Replication promiscuity of DNA-β satellites associated with monopartite begomoviruses; deletion mutagenesis of the Ageratum yellow vein virus DNA-β satellite localizes sequences involved in replication

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Pseudorecombination studies in Nicotiana benthamiana demonstrate that Ageratum yellow vein virus (AYVV) and Eupatorium yellow vein virus (EpYVV) can functionally interact with DNA-β satellites associated with AYVV, EpYVV, cotton leaf curl Multan virus (CLCuMV) and honeysuckle yellow vein virus (HYVV). In contrast, CLCuMV shows some specificity in its ability to interact with distinct satellites and HYVV is able to interact only with its own satellite. Using an N. benthamiana leaf disk assay, we have demonstrated that HYVV is unable to trans-replicate other satellites. To investigate the basis of trans-replication compatibility, deletion mutagenesis of AYVV DNA-β has been used to localize the origin of replication to approximately 360 nt, encompassing the ubiquitous nonanucleotide/stem-loop structure, satellite conserved region (SCR) and part of the intergenic region immediately upstream of the SCR. Additional deletions within this intergenic region have identified a region that is essential for replication. The capacity for DNA-β satellites to functionally interact with distinct geminivirus species and its implications for disease diversification are discussed.

INTRODUCTION

Until recently, the majority of the members of the genus Begomovirus (family Geminiviridae) that have been characterized were reported to have bipartite genomes, designated DNA-A and DNA-B, both of which are essential for a productive infection. However, since the characterization of tomato yellow leaf curl virus and tomato yellow leaf curl Sardinia virus (Kheyr-Pour et al., 1991; Navot et al., 1991), a large number of begomoviruses have been isolated for which only a single genomic component, equivalent to DNA-A, has been identified (Stanley et al., 2005). Many of these apparently monopartite begomoviruses are associated with a small satellite, designated DNA-β, which is essential for maintenance of the disease. Since the description of a DNA satellite, referred to as satDNA, associated with the monopartite begomovirus tomato leaf curl virus (ToLCV) (Dry et al., 1997) and the isolation of the first DNA-β satellite from Ageratum conyzoides plants infected with Ageratum yellow vein virus (AYVV) (Saunders et al., 2000), more than 200 satellite sequences have been established from a range of weeds, vegetable and fibre crops in Africa and Asia, reflecting their diversity and impact on agriculture throughout the Old World (Briddon & Stanley, 2006).

Replication of DNA-A and DNA-B components relies on an interaction between the DNA-A-encoded replication-associated protein (Rep) and reiterated motifs (termed iterons) located within the origin of replication (Argüello-Astorga et al., 1994; Fontes et al., 1992, 1994; Laufs et al., 1995; Gladfelter et al., 1997), which usually limits Rep trans-replication to its own DNA-A and DNA-B components. In contrast, the ability of intact and defective DNA-β satellites to be trans-replicated by distinct begomoviruses (Dry et al., 1997; Saunders et al., 2002; Mansoor et al., 2003) indicates that productive origin recognition is more relaxed for the satellite. Here, we have used infectious clones of AYVV, cotton leaf curl Multan virus (CLCuMV), Eupatorium yellow vein virus (EpYVV) and honeysuckle yellow vein virus (HYVV), together with their associated DNA-β satellites, to examine replication compatibility between monopartite begomoviruses and satellites, and deletion mutagenesis has localized satellite sequences that are required for replication.
METHODS

Construction of infectious clones of begomoviruses and satellites. The construction of infectious clones of AYVV and its associated satellite from A. conyzoides (pHNBin419 and pBin-AVVβl) has been described by Tan et al. (1995) and Saunders et al. (2000). The BamHI insert of EpVV clone pGEM-EYVVAM1 isolated from Eupatorium makinana (Saunders et al., 2003) was cloned into BamHI/BglII-digested pIC19H (Marsh et al., 1984), and the insert was subsequently cloned into this BamHI-digested intermediate to produce pIC-EYVV2AM. The tandem repeat of the EpVV genomic DNA was excised with HindIII and cloned into pBinPlus (van Engelen et al., 1995) to produce pBin-EYVV. The BamHI/Sall fragment of the EpVV satellite clone pGEM-EYVVJM10 (Saunders et al., 2003) was inserted into pIC19H and the full-length Ncol insert was subsequently cloned into this intermediate to produce pIC-EYVV1.9JM. The partial repeat of the satellite DNA was excised with HindIII and cloned into pBinPlus to produce pBin-EYVVβl. The construction of an infectious clone of CLCuMV from cotton (Gossypium hirsutum) has been described by Mansoor et al. (2003). The CLCuMV satellite (Briddon et al., 2001) was cloned as a SalI fragment into SalI/XhoI-digested pIC19H, and the fragment was subsequently cloned into this SalI-digested intermediate to produce pIC-CLCuMVβl. The insert was excised with HindIII and cloned into pBinPlus to produce pBin-CLCuMVβl.

To construct infectious begomovirus and satellite clones from honeysuckle, viral supercoiled DNA (scDNA) was isolated from an ornamental plant exhibiting a yellow vein phenotype (Lonicerajaponica var. aureoreticulata) growing in a garden in Deepham, UK, essentially as described by Stanley et al. (1997). A full-length fragment of the HYVV genomic component was PCR-amplified from the scDNA using overlapping primers V4594 and V4595 containing an HI-digested intermediate to produce pBS-AYVV. The pBS-AVVβl insert was excised with HI and cloned into pBluescript to produce pBS-AVVβl. The construction of an infectious clone of HYVV from honeysuckle, viral supercoiled DNA (scDNA) was isolated from an ornamental plant exhibiting a downward leaf curl phenotype, as described by Saunders et al. (2004). The sequences that had been deleted from these mutants were PCR-amplified as BglII fragments from wild-type DNA-βl using primer combinations V4713/V4723, V4724/V4732, V4727/V4737 and V4725/V4738 (Supplementary Table S1), and reinserted in the sense and antisense orientations. The resulting Spel clones were subcloned as tandem repeat sequences as described above for pBin-AYVVβl to produce pBin-AYVVβl(±) (Fig. 3a) and pBin-AYVVβlI1268 containing a newly introduced BglII I1268 site.

The ΔA, Δ7 and Δ9 mutant inserts were excised as Spel fragments and cloned into pBluescript to produce pBS-ΔA, pBS-Δ7 and pBS-Δ9, respectively. The sequences that had been deleted from these mutants were PCR-amplified as BglII fragments from wild-type DNA-βl using primer combinations V4713/V4723, V4724/V4732, V4727/V4737 and V4725/V4738 (Supplementary Table S1), and reinserted in the sense and antisense orientations. The resulting Spel clones were subcloned as tandem repeat sequences as described above for pBin-AYVVβl to produce pBin-AYVVβl(±) (Fig. 3a) and pBin-AYVVβlI1268 containing a newly introduced BglII I1268 site.

Using the scDNA isolated from honeysuckle, PCR-amplification with primers V4581 and V4582 (Supplementary Table S1), corresponding to nt 27–47 (virion-sense) and 2666–2693 (complementary-sense) of the established HYVV sequence, produced fragments from the expected region of the viral DNA as well as from DNA-βl recombinants containing the HYVV intergenic region, similar to those associated with AYVV infection (Stanley et al., 1997). The recombinant fragments were cloned into pGEM-T Easy and sequenced. Overlapping primers V4584 and V4585 (Supplementary Table S1), encompassing a unique Nhel site, were designed on the basis of the recombinant sequence and used to PCR-amplify a full-length fragment of DNA-βl. The fragment was cloned into pGEM-T Easy to produce pGEM-HYVVβl and the nucleotide sequence was subsequently deposited in GenBank (accession no. AJ543429). The Nco–BamHI fragment (restriction sites at nt 1997 and 164, respectively) of pGEM-HYVV was cloned into pCR18 (constructed by Dr Li Liu, John Innes Centre) to produce pIC-EYVV2AM. The tandem repeat of the EpVV genomic DNA was excised with HindIII and cloned into pBluescript to produce pBS-AVVβl. The pBS-ΔA and pBS-Δ7 inserts were excised with Spel and cloned into SpeI/BglII-digested pBS-AVVβlmonomer and pBS-Δ7monomer, respectively. The appropriate Spel inserts were then cloned into these Spel-digested intermediates to produce pBS-ΔA and pBS-Δ7dimer. Using pBS-AVVβlβl (Saunders et al., 2000) as a template, primers V4713 and V4723 (Supplementary Table S1) were used to mutate A1146–G, A1147–G, C1148–A, G1147–C, G1148–A, C1147–G, C1146–C and G1145–C to introduce BglII sites at positions 801 and 1047. The BglII/1146–1047–BglII fragment was cloned into pGEM-T Easy to produce pGEM-βlA3. The pGEM-βlA3 insert was excised with BglII and cloned into BglII/BamHI-digested pIC19H to produce pIC-βlA3monomer. The BglII insert was then cloned into the BglII-digested intermediate to produce pIC-βlA3dimer. The pBS-ΔA and pBS-Δ7 inserts were excised with Sall/Sall and the pIC-βlA3dimer was excised with HindIII. Inserts were cloned into appropriately digested pBinPlus to produce pBin-ΔA, pBin-Δ7 and pBin-Δ9.

RESULTS AND DISCUSSION

Co-agroinoculation experiments in N. benthamiana showed that AYVV could support the replication and systemic spread of satellites associated with CLCuMV, EpVV and HYVV (Fig. 1a–d). The upward leaf curl symptom associated with AYVV (Tan et al., 1995) was altered to a downward leaf curl phenotype in the presence of these satellites, indicative of a functional interaction between AYVV and DNA-βl (Saunders et al., 2000). Similarly, EpVV was compatible with AYVV, CLCuMV and HYVV satellites. However, CLCuMV could functionally interact only with AYVV DNA-βl and HYVV compatibility was confined to its own DNA-βl, suggesting that at least some specificity exists between the begomovirus and its satellite during trans-replication. Because begomo-
Viruses can systemically infect *N. benthamiana* in the absence of DNA-β (Fig. 1a–d) and non-functional satellites can be maintained in plants (Cui et al., 2004; Saunders et al., 2004; Saeed et al., 2005), the inability to detect a satellite in systemically infected tissues suggests trans-replication incompatibility rather than a defect in satellite systemic movement mediated by the helper virus. With the exception of CLCuMV, which could maintain a low level of EpYVV DNA-β replication that was not detected in systemically infected tissues (Fig. 1c), begomovirus trans-replication of the satellites in *N. benthamiana* leaf disks largely reflected the systemic infectivity data in plants (Fig. 1a–c). The leaf disk assay confirmed that HYVV was unable to trans-replicate satellites associated with AYVV, CLCuMV and EpYVV. *L. japonica* var. aureoreticulata is an ornamental plant that has been propagated vegetatively for many years. Such a prolonged association with a single host might account for the specificity of HYVV for its DNA-β, although it is noticeable that the satellite has retained the ability to be trans-replicated by AYVV (poorly) and EpYVV.

It is known that recombination occurs frequently between begomoviruses and their satellites (Stanley et al., 1997; Briddon et al., 2001; Saunders et al., 2001; Tao & Zhou, 2008). To ensure that the begomovirus origin of replication had not been transferred to DNA-β in these experiments, thereby enabling trans-replication of the satellite, DNA was isolated from an *N. benthamiana* plant systemically infected with AYVV and CLCuMV DNA-β, and full-length copies of the progeny satellite were PCR-amplified and cloned. Sequence analysis of seven independent clones indicated that recombination had not occurred, confirming that AYVV was able to maintain intact CLCuMV DNA-β.

**Fig. 1.** Replication compatibility of begomovirus components and their satellites. Combinations of AYVV, CLCuMV, EpYVV and HYVV were co-inoculated with DNA-β satellites associated with AYVV (a), CLCuMV (b), EpYVV (c) and HYVV (d) into either *N. benthamiana* plants (top two panels, in each case showing four replicates of viral and satellite DNA extracted from systemically infected tissues) or *N. benthamiana* leaf disks (bottom panel in each case). Days p.i., days post-inoculation; input, residual inoculum DNA. Blots were hybridized to probes for AYVV, CLCuMV, EpYVV, HYVV and their respective satellites as indicated.
As a first step towards understanding the determinants of satellite specificity, deletion mutagenesis was used to identify regions of AYVV DNA-β that participate in replication. A comparison of the sequences of satellites associated with AYVV, CLCuMV, EpYVV and HYVV identified regions of highly conserved sequences within a 200 nt fragment that includes the satellite conserved region (SCR), located immediately upstream of the ubiquitous nonanucleotide motif (Briddon & Stanley, 2006). As such conserved sequences could reflect their participation in replication, the first DNA-β mutants that were constructed (Δ1, Δ2 and Δ3; Fig. 2a) contained large deletions outside of this region. All N. benthamiana plants co-agroinoculated with AYVV, and mutants Δ1 and Δ2 (five plants in each case) produced upward leaf roll symptoms indistinguishable from those associated with AYVV alone, consistent with disruption of the βC1 gene (Saunders et al., 2004). Mutant Δ3 retained an intact βC1 gene and produced symptoms identical to those produced by wild-type DNA-β in all five co-agroinoculated plants. Despite the different phenotypes, Southern blot analysis indicated that all DNA-β mutants accumulated in systemically infected tissues and retained their deletions (Fig. 2b). The results indicated that some variation in the size of the satellite is tolerated, at least in N. benthamiana, and that sequences between the introduced SpeI114 and BglII1047 sites play no essential role in DNA-β replication.

To further define sequences that contribute to replication, BglII sites were introduced within the BglII1047–SpeI114 fragment at positions 1146, 1229 and 1268 to facilitate the progressive removal of blocks of highly conserved DNA-β sequences from the 5′ end of this region in mutants Δ4, Δ5 and Δ6 (Fig. 3a). All N. benthamiana plants co-agroinoculated with AYVV, and these mutants (five plants in each case) produced upward leaf roll symptoms indistinguishable from those associated with AYVV alone. However, in contrast with the results obtained for mutants Δ1 and Δ2, mutants Δ4, Δ5 and Δ6 could not be detected in systemically infected tissues by Southern blot analysis (data not shown). Furthermore, AYVV was unable to trans-replicate each of these mutants in an N. benthamiana leaf disk assay (Fig. 3b), indicating that sequences important for replication are located within the deleted region common to all three mutants, namely between the introduced BglII1047 and BglII1146 sites (mutant Δ4).

To demonstrate that no other part of DNA-β had been disrupted during mutant construction, the deleted BglII1047–BglII1146 fragment was reintroduced into mutant Δ4 in either the sense or antisense orientation (mutants Δ4+ and Δ4−, respectively). Co-agroinoculation of four N. benthamiana plants with AYVV and mutant Δ4+ produced a downward leaf curl phenotype indicative of functional DNA-β in each case, and Southern blot analysis confirmed that restoration of the deleted sequences conferred on mutant Δ4+ the ability to be trans-replicated and maintained in systemically infected tissues to wild-type levels (Fig. 3c). Consistent with this, all four A. conyzoides plants co-agroinoculated with AYVV and mutant Δ4+ produced typical yellow vein symptoms associated with wild-type levels of both AYVV and DNA-β (Fig. 3d). Interestingly, reintroduction of the deleted sequences in the opposite orientation also permitted a low level of mutant Δ4− accumulation in two of four co-agroinoculated N. benthamiana plants (Fig. 3c) associated with a mild downward leaf curl phenotype. Co-agroinoculation of four A. conyzoides plants with AYVV and mutant Δ4− also produced a mild yellow vein phenotype in one plant associated with a wild-type level of AYVV DNA accumulation but only a low level of DNA-β (Fig. 3d).

To investigate a possible contribution of the additional downstream sequences that had been deleted in mutants Δ5 and Δ6, deletion mutants Δ7, Δ8 and Δ9 were constructed using the introduced BglII sites (Fig. 3a). All N. benthamiana plants co-agroinoculated with AYVV and either mutant Δ7 or Δ8 (three plants each) produced upward leaf roll symptoms, and Southern blot analysis failed to detect the DNA-β mutants in systemically infected tissues (Fig. 3c), suggesting that at least the region between

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**Fig. 2.** Deletion mutagenesis of AYVV DNA-β. (a) Deletions in mutants Δ1, Δ2 and Δ3 are shown relative to βC1, the A-rich region and SCR of AYVV DNA-β. The positions of the naturally occurring SpeI129 site and introduced restriction sites are indicated. (b) Southern blot analysis of viral and satellite DNA extracted from tissues agroinoculated with either AYVV alone (lane 1) or in the presence of wild-type DNA-β (lane 2) and mutants Δ1 (lanes 3–5), Δ2 (lanes 6–8) and Δ3 (lanes 9–11) (three replicates for each mutant).

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**Fig. 3.** (a) Deletion mutants of AYVV DNA-β obtained by progressive restriction enzyme digestion of AYVV DNA. Mutants Δ1, Δ2 and Δ3 were constructed (Δ1, Δ2 and Δ3; Fig. 2a) containing large deletions outside of this region. All N. benthamiana plants co-agroinoculated with AYVV, and mutants Δ1 and Δ2 (five plants in each case) produced upward leaf roll symptoms indistinguishable from those associated with AYVV alone, consistent with disruption of the βC1 gene (Saunders et al., 2004). Mutant Δ3 retained an intact βC1 gene and produced symptoms identical to those produced by wild-type DNA-β in all five co-agroinoculated plants. Despite the different phenotypes, Southern blot analysis indicated that all DNA-β mutants accumulated in systemically infected tissues and retained their deletions (Fig. 2b). The results indicated that some variation in the size of the satellite is tolerated, at least in N. benthamiana, and that sequences between the introduced SpeI114 and BglII1047 sites play no essential role in DNA-β replication.

To further define sequences that contribute to replication, BglII sites were introduced within the BglII1047–SpeI114 fragment at positions 1146, 1229 and 1268 to facilitate the progressive removal of blocks of highly conserved DNA-β sequences from the 5′ end of this region in mutants Δ4, Δ5 and Δ6 (Fig. 3a). All N. benthamiana plants co-agroinoculated with AYVV, and these mutants (five plants in each case) produced upward leaf roll symptoms indistinguishable from those associated with AYVV alone. However, in contrast with the results obtained for mutants Δ1 and Δ2, mutants Δ4, Δ5 and Δ6 could not be detected in systemically infected tissues by Southern blot analysis (data not shown). Furthermore, AYVV was unable to trans-replicate each of these mutants in an N. benthamiana leaf disk assay (Fig. 3b), indicating that sequences important for replication are located within the deleted region common to all three mutants, namely between the introduced BglII1047 and BglII1146 sites (mutant Δ4).

To demonstrate that no other part of DNA-β had been disrupted during mutant construction, the deleted BglII1047–BglII1146 fragment was reintroduced into mutant Δ4 in either the sense or antisense orientation (mutants Δ4+ and Δ4−, respectively). Co-agroinoculation of four N. benthamiana plants with AYVV and mutant Δ4+ produced a downward leaf curl phenotype indicative of functional DNA-β in each case, and Southern blot analysis confirmed that restoration of the deleted sequences conferred on mutant Δ4+ the ability to be trans-replicated and maintained in systemically infected tissues to wild-type levels (Fig. 3c). Consistent with this, all four A. conyzoides plants co-agroinoculated with AYVV and mutant Δ4+ produced typical yellow vein symptoms associated with wild-type levels of both AYVV and DNA-β (Fig. 3d). Interestingly, reintroduction of the deleted sequences in the opposite orientation also permitted a low level of mutant Δ4− accumulation in two of four co-agroinoculated N. benthamiana plants (Fig. 3c) associated with a mild downward leaf curl phenotype. Co-agroinoculation of four A. conyzoides plants with AYVV and mutant Δ4− also produced a mild yellow vein phenotype in one plant associated with a wild-type level of AYVV DNA accumulation but only a low level of DNA-β (Fig. 3d).

To investigate a possible contribution of the additional downstream sequences that had been deleted in mutants Δ5 and Δ6, deletion mutants Δ7, Δ8 and Δ9 were constructed using the introduced BglII sites (Fig. 3a). All N. benthamiana plants co-agroinoculated with AYVV and either mutant Δ7 or Δ8 (three plants each) produced upward leaf roll symptoms, and Southern blot analysis failed to detect the DNA-β mutants in systemically infected tissues (Fig. 3c), suggesting that at least the region between...
Fig. 3. Deletion mutagenesis of AYVV DNA-β. (a) The introduced BglI sites that were used to produce deletions in mutants Δ4–Δ9. The nucleotide sequence of the deleted region in mutant Δ4 (top) has been aligned with ToLCV sat-DNA sequences (bottom) that are implicated in replication (Dry et al., 1997). Sequences with the potential to form a stem–loop structure are indicated by arrows above the sequence. AYVV DNA-β repeat and inverted repeat sequences and the sat-DNA sequence that is identical to the ToLCV iteron are shown in bold font. (b) Southern blot analysis of viral and satellite DNA extracted from N. benthamiana leaf disks inoculated with AYVV together with either wild-type DNA-β (wt) or deletion mutants Δ4–Δ6. (c) Southern blot analysis of viral and satellite DNA extracted from N. benthamiana tissues agroinoculated with either AYVV alone (−) or in the presence of wild-type DNA-β (wt) and the indicated mutants (three replicates for each). (d) Southern blot analysis of viral and satellite DNA extracted from N. benthamiana (left and centre panels) and A. conyzoides (right panel) agroinoculated with either AYVV alone (−) or in the presence of wild-type DNA-β (wt) and the indicated mutants.
the BglI\textsuperscript{1146} and BglI\textsuperscript{1229} sites (mutant A\textgreek{7}) contains additional sequences required for replication. To ensure the integrity of mutant A\textgreek{7}, the deleted region was reintroduced in the sense orientation in mutant A\textgreek{7}+. All five \textit{N. benthamiana} plants co-agroinoculated with AYVV and mutant A\textgreek{7}+ produced a typical downward leaf curl phenotype; the mutant accumulated at high levels in systemically infected tissues (Fig. 3d) and induced typical yellow vein symptoms in \textit{A. conyzoides} (data not shown), confirming that sequences within this region are required for replication.

All three \textit{N. benthamiana} plants co-agroinoculated with AYVV and mutant A\textgreek{9} produced an upward leaf roll phenotype and the DNA-\textgreek{b} mutant was not detected in systemically infected tissues (Fig. 3d). However, replacement of the deleted sequences in either the sense or antisense orientation (mutants A\textgreek{9}+ and A\textgreek{9}−, respectively) failed to restore the wild-type DNA-\textgreek{b} phenotype. Sequence analysis confirmed that the only changes in mutant A\textgreek{9}+ were at the BglI\textsuperscript{1229} and BglI\textsuperscript{1268} sites. As the BglI\textsuperscript{1229} mutation was tolerated in mutant A\textgreek{7}+, this implied that the BglI\textsuperscript{1268} mutation was responsible for the inability of mutant A\textgreek{9}+ to be trans-replicated by AYVV. To investigate this, two \textit{N. benthamiana} plants were co-agroinoculated with AYVV and a mutant containing just the introduced BglI\textsuperscript{1268} site. Both plants produced an upward leaf roll phenotype and the DNA-\textgreek{b} mutant was again undetectable in systemically infected tissues (data not shown), consistent with alteration of nucleotides 1269–1271 from CCC (wild-type) to GAT (introduced BglI\textsuperscript{1268} site) preventing trans-replication of the satellite.

We have demonstrated that the region between the introduced SpeI\textsuperscript{114} and BglI\textsuperscript{1047} sites is not required for DNA-\textgreek{b} replication. This region includes the \textgreek{b}1 open reading frame (ORF), which encodes a gene essential for pathogenicity (Saunders et al., 2004), and an A-rich region that may serve to maintain the size integrity of the satellite (Saunders et al., 2000). We have previously shown that an intact \textgreek{b}1 ORF is not required for systemic infection of \textit{N. benthamiana} (Saunders et al., 2004) and our current finding that the entire ORF is dispensable (mutants A1 and A2) is consistent with the findings of Qian & Zhou (2005) for the satellite associated with tomato yellow leaf curl China virus (TYLCCNV). In addition, removal of the A-rich region from TYLCCNV DNA-\textgreek{b} was tolerated, although the deletion mutant was associated with milder symptoms than those produced by the wild-type satellite (Tao et al., 2004). In contrast, deletion of this region in AYVV DNA-\textgreek{b} (mutant A\textgreek{3}) did not affect the phenotype, at least in \textit{N. benthamiana}, although the phenotype of this deletion mutant remains to be investigated in the natural host of the disease complex, \textit{A. conyzoides}. Large deletions within non-essential regions of the begomivirus genome are not tolerated and the deletion mutants revert to wild-type size by both intra- and intermolecular recombination during systemic movement (Etessami et al., 1989; Gilbertson et al., 2003). In contrast, removal of 361 nt of DNA-\textgreek{b} in mutant A2, representing 27\% of the satellite, was tolerated, although it remains to be seen if multiple passages of the mutant progeny result in reversion to wild-type size. This might be expected as the majority of naturally occurring DNA-\textgreek{b} satellites, nanovirus-like DNA-1 satellites (Briddon et al., 2004) and various recombinants between these satellites and their associated begomoviruses (Stanley et al., 1997; Briddon et al., 2001) tend to fall within an extremely narrow size range. Indeed, using deletion mutants of tobacco curly shoot virus DNA-\textgreek{b}, Qian et al. (2008) have recently shown that selection for wild-type size can occur in some instances.

Our findings indicate that the region between the introduced BglI\textsuperscript{1047} and BglI\textsuperscript{1146} sites is important for DNA-\textgreek{b} replication. Sequence comparison shows that this region aligns with that part of the ToLCV sat-DNA (Dry et al., 1997) containing an inverted repeat flanking a sequence that is identical to the ToLCV iteron. Mutagenesis and protein binding assays have demonstrated that this motif in both ToLCV and sat-DNA represents a high affinity Rep binding site, although it is not required for replication of either the begomovirus or its satellite (Lin et al., 2003). Interestingly, the AYVV DNA-\textgreek{b} sequence in this region also contains an inverted repeat and multiple copies of the sequence GTCTCC and its complement GGAGAC (Fig. 3a). While this differs from the predicted AYVV iteron GGTACTCA (Saunders et al., 2000), it suggests that these elements may play a role in replication. Consistent with this idea, a DNA-\textgreek{b} derivative containing an inverted BglI\textsuperscript{1047}–BglI\textsuperscript{1146} fragment (mutant A\textgreek{4}−) was replication-competent in both \textit{N. benthamiana} and \textit{A. conyzoides}, albeit less efficiently than the wild-type satellite, suggesting that a specific structure and/or inverted repeat sequence is recognized during replication. Since the initiation of this work, many more DNA-\textgreek{b} sequences have become available. Comparison of these sequences shows that many, but not all, have inverted repeat sequences in this region.

We have additionally shown that the region between the introduced BglI\textsuperscript{1146}–BglI\textsuperscript{1229} sites and sequences across the introduced BglI\textsuperscript{1268} site of AYVV DNA-\textgreek{b} are also required for replication. Although the contribution of the SCR to replication has not been investigated here, its highly conserved nature between distinct satellites [typically above 65\% sequence identity with blocks of absolutely conserved sequence (Briddon et al., 2003)] strongly suggests that it also plays an important role in the virus replication cycle. In addition, the adjacent stem–loop and conserved nonanucleotide sequence would be expected to participate in replication. Li et al. (2007) recently demonstrated that approximately the 386 nt upstream of the stem–loop structure in ToLCV sat-DNA, as well as the stem–loop structure itself, are essential for replication. This includes the predicted stem–loop structure encompassing the ToLCV iteron described above (Dry et al., 1997; Lin et al., 2003). Hence, in many respects, the DNA-\textgreek{b} replication origin resembles the multiple domains of bipartite begomoviruses (Argüello-Astorga et al., 1994; Argüello-Astorga & Ruiz-Medrano, 2001), although the specificity of Rep recognition seems more relaxed. Whether this relaxed
specifity is a feature only of begomoviruses that associate with DNA-β or is common to all monopartite begomoviruses remains to be determined. Such promiscuity will undoubtedly facilitate diversification and dissemination of satellites. For example, a single DNA-β is known to be associated with several distinct cotton begomoviruses (Mansoor et al., 2003) and a resistance breaking strain of the disease emerging in Pakistan is associated with a re-combinant DNA-β (Amin et al., 2006). Also, Sri Lankan cassava mosaic virus, a bipartite begomovirus, has been shown to function as a monopartite begomovirus and alter its host range in the presence of AYVV DNA-β (Saunders et al., 2002). However, the monopartite beet curly top virus (genus Curtovirus) is unable to support the replication of AYVV DNA-β in N. benthamiana (unpublished data), suggesting that satellite compatibility may be confined to begomoviruses. Currently, there is no clear indication why some DNA-β satellites are recognized by a particular begomovirus Rep while others are not. This may be addressed by constructing chimeras using infectious cloned components such as those described here.

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