Gene I Mutants of Peanut Chlorotic Streak Virus, a Caulimovirus, Replicate in Plants but Do Not Move from Cell to Cell

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Gene I of peanut chlorotic streak virus (PCISV), a caulimovirus, is homologous to gene I of other caulimoviruses and may encode a protein for virus movement. To evaluate the function of gene I, several mutations were created in this gene of an infectious, partially redundant clone of PCISV. Constructs with an in-frame deletion and a single amino acid substitution in gene I were not infectious. To test for replication of these mutants in primarily infected cells, an immunosorbent PCR technique was devised. Virus particles formed by mutants in plants were recovered by binding to antiviral antibodies on a solid matrix and DNase treated to discriminate against residual inoculum, and DNA of trapped virions was subjected to PCR amplification. Gene I mutants were shown to direct formation of encapsidated DNA as revealed by a PCR product. Control gene V mutants (reverse transcriptase essential for replication) did not yield a PCR product. Quantitative PCR allowed estimation of the proportion of cells initially infected by gene I mutants and the amount of extractable virus per cell. It is concluded that PCISV gene I encodes a movement protein and that the immunoselection-PCR technique is useful in studying subliminal virus infection in plants.

The entry of a plant virus into a plant cell, followed by its replication, does not necessarily lead to systemic infection by the virus. Frequently, to invade cells throughout the plant, virus progeny must be able to move successively into neighboring cells. Failure to move beyond one or a few primarily infected cells results in a subliminal, localized infection. A body of evidence is now available to support the notion that virus movement from cell to cell is controlled by the viral genome, in particular by specific transport proteins which interact with plant cells to facilitate virus movement (for reviews, see references 3 and 8).

Identification of viral transport functions requires that one be able to distinguish infection by viruses that are competent for replication but not movement. This requires detection of virus in single cells. When viruses were inoculated to produce infection of subliminal host plants, for example, the common strain of tobacco mosaic virus in cowpea (*Vigna sinensis*), preparation and staining of protoplasts from leaves soon after mechanical inoculation showed that one cell of each 50,000 to 150,000 became infected (32). Alternatively, one can infect mesophyll or suspension cell protoplasts rather than intact plants to evaluate competence for replication. To evaluate artificially engineered virus mutants, it is necessary to inoculate cloned viral cDNAs or RNA transcripts of viral cDNAs. This approach has not always been successful, unfortunately, with the cloned DNAs of caulimoviruses, the subject of interest in this investigation. Consequently, we sought alternative methods for identification of the transport functions of these viruses.

In this investigation, we have used a newly described caulimovirus, peanut chlorotic streak virus (PCISV) (25), in an effort to define the movement function of caulimoviruses. The

DNA sequence of this caulimovirus shows that five of the six major conserved genes of other caulimoviruses are present in the PCISV genome (26). However, the latter is devoid of gene II, which functions in aphid transmission of the caulimoviruses (2, 7, 36).

Several authors have pointed out sequence similarities between the tobacco mosaic virus 30K protein, the tobacco mosaic virus gene product that potentiates cell-to-cell movement of virus (4, 9), and gene I protein of cauliflower mosaic virus (CaMV), the type member of the caulimovirus group (16, 21). This has led to the proposal that gene I specifies the transport factor of the caulimoviruses (5). This suggestion has been strengthened by observations that the gene I product of CaMV is localized in the cell wall of infected plants (1) and, even more specifically, that this protein is associated with tubules in the plasmodesmata (19, 24).

In this investigation, we have constructed gene I mutants of PCISV which were inoculated to intact plants as cloned DNA. The competence of these mutants for replication was evaluated by capturing progeny virus by a solid-phase immunological procedure and then developing a quantitative PCR to demonstrate that these mutants replicate but do not move out of inoculated leaves.

MATERIALS AND METHODS

Infectivity assays. All infectivity assays were done on young seedlings of *Datura stramonium* held for 2 days at 25°C and then placed at 33°C for about 25 days (11). Before inoculation, seedlings in the three- to four-leaf stage were sprayed with 1% Celite. After allowing the leaf surfaces to dry, cloned viral DNA preparations were inoculated by dipping small sterile ground glass spatulas into the DNA solution and rubbing this gently over the leaf surface. The plants were then covered with a canopy of moist newspapers for 24 h. Symptoms of PCISV infection on *D. stramonium* are illustrated in reference 25.

Plasmids. All DNA manipulations were done by standard procedures (27). Partially redundant clones were maintained in *Escherichia coli* DH5; nonredundant clones were maintained in strain JM101.

Infectious single-length and partially redundant clones of PCISV. A full-length clone of PCISV (pPCISV-KI), generated by cloning viral DNA at its unique *Kpn*I site into the *Kpn*I site of pUC119 (35), as described in reference 25, was infectious when the viral genome was released by *Kpn*I digestion. To obviate the need for excision, a partially redundant PCISV clone was constructed (Fig. 1). pPCISV-KI was digested to completion with *Pst*I and *Nsi*I. The fragment containing the cloning vector was ligated by a *Pst*I-*Nsi*I fusion to generate an

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FIG. 1. Construction of a redundant end clone of PCISV. pPCISV-KI, an infectious clone of PCISV, was digested with restriction endonucleases *Pst*I and *Nsi*I and religated to create the clone pUC-R. The clone pDuc 4648, containing the kanamycin resistance gene inserted at position 4648, was excised from its cloning vector, by digestion with *Kpn*I, and recloned in the unique *Kpn*I site of pUC-R. The kanamycin resistance gene was later excised by digestion with *Sall*I, generating the clone pPCISV-KIR. Note the redundant portion of the genome. This clone is infectious without cleavage from the cloning vector. CAT, chloramphenicol acetyltransferase; FMV, figwort mosaic virus.

intermediate plasmid, pUC-R, which contains in succession the 3' end of open reading frame (ORF) V, a complete ORF VI, the promoter for the viral polycistronic mRNA, the polyadenylation signal, and the 5' end of ORF VII (Fig. 1). To generate a partially redundant clone of PCISV, a full-length PCISV genome was cloned in the proper orientation at the unique *Kpn*I site of pUC-R. For this, the plasmid pDuc 4648 (a full-length PCISV clone with the kanamycin resistance gene [*Kn*⁺] inserted at nucleotide 4648 of the PCISV genome [26]) was used. Both clones, pUC-R and pDuc 4648, were digested to completion with *Kpn*I, and the 9.6-kb *Kpn*I-*Kpn*I fragment from pDuc 4648 was cloned into the *Kpn*I site of pUC-R (Fig. 1). The *Kn*⁺ gene was removed later by a complete *Sall*I digestion. This partially redundant PCISV clone was designated pPCISV-KIR and was shown to be infectious by mechanical inoculation onto plants as covalently closed circular (uncut) DNA.

PCISV partially redundant gene I mutants. A point mutation and an in-frame deletion mutation were introduced within the coding region of gene I of PCISV. In each case, the mutant genome was inserted into a partially redundant clone to accomplish efficient inoculation with nonrestricted DNA.

(i) **In-frame deletion mutant.** A mutant with an in-frame deletion in the 5'-terminal region of the gene I coding region was designated pDuc 83. *Xba*I cuts at positions 6851 and 6973 were made within the coding region of gene I. The 5' overhangs left by *Xba*I digestion were made flush by mung bean nuclease and ligated to maintain the native reading frame. This mutant was designated pDuc 83.

amino acid No → 114 123
 PCISV gene I 5' CAT-GTT-AAT-GTT-GTT-CAG-ATA-GTA-ATT-AGA 3'
 his-val-aan-val-val-gln-ile-val-ile-arg
 Oligonucleotide 3' GTA-CAA-TTG-CAA-CAA-TTC-TAT-CAT-TAA-TC 5'
 pDuc 80
 Gln¹¹⁹ → Lys 5' CAT-GTT-AAC-GTT-GTT-AAG-ATA-GTA-ATT-AGA 3'
 and creation his-val-aan-val-val-lys-ile-val-ile-arg
 of a *Hpa*I site (underlined)

Consensus U H U x x U x U U x x x F x x G U x T x U x U x U x D x R U x x
 S * * * * *
 CaMV ORF I 127 VHLGAVKILLKAQFRNGIDTPIKIALIDRRINS 159
 FMV ORF I 123 IHLGAVKILLTAQFRQIGDTSVKMALIDRRIVN 155
 CERV ORF I 118 IHFGAIKVLKARFREGINSPIKMALIDRRITD 150
 SoCMV ORF I 100 VHI STLQVLKSTFLKGLDTPLELTLDNRLLN 122
 PCIMV ORF I 113 IHVNVVQIVIRSTFREGITTPVIRVEDNRIQD 135
 pDuc80 ORF I 113 IHVNVV**K**IVIRSTFREGITTPVIRVEDNRIQD 135

FIG. 2. Creation of a point mutation in gene I of PCISV. The top portion illustrates how a segment of gene I was subjected to oligonucleotide mutagenesis to change one amino acid residue and create a new restriction endonuclease site. At the bottom, segments of gene I of various caulimoviruses are shown within the borders of the 30K motif delineated in the work of Mushegian and Koonin (22). Residues conserved or similar in all five caulimovirus sequences are shown in uppercase in the consensus line; U stands for a hydrophobic residue (aliphatic or aromatic), and x stands for any residue. Residues that are shared by movement proteins from all 17 virus groups comprising the 30K superfamily are underlined. Residues that are conserved in CaMV, figwort mosaic virus (FMV), and carnation etched ring virus (CERV) but are changed to another particular residue in both soybean chlorotic mottle virus (SoCMV) and PCISV are marked by the asterisks. The residue that is changed in pDuc 80 is shown in boldface.

(ii) **Point mutation.** The point mutation was constructed in gene I of PCISV by the oligonucleotide site-directed mutagenesis technique described by Kunkel (18). The oligonucleotide was designed in such a way as to create (i) a codon change to produce the desired amino acid modification and (ii) a new restriction enzyme site close to the modified codon. The latter affected only the nucleotide sequence without altering the amino acid sequence and provided an easy way to screen for the mutant (Fig. 2). The point mutation introduced within the ORF I of PCISV resulted in the Gln-119→Lys amino acid change and was designated pDuc 80 (Fig. 2 and 3).

The parent clone for both mutants was pDuc 43, a subclone containing most of gene I (11).

PCISV partially redundant gene V mutants. Four nucleotides were inserted

FIG. 3. Summary of mutations introduced into gene I of PCISV and an alignment of a gene I segment of caulimoviruses (bottom). The top diagram shows the complete PCISV genome as it appears on the full-length virus RNA intermediate (26). The strategy of cloning and mutagenesis is detailed in Materials and Methods. Restriction sites used or modified upon construction are shown along with amino acid residues that were changed.

within the coding region of gene V, the gene that encodes viral reverse transcriptase. This frame-change mutation destroyed viral infectivity. The mutation was made as follows. The clone pPCISV-KIR was digested to completion at its unique *Bam*HI site (position 4310) within gene V. The 5' overhangs left by the *Bam*HI digestion were filled in with T4 polymerase in the presence of deoxynucleotides. After religation, the clone was designated pDuc 33.

Immunoselection (IS) PCR assay. Groups of four to six *D. stramonium* seedlings were inoculated with plasmid DNA of each partially redundant gene I mutant of PCISV. Control groups were inoculated with pDuc 70 (an infectious clone derived from pPCISV-KIR) and pDuc 33, the gene V frame-change mutant.

Twelve to 15 days after inoculation, the inoculated leaves from each group of plants were harvested separately. After an additional 15-day period, the top leaves were collected. The PCR procedure used for simple detection of virus (10) was as follows. Two grams of leaves was ground with a sterile mortar and pestle in 10 ml of enzyme-linked immunosorbent assay (ELISA) grinding buffer: 1× phosphate saline buffer (PBS), 2% (wt/vol) polyvinylpyrrolidone (molecular weight, 40,000), 0.2% (wt/vol) bovine serum albumin, and 0.05% (vol/vol) Tween 20 (6). The samples were centrifuged at 10,000 rpm in a Beckman JA-20 rotor for 15 min, and the supernatant was reserved for immediate IS of virus particles and amplification by the PCR as follows. ELISA plates were coated with purified gamma-globulin anti-PCISV as described by Clark and Adams (6). After washing the coated plates three times with PBS-Tween 20, the clarified sap was applied. Separate ELISA plates were used for each sample. After overnight incubation at 4°C, the plates were washed three times with PBS-Tween 20 and filled to overflowing with a solution containing 20 µg of DNase I per ml (Sigma Chemical Co., St. Louis, Mo.) in 1× nick translation buffer (0.05 M Tris-HCl [pH 7.5], 0.01 M MgSO₄, 0.1 mM dithiothreitol, 50 mg of bovine serum albumin per ml) (27). This step was introduced to ensure that the DNA recovered from the inoculated leaves was authentic, encapsidated, viral DNA and not a contamination from input unencapsidated DNA. Thus, if the mutant did not replicate, its DNA would not be recovered after the DNase treatment and no product would be observed after PCR amplification. Following a 90-min incubation with DNase I at 37°C, the plates were washed three times with PBS-Tween 20. A total of 100 µl of 0.05 M EDTA (pH 8.0) and 100 µl of 0.4 M NaOH were sequentially applied to each well. This released the viral DNA from the capsid and denatured it. The solution from each well was removed, pooled, and applied in 2-ml aliquots into a Centricon 30 microconcentrator (Amicon Division, Beverly, Mass.). The Centricon device was placed in a JA-20 Beckman rotor and centrifuged at 4,500 × g for 20 min. The concentrated sample was washed three times by the addition of 2 ml of sterile deionized water and recovered in the same Centricon device in a volume of ca. 40 µl.

The PCRs were performed with an MJ Research Thermocycler. The primers for the PCR amplification were designed to amplify the region where the mutation was introduced. Thus, the nature of the amplified DNA could be verified by restriction analysis and sizing of the fragments. The following temperature profile was followed: denaturation, 94°C for 30 s; primer annealing, 55°C for 30 s; and primer extension, 72°C for 1 min. Before the first cycle, a 2-min denaturation at 95°C was included. Fifteen microliters of the reaction product was analyzed by electrophoresis in a 1.2% agarose gel.

Quantitation of PCISV gene I mutant pDuc 80 in primarily infected cells. In an attempt to relate virus detection by the IS-PCR method to the amount of purified virus added to a known number of cells, several steps of IS-PCR were eliminated.

For these modified trials, cloned DNA was inoculated onto Celite-coated leaves of *D. stramonium* in the usual manner but by using the lower epidermis, which can be more readily peeled from intact leaves. After incubation (15 to 20 days), the epidermis of mutant DNA inoculated leaves was peeled, washed in PBS-Tween 20, and homogenized for IS-PCR tests. Before homogenization, epidermal cells were counted with the light microscope. The number of cells per leaf area was calculated as follows. Strips of peeled epidermis (ca. 500 mm²) were spread on a glass slide, and their area was calculated with a dissection microscope and grid paper. The number of cells obtained by direct counting was compared with the value calculated by counting cells in 20 microscope fields and adjusting for the number of fields in the strip. The two values were consistently found to differ by not more than 15%. On this basis, in further experiments the number of cells in the sample was estimated by using the calculated area of the strips and the known value of the cell density per square. Homogenate volumes were adjusted so that 10⁴ cells were extracted in each 10-µl aliquot. Different known amounts of purified wild-type virus were added to some extracts to serve as an internal standard during PCR amplification.

In these tests, instead of polystyrene plates, 500-µl conical polypropylene microcentrifuge tubes were used. These were coated with virus antibodies in the same manner as polystyrene plates used for other tests and then washed, and the virus extract was applied in 10-µl aliquots. After initial IS with 20 µl of plant homogenate, 30 µl of DNase solution in 2× nick translation buffer was added. After this treatment, samples were carefully removed, and the tubes were washed twice with 40 µl of sterile water. The PCR mix was added directly to the tubes; [α -³²P]dCTP (1 to 2 µCi per reaction mixture) was included. PCR was run for 35 cycles. An initial 5-min, 95°C step was found to be sufficient for releasing virion DNA without any further treatment. After PCR, the amplified products were digested with the appropriate restriction endonuclease to resolve the standard

and sample DNA bands. These amplified segments were resolved by electrophoresis and excised individually after agarose gel separation. The excised DNA samples were then subjected to liquid scintillation counting. The sample-to-standard ratio was plotted against known sample concentrations, and the point of equivalency was determined as described by Telenti et al. (33) and Pannetier et al. (23).

RESULTS

Infectivity of gene I mutants. pPCISV-KI (single length) as well as pPCISV-KIR (partially redundant clone of PCISV) was infectious, and the plants inoculated with those clones developed symptoms indistinguishable from those of the virus-inoculated plants. The partially redundant clone generally gave 75 to 95% infection on *D. stramonium* without enzymatic release from its cloning vector.

In the mutant pDuc 83, 126 nucleotides were deleted from the 5' end of PCISV ORF I as explained in Materials and Methods (Fig. 2). This in-frame deletion mutation results in a truncated gene I protein with amino acid numbers 2 to 43 being replaced by a single Tyr residue. This mutation was verified by DNA sequencing.

The choice of the point mutations introduced in pDuc 80 was based on limited similarities between segments of CaMV ORF I and the ATP binding sites of some kinases (20). In particular, the N-proximal residue (Fig. 2, in boldface) is the residue that was thought to correspond to the ATP-binding lysine in the bovine cyclic AMP-dependent protein kinase. This Lys residue is conserved in ORF I of cauliflower mosaic, figwort mosaic, and carnation etched ring viruses but not in gene I of PCISV or gene I of soybean chlorotic mottle virus (15) where this residue has been replaced by Gln. In the mutant pDuc 80, the Gln-119 residue was replaced with a Lys residue by oligonucleotide site-directed mutagenesis of the ORF I of PCISV (Fig. 2) to restore the sequence exhibited by other caulimoviruses.

All PCISV gene I mutants were tested by mechanical inoculation onto *D. stramonium*, *Datura innoxia*, and *Nicotiana tabacum* at least five times on 10 plants each time. None of the gene I mutants appeared to be infectious in these tests. Moreover, PCISV could not be detected in these inoculated plants by ELISA tests either of the inoculated leaves (15 days postinoculation) or of the top leaves (45 days postinoculation).

Replication competence. After confirming that the deletion mutant, pDuc 83, and the point mutation, pDuc 80, were not infectious in whole plant assays, an effort was made to determine whether these mutants were able to replicate in primarily infected cells of inoculated leaves. For this purpose, we tested an immunosorbent procedure selective for virus particles combined with a PCR method for amplification of viral DNA. Virus particles were trapped by immunosorbent binding to polystyrene plates or polypropylene tubes. Viral DNA was then released by alkali or heat, and when necessary, it was concentrated by filtration under centrifugal force. An aliquot of the recovered viral DNA was then amplified by PCR. The primers in the PCR amplification were designed to amplify the region where the mutation was introduced. Thus, the nature of the amplified DNA could be verified. As a negative control, a frame-change mutant of the region V reverse transcriptase gene (pDuc 33) was subjected to the same procedure. To ensure that the DNA recovered from the inoculated leaves was authentic viral DNA, and not a contamination from the cloned DNA used for inoculation, a DNase I digestion was performed before viral DNA extraction by alkali or heat. Thus, if the mutant did not replicate, its DNA would not be recovered after the DNase I treatment. Hence, no product would be observed after PCR amplification. DNA of the gene V frame-change

FIG. 4. Detection of the DNA of different PCISV gene I mutants, pDuc 80 and pDuc 83, by IS-PCR. Wild-type and mutant viral particles were immunoselected, and the genomic DNA was extracted as described in Materials and Methods. The viral DNA was amplified by PCR, and 15 μ l of the product was loaded onto a 1.2% agarose gel. (A) pDuc 80, a point mutation. Lanes 1 and 8 contain a molecular size standard 123-bp DNA ladder. Lanes 2 to 7 contain PCR-amplified products with the templates described below. Lane 2 is viral DNA immunoselected from leaves inoculated with pDuc 33 (a mutant incapable of replication); lane 3 is PCISV cloned DNA; lanes 4 and 6 are wild-type viral DNA immunoselected from leaves inoculated with pDuc 70 (an infectious PCISV clone); and lanes 5 and 7 are viral DNA immunoselected from leaves inoculated with pDuc 80. Before electrophoresis, the DNA fragments loaded in lanes 6 and 7 were digested with *Hpa*I restriction endonuclease. No change in the size of the wild-type PCISV amplified DNA was observed when it was digested with *Hpa*I (compare lanes 3, 4, and 6). However, as expected a reduction in size was produced by *Hpa*I digestion of the amplified DNA immunoselected from leaves inoculated with pDuc 80 (compare lanes 5 and 7). (B) pDuc 83, a deletion mutant. Lanes 1 and 6 contain a molecular size standard 123-bp DNA ladder. Lanes 2 to 5 contain the PCR-amplified product with the templates described below. Lane 2 is PCISV cloned DNA; lane 3 is viral DNA immunoselected from leaves inoculated with pDuc 33 (a mutant incapable of replication); lane 4 is wild-type viral DNA immunoselected from leaves inoculated with pDuc 70 (an infectious PCISV clone); and lane 5 is viral DNA immunoselected from leaves inoculated with pDuc 83.

mutant was invariably negative in PCR tests under these conditions.

IS-PCR amplification was performed with *D. stramonium* inoculated leaves 12 to 15 days postinoculation and with the top leaves 30 days postinoculation. In plants inoculated with pDuc 80 and pDuc 83, a specific PCR product was observed consistently in the inoculated leaves (Fig. 4) but not in the top noninoculated leaves of the same plants. In each case, the amplified fragment was confirmed as mutant viral DNA either by restriction enzyme analysis for pDuc 80 digested with *Hpa*I or by the size of the fragment (126 bp smaller in pDuc 83 than in the wild-type PCISV) (Fig. 4). No encapsidated DNA could be recovered, and no PCR-amplified product was obtained from plants inoculated with the pDuc 33 viral polymerase gene frameshift mutant. Thus, the IS-PCR procedure could unequivocally discriminate between input (naked cloned) DNA and progeny virion DNA following inoculation of plants.

Quantitation of PCISV gene I mutant pDuc 80 in primarily infected cells. The qualitative IS-PCR procedure used in the initial phases of this investigation gave unambiguous results, discriminating against the inoculum DNA background. However, the procedure involved dilution of sample, disruption of virions, washing, and concentration steps with variable losses at every stage. To make the procedure more precise for virus

quantitation, we eliminated several steps as pointed out in Materials and Methods.

In a typical quantitative experiment, extracts of 10^4 cells from the lower epidermis of plants inoculated with pDuc 80 were mixed with various amounts of purified, wild-type virus prior to IS. For 10^7 , 10^6 , 10^5 , 10^4 , and 10^3 wild-type virions added to extract, the mutant/wild-type ratios of amplified DNA were 0.0, 0.14, 0.69, 1.43, and 1.69, respectively.

From a number of trials giving data similar to those of the experiment described above, we estimated that the standard and sample DNA concentrations were equal when ca. 6×10^4 virus particles were added to the extract of 10^4 epidermal cells inoculated with pDuc 80. This was an estimate of the amount of virus in extracts of about 10^4 epidermal cells inoculated with pDuc 80.

In addition, we estimated the number of cells actually infected with pDuc 80 by coamplifying extracts of 10^4 epidermal cells peeled from pDuc 80-inoculated leaves. To these extracts, we added similar extracts from a known number of epidermal cells from leaves infected with wild-type virus (pDuc 70 inoculation). For the latter, the epidermis was peeled 10 to 15 days after inoculation. Staining of these epidermal strips from the wild-type viral DNA inoculations with 1% phloxine (29) showed that over 50% of the cells contained viral inclusion bodies. In another experiment, detection was on a cellular basis with cells from systemically infected plants (pDuc 70) as a source of wild-type virus in the coamplified samples. These infected cells were added to samples of the lower epidermis of 10^4 cells of plants inoculated with pDuc 80. For 10^3 , 10^2 , 10^1 , 10^0 , and 10^{-1} cells infected with wild-type virus, the mutant/wild-type ratios of amplified DNA were 0.0, 0.11, 0.45, 1.12, and 1.49, respectively. As shown, equal amounts of virus-specific DNA were recovered from about 10^4 cells of pDuc 80-inoculated leaves and 1 to 10 cells known to be infected with wild-type virus. Consequently, this value indicates that one cell of every 10^3 to 10^4 cells of epidermis inoculated with pDuc 80 contained an equal quantity of virus. Although we cannot assume that this is present in one or a few primarily infected cells, this seems the most likely explanation for this observation.

The fact that we could detect virus in inoculated leaves with both gene I mutants but not with a gene V mutant of PCISV by the IS-PCR method argues strongly that both gene I mutants are competent for replication.

DISCUSSION

The clone of PCISV redundant for gene VI and the large intergenic region of the viral genome (Fig. 1) showed good infectivity on *D. stramonium* without excision from the cloning vector. Recent transcriptional data for PCISV (26) indicate that a full-length RNA transcript can arise from this clone without recombination to regenerate the wild-type viral genome. Transcription initiated in the large intergenic region can proceed around the entire viral genome to its natural termination point without intervention by the cloning vector. Perhaps the full-length RNA is reverse transcribed to establish infection. This would be similar to the mechanism suggested by Geldreich et al. (13) to account for the origin of recombinant viruses when linearized cloned genomes of different strains of CaMV are inoculated onto plants simultaneously. They suggested that cohesive ends of the linear DNAs were probably ligated in host cells to produce a dimer from which a complete full-length RNA transcript could be generated without need for an intermolecular recombination event. Unique restriction sites of the parent strains were present in selected regions of

the recombinant strains, which indicated that dimerization had occurred (13). Others have shown that multimeric constructs of CaMV introduced into plants by agroinfection (via an *Agrobacterium* Ti-plasmid vector) can generate infections when a full-length 35S transcript can arise from the redundant viral genome (14).

Mutation of gene I of PCISV, even some point mutations, destroyed viral infectivity on whole plants as one might expect from disruption of a gene with a putative movement function. However, it was deemed possible that gene I mutants might replicate locally when introduced into plants. Our IS-PCR results indicated that this is the case. Viral mutants that were inactive on whole plants gave amplified products with the characteristic properties of the virus mutants following the IS-PCR procedure. This was true for mutants with both types of changes in gene I, viz., a point mutation of a single amino acid residue (pDuc 80) and a 126-bp deletion were both detectable in inoculated leaves of *D. stramonium* 12 to 15 days after the plants were inoculated with DNA of these mutants (Fig. 4). No similar amplified product was ever detected in the tip leaves of these same plants. Identical experiments showed that DNA of a PCISV gene V (reverse transcriptase) mutant was not detectable by the IS-PCR procedure. These results argue strongly for multiplication of the gene I mutants in the inoculated leaves. We interpret these results as a probable failure of cell-to-cell movement without impairment of virus multiplication in the inoculated leaves. Consequently, it seems likely that the function of gene I is not required for viral DNA replication or accumulation. The fact that amplifiable viral DNA can be immunoselected indicates that viral DNA is encapsidated as complete virus particles. Removal of an extensive region (encoding 41 amino acids) of the protein coding region of gene I does not affect functions that are needed for virus multiplication and assembly. However, this large deletion did not change the reading frame for translation and hence would not interfere with the continuity of translation of downstream genes of the full-length transcript. This RNA probably serves as a polycistronic mRNA for expression of the genes downstream of gene I (28).

The assay we described here is suitable for detection of a very small amount of encapsidated virus DNA. It relies on the extremely high sensitivity of PCR which is thought to be sufficient to detect a single target DNA molecule per standard, 30-cycle assay (17). With this level of sensitivity of PCR, it was essential to discriminate among progeny, encapsidated DNA, and inoculum, which is naked, plasmid DNA. We exploited antiviral antibodies to trap virions on a polystyrene support; as caulimovirus virions are DNase resistant, only unencapsidated DNA, including inoculum input, was destroyed by subsequent DNase treatment. Control inoculations with nonreplicating mutant pDuc 33 demonstrated that input DNA was effectively removed prior to the PCR step. This is a crucial distinction between our approach and the assay described in the work of Thomas et al. (34). In the latter work, reproduction of CaMV gene I mutants was assayed by Southern blotting which detected the prevalent amounts of input DNA. In contrast, we believe that virtually all DNA we detected was derived from virions formed by gene I mutants in primarily infected cells.

Although replication of PCISV gene I mutants in primarily infected cells was unequivocally demonstrated, the actual amount of virus in these cells as well as the actual number of cells that were infected by the cell-to-cell defective mutants could not be determined in a precise manner. A moderate level of quantitation can be obtained by inclusion of an internal standard that is closely similar to the gene I target sequence

during the coamplification process (31, 33, 37). The competition by amplified internal standard DNA could be discriminated from the target sequence by the presence of a new restriction enzyme site. However, even a closely similar standard can be subject to small variations in amplification efficiency in the early steps of the PCR process. With the exponential nature of the PCR process, these small differences can be greatly magnified in the product yields (30). Nevertheless, the addition of a known amount of a closely similar internal standard to spike the PCR sample to be amplified is the only feasible way to quantify the process. The use of this procedure for detection of human immunodeficiency virus proviral DNA has been estimated to permit quantitation of human immunodeficiency virus with a 20 to 30% variation coefficient (33).

Thus we estimated that about 3×10^4 virions of PCISV were present in a total of 10^4 epidermal cells of leaves about 2 weeks after inoculation with DNA of pDuc 80, which contains a point mutation in gene I of PCISV (see above). However, we cannot directly translate this into the number of infected cells. Either a single infected cell or several could account for these 3×10^4 particles. In addition, we used the method to estimate the probable number of infected cells per set of pDuc 80-inoculated leaves under the assumption that a single infected cell of the latter would contain the same amount of PCISV as similar cells from plants systemically infected with wild-type virus.

From these experiments, we estimated that one of every 10^3 to 10^4 epidermal cells of pDuc 80-inoculated leaves was infected (see above). Assuming that the accumulation of virus per single cell is the same for mutant and wild-type virus, and seeing from results described above that the amount of viral DNA amplified from 10^4 potentially mutant-infected cells equals the amount of DNA amplified from about one (10^0) wild-type cell, we concluded that probably only one of the 10^4 potentially infected cells was actually infected. Moreover, it should be pointed out that this number of cells is intermediate between estimates of the number of cells initially infected by an RNA virus (tobacco mosaic virus) inoculated as intact virions (12, 32).

These experiments reinforce the observations of other investigators that suggest that gene I of the caulimoviruses functions in cell-to-cell transport of these viruses. Until recently, no direct evidence for this hypothesis was available. In a related investigation, Thomas et al. (34) have reported that a gene I mutant of CaMV that causes no apparent infection on whole plants may be complemented for systemic infection by a wild-type strain of CaMV.

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