

RESEARCH ARTICLE

A Single Amino Acid Substitution in the Coat Protein of Cucumber Mosaic Virus Induces Chlorosis in Tobacco

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Some strains of cucumber mosaic virus (CMV) induce a bright yellow/white chlorosis in tobacco instead of the light green/dark green mosaic induced by most CMV strains. This property is controlled by RNA 3 of this tripartite virus. Recombination between cDNA clones of RNA 3 from a green mosaic strain, Fny-CMV, and a chlorotic strain, M-CMV, and inoculation of infectious transcripts of the chimeric RNAs 3, together with RNAs 1 and 2 of Fny-CMV, localized the chlorosis induction domain to a region of the coat protein gene containing two nucleotide differences. Site-directed mutagenesis of one nucleotide to change the codon for Leu¹²⁹ in the M-CMV coat protein to Pro¹²⁹ of Fny-CMV changed the phenotype from chlorotic to green mosaic, whereas the opposite change in phenotype was observed when the Pro¹²⁹ in the Fny-CMV coat protein was altered to Ser¹²⁹. Thus, the local secondary structure surrounding amino acid 129 rather than a particular amino acid per se is involved in chlorosis induction.

INTRODUCTION

Chlorosis, the loss of chlorophyll from plants, can be caused by a number of genetic and environmental factors including virus infection (Jones, 1987). Several strains (biotypes) of cucumber mosaic virus (CMV) are known that can induce chlorosis, although most strains induce a light green/dark green mosaic (Kaper and Waterworth, 1981). The M strain of CMV (M-CMV) induces chlorosis on many host species; on *Nicotiana* species it induces an extreme yellow/white chlorosis (Rao and Francki, 1982). The chlorosis domain for M-CMV was mapped to RNA 3 of this tripartite virus by reassorting the genomes of M-CMV and several nonchlorotic strains and inoculating plants with the corresponding pseudorecombinant viruses (Rao and Francki, 1982; Shintaku and Palukaitis, 1990).

RNAs 1 and 2 of CMV each encode one gene product, which together with a host protein of approximately 50 kD form the CMV replicase (Hayes and Buck, 1990). RNA 3 encodes two gene products: a 30-kD protein, believed to be involved in potentiating the cell-to-cell movement of CMV, and the 25-kD viral coat protein (Davies and Symons, 1988). We have determined the nucleotide sequence of RNA 3 of two CMV strains (Owen et al., 1990): a chlorotic strain, M-CMV, and a green mosaic strain, Fny-CMV (Roossinck and Palukaitis, 1990), a New York isolate producing a "fast" systemic infection in cucurbits. These two RNAs differ by only about 1% in nucleotide sequence (26 changes out of 2214 to 2216 nucleotides),

yet M-CMV shows considerable biological variation that has been ascribed to RNA 3 by pseudorecombination (Rao and Francki, 1982). Previously, we used biologically active cDNA clones of the three RNAs of Fny-CMV and RNA 3 of M-CMV to confirm the association of several phenotypes with M-CMV RNA 3 (Shintaku and Palukaitis, 1990). Here we show, by exchanging sequences between the cDNA clones of the corresponding RNAs 3 and site-directed mutagenesis, that either of two adjacent, single-nucleotide alterations in the coat protein gene is involved in determining the chlorosis induction phenotype in tobacco. By comparing our results with the nucleotide sequences of several chlorotic strains and several green mosaic strains (Shintaku, 1991), we conclude that the chlorosis phenotype is associated with a particular local secondary structure in the coat protein, rather than either the nucleic acid sequence itself or the identity of a particular amino acid per se.

RESULTS

Delimitation of the Chlorosis Induction Domain

A series of chimeric cDNAs was constructed between cloned cDNAs of Fny-CMV RNA 3 and M-CMV RNA 3. RNA transcripts derived from these chimeric cDNA clones were inoculated onto tobacco plants together with RNA transcripts of cDNA clones of Fny-CMV RNAs 1 and 2 (Rizzo and Palukaitis, 1990). The

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various recombinants and the phenotypes induced on tobacco are listed in Figure 1. The symptoms induced on tobacco plants inoculated with several of these constructs are shown in Figure 2.

All chimeras containing most or all of the coat protein gene of M-CMV (FMNhe, FMSal, and MFXho in Figure 1) elicited the same phenotype as induced by infection with either M-CMV RNAs 1 to 3 or Fny-CMV RNAs 1 and 2 and M-CMV RNA 3 (Figure 2 and results not shown). All of the reciprocal chimeras

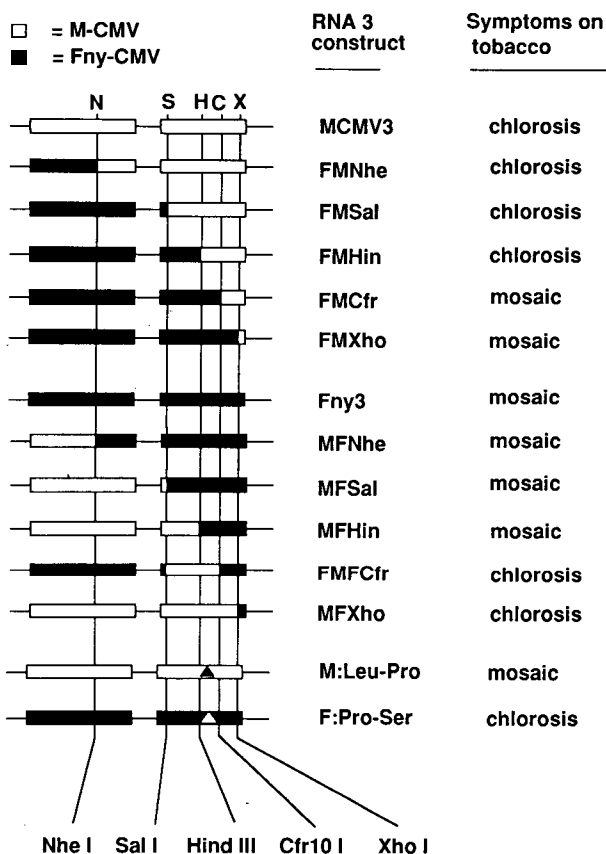


Figure 1. Schematic Diagram of RNA 3 of Fny-CMV and M-CMV and of the Various Chimeric Fny/M-CMV RNAs 3 Constructed.

The pathology induced on tobacco by RNA transcripts of each construct coinoculated with cDNA-derived transcripts of Fny-CMV RNAs 1 and 2 is also shown. The 3a gene (left) and coat protein gene (right) are represented as open or solid rectangles, depending on the viral source. The upper set and lower set are reciprocal recombinants, except for the pair listed as FMCfr and FMFCfr; the latter is a double recombinant. The construct M:Leu-Pro is a mutant of M-CMV RNA 3 generated by site-directed mutagenesis of a cDNA clone of M-CMV RNA 3, with a C at nucleotide 1642 replacing the T at the same position. The construct F:Pro-Ser was created by site-directed mutagenesis of a cDNA clone of Fny-CMV RNA 3 to replace a C with a T at nucleotide 1641. These mutations leading to amino acid substitutions of proline for leucine and serine for proline, respectively, are indicated by triangles.

(MFNhe, MFSal, and MFXho in Figure 1) elicited the light green/dark green mosaic symptoms typical of infection with Fny-CMV (Figure 2 and results not shown). Thus, the chlorosis-inducing phenotype is associated with elements of the viral coat protein gene.

Chimeras formed within the coat protein gene (FMHin and FMFCfr versus MFHin and FMCfr in Figures 1 and 2) showed that the chlorosis induction domain is associated with a small region encoding 46 amino acids of the 218-amino acid coat protein (Owen et al., 1990; Shintaku, 1991). Interestingly, chimeras that did not elicit the chlorosis phenotype (e.g., MFHin and FMCfr in Figures 1 and 2) always showed the green mosaic typical of infection by Fny-CMV. Thus, the two phenotypes are mutually exclusive, and chlorosis is associated with sequence changes in this region.

Localization of the Chlorosis Induction Sequences

Figure 3 shows the nucleotide and encoded amino acid sequence differences between the coat protein genes of M-CMV and Fny-CMV. The domain bordered by the HindIII site and the Cfr10I site (within the coat protein gene) has only two nucleotide sequence differences altering the encoded amino acid sequence of the coat protein. As a starting point for further analysis, we made two assumptions: (1) chlorosis is induced by the coat protein rather than the RNA encoding the coat protein, and (2) the Pro¹²⁹ to Leu¹²⁹ substitution was more likely to be associated with a change in phenotype than the conservative Ile¹⁰⁷ to Val¹⁰⁷ substitution. Thus, we altered nucleotide 1642 of the cDNA clone of M-CMV RNA 3 by polymerase chain reaction (PCR)-based, site-directed mutagenesis to convert the U to the C found in Fny-CMV RNA 3 (Figure 3). The effect on the amino acid sequence was to convert the Leu¹²⁹ of the M-CMV coat protein to the Pro¹²⁹ found in the Fny-CMV coat protein. Figure 2 shows that plants inoculated with transcripts of this mutant (M:Leu-Pro) cDNA clone of M-CMV RNA 3 developed symptoms identical to plants infected with Fny-CMV. Thus, a single amino acid substitution at position 129 of the coat protein is associated with chlorosis induction. In the absence of chlorosis, the green mosaic symptom typical of infection by most strains of CMV is induced.

Association of Coat Protein Structure with Chlorosis Induction

To establish that chlorosis is induced by the viral coat protein rather than by the viral RNA sequences within the coat protein gene, a comparison was made between the published nucleotide sequences of the coat protein genes of eight CMV strains: four that induce a green mosaic and four that induce chlorosis in tobacco (Shintaku, 1991). There was no correlation between changes at any particular nucleotide position and chlorosis induction; however, there was a correlation between the identity of the amino acid at position 129 and chlorosis

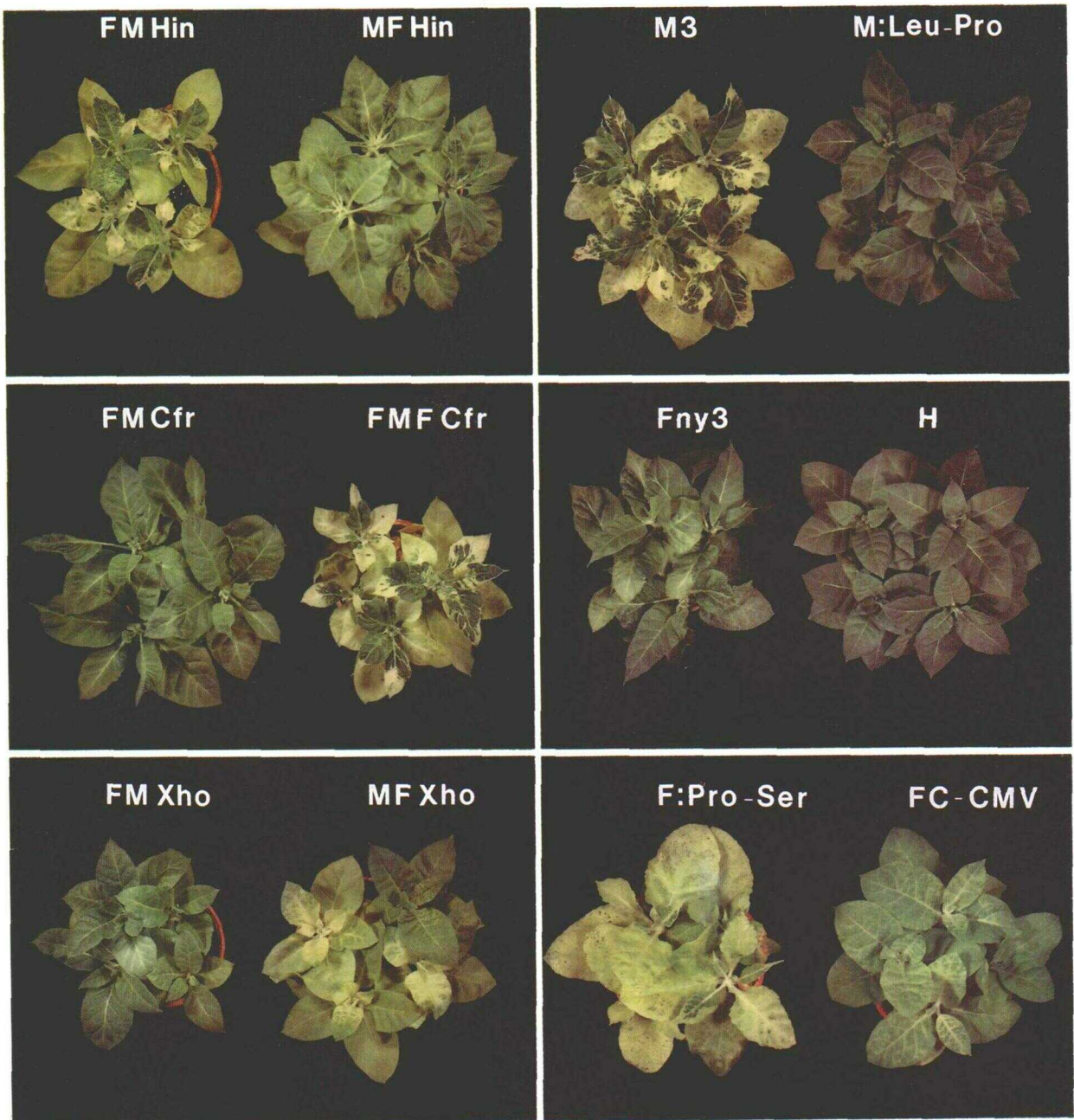


Figure 2. Symptoms Induced on Tobacco Plants by RNA Transcripts of Chimeric cDNAs of M-CMV RNA 3 and Fny-CMV RNA 3 Coinoculated with cDNA-Derived Transcripts of Fny-CMV RNAs 1 and 2, 2½ Weeks Postinoculation.

Note that the newly emerging leaves on the chlorotic tobacco plants have very little chlorosis (see text). The nature of the various chimeric constructs is indicated in Figure 1. M:Leu-Pro is a site-directed mutant of M-CMV RNA 3 (converting coat protein amino acid 129 from leucine to proline), and F:Pro-Ser is a site-directed mutant of Fny-CMV RNA 3 (converting coat protein amino acid 129 from proline to serine). FC-CMV indicates plants infected with Fulton's C strain of CMV, and H represents mock-inoculated plants.

induction (Shintaku, 1991). Four CMV strains that induce a green mosaic (C-, D-, Fny-, and O-CMV) contain a proline at this position, whereas two of the four chlorosis-inducing strains (M- and P6-CMV) contain a leucine and the other two chlorosis-

inducing strains (FC- and Y-CMV) contain a serine at position 129. In fact, the FC-CMV coat protein only differs from the Fny-CMV coat protein in this one amino acid. Therefore, the cDNA clone of Fny-CMV RNA 3 was mutated at nucleotide position

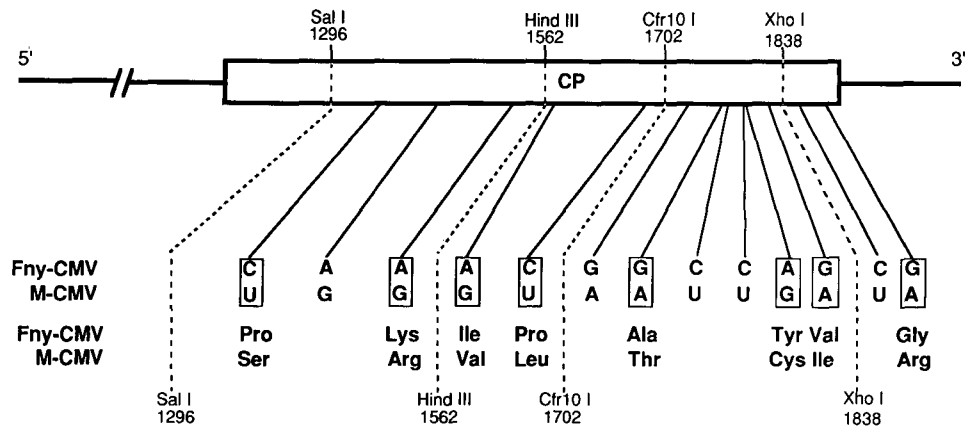


Figure 3. Schematic Diagram of the Coat Protein Gene of CMV with Flanking Sequences.

Also shown are the locations of various restriction endonuclease cleavage sites and the differences in nucleotide and encoded amino acid sequences between Fny-CMV and M-CMV for this region of RNA 3 (Owen et al., 1990). Note that there are only two nucleotide differences between the HindIII and Cfr10I sites and that both result in amino acid substitutions. Rectangle, CMV coat protein (CP); horizontal lines, flanking sequences.

1641 to convert the C to the U found in FC-CMV RNA 3. This mutant (F:Pro-Ser in Figure 1) should encode a serine at amino acid 129. Figure 2 shows that tobacco plants inoculated with transcripts of F:Pro-Ser developed yellow chlorosis. Thus, neither the particular nucleotide position (1641 for serine and 1642 for leucine) nor the particular amino acid substitution for proline (i.e., serine or leucine) seems to directly mediate the chlorotic response. Rather, the loss of the proline at position 129 results in chlorosis induction.

Computer-generated secondary structure maps of the coat proteins of M-CMV and Fny-CMV (not shown) indicated that Pro¹²⁹ disrupts a predicted β -pleated sheet in this region of the coat protein. Thus, chlorosis induction appears to be associated with a change in the local secondary structure in the coat protein of CMV.

DISCUSSION

The association of yellow chlorosis with alterations in the viral coat protein has been made previously. Mild chlorotic versus necrotic local lesions in tobacco were controlled by a single amino acid exchange in the coat protein of alfalfa mosaic virus (Neeleman et al., 1991). Artificial mutants of tobacco mosaic virus (TMV) containing in-frame deletions within the coat protein gene induced varying degrees of local or systemic chlorosis in tobacco (Dawson et al., 1988). Such plants did not produce virus particles; however, deleted coat protein molecules could be detected, albeit not accumulating within chloroplasts in the inoculated leaves (Lindbeck et al., 1991). In an earlier study, a correlation had been noted between the destruction of chloroplasts in tobacco plants infected with yellow strains and

mutants of TMV and the presence of insoluble coat protein in such plants (Jockusch and Jockusch, 1968). Virus particle formation and yellow chlorosis induction were inversely related and depended upon the temperature at which the inoculated plants were incubated (Jockusch, 1966a, 1966b). However, not all mutants of TMV with defective coat proteins induce chlorosis (Dawson et al., 1988), and not all chlorosis-inducing mutants produce insoluble coat protein (Siegel et al., 1962), although the extent of chlorosis may correlate with the solubility of the coat protein.

TMV coat protein from systemic symptom-inducing strains has been found to become associated with thylakoid membranes in leaves of systemically infected tobacco plants at 10 to 50 times the level of coat protein from a masked strain (Reinero and Beachy, 1986), although there was no difference in the level of associated coat protein from white or yellow chlorosis-inducing strains versus a light green/dark green mosaic strain. (The ratio of soluble to insoluble protein was not reported.) The white, chlorosis-inducing strain inhibited electron transport and also reduced the level of a 44-kD protein associated with photosystem II (Reinero and Beachy, 1989).

Y-CMV, which also induces a systemic, yellow chlorosis, elicits diffuse chlorotic spots on the inoculated leaves of tobacco plants. In these organs, the levels of two related proteins (22 and 23 kD) in the oxygen-evolving complex of photosystem II were reduced (Takahashi et al., 1991). Overall, however, yellow chlorosis appears to be associated with alterations in chloroplast development and the presence of virus in infected cells because the inoculated leaf and fully expanded leaves above the inoculated leaf do not develop severe chlorosis, although they contain virus (Rao and Francki, 1982; M. H. Shintaku, L. Zhang, and P. Palukaitis, unpublished observations). Chloroplasts in tobacco infected with the chlorotic CMV

strain P6-CMV (a strain of CMV from which the mutant M-CMV was derived [Price, 1934; Mossop et al., 1976]) showed fewer grana and numerous small vacuoles that sometimes were associated with the inner chloroplast membrane (Roberts and Wood, 1982). In addition, myelinlike membrane structures, presumably related to chloroplasts, were also seen in infected cells (Roberts and Wood, 1982). Chlorosis was also correlated with decreases in cytoplasmic and chloroplast ribosome concentrations but neither with a decrease in chloroplast DNA-dependent RNA polymerase activity found in normally expanding leaves nor with any increase in either protease or ribonuclease activity (Roberts and Wood, 1981). However, in these and other biochemical studies (Kato and Misawa, 1974), it was not possible to differentiate cause from effect.

The nature of the interactions between CMV coat protein and the host plant that lead to chlorosis is unknown. Whether the severe, yellow chlorosis is induced by free coat protein or by assembled virus particles is also not known, although the work with TMV would suggest the former (see above). This is supported by work involving turnip crinkle virus, in which a single amino acid exchange reduced the extent of chlorosis in *Nicotiana benthamiana* but did not appear to affect the structure of the virus particle (Heaton et al., 1991). This altered amino acid is not located on the surface of the virus particle but rather at a hinge between the shell and the protruding domain of each subunit in the virus, blocking its interaction with the cellular environment. Hence, in these cases the free coat protein subunits are the most likely elicitors. It would be interesting to determine if the model of free, insoluble coat protein being involved in yellow chlorosis induction by TMV strains (Jockusch and Jockusch, 1968) could be extended to other viruses and also if such "insoluble" coat protein then becomes associated with thylakoid membranes and somehow disrupts electron transport mediated by photosystem II (Reinero and Beachy, 1986, 1989; Takahashi et al., 1991), or if chlorosis is associated with effects on nuclear genes involved in chloroplast maintenance and/or development.

In addition to viral factors, some degree of host specificity is involved in yellow chlorosis induction by CMV because the extent of chlorosis on other host species is not as great as on *Nicotiana* species (Rao and Francki, 1982). Also, in tobacco there is a recovery phenomenon after CMV infection in which several layers of leaves are often symptomless and contain very little virus (see new leaves in Figure 2). This is followed by an acute infection phase, which cycles with a symptomless phase (Loebenstein et al., 1977). The temperature of incubation does not significantly affect the extent of chlorosis (at temperatures below which virus replication proceeds normally) (M. H. Shintaku, L. Zhang, and P. Palukaitis, unpublished observations), in contrast to chlorosis induced by specific sequences in satellite RNAs associated with some strains of CMV (Palukaitis, 1988). In the latter case, a few nucleotide changes in an RNA molecule also affect chlorosis (Sleat and Palukaitis, 1992), although these effects are not mediated via a protein intermediate (Masuta and Takanami, 1989; Jaegle et al., 1990).

Thus, numerous factors are involved in the induction of chlorosis. The viral coat protein sequence/structure alteration involved in chlorosis induction identified here is but one factor.

METHODS

Construction of Chimeric cDNA Clones

Full-length cDNA clones of Fny-cucumber mosaic virus (CMV) RNA 3 (Rizzo and Palukaitis, 1990) and the M strain of CMV (M-CMV) RNA 3 (Shintaku and Palukaitis, 1990) were used to create chimeric cDNA clones by standard procedures (Maniatis et al., 1982). The restriction endonuclease sites indicated in Figure 1 and a unique PstI site downstream of the 3' end were used to exchange segments in most of the chimeric constructs. The HindIII chimeras were made using HindIII alone (which also has one additional site in the downstream polylinker), whereas the Cfr10I constructs involved ligations of three fragments: FMCfr was constructed by replacing the Sall to PstI fragment of the Fny-CMV RNA 3 clone with the Sall to Cfr10I fragment of the Fny-RNA 3 clone and the Cfr10I to PstI fragment of the M-RNA 3 clone (Figure 1); and FMFCfr was constructed by replacing the Sall to PstI fragment of the Fny-RNA 3 clone with the M-RNA 3 Sall to Cfr10I fragment and the Fny-RNA 3 Cfr10I to PstI fragment (Figure 1).

Characterization of Chimeric cDNA Constructs

Verification of the nature of the constructs was done by sequencing various regions of the chimeric cDNAs using oligonucleotide primers complementary to (+) Fny/M-CMV RNA 3 (Owen et al., 1990; Shintaku, 1991).

Site-Directed Mutagenesis

The polymerase chain reaction (PCR)-based, site-directed mutagenesis procedure of Higuchi et al. (1988) was used to alter nucleotide 1642 of a cDNA clone of M-CMV RNA 3 (pMCMV3). A subclone representing nucleotides 1562 to 1838 (HindIII to XhoI sites) was constructed in pBluescript SK- (Stratagene, La Jolla, CA). Two complementary, mutagenic oligonucleotides, 1642+ (5'-CGTAAAG-TTCTGCCTCCTC-3') and 1642- (5'-GAGGAGGCAGGAACCTT-ACG-3'), and the T7 and T3 promoter primers (Stratagene) were used in the PCR (Higuchi et al., 1988). Thirty-four cycles of amplification were performed with the following parameters: denaturation at 92°C for 1 min, annealing at 42°C for 1 min, and synthesis at 72°C for 1 min. All PCR reactions were performed in 100 μ L using the buffers and protocols supplied by the enzyme manufacturer (New England Biolabs, Beverly, MA). The cycling parameters were identical in the primary and secondary reactions. The product of the secondary reaction was phenol extracted, ethanol precipitated, digested with HindIII and XhoI, and cloned into pBluescript SK- by standard procedures (Maniatis et al., 1982). The nucleotide sequence of the entire cloned fragment was determined to verify the presence of the expected mutation, and the cloned fragment was then subcloned back into pMCMV3. The presence of the mutagenized fragment was verified by sequencing the region around nucleotide 1642. The same procedure was used to alter nucleotide 1641 of a cDNA clone of Fny-CMV RNA 3 (pFny309),

using the mutagenic oligonucleotides 1641+ GTCCTGAAAGTTTCT-GCCTCCTCGG and 1641- CCGAGGAGGCAGAACTTTACGGAC to convert nucleotide 1641 from a C to a U.

Biological Assays

Six *Nicotiana tabacum* plants at the four- to six-leaf stage were incubated in the dark for 24 hr and dusted with Carborundum. They were inoculated with viral RNA transcripts as previously described (Rizzo and Palukaitis, 1990). The inoculated plants were incubated in an environmentally regulated growth chamber at 25°C under a 16-hr photoperiod. Symptom development occurred after 5 to 7 days and continued for several weeks before symptomless leaves began to appear. Viral RNA transcribed from the chimeric RNA 3 constructs was coinoculated with transcripts of cDNA clones of Fny-CMV RNAs 1 and 2 at least twice (and usually more often) for each construct.

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