Infection of *Nicotiana benthamiana* with Uncut Cloned Tandem Dimers of Tomato Golden Mosaic Virus DNA

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SUMMARY

Recombinant plasmids containing dimeric inserts of tomato golden mosaic virus (TGMV) DNA component A (pA2), DNA component B (pB2) and both DNA components (pA2B2) were constructed. When inoculated onto *Nicotiana benthamiana* plants, mixtures of uncut pA2 and pB2, or uncut pA2B2 alone, induced symptoms typical of TGMV infection. Infections induced by the uncut dimeric clones were very similar to those induced by the excised monomeric clones, as judged by symptom development, production of capsid protein, immunological reaction with antiserum to TGMV, production of circular double-stranded DNA and single-stranded DNA of genome size, and restriction analysis of supercoiled DNA. Infection with the dimeric DNA clones gave rise to a subgenomic DNA, derived from DNA A, which was not observed in infections with monomeric clones. The infectivity of excised monomeric DNA was shown to be dependent on the cloning site, but the uncut dimeric clones were as infectious as the most infectious monomeric clones.

INTRODUCTION

The geminiviruses are a unique group of plant viruses characterized by their twin icosahedral particles, genomes of circular single-stranded DNA molecules, 2.5 to 3.0 kb in size, and capsids composed of a single polypeptide species, mol. wt. 28000 to 30000 (for reviews, see Stanley, 1985; Harrison, 1985; Lazarowitz, 1987). The genome can be a single component as in the leafhopper-transmitted viruses, maize streak virus (Grimsley et al., 1987) and beet curly top virus (Stanley et al., 1986) or two components as in the whitefly-transmitted viruses, tomato golden mosaic virus (TGMV) (Hamilton et al., 1983), African cassava mosaic virus (ACMV) (synonym: cassava latent virus) (Stanley, 1983) and bean golden mosaic virus (Morinaga et al., 1983).

Double-stranded forms of the two species of TGMV DNA (referred to as the A and B components) are found in extracts of infected plant tissue (Hamilton et al., 1982) and have been cloned into the bacterial plasmid vector pAT153 (Bisaro et al., 1982; Hamilton et al., 1983). Inoculation of *Nicotiana benthamiana* plants with the cloned A and B components together, but not separately, results in systemic infection, but only when the viral DNA components are excised from the recombinant plasmids. Plants inoculated with this excised linear DNA produce the circular forms of the genome indicating that the linear DNA is circularized during the course of infection in the plant (Hamilton et al., 1983).

We report here that plants can be readily infected with mixtures of uncut recombinant plasmids containing tandem dimers of the TGMV A and B components respectively, and with a single uncut recombinant plasmid containing tandem dimers of both A and B components. The infectivity of such dimer clones is compared with that of the monomer dsDNA components cloned and linearized at different sites in the genome.

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METHODS

Plant growth and virus isolation. TGMV was propagated in *N. benthamiana* by inoculating the expanding upper leaves of plants grown to the four-leaf stage. Virus was isolated from infected plants by the procedure of Stein et al. (1983), except that the final 10 to 50% sucrose gradient was omitted. The virus samples were then used for immunodiffusion as described by Stein et al. (1983), or denatured and electrophoresed in 10% SDS–polyacrylamide gels (Laemmli, 1970).

DNA isolation and Southern blot analysis. Crude TGMV was prepared as described by Hamilton et al. (1983). Pellets prepared by ultracentrifugation of extracts from homogenized plants were resuspended in TE buffer (10 mM-Tris–HCl pH 7.5, 1 mM-disodium EDTA) and extracted twice with an equal volume of 25:25:1 (v/v/v) phenol:chloroform:isoamyl alcohol prior to precipitation from 70% ethanol. Alternatively, supercoiled DNA was prepared from the resuspended pellets as described by Sunter et al. (1985).

DNA was electrophoresed in 1.2% agarose gels in TBE buffer (90 mM-Tris–HCl pH 8.2, 90 mM-boric acid, 2 mM-disodium EDTA), depurinated by soaking the gel in 0.25 M-HCl for 15 min and then transferred to Genescreen Plus (New England Nuclear) either in 0.6 M-sodium chloride, 0.4 M-sodium hydroxide, or in 10 × SSC (1.5 M-sodium chloride, 0.15 M-trisodium citrate) without prior depurination. Alkaline blot transfers were neutralized for 15 min by soaking in 0.5 M-Tris-HCl pH 7.5, 2 × SSC and then dried at room temperature. Neutral blots were washed for 15 min in 2 × SSC and dried. In both cases the blots were then prehybridized and hybridized to nick-translated DNA (Rigby et al., 1977) according to the manufacturer’s instructions.

Plasmids and cloning. Plasmids pBH401 and pBH604 containing TGMV DNA A and DNA B cloned into the EcoRI and *Cla*I sites respectively of the vector pAT153 have been described previously (Bisaro et al., 1982; Hamilton et al., 1983). After excision from recombinant plasmids, TGMV DNA inserts were separated from vector molecules by electrophoresis in 1% agarose gels in TBE buffer and eluted according to Dretzen et al. (1981).

DNA manipulations were as described by Maniatis et al. (1982) unless otherwise stated. Plasmid DNA was purified from *Escherichia coli* by the alkaline lysis method (Birnboim & Doly, 1979) and further purified by centrifugation to equilibrium in caesium chloride density gradients containing ethidium bromide. *E. coli* DH5 or DH5α cells (Hanahan, 1985; Jesse, 1986) were used as recipients for transformation with recombinant pUC9 (Vieira & Messing, 1982) or pEMBL9 (Dente et al., 1985) plasmids.

For recircularization of A component DNA, 10 µg of pBH401 was digested with *Eco*RI and then incubated with 10 units of T4 DNA ligase for 16 h at 4°C in 5 ml of 50 mM-Tris–HCl pH 7.6 containing 10 mM-MgCl₂, 1 mM-dithiothreitol and 1 mM-ATP. After precipitation with ethanol and resuspension in TE buffer, the ligation products were electrophoresed through a 1% agarose gel in TBE buffer containing 0.5 µg/ml ethidium bromide and the band with the mobility of supercoiled TGMV DNA (Hamilton et al., 1982) was eluted from the gel and purified on Elutip-D (Schleicher & Schuell) minicolumns according to the manufacturer’s instructions.

A pEMBL/XhoI vector was constructed by digesting pEMBL9 with *Sma*I and inserting *Xho*I linkers (8-mer from New England Biolabs) by linker tailing. The X-gal colour selection (Vieira & Messing, 1982) was restored by digesting the resultant clone with *Pst*I and *Hind*III, treating it with T4 DNA polymerase and religating it.

An A component-specific clone was constructed by ligating the large *Eco*RI–*Xho*I fragment from pBH401 into *Eco*RI–*Sal*I-digested pUC9, and a B-specific clone was constructed by ligating the large *Pst*I–*Cla*I fragment of pBH604 into *Pst*I–*Acc*I-digested pUC9. These clones lacked the 230 bp sequence which is almost identical inDNAs A and B (Fig. 1).

RESULTS

Construction of dimeric and monomeric clones of TGMV DNA components A and B in pEMBL9

TGMV DNA components A and B were excised from recombinant plasmids pBH401 and pBH604 by digestion with *Eco*RI and *Cla*I respectively and separated from the pAT153 vector by gel electrophoresis. The separate components were introduced into pEMBL9 cleaved with *Eco*RI or *Cla*I. When the A or B component DNA was present in a fivefold molar excess during ligation, recombinants were obtained that contained dimeric inserts of DNA A (pA2) or DNA B (pB2) as well as recombinants that contained monomeric inserts of A (pA/*Eco*) or B (pB/*Cla*). Restriction mapping showed that the dimeric inserts were all in tandem.

A recombinant plasmid containing dimers of both A and B components was constructed by excising the B dimer from pB2 by a partial *Cla*I digestion and inserting it into the *Acc*I cloning site in the vector moiety of pA2. Since DNA A contains *Acc*I sites (Fig. 1), this was achieved by first subcloning the excised B dimer by cutting on each side with *Hind*III and *Sma*I and cloning this dimer fragment into pA2 cut with *Hind*III and *Sma*I for which there are no restriction sites in DNA A.
Infection with cloned dimeric geminivirus DNA

A monomeric clone of DNA A at its XhoI site (pA/Xho) was obtained by recircularization and ligation of A component DNA, cutting with XhoI and insertion into the XhoI site of a pEMBL/Xho vector.

Restriction sites in TGMV DNA components A and B, their relationship to open reading frames (ORFs) (Hamilton et al., 1984) and the structures of the recombinant pEMBL plasmids containing A and B dimeric inserts are shown in Fig. 1 and 2 respectively.

Infectivity of uncut dimeric clones of TGMV DNA components A and B

Inoculation of N. benthamiana plants with a mixture of the uncut dimer clones, pA2 and pB2, induced the development of symptoms typical of systemic infection with TGMV (Buck & Coutts, 1985). Similar symptoms were induced when plants were inoculated with the single uncut clone containing dimers of both A and B components (pA2B2). The level of infectivity,
Table 1. Infectivity of dimer and monomer clones of TGMV

<table>
<thead>
<tr>
<th>Inoculum*</th>
<th>Infected/inoculated plants</th>
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<tr>
<td>pA2B2 (5)</td>
<td>17/20</td>
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<tr>
<td>pA2 (5) + pB2 (5)</td>
<td>15/20</td>
</tr>
<tr>
<td>pA2 (5) + pB/Cla (5)</td>
<td>12/20</td>
</tr>
<tr>
<td>pA/Xho (5) + pB/Cla (5)</td>
<td>16/20</td>
</tr>
<tr>
<td>pA/Eco (25) + pB/Cla (5)</td>
<td>5/20</td>
</tr>
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* The dimeric clones pA2B2, pA2 and pB2 were uncut, the monomeric clones were cut with EcoRI (pBH401 and pA/Eco), ClaI (pBH604 and pB/Cla) or XhoI (pA/Xho). Amounts of DNA (μg) are shown in parentheses. The total volume of each inoculum was 50 μl.

measured as the number of plants showing symptoms after 21 days, using 5 μg of pA2B2 was similar to that using 5 μg each of pA2 and pB2. Over half of the plants also became infected when inoculated with mixtures of 5 μg each of pB2 and pA/Xho or pA2 and pB/Cla in which the dimer clone was uncut and the monomer clone was excised with the appropriate restriction endonuclease (Table 1).

Infectivities of the uncut dimeric clones were considerably higher than those of mixtures of the monomer clones EcoRI-cut pBH401 and ClaI-cut pBH604 or EcoRI-cut pA/Eco and ClaI-cut pB/Cla. With these monomer clones a significantly lower proportion of plants was infected using five times as much A component DNA as with the dimer clones. However it is noteworthy that the combination XhoI-cut pA/Xho plus ClaI-cut pB/Cla was as infectious as the uncut dimeric clones (Table 1).

Capsid protein production

Semi-purified preparations of virus were made from plants infected with each of the combinations of plasmids listed in Table 1 and a comparable extract was made from healthy plants. The preparations were denatured by heating with SDS and 2-mercaptoethanol and subjected to electrophoresis through a 10% SDS–polyacrylamide gel. When the gel was stained with Coomassie Brilliant Blue, a polypeptide of the same size as TGMV capsid polypeptide (mol. wt. 28000) was detected in preparations from infected plants but not in preparations from healthy plants (Fig. 3).

The presence of TGMV in the virus preparations was confirmed by gel immunodiffusion. All the samples, except the healthy one, gave a strong precipitin line against a TGMV-specific antiserum of titre 1/256 (Stein et al., 1983).

DNA forms produced during infection

Nucleic acid preparations from tissue infected with pA2B2, pA2 + pB2, pA2 + pB/Cla, pB2 + pA/Xho and pA/Xho + pB/Cla were electrophoresed in an agarose gel, Southern-blotted under alkaline conditions and probed with either A-specific or B-specific clones. All the preparations contained the TGMV-specific DNA species of both components, previously described (Hamilton et al., 1982), including double-stranded (supercoiled, open circular and linear) and single-stranded forms (Fig. 4 a). Blotting of a similar gel under non-denaturing conditions in which only single-stranded DNA binds to the membrane (Stachel et al., 1986) confirmed the nature of the single-stranded DNA species (Fig. 4b), proving that infection with dimeric clones leads to the production of genome-length single-stranded DNA of both components. None of the species detected hybridized with a pEMBL9 probe, indicating that the DNA species arising from the infection contained no vector sequences.

The identity of the supercoiled band in nucleic acid preparations from each type of infection was confirmed by its isolation and purification by caesium chloride density gradient centrifugation (Sunter et al., 1985), followed by gel electrophoresis, alkaline blotting and probing with A and B component-specific clones (Fig. 5). A major band of supercoiled DNA
Infection with cloned dimeric geminivirus DNA

Fig. 3. 10% SDS-polyacrylamide gel of preparations of partially purified virus (lanes 2 to 6) or equivalent material from healthy plants (lane 1), after denaturation by heating with SDS and 2-mercaptoethanol. Lane M contains human gamma globulin (bands a and c, mol. wt. 55000 and 23500 respectively) and lactate dehydrogenase (band b, mol. wt. 36000). Plants were infected with pA/Xho + pB/Cla (lane 2), pA2 + pB/Cla (lane 3), pA/Xho + pB2 (lane 4), pA2 + pB2 (lane 5) and pA2B2 (lane 6); cp, capsid polypeptide.

Fig. 4. Autoradiograph of TGMV DNA forms produced during infection. Total DNA was prepared from resuspended pellets produced by high speed centrifugation of homogenized plant material and electrophoresed on a 1.2% agarose gel. The DNA was transferred to Genescreen Plus in alkali (a) or 10 x SSC (b) and hybridized with 32p-labelled, nick-translated pA/Xho and pB/Cla. Lane 1 contains purified supercoiled DNA. Lanes 2 to 6 contain DNA from plants infected with pA/Xho + pB/Cla, pA2 + pB/Cla, pA/Xho + pB2, pA2 + pB2 and pA2B2 respectively; oc, open circular DNA; lin, linear DNA; sc, supercoiled DNA; ss, single-stranded DNA.

and some open circular double-stranded DNA were clearly detected in each preparation. The supercoiled species were resistant to treatment with exonuclease III, but sensitive to digestion with S1 nuclease (data not shown), as found previously for supercoiled DNA isolated from plants infected with TGMV (Hamilton et al., 1982; Sunter et al., 1985).

Restriction analysis of the supercoiled DNA confirmed its size to be that expected for genome-length TGMV DNA and that there had been no major sequence alterations. Digestion of the DNA isolated from all the infections with restriction enzymes having single sites in DNA A (PvuI, XhoI, HpaI, BstXI, ScaI, AsuII, BamHI, ApaI, SalI, EcoRI and HgiAI) or DNA B
Fig. 5. Autoradiograph of supercoiled DNA prepared from infected plants. Supercoiled DNA was prepared as described in Methods and then electrophoresed on a 1.2% agarose gel. The DNA was transferred to Genescreen Plus and hybridized with (a) A component- or (b) B component-specific nick-translated clones. Lanes 1 to 5 contain DNA from plants infected with pA/Xho+pB/Cla, pA2+pB/Cla, pA/Xho+pB2, pA2+pB2 and pA2B2 respectively; oc, open circular DNA; sc, supercoiled DNA; X, see text.

(PvuI, HpaI, ClaI, AccI, EcoRI, HgiAl, ScaI, NeoI, BglII and NaeI) yielded linear A and B molecules with the same mobility as those produced in the standard pA/Xho plus pB/Cla infections. Digestion with pairs of enzymes, or enzymes with more than one site in a TGMV DNA component, yielded fragments of the size expected from the nucleotide sequences of the cloned A and B components (Hamilton et al., 1984). Selected samples of restriction digests are shown in Fig. 6. The A-specific probe revealed fragments of 1690 and 900bp after BamHI + EcoRI digestion and 1630 and 960 bp after BamHI + XhoI digestion, whereas the B-specific probe detected fragments of 1070 and 1450 bp after BamHI digestion and 2190 and 330 bp after EcoRI + ClaI digestion (the 330 bp fragment was visible on the blot but is not visible on the photograph). Partial products were observed in some digestions, especially those done with ClaI.

**Formation of a TGMV A component subgenomic DNA**

Preparations of supercoiled DNA from plants infected with pA2B2 or pA2 + pB2 were observed to contain an A-specific species (band X) which migrated faster than genomic length supercoiled DNA (Fig. 5a). When supercoiled DNA isolated from plants infected with pA2B2 was digested with EcoRI or XhoI, band X disappeared and was replaced by a more slowly moving species (band Y), corresponding to a linear double-stranded DNA of about 1800 bp (Fig. 6a). Band Y presumably results from restriction endonuclease cleavage of band X, since it was absent from EcoRI or XhoI digests of supercoiled DNA from plants infected with pA/Xho + pB/Cla, which does not contain band X. It is deduced that band X is the supercoiled form of a subgenomic DNA of about 1800 bp derived from DNA A, which was linearized by EcoRI or XhoI to form band Y.

**DISCUSSION**

We have shown that uncut recombinant plasmids containing dimers of TGMV DNA A and B are infectious for *N. benthamiana* plants. The symptoms elicited, the virus capsid produced and the DNA forms present are the same as those produced by infection with the cloned linear double-stranded DNA components excised from recombinant plasmids containing monomeric virus DNA inserts. Although the supercoiled molecules produced by infection with the dimeric
clones were not sequenced, analysis by cleavage with a range of restriction endonucleases could not detect any difference in size or sequence from supercoiled DNA produced by infection with TGMV or the monomeric clones.

It is clear that an efficient mechanism must exist to allow the formation of unit length viral DNA from the dimeric clones soon after their inoculation. Intermolecular recombination, which was invoked to explain the products of infection induced with an intact monomeric clone of ACMV DNA 1 plus an excised monomeric clone of ACMV DNA 2 (Stanley & Townsend,
1986), is unlikely because unlike the infections with the ACMV clones (i) no vector sequences were detected in the progeny DNA and (ii) when the supercoiled molecules were digested with restriction endonucleases none of the fragments hybridized to both A- and B-specific probes. Monomer formation in infections with the TGMV dimeric clones is more likely to result from intramolecular recombination between homologous sequences in the dimer inserts or from a replicative mechanism.

Although the mechanism of geminivirus DNA replication is not known, Rogers et al. (1986) have pointed out the similarity of the sequence TAATATTAC found in the DNA components of all geminiviruses that have been sequenced (MacDowell et al., 1985; Lazarowitz, 1987) to the recognition and cleavage site for the gene A proteins of φX174 and other single-stranded DNA coliphages, which acts as an origin for rolling circle replication (Kornberg, 1982). If geminiviruses replicate in a similar way, the presence of two such origins in a dimeric clone could lead to endonucleolytic cleavage of progeny single-stranded DNA to produce unit-length molecules. In this context it would be interesting to compare the infectivity of partial dimers lacking a second putative replication origin.

Although this is the first report of direct infection of plants with uncut recombinant plasmids containing dimeric inserts of a geminivirus DNA, and indeed the first example of the two components of a virus bipartite genome being combined together to produce a single infectious molecule, geminivirus infections resulting from the introduction of dimeric clones into plant cells using Agrobacterium strains containing Ti plasmid vectors have been described. Rogers et al. (1986) produced transgenic plants containing direct repeats of TGMV A, B and A plus B integrated into the chromosomal DNA and demonstrated the formation and replication of unit-length viral DNA molecules and the production of virus particles (Sunter et al., 1987) in plants transgenic for the A component or for both components, but not in those transgenic for B alone. It was deduced that DNA A carries the information for virus structure and replication and that DNA B is required for virus cell-to-cell transmission. Grimsley et al. (1987) infected maize plants by inoculation with an Agrobacterium strain carrying a Ti plasmid vector containing a dimer of maize streak virus DNA. In this method, termed agroinfection, it is not known whether the dimeric DNA became integrated into the plant chromosomal DNA prior to the formation and replication of unit-length viral DNA molecules. Whatever the method of introducing dimeric geminivirus DNA into plant cells it is likely that the mechanism of monomer formation is similar.

It has been shown that the infectivity of the monomer clone pA/Xho is higher than that of monomer clones pA/Eco and pBH401 (all in conjunction with pB/Cla). This could be because the viral DNA from pA/Eco and pBH401 is linearized at the EcoRI site which lies within ORF A1 (Fig. 1) the sequence of which is highly conserved in diverse geminiviruses (MacDowell et al., 1985) and which is believed to be important for virus DNA replication. Removal of a single nucleotide from the single-stranded tails prior to recircularization could be lethal to the virus. On the other hand, viral DNA from pA/Xho is linearized at the XhoI site which lies within ORF A1 (Fig. 1), the putative TGMV coat protein gene (Hamilton et al., 1984). Alterations within the ACMV coat protein gene do not abolish infectivity (Stanley & Townsend, 1986) and hence alterations around the XhoI site of TGMV may also be tolerated. The cloning site of pB/Cla (ClaI) lies within an intergenic region (Fig. 1) which may not be sensitive to small deletions. Recloning DNA B at other single sites (EcoRI or Bg/II) has not resulted in any increased infectivity (C. L. Brough, unpublished observation). The dimeric clones were as infectious as pA/Xho + pB/Cla and the high infectivity probably resulted from the introduction into plant cells of intact copies of all the virus genes.

The formation of an A-specific subgenomic DNA has not been observed previously in infections with any of the bipartite geminiviruses. The Brazilian isolate of TGMV contains B-specific subgenomic DNAs (MacDowell et al., 1986) and the Kenyan isolate of ACMV contains a subgenomic DNA derived from ACMV DNA 2 (analogous to TGMV B) (Stanley & Townsend, 1985). No subgenomic DNA species have been observed by direct infection of plants with cloned monomeric components of TGMV (Hamilton et al., 1983) or ACMV (Stanley & Townsend, 1985), although B-specific subgenomic species were detected after infection of plants
with the progeny of infection with cloned TGMV DNA (MacDowell et al., 1986). The formation of A-specific subgenomic DNA species may be an event specific to infection with dimeric clones.

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