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Identification of a Novel Circular Single-Stranded DNA Associated with Cotton Leaf Curl Disease in Pakistan

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Recent reports have suggested that cotton leaf curl virus (CLCuV), a geminivirus of the genus *Begomovirus*, may be responsible for cotton leaf curl disease in Pakistan. However, the causal agent of the disease remains unclear as CLCuV genomic components resembling begomovirus DNA A are unable to induce typical disease symptoms when reintroduced into plants. All attempts to isolate a genomic component equivalent to begomovirus DNA B have been unsuccessful. Here, we describe the isolation and characterisation of a novel circular single-stranded (ss) DNA associated with naturally infected cotton plants. In addition to a component resembling DNA A, purified geminate particles contain a smaller unrelated ssDNA that we refer to as DNA 1. DNA 1 was cloned from double-stranded replicative form of the viral DNA isolated from infected cotton plants. Blot hybridisation using probes specific for either CLCuV DNA or DNA 1 was used to demonstrate that both DNAs co-infect naturally infected cotton plants from different geographical locations. DNA 1 was detected in viruliferous *Bemisia tabaci* and in tobacco plants infected under laboratory conditions using *B. tabaci*, indicating that it is transmitted by whiteflies. Sequence analysis showed that DNA 1 is approximately half the size of CLCuV DNA but shares no homology, indicating that it is not a defective geminivirus component. DNA 1 has some homology to a genomic component of members of Nanoviridae, a family of DNA viruses that are normally transmitted by aphids or planthoppers. DNA 1 encodes a homologue of the nanovirus replication-associated protein (Rep) and has the capacity to autonomously replicate in tobacco. The data suggest that a nanovirus-like DNA has become whitefly-transmissible as a result of its association with a geminivirus and that cotton leaf curl disease may result from a mutually dependent relationship that has developed between members of two distinct DNA virus families that share a similar replication strategy. © 1999 Academic Press

INTRODUCTION

Cotton is the most important cash crop of Pakistan, contributing >60% of the total foreign exchange earnings of the country. During the last 10 years, cotton leaf curl disease has caused heavy losses to the cotton crop in the country. The disease has been known to exist for ≥30 years but was never considered economically important until 1988, when it occurred as an epidemic in the Punjab province. Since 1992, the disease has spread to all cotton-growing districts of the Punjab, resulting in the loss of >1.3 million tonnes of cotton with an estimated value of $5 billion. Since 1997, the disease has spread to Sindh province, which previously was free of the disease (Mansoor *et al.*, 1998a).

Symptoms of the disease include upward or downward leaf curling, thick dark green veins and leaf-like growths (enations) emerging from these veins (Mansoor *et al.*, 1993; Harrison *et al.*, 1997). The disease is transmitted by the whitefly *Bemisia tabaci* (Genn.) and is associated with members of the *Geminiviridae* genus *Begomovirus*, collectively referred to as cotton leaf curl virus (CLCuV). Most begomoviruses have two genomic components (DNA A and DNA B), both of which are essential for infection. However, several viruses from the Old World have been described for which only a single component, analogous to DNA A, has been isolated and shown to induce disease symptoms (Navot *et al.*, 1991; Dry *et al.*, 1993; Tan *et al.*, 1995). CLCuV DNA isolated from diseased cotton shows extensive homology with DNA A components of other whitefly-transmitted geminiviruses found in the Indian subcontinent (Zhou *et al.*, 1998). The fact that symptom severity of the disease correlated with the level of CLCuV DNA in susceptible cotton genotypes has suggested that a whitefly-transmitted geminivirus is the causative agent of the disease (Mansoor *et al.*, 1998b). However, although cloned copies of the genomic component cause a systemic infection when reintroduced into *Nicotiana benthamiana* and cotton, symptoms are delayed, extremely mild, and not typical of cotton leaf curl disease (manuscript in preparation). All attempts to isolate a genomic component equivalent to DNA B have been unsuccessful and so the causal agent of cotton leaf curl disease remains obscure.

Here we report the cloning and characterisation of a novel whitefly-transmitted circular single-stranded (ss)

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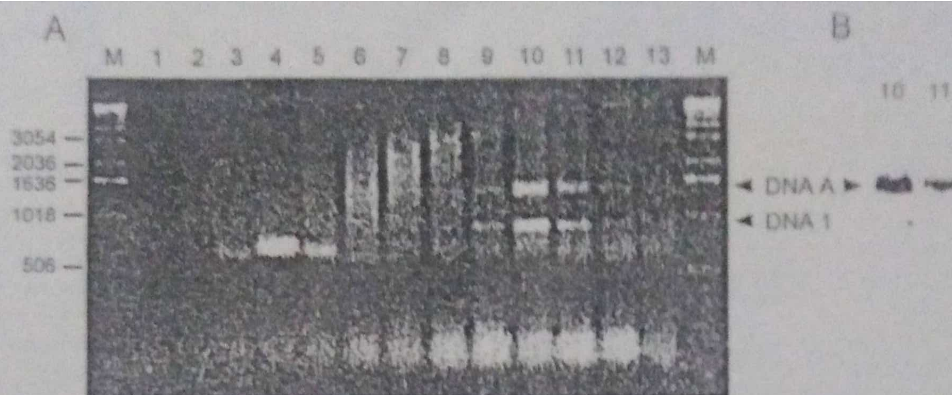


FIG. 1. Characterisation of DNA forms associated with geminivirus particles isolated from cotton showing leaf curl disease. (A) DNA was extracted from sucrose gradient fractions (lanes 1 (top) to 13), fractionated by agarose gel electrophoresis and stained using ethidium bromide. The sizes (bp) of markers (Gibco BRL) (lane M) are indicated. (B) Samples 10 and 11 were analysed by blot hybridisation using full-length CLCuV DNA as a probe. The positions of CLCuV DNA (DNA A) and DNA 1 are indicated.

DNA associated with cotton leaf curl disease in Pakistan. The ssDNA shows a close relationship to a genomic component of members of the Nanoviridae family of DNA viruses. The impact of this observation on our understanding of the aetiology of cotton leaf curl disease is discussed.

RESULTS

Small viral DNAs are associated with geminate particles isolated from infected cotton

Virus particles were purified by sucrose gradient centrifugation from cotton plants growing in the field and that were showing typical leaf curl disease symptoms. Fractions were analysed for the presence of characteristic geminivirus particles, and DNA was isolated from each fraction and analysed by agarose gel electrophoresis. The DNA associated with fractions containing virus particles (Fig. 1A, peak fractions corresponding to lanes 10 and 11) contained two DNA species that were present at similar levels. Of these, only the slower migrating DNA hybridised efficiently with a full-length CLCuV DNA probe (Fig. 1B), whereas a weak signal was associated with the faster migrating DNA that we refer to as DNA 1. The weak signal associated with DNA 1 is probably due to hybridisation of the probe to a low level of defective CLCuV DNA that is approximately half the length of the genomic component (Liu *et al.*, 1998, unpublished data).

To characterise DNA 1, viral supercoiled (sc) DNA was isolated from symptomatic cotton plants and purified by CsCl density gradient centrifugation. The DNA was cloned after digestion with *Bam*HI. Clones were screened for viral DNA using probes made from either scDNA or full-length CLCuV DNA. Clones that were detected using the scDNA probe, but not the CLCuV DNA probe, were selected for further analysis. Sequence analysis of one such clone, pBS-CLCV1, showed that its ~1.4-kb insert shared no homology with CLCuV DNA or to DNA B sequences of other begomoviruses, indicating that it was not derived from a defective genomic component. On the basis of the established sequence, abutting primers PB2-F and PB2-R were designed to PCR-amplify a full-length copy of DNA 1 from an infected cotton extract to produce clone pGEM-CLCV1.

To verify that DNA 1 was associated with geminivirus particles, as suggested from the sucrose gradient centrifugation data, virus was isolated from infected *N. benthamiana* extracts by immunotrapping using polyclonal antiserum raised against African cassava mosaic virus (ACMV) coat protein. The coat protein is the most highly conserved begomovirus gene product, and ACMV coat protein polyclonal antiserum is known to cross-react with other begomoviruses (Thomas *et al.*, 1986; Tan *et al.*, 1995). The ACMV antiserum used in this experiment specifically detects CLCuV coat protein on Western blots of diseased cotton extracts (M. Pinner, personal communication). To avoid false positive results arising from the amplification of small quantities of DNA adsorbed onto the surface of the plastic tubes (Wyatt and Brown, 1996), Southern blot analysis rather than PCR amplification of viral DNA fragments was used to analyse immunotrapped DNA. Such an analysis showed the presence of DNA 1 associated with immunotrapped particles (Fig. 2, lane 2). This DNA was not detected when the polyclonal antiserum was omitted from the assay. The DNA migrated to the approximate position of ssDNA extracted from a symptomatic cotton plant (lane 1). The slight difference in position is attributable to plant contaminants within cotton extracts that frequently affect the electrophoretic mobility of the viral DNAs. The DNA was detected using a probe for virion-sense DNA (lane 2) that also detected single- and double-stranded DNA forms normally associated with geminivirus-infected plants (lane 1). However, it was not detected using a probe for complementary-sense DNA (lane 4) that also failed to detect ssDNA in the plant extract (lane 3). This confirmed that a single-stranded form of DNA 1 was immunotrapped by the ACMV coat protein antiserum.

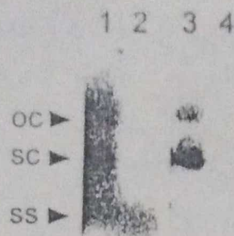


FIG. 2. Immunotrapping of DNA 1 and determination of the polarity of encapsidated DNA. DNA samples were extracted either from symptomatic cotton (lanes 1 and 3) or from virus particles immunotrapped from *N. benthamiana* extracts using polyclonal antiserum raised against ACMV (lanes 2 and 4) and fractionated by agarose gel electrophoresis. The blot was hybridised to a riboprobe produced by transcription of the DNA 1 insert of pGEM-CLCV1 using the T7 promoter (lanes 1 and 2). After autoradiography, the membrane was stripped and rehybridised to a riboprobe produced from the SP6 promoter of pGEM-CLCV1 (lanes 3 and 4). The positions of single-stranded (ss), supercoiled (sc), and open-circular (oc) DNA forms are indicated.

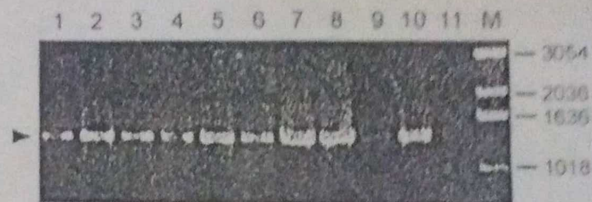


FIG. 4. PCR amplification of a fragment of DNA 1 from extracts of infected cotton and viruliferous whiteflies collected from the field. The primer pair DNA1-F and DNA1-R was used to amplify full-length DNA 1 (arrowhead) from extracts of individual symptomatic cotton plants (lanes 1–6) and viruliferous whiteflies (lanes 7–10). An extract from a healthy cotton plant was used as a control (lane 11). The sizes (bp) of markers (Gibco BRL) (lane M) are indicated.

CLCuV DNA and DNA 1 coexist in infected cotton and are whitefly-transmitted

Viral DNAs associated with symptomatic cotton that had been collected from the Multan region in 1992 were investigated by Southern blot analysis using full-length CLCuV DNA and DNA 1 probes (Fig. 3). All three samples contained viral DNAs that cross-hybridised to both probes (lanes 1–3; the CLCuV DNA signal in lane 3 is weak but clearly visible on the original autoradiograph). Both CLCuV DNA and DNA 1 were transmitted by whiteflies to tobacco (lanes 4–6), which developed symptoms typical of cotton leaf curl disease.

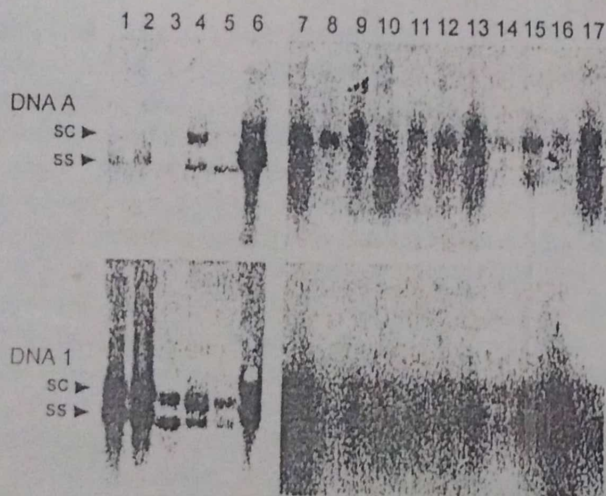


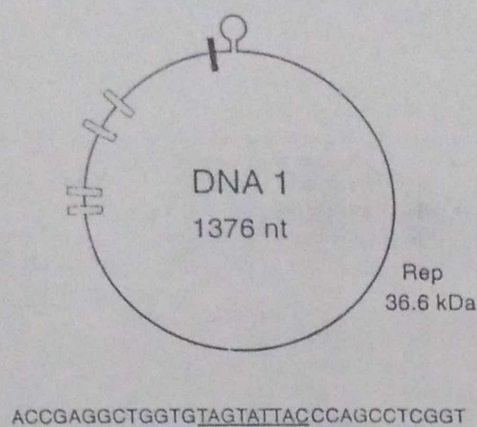
FIG. 3. Detection of viral DNAs in cotton and tobacco plants showing typical leaf curl disease symptoms. DNA samples were extracted from symptomatic cotton collected from Multan (lanes 1–3), tobacco infected using whiteflies fed on symptomatic cotton (lanes 4–6), cotton collected from Dunyapur, district Lodhran (lanes 7 and 17), the Cotton Research Institute, Rahim Yar Khan (lane 8), Tirinda Swai Khan, district Rahim Yar Khan (lane 9), Sahiwal (lane 10), the Cotton Research Station, Sahiwal (lane 11), Khanpur, district Rahim Yar Khan (lane 12), Ahmedpur East, district Bahawalpur (lane 13), Faisalabad (lanes 14 and 16) and Rahim Yar Khan (lane 15). All samples were collected in 1998 except those from Multan (lanes 1–3) and Faisalabad (lane 16), which were collected in 1992 and 1997, respectively. Blots were hybridised to either CLCuV DNA (DNA A) or DNA 1 probes. The positions of single-stranded (ss) and supercoiled (sc) DNA forms are indicated.

Symptomatic cotton plants were subsequently collected in 1997 and 1998 from locations in Pakistan between Faisalabad and Rahimyar Khan, encompassing regions that are separated by >400 km. Both CLCuV DNA and DNA 1 probes hybridised to viral DNAs extracted from all of the plants tested (Fig. 3, lanes 7–17; the DNA 1 signal in lane 8 is weak, but this DNA could be detected by PCR amplification). The levels of both DNAs as well as the relative amounts of ssDNA and scDNA varied considerably between samples. This is attributable to differences in the age of the sampled tissues and the extent of infection, as well as difficulties inherent in extracting intact DNA from cotton. Nonetheless these data indicate that both CLCuV DNA and DNA 1 have been associated with cotton leaf curl disease over a number of years, and both occur throughout the sampling region that covers a large part of the cotton growing region of Pakistan.

DNA 1 is related to nanovirus DNA

To confirm that the pBS-CLCV1 insert originated from a circular DNA and represents a full-length copy of DNA 1, partially overlapping primers PB1-F and PB1-R were designed to anneal to DNA 1 sequences adjacent to the *Bam*HI cloning site. Using these primers, a DNA fragment of similar size to the pBS-CLCV1 insert was PCR-amplified from extracts of individual cotton plants (Fig. 4, lanes 1–6). Cloning and sequence analysis confirmed that the termini of the pBS-CLCV1 insert were contiguous in the amplified DNA fragment, verifying the circular nature of the DNA. These primers were also used to amplify a full-length copy of DNA 1 from extracts of viruliferous whiteflies collected from the field (lanes 7–10).

The sequences of the pGEM-CLCV1 and pBS-CLCV1 inserts were established (GenBank Accession numbers AJ132344 and AJ132345) and shown to be 1375 and 1376 nucleotides in length, respectively, and closely related to



ACCGAGGCTGGTGTAGTATTACCCAGCCTCGGT

FIG. 5. Genome organisation of DNA 1 (pBS-CLCV1 insert). The position and orientation of the 36.6-kDa Rep protein, encoded on the virion-sense DNA strand, is shown in relation to a consensus TATA box sequence (TATAAA; filled box) and polyadenylation signals (AATAAA; open boxes). The conserved nonanucleotide (TAGTATT↓AC, underlined) containing the putative nick site (↓) for the initiation of virion-sense DNA replication, that is flanked by complementary sequences (arrows), is shown below the genome map. Nucleotide numbering in the text follows geminivirus convention, beginning with the nucleotide immediately downstream of the putative nick site within the nonanucleotide.

each other (95% identity). The orientation of DNA 1 was determined using the strand-specific riboprobes synthesised from pGEM-CLCV1 (Fig. 2). The ssDNA was detected only using the riboprobe synthesised from the T7 promoter, defining the probe sequence as complementary-sense. Comparison with database nucleotide sequences indicated that DNA 1 has between 43 and 53% identity to a genomic component of members of the Nanoviridae family of plant DNA viruses and is most closely related to milk vetch dwarf virus (MVDV). The most highly conserved nucleotide sequences are those encompassing a virion-sense open reading frame (ORF) with the capacity to encode a protein of 36.6 kDa (Fig. 5). This putative protein is referred to as the replication-associated protein (Rep) because it shows strong homology (34–47% identity, 57–64% similarity) to nanovirus Reps (Fig. 6), of which MVDV Rep is the most closely related. There are 57 nucleotide differences between the Rep ORFs of pGEM-CLCV1 and pBS-CLCV1, resulting in 11 amino acid changes (Fig. 6). The DNA 1 Rep contains consensus motifs that are characteristic of proteins involved in rolling circle replication (Koonin and Ilyina, 1992), including a tyrosine residue at position 90 that may be involved in binding to nascent DNA during the initiation of rolling circle replication (Koonin and Ilyina, 1992; Laufs *et al.*, 1995a) and a consensus NTP-binding motif, GGEGKS (amino acids 188–193), that occurs in nanovirus and geminivirus Reps (Gorbalenya *et al.*, 1990; Katul *et al.*, 1997).

Other features of DNA 1 include the sequence TAGTATTAC located within a potential stem-loop structure (nucleotides 1357–1376/1–13; numbering according to the pBS-CLCV1 insert) (Fig. 5). This sequence is identical to that found within stem-loop structures of many nanovirus DNA components (Sano *et al.*, 1998 and references therein), and closely resembles the TAATATTAC sequence that is found within similar structures in geminivirus DNA components. The virion-sense DNA strand is nicked within this sequence during the initiation of rolling circle replication of both nanoviruses and geminiviruses (Laufs *et al.*, 1995b; Stanley 1995; Hafner *et al.*, 1997). Of note, the sequence CCTCGGTTCCCTC (nucleotides 7–19) partially overlaps the stem-loop sequence and is repeated immediately downstream (nucleotides 21–33; the insert of pGEM-CLCV1 contains two mismatches in this downstream sequence). Such a reiterated sequence may participate in replication or the control of Rep expression. A consensus TATA box occurs upstream of the Rep ORF although, interestingly, it is located on the opposite side of the stem-loop sequence. Several consensus transcript polyadenylation signals (AATAAA) occur downstream of the ORF (Fig. 5). However, an (A/T)TGTA motif (nucleotides 996–1000) that is frequently associated with functional polyadenylation signals (Rothnie *et al.*, 1994; Sanfaçon, 1994), including that of the nanovirus coconut foliar decay virus (CFDV) (Merits *et al.*, 1995), is suitably positioned only upstream of the first motif, suggesting that one of the first two adjacent motifs (nucleotides 1020–1025 and 1032–1037) participates in transcript polyadenylation. Also, the proposed noncoding region contains two noticeable A-rich sequences [nucleotides 1117–1156 (73%) and 1195–1226 (81%)] flanking imperfect inverted repeat sequences (nucleotides 1162–1178 and 1180–1194). An increase in the size of the noncoding region between the 3′ terminus of the Rep ORF and the stem-loop sequence largely accounts for the difference in size of DNA 1 and the majority of nanovirus components that are between 1000 and 1100 nucleotides in length.

DNA 1 can replicate autonomously

A partial repeat of CLCuV DNA (pCLCV-Pak1; manuscript in preparation) or a dimer of DNA 1 (pGEM-CLCV1d) were introduced biolistically into tobacco leaf explants together with an expression cassette containing the *uidA* gene. Full-length circular copies of the genomic DNAs are produced from the cloned DNA either by homologous recombination between the repeat sequences or by a replicative mechanism if two copies of the origin of replication are present (Stenger *et al.*, 1991). Leaf explants harvested 24 h after inoculation showed blue spots when stained for *uidA* activity (data not shown), confirming the delivery of the inoculum into viable cells. Southern blot analysis showed that ssDNA and scDNA forms of both CLCuV DNA and DNA 1 are produced *de novo* in the inoculated tissue (Fig. 7), indicating that both DNAs can replicate autonomously.

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                                   •
DNA 1   MPTIQSQWWC FTVFFLSAT. APDLVPFFEN THVSYACWQE EESPTTRRRH  49
        MPTIQSQWWC FTVFFLSAT. APDLVPLFEN THVSYACWQE EESPTTRRRH  49
CFDV    MGSSIRR.WC FTLNYETEEE AANVVRRIES LNLVYAIVGD EVAPSTGQRH  49
FBNYV   MASKR...WC FTLNYKTAVE RESFISLFSR DELNYFVCGD ETAPTTNQKH  47
SCSV    MPTRQSTSWV FTLNF..... EGEIPILPFN ESVQYACWQH ERVG...HDH  42
MVDV    MPTLQGTFWC FTLNF..... SGDAPSLSFN ERVQYACWQH ERVS...HDH  42
BBTV    MARYVVC.WM FTINN..... ..PTTLPVMR DEIKYMVYQV ERGQEG.TRH  41
        *       *  **                        *      *        *

         •                 •
DNA 1   LQGYLQLKGK RS.LAQVKSL FGDLNPHLEK QRARKTDEAR DYCMKEETRV  98
        LHGYLQLKGK RS.LAQVKAL FGDLNPHLEK QRARKTDEAR DYCMKEETRV  98
CFDV    LQGFIHLKTG RR.LQGLKTV LGNDRIHLEP TRG.SDEQNR DYCSKE..RV  95
FBNYV   LQGYVSLKKM IR.LGGLKKK FGY.RAHWEI AKG.DDFQNR DYCTKETL..  92
SCSV    LQGFIQFKSR NTTLRQAKYI FNGLNPHLEI ARD.V.EKAQ LYAMKEDSRV  90
MVDV    LQGYIQMK.K RSTLKMMKEL LPG..AHLEV SKG.TPEEAS DYAMKEETRV  88
BBTV    VQGYVEMK.R RSSLKQMRGF FPG..AHLEK RKG.SQEEAR SYCMKEDTRI  87
          *    *       *            * *              *  **

               • • •
DNA 1   SGPFEFGDYC PAGSHKRRQR ESVIR..... .SPVRMAEEN PSVFRRVKAK 142
        SGPFEFGEYT AAGSHKRRQR ESVIR..... .SPVRMAEEN PSVFRRVKAK 142
CFDV    ..LLEHGVPT RPGVKRPRLA QRFAE..... .EPDELRLED PGGYRRCVVH 137
FBNYV   ..ISEIGAPV KKGSNQRKIM DLYLQ..... .DPEEMQLKD PDTALRCNAK 134
SCSV    AGPWEYGLFI KRGSHKRKLM ERFEE..... .DGEEMKIAD PSLYRRCLSR 134
MVDV    AGPWTYGELL KKGSNKRKLL DRYKE..... .NPEDMELED PAKARRC..R 130
BBTV    EGPFEFGSF. .KLSCNDNLF DVIQDMRETH KRPLEYLYDC PNTFDRSKDT 135
              *                                     *    *

                     •             •
DNA 1   ..IAEEDFQK TAKEIQI.SN LKSWQLRLKT LLERDPDDRT IFWVYGPDGG 189
        ..IAEEDFQK TANEIQI.SN LKSWQSRLKT LLERDPDDRT IFWVYGPDGG 189
CFDV    ..GASVEWTR WAAENPFPFP YHNWQLEVLS AIGEPADDRT ILWICGRDGG 185
FBNYV   ..RLRIEYCS SFAVI....S LRPWQSELHR VLMAEPDDRT IIWVYGSDGG 178
SCSV    ..KMAEE.QR CSSEWNY..D LRPWQEEVMH LLEEEPDYRT IIWVYGPAGN 179
MVDV    ..AKIDK.EK FIAEFKVEDD EQEWKKILEK EIEKIASPRS ILWVYGPQGG 177
BBTV    LYRVQAEMNK TKAMNSWRTS FSAWTSEVEN IMAQ.PCHRR IIWVYGPNGG 184
                                 *               *  * *  *  *

                       •                    •
DNA 1   EGKSTFARDL YRSGSWFYTR GGSADNVSYQ YIGCLGNNIV FDIPRDKKDY 239
        EGKSTFARDL YRSGTWFYTR GGSADNVSYQ YIGQLGNNIV FDIPRDKKDY 239
CFDV    DGKSVFAKYL GLKPDWFYTC GGTRKDVLYQ YIEDPKRNLI LDVPRCNLEY 235
FBNYV   EGKSTFAKEL .IKYGWFYTA GGKTQDILYM YAQDPERNIA FDVPRCSSEM 227
SCSV    EGKSTFARHL SLKDGWGYLP GGKTQDMMHL VTAEPKNNWV FDIPRVSSEY 229
MVDV    EGKTSKAKEL .ITRGWFYTR GGKKDDVAYS YVEDPTRHVV FDIPRDMQEY 226
BBTV    EGKTTYAKHL MKTRNAFYSP GGKSLDICRL Y..NYEDIVI FDIPRCKEDY 232
         **   *  *        *   **                     * **

DNA 1   LQYSLIEMFK DRLIVSNKYE P.LMAPLINC IHVVVMSNFL PDFEKISVDR 288
        LQYSLIEMFK DRLIVSNKYE P.LMAPLINC IHVVVMSNFL PDFEKISVDR 288
CFDV    LNYALLECVK NRAFSSDKYE P.LSYLGFDH VHVLVFANVL PDYLKISRDR 284
FBNYV   MNYQAMEMLK NRVFASTKYR P.VDLCVRKK VHLIVFANVS PDPTKISEDR 276
SCSV    VNYGVIEQVK NRVMVNTKYE PCVMRDDNHP VHVIVFANVL PDLGKLSEDR 279
MVDV    CNYSLIEMLK DRIIISNKYE P.ITNCQVYN IHVIVMANFL PDVTKISEDR 275
BBTV    LNYGLLEEFK NGIIQSGKYE PVLK..IVEY VEVIVMANFL PKEGIFSEDR 280
          *   *  *        **  *              *  *   *     * **

                     •
DNA 1   VHVIPCIPCG VCVKHHSADV MCGEYME 315
        VHVIPCIPCG VCLKHHSADV MCGEYME 315
CFDV    IKLWNI.... .......... ....... 290
FBNYV   IVIINC.... .......... ....... 282
SCSV    IKLIRC.... .......... ....... 285
MVDV    IKIIYC.... .......... ....... 281
BBTV    IKLVSC.... .......... ....... 286
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FIG. 6. Comparison of the DNA 1 Rep protein sequences with those encoded by nanoviruses. Sequences were compared using the program PILEUP. DNA 1 Rep sequences predicted from pGEM-CLCV1 (top line) and pBS-CLCV1 (second line) are compared with those of coconut foliar decay virus (CFDV; Q66005), faba bean necrotic yellows virus (FBNYV; O91254), subterranean clover stunt virus (SCSV; Q87013), milk vetch dwarf virus (MVDV; D1034955), and banana bunchy top virus (BBTV; Q86567). Differences between the two DNA 1 Rep sequences are indicated by black circles. Amino acids in bold are identical to those within one or both of the DNA 1 Reps, and asterisks indicate residues that are conserved throughout. Amino acid numbering is indicated on the right.

DISCUSSION

Cotton leaf curl disease is a major constraint to cotton production in Pakistan. The disease is transmitted by the whitefly *B. tabaci*, but the causal agent of the disease remains to be established. Geminate particles typical of the *Geminiviridae* are the only virus-like particles that

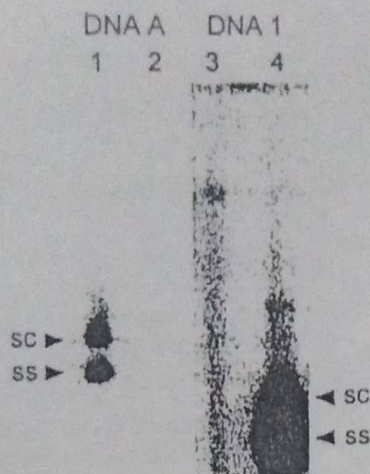

FIG. 7. Replication of CLCuV DNA and DNA 1 in *N. tabacum* leaf explants following biolistic delivery of cloned DNAs. DNA samples were extracted from leaf explants 5 days after inoculation with either CLCuV DNA (lanes 1 and 3) or DNA 1 (lanes 2 and 4). Blots were hydridised to CLCuV DNA (DNA A) and DNA 1 probes. The positions of single-stranded (ss) and supercoiled (sc) DNA forms of each viral DNA are indicated.

have been purified from infected plants (A. Bashir, unpublished data). Several infectious viral DNA clones have been produced from cotton plants exhibiting leaf curl disease and have been shown to have a genomic arrangement similar to begomovirus DNA A although they differ sufficiently in their sequences to be recognised as three distinct virus species (manuscript in preparation). These clones are systemically infectious in *N. benthamiana* and cotton, but produce only mild symptoms and not the thickened dark green veins and enations typical of the disease. Other reports have described similar components, collectively referred to as CLCuV, but none have been associated with infectivity (Harrison *et al.*, 1997; Nadeem *et al.*, 1997; Zhou *et al.*, 1998). Begomoviruses such as tomato leaf curl virus (TLCV) and Mediterranean isolates of tomato yellow leaf curl virus (TYLCV) have single genomic components that produce severe symptoms (Navot *et al.*, 1991; Dry *et al.*, 1993). The DNA A component of a Thailand isolate of TYLCV also can produce a systemic infection, but symptoms are more severe in the presence of a DNA B component (Rochester *et al.*, 1990). However, most begomoviruses are unable to systemically infect plants in the absence of a DNA B component. Repeated attempts to identify such a component for CLCuV using blot hybridisation, PCR amplification, and shotgun cloning strategies have been unsuccessful. During the course of our investigation, we have identified a novel circular viral DNA that co-exists with CLCuV DNA in naturally infected cotton plants growing in the field and that show typical symptoms of leaf curl disease. This novel DNA, that we refer to as DNA 1, is whitefly transmissible and can be immunotrapped using antiserum raised against begomovirus coat protein, suggesting that it is encapsidated in geminate particles. This is consistent with our observation that both CLCuV DNA and DNA 1 co-sediment with geminate particles in sucrose gradients.

The nucleotide sequence of DNA 1, cloned either from scDNA isolated from naturally infected cotton or from DNA that had been PCR-amplified from a cotton extract, has been established. DNA 1 (1375 and 1376 nucleotides in pGEM-CLCV1 and pBS-CLCV1, respectively) is almost exactly half the size of CLCuV DNA (~2750 nucleotides; Zhou *et al.*, 1998) but shows no significant homology to either the CLCuV DNA or its defective forms, which are also approximately half the size of the genomic component (Liu *et al.*, 1998). The defective DNAs are produced by deletion and rearrangement of the genomic DNA, but they all retain intergenic region sequences containing *cis* elements necessary for replication *in trans* by the Rep protein encoded by the full-length component. They are produced *de novo* during chronic infection of laboratory hosts such as tobacco and tomato (Liu *et al.*, 1998), and occur in cotton plants infected in the field (S. Mansoor, unpublished data).

DNA 1 is unrelated to other geminivirus DNAs and to the satellite DNA associated with TLCV (Dry *et al.*, 1997), which is significantly smaller (682 nucleotides) and depends on the geminivirus for its replication. However, it shows a close relationship to some genomic components of members of the *Nanoviridae*, specifically those that encode the Rep protein. It has the capacity to encode a 36.6-kDa Rep protein that is highly homologous to counterparts encoded by nanoviruses. Consistent with this observation, we have demonstrated that DNA 1 can replicate autonomously in tobacco to produce both single- and double-stranded DNA forms. DNA 1 differs from nanovirus components in two main respects. Firstly, it is whitefly-transmitted, whereas all known nanoviruses are transmitted by either aphids or planthoppers (Magee, 1940; Grylls and Butler, 1959; Julia, 1982). Secondly, it is significantly larger than the multiple components of banana bunchy top virus (BBTV), faba bean necrotic yellows virus (FBNYV), subterranean clover stunt virus (SCSV), and MVDV, which are all between 1000 and 1100 nucleotides in length (Boevink *et al.*, 1995; Burns *et al.*, 1995; Katul *et al.*, 1997, 1998; Sano *et al.*, 1998). The exception is the genomic component of CFDV that is only slightly smaller at 1291 nucleotides (Rohde *et al.*, 1990). Interestingly, CFDV is planthopper-transmitted while the other nanoviruses are aphid-transmitted, suggesting a correlation between genome size and encapsidation constraints resulting from adaptation to different insect vectors. This, coupled with the fact that only a single CFDV genomic component has been isolated (Rohde *et al.*, 1990), raises the interesting possibility that it, too, has an associated geminivirus component. Defective DNAs associated with begomoviruses are maintained at approximately half the size of the genomic components (Stanley and Townsend, 1985), presumably to facilitate encapsidation and whitefly transmission. Hence DNA 1 may have adapted its size to allow encapsidation within

geminate particles. If this proves to be the case, our results support the hypothesis that the size of the DNA rather than its sequence is important for encapsidation.

Whitefly-transmitted diseases similar to cotton leaf curl disease were reported for tobacco, hibiscus, and other plants long before they were associated with cotton (Pal and Tandon, 1937; Pruth and Samuel, 1942). This suggests that the nanovirus-like component is indigenous to Pakistan rather than being a recent import, but the disease has only recently had a major impact due to intensive cultivation of susceptible cotton cultivars coupled with the emergence of insecticide-resistant whiteflies resulting from extensive spraying regimes to control cotton leaf curl disease. DNA 1 may represent one component of a multipartite virus that belongs to a novel *Nanoviridae* genus comprising members that are transmitted by the whitefly *B. tabaci*. The fact that it is encapsidated by coat protein that is immunogenically related to begomovirus coat protein may mean that the nanovirus has acquired the gene by recombination with geminivirus DNA during a co-infection event. Recombination between species belonging to the same or different *Geminiviridae* genera has been proposed (Stanley *et al.*, 1986; Hou and Gilbertson, 1996; Zhou *et al.*, 1997) and, more significantly, this has been extended recently to recombination between geminivirus and nanovirus-like components (manuscript in preparation). However, if DNA 1 represents one component of an autonomous nanovirus, the occurrence of both CLCuV DNA and DNA 1 in all symptomatic cotton plants that were sampled must be fortuitous. This implies an extremely high incidence of infection that has been maintained over a period of several years (plants were sampled between 1992 and 1998) and throughout regions of Pakistan separated by as much as 400 km. An alternative hypothesis, and one that we currently favour, is that the geminivirus and nanovirus-like components are mutually dependent and, hence, invariably occur together within infected plants. We suggest that, although both CLCuV DNA and DNA 1 can replicate autonomously, DNA 1 relies on coat protein encoded by CLCuV DNA for encapsidation and whitefly transmission (rather than having acquired the coat protein by a recombination event as suggested above). In this respect, the nanovirus-like component would resemble members of the *Umbraviridae*, which rely on a distinct helper virus for encapsidation and transmission by aphids (Murant *et al.*, 1995). In turn, CLCuV DNA must rely on a gene product(s) encoded by either DNA 1 or another nanovirus-like component. Preliminary infectivity studies using cloned DNA components have demonstrated that DNA 1 moves systemically throughout cotton when co-inoculated with CLCuV DNA but is unable to confer typical leaf curl symptoms, suggesting that one or more additional viral components are associated with the disease. Thus it is likely that the putative nanovirus-like gene product will be an important pathogenic determinant, as has been demonstrated for DNA B-encoded movement proteins (von Arnim and Stanley, 1992; Pascal *et al.*, 1993; Duan *et al.*, 1997) and the gene *C4* product of beet curly top virus and TLCV (Latham *et al.*, 1997; Krake *et al.*, 1998).

The number of components that make up a nanovirus genome remains to be determined, but as many as 10 distinct components have been isolated for FBNYV and MVDV (Katul *et al.*, 1998; Sano *et al.*, 1998). The aetiology of these viruses has been complicated by the isolation of components that may encode functionally related but distinct gene products. Clearly, the next major advance in nanovirus research will be to characterise the genome by infectivity and transmission studies using cloned DNA components. Currently we are attempting to isolate additional nanovirus-like components associated with symptomatic cotton plants for the purpose of defining the causal agent of cotton leaf curl disease.

MATERIALS AND METHODS

Virus isolates, DNA extraction, and isolation of viral scDNA

Cotton (*Gossypium hirsutum* L.) plants showing typical symptoms of cotton leaf disease were originally collected from fields near Multan, Pakistan in 1992 and have been maintained at the John Innes Centre by grafting onto the susceptible cotton variety S-12. The disease was transmitted from these plants to tobacco (*Nicotiana tabacum* var. Samsun) using viruliferous whiteflies and maintained at the John Innes Centre by grafting. Symptomatic cotton plants originating from other locations within Pakistan, and collected in 1997 and 1998, were maintained at NIBGE, Faisalabad.

Virus particles were isolated from infected cotton plants and purified by sucrose gradient centrifugation, and DNA was extracted from purified virus using a modification of the method described by Czosnek *et al.* (1988) (manuscript in preparation). DNA was extracted from viruliferous whiteflies according to the method of Zeidan and Czosnek (1991).

DNA was isolated from cotton plants growing in the field and from cotton and tobacco plants maintained in a glasshouse using a CTAB method (Doyle and Doyle, 1987). ScDNA was purified from 25- to 50-g leaves by cesium chloride density gradient centrifugation as described by Stanley and Townsend (1985). Fractions collected from the gradient were analysed on an agarose gel, and those containing scDNA were identified by Southern blotting using a probe produced by labelling the full-length CLCuV DNA insert of clone pS1 (manuscript in preparation) using the method of Feinberg and Vogelstein (1983).

Construction of viral DNA clones

Attempts were made to clone additional genomic components after digesting the scDNA isolated from infected

cotton with *Bam*HI, *Bgl*II, *Pst*I, or *Eco*RI. DNA fragments were cloned into pBluescript SK- (Stratagene). Purified scDNA was labeled (Feinberg and Vogelstein, 1983) and used as a probe to identify clones containing inserts. Clones containing CLCuV DNA were identified using a probe prepared from pS1. A *Bam*HI clone, pBS-CLCV1, which hybridised to the scDNA probe but not the CLCuV DNA probe, was chosen for further analysis. Abutting primers PB2-F (nucleotides 1361–1376/1–13; numbering according to the pBS-CLCV1 sequence) and PB2-R (complementary to nucleotides 1331–1360 and encompassing the unique *Bam*HI site), designed on the basis of sequences adjacent to the cloning site, were used PCR-amplify full-length DNA 1 from infected cotton, which was cloned into pGEM-T (Promega) to produce pGEM-CLCV1.

Characterisation of DNA 1

The sequences of both strands of the pBS-CLCV1 and pGEM-CLCV1 inserts were derived using an ABI PRISM Big Dye Terminator, Cycle Sequencing, Ready Reaction Kit (Perkin–Elmer) together with standard forward and reverse sequencing primers and DNA 1-specific primers based on established sequence. Sequences were established using an ABI 373 automated sequencer. To ensure that the pBS-CLCV1 insert represented a full-length copy of the viral DNA, a DNA fragment was PCR-amplified using primers PB1-F (nucleotides 1294–1321) and PB1-R (complementary to nucleotides 1272–1299), both containing an additional GG dinucleotide mismatch at their 5′ termini. The sequence across the *Bam*HI site of the amplified fragment was determined. To determine the orientation of the DNA sequence (virion-sense or complementary-sense), viral DNAs were fractionated on agarose gels, Southern blotted, and hybridised to strand-specific riboprobes transcribed from either the T7 or the SP6 promoter of pGEM-CLCV1. Sequences were analysed using version 7 of the program library of the Genetics Computer Group (Devereaux *et al.*, 1984). Nanovirus sequences used for DNA and Rep protein comparisons (database Accession numbers in parentheses) were coconut foliar decay virus (M29963), faba bean necrotic yellows virus (AJ005968), subterranean clover stunt virus (U16735), milk vetch dwarf virus (AB000920), and banana bunchy top virus (S56276).

Analysis of encapsidated DNAs

N. benthamiana plants were infected by graft-inoculation from tobacco. Virus particles were isolated from infected *N. benthamiana* extracts by immunotrapping using polyclonal antiserum raised against ACMV coat protein that had been expressed in *Escherichia coli* (Liu, 1996). Microfuge tubes were coated with 200 μl antiserum for 3 h at 37°C. An extract of an infected leaf, prepared according to the method of Harrison *et al.* (1997), was incubated for 3 h at 37°C in treated and untreated (control) tubes. Tubes were rinsed three times in PBS, and bound material was eluted by incubation in 200 μl 10 mM Tris–HCl (pH 7.5), 1 mM EDTA containing 5 mg/ml proteinase K and 1% SDS for 0.5 h at 37°C. The eluent was phenol extracted and nucleic acids were precipitated with ethanol. Nucleic acids were analysed by Southern blotting using DNA 1 strand-specific riboprobes transcribed from either the T7 or the SP6 promoter of pGEM-CLCV1.

Detection of viral DNAs in plant extracts and insects

Samples (10 μg) of DNA isolated from cotton and tobacco plants were fractionated by agarose gel electrophoresis in TNE buffer [40 mM Tris acetate (pH 7.5), 20 mM Na acetate, 2 mM EDTA] and transferred to Hybond-N membranes (Amersham Life Science). Duplicate blots were probed using radiolabelled inserts of either pS1 or pBS-CLCV1. Full-length DNA 1 fragments were PCR-amplified from extracts of plants and viruliferous insects using partially overlapping primers DNA1-F (TgGGGATCCTAGGATATAAATAACACGTC, nucleotides 1328–1356) and DNA1-R (CTAGGATCCgGACAAATTACAAGCGTACC, complementary to nucleotides 1311–1339). *Bam*HI sites in these primers are underlined and single base mismatches are shown in lower case.

Investigation of viral DNA replication

Clone pGEM-CLCV1 was linearised using *Bam*HI, and a full-length copy of DNA 1, amplified using primers DNA1-F and DNA1-R, was inserted as a *Bam*HI fragment to produce a tandem repeat in pGEM-CLCV1d. To investigate replication of the cloned DNA, pGEM-CLCV1d and a clone containing a partial repeat of CLCuV DNA (pCLCV-Pak1; manuscript in preparation) were introduced into tobacco leaf explants using a helium-driven gun (PDS 1000, BioRad) with a 1100 psi rupture disc, essentially as described by Kikkert (1993). The expression cassette pJIT166 (Guerineau *et al.*, 1992), containing the *uidA* gene under the control of 35S promoter, was included with the viral DNA clones to monitor the biolistic delivery of the DNA. Leaf explants were incubated on MS medium in a growth chamber at 28°C with 16-h day length, and were harvested either after 24 h to assay *in situ* for GUS expression (Jefferson, 1987) or after 5 days to assay for viral DNA replication. DNA was extracted from leaf explants (Doyle and Doyle, 1987), and viral DNAs were analysed by Southern blotting using probes corresponding to full-length CLCuV DNA and DNA 1.

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