A single-stranded loop in the 5′ untranslated region of cucumber mosaic virus RNA 4 contributes to competitive translational activity

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Abstract The 5′ untranslated region (UTR) of cucumber mosaic virus (CMV) RNA 4 confers a highly competitive translational advantage on a heterologous luciferase open reading frame. Here we investigated whether secondary structure in the 5′ UTR contributes to this translational advantage. Stabilization of the 5′ UTR RNA secondary structure inhibited competitive translational activity. Alteration of a potential single-stranded loop to a stem by substitution mutations greatly inhibited the competitive translational activity. Tobacco plants infected with wild type virus showed a 2.5-fold higher accumulation of maximal coat protein than did plants infected with a loop-mutant virus. Amplification of viral RNA in these plants could not explain the difference in accumulation of coat protein. Phylogenetic comparison showed that potential single-stranded loops of 12–23 nucleotides in length exist widely in subgroups of CMV.

Key words: mRNA competition; Competitive translational activity; 5′ Untranslated region; Cucumber mosaic virus; RNA secondary structure

1. Introduction

Plant tripartite RNA viruses, including cucumber mosaic virus (CMV), brome mosaic virus (BMV), and alfalfa mosaic virus (AMV), show similar in vitro translational behavior. Each of these viruses has four 5′-capped viral RNAs that are translated with distinct efficiencies, particularly in the presence of a high concentration of virion RNA [1–4]. These observations have been interpreted as examples of mRNA competition/discrimination [5], which is postulated to be important within the cellular environment, especially during virus infection [6,7] and development [8,9].

Among the plant RNA virus RNAs, RNA 4, the mRNA which encodes the coat protein, is the most competitive [1–4]. However, these viruses appear to employ distinct strategies to achieve strong competitive translational activities. For AMV RNA 4, a competitive translational determinant was identified in the 3′ untranslated region (UTR) [10]. Meanwhile, we found that the 5′ UTR of CMV RNA 4 could confer a competitive translational advantage upon a heterologous luciferase open reading frame (ORF) [2].

It is well documented that the presence of a stable RNA secondary structure in the 5′ UTR inhibits translation of an mRNA (reviewed in [11]). In particular, stable RNA secondary structure in the vicinity of the mRNA cap inhibits the competitive translational activity of an mRNA [12–14]. Because the 5′ UTR of CMV RNA 4 has been proposed to form a moderately stable RNA secondary structure [15], we investigated the role of RNA secondary structure in competitive translational activity. We found that stabilization of a predicted RNA secondary structure in the 5′ UTR inhibited competitive translational activity in a structure stability-dependent manner. Surprisingly, a single-stranded loop in the 5′ UTR of RNA 4 contributed strongly to competitive translational activity and maximal coat protein synthesis.

2. Materials and methods

2.1. Virus strains and plasmids

Kor-CMV (Korean isolate) and pT7Kor2T, both used in this study, have been described previously [16]. The construction of pT7Kor3, pT7Kor4, pT7Kor4MUT, and pT7Kor4MLUC is described in [2]. Tobacco (*Nicotiana tabacum* cv. W38) plants were used in the virus infection experiment.

2.2. Plasmid constructs

pT7Kor1, pT7Kor2, pT7Kor3 and pT7Kor4 contain infectious full-length cDNA clones of Kor-CMV RNAs 1, 2, 3 and 4, respectively, immediately downstream of a synthetic T7 promoter (unpublished results). pT7Kor2 was constructed from the deletion of one T in the 5′-terminal GTTTT of pT7Kor2T by oligonucleotide site-directed mutagenesis, and contains the authentic sequence of Kor-CMV RNA 2 [16]. pT7Kor4MLIGHT was constructed by insertion into the *Aff*II- and *Apa*I-digested pT7Kor4MUT [2] of the polymerase chain reaction (PCR)-amplified ORF of the cDNA encoding the A/J mouse light chain, which was used to produce the Ars-A monoclonal antibody [17]. The inserted DNA of pT7Kor4MLUC was transferred to pSELECTKor4 [2] after *Xba*I and *Sac*I digestion and then mutated by oligonucleotide site-directed mutagenesis with the oligomer 5′-TGGCGTCTTCCATGACTCGACTCAATTCT-3′ according to the manufacturer's protocol (Promega). The resulting construct was named pSELECTKor4LLUCT34, and contains the exact native 5′ UTR sequence of Kor-CMV RNA4. After religation of the insert DNA into pSELECT-1 (Amp^s, Promega) after *Xba*I and *Sac*I digestion, the insert DNA was mutated with the use of oligonucleotide site-directed mutagenesis to construct various RNA 4-derived 5′ UTR mutants. The mutants were constructed using the oligomers: 5′-CACTCTCTATATAGTCGTAGACAATAACTATAGTG-3′ for 4ST1; 5′-TCGACTCAATTCTACTGACACAAAAGAGAAAAC-3′ for 4ST2; 5′-ACTCAATTCTACGACTACAAAAGAGAAAACAC-3′ for 4ST3; and 5′-CTACGACACAAAAGAGAGTACACAACA-CACACACT-3′ for 4ST4. The resulting constructs were digested with *Xba*I and *Sac*I and inserted into the *Xba*I and *Sac*I sites of pT7Kor4-MUT [2]. The resulting constructs were pT7Kor4LLUCT34, pT7Kor4ST1LUCT34, pT7Kor4ST2LUCT34, pT7Kor4ST3LUCT34, and pT7Kor4ST4LUCT34, respectively. The pT7Kor4ST5LUCT34 and pT7Kor3IRMUT mutants were made using the Quick mutagenesis Stratagene kit following the manufacturer's instructions with sense primer 5′-AGAGAGTGTGTGTTGCACTTTCTCTTTTG-TGTCGT-3′ and antisense primer 5′-ACGACACAAAAGAGAAA-GTGCAACACACACACTCTCT-3′, using pT7Kor4LLUCT34 and pT7Kor3 as plasmid templates, respectively.

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2.3. In vitro transcription and RNA preparation

All of the transcripts used in this study were prepared as capped forms by in vitro transcription using the mMESSAGEmMACHINE kit (Ambion). The transcripts 4L-, 4ST1-, 4ST2-, 4ST3-, 4ST4-, and 4ST5-LUC-T34 were generated by in vitro transcription of the *Mlu*I-linearized pT7Kor4LLUCT34, pT7Kor4ST1LUCT34, pT7Kor4ST2LUCT34, pT7Kor4ST3LUCT34, pT7Kor4ST4LUCT34, and pT7Kor4ST5LUCT34, respectively. Viral RNAs 1, 2 and 3 were obtained by in vitro transcription of pT7Kor1, pT7Kor2, and pT7Kor3. Mutant RNA 3 was transcribed from pT7Kor3IRMUT. L4-LIGHT-T34 was generated from the *Mlu*I-linearized pT7Kor4MLIGHT. Purification and measurement of transcripts are described in our previous paper [2].

2.4. Preparation of crude wheat germ extract and translation

Crude wheat germ extract was prepared according to published procedures [18,19]. Wheat germ (5 g) (Sigma) was ground in liquid nitrogen in a pre-chilled mortar, and the resulting powder was ground for an additional 2 min in homogenization buffer (40 mM HEPES/KOH, pH 7.6, 1 mM magnesium acetate, 100 mM potassium acetate, 2 mM calcium chloride, 4 mM dithiothreitol). After the homogenate was centrifuged at 20 000 × *g* for 10 min, the supernatant was passed through a Sephadex G-25 (Sigma) column saturated with column buffer (40 mM HEPES/KOH, pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 4 mM dithiothreitol), and the eluate was collected. Translation reactions were performed at 25°C for 90 min in wheat germ extracts ($A_{260} = 7$) under the following reaction conditions [17]: 20 mM HEPES/KOH pH 7.6, 0.1 mM GTP, 1.2 mM ATP, 5 mM dithiothreitol, 0.5 mM spermidine, 10 mM phosphocreatine, 50 μg/ml creatine phosphokinase, 50 μg/ml wheat tRNA, 0.5 U/μl human placental RNasin (Amersham), 80 μM amino acid mixtures complete (Promega), 2.25 mM magnesium acetate, and 105 mM potassium acetate. After termination of translation, an aliquot of the resultant reaction mixture was mixed with a 5× volume of luciferase assay reagent (Promega), and the translation level of luciferase was measured indirectly with a MicroLumat LB 96 P luminometer (E.G. and G. Berthold).

2.5. Computer analysis

Optimal RNA secondary structure could be predicted directly using mfold ver 3.0 on the mfold server (http://mfold1.wustl.edu/~mfold/rna/form1.cgi) [20,21]. The program also predicts various suboptimal structures from a given RNA sequence [22]. The temperature parameter was typically set at 37°C, the only temperature option in program ver 3.0 [20].

2.6. Inoculation of viral transcripts in tobacco plants

Viral transcripts (5 μg/ml, 200 μl) containing viral RNAs 1 (250 ng) and 2 (250 ng), and either RNA 3 (500 ng) or a mutant version of 3 (500 ng), were inoculated in an expanded leaf per tobacco plant. Virus infection was performed on 10 tobacco plants for each kind of viral transcript mixture. All tobacco plants showed mild mosaic symptoms in young systemic leaves, thus indicating a successful infection. Non-infected control plants were obtained with the same inoculation procedure, except that the inoculation solution did not contain viral RNA.

2.7. Double antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

Total soluble protein was prepared individually from leaf disks of the inoculated leaves and first systemic leaves at different time points using general extraction buffer (Bioreba, Switzerland). The individually prepared proteins from 10 leaf disks of the same condition from 10 plants were then mixed together, thus minimizing the possible variation in extraction procedure. DAS-ELISA was performed using CMV coat protein antibody from the manufacturer's protocol (Bioreba, Switzerland). Serial dilution of extracted total protein from a native CMV transcript-infected systemic leaf was used as standard curve for the estimation of relative level of coat protein.

2.8. RNA blot analysis

From five native and five mutant virus transcript-inoculated tobacco plants, total RNA was isolated individually from leaf disks at various stages of infection. The individually prepared total RNAs from five leaf disks were mixed together and quantitated by measuring the absorbance at A_{259}. RNA blot analysis was performed with the RNA mixtures using the radiolabeled 3′ UTR cDNAs of RNAs 1, 2, and 4 as probes. The 3′ UTR of RNA 4 is present also in the 3′-terminal region of RNA 3. Radioactivity on the blots was measured with a Bio Imaging Analyzer (Fuji BAS 1000, Fuji, Japan).

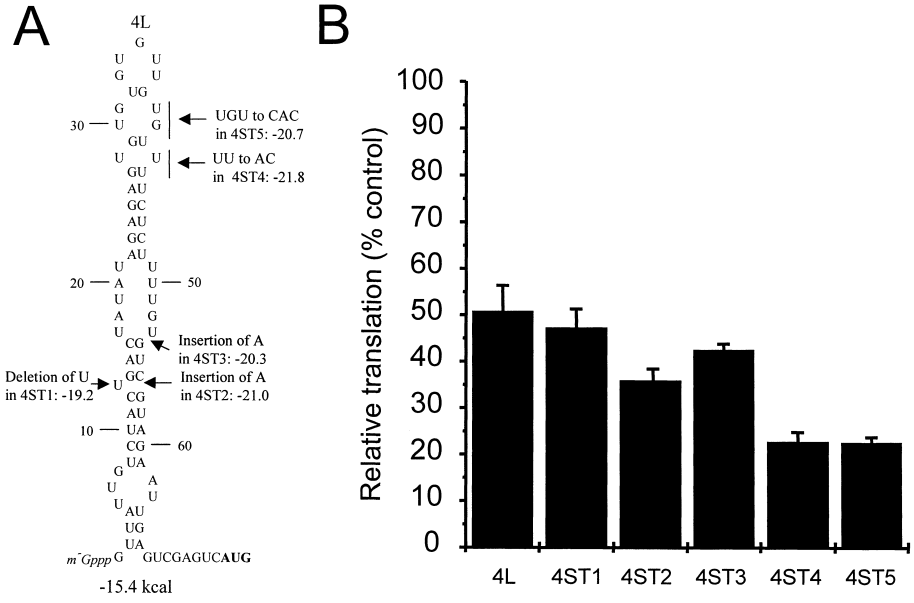

Fig. 1. Effects of stabilization and/or extension of RNA secondary structure on competitive translational activity. A: Schematic diagram of the predicted secondary structures of the 5′ UTR of Kor-CMV RNA 4. The sequence changes from that of 4L in 4ST1, 4ST2, 4ST3, 4ST4, and 4ST5 are indicated by arrows with descriptions of the mutations and the Gibbs free energy (kcal/mol). B: The relative level of translation of each transcript (3.75 nM) was measured in the presence of a 10-fold molar excess of L4-LIGHT-T34. Three independent translation reactions with and without competitor were performed, and the relative translational activities in the presence of competitor are shown with the standard error.

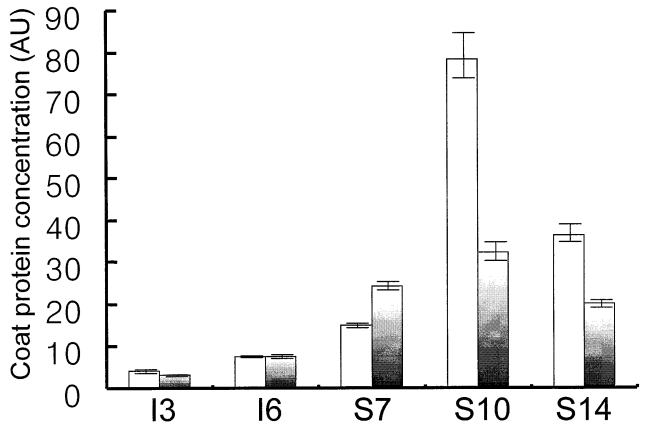

Fig. 2. Accumulation of coat protein in the inoculated and first systemic leaves of native and mutant CMV RNA-infected tobacco plants. The relative concentrations of coat protein in 10 native and 10 mutant CMV-infected tobacco plants were calculated by DAS-ELISA using coat protein antibody (Bioreba). Coat protein levels from tobacco plants inoculated with native and mutant viral RNAs are shown with white and dark gradient bars in arbitrary units (AU). Standard errors from six independent ELISA assay are shown. I3 and I6 indicate the viral RNA-inoculated leaves at 3 and 6 days post-inoculation, respectively. S7, S10 and S14 indicate the first upper systemic leaf at 7, 10 and 14 days post-inoculation, respectively.

3. Results and discussion

3.1. The effect of structure-stabilizing mutations on competitive translational activity

It has been proposed that the 5′ UTR of Y-CMV RNA 4 can be folded to form a moderately stable RNA secondary structure [15]. The 5′ UTR of Kor-CMV RNA 4 is predicted to form a very similar RNA secondary structure (Fig. 1A) with no probable competing structure predicted within 30% suboptimality. The tandem GU sequence that occurs around nucleotide positions 30–40 is predicted not to form a stem because of the broken stacking of the alternate GU pairing [20]. Thus this region is believed to form a single-stranded loop of about 15 nucleotides in size. Because stable RNA secondary structure in the cap-proximal region of a 5′ UTR inhibits the competitive translational activity of an mRNA [12–14], we attempted to inhibit the competitive translational activity of RNA 4 by introducing several structure-stabilizing mutations in its 5′ UTRs. Distinct mutations were introduced in several positions of the stem-loop structure of 4L-LUC-T34 (4L), yielding 4ST1-, 4ST2-, 4ST3-, 4ST4- and 4ST5-LUC-T34 (Fig. 1A), each of which contained a mutated 5′ UTR, a luciferase-encoding ORF, and the 3′ UTR of RNA 4. Deletion of the bulged U_{13} in 4L (to yield 4ST1-LUC-T34) increased structure stability by −3.8 kcal/mol. Insertion of an A after C_{56} in 4L (to yield 4ST2-LUC-T34) resulted in base pairing with the bulged U_{13}, thus stabilizing the stem by −5.6 kcal/mol. Insertion of an A after U_{53} in 4L (to yield 4ST3-LUC-T34) resulted in base pairing of $U_{17}A_{18}U_{19}$ with $G_{52}U_{53}A$, thus stabilizing the structure by −4.9 kcal/mol. Substitution of $U_{42}U_{43}$ with $A_{42}C_{43}$ (to yield 4ST4-LUC-T34) resulted in base pairing with $G_{27}U_{28}$, thus stabilizing the structure by −6.4 kcal/mol. Finally, substitution of $U_{39}G_{40}U_{41}$ with $C_{39}A_{40}C_{41}$ (to yield 4ST5-LUC-T34) resulted in base pairing with $G_{29}U_{30}G_{31}$, thus stabilizing the structure by −5.4 kcal/mol.

Unsaturating amounts of 4L-LUC-T34 and its derivatives (3.75 nM or ∼2.5 ng/μl) were translated individually in a wheat germ translation system with and without a 10-fold molar excess of L4-LIGHT-T34 (competitor), which encodes an unrelated immunoglobulin light chain. Luciferase activities from the translation of 4L and 4L derivatives in the absence of competitor were not significantly different and were normalized to 100% respectively (unpublished results) following the mRNA competition assay method [23]. The translational activities of 4L-LUC-T34 and 4ST1-LUC-T34 were inhibited ∼50% in the presence of a 10-fold molar excess of L4-LIGHT-T34 (Fig. 1B). Because the substantial difference in Gibbs free energy between 4L and 4ST1 did not differentiate clearly these competitive translational activities, the lesser structural stability of 4L appears not to inhibit substantially its competitive translational activity (Fig. 1B). In the presence of competitors, the translational activities of 4ST2- and 4ST3-LUC-T34 were inhibited more than that of 4L-LUC-T34 in a structure stability-dependent manner. The strong inhibition of 4ST4- and 4ST5-LUC-T34 in the presence of competitor indicated that the single-stranded nature of the loop region in 4L is an important feature that contributes to the competitive translational activity of the RNA. A structure stability comparison between 4ST2 (−21.0 kcal/mol) and 4ST5 (−20.7 kcal/mol) indicates that the strong inhibition of 4ST5-LUC-T34 translation cannot be explained simply by the stability difference. In addition, the inhibition of 4ST4- and 4ST5-LUC-T34 translation was almost equal, despite the differences in structure stability and in mutation position. All transcripts were stable during the course of the translation reaction, thereby excluding any possible contribution by differences in mRNA stabilities (unpublished results). Therefore, it can be concluded that the single-stranded loop region is an important RNA feature for contributing to competitive translational activity of 4L.

3.2. In vivo function of the single-stranded loop of RNA 4 in coat protein synthesis

RNA 4 is synthesized from RNA 3 during viral RNA replication in infected plants (reviewed in [24,25]). In order to determine the in vivo function of the single-stranded loop region in RNA 4 translation, we constructed a mutant version of RNA 3 that contains the 4ST5 mutation within RNA 3. Viral transcript mixtures that contained native RNAs 1, 2, 3 or native RNAs 1 and 2, and mutant RNA 3 were each inoculated on 10 tobacco plants. In the inoculated and first upper systemic leaves, viral coat protein, the translation product of RNA 4, was quantitated by DAS-ELISA. In the native and mutant virus-inoculated leaves, no significant difference in coat protein level was observed at 3 and 6 days after infection (Fig. 2). This suggests that the cellular environment supplies sufficient translation factor(s) for the entire pool of translatable viral RNA, whether native or mutant.

From the in vitro translation experiments, we envisaged that the effect of mRNA competition would be more obvious in a cellular environment containing higher concentrations of translatable mRNA. We also noticed that in the first upper systemic leaf, the accumulation of coat protein was substantially higher than in the inoculated leaf, suggesting a higher level of translation. Therefore, we used maximal coat protein

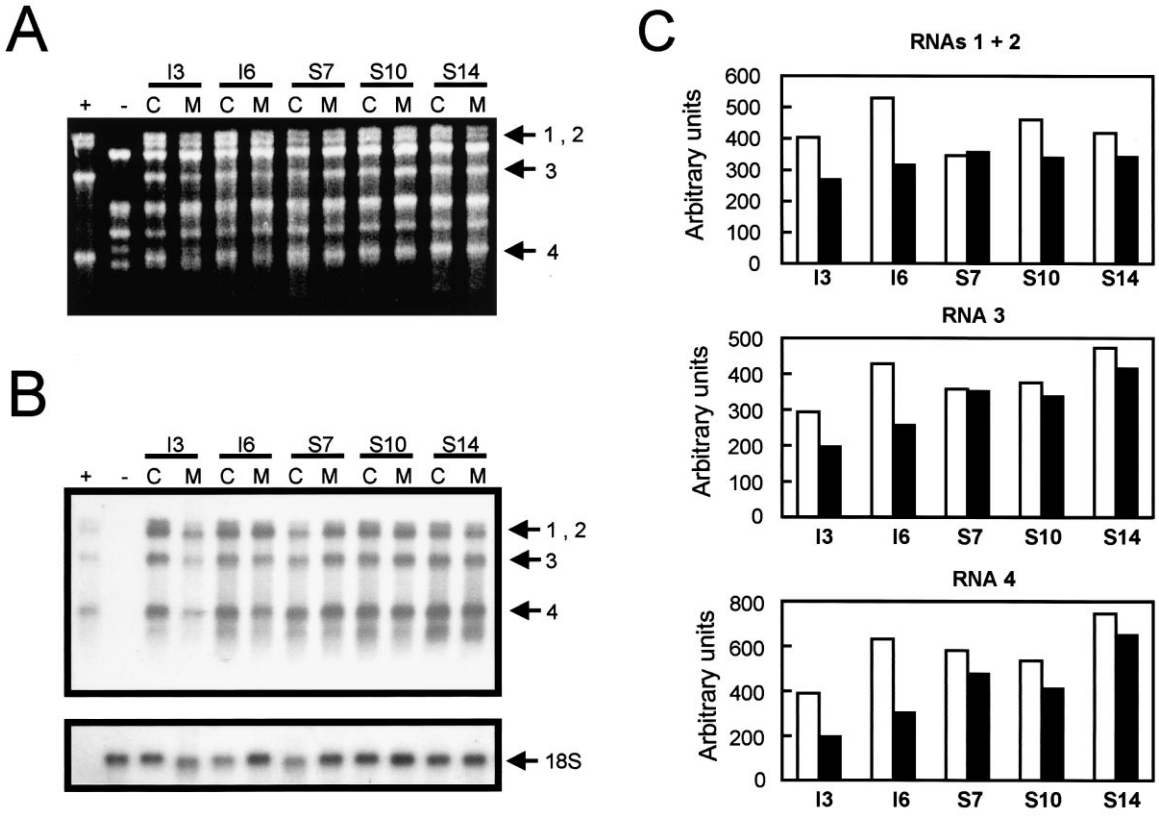

Fig. 3. Amplification of CMV RNAs in native and mutant virus-infected plants. I3, I6, S7, S10 and S14 are the same as in Fig. 2. 'C' and 'M' indicate the native and mutant viruses, respectively. The 1, 2, 3 and 4 at the right of panels A and B indicate RNAs 1, 2, 3 and 4 of CMV. 18S indicates ribosomal 18S RNA. '+' and '−' indicate CMV virion RNA and total RNA of a non-infected tobacco leaf. A: Agarose gel electrophoresis of total RNAs from native and mutant virus-infected plants. B: RNA blot analysis of CMV RNAs 1, 2, 3 and 4. C: Relative RNA levels of native and mutant viruses normalized by 18S rRNA were calculated using a Bio Imaging Analyzer from the RNA blot shown in B. White and black bars indicate the RNA level of native and mutant viruses in tobacco plants, respectively.

synthesis in the first upper systemic leaf as a read-out to estimate the competitive translational activity of the wild type and mutant viral mRNAs. At day 10, the maximal coat protein level in the first systemic leaf of a plant infected with native viral RNA was ~2.5-fold higher than in plants infected with the mutant viral RNA. Interestingly, the difference in the coat protein level between plants infected with the native and mutant viral RNAs was similar to the difference of translational activities between 4L and 4ST5-LUC-T34 in the presence of competitor (compare Figs. 1B and 2). Meanwhile, the coat protein level in plants infected with the mutant virus was substantially higher at 7 days than that of plants infected with the native virus. One explanation for this is that the systemic infection of the two viruses was not synchronous, probably because the mutation in mutant RNA 3 resulted in an alteration of RNA replication (Fig. 3) or synthesis of other viral proteins. Despite the putative non-synchronous infection of systemic leaves by these two viruses, the difference in coat protein synthesis at 10 days indicates that in the cellular environment the translational activities of native and mutant RNA 4 are clearly different.

3.3. Viral RNA levels amplified in plants

In order to investigate whether the observed difference in maximal coat protein synthesis for the native and mutant viruses was the consequence of differential accumulation of viral RNA, the relative quantities of viral RNA amplified in plants were estimated by ethidium bromide staining (Fig. 3A) and RNA blot analysis (Fig. 3B). Total RNA from virus-infected plants showed that the levels of viral RNA in plants are near to that of rRNA in both inoculated and systemic leaves (Fig. 3A). In order to compare the quantities of viral RNA in two kinds of virus RNA-infected plants, RNA blot analysis was performed for RNAs, 1, 2, 3 and 4 using 3′ UTR cDNA mixtures as the probe (Fig. 3B). The relative viral RNA level normalized with 18S rRNA showed that the amounts of viral RNAs in the inoculated leaf were higher in plants infected with native virus than in plants infected with mutant virus (Fig. 3C). This indicates that the mutation around nucleotide position +40 contributed to viral RNA 4 replication even though the major *cis*-acting element affecting RNA 4 synthesis was found to exist in the −70 to +20 region (+1 indicates the first nucleotide of RNA 4) [26]. It should be noted that the major *cis*-acting element affects RNA replication in an almost all-or-none fashion. The differences in the amounts of other viral RNAs in the plants infected with native and mutant virus RNAs are thought to be the result of the differences in RNA 4 and/or its translation product level. In any case, the abundance of RNA 4 at 10 days post-inoculation in the first upper systemic leaf (Fig. 3C) did not alleviate the differential accumulation of maximal coat protein (Fig. 2).

It has been observed that CMV infection does not shut off

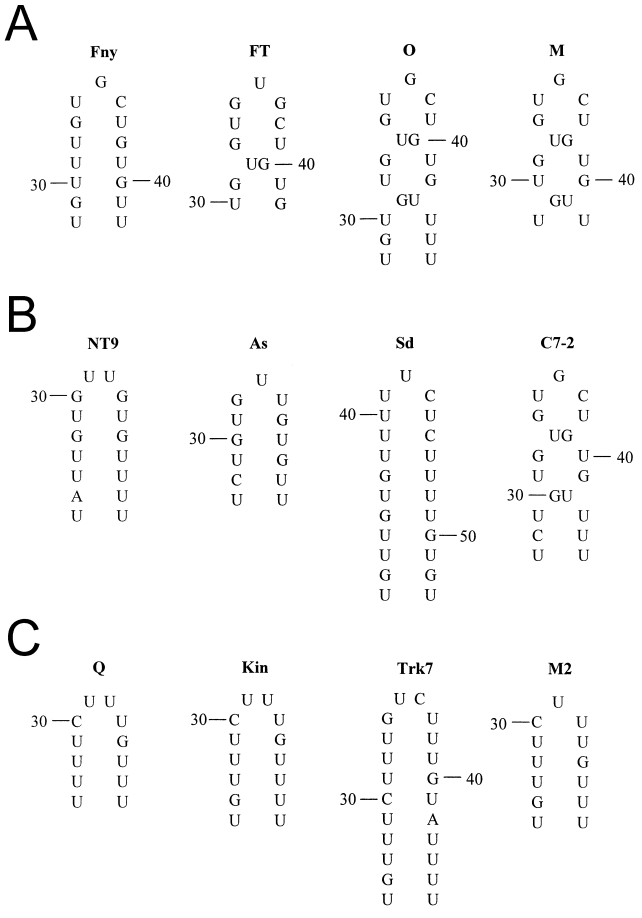

Fig. 4. RNA loop models predicted for representative CMV strains in subgroups IA (Fny, FT, O, M), IB (NT9, As, Sd, C7-2) and II (Q, Kin, Trk7, M2). RNA secondary structures were predicted using the mfold 3.0 server from the 5′ UTR sequences of the IA (A), IB (B), and II (C) subgroup strains of CMV, and the potential loop regions are shown with two nucleotide positions indicated. GenBank accession numbers are: Fny, D10538; FT, D28487; O, D00385; M, D10539; NT9, D28780; As, AF013291; Sd, AB008777; C7-2, D42079; Q, M21464; Kin, Z12818; Trk7, L15336; M2, AB006813.

the translation of cellular mRNA [27]. Thus, it is thought that the translatable portion of amplified viral RNA in the virus-infected plant is small, because the competitive translational activity of BMV RNAs, which are highly similar to CMV, is similar to that of polyadenylated cellular mRNA in vitro [28]. Large amounts of viral RNA are thought to been capsidated in the virion, and replicating viral RNA is not likely to be a template for translation [29]. Thus, a simple abundance of viral RNA does not reflect the size of the translatable viral RNA pool. Rather, the stage of virus infection appears to be an important parameter.

3.4. Phylogenetic conservation of single-stranded loops in CMV RNA

Because the single-stranded loop of Kor-CMV RNA 4 contributed to competitive translational activity, this RNA feature may be preserved in other subgroup strains of CMV. RNA secondary structure prediction of the recently categorized IA, IB and II subgroups of CMV [30] showed that single-stranded loop regions in these viruses are predicted to exist widely with a loop size of 12–23 nucleotides (Fig. 4). The overall shapes of the RNA secondary structures of the 5′ UTRs in these related viruses are also similar to that of Kor-CMV RNA 4 (unpublished results). Although we do not know its exact mechanistic role, the single-stranded loop region may constitute a binding site for a cellular translation factor that exists in the limiting quantities within the host cell.

It has been proposed that the melting step of RNA secondary structure in the cap-proximal region of mRNA by eIF4A can be rate-limiting only if the secondary structure is extraordinarily stable [12–14]. It has also been shown that eIF4A requires a single-stranded region of about 12–18 nucleotides to achieve efficient RNA binding [31–33]. Thus, we propose that the single-stranded loop region in the 5′ UTR of RNA 4 is a potential binding site for eIF4A and that the efficiency of binding of eIF4A to an RNA is an important step in mRNA discrimination. It is also possible that eIF4A functions in the context of the cap binding complexes, eIF4F and/or eIFiso4F (reviewed in [34]).

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