Lethal mutations within the conserved stem–loop of African cassava mosaic virus DNA are rapidly corrected by genomic recombination

Siân Roberts and John Stanley*

Department of Virus Research, John Innes Institute, John Innes Centre for Plant Science Research, Norwich NR4 7UH, U.K.

The nonanucleotide motif TAATATTAC occurs in the intergenic region of all geminiviruses that have been examined to date. The motif is invariably located within the loop of a potential stem–loop structure that has been implicated in viral DNA replication. To investigate the contribution of these sequences to virus proliferation, African cassava mosaic virus (ACMV) DNA B mutants have been screened for their ability to infect Nicotiana benthamiana when co-inoculated with DNA A. Mutants in which the putative stem structure was altered by the introduction of single nucleotide mismatches remained as infectious as the wild-type virus and the mutations were retained in the progeny. Mutants containing nucleotide substitutions within the loop sequences were similarly infectious but analysis of progeny showed that in most cases wild-type sequences were restored by recombination with DNA A. Stem–loop deletion mutants of both genomic components were not infectious when co-inoculated, although they were once again efficiently rescued by recombination when inoculated with the wild-type components. Co-inoculation of genomic components containing the motif TAGTATTAC did not result in a systemic infection while mutants containing the motif TAATACTAC were infectious and the mutation was stable. The results demonstrate that ACMV will tolerate some modification to this highly conserved region of the genome that might allow more precise mapping of the position at which the viral DNA is nicked during replication.

African cassava mosaic virus (ACMV) is typical of the majority of geminiviruses that infect dicotyledonous plants. Two genomic components (DNAs A and B) are essential for systemic infection (Stanley, 1983; Stanley & Gay, 1983) and genetic analysis has revealed that DNA A is responsible for viral DNA replication, coat protein synthesis and the production of virus particles (Townsend et al., 1985, 1986; Klinkenberg & Stanley, 1990) while DNA B is required for cell-to-cell and systemic movement of virus (von Arnim et al., 1993). The genomic components have a highly conserved sequence of approximately 200 nucleotides (common region) that is located within their intergenic regions (Stanley & Gay, 1983). Within the common region is a sequence that can potentially form a stem–loop structure in single-stranded DNA or a cruciform structure in double-stranded DNA (nucleotides 133 to 165, Fig. 1; here referred to as the stem–loop sequence), and at the apex of this structure is the nonanucleotide sequence TAATATTAC that has been found in all geminiviruses characterized to date.

Characterization of replicative intermediates of ACMV has suggested that double-stranded DNA amplification and the accumulation of single-stranded DNA proceeds by a rolling circle mechanism (Saunders et al., 1991), resembling the strategies adopted by bacteriophages and some prokaryotic plasmids (reviewed by Baas & Jansz, 1988; Gruss & Ehrlich, 1989). The product of gene AC1 is the only virus-encoded protein essential for ACMV DNA replication (Etessami et al., 1991). The protein from the related virus tomato golden mosaic virus (TGMV) binds in vitro to TGMV sequences located upstream of the stem–loop, but not to the stem–loop itself (Fontes et al., 1992). However, the analysis of TGMV and squash leaf curl virus (SqLCV) recombinants containing chimeric common region sequences has demonstrated that the stem–loop structure is also an integral part of the replication origin (Lazarowitz et al., 1992). AC1 might function in association with host proteins by binding to the double-stranded DNA and destabilizing the adjacent stem–loop sequences to allow the introduction of a nick in the virion-sense strand prior to the initiation of rolling circle replication.

Although the stem–loop sequence probably plays an important role in replication, no attempts have been made to introduce subtle changes into this region. For dicot-infecting geminiviruses, analysis is currently confined to the insertion of an 8 bp fragment into the nonanucleotide of TGMV, which served to abolish
ACMV components used in this investigation (DNA A, infectivity in *Nicotiana benthamiana* (Revington *et al.*, 1989). Here, we examine the contribution of stem–loop sequences by introducing nucleotide substitutions in this region and screening mutants for infectivity in *N. benthamiana*.

The construction in M13 vectors of infectious cloned ACMV components used in this investigation (DNA A, *MluI* clone pJS092; DNA B, *PstI* clone pJS094) has been described previously (Stanley, 1983). Because modification of DNA A common region sequences could prevent viral DNA replication by disrupting AC1 expression, the stem–loop sequences of DNA B were targeted. The mutations summarized in Fig. 1 were introduced by oligonucleotide-directed second strand synthesis (Curtis & James, 1991). A transversion, from C to G, was introduced at position 162, using a primer corresponding to nucleotides 118 to 121. This mutation (mutant B G162) was introduced into mutants B G162 and B G136/G162. The number of examples of each progeny type, determined by sequence analysis of cloned PCR fragments, is indicated on the right.

**PROGENY**

A155  
G162  
C136/G162  
Δ  
C151  
G145/C151  
A146/C151  
G148

<table>
<thead>
<tr>
<th>MUTANTS</th>
<th>PROGENY</th>
</tr>
</thead>
<tbody>
<tr>
<td>A155</td>
<td>A-G</td>
</tr>
<tr>
<td>G162</td>
<td>G-C</td>
</tr>
<tr>
<td>C136/G162</td>
<td>C-G</td>
</tr>
<tr>
<td>Δ</td>
<td>C-G</td>
</tr>
<tr>
<td>C151</td>
<td>G-T</td>
</tr>
<tr>
<td>G145/C151</td>
<td>G-T</td>
</tr>
<tr>
<td>A146/C151</td>
<td>G-T</td>
</tr>
</tbody>
</table>

Fig. 1. Nucleotide sequences of ACMV DNA B stem–loop mutants produced *in vitro* and their progeny detected in infected *N. benthamiana*. Part of the common region sequences of DNA A and DNA B have been aligned to indicated nucleotide differences (:). Gaps have been introduced into the DNA A sequence to maximize the alignment. The positions of stem sequences (underlined) and the conserved nonanucleotide (bold) located within the loop sequence are shown in relation to *SspI* and *ApaI* sites (the latter have been introduced into mutants B G162 and B G136/G162). The number of examples of each progeny type, determined by sequence analysis of cloned PCR fragments, is indicated on the right.

To investigate mutant viability, wild-type DNA A and DNA B mutants were excised from their cloning vectors by digestion with *MluI* and *PstI*, respectively, and mechanically co-inoculated onto *N. benthamiana* as described previously (Stanley, 1983). Somewhat surprisingly, all DNA B mutants tested retained the ability to produce a systemic infection and, with the exception of mutant B G162, their infectivity was comparable to wild-type DNA B (Table 1, experiment I). Furthermore, in all
Table 1. Infectivity of ACMV stem–loop mutants following mechanical inoculation onto N. benthamiana

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Inoculum</th>
<th>Plants infected/inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A + B</td>
<td>27/28</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G155&lt;/sup&gt;</td>
<td>22/26</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G155&lt;/sup&gt;/B&lt;sup&gt;G152&lt;/sup&gt;</td>
<td>21/26</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G156/G162&lt;/sup&gt;</td>
<td>20/24</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G162&lt;/sup&gt;</td>
<td>4/25</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G162&lt;/sup&gt;/B&lt;sup&gt;G151&lt;/sup&gt;</td>
<td>22/26</td>
</tr>
<tr>
<td></td>
<td>A + B&lt;sup&gt;G146/G151&lt;/sup&gt;</td>
<td>27/28</td>
</tr>
<tr>
<td>III</td>
<td>A + B</td>
<td>7/10</td>
</tr>
<tr>
<td></td>
<td>A&lt;sup&gt;G&lt;/sup&gt; + B&lt;sup&gt;G&lt;/sup&gt;</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td>A&lt;sup&gt;G&lt;/sup&gt; + B&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>A&lt;sup&gt;G146&lt;/sup&gt; + B&lt;sup&gt;G146&lt;/sup&gt;</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td>A&lt;sup&gt;G151&lt;/sup&gt; + B&lt;sup&gt;G151&lt;/sup&gt;</td>
<td>4/10</td>
</tr>
</tbody>
</table>

cases symptom development was neither delayed nor attenuated when compared with wild-type infection. Total nucleic acids were extracted from systemically infected leaves (Etessami et al., 1991), avoiding the lower inoculated leaves that might have retained residual inoculum DNA. Viral DNA was analysed by agarose gel electrophoresis, Southern blotting onto Hybond-N membranes (Amersham) and hybridization to probes specific to DNA A or B (Etessami et al., 1991). The results indicated that levels of both genomic components in all mutant-inoculated plants were similar to those associated with wild-type infection (data not shown).

The nucleotide substitutions in mutants B<sup>G162</sup> and B<sup>G152</sup> were designed to alter the stability of the putative stem–loop structure by introducing mismatches within the stem (Fig. 2a). The two nucleotide substitutions in mutant B<sup>G136/G162</sup> serve to restore the stability of the stem–loop structure to the wild-type level by creating a C–G pair (inverted with respect to the wild-type sequence) within the stem. Our initial premise, that decreasing the amount of base-pairing within the stem would affect the ability of the mutants to infect N. benthamiana, is clearly invalid. To investigate whether the mutant progeny had retained the mutations, a 1.1 kbp fragment of DNA B (nucleotides 2232 to 2724/1 to 620) was amplified by PCR using a Perkin-Elmer thermocycler (100 ng total nucleic acid in a 50 µl reaction, amplified using the following conditions: 95 °C for 7 min; 30 cycles of 95 °C for 30 s, 42 °C for 30 s, 72 °C for 60 s; 72 °C for 10 min). For sequence analysis, fragments were cloned into M13mp19 after digestion with XbaI and HindIII (unique sites are present in DNA B at positions 2281 and 608, respectively). Digestion of the PCR fragments derived from the progeny of mutants B<sup>G162</sup> and B<sup>G136/G162</sup> with ApaI produced fragments of 0.6 kbp and 0.5 kbp, indicating that the mutations had been maintained during infection. This was confirmed by sequence analysis of 10 B<sup>G162</sup> clones and 20 B<sup>G136/G162</sup> clones (Fig. 1), all of which had retained the mutation. Although a diagnostic restriction site was not present in mutant B<sup>G136</sup>, sequence analysis of 10 clones showed that this mutation was also stable. The stem sequences are highly conserved between dicot-infecting geminiviruses and there has been only a single report of a mismatch occurring in a natural virus isolate, for bean dwarf mosaic virus (BDMV), in which a T–G pair is substituted for the usual C–G pair (Fig. 2b; Hidayat et al., 1993).

While this suggests that the maintenance of complementary sequences confers a selective advantage on the virus, our results demonstrate that modifications introduced at the otherwise invariant positions 136 (G), 155 (C) and 162 (C) are tolerated.

Removal of most of the DNA B stem–loop sequences did not prevent infection when mutant B<sup>B</sup> was co-inoculated with DNA A. Analysis of the 1.1 kbp fragment amplified by PCR showed that it had lost the introduced ApaI site and regained an SspI site, suggesting that the stem–loop sequences had been restored by recombination with DNA A. This was subsequently confirmed by sequence analysis of four clones (Fig. 1). In three of these clones, DNA B-specific nucleotides were found upstream at position 102 and downstream at position 171, implying that recombination must have occurred somewhere between these nucleotides. In the reciprocal experiment, a DNA A mutant containing a stem–loop deletion (mutant A<sup>B</sup>) also retained a low level of infectivity (Table 1, experiment II) and produced wild-type levels of viral DNA in systemically infected leaves. Restriction analysis of a 1.8 kbp fragment of DNA A (nucleotides 2685 to 2779/1 to 1711) produced by PCR amplification indicated that the progeny of mutant A<sup>B</sup> had regained an SspI site, implying that the mutation had once again been corrected by recombination between the genomic components. Because the stem–loop sequences participate in viral DNA replication (Lazarowitz et al., 1992), recombination in these instances must have occurred by a non-replicative mechanism involving inoculum DNA, a fact that might account for the decrease in infectivity of these particular mutants. Co-inoculation of mutants A<sup>B</sup> and B<sup>B</sup> did not produce a systemic infection (Table 1, experiment II) because the absence of identical stem–loop sequences from both genomic components precludes recombinational rescue of this essential region.

A DNA B loop mutant in which the nonanucleotide motif had been changed to TAATACTAC (mutant B<sup>B154</sup>) was infectious when co-inoculated with DNA A. The mutation was retained in nine clones analysed after PCR amplification of progeny viral DNA and a tenth clone showed reversion to the wild-type sequence.
Fig. 2. (a) Possible stem–loop structures for wild-type and mutant single-stranded DNAs. The positions of the conserved nonanucleotide (bold) and altered nucleotides (circled) are shown. (b) Comparison of stem–loop sequences of dicot-infecting geminiviruses. Sequences are from the following sources: African cassava mosaic virus (ACMV) (Stanley & Gay, 1983); Indian cassava mosaic virus (ICMV) (Hong et al., 1993); tomato leaf curl virus (TLCV) (Dry et al., 1993); tomato yellow leaf curl virus (TYLCV) (Navot et al., 1991); tomato golden mosaic virus (TGMV) (Hamilton et al., 1984); tomato mottle virus (TMoV) (Abouzid et al., 1992); abutilon mosaic virus (AbMV) (Frischmuth et al., 1990); bean golden mosaic virus (strains BGMV-PR and BGMV-BZ) (Howarth et al., 1985; Gilbertson et al., 1993); bean dwarf mosaic virus (BDMV) (Hidayat et al., 1993); squash leaf curl virus (SqLCV) (Lazarowitz & Lazdins, 1991); potato yellow mosaic virus (PYMV) (Coutts et al., 1991); pepper huasteco virus (PHV) (Torres-Pacheco et al., 1993); beet curly top virus (BCTV) (Stanley et al., 1986). TLCV, TYLCV and BCTV are all single component viruses. With the exception of TGMV, BGMV-BZ and BDMV, sequences are identical between genomic components of individual viruses. Nucleotides that differ from the consensus sequence are shown in lower case. Stem (underlined) and loop sequences (bold) are indicated. Modifications to the ACMV sequence that are tolerated are shown at the top.
Having demonstrated that modification of the nonanucleotide in DNA B is tolerated, an identical mutation was introduced into DNA A, using a primer corresponding to nucleotides 133 to 165 with a single mismatch at position 151, to produce mutant A°151. Coinoculation of mutants A°151 and B°151 produced an infection in N. benthamiana (Table 1, experiment III), confirming that the mutation at nucleotide 151 is not lethal to the virus. Chlorotic lesions developed at the same time on leaves inoculated with either mutant or wild-type virus, although the appearance of systemic symptoms associated with the mutant was delayed by 1 to 2 days. As a consequence, plants became slightly less severely infected. This suggests that although conservation of the motif at this position is not essential for replication it is advantageous to the virus. A similar conclusion has been reached concerning an identical nonanucleotide mutant of the monocot-infecting gemivirus maize streak virus (MSV) that was infectious but exhibited a slight delay in symptom appearance (Schneider et al., 1992). In this instance, the mutation arose spontaneously in vivo when maize was inoculated with a mutant containing a 40 nucleotide insert within the nonanucleotide motif.

When co-inoculated with DNA A, DNA B mutants containing modifications to the loop sequences in addition to the tolerated mutation at position 151, either external to the nonanucleotide (mutant B°145/c151) or within the motif itself (mutant B°146/c151), were as infectious as wild-type virus (Table 1). Sequence analysis of 24 B°145/c151 clones and 15 B°146/c151 clones derived from the mutant progeny once again demonstrated that the mutations had been corrected by recombination with DNA A (Fig. 1). Comparison with other geminiviruses shows that while there is a strong preference for A at the position preceding the nonanucleotide (equivalent to ACMV position 145), variation is tolerated in TGMV and tomato yellow leaf curl virus (TYLCV) (Fig. 2b). In the absence of mutants containing single nucleotide substitutions at positions 145 and 146, we are unable to determine if the lack of mutant stability is due to the disruption of these specific nucleotides or to the cumulative effect of the double mutations.

Having demonstrated that a mutant modified at position 151 remained viable, we were interested to know whether the nonanucleotide motif could be altered elsewhere without adversely affecting virus infectivity. For this purpose, A to G transitions were introduced into both genomic components, using a primer corresponding to nucleotides 133 to 165 with a single mismatch at position 148, to produce mutants A°148 and B°148 containing the altered nonanucleotide TATATATTAC. This position was targeted because nonanucleotide variants TAGTATTAC and TATTATTAC, also located at the apex of comparable hairpin-loop structures, have been described for the monocot-infecting viruses coconut foliar decay virus (CFDV) (Rohde et al., 1990) and banana bunchy top virus (BBTV) (Harding et al., 1993), respectively. Co-inoculation of mutants A°148 and B°148 failed to produce either chlorotic lesions on the inoculated leaves or systemic symptoms in N. benthamiana (Table 1, experiment III), demonstrating that the mutations in ACMV are lethal. Although both CFDV and BBTV have circular single-stranded DNA genomes, neither has been assigned to the geminivirus group because of fundamental differences in particle structure, genome size and organization. However, in view of their overall similarities, it is likely that the CFDV and BBTV motifs play the same role as their geminivirus counterparts but have become adapted for specific interactions with virus- and/or host-encoded proteins. In comparison, all naturally occurring monocot-infecting geminiviruses characterized to date have retained the conserved TAATATTAC motif (Mullineaux et al., 1984; MacDowell et al., 1985; Donson et al., 1987; Andersen et al., 1988; Chatani et al., 1991; Briddon et al., 1992) and none are known to infect coconut or banana, all having host ranges confined to Gramineae.

Our results demonstrate two novel characteristics associated with ACMV. Firstly, stem–loop nucleotide substitutions and deletions that are presumed to incapacitate the virus are corrected by homologous recombination between common region sequences. This contrasts with a previous report in which a TGMV DNA B loop mutant containing an 8 bp insert was unable to infect N. benthamiana when co-agroinoculated with wild-type DNA A (Revington et al., 1989). The different behaviour of ACMV and TGMV mutants may be attributable to the nature of the mutation (nucleotide substitutions and deletions in ACMV, an insertion in TGMV), the method of introduction of the viral DNA into plants (mechanical inoculation of ACMV linearized double-stranded DNAs, agroinoculation of partial repeats of the TGMV genomic DNAs), variation in their host adaptation, or a combination of these factors. Interestingly, ACMV DNA A deletion mutants rapidly reverted to wild-type size during infection of N. benthamiana by processes involving either intramolecular or intermolecular recombination (Etessami et al., 1989; Klinkenberg et al., 1989) while a comparable TGMV deletion mutant remained unaffected during infection of this host (Gardiner et al., 1988), suggesting that ACMV stem–loop mutants are infectious because the virus has a greater propensity for recombination than TGMV. The efficient rescue of ACMV stem–loop mutants, as judged by the rapid onset of infection, indicates that recombination occurs frequently during virus proliferation. The virus will undoubtedly benefit from this process...
because it ensures that advantageous common region sequences are selectively maintained on both components. Furthermore, recombination events of this nature could promote the exchange of genetic material between distinct viruses, encouraging diversity and adaptation within geminivirus populations. Secondly, the virus can tolerate nucleotide substitutions within the stem-loop sequences that reduce the extent of base-pairing within the stem, and, in one case, alters the otherwise ubiquitous nonanucleotide motif located within the loop. The fact that stem mutants remain viable indicates that conservation of the stem sequences per se is not critical for replication and suggests that the residual base-pairing remains sufficient to promote destabilization of the double-stranded DNA in this region, if this is indeed its function. Additional mutants might be used to investigate the contribution of stem sequences to virus replication although further modification could encourage recombinational rescue, making it necessary to introduce mutations into both genomic components.

The analysis of ACMV size revertants produced during infection of N. benthamiana with deletion mutants identified a recombinational hot-spot at the 3’ terminus of the conserved nonanucleotide, within the tetranucleotide TACC (positions 152 to 155; Fig. 1) (Etiessami et al., 1989). Because origin-nicking enzymes are known to mediate recombination during rolling circle replication of bacteriophages and plasmids (Michel & Ehrlich, 1986a, b), the result suggested that ACMV DNA replication was initiated by nicking the virion-sense strand at this position. The analysis of wheat dwarf virus (WDV) recombinants produced in vivo has recently allowed the nick site to be mapped to the equivalent region of the genome within the pentanucleotide sequence TACCC (Heyraud et al., 1993), consistent with this proposal. Using genotypically distinct beet curly top virus (BCTV) strains, the nick site has been mapped to 20 nucleotides within the stem-loop, including the tetranucleotide sequence (Stenger et al., 1991). Having identified viable mutants containing nucleotide substitutions within this region, we are now in a position to apply a similar approach to map the initiation site of ACMV DNA replication.

We are grateful to Professor J. W. Davies for helpful discussion. S.R. was supported by a studentship from the Gatsby Charitable Foundation.

References


(Received 3 June 1994; Accepted 22 July 1994)