Replication of a geminivirus derived shuttle vector in maize endosperm cells

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ABSTRACT

A maize (*Zea mays* L.) endosperm cell culture has been shown to efficiently replicate DNA sequences derived from wheat dwarf virus (WDV), a monopartite monocot geminivirus. To analyze sequences necessary for viral replication and to verify their application for a plant gene expression vector, we have developed a 3.7 kilobase pairs *Escherichia coli*—plant cell shuttle vector, pWI-11. The p15A origin of replication, functional in *E. coli*, was introduced into the viral sequences. We have replaced the coding region of the coat protein gene by that of bacterial neomycin phosphotransferase II (NPT II) gene. The resulting NPT II gene fusion can serve as a selectable marker in both plant and *E. coli* systems. Into a unique cloning site in this pWI-11 vector, we introduced a gene fusion carrying the bacterial β-glucuronidase (GUS) coding region under control of the cauliflower mosaic virus 35S (CaMV35S) gene promoter and terminator. By transferring these viral sequences into protoplasts derived from maize endosperm cell cultures, we have demonstrated that the plasmid pWI-11 can replicate in maize endosperm cells, that the GUS reporter gene introduced into pWI-11 can be expressed at high level in the transformed cells, and that the replicating viral DNA can be rescued from endosperm cells by transforming *E. coli* in the presence of kanamycin. The level of GUS gene expression increased progressively in transformed endosperm cells during a prolonged culture period, coinciding with replication of the viral sequences in these cells.

INTRODUCTION

Geminiviruses are a group of plant DNA viruses whose genome consists of a single stranded circular DNA. They are characterized by twinned (geminate) icosahedral capsids (1,2,3,), and depend on DNA-dependent DNA polymerases for their replication. They are generally classified into two subgroups (4): monopartite geminiviruses that infect monocotyledonous plants via transmission by leafhoppers, and bipartite geminiviruses that infect dicotyledonous plants via transmission by whiteflies, though a few intermediate species have also been found. The geminiviruses propagate in the plant cell nucleus in high copy numbers. Their single-stranded DNA genomes are small (2.5 to 3 kilobases (kb)), making possible the cloning of their double-stranded replicative intermediates in *Escherichia coli* vectors. Thus, geminiviruses have attracted much attention as a source for an origin of replication in plant cells. Recent demonstration of replication of cloned geminivirus sequences in cultured plant cells has advanced the characterization of the viral genome. It has also been shown that viral replication is not restricted to cultured cells derived from plants within its host range. These findings have encouraged us to employ wheat dwarf virus (WDV), a geminivirus belonging to the first subgroup described above (5), for the construction of an extrachromosomal gene expression vector for maize (*Zea mays* L.) cells.

Analyses of WDV (6) and closely related maize streak virus (7,8) DNAs revealed two intergenic regions and several open reading frames encoding at least three proteins (Fig. 1A). The 10 kilodalton (kDa) protein (9), responsible for virus movement between host cells, and the coat protein (10), responsible for virus protection and insect transmission (11), are encoded by genes present on the plus-strand. The replication-associated protein (12) is encoded by a gene present on the minus-strand. This gene is comprised of two distinct overlapping open reading frames (ORFs III and IV) and contains an intron (12). The region between 10 kDa protein gene and replication-associated protein gene, called starting intergenic region, contains promoter sequences for these genes and a characteristic stem-loop structure conserved in all geminiviruses. The other intergenic region, called terminating intergenic region, contains potential polyadenylation signals and a region to which a short complementary primer for second strand synthesis binds (13, 14). Deletion analyses of WDV (15) and maize streak virus DNA (8, 16) demonstrated that both of these two intergenic regions are essential for the viral replication, while neither the 10 kDa protein nor the coat protein genes are needed.

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These experiments depended on the introduction of the viral DNA in the form of monomers with all *E. coli* vector sequences removed or dimers which upon recombination in plant cells released a functional monomeric viral genome.

To simplify reconstruction of functional viral genome in plant cells and molecular analyses of viral sequences, we have developed bifunctional plasmids or 'shuttle vectors' (17). The region encoding the 10 kDa protein and the coat protein was removed from WDV DNA and replaced by the replication origin of an *E. coli* plasmid and a selectable marker gene functional in both plant and *E. coli* cells. While making the viral genome defective in plant infection via insect vectors, this replacement allows replication of viral sequences in plant cells without removing the *E. coli* vector sequences. We have also created unique cloning sites on this shuttle vector.

To test replication of our WDV-derived vector in maize cells, we have chosen endosperm-derived tissue culture cells as a host. Endosperm develops from a triploid cell after double fertilization. During endosperm development, their genomes undergo DNA amplification in the absence of mitosis, rendering nuclear DNA content as high as 90 C (18). Since cultured endosperm cells remain differentiated as shown by their organ-specific gene expression (19), their nuclei may be capable of highly active DNA synthesis under appropriate culture conditions.

The work presented here demonstrated that the WDV-derived shuttle vector can replicate efficiently in cultured maize endosperm cells and that the introduced viral sequences can be rescued in *E. coli* via the *E.coli* replicon. The chimeric reporter gene introduced into the shuttle vector was expressed at high level in the transformed endosperm cells and its expression level increased progressively during a prolonged culture period, coinciding with replication of viral sequences in these cells.

MATERIALS AND METHODS

Construction of Plasmids

A plasmid pOK9 was constructed by ligating the MboII fragment of *E. coli* plasmid pACYC177 (20), containing the p15A replication origin (21), to the *Pst*I-*Hin*dIII fragment of pIC20h5K′ (22). The latter contains the Tn5 neomycin phosphotransferase II (NPT II) coding region flanked by several unique restriction sites. Plasmid pOK9 expresses NPT II by translation of a read-through transcript from the p15A origin, conferring kanamycin resistance (25 μg/ml) to *E. coli*. The upstream ATG located 16 bp 5′ of the NPT II translation start site was deleted by ligating *Xho*I/*Pst*I digested pOK9 to the 250 bp *Sal*I−*Pst*I fragment from pIC250neo (22), yielding pOK10.

Plasmid pW-11, a shuttle vector between *E. coli* and maize cells, was constructed in the following manner. From WDV DNA (6), we deleted 0.7 kb *Bst*EII-*Mlu*I fragment containing the 3′ end of the 10 kDa protein gene and most of the coat protein coding region (Fig. 1A). Based on the findings from the closely related maize streak virus, the resulting 2.0 kb fragment is assumed to contain DNA sequences necessary for viral replication (16) and the coat protein promoter (23). This fragment was ligated to *Hin*dIII-digested pOK10 DNA after treating both fragments with T4 DNA polymerase to generate blunt ends. The resulting plasmid, pWI-10, has the NPT II coding region downstream of the WDV coat protein promoter and transcription initiation site. Plasmid pWI-11 was generated by inserting the cauliflower mosaic virus 35S (CaMV35S) gene terminator (24) between the NPT II coding region and the p15A replication origin. For this, the *Sph*I−*Eco*RI fragment from pFF19 (22) was treated with T4 DNA polymerase to produce blunt ends and ligated to SmaI digested pWI-10 (Fig. 1B). The plasmid pWI-11 has unique *Acc*I, *Bam*HI and *Sal*I sites in between CaMV35S gene terminator and p15A origin, which facilitate cloning foreign genes into this vector.

A replication-defective control plasmid, pWI-del, was constructed from pWI-11 by deleting part of the replication-associated gene and part of the starting intergenic region. The 3.3 kb *Eco*O109I-*Hin*dIII fragment from pWI-11 was recircularized after removing the protruding ends (Fig. 1C). Plasmid pFF19G, a pUC derived plasmid containing the β-glucuronidase (GUS) gene under the control of regulatory regions of the CaMV35S gene (22) also served as a replication-defective control.

Plasmid pWI-GUS, a derivative of pWI-11 containing the GUS expression cassette from pFF19G, was constructed by inserting the 3.0 kb *Eco*RI−*Hin*dIII fragment of pFF19G into the unique *Bam*HI site of pWI-11 (Fig. 1D). Again, both DNA fragments were repaired with polymerase to generate blunt ends prior to ligation.

All plasmids were constructed and propagated in *E. coli* strain MV1184 (25). Large scale amplification and purification of closed circular plasmids was performed by the alkali lysis method followed by CsCl ultracentrifugation as described previously (26).

Transformation of protoplasts from maize endosperm culture with plasmid DNA

A suspension culture has been established from endosperm tissue of maize inbred line A636. This suspension cell line is differentiated and express endosperm-specific genes as described elsewhere (19). Protoplasts were isolated enzymatically from suspension cells as described (19). Typically, 50 μg of purified closed circular plasmid DNA was electroporated into 10^6 protoplasts at 150V with a 1450 μF capacitor (19). After electroporation, protoplasts were cultured in Murashige and Skoog (27) salts supplemented with 0.15 g/l L-asparagine, 0.5 mg/l thiamine HCl, 30 g/l sucrose, and 0.65 M D-mannitol, pH 5.8, at 25°C in the dark.

Analysis and rescue of pWI-11 plasmid replicating in endosperm protoplasts

At various intervals during culture period, endosperm protoplasts transformed with pWI-11 or pWI-del plasmid DNA were collected and washed twice with 15 ml of CPW solution (28) containing 0.65 M D-mannitol. DNA was extracted from the protoplasts as described (29). Five micrograms of undigested DNA isolated from transformed protoplasts was fractionated on a 1.0% agarose gel and analyzed by Southern blot hybridization using ^{32}P-labeled pWI-11 DNA as a probe as described (30).

Plasmid pWI-11 replicating in the endosperm protoplasts was recovered and amplified by directly transforming *E. coli* strain DH5α (BRL) with 5 μg of the total protoplast DNA in the presence of kanamycin (50 μg/ml), according to the conditions recommended by the manufacturer.

Determination of β-glucuronidase activity in transformed endosperm protoplasts

GUS activity was determined in the endosperm protoplasts transformed with 50 μg of pWI-GUS or pFF19G plasmid DNA, according to (31). As a negative control, protoplasts were also electroporated in the absence of plasmid DNA to determine the background level of GUS activity derived from the non-specific hydrolysis of the GUS substrate by the protoplast extracts. After

transformation, the cultured endosperm protoplasts were collected at various intervals, pelleted and resuspended in 250 μl of cold GUS extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% SDS, 10 mM β-mercaptoethanol). They were homogenized in the GUS extraction buffer in sterile 1.5 ml microfuge tubes, and the homogenate was spun for 10 minutes at 4°C in a microfuge. The supernatant was collected and stored at −70°C until the protoplast extracts had been prepared from all time points. The protein concentrations in the protoplast extracts were determined with BioRad Protein Assay Kit. One hundred μg of proteins from each protoplast extract was assayed for GUS enzymatic activity in a final volume of 400 μl in the GUS extraction buffer by the addition of 2 mM 4-methyl umbelliferyl glucuronide (MUG). They were incubated at 37°C for 1 hour, and the reaction was terminated by the addition of 900 μl of 0.2 M Na_2CO_3 to 100 μl of the reaction mixture. Fluorescence was measured with a Perkin-Elmer Fluorescence Spectrometer (model LS-3B) with excitation at 365 nm and emission at 455 nm. Fluorescence intensity was calibrated with 4-methyl umbelliferone (Sigma) standards.

RESULTS

Construction of plant-*E. coli* shuttle vector, pWI-11

The plant-*E. coli* shuttle vector, pWI-11 consists of three essential components: 1) the WDV genome lacking the 3′ region of the 10 kDa protein gene and most of the coat protein gene, 2) a gene fusion between the bacterial NPT II gene and the CaMV35S gene terminator, and 3) the p15A origin of replication from *E. coli* plasmid pACYC177. Construction of the intermediate plasmid, pWI-10 demonstrated two important aspects of shuttle vectors. As expected, the p15A origin of replication from the plasmid allowed replication of the WDV sequences in *E. coli*. Since pWI-10 plasmid DNA was amplified in *E. coli* under kanamycin selection, it has become clear that the NPT II gene is expressed in *E. coli*. This chimeric NPT II gene can also serve as selectable marker in plant cells.

To examine the level of kanamycin resistance of our final construct, pWI-11 was introduced into *E. coli* strain JV30 or DH5α and plated on medium with different levels of kanamycin. Cell growth continued even in the presence of 70 μg/ml kanamycin, a level sufficient for most cloning experiments. When pWI-11 plasmid was amplified in *E. coli* cells and isolated by the alkaline lysis method followed by CsCl ultracentrifugation, typical plasmid yields of 1−2 mg/500 ml *E. coli* culture were obtained, suggesting that the copy number of pWI-11 in *E. coli* is comparable to that of the plasmid pACYC177.

Usually, purified pWI-11 DNA exhibited the predicted mobility when electrophoresed in an agarose gel (Figure 2B, Lane 14), but occasionally different forms of slower migration were observed instead of the monomeric forms (Figure 2B, Lane 12). Linearization by restriction enzyme digestion (Figure 2B, Lane 13) showed that they correspond to either dimeric or concatameric forms of pWI-11 DNA accumulated in *E. coli*, as it has been observed for the parental pACYC177 plasmid.

Replication of pWI-11 in maize endosperm protoplasts

The pWI-11 DNA and a replication-defective pWI-del DNA were electroporated into maize endosperm protoplasts. Their fate in the transformed protoplasts was monitored by Southern blot analysis on the total DNA isolated from the transformed protoplasts, using ^{32}P-labeled pWI-11 DNA as a probe (Figure 2A). The pWI-11 DNA used for protoplast transformation consisted of a mixture of closed circular (cc) and open circular (oc) forms (Lane 18). By the end of the 2.4-hour (0.1 day) incubation period, the introduced DNA had undergone extensive degradation, giving rise to a smear on the gel (Lane 7). The cc form of pWI-11 could no longer be seen at this time, while the oc and linear forms were still detectable. One day after transformation, both pWI-11 (Lane 8) and pWI-del (Lane 3) DNA were barely detectable in the transformed protoplasts. After 3-day incubation, the copy number of pWI-11 DNA had increased, as indicated by the strong intensity of hybridization signals for cc, oc and linear DNA forms in the autoradiogram (Lane 9). Intensities of hybridization signals continued to increase during the 6-day incubation period (Lane 10), suggesting the continuous replication of pWI-11 DNA in the transformed endosperm protoplasts. The intensity of hybridization signals did not change significantly between 6- (Lane 10) and 9-day (Lane 11) incubation period. On the other hand, the replication-defective pWI-del DNA could not be detected in transformed protoplasts during the corresponding periods (Lanes 4, 5 and 6). Absence of hybridization of the probe to the DNA prepared at 0.1 day incubation period from control protoplasts (Lane 1) (to which

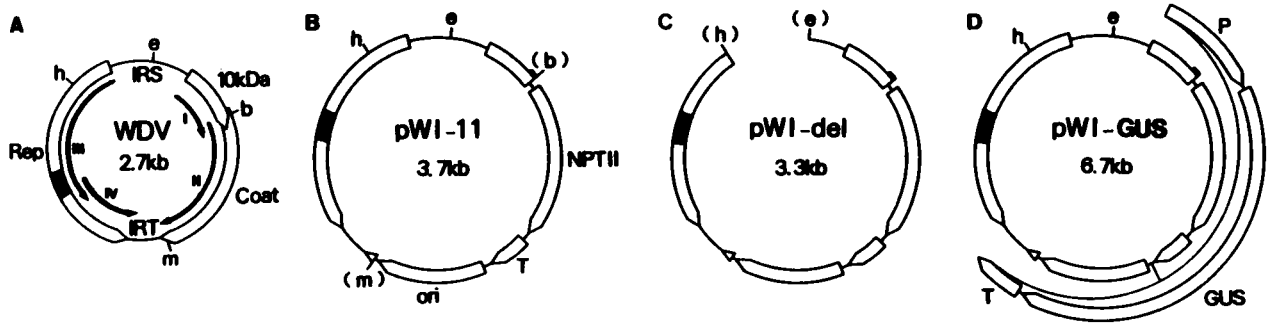

Fig. 1. Schematic representations of WDV genome and WDV-derived vectors. (*A*) WDV genome. ORFs of WDV are numbered I through IV and their orientations indicated by arrows. Three WDV genes are also indicated: Rep, replication-associated protein gene containing one intron (shaded); 10 kDa, 10 kDa protein gene; Coat, coat protein gene; IRS, starting intergenic region containing promoters; IRT, terminating intergenic region; h, *Hin*dIII; e, one of five *Eco*O109I sites. Other four sites are located between this site and the *Hin*dIII site; b, *Bst*EII; m, *Mlu*I. (*B*) pWI-11. NPT II, neomycin phosphotransferase II gene coding region; ori, replication origin of plasmid p15A; T, terminator of CaMV35S transcript. (*C*) pWI-del. DNA fragment shown here was made blunt-ended and religated. (*D*) pWI-GUS. P, duplicated promoter of CaMV35S transcript; GUS, β-glucuronidase gene coding region.

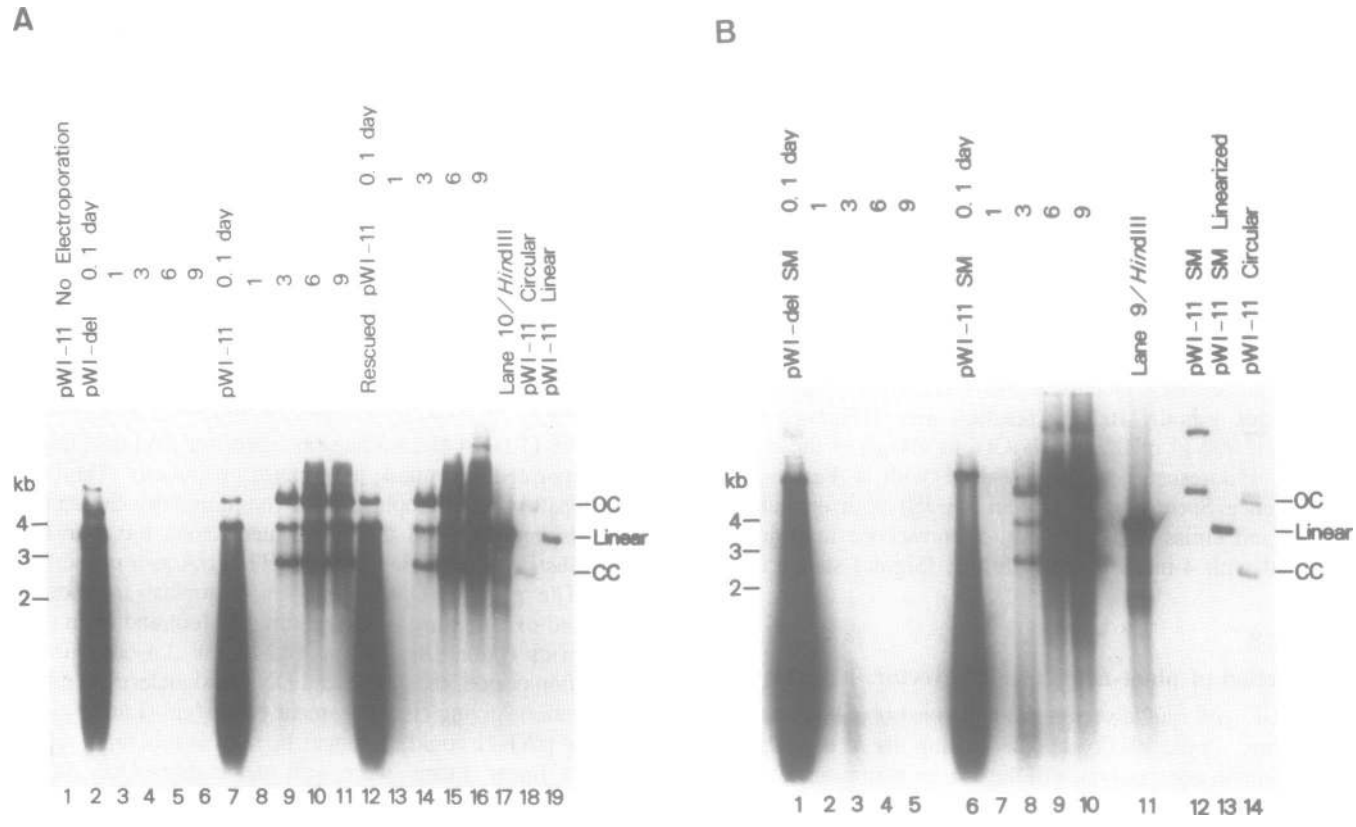

Fig. 2. Southern blot analysis of pWI-11 plasmids. (*A*) Monomeric form of pWI-11 plasmids were used. Lane 1, pWI-11 DNA was added to the protoplasts but not electroporated; Lanes 2 to 16, pWI-11, pWI-del and rescued pWI-11 DNA were electroporated and incubated for indicated period; Lane 17, DNA in Lane 10 was digested with *Hin*dIII; Lane 18, circular pWI-11 DNA was used for electroporation; Lane 19, linearized pWI-11 DNA. The amounts of pWI-11 plasmid in Lanes 18 and 19 correspond to 10 copies per genome. Five mg of DNA isolated from protoplasts was analyzed for each sample. (*B*) Slowly-migrating form of pWI-11 plasmids were used. Lanes 1 to 10, pWI-11 and pWI-del in slowly-migrating form were electroporated and incubated for the indicated period; Lane 11, DNA in Lane 9 was digested with *Hin*dIII; Lane 12, slowly-migrating form of pWI-11 was used for electroporation; Lane 13, linearized slowly-migrating form of pWI-11; Lane 14, circular pWI-11 DNA. The amounts of pWI-11 plasmid in Lanes 12 to 14 correspond to 10 copies per genome. Five μg of DNA isolated from protoplasts was analyzed for each sample.

pWI-11 DNA had been added but not electroporated) confirmed the efficiency of the DNA isolation method used for the exclusion of the plasmid DNA remaining in the culture medium.

To examine the physical integrity of the pWI-11 DNA during its replication in endosperm protoplasts, an aliquot of the total protoplast DNA prepared from the 6-day sample (represented in Lane 10) was digested with *Hin*dIII and analyzed by Southern blot hybridization. A single hybridizing band with the molecular size corresponding to that of the linearized double-stranded pWI-11 plasmid was observed in the autoradiogram (Lane 17). Since the pWI-11 contains a unique *Hin*dIII site, this result suggests the absence of gross rearrangement of the pWI-11 DNA during its replication in endosperm protoplasts.

The diffused hybridization backgrounds observed for the DNA sample taken from the 2.4 hour incubation period (Lanes 2 and 7) seem to represent rapid degradation of WDV DNA rather than an artifact created during DNA preparation, since they were not observed in protoplast DNA samples taken from 1-, 3-, 6- and 8-day incubation periods (Lanes 8, 9, 10, and 11). Similar degradation of WDV DNA immediately after its uptake into wheat protoplasts has been observed (15). No single-stranded form of pWI-11 DNA was detected in transformed endosperm protoplasts during the 9-day incubation period. This may be partly because the coat protein, which protects single-stranded form of viral DNA in the infected plants, are not produced from our plasmids. Another possibility is that the protoplast system is not favorable for single-stranded DNA accumulation, for it is reported that single-stranded form of WDV DNA is abundant in infected whole plants (32) but very scarce in infected plant protoplasts (15). The minor high-molecular-weight forms of WDV sequence detected during longer incubation period (Lanes 10 and 11) have also been observed for tomato golden mosaic virus in infected host plants (33). They are considered to represent multimeric forms of viral DNA.

As described earlier, we obtained the dimeric or concatameric forms of pWI-11 DNA (shown in Lane 12, Figure 2B) during amplification in *E. coli* cells. We have also tested these dimeric or concatameric forms of pWI-11 DNA for replication in maize endosperm protoplasts (Figure 2B). After transformation of endosperm protoplasts, these dimeric or concatameric forms of pWI-11 DNA also exhibited efficient replication (Lanes 8, 9 and 10). However, during their replication, these pWI-11 DNA were converted into monomeric forms as evident from their molecular sizes observed in the Southern blot analysis (compare Lanes 9 and 10 with Lane 14).

Since the coat protein promoter, fused to the NPT II gene in pWI-11, is a strong promoter producing the most abundant viral transcripts in infected cells, we have tried to select the transformed endosperm protoplasts by kanamycin. Unfortunately, since these maize endosperm protoplasts failed to undergo rapid

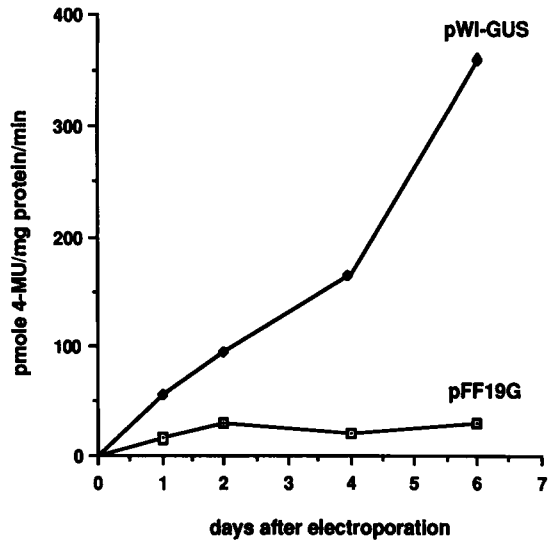

Fig. 3. GUS activity in the maize endosperm protoplasts electroporated with plasmids. ⊙, pFF19G, GUS gene cloned into a pUC plasmid; ●, pWI-GUS, GUS expression cassette cloned into pWI-11. Activity is expressed in pmoles of 4-methyl umbelliferone (4-MU), the product of the GUS enzymatic reaction, per mg protein in the protoplast extract per hour of assay. The GUS activity in the untransformed control protoplasts was substracted. The graph indicates average of the data obtained from two independent experiments.

cell divisions in culture, this selection was not possible in our studies. Thus, protoplasts used for our DNA analysis consisted of a mixture of transformed and untransformed protoplasts, and we could not accurately estimate the copy number of pWI-11 DNA molecules replicating per cell. However, since replication of the vector depends on the same machinery used for geminivirus DNA, its copy number may be assumed to be in the order of several hundred per cell, as reported for unmodified geminivirus DNA (34).

The results described above have clearly demonstrated that the pWI-11 DNA replicates efficiently in cultured endosperm cells and that the region deleted in pWI-del (a part of intergenic region containing the conserved stem-loop structure and a part of replication-associated gene) is needed for viral replication.

Rescue of pWI-11 from plant cells in *E. coli*

The pWI-11 DNA replicating in the transformed endosperm protoplasts was rescued in *E. coli*. Six days after electroporation, total DNA was prepared from protoplasts transformed with pWI-11 (DNA sample shown in Lane 10 in Figure 2A). Five micrograms of the DNA was used to transform *E. coli* strain DH5α cells in the presence of 50 μg/ml kanamycin. Several hundred colonies have been obtained with 5 μg of the total protoplast DNA. On the other hand, DNA prepared from protoplasts transformed with replication-deficient pWI-del (Lane 6 in Figure 2A) or DNA from untransformed control protoplasts failed to yield any *E. coli* transformants. pWI-11 DNAs were prepared from 29 of the rescued colonies. Extensive restriction enzyme analysis of these plasmid DNAs revealed no significant alterations in fragment sizes (data not shown), suggesting that pWI-11 had not undergone gross sequence rearrangement during its replication in maize endosperm protoplasts. One of the rescued DNA sample was reintroduced into maize endosperm protoplasts. Southern blot analysis of DNA prepared from transformed protoplasts showed that the rescued pWI-11 DNA replicated in the protoplasts in a manner similar to the authentic pWI-11 (Lanes 7 through 16 in Figure 2A). These results demonstrate that pWI-11 DNA 'shuttles' between *E. coli* and maize cells without any significant rearrangement.

Amplified expression of foreign gene by pWI-11

Based on the previous demonstration that the WDV-derived pWI-11 plasmid can efficiently replicate in maize endosperm cells, we further tested its application as an extrachromosomal expression vector for plant genes. As a model system, we have constructed pWI-GUS by cloning GUS expression cassette from pFF19G (22) into pWI-11. To distinguish transient expression of the fusion cassette from expression amplified through replication of the pWI-11 plasmid, we compared the level of GUS activity derived from replication-defective pFF19G and that from pWI-GUS in transformed endosperm protoplasts (Figure 3).

Endosperm protoplasts transformed with replication-defective pFF19G reached maximum GUS activity around two days after electroporation, and the level of GUS activity did not change significantly during the subsequent culture period. Endosperm protoplasts transformed with pWI-GUS, on the other hand, continued to express high level of GUS gene during the 6-day culture period. The increase in the GUS expression was correlated with the active replication of pWI-11 DNA in transformed protoplasts as described earlier.

These results demonstrated that a foreign gene as large as 3.0 kb can replicate stably in pWI-11 plasmid and that their expression can be amplified through the replication machinery of WDV.

DISCUSSION

For its small genome size and its ability to replicate to high copy number in plant cells, geminiviruses have gained much attention as an efficient extrachromosomal expression vector in plants. We have further extended this scope by developing a plant-*E. coli* shuttle vector, pWI-11. It utilizes the replication machineries of WDV and an *E. coli* plasmid which allow extrachromosomal replication of the vector in both plant and *E. coli*, respectively. To facilitate the amplification of WDV sequences in *E. coli*, the p15A replication origin from the *E. coli* plasmid pACYC177 is employed in pWI-11. Although it yields a lower copy number of plasmid in *E. coli* as compared with the pUC origin of replication, its compatibility with pUC-derived vectors offers other advantages.

We have deleted from the WDV genome the sequences which are not essential to viral replication, including the 10 kDa protein gene and the coding region of the coat protein gene. The coding region of the coat protein gene has been replaced by that of bacterial NPT II gene. The resulting NPT II gene fusion can be expressed in both plant and *E. coli* cells. However, it is not clear, at present, whether the WDV coat protein promoter is functional in *E. coli* or whether a cryptic promoter present on this plasmid vector is responsible for the NPT II gene expression. However, this fusion is essential not only for providing a selectable marker in both host systems, but also for keeping the vector compact in size (3.7 kb).

Wheat dwarf virus sequences have been shown to replicate in cultured cells derived from various graminaceous monocot species (12, 15). These cultured cells are derived from diploid somatic tissues and are undifferentiated. We are interested in applying our WDV-derived shuttle vector in differentiated plant cells,

particularly in maize endosperm cells, to study gene amplification and organ-specific regulation of genes taking place during endosperm development. Endosperm is a highly specialized tissue, consisting of triploid cells. Replication of geminivirus in these specialized cells has not been observed to date. Isolation of large quantity of protoplasts from developing endosperm tissues is not easy and it requires maintenance of large quantities of plants in a greenhouse or in the field throughout a year. Based on the previous observations that cultured maize endosperm cells remain differentiated, expressing organ-specific genes including genes encoding zeins (19) and pigment and starch biosynthesis (35,36), we have tested the ability of pWI-11 vector to replicate in these cells.

pWI-11, when introduced into protoplasts derived from maize endosperm suspension cells by electroporation, exhibits active replication during the 9-day culture period. The pWI-del construct showed that the 400 bp fragment containing a part of the starting intergenic region and a part of the replication-associated gene is necessary for viral replication. This observation is in agreement with the results obtained in deletion analysis of other geminiviruses that intergenic region and the replication-associated gene play some roles in viral replication. No gross sequence rearrangement was observed in pWI-11 during its replication in endosperm cells. In our study, no single stranded form of pWI-11 DNA was observed in transformed endosperm protoplasts, which is also in agreement with the published observation that single stranded form of WDV DNA is abundant in infected whole plants (32) but scarce in infected protoplasts (15). It is interesting to note that the dimeric or concatameric form of pWI-11 DNA, when introduced into maize endosperm protoplasts, was converted into the monomeric form during its replication.

In addition to the active replication of pWI-11 vector in maize endosperm cells, we have also demonstrated that a cloned reporter gene as large as 3.0 kb can be stably maintained on the vector during its replication. Furthermore, high expression level of the reporter gene can be attained through the extrachromosomal replication of the vector. It is clear that the level of reporter gene expression is correlated with the copy number of pWI-11 plasmid DNA during its replication. This high expression level of a cloned gene should be maintained as long as the vector sequence replicates in the cell unlike the transient expression a gene cloned in conventional *E. coli* vector. Although the chimeric WDV coat protein promoter—NPT II gene present on the pWI-11 vector could have been used to select for the transformed endosperm protoplasts in culture, the slow growth of cultured endosperm protoplasts made such selection not feasible during the relatively short culture period used in our studies. We are currently testing our pWI-11 vector for the selection of transformed protoplasts in other maize tissue culture cells such as Black Mexican Sweet cell lines since their protoplasts are capable of rapid growth in culture (37).

Finally, the features of the pWI-11 shuttle vector can be summarized as follows. Similar to reported geminiviral vectors (8, 34, 38, 39, 40, 41), its desirable feature as a plant vector includes 1) its high copy number in the plant cell, 2) its ability to express cloned foreign genes at high level, and 3) its ability to tolerate a large insert. Yet, the novel and advantageous feature of this shuttle vector resides in its ability to replicate both in *E. coli* and plant cells, which allows its easy manipulation and recovery in *E. coli* from plant cells. These aspects make the pWI-11 shuttle vector useful for the studies of viral replication and organ-specific gene regulation in host cells. Application of this shuttle vector for selection of transformed cell lines or whole plants still remains to be demonstrated.

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