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Studies on Plant-Virus Cell-to-Cell Movement Using Chimeric Viruses

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ABBREVIATIONS

B3(Ccp): a chimeric BMV RNA 3 with the CMV CP gene

B3(Cmp/Ccp): a chimeric BMV RNA 3 with both MP and CP genes of CMV

B3(Cmp): a chimeric BMV RNA 3 with the CMV MP gene

B3(CmpDC33): a derivative of B3(Cmp) which has a stop codon promote the deletion of C-terminal 33 amino acids from the CMV MP.

B3: BMV RNA 3

BMV-KU2: the BMV KU2 strain

BMV: *brome mosaic virus*

C-terminus: carboxyl terminus

C. quinoa: *Chenopodium quinoa*

C3: CMV RNA 3

CCMV: *cowpea chlorotic mottle virus*

cDNA: complementary DNA

CMV-Kin: the CMV Kin strain

CMV-Y: the CMV Y strain

CMV: *cucumber mosaic virus*

CP: coat protein

del-SN: deletion in the N-terminal region of the CMV CP

E. coli: *Escherichia coli*

ER: endoplasmic reticulum

MP: movement protein

N-terminus: amino terminus

ORF: open reading frame

PCR: polymerase chain reaction

pi: postinoculation

RNA: ribonucleic acid

RT-PCR: reverse transcriptase PCR

SDS-PAGE: polyacrylamide gel-electrophoresis containing 0.1% SDS

SDS: sodium dodecyl sulfate

TMV: *tobacco mosaic virus*

tRNA: transfer RNA

GENERAL INTRODUCTION

Systemic infection of most plant viruses is established by repetition of viral amplification in an infected cell and movement to uninfected cells. In most cases, viral movement occurs in two forms; a slow cell-to-cell spread through intercytoplasmic connections known as plasmodesmata, and a fast, long-distance spread through the vasculature (reviewed by Carrington *et al.*, 1996), following the source-sink route of the metabolites in the phloem (Fisher *et al.*, 1992).

The viral factors involved in each type of movement and the manner in which the virus moves are not the same for different viruses (reviewed by Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999). Most plant viruses contain a gene, called as the movement protein (MP) gene, which plays an essential role for viral intra- and inter-cellular movement. Some viruses additionally require the coat protein (CP) for cell-to-cell movement, and others not. Besides, viral replicase and other proteins affect the movement of some viruses.

The ability of a virus to move from cell to cell is considered as an important factor to determine its host range, since most plant viruses which have a positive-sense single-strand RNA genome can replicate in protoplasts isolated from plants which are either host or nonhost for the viruses (reviewed in Dawson and Hilf, 1992). Cell-to-cell movement of plant viruses is an active process that requires successful interactions between viral MP and an intercellular trafficking system of a host plant. Therefore, the MP can be considered as an important factor to determine viral host range.

The MPs of different viruses share their functions. This was first suggested from experimental data in which movement of a virus in its nonhost plant is complemented by coinfection of another virus that can systemically move within the plant (reviewed in Atabekov and Taliansky, 1990). Progress in genetic engineering techniques enables us to create chimeric viruses in which the naturally encoded MP gene is replaced with the corresponding gene of some other viruses. Such chimeric viruses usually can move from cell to cell and even systemically to distant tissues (e.g., Nejidat *et al.*, 1991; De Jong and Ahlquist, 1992; Mise *et al.*, 1993; Deom *et al.*, 1994; Giesman-Cookmeyer *et al.*, 1995; Ryabov *et al.*, 1999). This also

evidences that the MPs share their functions among viruses.

Cucumber mosaic virus (CMV) and *brome mosaic virus* (BMV) are type members of the genera *Cucumovirus* and *Bromovirus*, respectively (Alquist, 1999; Roossinck, 1999). Both genera are characterized by their icosahedral virions and belong to the *Bromoviridae*, a family of tripartite, positive-strand RNA viruses, together with the genera *Alfamo*-, *Ilar*-, and *Oleaviruses* (Rybiki, 1995; Pringle, 1998). CMV is one of the most common plant viruses of substantial agricultural significance and has the broadest host range of any known virus, infecting more than 1000 species of plants, shrubs, and trees, and both monocots and dicots (Roossinck, 1999). On the other hand, BMV does not cause major economic losses and its host range is restricted to monocots including cereals and other grasses, and several dicots. However, this virus has been extensively used as an experimental model to study virus replication, gene expression, virus-host interactions, virion assembly, and general molecular biology.

CMV and BMV share many properties, including similar virion structure and genome organization. Their virions are nonenveloped particles of approximately 29 (CMV) or 26 (BMV) nm in diameter. They consist of RNA (about 18%) and protein, with 180 copies of a single CP per capsid being arranged with T=3 quasi-icosahedral symmetry. The capsid structure of CMV has been solved to high resolution by X-ray crystallography and found to be similar to the X-ray structure of *cowpea chlorotic mottle bromovirus* (CCMV; Wikoff *et al.*, 1997). Although the X-ray structure of BMV has not been determined, cryoelectron microscope reconstructions show that BMV and CCMV have extremely similar capsid structures (Wikoff *et al.*, 1997; Krol *et al.*, 1999).

The CMV virions contain the genomic RNAs, encapsidated separately, as well as at least two subgenomic RNAs. In addition, the satellite RNAs, when present, are encapsidated in the virion. On the other hand, the BMV virions contain the genomic RNAs, encapsidated separately as is the case of CMV, as well as one subgenomic RNA. The genomic RNAs of CMV and BMV are designated as RNAs 1, 2, and 3, by diminishing size (Palukaitis *et al.*, 1992; Ahlquist, 1994). All the RNAs have a cap structure at the 5' terminus. The 3' portion of all the RNAs is also highly conserved in virus-specific manner and can form a tRNA-like

structure that can be aminoacylated with tyrosine. RNAs 1 and 2, encoding 1a and 2a proteins, respectively, are involved in viral replication (French *et al.*, 1985; Nitta *et al.*, 1988; Hayes and Buck, 1990). CMV RNA 2 encodes a second protein, 2b, which is translated from a subgenomic RNA, RNA 4A, and plays a role in systemic spread of the virus and virulence determination, possibly by suppressing a host RNA silencing mechanism (S. Ding *et al.*, 1995; Brigneti *et al.*, 1998). However, this 2b gene is not found in BMV RNA 2. RNA 3 encodes two proteins dispensable for viral replication in protoplasts. The 5' proximal open reading frame (ORF) on RNA 3 is for the 3a protein, designated the MP of these viruses (Mise *et al.*, 1993; B. Ding *et al.*, 1995), and the 3' proximal ORF the CP. The CP is translated from a subgenomic RNA 4 that is synthesized from negative strand RNA 3 (Miller *et al.*, 1985; Bocard and Baulcombe, 1993) and coencapsidated in virion together with RNA 3.

The MPs of CMV and BMV have common biochemical and biological properties with those of other viruses. They have the binding activity for single-stranded nucleic acids without sequence specificity (Li and Palukaitis, 1996; Jansen *et al.*, 1998; Fujita *et al.*, 1998). Domains essential for the nucleic acid-binding property have been determined (Vaquero *et al.*, 1997; Fujita *et al.*, 1998). Besides, the MPs of both CMV and BMV have the affinity for plasmodesmata (Itaya *et al.*, 1997; Blackman *et al.*, 1998; Fujita *et al.*, 1998) and ability to form tubular structures protruding from the surface of protoplasts (Kasteel *et al.*, 1997; Canto and Palukaitis, 1999). The CMV MP is more characterized than the BMV MP. In addition to the properties described above, the CMV MP has been shown to complement a movement defective CMV mutants when expressed in a transgenic plant (Kaplan *et al.*, 1995; Cooper *et al.*, 1996), to increase the size exclusion limit of plasmodesmata (Vaquero *et al.*, 1994; B. Ding *et al.*, 1995), to move through plasmodesmata (B. Ding *et al.*, 1995; Itaya *et al.*, 1997) and to traffic RNA from cell to cell (B. Ding *et al.*, 1995). However, several other properties known in the MPs of some viruses, e.g., phosphorylation by cellular kinase (Watanabe *et al.*, 1992; Citovsky *et al.*, 1993) or interaction with the cytoskeleton or the cortical ER (reviewed in Lazarowitz and Beachy, 1999), have not been found for the MPs of both CMV and BMV.

Although the MP plays an essential role in cell-to-cell movement of both CMV and

BMV, all the other viral encoded proteins are also involved in movement of the viruses. For example, the CP of both CMV and BMV is indispensable for not only formation of the virion particles, but also viral cell-to-cell (Schmitz and Rao, 1996; Canto *et al.*, 1997) and long-distance movement (Suzuki *et al.*, 1991; Taliansky and Garcia-Arenal, 1995; Rao and Grantham, 1995, 1996). However, it has not been made clear how the viral proteins are involved in viral movement.

As described above, CMV has extremely wide host range which is due to its ability to move within plants. To solve the mechanism how CMV moves from cell to cell, chimeric viruses in which the MP genes of BMV was replaced with that of CMV were used in this study. This experimental approach can be expected to reveal what is veiled when the study is achieved only with CMV derivatives. In Chapter I, it will be mentioned that although the replacement of the BMV MP gene with that of CMV abolishes viral cell-to-cell movement, deletion of the 33 amino acids from the C-terminus of CMV MP enables such a chimeric virus to move from cell to cell. The reasons of these experimental results will be manifested in Chapters II and III; that is, the movement-incompetency of the chimeric virus with the intact CMV MP gene is due to a character of the CMV MP requiring the cognate CP to function in viral movement (Chapter II), and the movement-competency of the chimeric virus with the deleted CMV MP gene is owing to the loss of the CP requirement of CMV MP by the C-terminal deletion (Chapter III).

ABSTRACTS

CHAPTER I.

The MP gene of BMV was precisely replaced with that of 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 mutation in the CMV MP gene resulting in deletion of the C-terminal 33 amino acids of the MP. Directed mutagenesis of 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*.

CHAPTER II.

CMV and BMV show many similarities including the three dimensional structure of virions, genome organizations and requirement of the CP for cell-to-cell movement. I have shown that a chimeric BMV having the CMV MP gene instead of its own can not move from cell to cell in *C. quinoa*, a common permissive host for both BMV and CMV. Another chimeric BMV RNA 3 was constructed by replacing both MP and CP genes of BMV with those of CMV (B3(Cmp/Ccp)) and tested for infectivity of the chimeric virus containing it in *C. quinoa*, to determine whether the CMV CP has some functions required for the CMV MP-mediated cell-to-cell movement and to exhibit functional difference between CPs of BMV and CMV. Cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp) occurred and small local lesions were induced on the inoculated leaves. A frameshift mutation introduced in the CMV CP gene of B3(Cmp/Ccp) resulted in a lack of cell-to-cell movement of the chimeric virus. These results

indicate that the viral movement mediated by the CMV MP requires its cognate CP. Deletion of the N-terminal region in CMV CP which is not obligatory for CMV movement also abolished cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp). This may suggest some differences in cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp) and CMV. On the other hand, the sole replacement of BMV CP gene with that of CMV abolished viral cell-to-cell movement, suggesting a possibility that the viral movement mediated by the BMV MP may also require its cognate CP. Functional compatibility between MP and CP in viral cell-to-cell movement is discussed.

CHAPTER III.

Viral cell-to-cell movement mediated by the CMV MP requires its cognate CP. However, a variant of the chimeric BMV in which the CMV MP with deletion of the C-terminal 33 amino acids is encoded moves from cell to cell though it does not encode the CMV CP. The C-terminus was further analyzed with variants of the chimeric BMV or CMV in which translation of the CMV MP was prematurely terminated by an introduced stop codon at various position. It was revealed that although deletion of only three C-terminal amino acids abolished ability of the MP to mediate viral movement, deletion from 33 to 36 C-terminal amino acids enabled both the chimeric BMV and CMV to move from cell to cell. Infectivity tests of the CP-defective mutants of the chimeric BMV and CMV containing the CMV MP truncation of its C-terminal 33 amino acids revealed that the CMV MP was altered by the C-terminal deletion to the MP that functions in cell-to-cell movement by itself. I created four chimeric BMV RNA 3 in which the MP and/or CP genes were replaced with the intact and truncated CMV MP genes. While a chimeric virus containing the chimeric RNA 3 with two truncated CMV MP genes can infect plants, other three chimeric viruses containing the RNA 3 with at least one intact CMV MP gene remarkably lost their ability to move from cell to cell. These results suggests that the intact CMV MP is nonfunctional without the cognate CP. Besides, I found the CMV CP can be bound to the CMV MP *in vitro*. Taken together, the mechanism of CMV cell-to-cell movement is discussed.

CHAPTER I: 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.

INTRODUCTION

The MPs of diverse plant viruses share their function. The chimeric viruses created by replacing the MP gene of a virus with that of another can move not only from cell to cell but also systemically (e.g., Nejdat *et al.*, 1991; De Jong and Ahlquist, 1992; Mise *et al.*, 1993; Deom *et al.*, 1994; Giesman-Cookmeyer *et al.*, 1995; Ryabov *et al.*, 1999). Cell-to-cell movement of such chimeric viruses requires interactions between the host intercellular transport system, the replaced MP, and the other recipient-viral factors including viral genome and its encoding proteins. The movement-competency of those chimeric viruses has been tested at least in plants which are common host for the donor and recipient viruses of the MP gene. In such plants, all factors of the chimeric virus should be compatible with the host elements. The successful movement of chimeric viruses thus also reflects compatibility between the replaced MP gene and the other recipient-viral factors. Since the MP-replaced chimeric viruses can move even when the gene replacement was done between taxonomically divergent viruses (De Jong and Ahlquist, 1992; Giesman-Cookmeyer *et al.*, 1995; Ryabov *et al.*, 1999), functions of the MP are presumed to be generally shared among plant viruses.

The genomes of both CMV and BMV are divided among three positive-sense RNAs (Palukaitis *et al.*, 1992; Ahlquist 1994). Replication of the genomic RNA depends on proteins 1a and 2a encoded by RNAs 1 and 2, respectively, whereas two gene products encoded by dicistronic RNA 3 are dispensable for viral RNA replication. A nonstructural MP is encoded in the 5' proximal of RNA 3 and potentiates the cell-to-cell movement of the viruses. The CP is

encoded in the 3' proximal of RNA 3 and is translated from a subgenomic RNA 4, produced by partial transcription of the negative-sense RNA 3 replication intermediate.

As reviewed in the General Introduction section, MP of both CMV and BMV have been shown to share some biochemical and biological properties with other viruses. However, the mechanism of cell-to-cell movement of either CMV or BMV is not clearly understood. In this chapter, a chimeric BMV was created by replacement of the BMV MP gene with that of CMV to test the functional interchangeability of the two genes. While the CMV MP gene did not mediate cell-to-cell movement of BMV genome, movement-competent pseudorevertants were obtained during experiments. The factor responsible for the competency was determined in the CMV MP gene.

This chapter corresponds to a paper published (Nagano *et al.*, 1997).

RESULTS

The CMV MP gene can not substitute for the function of the BMV MP gene.

Before replacing the MP gene of BMV with that of CMV, pT7CKY3, a plasmid that contains a full-length RNA 3 cDNA of the CMV Y strain (CMV-Y) was first constructed. To assess if the MP gene contained in this cDNA has biological activity in viral cell-to-cell movement, CMV RNA 3 (C3) synthesized from this plasmid were inoculated onto tobacco plants together with CMV RNAs 1 and 2 transcripts. Yellow mosaic symptoms developed on uninoculated upper leaves at 6 days postinoculation (pi) and were indistinguishable from those on plants inoculated with the parent CMV-Y. Accumulation of CMV-RNA in the inoculated and uninoculated upper leaves was confirmed by tissue printing analysis (data not shown). These results indicate that the CMV MP gene contained in the RNA 3 cDNA has the biological activity. The nucleotide sequence of the RNA 3 clone (accession number D83958 in the

EMBL/Genebank DNA databases) was 99.0% identical to RNA 3 of CMV-Y previously published (Nitta *et al.*, 1988); the only difference detected in the CMV MP gene of pT7CKY3 was a $^{351}\text{T} \rightarrow \text{C}$ change, which resulted in a valine to alanine substitution 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 [B3(Cmp)] cDNA clone. Although the CMV MP gene has a nucleotide substitution resulted from the construction, the protein translated from the replaced MP gene has amino acid sequence identical to that from pT7CKY3. To test the ability of replication in infected cells, the chimeric virus containing B3(Cmp) were inoculated into protoplasts isolated from *Chenopodium quinoa* plants, a common host for CMV and BMV. As shown in Fig. I-1, panels A and B, the genomic RNAs of the chimeric virus accumulated in protoplasts, although the accumulation level of positive and negative strand B3(Cmp) normalized against the signals of RNA 2 were 12-16% and 5-8%, respectively, of the levels of wt BMV RNA3 (B3) that accumulated in BMV-infected protoplasts. Immunoblot analysis showed that the CMV MP accumulated in the protoplasts infected with the chimeric virus containing B3(Cmp) (Fig. I-1 C). The level of accumulation of the CMV MP was comparable to that of CMV-infected protoplasts, when compared using equal number of protoplasts (data not shown).

The chimeric virus containing B3(Cmp) was then tested for infectivity in *C. quinoa* plants. Control inoculations of wt BMV and wt CMV induced more than 100 chlorotic lesions and more than 50 necrotic lesions on the inoculated leaves, respectively. These lesions appeared at 3-4 days pi, continued to enlarge, and began to coalesce at 6-8 days pi. The inoculated leaves dropped from the base of their petioles at 12-16 days pi. In contrast, no symptoms were induced on plants inoculated with the chimeric virus containing B3(Cmp). No viral RNA was detected in these inoculated leaves by press-blot analysis at 3 days pi (Fig. I-2) and 14 days pi

(data not shown) whereas distributed viral RNA was detected in the leaves inoculated with wt BMV at 3 days pi (Fig. I-2). Since the chimeric virus successfully infected protoplasts, these results indicate the inability of the chimeric virus to move from cell to cell. The infectivity tests of the chimeric virus containing B3(Cmp) have been repeated with more than 150 leaves and the same results were obtained except in one experiment described below.

Spontaneous mutants of the chimeric virus that infect *C. quinoa* plants.

During the infectivity tests of the chimeric virus containing B3(Cmp) in *C. quinoa* plants, four chlorotic lesions appeared on every ten inoculated leaves at 4 days pi in one experiment. Each of the lesions were analyzed by reverse transcriptase PCR (RT-PCR) with the 3' primer specific for the CMV MP gene and the 5' primer specific for the 5'-end of B3. 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 suggest the possibility that mutants of the chimeric virus was generated. Further analysis of the 38 RT-PCR-negative lesions by Northern hybridization of RNA samples extracted from *C. quinoa* leaves back-inoculated with extracts of individual lesions using probes each of which is specific for the BMV-3' end, the BMV MP gene or the CMV MP gene led me a conclusion that the inoculum used in the experiment had been contaminated by the wt B3.

To analyze the remaining two putative spontaneous mutants of the chimeric virus, full-length RNA3 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 and the wt RNA3 of BMV could be cloned using primers specific for the 3' and 5' ends of B3, the cDNA clones were analyzed by digestion with restriction-enzymes. The two cDNA clones obtained from one of the lesions were digested successfully with *DraI* and *HpaI*, which is diagnostic for the CMV MP gene, but were not digested with *ClaI*, 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 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 both the chimeric and the wt B3 can exist together in a single local lesion.

Biological activity of the cB3R#1, cB3R#2, and cB3R#3 were individually tested in *C. quinoa* plants by coinoculating with BMV RNAs 1 and 2 transcripts. More than 100 chlorotic lesions similar to those of wt 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 the cB3R#1 and cB3R#2 contained mutation(s) that allow the the chimeric BMV to move from cell to cell.

The nucleotide sequences of the cB3R#1 and cB3R#2 were found to be identical and therefore I hereafter refer to either the cB3R#1 or cB3R#2 as B3(Cmp)-R. When compared with the sequence of originally constructed B3(Cmp), the sequence of B3(Cmp)-R differed at four positions (Fig. I-3). One of the mutations was a four-base deletion of ⁸³⁰GAGT⁸³³ (equivalent to ⁸²⁷AGTG⁸³⁰, ⁸²⁸GTGA⁸³¹, or ⁸²⁹TGAG⁸³² 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 CMV MP and that three out-of-frame amino acids were added to the C-terminus of the truncated CMV MP. Three other mutations were present in B3(Cmp)-R: 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.,

$^{1193}\text{ACT}^{1195} \rightarrow \text{ATT}$) and the second was silent (i. e., $^{1610}\text{ACT}^{1612} \rightarrow \text{ACC}$).

The C-terminal truncation of CMV MP is responsible for cell-to-cell movement of the spontaneous mutant.

To determine which mutation(s) in B3(Cmp)-R were responsible for infectivity of the chimeric virus, the *HpaI* fragment containing the deletion was exchanged between B3(Cmp)-R and B3(Cmp). The resulting chimeric RNA 3, B3(Cmp) with the four-base deletion and B3(Cmp)-R without the four-base deletion was designated as B3(CmpD4b) and B3(Cmp)-R(-D4), respectively. *In vitro* transcripts of these chimeric RNA 3 derivatives were inoculated separately with BMV RNAs 1 and 2 transcripts onto *C. quinoa* plants. Chlorotic lesions similar to those of the wt BMV infection were induced by the chimeric virus containing B3(CmpD4b), but not by that containing B3(Cmp)-R(-D4b). Time-course press-blot analyses revealed that diffusion of viral RNA signals in the leaves showing such lesions was similar to that in the leaves inoculated with the chimeric virus containing B3(Cmp)-R (Fig. I-2). The number of signals detected from the leaves infected with these chimeric viruses were fewer than those from wt BMV-infected leaves, though the number of induced chlorotic lesions was indistinguishable between the chimeric viruses and wt BMV. Immunoblot analysis using anti-CMV MP antiserum showed that a protein with a molecular weight smaller than the intact CMV MP accumulated in leaves infected with the chimeric virus containing B3(CmpD4b) (Fig. I-4). On the other hand, no viral RNA was detected at 3 days pi (Fig. I-2) and 14 days pi (data not shown) from the leaves inoculated with the chimeric virus containing B3(Cmp)-R(-D4b). These results indicated that the four-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 four-base deletion in the chimeric RNA3 on viral RNA- and MP-accumulation, *C. quinoa* protoplasts were inoculated with the chimeric virus containing B3(Cmp) or B3(CmpD4b) and analyzed by Northern hybridization and immunoblot methods. As shown in Figs. I-1A and I-1B, accumulation levels of both positive and negative strands of viral RNAs were comparable between protoplasts inoculated with these chimeric viruses. Results from immunoblot analysis (Fig. I-1C) showed that the accumulation level of the truncated CMV MP from B3(CmpD4b) was also comparable to that of the intact CMV MP from B3(Cmp). These results suggested that accumulation levels of neither viral RNA nor CMV MP were affected by the four-base deletion and that the intact and truncated CMV MPs differ only for functions involved in intercellular transport of the viral genome.

To confirm that the truncation of CMV MP, rather than structural change of the chimeric RNA3, was essential for cell-to-cell movement of the chimeric virus, a stop codon was introduced into the MP gene of B3(Cmp) so as to encode the CMV MP lacking the C-terminal 33 amino acids. Transcripts of the resulting chimeric RNA3 [B3(CmpDC33)] clone were coinoculated with BMV RNAs 1 and 2 transcripts onto *C. quinoa* plants. The inoculated leaves showed chlorotic lesions which were similar to those induced by infection with other movement-competent variants of the chimeric virus (data not shown). Time-course press-blot analysis showed that cell-to-cell movement of the chimeric virus containing B3(CmpDC33) was comparable to that of the other movement competent variants (Fig. I-2). Thus it was strongly suggested that the deletion of the C-terminal amino acids from CMV MP was responsible for the cell-to-cell movement of the chimeric virus rather than structural change of the chimeric RNA3 caused by the four-base deletion.

The C-terminal deletion in CMV.

A CMV RNA 3 derivative with the four-base deletion in the MP gene [C3(mpD4b)] was created. *In vitro* transcripts of C3(mpD4b) were inoculated onto *C. quinoa* plants together with

CMV RNAs 1 and 2 transcripts. Necrotic lesions similar in number and morphology to those of wt CMV infection were induced in the inoculated leaves. The lesions appeared on the inoculated leaves at 5-6 days pi, while lesions by wt CMV infection appeared at 3-4 days pi. The expanding rate of lesions was slower for the mutant than the wt CMV; the diameter of lesions induced by the CMV variant containing C3(mpD4b) reached 3 mm at 14 days pi while those induced by wt CMV reached a comparable size at 6 days pi. Press-blot analyses at 1, 3 and 14 days pi of the leaves inoculated with either wt C3 or C3(mpD4b) together with CMV RNAs 1 and 2 showed that RNA signals of each virus diffused with time (Fig. I-5), while the rate of the signal-diffusion of the CMV mutant was slower than that of wt CMV. Since the viral RNA accumulation level of the CMV mutant was similar to that of wt CMV in *C. quinoa* protoplasts (data not shown), the results indicated that the CMV mutant moved from cell to cell less efficiently than wt CMV. Immunoblot analysis showed that the truncated CMV MP accumulated in leaves inoculated with the CMV variant containing C3(mpD4b) (Fig. I-4). Similar results were obtained when another derivative of CMV RNA 3 in which the C-terminal deletion of the CMV MP was induced by a stop codon [C3(mpDC33)] was used instead of C3(mpD4b) (data not shown). These results indicated that the C-terminal deletion was preserved in the CMV mutant. Furthermore, it appears that the C-terminal region of the CMV MP 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 the CMV variant was reduced when compared with wt CMV.

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 (De Jong and Ahlquist, 1992; Giesman-Cookmeyer *et al.*, 1995; Ryabov *et al.*, 1999). In this chapter, I 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 *Bromoviridae*) and the MPs of BMV and CMV show significant amino acid sequence homology (approx. 34%; Savithri and Murthy, 1983; Melcher, 1990). The homology between the MPs of BMV-KU2 and CMV-Y strains used in my 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 (Davies and Symons, 1988). In spite of these similarities, a chimeric virus 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. In contrast, mutants of the chimeric virus lacking the C-terminal 33 amino acids of the CMV MP were viable in plants and did move from cell to cell (Fig. I-2). I noted, however, that the numbers of press-blot signals in the leaves infected with such variants of the chimeric viruses were fewer than those in the wt BMV-infected leaves (Fig. I-2), although the number of lesions induced on the inoculated leaves were comparable between the wt BMV and the viable chimeric viruses. This may be reflective of the low RNA accumulation level of the chimeric virus in some lesions since the accumulation of the chimeric RNA 3 and the subgenomic RNA 4 in protoplasts was less than that of the corresponding RNAs of the wt BMV (Figs. I-1A and I-1B).

My results differed from those of Kaplan *et al.* (1995) 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 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 (1995)

reported that the CMV MP in CMV-infected cells accumulated in different manner from that 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. 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 the chimeric virus that contains with either the intact or truncated CMV MP gene infected *C. quinoa* protoplasts similarly (Fig. I-1), the viability of the chimeric BMV in plants most likely reflect the degree of compatibility between the CMV MP and the BMV genome. Cooper *et al.* (1996) 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 (TMV). They addressed the possibility that the CMV MP may not interact with the TMV genome in planta. This finding and my results strongly suggest that CMV MP has specificity for its viral genome. Furthermore, my results suggest that it is the C-terminal 33 amino acids 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 (Allison *et al.*, 1990; Suzuki *et al.*, 1991; Traynor *et al.*, 1991; Bocard and Baulcombe, 1993; Gal-On *et al.*, 1994; S Ding *et al.*, 1995; Rao and Grantham, 1995). 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* (Li and Palukaitis, 1996). 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 is involved in the specificity. Alternatively, the CMV MP might require some functions of other viral component to establish cell-to-cell movement of viral genome, while the C-terminal deletion might change such requirement.

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 (Figs. I-2 and I-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 (Kaplan *et al.*, 1995). However, the removal of the C-terminal region from the CMV MP decreased the efficiency of CMV movement (Fig. I-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 (98.0-99.3%) among the members of CMV belonging to subgroup I (Palukaitis *et al.*, 1992) while, compared with the MPs of other members of cucumovirus, the C-terminal region exhibits variability (data not shown). This observation supports my suggestion that the C-terminal 33 amino acids of the CMV MP are dispensable for viral cell-to-cell movement but involved in the specificity for the CMV genome.

MATERIALS AND METHODS

Plants

C. quinoa and tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) plants were grown in a commercially available soil mixtures in a plant growth room at $24\pm 1^{\circ}\text{C}$ 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 wt BMV, respectively, from which infectious transcripts can be produced using T7 RNA polymerase (Mori *et al.*, 1991; Mise *et al.*, 1992; Mise *et al.*, 1994). *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, Japan Tobacco Incorporated, Japan, and M. Suzuki, The University of Tokyo) contain the full-length cDNAs of CMV-Y RNAs 1 and 2, respectively, and infectious viral RNA transcripts can be transcribed *in vitro* by T7 RNA polymerase from these plasmids (Suzuki *et al.*, 1991). A full-length RNA 3 cDNA clone of the wt 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., Japan). The full-length RNA 3 cDNA fragment was inserted into pUCT7 (Mori *et al.*, 1991) to 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 B3(Cmp), 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 modified pBB3 (Mori *et al.*, 1991) 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 pCMP 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 (Mori *et al.*, 1993). The 0.6-kb *Bgl*III-*Eco*RI fragment of the plasmid was replaced with the corresponding 0.9-kb *Bgl*III-*Eco*RI fragment of pBTF3W to generate pB3CY3a. Then the 2.1-kb *Sna*BI-*Eco*RI fragment of pB3CY3a was inserted into pUCT7 (Mori *et al.*, 1991) to attach the T7 promotor sequence at the 5' end of the RNA3 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 RT-PCR. Total RNA was extracted from leaf-disks (2 mm x 2 mm) containing a single local lesion produced on the inoculated leaves of *C. quinoa* at 6 days pi as described (Kaido *et al.*, 1995). 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 using the 3' primer, 5'-CACGAATTCCCTGGTCTCTTTTAGAGAT-3', which is complementary to the 3' terminal 17 nucleotides of the B3 (*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'-CACTGCAGTAATACGACTCACTATAGTAAAATACCAACTAATTC-3', which corresponds to the 5' terminal 19 nucleotides of B3 (*italics*), phage T7 promotor sequence (**bold**), and *Pst*I site (underlined). The reaction was carried out with ExTaq DNA polymerase (Takara Shuzo) under conditions previously described (Fujita *et al.*, 1996). The amplified full-length RNA3 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 RNA3 cDNA clone (designated pB3C3aR). pB3C3aRR4 was constructed by replacing the *HpaI* fragment in pB3C3aR with the corresponding fragment from pT7B3CY3a. A plasmid, pB3C3a247T, containing B3 (CmpDC33) cDNA, was constructed by PCR-based *in vitro* mutagenesis (Ito *et al.*, 1991) using an oligonucleotide 5'-CCTCGGACTAACTGCGCGC-3'. Absence of undesired mutations was confirmed by nucleotide sequencing. A plasmid, pC3-247T, containing C3(mpDC33) cDNA was created by replacement the *HpaI* fragment of pT7CKY3 with that of pB3C3a247T.

Inoculation of plants and protoplasts

Capped full-length transcripts were synthesized *in vitro* using T7 RNA polymerase (Takara Shuzo) as described (Kroner and Ahlquist, 1992). RNA3 variants of BMV and CMV were always coinoculated with the wt RNAs 1 and 2 of BMV and CMV, respectively. Control inoculations always contained all three transcripts of the wt BMV or CMV RNAs. Crude viral extracts were prepared from individual leaf-tissue sections (2 mm x 2 mm) of *C. quinoa* by grinding with a mortar and pestle in 15 µl of 0.1 M sodium-phosphate buffer (pH7.0). 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 (de Varennes *et al.*, 1984) with some alterations. The lower epidermis of fully expanded leaves of 5-week-old plants were 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 CaCl₂ (pH 5.6) for 3 h at 25 °C. Freshly prepared protoplasts were separated from tissue debris by filtration through 4 layers of gauze. Approximately 3 x 10⁵ protoplasts were inoculated by means of polyethylene glycol with a mixture of transcripts (Kroner and Ahlquist, 1992). Protoplasts were incubated at 25° C for 24

h (Okuno and Furusawa, 1977).

RNA analysis

Total RNA from infected protoplasts or from infected leaves was extracted and hybridizations were performed as described (Kroner and Ahlquist, 1992; Kaido *et al.*, 1995). Positive- and negative-strand BMV genomic RNAs were detected with ³²P-labeled transcripts from *Hind*III-linearized pBSPL10 and pBSMI10, respectively (Kaido *et al.*, 1995). The BMV MP gene was detected with ³²P-labeled transcripts from *Eco*RI-linearized pBSL3DN4, which contains cDNA sequence complementary to the BMV MP gene encoded in pBTF3 (Mori *et al.*, 1991) downstream of SP6 promotor sequence. The CMV MP gene was detected with ³²P-labeled T7 RNA polymerase transcripts from *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 (Mansky *et al.*, 1990) of *C. quinoa* leaves were prepared as described (Mise *et al.*, 1993) using a flat aluminum board to apply pressure at $1.7 \times 10^2 \text{ kg/m}^2$ for 1 h. BMV RNAs and CMV RNAs were detected with ³⁵S-labeled transcripts from *Hind*III-linearized pBSPL10 and *Xba*I-linearized pCY200T (Kaido *et al.*, 1995), respectively. Tissue-printing of tobacco leaves was performed as described (Mise *et al.*, 1993) and CMV RNAs were detected with ³²P-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 using an oligonucleotide, 5'-GCTAAAGACCGTTAACC-ACCTGCGGTC-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 (Okuno *et al.*, 1993) and from 1.5×10^5 protoplasts (Mori *et al.*, 1993). The proteins were separated by electrophoresis on 12.5% polyacrylamide gels containing 0.1% SDS (i.e. by SDS-PAGE; Laemmli, 1970). Immunoblot analysis was carried out as described (Towbin *et al.*, 1979) 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.

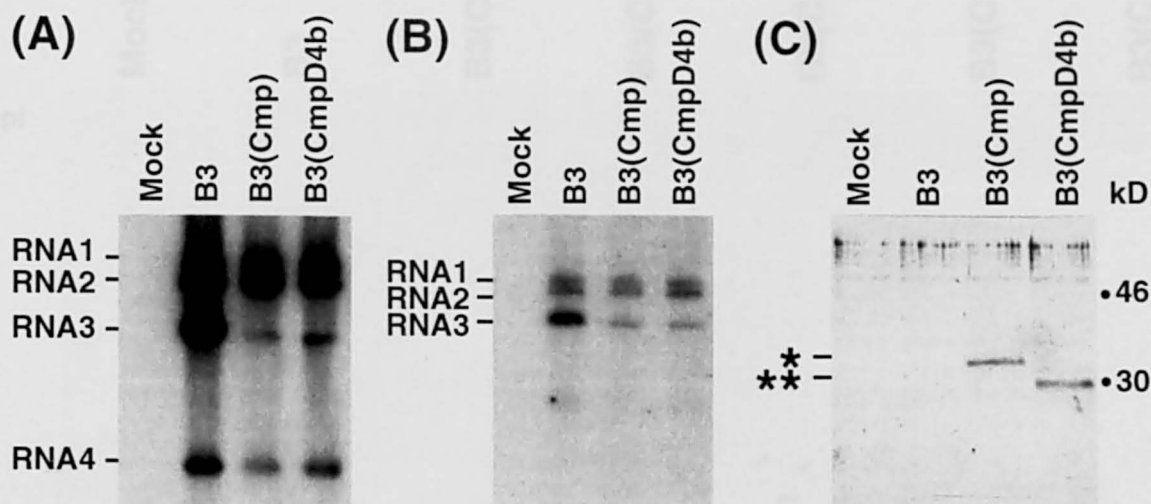


Fig. I-1. Northern hybridization and immunoblot analysis of viral RNAs and CMV MP in *C. quinoa* protoplasts. Protoplasts (3×10^5) were inoculated with *in vitro* transcripts from plasmid pBTF3W (B3), pT7B3CY3a [B3(Cmp)], or pB3C3aD4 [B3(CmpD4b)] together with *in vitro* transcripts from BMV RNAs 1 and 2 cDNA clones. Mock, inoculated with buffer only. Detection of positive- and negative-strand viral RNAs by Northern hybridization with riboprobes are shown in panels A and B, respectively. Total RNA was extracted at 24 h pi. Panel C shows immunodetection of the CMV MP using rabbit anti-CMV-MP antiserum. Total proteins were extracted at 24 h pi and fractionated by SDS-PAGE. An asterisk and a double-asterisk denote the positions of the full-length CMV MP and the truncated CMV MP lacking the C-terminal 33 amino acids, respectively. Numbers at the right of the panel indicate the size of molecular weight markers. kD, kilodaltons.

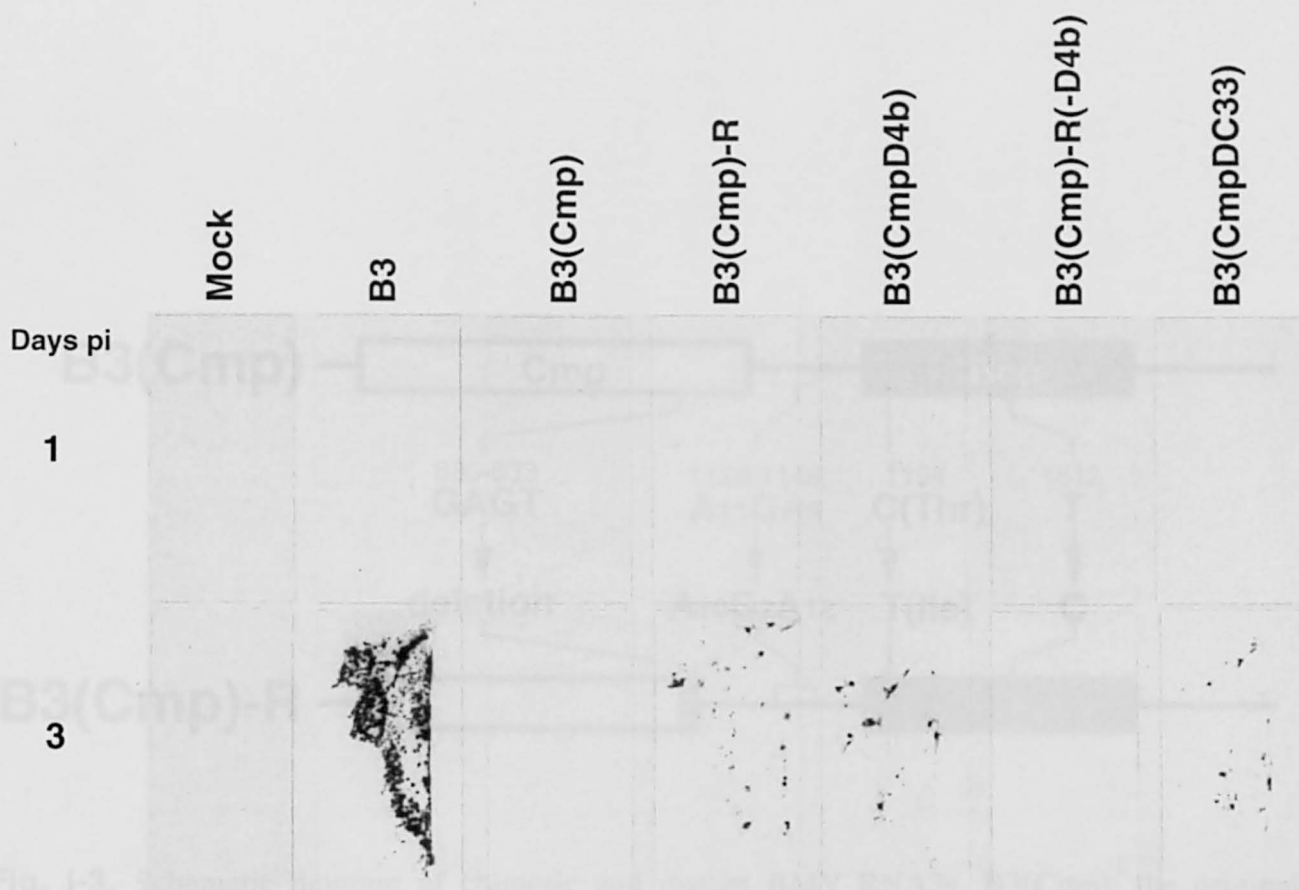


Fig. I-2. Press-blot analysis of viral RNA accumulation and distribution in inoculated leaves of *C. quinoa* at 1 and 3 days pi. *C. quinoa* leaves were inoculated with *in vitro* transcripts from plasmid pBTF3W (B3), pT7B3CY3a [B3(Cmp)], pB3C3aR [B3(Cmp)-R], pB3C3aD4 [B3(CmpD4b)], pB3C3aRR4 [B3(Cmp)-R(-D4b)], or pB3C3a247T [B3(CmpDC33)] together with *in vitro* transcripts from BMV RNAs 1 and 2 cDNA clones. Press-blots of the half-leaves whose abaxial epidermis were peeled off were analyzed by hybridization using a ^{35}S -labeled riboprobe complementary to the positive-strand conserved 3' terminal sequence of all BMV RNAs.

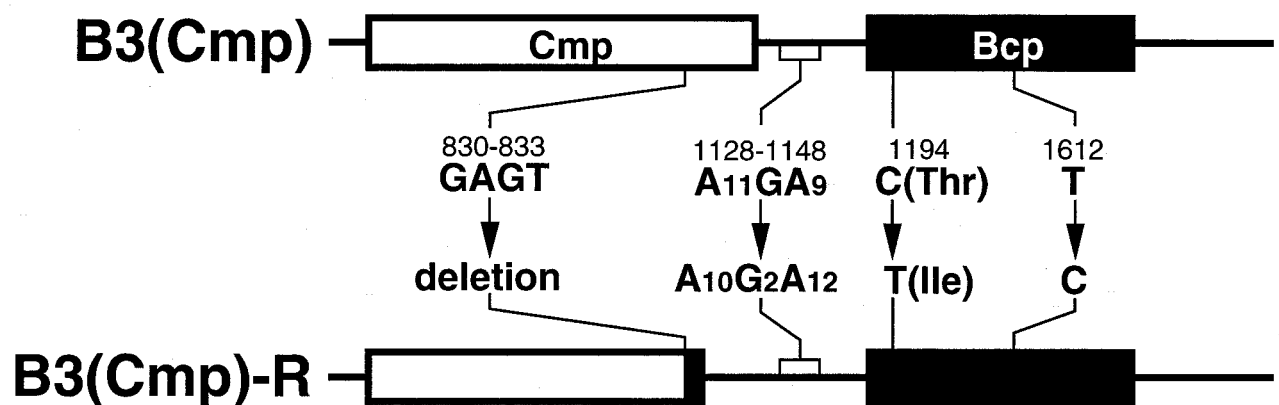


Fig. I-3. Schematic diagram of chimeric and mutant BMV RNA3s. B3(Cmp), the originally constructed chimeric RNA3; B3(Cmp)-R, the chimeric RNA3 cloned from a local lesion on *C. quinoa*. Differences in nucleotide sequence between B3(Cmp) and B3(Cmp)-R are denoted with position numbers. The MP gene of CMV (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 black box within the CMV mp gene of B3(Cmp)-R indicates the three changed amino acid residues resulting from a frameshift mutation.

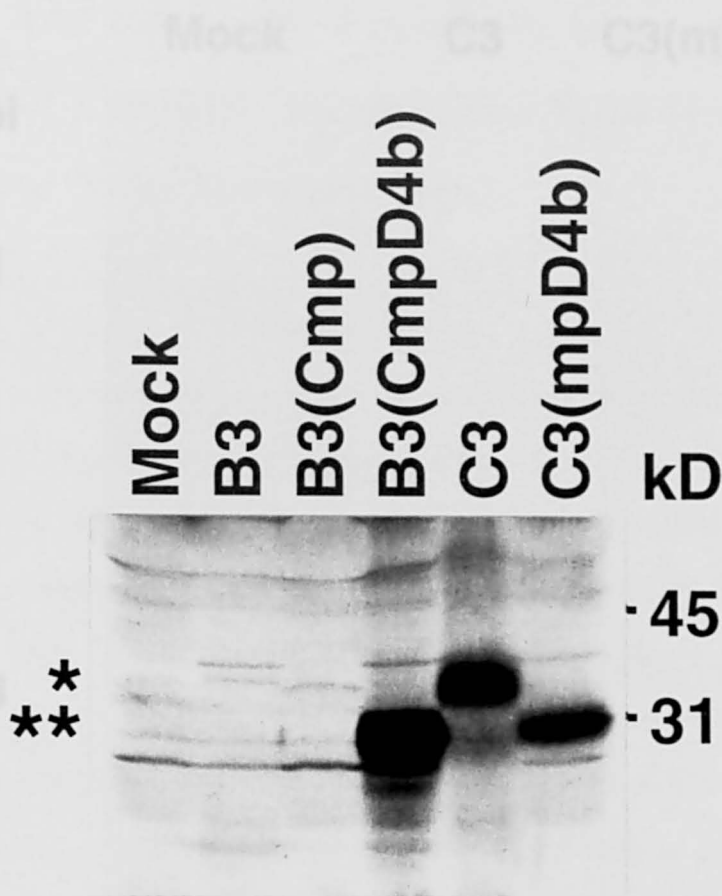


Fig. I-4. Immunodetection of CMV MP in inoculated leaves of *C. quinoa*. Leaves were inoculated with *in vitro* transcripts of the wild-type BMV RNA3 (B3), chimeric RNA3 with the intact CMV-MP gene (B3(Cmp), or chimeric RNA3 with the four-base deletion in the CMV-MP gene [B3(CmpD4b)] together with *in vitro* transcripts from BMV RNAs 1 and 2 cDNA clones. Also shown are results with *in vitro* transcripts of the wild-type CMV RNA3 (C3) and mutant CMV RNA3 with the four-base deletion [C3(mpD4b)] together with *in vitro* transcripts of CMV RNAs 1 and 2 cDNA clones. Mock, inoculated with buffer only. Proteins extracted from the inoculated leaves (fresh weight 0.1 g) were fractionated by SDS-PAGE. An asterisk and a double-asterisk denote the positions of the intact CMV MP and the CMV MP lacking the C-terminal 33 amino acids, respectively. Numbers at the right of the panel indicate the size of molecular weight markers.

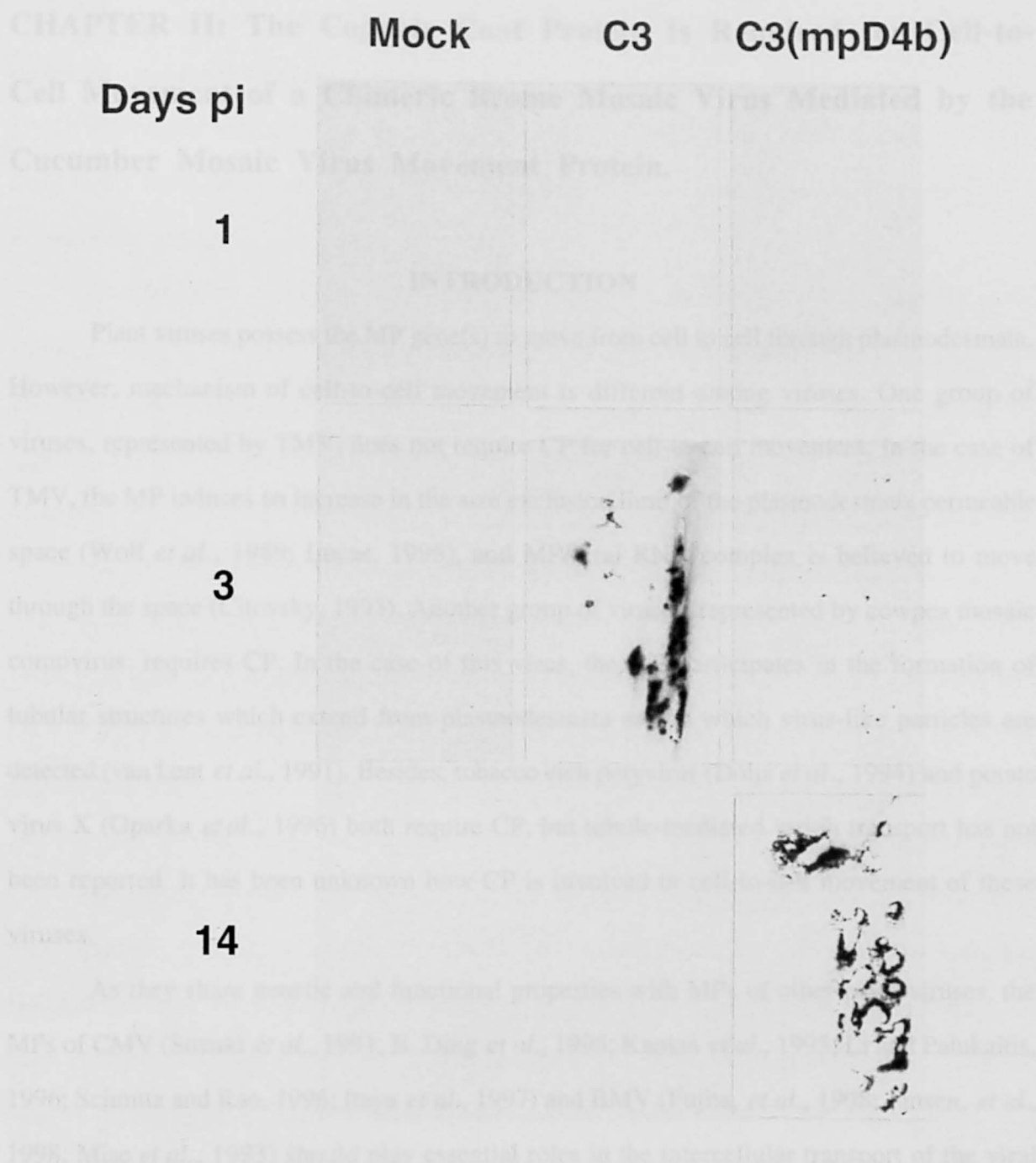


Fig. I-5. Press-blot analysis of viral RNA accumulation and distribution in the inoculated leaves of *C. quinoa* at 1, 3 and 14 days pi. *C. quinoa* leaves were inoculated with *in vitro* transcripts from plasmid pT7CKY3 (C3) or pC3-D4 [C3(mpD4b)] together with *in vitro* transcripts from CMV RNAs 1 and 2 cDNA clones. Press-blots of the half-leaves whose abaxial epidermis was peeled off were analyzed by hybridization using a ³⁵S-labeled riboprobe specific for the positive-strand conserved 3' terminal sequence of CMV RNAs.

CHAPTER II: The Cognate Coat Protein Is Required for Cell-to-Cell Movement of a Chimeric Brome Mosaic Virus Mediated by the Cucumber Mosaic Virus Movement Protein.

INTRODUCTION

Plant viruses possess the MP gene(s) to move from cell to cell through plasmodesmata. However, mechanism of cell-to-cell movement is different among viruses. One group of viruses, represented by TMV, does not require CP for cell-to-cell movement. In the case of TMV, the MP induces an increase in the size exclusion limit of the plasmodesmata permeable space (Wolf *et al.*, 1989; Lucas, 1995), and MP/viral RNA complex is believed to move through the space (Citovsky, 1993). Another group of viruses, represented by cowpea mosaic comovirus, requires CP. In the case of this virus, the MP participates in the formation of tubular structures which extend from plasmodesmata and in which virus-like particles are detected (van Lent *et al.*, 1991). Besides, tobacco etch potyvirus (Dolja *et al.*, 1994) and potato virus X (Oparka *et al.*, 1996) both require CP, but tubule-mediated virion transport has not been reported. It has been unknown how CP is involved in cell-to-cell movement of these viruses.

As they share genetic and functional properties with MPs of other plant viruses, the MPs of CMV (Suzuki *et al.*, 1991; B. Ding *et al.*, 1995; Kaplan *et al.*, 1995; Li and Palukaitis, 1996; Schmitz and Rao, 1996; Itaya *et al.*, 1997) and BMV (Fujita, *et al.*, 1998; Jansen, *et al.*, 1998; Mise *et al.*, 1993) should play essential roles in the intercellular transport of the viral genomes. In addition to the MP, the CP is also indispensable for the movement of either CMV (Canto *et al.*, 1997) or BMV (Schmitz and Rao, 1996). However, there are several instances suggesting that the required forms of CP in virus movement are distinct between the viruses. BMV mutants unable to form virions fail to move from cell to cell (Rao, 1997; Rao and Grantham, 1995; Schmitz and Rao, 1996), and wt BMV induces tubular structures containing virion-like particles on the surface of the infected protoplasts (Kasteel *et al.*, 1997). Thus, BMV

is most likely to move from cell to cell as a virion form. On the other hand, CMV mutants incapable of virion formation can move from cell to cell (Kaplan *et al.*, 1998; Schmitz and Rao, 1998), and tubular structures have not been found between cells in wt CMV-infected plants (B. Ding *et al.*, 1995; Blackman *et al.*, 1998). Therefore, CMV has been considered to move as a non-virion form.

The MP gene of BMV has been precisely replaced with that of CMV-Y (Nagano *et al.*, 1997; Chapter I). The chimeric BMV containing B3(Cmp) is not infectious in *C. quinoa* since the intact CMV MP cannot promote efficient cell-to-cell movement of the chimeric BMV genome. However, deletion of the C-terminal 33 amino acids of CMV MP enables the mutant chimeric virus to move from cell to cell in the plant. It is generally accepted that cell-to-cell movement of plant viruses occurs as a result of successful interactions between the MP, the other viral components and host factors (Carrington *et al.*, 1996). Since *C. quinoa* is a permissive host for efficient cell-to-cell movement of both BMV and CMV, it is most likely that each of the viral encoded proteins successfully interacts with the host counterparts. The CMV MP has binding activity to single stranded RNA *in vitro* in a sequence non-specific manner (Li and Palukaitis, 1996). Together with the results described above, it is suggested that the intact CMV MP may be unable to interact successfully with BMV-encoded proteins, but removal of the C-terminal 33 amino acids from the CMV MP may make such interaction(s) successful and facilitate viral cell-to-cell movement (Nagano *et al.*, 1997). On the other hand, the CP gene has been exchanged between BMV and CMV (Osman *et al.*, 1998). The chimeric CMV with the BMV CP gene failed to infect *C. quinoa* plants, suggesting that the BMV CP gene is not able to successfully substitute for the functions of the CMV CP. Taken together, these results raise the possibility that the cognate CP may be required for the establishment of cell-to-cell movement mediated by the CMV MP.

In this chapter, I show a chimeric BMV in which both the MP and CP genes were precisely replaced with the corresponding genes of CMV-Y moves from cell to cell in *C. quinoa*. This suggests that the cognate CP is required for cell-to-cell movement mediated by the CMV MP. Functional compatibility between MP and CP in viral cell-to-cell movement is

discussed.

This chapter corresponds to a paper published (Nagano *et al.*, 1999).

RESULTS

RNA 3 derivatives.

RNA 3 derivatives used in this study and their abbreviations were summarized in Fig. II-1. Each of the derivatives was inoculated together with the appropriate parental RNAs 1 and 2 as a mixture of *in vitro* transcripts. The inoculum and its progeny were named after the character of the RNA 3, e.g. BMV RNAs 1 and 2 and B3(Cmp/Ccp) as a chimeric virus containing B3(Cmp/Ccp). Either MP or CP gene of BMV was precisely replaced with the corresponding gene of CMV. However, the replaced CMV MP gene had one translationally silent mutation at the codon encoding the last amino acid residue of the CMV MP, CUU → CUC, and the replacement of CP gene resulted in a single nucleotide substitution, U → G, at the 3' noncoding region next to the termination codon of the CMV CP gene.

Establishment of cell-to-cell movement of the chimeric BMV by replacing both MP and CP genes with the corresponding genes of CMV.

In Chapter I, I demonstrated that a chimeric virus containing B3(Cmp) failed to move from cell to cell in *C. quinoa* plants (Nagano *et al.*, 1997). To verify the possibility that the cognate CP (CMV CP), instead of BMV CP, is required for establishment of cell-to-cell movement mediated by the CMV MP, another chimeric virus containing B3(Cmp/Ccp) was tested for its infectivity on *C. quinoa* plants. In this plant, wt BMV induces more than 200 chlorotic lesions whereas wt CMV induces 20 to 50 necrotic lesions in the inoculated leaves (Nagano *et al.*, 1997). No visible symptoms were observed in control plants inoculated with the chimeric virus containing B3(Cmp). By contrast, local lesions appeared at 6-7 days pi on the leaves inoculated with the chimeric virus containing B3(Cmp/Ccp). However, the number of local lesions (10 to 50) induced by the chimeric virus containing B3(Cmp/Ccp) was much fewer than that by wt BMV (Fig. II-2). These lesions did not enlarge with time and remained pin-point size (0.5-1.0 mm in diameter). These inoculated leaves were analyzed by press-blot

hybridization. Although 10 to 50 visible lesions were consistently observed, only several dispersed signals were detected in the press-blot of leaves inoculated with the chimeric virus containing B3(Cmp/Ccp) at 7 days pi (Fig. II-3). Such signals, however, were not detected at 1 day pi (data not shown). Signals at 14 days pi were weaker than at 7 days pi (data not shown). No signal was detected in leaves inoculated with the chimeric virus containing B3(Cmp) at any time tested (Fig. II-3). These results indicate that the chimeric virus containing B3(Cmp/Ccp) has the ability to move from cell to cell.

Effects of mutation in the CP gene of the MP/CP chimera.

To verify that cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp) was dependent on the CMV CP itself, a CP-defective frameshift mutation was introduced in the CMV CP gene of B3(Cmp/Ccp) (Fig. II-1 B). The CP-defective mutant of the chimeric virus containing B3(Cmp/Ccp) did not induce any symptom on *C. quinoa* leaves during the experimental period (14 days). No viral RNA was detected from the inoculated leaves by press blot analysis (data not shown). The corresponding frameshift mutation in CMV abolished viral cell-to-cell movement in *C. quinoa* leaves (data not shown), supporting the requirement of functional CP for CMV movement. These results indicated that the CP-defective frameshift mutant of the chimeric virus containing B3(Cmp/Ccp) was not able to move from cell to cell to detectable levels and further that the intact CP was essential to establish viral cell-to-cell movement mediated by the CMV MP.

The results obtained above raise a question whether the movement of the chimeric virus containing B3(Cmp/Ccp) occurred by the same mechanism as that of wt CMV. If so, a mutant of the chimeric virus containing B3(Cmp/Ccp) encoding a truncated CMV CP, in which the N-terminal proximal region dispensable for CMV movement (Kaplan *et al.*, 1998; Schmitz and Rao, 1998; Suzuki *et al.*, 1991) was deleted, should move from cell to cell. To test this possibility, a del-SN-mutant of the chimeric virus containing B3(Cmp/Ccp) was constructed (Fig. II-1B) and tested for its infectivity in *C. quinoa* plants. The del-SN-mutant of the chimeric virus containing B3(Cmp/Ccp) induced no symptom during the experimental period (14 days), and viral RNA was not detected by press blot analysis at any time tested (1, 7, 14 days pi; data

not shown). Control inoculation of a CMV mutant with the corresponding deletion in the CP gene (del-SN-CMV) induced local lesions in the inoculated leaves. These lesions appeared at 6 days pi, 2 days later than for wt CMV, and enlarged during the experimental period. Viral RNAs were detected by press blot analysis in the inoculated leaves (data not shown). These results may suggest some differences between cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp) and CMV.

Failure of the CP gene of CMV-Y to functionally substitute for that of BMV in cell-to-cell movement.

CMV is classified into two subgroups, I and II, on the basis of serological relationships, peptide mapping of the viral CP, and nucleic acid hybridization analysis (Palukaitis *et al.*, 1992). It has been shown that the CP gene of CMV does not substitute functions of that of BMV using the CP gene of the CMV Kin strain (CMV-Kin), which belongs to the subgroup II (Osman *et al.*, 1998). I tested the CP gene of CMV-Y, belonging to the subgroup I, for the ability to substitute functions of the BMV CP gene. A chimeric BMV containing B3(Ccp), in which the CP gene alone was precisely replaced with that of CMV-Y, induced no visible symptom on *C. quinoa* plants during the experimental period (14 days; Fig. II-2). The possibility of a symptomless infection was examined by press-blot hybridization analysis of the inoculated leaves. No signals were detected from these leaves at any time tested (Fig. II-3 and not shown). These results indicate that the CMV-Y CP gene, as well as that of the CMV-Kin (Osman *et al.*, 1998), did not substitute for the functions of the BMV CP gene in viral cell-to-cell movement mediated by the BMV MP.

Comparison of the chimeric viruses in protoplast infection.

Viral RNA accumulations in infected *C. quinoa* protoplasts were compared among the chimeric viruses by Northern blot hybridization. The relative levels of RNA accumulation was estimated on the basis of data from Fig. II-4 and two other experiments. The sum accumulation of all four positive-strand viral RNAs decreased to 85%, 10%, and 10% of the level of wt BMV in protoplasts inoculated with the chimeric viruses containing B3(Cmp), B3(Ccp) and B3(Cmp/Ccp), respectively (Fig. II-4 A). In protoplasts inoculated with the chimeric virus

containing B3(Cmp), the accumulation level of RNA 3 compared to RNAs 1 and 2 was reduced to 13% of the level of the wt BMV as previously described (Nagano *et al.*, 1997). In protoplasts inoculated with the chimeric virus containing either B3(Ccp) or B3(Cmp/Ccp), the accumulation levels of RNA 4 as well as those of RNA 3 compared to RNAs 1 and 2 decreased. The relative levels of B3(Ccp) and B3(Cmp/Ccp) were 21% and 16%, respectively, whereas those of RNA 4 derived from the respective chimeric RNA 3s were 40% and 34%, respectively. On the other hand, accumulation of negative-strand viral RNAs was also compared (Fig. II-4 B). Although negative-strand RNAs 1 and 2 of the chimeric viruses accumulated to the level comparable to those of wt BMV, accumulation of negative-strand RNA 3s decreased compared to that of wt B3. The levels of the chimeric negative-strand RNA 3 in protoplasts inoculated with the chimeric virus containing B3(Cmp), B3(Ccp), and B3(Cmp/Ccp) were 47, 20, and 10% of that of wt B3, respectively. These results suggest that the CMV CP gene affected the accumulation of the chimeric viral RNA. However, there was, no correlation between the levels of viral RNA accumulation in protoplasts and the ability in cell-to-cell movement of the chimeric viruses.

Expression of CMV MP and CP in the infected protoplasts was investigated by immunodetection (Figs. II-5 A and B). Using antiserum specific for CMV MP, an infection-specific band was detected from protoplasts inoculated with either the MP- or the chimeric virus containing B3(Cmp/Ccp) (Fig. II-5 A). The accumulation level of the CMV MP of either chimeric virus was not less than for wt CMV. Immunodetection using antiserum specific for CMV virions showed that the accumulation levels of CMV CP were similar in either the CP- or the chimeric virus containing B3(Cmp/Ccp) and were comparable to that of wt CMV (Fig. II-5 B). No positive correlation was observed between the accumulation levels of either CMV MP or CP and the establishment of viral cell-to-cell movement. Immunoblot analyses of these infected protoplasts showed that BMV CP of the chimeric virus containing B3(Cmp) and BMV MP of the chimeric virus containing B3(Ccp) accumulated to the levels comparable to those of wt BMV (Figs. II-5 C and D).

DISCUSSION

The CP is indispensable for cell-to-cell movement of both CMV and BMV. However, it is unclear how the CPs are involved in the process of intercellular transport of the viral genome. I have shown that the replacement of the BMV MP gene with that of CMV abolishes cell-to-cell movement of the chimeric virus [the chimeric virus containing B3(Cmp) in Chapters I and II]. The additional replacement of the BMV CP gene in the chimeric virus containing B3(Cmp) with that of CMV enables the resulting chimeric virus [the chimeric virus containing B3(Cmp/Ccp)] to move from cell to cell. On the other hand, the replacement of the CMV CP gene with that of BMV also abolishes cell-to-cell movement of the chimeric virus (Osman *et al.*, 1998). These results suggest that some function of the CMV CP gene is required for the establishment of viral movement mediated by the CMV MP. A CP-frameshift mutation in the CMV CP gene of the chimeric virus containing B3(Cmp/Ccp) resulted in failure of the chimeric virus to move from cell to cell, indicating that the CP itself, but not its encoding nucleotide sequence, is required for establishing viral cell-to-cell movement mediated by the CMV MP. Thus, it was suggested that the CMV CP has some functions specifically required for the CMV MP-mediated viral cell-to-cell movement. Similarly, the BMV CP may have some functions specific for the BMV MP-mediated movement. Replacement of the BMV CP genes with that of CMV-Y (subgroup I) as well as CMV-Kin (subgroup II, Osman *et al.*, 1998) results in failure of viral cell-to-cell movement in the inoculated leaves. In the case of BMV movement, however, functions of virus particles consisting of the BMV CP might be required since virion formation is considered to be necessary for the movement (Rao and Grantham, 1995; Schmitz and Rao, 1996; Rao, 1997). Although the CMV CP can encapsidate chimeric BMV RNAs (Osman *et al.*, 1998), virus particles consisting of the CMV CP may not be able to substitute for the functions of BMV virion.

The requirement of the cognate CP is a unique character of the CMV MP and possibly the BMV MP. MPs encoded by diverse families of plant viruses share their functions and are genetically interchangeable in many cases. Chimeric viruses which can systemically infect common host plants for the parental viruses have been created by replacing the MP gene of

CCMV with that of sunn-hemp mosaic tobamovirus (De Jong and Ahlquist, 1992) or by replacing the MP gene of TMV with that of red clover necrotic mosaic dianthovirus (Giesman-Cookmeyer *et al.*, 1995). These viruses are taxonomically divergent, but none of them require CP for cell-to-cell movement (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Xiong *et al.*, 1993; Rao, 1997). In these cases, it is most likely that the translocated MP genes function successfully without their cognate CP. Although BMV requires its CP in cell-to-cell movement, movement-competent chimeric virus can be created by exchanging the MP genes between members of the genus *Bromovirus*, BMV and CCMV (Mise *et al.*, 1993). CPs of these closely related viruses show significant amino acid sequence homology (70%; Speir *et al.*, 1995), and these CP genes are freely exchangeable (Osman *et al.*, 1997). Movement-competent reassortants can be created by exchanging RNA 3 between BMV and CCMV (Allison *et al.*, 1988). Therefore, it is likely that the BMV MP is compatible with other CCMV encoded proteins.

BMV and CMV are categorized into different genera belonging to the family *Bromoviridae* and both require their CP in cell-to-cell movement. In contrast to the cases described above, our previous study showed that replacement of the BMV MP gene with that of CMV abolished cell-to-cell movement (Nagano *et al.*, 1997; Chapter I). As shown in this paper, additional replacement of the BMV CP gene with the CMV CP gene in the chimeric RNA 3 was necessary for establishment of cell-to-cell movement of the chimeric virus. Thus, the noninterchangeability of the CMV MP gene with that of BMV is most likely due to the requirement of the cognate CP by the CMV MP. On the other hand, deletion of the C-terminal 33 amino acids of the CMV MP enables the chimeric virus to move from cell to cell (Nagano *et al.*, 1997; Chapter I). The intact CMV MP with support of its cognate CP can mediate the cell-to-cell movement of a heterologous viral genome. These results suggest that the N-terminal portion of the CMV MP shares similar functions with the BMV MP. Although the reason why the truncated CMV MP can facilitate the chimeric virus movement is not clear, it is possible that the C-terminal 33 amino acids of the CMV MP may mask the ability of the protein to transport a heterologous viral genome, and that the CMV CP can cancel the masking. Alternatively, the

intact CMV MP, but not the truncated one, might induce a host resistance that could be suppressed by the CMV CP.

The local lesions induced by the chimeric virus containing B3(Cmp/Ccp) were much fewer than those induced by wt BMV and did not enlarge. Besides MP and CP, replicase contributes to BMV movement (Traynor *et al.*, 1991). Similarly, factors encoded in either RNA 1 or 2 affect CMV movement (Gal-On *et al.*, 1994; S. Ding *et al.*, 1995; Hellwald and Palukaitis, 1995). The inadequate cell-to-cell movement of the chimeric virus containing B3(Cmp/Ccp) may be caused by either the absence of the required CMV factor(s), or an incompatibility between the BMV and CMV factors. I have shown that the chimeric virus containing B3(Cmp/Ccp) moves from cell to cell, while not the del-SN-mutant of the chimeric virus containing B3(Cmp/Ccp). The deletion of N-terminal proximal region in the CMV CP that resulted in failure of CMV to form virus particles (Suzuki *et al.*, 1991; Kaplan *et al.*, 1998; Schmitz and Rao, 1998) abolished the movement of the chimeric virus mutant containing B3(Cmp/Ccp) in inoculated leaves, but not that of CMV. These unparallel results may suggest difference between the chimeric virus containing B3(Cmp/Ccp) and CMV in the mechanism of intercellular movement. The cumulative data have suggested that BMV moves in a virion form (Rao and Grantham, 1995; Schmitz and Rao, 1996; Rao, 1997) while CMV in a nonvirion form (Suzuki *et al.*, 1991; Kaplan *et al.*, 1998; Schmitz and Rao, 1998) although both requires the CP in cell-to-cell movement. Osman *et al.* (1998) demonstrated that the BMV genome can be encapsidated by the CMV CP into virion-like particles *in vivo*. Thus, it is likely that the chimeric virus containing B3(Cmp/Ccp) can form virus particles in the infected plant cells similarly to the chimeric virus containing B3(Ccp). This idea may be supported by our data that in protoplasts viral RNAs of the chimeric virus containing B3(Cmp/Ccp) accumulated to the level similar to that of the chimeric virus containing B3(Ccp). To date, the intercellular trafficking forms of plant viruses have not been clearly determined. Similarly, it is very difficult to make it clear whether the chimeric virus containing B3(Cmp/Ccp) moves as virions or not. Osman *et al.* (1998) have described that the encapsidation of the chimeric BMV by the CMV CP is inefficient. Thus, the putative virions of the chimeric virus containing B3(Cmp/Ccp) would

also be inefficiently assembled. This might explain for the limited spread of the chimeric virus containing B3(Cmp/Ccp).

Although the del-SN-mutant of the chimeric virus containing B3(Cmp/Ccp) did not induce local lesions and did not accumulate its viral RNAs in the inoculated leaves to the level detectable by press-blot analysis, the possibility that it might move through several cells can not be excluded. The truncated CMV CP is less efficient than the wt CP in facilitating the cell-to-cell movement of CMV, since the local lesions appeared later in leaves inoculated with the del-SN-CMV than with wt CMV. Therefore, the gene replacement and the truncation of the N-terminal proximal region in the del-SN-mutant of the chimeric virus containing B3(Cmp/Ccp) may synergistically affect viral movement. Inefficient cell-to-cell movement may induce host responses that restrict viral infection within several cells (Mise and Ahlquist, 1995). Cooperation with the other CMV factors might be required for the movement of the del-SN-mutant of chimeric virus containing B3(Cmp/Ccp) to the detectable levels.

MATERIALS AND METHODS

cDNA clones and *in vitro* transcription

Plasmids pBTF1, pBTF2, and pBTF3W contain the full-length cDNAs of RNAs 1, 2, and 3 of the wt BMV (the KU2 strain), respectively (Mise *et al.*, 1992, 1994; Mori *et al.*, 1991). The plasmid pT7B3CKY3 contains the full-length cDNA of chimeric BMV RNA 3 with the CMV MP gene instead of the BMV MP gene [B3(Cmp); Nagano *et al.*, 1997]. Plasmids pCY1-T7 and pCY2-T7 (generous gifts from S. Kuwata and M. Suzuki) contain the full length cDNAs of CMV-Y RNAs 1 and 2, respectively (Suzuki *et al.*, 1991). pT7CKY3 contains the full-length cDNA of RNA 3 of CMV-Y (Nagano *et al.*, 1997).

Substitution of the CP gene of BMV with that of CMV was done according to the strategy used for replacing BMV genes with foreign genes (Mori *et al.*, 1993). Restriction sites *Nsi*I and *Bln*I were introduced into the initiation and termination codons of the BMV MP gene encoded in pBTF3W, respectively, using site-directed mutagenesis (Kunkel, 1985). Similarly, *Nsi*I and *Xba*I sites were introduced into the initiation and termination codons, respectively, of

the CMV CP gene encoded in pT7CKY3 (Nagano *et al.*, 1997). Replacement of these CP genes was done using standard molecular biology techniques (Sambrook *et al.*, 1989). The resulting plasmid was designated as pT7B3CYcp, in which cDNA of B3(Ccp) was contained. The precise replacement but one nucleotide substitution described in the Results section was confirmed by sequencing of pT7B3CYcp.

A plasmid pB3C3a/Ccp containing B3(Cmp/Ccp) cDNA was created by replacing the *Bgl*III-*Eco*RI fragment containing the CP gene of BMV in pT7B3CY3a with that in pT7B3CYcp. Frame-shift mutants of C3 and B3(Cmp/Ccp) was made from pT7B3C3a/Ccp and pT7CKY3, respectively, by duplicating 4 bases (TCGA) within *Sal*I site in the CMV CP gene. To generate the del-SN-mutants of B3(Cmp/Ccp) and C3, either pT7B3C3a/Ccp or pT7CKY3 was digested with *Sal*I. After the protruding ends were filled-in with T4 DNA polymerase and dNTPs, the plasmid was digested with *Nru*I and then self-ligated.

In vitro transcripts were synthesized from these plasmids by T7 RNA polymerase after linearizing with appropriate restriction endonuclease (Mori *et al.*, 1991; Suzuki *et al.*, 1991; Mise *et al.*, 1992, 1994; Nagano *et al.*, 1997).

Isolation of protoplasts and inoculation of plants and protoplasts

Isolation of protoplasts from *C. quinoa* plants was performed essentially as described previously (Nagano *et al.*, 1997) with some modifications. Protoplasts were isolated from fully expanded leaves of 6 to 7-week-old *C. quinoa* plants. The grade of cellulase was changed to Onozuka RS (Yakult Honsha). The isolated living protoplasts were collected by centrifugation of the suspension layered onto a 20% sucrose cushion. Protoplasts were incubated at 25° C for 24 h with continuous lightning in the protoplast medium (Kroner and Ahlquist, 1992) buffered by addition of 5 mM MES (Good's buffer; 4-morpholineethanesulfonic acid).

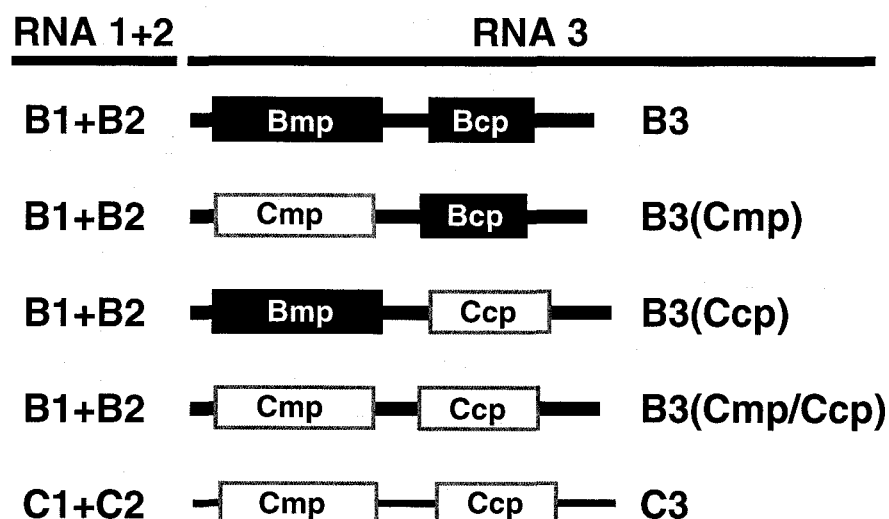
The two youngest fully-expanded leaves of 4-week-old *C. quinoa* plants were dusted with carborundum and mechanically inoculated with a mixture of *in vitro* transcripts corresponding to RNAs 1, 2, and 3 at a concentration of 0.1 mg/ml each per leaf. The inoculated leaves were then rinsed by water. The inoculated plants were cultured in a plant growth room at 24±1° with illumination for 16 h per day.

Analysis of RNA and protein

Northern hybridization and press blot was done as described previously (Nagano *et al.*, 1997). Inoculated leaves were press-blotted at 1, 3, 7, and 14 days pi. Probes specific for positive- or negative-strand BMV RNAs (Kaido *et al.*, 1995) and for CMV RNAs (Nagano *et al.*, 1997) were described previously. These probes were labeled with [α - 32 P]-UTP, and the radioactive signals on the membrane were quantified with a digital radioactive imaging analyzer (Fujix BAS 2000, Fuji Photo Film).

Infected protoplasts (1.5×10^5) were collected and resuspended in Laemmli's sample buffer (Laemmli, 1970). Proteins in the suspension were separated by electrophoresis on 12.5% polyacrylamide gels containing 0.1% SDS (SDS-PAGE). Immunoblot analysis (Towbin *et al.*, 1979) was carried out using an immobilon-P transfer membrane (Millipore). Polyclonal rabbit antiserum raised against BMV, CMV (Nagano *et al.*, 1997), the CMV MP (generous gift from P. Palukaitis and I. B. Kaplan), or mouse antiserum against the BMV MP (Fujita *et al.*, 1998) was used for the first antibody and they were labeled by alkaline phosphatase-conjugated goat secondary antibody, appropriate for the first antibody.

(A)



(B)

CMV CP

WT	MDKSEST SAGRNRRRLRRGSRSSSSSDANFRVLSQQLSRINK...
FS	MDKSEST SAGRNRRstsfaswfprrllllgc*
del-SN	MDKSEST SAGRNRRR-----LNK...

Fig. II-1. Characteristics of BMV or CMV RNA 3 mutants. (A) Schematic diagrams and abbreviations of chimeric RNA 3. Wild-type BMV RNA 3 (B3) and CMV RNA 3 (C3) are also shown. The noncoding sequences of B3 and C3 are represented as thick and thin lines, respectively. The open reading frames encoded by B3 and C3 are represented as closed and open boxes, respectively. Coinoculated wild-type RNAs 1 and 2 are shown at the left, and abbreviations of the inoculum and its progeny virus at the right. (B) Amino terminal sequences of translational products from the CMV CP gene of wild-type (WT), frameshift mutant (FS), and del-SN- mutant (del-SN). The amino acid residues different from those of wild-type due to a frameshift mutation are indicated in lower-case letters. An asterisk indicates the termination codon, and hyphens indicate amino acid residues deleted from amino-terminal proximal region of the CMV CP.

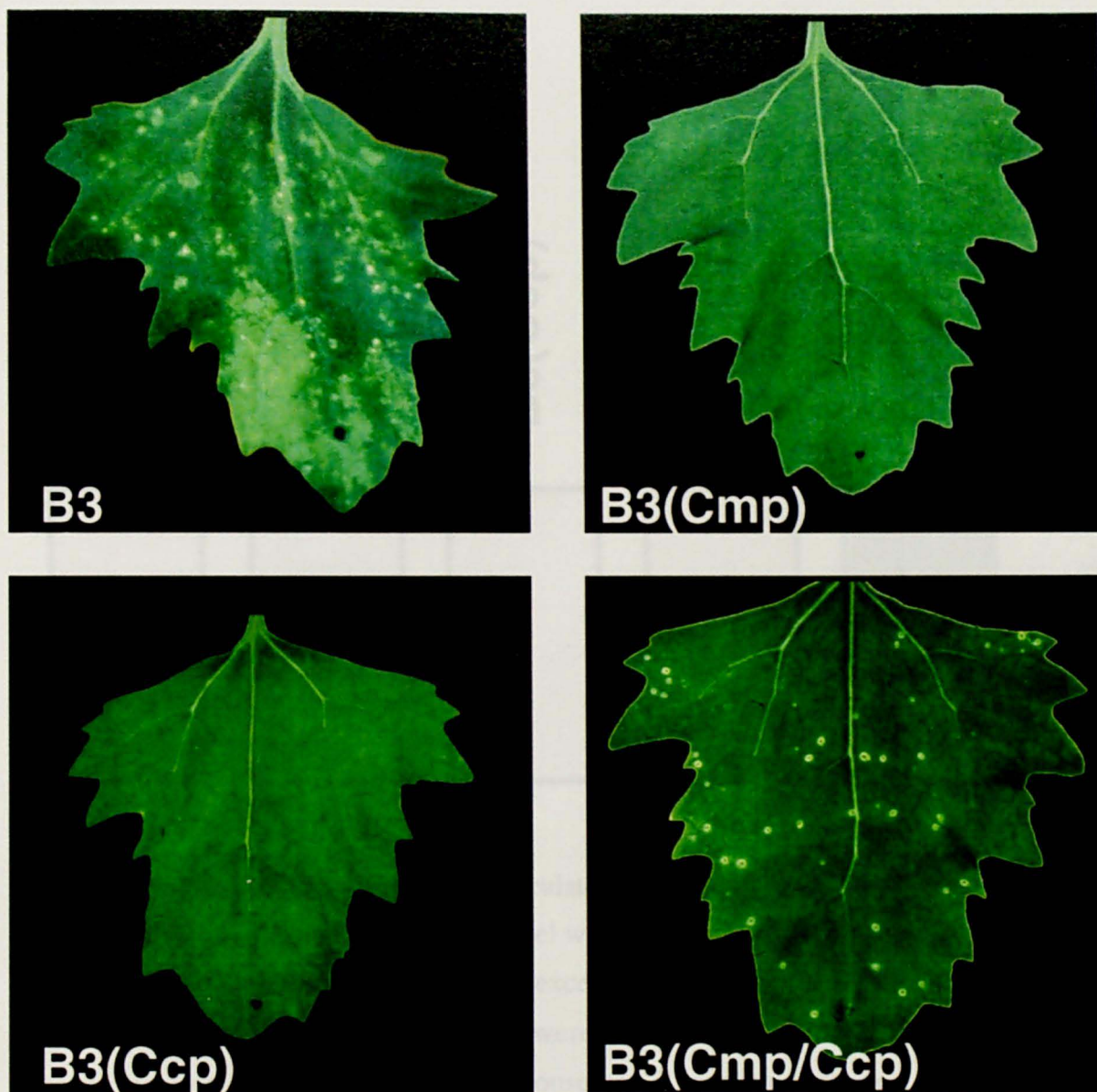


Fig. II-2. Local lesion formation on the inoculated *C. quinoa* leaves. The inocula contained wild type (B3) and the chimeric BMV RNA 3s, B3(Cmp), B3(Ccp), and B3(Cmp/Ccp) together with wt BMV RNAs 1 and 2. The photographs were taken at 6 (B3), 7 [B3(Cmp/Ccp)], and 14 [B3(Cmp) and B3(Ccp)] days pi.



Fig. II-3. Press blot analysis of the inoculated *C. quinoa* leaves. RNA 3 contained in each inoculum was indicated above each panel with the abbreviations identical to Fig. II-2. The press-blot was prepared at 7 days pi except for the blot of a B3-inoculated leaf, which was prepared at 3 days pi. They were hybridized with a ^{32}P -labeled riboprobe complementary to the positive-strand conserved 3' terminal sequence of all BMV RNAs, and then exposed to an X-ray film. Mock: inoculated with buffer only. B3*: the 24-fold shorter exposed image of "B3".

(A) Positive-strand

(B) Negative-strand

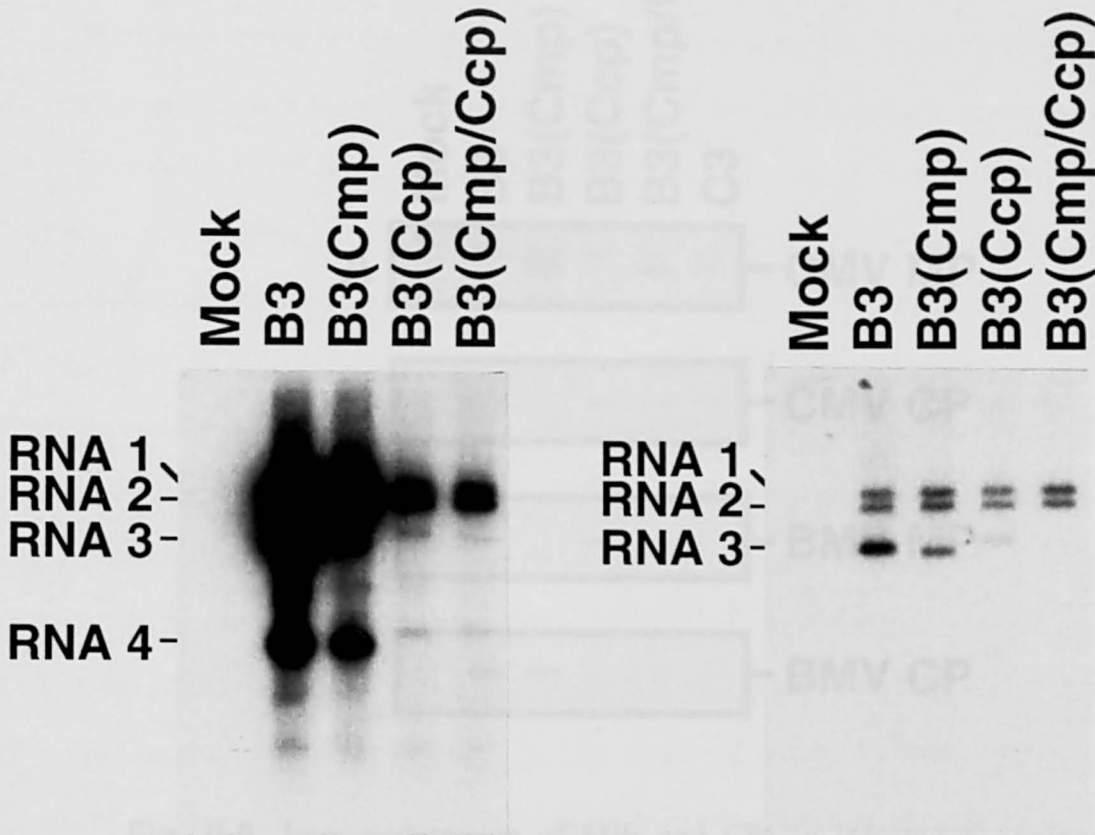


Fig. II-4. Accumulation of viral RNA in infected *C. quinoa* protoplasts. Total RNA extracted from 5×10^4 infected protoplasts was applied to each lane. Mock, inoculated with buffer only. Detection of positive or negative strand viral RNAs by Northern blot hybridization with ^{32}P labeled riboprobes are shown in panels A and B, respectively. Total RNA was extracted at 24 h pi. Positions of BMV RNA are indicated.

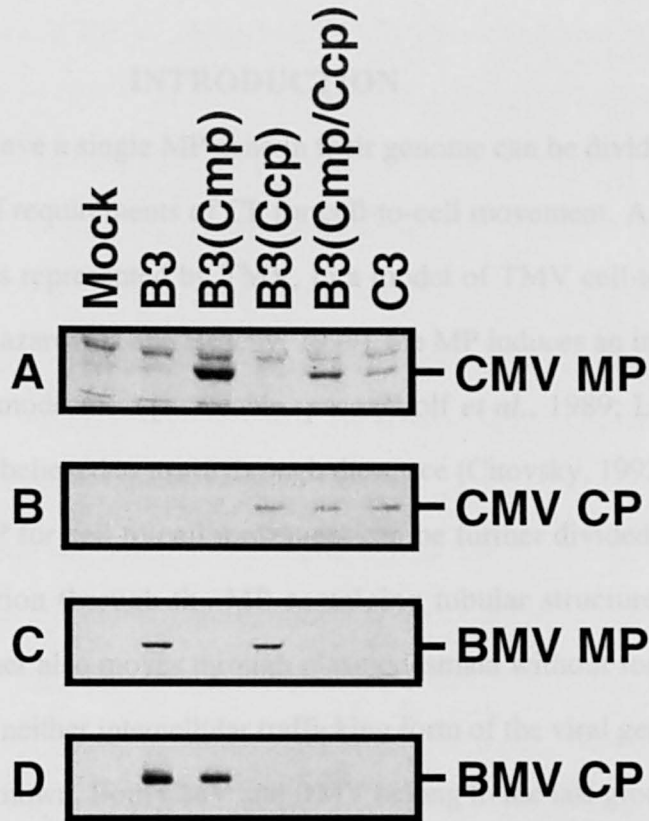


Fig. II-5. Immunodetection of MPs and CPs in infected *C. quinoa* protoplasts. Total proteins extracted from 3×10^4 infected protoplasts at 24 h pi were loaded onto each lane and fractionated by SDS-PAGE. Mock, inoculated with buffer only. Panels A and B show immunodetection of the MP and CP of CMV using anti-CMV-MP and anti-CMV antisera, respectively. Panels C and D show immunodetection of the MP and CP of BMV using anti BMV-MP and anti-BMV antisera, respectively.

CHAPTER III: The Requirement of Coat Protein for Cell-to-Cell Movement of Cucumber Mosaic Virus is Conferred by the C-terminus of Movement Protein.

INTRODUCTION

Plant viruses that have a single MP gene in their genome can be divided primarily into two groups on the basis of requirements of CP for cell-to-cell movement. A group of viruses that does not require CP is represented by TMV. In a model of TMV cell-to-cell movement (Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999), the MP induces an increase in the size exclusion limit of the plasmodesmata permeable space (Wolf *et al.*, 1989; Lucas, 1995), and MP/viral RNA complex is believed to move through the space (Citovsky, 1993). Another group of viruses that requires CP for cell-to-cell movement can be further divided into at least two groups; one moves as virion through the MP-containing tubular structures formed within plasmodesmata, and another also moves through plasmodesmata without forming the tubular structures. In the last case, neither intercellular trafficking form of the viral genome nor the role of CP in the process is unknown. Both CMV and BMV belong to the last group of viruses.

In Chapter I, I have shown that a chimeric BMV containing B3(Cmp) is incompetent for cell-to-cell movement whereas a derivative of the chimeric virus containing B3(CmpDC33) is competent. The reason for the incompetency of the chimeric virus to move from cell to cell has been shown in Chapter II that the cognate CP is required for establishment of the movement mediated by the CMV MP. In this Chapter, I investigated to know the reason why the derivative of the chimeric virus expressing the CMV MP from which the C-terminal 33 amino acids were deleted (CMP-DC33) can move from cell to cell. In addition, the role of CP played in viral cell-to-cell movement mediated by the CMV MP is discussed.

RESULTS

Effect of various length of C-terminal deletions in CMV MP on viral cell-to-cell movement.

I previously showed that the wild-type CMV MP can function in cell-to-cell movement of CMV, but not of the chimeric BMV, while the CMP-DC33 can function in the movement of both viruses (Nagano *et al.*, 1997; Chapter I). This raised a question how many residues of deletion from the C-terminus of the CMV MP allowed the chimeric BMV to move from cell to cell. The C-terminal 33 amino acid region was calculated to be dividable into three parts by using a computer program for protein secondary structure prediction (Prediction method; Chou and Fasman, Beta-turn Probability Value; 75×10^{-6} ; Fig. III-1). According to this, I created the chimeric BMV RNA 3 variants encoding the CMV MP from which the C-terminal 10, 19, and 25 amino acids were deleted by introducing of a stop codon.

When inoculated onto *C. quinoa* plants together with BMV RNAs 1 and 2 transcripts, none of these chimeric RNA 3 variants induced local lesions (Table III-1). No accumulation of viral RNA was detected in these leaves by press-blotting hybridization analyses (Table III-1). Similar results were obtained when concentration of the inocula increased up to threefold. To confirm these variants of the chimeric virus replicate in the single-cell level, *C. quinoa* protoplasts were inoculated with the variants. Northern hybridization analysis of the infected protoplasts revealed that viral RNA of these variants accumulated similarly to that of the chimeric BMV containing B3(CmpDC33) that is competent for cell-to-cell movement (data not shown). It is thus suggested that none of the variants of chimeric virus with the C-terminal deletion of 10, 19, and 25 amino acids were able to move from cell to cell.

I previously showed that the intact CMV MP was not able to function in cell-to-cell movement of the chimeric virus without help of the CMV CP. To test whether the CMV MP with the truncation of C-terminal 10, 19, or 25 amino acids functions in the movement when the CMV CP is present, I created CMV variants encoding these C-terminal deleted MP. These CMV variants all infected *C. quinoa* protoplasts and viral RNA accumulation was observed by Northern hybridization analysis (Fig. III-2). However, none of these CMV variants induced

any symptom and no viral RNA accumulation was detected in the inoculated leaves by press-blot hybridization analyses (Table III-1). Therefore, it is rather suggested that the three truncated forms of CMV MP lose their function for viral movement although the larger 33 amino acids can be deleted from the C-terminus of the CMV MP without losing the function.

I further created CMV variants with variable size of deletion from the C-terminus of MP. These variants were all similarly able to replicate in *C. quinoa* protoplasts (data not shown). The infectivity of the variants were then tested in *C. quinoa* plants. The abilities of the variants to induce symptom and to move from cell to cell estimated by press-blot hybridization were summarized in Table III-1. In short, while deletion of the C-terminus of CMV MP to the extent from 33 to 36 amino acids did not abolish either cell-to-cell movement or symptom induction of CMV, longer and shorter deletion from the C-terminus made me fail to detect viral movement by press-blot analyses. It is noteworthy that the function of CMV MP to mediate viral cell-to-cell movement is abolished if only 3 amino acids are deleted from its C-terminus although 33 amino acids can be deleted without abolishing the function. This suggests that the C-terminal region is not completely dispensable for cell-to-cell movement of CMV. Previously, a CMV mutant lacking the C-terminal 43 amino acids of the MP was reported to infect tobacco plants systemically (Kaplan *et al.*, 1995). The mutant was created by deletion of a region between two *HpaI* sites in the MP ORF. I also created and tested two corresponding CMV variants; one by introducing a stop codon and the other by deletion of the region between two *HpaI* sites. These variants, however, did not move from cell to cell and did not induce any symptom in *C. quinoa* plants throughout these experiments (Table III-1, data not shown).

On the other hand, the truncated forms of CMV MP were tested for the ability to mediate cell-to-cell movement of the chimeric BMV genome (Table III-1). The MPs that had been able to mediate the CMV movement were also able to mediate the chimeric BMV movement. While the MPs that had not functioned in the CMV movement also did not function in the movement of the chimeric BMV, there were a few exceptions such as the truncated MPs without its C-terminal 31 and 32 amino acids.

The C-terminal region of CMV MP is involved in the requirement for CP to

mediate cell-to-cell movement.

To test a possibility that the chimeric BMV having the gene of CMP-DC33 was able to move from cell to cell due to some changes in the CP requirement, I first constructed a CP-defective variant of the chimeric virus with the CMP-DC33 gene. This CP-defective variant induced chlorotic lesions in the inoculated leaves of *C. quinoa*. Press-blot analysis of the inoculated leaves showed that viral RNA accumulated in the leaves (Fig. III-3). Such RNA accumulation have never been observed in the leaves inoculated with a CP-defective variant of the chimeric BMV with the intact CMV MP gene (Fig.III-3). Immuno-blot analysis of the inoculated leaves revealed that BMV CP did not accumulate in the inoculation of the CP-defective variant (data not shown). These results indicate that the truncated CMV MP functions in cell-to-cell movement of the chimeric virus independent of the BMV CP.

The results described above raised a possibility that the truncated CMV MP can mediate the CMV movement without any help of the CMV CP. Thus I tested infectivity of a CP-defective CMV variant with the truncated MP gene. When inoculated onto *C. quinoa* plants, the CP-defective CMV variant with the CMP-DC33 gene induced necrotic lesions on the inoculated leaves, while a CP-defective CMV variant with the gene of intact CMV MP did not (data not shown). Dispersed viral RNA was detected in the leaves inoculated with the CP-defective CMV variant with the CMP-DC33 gene by press-blot hybridization, but not with the CP-defective CMV variant with the gene of intact CMV MP (Fig. III-3). Accumulation of CMV CP was not detected in the leaves inoculated with these CP defective variants by immuno-blot analysis (data not shown). These results indicate that the CMP-DC33 does not require the CP for mediating cell-to-cell movement of CMV as well as chimeric BMV.

The intact CMV MP inhibits viral cell-to-cell movement mediated by the truncated one.

Since the truncated CMV MP does not require CP to mediate viral movement, the BMV CP gene could be replaced with other genes in the chimeric RNA 3 having the CMP-DC33 gene. I replaced the BMV CP gene in the chimeric BMV RNA 3 containing the CMP-DC33 gene with the gene of intact or truncated CMV MP. Each of the RNA 3 derivatives containing

two CMV MP genes in tandem were inoculated onto *C. quinoa* plants together with BMV RNAs 1 and 2. While the chimeric virus containing a chimeric BMV RNA 3 with two CMP-DC33 genes [B3(CmpDC33/CmpDC33)] induced more than 50 chlorotic lesions in every inoculated leaf, that containing another chimeric BMV RNA 3 with the genes of truncated and intact CMV MP [B3(CmpDC33/Cmp)] induced 1 to 6 lesions in every inoculated leaf (Fig. III-4). Viral RNA accumulation in the inoculated leaves was detected by Northern hybridization analysis of leaf disks showing lesions, although it was not detected by press-blot hybridization (data not shown). I created two more chimeric RNA 3 derivatives by replacing the BMV CP gene in the chimeric RNA 3 containing the gene of intact CMV MP with the gene of intact or truncated CMV MP and tested their infectivity by inoculating onto *C. quinoa* plants together with BMV RNAs 1 and 2. These chimeric viruses did not induce symptoms (Fig. III-4), and no viral RNA accumulation was detected in those leaves (data not shown). These results indicate that the intact CMV MP inhibits viral cell-to-cell movement mediated by the truncated one.

The CMV CP binds to the CMV MP *in vitro*.

As described above, truncation of the CMV MP C-terminus made a change in the CP-requirement for viral cell-to-cell movement. To investigate how the CMV CP is required for the movement mediated by the intact CMV MP, I tested binding activity of the CMV CP to the CMV MP by glutathione-S-transferase (GST) pull-down method. The CMV CP was synthesized using rabbit reticulocyte lysate *in vitro* translation system in the presence of ³⁵S-methionine. The ³⁵S-labeled CMV CP was incubated with the CMV MP which had been purified from *E. coli* as a protein fusion with GST (GST:CMV). After washing, the CMV CP bound to the glutathione-GST:CMV complex was analyzed by SDS-PAGE and fluorography. As shown in Fig. III-5, a band comigrating with that of the product of cell-free translation system was detected, indicating that the CMV CP was bound to GST:CMV. When, as a control, the labeled CMV CP was incubated with free GST of which molecular-concentration was adjusted to the equal level to that of GST:CMV by immunoblot method using anti-GST antibody, no such band was detected (Fig. III-5). These results indicate that the CMV CP is

bound to the CMV MP *in vitro*.

Binding activity of CMV CP to the CMP-DC33 was also tested. The molecular-concentration of the fusion protein between GST and CMP-DC33 (GST:CMV-DC33) was adjusted by immunoblot method using anti-GST antibody. The CMV CP was also bound to the truncated CMV MP (Fig.III-5), suggesting that the CMV CP did not interact exclusively with the C-terminal region of the CMV MP.

To investigate specificity of the binding between the CP and MP, *in vitro* interaction between BMV CP and the intact or truncated CMV MP was also tested by using labeled BMV CP instead of CMV CP. BMV CP was bound to either the intact or the truncated CMV MP (Fig. III-5). However, the level of the BMV CP-binding was lower than that of the CMV CP (Fig. III-5).

DISCUSSION

Role of the C-terminus of CMV MP.

I have shown that the CMV MP with truncation of its C-terminal 33 amino acids is competent to mediate viral cell-to-cell movement (Nagano *et al.*, 1997). This study demonstrates that the C-terminus can be deleted further up to 36 amino acids without losing the competency for viral cell-to-cell movement. This is not coincide with the observation that a CMV mutant, lacking the C-terminal 43 amino acids of the 3a protein, is able to infect tobacco plants systemically (Kaplan *et al.*, 1995). The inconsistency may be due to difference in experimental system including the viral strain and the host plant. On the other hand, shorter deletion in the C-terminal region made the MP incompetent to mediate viral movement (Table III-1). This suggests that the C-terminal region is not completely dispensable for viral movement but play a role in the process.

The CMV MP requires its cognate CMV CP to establish viral cell-to-cell movement (Nagano *et al.*, 1999). However, several variants of the MP-chimera with C-terminal deletion in the CMV MP gene can move from cell to cell, although they do not have the CMV CP gene. I find the reason for it that the CMP-DC33 can mediate viral movement without any help of CP.

To our knowledge, this is the first finding of the MP that has two aspects in the CP-requirement for cell-to-cell movement. Since biological and biochemical properties are shared among MPs of plant viruses, it may be possible that as the case of CMV MP, the MPs of other viruses that require CP for cell-to-cell movement also convert by some mutations to MP that can function in the process without CP. It has been unclear why the CMV MP has the C-terminal region involved in the CP-requirement for cell-to-cell movement although the remaining part is sufficient for the process. Possible inferences are discussed below.

The intact CMV MP could be an inactivated form.

The CMV MP has been compared for their functions with that of TMV, one of the best characterized virus in cell-to-cell movement. They share ability to (i) traffic through plasmodesmata (Waigmann *et al.*, 1994; B. Ding *et al.*, 1995), (ii) increase plasmodesmal size exclusion limit (Waigmann *et al.*, 1994; B. Ding *et al.*, 1995), (iii) bind single-stranded nucleic acids *in vitro* (Citovsky *et al.*, 1990; Li and Palukaitis, 1996), and (iv) complement their respective movement-deficient mutants (Deom *et al.*, 1987; Kaplan *et al.*, 1995). However, there are several differences between MPs of the two viruses. For example, movement-competency of heterogeneous chimeric viruses in which the original MP gene was replaced with the respective MP gene of TMV (Giesman-Cookmeyer *et al.*, 1995; Solovyev *et al.*, 1996) and CMV (Nagano *et al.*, 1997) is different. Besides, transgenic plants expressing the CMV MP are unable to support cell-to-cell movement of TMV mutants in which the MP gene is disrupted (Kaplan *et al.*, 1995; Cooper *et al.*, 1996), while transgenic plants expressing the TMV MP support cell-to-cell movement of movement-defective CMV mutants (Cooper *et al.*, 1996; Rao *et al.*, 1998). These differences are considered to be resulted from a difference that TMV does not require CP for viral cell-to-cell movement, whereas CMV does. Coexistence of the CMV CP gene enables the chimeric virus with the CMV MP gene to move from cell to cell (Nagano *et al.*, 1999). Therefore, although sharing biological and biochemical characters with the TMV MP, the intact CMV MP may be non-functional in the absence of the CMV CP.

It is known that a non-functional MP expressed in transgenic plants confers resistance to multiple viruses (Malysenko *et al.*, 1993; Beck *et al.*, 1994; Cooper *et al.*, 1995). Similarly,

the chimeric viruses containing both intact and truncated CMV MP genes had remarkably inferior ability to move from cell to cell when compared to the chimeric virus containing two truncated CMV MP genes (Fig. III-4). This can be explained if the intact CMV MP is non-functional in the absence of the CMV CP; the intact CMV MP inhibits viral movement mediated by the truncated one.

Role of CMV CP in cell-to-cell movement mediated by the CMV MP.

Coexistence of the CMV CP with the CMV MP can mediate cell-to-cell movement of a heterogeneous viral genome (Nagano *et al.*, 1999). This suggests a possibility that the CMV CP plays a role to convert the CMV MP from a non-functional to a functional state. I found the CMV CP had binding activity for the CMV MP *in vitro*, suggesting *in vivo* interaction between these two proteins. This binding activity of the CMV CP to the CMV MP might be involved in the conversion of CMV MP, possibly affecting its conformation. If this is true, CMV might move through plasmodesmata by a mechanism similar to that of TMV (Carrington *et al.*, 1996; Lazarowitz and Beachy, 1999) with some modification of the conformational change of the MP by binding of the CP. However, it is unlikely that the CP specifically binds to the C-terminal region of the CMV MP, since deletion of the C-terminal 33 amino acids from the CMV MP did not attenuate the binding capacity of the CMV CP. Besides, BMV CP was also bound to either intact or truncated CMV MP. Therefore, the possibility that the binding might be artificial events irrelevant to viral cell-to-cell movement has not been excluded. Further experiments will be needed to clarify the relationship between the *in vitro* binding and CMV movement.

MATERIALS AND METHODS

cDNA clones and *in vitro* transcription

Plasmids pBTF1, pBTF2, and pBTF3W contain the full-length cDNAs of RNAs 1, 2, and 3 of the wt BMV (the KU2 strain), respectively (Mori *et al.*, 1991; Mise *et al.*, 1992, 1994). The plasmid pT7B3CKY3 and pB3C3a247T contains the full-length cDNA of chimeric BMV RNA 3 with the genes of intact and the 33 C-terminal amino acid-truncated CMV MP, respectively (Nagano *et al.*, 1997). Plasmids pCY1-T7, pCY2-T7 (these are generous gifts

from S. Kuwata, Japan Tobacco Incorporated, Japan, and M. Suzuki, The University of Tokyo), and pT7CKY3 contain the full length cDNAs of CMV-Y RNAs 1, 2, and 3, respectively (Suzuki *et al.*, 1991; Nagano *et al.*, 1997).

The RNA 3 variants of chimeric BMV and CMV with various length of C-terminal deletion of CMV MP were created as follows. A translational stop codon, UGA, was introduced into the subcloned 124 bp *HpaI* fragment in the CMV MP gene by PCR-based *in vitro* mutagenesis (Ito *et al.*, 1991) using appropriate oligonucleotides. After confirmation of successful introduction of the mutation without any undesired change by reading nucleotide sequences, the wt 125 bp *HpaI* fragment of pT7B3CKY3 or pT7CKY3 was replaced with those containing a stop codon at the various position. Direction of inserted fragments were confirmed by reading nucleotide sequences. Exceptions were the variants with the 3 amino acid-deletion from the CMV MP C-terminus. Since the codon encoding the 277th amino acid is overlapped with the 3'-proximal *HpaI* site and since *SalI* site exists at the 5' terminus of the CP gene of either BMV or CMV, mutagenesis was done in the 566 bp *NheI-SalI* fragment of pT7B3CKY3 or pT7CKY3. After confirmation of successful introduction of the mutation without any undesired change by reading nucleotide sequences, the wt *HpaI-SalI* fragment of pT7B3CKY3 (385 bp) or pT7CKY3 (472 bp) was replaced with those containing a stop codon at the 277th codon.

The CP-defective variants of CMV RNA 3 and chimeric BMV RNA 3 with the CMV MP genes were made by duplicating 4 bases (TCGA) within *SalI* site in the CP gene of CMV and BMV, respectively.

The chimeric BMV RNA 3 derivatives in which the CMV MP genes were in tandem were created as follows. First, the BMV CP gene in the *NsiI/BlnI*-introduced pBTF3W (Nagano *et al.*, 1999) was replaced with the CMV gene from pCMP-NX (Nagano *et al.*, 1997) as described previously (Mori *et al.*, 1993), to create pB3(CPtoCMP). The *BglIII/EcoRI* fragment of pT7B3CKY3 and pB3C3a247T were replaced with the corresponding fragment containing the CMV MP gene from pB3(CPtoCMP). The resulting plasmids were designated as pB3(Cmp/Cmp) and pB3(CmpDC33/Cmp), respectively. On the other hand, the 124 bp *HpaI*

fragment in pB3(CPtoCMP) was replaced with that from pB3C3a247T, to create pB3 (CPtoCMPDC33). The *Bgl*III/*Eco*RI fragment of pT7B3CKY3 and pB3C3a247T were replaced with the corresponding fragment containing the CMV MP gene with mutation from pB3 (CPtoCMPDC33). The resulting plasmids were designated as pB3(Cmp/CmpDC33) and pB3(CmpDC33/CmpDC33), respectively.

In vitro transcripts were synthesized from these plasmids by T7 RNA polymerase after linearizing with appropriate restriction endonuclease (Mori *et al.*, 1991; Suzuki *et al.*, 1991; Mise *et al.*, 1992, 1994; Nagano *et al.*, 1997).

Inoculation of plants and protoplasts

Isolation and inoculation of protoplasts from *C. quinoa* plants was performed as described previously (Nagano *et al.*, 1999).

The two youngest fully-expanded leaves of 4-week-old *C. quinoa* plants were dusted with carborundum and mechanically inoculated with a mixture of *in vitro* transcripts corresponding to RNAs 1, 2, and 3 at a concentration of 0.1 mg/ml each per leaf. The inoculated leaves were then rinsed by water. The inoculated plants were cultured in a plant growth room at 24±1° with illumination for 16 h per day.

Analysis of RNA

Northern hybridization and press blot was done as described previously (Nagano *et al.*, 1997). Probes specific for positive- or negative-strand BMV RNAs (Kaido *et al.*, 1995) and for CMV RNAs (Nagano *et al.*, 1997) were described previously. The radioactive signals by ³²P on the membrane were quantified with a digital radioactive imaging analyzer (Fujix BAS 2000, Fuji Photo Film).

Expression and purification of the fusion proteins

A plasmid pCMP-NX (Nagano *et al.*, 1997) and its derivative with a stop codon at the position of the 247th amino acid residue were digested with *Nsi*I and then the protruding ends were filled-in with T4 DNA polymerase and dNTPs. These plasmids were digested with *Xba*I and then ligated into the expression vector pET42a (Novagen) previously digested with *Eco*RV and *Xba*I. The ligated DNAs were transformed into *E. coli* strain BL21(DE3). The transformed

E. coli was cultured for 3 h at 25°C and then protein-expression was induced by adding IPTG as indicated by the supplier (Novagen). The expressed fusion proteins were purified with glutathione beads as indicated by the supplier (Amersham-Pharmacia). The purified GST and fusion proteins between GST and the CMV MPs were electrophoresed in 12.5% polyacrylamide gels containing 0.1% SDS (SDS-PAGE, Laemli, 1970) and then analyzed using anti-GST antiserum (Amersham-Pharmacia) after transferred to an immobilon-P transfer membrane (Millipore) as described (Towbin *et al.*, 1979). The proteins appeared as bands on the membrane were quantified using a free-available software, NIH-Image, ver. 1.62 (by W. Rasmand, National Institute of Health, USA).

GST pull-down assay

Labeled CP of either CMV or BMV was synthesized in rabbit reticulocyte lysate cell-free translation system (Pelham and Jackson, 1976) using ³⁵S-Methionine and incubated for 1 h at 4°C with the fusion protein which had been purified from *E. coli* using glutathione beads (Amersham-Pharmacia). After the incubation, the beads were washed by phosphate-buffered saline (pH 7.5) containing 1% Triton X-100 and 5 mM DTT. The washed beads were resuspended in Laemmli's sample buffer (Laemmli, 1970) and then electrophoresed in 15% polyacrylamide gels containing 0.1% SDS. The gels were analyzed by fluorography (Chamberlain, 1979).

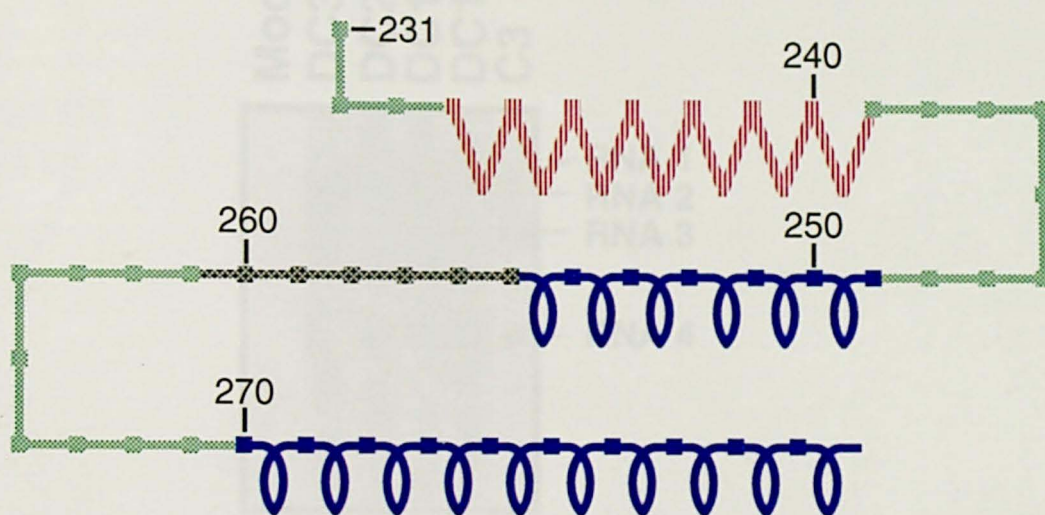
TABLE III-1. Ability of CMV and chimeric BMV derivatives containing the gene of CMV MP with various length of C-terminal truncations.

Number of amino acids deleted from the C-terminus	CMV		Chimeric BMV	
	Symptom induction ^a	Movement ^b	Symptom induction ^a	Movement ^b
0	++	+	-	-
3	-	-	-	-
5	-	-	-	-
7	-	-	-	-
10	-	-	-	-
19	-	-	-	-
25	-	-	-	-
27	-	-	-	-
29	-	-	-	-
31	+	-	+	+
32	+	-	+	+
33	++	+	+++	+
34	+	+	nt ^c	nt ^c
35	+	+	++	+
36	+	+	+	+
37	-	-	-	-
39	-	-	-	-
43	-	-	-	-

a. Number of local lesions per leaf were indicated by - (0), + (<10), ++ (<50), and +++ (>50).

b. Movement was estimated by whether the signals were detected by press-blot hybridization analyses or not.

c. nt, not tested.



 : HELIX
  : SHEET
  : TURN
  : COIL

Fig. III-1. Prediction of secondary structure of the CMV MP C-terminus. The prediction was done by a program included in DNASISTM v.3.6 (Hitachi co.) according to Chou and Fasman method. Beta-turn probability value was established at 75×10^{-6} . Rectangles indicate amino acid residues. The number indicates that of the residue in the CMV MP.

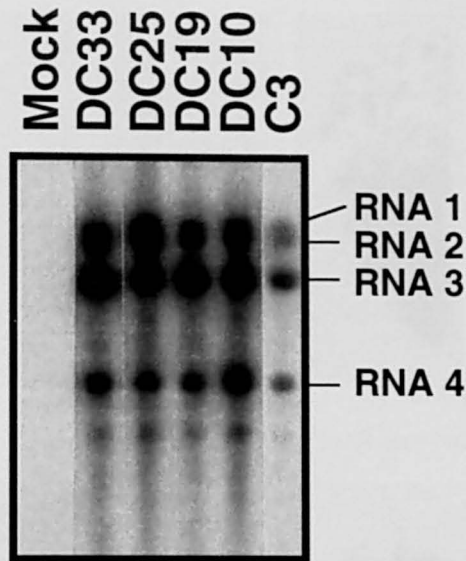


Fig. III-2. Viral RNA accumulation in *C. quinoa* protoplasts inoculated with the CMV derivatives containing the gene of CMV MP with various length of C-terminal deletions. Total RNA was extracted from the infected protoplasts at 24 h pi and analyzed by Northern hybridization using a ^{32}P -labeled riboprobe complementary to the 3' ends of CMV RNA. RNA 3s included in the inocula were indicated above the panell. Mock, inoculated with buffer only. DC33, DC25, DC19, and DC10, derivatives of C3 in which the C-terminal 33, 25, 19, and 10 amino acids were truncated from the MP. Positions of CMV RNA are indicated.

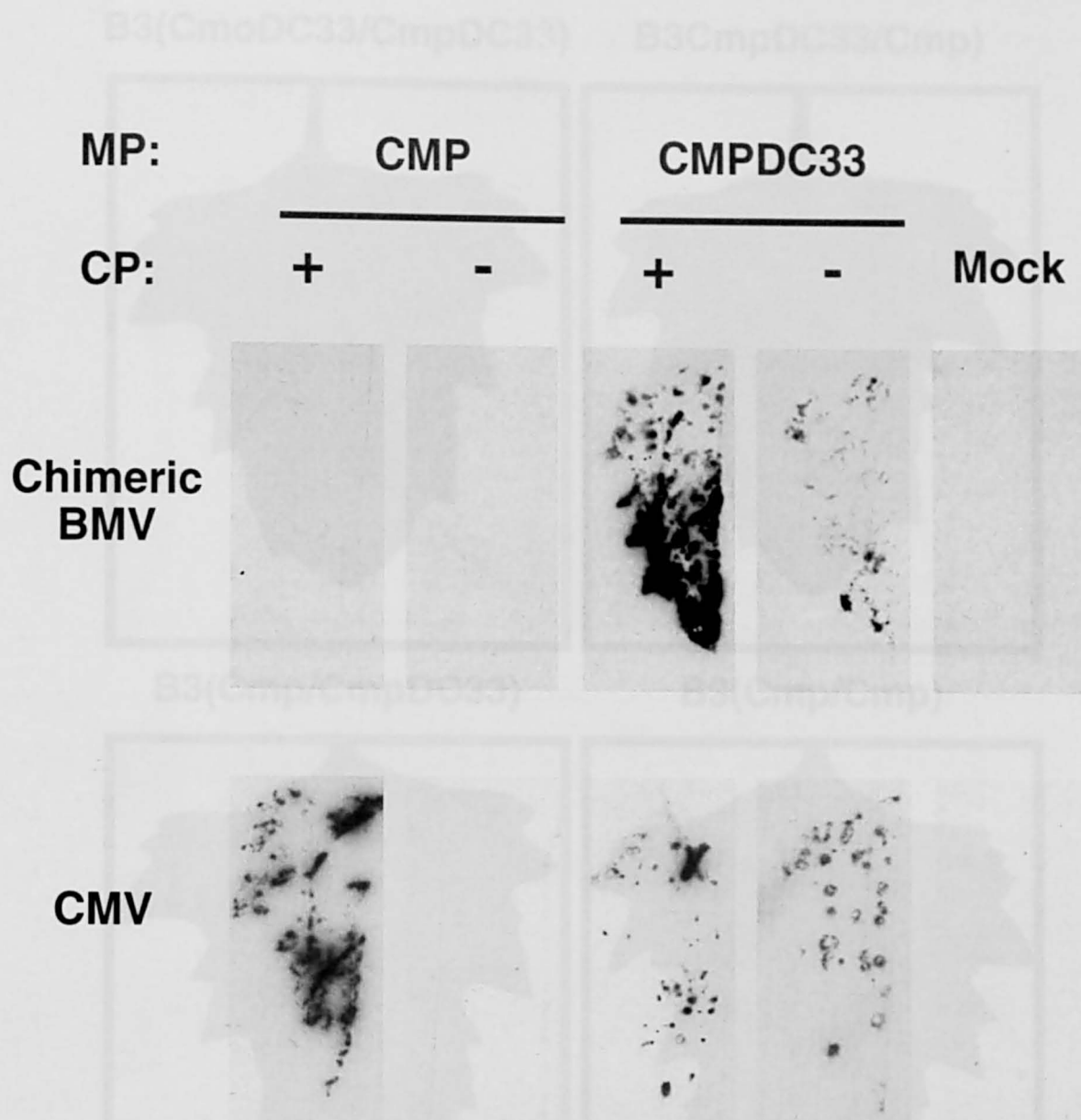


Fig. III-3. Press blot analyses of the *C. quinoa* leaves inoculated with the chimeric BMV or CMV derivatives at 7days pi. Only the inoculated leaf of wt CMV was analyzed at 3 days pi. The chimeric BMV derivatives have the CMV MP and the BMV CP genes. The intact (CMP) and the 33 C-terminal amino acid-truncated (CMPDC33) CMV MPs encoded in the viral RNAs and expression of the CP are indicated the lower side above the panels.

B3(CmoDC33/CmpDC33)

B3CmpDC33/Cmp)



B3(Cmp/CmpDC33)

B3(Cmp/Cmp)



Fig. III-4. The *C. quinoa* leaves inoculated with the chimeric RNA 3 containing two CMV MP genes together with BMV RNAs 1 and 2. "Cmp" and "CmpDC33" indicates the genes of intact and truncated CMV MP, respectively. Positions of the MP genes are indicated in order of 5' to 3' in RNA 3. The photographs were taken at 7 days pi.

CONCLUSION

The CMV MP plays an essential role in cell-to-cell movement of CMV. As an approach

to solve the mechanism how CMV moves from cell to cell, cloning vectors in which the CMV

genes were replaced with those of CMV were analyzed in this study. These cloning vectors

were able to replicate in prokaryotic cells and to complement machinery of BMV. It was a desirable

view in which the BMV MP was replaced with that of CMV MP. In this study, we found that

cell in *C. guinea*, a vector in which the CMV MP was replaced with that of BMV MP, was able to

character of the CMV MP. The CMV MP requires to interact CP and the BMV MP requires to

movement, since the BMV MP is a protein which is not able to move with the

corresponding protein. These results suggest that the BMV MP is not able to move with the

demonstrate that the BMV MP is not able to move with the corresponding protein. These

genes. On the other hand, the BMV MP is not able to move with the corresponding protein.

MP gene. The BMV MP is not able to move with the corresponding protein. These results

to cell (CMV). The BMV MP is not able to move with the corresponding protein. These

require the CP and the BMV MP is not able to move with the corresponding protein. These

interacts with CMV MP in vitro although it also interacts with the truncated CMV MP.

Moreover, the BMV MP is not able to move with the corresponding protein. These results

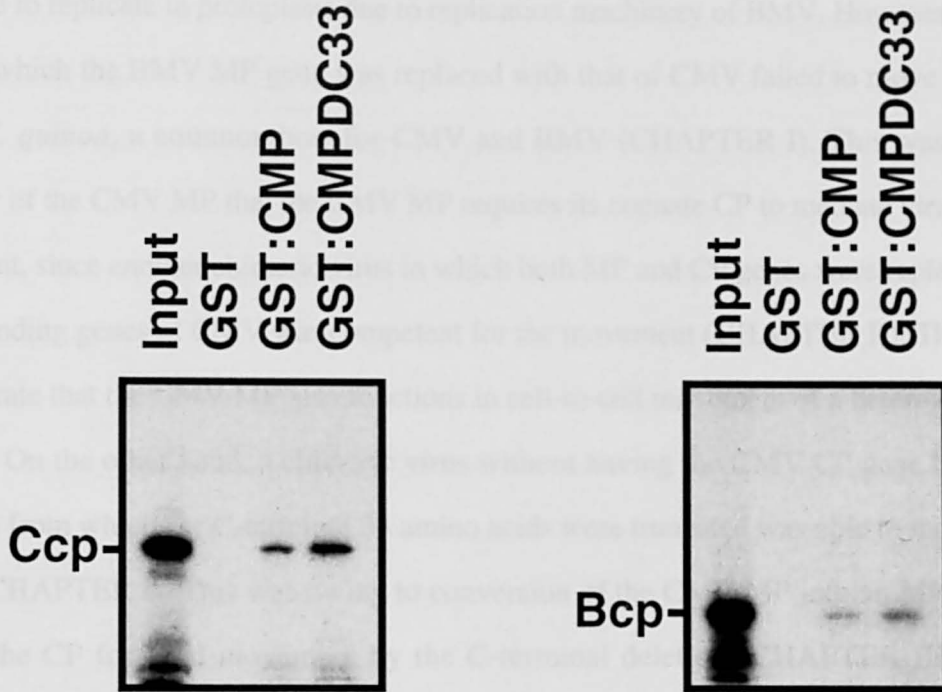


Fig. III-5. *In vitro* binding of the CPs to the intact and truncated CMV MPs. The binding of CMV CP (Ccp) and BMV CP (Bcp) was assayed by GST pull-down method and SDS-PAGE. The ^{35}S -labelled CP bound to the CMV MPs was visualized by fluorography. Input, the CP synthesized in the rabbit reticulocyte lysate cell-free translation system. GST, glutathione-S-transferase. GST:CMP, a fusion protein of GST with the intact CMV MP. GST:CMPDC33, a fusion protein of GST with the CMV MP with truncation of its C-terminal 33 amino acids.

CONCLUSION

The CMV MP plays an essential role in cell-to-cell movement of CMV. As an approach to solve the mechanism how CMV moves from cell to cell, chimeric viruses in which the BMV genes were replaced with those of CMV were analyzed in this study. Those chimeric viruses were able to replicate in protoplasts due to replication machinery of BMV. However, a chimeric virus in which the BMV MP gene was replaced with that of CMV failed to move from cell to cell in *C. quinoa*, a common host for CMV and BMV (CHAPTER I). This was owing to a character of the CMV MP that the CMV MP requires its cognate CP to mediate viral cell-to-cell movement, since another chimeric virus in which both MP and CP genes were replaced with the corresponding genes of CMV was competent for the movement (CHAPTER II). These findings demonstrate that the CMV MP also functions in cell-to-cell movement of a heterogeneous viral genome. On the other hand, a chimeric virus without having the CMV CP gene but the CMV MP gene from which the C-terminal 33 amino acids were truncated was able to move from cell to cell (CHAPTER I). This was owing to conversion of the CMV MP into an MP that did not require the CP for viral movement by the C-terminal deletion (CHAPTER III). CMV CP interacts with CMV MP in vitro although it also interacts with the truncated CMV MP. Moreover, coexistence of the intact CMV MP inhibited viral cell-to-cell movement mediated by the truncated CMV MP. These results suggest a possible scenario of CMV movement in which the binding of CP alters the CMV MP to a functional state for transporting viral genome.

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