Nucleic Acids Research, Vol. 20, No. 15 4047–4054

Trans replication and high copy numbers of wheat dwarf virus vectors in maize cells

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Received April 2, 1992; Revised and Accepted July 2, 1992

ABSTRACT

The replication of shuttle vectors derived from Wheat Dwarf Virus, a monopartite geminivirus, was studied in cultured maize endosperm cells, and in the Black Mexican Sweet (BMS) maize cell line. Using *in vivo* labeling and DNA methylation analysis, we showed that replication was initiated within 24 hrs after transfection, and did not require cell division in both cell lines. Copy numbers of 30,000 ds DNA molecules per cell were observed in endosperm cells after three days. The replication protein was shown to act in *trans*, since the wild type gene of the shuttle vector enabled replication-deficient vectors carrying mutated genes to replicate. These properties suggest that WDV may have similar applications in plants as SV40 in mammalian cells.

INTRODUCTION

Geminiviruses are characterized by one (monopartite) or two (bipartite) circular single stranded (ss) genomes approximately 2.5–3.0 kilobases (kb) in size (1–3). The monopartite genomes of wheat dwarf virus (WDV), maize streak virus (MSV), and digitaria streak virus (DSV) are similarly organized (4–8). Four open reading frames (ORFs) diverge bidirectionally from a large intergenic region. ORF I, encoding a 10 kilodalton (kDa) protein (9), and ORF II, encoding the viral coat protein (10), are present on the viral- or plus-strand. Neither protein is required for replication but both are essential for systemic spread (11–13). A gene present on the minus-strand, comprised of ORFs III and IV, encodes the replication protein. Joining of the ORFs by intron splicing results in a 40 kDa fusion protein necessary for viral replication (14, 15). Two intergenic regions are present in the monopartite genome. The starting intergenic region (IRS) contains promoter sequences for bidirectional transcription (14, 16, 17), and a potential stem-loop structure. This region of the genome is thought to function as an origin of replication for viral strand synthesis, in analogy to the bipartite tomato golden mosaic virus (TGMV) (18). The other intergenic region, the terminating intergenic region (IRT), contains polyadenylation signals, and a region to which a short complementary primer for second strand synthesis binds (19, 20).

Geminiviruses have attracted interest as replicating vectors, because they are the only class of plant viruses that propagate in the plant cell nucleus and depend solely on DNA-dependent DNA polymerases for their replication. The presence of double-stranded (ds) circular intermediates has suggested a rolling circle mode of replication. Evidence consistent with this mechanism was recently reported for beet curly top virus (BCTV) (21) and African cassava mosaic virus (ACMV) (22), a monopartite and bipartite virus, respectively. In this regard, geminiviruses may be analogous to the coliphages ϕX174 and M13, and their replication proteins may resemble the ϕX174 gene A or M13 gene II products. These are site-specific topoisomerases required for the production of ds replicative forms, and ss DNA (23). For bipartite geminiviruses, which contain two genomes (A and B), DNA A can replicate in single cells, but DNA B requires a gene product of DNA A for replication (24, 25). Therefore, the replication protein encoded by DNA A must act in *trans*. Mutations in the replication protein gene of TGMV and ACMV can be complemented in *trans* by the wild type gene (26–28). These mutations are thus similar to M13 gene II mutations. In contrast, mutations in the ϕX174 gene A have the unique property of being *cis* dominant (29).

Infection without the insect vector is possible only on dicotyledonous hosts, and occurs at low efficiency. Therefore, viral DNA(s) is usually introduced by agroinfection or transfection. In agroinfection, the viral genome is introduced into the plant as a tandem repeat that yields monomers either by homologous recombination, or via a replicative mechanism (e.g. 30). In transfection, linear monomers that require circularization by cellular ligases, or dimers are used (e.g. 31). Monomers require release from the *E. coli* vector prior to transfection. To simplify transfection, we developed an *E. coli*-plant cell shuttle vector utilizing WDV sequences and the p15A plasmid origin of replication (32).

Here we present a detailed analysis of the replication of this shuttle vector and its derivatives in maize tissue culture cells. We show that replication is independent of cell division and that the replication protein of WDV can function in *trans*. Preliminary data supporting *trans*-acting replication has been reported elsewhere (33). More interestingly, we found that the copy number of the shuttle vector was very high, of the order of 30,000 copies per cell.

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METHODS

Construction of plasmids

Plasmid pFF19G is a pUC120 (34) based vector containing the β-glucuronidase (GUS) coding region under control of the CaMV 35S regulatory sequences (35). The construction of plasmids pWI-11, pWI-Δ and pWI-GUS was described previously (32). The latter was constructed by insertion of the GUS expression cassette from pFF19G into the unique *Bam*HI site of pWI-11. Plasmid pWIΔ2G, a derivative of pWI-GUS, contains a frameshift mutation in ORF III of the WDV replication protein gene. This mutation was created by digesting pWI-GUS at its unique *Hin*dIII site and filling in the 4 nucleotide protruding ends with Klenow fragment followed by religation of the obtained fragment. A second replication-deficient vector, pWIΔ3G, was constructed in the following manner. pWI-11 was digested partially with *Aha*II and completely with *Hin*dIII so that a 920 bp fragment containing ORF IV and most of ORF III was removed. The remaining 2.8 kb fragment was ligated to the *Cla*I-*Hin*dIII flanked GUS cassette from p20R-35SG, a pIC20R (36) derivative containing the same CaMV 35S-GUS fusion as pFF19G. Plasmids were constructed and propagated in *E. coli* strain JV30 (37).

Protoplast preparation and transfection

The maize endosperm tissue culture was propagated as described (38). Protoplasts were isolated, electroporated and cultured as described (32, 38). The BMS suspension culture was maintained by subculturing every 2−3 days and protoplasts were prepared, electroporated and incubated according to Fromm et al. (39). Typically, 50 μg of CsCl-purified DNA was electroporated into 10^6 protoplasts at 150 V with a 1450 μF capacitor. In experiments where two constructs were co-electroporated, 50 μg of each DNA was used.

DNA isolation and southern analysis

Protoplasts were collected after the appropriate culture period, washed once with protoplast isolation solution, and total protoplast DNA was prepared (40). Supercoiled DNA was isolated by CsCl equilibrium centrifugation (41). Routinely, 1/20 or 1/40 of the DNA sample was used for Southern analysis. The DNA was fractionated on a 1.0% agarose gel, transferred to Nytran membrane (Schleicher and Schuell) and analyzed by hybridization using the appropriate ^{32}P-labeled probe as described (41).

In vivo labeling of protoplast DNA

For *in vivo* labeling, 10^6 protoplasts were electroporated and cultured as above, and 18 hrs prior to harvesting, 0.5 mCi ^{3}H-thymidine (70−90 Ci/mmol) was added. Supercoiled DNA was isolated, and microdialysed against TE-buffer (10 mM Tris pH = 8.0, 1 mM EDTA) for 30 minutes. The DNA samples were subsequently digested with *Bam*HI. Usually, 1/2 or 1/4 of the sample was electrophoresed on 1% agarose and blotted onto Nytran membrane. The membrane was sprayed with En^{3}hance (NEN DuPont) to enhance the radioactive signal, and exposed to x-ray film for 4 days−2 weeks. After exposure the membrane was washed twice in 2×SSC, 0.5% SDS solution for 20 min at room temperature and was subsequently used for hybridization.

β-Glucuronidase (GUS) enzyme assays

Transfected protoplasts were collected at various timepoints and cell lysates were prepared as described (32). The protein concentrations in the homogenates were determined using the Biorad Protein Assay Kit. The GUS activity was determined fluorimetrically, using 4-methyl umbelliferyl glucuronide (MUG) as a substrate. One hundred μg of protein from each protoplast extract was assayed (42). Fluorescence was measured with a Hoefer Fluorometer (model TKO 100) with excitation at 365 nm and emission at 460 nm. Fluorescence intensity was calibrated with 4-methyl umbelliferone standards. Since a background level of GUS activity is present in some tissues, the GUS activity of all samples was standardized against the activity obtained from mock-electroporated protoplasts. The histochemical GUS assay was also performed according to Jefferson (42). 10^6 protoplasts electroporated with 50 μg pWI-11 or pWI-GUS were aliquoted over 5 Petri-dishes containing 5 ml of the appropriate culture medium. After a 3 day culture period, 500 μμ of a 10×GUS substrate mixture ($NaPO_4$ buffer 0.5 M, pH 7.0, potassium ferricyanide 1 mM, potassium ferrocyanide 1 mM and X-Gluc 3% w/v) was pipetted onto the cells. Following an overnight incubation at 37°C, cells expressing GUS were counted under a microscope.

RESULTS

Replication of the shuttle vector in endosperm cells is independent of cell division

The construction of the shuttle vector, pWI-11, is shown in Figure 1 (32). The coat protein coding region of WDV was replaced by the neomycin phosphotransferase type II (NPT II) gene, creating a gene fusion which can serve as a selectable marker in both plant cells and *E. coli* (Fig. 1B). A replication-deficient control plasmid, pWI-Δ (Fig. 1C), was constructed by a deletion that removes the 5′-end of the replication protein gene and disrupts the conserved stem-loop structure in the intergenic region.

The replication of these vectors was tested by *in vivo* labeling and DNA methylation analysis in non-dividing protoplasts from maize endosperm tissue culture (38). For all *in vivo* labeling experiments, electroporated protoplasts were labeled with ^{3}H-thymidine for the last 18 hrs of culture. Supercoiled DNA was isolated from all samples to reduce the background from genomic DNA, and digested with *Bam*HI, which reduced open circular, closed circular, and multimeric forms to a single fragment. Plasmids pWI-11 and pWI-Δ were linearized by *Bam*HI resulting in 3.7 kb and 3.3 kb bands, respectively. The left panel in Figure 2 shows that after 3 days of culture, only pWI-11, and not pWI-Δ, incorporated ^{3}H-thymidine, as indicated by the 3.7 kb band (lane 11). A faint band was observed at the corresponding position with pWI-Δ (lane Δ), but this did not correspond to pWI-Δ DNA since the size of this plasmid was 3.3 kb. In addition, when the same filter was probed with pWI-11, no hybridization signal was obtained at the appropriate position (Fig. 2 middle panel). A major labeled product in these experiments was the 1.9 kb mitochondrial plasmid, which gave 1.6 kb and 0.3 kb bands with *Bam*HI (43). The signal obtained from the mitochondrial plasmid was similar in the two lanes, ruling out differences in sample loading and cell viability (Fig. 2 left and right panels).

Similar experiments showed that newly synthesized pWI-11 molecules could be detected one day after electroporation, and that replication continued for at least 10 days (data not shown). No ^{3}H-thymidine incorporation into the replication-deficient plasmid pWI-Δ was observed. The onset of replication was further determined by Southern analysis using the methylation-

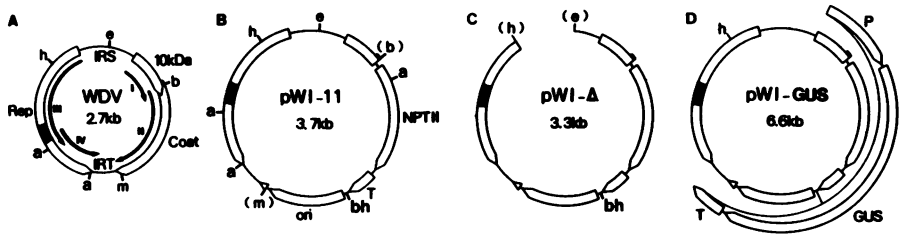

Fig. 1. The WDV Genome and Derived Shuttle Vectors. (**A**) The WDV genome. ORFs are numbered I through IV and their orientations are indicated by arrows. Three WDV genes are also indicated: Rep, replication protein gene containing one intron (shaded); 10 kDa, 10 kDa protein gene; Coat, coat protein gene; IRS starting intergenic region; IRT, terminating intergenic region; a, *Aha*II sites; b, *Bst*EII; e, one of five *Eco*O109I sites, other four sites are located between this site and the *Hin*dIII site; h, *Hin*dIII; m, *Mlu*I. (**B**) pWI-11. NPT II, neomycin phosphotransferase type II coding region; ori, replication origin of plasmid p15A; T, terminator of CaMV 35S transcript; bh, *Bam*HI. (**C**) pWI-Δ. DNA fragment shown here was blunt-ended and religated. (**D**) pWI-GUS. P, CaMV 35S promoter with duplicated enhancer; GUS, β-glucuronidase coding region; T, terminator of CaMV 35S transcript.

sensitive restriction enzymes *Bcl*I and *Dpn*I. *Bcl*I cannot digest DNA from *dam*$^+$ *E. coli*, whereas *Dpn*I requires methylation by *dam* methylase. Using these enzymes, replicated DNA may be distinguished from input DNA. One day post transfection, about a third of the pWI-11 DNA was digested with *Bcl*I (B), and DNAs resistant to *Dpn*I (D) were found (Fig. 3A). Upon further culturing, the vector DNA became fully cleavable with *Bcl*I and fully resistant to *Dpn*I. The replication-deficient vector pWI-Δ did not show this effect (Fig. 3B). This control was required since demethylation can occur in the absence of replication (44, and references therein). Concatenated forms of pWI-11 found in *E. coli* (Fig. 3C; 32) were resolved by replication into monomers (Fig. 3A), whereas pWI-Δ remained concatenated (Fig. 3B). These results confirmed that replication was initiated within 24 hrs post electroporation.

Since pWI-11 continued to replicate for 10 days after electroporation, we determined whether this resulted in a corresponding increase in copy number. Endosperm protoplasts electroporated with pWI-11 or pWI-Δ were harvested after culturing periods of 1, 2, 3, 5, 7 or 10 days. Supercoiled DNA was digested with *Bam*HI and probed sequentially with pWI-11 (Fig. 4A) and the mitochondrial plasmid (Fig. 4B). The copy number of pWI-Δ decreased with time relative to the mitochondrial plasmid, but remained detectable for approximately 3 days after electroporation. In contrast, pWI-11 accumulated relative to the mitochondrial plasmid. Southern analysis using a probe for a nuclear gene, the 10 kDa zein gene (45), showed that the copy number of the mitochondrial plasmid did not change over time (data not shown). Therefore, this plasmid was a good indicator of the number of protoplasts. Visual observations confirmed that protoplasts did not divide during the 10 day culturing period, and that the number of viable cells decreased slightly over time, consistent with the decrease in hybridization seen in Figure 4B. These experiments indicated that pWI-11 replicated and accumulated in endosperm cells in the absence of cell division.

Replication of pWI-11 in BMS protoplasts

During development, the triploid endosperm genome in maize undergoes DNA amplification in the absence of mitosis (endoreduplication), resulting in nuclear DNA content as high as 90 C (46). Since cultured endosperm cells remain differentiated (38), their nuclei may be capable of endoreduplication. This could enhance replication of pWI-11, which depends on cellular DNA polymerases. We therefore tested if pWI-11 could replicate

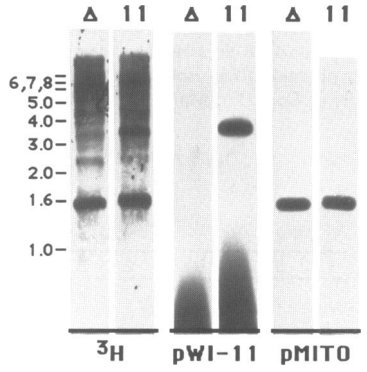

Fig. 2. *In Vivo* Labeling in Maize Endosperm Protoplasts. Protoplasts were electroporated with pWI-Δ (Δ) or pWI-11 (11) and incubated for 3 days. ^{3}H-Thymidine was added during the last 18 hrs of culture. Supercoiled DNAs were isolated, digested with *Bam*HI, separated on a 1% agarose gel and blotted onto Nytran membrane. Left panel (^{3}H), direct exposure of the filter to x-ray film. Middle panel (pWI-11), the same membrane probed with ^{32}P-labeled pWI-11 DNA. Right panel (pMITO), the filter was reprobed with mitochondrial plasmid DNA. The total supercoiled DNA fraction isolated from 10^6 protoplasts was analyzed for each sample. Molecular weight standards are indicated.

similarly in cells that do not undergo endoreduplication, using protoplasts derived from the maize Black Mexican Sweet (BMS) cell line. This cell line is derived from meristem tissue, and should represent normal mitotic cells.

BMS protoplasts were electroporated with pWI-11 or pWI-Δ and cultured for 1, 2, 3, 5, and 7 days. A timepoint of 10 days was not included since cell division occurred around 7–8 days in these protoplasts, as monitored visually. Similar results were obtained with *in vivo* labeling transfected BMS protoplasts as with endosperm protoplasts (Fig. 5A). ^{3}H-Thymidine incorporation was limited to pWI-11 DNA, demonstrating the ability of the shuttle vector to replicate in these cells. Again, replication was initiated within 24 hrs after electroporation, and thus appeared to be independent of cellular division. The same filter as in Figure 5A was sequentially probed with pWI-11 (Fig. 5B), and the mitochondrial plasmid (Fig. 5C). In constrast to the increase in hybridization intensity seen for pWI-11 in Figure 4A, a decrease was observed. This was due to a more rapid decrease in cell viability for BMS protoplasts than for endosperm protoplasts, as determined by microscopic analysis (not shown), and hybridization to the mitochondrial plasmid (Fig. 5C). The

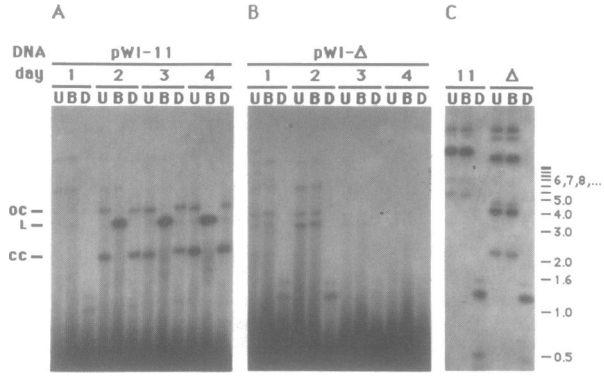

Fig. 3. Onset of pWI-11 Replication in Endosperm Cells. (**A**) Protoplasts were electroporated with pWI-11 and incubated for the indicated period. Supercoiled DNA was isolated and electrophoresed without digestion (U) or after digestion with *Bcl*I (B) or *Dpn*I (D). WDV-derived sequences were visualized by hybridization to pWI-11. OC, open circular; L, linear; CC, closed circular. (**B**) Same as (A) but protoplasts were electroporated with pWI-Δ DNA. (**C**) pWI-11 (11) and pWI-Δ (Δ) plasmid DNAs isolated from *E. coli*, undigested (U) or digested with *Bcl*I (B) or *Dpn*I (D). Molecular weight standards are indicated.

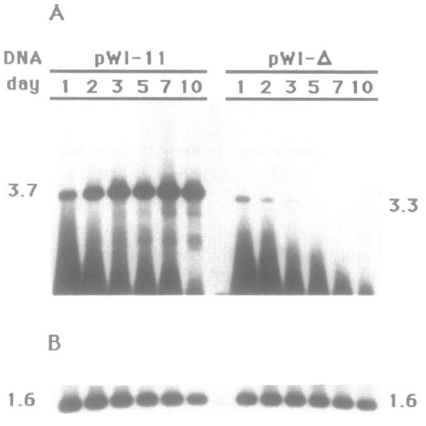

Fig. 4. Accumulation of pWI-11 DNA in Endosperm Protoplasts. (**A**) Southern blot analysis of endosperm protoplasts electroporated with pWI-11 or pWI-Δ and incubated for the indicated time period. DNA was digested with *Bam*HI and hybridized to ^{32}P-labeled pWI-11 DNA. The molecular weights of the DNA fragments are indicated. (**B**) Same filter as in (A) was reprobed with the 1.9 kb mitochondrial plasmid.

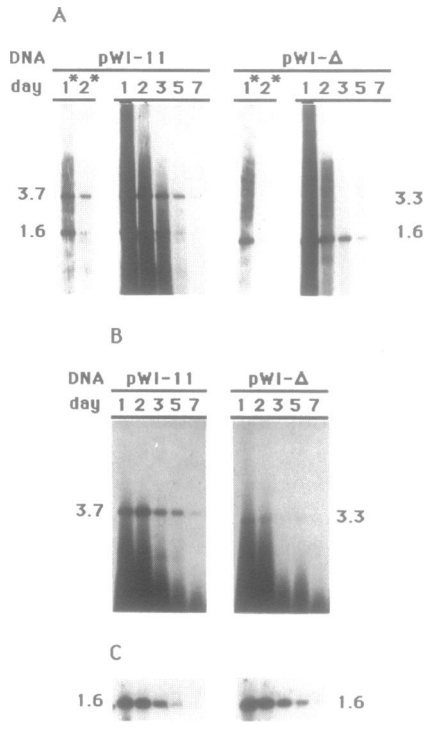

Fig. 5. Replication and Accumulation of pWI-11 in BMS Cells. (**A**) Direct exposures of *Bam*HI digested supercoiled DNA isolated from BMS protoplasts labeled with ^{3}H-thymidine for the last 18 hrs of the incubation periods shown above each lane. The constructs used for electroporation are indicated (pWI-11 and pWI-Δ). The two lanes on the left of each set, marked with asterisks, show lower exposures of the first two lanes to visualize the labeled bands clearly. Molecular weights of the DNAs are indicated. (**B**) Same as in (A) but exposed after hybridization to pWI-11 DNA. (**C**) Same filter as in (A) but the mitochondrial plasmid was used as a probe.

hybridization signal for pWI-11, however, increased over time relative to the signal for the mitochondrial plasmid. These results confirmed that pWI-11 could accumulate independent of cell division, and that endoreduplication was not necessary for accumulation.

The replication protein of WDV is *trans*-acting

Ugaki et al. (32) reported the construction of pWI-GUS, a derivative that contains a CaMV 35S-GUS gene fusion inserted into the unique *Bam*HI site of pWI-11 (Fig. 1D). When endosperm protoplasts were electroporated with pWI-GUS, GUS activity increased with culturing, unlike the transient expression obtained from non-replicating plasmids containing the same GUS cassette (32). This was also true of BMS protoplasts (data not shown). GUS activity in BMS cells electroporated with pWI-GUS increased between day 1 and day 4 relative to a non-replicating control. At day 6 the GUS activity was less than at day 4, probably owing to a decrease in the number of viable cells. This suggested that the GUS enzyme assay could be used to monitor replication.

This assay was used to test whether the replication protein from WDV was *trans*-acting. A replication-deficient vector was constructed by inactivating the replication protein gene of pWI-GUS. The unique *Hin*dIII site in ORF III of pWI-GUS (Fig. 1D) was filled in with Klenow fragment, creating a frameshift mutation (plasmid pWIΔ2G). This plasmid was co-electroporated with either pWI-11, which can provide the replication protein in *trans*, or pWI-Δ, which cannot. pWI-GUS, and pFF19G, a pUC derivative containing the same GUS cassette (35) provided replicating and non-replicating controls, respectively. The amount of DNA per electroporation was kept constant since differences in the amount of carrier DNA affects the enzyme activity (39). Figure 6 shows GUS activities in endosperm (Fig. 6A) and BMS (Fig. 6B) protoplasts. Over the tested period in both cell types, the GUS activity from pWIΔ2G/pWI-Δ was similar to the non-replicating control. This showed that the frameshift mutation rendered pWIΔ2G replication-deficient, and that no gene product from pWI-Δ could complement this defect. On the other hand, when pWIΔ2G was co-electroporated with pWI-11, the GUS activity was higher. In BMS protoplasts the activity was intermediate, but in endosperm cells the GUS activity was as high as that obtained from pWI-GUS, the replicating control.

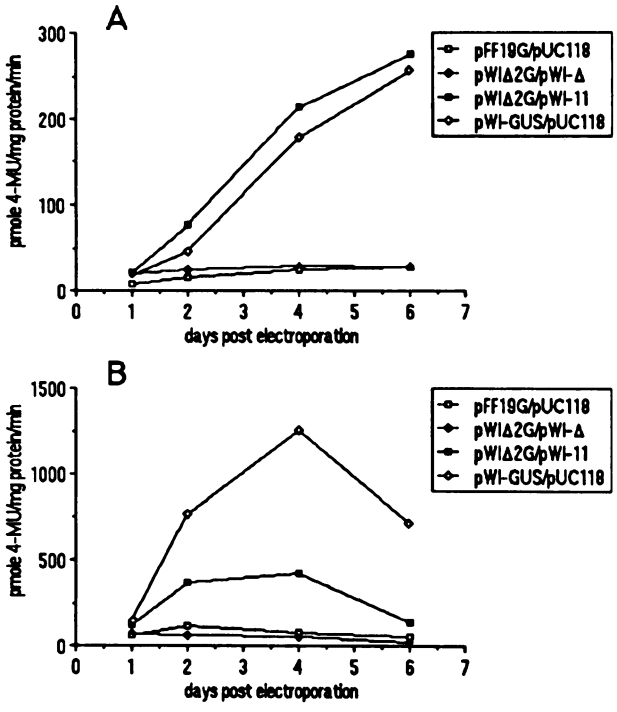

Fig. 6. *Trans*-Activity of the Replication Protein: GUS Assays. (**A**) The GUS activity in endosperm protoplasts electroporated with plasmids pFF19G/pUC118, pWIΔ2G/pWI-Δ, pWIΔ2G/pWI-11 or pWI-GUS/pUC118 and incubated for 1, 2, 4, or 6 days is shown. Activity is expressed in pmoles 4-methyl umbelliferone (4-MU)/mg protein/min. The GUS activity in untransfected control protoplasts was subtracted from each sample. The average of two independent experiments is shown. (**B**) Same analysis as in (A) but protoplasts derived from the BMS cell line were used.

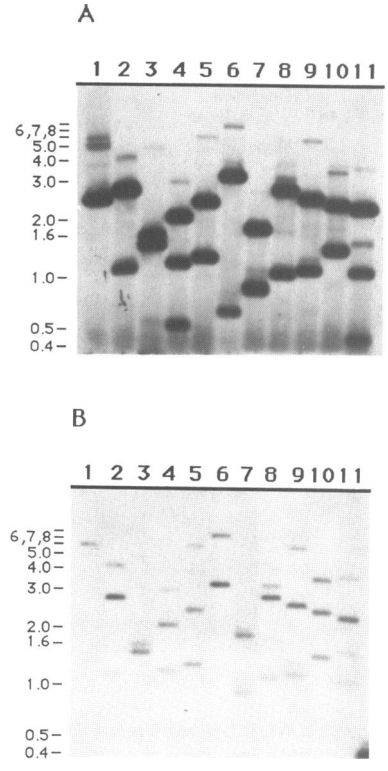

Fig. 7. *Trans*-Activity of the Replication Protein: Restriction Analysis. (**A**) Endosperm protoplasts were co-electroporated with 50 μg each of the plasmids pWIΔ2G and pWI-11, and incubated for 3 days. Supercoiled DNA was isolated and subjected to Southern blot analysis using pWI-11 DNA as a probe. Lane 1, undigested DNA; lanes 2–11, double digests of *Hin*dIII and *Bsm*I (lane 2), *Eco*RI (lane 3), *Hin*cII (lane 4), *Nco*I (lane 5), *Nde*I (lane 6), *Nru*I (lane 7), *Pst*I (lane 8), *Pvu*II (lane 9), *Sph*I (lane 10) or *Ssp*I (lane 11). The molecular weight standards are indicated. (**B**) A mixture of plasmids pWIΔ2G and pWI-11, 50 pg each, subjected to the same restriction digests as in (A) and probed with the same probe as in (A).

This suggested that the replication protein from pWI-11 could act in *trans*. An alternate possibility was recombination between homologous regions of pWI-11 and pWIΔ2G, that either restored the reading frame of the replication protein gene of pWIΔ2G or transferred the GUS expression cassette from pWIΔ2G to pWI-11. Either pathway of recombination should link the unique *Hin*dIII site in a functional replication protein gene to a molecule larger than pWI-11. Therefore, DNA from protoplasts electroporated with pWIΔ2G/pWI-11 was digested with *Hin*dIII plus various other enzymes (Fig 7A). A mixture (1:1) of pWIΔ2G and pWI-11 DNAs isolated from *E. coli* was also digested similarly (Fig. 7B). On hybridization with pWI-11, no new bands were found in the endosperm samples compared to bacterial DNA (the faint bands of 3.7 kb in lanes 6 and 8 were from partial digestion of pWI-11). The difference in the intensity of hybridization between the two plasmids resulted from intrinsic copy number differences between the plasmids, as shown below. However, longer exposures confirmed that no rearranged products accumulated to detectable levels in protoplasts. Also reprobing the filter with GUS coding sequences revealed the same DNA fragments for both the endosperm samples and the bacterial plasmids. Since GUS activity in this experiment was slightly higher than with pWI-GUS alone, DNA rearrangement was not the likely reason for the results in Figure 6A.

In further tests, we replaced the *Aha*II to *Hin*dIII fragment containing ORF IV and most of ORF III of pWI-11 (Fig. 1B) with a GUS cassette. Here, the intergenic regions were separated 1.9 kb further than in WDV. GUS expression from this vector was low when co-transfected with pWI-Δ, but when co-electroporated with pWI-11, GUS activity was comparable to the replicating control (data not shown). This was consistent with *trans* activity of the replication protein, and showed that most of the replication protein gene could be deleted. This also suggested that the spacing between the two intergenic regions was not critical for replication.

Copy numbers of the WDV-derived vectors in endosperm cells

Figure 7A revealed that *trans* replication resulted in a 13-fold reduction in copy number of pWIΔ2G relative to pWI-11. Since equal amounts of both plasmids were transfected, only a 2-fold difference, as in Figure 7B, was expected. The greater difference could be due to inefficient replication in *trans*, or to an intrinsic property of pWIΔ2G that reduced its copy number. A third possibility was interference, in which replication of a defective virus affects the replication of the wild type giving reduced copy numbers for both. These alternatives were tested by comparing the copy numbers of the plasmids under *trans* replication conditions to the autonomously replicating plasmids pWI-11 and pWI-GUS (Fig. 8). When pWIΔ2G was co-electroporated with pWI-11, the respective copy numbers of these two plasmids (lane 3) were similar to the copy numbers of pWI-GUS (lane 4) and pWI-11 (lane 1). The absence of a hybridization signal in lane

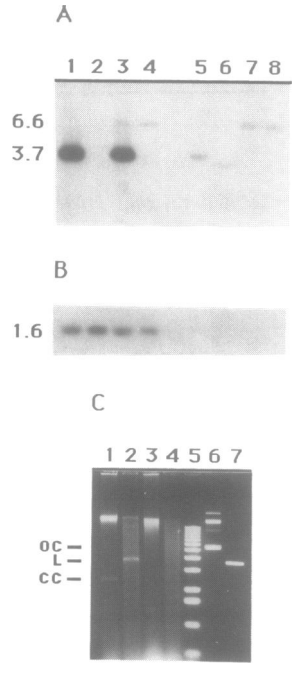

Fig. 8. Copy Numbers of WDV-Derived Vectors in Endosperm Cells. (**A**) Supercoiled DNA isolated from endosperm protoplasts after a 3 day incubation period (lanes 1–4) or from *E. coli* (lanes 5–8) was digested with *Bam*HI and probed with pWI-11 DNA. Lane 1, pWI-11/pUC118; lane 2, pWIΔ2G/pWI-Δ; lane 3, pWIΔ2G/pWI-11; lane 4 pWI-GUS/pUC118; lane 5, pWI-11; lane 6, pWI-Δ; lane 7, pWIΔ2G; lane 8, pWI-GUS. 50 μg of each plasmid were used during the electroporation procedure. The DNAs in lanes 1–4 was obtained from 5×10^4 protoplasts and in lanes 5, 7, and 8 it is equivalent to 250 copies per cell for 5×10^4 protoplasts. (**B**) Same blot as in (A) was reprobed with the mitochondrial plasmid. (**C**) DNA from 2.5×10^5 protoplasts from the same samples as shown in panel A lanes 1 and 2 was used for agarose gel electrophoresis either undigested (lanes 1 and 3) or after digestion with *BclI* (lanes 2 and 4). Lane 1–2, pWI-11/pUC118; lanes 3–4, pWIΔ2G/pWI-Δ; lane 5, 1 kb ladder (BRL); lane 6, undigested pWI-11 DNA isolated from *E. coli*; lane 7, same as lane 6 but after digestion with *Bam*HI. The amount of DNA in lane 7 is 25 ng.

2 confirmed that pWIΔ2G was unable to replicate in the presence of pWI-Δ. Sample loading was similar for all lanes as indicated by hybridization to the mitochondrial plasmid (Fig. 8B). This suggested that interference or less efficient replication in *trans* were not the reasons for the low copy number of pWIΔ2G in endosperm, but that an intrinsic property of the vector carrying the GUS cassette was more likely. This will be addressed further in the Discussion.

We also determined the copy numbers of the replicating plasmids pWI-GUS and pWI-11. The hybridization intensities in Figure 8A were quantitated by densitometry, and the transformation efficiency for this experiment was determined by the histochemical GUS assay. With pWI-GUS, approximately 10% of the electroporated cells stained blue. No cells in the control samples with pWI-11 showed the indigo stain. Taking into account the 10% electroporation efficiency, and that the equivalent of 250 copies per protoplast was loaded in lane 8 of Figure 8A, the copy number of plasmid pWI-GUS was estimated to be around 2,500 copies per transfected cell. The copy number of pWI-11 was at least 30,000 in 3 experiments. Copy numbers in BMS protoplasts were found to be comparable (data not shown).

With a copy number of this magnitude, the amount of pWI-11 DNA in 10^6 protoplasts electroporated with a 10% efficiency should be approximately 12 ng, and should thus be detectable on an ethidium-bromide stained agarose gel. This is shown in Figure 8C, where supercoiled DNA from 2.5×10^5 protoplasts was used per lane, with or without digestion with *BclI*. As shown above, *BclI* can distinguish between replicated and bacterial pWI-11 DNA, and should rule out plasmid contamination. A unique band was apparent in the sample electroporated with pWI-11 (lanes 1 and 2), which was absent in the replication-deficient control (lanes 3 and 4). This DNA could be cleaved with *BclI* (lane 2), and gave the same mobility as linear pWI-11 DNA (lane 7). In addition, the mobility of the undigested supercoiled DNA from protoplasts (lane 1) was greater than the concatenated pWI-11 DNA from *E. coli* (lane 6). Despite the transformation efficiency of 10% we isolated more pWI-11 DNA than mitochondrial plasmid, visualized at approximately 1.4 kb (lanes 1 and 3). This was consistent with *in vivo* labeling where the ^{3}H-incorporation was similar for both DNAs (Fig. 2 and Fig. 5A), though transformation efficiencies were lower in these experiments. Based on our copy number estimate, the amount of pWI-11 DNA in lane 2 should be 3 ng (lane 7 contains 25 ng of plasmid DNA).

DISCUSSION

Though *trans* replication was expected for bipartite geminiviruses, we showed that it applies to WDV as well. WDV is the first monopartite geminivirus for which this was shown, and it is possible that *trans* replication is a general phenomenon for monopartite geminiviruses. The *trans*-acting function of the replication protein should enable the definition of sequences required in *cis* for replication. These are most likely located in one or both of the intergenic regions of the viral genome. Vectors carrying these *cis* sequences can be replicated in *trans* either by a co-introduced vector like pWI-11, or by a chromosomal copy of the replication protein gene. Transgenic tobacco plants carrying the TGMV replication protein gene driven by the 35S promoter support replication of TGMV DNA B (47). Although the WDV host range is limited to monocotyledonous plants for which transformation is difficult, it should be possible to construct cell lines with the replication protein gene stably integrated in the genome, analogous to the cos cell system for SV40 (48).

Unlike endosperm protoplasts, the GUS activity obtained by *trans* complementation in BMS was consistently less than that obtained from the replicating control. This could be due to interference between the two vectors. Interference has been previously observed when full length ACMV was coinfected with subgenomic forms of its DNA B (49, 50). The apparent absence of interference in endosperm protoplasts suggests that one of the components needed for viral replication may be limiting in BMS, but not in endosperm cells. One possibility may be that endoreduplication in endosperm leads to a high level of proteins necessary for DNA synthesis. Alternately, the replication protein may be expressed at higher levels in this tissue. A rate-limiting amount of replication protein has been considered a possible basis for interference (50).

Since geminiviruses depend on host functions for replication, viral replication has been speculated to require host cell division. This notion was substantiated by transfection experiments with ACMV, BCTV, and WDV (25, 31, 51, 52). When WDV was

introduced into *T. monococcum* protoplasts, no DNA accumulation was detected before the first cell divisions. In contrast, we observed WDV replication within 24 hrs after transfection of maize protoplasts without cell division. Similarly, TGMV replication in *N. tabacum* protoplasts occurred prior to cell division (53). It is not understood what causes these differences in the onset of replication, but the findings on WDV suggest a possible role for the protoplast source.

Introduction of the GUS expression cassette into pWI-11 resulted in a 13-fold reduction in copy number. Analogous observations were reported for TGMV DNA A derivatives when the GUS and chloramphenicol acetyltransferase (CAT) coding sequences replaced the coat protein coding region (54). This was considered to be due to a deletion of a *cis*-acting element located in the coat protein gene that enhanced virus replication. The GUS cassette in pWI-GUS did not replace any of the pWI-11 sequences, but increased the size of the vector by ~3 kb (32). The copy number reduction in our case may be due either to the insertion of sequences that affect viral replication, or due to the increase in size from 3.7 to 6.6 kb. A correlation between plasmid size and copy number has been suggested previously (55, 56), although the CAT construct used by Hanley-Bowdoin et al. (54) was virtually identical in size to the wild type DNA A.

We showed that the shuttle vector can accumulate to a high copy number of 30,000 copies per cell. Although geminivirus replication has been compared to the replication of bacteriophage like M13 and ϕX174, our copy number is much higher than the 100–200 RF molecules present in a single infected *E. coli* cell (57). This copy number is comparable to those obtained for viruses like SV40, which give up to 30,000–200,000 copies per cell (48). It is also higher than other geminivirus vectors, with estimated copy numbers of approximately 500–1,000 copies per cell (e.g. 58, 59). Several explanations are possible for the high copy numbers. For instance, a negative control element in viral replication may be deleted in the shuttle vector. Alternately, the lack of coat protein may increase ds intermediates. A mechanism for this could be that the coat protein sequesters ss DNA by packaging; in the absence of packaging, ss DNA can be converted into new ds molecules. Similar observations have been made for bacteriophage when the phage encoded ss DNA binding protein was absent (60). However, coat protein mutants of TGMV show only a 2-fold increase in ds DNA (61). Another possibility is that our copy numbers are indeed comparable to those obtained during normal plant infection, since these have not been measured accurately.

The combination of high copy number and *trans* replication render pWI-11 a useful shuttle vector for plants. Several potential applications may be envisioned. One would be to overexpress genes in transfected cells. In this regard, although the replicating GUS constructs are present at 2,500 copies per cell, the GUS expression increased less than 10-fold, suggesting that the vector might titrate out factors required for gene expression. *In vivo* competition experiments for transcriptional and other factors may therefore be feasible. The high copy number may enable the isolation of transcripts and processing intermediates from transfected genes. Another use would be to generate high levels of antisense RNA for particular genes. Homologous recombination between two vector molecules, or between the vector and the resident gene copy may also be studied with this vector. *Trans* replication allows for the construction of cell lines carrying a chromosomal copy of the replication protein gene, in which such experiments may be conducted easily.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institutes of Health (GM 43261).

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