Genetic analysis of tomato golden mosaic virus: the coat protein is not required for systemic spread or symptom development

William E. Gardiner, Garry Sunter,
Leslie Brand¹, J. Scott Elmer¹,
Stephen G. Rogers¹ and David M. Bisaro

Department of Molecular Genetics and Ohio State Biotechnology Center, Rightmire Hall, Ohio State University, Columbus, OH 43210 and ¹Plant Molecular Biology, Biological Sciences, Monsanto Company, St Louis, MO 63198, USA

Communicated by J. Schell

The geminiviruses are a unique group of higher plant viruses that are composed of twin isometric particles which contain circular, single-stranded DNA. Tomato golden mosaic virus (TGMV), a whitefly-transmitted agent, belongs to the subgroup of geminiviruses whose members possess a bipartite genome. The TGMV A genome component has the capacity to encode at least four proteins. One of these is the viral coat protein, as inferred by homology with coat-protein genes of other geminiviruses and by the observation of typical geminate particles in transgenic plants that contain inserts of TGMV A DNA. We have investigated the role of the coat protein in TGMV replication and report here that its coding sequence may be interrupted or substantially deleted without loss of infectivity. However, certain coat-protein mutants showed reproducible delays in time of symptom appearance as well as reduced symptom development, when inoculated onto transgenic *Nicotiana benthamiana* plants containing the TGMV B component. The most attenuated symptoms were seen with a mutant in which the coat-protein coding sequence was almost entirely deleted. The significance of these findings for the development of plant vectors from TGMV DNA is discussed.

Key words: geminivirus/Ti plasmid/agroinfection/plant vectors

Introduction

The geminiviruses are unique among eukaryotic viruses in having circular, single-stranded DNA genomes packaged within small, twin isometric particles (for review see Harrison, 1985; Stanley, 1985; Lazarowitz, 1987). One subgroup of the geminiviruses includes cassava latent virus (CLV), bean golden mosaic virus (BGMV) and tomato golden mosaic virus (TGMV), which are all whitefly-transmitted and have genomes divided between two DNA molecules of nearly identical size. Much of our knowledge of geminivirus molecular biology derives from this subgroup because its members are mechanically transmissible and their cloned genome components have been shown, in combination, to be infectious (Hamilton *et al.*, 1983; Morinaga *et al.*, 1983; Stanley, 1983). The nucleotide sequences of CLV (Stanley and Gay, 1983), TGMV (Hamilton *et al.*, 1984)

and BGMV (Howarth *et al.*, 1985) have been determined. Computer analysis of these sequence data indicates that the two genome components of each virus could encode a total of six proteins, identified as open reading frames (ORFs) that share substantial homology between the three viruses. In TGMV, four of the ORFs reside on the A genome component. Three of these are in the leftward direction (ORFs AL-1, AL-2 and AL-3) and one (ORF AR-1) is in the rightward direction (virion sense). The two remaining ORFs are located on the B component, one in the leftward (BL-1) and the other in the rightward (BR-1) direction. The function of only one of these six proteins has been determined. Amino acid analysis of isolated CLV coat protein (Stanley and Gay, 1983) and *in vitro* translation experiments (Townsend *et al.*, 1985) have shown that the rightward 30.2-kd ORF of CLV-1 DNA (equivalent to TGMV ORF AR-1) encodes the CLV capsid protein. Phenotypic mixing experiments have provided further support for the location of the coat protein cistron on CLV DNA-1 (Stanley *et al.*, 1985), while analysis of transgenic plants containing the equivalent TGMV A DNA (Rogers *et al.*, 1986) has shown that the products of this genome component are sufficient to encapsidate single-stranded DNA into virus particles (Sunter *et al.*, 1987).

Recent data suggest that a functional coat protein is not required for geminivirus infectivity or symptom development. Stanley and Townsend (1986) have described a viable mutant of CLV DNA-1 generated by *in vivo* deletion of an M13 cloning vector inserted into the coat-protein (30.2 kd) ORF. The mutation consists of a small deletion of 76 nucleotides which separates the coat protein sequence into two non-overlapping ORFs that could produce polypeptides with predicted mol. wts of 8.8 and 11.6 kd. The CLV-1 DNA mutant, in combination with CLV-2 DNA, is infectious when mechanically inoculated onto healthy *Nicotiana*

Fig. 1. Diagrams of TGMV coat-protein mutants. The position of the AR-1 open reading frame, which encodes the TGMV coat protein, is indicated by the darkened arrow labeled WT (wild-type). Proteins predicted from the nucleotide sequences of mutants CP-1, -2, -3 and -4 are shown as darkened areas within the appropriate arrows. A detailed description of these mutants appears in the text. The common region (cross-hatched box) is a sequence of 230 bp that is nearly identical in the A and B genome components.

benthamiana plants and generates symptoms which are not obviously different from those produced by wild-type CLV.

We have further investigated the role of the coat protein in geminivirus replication using several TGMV AR-1 mutants. The results reported here confirm that the coat-protein coding sequence may be interrupted without loss of infectivity. However, certain of these coat-protein mutants show characteristic delays in the time of symptom appearance and decreased symptom severity in *N.benthamiana*. The most attenuated symptoms were observed with a mutant in which nearly the entire coat-protein ORF was deleted.

Results

Coat protein mutants and their assay

To define clearly the role of the TGMV coat protein in the infection process, several mutations within or near ORF AR-1 were constructed. The AR-1 ORF is defined by its position in the TGMV DNA sequence of Hamilton *et al.* (1984), and lies between nucleotides 327 and 1068 of the 2588-nucleotide A genome component. TGMV A mutants CP-1 to CP-4 and the coat proteins they might produce are shown diagrammatically in Figure 1 and are described briefly below. Modified coat proteins have been predicted from sequence alterations and have not been identified in extracts from infected plants.

Mutation CP-1 lies outside the AR-1 ORF and consists of a 13-nucleotide insertion after nucleotide 326, which

results in the following sequence (insertion underlined): 5'-AGATCTGGATCCATATG. This introduces a *Bgl*II, a *Bam*HI and an *Nde*I cleavage site 5' to the coat-protein coding sequence, which initiates with the ATG that is part of the new *Nde*I (5'-CATATG) site. Hence no alteration of the TGMV coat protein is predicted. The *Bgl*II site begins at nucleotide 326.

Mutation CP-2 contains a four-base insertion (underlined) within a unique *Xho*I site which begins at nucleotide 400: 5'-CTCGATCGAG. The insertion generates a new *Pvu*I site (5'-CGATCG), and should result in a short, 31-amino acid N-terminal coat protein fragment that terminates at the TAA at nucleotide 416. There is also potential for formation of a truncated coat protein by translation re-initiation at an ATG at position 444. This would result in a protein consisting of the C-terminal 84% of the coat protein (208 amino acids).

Mutation CP-3 was created by insertion of an eight nucleotide *Hind*III linker (underlined) in the *Sca*I site beginning at nucleotide 789: 5'-AGTCAAGCTTGACT. This results in a frameshift mutation which should terminate immediately after the linker insertion at a TGA created by the linker and flanking sequences. An N-terminal fragment consisting of 63% of the coat protein is the predicted product.

Mutation CP-4 was constructed from CP-1 by deletion of 711 nucleotides between the newly introduced *Bgl*II cleavage site at nucleotide 326 and the *Asu*II cleavage site at nucleotide 1037. Only the 30 3'-terminal nucleotides of the coat-protein ORF remain.

The CP mutants were introduced into an appropriate binary T-DNA vector containing approximately one-half of the TGMV A sequence (Elmer *et al.*, 1988) to give one and one half tandemly repeated copies of TGMV A DNA with only a single copy of the region of interest. These plasmids were mated into *Agrobacterium tumefaciens*, and the resulting strains were used to inoculate transgenic *N.benthamiana* plants containing tandem inserts of TGMV-B DNA (Rogers *et al.*, 1986). The mutants were compared with controls carrying a wild-type one and one half copies of the TGMV A sequence in the binary vector.

Plants transgenic for the TGMV B component were chosen for infectivity experiments because their use provides an increase in efficiency over co-inoculation of healthy plants with the two DNA components (one versus two-hit kinetics). Further increases in inoculation efficiency have been obtained by employing *agrobacterium*-mediated delivery of the altered

Table 1. Infectivity of TGMV coat-protein mutants in *N.benthamiana* plants transgenic for the TGMV B component

Mutation	Inoculum ^a	No. experiments	No. inf./ No. inoc.	Days until symptoms		
				Range	Av.	SD
Wild type	341-SE	4	51/54	10–29	16.7	3.9
CP-1	440-SE	3	29/30	12–21	16.2	2.5
CP-2	TGA9-SE	2	12/15	11–20	15.1	2.6
Wild type	337-SE	11	100/102	7–27	13.3	2.6
CP-3	356-SE	3	49/51	11–29	18.2	5.0
CP-4	458-SE	4	34/35	14–24	19.1	2.4

^aStrain 341-SE is the appropriate wild-type control for mutants CP-1 and CP-2. Strain 337-SE is the appropriate control for mutants CP-3 and CP-4. Strain numbers indicate Ti plasmid contained in *Agrobacterium* (Materials and methods).

Fig. 2. Symptomatic leaves from TGMV B tobacco plants infected with wild-type TGMV and coat-protein mutants. Comparable leaves were taken from plants inoculated with wild-type (W) TGMV A or mutants CP-1, CP-2, CP-3 or CP-4. The leaf labeled H is from a healthy control. All leaves were selected from near the apex of the infected plants. Note the extreme curling and chlorosis which results from infection with wild-type TGMV and the CP-1 mutant.

TGMV A DNAs. Both of these procedures have been discussed in detail elsewhere (Grimsley *et al.*, 1986; Elmer *et al.*, 1988; Grimsley and Bisaro, 1987).

Mutations in the coat protein coding sequence reduce symptom severity

The results of infectivity experiments with mutants CP-1 to CP-4 appear in Table I. All of the coat-protein mutants were capable of replicating and producing symptoms in *N. benthamiana* plants that supply TGMV B functions. The CP-1 mutant, which contains an insertion 5' to the coat-protein

Fig. 3. Delay in symptom appearance in TGMV B tobacco plants inoculated with mutant CP-4. *N. benthamiana* plants transgenic for the TGMV B genome component were agroinoculated with wild-type TGMV A (■, 337-SE, 102 plants) or the coat-protein mutant CP-4 (□, 458-SE, 35 plants). Plants were scored positive for symptoms when obvious leaf curl or chlorosis was evident.

Fig. 4. Southern blot analysis of TGMV A DNAs produced in leaves of TGMV B tobacco plants inoculated with CP mutants. Total DNA (2–5 µg) from leaves of inoculated plants was electrophoretically separated and hybridized with a nick-translated component-A-specific probe as described in Materials and methods. Lane 3 contains total leaf DNA from a CP-1 inoculated plant; lane 4 from a CP-2 inoculated plant; lane 5 from a CP-3 inoculated plant; lane 6 from a CP-4 inoculated plant; and Lanes 1 and 2 from plants inoculated with wild-type TGMV A controls 341-SE and 337-SE respectively. The DNA forms identified include open circular (oc) and covalently closed circular (ccc) double-stranded as well as single-stranded (ss) TGMV A DNAs.

coding sequence, produced symptoms similar to those seen with wild-type controls (Figure 2). In contrast, mutants CP-2, -3 and -4 gave rise to attenuated disease symptoms characterized by a reduced amount of chlorosis and leaf curl (Figure 2). The symptoms produced by mutant CP-2 were usually more obvious than those generated by CP-3. The greatest attenuation of disease symptoms was observed following agroinfection of *N. benthamiana* plants with the CP-4 deletion mutant (Figure 2). Mutants CP-3 and -4 also showed characteristic delays in time symptom appearance which could likewise be correlated with the position of the mutation (Table I). Again, mutant CP-4 exhibited the greatest delay in symptom appearance; in four experiments the delay was an average of 6 days compared with wild-type (Figure 3). However, it should be noted that since symptoms produced by the mutants were not as obvious as those produced by wild-type virus, their onset was sometimes difficult to ascertain with confidence.

Analysis of TGMV DNA in infected plants

Total leaf DNA prepared from symptomatic plants inoculated with TGMV coat-protein mutants was analyzed by Southern blot hybridization using a probe specific for the TGMV A component. The results of this analysis (Figure 4) clearly demonstrate that the mutants CP-1, -2 and -3 produce open circular and covalently closed circular dsDNA forms characteristic of TGMV infection (lanes 3–5) (Hamilton *et al.*, 1982). Mutant CP-4 produced similar, but smaller TGMV A forms (lane 6). Importantly, extracts from plants infected

Fig. 5. Southern blot analysis of restricted TGMV A DNAs produced in leaves of TGMV B tobacco plants inoculated with CP mutants. Total DNA (2–5 µg) from leaves of inoculated plants was run on agarose gels following digestion with the appropriate enzyme and hybridized with a component-A-specific probe as described in Materials and methods. Lanes 1 and 3 contain DNA from plants inoculated with wild-type control 341-SE; lanes 5 and 7 from plants inoculated with wild-type control 337-SE; and lanes 2, 4, 6 and 8 from plants inoculated with CP-1, CP-2, CP-3 and CP-4 respectively. DNAs in lanes 1 and 2 were digested with *Bgl*III, in lanes 3 and 4 with *Pvu*I, in lanes 5 and 6 with *Hind*III, and in lanes 7 and 8 with *Eco*RI. The DNA forms identified are open circular (oc), linear (lin), and covalently closed circular (ccc) double-stranded TGMV A DNA. DNA forms that appear below the position of ccc DNA are single stranded; in most samples this DNA has been lost during digestion. The 240-bp *Pvu*I fragment expected from CP-2 DNA (lane 4) was not retained on the gel. Relevant mol. wt markers are shown to the right of lane 8.

with coat-protein mutants also contained single-stranded TGMV DNA in amounts comparable to those seen in plants infected with wild-type controls. A similar analysis of infected plant DNAs using a component-B-specific probe did not reveal any detectable difference between wild-type and any CP-mutant component-B DNA forms (not shown).

The attenuated symptoms associated with the coat-protein mutants were not due to reversion, since dsDNAs produced by the mutants still contained the mutation. This was clearly demonstrated by restriction of CP-1 DNA with *Bgl*III (lane 2), CP-2 DNA with *Pvu*I (lane 4) and CP-3 DNA with *Hind*III (lane 6) as shown in Figure 5. The *Bgl*III and *Hind*III sites generated by the insertions present in CP-1 and CP-3 respectively, are unique so that digestion with these enzymes results in the production of linear molecules. The four nucleotide insertion in CP-2 DNA introduces a second *Pvu*I site into the TGMV A DNA sequence; therefore two fragments of 2350 and 240 bp are expected. Since all of the TGMV A DNAs found in CP-mutant-infected plants were cleaved as predicted, all of these mutants have retained their respective insertions. DNA from plants infected with deletion mutant CP-4, which cannot revert, migrated at a rate consistent with its predicted 1.9-kb size upon digestion with *Eco*RI (lane 8).

Discussion

The experiments presented in this report clearly demonstrate that the TGMV coat protein is not required for viral DNA replication, systemic spread of the virus, or symptom development. Our observations thus extend those of Stanley and Townsend (1986), who selected a CLV coat-protein mutant that is as infectious as wild-type CLV and generates wild-type disease symptoms in *N.benthamiana*. In contrast, we have found that TGMV coat protein clearly influences pathogenesis in the same host since certain coat-protein mutants produce delayed and attenuated disease symptoms compared with those produced by wild-type TGMV. The most attenuated and delayed symptoms are seen in a mutant which lacks nearly the entire coat-protein coding sequence.

Our conclusion that the coat protein is not essential for symptom production and systemic spread does not rule out the association of other proteins with the TGMV DNA during infection or the possibility that another protein may substitute in some way for the coat protein. One possible candidate could be the product of the BR1 ORF which has been shown to be 43% homologous to the coat protein at the conserved amino acid level by computer analysis of the CLV sequence (Kikuno *et al.*, 1984). This protein cannot simply replace the coat protein in virions since we have, so far, been unable to detect geminate particles of TGMV in transgenic B plants infected with the coat-protein deletion mutant (A. Rushing, unpublished data).

Previous studies of TGMV infection produced by cross pollination of transgenic plants indicate that viral functions essential for systemic spread and symptom development reside on the B-genome component (Rogers *et al.*, 1986), and that these processes depend primarily on factors other than viral DNA encapsidation (Sunter *et al.*, 1987). Nevertheless, one possible interpretation of the results we have obtained with TGMV coat-protein mutants is that the encapsidation of viral DNA facilitates spread through the various tissues of the host and the rapid spread of virus to

a minimum number of cells is necessary for the onset of observable disease. In the absence of functional coat protein, systemic spread might not occur quickly enough to cause the extensive chlorosis and curling that is characteristic of newly expanded, TGMV-infected leaves. The observation that older *N.benthamiana* plants, and expanded leaves of young plants, do not exhibit severe disease symptoms is consistent with this idea (unpublished data). Alternatively, the coat protein may directly influence symptom production, perhaps through a currently undefined interaction with other viral or host-coded function. Whatever the mechanism, it is clear that pathogenesis is a complex process that involves functions encoded by both viral genome components, including the coat protein.

It is not clear why the TGMV mutants described here and the CLV mutant described previously (Stanley and Townsend, 1986) induce disease symptoms that differ in severity. Perhaps the degree of symptom development observed with a particular mutant depends upon the ability of the truncated protein(s) the CLV mutant might synthesize to associate with specific nucleic acids or proteins in the infected cell. Furthermore, the wild-type symptoms observed in the CLV mutant could be a consequence of *in vivo* selection for a variant whose ability to move through the host plant and cause disease was not significantly impaired. Additional investigation of these mutants, and in particular the identification in infected plants of truncated proteins predicted from mutant DNA sequences, should help to clarify the role of the geminivirus coat protein in pathogenesis.

Since the coat protein is not absolutely required for viral DNA replication, systemic spread or symptom development, the function of the peculiar geminivirus capsid in the viral multiplication cycle remains uncertain. It may be that the capsid is necessary for the acquisition and/or transmission of the virus by its natural whitefly vector. In this regard, we note that a natural isolate of CLV exists that does not appear capable of producing virus particles (Robinson *et al.*, 1984). It may be that this variant CLV is transmitted solely by vegetative propagation of cassava. At this time, the ability of the whitefly to transmit this isolate has not been tested (Harrison, 1985).

In contrast to the results reported here for TGMV, the virus coat protein plays an essential role in the infection and/or systemic spread of other plant viruses such as cauliflower mosaic virus (CaMV). Daubert *et al.* (1983) showed that insertions of 250 bp resulting in frameshifts in ORF IV, which encodes the coat protein, results in complete loss of symptom appearance. The tobacco mosaic virus (TMV) coat protein is essential for systemic spread in infected tobacco plants. Takamatsu *et al.* (1987) created a deletion of the TMV coat protein which produces local lesions but does not spread from the inoculated leaf.

Plants inoculated with TGMV coat-protein mutants contain both double- and single-stranded viral DNAs in relative proportions that are within the range of variability seen in wild-type TGMV infections. This suggests that encapsidation is not necessary for the accumulation of the single-stranded genomic form in contrast to the decrease in ssDNA reported for the coat protein mutant of CLV (Stanley and Townsend, 1986). Our results also demonstrate that a TGMV A DNA molecule as small as 1.9 kb can be stably replicated and spread throughout the host contrary to the results of Stanley and Townsend using a variant that was

only 76 bp smaller than the intact CLV-1 component. Naturally occurring deletion variants of the CLV-2 and TGMV B component arise during passage of these viruses (Stanley and Townsend, 1985; MacDowell *et al.*, 1986) that are as small or smaller than the coat-protein deletion. However, the CP-4 deletion is the first report that a large deletion derivative of the A component can stably replicate. We conclude that the removal of coat-protein sequences, which results in the attenuation of disease symptoms, may be useful for the development of whole plant vectors from TGMV DNA. We have not tested the upper limit of the size of DNA that may be inserted in place of the coat-protein coding sequence and retain stable replication and spread of TGMV DNA. In view of our results, the size of the inserted DNA should not be limited by encapsidation constraints.

Materials and methods

DNA techniques

All restriction endonucleases and other DNA-modifying enzymes were used as recommended by the suppliers. Other techniques were performed according to Maniatis *et al.* (1982) if not stated otherwise. Sequence alterations in the mutants described below were confirmed either by direct DNA sequencing (Messing *et al.*, 1981) or by digestion with appropriate restriction endonucleases to demonstrate the presence of new cleavage sites.

TGMV-Ti plasmid vectors

The construction of pMON336 and pMON351, the binary *A. tumefaciens* Ti plasmid based vectors for the introduction of TGMV-DNA into permissive host plants, has been described previously (Elmer *et al.*, 1988). Briefly, pMON336 is a derivative of pMON505 that contains the 1.5-kb *EcoRI*–*HindIII* (*ScaI*) fragment of TGMV A derived from pMON344 (see below). The pMON351 plasmid is a derivative of pMON505 that carries the 1.1-kb common-region-containing *EcoRI*–*HindIII* (*ScaI*) fragment of TGMV A derived from pMON344. Both plasmids retain unique *EcoRI* and *HindIII* sites for the insertion of full-length TGMV A DNA so that greater than unit-length copies of TGMV may be generated for *Agrobacterium*-mediated delivery to plants.

Plasmid pMON341 consists of a wild-type TGMV A DNA sequence inserted into pMON336, and pMON337 contains the wild-type sequence inserted into pMON351. *A. tumefaciens* carrying these plasmids were employed as wild-type controls for infectivity experiments.

Construction of coat-protein ORF mutations

The following details the construction of four mutations created in or near the TGMV AR-1 (coat protein) ORF. All nucleotide coordinates used refer to the TGMV A DNA sequence of Hamilton *et al.* (1984). The mutations are described further in Results and appear in diagrammatic form in Figure 1.

Mutation CP-1 was constructed by site-directed mutagenesis on an *EcoRI* insert of TGMV A DNA in pUC119 (provided by J. Messing, Waksman Institute) according to the procedure of Kunkel (1985). Plasmid pUC119 is a derivative of pUC19 (Yanisch-Perron *et al.*, 1985) that can be isolated in single-stranded form after superinfection of *Escherichia coli* with M13 K07, a variant of M13 impaired in its ability to replicate. The mutagenesis was performed such that a 13-base sequence was introduced between nucleotides 326 and 327 in TGMV A DNA, and the altered TGMV A *EcoRI* fragment was transferred into the TGMV-Ti vector pMON336. The resulting plasmid is called pMON440.

Mutation CP-2 was created in plasmid pBH404, which contains a single TGMV A DNA inserted into the unique *EcoRI* site of pAT153 (Bisaro *et al.*, 1982). Plasmid pBH404 was cleaved at its unique *XhoI* site located at nucleotide 400 within ORF AR-1, filled in using the Klenow fragment of DNA polymerase I in the presence of the four nucleotide triphosphates, and religated. A plasmid was isolated which lacked the *XhoI* site and contained a new *PvuI* site, and the TGMV A DNA *EcoRI* fragment containing the *XhoI* fill-in mutation was subsequently transferred to pMON336 to yield plasmid pTGA9.

Mutation CP-3 was generated by insertion of a synthetic eight-base *HindIII* linker (5'-CAAGCTTG; New England Biolabs, Beverly, MA) at the *ScaI* site at nucleotide 788. This was accomplished by cleavage of pMON305, a pMON200 derivative which contains two tandem copies of TGMV A DNA inserted at the *EcoRI* site (Rogers *et al.*, 1986), with *ScaI* followed by addition of the synthetic linker and ligation. The reaction mixture was

then digested to completion with *HindIII*, and the resulting 2.6-kb TGMV fragment was transferred to *HindIII*-cleaved pUC18 DNA (Yanisch-Perron *et al.*, 1985). The resulting plasmid was designated pMON344. Finally, the 2.6-kb TGMV *HindIII* fragment was transferred to pMON351 to give pMON356.

Mutation CP-4 was created by deletion of the 711-bp fragment of TGMV A DNA between the *BglII* site (nucleotide 326) of the CP-1 mutant and the *AsuII* site (nucleotide 1037). This deletion was performed on a full-length *EcoRI* insert of TGMV A in pUC18 in such a way that the *BglII* site was retained but the *AsuII* site was lost. The modified *EcoRI* fragment (~1.9 kb) was then inserted into pMON351 to give plasmid pMON458.

Infectivity experiments

Transgenic *N. benthamiana* plants containing chromosomally integrated, tandem copies of the TGMV B component (Rogers *et al.*, 1986) were inoculated with TGMV A DNA by agroinfection (Elmer *et al.*, 1988). *A. tumefaciens* carrying a greater than unit length copy of TGMV A DNA (wild-type or CP mutant) in binary transformation vectors pMON336 or pMON351 was applied to cut main stems of 3–4-week-old plants using a sterile toothpick.

Biosafety considerations

All of the experiments described were conducted according to the NIH Guidelines for Recombination DNA Research. Transgenic plants with tandem repeats of geminivirus components in their genomes were propagated in limited-access growth chambers. For experiments involving *Agrobacterium* inoculations, the runoff from watering was collected and treated with hypochlorite (Chlorox, 1%) before disposal to kill any *Agrobacterium* that might wash off the inoculated plants. No *Agrobacterium* strains were created that carried more than one component (~50% of the TGMV genome) in accordance with the NIH guidelines for the cloning of virus genomes.

Acknowledgements

The authors thank Linda Hanley-Bowdoin for suggestions and Lissa Bisaro and Barbara Schiermeier for expert preparation of the manuscript. D.M.B., G.S. and W.E.G. were supported in part by USDA grant no. 84-CR-CR-11381, and by a grant from the Monsanto Company.

References

- Bisaro, D.M., Hamilton, W.D.O., Coutts, R.H.A. and Buck, K.W. (1982) *Nucleic Acids Res.*, **10**, 4913–4922.
- Daubert, S., Shepherd, R.J. and Gardner, R.C. (1983) *Gene*, **25**, 201–208.
- Elmer, J.S., Brand, L., Sunter, G., Gardiner, W.E., Browning, C.K., Bisaro, D.M. and Rogers, S.G. (1988) *Plant Mol. Biol.*, **10**, 225–234.
- Grimsley, N. and Bisaro, D.M. (1987) In Hohn, Th. and Schell, J. (eds), *Plant DNA Infectious Agents*. Springer-Verlag, Vienna, pp. 87–107.
- Grimsley, N., Hohn, B., Hohn, T. and Walden, R. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 3282–3286.
- Hamilton, W.D.O., Bisaro, D.M. and Buck, K.W. (1982) *Nucleic Acids Res.*, **10**, 4901–4912.
- Hamilton, W.D.O., Bisaro, D.M., Coutts, R.H.A. and Buck, K.W. (1983) *Nucleic Acids Res.*, **11**, 7387–7396.
- Hamilton, W.D.O., Stein, V.E., Coutts, R.H.A. and Buck, K.W. (1984) *EMBO J.*, **3**, 2197–2205.
- Harrison, B.D. (1985) *Annu. Rev. Phytopathol.*, **23**, 55–82.
- Howarth, A.J., Caton, J., Bossert, M. and Goodman, R.M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3572–3576.
- Kikuno, R., Toh, H., Hayashida, H. and Miyata, T. (1984) *Nature*, **308**, 562.
- Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.
- Lazarowitz, S.G. (1987) *Plant Mol. Biol. Rep.*, **4**, 177–192.
- MacDowell, S.W., Coutts, R.H.A. and Buck, K.W. (1986) *Nucleic Acids Res.*, **14**, 7967–7984.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Messing, J., Crea, R. and Seeburg, P.H. (1981) *Nucleic Acids Res.*, **9**, 309–321.
- Morinaga, T., Ikegami, M. and Miura, K. (1983) *Proc. Jap. Acad.*, **59**, 363–366.
- Robinson, D.J., Harrison, B.D., Sequeira, J.C. and Duncan, G.H. (1984) *Ann. Appl. Biol.*, **105**, 483–493.
- Rogers, S.G., Bisaro, D.M., Horsch, R.B., Fraley, R.T., Hoffmann, N.L., Brand, L., Elmer, J.S. and Lloyd, A.M. (1986) *Cell*, **45**, 593–600.
- Stanley, J. (1983) *Nature*, **305**, 643–645.
- Stanley, J. (1985) *Adv. Virus Res.*, **30**, 139–177.

- Stanley, J. and Gay, M. (1983) *Nature*, **301**, 260–262.
- Stanley, J. and Townsend, R. (1985) *Nucleic Acids Res.*, **13**, 2189–2206.
- Stanley, J. and Townsend, R. (1986) *Nucleic Acids Res.*, **14**, 5981–5998.
- Stanley, J., Townsend, R. and Curson, S.J. (1985) *J. Gen. Virol.*, **66**, 1055–1061.
- Sunter, G., Gardiner, W.E., Rushing, A.E., Rogers, S.G. and Bisaro, D.M. (1987) *Plant Mol. Biol.*, **8**, 477–484.
- Takamatsu, N., Ishikawa, M., Meshi, T. and Okada, Y. (1987) *EMBO J.*, **6**, 307–311.
- Townsend, R., Stanley, J., Curson, S.J. and Short, M.N. (1985) *EMBO J.*, **4**, 33–37.
- Yanisch-Perron, C., Viera, J. and Messing, J. (1985) *Gene*, **33**, 103–119.

Received on October 15, 1987; revised on February 1, 1988