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Replicative intermediates of *Tomato leaf curl virus* and its satellite DNAs

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Abstract

Several plant geminiviruses have been shown recently to utilize both rolling-circle replication (RCR) and recombination-dependent replication (RDR) strategies. A highly specific binding of the viral replication-associated protein (Rep) to its cognate DNA is essential for initiation of viral DNA replication and for the recognition of DNA components of the bipartite geminiviruses of the *Begomovirus* genus. We have extended the replication analysis to the monopartite Australian *Tomato leaf curl virus* (ToLCV), its Rep binding deficient mutants, and the satellite DNAs it supports. Analyses of viral DNA by two-dimensional agarose gel electrophoresis after fractionation by single-stranded (ss) DNA-selective cellulose chromatography revealed that DNA intermediates of ToLCV and its mutant were identical. Both RCR and RDR intermediates were identified. New ToLCV DNA forms were observed and characterized as subgenomic topoisomers, heterogeneous open circular double-stranded (ds) DNA, and degradation products. A 1350-nt DNA β satellite associated with the unrelated *Cotton leaf curl Multan virus* (CLCuMV) was supported by ToLCV and produced intermediates of both RCR and RDR, suggesting that replication strategies of satellites are determined by the helper virus. Replicative intermediates of the 682 nt ToLCV satellite DNA could not be resolved; however, concatemers of up to octamer were detected, together with a field of hybridizing material suggestive of complementary strand replication on heterogeneous circular ssDNA templates.

Keywords: Two-dimensional electrophoresis; Geminivirus; Replication; Recombination

Introduction

Geminiviruses are important plant pathogens containing circular single-stranded (ss) DNA genomes ranging in size from 2.5 to 3.0 kb (Böttcher et al., 2004; Zhang et al., 2001). They have twin-shaped particles encapsidating either a monopartite or bipartite genome, and are transmitted by different insect vectors. Based on insect vectors and plant hosts, the *Geminiviridae* family has been classified into the genera *Begomovirus*, *Curtovirus*, *Mastrevirus*, and *Topocuvirus* (Rybicki et al., 2000).

Of the geminiviral encoded proteins, only the replication-associated protein (Rep) is indispensable for DNA replication. This multifunctional protein specifically binds the cognate viral DNA, introduces a sequence-specific single-stranded DNA break and religates the free ends, activates the host cell machinery for DNA synthesis, and autorepresses its own transcription (reviewed by Hanley-Bowdoin et al., 1999).

After entering a susceptible host plant, geminiviral DNA is released and is converted to a double-stranded (ds) form with the help of host factors. While mastreviruses package a complementary-sense primer into the virion, complementary strand replication (CSR) of begomoviruses is initiated by an RNA primer generated by a host RNA polymerase or DNA primase (Saunders et al., 1992). The dsDNA circular molecule is then assembled, with host histones, into nucleosomes resulting in a viral minichromosome (Abouzid et al., 1988; Pilartz and Jeske, 1992, 2003). In this state, the DNA serves as template for transcription as well as replication. Previously, rolling circle replication (RCR) was believed to be the sole mechanism of geminivirus

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DNA replication (Saunders et al., 1991; Stenger et al., 1991). The initial step of RCR is specific binding of Rep to the cognate DNA involving a directly repeated Rep-binding motif, within the intergenic region (Arguello-Astorga et al., 1994; Behjatnia et al., 1998; Fontes et al., 1992, 1994a,b). Downstream of this motif, the virion-sense strand is nicked by Rep within the universally conserved nonanucleotide sequence (TAATATTAC) within the loop of a hairpin structure (Laufs et al., 1995; Stanley, 1995) and virus genome multiplication proceeds by RCR (reviewed by Hanley-Bowdoin et al., 1999). The viral genes are encoded in both, the virion-sense and complementary-sense strands of the dsDNA form and transcription takes place bidirectionally (Dry et al., 1993; Frischmuth et al., 1991; Petty et al., 1988; Townsend et al., 1985). There may be therefore an apparent conflict between replication and transcription processes if the templates are used at the same time that may lead to the generation of damaged viral DNA (Brewer, 1988). Recent investigations by two-dimensional gel electrophoresis and electron microscopy have indicated geminiviral replication also involves a recombination-dependent replication (RDR) mechanism (Jeske et al., 2001). RDR provides a mechanism by which damaged and incomplete geminivirus DNA can be rescued. Unlike RCR, where open circular (oc) DNA is the template for the synthesis of ssDNA, the template for RDR is covalently closed circular (ccc) DNA. According to this conservative model of replication, a viral DNA fragment recombines at a homologous site within an intact cccDNA molecule and is subsequently extended. The newly produced ssDNA may be converted to dsDNA by a mechanism similar to viral CSR. RDR can go through many rounds of replication before the product is displaced leading to a large population of heterogeneous linear dsDNA molecules.

Previous investigations have focused on geminiviruses from the genera *Begomovirus* and *Curtovirus* that originate from the new and old world and have revealed replicative intermediates consistent with both RCR and RDR (Preiss and Jeske, 2003).

During the past few years, a new class of virus-associated DNA molecules has been described. Initially, a 682-nt DNA-satellite associated with the Australian *Tomato leaf curl virus* (ToLCV, 2766 nt), a monopartite begomovirus, was discovered. Sequence similarity between the helper virus and satellite was limited to the universally conserved nonanucleotide sequence and the Rep-binding motifs (Dry et al., 1993, 1997). Subsequently, a number of DNA satellites of about 1350 nt, named DNA β, were isolated in association with monopartite begomoviruses (Briddon et al., 2000, 2001, 2002, 2003; Saunders et al., 2000). The DNA β molecules are characterized by a satellite conserved region (SCR), the universally conserved nonanucleotide sequence in a hairpin structure similar to that of geminiviruses, and an open reading frame that influences the severity of symptoms in infected plants. Geminivirus DNA satellites are dependent on the respective helper virus for replication and packaging. A DNA β molecule naturally associated with *Cotton leaf curl Multan virus* (CLCuMV), which in combination with its helper virus causes the severe cotton leaf curl disease (CLCuD), is known to be supported by ToLCV as a helper virus (Saeed et al., 2004). No information about satellite replication has so far been available. This has prompted the current study about the mechanism of helper virus-Rep-mediated satellite DNA replication, where replication is achieved in the absence of a cognate high-affinity Rep binding (Lin et al., 2003).

Results

ToLCV DNA intermediates are consistent with CSR, RCR, and RDR

ToLCV has only been found in the northern coastal region of Australia and is the only begomovirus so far discovered on this continent (Stonor et al., 2003). Its geographical isolation and the ability of this virus and its satellite DNA to replicate in the absence of a high-affinity Rep binding site raised the question as to whether ToLCV is utilizing the same replication mechanisms identified for *Abutilon mosaic virus* (AbMV), *Tomato golden mosaic virus* (TGMV), *African cassava mosaic virus* (ACMV), *Tomato yellow leaf curl virus* (TYLCV), and *Beet curly top virus* (BCTV).

Replicative DNA intermediates of ToLCV were resolved using two-dimensional gel electrophoresis (Jeske et al., 2001) only when the samples were fractionated by a chromatography step prior to analyses (Preiss and Jeske, 2003). Electrophoresis in the first dimension separated DNA species mainly on the basis of molecular mass whereas in the second dimension the dsDNA forms were further resolved due to the retarded electrophoretic mobility caused by the intercalating chloroquine. The cccDNA was affected in such a way that the negative superhelicity was converted into positive superhelicity depending on the chloroquine concentration (Olavarrieta et al., 2002; Snapka et al., 1991). The patterns obtained for the two DNA fractions of ToLCV-infected tissues are shown in Fig. 1. The DNA profiles are similar to those of geminiviruses studies previously (Jeske et al., 2001; Preiss and Jeske, 2003) and are outlined briefly as follows.

The main DNA forms

ssDNA of genomic length (1xss) is more prominent in the eluate fraction (Figs. 1B,C) and is rather faint in the wash fraction (Fig. 1A), indicating a slightly overloaded column. Heterogeneous, circular ssDNA forms a straight line in which multimers (2xss, 3xss) are pronounced. ssDNA multimers are clearly divided into spots of circular (c) and linear (l) DNA. dsDNA of linear (1xlin), open circular (1xoc) or covalently closed circular (1xccc) form migrate as distinct species where cccDNA is separated into

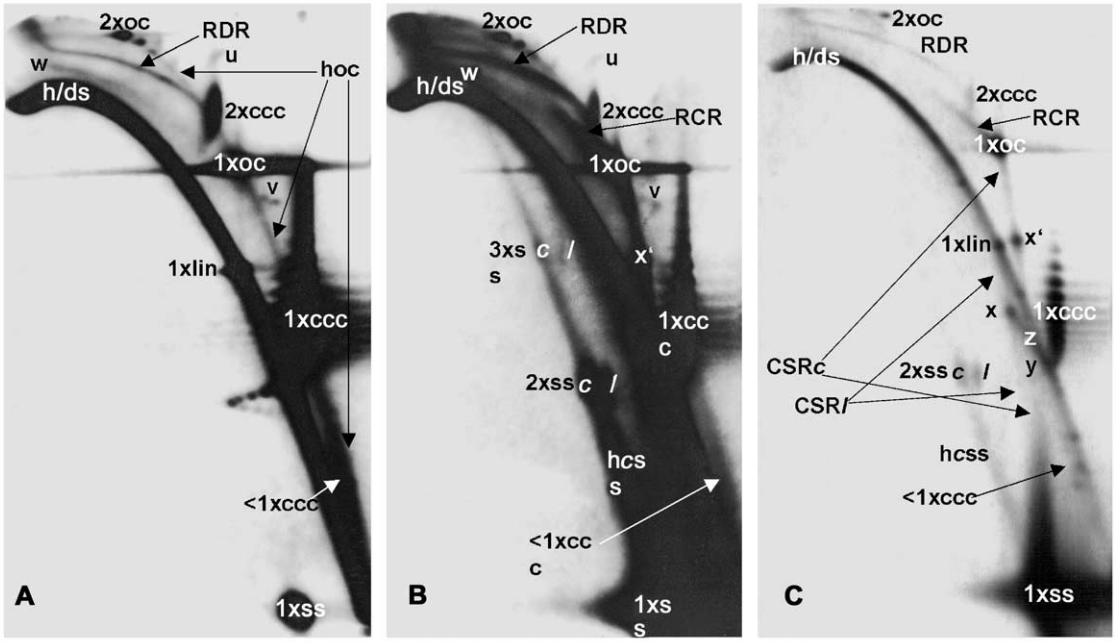

Fig. 1. Two-dimensional gel analysis of ToLCV DNA forms (isolated from tomato) after fractionation by BND-cellulose chromatography. (A) Wash fraction. (B, C) Eluate fraction. Detection was performed with a DIG-labeled full-length ToLCV probe. Parameters used for electrophoresis in the first dimension: 0.5% agarose, 0.03% SDS in the running buffer, 19-h running time at 10 V. Parameters for the second dimension: 1.4% agarose, 50 μg/ml chloroquine, 19-h running time at 45 V. Approximately 11 μg DNA (from 1 g of leaf tissue) was fractionated by BND-cellulose chromatography. Twenty percent of the resulting fractions were applied to the gels (A) or (B) and 6% of the eluate fraction was applied to gel (C). The DNA forms identified are single-stranded (ss) or double-stranded linear (lin), open circular (oc), and covalently closed circular (ccc). DNA intermediates are designated by their respective replication mechanisms: recombination-dependent replication (RDR), rolling circle replication (RCR), complementary strand replication (CSR). Heterogeneous DNA is marked with h. Numbers 1× or 2× refers to monomeric or dimeric genome length. Circular forms are marked with c, and linear forms with l. Forms u, v, w, x, x′, y, and z are explained in the text.

its topoisomers. Dimers of ocDNA and cccDNA (2xoc, 2xccc) are also present but the dimer of linear dsDNA cannot be discriminated from heterogeneous dsDNA (h*l*ds), which is represented by a strong arc-shaped signal.

Intermediate DNA forms indicating different replication mechanisms

CSR, RCR, and RDR are characterized by partially single-stranded intermediates that are enriched in the eluate fraction (Fig. 1B).

CSR begins with a single-stranded template (1xss), which may be linear or circular. The respective end products are linear dsDNA (1xlin) or ocDNA (1xoc). The two lines connecting those end products with the template DNA (1xss), therefore, represent forms containing different proportions of dsDNA synthesized progressively during complementary strand replication. One stage during linear and circular CSR appears to be more conserved (x, x′). CSR was resolved only for the monomeric and not for the dimeric genome length DNA, as it had been found for AbMV, TGMV, TYLCV, and BCTV (Preiss and Jeske, 2003). The signal corresponding to RCR intermediates originates from 1xocDNA and forms a straight line that ends abruptly, suggesting that molecules at the terminal point of this signal consist of ocDNA linked with one monomeric length of ssDNA.

RDR intermediates migrate as an arc starting from 2xcccDNA (Figs. 1A–C). In the case of single genome length DNA, it is not possible to distinguish this arc from heterogeneous linear dsDNA as explained previously (Jeske et al., 2001), but RDR intermediates were visualized by electron microscopy (Jeske et al., 2001).

These replicative DNA forms of ToLCV correspond to the forms identified previously for other geminiviruses. Therefore, it is likely that there is no significant difference with respect to their replication.

New forms

Apart from the previously known DNA forms, new intermediate DNAs specific to ToLCV were identified. Firstly, a line connecting monomer, dimer, and trimer of ocDNA and reaching to the bottom of the image was found reproducibly (Fig. 1A). Due to its appearance in the wash fraction, we conclude that ToLCV accumulates heterogeneous ocDNA (hoc). Secondly, a new population of topoisomers was found (Fig. 1A) that comprises less than one genomic length (<1xccc). Thirdly, a short but clear line (z; Fig. 1C) extends from the conserved spot (x) on the line of linear CSR. It also ends at a conserved spot (y) on the arc of h*l*dsDNA. These intermediates may be products of degradation of the conserved form x. Finally, some other

signals appeared reproducibly (u, v, w), but their identity remains unclear.

ToLCV Rep binding sites have no influence on replicative intermediates

The specific binding of Rep to its cognate binding site was considered to be an essential step for initiation of geminivirus DNA replication (Chatterji et al., 2000; Choi and Stenger, 1995, 1996; Fontes et al., 1994a; Gutierrez, 2000; Hanley-Bowdoin et al., 1999). In contrast, a ToLCV mutant that was impaired in its ability to bind Rep was still capable of replication (Lin et al., 2003).

To investigate whether the absence of a cognate Rep-binding site has an impact on one or more of the replication modes, a ToLCV DNA mutant lacking both the 5′ and the 3′ Rep binding motifs (ToLCV 5′3′ mutant) was analyzed in parallel with wild-type ToLCV. The resulting pattern produced by the mutant and wild-type DNA showed no obvious differences (Figs. 2A,B). The signals representing typical DNA forms as well as replicative intermediates and the new forms mentioned above were reproduced in indistinguishable shape and strength. Consequently, we conclude that Rep-binding motifs are not essential in any of the ToLCV replication modes under the experimental conditions described.

CLCuD DNA β replicates via RCR and RDR

CLCuD DNA β, originally isolated in Pakistan, is not restricted to its natural helper virus (*Cotton leaf curl Multan virus*, CLCuMV) and can be transreplicated by at least two other viruses, namely *Ageratum yellow vein virus* (AYVV; (Briddon et al., 2003) and ToLCV (Saeed et al., 2004). Furthermore, tomato is a suitable host plant for this DNA β (Briddon et al., 2003). We co-inoculated tomato plants with ToLCV and CLCuD DNA β and analyzed the replicative intermediates of DNA β. The 2-D gel analysis system was adjusted to the size of DNA β, which is approximately half that of ToLCV. Optimized conditions are given in the legend to Fig. 3. A representative pattern of the 2D analysis of CLCuD DNA β is shown in Fig. 3. The site resolution of the hybridization signals was weaker obtained by auto-radiography than by DIG detection, but all the main forms described above for ToLCV were also found for DNA β. Interestingly, intermediates of both RDR and RCR are clearly visible. In addition, DNA β also generates heterogeneous ocDNA (hoc) represented by a line, which links ocDNA spots, and accumulates linear dsDNA (y) of a certain size, smaller than unit length (Fig. 3B). The signals for linear and circular CSR were relatively weak, but the conserved forms x and x′ confirm the presence of these intermediates. We conclude that CLCuD DNA β is replicated by the same multiple modes as its helper virus.

ToLCV satellite DNA intermediates

In contrast to DNA β, ToLCV satellite DNA from Australia does not encode any ORFs and is considerably smaller (682 nts). Previous studies had demonstrated that replication of the satellite is not restricted to ToLCV and can be supported by taxonomically distinct geminiviruses (Dry

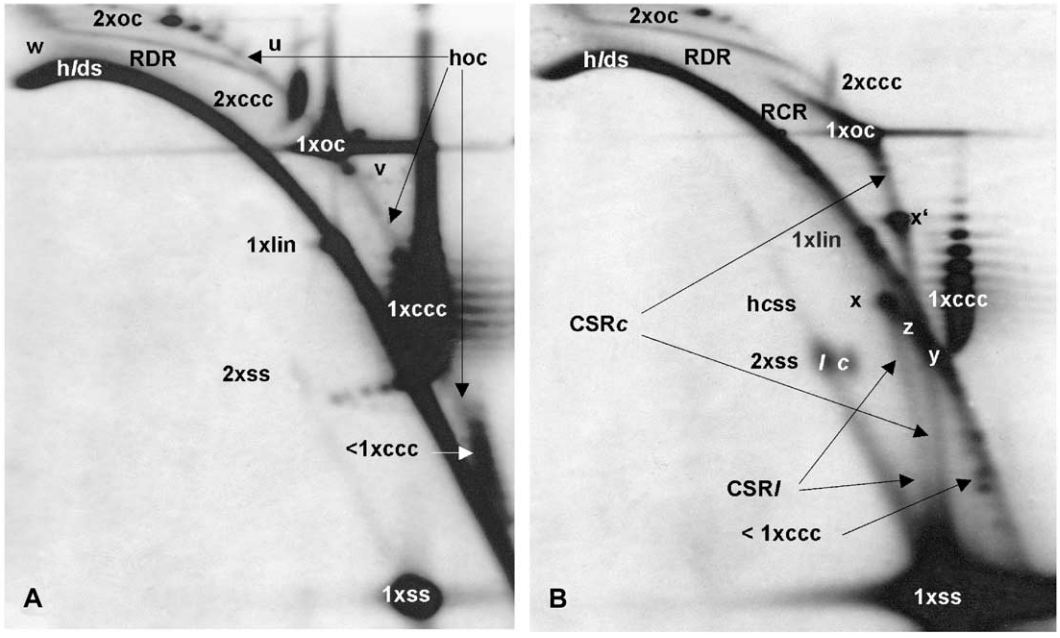

Fig. 2. Two-dimensional gel analysis of ToLCV-Rep binding site mutant (isolated from tomato) after fractionation by BND-cellulose chromatography. (A) Wash fraction. (B) Eluate fraction. Electrophoresis conditions and abbreviations as in Fig. 1. The amounts of DNA applied correspond to those of Figs. 1A and B.

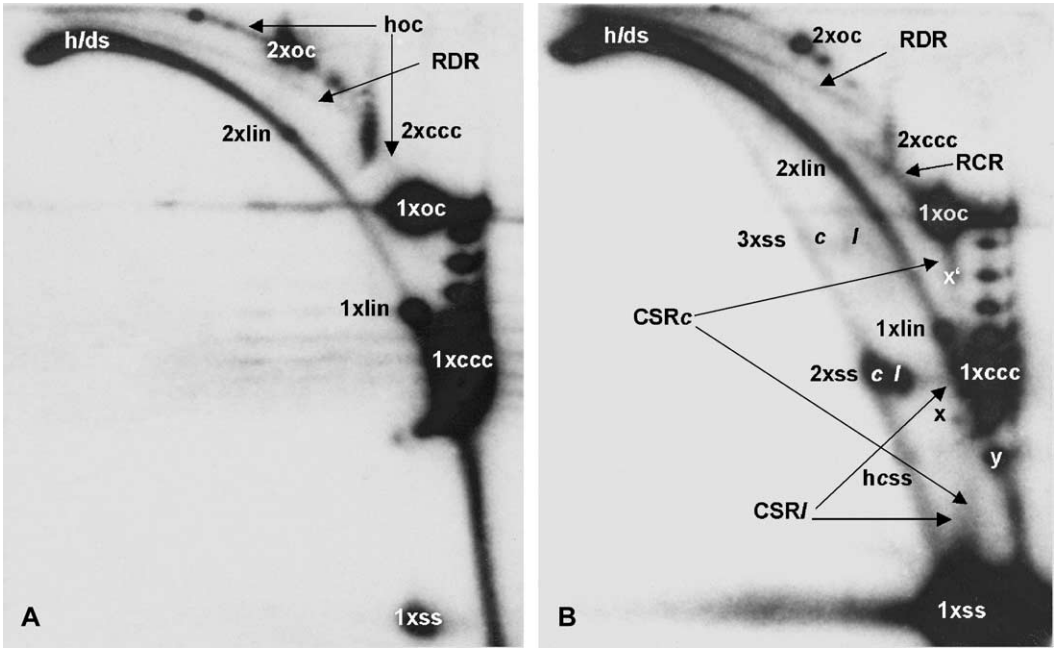

Fig. 3. Two-dimensional gel analysis of *Cotton leaf curl virus* (CLCuV) DNA β forms isolated from tomato, after fractionation by BND-cellulose chromatography. (A) Wash fraction. (B) Eluate fraction. The detection was performed with a radioactively labeled CLCuD-DNA β probe. Electrophoresis parameters in the first dimension were 0.65% agarose, 0.03% SDS in the running buffer, 15-h running time at 10 V. Parameters for the second dimension: 2% agarose, 60 μg/ml chloroquine, 19-h running time at 45 V. Approximately 9 μg DNA (from 1 g of leaf tissue) was fractionated by BND-cellulose chromatography. Fifty percent of the resulting fractions were applied to the gels (A) or (B), respectively. For abbreviations, see Fig. 1.

et al., 1997). Therefore, we investigated the mechanisms of its replication. Due to its smaller size, the gel system was modified to resolve the DNA forms. The agarose concentration for the second dimension was increased to 2% (Figs. 4A,B). Nevertheless, 1xocDNA and 1xcccDNA migrated too close to each other and to the arc of h/dsDNA to allow distinguishing possible RCR and RDR intermediates from the h/dsDNA arc. The identity of this arc was confirmed by

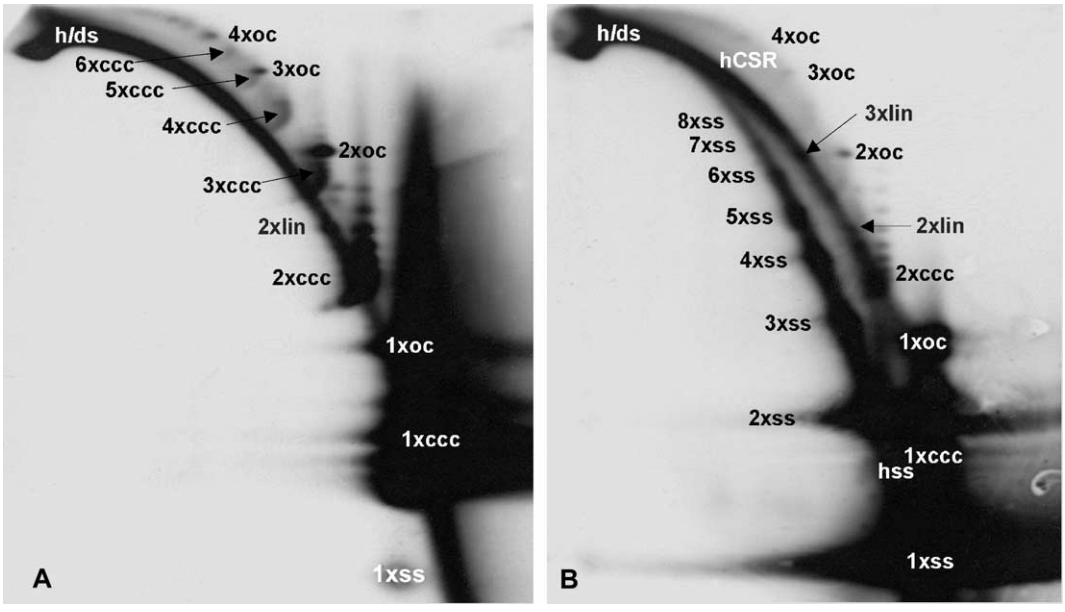

Fig. 4. Two-dimensional gel analysis of ToLCV satellite DNA forms isolated from tomato, after fractionation by BND-cellulose chromatography. (A) Wash fraction. (B) Eluate fraction. The detection was performed with a DIG-labelled ToLCV-sat probe. Electrophoresis parameters used in the first dimension: 0.65% agarose, 0.03% SDS in the running buffer, 14-h running time at 10 V. Parameters for the second dimension: 2% agarose, 75 μg/ml chloroquine, 14.5-h running time at 45 V. Approximately 11 μg DNA (from 1 g of leaf tissue) was fractionated by BND-cellulose chromatography. Twenty percent of the resulting fractions were applied to the gels (A) or (B), respectively. For abbreviations, see Fig. 1. Numbers 1× to 8× refer to concatemeric DNA forms of up to eight times the genomic length.

co-electrophoresis of satellite DNA with *Acc*I-cut bacteriophage λ DNA, followed by hybridization with a satellite-specific and rehybridization with a λ-specific probe. The spots of λ DNA were overlapping the arc (data not shown). A field of hybridizing material between heterogeneous circular ssDNA (h*c*ss) and heterogeneous ocDNA (hoc; Fig. 4B) indicated CSR of heterogeneous circular ssDNA templates (hCSR). How h*c*ssDNA may be generated remains a question and will be discussed below. Remarkably, ssDNA multimers (Fig. 4B) of lin, oc, and ccc forms (Fig. 4A) were up to eight times the satellite genome, elucidating a greater variability of satellite DNA forms compared to those evident from 1D gel analysis.

Discussion

Two-dimensional gel electrophoresis has been shown to be extremely useful for resolving replication intermediates of viral DNA, analyzing transcription, and packaging of plant geminiviruses (Jeske et al., 2001; Pilartz and Jeske, 2003; Preiss and Jeske, 2003) and viruses of other organisms (Snapka et al., 1991). The gel parameters optimized here for DNA β may also fit the requirements for the examination of nanoviruses, circoviruses, and anelloviruses (Hino, 2002; Meehan et al., 1997). The results presented here extend this knowledge in showing how 2D electrophoresis can be applied to characterize viral mutants. They confirm that a virus from a distant geographical origin utilizes the same multitasking replication mechanisms common to other geminiviruses. It also provides the first evidence that satellite-like DNAs β follow the same strategy.

Our results clearly establish that replication of CLCuD DNA β takes place via RCR and RDR. However, the replicative intermediates of the Australian ToLCV satellite DNA appeared to escape detection because of their relatively small size and limitations in the agarose gel system used. Polyacrylamide gels may be more suitable in future experiments to resolve the replicative intermediates of the 682 nt satellite. Nevertheless, there are some indications for the existence of RDR for ToLCV satellite. As discussed previously (Jeske et al., 2001), the prominent arc of heterogeneous linear dsDNA probably represents final products of RDR rather than RCR. This heterogeneous linear dsDNA may be the template for further replication either by a RCR-like or RDR-like (displacement) mode (Preiss and Jeske, 2003). Intermediates of such a replication mechanism, however, would not appear in hybridizing arcs or lines but in fields of heterogeneous signals (Preiss and Jeske, 2003). The conservation of the nonanucleotide sequence within satellite DNAs suggests that a RCR-like nicking-closing mechanism exists, despite the technical limitation to detect possible corresponding intermediates starting from 1xocDNA for ToLCV satDNA. Such intermediates have been found for geminiviruses in general (Preiss and Jeske, 2003) and DNA β in particular (this study).

The only functional ORF encoded by CLCuD DNA β is responsible for symptom severity (Briddon et al., 2000, 2001, 2003; Saeed et al., 2004; Saunders et al., 2004), but this function is not related to replication. DNA β depends on the Rep protein of the helper virus for replication.

How DNA β is recognized by the helper viral Rep remains unclear. It has been reported that CLCuD DNA β, as well as ToLCV satellite DNA, accept different helper viruses for replication (Briddon et al., 2003; Dry et al., 1997). A virus-specific recognition is therefore unlikely to be involved. In the absence of known recognition sites, the nonanucleotide sequence common to both helper viruses and DNA satellites may be the only common motif required for their replication.

In bipartite begomoviruses, however, a substantial body of evidence suggests that a specific recognition of the cognate Rep-binding sites by Rep is essential for co-replication of DNA A and B. Aside from ToLCV (Behjatnia et al., 1998), high-affinity Rep-binding sites have been identified in the genomes of many geminiviruses (Choi and Stenger, 1995, 1996; Fontes et al., 1992, 1994b; Jupin et al., 1995). Studies with *Tomato leaf curl virus* from New Delhi (Chatterji et al., 2000) and *Tomato golden mosaic virus* (Fontes et al., 1994a) revealed that mutation of these Rep-binding motives impaired their ability to replicate. In contrast, mutants of the monopartite ToLCV deficient in Rep binding accumulate normally (Lin et al., 2003) by the same replication mechanisms as used by the wild-type virus. There was no evidence that replication had changed in favor of either RCR or RDR. The existence of an undiscovered low-affinity binding site for Rep could not be completely ruled out, but trans-replication of the unrelated DNA β indicates that this explanation is unlikely.

It may be possible that bipartite begomoviruses need a more stringent control for trans-replication and they achieve this through Rep recognition of the binding sites. Apart from replication, Rep plays a role in transcriptional control. The Rep binding motif is located within the promoter region in front of the C1 ORF that encodes Rep. For TGMV, a negative autoregulation of Rep transcription has been reported (Eagle et al., 1994; Gröning et al., 1994; Sunter et al., 1993). Further studies are required to examine whether ToLCV Rep plays a similar role in regulation of transcription.

Transcriptional regulation may also influence replication, especially for bipartite geminiviruses that may have higher constraints on cooperative multiplication versus transport and packaging processes. In this regard, it makes sense that different geminiviruses have developed different promiscuity levels for unrelated DNA molecules. An obligate maintenance of a second component as in the case of bipartite geminiviruses might have more stringent requirements than the facultative maintenance of a satellite by a helper virus.

Materials and methods

Plants, viruses, and satellites

Agrobacterium tumefaciens C58 transfected with infectious clones of *Tomato leaf curl virus* (ToLCV) (=TLCV in (Rigden et al., 1996), ToLCV-3′-Rep binding site mutant (ToLCV-3′mut) (Lin et al., 2003), ToLCV-sat-DNA (Dry et al., 1997), or CLCuD-DNA β (Saeed et al., 2004) were used in this study. ToLCV-WT, ToLCV-3′mut, ToLCV-WT and ToLCV-sat-DNA or CLCuD DNA β were agroinoculated onto *Solanum lycopersicon* cv. Grosse Lisse plants as described (Dry et al., 1997). Youngest leaves were sampled at the first onset of symptom development 14 days post infection (dpi).

DNA extraction and purification

Plant nucleic acids enriched for viral and satellite DNA were prepared as described (Preiss and Jeske, 2003). DNA was quantified at the South Australian Research and Development Institute, Adelaide using either 4′,6-diamidino-2-phenylindole (DAPI; Tanious et al., 1992) or PicoGreen. Nucleic acids were fractionated by chromatography on benzoylated naphtoylated DEAE (BND) Cellulose (Sigma B6385) (Dijkwel et al., 1991) as described (Preiss and Jeske, 2003) except that 9–11 μg DNA was applied to the columns. Following this procedure, the "wash fraction" was enriched in dsDNA and the "eluate fraction" in ssDNA.

Two-dimensional gel electrophoresis and Southern hybridization

A portion of 20% (for ToLCV-WT, ToLCV-3′mut, ToLCV-sat-DNA) or 50% (for CLCuD DNA β) of fractionated DNA were used for two-dimensional agarose gel electrophoresis as described (Jeske et al., 2001). The concentrations of agarose and chloroquine, field strengths, and running times were varied depending on the viral agent to be examined as indicated in figure legends. Alkaline transfer was performed according to Chomczynski and Qasba (1984). Probes were generated from full-length genomic clones of viral and satellite DNA and labeled with digoxigenin (DIG) (Jeske et al., 2001) or [α-^{32}P]dCTP (Dry et al., 1993) as indicated in figure legends.

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