Two distinct sites are essential for virulent infection and support of variant satellite RNA replication in spontaneous beet black scorch virus variants

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INTRODUCTION

Beet black scorch virus (BBSV) is a member of the genus Necrovirus in the family Tombusviridae (Cao et al., 2002; Rochon et al., 2011). BBSV isolates reported in China (Cao et al., 2002; Xi et al., 2006), the USA (Weiland et al., 2006, 2007), Iran (Koenig & Valizadeh, 2008) and Spain (González-Vázquez et al., 2009) exhibit nucleotide sequence similarities ranging from 93.0 to 99.5 %. Mechanical inoculation with virus preparations has shown that BBSV can infect 13 plant species within four families, including Chenopodium amaranticolor, which develops necrotic lesions at 3 days post-inoculation (p.i.) (Cai et al., 1993).

BBSV has a single positive-stranded RNA genome of 3644 nt and shares 61 % nucleotide sequence identity with tobacco necrosis virus D (TNV-D) (Coutts et al., 1991) and 44 % sequence identity with TNV-A (Meulewaeter et al., 1990) in the genus Necrovirus (Rochon et al., 2011). BBSV is predicted to encode six viral proteins and cause is serious necrotic symptoms on the upper leaves of host plants. The 5'-proximal ORF of BBSV encodes a 23 kDa protein (P23) terminated by an amber stop codon that can be circumvented by translational read-through to produce a low-abundance 82 kDa protein (P82) containing a conserved ‘GDD’ polymerase motif (Argos, 1988). Three small BBSV proteins (P7a, P7b and P5') expressed from subgenomic (sg) RNA1 are essential for viral cell-to-cell movement, and the viral coat protein (CP) is translated from sgRNA2 (Yuan et al., 2006). The wild-type (wt) CP has been shown to have a nuclear-localization signal (Zhang et al., 2011) that is dispensable for virus pathogenicity, because deletion of the N-terminal 23 aa leads to the same symptom phenotype as that caused by the original cloned virus (Cao et al., 2006).

Recently, we observed a barley yellow dwarf virus (BYDV)-like translation enhancer (BTE) element harbouring a 17 nt conserved sequence predicted in the 3'UTR of BBSV, in which two nucleotides at positions 3469 and 3477 differed from the BTE consensus sequences of other members of the genus Necrovirus, including TNV-A and TNV-D (Shen & Miller, 2004; Wang et al., 2010). Several studies have shown

Three figures, a table and references are available as supplementary material with the online version of this paper.
that the 3’UTR sequences of many plant viruses are involved in long-distance RNA–RNA interactions that affect virus gene expression and replication (Miller & White, 2006). These elements have distinct structures and have been designated cap-independent translation elements (CITE) or, in the case of luteoviruses, BTE (Miller & White, 2006; Miller et al., 2007).

A single-stranded, 615 nt satellite RNA (satRNA) has been identified in infections by the BBSV Xinjiang isolate in China (Guo et al., 2005), and satRNAs have also been detected by RT-PCR with other BBSV isolates in Iran (Mehrvar & Bragard, 2008). It was also shown that multimeric forms of satRNA have different roles in their replication (Guo et al., 2005).

Spontaneous point mutations of viral genomes are important in RNA virus evolution (Ramirez et al., 1995; Roossinck, 2008). Mutations in the coding regions of different viruses (de Assis Filho et al., 2002; Gal-On, 2000; Hagiwara et al., 2002; Suzuki et al., 1995; Tsai & Drehé, 1993; Yamaba et al., 2008), or within the 5′ UTR of alfalfa mosaic virus RNA 3 (van der Vossen et al., 1996), can result in modifications of the biological properties of viruses, and both classes of mutation have been shown to affect symptom severity. Also, mutagenesis of other viral genes has been shown to be responsible for variations in the helper-dependent replication of subviral RNAs, including satellite viruses (Andriessen et al., 1995) and satRNAs (Célix et al., 1999; Hu et al., 1998; Rasochová et al., 1997; Roossinck et al., 1997; Routh et al., 1997; Yamaguchi et al., 2005). In this paper, we have conducted a comparative analysis of spontaneous variants of BBSV and its satRNA with wtBBSV. Variations in the BBSV genome that affect symptom severity and variant satRNA replication have been identified and we have discussed mechanisms involved in maintaining the plant–virus–subvirus interactions.

RESULTS

Sequence divergence among the variants of BBSV and its satRNA

We initially isolated the wtBBSV (GenBank accession no. AF452884) from field-grown sugar beets (Beta vulgaris L.) in the Ningxia province of China (Cao et al., 2002), and later recovered the wt satRNA (NC006460) from sugar beets grown in Xinjiang province (Guo et al., 2005). The wtBBSV (Cao et al., 2006) and satRNAs (Guo et al., 2005) were cloned and the respective clones were designated pUBF52 and pMBS-M4. In 2005, during the course of a serial propagation study of BBSV and its satRNA in Chenopodium, we observed a variant population of BBSV and its satRNAs that elicited mild chlorotic mottling symptoms when maintained in Nicotiana benthamiana at 16–20 °C. We continued to propagate this population in N. benthamiana by about two mechanical inoculations month⁻¹ for the next 2 years. In 2007, the serially propagated variant population began to develop more severe symptoms, consisting of leaf curling in newly emerging leaves and stunting of N. benthamiana plants (Fig. 1a). The increased virulence of the serially propagated virus suggested that, during N. benthamiana propagation, highly virulent spontaneous mutants (S-Muts) had emerged. To analyse the S-Mut population, 77 BBSV and 45 satRNA biologically active cDNA clones were generated by RT-PCR and sequenced. None of these had either the wtBBSV or the wt satRNA sequence (GenBank accession no. NC006460) (Figs S1 and S2, available in JGV Online), but there was 99.2 % identity among the 45 satRNA cDNAs, typified by a representative clone (M satRNA, JN635326).

In marked contrast to the uniformity of the variant satRNA population, two distinct groups of variant BBSV cDNA clones were isolated from the S-Mut population. The first group consisted of 42 minor variant cDNA clones that contained seven or fewer nucleotide substitutions compared with the wt BBSV sequence. However, the second group comprised 35 variant clones that had at least 26 altered nucleotides.

Four types of the second group of BBSV variants, represented by BBSV-m81 (GenBank accession no. JN635329), BBSV-m149 (JN635327), BBSV-m163 (JN635328) and BBSV-m294 (JN635330), were classified based on their divergent sequence patterns (Figs 1b and S1). These variants differed in five major blocks along the genomic (g) RNA compared with the wtBBSV (Fig. 1b; B1–5). The four second-group variants also showed divergence in N. benthamiana pathogenicity and varied in their ability to maintain satRNA variants.

Divergent virulence among BBSV variants and precise localization of a symptom-severity determinant

To determine the biological activity of the 77 BBSV variants recovered after cloning of the serially passed population, viral RNAs synthesized in vitro from all 77 BBSV cDNAs were inoculated to N. benthamiana. Within these variants, all 42 cDNA clones of the first group and nine in the second group, represented by BBSV-m81 and BBSV-m149, induced mild leaf mottle symptoms similar to those initially produced with wtBBSV after the first successful transfer from Chenopodium (Fig. 1a). However, another 26 variants from the second group, typified by BBSV-m163 and BBSV-m294, elicited severe curling symptoms in the newly emerging leaves and plant stunting similar to those of the propagated S-Muts (Fig. 1a). These results suggest that the BBSV variants represent constituents of a quasispecies population that arose during serial propagation as the virus evolved to confer increased population virulence. Because sequence alignments of the second group (Fig. 1b) revealed a clear correlation between the nucleotide substitutions in the 3’ UTR and the severe symptoms caused by BBSV-m294 and BBSV-m163 (Fig. 1a), reverse-genetic analyses were carried out to identify determinants responsible for symptom severity.
Recombinants of the 3′UTR fragments were generated between the wtBBSV and BBSV-m294 clones (Fig. 2a). After inoculation with recombinant viral RNAs transcribed in vitro from the WT-3′UTR<sub>m294</sub> chimera, severe plant stunting and leaf curling symptoms appeared on <i>N. benthamiana</i> at about 12 days p.i. This phenotype is similar to that elicited by BBSV-m294, and contrasts with the mild yellow spots appearing on leaves inoculated with the m294-3′UTR<sub>WT</sub> recombinant and wtBBSV (Fig. 2b). Consistently higher levels of BBSV RNA, CP and P23 replicase subunit were detected in systemically infected leaves infected with WT-3′UTR<sub>m294</sub> and BBSV-m294, compared with those resulting from m294-3′UTR<sub>WT</sub> and wtBBSV infections (Fig. 2c). These results confirm that

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**Fig. 1.** Symptoms and genetic variation of BBSV after passage propagation. (a) Symptoms on <i>N. benthamiana</i>, showing phenotypic variations resulting at 12 days p.i. with the wt BBSV (pUBF52) or variant isolates. Symptoms on plants representing the four BBSV variant types and the purified viral RNAs of S-Muts were photographed; newly emerging leaves are enlarged on the upper left. (b) Classification and illustration of genetic heterogeneity among four BBSV variants falling into the second group of variants. Clones representative of the four types are identified on the far left and the number of individual clones falling into each type are shown in parentheses. The typical phenotypes are characterized as severe (S) or mild (M) symptoms, and + or − indicates the ability of the variants to support wt or M satRNA replication. Boxes on the right side under the BBSV gRNA cartoon highlight five sequence blocks (B1–5) that are characteristic of the four types. Variant nucleotides of the sequenced clones falling into each type are identified by vertical lines.
altered nucleotides nested within the 3′ UTR domain of BBSV-m294 conferred an enhanced virulence phenotype on N. benthamiana.

The 3′ UTR contains four residues that differ between the wtBBSV clone (pUBF52) and the BBSV-m294 variant clone (m294). To identify the precise sequences that contribute to the severe-symptom phenotype, site-directed mutagenesis was carried out between virulent BBSV-m294 and the wtBBSV (Fig. 3a, b). Single- or double-nucleotide substitutions at variant positions 3365, 3441, 3446 and 3477 were constructed and RNA transcripts from the mutated derivatives were inoculated to N. benthamiana. When the variant BBSV-m294 clone was used as a backbone for constructions, and guanine (G) residues were replaced with thymine (T) residues at nucleotide positions 3446 and 3477 to produce the double mutant V-3/4 or the single substitution at residue 3477 (V-4) (Fig. 3a), milder symptoms and lower levels of RNA and CP accumulation could be detected in plants infected with the mutated viral RNAs (Fig. 3c), while the mutants at nt 3365 (V-1), nt 3441 (V-2) or both residues (V-1/2) exhibited the same symptoms and similar levels of RNA and CP accumulations as BBSV-m294. In contrast, severe symptoms were seen and high levels of viral RNAs and CP accumulated in plants inoculated with wtBBSV backbone mutants harbouring a single G residue substitution at W-4 residue 3477, or the W-3/4 double-residue mutants at positions 3446 and 3477 (Fig. 3b, d), but no changes in symptoms or virus accumulation were found in variants with mutations at nt 3365 (W-1), nt 3441 (W-2) or the double sites (W-1/2), which are the same as wtBBSV. These results thus demonstrate that a G residue located at nt 3477 in the 3′ UTR in block 5 is able to confer the severe-symptom phenotype and to elicit increases in virus replication and accumulation in N. benthamiana. Hence, a single nucleotide residue in the 3′ UTR has a critical effect on the BBSV symptom phenotype and levels of replication.

Comparisons of homologous loci in the 3′ UTRs of BBSV, TNV-A and TNV-D

In order to verify and understand further the significance of the BBSV BTE element, sequences of the 17 nt region were aligned using the BBSV-m294 variant, wtBBSV, TNV-AC (GenBank accession no. AY546104.1) and TNV-DH (NC003487.1), as shown in Fig. 4(a). Interestingly, the BTE element of the mild-symptom-eliciting wtBBSV has a U residue at nt 3477, whereas a G residue occupies nt 3477 of the severe BBSV-m294 variant, and G residues are also present in the consensus BTE elements of TNV-A C and TNV-D H, both of which cause severe symptoms on N. benthamiana. Hence, a single nucleotide residue in the 3′ UTR has a critical effect on the BBSV symptom phenotype and levels of replication.
of the TNV strains were replaced by T residues to create constructs TNV-AC-M and TNV-DH-M (Table S1) for in vitro transcription. After mechanical inoculation to N. benthamiana, the plants infected by mutated RNAs from TNV-AC-M and TNV-DH-M developed symptoms milder than those caused by wtTNV isolates (Fig. 4b). Moreover, lower levels of CP accumulated in plants infected with TNV-AC-M and TNV-DH-M (Fig. 4c). This result provides strong evidence supporting the hypothesis that the G residue at nt 3477 of BBSV variant m294 and the G residues at homologous BTE sites of TNV-A and TNV-D have similar roles in N. benthamiana infection processes. Thus, we suggest that this single nucleotide mediates a key replication- and symptom-modulation function in the BTE element that is conserved in members of the genus Necrovirus.

**Mutant satRNA replication is affected by a variant of the BBSV P82 replicase**

As mentioned above, 45 satRNA cDNA clones were obtained from the S-Muts. Unexpectedly, these mutant satRNAs, represented by the M satRNA, could not replicate when co-inoculated with wtBBSV (Figs 1b, 5b; lane 1, M satRNA panel), as opposed to the wt satRNA (Figs 1b, 5c; lane 1, wt satRNA panel). Co-inoculation of N. benthamiana with each of the 77 BBSV variants revealed that M satRNA replication was supported by 33 variants among those classified in the second group of BBSV classes, including the typical m81, m149 and m294 variants, representing three types of BBSV variant, but not the m163 representative (Fig. 1b). Sequence comparisons among the BBSV-m81, BBSV-m149, BBSV-m163 and BBSV-m294 types indicated that mutations in block 2, encompassing the central portion of the P82 replicase gene (Fig. 1b), were probably coupled with the ability to support M satRNA replication.

To confirm the requirement for the block 2 region of P82 in replication of M satRNA, two chimeric recombinants were constructed by reciprocal substitutions of KpnI–XhoI (Kp-Xh) restriction fragments. These fragments encompass the entire 725 nt (nt 1013–1737) region of wtBBSV and BBSV-m294 to produce the WT-m294Kp-Xh and m294-WT<sub>Kp-Xh</sub> clones (Fig. 5a) for inoculation of the local lesion host, C. amaranticolor, with combinations of either the wt
or M satRNAs. Under the inoculation conditions, a similar dense array of small lesions appeared on leaves infected with all of the viral and satRNA mixtures (data not shown). Northern blot hybridizations of RNAs extracted from these leaves verified our previous results by demonstrating that the wtBBSV supported the wt satRNA, but not the M satRNA variant, and that BBSV-m294 could support both satRNAs (Fig. 5b, c; lanes 1 and 2). Moreover, as anticipated, the wtBBSV chimera containing the Kp-Xh fragment from BBSV-m294 (WT-m294 Kp-Xh) could support the replication of both satRNAs, whereas the reciprocal substitution of m294-WTKp-Xh could support only the wt satRNA (Fig. 5b, c; lanes 3 and 4). These results provide additional evidence that variant nucleotides dispersed throughout the 725 nt region of BBSV-m294 are essential for support of the M satRNA. However, it was of considerable interest that the WT-m294 Kp-Xh substitution greatly enhanced the replication of both satRNAs. The enhanced bands were not due to variations in the levels of extracted RNAs, because the rRNA-loading controls were nearly identical; also, the bands were not the results of major differences in the number of lesions on the inoculated leaves.

**The ^{516}R residue of the BBSV-m294 P82 replicase is essential for enhanced replication of M satRNA**

After more refined analyses of sequences within the 725 nt region, we noted that the BBSV-m294 harboured 15 silent nucleotide substitutions, and also had two codon alterations that accounted for two amino acid (R455H and Q516R) differences from wtBBSV (Fig. 5a). To determine more precisely whether the functional requirement for support of both satRNAs is a consequence of sequence or structural changes in the 725 nt region, or is due to the two amino acid alterations within the BBSV-m294 P82 protein, we conducted site-directed mutagenesis for additional genetic mapping (Fig. 5a). Four reciprocal substitutions at nt 1402 and 1585 were implemented to generate the WT-R455H, WT-Q516R-I, m294-H455R and m294-R516Q mutants (Fig. 5a), and the resulting RNA transcripts were used for co-inoculations of *C. amaranticolor* with wt satRNA
or the M satRNA variant. Northern blot analyses revealed that M satRNA accumulated to very high levels in plants co-inoculated with the m294, WT-m294Kp-Xh and WT-Q516R-I combinations (Fig. 5b; lanes 2, 3 and 6) and to a lesser extent in co-inoculations containing the m294-H455R mutant (Fig. 5b; lane 7). However, accumulation declined significantly and hybridizing bands were difficult to discern in co-inoculations containing the WT-R455H and m294-R516Q constructs (Fig. 5b; lanes 5 and 8) and, in the mutant m294-WTKp-Xh mutant co-inoculation, a hybridizing M satRNA band was not evident (Fig. 5b; lane 4). In contrast, wt satRNA replication was supported to at least moderate levels by all BBSV combinations, although the intensity of their bands varied considerably (Fig. 5c). The mutant experiments shown in Fig. 5 with *C. amaranticolor* were repeated by infection of the *N. benthamiana* systemic host and very similar results were obtained (data not shown). These results thus demonstrate that nt 1585 in BBSV-m294 is essential for support of M satRNA replication.

Although the Q516R substitutions facilitating M satRNA replication resulted in an amino acid change, it is possible that sequence differences in the codon per se, and a corresponding alteration in the structure of BBSV RNA, might have affected M satRNA replication. In order to resolve this issue, 12 mutant combinations of codon 516 were introduced into wtBBSV cDNA by inverse PCR to produce amino acids with different chemical properties. Among these, four mutants, WT-Q516R-I, WT-Q516R-II, WT-Q516R-III and WT-Q516R-IV, were designed to substitute R codons that might affect RNA structural interactions (Fig. 6a). Viral RNAs synthesized in vitro from these cDNA constructs were co-inoculated to *C. amaranticolor* with wt satRNA or the M satRNA. Remarkably, both the M satRNAs and the wt satRNAs accumulated to extremely high levels in plants co-infected by the four R residue mutants WT-Q516R-I, WT-Q516R-II, WT-Q516R-III and WT-Q516R-IV (Fig. 6b, c; lanes 2, 4, 5 and 6), and additional data suggest that the M satRNA levels were higher than those of wt satRNAs (not shown). In contrast, except for WT-Q516P and WT-Q516E mutant (Fig. 6a, c; lanes 12 and 13), all of the remaining mutants supported higher levels of wt satRNA than M satRNA (Fig. 6a, c). Moreover, no M satRNA accumulation was evident in co-inoculations with wt BBSV or the WT-Q516Q-I mutant (Fig. 6b; lanes 1 and 3), in which the Q residue was encoded by a degenerate codon (Fig. 6a). These results thus point to a specific role of P82 R516 in mediating replication of M satRNA.

In marked contrast to the $^{516}$R mutants, M satRNA abundance declined dramatically in leaves co-infected with
the WT-Q561H, WT-Q516K, WT-Q516G, WT-Q516A, WT-Q516L, WT-Q516P and WT-Q516E mutants that were designed to elicit other amino acid changes in P82 at position 516 (Fig. 6b; lanes 7–13). The low M satRNA abundance was most pronounced in the WT-Q516H, WT-Q516G and WT-Q516L mutants (Fig. 6b; lanes 7, 9 and 11), in which M satRNA accumulations were much lower than the others. However, low levels of M satRNA were supported by the WT-Q516K, WT-Q516A, WT-Q516P and WT-Q516E mutants (Fig. 6b; lanes 8, 10, 12 and 13).

In summary, these results provide a strong argument that the R residue at position 516 of P82 per se is critical for the gain-of-replicase function enabling high-efficiency M satRNA replication, and suggest that the viral RNA sequence or structural elements within this region have only a minor, if any, role in altering the ability of BBSV to support M satRNA replication.

**DISCUSSION**

Our sequencing results revealed that numerous dispersed nucleotide mutations have been generated throughout the BBSV and satRNA quasispecies genomes during replication and serial propagation in *N. benthamiana* (Figs S1 and S2). Since the RNA-silencing defence system of *N. benthamiana* is compromised at low temperature (Szittya *et al.*, 2003), the BBSV and satRNA variants may have arisen through interactions with temperature-sensitive defence responses. Under our experimental growth conditions, systemic infections could be established successfully in *N. benthamiana* at low temperature and, over time, these resulted in different symptom phenotypes, whereas only inefficient systemic infections and mild symptoms resulted at 24 °C (data not shown). At 18 °C, new leaf curling and plant stunting were elicited by the virulent BBSV variants that evolved during serial transfers (Fig. 1a) and the severe symptoms correlated positively with virus accumulation (Figs 2 and 3), corresponding to previous reports with cucumber mosaic virus (Du *et al.*, 2007), bean pod mottle virus (Gu & Ghabrial, 2005), apple stem grooving virus (Hirata *et al.*, 2003) and *Alternanthera* mosaic virus (Lim *et al.*, 2010). Irrespective of the evolutionary mechanisms, the low-temperature conditions appear to permit co-evolution of spontaneous mutations or viral genome recombination events (Aranda *et al.*, 1997; Sztuba-Solińska *et al.*, 2011) and these conditions have contributed to the genetic heterogeneity and functional diversity among the BBSV and satRNA variants observed in our experiments.

Because nt 3477 in the 3′UTR of the BBSV genome affects symptom severity and viral RNA accumulation in infected
N. benthamiana, we postulated that RNA–RNA interactions in the genome might be involved in these processes. Prediction of the secondary structure of BBSV 3’UTR by using Mfold (Zuker, 2003) revealed a stem–loop structure consisting of the pentanucleotide 340 GUAAA 480. Further analysis of the sequence indicated that the stem–loop structure has a consensus with the BTE 17 nt consensus sequence of BYDV (Wang et al., 2010). Although considerable differences in BTE sequences are evident among members of the genera Luteovirus and Necrovirus, specific BBSV BTE structural elements appear to be required to mediate replication in N. benthamiana and it is of considerable interest that related sequences are involved in the symptom modulation in TNV-D and TNV-A (Shen & Miller, 2004, 2007). Hence, it is possible that the BTE-like structure formed by sequences surrounding nt 3477 facilitates translational enhancements that lead to symptom intensification in N. benthamiana; we are testing this hypothesis in ongoing research.

To determine to what extent amino acid mutations might affect the structure of the P82 replicase protein, we predicted the three-dimensional structure of a portion of the wt BBSV replicase by comparisons with the structure of bovine viral diarrheaa virus replicase (Choi et al., 2004, 2006). Interestingly, residue 516 is located at the bottom of the palm domain and a considerable distance (>20 Å (2 nm)) from the GDD catalytic core, which is 12 aa upstream of the GDD in the primary sequence (Fig. S3), and appears not to interact closely with other putative functional residues. These results support our hypothesis that the Q to R mutation may affect catalytic replicase activity indirectly. Consideration of the 516R residue is predicted to be at the protein surface and has a large size with an abundance of hydrogen bond donor groups, the Q to R mutation may introduce more efficient RNA-binding affinities (Terribilini et al., 2006) on the protein surface and may contribute to affinity between the replicase and M satRNAs. In addition to the variant BBSV functions required for support of the non-coding satRNA variants described above, the genetic differences between wt and M satRNAs have major roles in their ability to be replicated by the BBSV variants. Perusal of the variant sequences reveals that a sequence identity of 91.5% is shared between M and wt satRNAs, but only two nucleotide mutations in unpaired sequences were found in the highly structured region of nt 431–523 (comparable between the satRNAs), supporting the concept that a need to maintain functional structure may limit genetic divergence of viral RNAs (Aranda et al., 1997; Fraile & Garcia-Arenal, 1991). We are currently investigating whether molecular mimicry of helper RNA structural elements (Huang et al., 2010) or structure and sequence integrity between the two populations contributes to their co-evolution (Gellatly et al., 2011).

METHODS

Plant growth and virus inoculation. N. benthamiana plants used for serial passage of BBSV and satRNAs were grown in growth chambers maintained at 18 °C with a 13/11 h light (approx. 75 µE m⁻² s⁻¹)/dark photoperiod and 60% relative humidity, and C. amaranticolor plants used for local lesion infections and wtBBSV maintenance were grown in the chambers under the same conditions except at 24 °C. BBSV RNA and satRNA, transcribed in vitro using a T7 RNA polymerase kit (Promega), were mixed in a ratio of 1:6, and 1–2 µg mixture was diluted with an equal volume of inoculation buffer (50 mM glycine, 30 mM K2HPO4, 1% bentonite, 1% celite, pH 9.2) for use in mechanical inoculation as described previously (Yuan et al., 2006). The viral transcripts were inoculated to C. amaranticolor, the resulting virus and accompanying satRNAs were routinely maintained in Chenopodium by weekly transfers, and each month the transfers were inoculated to other hosts to determine possible changes in virulence.

Viral cDNA cloning and sequencing. BBSV and satRNA were purified from N. benthamiana after serial passages by sucrose density-gradient centrifugation (Bo et al., 1996) and extracted as described previously (Guo et al., 2005). A mixture of BBSV RNAs and satRNAs (approx. 2 µg) were used for cDNA synthesis by RT-PCR with primer pairs that flanked the both ends of the BBSV or satellite RNA genomes, respectively (Table S1). For accurate amplification of long products, high-fidelity PCRs were carried out by use of PrimeSTAR HS DNA polymerase (TaKaRa). The products were ligated into the pMD20-T plasmid (TaKaRa) and propagated in Escherichia coli DH5α (TaKaRa). Nucleotide variations in the cDNA clones were identified by sequencing.

To create BBSV mutant recombinants, the SceI restriction site located at nt 3314 in the BBSV genome (GenBank accession no. AF452884) and the XbaI site in the vector sequences were used to exchange the 3’-proximal ends of the viral variants as shown in Fig. 2(a). In addition, the KpnI and XhoI restriction sites were used to exchange a 725 nt fragment (nt 1013–1737) containing a portion of the P82 replicase (Fig. 5a). Site-directed mutagenesis of the BBSV genome was carried out by inverse PCR amplifications (Ochman et al., 1988) using primer pairs corresponding to specific sites on the BBSV genome (Table S1). After reaction, the mixtures were digested with DpnI to remove the template DNAs (Geier & Modrich, 1979) and the PCR products, high-fidelity PCRs were carried out by use of PrimeSTAR HS DNA polymerase (TaKaRa). Nucleotide variations in the cDNA clones were identified by sequencing.

Analysis of viral RNAs and proteins. After inoculation, total RNAs were extracted from systemically infected leaves of N. benthamiana or inoculated leaves of C. amaranticolor by precipitation with 4 M LiCl (Barlow et al., 1963). After separation in 1% agarose gels, the RNAs were transferred to nylon membranes (Amersham Hybond-N+) for Northern blot analysis with 32P-labelled probes produced with the Prime-a-Genie Labelling system (Promega). Replication of BBSV RNAs was assessed with a cDNA probe complementary to the 3’-proximal 300 nt of the BBSV genome (nt 3345–3644) and satellite RNAs were probed with the full-length 615 nt cDNA of the wt satRNA (Guo et al., 2005). The relative amounts of helper virus RNA, satRNA, or rRNA accumulation were estimated by Quantity One software (Bio-Rad).

Western blots were performed with polyclonal rabbit antibodies raised against BBSV CP (Cao et al., 2006) or the P23 replicase subunit (unpublished), and TNV-A 20 and TNV-D 23 CP (Xi et al., 2007). Leaves recovered from multiple inoculations made at the same time were pooled and homogenized in liquid nitrogen for protein extractions with equal volumes of gel loading buffer (100 mM Tris base, pH 6.8; 20% glycerol; 4% SDS; 200 mM β-mercaptoethanol; 0.2% bromophenol blue). Proteins remaining in the supernatant after boiling and centrifugation at 12 000 g for 5 min were resolved by SDS-PAGE [12.5% (w/v) acrylamide] either by staining with Coomassie brilliant
blue or by transferring to nitrocellulose filters for Western blot analysis (Zhang et al., 2010).

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