Nucleic Acids Research, Vol. 18, No. 8 2001

Selection for wild type size derivatives of tomato golden mosaic virus during systemic infection

Scott Elmer* and Stephen G.Rogers
Plant Molecular Biology, Corporate Research Laboratories, Monsanto Co., AA2G, 700 Chesterfield Village Parkway, St Louis, MO 63198, USA

Received January 23, 1990; Revised and Accepted March 7, 1990

ABSTRACT

A chimeric tomato golden mosaic virus (TGMV) A component DNA, which results from replacement of the coding region of the viral coat protein gene (CP) with the larger bacterial beta-glucuronidase coding sequence (GUS), can replicate in agroinoculated leaf discs but is unstable in systemically infected plants (1). We have made similar replacements of the TGMV CP gene with the GUS coding sequence in both the sense and antisense orientations. Both derivatives replicated in leaf discs inoculated via *Agrobacterium*. However, systemic movement of the GUS substituted vectors was not detected in agroinoculated *Nicotiana benthamiana* plants. The only TGMV A derivatives detected in systemically infected leaves of inoculated plants were similar in size to the wild type viral component. Sequence analysis of derivatives from six independently inoculated plants revealed that they did not result from internal deletions of the larger replicons detected in leaf discs but, instead, were generated by fusion events occuring within the original T-DNA insert. These results indicate that systemic movement of TGMV in *N. benthamiana* plants provides a strong selective pressure favoring viral derivatives similar in size to the wild type virus components.

INTRODUCTION

Geminiviruses are plant viruses characterized by their twinned particle morphology and their genomes, which may consist of one or two circular single-stranded (ss) DNA molecules from 2.5−3.0 kb in size. Geminivirus DNAs also exist as double-stranded (ds) forms which probably function as replicative intermediates and templates for transcription. The dsDNA forms of several one and two genome component geminiviruses have been cloned. The cloned geminivirus DNAs are infectious upon reintroduction into host plants by mechanical inoculation of free DNA and by inoculation of *Agrobacterium* containing the cloned viral genomes in their T-DNA [agroinoculation; (2,3)].

The availability of cloned, infectious viral DNA has facilitated functional analysis of geminivirus genomes (4). For the two component geminivirus tomato golden mosaic virus (TGMV), both the A and B viral components are required for infectivity (5). However, the A component of TGMV can replicate autonomously in plant cells in the absence of the B component (6). Further analysis of TGMV and African cassava mosaic virus (ACMV) revealed that the viral coat protein (CP) is not required for replication or systemic spread of these two component geminiviruses (7,8). For TGMV, a derivative of the A viral component with most of the CP coding region deleted can replicate and systemically spread when inoculated with the TGMV B genome component (7).

These discoveries provided the foundation for the construction of geminiviruses vectors engineered to express foreign genes in plants or cultured plant cells. For several geminiviruses, functional vectors have been constructed by replacement of the CP coding region with various foreign genes. Derivatives of the ACMV 1 component (analogous to the TGMV A component), in which the CP coding sequence was replaced by coding sequences of similar size, can replicate in plant cells and express the substituted protein (9). Similarly, vectors containing the chloramphenicol acetyl transferase (CAT) or hygromycin phosphotransferase (HPT) coding sequences in place of the coat protein coding sequence of maize streak virus, a single genome component geminivirus, can replicate in maize cells (10). Recently Hayes et al. (1) showed that a derivative of the TGMV A component, with the CP coding sequence replaced by a 1.2 kb larger sequence encoding bacterial beta-glucuronidase (GUS), can replicate in transgenic plants, express GUS, and move systemically. However, these studies also showed that the larger GUS replacement vector exhibits instability in agroinoculated plants.

In the analyses reported here, we have examined the stability of geminivirus vectors larger than the wild type viral component in greater detail. TGMV A derived vectors in which the coat protein coding sequence has been replaced by the GUS coding sequence in both orientations are described. We show that these vectors can replicate in plant cells but exhibit instability during systemic movement. Agroinoculation of plants with the TGMV-GUS vectors resulted in the accumulation of TGMV A derivatives similar in size to the wild type viral component in systemically infected tissue. Implications regarding geminivirus vector instability and agroinoculation are discussed.

* To whom correspondence should be addressed

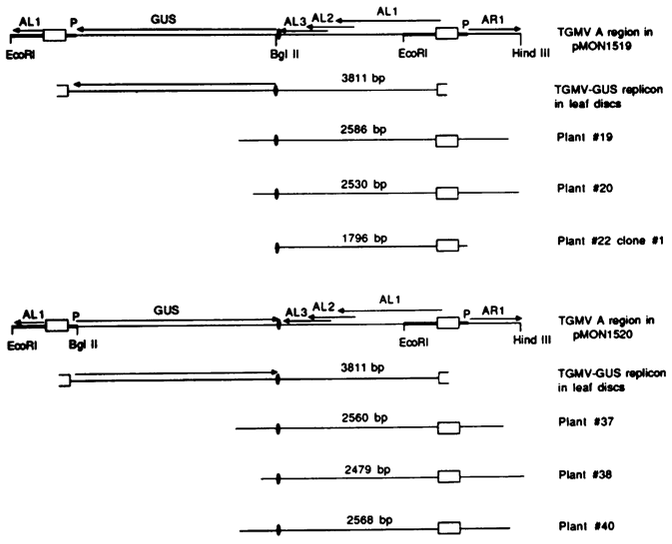

Figure 1. Diagram of pMON1519 or pMON1520 and the replicons derived from them. TGMV A regions in the binary pMON1519 and pMON1520 plasmids are shown. Replicons identified in agroinoculated leaf discs or individual infected plants are listed at right and shown in linearized form directly below the region from which they are derived. The size of each replicon as estimated from sequence information and electrophoretic mobility is given. P represents the AR1 promoter, the boxes represent the common region of TGMV, and the filled oval represents the bidirectional polyadenylation signal. Repeated TGMV A sequences (including the common region) in pMON1519 and pMON1520 are shown as bold lines. Open reading frames AR1, AL1, AL2, AL3, and GUS and their orientations are shown by the arrows; restriction sites relevant to cloning and analysis are given.

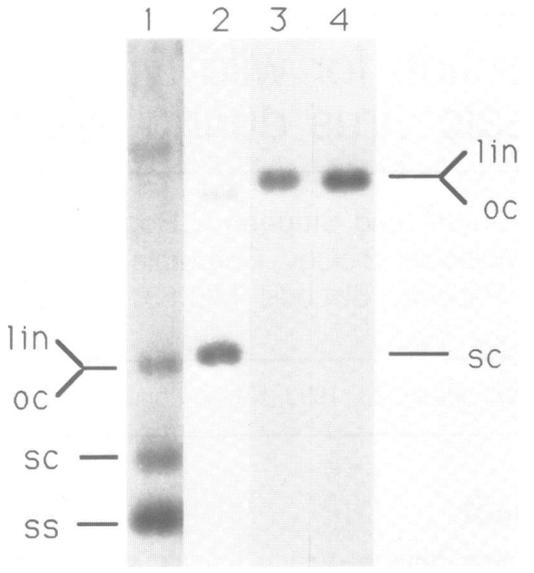

Figure 2. Southern analysis of inoculated leaf discs. Total DNA isolated from petunia leaf discs 4 days after agroinoculation with pMON337 (lane 1), pMON1520 (lanes 2–3), and pMON1519 (lane 4) were analyzed by southern hybridisation. The DNAs in lanes 3 and 4 were digested with Bgl-II. Lane 1 was hybridised with a probe specific for the TGMV-A component. Lanes 2–4 were hybridised with a probe specific for the GUS gene. Single stranded (ss) DNA and the linear (lin), open circular (oc), and supercoil (sc) forms of double stranded viral DNA are designated on the left margin for lane 1 and on the right margin for lanes 2–4.

MATERIALS AND METHODS

Plasmids pMON1519 and pMON1520 were constructed by insertion of a 1.9 kb BglII-BamHI fragment containing the GUS coding sequence (11) into the BglII site of pMON458, a TGMV coat protein gene (CP) deletion construct (7). The vectors contain the GUS coding sequence in the sense (pMON1520) or antisense (pMON1519) orientation relative to the CP promoter (Fig. 1). A BglII site is retained at the CP promoter-GUS junction of pMON1520 and at the GUS-TGMV poly A junction of pMON1519. These vectors are analogous to pMON337 (12) and carry tandemly repeated sequences of the TGMV A component flanking a single copy of the substituted coding sequence.

The TGMV A derivatives were tested in infectivity assays using the transgenic *Nicotiana benthamiana* line 3427 (13), which contains three tandem copies of the TGMV B component. Inoculation was performed by gently rubbing 20 μl of a saturated overnight culture of *Agrobacterium tumefaciens* containing the desired binary Ti plasmid vector onto two carborundum treated leaves of a 3–5 week old host plant. Inoculated plants were monitored for the timing and severity of viral symptoms. Leaf discs from Mitchell diploid petunia (*Petunia hybrida*) plants were inoculated with *Agrobacterium* containing the appropriate binary Ti plasmid vector and assayed for the presence of viral replicons as previously reported (12).

Plant DNA isolation, radio-labelling of DNA probes and southern hybridizations were performed as described by Rogers et al. (6). Total plant DNA (ca. 10 μg) from infected tissue or inoculated leaf discs was digested and separated by electrophoresis through a 1% agarose gel for 16 hours at 30 volts prior to Southern analysis. Restriction endonucleases were used as recommended by the suppliers. Mung bean nuclease digestions were performed under conditions described by the supplier (Pharmacia-PL, Piscataway,NJ) using 10 units of enzyme for 15 minutes at 37°C to digest 10 μg of total leaf DNA. Blots were hybridized to probes specific to either the TGMV A component or the GUS coding sequence. The TGMV A specific probe was a 1460 bp ScaI-EcoRI fragment derived from pMON349 (6). The GUS probe consisted of the entire coding sequence.

Inoculated leaf discs and plants were assayed for GUS activity by the 5-bromo-4-chloro-3-indolyl-B-glucuronide (X-gluc) histochemical staining assay (14). Tissue was incubated with X-gluc solution for 2 hours at 37°C and scored for the development of blue stain.

Polymerase chain reactions (PCR) were performed using a kit supplied by Perkin-Elmer. Total DNA (1 μg) from plants inoculated with *Agrobacterium* containing pMON1519 or pMON1520 and 40 pmoles of each primer were used in a reaction. The 20 nucleotide primers hybridize to sequences encompassing either the EcoR1 site in the TGMV AL1 open reading frame (ORF) at position 2263 or the BAMH1 site in the TGMV AL2,3 ORFs at position 1357 (15). The primers were designed to amplify the region between these sites that contains the TGMV A common region. Reactions were repeated for 28 cycles with each cycle consisting of denaturation at 90°C for 1 minute, hybridization at 60°C for 2 minutes, and polymerization at 72°C for 3 minutes. Following amplification, each PCR reaction was extracted once each with chloroform and phenol, precipitated by 2M potassium acetate and one volume of isopropanol, and resuspended in 50 μl water. The PCR products were then digested with EcoRI and BamHI and cloned into pUC119; isolated clones were sequenced by the dideoxy method using single-stranded DNA templates (16). Single-stranded templates were also generated in PCR reactions as described above except that reactions were initiated with approximately 100

ng of a double stranded PCR product and contained only 1 pmole of the primer covering the EcoR1 site. The single-stranded PCR products were separated from the primers by low speed centrifugation through a centricon 30 membrane (Amicon) and sequenced directly by the dideoxy method.

RESULTS

TGMV-GUS vectors replicate in leaf discs and develop attenuated symptoms in inoculated plants

Petunia leaf discs were inoculated with *Agrobacterium* cultures containing either pMON337, pMON1519, or pMON1520. Freely replicating TGMV A component derivatives of the ds DNA form were detected by southern hybridization in the leaf discs 4 days after agroinoculation (Fig. 2, lanes 2–4). Restriction analysis of the TGMV-GUS replicons indicated that they are the size expected for plant plasmids released by homologous recombination from their respective *Agrobacterium* constructs (Fig. 2, lanes 3 and 4). The absence of detectable ss DNA in the autoradiogram most likely reflects variability in recovery and detection of this form in DNA prepared from leaf discs.

Transgenic *N. benthamiana* plants containing tandemly integrated copies of the TGMV B component were inoculated with *Agrobacterium* carrying pMON1519, pMON1520, or pMON337 and monitored for the appearance of symptoms. All 12 plants inoculated with pMON1519 or pMON1520 (6 with each construct) developed attenuated symptoms after 22–26 days. In contrast, all 6 plants agroinoculated with pMON337, which contains the wild type TGMV A component, developed normal viral symptoms within 10 days after agroinoculation. The development of normal viral symptoms after agroinoculation with pMON337 typically occurs within 7–18 days (13).

Accumulation of wild type size replicons in symptomatic tissue

Leaves from systemically infected plants inoculated with pMON1520, which contain the GUS gene replacement of the TGMV CP gene in the sense orientation, were assayed for GUS expression by histochemical staining with X-gluc. No GUS activity was detected in the systemically infected leaves even though it was readily detected in primary inoculated leaves 2 days after inoculation (data not shown). As expected, no GUS activity was detected in either inoculated or systemically infected leaves from plants inoculated with pMON1519, which contains the GUS gene replacement of the TGMV CP gene in the antisense orientation.

Southern blot analysis revealed that systemically infected tissue from pMON1519 and pMON1520 inoculated plants did not contain the 3.8 kb TGMV A derivatives that were released from the same constructs in leaf discs. Instead, replicons almost identical in size to the normal TGMV A component (2.6 kb) were detected (Fig. 3). Longer exposure revealed bands in lanes 2 and 3 that comigrated with the ss form of the normal TGMV-A component and were sensitive to mung bean nuclease digestion (data not shown).

The ds TGMV A derivatives in three plants inoculated with pMON1519 and three plants inoculated with pMON1520 were characterized by digestion with BglII. Both pMON1519 and pMON1520 contain a single BglII site, but at different locations relative to viral sequences. The BglII site in pMON1519 is at the viral polyadenylation site/GUS junction while the BglII site in pMON1520 is at the CP promoter/GUS junction (Fig.1). Replicons present in pMON1519 inoculated plants were digested with BglII (Fig. 4, lanes1–3). In contrast, replicons present in pMON1520 inoculated plants were not sensitive to BglII digestion (Fig. 4, lanes 4–9). The small amount of open circular or linear DNA seen in lanes 4–6 was also present in undigested samples in lanes 7–9. These results indicate that the BglII site is retained

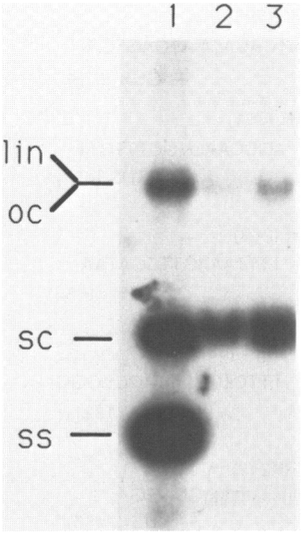

Figure 3. Southern analysis of infected plants. Total DNA isolated from pooled symptomatic tissue of transgenic *N. benthamiana* plants containing tandem integrated inserts of the TGMV B component agroinoculated with pMON337 (lane 1), pMON1519 (lane 2), or pMON1520 (lane 3) were analyzed by southern hybridisation. All lanes were hybridised with a probe specific for the TGMV A component. Single stranded (ss) DNA and the linear (lin), open circular (oc), and supercoil (sc) forms of double stranded viral DNA are designated on the left margin.

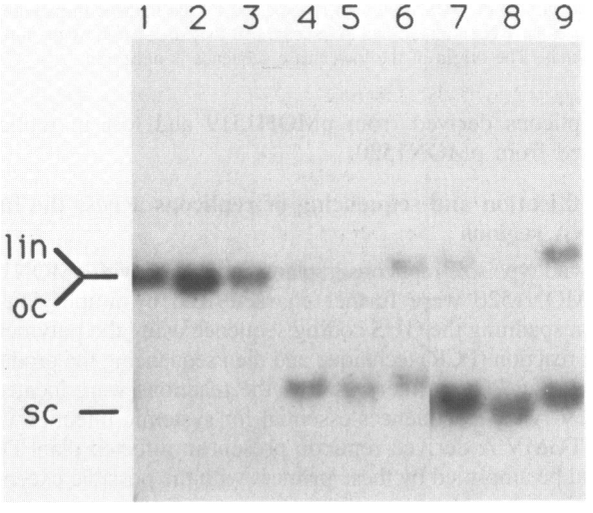

Figure 4. TGMV A derivatives in infected tissue digested with Bgl II. Total DNA from symptomatic tissue of individual transgenic *N. benthamiana* plants containing tandem integrated inserts of the TGMV B component agroinoculated with pMON1519 (lanes 1–3) or pMON1520 (lanes 4–9) were analyzed by southern hybridisation. The DNAs in lanes 1–6 were digested with Bgl II. The DNAs in lanes 1–6 are from plant # 19, 20, 22, 37, 38, and 40, respectively. Undigested DNAs corresponding to lanes 4–6 were analyzed in lanes 7–9. The blot was hybridised to a probe specific to the TGMV A component. The linear (lin), open circular (oc), and supercoil (sc) forms of double stranded viral DNA are designated on the left margin.

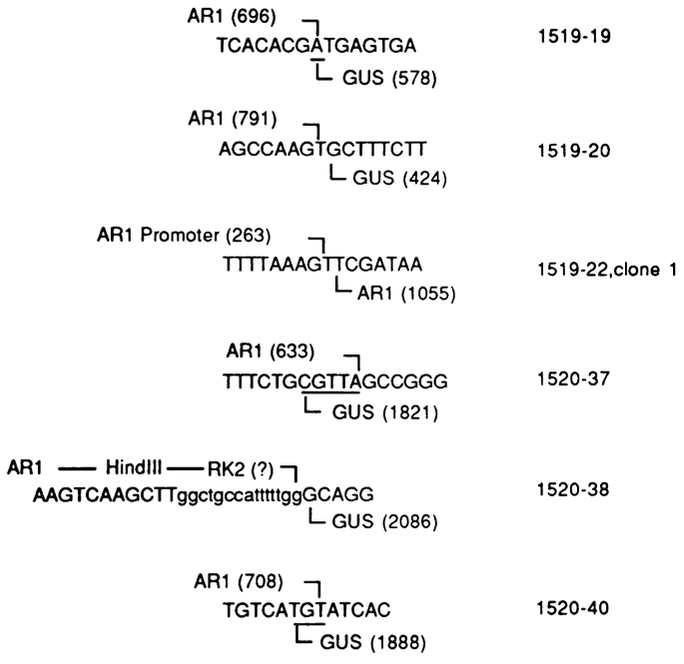

Figure 5. Fused regions in replicons from infected plants. Sequences flanking the fusion of distal T-DNA regions in replicons present in pMON1519 and pMON1520 inoculated plants are shown. The numbers at right designate the inoculated construct and the plant from which the replicon was isolated. The position of each sequence relative to published sequence of the TGMV A component (15) or GUS gene (23) is indicated by the number in parentheses. Sequences have been underlined to indicate that they are shared by the fused T-DNA ends. The origin of the lower case sequence is uncertain.

in replicons derived from pMON1519 and lost in replicons derived from pMON1520.

Amplification and sequencing of replicons across the fused T-DNA regions

The wild type size replicons in plants inoculated with pMON1519 or pMON1520 were further characterized by amplifying the region spanning the GUS coding sequence using the polymerase chain reaction (PCR) technique and then sequencing the products. Both of the PCR primers used in the reactions were located in TGMV A ORF sequences essential for systemic infection (12). Any TGMV A derived replicon present in infected plant DNA should be amplified by these primers with the possible exception of replicons with defects in the primer binding regions of these ORFs. Such defective replicons would be dependent upon the presence of derivatives with intact ORFs to complement their deficiencies in *trans* (17).

DNAs isolated from the six plants shown previously (Fig. 3) to contain GUS derived replicons were amplified by PCR. The major PCR products from each of these plants were 1.6−1.7 kb, as expected for replicons similar in size to the wild type TGMV A component. A single clone from the PCR product of each plant was sequenced except for plant 22. In this case, two separate subclones which differed significantly in size were sequenced. In addition, a single-stranded PCR product was synthesized from plant 19 DNA and sequenced directly to determine whether the PCR product was homogeneous or heterogeneous. A Homogeneous PCR product indicates amplification of a population of identical replicons while a heterogeneous PCR product indicates amplification of a mixed population of unique replicons of the same size.

The sequencing results revealed that six of the seven replicons derived from pMON1519 or pMON1520 infected plants contain a single fusion between sequences in the T-DNA (Fig. 5). The fused sequences were different for every replicon. Heterogeneity within a population of replicons from the same plant was also seen in the case of plant 22 inoculated with pMON1519 (Fig. 5). The replicon sequenced from plant 38 contains a fusion of TGMV CP sequences with 14 bp of unknown sequence upstream of GUS sequences. If this replicon is the result of a single fusion event, the unknown sequence would be from the RK2 region adjacent to the TGMV CP ORF in pMON1520.

One of the replicons from the pMON1519 inoculated plant 22 appears to be the product of multiple fusion events. It contains TGMV CP promoter DNA fused at TGMV A nucleotide 259 with 39 bp of unknown sequence. This unknown sequence is then fused to 251 bp of DNA derived from the nopaline synthase gene (NOS; nucleotides −264 to −14 of the published sequence (18)) in pMON1519. The NOS gene region is then fused to another unknown sequence. This region is shown below with the endpoints of the known sequences in bold type:

```
TGMV-CP-----|------------------unknown--------------
CTAAGATATTTT T CATCGATGAT AAGCTGTCAA ACATGAGAAT

-----------|----------NOS(251bp)--------|--unknown--
TATTCCCCGGATCATGAGCG ..... AAATTCCCCTGGAACGAGTGT
```

A second replicon derived from plant 22 contains a fusion of TGMV CP promoter DNA to TGMV sequences near the bidirectional polyadenylation site. This replicon is only 1.8 kb in size based on both sequence analysis and the size of the cloned PCR product spanning the fused region. Only this sequenced replicon could have arisen by deletion of the full size TGMV-GUS replicon present in leaf discs agroinoculated with pMON1519 (Fig. 1). The absence of a detectable band analogous to this smaller replicon by southern analysis (Fig. 4, lane 3) suggests that it represents a very small proportion of the total population of TGMV A derived replicons in plant 22. This replicon was probably detected by PCR because of the kinetics of the polymerase chain reaction which results in amplification of smaller products to a greater extent than larger ones.

The sequence determined directly from the single-stranded PCR product from plant 19 was identical to that obtained from the cloned double stranded PCR product. The predominance of a single sequence obtained directly from the PCR product indicates that the majority, if not all, of the ss PCR product is identical. Since any TGMV A derived replicon present in plant 19 would be amplified by the PCR reaction performed, uniformity of the ss PCR product indicates a corresponding uniform population of TGMV A derived replicons in plant 19.

DISCUSSION

The pMON1519 and pMON1520 *Agrobacterium* vectors used in these experiments were designed to allow release and replication of 3.8 kb TGMV-GUS replicons in inoculated plant cells. A single copy of the TGMV A component with the GUS coding sequence in place of the CP coding sequence can be released by homologous recombination between TGMV A sequences from the pMON1519 or pMON1520 T-DNA. Release may also occur by a postulated replicative mechanism involving

the two TGMV common regions (6). The T-DNA regions from both of these constructs containing the relevant TGMV A and GUS sequences are shown in Fig. 1. The TGMV A replicons released from these constructs by homologous recombination are also shown in Fig. 1. We detected the release and replication of these replicons in inoculated petunia leaf discs in agreement with the results reported by Hayes et al. (1). Our earlier report of high levels of GUS transcripts in *N. benthamiana* leaf discs agroinoculated with pMON1520 indicate that TGMV-GUS vectors also replicate in this species (19).

In contrast to the inoculated leaf discs, systemically infected leaves of *N. benthamiana* plants agroinoculated with pMON1519 or pMON1520 contained TGMV A derivatives that were similar in size (ca. 2.6 kb) to the wild type component. Replicons of this size were not predicted to arise from either construct via homologous recombination or replicative release. The intact 3.8 kb TGMV-GUS replicon was not detected in systemically infected tissue either by southern analysis of pMON1519 and pMON1520 inoculated plants or by histochemical staining for GUS activity in pMON1520 inoculated plants.

Further characterization of these wild type size replicons indicated that they are the products of nonspecific fusion events occuring within the T-DNA transferred from the *Agrobacterium* to the plant cell during agroinoculation. Sequencing across fused regions of these replicons after PCR amplification revealed that all but one contain a single junction of distal T-DNA regions. In each case, the fusion results in the release of a wild type size replicon containing all of the viral sequences and ORF's encoded by the TGMV A component essential for replication and systemic spread (12) and additional sequences adjacent to these on the T-DNA (Fig. 1).

Although replicons from different plants are similar in size and are derived from the same region of the T-DNA, each contains distinct junction sequences (Fig. 5). This diversity indicates that these replicons are generated through a nonspecific mechanism with respect to the particular sequences that are fused. Such a mechanism may involve illegitimate recombination or attenuated viral replication as postulated for the generation of subgenomic geminivirus components that contain similar fusions (20–22).

The events which generate these wild type size replicons occur at a low frequency in inoculated plants. The absence of detectable replicons other than the 3.8 kb TGMV-GUS vector in leaf discs agroinoculated with pMON1519 or pMON1520 indicates that wild type size replicons are rarely generated in primary inoculated cells. These replicons must arise in primary inoculated cells since the T-DNA from which they are derived is present only in these cells and does not move systemically. The long delay in symptom appearance after agroinoculation of plants with pMON1519 or pMON1520 also supports the hypothesis that the wild type size replicons which systemically infect these plants are generated from rare events. Finally, if the fusion events that generate wild type size replicons from the T-DNA occur frequently, individual inoculated plants should contain a variety of wild type size replicons similar to the variety observed among replicons isolated from different plants. Instead, the uniform sequence obtained from a PCR amplified population of replicons in plant 19 suggests that the majority (or all) of the replicons in an individual infected plant are identical and most likely arise from a single event.

Our results also indicate that wild type size replicons are enriched during systemic movement because of their size. The same rare fusion events that produce wild type size replicons should also give rise to larger and smaller TGMV A derivatives capable of systemic movement (1,7). However, the predominance of wild type sized replicons in systemically infected tissue suggests that they are selected preferentially. The presence of a 1.8 kb replicon as a minor species in the systemically infected tissue of a single pMON1519 inoculated plant suggests that derivatives of various sizes are generated but do not compete efficiently with wild type sized derivatives during systemic infection. The hypothesis of size selection of geminivirus derivatives during infection is supported by results which show that systemic infection of ACMV 1 derivatives carrying large deletions (0.7 kb) of the coat protein coding sequence occurs only after they are restored to wild type size by incorporation of foreign sequences via illegitimate recombination (8). Taken together these results suggest that the wild type geminivirus components represent an optimum size for systemic movement.

Preferential accumulation of wild type sized replicons in systemically infected tissue was not observed in a similar experiment reported by Hayes et al. (1). They detected TGMV derivatives ranging in size from 2.6 kb to 3.8 kb (the full length TGMV GUS replicon) in systemically infected leaves of individual *N. tabacum* cv Samsun plants which were stem injected with an *Agrobacterium* vector similar to pMON1520. These results may be due to the different host species used in these two studies. *N. benthamiana* plants may exert a stronger selection for wild type size TGMV A replicons or totally restrict the movement of larger derivatives. Alternatively, these results may reflect a difference in the frequency with which wild type size replicons are generated in inoculated plants. It is possible that the derivatives which accumulate in systemically infected tissue represent those closest to the wild type component in size that are generated in the inoculated cells of each individual plant. This interpretation would suggest that wild type size derivatives were generated at a lower frequency in the experiments reported by Hayes et al. (1) as compared to those reported here. Differences in the inoculation procedure, the host plant, and the constructs might lead to variability in the generation of wild type size replicons through rare fusion events.

The majority, possibly all, of the replicons in infected tissue were derived from the original input T-DNA and not from the 3.8 kb TGMV-GUS vector present as a high copy replicon in inoculated discs. This was unexpected because geminivirus derivatives generated by deletion of larger replicons have been previously reported (20–22) and were also expected to result from the TGMV-GUS replicon. It is possible that wild type size derivatives of the TGMV-GUS replicon simply are not generated, or they may be generated but cannot compete with T-DNA derivatives.

The presence of TGMV CP sequences in 5 of the 7 sequenced replicons suggests that this region may be important in the generation of TGMV A derivatives or their selection during systemic infection. These sequences, while not required for systemic infection (7), may contain cis acting elements which confer a selective advantage on the replicons that contain them. Alternatively, the presence of these sequences may increase the frequency of the fusion events that give rise to wild type size TGMV A derivatives. In this regard, it is interesting that in the similar experiment reported by Hayes et al. (1), the *Agrobacterium* vector did not contain these TGMV CP sequences.

Our agroinoculation results indicate that systemic infection of TGMV A derivatives exerts a strong selective pressure in favor of replicons similar in size to the wild type viral component. This selective pressure allows TGMV A derivatives, which arise from *Agrobacterium* vectors through rare events, to accumulate during systemic infection to the exclusion of the larger replicons that

the vectors were designed to release. Comparison of these results with those reported by Hayes et al. (1) suggests that a number of factors such as the host plant, inoculation procedure, and vector construct may affect the generation or selective accumulation of rare TGMV derivatives in inoculated plants. Further investigation of these factors may allow the development of more stable geminivirus vectors in the future.

ACKNOWLEDGEMENTS

We thank Nancy Mathis for constructing the *N. benthamiana* line 3427 used in this study. We also thank Linda Hanley-Bowdoin for her critical reading of this manuscript.

REFERENCES

1. Hayes, R.J., Coutts, R.H.A. and Buck, K.W. (1989) Nucleic Acids Res. 17, 2391–2403.
2. Grimsley, N., Hohn, T., Davies, J.W. and Hohn, B. (1987) Nature 325, 1 77–179.
3. Stanley, J. and Gay, M.R. (1983) Nature 301, 260–262.
4. Davies, J.W. and Stanley, J. (1989) TIG 5, 77–81.
5. Hamilton, W.D.O., Bisaro, D.M., Coutts, R.H.A. and Buck, K.W. (1983) Nucleic Acids Res. 11, 7387–7396.
6. Rogers, S.G., Bisaro, D.M., Horsch, R.B., Fraley, R.T., Hoffman, N.L., Brand, L., Elmer, J.S. and Lloyd, A.M. (1986) Cell 45, 593–600.
7. Gardiner, W.E., Sunter, G., Brand, L., Elmer, J.S., Rogers, S.G. and Bisaro, D.M. (1988) EMBO J. 7, 899–904.
8. Etessami, P., Watts, J. and Stanley, J. (1989) J. Gen. Virol. 70, 277–289.
9. Hayes, R.J., Petty, I.T.D., Coutts, R.H.A. and Buck, K.W. (1988) Nature 334, 179–182.
10. Lazarowitz, S.G., Pinder, A.J., Damsteegt, V.D. and Rogers, S.G. (1989) EMBO J. 8, 1023–1032.
11. Hyde-DeRuyscher, R. and Carmichael, G.C. (1988) Proc. Natl. Acad. Sci. USA 85, 8993–8997.
12. Elmer, J.S., Brand, L., Sunter, G., Gardiner, W.E., Bisaro, D.M. and Rogers, S.G. (1988) Nucleic Acids Res. 16, 7043–7060.
13. Elmer, J.S., Sunter, G., Gardiner, W.E., Brand, L., Browning, C.K., Bisaro, D.M. and Rogers, S.G. (1988) Plant Mol. Biol. 10, 225–234.
14. Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) EMBO J. 6, 3901–3907.
15. Hamilton, W.D.O., Stein, V.E., Coutts, R.H.A. and Buck, K.W. (1984) EMBO J. 3, 2197–2205.
16. Sanger, F.S., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
17. Hanley-Bowdoin, L., Elmer, J.S. and Rogers, S.G. (1989) Plant Cell 1, 1057–1067.
18. Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman, H.M. (1982) J. Mol. Appl. Genet. 6, 561–573.
19. Hanley-Bowdoin, L., Elmer, J.S. and Rogers, S.G. (1988) Nucleic Acids Res.16, 10511–10528.
20. Macdonald, H., Coutts, R.H.A. and Buck, K.W. (1988) J. Gen. Virol. 69, 1339–1344.
21. MacDowell, S.W., Coutts, R.H.A. and Buck, K.W. (1986) Nucleic Acids Res.14, 7967–7984.
22. Stanley, J. and Townsend, R. (1985) Nucleic Acids Res. 13, 2189–2206.
23. Jefferson, R.A., Burgess, S.M. and Hirsh, D. (1986) Proc. Natl. Acad. Sci. USA 83, 8447–8451.