Plasmid DNA Containing a Copy of RNA3 Can Substitute for RNA3 in Alfalfa Mosaic Virus RNA Inocula

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SUMMARY
The infectivity of a DNA copy of RNA3 from strain S of alfalfa mosaic virus inserted in a pT7-1 plasmid vector (clone 3pT7-1) was tested as circular DNA by co-inoculation with RNAs 1, 2 and 4 from strain B (mix I₀) on young tobacco leaves. Virus multiplication was monitored by serological detection of the coat protein. Progeny RNA3 derived from circular plasmid DNA 3pT7-1 was of the same length as naturally occurring RNA3-S. Analysis by primer extension demonstrated that it had the 5’-terminal sequence of RNA3-S and not that of RNA3-B. Mix I₀ also became infectious when mixed with M13 single-stranded DNA carrying RNA3-S inserted in the (−) strand orientation, probably because traces of RNA3-B present in mix I₀ were protected from degradation by base-pairing and were therefore active.

INTRODUCTION
Construction of cDNA clones corresponding to the genomic RNA of several plant or animal viruses and the use of in vitro transcription have yielded synthetic RNA transcripts capable of infecting host cells (Ahlquist et al., 1984; Kaplan et al., 1985; Mizutani & Colonno, 1985; Dasmahapatra et al., 1986; Meshi et al., 1986). This has also been so for multipartite genome viruses such as brome mosaic virus (BMV), for which transcripts corresponding to each of the three genomic RNAs were mixed to give an infective inoculum (Ahlquist et al., 1984). For alfalfa mosaic virus (AlMV), a plant virus with a genome similar in size and organization to BMV, the genomic RNAs (RNA1, RNA2 and RNA3) require the addition either of the subgenomic coat protein (P4) messenger RNA4 derived from RNA3, or P4 itself, to become infective (Jaspars, 1985). Transcripts corresponding to RNA4, capable of activating the genomic RNAs, have been reported (Loesch-Fries et al., 1985; Langereis et al., 1986a).

Infection with plasmids containing cDNA clones of viral RNA has been reported for poliovirus (Racaniello & Baltimore, 1981), but not for plant viruses. Successful results were, however, obtained with satellites (Collmer & Kaper, 1986; Gerlach et al., 1986; Van Emmelo et al., 1987) and with viroid clones (Cress et al., 1983; Meshi et al., 1984; Visvader et al., 1985). We report here expression of plasmid DNA carrying copies of the genomic RNA3 of AlMV in plants, observed after co-inoculation with the other genomic RNAs and subgenomic RNA.

METHODS
AlMV strains and RNA fractionation. The AlMV strains B and S were multiplied in Nicotiana tabacum var. Xanthi nc. Virus preparation and RNA fractionation were as described by Pinck & Fauquet (1975).

cDNA cloning. RNA3-S, purified by PAGE, was polyadenylated (Devos et al., 1976) and cloned in a pUC9 vector (Heidecker & Messing, 1983). A full-length cDNA was constructed by ligation of two pUC9 clones and inserted into a pT7-1 transcription vector (Genescibe, Cleveland, Ohio, U.S.A.). This full-length DNA copy, i.e. the EcoRI-PstI insert, was also cloned in the replicative forms of bacteriophages M13mp10 and M13mp11 so that ssDNA of both polarities could be produced. In vitro transcription of the pT7-1 clone from the T7 promoter was carried out as described by Melton et al. (1984) and yielded (+) strand RNA3 with additional nucleotides at each end.

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Biological activity controls. One leaf (3 to 4 cm long) of *N. tabacum* var. Xanthi nc. was dusted with Celite and inoculated with 40 μl of a mixture containing RNA1 (3.2 μg), RNA2 (2.4 μg) and RNA4 (0.9 μg) in 20 mM-phosphate buffer pH 6.8. This RNA mixture was added either to synthetic RNA3-S or to various plasmid DNA forms carrying a copy of RNA3-S. Similar experiments with authentic RNA3-S or RNA3-B produce symptoms of infection after 3 to 4 days at 24 °C.

Detection of coat protein in inoculated tobacco leaves. A leaf was harvested 4 days after inoculation and was ground quickly in liquid nitrogen. A portion (100 mg) of the resulting fine powder was mixed with 200 μl 200 mM-Tris-HCl pH 8.0, 5 mM-EDTA, and centrifuged for 5 min at 12000 g in a microcentrifuge. Eight μl of the supernatant fraction was diluted with 4 μl 4% SDS, 9 mM-urea and 2% mercaptoethanol in 75 mM-HCl pH 6.8, boiled for 2 min and loaded on a 12.5% polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose gel by electrophoretic blotting and reacted with serum raised against coat protein. The PAGE conditions, transfer procedure and immunoblotting reactions were as described by Berna et al. (1985).

Primer extension and RNA sequencing. Synthetic oligomers of 17 deoxynucleotides were used to analyse the 5' end region of RNA3 present in total RNA from inoculated leaves, by primer extension with reverse transcriptase (Life Sciences, St. Petersburg, Fla., U.S.A.) (Qu et al., 1983). Extension was either after 5' end-labelling of the primer with polynucleotide kinase and [γ-32p]ATP (3000 Ci/mmol) (Boseley et al., 1978) or by reverse transcription in the presence of [α-32p]dATP (400 Ci/mmol) for 10 min at 37 °C followed by a 20 min chase, essentially as in Qu et al. (1983). The cDNAs thus obtained were analysed on sequencing gels according to Ansorge & Labeit (1984).

Northern blot analysis. Total RNA from tobacco leaves was extracted according to Jackson & Larkins (1976). Samples of RNA (10 μg) were heated for 5 min at 65 °C in 10 μl of 20 mM-HEPES, 1 mM-EDTA, pH 7.8, containing 50% deionized formamide and 6% formaldehyde, electrophoresed in a 1% agarose slab gel prepared in the same buffer lacking formamide and transferred to GeneScreen Plus membranes (New England Nuclear) by blotting with 20 x SSC (0.3 M-sodium citrate, 3 M-NaCl), as described by Gustafson et al. (1982). The blots were hybridized for 14 h at 42 °C with cDNA labelled by reverse transcription using [α-32p]dATP (3000 Ci/mmol) without a chase (Qu et al., 1983). Processing of the membranes was according to manufacturer's instructions.

RESULTS

Constructions containing a full-length DNA copy of RNA3 from AlMV strain S

A full-length cDNA of RNA3-S was assembled from two pUC9-derived partial clones, one containing nucleotides 1 to 552, and the other the 3' part of RNA3-S from nucleotides 285 to 2111. The nucleotide numbering used takes into account the leader sequence repeat reported in Langereis et al. (1986b). The clones share a unique XhoI site in RNA3-S, which allowed ligation of the two parts of the DNA copy. The full-length cDNA of RNA3-S was inserted into a pT7-1 expression vector between the PstI and the EcoRI sites, yielding clone 3pT7-1. Transcription with T7 polymerase gave transcripts of (+) polarity corresponding to RNA3-S, with additional nucleotides: 17 nucleotides were introduced during the cloning procedures at the 5' end (GGGAGACCGGAAUCCCG...) and 90 nucleotides, of which 80 corresponded to a poly(A) sequence [...] (A₈₀)UGCAGGUCGACC] at the 3' end. The full-length cDNA (EcoRI-PstI fragment) was inserted in the replicative forms of M13mpl0 and M13mpl1 in order to obtain single-stranded forms of the insert corresponding to viral RNA3 polarity in (+) strand orientation [M13.3(+) and in (−) strand orientation [M13.3(−)], respectively. The biological activity of the synthetic RNA3-S transcribed from 3pT7-1, of the 3pT7-1 plasmid itself (circular dsDNA) and of the ssDNAs of M13.3(+) and of M13.3(−) was tested.

Biological activity of ssDNAs containing a copy of RNA3 [M13.3(+) and M13.3(−)]

Infectivity of AlMV RNAs is dependent on the presence of RNA1, RNA2, RNA3 and RNA4 in the inoculum (Jaspars, 1985). Consequently, the infectivity of a mixture containing only RNA1, RNA2 and RNA4 (mix I₀) plus the material to be tested is a sensitive assay for the detection of RNA3. The presence of coat protein in inoculated leaves was used to show that virus synthesis had occurred, i.e. that mix I₀ had been complemented with a source of biologically active RNA3.

The AlMV RNA1, RNA2 and RNA4 of strain B (mix I₀) that were used for inoculation were purified from total virion RNA by separation in 2.8% polyacrylamide slab gels. No traces of RNA3-B could be detected by staining after PAGE fractionation of the purified RNA1, RNA2 or RNA4 used to make the inoculation mixture. For assays *in vivo* the standard concentration of...
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Fig. 1. Detection of P4 protein in tobacco leaves infected with AlMV RNA (a) and analysis of the cDNA reverse-transcribed after priming with P438 on RNA extracted from the inoculated leaves (b). (a) Protein extracts from leaves inoculated with mix I0 alone (lane 1) or mixed with 4 μg M13.3(+) ssDNA (lane 2) or with 4 μg M13.3(+) ssDNA alone (lane 3) were analysed. In lane 4 the inoculum was mix I0 containing 4 μg M13.3(−) ssDNA. In lane 5, 4 μg M13.3(−) ssDNA was inoculated alone. In lane 6 the inoculum was AlMV RNA-B containing 2.7 μg RNA1, 1.4 μg RNA2, 0.9 μg RNA3 and 2 μg RNA4. (b) Autoradiogram of a sequencing gel of cDNA reverse-transcribed after priming with P438 on 10 μg RNA extracted from leaves harvested 5 days after inoculation with the mixtures described above. Lane 7 shows cDNA obtained after priming in RNA of strain S. Full-length cDNA of RNA3-S and RNA3-B are indicated at the side by S and B and the characteristic shorter products caused by pauses in reverse transcription by S', B' and B".

Fig. 2. Location of the primers along the leader sequences of RNA3-S and RNA3-B. The homologous repeated sequences in RNA3-S and RNA3-B of 27 to 30 nucleotides long are shown by dotted areas, the 56 nucleotide repeat by hatched areas and the P3 coding region in black. The primer sequences indicated in the upper lines of each large box are shown base-paired with the RNA3-S sequence underneath; the GC pairings are indicated by heavy lines. The lower sequence corresponds to RNA3-B. Mismatches between S and B are boxed.

purified RNAs in mix I0 was RNA1 3.2 μg, RNA2 2.4 μg and RNA4 0.9 μg in 40 μl. Using this inoculum, no infection developed in the inoculated leaf after 5 days, as deduced from the absence of detectable P4 protein (Fig. 1 a, lane 1). All complementation assays described hereafter were carried out in triplicate with the same mix I0.
Inoculation of 4 µg M13.3(+) ssDNA, either with the above RNA mixture or alone, did not produce any detectable P4 (lanes 2 and 3, respectively). P4 was detected (lane 4) when the same inoculum to which 4 µg M13.3(−) ssDNA had been added was used, but not when M13.3(−) ssDNA was inoculated alone (lane 5). Inoculation of virion RNAs yielded P4 (lane 6), as in lane 4. The presence of P4 protein in lane 4 was therefore directly related to the presence of M13.3(−) ssDNA in the inoculum. Since the amount of RNA4 in mix I0 was below the level at which it could give rise to detectable amounts of protein P4 (lane 1), the presence of P4 in lane 4 must be an indication of the presence of RNA3. This RNA could result either from synthesis of RNA3 by a polymerase using M13.3(−) ssDNA as its template, or from a 'protective' effect of M13.3(−) ssDNA due to DNA–RNA hybrid formation with traces of RNA3 of B strain present in the original mix of purified RNAs. The first possibility implies that RNA3 of strain S would be produced, while in the latter case RNA3 would correspond to that of strain B. These two cases were distinguished by primer extension as described below, since the serological method could not discriminate between the P4 protein of strains S and B.

Analysis of RNA3 by primer extension is based on sequence homology between RNA3 of strains S and B and on the significant differences in length of the 5′-terminal non-coding regions. The leader sequence of strain B is 74 nucleotides shorter than in strain S (Ravelonandro et al., 1983; Langereis et al., 1986b). Thus the two RNA species can be distinguished on the basis of the length of cDNA produced by primer extension from an appropriate RNA3-specific primer. The sequences of the two primers are indicated in Fig. 2. The name of each primer indicates the distance in nucleotides from the priming site to the 5′ end of RNA3-S, and thus also indicates the length of cDNA that can be synthesized for RNA3-S. Primer P171 hybridized only with RNA3-S and was used to determine the precise length of its leader. Primer P438 was fully homologous to the S sequence and had only one nucleotide mismatch with strain B. P438 primed efficiently on both RNA3-S and RNA3-B and was used to identify the origin of RNA3 based on the length of the reverse-transcribed cDNA. The size of full-length cDNA after priming on RNA3-B and RNA3-S was 381 and 455 nucleotides respectively.

The RNAs from leaf samples of the tests shown in Fig. 1(a) were extracted and primed with P438 for reverse transcription. The resulting cDNAs were analysed on sequencing gels as shown in Fig. 1(b). In addition, the full-length cDNA reverse-transcribed after priming on RNA3 from strain S virions is shown in lane 7 (S), while full-length cDNA of RNA3-B is shown in lane 6 (B). The shorter cDNA bands B' and B", characteristic of RNA3-B, result from stops or pauses in reverse transcription of this molecule and are not present in cDNA of RNA3-S (lane 7). These patterns of bands allow easy discrimination of RNA3 originating from S and B. For example, the pattern of bands in lane 4 indicate that RNA3-B is present and its origin can only be explained by the presence of contaminating RNA3-B in the inoculum. Since no virus multiplication was detected in leaves inoculated with mix I0 alone (lane 1) or together with M13.3(+) ssDNA (lane 2), it can be concluded that (i) M13.3(−) ssDNA present in the inoculum of lane 4 has a 'protective' effect on traces of RNA3-B present in the inoculum used and (ii) there is no synthesis of RNA3-S derived from single-stranded M13.3 DNA inoculated alone or in complementation assays. This result demonstrates that primer extension is the most reliable way to determine the biological activity of the various constructs and to analyse the origin of RNA3 molecules appearing after inoculation. It was therefore routinely used to analyse RNA3 produced by various inocula.

**Biological activity of 3pT7-1 plasmid**

Complementation assays of mix I0 were carried out under the same conditions as used previously with 2.5 µg circular dsDNA. Fig. 3(a) shows the analysis of cDNA reverse-transcribed after P438 priming on RNA from an inoculated leaf extracted 5 days after inoculation (lanes 1 to 4). The cDNAs obtained from priming on virion RNA of strain B (lane 5) and strain S (lane 6) showed a characteristic band pattern similar to that of Fig. 1(b) (lane 6 and 7) and serves as a length reference. In lane 4, the RNAs extracted after inoculation of mix I0 alone did not contain RNA3. If mix I0 was complemented with both RNA3s in a ratio S : B of 1 : 10 (0.1 µg RNA3-S and 1 µg RNA3-B), the extracted RNA clearly contained both full-length
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Fig. 3. Autoradiogram of analysis on sequencing gels of cDNA reverse-transcribed after priming with P438 on ALMV RNA and on RNA from inoculated and uninoculated leaves positioned above the inoculated leaves. (a) Lanes 1 to 6 show the cDNAs reverse-transcribed after priming with labelled primer on RNA. In lanes 1 to 4 priming was on 10 µg of total RNA extracted from one leaf inoculated with 30 µl containing 1.9 µg RNA1, 1.5 µg RNA2 and 0.5 µg RNA4 from strain B mixed with 1 µg RNA3-B and 0.1 µg RNA3-S (lane 1), with 2.5 µg 3pT7-1 circular DNA (lane 2), with 2.5 µg M13 3(−) ssDNA (lane 3) or without addition (lane 4). RNA (1.5 µg) purified from ALMV B (lane 5) or from ALMV S (lane 6) served as references. The bands corresponding to full-length cDNA of S and B RNA3 are indicated at the side by S and B and the products characteristic of pauses in reverse transcription are indicated by S', B' and B". Electrophoresis was performed in an 8% sequencing gel. Autoradiography was for 4 days with an intensifying screen. (b, c) The autoradiogram shows similar analysis after priming on RNA extracted from inoculated (b) and uninoculated leaf from above the inoculated leaf (c) from the same plant, analysed 5 days after inoculation with the RNAs described for lanes 1 to 3 of (a).

cDNA-S and cDNA-B corresponding to RNA3-S and RNA3-B and the shorter characteristic bands S' and B', B" (lane 1). As noted above, complementation with M13 3(−) ssDNA (lane 3) gave the same results as in Fig. 1 (b) (lane 4). Comparison of lanes 1 and 6 shows that smaller amounts of intermediate cDNA bands were found in RNA extracted from infected leaves than in virion RNA of strain S. The larger amounts of ‘incomplete’ cDNA in the latter case probably resulted from extension on molecules damaged during virus purification. Such damage occurred only with strain S. Complementation with pT7-1 plasmid DNA yielded RNA whose cDNA produced bands characteristic of RNA3-S in addition to bands characteristic of RNA3-B (lane 2).

The 5' terminus of the full-length cDNA corresponding to RNA3-S (Fig. 3a, lane 2) was analysed with primer P171 to obtain the precise length of this RNA. Fig. 4 shows the full-length cDNA of virion RNA of strain S used as a control (lane 2) and the absence of priming on RNA of strain B (lane 1). DNA made by extension in the presence of dideoxycytidine triphosphate shows the position of the C residues in the leader of RNA3-S (lane 4); resolution was high in this region with C 13 and C 15 separated well. The double bands, identical in lanes 2 to 4, indicate that the 5'-terminus was capped. The migration pattern in lane 3 indicates that RNA3-S derived from 3pT7-1 and virion RNA3-S were identical in length.

Virion RNAs of strain B, strain S and the RNA shown in lane 2 (Fig. 3a) were separated on a denaturing agarose gel (Fig. 5, lanes 1, 2 and 3, respectively) and the Northern blot was hybridized with a labelled cDNA probe made by P438 extension. Lane 1 shows the position of RNA3-B. Lane 2 shows the two bands correspond to RNA3 and RNA3'. The latter is shorter than RNA3 by 210 nucleotides at the 5' end and is always present in virion RNA of strain S.
Fig. 4. Autoradiogram from sequence gel analysis of cDNAs reverse-transcribed after priming with the S strain-specific primer P171. The RNAs analysed were virion RNA of strain B (lane 1), of strain S (lanes 2 and 4) and the RNA analysed in lane 2 of Fig. 3(a) (lane 3). Products in lane 4 were made in the presence of dideoxyCTP. Numbering at the side indicates the positions of C13 and C15 in the leader.

Fig. 5. Autoradiogram of Northern blot after analysis of virion RNA of strains B (lane 1) and S (lane 2) and of RNA analysed in lane 2 of Fig. 3(a) (lane 3) on a denaturing 1% agarose gel in the presence of formaldehyde. The probe used was labelled cDNA obtained after priming with P438 on RNA3-S. Position of viral RNA detected by staining is indicated at the side.

(Ravelonandro et al., 1983). The single band in Fig. 5 lane 3 has a mobility close to RNA3-S and RNA3-B and no shorter bands were detected. This indicates that 3pT7-1-derived RNA has few, if any, additional nucleotides at the 3' end.

The presence of RNA3-S derived from the complementation assay with 3pT7-1 is evidence for the presence of biologically active RNA. To investigate if this RNA3-S, present along with RNA3-B in the RNA extracted from the inoculated leaf, was able to multiply, the total RNA from the inoculated leaf was re-inoculated. Three sets of inoculations were made, each being of the RNA shown in lanes 1, 2 or 3 of Fig. 3(a). Five days after inoculation the RNA was extracted from the inoculated (Fig. 3 b) and the uninoculated leaves above the inoculated leaf (Fig. 3 c) and analysed. RNA3-S alone was present in lane 1 of Fig. 3(b) and 3(c). The RNA3-B initially present in low amounts in the inoculum (lane 1, Fig. 3a) was undetectable, even after longer autoradiographic exposure. This indicates that RNA3-S replicated at a greater rate than RNA3-B after the second inoculation, despite an initial 1:9 ratio of RNA3-S to RNA3-B in the first inoculum. In each lane 3, only RNA3-B was found. In each lane 2, RNA3-S was present in large amounts in both the inoculated and uninoculated upper leaves, while RNA3-B was not detected in either. This behaviour of RNA3-S, which was similar to that described for the RNA in lane 1, proves that the RNA3-S derived from 3pT7-1 was fully functional.

**DISCUSSION**

Primer P438 was hybridized on RNA3-S and RNA3-B, despite a mismatch of one nucleotide with RNA3-B, and enabled specific identification of RNA3 of each strain (Fig. 1 b, lanes 6 and 7), even in a mixture of both (Fig. 3a, lane 1). The identification of newly synthesized RNA3 was therefore based on hybrid extension with this primer.

Virion RNA of AlMV strain B was free of contaminants from other strains: multiplication of mix 10, complemented with RNA3-B or with M13.3(−) ssDNA always yielded RNA3-B in the
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inoculated leaf (Fig. 1 b, lanes 4 and 6) and in systemically infected leaves (Fig. 3, lanes 3). If mix I₀ was inoculated with a large excess of RNA3-B over RNA3-S, the progeny isolated from the inoculated and uninoculated upper leaves indicated more efficient production of RNA3-S (Fig. 3a, lanes 1). Comparison of the intensity of label in bands S, S' and B, B' corresponding to the cDNA extended from P438 on RNA3-S and RNA3-B, respectively, in these assays showed that after the second inoculation the level of RNA3-B decreased gradually in the systemically infected leaves. This implies that efficient replication of RNA3-S had occurred and also showed that in mixed inoculation, using mix I₀ and RNA3-S for complementation, RNA3-S was replicated more efficiently than RNA3-B by the B strain replicase. Complementation of mix I₀ with single- or double-stranded plasmid DNA carrying an RNA3-S cDNA insert therefore enabled detection of RNA3-S expression from these constructs, despite the existence of traces of RNA3-B present in mix I₀. The reverse situation, using dsDNA forms with RNA3-B insertions and an RNA-S-derived I₀ mix, would be less favourable.

Neither of the two ssDNA constructs, either alone or complemented with mix I₀, allowed expression of the insert in inoculated or systemically infected leaves. Addition of M13.3(-) ssDNA to mix I₀ increased its infectivity (Fig. 1a, b), most likely by RNA-DNA hybrid formation which protected traces of RNA3-B in mix I₀. Synthesis of RNA3 in the presence of complementary ssDNA implied that the DNA–RNA3 hybrid, even if not translatable in vivo [assuming hybrid-arrested translation as observed in vitro (Paterson et al., 1977)] could be replicated, thus allowing translation from the newly synthesized RNA3.

Synthesis of RNA3-S after complementation of mix I₀ with 3pT7-1 DNA implies that a first transcription step occurs under control of one of the three cellular RNA polymerases, since no DNA-dependent step during replication of plant RNA viruses has been reported until now. RNA transcription presumably takes place in the nucleus and is followed by export of the newly synthesized RNA into the cytoplasm where viral replication would take place. The polarity of this transcribed RNA is unknown. If this RNA is of (+) polarity it should not contain many additional nucleotides, especially at the 5' end, because their presence would be expected to decrease replication by viral replicases dramatically, as shown for BMV RNAs (Janda et al., 1987) and tobacco mosaic virus RNAs (Dawson et al., 1986; Mashi et al., 1986). Complementation assays made with T7 transcripts from 3pT7-1 were unsuccessful in the standard conditions used here. The additional nucleotides present at the ends of these transcripts were probably responsible for the lack of infectivity.

The length of RNA3 produced from 3pT7-1 was identical to that of RNA3-S in Northern blot analysis (Fig. 5) and had the same 5' end as natural RNA3-S (Fig. 4). The possible presence of additional nucleotides remaining at the 3' end could not be investigated precisely with the methods used here but the number of extra 3' nucleotides, if any, cannot be large since a difference of 210 nucleotides was easily visible (Fig. 5, lane 2).

The occurrence of RNA3-S synthesis derived from double-stranded DNA (3pT7-1) circular form was detected in three out of 20 plants tested in four separate assays. This rather low frequency might be due to the nuclear step thought to be necessary during synthesis of this RNA.

Preliminary results indicate that in assays with linearized forms of 3pT7-1 DNA synthesis of RNA3-S could also be observed. Additional experimentation with various forms of cleaved 3pT7-1 and with other constructs containing the same insert should provide information on the mechanism involved in this phenomenon. Definitive confirmation that 3pT7-1 is indeed the template for RNA3-S found in the progeny can only be provided by genetic labelling of the insert. Work is in progress to obtain a construct that meets this requirement.

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REFERENCES

