In vitro transcripts of a full-length cDNA of a naturally deleted RNA2 of barley mild mosaic virus (BaMMV) replicate in BaMMV-infected plants

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The RNA2 of a naturally deleted RNA2 of barley mild mosaic virus (BaMMV) replicates in BaMMV-infected plants.

Barley mild mosaic virus (BaMMV) is a member of the Bymovirus genus of the family Potyviridae (Barnett, 1991; Usugi et al., 1989) and is one of the agents causing yellow mosaic disease of winter barley. The virus has a bipartite genome and is transmitted by the soil-borne fungus Polymyxa graminis Ledingham (Adams et al., 1989).

Recently, the complete nucleotide sequences of RNA2 of a German isolate (BaMMV-ASL1; Timpe & Kühne, 1994) and a deleted form of a French isolate (Desserts et al., 1995) of BaMMV have been determined. The former is 3524 nucleotides long excluding the 3’-terminal poly(A) tail and has one large open reading frame (ORF) encoding a polyprotein with a molecular mass of approximately 98 kDa. The N-terminal part of the polyprotein contains the amino acid (aa) motif GFCY...70aa...H, which is similar to a proteolytically active site found in the proteinase HC-Pro (helper component protein) of aphid-transmissible potyviruses (GYCY...71aa...H; Oh & Carrington, 1989) and in barley yellow mosaic bymovirus (BaYMV; GYC...70aa...H; Kashiwazaki et al., 1991; Davidson et al., 1991). Cleavage of the polyprotein would result in a 25 kDa protein (putative proteinase) and a 73 kDa protein of unknown function.

BaMMV-ASL1 was passaged every 6 weeks by mechanical inoculation of barley plants (Hordeum vulgare L. cv. ‘Maris Otter’) and maintained in a climate chamber at 14 to 16°C. The first viral RNA purification was performed ten passages after initial isolation of the virus from the field (Fig. 1, lane 1) and a second viral RNA purification was performed five passages later (Fig. 1, lane 2). In the first RNA preparation a band that stained very weakly and migrated faster than the major RNA2 was visible. This shortened form of RNA2 became predominant in the second and all subsequent preparations. The isolate that contained this altered form was called BaMMV-ASL1a.

BaMMV-ASL1a RNA2 was transcribed into cDNA and cloned, using the RT-PCR technique. First strand cDNA was synthesized after annealing of the poly(T) primer P1 (Fig. 2, Table 1), which has additional nucleotides representing recognition sites for ClaI, DraI and HindIII. P1 and a second primer, P2 (Fig. 2, Table 1), were used to amplify the 3’ region of RNA2. Following cleavage with HindIII and KpnI the amplification product was ligated into the analogous sites in the plasmid pGEM-3Zf(+) (Promega). The recombinant plasmid pG-3'-ASL1a was cloned in Escherichia coli NM522. Partial sequencing of this clone confirmed that it had a short poly(A) track of 20 residues.

The precise location of the deletion was determined by sequencing subclones of pG-3'-ASL1a. The deletion is

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Fig. 1. BaMMV RNA preparations. Viral RNA purifications were performed ten passages after initial isolation of the virus from the field (lane 1) and for a second time five passages later (lane 2). M, RNA molecular mass marker.

630 nt long corresponding to nt 2154 to 2783 of BaMMV-ASL1 RNA2. The deletion occurs in the C-terminal part of the region coding for the 73 kDa protein (Fig. 2, Del-630). Sequence analysis of the flanking regions did not reveal any differences between these and those of the initial field isolate.

A full-length cDNA clone of the deleted RNA2 of BaMMV was created in two steps. Firstly, the primer P3 was used for cDNA synthesis and a subsequent amplification was performed with the nested primers P4 and P5 (Fig. 2, Table 1). P5 is complementary to the first 15 nt of the 5' terminus of BaMMV-ASL1 RNA2 with additional nucleotides providing EcoRI and Dral recognition sites. Cleavage of the amplification product with Dral and KpnI produced a fragment that was ligated into the vector pT7E19(+) (Petty, 1988) which had had its SacI site removed with T4 DNA polymerase and had been restricted with KpnI. The resulting clone was named pT-5'-ASL1a.

In the second step the KpnI-HindIII fragment of pG-3'-ASL1a was ligated into the KpnI-HindIII site of pT-5'-ASL1a. The resulting clone was named pT-ASL1a. Subsequent to HindIII cleavage, a run-off transcript of the full-length clone was synthesized in vitro with T7 RNA polymerase. Compared with the virus RNA there are minor modifications at the extremities of the transcript. At the 5' terminus there is an additional G that is essential for the T7 RNA polymerase, and the short poly(A) sequence at the 3' terminus is followed by a GCT triplet that forms part of the HindIII recognition sequence.

The in vitro transcript of the full-length clone of BaMMV-ASL1a RNA2 was tested for infectivity in

Fig. 2. Scheme for the organization of BaMMV RNA2. The location of the natural deletions (Del-630, Del-JG and Del-GI/GB), of the primers used for cloning (P1 to P5) and detection of the deletions (P6 to P8), are indicated. VG/A is the putative cleavage site for the potential proteinase that is located in the N-terminal part of the polyprotein.
susceptible 'Maris Otter' barley plants. It was not expected that RNA2 could replicate alone and therefore a source for RNA1 was required. Since a full-length cDNA clone of RNA1 was not available, a wild-type-like BaMMV isolate with an RNA2 that is clearly distinguishable from the deleted form was required.

In order to select an appropriate isolate for co-inoculation, total RNA was extracted from single side shoots of barley plants infected with a number of different BaMMV isolates which had been propagated by mechanical inoculation for several years. First-strand cDNA was synthesized after annealing of primer P6 and the subsequent amplification of the cDNAs was performed with the nested primers P7 and P8 (Fig. 2, Table 1). The resulting products were compared electrophoretically with the corresponding fragment (1080 nt) of BaMMV-ASL1a. Two of the three isolates, BaMMV-GI and BaMMV-GB, contained deletions of approximately the same size in the region of their RNA2 that was amplified (Del-GI/GB, Figs 2 and 3). A third isolate, BaMMV-JG, the only one that was exclusively propagated at 10 °C, appeared to have no deletion in this amplified region of RNA2.

BaMMV-JG was chosen for co-inoculation experiments with in vitro transcripts of pT-ASL1a. The RNA was synthesized with and without the cap structure m7G(5')ppp(5')G in a volume of 20 μl using a T7 transcription kit (Boehringer Mannheim) and then immediately diluted to 500 μl with 0·5 M-K2HPO4. BaMMV-JG leaf pieces that were frozen for a short amount of time at −80 °C, were then mixed at a ratio of 1:3 (w/v) with 0·5 M-K2HPO4 and carborundum and

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Table 1. Nucleotide sequences of primers used for full-length cloning of BaMMV RNA2 and for the detection of deletions in BaMMV isolates

<table>
<thead>
<tr>
<th>Name</th>
<th>Position*</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>P1</td>
<td>Poly(A) tail (+ additional)</td>
<td>5' d[GGATCGATTTAAGCT] 3'</td>
</tr>
<tr>
<td>P2</td>
<td>383 to 404</td>
<td>5' d[GTCCTTCCATGGGTTTTTCTC] 3'</td>
</tr>
<tr>
<td>P3</td>
<td>1133 to 1152</td>
<td>5' d[GTGTGATACGACAGTGCG] 3'</td>
</tr>
<tr>
<td>P4</td>
<td>1105 to 1122</td>
<td>5' d[GTCCGACTAGGAGTACC] 3'</td>
</tr>
<tr>
<td>P5</td>
<td>1 to 15 (+ additional)</td>
<td>5' d[GTGAATTCTTTAAAAAATTTTTCAC] 3'</td>
</tr>
<tr>
<td>P6</td>
<td>2996 to 3015</td>
<td>5' d[CCCCGTGAACCCCTTCAATGC] 3'</td>
</tr>
<tr>
<td>P7</td>
<td>2973 to 2992</td>
<td>5' d[TGCAGAGCAGCAGACACTCTAG] 3'</td>
</tr>
<tr>
<td>P8</td>
<td>1283 to 1302</td>
<td>5' d[CGGCCCTCAACAGAACCG] 3'</td>
</tr>
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* Primer positions are related to the nt sequence of BaMMV-ASL1 (Timpe & Kühne, 1994).
then were homogenized in a mortar. For inoculation 100 µl of the diluted transcript was dropped on the surface of the youngest leaf of each of five barley seedlings at the three-leaf stage and virus-containing sap was then applied. All inoculated plants and healthy control plants were kept in a climate chamber at 14 to 16 °C.

Six and seven weeks after inoculation, five plants from each treatment set were assayed to detect the occurrence and length of RNA2 by RT-PCR using primers P7 to P8 as described above and total RNA was prepared from leaves taken from a single side shoot of each plant following the procedure of Verwoerd et al. (1989). No amplification product was detected in extracts from non-inoculated or mock inoculated plants, or in extracts from plants inoculated only with a capped transcript (data not shown). The results obtained after co-inoculation of plants with the in vitro transcript of pT-ASL1a and BaMMV-JG are shown in Table 2 and the resulting amplification products are presented in Fig. 3. Surprisingly, co-inoculated plants contained the wild-type-like amplification product of BaMMV-JG (1710 nt) only in 50% of the tested shoots whereas the other 50% contained an RNA2 with a deletion of approximately 150 nt (Del-JG, Fig. 2 and Fig. 3). Additional tests on the virus source performed with the same method described above revealed that the shorter form of RNA2 of BaMMV-JG was already present and did not result from the co-inoculation assay. With two out of five co-inoculated plants an amplification product was obtained that corresponded to the expected size for the pT-ASL1a transcript progeny. To exclude the possibility that these products were the result of a spontaneous deletion of BaMMV-JG, the RT-PCR products of BaMMV-JG control plants and of co-inoculated plants I, II, and III (Table 2) were purified with MicroSpin S-400 columns (Pharmacia) and partially sequenced with primer P8 (Table 1). Base exchanges at nt 2559 (G, loss of HincII site) and nt 2809 (A) as compared to BaMMV-ASL1 isolate. This result clearly indicates that in vitro transcripts of pT-ASL1a were infectious.

Transcripts from pT-ASL1a with or without a 5'-terminal cap structure were able to replicate in BaMMV-infected plants. Surprisingly, the two different types of RNA2 were detected simultaneously only in one preparation (plant I, Table 2).

The results indicate that RNA2 of BaMMV occurs both as the wild-type length and also with various deletions, thus supporting the findings of Dessens et al. (1995). All the deletions found so far are of varying length and occur exclusively in the 3'-terminal half of the ORF and affect the 73 kDa protein, the function of which is still unknown. No deletions were detected in other regions of the RNA2 (data not shown). Once a deletion has appeared, it seems to be stable when the virus is transmitted mechanically. Moreover, in this case, the deleted RNA2 replaces the initial form in infected plants indicating that it is more efficient in replication than wild-type RNA2. Recently, a similar observation has been described for another P. graminis-transmitted virus, the soil-borne wheat mosaic virus (Chen et al., 1994). Because wild-type RNA2 was predominant in all field isolates of BaMMV that have been investigated so far (unpublished results and Dessens et al., 1995), a selective mechanism for complete RNA2 exists. This suggests that the 73 kDa protein may be important for enabling the transmission of BaMMV by its natural vector. This hypothesis is supported by the findings of Adams et al. (1988) who have demonstrated that repeated mechanical transmission of the Streptley isolate of barley yellow mosaic virus, which has now been classified as BaMMV (Andersen et al., 1993), resulted in a strain that could not be acquired and/or transmitted by the vector. It seems reasonable to speculate that the 73 kDa protein possesses at least one additional function because all the deletions known so far are restricted to the C-terminal half of the protein. There are some sequence identities between this protein of BaMMV and the capsid readthrough proteins of furoviruses that are assumed to be involved in fungus transmission (Dessens et al., 1995).

In vitro transcripts of a full-length cDNA of deleted BaMMV-RNA2 (pT-ASL1a) replicate in barley plants when co-inoculated with wild-type-like BaMMV. A 5'-
terminal cap structure is not necessary for infectivity. The full-length cDNA clone of BaMMV RNA2 provides an excellent tool for further investigation of specific interactions between virus, vector and host.

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References


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