereas no affect on satBaMV replication was observed when the same mutation was introduced (17). Although non-interfering satBaMV could compete with the low replicating BaMV mutant (BaMV-C64U) (Figure 6), the C64U and C86U mutants were not found in natural BaMV isolates (Figure 4), suggesting a restriction of nucleotide change imposed by the AHSL formation. It has been shown that a single C-to-U substitution in loop B of the stem loop IV of an enterovirus internal ribosome entry site greatly changes the shape and flexibility of RNA (58). In the present study, a single C-to-U substitution in the AHSLs of BaMV and satBaMV did not change the secondary structure, but may possibly have altered the tertiary structure of the AHSL. It is also likely that the nucleotides changed at the equivalent positions of BaMV and satBaMV AHSLs differ in their effects on the AHSL structures. The differential requirements for nucleotides in the AHSL of BaMV and satBaMV suggest that other sequences or structures in their genomes are also involved in the stability of the AHSL structure. Whether the AHSL like 5′ termini of other RNA viruses can bind the viral and/or host factors to facilitate genome synthesis (59), or recruit to the replication site on the membranes (60,61) remains to be further investigated. However, we have excluded the potential long-range RNA-RNA interactions between BaMV and satBaMV by BaMV-free satBaMV replication system. The roles of AHSL structures in BaMV and satBaMV replication still need to be explored.

Satellite RNA-mediated downregulation of BaMV replication is dose-dependent

Previously, the reduction of CMV replication by satRNA was shown to compete with the helper virus for replication by way of the RdRp complex (62). Moreover, the symptom expression patterns in tomato plants infected with CMV-77.2 and the mixture of Tfn- and 77-satRNA are dependent on the ratio of two satRNA variants (24). Our results also show that the level of BSL6’s interference with BaMV replication depends on the molar ratio of satBaMV/BaMV, and that the accumulation level of BSL6 RNA is inversely proportional to the inoculated satBaMV/BaMV ratio (Figure 7). Based on their genome size and a ratio of BaMV/satBaMV of 8:1, the rate of minus-strand satBaMV RNA synthesis is 8-fold faster than that of BaMV. Thus, the progeny of BSL6 could compete for more replication factors and downregulate BaMV replication even when the molar ratio of BaMV/satBaMV was reduced to 1 in co-inoculated N. benthamiana protoplasts (Figure 7). However, more
RdRp was translated from increasing levels of BaMV when the molar ratio of satBaMV/BaMV was decreased below 1. Subsequently, more satBaMV RNAs were amplified at the early stage of co-infection (e.g. 24 hpi). After 24 hpi, the high accumulation level of BSLG RNA may likely continue to downregulate BaMV replication.

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