A Geminivirus, Serologically Related to Maize Streak Virus, from Digitaria sanguinalis from Vanuatu

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SUMMARY

Electron microscopy of purified particles of a virus found in Digitaria sanguinalis from Vanuatu (formerly New Hebrides) indicated that it is a geminivirus. Preparations of virus particles contained one coat protein of mol. wt. about 27500 and circular and linear single-stranded DNA about 2350 nucleotides in length. In thin sections of infected cells, geminate particles were found in crystalline arrays both in nuclei and in the cytoplasm. The virus is serologically related to maize streak virus but differs from it with a serological differentiation index of 3.

A striate mosaic disease of Digitaria sanguinalis in Vanuatu (formerly New Hebrides) was described some years ago (Dollet & De Taffin, 1979), but the causal agent was not identified. In this paper, we show that the diseased plants contain geminate virus particles (Francki et al., 1985; Harrison, 1985) that are serologically related to maize streak virus (MSV) (Bock et al., 1974).

Infected D. sanguinalis plants were collected in the field and vegetatively propagated in an insect-proof glasshouse. Healthy D. sanguinalis was grown from seed in sterilized soil in the same glasshouse.

Pieces of diseased leaf were fixed in glutaraldehyde and osmium tetroxide and embedded in epoxy resin (Dollet et al., 1983). Sections were stained in uranyl acetate and lead citrate. Linear aggregates of geminate particles (16 to 21 nm) were observed in the nuclei of companion and phloem parenchyma cells, often in large amounts (Fig. 1). Similar arrays of particles were also found in intact areas of cytoplasm, in cells in which nuclei appeared to be intact. Among other geminiviruses, this unusual location has been reported only for chloris striate mosaic virus (CSMV) (Francki et al., 1979).

Virus particles were purified by a method derived from that of Larsen & Duffus (1984). Frozen leaves were ground in 0.1 M-potassium phosphate buffer pH 7, containing 10 mM-EDTA, 10 mM-sodium diethyldithiocarbamate and 20 mM-Na2SO3. The sap was treated with 1% Triton X-100 and clarified with 1/10 vol. chloroform/butanol (1:1, v/v). Virus particles were concentrated with 12% polyethylene glycol and 0.2 M-NaCl and a cycle of differential centrifugation. The pellets were dissolved in 0.1 M-phosphate buffer pH 7 containing 10 mM-EDTA, and the virus preparation ultracentrifuged in a 10 to 40% sucrose density gradient prepared in the same buffer. The virus-containing band was concentrated by ultracentrifugation, and the sucrose density gradient centrifugation was then repeated. The yield was about 6 mg of virus per kg of fresh leaf tissue (a relatively large amount for a geminivirus), estimated assuming that A1%260nm is 7.7 (Goodman & Bird, 1978). Most particles in the purified virus preparations were geminate (Fig. 2), and the remaining few were single. The methods of
Fig. 1. Crystalline arrays of geminate particles in the cytoplasm of a parenchyma cell. Bar marker represents 200 nm.

Fig. 2. Purified virus negatively stained in 2% aqueous uranyl acetate. Bar marker represents 50 nm.

Fig. 3. Ouchterlony pattern of the *Digitaria* virus (D) and MSV-N (N) with *Digitaria* virus antiserum (A). H, Concentrated healthy plant sap.
Table 1. Homologous and heterologous titres of MSV-N and Digitaria virus antisera, measured by electron microscope decoration of purified particles

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>MSV-N</th>
<th>Digitaria virus</th>
<th>Difference (twofold steps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSV-N</td>
<td>2048*</td>
<td>256</td>
<td>3</td>
</tr>
<tr>
<td>Digitaria virus</td>
<td>512</td>
<td>4096</td>
<td>3</td>
</tr>
</tbody>
</table>

* Reciprocal value of the highest reacting twofold dilution.

purification described for MSV (Bock et al., 1974) and for CSMV (Francki et al., 1979) yielded no detectable virus.

To obtain an antiserum, a rabbit was given three intramuscular injections of purified preparations containing 0.4, 0.75 and 1.5 mg of virus in 1.3, 1.5 and 1 ml of buffer respectively. Virus preparations were emulsified with Freund's adjuvant, complete for the first injection and incomplete for the others, and given at intervals of about 10 days. Blood was taken at 3 week intervals after the first injection. Serological tests were made with purified virus preparations by agar gel double diffusion (Ouchterlony test) or electron microscope decoration (Milne, 1984). The serum had a homologous titre of 1:256 in agar gel double diffusion tests and did not react with healthy plant components. The virus was tested with sera to African cassava (latent) mosaic virus (ACMV) (C. Faquet, Abidjan, Ivory Coast), CSMV (R. I. B. Francki, Adelaide, Australia), MSV from Nigeria, severe strain (MSV-N) (M. Boulton, Norwich, U.K.), MSV from South Africa (MSV-SA) (M. B. von Wechmar, Cape Town, South Africa) and tobacco leaf curl virus (T. Osaki, Osaka, Japan). Reactions were observed only with sera to MSV isolates. In gel diffusion the dilution endpoint of reaction of MSV-SA antiserum (homologous titre 1:128 in gel diffusion, M. B. von Wechmar, personal communication) was 1:16 and of MSV-N antiserum (homologous titre 1:512 in tube precipitin test, M. Boulton, personal communication) was 1:64. In decoration tests a serological differentiation index (SDI) of 3 was obtained between the Digitaria virus and MSV-N (Table 1). In Ouchterlony tests the serum to the Digitaria virus formed a spur between homologous and heterologous viruses (Fig. 3); a similar spur formed in tests with serum to MSV-N.

Virus coat protein was extracted by two methods: (i) pellets of virus were resuspended in 0.1 M-potassium phosphate buffer pH 7 containing 10 mM-EDTA, the suspension was mixed with 3 vol. acetone, kept at -18 °C overnight, then the protein was recovered by low-speed centrifugation and resuspended in water; (ii) virus suspensions were treated with 8 M-urea and 1% 2-mercaptoethanol, as described by Boccardo & Milne (1975). Polyacrylamide gel electrophoresis (PAGE) was in the discontinuous system of Laemmli (1970) and in the continuous system of Swank & Munkres (1971), using in each case 15 and 12.5% polyacrylamide gels. By either method, Digitaria virus protein formed a single band that corresponded to a mol. wt. of about 27500 (Fig. 4b). We have not, however, estimated the mol. wt. using lower concentration gels.

To isolate the nucleic acid, purified virus was mixed with 1 mg/ml protease (type XI from Tritirachium album, Sigma) in 0.1 M-sodium acetate pH 7.5, 0.5% SDS, and dialysed overnight at room temperature against the same buffer. The nucleic acid was then extracted by treatment with phenol in the presence of 1% (w/v) bentonite and precipitated from the aqueous phase by adding 2.5 vol. ethanol. The yield was about 0.1 mg nucleic acid per mg of purified virus (A260nm, 1cm is 25). The nucleic acid was destroyed by treatment with DNase and S, nuclease showing that it was single-stranded DNA (data not shown). It was analysed by electrophoresis in 1% agarose gels in 40 mM-Tris–HCl pH 8.1, 20 mM-sodium acetate, 2 mM-EDTA, for 16 h at 30 V. The markers used were pBR322 plasmid DNA digested with either PvuII and HindIII, or with PvuII and EcoRV, and heat-denatured to generate single-stranded fragments of 2481 and 2325 nucleotides respectively. The Digitaria geminivirus DNA gave two closely spaced major bands. If this DNA behaves as ACMV DNA, the slower band corresponds to the circular form, and the faster one to linear molecules (Harrison et al., 1977). The linear DNA was estimated to consist of 2350 (+20) nucleotides. When the slot was overloaded two minor bands of DNA
Fig. 4. Electrophoresis of *Digitaria* virus coat protein (b) in a 15% continuous gel. (a, c) Mol. wt. markers in order of increasing mobility (mol. wt. in parentheses): rabbit muscle pyruvate kinase type M (57000), ovalbumin H chain (43000), bovine erythrocyte carbonic anhydrase (29000), bovine pancreas chymotrypsinogen (25700), tobacco mosaic virus coat protein (17500) and horse heart cytochrome c type VI (11700).

Fig. 5. Electrophoresis of *Digitaria* virus DNA in 1% agarose gel (a, d), showing circular (c) and linear (l) forms. (e) Overloading to show the circular (dc) and linear (dl) dimers. The markers (nucleotides) used were pBR322 plasmid DNA digested with *Pvu*II and *Eco*RV (b) and with *Pvu*II and *Hind*III (c).

appeared (Fig. 5e). The DNA length estimation on agarose gel (4700 ± 80 nucleotides) is consistent with circular and linear dimeric molecules. These dimeric molecules represent about 1 to 3% of the total DNA, according to the ratio of the absorbance of the two bands.

Electron microscopy of the DNA was done after spreading, using cytochrome c and 50% formamide, according to Davis *et al.* (1971). A mixture of circular and linear molecules was found (Fig. 6). Length measurements of 26 circular molecules and comparison with φX174 DNA (5386 nucleotides) included as an internal standard gave an estimate of 2380 nucleotides for the size of *Digitaria* virus DNA. Circular molecules having about twice the contour length of the others were also routinely seen (Fig. 6, inset); they were probably dimers. Similar dimer molecules have been described with ACMV DNA (Stanley & Townsend, 1985). No defective molecules (minicircles) were observed.

Geminivirus DNAs which have been sequenced have 2508 to 2779 nucleotides (see Harrison, 1985). The size of MSV DNA itself is known from sequencing: 2687 nucleotides (Mullineaux *et al.*, 1984). The *Digitaria* virus DNA appears, by two different methods, to be significantly smaller, although further work will be necessary to confirm this. The virus could prove to be a deletion mutant which would be of interest as a gene vector.

We have not given the *Digitaria* geminivirus a name because it is probably best considered as a strain of MSV (MSV *Digitaria* strain). Should the vector (so far unknown) prove to be unusual, or the nucleotide sequence be found to differ substantially from that of MSV, the *Digitaria* virus should then be considered as distinct.
Fig. 6. Circular and linear forms of *Digitaria* virus DNA. Inset: a larger circular molecule, probably a dimer. Bar marker represents 100 nm.

REFERENCES


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