Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco

Lee Zhang, Kaoru Hanada† and Peter Palukaitis*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853, U.S.A.

Cucumber mosaic cucumovirus (CMV) can be divided into two subgroups, I and II. LS-CMV and most other subgroup II strains cause a mild, systemic mottle on tobacco and can induce a necrotic etching (necrotic rings) symptom on inoculated tobacco leaves. In contrast, Fny-CMV and most other subgroup I strains cause severe, systemic mosaic symptoms on tobacco, but do not induce the necrotic etching symptom. Full-length cDNA clones of all three genomic RNAs of LS-CMV were constructed and infectious RNAs were generated from these clones. Using pseudorecombinants constructed from the infectious transcripts of LS-CMV and Fny-CMV, we found that both RNAs 1 and 2 of Fny-CMV are involved in determining the severity of systemic symptom on tobacco, and that LS-CMV RNA 3 contains the determinant for the necrotic etching symptom. Chimeras formed between Fny- and LS-CMV RNA 3 were used to demonstrate that the inducer of the necrotic etching symptoms mapped to the 5' 618 nucleotides of LS-CMV RNA 3, and required sequences in both the 5' non-translated region, as well as the 3a gene of CMV.

Introduction

Cucumber mosaic cucumovirus (CMV) has a positive-sense, single-stranded genome consisting of three RNAs, namely RNA 1, RNA 2 and RNA 3. RNAs 1 and 2 encode one protein each, both of which are components of the viral replicase (Nitta et al., 1988; Hayes & Buck, 1990a), while RNA 3 encodes two proteins: the 3a putative movement protein and the 25-5K viral coat protein (reviewed in Palukaitis et al., 1992). CMV is one of the most widespread viruses known. CMV exists as a collection of strains with overlapping host ranges encompassing over 800 plant species (Douine et al., 1979; Kaper & Waterworth, 1981; Palukaitis et al., 1992). The numerous strains of this virus, with a worldwide distribution, show differences in pathology and/or host range (Kaper & Waterworth, 1981). CMV strains can be divided into two subgroups, I and II (Palukaitis et al., 1992). The Fny strain of CMV (Fny-CMV), like most subgroup I strains, causes severe, systemic mosaic symptoms on tobacco. The full-length cDNA clones of all three RNAs of Fny-CMV were constructed previously in this laboratory (Rizzo & Palukaitis, 1990). The LS strain of CMV (LS-CMV) is a subgroup II strain of CMV and causes milder, systemic symptoms on tobacco (Wahyuni et al., 1992) as well as symptoms on the inoculated leaves consisting of numerous necrotic rings, or 'etching' (Fig. 1a). This latter symptom is specific to subgroup II strains, and is exhibited by over 20 subgroup II isolates in our collection (unpublished observation). By contrast, Fny-CMV (Fig. 1b) and over 30 subgroup I isolates in our collection do not cause the necrotic etching symptom (unpublished data). This symptom was also induced by the subgroup II strains W (Roberts & Wood, 1982) and R-CMV (Marchoux et al., 1975). Using pseudorecombinants involving gel-purified viral RNAs between D-CMV (subgroup I) and R-CMV (subgroup II), Marchoux et al. (1975) demonstrated that the induction of necrotic etching mapped to R-CMV RNA 3. Here we describe the cloning of cDNAs representing the RNAs of LS-CMV, as well as the construction of pseudorecombinants and recombinants between LS-CMV and Fny-CMV and their use to delimit which viral sequences are involved in the above pathogenic responses in tobacco.

Methods

Construction of full-length cDNA clones of LS-CMV RNAs 1, 2 and 3. The construction of the full-length cDNA clones of all three RNAs of LS-CMV was done as follows. Total LS-CMV RNAs were used as a template for the first-strand cDNA synthesis. First- and second-strand cDNA were synthesized following the protocol and materials provided in the cDNA synthesis kit (Amersham). An oligonucleotide

† Present address: Kyushu National Agricultural Experiment Station, Kikuchi-gun, Kumamoto 861-11, Japan.
LS-CMV RNA. This primer contains an *NheI* site (underlined). After first-strand cDNA synthesis, second-strand cDNA was primed using oligonucleotide 5′GGGATCCCTAATACGACTCACTATAGTTAA

TCTTAC 3′. This primer was also used in the construction of the full-length cDNA clone of Fny-CMV RNA 3 (Rizzo & Palukaitis, 1990).

The cDNAs representing the 5′ termini of LS-CMV RNAs 1, 2 and 3 were amplified by the polymerase chain reaction (PCR) (Sambrook et al., 1989) and then cloned into the plasmid vector pUC18. The full-length cDNA clones of LS-CMV were constructed by ligation of 5′-proximal PCR cDNA products of each RNA to the corresponding 3′-coterminal cDNA clones, using the restriction enzyme sites specified by the primers.

**Construction and characterization of pseudorecombinants of Fny- and LS-CMV.** The Fny-CMV cDNA clones were linearized with *PstI* (Rizzo & Palukaitis, 1990), while pLS-CMV 1, pLS-CMV 2 and pLS-CMV 3 were linearized with *SacI*, *SphI* and *BamHI*, respectively.

The cDNA-derived infectious RNAs were generated according to the methodology of Ahlquist (1986). The nucleotide sequence of the 5′-proximal 700 nucleotides [containing the 5′ end nontranslated region (NTR) and the N-terminal 2/3 of the 3a gene] of Fny-CMV RNA 3 was determined by partial RNA sequencing and by a ribonuclease protection assay following the protocols and materials provided in the RT RNA sequencing kit (United States Biochemical) and the RPA II kit (Ambion), respectively.

**Construction of RNA 3 chimeras between Fny- and LS-CMV.** The first pair of reciprocal chimeras was constructed using the common *BamHI* site upstream of the T7 promoter and the *NheI* site which is in the 3a gene, 618 nucleotides from the 5′ end of LS-CMV RNA 3 and 641 nucleotides from the 5′ end of Fny-CMV RNA 3. The second pair of chimeras was constructed by exchanging *KpnI* fragments, located at nucleotides 132 and 633 in Fny-CMV RNA 3 and nucleotides 108 and 609 in LS-CMV RNA 3. In vitro transcripts were generated from these RNA 3 RNA 3 chimeras, *FLFK* 3 and *F1L2L~* 3, *LFLK* 3 and *LF1L~* 3, *LFL2L~* 3 and *LFL1L~* 3. Each parental and pseudorecombinant RNA, without further purification, was diluted with 1 vol. 50 mM-sodium phosphate, pH 8.6, and inoculated (at 100 to 250 μg/ml) onto tobacco plants (*Nicotiana tabacum* cv. Xanthi nc). All the tobacco plants to be inoculated with transcripts were kept in the dark for 24 to 48 h and lightly dusted with carborundum before inoculation. Virus from the infected tobacco plants was purified (Palukaitis & Zailtin, 1984) and the progenies of all pseudorecombinants were characterized by partial RNA sequencing (data not shown) and by a ribonuclease protection assay following the protocols and materials provided in the RT RNA sequencing kit (United States Biochemical) and the RPA II kit (Ambion), respectively.

**Partial RNA 3 sequence comparison and secondary structure analysis.** The nucleotide sequence of the 5′-proximal 700 nucleotides [containing the 5′ end nontranslated region (NTR) and the N-terminal two-thirds of the 3a gene] of LS-CMV RNA 3 was determined by partial dioxygenynucleotide sequencing of LS-CMV RNA 3 as well as cDNA clones of LS-CMV RNA 3 following the protocols and materials provided in the RT RNA sequencing kit and Sequenase kit, respectively.

The nucleotide sequences of the 5′-proximal 350 nucleotides (containing the 5′ end NTR and N-terminal part of 3a gene) of Fny-, LS-CMV RNA 3 and two RNA 3 chimeras *FLFK* 3 and *LFLK* 3 were folded into secondary structures by a computer program (GGC: Squiggles).
Results and Discussion

Construction of full-length cDNA clones of LS-CMV and formation of six pseudorecombinants between Fny- and LS-CMV

Attempts to make full-length cDNA clones of the LS-CMV RNAs using the PCR and primers corresponding to the termini of each CMV RNA (Hayes & Buck, 1990b; Boccard & Baulcombe, 1992) were unsuccessful in our hands. However, since terminal primers could be used to PCR amplify full-length cDNA of Fny-CMV RNA 2 using the cDNA clone pFny209 as a template (unpublished data) we assume the problem lies in the cDNA synthesis step from the RNA template. Thus, we used a three-step approach to make full-length cDNAs to each LS-CMV RNA. (i) A cDNA library was made using a primer specific to the 3’ termini of each CMV RNA and a commercial cDNA synthesis kit. These cDNA clones contained sequences of either 50 % (RNA 1) or 80 to 95 % (RNAs 2 and 3) of the LS-CMV RNAs. (ii) Primers complementary to internal sequences (deduced from the 3’-coterminal cDNA clones) and anti-complementary to the 5’ terminus of each corresponding (subgroup II) Q-CMV RNA were used to prepare double-stranded cDNA to the 5’-proximal regions. These cDNAs were amplified by the PCR prior to cloning. (iii) The corresponding cDNA clones of the 5’-proximal and 3’-coterminal sequences of each LS-CMV RNA were ligated together to construct full-length cDNA clones.

RNAs transcribed from each full-length cDNA clone were infectious when combined and inoculated to tobacco plants (Table 1). The RNA transcripts from the full-length cDNA clones of LS- and Fny-CMV RNAs 1,

Table 1. Properties of Fny-CMV, LS-CMV and their pseudorecombinants in tobacco

<table>
<thead>
<tr>
<th>Virus*</th>
<th>Severity of systemic symptoms†</th>
<th>Local etching symptoms</th>
<th>Virus yield (μg/g)‡</th>
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<tr>
<td>F1F2F3</td>
<td>++ + +</td>
<td>No</td>
<td>176</td>
</tr>
<tr>
<td>L1L2L3</td>
<td>+ + +</td>
<td>Yes</td>
<td>80</td>
</tr>
<tr>
<td>F1L2F3</td>
<td>++ + +</td>
<td>Yes</td>
<td>80</td>
</tr>
<tr>
<td>L1L2F3</td>
<td>++ No</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>F1L2F3</td>
<td>++ +</td>
<td>No</td>
<td>73</td>
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<tr>
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</tr>
<tr>
<td>LS</td>
<td>+</td>
<td>Yes</td>
<td>106</td>
</tr>
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* Purified virus at 50 μg/ml was inoculated to eight tobacco plants in each of four separate experiments.
† ++ + +, Severe symptoms; ++ +, intermediate symptoms; +, mild symptoms.
‡ Yield data are from one experiment.

Fig. 2. Autoradiogram of a ribonuclease protection assay of RNAs from Fny-CMV, LS-CMV and all six pseudorecombinants. The 32P-labelled probes were transcripts complementary to LS-CMV RNA 1 (nucleotides 2011 to 2425) (a), LS-CMV RNA 2 (nucleotides 2003 to 2434) (b) or LS-CMV RNA 3 (nucleotides 1523 to 1845) (c). The probes were hybridized to the viral RNAs, digested with ribonuclease and analysed by denaturing polyacylamide gel electrophoresis and autoradiography.

Fig. 3. Schematic diagram of the various chimeric Fny-/LS-CMV RNAs 3 and their ability to induce etching symptoms on tobacco. All RNA 3 constructs were inoculated on tobacco in the presence of cDNA derived transcripts of Fny-CMV RNAs 1 and 2. The 3a and coat protein (cp) genes are represented as open (LS-CMV) or filled (Fny-CMV) boxes. The restriction enzyme sites (KpnI and NheI) used for construction of the chimeras (FLN3, LFN3, FLFK3 and LFLK3) are indicated.
2 and 3 were combined to form six different pseudorecombinants: F1F2F3, F1L2F3, F1F2L3, L1F2F3, L1F2L3, and L1L2F3. The composition of these pseudorecombinants were confirmed by partial RNA sequencing (data not shown) and by a ribonuclease protection assay (Fig. 2). Thus, all of the pseudorecombinants containing either RNA 1 of LS-CMV (Fig. 2a), RNA 2 of LS-CMV (Fig. 2b) or RNA 3 of LS-CMV (Fig. 2c) protected probes specific to those RNAs, whereas the other pseudorecombinants did not. These results were confirmed using the reciprocal Fny-CMV RNA specific probes (data not presented).

**Systemic symptom determinants of Fny-CMV**

The Fny-CMV infected tobacco plants usually show severe symptoms on the systemically infected leaves and no symptoms on the inoculated leaves. The symptoms of both Fny-CMV and F1F2F3 appear as a light-green/dark-green mosaic with leaf distortions (see Shintaku et al., 1992). In contrast to Fny-CMV, LS-CMV causes mild mottle symptoms in systemically infected tobacco. To map which of the three RNAs of Fny-CMV determines the systemic symptom on tobacco, we inoculated tobacco with the Fny-/LS-CMV virus and observed the symptoms on the inoculated leaves and the systemically infected leaves of tobacco. The symptoms observed were consistent with those of Fny-CMV, suggesting that the 3a gene of Fny-CMV is the systemic symptom determinant.
Symptom determinants of CMV

Local (necrotic etching) symptom determinants of LS-CMV

LS-CMV causes symptoms on the inoculated leaves of tobacco consisting of numerous necrotic rings, or ‘etching’ (Fig. 1 a). Fny-CMV does not cause the necrotic etching symptom (Fig. 1 b). Using the same pseudorecombinants (i.e. \( F_1 F_2 L_3, F_1 L_2 F_3, F_1 L_2 L_3, F_1 F_2 L_3, L_1 F_2 F_3, L_1 F_2 L_3 \) and \( L_1 L_2 F_3 \)) the necrotic etching symptoms on the inoculated leaves were induced when the inoculum consisted of any combination that included LS-CMV RNA 3 (i.e. \( F_1 F_2 L_3, F_1 L_2 L_3 \) and \( L_1 L_2 F_3 \); Table 1). Thus, RNA 3 of LS-CMV determines the production of the necrotic etching symptom on the inoculated leaves of tobacco.

To delimit which sequences within RNA 3 determine the induction of the necrotic etching symptom on the inoculated leaves of tobacco, chimeric recombinants were constructed between cDNA clones of RNA 3 of Fny- and LS-CMV. A pair of reciprocal chimeras was constructed using the common \( NheI \) site, 618 nucleotides from 5’ end of LS-CMV RNA 3, and 641 nucleotides from the 5’ end of Fny-CMV RNA 3. In vitro transcripts of these chimeras (FLN\(_3\) and LFN\(_3\)) were combined with pseudorecombinants, \( F_1 F_2 L_3, F_1 L_2 F_3, F_1 L_2 L_3, L_1 F_2 F_3, L_1 F_2 L_3 \) and \( L_1 L_2 F_3 \). The results showed that the \( F_1 F_2 L_3 \) infected tobacco plants produced severe systemic symptoms and \( L_1 L_2 F_3 \) infected plants exhibited mild systemic symptoms. While the severity of symptoms caused by \( F_1 L_2 F_3 \) and \( L_1 F_2 L_3 \), \( F_1 L_2 L_3 \) and \( L_1 F_2 F_3 \) were very similar, they were not as severe as those caused by \( F_1 F_2 F_3 \) or \( F_1 F_2 L_3 \), but were more severe than those caused by either LS-CMV, \( L_1 L_2 F_3 \) or \( L_1 L_2 L_3 \) (Table 1). Therefore, we conclude that RNAs 1 and 2 of Fny-CMV are both involved in determining the severity of the systemic symptoms in tobacco.

The yields of purified virus for the six pseudorecombinants, LS-CMV and the reconstituted parental virus, \( L_1 L_2 L_3 \), were similar (Table 1), and all were less than for the parental virus, \( F_1 F_2 F_3 \). Thus, since \( F_1 F_2 L_3 \) and \( L_1 L_2 L_3 \) produced identical yields of virus, but \( F_1 F_2 L_3 \) produced systemic symptoms identical to \( F_1 F_2 F_3 \), the severity of the systemic symptoms does not correlate with the level of accumulation of the virus. The yield data also suggest there is no incompatibility between the encoded 1a and 2a proteins of the two subgroups that might affect RNA accumulations, and indirectly the pathology.

Fig. 5. Secondary structure models of the 5’ terminal 350 nucleotides of Fny-CMV RNA 3 (a) and LS-CMV RNA 3 (b) and two reciprocal chimeras of Fny- and LS-CMV RNA 3, FLFK\(_3\) (c) and LFLK\(_3\) (d).
transcripts of Fny-CMV RNAs 1 and 2 and then inoculated onto tobacco plants. The chimera containing LS-CMV RNA 3 sequences 5' of the NheI site (F3,F3LFN3) induced necrotic ringspots (etching) on the inoculated leaf, whereas the reciprocal chimera (F3,F3FLN3) did not (Fig. 3). A second pair of chimeras was constructed by exchanging KpnI fragments to determine whether the 5'NTR or the 3a gene contained the sequences eliciting the necrotic etching symptoms. (Although the first KpnI site is within the amino terminus of the 3a gene, there are no nucleotide differences between the initiation codon and the first KpnI site.) In vitro transcripts were generated from these two reciprocal RNA 3 cDNA constructs, FLFK3 and LFLK3, separately mixed with the Fny-CMV RNAs 1 and 2 transcripts, and inoculated onto tobacco plants. No tobacco plants inoculated with either of the KpnI chimeras (FLFK3 or LFLK3) showed the necrotic etching symptom (Fig. 3), indicating that either sequences in both the 5'NTR and the amino-terminal two-thirds of the LS-CMV 3a gene, or a secondary structure involving both regions and unique to the parental LS-CMV RNA 3, are important for the induction of the necrotic etching symptom in tobacco (Fig. 1a).

**Partial RNA 3 sequence comparison and secondary structure analysis**

To further delimit the mechanism of 'etching' induction by the 5'-proximal ca. 650 nucleotides of LS-CMV RNA 3, but not by the corresponding sequences of Fny-CMV RNA 3, the nucleotide sequence of the 5'-proximal 700 nucleotides (containing the 5'NTR and the amino-terminal two-thirds of the 3a gene) of LS-CMV RNA 3 was determined and aligned to the sequence of the same region of Fny-CMV RNA 3 (Fig. 4). The 5'NTR of Fny-CMV RNA 3 contains additional nucleotides compared to the 5'NTR of LS-CMV RNA 3. In addition, there are 51 nucleotide differences (including deletions) between the 5'NTRs of the two RNAs 3, as well as 110 nucleotide differences within the 3a gene up to the NheI site. There are also 22 amino acid sequence differences in the encoded 3a protein up to the NheI site (Fig. 4). The differences between Fny- and LS-CMV RNA 3 from the 5' end to the NheI site are too numerous to indicate which RNA or amino acid sequences could be the determinants for the induction of the necrotic etching symptom on the inoculated leaves of tobacco.

The 5'-proximal 350 nucleotide termini of RNA 3 of Fny-CMV and LS-CMV, and of the RNA 3 chimeras, FLFK3 and LFLK3, were folded into putative secondary structures (Fig. 5). The predicted secondary structures of Fny-CMV RNA 3 (F3) and LS-CMV RNA 3 (L3) are very different from each other (Fig. 5a, b). The predicted secondary structure of FLFK3 (Fig. 5c) is similar to that of F3, but not to L3. This is consistent with FLFK3 being unable to induce the necrotic etching symptom (Fig. 3). The predicted secondary structure of LFLK3 (Fig. 5d) is more similar to that of L3, but they are still significantly different from each other in some regions. Another difference between L3 and LFLK3 is that the amino-terminal two-thirds of the 3a protein of LFLK3 is from F3. Thus, if both the secondary structure of the RNA, and also the 3a protein are important factors in the induction of the necrotic etching symptom, then LFLK3 also would not be able to induce necrotic etching. Hence, the necrotic etching symptom, which shows a distribution pattern suggestive of the path of virus movement (radial from the sites of infection and along veins; Fig. 1a), may be induced by interactions involving host factors with RNA sequences folded into specific structures and also with virus-encoded proteins, but not the latter alone. Experimental determination of the RNA secondary structure in the 5' 350 nucleotides of the various RNAs shown in Fig. 5 may help to delimit the various models for the nature of the inducer of the necrotic etching symptom.

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**References**


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