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Positional Effect of Deletions on Viability, Especially on Encapsidation, of *Brome mosaic virus* D-RNA in Barley Protoplasts

Tri Asmira Damayanti, Hideaki Nagano, Kazuyuki Mise, Iwao Furusawa, and Tetsuro Okuno[1]

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

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Brome mosaic virus (BMV), a tripartite RNA plant virus, accumulates RNA3-derived defective RNAs (D-RNAs) in which 477–500 nucleotides (nt) are deleted in the central region of the 3a protein open reading frame (ORF), after prolonged infection in barley. In the present study, six artificial D-RNAs (AD-RNAs), having deletions of the same size as the naturally occurring D-RNA but at different positions in the 3a ORF, were constructed and tested for their amplification and encapsidation in barley protoplasts by coinoculation with BMV RNA1 and 2, or RNA1, 2, and 3. Northern blot analysis of RNA accumulation in total and virion fractions showed that deletions of 492 nt in the 3′-proximal and the 5′-proximal regions of the 3a ORF decreased encapsidation efficiency of the AD-RNAs compared with that of RNA3, whereas deletions in the central region enhanced encapsidation efficiency. The present results also show that deletion positions affect competition with RNA3 in the amplification and encapsidation of AD-RNAs.

Key Words: plant virus; bromovirus; *Brome mosaic virus*; defective RNA; RNA replication; encapsidation; RNA packaging; competition.

INTRODUCTION

Defective RNAs (D-RNAs) are deleted forms of a viral genome that are generated by replicase errors during viral RNA replication. They are incapable of autonomous replication and require the parental helper virus, which supplies essential components in *trans* (Roux *et al.,* 1991; White *et al.,* 1992). D-RNAs maintain *cis*-acting elements required for viral replication and encapsidation (Roux *et al.,* 1991; White *et al.,* 1992; Strauss and Strauss, 1997). D-RNAs that interfere with the replication of the helper virus genome are referred to as defective interfering RNAs (DI-RNAs) (Holland, 1990).

Because D- and DI-RNAs are a deleted form of the parental viral genomic RNAs, they provide a useful approach to identify *cis*-acting sequences for viral RNA replication and encapsidation (Holland, 1990). These studies have been done by using either naturally occurring or artificially constructed D- or DI-RNAs of many viruses, including *Sindbis virus* (Levis *et al.,* 1986), *Mouse hepatitis virus* (Kim *et al.,* 1993), *Brome mosaic virus* (BMV) (Duggal and Hall, 1993), *Cymbidium ringspot virus* (Havelda *et al.,* 1995), *Turnip crinkle virus* (Qu and Morris, 1997), and *Broad bean mottle virus* (Pogany *et al.,* 1997).

BMV, a member of the Bromoviridae, is an icosahedral, tripartite RNA plant virus. The genome of BMV consists of three species of messenger-sense single-stranded RNAs (Ahlquist, 1992). RNA1 (3.2 kb) and RNA2 (2.9 kb) encode the 1a and 2a proteins, respectively, which are required for viral RNA replication (French *et al.,* 1986; Kibertis *et al.,* 1981). RNA3 (2.1 kb) encodes the nonstructural 3a protein, which is required for cell-to-cell movement of the virus (Schmitz and Rao, 1996). Subgenomic RNA4 (0.9 kb), which encodes the coat protein (CP), is synthesized by the viral replicase from a promoter present in the minus strand of RNA3 (Miller *et al.,* 1985). The two gene products encoded by dicistronic RNA3 are dispensable for viral RNA replication, but are required for systemic infection in plants (Allison *et al.,* 1988; Rao and Grantham, 1995). RNA1 and RNA2 are encapsidated separately into individual particles, and RNA3 is encapsidated into a single particle together with subgenomic RNA4 (Lane and Kaesberg, 1971). Studies of BMV RNA encapsidation using CP deletion mutants have shown that a highly conserved N-terminal arginine-rich motif (ARM) plays an important role in RNA–CP interactions (Sacher and Ahlquist, 1989; Rao and Grantham, 1996). Recent studies further showed that amino acid residues located between residues 7 and 19 in the N-terminal ARM are intimately involved in interactions with each of the genomic and subgenomic RNAs of BMV in their encapsidation (Choi *et al.,* 2000).

Previously, we characterized RNA3-derived D-RNAs which were obtained from BMV-infected barley plants after prolonged infection (Damayanti *et al.,* 1999). The D-RNAs have several common features. The length of the deleted sequences ranges from 477 to 500 nucleotides (nt) in the central region of the 3a open reading

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-75-753-6131. E-mail: okuno@kais.kyoto-u.ac.jp.

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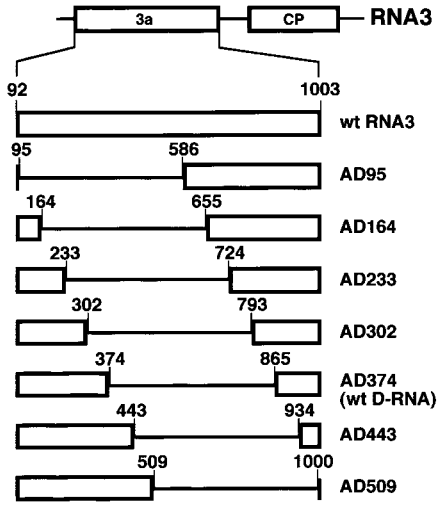

FIG. 1. Schematic representation of deleted regions in AD-RNAs. Nucleotide deletions (length 492 nt) from the 3a ORF (open box) are indicated by a horizontal thin line with the nucleotide numbers at their 5′ and 3′ ends. Wild-type RNA3 is also shown at the top of this figure. All AD-RNAs (AD95, AD164, AD233, AD302, AD443, and AD509) were constructed by *in vitro* mutagenesis to create in-frame deletions. AD374 corresponds to a wt D-RNA.

frame (ORF). The 5′-proximal sequences (365–373 nt) and the 3′-proximal sequences (135–153 nt) in the 3a ORF are retained. The similarity of deleted regions suggests that the nondeleted regions of the 3a gene are essential for efficient replication, encapsidation, or stability of the D-RNAs. To investigate these, we constructed artificial D-RNAs (AD-RNAs) with deletions of the same length but at different positions in the 3a ORF. These AD-RNAs were tested for their ability to replicate and be encapsidated in barley protoplasts by coinoculation with BMV RNA1 and 2, or RNA1, 2, and 3. The results show that deletion position did not affect the ability of AD-RNAs to be replicated or the stability of RNA molecules, but it did affect encapsidation efficiency of AD-RNAs. Deletion position also affected competition with RNA3 in replication, encapsidation, or both.

RESULTS

Effects of deletion position on the amplification and encapsidation of AD-RNAs

Figure 1 shows the deleted regions (492 nt) of the AD-RNA cDNA clones. AD374 corresponds to the cDNA clone of the wild-type (wt) D-RNA. *In vitro* generated transcripts from the AD-RNA clones, together with transcripts from cDNA clones of BMV RNA1 and 2, were inoculated into barley protoplasts. Total and virion fraction RNAs were extracted at 24 h postinoculation (hpi) and were analyzed by the Northern blot method using a probe complementary to the conserved 3′-terminal sequence of BMV RNAs. In total RNA, accumulation levels of AD-RNAs relative to those of RNA1 and 2 differed depending on the deletion position: accumulation of AD509 was significantly low compared with that of other AD-RNAs and RNA3, while accumulations of AD164, AD233, AD302, and AD374 and those of AD95 and AD433 were higher than or similar to that of RNA3, respectively (Fig. 2). In the virion fraction, accumulation levels of AD95, AD433, and AD509 were extremely low compared with RNA3 and other AD-RNAs (Fig. 3). These results indicate that the accumulation of AD-RNAs was affected by the position of the deletion and suggest that the effect of the deletions was more prominent on encapsidation than on accumulation.

To investigate whether the deletion of these regions affects replication or stability of AD-RNAs, a series of AD-RNA derivatives [AD-RNA(CP-fs)s] was constructed by introducing a frameshift mutation into the 5′-proximal region of the CP gene. The mutation leads to premature translational termination of the BMV CP, which results in production of polypeptides consisting of 19 amino acids. This allows the observation of the replication and/or stability of AD-RNAs independently from the encapsidation factor. Barley protoplasts were inoculated with BMV RNA1 and 2 and a series of AD-RNA derivatives [AD-RNA(CP-fs)s]. Viral RNA accumulation was analyzed at 24 hpi. Lack of CP accumulation in these protoplasts was confirmed by immunoblot analysis using BMV CP antisera (data not shown). The accumulation levels of AD-RNA(CP-fs)s and RNA3 (CP-fs) relative to those of RNA1

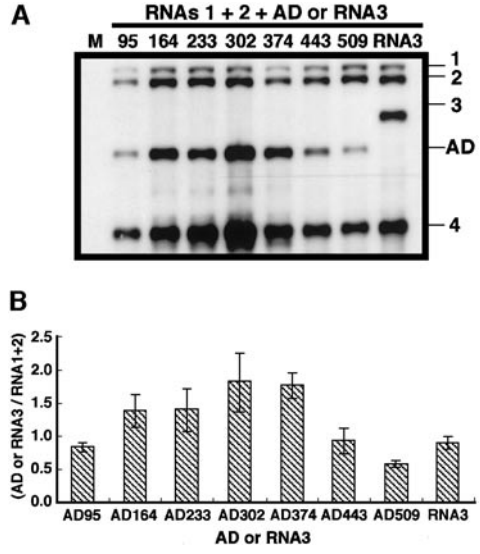

FIG. 2. Northern blot analysis of total RNA extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1 and 2 and AD-RNAs. (A) Raw data of an experiment. The complete set of inocula is shown above the photograph with the designation of individual AD-RNA and wt RNA3 lane by lane. Lane M, inoculated with water only. Positions of RNA1, 2, 3, 4, and AD are indicated on the right. Total RNA was extracted from 1.5×10^4 protoplasts. (B) Relative value of AD-RNA accumulation. Coinoculated RNA1+2 was used as an internal standard. The mean value (shaded columns) and the standard deviations (thin vertical lines) were calculated from three independent experiments.

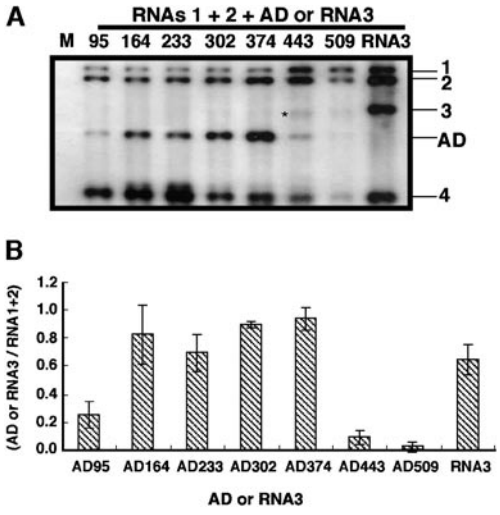

FIG. 3. Northern blot analysis of virion RNA fraction extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1 and 2 and AD-RNAs. (A) Raw data of an experiment. The virion RNA fraction was prepared from an aliquot of the protoplast samples used in the experiment shown by Fig. 2A. The asterisk indicates the position of a band that is undetectable by RT-PCR using primers specific to the 5′- and 3′-terminus of RNA3. (B) Relative value of AD-RNA accumulation. Coinoculated RNA1+2 was used as an internal standard. The mean value (shaded columns) and the standard deviations (thin vertical lines) were calculated from three independent experiments. For other notations, refer to the legend in Fig. 2.

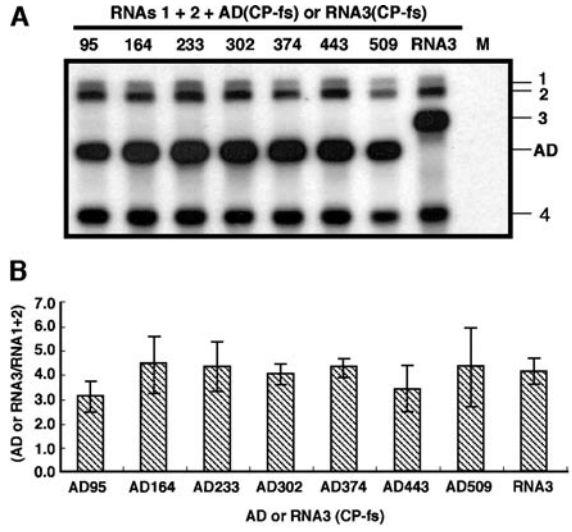

FIG. 4. Northern blot analysis of total RNA extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1 and 2 and AD-RNAs(CP-fs) or BMV RNA1 and 2 and RNA3 (CP-fs). (A) Raw data of an experiment. The complete set of inocula is shown above the photograph with the designation of individual AD-RNA(CP-fs) and RNA3(CP-fs) lane by lane. Total RNA was extracted from 3.0×10^4 protoplasts. (B) Relative value of AD-RNA accumulation. Coinoculated RNA1+2 was used as an internal standard. The mean value (shaded columns) and the standard deviations (thin vertical lines) were calculated from three independent experiments. For other notations, refer to the legend in Fig. 2.

and 2 did not differ significantly (Fig. 4). These results indicate that the deletions at various positions in the 3a gene did not significantly affect the replication and the stability of the AD-RNAs.

In addition, it was noteworthy that accumulation ratio of AD-RNAs or RNA3/RNAs 1 and 2 was high in the absence of CP (Fig. 4) compared to that in the presence of CP (Fig. 2). This was also observed in protoplasts inoculated with both AD-RNA and RNA3 together with RNAs 1 and 2 as described below (Figs. 5 and 7). The absence of CP may up-regulate the accumulation of RNA3 or down-regulate the accumulation of RNAs 1 and 2. It has been shown that in the absence of functional CP, expression of BMV 1a protein is stimulated in barley protoplasts (Sacher and Ahlquist, 1989) and that BMV RNA3 is stabilized by BMV 1a protein in yeast (Janda and Ahlquist, 1998). The stabilization of RNA3(CP-fs) or its derivative AD-RNA(CP-fs)s by 1a protein may lead to their higher accumulation in the absence of CP.

Competition with RNA3 on the amplification of AD-RNAs

Because AD-RNAs are deletion mutants of RNA3, AD-RNAs and RNA3 may compete with each other for replication, encapsidation, or stability. To examine the effects of competition between AD-RNAs and RNA3, barley protoplasts were inoculated with AD-RNAs together with BMV RNA1, 2, and 3, and viral RNA accumulation was analyzed as described above. RNA3 accumulated to similar levels in protoplasts coinoculated with any of the AD-RNAs, but accumulation levels of AD-RNAs differed

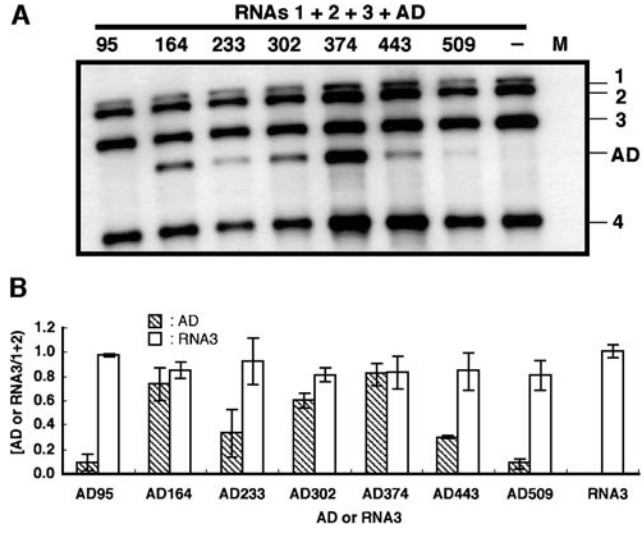

FIG. 5. Northern blot analysis of total RNA extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1, 2, and 3 and AD-RNAs. (A) Raw data of an experiment. (B) Comparison of relative value of accumulation between AD-RNAs (shaded columns) and RNA3 (open columns). Coinoculated RNA1+2 was used as an internal standard. The mean value (columns) and the standard deviations (thin vertical lines) were calculated from three independent experiments.

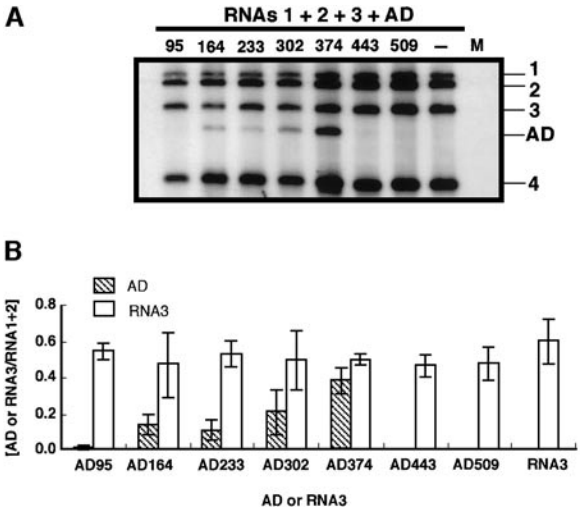

FIG. 6. Northern blot analysis of the virion RNA fraction extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1, 2, and 3 and AD-RNAs. (A) Raw data of an experiment. The virion RNA fraction was prepared from separate aliquots of the protoplast samples used to obtain the Northern blots in Fig. 5A. (B) Comparison of relative value of accumulation between AD-RNAs (shaded columns) and RNA3 (open columns). Coinoculated RNA1+2 was used as an internal standard. The mean value (columns) and the standard deviations (thin vertical lines) were calculated from three independent experiments. For other notations, refer to the legend in Fig. 2.

depending on the position of the deletion. Accumulation levels of all AD-RNAs except for wt AD374 were significantly lower than that of RNA3 (Fig. 5). Similar results, but with more distinct effects of RNA3 coexistence on accumulation of AD-RNAs, were observed when analyzing the virion fraction RNA prepared from aliquots of protoplasts used for the analysis of total RNA (Fig. 6). These results suggest that there was competition between RNA3 and AD-RNAs in replication, stability, or encapsidation.

To exclude the effect of encapsidation on competition between RNA3 and AD-RNAs, barley protoplasts were inoculated with AD-RNA(CP-fs)s together with RNA1 and 2, and RNA3(CP-fs), which had the same frameshift mutation in the CP gene as AD-RNA(CP-fs). There was significant difference in accumulation levels between AD-RNA(CP-fs)s and RNA3(CP-fs) in protoplasts inoculated with AD(CP-fs)s except for the wild-type AD374(CP-fs) (Fig. 7). The difference was especially prominent in AD95(CP-fs), AD443(CP-fs), and AD509(CP-fs), suggesting that the deletion positions affected competition with RNA3 in replication or stability of AD-RNAs. In contrast, accumulation levels of RNA3(CP-fs) was not so different as was observed in those of AD-RNA(CP-fs) among protoplasts coinoculated with any AD-RNA(CP-fs)s or inoculated with RNA3(CP-fs) alone, although RNA3(CP-fs) accumulation was relatively low in the presence of the wild-type AD-RNA(CP-fs). This indicates that accumulation of RNA3(CP-fs) was not as much affected by the presence of AD-RNA(CP-fs)s as that of AD-RNA(CP-fs)s was affected by RNA3(CP-fs).

DISCUSSION

The present study showed that the deletions of 492 nt in the 5′- and 3′-proximal regions of the 3a ORF impaired encapsidation efficiency of the AD-RNAs. This suggests that the regions deleted in these AD-RNAs contain *cis*-acting sequences required for efficient encapsidation of RNA3, or that deletions render RNA structure unsuitable for packaging. Alternatively, alterations of protein products caused by deletions might affect packaging efficiency of mutant RNA3, as discussed below.

Various regions of RNA3 are required for its efficient amplification in barley protoplasts. The regions include the 3′- and the 5′-noncoding regions and the intercistronic region, but not the 3a ORF; deletion of the 5′- or the 3′-half of the 3a ORF or of the entire 3a ORF in BMV RNA did not affect accumulation of the mutant RNA3 (French and Ahlquist, 1987). Our present results show that, depending on position, deletions of 492 nt in the 3a ORF significantly affect the accumulation of the AD-RNAs by either reducing or enhancing the accumulation compared with that of RNA3. This seems to be due to a difference in packaging efficiency of the AD-RNAs, because all AD-RNAs accumulated to a level similar to RNA3 in the absence of CP (Fig. 4). Deletions might remove the sequences that are involved in the mainte-

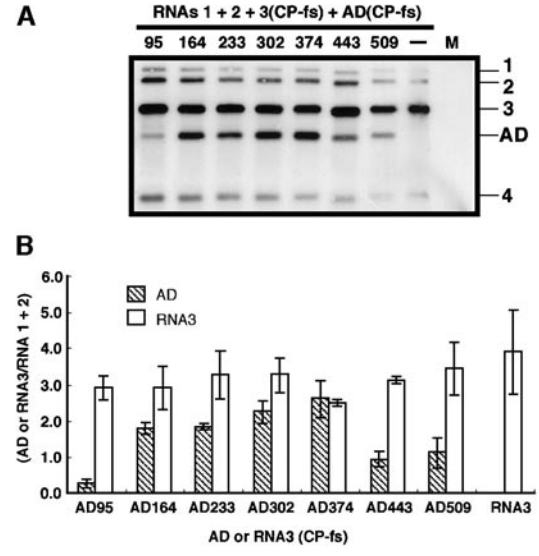

FIG. 7. (A) Northern blot analysis of total RNA fraction extracted from barley protoplasts at 24 h after inoculation with a mixture of *in vitro* transcripts of BMV RNA1, 2, and 3 (CP-fs) and AD-RNAs(CP-fs). Northern blot hybridization was carried out with a probe as described in the legend to Fig. 2A. (B) Accumulation of AD-RNAs (shaded columns) and RNA3 (CP-fs) (open columns) relative to the internal standard of coinoculated RNA1+2. Columns represent the mean value from three independent experiments. For other notations, refer to the legend in Fig. 4.

nance of RNA structure, and the structural change in RNA molecules caused by the deletions might influence the interaction with CP and other factors that interact with CP or virus RNA. Alternatively, deleted forms of the 3a protein translated from AD-RNAs might affect the encapsidation of AD-RNAs, because all AD-RNAs used in the experiments have an in-frame deletion, although the nucleotide binding domain of 3a protein (amino acids from 189 to 242) (Fujita *et al.,* 1998) did not seem to be involved. In this case, the interaction must be specific between truncated 3a protein and the AD-RNA from which the truncated 3a protein is translated, because accumulation levels of RNA3 in the virion fraction were not affected by the presence of any AD-RNAs (Fig. 6).

In vitro assembly experiments have shown that BMV CP has some specificity for recognition and encapsidation of BMV RNAs (Cuillel *et al.,* 1979). BMV CP expressed from chimeric *Cucumber mosaic virus* (CMV) RNA3 constructs is unable to package either the chimeric RNA3 harboring the BMV CP gene or other CMV RNAs (Osman *et al.,* 1998), suggesting that there is specificity between BMV CP and BMV RNAs in encapsidation. On the other hand, Choi and Rao (2000) recently demonstrated that chimeric *Tobacco mosaic virus* (TMV) having the BMV CP gene produces icosahedral virus-like particles that contain subgenomic RNAs with molecular sizes of 3138, 1650, and 875 nt. This indicates that BMV CP can encapsidate chimeric RNAs having sequences unrelated to BMV in addition to the BMV CP gene. These results suggest that nucleotide sequence and/or size of RNA molecules influence RNA packaging by BMV CP. Deletions of 492 nt in the 3a ORF did not decrease the packaging efficiency of AD164, AD233, AD302, and AD374 compared with that in BMV RNA3 (Fig. 3), suggesting that the regions from nt position 164 to 865, which were deleted in these AD-RNAs, do not contain *cis*-acting sequences to encapsidate RNA3. Furthermore, nucleotide sequences from nt position 95 to 163 and from nt 866 to 1000 alone do not function as *cis*-acting sequences that directly interact with BMV CP to encapsidate RNA3, because either one of these sequences is present in AD95, AD443, or AD509, which are poorly encapsidated. Taken together, structural features of RNA rather than size seem important for encapsidation of AD-RNAs by BMV CP.

BMV D-RNAs generated in natural conditions have a deletion similar to AD374 (Damayanti *et al.,* 1999), and no other types of BMV D-RNAs have been reported. This implies that the D-RNA has some advantages over other types in nature. The present results show that there is strong competition between AD-RNAs (except for wt AD374) and RNA3, especially in encapsidation as well as in replication and/or stability (Figs. 6 and 7). The competition with RNA3 may explain why natural BMV D-RNAs have deletions similar to that of AD374 but not of AD164, AD233, and AD302, which can be amplified as efficiently as AD374 in the absence of RNA3 (Fig. 2).

MATERIALS AND METHODS

Plasmid clones

Plasmids pBTF1, pBTF2, and pBTF3WSS5R25 contain the full-length cDNAs of BMV RNA1, RNA2, and RNA3, respectively (Mori *et al.*, 1991; Damayanti *et al.*, 1999).

Construction of D-RNA mutants and *in vitro* transcription

All cDNA clones for D-RNA mutants constructed in this study (Fig. 1) were derived from a plasmid pBTF3WSS5R25 (Damayanti *et al.,* 1999). Polymerase chain reaction (PCR)-based *in vitro* mutagenesis (Ito *et al.,* 1991) with appropriate combinations of deoxyoligonucleotides was used to construct cDNA fragments with appropriate deletions. The amplified cDNA products were digested with *Pst*I and *Eco*RI and cloned into pUC119 (Takara, Otsu, Japan) at the corresponding enzyme sites. To facilitate subcloning by evading putative undesired mutations derived from PCR, the cDNA clones were digested with appropriate restriction enzymes that generated small cDNA fragments having deletions. The resulting cDNA fragments were substituted for the corresponding region in pBTF3WSS5R25 to create mutant clones. The deleted length in all the mutants was 492 bases, leading to in-frame deletion. To construct pBTF3WSS5R25(CP-fs), four bases were inserted into the pBTF3WSS5R25 cDNA clone by digestion with *Bss*HII, treatment with T4 DNA polymerase, and relegation. The frameshift results in premature translational termination of the BMV CP, which results in production of polypeptides consisting of 19 amino acids. The AD cDNA clones were digested with *Bgl*II and *Eco*RI and the resulting cDNA fragments were substituted for the corresponding region in pBTF3WSS5R25(CP-fs) to create a series of AD(CP-fs) mutant clones. The presence of expected deletions and/or mutations was verified by sequencing plasmid DNA with an automated DNA sequencer, as described previously (Damayanti *et al.,* 1999).

All plasmids were linearized with *Eco*RI, and RNA transcripts were synthesized *in vitro* with T7 RNA polymerase, as previously described (Mori *et al.,* 1991).

Protoplast inoculation, RNA extraction, and analysis of viral RNA

Isolation and inoculation of barley protoplasts (*Hordeum vulgare* cv. Gose-shikoku) and extraction of total and virion fraction RNAs were as described (Damayanti *et al.,* 1999). RNAs were subjected to Northern blot analysis essentially as described (Damayanti *et al.,* 1999), except that probes for detection of (+)-strand RNAs were la-

beled with DIG-UTP (Roche, Indianapolis, IN) (Sasaki *et al.,* 2001).

RNA accumulation was quantified as follows: X-ray films were scanned with a scanner (GT-8700F, Epson) and the image was saved into the TIFF file. The RNA signals were counted lane by lane with a computer soft NIH Image version 1.61 (National Institutes of Health, Bethesda, MD), after subtracting background and calibrating the image with uncalibrated optical density. The RNA intensities were quantified using the image for densitometry analysis of gel plotting macros according to the standard instructions from Division of Computer Research and Technology (NIH). The quantified values of AD-RNA and RNA3 relative to that of the internal standard of RNA1+2 in each lane were used to obtain the mean values with standard deviation from at least three independent experiments.

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