

The C Terminus of Brome Mosaic Virus Coat Protein Controls Viral Cell-to-Cell and Long-Distance Movement

YASUSHI OKINAKA,[†] KAZUYUKI MISE,* ERI SUZUKI, TETSURO OKUNO, AND IWAO FURUSAWA

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

Received 28 September 2000/Accepted 9 March 2001

To investigate the functional domains of the coat protein (CP; 189 amino acids) of *Brome mosaic virus*, a plant RNA virus, 19 alanine-scanning mutants were constructed and tested for their infectivity in barley and *Nicotiana benthamiana*. Despite its apparent normal replicative competence and CP production, the C-terminal mutant F184A produced no virions. Furthermore, virion-forming C-terminal mutants P178A and D182A failed to move from cell to cell in both plant species, and mutants D181A and V187A showed host-specific movement. These results indicate that the C-terminal region of CP plays some important roles in virus movement and encapsidation. The specificity of certain mutations for viral movement in two different plant species is evidence for the involvement of host-specific factors.

One viral component, coat protein (CP), encoded by several positive-strand RNA plant viruses, is multifunctional. As well as protecting viral RNAs from degradation, the CP plays a major role in symptom modulation (12, 36, 43), replication (6, 20, 21), long-distance movement, and cell-to-cell movement (7, 29) in some viruses. To function in these roles, the CP is thought to interact physically with putative host-derived factors, as well as with viral components such as viral RNAs and CP itself (7, 29). However, not all RNA viruses share the requirement for CP in order to spread systemically, because deletion of the CP gene from either *Tomato bushy stunt virus* (44) or *Barley stripe mosaic virus* (35) has no significant effect on viral spread in plants. Interestingly, the CP is one of the most abundant proteins in virus-infected plants. The ability of CP to accumulate to high levels in host cells suggests that the CP may suppress or evade some plant defense responses, perhaps through interaction with host factors.

Brome mosaic virus (BMV) is a well-studied, tripartite, single-stranded, positive-sense RNA plant virus (3). RNA1 and RNA2 encode viral replicase proteins 1a and 2a, respectively, whereas RNA3 codes for the 3a movement protein (MP) and CP. The CP gene is expressed through a subgenomic mRNA, RNA4 (3). BMV replicase proteins have been characterized extensively by genetic and biochemical approaches (2, 46), and the 3a MP has also been well examined by several mutation analyses linked to phenotypic investigations (17–19, 31, 32, 36, 41) and by biochemical and cytological techniques (16, 23). On the other hand, the study of CP has progressed mostly in terms of capsid architecture (28), although information about its roles in viral infection has recently been accumulating. Flasin-ski et al. (15) reported that mutations, mainly introduced into the N-terminal and hydrophobic domains of BMV CP (BCP), affect multiplication as well as movement of the virus in barley

and in a variety of *Chenopodium hybridum*. More detailed examinations of the N-terminal region were performed (8, 36, 37, 39) and indicate that the N-terminal region, especially the arginine-rich domain, is important for virus infection in barley and *C. quinoa*. The seven N-terminal residues of BCP have significant effects on lesion formation in *Chenopodium* species. Whereas these studies have predominantly revealed some roles of the N-terminal and hydrophobic regions of BCP, the C-terminal and internal hydrophilic regions have been less well studied. These regions may be particularly important because they are probably displayed on the surface of the BCP molecule (45) and are therefore likely to interact with putative host plant factors and/or viral components. A deletion mutant study of BCP suggested that the loss of 12 C-terminal residues affected encapsidation, as well as virus infectivity, in barley and *Chenopodium* species (36). Moreover, all of the BCP-interacting barley proteins that we have recently identified (Y. Okinaka, K. Mise, and I. Furusawa, Abstr. 9th Internatl. Cong. Mol. Plant-Microbe Interact., abstr. 136, 1999) require at least the C-terminal portion of BCP for binding. Therefore, in this study, we investigated the roles of putative C-terminal and internal surface regions of BCP in virus infectivity by using BCP mutants, each of which bears a single or double consecutive amino acid substitution with alanine (9).

The plasmids used in this study are summarized in Table 1. The cDNA clones of wild-type BMV strain M1 (pB1TP3, pB2TP5, and pB3TP8) (22) were kindly provided by P. Ahlquist (University of Wisconsin—Madison). All BMV RNA3 mutants were made by site-directed mutagenesis (5) of pB3TP8 with sets of mutagenized forward primers (Table 1) and reverse primers that completely matched the corresponding BMV RNA3 sequences. The PCR products amplified with these primer sets were digested with restriction enzymes. The resulting DNA fragments contained the following mutagenized sequences: 251-bp *SlyI-SlyI* fragments in pB3SK052053AA, pB3EQ110112AA, and pB3SS128129AA; 244-bp *SacI-AvaIII* fragments in pB3SS078079AA, pB3NK082083AA, and pB3YL155156AA; a 59-bp *AvaIII-StuI* fragment in pB3HV-175176AA; and 138-bp *StuI-HindIII* fragments in pB3P178A, pB3T179A, pB3F180A, pB3D181A, pB3D182A, pB3F183A,

* Corresponding author. Mailing address: Laboratory of Plant Pathology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan. Phone: 81-75-753-6132. Fax: 81-75-753-6131. E-mail: kmise@kais.kyoto-u.ac.jp.

[†] Present address: Department of Plant Pathology, University of California, Riverside, CA 92521.

TABLE 1. Summary of plasmids used in this study

Plasmid ^a	Encoded polypeptide	Amino acid substituted ^b /relevant RNA sequence ^c
pB1TP3	BMV wild-type 1a	
pB2TP5	BMV wild-type 2a	
pB3TP8	BMV wild-type 3a and wild type CP	
pB3TP8-derived mutants	BMV wild-type 3a and CP variant	
pBSK052053AA	SK052053AA	52(S), 53 (K)/UCA AAG→GCA GCG
pB3SS078079AA	SS078079AA	78(S), 79 (S)/UCU UCU→GCU GCU
pB3NK082083AA	NK082083AA	82(N), 83 (K)/AAU AAG→GCU GCG
pB3EQ110112AA	EQ110112AA	110(E), 112 (Q)/GAG AAA CAG→GCG AAA GCG
pB3SS128129AA	SS128129AA	128(S), 129 (S)/UCC UCG→GCC GCG
pB3YL155156AA	YL155156AA	155(Y), 156 (L)/UAU CUG→GCU GCG
pB3HV175176AA	HV175176AA	175(H), 176 (V)/CAC GUA→GCC GCA
pB3P178A	P178A	178 (P)/CCU→GCU
pB3T179A	T179A	179 (T)/ACG→GCG
pB3F180A	F180A	180 (F)/UUC→GCC
pB3D181A	D181A	181 (D)/GAU→GCU
pB3D182A	D182A	182 (D)/GAC→GCC
pB3F183A	F183A	183 (F)/UUC→GCC
pB3F184A	F184A	184 (F)/UUC→GCC
pB3T185A	T185A	185 (T)/ACC→GCC
pB3P186A	P186A	186 (P)/CCG→GCG
pB3V187A	V187A	187 (V)/GUU→GCU
pB3Y188A	Y188A	188 (Y)/UAU→GCU
pB3R189A	R189A	189 (R)/AGG→GCG

^a pB1TP3, pB2TP5, and pB3TP8 were described in detail previously (22).

^b The amino acid residues substituted for with alanine are shown by the standard one-letter codes in parentheses accompanied by the numbers that indicate their position in BCP.

^c The short RNA sequences represent codon changes that induce amino acid substitutions. As an exception, the mutant EQ110112AA accompanies the unchanged AAA codon.

pB3F184A, pB3T185A, pB3P186A, pB3V187A, pB3Y188A, and pB3R189A. These DNA fragments were recovered after agarose gel electrophoresis. The corresponding restriction fragments in pB3TP8 were replaced with these fragments to produce the pB3TP8 derivatives, and the introduced mutations were then verified by automated DNA sequencing. Enzymatic DNA digestions, ligations, and transformations were performed by standard methods (40).

Barley [*Hordeum vulgare* L. cv. Hinodehadaka] and *Nicotiana benthamiana* plants were planted in a growth room at 25°C with 16 h of illumination per day and daily watering with half-strength Hoagland's solution (13). Synthesis of capped transcripts from *Eco*RI-linearized full-length cDNA plasmids pB1TP3 and pB2TP5, as well as pB3TP8 and its derivatives (26); inoculation of whole plants and protoplasts with transcripts (26, 31); extraction of total nucleic acids from plant leaves (4) and protoplasts (26); and preparation of samples for tissue printing analysis (31) were performed as described previously. Each experiment was repeated at least two or three times with independently synthesized *in vitro* transcripts.

For the identification of virion RNAs, viral RNAs were extracted from virion fractions isolated from infected barley protoplasts by polyethylene glycol precipitation (26) 24 h after inoculation. Northern blot analysis to detect positive-sense BMV RNAs was performed as described previously (24), except that the DIG (digoxigenin) Labeling and Detection kit (Roche, Indianapolis, Ind.) was used. Northern blotting patterns were densitometrically quantified with the NIH Image program v. 1.61. Western blot analysis of BCP was performed

as described previously (10) after suspension of infected protoplasts directly in Laemmli's sample buffer (27). Enzyme-linked immunosorbent assays (ELISAs) were carried out as described previously (34), and virus yields were estimated by using a serial dilution of purified BMV virion as the standard. In both assays, BCP was detected with a rabbit anti-BMV antiserum (ATCC PVAS-178; American Type Culture Collection).

Design of alanine-scanning mutagenesis for the putative external BCP regions. Virus infection of plants requires the association of host and viral factors, which is likely to occur between the external structures of the molecules. To verify whether the external regions of BCP are required for the viral infection of plants, we searched predicted surface sites on the BCP molecule by the method of Emini et al. (14) (Fig. 1) and performed their alanine-scanning mutagenesis (9), excluding the putative N-terminal surface regions previously investigated in detail (8, 15, 30, 36, 37, 39). In this way, 19 site-directed mutations were successfully introduced into the internal and C-terminal regions of BCP (Table 1). The intensive mutagenesis at the C terminus was performed because this region was expected to be important for BMV infection (36) and to be displayed outside of the BCP β -barrel conformation (45).

Effects of internal mutations of BCP on virus infectivity in barley. RNA3 derivatives with mutations causing double amino acid substitutions in the internal sites of BCP were tested for their infectivity in barley. RNA transcripts of the mutants were coinoculated with wild-type BMV RNA1 and RNA2 into both barley plants and protoplasts. Transfection of

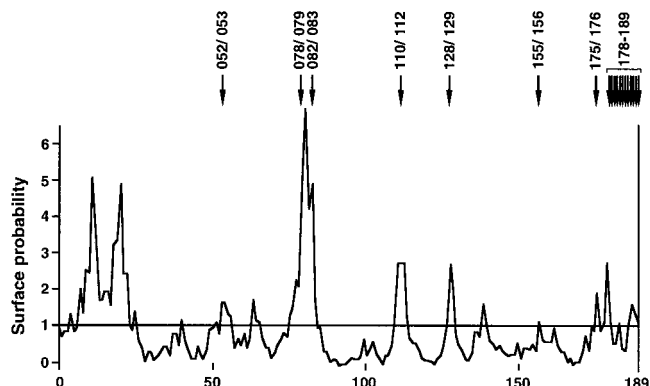


FIG. 1. Surface probability plot of BCP. The surface probability of BCP (189 amino acids) was analyzed by the method of Emini et al. (4). Arrows accompanied by numbers indicate the positions at which amino acid substitutions were introduced.

barley protoplasts with all of these mutants resulted in efficient accumulation of progeny viral RNAs. In addition, both the full-length CP (CP1) and the truncated CP (CP2), which is translated from the second AUG codon of RNA4 (39), were

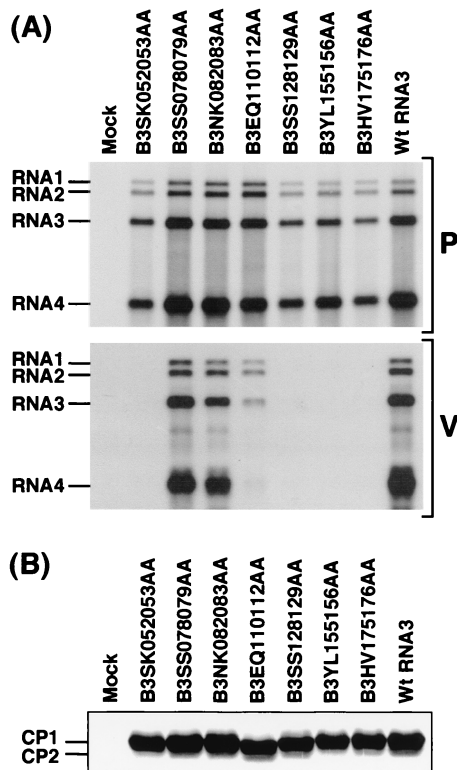


FIG. 2. (A) Replicative competence and encapsidation assays of BCP internal mutants. Barley protoplasts were transfected with the indicated wild-type (Wt) or variant RNA3 transcripts, together with wild-type RNA1 and RNA2. Progeny RNAs were extracted from the infected protoplasts (P) and purified virions (V) and subjected to Northern blot analysis. The positions of the four BMV RNAs are indicated on the left. (B) Western blot analysis of CP accumulation and integrity in the internal mutants. The positions of CP1 and CP2 (39) are indicated on the left.

TABLE 2. Analyses of BCP mutants bearing amino acid substitution in the putative internal surface regions

BCP mutant ^a	Encapsidation (arbitrary unit) ^b	Movement ^c		Virus yield (mg/g FW) ^d
		I	S	
Mock	0.0	-	-	0.0 ± 0.0
B3SK052053AA	0.0	-	-	0.0 ± 0.0
B3SS078079AA	1.1	+	+	2.5 ± 0.1
B3NK082083AA	0.8	+	+	1.3 ± 0.2
B3EQ110112AA	0.3	+	+	1.4 ± 0.0
B3SS128129AA	0.0	-	-	0.0 ± 0.0
B3YL155156AA	0.0	-	-	0.0 ± 0.0
B3HV175176AA	0.0	-	-	0.0 ± 0.0
Wild-type B3	1.0	+	+	4.0 ± 0.3

^a Wild-type BMV RNA3 or its derivatives were used as the inoculum together with wild-type BMV RNAs 1 and 2.

^b The Northern blotting data in Fig. 2 and from two other experiments were densitometrically analyzed, averaged, and indicated as an arbitrary unit.

^c The transcripts were inoculated on 6-day-old barley seedlings, and virus infectivity was measured by the existence of viral RNAs 2 weeks after inoculation. BMV RNAs in inoculated (I) and systemic (S) leaves were detected by tissue printing assay (31).

^d CP accumulation in secondary leaves (systemic leaves) was measured by ELISA and expressed as virion concentration. Data are means ± standard deviation for three replicates. FW, fresh weight.

accumulated, although the electrophoretic mobilities of the two CPs differed slightly (Fig. 2). However, encapsidation competence was reduced by 70% in B3EQ110112AA and abolished in B3SK052053AA, B3SS128129AA, B3YL155156AA, and B3HV175176AA. Northern blot analysis of the barley plants inoculated with these mutant transcripts revealed that B3SS078079AA, B3NK082083AA, and B3EQ110112AA produced progeny viral RNAs in both inoculated and systemic leaves, although no viral RNAs were observed with B3SK052053AA, B3SS128129AA, B3YL155156AA, or B3HV175176AA (Table 2). Virus accumulation was also estimated by using ELISA to measure the CP content in the systemically infected leaves of inoculated plants. In plants inoculated with B3SS078079AA, virus accumulation was found to be two-thirds that of plants inoculated with wild-type RNA3, and B3NK082083AA and B3EQ110112AA accumulated progeny viruses to a level one-third that of the wild type (Table 2). No CP accumulation was detected with B3SK052053AA, B3SS128129AA, B3YL155156AA, or B3HV175176AA. One interesting observation was that the infectivity of mutants B3SS078079AA and B3NK082083AA was significantly reduced in barley plants, although these mutants showed good encapsidation competence in protoplasts, indicating their defects in cell-to-cell and/or long-distance movement. No virus infectivity in plants inoculated with B3SK052053AA, B3SS128129AA, B3YL155156AA, or B3HV175176AA was observed, which is probably attributable to their lack of encapsidation competence. Sequence analysis of the progeny viral RNAs purified from the systemically infected leaves of barley was performed, as previously described (33), and demonstrated that all of the mutagenized positions shown in Table 1 were conserved after virus multiplication (data not shown).

Encapsidation competence of BCP C-terminal mutants. It was previously demonstrated that the deletion mutant of BCP that lacks the C-terminal residues 178 to 189 is deleteriously affected in its encapsidation competence and infectivity in plants of *Chenopodium* species (36). To further these investi-

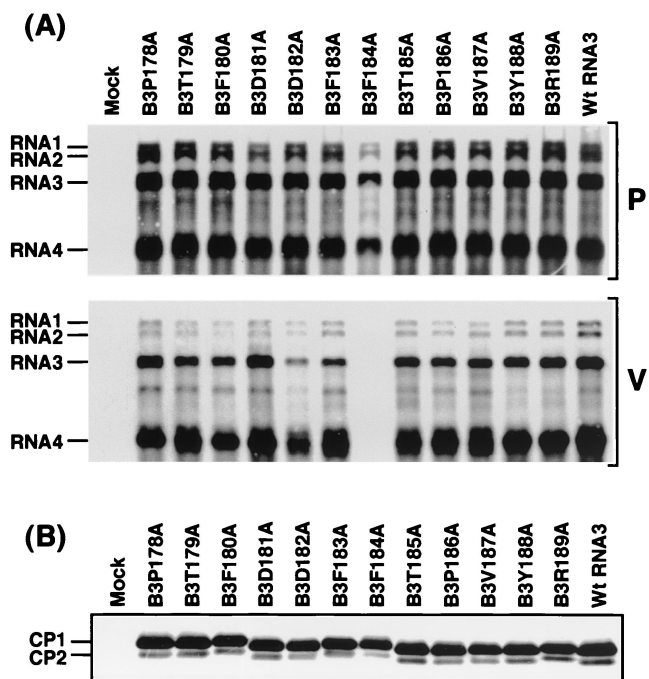


FIG. 3. (A) Replicative competence and encapsidation assays of BCP C-terminal mutants. (B) CP accumulation and integrity of BCP C-terminal mutants. All procedures were performed as described in the legend to Fig. 2.

gations, BCP mutants were constructed by introducing single amino acid substitutions with alanine at the C terminus (Table 1). Inoculation of barley protoplasts with these mutant transcripts, together with wild-type RNA1 and RNA2, revealed that progeny RNAs and CP accumulated to levels similar to those in protoplasts inoculated with wild-type RNA3 for all of the mutants tested (Fig. 3), although the electrophoretic mobilities of the mutated CPs differed slightly from one another.

Interestingly, in the encapsidation assay, progeny RNAs were not detected in the virion fraction when protoplasts were inoculated with B3F184A, whereas they were detected with all of the other mutants tested (Fig. 3A and Table 3). These results indicate that Phe¹⁸⁴ and/or the corresponding RNA sequence is important for the encapsidation process or the stability of the virus particles. A fine X-ray crystallographic study of the CP from a closely related bromovirus, *Cowpea chlorotic mottle virus* (CCMV) (45), indicates that Phe¹⁸⁴ (Phe¹⁸⁶ in CCMV CP) plays an essential role in dimer formation, an initial step in virion assembly. In dimer formation, Phe¹⁸⁴ may interact hydrophobically with many different amino acid residues in the parallel β structures of BCP. Furthermore, the conservation of this phenylalanine residue among four bromoviruses, BMV (1), CCMV (11), *Broad bean mottle virus* (38), and *Spring beauty latent virus* (K. Fujisaki, K. Mise, and I. Furusawa, unpublished data), suggests that this phenylalanine residue may be important in their encapsidation processes. The X-ray crystallographic data also suggest that Asp¹⁸² in BCP may contribute to hydrophilic CP dimer contacts. However, unexpectedly, the B3D182A mutant still displayed good encapsidation competence (Fig. 3, and Table 3).

Effects of C-terminal mutations of BCP on virus infectivity in different hosts. The infectivity of BCP mutants, each bearing a single amino acid substitution at the C terminus, was examined in both barley and *N. benthamiana* plants. Data are summarized in Table 3. Among the 12 C-terminal mutants, B3T179A, B3F180A, B3F183A, B3T185A, B3P186A, B3Y188A, and B3R189A showed vigorous systemic infections in both plant species and gave 49 to 102% virus yields relative to wild-type RNA3 in barley and 57 to 89% of wild-type yields in *N. benthamiana*. The effects of these seven mutations on virus accumulation were relatively minor compared with the effects of the other mutations described below. Interestingly, the phenotypic symptoms observed with B3T185A infection were mild (data not shown), even though the level of accumulated virus was similar to that with wild-type RNA3 infection.

TABLE 3. Analyses of BCP C-terminal mutant^a

BCP mutant	Result for:							
	Encapsidation (arbitrary unit) ^b	Barley		Virus yield	<i>N. benthamiana</i>			
		Movement			Movement		Virus yield	
		I	S		I	S	I	S
Mock	0.0	–	–	0.0 ± 0.0	–	–	0.0	0.0 ± 0.0
B3P178A	1.1	–	–	0.0 ± 0.0	–	–	0.0	0.0 ± 0.0
B3T179A	1.1	+	+	4.1 ± 1.8	+	+	7.6	2.9 ± 0.5
B3F180A	0.8	+	+	3.6 ± 0.5	+	+	4.4	2.6 ± 0.5
B3D181A	1.3	+	+	5.4 ± 2.2	+	–	0.4	0.0 ± 0.1
B3D182A	0.9	–	–	0.0 ± 0.0	–	–	0.0	0.0 ± 0.0
B3F183A	1.0	+	+	2.5 ± 0.8	+	+	1.5	2.6 ± 0.7
B3F184A	0.0	–	–	0.0 ± 0.0	–	–	0.0	0.0 ± 0.0
B3T185A	1.0	+	+	5.2 ± 1.6	+	+	4.7	4.0 ± 1.0
B3P186A	1.0	+	+	2.7 ± 0.1	+	+	2.6	2.9 ± 1.4
B3V187A	1.0	+	+	2.4 ± 0.5	+	+	0.3	0.6 ± 0.3
B3Y188A	0.9	+	+	3.7 ± 0.5	+	+	1.3	2.8 ± 0.3
B3R189R	0.8	+	+	3.4 ± 0.7	+	+	3.1	3.4 ± 1.1
Wild-type B3	1.0	+	+	5.1 ± 1.4	+	+	6.2	4.5 ± 1.0

^a All of the procedures and directions follow those described in the footnotes to Table 2 with the following exceptions: encapsidation data were obtained from Fig. 3 and two other experiments, and virus yield data obtained with the inoculated leaves of *N. benthamiana* are means of two independent experiments.

In contrast, no infectivity was detected with mutant B3P178A, B3D182A, or B3F184A in either plant species, even in the inoculated leaves (data not shown in part). However, the accumulation and encapsidation of viral RNAs occurred normally in barley protoplasts after inoculation with B3P178A and B3D182A, as mentioned earlier. For further confirmation of this causation, the virion fractions prepared from protoplasts infected with either of three movement-incompetent mutants, B3P178A, B3D182A, or B3F184A, were observed under the electron microscope as previously described (36). Icosahedral virions, apparently identical to those of wild-type BMV, were observed with inoculations of B3P178A and B3D182A, whereas no virions were detected with B3F184A (data not shown). A plausible explanation for the loss of infectivity in B3F184A is that the lack of encapsidation competence mentioned above interferes with virus multiplication in the host plants because BMV has been reported to move from cell to cell in virion form (25, 39, 42). The mutants B3P178A and B3D182A perhaps lack the ability of cell-to-cell movement, due to the defective interactions of the virions with BMV proteins or with host factors that are functionally similar in the two hosts. The former could include putative virion-MP interactions during transport through tubular structures (25). Our data are also consistent with the previous observation that cell-to-cell movement of BMV requires the CP, together with the 3a protein (42). By comparison with the X-ray crystallographic data of CCMV CP (45), the C-terminal residues of BCP (positions 178 to 189) would not be displayed either on the surface of the virion particle or on the dimer molecule. Therefore, residues Pro¹⁷⁸ and Asp¹⁸² may not mediate direct interaction of the virions with viral or plant factors, but may affect such interactions indirectly through a change in virion shape. However, our electron microscopy observations of B3P178A and B3D182A virions suggest that the change must be slight. Alternatively, nonassembled BCP monomer itself may play some role in virus movement, in which Pro¹⁷⁸ and Asp¹⁸² interact directly with BMV RNA, BMV proteins, and/or host factors. Of these interactions, the former two may involve the formation of a putative CP-MP-BMV RNA complex during the intra- and/or intercellular movement of BMV (7, 29). Mechanisms for these interactions are suggested by the fact that BMV 3a MP binds to BMV RNAs (16, 23). Finally, it is also possible that the changes at Pro¹⁷⁸ and Asp¹⁸² may elicit defense responses in the initially infected host cells (47).

Other noteworthy observations are that, with respect to the mutants B3D181A and B3V187A, systemic infections occurred in barley with virus yields of 106 and 47%, respectively, relative to that with wild-type RNA3, but were abolished and significantly reduced (13% wild-type virus yield), respectively, in *N. benthamiana*. With the mutants B3D181A and B3V187A, the virus yields were approximately 6 and 5% of that with wild-type RNA3, respectively, in the inoculated leaves of *N. benthamiana*. These results indicate that the B3D181A mutation does not affect virus infectivity in barley, but may inhibit both cell-to-cell and long-distance movement in *N. benthamiana*. Similarly, the infectivity of the B3V187A mutant was still high in barley, but its cell-to-cell movement ability in *N. benthamiana* may be reduced. Sequence analysis of the progeny viral RNAs purified from the systemically infected leaves (except in the case of *N. benthamiana* infected with B3D181, in which the

inoculated leaves were analyzed) demonstrated that all of the mutagenized nucleotides listed in Table 1 were conserved after virus multiplication (data not shown). A threshold of virus concentration required for successful long-distance movement might explain this phenomenon in part, but this possibility can be eliminated here, because B3V187A, which gave virus yields in inoculated leaves similar to or lower than that of B3D181A, still exhibited systemic infection. A possible explanation for these host-specific infections with B3D181A and B3V187A is that viral infection in *N. benthamiana* is supported by some host-specific factors that interact with wild-type BCP, but not with the mutant CP of either B3D181A or B3V187A. Alternatively, as discussed above, some defense response may be induced by these two mutants only in *N. benthamiana*.

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