VIROLOGY 248, 323–331 (1998)
ARTICLE NO. VY989257

Deletions in the Conserved Amino-Terminal Basic Arm of Cucumber Mosaic Virus Coat Protein Disrupt Virion Assembly but Do Not Abolish Infectivity and Cell-to-Cell Movement

Isabelle Schmitz and A. L. N. Rao[1]

Department of Plant Pathology, University of California, Riverside, California 92521-0122

Received April 9, 1998; returned to author for revision May 6, 1998; accepted May 26, 1998

The N-terminal basic arm of cucumber mosaic cucumovirus (CMV) coat protein (CP) contains a conserved arginine-rich motif, which is characteristic of RNA binding proteins of several plant and nonplant viruses. To identify regions of the CMV CP N-terminus that are essential for interacting with viral genomic RNA, a comprehensive set of mutations was engineered into biologically active clones of CMV RNA3 and the behavior of each variant with respect to infectivity, packaging and movement was examined. Biological assays conducted in *Chenopodium quinoa* (local lesion host) and *Nicotiana benthamiana* (systemic host) revealed that variants lacking either 12 N-proximal amino acids or a region containing four consecutive arginine residues of the CP N-terminus were competent for assembly into virions and remained infectious in plants. Interestingly, two other variants, lacking either 19 N-proximal amino acids or a domain containing a cluster of six arginines in the arginine-rich motif, were incompetent for virion assembly but retained the ability to move cell to cell. Taken together, these results indicate that a major portion of the N-terminal basic arm of CMV CP is dispensable for CP-RNA interactions and also establish that CMV can move cell to cell in a nonvirion form. The distinctive role played by the viral CP in movement and specifically, the extent to which the CP N-terminal basic arm is involved in the infection cycle of CMV are discussed.

INTRODUCTION

Many plant icosahedral RNA viruses such as members of the *Bromoviridae* (Sacher and Ahlquist, 1989; Rao and Grantham, 1995; Yusibov and Loesch-Fries, 1995), tomato bushy stunt tombusvirus (Harrison *et al*., 1978), turnip crinkle carmovirus (Hogle *et al*., 1986), and southern bean mosaic sobemovirus (Erickson and Rossmann, 1982), have coat proteins (CPs) with highly basic N-terminal arms that are thought to interact with the viral RNA inside the capsid shell. For bromoviruses, *in vitro* cross linking experiments (Sgro *et al*., 1986) showed that N-terminal peptides encompassing brome mosaic bromovirus (BMV) CP residues 11 to 19, but not residues 1 to 7, bind to viral RNA. Consequently, deletion of the first 7 N-terminal amino acids had no detectable effect on replication, packaging and infectivity in barley plants (Sacher and Ahlquist, 1989; Rao and Grantham, 1995;) but changed the symptom phenotype in *Chenopodium quinoa* (Rao and Grantham, 1995). The N-terminal basic arm of BMV CP has an arginine-rich motif (6 arginines between positions 9–21) and is conserved among bromoviruses, human immunodeficiency virus (HIV) Tat and Rev proteins, λN and yeast PR6 (Tan and Frankel, 1995). Our recent functional analysis of the arginine-rich motif of BMV CP revealed that, a deletion encompassing the first 19, but not 18, N-terminal amino acids rendered the BMV CP incompetent for virion formation and infectivity (Rao and Grantham, 1996).

When the amino acid sequence of the N-terminal basic arm of BMV CP was compared to that of other plant viruses with similar basic arms, we found that only the CPs of cucumoviruses (belonging to the *Bromoviridae*) also contained the conserved arginine-rich motif (Rao and Grantham, 1996). Like BMV, cucumber mosaic cucumovirus (CMV) is a tripartite icosahedral virus and represents the type member of the cucumovirus genus (Palukaitis *et al*., 1992). Its genome is divided among three single-stranded positive sense RNAs (ssRNA), encoding four nonstructural and one structural protein. CMV RNAs 1 and 2 encode replicase subunits 1a and 2a, respectively (Hayes and Buck, 1990). In addition, CMV also encodes another gene product, 2b, that has been shown to regulate viral movement in tobacco (Ding *et al*., 1995b). The two gene products encoded by dicistronic RNA3 are dispensable for viral replication but are required for infectivity in plants (Suzuki *et al*., 1991; Boccard and Baulcombe, 1993). A nonstructural protein of 30 kDa, encoded by the 5′ half of RNA3 is the designated movement protein (MP) required for cell-to-cell movement (Ding *et al*., 1995a). The 20-kDa CP, encoded by the 3′ half of CMV RNA3, is translated from subgenomic RNA4 transcribed from progeny minus-sense RNA3 (Boccard

[1] To whom correspondence should be addressed. Fax: (909) 787-4294. E-mail: arao@ucrac1ucr.edu.

0042-6822/98 $25.00

and Baulcombe, 1993). The structure of CMV was recently determined by cryoelectron microscopy and X-ray crystallography and found to be similar to that of cowpea chlorotic mottle bromovirus (CCMV; Wikoff *et al*., 1997). As observed for CCMV, the N-terminal basic arm of CMV CP is internal and therefore not visible in the electron density map (Wikoff *et al*., 1997). Although CMV CP has been extensively studied in relation to symptom expression and infection spread (Shintaku *et al*., 1992; Suzuki *et al*., 1995; Taliansky *et al*., 1995; Canto *et al*., 1997), the contribution of the CP's conserved N-terminal basic arm to RNA-CP interactions has not been investigated. This study reports the functional analysis of the CMV CP N-terminal basic arm and specifically the arginine-rich motif contained therein by engineering a series of variants targeting this region and testing their biological activities in susceptible host plants.

RESULTS

Characteristics and replication of N-terminal variants of CMV CP

The first 25 amino acids of the CMV CP, which constitute the N-terminal basic arm, are rich in positively charged residues (Fig. 1A). The region between positions 10 and 22 is defined as the conserved arginine-rich motif (boxed region in Fig. 1A), which shares a high degree of homology to other arginine-rich motifs found in genetically related bromoviruses (Rao and Grantham, 1996). Six arginine (Arg) residues at positions 14 to 17, 19, and 20 of this motif are referred to as the Arg cluster. A lysine (Lys) at position 3 and another Arg at position 23 also contribute to the positively charged environment of the CP N-terminal arm. In bromoviruses, the arginine-rich motif has been shown to be important in RNA-protein interactions during virus assembly (Sgro *et al*., 1986; Rao and Grantham, 1996). To identify the regions of the CMV CP N-terminal arm involved in such interactions, we engineered a series of mutations into the CP gene sequence coding for the first 20 N-proximal amino acids and examined their effect on RNA packaging, infectivity and movement characteristics in two susceptible hosts. The variants constructed in this study fall into two categories. The first set, which includes variants ΔN12 and ΔN19 (Fig. 1A) was designed to examine the minimal N-proximal amino acids required to retain biological activity. CP variant ΔN12 is characterized by the deletion of 12 N-proximal amino acids, which include the single Lys and Arg present at positions 3 and 11, respectively, leaving the Arg cluster (residues 14 to 20) intact (Fig. 1A). In CP variant ΔN19, the deletion present in ΔN12 was extended to include the Arg cluster (Fig. 1A). The second set, which includes variants 15P, Δ4R, and ΔR (Fig. 1A), was intended to establish the specific role played by the Arg cluster in RNA-CP interactions. To examine the significance of four consecutive Arg residues at positions 14 to 17, two variants were constructed. These are variant 15P, in which a Pro was substituted for an Arg at position 15, and variant Δ4R, in which all four Arg residues were deleted (Fig. 1A). In variant ΔR, the sequence encoding the first N-terminal 13 amino acids was fused to the sequence encoding amino acid 21, resulting in a deletion of the entire Arg cluster located at positions 14 to 20 (Fig. 1A).

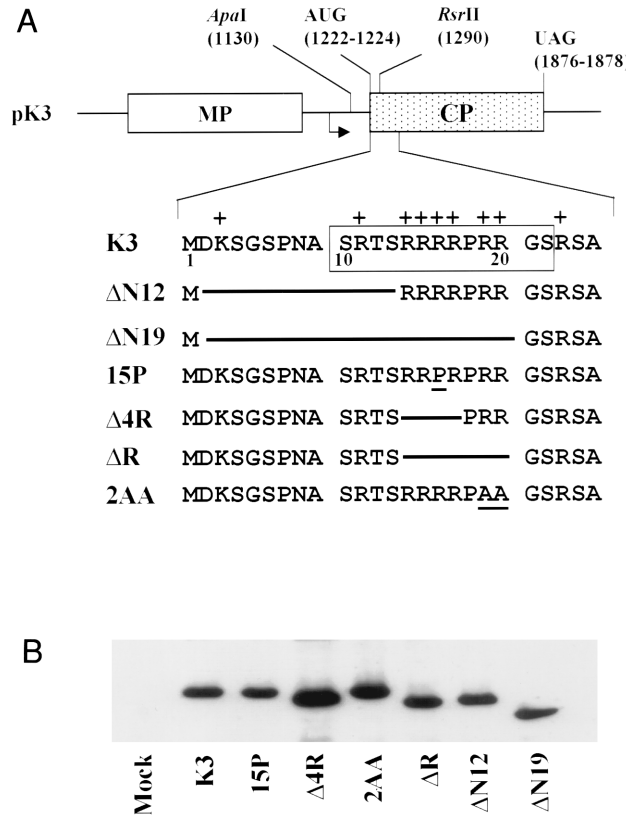

FIG. 1. Mutational analysis of the N-terminal basic arm of CMV CP. (A) The structure of wild type (wt) CMV-Kin RNA 3 (pK3) is shown, with noncoding sequences represented as single lines and the movement protein (MP) as an open box. The stippled box represents the coat protein (CP). The positions of the CMV CP start codon (AUG present at positions 1222–1224) and stop codon (UAG present at 1876–1878) are shown. The transcription start site of CMV CP subgenomic RNA4 (at base 1167) is indicated by a bent arrow. The restriction sites, *Apa*I and *Rsr*II, used to manipulate the introduced mutations are shown. The first 25 N-terminal amino acids encoded by wt CMV CP gene are shown. Positively charged side chains are indicated by a (+). The boxed region indicates the arginine-rich motif. The extent of the engineered deletions for variants ΔN12, ΔN19, Δ4R, and ΔR are shown by solid lines, whereas substituted amino acids for variants 15P and 2AA are underlined. (B) Western blot analysis of CP accumulation. Polyacrylamide gel electrophoresis of CP from *C. quinoa* protoplasts transfected with transcripts of wt K1 and K2 and either wt K3 or the indicated K3 variant. Protoplasts were suspended in SDS-PAGE sample buffer, denatured by boiling for 8 min, and subjected to 16% SDS-PAGE. After proteins were transferred to a nitrocellulose membrane, the blot was probed with antibodies specific for CMV CP (serogroup S) and detected with an Enhanced Chemoluminescence (ECL) kit (Amhersham). Note the difference in mobility of CP for wt K3 and deletion variants.

In CMV, replication is dependent on gene products encoded by RNAs 1 and 2, but does not require RNA3 (Hayes and Buck, 1990; Boccard and Baulcombe, 1993). Consequently, transfection of *C. quinoa* protoplasts with each CP variant resulted in efficient replication and synthesis of CPs of expected sizes (Fig. 1B).

Symptom expression and movement characteristics of N-terminal variants of CMV CP

Although CMV has a wide host range (Palukaitis *et al*., 1992), we chose to analyze the effect of our CP mutants in *C. quinoa* because this host has been previously reported to exhibit symptom modulation in response to inoculation of several CP variants of genetically related BMV (Rao and Grantham, 1995, 1996). In *C. quinoa*, infection by wild-type (wt) CMV-Kin results in the induction of expanding chlorotic local lesions 2 mm in diameter 3–5 days postinoculation (dpi) and lesions turn necrotic by 8–10 days p.i. (Fig. 2A; Table 1). No systemic infection was observed (Table 2). Irrespective of the engineered mutation, all CMV CP variants were infectious to *C. quinoa* and induced local lesions, however, to varying degrees. The symptom phenotypes induced in *C.quinoa* are summarized in Table 1, and representative examples are shown in Fig. 2A. Local lesions induced by 15P were indistinguishable from those of wt CMV (Table 1). Other CP variants, ΔN12, ΔN19, Δ4R, and ΔR, also induced local lesions but they remained small in size ($<$0.5 mm), and their appearance was delayed by at least 3 days (Table 1; Fig. 2A). These lesions did not expand with time. Western blot analysis of CP recovered from these symptomatic leaves (Table 2) revealed a profile similar to those observed in protoplast transfections (Fig. 1B), suggesting that the observed symptoms resulted from infection by respective CP variants and are not due to contamination. As observed for wt CMV-Kin, none of noninoculated systemic leaves of these plants displayed any symptoms and neither viral RNA nor CP was detected in these leaves when sensitively probed by Northern and Western blot analysis, respectively (Table 2).

Because wt CMV-Kin is inherently limited to localized infections in *C. quinoa* (Tables 1 and 2), the competence of each CMV CP variant for long distance movement could not be assessed in this host. CMV-Kin infects *Nicotiana clevelandii* and *N. benthamiana* systemically. In *N. clevelandii* CMV-Kin induces a mild systemic mosaic, whereas in *N. benthamiana*, an infection is characterized by more obvious chlorotic mosaic symptoms in the systemic leaves 10–14 days p.i. (Fig. 2B). Therefore, *N. benthamiana* plants were inoculated with wt CMV-Kin and each CMV CP variant and observed for symptom expression and systemic infection. Among the CP variants examined, three variants, 15P, ΔN12, and Δ4R, infected *N. benthamiana* systemically on a time scale similar to that of wt CMV-Kin. Symptoms induced by variants 15P and Δ4R were indistinguishable from those of wt CMV-Kin, while those induced by variant ΔN12 were more severe (Fig. 2B; Table 1). Neither ΔR nor ΔN19 induced visible symptoms on noninoculated systemic leaves and plants remained symptomless even 4–5 weeks post inoculation. Northern and Western blot analysis of progeny RNA and CP obtained from inoculated and noninoculated leaves revealed that variants 15P, ΔN12 and Δ4R were competent for systemic movement, whereas infections resulting from variants ΔR and ΔN19 remained local (Fig. 3; Table 2).

From comparing the different symptom phenotypes and movement behavior of variants Δ4R and ΔR in *N. benthamiana*, we surmised that the lack of systemic movement exhibited by variant ΔR was perhaps due to the extended deletion including two additional Arg at positions 19 and 20 (Fig. 1A). Thus, to ascertain the importance of these Arg residues, another CP variant, 2AA, in which Arg 19 and 20 were replaced with Ala (Fig. 1A), was constructed. This variant replicated to near wt levels in *C. quinoa* protoplasts (data not shown) and synthesized CP of expected size (Fig. 1B). Inoculation of variant 2AA to *C. quinoa* and *N. benthamiana* resulted in symptom phenotypes indistinguishable from those of wt CMV-Kin (Table 1) and infection was confirmed by the detection of progeny RNA in Northern blots (Fig. 3; Table 2) and CP in Western blots (Table 2). Taken together, these observations indicate that the lack of systemic movement exhibited by ΔR could be due to either the cumulative effect of the introduced deletion or the inability to form virions (see below).

Virion-assembly defective CP variants of CMV-Kin are competent for cell-to-cell movement

Previous mutational analyses of CMV MP and CP revealed that both genes are essential for cell-to-cell movement (Suzuki *et al*., 1991; Boccard and Baulcombe, 1993; Kaplan *et al*., 1995; Canto *et. al.,* 1997). However, the CP form in which CMV is transported between cells is not known. Thus, we sought to examine whether infections resulting from inoculations with each CP variant examined in this study (Fig. 1A) were due to the transport of CMV in a virion form or as a CP-RNA complex. To address this question, virions were purified from inoculated and noninoculated systemic leaves of *N. benthamiana* and examined by electron microscopy. The results are summarized in Table 2 and representative examples are shown in Fig. 4. Virions characteristic of CMV were consistently observed in preparations obtained from symptomatic leaves of plants infected with 15P, ΔN12, Δ4R, and 2AA (Table 2; Fig. 4). No virions could be purified from leaves infected with either ΔN19 or ΔR (Table 2). Nonetheless, if any virions had assembled from truncated CP variants, ΔN19 or ΔR, they may have been unstable during the purification process. To verify this possibility, leaf dip preparations were made for

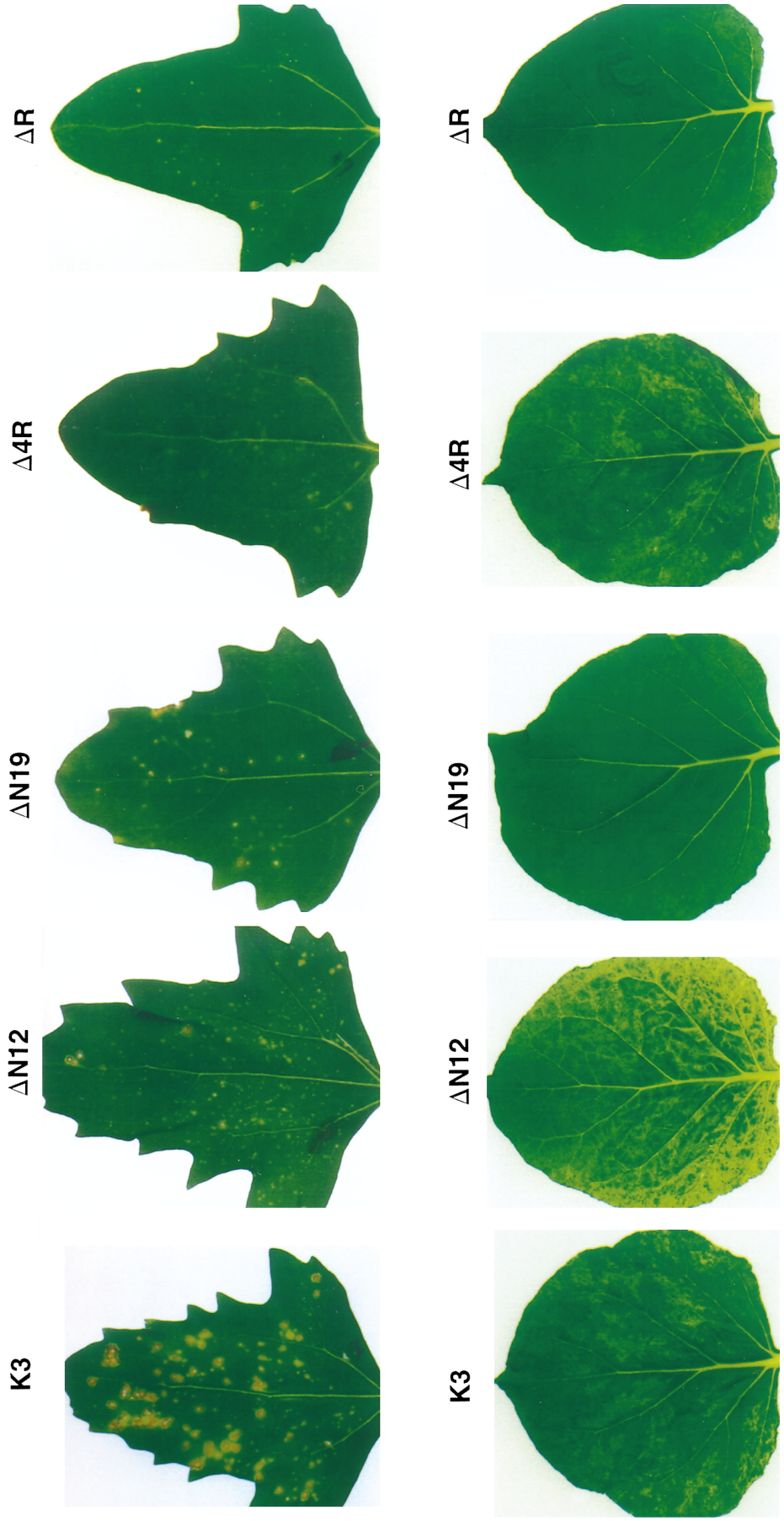

FIG. 2. Representative examples of symptom phenotypes induced on the inoculated leaves of *C. quinoa* (A) and noninoculated systemic leaves of *N. benthamiana* (B). Plants were inoculated with *in vitro* transcripts of wt K1 and K2 and either wt K3 or the indicated K3 variant. For *C. quinoa*, leaves were photographed 10 days p.i. and for *N. benthamiana* 14 days p.i.

TABLE 1

Symptom Phenotypes Induced by CMV CP Variants in *C. quinoa* and *N. benthamiana*

Inoculum[a]	*C. quinoa*[b] Local	*N. benthamiana* Systemic
K3 (wt)	Necrotic local lesions (2)	Chlorotic mosaic
ΔN12	Small necrotic local lesions (<0.5)	Severe chlorotic mosaic
ΔN19	Small necrotic local lesions (<0.5)	Not infected[c]
15P	Necrotic local lesions (2)	Chlorotic mosaic
Δ4R	Small necrotic local lesions (<0.5)	Mild chlorotic mosaic
ΔR	Small necrotic local lesions (<0.5)	Not infected[c]
2AA	Necrotic local lesions (2)	Chlorotic mosaic

[a] Each inoculum (300 μg/ml) contained a mixture of *in vitro* transcripts of wt K1 and K2 and either wt K3 or the indicated K3 variant.
[b] The numbers in parentheses represent the average size (in millimeters) of the local lesions induced in *C. quinoa.*
[c] Absence of infection was confirmed by Western and Northern blot analysis.

ISEM from *N. benthamiana* and *C. quinoa* leaves inoculated with ΔN19 or ΔR. As a positive control, a similar preparation was made from leaves inoculated with wt CMV-Kin. Although virus particles characteristic of CMV were observed in the control samples, no virions were observed in preparations of either *N. benthamiana* or *C. quinoa* inoculated with ΔN19 or ΔR (data not shown). Therefore, we conclude that, both variants were incompetent for virion assembly and moved cell to cell in a nonvirion form, possibly as a ribonucleoprotein complex.

DISCUSSION

Contribution of N-terminal basic arm of CMV CP to RNA interactions and virion assembly

Previous studies have examined the importance of the N-terminal basic arms found in the CPs of bromoviruses and alfalfa mosaic virus (AMV, a member of the *Bromoviridae*). The onset of AMV infection is dependent on the CP and its N-terminal basic arm is required to initiate this process (Yusibov and Loesch-Fries, 1995). By contrast, bromoviruses do not require CP for infectivity, but their CP N-terminal basic arm is essential to interact with viral RNA during the encapsidation process *in vivo* (Argos, 1981; Sgro *et al*., 1986; Vriend *et al*., 1986). Although CMV CP also contains an N-terminal basic arm (Fig. 1A), its specific role in the CMV infection cycle has not been investigated. Therefore, the major objective of this study was to examine whether the conserved N-terminal basic arms of BMV and CMV CPs are functionally similar. The results of this study demonstrate that the absence of a major portion of the CMV CP N-terminal basic arm does not prevent the truncated CP from interacting with viral RNA during the infection process. This is exemplified by

TABLE 2

Characteristics of CMV CP Progeny

	C. quinoa				*N. benthamiana*					
	Northern blot[a]		Western blot[b]		Northern blot[a]		Western blot[b]			
Inoculum	L	S	L	S	L	S	L	S	EM[c]	Sequence[d]
K3 (wt)	+	−	+	−	+	+	+	+	+	Conserved
ΔN12	+	−	+	−	+	+	+	+	+	Conserved
ΔN19	+	−	+	−	+	−	+	−	−	Conserved
15P	+	−	+	−	+	+	+	+	+	Conserved
Δ4R	+	−	+	−	+	+	+	+	+	Conserved
ΔR	+	−	+	−	+	−	+	−	−	Conserved
2AA	+	−	+	−	+	+	+	+	+	Conserved

[a] Detection of CMV RNAs by Northern hybridization; L = local; S = Systemic. +, Presence of CMV RNAs; −, Absence of CMV RNAs.
[b] Detection of CMV CP by Western analysis; L = local; S = systemic; +, presence of CMV CP; −, absence of CMV CP.
[c] Virions were purified from either inoculated leaves (for variants ΔR and ΔN20) or symptomatic systemic leaves for all other variants, negatively stained and examined under an electron microscope. +, presence of CMV virions; −, absence of CMV virions.
[d] Sequence of progeny RNA was determined from either inoculated leaves (for variants ΔR and ΔN20) or symptomatic systemic leaves for all other variants, as described under Materials and Methods.

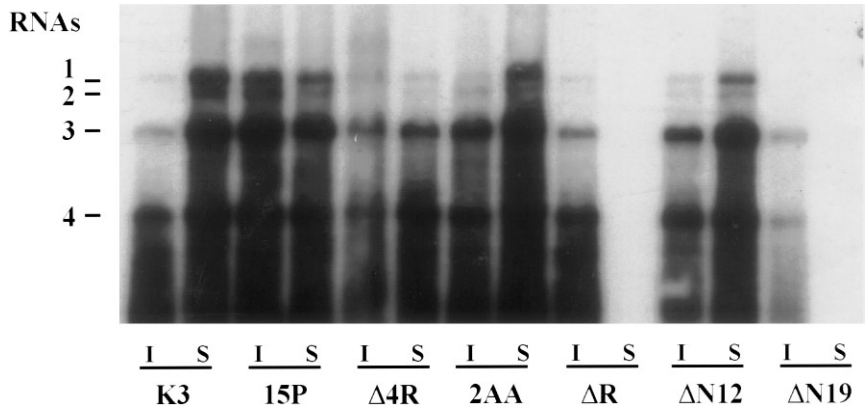

FIG. 3. Northern blot analysis of progeny RNA. Autoradiograph showing progeny RNA recovered from either inoculated (I) or noninoculated systemic (S) leaves of *N. benthamiana* infected with transcripts of wt K1 and K2 and either wt K3 or the indicated K3 variant. Total RNA was isolated and denatured with glyoxal prior to being subjected to electrophoresis in a 1% agarose gel. RNA was then electrophoretically transferred to nylon membranes. The membranes were then hybridized with ^{32}P-labeled (−) sense RNA probes representing the homologous 3′ 300 nt region present on each of the four CMV RNAs. The positions of the four CMV RNAs are shown to the left. The blot shown was exposed for 3 h at room temperature.

the infectious nature in inoculated leaves of *C. quinoa* and *N. benthamiana* of variant ΔN19 (Figs. 2 and 3), which lacks 19 N-proximal amino acids (Fig. 1A). Similarly, another variant, ΔR, characterized by the deletion of the Arg cluster located at positions 14 to 20 (Fig. 1A), was also infectious to the inoculated leaves of both host plants examined in this study (Figs. 2 and 3). As observed with variant ΔN19, a truncated BMV CP having a deletion of the first 18 N-terminal amino acids, encompassing five of the eight basic residues located within the first 25 N-terminal amino acids, is infectious to *C.quinoa* (Rao and Grantham, 1996). These observations underscore the functional similarities between the N-termini of BMV and CMV. Previous *in vitro* studies found that the first 25 N-terminal amino acids of BMV CP contain determinants for RNA binding (Sgro *et al*., 1986;

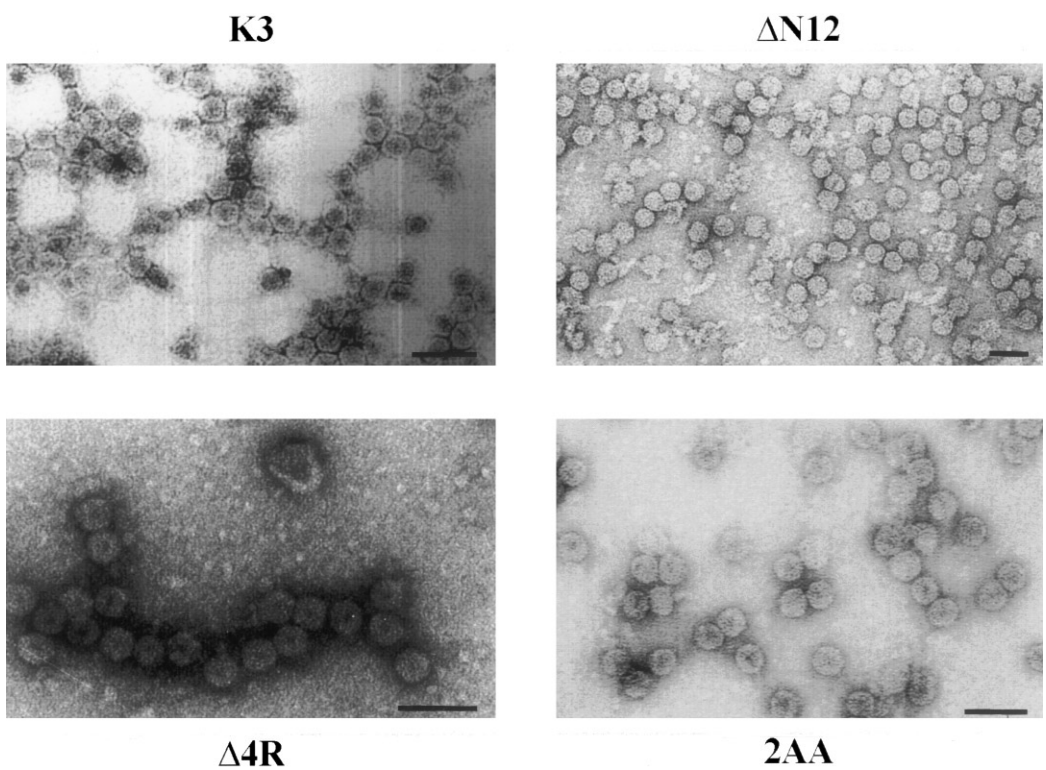

FIG. 4. Electron micrographs of purified virions. Virions were isolated from symptomatic leaves of *N. benthamiana* infected with wt K1 and K2 and either wt K3 or the indicated K3 variant. Purified virus preparations were negatively stained with uranyl acetate and photographed when viewed under a Hitachi transmission electron microscope. Bar: 75 nm.

Vriend *et al*., 1986; Duggal *et al*., 1993). However, recent additional mutational analysis of the BMV CP N-terminus revealed that deletion of the entire arginine-rich motif or neutralization of all positively charged amino acids that contribute to the basic environment of the N-terminus do not affect infectivity and packaging (Choi and Rao, unpublished data). Likewise, present *in vivo* analysis (Fig. 2; Table 2) using full-length CMV CP demonstrated that the N-terminal basic arm of the CP is in fact not the major RNA-binding domain to form an infectious ribonucleoprotein complex. Thus we speculate that additional RNA binding domains exist elsewhere in the CP gene and remain to be identified by *in vitro* binding assays.

Although our data clearly establish that a major portion of the CMV CP N-terminus can be deleted without abolishing infectivity, results of this study also indicate that specific amino acids contained within the N-terminus play a crucial role in virion assembly. For example, CPs derived from variants ΔN12, 15P, Δ4R, and 2AA were competent for virion assembly (Table 2; Fig. 4), whereas variants ΔN19 and ΔR lacking the Arg cluster were not (Table 2). This finding is further supported by the ability of variant ΔN12, which retains the Arg cluster, to assemble into virions (Figs. 1A and 4). Another likely function for the N-terminal basic arm of CMV is to maintain the overall structural integrity of the CP to promote a compatible interaction with the host to overcome the host defense response (Rao, 1997; Osman *et al*., 1997). For example, although CP variants ΔN12 and Δ4R were capable of assembling into virions, local lesions induced in *C. quinoa* infected by these variants were significantly reduced in size when compared to those of wt (Fig. 2A, Table 1). The reduced spread could be attributed to the lack of proper interaction between the host and the modified viral CP.

The required forms of CP for cell-to-cell movement of CMV and BMV are distinct

Previous genetic analyses of CMV and BMV revealed that, in addition to MP, CP is also required to mediate cell-to-cell movement (Schmitz and Rao, 1996; Canto *et al*., 1997; Rao, 1997). However, the required form(s) in which the CP assists in the local spread of these two viruses appears to be distinct. Kasteel *et al.* (1997) reported the presence of virus-like particles in tubular structures extending from protoplasts infected with BMV, implying that a tubule-guided mechanism is conceivable for BMV movement. We also observed that several virion-assembly defective variants of BMV failed to move cell to cell (Schmitz and Rao, 1996; Rao, 1997), suggesting that BMV can be transported between cells as intact particles. This form of viral movement, however, needs to be verified in BMV-infected plants. Unlike the situation described above for BMV, the data presented in this study indicate that, although CP is obligatory for cell-to-cell movement of CMV (Canto *et al*., 1997), assembly of the CMV CP into virions is not a prerequisite for this active process. This is exemplified by the induction of local lesions by two truncated CP variants, ΔR and ΔN19 that are defective in virion assembly. Because a visible local lesion requires the infection of many cells by the invading virus (Schmitz and Rao, 1996) and the fact that no virions could be recovered, we speculate that both ΔR and ΔN19 are transported locally as a ribonucleoprotein complex. However, virion formation appears to be essential for long distance movement because ΔR and ΔN19 failed to infect *N. benthamiana* systemically (Fig. 3; Table 2). Additional experiments using hybrids of bromo- and cucumoviruses constructed by exchanging either their entire CP genes or specifically their N-termini are likely to further delineate their role in virus-host interactions.

MATERIALS AND METHODS

Virus strain and cDNA clones

In this study, we used CMV-Kin strain belonging to subgroup II of CMV (Boccard and Baulcombe, 1992). Full-length cDNA clones corresponding to the three genomic RNAs of CMV-Kin, pK1, pK2, and pK3, from which wt RNAs 1 (K1), 2 (K2), and 3(K3) can be transcribed *in vitro,* have been described previously (Boccard and Baulcombe, 1992, 1993).

Construction of N-terminal CP variants of K3

Using pK3 cDNA as a template in a polymerase chain reaction (PCR) and a desired combination of oligonucleotide primers (Table 3), a series of mutations was engineered into the first 60 bases of the CMV CP gene, which encode the first 20 N-terminal amino acids (Fig. 1A). Each PCR product was digested with *Apa*I and *Rsr*II and subcloned into similarly treated pK3 (Fig. 1A). The nucleotide sequence of the subcloned fragments was determined to verify introduced mutations. Six different CP variants were constructed and are described as follows: ΔN12 (N-proximal residues 2 to 13 are deleted); ΔN19 (N-proximal residues 2 to 20 are deleted); 15P [arginine (Arg) at position 15 is replaced with proline (Pro)]; Δ4R (four Arg at positions 14 to 17 are deleted); ΔR (six Arg and one Pro at positions 14 to 20 are deleted); and 2AA [two Arg at positions 19 and 20 are replaced with alanine (Ala)].

In vitro transcription, isolation, and transfection of *Chenopodium quinoa* protoplasts

Prior to transcription, parental and variant cDNA clones were linearized with *Bgl*II and transcribed with T7 RNA polymerase using a MEGAscript T7 kit (Ambion Inc., TX). For protoplast transfections, template cDNA was removed by LiCl precipitation (Rao *et al*., 1994). Protoplasts were isolated from approximately 8-week-old *C. quinoa* leaves (Rao and Grantham, 1996) and transfected

TABLE 3

Primers Used in PCR for Creating Mutations in the N-terminal Basic Arm of CMV Coat Protein[a]

Oligonucleotide	Sequence (5′ → 3′)	Comments[b]
K3/5′ Primer	GTTGGGCCCCTTACTTTC	Used as a 5′ primer in all PCR reactions
ΔN12	ACCAGAAGCGGACCGAGAACCTCCACGCGGGGCGACGACGCCGCATAGGTAC	Deletes residues at positions 2 to 13
ΔN19	ACCAGAAGCGGACCGAGAACCCATAGGTAC	Deletes residues at positions 2 and 20
15P	ACCAGAAGCGGACCGAGAACCTCTACGCGGGCGACGGGGCCGGGAGGT	Substitutes a Pro for Arg at position 15
Δ4R	ACCAGAAGCGGACCGAGAACCTCTACGCGGGGAGGTTCT	Deletes 4 Arg residues at positions 14 to 17
ΔR	ACCAGAAGCGGACCGAGAACCGGAGGTTCT	Deletes Arg cluster at positions 14 to 20
2AA	ACCAGAAGCGGACCGAGAACCTGCAGCCGGGCGACGACG	Substitutes two Ala for Arg at positions 19 and 20

[a] For generating all variant sequences, pK3 was used as a template.
[b] To identify the amino acid position, refer to Fig. 1A.

(2.5×10^5 /sample) using the polyethyleneglycol method as described by Rao *et al.* (1994). Transfected protoplasts were incubated under lights for 30 to 36 h.

Whole plant inoculations

C. quinoa and *Nicotiana benthamiana* plants were kept in the dark for at least 18 h prior to inoculation. They were mechanically inoculated with 10 μl/leaf (300 μg/ml) of a mixture containing wt K1 and K2 and either wt K3 or the desired K3 variant. Each experiment was repeated at least three times using four to six plants. The inoculated plants were held at 25°C in the greenhouse and observed for symptom development over a period of 3–4 weeks. Inoculated and noninoculated leaves of symptomatic and asymptomatic plants were screened for viral RNA and CP by Northern and Western blot analysis, respectively.

Virus purification and electron microscopy

The purification procedure used for CMV-Kin was adapted from Mossop *et al.* (1976). Virus was purified from inoculated or systemic leaves of *N. benthamiana*, by grinding fresh leaf tissue in 3 vol (w/v) of 0.1M Na_2HP0_4, pH 8.0. Particulate material was removed by a low speed centrifugation at 8,000 *g* for 10 min at 4°C. Triton-X 100 was added to a final concentration of 2% to clarify the supernatant. The mixture was stirred at 4°C for 15 min and centrifuged at 78,000 *g* for 90 min at 4°C. The virus pellets were suspended in 1/10 of the original volume of extraction buffer and subjected to a low speed centrifugation at 5,500 *g* for 5 min at 4°C. The supernatant was loaded onto a 10% sucrose (prepared in 0.1 M Na_2HPO_4, pH 8.0) cushion and centrifuged at 144,000 *g* for 45 min at 4°C. Finally, the virus pellets were suspended in an appropriate volume of 10 mM sodium borate buffer, pH 7.5. For electron microscopic analysis, virus preparations were negatively stained with uranyl acetate and examined under a Hitachi (H-600) transmission electron microscope. For immunosorbent electron microscopy (ISEM), grids were initially coated with CMV CP antibody at a dilution of 1:1000 and allowed to react with leaf dip preparations from symptomatic leaves. The grids were then negatively stained and observed under an EM as described above.

Northern, Western, and progeny sequence analysis

Total RNA was extracted for Northern analysis using SDS-phenol (Rao *et al*., 1994, Rao and Grantham, 1995) and suspended in sterile water. RNA samples were denatured with glyoxal, electrophoresed on 1% agarose gels and electrophoretically transferred to nylon membranes (Rao *et al*., 1994). Blots were hybridized with ^{32}P-labeled riboprobe to detect positive-strand CMV RNA progeny. A T7 RNA polymerase transcript of pT73KT, constructed by cloning a *Hin*dIII-*Bam*HI fragment corresponding to the 3′ 300 nucleotide (nt) sequence of pK3 (Boccard and Baulcombe, 1992) into a similarly treated pT7T3–18U (Pharmacia), was used to detect progeny positive-strand sequence common to all four CMV RNAs. For Western blot analysis, proteins were obtained from either leaf tissue or transfected protoplasts, suspended in SDS-PAGE buffer and fractionated on 16% SDS-PAGE according to Laemmli (1970). Fractionated proteins were electrophoretically transferred to a nitrocellulose membrane and detected with CMV CP antibody (serogroup S; 1:3000 dilution) using an Enhanced Chemoluminescence (ECL) kit (Amhersham). For sequencing, progeny RNA was subjected to reverse transcriptase-PCR (RT-PCR) and PCR products were directly sequenced as described previously (Rao and Grantham, 1995).

ACKNOWLEDGMENTS

We thank David Baulcombe for providing cDNA clones of CMV-Kin, Allan Dodds for CMV coat protein antiserum, Yoon Gi Choi for assisting with the electron microscopy, and George Grantham for technical help. Research in this laboratory was supported in part by a grant from the USDA NRICGP (9600968).

REFERENCES

Argos, P. (1981). Secondary structure prediction of plant virus coat proteins. *Virology* 110, 55–62.

Boccard, F., and Baulcombe, D. C. (1992). Infectious *in vitro* transcripts from amplified cDNAs of the Y and Kin strains of cucumber mosaic virus. *Gene* 114, 223–227.

Boccard, F., and Baulcombe, D. C. (1993). Mutational analysis of *cis*-acting sequences and gene Function in RNA3 of cucumber mosaic virus. *Virology* 193, 563–578.

Canto, T., Prior, D. A. M., Hellwald, K.-H., Oparka, K. J., and Palukaitis, P. (1997). Characterization of cucumber mosaic virus. IV. Movement protein, and coat protein are both essential for cell-to-cell movement of CMV. *Virology* 237, 237–248.

Ding, B., Li, Q., Nguyen, L., Palukaitis, P., and Lucas, W. J. (1995a). Cucumber mosaic virus 3 a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. *Virology* 207, 345–353.

Ding, S. W., Li, W. X., and Symons, R. H. (1995b). A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J.* 14, 5762–5772.

Duggal, R., and Hall, T. C. (1993). Identification of domains in brome mosaic virus RNA1 and coat protein necessary for specific interaction and encapsidation. *J. Virol.* 67, 6406–6412.

Erickson, J. W., and Rossmann, M. G. (1982). Assembly and crystallization of T=1 icosahedral particles from trypsinized southern bean mosaic virus coat protein. *Virology* 116, 128–136.

Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K., and Bricogne, G. (1978). Tomato bushy stunt at 2 A resolution. *Nature* 276, 368–373.

Hayes, R. J., and Buck, K. W. (1990). Complete replication of a eukaryotic virus RNA *in vitro* by a purified RNA-dependent RNA polymerase. *Cell* 63, 363–368.

Hogle, J. M., Maeda, A., and Harrison, S. C. (1986). Structure and assembly of turnip crinkle virus. I. X-ray crystallographic structure analysis at 3.2 A resolution. *J. Mol. Biol.* 159, 93–108.

Kaplan, I. B., Shintaku, M. H., Li, Q., Marsh, L. E., and Palukaitis, P. (1995). Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* 209, 188–199.

Kasteel, D. T. J, Van der Wel, N., Jansen, K. A. J., Goldbach, R. W, and Van Lent, J. W. M. (1997). Tubule-forming capacity of the movement proteins of alfalfa mosaic virus, and brome mosaic virus. *J. Gen. Virol.* 78, 2089–2093.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 488–492.

Mossop, D. W., Francki, R. I. B., and Grivell, C. J. (1976). Comparative studies on tomato aspermy and cucumber mosaic viruses. V. Purification and properties of a cucumber mosaic virus inducing severe chlorosis. *Virology* 74, 544–546.

Osman, F., Grantham G. L., and Rao, A. L. N. (1997). Molecular studies on bromovirus capsid protein. IV. Coat protein exchanges between brome mosaic and cowpea chlorotic motlle viruses exhibit neutral effects in heterologous hosts. *Virology* 238, 452–459.

Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., and Francki, R. I. B. (1992). Cucumber mosaic virus. *Adv. Virus Res.* 41, 281–348.

Rao, A. L. N. (1997). Molecular studies on bromovirus capsid protein. III. Analysis of cell-to-cell movement competence of coat protein defective variants of cowpea chlorotic mottle virus. *Virology* 232, 385–395.

Rao, A. L. N., Duggal, R., Lahser, F., and Hall, T. C. (1994). Analysis of RNA replication in plant viruses. *In* "Methods in Molecular Genetics: Molecular Virology Techniques" (K. W. Adolph, Ed.), Vol. 4, pp. 216–236. Academic Press, Orlando.

Rao, A. L. N., and Grantham, G. L. (1995). Biological significance of the seven amino-terminal basic residues of brome mosaic virus coat protein. *Virology* 211, 42–52.

Rao, A. L. N., and Grantham, G. L. (1996). Molecular studies on bromovirus capsid protein. II. Functional analysis of the amino-terminal arginine-rich motif, and its role in encapsidation, movement, and pathology. *Virology* 226, 294–305.

Sacher, R., and Ahlquist, P. (1989). Effect of deletions in the N-terminal basic arm of brome mosaic virus coat protein on RNA packaging and systemic infection. *J. Virol.* 63, 4545–4552.

Schmitz, I., and Rao, A. L. N. (1996). Molecular studies on bromovirus capsid protein. I. Characterization of cell-to-cell movement defective RNA3 variants of brome mosaic virus. *Virology* 226, 281–293.

Sgro, J., Jacrot, B., and Chroboczek, J. (1986). Identification of regions of brome mosaic virus coat protein chemically cross-linked in situ to viral RNA. *Eur. J. Biochem.* 154, 69–76.

Shintaku, M. H., Zhang, L., and Palukaitis, P. (1992). A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. *Plant Cell* 4, 751–757.

Suzuki, M., Kuwata, S., Kataoka, J., Masuta, C., Nitta, N., and Takanami, Y. (1991). Functional analysis of deletion mutants of cucumber mosaic virus RNA3 using an *in vitro* transcription system. *Virology* 183, 106–113.

Suzuki, M., Kuwata, S., Masuta, C., and Takanami, Y. (1995). Point mutations in the coat protein of cucumber mosaic virus affect symptom expression and virion accumulation in tobacco. *J. Gen. Virol.* 76, 1791–1799.

Taliansky, M. E., and Garcia-Arenal, F. (1995). Role of cucumovirus capsid protein in long distance movement within the infected plant. *J. Virol.* 69, 916–922.

Tan, R., and Frankel, A. D. (1995). Structural variety of arginine-rich RNA binding peptides. *Proc. Natl. Acad. Sci. USA* 92, 5282–5286.

Vriend, G., Verduin, B. J. M., and Hemminga, M. A. (1986). Role of the N-terminal part of the coat protein in the assembly of cowpea chlorotic mottle virus: A 500 MHz proton nuclear magnetic resonance study and structural calculations. *J. Mol. Biol.* 191, 453–460.

Wikoff, W. R., Tsai, C. J., Wang, G., Baker, T. S., and Johnson, J. E. (1997). The structure of cucumber mosaic virus: Cryoelectron microscopy, x-ray crystallography, and sequence analysis. *Virology* 232, 91–97.

Yusibov, V. M., and Loesch-Fries, L. S. (1995). N-terminal basic amino acids of alfalfa mosaic virus coat protein involved in the initiation of infection. *Virology* 208, 405–407.