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Identification of novel DNA forms in tomato golden mosaic virus infected tissue. Evidence for a two component viral genome

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ABSTRACT

Extracts obtained from cells infected with the geminivirus tomato golden mosaic (TGMV) are shown to contain, in addition to viral single-stranded DNA, several novel species of virus-specific single- and double-stranded DNA (ss and ds DNA). The results of nuclease studies and electron microscopy suggest that three of the intracellular DNAs are unit-genome length duplexes of closed circular, relaxed circular, and linear form. The remaining ds DNA species are of high molecular weight and appear to be concatamers consisting of two or more unit-length genomes. A low molecular weight virus-specific DNA species was also detected.

Restriction endonuclease digestion of unit-length circular ds TGMV DNA resulted in fragments whose combined size is twice the unit-genome length. Thus ds TGMV is composed of two components of nearly identical size but different nucleotide sequence.

INTRODUCTION

Tomato golden mosaic virus (TGMV) is a DNA containing plant virus which belongs to the recently described geminivirus group (1, 2). Geminiviruses consist of siamese twin, isometric particles which contain single-stranded (ss) circular DNA of molecular weight 0.7 - 0.8 x 10^6 (see 3 for review). It has been reported that the genome of one member of this group, bean mosaic virus (BGMV), is divided between two ss DNA components of different sequence but identical size (4). A virus-specific double-stranded (ds) DNA has also been identified in BGMV-infected tissue and shown to contain the sequences of both types of genomic DNA. On the basis of denaturation and nuclease tests this ds DNA was shown to have a circular structure with a discontinuity in one strand (5).

In this paper we present evidence which indicates that TGMV DNA, like that of BGMV, is composed of two components of nearly identical size. The identification in infected cell extracts of six novel species of virus-specific ss and ds DNAs is likewise reported. Five of these

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species have not been previously reported for any geminivirus, and their possible significance in viral DNA replication is discussed.

MATERIALS AND METHODS

Cellular extracts and viral DNA. Cellular extracts enriched in viral DNA (crude TGMV DNA) were prepared as follows. Leaf material, obtained from either healthy Nicotiana benthamiana plants or plants infected with TGMV 14 days previously, was homogenised in buffer (0.5 M KH_2PO_4, 0.75%, Na_2SO_3, pH 7.0, 2 ml/g leaf tissue). The homogenate was made to 2.5% by volume in Triton X-100, stirred for 16 hr at 4°, and filtered through muslin. After centrifugation at 10,000 x g for 10 min, the supernatant fluid was further centrifuged at 40,000 rpm for 3 hr in a Beckman Ti45 rotor. The resulting pellets were resuspended in TAE buffer (40 mM Tris, 5 mM acetic acid, 10 mM Na_2EDTA, pH8.2, 1 ml/20 g leaf tissue). Following adjustment to 0.1% in SDS, samples were extracted with an equal volume of buffer-saturated phenol and chloroform-isoamylalcohol (24:1). Nucleic acids were precipitated from the final aqueous phase with ethanol and resuspended in a small volume of TAE buffer.

Viral DNA was isolated from purified preparations of TGMV as previously described (2).

Gel electrophoresis. Electrophoresis was performed in horizontal slab gels containing 1% agarose and 0.5µg/ml ethidium bromide in TAE buffer. DNA species were extracted from gels by a modification of the procedure of Dretzen et al.(6). Briefly, agarose gel bands were excised and placed in a tube gel cylinder closed at one end with a small agar plug to prevent buffer drainage. A disc of DE-81 paper (Whatman, previously soaked in 3 M NaCl) was fitted to the plugged end of the cylinder and held in place with a perforated cap. The cylinder was filled with TAE buffer, and DNA was electroeluted from the excised bands (2-10 mA per cylinder for 4-16 hr) onto the DE-81 paper in a Shandon tube gel apparatus. Further processing of the DE-81 paper was as described (6).

In some cases, electrophoresis of DNA was carried out in 4% polyacrylamide slab gels containing 8 M urea and 0.1% SDS (urea-PAGE) (7). DNA components were extracted from gels by the method of Schuerch et al. (8).

Glyoxalation of DNA samples was done according to the procedure of McMaster and Carmichael (9), and in all cases DNA molecular weight was determined by comparison with phage λ DNA Hind III fragments and

Øx174 RF Hae III fragments included on the gels.

Electron microscopy. Electron microscopy of urea-PAGE separated DNA was accomplished by the Kleinschmidt cytochrome c technique (10) as previously described (2).

Nuclease digestions. Nuclease treatments of crude TGMV DNA samples were performed in the following buffers : 10 mM Tris, 10 mM $MgCl_2$, pH 7.4 (DNase and RNase reactions), 10 mM Na acetate, 0.5 mM Zn SO_4, pH 5.0 (nuclease S_1 reactions) and 66 mM Tris, 77 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol, pH 8.0 (exonuclease III reactions). Reactions contained about 3µg of nucleic acid and either 1µg DNase I (Worthington), 1µg RNase A (Sigma), 15 units nuclease S_1 (Sigma) or 25 units exonuclease III (BRL).

All restriction endonuclease digestions of gel purified DNA were carried out in 200 mM Tris, 1 mM $MgCl_2$, 100 mM KCl, 100µg/ml gelatin, 1 mM dithiothreitol, pH 7.5. Reactions contained about 1µg of DNA and 15 units of Eco RI and/or Bam HI (BRL). All treatments were at 37°C for 1-2 hr.

DNA-DNA hybridisation. Crude TGMV DNA or agarose gel purified DNA species were electrophoresed on agarose gel (1% agarose - 0.5µg/ml ethidium bromide) and transferred to nitrocellulose paper (11). Immobilised DNA was then hybridised with ^{32}P - DNA complementary to ss viral DNA (TGMV cDNA) according to the method of Wahl *et al.* (12). TGMV cDNA was prepared by the random primer procedure (13) as previously described (14).

Hybridisation reactions were performed in the presence of dextran sulfate (12), and it is important to note that a considerable amount of hybridisation occurs between the randomly synthesised cDNA molecules and intact ss DNA template strands in this system. The net effect is a decrease in the signal intensity of ss DNA bands relative to their ds DNA counterparts on nitrocellulose blots (see Results).

In some cases, immobilised DNA species were hybridised with ^{32}P labelled DNA prepared by the nick translation procedure of Rigby *et al.* (15). Cloned ds TGMV DNA, prepared as described in the following communication (16, this volume) was used as probe in these experiments.

RESULTS

Identification of nucleic acid species in TGMV-infected tissue. DNA obtained from either healthy or TGMV infected plant tissue (crude TGMV DNA) was electrophoresed on agarose gel in the presence of ethidium bromide

in order to detect discrete and possibly virus-related DNA species. As evident in Fig. 1, crude TGMV DNA preparations contained a considerable amount of host DNA which migrated as a smear from near the top of the well to the region just above the viral ss DNA band (band 4). However, at least three discrete bands can be detected within the host background, and another below the viral DNA. None of these were seen in extracts obtained from healthy cells.

Hybridisation of DNA from infected cells with TGMV cDNA. The nature of the new nucleic acid species and their viral origin was confirmed in the following manner. Native crude TGMV DNA samples and samples previously treated with either DNase I, RNase A or nuclease S_1 were immobilised on nitrocellulose paper after agarose gel electrophoresis and tested for complementarity with ^{32}P - cDNA prepared against viral DNA (TGMV cDNA). As shown in Fig. 2, crude TGMV preparations contain (in addition to viral DNA) at least six species capable of hybridising with TGMV cDNA. No species homologous to the probe were detected in extracts from healthy plants.

All of the virus-specific components found in infected tissue were

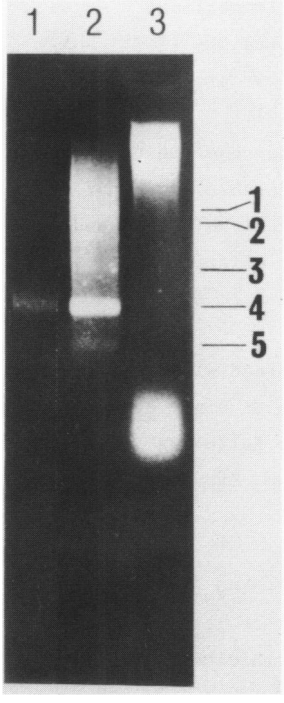

Figure 1. Electrophoresis of viral DNA and infected cell DNA. (1) Purified TGMV DNA; (2) infected cell DNA; (3) healthy cell DNA.

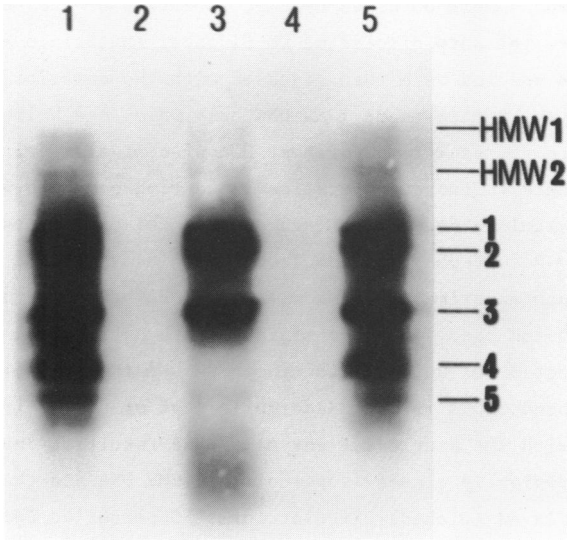

Figure 2. Autoradiogram of a blot probed with ^{32}P - TGMV cDNA. (1) infected cell DNA; (2) healthy cell DNA; infected cell DNA digested with (3) nuclease S_1, (4) DNase I, (5) RNase A. Due to hybridisation of probe molecules with template DNA, ss DNA bands appear fainter relative to ds DNA bands.

hydrolysed by DNase I but resistant to RNase digestion (Fig. 2). As expected, viral DNA (band 4) was sensitive to digestion by single-strand specific nuclease S_1, as was most of the faster migrating band 5 material. Thus the remaining five species, bands 1-3 and the high molecular weight (HMW) bands 1 and 2, are apparently virus-specific ds DNA.

Virus-specific DNA species have the following apparent molecular weights: HMW-1, 6.8 x 10^6; HMW-2, 3.5 x 10^6; band 2, 1.6 x 10^6; band 5 0.6 x 10^6. Bands 1 and 3 are ds circular molecules identical in size to band 2 (discussed below). It should be noted that two S_1-sensitive bands seen on the blot presented here (Fig. 2) between bands 3 and 4 and below band 5 were found in some but not all crude TGMV preparations. The amount of HMW ds DNAs 1 and 2 and of the ss DNA band 5 also varied between preparations, although these were consistently detected. The reason for the variable appearance of some bands is presently unknown, although it may be due to the stage of infection prevalent in the plants from which the extracts were obtained. We have chosen to further characterise only the unit-length ds DNA species (bands 1, 2 and 3) in this communication.

Characterisation of unit length ds TGMV DNAs. Separated ds DNA

components 1, 2 and 3 were obtained by electroelution of the relevant bands from agarose gel onto circles of DE-81 paper (Materials and Methods). The extracted DNA species were then digested with the restriction endonuclease Eco RI, which cleaves ds TGMV DNA only once (see below). In order to eliminate the background of host DNA present along with the separated components in these preparations, reaction products were transferred to nitrocellulose paper after agarose gel electrophoresis and hybridised with ^{32}P-TGMV DNA prepared from cloned ds viral DNA (16). The results of one such experiment are shown in Fig. 3. The cleavage product of band 3 and band 1 DNAs co-migrated with native band 2 at the position expected for unit-length linear ds DNA (MW 1.6 x 10^6). Thus the mobility of band 3 DNA is decreased while that of band 1 is increased upon incubation with the single-cut enzyme. This result strongly suggests that band 3 is covalently closed (supercoiled) TGMV DNA and that band 1 represents the relaxed (nicked) circular form. Supercoiled DNA is a compact structure and would be expected to migrate more rapidly in agarose gel than either relaxed circular or linear forms. Relaxed circular DNA migrates more slowly than linear DNA because of its extended configuration (17). The band 1 material present in native band 3 preparations (Fig. 3, lane 1) can hence be regarded as circles which were

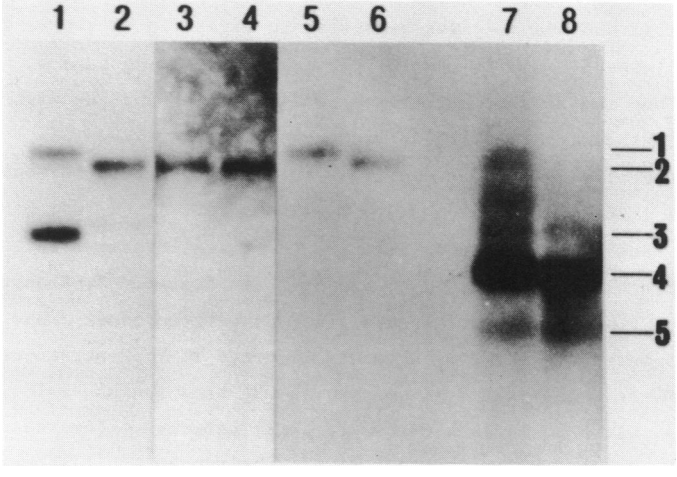

Figure 3. Autoradiogram of a blot probed with ^{32}P-TGMV DNA.
(1) Band 3 DNA; (2) Eco RI digested band 3; (3) band 2 DNA; (4) Eco RI digested band 2; (5) band 1 DNA; (6) Eco RI digested band 1 DNA; (7) infected cell DNA; (8) exonuclease III digested infected cell DNA.

nicked during the electroelution process.

Further evidence for the covalently closed structure of band 3 lies in its resistance to digestion by exonuclease III. Only single-stranded and covalently closed double-stranded DNAs are resistant to the 3'-exonucleolytic activity of this enzyme (18). As expected, band 1 and 2 DNAs present in crude TGMV DNA preparations are hydrolysed by exonuclease III while bands 3, 4 and 5 are not (Fig. 3, lane 8).

It is curious that the linear band 2 DNA was not cleaved by Eco RI in this experiment (Fig. 3, lane 8), and was not cleaved by Eco RI in several other attempts (not shown). While this may be the result of a contaminant present in DNA preparations which inhibits the activity of this enzyme, it should be pointed out that both band 1 and 2 DNAs used in the experiment presented here were isolated from the same preparative gel, and further were incubated with Eco RI in parallel reactions. An alternative explanation for this apparent resistance to restriction may be that linear ds TGMV DNA arises from specific cleavage (or breakage) of circular molecules, and that the site of this cleavage lies in close proximity to the Eco RI cleavage site. Further work on this point is in progress.

Further characterisation of band 1. The data presented above provide evidence which indicates that band 1 is the relaxed circular form of ds TGMV DNA. In order to directly determine the size of this species, and indirectly the size of the closed circular form from which it can be generated, the component was directly visualised. Band 1 DNA was separated from other viral DNA forms by urea-PAGE (since in this system it is also well separated from host DNA) and after extraction from the gel was examined by electron microscopy (Fig. 4). The component was found to consist of predominantly open circular molecules of the size expected for unit-length viral DNA. TGMV circles had a mean contour length of 3.81±0.03 units (n=128 molecules); SV-40 circular molecules included as an internal standard had a mean contour length of 7.86±0.07 units (n=89 molecules). SV-40 DNA contains 5241 base pairs and has a molecular weight of about 3.40×10^6 (19). The corresponding values for ds TGMV circular DNA molecules are 2540 base pairs and 1.63×10^6, in good agreement with those previously obtained for circular ss-TGMV DNA (2470 nucleotides, MW 0.79×10^6) (2).

Glyoxalation followed by urea-PAGE of band 1 material yielded two discrete bands of roughly equal intensity (not shown). One of these co-migrated with circular and the other with linear ss viral DNA; these

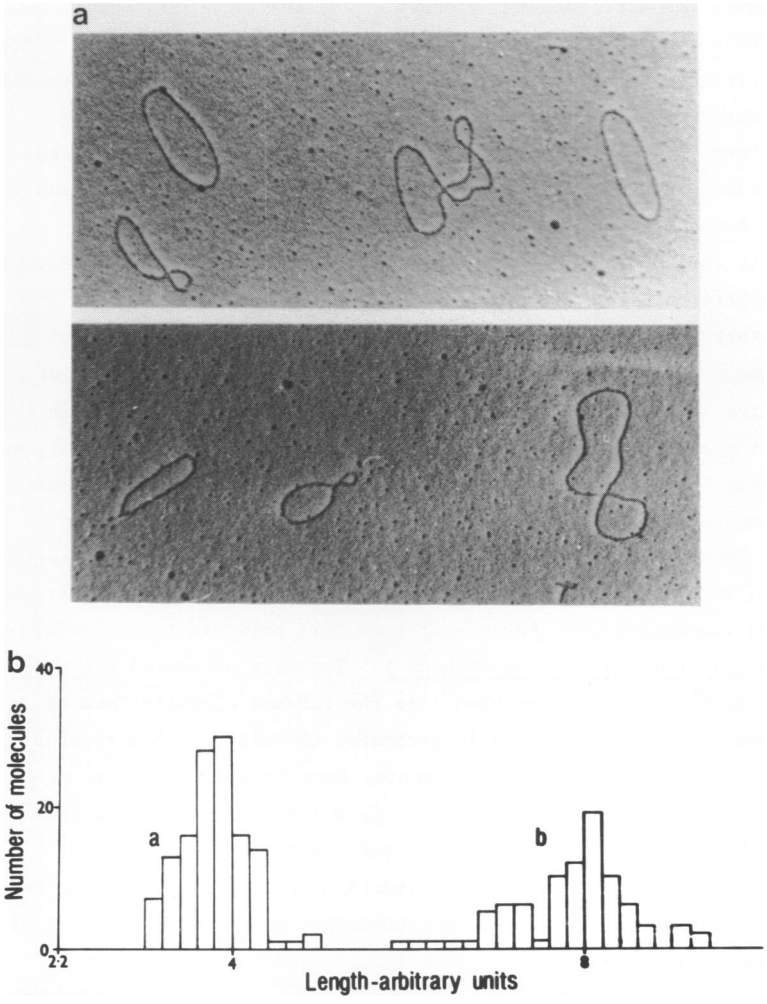

Figure 4. (A) Electron micrographs of TGMV ds DNA molecules. Relaxed circular TGMV molecules with larger SV-40 circles included for comparison. (B) Length distributions of ds DNA molecules. (a) TGMV ds DNA; (b) SV-40 ds DNA.

two forms have previously been shown to be the same size (2). This result is consistent with a double-stranded circular structure containing a single nick in one of the strands. Further analysis will be necessary before it can be determined if the nick occurs at a specific site in the nucleotide sequence.

Restriction endonuclease analysis. It is known that the ds DNA

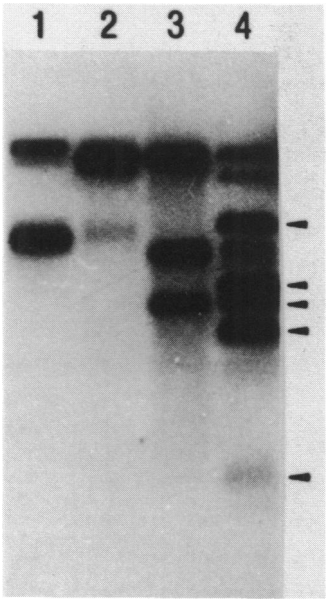

Figure 5. Autoradiogram of a blot probed with ^{32}P-TGMV DNA. (1) Band 3 DNA; band 3 DNA digested with Eco RI (2), Bam HI (3), and Eco RI + Bam HI (4). Eco RI + Bam HI digestion products are indicated. Some fragments resulting from partial digestion are visible (lane 4).

of BGMV is composed of two identical-size components with different nucleotide sequence (5). Agarose gel purified circular ds TGMV DNA was therefore digested with restriction enzymes to determine whether the same might be the case for this virus. As before, reaction products were transferred to nitrocellulose paper after agarose gel electrophoresis and hybridised with ^{32}P-TGMV DNA. As indicated in Fig. 5, circular viral DNA (band 3) was cleaved only once by Eco RI, yielding a linear fragment whose size corresponds to full-length ds viral DNA. In contrast, Bam HI digestion gave rise to three fragments whose combined size was approximately twice that expected for viral DNA (Table 1). The largest of these also corresponded in size with full-length viral DNA. Double digestion of circular DNA with Eco RI and Bam HI similarly resulted in fragments

Table 1 Digestion of ds TGMV DNA with Eco RI and Bam HI. Data are expressed in base pairs.

Treatment	Eco RI	Bam HI	Eco RI + Bam HI
Fragment size	2660	2660	1730
		1455	1190
		1080	1080
			930
			310
Total size	2660	5195	5240

totalling twice the unit-genome length, thus showing that those obtained with Bam HI alone were not the products of partial digestion. Identical fragments were generated by restriction of band 1 DNA with the two enzymes (not shown). These results indicate that ds TGMV DNA consists of two populations of molecules with nearly identical physical size but different nucleotide sequence. Both populations contain only one Eco RI site, while one population contains a single Bam HI site and the other two. Our observation of two TGMV DNA components extends the initial finding of two components in BGMV DNA (4,5) to another member of the geminivirus group.

DISCUSSION

Cells infected with TGMV contain several discrete species of virus-specific DNA in addition to genomic viral DNA. At least one ss DNA and five ds DNA species are consistently detected. The two largest are double-stranded (HMW-1 , MW 6.8 x 10^6; HMW-2, MW 3.5 x 10^6) and are possibly concatamers composed of tandemly arranged viral genomes. The remaining three double-stranded components are of unit-genome length (MW 1.6 x 10^6) and comprise the closed circular (band 3), relaxed circular (band 1), and linear (band 2) forms of ds TGMV DNA. The conformation and significance of the single-stranded band 5 (MW 0.6 x 10^6) is presently unknown. With the exception of the relaxed circular form (5), none of the DNA species identified here have been previously reported for any geminivirus.

It is interesting that Ikegami et al.(5) were unable to detect closed circular molecules or molecules of greater than unit-length in BGMV-infected tissue, despite the fact that closed circular ds M 13 DNA included as a control survived their purification procedure. This difference suggests that either BGMV and TGMV replicate by a different mechanism or that the purification procedure described in this communication, which includes treatment of cell lysates with Triton X-100, may be a more efficient method for isolating these DNA species.

Double-stranded DNA forms described here are not found in DNA preparations obtained from purified virus. We presume, then, that they are replicative intermediates which exist in a free state in infected cells. The presence of closed and relaxed circles of unit-length indicates that TGMV DNA replicates via circular ds replicative form (RF) DNAs analagous to those of the ss DNA bacteriophages (for review see 20).

Further, the large and possibly concatameric ds DNAs detected here may prove to be intermediates in the replication of viral RF DNA. The low molecular weight band 5 DNA component with its apparent ss-character might represent a helper-dependent defective TGMV-virus analogous to the mini-phages arising during replication of ss-DNA phages (20). Experiments designed to test these ideas and to further characterise all forms of intracellular TGMV DNA are currently in progress.

Restriction endonuclease digestion of ds circular TGMV DNA gives rise to fragments whose combined size is twice that expected for unit length viral DNA. Such a result indicates that ds TGMV DNA is composed of two populations of molecules with different sequence but nearly identical physical size, and further suggests a divided viral genome. This has already been found to be the case for one other geminivirus (4), and perhaps all geminivirus genomes are organised in this way. Both ds TGMV DNA components contain a single Eco RI cleavage site, and we have used this information to advantage in the characterisation of virus-specific DNA species (discussed above). In subsequent work, we have used this site to insert and clone viral DNA in the E. coli plasmid pAT 153 (16). The results of this work show conclusively that genomic TGMV DNA consists of two components of almost, but not exactly, the same size.

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