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# Deletion of the C-Terminal 33 Amino Acids of Cucumber Mosaic Virus Movement Protein Enables a Chimeric Brome Mosaic Virus To Move from Cell to Cell

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**The movement protein (MP) gene of brome mosaic virus (BMV) was precisely replaced with that of cucumber mosaic virus (CMV). Infectivity tests of the chimeric BMV on *Chenopodium quinoa*, a permissive host for cell-to-cell movement of both BMV and CMV, showed that the chimeric BMV failed to move from cell to cell even though it replicated in protoplasts. A spontaneous mutant of the chimeric BMV that displayed cell-to-cell movement was subsequently obtained from a local lesion during one of the experiments. A cloned cDNA representing the genomic RNA encoding the MP of the chimeric BMV mutant was analyzed and found to contain a mutation in the CMV MP gene resulting in deletion of the C-terminal 33 amino acids of the MP. Directed mutagenesis of the CMV MP gene showed that the C-terminal deletion was responsible for the movement capability of the mutant. When the mutation was introduced into CMV, the CMV mutant moved from cell to cell in *C. quinoa*, though the movement was less efficient than that of the wild-type CMV. These results indicate that the CMV MP, except the C-terminal 33 amino acids, potentiates cell-to-cell movement of both BMV and CMV in *C. quinoa*. In addition, since *C. quinoa* is a common host for both BMV and CMV, these results suggest that the CMV MP has specificity for the viral genomes during cell-to-cell movement of the virus and that the C-terminal 33 amino acids of the CMV MP are involved in that specificity.**

Cucumber mosaic virus (CMV) and brome mosaic virus (BMV) are the type members of the *Cucumovirus* and *Bromovirus* genera, respectively. Both are isometric plant viruses with a tripartite single-stranded, messenger-sense RNA genome (1, 41). Replication of the genomic RNA depends on proteins 1a and 2a encoded by RNAs 1 and 2, respectively. In CMV, the 2b protein encoded by RNA 2 is translated from a subgenomic RNA 4A (15). Two gene products encoded by dicistronic RNA 3 are dispensable for viral RNA replication. A nonstructural 3a protein encoded in the 5′-proximal open reading frame (ORF) of RNA 3 potentiates the cell-to-cell movement of the viruses (10, 14, 23, 31, 44) and is called the movement protein (MP). The coat protein (CP) is encoded in the 3′-proximal ORF of RNA 3 and is translated from a subgenomic RNA 4.

The mechanism of cell-to-cell movement of CMV and BMV is not clearly understood. However, several characteristics of the CMV MP have been revealed. For example, *Escherichia coli*-expressed CMV MP can move from cell to cell when injected into leaf mesophyll cells and potentiates the movement of coinjected CMV RNA (14). Also, the CMV MP binds single-stranded nucleic acid in vitro (26). Thus, it is proposed that CMV MP interacts with the viral genome as well as host factors that are involved in the intercellular transport system. CP and other virus-encoded elements are also required for the efficient cell-to-cell movement of CMV (4, 16, 18, 44). Similarly, interaction between the MP and viral genome is thought to be required for cell-to-cell movement of BMV.

Chimeric viruses have been created by substituting the MP gene into viruses belonging either to the same genus (12, 31, 37) or to taxonomically divergent genera (9, 19). The chimeric viruses systemically infect plants if the plants are a common host for the donor and recipient viruses of the MP gene. Thus, the MPs of several plant viruses apparently function in a similar way.

*Chenopodium quinoa* is a permissive host for cell-to-cell movement of both CMV and BMV, and it is presumed that the MP and other components of the viruses are adapted to the plant. Thus, *C. quinoa* is a suitable host to evaluate the interactions between the MP and the viral genome and their role in viral cell-to-cell movement of CMV and BMV.

In this study we constructed a chimeric BMV whose MP gene was precisely replaced with that of CMV. Although this chimeric BMV failed to move from cell to cell in *C. quinoa*, we identified a spontaneous mutant of the virus that did. Analysis of the mutant showed that it contained a deletion of the C-terminal 33 amino acids of the CMV MP and was apparently responsible for the cell-to-cell movement. When the mutant MP was introduced into CMV, the mutant virus also moved from cell to cell in *C. quinoa*. The efficiency of the movement of the mutant CMV, however, was less than that of the wild-type CMV. These results are discussed in terms of the specificity of the CMV MP for the genomes of BMV and CMV and the role of this specificity in viral cell-to-cell movement.

## MATERIALS AND METHODS

**Plants.** *C. quinoa* and tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) plants were grown in commercially available soil mixtures in a plant growth room at 24 ± 1°C with illumination for 16 h per day.

**cDNA clones.** Plasmids pBTF1, pBTF2, and pBTF3W contain the full-length cDNAs of RNAs 1, 2, and 3 of the wild-type BMV, respectively, from which infectious transcripts can be produced by using T7 RNA polymerase (32–34). In vitro transcripts from these plasmids and their progenies will be referred to as the BMV KU2 strain. Plasmids pCY1-T7 and pCY2-T7 (generous gifts from S. Kuwata and M. Suzuki, Japan Tobacco Incorporated) contain the full-length cDNAs of CMV strain Y RNAs 1 and 2, respectively, and infectious viral RNA transcripts can be transcribed in vitro by T7 RNA polymerase from these plasmids (44). A full-length RNA 3 cDNA clone of the wild-type CMV (an isolate of the Y strain) was constructed by joining partial cDNA fragments from pCY32C1 and pCY78 (generous gifts from M. Nakayama, Takeda Chemical Industries, Ltd.). The full-length RNA 3 cDNA fragment was inserted into pUCT7 (34) to

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attach a T7 promoter sequence at the 5′ end. The resulting plasmid, designated pT7CKY3, was linearized at the 3′ end of RNA 3 with *Nsi*I and made blunt prior to in vitro transcription.

A plasmid containing the full-length cDNA of the chimeric BMV RNA 3 with the CMV MP gene, designated pT7B3CY3a, was constructed as follows. *Nsi*I and *Bln*I sites were introduced into the initiation and termination codons of the BMV MP gene, respectively, in a modified pBB3 plasmid (34) from which the 0.3-kb *Sac*I-*Stu*I fragment had been eliminated, to produce pBMP-NB. Similarly, *Nsi*I and *Xba*I sites were introduced into the initiation and termination codons of the CMV MP gene, respectively, in a pCMP plasmid that had been constructed by inserting the 1.3-kb 5′ half of the RNA 3 cDNA fragment of pCY32C1 into pUC118, to produce pCMP-NX. The BMV MP gene in pBMP-NB was precisely replaced with the CMV gene in pCMP-NX according to the strategy used for replacing BMV genes with foreign genes (35). The 0.6-kb *Bgl*II-*Eco*RI fragment of the plasmid was replaced with the corresponding 0.9-kb *Bgl*II-*Eco*RI fragment of pBTF3W to generate pB3CY3a. Then, the 2.1-kb *Sna*BI-*Eco*RI fragment of pB3CY3a was inserted into pUCT7 (34) to attach the T7 promoter sequence at the 5′ end of RNA 3 to produce pT7B3CY3a. Precise replacement of the BMV MP gene with the CMV MP gene and absence of undesired mutations were confirmed by nucleotide sequencing of pT7B3CY3a.

RNA 3 cDNA clones of progeny viruses from infected *C. quinoa* plants were constructed by reverse transcriptase PCR (RT-PCR). Total RNA was extracted from leaf disks (2 by 2 mm) containing a single local lesion produced on the inoculated leaves of *C. quinoa* at 6 days postinoculation (6 dpi) as described elsewhere (22). The RNA samples were further purified twice by phenol-chloroform extraction and RQ1 DNase I (Promega) treatment. First-strand cDNA was synthesized from each of the RNA samples by SuperScript II (GIBCO BRL) according to the manufacturer's recommendations by using the 3′ primer, 5′-C ACGAATTCC*CTGGTCTCTTTTAGAGAT*-3′, which is complementary to the 3′-terminal 17 nucleotides of BMV RNA 3 (italics) and contains a unique *Eco*RI site (underlined). The resulting cDNA was amplified by PCR with the 3′ primer and a 5′ primer, 5′-CACTGCAG**TAATACGACTCACTATAG***TAAAATACCAA CTAATTC*-3′, which corresponds to the 5′-terminal 19 nucleotides of BMV RNA 3 (italics), phage T7 promoter sequence (bold), and *Pst*I site (underlined). The reaction was carried out with ExTaq DNA polymerase (Takara Shuzo) under conditions previously described (17). The amplified full-length RNA 3 cDNA was digested with *Pst*I and *Eco*RI and cloned into pUC119 at the *Pst*I-*Eco*RI sites.

Plasmids pB3C3aD4 and pC3D4 were constructed by replacing the *Hpa*I fragment in the CMV MP gene in pT7B3CY3a and pT7CKY3, respectively, with the corresponding fragment from a cloned PCR-amplified chimeric RNA 3 cDNA clone (designated pB3C3aR). pB3C3aRR4 was constructed by replacing the *Hpa*I fragment in pB3C3aR with the corresponding fragment from pT7B3CY3a. pB3C3a247T, containing the chimeric RNA 3 cDNA with a stop codon resulting in the truncation of the C-terminal 33 amino acids in the CMV MP, was constructed by PCR-based in vitro mutagenesis (21) using oligonucleotide 5′-CCTCGGACTAACTGCGCGC-3′. Absence of undesired mutations was confirmed by nucleotide sequencing.

**Inoculation of plants and protoplasts.** Capped full-length transcripts were synthesized in vitro by using T7 RNA polymerase (Takara Shuzo) as described elsewhere (24). RNA 3 variants of BMV and CMV were always coinoculated with the wild-type RNAs 1 and 2 of BMV and CMV, respectively. Control inoculations always contained all three transcripts of the wild-type BMV or CMV RNA. Crude viral extracts were prepared from individual leaf tissue sections (2 by 2 mm) of *C. quinoa* by grinding with a mortar and pestle in 15 μl of 0.1 M sodium-phosphate buffer (pH 7.0). Either a mixture containing viral RNA transcripts or crude extract of leaf tissue was inoculated mechanically with Carborundum onto the two youngest fully expanded leaves of 4-week-old *C. quinoa* and 14-week-old tobacco plants.

*C. quinoa* protoplasts were prepared as described elsewhere (13) with some alterations. The lower epidermis of fully expanded leaves of 5-week-old plants was peeled off, and leaf tissue was digested with 2% cellulase (Onozuka R-10) and 0.1% pectolyase (Y-23) in 0.6 M mannitol containing 10 mM $CaCl_2$ (pH 5.6) for 3 h at 25°C. Freshly prepared protoplasts were separated from tissue debris by filtration through four layers of gauze. Approximately $3 \times 10^5$ protoplasts were inoculated by means of polyethylene glycol with a mixture of transcripts (24). Protoplasts were incubated at 25°C for 24 h (39).

**RNA analysis.** Total RNA from infected protoplasts or from infected leaves was extracted and hybridizations were performed as described elsewhere (22, 24). Plus- and minus-strand BMV genomic RNAs were detected with $^{32}$P-labeled transcripts from *Hin*dIII-linearized pBSPL10 and pBSMI10, respectively (22). The BMV MP gene was detected with $^{32}$P-labeled transcripts from *Eco*RI-linearized pBSL3DN4, which contains cDNA sequence complementary to the BMV MP gene encoded in pBTF3 (34) downstream of the SP6 promoter sequence. The CMV MP gene was detected with $^{32}$P-labeled T7 RNA polymerase transcripts from an *Eco*RI-linearized pPCMP which contains the 0.9-kb *Nsi*I-*Xba*I fragment from pCMP-NX at the *Pst*I-*Xba*I sites of pBluescript KS(−) (Stratagene). The signals were quantified with a digital radioactive imaging analyzer (Fujix BAS 2000; Fuji Photo Film).

Press blots (28) of *C. quinoa* leaves were prepared as described elsewhere (31) by using a flat aluminum board to apply pressure at $1.7 \times 10^2$ kg/m$^2$ for 1 h. BMV RNAs and CMV RNAs were detected with $^{35}$S-labeled transcripts from *Hin*dIII-linearized pBSPL10 and *Xba*I-linearized pCY200T (22), respectively. Tissue printing of tobacco leaves was performed as described elsewhere (31), and CMV RNAs were detected with $^{32}$P-labeled transcripts from *Xba*I-linearized pCY200T.

Detection of the CMV MP gene in progeny from local lesions on *C. quinoa* was carried out by RT-PCR as described above by using oligonucleotide 5′-GC TAAAGACCGTTAACCACCTGCGGTC-3′ as the 3′ primer. This oligonucleotide contains sequence complementary to the last 26 nucleotides of the CMV MP ORF and an additional G residue at the 5′ end. The RT-PCR products were separated by electrophoresis on 1.0% agarose gels and visualized with UV illumination after ethidium bromide staining.

**Protein analysis.** Proteins were extracted from whole inoculated leaves (40) and from $1.5 \times 10^5$ protoplasts (35). The proteins were separated by electrophoresis on 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) (i.e., by SDS-PAGE [25]). Immunoblot analysis was carried out as described elsewhere (45) by using an Immobilon-P transfer membrane (Millipore). The CMV MP was detected with a polyclonal rabbit antiserum raised against the purified *E. coli*-expressed fusion protein composed of the CMV MP and protein A derived from *Staphylococcus aureus* (a gift from M. Nakayama) and with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

## RESULTS

**The chimeric BMV containing the CMV MP gene fails to move from cell to cell.** Before replacing the MP gene of BMV with that of CMV, we first constructed pT7CKY3, a plasmid that contains a full-length cDNA of the CMV Y RNA 3. Biological activity of this MP gene was assessed in tobacco plants following inoculation with in vitro transcripts from the plasmid (C3) and CMV RNA 1 and 2 cDNA clones (C1+2). Yellow mosaic symptoms developed on uninoculated upper leaves at 6 dpi and were indistinguishable from those on plants inoculated with the parent CMV Y. CMV RNA accumulation was detected in the inoculated and uninoculated upper leaves by tissue printing analysis (data not shown). Thus, we concluded that the CMV MP gene of the clone was biologically active. The nucleotide sequence of the clone (accession number D83958 in the EMBL and GenBank DNA databases) was 99.0% identical to that of the corresponding RNA of CMV Y previously published (38); the only difference detected in the CMV MP gene of pT7CKY3 was a $^{351}$T→C change, which resulted in a valine-to-alanine change at amino acid 78 in the MP.

The BMV MP gene was precisely replaced with the CMV MP gene to create the chimeric BMV RNA 3 (cB3) cDNA clone. The construction resulted in a silent mutation, CTT→CTC, immediately before the termination codon of the CMV MP gene. To assess infectivity of the chimeric BMV containing the CMV MP gene, in vitro transcripts of BMV RNAs 1 and 2 (B1+2) and cB3 were inoculated into *C. quinoa* protoplasts. As shown in Fig. 1A and B, the genomic RNAs of the chimeric virus accumulated in protoplasts, although the accumulation levels of positive- and negative-strand cB3 normalized against the signals of RNA 2 were 12 to 16 and 5 to 8%, respectively, of the levels of wild-type BMV RNA 3 (B3) that accumulated during infection with BMV. Immunoblot analysis showed that the CMV MP accumulated in the protoplasts inoculated with B1+2 and cB3 (Fig. 1C). The level of accumulation of the CMV MP was comparable to that of protoplasts infected with CMV, when equal number of protoplasts were compared (data not shown).

The chimeric BMV was then tested for infectivity in *C. quinoa* plants, a common host for both BMV and CMV. Under our experimental conditions, transcript mixtures of the wild-type BMV and CMV induced more than 100 chlorotic lesions and more than 50 necrotic lesions on the inoculated leaves, respectively. The lesions induced by either virus appeared at 3 to 4 dpi, continued to enlarge, and began to coalesce at 6 to 8 dpi. The inoculated leaves dropped from the base of their petioles at 12 to 16 dpi. In contrast, no symptoms were induced

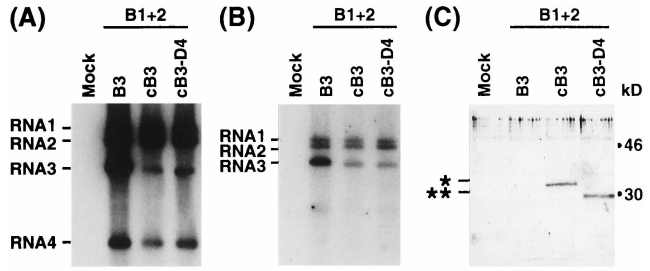


FIG. 1. Northern hybridization and immunoblot analysis of viral RNAs and CMV MP in *C. quinoa* protoplasts. Protoplasts ($3 \times 10^5$) were inoculated with in vitro transcripts from plasmid pBTF3W (B3), pT7B3CY3a (cB3), or pB3C3aD4 (cB3-D4) together with in vitro transcripts from BMV RNA 1 and 2 cDNA clones (B1+2). Mock, inoculated with buffer only. (A and B) Detection of plus-strand or minus-strand viral RNAs, respectively, by Northern hybridization with riboprobes. Total RNA was extracted at 24 h postinoculation. (C) Immunodetection of the CMV MP using rabbit anti-CMV-MP antiserum. Total proteins were extracted at 24 h postinoculation and fractionated by SDS-PAGE. Asterisk and double asterisk, positions of the full-length CMV MP and the truncated CMV MP lacking the C-terminal 33 amino acids, respectively. Sizes of molecular weight markers are indicated on the right.

on plants inoculated with a transcript mixture composed of B1+2 and cB3, and no viral RNA was detected in the inoculated leaves by press blot analysis at 3 dpi (Fig. 2) and 14 dpi (data not shown); infection with BMV yielded wild-type BMV RNA at 3 dpi (Fig. 2). Since the chimeric BMV successfully infected protoplasts, these results indicate that the chimeric BMV did not move from cell to cell. We have repeated the infectivity tests with more than 150 leaves, and the same results were obtained except in one experiment described below.

**Spontaneous mutants of the chimeric BMV that infect *C. quinoa* plants.** During the infectivity tests of the chimeric BMV in *C. quinoa* plants, 40 chlorotic lesions appeared on 10 inoculated leaves at 4 dpi in one experiment. Each of the lesions was analyzed by RT-PCR with the 3′ primer specific for the CMV MP gene and the 5′ primer specific for the BMV RNA 3 5′ end. Two of the 40 lesions produced a PCR product with the size expected (957 bp) for the chimeric RNA 3 with the CMV MP gene, while the other 38 lesions did not (data not shown). These results suggested the possibility that mutants of the chimeric BMV had been generated. Upon further analysis of the 38 RT-PCR-negative lesions by back-inoculation of leaf extracts onto *C. quinoa* leaves and by RNA hybridization reactions using probes each of which is specific for the BMV 3′ end, the BMV MP gene, or the CMV MP gene, we concluded that the inoculum used in the experiment had been contaminated by the wild-type B3.

To analyze the remaining two putative spontaneous mutants of the chimeric BMV, full-length RNA 3 cDNA clones were constructed from RNA samples of the two RT-PCR-positive lesions. Two and four clones were obtained from the two lesions, respectively. Since both the chimeric RNA 3 and the wild-type RNA 3 of BMV could be cloned by using primers specific for the 3′ and 5′ ends of BMV RNA 3, the cDNA clones were analyzed by digestion with restriction enzymes. The two cDNA clones obtained from one of the lesions were digested successfully with *Dra*I and *Hpa*I, which is diagnostic for the CMV MP gene, but were not digested with *Cla*I, which is diagnostic for the BMV MP gene. This suggested that the two clones contained the CMV MP gene. The clones were designated cB3R#1 and cB3R#2, respectively. The four

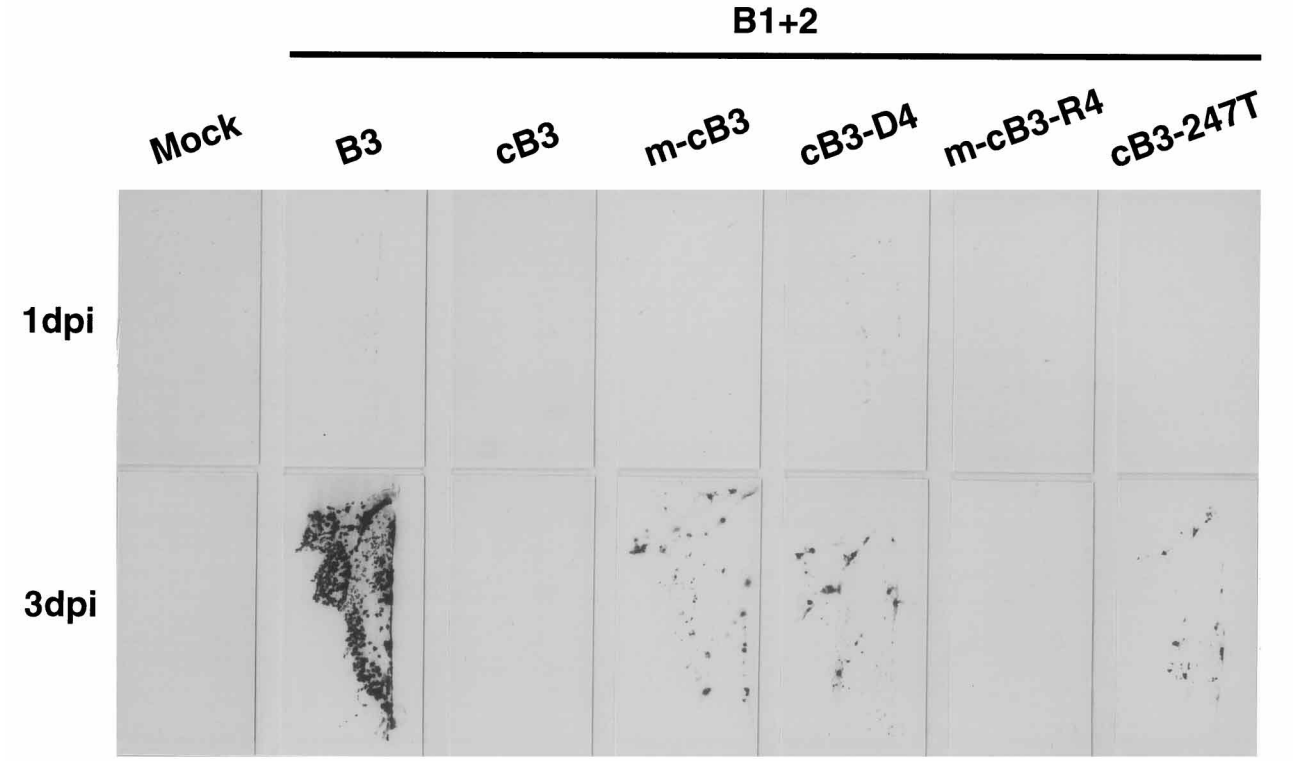


FIG. 2. Press blot analysis of viral RNA accumulation and distribution in inoculated leaves of *C. quinoa* at 1 and 3 dpi. *C. quinoa* leaves were inoculated with in vitro transcripts from plasmid pBTF3W (B3), pT7B3CY3a (cB3), pB3C3aR (m-cB3), pB3C3aD4 (cB3-D4), pB3C3aRR4 (m-cB3-R4), or pB3C3a247T (cB3-247T) together with in vitro transcripts from BMV RNA 1 and 2 cDNA clones (B1+2). Press blots of the half-leaves whose abaxial epidermis was peeled off were analyzed by hybridization using a $^{35}$S-labeled riboprobe complementary to the plus-strand conserved 3′-terminal sequence of BMV RNAs.

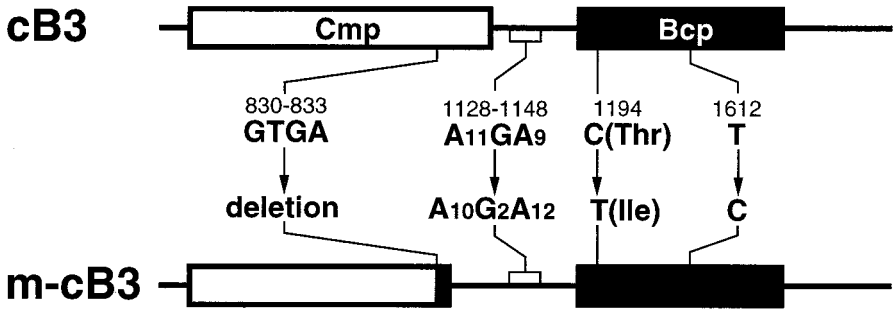


FIG. 3. Schematic diagram of chimeric and mutant BMV RNAs 3. cB3, originally constructed chimeric RNA 3; m-cB3, chimeric RNA 3 cloned from a local lesion on *C. quinoa*. Differences in nucleotide sequence between cB3 and m-cB3 are denoted with position numbers. The MP gene of CMV (*Cmp*) and the CP gene of BMV (*Bcp*) are denoted by open and shaded boxes, respectively. The surrounding noncoding regions are denoted by horizontal lines. The filled box in m-cB3 represents the three changed amino acid residues resulting from a frameshift mutation.

cDNA clones obtained from the second lesion were similarly analyzed. One of these four clones was also digested successfully with *Dra*I and *Hpa*I but not with *Cla*I. This clone was designated cB3R#3. The remaining three clones, however, were not digested with either *Dra*I or *Hpa*I but were digested with *Cla*I, suggesting that they contained the BMV MP gene. These results suggested that the chimeric RNA 3 and the wild-type RNA 3 of BMV can exist together in a single local lesion.

Biological activities of the cB3R#1, cB3R#2, and cB3R#3 transcripts were individually tested in *C. quinoa* plants by coinoculating with B1+2. More than 100 chlorotic lesions similar to those of wild-type BMV infection were induced on inoculated leaves with cB3R#1 and cB3R#2 transcripts, while no symptoms were induced by cB3R#3. These results suggest that cB3R#1 and cB3R#2 contained a mutation(s) that allows the chimeric BMV to move from cell to cell.

The nucleotide sequences of cB3R#1 and cB3R#2 were found to be identical, and therefore, we hereafter refer to either cB3R#1 or cB3R#2 as m-cB3. Compared with the sequence of cB3 (original construct), the sequence of m-cB3 differed at four positions (Fig. 3). One of the mutations was a 4-base deletion of $^{830}$GAGT$^{833}$ (equivalent to $^{827}$AGTG$^{830}$, $^{828}$GTGA$^{831}$, or $^{829}$TGAG$^{832}$, considering the nucleotide sequence around this position). The deletion was followed by a frameshift resulting in premature translational termination of the CMV MP three codons downstream of the deletion. The sequence suggested that the C-terminal 33 amino acids were removed from the mutant CMV MP and that 3 out-of-frame amino acids were added to the C terminus of the truncated CMV MP. Three other mutations were present in m-cB3: an elongation of the poly(A) sequence in the intercistronic region by one guanine and two adenine residues and two point mutations in the CP gene. One point mutation resulted in a substitution of threonine for isoleucine (i.e., $^{1193}$ACT$^{1195}$→ ATT), and the second was silent (i.e., $^{1610}$ACT$^{1612}$→ACC).

**The truncation of CMV MP is responsible for cell-to-cell movement of the chimeric BMV.** To determine which mutation(s) in m-cB3 was responsible for infectivity of the chimeric virus, the *Hpa*I fragment containing the deletion was exchanged between m-cB3 and cB3. In vitro transcripts from either of these recombinant clones, that is, cB3 with the 4-base deletion (cB3-D4) and m-cB3 having the intact CMV MP gene (m-cB3-R4), were inoculated separately with B1+2 transcripts onto *C. quinoa* plants. Chlorotic lesions similar to those of the wild-type BMV infection were induced by the chimeric BMV containing cB3-D4 transcripts but not by the chimeric BMV containing m-cB3-R4 transcripts. Time course press blot analyses showed that diffusion of viral RNA signals in the inoculated leaves of B1+2 and cB3-D4 transcripts was similar to that of B1+2 and m-cB3 transcripts (Fig. 2). Signals detected from the leaves infected with these chimeric viruses were fewer than those from the wild-type BMV-infected leaves, though the number of induced chlorotic lesions was indistinguishable between the chimeric viruses and the wild-type BMV. Immunoblot analysis using anti-CMV MP antiserum showed that a protein with a molecular weight lower than that of the intact CMV MP accumulated in leaves inoculated with B1+2 and cB3-D4 transcripts (Fig. 4). On the other hand, no viral RNA was detected at 3 dpi (Fig. 2) and 14 dpi (data not shown) from the leaves inoculated with B1+2 and m-cB3-R4 transcripts. These results indicated that the 4-base deletion in the CMV MP gene was responsible and sufficient for cell-to-cell movement of the chimeric BMV.

It is generally accepted that cell-to-cell movement of viruses reflects both virus replication in the infected cells and the MP-mediated intercellular transport of the viral genome. To investigate the effects of the 4-base deletion in the chimeric RNA 3 on viral RNA and MP accumulation, *C. quinoa* proto-

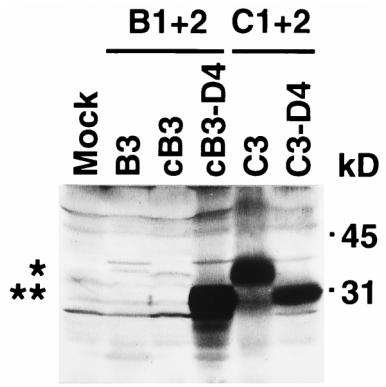


FIG. 4. Immunodetection of CMV MP in inoculated leaves of *C. quinoa*. Leaves were inoculated with in vitro transcripts of the wild-type BMV RNA 3 (B3), chimeric RNA 3 with the intact CMV MP gene (cB3), or chimeric RNA 3 with the 4-base deletion in the CMV MP gene (cB3-D4) together with in vitro transcripts from BMV RNA 1 and 2 cDNA clones (B1+2). Also shown are results with in vitro transcripts of the wild-type CMV RNA 3 (C3) and mutant CMV RNA 3 with the 4-base deletion (C3-D4) together with in vitro transcripts of CMV RNA 1 and 2 cDNA clones (C1+2). Mock, inoculated with buffer only. Proteins extracted from the inoculated leaves (fresh weight, 0.1 g) were fractionated by SDS-PAGE. Asterisk and double asterisk, positions of the intact CMV MP and the CMV MP lacking the C-terminal 33 amino acids, respectively. Sizes of molecular weight markers are indicated on the right.

plasts were inoculated with cB3 or cB3-D4 together with B1+2 and analyzed by Northern hybridization and immunoblot methods. As shown in Fig. 1A and B, accumulation levels of both plus and minus strands of viral RNAs were comparable between cB3- and cB3-D4-inoculated protoplasts. Results from immunoblot analysis (Fig. 1C) showed that the accumulation level of the truncated CMV MP from cB3-D4 was also comparable to that of the intact CMV MP from cB3. These results suggested that accumulation levels of neither viral RNA nor CMV MP were affected by the 4-base deletion and that the intact and truncated CMV MPs differ only in functions involved in intercellular transport of the viral genome.

To confirm that the truncation of CMV MP, rather than structural change of the chimeric RNA 3, was essential for cell-to-cell movement of the chimeric BMV, a stop codon was introduced into the MP gene of cB3 so as to encode the CMV MP lacking the C-terminal 33 amino acids. Transcripts of the resulting chimeric RNA 3 clone, cB3-247T, were coinoculated with B1+2 transcripts onto *C. quinoa* plants. The inoculated leaves showed chlorotic lesions which were similar to those induced by infection with the chimeric BMVs containing either m-cB3 or cB3-D4 (data not shown). Time course press blot analysis showed that cell-to-cell movement of the chimeric BMV containing cB3-247T was comparable to that of the chimeric BMV containing m-cB3 or cB3-D4 (Fig. 2). Thus, it was strongly suggested that the deletion in the amino acid sequence of the C-terminal region of CMV MP was responsible for the cell-to-cell movement of the chimeric BMV rather than structural change of the chimeric RNA 3 caused by the 4-base deletion.

**Four-base deletion in CMV.** The 4-base deletion was introduced into the MP gene in C3 to create the CMV RNA 3 mutant (C3-D4) clone. C3-D4 transcripts were coinoculated with C1+2 transcripts onto *C. quinoa* plants. Necrotic lesions similar in number and morphology to those caused by the wild-type-CMV (C1+2 and C3) infection were induced. The lesions appeared on the inoculated leaves at 5 to 6 dpi, while lesions caused by the wild-type-CMV infection appeared at 3 to 4 dpi. The rate of spread of the infection site for the mutant was lower than that for the wild-type CMV; the diameter of lesions induced by the mutant CMV with the 4-base deletion reached 3 mm at 14 dpi, while those induced by wild-type CMV reached a comparable size at 6 dpi. Press blot analysis, at 1, 3, and 14 dpi, of the leaves inoculated with C1+2 and C3 or with C1+2 and C3-D4 showed that RNA signals of each virus diffused with time (Fig. 5), while the rate of the signal diffusion of the CMV mutant was lower than that of the wild-type CMV. Since the viral RNA accumulation level of the CMV mutant was similar to that of the wild-type CMV in *C. quinoa* protoplasts (data not shown), the results indicated that the CMV mutant moved from cell to cell less efficiently than the wild-type CMV. Immunoblot analysis showed that the truncated CMV MP accumulated in leaves inoculated with in vitro transcripts of C3-D4 and C1+2 (Fig. 4). These results indicated that the 4-base deletion was preserved in the CMV mutant. Furthermore, it appears that the C-terminal region of the CMV MP lost due to the 4-base deletion was dispensable as regards the ability of CMV to spread from cell to cell in *C. quinoa*, although efficiency of cell-to-cell movement of CMV was reduced compared with wild-type CMV.

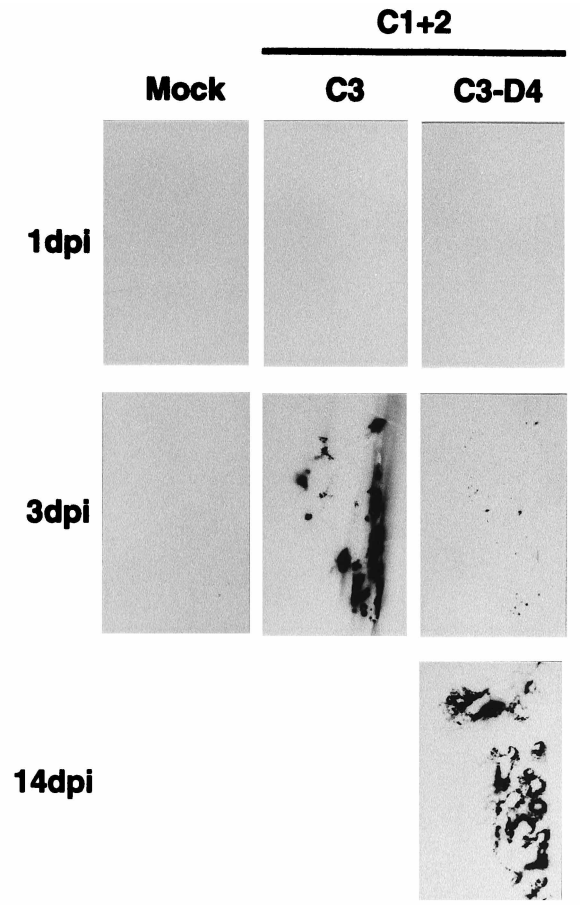


FIG. 5. Press blot analysis of viral RNA accumulation and distribution in the inoculated leaves of *C. quinoa* at 1, 3, and 14 dpi. *C. quinoa* leaves were inoculated with in vitro transcripts from plasmid pT7CKY3 (C3) or pC3D4 (C3-D4) together with in vitro transcripts from CMV RNA 1 and 2 cDNA clones (C1+2). Press blots of the half-leaves whose abaxial epidermis was peeled off were analyzed by hybridization using a $^{35}$S-labeled riboprobe specific for the plus-strand conserved 3′-terminal sequence of CMV RNAs.

## DISCUSSION

Viable chimeric viruses can be created by replacing the MP genes of the viruses, even though the viruses are taxonomically divergent and their MPs share little sequence homology (9, 19). In this study, we investigated whether the CMV MP functions in cell-to-cell movement of BMV by creating and testing chimeric viruses. BMV and CMV are taxonomically related (both are members of the family *Bromoviridae*), and the MPs of BMV and CMV show significant amino acid sequence homology (approximately 35%) (30, 43). The homology between the MPs of the BMV KU2 strain and CMV Y strain used in our experiments is approximately 33%. In addition to the sequence homology, an ancestral relationship and possible functional similarities between the MPs are suggested from similarities in their charge distributions; hydrophobicity profiles; and α-helix, β-sheet, and β-turn propensities (8). In spite of these similarities, a chimeric BMV whose MP gene was precisely replaced with that of CMV was not viable in plants due to its inability to move from cell to cell. However, mutants of the chimeric BMV lacking the C-terminal 33 amino acids of the CMV MP were viable in plants and did move from cell to cell (Fig. 2). We noted, however, that press blot signals in the leaves infected with the chimeric viruses containing m-cB3 or cB3-D4 were fewer than those in the wild-type-BMV-infected leaves (Fig. 2), although the numbers of lesions induced on the inoculated leaves by the wild-type and the chimeric BMVs were comparable. This may be reflective of the low level of

accumulation of chimeric BMV RNAs in some lesions since the accumulation of the chimeric BMV RNA 3 and the subgenomic RNA 4 in protoplasts was less than that of the corresponding RNAs of the wild-type BMV (Fig. 1A and B).

Our results differed from those of Kaplan et al. (23), who reported that cell-to-cell movement of BMV can be complemented in transgenic tobacco plants expressing the MP from the CMV Fny strain. It should be noted, however, that the BMV that moved from cell to cell in transgenic tobacco had all the components of the BMV genome. It is likely that the BMV MP is more compatible with the BMV genome than is CMV MP. Cell-to-cell movement of the BMV genome may become more efficient by participation of the BMV MP. Cooper and Dodds (6) reported that the CMV MP in CMV-infected cells accumulated in a manner different from that of the MP expressed in transgenic plants. Differences in accumulation may account for the difference in the complementation of BMV movement by the CMV MP. In addition, the difference in the complementation could also result from the differences in sequences of CMV MP and/or BMV RNA used in the experiments as well as experimental conditions.

It is generally accepted that cell-to-cell movement of plant viruses occurs as a result of successful interactions between the MP, the viral genome, and host factors (3, 5, 11, 20, 27, 29, 36). Because *C. quinoa* is a common host for both BMV and CMV, the MPs and the other genomic elements of the viruses are certainly adapted to the host plant. Since mutants of chimeric BMV that contains either the intact or truncated CMV MP gene infected *C. quinoa* protoplasts similarly (Fig. 1), the viability of the BMV chimeras in plants most likely reflects the degree of compatibility between the CMV MP and the BMV genome. Cooper et al. (7) reported that transgenic tobacco plants expressing the CMV MP gene complemented the cell-to-cell and systemic spread of a movement-defective CMV mutant but not the local or systemic spread of a movement-defective mutant of tobacco mosaic virus. They addressed the possibility that the CMV MP may not interact with the tobacco mosaic virus genome in plants. This finding and our results strongly suggest that CMV MP has specificity for its viral genome. Furthermore, our results suggest that it is the C-terminal 33-amino-acid portion of the CMV MP that is involved in conferring specificity for the viral genome. In addition to the MP, CP and other viral genome components are involved in efficient cell-to-cell movement of bromo- and cucumoviruses (2, 4, 16, 18, 42, 44, 46). Therefore, a successful interaction between the MP and other viral factors is likely to be required for the cell-to-cell movement of the viruses. The intact CMV MP may be unable to interact successfully with BMV factors, but removal of the C-terminal 33 amino acids from the CMV MP may make the interaction(s) successful and facilitate cell-to-cell movement. This suggests that CMV MP interacts with viral RNAs in vivo, though the binding is not sequence specific in vitro (26). It is tempting to speculate that, if specific binding between the MP and RNAs of CMV occurs in vivo, the C-terminal 33 amino acids of CMV MP are involved in the specificity.

The truncated CMV MP lacking the C-terminal 33 amino acids supported the cell-to-cell movement of CMV as well as the chimeric BMV (Fig. 2 and 5). This indicates that the cell-to-cell transport function is encoded in the remaining sequences of the CMV MP. This is in accord with previous results that the C-terminal 43 amino acids can be successfully removed from the CMV MP and the truncated MP remains functional (23). However, the removal of the C-terminal region from the CMV MP decreased the efficiency of CMV movement (Fig. 5). The reduced efficiency of cell-to-cell movement may be explained by the specificity of the CMV MP for the CMV genome. By the deletion of the C-terminal region that plays an important role(s) in the specificity, cellular components may compete with the viral genome for CMV MP. Alternatively, the removal of the CMV-MP C-terminal region may cause a conformational change of the protein that affects the efficiency of CMV cell-to-cell movement.

The amino acid sequence of the CMV-MP gene, including the C-terminal region, is well conserved (98.0 to 99.3%) among the members of CMV belonging to subgroup I (41), while compared with the MPs of other cucumoviruses, the C-terminal region exhibits variability (data not shown). This observation supports our suggestion that the C-terminal 33 amino acids of the CMV MP are dispensable for viral cell-to-cell movement but are involved in the specificity for the CMV genome.

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