Infectious transcripts from PCR-amplified broad bean mottle bromovirus cDNA clones and variable nature of leader regions in RNA 3 segment

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The genome of broad bean mottle bromovirus (BBMV) contains three positive-sense ssRNA segments, each capped with m7GpppA. Full-length transcribable cDNA clones for four strains of BBMV were constructed by employing reverse transcriptase–PCR (RT-PCR) and a high fidelity Vent DNA polymerase. The transcribed BBMV RNAs contained a 5' non-viral G residue and, although delayed, produced symptoms similar to those observed in plants infected with authentic virion RNAs. The transcripts replicated inefficiently in protoplasts. In contrast, transcript-derived progeny BBMV RNAs had the repaired termini, were as infectious as the authentic BBMV RNAs and replicated to high levels in protoplasts. In vitro translation of synthetic RNAs confirmed the previously proposed gene expression strategy for BBMV. Sequencing of virion RNAs from the Bawden strain revealed two forms of BBMV RNA 3 components, the longer form containing 21 5' extra nucleotides derived by the duplication of two short 5' leader regions. The relative concentration of the two RNA 3 forms was found to be host-dependent, with the longer form prevailing in broad bean and Nicotiana clevelandii infections and the shorter form in bean infections.

Broad bean mottle virus (BBMV) is a member of the group of bromoviruses (Lane, 1981), which have a tripartite, positive-sense RNA genome. RNA 1 (3.1 kb), RNA 2 (2.8 kb) and RNA 3 (2.3 kb) of BBMV are each capped and, unlike other bromovirus RNAs, each has an A residue at the 5' terminus (Dzianott & Bujarski, 1991; Romero et al., 1992). Several BBMV strains, including those from England [Bawden (Ba)], Morocco (Mo), Sudan (Su), Syria (Suv) and Tunisia (Tu) have been described (Bawden et al., 1951; Makkouk et al., 1988). These strains are serologically indistinguishable but show distinct reactions with selected plant hosts (Makkouk et al., 1988; J. Romero, Q. Huang & J. Bujarski, unpublished results). In addition to BBMV, the bromovirus group includes brome mosaic virus (BMV) and cowpea chlorotic mottle virus (CCMV) (Kaesberg, 1987). All three bromoviruses demonstrate a high degree of nucleotide sequence similarity and are closely related morphologically and functionally (Ahlquist et al., 1984a; Allison et al., 1989; Dzianott & Bujarski, 1991; Romero et al., 1992). Despite these similarities, the systemic host ranges of BMV, BBMV and CCMV are significantly different (Lane, 1981). These features make comparative molecular studies of bromoviruses important for the understanding of host–virus interactions.

Infectious RNA transcripts from full-length cloned genomic cDNAs of several tricornaviruses have been reported: BMV (Ahlquist et al., 1984b), CCMV (Allison et al., 1988), cucumber mosaic virus (CMV; Hayes & Buck, 1990) and alfalfa mosaic virus (AlMV; Neelaman et al., 1991). For a review, see Bujarski & Miller (1992). In this paper we describe the use of reverse transcriptase–PCR reactions (RT–PCR) and a high fidelity Vent DNA polymerase (Mattila et al., 1991) for construction of full-length cDNA clones of Ba, Mo, Suv and Tu strains of BBMV RNA components. Previously, Hayes & Buck (1990) have used a low fidelity Taq DNA polymerase for RT–PCR amplification of full-length CMV cDNAs. Transcribed BBMV RNAs have been used to determine the translational capacity of the individual BBMV segments and to demonstrate the repair of 5' termini in transcript-derived progeny BBMV RNAs. Also, we describe sequence heterogeneity among the RNA 3 components of a greenhouse Ba BBMV isolate.

The Ba strain was obtained from R. Hull (John Innes Institute, Norwich, U.K.), whereas Mo, Su, Suv and Tu strains were obtained from K.M. Makkouk (International Center for Agricultural Research of Dry Areas, Aleppo, Syria). Virion RNAs were extracted using a proteinase K–SDS–phenol procedure, according to

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Romero et al. (1993). The first-strand cDNA was synthesized with Moloney murine leukaemia virus reverse transcriptase as described by Romero et al. (1993). The amplification of double-stranded cDNA was accomplished by 25 PCR thermocycles (92°C, 1 min; 57°C, 2 min; 72°C, 2.5 min) in a reaction mixture as described by Romero et al. (1993), using Vent DNA polymerase (New England Biolabs). One difficulty in designing the correct transcription initiation sites was that BBMV RNAs initiated with an A residue. Earlier attempts with other positive-strand RNA viruses initiating with an A residue, such as tobacco rattle virus, tobacco vein mottling virus or red clover necrotic mosaic virus (Angenent et al., 1989; Domier et al., 1989; Xiong & Lommel, 1991; respectively), demonstrated that no detectable quantity of transcripts was produced by T7 polymerase with an A as the first nucleotide (Dunn & Studier, 1983). Therefore, the 5' primers contained an extra G preceding the viral 5' A residue. The 3' end primers contained the 3' BBMV RNA sequences, common among three BBMV RNAs, and an overhang primers contained the 3' BBMV RNA sequences, polymerase with an A as the first nucleotide (Dunn & Lommel, 1991; respectively), demonstrated that no detectable quantity of transcripts was produced by T7 polymerase with an A as the first nucleotide (Dunn & Studier, 1983). Therefore, the 5' primers contained an extra G preceding the viral 5' A residue. The 3' end primers contained the 3' BBMV RNA sequences, common among three BBMV RNAs, and an overhang that created a unique BamHI restriction site for RNAs 1 and 2, and a HindIII site for RNA 3. This produced five 3' non-viral nucleotides in corresponding in vitro transcripts. The following oligodeoxynucleotide primers were used to initiate first-strand cDNA synthesis: 5' CGC GGATCCTGTTCTCCCCTAAGAG 3' (primer 223 for RNA 1) and RNA 2 containing a BamHI site (underlined), and 5' CCAAGCTTGGTCTCCCCTAAGAG 3' (primer 222 for RNA 3) containing a HindIII site (underlined). Second-strand synthesis primers contained upstream T7 promoter sequences (underlined) and downstream sequences specific for each BBMV RNA: 5' ATTAATACGACTCACTATAGATTAACACTGAGAGACCGAGTT 3' (primer 318 for RNA 1), 5' ATTAATACGACTCACTATAGATTAAGTGATAGCGG 3' (primer 319 for RNA 2), and 5' ATTAATACGACTCACTATAGATTAACACTGAGAGACCGAGTT 3' (primer 321 for RNA 3). The full-length products of RT-PCR were purified by electrophoresis in a low melting point agarose gel. DNA was recovered from the gel by incubation at 65°C for 10 min followed by SDS-phenol-chloroform extraction as described by Sambrook et al. (1989).

In contrast to Taq DNA polymerase, the Vent enzyme produces blunt-ended cDNA products (Lohff & Cease, 1992). This allowed direct ligation of the amplified BBMV cDNAs into a SmaI-linearized pUC19 cloning vector. To confirm that the resulting constructs read correctly, the 5'-terminal portions of each clone were sequenced (not shown). Several full-length clones were routinely obtained for each genomic RNA segment of Ba, Mo, Suv and Tu strains of BBMV. All clones analysed were transcriptionally active (data not shown).

Capped BBMV RNA transcripts were synthesized from BamHI- (RNAs 1 and 2) or HindIII- (RNA 3) linearized cDNA clones by using T7 RNA polymerase, as described by Janda et al. (1987). Direct RNA sequencing confirmed the presence of one extra 5' G residue in transcribed BBMV RNAs (data not shown). It also confirmed efficient capping during in vitro transcription.

By means of an in vitro translation analysis it has been demonstrated previously that in BMV and in CCMV, RNAs 1 and 2 were monocistronic whereas RNA 3 was dicistronic (Kaesberg, 1987; Allison et al., 1989). Sequence analysis suggested a similar organization of the BBMV genome (Dzianott & Bujarski, 1991; Romero et al., 1992). To confirm the expression strategy of the BBMV genome, transcribed BBMV RNAs were translated in vitro in a rabbit reticulocyte lysate system (Promega). Fig. 1 shows that translation from BBMV RNA 1 and 2 transcripts yielded a single protein that

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**Fig. 1.** Autoradiograph of in vitro translation products of 1 μg in vitro transcripts of Ba RNAs 1, 2 and 3 (lanes 1, 2 and 3, respectively). 1 μg wt Ba BBMV RNA (lane BBMV) and 1 μg of wt BMV RNA (lane BMV). The positions of viral polypeptides, separated in a 12.5% polyacrylamide gel (Laemmli, 1970), are indicated: 1a and 2a, polypeptides encoded by RNAs 1 and 2, respectively; 3a and CP, polypeptides encoded by RNA 3. An intense additional protein product (indicated by an asterisk) between 2a and 3a (lane BBMV) represents a truncated 2a protein translated from defective RNA 2 molecules present in this isolate of the Ba strain. Other additional bands represent either products of premature termination of translation, or degradation products.
Table 1. Comparison of the infectivity of in vitro transcribed genomic RNAs, transcript-derived progeny RNAs and authentic virion RNAs of four BBMV strains in selected systemic hosts*

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Strain</th>
<th>Transcipt-derived wt virion RNAs (15 µg/plant)</th>
<th>Transcript-derived progeny RNAs (0.2 µg/plant)</th>
<th>wt virion RNAs (0.2 µg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. faba</td>
<td>Ba</td>
<td>75</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>cv. Windsor</td>
<td>50</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Tu</td>
<td>50</td>
<td>87.5</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Suv</td>
<td>50</td>
<td>NA†</td>
<td>100</td>
</tr>
<tr>
<td>Pisum sativum</td>
<td>Ba</td>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>cv. Sugar Ann</td>
<td>NA</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Tu</td>
<td>NA</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Suv</td>
<td>NA</td>
<td>50</td>
<td>75</td>
</tr>
<tr>
<td>N. clevelandii</td>
<td>Ba</td>
<td>NA</td>
<td>87.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Mo</td>
<td>NA</td>
<td>NA</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Tu</td>
<td>NA</td>
<td>NA</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Suv</td>
<td>NA</td>
<td>NA</td>
<td>100</td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td>Ba</td>
<td>NA</td>
<td>62.5</td>
<td>62.5</td>
</tr>
<tr>
<td></td>
<td>cv. Canadian Wonder</td>
<td>NA</td>
<td>NA</td>
<td>50</td>
</tr>
</tbody>
</table>

* The table shows mean percentage of infected plants from two independent experiments, each involving a separate set of RNAs synthesized from a separate set of plasmids, and four host plants. Plants were inoculated mechanically with the RNA inocula as described in the text and the symptoms of BBMV infection were examined 2 weeks (V. faba, P. sativum and N. clevelandii) or 4 weeks (P. vulgaris) post-inoculation.
† NA, Not analysed.

comigrated with the corresponding proteins synthesized from BBMV virion RNA, somewhat smaller in size than the corresponding BMV 1a and 2a proteins (110K and 94K). The transcribed BBMV RNA 3 produced a single protein of approximately the same size as the BMV 32K protein. As in BMV and CCMV, the coat protein (CP) ORF was not functional in BBMV RNA 3 transcripts but was synthesized from unfractionated virion RNA (lane BBMV), suggesting that CP was expressed from subgenomic RNA 4. The in vitro translation results confirmed that unwanted translational stop codons had not been introduced during RT–PCR amplification.

In addition to the expected proteins, a novel in vitro translation product that migrated between the BBMV 2a and 3a proteins was synthesized when an unfractionated virion Ba BBMV RNA preparation was analysed (lane BBMV in Fig. 1). Further experiments demonstrated that this particular RNA preparation of the Ba strain contained a defective interfering (DI) RNA component that encoded a truncated 2a protein (J. Pogany & J. Bujarski, unpublished results; Romero et al., 1993).

To determine the infectivity of BBMV transcripts, broad bean seedlings were inoculated mechanically with a mixture of 3 to 5 µg of each of the three genomic synthetic RNAs, using a buffer containing 10 mM-Tris–HCl pH 7.4, 1 mM-EDTA, 0.2% bentonite and 0.2% Celite. Two separately inoculated sets of capped transcripts, made from two separate sets of cDNA clones, produced a systemic mottling typical for BBMV infection in broad bean, although the appearance of symptoms was delayed by 2 to 4 days compared to those induced with the corresponding wild-type (wt) virion RNAs (Table 1). Corresponding uncapped transcripts were not infectious, even after inoculation with a high amount (40 to 50 µg/plant) of RNA (not shown). The presence of a single nucleotide marker mutation that was introduced during the synthesis of the cDNA of Ba RNA 3 (A to G substitution at position 14) was recovered in the progeny BBMV Ba RNA 3 component. This excluded the possibility that the observed infections arose from contaminations with wt BBMV. Fifteen µg per plant of transcribed RNA inocula was required to infect 50 to 75% of inoculated broad bean plants, as compared to 75 to 100% infectivity routinely obtained with 0.2 µg wt virion RNAs. BBMV transcripts were also infectious in Chenopodium quinoa, causing small chlorotic lesions similar to those obtained with wt virus on the inoculated leaves (data not shown).

Sequencing of the 5'-terminal region in transcript-derived progeny RNAs demonstrated that the progeny virion RNA initiated correctly with an A residue (not shown). To determine how the repair of terminal
Table 2. Comparison of the infectivity of in vitro transcribed genomic RNAs, transcript-derived progeny RNAs and authentic virion RNAs of BBMV strain Ba in protoplasts

<table>
<thead>
<tr>
<th>Source of protoplasts</th>
<th>Inoculum</th>
<th>Transcript-derived wt virion RNA</th>
<th>wt virion RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley (cv. Dixon)</td>
<td>Transcripts</td>
<td>+ + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>N. benthamiana</td>
<td>+</td>
<td>+ + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Tobacco (cv. Xanthi)</td>
<td>-</td>
<td>+ + + + + + + +</td>
<td></td>
</tr>
<tr>
<td>Broad bean (cv. Windsor)</td>
<td>-</td>
<td>+ + + + + + + +</td>
<td></td>
</tr>
</tbody>
</table>

* The symbols +, + + + and – respectively indicate low, high and undetectable levels of accumulation of viral RNA, as detected by Northern blot analysis.

sequences affected infectivity, 0.2 μg of transcript-derived progeny BBMV RNA was inoculated onto broad bean plants. Table 1 illustrates that the infectivity was similar to that of authentic virion RNA preparations. The induced symptoms were indistinguishable from those obtained with the authentic BBMV RNAs (not shown).

To study the replication of BBMV transcripts in protoplasts, samples of protoplast cells were inoculated by means of a polyethylene glycol-mediated procedure (according to Kroner et al., 1989), using mixtures of capped Ba or Mo transcribed RNAs 1 to 3. Protoplasts were prepared from leaves of Nicotiana benthamiana, N. tabacum (cv. Xanthi) and Vicia faba (broad bean, cv. Windsor) according to Jones et al. (1990), except that the protoplast medium contained Aoki salts (Aoki & Takebe, 1969). Protoplasts from Hordeum vulgare (barley, cv. Dixon) were isolated according to the procedure of Kroner et al. (1989). As shown in Table 2, transcribed BBMV RNAs replicated to a detectable level only in N. benthamiana protoplasts. A Northern blot revealed the appearance of a subgenomic RNA 4 (not shown), indicating that RNA replication processes were not grossly altered. In contrast to synthetic BBMV RNAs, transcript-derived progeny as well as wt virion RNAs replicated to high levels in all protoplast systems studied.

The reduction, of almost 100-fold, observed for the infectivity of BBMV transcripts, as well as the reduction in RNA replication as compared with authentic virion RNAs revealed that a natural isolate of the Ba strain, which was maintained in N. clevelandii plants, appeared to have two types of RNA 3 molecules (Fig. 2a, panel I). A major fraction (designated RNA 3-A) had the previously published 5’ end sequence (Romero et al., 1992) whereas a minor fraction (designated RNA 3-B) contained additional nucleotides at the 5’ end. Following two subsequent passages through either broad bean (panels IV and V) or N. clevelandii (not shown) plants, the RNA 3-B molecule became dominant over RNA 3-A. In contrast, one passage through broad bean eliminated RNA 3-B molecules (panel III) whereas one passage through pea did not significantly affect the RNA 3-A:RNA 3-B ratio (panel II).

Sequence analysis of the broad bean-passaged Ba strain revealed that the 5’ end of RNA 3-B contained 21 extra nucleotides (Fig. 2b) which resulted from the duplication of two overlapping regions of the leader sequence (Fig. 2c). This sequence rearrangement introduced an additional hairpin loop structure at the 5’ terminus (Fig. 2d). The 5’ end sequences of RNA 3 molecules in natural Mo, Su, Suv and Tu isolates maintained in N. clevelandii or in broad bean were also determined (data not shown). None of the analysed isolates contained RNA 3-B-type molecules. This suggested that only the Ba strain can support 5’ leader sequence heterogeneity in RNA 3 components or that it represents a rare event.

We observed previously that the 5’-terminal BBMV RNA 3 sequences do not align well with the internal control region-like (ICR-like) sequences (Romero et al., 1992) that are present in BMV and CCMV (Pogue et al., 1990) and which play a role in positive-strand BMV RNA synthesis (Pogue & Hall, 1992). The ICR-like motifs were found to be exposed in the 5’ end hairpin loop structures of BMV (Pogue & Hall, 1992) and AIMV (van der Vossen et al., 1993). The presence of an additional hairpin structure in BBMV RNA 3-B did not create a closer alignment of ICR-like sequences. Therefore, it is unclear whether synthesis of BBMV RNA 3 plus-strands utilizes ICR motifs, as found for BMV RNAs.

The variable nature of the 5’ leader sequences observed here for BBMV Ba RNA 3 has been documented previously for the RNA 3 segment among several strains of AIMV (Langereis et al., 1986). As in BBMV RNA 3, the differences among 5’ non-coding regions in AIMV RNA 3 arose as a result of repeats of various 5’
sequences, differing both in the length and in the copy number. The mechanism of generation of the 5' duplications is not known. Possible explanations include viral replicase stuttering at highly structured 5' ends or aberrant homologous recombination (Lai, 1992) between two BBMV RNA 3 molecules.

The mechanism by which BBMV hosts select between RNA 3-A and RNA 3-B variants is not known. One can speculate that an additional hairpin at the 5' end of the RNA 3-B molecule affects the rate of plus-strand RNA synthesis. Indeed, regardless of the presence of ICR-like motifs, the importance of the 5' end secondary structure in virus RNA replication was revealed by Gilmer _et al._ (1993) in beet necrotic yellow vein virus. That different BBMV Ba RNA 3 variants prevailed in different hosts may reflect the involvement of host factors during synthesis of plus strands. Bean and broad bean host factors may differ in their affinity for various 5' leader
sequences. Also, the presence of additional 5’ secondary structure elements may affect the kinetics of translation of the 3a movement protein from RNA 3-B molecules, as proposed before for AINV RNA3 by van der Vossen et al. (1993). This, in turn, may influence the patterns of selection of BBMV RNA segments. Consistent with the removal of RNA 3-B molecules in bean plants, we reported previously the removal of DI RNAs in BBMV-infected bean plants (Romero et al., 1993). The putative effect of BBMV RNA 3 leader sequences on RNA replication, on the kinetics of translation initiation, or on the interaction with host factors requires experimental confirmation.

In this work we demonstrated that the use of Vent polymerase in PCR reactions readily produced clonable full-length cDNA copies from which infectious BBMV transcripts could be synthesized. Both its proofreading activity (Mattila et al., 1991) and its ability to generate blunt-ended cDNA products (Lohff & Cease, 1992) make Vent polymerase an enzyme of choice for generating transcribable infectious cDNA clones of positive-stranded RNA viruses. The availability of infectious transcripts has allowed us to initiate BBMV infections from a homogeneous population of genomic RNA molecules. This will be useful for investigation of the mechanisms involved in generation of heterogeneous RNA 3 leader sequences and in the formation of DI RNAs in BBMV (Romero et al., 1993).

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References


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