Complementation of African cassava mosaic virus AC2 gene function in a mixed bipartite geminivirus infection

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We have previously demonstrated that African cassava mosaic virus (ACMV) DNAs A and B efficiently complement the systemic spread of tomato golden mosaic virus (TGMV) DNA A when co-agroinoculated onto *Nicotiana benthamiana*. Here, we show that a mixture of an ACMV DNA A AC2 mutant and DNA B that is normally unable to systemically infect *N. benthamiana* can do so at low frequency when co-agroinoculated with TGMV DNA A. Analysis of viral DNA showed that the AC2 mutation was retained during infection. The mixture of genomic components was sap transmissible, indicating that systemic infectivity is not specifically attributable to the use of agroinoculation. In the presence of TGMV DNA A, ACMV coat protein as well as the DNA B gene products BV1 and BC1 were detected in systemically infected tissues. The results demonstrate that dysfunctional AC2 can be complemented *in planta* by its TGMV homologue AL2.

The majority of whitefly-transmitted geminiviruses, such as African cassava mosaic virus (ACMV) (Stanley, 1983; Stanley & Gay, 1983) and tomato golden mosaic virus (TGMV) (Hamilton et al., 1984), have bipartite genomes (DNAs A and B). ACMV DNA A encodes the coat protein (Townsend et al., 1985), gene AC1 required for viral DNA replication, and the regulatory genes AC2 and AC3 (Townsend et al., 1986; Etessami et al., 1991). DNA A alone is able to produce virus particles (Klinkenberg & Stanley, 1990) but DNA B genes BV1 and BC1 are essential for efficient virus spread and symptom induction (Etessami et al., 1988).

Disruption of AC2 expression prevents systemic infection of *Nicotiana benthamiana* although AC2 mutants retain the ability to replicate in *N. tabacum* protoplasts (Etessami et al., 1988). Studies in which the TGMV coat protein coding sequences have been replaced by the reporter genes β-glucuronidase (GUS) and neomycin phosphotransferase have indicated that coat protein expression is dependent on the activity of AL2 (the TGMV homologue of ACMV AC2) (Sunter et al., 1990; Sunter & Bisaro, 1991; Gröning et al., 1994). The product of this gene acts as a transcription factor for the expression of the coat protein gene and BR1 (Sunter & Bisaro, 1992), which are located in similar positions on their respective genomic components. Transient expression studies using GUS under the control of ACMV promoters have suggested that the product of AC2 is additionally required for the expression of BC1 (Haley et al., 1992).

The production of viable pseudorecombinants by reassortment of genomic components of bipartite geminiviruses is usually confined to closely related strains (Stanley et al., 1985; Lazarowitz, 1991; von Arnim & Stanley, 1992a; Frischmuth et al., 1993) because of the highly specific nature of the interaction of AC1 with the origin of viral DNA replication (Fontes et al., 1992; Lazarowitz et al., 1992; Gilbertson et al., 1993). This precludes the use of pseudorecombinants for the study of gene complementation between distinct viruses. However, experiments in which mixtures of genomic components were introduced into *N. benthamiana* have shown that TGMV DNA A spreads efficiently in the presence of ACMV DNAs A and B (Frischmuth et al., 1993), demonstrating that ACMV DNA B genes can functionally interact with the TGMV genomic component. Here, we assess the feasibility of using this type of approach to investigate DNA A gene complementation. Because ACMV AC2 is essential for infectivity, we have tested the ability of TGMV AL2 to complement dysfunctional AC2 simply by screening for the development of systemic symptoms in *N. benthamiana*.

The construction of pBin19 clones containing either partial or tandem repeats of ACMV DNA A (AA) and DNA B (AB) and common strain TGMV DNA A (TA) and DNA B (TB) in *Agrobacterium tumefaciens* strain LBA4404 has been described (von Arnim & Stanley, 1992a; Klinkenberg et al., 1989). Nucleotide numbering of ACMV and TGMV is according to Stanley & Gay.
Table 1. Infectivity of combinations of ACMV and TGMV genomic components in N. benthamiana

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA + AB</td>
<td>6/11</td>
<td>—</td>
<td>2/3</td>
<td>10/10</td>
<td>4/4</td>
<td>4/4</td>
<td>—</td>
</tr>
<tr>
<td>AA + AB + TA</td>
<td>9/11</td>
<td>10/15</td>
<td>—</td>
<td>—</td>
<td>3/4</td>
<td>3/5</td>
<td>—</td>
</tr>
<tr>
<td>AA-AC2-2 + AB</td>
<td>0/11</td>
<td>0/5</td>
<td>—</td>
<td>—</td>
<td>0/4</td>
<td>0/4</td>
<td>0/50</td>
</tr>
<tr>
<td>AA-AC2-2 + AB + TA</td>
<td>1/11</td>
<td>0/15</td>
<td>1/24</td>
<td>2/20</td>
<td>1/15</td>
<td>1/25</td>
<td>1/50</td>
</tr>
<tr>
<td>TA-ARI-1 + TB</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4/4</td>
<td>4/4</td>
<td>—</td>
</tr>
<tr>
<td>AA-AC2-2 + AB + TA-ARI-1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0/15</td>
<td>1/50</td>
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</table>

* Virus genomic components were introduced either by agroinoculation (experiments I, II, III, V, VI and VII) or by sap transmission using infected plants from experiment III (experiment IV).

(1983) and Hamilton et al. (1984), respectively. The ACMV mutant AC2-2, containing a HpaI(1651) site and a premature termination codon within the AC2 coding sequence (Etessami et al., 1991), was cloned as a partial repeat in pBin19 as described for ACMV DNA A (Klinkenberg et al., 1989) to produce AA-AC2-2. A TGMV coat protein mutant was made by digestion of clone cTVA (von Arnim & Stanley, 1992) at the unique XhoI(400) site, infill of protruding 5’ termini and religation to create an additional PvuI site. Partial repeats of this clone were constructed in pBin19 as described for TGMV DNA A (von Arnim & Stanley, 1992) to produce TA-ARI-1. AA-AC2-2 and TA-AR1-1 were mobilized into A. tumefaciens strain LBA4404 by triparental mating (Hoekema et al., 1983; Ditta et al., 1980). ACMV and TGMV were held and manipulated under MAFF licence numbers PHF 1185A/68(21) and PHF 1185B/17(111) under the Plant Pests (Great Britain) Order 1980.

N. benthamiana was maintained in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse at 25 °C with supplementary lighting to give a 16 h photoperiod. Combinations of ACMV and TGMV genomic components were introduced into plants by stem agroinoculation (Klinkenberg & Stanley, 1990). As expected, both ACMV (AA + AB and AA + AB + TA) and TGMV (TA + TB) were highly infectious and induced severe leaf curl and chlorotic symptoms typical of these viruses in this particular host (Table 1). In contrast, the ACMV AC2 mutant (AA-AC2-2 + AB) was unable to systemically infect N. benthamiana, consistent with earlier results when the mutant was introduced as a linearized clone insert by mechanical inoculation (Etessami et al., 1991). However, when the ACMV AC2 mutant was co-agroinoculated with TGMV DNA A (AA-AC2-2 + AB + TA), mild systemic symptoms consisting of chlorosis around the main mid-rib and occasionally around the minor veins of leaves were induced at low frequency (five plants out of 140, over five experiments), suggesting that the ACMV mutant could be complemented by TGMV DNA A. In addition, when the AC2 mutant was co-agroinoculated with a TGMV coat protein mutant (AA-AC2-2 + AB + TA-ARI-1) a single plant (out of 65) showed mild symptoms. Similar symptoms were prevalent in plants which had been inoculated by sap transmission (experiment IV), consistent with the proposal that reversion or recombination in gene AC2 to produce wild-type virus had not occurred.

To investigate the viral DNA forms associated with these infections, total nucleic acids were extracted from systemically infected leaves (Etessami et al., 1991) and equal amounts were fractionated by agarose gel electrophoresis in 40 mM-Tris-acetate pH 7.5, 20 mM-sodium acetate, 2 mM-EDTA (TNE buffer). When appropriate, samples were digested with restriction endonucleases in buffers supplied by the manufacturer and subsequently with 5U mungbean nuclease for 30 min at 37 °C to remove viral single-stranded (ss) DNA. Fragment sizes were estimated using BRL 1 kb marker DNA standards. Viral DNAs were detected by Southern hybridization with oligolabelled probes (Feinberg & Vogelstein, 1983) specific for ACMV DNA A [DraI(223)—SphI(2585)], ACMV DNA B [PstI(249)—EcoRV(2552)], TGMV DNA A [NcoI(345)—NcoI(2059)] and TGMV DNA B [AflII(1264)—NcoI(1858)], and membranes were washed finally in 0·1 x SSC, 0·1% SDS at 65 °C to avoid cross-hybridization between genomic components. As is generally the case, more ssDNA than double-stranded, supercoiled (sc) DNA accumulated in tissues infected with wild-type ACMV (AA + AB) and TGMV (TA + TB) although the relative level of ssDNA associated with the TGMV coat protein mutant (TA-AR1-1 + TB) was greatly reduced (Fig. 1).

No viral DNAs were detected in symptomless plants inoculated with the ACMV AC2 mutant (AA-AC2-2 + AB) (data not shown), consistent with previous results obtained following the introduction of the mutant
by mechanical inoculation (Etessami et al., 1991). However, when the mutant was co-agroinoculated in the presence of TGMV DNA A (AA-AC2-2 + AB + TA), both ACMV genomic components as well as TGMV DNA A were detected in systemically infected symptomatic tissues. When TGMV DNA A was replaced by a coat protein mutant (AA-AC2-2 + AB + TA-AR1-1), all three genomic components were detected although the relative level of ssDNA in each case was again reduced.

To ensure that the mutation had been retained in mutant AA-AC2-2 during systemic infection, total nucleic acids extracted from leaves of three plants infected with the clone combinations AA-AC2-2 + AB + TA and AA-AC2-2 + AB + TA-AR1-1 were digested with HpaI and MluI (Fig. 2). Wild-type ACMV, containing only a single MluI site, was linearized under these conditions (lane 1) and the AA-AC2-2 mutant, containing an additional HpaI site, was linearized under these conditions (lane 2) and the AA-AC2-2 mutant, containing an additional HpaI site, was linearized under these conditions (lane 2). This produced fragments of the expected sizes of 1862 and 917 bp (lanes 2 and 3), indicating that the mutation had been retained. A DNA A fragment (nucleotides 1329–1782) encompassing the AC2 coding sequence was PCR-amplified from total nucleic acids extracted from a plant infected with the clone combination AA-AC2-2 + AB + TA essentially as described (Stanley, 1995). Direct sequence analysis of the fragment using component-specific primers confirmed that the mutation had been retained and indicated that no additional compensating mutation had been introduced within the coding sequence.

Previous investigations have indicated that agroinoculation facilitates the amplification of DNA A alone in systemically infected tissues (Klinkenberg & Stanley, 1990; Evans & Jeske, 1993). To eliminate the possibility that agroinoculation contributes significantly to the infectivity of the mixed components, an attempt was made to transmit the progeny by mechanical means. Virus from systemically infected leaves was sap transmissible at low frequency (Table 1, experiment IV) and induced mild symptoms similar to those produced after agroinoculation. DNA extracted from systemically infected leaves of such plants gave a similar hybridization profile to those shown in Fig. 1, and restriction analysis using HpaI and MluI once again demonstrated that the AC2 mutation had been retained (data not shown). The ability to sap transmit the mixture of genomic components indicates that the use of agroinoculation to introduce the cloned components into plants does not contribute significantly to the complementation phenomenon. Interestingly, dot blot analysis of plants that remained asymptomatic after sap transmission revealed the presence of TGMV DNA A alone in the upper leaves.
of two of the five plants examined. Presumably, on these occasions, ACMV DNA B gene products had facilitated the systemic spread and amplification of the TGMV component while the ACMV components themselves remained below the level of detection in, or had not reached, these tissues.

Wild-type TGMV DNA A, containing unique BamHI and PvuI sites, produced the expected fragments of 1388 and 1200 bp when total nucleic acid extracts of plants agroinoculated with clone combinations TA + TB and AA-AC2-2 + AB + TA were digested with these enzymes (Fig. 2, lanes 4 and 6). The loss of the 1200 bp fragment and its replacement by a 956 bp fragment verified the presence of the additional PvuI site associated with the coat protein mutation within TGMV DNA A in extracts of plants agroinoculated with the clone combinations TA-AR1-1 + TB and AA-AC2-2 + AB + TA-AR1-1 (lanes 5 and 7). Digestion with XhoI and BamHI linearized the viral DNA, confirming the loss of the unique XhoI site in the progeny of TA-AR1-1 (data not shown).

Proteins were extracted from leaves displaying systemic symptoms, resolved by SDS–PAGE and transferred to nitrocellulose for immunoblotting. The supernatant fraction S1 was used for the detection of coat protein and extract E2 for the detection of BV1 and BC1 as previously described (von Arnim et al., 1993). Blots were probed using polyclonal antisera raised against purified ACMV (Townsend et al., 1985) or synthetic oligopeptides specific to BV1 and BC1 (von Arnim et al., 1993), and developed using phosphatase-conjugated goat anti-rabbit IgG (Sigma) as described (Blake et al., 1984). ACMV coat protein was detected in plants inoculated with wild-type ACMV (AA + AB) and in plants co-agroinoculated with the clone combination AA-AC2-2 + AB + TA (Fig. 3 a), implying that transactivation of ACMV coat protein expression occurs in the presence of complementing AL2. The band detected in extracts from

![Fig. 2. Restriction endonuclease analysis of ACMV and TGMV DNA A genomic components extracted from systemically infected N. benthamiana leaves. Plants were co-agroinoculated with AA and AB (lane 1); AA-AC2-2, AB and TA (lanes 2 and 6); AA-AC2-2, AB and TA-AR1-1 (lanes 3 and 7); TA and TB (lane 4); TA-AR1-1 and TB (lane 5). Samples in lanes 1–3 were digested with HpaI and MluI and probed for ACMV DNA A and those in lanes 4–7 were digested with BamHI and PvuI and probed for TGMV DNA A. Fragment sizes are indicated.](image)

![Fig. 3. Western blot analysis of ACMV-encoded proteins extracted from systemically infected N. benthamiana leaves. Proteins were extracted from either a healthy plant (H) or plants co-agroinoculated with combinations of ACMV (AA + AB) and TGMV (TA + TB) genomic components, ACMV AC2 mutant (AA-AC2-2) and TGMV coat protein mutant (TA-AR1-1). Blots were probed using polyclonal antiserum raised against purified ACMV (a) and synthetic oligopeptides specific to ACMV BV1 (b) and BC1 (c). The positions of coat protein (CP), BV1 and BC1 are indicated. Prestained markers (Bio-Rad) were used as molecular mass standards (kDa).](image)
infectivity and symptom development (Etessami et al., 1991). However, coat protein was also detected in plants co-agroinoculated with the clone combination that included the TGMV coat protein mutant (AA-AC2-2 + AB + TA-AR1-1), ensuring that antiserum was detecting ACMV rather than TGMV coat protein in this instance and confirming that AL2-mediated transactivation of ACMV coat protein expression had occurred. An extremely faint band comigrating with ACMV coat protein, associated with the TGMV coat protein mutant (TA-AR1-1 + TB), was not visible on other blots and may have arisen as a result of slight contamination from the adjacent lane on this occasion.

Because both ACMV BV1 and BC1 are essential for infectivity and symptom development (Etessami et al., 1988), it is reasonable to assume that AL2 additionally transactivates one or both of these genes during the mixed infection, as suggested from earlier work (Sunter et al., 1990; Sunter & Bisaro, 1991, 1992; Gröning et al., 1994; Haley et al., 1992). The presence of both DNA B gene products was confirmed in plants infected with the clone combination AA-AC2-2 + AB + TA-AR1-1 (Fig. 3b, c). As previously reported (von Arnim et al., 1993), BV1 migrated as a single band and BC1 as multiple bands during SDS-PAGE. BC1 was also detected in plants co-agroinoculated with the clone combination AA-AC2-2 + AB + TA, albeit at reduced levels, although BV1 was not detected in the same extract. Coat protein was also expressed at a reduced level (Fig. 3a) and correlated with the low levels of scDNA found in these tissues (Fig. 1). In contrast, co-infection with the TGMV coat protein mutant resulted in a reduction in the relative abundance of TGMV ssDNA and concomitant increase in scDNA, offering an explanation for the presence of relatively high levels of the three ACMV proteins in the systemically infected tissues that were sampled.

A reduction in the level of ssDNA associated with coat protein mutants in N. benthamiana has previously been described for both ACMV and TGMV (Stanley & Townsend, 1986; Elmer & Rogers, 1990). Interestingly, ssDNA accumulates in tissues infected with the ACMV AC2 mutant in the presence of wild-type TGMV DNA A (Fig. 1, clone combination AA-AC2-2 + AB + TA) but not in the presence of the TGMV coat protein mutant (clone combination AA-AC2-2 + AB + TA-AR1-1). The accumulation of ssDNA in wild-type infections has been attributed to the availability of coat protein to encapsidate it, making it unavailable for re-entry into the replication cycle (Stanley & Townsend, 1986). On this basis, however, it is not clear why reduced levels of ssDNA are found in plants co-infected with ACMV AC2 mutant and TGMV coat protein mutant even though these tissues contain reasonable quantities of ACMV coat protein. One possibility is that virus particle formation by intact ACMV coat protein may be disrupted in the presence of the amino-terminal portion of the defective coat protein encoded by the TGMV mutant.

The ability of ACMV to mediate the systemic infection of N. benthamiana by TGMV DNA A following co-agroinoculation of genomic components (Frischmuth et al., 1993) has allowed us to test in planta whether TGMV gene AL2 can functionally complement an ACMV AC2 mutant. The ACMV mutant is unable to systemically infect this host when co-inoculated with DNA B (Etessami et al., 1991). By screening for the production of systemic symptoms, we have demonstrated that the ACMV AC2 mutant can systemically infect N. benthamiana in the presence of co-agroinoculated DNA B and TGMV DNA A, showing that AL2 has the ability to fully complement defective AC2 expression albeit at low frequency. Recently, ACMV AC2 has been shown to transactivate GUS expression from the TGMV coat protein and BR1 promoters in tobacco protoplasts (Sunter et al., 1994). Our observation using the reciprocal combination (i.e. AL2 complementation of AC2 function) serves to emphasize the functional homology of these two proteins and additionally demonstrates that complementation extends to the infection of whole plants. Such reciprocal compatibility of gene function does not always occur; previous work on these particular viruses has shown that although ACMV DNA B genes facilitate the spread of TGMV, TGMV DNA B genes are not only unable to spread ACMV but also actively inhibit this process (von Arnim & Stanley, 1992b; Frischmuth et al., 1993).

Having demonstrated that ACMV-mediated spread of TGMV DNA A occurs efficiently and at high frequency (Frischmuth et al., 1993) we consider it unlikely that the requirement for co-infection of three genomic components is solely responsible for the low level of infectivity and mild symptoms associated with the complemented ACMV AC2 mutant. Hence, our results suggest that AL2 transactivates expression of coat protein and one or both of the essential ACMV DNA B genes with reduced efficiency compared with AC2. This is consistent with the observed decrease in the level of transactivation of TGMV BR1 by AC2 in tobacco protoplasts (Sunter et al., 1994) and probably reflects the divergent nature of Old World and New World geminiviruses (Padidam et al., 1995), represented by ACMV and TGMV, respectively.

ACMV differs from TGMV in that it has a putative gene (AV2) of unknown function that overlaps the 5'
terminus of the coat protein gene. The most abundant virion-sense transcript initiates immediately upstream of AV2 suggesting that it might function as a polycistronic message for both AV2 and coat protein expression (Townsend et al., 1985). Although ACMV AC2 and TGMV AL2 have been shown to be functionally interchangeable, it remains to be seen if AC2 specifically transactivates expression of coat protein, as has been demonstrated for AL2, or if it has an additional effect on AV2 expression. The control of ACMV DNA A virion-sense gene expression is the subject of a current investigation.

References


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