Role of the 5' leader sequence of alfalfa mosaic virus RNA 3 in replication and translation of the viral RNA

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ABSTRACT

RNA 3 of alfalfa mosaic virus (AIMV) encodes the movement protein P3 and the viral coat protein which is translated from the subgenomic RNA 4. The 5'-leader sequences of RNA 3 of AIMV strains S, A, and Y differ in length from 314 to 392 nucleotides and contain a variable number of internal control regions of type 2 (ICR2 motifs) each located in a 27 nt repeat. Infectious cDNA clones were used to exchange the leader sequences of the three strains. This revealed that the leader sequence controls the specific ratio in which RNAs 3 and 4 are synthesized for each strain. In addition, it specifies strain specific differences in the kinetics of P3 accumulation in plants. Subsequent deletion analysis revealed that a 5'-sequence of 112 nt containing one ICR2 motif was sufficient for a 10 to 20% level of RNA 3 accumulation in protoplasts and a delayed accumulation in plants. An additional leader sequence of maximally 114 nt, containing two ICR2 motifs, was required to permit wildtype levels of RNA 3 accumulation. The effect of deletions in the leader sequence on P3 synthesis *in vitro* and *in vivo* was investigated.

INTRODUCTION

The proposed group of plant tricornavirus includes the bromoviruses, cucumoviruses, ilarviruses and alfalfa mosaic virus (AIMV). The overall structure of the tripartite RNA genome of these viruses is similar: RNAs 1 and 2 encode the replicase proteins P1 and P2, respectively, whereas RNA 3 encodes the movement protein P3 and the viral coat protein (CP; the nomenclature of the proteins is that used for AIMV). *Cis*-acting sequences involved in replication have extensively been analysed in the RNAs of the bromoviruses brome mosaic virus (BMV; 1, 2, 3) and cowpea chlorotic mottle virus (CCMV; 4). In RNA 3 of these viruses they have been mapped in the 5'-terminal 90 nucleotides (nt) and in the 3'-terminal 134 nt which contain a tRNA-like structure. In addition, part of the intercistronic region of BMV RNA 3 is required for replication of this genome segment (2).

In contrast to bromo- and cucumoviruses, the 3'-termini of the RNAs of AIMV and ilarviruses do not contain a tRNA-like structure. Instead, these 3'-termini contain a binding site with a high affinity for CP, and a mixture of the three genomic RNAs of these viruses is infectious only when a few molecules of CP are added per RNA molecule to the inoculum (5). We have shown that tobacco plants transformed with a combination of the P1 and P2 genes of AIMV (P12 plants) are able to support replication of RNA 3 of this virus without the requirement for CP in the inoculum (6). This system has been used to study the sequences of RNA 3 required for replication and synthesis of the subgenomic mRNA for CP, RNA 4. *Cis*-acting elements involved in replication were preliminarily mapped to the 5'-terminal 169 nt and 3'-terminal 210 nt of AIMV RNA 3 (7). Evidence was obtained that CP has a *trans*-acting function in regulation of the balance of viral plus- and minus-strand RNA synthesis (8). A role of CP in RNA replication was further supported by the finding that, in addition to P1 and P2, CP is present in the purified AIMV replicase complex (9). Such a role is probably specific for AIMV and ilarviruses as no CP was found in the purified replicase of the cucumovirus cucumber mosaic virus (CMV; 10).

In the present study we have analysed in more detail the role of the 5'-noncoding region of AIMV RNA 3. When different strains of AIMV were compared this leader sequence was found to be highly variable (11). The leader sequence of RNA 3 of the M strain is 240 nt long and contains three copies of a repeat of 27 nt. In the leader sequence of strain S (314 nt) a sequence of 56 nt, containing one of the 27 nt repeats, is duplicated. In the leader of the A strain (345 nt) this duplication is increased to 75 nt, whereas in the Y strain (392 nt) it is increased to 149 nt (12). In this latter strain the long repeat overlaps with the initiation codon of the P3 gene and contains two copies of the 27 nt repeat. Another variable element is the presence of the A-rich sequence A₅GA₁₄GA₅CA₅GGA₆ in the leader of strain A; this sequence is reduced to A₅GA₅ or A₅GA₄ in the other strains. Finally, the 5'-terminal 22 nt of RNA 3 of the S strain are apparently exchanged for a sequence of 38 nt derived from the 5'-end of RNA 1 of this strain (11). The overall sequence similarity between RNA 3 of strains S, A and Y is about 97%. A schematic representation of their leader sequences is shown

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Figure 1. Schematic representation of a restriction map of RNA 3 of AIMV strain YSMV and the leader sequences of RNA 3 of AIMV strains YSMV (Y), 425 (A) and Strasbourg (S). Arrows 1–5 represent repeats of 27 nt. Arrows numbered 149, 75 and 56 represent repeats of the indicated length in nt. The location of an A-rich sequence is indicated.

in Fig. 1. We have exchanged the leader sequences of these three strains and we have made deletions in the leader sequence of the Y strain to determine the role of its various sequence elements in the replication of RNA 3, the synthesis of subgenomic RNA 4 and the expression of the P3 gene.

MATERIAL AND METHODS

Construction of mutants

Plasmids pAL3 (12), pX.Y037, formerly named pYSMV3 (12) and p3BS(1,0)18 (13) (kindly provided by Dr L.Pinck, Strasbourg) contain full-length cDNA copies of RNA 3 of AIMV strains 425 (A), YSMV (Y) and Strasbourg (S), respectively, fused to the T7 RNA polymerase promoter. A unique *Pst*I site is present at the 3'-end of the cDNA. The location of other restriction enzymes used to generate mutants is indicated in Fig. 2.

The leader sequences of strains A, Y and S were exchanged using the *Xho*I site near the 5' end of the P3 gene. In construct YA the RNA 3 sequence upstream of the *Xho*I site is from strain Y and the sequence downstream of this site is from strain A. It was made by replacing the *Xho*I–*Pst*I fragment of pX.Y037 with the corresponding fragment of pAL3. Constructs AS and SA were made in a similar way by exchanging the appropriate *Xho*I–*Pst*I fragments.

Mutants DD1, HD, SH and SD were constructed by deleting from pX.Y037 the restriction fragments indicated in Fig.2. Subsequently, the *Xho*I–*Pst*I fragment of the resulting mutants was replaced with the corresponding fragment of plasmid pAL3. For the construction of DD1, pX.Y037 was digested with *Dra*I which cuts in the leader sequence at positions 169 and 335 and at one site in the vector. The two large digestion products were purified by agarose gel electrophoresis and religated with T4 DNA ligase (Promega). To obtain mutant HD, pX.Y037 was cut at the *Pvu*II site in the vector just upstream of the cDNA

Figure 2. Schematic representation of RNA 3 of AIMV strain Y and deletions made in the leader sequence of RNA 3. Solid and broken lines represent sequences of strains Y and A, respectively. In the deletion mutants, numbering indicates the bordering nucleotides of the restriction fragment removed.

and at the *Xho*I site in the P3 gene. The fragment with the leader sequence was purified and digested with *Hae*III at positions 112 and 262. The 115 bp *Pvu*II–*Hae*III fragment was ligated in DD1 from which the *Pvu*II–*Dra*I fragment had been removed by digestion with *Pvu*II and partial digestion with *Dra*I. To construct mutant SH, pX.Y037 was digested with *Xho*I and subsequently with *Ssp*I. The fragment containing the cDNA sequence from nt 20–415 was digested with *Hae*III. The *Hae*III–*Xho*I fragment (nt 262–415) was ligated into pX.Y037 from which the sequence from nt 20–415 had been removed by partial digestion with *Ssp*I followed by digestion with *Xho*I. To obtain mutant SD, pX.Y037 was partially digested with *Ssp*I and the linearised plasmid was cut with *Pst*I. The 2397 bp *Ssp*I–*Pst*I fragment which contains the RNA 3 sequence without the 5'-terminal 20 nt was digested with *Dra*I at position 335 and the 1853 bp *Dra*I–*Pst*I fragment was ligated in pX.Y037 digested with *Ssp*I and *Pst*I.

To permit the construction of mutants DN, SN and HN an *Nco*I restriction site was engineered by site directed mutagenesis around the AUG start codon of the P3 gene in plasmid pAL3, yielding pAL3NcoP3. For the construction of DN, pX.Y037 was digested with *Dra*I and the 1881 bp *Dra*I–*Dra*I fragment isolated. pAL3NcoP3 was digested with *Dra*I and subsequently with *Nco*I. The overhanging *Nco*I end was filled up with T4 DNA polymerase (Promega) and the resulting 2400 bp *Dra*I–*Nco*I fragment ligated to the 1881 bp pX.Y037 *Dra*I–*Dra*I fragment. SN was obtained by removing the *Ssp*I–*Nco*I restriction fragment in pAL3NcoP3 by partial digestion with *Ssp*I followed by digestion of the linear fragment with *Nco*I. The larger fragment was recircularized after blunting the noncompatible ends. HN was constructed by digestion of pAL3NcoP3 with *Pvu*II and *Nco*I. After blunting the *Nco*I overhanging ends, recovery of the 4200

Figure 3. Effect of the leader sequence on the accumulation of AIMV RNAs 3 and 4 in P12 plants. P12 plants were inoculated with RNA 3 of wt strains YSMV (YY), 425 (AA) and Strasbourg (SS) or mutants YA, AS and SA which contain the leader sequences of strains Y, A and S, and the remaining sequences of strains A, S and A, respectively. Accumulation of encapsidated RNA was analysed by Northern blot hybridisation; the position of RNAs 3 and 4 is indicated in the left margin.

Figure 4. Western blot analysis of the accumulation of P3 in P12 plants inoculated with RNA 3 transcripts YY, YA and AA. Protein was extracted 1, 2 or 3 days after inoculation. H: extract from healthy plants. The position of P3 is indicated in the right margin.

bp *NcoI*–*PvuII* was carried out by the freeze–squeeze method. pX.Y037 was digested with *PvuII* and *XhoI*. The resulting 409 bp *PvuII*–*XhoI* fragment was then digested with *HaeIII* and the 152 bp *PvuII*–*HaeIII* fragment ligated into pAL3NcoP3 digested with *PvuII* and *NcoI*.

DD2 was constructed by digesting both pAL3 and pX.Y037 with *DraI*. The 2370 bp *DraI*–*DraI* fragment of the first plasmid was ligated to the 1881 bp *DraI*–*DraI* fragment of the second plasmid.

All restriction enzymes used were from GIBCO/BRL and all enzymatic incubations were performed under conditions recommended by the supplier.

In vitro RNA transcription and translation

All cDNA clones were linearized with *PstI*, and blunt ends were generated using T4 DNA polymerase prior to T7 RNA polymerase transcription. Transcription was carried out as described previously (7), giving rise to uncapped transcripts.

In vitro translation was carried out with a rabbit reticulocyte lysate translation system and protocol from GIBCO-BRL. Instead of ³⁵S-methionine, a final concentration of 50 μ M non-labeled methionine was added. 200 ng of transcript was added per 10 μ l reaction mixture. Protein synthesis was monitored via Western blot analysis (14) using 10 μ l of reaction mixture per sample and antiserum prepared against AIMV P3.

Inoculation of protoplasts and infection of plants

Protoplasts from P12 plants were prepared according to van Dun *et al.* (15). Per sample, 10⁵ protoplasts were inoculated by means of the PEG method (16) with 20–30 μ l of the transcription

Figure 5. Effect of leader deletions on the accumulation of RNA 3 and 4 in P12 protoplasts. The protoplasts were inoculated with RNA 3 transcripts containing wt leader sequences (YY, YA and AA) or inoculated with the indicated mutants with deletions in the leader sequence of transcript YA (see Fig. 2). Encapsidated RNAs were analysed by Northern blot hybridisation. The position of wt RNAs 3 and 4 is indicated in the right margin.

mixture. The protoplasts were collected after 24 hours incubation at 25°C under constant illumination.

P12 plants were grown and inoculated as described by van Dun *et al.* (15). Per plant, three half-leaves were inoculated with 500 ng per half-leaf of each of the indicated transcripts. Two to three plants were inoculated with each transcript.

RNA analysis

Total RNA was isolated from protoplast samples as described by van der Kuyl *et al.* (7). Inoculated leaves from infected plants were collected 1–10 days after inoculation. Virus particles were isolated from these leaves as described by van Vloten-Doting and Jaspars (17). RNA extracted from protoplast samples and purified virus particles was analysed, after glyoxylation and electrophoresis in 1% agarose gels by Northern blot hybridization using random primed cDNA3 as probe (18). Differences in RNA accumulation were quantitated by using a Betascope blot analyser (Betagen Corp. Waltham, MA).

Protein analysis

Total plant protein extracts were prepared by grinding 0.1 mg of leaf material in 200 μ l PE buffer (10 mM NaH₂PO₄, 1 mM EDTA, pH 7.0). 100 μ l of this homogenate was added to 200 μ l of Laemmli loading buffer (19). To remove excess plant debris the mixture was subsequently centrifuged at 6500 rpm for 5 minutes in an Eppendorf centrifuge. 50 μ l of the supernatant was loaded onto an SDS polyacrylamide gel and analysed by the Western blot technique (14) using antiserum prepared against AIMV P3.

RESULTS

Role of the leader sequence in the synthesis of RNAs 3 and 4

AIMV strains Y, A and S show characteristic differences in the ratio in which RNA 3 and 4 are synthesised. Strain A produces substantially more RNA 4 than RNA 3. In strain S this ratio is reversed whereas strain Y holds an intermediate position. To investigate a possible role of the 5'-noncoding region of RNA 3 in this phenomenon, the leader sequences of the three strains were exchanged using the common *XhoI* restriction site (position 415 in strain Y) indicated in Fig. 1. As the three strains do not show any nucleotide differences between the initiation codon of the P3 gene and this *XhoI* site, these exchanges did not affect the sequence of the P3 genes. In mutants YA, AS and SA, the leader sequence is from strains Y, A and S, respectively. Fig. 3

shows a Northern blot analysis of the production of RNAs 3 and 4 five days after inoculation of P12 plants with the three mutants or the wild type strains Y, A and S, indicated as YY, AA and SS, respectively. It is clear that the RNA patterns of each mutant is similar to that of the wild type strain from which its leader sequence is derived.

Role of the leader sequence in the synthesis of P3

The leader sequences of strains Y, A and S are of different length and contain A-rich sequences ranging in size from 10 to 40 nt. Moreover, the leaders of strains A and Y contain one or two internal AUG codons, respectively. These differences could affect the expression of the P3 gene in the respective RNAs. Fig. 4 shows a Western blot analysis of the accumulation of P3 protein in P12 plants, one, two and three days after inoculation with RNA 3 transcripts of wild type strains Y (YY) and A (AA) and mutant YA. For strain Y, P3 accumulation is maximal one day after inoculation and rapidly drops to lower levels on days two and three. For strain A, P3 accumulation remains relatively constant during the first three days after infection. The time course of P3 accumulation induced by mutant YA is similar to that of strain Y. This indicates that the leader sequence of AIMV RNA 3 affects the kinetics of P3 accumulation. No significant differences were seen in the time course of the P3 accumulation induced by strains A and S or their mutants AS and SA (result not shown).

Replication of leader mutants in P12 protoplasts

To analyse the role of sequence elements in the leader of RNA 3 in more detail, the deletion mutants shown in Fig. 2 were constructed. With the exception of mutant DD2, the deletions were made in the leader sequence of strain Y and the leader constructs were fused to downstream sequences of strain A using the common *Xho*I site (mutants DD1, HD, SH and SD) or the *Nco*I site that was engineered around the initiation codon of the P3 gene in the A strain clone pAL3 (mutants DN, HN and SN).

Figure 6. Time course of the accumulation of RNAs 3 and 4 in P12 plants inoculated with leader deletion mutants. Panels A and B represent duplicate experiments. P12 plants were inoculated with transcripts containing a wt leader sequence (YA and AA in panel A; YA in panel B) or the indicated mutants with deletions in the leader sequence of transcript YA (see Fig. 2). Mutant HN was tested only in panel B. Virus particles were purified 3, 6 and 10 days after infection (d.p.i.) and RNA extracted from these particles was analysed by Northern blot hybridisation. The position of wt RNAs 3 and 4 is indicated in the right margin.

In mutant DD2, the sequence downstream of the *Dra*I deletion is completely derived from the A strain and starts from the corresponding *Dra*I site in the leader of this strain. Thus, the main difference between the leaders of mutants DD1 and DD2 is the presence of an A-rich sequence of 11 nt in the former and an A-rich sequence of 40 nt in the latter.

Fig. 5 shows a Northern blot analysis of the accumulation of RNA 3 and 4 in protoplasts from P12 plants after inoculation with wild type and mutant transcripts. The proper control for the mutants is construct YA (lane 2) which, at the protoplast level, shows an RNA accumulation that is quantitatively similar to that induced by strains Y (lane 1) and A (lane 10). Mutants DD1 (lane 3) and DD2 (lane 9) accumulate at wild type levels. Apparently, deletion of the 149 nt sequence that is duplicated in the leader of strain Y does not affect accumulation, nor does the difference in length of the A-rich sequence in the two mutants. Compared to mutant DD1, mutants DN and HD have additional deletions of leader sequences containing the short 27 nt repeat number 5 (DN) and the short 27 nt repeat number 2 (HD), respectively. Compared to the wild type and mutant DD1, the RNA accumulation induced by mutants DN and HD is reduced five to ten fold (lanes 4 and 5). Deletion of both these sequences in mutant HN, leaving only the 5' 112 nt of the leader sequence, also resulted in a 10 fold reduction of mutant RNA accumulation (result not shown). Deletion of nucleotides 20–262 and 20–335 in mutants SH and SD, respectively, resulted in a 100 fold reduction of the RNA accumulation in P12 protoplasts. The

Figure 7. Western blot analysis of the accumulation of P3 in P12 plants inoculated with leader deletion mutants. P12 plants were inoculated with transcripts containing a wt leader sequence (YA and AA) or the indicated mutants with deletions in the leader sequence of YA (see Fig. 2). Protein was extracted 1, 3 and 6 days after inoculation. H: extract from healthy plants. The position of P3 is indicated in the right margin.

Figure 8. Translation of wt and mutant RNA 3 transcripts *in vitro*. A western blot was loaded with translation products of transcripts YY (lane 2), YA (lane 3), DD1 (lane 4), DN (lane 5), HD (lane 6), SH (lane 7), SD (lane 8), SN (lane 9), DD2 (lane 10) and AA (lane 11). In lane 12 no transcript was added to the reticulocyte lysate. Lanes 1 and 13 were loaded with an extract from P12 plants inoculated with RNA 3 of the A strain. Translation products were detected with an antiserum to P3; the position of P3 is indicated in the right margin.

RNAs of these mutants became detectable in lanes 6 and 7 of Fig. 5 only after a longer exposure of the autoradiograph (result not shown). Accumulation of mutant SN, which contains only the first 20 nt of the leader sequence, was not detectable in P12 protoplasts (Fig. 5, lane 8) even after a longer exposure of the autoradiograph.

Replication of leader mutants in P12 plants

The P3 gene has no *cis*- or *trans*-acting function in the replication of AIMV RNA 3 in P12 protoplasts but is essential for efficient spread of viral material in P12 plants (20). To see whether deletions in the leader sequence of RNA 3 would affect the role of P3 in cell-to-cell transport, P12 plants were inoculated with the various mutant transcripts and the accumulation of viral RNA was monitored by Northern blot hybridisation 3, 6 and 10 days after inoculation (d.p.i.). Because the susceptibility to infection of different batches of plants is slightly variable the results of two independent experiments are presented in Fig. 6. In the first experiment (Fig. 6A) the level of RNA accumulation obtained 3 d.p.i. resembles the pattern obtained in P12 protoplasts (Fig. 5). Accumulation of DD1 and DD2 is similar to the control YA (Fig. 6A, lanes 2, 8 and 1, respectively). The accumulation of DN (lane 3) and HD (lane 4) is slightly reduced and the accumulation of SH, SD and SN (lanes 5, 6 and 7) is not detectable (mutant HN was not included in this experiment). Six

d.p.i. the accumulation of mutants DN and HD (lanes 12 and 13) have reached wild type levels and the accumulation of mutants SH and SD (lanes 14 and 15) becomes detectable. A further increase in the levels of mutants SH and SD is observed 10 d.p.i. (lanes 19 and 20). Mutant SN was not detectable at any time. This indicates that the leader sequences of mutants SH and SD permit a reduced level of replication at the single cell level, resulting in a delayed spread of these mutants. A delay in the accumulation of mutants SH and SD is also apparent in the second experiment shown in Fig. 6B. However, in the second experiment SH and SD are already detectable at 3 d.p.i. (Fig. 6B, lanes 6 and 7). In both experiments the delay of SH is more severe than that of SD. The delay in the accumulation of mutant HN (Fig. 6B, lane 5) resembled that of mutant SD. In the second experiment mutants HN, SH and SD had already approached wild type levels of accumulation at 6 d.p.i. (Fig. 6B, lanes 14, 15 and 16). A very faint signal of RNA 4 was detectable in this experiment in plants inoculated with mutant SN (Fig. 6B, lane 17) suggesting that a leader sequence of only 20 nt may permit a low level of replication.

The Western blot presented in Fig. 7 shows the level of P3 accumulation one, three and six days after inoculation of P12 plants with various mutant and wild type transcripts. The time course of P3 accumulation induced by mutants DD1, DN, HD and DD2 resembled that of the wildtype. Accumulation of P3 of mutants SH and SD was delayed and more or less paralleled the accumulation of RNA induced by these mutants. P3 accumulation induced by mutant HN resembled that of mutants SH and SD (result not shown). Mutant SN did not accumulate P3 at significant levels.

In vitro translation of leader mutant RNAs

To further analyse a possible regulatory role of the leader sequence of AIMV RNA 3 in the expression of the P3 gene, transcripts with deletions in the leader sequence were translated *in vitro* into P3 protein. Fig. 8 shows that the efficiency of translation is inversely related to the length of the leader sequence. Translation directed by the wt leader is shown in lane 3. In lanes 4 to 9 the leader sequence contains deletions of 166, 223, 223,

Figure 9. Similarity between the consensus sequence of the ICR2 motif and the sequence of 27 nt repeats 1 and 2 from the leader sequence of strain Y. The sequence of repeat 2 is identical to that of repeats 3, 4 and 5. Putative ICR2 motifs are underlined.

Figure 10. Predicted secondary structure of the leader sequence of the strain Y. The 27 nt repeats 1 to 5 are overlined.

242, 315 and 372 nucleotides, respectively. Transcript SN with a leader sequence of only 20 nt is the most efficient messenger for translation of P3 *in vitro* (Fig. 8, lane 9). Possibly, the additional sequences present in the wt leader pose a barrier to scanning ribosomes. The translation efficiencies of mutants DD1 and DD2 (Fig. 8 lanes 4 and 10, respectively) appeared to be similar. Thus, the A-rich sequence of 40 nucleotides that is present in the leader of DD2 does not affect *in vitro* translation of P3. Capping of the transcripts did not affect their messenger activity *in vitro* (result not shown).

DISCUSSION

As the N-terminal sequence of the P3 protein has not been determined, uncertainty exists about the actual length of the leader sequence of AIMV RNA 3. The first AUG codon in the P3 open reading frame is followed after 12 triplets by a second in frame AUG codon. Recently, we observed that a mutant with a frameshift in the *XhoI* site, which is located between the first and second AUG codon, was able to replicate in P12 plants but produced a truncated P3 protein which had probably lost the N-terminal 12 amino acids (Neeleman *et al.*, unpublished results). This unequivocally identifies the AUG triplet at position 392 of RNA 3 of the Y strain as the initiation codon.

Sequence motifs in the RNAs of BMV and a number of other plant viruses were noted to resemble internal control regions (ICRs) 1 and 2 that promote transcription of tRNA genes (21, 22). Fig. 9 shows that the consensus ICR2 sequence has significant similarity with a motif that is present two times in the 27 nt repeats that occur in the leader sequence of AIMV RNA 3. The repeats shown in Fig. 9 in the DNA notation are those of strain Y. The sequence of repeat 2 is identical to that of repeats 3, 4 and 5 (Fig. 1). Compared to the other repeats, repeat 1 shows two nt differences, one creating an AUG codon that is followed by a termination codon three triplets downstream in the leader sequence. A model for the secondary structure of the leader of strain Y, generated by the UWGCG computer programme Fold (23), is shown in Fig. 10. The 27 nt repeats 1, 2 and 4 (overlined in Fig. 10) constitute the upper parts of similar hairpin structures with the 3'-ICR2 motif in these repeats being exposed in the loop. Also in BMV RNAs the ICR2 motif is part of a stem-loop structure and a mutational analysis indicated that it is the stem-loop structure in the viral plus-strand that is essential for RNA replication and not its complement in the viral minus-strand (24). It has been proposed that a host protein, possibly a polymerase III transcription factor, binds to the ICR2 motif in a duplex of plus- and minus-strand RNA representing a putative replicative intermediate. This binding would induce hairpin formation in the plus-strand thereby making the 3'-end of the minus-strand accessible as a promoter for the initiation of the plus-strand RNA synthesis (25).

In BMV RNA 3 one ICR2 motif is found in the 92 nt leader sequence and two are present in the intercistronic region of 248 nt. Deletion of the 5'-terminal ICR2 motif had little effect on RNA 3 accumulation in protoplasts but debilitated systemic spread in barley. Deletion of the internal most downstream ICR2 motif reduced BMV RNA 3 accumulation in protoplasts substantially, affected the ratio in which plus- and minus-strand RNA 3 are synthesized and abolished systemic infectivity (25). In AIMV RNA 3 the intercistronic region is only 42 nt long and does not contain detectable ICR motifs. In mutant DD1 the 149 nt repeat in the leader of strain Y is precisely removed, resulting in a leader

organisation similar to that of the M strain of AIMV (11). Mutant DD1 accumulates at wt levels in P12 protoplasts and plants indicating that under greenhouse conditions this long repeat does not offer a selective advantage to the virus. Deletion of the sequence with the 27 nt repeats 3 and 4 in DD1 does not affect the structure of the hairpins containing repeats 1 and 2 as shown in Fig. 10 but permit a folding of the sequence containing repeat 5 into a hairpin structure with the ICR2 motif exposed in the loop (result not shown). Extension of the A-rich sequence of 11 nt (A₅GA₅) in mutant DD1 to a sequence of 40 nt in the mutant DD2 does not affect the structure of the hairpins with repeats 1, 2 and 5. As mutants DD1 and DD2 accumulate at similar levels in P12 protoplasts and plants, a possible role of this A-rich sequence remains unclear.

Mutants DN, HD and HN all accumulate at five to ten fold reduced levels in P12 protoplasts and show a delayed accumulation in P12 plants. All three mutants contain 27 nt repeat 1. In addition, mutants DN and HD contain repeats 2 and 5, respectively. Folding of the leaders of these mutants predicts that the ICR2 motif in each repeat is located in the loop of a hairpin structure similar to that shown in Fig. 11 (result not shown). The results indicate that a hairpin structure with motif 1 is sufficient to permit accumulation of the mutants at a 10 to 20% level of that of the wt or DD1. Apparently, the reduction is caused by the deletion of motif 2, motif 5 or both from the leader sequence of DD1. The fact that the RNAs analysed in Fig. 6 were extracted from purified virus demonstrates that all mutants are encapsidated. This indicates that the accumulation level in protoplasts reflects replication rather than the stability of the mutant RNAs.

Mutant SH contains repeats 4 and 5 whereas mutant SD contains repeat 5 only. Both mutants accumulate in P12 protoplasts at a level that is about 1% of that of wt. The difference in accumulation of mutant HN (repeat 1, 10–20% level) and mutant SD (repeat 5, 1% level) suggests that sequences flanking repeat 1 (nt 56–82) in the 112 nt leader sequence of mutant HN represent important *cis*-acting elements. These elements are currently being analysed.

In BMV and CCMV RNA 3 5'-terminal elements required for replication are completely confined to the first 90 nucleotides (2, 26). The first 112 nt of AIMV permit a reduced level of accumulation and a maximum of 226 nt with three ICR2 motifs is required for a wt phenotype (mutant DD1). Possibly the sequence between nt 112 and 226 is equivalent to the intercistronic sequence of 150 nt in BMV RNA 3 that is required for replication (2) and contains two ICR2 motifs (25). CCMV RNA 3 sequences of 145 nt and 125 nt could be deleted from the leader and intercistronic region, respectively, without any effect on replication in protoplasts or plants. However, when plants were co-inoculated with mutant and wt virus the mutants were completely lost, indicating that these sequences contribute to the competitive fitness of CCMV (27). In contrast, viable AIMV leader mutants competed efficiently with wt virus (van der Vossen, unpublished results).

The sequence preceding the movement gene in CCMV RNA 3 shows similarity to the subgenomic promoter for RNA 4 synthesis (28). Evidence for a similar cryptic subgenomic promoter in the leader of AIMV RNA 3 was obtained in *in vitro* assays using minus-strand RNA 3 as a template for the purified AIMV RNA polymerase (29). Such sequences can influence the activity of down stream subgenomic promoters in an unknown way (30). Differences in these cryptic subgenomic promoters

could explain the observation that the leader sequence is responsible for the characteristic differences in the ratio of RNA 3/4 accumulation of AIMV strains A, Y and S (Fig. 3). The leader sequence also affects the kinetics of P3 accumulation in P12 plants (Fig. 4). The time course of the accumulation of P3 in subcellular fractions of leaf homogenates from nontransgenic plants infected by AIMV strain S that has been reported by others (31), differed somewhat from the kinetics of the overall P3 accumulation observed in this study. This may be due to differences in the experimental conditions.

In wheat germ and reticulocyte cell free systems the relative messenger activities of AIMV RNAs 1, 2, 3 and 4 were found to be 10, 60, 1 and 150, respectively, indicating that RNA 3 is a poor messenger (32). Deletion of the putative hairpin structures in the leader sequence of RNA 3 greatly enhanced its *in vitro* messenger activity (Fig. 8). In P12 plants P3 accumulation paralleled the accumulation of mutant RNA and no indication for a regulatory role of the leader sequence in translation *in vivo* was obtained. We have not been able to detect P3 accumulation in infected P12 protoplasts so far. Transient expression of a reporter gene in protoplasts would be the system of choice to study elements in the leader sequence of RNA 3 that govern translation *in vivo*. In this way motifs responsible for enhancing translation were recently identified in the 5'-leader of tobacco mosaic virus (33).

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