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Evolution

Plant virus DNA replication processes in *Agrobacterium*: Insight into the origins of geminiviruses?

(tomato leaf curl virus/rolling-circle replication/molecular evolution)

J. E. RIGDEN*, I. B. DRY, L. R. KRAKE, AND M. A. REZAIAN†

Division of Horticulture, Commonwealth Scientific and Industrial Research Organization, GPO Box 350, Adelaide 5001, Australia

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ABSTRACT ***Agrobacterium tumefaciens*, a bacterial plant pathogen, when transformed with plasmid constructs containing greater than unit length DNA of tomato leaf curl geminivirus accumulates viral replicative form DNAs indistinguishable from those produced in infected plants. The accumulation of the viral DNA species depends on the presence of two origins of replication in the DNA constructs and is drastically reduced by introducing mutations into the viral replication-associated protein (Rep or C1) ORF, indicating that an active viral replication process is occurring in the bacterial cell. The accumulation of these viral DNA species is not affected by mutations or deletions in the other viral open reading frames. The observation that geminivirus DNA replication functions are supported by the bacterial cellular machinery provides evidence for the theory that these circular single-stranded DNA viruses have evolved from prokaryotic episomal replicons.**

Geminiviruses are a group of plant viruses characterized by twin-shaped particles that are transmitted in the wild by insects and cause major crop losses worldwide. Their genomes consist of either one or two circular single-stranded (ss) DNA species of 2.5–3.0 kb that are thought to replicate via a double-stranded (ds) intermediate within the plant cell by a rolling circle replication (RCR) mechanism (1, 2) analogous to that found in a class of eubacterial plasmids (3) and in some bacteriophages (4). The only viral ORF absolutely required for replication, the geminiviral replication-associated protein (Rep or C1), has been shown by computer-assisted analysis of all geminiviruses characterized to date to contain three sequence motifs common to RCR initiator proteins of the same eubacterial plasmids and bacteriophages mentioned above (5, 6). However, experimental evidence supporting an evolutionary origin for these DNA viruses as mobilized prokaryotic episomes has so far been lacking.

Most geminiviruses are not mechanically transmissible and are routinely inoculated into host plants in the laboratory by a process that utilizes the ability of the bacterium *Agrobacterium tumefaciens* to transfer cloned tandem repeats of the viral DNA into host cells (agroinoculation; ref. 7). Following agroinoculation, circular, monomeric DNA forms are generated from the tandem repeats by recombination or a replicational release mechanism (1). During the course of our work on the mutational analysis of various ORFs in the geminivirus tomato leaf curl virus (TLCV; refs. 8 and 9), we observed that DNA preparations taken from strains of *A. tumefaciens* that contained cloned tandem repeats of the viral genome in the vector pBin19 also contained monomeric, circular forms of the viral DNA. In the present study we examine the accumulation of these TLCV-specific DNA species in *A. tumefaciens* and demonstrate that the bacterial replication machinery, in association with the viral Rep protein, provides functions to allow the accumulation of these infectious, monomeric viral DNA species within the bacterial cell.

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MATERIALS AND METHODS

Construction of Clones. TLCV ORFs are present on both the virion-sense (encapsidated strand) and the complementary strand of the viral ds replicative form (RF) as shown in Fig. 1. Construction of virion-sense ORF mutants is described in detail in Rigden *et al.* (8). These include frame-shift mutations in the V1 ORF at nucleotide position 153 (V1 mutant), in the V2 ORF at position 663 (V2 mutant) or in both (V1/V2 mutant), and deletion or inversion of nucleotide sequence between these two positions (Fig. 1).

Generation of mutants of the four complementary-sense ORFs (Fig. 1) were carried out by site-directed mutagenesis using an Altered Sites kit (Promega). The C1-mutant was created by changing the ATG initiation codon at nucleotide position 2615 to a TAG termination codon. A premature TAA stop codon was inserted into the C2 ORF by addition of AA after the T nucleotide at position 1525 (Fig. 1*A*). The C3-mutant was produced by a G to A mutation at position 1418 to create a premature TAG stop codon. The C4-mutant was produced by disruption of the ATG initiation codon at 2457, without affecting the coding of the overlapping C1 gene, as described in Rigden *et al.* (9).

Mutants were initially produced as monomers and then cloned as head to tail repeats of 1.1, 1.5, or 2 units, containing either one or two origins of replication (Fig. 1*B*), into the binary transformation vector pBin19 (11). TLCV dimers (2.0 mer) were constructed by ligation of an *Xba*I (wild type) or *Bam*HI dimer (C1-mutant, not shown in Fig. 1) into the complementary cloning sites of pBin19. V1 and V2 mutant (Fig. 1*A*), 1.5-mer, and wild-type 1.5-mer constructs were produced by cloning a *Kpn*I monomer (containing the specific mutations/deletions) into the *Kpn*I site of a pBin19 clone containing a 1246-bp *Kpn*I/*Sal*I fragment of wild-type TLCV, thereby ensuring that the constructs contained only a single copy of the mutated ORF. In the case of the C1-mutant 1.5-mer, the mutant *Kpn*I monomer was cloned into a pBin19 clone containing a 1246-bp *Kpn*I/*Sal*I fragment obtained from the C1-mutant TLCV monomer. Wild-type and complementary-sense ORF mutant 1.1-mer constructs were produced by cloning a *Bam*HI monomer (containing the specific mutations) into the *Bam*HI site of a pBin19 clone containing a 276-bp *Bam*HI/*Hin*dIII fragment of TLCV DNA. The *Hin*dIII site is not present in the wild-type TLCV DNA and was introduced by site-directed mutagenesis.

Abbreviations: TLCV, tomato leaf curl virus; RCR, rolling circle replication; RF, replicative form; Rep, replication-associated protein; ss, single stranded; ds, double stranded.

*Present address: R. W. Johnson Pharmaceutical Research Institute, P.O. Box 3331, Sydney 2001, Australia.
†To whom reprint requests should be addressed. e-mail: ali.rezaian@adl.hort.csiro.au.

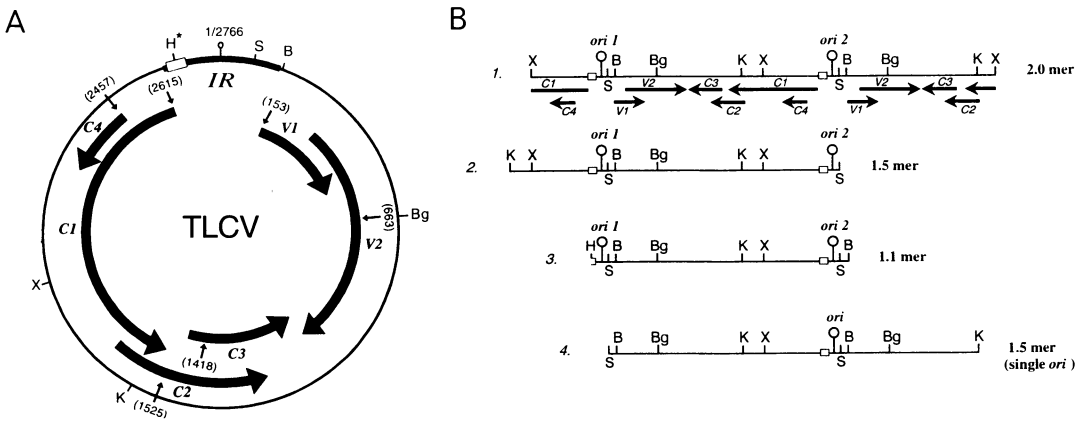

FIG. 1. Genome organization of TLCV and structures of linear tandem repeats used to produce pBin19-based constructs. (*A*) ORFs shown by thick arrows are on both the virion-sense (encapsidated strand) of the viral DNA (V1 and V2) and on the complementary-sense strand of the dsRF (C1, C2, C3, and C4). Small arrows pointing to the ORFs indicate the positions of introduced mutations (nucleotide positions shown in brackets). IR is the intergenic region containing the origin of replication. The open box (□) just upstream of the stem–loop motif (|) represents a region containing three iterative elements proposed as the binding site of the Rep (C1) protein (10). B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; S, *Sal*I; X, *Xba*I. A *Hin*dIII site (H*) was introduced by site-directed mutagenesis at position 2642 to enable construction of the 1.1 mer. (*B*) Linear tandem repeats of TLCV DNA cloned into pBin19. Note that constructs 1, 2, and 3 each contain two *ori* but that the 1.1-mer (no. 3) contains only two of the three iterative elements within the Rep binding region of *ori* 1 (⊐). There is only a single *ori* in construct 4.

Constructs were introduced into *A. tumefaciens* strains C58 and C58C1 and *Escherichia coli* strains DH5α and JM 101 by electroporation with a Gene Pulser apparatus (Bio-Rad) according to the manufacturer's specifications.

Extraction and Analysis of DNA. Total nucleic acids were extracted from TLCV-infected tomato plants (*Lycopersicon esculentum* Mill cv. Rutgers) as described (12). DNA was extracted from bacteria using the miniprep method of Holmes and Quigley (13). Circular, RF viral DNA species were isolated from DNA preparations of *A. tumefaciens* by electrophoresis through 1.2% agarose Tris/acetate/EDTA gels and the excised bands were purified using Geneclean (Bio 101) according to the manufacturers specifications. Southern blot hybridization was performed using 1.2% agarose gels in Tris/borate/EDTA and blotting with 0.4 M NaOH, unless specified otherwise, as described (12). ^{32}P-labeled DNA probes were prepared by a random decamer priming kit (Bresatec, Adelaide, Australia) using a full-length cloned TLCV DNA insert or pBin19 DNA as the templates. TLCV strand-specific probe was synthesized by the same procedure using a mixture of six virion-sense primers interspaced on the viral DNA. The probes were used at a concentration of ≈4000 cpm/ml in hybridization reactions (12).

RESULTS

TLCV DNA Forms in *Agrobacterium*. Southern blot analysis of DNA from *A. tumefaciens* strain C58 harboring the binary vector pBin19 containing tandem repeats of TLCV DNA indicated that the replication of the plasmid constructs within the *Agrobacterium* cell was accompanied by the accumulation of both ss- and dsRF of TLCV DNA (Fig. 2*A*, lanes 6 and 8) identical in size to those found in infected plant tissues (Fig. 2*A*, lanes 2 and 9). The generation of TLCV-specific DNA species occurred reproducibly with 10 different TLCV constructs in *Agrobacterium* (see below), and there was no evidence for the production of these DNA forms in *E. coli* (Fig. 2*A*). The detection of the DNA species designated as single-stranded (Fig. 3*A*) was variable and later found to relate to the condition of electrophoresis. The low level of ssDNA was not related to the method of extraction as phenol extraction of total DNA from lysed cells contained similar relative amount of the DNA forms. The identity of the ssDNA species (Fig. 3*A*) was confirmed by (*i*) strand-specific probing where, unlike other TLCV DNA forms, it did not hybridize to a virion-sense DNA probe (Fig. 3*B*) and (*ii*) by blot hybridization without denaturation where it was the only TLCV DNA form that hybridized to the TLCV probe (Fig. 3*C*).

TLCV-specific supercoiled dsDNA as shown in Fig. 2 was purified from *A. tumefaciens* harboring pBin19 containing a 1.5-mer tandem repeat of the viral DNA and found to give restriction digest patterns identical to those of wild-type TLCV dsDNA with five restriction enzymes (data not shown). Furthermore, agroinoculation of host plants with a tandem dimer of this dsDNA purified from *Agrobacterium* produced infections in host plants identical to those previously described for wild-type virus (12).

Products of Ti Plasmid Vir Genes Are Not Involved in TLCV DNA Generation in *Agrobacterium*. We originally suspected that the appearance of the TLCV DNA forms in the bacterial cells might involve the VirD1,D2 endonuclease encoded by the pTiC58 plasmid from *A. tumefaciens*. The VirD1,D2 endonuclease is responsible for the cleavage of the transferred DNA borders and VirD2 is known to accompany ss transferred DNA covalently linked to its 5′ end into the plant nucleus during *Agrobacterium*-mediated transformation (14). To test for involvement of VirD1,D2, or indeed any other Vir genes of the Ti plasmid, in TLCV DNA production from pBin19, we transformed an identical strain of *A. tumefaciens* that lacked the pTiC58 plasmid (*A. tumefaciens* C58C1) with a wild-type TLCV 1.5-mer construct cloned in pBin19. Southern blot analysis of DNA from this culture produced a pattern of DNA forms (data not shown) indistinguishable from those of the *A. tumefaciens* C58 culture containing the same 1.5-mer construct (Fig. 2, lane 6). Therefore, we conclude that the appearance of the TLCV genomic DNA is independent of the Ti plasmid-encoded DNA processing machinery involved in *Agrobacterium*-mediated transformation.

The Viral *rep* Gene Is Involved in the Production of TLCV DNA Forms in *Agrobacterium*. To investigate the role of viral genes in the accumulation of TLCV DNA in *Agrobacterium*, we examined DNA from *A. tumefaciens* harboring pBin19 containing tandemly repeated TLCV DNA inserts with mutations introduced, separately, into each of the six ORFs (Fig. 1). Constructs containing frame shift mutations, deletions, and inversions within the virion-sense ORFs V1 and V2 accumulated the same TLCV DNA species in *A. tumefaciens* as the wild-type constructs, with the deleted construct producing correspondingly smaller viral DNA forms (Fig. 4*A*). We have previously used these same mutant constructs to demonstrate

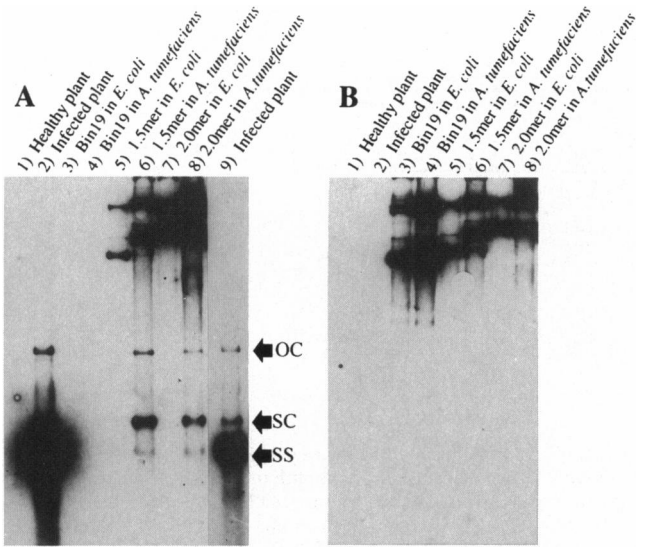

FIG. 2. TLCV-specific DNA species produced in *A. tumefaciens* harboring pBin19 containing tandem repeat copies of TLCV DNA. DNA was extracted (13) from *A. tumefaciens* C58 or *E. coli* DH5α cells containing pBin19/TLCV constructs and analyzed by Southern blotting (12). Blots were probed with either full-length TLCV probe (*A*) or full-length pBin19 probe (*B*) as described. TLCV DNA forms are marked ss, sc (supercoiled double-stranded), and oc (open circular double-stranded).

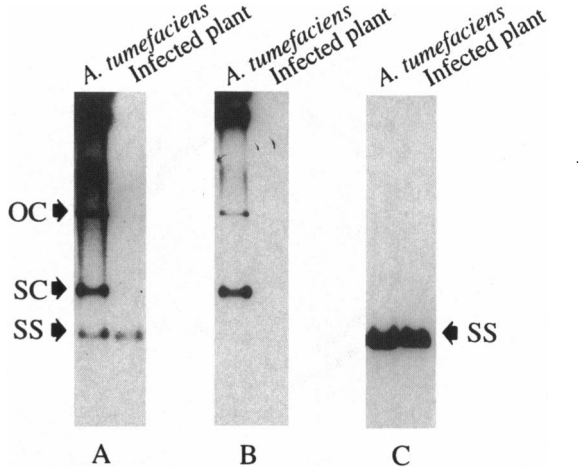

FIG. 3. Identification of TLCV ssDNA produced in *Agrobacterium*. DNA samples from infected plant and from *A. tumefaciens* containing TLCV DNA forms, generated from a 1.5-mer construct, were analyzed by Southern blotting. (*A*) Alkali blotting and probing the denatured DNA with a mixture of virion-sense and complementary-sense ^{32}P-DNA. (*B*) Alkali blotting and probing the denatured DNA with virion-sense ^{32}P-DNA. (*C*) Blotting under nondenaturing conditions with 10× SSC (1.5 M NaCl/0.15 M trisodium citrate) and probing with a mixture of virion-sense and complementary-sense ^{32}P-DNA.

that V1 and V2 are not involved in viral replication in host plants—V1 playing a role in DNA accumulation and/or viral spread and V2, the coat protein, being required for encapsidation of ssDNA and viral spread (8). Similarly, mutations that disrupt the ORFs C2, C3, or C4 (Fig. 1) do not affect TLCV DNA accumulation in *A. tumefaciens* (Fig. 4*B* and data not shown). Previous work has indicated the C4 protein to be a determinant of symptom severity (9), while the C2 is believed to trans-activate the expression of the virion-sense genes (15) and the C3 has been implicated in the regulation of the level of DNA in infected plants (reviewed in ref. 16).

In contrast, mutagenesis of the complementary-sense C1 (*rep*) ORF, which rendered the construct incompetent for replication in host plants when tested by agroinoculation, resulted in a dramatic decrease in the accumulation of TLCV DNA in *A. tumefaciens* (Fig. 4*B*). This observation with two independent constructs of the *rep* gene mutant indicates that the *rep* gene of TLCV is required for the generation of these RFs in agrobacteria. Furthermore, it indicates surprisingly that the TLCV *rep* gene is both transcribed and translated within *A. tumefaciens* and that it forms an active product that functions within the context of the bacterial cell. This suggestion is supported by the high level expression of a bacterial β-glucuronidase reporter gene fused to the putative promoter element of the TLCV *rep* gene. Extracts of agrobacteria harboring the *rep* promoter–β-glucuronidase fusion converted the β-glucuronidase substrate, 4-methylumbelliferyl β-glucuronide, at a rate of 731 μmol/min per mg of protein extracted compared with a corresponding figure of 2.1 for the cells containing pBin19 alone (M.A.R. and I.B.D., unpublished work).

Are the Viral DNA Forms Produced by Homologous Recombination? Although efficient production of TLCV DNAs in *Agrobacterium* was *rep*-gene-dependent, we examined the possible role of DNA recombination in this process. Involvement of recombination would be consistent with the lower level of the viral DNA forms generated by constructs containing a shorter tandem repeat (1.1-mer) of the TLCV genome vs. those containing either a 1.5-mer or a 2-mer. The possible role of recombination was examined by producing a 1.5-mer TLCV DNA containing only a single copy of the viral origin of replication (*ori*) in pBin19 (Fig. 1*B*, construct 4). As expected, this construct in *Agrobacterium* was fully infectious in host plants, but the bacteria did not produce detectable amount of ssDNA or super-coiled RF (Fig. 5). However, a trace amount of TLCV DNA at the position of open circles was observed (Fig. 5). This finding indicates that recombinational release of TLCV DNA may occur in the *rec*A$^+$ *A. tumefaciens* but is not responsible for the production of relatively high levels of TLCV replicative DNA forms.

DISCUSSION

We have observed that *A. tumefaciens* containing tandem repeats of TLCV DNA in the plasmid Bin19 can support processes resembling the viral DNA replication in plants. The TLCV DNAs are produced efficiently in the bacterium only when the constructs contain two copies of the viral *ori* and a wild-type *rep* gene.

The *rep* gene mutant examined contained two base substitutions in its initiation codon. This mutation does not appear completely to prevent the accumulation of TLCV-specific DNAs in *Agrobacterium* (see Fig. 4*B*, lanes 2 and 4). These low levels of circular TLCV DNA species (or indeed the higher levels seen with the wild type) are unlikely to be due to the action of any of the proteins involved in the replication of pBin19, as no TLCV-specific DNAs are seen in *E. coli* strains DH5α or JM101 (*rec*A$^-$ and *rec*A$^+$ respectively) harboring the same pBin19 constructs (Fig. 2). A more likely explanation for the low levels of TLCV DNA forms produced by C1-mutant constructs in *Agrobacterium* is a low frequency recombinational event that has been observed during agroinfection with some geminivirus constructs (1) although internal initiation of translation within the C1 ORF leading to a truncated Rep protein with significantly reduced activity has not been ruled out. It is interesting that the 1.5-mer construct that contains a single copy of the TLCV *ori* shows a similar DNA profile to the C1 mutant. This is consistent with the view that the low level of DNA accumulation is independent of viral functions.

The Rep proteins of geminiviruses have been shown to recognize their respective viral DNAs specifically (17–19). They introduce site-specific nicks into the conserved non-

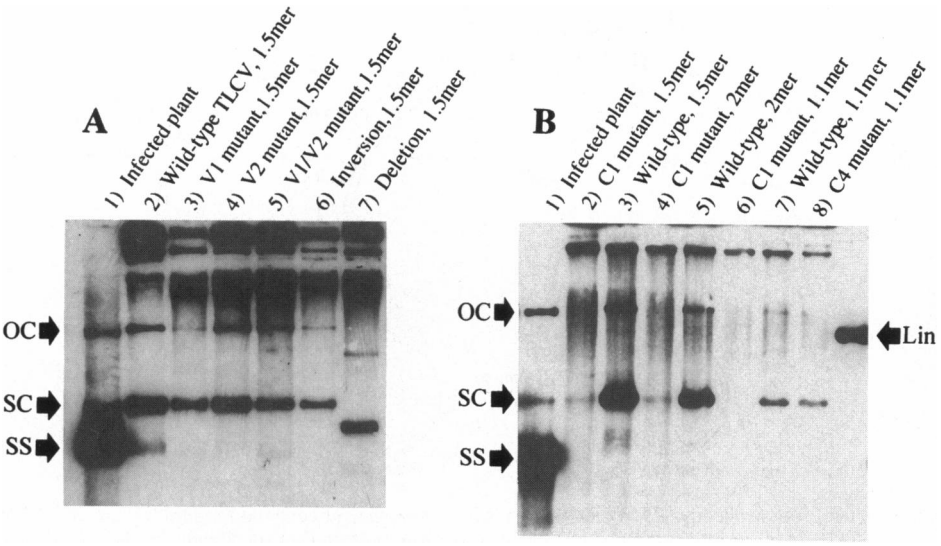

FIG. 4. Rep is the only gene required for the accumulation of TLCV DNA species in *A. tumefaciens* harboring pBin19 containing cloned tandem repeats of TLCV. Southern blot showing DNA from *A. tumefaciens* carrying pBin19/TLCV constructs containing mutations in the virion-sense ORFs (*A*) or complementary-sense ORFs (*B*), hybridized to the full-length TLCV probe. C2- and C3-mutant 1.1-mer constructs in *A. tumefaciens* produced a pattern of TLCV-specific DNA as shown in lanes 7 and 8 (*B*).

anucleotide of a stem-loop motif within the plus strand origin of replication (Fig. 1) and become linked to the 5′ end of the cleaved strand (20). This nicking process is analogous to the initiation of RCR in prokaryotic phage and ssDNA plasmids (3) and the conserved nonanucleotide is similar to the sequence of the nicking site of the DNA of coliphage ΦX174, that is also located in a hairpin structure (21). Synthesis of the geminiviral plus strand DNA is then performed by host polymerases, as is the case for phage and plasmid DNA replication in bacteria (3, 4). It seems likely, therefore, that

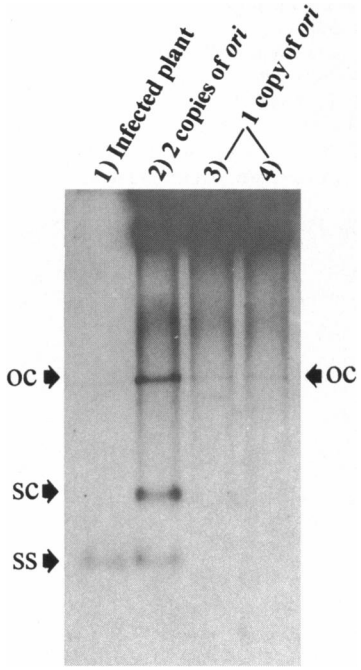

FIG. 5. The presence of two copies of the TLCV *ori* is required for efficient production of the viral DNA forms in *A. tumefaciens*. DNA extracts of agrobacteria containing 1.5-mer TLCV constructs either with two (lane 2) or one copy of the *ori* (lane 3) were analyzed by Southern blotting as in Fig. 1. A separate clone of the construct containing one *ori* was used as a duplicate in lane 4. An extract of TLCV-infected plant was used as ssDNA marker. Structures of the two 1.5-mer DNA constructs have been shown in Fig. 1.

Rep protein of TLCV expressed in *Agrobacterium* initiates the synthesis of plus strand DNA by a mechanism analogous to the initiation of RCR of some bacterial plasmids (3). Furthermore, the presence of both ss- and dsDNA forms implies that like the plant host, *Agrobacterium* also provides functions necessary for the conversion of plus strand DNA to dsRF, involving the recognition of an origin of replication for the minus strand.

Based on these analogies, we propose a model (Fig. 6) for the replicational release of TLCV DNA from pBin19 plasmid in *Agrobacterium* that is consistent with the production of TLCV DNA forms observed. A distinction between TLCV DNA replication in the plant host and its accumulation in *Agrobacterium* is the lower relative levels of the ssDNA forms observed in the bacterium (Fig. 2). This resembles ssDNA levels in plants infected with coat protein mutants of TLCV (8) and may be due to the lack of DNA encapsidation within *Agrobacterium*.

We found that constructs containing the 1.1-mer tandem repeat of TLCV DNA consistently produced lower levels of dsRF in *Agrobacterium* than those containing larger repeats (Fig. 4*B*). Comparison of the constructs in Fig. 1*B* shows that while all contain two copies of the stem–loop structure, the origin of replication preceding the full-length TLCV sequence in the 1.1-mer constructs is truncated. This region of the geminiviral *ori* has been proposed as the binding site for the Rep protein (18) and contains three iterative sequence elements implicated in Rep recognition and binding (10). The 1.1-mer constructs used here appear to lack one of these sequence elements within the *ori* resulting in inefficient initiation of replication from these constructs.

The observation that elements capable of replicating geminiviral DNA are active in prokaryotic cells strongly suggests that these important and often devastating plant pathogen, have evolved from prokaryotic episomal replicons employing RCR. Possible involvement of *Agrobacterium*-like organisms in this transition is a plausible idea. These widely distributed soil-borne plant pathogens are capable of inserting exogenous DNA into the plant genome. An integrated bacterial replicon carrying its cognate *rep* gene would have the possibility of adapting to plant polymerases for replication. Surprisingly, remnants of geminivirus DNA have been identified in the genome of certain geminivirus host plants (22). It is possible that geminivirus progenitors arose from integrated replicons that escaped from the plant genome, by a mechanism similar

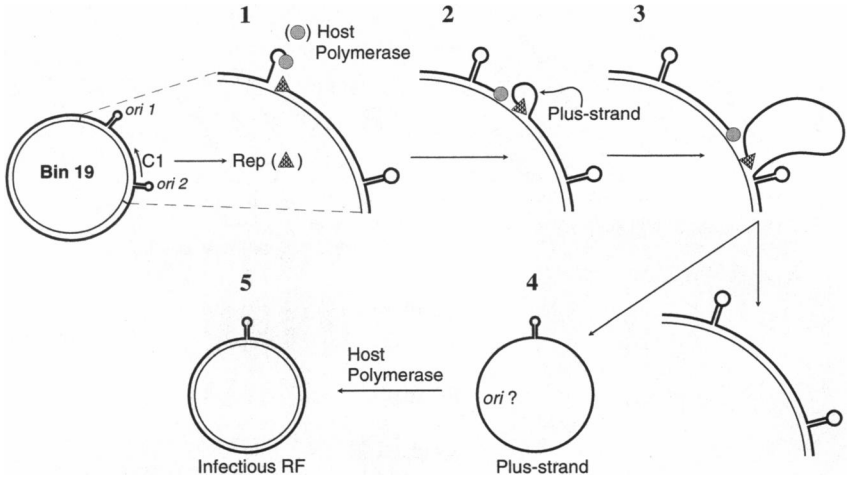

FIG. 6. A model for the production of replicative TLCV DNA forms in *A. tumefaciens* carrying tandem repeats of TLCV DNA in the plasmid pBin19. Expression of the C1 ORF (Fig. 1) results in the production of active Rep protein that specifically binds to a site within the *ori*. Only Rep molecules initiating replication at *ori 1* would generate TLCV DNA copies. Rep nicks the DNA within a conserved nonanucleotide in the hairpin (♀), and binds to the resulting 5′ end (step 1) allowing the extension of the 3′ end by host polymerases and displacement of the plus strand. When the region of plus strand DNA containing *ori 2* is displaced, the Rep molecule introduces a second nick into the DNA while simultaneously ligating the ends of the displaced full-length strand giving rise to circular ssDNA (steps 3 and 4). Production of dsRF by host enzymes may involve an unidentified minus-strand *ori* (step 5).

to viral DNA release that occurs readily during agroinoculation (1). The encapsidation of such replicons may have provided stability in plant cellular environment, and virus spread to other plant hosts may have brought about the diversification observed in today's geminiviruses.

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