Analysis of the subgenomic RNAs and the small open reading frames of Beetle black scorch virus

Xuefeng Yuan,† Yunhe Cao,† Dehui Xi, Lihua Guo, Chenggui Han, Dawei Li, Yafeng Zhai and Jialin Yu

State Key Laboratory of Agro-Biotechnology, College of Biological Sciences, China Agricultural University, Beijing 100094, PR China

A full-length cDNA of the genome of Beetle black scorch virus (BBSV), isolate Ningxia, was constructed and modified by site-directed mutagenesis to permit in vitro transcription of mutant viral RNAs. Two subgenomic (sg) RNAs (sgRNA1 and sgRNA2) appeared during BBSV replication. Mutagenesis revealed that sgRNA1 transcription was initiated at G^{2209} within the P82 polymerase subunit open reading frame (ORF) and that transcription of sgRNA2 began at G^{2628} within the nested p7b/p5′ ORF. Initiation-codon shifting or premature termination of translation of the three ORFs (P7a, P7b and P5′) encoded by sgRNA1 indicated that each of the genes was required for localized movement, accumulation of viral RNAs and formation of local lesions on the leaves of Chenopodium amaranticolor. Microscopic observations of the distribution of green fluorescent protein fused to the N-terminal portion of the capsid protein provided additional evidence that the P7a, P7b and P5′ proteins are each required for cell-to-cell movement. In contrast, elimination of sgRNA2 showed that the BBSV coat protein was not required for viral RNA accumulation or the appearance of local lesions on C. amaranticolor. In addition, disruption of the small P5 ORF previously predicted by computer analysis to originate at the C terminus of the P82 ORF had no effect on disease phenotype, suggesting that this ORF may represent a cryptic, non-essential gene. These results show that BBSV has a novel cell-to-cell movement protein organization that differs in size and sequence from that of other viruses.

INTRODUCTION

Beetle black scorch virus (BBSV) was first identified in China in the late 1980s (Cui et al., 1988; Liu & Xian, 1995; Zhang et al., 1996) and represents a new species of the genus Necrovirus (Cao et al., 2002; Lommel et al., 2005). The virus elicits severe, systemic disease symptoms typified by black scorched leaves and necrotic fibrous roots in sugar beet (Beta vulgaris L.) fields throughout north-west and eastern China. The virus has also been shown to be transmitted efficiently through the soil in a non-persistent manner by zoospores of Olpidium brassicae (liang et al., 1999). After mechanical inoculation of BBSV in the greenhouse, necrotic local lesions appear on the leaves of Chenopodium spp., Tetragonia expansa and Spinacia oleracea, and symptomless infections are observed on many Nicotiana spp., Solanum lycopersicum, Physalis floridana and Lactuca sativa (Cai et al., 1993). Virus can be detected in each of these hosts by ELISA and by back-inoculation to Chenopodium amaranticolor.

BBSV comprises an icosahedral particle of 28 nm that encapsidates a positive-sense, single-stranded (ss) genomic (g) RNA of 3644 nt. BBSV isolates from Ningxia Province (BBSV-N) (Cao et al., 2002) and Xinjiang Province (BBSV-X) in China have 99-45 % nucleotide identity, and a 615 nt satellite RNA has been found in the BBSV-X isolate (Guo et al., 2005). As a member of the genus Necrovirus, BBSV shares the highest sequence identity (61 %) with Tobacco necrosis virus D (TNV-D) (Coutts et al., 1991), but lacks extensive sequence relatedness to other viruses deposited in DDBJ/EMBL/GenBank.

In a previous report, six open reading frames (ORFs) were predicted in the BBSV genome by computer analysis (Cao et al., 2002). The P23 and P82 ORFs are thought to encode the viral RNA polymerase subunits, as the P82 protein contains a GDD motif that is conserved in RNA-dependent RNA polymerases (Argos, 1988). The P82 ORF, residing between nt 36 and 2210, is probably expressed directly from the gRNA via translational readthrough of an amber stop codon. The functions of the small P5, P7a and P7b ORFs located in the central region of the BBSV RNA genome have

†These authors contributed equally to this paper.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this work are AF452884 (BBSV-N) and AY626780 (BBSV-X).

Details of the primers used in this study are available as supplementary material in JGV Online.
not been identified, and similar proteins encoded by a subgenomic (sg) RNA in TNV-D (Coutts et al., 1991) and TNV-DH (Drouzas et al., 1996; Molnár et al., 1997) are required for cell-to-cell movement. The 3′-proximal ORF (nt 2647–3345) in BBSV is predicted to encode the capsid protein (P24) due to its 24-5 kDa mass, as estimated by SDS-PAGE. The capsid proteins of TNV-DH (Molnár et al., 1997) and Turnip crinkle virus (TCV) (Cohen et al., 2000a) have been demonstrated to be involved in long-distance movement during systemic infection of Nicotiana benthamiana, but no information is yet available about this requirement for BBSV.

We have shown previously that two sgRNAs along with the viral gRNA are present in plant tissues infected with BBSV, but the sgRNAs are not encapsidated in viral particles (Guo et al., 2005). We presume that the ORFs located downstream of the putative BBSV RNA polymerase subunits use these sgRNAs for their translation, as is the case for many positive-strand RNA viruses (Miller & Koev, 2000). This study reports mutagenesis experiments that were carried out to investigate the requirements of the downstream BBSV ORFs and their encoded proteins for cell-to-cell movement and local lesion formation in C. amaranticolor.

METHODS

Virus preparation and RNA extraction. BBSV-N was collected from Ningxia Province and propagated in C. amaranticolor by mechanical inoculation in the greenhouse. The BBSV-N sequence reported previously (Cao et al., 2002) was used to design mutagenesis strategies throughout this study. When local lesions first appeared at 3–4 days post-inoculation (p.i.), virus particles were purified from infected leaves by sucrose density-gradient centrifugation (Bo et al., 1996) and used for viral RNA extraction as described previously (Guo et al., 2005). One microgram of purified virus was used directly to determine the amino acid sequence of the coat protein (CP) by Edman degradation (Percy & Buchwald, 1972) on an Applied Biosystems (Procise 491) protein sequencer.

Amplification of the 5′ regions of sgRNAs. In order to confirm the 5′ sequences of the BBSV sgRNAs, double-stranded (ds) RNAs were extracted from infected C. amaranticolor leaves on CF-11 cellulose columns as described by Dodds et al. (1984). The dsRNA templates corresponding to BBSV sgRNA1 and sgRNA2 were recovered separately from 0.8% agarose gels by a freeze–thaw procedure (Benson et al., 1985). Two primers designated BB-20 and BB-25 that were complementary to BBSV RNA at nt 2646–2626 and 3264–3247 (see Supplementary Table S1, available in JGV Online) were used for initiation of RACE RT-PCR (Frohman et al., 1988). The ss cDNAs complementary to sgRNA1 and sgRNA2, respectively, were purified with a High Pure PCR Products Purification kit (Roche). Oligo(dC) was ligated to the 5′ ends and the cDNAs were used as templates for PCR amplification of ds cDNAs using appropriate oligonucleotides and an oligo(dG)16 primer complementary to the anchor sequence (Supplementary Table S1). The 400 and 700 bp PCR products corresponding to sgRNA1 and sgRNA2 were ligated into the pUCm-T vector (Sangon) and four individual cDNA clones were selected for sequencing after screening of Escherichia coli transformants. Two BBSV mutant cDNAs, p8r1-Mut-A3290 and p8r2-Mut-C2526, were constructed for in vitro transcription using the primer pairs BB-88/BB-82 and BB-83/BB-84, respectively (Supplementary Table S1; Fig. 1a).

Construction of BBSV infectious cDNAs. Full-length BBSV cDNA was synthesized by RT-PCR using 2 μg viral RNA as template in reactions containing the BB-18 and BB-14 primers (see Supplementary Table S1, available in JGV Online) and 200 U Superscript reverse transcriptase II (Invitrogen). The amplified full-length cDNA contained a bacteriophage T7 RNA polymerase promoter (Quillet et al., 1989) that was designed to start in vitro transcription of gRNA with one additional G residue at the 5′ end and a Smal restriction site at the 3′ end to facilitate termination of transcription. After blunt-ending with T4 DNA polymerase, the 3.6 kb ds cDNA was ligated into pUC19 at the Smal site for transformation of E. coli strain DH5α. The cDNA clone of the wild-type BBSV-N isolate was confirmed by nucleotide sequencing of both strands and was designated pUBF52 (Fig. 1a).

Using the methods described by Ho et al. (1989), BBSV mutants containing substituted nucleotide(s) were derived from pUBF52 by site-directed mutagenesis of PCR amplifications using primer pairs corresponding to specific regions of the BBSV genome (Fig. 1a; Supplementary Table S1). PCR products were digested with DpnI to remove the template DNAs from the mixtures (Geier & Modrich, 1979) and religated for transformation of E. coli. Nine single- or double-nucleotide site-specific BBSV mutants were constructed in the small ORFs of P5, P7a, P7b and P5′ (Fig. 1a) using the oligonucleotide pair combinations shown in Supplementary Table S1, available in JGV Online.

To facilitate analysis of BBSV cell-to-cell movement, the green fluorescent protein (GFP) gene (Cormack et al., 1996) was amplified with the GFP-1 and GFP-2 primers (Supplementary Table S1). The GFP gene was fused downstream of the EcoNI site (nt 2715) in the 5′-proximal region of the BBSV P24 gene in pUBF52 to create the plasmid pBGFP, in which most sequences of the BBSV CP were truncated by PCR using the primer pair BB-21 and BB-46 (Supplementary Table S1). In addition, three other mutants (p7a-Mut-GFP, p7b-Mut-GFP and p5′-Mut-GFP; Fig. 1b) were derived by modification of plasmid pBGFP with the p7a-Mut-G2229, p7b-Mut-G2422 or p5′-Mut-C2455 derivatives, respectively (Fig. 1a). These plasmids contained a GFP fusion to an N-terminal 22 aa (excluding Met) fragment of the capsid protein that was introduced by substituting the Xhol–SspI fragment (nt 1737–2595) of pBGFP with the same fragments from p7a-Mut-G2229, p7b-Mut-G2422 or p5′-Mut-C2455, respectively, to create p7a-Mut-GFP, p7b-Mut-GFP and p5′-Mut-GFP. These plasmids thus contained a reporter gene to assess expression of the mutated translational initiation codons of ORF P7a, P7b and P5′, respectively. Each mutant was verified by diodeoxyxynucleotide chain-termination sequencing (Sanger et al., 1977) and RNAs transcribed from the mutant plasmids were inoculated into plants. The MOLD web server of M. Zucker (http://www.bioinfo.rpi.edu/applications/mfold, version 2.3) was used to predict the secondary structure of the viral RNAs.

Inoculation with in vitro-synthesized RNAs. Plasmids (100 ng) were linearized with Smal and used for run-off transcription at 37°C for 1.5 h with a T7 RNA polymerase kit as described by the manufacturer (Promega). The synthesized RNAs (1–2 μg) were mixed with an equal volume of inoculation buffer (50 mM glycine, 30 mM K2HPO4, 1% bentonite, 1% celite, pH 9-2) and rubbed on C. amaranticolor leaves. After separation on 1% agarose gels, the RNAs were transferred to nylon membranes (Hybond N+; Amersham Biosciences) for Northern blot analysis. Replication of BBSV RNAs was assessed with a cDNA probe complementary to the 300 nt (nt 3345–3644) at the 3′-proximal end of the BBSV genome. Digoxigenin-labelled probes were produced with a DIG-High Prime DNA Labelling and Detection Starter kit I (Roche) and 35S-labelled probes were generated with the Prime-a-Gene Labelling system.
(Promega) and [α-32P]dCTP according to the manufacturer’s instructions. C. amaranticolor leaves were inoculated with transcripts from pBGFP or the mutant derivatives (Fig. 1b) by bombardment (PDS-1000/He system; Bio-Rad) and GFP fluorescence was visualized under a laser-scanning microscope (LSM 510; Carl Zeiss) (10× objective, 488 nm laser excitation with a 505–530 nm filter).

RESULTS

N-terminal sequencing of BBSV CP

The BBSV CP was initially presumed to start at an AUG codon at nt 2647–2649 (Cao et al., 2002), but another in-frame AUG codon exists at nt 2686–2688 that could potentially initiate translation of a smaller protein. To determine the authentic initiation codon, 5 aa at the N terminus of the purified viral protein were sequenced and determined to be Ala–Pro–Lys–Arg–Asn (data not shown). As predicted earlier, this result was consistent with initiation of translation at the first AUG.

Identification of the start sites of BBSV sgRNAs

Full-length BBSV RNAs produced by run-off transcription in vitro from the plasmid pUBF52 were used for inoculation of C. amaranticolor. Local lesions appearing on the leaves at 3–5 days p.i. were similar in timing and appearance to those caused by wild-type BBSV (Fig. 2a). Northern blot analysis of RNA from leaves containing the lesions revealed that the viral RNAs included an abundant gRNA and two smaller lower-abundance RNAs, designated sgRNA1 and sgRNA2. These RNAs accumulated to about the same levels as in leaves infected with wild-type BBSV RNA (Fig. 2b). Thus, these results showed that the in vitro-synthesized viral RNAs from pUBF52 faithfully represented the biological activity of wild-type BBSV and produced similar patterns of viral and sgRNAs.

To define the two sgRNAs, the 5′ regions of the sgRNAs were amplified by RACE (rapid amplification of cDNA ends) RT-PCR using dsRNA templates corresponding to the sgRNAs (data not shown). Sequencing from both directions of the cloned PCR products indicated that the 5′-proximal nucleotide was a guanine corresponding to nt 2209 (G2209) of the BBSV genome. This position was postulated to represent the transcription start site of the sgRNA1. To verify this hypothesis, the BBSV cDNA mutant psR1-Mut-A2209 was constructed for in vitro transcription of mutant viral RNAs. This mutant contained an adenine (A) substituted for the G2209 residue in pUBF52, which did not alter the stop codon of the P82 polymerase found at nt

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Fig. 1. Structure of BBSV cDNAs used for in vitro transcription and GFP expression. (a) Wild-type BBSV cDNA (pUBF52) is shown with the central region expanded below. The boxes show predicted ORFs in the BBSV genome. A stop codon readthrough between P23 and P82 is illustrated by a vertical dashed line. The nine BBSV mutants were prepared by site-directed mutagenesis using appropriate oligonucleotides (see Supplementary Table S1, available in JGV Online). In these cDNA mutants, the AUG codon and other residue substitutions of the wild-type BBSV are linked by a dashed line across the expanded region to identify the sequence position. (b) The recombinant BBSV cDNA (pBGFP) is shown with the central region expanded below. The sequence lengths of the GFP fusion proteins are indicated in parentheses. The three mutants were generated by substituting the Xhol–SpeI fragment (nt 1737–2595) of pBGFP with the same fragments from p7a-Mut-G2229, p7b-Mut-G2422 or p5′-Mut-C2435, respectively.
2208–2210 (Fig. 1a). After mechanical inoculation with transcripts from psR1-Mut-A2209, local lesions failed to appear on the leaves of C. amaranticolor (Fig. 2a). Northern blot analysis of highly concentrated total RNA preparations from these leaves failed to visualize sgRNA1, but the presence of the gRNA and sgRNA2 could be detected in the heavily overloaded lane (Fig. 2b). These results and the nucleotide sequence from the RACE-PCR product amplified from the dsRNA template provided persuasive evidence that the G2209 residue represented the transcriptional start site of sgRNA1. In addition, as no local lesions were formed on the leaves infected by in vitro transcripts of psR1-Mut-A2209, and the CP was not evident in Western blot analysis of leaf extracts (data not shown), one or more of the P7a, P7b or P5’ proteins potentially encoded by sgRNA1 were presumed to be required for cell-to-cell movement and virus accumulation in the infected tissues.

The smaller dsRNA corresponding to sgRNA2 was recovered from gels and a cDNA fragment was synthesized by RACE RT-PCR as outlined for sgRNA1. Sequencing data from four individual clones suggested that a guanosine at position 2526 (G2526) was the transcriptional start site of BBSV sgRNA2. To confirm this result, G2526 in the plasmid pUBF52 was replaced by a cytosine (C) residue to produce the plasmid psR2-Mut-C2526. This substitution did not affect the P7b (nt 2421–2618) or P5’ (nt 2423–2577) ORFs, but the codon for glutamic acid at nt 2526–2528 in the p7b gene was altered to a glutamine. The latter change did not appear to affect infection, as inoculation of C. amaranticolor with in vitro transcripts of psR2-Mut-C2526 produced local lesions within 3–4 days p.i. that had the same timing and appearance as those caused by wild-type BBSV (Fig. 2a). Northern blot analysis of the viral RNAs from inoculated leaves demonstrated that the C2526 mutation abolished sgRNA2 (Fig. 2b) and eliminated the ability to detect the CP by Western blot analysis (data not shown). However, in contrast to the reduced RNA abundance noted when leaves were inoculated with the psR1-Mut-A2209 transcripts, the gRNA and sgRNA1 of the mutated BBSV accumulated to levels similar to those in plants infected by wild-type BBSV or the RNA transcripts from pUBF52 (UBF52) and psR2-Mut-C2526 (Mut-C2526), as shown by the ethidium bromide-stained gel of rRNA in the lower panel. The positions of the full-length gRNA and sgRNA1 and sgRNA2 are indicated.

Fig. 2. Lesion phenotypes and Northern blot analysis of BBSV RNAs in C. amaranticolor leaves inoculated with in vitro transcripts at 3–4 days p.i. (a) Leaves were inoculated mechanically with wild-type BBSV or in vitro-synthesized viral RNAs from the cDNA clones pUBF52 (UBF52), psR1-Mut-A2209 (Mut-A2209) or psR2-Mut-C2526 (Mut-C2526) (see Fig. 1a), respectively. (b) For Northern blot analysis, a 300 nt cDNA probe specific for the BBSV 3’ end was used for detection of gRNA and sgRNAs. The total RNA sample loaded from plants inoculated with the RNA transcripts of psR1-Mut-A2209 (Mut-A2209) was 50-fold more concentrated than those from plants infected by wild-type BBSV or the RNA transcripts from pUBF52 (UBF52) and psR2-Mut-C2526 (Mut-C2526), as shown by the ethidium bromide-stained gel of rRNA in the lower panel. The positions of the full-length gRNA and sgRNA1 and sgRNA2 are indicated.
Translational mutagenesis of the P5 ORF

As elimination of sgRNA1 resulted in a drastic reduction in viral RNA accumulation and lesion development in C. amaranticolor leaves, we postulated that one or more of the small, centrally located ORFs that encoded the P5, P7a, P7b and P5’ proteins were required for cell-to-cell movement and local lesion induction. To clarify these functions, seven site-directed modifications of the translation initiation codons in each of the small ORFs were constructed (Fig. 1a) and used for in vitro transcription of BBSV RNAs. The P5 ORF (nt 2107–2244) overlaps the start site of sgRNA1 (G2209), the P82 ORF (nt 36–2210) and the P7a ORF (nt 2228–2419) (Fig. 1a). Two in-frame AUGs at nt 2107–2109 and 2128–2130 were potential initiation codons for translation of the 5 kDa protein. Therefore, two simultaneous site-specific mutations were introduced to convert the two AUG codons into AC2108G and AC2129G to produce the plasmid p5-Mut-C (Fig. 1a). Another mutant plasmid, p5-Mut-G2144 (Fig. 1a), was constructed by substitution of the UCA for UGA at nt 2143–2145 of the RNA to produce a premature termination of the P5 ORF. In these two mutants, the codons for aspartic acid (nt 2106–2108), asparagine (nt 2127–2129) and leucine (nt 2142–2144) in the P82 ORF were maintained after the nucleotide substitutions. After inoculation with transcripts from p5-Mut-C or p5-Mut-G2144, local lesions indistinguishable from those induced by the wild-type virus appeared on C. amaranticolor within 3–4 days p.i. Northern blot analysis also revealed that the viral RNA components from infected leaves accumulated to levels similar to those produced by wild-type RNA transcripts (Fig. 3). An additional mutant (p5-Mut-D) in the P5 ORF was prepared by site-directed mutagenesis to replace the putative AUG initiation codons with two AGGs (AG2108G and AG2129G). This mutant contained glutamic acid and lysine codon substitutions for aspartic acid (nt 2106–2108) and asparagine (nt 2127–2129), respectively, in the P82 ORF. After inoculation with in vitro transcripts, only a few local lesions appeared on C. amaranticolor leaves and very low levels of viral RNAs were detected in infected tissue by Northern blot analysis (data not shown). These results, when compared with those resulting from inoculation with the p5-Mut-C or p5-Mut-G2144 transcripts (Fig. 3), suggested that the reduction of symptoms and viral RNA accumulation in the AG2108G and AG2129G mutant (p5-Mut-D) infections resulted from amino acid substitutions in the overlapping P82 replicase sequence. Based on these results, the P5 ORF of BBSV appeared not to be essential for the infection phenotype or for replication of BBSV RNA in C. amaranticolor.

Translational mutagenesis of other small ORFs

In order to identify more precisely the functions of the sgRNA1-encoded proteins, P7a, P7b and P5’ ORF mutants were constructed by site-directed mutagenesis. In the plasmid p7a-Mut-G2229, the putative translational start codon of ORF P7a (nt 2228–2230) was changed from AUG to AGG (Fig. 1a). Similarly, the translational start codons of ORF P7b and ORF P5’ were mutated to produce the plasmids p7b-Mut-G2422 and p5’-Mut-C2435. These manipulations introduced single-nucleotide substitutions to replace the AUG codons with AGG and ACG, respectively (Fig. 1a). As the whole P5’ ORF was nested within the P7b ORF, the tyrosine codon (nt 2433–2435) of ORF P7b was maintained in p5’-Mut-C2435. Two additional mutant plasmids (p7b-Mut-U2529 and p5’-Mut-U2527) were constructed for premature termination of the P7b and P5’ ORFs. In these cases, the P5’ ORF had a lysine codon replaced with aspartic acid at nt 2527–2529 in p7b-Mut-U2529 and the P7b ORF had a glutamic acid codon at nt 2526–2528 replaced with valine in p5’-Mut-U2527, respectively.

After mechanical inoculation of C. amaranticolor with in vitro transcripts from each of the five mutants p7a-Mut-G2229, p7b-Mut-G2422, p5’-Mut-C2435, p7b-Mut-U2529 and p5’-Mut-U2527, inoculated leaves remained symptomless for several weeks. Furthermore, leaves inoculated with the mutants all contained very low levels of viral RNA compared with those inoculated with wild-type BBSV (Fig. 4), although the mutants and the wild-type virus had similar ratios of gRNA and sgRNAs. In conclusion, the results from site-directed mutagenesis of the translational codons of the P7a, P7b and P5’ ORFs provided evidence that each of the three proteins encoded by the small ORFs was required for production of visible local lesions and suggested that the low
levels of viral RNAs observed resulted from replication in primary infected cells from which the virus was unable to move.

To determine whether mutations in the P7a, P7b and P5’ ORFs disrupted movement, virus infection was evaluated in leaf tissues by expression of GFP–CP fusions that expressed GFP fused to a 22 aa N-terminal fragment of the CP. For this purpose, the pBGFP reporter plasmid and derivative mutants (p7a-Mut-GFP, p7b-Mut-GFP and p5’-Mut-GFP; Fig. 1b) were transcribed in vitro for plant inoculations. Young leaves of C. amaranticolor were harvested and bombarded with the viral RNAs. After incubation for 2–3 days in moist Petri dishes at room temperature, the inoculated leaves were examined by confocal microscopy. Leaves inoculated with the pBGFP (wild-type GFP derivative) transcripts produced clusters of several cells exhibiting green fluorescence that was prominent in the cytoplasm and nuclei of infected cells (Fig. 5a). In contrast, fluorescence was limited to single cells when the plants were inoculated with the mutant derivatives, although inexplicably the fluorescence was localized predominantly in the nuclei of each of the single infected cells (Fig. 5b–d). These results demonstrated that each of the P7a, P7b and P5’ proteins encoded by the small, centrally located ORFs was essential for the cell-to-cell movement of BBSV.

**DISCUSSION**

BBSV has strong similarities in RNA sequence, length (3·6–3·8 kb) and genome organization to other species in the genus *Necrovirus*, including TNV-A (Meulewaeter et al., 1990), TNV-D (Couuts et al., 1991; Molnár et al., 1997), *Olive latent virus* 1 (*OLV-1*) (Grieco et al., 1996), *Leek white stripe virus* (Lot et al., 1996) and olive mild mosaic virus (Cardoso et al., 2005). Our sequencing results also suggest that BBSV, like other necroviruses, expresses the upstream P23 and P82 ORFs by direct translation of gRNA from the first initiation codon and that the P82 protein is probably produced by a translational readthrough. The results presented in this paper have shown that the genes positioned downstream of the viral RNA polymerase are expressed via translation of sgRNAs, as is the case for a large number of positive-strand RNA viruses (Miller & Koev, 2000), including TNV-A and TNV-D (Meulewaeter et al., 1992; Molnár et al., 1997). In the case of BBSV, site-directed mutagenesis of the transcriptional start sites demonstrated that two sgRNAs are involved in expression of the 3’-terminal ORFs. The mutation at G<sup>2229</sup> eliminated sgRNA1 expression and translation of the three small proteins P7a, P7b and P5’, whereas the G<sup>2526</sup> mutation abolished expression of sgRNA2 and expression of the CP. All of these properties of BBSV are similar to those of TNV-D (Offei & Couuts, 1996), including the lengths of the sgRNAs and their untranslated leader sequences and the functional similarities of their respective proteins. Based on these results, a model for the BBSV genomic organization and sgRNA expression strategy is proposed in Fig. 6. These results suggest that the P7a and the P7b/P5’ proteins are probably translated through unusual ribosomal-scanning mechanisms, whereas the P24 CP is expressed from a monocistronic sgRNA.

As elimination of sgRNA1 prevented the appearance of local lesions on *C. amaranticolor*, we questioned whether the small proteins encoded by sgRNA1 were essential for cell-to-cell movement. In leaves inoculated with sgRNA1 mutants, the BBSV RNAs, including gRNA and sgRNA2, were present at much lower levels than in plants inoculated with wild-type virus (Fig. 2b). Our results with the GFP reporter derivatives suggested that the reduced accumulation of BBSV RNAs was due to failure of the virus to move from the cells that were initially infected, rather than as a result of interference with virus replication. Thus, the P7a, P7b and P5’ proteins were each required for cell-to-cell movement (Fig. 5). This requirement for P7a, P7b and P5’ also reflects the requirement for the small proteins encoded by TNV-A or TNV-D, which are involved in cell-to-cell movement (Meulewaeter et al., 1992; Offei et al., 1995; Drouzas et al., 1996; Molnár et al., 1997). The P7a and P7b ORFs are similar to those of TNV-D<sup>14</sup>. However, in BBSV, the P5’ ORF differs by being nested within the P7b ORF in a different reading frame and this feature distinguishes BBSV from all other necroviruses. In contrast, the P5 ORF of BBSV spanning the P82 and P7a ORFs is positioned similarly to that of the P7<sub>1</sub> ORF in TNV-D and TNV-D<sup>14</sup> (Molnár et al., 1997). As P5 is not encoded by sgRNA1, it is unlikely that this ORF is expressed during infection. Irrespective of this, P5 is dispensable for cell-to-cell movement, whereas the P7<sub>1</sub>
ORF of TNV-D\(^{11}\) (Molnár et al., 1997) is required for movement. These results suggest that the BBSV P5 ORF may represent a cryptic non-essential gene, or that, if a P5 protein is encoded, its role in the BBSV life cycle differs from that of the P7\(^1\) ORF of TNV-D\(^{11}\).

Compared with the triple gene block movement proteins present in some plant viruses (Morozov & Solovyev, 2003), BBSV has a novel cell-to-cell movement protein organization that differs in size and sequence from those of other viruses. The genera Avenavirus (Boonham et al., 1995), Necrovirus (Meulewaeter et al., 1990; Coutts et al., 1991; Molnár et al., 1997; Castellano et al., 2005), Carmovirus (Hacker et al., 1992; Li et al., 1998), Panicovirus (Turina et al., 2000) and Machlomovirus (Scheets, 2000) of the family Tombusviridae encode a 7–9 kDa movement protein that is associated with another small ORF encoding an 8–9 kDa polypeptide. The genera Tombusvirus (Hearne et al., 1990) and Aureivirus (Rubino & Russo, 1997) encode a 22 and a 27 kDa movement protein, respectively, and members of the genus Dianthovirus utilize a third type of movement protein of around 35 kDa (Boonham et al., 1995; Lommel et al., 2005). The small proteins of the genera Aureivirus, Avenavirus, Panicovirus and Tombusvirus usually have overlapping coding regions. However, the proteins encoded by the nested genes of the genera Tombusvirus and Aureivirus, respectively, are associated with the suppression of virus-induced gene silencing (Lakatos et al., 2004; Mérai et al., 2005) and appear to affect movement only indirectly. From our results, the three small proteins P7a, P7b and P5‘ are each required for cell-to-cell movement of BBSV. The P5‘ ORF is fully nested within the P7b ORF and thus the BBSV movement protein organization differs from that of other members of the family Tombusviridae. Therefore, the P5‘ movement complex comprising P7a/ P7b/P5‘ of BBSV may represent a new class of cell-to-cell movement protein in the variable family Tombusviridae.

Plant RNA virus CPs are often multifunctional proteins that have been reported to be involved in virus movement, vector transmission, genome activation or elicitation of symptoms (Callaway et al., 2001). In addition, suppressor functions of the CPs of TCV and Citrus tristeza virus in RNA silencing have been reported (Qu et al., 2003; Thomas et al., 2003; Lu et al., 2004). Our results with the mutant psR2-Mut-C\(^{526}\), which destroyed sgRNA2 transcription, demonstrated that the local lesion phenotype in C. amaranticolor (Fig. 2a) is not affected by the absence of the viral CP and this result is consistent with the results produced by inoculation with mutants with a single base substitution that altered the CP initiation codon (data not shown). Therefore, BBSV is similar to TNV-D\(^{11}\), but not to TCV, in the family Tombusviridae (Hacker et al., 1992; Molnár et al., 1997; Li et al., 1998; Cohen et al., 2000), as the BBSV CP is not essential for cell-to-cell spread in C. amaranticolor. However, it is not clear whether efficient systemic movement of BBSV in other host plants, such as sugar beet, is compromised by deletion of the CP.

In experiments using GFP expression to assess the movement of pBGFP, where most of the BBSV CP sequences were replaced by the GFP–CP fusion, a substantial decrease in the number of local lesions on the leaves of C. amaranticolor was
observed following inoculation with in vitro transcripts. However, the sizes of the local lesions were slightly larger than the wild-type local lesions. This result differed from our site-directed mutagenesis experiments with sgRNA2, which suggested that expression of the CP was not essential for BBSV infection. Thus, the reduced lesion numbers noted on *C. amaranticolor* infected by the pBGFP transcripts may have been due to the destruction of cis-acting sequences required for high levels of virus replication during substitution of GFP for most of the P24 sequence. This result thus may be reminiscent of studies with *Tomato bushy stunt virus* (TBSV) in which long-distance interactions were shown to be required for efficient replication (Lin & White, 2004). If so, the few lesions produced should contain compensatory mutations to restore these putative interactions, and this possibility is being investigated.

In contrast to the P7a, P7b and P5' mutants, where the GFP–CP fluorescence was confined largely to the nuclei of the isolated infected cells (Fig. 5b–d), GFP fluorescence from pBGFP appeared throughout the cytoplasm of the clusters of infected cells (Fig. 5a), mainly in association with the plasma membrane. This subcellular-localization difference appeared to be due to mutations introduced into the movement proteins, but the reasons for the shift in fluorescence are not obvious. As GFP was fused to a 22 aa fragment of the CP, the nuclear localization differed from that associated with movement-associated proteins of the necroviruses TCV, OLV-1 and TNV-D (Drouzas et al., 1996; Cohen et al., 2000b; Castellano et al., 2005).

As BBSV infections were initiated with uncapped transcripts, it is likely that BBSV is translated by a
cap-independent mechanism, similar to that of other members of the family Tombusviridae. During sequence alignments, we noted a conserved 5′-ACCA-3′ sequence in the 5′ regions of BBSV gRNA and the two sgRNAs. This sequence is complementary to a 5′-UGGU-3′ sequence in the 3′ untranslated regions (UTRs) of the RNAs (Fig. 7a) that may act as a cap-independent translational enhancer (Wu & White, 1999). In addition, Mfold structural analysis of the entire BBSV sequence indicated that both the gRNA and sgRNAs contain potential stem-loop structures at their 5′ and 3′ termini (Fig. 7b; –237·5 kcal mol−1 at 25 °C). These results suggest that more energetically favourable, long-distance, 5′-3′ RNA–RNA interactions may be involved in translation of BBSV RNAs, as is the case with TBSV in the family Tombusviridae (Fabian & White, 2004). In the 3′ UTR of BBSV RNAs, a Y-shaped domain formed by the pentaloop 5′-GUAA-3′ is similar to the tetraloop 5′-GAAA-3′ in TBSV and other necroviruses (Fabian & White, 2004; Meulewaeter et al., 2004). Interestingly, a similar conserved sequence and a terminal stem–loop structure are also found in the 615 nt satellite RNA associated with BBSV (Guo et al., 2005; Fig. 7a, b). As no protein is encoded by the satellite RNA and no unique proteins have been detected in satellite RNA infections with BBSV, this observation suggests that the 5′-3′ RNA–RNA interactions may be involved in other aspects of BBSV replication, in addition to a translational enhancer function to recruit the host protein-synthesis machinery (Guo et al., 2001; Meulewaeter et al., 2004).

ACKNOWLEDGEMENTS

We thank Professor Andrew O. Jackson (Department of Plant and Microbial Biology, University of California at Berkeley, CA, USA) for his helpful suggestions and constructive criticism. This work was supported by grants 30325001 and 30270063 from the National Natural Science Foundation of China (NSFC) and grant 2002AA206641 from the National High Technology R&D Program of China.

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