

Expression of the Oligomerization Domain of the Replication-associated Protein (Rep) of *Tomato Leaf Curl New Delhi Virus* Interferes with DNA Accumulation of Heterologous Geminiviruses*

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Anju Chatterji, Roger N. Beachy, and Claude M. Fauquet‡

From the International Laboratory for Tropical Agricultural Biotechnology, Donald Danforth Plant Science Center, St. Louis, Missouri 63121

The minimal DNA binding domain of the replication-associated protein (Rep) of *Tomato leaf curl New Delhi virus* was determined by electrophoretic mobility gel shift analysis and co-purification assays. DNA binding activity maps to amino acids 1–160 (Rep-(1–160)) of the Rep protein and overlaps with the protein oligomerization domain. Transient expression of Rep protein (Rep-(1–160)) was found to inhibit homologous viral DNA accumulation by 70–86% in tobacco protoplasts and in *Nicotiana benthamiana* plants. The results obtained showed that expression of N-terminal sequences of Rep protein could efficiently interfere with DNA binding and oligomerization activities during virus infection. Surprisingly, this protein reduced accumulation of the *African cassava mosaic virus*, *Pepper huasteco yellow vein virus* and *Potato yellow mosaic virus* by 22–48%. Electrophoretic mobility shift assays and co-purification studies showed that Rep-(1–160) did not bind with high affinity *in vitro* to the corresponding common region sequences of heterologous geminiviruses. However, Rep-(1–160) formed oligomers with the Rep proteins of the other geminiviruses. These data suggest that the regulation of virus accumulation may involve binding of the Rep to target DNA sequences and to the other Rep molecules during virus replication.

Geminiviruses cause economically significant diseases in a wide range of cereal, vegetable, and fiber crops (1). These viruses have a single-stranded DNA genome that is replicated in nuclei of infected cells by a rolling circle mechanism (2, 3). Of the different gene products encoded by the virus, only *AC1*, the replication-associated protein (Rep), is essential for viral DNA replication. The first step in the replication process involves recognition of specific DNA sequences referred to as iterons, (4), by the Rep protein in the common region (CR)¹ of the virus genome. Most iteron sequences occur as direct repeat motifs of 6–12 base pairs between the TATA box and the start site of

transcription of the *AC1* gene. The iterons serve as high affinity binding sites of the Rep protein and therefore function as the origin recognition sequences. Specific regions on the N terminus of Rep protein are involved in DNA binding and have been identified for *Tomato golden mosaic virus* (TGMV) (5, 6), *African cassava mosaic virus* (ACMV) (7), and *Tomato yellow leaf curl virus* (8).

The potential binding site sequences in the common region of the *Tomato leaf curl New Delhi virus* (ToLCNDV) (9) genome were identified by site-directed mutagenesis (10). Further analyses using gel shift assays confirmed that the Rep protein specifically binds to the iterated motifs GGTGTCTGGAGTC (nucleotides 2640–2653) in the origin of replication (11). In the present study, our objective was to identify the DNA binding domain of the Rep protein and to determine the nature and contribution of DNA binding and protein oligomerization properties of the Rep protein to limit viral DNA accumulation in plants. In two cases, truncated Rep proteins have been shown to confer resistance to other geminiviruses (7, 12), and the resistance was specific and limited to the homologous virus. We based our choice of truncated Rep protein on the knowledge of overlapping sites for DNA cleavage, domains for DNA binding, and domains for protein oligomerization (13, 14). We hypothesized that a truncated Rep protein that was competent for DNA binding and oligomerization domain might have a greater probability to interfere with the virus replication and might be effective against both homologous and heterologous viruses.

In this study, we mapped the minimal binding domain on the Rep protein by electrophoretic mobility shift assays (EMSAs). We also tested the effect of truncated and full-length *AC1* sequences on DNA replication of ToLCNDV and other geminiviruses in transient assays using BY2 protoplasts and *Nicotiana benthamiana* plants. These studies revealed that transient expression of the ToLCNDV-truncated Rep protein encoding the DNA binding and the oligomerization domains could significantly inhibit replication of ToLCNDV viral DNA and to some extent the replication of other geminiviruses having similar iteron sequences.

MATERIALS AND METHODS

Plasmid Constructs—The full-length *AC1* genes from the severe and the mild strains of ToLCNDV were amplified by PCR from pMPA1 (DNA-A of the severe strain ToLCNDV) and pMPA2 (DNA-A of the mild strain ToLCNDV) (15), cloned in the bacterial expression vector pGEX-4T-3 (Amersham Pharmacia Biotech), and overexpressed in *Escherichia coli* cells. The recombinant proteins were named according to the number of amino acids at the N or C terminus of the Rep protein. The C-terminal truncations were made by inserting an in-frame stop codon at positions 2436 (pAC1-(1–52)), 2250 (pAC1-(1–114)), and 2110 (pAC1-(1–160)). The truncated *AC1* sequences were subcloned as a *Bam*HI–*Xho*I fragment in the pGEX-4T-3 vector, generating pAC1-(1–52), pAC1-(1–114), and pAC1-(1–160), respectively. At the N terminus, the

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‡ To whom correspondence should be addressed: International Laboratory for Tropical Agricultural Biotechnology, Donald Danforth Plant Science Center, 8001 Natural Bridge Rd., CME, M308, University of Missouri, St. Louis, MO 63121. Tel.: 314-516-4585; Fax: 314-516-4582; E-mail: iltab@danforthcenter.org.

¹ The abbreviations used are: CR, common region; TGMV, *Tomato golden mosaic virus*; ACMV, *African cassava mosaic virus*; ToLCNDV, *Tomato leaf curl New Delhi virus*; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; PHYVV, *Pepper huasteco yellow vein virus*; PYMV, *Potato yellow mosaic virus*.

first 21 amino acids of the protein were deleted, and an *NheI* site was inserted to create an in-frame start codon. The truncated fragment was cloned as a *NheI*-*XhoI* fragment in the vector pGEX-4T-3 to produce pAC1-(22–360). The plasmids pAC1-(52–360) and pAC1-(114–360) were produced similarly but had a deletion of the first 51 and 113 amino acids, respectively, from the N terminus of the AC1 gene.

Protein Expression and Analysis—The truncated Rep proteins were expressed from plasmids mentioned above in *E. coli* cells. The glutathione *S*-transferase (GST)-tagged AC1 fusion proteins were purified by glutathione affinity chromatography on glutathione-Sepharose beads according to the manufacturer's recommendations.

Briefly, the cells were grown to a density of 0.75–0.8 A_{600} . The cultures were induced by the addition of isopropyl- β -D thiogalactoside at a final concentration of 1 mM and grown further for 2 h. The cells were finally harvested at 4000 rpm (Beckman, JS 10.5 rotor) for 10 min. The pellets were suspended in ice cold 1 \times PBS (10 mM KH_2PO_4 , 100 mM NaCl) and lysed by sonication. The lysate was clarified at 17,000 $\times g$ for 30 min. The resulting supernatant was loaded on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) previously equilibrated with 1 \times PBS. After repeated washing of the column with 1 \times PBS, the protein was eluted with glutathione elution buffer (Amersham Pharmacia Biotech). The eluted fractions were dialyzed against 1 \times PBS to remove glutathione and concentrated using centricon filters (Amicon, Centricon). Protein concentrations were estimated using Bradford's reagent (Bio-Rad).

Protein extracts from *E. coli* cells co-expressing the untagged, wild type AC1 and GST fusion of truncated Rep proteins were tested for AC1 oligomerization by co-purification on glutathione-Sepharose. Co-purification of proteins was monitored by resolving the eluted fractions on SDS-PAGE and by immunoblotting. The full-length and truncated Rep proteins were detected using the polyclonal anti-AC1 antibody. A similar procedure was used to assess the oligomerization of full-length Rep proteins from other geminiviruses with the truncated Rep protein of ToLCNDV (pAC1-(1–160)). Protein extracts from *E. coli* cells co-expressing wild type AC1 of *Pepper huasteco yellow vein virus* (PHYVV), *Potato yellow mosaic virus* (PYMV), and ACMV and the GST-tagged pAC1-(1–160) were incubated with GST-Sepharose beads, washed thoroughly with 1 \times PBS, and eluted with glutathione elution buffer (Amersham Pharmacia Biotech). The eluate was resolved on SDS-PAGE gels and then transferred to nitrocellulose membranes and detected by immunoblotting using polyclonal anti-AC1 antibody and anti-GST antibody.

Construction of Expression Cassettes—For expression of the truncated AC1 gene in plant cells, the mutants described above were subcloned as *Bam*HI fragments in the plant expression vector pILTAB 350. This placed the DNA fragment 3' of the cassava vein mosaic virus promoter (16) upstream of the AC1 gene sequences to produce the gene expression cassettes, pILTAB 401 (encoding AC1-(1–52)), pILTAB 402 (encoding AC1-(1–114)), and pILTAB 403 (encoding AC1-(1–160)), respectively.

Constructions of infectious clones of plasmids containing full-length DNA of ToLCNDV are named pMPA1 and pMPB1 and were previously described (15). John Stanley (John Innes Institute, Norwich, United Kingdom) generously provided full-length infectious dimers of ACMV-Kenya, pCLV 1.3A, and pCLV 2B (17). Infectious monomers of PHYVV (18) were kindly provided by Riviera Bustamante (CINVESTAV, Irapuato, Mexico). The PYMV clones have been described (19).

EMSAs—The sequences of the synthetic oligonucleotides used as probes or competitors in EMSAs are given in Table III. In the case of the severe and mild strains of ToLCNDV, the 18-mer oligonucleotides corresponding to the binding sites of the Rep protein were used as probe (11). For the geminiviruses, ACMV, PHYVV, and PYMV, fragments of their CR sequences were synthesized and used as competitors in EMSAs. All oligonucleotides were synthesized commercially by Life Technologies, Inc.

The single-stranded 18-mer oligonucleotides containing the potential binding sites of the Rep protein of ToLCNDV were annealed to their complementary strands. The oligonucleotides were end-labeled with [γ - ^{32}P]ATP using T4 polynucleotide kinase and purified on polyacrylamide gels. The final concentration of the probes was 500 pM (30,000 cpm). The concentration of competitor DNA used was 50 nM per reaction. Both the probe and the competitor DNAs were purified on Sephadex G-25 columns, quantified by scintillation counting, and diluted to 30,000 cpm for each binding reaction.

The binding assays were performed using purified Rep protein. Typically, the binding reactions contained 500 ng of pure protein, 1 ng of labeled DNA, and 0.2 μ g of poly(dI-dC). Binding buffer contained 20 mM HEPES, pH 7.5, 60 mM KCl, 1 mM dithiothreitol, and 15% glycerol.

Reactions were incubated at 25 °C for 30 min, and the complexes were resolved on 4% polyacrylamide gels in 0.25 \times TBE buffer. The gels were dried on Whatman paper and exposed to x-ray film. Comparative efficiency of binding was analyzed by quantifying the amount of radioactivity in the retarded bands using a PhosphorImager (Molecular Dynamics).

Transient Replication Assays in Protoplasts and Plants—Protoplasts derived from *Nicotiana tabacum* BY-2 suspension cultures were used for transfection with viral DNA (20). Protoplasts were collected from cultures 48 h postinoculation for DNA isolation and analysis. One million protoplasts were inoculated by electroporation (250 V, 500 microfarads) with 2 μ g each of A and B component DNAs and 40 μ g of sheared herring sperm DNA (10). For co-inoculation experiments, 2 μ g of the plasmid DNA containing the expression cassettes with truncated AC1 gene sequences were used. Total DNA from the protoplasts was extracted 48 h after transfection (21, 22). Viral DNA accumulation was analyzed by Southern blotting (10).

Total proteins were extracted from the protoplasts 48 h after transfection by sonication of the cell pellets in ice cold 1 \times PBS (10 mM KH_2PO_4 , 100 mM NaCl). The lysate was clarified at 17,000 $\times g$ for 15 min, and the resulting supernatant was used for immunoprecipitations. Immunoprecipitations were done by incubating 50 μ g of total protein extracts with polyclonal anti-AC1 antiserum (1 mg) overnight at 4 °C. Protein-antibody complexes were incubated with protein A-agarose for 2 h at 4 °C and then washed with 1 \times PBS. Bound proteins were eluted from the agarose beads in SDS-PAGE sample buffer by boiling at 100 °C for 5 min. Proteins resolved on the gel were transferred on nitrocellulose membranes and analyzed by immunoblotting with polyclonal anti AC1 antibody using 3,3'-diaminobenzidine tetrahydrochloride for colorimetric quantitation of the expressed Rep levels in the cell extracts.

Two-week-old seedlings of *N. benthamiana* were grown in magenta boxes and inoculated with partial tandem dimers of viral DNA using a Bio-Rad helium-driven particle gun (10). Ten plants were inoculated with each mutant using 0.5 μ g each of DNA-A and DNA-B genomic components per plant. Plants were observed for symptom development, and newly emerging leaves were harvested for Southern blot analysis 4 weeks postinoculation.

Southern Blot Analysis—DNA extractions from systemically infected leaf samples were completed as described by Dellaporta *et al.* (21) and from protoplasts by following the procedure of Mettler (22). Total DNA (4 μ g) was fractionated on 1% agarose gels without ethidium bromide and transferred to nylon membranes. Viral DNA was detected by using a 900-base pair *Afl*III-*Pst*I fragment of the A component containing sequences from the open reading frames of the AC1, AC2, and AC3 genes or a probe specific for the B component (878-base pair PCR-amplified BC1 gene). The amount of viral DNA was quantified as previously described (10). In the case of geminiviruses other than ToLCNDV, fragments of their AC1 and BC1 genes were amplified and used as probes to analyze the replication levels of viral DNA.

RESULTS

Determination of a Minimal Binding Domain of the Rep Protein of ToLCNDV—The Rep protein binds specifically to a directly repeated DNA sequence motif in the common region of the ToLCNDV genome (11). Purified Rep proteins truncated at amino acids 160, 114, and 52 were used to map the C-terminal boundary of the Rep DNA binding domain *in vitro*. As a control, full-length Rep protein (amino acids 1–360) was used in all assays. The truncated and full-length Rep proteins were expressed in *E. coli* with a GST tag and affinity-purified on a glutathione-Sepharose 4B column. The affinity-purified proteins were highly enriched as determined by Coomassie staining following electrophoresis on SDS-PAGE gels. The proteins were detected in immunoblots using anti-GST antibody (data not shown).

The purified Rep proteins were tested for their ability to bind a radiolabeled 18-mer (nucleotides 2632–2653) that contains the Rep binding site sequence, 5'-GGTGTCTGGAGTC-3'. DNA-protein complexes that contained Rep-(1–360) and Rep-(1–160) were detected. No binding was observed for Rep-(1–52) or Rep-(1–114) (Fig. 1A, lanes 1–4). These results located the C-terminal boundary of the DNA binding domain of the Rep protein between amino acids 115 and 160.

The N-terminal boundary of the DNA binding domain was

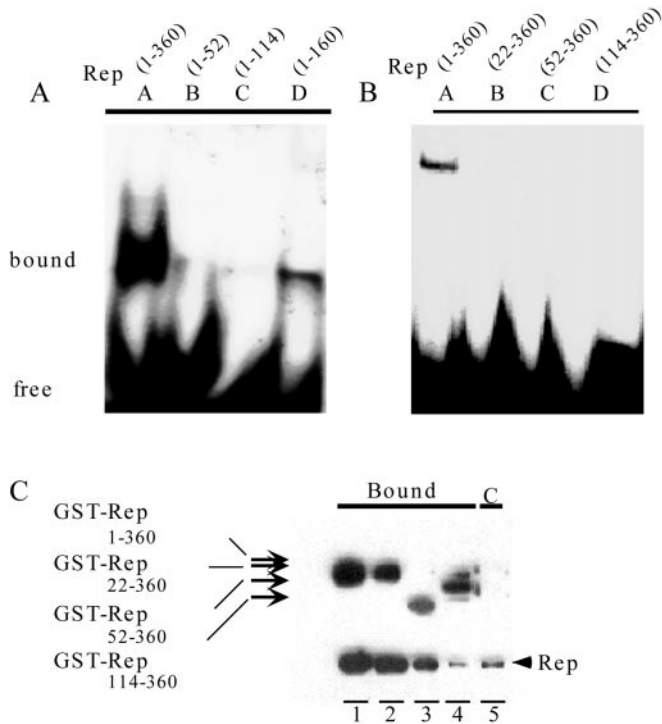


FIG. 1. Determination of the DNA binding domain of the Rep protein of ToLCNDV. A, binding of full-length and C-terminal truncated Rep proteins to origin DNA sequences. Highly enriched preparations of GST-AC1 fusion Rep proteins were analyzed for their ability to bind to radiolabeled iteron sequences containing the ToLCNDV Rep protein binding site in EMSAs. Typically, the binding reactions contained 500 ng of pure protein, 1 ng of labeled DNA, and 0.2 μ g of poly(dI-dC). Binding buffer contained 20 mM HEPES, pH 7.5, 60 mM KCl, 1 mM dithiothreitol, and 15% glycerol. Reactions were incubated at 25 $^{\circ}$ C for 30 min, and the complexes were resolved on 4% polyacrylamide gels in 0.25 \times TBE buffer. Lane 1 represents the binding observed *in vitro* with a full-length Rep protein. Lanes 2–4 show the same binding assays with the Rep protein truncated at its C terminus at amino acids 1–52 (lane 2), 1–114 (lane 3), and 1–160 (lane 4). B, mapping the N-terminal boundary of the DNA binding domain of the Rep protein of ToLCNDV. Highly enriched preparations of GST-AC1 fusion Rep proteins were analyzed for their ability to bind to radiolabeled iteron sequences containing the ToLCNDV Rep protein binding site in EMSAs. Typically, the binding reactions contained 500 ng of pure protein, 1 ng of labeled DNA, and 0.2 μ g of poly(dI-dC). Binding buffer contained 20 mM HEPES, pH 7.5, 60 mM KCl, 1 mM dithiothreitol, and 15% glycerol. Reactions were incubated at 25 $^{\circ}$ C for 30 min, and the complexes were resolved on 4% polyacrylamide gels in 0.25 \times TBE buffer. Lane 1 represents the binding observed *in vitro* with a full-length Rep protein. Lanes 2–4 show the same binding assays with the Rep protein truncated at its N terminus at amino acids, 22–360 (lane 2), 52–360 (lane 3), and 114–360 (lane 4). C, co-purification of the N-terminal truncated GST-AC1 fusion proteins with the full-length untagged wild type Rep protein. Bacterial cell lysates co-expressing a truncated GST-AC1 fusion protein with a full-length Rep protein were passed over a glutathione-Sepharose 4B column. After washing, the eluted fractions were resolved on SDS-PAGE and detected in immunoblots using a polyclonal anti AC1 antiserum. Lanes 1–4 represent the different GST-tagged Rep fusion proteins truncated at their N termini that bound with the untagged full-length Rep protein. Lane 1, GST-AC1-(1–360); lane 2, GST-AC1-(22–360); lane 3, GST-AC1-(114–360); lane 4, GST-AC1-(52–360); lane 5, untagged full-length Rep protein used as a control.

determined *in vitro* by comparing the binding of full-length Rep-(1–360) and Rep-(22–360), Rep-(52–360), and Rep-(114–360) to the 18-base pair iteron sequence, 5'-GGTGTCTG-GAGTC-3' in EMSAs. The DNA-protein complexes were observed in case of full-length Rep protein, while no DNA-protein complexes were detected for the Rep-(22–360), Rep-(52–360), or Rep-(114–360) (Fig. 1B, lanes 2–4). These results demonstrated that the sequences within the first 21 amino acids of the Rep protein are essential for protein-DNA interactions.

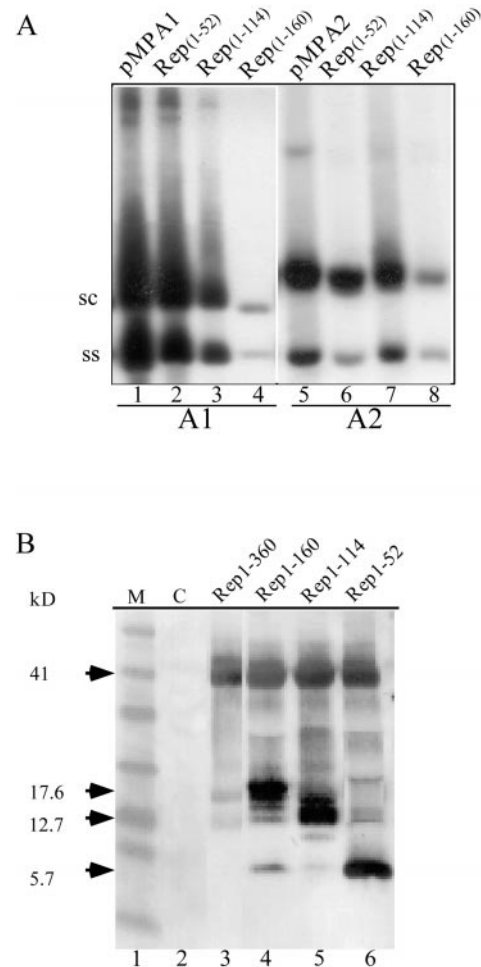


FIG. 2. Effect of Rep-(1–160) on accumulation of viral DNA in tobacco protoplasts. A, Southern blot analysis of total DNA extracted from protoplasts 48 h after co-transfection with wild type, infectious dimers of the DNA-A of the severe strain pMPA1 alone (lane 1) or with Rep-(1–52) (lane 2), Rep-(1–114) (lane 3), or Rep-(1–160) (lane 4). Lanes 5–8 represent viral DNA accumulation in protoplasts co-infected with the DNA-A of the mild strain pMPA2 alone (lane 5) or with Rep-(1–52) (lane 6), Rep-(1–114) (lane 7), and Rep-(1–160) (lane 8) of the homologous strain. B, immunoblot analysis of the proteins extracted from tobacco protoplasts co-transfected with various Rep constructs and detected by anti-AC1 antibody. 50 μ g of total protein extracts were incubated with 1 mg of anti-AC1 antibody overnight at 4 $^{\circ}$ C. Bound proteins were recovered from the agarose beads after extensive washing of protein-antibody complexes in 1 \times PBS and boiling the beads in SDS-PAGE sample buffer. Proteins resolved on the gel were transferred on nitrocellulose membranes and analyzed by immunoblotting with polyclonal anti-AC1 antibody using the 3,3'-diaminobenzidine tetrahydrochloride (DAB) that provided a quantitative estimation of the precipitated protein-antibody complex in the samples. The protoplasts were transfected with wild type infectious dimer of the DNA-A of the severe strain pMPA1 with Rep-(1–360) (lane 3), Rep-(1–160) (lane 4), Rep-(1–114) (lane 5), or Rep-(1–52) (lane 6). The extracts from the uninfected protoplasts served as the negative control (lane 2). The molecular masses of the truncated and full-length Rep (kDa) are shown on the left.

Together, these results placed the DNA binding domain of ToLCNDV Rep protein between amino acids 1 and 160.

To determine if truncations at the N and the C termini of Rep protein affect its ability to oligomerize, GST-tagged truncated Rep proteins were co-expressed with untagged wild type full-length Rep protein in bacterial cells and co-purified on glutathione-Sepharose beads. The bound fractions were eluted and analyzed in immunoblots using polyclonal anti-AC1 antiserum. The wild type Rep-(1–360) co-purified with GST-tagged truncated proteins Rep-(22–360), Rep-(52–360), and Rep-(114–360)

TABLE I

Virus replication in BY2 protoplasts and *N. benthamiana* plants co-inoculated with truncated Rep protein gene constructs and the viral DNA of the severe strain of ToLCNDV

Virus construct	Symptom expression ^a	Replication ^b		
		Protoplasts	Plants	
			DNA-A	DNA-B
		%	%	%
A1+B	Severe, 10/10	100	100	100
A2+B	Mild, 10/10	55	48–50	10–15
A1+B+Rep-(1–52)/A1	Severe, 10/10	100	92–98	92–110
A1+B+Rep-(1–114)/A1	Severe, 10/10	90–92	89–92	90–98
A1+B+Rep-(1–160)/A1	Very mild, ^c 10/10	22–28	14–30	5–14
A1+B+Rep-(1–52)/A2	Severe, 10/10	100	94–96	100
A1+B+Rep-(1–114)/A2	Severe, 10/10	100	89–93	98–100
A1+B+Rep-(1–160)/A2	Severe, 10/10	78–80	70–74	94–98
A2+B+Rep-(1–160)/A2	No symptoms, 10/10	50	56	12–14

^a A total of 10 plants were inoculated per experiment, and each experiment was repeated three times. Shown are the number of plants infected/number of plants inoculated. Plants were scored for symptom expression 3 weeks postinoculation.

^b The numbers refer to the amount (in percentage) of viral DNA replication in protoplasts electroporated with similar constructs. The viral DNA was quantified using a PhosphorImager (Molecular Dynamics).

^c About 55% of plants were asymptomatic, 30% showed mild chlorosis, and only 15% of plants expressed mild symptoms of leaf curl (Table I). None of the plants showed severe infection or stunted growth found in wild type infection. Most of the plants inoculated with AC1–1(1–52) and AC1–2(1–114) developed severe symptoms 7 days post inoculation (Table II).

(Fig. 1C, lanes 1–5), suggesting that truncations made at the N terminus in the Rep did not affect the ability of the Rep protein to oligomerize with itself, although each of the truncated proteins was deficient for DNA binding.

ToLCNDV Replication Is Inhibited by Transiently Expressed Rep Protein—The effect of Rep protein on viral DNA replication was investigated by co-inoculating *N. tabacum* BY2 protoplasts with DNA-A and various cassettes that express truncated AC1 gene sequences from the CsVMV promoter. ToLCNDV DNA-A replicated in BY-2 cells and accumulated high levels of single-stranded (ss) and supercoiled (sc) DNA (Fig. 2A, lane 1). In contrast, there was a significant decrease in the level of viral DNA replication (78% drop) in the presence of Rep-(1–160) (Fig. 2A, lane 4, Table I). Reduction in replication was estimated by quantifying the amount of radioactivity using a PhosphorImager (Storm 860; Molecular Dynamics). The reduction in virus replication was not as dramatic in the presence of Rep-(1–52) (Fig. 2A, lane 2) or Rep-(1–114) (Fig. 2A, lane 3) when compared with Rep-(1–160) (Fig. 2A, lane 4). EMSAs showed that the Rep-(1–52) and Rep-(1–114) do not bind DNA (Fig. 1A, lanes 2 and 3), implying that an intact DNA binding domain and/or a protein oligomerization domain is essential for inhibition of replication.

Similar experiments were conducted with the mild strain of ToLCNDV in which analogous truncated mutations of the Rep gene were co-introduced in tobacco protoplasts with DNA-A from the mild strain. In these studies, an analogous inhibition of viral DNA accumulation in BY2 protoplasts was detected (Fig. 2A, lanes 5–8, and Table II).

To determine the relative expression levels of the three truncated Rep proteins in transfected tobacco protoplasts, total proteins were extracted from the tobacco protoplasts 48 h after infection and immunoprecipitated with the anti-AC1 antibody, and the protein-antibody complexes were resolved on SDS-PAGE gels. All of the three truncated proteins could be detected in immunoblots from the transfected protoplasts when developed using 3,3'-diaminobenzidine tetrahydrochloride. 3,3'-Diaminobenzidine tetrahydrochloride produces a brown precipitate with the peroxidase and thereby provided a direct measure of the amount of antibody bound to the expressed protein in the samples, revealing that all three truncated Rep proteins were expressed stably and in equivalent amounts in the protoplasts (Fig. 2B, lanes 2–6).

Infection of *N. benthamiana*—Two-week-old seedlings of *N. benthamiana* plants were co-bombarded with 2 μ g each of

infectious dimers of ToLCNDV DNA-A and DNA-B in the presence or absence of genes encoding Rep-(1–160). The plants were observed daily for symptom development. All of the plants inoculated only with the wild type virus DNAs developed severe symptoms 5 days after inoculation. In contrast, plants co-inoculated with the virus and the genes encoding Rep-(1–160) developed milder symptoms of ToLCNDV infection (Table I). About 55% of the plants were asymptomatic, 30% showed mild chlorosis, and 15% expressed mild leaf curl symptoms (Table I). None of the plants showed severe infection or stunted growth as in plants infected only with ToLCNDV. Most of the plants co-inoculated with Rep-(1–52) and Rep-(1–114) developed severe symptoms by 7 days postinoculation (Tables I and II).

The levels of viral DNA in ToLCNDV-infected plants were analyzed by Southern blot analysis of young leaves sampled 28 days postinoculation using probes that detected DNA-A and DNA-B (see “Materials and Methods” and Fig. 3, A and B, respectively). The amount of viral DNA ranged from undetectable to very low (an average of 15% of the wild type levels) in asymptomatic plants, and the accumulation of both genomic components increased with increasing severity of symptom expression. Plants co-inoculated with expression cassettes Rep-(1–52) and Rep-(1–114) developed intermediate to severe symptoms in most of the plants and accumulated viral DNA between 85 and 92% of wild type infection.

Co-infection with Rep-(1–160) of ToLCNDV (Severe) Reduces the Viral DNA Accumulation of Other Geminiviruses—To investigate the potential of truncated Rep protein to inhibit the replication of other geminiviruses, we selected examples of viruses that belonged to the Old World (ACMV) and New World geminiviruses (PHYVV and PYMV-TT). We reasoned that for the Rep to be able to interfere in replication of heterologous geminiviruses, it must (a) bind to the origin sequences of these viruses and (b) oligomerize with their Rep proteins. For the EMSA studies, fragments of the intergenic region sequences of the selected heterologous geminiviruses close to the TATA box were chosen. The coordinates of these sequences are given in Table III. To determine if the putative iteron sequences of the other geminiviruses could compete with the cognate iteron sequences of ToLCNDV for binding to ToLCNDV Rep protein, synthetic oligonucleotides encoding the CR sequences from each virus were synthesized and used as competitors in EMSAs. None of the CR sequences were effective competitors in EMSA with the ToLCNDV Rep protein (Fig. 4A, lanes 3–6) and did not affect binding of the Rep protein with its cognate

TABLE II

Virus replication in BY2 protoplasts and *N. benthamiana* plants co-inoculated with truncated Rep protein constructs and viral DNA of the mild strain of ToLCNDV

Virus construct	Symptom expression ^a	Replication ^b		
		Protoplasts	Plants	
			DNA-A	DNA-B
A1+B	Severe, 10/10	100	100	100
A2+B	Mild, 10/10	55	52	10–12
A2+B+Rep-(1–52)/A2	Mild, 10/10	55	56	10–13
A2+B+Rep-(1–114)/A2	Mild, 10/10	45	42–45	8–10
A2+B+Rep-(1–160)/A2	No symptoms, 10/10	12	20	10–14
A2+B+Rep-(1–52)/A1	Mild, 10/10	50	60	10–12
A2+B+Rep-(1–114)/A1	Mild, 10/10	52	58	10–13
A2+B+Rep-(1–160)/A1	Mild, 10/10	28–30	36–40	10–12

^a A total of 10 plants were inoculated per experiment, and each experiment was repeated three times. Shown are the number of plants infected/number of plants inoculated. Plants were scored for symptom expression 3 weeks postinoculation.

^b The numbers refer to the amount (in percentage) of viral DNA replication in protoplasts electroporated with similar constructs. The viral DNA was quantified using a PhosphorImager (Molecular Dynamics).

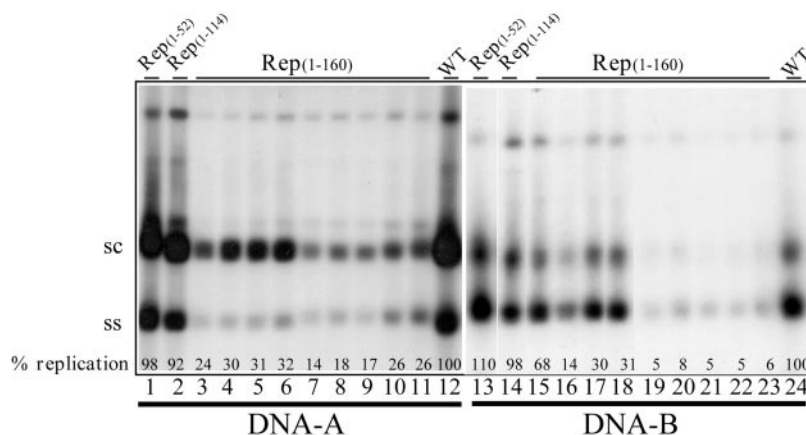


FIG. 3. Expression of the truncated Rep protein containing the minimal DNA binding domain (Rep-(1–160)) of the Rep protein from two strains of ToLCNDV interferes in virus accumulation in *N. benthamiana* plants. Two-week-old *N. benthamiana* plants were bombarded with 2 μ g each of DNA-A and DNA-B of ToLCNDV, and plasmids encoding truncated Rep proteins were expressed from the CsVMV promoter. Total DNA was extracted from *N. benthamiana* plants 21 days postinoculation and analyzed by Southern blots. The blots were probed for accumulation of DNA A (lanes 1–12) and DNA-B (lanes 13–24) as described under “Materials and Methods” using AC1- and BC1-specific probes, respectively. The levels of viral DNA accumulation in plants co-infected with Rep-(1–160) ranged from 8 to 23% as depicted in lanes 3–11 (DNA-A) and lanes 15–23 (DNA-B). Wild type (WT) controls are shown in lanes 12 and 24.

TABLE III

The sequence of synthetic oligonucleotides used in EMSAs

Virus	Sequence in the CR of the viral genome ^a	GenBank™ accession no.
ToLCNDV (s)	ATTGGTGTCTGGAGTCCC (2632–2653)	U15015, U15017
ToLCNDV (m)	ATTGGCGTCTGGCGTCCC (2632–2653)	U15016
PHYVV	ATCGGTGTATTGGTAGCCAAT (2554–2574)	PHV-mex, X70418, 70419
PYMV/TT	ATCGGTGTATTGGGGTACTAT (2508–2528)	PYMV/TT, AF039031
ACMV	ATTGGAGA-(40 bp)-GGAGACAT (2625–2682)	ACMV-K, j02057, j02058

^a The numbers within parenthesis indicate the coordinates of the CR sequences used as competitor in EMSAs for the respective virus.

13-mer iteron sequences to a significant degree.

The crude lysates of *E. coli* cells co-expressing wild type Rep proteins from ACMV, PHYVV, or PYMV and the GST-tagged ToLCNDV Rep-(1–160) were tested for the ability to bind to each other. Crude protein extracts from bacterial cells co-expressing the target proteins were loaded on a GST-Sepharose column, washed extensively with 1 \times PBS, and eluted with glutathione elution buffer. The resulting fractions were detected in immunoblots using polyclonal anti-AC1 and anti-GST antibodies. In immunoblots, the anti-AC1 antibody detected wild type untagged Rep proteins from ACMV, PHYVV, and PYMV-TT that co-purified with the ToLCNDV Rep-(1–160) (Fig. 4B, lanes 2–6). When the same blot was washed and reprobed with anti-GST antibody, only the truncated Rep protein of ToLCNDV (Rep-(1–160)) in each of the samples was detected (Fig. 4C, lanes 2–6).

To determine if Rep-(1–160) could reduce accumulation of

other geminiviruses, *in vivo* replication assays were conducted by co-bombarding the *N. benthamiana* plants with partial/tandem dimers of full-length A and B components of ACMV, PHYVV, and PYMV-TT with genes encoding Rep-(1–160) of ToLCNDV. Most plants developed typical symptoms of virus infection within 21–27 days postinoculation as opposed to 7–10 days required for the symptoms on control plants to develop infection. Southern blot analysis of viral DNA extracted from the plants harvested 28 days postinoculation showed a minor reduction in the levels of virus accumulation as compared with the control plants (Table IV and Fig. 5, A and B). However, the decrease in replication was not as significant as the inhibition observed in the case of ToLCNDV DNA.

DISCUSSION

We determined the nature and the significance of the DNA binding and protein oligomerization functions of a truncated

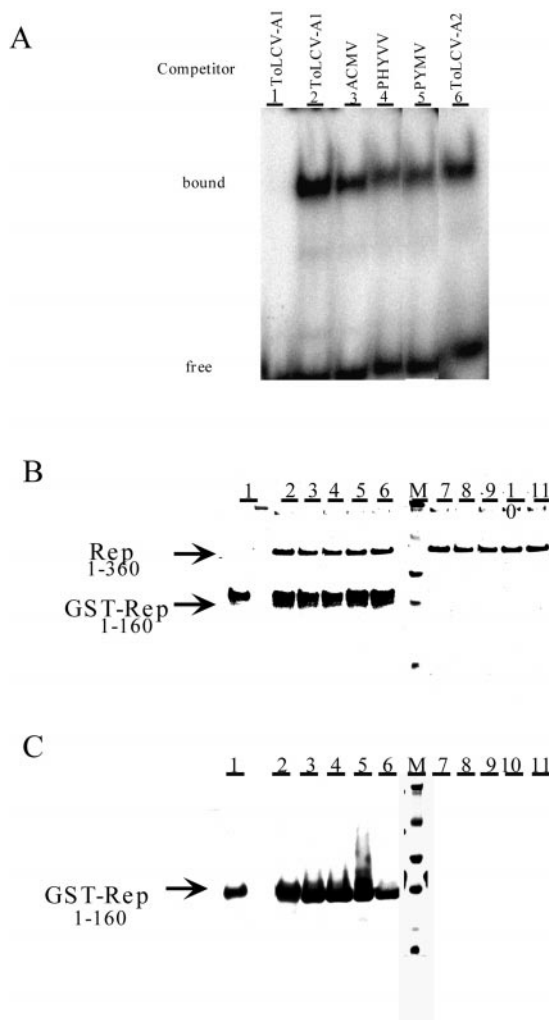


FIG. 4. Interaction of ToLCNDV Rep(1-160) with the Rep protein of other geminiviruses. A, competition of heterologous geminivirus CR sequences in EMSAs with the Rep(1-160) of ToLCNDV. Each reaction contained 500 ng of purified Rep(1-160) incubated with a 100-fold excess of CR sequences of different geminiviruses for 5 min on ice before the addition of the ^{32}P -labeled probe comprising the iteron sequences of ToLCNDV. After 30 min, the DNA-protein complexes were resolved on 4% polyacrylamide gels. Lane 1, the control reaction containing the iteron sequences of the severe strain of ToLCNDV as the competitor; lane 2, binding by the Rep(1-160) to iteron sequences of the severe strain of ToLCNDV-A1 without any competitor; lane 3, binding by the Rep(1-160) in the presence of CR sequences of ACMV; lane 4, binding by the Rep₁₋₁₆₀ with CR sequences of PHYVV as competitor; lane 5, binding by the Rep(1-160) in the presence of CR sequences from PYMV; lane 6, binding by the Rep(1-160) in the presence of iteron sequences of the mild strain of ToLCNDV-A2. B, immunoblots showing co-purification of Rep protein from other geminiviruses with the Rep(1-160) of ToLCNDV as detected by polyclonal anti-AC1 antibody. Lanes 2 and 7, Rep protein of ACMV; lanes 3 and 8, Rep protein of PHYVV; lanes 4 and 9, Rep protein of PYMV; lanes 5 and 10, Rep protein of ToLCNDV, mild strain; lanes 6 and 11, Rep protein of ToLCNDV, severe strain. Lane 1, control showing the relative mobility of GST-tagged Rep(1-160) protein in the gel. C, immunoblots showing co-purification of Rep protein from other geminiviruses with the Rep(1-160) of ToLCNDV as detected by polyclonal anti-GST antibody. Lanes 2 and 7, ACMV; lanes 3 and 8, PHYVV; lanes 4 and 9, PYMV; lanes 5 and 10, ToLCNDV, mild strain; lanes 6 and 11, ToLCNDV, severe strain. Lane 1, control showing the relative mobility of GST-tagged Rep(1-160) protein in the gel.

Rep(1-160) protein to interfere in DNA accumulation of homologous and heterologous geminiviruses. Our studies show that while both activities of Rep(1-160) contribute to interference in DNA accumulation, protein oligomerization, unlike DNA binding, is nonspecific and can occur between the Rep

proteins of two unrelated geminiviruses.

We mapped the DNA binding domain on the Rep protein of ToLCNDV to amino acids 1-160 and showed that the transient expression of this Rep sequence significantly inhibits ToLCNDV DNA accumulation in inoculated tobacco protoplasts and plants. Of the three C-terminal truncations made in the *AC1* gene, only Rep(1-160) bound the iteron DNA sequences *in vitro*, and the two truncations Rep(1-52) and Rep(1-114) were not competent to bind viral DNA *in vitro*. None of the N-terminal truncations tested (*i.e.* Rep(22-360), Rep(52-360), or Rep(114-360)) bound viral DNA, indicating that an intact N terminus is required for the Rep protein to bind the origin sequences. In co-purification assays, each of the three N-terminal truncated proteins bound with the wild type Rep protein as detected by immunoblotting of the bound fractions. The co-immunoprecipitation assays indirectly suggested that the oligomerization domain might overlap the DNA binding domain. In TGMV, it is known that the oligomerization domain overlaps the DNA binding domain (13).

In co-infection studies, the sequences comprising the Rep(1-52) or Rep(1-114) amino acids of the Rep protein did not cause a significant reduction in ToLCNDV accumulation. However, Rep(1-160) bound with high affinity to the iteron sequences and reduced viral replication in protoplasts and plants. Colorimetric quantification of the truncated proteins revealed that all three proteins were expressed in equivalent amounts, ruling out the possibility that poor expression and/or instability of Rep(1-52) and Rep(1-114) in tobacco protoplasts may have compromised their ability to inhibit virus replication. In related studies, we observed that *N. benthamiana* plants co-bombarded with plasmids that produced Rep(1-160) and infectious ToLCNDV produced a range of symptoms from asymptomatic to a mild leaf curl. None of the plants developed the severe puckering and blistering associated with wild type virus infection. Southern blot analysis of the infected plants showed that accumulation of viral DNA in plants with mild or no symptoms was much less than in plants showing severe symptoms. More importantly, the degree of inhibition in plants was similar to those observed in BY-2 protoplasts, indicating that the impact is probably on virus replication.

Rep(1-160) contains the DNA binding domain of the ToLCNDV-Rep. By analogy with the Rep proteins of TGMV and *Tomato yellow leaf curl virus*, this fragment is expected to contain the domains for DNA cleavage and ligation, as well as protein oligomerization domain (13, 23). This region of the Rep protein of ToLCNDV is involved in the specificity of origin recognition and binding (10). Considering the various activities that are associated with Rep(1-160), it is possible to suggest the mode of action of Rep(1-160) in limiting virus DNA accumulation.

One possibility is that the Rep(1-160) protein reduces replication by competing with the viral Rep protein for binding the iteron sequences in the origin. The truncated Rep protein may therefore behave as a dominant negative mutant (24) and block virus replication.

Another possibility is that the truncated Rep protein does not contain the NTP binding domain present on the C terminus of the Rep protein. The NTP binding domain is required for replication (25), and the lack of this region may interfere with the normal replication process of the virus.

The fact that the Rep protein represses its own transcription may be yet another explanation for the inhibition of virus replication. Presumably, binding by the Rep protein to the origin is responsible for the repression of *AC1* gene transcription in TGMV (26, 27) and ACMV (7). Constitutive expression of the truncated viral Rep protein could repress the transcrip-

TABLE IV
Regulation of virus DNA replication in BY2 protoplasts by the N-terminal sequences of AC1 gene of ToLCNDV

Virus	Putative iteron ^a	N-Rep sequence	EMSA	Replication ^b	
				A	B
			%	%	%
ACMV	GGAGA	MRTPPRFRIQANKYFLTYPKC		48–62	25
PHV	GGTGA	MPLPKRFLNAKNYFLTYPQC		26–52	19
PYMV	GGTGT	MP-PKRFRINANKYFLTYPKC		43–22	27
ToLCNDV (s)	GGTGT	MAPPFRFRVNANKYFLTYPKC	100	70–86	86–95
ToLCNDV (m)	GGCGT	MASPRFRIDANKYFLTYPKC		36–40	12–14

^a The sequences indicate putative iteron sequences.

^b Only Rep-(1–160) was tested in competition experiments. The numbers indicate relative inhibition of virus replication levels as compared with a wild type ToLCNDV Rep by Rep-(1–160) as determined by Southern blotting and PhosphorImager analysis.

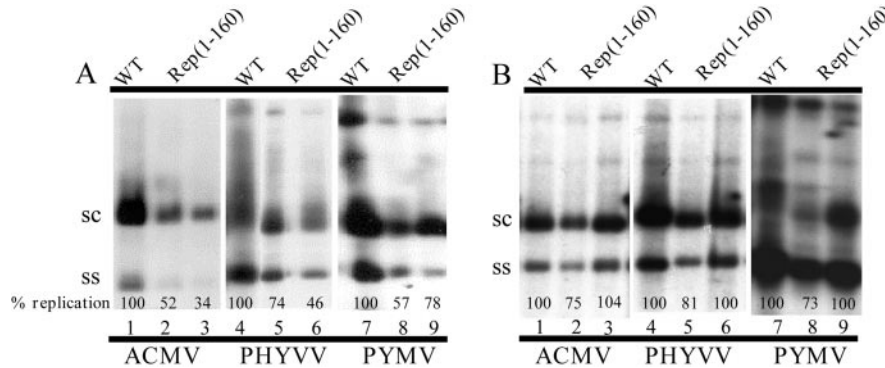


FIG. 5. **Effect of Rep-(1–160) expression on DNA accumulation of other geminiviruses.** Two representative samples from a set of 30 inoculated plants are shown to reflect the variation in DNA accumulation. **A**, Southern blots showing relative levels of DNA accumulation in *N. benthamiana* plants co-inoculated with infectious dimers of DNA-A and DNA-B of ACMV (lanes 2 and 3), PHYV (lanes 5 and 6), and PYMV (lanes 8 and 9) in the presence of Rep-(1–160) and probed with the AC1 gene sequences of different geminiviruses. Lanes 1, 4, and 7 represent the wild type (WT) level of DNA-A accumulation in the absence of Rep-(1–160) for ACMV, PHYVV, and PYMV, respectively. **B**, Southern blots showing relative levels of DNA accumulation in *N. benthamiana* plants co-inoculated with infectious dimers of DNA-A and DNA-B of ACMV (lanes 2 and 3), PHYVV (lanes 5 and 6), and PYMV (lanes 8 and 9) in the presence of Rep-(1–160) and probed with the *BC1* gene sequences of different geminiviruses. Lanes 1, 4, and 7 represent the wild type (WT) level of DNA-B accumulation in the absence of Rep-(1–160) for ACMV, PHYVV, and PYMV, respectively.

tion of the wild type *AC1* gene by binding to the origin, thereby influencing viral accumulation levels. Alternately, as suggested in the case of wheat dwarf virus, constitutively expressed Rep may adversely affect the integrity of the viral DNA by introducing nicks at cryptic motifs (28).

Comparison of co-infection experiments performed using truncated Rep proteins of the mild and the severe strains of ToLCNDV in protoplasts and plants suggested that only homologous Rep sequences could reduce virus accumulation with high efficiency. The Rep-(1–160) from the severe strain did not significantly restrict the virus replication of the mild strain and vice versa. Our previous work showed that the mild and the severe strains of ToLCNDV exhibit selectivity in binding to their cognate iteron sequences (11). Hence, the inability of Rep-(1–160) of one strain to limit virus DNA accumulation of the heterologous strain may reflect specificity of interaction by the Rep protein for its cognate origin DNA sequences. Since the Rep-(1–160) can bind to DNA and form oligomers, these results support the hypothesis that DNA binding and protein oligomerization are important in inhibition of virus replication by Rep-(1–160).

Notwithstanding that Rep-(1–160) of the severe strain of ToLCNDV had a modest effect on the replication of the mild strain, we were interested to know if Rep-(1–160) can interfere with the replication of related geminiviruses that have similar sequences in their origins of replication. The reduction in virus accumulation in the case of unrelated geminiviruses was rather surprising, considering that none of the CR sequences from these viruses were effective competitors for Rep-(1–160) in EMSAs. However, the results were not unexpected because the co-purification experiments revealed the ability of Rep-(1–160) to interact with the Rep proteins of heterologous gemini-

viruses. These data suggest that since the Rep-(1–160) protein does not bind to the heterologous CR sequences, reduction in virus accumulation may result from oligomerization of Rep-(1–160) with the Rep proteins of the unrelated geminiviruses. This level of virus reduction was similar to the reduction of mild strain ToLCNDV accumulation by the Rep-(1–160). We suggest that oligomers of Rep-(1–160) could potentially interfere with the replication complexes formed during infection by PHYVV, PYMV, ACMV, or the mild strain of ToLCNDV. Formation of heteromultimers via protein-protein interactions (29) has been reported between TGMV and BGMV. The formation of heteromultimeric complexes might sequester the wild type Rep oligomers that otherwise would participate in the formation of a replication complex or might prevent recognition of origin sequences.

Several approaches to control replication of geminiviruses have been developed. Transgenic *N. benthamiana* plants that accumulate defective interfering DNA (30, 31) of ACMV were less susceptible to ACMV infection, but resistance was confined to closely related strains of ACMV. Transgenic *N. tabacum* expressing antisense RNA targeted against TGMV AL1 (32) or *Tomato yellow leaf curl virus* (33) showed that specificity of resistance depended on the level of homology between the antisense RNA and the target virus sequence. Finally, the possibility of expressing a full-length *AC1* transgene in ACMV (34) and the N-terminal sequences of *Tomato yellow leaf curl virus* Rep (12) in virus resistance has also been documented. Our studies demonstrate the potential of using Rep proteins that are mutated in the oligomerization and DNA binding domain to interfere with viral DNA replication. Experiments are in progress to test the stable expression and efficiency of

Rep-(1–160) in transgenic tobacco and tomato plants for resistance to ToLCNDV.

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