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Molecular cloning and characterisation of the two DNA components of tomato golden mosaic virus

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ABSTRACT

We report the molecular cloning of the tomato golden mosaic virus (TGMV) genome in the E. coli plasmid pAT 153. The results of this work conclusively show that TGMV DNA consists of two components (designated A and B) of almost, but not exactly, the same size. Four different recombinant plasmids are described, two containing component A in opposite orientation and two containing component B in opposite orientation. Southern blot analysis revealed little sequence homology between A and B and showed both components to be equally represented in viral and intracellular DNA forms. Detailed restriction maps of the cloned DNAs are presented, and a comparison of these with digests of intracellular viral dsDNA indicates that the former are full-length faithful copies of TGMV DNA. This is the first report of the cloning of a geminivirus genome.

INTRODUCTION

Geminiviruses are unique among the plant viruses in having a genome of covalently closed single-stranded DNA (see 1 for review). Moreover, recent evidence indicates that the genomes of at least two members of this group are divided between two DNA components of identical size but different nucleotide sequence (2, 3). We have previously reported the isolation and characterisation of virus-specific double-stranded DNA (dsDNA) found in cells infected with tomato golden mosaic virus (TGMV) (3). Here we report the molecular cloning of both components of TGMV DNA from ds viral DNA. The cloned DNAs are characterised with respect to their physical size, restriction endonuclease cleavage sites, sequence homology with the opposite component, and representation in intracellular viral DNA forms. The results provide strong evidence for a divided genome, and the possible significance of such a genomic arrangement is discussed.

MATERIALS AND METHODS

Isolation of ds viral DNA. The procedures used to isolate relaxed circular ds TGMV DNA have been previously described (3). Briefly, extracts

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obtained from infected cells (crude TGMV DNA) were electrophoresed on 4% polyacrylamide gel (containing 8M urea and 0.1% SDS) (4), and the ds DNA bands extracted from the gel by the method of Schuerch et al. (5). Following extraction, ds DNA samples were tested for homogeneity by electrophoresis on either polyacrylamide gel or 1% agarose gels containing 0.5 µg/ml ethidium bromide.

Insertion and cloning of TGMV DNA in plasmid pAT 153. Circular ds TGMV DNA linearised by cleavage at the unique Eco R1 sites common to both of its components (3) was inserted into the Eco R1 site of the E. coli plasmid pAT 153 (6) as described below. Plasmid pAT 153 in E. coli strain HB-101 and E. coli strain DH-1 were generously provided by P.W.J. Rigby and D.M. Glover.

Removal of free 5'-phosphate groups from Eco R1 cleaved pAT 153 DNA was accomplished by the action of calf intestinal phosphatase (Boehringer Mannheim), further purified before use by the method of Efstratiadis et al. (7). Phosphatase reactions were performed in 10 mM Tris, 10 mM $MgCl_2$, pH 8.6 for 1 hr at 37°. After heating briefly to 80°, preparations were extracted once with phenol and once with chloroform-isoamylalcohol (24:1). The plasmid DNA was precipitated from the final aqueous phase with 2 vol of ethanol and resuspended in a small volume of water.

Eco R1 cleaved TGMV DNA and Eco R1 cleaved-phosphatase treated pAT 153 DNA was annealed for 16 hr at 16° in the presence of T4 DNA ligase (kindly provided by W. Fiers). Reactions were carried out (final volume = 16 µl) in 67.5 mM Tris, 10 mM $MgCl_2$, 15 mM dithiothreitol, 1 mM ATP, 1 mM spermidine, pH 7.6 and contained 140 ng pAT 153 DNA and ~ 120 ng TGMV DNA. E.coli DH-1 cells (D. Hanahan) were transformed as described by Morrison (8), and plated on agar containing 20 µg/ml tetracycline. Under these conditions, about 70% of transformants contained recombinant plasmid.

Isolation of plasmid DNA. Transformed colonies were directly screened for the presence of recombinant plasmid. Plasmid DNA was isolated from 1 ml cultures ("mini preps") by the protocol of Ish-Horowicz and Burke (9), and digested with restriction enzymes after an initial treatment with RNase. For larger cultures, the same isolation method was employed scaled up to the appropriate volume. A LiCl precipitation step was included to remove the bulk of contaminating RNA (M. Koziel, K. Kolacz and A. Siegel, personal communication).

Restriction endonuclease digestion and agarose gel electrophoresis. All restriction endonuclease digestions were carried out in 20 mM Tris, 7 mM $MgCl_2$, 100 mM KCl, 100 µg/ml gelatin, 1 mM dithiothreitol, pH 7.5.

Incubations were performed at 37°, except in the case of Pst I (30°).

Reaction products were analysed on 1% agarose gels (containing 0.5 µg/ml ethidium bromide) in TAE buffer (40 mM Tris, 5 mM acetic acid, 1 mM EDTA, pH 8.2). In some cases, DNA species were purified from agarose gel by the method of Dretzen et al. (10) as previously modified (3).

DNA-DNA hybridisation. Hybridisation of ^{32}P-cloned DNAs with DNA samples transferred to nitrocellulose paper after agarose gel electrophoresis (11) was accomplished according to the procedure of Wahl et al. (12). Labelled DNA was prepared by the combined action of DNase I (Worthington) and DNA polymerase I (Sigma) according to the nick translation procedure of Rigby et al. (13). Either agarose gel purified component A or component B DNA was labelled, except in the case of one experiment (see Fig. 1, Results). This probe was prepared by including equal amounts of both components in the nick translation reaction.

RESULTS

Cloning of TGMV DNA. Circular ds TGMV DNA was converted to linear form by cleavage at the unique Eco R1 sites present in both of its DNA components (3) and inserted into the corresponding site of plasmid pAT 153. Eco R1 cleaved plasmid DNA was treated with calf intestinal phosphatase prior to annealing with TGMV DNA to prevent self-ligation (Materials and Methods). Following transformation, 402 tetracycline-resistant colonies were obtained and the plasmid DNA of 102 of these was directly analysed by digestion with Eco RI and Bam HI. The latter enzyme is diagnostic for the two viral DNA components since one of them (designated component A) contains a single Bam HI site while the other (designated component B) contains two sites (3). 65% of the transformed colonies examined (66 of 102) contained inserts corresponding in size to full-length TGMV DNA. Of these inserts, 47% (31 of 66) were found to be component A and 53% (35 of 66) to be component B (Fig. 1).

There is an exact correspondence between the fragments resulting from digestion of insert and intracellular viral ds DNA (Fig. 1). The total length of viral DNA fragments is twice that expected for unit-length TGMV DNA, indicating the presence of two components (3). The fragments which arise from digestion of cloned A and B DNA, however, clearly show each to be a subset of viral DNA with unit length size (Table I). On the basis of this evidence, we conclude that the inserts described here are faithful and complete copies of viral DNA.

The A and B inserts appear in both possible orientations relative to

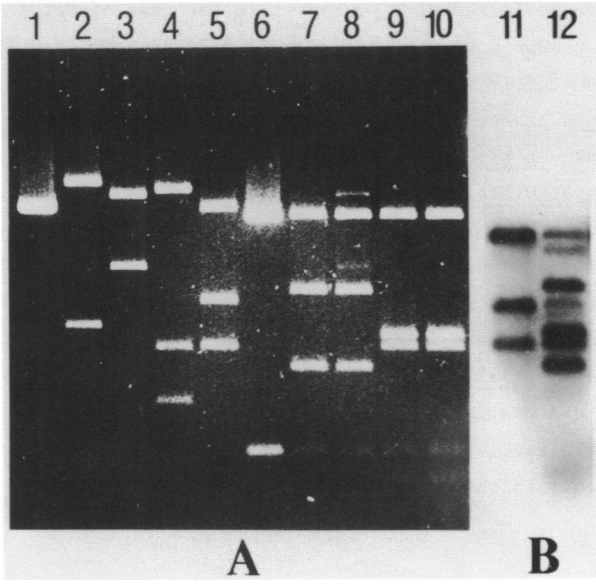

Figure 1

(A) Bam HI digests of pAT 153, pBH 404, pBH 401, pBH 405 and pBH 407 DNA (1-5); Eco RI + Bam HI digests of these plasmids (6-10) run on a 1% agarose gel. See Fig. 2 for plasmid nomenclature.

(B) An autoradiogram of Bam HI (11) and Eco RI + Bam HI (12) digests of circular ds viral DNA probed with ^{32}P-pBH 401 and pBH 405 insert DNA. Partial digestion products are present in lanes 8 and 12.

the single Bam HI site of pAT 153, and a diagram of these four types of recombinant plasmid is presented in Fig. 2.

Physical size of components A and B. The liklihood of the components of any divided genome virus being exactly the same size is remote. Consequently, the mobilities of the two components of TGMV DNA on agarose gel were subjected to close inspection. Fig. 3 shows the results of an experiment in which Eco RI restricted pBH-404 and pBH-407 DNA was run in the same and separate lanes of a gel. After prolonged electrophoresis, a difference was seen in the mobilities of components A and B (lanes 1 and 3) and the mixed components were clearly resolved into a doublet (lane 2). The size difference, about 75 base pairs, can also be discerned from the averaged sum of the molecular weights of restriction fragments used to prepare restriction maps of components A and B (discussed below). Thus, the differential mobility of A and B on agarose gel reflects a real difference in the size of these molecules.

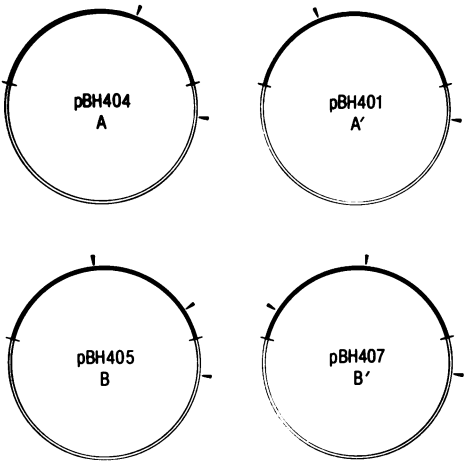

Figure 2

Structure of recombinant plasmids. The orientation of insert DNA (shaded) relative to the single Bam HI site of pAT 153 is shown. Bam HI sites are indicated by arrows.

Restriction maps. Cloned TGMV DNA was tested for restriction with twenty-one different enzymes. A catalogue of the fragments generated by the twelve enzymes which cut either or both components A and B is given in Table 1. The molecular weight of component A, calculated as the averaged sum of the fragments for each enzyme, is 1.70×10^6 (ca. 2660 bp). The molecular weight of component B is 1.65×10^6 (ca. 2580 bp). Both values are in good agreement with those previously obtained for single (14) and double-stranded (3) TGMV DNA, and provide further evidence fora small

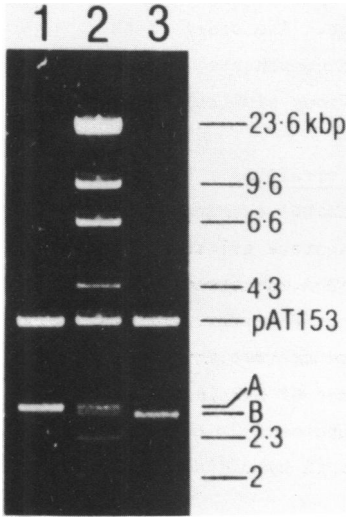

Figure 3

Agarose gel electrophoresis of Eco RI cleaved recombinant plasmids.
(1) pBH 404; (2) a mixture of pBH 404 and pBH 407 (λDNA digested with Hind III is included as marker); (3) pBH 407.

Table 1

Restriction fragments of TGMV DNA. Fragments listed result from digestion of purified insert DNA (i.e. obtained by Eco RI cleavage of recombinant plasmids). Sizes were calculated as a percentage of the unit-genome length. Unit-genome length is the averaged sum of the fragments for each enzyme.

Component A		Component B	
Enzyme	Fragment sizes (bp)	Enzyme	Fragment sizes (bp)
Eco RI	2660	Eco RI	2580
Bam HI	1730,930	Bam HI	1190,1080,310
Xho I	1915,745	Hha I	1160,620,490,310
Acc I	1360,1300	Hind II	1470,670,440
Hha I	1860,505,290	Cla I	2220,360
Hind II	1860,800	Bgl II	1290,1290
Cla I	2180,480	Hinf I	1160,620,490,310
Hinf I	1890,505,270	Hae II	1190,1080,310
Ava II	1915,560,190	Ava II	1445,465,335,335,
Pst I	1675,750,240	Pst I	1700,440,440
Hpa II	1860,480,320	Hpa II	1085,540,385,385,180
No restriction with : Bgl II, Hae II, Sal I, Kpn I, Pvu II, Sma I, Xba I, Sst I, Bcl I, Xma I, Hind III		No restriction with : Sal I, Xho I, Kpn I, Pvu II, Sma I, Xba I, Sst I, Bcl I, Xma I, Acc I, Hind III	

Failure to cleave components A and B with Bcl I and Xba I may be due to methylation of the DNA by bacterial modification enzymes, rather than absence of the recognition sequence for these two restriction endonucleases.

size difference between the two components (see above).

The relative position of restriction sites was determined by digestion with pairs of enzymes after initial Eco RI cleavage. The order of the fragments with respect to the Eco RI sites of both components (arbitrarily chosen as zero points) is shown in Fig. 4. No obvious similarities are observed when the two maps are compared.

Sequence homology between components A, B and viral DNA. Eco RI restricted DNA of plasmids pBH 401 and pBH 405, and DNA extracted from cells infected with TGMV, was electorophoresed on agarose gel and transferred to nitrocellulose paper. Following transfer, the DNA was tested for homology with ^{32}P-pBH 401 (component A) or pBH 405 (component B) insert DNA (Fig. 5). It is evident from these experiments that few sequences are common to both component A and component B, although a small amount of hybridisation is consistently observed. Both inserts comigrated with band 2, the species previously identified as linear ds TGMV DNA, and both hybridised with

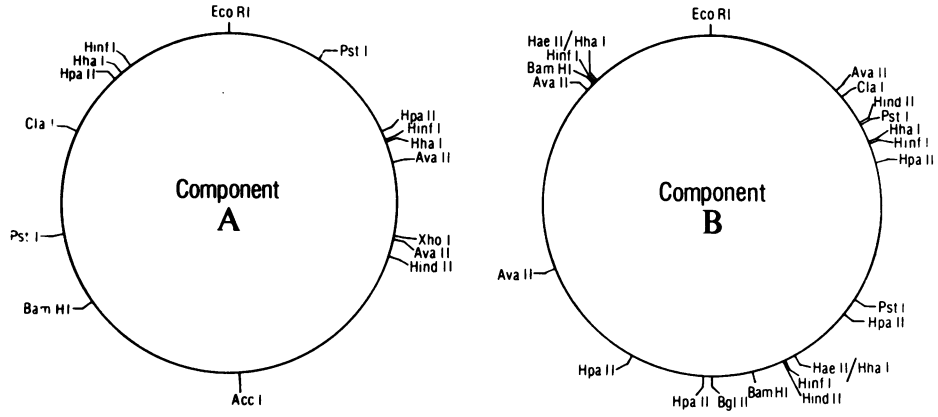

Figure 4

The restriction maps of cloned TGMV components.

the same DNA species previously detected in infected cell extracts using TGMV cDNA probes (3).

Component A and B probes hybridise to roughly the same extent with single-stranded viral DNA (band 4 of the infected cell extract), and the same is true for all other virus-specific DNAs (both single- and double-stranded) present in infected tissue. This is consistent with the relative amounts of each type of recombinant plasmid obtained in the initial cloning experiments from ds circular DNA (47% A, 53% B, see above), and suggests that both components are replicated to the same extent in infected cells. The equivalent hybridisation of both probes with ss viral DNA (band 4) also provides conclusive evidence for the presence of two components in genomic TGMV DNA. No hybridisation with DNA extracts from healthy cells was detected with either probe.

DISCUSSION

The results of experiments presented here demonstrate the successful molecular cloning of both DNA components of TGMV in E. coli. Infectivity studies, which must provide the final proof for complete and faithful amplification are currently in progress.

The two components of TGMV DNA are only slightly different in size, component A being about 75 base pairs longer than B. The restriction maps of these molecules on the other hand are completely different, and Southern blot analysis indicates that A and B have few nucleotide sequences in

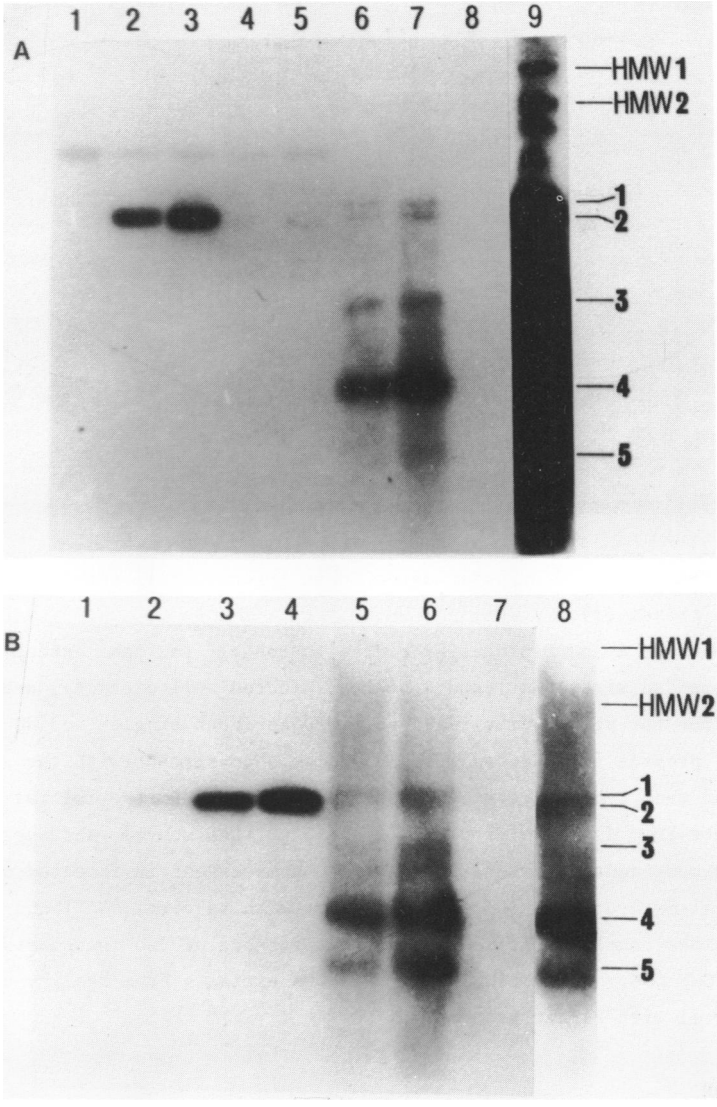

Figure 5

Sequence homology between components A and B. (A) Southern blot probed with ^{32}P-pBH 401 insert DNA (which contained a small amount of contaminating pAT 153 DNA). Plasmid DNAs were restricted with Eco RI. (1) pAT 153 (0.2 µg); (2) pBH 401 (0.2 µg); (3) pBH 401 (0.4 µg); (4) pBH 405 (0.2 µg); (5) pBH 405 (0.4 µg); (6) infected cell DNA (2 µg); (7) infected cell DNA (4 µg); (8) healthy cell DNA (4 µg); (9) longer exposure of lane 7. (B) Southern blot probed with ^{32} P-pBH 405 DNA. (1) pBH 401 (0.2 µg); (2) pBH 401 (0.4 µg); (3) pBH 405 (0.2 µg); (4) pBH 405 (0.4 µg); (5) infected cell DNA (2 µg), (6) infected cell DNA (3 µg); (7) healthy cell DNA (3 µg); (8) longer exposure of lane 6. Bands previously identified in infected cell extracts are indicated.

common. Both components appear to be represented to an equal extent in viral and virus-specific intracellular DNA forms. Together, these results provide strong evidence for a divided viral genome. Such a genomic arrangement has already been postulated for one other geminivirus (2), and may prove to be the case for all members of this group.

The functional significance of a divided genome in this or any plant virus is obscure, although one might speculate that the expression of early genes (i.e. replicative functions) and late genes (i.e. virion proteins or assembly functions) are regulated in this way. It is noteworthy that in plant viruses with bipartite RNA genomes, such as tobacco rattle virus (15) and cowpea mosaic virus (16), independent replication and expression of one RNA component proceeds in the absence of a second component which encodes virus structural protein(s). Perhaps a similar segregation of replicative and structural functions exists between the two DNA components of geminiviruses.

Geminiviruses themselves have considerable potential as vectors for the introduction of foreign genetic material into plants. As a group, they infect a wide range of moncotyledonous and dicotyledonous hosts. They, therefore, have an advantage over other potential vectors for plant genes, such as caulimoviruses (17) and Agrobacterium tumefaciens Ti plasmid (18), which may be limited to dicots. We are currently studying the basic features of TGMV genome organisation in order to construct such a vector.

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