Crucial Role of the 5' Conserved Structure of *Bamboo Mosaic Virus* Satellite RNA in Downregulation of Helper Viral RNA Replication

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Satellite RNA of *Bamboo mosaic virus* (satBaMV), a single-stranded mRNA type satellite encoding a protein of 20 kDa (P20), depends on the helper BaMV for replication and encapsidation. Two satBaMV isolates, BSF4 and BSL6, exhibit distinctly differential phenotypes in *Nicotiana benthamiana* plants when coinoculated with BaMV RNA. BSL6 significantly reduces BaMV RNA replication and suppresses the BaMV-induced symptoms, whereas BSF4 does not. By studies with chimeric satBaMVs generated by exchanging the components between BSF4 and BSL6, the genetic determinants responsible for the downregulation of BaMV replication and symptom expression were mapped at the 5' untranslated region (UTR) of BSL6. The 5' UTR of BSL6 alone is sufficient to diminish BaMV RNA replication when the 5' UTR is inserted in *cis* into the BaMV expression vector or when coinoculation with mutants that block the synthesis of P20 protein takes place. Further, the 5' UTR of natural satBaMV isolates contains one hypervariable (HV) region which folds into a conserved apical hairpin stem-loop (AHSL) structure (W. B. Yeh, Y. H. Hsu, H. C. Chen, and N. S. Lin, *Virology* 330:105-115, 2004). Interchanges of AHSL segment of HV regions between BSF4 and BSL6 led to the ability of chimeric satBaMV to interfere with BaMV replication and symptom expression. The conserved secondary structure within the HV region is a potent determinant of the downregulation of helper virus replication.

Satellite RNAs (satRNAs) depend on their helper viruses for replication, encapsidation, and systemic movement, but they share little or no sequence similarity with the helper virus genome (reviewed in reference 38). Replication of satRNA can interfere with helper virus replication and modify the symptoms induced by the helper virus. The molecular interactions depend on the host plants, the strain of helper viruses, and the satRNA (6). For instance, some cucumber mosaic virus (CMV) satRNAs could attenuate the symptoms induced by CMV in tomato plants and others could intensify chlorotic symptoms in tomato and tobacco or necrosis in tomato (6). Sequences specifying chlorosis or necrosis were delimited to the 5' and/or 3' half of the satRNA (11, 29). The 5' half of minus-stranded satRNAs could induce tomato necrosis, the necrogenicity domain located in an octanucleotide loop and adjacent stem of a hairpin structure (46). Another example is the satRNA C of *Turnip crinkle virus* (TCV) either intensifying or attenuating symptoms, depending on the host and the level of coat protein (CP) in TCV-infected plants (16, 19, 39, 53). Since the CP of TCV is a silencing suppressor, satRNA C can reduce the encapsidation of genomic RNA and increase the level of free CP, which implies that satRNA C may enhance TCV to overcome posttranscriptional gene silencing (47).

In many cases, attenuation of symptoms is usually accompanied by a reduction in the helper virus titer (38). The attenuation associated with CMV satRNA is due to competition with

the helper virus for replication by RNA-dependent RNA polymerase (RdRp) (13, 55). However, when tomato aspermy cucumovirus (TAV) was the helper virus, some satRNAs could attenuate the TAV-induced symptoms but not the level of TAV RNAs (31). Evidence also suggests that subviral RNAs can enhance the resistance of host plants (40).

Bamboo mosaic virus (BaMV), a member of the potexvirus group, contains a single-stranded positive-sense RNA genome with five conserved open reading frames (ORFs) (26, 56). The satRNA associated with BaMV (satBaMV) is a linear RNA molecule of 836 nucleotides (nt) which contains an ORF for a protein of 20 kDa (P20) flanked by a 5' untranslated region (UTR) of 159 nt and a 3' UTR region of 129 nt (23). P20 is an RNA-binding protein (49) but is dispensable for satBaMV replication (25). The satBaMV isolates collected worldwide were classified into two major phylogenetic groups, A and B, in which one hypervariable (HV) region with divergence of up to 20% was identified in the 5' UTRs of those satBaMV isolates (57). Enzymatic probing and mutational analysis revealed that the 5' UTR of prototype BSF4 satBaMV RNA folds into a long stem loop (LSL) and a small stem loop (1). Interestingly, regardless of phylogenetic group A or B, the HV regions of most of the satBaMV isolates fold into a conserved apical hairpin stem loop (AHSL) structure located at the top of an LSL (58).

Two isolates of satBaMV, BSF4 and BSL6, originally from different bamboo hosts, exhibited differential interactions when *Nicotiana benthamiana* plants were coinoculated with the BaMV helper RNA (9). BSF4 did not significantly affect the BaMV replication, but BSL6 markedly reduced the accumulation of genomic and subgenomic RNAs of BaMV and suppressed the BaMV-induced symptom expression (9). The mo-

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lecular determinants of BSL6, which downregulates BaMV RNA replication, remained to be identified.

In this study, the chimeric full-length infectious cDNA clones of the two satBaMV isolates between BSF4 and BSL6, with an interchange of a 5' UTR, P20 coding region, and 3' UTR, revealed that only those with the 5' UTR from BSL6 interfered with BaMV replication and attenuated symptom expression. The 5' UTR of BSL6 inserted into the infectious cDNA vector of BaMV in *cis* also inhibited BaMV replication. Concurrent analyses of natural satBaMV isolates and mutational analyses further demonstrated that the determinants are located in the secondary AHSL structure derived from the HV region.

MATERIALS AND METHODS

Virus isolates, purification, and RNA extraction. BaMV-S, a mutant derived from the isolate BaMV-O, causes severe necrotic and mosaic symptoms on *N. benthamiana* (4, 20). BaMV-V is an isolate harboring BSF4 satBaMV (23). Procedures for virus purification and RNA extraction were described previously (22).

Construction of the BaMV expression vector. Plasmid pBV was constructed by modification of a pBaMV infectious clone (48) by inserting the duplicated BaMV CP subgenomic promoter sequences. A 125-nt region upstream of the CP gene from BaMV-V (18) was duplicated and inserted between the TGBp3 and CP with multiple cloning sites (EcoRI, NcoI, and NotI). The duplicated CP gene start codon was mutated from AUG to AGG for insertion of the 5' UTR of satBaMV variants. For pBV4-5' and pBV6-5', 159 or 160 nt of the 5' UTR of pBSF4 and pBSL6 (23, 27), respectively, were inserted at the EcoRI site. This vector was able to express the 5' UTR as newly synthesized subgenomic RNA. All constructs were sequenced to confirm the nature of engineered insertions.

Construction of satBaMV variants. Plasmids pBSF4 and pBSL6, from which biologically active satBaMV can be transcribed, have been described previously (9, 25). Six chimeric satellite cDNA clones were generated by exchanging each 5' UTR, P20 protein coding region, and 3' UTR with the progenitor plasmids pBSF4 and pBSL6 (see Fig. 1A). Hybrids pBS466 and pBS644, which exchanged the 5' UTR segments, were constructed by replacing the fragments between the HindIII site external to the T7 promoter and BstXI restriction sites in pBSL6 and pBSF4, respectively. Hybrids pBS464 and pBS646, which exchanged the coding region of P20 protein, were constructed by replacing the 493-bp DNA fragment by cleavage with BstXI and EcoNI. Similarly, hybrids pBS446 and pBS664, which exchanged the 3' UTR, were obtained by exchanging the restriction fragments of EcoNI and EcoRI.

Two mutants of BSL6 satBaMV, which blocked the synthesis of the P20 protein, were constructed by the method of Kunkel et al. (17). Mutant BS23 was produced by using primer BS-21-1 (5'-CTCTCCCTACCTACGCTTGGTA AG-3') to change the initiation codon ATG of the P20 protein to the stop codon TAG, and the second in-frame ATG might serve as the initiation codon to synthesize the putative 18-kDa protein (23, 25). Mutant BS24 was constructed by using the primer BS-22 (5'-TCTCCTCCGAGCCATCGTCTT-3') to make an insertion of C after the initiation codon, which led to a frameshift resulting in the premature termination of a 5.4-kDa protein (see Fig. 2A).

Chimeric mutants pBSF20 and pBSF21 were generated from the interchanged hypervariable sequences of pBSF4 and pBSL6, respectively, and have been described previously (57).

In vitro transcription. In vitro transcription was carried out as described previously (25). Plasmids pBSF4, pBS464, pBS664, and pBS644 and natural isolates of DL11, DL6V6, DL6V1, DLIV, and DL16 (58) were linearized with XbaI, and plasmids pBSL6, pBS466, pBS466, and pBS446 and mutants of pBSL6 were linearized with XhoI. Plasmids pBV, pBV4-5', and pBV6-5' were linearized with SacI and subsequently synthesized with T7 RNA polymerase (New England Biolabs, Inc., Beverly, MA).

Inoculation of protoplasts and plants and Northern blot analyses. Preparation of protoplasts from *N. benthamiana*, inoculation with BaMV and satBaMV transcripts, and isolation of total RNA from protoplasts were performed as described previously (24). Inoculation of *N. benthamiana* and *Chenopodium quinoa* plants was done as described by Hsu et al. (9), and total extraction of RNA from plants was done as described by Verwoerd et al. (51). Replication of BaMV and satBaMV RNAs in the inoculated protoplasts or plants was assayed by Northern blot hybridization. Probes to detect genomic and satBaMV RNAs,

designated L and S probes, respectively, were P^{32} -labeled in vitro transcripts from cloned HindIII-linearized pBaHB (21) or EcoRI-linearized pBSHE (25). Likewise, probe for the detection of minus strands of BaMV was transcribed from BamHI-cut pBaHB (18). Hybridization signals were detected and quantified with the use of a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Determinants of BSL6-mediated interference were mapped in the 5' UTR. The two full-length infectious cDNA clones pBSF4 and pBSL6 showed a 7% difference in nucleotide sequence that dispersed in the whole molecule (27). To map the region affecting the genomic RNA replication and symptom formation by BaMV, six chimeric satellites were generated with cDNA clones derived from pBSF4 and pBSL6 by using their common BstXI and EcoNI sites at positions 159 and 652, respectively (23) (Fig. 1A). Initially, each of the six possible chimeric satBaMVs and two parental satBaMVs were coinoculated onto protoplasts of *N. benthamiana* with helper BaMV-S RNA. Northern blot analyses of total RNAs from infected protoplasts with the use of a satBaMV-specific probe (S probe) revealed that these six chimeric transcripts were biologically active and were accumulated to substantial levels as parental transcripts 24 h postinoculation (hpi) (Fig. 1B). As shown previously (9) and in Fig. 1B, BSL6 satBaMV greatly reduced the accumulation level of the BaMV genomic RNAs (6.4 kb) and the two subgenomic (2.0 and 1.0 kb) RNAs as detected with the BaMV-specific probe (L probe) (Fig. 1B, lane 3). The diminished accumulation of genomic and subgenomic RNAs was also observed in protoplasts coinoculated with chimeric BS644, BS646, and BS664 satBaMVs (lanes 7, 8, and 9, respectively). However, chimeric satBaMVs with a 5' UTR derived from BSF4, such as BS446, BS464, and BS466, like BSF4, had slight effects on BaMV replication (lanes 4, 5, and 6, respectively). In six independent experiments, chimeric satBaMVs in protoplasts containing the 5' UTR of BSL6 (e.g., BS646, BS664, and BS644) were able to reduce the level of genomic and subgenomic RNAs to the range of 1% to 10% compared with that of protoplasts inoculated with BaMV RNA alone (Fig. 1C).

Since the negative strand is the intermediate in BaMV replication, we detected the accumulation of BaMV minus-strand RNA (Fig. 1B). Consistent with the results described above, a substantially lower level of minus-strand RNA accumulation was observed in protoplasts coinoculated with BSL6 and its 5' UTR derivatives BS646, BS664, and BS644. The result implies that the primary genetic determinants of BSL6 satBaMV, which is responsible for the diminished level of both the plus and minus strands of BaMV RNA, resides in the 5' UTR.

Next, we examined the effect of the chimeric satBaMVs on symptom expression in planta. Plants of *Chenopodium quinoa* and *N. benthamiana* were inoculated with BaMV-S RNA or together with either of the chimeric satBaMV isolates. Coinoculation with BSL6 satBaMV greatly reduced the number of local lesions in leaves of *C. quinoa*, as was shown previously (9). Likewise, BS644, BS646, and BS664 satBaMVs reduced the number of lesions to approximately 10% to 20% of that with BaMV RNA alone, whereas coinoculation of BSF4, BS464, BS446, and BS466 resulted in a similar or even higher number of lesions (not shown).

FIG. 1. Schematic diagram of chimeric cDNAs of pBSF4 and pBSL6 and their effects on BaMV replication and BaMV-induced symptoms. (A) Chimeric cDNAs were made up of parts from either pBSF4 (red) or pBSL6 (green) cDNA using the restriction sites BstXI and EcoNI. Open boxes represent the UTRs of satBaMVs. (B) Protoplasts of *N. benthamiana* were inoculated with BaMV-S RNA alone (lane 1) or coinoculated with the following satBaMV variants: BSF4 (lane 2), BSL6 (lane 3), BS446 (lane 4), BS464 (lane 5), BS466 (lane 6), BS644 (lane 7), BS646 (lane 8), or BS664 (lane 9). At 24 hpi, total RNAs extracted from 3×10^4 protoplasts were glyoxylated, electrophoresed in a 1% agarose gel, and transferred to a nylon membrane. Blots were hybridized with 32 P-labeled BaMV-specific L (top panel) (21) and satBaMV-specific S (middle panel) (25) probes. Additionally, a slot blot of total RNA from inoculated protoplasts was hybridized with a BaMV-specific probe for minus-strand RNA (18). Positions of BaMV genomic RNA (6.4 kb), subgenomic RNAs (2.0 and 1.0 kb), and satellite (7) are indicated on the left. (C) Each bar represents average accumulation levels of genomic RNA and two subgenomic RNAs obtained from six independent experiments with standard deviation. Numbers along the x axis represent lane numbers shown in panel B. (D and E) Effects of chimeric satBaMVs on symptom formation. Relative numbers of *N. benthamiana* plants showing symptoms after 12 and 24 dpi (D) and appearance of symptoms at 24 dpi (E).

Visual symptoms on *N. benthamiana* leaves were monitored for 24 days postinoculation (dpi). BaMV-infected *N. benthamiana* showed severe mosaic and necrotic symptoms in all 12 inoculated plants (Fig. 1D and 1E). When BSF4 or chimeric satBaMVs with the 5' end derived from BSF4 (e.g., BS464, BS446, and BS466) were coinoculated, similar severe symptoms were frequently observed. In contrast, leaves coinocu-

lated with BSL6 or chimeric satBaMV with the 5' end derived from BSL6 (e.g., BS644, BS646, and BS664) showed a complete or nearly complete absence of symptoms (Fig. 1E). Approximately 10% to 20% of the inoculated plants showed mild mosaic symptoms at 12 dpi, and 20% to 30% became systematically mosaic at 24 dpi (Fig. 1D). These results were reproducible, and Northern blot analyses confirmed the low level of

FIG. 2. Schematic diagram of satBaMV mutants derived from pBSL6 (A) and their effects on BaMV replication (B and C). (A) Mutants of BSL6 satBaMV in which the P20 protein gene was blocked. The first ATG of the P20 protein gene was changed to TAG in mutant BS23, whereas the additional C was inserted after the first ATGG, causing the frameshift in mutant BS24. Open box, noncoding sequence; filled box, coding region of satBaMV; hatched box, altered reading frame of the coding region. (B) Northern blot analysis of BaMV and satBaMV RNA accumulation in inoculated protoplasts, as described in the legend to Fig. 1. (C) Each bar represents relative average accumulation levels of genomic RNA and two subgenomic RNAs from six independent experiments with standard deviation.

BaMV genomic and subgenomic RNAs in those asymptomatic plants (not shown). Consistent with another report (38), the attenuation of symptoms is associated with the reduced level of BaMV genomic and subgenomic RNAs in the infected cells.

Taken together, the results indicate that the genetic determinants responsible for the downregulation of BaMV positive- and minus-strand RNA synthesis and symptom suppression reside in the 5' UTR of BSL6 satBaMV RNA.

BSL6-mediated interference of BaMV RNA replication is independent of P20 protein expression. Since all the chimeric satBaMVs constructed contained the ORF for the P20 protein, the possible effect of P20 on the BSL6-mediated interference of BaMV replication was determined by use of two mutants, BS23 and BS24, both derived from BSL6. For BS23, the first ATG of the P20 gene was changed to TAG, and subsequently by an 18-kDa protein was synthesized from the downstream initiation codon. An additional C was inserted after ATGG of the P20 gene, causing the frameshift in mutant BS24 (Fig. 2A). In the *N. benthamiana* protoplast assays, BS23 and BS24 replicated to a level approximately 30% less than that of BSF4 and BSL6 satBaMVs (Fig. 2B, lower panel); however, they greatly reduced the level of BaMV genomic and two subgenomic RNAs, as did BSL6 at 24 hpi, being only about 5% to 15% of that in protoplasts inoculated with BaMV-S RNA alone (Fig. 2B and 2C).

BS23 and BS24 satBaMVs also greatly reduced the production of local lesions on *C. quinoa* to 10% to 15% of the wild-type level, a value close to that of the BSL6-induced reduction (not shown). Northern blots of total RNAs extracted from inoculated leaves of *C. quinoa* also confirmed the results obtained from infected protoplasts, except that BSL6 (9) and the two mutants, BS23 and BS24, were accumulated to a lower level than that of BSF4 in the inoculated leaves (not shown).

We conclude that the determinants of BSL6-mediated interference of BaMV replication reside only at the 5' UTR and that the interference is independent of P20 translation.

The 5' UTR of BSL6 satBaMV alone is sufficient to interfere with BaMV replication *in cis*. To further confirm the 5' UTR of BSL6 as a downregulation determinant, the 5' UTRs of satBaMVs were cloned downstream of the CP subgenomic promoter of pBV to give rise to the genomic clone variants pBVF4-5' and pBVL6-5' (Fig. 3A). This vector was able to express the 5' UTR of satBaMV, as evidenced by newly synthesized subgenomic RNA in the infected *C. quinoa* (Fig. 3B, lane 1). Compared to inoculation with the wild-type pBV vector, that with insertion of the 5' UTR of BSF4 reduced the level of genomic and 1.0-kb subgenomic RNA of pBVF4-5' to approximately half (Fig. 3B, lane 2, and 3E). However, a stronger reduction in the level of genomic and 1.0-kb subgenomic RNA of pBVL6-5' with the 5' UTR of BSL6 was noted to be less than 5% of that of the wild type (Fig. 3B, lane 3, and 3E). Interestingly, the level of the additionally synthesized 1.2-kb subgenomic RNA was not proportionally reduced. A BaMV-specific probe for minus-strand RNA detected the abundance of minus-strand 1.2-kb subgenomic RNA in both the pBVF4-5' and pBVL6-5'-inoculated leaves (Fig. 3C), which indicates that the newly synthesized 1.2-kb subgenomic RNA might be replication competent. Moreover, pBVL6-5' did not produce any visible lesions in the inoculated *C. quinoa* plants (Fig. 3D), nor in *N. benthamiana* (not shown). These results are consistent with earlier findings that the 5' UTR of BSL6 alone not only interferes with BaMV replication but also attenuates symptoms.

Other natural satBaMV isolates are capable of interfering with BaMV replication. The 5' UTRs of BSF4 and BSL6 satBaMVs differ in 13 nt (27). To further map the key determinant in the 5' UTR of BSL6, we attempted to construct chimera representing this region between the two isolates and

FIG. 3. Replication and symptoms of BaMV expression vector carrying 5' UTR of satBaMV variants in *C. quinoa* plants. (A) Schematic representation of the pBV, pBVF4-5', and pBVL6-5' genomes. ORFs are denoted by open boxes, and the solid green circle denotes insertion of the duplicated subgenomic promoter. A small solid rectangular box represents the 5' UTR derived from BSF4 or BSL6 satBaMV, respectively. (B and C) Northern blot of total RNA extracted from plants inoculated with these three constructs and hybridized with a probe specific to detect plus- (B) and minus- (C) strand BaMV RNA. The arrow represents the accumulation of additional synthesized subgenomic RNA under the control of a duplicated subgenomic promoter. (D) Development of local lesions on *C. quinoa* leaves inoculated with pBV, pBVF4-5', or pBVL6-5' at 10 dpi. (E) Each bar represents relative average accumulation of genomic RNA obtained from three independent experiments with standard deviation.

to assay their ability to interfere with BaMV replication. However, no definite results were obtained, probably because of the complete changes of secondary structures of chimeric 5' UTRs as predicted by *mfold* (59). Even the most conserved AHSL structures within the 5' HV region found in natural satBaMV isolates of two evolutionarily phylogenetic groups (57) were not attained. Therefore, attempts were switched to test our collected natural satBaMV isolates.

Features of isolate BS6V6 were first noted since its 5' UTR sequence is completely identical to that of BSL6, although some variations occur in the rest of the satBaMV genome. As expected, coinoculation with BS6V6 revealed a great reduction of genomic and subgenomic RNAs of BaMV, as did coinoculation with BSL6 in the BaMV-infected protoplasts (Fig. 4B). Since the 5' UTR shows the most divergence among the satBaMV genome (57), we next chose the two isolates in the 5' UTR most similar to BSL6. Isolate DL16 shows only a 1-nt difference, while DL11 shows a 3-nt difference, all of which are located within the HV region. Further, the HV sequence-folding AHSL structures are conserved and nearly identical to those of BSL6 (Fig. 4A). Protoplast assays revealed that DL11 and DL16 greatly diminish BaMV replication, similar to BSL6 (Fig. 4B). As control isolates, DLIV and 6V1 isolates were chosen, since they have less conserved AHSL structures than the other isolates (Fig. 4A). Like BSF4, DLIV and 6V1 had little or no effect on the accumulation of genomic RNAs or the two subgenomic RNAs in the protoplast inoculation assay (Fig. 4B). The results imply that the conserved AHSL structure may be involved in the downregulation of BaMV replication.

Conserved AHSL structure is responsible for BSL6 satBaMV-mediated interference in BaMV replication. Since the conserved AHSL structure might be involved in BSL6 satBaMV-mediated interference, the 5' UTR of BSL6 was subjected to computational structure prediction with *mfold* (59). The structure of the 5' UTR of BSL6 formed five small hairpins at 25°C in which the HV region folds conserved AHSL structures located in the upper part of the third hairpin (Fig. 5A). Therefore, the entire HV regions between BSF4 and BSL6 were interchanged and chimeric BSF20 and BSF21 satBaMVs in which the interchanging AHSL structures were stably maintained were generated (Fig. 5A). Similar to BSL6, BSF20 but not BSF21 exhibited nearly complete suppression of genomic and subgenomic RNA accumulation in *N. benthamiana* protoplasts (Fig. 5B). Symptomless infection was also observed in the BSF20 coinoculated *N. benthamiana* plants, whereas plants coinoculated with BSF21 showed mosaic and necrotic infection (Fig. 5E). The interchange of the HV region between BSF4 and BSL6 altered the ability of satBaMV to affect BaMV accumulation, which suggests that the HV sequence in the 5' UTR of BSL6 plays a crucial role to downregulate the BaMV accumulation and symptom expression.

DISCUSSION

Interference of viral replication and attenuation of virus-induced symptoms by satellite RNA is one of the main interests in studying the molecular interactions among satellite RNA, helper viruses, and host plants. We previously isolated and characterized two satBaMV isolates, BSF4 and BSL6, which differ in interference with BaMV RNA replication and suppression of BaMV-induced symptoms (9). In the present

FIG. 4. Effect of natural satBaMV isolates on the accumulation of BaMV in *N. benthamiana* protoplasts. (A) AHSL structures of HV regions from different natural satBaMV isolates, predicted by the *mfold* program (59). Nucleotides in the HV regions different from those of BSL6 satBaMV are shown in red. (B and C) Northern analysis of BaMV and satBaMV RNA accumulation in *N. benthamiana* protoplasts, as described in the legend to Fig. 1. (D) Total RNA in ethidium bromide-stained agarose gel showing equal loading on each lane.

study, we expanded our studies to characterize the genetic determinants of downregulation of BaMV replication. Our results indicate that a conserved AHSL secondary structure within the HV region, comprising an apical loop followed by two internal loops interwoven by 3- to 4-, 2-, and 5-bp stems (1, 57), plays a crucial role for this interference.

By use of recombinant chimeric satBaMV RNAs between BSF4 and BSL6, we first determined that the primary genetic determinants of the BSL6 satBaMV responsible for interference of BaMV RNA replication resided in the 5' UTR (Fig. 1) and that the P20 protein dispensable for satBaMV replication (25) is not involved (Fig. 2). Inhibition of BaMV RNA replication by BSL6 results in not only a great reduction in the level of BaMV genomic and subgenomic RNAs and minus-strand RNA, a replication intermediate as a template for the synthesis of genomic and 3'-coterminal subgenomic RNAs (3, 12), but also symptom attenuation (Fig. 1). The role of the BSL6 5' UTR alone as a downregulating determinant for BaMV replication was further strengthened by the findings that the insertion of the 5' UTR of BSL6 into the BaMV vector pBV resulted in a dramatic elimination in chimeric BaMV replication and symptom formation in *C. quinoa* (Fig. 3) and *N. benthamiana* (not shown) plants. These results clearly demonstrate that the 5' UTR alone, without encoding proteins or intramolecular interactions with its 3' end, is a potent determinant. However, the chimeric subgenomic RNA from pBVL6-5', consisting of the 5' UTR of BSL6, the CP coding region, and the 3' UTR of BaMV, might be replication competent during the infection (Fig. 3B). It accumulated substantially both positive- and negative-strand RNAs (Fig. 3C), while the genomic and other subgenomic RNAs were greatly diminished in level in the infected cells (Fig. 3B). This chimeric subgenomic RNA, like BSL6 satBaMV, may outcompete BaMV genomic RNA for the RdRp complexes and thus decrease the genomic RNA replication. Satellite RNAs, such as

CMV (2, 10, 29) or groundnut rosette virus (45), flanked by a nonsatellite sequence expressed in transgenic plants could be rescued into the replication-compatible unit length. Our results also support this notion, because protoplasts coinoculated with the 5' UTR of BSL6 (nt 1 to 160) and BaMV RNA showed no interference of BaMV accumulation (not shown), which implies that a replication-competent RNA molecule is required for this downregulation event. Similarly, the downregulation of groundnut rosette virus replication by a variant satellite RNA (NM3c) is also controlled by an R domain near its 5' end (43, 44); however, this R domain cannot function by itself when expressed in transgenic plants (45).

The 5' UTR of the satBaMV genome exhibits significant genetic heterogeneity within an HV region, which allows for diversity of up to 20% variation for adaptation (57). A number of *cis*-acting elements important for satBaMV replication have been identified within HV regions in protoplast and whole-plant assays (1). Strikingly, the 5' HV region folds into an evolutionarily conserved AHSL secondary structure whose intramolecular base pairing is more important for satBaMV replication than the primary sequence (57). Taking advantage of the AHSL structure being functionally interchangeable between the two phylogenetic satBaMV groups (57), the HV region was further mapped as the key downregulating determinant by exchanging the HV region between the BSL6 and BSF4 satBaMVs (Fig. 5).

Although the HV region provides the greatest sequence diversity among the whole genome, some of the natural isolates of satBaMV contain identical sequences in this region. For instance, the natural isolate 6V6 has a 5' UTR sequence that is completely identical to that of BSL6, but DL11 and DL16, with only a 0.6% to 1.9% difference and carrying an identical or nearly identical AHSL structure, are also BaMV-interfering isolates (Fig. 4). Earlier studies indicated that a putative secondary structure, rather than the primary se-

FIG. 5. Secondary structure of 5' UTR of satBaMV variants and their effect on BaMV replication and symptom expression. (A) Schematic representation of the secondary structure of 5' UTR of pBSF4 (green), pBSL6 (red), and two satBaMV mutants, BSF20 and BSF21, in which the entire HV regions were interchanged between BSF4 and BSL6. (B and C) Accumulation of BaMV and satBaMV RNAs in the inoculated protoplasts of *N. benthamiana* at 24 hpi, as shown at the top of each lane. (D) Total RNA as loading controls. (E) Symptom formation in the *N. benthamiana* plants inoculated with BaMV or coinoculated with satBaMV. Photos were taken 24 days after inoculation.

quence, is a necrogenicity domain of CMV satellite RNA (46). Similarly, one or few nucleotide variations can greatly change the viral or satellite RNA pathogenicity and/or phenotype (15, 33, 41, 42), which implies that the AHSL with refined structural features may determine whether an RNA is predisposed toward RNA-RNA formation and/or a critical interaction with a host protein or RdRp complexes. Recently, a 54-kDa host protein was shown to bind in the 5' UTR of potato virus X, which is important for potato virus X replication (14). Thus,

the process of reduction in helper virus replication and symptom formation might involve a novel type of interaction between satRNA, the helper virus, and the host plant, although it is unclear whether satRNA sequences are directly involved in the interaction with components of the host plant or whether they function through interaction with the helper virus (43, 44). Yet, likely, the AHSL structures of satBaMV isolates determine the selective interactions with replication factors of viral and/or cellular origins and, thus, the interference was simply

attributed to the main competition for limited quantities of replication factors (40).

All RNA viruses studied so far contain at least some evolutionarily conserved RNA structures, particularly in the 3' UTRs of viral or subviral genomes, which are essential in viral replication and transcription. The conserved RNA structures also commonly occur in the 5' UTR, or even within coding regions (8, 35), involved in RNA replication (5, 7, 32, 36, 37, 50, 52, 54, 58), translation (28, 30, 58), virion assembly (7), adaption (5), virulence (15), or even small interfering RNA- or microRNA-mediated destruction (34). The HV region in the 5' UTR of satBaMV isolates identified phylogenetic groups (57) whose divergence may be a result of independent evolution in a distinct host-helper-satBaMV relationship. Conservation of RNA structures within the HV region may evolve as a functional unit that not only retains its own features required for efficient interactions but also allows genetic variability for virus adaptation.

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