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Maize streak virus genes essential for systemic spread and symptom development

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The entire genome of single component geminiviruses such as maize streak virus (MSV) consists of a single-stranded circular DNA of ~2.7 kb. Although this size is sufficient to encode only three average sized proteins, the virus is capable of causing severe disease of many monocots with symptoms of chlorosis and stunting. We have identified viral gene functions essential for systemic spread and symptom development during MSV infection. Deletions and gene replacement mutants were created by site-directed mutagenesis and insertion between flanking MSV or reporter gene sequences contained in *Agrobacterium* T-DNA derived vectors. Following *Agrobacterium*-mediated inoculation of maize seedlings, the mutated MSV DNAs were excised from these binary vectors by homologous recombination within the flanking sequences. Our analyses show that the capsid gene of MSV, while not required for replication, is essential for systemic spread and subsequent disease development. The '+' strand open reading frame (ORF) located immediately upstream from the capsid ORF and predicted to encode a 10.9 kd protein was also found to be dispensable for replication but essential for systemic spread. By this analysis, MSV sequences that support autonomous replication were localized to a 1.7 kb segment containing the two viral intergenic regions and two overlapping complementary '−' strand ORFs. Despite the inability of the gene replacement mutants to spread systemically, both inoculated and newly developed leaves displayed chlorotic patterns similar to the phenotype observed in certain developmental mutants of maize. The similarity of the MSV mutant phenotype to these developmental mutants is discussed.

Key words: disease development/geminiviruses/maize streak virus/site-directed mutagenesis/systemic spread

Introduction

In contrast to the bipartite whitefly-transmitted geminiviruses, the leafhopper-transmitted geminiviruses contain only a single ~2.7−2.9 kb circular single-stranded (ss)DNA genomic component (Howell, 1984; Millineaux *et al.*, 1984; MacDowell *et al.*, 1985; Stanley *et al.*, 1986; Donson *et al.*, 1987; Lazarowitz, 1988), making these the smallest known viruses. Based on sequence analyses, the organization of this single genomic component resembles that of the A component of the bipartite geminiviruses (see reviews Davies *et al.*, 1987; Lazarowitz, 1987). Thus, in the monocot viruses such as maize streak (MSV) the open reading frames (ORFs) for the capsid (R1) and homolog of AL1 (L1′ + L1″) are found in similar positions on the virion (+) and complementary (−) strains, respectively; and there is an intergenic region, IR_c, which is the analog of the bipartite virus Common Region based both on its position between divergent ORFs and the presence of a conserved viral sequence element (Figure 1) (Howell, 1984; Millineaux *et al.*, 1984; Lazarowitz, 1987, 1988). Analyzed in this manner, the essential difference between the single component and bipartite geminiviruses is that the latter require B-encoded functions for systemic spread and disease development (Rogers *et al.*, 1986; Sunter *et al.*, 1987; Etessami *et al.*, 1988) whereas the single component virus does not.

Mutational analysis of the bipartite tomato golden mosaic virus (TGMV) has delineated the role of viral A-encoded gene products in replication, systemic spread and symptom

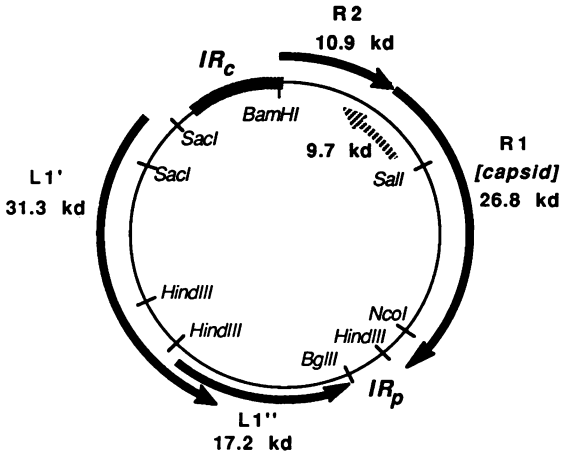

Fig. 1. Genomic organization of MSV. Diagrammatic representation of the single genomic component of MSV-S based on sequence analysis of infectious, cloned viral DNA (Lazarowitz, 1988). Arrows indicate limits and directions of potential ORFs encoding predicted proteins as shown (kd). '+' strand (R1, R2) and '−' strand (L1′, L1″) ORFs have been named according to the convention adopted for the bipartite geminiviruses and to reflect homologies with corresponding ORFs in the A component of the bipartite viruses, where these exist. Identification of the capsid gene (R1) is by homology to a Nigerian isolate of MSV (Morris-Krsinich *et al.*, 1985). The L1′ and L1″ encoded proteins are homologous to the NH_2- and COOH-terminal ends, respectively, of the product encoded by AL1 in the bipartite geminiviruses (Mullineaux *et al.*, 1985). IR_c is the analog of the bipartite virus Common Region. IR_p is the location of an 80 base DNA primer for dsDNA synthesis found hybridized to the virion ssDNA (Donson *et al.*, 1984; Lazarowitz, 1988). Based on strain differences which drastically alter the ORF for the 9.7 kd protein, it is unlikely that this ORF in fact exists. Positions of restriction sites are: *Bam*HI = 1, *Sal*I = 576, *Nco*I = 1004, *Bgl*II = 1205, *Sac*I = 2264, 2418, *Hin*dIII = 1091, 1720, 1841.

development (Elmer *et al.*, 1988; Gardiner *et al.*, 1988; Rogers *et al.*, 1988). The A component is capable of autonomous replication, encoding a product which is also required in *trans* for the replication of the B component (Rogers *et al.*, 1986). The protein encoded by AL1 is essential for this replication (Elmer *et al.*, 1988). AL2 and the B component are required for systemic spread (Elmer *et al.*, 1988). Encapsidation, however, is not essential for systemic spread and symptom development (Gardiner *et al.*, 1988). Mutants lacking the capsid gene do spread systemically, with resultant symptoms that appear later and are attenuated, but clearly present. A deletion in AL3 also results in attenuation of symptoms (Elmer *et al.*, 1988).

While S1 nuclease and primer extension analyses of MSV (Morris-Krsinich *et al.*, 1985) have identified possible transcripts for the '+' strand encoded 10.9 kd (R2) and capsid proteins, and the '−' strand encoded 31.3 kd (L1′) and 17.2 kd (L1″) proteins, mutational analysis of the single component geminiviruses to delineate essential viral gene functions has not been done. The pattern of transcription is complex, and those transcripts detected for MSV do not correlate with the individual ORFs. A single '−' strand ~1.2 kb transcript appears to traverse the entire region of the overlapping L1′ and L1″ ORFs (Figure 1). The major ~0.9 kb and less abundant ~1.1 kb '+' strand transcripts are co-terminal at their 3′-ends near position 1114 (Morris-Krsinich *et al.*, 1985). The 5′-end of the ~0.9 kb transcript, the one likely to function in capsid synthesis (Fenoll *et al.*, 1988), maps at position 163 in the middle of R2, while that for the ~1.1 kb transcript maps only four nucleotides before the ATG start codon for the 10.9 kd protein potentially encoded by R2 (Morris-Krsinich *et al.*, 1985) (Figure 1). However, mutational studies to define the functional roles of viral genes in replication, systemic spread and disease development remain to be done.

We report here a mutational analysis of MSV, using deletion, gene replacement and point mutants to define viral genes essential for systemic spread and symptom development in this single component geminivirus. Our results demonstrate that, unlike the bipartite viruses, the capsid of MSV is essential for systemic spread and classic disease development. Furthermore, we find that the '+' strand R2 ORF is also essential for viral movement. Finally, our analyses show that all of the information required for both viral ssDNA and dsDNA replication resides in the 1.7 kb genomic segment containing the two viral intergenic regions, IR_c and IR_p (location of the virion primer DNA), and the overlapping '−' strand L1′ and L1″ ORFs homologous to AL1 (Figure 1).

Results

Biological properties of deletion mutants MSV-CPΔ and MSV-(CP+R2)Δ

Mutants MSV-CPΔ and MSV-(CP+R2)Δ, deleted, respectively, for the capsid coding sequences alone or in combination with those for the 10.9 kd protein, were constructed by site-directed mutagenesis and enzymatic excision of appropriate fragments from the MSV genome (Figure 2; Materials and methods). To delete the capsid coding sequences and insert a unique *Bam*HI site convenient for cloning (MSV-CPΔ) the original *Bam*HI site which forms part of the start codon for R2 was eliminated by point mutation (Figure 2a). The resultant mutant, MSV-R2(D1), contains a conservative third position codon change in the putative 10.9 kd protein encoded by R2 (Figures 1 and 2). Plants agro-inoculated with a dimeric construct of MSV-R2(D1) (see Materials and methods) developed typical symptoms of maize streak disease with an efficiency and time course indistinguishable from wild-type MSV105 (Table I, Figure 7c). MSV-R2(D1) was also leafhopper transmissible (Table I). MSV-CPΔ was constructed from MSV-R2(D1) by creation of a new *Bam*HI site just upstream from the start of R1 and deletion of the capsid coding sequences by digestion with *Bam*HI and *Nco*I (Figure 2a). Both R1 and R2 were deleted to create MSV-(CP+R2)Δ by elimination of the ATG start codon for R2 and digestion of the resultant altered genome with *Bam*HI and *Nco*I directly (Figure 2b; Materials and methods).

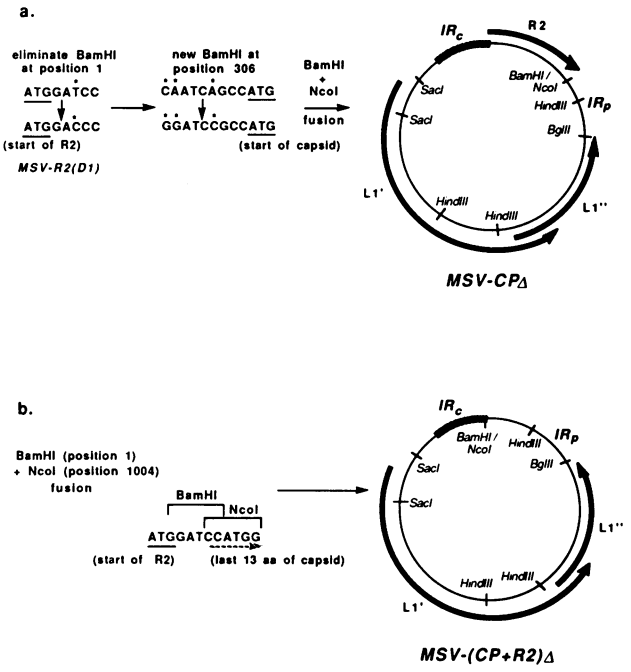

Fig. 2. Deletion mutants MSV-CPΔ and MSV-(CP+R2)Δ. Scheme for construction and genomic structure of (**a**) MSV-CPΔ, from which the capsid coding sequence was deleted; and (**b**) MSV-(CP+R2)Δ, from which both the capsid and 10.9 kd protein coding sequences were deleted. CP = coat protein. See Figure 1 for map of MSV-S and text for details.

Neither MSV-CPΔ nor MSV-(CP+R2)Δ DNAs (nor bacterial sequences) could be detected in extracts from either inoculated leaves (defined as those existing at the time of inoculation of the apical meristem and having needle marks on their expanded surfaces), or in the subsequently developing 'systemic leaves' of maize seedlings which had been agro-inoculated with dimeric constructs of either deletion mutant (Table I). These plants did not develop any visible disease symptoms during the course of the experiments (8 weeks). Nor did leafhoppers (*Cicadulina mbila*) fed on these agro-inoculated plants transmit disease (Table I). While these studies with MSV-CPΔ and MSV-(CP+R2)Δ demonstrated that deletion of the capsid sequences impaired some essential function, interpretation of these negative results was difficult. We further investigated the roles of the capsid and R2 ORFs in viral infection by analyzing MSV mutants containing bacterial reporter gene sequences directly substituted for those which had been deleted in MSV-CPΔ and MSV-(CP+R2)Δ.

Table I. Biological activity of MSV mutants

Mutant	Mutation	Replication	Systemic spread	Symptoms[a]	Insect[b] transmission
MSV-R2(D1)	Third position codon change to eliminate *Bam*HI site (ATGGATCC/ATGGACCC)	+	+	wt[c]	+
MSV-CPΔ	Deletion of capsid in MSV-R2(D1)	−	−	−	−
MSV-CPΔ−CAT	CAT replacement of capsid in MSV-CPΔ	+[d]	−	cl[e]	−
MSV-CPΔ−Hph	Hph replacement of capsid in MSV-CPΔ	+[d]	−	cl[e]	−
MSV-(CP+R2)Δ	Deletion of capsid +R2 ORFs	−	−	−	−
MSV-(CP+R2)Δ−CAT	CAT replacement of capsid +R2 ORFs in MSV-(CP+R2)Δ	+[d]	−	cl[e]	−
MSV-(CP+R2)Δ−Hph	Hph replacement of capsid +R2 ORFs in MSV-(CP+R2)Δ	+[d]	−	cl[e]	−
MSV-R2(D2)	Elimination of ATG for 10.9 kd protein (TCATG/TAGTG)	+[d]	−	cl[e]	−

[a]Symptoms first appearing and evident systemically by 5−7 days post-inoculation. In different experiments, 25−50% of the plants developed systemic lesions. These efficiencies were always comparable to those observed for wild-type MSV105 (25−60%) controls which were included in each experiment.
[b]Transmission to maize seedlings by the leafhopper *C.mbila*.
[c]Wild-type.
[d]Excised, freely replicating viral DNA detected only in inoculated leaves, not systemically.
[e]Cell lineage patterns (see Figure 7 and text for details).

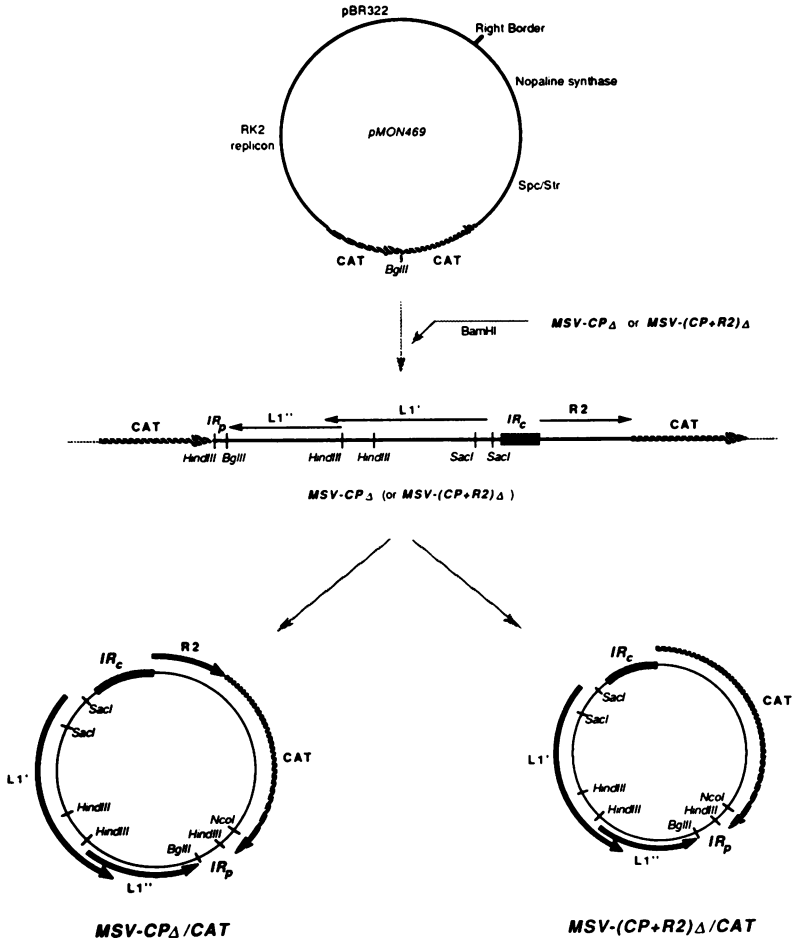

Fig. 3. Scheme for generation of MSV gene replacement mutants. Diagrammed is the approach for generating CAT replacements for the sequences deleted from MSV-CPΔ and MSV-(CP+R2)Δ (see Figures 1 and 2 for reference). Shown are: (**top**) the pMON469 binary vector, containing direct, tandem repeats of the CAT coding sequences separated by a unique *Bgl*II site; (**middle**) insertion of *Bam*HI linear of MSV-CPΔ into the *Bgl*II site of pMON469 in correct orientation so as to generate a direct replacement of the CAT sequence for R1; and (**bottom**) genomic structure of direct CAT gene replacements for R1 (left) or R1 + R2 (right). Fusion plasmids MSV-CPΔ/CAT and MSV-(CP+R2)Δ/CAT are referred to as MSV-CPΔ−CAT and MSV-(CP+R2)Δ−CAT, respectively, in the text.

Generation and biological properties of MSV gene replacement mutants

To facilitate the substitution of reporter genes for those viral sequences eliminated from MSV deletion mutants, the deleted viral genomes were inserted between two tandem direct repeats of a bacterial gene coding sequence contained in a binary vector. MSV-CPΔ and MSV-(CP+R2)Δ were cloned into the binary vector pMON469 containing direct repeats of the *Escherichia coli* chloramphenicol acetyltransferase (CAT) coding sequences (Figure 3), or the analogous pMON464 vector containing direct repeats of the bacterial hygromycin phosphotransferase (Hph) coding sequences (see Materials and methods). In this manner, we tested whether MSV gene substituted mutants containing these reporter genes inserted in correct (MSV-CPΔ−CAT, MSV-CPΔ−Hph, MSV-(CP+R2)Δ−CAT, MSV-(CP+R2)Δ−Hph) or inverted (MSV-CPΔ−CAT′, MSV-CPΔ−Hph′, MSV-(CP+R2)Δ−CAT′, MSV-(CP+R2)Δ−Hph′) orientation would be generated by homologous recombination within the flanking bacterial gene coding sequences following agro-inoculation of maize seedlings. It is unlikely that excision would occur by a replicative process since no viral sequences are duplicated in these constructs. An advantage of this approach was that only excised, freely replicating gene substituted MSV mutants would contain a functional reporter gene inserted between viral promoter and polyadenylation signals necessary for expression in the plant.

In contrast to the findings with the corresponding MSV deletion mutants, excision and replication of the gene substituted derivatives of MSV-CPΔ and MSV-(CP+R2)Δ was detected in maize plants within ~7 days following agro-inoculation with the pMSV::pMON469 or pMSV::pMON464 constructs described above (Table I). Efficiency was comparable to that of wild-type MSV-S, with mutant MSV DNA being detected in 25−40% of inoculated plants (Table I) (Lazarowitz, 1988). Replication of these MSV gene replacement mutants was detected in the inoculated leaves of the plants, but not in the subsequently developing 'systemic' leaves.

As shown for MSV-(CP+R2)Δ−CAT (Figure 4), DNA of the expected size (~2.5 kb) was detected in extracts from inoculated leaves. That this was indeed MSV-(CP+R2)Δ−CAT was shown by hybridization of Southern blots (Southern, 1975) with appropriate probes. This DNA hybridized with both the 1.7 kb *Bam*HI−*Bgl*II fragment and the ~200 bp *Nco*I−*Bgl*II fragment of MSV (Figures 3 and 4a and 4d). It did not hybridize with the 576 bp *Bam*HI−*Sal*I fragment of MSV which had been replaced by CAT coding sequences (Figure 4b). Nor did it hybridize with pMON464 which contains all of the binary vector sequences present in pMON469 but lacks the CAT sequences (Figure 4c), although it did hybridize with pMON469 (not shown). Hence, gene replacement mutant MSV-(CP+R2)Δ−CAT containing CAT coding sequences substituted for those of the 10.9 kd and capsid proteins (Figure 3) was generated by homologous recombination within the CAT coding sequences of the binary pMSV-(CP+R2)Δ::pMON469 plasmid following agro-inoculation of maize. Replicating MSV-(CP+R2)Δ−CAT was found in inoculated leaves, but did not move systemically (Figure 4). That this was replication and not simply excision is concluded from the negative findings with the corresponding deletion mutants,

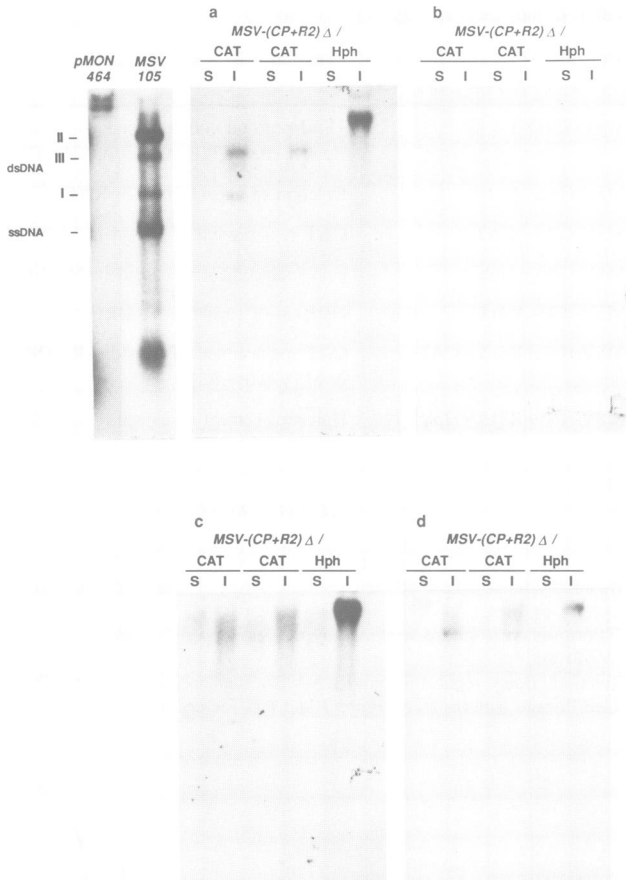

Fig. 4. Southern blot analysis of MSV-(CP+R2)Δ gene substitution mutant DNA in inoculated (I) and systemic (S) leaves from agro-inoculated plants. MSV-(CP+R2)Δ−CAT is the CAT gene replacement mutant. MSV-(CP+R2)Δ−Hph is the hygromycin gene replacement. (**a**) The probe was MSV 1.7 kb *Bgl*II−*Bam*HI fragment (see Figure 1). (**b**) The blot shown in (a) was eluted and hybridized with the 576 bp *Bam*HI−*Sal*I fragment of MSV, which was deleted in these mutants. (**c**) The probe was pMON464 which contains the same bacterial sequences as pMON469 with an insert of the hygromycin coding sequences instead of the CAT coding sequences. (**d**) Blot shown in (c) was eluted and hybridized with the 630 bp *Sal*I−*Bgl*II fragment of MSV. Shown top left are undigested pMON464 and an extract from wild-type pMSV105(*ASE*) inoculated maize.

and the fact that we could not detect unexcised MSV or binary vector DNA (pMON505, pMON469 or pMON464) in inoculated leaf extracts from appropriately agro-inoculated plants.

The MSV-(CP+R2)Δ−CAT′, MSV-(CP+R2)Δ−Hph and MSV-(CP+R2)Δ−Hph′ gene replacement mutants were also detected as freely replicating MSV mutant DNA of the expected size and structure in inoculated leaves and did not move systemically. Shown in Figure 4 is DNA from a unique plant in which a MSV-(CP+R2)Δ−Hph substitution mutant of ~8 kb was detected replicating in the inoculated leaves. This DNA was apparently generated by an illegitimate recombination event and contains all of the expected MSV sequences (Figures 4a,b and d) as well as binary vector sequences in addition to the Hph sequences (Figure 4c, and additional data not shown). Thus, DNA much larger than viral size can be stably replicated. Furthermore, the results with these gene replacement mutants

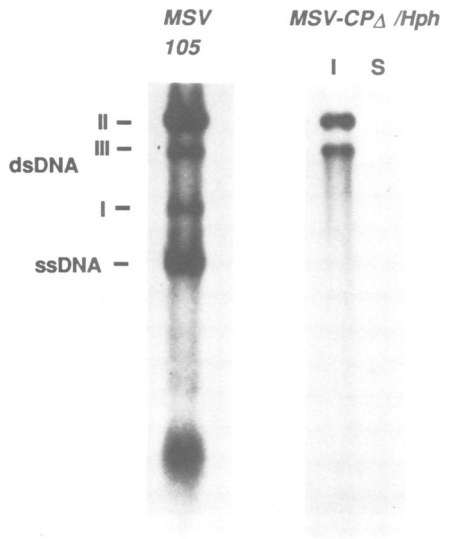

Fig. 5. Southern blot analysis of hygromycin gene substitution mutant MSV-CPΔ−Hph DNA in inoculated (I) and systemic (S) leaves from agro-inoculated plants. The probe used was the 1.7 kb *Bgl*II−*Bam*HI fragment of MSV. Shown on the left is an extract from wild-type MSV105(*ASE*) inoculated maize.

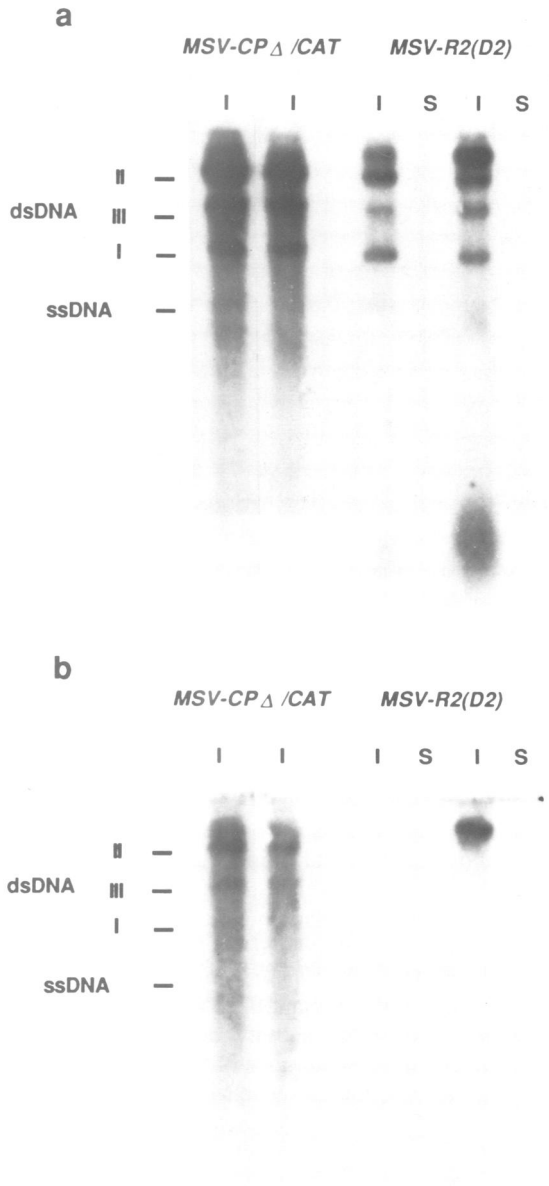

Fig. 6. Southern blot analysis of CAT gene substitution mutant MSV-CPΔ−CAT and point mutant MSV-R2(D2) in inoculated (I) and systemic (S) leaves from agro-inoculated maize plants. Probes were (**a**) MSV 1.7 kb *Bgl*II−*Bam*HI fragment and (**b**) binary plasmid pMON469, which contains the CAT coding sequences. Marked are the positions of wild-type MSV ssDNA and dsDNA forms found in extracts from MSV105(*ASE*) inoculated maize.

of MSV-(CP+R2)Δ, as well as point mutants (see below), demonstrate that all of the information required for viral DNA replication is located in the 1.7 kb *Bam*HI−*Nco*I fragment (Figures 1 and 2) which contains the intergenic regions IR_c and IR_p, and the L1′ and L1″ ORFs.

Similar results were obtained for derivatives of MSV-CPΔ containing CAT or Hph coding sequences substituted for those of the capsid alone. Shown in Figures 5 and 6 are the results for MSV-CPΔ−Hph and MSV-CPΔ−CAT. DNA of the gene replacement mutants was again found excised and freely replicating in inoculated leaves, but did not move systemically. This DNA contained all of the expected MSV sequences and appropriate Hph or CAT coding sequences (Figures 5, 6 and additional data). Binary vector sequences exclusive of Hph or CAT could not be detected. The same was found for each of these gene replacements in inverted orientation. Based on these findings with gene replacement mutants of MSV-CPΔ, it is clear that, in contrast to the bipartite geminiviruses (Stanley and Townsend, 1986; Gardiner *et al.*, 1988), the capsid protein of MSV is essential for systemic spread.

Although the gene replacement mutants derived from MSV-CPΔ and MSV-(CP+R2)Δ were confined to inoculated leaves and classical viral disease symptoms were not observed, the agro-inoculated plants were usually not phenotypically normal (Table I). Instead of the typical disease symptoms of punctate chlorosis coalescing into the characteristic streaks (Figure 7a), these plants displayed chlorotic patterns that appeared to follow cell lineages on both inoculated and the later developing 'systemic' leaves (Figure 7b). This systemic development of cell lineage patterns was dependent on replication of the MSV mutants. It was not observed in plants agro-inoculated with binary vectors lacking MSV sequences. Nor was it observed in plants agro-inoculated with the MSV deletion mutants which did not replicate (Table I). Thus, as well as being essential for systemic spread of the virus, the MSV capsid subunit is also required for the appearance of classic disease symptoms in the plant. When leafhoppers were fed on the inoculated leaves of these plants, none of the gene substituted mutants derived from MSV-CPΔ or MSV-(CP+R2)Δ were leafhopper transmissible (Table I), suggesting a role for the capsid in insect transmission as well.

Expression of CAT by MSV gene replacement mutants

MSV-CPΔ−CAT and MSV-(CP+R2)Δ−CAT were tested for expression of the bacterial CAT gene. CAT activity was

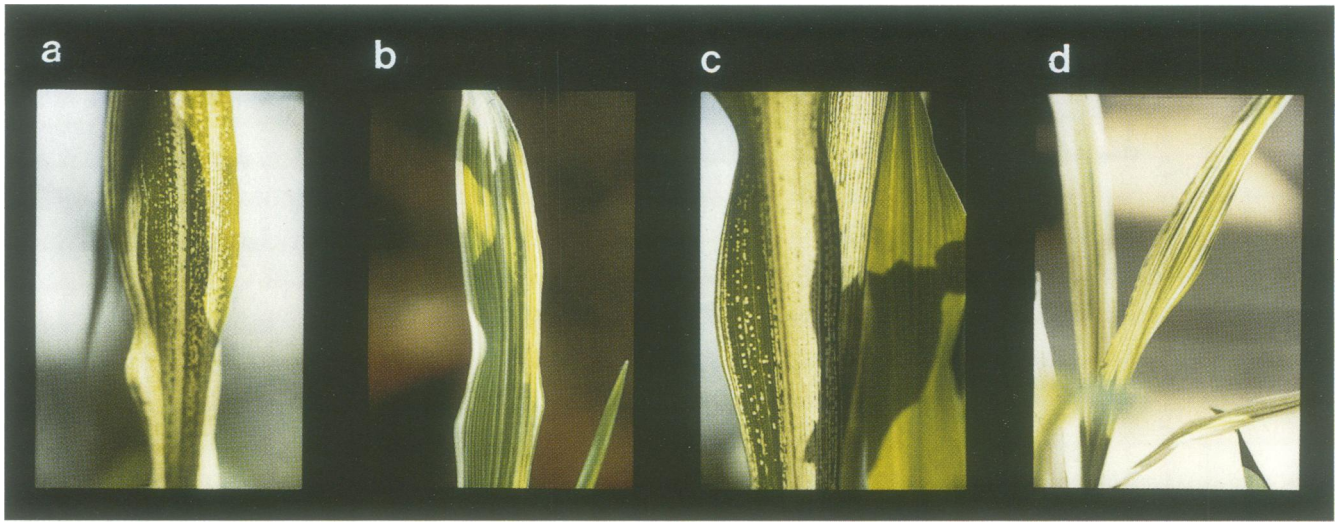

Fig. 7. Appearance of maize agro-inoculated with wild-type or mutant MSV. (**a**) Maize inoculated with MSV105 showing classic disease symptoms of punctate chlorosis coalescing into streaks; (**b**) plant inoculated with gene replacement mutant MSV-CPΔ−Hph showing cell lineage phenotype. (**c**) maize inoculated with point mutant MSV-R2(D1), containing the conservative change GAT/GAC in the second codon of the R2 ORF; and (**d**) plant inoculated with point mutant MSV-R2(D2) containing the mutation TCATG/TAGTG at the start codon in R2.

detected in extracts of inoculated leaves which contained excised, freshly replicating MSV-CPΔ−CAT DNA (Figures 6 and 8). Inoculated leaves in which excised MSV-CPΔ−CAT DNA was not detected did not contain enzymatic activity above background levels. Nor was activity detected above background in inoculated leaves containing MSV-CPΔ−CAT′ in which the CAT sequences were inserted in inverted orientation (Figure 8). Thus, the CAT activity detected was expressed by MSV-CPΔ−CAT and not by residual *Agrobacterium tumefaciens* in the inoculated leaves. Given that this mutant does not move in the plant, the low levels of CAT activity detected are likely the consequence of the low number of cells initially infected.

In contrast to these findings with MSV-CPΔ−CAT, MSV-(CP+R2)Δ−CAT did not express CAT activity above background (Figure 8). Although this might be expected given the low level of activity detected for MSV-CPΔ−CAT, R2 has not been shown to encode an essential viral function. While our gene replacement studies demonstrated the importance of the capsid sequence in viral movement and disease development, they did not provide information on the functions of R2 since all of these mutants were phenotypically indistinguishable from each other. Therefore, to investigate the role of R2 in infection further mutational and genetic analyses were done.

R2 encodes an essential viral function

To determine the function of R2, we altered two nucleotides at the predicted start codon for the 10.9 kd protein by site-directed mutagenesis, changing the sequence from TCATGGATCC to TAGTGGATCC. This mutation [MSV-R2(D2)] eliminated the ATG start codon, replacing it with a GTG and overlapping UAG stop codon. This mutation did not affect other potential ORFs, and the next ATG in the sequence is the start codon for the capsid protein (Lazarowitz, 1988). Thus, both by location and conservation of spacing, this mutation should precisely test the function of R2 without potentially interfering with processing of the transcript for the capsid which has its 5′-end in the middle of R2 at position 163 (Fenoll *et al.*, 1988) (Figure 1).

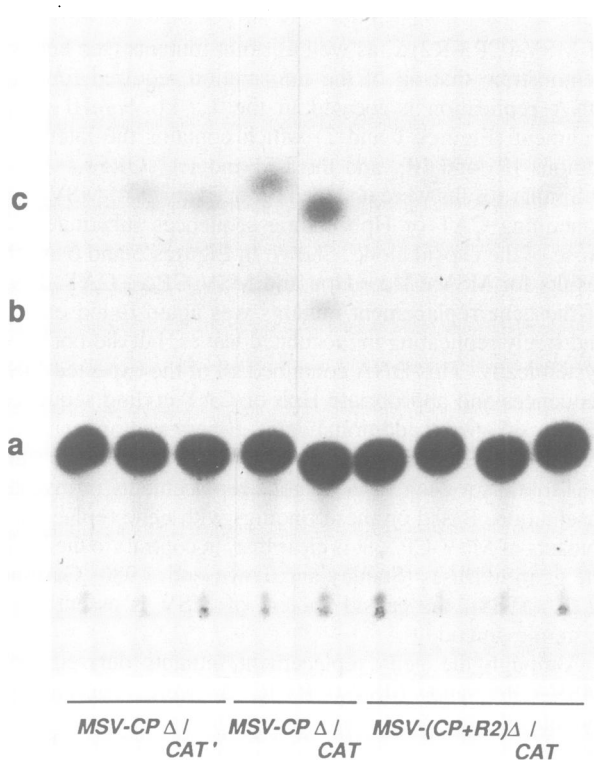

Fig. 8. CAT activity of extracts from inoculated leaves of maize containing MSV-CPΔ−CAT′, MSV-CPΔ−CAT and MSV-(CP+R2)Δ−CAT. All MSV-CPΔ−CAT′ and MSV-(CP+R2)Δ−CAT extracts contained excised viral DNA in amounts equal to or greater than those detected in the MSV-CPΔ−CAT samples. See Figure 6 for MSV-CPΔ−CAT extracts used. (**a**) Unreacted, (**b**) 1-acetate and (**c**) 3-acetate chloramphenicol.

Maize seedlings agro-inoculated with MSV-R2(D2) did not develop classic maize streak disease. Nor was MSV-R2(D2) leafhopper transmissible (Table I). As found for MSV gene replacement mutants, replicating MSV-R2(D2) ssDNA and dsDNA was detected in the inoculated leaves

of the plant, but not systemically (Figure 6). Furthermore, these plants developed cell lineage patterns of chlorosis, as observed for the gene replacement mutants derived from MSV-(CP+R2)Δ and MSV-CPΔ (Figure 7d).

To demonstrate that this mutation did not affect transcription of the capsid gene we attempted genetic complementation, co-inoculating maize seedlings with MSV-R2(D2) and MSV-CPΔ−CAT. If the mutation in MSV-R2(D2) only affected the 10.9 kd protein, then this mutant should synthesize capsid protein and complement the defect in the capsid gene replacement mutant MSV-CPΔ−CAT. Since MSV-CPΔ−CAT is only 114 nucleotides longer than wild-type MSV it should be encapsidated. Thus, if the 10.9 kd protein functions in *trans*, this complementation should result in the systemic appearance of maize streak disease and CAT activity expressed by MSV-CPΔ−CAT. However, in repeated attempts we did not observe complementation, obtaining only a single symptomatic plant out of ~500 which proved to contain a recombinant MSV derived from the two injected mutant genomes. This result is not unexpected since recombination rather than complementation has been the outcome in similar studies with other geminiviruses (Etessami *et al.*, 1988) as well as with cauliflower mosaic virus (CaMV) (Howell *et al.*, 1981).

Based on these results, we cannot directly exclude the possibility that the mutation in MSV-R2(D2) is exerting a transcriptional or polar effect on the downstream capsid gene. However, the mutant MSV-R2(D1) contains a third position conservative change in the second codon of R2 (TCATGGATCC to TCATGGACCC) (Figure 2) located five nucleotides downstream from the mutation in MSV-R2(D2), and MSV-R2(D1) is completely wild-type in its properties (Figure 6c, Table I). These results with MSV-R2(D2) and MSV-R2(D1), given the transcript mapping (Morris-Krsinich *et al.*, 1985), suggest the simplest conclusion, namely that R2, the gene for the 10.9 kd protein in MSV, exists and encodes a function essential for systemic spread and classic symptom development. This protein has recently been detected in MSV-infected tissue (Mullineaux *et al.*, 1988).

Discussion

We have undertaken a mutational analysis of MSV, initially concentrating on the virion '+' strand ORFs in an attempt to define similarities and differences between the single component and bipartite geminiviruses in functions essential for systemic spread and the development of disease symptoms. Using site-directed mutagenesis to construct MSV mutants containing point mutations, deletions and gene replacements we have identified regions of the viral genome encoding functions for systemic spread, symptom development and viral replication.

We used a novel approach to generate gene replacement mutants of MSV following introduction of the recombinant binary vector construct into the plant. This method involved insertion of the viral DNA in the appropriate orientation between two copies of a gene coding sequence contained in the binary vector (Figure 3) and relied on excision of the desired gene substituted mutant occurring in the agro-inoculated plants by homologous recombination within these flanking coding sequences. Using both the CAT and Hph coding sequences we did generate MSV mutants containing these reporter gene sequences in place of either the capsid coding sequences alone (MSV-CPΔ derivatives) or in combination with the R2 ORF [MSV-(CP+R2)Δ derivatives] (Figures 3−6). Thus, repeats of any non-viral sequences can substitute for viral sequences in excision of the viral genome following introduction into the plant. Furthermore, this provides a convenient method for the construction of substitution mutants containing a particular reporter gene inserted at different sites in the viral genome and, with the demonstration of CAT activity by MSV-CPΔ−CAT, provides a potential assay for identifying viral sequence elements important in gene expression.

Analysis of deletion mutants MSV-CPΔ and MSV-(CP+R2)Δ, and their gene substituted derivatives, demonstrated that the capsid of MSV is essential for systemic spread and the development of classic disease symptoms (Table I, Figures 4−7). ssDNA and dsDNA of gene substitution mutants of MSV-CPΔ and MSV-(CP+R2)Δ, as well as a point mutant in R2 [MSV-R2(D2)], were detected excised and freely replicating in extracts from inoculated leaves of maize plants, but not in systemic leaves from these same plants (Figures 4−6). This is in contrast to the bipartite geminiviruses in which the capsid is not essential for movement and symptom development (Gardiner *et al.*, 1988; Hayes *et al.*, 1988; Ward *et al.*, 1988). Hence, encapsidation of MSV plays an essential role in addition to protection of the genome and insect transmission of the virus, and is not the precise functional equivalent in the single component and bipartite geminiviruses. Analysis of beet curly top virus capsid replacements would determine whether this truly is a single component/bipartite geminivirus distinction rather than a difference between monocot and dicot geminiviruses. In these studies, we found the amount of viral ssDNA detected to be variable. ssDNA was difficult to detect for the MSV gene replacement mutants, possibly because none of these synthesized the capsid. However, ssDNA was always found with MSV-R2(D2) and its presence in some preparations of the gene substituted mutants indicated that it was synthesized. This variability in detecting viral ssDNA has been well documented as a common problem in all geminivirus studies (Rogers *et al.*, 1986; Stanley and Townsend, 1986; Grimsley *et al.*, 1987).

Neither deletion mutant MSV-CPΔ nor MSV-(CP+R2)Δ was found to replicate in maize. However, mutational analysis of the bipartite geminiviruses (Elmer *et al.*, 1988) as well as our own studies on a narrow streak variant of MSV (S.G.Lazarowitz and A.J.Pinder, unpublished data) have detected replicating viral DNA as small as 1.6−1.9 kb. The deleted MSV DNA replicating in the narrow streak infected tissue did not change the rightward distance between IR_c and IR_p. Thus, its seems that the replication defect in our two MSV deletion mutants is likely due to the two viral intergenic regions or other sequences being brought into close proximity and interfering with replication, rather than the result of torsional constraints. For example, replication or transcription initiating within IR_c in these deletion mutants may interfere with synthesis of the viral DNA primer located in IR_p. The ability to replicate was restored upon the addition of sequences to bring the viral DNA back to about wild-type size. CAT or Hph coding sequences inserted in either orientation into MSV-CPΔ or MSV-(CP+R2)Δ functioned in this capacity. Thus, the precise nature of the inserted sequences is apparently not critical. Similar results have recently been reported for CAT gene replacements of the capsid coding sequences in the A component of cassava

latent virus (CLV) (Ward *et al.*, 1988).

The relationship between size and ability to replicate was also addressed by our findings with MSV gene substitution mutants. At the extremes, MSV-(CP+R2)Δ−CAT (~2.5 kb) is 200 nt smaller than MSV, and MSV-CPΔ−Hph (~3.2 kb) is ~500 nt longer than the wild-type genome. Over this size range we did not detect any difference in the ability to replicate in inoculated leaves. Furthermore, we did detect an ~8 kb MSV substitution mutant replicating in the inoculated leaves from a single plant which had been agro-inoculated with pMSV-(CP+R2)Δ::pMON464 (Figure 4). This mutant contained binary vector sequences in addition to the expected MSV-(CP+R2)Δ and Hph sequences, and was probably generated by an illegitimate recombination event. This suggests that there is no inherent size limitation in the absence of encapsidation, in contrast to earlier suggestions based on studies with CLV (Stanley and Townsend, 1986). Substitution mutants several times larger than viral size can replicate.

The ability of the gene substitution mutants derived from MSV-CPΔ and point mutant MSV-R2(D2) to replicate lead to the conclusion that all of the information required for viral ssDNA and dsDNA replication is located in the 1.7 kb *Bam*HI−*Bgl*II segment of the viral genome. This segment contains both viral intergenic regions IR_c and IR_p, and the overlapping '−' strand L1′ and L1″ ORFs homologous to AL1 in the bipartite viruses (Figure 1), thus strengthening the functional analogy between the single component viral genome and the A component of the bipartite geminiviruses. In TGMV, AL1 has been shown to be the only viral gene essential for replication of both viral DNA components (Elmer *et al.*, 1988).

Elimination of the ATG start codon for the '+' strand R2 ORF [(MSV-R2(D2)] suggests that the encoded 10.9 kd protein is essential for systemic spread and symptom development. The mutation in MSV-R2(D2) does not alter spacing; nor does it affect other potential ORFs (Figure 1) (Lazarowitz, 1988). Furthermore, it is located 162 bases upstream of the 5′-end of the major '+' strand transcript which is likely to function in the synthesis of the capsid subunit (Morris-Krsinich *et al.*, 1985). A recent study utilizing a maize protoplast transient expression system and CAT reporter gene to analyze the MSV '+' strand promoter supports the notion that this is the transcript for the capsid subunit (Fenoll *et al.*, 1988). Thus, the defective MSV-R2(D2) phenotype should not be the result of a polar effect of the mutation on expression of the capsid gene since the mutated sequence is not present in the capsid transcript. Based on primer extension analysis of CaMV 'promoter-enhanced' transcription in this transient expression system, Fenoll *et al.* (1988) raised the possibility that the R2 sequences are part of a large, diffuse promoter for the capsid gene rather than a gene encoding a protein. While our attempts at genetic complementation between MSV-R2(D2) and MSV-CPΔ−CAT did not completely eliminate the possibility of the MSV-R2(D2) mutation affecting the synthesis of the capsid gene transcript, MSV-R2(D1) containing a third position conservative mutation five bases downstream from that in MSV-R2(D2) was phenotypically wild-type (Figure 7c, Table I). In light of this, it seems highly unlikely that the 2 nt change in MSV-R2(D2) would completely abolish promoter function (Meyers *et al.*, 1986) particularly in a large, diffuse promoter as postulated by these authors (Fenoll *et al.*, 1988).

Our analysis of R2, in combination with transcript mapping (Morris-Krsinich *et al.*, 1985), suggests that the gene R2 exists and encodes a protein essential for systemic spread of the virus and subsequent disease development. This conclusion strengthens the functional analogy between the single component and bipartite geminiviruses. There are three identified functions essential for systemic spread in the bipartite geminiviruses, two encoded on the B component and one provided by AL2 (Rogers *et al.*, 1986, 1988; Elmer *et al.*, 1988; Etessami *et al.*, 1988). Our results suggest two essential gene functions for movement in the single component geminiviruses provided by the capsid and the 10.9 kd protein. A direct assay for whether the MSV-R2(D2) mutation exerts a polar effect on the capsid gene will be expression of CAT activity by the double mutant containing both this mutation and the CAT coding sequences substituted for those of the capsid. Recent immunological studies have also provided evidence for the existence of the 10.9 kd protein in MSV (Mullineaux *et al.*, 1988).

Although we did not detect systemic movement of the gene substituted derivatives of MSV-CPΔ and MSV-(CP+R2)Δ, or the point mutant MSV-R2(D2), maize containing these mutants developed systemic patterns of streaks which followed cell lineages (Table I, Figure 7). The phenotypes of these plants ranged from very few 'breaks' to completely white leaves, and the chlorosis appeared to follow the vascular system, reminiscent of phenotypes observed for the *Argentia* mutation in maize (Langdale *et al.*, 1987). These systemic patterns were dependent on replication of the mutant MSV DNA and not simply the result of needle damage to the plant. The normal phloem restriction of the virus and the similarity in the appearance of this mutant phenotype to patterns seen in the *Argentia* mutation leads to the speculation that these systemic cell lineage patterns may result from mutant MSV DNA replicating in and damaging meristematic cells in tissue such as the procambium which are destined to become the vascular system of the plant. Such damage of precursor cells could result in abnormal development of the vascular system and subsequent abnormal development of bundle sheath and/or mesophyll cells involved in photosynthesis (Langdale *et al.*, 1987), thus leading to the observed cell lineage patterns. This MSV mutant phenotype further suggests the possibility that in the absence of encapsidation the viral DNA may retain its cell and/or tissue specificity.

In conclusion, our mutational analysis of MSV has identified two essential genes for systemic spread and subsequent disease development, those for the capsid subunit and the 10.9 kd (R2) protein. These results establish a functional distinction between the single component and bipartite geminiviruses, namely that the capsid subunit is essential for systemic spread in the former as opposed to the latter. However, they also suggest a functional analogy, with both types of geminiviruses encoding multiple essential functions for movement. This functional analogy is further underscored by our demonstration that all of the information required for viral DNA synthesis is located in that segment of the viral genome containing IR_c and IR_p and the '−' strand L1′ and L1″ ORFs homologous to AL1 in the bipartite viruses. Further mutational studies should directly demonstrate the role of these latter ORFs, as well as sequence elements in the two intergenic regions, in viral replication and investigate their potential processing to produce a single product. Finally, biochemical studies utilizing native or

fusion proteins synthesized in *E.coli* should begin to define the nature of these viral proteins involved in movement and replication.

Materials and methods

Cloned MSV DNAs and infectivity assays

Infectious clones of a South African isolate of MSV (MSV-S) (Lazarowitz, 1988) were used in the mutant constructions. Infectivity on maize (*Zea mays* var. Golden Bantam) was assayed by agro-inoculation (Grimsley and Bisaro, 1987; Grimsley *et al.*, 1987) using *A.tumefaciens* carrying a disarmed nopaline-type Ti plasmid, pTiT37-*SE* (strain *ASE*) (Rogers and Klee, 1987). MSV deletion and base substitution mutants were cloned as direct dimeric repeats in the binary vector pMON505 (Lazarowitz, 1988). Gene substitution mutants were constructed by insertion of MSV deletion mutants into the binary vectors pMON464 and pMON469 (see below). Extracts were prepared from agro-inoculated plants and analyzed for the presence of viral DNA as previously described (Lazarowitz, 1988). Leafhopper transmissions with *C.mbila* were done using acquisition periods of 48 h (Damsteegt, 1983). All infectivity studies and insect transmissions were done under an APHIS-USDA permit in the self-contained quarantine greenhouse facility at Frederick, MD. Materials were autoclaved and treated according to quarantine regulations prior to disposal.

Site-directed mutagenesis

Site-directed mutagenesis was performed using altered 15–21 base synthetic oligonucleotides as primers (Smith and Gillam, 1981) and uracil-containing (Kunkel, 1985) cloned template MSV DNA. Synthetic oligonucleotides were produced on an ABI380A synthesizer (Applied BioSystems) and purified by HPLC. T7 polymerase altered to have low exonuclease activity ('Sequenase', United States Biochemical Corp.) was used at sites with secondary structure which caused Klenow DNA polymerase (Klenow and Henningsen, 1970) to produce specific deletions or duplications rather than simple base substitutions (S.G.Lazarowitz, unpublished data). Sequencing of rescued recombinant ssDNA or restriction analysis of extracted dsDNA showed the mutation efficiency to be 30–50%. To ensure that any phenotypic change was caused by the introduced mutation, a 100–200 base restriction fragment containing the mutation was ligated into appropriately digested cloned MSV DNA which had not been subjected to mutagenesis, and the reconstructed mutant DNA was sequenced across the junctions and through the length of the transferred fragment.

Construction of deletion and base substitution mutants

To delete the capsid coding sequences (MSV-CPΔ), the unique *Bam*HI site located at the start codon for the 10.9 kd protein ORF (ATGGATCC) was eliminated by site-directed mutagenesis to ATGGACCC [MSV-R2(D1)] (Figure 2a). This is a third position codon change (GAT/GAC) which retains the Asp in the predicted polypeptide. A new unique *Bam*HI site was then introduced at position 306 in MSV-R2(D1), just upstream of the start of the capsid coding sequence at nt 315 (Morris-Krsinich *et al.*, 1985; Lazarowitz, 1988), and the capsid coding sequence was deleted by digestion with *Bam*HI and *Nco*I (Figure 2a). Following incubation with Klenow polymerase and deoxynucleotide triphosphates to fill-in the protruding 5'-ends (Maniatis *et al.*, 1982), the DNA was isolated on a low melting temperature agarose gel (SeaPlaque Agarose, FMC Bioproducts) (Struhl, 1983) and blunt-end ligated using T4 polynucleotide ligase (Weiss *et al.*, 1968) to regenerate the unique *Bam*HI and *Nco*I sites (Figure 2a). MSV-CPΔ retains only the last 13 amino acids of the capsid protein.

To delete both '+' strand R1 and R2 ORFs [MSV-(CP+R2)Δ], MSV DNA was digested with *Bam*HI and *Nco*I (Figure 2b) and the ends were filled-in and blunt-end ligated as described above. MSV-(CP+R2)Δ also retains the unique *Bam*HI and *Nco*I sites (Figure 2b). Since the *Bam*HI site in MSV-S forms part of the start codon for the 10.9 kd protein, this potentially interfering upstream ATG was eliminated by introduction of a second *Bam*HI site 10 nt upstream and digestion with *Bam*HI. This construction recreated the cap site sequence 2 nt closer to the potential TATA consensus sequence at position +26.

The start codon for R2 was specifically eliminated by mutation from TCATG to TAGTG [MSV-R2(D2)]. This changed the start codon from ATG to GTG and introduced a UAG stop codon at the beginning of R2.

Construction of gene substitution mutants

To construct gene substitution mutants of MSV-CPΔ and MSV-(CP+R2)Δ, MSV DNA was cloned into the binary vectors pMON464 or pMON469, each of which contained two tandem direct repeats of a particular non-viral gene coding sequence separated by a unique restriction site. pMON469, containing two copies of the bacterial CAT coding sequences, was constructed by insertion of these CAT sequences into the polylinker region of the binary vector pMON505 (Horsch and Klee, 1986) and retains the Ti plasmid right border sequence and nopaline synthase gene, and the RK2 *trfA*, *oriB* and *oriT* functions. pMON464 is analogous to pMON469 but contains two copies of the bacterial Hph coding sequences. Details of the construction of pMON469 and pMON464 will appear elsewhere (Elmer *et al.*, in preparation).

To substitute the CAT coding sequence for the corresponding deleted coding sequences, MSV-CPΔ or MSV-(CP+R2)Δ was linearized at the unique *Bam*HI site (Figure 2), and cloned into the unique *Bgl*II site of pMON469 (Figure 3). Clones containing the viral DNA inserted in both orientations were identified by restriction analysis. In one orientation, MSV-CPΔ–CAT or MSV-(CP+R2)Δ–CAT with a single copy of the CAT coding sequences directly substituted for those of either the capsid subunit alone or in combination with the 10.9 kd protein would be generated in the plant by excision via homologous recombination within the flanking CAT sequences (Figure 3). MSV-CPΔ or MSV-(CP+R2)Δ inserted in the opposite orientation would generate MSV-CPΔ–CAT′ or MSV-(CP+R2)Δ–CAT′ containing the CAT sequence in inverted orientation relative to the direction of transcription of the replaced MSV ORFs. By the same approach, pMON464 was used to construct the analogous gene replacement mutants MSV-CPΔ–Hph, MSV-CPΔ–Hph′, MSV-(CP+R2)Δ–Hph and MSV-(CP+R2)Δ–Hph′ containing, respectively, direct and inverted substitutions of Hph coding sequences for either the capsid coding sequences or both the R2 and capsid ORFs.

CAT assays

CAT assays were performed according to published protocols (Gorman *et al.*, 1982). A 100 mg sample of tissue was ground under liquid N_2 in a mortar and pestle and extracted by Dounce homogenization in 200 μl 50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Triton X100, 0.1% Sarkosyl (Masson and Fedoroff, 1989). A 25 μl aliquot of extract was assayed.

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