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II. Functional Analysis of the Amino-Terminal Arginine-Rich Motif and Its Role in Encapsidation, Movement, and Pathology

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The N-terminal region of the brome mosaic bromovirus (BMV) coat protein (CP) contains an arginine-rich motif that is conserved among plant and nonplant viruses and implicated in binding the RNA during encapsidation. To elucidate the functional significance of this conserved motif in the BMV CP, a series of deletions encompassing the arginine-rich motif was introduced into a biologically active clone of BMV RNA3, and their effect on replication, encapsidation, and infection in plants was examined. Analysis of infection phenotypes elicited on *Chenopodium quinoa* revealed the importance of the first 19 N-proximal amino acids of BMV CP in encapsidation and pathogenicity. Inoculation of *C. quinoa* with three viable variants of BMV RNA3 lacking the first 11, 14, and 18 N-terminal amino acids of the CP resulted in the development of necrotic local lesions and restricted the spread of infection to inoculated leaves. Progeny analysis from symptomatic leaves revealed that, in each case, virus accumulation was severely affected by the introduced mutations and each truncated CP differed in its ability to package genomic RNA. In contrast to these observations in *C. quinoa,* none of the CP variants was able to establish either local or systemic infections in barley plants. The intrinsic role played by the N-terminal arginine-rich motif of BMV CP in packaging viral RNAs and the interactions between the host and the truncated CPs in modulating symptom expression and movement are discussed.

INTRODUCTION

Bromoviruses are a group of icosahedral, multipartite, positive-strand RNA viruses that infect plants (Ahlquist, 1994). The bromovirus group contains three members: the monocot-adapted brome mosaic virus (BMV; type member) and the dicot-adapted cowpea chlorotic mottle (CCMV) and broadbean mottle viruses (Ahlquist, 1994). BMV has a narrow host range and mostly infects monocotyledonous plants (Lane, 1981). Essential functions required for replication and subsequent establishment of infection of BMV are partitioned among three RNA components. Viral RNA replication is dependent on efficient interaction between two nonstructural proteins, 1a and 2a, encoded by the monocistronic RNAs 1 and 2, respectively (Kao *et al.,* 1992). These replication factors share amino acid sequence similarity with proteins encoded by many morphologically and genetically diverse (+) strand RNA viruses of plants, such as tobacco mosaic tobamovirus, and of animals, such as Sindbis virus (Ahlquist *et al.,* 1985). The two gene products encoded by the dicistronic RNA3 are dispensable for viral replication, but are required for infection in plants (De Jong and Ahlquist, 1991). Genomic RNA3 encodes a nonstructural protein of 32 kDa, the designated movement protein (Mise *et al.,* 1993), and a 19-kDa coat protein (CP) that is translated from subgenomic RNA4 derived from minus-strand RNA3 (Miller *et al.,* 1985).

Many plant icosahedral RNA viruses such as BMV (Sacher and Ahlquist, 1989), tomato bushy stunt (Harrison *et al.,* 1978), turnip crinkle (Hogle *et al.,* 1986), and southern bean mosaic viruses (Erickson and Rossmann, 1982), have coat proteins with highly basic N-terminal regions that are thought to interact with viral RNA inside the capsid shell. Thus, deletion of the N-proximal basic arm by protease treatment rendered the truncated proteins of these viruses incapable of *in vitro* assembly with RNA (Erickson and Rossmann, 1982; Sgro *et al.,* 1986; Tremaine *et al.,* 1977; Vriend *et al.,* 1986). For bromoviruses, *in vitro* cross-linking experiments (Sgro *et al.,* 1986) showed that peptides encompassing BMV CP residues 11 to 19, but not residues 1 to 7, bind to viral RNA. Consequently, the first 7 N-terminal amino acids have no detectable effect on replication, packaging, and infection in barley plants (Flasinski *et al.,* 1995; Rao and Grantham, 1995b; Sacher and Ahlquist, 1989) but act as a host range determinant in *Chenopodium* spp. (Rao and Grantham, 1995b). Recently, Tan and Frankel (1995) reported that a short region of basic amino acids of approximately 8–20 residues particularly rich in arginine and characteristic of RNA binding proteins is conserved among bromoviruses, human immunodeficiency virus Tat and Rev proteins, λN,

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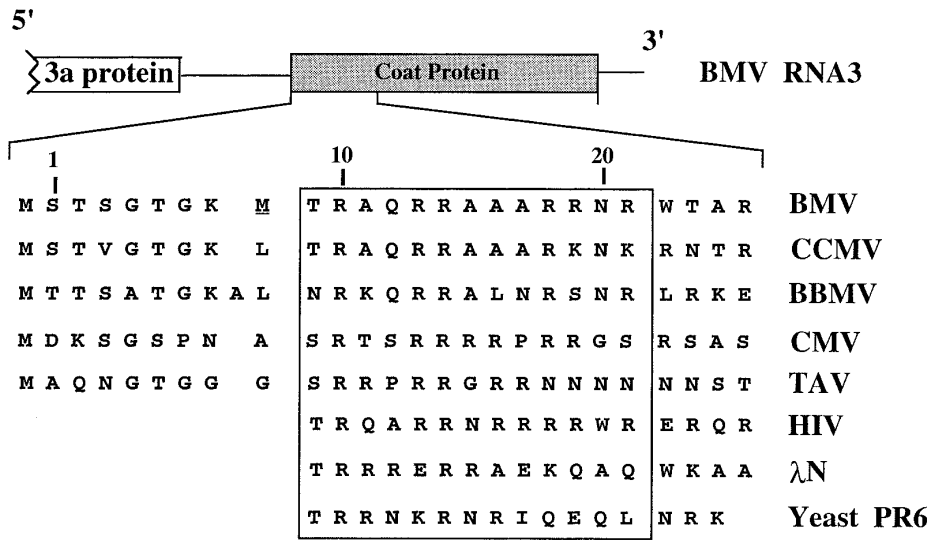

FIG. 1. Location of the coat protein gene on BMV RNA3 and sequence of the first 25 N-terminal amino acid region. Boxed region indicates the NARM conserved among the indicated viruses, λN, and yeast PR6. BMV, brome mosaic bromovirus; CCMV, cowpea chlorotic mottle bromovirus; BBMV, broad bean mottle bromovirus; CMV, cucumber mosaic cucumovirus; TAV, tomato aspermy cucumovirus; HIV, human immunodeficiency virus.

and yeast PR6 (Fig. 1). We have extended these comparisons to other plant viruses with basic N-terminal domains and found that only the CPs of cucumoviruses (belonging to the *Bromoviridae* family) contained the conserved arginine-rich motif (Fig. 1, boxed region). To continue our previous studies on the BMV CP (Rao and Grantham, 1995b) and to evaluate the function of the N-terminal arginine-rich motif (NARM) of BMV CP in the encapsidation process *in vivo,* we have engineered a series of defined deletions in the NARM region to examine their effect on *in vivo* packaging, spread of infection, and pathology in susceptible host plants.

MATERIALS AND METHODS

Construction of N-terminal deletion mutants of BMV CP

All CP deletion mutants constructed in this study (Fig. 2) are derived from a plasmid, pT7B3(-Tth), which contains a cDNA clone of full-length wild-type (wt) BMV RNA3 (B3; Fig. 2A) from which biologically active transcripts can be synthesized *in vitro* (Dreher *et al.,* 1989). Using polymerase chain reaction (PCR) and the desired combination of template DNA and mutagenic oligonucleotides (Table 1), several variants of pT7B3 were constructed to contain deletions in the amino-terminal region of the BMV CP gene (Fig. 2B). Each PCR product was digested with *Bgl*II and *Stu*I and subcloned into similarly treated pT7B3. The nucleotide sequences of the subcloned fragments were determined to verify the presence of the engineered deletions. Variant B3 transcripts derived from these clones were B3ΔCP11 (N-terminal amino acids 1–11 deleted), B3ΔCP14 (N-terminal amino acids 1–14 deleted), B3ΔCP18 (N-terminal amino acids 1–18 deleted), B3ΔCP19 (N-terminal amino acids 1–19 deleted), and B3ΔCP22 (N-terminal amino acids 1–22 deleted) (Fig. 2B).

In vitro transcription, transfection of protoplasts, and Northern and Western analysis

Full-length cDNA clones corresponding to the three genomic RNAs of BMV, pT7B1, pT7B2, and pT7B3, from which wt infectious RNAs 1 (B1), 2 (B2), and 3 (B3), respectively, can be transcribed *in vitro,* have been described previously (Dreher *et al.,* 1989). All wt and variant clones were linearized with *Bam*HI prior to transcription. Capped full-length transcripts were synthesized *in vitro* using a MEGAscript T7 kit (Ambion, Inc., Austin, TX). Unless specified otherwise, for replication and infectivity assays, each of the B3 variant transcripts were coinoculated with transcripts of wt B1 and B2. Control inoculations contained *in vitro* transcripts of all three wt BMV RNAs. *Chenopodium quinoa* protoplasts were isolated by the procedure used for barley (Rao *et al.,* 1994), except that the enzyme solution contained 1% cellulose, 0.5% macerozyme, 0.1% driselase, and 0.55 *M* mannitol, pH 5.9. The procedures used to extract progeny RNA from transfected protoplasts and their analysis by Northern hybridization using riboprobes of desired specificity were performed as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995b). Northern blots were quantitated by densitometry and data were corrected for differences in inoculation efficiency and protoplast variability by normalizing the yield of progeny B3 against the value obtained for B1 and B2 (French *et al.,* 1986; Rao *et al.,* 1989).

Proteins for Western blot analysis were obtained from

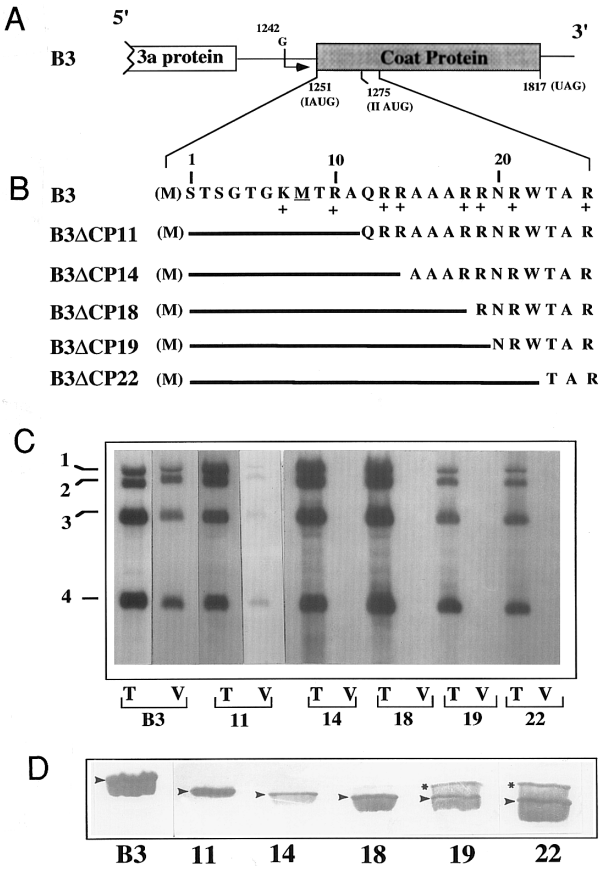

FIG. 2. Deletion analysis of the NARM of BMV CP. (A) The structure of wt BMV RNA3 (B3) is shown, with noncoding sequences represented as single lines and a portion of the 3a gene as an open box. The stippled box represents the CP gene. The position of the first methionine (present at 1251–1253), the second methionine (present at 1275–1277), and the CP stop codon (present at 1817–1819) are shown. The start of the CP subgenomic RNA4 on RNA3 (at base 1242) is shown by a bent arrow. (B) The basic 25 N-terminal amino acids of wt BMV CP (B3) gene are shown. Positively charged side chains which are also trypsin cleavage sites are indicated (+). In bromoviruses, the initiating methionine (enclosed in parentheses) is removed and the resultant N-terminal serine is acetylated in the mature coat protein (Moosic *et al.,* 1983). The extent of engineered N-terminal deletions in each B3 variant clone is shown by solid lines. (C) Replicative competence and encapsidation assays of BMV CP N-terminal deletion mutants in *C. quinoa* protoplasts. Protoplasts were transfected with *in vitro* transcripts of wt B1 and B2 and the indicated B3 variant. Transfected protoplasts were incubated for 48 hr and RNA was extracted from either protoplasts (T) or purified virions (V). RNAs were analyzed on 1% agarose gels after denaturation with glyoxal and transferred to nylon membranes. Blots were hybridized with ^{32}P-labeled (−) sense RNA probes representing the homologous 3′ region present on each of the four BMV RNAs. Lanes containing virion RNAs were exposed overnight at −80°, whereas lanes containing total RNA were exposed for 2 hr at −80°. The positions of the four wt BMV RNAs are shown to the left. (D) Western blot analysis of CP accumulation. Polyacrylamide gel electrophoresis of CP from *C. quinoa* protoplasts inoculated with transcripts of wt B1 and B2 and the following B3 transcripts: wt (B3), B3ΔCP11 (11), B3ΔCP14 (14), B3ΔCP18 (18), B3ΔCP19 (19), and B3ΔCP22 (22). Protoplasts were suspended in SDS–PAGE sample buffer, denatured by boiling for 5 min, and subjected to 16% SDS–PAGE. After the proteins were transferred to a nitrocellulose membrane, the blot was probed with antibodies specific for BMV CP. Note the difference in mobility between the CPs synthesized from wt B3 and the five B3 N-terminal variants. Arrowheads point to the major CP bands and asterisks indicate a CP band of unknown origin for variants B3ΔCP19 and B3ΔCP22.

purified virus preparations or transfected protoplasts (2.5 × 10^5) collected 48 hr postinoculation. Samples were suspended in SDS–PAGE sample buffer (final concentration 125 m*M* Tris, pH 6.8, 10% [w/v] glycerol, 2.5% [w/v] dithiothreitol, 2% SDS, 0.01% bromophenol blue), denatured at 100° for 5 min, and fractionated on a 16% SDS–PAGE according to Laemmli (1970). Fractionated proteins were electrophoretically transferred to a nitrocellulose membrane and detected with BMV CP antibody (1:1000 dilution) and goat anti-rabbit antibody conjugated with alkaline phosphatase (Bio-Rad).

Encapsidation assays

C. quinoa protoplasts (1 × 10^6) transfected with RNA transcripts were collected 48 hr after inoculation and emulsified with a mixture containing an equal volume of virus purification buffer (0.5 *M* sodium acetate, 0.08 *M* magnesium acetate, pH 4.5, containing 1% β-mercaptoethanol) and chloroform. The solution was centrifuged for 15 min at 10K rpm at 4° and the supernatant was centrifuged at 95K rpm for 90 min in a Beckman TL 100.2 rotor. The virus pellet was suspended in buffer (0.05 *M* sodium acetate and 0.008 *M* magnesium acetate, pH 4.5) and treated with micrococcal nuclease prior to RNA extraction by the SDS and phenol method (Rao *et al.,* 1994).

Biological assays, virus purification, and sequence analysis of progeny RNA

Barley (*Hordeum vulgare* cv. Dickson) and *C. quinoa* plants were kept in the dark for at least 18 hr and dusted with carborundum prior to inoculation. They were mechanically inoculated with 10 μl/leaf of a mixture containing viral RNA transcripts in desired combinations (150 μg/ml; Rao *et al.,* 1994). Each experiment was repeated at least three times with independently synthesized *in vitro* transcript preparations. The inoculated plants were kept in the greenhouse at 25° and observed for symptom expression over a period of 2–3 weeks. Absence of visible symptoms was not considered to be conclusive evidence for the noninfectious nature of the inoculum; therefore total RNA isolated from asymptomatic leaves was analyzed by Northern blots. Virus was purified from symptomatic leaves as described above and quantitated by measuring the absorbance at 260 nm using a Beckman spectrophotometer.

Progeny viral RNA was suspended in sterile distilled water and subjected to reverse transcription-PCR (RT-PCR) as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995a). The PCR products were either directly

TABLE 1

Oligonucleotides Used in PCR for Creating Deletions in the N-terminal Region of BMV Coat Protein

Oligonucleotide	Sequence (5′ → 3′)	Template	Comments[a]
B3 3′	38-mer CAGGATCCCGACATGGTCTTCTTTAGAGATTTACAGTG	3′ primer used along with the mutagenic oligo in PCR	
ΔCP11	42-mer AAAAGATCTATGTCCTAATTCAGCGTATTAATAATGCAGCGT	pT7B3ΔCP7[b]	To delete the first 11 N-terminal amino acids.
ΔCP14	42-mer AAAAGATCTATGTCCTAATTCAGCGTATTAATAATGGCTGCC	pT7B3ΔCP7	To delete the first 14 N-terminal amino acids.
ΔCP18	42-mer AAAAGATCTATGTCCTAATTCAGCGTATTAATAATGAGAAAT	pT7B3ΔCP7	To delete the first 18 N-terminal amino acids.
ΔCP19	42-mer AAAAGATCTATGTCCTAATTCAGCGTATTAATAATGAATCGT	pT7B3ΔCP18	To delete the first 19 N-terminal amino acids.
ΔCP22	45-mer AAAAGATCTATGTCCTAATTCAGCGTATTAATAATGACCGCTAGG	pT7B3ΔCP19	To delete the first 22 N-terminal amino acids.

[a] To identify the amino acid position, refer to Fig. 2.

[b] Plasmid pT7ΔCP7 is characterized by lacking the first 7 N-terminal amino acids of BMV CP (Rao and Grantham, 1995b).

sequenced (Rao and Grantham, 1995a) or subcloned into the *Sma*I site of pT7/T3*lac*Z and sequenced with Sequenase (Rao and Hall, 1993).

RESULTS

Replication and encapsidation competence of BMV CP N-terminal variants in protoplasts

To analyze the significance of the NARM of BMV CP, which previously had been shown to be important in RNA–protein interactions during virus assembly (Sgro *et al.,* 1986), a series of nested deletions was engineered in the NARM of a wt B3 clone (Fig. 2B). The extent of the introduced deletions is shown in Fig. 2B and the transcripts derived from these variant clones are designated as B3ΔCP11, B3ΔCP14, B3ΔCP18, and B3ΔCP22. It was anticipated that CP subgenomic mRNA4 produced *in vivo* from the minus-strand B3 progeny of B3ΔCP11, B3ΔCP14, B3ΔCP18, and B3ΔCP22 transcripts would translate to yield mature CPs lacking the N-terminal 11, 14, 18, and 22 amino acids, respectively. Although barley protoplasts are routinely used in our laboratory for replication studies, in this study it was necessary to analyze the replicative competence of each B3 variant in *C. quinoa* protoplasts, since most of our infectivity assays were carried out in this host (see below). Transfection of *C. quinoa* protoplasts with each B3 variant resulted in efficient replication and accumulation of progeny RNA (Fig. 2C). Densitometric analysis of Northern blots revealed that the amount of viral RNAs recovered from protoplasts transfected with each variant was approximately 25% lower than that of wt (data not shown). The subgenomic RNAs produced *in vivo* from B3ΔCP11, B3ΔCP14, B3ΔCP18, and B3ΔCP22 variants synthesized truncated CPs of expected size (indicated by arrowheads in Fig. 2D). However, for variant B3ΔCP22, an additional band migrating slower than the major predicted band (indicated by an asterisk in Fig. 2D) occurred in several repeated trials. The nature of this band is unknown.

To examine the encapsidation competence of each truncated CP, resulting from inoculations with B3ΔCP11, B3ΔCP14, B3ΔCP18, and B3ΔCP22, virions were purified from transfected *C. quinoa* protoplasts and viral RNA was extracted and analyzed on Northern blots using a probe complementary to a 3′-terminal sequence that is highly conserved among all four BMV RNAs (Rao *et al.,* 1989). As expected, CP synthesized from wt B3 encapsidated all four RNAs (Fig. 2C). Among the four B3 variants examined, encapsidated RNAs were observed only for variant B3ΔCP11 and their detection required prolonged exposure of Northern blots (Fig. 2C). By contrast, no RNA was recovered from virions obtained from protoplasts transfected with any of the other three variants B3ΔCP14, B3ΔCP18, and B3ΔCP22 (Fig. 2C). Our inability to detect virion RNA in these samples was attributed

FIG. 3. Representative examples of phenotypes induced on *C. quinoa* by wt and five B3 variants. Local lesions induced on *C. quinoa* after inoculation with *in vitro* transcripts of wt B1 and B2 and the following: B3, B3ΔCP11, B3ΔCP14, and B3ΔCP18. Asymptomatic leaves of B3ΔCP19 and B3ΔCP22 are also shown. Leaves were photographed 10 dpi.

to a severe reduction in virus yield for variants B3ΔCP14 and B3ΔCP18 and the inherently defective virion assembly *in vivo* for variant B3ΔCP22 due to the lack of the first 22 N-terminal amino acids (see below).

Effect of N-terminal deletions on pathogenicity and infection spread in *C. quinoa*

We (Rao and Grantham, 1995b) and others (Flasinski *et al.,* 1995; Sacher and Ahlquist, 1989) have previously demonstrated that deletion of the first 7 N-terminal amino acids from the mature BMV CP has no effect on replication, encapsidation, symptom expression, or rate of spread in barley. However, the deletion modified the lesion phenotype and prevented systemic spread of infection in *C. quinoa* (Rao and Grantham, 1995b). To examine whether additional N-terminal deletions constructed in this study (Fig. 2B) would exert similar constraints on systemic infection, *C. quinoa* plants were coinoculated with wt transcripts of B1 and B2 and B3ΔCP11, B3ΔCP14, B3ΔCP18, or B3ΔCP22. Among the four B3 variants, three variants (B3ΔCP11, B3ΔCP14, and B3ΔCP18) were infectious and produced local lesions on a time scale similar to that of wt B3 control inoculations. However, the appearance and morphology of the lesions induced by B3ΔCP11, B3ΔCP14, and B3ΔCP18 differed from that of wt B3 (Fig. 3; Table 2). In marked contrast to chlorotic expanding lesions induced by wt B3 infections, the variant lacking the first 11 N-terminal amino acids (i.e., B3ΔCP11) induced necrotic instead of chlorotic local lesions (Fig. 3; Table 2). By 8 days postinfection (dpi), these necrotic lesions expanded and coalesced, resulting in complete leaf necrosis. Although the lesions induced by two other variants lacking the first 14 and 18 N-terminal amino acids (i.e., B3ΔCP14 and B3ΔCP18) were also necrotic, they did not expand as rapidly as those induced by B3ΔCP11 (Fig. 3; Table 2). *C. quinoa* leaves coinoculated with transcripts of wt B1 and B2 and the B3ΔCP22 variant failed to induce any visible symptoms (Fig. 3; Table 2). All plants were held in the greenhouse for an additional 2- to 3-week period for further symptom observation. Plants inoculated with all three wt transcripts developed systemic mottling symptoms by 8 dpi (Table 2). None of the plants inoculated with transcripts of wt B1 and B2 and any of the four B3 variants displayed systemic symptoms on uninoculated leaves (Table 2). These results were consistent in repeated inoculations with independently synthesized transcripts. Northern blot analysis of total RNA isolated from symptomatic leaves of *C. quinoa* infected with variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 revealed an RNA profile resembling that of BMV (Table 3). No viral RNA characteristic of BMV was detected either in asymptomatic systemic leaves of plants infected with B3ΔCP11, B3ΔCP14, and B3ΔCP18 or in any leaves of plants inoculated with B3ΔCP22 (Table 3).

While the B3 variant which had the first 18 N-terminal amino acids deleted remained infectious, another B3 variant containing a deletion extended to include the first 22 amino acids lost infectivity (Table 2; Fig. 3). To determine the exact number of N-proximal amino acids of BMV CP that could be deleted without having an effect on infectivity, an additional B3 variant, referred to as B3ΔCP19 (Fig. 2B) and capable of synthesizing a mature CP lacking the first 19 N-terminal amino acids, was constructed (Fig. 2B; Table 1). This variant replicated efficiently in *C. quinoa* protoplasts (Fig. 2C) and synthesized a truncated CP with expected electrophoretic mobility as well as another protein migrating as a slower band similar to the additional protein band observed for variant B3ΔCP22 (Fig. 2D). Like B3ΔCP14, B3ΔCP18, and B3ΔCP22, variant B3ΔCP19 also failed to encapsidate detectable levels of viral RNA (Fig. 2C). Coinoculation of B3ΔCP19 transcripts with wt B1 and B2 onto *C. quinoa* failed to induce any visible local lesions (Fig. 3) and no viral RNA was detected in inoculated or uninoculated leaves by Northern blot analysis (Table 3). These observations suggest that the first 19 N-terminal amino acids are critical for infectivity.

Stability *in vivo* of B3ΔCP11, B3ΔCP14, and B3ΔCP18 variants

To verify the genetic stability of engineered deletions during the infection process in *C. quinoa* and to justify that symptom modifications observed in *C. quinoa* plants induced by B3ΔCP11, B3ΔCP14, and B3ΔCP18 (Fig. 3) were the manifestation of the starting genotype, total RNA was isolated from symptomatic leaves infected with wt B1 and B2 and the respective B3 variants and the region encompassing the entire CP gene was amplified by RT-PCR (Rao and Grantham, 1995b). Direct sequencing of the PCR product revealed that, in each case, introduced deletions were stably maintained (Table 3). These results suggest that symptom phenotypes induced by B3ΔCP11, B3ΔCP14, and B3ΔCP18 were not due to inadvertent selection of pseudorevertants.

N-terminal deletions affect packaging of BMV RNAs

The yield of virus purified from symptomatic inoculated leaves of *C. quinoa* was 50% less for variant B3ΔCP11 and 60 and 80% less for variants B3ΔCP14 and B3ΔCP18, respectively, when compared to yield from leaves inoculated with wt BMV (Table 3). When purified preparations of all three variants were negatively stained and examined under the electron microscope, they were shown to contain icosahedral virions, resembling those of wt BMV (Fig. 4). However, unlike wt BMV virions, the particles assembled from each of the truncated CPs (confirmed by Western blot analysis, data not shown) had electron-dense centers indicating that the uranyl acetate had penetrated into the particles (Fig. 4). To examine

TABLE 2

Symptom Phenotypes Induced by BMV CP Variants in *C. quinoa* and Barley Plants

Inoculum[a]	*C. quinoa*		Barley
	Local	Systemic	Systemic
B3 (Wt)	CLL/MVN	SM	M
B3ΔCP11	NLL/MVN	NI	NI
B3ΔCP14	SNLL/MVN	NI	NI
B3ΔCP18	SNLL/MVN	NI	NI
B3ΔCP19	NI	NI	NI
B3ΔCP22	NI	NI	NI

Note. CLL, chlorotic local lesions; MVN, mild venial necrosis; NLL, necrotic local lesions; SNLL, small necrotic local lesions; SM, systemic mottling; M, mosaic; NI not infected.

[a] Each inoculum (150 μg/ml) contained a mixture of *in vitro* transcripts of wt B1 and B2 and the indicated B3 variant.

whether these particles contained viral RNA or if they were empty, virion RNA profiles for each B3 variant were analyzed by Northern hybridization using multiple blots and riboprobes specific for BMV RNAs 1 and 2 (designated B1 and B2 probes, respectively), the CP gene (detects both RNA3 and RNA4, designated CP probe), and the tRNA-like conserved 3′ noncoding region (designated tRNA probe which detects all four BMV RNAs). The virion RNA profiles of each of the three B3 variants were compared to respective total RNA preparations obtained from symptomatic leaves of *C. quinoa* (Figs. 5A and 5B).

In protoplasts, the replication of B1 and B2 is independent of B3 and, therefore, the accumulation level of each variant B3 progeny was quantitated by using B1 and B2 as internal standards (French *et al.,* 1986; Rao *et al.,* 1989). By contrast, gene products encoded by B3 are obligatory for whole plant infections (De Jong and Ahlquist, 1991) and consequently any reduction in the replication of B3 and its gene products might result in impaired movement, eventually affecting the accumulation of total progeny RNA. Therefore, the use of B1 and B2 as internal standards would be inappropriate to quantitate the accumulation of variant progeny in plants. The accumulation levels of viral progeny from either purified virions or total RNA recovered from *C. quinoa* plants infected with B3 variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 were assessed based on the exposure times required for the Northern blots to reveal the RNA bands. The exposure time required to detect BMV-specific RNAs in a given wt or variant sample remained consistent in three or more independent Northern blot assays.

Riboprobes complementary to either the 3′ noncoding region or a specific region for each genomic RNA detected all four BMV RNAs in total RNA and virion RNA preparations from plants infected with the wt control (Figs. 5A and 5B). Their detection in Northern blots usually required only 10 to 15 min of exposure. When riboprobes of similar specific activity were used to probe the blots containing B3 variant progeny, they displayed discernable RNA profiles. For variants B3ΔCP11 and B3ΔCP14, RNA2 was the most prominent component, while levels of RNAs 1, 3, and 4 remained lower and their detection required prolonged exposure times of the Northern blot (Figs. 5A and 5B). In contrast to B3ΔCP11 and B3ΔCP14, no progeny of RNA1 in total as well as virion RNA preparations of B3ΔCP18 could be detected with either the tRNA- or the B1-specific probes (Figs. 5A and 5B). Prolonged exposure of the blots did not reveal the presence of RNA1. Since virion RNA of B3ΔCP18 is infectious to *C. quinoa* plants (data not shown), we speculate that RNA1 is encapsidated by the truncated CP of B3ΔCP18, but to a degree below the sensitivity afforded by Northern blot analysis. However, RNA2 progeny of B3ΔCP18 were encapsidated to detectable levels (Fig. 5B). An interesting and unexpected observation made from the Northern analysis of all B3 variant progeny was the presence of additional truncated RNA species (indicated by arrowheads in Figs. 5A and 5B) derived from each genomic BMV RNA when the blots were hybridized with the respective riboprobes. These truncated RNAs were consistently detected in total as well as virion RNA preparations of B3 variant inoculations, but were never observed in wt control infections (Figs. 5A and 5B). The nature of these truncated RNA species is currently unknown.

TABLE 3

Analysis of BMV CP Variant Progeny Obtained from *C. quinoa*

Inoculum[b]	Characteristics of progeny[a]				
	Virus yield[c]	EM[d]	Northern blots[e]		Sequence[f]
			I	S	
B3 (Wt)	2.40	+	+	+	Conserved
B3ΔCP11	1.40	+	+	−	Conserved
B3ΔCP14	0.96	+	+	−	Conserved
B3ΔCP18	0.46	+	+	−	Conserved
B3ΔCP19	0.0	−	−	−	ND
B3ΔCP22	0.0	−	−	−	ND

[a] Progeny were obtained from inoculated leaves of *C. quinoa.*

[b] Each inoculum (150 μg/ml) contained a mixture of *in vitro* transcripts of wt B1 and B2 and the indicated B3 variant transcript.

[c] Virus yield was estimated using a spectrophotometer and expressed as mg/ml of virus recovered from a milligram of leaf tissue.

[d] Virions were purified from symptomatic leaves of *C. quinoa* for variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 and from *C. quinoa* protoplasts for variants B3ΔCP19 and B3ΔCP22 and examined under an electron microscope; (+) presence of virions characteristic of BMV, (−) absence of virions.

[e] Detection of BMV RNAs by Northern hybridization; I, inoculated leaves; S, systemic leaves. (+) Presence of BMV RNAs, (−) absence of BMV RNAs.

[f] Sequence of progeny RNA was determined as described under Materials and Methods; ND, not determined.

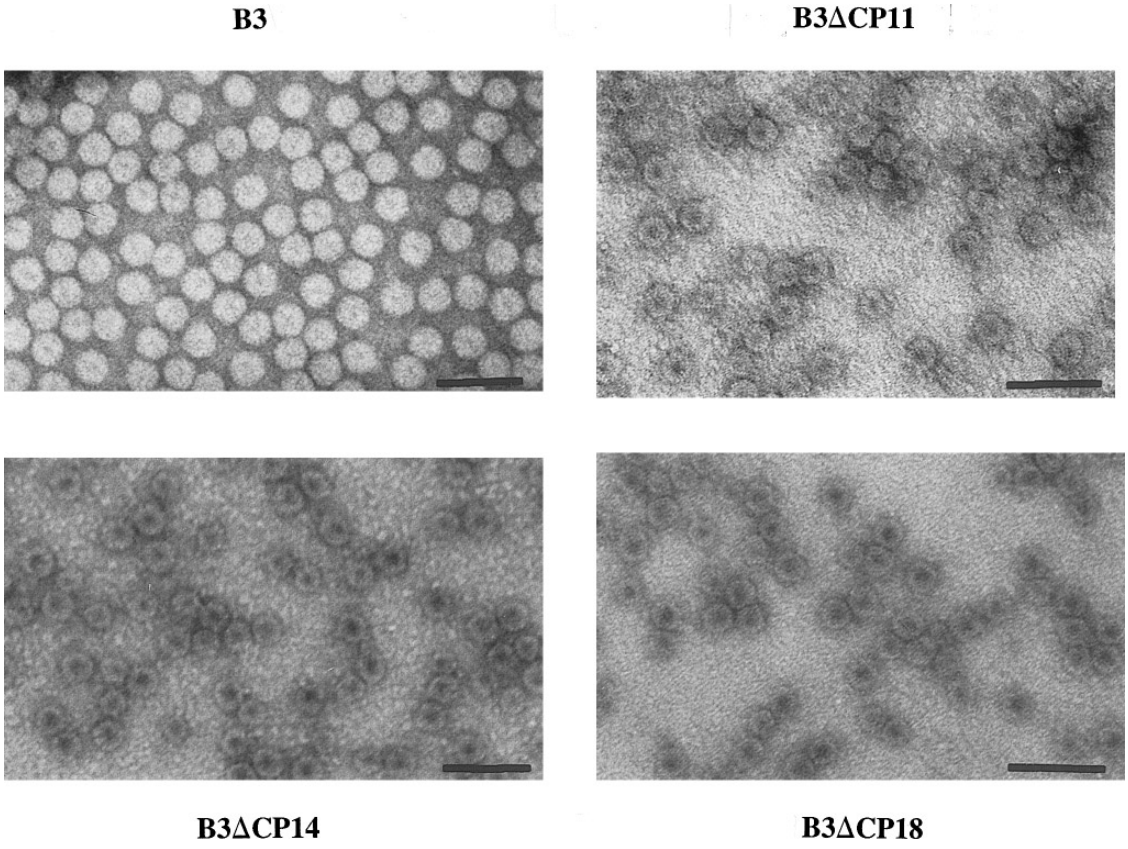

FIG. 4. Electron micrographs of purified virus preparations. Virions were purified from symptomatic primary leaves of *C. quinoa* infected with wt B1 and B2 and either wt B3 or the indicated B3 variant. Purified virus preparations were negatively stained with uranyl acetate and photographed at a magnification of 60K. Bar, 100 nm.

NARM mutants are noninfectious in barley

The deletion of residues 1 to 7 from the amino terminus of mature BMV CP resulted in the loss of systemic spread in *C. quinoa* (Rao and Grantham, 1995b), but not in barley (Flasinski *et al.,* 1995; Rao and Grantham, 1995b; Sacher and Ahlquist, 1989). To examine whether variants of BMV CP containing additional N-terminal deletions constructed in this study (Fig. 2B) would behave differently in barley, individual transcripts of B3ΔCP11, B3ΔCP14, B3ΔCP18, B3ΔCP19, and B3ΔCP22 were mixed with wt B1 and B2 and inoculated onto barley. None of the barley plants inoculated with wt B1 and B2 and each B3 variant developed local or systemic symptoms (Table 2). Northern blot analysis of total RNAs from inoculated and uninoculated leaves failed to reveal RNAs characteristic BMV (Table 3).

DISCUSSION

Role of the NARM region in RNA–CP interactions

The first 25 N-proximal residues of BMV CP contain 7 arginine, 1 lysine, and no acidic residues (Fig. 2B). Previous studies speculated that these positively charged amino acid residues interact with viral RNA during the assembly process (Argos, 1981; Sgro *et al.,* 1986; Vriend *et al.,* 1986). *In vivo* studies confirmed that the region of the first 25 N-terminal amino acids of the BMV CP plays a major role in RNA–CP interactions, since its deletion completely abolishes virion formation and subsequent infectivity (Sacher and Ahlquist, 1989). The *in vivo* studies presented here further delineate the minimum number of N-proximal amino acids required for virion formation and identify the first 19 N-terminal amino acids as being involved in RNA–CP interactions. This is exemplified by the biological activity expressed in plants by the five N-terminal B3 variants: B3ΔCP11, B3ΔCP14, B3ΔCP18, B3ΔCP19, and B3ΔCP22 (Table 2; Fig. 3). Because these variants are characterized by large deletions, it is difficult to precisely deduce whether the loss of infectivity in inoculations with wt B1 and B2 and the variant B3ΔCP19 resulted from the cumulative effect of the deleted 6 positively charged amino acid residues or was due to the deletion of any 1 specific amino acid located within the region of the first 19 N-terminal amino acids. In bromoviruses, minor alterations (either deletions or substitutions involving 1 or 2 amino acids) have been shown to have a profound effect on the resulting virus. A single lysine to arginine change at position 42 of CCMV CP resulted in a salt-stable virion phenotype (Fox *et al.,* 1996). For

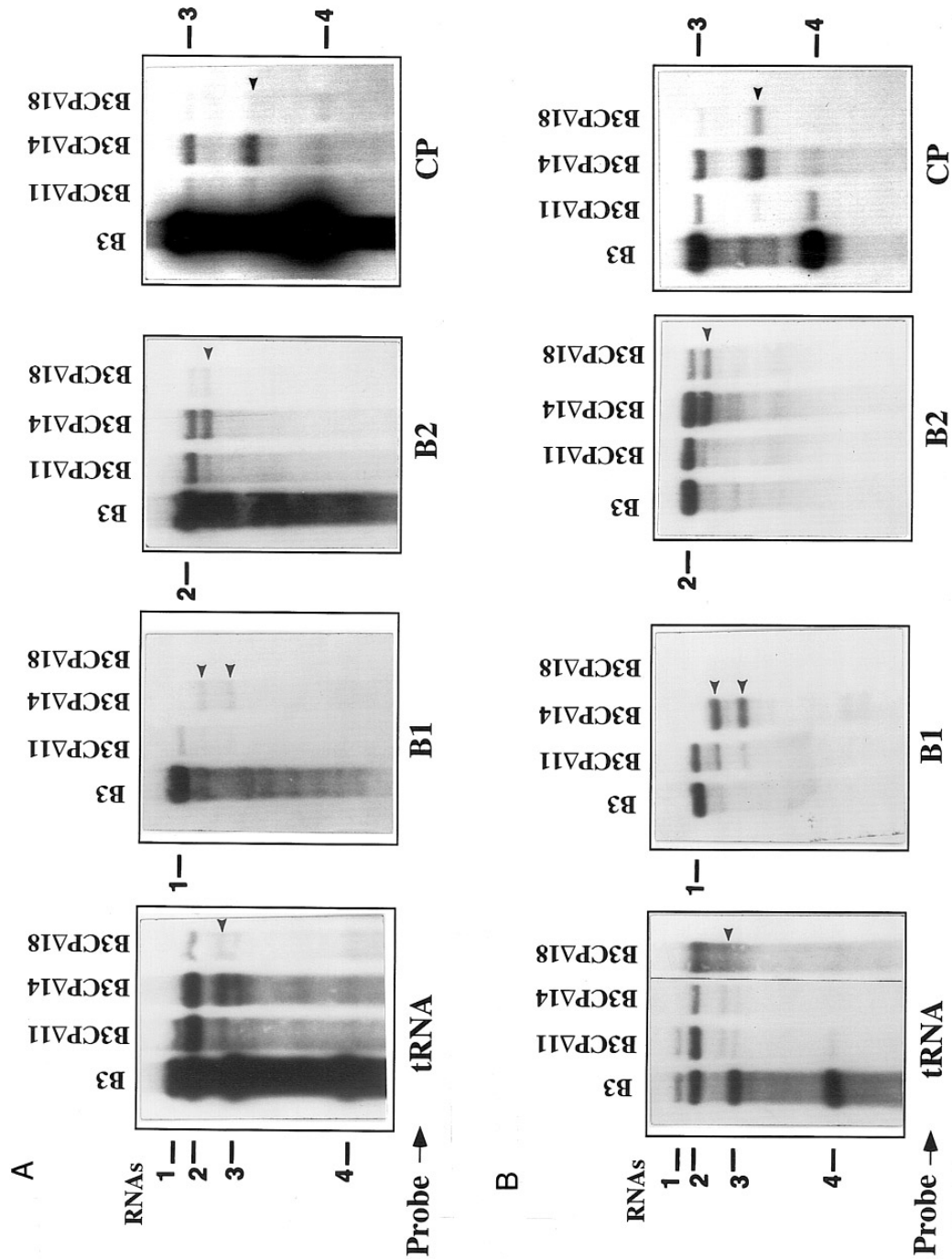

FIG. 5. Progeny RNA analysis of wt BMV and three B3 variants from *C. quinoa* plants. (A) Northern blot analysis of total RNA isolated from leaves inoculated with transcripts of wt B1 and B2 and indicated B3 variants. Multiple blots containing either 1 μg (for wt) or 10 μg (for all three B3 variants) of total RNA isolated from the symptomatic leaves of *C. quinoa* were produced as described for Fig. 2. Each blot was hybridized with indicated riboprobes of equal specific activity. (B) Northern blot analysis of virion RNA of wt and the indicated B3 variants. Approximately 50 ng of virion RNA was subjected to electrophoresis on multiple gels and each blot was hybridized with the indicated riboprobe. Truncated RNA bands (shown in A and B) of unknown origin detected by riboprobes specific for B1, B2, and the CP gene are indicated by arrowheads. The positions either of all four genomic BMV RNAs detected by the tRNA probe or of the individual genomic RNA detected by either B1 or B2 probes are shown to the left. The positions of genomic B3 and subgenomic RNA4 detected by the CP probe are shown to the right. Blots shown in A and B were exposed 20 hr at room temperature.

BMV, CP variants harboring a deletion of the first 7 N-terminal amino acids exhibited wild-type properties with respect to packaging and infectivity in plants (Flasinski *et al.,* 1995; Rao and Grantham, 1995b; Sacher and Ahlquist, 1989), while two other variants, each having consecutive deletions of two amino acids, both lacking a positively charged lysine which is located at position 7 (Fig. 2B), rendered the CP incompetent for encapsidation and proved to be noninfectious (Rao and Grantham, 1995b). Furthermore, recent *in vitro* filter-binding assays between BMV RNA and synthetic oligopeptides revealed that an arginine-rich oligopeptide encompassing wt amino acids 8–25 has a high affinity for RNA binding, whereas mutations altering arginines to alanines (for example $R_{13}R_{14} \rightarrow A_{13}A_{14}$) and introduction of a proline mutation (for example $R_{18} \rightarrow P_{18}$) greatly decreased binding of RNA (C. Kao, personal communication). Therefore, incorporation of these and additional mutations into a biologically active B3 clone and their *in vivo* analysis is likely to elucidate which positively charged amino acids precisely interact with the RNA.

In bromoviruses, the three genomic and the single subgenomic RNA are packaged into three separate particles (Lane, 1981). Encapsidation assays for variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 revealed that mutations in the basic N-terminal arm of BMV CP affected the packaging ability of each genomic RNA (Figs. 5A and 5B). For example, the truncated CP lacking 11 N-terminal amino acids derived from variant B3ΔCP11 packaged all four RNAs, however at a lower efficiency than wt, despite the fact that progeny RNAs of B3ΔCP11 accumulated to 75% of wt level in protoplasts (Fig. 2C). The packaging efficiency of each genomic RNA further decreased as N-terminal truncations increased (Figs. 5A and 5B). Irrespective of the extent of the deletion, genomic RNA2 was packaged efficiently by all three variants, suggesting that different amino acids present within the region of the first 19 N-terminal amino acids interact differently with each genomic RNA during encapsidation. Interestingly, although the truncated CPs of B3ΔCP11, B3ΔCP14, and B3ΔCP18 encapsidated RNAs, the virions resembled empty particles when examined under the electron microscope (Fig. 4). It is possible that, unlike wt, the decreased efficiency to encapsidate the progeny RNAs (Fig. 5B) enabled the truncated CPs to assemble into virions reminiscent of empty capsids.

In this study, full virions were recovered from symptomatic leaves of *C. quinoa* infected with variants B3ΔCP11, B3ΔCP14, and B3ΔCP18. Previous *in vitro* assembly studies with CCMV showed that CP lacking the first 26 N-terminal amino acids assembled only into empty particles (Zhao *et al.,* 1995). Therefore, we anticipated that CP derived from variants B3ΔCP19 or B3ΔCP22 would assemble into empty particles. However, electron microscopic examination of protoplast preparations transfected with wt B1 and B2 and B3ΔCP19 or B3ΔCP22 could not identify any virions characteristic of BMV (Table 3), although control transfections containing either B3ΔCP11 or B3ΔCP14 revealed the presence of BMV virions. It is conceivable that several undetermined conditions, such as salt concentration, pH, and the compartmentalization of viral gene products during the infection process, are likely to influence the assembly process *in vivo.*

Function of BMV CP in symptom modulation

The symptom phenotypes elicited by several plant viruses can be modified by a variety of altered interactions between the gene products of the virus and the host. In plant viruses, CP has been shown to influence symptom expression (Heaton *et al.,* 1991; Hilf and Dawson, 1993; Kong *et al.,* 1995; Shintaku *et al.,* 1992; Suzuki *et al.,* 1995). In BMV, CP has a specific host determinant for *C. quinoa,* since inoculation with a CP variant lacking the first 7 N-terminal amino acids from matured BMV CP altered the lesion phenotype from chlorotic to necrotic (Rao and Grantham, 1995b). Similarly, in alfalfa mosaic virus, a tripartite virus belonging to *Bromoviridae* family, deletion of five N-proximal amino acids induced a hypersensitive response in tobacco plants (Van Der Vossen *et al.,* 1994), suggesting that the function of N-terminal amino acids in regulating virus–host interactions is not restricted to BMV alone. In the present study, inoculation of BMV CP variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 onto *C. quinoa* also resulted in the development of necrotic local lesions (Fig. 3). In bromoviruses, the first 25 N-terminal basic amino acids of the CP are internal and interact with the RNA and are therefore not visible in the electron density map (Speir *et al.,* 1995). Thus, the observed symptom modifications in *C. quinoa* by several N-terminal variants of BMV CP analyzed in previous studies (Rao and Grantham, 1995b) as well as those analyzed in this study (Fig. 3) must have resulted from the interaction between the host and the free coat protein subunits with altered N-terminus prior to encapsidation. It is also likely that a change in the confirmation of resulting CP, affecting RNA–CP interactions or protein–protein (viral vs host) interactions, must be involved in altering symptom expression.

The lack of systemic infection in *C. quinoa* inoculated with wt B1 and B2 and the B3 variants B3ΔCP11, B3ΔCP14, or B3ΔCP18 can be attributed to the onset of necrosis in the inoculated leaves preventing the virus from spreading to healthy cells. This development accounts for the low titer associated with each B3 variant (Table 3). The fact that neither necrosis nor infection occurred in barley plants inoculated with wt B1 and B2 and the variants B3ΔCP11, B3ΔCP14, and B3ΔCP18 (Table 3) suggests that different plant species respond differently to altered viral gene products.

Function of BMV CP in cell-to-cell movement

The mechanistic role played by the BMV CP in viral transport is currently unknown. Mutational and genetic analysis of bromovirus MP and CP suggest that both genes are essential for cell-to-cell movement. Mutants of BMV that either failed to produce functional CPs or produced truncated CPs did not infect permissive hosts (Rao and Grantham, 1995b; Sacher and Ahlquist, 1989). Similar genetic analysis of the CMV CP also confirmed the requirement of CP for cell-to-cell and long distance movement (Boccard and Baulcombe, 1993; Suzuki *et al.,* 1991; Taliansky and Garcia-Arenal, 1995). The lack of infection manifested by the encapsidation-incompetent BMV CP variants B3ΔCP19 and B3ΔCP22 is in agreement with previous observations (Allison *et al.,* 1990; Rao and Grantham, 1995b) that bromovirus CP contributes to efficient cell-to-cell movement. Additional evidence obtained by our recent fluorescence *in situ* hybridization analysis of infections of several BMV CP variants, defective in encapsidation, confirmed the requirement of CP for efficient cell-to-cell spread (Schmitz and Rao, 1996). The recent detection of virus-like particles of BMV in tubules extending from transfected protoplasts (Kasteel *et al.,* 1996), a situation reminiscent of the cytological evidence seen in the wake of cell-to-cell movement in comoviruses (Van Lent *et al.,* 1991) and nepoviruses (Ritzenthaler *et al.,* 1995) further implicates the role of encapsidation-competent CP in cell-to-cell transport.

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