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ARC-1, a sequence element complementary to an internal 18S rRNA segment, enhances translation efficiency in plants when present in the leader or intercistronic region of mRNAs

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ABSTRACT

The sequences of different plant viral leaders with known translation enhancer ability show partial complementarity to the central region of 18S rRNA. Such complementarity might serve as a means to attract 40S ribosomal subunits and explain in part the translation-enhancing property of these sequences. To verify this notion, we designed β-glucuronidase (GUS) mRNAs differing only in the nature of 10 nt inserts in the center of their 41 base leaders. These were complementary to consecutive domains of plant 18S rRNA. Sucrose gradient analysis revealed that leaders with inserts complementary to regions 1105–1114 and 1115–1124 ('ARC-1') of plant 18S rRNA bound most efficiently to the 40S ribosomal subunit after dissociation from 80S ribosomes under conditions of high ionic strength, a treatment known to remove translation initiation factors. Using wheat germ cell-free extracts, we could demonstrate that mRNAs with these leaders were translated more than three times more efficiently than a control lacking such a complementarity. Three linked copies of the insert enhanced translation of reporter mRNA to levels comparable with those directed by the natural translation enhancing leaders of tobacco mosaic virus and potato virus Y RNAs. Moreover, inserting the same leaders as intercistronic sequences in dicistronic mRNAs substantially increased translation of the second cistron, thereby revealing internal ribosome entry site activity. Thus, for plant systems, the complementary interaction between mRNA leader and the central region of 18S rRNA allows cap-independent binding of mRNA to the 43S pre-initiation complex without assistance of translation initiation factors.

INTRODUCTION

Plant cytoplasmic mRNAs are typically monocistronic and possess a 5′-terminal cap structure. The cap is followed by a leader that ends at the first AUG codon of the first open-reading frame. Translation initiation on such mRNAs is well explained by the scanning model (1–3). However, cap-independent initiation and exceptional modes of cap-dependent initiation of translation (shunting) of some plant viral mRNAs have also been found (reviewed in 4–7).

Messenger RNAs of many plant viruses are translated with greater efficiency than host mRNAs. This phenomenon is ascribed to *cis*-acting translational enhancers within their leaders (4,5,7). The first leaders examined for the ability to enhance translation of reporter mRNA were the 68 base long 'Ω' leader from tobacco mosaic virus (TMV) genomic RNA and the 36 base leader from alfalfa mosaic virus RNA 4 (8,9).

Some plant viral mRNAs naturally lack a cap and yet are efficiently translated (4,7). Examples include tobacco etch virus (TEV) (10–12), potato virus Y (PVY) (13), turnip mosaic (poty)virus (14), pea seed borne mosaic virus (15) and others (4,7).

Plant virus RNAs compete efficiently with cellular mRNAs seemingly by the ability of their leaders to reduce the requirement for (16,17), or increase the affinity to, initiation factors (18) and other helper proteins (*trans*-factors) (19).

Although many data suggest that initiation factors and additional specific proteins mediate the translational enhancement conferred by viral leaders, there are only few indications that the ribosome itself may also be responsible for this phenomenon (20). Consistent with this notion is the ability of TMV RNA to be cross-linked to 18S rRNA in a wheat germ (WG) ribosome–mRNA complex (21).

Computer analysis of eukaryotic 18S rRNAs revealed up to 13 relatively short (7–14 nt) GC-rich segments that have the potential to base pair with the complementary ('clinger') segments often present in eukaryotic mRNAs (22). It has been suggested that 'clinger' segments may function by attaching mRNA to 18S rRNA within the 40S ribosomal subunit, thus

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increasing the chance of that mRNA being translated. More recently, an extensive computer search has revealed that large numbers of mRNAs contain segments that are similar to, or complementary to, 18S rRNA and it was suggested that the rRNA-like sequences of mRNA might bind ribosomal proteins, whereas the complementary sequences may base pair to the rRNA itself (23).

Experiments investigating the importance of mRNA complementarities to 18S rRNA have shown that a segment containing nucleotides 133–141 of the 196 base leader of the mRNA encoding the Gtx-homeodomain protein could directly bind the 40S ribosomal subunit by base paring to 18S rRNA (hairpin 29, nucleotides 1124–1132) (24). Interestingly, this region of 18S rRNA coincides with 'clinger' segment no. 9 from Matveeva and Shabalina (22). Moreover, the same fragment of Gtx-mRNA was shown to function as an internal ribosome entry site (IRES) module when tested in the intercistronic region of a dicistronic mRNA, and multiple linked copies of this module greatly enhanced IRES activity (25).

In the present article we demonstrate that introduction of limited complementarities to the central domain of plant 18S rRNA into the mRNA leader can substantially enhance the translational efficiency of a reporter mRNA both *in vitro* and *in vivo*, to levels exceeding those attributable to natural enhancers of viral origin. Moreover, the translation-enhancing ability of a given short 18S complementary insert directly correlates with its ability to bind 40S ribosomal subunits washed with high-salt buffer, indicating that mRNA and plant 18S rRNA may interact without the assistance of initiation factors. The ability of these artificial inserts to function as an IRES suggests that such complementarity may constitute one of the mechanisms of cap-independent, and internal, translation initiation.

MATERIALS AND METHODS

Recombinant DNA constructs

All constructs for *in vitro* expression were based on plasmids 'pl-GUS' and 'Ω-GUS', encoding bacterial β-glucuronidase (GUS) (26), which were kindly provided by Dr D. R. Gallie (pl, polylinker-derived leader; Ω, leader of TMV genomic RNA). The construct 'Y-GUS' was constructed by inserting a DNA fragment corresponding to the leader of PVY genomic RNA into pl-GUS between the HindIII and NcoI sites. Six sets of synthetic complementary oligonucleotides were used to create constructs with artificial complementarities to plant 18S rRNA in their leaders. Each annealed pair of oligonucleotides had 10 base complementarities to consecutive adjacent regions of 18S rRNA in the interval from nucleotides 1073 to 1134 (Fig. 1C and D) and was flanked by the sticky ends of restriction endonuclease HindIII such that, after insertion into the HindIII site of pl-GUS plasmids, only the 5′-proximal HindIII site was recovered, permitting sequential insertion of several copies of the same oligo-DNA. The correct orientation of inserts was verified by restriction and PCR analyses and finally by sequencing.

Numbering of the rice 18S rRNA (EMBL accession no. X00755) is according to De Rijk and De Wachter (27). The secondary structure model (Fig. 1A and B) was drawn using the program RnaViz (28). Primary structures of all constructs were verified by sequencing.

RT–PCR was carried out using the AccessQoick RT-PCR System from Promega; 0.2 optical units (260 nm) of 40S ribosomal subunits were used as a template for the 50 µl reaction. Primers: oligo complementary to the region 1115–1124, ATACTCCCCC; primer 1043–1052, ACCAGGGATC; primer 1009–1018, GATACCGTCC; primer 923–932, TTTATGAAAG.

For transient expression, the various leader constructs (flanked by HindIII and NcoI sites) were transferred into a cauliflower mosaic virus (CaMV) 35S promoter-based plasmid [kindly provided by S. Pauli and A. Karsies, FMI, Basel (29)] upstream of the chloramphenicol acetyltransferase (CAT) reporter gene followed by the *nos*-termination (polyadenylation) signal. Dicistronic plasmids contained the GUS and CAT genes as the first and second cistrons, respectively, separated by various intercistronic sequences (ICS) corresponding to the set of leaders described above.

In vitro transcription was carried out using bacteriophage T7 RNA polymerase as described previously (30). For synthesis of uncapped leaders only, the plasmids were cleaved before transcription at the unique NcoI site and then transcribed in the presence of [α-^{32}P]UTP. To transcribe uncapped full-length mRNAs encoding the GUS protein, the same plasmids were linearized with EcoRI (Fig. 1D). RNA integrity was assessed by urea-agarose gel electrophoresis.

In vitro translation was carried out in a WG cell-free system as described previously (31) with slight modifications. The standard reaction mixture in 25 µl contained: 20 mM Tris–acetate pH 7.6; 90 mM potassium acetate; 2.5 mM magnesium acetate; 1.6 mM DTT; 0.1 mM spermine; 1 mM ATP; 0.2 mM GTP; 10 mM creatine phosphate; 0.12 mg/ml creatine kinase; 0.1 mM of each amino acid; 2 µg of mRNA and 7 µl of WG extract. Incubation was for 60 min at 26 or 37°C. Translation efficiency was estimated by fluorometric measurement of GUS activity as described (26).

Protoplast preparation and transient expression assay

Protoplasts were isolated from *Orychophragmus violaceus* suspension culture and transfected by electroporation with 3 or 10 µg of circular DNA constructs as described (32). After overnight incubation (15 h) a protoplast protein extract was prepared and the activity of the GUS enzyme was measured using a fluorometric assay while the amount of CAT protein was estimated using the ELISA assay kit obtained from Roche.

Sedimentation analysis

Sedimentation analysis and isolation of ribosomal subunits were carried out as described previously (33). The WG ribosomal fraction pellet was suspended in high-salt buffer (50 mM HEPES, pH 7.6; 500 mM KCl; 0.1 mM $MgCl_2$; 5 mM β-mercaptoethanol) and applied to a 5–20% linear sucrose gradient prepared in the same buffer and centrifuged in an SW-27 rotor (Beckman) for 20 h at 30 000 *g* and 4°C. Fractions containing dissociated 40S subunits were combined, diluted 1:1 with the standard buffer (50 mM HEPES, pH 7.6; 100 mM KCl; 2.5 mM $MgCl_2$; 5 mM β-mercaptoethanol) and collected by centrifugation in a Ti-50 rotor for 4 h at 105 000 *g* and 4°C. Pellets of 40S subunits were resuspended in standard

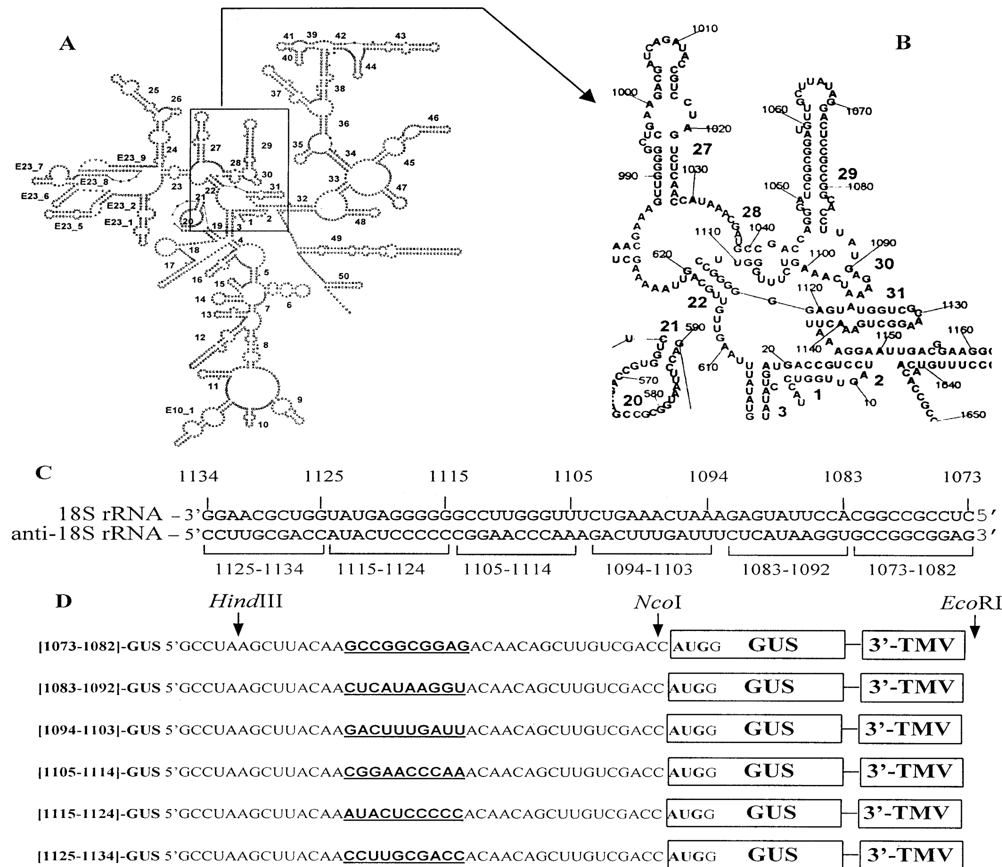

Figure 1. Secondary structure model of rice (*Oryza sativa*) 18S rRNA (**A**) with an enlargement of the central domain (**B**). Numbering of nucleotides in rice 18S rRNA is according to the sequence with accession no. X00755. (**C**) Primary structure of rice 18S rRNA segment from nucleotides 1073 to 1134 (in antisense orientation) divided into six consecutive 10 base subsegments. (**D**) Schematic structure of mRNAs containing in their leaders 10 base inserts (bold and underlined) complementary to adjacent consecutive segments of 18S rRNA from nucleotides 1073 to 1134. Arrows indicate positions of restriction enzymes used for insertion (HindIII) and for linearization (NcoI or EcoRI). 3′-TMV corresponds to the TMV 3′-UTR present in all mRNAs tested. GUS is the β-glucuronidase coding region, with the AUG as shown.

buffer, centrifuged once more for 20 min at 15 000 *g* to remove aggregates and adjusted to 10 A_{260}/ml with standard buffer. To test the binding ability, 2 µl of 100 000 c.p.m./µl of each radiolabeled leader was mixed with 100 µl of 40S subunit suspension, incubated for 20 min at 20°C, applied to a 5–20% sucrose gradient prepared in standard buffer and centrifuged in an SW-41 rotor for 4 h at 105 000 *g* and 4°C. The A_{260} profile and trichloroacetic acid-precipitable radioactivity in gradient fractions was determined.

RESULTS

Complementarity of a mRNA leader to the central domain of plant 18S rRNA can enhance translation efficiency *in vitro*

Computer analysis of different plant viral leaders with known translation enhancer ability using the program Mfold (34), revealed that many of them had substantial complementarities to the central domain of plant 18S rRNA in the 1080–1135 nt interval. For instance, nucleotides 130–180 of the 184 base long leader of PVY genomic RNA have strong (–16.7 kJ/mol), although imperfect, complementarities to the 18S rRNA within the 1080–1135 interval. The PVY RNA leader has been shown to possess cap-independent translation enhancer ability in rabbit reticulocyte lysate (13) and tobacco protoplasts (35). This property is revealed also in a WG cell-free system (Table 1).

Table 1. Translation enhancer property of the leader of PVY genomic RNA in a WG cell-free system at different temperatures

mRNA	GUS activity (%) Incubation at 26°C	Incubation at 37°C
pl-GUS	100	4 ± 2
Y-GUS	1064 ± 11	1104 ± 10

Average of at least five experiments. pl, 17 base polylinker-derived leader; Y, leader of PVY genomic RNA.

At 26°C, the 'Y-GUS' mRNA was translated approximately 10 times more efficiently than the control mRNA 'pl-GUS', confirming the translation enhancer properties of the PVY RNA leader. Increasing the incubation temperature to 37°C had no effect on the translation rate of 'Y-GUS' while 'pl-GUS' translation was almost completely inhibited. As a result, the *de facto* translation enhancement increased 250-fold. Significantly, deletion of the 130–184 nt region of the PVY

Table 2. Binding to 40S ribosomal subunits of uncapped leaders with 10 base inserts complementary to different regions of 18S rRNA (left) and messenger activity of mRNAs with the corresponding leaders (right)

Leader	Binding to 40S ribosomal subunit (%)	mRNA	GUS activity (%)
Pl	100	pl-GUS	100
[1073–1082]	107 ± 5	[1073–1082]-GUS	113 ± 4
[1083–1092]	114 ± 3	[1083–1092]-GUS	131 ± 7
[1094–1103]	286 ± 5	[1094–1103]-GUS	228 ± 6
[1105–1114]	550 ± 4	[1105–1114]-GUS	281 ± 8
[1115–1124][a]	572 ± 4	[1115–1124]-GUS	343 ± 5
[1125–1134]	267 ± 4	[1125–1134]-GUS	205 ± 6

Average of at least five experiments. Translation reactions were carried out at 26°C. The stability of leader secondary structures predicted using the Mfold program are calculated to be as follows (in kJ/mol): [1073–1082], –10.5; [1083–1092], –4.3; [1094–1103], –4.4; [1105–1114], –3.3; [1115–1124], –2.6; [1125–1134], –2.6. pl, 17 base polylinker-derived leader.
[a]Named 'ARC-1'.

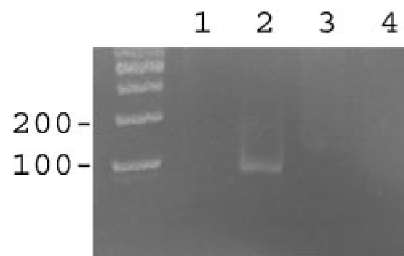

Figure 2. Electrophoresis of products of RT–PCR. WG 40S ribosomal subunits were used as a template. Lane 1, RT–PCR from primers AS(1115–1124) and (1043–1052) without reverse transcriptase; lane 2, RT–PCR from primers AS(1115–1124) and (1043–1052); lane 3, RT–PCR from primers AS(1115–1124) and (1009–1018); lane 4, RT–PCR from primers AS(1115–1124) and (923–932).

leader (35), or blocking it with antisense oligonucleotides (13), significantly reduces its enhancer activity.

The potential importance of the 18S rRNA central region in binding of mRNA was suggested by both computer analysis (22) and hybridization experiments using synthetic oligonucleotides (36). It has also been demonstrated that this region is highly exposed and located on the interface between the 40S and 60S subunits (36).

In order to localize more precisely the region of plant 18S rRNA involved in complementary interaction with mRNA, we prepared six variants of 40 nt long radiolabeled leaders. These variants were identical with the exception of 10 base inserts 17 nt upstream of the AUG codon, which were complementary to adjacent consecutive regions of 18S rRNA between nucleotides 1073 and 1134 (Fig. 1C). Each of these leaders was mixed with 40S ribosomal subunits, which were dissociated from 80S ribosomes under high-salt conditions, and the resulting complexes were analyzed in sucrose gradients. Radioactivity contained in the 40S zone of gradients was counted (Table 2, left). In parallel, six variants of full-length mRNAs were prepared with the same set of leaders preceding the GUS coding sequence. These mRNAs were translated in a WG cell-free system and their translation activities estimated by measuring the activity of newly synthesized GUS enzyme (Table 2, right).

The results presented in Table 2 demonstrate that the affinity of a given leader for the 40S ribosomal subunit directly correlates with its ability to promote the translation of reporter mRNA. Importantly, the leaders tested did not bind to 60S ribosomal subunits (data not shown), indicating a specific interaction with 40S ribosomal subunits. Region 1073–1082 of plant 18S rRNA overlaps 'clinger' segment no. 9 (22) in mammalian 18S rRNA (position 1124–1135; here numbering is according to human 18S rRNA, accession no. X03205), which is also complementary to the Gtx-mRNA leader (24,25). In our experiments, this region of plant 18S rRNA did not support substantial interaction with the corresponding complementary leader, which was neither stimulatory nor inhibitory to translation of reporter mRNA. Thus, this region of plant 18S rRNA, as well as the adjacent one (1083–1092), seems to be inaccessible for complementary interactions with exogenous RNAs.

The leaders with complementarities to regions 1105–1114 and 1115–1124 of plant 18S rRNA display the highest affinity to 40S subunits and stimulate translation most efficiently. It is noteworthy that these regions of plant 18S rRNA overlap with the purine (especially G)-rich 'clinger' segment no. 10 (22) in mammalian 18S rRNA (position 1162–1172; numbering according to human 18S rRNA).

In order to check ability of region 1115–1124 of plant 18S rRNA to complementary interact with another nucleic acid we performed RT–PCR on purified 40S ribosomal subunits. As a primer for reverse transcription we used 10 nt long oligo complementary to the region 1115–1124 of plant 18S rRNA. Reverse transcription was performed at 37°C in order to avoid conformational change or denaturation of 18S rRNA. As a second primer we used oligos corresponding to regions 1043–1052, 1009–1018 and 923–932 of 18S rRNA (Fig. 2).

It was possible to perform an RT–PCR using 40S ribosomal subunit as a template (Fig. 2, lanes 2 and 3). PCR products from primers 1043–1052 and 1009–1018 were sequenced. These sequences corresponded to regions of rRNA in intervals 1043–1124 and 1009–1124. The PCR product 1009–1124 is weaker than 1043–1124. We could not get PCR product from primer 923–932. This result showed that the region 1115–1124 of rRNA is exposed and accessible for oligos and reverse transcriptase. The region 923–1009 is more structured or covered with proteins. These data do not exclude the possibility of interaction of the 10 base insert within the 5′-UTR complementary to the 1115–1124 nt region of plant 18S rRNA (hereafter referred to as 'active ribosomal RNA complementary' sequence, ARC-1) with some other regions of 18S rRNA, but prove the possibility of interaction of ARC-1 with region 1115–1124.

Our data confirm that these regions of plant 18S rRNA are indeed exposed and accessible to complementary interaction with other RNA molecules. Such interactions most likely occur without the assistance of extra protein factors, which would have been washed out by the treatment with 500 mM KCl (20). However, it cannot be ruled out that some unknown ribosome-associated factor is resistant to removal by the high-salt wash procedure.

We compared the sequence of 18S rRNA from rice with those from tobacco, maize, wheat and human. In all cases the

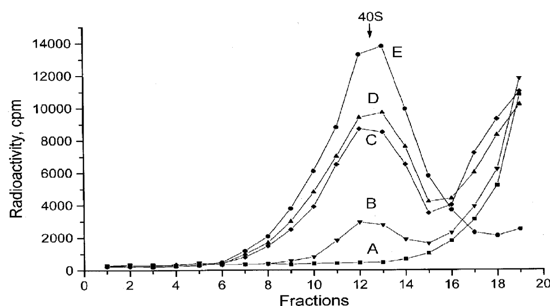

Figure 3. Ability of different radioactively labeled leaders to bind to 40S ribosomal subunits dissociated from 80S ribosomes under high ionic strength conditions. A, plasmid polylinker-derived leader; B, ARC-1; C, 2xARC-1; D, PVY viral genomic RNA leader; E, 3xARC-1. Preparation of 40S subunits and sucrose gradient analysis was performed as described in Materials and Methods. The arrow indicates the position of the 40S subunit.

Table 3. Messenger activity in a WG cell-free system of uncapped reporter mRNAs with different leaders

mRNA	GUS (%)
pl-GUS	100
Ω-GUS	1403 ± 9
Y-GUS	1011 ± 7
ARC-1-GUS	315 ± 5
2xARC-1-GUS	929 ± 9
3xARC-1-GUS	1470 ± 7

Average of at least three experiments. Translation reactions were carried out at 26°C. pl, 17 base polylinker-derived leader; Ω, 68 base leader of TMV genomic RNA; Y, 184 base leader of PVY genomic RNA; ARC-1, 40 base leader with 10 base insert complementary to 1115–1124 region of plant 18S rRNA as shown in Figure 1D; 2xARC-1, 62 base leader of the following structure: GCCUAAGCUUACAAUACUCCCCCACACAGCUUACAAAUACUCCCCCACAACAGCUUGUCGAC; 3xARC-1, 86 base leader of the following structure: GCCUAAGCUUACAAAUACUCCCCCACAACA-GCUUACAAUACUCCCCCACACAGCUUACAAAUACUCCCCCACAA-CAGCUUGUCGAC. The 10 base inserts complementary to 1115–1124 region of plant 18S rRNA are underlined.

region of 18S rRNA corresponding to ARC-1 was absolutely conserved.

We then tested the ability of uncapped leaders containing reiterated copies of a 10 base insert complementary to the 1115–1124 nt region of plant 18S rRNA to bind to the 40S ribosomal subunit (Fig. 3) and to promote translation of reporter mRNA (Table 3).

The affinity for the 40S ribosomal subunit of leaders harboring the ARC-1 sequence directly correlated with the number of inserted copies of ARC-1 (Fig. 3). With two copies, the binding strength reaches that of the PVY RNA leader, and with three copies even exceeds it. The distribution of labeled material in the sucrose gradient suggests that the 40S subunits form specific complexes with the corresponding leaders in each case.

The translation efficiency of reporter mRNAs also increased with the number of complementary inserts in their leaders (Table 3). Indeed, the translation-enhancer property of the artificial leader with three consecutive ARC-1 copies exceeded that of the natural leaders of TMV and PVY viral genomic RNAs.

Table 4. Transient expression in *O.violaceus* protoplasts of the CAT reporter gene preceded by different leaders

No.	Leader	Schematic structure of monocistronic mRNA	Amount of CAT protein (ELISA test)
1	Ω	CAT	0.343 ± 25
2	Y	CAT	0.984 ± 47
3	4x[1083-1092]	CAT	0.406 ± 38
4	2xARC-1	CAT	1.483 ± 45
5	3xARC-1	CAT	1.561 ± 53
6	4xARC-1	CAT	1.911 ± 49
7	5xARC-1	CAT	2.019 ± 57
8	4x[ARC-S]	CAT	1.569 ± 51

Average of at least three experiments. Ω, 68 base leader of TMV genomic RNA; Y, 184 base leader of PVY genomic RNA; ARC-1-S, without spacer sequence.

The nucleotide sequences flanking the inserts were identical in all artificial leaders, so they cannot be responsible for the difference in the ability of a given leader to bind to the 40S ribosomal subunit or to enhance the translation of reporter mRNA. Similarly, the sequence of a 13 base spacer between the first and the second complementary inserts in the leader '2xARC-1' was identical to that between the second and third inserts in '3xARC-1' and almost identical to that between first and second inserts in the latter case. Thus, the 10 base complementary inserts must be the main determinants underlying the differences in binding to 40S subunits and in translational enhancement. A GUS-encoding reporter mRNA with a leader carrying 5xARC-1 was also efficiently translated in rabbit reticulocyte lysate, as well as in yeast and *Escherichia coli* extracts (data not shown).

An increase in the affinity of the leader for 40S ribosomal subunits with an increasing number of complementary inserts is most likely explained by the increased concentration of ARC-1 in the solution which increases the probability of complementary interaction between leader and 18S rRNA in the absence of translation initiation factors. A more efficient recruiting of 40S subunits to the leader may increase the translation efficiency of a reporter mRNA. Such a mechanism of recruitment does not require a 5′ cap (or cap-binding factor eIF4E), since the 40S subunit can efficiently bind to internal (cap-distal) sites in the leader.

It is known that translation-enhancing leaders of some plant viral mRNAs are able to confer on reporter mRNAs the ability to be efficiently translated under heat shock conditions (37) (Table 1). We also tested this ability of our artificial leaders in a WG cell-free system incubated at normal (26°C) and elevated (37°C) temperatures (Fig. 4). Translation of uncapped reporter mRNAs with different leaders was carried out in the presence of [^{35}S]methionine and translation products were analyzed by SDS–polyacrylamide gel electrophoresis with subsequent autoradiography.

The reporter mRNA with the 2xARC-1-containing leader, which is efficiently translated under normal temperature, largely retained its translational activity at elevated temperature (Fig. 4). This suggests that the presence of the ARC-1 sequences enables the mRNA to partially escape the high-temperature-dependent translational repression observed with the leaders of ordinary mRNAs.

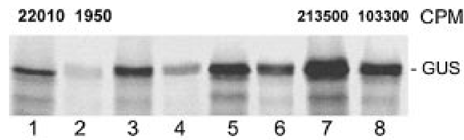

Figure 4. Autoradiogram of an SDS–polyacrylamide gel after electrophoresis of translational products synthesized in a WG cell-free system incubated for 1 h at 26°C (odd lanes) or 37°C (even lanes). Translated mRNAs: lanes 1 and 2, pl-GUS; lanes 3 and 4, [1073–1082]-GUS; lanes 5 and 6, ARC-1-GUS; lanes 7 and 8, 2xARC-1-GUS.

Table 5. Transient expression in *O.violaceus* protoplasts of the CAT gene located as the second cistron preceded by various ICS in dicistronic mRNA

No	Intercistronic sequences	Schematic structure of dicistronic mRNA	CAT (ELISA test)
1	Ω	GUS CAT	0.002 ± 2
2	Y	GUS CAT	0.044 ± 7
3	4x[1083-1092]	GUS CAT	0.006 ± 3
4	3xARC-1	GUS CAT	0.052 ± 7
5	4xARC-1	GUS CAT	0.090 ± 10
6	5xARC-1	GUS CAT	0.111 ± 12
7	crTMV-$IRES_{MP,75}$	GUS CAT	0.024 ± 6

Typical results of at least three experiments. Ω, 68 base leader of TMV genomic RNA; Y, 184 base leader of PVY genomic RNA; crTMV-$IRES_{MP,75}$, 75 base sequence preceding the movement protein gene of crucifer-infecting TMV subgenomic RNA I_2 (38).

Artificial leaders containing the ARC-1 sequence are able to enhance translation efficiency and to serve as IRES elements *in vivo*

The translation enhancer ability of the leaders with artificially introduced complementarity to 18S rRNA was also tested *in vivo*. For this purpose we created a series of plasmids bearing the CAT reporter gene under the control of the CaMV 35S promoter. All constructs were identical with the exception of the leader sequences as depicted in Table 4. The leaders of TMV (Ω) and PVY (Y) genomic RNAs, with known translation enhancer abilities, served as positive controls (structures 1 and 2, respectively). A leader with four copies of an insert complementary to the 1083–1092 region of plant 18S rRNA served as negative control (structure 3). As described above (Table 2), complementarity to this region did not endow the leader with any substantial translation enhancer ability. The constructs under consideration contained in their leaders from two to five copies of ARC, separated by 13 nt long spacers (structures 4–7). Additionally, a construct containing four directly adjacent copies of the ARC-1 insert (structure 8 in Table 4) was analyzed.

All plasmids were introduced into *O.violaceus* protoplasts by electroporation and, after overnight incubation, the amount of CAT protein synthesized was measured by ELISA assay. To provide an internal standard, protoplasts were co-transfected with a plasmid containing the GUS reporter gene with a polylinker-derived leader (pl), and GUS activity was measured (Table 4).

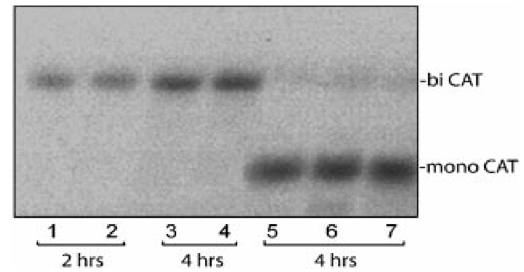

Figure 5. Northern blot hybridization of total RNA purified from *O.violaceus* protoplasts at different time intervals after transfection with dicistronic (lanes 1–4) and monocistronic (lanes 5–7) constructs and probed with a ^{32}P-labeled RNA complementary to the CAT gene. Total RNA was extracted at 2 (lanes 1 and 2) and 4 h (lanes 3–7) post-transfection. Constructs used for transfection: lanes 1 and 3, dicistronic 'pl-GUS-4x[1083–1092]-CAT'; lanes 2 and 4, dicistronic 'pl-GUS-4xARC-1-CAT'; lane 5, monocistronic 'Y-CAT'; lane 6, monocistronic '4x[1083–1092]-CAT'; lane 7, monocistronic '4xARC-1-CAT'.

Similar to the *in vitro* results (Table 3), ARC-1 enhanced mRNA translation *in vivo* to a much greater extent than the inserts complementary to the 1083–1092 region of plant 18S rRNA. Multiple copies of reiterated ARC-1 sequences increased the mRNA translation efficiency to a level exceeding that attributable to natural leaders of viral origin. Moreover, spacer sequences between the ARC-1 inserts are not required, although they increase the translation rate to a certain extent. Very similar results were obtained with protoplasts from *Nicotiana plumbaginifolia* leaves (data not shown).

It is known that leaders of naturally uncapped potyviral genomic RNAs may not only enhance translation of monocistronic reporter mRNAs in a cap-independent manner, but also function as IRESs (11–14). Our observation that the artificial leaders containing ARC-1 sequences are able to enhance translation of monocistronic reporter mRNA in a cap-independent manner (Tables 2 and 3), prompted us to investigate their IRES activity when placed as an intercistronic sequence (ICS) in dicistronic mRNAs. Several dicistronic constructs under CaMV 35S promoter control, differing only in the primary structure of the ICS were designed (Table 5). The first cistron (GUS) was preceded by the standard polylinker-derived leader (pl) while the second cistron (CAT) was preceded by different ICSs. The TMV genomic RNA leader (Ω) and the artificial non-enhancer sequence containing four inserts complementary to the 1083–1092 region of plant 18S rRNA served as negative controls, with the PVY genomic RNA leader (Y) (13) and crucifer-infecting (cr)TMV-$IRES_{MP,75}$ sequences (38) with known IRES activities as positive controls. The test constructs contained three to five copies of ARC-1 in their ICS (structures 4–6 in Table 5). All the plasmids were electroporated into *O.violaceus* protoplasts and the amounts of CAT protein synthesized after overnight incubation were measured using the ELISA assay (Table 5).

As expected, the results showed that 'Ω' and '4x[1083–1092]' ICS displayed negligible IRES activity, while confirming that 'Y' and 'crTMV-$IRES_{MP,75}$' sequences possess considerable IRES activity. Interestingly, the latter sequence contains two oligopyrimidine stretches that potentially may

base pair with the G-rich segment 1114–1126 of plant 18S rRNA.

The artificial ICS containing three to five copies of ARC-1 supported relatively high levels of expression of the second (CAT) cistron. Increasing the number of complementary inserts to five increased the IRES activity to a level exceeding that of either natural viral IRES element several fold. Very similar results were obtained with *N.plumbaginifolia* leaf protoplasts (data not shown). These data indicate that complementarity to a defined segment in the central domain of 18S rRNA may support considerable binding of 40S subunits to mRNA sequences located far from the 5′ end.

The translation-promoting effect of the ARC-1 sequence is much higher when present within a leader than within an ICS (Tables 4 and 5). Probably, an ARC-1 element in the leader is better able to initiate an interaction with 18S rRNA than one in an internal position of the mRNA.

An alternative possible explanation of enhanced expression of the CAT cistron may be that fragmentation or unusual splicing of the dicistronic mRNA results in the second (CAT) cistron becoming a truncated monocistronic mRNA. Moreover, the ICS possibly contain transcriptional promoter (or enhancer) elements that could direct synthesis of some amount of short CAT-encoding monocistronic mRNA. To exclude these possibilities, we performed northern blot analysis of total RNA extracted from transfected protoplasts at different time intervals after transfection (Fig. 5). Protoplasts were transfected with either monocistronic (Table 4, constructs 2, 3 and 6) or dicistronic (Table 5, constructs 3 and 5) plasmids and a ^{32}P-labeled RNA complementary to the CAT gene was used as a probe.

The results demonstrate that the dicistronic mRNAs are intact (Fig. 5, lanes 1–4) so translation of the second cistron in Table 5 cannot be accounted for by initiation from truncated mRNAs.

In summary, we have shown that complementary interaction of certain mRNA sequences with the central domain of plant 18S rRNA (especially nucleotides 1115–1124; ARC-1) can play an important role in mRNA sequestration. As a consequence, such sequences can serve as cap-independent translational enhancers and IRES elements and provide for heat-shock-insensitive translation.

DISCUSSION

Binding of mRNA to the 43S pre-initiation complex is one of the rate-limiting steps in translation initiation (39). In most cases, such binding occurs through cap recognition by the eIF4 initiation factors, hydrolysis of ATP (3) and subsequent scanning until the first AUG codon is encountered (1).

Nevertheless, many eukaryotic and viral mRNAs can escape the need for cap binding (5,40). For instance, the naturally uncapped plant potyviral mRNAs may initiate translation in a cap-independent manner and their leaders contain translational enhancers that may efficiently recruit 40S ribosomal subunits as IRESs (10–15). The general mechanism explaining these properties of potyviral leaders is not known, although the common feature of such leaders is the presence of several oligopyrimidine stretches.

In the case of TEV RNA, the 143 nt long leader confers cap-independent translation to reporter mRNAs (10,11). Deletion analysis has revealed that a centrally located (28–118 nt) cap-independent regulatory element (CIRE) retains almost all the translation enhancer ability. This CIRE contains two oligopyrimidine stretches (58)UUCUACUUCU(67) and (84)UCAUUUCUUUU(94), each interrupted by a single A. Cleavage of the TEV RNA leader at nucleotide 65 results in two fragments (CIRE-1 and CIRE-2), each retaining much less translation enhancer ability than the complete element (11). The oligopyrimidine stretches of the TEV RNA leader may hybridize to the G-rich segment 1114–1126 of 18S rRNA.

In this paper, we have demonstrated that complementarity to the 1115–1124 region (ARC-1) of plant 18S rRNA, artificially introduced into a mRNA leader, considerably increases cap-independent recruitment of 40S subunits as well as mRNA translation efficiency. Moreover, two or more copies of ARC-1 in the leader have a considerably higher effect than a single copy (Table 3 and Fig. 4).

Not every region of 18S rRNA is accessible for complementary interaction with mRNA leaders (41). Even if interaction does occur, its effect on reporter mRNA translation may range from strong inhibition (24,41) to substantial stimulation (41). Importantly, the translation rate of a reporter (CAT-encoding) mRNA with leaders complementary to the 928–1159 region of mammalian 18S rRNA was increased several times with respect to a control lacking such complementarities, as shown in transient expression experiments in human cells (41). After correction of difference in nucleotide numbers caused by the difference in the length between plant and mammalian 18S rRNAs, it becomes evident that the critical region in this latter study partially coincides with the region mapped in the present work.

In different leaders complementary to the exposed 1094–1134 region of plant 18S rRNA mapped here, the extent of stimulation of translation varied (Table 2). One might conclude from this observation that the complementary interactions between the mRNA leader and the central domain of the 18S rRNA might not only stabilize the 40S mRNA complex but also switch the conformation of 18S rRNA (and consequently the 40S subunit) to an activated state, in which the 40S subunit becomes competent to perform the subsequent stages of translation more efficiently. Interestingly, the 1100–1130 region of plant 18S rRNA is highly conserved among other eukaryotes and is also able to base pair with 5S rRNA (36). We suggest that the complementary interaction of an mRNA leader with the central domain of 18S rRNA may mimic that of 5S rRNA, rendering the 40S subunit more competent to join the 60S ribosomal subunit, and promoting efficient transition into the elongation stage of translation.

As mentioned above, the region 1114–1126 of plant 18S rRNA is rich in purines (especially G) and is well suited to strong complementary interaction with the oligopyrimidine stretches often present in plant viral (9–11,13–15,38) and cellular (42–44) mRNA leaders with known translation enhancer properties. Other mRNAs capable of complementary interaction with 18S rRNA might be the numerous cellular *TOP* mRNAs with 5′-terminal oligopyrimidine tract (5′TOP), comprising the core of their translational *cis*-regulatory element (45).

Thus, direct base pairing of some mRNA leaders to the central domain of 18S rRNA may partially explain their translation enhancer abilities and cap independence. However, such mechanisms cannot explain all cases of translation enhancement caused by mRNA 5′- and especially by 3′-UTRs. Additional research is required to elucidate the mechanisms of their action. Nevertheless, the results presented in this work permit the design of efficiently translatable mRNAs for use in different biotechnological projects.

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