Volume 264, number 2, 183-186 FEBS 08425 May 1990

Biologically active transcripts of alfalfa mosaic virus RNA3

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Received 28 March 1990

Transcripts of the bicistronic RNA3 of alfalfa mosaic virus were synthesized using the in vitro T7 run-off transcription system. Synthetic RNA3 containing one additional G nucleotide at the 5' end were found to be infectious when coinoculated with RNA1 and RNA2 and coat protein.

Alfalfa mosaic virus; Infectious RNA3 transcript; Strain discrimination

1. INTRODUCTION

Alfalfa mosaic virus (AlMV) is a plant virus with a genome composed of three capped messenger RNAs: RNA1, RNA2 and RNA3. RNA1 and RNA2 are monocistronic [1,2] and code for proteins P1 and P2, respectively, proteins which are implicated in viral RNA replication. RNA3 is a bicistronic molecule [3-5] expressing protein P3 encoded by the 5' end cistron, whereas the 3' end cistron, encoding the coat protein, is expressed through RNA4, a subgenomic RNA. P3 protein is supposed to be involved in the movement of the virus through the plant [6]. Plant virus infection requires the three genomic RNAs plus RNA4 or the coat protein [7].

Development of in vitro run-off transcription systems was used to synthesize infectious RNA transcripts of the plant viruses including beet necrotic yellow vein virus [8,9], brome mosaic virus [10,11], cowpea chlorotic mottle virus [12], cowpea mosaic virus [13,14], tobacco mosaic virus [15,16], tomato rattle virus [17] and turnip crinkel virus [18], in order to study the molecular biology of these viruses. Biologically active AlMV RNA4 transcripts have been described [19,20] and we gave evidence that a DNA plasmid carrying RNA3 cDNA of strain S was biologically active at a very low level [21]. Here we describe the construction of infectious RNA3 transcripts of strain S (RNA3-S).

2. MATERIAL AND METHODS

2.1. *Modification of the 3' end of the transcripts*

A *Pst*I restriction site was introduced downstream of the extremity of the RNA3-S cDNA (cDNA3-S) opposite to the T7 promoter in the 3pT71 plasmid containing the complete cDNA3-S [21]. For this purpose we cloned the 3' end of RNA4, the subgenomic RNA of RNA3, with a synthetic oligodeoxynucleotide used as primer for the synthesis of a double-stranded (ds) DNA. The 3' part of this primer hybridized to the last 19 nucleotides of the 3' end of RNA4 and the 5' part contained the *Pst*I restriction site. The first strand synthesis was for 30 min at 37°C [22]. Then the RNAs were hydrolyzed by 0.3 M KOH for 3 min at 90°C. The second strand was synthesized by reverse transcription in the same buffer used for the first strand synthesis for 2 h at 20°C, followed by a 30-min incubation at 37°C. The ds DNA produced was digested by *Pst*I and *Apa*I enzymes to get a fragment of 241 base pairs. *Apa*I was the closest unique restriction site to *Pst*I in cDNA3-S. This fragment was introduced in a M13 vector containing the *Eco*RI-*Pst*I DNA fragment of the 3pT71 plasmid, corresponding to the complete cDNA3-S insert, cut by *Apa*I and *Pst*I enzymes. The *Pst*I-*Apa*I fragment from a positive clone containing the modification was introduced in a 3pT71 plasmid digested by these two enzymes to give plasmid 3pT71(17,0).

2.2. *Modification of the 5' end of the transcripts*

The junction between the T7 promoter and the cDNA3-S was modified by in vitro-directed mutagenesis [23] without enrichment for covalently close ds DNA. For this purpose the *Eco*RI-*Pst*I DNA fragment from 3pT71(17,0), containing the 3' modified complete cDNA3-S, was inserted in the vector pBS- (Stratagene) to get clone 3BS-(12,0). All nucleotides between the first nucleotide transcribed from the T7 promoter and the first nucleotide of the cDNA3-S were removed using the single-stranded DNA form of this clone and an oligodeoxynucleotide as primer. The single-stranded form of plasmid 3BS-(12,0) isolated as described by the manufacturer contained the DNA sequence corresponding to the RNA3 of plus polarity. The synthetic oligodeoxynucleotide used, GTGTAAGATGAAAACCTATAGGAGTCGTA, contained 15 nucleotides at the 5' end complementary to the 5' end of RNA3-S and 15 nucleotides at the 3' end complementary to the T7 promoter. Positive clones were identified with the labelled mutagenic primer. The presence of the modification was checked by sequencing for two clones: 3BS-(1,0)a and 3BS-(1,0)b.

2.3. *In vitro transcription*

Plasmids were digested by *Pst*I enzyme. The protruding 3' end generated by *Pst*I was then removed with T4 DNA polymerase. The latter reaction was carried out for 30 min at 37°C in 50 mM Tris-HCl, pH 8.3, 15 mM $(NH4)_2SO_4$, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM β-

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

mercaptoethanol, 0.2 μg/μl BSA, 50 μM each of dATP, dCTP, dGTP, dTTP containing 0.2 μg/μl of *Pst*I-digested DNA and 0.1 unit/μl of T4 DNA polymerase (Strategene). The transcription of 5 μg of plasmid digested by *Pst*I and treated by T4 DNA polymerase was in 100 μl of 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 0.1 μg/μl BSA, 1 unit/μl RNasin (Pharmacia), 50 μM GTP, 500 μM each of ATP, CTP and UTP, 500 μM m^7GpppG, containing 50 units of bacteriophage T7 RNA polymerase (Strategene) for 1 h. After 30 min the GTP concentration was raised to 100 μM. After transcription nucleic acids were phenol-extracted, precipitated from ethanol, and aliquots corresponding to the transcription of 0.6 μg of DNA were used for the inoculations.

2.4. *Infectivity test and RNA3 detection*

The inoculation was done with 20 μl of a mixture containing 1.6 μg RNA1, 1.2 μg RNA2 prepared from the AlMV B strain as previously described [24] and coat protein from strain L prepared according to [25]. Young tobacco plants with three leaves (2-3 cm width) were used. RNAs were extracted from the inoculated leaf 6 days after inoculation and from a systemic upper leaf 8 days after inoculation as indicated in [26]. RNA3 detection and strain characterization by primer extensions were done as in [24].

3. RESULTS

T7 transcripts obtained with plasmid 3pT71, containing the complete cDNA3-S and cut by a *Pst*I enzyme were not infectious. These transcripts carry 17 non-viral nucleotides and the 5′ end and about 90 extra nucleotides including a non-viral polyA track at the 3′ end. However, both cistrons were functional in vitro (results not shown). In order to get infectious transcripts, both extremities of the cDNA3-S were modified to get T7 transcripts with only one additional G residue at the 5′ end and with a 3′ end identical to the natural RNA3.

To synthesize transcripts without additional non-viral nucleotides at the 3′ end a *Pst*I restriction site was created immediately downstream to the cDNA3-S at the extremity opposite to the T7 promoter in plasmid 3pT71 (see section 2 and Fig. 1) to get plasmid 3pT71-(17,0). The numbers in parentheses indicate the number of additional non-viral nucleotides present in the T7 transcripts at the 5′ and the 3′ end. A *Pst*I site was introduced to linearize the plasmid by cutting the DNA strand used as template by the T7 polymerase just after the last nucleotide of the cDNA3-S (Fig. 1).

The extremity of the cDNA3-S opposite to the *Pst*I restriction site was fused to the first nucleotide transcribed from the T7 promoter to remove all additional nucleotides, but one G, at the 5′ end of the transcripts. The final construction was made in vector BS containing the *Eco*RI-*Pst*I DNA fragment from plasmid 3pT71(17,0) (section 2).

Two clones 3BS-(1,0)a and b producing transcripts (1,0)a and (1,0)b, respectively, were selected. Prior to transcription, plasmids linearized with *Pst*I were treated by T4 DNA polymerase to remove the 3′ protruding end. This treatment enhanced the amount of RNA3-S transcripts produced by the T7 polymerase and prevented the synthesis of RNA products of larger

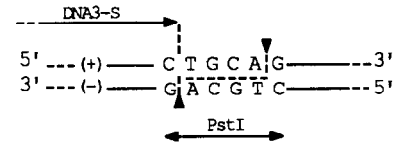

Fig. 1. Schematic representation of the *Pst*I site in clone 3BS-(1,0). The upper strand corresponds to the DNA sequence of plus-strand RNA3-S. The positions of the *Pst*I cuts in the two strands are indicated by arrowheads.

molecular weight ([27] and personal communication). Analysis of the transcription is shown in Fig. 2. The transcription of plasmids 3BS-(1,0)a (lane 4) and b (lane 6) producing transcripts (1,0)a and (1,0)b, respectively, was as efficient as transcription from plasmid 3pUC9(2,0) (construction not shown) giving RNA3-S transcripts with two additional G nucleotides at the 5′ end (lane 2), but less efficient than with the 3pT71 plasmid (result not shown). This is probably due to the nature of the sequence present downstream of the T7 promoter [8,14,17].

Capped transcripts were used to complement a mixture of RNA1,2 of strain B and coat protein of strain 425L. RNA 1 and 2 were purified from strain B of AlMV and coat protein from strain 425L because it is easy to distinguish RNA3 molecules of strain S, B and 425L by primer extension [24] since the length of their leader is different ([4,5] and unpublished results). An oligodeoxynucleotide hybridizing specifically on all RNA3s in the P3 open reading frame near the AUG initiation codon was used as primer to synthesize full-length cDNA on RNA3 present in total RNA extracted from leaves. Then the length of the cDNA was analyzed in a sequencing gel to identify the strain origin of the RNA3 reverse transcribed. The full-length cDNA is in increasing order RNA3-B, RNA3-S, RNA3-L [24].

In Fig. 3A, full-length cDNA synthesized on purified RNA of strain S is indicated with an S. Minor bands of strong stop products are also visible. When the mixture of RNA1, 2 and CP was not complemented (lane 1) or complemented with transcripts (2,0) (lane 2), no RNA3 was detected in phenol extracts from inoculated leaf,

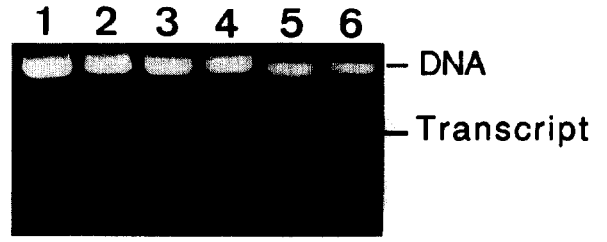

Fig. 2. Analysis of the transcripts. Transcripts were analyzed on agarose gel stained with ethidium bromide. Analysis of plasmids 3pUC9(2,0) (lanes 1 and 2), 3BS-(1,0)a (lanes 3 and 4) and 3BS-(1,0)b (lanes 5 and 6) is shown before (lanes 1, 3 and 5) and after (lanes 2, 4 and 6) transcription.

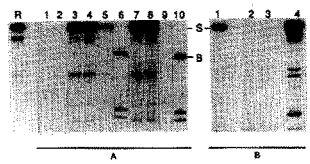

Fig. 3. Detection of RNA3 in tobacco leaves infected with RNA3 transcripts complemented with RNA1 and RNA2 of AlMV-B and coat protein of AlMV-L. (A) Analysis by primer extension of RNA extracted from virions of strain S is shown as a reference (lane R). The analyzed RNA were from inoculated leaves (lanes 1–6) and from systemic non-inoculated leaves (lanes 7–10). The RNA were from plants inoculated with the mixture of RNA 1, 2 and CP (lane 1), the same mixture complemented with transcripts (2,0) (lane 2), with transcripts (1,0)a (lanes 3 and 7), with transcripts (1,0)b (lanes 4 and 8), with equal amounts of transcripts (2,0), (1,0)a and (1,0)b (lanes 5 and 9) and with RNA3-B (lanes 6 and 10). S and B indicate the position of the full-length cDNA obtained with RNA3 of strain S and strain B, respectively. (B) Same analysis as in (A) on RNA extracted from the inoculated leaf of plants inoculated with the mixture of RNA1, 2 and coat protein complemented with 3BS-(1,0)a plasmid after (lane 1) and before (lanes 2 and 3) transcription. As a control the analysis was done with RNA extracted from virus of strain S (lane 4).

suggesting that RNA1 and RNA2 were not contaminated by RNA3-B, that coat protein was not contaminated by RNA3-L and that transcripts (2,0) were not able to complement this mixture. The mixture became infectious after complementation with RNA3-B. RNA3-B was detected in the inoculated (lane 6) and the upper non-inoculated leaf (lane 10). The addition of transcripts (1,0)a or (1,0)b to the mixture rendered the mixture infectious. In these cases the RNA3 detected in the inoculated and non-inoculated leaf was exclusively of strain S (lanes 3, 4, 7 and 8), indicating that these transcripts were biologically active. The mixture complemented with equal amounts of transcripts (2,0), (1,0)a and (1,0)b was also infectious but RNA3 of strain S was only detected in the inoculated leaf (lanes 5 and 9). This lower infectivity could be due to: (i) an inhibitory effect of DNA present in a three times higher amount in the inoculum than in the inoculum rubbed on the leaves analyzed in lanes 3 and 4; (ii) the presence of transcripts (2,0); or (iii) both reasons. The second hypothesis is likely because all transcripts have the same 3′ end extremity. As transcripts (2,0) were not infectious in lane 2, they could compete with transcripts (1,0) for the first step of the RNA3 replication by the synthesis of non- or less functional minus-strand RNA3. These results indicate that only transcripts (1,0) were infectious. However, transcripts (2,0) could be infectious at a lower extent, not detected under the conditions of experimentation used here. As expected, transcripts (1,0) were also infectious when coat protein was replaced by RNA4 (result not shown).

The mixture of RNA1, 2 and coat protein was complemented by BS-(1,0)DNA after (Fig.3B, lane 1) or before (lanes 2 and 3) transcription. As a reference, the primer extension was done with RNA extracted from virus of strain S (lane 4). The analysis of the inoculated leaf showed the presence of RNA3-S only in lane 1, indicating that the infectivity was related to the presence of the transcripts (1,0).

4. DISCUSSION

According to these results, the infectivity of the transcripts corresponding to RNA3 of AlMV strain S was very sensitive to the number of non-viral nucleotides present at the 5′ end, as it was also noted for other plant viruses [8,11,14,15,17,18].

Only the RNA3-S transcripts with one additional G nucleotide were infectious. They will be used to study the molecular aspects of the virus multiplication. As previously reported, the amount of RNA3 relative to that of RNA4 extracted from the virions of strains S, B, and 425 L differed [24], and furthermore this ratio seems not to be influenced by the strain from which RNA1 and RNA2 originated (to be published).

Since the major sequence differences between the RNA3 in the three AlMV strains are located in the 5′ non-coding region and because the intercistronic region is highly conserved, variations in the length or the structure of the leader sequence will be created. The consequences of these modifications will be assessed by the study of the virus multiplication initiated by complementation of RNA1, RNA2 and RNA4 with transcripts from modified 3BS-(1,0) clones.

Acknowledgments: The authors are grateful to Dr E.M.J. Jaspars for improving the manuscript and to Mrs Sandra Blumenfeld for skillful technical assistance.

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