Genome organization of ageratum yellow vein virus, a monopartite whitefly-transmitted geminivirus isolated from a common weed

Priscilla H. N. Tan, Sek Man Wong, Mian Wu, Ian D. Bedford, Keith Saunders and John Stanley

1 Department of Botany, National University of Singapore, Lower Kent Ridge Road, Singapore 0511 and 2 Department of Virus Research, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

A full-length copy of a single genomic component of the whitefly-transmitted geminivirus ageratum yellow vein virus (AYVV) has been cloned from an extract of infected Ageratum conyzoides originating from Singapore. Sequence analysis shows that the genomic component encodes two virion-sense (V1 and V2) and four complementary-sense open reading frames (C1-C4), typical of DNA A of whitefly-transmitted geminiviruses from the Eastern hemisphere. A genomic component equivalent to DNA B was not detected in extracts of infected A. conyzoides. The cloned genomic component produced a systemic infection in Nicotiana benthamiana, Phaseolus vulgaris and Lycopersicon esculentum when introduced into plants by agroinoculation, and symptoms were identical to those produced by wild-type virus introduced into these hosts using viruliferous whiteflies. However, attempts to re-establish a systemic infection in A. conyzoides either by agroinoculation or by whitefly transmission of the cloned progeny were unsuccessful, suggesting that additional factors are required for infection of the natural host. The significance of A. conyzoides as a reservoir host for the economically important geminivirus diseases is discussed.

Introduction

The tobacco whitefly Bemisia tabaci (Gennadius) transmits around 60 different plant viruses (Bedford et al., 1994), the majority of which are members of the geminivirus group. Geminiviruses are unique plant viruses characterized by twinned (geminate) icosahedral virions (Harrison et al., 1977) that encapsidate circular single-stranded (ss) DNA ranging in size from 2.6-3.0 kb (reviewed by Stanley, 1985; Lazarowitz, 1987). Whitefly-transmitted geminiviruses such as African cassava mosaic virus (ACMV) have genomes comprising two DNA components (DNAs A and B) (Stanley & Gay, 1983). ACMV DNA A can autonomously replicate in protoplasts and synthesize virus particles (Townsend et al., 1986; Klinkenberg & Stanley, 1990) although both genomic components are essential for systemic infection (Stanley, 1983), implicating DNA B in virus movement. However, several whitefly-transmitted geminiviruses have recently been isolated that have only single DNA components equivalent to DNA A. These interesting exceptions include tomato yellow leaf curl virus from Israel (TYLCV-I) (Navot et al., 1991) and Sardinia (TYLCV-S) (Kheyr-Pour et al., 1991), and tomato leaf curl virus from Australia (TLCV-Au) (Dry et al., 1993).

Not surprisingly, research has focused on viruses that infect economically important crop plants, exemplified by the above-mentioned single component viruses that were all isolated from tomato. This has meant that research on virus-infected weeds, which are commonly found in areas where crop plants are cultivated, has been neglected. However, a whitefly-transmitted virus from Singapore has recently been identified in a common weed, Ageratum conyzoides L., which thrives in moist, shady areas in many parts of south-east Asia. The virus, ageratum yellow vein virus (AYVV), has been identified as a geminivirus on the basis of host range, transmission, virion morphology, serology and cytopathology (Tan & Wong, 1993; Wong et al., 1993). Although the virus was shown to have a rather limited host range, a feature typical of many dicotyledon-infesting geminiviruses, it could be transmitted by whiteflies to tomato. Because AYVV infection has no obvious detrimental effect on the growth and development of A. conyzoides, this particular
weeds could act as an important reservoir host from which the virus can spread to important crop plants.

To our knowledge, AYVV is the only weed-infesting geminivirus that is currently under intensive research. Here, we describe the construction and characterization of an infectious clone of AYVV, and the relationship of this virus to other geminiviruses. The results are discussed in terms of geminivirus evolution and the importance of reservoir hosts to geminivirus disease epidemiology.

**Methods**

*Source and maintenance of virus isolate.* *A. conyzoides* plants showing typical yellow vein symptoms were collected from wastelands in Singapore and the virus was maintained in *A. conyzoides* by whitefly transmission in insect-proof cages. AYVV was held and manipulated at the John Innes Centre under MAFF licence numbers PHF 1185A/68(21) and PHF 1185B/17(111) under the Plant Pests (Great Britain) Order 1980.

*Construction of cloned copies of AYVV DNA A.* Total cellular nucleic acids were extracted from AYVV-infected *A. conyzoides* using the procedure of Covey & Hull (1981). Fragments of the viral genome were PCR-amplified from 100 ng total nucleic acids using a Perkin Elmer Cetus DNA thermal cycler programmed for nine cycles of 1 min at 94 °C, 2 min at 55 °C and 4 min at 72 °C, and 40 cycles of 3 min at 94 °C, 2 min at 60 °C and 4 min at 72 °C.

Initially, a 1 kb fragment of AYVV DNA A was amplified using a virion-sense primer encompassing conserved sequences in the stem-loop region (primer P1; 5' CGGAATTC(A/G)TA(C/T)TT(A/C/G/T)CC(A/C/G/T)GC-3'). Both P1 and P2 were designed with unique sequence obtained from the pHN1 insert. A full-length copy of the genome was amplified using these primers, digested with restriction endonucleases and the products were fractionated on agarose gels, Southern blotted and sequenced to identify regions conserved across different geminivirus genomes. The full-length clone was assembled and analysed using University of Wisconsin Genetics Computer Group software (Devereux et al., 1984).

*Analysis of AYVV DNA A.* Nucleotide sequences of cloned DNA were derived by the deoxyxynucleotide chain termination method of Sanger et al. (1977), using the TaqTrack sequencing system (Promega) and [α-35S]dATP (DuPont NEN). To avoid sequencing ambiguities, sequences were determined for both DNA strands. The sequence of the full-length clone was assembled and analysed using University of Wisconsin Genetics Computer Group software (Devereux et al., 1984).

*Attempts to detect a putative AYVV DNA B component.* Virion-sense primer V2127 (5' GAATGGTACCCCATTTAGTTGA 3') and complementary-sense primer V2128 (5' AT TTGGA TTCATCCCCCAATTGGA 3') were designed on the basis of the nucleotide sequence of the AYVV DNA A intergenic region. A single mismatch was introduced into each primer to create KpnI sites (underlined). The primers were used to PCP-amplicot viral DNA using total nucleic acids extracted from infected *A. conyzoides* in a thermal cycler programmed for 40 cycles of 1 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C. The amplified fragment was digested with restriction endonucleases and the products were fractionated on agarose gels, Southern blotted and probed using either an [α-32P]dCTP-radio-labelled 2.5 kbp BamHI-A-specific fragment or a 301 bp DraI-PstI fragment from the intergenic region.

In a second experiment, an attempt was made to amplify a DNA B fragment using the universal DNA B primers PCRc1 and PBL1 (2004) described by Rojas et al. (1993). Total nucleic acids extracted from infected *A. conyzoides*, *Phascolus vulgaris* (French bean cv. Top Crop) and *Nicotiana benthamiana* were used in a thermal cycler programmed for nine cycles of 1 min at 94 °C, 2 min at 55 °C and 4 min at 72 °C, followed by 40 cycles of 1 min at 94 °C, 2 min at 60 °C and 4 min at 72 °C. A total nucleic acid extract from ACMV-infected *N. benthamiana* was used as a positive control for the presence of DNA B.

*Agroinoculation of AYVV DNA A.* AYVV PsH-BamHI fragments of approximately 400 bp, encompassing the intergenic region, were isolated from pHN419 and pHN429, and cloned into pIC19H (Marsh et al., 1984). Full-length copies of AYVV DNA from pHN419 and pHN429 were inserted into these clones at their unique BamHI sites to produce partial repeats of the viral DNA in clones pHNIC419 and pHNIC429. The partial repeats were excised using flanking HindIII sites and cloned into pBin19 (Bevan, 1984) to produce pHNBin419 and pHNBin429. Clones were conjugated into *Agrobacterium tumefaciens* strains A3489, C58, LAB4404 and PGV3850 (Garfinkel et al., 1981; Hoekema et al., 1983; Zambryski et al., 1983; Hupburn et al., 1985).

For the purpose of agroinoculation, *A. tumefaciens* cultures were grown on plates at 28 °C for 48 h. Cells were scraped from the plates with a sterile toothpick and applied to seedlings at the three to five leaf stage, and an inoculation needle was used to prick through the inocula into the plant four to six times. Plants were maintained in the UK in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse maintained at 25 °C with supplementary lighting to give a 16 h photoperiod.

*Whitefly transmission experiments.* *B. tabaci* was originally obtained in 1991 from Florida, USA and identified as the polyphagous B biotype (Bedford et al., 1994). Insects were held in the UK under MAFF licence number PHF 1419/819/14, and maintained as a viruliferous colony on AYVV-infected *A. conyzoides* in cages within a controlled environment growth room kept at 25 °C with supplementary lighting to give a 12 h photoperiod. For transmission studies from *A. conyzoides*, groups of 50 viruliferous insects were allowed a minimum 48 h transmission access period on healthy test seedlings. Insects were then removed from the plants, which were transferred to an insect-proof glasshouse and fumigated twice weekly with the carbamate-based insecticide propoxur (Octavius Hunt). Transmission tests from alternative hosts were accomplished by allowing groups of 50 non-viruliferous insects a minimum acquisition access period of 48 h on infected plants before transfer to healthy seedlings.

*Analysis of infected plants.* Plants were monitored for the development of symptoms and tested for the presence of viral DNA by dot hybridization (Maule et al., 1983). Total cellular nucleic acids were extracted from systemically infected tissues or ACMV using the procedure of Covey & Hull (1981), and fractionated by agarose gel electrophoresis in TNE buffer (40 mM-Tris acetate pH 7.5, 20 mM-sodium acetate, 2 mM-EDTA). Viral DNA forms were investigated by Southern blot analysis using randomly primed AYVV probes. The presence of coat protein was examined by immuno blot analysis of plant extracts using a polyclonal antiserum raised against purified ACMV as described by Stanley & Townsend (1986).

**Results and Discussion**

Attempts to isolate virus-specific supercoiled (sc) DNA for the purpose of cloning copies of the AYVV genomic components, as described for ACMV (Stanley &
were predicted to hybridize with sequences in the stem-loop region and at the 3' terminus of the coat protein gene that are reasonably conserved between whitefly-transmitted geminiviruses. Sequence analysis of the fragment confirmed that it had originated from DNA A, and facilitated the design of overlapping DNA A-specific primers (V957 and V958) that were used to generate two full-length DNA A clones containing two copies of the intergenic region were cloned into the binary vector pBinl9 (pHNBin419 and pHNBin429). The reason for this anomalous behaviour has not yet been investigated.

AYVV coat protein was readily detected in extracts from A. conyzoides infected using viruliferous whiteflies (Fig. 2, lane 2) as well as from agroinoculated N. benthamiana (lane 4) when immunoblots were probed with a polyclonal antiserum raised against ACMV. In addition, typical geminate particles were detected in agroinoculated N. benthamiana extracts by electron microscopic examination of negatively stained samples (data not shown).

Repeated attempts to infect A. conyzoides using A. tumefaciens strains A348N, C58, LBA4404 and PGV3850, by inoculation into the stem, apical meristem or onto decapitated shoot tips, were unsuccessful as judged by the lack of symptom development and inability to detect viral DNA by dot blot analysis. A. tumefaciens strains C58 and A348N induced tumours in A. conyzoides, ensuring that the T-DNA, and hence the
The wild-type isolate of AYVV was routinely maintained in A. conyzoides by whitefly transmission from symptomatic French bean or tomato to either of these hosts or to A. conyzoides as judged by the lack of symptom development and inability to detect viral DNA by Southern blot analysis. While reasonable levels of ssDNA are produced in infected French beans and tomato, little or no coat protein could be detected by immunoblot analysis of samples from agroinoculated plants (Fig. 2, lanes 6 and 8), suggesting that the virus is poorly adapted to these hosts. It should be noted that coat protein detection in extracts of beans infected either by agroinoculation or using viruliferous whiteflies (lanes 8 and 9, respectively) was hampered by a comigrating host protein present in healthy extracts (lane 7). However, the results suggest that our inability to whitefly-transmit the disease from these hosts may be due to low abundance or complete absence of virus particles. Unfortunately, although virus particles accumulated in infected N. benthamiana, transmission experiments were not possible because whiteflies could not be maintained on this host.

The nucleotide sequence of the insert of clone pHN419, determined in both orientations, comprises 2741 nucleotides and has a base composition of 27.2% A, 20.6% C, 31.6% T and 20.6% G (Fig. 3). Comparison of sequences at the cloning site with those adjacent to the BamHI site in pHN1 confirmed that the pHN419 insert represented a full-length copy of the genomic component.

Analysis of potential coding sequences that have the capacity to encode proteins in excess of 10 kDa shows that the component is organized on the same basis as DNA A of other whitefly-transmitted geminiviruses (Fig. 4), having two overlapping virion-sense open reading frames (ORFs) (V1 and V2) and four overlapping complementary-sense ORFs (C1–C4) that are conserved between these viruses. An additional complementary-sense ORF, C5, has not been observed previously and

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viral DNA contained within it, was being transferred to the plant during the inoculation process. Viral DNA replication was readily detected in A. tumefaciens-induced tumours from N. benthamiana (Fig. 1, lane 6) but could not be detected in equivalent tissue from A. conyzoides although high molecular mass DNA containing viral sequences, probably A. tumefaciens DNA, occurred at low levels (lane 1). The inability to detect viral DNA accumulation in A. conyzoides tumour extracts could mean that the cloned DNA has a host-specific defect in replication and/or movement that prevents infection of this plant species. However, we feel that this explanation is unlikely because we have tested two independently derived clones isolated directly from an infected A. conyzoides extract, and both were found to be highly infectious in N. benthamiana and French beans although neither reinfect the natural host. Alternatively, the lack of infectivity in A. conyzoides may be attributable to the introduction of the viral DNA into tissues unsuitable for virus multiplication and/or at the wrong time during plant development. Preliminary experiments using biolistic bombardment of A. conyzoides seedlings with the cloned DNA have also been unsuccessful although N. benthamiana plants became infected in control experiments (data not shown).

The wild-type isolate of AYVV was routinely maintained in A. conyzoides by whitefly transmission from symptomatic plants to healthy seedlings, demonstrating that the plants were being kept under physiological conditions suitable for infection. The virus was also whitefly-transmissible from A. conyzoides to French bean and tomato, in which it induced symptoms indistinguishable from those produced by agroinoculation of the cloned DNA. However, repeated attempts to whitefly-transmit virus from agroinoculated symptomatic French bean either to A. conyzoides or French bean were unsuccessful. Surprisingly, the wild-type virus could not be whitefly-transmitted from symptomatic French bean or tomato to either of these hosts or to A. conyzoides as judged by the lack of symptom development and inability to detect viral DNA by immunoblot analysis. While reasonable levels of ssDNA are produced in infected French beans and tomato, little or no coat protein could be detected by immunoblot analysis of samples from agroinoculated plants (Fig. 2, lanes 6 and 8), suggesting that the virus is poorly adapted to these hosts. It should be noted that coat protein detection in extracts of beans infected either by agroinoculation or using viruliferous whiteflies (lanes 8 and 9, respectively) was hampered by a comigrating host protein present in healthy extracts (lane 7). However, the results suggest that our inability to whitefly-transmit the disease from these hosts may be due to low abundance or complete absence of virus particles. Unfortunately, although virus particles accumulated in infected N. benthamiana, transmission experiments were not possible because whiteflies could not be maintained on this host.

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**Fig. 2.** Immunoblot analysis of coat protein synthesis in AYVV-infected plants. Samples were from healthy A. conyzoides (lane 1) and A. conyzoides infected by whitefly transmission of wild-type virus (lane 2), healthy and agroinoculated symptomatic N. benthamiana (lanes 3 and 4, respectively), healthy and agroinoculated symptomatic tomato (lanes 5 and 6, respectively), healthy French bean (lane 7) and symptomatic French bean infected either by agroinoculation (lane 8) or by whitefly transmission of wild-type virus (lane 9). A sample from ACMV-infected N. benthamiana (lane 10) has been included to show the mobility of ACMV coat protein (cp). The membrane was probed for coat protein using an antiserum raised against purified ACMV. Prestained markers (Bio-Rad) were used as molecular mass standards.
may have arisen fortuitously. Comparison with other
geminiviruses suggests that ORF V1 encodes the coat
protein (Townsend et al., 1985), ORF C1 encodes the
replication-associated protein (Hanley-Bowdoin et al.,
1990), ORFs C2 and C3 regulate virion-sense gene
expression and DNA replication respectively (Sunter et
al., 1990; Sunter & Bisaro, 1992), and ORF C4 is a
pathogenicity determinant that may affect host cell

Fig. 3. Nucleotide sequence of the insert of clone pHN419 (EMBL accession number X74516). The sequence of the virion-sense strand
is given and position 1 has been defined as the A residue immediately downstream of the nick site within the conserved nonanucleotide
(TAATATT~AC), as defined for ACMV (Stanley, 1995).
division (Stanley & Latham, 1992). ORF V2 is apparently confined to subgroup III geminiviruses (Mayo & Martelli, 1993) from the Eastern hemisphere. Its function is not known although the AYVV ORF V2 is highly homologous to a counterpart in ACMV, that is dispensable for infection of N. benthamiana (Etessami et al., 1989), and one in TLCV, that is required for the accumulation of normal levels of viral DNA and symptom development in tomato (Rigden et al., 1993).

The intergenic region contains the ubiquitous nonanucleotide TAATATTAC required for DNA replication (Stanley, 1995) located within a potential stem-loop sequence. The sequence motif GGTACTCA, occurring at positions 2617–2624 and 2652–2659 and in the complementary-sense at position 2669–2676, may participate in C1 binding (Fontes et al., 1994). The arrangement of these iterated motifs relative to the putative TATA box for complementary-sense transcription, as defined by Argüello-Astorga et al. (1994), is unique to AYVV but most closely resembles those of Eastern hemisphere geminiviruses. Comparison of the entire nucleotide sequence with other geminiviruses also places AYVV with subgroup III geminiviruses originating from the Eastern hemisphere (Padidam et al., 1995a), and it is most closely related to the Australian monopartite virus TLCV (Dry et al., 1993) with which it shows 73.5% identity. Comparison of amino acid sequences encoded within each ORF, particularly coat protein and replication-associated protein sequences, with those from other subgroup III viruses serves to emphasize the close relationship of AYVV with viruses from the Eastern hemisphere.

In an attempt to resolve the problem of lack of infectivity of AYVV DNA A in its natural host, infected A. conyzoides was analysed for the presence of a genomic component equivalent to DNA B. Because of the low levels of viral scDNA in infected plants, a full-length viral DNA fragment was PCR-amplified using primers located within the putative common region that should hybridize equally well to DNA A and DNA B (if present). KpnI, XhoI and PstI sites occur only once within DNA A at positions 1570, 1591 and 2459, respectively. BgIII sites occur twice at positions 1252 and 1865, and there is no HindIII site. Southern blot analysis using a DNA A-specific probe showed that digestion of the 2.8 kbp PCR-amplified fragment with either KpnI or XhoI produced fragments of approximately 1.7 and 1.1 kbp, PstI produced a fragment of approximately 2.6 kbp (the anticipated 0.2 kbp fragment was not detected by Southern blot analysis), BgIII produced fragments of approximately 1.4, 0.8 and 0.6 kbp, and the fragment remained intact following HindIII digestion. With the exception of the 0.6 kbp BgIII produced fragment, all of these fragments were also detected using a probe encompassing the intergenic region. The inability to detect fragments in addition to those predicted for DNA A using either probe suggests the presence of only one AYVV genomic component. Furthermore, we were unable to amplify a DNA B fragment from an infected A. conyzoides extract using a PCR-based detection

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<th>Table 1. Comparison of amino acid sequences encoded by conserved ORFs of AYVV and other subgroup III geminiviruses</th>
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<td><strong>Percentage identity of derived amino acid sequence</strong></td>
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* ORF V2 is present only in viruses originating from the Eastern hemisphere.
technically similar to that described for the identification of whitefly-transmitted geminiviruses (Rojas et al., 1993), although such a fragment was readily obtained from an ACMV-infected *N. benthamiana* extract. Hence, the data suggest that *AYVV* is a monopartite geminivirus resembling some isolates of TYLCV (Navot et al., 1991; Kheyry-Pour et al., 1991) and the closely related Australian isolate of TLCV (Dry et al., 1993).

Although most research has focused on geminiviruses that have been isolated directly from infected crop plants, it is generally accepted that alternative endemic hosts can play an important role in disease epidemiology. For example, *Hewititia sublobata* (Euphorbiaceae) is widespread throughout the tropics and serves as a host for East African cassava mosaic virus (Bock et al., 1981; Hong et al., 1993), and *Sida acuta* (Malvaceae) is believed to be a host in the wild for okra leaf curl virus (Konat et al., 1991). The fact that the single *AYVV* genomic component infects French bean and tomato suggests that *A. conyzoides* may act as a reservoir host for the pathogen. However, comparison of epitope profiles of viruses from *A. conyzoides* and tomato originating from Singapore does not support this idea (Wong et al., 1993). Nonetheless, it is worth noting that symptomatic *A. conyzoides* frequently occurs in fields of tomatoes infected with Indian tomato leaf curl virus (Muniyappa et al., 1991), and viruses from both hosts in India appear to be closely related (Wong et al., 1993). We observed very little cross-hybridization on dot blots between *AYVV* from Singapore and viruses from symptomatic *A. conyzoides* originating from Pakistan and Nepal (J. Stanley, I. D. Bedford & P. G. Markham, unpublished), and the epitope profiles of Singapore and Indian virus isolates have been shown to differ (Wong et al., 1993), suggesting that more than one distinct geminivirus has become adapted to this host.

We have been unable to formally demonstrate that *AYVV* is the causal agent of yellow vein disease in *A. conyzoides* because of the failure to reintroduce the virus into this host, either by agroinoculation of the cloned component or by whitefly-transmission of cloned progeny. It is conceivable that an additional factor, for example a second genomic component present at a level below the limit of detection in our experiments or even a co-infecting virus, is necessary for infection of a less permissive host like *A. conyzoides*. In this respect, it is interesting to note that an isolate of TYLCV from Thailand is considered to be a bipartite virus even though DNA A alone causes a systemic systemic infection in *N. benthamiana* and tomato (Rochester et al., 1994). In this particular case, DNA B may have been retained because it is required for infection of less permissive hosts that play an important role in maintaining the virus population. Since all other subgroup III monopartite viruses have been isolated from tomato, this raises the intriguing possibility that cognate DNA B components exist for these viruses, but have been lost from regions where permissive hosts are abundant throughout the year as a result of monocultural farming practices.

Having demonstrated that a single genomic component of *AYVV* is infectious in certain hosts, we are now continuing to study the aetiology of *A. conyzoides* yellow vein disease which should ultimately contribute to our understanding of geminivirus epidemiology.

We would like to thank Marion Pinner for her excellent technical assistance. We also thank Andrew P. Lucy and Dixon C. Y. Yeong for accessing the GenBank database and sharing molecular biology techniques. This research project was supported in part by grant RP 3910399 from the National University of Singapore, Republic of Singapore.

**References**


(Received 8 June 1995; Accepted 4 September 1995)