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Genetics

Replicational release of geminivirus genomes from tandemly repeated copies: Evidence for rolling-circle replication of a plant viral DNA

(beet curly top virus/plus-strand origin/conserved hairpin/agroinoculation)

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ABSTRACT **_Agrobacterium_-mediated inoculation of _Nicotiana benthamiana_ plants with Ti plasmids containing tandem genome repeats derived from different strains of the geminivirus beet curly top virus (BCTV) resulted in the production of unit-length recombinant progeny genomes in systemically infected plants. When two putative plus-strand origins of replication were present in constructs used as inocula, a replicational escape mechanism was favored that resulted in progeny genomes of a single predominant genotype. The genotype was dependent upon the arrangement of repeated parental genomes in the inocula. Sequencing across the junction between parental BCTV strains in the recombinant progeny allowed mapping of the plus-strand origin of replication to a 20-base-pair sequence within the conserved hairpin found in all geminivirus genomes. In contrast, when inocula contained tandemly repeated BCTV genome sequences but only a single conserved hairpin, a number of different progeny genotypes were simultaneously replicated in infected plants, a result expected if unit-length viral genomes were generated by random intramolecular recombination events. These results and other considerations indicate that geminivirus DNA replication occurs by a rolling-circle mechanism.**

Members of the geminivirus group of plant viruses package their genetic information as circular 2.5- to 3.0-kilobase (kb) single-stranded DNA (ssDNA) molecules within capsids formed from two fused icosahedra (for review, see refs. 1 and 2). Geminiviruses replicate in the nucleus of the infected cell and are thought to employ a rolling-circle mechanism similar to one used by the ssDNA-containing coliphages (e.g., ΦX174) and certain *Staphylococcus aureus* and *Bacillus subtilis* plasmids (e.g., pC194). A number of observations support this hypothesis. (*i*) Circular unit-length and concatameric double-stranded DNA (dsDNA) replicative forms are produced during geminivirus infection (3–5). (*ii*) Putative minus-strand replication origins have been identified in the encapsidated ssDNA (plus strand) of geminiviruses that infect monocots. These are hydrogen bonded to complementary oligonucleotide primers containing several ribonucleotide residues at the 5′ end (6–8). (*iii*) All geminivirus genomes so far examined contain a conserved inverted repeat that may form a hairpin structure. Within the loop of the hairpin is an invariant sequence (5′-TAATATTAC) that resembles the gene A protein cleavage sites of ΦX174 (5′-TG ↓ ATATTAT, where underlined bases are conserved; ref. 9) and pC194 (5′-TG ↓ ATAATAT; ref. 10). In ϕX174 and pC194, site-specific single-strand cleavage provides the 3′-OH terminus needed for the initiation of plus-strand DNA synthesis, and the nick site is, therefore, part of the plus-strand origin of replication. (*iv*) Mutational analysis has demonstrated that the invariant sequence in the geminivirus hairpin is an essential cis element required for viral DNA replication (11). However, despite these observations, the mechanism of geminivirus DNA replication remains to be established.

Previous studies have demonstrated that cloned geminivirus DNAs are infectious as tandemly repeated copies within a plasmid DNA and may be delivered to plants by mechanical inoculation (12–14) or by *Agrobacterium*-mediated inoculation (agroinoculation) when the repeats are present on a Ti plasmid (15–17). In both cases, a unit-length circular viral genome is released that is capable of initiating a systemic infection. Intramolecular homologous recombination or single-strand replication have been proposed as alternative mechanisms for release, and these mechanisms are not mutually exclusive (Fig. 1; ref. 17). Intramolecular homologous recombination resulting from a single cross-over event could occur at random locations within the tandem viral genome repeats. Consequently, a number of recombinant progeny genotypes would be expected if the parental repeated genomes were derived from different distinguishable strains. Direct evidence for release of geminivirus DNA by homologous recombination has been reported (18). Replicational release is proposed to occur by displacement DNA synthesis initiating at a specific nick (the plus-strand origin of replication). This process is functionally equivalent to rolling-circle replication from the native circular dsDNA replicative form, in that a linear ssDNA is generated that is subsequently cleaved from the replication intermediate and ligated to form unit-length circular ssDNA. In a unit-length circular molecule, initiation and termination of ssDNA synthesis should occur at the same site within the plus-strand origin of replication. If two plus-strand origins of replication are present in the same molecule, as is the case in molecules containing tandem viral genome repeats, plus-strand synthesis may initiate at one origin and terminate at the other (19–22). Single-strand replication from a molecule containing tandem genome repeats derived from different viral strains is predicted to generate unit-length progeny genomes of a single predominant genotype.

In this study, we constructed tandemly repeated copies of the beet curly top virus (BCTV) genome in which the repeated copies were derived from the related Logan and Worland strains that differ in nucleotide sequence.† This strategy is based on one used by Grimsley *et al.* (23) to map template-switching events during reverse transcription of cauliflower mosaic virus. Examination of unit-length progeny virus genotypes allowed us to distinguish between prog-

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Abbreviations: BCTV, beet curly top virus; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M74562 for BCTV-Logan and M74563 for BCTV-Worland).

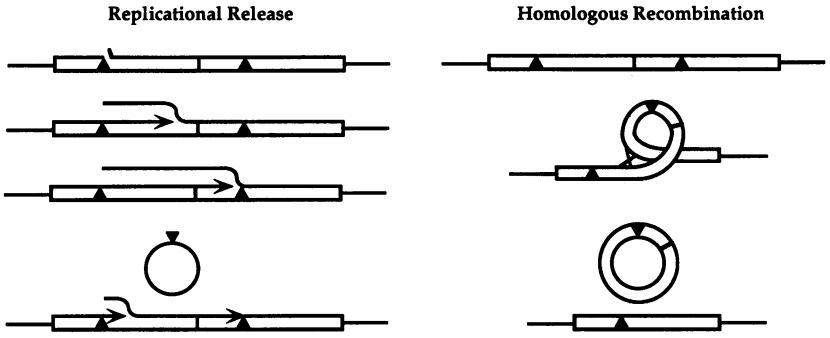

FIG. 1. Models for release of unit-length viral DNA from tandem repeats. The diagram presents two potential mechanisms for the release of unit-length geminivirus DNAs. Tandemly repeated copies in the inoculum can give rise to a circular dsDNA by recombination. Alternatively, replication between plus-strand origins (the conserved hairpins, indicated by solid triangles) may generate a ssDNA that can be converted to circular dsDNA. Replication is initiated at a specific nick in the plus-strand origin. Note that a number of identical progeny genomes could be released by replication from a single tandem repeat.

eny genomes produced from the tandem repeats by intramolecular homologous recombination or replicational release and to map the plus-strand origin in those generated by replication. The results indicate that geminivirus DNAs replicate by a rolling circle.

MATERIALS AND METHODS

Construction of Clones. All restriction endonucleases and DNA-modifying enzymes were used as recommended by the manufacturers. Other techniques were performed essentially as described by Ausubel *et al.* (24). Infectious DNA clones containing one (pLogan and pWorland) or two (pLogan-D and pWorland-D) copies of the BCTV Logan or Worland strain genome have been described (13). Plasmids containing chimeric tandem repeats were constructed by inserting a genome-length *Csp*45I fragment from pLogan-D or pWorland-D into *Csp*45I-digested pLogan or pWorland DNA. The resulting constructs (pWLW.1 and pLWL.1) thus contained two BCTV genome copies derived from different strains (Fig. 2). A plasmid (pWL.1) containing a partial chimeric repeat of the BCTV genome with only a single copy of the conserved hairpin was constructed by deletion of a 1.5-kb *Sal* I–*Csp*45I fragment of pLWL.1 that contained the Logan conserved hairpin (Fig. 2). The chimeric tandem repeats of pWLW.1, pLWL.1, and pWL.1 were transferred into the Ti plasmid binary vector pMON521, a derivative of pMON505 (25), to produce pLWL, pWLW, and pWL. Ti plasmid constructs bearing tandem repeats of Logan or Worland DNA were prepared by transferring the inserts of pLogan-D or pWorland-D into pMON521 to produce pMLogan and pMWorland. The Ti plasmid derivatives were introduced into *Agrobacterium tumefaciens* strain GV3111 containing the disarmed Ti plasmid pTiB6S3 by triparental mating (26). *Nicotiana benthamiana* plants were agroinoculated with Ti plasmids containing wild-type or chimeric tandem repeats as described (17, 27).

Progeny virus genomes were linearized by restriction endonuclease cleavage with *Sal* I (pWLW), *Bam*HI (pLWL), or *Csp*45I (pWL) and recovered from DNA extracts (see below) by gel purification. Linearized genomes were then ligated to pUC8 previously digested with *Sal* I (pWLW), *Bam*HI (pLWL), or *Acc* I (pWL) and used to transform *Escherichia coli* strain DH5αF′. This cloning strategy allowed the recovery of virtually all possible recombinants from pWLW and pWL. However, progeny virus derived from pLWL by homologous recombination within a 1-kb region between the *Eco*RI–*Sal* I sites and containing the *Sst* I site would not have been recovered. pLWL progeny derived from this region were not abundant, since most were resistant to *Sst* I digestion. Infectivity assays of progeny virus clones were performed as described above.

Analysis of BCTV Progeny Genomes. Total nucleic acid was isolated from infected plants essentially as described (28). Systemically infected tissue (5 g), collected and pooled from six to eight plants, was used in each extraction. RNA was precipitated by the addition of 2 M LiCl, and DNA was precipitated from LiCl supernatants with ethanol. The behavior of ssDNA in LiCl was variable; in some cases, LiCl precipitation also removed viral ssDNA. Residual RNA was removed from DNA preparations by digestion with RNase A. DNA (1 μg), with or without prior restriction enzyme cleavage, was analyzed by Southern blot hybridization using ^{32}P-labeled pWLW.1 as a probe. DNA samples (5 μg) were digested overnight with 5–10 units of restriction endonuclease per μg of DNA. To verify complete digestion, 25 μl taken from a 50-μl digest was incubated under the same conditions after the addition of 1 μl (40 ng) of ^{32}P-end-labeled *Hin*dIII-digested λ DNA. The resulting digestion products were

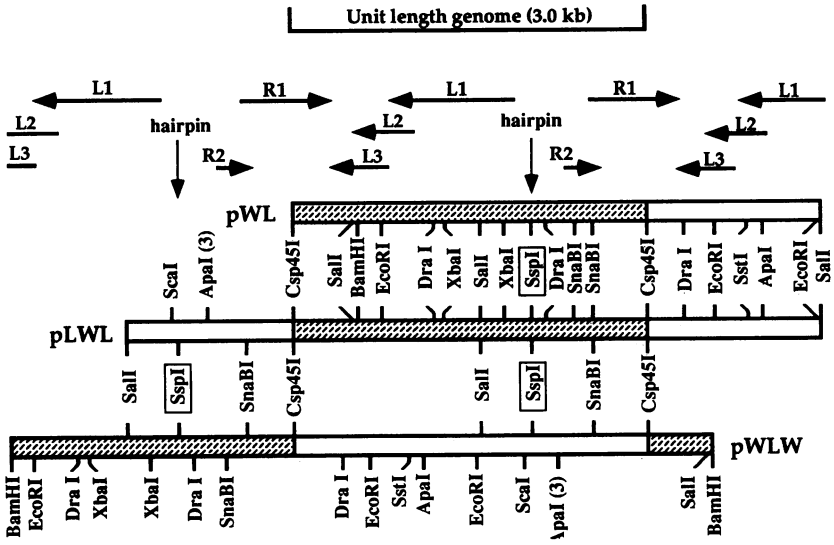

FIG. 2. Maps of BCTV-Logan/BCTV-Worland tandem repeats. Chimeric tandemly repeated copies of BCTV DNA were inserted into the Ti plasmid vector pMON521 (25) for use as inocula. The vector sequences are not shown. Shaded regions represent BCTV-Worland DNA, and open regions denote BCTV-Logan DNA. Horizontal arrows mark the positions of open reading frames as determined from the nucleotide sequence of the Logan strain (unpublished data). Vertical arrows show the locations of conserved hairpins. The boxed *Ssp* I site is contained within the loop of the hairpin.

separated by electrophoresis and visualized by autoradiography.

DNA Sequencing. Selected fragments were subcloned into bacteriophage M13mp18 or M13mp19 vectors (29) and sequenced by the dideoxynucleotide chain-termination procedure (30) using Sequenase version 2.0 (United States Biochemical). The nucleotide sequence of BCTV Logan DNA has been determined in this laboratory (unpublished data) and is similar to the California isolate of BCTV (31). The DNA sequence of the hairpin region of BCTV Worland was determined in this study.

RESULTS

Infectivity of Recombinant BCTV DNAs. Two plasmids containing the complete genome of BCTV-Logan and BCTV-Worland in inverse order (pWLW and pLWL) and a third plasmid containing the complete Worland sequence but only half of the Logan sequence (pWL) were constructed and are shown in Fig. 2. All three plasmids were infectious when delivered to *N. benthamiana* plants by agroinoculation (Table 1) and produced symptoms similar in appearance to those observed with wild-type BCTV-Logan or BCTV-Worland. The results of Southern blot hybridization analysis of DNA isolated from plants inoculated with pWLW or pLWL are shown in Fig. 3. In both DNA preparations, genome-length (3.0 kb) supercoiled and open circular forms of BCTV dsDNA were detected. In this experiment, LiCl precipitation removed ssDNA from the preparation obtained from pLWL-inoculated plants but not from the preparation obtained from pWLW-inoculated plants. Plants inoculated with pLWL also contained additional virus-specific subgenomic DNA forms that migrated more rapidly than unit-length BCTV DNAs. The subgenomic DNAs exist in at least two major size classes (≈1.5 kb and 1.7 kb), each of which is represented by circular ss- and dsDNA forms. We have observed that subgenomic viral DNA molecules routinely arise *de novo* after inoculation of *N. benthamiana* with wild-type BCTV-Logan. In a separate communication, we characterize (unpublished data) BCTV subgenomic DNAs as specific deletion derivatives that likely are produced after the initial release of unit-length viral genomes from the inoculum. The presence of these subgenomic DNAs somewhat complicated restriction analysis of progeny virus populations from pLWL-inoculated plants (see below).

One Progeny Virus Genotype Predominates When Inoculum DNA Contains Two Conserved Hairpins. Restriction endonuclease analysis of DNA from plants inoculated with pWLW and pLWL indicated that progeny virus genomes were comprised of nearly homogeneous populations with little restriction fragment polymorphism evident within a sample (Fig. 3). The virus populations derived from pWLW and pLWL were distinct, but in both restriction sites located between the two conserved hairpins in the inoculum DNA were present in nearly all progeny genomes, whereas restriction sites flanking the hairpins were not. For example, progeny virus DNA of pWLW was linearized by digestion with *Sca* I, but progeny virus DNA of pLWL was not cleaved by this enzyme (Fig. 3, lanes 6). Progeny virus of pWLW contained two *Sna*BI sites, resulting in the production of a 2.7-kb fragment and a 0.3-kb fragment (visible only upon overexposure), whereas progeny virus of pLWL contained a single *Sna*BI site resulting in a 3.0-kb full-length linear fragment (Fig. 3, lanes 7). Progeny DNA of pWLW did not contain either of the *Xba* I sites unique to BCTV-Worland DNA, whereas *Xba* I digestion of pLWL progeny virus DNA resulted in the appearance of the 2.4-kb and 0.6-kb fragments expected from BCTV-Worland (Fig. 3, lanes 5). Thus, restriction enzyme digestion indicated that viral genomes released from pWLW and pLWL inoculum DNAs were predominantly of a single genotype and contained BCTV sequences between the conserved hairpins, regardless of the strain of origin.

Table 1. Infectivity of Ti plasmids containing wild-type or chimeric tandem repeats of cloned BCTV DNA delivered to *N. benthamiana* plants by agroinoculation

Ti plasmid	BCTV strain	Infectivity*
pMLogan	Logan wild type	16/16
pMWorland	Worland wild type	16/16
pWLW	Worland/Logan	16/16
pLWL	Worland/Logan	16/16
pWL	Worland/Logan	14/16
pMON521	None	0/8
pMWLR10	Worland/Logan	3/8
pMLWR2	Worland/Logan	8/8

*Number of plants infected/number of plants inoculated.

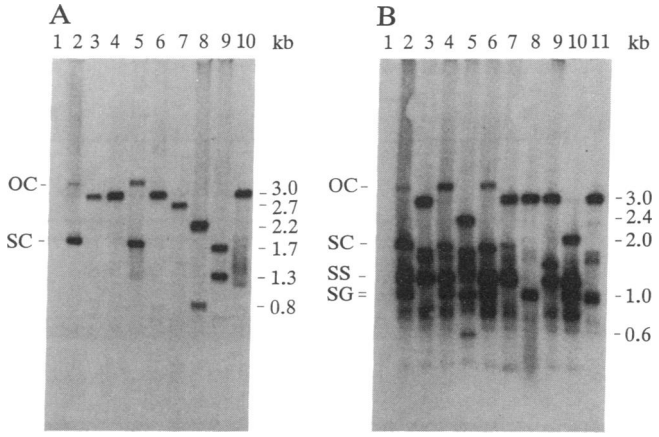

FIG. 3. Southern blot hybridization analysis of progeny virus DNA populations. DNA samples (1 μg) isolated from infected plants were hybridized with a ^{32}P-labeled nick-translated probe prepared with pWLW.1 DNA. (*A*) DNA from *N. benthamiana* plants inoculated with pWLW. (*B*) DNA from plants inoculated with pLWL. Lanes: 1, DNA isolated from mock-inoculated plants; 2, uncut DNA from inoculated plants. Remaining lanes contain DNA samples digested with the following restriction enzymes. Lanes: 3, *Csp*45I; 4, *Sst* I; 5, *Xba* I; 6, *Sca* I; 7, *Sna*BI; 8, *Eco*RI; 9, *Dra* I; 10, *Sal* I; 11, *Bam*HI. The positions of supercoiled (SC), open circular (OC), single-stranded (SS), and supercoiled subgenomic (SG) viral DNA forms are indicated to the left. The sizes of restriction fragments generated from unit-length BCTV genomes are indicated to the right. Lanes 3–11 in *B* contain a number of fragments generated from subgenomic DNA. For example, full-length linear subgenomic DNAs (≈1.5 and 1.7 kb) are apparent in lanes 3, 4, 5, 7, and 11. Other lanes may contain open circular subgenomic DNAs or fragments arising from multiple cleavage.

The presence of minor bands in some restriction digests indicates that a small portion of progeny virus genomes (<5%) had a genotype different from the majority of the population. This is most clearly seen in the pWLW progeny (Fig. 3*A*), since the issue of minor bands is confused by the appearance of restriction products derived from subgenomic DNAs in progeny from pLWL (Fig. 3*B*). In any event, genotype variants within a population are most likely generated by homologous recombination between various sites in the repeated parental genomes of the inoculum DNA.

A Parental Strain Sequence Junction Is Located in the Conserved Hairpin. The genotypes of progeny virus from plants inoculated with pWLW and pLWL were determined at higher resolution by restriction analysis of unit-length clones recovered from infected plants. The results of this analysis are summarized in Fig. 4. Progeny virus clone pWLR10 and five similar but independent clones recovered from pLWL-inoculated plants contained a Logan/Worland sequence

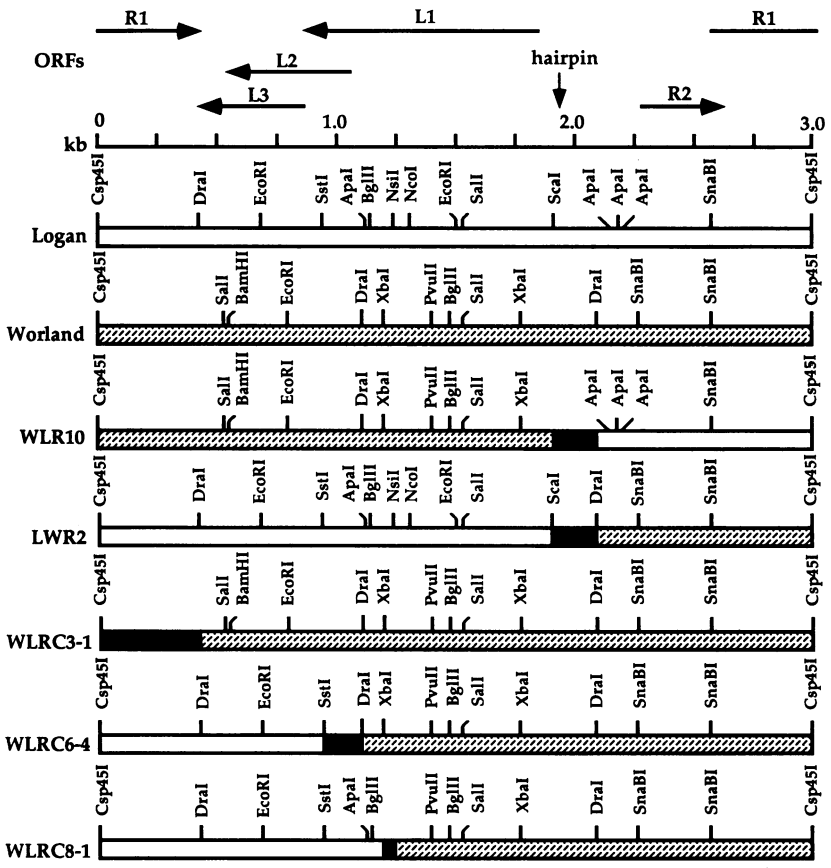

FIG. 4. Restriction maps of unit-length progeny virus genomes recovered from inoculated plants. Unit-length BCTV genomes cloned from infected plants are shown in linear form. Linear maps of wild-type BCTV-Logan (open regions) and BCTV-Worland (shaded regions) are shown for comparison. Solid boxes denote regions where strain junction sequences were mapped by the presence or absence of restriction sites. Horizontal arrows above the maps show the positions of BCTV open reading frames (ORFs), and the location of the conserved hairpin is indicated by the vertical arrow.

junction that mapped to a 200-base-pair (bp) region that includes the conserved hairpin. Restriction analysis of progeny virus clone pLWR2 and five independent clones recovered from pWLW-inoculated plants indicated that these also contained a Logan/Worland sequence junction within the same 200-bp region. However, the genomes represented by pWLR10 and pLWR2 were clearly distinct in that the contribution of DNA sequences from the parental strains was reversed. A second sequence junction between Logan and Worland DNA was located in all progeny virus clones at the *Csp*45I site used to construct the chimeric tandem repeats in the inoculum molecules. The recombinant BCTV DNA inserts of pWLR10 and pLWR2 were reintroduced as tandem repeats into the Ti plasmid vector pMON521, and the resulting constructs (pMWLR10 and pMLWR2) were found to be infectious after agroinoculation of *N. benthamiana* plants (Table 1).

Nucleotide sequencing of the conserved hairpin region in progeny virus clones allowed Logan/Worland sequence junctions to be determined to the nearest different base. As shown in Fig. 5, the junction was mapped to a 20-bp sequence within the conserved hairpin itself. In pLWR2, the sequence upstream of this 20-bp sequence is identical to strain Logan, whereas the sequence downstream is identical to strain Worland. Precisely the inverse result was observed with pWLR10.

In contrast to the homogeneity observed in progeny virus clones recovered from plants inoculated with pWLW and pLWL, those recovered from plants inoculated with a construct containing a single conserved hairpin (pWL; Fig. 2) were not of a single predominant genotype. Instead, the location of the junction between parental virus strains was variable. Three of five independent progeny virus clones examined, pWLRC3-1, pWLRC6-4, and pWLRC8-1, contained a Logan/Worland sequence junction at different locations, and two additional clones appeared to be similar to pWLRC6-4 at the level of restriction analysis (Fig. 4).

DISCUSSION

We have investigated the mechanism by which unit-length geminivirus genomes are released from inoculum molecules containing tandemly repeated viral genome copies. The experiments were performed using the repeated genomes of two

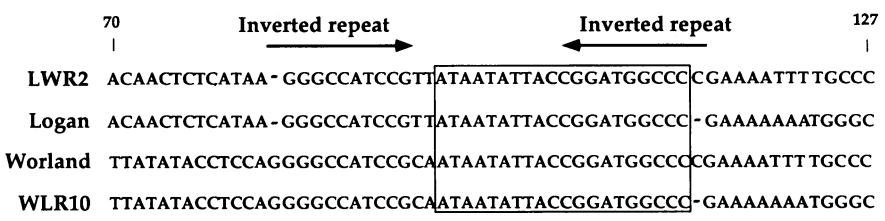

FIG. 5. Nucleotide sequences of progeny virus conserved hairpin regions. The conserved hairpin regions (delimited by inverted repeats) of wild-type BCTV strains Logan and Worland and chimeric progeny virus genomes LWR2 and WLR10 (three clones of each type) are shown. The box encloses the 20-bp sequence in which the strain junctions in LWR2 and WLR10 are located. This sequence contains the TAATATTAC motif found in all geminivirus genomes. The nucleotide sequence presented represents the encapsidated viral (plus) strand. Nucleotide 1 is the first nucleotide upstream of the open reading frame L1 start codon in BCTV-Logan.

BCTV strains that are distinguishable by restriction site and DNA sequence differences, and so it was possible to determine the genotypes of progeny virus molecules with precision. It was found that when the inoculum DNA contained two copies of the conserved hairpin present in all geminivirus genomes, progeny virus populations were comprised of a single predominant genotype. This result is predicted if viral genomes are released by a replicative mechanism. The junction between parental strain sequences in progeny virus genomes generated by replication was located within a 20-bp region that includes part of the conserved hairpin. In contrast, when the inoculum DNA contained repeated viral genome sequences but only a single copy of the conserved hairpin, virus populations consisted of a mixture of genotypes, suggesting that progeny virus was released from the inoculum by intramolecular homologous recombination. Similar results, but with lower resolution, have been obtained in experiments with tandemly repeated copies of tomato golden mosaic virus DNA, where one copy was physically marked by the removal or introduction of restriction sites (32).

These data suggest that unit-length viral genomes are released from the tandem genome repeats by rolling-circle replication and that the conserved hairpin is part of the plus-strand origin of replication. Several lines of evidence support this hypothesis. (*i*) The 20-bp junction sequence identified in recombinant BCTV progeny genomes includes a portion of the conserved hairpin that contains the invariant geminivirus sequence motif TAATATTAC, which is an essential cis element required for DNA replication (11). The requirement of this sequence for replication and its presence within the sequence junction is unlikely to be a coincidence. (*ii*) The TAATATTAC sequence strongly resembles sequences found at the plus-strand replication origins of bacteriophages and plasmids that are known to replicate by a rolling-circle mechanism (9, 21, 22, 33). (*iii*) The replication of sequences located between two plus-strand origins present in the same DNA molecule is an established property of rolling-circle replication (19–22).

There are perhaps other possible reasons why a single genotype might predominate when two conserved hairpins are present in the inoculum. First, the 20-bp sequence within the hairpin may be a "hot spot" for recombination. Although this cannot entirely be ruled out, some experimental evidence argues against this possibility. We have observed that tandem repeats in pWLW are occasionally unstable in *recA*$^+$ *E. coli* (strain MM294), and plasmids that have suffered a spontaneous deletion of a unit-length copy of BCTV DNA are generated at low frequency. The deletions are presumably the result of intramolecular homologous recombination, and restriction mapping has located the recombination sites in two such plasmids to different sequences within the L1 open reading frame (unpublished data). This suggests that, in *E. coli* at least, the conserved hairpin is not recognized as a recombination hot spot. The predominance of a single genotype in progeny virus populations might also be due to enhanced replication of progeny genomes generated by recombination within the conserved hairpin. Again, this possibility is considered unlikely. Viral replication is expected to be a selective and highly competitive process, and one would not expect alterations within a highly conserved sequence necessary for DNA replication to provide some selective advantage.

Results obtained using pWL as inoculum demonstrated that when replicational release was precluded, unit-length viral genomes were released from tandem genome repeats by intramolecular homologous recombination. It is important to note that even in cases where replicational release was possible evidence of minor progeny genotypes that probably arose by recombination was found. The simplest interpretation of these results is that replication is a more efficient method for generating unit-length viral genomes than recombination and, when both are possible, release by replication prevails. This is in accord with earlier tomato golden mosaic virus agroinoculation studies, in which plants inoculated with constructs containing two conserved hairpins displayed symptoms of infection more rapidly than those inoculated with constructs containing only one conserved hairpin (17).

In summary, the results presented in this communication indicate that infectious geminivirus genomes may be released from tandemly repeated genome copies by a single-strand replication mechanism. We conclude that the mechanism employed is rolling-circle replication and that initiation of plus-strand synthesis occurs at some point within a 20-bp sequence that is part of the conserved hairpin and includes the invariant TAATATTAC sequence found in all geminiviruses.

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