Adaptation from whitefly to leafhopper transmission of an autonomously replicating nanovirus-like DNA component associated with ageratum yellow vein disease

Keith Saunders, Ian D. Bedford and John Stanley

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

Ageratum yellow vein disease is caused by the whitefly-transmitted monopartite begomovirus *Ageratum yellow vein virus* and a DNA β satellite component. Naturally occurring symptomatic plants also contain an autonomously replicating nanovirus-like DNA 1 component that relies on the begomovirus and DNA β for systemic spread and whitefly transmission but is not required for maintenance of the disease. Here, we show that systemic movement of DNA 1 occurs in *Nicotiana benthamiana* when co-inoculated with the bipartite begomovirus *Tomato golden mosaic virus* and the curtovirus *Beet curly top virus* (BCTV), but not with the mastrevirus *Bean yellow dwarf virus*. BCTV also mediates the systemic movement of DNA 1 in sugar beet, and the nanovirus-like component is transmitted between plants by the BCTV leafhopper vector *Circulifer tenellus*. We also describe a second nanovirus-like component, referred to as DNA 2, that has only 47% nucleotide sequence identity with DNA 1. The diversity and adaptation of nanovirus components are discussed.

There are two groups of plant viruses with single-stranded DNA components. DNA A encodes the coat protein and replicates autonomously while DNA B is required for systemic infection and disease production. Some begomoviruses, for example *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (ToLCV), lack a DNA B component yet produce a systemic symptomatic infection in plants, and the disease is transmissible between plants by the whitefly vector (Kheyr-Pour et al., 1991; Navot et al., 1991; Dry et al., 1993). In contrast, the monopartite begomoviruses *Ageratum yellow vein virus* (AYVV; Fig. 1) (Tan et al., 1995) and *Cotton leaf curl virus* (CLCuV) (Briddon et al., 2000) are able to systemically infect *Ageratum conyzoides* (ageratum) and cotton, respectively, although they are unable to induce symptoms typical of ageratum yellow vein disease (AYVD) and cotton leaf curl disease (CLCuD) in these hosts. Recently, a variety of small (~1300 nucleotide) recombinant DNAs (recDNAs) and satellite DNAs were isolated from ageratum exhibiting AYVD (Stanley et al., 1997; Saunders & Stanley, 1999; Saunders et al., 2001). One such satellite, referred to as DNA β, is an essential component of the AYVD complex (Saunders et al., 2000), and a similar satellite has since been isolated from CLCuD-affected cotton (Briddon et al., 2001).

The majority of nanoviruses, including *Faba bean necrotic yellows virus* (FBNYV), *Subterranean clover stunt virus* (SCSV), *Milk vetch dwarf virus* (MVDV) and *Banana bunchy top virus* (BBTV), are transmitted by aphids. The exact number of components associated with these viruses remains to be established, but between six and eleven components have been isolated, each approximately 1000–1100 nucleotides in length (Boevink et al., 1995; Katul et al., 1998; Sano et al., 1998; Horser et al., 2001). The exception is the planthopper-transmitted *Coconut foliar decay virus* (CFDV), for which only a single component has been isolated that, at 1291 nucleotides, is significantly larger than its counterparts in other nanoviruses (Rohde et al., 1990). Diseased plants contain a number of related components that encode replication-associated proteins (Reps), only one of which (the master Rep component) is responsible for trans-replication of the components that encode non-Rep proteins (Timchenko et al., 1999, 2000; Horser et al., 2001). The other Rep-encoding components are presumably not essential for the disease but depend on the *bona fide*
nanovirus components for their systemic spread and insect transmission. Whether these components have a direct role in the aetiology of the disease or simply contribute to the diversity and evolution of nanoviruses remains to be determined.

In addition to a begomovirus and DNA $\beta$, both AYVD and CLCuD are associated with autonomously replicating DNAs, referred to as DNA 1 (Fig. 1) (Mansoor et al., 1999; Saunders & Stanley, 1999). These DNAs are related to nanovirus components that encode Rep proteins, and have probably evolved by adaptation to whitfly transmission during co-infection of plants with a begomovirus and a nanovirus. They play no direct role in the disease aetiology, but may help to modulate the accumulation of the essential begomovirus and DNA $\beta$ components (Saunders et al., 2000). It has been suggested that the single CFDV Rep-encoding component may simply be a nanovirus-like DNA that has become associated with an unidentified planthopper-transmitted virus (Mansoor et al., 1999).

We have previously demonstrated that the systemic movement of DNA 1 in Nicotiana benthamiana can be mediated by the begomovirus ACMV (Saunders & Stanley, 1999). To extend this investigation, N. benthamiana plants were agroinoculated with DNA 1 and either the begomovirus TGMV, the mastrevirus BeYDV or the curtovirus BCTV, using partial or tandem repeats of these components in agrobacterium binary vectors (Stanley et al., 1986; Briddon et al., 1989; von Arnim & Stanley, 1992; Tan et al., 1995; Liu et al., 1997; Saunders & Stanley, 1999). Nucleic acids isolated from plants (Covey & Hull, 1981) were analysed by blot hybridization as described by Saunders & Stanley (1999) using oligolabelled probes (Feinberg & Vogelstein, 1983) specific for each component. Of six plants infected with TGMV, one contained detectable levels of DNA 1 in the upper symptomatic leaves at 16 days post-infection (p.i.) (Fig. 2A, lanes 1–6). In comparison, DNA 1 was found in systemically infected tissues in one out of three plants when agroinoculated with ACMV, and in two out of three plants following mechanical inoculation with this virus (Saunders & Stanley, 1999). However, we were unable to detect DNA 1 in three samples taken from upper leaves of plants infected with BeYDV at 16 days p.i. (Fig. 2A, lanes 9–11) and in two samples from plants showing severe symptoms at 35 days p.i. (Fig. 2A, lanes 12 and 13). Dot-blot analysis (Maule et al., 1983) of a further 15 symptomatic plants failed to detect DNA 1 in association with BeYDV (data not shown). In contrast, DNA 1 was readily detected in all three samples from plants infected with BCTV at both 16 and 35 days p.i. (Fig. 2A, lanes 16–21). Although plants containing DNA 1 accumulated less BCTV DNA (compare lanes 14 and 15 with lanes 16–21), there were no obvious differences in the onset and severity of symptoms during the course of the experiment.

To investigate whether the association of DNA 1 with BCTV may affect its insect transmission characteristics, ten sugar beet plants (Beta vulgaris cv. Giant Western) were co-inoculated with both components. Symptoms started to appear in all plants by 6 days p.i., and all ten symptomatic plants screened by dot-blot analysis contained detectable levels of DNA 1 (data not shown). Blot hybridization analysis of extracts from the upper systemically infected leaves of two
such plants, sampled at 12 days p.i., confirmed the presence of both components (Fig. 2B, lanes 2 and 3). Twenty non-viruliferous *Circulifer tenellus* leafhoppers were allowed a 48 h access period on a symptomatic plant co-infected with both components and were then maintained on three sugar beet seedlings, essentially as described by Bridden et al. (1990). After 20 days, two plants that developed severe symptoms of stunting and leaf curl following leafhopper transmission contained BCTV DNA and high levels of DNA 1 (Fig. 2B, lanes 4 and 5). The third plant, which developed much milder symptoms, also contained BCTV DNA and DNA 1, although the latter was detectable only after an extended exposure of film to the blot (lane 6).

The recombinant recDNA19 (previously called def19; Stanley et al., 1997) contains two fragments (A and B) from AYVV DNA A separated by two distinct regions (C and D) that do not originate from the begomovirus component (Fig. 1). PCR-amplification using viral supercoiled DNA extracted from naturally infected ageratum plants and divergent primers based on the sequence of fragment C allowed the identification of DNA 1 (Saunders & Stanley, 1999). Because the sequences of fragments C and D show little homology, we were interested in identifying the source of the DNA from which fragment D derives. Divergent primers based on the sequence of fragment D were used to PCR-amplify a product of approximately the same size as DNA 1, suggesting that the recombinant fragment

---

**Fig. 2.** Southern blot analysis of extracts of plants co-inoculated with DNA 1 and either TGMV, BeYDV or BCTV. (A) *N. benthamiana* plants were inoculated with TGMV and DNA 1 (lanes 1–6), BeYDV either alone (lanes 7 and 8) or with DNA 1 (lanes 9–13), and BCTV either alone (lanes 14 and 15) or with DNA 1 (lanes 16–21). Samples were taken at either 16 days p.i. (lanes 1–11 and 14–18) or 35 days p.i. (lanes 12 and 13, and 19–21). Blots were hybridized to probes produced from either the appropriate geminivirus DNA (upper panels) or DNA 1 (lower panels). (B) *B. vulgaris* plants were either mock-inoculated (lane 1) or co-inoculated with BCTV and DNA 1 (lanes 2 and 3). Samples were taken at 12 days p.i. Alternatively, plants were infected using viruliferous leafhoppers fed on a plant that had been co-infected with BCTV and DNA 1 (lanes 4–6). An extract from an ageratum plant containing DNA 1 has been loaded in lane M. The positions of geminivirus and nanovirus-like single-stranded and supercoiled DNAs (ssDNA and scDNA, respectively) are indicated.
Fig. 3. Diversity of nanovirus and nanovirus-like components. (A) Comparison of Rep amino acid sequences encoded by nanovirus-like DNAs associated with ageratum yellow vein disease (AYVD) and cotton leaf curl disease (CLCuD). Identical amino acids are indicated ( ), and spaces ( ) have been introduced for maximum alignment. The conserved tyrosine that may be involved in covalent linkage to Rep is indicated by an arrow and the putative NTP-binding motif (Gorbalenya et al., 1990) is underlined.

(B) Phylogenetic analysis of Reps encoded by nanovirus and nanovirus-like components. Boxes indicate clustering of Reps encoded by AYVD DNA 1 and DNA 2 (AYVV1 and AYVV2, respectively) and the DNA 1 homologue associated with CLCuD (CLCV1), and the Reps encoded by nanovirus master components. Sequences of nanovirus and nanovirus-like components were aligned using PILEUP and CLUSTALX programs, and an unrooted phylogenetic tree was constructed using the programs NEIGHBOR and CONSENSE, based on the distance matrix derived by DNADIST (PHYLIP v3.5 suite of programs). Components used for Rep comparisons (accession numbers in parentheses) were AYVD DNA 1 (AJ238493) and DNA 2 (AJ416153), CLCuD DNA 1 (AJ132345), BBTV components 1A, 1t, S1, S2, W1 and W2 (AR010225, ... respectively), CFDV (M29963), FBNYV components C1, C2, C7 and C9 (X80879, Y11405, AJ005964 and AJ005966, respectively), MVDV components C1, C2, C3, C10 and C11 (AB000920, AB000921, AB000922, AB009047 and AB027511, respectively), and SCSV components C2, C6 and C8 (U16731, U16736 and AJ290434, respectively).
also derives from a circular DNA component. Sequence analysis of the PCR product allowed the identification of a unique EcoRI site. Divergent primers overlapping this site and corresponding to nucleotides 342–371 (primer V4547) and complementary sense nucleotides 317–347 (primer V4548) were used to PCR-amplify a full-length copy of the component, which was subsequently cloned into pGEM-T Easy (Promega) and sequenced (clone pGEM-AYVV2; database accession no. AJ416153). Sequences were analysed using version 8 of the program library of the Genetics Computer Group (Devereux et al., 1984). The sequence of recDNA19 fragment D is identical to the complementary strand of the component, referred to as DNA 2, between nucleotides 1176–1295 (Fig. 1). DNA 2 is similar in length to DNA 1 (1360 and 1367 nucleotides, respectively), it has a typical stem–loop sequence incorporating the nonanucleotide TAGTATTAC that is the hallmark of many nanovirus components, and encodes a Rep that facilitates its autonomous replication in an N. benthamiana leaf disk assay (data not shown). Like DNA 1, it also has an extensive A-rich region located immediately downstream of the Rep coding sequence. However, DNA 2 shows only 47% nucleotide sequence identity with AYVD DNA 1 and 49% with the CLCuD DNA 1 homologue (Mansoor et al., 1999). In contrast, the two DNA 1 homologues are much more closely related (73% nucleotide identity; Saunders & Stanley, 1999). This diversity is also reflected at the protein level (Fig. 3A). The Reps encoded by the DNA 1 homologues are much more closely related to each other (87% identity, 91% similarity) than to DNA 2 (AYVD DNA 1: 40% identity, 61% similarity; CLCuD DNA 1: 43% identity, 51% similarity). Nonetheless, phylogenetic analysis shows that the Reps encoded by DNA 2 and both DNA 1 homologues are more closely related to each other than to their counterparts encoded by nanovirus components (Fig. 3B). However, they show a closer relationship with nanovirus Reps that are responsible simply for the autonomous replication of their own component than with the master Reps (Timchenko et al., 1999, 2000; Horser et al., 2001) that form a cluster in this analysis.

It is likely that DNA 1 and DNA 2 evolved from nanovirus components by association with a begomovirus, necessitating an increase in size to approximately half that of the begomovirus component to allow encapsidation and systemic movement. The increase in size has been attributed to the inclusion of an A-rich sequence within the intergenic region (Mansoor et al., 1999). Encapsidation by the begomovirus coat protein allowed adaptation of DNA 1 and DNA 2 from aphid transmission to whitefly transmission. In the current investigation, we have demonstrated that DNA 1 can also readily adapt to leafhopper transmission as a consequence of its association with the curtovirus BCTV. This implies not only that BCTV can facilitate the systemic movement of DNA1 in N. benthamiana and sugar beet, but also that DNA 1 is encapsidated by the BCTV coat protein to allow insect transmission. Trans-encapsidation in this manner is not without precedent. It has previously been demonstrated that the BCTV coat protein can encapsidate the genomic components of the
begomovirus ACMV, and in doing so allow ACMV to be transmitted by the BCTV leafhopper vector (Briddon et al., 1990).

Nanovirus-like components are associated with monopartite begomovirus infections in the field, and we have demonstrated that DNA 1 can accumulate in plants when co-inoculated with the Old and New World bipartite begomoviruses ACMV and TGMV, and with the curtovirus BCTV (Mansoor et al., 1999; Saunders & Stanley, 1999; this paper). This raises the intriguing possibility that the association of nanovirus-like components with geminiviruses may be a widespread phenomenon in the field. Our demonstration of adaptation to a leafhopper vector may have significant implications for the dispersal, diversity and evolution of nanovirus components, particularly in view of their propensity to undergo recombination. Certainly, this observation supports the suggestion that the CFDV component is in fact a nanovirus-like component associated with a geminivirus that has become adapted to planthopper transmission by a size increase (Mansoor et al., 1999). Reports of nanovirus-like components associated with geminivirus diseases are so far confined to the Old World. However, it is worth noting that BCTV and, more recently, the monopartite begomovirus TYLCV have been introduced into the New World from the Middle East (Briddon et al., 1998; Polston et al., 1994), where geminivirus and nanovirus diseases are prevalent and provide the opportunity for mixed infections and component adaptation. It remains to be seen if additional distinct nanovirus-like components are associated with AYVD and CLCuD, and detailed surveys are required to search for nanovirus-like components associated with other geminivirus diseases to determine the full extent of their dissemination.

This work was funded by a grant-in-aid to the John Innes Centre from the BBSRC. Viruses were maintained and manipulated with the authorization of the Department for Environment, Food and Rural Affairs under licence number PHL 11D/3825(7/2001).

References


K. Saunders, I. D. Bedford and J. Stanley


Received 22 October 2001; Accepted 10 December 2001