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Wheat Dwarf Virus Vectors Replicate and Express Foreign Genes in Cells of Monocotyledonous Plants

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Wheat dwarf virus (WDV) is a geminivirus that infects monocotyledonous plants. To exploit the potential of WDV as a replicative gene vector, we developed a transient replication and expression system based on the transfection of protoplasts derived from *Triticum monococcum* suspension culture cells. Cloned genomic copies of various WDV isolates as well as mutants constructed in vitro were introduced into the protoplasts and assayed for their ability to replicate. As a result, regions of the WDV genome necessary or dispensable for the viral DNA replication could be defined. In addition, the gene encoding the viral capsid protein was replaced by three different bacterial marker genes, neomycin phosphotransferase, chloramphenicol acetyltransferase, and β-galactosidase. The β-galactosidase gene doubled the size of the WDV genome. The replication of the recombinant WDV genomes and the expression of these genes were monitored in suspension culture cells of *T. monococcum*. The potential of replicative expression vectors based on the WDV genome is discussed.

INTRODUCTION

Geminiviruses are characterized by a twinned particle structure and a single-stranded DNA genome of one or two circular molecules ranging from 2.6 kb to 3.0 kb in size (for reviews, see Stanley and Davies, 1985; Lazarowitz, 1987). Based on different types of insect vectors and the organization of the genome, the geminiviruses can be divided into two subgroups (Harrison, 1985).

Geminiviruses transmitted by leafhoppers are characterized by a single component genome (Mullineaux et al., 1984; MacDowell et al., 1985; Donson et al., 1987; Andersen et al., 1988; Lazarowitz, 1988; Woolston et al., 1988). With the exception of beet curly top virus (Stanley et al., 1986), these geminiviruses infect monocotyledonous plants. The geminiviruses that are transmitted by whiteflies have a bipartite genome (Stanley and Gay, 1983; Hamilton et al., 1984; Howarth et al., 1985; Morris et al., 1990) and infect a wide variety of dicotyledonous plants. Mechanical transmission has only been achieved for the geminiviruses that infect dicotyledonous plants, and in these cases the single-stranded or double-stranded cloned DNA has also been shown to be infectious (Hamilton et al., 1983; Stanley, 1983). For the geminiviruses that infect monocotyledonous plants, the ultimate proof that only one circle of DNA represents the viral genome was provided by the "agroinfection" (or "agroinoculation") of plants with agrobacteria containing a cloned dimer of the genome of maize streak virus (MSV) within the T-DNA (Grimsley et al., 1987). Successful agroinoculation has been demonstrated meanwhile for a different isolate of MSV (Lazarowitz, 1988), digitaria streak virus (Donson et al., 1988), beet curly top virus (Briddon et al., 1989), and two isolates of wheat dwarf virus (WDV) (Hayes et al., 1988a; Woolston et al., 1988).

We have cloned and sequenced three different isolates of WDV: WDV-S from Sweden (Lindsten, 1980), WDV-Ru isolated in Czechoslovakia (kindly provided by J. Vacke), and a barley-adapted strain (WDV-Er, K. Lindsten, personal communication). From the DNA sequences, which are similar to those obtained by MacDowell et al. (1985) and Woolston et al. (1988), four open reading frames (ORFs) can be deduced, as shown in Figure 1A: two in the orientation of the viral plus strand (ORF I and ORF II) and two with the polarity of the minus strand (ORF III and ORF IV). A 35% direct amino acid sequence homology to the sequence of the maize streak virus capsid protein suggests that ORF II codes for the capsid protein of WDV. Based on the occurrence of promoter elements in the large intergenic region, a bidirectional mode of transcription was inferred from the DNA sequence. This has been verified for WDV-S by transcript mapping (Schalk, 1989) and

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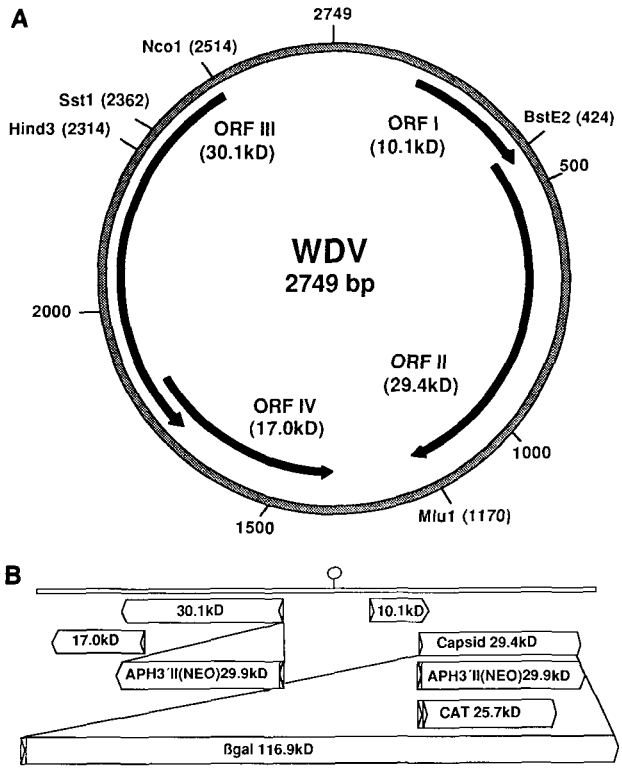


Figure 1. Organization of the WDV Genome and Insertion of Reporter Genes into the Viral Genome.

(A) Schematic map of the WDV-S genome. ORFs are represented as arrows containing the protein size deduced from the DNA sequence. The zero point of the DNA map has been located in the loop of a stem-loop structure conserved throughout all geminivirus genomes sequenced so far. The T underlined in this short sequence (TTA $^{1}\underline{T}$ AATATT) has been defined as the first nucleotide of the WDV-S DNA sequence. The locations of some representative restriction enzyme recognition sequences are indicated. **(B)** Gene replacements and insertions into the WDV-S genome. The expanded linear diagram shows the replacement of the coding region for the capsid protein gene by different marker genes and the insertion of the NPT II gene in front of the 30.1-kD ORF III on the minus strand. The conserved stem-loop structure in the large intergenic region is indicated. The first ATG codons of the genes are symbolized as triangles. In all cases, the marker genes were cloned as translational fusions behind the corresponding WDV ATG codon leading to fusion proteins with small amino-terminal additions.

seems to be common for all geminiviruses known (Morris-Krsinich et al., 1985; Townsend et al., 1985; Accotto et al., 1989; Sunter and Bisaro, 1989; Sunter et al., 1989). To examine regions of the viral genome necessary for replication and to assess whether the WDV genome could be adapted as a replicating expression vector, we have established a transient replication and expression system for the cloned WDV DNA based on the transfection of protoplasts derived from suspension culture cells of *Triticum monococcum*. This system enabled us to analyze mutations introduced into a cloned copy of the WDV genome.

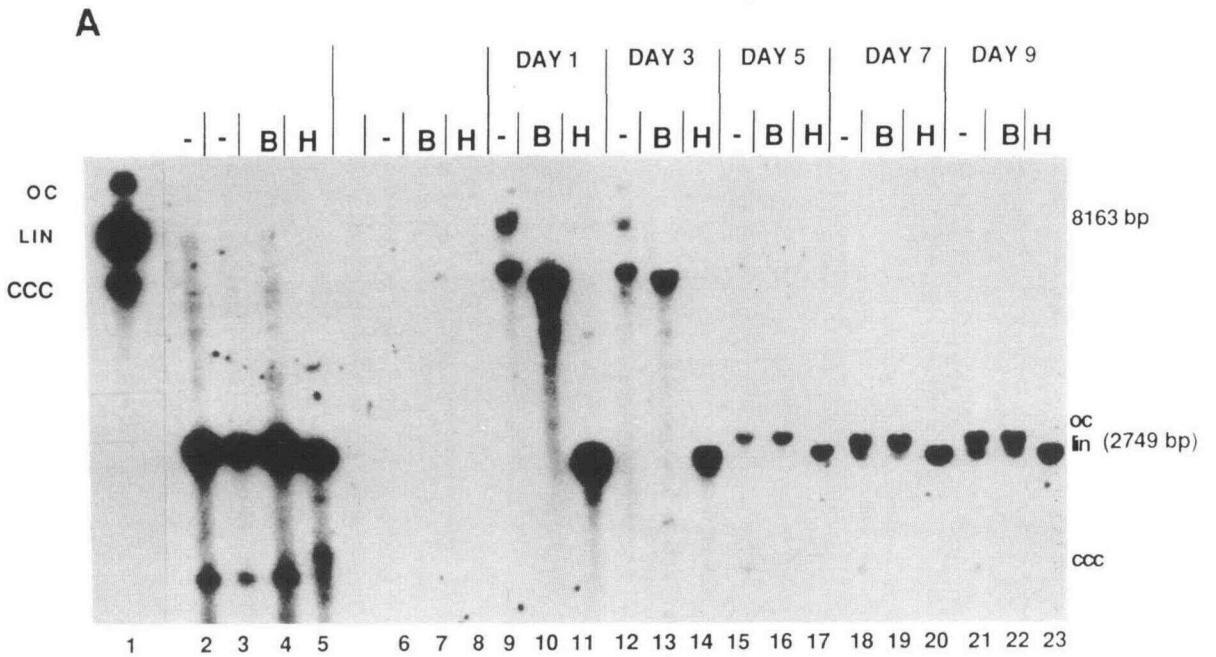
In addition, the viral capsid protein was replaced by three different marker genes: the neomycin phosphotransferase (NPTII) gene from Tn5 (Beck et al., 1982), the chloramphenicol acetyltransferase (CAT) gene from Tn9 (Alton and Vapneck, 1979), and the β-galactosidase (βgal) gene from *Escherichia coli* (Kalnins et al., 1983). Here we show that recombinant WDV genomes are capable of autonomous replication and that they express foreign genes in cells of various graminaceous plants. These results illustrate that a recombinant WDV may serve as a gene vector for monocotyledonous plants.

RESULTS

Replication of a Cloned WDV Genome in Suspension Culture Cells

A tandem dimer of the WDV genome (pWDV2), inserted into the HindIII site of pUC8 (Vieira and Messing, 1982), was used to transfect protoplasts of *T. monococcum* cells, as described in Methods. Starting 24 hr post-transfection, cells were collected over a period of 3 weeks, and the DNA isolated from the protoplasts was probed in DNA gel blot experiments for virus-specific sequences. A typical experiment is shown in Figure 2A. The input plasmid was detected up to 3 days post-transfection. At day 5, open circular and covalently closed circular viral DNA accumulated. The appearance of the replicative forms of the WDV DNA coincided with the first divisions of the *T. monococcum* cells. Under the conditions used for the preparation of the DNA, single-stranded and supercoiled viral DNAs were only detected upon prolonged exposure of the blots. DNA from infected plants showed a reduced level of single-stranded WDV DNA when isolated late in the infection cycle. The DNA isolated from infected plants used as a reference in Figure 2A was isolated 6 weeks to 8 weeks after infection. Digestion of DNA isolated from protoplast-derived cells with BamHI discriminated between input plasmid DNA and the replicative forms of the WDV DNA. BamHI linearized the plasmid DNA in the polylinker of pUC 8, and HindIII, which released the viral sequences from the input DNA, was used to linearize the replicative forms of the WDV DNA appearing at day 5 after transfection (Figure 2A). During infection, the input plasmid disappeared, whereas the double-stranded viral DNA could be followed over a period of 2 weeks.

To demonstrate further that the newly appearing monomeric forms of the viral DNA represented replicating



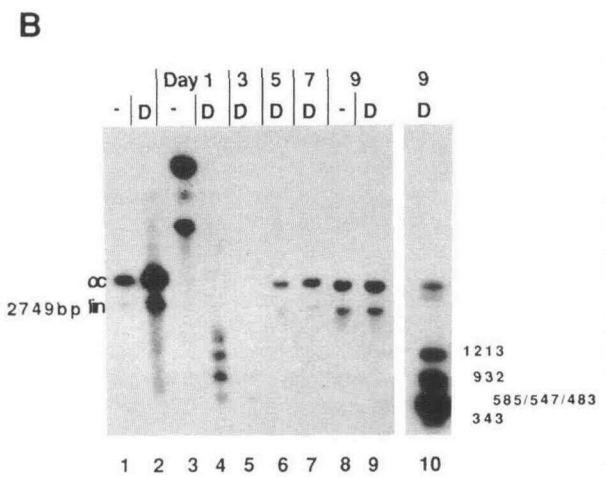


Figure 2. Replication of the WDV Genome in Protoplasts Derived from a *T. monococcum* Cell Suspension Culture.

(A) Replication of WDV-S in monocotyledonous cells. A typical DNA gel blot of DNA prepared from *T. monoccocum* cells is shown. In this experiment, the single-stranded WDV genome was used to prepare a probe by random priming for the viral DNA in transfected protoplasts of *T. monococcum*. All lanes of the 0.8% agarose gel were loaded with the same amount of total protoplast DNA (10 μg). As a reference, 10 ng of the input plasmid DNA (pWDV2) are shown in lane 1. In lanes 2 to 5, total DNA from infected wheat plants was run undigested (–) and digested with BamHI (B) and HindIII (H). Lanes 6 to 8 contain DNA from uninfected plants. All of the following lanes (9 to 23) demonstrate the appearance of the viral DNA in the transfected protoplasts beginning from day 1 to day 9 post-transfection. OC, open circular; CCC, covalently closed circular; LIN, linear.

(B) The same DNA as in **(A)** was run on an 0.8% agarose gel to demonstrate the de novo synthesis of the WDV DNA in protoplasts. Again, single-stranded viral DNA was labeled by random priming and used to probe the DNA gel blot. Lanes 1 and 2 contain DNA from infected plants undigested (–) and digested with DpnI (D). In lanes 3 to 9, the same DNAs as used in **(A)** were digested with DpnI. As a control for the DpnI digestion protoplast, DNA prepared 9 days after transfection was mixed with 10 ng of the input plasmid DNA (pWDV2) and digested with DpnI (lane 10).

molecules and did not arise merely from ligated input DNA, the DNA of transfected cells was digested with DpnI. This restriction enzyme cleaves DNA only if the recognition sequence GA/TC is methylated at the N^6 of the adenine residue (Peden et al., 1980). The dimeric input DNA derived from *E. coli* could be cleaved by DpnI (Figure 2B), whereas the replicative WDV DNA detectable at day 5 was resistant to digestion with DpnI. This result indicated that the WDV DNA was newly synthesized in the transfected *T. monococcum* cells. In contrast to the results obtained with the cloned dimer of the WDV DNA, no replication was observed when protoplasts were transfected with the DNA of a cloned monomer. However, when the monomeric WDV DNA was released from the plasmid, replicative WDV

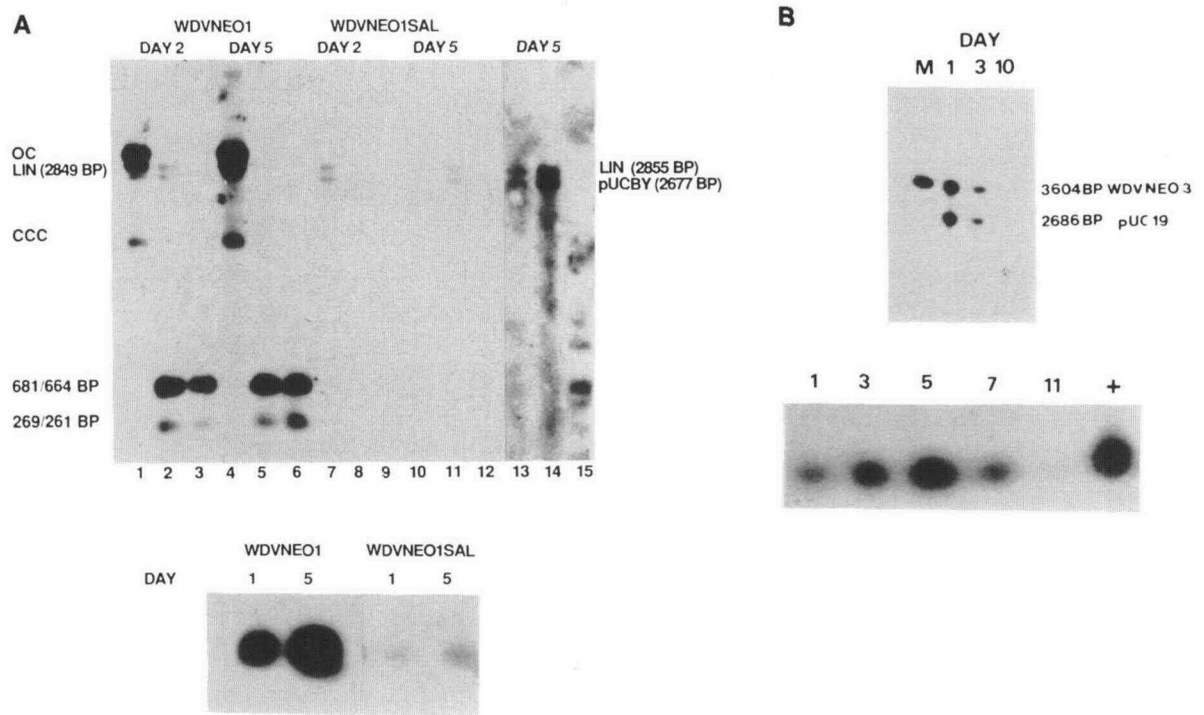


Figure 3. *Replication and Expression of the NPT II Gene by Recombinant WDV Genomes.*

(A) Replication of WDVNEO1 and WDVNEO1SAL in protoplasts derived from the cell suspension culture of *T. monococcum*. Before transfection, the DNA was excised from the vector and the linearized molecules were used to transfect the protoplasts. Total DNA prepared from the transfected protoplasts was run on a 1% agarose gel. A nick-translated full-length copy of the cloned WDV-S genome was used to probe the DNA gel blot. The lengths of the recombinant WDV genomes (2849 bp and 2855 bp) and the bacterial vector pUCBY (2679 bp) are indicated. Lanes 1 and 4 show total DNA from transfected protoplasts isolated at day 2 (lane 1) and day 5 (lane 4) after transfection. Lanes 2 and 5 are MboI and lanes 3 and 6 are Sau3A digestions of the corresponding DNA. In lanes 7 and 11, the total DNA from protoplasts transfected with WDVNEO1SAL was run on the agarose gel. Lanes 8 and 10 and 9 and 12 are the corresponding MboI and Sau3A digestions. Lanes 1 to 12 show a 16-hr exposure of the filter. To demonstrate that no replicative forms are detected, lanes 13 to 15 show a 15-day overexposure of lanes 10 to 12. The autoradiogram of an NPT II filter (P81 paper) below the DNA gel blot shows the reporter gene expression by WDVNEO1 and WDVNEO1SAL. The NPT II expression was assayed with protoplasts transfected in the same experiment as analyzed in the DNA gel blot. The autoradiogram of the NPT II assay with WDVNEO1 was exposed for 1.5 hr, and with WDVNEO1SAL for 15 hr. OC, open circular; CCC, covalently closed circular; LIN, linear.

(B) The DNA gel blot with the nonreplicating mutant pWDVNEO3 is shown. In this recombinant, the NPT II gene is inserted in front of the ORF III. The same probe as in **(A)** was used. Again, the NPT II expression in the corresponding experiment is shown below the DNA gel blot. The autoradiogram of the NPT II filter was exposed for 16 hr. (+) is a 1:10 dilution of an NPT II-containing extract derived from protoplasts transfected with the replicating WDVNEO1 vector.

forms appeared at the same time after transfection as with the dimeric WDV clones (data not shown). This finding indicates that the disruption of ORF III by the insertion of the bacterial vector into the unique HindIII site (Figure 1A) leads to a replication-deficient mutant. Thus, a double-stranded copy of the WDV genome introduced into *T. monococcum* protoplasts, either as closed circular DNA of a cloned WDV dimer or as a linear WDV DNA, is sufficient to initiate the de novo synthesis of the viral genome in cells developing from the protoplasts.

Replication and Expression of Foreign Genes by WDV

Two open reading frames are located on the plus strand of WDV (Figure 1A). ORF I (10.1 kD) overlaps ORF II by 10 codons. In the Swedish isolate (WDV-S), two potential translational start codons of the capsid protein gene occur at a distance of 14 codons on the DNA sequence. To test whether the WDV genome might serve as an autonomously replicating gene expression vector, three different marker genes, NPT II, CAT, and βgal, were used to replace

the capsid protein gene. They were inserted into a unique BstEII restriction site downstream of the first ATG codon of ORF II. After insertion of the foreign sequences, the WDV capsid protein gene was deleted up to the MluI site at the 3′ end of the gene, generating pWDVNEO1, pWDVCAT, and pWDVβgal (Figures 1A and 1B).

Protoplasts of the *T. monococcum* suspension culture cells were transfected using either dimeric clones of the mutant WDV genomes or the corresponding linear monomers released from the bacterial plasmids. The appearance of the replicating viral DNA was assayed as described for the wild-type genome, including digests with either DpnI or MboI/Sau3A. Whereas MboI only cleaved de novo synthesized unmethylated DNA, the activity of its isoschizomer Sau3A was not affected by DNA methylation and, thus, also cleaved the transfected input DNA. Figure 3A shows that the NPT II gene, which is about the same size as the viral capsid protein gene, is replicated as part of the recombinant WDV genome. Total DNA isolated from protoplasts transfected with WDVNEO1 (lanes 1 to 6) and WDVNEO1SAL (lanes 7 to 15) was assayed for virus-specific DNA. WDVNEO1SAL is a frameshift mutant of ORF III, which is derived from WDVNEO1 by insertion of an 8-bp SalI linker into the NruI site at position 2074 of the viral genome. After transfection of WDVNEO1 at day 2, the typical replicating viral forms are detected and can be cleaved with MboI and Sau3A, whereas the linear input DNA is resistant to cleavage with MboI (lanes 2 and 5). In contrast to WDVNEO1, even in overexposures (lanes 13 to 15), no replicative intermediates are identified after transfection of the protoplasts with WDVNEO1SAL. The hybridizing viral DNA is resistant to MboI digestion and only cleaved by Sau3A, indicating that this DNA is residual input DNA. Thus, interfering with the synthesis of the minus strand-encoded proteins blocks the replication of the viral genome.

Replication was also observed when the capsid protein gene was not deleted after insertion of the NPT II gene, thereby adding 845 bp to the viral genome (data not shown). The successful replication of this insertion mutant (WDVNEOCOAT) suggests that there is no narrow limit on the size of the DNA that can be replicated as part of the WDV genome in suspension culture cells. In parallel to the analysis of the replication of the WDV gene replacement mutants, protoplasts from the same transfection experiments were assayed for the expression of the reporter genes. Samples of the transfected cells were collected and assayed for NPT II activity, as described in Reiss et al. (1984) and Schreier et al. (1985). The expression of the NPT II gene by chimeric WDV-S genomes in protoplast-derived cells is illustrated in Figure 3 beneath the corresponding DNA gel blots. The level of expression measured at day 5 for the replicating NPT II replacement WDVNEO1 in Figure 3A was about 20 times higher than the expression measured for the nonreplicating mutant WDVNEO1SAL.

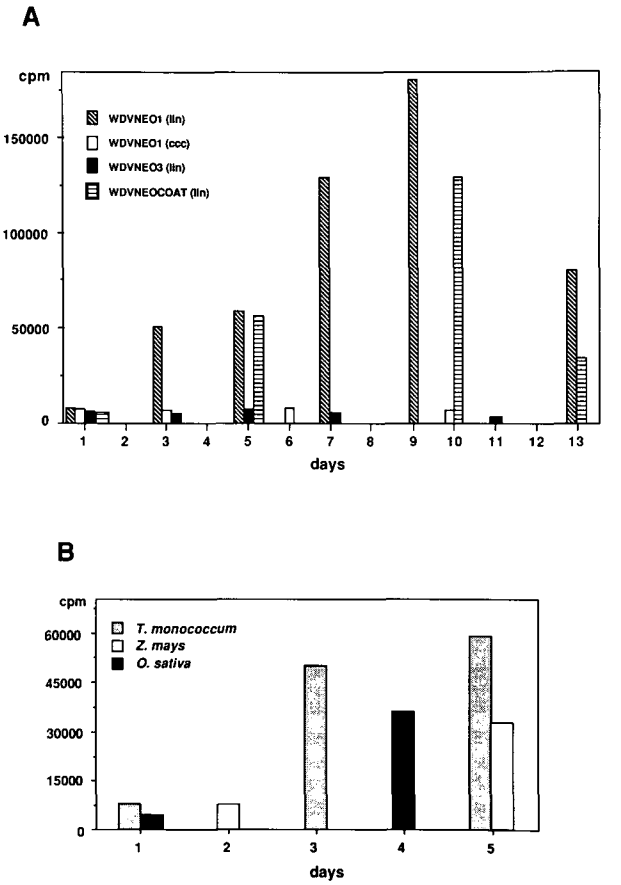


Figure 4. Comparison of the NPT II Activity of Different Recombinant WDV Vectors.

(A) Histogram of NPTII expression data of different recombinant WDV genomes. WDVNEO1(lin) and WDVNEOCOAT(lin) were shown to replicate in the protoplast-derived cells, whereas WDVNEO1(ccc) and WDVNEO3(lin) were deficient for replication. Samples were taken at the indicated time points after transfection and assayed for the expression of the NPT II gene. Every bar represents the result of at least two independent experiments. The spots of phosphorylated kanamycin were excised from the P81 paper and counted in a liquid scintillation counter. lin, linear; ccc, covalently closed circular.

(B) Expression of the NPT II marker gene in nonhost cell suspension culture cells. Protoplasts of a maize (*Z. mays*) and rice (*O. sativa*) cell suspension culture were transfected with WDVNEO1 excised from the bacterial vector to allow the replication of this mutant. For the transfection of the protoplasts and the assay of the NPT II gene expression, the procedure used for the *T. monococcum* cells was followed. For comparison, the data from the pWDVNEO1-transfected *T. monococcum* protoplasts shown in **(A)** were included.

As summarized in Figure 4A, the enzyme activity was first detected about 24 hr after transfection and could be followed at least until day 13, with a maximum of expression at day 9 for the replicating mutants WDVNEO1 and WDVNEOCOAT. Thus, a foreign gene replacing the capsid

protein gene of WDV is not only replicated along with the viral genome, it is also expressed as a part of it. In addition, the expression of the NPT II gene was assayed in transfection experiments using the capsid protein gene replacement mutant without removal of the bacterial plasmid (WDVNEO1ccc). Comparing the maxima of NPT II expression, the observed enzyme activity was about 20 times lower than the NPT II expression derived from the replicating mutant (Figure 4A).

To assay whether the minus strand transcription unit of WDV is also capable of expressing additional genes, the NPT II gene was fused to the ATG codon of the 30.1-kD ORF (pWDVNEO3). Figure 3B shows that no replicative forms were detected in cells transfected with DNA of this mutant. Only the linearized input DNA persisted for about 10 days. At its maximum, the expression of the NPT II activity obtained by this replication-deficient recombinant WDV genome was approximately 20 times less than that with replicating replacement vectors. It was about the same as ORF II replacements (Figure 4A) when no replication of the vector was allowed.

As an additional marker gene, CAT was fused to the viral capsid protein promoter to yield pWDVCAT. The replication of this mutant and the corresponding gene expression are illustrated in Figure 5A. Protoplast-derived cells transfected with this recombinant WDV genome after its release from the bacterial plasmid showed the same kinetics of CAT gene expression as the WDV NPT II replacement vectors (Figures 5B and 5C).

To exploit further the potential of the WDV genome as a gene vector and to delineate its size tolerance toward additional DNA to be replicated, the 3007-bp βgal gene (*lac Z*) of *E. coli* was inserted into the viral genome (pWDVβgal). The chimeric geminivirus genome containing the βgal gene is about 5.7 kb (Figure 1B). The appearance of the open circular form of the chimeric WDVβgal, as illustrated in Figure 6, suggests that genes up to 3 kb can be replicated as part of the viral genome. During the experiment, no rearrangement of the chimeric WDV genome was observed. The finding that inserts of that size are replicated in wheat cells is supported by the replication of a recombinant WDV mutant carrying a 2.9-kb insertion derived from the transposable element *Ac/Ds* (Laufs et al., 1990). The expression of functional βgal was assayed by immobilizing the enzyme from a crude cellular extract by a specific antibody directed against the bacterial βgal to the surface of a microtiter plate. Enzyme activity was measured directly in the wells using the substrate *o*-nitrophenyl galactoside (Miller, 1972). As early as 3 days after transfection, βgal activity could be detected (data not shown).

In addition to the natural hosts described by Vacke (1972), we analyzed the replication and expression of the NPT II gene of linearized pWDVNEO1 DNA in suspension culture cells of maize and rice. Protoplasts of these cell lines were transfected and analyzed in the same way as

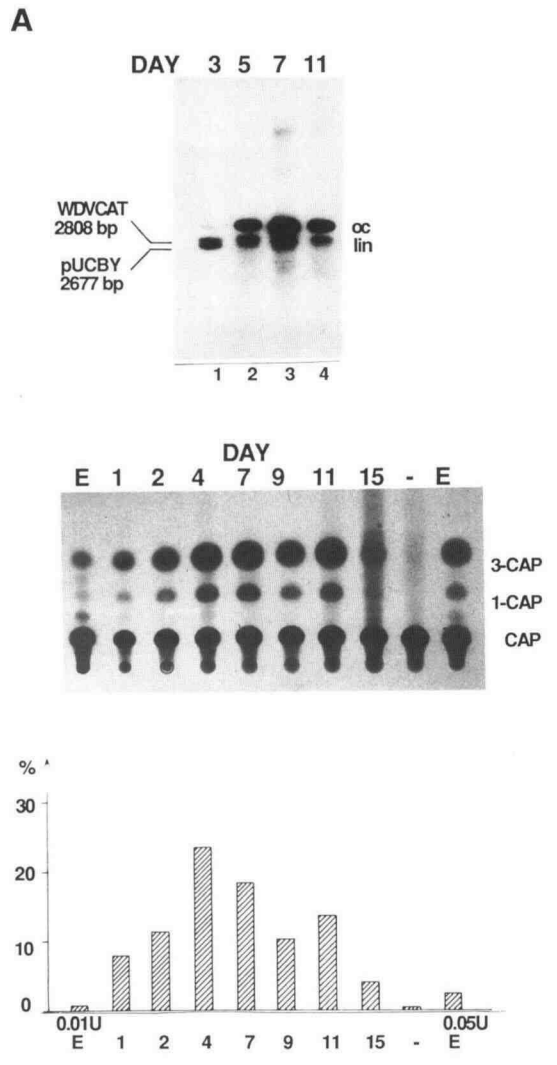


Figure 5. Replication and Expression of the CAT Gene by the WDV-S Genome.

(A) Replication kinetics of pWDVCAT in protoplasts of the *T. monococcum* cell suspension culture. The DNA gel blot was probed with a nick-translated full-length cloned copy of the wild-type WDV-S genome. The positions of the linear WDV mutant (WDVCAT: 2809 bp) and the linearized plasmid vector (pUCBY: 2677 bp) are indicated. OC, open circular; LIN, linear.

(B) Autoradiogram of a TLC separating the different acetylated forms of chloramphenicol. Protoplasts from the same transfection experiment were used for the chromatography. (–), 0.5 μCi of ^{14}C-chloramphenicol; CAP, chloramphenicol; 1-CAP, 1-acetylchloramphenicol; 3-CAP, 3-acetylchloramphenicol.

(C) Quantitation of chloramphenicol acetylation was determined by scintillation counting of the excised spots (1-CAP, 3-CAP). E, activity of 0.01 and 0.05 units of purified chloramphenicol acetyltransferase (Pharmacia).

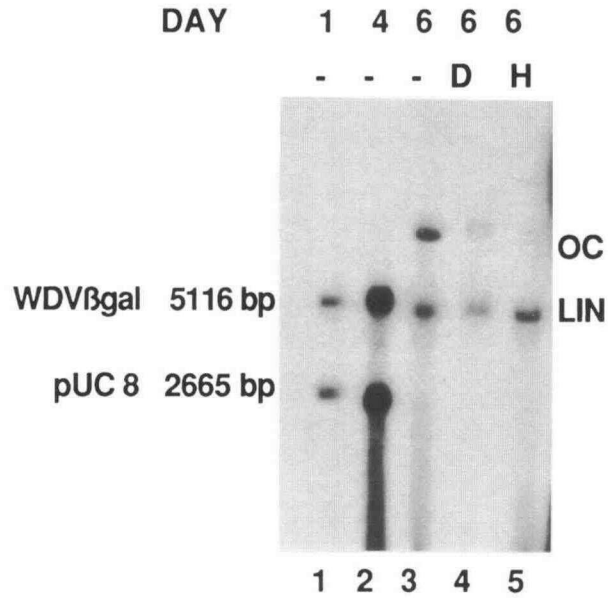


Figure 6. Replication of the βgal Gene in Suspension Culture Cells.

The DNA from transfected protoplasts was prepared and digested as described in Methods and run on a 1% agarose gel. The filter was probed with the nick-translated plasmid pWDVβgal. Lanes 1 to 3 show the virus-specific DNAs detected between day 1 and 6. In lane 4, total DNA isolated at day 6 was digested with DpnI (D) and in lane 5 with HindIII to assay the correct religation of the cloning site that was used to release the viral insert from the vector. OC, open circular; LIN, linear.

described for the *T. monococcum* cells. Although the level of expression was only half of that observed in *T. monococcum* cells, the replication of WDVNEO1 was readily detected in these nonhost cells (data not shown). The expression of the NPT II gene followed the same kinetics as the *T. monococcum* cells (Figure 4B), which indicates that the natural host range of WDV is not determined by its limited ability to replicate only in these plants.

DISCUSSION

Transient expression assays have become a convenient tool to study the function and regulation of plant and animal promoters (Fromm et al., 1985), but they have also been used for the analysis of the replication requirements of a viral genome (Lusky and Botchan, 1986). We have established a protoplast-derived system based on *T. monococcum* suspension culture cells to study the replication and expression of WDV DNA and WDV gene replacement mutants. Protoplasts of *T. monococcum* cells readily initiate cell wall formation, divide, and form microcalli (Lörz et al., 1985). Here we concentrated on a transient assay system to study the functions needed for the replication of the WDV genome and the expression of foreign genes by WDV-based vectors.

The arrangement of ORFs on the genome of unicomponent geminiviruses is strikingly similar to that of the component A (or DNA 1) of the bipartite geminiviruses. This structural similarity can be extended further to a functional level in that both DNA 1 of cassava latent virus (CLV) and DNA A of tomato golden mosaic virus (TGMV) are capable of replicating independent of DNA 2 or DNA B. This has been demonstrated in protoplasts of *Nicotiana plumbaginifolia* (Townsend et al., 1986) or in plants (Rogers et al., 1986).

Using a cell suspension culture, we have shown that the single component of the WDV genome contains all information for sustained replication in monocotyledonous cells. Similar results were obtained recently by using a Czechoslovakian isolate of WDV (Woolston et al., 1989). It has also been demonstrated by agroinfection that the single DNA of WDV is sufficient to elicit an infection in host plants (Hayes et al., 1988a; Woolston et al., 1988; Schalk, 1989).

Replication of the WDV genome is only detected if the cloned copy is able to form a complete replicative unit in the protoplast-derived cells. With the WDV clones used in this study, this was achieved either by using a cloned tandem dimer of WDV that leads to the release of a replicating unit either by way of homologous recombination or some other replicative mechanism, or by transfection of the protoplasts with a monomeric linear WDV genome. This linear WDV monomer becomes circularized by the action of cellular ligases and, hence, serves as a replicative intermediate. Previous work on the replication of CLV component 1 in mesophyll protoplasts of *N. plumbaginifolia* (Townsend et al., 1986) led to the suggestion that the viral DNA replication is linked to the cell division of the host cell. Similar observations were made with WDV replicating in *T. monococcum* suspension culture cells.

The insertion of the NPT II gene in front of ORF III of WDV resulted in the loss of viral DNA replication in the *T. monococcum* cells. WDV mutants in which the ATG codon of ORF III was deleted or mutants with deletions of ORF III or ORF IV were also deficient in DNA replication (Schalk et al., 1989). A similar result was obtained for the dicotyledonous geminiviruses, where mutations in the coding region for the AL1 protein of TGMV led to the loss of replication both in plants and in leaf discs of petunia agroinoculated with redundant copies of the TGMV genome (Elmer et al., 1988).

The replication of the WDV genome, however, is not affected when the viral capsid protein gene is replaced by marker genes. Even an insertion of about 3000 additional base pairs (WDVβgal) seems to be tolerated by the viral replicon in protoplast-derived cells. This indicates that the genome size of WDV is not strictly limited regarding the

replication of its DNA at the cellular level. This has been confirmed recently by demonstrating that a recombinant WDV mutant carrying a 2.9-kb insertion derived from the transposable element *Ac/Ds* is replicated in the *T. monococcum* cell suspension culture (Laufs et al., 1990). For TGMV, the replication of a recombinant genome containing the β-glucuronidase gene has been observed in transformed petunia leaf discs (Hanley-Bowdoin et al., 1988) and in transgenic tobacco plants (Hayes et al., 1989). These results are in contrast to infection experiments with CLV, where the insert size has been shown to be very restricted (Stanley and Townsend, 1986; Ward et al., 1988; Etessami et al., 1989). Replication of the WDV genome in *T. monococcum* cells was also observed when most of the ORF I was deleted, indicating that ORF I is not necessary for viral DNA replication (M. Kammann, unpublished results; Laufs et al., 1990). In addition, the distance between the large intergenic region containing the bidirectional promoters and the small intergenic region harboring the transcription termination signals and the primer binding site (Hayes et al., 1988b) seems not to be critical for the DNA replication of WDV. This result is in contrast to the interpretation of data obtained by Lazarowitz et al. (1989), who hypothesized, based on the analysis of deletion mutants of ORF I and ORF II of MSV, that changing the distance between the two intergenic regions might result in a defect of replication in maize plants.

Using recombinant WDV vectors, we have shown that different marker genes can be expressed in *T. monococcum* cells under the control of WDV promoters. Maximal gene expression was achieved when replicating viral vectors were used, resulting in a 20-fold amplification of the marker gene expression as compared with the level obtained with nonreplicating WDV vectors. This elevated level of expression was comparable with the activity of the NPT II gene driven by the cauliflower mosaic virus 35S promoter in *T. monococcum* cells transfected under the same conditions (data not shown). A similar amplification of gene expression was described in *N. tabacum* plants agroinoculated with recombinant derivatives of TGMV carrying the NPT II gene (Hayes et al., 1988c). An alternative approach of transient expression was recently used by Hanley-Bowdoin et al. (1988), who assayed the transcription of the CAT and the β-glucuronidase marker genes replacing the capsid protein gene of TGMV in petunia leaf discs after inoculation with agrobacteria carrying redundant copies of the recombinant TGMV genomes.

When the NPT II gene was inserted in front of ORF III of WDV, the level of NPT II expression was comparable with the nonreplicating WDV ORF II replacement vectors. With respect to the different amounts of transcripts derived from the rightward and the leftward transcription unit, it has to be further analyzed whether this similar level of expression might either be a result of differences in promoter strength or reflect different transcript stabilities of the chimeric transcripts.

The NPT II gene replacement vector pWDVNEO1 was further used to investigate the replication and expression of this marker gene in suspension culture cells of maize and rice. The replication of WDVNEO1 and the corresponding NPT II expression in these cell lines indicate that replication is not the limiting factor determining the natural host range in these plants.

Replication and expression of various marker genes by a WDV replicon in protoplast-derived suspension culture cells demonstrate the potential of the WDV genome as a plant gene vector for monocotyledonous plants. This potential is emphasized further by the observation that such a WDV-based vector can be also used in cells derived from nonhost plants.

METHODS

Recombinant DNA Techniques

Restriction endonucleases and other DNA-modifying enzymes were used as recommended by the suppliers. Standard techniques in molecular biology were applied as summarized by Maniatis et al. (1982). The DNA sequence of the mutants was verified either by dideoxy sequencing (Sanger et al., 1977) or by diagnostic digests with appropriate restriction endonucleases.

Construction of Plasmids

pWDVNEO1

Clones containing dimers of WDV-S or recombinant WDV-S DNA were generated by restriction by either HindIII or SstI and ligation of the respective vectors and the WDV-S DNA. The correct structure of the recombinants was verified by restriction analysis and sequencing. The NPT II (or NEO) gene was inserted into plasmid pWDV, which contains one copy of WDV-S DNA cloned by way of the unique SstI site in pUCBY, a derivative of pUC18 in which an 8-bp XhoI linker replaces the sequence between the SmaI and HincII sites of the polylinker (plasmid collection of B. Gronenborn, unpublished results). After linearization of pWDV with BstEII (position 424 on the WDV-S genome) and filling in the ends with Klenow fragment of DNA polymerase I, the coding sequence of NPT II was inserted as a HincII fragment. This results in a translational fusion of the NPT II gene 5 bases downstream of the ATG codon of the WDV capsid protein gene and generates the plasmid pWDVNEOCOAT.

The HincII fragment containing the NPT II coding sequence was taken from an intermediate vector (pUC18 Bam Kan; B. Gronenborn, unpublished plasmid). It contains the NPT II coding sequence between the HindIII linker of pKM 22 and the HindIII linker of plasmid pKM 243a (inserted 11 bp 3′ of the insertion of pKM 243; Beck et al., 1982). After cloning into the HindIII site of pJPAX, a derivative of pUC8 (Paszkowski et al., 1984), the marker gene was transferred as a BamHI fragment ("Bam Kan") into pUC18. In this plasmid, the NPT II coding sequence devoid of its ATG codon but including the stop codon is bracketed by the following restric-

tion sites: EcoRI, SstI, KpnI, SmaI, BamHI, SalI, PstI, HindIII, **NEO**, HindIII, BamHI, XbaI, SalI, PstI, SphI, HindIII, which allows an easy transfer of the gene with a variety of different ends.

After the insertion of the HincII **NEO** fragment, the capsid protein gene of pWDVNEOCOAT was deleted by cleavage with XbaI and MluI (position 1170 near the 3′ end of the capsid gene), followed by a fill-in reaction and subsequent ligation to yield plasmid pWDVNEO1. The sequences linking the NPT II gene to the WDV genome are shown below. The numbers give the position on the WDV genome according to the coordinates defined by Schalk et al. (1989).

422
|
...atg gtg acg/ac**c tgc ag**c caa gct tgg **att... NPT II gene**
...tga gcg ggacccaagcttcgac***ggatcc***tctag**cgcgtgtgttattt...**
|
1171

Boldface type indicates the ATG codon of the WDV capsid protein gene, now serving as initiation codon for NPT II gene translation, the filled BstEII site and the bases derived from the original NPT II gene, bracketed by the HindIII sites (underlined) of plasmids pKM 22 and pKM 243a. A PstI and a BamHI site located 5′ and 3′ of the HindIII sites are displayed in italics. These sites were used to construct the plasmid pWDVβgal (see below). The WDV sequence, marked by the filled MluI site (position 1171), is again displayed in boldface type.

pWDVNEO1SAL

To insert a frameshift mutation into the ORF III, the plasmid pWDVNEO1 was digested with NruI. Into this unique blunt-end site at position 2074 of the viral genome, an 8-bp SalI linker (New England Biolabs) was inserted. The proper insertion of the linker was assayed by digestion with SalI. This insertion leads to a truncated ORF III shortened by 51 amino acid codons and changing the amino acid sequence starting from the NruI site.

pWDVNEO3

To insert the NPT II gene in front of ORF III, the NPT II fragment from pUC18 Bam Kan (see pWDVNEO1) was cloned as a blunt-ended SalI fragment into the filled in NcoI site of pWDV. The recognition sequence of the NcoI site at position 2514 of the viral genome contains the ATG start codon for ORF III and, by filling in the protruding ends, the NPT II gene translation is initiated at the WDV ORF III ATG codon.

2516
|
...ccatg tcg acc *tgc agc* caa gct tgg **att... NPT II gene ...tga**
gcggga cccaagcttcgac*ggatcc*tctagagtcgacatg**gcctct...**
|
2516

The WDV sequence and the nucleotides derived from the NPT II gene are displayed in boldface type. The PstI and a BamHI site located 5′ and 3′ of the HindIII (underlined) sites are displayed in italics (see pWDVNEO1).

pWDVCAT

To replace the WDV capsid protein gene by the CAT gene, pWDV DNA was cut with BstEII and MluI, followed by an incomplete fill-in reaction with Klenow fragment of polymerase I and subsequent ligation. This restored the MluI site into which the CAT gene derived from plasmid pCAP 212 (Velten and Schell, 1985) was inserted as a BamHI fragment. All ends had been made flush by filling in with Klenow fragment of polymerase I. As a result, the ATG codon of the WDV capsid gene becomes fused to sequences immediately preceding the ATG codon of the CAT gene, and both initiation codons are in the same frame. Whether this leads to a CAT fusion protein with 19 additional amino acids at the N terminus and whether this fusion protein also contributes to the CAT activity remain open to question. The relevant sequences of pWDVCAT are shown below.

422
|
...atg gtg acg c*gg atc* c cc ggg atc ttg cga gat ttt tca gga gct aag gaa gct aaa **atg...CAT gene...ggg gcg taa** ttttttt...70 bp
...agaaatcg*aggatc* **cgcgtgt**
|
1171

The BamHI sites are displayed in italics; the coordinates refer to the WDV sequence.

pWDVβgal

The βgal gene was cloned from pHWG5 (von Wilcken-Bergmann et al., 1986) as a PstI-BamHI fragment into a frameshift derivative of pWDVNEO1. In this mutant, the manipulation of the BstEII site had led to a 2-bp deletion after the ATG codon of the WDV capsid protein gene and restored the BstEII site. The resulting shift of the cutting position of the PstI site provided an easy means to replace the NPT II gene by the *Escherichia coli lac Z* gene as a PstI-BamHI fragment in the appropriate frame for the translation of β-galactosidase initiated at the ATG codon of the WDV capsid protein gene. Finally, the chimeric WDV genome was cloned into the HindIII site of pUC8. The sequence below shows the flanking sequences. Note the change in register of the PstI site in relation to the ATG codon (see pWDVNEO1). The nucleotides of the BstEII site, the PstI site, and the BamHI site are displayed in italics.

422
|
...atg *gtg* acc *ctg cag* caa gat cta ccc ggg aat tca **ctg gcc...**
***lacZ* gene (3 kb)...aac aaa taa**taataa ccggcaag*ggatcc*
tctag**cgcgtg**
|
1171

Tissue Culture and Preparation of Protoplast DNA

Propagation of the *Triticum monococcum* cell suspension culture, protoplast isolation, and culture of the transfected protoplasts were carried out as described in Lörz et al. (1985). Routinely, 1 to 3 $\times$ 10^6 protoplasts were transfected with 10 μg to 20 μg of

the corresponding CsCl-purified DNA according to Meyer et al. (1985). After transfection, the protoplasts were resuspended in C8/IV medium (Dudits et al., 1977), distributed to two Petri dishes, and incubated at 26°C in the dark. Cells were harvested by centrifugation at 90*g* for 5 min in a Haereus Christ centrifuge. Depending on their amount, the cells were resuspended in a volume of 100 μL to 200 μL of 0.1 M NaCl, 0.1 M Tris/HCl, pH 8, 0.5% SDS, 0.02 M EDTA. They were either frozen at −20°C or directly used for DNA purification by grinding them with a glass pestle followed by multiple extractions with phenol and phenol/chloroform. After ethanol precipitation, the precipitated DNA was washed with 70% ethanol and resuspended in 10 mM Tris/HCl, 1 mM EDTA, pH 7.5.

Neomycin Phosphotransferase Assay

The activity of the neomycin phosphotransferase was assayed as originally described by Reiss et al. (1984) and adapted to plant tissue by Schreier et al. (1985). Transfected cells of one Petri dish (about 0.5 to 1.5 × 10^6 protoplasts) were ground in 50 μL of 62.5 mM Tris/HCl, pH 6.8, 0.1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue-xylencyanol dye marker. After sedimentation for 10 min in an Eppendorf centrifuge, 50 μL of the supernatant were loaded onto a 10% nondenaturing Laemmli-type polyacrylamide gel and run with 12 mA for 13 hr at 4°C in the cold room. The gel was preincubated for 30 min in 67 mM Tris/maleate, pH 7.1, 42 mM $MgCl_2$, 400 mM NH_4Cl_2, and then covered with 1% agarose dissolved in the same buffer containing 100 μCi of γ-^{32}P-ATP. After a 30-min incubation, the in situ phosphorylated ^{32}P-labeled kanamycin was blotted onto Whatman P81 paper. After a proteinase K treatment by incubation of the P81 paper for 30 min at 65°C in a 0.1% SDS solution within a sealed plastic bag to remove the proteins and several washings at 80°C, the paper was dried and autoradiographed.

CAT Assay

The determination of CAT activity was performed essentially as described by Gorman et al. (1982). About 60 mg of cells were extracted in 60 μL of 0.25 M Tris/HCl, pH 7.5, heated for 10 min at 65°C to reduce unspecific activities, and half of the volume was assayed by adding 0.5 μCi of ^{14}C-chloramphenicol and acetyl-CoA to a final concentration of 1 mM. After 60 min at 37°C, the reaction mix was extracted three times with ethyl acetate and concentrated by evaporation. The different acetylated forms of chloramphenicol were separated by ascending TLC on silica gel plates in chloroform/methanol (95/5) and visualized by autoradiography. For quantitation, the spots were excised and the amount of radioactivity was determined in a liquid scintillation counter.

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