Encapsidation and spread of African cassava mosaic virus DNA A in the absence of DNA B when agroinoculated to *Nicotiana benthamiana*

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Agroinoculation of African cassava mosaic virus DNA A alone into *Nicotiana benthamiana* resulted in the occasional spread of viral DNA throughout the stem, leaves and root. The amount of DNA A reached 5% of that associated with full infection following agroinoculation of both DNAs A and B, although the plants remained asymptomatic. Detection of virus particles in the upper leaves indicates that DNA B is not essential for virus assembly. The predominant form of the virus-specific DNA was single-stranded, which is possibly encapsidated within the virus particles. Double-stranded DNA forms could also be detected in the upper leaves.

Genes essential for the proliferation of the geminivirus African cassava mosaic virus (ACMV; also known as cassava latent virus) are distributed between two similarly sized genomic components, DNAs A and B (formerly DNAs 1 and 2 respectively; Stanley & Gay, 1983). DNA A encodes the coat protein (Townsend et al., 1985). When introduced into protoplasts it is capable of self-replication to produce both ss and ds DNA forms typical of infection which showed that the genes responsible for its replication are in this component (Townsend et al., 1986). Because both components are required for systemic infection in plants (Stanley, 1983), DNA B gene products are implicated in spread of the virus or viral DNA throughout the plant (Townsend et al., 1986; Etessami et al., 1988). In contrast to ACMV, beet curly top virus (BCTV) has a single genomic component, organized similarly to ACMV DNA A (Stanley et al., 1986). The absence of a component analogous to DNA B was correlated with the distribution of BCTV in plants, which appears to be tightly phloem-associated (Esau & Hoefert, 1973), unlike that of ACMV which extends to additional leaf tissues (Sequeira & Harrison, 1982). This suggested that DNA B gene products were responsible at least for movement to and from the phloem and explained why ACMV, but not BCTV, can be mechanically transmitted to *Nicotiana benthamiana* by leaf abrasion. However, recently BCTV was introduced into *N. benthamiana* by agroinoculation (Grimslay et al., 1986; Briddon et al., 1989), demonstrating that this technique efficiently delivers the inoculum directly into the tissues in which the virus proliferates and from where it can spread, and in doing so bypasses the need for DNA B-mediated spread. On the basis of this observation, and considering the fundamental similarity of ACMV DNA A to BCTV DNA, we have tested the ability of ACMV DNA A to spread systemically in the absence of DNA B, following agroinoculation.

The construction of a dimer of DNA A of the Nigerian isolate of ACMV (pBinN2A) and a partial repeat of DNA A (pBin1.3A) and a dimer of DNA B (pBin2B) of the Kenyan isolate in pBin19, and their agroinoculation into *N. benthamiana* seedlings were as described by Klinkenberg et al. (1989). Constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoeskema et al., 1983) which was introduced into plants either by pin-pricking through inocula into stems approximately 1 cm above soil level or by the application of inocula onto decapitated shoots. The former method has the advantage of reducing the possibility of externally contaminating developing leaves with the *A. tumefaciens* culture. Newly expanding leaves were screened for the presence of virus-specific DNA by dot blot analysis (Maule et al., 1983) using radiolabelled fragments (Feinberg & Vogelstein, 1983) specific to DNA A [MluI(734)–SphI(2581)] and DNA B [PstI(245)–EcoRV(2550)], with the results shown in Table 1. When agroinoculation was with DNA A alone, from either the Nigerian or Kenyan isolate, virus-specific DNA was first detected after approximately 2 weeks. In three experiments, 10 plants in a total of 49 tested contained viral DNA in the upper leaves. In parallel experiments,
Table 1. Systemic spread following agroinoculation of ACMV DNA A constructs into N. benthamiana

<table>
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<tr>
<th>Inoculum</th>
<th>Agroinoculation method</th>
<th>Systemic spread†</th>
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<tr>
<td></td>
<td></td>
<td>Expt. 1</td>
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<tr>
<td>pBinN2A</td>
<td>Cut</td>
<td>1/9</td>
</tr>
<tr>
<td></td>
<td>Pp</td>
<td>4/10</td>
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<tr>
<td>pBinN2A + pBin2B</td>
<td>Cut</td>
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<td>Pp</td>
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<tr>
<td>pBin1.3A</td>
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<tr>
<td>pBin1.3A + pBin2B</td>
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* Cut, inoculum applied to decapitated stems; Pp, pin-prick through inoculum into stems.
† Plants positive for DNA A spread/plants agroinoculated.

Severe symptoms developed in all plants between 1 and 2 weeks after agroinoculation with both genomic components (pBinN2A or pBin1.3A each together with pBin2B). Dot blot analyses for nine of the 10 plants agroinoculated with DNA A by pin-pricking (Table 1, experiment 1) are shown in Fig. 1. In no plant was DNA B detected (1% of the level of DNA A would be readily detected under these conditions). Because the amounts of each component are generally similar in wild-type ACMV-infected plants, the result indicates that DNA A movement was not facilitated by contaminating DNA B. The amounts of DNA A in the upper leaves reached approximately 5% of that associated with infection using both DNAs A and B, as estimated by Cerenkov counting of dot blots. The DNA was detectable throughout the plants and was present in highest amounts in the stems and roots, probably reflecting the relative abundance of vascular tissues. All plants in which DNA A was detected were asymptomatic. These results contrast with a previous report on the agroinoculation of ACMV DNA A into N. clevelandii in which this component could not be detected in newly developing leaves by dot blot analysis unless the plants also contained DNA B (Morris et al., 1988).

Total nucleic acids were extracted from upper leaves of two plants (lanes 3 and 8; Fig. 1) using the method of Covey & Hull (1981) and the virus-specific DNA forms were investigated by Southern blot analysis (Southern, 1975) following depurination to enhance the detection of supercoiled (sc) DNA (Wahl et al., 1979). The results shown in Fig. 2, demonstrate that in both plants by far the most abundant form was monomeric ssDNA (lanes 3 and 4) although lesser amounts of DNA that comigrate with dimeric ssDNA (Stanley & Townsend, 1985), scDNA and open-circular (oc) DNA produced during normal infection with DNAs A and B (lane 5) were also present. Both ssDNAs migrated as doublet bands, presumably representing circular and linear forms. The nature of the scDNA and ocDNA was confirmed by digestion with BsmHI (the enzyme used to clone full-length DNA A) which linearized the dsDNA forms to give products (lanes 6 and 7) that comigrate with the full-length DNA A clone insert (lane 8). In this experiment, the ssDNAs were degraded during restriction analysis.

Immunosorbent electron microscopy (Roberts & Harrison, 1979) using antiserum raised against the Kenyan isolate of ACMV, which is known to cross-react with the coat protein of the Nigerian isolate (Stanley et al., 1985), showed that geminate particles were present in extracts of plants agroinoculated with DNA A alone. This suggests that the ssDNA detected in the upper leaves was encapsidated. Furthermore, the result indicates that DNA B is not essential for the assembly of virus particles, as previously deduced for tomato golden mosaic virus (TGMV) from the analysis of plants transgenic for multiple copies of DNA A (Sunter et al., 1987).

It is possible that the presence of the DNA A in the upper leaves results from spread and subsequent reinfection by A. tumefaciens within the vascular system, as has been reported to occur in grapevine (Burr & Katz, 1983) and the legume Sesbania rostrata (Vlachova et al., 1987). Using stem segments, leaf discs, leaf veins and extracts thereof, we have been unable to recover A. tumefaciens on precalising plates (Horsch & Klee, 1986) or L agar plates with or without kanamycin selection (Klinkenberg et al., 1989) as was accomplished by Vlachova et al. (1987). In contrast, examination of the initial site of agroinoculation after 3 weeks resulted in the isolation of kanamycin-resistant bacteria that reacted positively to the ketolactose test (Bernaerts & De Ley, 1963). Therefore, it is considered unlikely that intravascular spread of A. tumefaciens accounts for the observed levels of viral DNA in the upper leaves.
Dilutions are indicated. Blots were probed with radiolabelled fragments pBinN2A (lanes 1 to 9) and pBinN2A and pBin2B (C). Extract dilutions are indicated. Blots were probed with radiolabelled fragments specific to DNA A (a) or DNA B (b).

Fig. 1. Dot blot analysis of extracts from plants agroinoculated with pBinN2A (lanes 1 to 9) and pBinN2A and pBin2B (C). Extract dilutions are indicated. Blots were probed with radiolabelled fragments specific to DNA A (a) or DNA B (b).

Fig. 2. Southern blot analysis of total nucleic acids extracted from plant 3 (3 µg; lanes 3 and 6) and plant 8 (3 µg; lanes 4 and 7) agroinoculated with pBinN2A alone and from a plant agroinoculated with pBinN2A and pBin2B (0.3 µg; lane 5). Extracts in lanes 6 and 7 had been treated with BamHI. Markers were purified ssDNA (lane 1), scDNA (lane 2) and a full-length DNA A clone insert from the Nigerian isolate (lane 8; Stanley et al., 1985). The blot was probed using a radiolabelled fragment specific to DNA A. The positions of open-circular (oc), linear (lin), supercoiled (sc) and monomeric (ss mon) and dimeric (ss dim) single-stranded DNA forms are indicated.

The fact that DNA A spread has not been observed after mechanical inoculation presumably reflects the ability of the agroinoculation technique to deliver the inoculum to tissues intimately associated with the vascular system. Since the amount of viral DNA is low in comparison with normal ACMV infection and the plants remain asymptomatic, the results emphasize the difference in behaviour of ACMV DNA A and BCTV when introduced using the same technique into a common host. The presence of dsDNA A in the upper leaves might be indicative of a low frequency of reinfection within these tissues which would imply that DNA B is not essential for long-distance spread of the virus. However, the presence of primarily ssDNA in the upper leaves suggests atypical behaviour following agroinoculation that might be due, not to de novo synthesis of viral DNA in these tissues, but to the release of virus and dsDNA into the vasular system from the primary point of infection. This might result from degeneration of infected cells, as has been observed in N. benthamiana tissues showing severe symptoms of TGMV infection (Rushing et al., 1987). Under such conditions unencapsidated DNA would be expected to be more susceptible to degradation, consistent with the observed reduction in the relative amount of dsDNA.

We thank Sue Ellwood for technical help and Drs Jeff Davies and Andrew Maule for interesting comments. F.A.K. was a visiting student from the Agricultural University, Wageningen. ACMV was held and manipulated under MAFF licence numbers PHF 49A/85 and PHF 49A/41 under the Plant Pests (Great Britain) Order 1980.

References


(Received 11 December 1989; Accepted 16 February 1990)