Mutational Analysis of the Coat Protein Gene of Brome Mosaic Virus: Effects on Replication and Movement in Barley and in *Chenopodium hybridum*

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The coat protein (CP) open reading frame (ORF) of brome mosaic virus (BMV) has been mutated to study host-related CP functions in barley, a systemic host, and in *Chenopodium hybridum* L., which supports both local lesion formation and systemic spread of BMV. To test the role of the N-terminal region of CP, mutants C1 to C3, which synthesized the CP lacking first seven amino acids, and mutant D1, which had Trp 22 and Thr 23 replaced with Phe-Gly-Ser, were generated. C1 to C3 inhibited virus systemic spread in *C. hybridum* but not in barley while D1 only reduced virus accumulation in noninoculated leaves of *C. hybridum*. More internal CP regions were tested by mutation of Lys 63 to Leu (mutant SP3) and Lys 129 to Arg (mutant SP1). SP1 behaved similarly to C1 to C3 while SP3 similarly to D1. In addition, SP3 reduced concentrations of RNA3 and RNA4 in both hosts. Apparently, various CP regions differentially affect, either directly or indirectly, virus translocation in different hosts, suggesting both the CP and host factors to be important for virus spread. Larger deletions in the CP ORF (mutants BB4 and SX1) or a decrease of CP production by using a frameshift mutant C, inhibited virus systemic spread in both hosts, and delayed the appearance of smaller local lesions on *C. hybridum*. Thus, the CP is not required for cell-to-cell movement but is required for systemic translocation of BMV.

Additional keywords: Bromoviridae, systemic and cell-to-cell movements, limiting host responses.

Brome mosaic virus (BMV) is a tripartite single-stranded, positive-sense RNA plant virus. RNA1 and RNA2 segments of BMV encode two viral replicate proteins (Ahliquist et al. 1991), whereas RNA3 encodes the movement protein and the coat protein (Sacher and Ahliquist 1989; Allison et al. 1990; De Jong and Ahliquist 1991). BMV replicate polypeptides have been characterized extensively (Ahliquist et al. 1991; Kao et al. 1992; Kao and Ahliquist 1992). Using BMV and cowpea chlorotic mottle virus (CCMV) pseudorecombinants, Allison et al. (1988) have shown that host specificity determinants are encoded by RNA3 as well as by RNA1 and/or RNA2. Traynor et al. (1991) demonstrated that mutations in the 2a protein affected virus spread and virus yield in both inoculated and uninoculated barley leaves. Hybrid BMV/CCMV viruses, in which the 3a protein genes have been precisely exchanged, systemically infected *Nicotiana benthamiana*, a permissive host for both parental viruses. However, the hybrids failed to systemically infect either barley or cowpea, selective hosts for the parental viruses, demonstrating that bromovirus movement protein plays a crucial role in host specificity (De Jong and Ahliquist 1992; Mise et al. 1993).

There are two in-frame AUG codons involved in the initiation of BMV CP synthesis, with the second one located eight codons downstream. The functionality of the second AUG codon in CP translation was demonstrated by finding that virion preparations of wild-type BMV isolated from barley contain a small amount of truncated CP (Sacher and Ahliquist 1989). This result has been confirmed by showing that deletion of the first eight codons allowed translation to start from the downstream AUG and did not affect RNA packaging and systemic infection in barley plants. A laboratory strain of BMV (ATCC 66) produced an equal ratio of two types of CP because mutations 5' to the first initiation codon reduced its translational activity and increased translation from the second AUG codon (Mise et al. 1992). A frameshift mutation between the first and the second AUGs did not prevent local lesion formation in *C. hybridum* (Bujarski et al. 1994) but abolished BMV infection in barley (Sacher and Ahliquist 1989). In barley protoplasts, however, this mutant produced a low concentration of truncated CP but did not form viroids (Sacher and Ahliquist 1989).

Within the first 25 amino acids of BMV CP there are eight basic residues and no acidic residues, suggesting that this region may neutralize the negative charge of RNA in encapsidation (Sacher and Ahliquist 1989). Involvement of the N-terminal CP sequences of BMV in virion RNA interactions was demonstrated by Sgro et al. (1986). It has been recently confirmed *in vitro* by Duggal and Hall (1993). Sacher and Ahliquist (1989) have shown that deletion of the first 25 amino acids inhibited RNA packaging in protoplasts and systemic infection in whole barley plants. Similarly, the N-
terminal arm of 25 amino acids of CCMV CP was able to bind certain oligonucleotides, indicating its role in virion assembly (Vriend et al. 1986).

In this work we have identified further host-dependent functions of the BMV CP gene. By testing a series of BMV CP variants bearing mutations at several locations, we demonstrate that different mutations have different effects on such virus features as systemic spread, the molar ratio of individual RNA components, and virion stability. We show that CP RNA sequences and/or CP molecules are not necessary for short-distance migration but are required for leaf-to-leaf spread. By using both local lesion and systemic hosts we demonstrate that different mutants interact differently with different plants. These data provide evidence that both CP and host factors are involved in the translocation of BMV.

RESULTS

Generation of BMV coat protein mutants and their stability during infection.

As shown in Figure 1, with the exception of basic terminal regions, other CP domains conserved among three bromoviruses are hydrophobic, alternating with more heterogeneous hydrophilic domains. To assess the role of CP in infection, we have generated three groups of CP mutations. The first group had limited amino acid changes positioned at several locations (Table 1). Two mutants had single amino acid substitutions at internal hydrophobic domains: A basic (Lys) amino acid at position 63 has been replaced by a neutral (Leu) residue (mutant SP3) as a result of a double transversion AA to CU; or, a basic (Lys) residue at position 129 was replaced by another basic (Arg) residue in mutant SP1 due to an A to G transition. Mutant SP3 was stable during prolonged infections or after several passages through C. hybridum or barley. Mutant SP1 was stable after one passage through these hosts. Mutant D1 had three U residues inserted in a region variable among three bromoviruses, between the basic N terminus and the first hydrophobic domain (Fig. 1). Since these three U residues were inserted at two separate locations, they caused the substitution of an aromatic (Trp) and a polar (Thr) residues at positions 22–23 with another aromatic (Phe) and polar (Ser) amino acids, and an insertion of a neutral (Gly) residue. Mutant D1 was selected as a pseudorevertant from a single local lesion on C. hybridum after inoculation with a frameshift mutant D. This mutant D contained an additional U residue between nucleotides 1320–21 (Bujarski et al. 1994).

The second group of mutants had larger modifications at the N terminus. Specifically, mutants C1, C2, and C3 were selected as pseudorevertants after two passages of a previously generated frameshift mutant C (Bujarski et al. 1994) through C. hybridum. Mutant C had a four-nucleotide insert (UCGA) between the first and second CP AUG codons (Table 1). Pseudoreversion events altered the first AUG codon of mutant C to ACG, GUG or UUG, respectively, so that a CP molecule missing the seven N-terminal amino acids was synthesized efficiently from the second in-frame AUG codon.

The third group of mutants contained two deletion mutants, BB4 and SX1 (Fig. 1 and Table 1). These mutants were generated to test the function of larger blocks of CP sequences in BMV-host interactions. BB4 had the entire CP ORF and part of the subgenomic RNA4 promoter region removed, while SX1 could synthesize only one two amino acid N-terminal fragment of BMV CP followed by four amino acids from another frame.

All the above mutants were infectious to at least one of the two hosts. Progeny RNA3 isolated from local lesions or systemic leaves of C. hybridum and barley were always checked by sequencing to confirm the presence of the original muta-
Table 1. Nucleotide and amino acid sequence alterations in individual BMV coat protein mutants and comparison of the reactions on two host plants*  

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nucleotide acid level</th>
<th>Amino acid level</th>
<th>C. hybrifum</th>
<th>Barley</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NLL</td>
<td>SI</td>
</tr>
<tr>
<td>C</td>
<td>5'AGUG  UCGA UC83'</td>
<td>Frameshift</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>C1</td>
<td>5'ACGG UCGA UC83'</td>
<td>_7 aa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C2</td>
<td>5'GUGG UCGA UC83'</td>
<td>_7 aa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C3</td>
<td>5'UGUG UCGA UC83'</td>
<td>_7 aa</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Wild-type</td>
<td>5'AGUG UCGA3 (125-7)</td>
<td>wt</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>D1</td>
<td>5'UUUG GGA UC3'</td>
<td>Phe Gly Ser</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trp Thr (22-23)</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>SP3</td>
<td>5'CUGA3'</td>
<td>Leu</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5'AAAGA3' (1440-2)</td>
<td>Lys (63)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SP1</td>
<td>5'AGA3'</td>
<td>Arg</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5'AAAGA3' (1638-40)</td>
<td>Lys (129)</td>
<td>_</td>
<td>+</td>
</tr>
<tr>
<td>Deletion mutants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB4</td>
<td>Δ(1225-1861 nt)</td>
<td>-CP</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SX1</td>
<td>Δ(1258-1760 nt)</td>
<td>-CP</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* NLL, necrotic local lesion; SI, systemic infection; +, virus detected; -, no virus detected. Mutated nucleotides and amino acids are shown in boldface. Numbers indicate positions of nucleotides (Nucleotide acid level) in RNA3, or positions of amino acid (Amino acid level) in the CP.

This demonstrated that all the mutants were stable during infection, at least after one passage. Revertants containing wild-type CP sequences have been found in the progeny of SP1, C, and C1 to C3 mutants after several passages through these hosts. The presence of _' marker mutations (Spe1 linker in SP3 or BamHI restriction site in C, C1, to C3) excluded the possibility of contamination with wild-type BMV. Those revertants were used to test the effect of marker mutations on BMV infectivity in barley and _C. hybrifum_. The presence of the Spe1 linker or additional AT residues (inserted to create BamHI site) had no detectable effect on BMV infectivity in both hosts (data not shown).

Effects of CP mutations on virus infectivity, accumulation, and spread.

In our experiments we used a cultivar of _C. hybrifum_ which has green stems and petioles. In this cultivar, BMV causes small local lesions on the inoculated leaves, yellow mottling on systemically infected leaves, and degeneration of the apical bud (Verduin 1978). In the cultivar with purple stems and petioles, BMV causes large local lesions but does not spread systemically (Bancroft 1972). Thus, the green cultivar was useful for estimating the effects of CP mutations on both short- (local lesion formation) and long-distance (systemic) movements of the virus. As shown in Table 1, all mutants induced local lesions on _C. hybrifum_. However, only mutants D1 and SP3 spread systemically to upper leaves of _C. hybrifum_, indicating that different factors, which are either virus or host encoded, participate in BMV cell-to-cell and systemic spread in this host. To test if the observed effects are host-specific, the C, C1 to C3, D1, SP1, SP3, BB4, and SX1 mutants were inoculated to barley plants. With the exception of mutants that do not produce virions (i.e., C, BB4, and SX1) all other mutants caused BMV systemic infection in barley (Table 1). This suggested that CP or CP sequences contribute differentially to the systemic spread of BMV in barley and in _C. hybrifum_ hosts. Typical symptoms on non-inoculated leaves of barley appeared 5 days after mechanical inoculation with either the wild-type or mutated RNA3 in conjunction with the wild-type RNA1 and 2. Timing of symptom appearance suggests rapid long-distance movement of BMV in barley vasculature rather than slow leaf-to-leaf movement in the cortex.
To test the involvement of CP in necrotic reactions in *C. hybridum*, the course of development of local lesions was studied. Some infectious CP mutants (D1, SP1, and SP3) did not change either the size or time of appearance of the lesions (5–7 days after inoculation) as compared to those induced by wild-type RNA3 (data not shown). This revealed that cell-to-cell transport was not affected by these mutations and/or that the activation of limiting host defenses was not changed. However, those mutants that synthesized none (BB4) or a very small amount of CP (frameshift mutant C), or a short CP fragment (SX1) gave smaller lesions that appeared 3–7 days later postinoculation than the wild type (data not shown). This demonstrated that the lack or low concentration of CP decreased the cell-to-cell virus transport. Alternatively, the host defense reactions were more destructive for the non-encapsidated BMV RNA.

Dot blot hybridization was used to find out whether virus RNA accumulation correlated with symptom formation in barley plants and *C. hybridum* (Fig. 2). In barley, the SP1 mutant accumulated significantly less RNA than wild-type or other mutants, but the symptoms induced by all these mutants were indistinguishable from wild-type BMV. Apparently, viral RNA concentration did not correlate with the intensity of symptoms. In *C. hybridum*, significant differences in viral RNA concentration were detected between the first and the fourth systemic leaves. While in the first uninoculated leaf only the wild-type virus accumulated, in the fourth leaf significant amounts of the wild-type, D1, or SP3, but not C1, C2, and SP1 mutants accumulated. SP3 and D1 mutations decreased the virus concentration at least twofold. The dot blot hybridization assays confirmed that the lack of systemic symptoms in *C. hybridum* correlated with the inability of C1, C2, and SP1 mutants to move from leaf to leaf. Accumulation of the C3 mutant has been caused by its reversion to the wild-type sequence. The possibility of contamination with wild-type BMV was excluded because *BamHI* marker mutation was found at the 3′ end of progeny RNA of the C3 mutant.

BMV RNA accumulation in barley protoplasts revealed

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**Fig. 3.** Northern blot analysis of accumulation of RNAs of selected BMV CP mutants in barley protoplasts (A) and in local lesions on *Chenopodium hybridum* (B). A, Barley protoplasts were inoculated as described in the text and total RNA isolated 24 hr postinoculation. The experiment was repeated twice. B, *C. hybridum* plants were inoculated as described in the text and total RNA was isolated from local lesion tissue 2 days after the lesions appeared (between 6 and 10 days postinoculation). The RNAs extracted from three local lesions were analyzed to assess possible variability of viral RNA accumulation. Note that the RNAs in wild-type infections accumulated to much higher levels than those in mutant infections. A purified wild-type virion BMV RNA was used as a positive control (0.1 μg; lanes "Control").
some differences among individual CP mutants (Fig. 3A). Frameshift mutant C accumulated smaller amounts of all RNA components than the wild-type virus, while mutants C1 and SP3 accumulated relatively smaller amounts of RNA4 than other variants.

The BMV mutants studied in this work can be divided into two phenotype groups based on the responses of C. hybridum to infection. The first phenotype group caused local lesions but did not spread systemically in this host (BB4, C1, C2, C3, SP1, and SX1), while the second one caused local lesions and spread systemically (D1 and SP3). One could speculate that the former mutants did not spread systemically because they accumulated with a lower rate, thus enhancing the efficiency of their localization in C. hybridum. To determine the rate of viral RNA accumulation in local lesions, total RNA was isolated from individual local lesion tissue (three lesions for each mutant) and analyzed by Northern blot hybridization (Fig. 3B). The accumulation of BMV RNAs for each analyzed CP mutant was smaller than that for the wild-type virus and, as expected, differences among individual lesions for a given mutant were observed. Although the lowest RNA concentration was found for mutants that did not spread systemically, the systemically spreading mutants D1 and SP3 also accumulated a significantly reduced amount of BMV RNA.

Mutant SP3 accumulated reduced levels of the RNA4 component not only in barley protoplasts but also—unlike with the RNA3 component—in local lesions on C. hybridum (Fig. 3B), and in systemically infected barley plants (Fig. 4). The relative concentrations of BMV RNA components in total and in virion RNA preparations of SP3 from inoculated barley leaves were compared to investigate the role of encapsidation. Northern blot hybridization revealed that the concentration of RNA3 and RNA4 segments in both RNA preparations was much lower for SP3 mutant than for the wild-type virus (Fig. 4). This suggested that the RNA3/RNA4 reduction might be caused by other than encapsidation defect(s) (see also discussion).

Characterization of virion stability in vitro.

The ability to protect encapsidated BMV RNAs was tested in vitro to determine if the lack of systemic spread in C. hybridum resulted from decreased virion stability. Overnight storage in an acidic buffer (pH 6.0), with or without RNase A, did not affect RNA patterns in any mutant (data not shown). However, after the pH was increased to 7.4, which causes the swelling of BMV particles (Lane 1981), the storage at room temperature or RNase A treatment resulted in degradation of RNA encapsidated in SP1 virions, but not in other mutants or in the wild-type virions (Fig. 5). This was probably due to a greater swelling of the less stable viral particles. Although the observed decreased stability of SP1 virions under these conditions suggested a correlation with the lack of systemic movement in C. hybridum, this correlation did not hold for mutants C1 and C2. This suggested that either there is no correlation of particle stability and long-distance movement in this host or the virion stability assay was not sensitive enough to detect the important differences. Mutant D1, which had modifications in the area responsible for virion assembly (amino acids 22–23; Sacher and Ahlquist 1989), did not show any difference in virion stability. This indicated a tolerance for modifications at the region of virion assembly.

**DISCUSSION**

Virus spread involves two processes: Movement between surrounding cells through plasmodesmata and long-distance migration through the vascular system (Hull 1991; Maule 1991). Our observation that BMV mutants that did not contain the CP gene (BB4 and SX1) were capable of inducing local lesions in C. hybridum, as well as the data of Allison et al. (1990) which demonstrated a limited replication of CCMV without CP expression, suggest that bromoviral CP molecules are not required for short-distance movement. We did not do cytological analysis of the local lesions to determine how many cells have been infected. However, the amounts of virion RNA isolated from lesions induced by CP-deficient mutants were easily detectable by Northern blot hybridization and were comparable to the amount of RNA isolated from 1.5 × 10^5 protoplasts (Fig. 3A and B), which indicated infection in a large number of cells. Therefore, our data suggest that BMV infection can spread from cell to cell as a non-virion infectious entity. Such an entity could be similar to the nucleoprotein proposed for TMV by Deom et al. (1992), for example. That BMV cell-to-cell movement does not require CP is in contrast to models that have been proposed for cowpea mosaic virus (CPMV; van Lent et al. 1991; Wellink et al. 1993), and for cauliflower mosaic virus (CaMV; Perbal et al. 1993), which suggest that whole virus particles move from cell to cell. However, our observation that the appearance of lesions with BB4 and SX1 mutants required longer times and larger amounts of mutant BMV RNA transcripts (see Materials and Methods), suggests that CP molecules participate in efficient cell-to-cell movement. One possibility is that BMV can spread to surrounding cells not only as a CP-deficient

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Fig. 4. Northern blot analysis of brome mosaic virus (BMV) RNA accumulation in total and in virion RNA preparations (indicated on top) in barley leaves infected with SP3 or with wild-type viruses. RNA was isolated from the same amount of infected leaf tissue 6 days post-inoculation (see Materials and Methods).
nucleoprotein but also as whole virus particles. Alternatively, CP molecules can contribute to the process of cell-to-cell translocation by interacting with other viral or host proteins to form an efficiently translocating entity. An effect of CP on BMV RNA replication or protection of viral RNAs from limiting host defense responses (causing intracellular degradation) could also be important. Thus the observed changes in local lesion appearance might reflect both active viral and active host processes. It is less likely that the absence of CP increases the activation of limiting host responses because the appearance of local lesions for BB4, C, and SX1 mutants was significantly delayed.

An association between the CP and virus long-distance spread has been noted before (Dawson 1992; Hull 1991; Maule 1991). It seems that virus movement to upper leaves involves factors other than those involved in spread within the inoculated leaves. For instance, no systemic infection has been established even for certain tobacco etch potyvirus (TEV) mutants were able to move to surrounding cells (Dolja et al. 1994). Apparently, short- and long-distance TEV translocations required distinct host-virus interactions. Similarly, in the work described here (see Table 1), all BMV CP mutants accumulated within local lesions, while only some of them were capable of systemic spread in C. hybridum.

We cannot distinguish between the possibilities of either direct or indirect effects of the CP on the long-distance movement. A direct CP interaction with unknown host factor(s) could be required for the stabilization and/or movement of the leaf-to-leaf translocation-competent entity. The observed pattern of preferred accumulation of the mutants within more distal leaves at the apex of C. hybridum (Fig. 2) is typical of movement of metabolites in phloem. Therefore, a possible scenario may include the requirement of specific receptors for the CP in the phloem, as suggested for tobamovirus spread in tobacco (Dawson 1992; Hull 1991). Since some CP mutants were able to move long distance in barley but not in C. hybridum, virus-host interaction requirements for long-distance movement of BMV are probably different in these two hosts.

Indirect inhibitory effects of CP on long-distance movement could be due to reduction of the concentration of BMV translocation components or to increasing the limiting host defense responses. The former could be through inhibition of BMV RNA replication. Indeed, Hirikosha et al. (1987) demonstrated the effect of CP on BMV RNA replication in vitro, while Marsh et al. (1991) found that the BMV CP can modify the ratio of positive to negative RNA strands. Here, we did not observe major effects of CP mutants on BMV RNA replication in protoplasts (Fig. 3A). Also, a decreased accumulation of BMV-specific RNAs in local lesion tissue was observed for all mutants (Fig. 3B). These data do not allow us to recognize clearly if virus concentration in local lesions is critical for BMV escape in C. hybridum. As pointed out by Hilf and Dawson (1993), the establishment of a systemic infection may require a precise coordination between the processes of RNA synthesis, movement between cells, and the long-distance translocation. Thus, even small effects on RNA replication, in conjunction with alterations of virus accumulation within local lesions, may synergistically affect the ability to establish systemic BMV infection in the C. hybridum host.

As with cell-to-cell movement, we do not know the nature of viral components involved in long-distance translocation. On the basis of the results obtained for SP, the mutant that does not spread systemically and exhibits a reduced virion stability (Fig. 5), as well as for CP-deficient mutants and frameshift mutant C, which do not move systemically in both hosts, one might assume that BMV virions are transported long distance. However, this is not conclusive because the nonspreading mutants C1 to C3 did not reveal a reduced virion stability under the conditions tested. Testing of other mutants with reduced capsid stability should allow us to distinguish between the effects coming from virion stability or from sequence modifications. That some plant RNA viruses do not require virion formation to establish a systemic infection has been demonstrated for barley stripe mosaic virus (Petty and Jackson 1990).

![Fig. 5. Analysis of the ability of mutated brome mosaic virus (BMV) virions to protect the encapsidated RNA against RNase A degradation. Virions were incubated for 24 hr in Na-phosphate (pH 7.4) buffer at room temperature without (lanes "24 h") or with the addition of 10 ng/ml RNase A (lanes "RNase A"). Isolated RNA was analyzed by electrophoresis in 1% agarose gels. Mutant BMV CP nomenclature (shown above each lane) is described in the text. Control incubations (lanes "O") were done without RNase A for 24 hr at 4°C.](image)
There are several findings that demonstrate the requirement of CP sequences for elicitation of the hypersensitive response (HR) by plant virus infections. For instance, the CP mutations affected the HR of *N. sylvestris* to TMV (Knorr and Dawson 1988; Saito et al. 1987) or in tobacco to AIMV (Neeleman et al. 1991). In contrast, Wheaten et al. (1991) and Hacker et al. (1992) reported that the CP of turnip crinkle virus (TCV) is not required for eliciting local lesion formation on the inoculated leaves of *C. amaranticolor*. This was also observed for TMV on Xanthi-nc tobacco plants by Dawson et al. (1988). In this regard, BMV is similar to TCV and TMV because BB4 and SX1 mutants (lacking CP ORF sequences) elicited local lesions in *C. hybridum*. This demonstrates that BMV components other than CP ORF sequences and/or CP itself are responsible for the induction of at least some plant genes involved in the HR. Indeed, we have isolated a BMV 3a protein mutant that did not affect virus infectivity in barley and did spread systemically in *C. hybridum* without local lesion formation (Bujarski et al. 1994). This mutant (designated Bx4) had four additional amino acids near the C terminus of the 3a protein, indicating that 3a nucleotide and/or amino acid sequences participate in triggering HR in *C. hybridum*. The production of CP, however, increases the HR against BMV in *C. hybridum*, because the HR induction was significantly delayed by CP-deficient mutants. All these findings reflect a more general notion that a similar type of reaction can be induced by different gene products of viruses in different plants (Dawson 1992).

The reduced levels of the RNA3 and RNA4 components, a phenotype of the SP3 mutation observed in both hosts, represented a viral rather than a host-related feature (Fig. 4). Since these reduced levels were shown in both total and virion RNA preparations, it might result from decreased replication or decreased stability of RNA3/RNA4 molecules. The former could be due to some yet to be determined CP-RNA interactions during viral RNA synthesis, while the latter could be caused by a poor encapsidation of RNA3/RNA4, e.g. due to modifications within the putative encapsidation signal. Also, the SP3 mutation might decrease only the synthesis of the subgenomic RNA4, as was shown in protoplasts (Fig. 3A). Since those RNAs are encapsidated together, a molar excess of RNA3 to RNA4 would not be encapsidated and might be more susceptible to degradation in planta. An increased susceptibility of both mutated RNA3/RNA4 molecules to a direct degradation at the SP3 locus by plant defense mechanisms has to be also considered.

Our data allow us to discuss the involvement of some regions of BMV CP (or its gene) in host-related functions. The very N-terminal hydrophilic domain might participate in long-distance movement and in systemic infection in *C. hybridum*, because modifications in mutants C1 through C3 caused a deficiency in these functions. This, however, requires more studies because C1 to C3 mutants contain sequence modifications near the subgenomic RNA4 promoter, which reduces the production of CP-encoding RNA4 (Fig. 3A, lane C1). The internal hydrophobic domain around amino acid 129 must be involved in stabilization of the virion structure because the SP1 mutation markedly destabilizes the capsids. A domain located around amino acid 63 (mutant SP3) seems to be involved in regulation of RNA3 and RNA4 accumulation. A more upstream CP region is probably not involved in the above functions because of the lack of effects for a three-amino acid modification in mutant D1. We are currently investigating whether various functions of CP in the BMV life cycle can be mutated separately and mapped more precisely.

**MATERIALS AND METHODS**

**Materials.**

MMLV reverse transcriptase, T4 DNA ligase, Klenow fragment DNA polymerase I, T4 DNA polymerase, T7 RNA polymerase, and restriction enzymes were from Gibco BRL, Gaithersburg, MD. Vent DNA polymerase was from New England Biolabs, Beverly, MA. AMV reverse transcriptase and T7 DNA polymerase (Sequenase Version 2.0) sequencing kits were from United States Biochemicals (USB), Cleveland, OH. RQ1 DNase, RNasin, and the Altered Sites in vitro Mutagenesis System were from Promega, Madison, WI. Diguanosine triphosphate cap analog was from Pharmacia LKB Biotechnology. Cellulysin and macerozyme were from Calbiochem, La Jolla, CA. All radioactive chemicals were from Amersham, Arlington Heights, IL. Deoxyoligonucleotides were synthesized in an Applied Biosystems DNA synthesizer at Northern Illinois University.

Plasmids pB1TP3, pB2TP5, and pB3TP7 (a generous gift from Paul Ahlquist, University of Wisconsin, Madison) contained full-length cDNA copies of wild-type BMV RNA components 1, 2, and 3, respectively, and were used to synthesize infectious viral RNA transcripts.

**Plants and virus strain.**

Barley (*Hordeum vulgare* ‘Morex’) and the green cultivar of *Chenopodium hybridum* L. were maintained in the greenhouse with summer temperatures of 25–35°C and winter temperatures of 21–28°C, with approximately 5,000 lux illumination for 12 hr per day. The M1 (Russian) strain of BMV was used in all experiments (Janda et al. 1987).

**Mutagenesis of BMV coat protein gene.**

All BMV CP mutants shown in Table 1 were generated in the pB3TP7 plasmid (Janda et al. 1987) and were verified by nucleotide sequencing. Mutants SP1 and SP3 were generated by site-directed mutagenesis using, respectively, the following primers: 5′AGACTCTTCAGAGAGAGGTTGTC3′ (which substituted a G residue for an A at position 1639) and 5′GCGATTACAGCGCTAGCCACCAATGC3′ (which substituted residues AA with residues CT at position 1400–1401). This mutagenesis created *NheI* and *XhoI* sites, respectively. As a marker mutation an *SpeI* linker (GACTAGTC) was introduced into SP1 and SP3 mutants at a *BanII* site present in the 3′ noncoding region at positions 1835–40 (after the removal of the AGGC 3′ overhang with T4 DNA polymerase).

Frameshift mutant C was produced by digestion of pB3TP7 with restriction enzyme *SalI* (positions 1253–58), repair of the ends with Klenow DNA polymerase, and religation. Pseudorevertants C1, C2, and C3, which had the first AUG codon changed to ACG, GUG, or UUG, respectively, were selected after two passages of mutant C through *C. hybridum*. To obtain transcriptionally active cDNA clones for mutants C1, C2, and C3, corresponding progeny RNA3 was amplified as...
cDNA by a reverse transcription-Vent polymerase chain reaction (RT-PCR, see also: Analysis of RNA) and ligated to pB3TP7 between the BglII (positions 1221–26) and SstI (positions 1476–81) sites. Pseudorevertant D1 has been selected from a single local lesion on C. hybrida after inoculation with mutant D (Bujarski et al. 1994). Mutant D was obtained by site-directed mutagenesis using primer 5’GCCTTGGATCCGCTAGGG3’ representing BMV RNA3 sequence between nucleotides 1314–1329 with one nucleotide insertion (underlined). A transcriptionally active cDNA clone of mutant D1 was obtained by RT-PCR amplification, similarly to mutants C1, C2, and C3. All the above mutants contained BamHI marker mutation within the 3’ noncoding region, generated by insertion of AT residues between nucleotides 1861–2 (Bujarski et al. 1994).

The BB4 deletion mutant was constructed by removal from pB3TP7 of a fragment between BglIII and BamHI sites (generated as described above) and religation (Table 1). This mutant lacked the entire CP sequence, a part of the subgenomic RNA4 promoter and a small part (41 nt) of the 3’ noncoding region. To create mutant SX1, a fragment between SstI (position 1253–58) and XbaI (position 1760–65) restriction sites was removed from pB3TP7, followed by repair of the ends with Klenow DNA polymerase I, and religation. This retained the CP AUG codon and created a stop codon six codons downstream.

In vitro transcription and RNA inoculation.

Infectious BMV transcripts were synthesized from plasmids pB1TP3, pB2TP5, pB3TP7, and from pB3TP7 variants containing the mutation within CP ORF. In vitro transcription reactions and inoculations were performed as described by Janda et al. 1987. Briefly, 1 μg of EcoRI linearized plasmid was transcribed in a total volume of 10 μl. After RQ1 DNase treatments and extraction with phenol/chloroform, the RNA1, RNA2, and RNA3 (or RNA3 mutants) were mixed together in inoculation buffer containing 50 mM Tris-HCl, pH 8.0, 0.2% bentonite and 0.2% Celite. The resulting mixtures (20 μl) were mechanically inoculated on 14-day-old C. hybrida plants (in a 4- to 6-leaf stage) or on 5-day-old barley plants. However, for inoculation of plants with CP-deficient mutants we used five times more RNA3 transcripts than for other mutants or the wild-type BMV. The leaf tissues were collected 10–14 days postinoculation and used for either isolation of BMV viroids, extraction of total nucleic acids, or reinoculations.

Protoplast cells were isolated from 6-day-old barley seedlings using the method described by Loesch-Fries and Hall (1980), and inoculated with in vitro transcripts by a polyethylene glycol procedure (Samac et al. 1983). Approximately 1.5 x 10^6 protoplasts were inoculated with about 1 μg of each of the BMV RNAs, incubated for 24 hr at 24°C under continuous light, and the progeny RNA was isolated according to the method of Nagy and Bujarski (1992).

Analysis of RNA.

Virion RNA or total cellular RNA were extracted from infected tissue by homogenization in a 50 mM glycine buffer, pH 9.0, containing 1% SDS, 50 mM NaCl, and 10 mM EDTA (Nagy and Bujarski 1992). RNAs were purified by standard phenol/chloroform extraction and ethanol precipitation. To perform Northern blot hybridizations, total or virion RNA preparations were separated in 1% agarose gels and nucleic acids were transferred to a nylon filter (Hybnd-N®, Amer sham), as described by Kroner et al. (1989). The filter was probed with a 32P-labeled BMV RNA-specific minus-strand probe transcribed from plasmid pGEM3-B3-3 containing a cDNA insert representing the last 200 nt of BMV RNA3 (Nagy and Bujarski 1992).

To determine the nucleotide sequence of the progeny BMV RNA3, the RNA was amplified from either total or virion RNA preparations by an RT-PCR procedure similar to that of Nagy and Bujarski (1992). The Taq DNA polymerase was substituted with Vent DNA polymerase (NEB). The first strand synthesis primer (5’CAGTGAAATCTGTGTTCACTGTTT-TTAGAGATT-TCAG3’) was complementary to the 3’ end of RNA3 (positions 2095–2117), whereas the second strand primer (5’ACATAAGTTCTCCCTTCCAGTGG3’) represented the 3a protein ORF sequence (position 99–120). The resulting cDNA was analyzed in 1% agarose gels. The amplified cDNA was digested with EcoRI and cloned between the Smal and EcoRI sites of the pUC-19 cloning vector, and sequenced. In some cases, virion RNA was sequenced directly by a primer extension protocol using the AMV reverse transcriptase (Hahn et al. 1989).

Analysis of virion stability in vitro.

BMV viroids were partially purified by a single polyethylene glycol 8,000 precipitation step from 1 g of tissue homogenized in 0.5 M Na-acetate buffer containing 0.8 M acetic acid and 0.01 M MgCl2 (Lane 1986 ). More tissue (3 g) was used for purification of SP1 mutant viroids due to its lower titer in barley plants. The virus was resuspended in 600 μl of 30 mM Na-phosphate buffer, pH 6.0 or 7.4, and divided into three aliquots. One part was incubated at room temperature for 24 hr, the second was treated with 10 mg/ml RNase A at room temperature for 24 hr and the third was a control from which RNA was isolated after 24 hr of incubation at 4°C. The ribonuclease activity was neutralized by incubation with 0.1 mg/ml proteinase K for 15 min at room temperature. RNA was isolated by SDS-phenol extraction followed by ethanol precipitation. RNA patterns for each mutant were determined by electrophoresis in 1% agarose gel.

Computer-assisted sequence analysis.

Nucleotide and amino acid sequences of bromoviruses were compared and analyzed using programs BESTFIT, GAP, and PEPSTRUCTURE, of the University of Wisconsin Genetics Computer Group (UWGCG) sequence analysis package (Devereux et al. 1984).

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LITERATURE CITED
