Agrobacterium tumefaciens supports DNA replication of diverse geminivirus types

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Abstract We have previously shown that the soil-borne plant pathogen *Agrobacterium tumefaciens* supports the replication of tomato leaf curl geminivirus (Australian isolate) (TLCV) DNA. However, the reproducibility of this observation with other geminiviruses has been questioned. Here, we show that replicative DNA forms of three other geminiviruses also accumulate at varying levels in *Agrobacterium*. Geminiviral DNA constructs that lacked the ability to replicate in *Agrobacterium* were rendered replication-competent by changing their configuration so that two copies of the viral *ori* were present. Furthermore, we report that low-level replication of TLCV DNA can occur in *Escherichia coli* containing a dimeric TLCV construct in a high copy number plasmid. These findings were reinforced by expression studies using β-glucuronidase which revealed that all six TLCV promoters are active in *Agrobacterium*, and two are functional in *E. coli*. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Geminivirus; Begomovirus; Replication; β-Glucuronidase; *Agrobacterium tumefaciens*; *Escherichia coli*

1. Introduction

Geminiviruses are plant pathogens which infect a wide range of crops and cause significant economic losses worldwide. Members of this family are characterized by twinned icosahedral virions encapsidating circular, single-stranded DNA genomes of 2.5–3.0 kb (for review, see [1]). Replication of geminiviral DNA is thought to occur by a rolling circle mechanism, analogous to that employed by some bacteriophages [2] and a class of eubacterial plasmids [3]. A hallmark of this replication strategy is the production of supercoiled, open circular, and linear double-stranded (ds) DNA species (for review, see [4]). In addition, recent evidence suggests that a recombination-related process is also involved in geminivirus DNA replication [5].

We have demonstrated in earlier studies that replication of tomato leaf curl virus (TLCV) (*Geminiviridae*: begomovirus) DNA occurs in vivo in *Agrobacterium tumefaciens* carrying a pBin19 plasmid vector harboring tandem copies of the TLCV genome [6]. This process required a functional C1 gene, which encodes the viral replication initiator protein (Rep), and two copies of the viral *ori*. None of the other viral genes were necessary for accumulation of TLCV DNA. The observation that TLCV DNA replication was supported by the bacterial cellular machinery provided the first experimental information supporting the hypothesis that geminiviruses may have evolved from prokaryotic episomal replicons.

Despite the novelty of the original finding, as yet no report of DNA replication in bacteria by any other geminivirus has appeared, suggesting that this ability may be specific to TLCV. This is an intriguing possibility since most characteristics of TLCV, including its genome organization, intergenic region, and gene functions, appear to be typical of the begomovirus genus of *Geminiviridae*. In an effort to elucidate whether DNA replication in *A. tumefaciens* is unique to TLCV or common among geminiviruses, pBin19 constructs carrying other geminiviral sequences were analyzed. Here we report that viral DNA species of two other begomoviruses, the monopartite tomato yellow leaf curl virus (TYLCV) and the bipartite African cassava mosaic virus (ACMV), also accumulate to significant levels in *A. tumefaciens*. In addition, a strain of TLCV recently discovered in the Northern Territory of Australia, termed TLCV-D1 [7], possesses the ability to replicate in *Agrobacterium*. We also tested whether TLCV DNA species could accumulate in *Escherichia coli*, and found that this bacterium could support viral replication when transformed with a pUC8 plasmid construct containing a TLCV dimer.

To provide further evidence for the occurrence of TLCV DNA replication in *Agrobacterium* and *E. coli*, the activity of the TLCV promoters in these bacteria was measured using the β-glucuronidase (GUS) gene as a reporter. All six promoters were active in *Agrobacterium*, while in *E. coli* only the C1 and C2 promoters produced detectable expression of GUS.

2. Materials and methods

2.1. Construction of clones

Tandem repeats of cloned TLCV DNA were inserted into pBin19 or pUC8 to create pBin19-TLCV2.0 and pUC8-TLCV2.0, respectively. The pUC8-TLCV2.0 plasmid was constructed by ligating a *Bam*HI monomer from TLCV clone pTLC4 [8] with *Bam*HI-linearized vector pUC8, and selecting a transformant containing a head-to-tail dimeric insert. Plasmid pBin19-TLCV2.0 is described in Rigden et al. [6]. The method used to create a dimeric clone of TLCV-D1 in pBin19 has been described [7].

To create a pBin19 construct containing a tandem repeat of the ACMV DNA A component, an *Eco*RV monomer from ACMV clone pBinCLV1.3A [9] was ligated into pBluescript SK (Stratagene), which

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Abbreviations: ACMV, African cassava mosaic virus; GUS, β-glucuronidase; TLCV, tomato leaf curl virus; TYLCV, tomato yellow leaf curl virus

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was then linearized with *Pfl*MI and ligated with a full length *Pfl*MI ACMV monomer. The dimeric viral DNA was then moved into pBin19 as a *Xho*I/*Xba*I fragment, generating pBin19-ACMV2.0 (Fig. 1).

The TYLCV construct was created by ligating a *Bgl*II monomer from TYLCV clone pBin19/TYLCV-S1.8 [10] into *Bam*HI-digested pBin19, resulting in loss of the vector *Bam*HI sites. This plasmid was then linearized with *Bam*HI (which cuts at nucleotide 152 of the TYLCV genome) and ligated with a *Bam*HI monomer, to generate pBin19-TYLCV2.0 (Fig. 1).

2.2. Extraction and analysis of DNA

DNA was extracted from *A. tumefaciens* C58 carrying the binary vector pBin19-TLCV2.0 or *E. coli* Dh5α carrying pUC8-TLCV2.0 using the boiling miniprep method [11]. The DNA was purified by either phenol:chloroform extraction or by adsorption onto a spin-column (Qiagen). Approximately 50 ng of samples were electrophoresed in 1.2% w/v agarose gels in Tris/borate/EDTA and blotted onto Zeta-Probe membrane (Bio-Rad) with 0.4 M NaOH, as described [8]. ^{32}P-labelled probes were prepared by a random decamer priming kit (Geneworks) using dimeric viral DNA fragments as the templates.

2.3. Analysis of GUS expression in E. coli

The construction of pBin19 plasmids containing the individual TLCV gene promoter regions fused to the GUS reporter gene has been described [12]. *A. tumefaciens* and *E. coli* cells harboring these constructs were grown for 36 h at 28°C and overnight at 37°C respectively. Cell pellets from 600 μl of culture were frozen in liquid nitrogen, and ground with the aid of sand using an electric screwdriver equipped with a grinding tip in Eppendorf tubes. The homogenate was extracted in 350 μl extraction buffer, spun for 10 min in a microfuge and two 150 μl samples of the supernatant withdrawn for analysis. All measurements were on duplicate starting cultures. GUS activity was determined by a fluorometric assay [13], and corrected for variations in extraction efficiency by measuring protein concentrations using a Bio-Rad protein assay reagent kit according to the manufacturer's instructions.

3. Results and discussion

From studies of TLCV DNA replication in *Agrobacterium* a model has been proposed postulating that the viral construct must contain an intact *rep* gene whose product would cleave the universal geminivirus nicking site (TAATATTAC) present in a structurally conserved hairpin-loop [6]. A replication-competent construct in *Agrobacterium* must also contain two copies of the viral *ori* including nicking sites so that a unit length viral DNA is released and circularized.

With this background in mind, we tested a number of geminiviral constructs which either complied with or were deficient in terms of the criteria outlined above. All viral constructs produced were tested by agroinoculation in tomato (for TYLCV) or tobacco (for ACMV) and viral symptoms were observed after 3 weeks (data not shown). When pBin19 vector constructs containing virus tandem dimers of TLCV-D1, TYLCV, and ACMV DNA were introduced into *Agrobacterium*, replicative viral DNA species were produced in overnight cultures (Fig. 2, lanes 2, 3, and 5 respectively). The accumulation of these viral DNAs was comparable to our control construct, the TLCV dimer (Fig. 2, lane 1). However, *A. tumefaciens* was not able to support DNA replication of the TYLCV 1.8-mer construct (Fig. 2, lane 4), which contains just one viral *ori* (Fig. 1). This construct was infectious in plants following agroinoculation (data not shown). We speculate that failure to reproduce our original findings with TLCV in other laboratories may have been due to the lack of two geminiviral origins of replication in the DNA constructs employed.

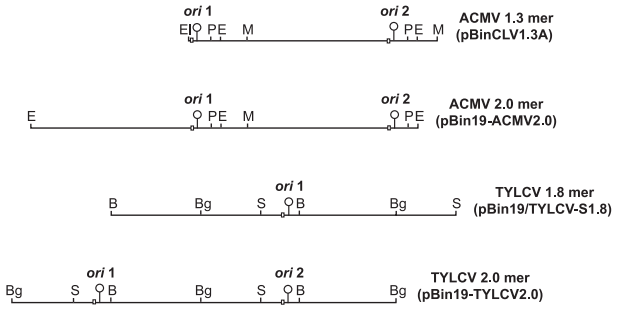

Fig. 1. Linear tandem repeats of ACMV (upper two panels) and TYLCV (lower two panels) DNA cloned into pBin19. The open box (□) upstream of the stem-loop (♀) represents a region containing three iterative elements thought to comprise the Rep protein binding site. Note that the TYLCV 1.8-mer contains only one origin of replication. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RV; EI, *Eco*RI; M, *Mlu*I; P, *Pfl*MI; S, *Sst*I.

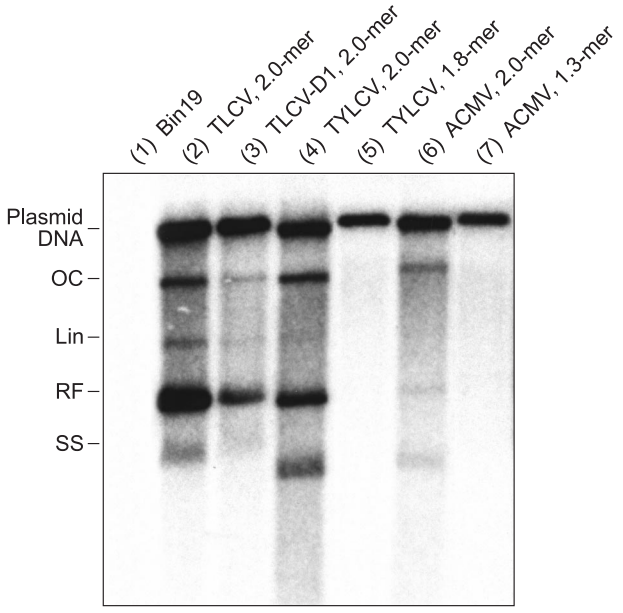

Fig. 2. Virus-specific DNA species produced in *A. tumefaciens* harboring pBin19 containing tandem repeat copies of TLCV, TLCV-D1, TYLCV, and ACMV DNA. DNA extracts from *A. tumefaciens* containing pBin19 geminivirus constructs were analyzed by Southern blotting [4]. Geminiviral DNA forms are marked OC (open circular double-stranded), Lin (linear), RF (supercoiled double-stranded), and SS (single-stranded).

The ACMV 1.3-mer construct (pBinCLV1.3A), which does contain two copies of the stem-loop nicking site, was also unable to replicate in *Agrobacterium* (Fig. 2, lane 6), although in a number of blots trace levels of viral DNA species were visible (data not shown). This result resembles that observed for a TLCV 1.1-mer [6], which lacks one of three iterative elements within the Rep binding region of *ori* 1. Both pBinCLV1.3A and the TLCV 1.1-mer have a relatively short copy of the repeat *ori* 1 (Fig. 1), but unlike the TLCV construct, pBinCLV1.3A appears to contain all of the predicted Rep binding iterons [14]. However, the iterons which make up the high-affinity Rep binding site are only a part of the entire geminivirus *ori* which has a modular structure containing multiple elements [15]. In fact, recent experiments in this laboratory have shown that the removal of these DNA sequences

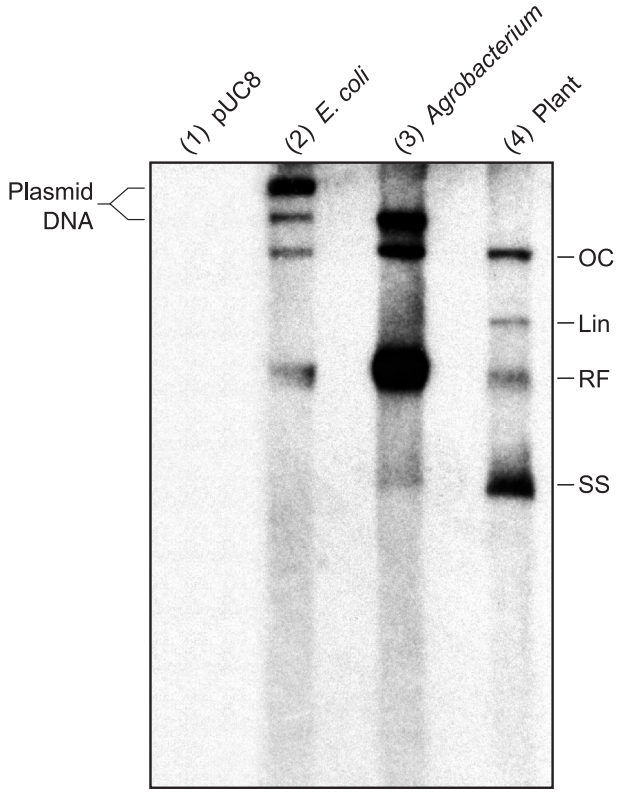

Fig. 3. Accumulation of TLCV DNA species in *E. coli* transformed with pUC8 containing a dimeric head-to-tail insert of TLCV. TLCV DNA obtained from *Agrobacterium* and infected plant tissue is also shown. Geminiviral DNA forms are labelled as in Fig. 2.

has no discernible effect on the replication of TLCV or its satellite in the host plants tested (B. Lin, personal communication). It is therefore possible that the restricted replicative ability of pBinCLV1.3A and the TLCV 1.1-mer is a result of these constructs lacking some specific DNA elements, apart from the Rep binding iterons, in *ori* 1.

In all experiments performed, the quantity of ACMV DNA observed, particularly the ds replicative form (RF), was significantly less than that of the monopartite viruses tested. The A component of ACMV encodes all of the information necessary for viral replication and encapsidation in planta [16]. It seems probable, therefore, that the reduced replicative ability of ACMV reflects some minor differences in the bacterial:viral interaction occurring among the two genera. However, we cannot rule out the possibility that for bipartite geminiviruses, one or both of the B DNA component genes, while unnecessary for efficient replication in planta, may be involved in accumulation of viral DNA in *Agrobacterium*.

The cellular machinery of another bacterium, *E. coli*, is also able to support processes resembling viral DNA replication in plants when transformed with a pUC8 construct containing a tandem dimer of TLCV (Fig. 3, lane 1). In comparison with TLCV DNA replication in *A. tumefaciens* (Fig. 3, lane 2), less replicative form (RF) DNA was observed in relation to the quantity of vector, suggesting that TLCV is less well adapted to the replicative environment of *E. coli*. This is consistent with our previous observations [6] that TLCV replicative DNA forms could not be detected in *E. coli* harboring the low copy number plasmid pBin19 containing a TLCV dimer. It has been proposed that geminivirus progenitors may have arisen from bacterial replicons which were inserted into the host plant genome by *Agrobacterium* and escaped via a DNA release process [6,17]. The finding of TLCV DNA replication in *E. coli*, although not to the level of *A. tumefaciens*, raises the possibility that geminiviruses may also replicate in other bacterial species. It is intriguing to consider that the future evolution of geminiviruses may involve episomal associations with new bacteria, allowing exposure to other species and possibly resulting in host-switching events.

The ability of geminiviruses to replicate in bacteria is further supported by the observation that TLCV promoters are active within these cells. Fig. 4 shows the relative level of expression of GUS by TLCV promoters in *E. coli* and *Agrobacterium*. Each of the promoters exhibit significant activity in *Agrobacterium*, with the putative promoter element of the *rep* gene able to convert the GUS substrate 4-methylumbelliferyl β-glucuronide (MU) at a rate of 7310 μmol/min/mg protein extracted.

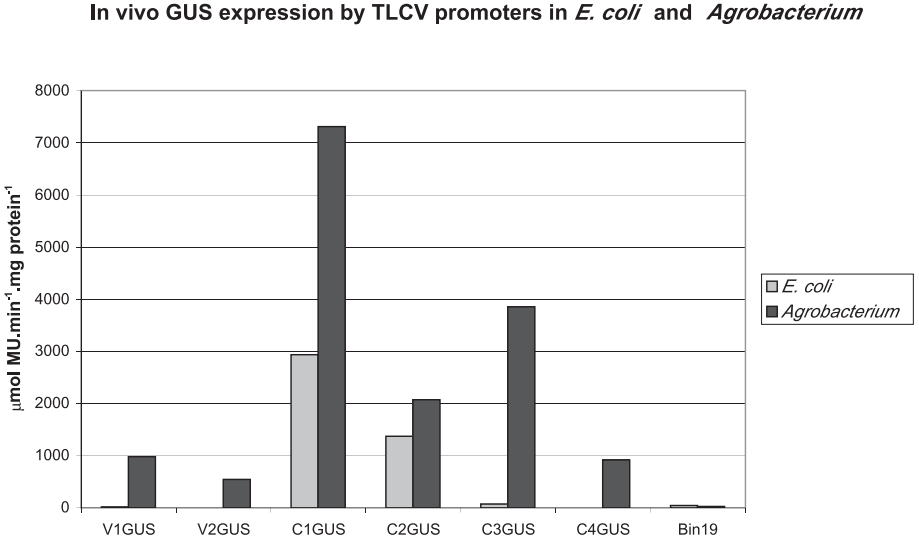

Fig. 4. TLCV promoters are active within bacterial cells. Extracts from *A. tumefaciens* and *E. coli* cells transformed with pBin19 constructs containing GUS-TLCV promoter fusions were analyzed for GUS activity by a fluorometric assay [10].

Detectable levels of GUS expression in *E. coli* were only obtained in cells containing the C1GUS and C2GUS fusions. This is in contrast to an early report which indicated that the tomato golden mosaic virus coat protein promoter actively drives expression of the kanamycin resistance gene in *E. coli* [18], although this discrepancy may simply be a result of the different methods used to measure promoter activity. Since only the C1 gene is required for replication of TLCV DNA in *Agrobacterium*, it is possible that the low-level accumulation of TLCV replicative DNA species in *E. coli* compared to *Agrobacterium* (Fig. 3) is a direct result of the differential expression of this gene in these bacteria. However, it cannot be ruled out that efficient replication of TLCV DNA in *E. coli* requires the presence of other viral gene products. In particular, the lack of GUS expression from the C3 promoter, which drives production of a protein which is known to greatly enhance geminiviral DNA accumulation in planta [19], may be the cause of reduced accumulation of TLCV DNA in *E. coli*.

Our results suggest that the ability of geminiviruses to replicate in *Agrobacterium* is not limited to TLCV and may be a common feature of these plant pathogens. This finding raises the possibility that an in vitro replication system for geminiviruses could be developed using bacterial cell extracts supplemented by viral Rep. Such a tool would prove invaluable for further characterization of geminivirus replication.

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