***Cucumber mosaic virus*–Plant Interactions: Identification of 3a Protein Sequences Affecting Infectivity, Cell-to-Cell Movement, and Long-distance Movement**

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Mutants of the *Cucumber mosaic virus* (CMV) movement protein (MP) were generated and analyzed for their effects on virus movement and pathogenicity *in vivo*. Similar to the wild-type MP, mutants M1, M2, and M3, promoted virus movement in eight plant species. Mutant M3 showed some differences in pathogenicity in one host species. Mutant M8 showed some host-specific alterations in movement in two hypersensitive hosts of CMV. Mutant M9 showed altered pathogenicity on three hosts and was temperature sensitive for long-distance movement, demonstrating that cell-to-cell and long-distance movement are distinct movement functions for CMV. Four mutants (M4, M5, M6, and M7) were debilitated from movement in all hosts tested. Mutants M4, M5, and M6 could be complemented *in trans* by the wild-type MP expressed transgenically, although not by each other or by mutant M9 (at the restrictive temperature). Mutant M7 showed an inability to be complemented *in trans*. From these mutants, different aspects of the CMV movement process could be defined and specific roles for particular sequence domains assigned. The broader implications of these functions are discussed.

The movement of most plant viruses within their hosts involves several distinct stages: translocation of a recently replicated virus within the inoculated cell to the intercellular connections and the plasmodesmata, trafficking through the plasmodesmata and through adjacent cells to bundle sheath cells, movement into and through different cell types of the vascular bundles from bundle sheath cells through vascular parenchyma cells and companion cells to the sieve elements, movement in the phloem through the sieve tube elements, exit into young companion cells of newly developing leaves, and translocation through cells of vascular bundles to the mesophyll and epidermal cells of the systemically infected tissues (Carrington et al. 1996; Lazarowitz and Beachy 1999; Lucas

and Gilbertson 1994; Reichel et al. 1999). The research focused on movement within and between cells, with plants being assessed for various cellular barriers to virus movement (Carrington et al. 1996; Lazarowitz and Beachy 1999; Nelson and Van Bel 1998). These barriers probably reflect the major form of resistance to virus infection: the inhibition of virus movement (Dawson and Hilf 1992; Gilbertson and Lucas 1996; Meshi and Okada 1987). The interactions between virus movement proteins (MPs) and the cell ultrastructure to promote movement within the cell (e.g., across the nuclear membrane or along the cytoskeleton within cells) and via plasmodesmata or tubules formed between cells have opened up new areas of research in plant virology, cell biology, and cell physiology (Ding et al. 1999; Lazarowitz and Beachy 1999; Lucas and Wolf 1999; Reichel et al. 1999). MPs may have evolved to either coopt or mimic native plant proteins or processes involved in translocation within and between plant cells (Gilbertson and Lucas 1996). It also has been suggested that plant virus MPs may have been this type of plant protein at one stage (Lucas and Gilbertson 1994; Lucas and Wolf 1999).

Depending on the virus system, plant viruses encode one to five proteins involved in their movement (Carrington et al. 1996; Lazarowitz and Beachy 1999; Maule 1991). For some viruses, the coat protein(s) (CP) of the virus as well as the MP(s) are involved in virus movement. In other viruses, there is not just one protein required for virus movement but a complex of three proteins encoded by overlapping genes, referred to as the “triple gene block.” In some cases, the movement of such viruses also may require CP (Carrington et al. 1996; Lazarowitz and Beachy 1999; Nelson and Van Bel 1998). For some nuclear-associated viruses, one MP has the role of translocation from the nucleus to the cytoplasm, whereas another functions in gating between cells (Lazarowitz and Beachy 1999). In other cases, the MP is associated with tubules that form between cells and contain isometric virus particles (Carrington et al. 1996; Lazarowitz and Beachy 1999; Maule 1991). In spite of the seeming multiplicity of forms of movement, many MPs show similar characteristics whether or not they share the same requirements for cell-to-cell (local) and long-distance (systemic) movement (Carrington et al. 1996; Lazarowitz and Beachy 1999; Melcher 2000; Mushagian and Koonin 1993). In addition, for some viruses gene products

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involved in virus replication appear to be involved in virus movement (Deom et al. 1997; Gal-On et al. 1994; Nelson and Van Bel 1998). Models for the movement of viral genomes from the site of replication (or assembly) to adjacent cells have been proposed (Carrington et al. 1996; Gilbertson and Lucas 1996; Lazarowitz and Beachy 1999), but there are still many unanswered questions, including why some viruses seem to have elements of different movement mechanisms and what is the nature of the molecular basis of the host specificity of virus movement.

To begin addressing some of these issues, we mutated the gene encoding the MP of *Cucumber mosaic virus* (CMV) to identify functional domains of the movement process. CMV occurs worldwide as biologically differentiable isolates, called strains. The collective host range of these strains is approximately 1,241 species in 101 plant families (Edwardson and Christie 1991). CMV contains five genes expressed from three genomic and two subgenomic RNAs. All five encoded proteins appear to have some effect on virus movement. This includes the two replication-associated proteins (98 and 111 kDa), the viral CP (25 kDa), a protein involved in suppressing plant responses (13 kDa), and the 3a protein (30 kDa) (Ding et al. 1995a; Ding et al. 1995b; Kaplan et al. 1995; Palukaitis et al. 1992). The 3a protein shares several of the above features common to a number of MPs and thus is referred to as the CMV MP (Canto et al. 1997; Ding et al. 1995a; Kaplan et al. 1995; Li and Palukaitis 1996). We constructed a series of alanine-scanning mutants in the 3a gene of the Fny strain of CMV with procedures first used to mutate the MP of the dianthovirus *Red clover necrotic mosaic virus* and delineate functional domains of this MP (Fujiwara et al. 1993; Giesman-Cookmeyer and Lommel 1993). The CMV 3a MP mutants were then assessed for their effects on CMV, vis-à-vis infectivity and pathogenicity on eight host species, and the ability of the mutants to be complemented in *trans* by CMV MP expressed in transgenic plants. A functional map of the CMV MP is presented, incorporating the effects of various mutations and alterations described here and previously. Ad-

ditional novel interactions suggested by the data presented here also are discussed.

RESULTS

Target selection for alanine scanning mutagenesis.

A series of seven alanine-scanning mutants (designated M1 to M7) initially was created by mutating adjacent or nearby pairs of codons to alanine within the CMV 3a protein. These mutations were made at sequences corresponding to 3a protein positions R8 plus T9 (M1), E38 plus D40 (M2), Y75 plus D76 (M3), R97 plus T98 (M4), Y144 plus D145 (M5), R156 plus F158 (M6), and N191 plus Y192 (M7) where at least one of each pair was a charged or polar residue. The mutation of such paired sequences reduced the likelihood of reversion. These sequences were selected on the basis of being in regions highly conserved between strains of CMV and three bromoviruses (Fig. 1), where sequences involved in common functions are more likely to be located. Two other mutants were then created in areas of less sequence similarity. Because mutant M8 (D20 and D21) is located in a region proximal to the N terminus where CMV and bromoviruses showed considerable sequence divergence, it was thought that this region would be more likely to affect specific rather than generic interactions. Mutant M9 (P60) involved a highly conserved proline that potentially could affect the folding of the MP. No mutations were made beyond position 192 (M7) for reasons discussed below. In addition, there is virtually no sequence similarity between CMV and the bromoviruses beyond position 209 (Fig. 1).

Infectivity and accumulation of alanine-scanning mutants in tobacco plants.

The nine MP mutants were assessed initially for their infectivity in tobacco. RNA 3 transcribed from the mutant cDNA clones of pFny309 was combined with CMV RNAs 1 and 2 transcribed from wild-type cDNA clones pFny109 and pFny209, and the RNAs were inoculated in tobacco. These

CMV	MAFQGTSTRT...LTQSSAATSDDLQKILFSPEAIKKMATECDLGRHHWMRADNAISVRPLVPEVTHGRIASF	70
BBMV	MSNISSLNP-S-S---LVE.NK-VG-SDAES-L-EM--L--Q-EY-K--N---YTELK-NRRLNHID---K-HGMKML-M	79
BMV	MSN.I.VSP-S-S---TSDVGK-AGGTSDEK-IES---EK-V-EI-A--K--CYNLKSNEPRNYID---KSHVSAWL-W	78
CCMV	MSN.TTFRP-T-S---VVE.GE-AG-QDDMS-LQS---DKSREEF-K--K--MYTNLSSN-RLNYID---KN-GS-IASF	78
	M1 M8 M2 M9	
CMV	FKSGYDVGELCSKGYMSVPQVLCVTRTVSTDAEGSLRIYLADLDGKE...LSPIDG.QCVSLHNHDLPALVSFQPTYD	145
BBMV	-R-E--K-NVP-S-VLNI-RI-LYLV--SHSSTV--IT-R-V-TYSASDCL-EA---EFTVDLSS--CMIGFS----	158
BMV	AT-K--K---P-R-F-N--RIV-FLV--TDSAES--ITVS-C-S-KAARAGV-EA--N.-EATIQLSA----IALT-S--	157
CCMV	F--E-EK-HIP-S-VL-I-R--VFLV--TTVTES--VT-R-V--ISASSVEI-E-V--T-EATIPISS---I-C-S-S--	158
	M3 M4 M5	
CMV	CPMETVG...NRKRCFAVVIERHGYIGYTGTASVCSNWQARFSSKNNNYTHIAAGKTLVLPFNRLAEQTKPSAVARL	220
BBMV	-KL-M-D...G-R---GI-T-LN-V--EGH-V-M-HAY-K-M-RT-PG---RVKPAAKFIA--D--KQLSSGQLD-FI	233
BMV	---VI-GDSG-N---GIATQLS-VV-T--SV-VTHAY---N-KA-P---KLHGPAITIM-M--D--RQLD-K-LKNI	235
CCMV	---QMI-...H--GL-TQLD-V-SSGS-VVMHAY-S-N-R--P---KQY-PMYKY-E--D--KRLSRKQLKNYV	233
	M6 M7	
CMV	LKSQLNNISSQYLLTNAKINQNARSESEDNLN.ESPPAAIGSSSASRSEAFRPQVVNGL	279
BBMV	KGISN-S-DHGYLMGSTINNKKKTN...VKE.---TSDPQ-GEV-SMTQSV-GAADTRIPKPRRR	295
BMV	RGISNQSVDDHGYL-GRPLQSVQVAQ-DLLVEES---S-LGRGV.KDSKSVSASS-AGLPVSSPTLRIK	303
CCMV	RGITNQSVNHGYL-GKPLLTDEQDPEMIV-EEESLT-TDSNGVGKDKIAVTAKS-AGLPASLSINRR	302

Fig. 1. Comparison of the amino acid sequences of the movement proteins (MPs) of *Cucumber mosaic virus* (CMV) and the three bromoviruses: *Brome mosaic virus* (BMV), *Broadbean mottle virus* (BBMV), and *Cowpea chlorotic mottle virus* (CCMV). Dashes indicate sequence identity of bromovirus MP sequences to that of the CMV MP. Dots indicate the absence of corresponding amino acids in the sequence. Amino acid sequences altered in the nine alanine-scanning mutants (M1 to M9) are underlined.

RNAs containing MP mutants M1, M2, M3, M8, and M9 (referred to below by the mutant number) were able to systemically infect tobacco plants (Table 1). All but mutant M9 showed a similar time course of symptom development and produced comparable yields of virus (0.23 to 0.28 mg of virus per g of leaves), somewhat less than what was obtained for wild-type Fny-CMV derived from infectious RNA transcripts (0.35 mg per g) (Table 1). Mutant M9 produced mild mosaic and vein-clearing symptoms on tobacco versus the severe mosaic and leaf distortion typical of wild-type Fny-CMV. The yield of virus (0.21 mg per g), however, was comparable to the yields for the other mutants described above.

In contrast, mutants M4, M5, M6, or M7 did not induce any symptoms on tobacco and no accumulating viral RNA could be detected by Northern blot hybridization of total RNAs extracted from either the inoculated or upper leaves (Fig. 2A and data not shown). Mutants M4, M5, and M6, however, were able to infect transgenic tobacco plants expressing the wild-type Fny-CMV 3a protein (Table 1 and Fig. 2B). The yield of virus was less than that observed for the other mutants (0.13, 0.18, and 0.17 mg per g for M4, M5, and M6, respectively). The apparent variation in accumulation of the various CMV RNAs visible in Figure 2 was not observed consistently in other experiments (data not presented). The symptoms elicited by mutants M4, M5, and M6 were limited to the vein-clearing previously observed with 3a deletion mutants (Kaplan et al. 1995). Except for some plants infected by mutant M4, virus purified from these 3a-transgenic tobacco was unable to infect nontransformed tobacco (data not presented), indicating that neither had the mutations reverted nor had the recombination that occurred with the wild-type 3a gene in the transgenic plants. The altered regions of the progeny virus RNAs were sequenced, and the sequence was as expected for each mutant (data not presented).

The various mutants M4, M5, M6, and M7 also were coinoculated in six pairwise combinations to nontransgenic tobacco plants. In no case was infection detected by symptoms or Northern blot hybridization of RNA extracted from the inoculated leaves (data not presented). This indicates that the mutants were unable to complement each other.

Accumulation of alanine-scanning mutants in tobacco protoplasts.

Mutant M7 was unable to infect either nontransformed or 3a-transgenic tobacco (Table 1 and Fig. 2). Because the 3a

gene is dispensable for the replication of CMV RNA 3, it did not seem likely that the RNA mutation in mutant M7 had a direct, *cis*-acting effect on the ability of RNA 3 to be replicated. It was possible, however, that the encoded 3a protein in mutant M7 had a *cis*- or *trans*-acting effect on the replication of the CMV RNAs. To examine this possibility, protoplasts were prepared from nontransformed and 3a-transgenic tobacco and transfected with transcripts containing either the wild-type RNA 3 or the various MP mutants. The RNAs extracted from the protoplasts were analyzed 24 h postinoculation by Northern blot hybridization (Fig. 3A). Here it is apparent that all three genomic CMV RNAs were able to replicate and accumulate for mutant M7 to levels similar to that of the wild-type virus or the other MP mutants. In addition, there was no spe-

Fig. 2. *Cucurbit mosaic virus* (CMV) accumulation in plants infected by wild-type (WT) CMV and the alanine-scanning mutants (M3–M7). Samples were obtained from inoculated plants: **A**, nontransformed tobacco plants; **B**, 3a-transgenic tobacco plants. RNAs were extracted from inoculated leaves (I) or leaves above the inoculated leaves (S) and analyzed by Northern blot hybridization with a probe specific to the 3' common nontranslated region of each viral RNA and the subgenomic RNA (4). The positions of the CMV RNAs 1, 2, 3, and 4 are indicated.

Table 1. Infectivity and complementation of *Cucurbit mosaic virus* movement protein mutants in non- and 3a-transgenic tobacco

Inoculum	Tobacco	3a-Tobacco ^a
WT ^b	+ ^c ; (0.35) ^d	+
M1 (R8, T9)	+	+
M2 (E38, D40)	+	+
M3 (Y75, D76)	+	+
M4 (R97, T98)	–	+; (0.13)
M5 (Y144, D145)	–	+; (0.18)
M6 (R156, F158)	–	+; (0.17)
M7 (N191, Y192)	–	–
M8 (D20, D21)	+	+
M9 (P60)	+	+

^a Transgenic tobacco expressing the 3a movement protein.

^b WT = wild-type 3a movement protein.

^c Local and systemic infection (+) versus no detectable infection (–).

^d Yield of virus in milligrams of virus per gram of tissue.

cific effect on the generation of the subgenomic RNA 4 encoding the CP gene for mutant M7 versus the other mutants. In the absence of CMV CP, CMV RNAs accumulate poorly or not noticeably in protoplasts (Canto et al. 1997; Suzuki et al. 1991). The expression and accumulation of the MP and CP in protoplasts for mutant M7 were similar to those of the other MP mutants in tobacco protoplasts (Fig. 3B). Thus, it is unlikely that the mutant M7 3a protein is less stable than the wild-type 3a protein. Moreover, any hypothetical reduced stability would not account for the inability of mutant M7 to be complemented by the transgenic 3a protein (Table 1 and Fig. 2B). In fact, the defective 3a protein of mutant M7 is *cis* dominant for movement of CMV RNA 3 and possibly *trans* dominant for movement of CMV RNAs 1 and 2 in the 3a-transgenic plants.

Infectivity of alanine-scanning mutants in other hosts.

Table 2 shows the results of infectivity analyses of the nine mutants on other CMV hosts, including plants in the families *Solanaceae* (tomato and pepper), *Cucurbitaceae* (cucumber and squash), *Compositae* (*Zinnia elegans*), *Chenopodiaceae* (*Chenopodium quinoa*), and *Leguminosae* (cowpea). In all of the systemic host species (excluding the hypersensitive hosts *C. quinoa* and cowpea in which Fny-CMV induces local lesions), those mutants that could infect tobacco also could in-

fect the other host species (compare Tables 1 and 2). None of the mutants defective for movement on tobacco (M4 to M7) could infect any of the other CMV hosts, indicating that the effects of those mutations were on the more generic functions of the MP rather than on its host-specific functions.

In tomato, mutant M9 induced more severe symptoms than the wild-type CMV, but the level of accumulated viral RNA for mutant M9 was similar to that of the wild-type CMV (data not shown). The symptoms induced by mutants M9 and M3 were milder than they were for the other mutants or for wild-type CMV in *Z. elegans*, also presumably reflecting some difference in host-specific interactions. Most strikingly, however, was the effect of the mutation in the 3a gene of mutant M8 on the infection of the two hypersensitive hosts, *C. quinoa* and cowpea (Table 2). In both of these species, mutant M8 did not induce a hypersensitive response and no viral RNA could be detected in the inoculated leaves by Northern blot hybridization (data not shown). Further experiments testing mutant M8 on *C. amaranticolor* showed that mutant M8 was unable to move out of the initially inoculated epidermal cells. Moreover, the requirement for cell-to-cell movement rather than just the coexpression of MP and CP is essential for the elicitation of a hypersensitive response in this host (Canto and Palukaitis 1999a).

Effects of mutations on long-distance movement.

Mutants M1 to M3, M8, and M9 were assessed for potential temperature-sensitive (ts) effects on virus movement in to-

Fig. 3. *Cucumber mosaic virus* (CMV) accumulation in tobacco protoplasts. Wildtype (WT) CMV and the nine alanine-scanning mutants were transfected into tobacco protoplasts. At 24 h posttransfection, **A**, total RNAs and **B**, proteins were extracted and analyzed separately. The RNAs were analyzed by Northern blot hybridization with a probe specific to the 3' end of each viral RNA (**A**). Proteins were analyzed by Western blotting and immunoprobing sequentially with antisera to the 3a MP and the CP, respectively, before photography (**B**). Hea = extracts from noninoculated protoplasts. Position of the CMV RNAs 1, 2, 3, and 4 (**A**) and the 3a MP and CP are indicated (**B**). Mr = protein molecular weight markers.

Fig. 4. Effect of temperature on *Cucumber mosaic virus* (CMV) accumulation in tobacco plants. Wild-type (WT) CMV and CMV containing movement protein mutant M9 were inoculated to tobacco plants maintained for 7 days at 26 or 34°C. Total RNAs were extracted from inoculated leaves (I) as well as leaves above the inoculated leaves (S) and analyzed by Northern blot hybridization with a CMV-specific probe. Positions of the four CMV RNAs are indicated. Increased mobility of RNAs in the sample from leaves infected systemically (S) by mutant M9 and maintained at 26°C is an electrophoretic artifact.

bacco. Mutant M9 showed a ts effect on virus movement at 34°C (Fig. 4), whereas the other mutants did not (data not presented). Below 32°C, however, mutant M9 did not show a ts effect on virus movement (Fig. 4 and data not presented). Mutant M9 could still move cell to cell in the inoculated leaf at 34°C but could not move long distance (Fig. 4). In contrast, wild-type Fny-CMV could move long distance at 34°C (Fig. 4). After shifting the temperature from 34 to 26°C, mutant M9 could again move long distance (systemically) in tobacco that was initially inoculated and maintained at 34°C (not shown). This confirms previous experiments that indicate a role for the CMV MP in long-distance and cell-to-cell movement (Kaplan et al. 1997; Ryabov et al. 1999).

Mutants M4, M5, and M6 were each mixed separately with mutant M9 and inoculated to tobacco plants that were maintained at 34°C. No complementation for long-distance movement was observed between the mutants affecting cell-to-cell movement (M4, M5, and M6) and the mutant M9, which affected systemic infection of tobacco (data not shown).

DISCUSSION

Functional map of the CMV MP.

A functional map of the CMV 3a MP can be constructed (Fig. 5) based on data presented here and elsewhere (Canto and Palukaitis 1999a; Canto and Palukaitis 1999c; Canto et al. 1997; Ding et al. 1995a; Gal-On et al. 1996; Kaplan et al. 1995; Kaplan et al. 1997; Li and Palukaitis 1996; Vaquero et al. 1997; You et al. 1999). Although this map, similar to those proposed for the functional maps of *Tobacco mosaic virus* (TMV) (Kahn et al. 1998; Mezitt and Lucas 1996), *Cauliflower mosaic virus* (Huang et al. 2000; Thomas and Maule 1999), *Cowpea mosaic virus* (Bertens et al. 2000), and *Red clover necrotic mosaic virus* (Mezitt and Lucas 1996), is linear, it is recognized that the three-dimensional structure of the MP is likely to be important for functionality, at the very least through coordination of the independently analyzed functions. Superimposed on this map of sites affecting various functions (Fig. 5) are several putative functional domains and conserved or variable sequences that are based on sequence analyses (De

Jong et al. 1995; Fujita et al. 1996; Gafny et al. 1992; Nagano et al. 1997; Romero et al. 1992). Thus, it can be seen that all of the mutants that exhibited an inhibition of virus movement are clustered in the center of the MP, whereas two conditionally dysfunctional mutants (M8 and M9) are grouped in the N-terminal region at amino acids 20 and 21 and 60. At position 51 is another site associated with a conditional dysfunction (S3), an inability to systemically infect cucurbit species (Kaplan et al. 1997). Position 51 is equivalent to BMV MP position 54 (Fig. 1), and mutations at this site are associated with a change in host range of BMV (De Jong et al. 1995). The sequences from amino acids 213 to 279 (the C terminus) showed virtually no similarity between CMV and the bromoviruses, whereas the MPs of CMV strains themselves showed considerable variation between amino acids 228 and 279 (Fig. 1 and data not shown). In addition, CMV MP sequences from position 237 to 279 could be deleted without affecting movement, whereas some or all of amino acids 209 to 236 were required for some aspect of the movement process (Kaplan et al. 1995). The sequences between amino acids 174 to 233 were shown to contain the core of the in vitro RNA binding domain (Vaquero et al. 1997). This domain includes mutant M7 (amino acids 191 and 192) as well as sequences required for movement (amino acids 209 to 236) (Kaplan et al. 1995). The deletion of amino acids 160 to 200 or 200 to 246, however, did not affect RNA binding in vitro (You et al. 1999). This may indicate the presence of more than one RNA binding site available within this region. While the C-terminal 40 amino acids of the CMV MP appeared to be dispensable, amino acid 240, located in this region, together with amino acid 51 conditioned for a cucurbit-specific, restricted-movement phenotype (Kaplan et al. 1997) and for increased spread within tobacco plants (Gal-On et al. 1996). Therefore, the C terminus of the 3a protein appears to contain sequences that have subtle effects on host-specific interactions (Nagano et al. 1997). This C-terminal region of the MP also appears to be involved in interactions involving the CP (Nagano et al. 1997).

The N-terminal region of the MP also contains sequences involved in host-specific interactions, as shown by mutant M8

Table 2. Infectivity of *Cucumber mosaic virus* (CMV) movement protein mutants in other host plant species

Inoculum	Host plants ^a						
	<i>Lycopersicon esculentum</i>	<i>Capsicum annum</i>	<i>Cucumis sativus</i>	<i>Cucurbita pepo</i>	<i>Zinnia elegans</i>	<i>Chenopodium quinoa</i>	<i>Vigna unguiculata</i>
WT ^b	+	+	+ ^d	+	+++	LL ^f	LL
M1 (R8, T9)	+	+	+	+	+++	LL	LL
M2 (E38, D40)	+	+	+	+	+++	LL	LL
M3 (Y75, D76)	+	+	+	+	+++ ^e	LL	LL
M4 (R97, T98)	-	-	-	-	-	-	-
M5 (Y144, D145)	-	-	-	-	-	-	-
M6 (R156, F158)	-	-	-	-	-	-	-
M7 (N191, Y192)	-	-	-	-	-	-	-
M8 (D20, D21)	+	+	+	+	+++	-	-
M9 (P60)	+++ ^c	+	+	+	+++ ^e	LL	LL

^a Host plants tested for infectivity: *L. esculentum* (tomato), *C. annum* (pepper), *C. sativus* (cucumber), *C. pepo* (squash), *Z. elegans*, *C. quinoa*, *V. unguiculata* (cowpea).

^b WT = wild-type 3a movement protein.

^c Symptoms more severe than for WT CMV.

^d Local and systemic infection (+) versus no detectable infection (-).

^e Symptoms of mutants M3 and M9 on *Z. elegans* were much milder than with WT CMV.

^f LL = local lesions with no systemic infection.

(Table 2). Deletion of the N-terminal 34 amino acids was shown to lead to a loss of virus movement, whereas mutations at amino acid 2, 3, 8, and 9 did not affect movement (Kaplan et al. 1995). Similarly, duplications of amino acid 4 and 5, generated in vivo by pseudoreversion of a frameshifted mutant, led to the production of a virus that appeared to move normally (Kaplan et al. 1995). Thus, in contrast to the situation with TMV (Gafny et al. 1992), the sequence of the N-terminal nine amino acids of the CMV MP does not appear to be critical for movement, whereas deletion of the first 34 amino acids affected either the stability or function of the MP (Kaplan et al. 1995; You et al. 1999).

Dysfunctional mutant M4 is located within a hydrophobic core domain (amino acids 86 to 108) conserved in putative MPs of 17 plant virus genera (Melcher 2000; Mushegian and Koonin 1993). This hydrophobic domain contains a putative transmembrane helix, which may be conserved in cellular transport proteins. In contrast, dysfunctional mutants M5, M6, and M7 are located in sequence blocks that resemble putative zinc fingers and possible RNA binding domains (Fig. 5). Within these putative zinc finger regions are putative nucleic acid binding domains previously identified in bromoviruses (Romero et al. 1992). MP mutant M5 is at the end of the first putative zinc finger region (Fig. 5), and was shown to be unable to traffic itself or viral RNA from cell to cell or to increase the size exclusion of fluorescent dextran (Ding et al. 1995a). The sequences in the second putative nucleic acid binding domain of BMV, equivalent to CMV amino acid 165 that is near mutant M6 (amino acids 156 and 158), was altered in a bromovirus hybrid that developed the ability to systemically infect cowpeas (Fijita et al. 1996). Thus, this region of the MP contains sequences affecting generic functions of the MP as well as host-specific functions.

Complementation of movement functions: models for virus movement.

The inability of mutant M7 to be complemented in *trans* by the transgenic 3a protein suggests that this mutant MP is inter-

fering with the movement of CMV. This particular mutation in RNA 3 did not affect its replication nor did the mutated MP interfere with the replication and accumulation of any of the other genomic RNAs or the expression of the subgenomic RNA required for CP production (Fig. 3). Rather, the effects of the mutation appeared to be on some aspect(s) of the movement process, per se. In this regard, mutant M7 behaved similar to the transdominant mutations made in the N-terminal region of the TMV 30-kDa MP (Lapidot et al. 1993) and within the 26-kDa MP of the potyvirus *White clover mosaic virus* (Beck et al. 1994). In those cases, transgenic expression of the mutant MPs led to resistance to infection by the homologous wild-type virus as well as provided some level of protection against infection by a range of distantly related and unrelated viruses (Beck et al. 1994; Cooper et al. 1995; Lapidot et al. 1993). It is of interest to determine whether mutant M7 could also exhibit such broad protection to other viruses if expressed in transgenic plants. The mechanism of such protection and the ability of mutant M7 to inhibit movement could provide valuable insight into the mechanisms of virus movement.

Complementation of CMV movement by infection with four CMV RNAs (RNA 1, RNA 2, and two dysfunctional forms of RNA 3) has been demonstrated (Canto and Palukaitis 1999a; Canto and Palukaitis 1999b; Canto and Palukaitis 1999c; Canto et al. 1997). In those instances, however, complementation involved dysfunction in different genes (MP and CP). The inability of mutants to complement each other here may be understandable for mutants M4 to M6, which are in close proximity and may affect the same or overlapping functional domains. Although mutant M9 could move cell to cell at the restrictive temperature (Fig. 4), the inability of mutants M4 to M6 to complement mutant M9 at the restrictive temperature could indicate that mutants M4 to M6 also have an effect on long-distance movement or that a single-movement protein must be able to interact with the viral RNA and various host components.

Mutant M8 was capable of systemic infection in the various systemic hosts tested (Tables 1 and 2). Moreover, MP

Fig. 5. Mutational and functional map of the *Cucurbit mosaic virus* (CMV) 3a movement protein (MP). Positions of MP mutants M1–M9 and S3 are indicated. Filled-pattern rectangles indicate various domains or conserved regions, described below. Plus sign indicates MP infectious mutants, whereas minus sign indicates noninfectious MP mutants. Conditionally infectious MP mutants (M8, M9, and S3) are indicated. MP S3 indicates two positions (51 and 240) that together influence movement in tobacco and cucurbit species. MP mutant M1 (positions 8 and 9) lies within an N-terminal region (amino acids 1–9) where deletion and duplication mutants do not affect CMV movement. MP mutant M2 (positions 38 and 40) lies between two regions (10–25 and 44–57) of strong sequence dissimilarity between the MPs of CMV and the bromoviruses (Fig. 1). MP mutant M8 (positions 20 and 21) lies within one of these regions. MP mutants M9 (position 60) and M3 (positions 75 and 76) lie between the above sequence-heterogeneous regions and a highly conserved hydrophobic core (positions 86–118) conserved in the putative MPs of 17 plant virus genera. MP mutant M4 lies within this hydrophobic core region. MP mutants M5 (positions 144 and 145), M6 (positions 156 and 158), and M7 (positions 191 and 192) lie within two putative zinc finger domains (positions 126–146 and 157–194), which also encompass two putative nucleic acid binding domains (black rectangles at positions 134–138 and 164–168). MP mutant M7 also is located within the in vitro RNA binding domain (diagonal pattern fill at positions 174–233). Domain bordered by amino acid positions 209–236 (horizontal-line pattern fill) overlaps the RNA binding domain and contains sequences essential for CMV movement. Adjacent domain bordered by amino acids 237–279 (C terminus) is dispensable for CMV movement. Sequences in this region also show the greatest variability between CMV and the bromoviruses (vertical-line pattern fill at positions 213–279) or between strains of CMV (diagonal-line pattern fill at positions 228–279).

mutant M8 was able to associate with the plasmodesmata of epidermal cells (Canto et al. 1999a), yet it was not able to promote movement of RNA or MP to other epidermal cells (Canto et al. 1999c). CMV containing mutant M8, however, was able to move systemically in most host species tested (Tables 1 and 2). Thus, MP mutant M8 must retain the ability to bind and interact with viral RNA. This indicates that there are viral-encoded, active processes that occur in CMV movement beyond the association of the RNA:MP complex with plasmodesmata. This could involve a conformational change in the MP, as previously proposed for the role of the CP in cell-to-cell movement (Ryabov et al. 1999), or it could involve plasmodesmata-associated proteins that differ slightly between epidermal-epidermal and epidermal-mesophyll cells.

A model has been proposed for the movement of CMV RNA into sieve elements (Blackman et al. 1998). This model predicts that CMV RNA and MP and CP interact to form a ribonucleoprotein complex that moves through the plasmodesmata between companion cells and the sieve element. Various data suggest that CMV RNA does not move cell to cell in the form of virus particles even though CP is required for CMV movement (Blackman et al. 1998; Kaplan et al. 1998; Schmitz and Rao 1998). Rather, the data suggest that RNA must be associated with MP for cell-to-cell movement and CP plays an important, but possibly indirect, role. No interaction has been detected directly between MP and CP (O'Reilly et al. 1997; D. Szilassy and P. Palukaitis *unpublished*). Because replication proteins (Deom et al. 1997; Gal-On et al. 1994; Nelson and Van Bel 1998) and the CMV 2b protein (Ding et al. 1995b) also affect virus movement, it is of interest to determine whether they also participate directly in the long-distance movement process.

MATERIALS AND METHODS

Viral constructs, alanine-scanning mutagenesis, transcription, and plant inoculation.

Plasmids pFny109, pFny209, and pFny309 contain full-length cDNA copies of RNA 1, RNA 2, and RNA 3, respectively, of the Fny strain of CMV (Rizzo and Palukaitis 1990). Nine alanine-scanning mutations were generated in the 3a gene of Fny-CMV by site-directed mutagenesis (Kunkel et al. 1987) with the use of synthetic oligonucleotide primers. The mutated DNA was digested with restriction enzymes and subcloned into pFny309 by standard procedures (Sambrook et al. 1989). The nucleotide changes were then confirmed by DNA sequencing. The plasmids pFny109, pFny 209, pFny309, and various derivatives of the last were transcribed *in vitro* with T7 RNA polymerase, and the viral RNA transcripts were inoculated to various plant species, as previously described (Zhang et al. 1994). The plant species inoculated were tobacco (*Nicotiana tabacum* cv. Xanthi nc), tomato (*Lycopersicon esculentum* cv. Rutgers), pepper (*Capsicum annum* cv. Jalapeño), cucumber (*Cucumis sativus* cv. Straight 8), zucchini squash (*Cucurbita pepo* cv. Black Beauty), *C. quinoa*, cowpea (*Vigna unguiculata* cv. Blackeye 5), and *Z. elegans*. Transgenic tobacco plants expressing the CMV 3a gene and competent for complementation of CMV defective for movement (Kaplan et al. 1995) also were used in some experiments.

Analysis of viral RNAs and viral-encoded proteins.

Tobacco protoplasts were prepared and transfected with CMV RNA transcripts as previously described (Gal-On et al. 1994; Gal-On et al. 1995). Proteins were extracted from infected protoplasts and were analyzed by electroblotting and immunoprobings proteins with antisera to the 3a and CP (sequentially). RNAs were extracted from infected protoplasts and analyzed by Northern blot hybridization with a probe specific to the 3' nontranslated and common to each of the four CMV RNAs. Protein and RNA levels were equalized prior to loading gels. All of the above were done as previously described by Gal-On et al. (1994). Total RNAs were extracted from either inoculated leaves or leaves above the inoculated leaves and analyzed for the presence of the CMV RNAs by Northern blot hybridization (Gal-On et al. 1994; Gal-On et al. 1995).

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