Research Note

The Rate of Cell-to-Cell Movement in Squash of Cucumber Mosaic Virus Is Affected by Sequences of the Capsid Protein

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The M strain of cucumber mosaic virus (CMV) does not infect squash plants systemically and moves very slowly in inoculated cotyledons. Systemic infection and an increase in the rate of local movement were observed when amino acids 129 or 214 of the M-CMV capsid protein (CP) were altered to those present in the Fny strain of CMV. While the opposite alterations to the CP of Fny-CMV inhibited systemic infection of squash, they did not show the same effects on the rates of both cell-to-cell and long-distance movement. However, the ability of CMV to infect squash systemically was affected by the rate of cell-to-cell movement.

Virus movement through plants occurs as several discrete phases: movement within the cell, movement between cells in the leaf (via plasmodesmata), movement from leaf to leaf (via the vasculature), and movement back into and throughout the leaf cells (reviewed by Carrington et al. 1996; Séron and Haenni 1996). Plants may contain barriers to the movement of particular viruses at any of these phases. In some cases, other host responses may be involved, which allow limited or reduced rates of movement (De Jong et al. 1995; Ding et al. 1995; Mise and Ahlquist 1995; Valkonen and Somersalo 1996; Canto et al. 1997; Kaplan et al. 1997; Kasschau et al. 1997; Carrington et al. 1998; Kaplan et al. 1998; Ryabov et al. 1998; Takeshita et al. 1998; Yoshii et al. 1998). The basis of such responses is unknown. Different viral genes may be either the elicitors or the inactivators of such host responses. In the case of cucumber mosaic virus (CMV), specific sequences in each of the five genes have been shown to influence the rate of virus movement (Carr et al. 1994; Gal-On et al. 1994; Ding et al. 1995; Canto et al. 1997; Kaplan et al. 1997, 1998), although usually in specific hosts. In most of those cases, the reduced rate of cell-to-cell movement in the inoculated leaf resulted in a lack of systemic infection. Many of the examples cited involved particular cucurbit species or cultivars (Gal-On et al. 1994; Ding et al. 1995; Kaplan et al. 1997, 1998). This

also applies to the M strain of CMV, which can infect cucumber (*Cucumis sativus*) but not squash (*Cucurbita pepo*). In this case, the inability to infect squash systemically mapped to the viral capsid protein (CP; Shintaku and Palukaitis 1990). However, it was not known whether the inability of the M-CMV CP to promote systemic infection was associated with an effect on the rate of cell-to-cell movement in the inoculated leaves. In this report, the specific amino acid sequences of the M-CMV CP that affect long-distance movement are identified and the effects of these sequence alterations on the rate of cell-to-cell movement in the inoculated leaves are examined.

Since the Fny strain of CMV infects both squash and cucumber systemically (Kaplan et al. 1997), CMV containing a series of chimeric RNA 3s was used to delimit the M-CMV CP sequences restricting systemic infection in squash. The chimeric RNA 3s were generated previously from cDNA clones of M-CMV RNA 3 and Fny-CMV RNA 3, using specific and common restriction endonuclease sites (Shintaku et al. 1992). Systemic infection was observed when either all or specific parts of the *cp* gene were derived from Fny-CMV (Fig. 1). However, as CMV containing chimeric RNA 3 from FMCfr and FMFCfr as well as FMXho and MFXho was able to infect squash plants systemically (Fig. 1), this indicates that there must be at least two CP domains affecting movement in squash.

The encoded CP of M-CMV contains seven amino acid differences from that of Fny-CMV (Perry et al. 1994). Between the *Hind*III and *Cfr*10I sites, there is only one encoded amino acid difference (¹²⁹Pro in Fny-CMV and ¹²⁹Leu in M-CMV), and between the *Xho*I site and the C terminus of the CP there is only one amino acid difference (²¹⁴Gly in Fny-CMV and ²¹⁴Arg in M-CMV). Thus, cDNA clones encoding Fny-CMV RNA 3 (pFny309) or M-CMV RNA 3 (pMCMV3) were modified by oligonucleotide-directed site mutagenesis (Kunkel et al. 1987) at nucleotide positions 1642 and/or 1896, which are within the codons for CP amino acids 129 and 214, respectively, creating the cDNA clones used to generate the RNA 3s shown in Table 1. The mutated nucleotides were all confirmed by DNA sequence analysis (Sanger et al. 1977). RNA transcripts generated from these cDNA clones were co-inoculated

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with transcripts of Fny-CMV RNAs 1+2 to tobacco plants. The systemically infected tobacco leaves were analyzed for the presence of CMV at 10 days post inoculation (d.p.i.) with the "Touchdown" reverse transcriptase-polymerase chain reaction (Seoh et al. 1998) on total nucleic acids extracted from the tissues (Gal-On et al. 1994), and a primer complementary to nucleotides 1983 to 2015, plus a primer corresponding to nucleotides 1149 to 1176. In each case, the expected 867-bp fragment was obtained and the DNA was analyzed by automated sequencing, confirming the presence of the introduced mutations and no other nucleotide sequence alterations (data not shown). Virus was also partially purified from infected leaves and was examined after negative staining (Christie et al. 1987) under a transmission electron microscope. In all cases, typical CMV particles were observed (data not presented). Sap from recently infected tobacco plants, containing similar levels of CMV (Perry et al. 1998; data not presented), was inoculated to squash cotyledons.

Except for squash plants inoculated with CMV containing RNA 3 derived from pMCMV3, or the mutants F-129L and F-

129L/214R, all squash plants inoculated by CMV containing RNA 3 derived from either pFny309 or any of the other mutant RNAs 3 were infected systemically (Table 1), although at different rates for the single mutants. Thus, alterations in the amino acid sequence at either position 129 or 214 could affect the ability of M-CMV to infect squash systemically.

To ascertain whether there were differences in the rate of cell-to-cell movement, inoculated cotyledons were harvested at 3, 5, and 8 d.p.i. with the wild-type viruses and the various CP-mutant viruses. The abaxial epidermis of each cotyledon was peeled and the remaining cotyledon was pressed onto a nitrocellulose membrane as previously described (Gal-On et al. 1994). The blots were probed with CMV antiserum (1:1,000 dilution). Each experiment was done in duplicate and independently at least four times.

At 3 d.p.i., the number of infection foci was similar for CMV containing RNA 3 of Fny-CMV, the CP mutant F-214R, and three of the CP mutant RNA 3s derived from pMCMV3: M-214G, M-129P, and M-129P/214G (Figs. 2 and 3). CMV containing RNA 3 of M-CMV showed many fewer infection foci than did the rest, while CMV containing RNA 3 of either the single CP mutant F-129L or the double CP mutant F-129L/214R of Fny-CMV did not show any visible infection foci at 3 d.p.i.

A general increase in the size of infection foci was observed for all mutant viruses as well as the wild-type viruses at 5 d.p.i. (Figs. 2 and 3). By 8 d.p.i., Fny-CMV had spread throughout the entire cotyledon (Fig. 2). By contrast, CMV containing the M-CMV CP showed only limited spread from the initial infection sites (Fig. 3), as did CMV containing

Fig. 1. Systemic infection of cucumber mosaic virus (CMV) on squash. The CMV inocula contained RNAs 1 + 2 of the Fny strain of CMV and RNA 3 derived from Fny-CMV (Fny3), M-CMV (M3), or chimeras formed between cDNA clones of Fny-CMV RNA 3 (open rectangles) and M-CMV RNA 3 (filled rectangles), with the restriction endonuclease sites *NheI* (N), *SalI* (S), *HindIII* (H), *Cfr10I* (C), or *XhoI* (X). Genes encoding the movement protein (MP) and the capsid protein (CP) on RNA 3 are shown as rectangles. Inocula were applied to tobacco plants and sap from infected tobacco was applied to squash plants (3 to 5 plants per trial; at least four trials for each chimera). Systemic pathology of the virus is indicated by "+" or "-". Number of "+" shows severity of symptoms: +++ = severe stunting and green-yellow mosaic appearing 3 to 4 days post inoculation (d.p.i.); ++ = stunting with chlorotic lesions on systemically infected leaves appearing 6 to 9 d.p.i.; + = chlorotic lesions only on systemically infected leaves appearing 6 to 9 d.p.i. Plants showing no systemic symptoms ("-") were confirmed as not infected by back inoculation to tobacco plants. Phenotypes indicated by + and ++ were maintained upon passage to other squash plants.

Table 1. Systemic infection of squash by cucumber mosaic virus (CMV) and various capsid protein mutants

Source of RNA 3 ^a	Systemic symptoms ^b
Fny-CMV	Sev St; Mos.; 3 to 4 d.p.i. ^c
F-129L ^d	None ^e
F-214R	St; Chll; 6 to 9 d.p.i.
F-129L/214R	None
M-CMV	None
M-129P ^d	Chll; 6 to 9 d.p.i.
M-214G	St; Chll; 6 to 9 d.p.i.
M-129P/214G	Sev. St.; Mos. 3 to 4 d.p.i.

^a Inocula consisting of transcripts of the Fny strain of CMV RNAs 1+2 and RNA 3 of Fny-CMV, the M strain of CMV, or one of the capsid protein (CP) mutants listed above. Inocula were applied to tobacco, and sap from infected tobacco was applied to squash cotyledons (3 to 5 plants per treatment). Each treatment was done at least 4 times.

^b Symptoms on newly grown leaves not present at the time of inoculation. Sev = severe; St = stunting; Mos = green-yellow mosaic; Chll = chlorotic lesions.

^c Systemic symptoms appeared at either 3 to 4 days post inoculation (d.p.i.) or 6 to 9 d.p.i. The same symptoms and timing of systemic infection were observed during three passages through squash.

^d F-129L = Fny-CMV RNA 3 with amino acid 129 of the encoded CP altered from Pro to Leu; F-214R = Fny-CMV RNA 3 with encoded CP amino acid 214 altered from Gly to Arg; F129L/214R = Fny-CMV RNA 3 with encoded CP amino acids 129 and 214 altered to Leu and Arg, respectively; M-129P = M-CMV RNA 3 with encoded CP amino acid 129 altered from Leu to Pro; M-214G = M-CMV RNA 3 with encoded CP amino acid 214 altered from Arg to Gly; M-129P/214G = M-CMV RNA 3 with encoded CP amino acids 129 and 214 altered to Pro and Gly, respectively.

^e None. No systemic symptoms. Lack of infection was verified in some experiments by either back inoculation to tobacco or dot blot hybridization analysis.

RNA 3 derived from the mutants of pFny309, where CP amino acid 129 had been altered from Pro to Leu (F-129L and F-129L/214G in Figure 2).

Alteration of Fny-CMV CP amino acid 214 from Gly to Arg (F-214R in Figure 2) consistently resulted in a noticeably reduced rate of CMV movement between 5 and 8 d.p.i. in the

infected cotyledon. However, this was not quantifiable when CP amino acid position 129 also had been altered from Pro to Leu (compare F-129L with F-129L/214R in Figure 2). On the other hand, alteration of either position in the CP of M-CMV led to an increase in the number of infection sites as well as the rate of movement, with CMV containing RNA 3 of the

Fig. 2. Immunoblots of squash cotyledons at 3, 5, and 8 days post inoculation (d.p.i.) after infection with the Fny strain of cucumber mosaic virus (CMV) and capsid protein (CP) mutants of Fny-CMV. Sap from tobacco infected by Fny-CMV or mutant viruses in which the CP was modified at amino acid positions 129, 214, or both was inoculated to squash cotyledons. At the times indicated, the cotyledons were subjected to leaf-press analysis and the membranes containing the expressed sap were probed with antiserum to the virus CP and a secondary antibody conjugated to alkaline phosphatase. Purple foci show the presence of CMV CP.

double mutant M-129P/214G showing a rate of systemic infection more rapid than those of either single mutant (Table 1) and identical to that of Fny-CMV (Table 1). The rate of cell-to-cell movement of the double mutant M-129P/214G was also more rapid than that of the single mutant M-214G (Fig. 3; M-129P/214G versus M-214G; 5 d.p.i. versus 8 d.p.i.). Thus, the amino acids 129 and 214 both affect the rate of cell-to-cell

movement, with the amino acid in position 129 exerting a reproducibly more pronounced effect than the amino acid at position 214. The magnitude of the effect of particular amino acid exchanges also was influenced by the other differences between the CP genes, since CMV containing the CP mutants F-129L and F-129L/214R showed less cell-to-cell movement than CMV containing the M-CMV CP.

Fig. 3. Immunoblots of squash cotyledons after infection with the M strain of cucumber mosaic virus (CMV) and capsid protein (CP) mutants of M-CMV. Sap from tobacco infected by M-CMV or mutant viruses in which the CP was modified at amino acid positions 129, 214, or both was inoculated to squash cotyledons. At times indicated, cotyledons were analyzed for the presence of CP, as in Figure. 2. Purple foci show the presence of CMV CP.

Finally, the rate of cell-to-cell movement may be a factor in determining whether or not systemic infection took place, since CMV containing RNA 3 of M-CMV or the CP mutants F-129L and F-129L/214R did not infect squash systemically, and all showed slow or poor cell-to-cell movement (Table 1 and Figs. 2 and 3). These effects were not due to differences in the titer or specific infectivity of the inocula, since all of the mutants contained the same movement protein and replicase genes. Moreover, differences in accumulation kinetics of Fny-CMV, due to the presence of satellite RNA, did not affect the rate of movement (Gal-On et al. 1995). A linkage between the rate of cell-to-cell movement and the ability to infect squash systemically also was observed with mutants of the 3a protein (Kaplan et al. 1997). However, it is not clear if there are similar barriers affecting cell-to-cell movement versus systemic infection, or if the barrier to systemic infection observed here is due to an induced host response.

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