Mutagenesis of the AC3 open reading frame of African cassava mosaic virus DNA A reduces DNA B replication and ameliorates disease symptoms

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Small insertions were made independently at each of four unique restriction sites on African cassava mosaic virus (ACMV) DNA A to disrupt the three overlapping complementary-sense open reading frames (ORFs) herein designated AC1, AC2 and AC3. The DNA A mutants were assayed for their infectivity by agroinoculation of monomeric constructs to Nicotiana benthamiana plants containing chromosomal insertions of ACMV DNA B. Disruption of the AC3 ORF alone resulted in a delay and amelioration of disease symptoms which correlated with reduced replication of DNA B. Normal replication of DNA A still carrying the AC3 ORF mutation was found in extracts from these plants. No ACMV DNA or symptoms were observed in corresponding inoculations with either the simultaneous disruption of the overlapping AC2 and AC3 ORFs or disruption of the AC1 ORF. Complementation by the inoculation of different mutant pairs produced a delay in disease symptoms followed by repair of mutated sites. A DNA A construct with the virus-sense AV1 (coat protein) ORF deleted was infectious producing typical ACMV disease symptoms. A similar construct with a larger deletion encompassing the complementary-sense AC3 ORF produced symptomless infections. The DNA recovered from plants revealed DNA A of normal size where the position of the deleted ORF was replaced with cloning vector DNA. Significantly reduced DNA B replication was observed for the AC3 deletion construct.

Introduction

The geminivirus group (for review see Davies & Stanley, 1989) contains a subgroup comprising viruses transmitted by whiteflies and having a bipartite genome (DNA 1 or A and DNA 2 or B). Included in this subgroup are African cassava mosaic virus (ACMV; synonym cassava latent virus) and tomato golden mosaic virus (TGMV). The cloned genome components of each of ACMV and TGMV have been shown to infect plants (Hamilton et al., 1983; Stanley, 1983). Plant infections by ACMV or TGMV mediated by the Ti plasmid of Agrobacterium tumefaciens containing constructs of the virus DNA (herein referred to as agroinoculation) have proven more efficient than mechanical inoculations with viral DNA (Elmer et al., 1988a; Morris et al., 1988). DNA A of ACMV (Townsend et al., 1986) or TGMV (Rogers et al., 1986) has the capacity for self replication in single cells; DNA B appears to be required for spread of the virus.

The nucleotide sequences of the bipartite genomes of ACMV (Stanley & Gay, 1983) and TGMV (Hamilton et al., 1984) have been determined. Comparison of the sequence data identifies four open reading frames (ORFs) on each of ACMV and TGMV DNA A and two ORFs on each of ACMV and TGMV DNA B. DNA A ORFs are referred to as AV1, AC1, AC2 and AC3 for ACMV and AR1, AL1, AL2 and AL3 for TGMV where V or R denote a virus-sense or rightward ORF and C or L denote a complementary-sense or leftward ORF. The DNA B ORFs are referred to as BV1 and BC1 or BR1 and BL1 for ACMV and TGMV, respectively.

The virus coat protein genes of ACMV and TGMV have been identified as virus-sense ORFs (AV1 and AR1) on DNA A (Townsend et al., 1985; Kallender et al., 1988). Mutational analyses have shown that a functional coat protein is not required for systemic spread or symptom development of either virus (Gardiner et al., 1988; Morris et al., 1988; Stanley & Townsend, 1986). However, some mutants in the coat protein ORF of ACMV or TGMV are not infectious with mechanical inoculation (Brough et al., 1988; Etessami et al., 1989), but the same coat protein mutants in TGMV are infectious by agroinoculation (Hayes et al., 1988).
Agroinoculation of TGMV constructs to tobacco plants and leaf discs determined that only the first (or largest) of three complementary-sense ORFs (AL1) on DNA A is required for viral DNA synthesis (Elmer et al., 1988a). Viral constructs with this ORF intact and disruptions to the remaining two ORFs (AL2 and AL3) were found to replicate in leaf discs but were unable to spread systemically in plants. AL2 was required in addition to AL1 for the systemic infection of plants. AL3 was not required for systemic infection however disruptions to this ORF produced both a delay and attenuation of disease symptoms.

Recently, transgenic plants expressing the complementary-sense ORFs from TGMV DNA A have been used to investigate the requirements for replication of TGMV DNA (Hayes & Buck, 1989; Hanley-Bowdoin et al., 1989, 1990). Hanley-Bowdoin et al. (1990) reported that expression of AL1 can direct synthesis of both single- and double-stranded forms of DNA B in leaf discs. In contrast Hayes & Buck (1989) reported that both AL1 and AL2 are required for replication of single- and double-stranded TGMV DNA in intact plants. The AL1 ORF in transgenic plants permitted replication of the TGMV dsDNA form with no ssDNA being produced. Constructs containing mutations in TGMV DNA complementary-sense ORFs co-inoculated with DNA A, DNA B and the AC1, AC2, AC3 and AV1 ORFs co-inoculated with normal DNA B could be complemented by the transgenic plant expressing the corresponding ORF.

The effect on infectivity of mutations to ACMV DNA A complementary-sense ORFs has not been examined, with the exception of an alteration to AC3 (Etessami et al., 1989). A coat protein gene deletion construct with a deletion in the AC3 ORF was not infectious by mechanical inoculation to plants.

Insertion and deletion mutagenesis of the two ORFs on both ACMV and TGMV DNA B destroyed infectivity (Etessami et al., 1988; Brough et al., 1988).

In this paper we determine which ORFs on the complementary-sense ACMV DNA A are required for the systemic infection of *Nicotiana benthamiana*. Four insertion mutants were constructed to disrupt individual ORFs by making small additions at selected restriction enzyme sites. Two deletion mutants of DNA A were also made. The infectivity of mutant constructs was assayed by agroinoculation of seedlings from selfed mother plants carrying chromosomal insertions of ACMV DNA B.

**Methods**

*Construction of mutants.* DNA manipulations were performed using standard procedures (Maniatis et al., 1982). Nucleotide numbering throughout refers to the ACMV DNA sequences of pJS092 (DNA 1 or A) and pJS094 (DNA 2 or B) (Stanley & Davies, 1985) irrespective of deletions. DNA A, DNA B and the AC1, AC2, AC3 and AV1 ORFs referred to throughout are according to suggested geminivirus nomenclature (Davies & Stanley, 1989). To facilitate the description of constructs, the positions of relevant restriction endonuclease sites in relation to the common region (CR) and ORFs of DNA A are indicated in Fig. 1. Constructs of monomer DNA A (pCL10 and pCL15) and monomer DNA B (pCL26) have been described (Morris et al., 1988).

The insertion mutant construct pCL10/BclI was prepared from pCL10 DNA digested with BclI and religated after filling in the protruding termini using DNA polymerase I (Klenow fragment). DNA of pCL10 was prepared from an *Escherichia coli* dam’ host. The effect of adding 4 bp at the BclI site (position 1291) was to create a new ClaI site and to cause a frameshift in the C-terminal region of the AC3 ORF (15-8K product) giving rise to a truncated 14-5K polypeptide. Truncated polypeptides are defined throughout from their first in-frame methionine codon ATG.

Insertion mutants pCL15/Acl, pCL15/NcI and pCL15/ClaI were made from pCL15. The DNA was digested separately with either Acl, NcoI or ClaI and self-ligated following infill reactions using DNA polymerase I. The effect of adding 2 bp at the Acl site (position 1365) in pCL15/Acl was to cause frameshifts in the overlapping AC2 (15-1K product) and AC3 (15-8K product) ORFs, potentially encoding a 16-1K fusion polypeptide encompassing the amino-terminal half of AC2 (69 of 135 amino acids) and all but the 19 amino-terminal amino acids of AC3. In addition, two truncated polypeptides of 7-6K and 2-5K, deriving from the 66 carboxy-terminal amino acids of the AC2 product and 19 amino-terminal amino acids of the AC3 product, respectively, were potentially formed. In pCL15/NcoI the effect of adding a further 4 bp at position 2124 was to form a novel NsiI site and to cause a frameshift in the AC1 ORF (40-3K product) giving rise to a putative truncated 24-1K polypeptide. The addition of 2 bp in pCL15/ClaI at position 2403 created a new NsiI site and caused a frameshift in AC1 to produce potentially a truncated 17-2K polypeptide.
The mutant DNA A clones were digested with the appropriate enzyme to confirm the presence of new restriction sites. The nucleotide sequences surrounding the mutation sites in pCL10/BclI, pCL15/AclI, pCL15/Neol and pCL15/ClaI were determined to verify changes made. For this, DNA restriction fragments were selected, subcloned into M13mp18 and M13mp19 (Messing, 1983) and template DNA was sequenced by the dideoxynucleotide chain termination method using the Sequenase kit (USB).

Two deletion constructs were prepared from DNA A restriction fragments excised from pCL10. The SmaI (position 1190) to BamHI (position 291) fragment deleting the coat protein gene (AV1) but containing the CR and intact AC1, AC2 and AC3, was cloned into pUC19 digested with BamHI and SmaI to make pCU65. The construct pCU94 was prepared with the BclI (position 1292) to BamHI (position 291) fragment of DNA A encompassing the CR, intact AC1 and AC2, but deleting AV1 and part of AC3. This fragment was inserted into a BamHI-cut pUC18 vector. The orientation of the DNA A fragment in pCU94 was the same as in pCL15.

The ACMV DNA A mutant constructs were individually recombined into pCGN587 and mated into A. tumefaciens LBA4404 (Morris et al., 1988) for the agroinoculation of transgenic plants.

DNA B transgenic plants. Leaf discs of N. benthamiana were transformed with A. tumefaciens strain LBA4404 (pAL4404:pCL587::pCL26; Morris et al., 1988) by the method of Horsch et al. (1985). Transformed cells were selected on a shoot-inducing medium containing kanamycin (100 μg/ml) and cefotaxime (100 μg/ml). Young shoots were transferred to root-inducing medium containing kanamycin (100 μg/ml).

Integration of the DNA B into plant chromosomal DNA was verified by restriction endonuclease digestion and Southern blot hybridization using the probe pCL26. The DNA was prepared from transgenic plants as described by Dellaporta et al. (1983). Inheritance of kanamycin resistance was determined by germinating self-pollinated seed, harvested from the transgenic plants, on medium containing 300 μg/ml kanamycin.

Agroinoculation and ACMV DNA analysis. The agroinoculation of plants and isolation of total leaf DNA was carried out as previously described (Morris et al., 1988). The DNA of ACMV in plant extracts was analysed by Southern blot hybridization in order to characterize the progeny DNA following agroinoculation.

Results

Production of transgenic plants containing DNA B

Transgenic plants with chromosomal insertions of DNA B were produced by transforming N. benthamiana with the Agrobacterium strain LBA4404 containing the binary vector pCGN587::pCL26. The construct pCL26 contained a full-length monomer of DNA B cloned into the Psfl site within the intergenic region (Morris et al., 1988). Transformed plants appeared phenotypically normal, and Southern hybridization of restricted genome DNA from these plants revealed that they contained inserted DNA B.

The results of Southern blot hybridization analyses, using the probe pCL26, on three plants (15, 16/1 and 16/2) are shown in Fig. 2, lanes 6 to 11. We compared the restriction digest pattern obtained for these three plants with that obtained from the binary vector construct pCGN587::pCL26 (Fig. 2, lanes 2 to 4) to determine whether the chromosomal insertions of DNA B in plants were intact. The number of T-DNA copies containing pCL26 integrated into the plant genome was estimated from the intensity of the hybridization signal relative to copy number standards (Fig. 2, lanes 2 to 4) and from the number of junction fragments between T-DNA and plant DNA. Using these two criteria, it was estimated that plant 15 contained at least three T-DNAs, both intact and truncated, plant 16/1 contained at least three intact T-DNAs, and plant 16/2 contained five or more T-DNAs, at least three of which were intact. Hybridization of the pCL26 probe was not detected in extracts from healthy plants (Fig. 2, lane 5).

Resistance to kanamycin conferred by the expression of the neomycin phosphotransferase (NPTII) gene in the T-DNA was used to select transgenic plants. Inheritance of kanamycin resistance was determined for plants 15, 16/1 and 16/2 by germinating self-pollinated seed
harvested from each plant on medium containing kanamycin. A ratio of almost 63:1 green (kanamycin-resistant) to white (kanamycin-sensitive) seedlings germinated was determined for plants 15 and 16/2 (ratios were 621:9 and 832:14, respectively). These results show the presence of three unlinked copies of functional NPTII genes in plants 15 and 16/2. For plant 16/1 a ratio of approximately 27:1 (844:31) kanamycin-resistant to kanamycin-sensitive seedlings was obtained. This value falls between the two expected ratios of 15:1 and 63:1 indicating that two of the three NPTII genes were probably linked. If the T-DNA associated with each NPTII gene is intact, we would expect similar ratios for the inheritance of ACMV DNA B in progeny from these plants.

Plants transgenic for DNA B support the replication of DNA A

To determine whether transgenic plants containing insertions of the DNA B monomer would support the replication of DNA A and could be used to test the infectivity of DNA A mutants, seedlings collected from selfed plants 15, 16/1 and 16/2 were agroinoculated using Agrobacterium strain LBA4404 (pAL4404.pCGN587::pCL10). The pCL10 construct contains a full-length monomer of DNA A cloned at the MluI site within the virus coat protein gene (Morris et al., 1988). Twenty seedlings were inoculated for each transgenic mother plant, resulting in 20 from plant 15, 17 from plant 16/1 and 19 from plant 16/2 becoming infected and giving rise to normal ACMV systemic symptoms within 12 days. The few uninfected plants may not have inherited the T-DNA containing DNA B or the agroinoculation procedure may not have been effective.

Southern hybridization of DNA from the infected plants showed the single- and double-stranded forms of DNA A and DNA B (Fig. 3a, b, lane 3) typical of wild-type virus infections (Fig. 3a, b, lane 1) in normal ratios. The progeny DNA A molecules from agroinoculation experiments usually retained the MluI cloning site (Fig. 3a, lane 4) whereas DNA B molecules had frequently lost the PstI cloning site (Fig. 3b, lane 4). The same restriction enzyme digests on a DNA extract from the wild-type virus infection are included for comparison (Fig. 3a, b, lane 2). No single- or double-stranded DNA B replication was observed in uninoculated transgenic mother plants or seedlings. This observation indicates that the extrachromosomal replication of DNA B was dependent on DNA A infection. It was concluded from the results that seedlings derived from the DNA B transgenic plants 15, 16/1 and 16/2 would be suitable for inoculation with the DNA A mutant constructs.

Fig. 3. Southern blot hybridization analysis of ACMV DNA forms produced in DNA B complementing plants following agroinoculation with the DNA A constructs. Plants were infected with wild-type virus (a and b, lanes 1 and 2), pCL10 (a and b, lanes 3 and 4) and pCL10/BoI (a, lanes 5 to 7; b, lanes 5 to 8). DNA from 100 mg of systemically infected leaves was untreated (a and b, lanes 1, 3 and 5), or digested with MluI (a, lanes 2 and 4), ClaI (a, lane 6), BstI (a, lane 7) and PstI (b, lanes 2, 4 and 8) and electrophoresed in a 1-2% agarose gel, transferred to nylon membrane and hybridized with probes randomly labelled with 32P specific to DNA A (a), or DNA B (b). Note that lanes 7 and 8 in (b) are a longer autoradiogram exposure of (b) lanes 5 and 6. The DNA forms shown are the double-stranded supercoiled (sc), linear (l) and open circular (oc) and virion single-stranded (ss). Molecular size markers (a, lane 8; b, lane 9) were pCL4 digested with ClaI, EcoRI and MluI and pCL3 digested with AccI, EcoRI and PstI, respectively (Morris et al., 1988). Fragment sizes (kb) are shown in the margin.

Agroinoculation of the DNA A insertion mutants

Five- to six-week-old seedlings derived from plants 15 and 16/1 were agroinoculated in their stems using Agrobacterium LBA4404 (pAL4404.pCGN587) containing one of the four DNA A insertion mutant constructs pCL10/BoI (AC3 mutant), pCL15/AccI (AC2 and AC3 mutant), pCL15/NcoI or pCL15/ClaI (AC1 mutants).
Twelve transgenic seedlings were inoculated with each construct. Symptom development was monitored on these plants, and Southern hybridizations were carried out on DNA extracted from upper leaves to characterize the progeny ACMV DNA forms produced in systemic infections.

Systemic symptoms of ACMV infection were produced on nine of the 12 seedlings agroinoculated with the AC3 mutant pCL10/BclI. Control inoculations using an unaltered pCL10 construct showed a more severe symptom response and confirmed a delay of between 2 and 5 days for the appearance of ameliorated disease symptoms on the pCL10/BclI-inoculated plants. Extracts from plants infected using the AC3 mutant construct showed normal replication of DNA A with both single- and double-stranded DNA forms being produced (Fig. 3a, lane 5). Retention in the progeny DNA A of the second ClaI site (Fig. 3a, lane 6), introduced at the deleted BclI site during the construction of pCL10/BclI, showed that the DNA A had not reverted to wild-type. As expected, the progeny dsDNA was not cut with BclI (Fig. 3a, lane 7) although this could also be the case if methylation of the site occurred during replication in the plant. Greatly reduced DNA B replication was observed in extracts from plants infected with the AC3 mutant (Fig. 3b, lanes 5 and 6). A longer autoradiogram exposure of these lanes (Fig. 3b, lanes 7 and 8) shows the much reduced level of dsDNA B in the absence of ssDNA B. No disease symptoms typical of ACMV were observed on plants inoculated with either pCL15/AccI (AC2 and AC3 mutant), pCL15/NcoI or pCL15/ClaI (AC1 mutants) and no virus DNA forms were detected in extracts from the upper leaves of these plants.

Recombination between the DNA A insertion mutants

To test for complementation between the DNA A insertion mutant constructs, pCL15/AccI, pCL15/ClaI and pCL15/NcoI were agroinoculated in pairs to DNA B transgenic seedlings. Ten seedlings were inoculated for each pair of mutant constructs. Symptoms typical of ACMV infection were produced on a few plants inoculated with each of the paired combinations. In all cases the progeny DNA A had the appearance of the wild-type with repair of the mutated AccI, ClaI or NcoI restriction sites as shown for one example in Fig. 4 (lanes 1 to 3). Normal DNA B replication was observed in extracts from these plants (not shown).

Agroinoculation of DNA A deletion mutants

Two deletion mutant constructs, pCU65 and pCU94 (see Fig. 1), were made to examine the limit of deletions possible in DNA A ORFs where infectivity was retained. Ten DNA B transgenic seedlings were agroinoculated with each deletion mutant. Nine seedlings became infected using the pCU65 construct (AV1 deleted) and showed disease symptoms typical of ACMV infection. Normal levels of normal size dsDNA A and B forms were found in these plants with reduced levels of ssDNA (Fig. 5a and b, lane 1). No ACMV disease symptoms were observed on plants inoculated with the pCU94 construct (deleted AV1 and C terminus of AC3). However, extracts from these plants contained normal size single- and double-stranded forms of DNA A (Fig. 5a, lane 4). Some reduction in the level of DNA A was observed compared with wild-type ACMV infections. A much greater reduction in the level of the DNA B was found in these plants (Fig. 5b, lanes 3 to 6). Progeny single- and double-stranded DNA A forms of normal size were found for the deletion mutant infections. The dsDNA A present in both pCU65 and pCU94 infections could be linearized with the restriction enzymes ClaI and SacI (Fig. 5a, lanes 2, 3, 5 and 6). The new SacI site in pCU65 and pCU94 was probably obtained with a fragment of pUC19 containing this site and situated close to the cloned DNA A fragment in each construct. Cloning of ACMV DNA from plants infected with pCU65 followed by DNA sequencing confirmed the presence of pUC19 sequences in the DNA A progeny.
Fig. 5. Southern blot hybridization analysis of ACMV DNA forms produced in DNA B complementing plants after agroinoculation with the deletion mutant constructs pCU65 (a, lanes 1 to 3; b, lanes 1 and 2) and pCU94 (a, lanes 4 to 6; b, lanes 3 to 6). DNA samples from 100 mg of the leaves were untreated (a, lanes 1 and 4; b, lanes 1 and 3), or digested with Clal (a, lanes 2 and 5; b, lanes 2 and 4) or SacI (a, lanes 3 and 6) and electrophoresed in a 1% agarose gel, transferred to nitrocellulose and hybridized with probes randomly labelled with 32p specific to DNA A (a) and DNA B (b). Note that lanes 5 and 6 in (b) are a longer autoradiogram exposure of (b) lanes 3 and 4. The molecular markers in (a and b) lane 7, and the band designations, are as for Fig. 3.

Discussion

The transformed mother plants examined in this study had multiple insertions of T-DNA; each plant had at least three copies of both the DNA B monomer construct and the kanamycin resistance gene. It is possible that the chimeric NPTII gene in single-copy insertions was not expressed at a sufficient level for the single event transformants to survive kanamycin selection. This inadvertent selection proved an advantage since most of the seedlings derived from the transformed mother plants could be infected by inoculation with DNA A. It is assumed the ratios obtained for inheritance of kanamycin resistance in seedlings are the same for plants having chromosomal insertions of DNA B.

It has previously been shown that monomer constructs of ACMV DNA A and DNA B cloned separately within the pCGN587 binary vector and agroinoculated together are infectious (Morris et al., 1988). These results contrast with those obtained using the pMON200 or pBin19 binary vectors for agroinoculation where head-to-tail repeats of the virus DNA are required in the vector to allow plant infections (Elmer et al., 1988a; Hayes et al., 1988). The 1 kb direct repeat of pUC DNA sequences surrounding the ACMV DNA monomer construct in pCGN587 may provide a mechanism for efficient recombination and excision of the ACMV DNA monomer.

Direct evidence has been provided to demonstrate that the AC1, AC2 and AC3 ORFs of ACMV DNA A are genes whose products are required for normal infection and symptom production. A delay and amelioration of disease symptoms was observed for a mutation disrupting the C terminus of the AC3 ORF. The mutation was stably maintained in the progeny DNA A and correlated with a marked reduction in the level of DNA B found in extracts from these plants. However, normal DNA A replication was observed for the AC3 mutation.

There are several reports of symptomless or mild ACMV infections being produced as a consequence of the replication of DNA A alone or through reduced replication of DNA A and/or DNA B. Symptomless replication and spread of ACMV DNA A in the absence of DNA B has been reported for agroinoculated N. benthamiana plants (Klinkenberg & Stanley, 1990). This type of inoculation was inefficient with between 10% and 40% of plants becoming infected and in those plants the amount of DNA A produced was only about 5% of that associated with full infection following the agroinoculation of both DNAs. A reduction in DNA B replication in some wild-type virus infections has been correlated with the presence of subgenomic or defective DNA B molecules (Stanley & Townsend, 1985). The defective DNA B molecules found were approximately half the normal size with deletions spanning the region between positions 200 and 1700. Inoculation of defective viral DNA B together with normal DNA A and DNA B not only decreases the number of plants showing disease symptoms but also increases the time for symptom development. Recently, transgenic N. benthamiana plants containing tandemly repeated copies of the defective DNA B have been used to demonstrate a
possible mechanism for ACMV disease control (Stanley et al., 1990). The transgenic plants showed ameliorated disease symptoms following ACMV inoculation but a disproportionate reduction in replication of DNA A by 20\% and DNA B by 70\%. Reduced DNA replication is concurrent with the mobilization of defective DNA B from the chromosome and its amplification.

We observed that the mutation of AC3 in DNA A caused both a reduction in the level of DNA B produced and a reduction in the severity of disease symptoms. It appears that an intact AC3 ORF is required for the efficient replication of DNA B. Whether this gene is directly involved in DNA B replication or in its control is unknown at this stage and requires further investigation. Prior to this report there had been some confusion over which of the ACMV DNAs (A or B) and their ORFs contributed most to disease symptoms. A study made with pseudorecombinants of the cloned DNAs from ACMV Kenyan and Nigerian strains reported a correlation between disease severity and the DNA A component of the Kenyan strain and further suggested that the coat protein gene (AV1) may be responsible since there were sequence differences between the two virus strains in this gene (Stanley et al., 1985). However, chimeric clones have been constructed in which the coat protein gene of ACMV has been replaced with that of beet curly top virus and this construct shows disease symptoms typical of ACMV when inoculated onto plants along with an unaltered ACMV DNA B (Briddon et al., 1990).

We have found a correlation of disease severity with the DNA B component of the Kenyan strain (Morris et al., 1988a) in contrast to the earlier report on ACMV pseudorecombinants. Other experiments again using pseudorecombinants between two virus strains (von Arnim et al., 1990), have shown that symptom development correlates with the DNA B component of TGMV.

The amelioration of disease symptoms for TGMV-infected plants has been reported for a mutation in the AL3 ORF (Elmer et al., 1988b), which corresponds to ACMV AC3. For the AL3 mutant-infected plants, the quantities and proportions of single- and double-stranded DNA A were similar to those seen in wild-type TGMV-infected plants. Unfortunately DNA B replication was not examined. In different work, TGMV DNA A with an N-terminal mutation in AL3 was inoculated to tobacco protoplasts and a reduction in both DNA A and DNA B single- and double-stranded forms was found (Sunter et al., 1990).

No DNA replication or disease symptoms were observed for ACMV constructs containing simultaneous disruptions to AC2 and AC3 following agroinoculation to transgenic DNA B plants. This is consistent with results reported for deletions and insertions made to the corresponding TGMV DNA A ORFs. Mutations affecting TGMV AL2 and AL3 ORFs abolish both systemic spread and symptom development in N. benthamiana plants (Brough et al., 1988; Elmer et al., 1988b), although replication without spread of the mutant DNA A has been observed for agroinoculated petunia leaf discs (Elmer et al., 1988b). The inoculation of tobacco protoplasts with a TGMV construct containing mutations to AL2 and AL3 has shown both reduced single- and double-stranded DNA A and B replication (Sunter et al., 1990). Transgenic plants containing the complementary-sense ORFs of TGMV DNA A have been used to examine virus DNA replication. These experiments have shown that AL2 is required for replication of single- and double-stranded TGMV DNAs in plants but is not required for the replication in leaf discs (Hayes & Buck, 1989; Hanley-Bowdoin et al., 1990). Given the differences between the three assay systems (intact plants, leaf discs and protoplasts), these results are entirely consistent with the idea that AC2 and AC3 are required for virus DNA replication at high levels.

An intact AC1 ORF appears to be a requirement for ACMV DNA A replication. Two mutant constructs disrupting AC1 were not infectious. Progeny DNA was not found in plants inoculated with either AC1 mutant. Disruptions to the corresponding AL1 ORF in TGMV DNA A totally abolished infectivity and viral DNA replication in N. benthamiana plants and petunia leaf discs (Brough et al., 1988; Elmer et al., 1988b). The expression of TGMV AL1 alone in transgenic plants is sufficient to direct replication of TGMV DNAs (Hanley-Bowdoin et al., 1990). The role of AC1 in ACMV is probably similar.

We observed recombination and repair in the agroinoculation of different mutant pairs of ACMV DNA A. A delay in the production of typical disease symptoms was observed for these plants but otherwise normal DNA replication was seen.

An AV1 deletion mutant of ACMV DNA A gave typical disease symptoms following agroinoculation with normal single- and double-stranded DNA levels. In contrast the larger deletion mutant encompassing AV1 and the C terminus of the AC3 product produced symptomless infections, with some reduction in the levels of single- and double-stranded forms of DNA A progeny; however the reduction in progeny DNA B was more pronounced. These results are consistent with those observed for the AC3 insertion mutant pCL10/BclI. Our results contrast with those of Etessami et al. (1989), who examined both the infectivity and size reversion of progeny DNA for a series of ACMV DNA A coat protein deletions. They reported that their AV1 and AC3 deletion mutant was not infectious by mechanical inoculation. We can only conclude that their result is a reflection of the lower efficiency of infection obtained for
mechanical inoculation compared with agroinoculation of ACMV constructs. This conclusion is supported by the fact that TGMV DNA A coat protein deletion mutants, shown not to be infectious following mechanical inoculation (Brough et al., 1988), were found to be infectious with agroinoculation (Hayes et al., 1988).

We noted size reversion in the progeny single- and double-stranded DNA for the two deletion mutants of ACMV DNA A. The deleted region in the progeny DNA A was replaced by cloning vector DNA sequenced from pUC DNA surrounding the DNA A fragment in the inoculated construct. Size reversion of ACMV DNA A has been shown previously for systemic infections resulting with coat protein deletion mutants (Etessami et al., 1989; Klinkenberg et al., 1989).

Klinkenberg et al. (1989) showed that the size reversion observed for ACMV DNA A with the coat protein deletion mutants was produced following systemic spread, whereas in dividing callus cells the deleted DNA A was replicated. Conservation of the size of DNA A and DNA B has been also observed after inoculations with monomeric constructs of ACMV DNA (Stanley & Townsend, 1986; Morris et al., 1988). However, there are two exceptions to this. Firstly, the deleted ACMV DNA B molecules previously mentioned can be stably maintained in plant infections (Stanley & Townsend, 1985). Secondly, size reversion of TGMV DNA A did not occur after agroinoculation of a coat protein deletion mutant (Gardiner et al., 1988), but plants systemically infected by agroinoculation using TGMV DNA A chimeric constructs, where the coat protein gene is replaced by a larger gene (bacterial β-glucuronidase), have progeny DNA A similar in size to the wild-type component (Elmer & Rogers, 1990). The deleted TGMV DNA A molecules are maintained in a deleted form and spread throughout the infected plant.

The results from this study are of practical significance for determining the smallest fragment of ACMV DNA A capable of autonomous replication to be used towards the development of a plasmid-like expression vector for plants. In addition we suggest there is a possible mechanism for the control of ACMV disease and other bipartite genome geminivirus diseases by manipulating AC3 expression through antisense RNA and reducing DNA B replication.

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References


