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Delimitation of essential genes of cassava latent virus DNA 2

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ABSTRACT

Insertion and deletion mutagenesis of both extended open reading frames (ORFs) of cassava latent virus DNA 2 destroys infectivity. Infectivity is restored by coinoculating constructs that contain single mutations within different ORFs. Although frequent intermolecular recombination produces dominant parental-type virus, mutants can be retained within the virus population indicating that they are competent for replication and suggesting that rescue can occur by complementation of trans acting gene products. By cloning specific fragments into DNA 1 coat protein deletion vectors we have delimited the DNA 2 coding regions and provide substantive evidence that both are essential for virus infection. Although a DNA 2 component is unique to whitefly-transmitted geminiviruses, the results demonstrate that neither coding region is involved solely in insect transmission. The requirement for a bipartite genome for whitefly-transmitted geminiviruses is discussed.

INTRODUCTION

Cassava latent virus (CLV, also known as African cassava mosaic virus) is typical of the geminiviruses transmitted by the whitefly Bemisia tabaci, having a genome comprising two circular single-stranded (ss) DNAs (reviewed by Stanley (1)). Cloned full-length copies of each genomic component are infectious only when both are mechanically inoculated to host plants, demonstrating the true bipartite nature of the genome (2). DNA 1 encodes the coat protein (3,4) in addition to all functions required for DNA replication, as demonstrated by its capacity for self replication in dividing Nicotiana plumbaginifolia protoplasts (5). As a consequence, DNA 2 is implicated in systemic spread of the virus thoughout the host plant.

A bipartite genome appears to be a unique feature of the whitefly-transmitted geminiviruses, having been demonstrated for

tomato golden mosaic virus (TGMV) (6) and bean golden mosaic virus (BGMV) (7) in addition to CLV. All leafhopper-transmitted members so far investigated, namely maize streak virus (8-10), wheat dwarf virus (11) and digitaria streak virus (12,13) infecting monocotyledonous plants and beet curly top virus (BCTV) (14) infecting dicotyledonous plants, have single component genomes showing varying degrees of homology with CLV DNA 1 (14, 15). On the strength of this, it is suggested that DNA 2 might encode a protein responsible for whitefly transmission. Such a gene product, if concerned solely with insect transmission, can be considered to be non-essential under conditions of mechanical transmission of cloned DNA components.

Nucleotide sequence data (3), transcript mapping (4) and comparison with closely related geminiviruses (1) has suggested that DNA 2 contains two non-overlapping genes with potential coding capacities of 29.3Kd and 33.7Kd, located on complementary DNA strands. In this paper we define the DNA 2 coding regions by cloning fragments into DNA 1 coat protein deletion vectors and investigate whether or not they are essential by screening for the capacity of insertion and deletion mutants to spread systemically and elicit disease symptoms when coinoculated with infectious cloned DNA 1.

MATERIALS AND METHODS

Construction of DNA 2 mutants

Full-length infectious copies of each CLV (Kenyan isolate) genomic component in bacteriophage M13 vectors (DNA 1, pJS092; DNA 2, pJS094) have been described (2). Clone pJS094 was linearised using either XbaI or NcoI which have unique sites at nucleotides 608 and 1918 respectively in DNA 2 (indicated in Figure 1) and no sites within the cloning vector. Protruding 5' termini were filled-in and religated according to Maniatis et al. (16). Alternatively, sequences were deleted using nuclease Bal31 (Boehringer Mannheim) using the supplier's protocol and termini filled-in and fragments religated as described (16). The integrity of the filling-in reaction and the extent of the deletions was investigated by nucleotide sequence analysis using the

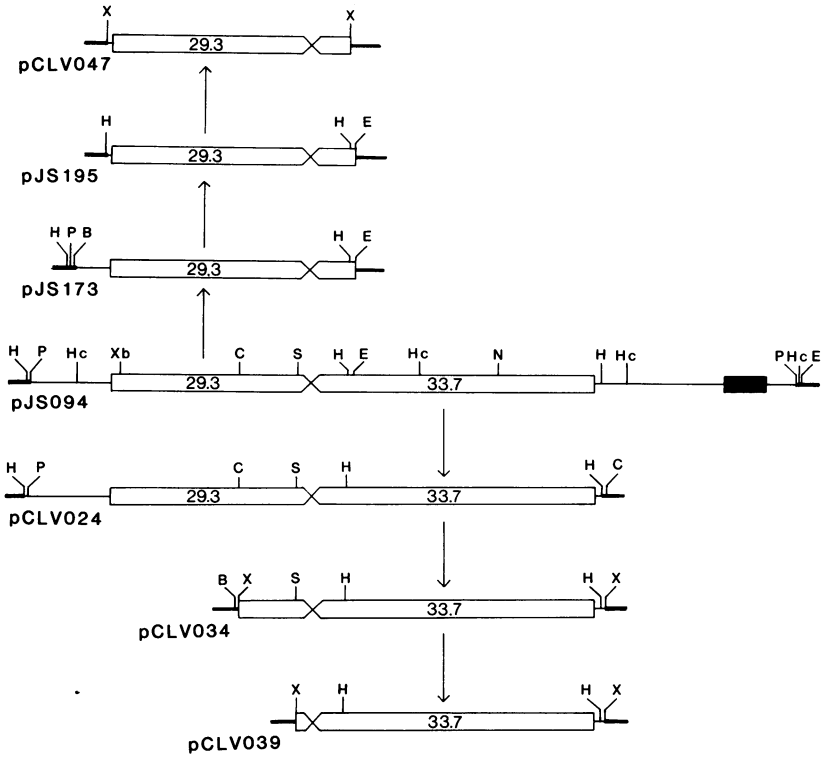


FIGURE 1. Scheme detailed in the text for the isolation of discrete fragments containing the DNA 2 coding regions. The position and orientation of the ORFs (coding capacities in kilodaltons) within pJS094 relative to the common region (black box) and selected restriction endonuclease sites are indicated. The orientation of the vector sequences for pJS172, pJS195 and pCLV024 are reversed. B, BamHI; C, ClaI; E, EcoRI; H, HindIII, Hc, HincII; N, NcoI; P, PstI; S, SphI; X, XhoI; Xb, XbaI.

dideoxy-termination procedure of Sanger et al. (17,18) after subcloning appropriate fragments into bacteriophage M13 vectors.

Isolation of DNA 2 gene sequences

The 29.3Kd and 33.7Kd open reading frames (ORFs) of DNA 2 were isolated from pJS094 as shown in Figure 1. The HincII(416) -EcoRI(1528) fragment (numbering throughout the text relates to that of the full-length components (3)) containing the 29.3Kd ORF was cloned into SmaI-EcoRI double-digested M13mp18 to produce pJS173. Sequences from the BamHI site in the polylinker of

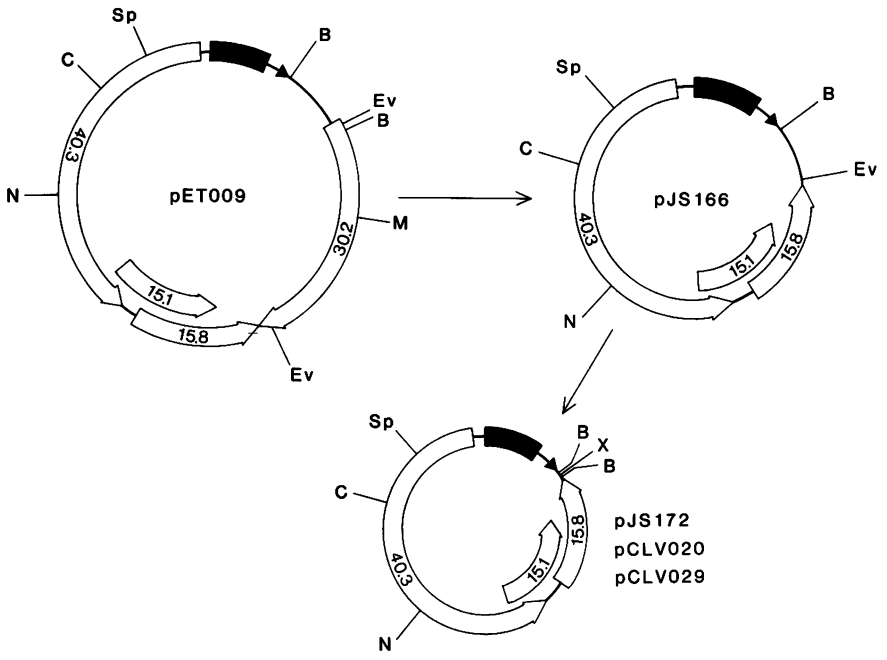
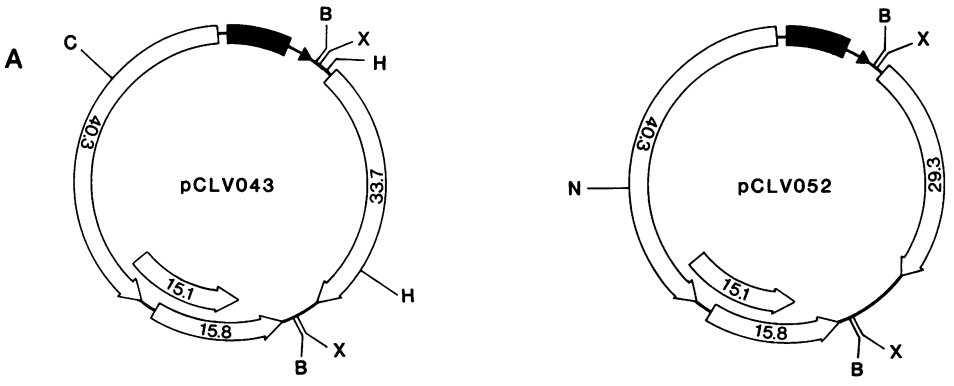


FIGURE 2. Construction of DNA 1 coat protein deletion mutants. The position and orientation of DNA 1 ORFs (coding capacities in kilodaltons) relative to the common region (black box), putative coat protein promoter sequence (black arrow) and selected restriction endonuclease sites are indicated. Only DNA 1 sequences are shown; cloning sites and vectors are described in the text. B, BamHI; C, ClaI; Ev, EcoRV; M, MluI, N, NcoI; Sp, SphI; X, XhoI.

pJS173 were deleted unidirectionally towards the start of the 29.3Kd ORF by ExoIII digestion (19) after double-digestion with BamHI and PstI. Sequence analysis showed that the leader sequence was deleted up to, but not including, nucleotide 581 in pJS195. In addition, nucleotides within the M13mp18 polylinker up to the HindIII site were deleted during this treatment, possibly as a result of overdigestion with nuclease S1 prior to religation. The HindIII fragment encompassing the ORF was excised from pJS195, the protruding 5' termini filled-in and the fragment blunt end-ligated to XhoI linkers (CCTGCAGG) before cloning into XhoI cut bluescript M13+ (Stratagene) to give pCLV047.

Clone pJS094 was partially digested with HindIII (0.1µg/µl DNA incubated with 0.05u/µl HindIII for 2h at 37°C in the presence of 40µg/ml ethidium bromide) and the HindIII (M13 polylink-



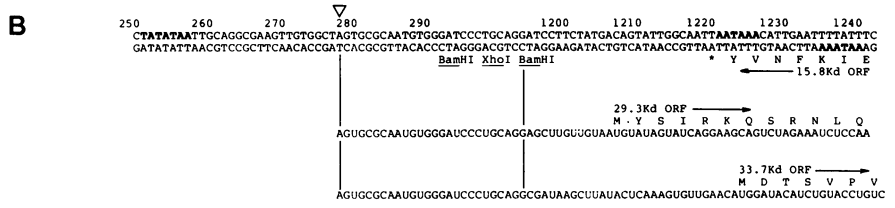


FIGURE 3. A. Insertion of the 29.3Kd and 33.7Kd ORFs of DNA 2 in the (+) orientation into DNA 1 coat protein deletion vectors, downstream of the putative coat protein promoter (black arrow). Only CLV sequences are shown; cloning sites and vectors are described in Figure 2 and the text. B, BamHI; C, ClaI; H, HindIII, N, NcoI; X, XhoI. B. Nucleotide sequence in the region of the deletion across the coat protein gene of DNA 1 shown as dsDNA. The positions of consensus TATA box (TATAT/AAT/A (27)) and polyadenylation sequences (AATAAA (35)) are indicated (bold letters) relative to the carboxy-terminus of the 15.8Kd ORF. The transcript start at nucleotide 278 (26) is arrowed. Below are given the 5' termini of the expected transcripts encoding the 29.3Kd and 33.7Kd gene products of DNA 2 when under the control of the coat protein promoter in pCLV052 and pCLV043 respectively.

er)-HindIII(2281) fragment was cloned into pAT153 in the (-) orientation with respect to the sense of the 33.7Kd ORF to produce pCLV024. The ClaI fragment encompassing the 33.7Kd ORF was excised, the protruding 5' termini filled-in and the fragment blunt end-ligated to XhoI linkers before cloning into XhoI cut bluescript M13+ to produce pCLV034. The BamHI(polylinker)-SphI (2581) fragment located downstream of the 33.7Kd ORF was excised and XhoI linkers attached to the residual construct after remo-

val of the protruding 3' termini using nuclease S1 to give pCLV039.

Construction of DNA 1 coat protein deletion mutants

The construction of pET009 (Figure 2) from pJS092 has been described (20). The EcoRV(1192)-2779/1-EcoRV(464) fragment was excised, circularised and cloned at the unique SphI(2581) site into a derivative of M13mp18 in which the BamHI-SmaI fragment of the polylinker has been deleted, to produce pJS166. The BamHI (291)-EcoRV(464) fragment of pSJ166 was excised, protruding 5' termini filled-in and the construct recircularised in the presence of XhoI linkers to produce pJS172. The XhoI site is flanked by reconstructed BamHI sites (see Figure 3b). The pJS172 insert was subcloned at its unique ClaI(2403) site into pAT153 or NcoI (2124) site into pNeo (Pharmacia) to give pCLV020 and pCLV029 respectively.

Insertion of DNA 2 genes into DNA 1 deletion mutants

The 29.3Kd ORF was excised from pCLV047 as a XhoI fragment and cloned into XhoI cut pCLV029 to give pCLV049 ((-) orientation) and pCLV052 ((+) orientation, Figure 3a). The 33.7Kd ORF was excised from pCLV039 as a XhoI fragment and cloned into XhoI cut pCLV020 to give pCLV043 ((+) orientation, Figure 3a) and pCLV044 ((-) orientation).

Infectivity studies

DNA inserts were excised from the cloning vector using MluI (pJS092), PstI (pJS094 and mutants thereof), ClaI (pCLV043 and pCLV044) or NcoI (pCLV049 and pCLV052) and inoculated onto two week-old Nicotiana benthamiana seedlings, maintained at 25°C in an insect-free greenhouse, by rubbing inocula onto celite-dusted leaves. No attempt was made to purify the linearised insert DNA from the cloning vector. Routinely, 1µg of each DNA insert in a total of 10µl sterile water was distributed between the two youngest leaves of each test plant. Virus was passaged from sap expressed from systemically infected N. benthamiana.

Characterisation of progeny viral DNA

Total nucleic acid was extracted from systemically infected leaf material and dot-blotted as described (2). Aliquots containing 200ng total nucleic acid were digested with various restriction endonucleases and subsequently treated with 5 units

TABLE 1. Description of DNA 2 mutants

Clone	Coordinates of deleted nucleotides	net change (bp)
pET003	1865-1951	-86
pET004	1837-1985	-148
pET005	1916-1921	-5
pET006	1903-1954	-51
pET018	infill (1919)	+4
pET007	570-662	-92
pET014	445-703	-258
pET015	549-633	-84
pET016	357-575	-218
pET031	infill (609)	+4

nuclease S1 at 4°C for 30 minutes in 150mM Na acetate, 1.5M NaCl (pH 4.6) to remove viral ssDNA. After agarose gel electrophoresis, DNA was depurinated (21) prior to blotting onto nitrocellulose (22). Blots were probed with a gel-purified radiolabelled (23) fragment (PstI(245)-EcoRV(2550)) specific to DNA 2.

RESULTS

In all infectivity experiments, mutants of DNA 2 were inoculated in the presence of infectious DNA 1 from pJS092. XbaI and NcoI cut at single sites in pJS094 within the 29.3Kd and 33.7Kd ORFs respectively (indicated in Figure 1). All nuclease Bal31 deletions created from either site, described in Table 1, rendered DNA 2 non-infectious as judged by the lack of symptom production in addition to the inability to detect viral DNA in newly expanding leaves by dot-blotting (data not shown). Previous observations concerning the infectivity of chimeric constructs containing CLV genomic DNAs have suggested that size might play an important role in determining whether or not the genomic DNA is infectious (24). While this might account for the non-infectious nature of the majority of deletion mutants (between 84 to 258bp removed), the fact that only 5bp have been removed from clone pET005 suggests a requirement at least for an intact 33.7Kd ORF. Protruding termini generated by XbaI and NcoI digestion were filled-in and religated in order to disrupt both the 29.3Kd and 33.7Kd ORFs by creating 4bp insertions at these positions (clones pET031 and pET018 respectively). Again, in

TABLE 2. Infectivity of DNA 2 mutant combinations

clone combination	infectivity* infected/inoculated I	II	III
pET003 + pET015	19/20	-	-
pET018 + pET007	-	1/10	-
pET018 + pET014	-	3/10	-
pET018 + pET015	-	4/10	-
pET018 + pET016	-	2/10	-
pET018 + pET031	-	4/10	7/10
pET003 + pET031	-	4/10	-
pET004 + pET031	-	4/10	-
pET005 + pET031	-	6/10	-
pET006 + pET031	-	2/10	-
pJS094	20/20	8/10	7/10

*In the presence of DNA 1 derived from pJS092

each case this had the effect of destroying infectivity. The ability of mutants containing single mutations within different ORFs to rescue one another was demonstrated for the combinations described in Table 2. For all of these mutant combinations, symptoms typical of CLV infection resulted that were noticeably delayed by a period of between 1 to 5 days relative to control plants inoculated with pJS094 insert.

To investigate the possibility that the deleted nucleotide sequences per se are involved in essential aspects of viral replication in addition to encoding gene products, progeny viral double-stranded (ds) DNA was examined by Southern blotting after the removal of viral ssDNA using nuclease S1. Mutant combinations of pET031 (4bp fill-in) with either pET018 (4bp fill-in) or pET004 (148bp deletion) were selected for this purpose. When compared with that of the parental clone pJS094, the onset of symptom expression is typically delayed by 1 to 2 days for the combination pET018/pET031 and 4 to 5 days for pET004/pET031. Similar levels of supercoiled (sc) DNA (Figure 4a) were produced in leaves infected with the parental clone pJS094 (lane 2) and the combination pET018/pET031 (lane 3). Digestion with either NcoI (lane 4) or XbaI (lane 6) resulted in the linearisation of the pJS094 progeny scDNA. However, while XbaI linearised most of the pET018/pET031 progeny scDNA (lane 7), the greater part of the scDNA remained undigested using NcoI (lane 5) suggesting that the mutations had been retained within the virus popula-

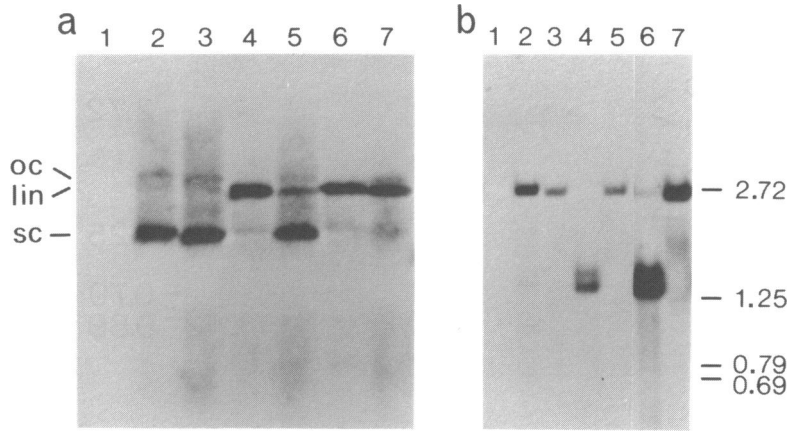


FIGURE 4. Southern blot analysis of total nucleic acid extracted from a healthy plant (lane 1) and plants inoculated with pJS092 together with either pJS094 (lanes 2, 4 and 6) or a mixture of pET018 and pET031 (lanes 3, 5 and 7). (a) Extracts were either untreated (lanes 1 to 3) or digested with NcoI (lanes 4 and 5) or XbaI (lanes 6 and 7). (b) Extracts were either untreated (lane 1) or digested with ClaI (lanes 2 and 3) or a mixture of NcoI and XbaI (lanes 4 and 5). Lanes 6 and 7 are a longer exposure of lanes 4 and 5 respectively. The blot was hybridised with a probe specific to DNA 2. The position of open circular (oc), linear (lin) and supercoiled (sc) DNA are indicated here and in subsequent figures. Size markers (in Kb) were derived from digestion of pJS094 with PstI or a mixture of PstI and HindIII.

tion. This suggestion was substantiated by the results of XbaI and NcoI double digestion (Figure 4b) which produced the expected fragments of 1415 and 1309bps from the pJS094 progeny scDNA (lane 4), absent for the combination pET018/pET031 (lane 5) which instead gave rise to a single fragment that comigrated with both pJS094 and pET018/pET031 progeny scDNA that had been linearised using ClaI (lanes 2 and 3). In this instance, the subgenomic fragments remained undetected even after extended autoradiography (lane 7). The reason for the low yield of mutant pET031 compared with pET018 shown in Figure 4a is unclear since the inocula containing the complementing mutants were produced using the same batch of restriction endonuclease and the mutants were coinoculated onto test plants in equimolar amounts. It is unlikely that this imbalance reflects the impaired replication

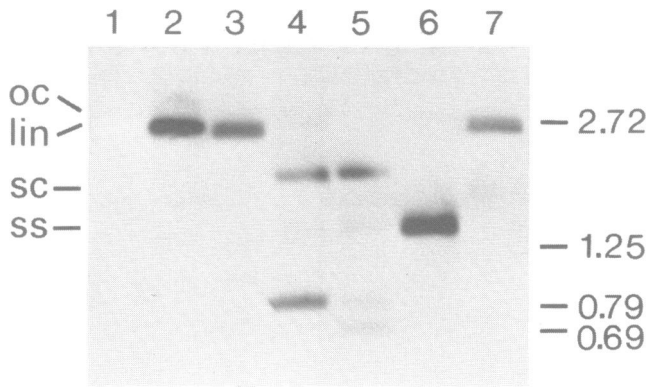


FIGURE 5. Southern blot analysis of total nucleic acid extracted from a healthy plant (lane 1) and plants inoculated with pJS092 together with either pJS094 (lanes 2, 4 and 6) or a mixture of pET004 and pET031 (lanes 3, 5 and 7). Extracts were digested with ClaI (lanes 2 and 3), HindIII (lanes 4 and 5) or a mixture of NcoI and XbaI (lanes 6 and 7). The blot was hybridised with a probe specific to DNA 2. Size markers (in Kb) are identical to those in Figure 2.

of pET031 as this mutant appears in similar amounts to that of pET004 (see Figure 5, lane 5) which together replicate to the same level as that of the parental clone pJS094 (lane 4).

Linearisation of the pET004/pET031 progeny scDNA using ClaI gave two products that could just be resolved by gel electrophoresis (Figure 5, lane 3; more evident on the original autoradiograph; see also lane 7), one comigrating with linearised pJS094 progeny scDNA (lane 2), the other with a slightly higher mobility. Digestion with HindIII produced the expected fragments of 1937 and 787bps from the pJS094 progeny (lane 4) and an additional fragment from the pET004/pET031 progeny (lane 5) corresponding to the 148bp deletion within the 787bp fragment HindIII(1494)-HindIII(2281) from pET004. The faint bands in lanes 4 and 5, corresponding to fragments of approximately 1400bp, probably contain residual ssDNA that has escaped digestion with nuclease S1. Double-digestion of the pJS094 progeny scDNA with NcoI and XbaI (lane 6) again gave the subgenomic fragments seen in Figure 4b, although in this instance the fragments have comigrated. However, double-digestion of the pET004/pET031 progeny

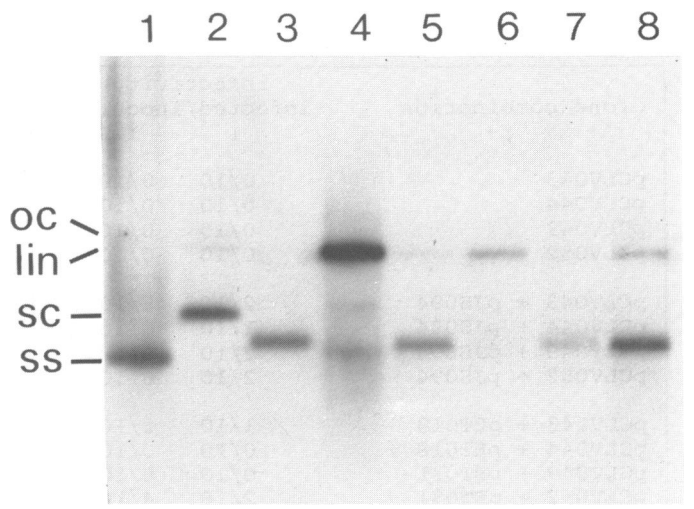


FIGURE 6. Southern blot analysis of total nucleic acid extracted from plants inoculated with pJS092 together with either pJS094 (lane 3) or a mixture of pET018 and pET031 (lane 4). Extracts from individual plants inoculated using sap expressed from leaves infected using the clone combination pET018/pET031 are shown in lane 5 to 8. All extracts had been double-digested with a mixture of NcoI and XbaI. Lanes 1 and 2 contain markers of virion ssDNA and scDNA respectively. The blot was hybridised with a probe specific to DNA 2.

scDNA (lane 7) produced two similar-sized fragments that comigrated with those linearised with ClaI in lane 3. The results demonstrate that the extensive deletion within the 33.7Kd ORF in pET004 is maintained within the population.

Progeny virus from the clone combination pET018/pET031, apparently devoid of recombinants (Figure 4b), was passaged to N. benthamiana by mechanical inoculation of expressed sap. ScDNA was extracted from four randomly chosen plants and double-digested using NcoI and XbaI (Figure 6). In each case (lanes 5 to 8) fragments that were representative of the progeny of both pJS094 (lane 3) and the combination pET018/pET031 (lane 4) were present in varying ratios. The subgenomic fragments were again not resolved on this blot. The two minor bands in lane 4, also observed in Figure 4b (lane 7) probably correspond to undigested scDNA and ssDNA.

TABLE 3. Infectivity of DNA 1 vectors containing DNA 2 coding regions

clone combination	infectivity infected/inoculated I	II
pCLV043	0/10	0/10
pCLV044	0/10	0/10
pCLV049	0/10	0/10
pCLV052	0/10	0/10
pCLV043 + pJS094	2/10	5/10
pCLV044 + pJS094	1/10	0/10
pCLV049 + pJS094	2/10	1/10
pCLV052 + pJS094	2/10	6/10
pCLV043 + pET018	1/10	1/10
pCLV044 + pET018	0/10	0/10
pCLV049 + pET031	0/10	0/10
pCLV052 + pET031	2/10	4/10
pCLV043 + pCLV049	0/10	0/10
pCLV044 + pCLV049	0/10	0/10
pCLV043 + pCLV052	10/10	10/10
pCLV044 + pCLV052	0/10	0/10
pJS092 + pJS094	5/10	9/10

Repeated attempts to infect N. benthamiana using the coat protein deletion mutants pJS172, pCLV020 and pCLV029 in the presence of DNA 2 from pJS094 have been unsuccessful. However, replacement of the deleted sequences with DNA 2 fragments containing either the 29.3Kd ORF (pCLV049 and pCLV052) or 33.7Kd ORF (pCLV043 and pCLV044) in either orientation serves to restore infectivity when coinoculated with DNA 2 (Table 3). DNA 2 mutants pET018 and pET031 could be rescued only when the appropriate DNA 2 gene was inserted into the DNA 1 deletion mutant in the correct orientation for expression from the coat protein promoter; thus pET018 (33.7Kd mutant) could be rescued by pCLV043 but not pCLV044 and pET031 (29.3Kd mutant) could be rescued by pCLV052 but not pCLV049. Individually, none of the clones pCLV043, pCLV044, pCLV049 or pCLV052 is infectious. Similarly, clone combinations pCLV043/pCLV049, pCLV044/pCLV049 and pCLV044/pCLV052, which contain one or both of the DNA 2 genes in the incorrect orientation for expression, are non-infectious. However, when pCLV043 and pCLV052 are coinoculated

onto N. benthamiana, they are at least as infectious as the parental clone combination pJS092/pJS094 and exhibit the same severity of symptoms.

DISCUSSION

We have demonstrated that disruption of either the 29.3Kd or 33.7Kd ORF of CLV DNA 2 renders the genomic component non-infectious. A similar conclusion has recently been reached concerning the equivalent ORFs of TGMV (25). Infectivity can be restored by supplying trans acting functions from complementing mutants. Although rescue by recombination of non-viable DNA 1 and 2 constructs containing large inserts has been described (24), on two occasions described here, when plants were inoculated with the mutant clone combinations pET018/pET031 (Figure 4, lanes 5 and 7) and pET004/pET031 (Figure 5, lane 7), recombinants remained undetected. We cannot of course eliminate the possibility that an extremely low level of recombination has occurred in these instances. However, if this were an essential step for the restoration of infectivity, it would occur early in the infection process and recombinants might be expected to become dominant, and hence readily detectable within systemically infected plant material, simply on the basis of only having to replicate a single parental-type recombinant compared with two complementing mutants. The shorter period required for the onset of symptom expression in plants inoculated using the parental clone pJS094 relative to the various mutant combinations might reflect this advantage. We therefore consider it unlikely that intermolecular recombination, to reconstruct parental-type viral DNA, is prerequisite for the maintenance of the mutants within the population. Similarly, complementation of TGMV DNA B (equivalent to CLV DNA 2) mutants has been observed although it remained unclear whether recombination had occurred in this instance (25). The results presented in Figure 6 demonstrate that during a single passage of the progeny of the mutant clone combination pET018/pET031, recombination frequently occurs and, as anticipated, the recombinants rapidly become dominant (lanes 4, 7 and 8). It is possible that the mutations affect the encapsidation of one or both mutants and as a conse-

quence, the mutant DNA is less well protected from degradation within the expressed sap inoculum, serving to select the packaged recombinants.

The fact that mutations can persist within the population demonstrates that sequence changes at the NcoI or XbaI sites, and in particular between nucleotides 1837-1985 in the 33.7Kd ORF (mutant pET004), do not affect DNA replication but instead disrupt essential genes. We have initiated an investigation of these genes by cloning specific DNA 2 fragments containing the extended ORFs predicted from the nucleotide sequence (3) into transcription fusion vectors constructed from a coat protein deletion mutant of DNA 1 (Figure 2). The start of the transcript encompassing the coat protein has been mapped to the vicinity of nucleotide 278 (26) which is located 27 nucleotides downstream of a consensus TATA box (27). We have deleted nucleotides 296-1194 inclusive from cloned DNA 1 and replaced them with a XhoI linker, resulting in a net deletion of 891bp. The XhoI site is flanked by reconstructed BamHI sites, shown in Figure 3b. The DNA was initially cloned at the SphI(2581) site into a modified version of M13mp18 (with the BamHI site deleted from the polylinker sequence) to allow fragment insertion at either the BamHI or XhoI site and transcription as an RNA fusion product from the coat protein promoter. To allow excision of intact chimeric DNA containing an SphI site, the CLV DNA insert from pJS172 was subcloned at the ClaI(2403) site into pAT153 (pCLV020) and at the NcoI(2124) site into pNeo (pCLV029). This arrangement offers greater flexibility over that of the CLV DNA 1-based translation fusion vector recently described for the expression of a bacterial gene in plants (20) which is reliant not only on fusing the input gene in-frame to the amino-terminus of the coat protein but also on retention of biological activity of the gene product as a fusion protein. The 29.3Kd ORF as a 931bp fragment from pCLV047, containing both ClaI and SphI sites, was by necessity cloned into pCLV029 (pCLV049, (-) orientation; pCLV052, (+) orientation) giving a net increase in the size of DNA 1 of 40bp. The 33.7Kd ORF as a 989bp fragment from pCLV039, containing an NcoI site, was cloned into pCLV020 (pCLV043, (+) orientation; pCLV044, (-) orientation) giving a

net increase of 98bp. Although a 76bp deletion within the CLV coat protein gene is tolerated (24), larger deletions are known to destroy infectivity (20). Clones pJS172, pCLV020 and pCLV029 are likewise non-infectious when each is coinoculated with DNA 2 derived from pJS094. We have demonstrated that when the DNA 2 fragments are inserted in either orientation within the coat protein deletion constructs, infectivity is restored in the presence of DNA 2. However, only when the 29.3Kd and 33.7Kd ORFs are cloned in the orientation compatible with expression from the coat protein promoter (pCLV043 and pCLV052; Figure 3a) can the appropriate DNA 2 mutant (pET018 or pET031) be rescued. Furthermore, inoculated individually, all four gene replacement constructs are non-infectious and in pairs, only the combination pCLV043/pCLV052 elicits an infection. The results are indicative of the presence of two essential genes on DNA 2, each delimited by the fragments cloned within the DNA 1-based vectors. It should be pointed out that, in addition to containing intact DNA 2 ORFs, the inserts from pCLV039 and pCLV047 also include the carboxy-termini of the complementary ORFs (Figure 1), together with their proposed downstream signals for transcript processing and polyadenylation. Therefore, when inserted in the wrong orientation for expression of the intact ORF from the coat protein promoter (pCLV044 and pCLV049), the constructs should theoretically produce truncated transcripts encompassing the carboxy-termini of the complementary ORFs and not 'antisense' transcripts of the intact ORFs which might otherwise interfere with gene expression. We have not yet screened infected plants for the presence of such truncated transcripts.

The predicted leader sequences of the fusion transcripts that encode the DNA 2 gene products, based on a transcript start at nucleotide 278 (26) are shown in Figure 3b. An AUG at position 9, which is normally in-frame with a 13.1Kd ORF that overlaps the coat protein gene in the intact genomic component (3), is located upstream of the start of both the 29.3Kd and 33.7Kd ORFs. A corresponding ORF is absent in other whitefly-transmitted geminiviruses and the results presented here indicate that it performs no essential function for CLV proliferation within plants. In both constructs shown in Figure 3b, the 5'

promixal AUGs are in-frame with nonsense triplets which are themselves located upstream of the start of both the 29.3Kd and 33.7Kd ORFs. As a consequence, it is considered likely that the 5' proximal in-frame AUGs of both the 29.3Kd and 33.7Kd ORFs are those recognised on the fusion transcripts and correspond to the authentic initiation codons of the gene products when located within DNA 2.

It has been proposed that the distribution of genes on complementary DNA strands reflects the temporal control of their expression (1). For example, the virus-coded replicative functions required early in infection and the coat protein gene required late in infection are located on complementary strands of DNA 1 (4,5). The fact that both DNA 2 gene products can apparently be expressed from the presumed 'late' coat protein promoter without affecting the time course of infection or symptom severity either implies that both DNA 2 products are required at a late stage in the infection cycle or that this level of control if it exists, is a refinement and not a necessity, at least for DNA 2.

While the results demonstrate that intact 29.3Kd and 33.7Kd ORFs of DNA 2 are essential for CLV proliferation, the precise function and mode of action of the gene products remains obscure. The production of geminate particles in plants transgenic for multiple copies of DNA A (equivalent to CLV DNA 1) of the closely related geminivirus TGMV (28) has demonstrated that DNA 2 gene products are not involved in the maintenance of particle morphology. Furthermore, it has been shown here and elsewhere (20,24) that encapsidation of the genomic DNA is not necessary for spread of CLV, implying that viral gene products in addition to the coat protein, which might be required for early events on infection of a cell, are not necessarily associated with intact capsids. The transported form of plant viruses remains open to conjecture (reviewed in 29); it is conceivable that one or both DNA 2 gene products interacts with the genomic DNA and in doing so sequesters it specifically for the purpose of transportation. Because there have been no reports of structures corresponding to protein shells devoid of DNA, it has been suggested that interaction between the coat protein and the genomic DNA is

important for particle stability (30). It is tempting to suggest that the amino acid sequence homologies detected between the coat protein and the 29.3Kd ORF (31) might indicate an affinity of the latter for the DNA components.

The results presented here indicate that neither DNA 2 coding region contributes uniquely to insect transmission other than to aid virus proliferation or spread within the host thereby allowing acquisition by the whitefly vector. On the basis of the comparison of mechanical transmission data of CLV and BCTV, it has been proposed that DNA 2 is responsible for cell-to-cell spread from parenchyma tissue into the phloem (15) and possibly for movement in the opposite direction after long distance spread within the vascular system, back into the parenchyma tissue in which CLV is sometimes found (32). Why then does CLV require a genomic component responsible for virus spread when a leafhopper-transmitted geminivirus such as BCTV, which shows a high degree of homology to CLV DNA 1 in addition to sharing common hosts (14), does not? In the field the viruses are transmitted by B. tabaci (CLV) or Circulifer tenellus (BCTV), both of which are phloem feeders. During feeding, C. tenellus lays down a pronounced sheath around the stylets which was considered by Bennett (33) to effectively seal off all contents of cells external to the phloem during stylet probing and withdrawal. Consequently, BCTV acquisition and transmission are possibly completely phloem-limited phenomena and a distinct barrier exists to movement of the virus between the phloem and the parenchyma tissue. In contrast, B. tabaci only rarely produces a stylet sheath, and while stylet penetration is predominantly intercellular, appreciable parenchyma cell damage has been observed (34). While this suggests that localised cell-to-cell spread, and hence DNA 2, might play an important role in CLV proliferation in the field it is difficult to appreciate the need for such a function considering the apparent efficiency with which B. tabaci probes the phloem tissue.

The demonstration that the DNA 2 mutants can be maintained within the virus population suggests that complementation of non-viable gene replacement constructs could provide an important tool for the investigation of the control of gene

expression in this genomic component. Owing to the frequency of recombination, however, it would be essential to incorporate a suitable selection mechanism into the system to ensure construct stability. Current work centres on the immunological detection of the DNA 2 gene products; in view of the relative abundance of the coat protein transcript compared with those specific to DNA 2 (4) it is possible that the gene replacement constructs described here will facilitate this investigation. Refinements of the DNA 1-based coat protein replacement vectors described here will undoubtedly prove useful for the investigation of expression of foreign genes in plants.

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