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Synthesis of viral DNA forms in *Nicotiana plumbaginifolia* protoplasts inoculated with cassava latent virus (CLV); evidence for the independent replication of one component of the CLV genome

Rod Townsend, John Watts and John Stanley

Departments of Virus Research and Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

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ABSTRACT

Totipotent leaf mesophyll protoplasts of Nicotiana plumbaginifolia, Viviani were inoculated with cassava latent virus (CLV) or with full length copies of CLV genomic DNAs 1 and 2 excised from replicative forms of M13 clones. Virus specific DNAs began to appear 48-72h after inoculation with virus or cloned DNAs, coincident with the onset of host cell division. Infected cells accumulated supercoiled forms of DNAs 1 and 2 as well as progeny single-stranded (ss) virion (+) sense DNAs representing each component of the genome. Both supercoiled and ss molecules were synthesised by cells inoculated with cloned DNA 1 alone but DNA 2 failed to replicate independently.

INTRODUCTION

Cassava latent virus (CLV) is typical of the whitefly transmitted geminiviruses which infect dicotyledonous hosts (1). It has a bipartite genome comprising two similarly sized circles of single-stranded (ss) DNA (DNA 1, 2779 nucleotides and DNA 2, 2724 nucleotides) which share a region of sequence encompassing approximately 200 nucleotides (2). The mechanism of geminivirus DNA replication has not been resolved. Unit length double-stranded (ds) open circular (oc) (3,4) and supercoiled DNAs (4,5) have been isolated from dicotyledonous hosts infected with different geminiviruses inviting speculation that the geminivirus genome is replicated via circular ds replicative form (RF) DNAs in a manner analogous to the replication of small ss DNA bacteriophages such as ØX174.

The capacity of each component of the bipartite genome of CLV or those of other whitefly transmitted geminiviruses, for independent replication, has not been determined. Infectivity studies using recombinant DNA clones containing full length

copies of CLV DNAs 1 and 2 inserted in bacteriophage M13 show that sequences from both DNAs are required to elicit a systemic infection in Nicotiana benthamiana plants (6), but this does not rule out the possibility that one or both components are able to replicate independently but are unable to spread systemically through the host and so remain undetected.

To facilitate studies on the replication of geminiviruses and in particular to determine the capacity of each component of the bipartite genome for self replication, we have investigated the potential of dividing leaf mesophyll protoplasts of Nicotiana plumbaginifolia, Viviani to support replication of CLV. Novel virus specific DNAs which were synthesised following inoculation of protoplasts with CLV or with full length recombinant DNA copies of the viral genome have been partially characterised and compared. Evidence is presented which demonstrates that one component of the CLV genome is capable of independent replication.

MATERIALS AND METHODS

Preparation of virus and DNAs for inoculation

Nicotiana benthamiana plants were inoculated with CLV (Kenyan isolate) derived from full length infectious genomic clones of DNAs 1 (pJS092) and 2 (pJS094) in M13 (6). Plants were maintained at 25°C and leaves showing symptoms of systemic infection were removed after 9 days. Virus was extracted from the leaves and precipitated with polyethylene glycol (PEG) 6000 (7). Precipitated virus was resuspended in 10mM Tris-HCl (pH 8.0) containing 1mM EDTA (TE) and pelleted through a 15% sucrose cushion by centrifugation at 160,000 xg for 1h at 4°C. Partially purified virus was resuspended in TE at an approximate concentration of 1mg ml^{-1} and sterilized by filtration through 0.45µm pore diameter membrane filters.

CLV DNAs were excised from RFs of M13 clones pJS092 and pJS094 by digestion with Mlu1 and Pst1 respectively (6) and the digests fractionated by sucrose density gradient centrifugation. Following ethanol precipitation, the DNAs were resuspended in TE at a concentration of 1mg ml^{-1}. Both preparations contained about 75% insert DNA and 25% linearised vector.

Preparation and inoculation of protoplasts

Leaves approximately 15cm long from plants of N. plumbaginifolia were surface sterilised by immersion in 10% bleach solution for 10 min and then washed in distilled water. The lower epidermis of each leaf was dusted with 600 mesh carborundum powder and brushed gently to abrade the surface without damaging the epidermal cells. The mid rib was removed, and the leaf cut into 1cm square pieces which were washed in 0.7M mannitol. Leaf pieces were digested overnight in the dark without agitation in a protoplast medium (PM) based on B5 medium (8) (pH 5.6) supplemented with 137g sucrose, 750mg $CaCl_2$, 250mg xylose, 1mg benzyl adenine and 3mg naphthalene acetic acid l^{-1} and containing 0.5% Onozuka R10, 0.1% Macerozyme R10 (Yakult Biochemicals Ltd) and 0.05% Driselase (Fluka).

Released protoplasts were filtered through 0.1mm nylon mesh, centrifuged at 800 xg for 10 min, washed by floatation in PM, and resuspended in PM at a concentration of approximately 10^6 protoplasts ml^{-1}. Each 5ml aliquot of protoplasts was mixed with 200µg of virus or 10µg of each gradient purified insert DNA and 5ml of 50% PEG 1500 containing 15mM $Ca(NO_3)_2$. The mixture was incubated for 20 min at 20°C before being diluted, in steps, with PM over a period of 5 min until the concentration of PEG was below 5%. The suspension was dispensed as 15ml aliquots in 9cm diameter plastic Petri dishes at a density of about 10^5 protoplasts ml^{-1}. The floating protoplasts were cultured at 25°C in the dark for two to three days and then subjected to continuous white fluorescent light. Samples were taken 2h post inoculation and thereafter at 24h intervals.

Extraction of DNA from protoplasts

The protoplasts from each dish were collected by filtration through Miracloth (Calbiochem) and the filtrate drawn under slight vaccuum, through GF/C filters to remove any remaining cells. Filters were washed with 4% $Ca(NO_3)_2$ and then placed in 5ml of 50mM Tris HCl (pH 8.0) containing 25mM EDTA at 4°C. Cells were lysed by the addition of 0.5% SDS, extracted three times with an equal volume of phenol/chloroform (1:1 v/v) and total nucleic acids precipitated with ethanol. The precipitate was

dissolved in TE and then digested with 10µg RNase A (rendered DNase free by heating at 100°C for 10 min) for 30 min at 4°C. SDS was added to 1% and EDTA to 10mM and the solution digested with 250µg of proteinase K (predigested for 30 min at 37°C) for 1h at 37°C. After extraction with phenol and ethanol precipitation, each sample of DNA was taken up in 50µl of TE.

Analysis of DNAs

Total protoplast DNA samples (10µl), as well as standards of CLV supercoiled DNA extracted from plants and circular ssDNA from virions (5) were fractionated on 1.4% agarose gels in 40mM Tris, 20mM Na acetate (pH 7.5) containing 2mM EDTA. After an initial depurination step to ensure efficient transfer of supercoiled DNA (9), gels were Southern blotted (10) onto Hybond N membranes (Amersham). Blots were probed with nick translated M13 RFs or inserts of clones pJS092, pJS094 or clone pJS055 which is specific for DNA 2 since it contains a Hind111 insert (1494 - 2286) which lacks any of the CLV common region. Hybridised probe DNAs were stripped from blots in 0.4M NaOH at 45°C according to the manufacturers protocol.

Characterisation of DNA forms

To distinguish between ds and ss molecules, 10µl samples of protoplast DNA were digested with 10 units of S_1 nuclease, in 280mM NaCl, 30mM Na acetate, 4.5mM Zn acetate (pH 4.4) for 30 min at 0°C. Digests were fractionated on a 1.4% agarose gel, blotted as above and probed with nick translated clone pJS092. A marker preparation containing supercoiled, oc, linear ds and circular ssDNA molecules was prepared by digesting CLV supercoiled DNA isolated from infected plants (5) with 100U of S_1 nuclease as above for 1h at 37°C. The digest was extracted with phenol, ethanol precipitated and the products taken up in TE containing circular ssDNA extracted from virions (5).

To confirm the identity of ssDNA molecules synthesised in protoplasts as virion (+) or complementary (-) sense, samples (10µl) of total protoplast DNA, as well as CLV supercoiled and virion ssDNAs were analysed on 1.4% agarose gels. These were blotted and probed with M13 ssDNA clones containing full length copies of DNA 1 in either orientation, pJS092 (viral (+) sense) and pJS091 (complementary (-) sense) at a concentration of 1µg

ml^{-1} of hybridisation solution. After washing in 2 x SSC with 0.1% SDS at 65°C (10) blots were probed with nick translated M13 RF to locate hybridised ss M13 clones.

RESULTS

Synthesis of DNAs following inoculation of protoplasts with CLV

In samples of protoplast DNA removed two hours post inoculation, a faint band 1i was apparent following probing with pJS092. It co-migrated with circular ssDNA extracted from CLV virions (Figure 1a) and may reflect residual inoculum virus within or closely associated with the protoplasts. By 24h it

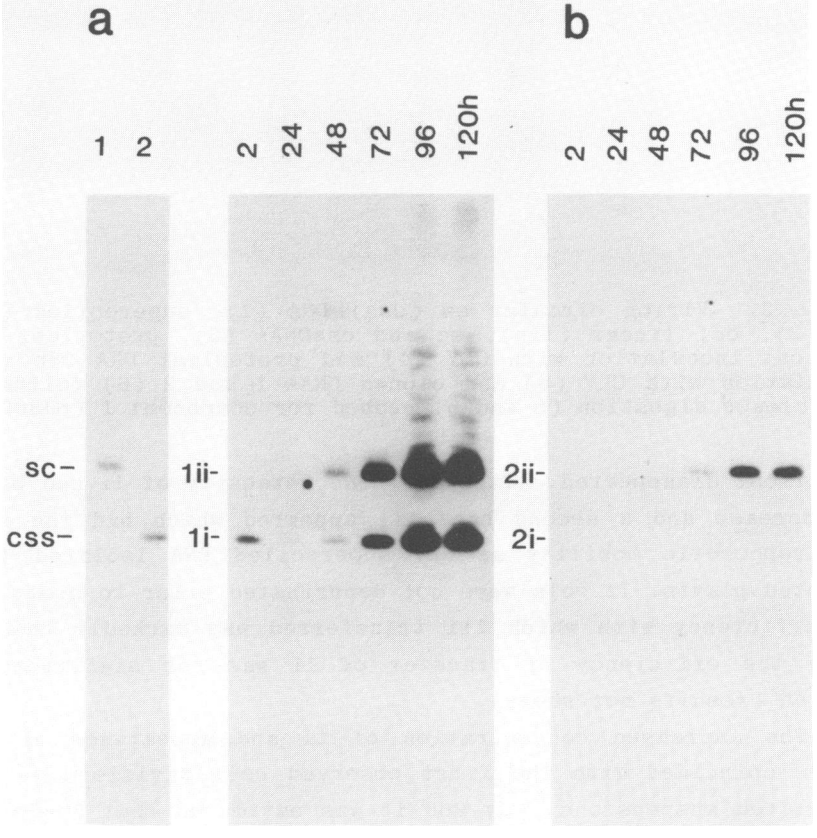

Figure 1. Supercoiled (sc) DNA (1), virion circular ssDNA (css) (2) and DNA extracted from protoplasts 2-120h post inoculation with CLV, probed for; (a), component 1 (pJS092) and (b), component 2 (pJS055).

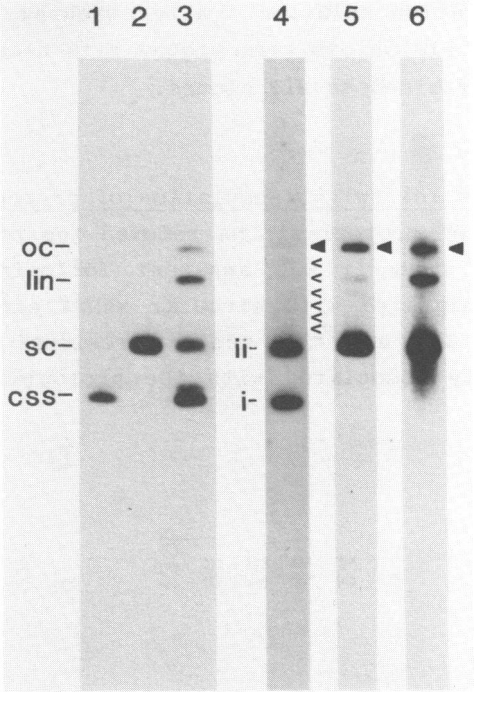

Figure 2. Virion circular ss (css) DNA (1), supercoiled (sc) DNA (2), oc, linear (lin), sc and cssDNAs (3), protoplast DNA 72h post inoculation with CLV (4) and protoplast DNA 96h post inoculation with CLV (5) and cloned DNAs 1 and 2 (6) following S_1 nuclease digestion (5 and 6) probed for component 1 (pJS092).

had almost disappeared. After 48h, the intensity of 1i had begun to increase and a second band 1ii appeared which had the same electrophoretic mobility as CLV supercoiled DNA isolated from infected plants. If gels were not depurinated prior to blotting, the efficiency with which 1ii transferred was markedly reduced while the efficiency of transfer of 1i was not significantly changed (results not shown).

The increased concentration of 1i and appearance of 1ii always coincided with the first observed cell divisions in the inoculated suspensions. By 96h it was estimated that 50-60% of the cells had undergone division and the concentrations of DNA forms 1i and 1ii had reached a maximum. A "ladder" of bands was often apparent above 1ii (Figure 2, lane 4, arrowed). The slow-

est migrating of these bands (solid arrow) co-electrophoresed with unit length ocDNA (lane 3).

Blots stripped and re-probed with pJS055 revealed component 2 DNAs, that is, 2i and 2ii (Figure 1b) equivalent to forms 1i and 1ii. Their synthesis followed the same time course as component 1 DNAs but both forms were apparently produced in substantially reduced amounts and band 2i could only be detected after extended autoradiography. Some of this apparent reduction could be accounted for on the basis of the smaller size of the insert in clone pJS055 since re-probing with pJS094, containing a full length insert, resulted in an increase in signal. However, synthesis of form 2i was still substantially less than that of 1i.

Characterisation of synthesised DNAs

Samples of DNA extracted from protoplasts 72h and 96h post inoculation with CLV were subjected to S_1 nuclease digestion and strand specific probing. DNA form i was completely sensitive to S_1 nuclease digestion (Figure 2, lane 5) and hybridised to the complementary (-) sense ssDNA probe pJS091 (Figure 3, lane 5) but not to the virion (+) sense ssDNA probe pJS092 (lane 2). On the basis of these results it is concluded that form i represents progeny virus (+) sense ssDNA which is presumably circular since it co-migrated with virion ssDNA that has been shown to be predominantly circular (5).

Form ii was partially sensitive to S_1 nuclease (Figure 2, lane 5) as indicated by an increase in the intensity of the band (arrowed) which co-migrated with CLV ocDNA (lane 3). Both virion (+) and complementary (-) sense probes hybridised with equal efficiency to band ii (Figure 3, lanes 2 and 5). These properties, coupled with the electrophoretic mobility and reduced blotting efficiency of the DNA when depurination was omitted, indicate that form ii represents CLV supercoiled DNA. Attempts to confirm this by restriction of the DNA with endonucleases (5) have so far been frustrated by the presence of endogenous nuclease activity in the protoplast extracts which was activated at 37°C. The 'ladder' of bands between the supercoiled form ii and the oc form (Figure 2, lane 4) presumably represent molecules with different numbers of supercoil turns.

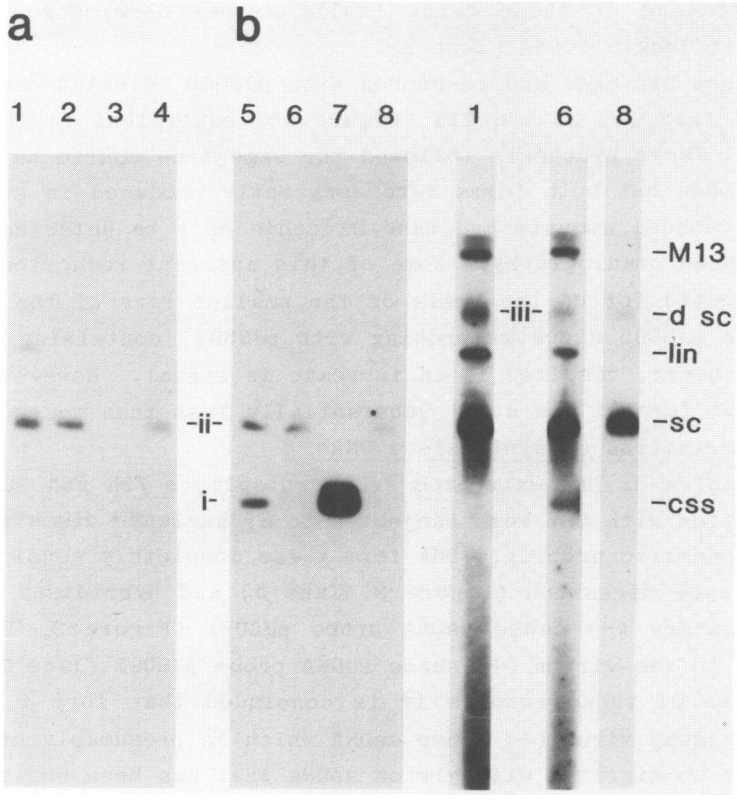

Figure 3. Virion circular ss (css) DNA (3 and 7), supercoiled (sc) DNA (4 and 8), and protoplast DNA 120h post inoculation with CLV (2 and 5) and cloned DNAs 1 and 2 (1 and 6) probed with; (a), ss M13 clones pJS092 (+ sense) and (b), ss clone pJS091 (- sense). The remaining three lanes show details revealed by extended autoradiography of lanes 1, 6 and 8 respectively, including dimeric sized supercoiled molecules (dsc).

Synthesis of novel DNAs following inoculation of protoplasts with genomic clones of CLV

A large proportion of the linear DNA inoculum remained associated with the protoplasts thereby masking the synthesis of novel molecules during the first 48h. By 72h it had largely degraded but a small amount of linear dsDNA persisted throughout the course of the experiment and may represent DNA protected from nuclease activity within protoplasts (Figure 4a and b). After 48h, supercoiled forms of DNAs 1 and 2 could be discerned. These co-migrated with CLV supercoiled DNA from plants, were

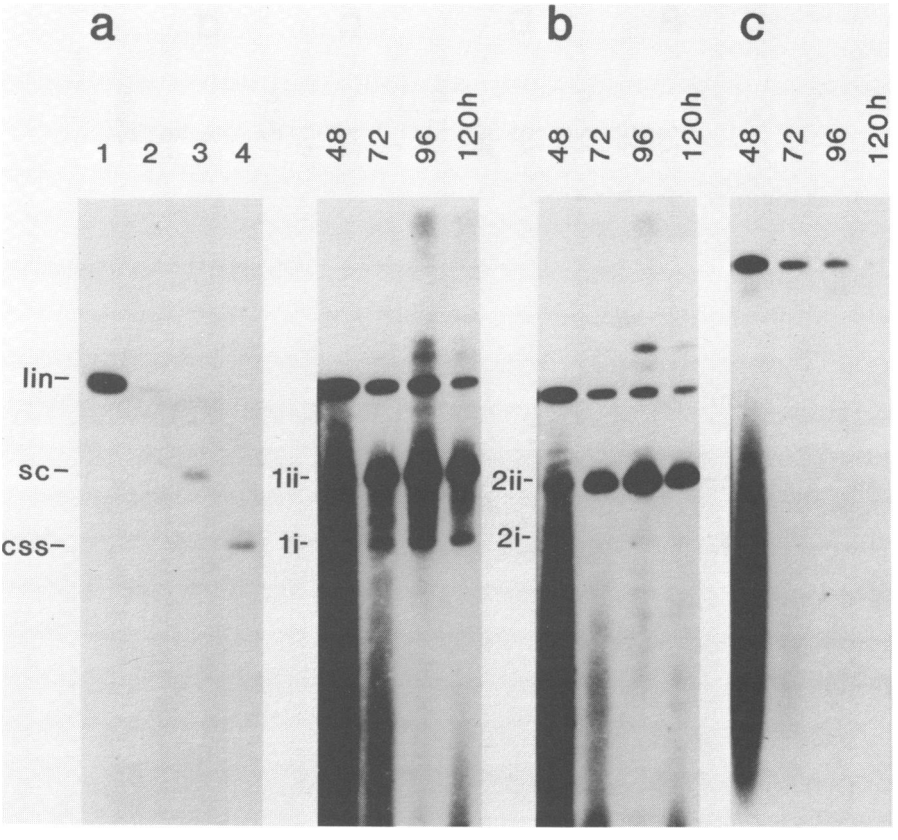

Figure 4. Inoculum DNAs 1 (1) and 2 (2), supercoiled (sc) DNA (3) and virion circular ss (css) DNA (4) and protoplast DNA 48-120h post inoculation with cloned DNAs 1 and 2, probed for; (a), component 1 (pJS092 insert); (b), component 2 (pJS094 insert) and (c), M13 vector (M13mp8).

partially sensitive to S_1 nuclease (Figure 2, lane 6) giving rise to unit sized oc molecules (arrowed), and hybridised to both (+) and (-) sense ss probes (Figure 3, lanes 1 and 6). By 72h, ss forms of DNA 1 had appeared (Figure 4a) which co-migrated with authentic virion circular ssDNA. They were completely digested by S_1 nuclease (Figure 2, lane 6) and only hybridised to the (-) sense ss probe (Figure 3, lanes 1 and 6). Corresponding ss circles of DNA 2 could barely be discerned above the background. Both supercoiled and circular ss forms reached a maximum concentration after 96h. Other minor bands migrating

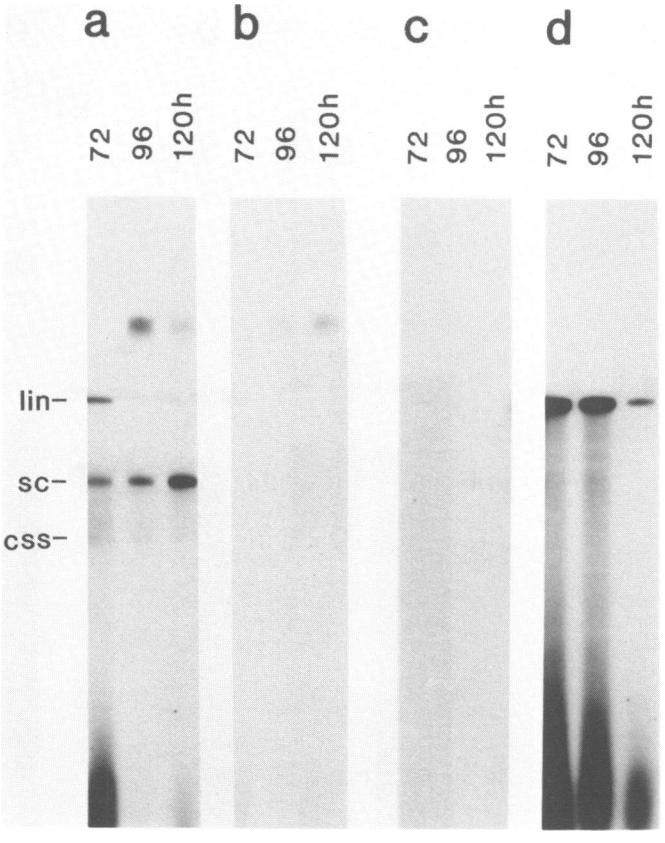

Figure 5. Protoplast DNA 72-120h post inoculation with; (a and c); cloned DNA 1 and (b and d), cloned DNA 2 probed for; (a and b), component 1 (pJS092) and (c and d), component 2 (pJS094).

more slowly than linear dsDNA were apparent but have not been characterised. One of these (iii) (lanes 1 and 6) co-electrophoresed with dimeric supercoiled DNA (lane 8) which occurs in small amounts in preparations of CLV supercoiled DNA from infected tissue (5). In all experiments the proportion of supercoiled DNA synthesised relative to ssDNA was greater than in comparable protoplast preparations inoculated with virus. Reprobing with M13 RF revealed that a small proportion of the linearised vector also persisted for the duration of the experiment but there was no evidence for the circularisation of the linear DNA or the formation of supercoiled or circular ss

forms of M13 (Figure 4c). Furthermore, when N. plumbaginifolia protoplasts were inoculated with a linearised dsDNA cloned copy of the genome of maize streak virus (MSV; a gift from P.M. Mullineaux), a geminivirus restricted to graminaceous hosts, no supercoiled or ss MSV DNAs could be detected (results not shown).

Independent replication of one DNA component

A single preparation of protoplasts was divided into three aliquots and inoculated with a mixture of excised DNAs 1 and 2 or with each DNA separately. Protoplasts inoculated with DNA 1 alone produced supercoiled and progeny viral ssDNAs (Fig. 5a). However, protoplasts inoculated with DNA 2 showed no evidence of the replication of that DNA. A small proportion of linear inoculum DNA 2 persisted thoughout the course of the experiment (Fig. 5d) but extended autoradiography failed to reveal any evidence of supercoiled or ssDNAs even after 120h incubation. Protoplasts divided normally in all these cultures and those inoculated with a mixture of DNAs supported replication of DNA 2.

DISCUSSION

The synthesis of progeny viral ssDNA in N. plumbaginifolia protoplasts inoculated with virus or cloned copies of the CLV genome shows that replication of the viral DNA occurred in at least a proportion of inoculated cells. Molecules with the same electrophoretic mobility as ocDNA were apparent in nucleic acid preparations from infected protoplasts but in view of the presence of endogenous nuclease activity these may have arisen as a consequence of nicking of supercoiled forms by endogenous nucleases and so their identity as replicative intermediates (3,4) remains to be established.

The abundance of supercoiled CLV molecules relative to ssDNAs in infected protoplasts suggests that DNA replication may be biased towards the synthesis of supercoiled forms at the expense of progeny ssDNA. Studies with the circular ssDNA bacteriophage ØX174 suggest that the parental RF with its conserved primosome is the only replicating unit and that the function of the numerous other supercoiled DNAs in the infected cell is transcriptional (11). The majority of supercoiled CLV

molecules are likely to be active in transcription rather than the synthesis of viral ssDNAs. In the later stages of ØX174 infection, accumulation of progeny viral ss (+) strands depends on the availability of virus encoded proteins to complex and encapsidate the DNA making it unavailable as a template for (-) strand synthesis leading to RF formation (12). Blocking protein translation with chloramphenicol results in the accumulation of supercoiled molecules (13). In view of the similarities in organisation and transcription of the CLV genome (14) and those of papovaviruses such as SV40 (15), it has been proposed that CLV may also exhibit temporal regulation of early and late functions according to transcriptional polarity (1). Our observations may indicate that supercoiled CLV DNAs accumulate because transcription and/or translation of late viral genes is partially inhibited in leaf mesophyll protoplasts. Tissue specific temporal expression of late genes is an interesting possibility in view of the localisation of CLV to the phloem cells of infected plants (7) and could have important implications in the exploitation of geminiviruses as gene vectors (16).

Our results strongly suggest that replication of the CLV genome is dependent upon cellular synthesis. In this respect geminiviruses may resemble autonomously replicating parvoviruses which have small genomes of linear ssDNA and depend on host replication functions expressed during the S-phase of cell growth (17,18). Previous attempts (unpublished) to infect non-dividing tobacco mesophyll protoplasts failed, presumably because these cells are normally arrested in G1 (19). Since the division cycle of cultured cells can be controlled by alterations in nutrient and growth factor concentrations (20) it should be possible to synchronise the replication of CLV in order to study the synthesis of viral DNAs on a chronological basis.

This study has shown that component 1 of the CLV genome encodes all the virus specific functions necessary to replicate the genome. Component 1 also encodes the coat protein (14) but, both components of the CLV genome are required to initiate an infection in plants (6). It is, therefore, highly probable that one of the functions encoded by DNA 2 is responsible for systemic spread of the virus in its host.

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