JOURNAL OF VIROLOGY, Aug. 2006, p. 7740–7743 Vol. 80, No. 15
0022-538X/06/$08.00+0 doi:10.1128/JVI.00436-06

NOTES

Compensatory Capsid Protein Mutations in Cucumber Mosaic Virus Confer Systemic Infectivity in Squash (*Cucurbita pepo*)

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Received 1 March 2006/Accepted 9 May 2006

Cucumber mosaic virus (CMV) systemically infects both tobacco and zucchini squash. CMV capsid protein loop mutants with single-amino-acid substitutions are unable to systemically infect squash, but they revert to a wild-type phenotype in the presence of an additional, specific single-site substitution. The D118A, T120A, D192A, and D197A loop mutants reverted to a wild-type phenotype but did so in combination with P56S, P77L, A162V, and I53F or T124I mutations, respectively. The possible effect of these compensatory mutations on other, nonsystemically infecting loop mutants was tested with the F117A mutant and found to be neutral, thus indicating a specificity to the observed changes.

The tripartite genome of *Cucumber mosaic virus* (CMV) encodes replicase functions in RNA1 and -2, with RNA3 encoding the movement protein (MP) and capsid protein (CP) (for a review, see reference 10). Both the CP and the MP of CMV are required for local cell-to-cell movement (1, 15) and systemic movement in squash (2, 3, 18). The functions of the CP in cell-to-cell and systemic movement are different, as shown by the ability of a CMV mutant lacking the CP and the C terminus of the MP to move from cell to cell but not systemically (8). A host-specific role for the CP in the systemic movement of CMV has also been demonstrated in recombination and complementation experiments using CMV and the cucumovirus *Tomato aspermy virus*, which does not systemically infect cucumber (4, 13, 16). In this study we demonstrate that in engineered CP mutants unable to systemically infect squash, spontaneous, compensatory, single-amino-acid substitutions in the CP confer the ability to systemically infect this host.

The construction of all mutants of a CMV-Fny RNA3 cDNA clone, pFny309, was carried out by PCR-mediated mutagenesis, as previously described (7). Capped in vitro transcripts from the full-length clones pFny109 (RNA1), pFny209 (RNA2), and pFny309 (RNA3) (12) and all derived RNA3 mutants were synthesized using an mMessage mMachine capped RNA transcription kit (Ambion). The sequences of all CP genes from all mutants were monitored throughout the course of experiments in both tobacco and squash leaves and inoculated cotyledon. Total RNA extracts were prepared using an RNeasy plant minikit (QIAGEN).

Single-amino-acid substitutions in the CP disrupt systemic movement. A panel of 16 CP single-amino-acid substitution mutants was screened for the ability to infect and move systemically in squash. All of these mutants have previously been shown to readily infect and move systemically in tobacco (7; A. J. Clark and K. L. Perry, unpublished data). Symptoms in squash cotyledons inoculated with all the mutant viruses were comparable to those observed for the wild-type virus. However, 12 of the mutants were impaired in their ability to systemically infect growth chamber-grown (19°C) squash, with less than 25% of the plants becoming infected (Table 1). Inoculations of these 12 mutants were repeated with greenhouse-grown (20 to 25°C) squash, and a reduced level of infection (less than 25% of the inoculated plants) was again observed for 9 of the mutants (the P77A, D81A, K116A, F117A, T120A, D191A, D192A, L194A, and D197A mutants); the remaining 3 mutants (the P78A, I80A, and D118A mutants) infected about half of the inoculated plants, compared with >90% for the wild-type virus. Thus, single-amino-acid substitutions in the CP can alter the ability of the virus to move systemically in squash.

Selection of revertants competent for systemic movement in squash. For the mutants that systemically infected squash inefficiently, hypothetically there would be strong selection pressure for reversion. Eleven mutants were serially passaged two to three times in squash (or until >90% of the plants became infected), with progeny virus from systemically infected leaves of greenhouse-grown plants used to mechanically inoculate the cotyledons of uninfected plants. Five mutants (the D81A, D118A, T120A, D192A, and D197A mutants) reverted to a wild-type phenotype, with >90% of the plants becoming infected (Table 1). RNAs were extracted separately from systemically infected leaves of at least two plants per revertant, and in all but one case, the original mutation had been retained; the exception was a simple reversion of D81A to A81D. A single additional encoded amino acid change was observed in five revertants (Table 1). In the case of the D197A parental mutant, three different revertants were recovered, including one with two additional amino acid changes.

Compensatory mutations in the capsid protein gene confer

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TABLE 1. Systemic infection of squash by CMV capsid protein surface loop mutants

CP mutant substitution[a]	No. of plants infected/no. of plants inoculated (%) in[b]:		Revertant CP amino acid changes[c]
	Growth chamber expts	Greenhouse expts	
P77A	0/44 (0)	0/50	
P78A	5/35 (14)	25/50	
K79A	28/30 (93)	NT	
I80A	0/33 (0)	21/50	
D81A	0/35 (0)	8/50	A81D
R82A	13/36 (36)	NT	
K116A	2/32 (6)	10/50	
F117A	0/33 (0)	3/50	
D118A	0/36 (0)	22/50	P56S
S119A	22/34 (65)	NT	
T120A	0/30 (0)	5/50	P77L
D191A	5/30 (17)	4/50	
D192A	2/32 (6)	10/50	A162V
L194A	8/34 (23)	5/50	
E195A	9/29 (31)	NT	
D197A	0/27 (0)	10/50	I53F + E198G, H55R, T124I

[a] Each mutant of cucumber mosaic virus strain Fny harbors a single-amino-acid substitution in the capsid protein. Mutations are given using the original amino acid followed by the numbered position in the capsid protein sequence followed by the introduced amino acid. Amino acid positions 77 to 82 are in the βB-βC loop, positions 116 to 120 are in the βD-βE loop, and positions 191 to 197 are in the βH-βI loop.

[b] Viruses were inoculated onto cotyledons. The infection of noninoculated true leaves was observed, and the presence of the virus in both cotyledons and true leaves was confirmed by reverse transcription-PCR and sequencing of the products. The values indicate the number of plants systemically infected relative to the total number of plants inoculated. The percentage of systemically infected plants is indicated in parentheses. NT, not tested.

[c] Any amino acids encoded by capsid protein genes from viruses present in systemically infected leaves that differed from the transcript-inoculated genes are indicated (revertant) along with the amino acid position. Unless indicated, the original engineered mutations were still present.

TABLE 2. Systemic infection of squash by engineered CMV capsid protein mutants with compensatory amino acid changes

Parental virus[a]	Mutation(s) in engineered mutant[b]	No. of systemically infected plants/no. of inoculated plants (%)[c]
F117A mutant	F117A	0/22
	F117A-P56S	0/21
	F117A-P77L	0/21
D118A mutant	D118A	16/16 (100)[d], 17/19 (89)[d], 19/22 (86)[d]
	D118A-P56S	15/16 (93)
	P56S	16/17 (94)
T120A mutant	T120A	0/16
	T120A-P77L	21/25 (84)
	P77L	16/16 (100)
D192A mutant	D192A	0/15
	D192A-A162V	21/21 (100)
	A162V	20/20 (100)
D197A mutant	D197A	0/15
	D197A-I53F/E198G	15/16 (93)
	I53F	19/19 (100)
	E198G	0/20
	D197A-I53F	16/16 (100)
	D197A-T124I	17/17 (100)
	T124I	24/24 (100)
	D197A-H55R	7/25, 19/23 (28, 83)
	H55R	22/23 (96)
Fny (wild type)		17/18 (94)

[a] Each of the parental capsid protein mutants of cucumber mosaic virus strain Fny harbors a single engineered amino acid change. The mutations are given using the original amino acid followed by the numbered position in the capsid protein sequence followed by the introduced amino acid.

[b] The first mutation listed in each series is that of the parental mutant. The engineered, putative reversions are named as for the parental mutations, joined by a hyphen.

[c] The cotyledons of squash (*Cucurbita pepo*) were inoculated with virus, and the values indicate the number of systemically infected plants relative to the total number of plants inoculated. Where systemic infections were observed, the percentage of infected plants is shown in parentheses. The capsid protein genes from viruses present in systemically infected leaves were sequenced and compared with those of the inoculated mutant. There were no additional amino acid changes except where noted in footnote *d* below.

[d] The capsid protein gene of the inoculated D118A mutant was observed unchanged in inoculated cotyledons but was modified in systemically infected leaves. In three independent experiments, the capsid protein gene in systemically infected tissues harbored an additional insertion of three nucleotides encoding an asparagine in between amino acid positions 164 and 165.

systemic movement. The observed second-site mutations were individually engineered into the CP gene of the respective parental mutants and inoculated onto tobacco and then squash in the growth chamber. Engineered T120A P77L, D192A A162V, D197A H55R, and D197A T124I double mutants were all able to infect squash systemically with nearly wild-type efficiency (Table 2). Second-site mutations (P77L, A162V, H55R, and T124I) engineered alone into the wild-type CP systemically infected squash relatively efficiently. For the two second-site mutations (I53F and E198G) observed in a single virus isolate derived from the D197A mutant, amino acid change I53F was able to compensate for both D197A and E198G. To test whether amino acid changes that compensate for one mutation might also compensate for proximal mutations in the same CP loop (Fig. 1), mutations P56S and P77L, found to compensate for T120A and D118A, respectively, were individually introduced in combination with F117A (βD-βE loop). The two mutants that resulted gave rise to wild-type infections in tobacco and on squash cotyledons, but neither was able to systemically infect squash (Table 2).

Strong selection for the insertion of a codon triplet in the CP gene. The second-site mutation P56S was observed in progeny of the D118A mutant (Table 1). This mutation alone and in combination with D118A gave rise to wild-type infections in tobacco and squash (Table 2); however, the control inoculation of squash with the D118A mutant generated an unusual revertant containing an insertion of three nucleotides encoding an asparagine between amino acid positions 164 and 165. This experiment was repeated twice with the same results.

In our experiments, we found that all isolates, systemic or not, developed characteristic clear chlorotic spots on the inoculated cotyledons around 7 days postinoculation, indicating a symptomatology similar to that of the wild-type CMV-Fny at the cell-to-cell stage. Differences in the infectivities of cucumoviruses between tobacco and squash reported elsewhere (3, 13) also emphasize the importance of host-virus adaptation. In tobacco, the diversity in the CMV RNA population is significantly reduced during systemic infection, with only a subset of genotypes being loaded into the vasculature (5). Of the five amino acid positions involved in compensatory mutations ob-

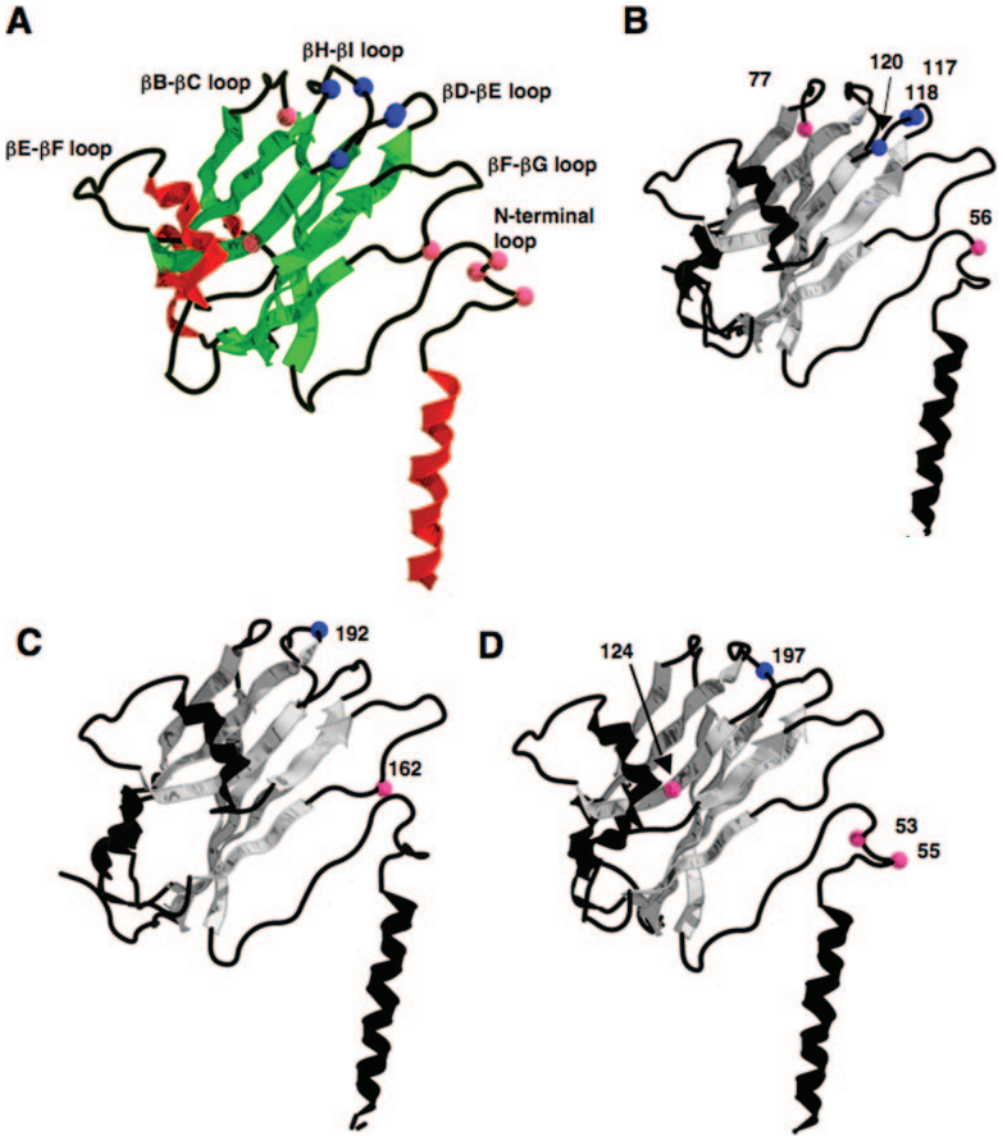

FIG. 1. Structural model of the cucumber mosaic virus capsid protein B subunit illustrating the relative amino acid positions of engineered and compensatory, second-site mutations. (A) Ribbon diagram with labeled loops. The orientation is such that the surface of the virion is at the top and the α-helix is at the bottom and pointed downward toward the interior of the virion. The color-coded structures are as follows: α-helices, red; β-strands, green; and coils or loops, black. The positions of engineered amino acid changes are indicated as blue spheres and those of the compensatory, second-site mutations as pink spheres. (B) Ribbon diagram with blue spheres showing the primary mutations in the βD-βE loop at amino acid positions 117, 118, and 120. Compensatory, second-site mutations at positions 56 and 77 are shown as pink spheres. (C) Ribbon diagram with a blue sphere indicating the primary mutation at amino acid position 192 in the βH-βI loop and a pink sphere indicating the compensatory, second-site mutation at position 162. (D) Ribbon diagram with a blue sphere indicating the primary mutation at amino acid position 197 in the βH-βI loop. Compensatory, second-site mutations at positions 53, 55, and 124 are indicated by pink spheres. Each of the ribbon diagrams have been rotated slightly differently in order to most clearly reveal the amino acid positions in the loops. The figures were created with Molview software (http://www.danforthcenter.org/smith/) (13).

served here, only one (position 162) has been reported before, identified as important in virion stability and aphid transmission (9, 11). Three of the five compensatory changes (I53F, H55R, and A162V) were localized in the same region of the folded polypeptide, above the N-terminal α helix. In particular, the βF-βG loop differs between subunits and may affect the plasticity of the CP (14). These changes and that of P77L are likely to affect the dynamic properties of virions, but it is not clear how they affect any critical interactions between CP and other factors. Second-site capsid protein mutations that affected systemic movement were also observed with hibiscus chlorotic ringspot virus, although in this case it was a converse relationship. Passaging of hibiscus chlorotic ringspot virus in a local lesion host was correlated with the development of second-site mutations that eliminated the capacity for systemic movement in the original host (6) Mechanisms underlying systemic infection by CMV are not clear, but subtle changes in CP conformation could affect interactions between the

movement, 2b, and host proteins and/or viral RNA, thereby disrupting virus trafficking through the plasmosdesmata of the bundle sheath cells to the intermediary cells and then onto the phloem (17).

In conclusion, the significance of this study's results are as follows: (i) a variety of single-amino-acid changes in the capsid protein of a virus can have a gross effect on systemic infectivity; (ii) this infectivity can be host specific; and (iii) the virus can adapt to different hosts with spontaneous, intramolecularly compensatory, single-amino-acid substitutions.

We thank Laura Miller and Caroline Josefsson for technical support, Anthony Clark for practical advice, and Mark Tepfer and Fernando García-Arenal for critically reviewing the manuscript.

This work was supported by USDA NRI grant 2002-00647.

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