Symptom induction by **Cowpea chlorotic mottle virus** on *Vigna unguiculata* is determined by amino acid residue 151 in the coat protein

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The type strain of **Cowpea chlorotic mottle virus** (CCMV-T) produces a bright chlorosis in cowpea (*Vigna unguiculata* cv. California Blackeye). The attenuated variant (CCMV-M) induces mild green mottle symptoms that were previously mapped to RNA 3. Restriction fragment exchanges between RNA 3 cDNA clones of CCMV-T and CCMV-M that generate infectious transcripts and site-directed mutagenesis indicated that the codon encoding amino acid residue 151 of the coat protein determines the symptom phenotypes of CCMV-T and CCMV-M. Amino acid 151 is within an α-helical structure required for calcium ion binding and virus particle stability. No differences in virion stability or accumulation were detected between CCMV-T and CCMV-M. Mutational analysis suggested that the amino acid at position 151 and not the nucleotide sequence induce the symptom phenotype. Thus, it is likely that subtle influences by amino acid residue 151 in coat protein–host interactions result in chlorotic and mild green mottle symptoms.

**Cowpea chlorotic mottle virus** (CCMV), a member of the genus *Bromovirus*, contains a tripartite, single-strand RNA genome of positive polarity (Ahlquist, 1999). The genome of the CCMV type strain (CCMV-T) has been cloned and sequenced (Allison et al., 1989; Dzianott & Bujarski, 1991). RNA 1 (3.2 kb) and RNA 2 (2.8 kb) encode proteins required for replication. The dicistronic RNA 3 (2.2 kb) contains open reading frames for the 3a movement protein (MP) and the coat protein (CP). The CP is translated from RNA 4, a subgenomic RNA transcribed from minus-strand RNA 3.

CCMV-T induces an extensive systemic chlorosis in cowpea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye') (Kuhn, 1964). Continuous propagation of CCMV-T in cowpea resulted in an attenuated variant, CCMV-M, which induces mild green mottle symptoms (Kuhn & Wyatt, 1979). The specific infectivities of CCMV-T and CCMV-M were similar, as were their levels of replication in cowpea (Kuhn & Wyatt, 1979). Using pseudorecombinants generated with isolated genomic RNAs from CCMV-T and CCMV-M, the determinant of systemic symptoms was mapped to RNA 3 (Kuhn & Wyatt, 1979). In this report, we exchanged regions between biologically active cDNA clones of RNA 3 from CCMV-T and CCMV-M and localized the symptom determinant to the CP gene. Further analysis by site-directed mutagenesis identified CP amino acid residue 151 as a determinant in systemic disease symptoms in cowpea.

Cowpea plants (cv. California Blackeye) were used in all experiments. Purified viruses, *in vitro* transcripts from infectious viral cDNA clones or homogenates from infected cowpea leaves were inoculated onto primary leaves of 8-day-old cowpea seedlings. Virus- or mock-inoculated plants were maintained in either a growth chamber (14 h light/10 h dark cycle) at 25 °C or a greenhouse at a similar temperature. Inoculated plants were evaluated for infection by symptomology and serology (Western blot analysis or protein-A enzyme-linked immunosorbent assay, PAS-ELISA) (Edwards & Cooper, 1985) using polyclonal antibodies specific for the CP of CCMV-T.

RT–PCR (Deom et al., 2000) was used to synthesize and amplify cDNA representing full-length CCMV-M RNA 3 from purified CCMV-M RNA (Kuhn & Wyatt, 1979). The 5′-prime was 5′ CGGGGTACCTATAGACTGATATTGGTGATCTTACCACAAAC 3′, which contains a T7 promoter (underlined) and a unique 5′-flanking *KpnI* site (italics). The 3′-primer was 5′ TGGCTAGATGTTCTTCTTAGATAGTCACC 3′, which is complementary to the 3′ end of CCMV-T RNA 3 and contains a unique 5′-flanking *XbaI* site (italics). The primers were based on the published sequence of CCMV-T (Allison et al., 1989). PCR products were ligated into the *KpnI*-XbaI sites of pUC19 (Life Technologies). Biologically active transcripts were synthesized from full-length cDNA clones of CCMV-T RNA 1, RNA 2 and RNA 3 (pCCT1, pCCT2 and pCCT3, respectively; S. Quan & C. Deom, 2000).
Table 1. Nucleotide differences in RNA 3 of CCMV type (T) and mild (M) strains and subsequent predicted amino acid substitutions

<table>
<thead>
<tr>
<th>Sequence domain</th>
<th>Base change T → M</th>
<th>Amino acid change T → M</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP gene</td>
<td>C&lt;sup&gt;913&lt;/sup&gt; → U&lt;sup&gt;913&lt;/sup&gt;</td>
<td>Silent</td>
</tr>
<tr>
<td>MP gene</td>
<td>A&lt;sup&gt;1098&lt;/sup&gt; → U&lt;sup&gt;1098&lt;/sup&gt;</td>
<td>Lys-286 → Arg-286</td>
</tr>
<tr>
<td>Intergenic</td>
<td>C&lt;sup&gt;1291&lt;/sup&gt; → A&lt;sup&gt;1291&lt;/sup&gt;</td>
<td>Nontranslated</td>
</tr>
<tr>
<td>Intergenic</td>
<td>Deletion of 7 A residues from poly(A) tract</td>
<td>Nontranslated</td>
</tr>
<tr>
<td>CP gene</td>
<td>C&lt;sup&gt;1813&lt;/sup&gt; → U&lt;sup&gt;1806&lt;/sup&gt;</td>
<td>Ala-151 → Val-151</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic of chimeric CCMV RNA 3 cDNA clones and symptoms induced by in vitro transcripts from each clone. Five unique restriction sites (B, BsuWI; H, HpaI; K, KpnI; M, MfeI; X, XbaI) were used for constructing chimeric RNA 3 cDNA clones. Four to six cowpea seedlings were co-inoculated with in vitro transcripts from pCCT1, pCCT2 and pCCM3; or pCCT1, pCCT2 and the chimeric RNA 3 cDNA clones. Each experiment was replicated three to five times with independently synthesized in vitro transcripts. Plasmid pCCT3 contains a full-length cDNA of CCMV-T RNA 3. Plasmid pCCM3 contains a full-length cDNA of CCMV-M RNA 3. MP, movement protein gene; CP, coat protein gene.

unpublished results), CCMV-M RNA 3 (pCCM3) and from chimeric RNA 3 cDNA clones using a RiboMAX Large Scale RNA Production System-T7 (Promega).

In vitro transcripts from each of five CCMV-M RNA 3 cDNA clones were co-inoculated onto cowpea plants with in vitro transcripts from pCCT1 and pCCT2. All induced mild green mottle symptoms that were indistinguishable from those induced by CCMV-M. The clone pCCM3 was chosen for further study. The nucleotide sequence of pCCM3 was determined and compared to the sequence of pCCT3 (S. Quan & C. Deom, unpublished results). Four nucleotide differences between pCCT3 and pCCM3 were identified (Table 1). In addition, there was a deletion of seven A residues from the polyadenylation [poly(A)] tract in the intergenetic region of RNA3.
CCMV-M RNA 3 between the MP and CP genes. With the seven deleted A-residues in CCMV-M, the nucleotide at position 1813 in CCMV-T is equivalent to position 1806 in CCMV-M.

To map the mild symptom determinant in CCMV-M RNA 3, chimeric cDNA clones were generated between cDNA clones of CCMV-T RNA 3 and CCMV-M RNA 3 (Fig. 1). RNA transcripts derived from each chimeric cDNA clone were inoculated onto cowpea together with transcripts from pCCT1, pCCT2 and from the RNA 3 cDNA clone of each mutant. Control plants were mock-inoculated, inoculated with purified CCMV-T or CCMV-M, inoculated with in vitro transcripts from pCCT1, pCCT2 and pCCT3 cDNA clones, or inoculated with in vitro transcript from pCCT1, pCCT2 and pCCM3 cDNA clones.

Table 2. Analysis of symptom phenotypes of CCMV CP mutants

<table>
<thead>
<tr>
<th>Mutant clone*</th>
<th>Nucleotide change</th>
<th>Amino acid at position 151</th>
<th>Symptoms on cowpea†</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCCT3</td>
<td>–</td>
<td>Ala</td>
<td>Severe</td>
</tr>
<tr>
<td>pCCM3</td>
<td>–</td>
<td>Val</td>
<td>Mild</td>
</tr>
<tr>
<td>pM151VA</td>
<td>U&lt;sup&gt;1806&lt;/sup&gt; → C&lt;sup&gt;1806&lt;/sup&gt;</td>
<td>Val → Ala</td>
<td>Severe</td>
</tr>
<tr>
<td>pT151AV</td>
<td>C&lt;sup&gt;1812&lt;/sup&gt; → U&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Val</td>
<td>Mild</td>
</tr>
<tr>
<td>pT151AA</td>
<td>C&lt;sup&gt;1812&lt;/sup&gt; → A&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Ala</td>
<td>Severe</td>
</tr>
<tr>
<td>pT151AG</td>
<td>C&lt;sup&gt;1812&lt;/sup&gt; → G&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Gly</td>
<td>Severe</td>
</tr>
<tr>
<td>pT151AS</td>
<td>G&lt;sup&gt;1812&lt;/sup&gt; → U&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Ser</td>
<td>Severe</td>
</tr>
<tr>
<td>pT151AD</td>
<td>C&lt;sup&gt;1812&lt;/sup&gt; → A&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Asp</td>
<td>Severe</td>
</tr>
<tr>
<td>pT151AK</td>
<td>C&lt;sup&gt;1812&lt;/sup&gt; → A&lt;sup&gt;1812&lt;/sup&gt;</td>
<td>Ala → Lys</td>
<td>Mild</td>
</tr>
</tbody>
</table>

* Mutations were generated in subclones containing the Hpa–Xba fragment from either pCCT3 or pCCM3 using a QuickChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenesis primers that spanned nucleotides 1791 through 1819 of the RNA 3 cDNA clone of CCMV-T or nucleotides 1785 through 1813 of the RNA3 clone of CCMV-M were used to introduce changes into the codon for amino acid residue 151. Mutations were confirmed by sequencing the mutated subclones in both directions. The mutant Hpa–Xba fragments were cloned back into pCCT3 or pCCM3 to generate the mutant RNA 3 cDNA clones. Mutants of the CCMV-T RNA 3 cDNA clone begin with 'pT'; a mutant of the CCMV-M RNA 3 cDNA clone begins with 'pM'.

† The symptoms were determined 3 weeks after inoculation. Plants were inoculated with a mixture of each capped in vitro transcript (15 µg). Transcripts were generated from pCCT1, pCCT2 and from the RNA 3 cDNA clone of each mutant. Control plants were mock-inoculated, inoculated with purified CCMV-T or CCMV-M, inoculated with in vitro transcripts from pCCT1, pCCT2 and pCCT3 cDNA clones, or inoculated with in vitro transcript from pCCT1, pCCT2 and pCCM3 cDNA clones.

When U<sup>1806</sup> in CCMV-M RNA 3 was mutated to C<sup>1806</sup> (pM151VA, Table 2), transcripts from pCCT1, pCCT2 and pM151VA elicited symptoms on cowpea indistinguishable from those induced by CCMV-T. In contrast, when C<sup>1812</sup> in CCMV-T RNA 3 was mutated to U<sup>1812</sup> (pT151AV, Table 2), transcripts from pCCT1, pCCT2 and pT151AV induced symptoms on cowpea indistinguishable from those induced by CCMV-M. Therefore, the nucleotide at position 1813 in CCMV-T (the equivalent position is 1806 in CCMV-M) or the resulting amino acid substitution is important in determining symptoms of CCMV-T and CCMV-M on cowpea.

To determine if the phenotype is induced by the nucleotide sequence or the subsequent amino acid substitution in the coat protein, additional mutations were made in the codon (nucleotide 1812–1814) of amino acid residue 151 in RNA 3 of CCMV-T (Table 2). While the substitution of C<sup>1813</sup> → U<sup>1813</sup> in RNA 3 of CCMV-T (pT151AV) resulted in mild green mottle symptoms identical to those induced by CCMV-M, the substitution of C<sup>1813</sup> → G<sup>1813</sup> (pT151AG) and C<sup>1813</sup> → A<sup>1813</sup> (pT151AD) resulted in severe symptoms identical to those induced by CCMV-M. However, when multiple substitutions were introduced into the codon (GCC<sup>1814</sup> → AAG<sup>1814</sup>), transcripts from the mutant pT151AK elicited mild green mottle symptoms. One of the substitutions in pT151AK was C<sup>1813</sup> → A<sup>1813</sup>, which alone (pT151AD) elicited severe symptoms.
Taken together, the results suggest that the amino acid residue at position 151 is the important factor in determining the symptom phenotype, not the nucleotide sequence.

The structure of native CCMV has been determined by X-ray crystallography to a resolution of 3.2 Å (Speir et al., 1995). Amino acid residue 151 lies within the zGH helix, one of two helices (the second is zGDII) that make up the putative calcium-binding site that helps stabilize intercapsomere interactions along the quasi-three-fold axis of the icosahedral shell. Amino acids within the zGH helix that coordinate calcium binding are Glu-148, Gln-149 and Asp-153. Not surprisingly, amino acid modifications at residue 151 might be expected to have an effect on calcium binding and, therefore, virion stability. The degree of stability between capsomeres mediated by calcium ions might play a role in symptom determination.

To determine if there is a correlation between symptoms induced by CCMV-T and CCMV-M and virus stability, a series of experiments was performed to evaluate particle stability in vitro under conditions that induce either a conformational change in virion structure (swelling) or virion dissociation. At pH < 6.0 and low ionic strength ([I] < 0.2), CCMV particles are contracted and sediment at 88S, while at pH > 7.0 and low ionic strength ([I] < 0.2) the peak of sedimentation changes to 78S as a result of swelling (Bancroft et al., 1967; Speir et al., 1995). Swelling is thought to result from the removal of Ca$^{2+}$ from the quasi-three-fold axis of the virion (Speir et al., 1995). Purified CCMV-T or CCMV-M was incubated in 0-1 M phosphate buffer at pH 5-0 or pH 7.5. Virus was centrifuged through 10-40% linear sucrose gradients for 4 h at 25000 g (Fox et al., 1996) and gradient profiles were monitored by absorbance at 254 nm. CCMV-T and CCMV-M were equally susceptible to swelling at pH 7.5, as indicated by identical changes in the gradient profiles of each virus, when incubation times prior to centrifugation were as short as 1 min (data not shown). Using a second approach, agarose gel electrophoresis, under conditions that result in pH-induced conformational changes (Heaton, 1992), CCMV-T and CCMV-M were found to be equally susceptible to swelling at pH 7.5, based on the slower migration pattern of each virus relative to contracted virus particles (data not shown).

Kuhn & Wyatt (1979) previously reported that CCMV-M could swell more easily than CCMV-T in low molarity phosphate buffer (0.01 M) at pH 7.5, but we detected no difference under the same conditions.

The stability of CCMV-T and CCMV-M was also compared on sucrose gradients under conditions that either induce or do not induce particle dissociation (Fox et al., 1996). Virions dissociate into genomic RNA and CP at pH > 7.4 and high ionic strength ([I] ≥ 1.0). Both CCMV-T and CCMV-M were stable under low salt (0.1 M) and low pH (pH 5.0) conditions, while both viruses dissociated under high salt (1.0 M) and high pH (pH 7.5) conditions. Taken together, the conformational and dissociation data indicate that there is no correlation between virion stability and symptoms. This conclusion is supported by research on mutants of the CP of Turnip crinkle virus, where there was no correlation detected between particle stability and symptom phenotype (Lin & Heaton, 1999).

Although residue 151 is in the zGH helix, no differences were detected in the stability of CCMV-T and CCMV-M under conditions that cause virus swelling or dissociation in vitro. We believe this indicates that Ca$^{2+}$ binding is similar in CCMV-T and CCMV-M and this conclusion is in agreement with virus accumulation data. At 10 days post-inoculation (p.i.), there was no significant difference in the levels of accumulation of CCMV-T and CCMV-M in inoculated or systemic leaf tissue as determined by virus purification or ELISA. Interestingly, CCMV-M was previously shown to be as fit as CCMV-T with a strong tendency for CCMV-M to become dominant when co-inoculated with CCMV-T (Kuhn & Wyatt, 1979). Furthermore, no significant difference was detected between the levels of viruses having substitutions at position 151 (Table 2) and the levels of CCMV-T and CCMV-M at 10 days p.i. as determined by ELISA.

The role of viral CP in symptom development has been demonstrated at the molecular level for several viruses (Banerjee et al., 1995; Culver & Dawson, 1989; Dawson et al., 1988; Heaton et al., 1991; Knorr & Dawson, 1988; Lin & Heaton, 1999; Neelman et al., 1991; Shintaku & Palukaitis, 1992). Significant structural changes to the zGH helix would be expected to disrupt the putative calcium-binding site that helps stabilize intercapsomere interactions. However, the similarity in stability of CCMV-T and CCMV-M and the similar accumulation levels of CCMV-T, CCMV-M and the viruses with mutations at residue 151 suggests that subtle influences by amino acid residues at position 151 likely play a role in the CP–host interactions that determine the respective symptom phenotypes.

Cowpea likely plays a host specific role in symptom induction by CCMV-T and CCMV-M, since symptoms were similar for CCMV-T and CCMV-M on other known hosts of CCMV-T, and no new hosts were identified for CCMV-M (Kuhn & Wyatt, 1979). This would not be surprising since CCMV-M was selected by continuous passage of CCMV-T in cowpea (Kuhn & Wyatt, 1979).

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References


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