Natural isolates of *Brome mosaic virus* with the ability to move from cell to cell independently of coat protein

Atsushi Takeda,† Wakako Nakamura,† Nobumitsu Sasaki, Kaku Goto, Masanori Kaido, Tetsuro Okuno and Kazuyuki Mise

Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

*Brome mosaic virus* (BMV) requires encapsidation-competent coat protein (CP) for cell-to-cell movement and the 3a movement protein (MP) is involved in determining the CP requirement for BMV movement. However, these conclusions have been drawn by using BMV strain M1 (BMV-M1) and a related strain. Here, the ability of the MPs of five other natural BMV strains to mediate the movement of BMV-M1 in the absence of CP was tested. The MP of BMV M2 strain (BMV-M2) efficiently mediated the movement of CP-deficient BMV-M1 and the MPs of two other strains functioned similarly to some extent. Furthermore, BMV-M2 itself moved between cells independently of CP, demonstrating that BMV-M1 and -M2 use different movement modes. Reassortment between CP-deficient BMV-M1 and -M2 showed the involvement of RNA3 in determining the CP requirement for cell-to-cell movement and the involvement of RNAs 1 and 2 in movement efficiency and symptom induction in the absence of CP. Spontaneous BMV MP mutants generated in planta that exhibited CP-independent movement were also isolated and analysed. Comparison of the nucleotide differences of the MP genes of BMV-M1, the natural strains and mutants capable of CP-independent movement, together with further mutational analysis of BMV-M1 MP, revealed that single amino acid differences at the C terminus of MP are sufficient to alter the requirement for CP in the movement of BMV-M1. Based on these findings, a possible virus strategy in which a movement mode is selected in plant viruses to optimize viral infectivity in plants is discussed.

INTRODUCTION

To establish systemic infection, plant viruses move from cell to cell via plasmodesmata and spread into upper leaves through the vasculature (Carrington et al., 1996). Virus movement proteins (MPs) play central roles in virus movement (Carrington et al., 1996; Lazarowitz & Beachy, 1999) and the roles of the coat protein (CP) in cell-to-cell movement differ among viruses (Callaway et al., 2001). *Tobacco mosaic virus*, *Red clover necrotic mosaic virus* and *Cowpea chlorotic mottle virus* (CCMV) do not require CP for cell-to-cell movement (Rao, 1997; Takamatsu et al., 1987; Xiong et al., 1993). These viruses have an MP that binds to nucleic acids (Citovsky et al., 1990; Fujita et al., 1999; Osman et al., 1992) and they are thought to move from cell to cell as an MP–RNA complex (Carrington et al., 1996; Lazarowitz & Beachy, 1999). On the other hand, many plant viruses require CP for cell-to-cell movement. There are several forms of cell-to-cell movement in this group. Viruses in the genera *Comovirus* and *Nepovirus* appear to move as mature virions through plasmodesmata that are structurally modified by tubular structures containing MP (van Lent et al., 1990; Wieczorek & Sanfaçon, 1993). *Cucumber mosaic virus* (CMV) requires CP, but not virion formation, for cell-to-cell and long-distance movement (Blackman et al., 1998; Kaplan et al., 1998; Schmitz & Rao, 1998). This virus is thought to move from cell to cell as a nucleoprotein complex distinct from the virion (Blackman et al., 1998).

*Brome mosaic virus* (BMV) is a positive-sense single-stranded RNA virus and the type species of the genus *Bromovirus* in the family *Bromoviridae* (Ahlquist, 1999). The genome of BMV consists of tripartite RNAs, designated RNA1, RNA2 and RNA3. RNA1 and RNA2 encode the 1a and 2a proteins, respectively, both of which are required for viral RNA replication (Kroner et al., 1989, 1990). RNA3 encodes the 3a protein and CP. The 3a protein is the MP of BMV, as it is essential for BMV movement (Rao, 1997; Schmitz & Rao, 1996; Takeda et al., 2004).

†These authors contributed equally to this work.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this paper are AB183259–AB183261.
Encapsidation-competent CP is also required for BMV movement (Okinaka et al., 2001; Rao & Grantham, 1995, 1996). BMV infection produces tubular structures containing virion-like particles on infected protoplasts (Kasteel et al., 1997). Collectively, BMV is thought to move from cell to cell as virions. Most studies on BMV infection have been undertaken using particular virus strains (e.g. strain M1) derived from the Russian strain (Ahluquist et al., 1984; Dreher et al., 1989), although many other strains of BMV have been isolated (Lane, 1974, 1981).

Plant viruses in a given family generally have similar features regarding the role of CP in cell-to-cell movement. However, at least one exception to that rule is found within the family Bromoviridae. BMV, CCMV and CMV show different types of requirement for CP and move from cell to cell as distinct forms, as mentioned above (Callaway et al., 2001). On the other hand, the form of cell-to-cell movement of these viruses is altered by mutations in the C termini of MPs (Nagano et al., 2001; Osman et al., 1999; Takeda et al., 2004). In CMV, truncation of the C terminus of MP confers the ability to mediate CP-independent movement (Nagano et al., 2001). In CCMV, truncation of the C terminus of MP causes the loss of the ability to mediate CP-independent cell-to-cell movement (Osman et al., 1999). Furthermore, a few amino acid substitutions in the C terminus of BMV-M1 MP are sufficient to alter its requirement for CP in cell-to-cell movement (Takeda et al., 2004). Interestingly, these substitutions do not affect the long-distance movement of BMV (Takeda et al., 2004), indicating that CP-dependent cell-to-cell movement is not a prerequisite for systemic infection by BMV. These observations led us to hypothesize that there are natural BMV strains that do not require CP for intercellular movement. In this study, to examine this hypothesis, we investigated whether the BMV MPs of five other strains mediate the cell-to-cell movement of the BMV M1 strain without CP.

**METHODS**

**Virus strains.** The MP genes of six BMV strains, M1 (Janda et al., 1987), M2 (De Jong & Ahluquist, 1995), KU1 (Mise et al., 1994), ATCC PV-47, ATCC PV-178 and ATCC PV-180 (American Type Culture Collection) were used in this study. Hereafter, BMV M1 and M2 strains are referred to as BMV-M1 and BMV-M2, respectively, and the three ATCC strains as ATCC47, ATCC178 and ATCC180.

**Construction of cDNA clones.** Plasmids pb1TP3, pb2TP5 and pb3TP8 (transcript name, B3/M1/CP) contain full-length cDNA clones of wild-type RNA1, RNA2 and RNA3, respectively, of BMV-M1 (Janda et al., 1987). Plasmids pb1WD1, pb2WD2 and pb3WD3 (transcript name, B3/M2/CP) contain full-length cDNA clones of wild-type RNA1, RNA2 and RNA3, respectively, of BMV-M2 (De Jong & Ahluquist, 1995). To construct pB3/KU1/GFP and pB3/M2/GFP, the 1.1 kb 5′–Aor–51HI fragment of pB3MAR7 (transcript name, B3/M1/GFP) (Sasaki et al., 2003) was replaced with the corresponding fragment of pB3/KU1/GFP (Mori et al., 1993) and pB3/M2/GFP (De Jong & Ahluquist, 1995), respectively. To construct pB3/M1(ACP/GFP) (transcript name, B3/M1/M2/GFP), we first determined the 3′ half of the cDNA sequence of pb3WD3 (GenBank/DDJ accession no. AB138261 for full-length M2-RNA3 sequence) and precisely replaced the CP gene in pB3WD3 with a green fluorescent protein (GFP) gene, using the same strategy as used to generate pB3/M1(ACP/GFP2) (transcript name, B3/M1/GFP2) (Takeda et al., 2004). The ATCC47, ATCC178 and ATCC180 strains of BMV were propagated in barley plants (Hordeum vulgare cv. Gose-shikokou) and cDNA fragments containing the respective MP genes of these strains were synthesized as described previously (Sasaki et al., 2001). The MP genes of these strains were then amplified by PCR using the appropriate primers and the amplified DNA fragments were sequenced directly by using a model 310 DNA sequencer (Applied Biosystems). DDBJ accession numbers for the MP genes of ATCC47 and ATCC180 are AB183259 and AB183260, respectively. To construct pB3/47/GFP and pB3/180/GFP, the MP gene of pB3MAR7 was precisely replaced with the MP genes of ATCC47 and ATCC180, respectively. The 0.2 kb Fba–Aor51HI fragment of pB3MAR7 was replaced with the corresponding fragments of pB3/KU1/GFP, pB3/M2/GFP and pB3/180/GFP to construct pB3/D281E/GFP, pB3/S297G + T299S/GFP and pB3/L275P/GFP, respectively. pB3/51RI + S81P/GFP, pB3/59Q/GFP, pB3/S297G/GFP and pB3/T299S/GFP were constructed by PCR-based site-directed mutagenesis of pB3MAR7 with the appropriate primers. Plasmids to be digested with FbaI, a dam methylation-sensitive restriction enzyme, were amplified in Escherichia coli JM110. The cDNA regions derived by PCR were sequenced to confirm the presence of only the desired mutations. All cDNA plasmids were linearized with EcoRI and capped viral RNA transcripts were synthesized as described previously (Kroner & Ahluquist, 1992). The transcript from the plasmid containing the RNA3 variant is referred to by its plasmid name without the prefix ‘p’, unless otherwise stated, as transcript names.

**Analysis of viral RNAs and proteins.** Transcripts of BMV RNA3 derivatives were inoculated together with BMV RNA1 and RNA2 transcripts onto Chenopodium quinoa plants as described previously (Nagano et al., 1999). Unless otherwise indicated, all RNAs 1 and 2 used as inocula were derived from BMV-M1 only throughout this study, except for one subsection in the Results section, where transcripts from BMV-M2, as well as those from BMV-M1, were used. GFP fluorescence in inoculated leaves of C. quinoa was detected as described previously (Sasaki et al., 2003). Tissue-print analysis to detect viral RNAs was performed as described previously (Mise et al., 1993). Distribution of CP in the inoculated leaves of C. quinoa was detected by hammer-blot analysis as described previously (Fujisaki et al., 2003). Accumulation of CP in the inoculated leaves of C. quinoa was examined as described previously (Takeda et al., 2004).

**In planta selection of MP mutants showing cell-to-cell movement independent of CP.** From C. quinoa leaves inoculated with B3/M1/GFP or B3/KU1/GFP, small pieces of leaf tissue that contained an infection site with multiple fluorescent cells were excised with a razor blade under an epifluorescence microscope. Progeny viral RNAs were then isolated from the collected pieces by using an RNeasy plant mini kit (Qiagen) according to the manufacturer’s instructions. DNA fragments containing the MP genes of these progeny viruses were amplified by RT-PCR using the appropriate primers and the MP gene of the amplified DNA fragments were sequenced directly. To construct pB3/E262A/GFP, pB3/Q524K/GFP and pB3/A44/GFP, the MP gene of pB3MAR7 was replaced with the MP genes of viruses recovered from B3/M1/GFP-inoculated leaves. To construct pB3/KU1/A23/GFP, the MP gene of pB3MAR7 was replaced with the MP gene of a virus recovered from B3/KU1/GFP-inoculated leaves. To construct pB3/A23/GFP, the 0.2 kb Fba–Aor51HI fragment of pB3MAR7 was replaced with the corresponding fragment of pB3/KU1/A23/GFP. To construct pB3/Q524K/CP, pB3/E262A/CP, pB3/A44/CP and pB3/A23/CP, the 0.5 kb ClaI–Aor51HI fragment of pB3MAR7 was replaced with the corresponding fragment of pB3/KU1/A23/GFP, respectively.
fragment of pB3TP8 was replaced with the corresponding fragments of pB3/Q254/K/GFP, pB3/E262A/GFP, pB3/D44/GFP and pB3/D23/GFP, respectively.

RESULTS

Differences in the MP gene sequence among six strains of BMV

To examine whether the dependence of MP on CP to mediate cell-to-cell movement is a general feature of BMV, we analysed six BMV MP genes derived from six BMV strains: M1 (Ahlquist et al., 1984), M2 (De Jong & Ahlquist, 1995), KU1 (Mise et al., 1994), ATCC47, ATCC178 and ATCC180. We determined the MP gene sequences of ATCC47, ATCC178 and ATCC180, and compared the nucleotide (Fig. 1) and the deduced amino acid (Table 1) sequences among the six strains. There are two to four amino acid differences between M1 and the five other strains (Table 1). Because there was no difference in the

<table>
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<th>ATCC180</th>
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<td>332</td>
<td>587</td>
<td>915</td>
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<tr>
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<td>59</td>
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Table 1. Differences in the deduced amino acid sequences of the MP’s of BMV strains

Bold letters indicate deduced amino acids different from those of the M1 strain.

MP gene sequence between ATCC47 and ATCC178, only the data for ATCC47 are shown in Fig. 1 and Table 1.

CP-independent cell-to-cell movement mediated by MPs from several BMV strains

To test whether the MPs of BMV strains other than the M1 strain require CP to mediate cell-to-cell movement, we used an infectious clone of the M1 strain as the genomic backbone for the following reasons: (i) bromovirus MP has been shown to determine the CP requirement for cell-to-cell movement independently of the genome context (Nagano et al., 1999; Sasaki et al., 2003), and (ii) no infectious clones of the three ATCC strains were available. To assess the CP requirement, we used an M1-RNA3 derivative, B3/M1/GFP, in which the CP gene is replaced by the GFP gene (Sasaki et al., 2003). Each MP gene of the M2, KU1, ATCC47 and ATCC180 strains was substituted for that of B3/M1/GFP, generating B3/M2/GFP, B3/KU1/GFP, B3/47/GFP and B3/180/GFP, respectively. Their infectivity was then examined on fully expanded (putative) leaves of C. quinoa by coinoculating them with BMV-M1 RNAs 1 and 2. In the C. quinoa leaves inoculated with these derivatives, GFP fluorescence was observed at 3 days post-inoculation (p.i.) under an epifluorescence microscope. In leaves inoculated with B3/47/GFP, most GFP fluorescence was confined to single cells as in B3/M1/GFP inoculation; we never detected fluorescent clusters containing over five fluorescent cells (Fig. 2). In contrast, in leaves inoculated with B3/KU1/GFP or B3/180/GFP, about 30% of fluorescent clusters with multiple cells contained three to five cells and some were expanded to six to ten cells (Fig. 2). These results imply that the MPs of strains KU1, ATCC180 and especially M2 have the ability to facilitate the cell-to-cell movement of BMV independently of CP.

Fig. 1. Differences in the nucleotide sequences of the MPs of BMV strains. Because no difference was found in the MP gene sequences of ATCC47 and ATCC178, only the data for ATCC47 are shown. The open box represents the MP gene. Lines in the box and numbers to the right of the box indicate the positions of the different nucleotides. Bold letters indicate nucleotides that are different from those in the MP gene of the M1 strain. The numbers shown at the left of the box (92 and 1003) are the first nucleotide in the initiation codon and the third nucleotide in the termination codon, respectively, of the MP gene on RNA3 of BMV-M1. Triangles indicate nucleotide differences that lead to amino acid differences.
Differences in the MP gene influence the requirement for CP in cell-to-cell movement

To determine which amino acid differences in BMV MP contribute to alterations in the requirement for CP in cell-to-cell movement, we constructed B3/M1/GFP derivatives that contained nucleotide substitutions in the MP gene and tested their ability to mediate cell-to-cell movement independently of CP by co-inoculation with BMV-M1 RNAs 1 and 2. Because we have recently shown that BMV RNA3 mutants with mutations at the C terminus of MP move from cell to cell independently of CP (Takeda et al., 2004), we first tested B3/S297G+T299S/GFP, B3/D281E/GFP and B3/L275P/GFP, which contain amino acid differences in the C-terminal region of MP (Table 1; Fig. 2). In the leaves inoculated with B3/S297G+T299S/GFP or

Fig. 2. Cell-to-cell movement of CP-defective, GFP-expressing BMV-M1 variants containing the MP genes of BMV strains. B3/M1/GFP, which is a BMV RNA3 derivative expressing GFP, was used for the analysis of cell-to-cell movement of MP variants. C. quinoa leaves were inoculated with B3/M1/GFP or its derivatives containing mutations in the MP gene, together with wild-type RNAs 1 and 2 of BMV-M1. B3/M1/GFP, B3/M2/GFP, B3/KU1/GFP, B3/47/GFP and B3/180/GFP encode wild-type MP of strains M1, M2, KU1, ATCC47 and ATCC180 of BMV, respectively. The data represent the numbers of infection foci containing the indicated number of green fluorescent cells at 3 days p.i. Refer to the legend to Fig. 1 for the schematic diagrams. Note that thick-lined boxes represent the MP gene of BMV strains other than BMV-M1 (thin-lined box) and the positions of nucleotide differences causing amino acid changes are presented as triangles and a bold line in the figure.

### Differences in the MP gene influence the requirement for CP in cell-to-cell movement

To determine which amino acid differences in BMV MP contribute to alterations in the requirement for CP in cell-to-cell movement, we constructed B3/M1/GFP derivatives that contained nucleotide substitutions in the MP gene and tested their ability to mediate cell-to-cell movement independently of CP by co-inoculation with BMV-M1 RNAs 1 and 2. Because we have recently shown that BMV RNA3 mutants with mutations at the C terminus of MP move from cell to cell independently of CP (Takeda et al., 2004), we first tested B3/S297G+T299S/GFP, B3/D281E/GFP and B3/L275P/GFP, which contain amino acid differences in the C-terminal region of MP (Table 1; Fig. 2). In the leaves inoculated with B3/S297G+T299S/GFP or
B3/D281E/GFP, 65–95% of infection sites with multiple fluorescent cells contained more than three cells, and some of those contained more than 20 cells at 3 days p.i. (Fig. 2). B3/D281E/GFP moved from cell to cell more efficiently than parental B3/KU1/GFP (Fig. 2). This suggests that a single amino acid difference (D281E) is sufficient to alter the CP requirement in cell-to-cell movement and that the effect of this amino acid difference is suppressed by other amino acid differences(s): I64L, S81P, and/or D166H in MP of the BMV KU1 strain. On the other hand, B3/180/GFP showed slightly more efficient movement than its derivative B3/L275P/GFP (Fig. 2), suggesting enhancing activity of S81P, as described below for MP of the M2 strain. We then tested B3/S297G/GFP and B3/T299S/GFP (Fig. 2). In leaves inoculated with B3/S297G/GFP, many infection foci containing multiple fluorescent cells were observed, whereas in those inoculated with B3/T299S/GFP, most GFP fluorescence was confined to one or two cells per infection focus at 3 days p.i. (Fig. 2). These results show that the amino acid differences in the C-terminal region of MP (especially S297G and D281E) alter the requirement for CP in the cell-to-cell movement of BMV-M1 and that single amino acid differences are sufficient for such an alteration.

As shown above, B3/S297G + T299S/GFP moved from cell to cell independently of CP, but not as efficiently as parental B3/M2/GFP (Fig. 2). The MP of BMV-M2 contains four amino acid differences from that of BMV-M1 (De Jong et al., 1995; Table 1), suggesting that the other two amino acid differences contribute to the altered requirement for CP in cell-to-cell movement of B3/M2/GFP. We then examined the infectivity of B3/E59Q + S81P/GFP, B3/E59Q/GFP and B3/S81P/GFP. However, all three mutants showed inefficient cell-to-cell movement (Fig. 2). Considering these data collectively, we conclude that a single amino acid difference (S297G) in the C-terminal region of the MP of BMV-M2 contributes mainly to altering the requirement for CP in cell-to-cell movement and that the other differences in the MP of BMV-M2 also function to enhance its ability to mediate cell-to-cell movement independently of CP.

**Table 2. Cell-to-cell movement of CP-deficient, GFP-expressing reassortants between BMV-M1 and BMV-M2**

C. quinoa leaves were inoculated with B3\(^{M2}/M2/GFP2\) or B3/M1/GFP2 together with wild-type RNAs 1 and 2 of BMV-M2 or of BMV-M1. The data represent the numbers of infection foci containing the indicated number of green fluorescent cells at 1 day p.i.

<table>
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<th>Inoculum</th>
<th>No. fluorescing cells</th>
<th>Total no. foci examined</th>
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<tr>
<td></td>
<td>1–2</td>
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</tr>
<tr>
<td>M1</td>
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**CP-defective BMV-M2 moves from cell to cell and induces necrotic local lesions in C. quinoa leaves**

To confirm that MP of BMV-M2 has the ability to mediate cell-to-cell movement independently of CP in the context of the BMV-M2 genome, as well as the BMV-M1 genome, C. quinoa leaves were inoculated with RNAs 1 and 2 of BMV-M2 together with B3\(^{M2}/M2/GFP2\), in which the CP gene of BMV-M2 RNA3 was precisely replaced with a GFP gene. At 3 days p.i., many infection foci containing multiple GFP-expressing fluorescent cells were observed. However, because most of the infection foci contained a small cluster of necrotized cells in their centres (data not shown), we could not record the precise number of fluorescent cells. Therefore, we counted GFP fluorescence at 1 day p.i. and found many infection foci containing multiple fluorescent cells (Table 2; Fig. 3a). At 5 days p.i., large necrotic local lesions were visible (Fig. 3b). These results show that BMV-M2 does not require CP in either cell-to-cell movement or the induction of necrotic local lesions on C. quinoa. We also carried out genome reassortment to investigate how genomic combinations influence the CP requirement for virus movement or symptom induction. We first inoculated B3\(^{M2}/M2/GFP2\) onto C. quinoa with RNAs 1 and 2 of BMV-M1. Many foci with multiple fluorescent cells were observed at 1 day p.i., whereas the cell-to-cell movement of this heterologous combination was slightly slower than that of B3\(^{M2}/M2/GFP2\) with RNAs 1 and 2 of BMV-M2 (Table 2). Interestingly, no necrotic local lesions were visible at 5 days p.i. on the leaves inoculated with B3\(^{M2}/M2/GFP2\) plus RNAs 1 and 2 of BMV-M1 (Fig. 3c). In contrast, neither multiple fluorescent cells nor necrotic local lesions were observed after inoculation with B3/M1/GFP2, in which the CP gene of BMV-M1 RNA3 was replaced with the GFP gene, together with RNAs 1 and 2 of BMV-M2 (Table 2; Fig. 3d) or of BMV-M1 (Table 2; Fig. 3e). These results suggest strongly that RNA3 (probably the MP gene), but not RNA1 and RNA2, play a crucial role in the CP requirement in BMV movement, but that RNA1 and RNA2 are involved.
in the rate of cell-to-cell movement and the induction of necrotic lesions on *C. quinoa*.

**Isolation of in planta-generated MP mutants showing cell-to-cell movement independently of CP**

During inoculation with B3/M1/GFP, we observed a few foci containing three to five fluorescent cells (Fig. 2). The development of these infection foci may occur due to either simultaneous inoculation of neighbouring cells or non-specific passive GFP movement, as discussed previously (Schmitz & Rao, 1996). Because a single nucleotide substitution in the MP gene of BMV-M1 is enough to alter the requirement for CP in cell-to-cell movement (Fig. 2), and because such single nucleotide substitutions are likely to occur in the course of bromoviral infections, as shown by our previous studies (Fujita et al., 1996; Sasaki et al., 2001), we considered the alternative possibility that mutants with the ability to move from cell to cell in a CP-independent manner had appeared in these infection foci. To test this alternative possibility, we searched for large fluorescent clusters containing more than 10 cells in *C. quinoa* leaves inoculated with B3/M1/GFP together with RNAs 1 and 2 of BMV-M1. We discovered such fluorescent clusters at a frequency of approximately one per 3000 foci, isolated the progeny virus RNAs from the fluorescent clusters and determined the nucleotide sequences of their MP genes by an RT-PCR-mediated direct sequencing method (Sasaki et al., 2003). We identified three B3/M1/GFP-derived mutants with a nucleotide substitution or an insertion in the MP gene of BMV-M1 (Fig. 4). The first mutant had a substitution at nt 851 from C to A, resulting in the amino acid substitution Q254K. The second mutant contained a substitution at nt 876 from A to C, resulting in the amino acid substitution E262A. The third mutant contained a 5 nt (GUCAG) insertion between nt 866 and 867, which resulted in seven amino acid changes from positions 260 to 266, the generation of a termination codon at amino acid position 267 and a total deletion of 44 authentic amino acids from the C terminus (Δ44). To examine whether these mutations in the MP gene are responsible for the requirement for CP in cell-to-cell movement, the transcripts of three B3/M1/GFP-derived cDNA clones with each mutation (B3/E262A/GFP, B3/Q254K/GFP and B3/Δ44/GFP) were inoculated together with RNAs 1

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**Fig. 3.** GFP fluorescence and symptoms in *C. quinoa* leaves inoculated with CP-deficient, GFP-expressing reassortants between BMV-M1 and BMV-M2. (a) A fluorescent focus on a *C. quinoa* leaf inoculated with B3/M2/GFP together with wild-type RNAs 1 and 2 of BMV-M2 at 1 day p.i. Bar, 200 μm. (b–f) Inoculated leaves of *C. quinoa* are viewed at 5 days p.i. Inocula for the leaves are B3/M2/GFP plus RNAs 1 and 2 of BMV-M2 (b), B3/M2/GFP plus RNAs 1 and 2 of BMV-M1 (c), B3/M1/GFP plus RNAs 1 and 2 of BMV-M2 (d), B3/M1/GFP plus RNAs 1 and 2 of BMV-M1 (e) or buffer only (f).
and 2 of BMV-M1 onto *C. quinoa* plants. Many fluorescent clusters containing several cells (more than six cells) were observed in these inoculations at 3 days p.i. (Fig. 4), showing that the B3/M1/GFP derivatives containing *in planta* mutations in the MP gene move from cell to cell independently of CP.

Furthermore, we accidentally discovered another infection site consisting of about 30 fluorescent cells in a leaf inoculated with B3/KU1/GFP plus RNAs 1 and 2 of BMV-M1. The size of this infection site was larger than that of other foci, which normally expanded up to six to ten cells at most. We inferred that a mutant with enhanced ability to move from cell to cell independently of CP had been generated. Therefore, we isolated progeny virus RNA from this infection site and determined the nucleotide sequence of its MP gene. This B3/KU1/GFP-derived mutant contained a deletion from nt 866 to 934 in the MP gene, resulting in an in-frame deletion of 23 aa from positions 259 to 281 of MP (Fig. 4 and data not shown). These results demonstrate that deletions in the C-terminal region of MP (*Δ23* and *Δ44*) interfere with systemic infection by BMV-M1. Moreover, only weak viral RNA signals were detected in the leaves inoculated with B3/Δ44/CP or B3/Δ23/CP, as well as B3/M1/CP, induced similar systemic symptoms (data not shown) and strong viral RNA signals were detected by tissue-print analysis in both inoculated and upper uninoculated leaves (Fig. 5a).

**Spread of MP mutants in the presence of CP**

To investigate the influence of the mutations identified in the *in planta* mutants on BMV-M1 infection in the presence of CP, four BMV RNA3 derivatives (B3/E262A/CP, B3/Q254K/CP, B3/ΔA44/CP, and B3/ΔA23/CP) were inoculated onto *C. quinoa* plants together with RNAs 1 and 2 of BMV-M1. Virus infectivity was estimated by the observation of symptoms and tissue-print analysis for the detection of viral RNAs, in inoculated and upper uninoculated leaves at 14 days p.i. *C. quinoa* plants inoculated with wild-type BMV-M1 (B3/M1/CP) developed systemic symptoms with leaf distortions (data not shown), as reported previously (Takeda et al., 2004). Inoculation of *C. quinoa* plants with B3/Q254K/CP or B3/E262A/CP, as well as B3/M1/CP, induced similar systemic symptoms (data not shown) and strong viral RNA signals were detected by tissue-print analysis in both inoculated and upper uninoculated leaves (Fig. 5a). These results demonstrate that the two point mutations (Q254K and E262A) in the C-terminal region of MP of BMV-M1 have little effect on either the cell-to-cell movement of or systemic infection by BMV-M1. On the other hand, B3/ΔA44/CP and B3/ΔA23/CP did not induce systemic symptoms even at 14 days p.i. (data not shown), nor did they cause the accumulation of detectable amounts of viral RNA in the upper uninoculated leaves (Fig. 5a). These results demonstrate that deletions in the C-terminal region of MP (*ΔA44* and *ΔA23*) interfere with systemic infection by BMV-M1. Moreover, only weak viral RNA signals were detected in the leaves inoculated with B3/ΔA44/CP or B3/ΔA23/CP. To investigate virus distribution in more detail, hammer-blot analysis of those deletion mutants was performed. BMV CP was detected throughout the leaves inoculated with B3/M1/CP (Fig. 5b), but was not detected in the leaves inoculated with B3/MP-fs/CP (Fig. 5b), which does not express functional MP because of a frameshift mutation in the MP gene ([B3B3a-FS in the paper by Takeda et al. (2004)]. In the leaves

**Fig. 4.** Cell-to-cell movement of BMV-M1 MP mutants with mutations that were generated *in planta*. *C. quinoa* leaves were inoculated with B3/M1/GFP or its derivatives containing mutations in the MP gene together with the wild-type RNAs 1 and 2 of BMV-M1. B3/E262A/GFP and B3/Q254K/GFP contain a single amino acid substitution in the C-terminal region of MP. B3/ΔA44/GFP contains a 5 nt (GUCAG) insertion leading to a frameshift mutation causing both a deletion of 44 authentic amino acids from the C terminus of MP and the addition of seven non-viral amino acids (260–266). B3/ΔA23/GFP contains an in-frame deletion of 23 amino acids from positions 259 to 281 of MP. The data represent the numbers of infection foci containing the indicated number of green fluorescent cells at 3 days p.i.
inoculated with B3/Δ44/CP or B3/Δ23/CP, small and faint BMV CP signals were detected (Fig. 5b). Moreover, Western blot analysis showed that BMV CP accumulated in leaves inoculated with B3/Δ44/CP or B3/Δ23/CP to levels about 50- to 100-fold lower than that accumulated after B3/M1/CP inoculation (Fig. 5c). These results demonstrate that B3/Δ44/CP and B3/Δ23/CP moved from cell to cell in the presence of CP, but more inefficiently than did the wild-type BMV-M1.

**DISCUSSION**

Based on previous studies of BMV-M1 and its related strain, it has generally been accepted that BMV requires encapsidation-competent CP for cell-to-cell movement (Callaway *et al.*, 2001). In this study, we demonstrate that BMV-M2 (De Jong & Ahlquist, 1995), which is derived from BMV-1 (Valverde, 1987), does not require CP for cell-to-cell movement (Table 2; Figs 2 and 3). Moreover, the MPs of the KU1 and ATCC180 strains showed a weak ability to potentiate the CP-independent movement of heterologous genomes of BMV-M1 (Fig. 2), suggesting that those strains themselves might move independently of CP. Our data indicate strongly that BMV may use different cell-to-cell movement modes, depending on the strain. To our knowledge, this is the first example of differences in the requirement for CP in plant virus cell-to-cell movement within one virus species. To date, the role of CP in cell-to-cell movement has been studied for many plant viruses (Callaway *et al.*, 2001). However, most studies have used only one strain for each virus species. Our results presented here indicate that several strains of a virus species should be analysed to generalize the role of CP of that virus species in cell-to-cell movement. We also need to...
specify the name of the strain of the virus species examined to precisely understand the nature of the species in terms of its CP requirement for cell-to-cell movement.

Sequence comparisons and mutational analyses of six strains of BMV demonstrated that the CP-dependent movement of BMV-M1 is controlled by MP. Furthermore, reassortment experiments further revealed that the difference in CP requirement between BMV-M1 and -M2 is attributable to RNA3, but not to RNA1 or RNA2, whereas RNA1 and/or RNA2 influences the efficiency of viral movement and the induction of host responses causing necrosis (Table 2; Fig. 3). These data confirm the essential role of MP in determining the movement modes of BMV. Moreover, the nucleotide sequences responsible for the conversion of the CP requirement, which were identified in this study, are located mainly in the C-terminal region of BMV MP (Figs 2 and 4), as reported previously in another system (Takeda et al., 2004). These results reinforce the essential role of the C terminus of BMV-M1 MP in forming a functional module that is involved in the CP requirement. In support of this, recent studies in CMV (Nagano et al., 2001), Alfalfa mosaic virus (Sánchez-Navarro & Bol, 2001) and Cowpea mosaic virus (Carvalho et al., 2003) have suggested or demonstrated an interaction between the C terminus of MP and the cognate CP.

We have identified several sequence changes in the MP gene that alter the requirement for CP in the cell-to-cell movement of BMV-M1 (Figs 2 and 4). How the requirement for CP is altered by such sequence changes remains unknown. However, recent studies of CMV may provide a clue to this unknown mechanism. Like BMV-M1, the Y and Fny strains of CMV require CP in cell-to-cell movement (Canto et al., 1997; Suzuki et al., 1991) and the requirement for CP is altered by a change in the MP sequence, i.e., deletion of the 33 C-terminal amino acids from the MPs of these CMV strains (Kim et al., 2004; Nagano et al., 2001). Andreev et al. (2004) and Kim et al. (2004) showed that the CMV MP with the 33 aa deletion binds viral RNAs more efficiently and strongly than does wild-type MP, and they suggest that CMV CP alters the MP conformation to increase its binding affinity for RNA through an indirect interaction between the two proteins. Similarly, some conformational change in the MP of BMV-M1 could be induced by the identified point mutations and deletions, leading to enhanced binding affinity for RNA and the formation of stable complexes of virus RNAs and MP. Such nucleoprotein complexes could somehow move between cells in a way distinct from the movement of virions or CP-associated complexes. Alternatively, the MP–RNA complexes could escape the host defence responses that are normally blocked by CP. Further studies are required to examine these hypothetical mechanisms.

The MP of BMV-M2 has a potent capacity to mediate cell-to-cell movement independently of CP, whereas those of the five other strains examined have no ability or only a weak ability to facilitate cell-to-cell movement in the absence of CP (Fig. 2). Why does BMV-M2 MP function in a way distinct from the other MPs? A unique feature of BMV-M2 is that it systemically infects the legume cowpea line TVu-612 (Valverde, 1987), although most known systemic hosts of BMV strains are monocotyledonous plants, such as barley. On the other hand, BMV-M1 does not systemically infect cowpea species, including TVu-612 (Mise et al., 1993; De Jong & Ahlquist, 1995). De Jong et al. (1995) examined the individual and synergistic effects of four amino acid differences in the MPs of BMV-M1 and BMV-M2 (Table 1) on local and systemic infection in TVu-612 and found that the differences enhance the rate of local and systemic infection synergistically. Interestingly, our results also show a synergistic effect of the four amino acid differences on CP-independent cell-to-cell movement (Fig. 2). These collective and correlative data suggest that CP-independent functions of BMV-M2 MP are involved in the faster local spread and successful systemic infection of the cowpea plant and that the change in the MP sequence of BMV-M2 optimizes its infectivity of the cowpea plant. Consistent with this possibility, we have recently found other amino acid changes in BMV-M1 MP that enable BMV-M1 to move partly or fully in the absence of CP (Sasaki et al., 2005). These amino acid changes have been identified as spontaneous mutations that allow a cowpea-non-adapted chimeric CCMV carrying the BMV-M1 MP gene to infect cowpea plants systemically (Fujita et al., 1996; Sasaki et al., 2001).

As shown by the MPs in BMV-M2, B3/Q254K/CP and B3/ E262A/CP, as well as in some alanine-scanning mutants (Takeda et al., 2004), one or a few nucleotide substitutions are enough to convert the requirement for CP in cell-to-cell movement of BMV-M1 without affecting its capacity for long-distance movement. Of these, the MPs of B3/ Q254K/CP and B3/E262A/CP appeared in planta during inoculation experiments in our greenhouse. These results imply that BMV MP mutants capable of CP-independent movement can appear in natural fields and survive by chance. Our data may also reflect a possible virus strategy in which a movement mode is selected in plant viruses, like BMV, that is between CP-dependent and -independent movements and optimizes virus infectivity of a particular range of plants. As discussed above, BMV-M2 may be an example of a strain in which a CP-independent cell-to-cell movement has been selected, allowing it to infect a leguminous plant, unlike other strains including BMV-M1.

ACKNOWLEDGEMENTS

We are grateful to Dr Paul Ahlquist for the cDNA clones of the BMV M1 and M2 strains. This work was supported in part by a Grant-in-Aid (12052201) for Scientific Research on Priority Area (A) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and a Grant-in-Aid (JSPS-RFTP96L00603) from the ‘Research for the Future’ programme, a Grant-in-Aid (15380035) for Scientific Research (B) and a Grant-in-Aid (13306005) for Scientific Research (A) from the Japan Society for the Promotion of Science.
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