Materials and Methods

Reagents

Oligonucleotide primers were purchased from Operon Technologies (Alameda, CA). Nucleoside triphosphates were purchased from Roche Molecular Biochemicals (Indianapolis, IN). Promega (Madison, WI) provided restriction endonucleases. All PCR and ligation reagents were purchased from Epicentre Technologies (Madison, WI). Mini-prep, plant genomic extraction, RT-PCR, and PCR product purification kits were purchased from Qiagen (Santa Clarita, CA). *E. coli* DH10B cells were obtained from Gibco-BRL (Rockville, MD). Bacterial, HeLa cell, and plant growth media components were all purchased from Gibco-BRL, Sigma Chemical Company (St. Louis, MO), or Fisher Scientific (Pittsburgh, PA). Wetsel (Harrisonburg, VA) provided potting soil. Electroporation equipment and SDS-PAGE reagents were from Bio-Rad (Hercules, CA). Western blot reagents were purchased from Pierce Chemical Company (Rockford, IL).

DNA fractionation

All DNA (plasmids and PCR products) samples were fractionated on 1% agarose (molecular screening grade, Roche Molecular Biochemicals) gels in TAE buffer (40 mM Tris base, 1% glacial acetic acid, 1 mM EDTA, pH 7.8) at 100 V.

rep78 plant vector construction

The *rep78* gene (1866 bp) was amplified from the AAV-2 genomic clone pSUB-201 (a gift of Jude Samulski, University of North Carolina Chapel Hill), that contains the entire AAV-2 genome within pEMBL-8 (Samulski et al, 1987). *rep*-specific primers sRep3 (5 -CTT**AGATCT**AATGCCGGGGTTTTACGAG-3) and sRep4 (5 -CTT**AGATCT**CCTTGTTCAAAGATGCAGTC-3) were used with the cycling

conditions outlined in Table I for 30 rounds of amplification. PCR buffer g (*MasterAmp* PCR optimization kit, Epicentre) was used in a 25 l reaction. Final primer and template concentrations were 0.6 uM and 2 ng/ l, respectively. sRep3 and sRep4 contain terminal *Bgl*II restriction sites (5 -AGATCT-3) for cloning of the ~1900 bp product into a binary plant vector. sRep3 has an added nucleotide to ensure in-frame cloning with a translation start present in the cloning region of the plasmid, resulting in the addition of four amino acids to the N terminus of *rep78*. Two nucleotides were added to sRep4 to provide in-frame insertion into the GFP fusion cassette.

Temp	Time
94°C	5 min
94°C	30 sec
60°C	45 sec
72°C	90 sec

Table I. Cycling conditions for amplification of *rep78*, 30 cycles total from step 2.

1 ug of this product was purified using the QIAQuick purification kit and digested with 4U *Bgl*II. 1 ug plasmid pCAMBIA-3302 (Hajdukiewicz et al., 1994) was digested similarly. This vector contains a universal bacterial *ori* and a kanamycin resistance gene. The T-DNA region contains an herbicide-resistance gene and *GFP* regulated by the CaMV 35S double-enhanced promoter and the Nos terminator. Digested insert and plasmid were ligated overnight at a molar ratio of 5:1 using the Fast-Link kit (Fast-Link buffer, 1 mM ATP, 1 U Fast-Link ligase, 15 l total). The resulting plasmid, termed

pSdDan (Fig. 4), yields a *GFP-rep78* fusion cassette flanked by the CaMV 35S promoter and the Nos terminator. *GFP* functions as a reporter gene in the plant. Bacterial transformants are selected by kanamycin resistance, first in *E. coli* and subsequently in *Agrobacterium*. Plant transformants are selected by resistance to the herbicide phosphinothrycin, a compound similar in structure to glutamine and an inhibitor of glutamine synthase. The *bar* gene encodes phosphinothricin acetyltransferase and provides herbicide resistance by modifying and inactivating phosphinothricin (this vector is a modified version of pCAMBIA 1302, and was kindly provided by Bonnie Wolfenden, Virginia Polytechnic Institute and State University). As a binary vector, pCAMBIA-3302 T-DNA, in this case the *rep78* expression cassette, is integrated into the plant genome by gene products inherent to the host *Agrobacterium*.

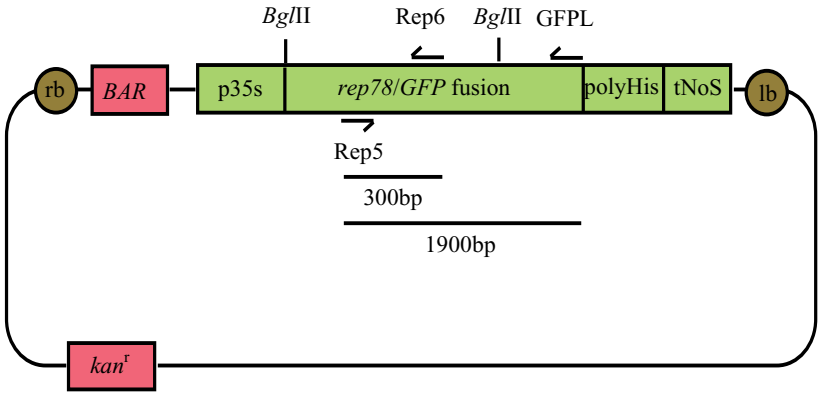

Figure 4. pSdDan. AAV-2 *rep78* was inserted into pCAMBIA-3302 using *Bgl*II sites, creating a *rep78-GFP* fusion cassette with a histidine tag (green). Flanked by signal borders (brown), the T-DNA also encodes phosphinothrycin resistance (*BAR*, red). Kanamycin resistance provides for bacterial selection (*kan*^{r}, red). Internal *rep* primers Rep5 and Rep6 yield a ~300 bp PCR product. Primers rep5 and GFPL yield a ~1900 bp PCR product.

Competent bacteria preparation

E. coli DH10B cells were prepared for electroporation using a procedure adapted from Dower et al., 1988. Cells were grown in 500 ml LB broth (10 g/l Bacto-Tryptone, 5 g/l Bacto-Yeast extract, 10 g/l NaCl, pH 7) overnight at 37°C. Upon reaching an OD_{600} of 0.6, the cells were pelleted in two 250 ml bottles at 5000 x *g* for 15 minutes at 4°C, washed twice in 1 volume (250 ml) cold 10% glycerol, and resuspended in cold 10% glycerol at 1% of the original culture volume (5 ml). 90 µl aliquots were stored in screw cap tubes at -70°C. *A. tumefaciens* GV3101 cells (a gift of Brenda Winkel, Virginia Polytechnic Institute and State University) were prepared similarly, but were grown in 2xYT broth (16 g/l Bacto-Tryptone, 10 g/l Bacto-Yeast extract, 5 g/l NaCl, pH 7) at 28°C.

Bacterial transformation

40 l *E. coli* cells were transformed by electroporation with 5 l ligation reaction in a 0.2 cm cuvette at 2.5 kV. Cells were immediately transferred to 1 ml SOC broth (20 g/l Bacto-Tryptone, 5 g/l Bacto-Yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, and 20 mM glucose) and shaken for 1 hour at 37°C. 100 l of this suspension was spread on LB agar (LB broth, 15 g/l agar, and 50 ug/ml kanamycin). *Agrobacterium* was transformed similarly, being transferred to 1 ml 2xYT broth and shaken at 28°C for 2 hours. This entire suspension was pelleted, resuspended in 100 l 2xYT broth, and spread on 2xYT agar (2xYT broth, 15 g/l agar, and 50 ug/ml kanamycin).

Bacterial clone analysis

Potential bacterial clones harboring pSdDan were screened using colony PCR. Single *E. coli* or *Agrobacterium* colonies were picked using a sterile toothpick and added

to a 25 l PCR reaction. *rep* -specific primers Rep5 (5 -CCATGGTTTTGGGACG-3 , see Fig. 5 for locations of all pSdDan primers) and Rep6 (5 -CTGATCACCGGCGC-3) were used with buffer g and the cycling conditions outlined in Table II for thirty rounds of amplification. Final primer and template concentrations were 0.6 M and 2 ng/ l, respectively. The expected product is ~300 bp.

Temp	Time
94°C	5 min
94°C	30 sec
50°C	30 sec
72°C	90 sec

Table II. Cycling conditions for amplification of ~300 bp *rep78* fragment, 30 cycles total from step 2.

Diagnostic PCR was also used to determine the orientation of the *rep78* insert in pCAMBIA-3302. *rep*-specific primer Rep5 and *GFP*-specific primer GFPL (5 -GTTGTGGGAGTTGTAGTTGTATTC-3) were used in a 25 l reaction with Epicentre buffer g and the cycling conditions outlined in Table III for 30 rounds of amplification. Final primer and template concentrations were 0.6 uM and 2 ng/ l, respectively. Given the correct 5 to 3 insert orientation, the expected product is ~1900 bp.

Positive colonies were then transferred to appropriate broth containing 50 g/ml kanamycin for growth prior to plasmid extraction using the QIAprep miniprep kit.

Temp	Time
94°C	5 min
94°C	30 sec
55°C	30 sec
72°C	90 sec

Table III. Cycling conditions for amplification of ~1900 bp *rep78/GFP* fragment, 30 cycles total from step 2.

Sequence analysis

The nucleotide sequence of cloned *rep78* was determined using an Applied Biosystems (Foster City, CA) sequencing kit. 30-90 ng purified PCR product was used with 1.6 pmol primer (see Materials and Methods, PCR Analysis of Transformed Plants for primer sequence information) and 3 l BigDye Terminator v3.0 Cycle Sequencing Ready Reaction in a 10 l reaction and the cycling conditions outlined in Tables IV and V. Extracted plant DNA was sequenced similarly. All sequence reactions were processed at the Virginia Bioinformatics Institute s Core Sequencing Facility, Blacksburg, VA.

Temp	Time
95°C	30 sec
50-60°C	30 sec
60°C	4 min

Table IV. Cycling conditions for sequencing of *rep78* from pSdDan and transgenic plants, 30 cycles total. Annealing temperatures ranged from 50-60°C depending on the primer used (see Table V).

Infiltration medium

A. tumefaciens GV3101 containing pSdDan was prepared for vacuum infiltration using a procedure adapted from Bechtold et al. (1993). Cells were grown in 200 ml 2xYT with 50 ug/ml kanamycin at 28°C. Upon reaching an OD_{600} of 0.8, cells were pelleted in 250 ml bottles at 5000 x *g* for 10 minutes. Cells were resuspended in 1 volume (200 ml) infiltration medium (0.5x Murashige and Skoog salts, 1x B5 vitamins, 0.044 uM benzylaminopurine (BAP), and 0.03% Silwet L-77).

***Arabidopsis thaliana* vacuum infiltration**

A. thaliana ecotype Columbia and *A. thaliana* harboring AAVS1 were grown in Metro Mix 200 potting soil in pots of varying sizes with a 12 hour light/dark cycle. Plants were routinely bottom-watered with tap water.

Plants were vacuum infiltrated with *A. tumefaciens* using a procedure adapted from Bechtold et al., 1993. Mature plants were trimmed of any flowers, buds, or siliques 3-7 days prior to infiltration. Plants were inverted, placed in the infiltration medium, and exposed to a vacuum of 25 mm Hg for 15 minutes. After rapid vacuum release, plants were returned to normal growth conditions. Seeds were collected 4-6 weeks later.

Plant Selection/Genomic DNA Extraction

Dried seeds were densely spaced in potting soil and the pots were placed in a cold room for 2d. The seeds were then transferred to the growth conditions outlined above for 5d. The plants were then sprayed once daily for 3d with Basta solution (1:10,000 dilution Liberty Herbicide and 0.005% Silwet L-77). This spraying cycle was repeated 1 week later. Herbicide resistant plants were harvested approximately 2-3 weeks after being sowed and observed for *GFP* expression using an Olympus AX-70 UV microscope (thanks to Jill Sible, Virginia Polytechnic Institute and State University). Total plant DNA was isolated using the Qiagen Dneasy plant mini kit.

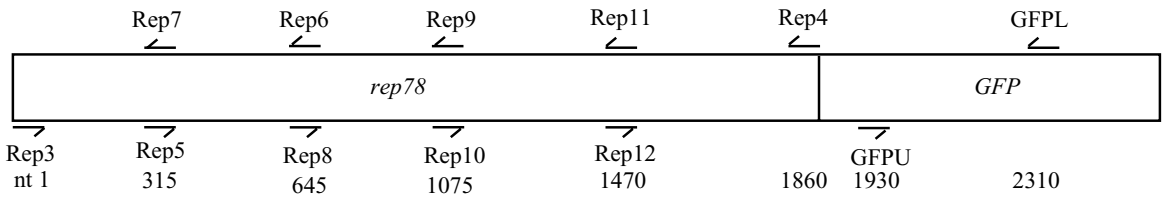

Figure 5. Locations of Rep and GFP primers within pSdDan.

primer	Tm°C
Rep3	55
Rep4	50
Rep5	50
Rep6	50
Rep7	52
Rep8	52
Rep9	52
Rep10	52
Rep11	50
Rep12	50
GFPU	52
GFPL	55

Table V. Primer annealing temperatures. When using primer pairs, the lower annealing temperature was used. See figure 5 for primer locations.

HeLa cell infection

HeLa cells (a gift of Giuseppe Attardi, California Institute of Technology) were grown in minimum essential medium (MEM) with 0.062 g/l penicillin, 0.1 g/l streptomycin, 0.6 g/l glutamine, and 0.075% bicarbonate supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Flasks were placed at 37°C in a water-jacketed incubator. Upon reaching confluent growth, cells were passed to new

flasks using a trypsin solution (1.4 M NaCl, 0.05 M KCl, 40 mM $NaHCO_3$, 60 mM glucose, 5 mM Na-EDTA, 5 g/l 1:250 trypsin).

A productive AAV-2 infection was achieved by infecting 50-70% confluent HeLa cells with AAV-2 and Ad-5 (a gift of Mathew Weitzman, Salk Institute for Biological Studies, La Jolla, CA) in a 25 cm^2 tissue culture flask. Medium was removed and replaced with 330 l unsupplemented medium (no fetal bovine serum) containing 20 AAV-2 particles/cell (multiplicity of infection, [MOI] = 20) and 5 Ad-5 particles/cell (multiplicity of infection, [MOI] = 5). After incubation at 37°C for 1 hour, this infectious suspension was replaced with complete medium. Infected and non-infected cells were harvested at 8, 12, 24, and 36 hours.

Cell lysate was prepared for SDS-PAGE by scraping the adherent HeLa cells from the 25 cm^2 flask. They were then pelleted in 50 ml polyethylene tubes at 5000 x *g* for 5 minutes, and washed twice with 2x Tamm s PBS (0.13 M NaCl, 2.7 mM KCl, 0.82 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.91 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.3). Cells were ultimately resuspended in 0.5 ml 1x Laemmli buffer (Laemmli, 1970; 63 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 10% β-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride, and 1 mM aprotinin). This suspension was sonicated until the viscosity was significantly reduced and stored at -20°C.

SDS-PAGE and Western blotting

20 l of the 0.5 ml infected HeLa cell lysate were fractionated on 7% polyacrylamide (1.17 ml 30% bis-acrylamide, 1.25 ml 1.5 M Tris-Cl, pH 8.8, 2.47 ml H_2O, 50 l 10% SDS, 30 l ammonium persulfate [APS], 10 l N,N,N ,N -Tetramethyl-ethylenediamine [TEMED]) with a 4% stacking gel (0.27 ml 30% acrylamide/Bis, 0.5 ml

0.5 M Tris-Cl, pH 6.8, 1.19 ml H_2O, 20 µl 10% SDS, 10 µl APS, 5 µl TEMED) at 100 V in running buffer (0.025 M Tris base, pH 8.3, 192 mM glycine, and 0.1% SDS).

Samples were then transferred to a nylon membrane (0.2 micron Biotrans, ICN, Irvine, CA) at 100 V for 30 minutes. Protein transfer was confirmed by Ponceau S staining. Upon destaining the blot, it was blocked with Blotto (5% nonfat dry milk in TTBS; 0.3 M NaCl, 20 mM Tris base, pH 7.4, 0.5 ml 100% Tween-20) for 1 hour at room temperature. Monoclonal anti-Rep78/68 antibody (Hunter and Samulski, 1992; a gift of Jude Samulski, University of North Carolina, Chapel Hill) was diluted 1:500 in 3% bovine serum albumin (fraction 5, Sigma Chemicals) and incubated with the blot for 1 hour at 37°. The blot was then washed for five minutes with three changes of TTBS. The Pierce West Pico kit was used for subsequent steps. Goat anti-mouse antibody conjugated to horseradish peroxidase was diluted 1:3000 in Blotto and incubated with the blot for 1 hour at room temperature. TTBS was again used to wash the blot three times, five minutes each. The blot was then placed in substrate for 10 minutes at room temperature. After exposure to the blot, film (Kodak *biomax* scientific imaging film, maximum resolution) was developed using an automated film processor.

Plant extracts were analyzed similarly. Plant samples were ground under liquid nitrogen and suspended in Laemmli buffer (0.5 ml buffer/gram tissue). This suspension was boiled for 15 minutes and spun twice at 14 K rpm/10,000 x g to remove unsuspended debris. 25-50 ug of the protein supernatant was electrophoresed and immunoblotted as described above. Anti-GFP antibody (Clontech Laboratories, Palo Alto, CA; a gift of Brenda Winkel, Virginia Polytechnic Institute and State University) was used at a concentration of 1:250, while HP-conjugated secondary antibody (Sigma

Immunochemicals, St. Louis, MO; also a gift of Brenda Winkel) was used at a concentration of 1:75,000.

PCR analysis of transformed plants

Genomic DNA extracted from wild type and transformed plants was used as template in PCR reactions with the following *rep*-specific primer pairs: Rep3 (5 -ATGCCGGGGTTTTACGAG-3) and Rep7 (5 -CGTCCCAAAACCATGG-3), Rep5 (5 -CCATGGTTTTGGGACG-3) and Rep6 (5 -CTGATCACCGGCGC-3), Rep8 (5 -GCGCCGGTGATCAG-3) and Rep9 (5 -GTTTACGCACCCGTAG-3), Rep10 (5 -CTACGGGTGCGTAAAC-3) and Rep11 (5 -ACCTTTTTGACGTAG-3), Rep12 (5 -CTACGTCAAAAAGGGT-3) and Rep4 (5 -TTGTTCAAAGATGCAGTC-3). Epicentre buffer g was used in a 25 l reaction with the conditions outlined in Tables V and VI for thirty rounds of amplification. Final primer and template concentrations were 0.6 M and 4 ng/ l, respectively. The expected size of each product is between 350 and 450 bp.

A *GFP* fragment was also amplified from transformed plants. *GFP*-specific primers GFPU (5 -CTTGTTGAATTAGATGGTGATGTT-3) and GFPL (5 -GTTGTGGGAGTTGTAGTTGTATTC-3) were used with the same reaction and cycling conditions as above. The expected size of the product is ~400 bp.

Temp	Time
94°C	5 min
94°C	30 sec
50-60°C	30 sec
72°C	90 sec

Table VI. Cycling conditions for amplification of *rep78* fragments and *GFP* from transformed plants, 30 cycles total from step 2. Annealing temperatures ranged from 50-60°C depending on the primer pair used (see Table V).

RT-PCR analysis of transformed plants

In order to further characterize Rep expression, RNA was isolated from 100 mg of frozen plant tissue using the Qiagen RNeasy plant mini kit. This plant RNA was used as template in Qiagen OneStep RT-PCR reactions with the following rep-specific primer pairs: Rep5 (see above for sequence information) and Rep6, Rep10 and Rep11, and Rep12 and Rep4. 25 l reactions were performed with the cycling conditions outlined in Tables VII and V with final primer and template concentrations of 0.6 uM and 30 ng/ l, respectively. The expected size of each product is between 350-450 bp, depending on the primer pairs used.

RNA from transformed plants was also used to amplify GFP. *GFP*-specific primers GFPU (see above for sequence information) and GFPL were used in 25 l reactions with the cycling conditions outlined in Tables V and VII, with final primer and template concentrations of 0.6 M and 120 ng/ l, respectively. The expected size of the product is ~400 bp.

Temp	Time
50°C	30 min
95°C	15 min
94°C	30 sec
50-60°C	30 sec
72°C	60 sec
72°C	10 min

Table VII. Cycling conditions for RT-PCR amplification of *rep* fragments and *GFP* from transformed plants, 40 cycles total from steps 3-5. Annealing temperatures ranged from 50-60°C depending on the primer pair used (see Table V).