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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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) 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 2640–2655) 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 BamHI–XhoI fragment in the pGEX-4T-3 vector, generating pAC1-(1–52), pAC1-(1–114), and pAC1-(1–160), respectively. At the N terminus, the
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 Nhel–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. Both the GST- and the native proteins were generated in 0.75–0.8 A280. 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× PBS (10 mM KH2PO4, 100 mM NaCl) and lysed by sonication. The lysate was clarified at 17,000 × g for 30 min. The resulting supernatant was loaded on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) previously equilibrated with 1× PBS. After repeated washing of the column with 1× PBS, the protein was eluted with glutathione elution buffer (Amersham Pharmacia Biotech). The eluted fractions were dialyzed against 1× PBS to remove glutathione and concentrated using centrifugation filters (Amicon, Centricron). Protein concentrations were estimated using Bradford's reagent, with 1× PBS as a blank.

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× 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 into BglII fragments in the pBILTAB vector (35). This placed the DNA fragment 3′ of the cassava vein mosaic virus promoter (16) upstream of the AC1 gene sequences to produce the expression cassettes, pLITAB 401 (encoding AC1-(1–52)), pLITAB 402 (encoding AC1-(1–114)), and pLITAB 403 (encoding AC1-(1–160)), respectively.

**Construction of Infectious Clones of Plasmids Containing Full-Length DNA of ToLCNDV**—The plasmids pMPA1 and pMPB1 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-32P]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 pm per reaction. 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 μg 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× 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.*

**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 Rep 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 [32P]-labeled 18-mer (nucleotides 2632–2653) that contains the Rep binding site sequence, 5′-GGTGTCCTGGAGTC-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...
Expression of Functional Domains of ToLCNDV Rep

**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 μg 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× 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 μg 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× 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 antibody. 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.

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.

**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 μg of total protein extracts were incubated with 1 μg of anti-AC1 antibody overnight at 4 °C. Bound proteins were recovered from the agarose beads after extensive washing of protein-antibody complexes in 1× 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.
Expression of Functional Domains of ToLCNDV Rep

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 | DNA-A | DNA-B |
| A1 + B          | Severe, 10/10             | 100   | 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   | 94–98 |
| 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 Act–2–2(3–14) 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-(1–160) (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 immunoblobs from the transfected protoplasts when detected 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 μg 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 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 PMYV-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
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 1/3 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 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...
Expression of Functional Domains of ToLCNDV Rep

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-
Expression of Functional Domains of ToLCNDV Rep

Table IV

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

| Virus         | Putative iteron* | N-Rep sequence | EMSA  | Replication* | A | B |
|---------------|------------------|----------------|-------|---------------|---|---|
| ACMV          | GGAGA            | MRTPRFRRIQANKYFLTPKC | 48–62 | % | 25 |
| PHV           | GGTGA            | MPLPRFSIAKNFVLTPKC | 26–52 | % | 19 |
| PYMV          | GGTGT            | MPYPRFRSIANKFLTPKC | 43–22 | % | 27 |
| ToLCNDV (s)   | GGTGT            | MAPRFRSNKAFYLETPKC | 100   | % | 87–95 |
| ToLCNDV (m)   | GGGCT            | MASPRFRDANKYFLTPKC | 36–40 | % | 12–14 |

*The sequences indicate putative iteron sequences.

*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), PHV (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), PHV (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.

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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), PHV (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), PHV (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.

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Expression of Functional Domains of ToLCNDV Rep

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

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