Identification of the replication-associated protein binding domain within the intergenic region of tomato leaf curl geminivirus

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ABSTRACT

The geminiviral replication-associated protein (Rep) is the only viral protein required for viral DNA replication. Tomato leaf curl virus (TLCV) Rep was expressed in Escherichia coli as a histidine-tagged fusion protein and purified to homogeneity in non-denaturing form. The fusion protein was used in in vitro binding experiments to identify the Rep-binding elements within the origin of replication of TLCV. Electrophoretic mobility shift assays demonstrated that the Rep binds specifically to a 120 bp fragment within the TLCV intergenic region. Fine resolution of the binding regions within the 120 bp fragment, using DNase I footprinting, demonstrated two footprints covering the sequences GGAATTGGTGTCTTCTCAAC and TGAATCTGTGTTGGG containing a direct repeat of the motif GGTGTCT (underlined). Our results suggest that the repeated motif is involved in virus-specific Rep-binding, but may not constitute the entire binding element. This is the first demonstration of geminivirus sequence elements involved in Rep-binding by direct protein–DNA interaction assays.

INTRODUCTION

Geminiviruses are characterised by their twinned isometric particles containing one or two circular single-stranded (ss) DNA genomes. They are transmitted by leafhopper or whitefly insects and infect a wide variety of crop plants throughout the world. Tomato leaf curl virus (TLCV) from Australia, a subgroup III geminivirus, has a monopartite genome of 2766 nucleotides (nt) containing six open reading frames (ORFs) (1). The two virion sense ORFs (V1 and V2) and four complementary sense ORFs (C1, C2, C3 and C4) are interspaced by an intergenic region (Fig. 1). This region includes a 13 bp element (GGTAGTTAA TTAC-3′) which is a high affinity binding site for the TGMV Rep (3).

Geminivirus Rep–ori interactions are virus-specific. Lazarowitz et al. (4) demonstrated that chimeric molecules derived from tomato golden mosaic virus (TGMV) and squash leaf curl virus, both subgroup III geminiviruses, could replicate in trans, as long as the Rep and ori were derived from the same virus. The Logan and CHF strains of beet curly top geminivirus, a monopartite subgroup II geminivirus (18), also possess specific replication factors that are not functionally interchangeable. Similarly, Jupin et al. (19) using the Sardinian and the Israeli isolates of tomato yellow leaf curl geminivirus, a monopartite subgroup III geminivirus, showed that Rep proteins were specific for their respective ori. Thus, specificity of Rep–ori interaction may be considered an essential trait defining individual biological species of geminiviruses (20).

Sequence analysis of the intergenic region of dicot-infecting geminiviruses has identified iterative elements of 8–12 nt which have been postulated to act as Rep-specific binding sites (2). Experimentally, it has been shown that the Rep-binding site of TGMV maps to a 52 bp region on the left of the intergenic region (15). This region includes a 13 bp element (GGTAGTAGGTTAACG) containing a 5 bp direct repeat which is suggested to be a high affinity binding site for the TGMV Rep (3).

The iterative elements of the monopartite subgroup III geminiviruses, identified by sequence alignment of the intergenic region, have a different organisation compared with the Rep-binding site in TGMV. To identify the Rep-binding domains in the monopartite DNA of TLCV, we have expressed and purified the viral Rep protein in non-denaturing form and have used the protein in electrophoretic mobility shift and DNase I footprinting.

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assays. The results identified two sequence elements within a 44 nucleotide region of the TLCV origin of replication as the Rep-binding site.

MATERIALS AND METHODS

Expression of TLCV Rep protein

The complete C1 ORF was amplified from a clone of TLCV (1) using two TLCV-specific oligonucleotide primers, P1 and P2 which contain introduced BamHI and HindIII sites at their respective 5′ ends (Table 1). The 1103 bp PCR product was digested with BamHI and HindIII and ligated into the expression vector pQE30 (QIAGEN, Germany). The resultant recombinant plasmid, pQE30-C1, contained the entire TLCV C1 ORF, excluding the initiation codon, fused in frame behind a vector sequence encoding the Rep-binding site.

Plasmid pQE30-C1 was transformed into Escherichia coli strain M15 using a Gene-Pulser electroporator (Bio-Rad, USA) and the cells grown overnight on an LB plate in the presence of 100 µg/ml kanamycin. Bacterial colonies were screened for the presence of the 450 bp plasmid insert using colony PCR and restriction enzyme analysis.

Electrophoretic mobility shift assay

A pair of TLCV-specific oligonucleotide primers, P3 and P4 (Table 1), was used to amplify a 342 bp fragment (Fig. 1, IR1) containing the 298 bp intergenic region (IR). DNA amplification was performed as described previously (22). Digestion of the PCR product with EcoRI generated two fragments of 120 and 222 bp (Fig. 1, fragments IR1 and IR2, respectively). Alternatively, the 120 bp fragment was amplified using the P4 and P5 primers (Table 1). Three other TLCV fragments referred to as fragments I, II and III (Fig. 1), were generated by double digestion of a TLCV clone by either XbaI and NcoI (387 bp), NcoI and XbaI (469 bp) or BamHI and NdeI (383 bp), respectively. A full-length 356 bp DNA of the Darwin strain of potato spindle tuber viroid (PSTVd-D), released by BamHI digestion of a PSTVd-D clone (22), was used as a heterologous DNA in binding assays. A 177 bp PSTVd-D DNA fragment was amplified using primers Pc and Pv (Table 1).

PCR products or restriction fragments were subjected to electrophoresis in 1.2 or 1.5% agarose gels in Tris-borate/EDTA (TBE) (23), and the excised bands purified using a QIAquick gel extraction kit (QIAGEN). The DNA fragments (100 ng) were end-labelled in the presence of 50 µCi [α-32P]dATP (3000 Ci/mmol) using Klenow fragment (5 U; Bresatec, Australia) in reaction buffer provided by the same source.

Extraction of Rep protein

Cells from an IPTG-induced culture were harvested and resuspended in extraction buffer (50 mM NaH2PO4 pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol). Cells were incubated with 1 mg/ml lysozyme on ice for 5 min, frozen in liquid nitrogen, thawed in cold water and then sonicated for 20 s. The lysates were centrifuged at 10 000 g for 5 min at 4°C. The supernatant (soluble fraction) was transferred to a new tube and the pellet (insoluble fraction) was resuspended in extraction buffer. Both protein fractions were denatured and analysed in SDS–polyacrylamide gel as described above.

To increase the level of protein solubilization under non-denaturing conditions, 10% glycerol and either 1% sarkosyl or 1% Tween 20 were added to the extraction buffer and Rep protein extracted as described above.

Purification of Rep protein under non-denaturing conditions

The soluble protein obtained from 250 ml of IPTG-induced culture was added to 4 ml of a 50% slurry of Ni2+-NTA resin (QIAGEN), previously equilibrated with extraction buffer. The mixture was stirred at room temperature for 60 min and then loaded onto a 1.2 cm diameter column. After washing the column with 10 column vol of extraction buffer, pH 8.0, supplemented with 20 mM imidazole and an additional 10% glycerol, the His–Rep fusion protein was eluted from the column by increasing the imidazole concentration to 250 mM. The eluate was concentrated 5-fold in 10 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM EDTA, 200 mM NaCl, 1 mM DTT and 1 mM phenylmethylsulfonyl fluoride (16) using Centricron concentrator columns (Amicon, USA). The concentrated protein was mixed with an equal volume of 100% glycerol and stored at −20°C. The concentration of the purified His–Rep fusion protein was estimated by comparison with the known concentrations of bovine serum albumin using Coomassie brilliant blue R-250 staining following SDS–PAGE.

Extraction of Rep protein

Cells from an IPTG-induced culture were harvested and re-suspended in extraction buffer (50 mM NaH2PO4 pH 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol). Cells were incubated with 1 mg/ml lysozyme on ice for 5 min, frozen in liquid nitrogen, thawed in cold water and then sonicated for 20 s. The lysates were centrifuged at 10 000 g for 5 min at 4°C. The supernatant (soluble fraction) was transferred to a new tube and the pellet (insoluble fraction) was resuspended in extraction buffer. Both protein fractions were denatured and analysed in SDS–polyacrylamide gel as described above.

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Electrophoretic mobility shift assay was performed using the purified solubile His–Rep fusion protein. Typical binding reactions contained ~150 ng of His–Rep fusion protein, 1–2 ng labelled DNA and 1 μg of either poly(dI–dC) or Salmon sperm DNA in 10 μl binding buffer (20 mM HEPES pH 7.4, 40 mM KCl, 10% glycerol and 1 mM DTT). Reactions were incubated at 25°C for 20 min and samples were analysed by electrophoresis in non-denaturing 4% polyacrylamide gels in 1× TBE (23). The gels were dried on Whatman paper and radioactive bands visualised by autoradiography.

**DNase I footprinting**

The protocol for DNase I footprinting was essentially as described in (24). TLCV P4 or P5 and PSTVd-D Pv primers (100 ng) were 5′-end-labelled using 25 μCi [γ-32P]ATP (4000 Ci/mmol) and 1.5 U T4 polynucleotide kinase (Bresatec, Australia) in the reaction buffer provided by the same source. Each 5′-end-labelled primer was used directly without purification with an unlabelled primer in PCRs. The labelled DNA fragments were purified using a QIAquick PCR purification kit (QIAGEN).

His–Rep fusion protein (150 ng) was incubated with 1–2 ng 32P-end-labelled DNA fragment in 10 μl of binding buffer containing 100 ng salmon sperm DNA for 20 min at 25°C. Control incubations contained all of the reaction components except for the His–Rep fusion protein. Reactions were diluted to 50 μl with water, supplemented first with 1 mM MgCl₂ and then with 5 U DNase I and incubated for 1 min at room temperature. The reaction was terminated by addition of an equal volume of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS) containing 10 μg E.coli tRNA (Boehringer Mannheim) as carrier. The reaction product was extracted once with an equal volume of phenol:chloroform (4:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1) and precipitated with 2 vol of ethanol. The DNA fragments produced by partial DNase I digestion were resolved in an 8% sequencing gel in 1× TBE in parallel with sequencing reactions of the corresponding labelled DNA fragments. Sequencing by the dideoxynucleotide chain termination method (25) was performed using a Sequenase kit (United States Biochemical) according to the manufacturer’s instructions and the same 32P-labelled primers used for amplification of the fragments.

**RESULTS**

Expression and purification of Rep protein

Cloning of the C1 ORF of TLCV into the bacterial expression vector pQE30, in-frame with the 6× His ORF, resulted in the production of significant amounts of His–Rep fusion protein after induction with IPTG (Fig. 2, compare lanes 1 and 2). The fusion protein migrated in SDS-containing gels to a position corresponding to an Mr of ~44 500 when compared with molecular weight markers. The Mr of the fusion protein is slightly greater than that expected for the combined C1 translation product and vector-derived amino acids which is calculated to be 42 469. Similar observations of higher than expected Mr estimates have also been made for the AC2 protein of TMGV (26) and of potato yellow mosaic virus (27) when expressed in E.coli.

The expressed His–Rep fusion protein was found almost entirely in the insoluble fraction of cell homogenates (Fig. 2, compare lanes 3 and 4). Attempts were made to solubilize the protein under non-denaturing conditions necessary for DNA-binding assays using either non-ionic detergent (Twee 20) or ionic detergent (sarkosyl). Lysing bacteria in extraction buffer containing 1% sarkosyl and 10% glycerol resulted in a high yield of soluble protein (results not shown). However, the solubilized

![Figure 2](image-url)
protein did not bind to the Ni-NTA resin used for affinity purification of the His–Rep fusion protein. In contrast, extraction in the presence of 1% Tween 20 and 10% glycerol produced a lower yield of soluble protein (Fig. 2, lanes 5 and 6), but the protein bound efficiently to the Ni-NTA resin allowing its purification to near homogeneity as judged by SDS–PAGE (Fig. 2, lane 7). Incubation of 32P-labelled dsDNA fragment of the TLCV IR T with purified His–Rep fusion protein resulted in production of a discrete complex with retarded electrophoretic mobility (Fig. 3, lane 11) demonstrating that the shift in probe mobility was protein-mediated. Furthermore, when His–Rep fusion protein was substituted with the same amount of bovine serum albumin in the binding reaction, no retarded complex was formed (data not shown) indicating the specificity of the binding reaction for the His–Rep fusion protein.

**TLCV Rep binds specifically to the viral ori**

The retarded complex was not present when the protein–DNA reaction product was incubated with 1.0 mg/ml proteinase K for 30 min at 37°C (Fig. 3, lane 11), demonstrating that the shift in probe mobility was protein-mediated. Furthermore, when His–Rep fusion protein was substituted with the same amount of bovine serum albumin in the binding reaction, no retarded complex was formed (data not shown) indicating the specificity of the binding reaction for the His–Rep fusion protein. The addition of divalent cations Mg2+, Ca2+ or Zn2+, each at 5 mM to the binding reaction prevented complex formation (results not shown), indicating that these cations induced unfavourable conformational changes to the protein and/or the probe. However, the complex was sufficiently stable in the presence of 1.0 mM Mg2+ which was required for DNase I activity in the footprinting experiments (see below).

The specificity of Rep-binding was examined using three different DNA fragments from the TLCV genome obtained from outside of the intergenic region (Fig. 1, fragments I, II and III) and from a DNA clone of PSTVd-D. His–Rep fusion protein failed to bind to any of these fragments (Fig. 3, lanes 3–10) indicating the specificity of Rep for binding sequence(s) within the TLCV intergenic region.

Sequence specificity of the binding reaction was further tested by competition assays in which labelled TLCV IR T dsDNA fragment was incubated in the presence of different molar ratios of unlabelled homologous or heterologous DNAs. The level of either poly(dI–dC) or salmon sperm DNA in competition assays was reduced from 1.0 µg to 100 ng per reaction as the level of competition was markedly improved in the presence of the lower level of DNA. Increasing the concentration of poly(dI–dC) or salmon sperm DNA from 1.0 µg to 100 ng in the binding reaction increased the number of retarded bands (compare lane 2 in Fig. 4 with lane 2 in Fig. 3) while complete absence of these heterologous DNAs shifted the labelled DNA fragment to the top of the gel (data not shown). The addition of a 150-fold excess of unlabelled homologous DNA to the binding reaction almost completely blocked complex formation with the labelled DNA fragment (Fig. 4, lane 3) whereas addition of the same amount of unlabelled heterologous PSTVd-D DNA had no significant effect on the formation of complexes of retarded mobility (Fig. 4, lane 4). These experiments further demonstrate that Rep interacts specifically with sequence(s) within the intergenic region of TLCV.

**The stem–loop structure is not essential for Rep-binding**

To delimit the Rep-binding site within the intergenic region, this region was subdivided into two fragments of 120 bp (IR L ) and 222 bp (IR R ), the latter containing the putative stem–loop structure (Fig. 1). Binding experiments using each of the two DNA fragments revealed that the electrophoretic mobility of the labelled 120 IR L fragment, but not the labelled 222 bp IR R fragment, was retarded by TLCV Rep (Fig. 5). These results indicated that the Rep-binding site maps to a region located between nt 2594 and 2713 of the left side of intergenic region, and that the binding is not mediated by the putative stem–loop structure. However, it can be seen that the efficiency of binding
Figure 5. TLCV Rep binds specifically to the left part of intergenic region. Labelled DNA fragments of the TLCV 120 bp IR_L fragment (lanes 1 and 2) and the 222 bp IR_R fragment (lanes 3 and 4) were incubated in the absence (–) or the presence (+) of His–Rep fusion protein. Binding reactions were carried out and analysed as described in Figure 3.

of Rep to IR_L (Fig. 5, lane 2) was significantly lower than with IR_R (Fig. 4, lane 2). This apparent difference in binding efficiency suggests that IR_R may have a role in enhancing complex formation with TLCV Rep.

Determination of the nucleotide sequence of Rep-binding domains within the TLCV ori

The 120 bp IR_L fragment was labelled at the 5′ end of the virion sense DNA strand and used in DNase I footprinting. Two zones of the DNA fragment were consistently protected from DNase I digestion in the presence of TLCV Rep compared with control digestions (Fig. 6a). Comparison with the TLCV sequence revealed these two footprints (A and B) to be positioned 9 nt apart in a region located between the TATA box and the initiation codon of the C1 (Rep) promoter (Fig. 6d). Footprint A includes the sequence GCAA TTGGTGTCTCAAA (nt 2625–2642), while footprint B covered the sequence TGAA TCGGTGTCTGGGG (nt 2652–2668) and both contain a direct repeat of the motif GGTGTCT (underlined). When DNase I footprinting was carried out with the same 120 bp IR_L fragment carrying the 32P label at the 5′-end of the complementary-sense strand, only footprint B was observed (Fig. 6b). Footprint A was difficult to resolve unambiguously because certain residues in this region of the complementary-sense DNA probe were not digested by DNase I in control reactions carried out in the absence of Rep protein. No protected zones were observed when an unrelated PSTVd-D DNA fragment was end-labelled and used as a control in parallel reactions (Fig. 6c).

It is interesting to note that three bases, GTC, of the repeated element in footprints A and B were not protected when the footprinting was carried out with IR_L fragment labelled in the virion-sense strand (Fig. 6a) compared with complementary-sense strand (Fig. 6b). This observation suggests differential exposure of the two DNA strands within the Rep-binding complex to DNase I digestion.

DISCUSSION

To date, studies of Rep-binding elements in geminiviral ori have focused on bipartite geminiviruses and have utilised Rep prepared by immunoprecipitation from plant or insect cell extracts (3,15). Using competitive DNA binding assays, Fontes et al. (3) were able to demonstrate that TGMV Rep binds specifically to a 13 bp element (GGTAGTAAGGTAG) located on the left side of the intergenic region between the transcription start site and the TATA box of the AL1 (Rep) promoter. However, direct characterisation of the Rep-binding domain by complementary methodologies such as mobility shift and footprinting assays was not possible due to potential interference from contaminating proteins immunoprecipitated from these crude cell extracts (3,15). In the present work, we have utilised a bacterial expression system to produce Rep of a monopartite geminivirus, TLCV, and have purified it to homogeneity under non-denaturing conditions (Fig. 2). This enabled us to carry out both DNA-binding

![Figure 6. Footprinting analysis of Rep-binding site within the TLCV ori.](image-url)
assays and DNase I footprinting to directly identify the sequence elements involved in Rep-binding within the TLCV ori.

Electrophoretic mobility shift assays demonstrated that TLCV Rep forms specific complexes with the intergenic region of TLCV (Fig. 3). The specificity of Rep-binding for the intergenic region is demonstrated by the fact that there was no binding observed with four heterologous DNA fragments, including three TLCV fragments derived from outside the intergenic region (Fig. 3). Furthermore, the ability of the His–Rep fusion protein to bind to the TLCV intergenic region was almost completely blocked by the addition of a 150-fold molar excess of unlabelled homologous competitor DNA, while the addition of a 150-fold molar excess of heterologous DNA had no significant effect on the pattern typically observed (Fig. 4). The direct demonstration of specific Rep-binding to the intergenic region of the monopartite TLCV, using electrophoretic mobility shift assays, is also consistent with the results of studies with bipartite geminiviruses (15,16,29).

Subdivision of the TLCV intergenic region into two fragments showed that the TLCV Rep-binding was targeted specifically to the 120 bp fragment covering the left-hand side of the intergenic region (Fig. 5). This confirms that the TLCV cis-acting sequences for Rep-binding are located in this part of the intergenic region as in other geminiviruses (4,15,18,19).

Further delineation of the TLCV Rep-binding sequence elements within the left-hand side of the intergenic region was achieved by DNase I footprinting. Footprinting of the 120 bp IR1 fragment indicated the presence of two protected regions in the presence of TLCV Rep (Fig. 6). Each footprint is ~17–18 nt in size and both contain the direct repeat element GGTGTCT as a core sequence. A closely related sequence element (GGGTGTC) is also located in an inverted orientation downstream of the TLCV C1 promoter TATA box (Fig. 6d, nt 2683–2689). However, there was no evidence of any protection of this sequence in our footprinting experiments, indicating that TLCV specific Rep-binding domains, within the viral ori, are confined to regions A and B (Fig. 6).

The observed protection of sequences bordering the TLCV repeat motifs in footprints A and B (Fig. 6) suggests that Rep-binding may not be limited to repeat elements. However, we cannot rule out the possibility that the protection of the bordering sequences results from exclusion of DNase I due to the formation of the DNA-Rep complex involving only the repeat elements. Alternative methods of DNA footprinting analysis, using smaller chemical cleavage agents (30,31), would help to resolve this question.

The GGGTGTCT repeat element has previously been postulated by Argüello-Astorga et al. (2) to be a Rep specific-binding site. Fontes and co-workers (3,32) have established that the recognition sequence required for Rep-binding to the cognate intergenic region of the bipartite geminiviruses TGMV and bean golden mosaic virus (BGMV), is a 12–13 bp sequence element containing the direct repeat elements GGTAG (TGMV) and TGAG (BGMV). Our results with the monopartite TLCV further support the suggestions that these direct repeats function as core elements in Rep recognition and binding and that the sequence of the repeat element is specific for each geminiviral Rep. The location of the Rep-binding elements between the TATA box and the initiation codon of the C1 (Rep) ORF appears to be highly conserved between the subgroup III viruses TGMV (3), BGMV (32) and TLCV (Fig. 6). This is also consistent with the observed Rep-mediated repression of C1 gene expression (33–35), as Rep binding at this position would interfere with transcription of the C1 ORF by RNA polymerase. However, the relative positioning of the two repeats appears to vary significantly between each of the two evolutionary branches of the subgroup III viruses (2). For example, the distance between these repeat elements in BGMV and TGMV, both New World geminiviruses, is 2 and 3 nt, respectively, while the repeat elements in TLCV, an Old World virus, are 20 nt apart. The significance of this variable spacing on Rep-binding remains unknown.

The presence of two retarded bands observed in electrophoretic mobility shift assay experiments (Fig. 4, lane 2) may indicate the possible binding of more than one Rep molecule to the TLCV intergenic region. This possibility is consistent with the recent findings that geminiviral Rep contains a domain specifying protein oligomerisation and that multimerisation may be a prerequisite for DNA binding (29).

The relative functions of the individual footprints A and B in TLCV replication remain unknown. In TGMV, two sequence elements have been shown by mutagenesis to be involved in Rep-binding and viral DNA replication (3). However, the existence of two Rep-binding domains within the TLCV ori contrasts with the presence of only a single putative Rep-binding sequence element in the recently described TLCV-satellite DNA (36). The 682 nt ssDNA satellite depends on TLCV for replication and has no significant sequence similarity to the TLCV helper virus genome other than the conserved TAATATAC motif, found in all geminiviruses and the sequence element TGAATCGGTTCT which is also present in footprint B (Fig. 6). Further analysis of the interaction of TLCV Rep with the satellite genome by electrophoretic mobility shift assay and DNase I footprinting will reveal the role of this single binding domain in TLCV Rep-mediated satellite replication (36).

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