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The open reading frame of bamboo mosaic potexvirus satellite RNA is not essential for its replication and can be replaced with a bacterial gene

(satellite-based vector/satellite-encoded protein)

NA-SHENG LIN*†, YUN-SHIEN LEE*, BIING-YUAN LIN*, CHIN-WEI LEE‡, AND YAU-HEIU HSU‡

*Institute of Botany, Academia Sinica, Taipei, Taiwan 115, Republic of China; and ‡Agricultural Biotechnology Laboratory, National Chung Hsing University, Taichung, Taiwan 402, Republic of China

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ABSTRACT **A satellite RNA of 836 nt depends on the bamboo mosaic potexvirus (BaMV) for its replication and encapsidation. The BaMV satellite RNA (satBaMV) contains a single open reading frame encoding a 20-kDa nonstructural protein. A full-length infectious cDNA clone has been generated downstream of the T7 RNA polymerase promoter. To investigate the role of the 20-kDa protein encoded by satBaMV, satBaMV transcripts containing mutations in the open reading frame were tested for their ability to replicate in barley protoplasts and in *Chenopodium quinoa* using BaMV RNA as a helper genome. Unlike other large satellite RNAs, mutants in the open reading frame did not block their replication, suggesting that the 20-kDa protein is not essential for satBaMV replication. Precise replacement of the open reading frame with sequences encoding chloramphenicol acetyltransferase resulted in high level expression of chloramphenicol acetyltransferase in infected *C. quinoa*, indicating that satBaMV is potentially useful as a satellite-based expression vector.**

To genetically engineer plants, one of the major goals is to express useful foreign genes in these plants. The use of plant viral vectors for this purpose provides some advantages over transgenic plants: not only does the virus replicate in high copy number but infection can be obtained within 1–2 weeks, rather than months or longer usually required for plant regeneration. Although several plant viral vectors are useful in this aspect (1–3), it has not been demonstrated for a helper-dependent vector system, such as defective interfering RNAs or satellite RNAs (sat RNAs).

Satellite RNAs are parasites associated with many groups of plant viruses. They are small RNA molecules dependent on helper viruses for their replication, encapsidation, and movement, but they have no sequence homology with the helper viruses (4–6). sat RNAs can be classified by size into four types (5). Types A and B are larger than 0.7 kb and encode a functional open reading frame (ORF): type A that encodes their own capsid protein is called satellite virus, and type B codes for a nonstructural protein. Many of the type B satellites have a nepovirus as helper (7) and encode proteins ranging from 38 to 48 kDa that are essential for the replication of these sat RNAs (8–10). Type C satellites are smaller than 0.7 kb and lack significant messenger activity. The cucumber mosaic virus associated RNA 5 (333–386 nt) is a well-known example (6).

Potexviruses have a 6- to 7-kb single-stranded, positive-sense RNA genome that includes five conserved ORFs (11–13). A sat RNA naturally associated with bamboo mosaic virus (BaMV) is the only one found in the potexvirus group (14). This RNA (satBaMV) is a linear molecule of 836 nt [excluding the poly(A) tail] and encapsidated with BaMV capsid protein into the rod-shaped particles of 60 nm (14). It contains an ORF for a protein of 183 amino acids (20 kDa) flanked by a 5′ noncoding region of 159 nt and a 3′ noncoding region of 129 nt. The 20-kDa protein shares 46% identity in amino acid sequence with the capsid protein of satellite viruses associated with the spherical panicum mosaic sobemovirus (15). The first 94 nt of satBaMV 5′ noncoding region show 63% identity with BaMV RNA near the 5′ terminus, with the first 6 nt GAAAAC in common (14). The conserved sequence ACCUAA, important for the replication of potexvirus genomic RNA (16), is also found at the 3′ end of satBaMV (14).

In this paper we report the construction of a full-length cDNA clone of satBaMV and clones in which the ORF has been mutated. The ability of satBaMV ORF mutants to replicate was analyzed in barley protoplasts and in *Chenopodium quinoa*. Our results indicate that the satBaMV-encoded protein is not essential for satBaMV replication. Moreover, the ORF of satBaMV can be replaced with a bacterial gene, chloramphenicol acetyltransferase (CAT), which was efficiently expressed in the infected leaves.

MATERIALS AND METHODS

Virus Isolates. BaMV isolate V (BaMV-V) contains satBaMV (14). BaMV-L is an isolate derived from BaMV-V and free of satBaMV (designated as BaMV-V/S$^-$ in ref. 14). Preparation of BaMV and its RNA and native satBaMV RNA has been described (14, 17).

Construction of a Full-Length cDNA Clone of satBaMV. cDNA to satBaMV was synthesized (14) with primer BSO [5′-GTCGA**CTCTAGA**$(T)_{15}$; *Xba* I site is in boldface type]. The second-strand cDNA was synthesized with primer BS19 (5′-TGC**CTGCAG**TAATACGACTCACTATA*GAAAACT-CACCGCAACGA*; Pst I site is in boldface type; underlined bases contain the T7 promoter; the italicized bases are the 5′ terminal sequence of satBaMV) and T4 DNA polymerase (Riboclone cDNA synthesis system, Promega). Full-length double-stranded cDNA was isolated from 1% low-melting-point agarose gels, cut with *Pst* I and *Xba* I, ligated to *Pst* I- and *Xba* I-cut pUC119, and introduced by transformation into *Escherichia coli* DH5α. The resulting plasmid, pBSF4, contains the full-length cDNA of satBaMV with 17 3′-terminal (A) residues and lacks nonviral sequence between the T7 promoter initiation site and the 5′ end of satBaMV.

Mutant Constructions. Oligonucleotide-directed mutagenesis in the N-terminal region of the 20-kDa protein gene was performed by using pBSF4 DNA as a template (18). To avoid

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Abbreviations: BaMV, bamboo mosaic virus; CAT, chloramphenicol acetyltransferase; ORF, open reading frame; satBaMV, satellite RNA associated with BaMV; BSCAT, *in vitro* transcripts of pBSCAT; p.i., postinoculation.

†To whom reprint requests should be addressed.

mutations that might have been introduced elsewhere in the cloned cDNA, the changes in the mutagenized clones were verified by sequencing the complete cDNA. Clone pBSF5 carries a single mutation in which the $A^{160}UG$ initiation codon was changed to $A^{160}UU$. pBSF6 is a frameshift mutant in which a cytidine was inserted after $A^{160}UGG$. pBSF9 is a deletion mutant of bases 160–711 (codons 1–183), in which the coding region of the 20-kDa protein was completely removed.

Internal deletions in the 20-kDa ORF were constructed by excising selected restriction fragments and religating the resulting ends. pBSF7 has a frameshifting deletion of bases 450–487, made by deleting the *Alu* I fragment from pBSF4. pBSF8 has an in-frame deletion of bases 280–559, made by deleting the *Eae* I fragment from pBSF4.

To generate the chimeric mutant pBSCAT, the DNA fragment corresponding to the CAT ORF was amplified from pCM7 (Promega) by PCR (19) using primers BSCAT1 (5′-TATCCAAGACG*ATGGAGAAAAAAATC*-3′; the *Bst*XI is in boldface type; the italicized bases are identical to the first 15 nt of the CAT ORF) and BSCAT2 (5′-CAG**CCTCTGGGAG**-*GTTACGCCCC-GCCCTG*-3′; the *Eco* NI is in boldface type; the italicized bases are complementary to the last 15 nt of the CAT ORF). pBSCAT was constructed by replacing the *Bst*XI and *Eco*NI ORF cassettes of pBSF4 with the amplified CAT ORF (see Fig. 5*A*).

***In Vitro* Transcription and Cell-Free Translation.** Conditions for *in vitro* transcription of linearized plasmids were as described for brome mosaic virus (20). RNA transcripts were translated in rabbit reticulocyte lysate (Promega), and translation products were analyzed on an 8% to 20% linear-gradient SDS/polyacrylamide gel (21).

Protoplast and Plant Inoculation, RNA Isolation, and Analysis. Barley protoplast and plant inoculation and total RNA extraction were as described (14, 22). Each inoculum contained a mixture of 1 μg of BaMV-L RNA and 1.5 μg of satBaMV mutant transcripts. The quantity and quality of synthesized transcripts were verified by agarose gel electrophoresis before inoculation.

Total RNA prepared from protoplasts at various times after incubation or virion RNA from inoculated leaves was analyzed by Northern hybridization as described (21), except that RNA probes were used instead. Genomic RNA specific probe (ORF-1 probe) was generated from T7 RNA polymerase transcripts of pORF1, constructed by cloning the *Eco*RI/*Hin*cII fragment of pBaBL2 (13) into the transcription vector pGEM4 (Promega). The probe specific to satBaMV (S probe) is a T7 polymerase transcript of a cDNA clone complementary to the 3′ terminal 822 nt of satBaMV.

Sequence Analysis of Progeny RNA. cDNA to satBaMV or mutant progenies was synthesized with the 3′ terminal primer BS0 (14). This cDNA was amplified by PCR (19) with a 5′ primer BS23 corresponding to the first 18 nt of satBaMV. The PCR products were then cloned into pCR vector (TA cloning kit, Invitrogen). The DNA sequences of the inserts were determined by the chain-termination procedure using Sequenase (United States Biochemical).

Immunoassay of BaMV Capsid Protein and CAT Protein in Infected *C. quinoa*. Inoculated leaves, harvested at 3-day interval after inoculation, were ground in liquid nitrogen, and 2.5 × (ml/g) sample buffer (10 mM Tris·1 mM EDTA, pH 8.0/0.5% 2-mercaptoethanol/1% Triton X-100) was added. After being stirred for 3 min, the filtrate was centrifuged at 10,000 × *g* for 5 min. To the supernatant, serial dilutions were made in sample buffer and immunoassayed for BaMV capsid protein (17) and CAT enzyme (CAT ELISA kit, Boehringer Mannheim).

RESULTS

Biological Activity of Synthetic Transcripts. Full-length DNA copies of satBaMV were synthesized using two primers that annealed to the terminal sequences of satBaMV. *In vitro* transcripts (BSF4) from *Xba* I-linearized pBSF4 contain only one nonviral nucleotide at the 3′ end.

For the activity assay, synthetic capped BSF4 transcripts were inoculated into barley protoplasts together with BaMV-L RNA. Northern blot analysis with the BaMV ORF-1-specific probe detected a high accumulation level of 6.4-kb genomic RNA in barley protoplasts inoculated with BaMV-L RNA alone (Fig. 1*A*, lane 1), or the mixture of BaMV-L and native satBaMV RNAs (lane 2), or BaMV-L RNA plus BSF4 transcripts (lane 3) 24-hr postinoculation (p.i.). The satBaMV-specific probe (S probe) detected the satBaMV-specific sequences in protoplasts coinoculated with BaMV RNA and BSF4 transcripts (Fig. 1*B*, lane 3), at levels similar or higher than those coinoculated with native satBaMV (Fig. 1*B*, lane 2). No satBaMV was detected in protoplasts inoculated with BaMV-L RNA only (Fig. 1*B*, lane 1), even after longer exposure, indicating the absence of satBaMV RNA in the BaMV-L RNA preparation. The results show that BSF4 transcripts are biologically active in protoplasts coinoculated with the genomic RNA.

Mutants of satBaMV. To determine whether the satellite-encoded protein is essential in satBaMV replication, a series of frameshift and deletion mutations were introduced into the ORF of pBSF4. Two mutants were constructed with mutations at the N terminus of the 20-kDa protein. In mutant pBSF5 the initiation codon $A^{160}UG$ was changed to $A^{160}UU$. This mutant contained a second in-frame initiation codon at nt 205 of satBaMV (Fig. 2*A*). pBSF6 is a translational frameshift mutant in which a cytidine insertion after $A^{160}UGG$ leads to the introduction of an alanine residue at amino acid 2 followed by a frameshift resulting in the premature termination of the satellite protein 53 codons downstream. In an *in vitro* translation system, BSF5 transcripts directed the synthesis of a major 18-kDa product as well as a minor 20-kDa protein (data not shown). The synthesis of the 20-kDa protein is probably the result of using AUU as an initiation codon as found in baculovirus (23). BSF6 encoded an altered ORF of 6 kDa.

Three deletion mutants were also constructed. In mutant pBSF7, the deletion of nt 449–488 led to the appearance of UAA termination at nt 499; in mutant pBSF8, the deletion of 282 nt from 279 to 560 led to a deletion of 94 amino acids; and in mutant pBSF9, sequences encoding the ORF of 20 kDa was completely removed. When the coding capacity of the transcripts BSF7, BSF8, and BSF9 was assayed by *in vitro* translation, it was found that proteins of the expected sizes were

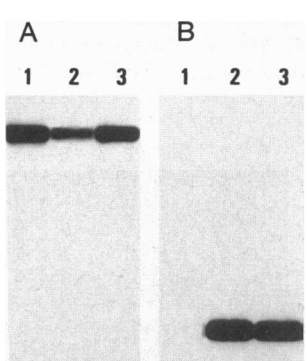

FIG. 1. Northern blot analyses of the replication of satBaMV synthetic transcripts in barley protoplasts coinoculated with BaMV-L RNA. Protoplasts were inoculated with BaMV-L RNA alone (lane 1), or BaMV-L RNA and native satBaMV (lane 2), or with BaMV-L RNA and BSF4 (lane 3). At 24-hr postinoculation (p.i.), 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 ORF-1 probe (*A*) and S probes (*B*).

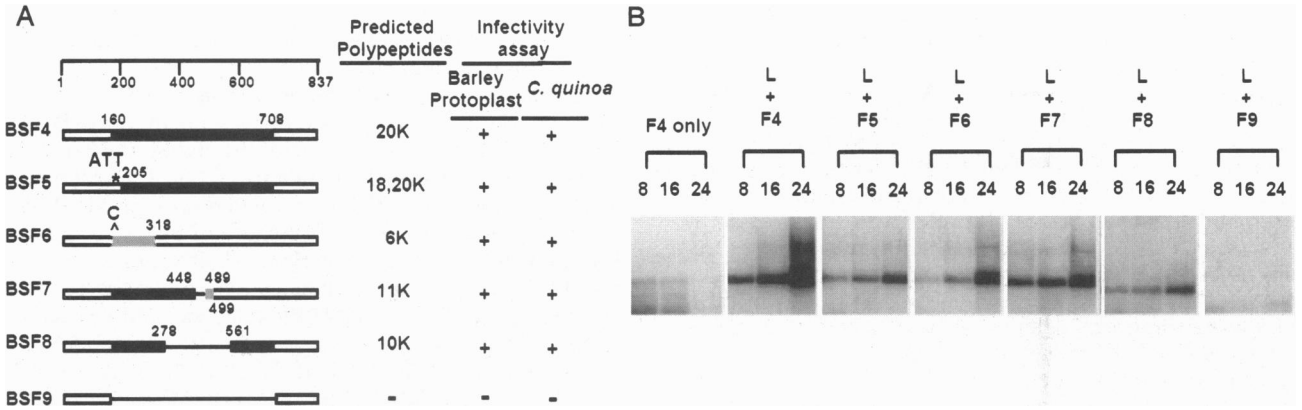

FIG. 2. Summary of the construction and activity of satBaMV mutants. (*A*) Schematic diagram of satBaMV mutants and their altered coding capacity and ability to replicate in barley protoplasts and *C. quinoa* plants. The empty box represents the noncoding sequence of satBaMV, and the thin line corresponds to the deleted sequences. The black box indicates the coding region of satBaMV, and the shaded box indicates the altered reading frame of the coding region. (*B*) Time course accumulation of satBaMV derivatives in infected protoplasts. Northern blot analyses with S probe of total RNAs isolated from 3×10^4 barley protoplasts 8, 16, and 24 hr p.i. with BaMV-L RNA and satBaMV mutants as indicated above each lane. F4 only, inoculation with BSF4 transcripts only.

synthesized: 11 kDa for BSF7, 10 kDa for BSF8, and no detectable products for BSF9 (data not shown).

Replication of satBaMV Mutants in Protoplasts and in *C. quinoa*. Northern blot analysis of total RNAs extracted from infected protoplasts revealed that all the mutants, except BSF9, replicated in cells in the presence of BaMV-L RNA, as evidenced by the increasing accumulation of satBaMV at the different time intervals p.i. (Fig. 2*B*). The amount of detectable satBaMV RNA transcripts for BSF4–BSF8 at 24 hr p.i. was ≈5- to 20-fold higher than that at 8 hr p.i. as quantitated by PhosphorImager (Molecular Dynamics) (Fig. 2*B*). However, there was no significant increase in hybridization signal observed for BSF9 between 8 and 24 hr. In contrast, in protoplasts inoculated with BSF4 transcripts alone, the accumulated RNA transcripts decreased over time (Fig. 2*B*).

Mutations at the N terminus or internal deletions of the satellite protein caused a slight decrease in activity compared with the wild-type BSF4. In six independent experiments, the activity decrease averaged from 21 to 50% for BSF5–BSF8 24 hr p.i. Deletion of the entire ORF in BSF9, however, completely abolished RNA synthesis. Genomic RNA was detectable at 8 hr p.i., and new synthesis continued to 48 hr p.i. in all inoculations (data not shown).

When *C. quinoa* plants were mixedly inoculated with BaMV-L RNA and satBaMV mutants, the results similar to those with protoplasts were obtained. The encapsidated progeny RNAs of satBaMV were detected in virion RNA preparations from infected leaves for BSF4–BSF8, but not for BSF9 (data not shown).

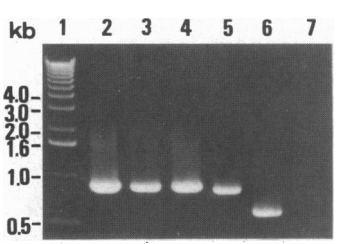

FIG. 3. Gel electrophoresis of reverse transcription-PCR products amplified from virion RNA of *C. quinoa* leaves inoculated with BaMV-L RNA and satBaMV mutants by primers BS0 and BS23. Reverse transcription-PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Sizes of the markers in lane 1 are indicated in kb. Lanes 2–7, products from cells inoculated with BaMV-L RNA and BSF4, BSF5, BSF6, BSF7, BSF8, or BSF9, respectively.

Detection of Progeny RNA in *C. quinoa*. To determine the stability of progeny RNA of mutated satBaMV in infected *C. quinoa*, virion RNA was extracted from infected leaves and cDNA of each mutant was synthesized and amplified by PCR. The recovered fragments from each of the inoculated samples, except BSF9, were shown to be of the expected sizes (Fig. 3). However, the cDNA samples from BSF4, BSF5, and BSF6 were identical in size and cannot be resolved from one another on 1% agarose gel. Therefore the recovered fragments were cloned and sequenced.

Sequence analysis revealed the presence of $A^{160}UG$, $A^{160}UU$, and $A^{160}UGGC$ in the progeny RNA of BSF4, BSF5, and BSF6 from coinoculated plants (Fig. 4), confirming that the mutant sequences were preserved during replication in plants and are apparently stable.

Activity of BSCAT and CAT Immunoassays. Since the complete satBaMV-encoded protein is not required for its replication, the chimeric plasmid pBSCAT was constructed by substituting the CAT coding sequence for the ORF of pBSF4 (Fig. 5*A*). The hybrid BSCAT RNA accumulated to near-wild-type BSF4 levels in protoplasts coinoculated with BaMV-L RNA (data not shown). Similar results were obtained from inoculating *C. quinoa* plants. In Fig. 5*B*, the encapsidated BSF4 (lane 2) and chimeric BSCAT (lane 3) were detected in ethidium bromide-stained agarose gel in virion preparations purified from *C. quinoa* leaves coinoculated with BaMV-L RNA. Northern analysis with the S probe confirmed the identity of these RNAs (Fig. 5*C*).

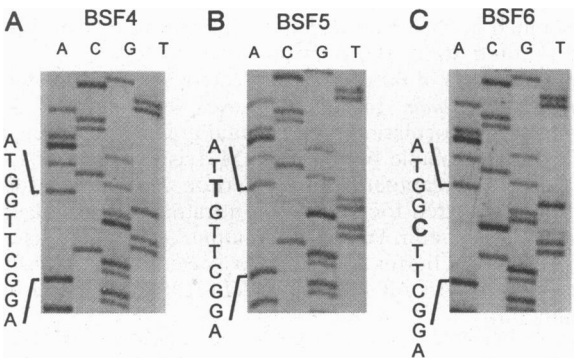

FIG. 4. Sequence analysis of PCR products generated from virion RNA of *C. quinoa* leaves inoculated with BaMV-L RNA and the indicated satBaMV mutants.

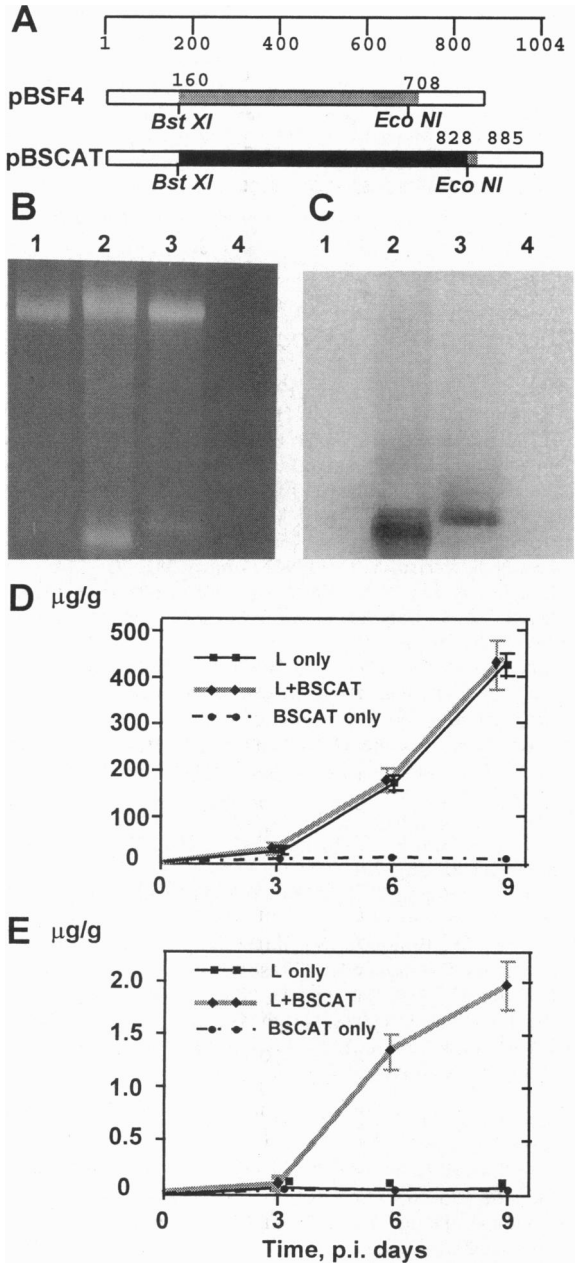

FIG. 5. Schematic diagram, gel electrophoresis, Northern analysis, and the CAT expression of chimeric BSCAT in *C. quinoa* leaves coinoculated with BaMV-L RNA. (*A*) Structure of wild-type pBSF4 and pBSCAT mutant. Light shading indicates BSF4 ORF coding region, and dark region indicates CAT coding region. (*B*) Virion RNAs from 0.2 g of *C. quinoa* leaves inoculated with BaMV-L RNA alone (lane 1), or the mixture with BSF4 (lane 2), BSCAT (lane 3), or mock-inoculated (lane 4) were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. (*C*) Northern blot analysis of virion RNA samples as in *B*. RNAs were transferred to a nylon membrane, hybridized with the S probe as in Fig. 1, except that the S probe was linearized with *Eco*NI before *in vitro* transcription. Accumulations of BaMV capsid protein (*D*) and CAT enzyme (*E*) detected by ELISA in *C. quinoa* leaves inoculated with BaMV-L RNA, BSCAT transcripts alone, or the mixture of BaMV-L RNA and BSCAT transcripts. Results are means ± SEM of three independent experiments.

To further investigate the expression of chimeric BSCAT in *C. quinoa*, the levels of capsid protein and CAT accumulation were determined by ELISA at 3-day intervals after inoculation. Capsid protein accumulation in plants inoculated with BaMV-L RNA alone or coinoculated with BaMV-L RNA and BSCAT reached a similar level (0.5 mg per g of leaves) 9 days p.i. (Fig. 5*D*), indicating that the presence of BSCAT transcripts did not affect helper-virus replication. CAT protein levels increased remarkably over time in extracts of leaves coinoculated with BaMV-L RNA and BSCAT transcripts (Fig. 5*E*). In assays using standard CAT enzyme as a standard, 2 μg of CAT enzyme was produced per g of *C. quinoa* leaves. Control samples inoculated with either BaMV-L RNA or BSCAT transcripts alone exhibited only background ELISA reading (Fig. 5*E*).

DISCUSSION

The ability to synthesize biologically active transcripts *in vitro* from cloned cDNA of satBaMV allows us to study the role of satBaMV-encoded protein involved in the replication cycle. Surprisingly, mutants with the altered ORF were able to replicate in barley protoplasts and *C. quinoa* plants in the presence of genomic RNA (Fig. 2). Analyses of progeny RNA of all the mutants by PCR amplification and nucleotide sequencing revealed that these mutants retained their expected sizes or sequences (Figs. 3 and 4). This result excluded the possibility of contamination by wild-type transcripts BSF4 or by the native satBaMV during infection. Thus, our results indicate that the intact satellite-encoded protein is not essential for the replication of satBaMV. However, it may play a supporting role in replication, as none of the mutants replicated as well as wild-type BSF4. This result contrasts with the results of several large satellite RNAs associated with nepoviruses in which the satellite-encoded proteins are required for their replication (8–10). In some of these cases, a single-amino acid substitution of the encoded protein eliminated the biological activity of the satellite RNAs. In this respect, satBaMV acquired properties similar to those of small satellite RNA groups. Collmer and Kaper (24) altered the AUG initiation codon of a small, putative ORF in cucumber mosaic virus-associated RNA5 and demonstrated that translation of this ORF was not required for satellite replication.

The satBaMV-encoded protein is expressed and immunologically detectable in both protoplasts and plants infected with BaMV-V (N.-S.L. and Y.-H.H., unpublished data). The satBaMV-encoded protein shares no significant homology with other large satellite proteins; however, they have some properties in common—e.g., a highly basic protein with strong net positive charges at the N-terminal region (7, 14). The presence of arginine-rich sequences at the N termini in these proteins might suggest a common RNA-binding motif (25). The 20-kDa protein may well be an RNA-binding protein that assists in replication, movement, or other functions of satBaMV.

The detectable activity of the large deletion mutant BSF8 in infected protoplasts and plants confirmed that the essential cis-acting elements required for satBaMV replication were mainly located at the 5′ and/or 3′ termini of satBaMV. The lack of replication of BSF9 may be due to size effect and/or stability of RNA after the complete removal of the ORF, or the requirement of ORF translability as found in some of defective interfering RNAs (26, 27). The activity of BSCAT could be restored, but not to the level of full-length BSF4, even when a totally unrelated sequence was inserted, as shown in Fig. 5*B*. Hybrid BSCAT containing both the 5′ and 3′ sequences of satBaMV and the inserted ORF sequences were encapsidated.

The CAT gene was expressed from the chimeric BSCAT in coinoculated local-lesion host *C. quinoa* as a replacement for the ORF in BSF4 (Fig. 5*E*), indicating that satBaMV can be a helper-dependent vector to express foreign gene in plants. The relatively high yield of encapsidated BSCAT as compared to the wild-type BSF4 (Fig. 5*B*) precludes the possibility that foreign gene sequence greatly affects the efficiency of virus or satellite RNA replication. The level of CAT protein expressed by BSCAT in plants (2 μg/g of leaf) was comparable to that

achieved by using tobacco mosaic viral vectors (1 μg/g of leaf; ref. 28) or transgenic tobacco (0.8–7.2 μg/g of leaf; ref. 29). However, some other foreign proteins expressed in transgenic plants can reach up to 2% of total soluble protein. The low level of CAT protein detected in this study might be due to the protein stability or unknown translational control mechanism of satellite protein on the satellite RNA. In one case, a large satellite RNA protein was not detectable in infected plants (7).

Systemic expression of foreign gene is one of the major concerns to evaluate the usefulness of plant viral vectors. The host range of this potexvirus is relatively narrow. Besides the natural host bamboos, BaMV-L systemically infects only barley and *Nicotiana benthamiana* among the nearly 50 plant species we tested so far. The expression level of BSCAT in systemic leaves of infected barley and *N. benthamiana* was only about 1/40–1/100th of that in inoculated leaves (data not shown). However, BSCAT did not accumulate to detectable levels under some growth conditions, a phenomenon similar to that of capsid protein mutants of satellite tobacco mosaic virus (30). This low level of BSCAT expression in systemic leaves is possibly related to the low efficiency of both virus replication and systemic movement in these two plants. It is also possible that the 20-kDa protein plays a supporting role in satellite movement.

The data presented above demonstrate that a satellite-based vector can express a foreign gene in the intact plants. The satellite-based vector provides some advantages over the plant viral vectors. Satellites usually replicate in higher copy numbers than the viral genomic RNA. Moreover, in the construct pBSCAT the ORF was replaced by the CAT gene, whereas in other viral vectors the foreign gene was either inserted in addition to the normal ORFs or fused to an expendable viral sequence. Thus, in our system, the factors associated with gene instability resulting from homologous or nonhomologous recombinations could be greatly reduced (2). This is provided by the fact that the CAT protein expressed by BSCAT was accumulated constantly to the level of 2 μg per g of *C. quinoa* leaves after three serial passages. The wide use of such a vector in the expression of foreign gene and in the study of molecular biology of BaMV and satBaMV will be expected.

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