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Arabidopsis tRNA-derived fragments as potential modulators of translation

Stéphanie Lalande[a], Rémy Merret[b], Thalia Salinas-Giegé[a], Laurence Drouard[a1]

[a]Institut de biologie moléculaire des plantes-CNRS, Université de Strasbourg, F-67084 Strasbourg, France

[b]Université de Perpignan Via Domitia, LGDP-UMR5096, 58 av. Paul Alduy, 66860 Perpignan, France

[1]To whom correspondence should be addressed. Email Laurence.drouard@ibmp-cnrs.unistra.fr

ABSTRACT

Transfer RNA-derived fragments (tRFs) exist in all branches of life. They are involved in RNA degradation, regulation of gene expression, ribosome biogenesis. In archaebacteria, kinetoplastid, yeast and human cells, they were also shown to regulate translation. In Arabidopsis, the tRFs population fluctuates under developmental or environmental conditions but their functions are yet poorly understood. Here, we show that populations of long (30-35 nt) or short (19-25 nt) tRFs produced from Arabidopsis tRNAs can inhibit *in vitro* translation of a reporter gene. Analyzing a series of oligoribonucleotides mimicking natural tRFs, we demonstrate that only a limited set of tRFs possess the ability to affect protein synthesis. Out of a dozen of tRFs, only two deriving from $tRNA^{Ala}$(AGC) and $tRNA^{Asn}$(GUU) strongly attenuate translation *in vitro*. Contrary to human tRF(Ala), the 4 Gs present at the 5' extremity of Arabidopsis tRF(Ala) are not implicated in this inhibition while the G_{18} and G_{19} residues are essential. Protein synthesis inhibition by tRFs does not require complementarity with the translated mRNA but, having the capability to be associated with polyribosomes, tRFs likely act as general modulation factors of the translation process in plants.

KEYWORDS: protein synthesis; polyribosomes; small non-coding RNAs; tRNAs; plant

Introduction

Beyond their primordial role in protein synthesis, transfer RNAs (tRNAs) appear to have many other multiple functions [1] [2]. Among them, the production of regulatory small non-coding RNAs (sncRNAs) called tRFs (tRNA-derived Fragments) has been put forward in the last few years. These tRFs have various origins within tRNA transcripts. They can originate from tRNA precursor molecules, but most of them derive from mature tRNAs (for reviews see *e.g.* [3] [4]). Mature tRNAs generate two main classes of tRFs depending on the cleavage site: long tRFs (l-tRFs, 30-35 nt in size) when cleavage occurs in the anticodon region and short ones (s-tRFs, 19-25 nt) if the cleavage is located in the D or T regions of tRNA molecules. Several nomenclatures exist and we will use here the nomenclature we have proposed in [3] where l-tRFs mainly correspond to tRF-5A or tRF-3A and s-tRFs to tRF-5D or tRF-3T whether the RNA fragments derive from the 5' or 3' extremities of mature tRNAs.

The cleavage of tRNAs likely represents an essential process to recycle molecules such as phosphate or nitrogen upon nutrient starvation. Nevertheless, in all evolutionary divergent organisms from bacteria to human cells, a growing list of other important functions have been assigned to tRFs (for a review, see [2]). For instance, numerous l-tRFs were often shown to be upregulated upon stress ([5] [6] [7] and references therein). Others are involved in tumorigenesis [8] and hematopoiesis [9]. The implication of tRFs to promote RNA degradation [10], to prime viral reverse transcription [11] or to regulate gene expression via the RNA silencing pathway (*e.g.* [12] [13]) has also been described. Recent work also showed that tRFs can regulate epigenetic inheritance [14], the expression of retro-elements [15], or plant nodulation [16].

Regulation of translation appears to be the most documented process where tRFs are involved. Upon abiotic stress, l-tRFs produced in human cells promote stress granule assembly and translation repression [17]. Reduction of translation was also observed when some human stress-induced l-tRFs, including tRF-5A (Ala) and tRF-5A (Cys), can displace the eIF4F factor of the initiation elongation complex [18]. Also in human, Sobala and Hutvagner [19] showed that some human s-tRFs (*e.g.* a tRF-5D (Gln) of 19 nt), can inhibit protein synthesis. More recently, a proteomic approach allowed us to demonstrate that this tRF can interact with the multi-synthetase complex (MSC), a complex interacting with ribosomes [20]. Furthermore, in the yeast *Saccharomyces cerevisiae* [21] and the archaea, *Haloferax volcanii* [22], stress-

induced tRFs can bind ribosomes and inhibit translation *in vitro.* By contrast, it was recently demonstrated that in the kinetoplastid *Trypanosoma brucei*, a tRF-3A (Thr) produced during nutrient deprivation can associate with ribosomes and subsequently stimulate translation [23].

The existence of tRFs has also been proven in various plants (*e.g.* [7] [13] [24] [25] [26] [27]). In *Arabidopsis thaliana*, the tRFs population varies upon stress and plant development and some are specifically enriched in Argonaute (AGO) immunoprecipitates [28] [29]. This suggests that tRFs likely also play key roles in photosynthetic cells. Currently, except for the study reporting that Arabidopsis AGO1-associated tRFs can target and cleave transposable elements, little is known about their functions [30].

Some work on pumpkin provided evidence that a phloem small non-coding RNA (sncRNA) population triggers translation inhibition *in vitro* [24]. This sncRNA population comprises many tRFs, and here we have addressed the question of whether plant tRFs could act as a regulator of protein synthesis. We observed that both tRFs purified from *A. thaliana* leaves and specific synthetic tRFs deriving from the 5' extremity of Arabidopsis $tRNA^{Ala}$ and $tRNA^{Asn}$ efficiently inhibit protein synthesis *in vitro*. This inhibition is cap-independent and does not require a sequence complementarity with the mRNA to be translated. Furthermore, analysis of Arabidopsis ribosomal fractions shows that tRFs can bind and be associated with heavy polysomes, suggesting their involvement in global regulation of the translation process in the land plant.

Results

Arabidopsis short and long tRFs inhibit protein synthesis in vitro

We previously showed that Arabidopsis tissues (roots, stems, leaves, flowers, siliques) contain complex and variable pools of tRFs [28]. Furthermore, several RNases T2, called RNS1, 2 and 3, expressed in these tissues are involved in the biogenesis of both l-tRFs and s-tRFs [31]. Indeed, incubation of *in vitro* synthesized cytosolic $tRNA^{Ala}$ transcript or Arabidopsis total tRNAs with a crude enzymatic extract from Arabidopsis leaves produces l-tRF (Ala) and s-tRF (Ala), (Figures 1A and 1B). As a first step to knowing whether plant tRFs can modulate translation, l- and s-tRFs pools were produced by incubating total Arabidopsis tRNAs in the presence of a crude enzymatic extract from Arabidopsis leaves (Figure 1C). According to our previous results, this enzymatic extract contains the RNS involved in tRFs production in Arabidopsis [31],

and the tRFs population must correspond to the previously identified one when analyzing sncRNA libraries [28]. Then, an *in vitro* coupled transcription/translation wheat germ system was used to test the effect of the tRFs pools on the translation of a mRNA coding for the pGFP (*i.e.* GFP fused at its N-terminal extremity to a mitochondrial targeting sequence) in the presence of ^{35}S methionine. No difference in protein synthesis efficiency, as compared to the mock sample, was observed when purified Arabidopsis tRNA fractions were added to the system (Figure 1D). By contrast, both types of tRFs efficiently inhibit the production of radiolabeled pGFP when added to the wheat germ system. Only two concentrations of tRFs (4 and 8 µM) were used, but the data obtained suggest that the inhibition displays dose-dependency. At a concentration of 8 µM, l- or s-tRFs can inhibit up to 95% of pGFP expression (Figure 1E). Globally, each set of Arabidopsis tRFs (long or short) inhibits translation. However, both the Arabidopsis l- and s-tRFs populations are generated from a vast collection of Arabidopsis tRNAs [32], and the question of whether all of them can modulate protein-coding gene expression needed to be addressed.

Only a limited set of Arabidopsis tRFs affects translation **in vitro**

The Arabidopsis tRFs are highly variable in origin, sequence, and length, with a majority deriving from the 5' or 3' extremities of mature tRNAs [28]. On the one hand, we previously identified a specific population of tRFs associated with AGO1, thus suggesting their implication at least in the regulation of gene expression *via* the RNA silencing pathway. On the other hand, abundant tRFs that were not found in AGO precipitates could be involved in other molecular processes, including regulation of translation as shown in other organisms. As a first attempt to know whether translation inhibition is a general function of tRFs, we synthesized ten sequences (Figure 2A; Supplemental Table S1) representative of the Arabidopsis tRFs population that we previously analyzed in [28]. These sequences correspond to three tRFs that were shown to be among the most abundant ones associated with AGO1. The two tRFs-3T reflect two different abundances: Thr-AGU is rather abundant whereas Gly-GCC is present in low amount in sncRNA libraries. Finally, among the five selected tRF-5, Ala-AGC, Gly-GCC, and Glu-CUC belong to the most abundant tRFs and by contrast, Asn-GUU and Cys-GCA are present in a low amount. We tested them at two concentrations (0.2 and 1 µM) for their ability to inhibit the translation of pGFP using the *in vitro* coupled transcription/translation wheat germ system (Figures 2B and 2C). As control

experiments, two random oligoribonucleotides of 15 and 21 nt corresponding to an internal sequence of GFP have no significant effect on expression (Supplementary Figure 1). The three tRFs previously found associated with AGO1 in leaves have no effect on protein expression (rather a slight positive effect of pGFP synthesis was noted). For five others [*e.g.* tRF-5D (Gly-GCC), tRF-5A (Cys-GCA) or tRF-3T (Thr-AGU)], inhibition of expression is also not observed. Only two tRFs of 20 nt, namely tRF-5D (Ala-AGC) and tRF-5D (Asn-GUU) strongly inhibit GFP expression already at a concentration of 0.2 µM, inhibition reaching 75-90% at 1 µM. It is important to note that several isomers exist for each of these tRFs *in vivo*. Indeed, from the 33 $tRNA^{Ala}$ and the 15 $tRNA^{Asn}$ Arabidopsis nuclear genes, various tRFs of 20 nt can be generated (Supplementary Figure 2) and are found in sncRNA libraries [28]. For simplification, these two tRFs are now called Ala20 and Asn20 and correspond to one specific isomer (see Supplementary Figure 2). To have an idea of the amount of Ala20 versus $tRNA^{Ala}$ *in vivo*, we quantified them by northern blot in an Arabidopsis total tRNA extract and we observed that roughly there is 10^4 more tRNAs than tRFs in the extract (Supplementary Figure 3). It is to note that tRFs-5D (Ala) are the most abundant tRFs found in sncRNA libraries, whereas tRF-5D (Asn) is 15 times less present than Ala20 [28]. In the wheat germ extract, the tRNA concentration is about 20 µM (Supplementary Figure 3), thus to keep physiological conditions, we should have a 2 nM tRF concentration. However, in our assays, we used 0.2 to 1 µM. There is an excess of tRFs but we also cannot exclude that, *in vivo,* there is a higher localized concentration within the cell. Though this analysis has been performed at a small scale, our observations indicate that Arabidopsis tRFs behave differently (from null to strong effect) in their capacity to modulate expression. Interestingly, none of the major tRFs associated with AGO1 affects mRNA expression and more, the tRF capacity to modulate expression is not correlated with their abundance in the total Arabidopsis tRFs population. Thus, likely only a limited set of tRFs are efficient inhibitors, among them, Asn20 and Ala20.

tRF Ala20 inhibits translation by a cap- and sequence-independent mechanism

Interestingly, a stress-induced tRF of 29 nt deriving from human $tRNA^{Ala}$ and called 5′-$tiRNA^{Ala}$, was shown to strongly inhibit translation [17, 18]. We thus wondered whether inhibition by tRF (Ala) is an evolutionary conserved molecular process and focused our work on Ala20. In Arabidopsis, under normal growth conditions, tRF-5 of 16, 20 and

33 nt deriving from $tRNA^{Ala}$ are abundant [28]. Thus, in addition to Ala20, we further tested two other synthetic oligoribonucleotides corresponding to tRF-5D of 16 nt (Ala16) and 33 nt (Ala33) deriving from $tRNA^{Ala}$ (Figures 3B, C and D). In contrast to Ala20, no significant inhibition of pGFP translation is observed when Ala16 is added in the transcription/translation system at a concentration of 1 µM, and a 40% decrease of expression is observed at a 4 µM concentration. When Ala33 is added in the transcription/translation system, an intermediate inhibitory effect is observed with a 40% decrease of expression at 1 µM. Altogether, this suggests that the size and/or the sequence are important for determining the efficiency of inhibition. Yet, no tRF was shown to be implicated in the inhibition of both transcription and translation. The coupled transcription/translation system does not allow us to discriminate at which step the tRF is implicated. We thus performed *in vitro* translation experiments with a wheat germ extract translation kit where no transcription is possible. In this case, in the presence of *in vitro* synthesized capped or uncapped pGFP mRNA reporter, a similar important decrease in pGFP synthesis is observed when Ala20 or Asn20 is present (Figure 3E). This further demonstrates that the decrease in protein synthesis is not due to inhibition of transcription but rather that the translation process is indeed affected by the addition of the tRF. Finally, to exclude the possibility that the Ala20 is inhibiting translation by directly interfering with the sequence of the pGFP mRNA (although no sequence similarity has been bioinformatically detected), an *in vitro* transcription/translation assay was performed in the presence of another recombinant plasmid coding for pDHFR (the mammalian DiHydroFolateReductase fused to a mitochondrial targeting sequence), [33]. As for pGFP translation, pDHFR synthesis is not affected by GFP15, GFP21 (Supplementary Figure 1) and Ala16 but is efficiently repressed by the addition of Ala20 (Figure 3F). This shows that plant tRF-5D Ala20 inhibits translation by a transcript sequence-independent mechanism.

Conserved G_{18} and G_{19}, and A_{16} are important for tRF-mediated translation inhibition in Arabidopsis

Several residues of mammalian tRFs (also called tiRNA) were identified as important for translation inhibition. On the one hand, the four G residues present at the 5'-end of 5′-$tiRNA^{Ala}$ and 5′-$tiRNA^{Cys}$ can form a G-quadruplex that binds the YB1 protein. This complex then displaces eIF4F complex from the cap and inhibits translation initiation [18]. Arabidopsis Ala20 also starts with four Gs (Figure 3A). To determine if these

residues are also involved in translation inhibition in plants, we replaced them by Us (Ala20-5U, Figure 4A). As shown in Figures 4B and 4C, the inhibition of translation is not affected when a synthetic Ala20-5U is added to the wheat germ system, thus demonstrating that in Arabidopsis, contrary to human tRF-5 (Ala), the G1 to G4 residues are not essential for tRF-mediated translation inhibition. This result is in agreement with the work of Nowacka and collaborators [34] showing that human tRF-5 Ala does not inhibit translation in a wheat germ extract. On the other hand, Sobala and Hutvagner showed that in several human tRFs-5D, the conserved two Gs present in the D loop at positions 18 and 19 of tRNAs are important for translation inhibition [19]. Therefore, we also replaced G_{18} and G_{19} by Us in Ala20 (Ala20-3U). As compared to the wild type sequence, the synthetic tRFAla20-3U has lost its capacity to promote translation inhibition (Figures 4B and 4C), suggesting that these two residues are important for tRF(Ala)-mediated translation inhibition. To confirm these data, when the two Gs located at the same position in Asn20 oligoribonucleotide (Figure 4A) are replaced by Us, the same loss in translation inhibition is observed (Figure 4D). Furthermore, sequence comparison between Ala20 and Asn20 (the two tRFs that efficiently inhibit translation) and the sequence of Arg19 (unable to reduce translation), reveals a high degree of conservation (Figure 4E). The major difference is that G_{18} and G_{19} are at positions 17 and 18 in Arg19 due to the absence of an additional A at position 16 in Ala20 and Asn20. To know whether A_{16} residue is also important for translation inhibition, other oligoribonucleotides were designed. When the A_{16} of Ala20 is deleted (Ala20-16A) or replaced by a U (Ala20-A16U), translation inhibition is strongly affected. Conversely, when an A is introduced at position 16 in Arg19 sequence (Arg19+A16), this tRF acquires the ability to inhibit translation as efficiently as Ala20 or Asn20 (Figures 4E and 4F). The introduction of a U rather than an A (Arg19+U16), although less efficient, still leads to a 50% inhibition. To sum up, among tRF sequences, G_{18} and G_{19} appear to be crucial to get the capacity of inhibiting translation; nucleotides such as A_{16} may have a synergistic effect, and the size of the tRF and/or the position of the nucleotides within the sequence may also be crucial.

tRF-5D (Ala) associates with polyribosomes

A subset of Arabidopsis s-tRFs appears to efficiently repress translation, raising the question at which level this regulation is achieved. In organisms such as Archaea [35] or human cells [19], it has been shown that regulation of translation by short tRFs can

be due to their association with ribosomal subunits and we hypothesize a similar mechanism in plants.

To determine if Arabidopsis tRF Ala20 can act similarly, *in vitro* binding studies were performed. For that purpose, crude lysates from Arabidopsis seedlings, pre-incubated in the presence of the synthetic oligoribonucleotide Ala20, were fractionated on sucrose gradient to separate free RNAs, ribosomal subunits and polyribosomes. The quality of the fractionation is attested by the ribosome profile (Figure 5A) and northern blots (Figure 5B). An enrichment of 5S rRNA was observed in the polyribosomes, ribosomes and free ribosomal subunits fractions, while few 5S rRNA is present in the free RNA fractions. As expected, most of tRNAs (as attested by $tRNA^{Ala}$) is found in the low-density fractions and in the polyribosomes where active translation occurs. When polyribosomes are disrupted in the presence of puromycin, an antibiotic causing premature chain termination of translation, the distribution of 5S rRNA and $tRNA^{Ala}$ is shifted towards the light fractions of the sucrose gradient (right panels of Figure 5A). The profile of the binding of tRF Ala20 follows the profile of $tRNA^{Ala}$ (Figure 5C). As expected, more than 98% of Ala20 is recovered in the low-density fractions. It corresponds to the excess of tRF Ala20 added to the Arabidopsis extract before fractionation on the sucrose gradient. The remaining 2% of Ala20 is mainly found in the heavy polyribosomal fractions compared to the 80S and ribosomal subunit fractions (Figures 5B and 5C). By contrast, when a puromycin treatment is performed, Ala20 is not detected anymore in the polyribosomal fractions. Altogether this indicates that Arabidopsis tRF Ala20 does not efficiently associate with 80S ribosomes or with free ribosomal subunits but rather binds active polyribosomes. Similar binding experiments were performed with Ala16 and Ala33, which were previously shown to not efficiently inhibit translation *in vitro*. Both tRFs can associate with polyribosomes as Ala20 does (Supplemental Figure 4). In the same way, the Arg19 that does not affect mRNA expression *in vitro* has the same binding profile than Ala20 (Supplemental Figure 5). Thus, the capacity of tRFs to bind polyribosomes *in vitro* cannot explain their differential translation inhibition observed in wheat germ extract.

In Arabidopsis seedlings and leaves, under normal condition of growth, the amount of tRF Ala20 is very low as compared to the amount of the corresponding tRNA. Nevertheless, we raised the question of whether endogenous tRFs can be detected in polyribosomal fractions in a crude lysate from Arabidopsis seedlings under unstressed conditions. As shown in Figure 5D, although Ala33 is visible in the light fractions, it is

not detected in the polyribosomal ones, thus suggesting that *in vivo* this l-tRF is weakly or not associated with active ribosomes. Two weak signals (called a and b; Figure 5D) of around 18-19 and 24-26 nt in size, abundant in the free fractions but also observed all along the gradient, are slightly enriched in the heavy fractions (Figures 5D and 5E). They represent less than 0.5% of the total tRF Ala of the same size found in the free fractions. As different tRF-5D (Ala) in the size range of 19-26 nt were previously identified in sncRNA libraries from Arabidopsis leaves [28], it is presently impossible to precisely identify which ones are detected in polyribosomes. *In vitro*, tRF-5D Ala20 strongly inhibits translation and can bind polyribosomes; however, *in vivo*, the amount of s-tRF-5D (Ala) associated with polyribosomes is very low. Thus, taking into account these two facets of Ala20, we propose that tRFs may rather act as modulators rather than strong inhibitors of translation.

Discussion

Among the functions attributed to tRFs, regulation of translation has been observed in various organisms such as Archaea, protozoan, yeast, and human. In plants, previous work on sncRNAs found in the phloem sap of pumpkin and on Arabidopsis tRNA degradants suggested that plant tRFs could also have an inhibitory effect on protein biosynthesis [24] [34]. Here, our work brings further evidence that indeed, in *Arabidopsis,* some specific tRFs possess the ability to modulate protein synthesis.

Out of a dozen of tested tRFs, only two tRF-5D, derived from $tRNA^{Ala}$ and $tRNA^{Asn}$, were shown to strongly inhibit translation. These two tRFs are 20 nt long, but we also showed that a total fraction of Arabidopsis l-tRFs can efficiently inhibit translation *in vitro*. Thus, the population of Arabidopsis tRFs able to negatively affect translation is likely more widespread. However, considering the high complexity of the Arabidopsis tRFs population [28], no rule can be established yet to predict which tRFs will affect the translation. Starting from total s-tRFs or l-tRFs populations of Arabidopsis, a SELEX approach may be a valuable tool to identify them. Besides, the *in vitro* synthesized tRFs lack modifications that can influence the affinity of nucleotide-binding, thus the use of natural post-transcriptionally modified tRFs as those generated from the Arabidopsis tRNA fraction is likely useful.

Intriguingly, while the function of tRFs as a translation inhibitor appears as a rather widespread process, the modes of action seem to differ depending on the organism and the type of tRF. Yet, only a few data on the mode of actions have been reported,

they are summarized in Figure 6. In the human alanine l-tRF, the four G residues of the 5' extremity enable the formation of a G-quadruplex structure essential for translation repression by interacting with the translational silencer YB-1 and displacing the eukaryotic initiation factor eIF4G/A from mRNAs, thus inhibiting the binding of the small ribosomal subunit [18] [36]. By contrast, we show here that the four Gs also present in Arabidopsis tRF (Ala) are not essential to affect protein synthesis. Interestingly, the G18 and 19 residues of tRF-5D (Ala) and tRF-5D (Asn) appear necessary. However, they belong to the conserved nucleotides of the D-loop present on mostly all tRNA molecules and are thus not sufficient to explain the specificity of inhibition. Nevertheless, similar observations were obtained in human cells with a global protein synthesis inhibition by s-tRFs, in particular, tRF-Gln19 [19]. Both in Arabidopsis and human cells, these tRFs are not or are weakly associated with AGO proteins and repression of translation is independent of the mRNA sequence, thus suggesting that their mode of action is independent of the silencing pathway. Rather, such tRFs can associate with actively elongating polyribosomes, but not efficiently with ribosomal subunits. This may suggest the existence of a still unknown but conserved process of global translation regulation by tRFs between animals and plants, and future work is required to verify this hypothesis. This is different to what has been described in the archaea *Haloferax volcanii* where s-tRFs inhibit translation by binding the small ribosomal subunit and competing with mRNA association [22] or in *Saccharomyces cerevisiae* where l-tRFs negatively affect protein synthesis by interacting with aminoacyl-tRNA synthetases and the small ribosomal subunit [37]. Finally, in *Trypanosoma brucei*, a l-tRF deriving from a $tRNA^{Thr}$ induced under nutrient starvation has an opposite effect, which is to stimulate translation by binding to ribosomes and polysomes [23].

How Arabidopsis tRFs associate with active polyribosomes is presently unknown. Interestingly, by providing the first tRF protein interactome, Keam et al demonstrated recently that the human tRF (Gln)19 which globally represses translation can associate with the Multisynthetase Complex MSC, a complex known to be associated with ribosomes, and paradoxically increase the translation of ribosomal and RNA-binding proteins [20]. Such a MSC complex that was shown to interact with polyribosomes in mammals or archaea also exists in protozoans and yeast [38] and is likely present in Arabidopsis. Deciphering the Arabidopsis tRF interactome will be required to see whether a similar process may be relevant in plants or whether other partners are

identified. In human, archaea and yeast, many tRFs are induced upon various stress conditions, from oxidative stress to nutrient deprivation or pH change. In these organisms, the regulation of translation by tRFs seems to be linked to the stress response. Here, in Arabidopsis, a small proportion of tRF (Ala) was detected in active polyribosomal fractions from young leaves of plants grown under normal conditions. This low amount of tRFs may represent a way to slightly and regularly modulate the global translation process under normal conditions and to play an important biological role by allowing a rapid change in translation efficiency upon demand, i.e. during plant development or under adverse growth conditions. Indeed, as already shown, the population of plant tRFs dynamically fluctuates upon various stresses or in different tissues or organs. For instance tRFs deriving from Arabidopsis Alanine tRNAs are among the most abundant in leaves [28] and their population may even be much higher in senescing tissues such as old leaves or senescing seeds where the RNases T2 responsible for tRFs production are upregulated [31]. Unfortunately, polyribosomes fractionation from such tissues has not been successful yet to allow tRFs analysis.

Materials and methods

Plant material and growth conditions

A. thaliana is of Columbia 0 (Col-0) ecotype. Eight-week old leaves were harvested from plants grown on soil at 22°C under a 16h light photoperiod. For *in vitro* grown seedlings, surface sterilized seeds stratified 2 days at 4°C in the dark were sown on agar plates containing MSP01 (Caisson Labs UT, USA) supplemented with 3% (wt/vol) sucrose and grown 8 days at 22°C under a 16h light photoperiod.

Crude leaf enzymatic preparation

The protocol used is from [31]. Briefly, 200 mg of leaves powder were resuspended in 1 ml of enzymatic extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM Phenylmethanesulfonyl fluoride, 1X proteases inhibitor cocktail Complete® (Roche)) and shaked at 4°C for 20 min. After centrifugation at 10 000 x g for 15 min, the supernatant was used for *in vitro* cleavage assays.

In vitro *synthesized tRNA and GFP transcript*

Plasmid containing *A. thaliana* cytosolic $tRNA^{Ala}$(UGC) gene sequence was obtained previously [39]. The tRNA gene was fused to the T7 RNA polymerase promoter at its 5' terminus and included a BstNI site at its 3' terminus. The BstNI linearized constructs was used as substrate to synthesize an *in vitro* transcript with T7 RNA polymerase using Ribomax™ transcription kit (Promega, Madison WI) under conditions previously described in [40]. Capped pGFP transcript was obtained using the same kit in the presence of a pSu9GFP recombinant plasmid [33]. Reaction was performed in a final volume of 10 µl following manufacturer's protocol, with slight differences: rGTP was added to a 0.9 mM final concentration, the Cap analog used was from New England Biolabs, and 50 µM BSA and 50 µM RNase OUT™ (Invitrogen) were added. Reaction was run 25 min at 37°C, then 0.75 µl of rGTP (100 mM) was added, and reaction was pursued during 2h30. To synthesize uncapped GFP transcript, no cap analog was added and rGTP was supplied to a final concentration of 7.5 mM. Plasmid DNA was eliminated by a 40 min incubation at 37°C after addition of 80 µl of water, 10 µl of DNase buffer (400 mM Tris-HCl pH 8.0, 100 mM $MgSO_4$, 10 mM $CaCl_2$) and 1 µl of DNase RQ1 (1U/µl, Promega). RNAs were then extracted with phenol/chloroform (1/1) and ethanol precipitated.

In vitro *tRNA cleavage assay*

Assays [31] were performed at 20°C in a 175 µl reaction volume containing 1 µg of synthesized $tRNA^{Ala}$ transcript, 12.5 µl of leaf enzymatic extract and completed with water. At time points 0, 5, 10, 15 and 30 min, 30 µl aliquots were rapidly transferred into a microtube containing 200 µl of water saturated phenol and 170 µl of tRNA extraction solution (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1% SDS) and vortexed until all samples were collected. After centrifugation (2300 x g, 10 min), the aqueous phase was ethanol precipitated, and RNAs were analyzed by northern blot as described in [28] using as a probe an oligonucleotide (Supplemental Table S1) complementary to the 5' extremity of most Arabidopsis $tRNAs^{Ala}$ (Supplementary Figure 2). As a negative control, $tRNA^{Ala}$ transcript was incubated in the absence of leaf enzymatic extract.

Arabidopsis *tRNAs and tRFs purification*

Total tRNA was prepared from *A. thaliana* Col-0 leaves as described in [41]. This protocol includes a LiCl precipitation step that allows enrichment, in the supernatant,

of RNAs of a size smaller than 150 nt (mainly 5S rRNA, tRNAs and sncRNAs). The tRNA fraction was further purified on 15% polyacrylamide gel by cutting out the piece of gel containing tRNAs after staining by methylene blue. tRNAs were eluted from the gel by a 16-hour incubation at 20°C in the presence of extraction solution (500 mM ammonium acetate, 10 mM magnesium acetate, 100 mM EDTA, 0.1% SDS). Eluted tRNAs were submitted to a phenol extraction, followed by ethanol precipitation. The tRNA pellet was dissolved in water. To produce l-tRFs and s-tRFs, 5 µg of purified tRNAs were incubated 30 min at 20°C in the presence of 15 µl of leaf enzymatic extract in a total volume of 90 µl, essentially as described above for *in vitro* tRNA cleavage assay. After phenol extraction and ethanol purification, the RNA sample was fractionated on 15% polyacrylamide gel. Purification of l-tRFs and s-tRFs was performed as described above for total tRNA sample preparation. As a negative control, tRNAs incubated in the absence of Arabidopsis leaf enzymatic extract were purified following the same protocol.

In vitro *protein synthesis assays*

In vitro radiolabeled protein synthesis assays were performed using the TnT-T7 Coupled Wheat Germ Extract System kit (Promega) following manufacturer instructions. Recombinant plasmids expressing pSu9GFP (pGFP) or pSu9DHFR (pDHFR) constructs under the control of T7 promoter [33] were used and synthetic oligoribonucleotides were added at different concentrations. After fractionation of the protein samples on 15% polyacrylamide gels, gels were further stained with Coomassie blue, dried on a Whatmann paper and analyzed by autoradiography and Phosphor imaging. For translation assays only, the TnT Wheat Germ Extract kit (Promega) was used following manufacturer instructions, in the presence of 1.5 µg of capped or uncapped GFP transcripts and synthetic oligoribonucleotides.

Polyribosomes extraction and RNA analysis

Polyribosomes were extracted from A*. thaliana* seedlings and analyzed as described previously [42], with few modifications. Crude polysomal extracts were obtained from 200 mg of seedlings powder (in 600 µl), and 300 µl were resolved on 15-60% sucrose gradient (9 ml) centrifuged for 3h at 178 000 x g (rotor SW41-Ti, Beckman Coulter). Analysis of polysome profiles was performed with an absorbance detector at 254 nm and sucrose gradients collected with a BioLogic Duoflow fractions collector (Biorad)

into 13 to 16 fractions of 600 µl each. When mentioned, 1.2 µl of a synthetic oligoribonucleotide (100 µM) was added to lysis buffer. For puromycin treatment, 200 mg of seedlings powder was homogenized in the presence of 600 µl of lysis buffer (200 mM Tris-HCl pH 9.0, 200 mM KCl, 35 mM $MgCl_2$, 1% Brij-35, 1% Triton X-100, 1% NP40, 1% Tween 20). After 10 min of incubation on ice and 10 min centrifugation at 16 000 x g at 4°C, supernatant (340 µl) was supplied with 85 µl of puromycin solution (3 mg/ml puromycin, 1.5 M KCl) and 75 µl of 2 M KCl. After 30 min of incubation at 37°C, 27.5 µl of 10% sodium deoxycholate and 22.5 µl of water were added. After centrifugation (5 min, 16 000 x g, 4°C), 485 µl of supernatant were loaded on a 15-60% sucrose gradient.

RNAs were extracted from sucrose gradient fractions or from crude polysomal extract by water-saturated phenol/chloroform (1/1), chloroform extracted, and ethanol precipitated. Then they were analyzed by northern blot as previously described [28].

Miscellaneous

The oligonucleotide sequences used in this study are listed in Supplementary Table S1. The tRFs were chemically synthesized and HPLC purified by the company Integrated DNA Technologies IDT.

Supplementary data

Supplementary Data are available at RNA Biology *online.*

Funding

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure legends

Figure 1. *A. thaliana* l- or s-tRFs can inhibit translation *in vitro*.

(**A**) Kinetics of cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$(UGC) transcript upon incubation in the presence (+) or absence (-) of a crude enzymatic extract from Arabidopsis leaves (Enz. Ex.). After incubation, RNAs were phenol extracted and fractionated on 15% polyacrylamide gel, followed by northern blot analysis with a radiolabeled oligonucleotide specific for the 5' extremity of cytosolic Arabidopsis $tRNA^{Ala}$. (**B**) Cleavage of Arabidopsis total tRNA (Tot RNA) upon incubation in the presence (+) or absence (-) of a crude enzymatic extract from Arabidopsis leaves (Enz. Ex.). After incubation, RNAs were phenol extracted and fractionated on a 15% polyacrylamide gel, followed by northern blot analysis with a radiolabeled oligonucleotide specific for the 5' extremity of cytosolic Arabidopsis $tRNA^{Ala}$. (**C**) Production of long (l-tRF) and short (s-tRF) tRFs by *in vitro* cleavage of total Arabidopsis tRNAs in the presence (+) of a crude enzymatic extract from Arabidopsis leaves (Enz. Ex.). After incubation, RNAs were phenol extracted, fractionated on 15% polyacrylamide gel and all l-tRFs and s-tRFs were purified separately from the gel. (**D**) Effect of the addition of total Arabidopsis tRNAs or tRFs purified in (**B**) on the synthesis of GFP in a wheat germ coupled transcription/translation system in the presence of ^{35}S methionine. Radiolabeled GFP was detected by autoradiography or phosphor imaging after fractionation on 15% acrylamide gel. Two concentrations of tRNAs or tRFs were used: 4 µM (tRNA-4 and tRF-4) and 8 µM (tRNA-8 and tRF-8). Mock = control

experiment without added tRNAs or tRFs. Coomassie blue staining of the gel is shown as a loading control. (**E**) Relative quantification of *in vitro* synthesized pGFP in experiments similar to that described in (**D**). Error bars show standard deviations (mean of three biological replicates). One-way ANOVA tests were used to calculate p-values. Asterisks indicate statistically significant differences between Mock and each treatment (****P*<0.001; ***P*<0.01; **P*<0.1). A value of 1 has been given to the Mock sample.

Figure 2. A subset of Arabidopsis tRFs can inhibit efficiently translation *in vitro*.
(A) Features of the ten tRFs analyzed in this work. Their sequences are presented in Supplemental Table 1 and the data derive from [28]. Besides, tRF-5A (Asp) was also found in AGO1 immunoprecipitates (L. Drouard, personal communication). (**B**) The synthetic tRFs presented in (**A**) were tested for their ability to inhibit the *in vitro* protein synthesis of pGFP. Experiments were performed essentially as described in Figure 1C. Two concentrations of oligoribonucleotides (0.2 and 1 µM) were used. Quantifications of the results are presented in (**C**). An arbitrary value of 1 (dotted vertical line) has been given to *in vitro* synthesized pGFP in the absence of added tRF (Mock experiment: M). Error bars represent standard errors of the mean of independent biological experiments (n= 3 to 13). One-way ANOVA tests were used to calculate p-values. Asterisks indicate statistically significant differences between Mock and each treatment (***P*<0.01; **P*<0.1). Dark grey: 0.2 µM, pale grey: 1 µM.

Figure 3. The tRF-5D (Ala) of 20 nt efficiently inhibits translation *in vitro*.
(**A**) Sequences of the three tRF-5D of 16, 20, and 33 nt (Ala16, Ala20, and Ala33) deriving from Arabidopsis $tRNA^{Ala}$(AGC). (**B**) Effect of the addition of Ala16, Ala20, and Ala33 on the *in vitro* synthesis of pGFP protein. The experiment was performed as described in Figure 1C. Increasing concentrations of synthetic tRFs were used: 0.2, 0.4, 1, 2 and 4 µM. M = Mock, control experiment without tRF. Coomassie blue staining of the gel is shown as a loading control. (**C**) Relative quantification of *in vitro* synthesized GFP in the experiment presented in (**B**). Ala16: white triangles, Ala20: black squares, Ala33: Grey circles. (**D**) Relative quantification of *in vitro* synthesized pGFP in experiments similar to that described in (**A**) and for a tRF concentration of 1 µM. Error bars show standard deviations (mean of three biological replicates). A value of 1 has been given to M. (**E**) *In vitro* protein synthesis in a wheat germ translation

system of synthesized pGFP transcript in the presence of ^{35}S methionine. Capped or uncapped transcript was used in the absence or presence of Ala20 or Asn20 oligoribonucleotide at 1 µM concentration. M = Mock, control experiment without tRF. Coomassie blue staining of the gel is shown as a loading control. (**F**) Effect of the addition of Ala16 and Ala20 oligoribonucleotides on the *in vitro* synthesis of DHFR protein. The experiment was performed as described in Figure 2B with the same concentration range.

Figure 4. Importance of A16, G18 and G19 of tRF-5D (Ala) for the inhibition of translation.

(**A**) Sequence alignment of Ala20, Ala16, Asn20 with the mutated versions Ala20-5U, Ala20-3U and Asn20-3U. Us replacing Gs are in bold and underlined (**B**) Effect of the addition of the Ala20-5U and Ala20-3U presented in (**A**) on the *in vitro* synthesis of pGFP. The experiment was performed as described in Figure 3B with the same concentration range. (**C**) Quantification of *in vitro* synthesized pGFP in experiments similar to that described in (**B**). Error bars represent standard errors of the mean of independent biological experiments (n= 3 to 13). One-way ANOVA tests were used to calculate p-values. Asterisks indicate statistically significant differences between Mock and each treatment (****P<0.0001). A value of 1 has been given to M (Mock, control experiment without tRF). (**D**) Effect of the addition of Ala20-3U or Asn20-3U on the *in vitro* synthesis of pGFP. Experiment was performed as in (**B)**. Two concentrations of oligoribonucleotides (0.2 and 1 µM) were used. (**E)** Sequence alignment of Ala20 and Arg19 with the mutated versions Ala20-A16, Ala20A16U, Arg19+A16, Arg19+U16. Mutated or added nucleotides are in bold and underlined. The A residue at position 16 in Asn20 and Ala20 is under gray background. *In vitro* synthesis of pGFP in the presence of these oligoribonucleotides and quantifications of the results are presented in (**F**) and (**G**) respectively. The experiments were performed as in Figure 3B, except that only two concentrations of oligoribonucleotides (0.2 and 1 µM) were used. Error bars represent standard errors of the mean of independent biological experiments (n= 3 to 13). A value of 1 has been given to M (Mock, control experiment without tRF). One-way ANOVA tests were used to calculate p-values. Asterisks indicate statistically significant differences between Mock and each treatment (****P<0.0001; **P<0.01).

Figure 5. tRF-5D (Ala) associates with polyribosomes *in vitro* and *in vivo*

(**A**) Polyribosomes profile of Arabidopsis seedlings pre-incubated *in vitro* with Ala20 was determined using sucrose gradient sedimentation and OD_{254nm} measurement (blue line) and conductivity (red line). Samples were treated or not with Puromycin. The positions of the heavy and light polyribosomes, 80S and ribosomal subunits and free RNAs are shown on the graphs. (**B**) RNAs extracted from each fraction shown in (**A**) were fractionated on 15% polyacrylamide gels and analyzed by northern blots using probes specific for 5S rRNA, $tRNA^{Ala}$ and tRF-5D (Ala). Ethidium bromide (EtBr) profiles are also shown. (**C**) Profiles obtained after quantification of the signals from the northern blots (5S rRNA in blue, tRNA in grey and tRF Ala20 in orange) performed with samples not treated with puromycin. Due to saturation of the free RNA fractions with the alanine probe, quantification was done only for fractions 1 to 11. An arbitrary value of 10 was given to the highest value obtained for each curve. Note that the intensity of the signals is not comparable from one curve to the other. In (**D**) a similar experiment was performed without adding any tRF to determine *in vivo* association of tRF-5D (Ala) with polyribosomes. The RNA fragments corresponding to the signals a and b are indicated by arrows. In (**E**) the profiles obtained after northern blots quantification with 5S rRNA probes (in blue) are compared to those obtained for a or b (in orange).

Figure 6. Representative examples of modulation of translation by tRFs

(**a**) Inhibition of translation by human l-tRF (Ala), via a G-quadruplex structure (Gq), by displacement of eIF4G/A and interaction with YB-1. (**b**) In human cells and this work in Arabidopsis shows that s-tRFs can associate with active polyribosomes. The two residues G18 and G19 (GG) are essential. Also, an interaction between the MultiSynthetases Complex (MSC) and tRFs has been characterized in humans. Note that in human cells, while global repression of translation has been observed [19], the protein synthesis of a specific set of mRNAs (coding for ribosomal and RNA-binding proteins) is, by contrast, stimulated [19]. (**c**) In the archaea *H. volcanii*, s-tRFs bind to the small ribosomal subunit and compete with mRNA for ribosome binding. (**d**) In the yeast *S. cerevisiae*, l-tRFs directly interact with the small ribosomal subunit and with aminoacyl-tRNA synthetases associated with ribosomes, thus impairing tRNA aminoacylation. (**e**) The $tRNA^{Thr}$ 3' half (depicted in green) stimulates protein synthesis in the protozoan *T. brucei*, by interacting with ribosomes.

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Lalande et al, Arabidopsis tRNA-derived fragments as potential modulators of translation

Response to reviewer 2

The authors have addressed most of my comments and suggestions. The revised manuscript with additional data is now improved. But I do still have unresolved concerns listed below.

Major points

1. In Supp. Fig. 3, the oligonucleotide Ala16 mentioned in the legend is missing. Amounts of each RNA used in the blotting should be provided in the figure legend. In the blot for total RNA, the signal for tRF20 appears to be much weaker than that of tRF20. The quantification data below the blot however do not seem to translate the signals in the blot.

The reviewer is right. In the Supp Fig.3, no Ala16 was loaded on the blot. “Ala 16” has been deleted from the legend. The amounts of each RNA used in the experiment are now provided in the figure legend. I imagine that the reviewer is comparing the signals obtained for Ala33 and Ala20 in the total RNA sample. We agree with the reviewer that the Ala20 signal is weaker than the signal observed for Ala33. However, when quantification has been performed in the linear part, the contrary was obtained. This is likely because the probe is better hybridizing to Ala33 than to Ala20 (e.g. when comparing hybridization signals obtained with 50 fmol of oligonucleotides, the signal is much stronger for Ala33 than for Ala20). In our opinion, this experiment only gives a rough estimation of tRFs in a total RNA sample.

Minor points

1. In response to the minor point #3, the authors mentioned that "This is now mentioned in the --- , page 13". This critical description is missing in the MM section.

The information is now added in the paragraph “Miscellaneous” in the MM section, page 14

2. Consistency in writing concentration and volume terms etc. needs to be maintained (e.g., page 13 and many places in the MM section, 2M vs. 200 mM, ul vs uL, rpm vs. x g).

Writing of concentrations, volumes and centrifugation speeds have been checked for homogeneity.

3. Style in the reference is still not consistent. Too many errors!

Style errors have been corrected.

4. Consistency in P values in all figure legends: e.g. *** P<0.001 vs. **** P<0.001

Consistency in P values has been given.

Figure 1

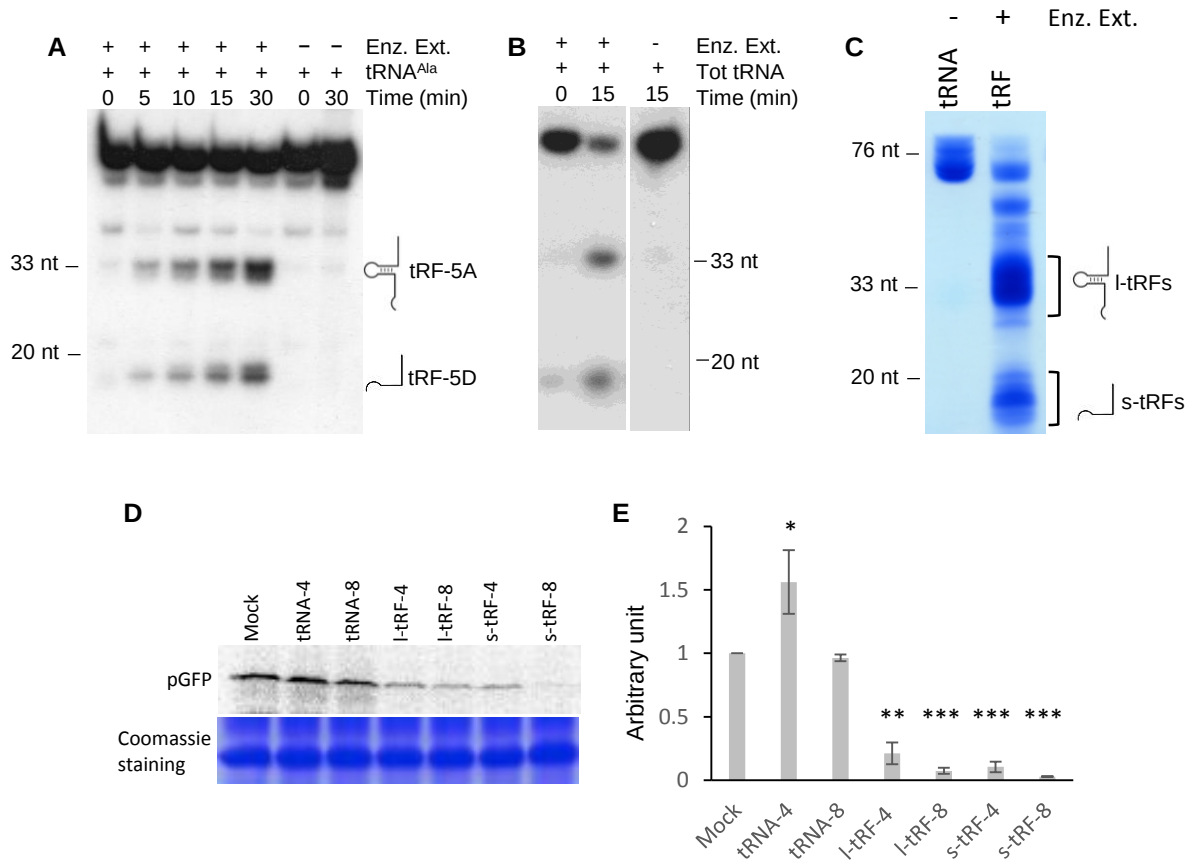
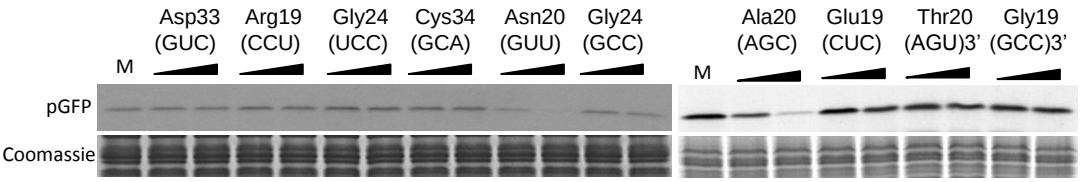

A
Enz. Ext.
tRNA^Ala
Time (min)
0 5 10 15 30 0 30
33 nt
20 nt
tRF-5A
tRF-5D
B
Enz. Ext.
Tot tRNA
Time (min)
0 15 15
33 nt
20 nt
C
- + Enz. Ext.
tRNA
tRF
76 nt
33 nt
20 nt
l-tRFs
s-tRFs
D
Mock
tRNA-4
tRNA-8
l-tRF-4
l-tRF-8
s-tRF-4
s-tRF-8
pGFP
Coomassie staining
E
Arbitrary unit
2
1.5
1
0.5
0
*
** *** *** ***
Mock
tRNA-4
tRNA-8
l-tRF-4
l-tRF-8
s-tRF-4
s-tRF-8

Figure 2

A

	tRFs									
AA	Asp	Arg	Gly	Cys	Asn	Gly	Ala	Glu	Thr	Gly
Anticodon	GUC	CCU	UCC	GCA	GUU	GCC	AGC	CUC	AGU	GCC
Size	33	19	24	34	20	24	20	19	20	19
Type	5A	5D	5D	5A	5D	5D	5D	5D	3T	3T
AGO1	+++	+++	+++	-	-	-	-	-	-	-

B



C

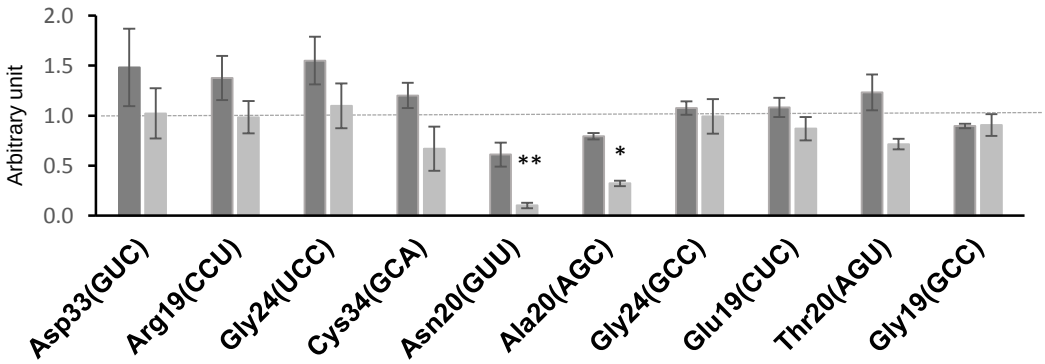


Figure 3

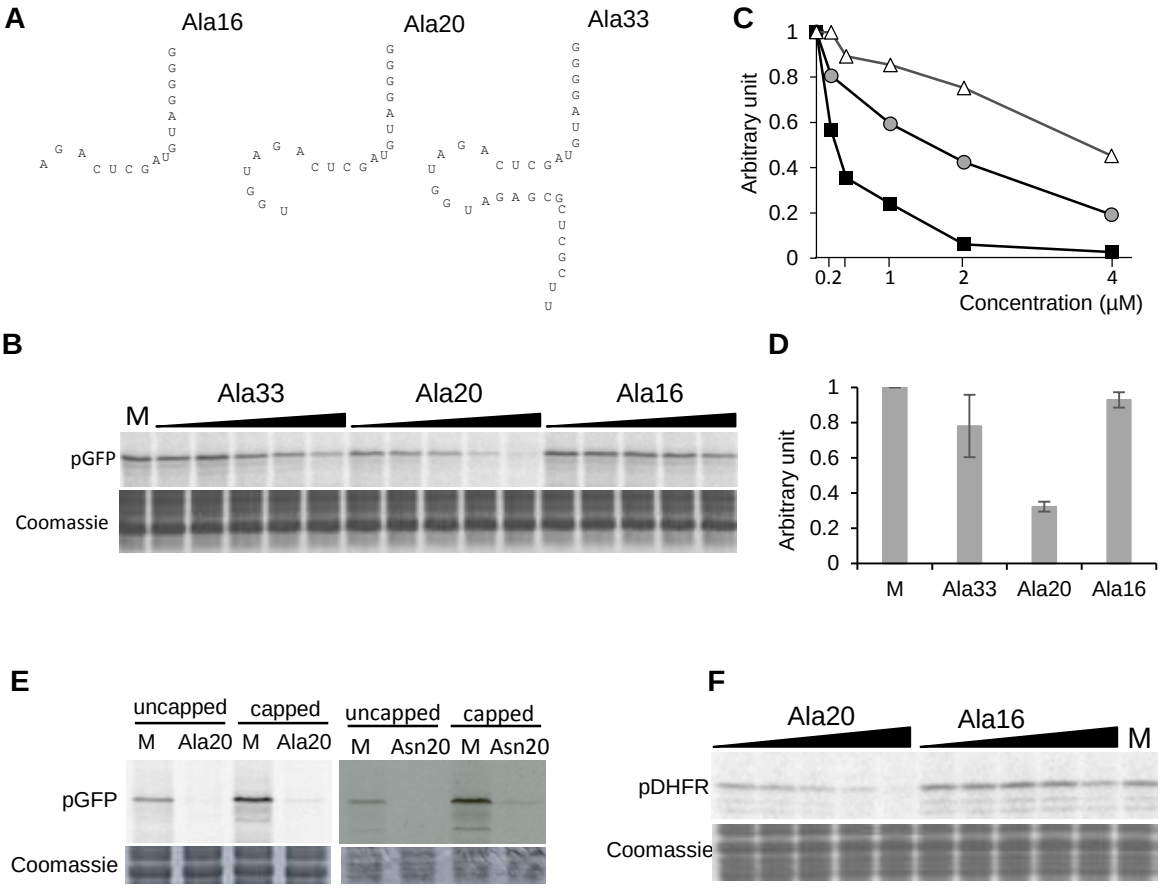

A
Ala16
Ala20
Ala33
C
Arbitrary unit
Concentration (μM)
B
M
Ala33
Ala20
Ala16
pGFP
Coomassie
D
Arbitrary unit
M
Ala33
Ala20
Ala16
E
uncapped
capped
M
Ala20
Asn20
pGFP
Coomassie
F
Ala20
Ala16
M
pDHFR
Coomassie

Figure 4

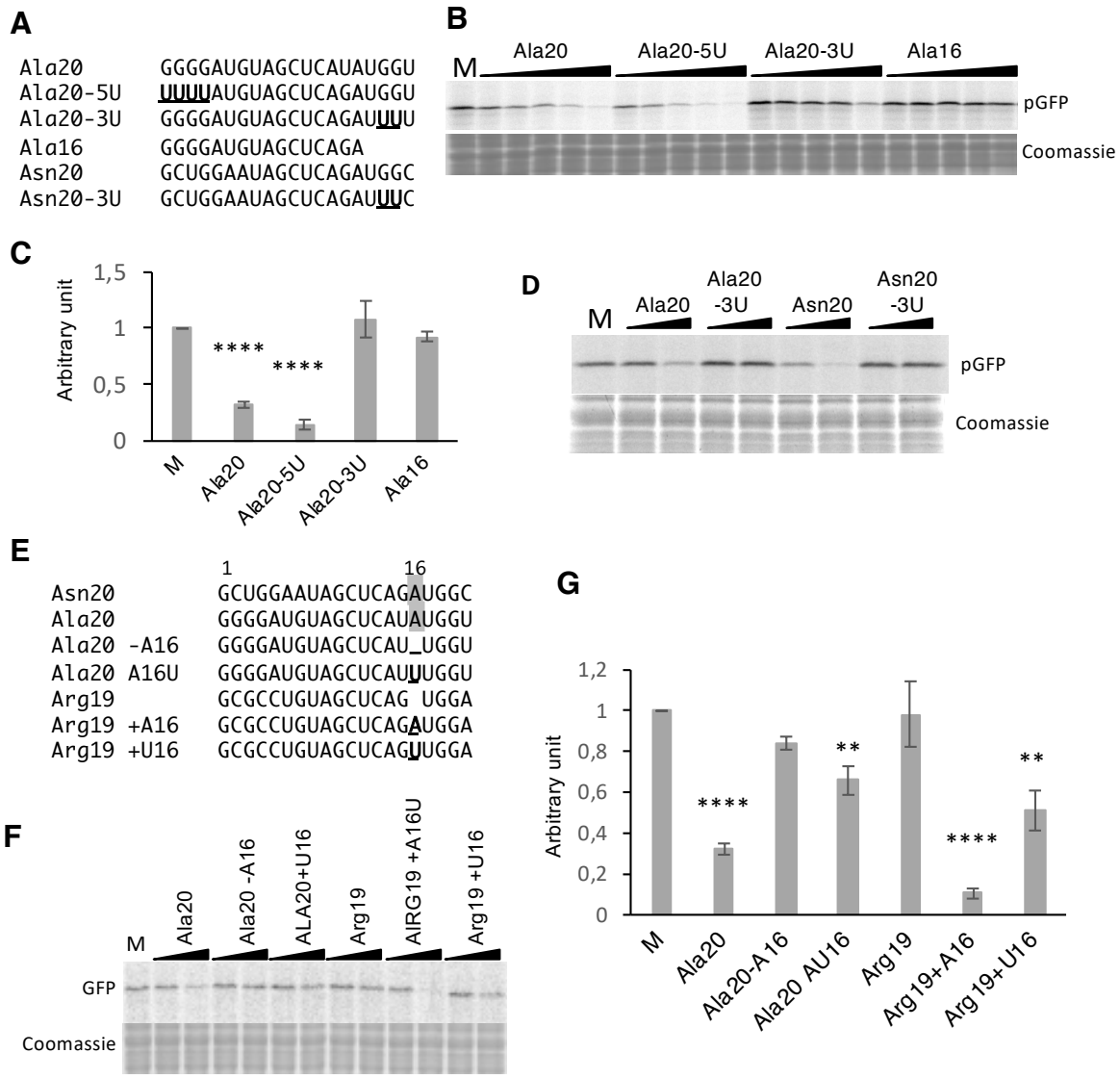

A
Ala20 GGGGAUGUAGCUCAUAUGGU
Ala20-5U UUUUAUGUAGCUCAGAUGGU
Ala20-3U GGGGAUGUAGCUCAGAUUUU
Ala16 GGGGAUGUAGCUCAGA
Asn20 GCUGGAAUAGCUCAGAUGGC
Asn20-3U GCUGGAAUAGCUCAGAUUUC
B
M
Ala20
Ala20-5U
Ala20-3U
Ala16
pGFP
Coomassie
C
Arbitrary unit
1,5
1
0,5
0
****
****
M
Ala20
Ala20-5U
Ala20-3U
Ala16
D
M
Ala20
Ala20 -3U
Asn20
Asn20 -3U
pGFP
Coomassie
E
1
16
Asn20 GCUGGAAUAGCUCAGAUGGC
Ala20 GGGGAUGUAGCUCAUAUGGU
Ala20 -A16 GGGGAUGUAGCUCAU_UGGU
Ala20 A16U GGGGAUGUAGCUCAUUUGGU
Arg19 GCGCCUGUAGCUCAG UGGA
Arg19 +A16 GCGCCUGUAGCUCAGAUGGA
Arg19 +U16 GCGCCUGUAGCUCAGUUGGA
F
M
Ala20
Ala20 -A16
ALA20+U16
Arg19
AIRG19 +A16U
Arg19 +U16
GFP
Coomassie
G
Arbitrary unit
1,2
1
0,8
0,6
0,4
0,2
0
****
**
****
**
M
Ala20
Ala20-A16
Ala20 AU16
Arg19
Arg19+A16
Arg19+U16

Figure 5

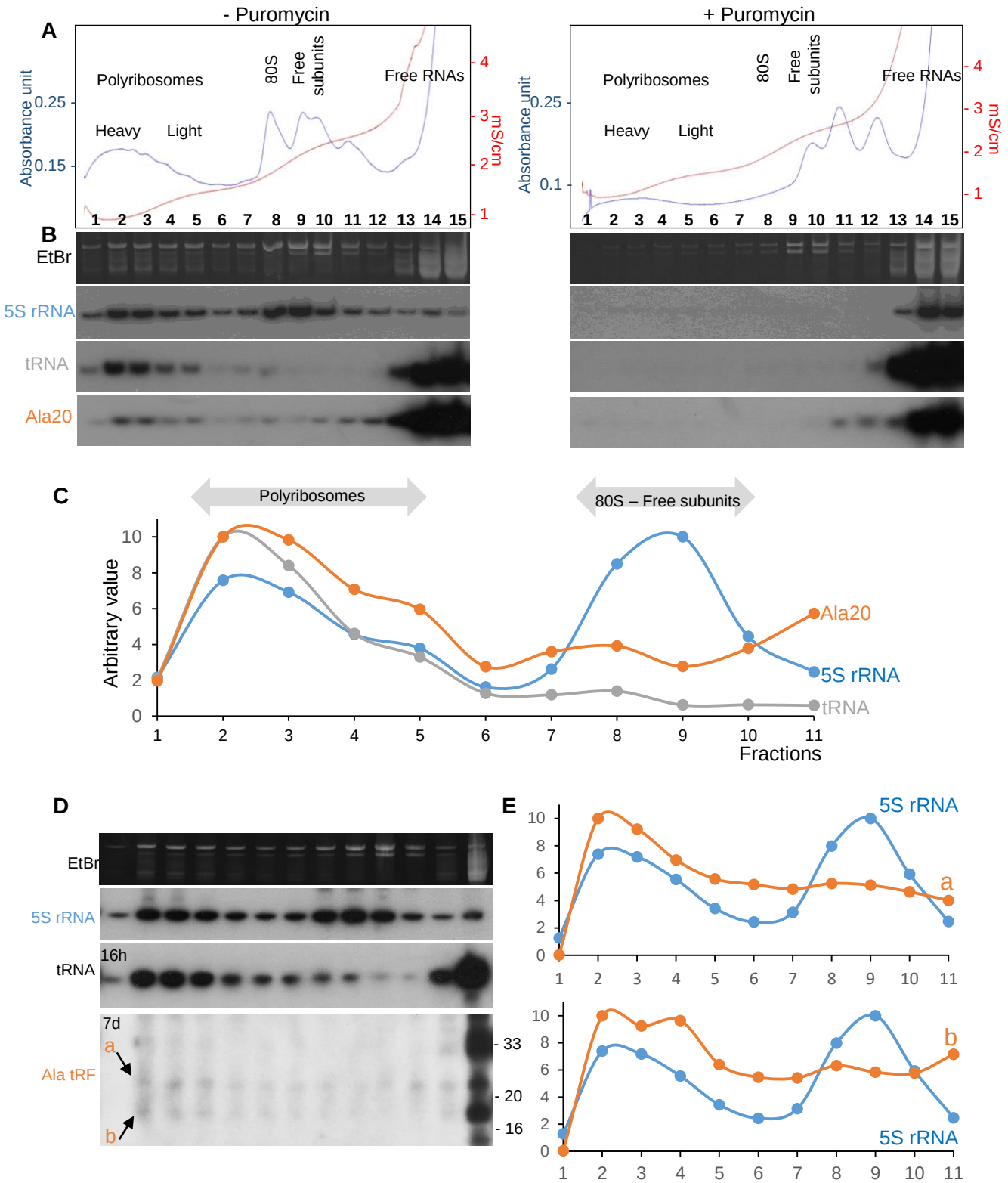
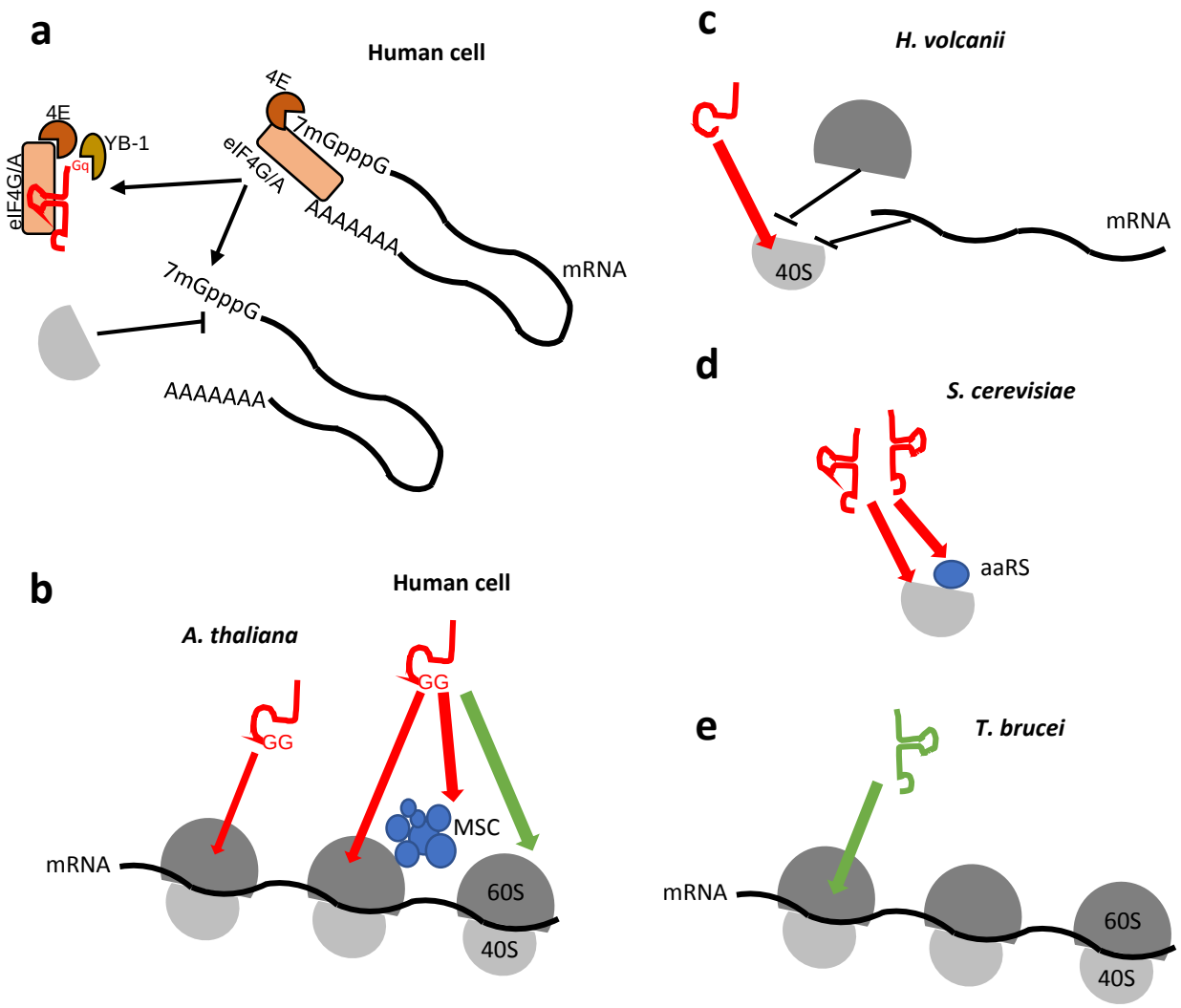
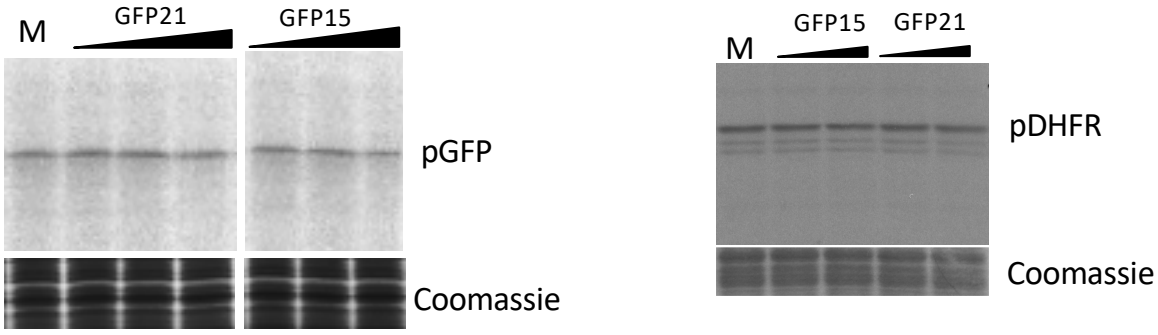

A
- Puromycin
+ Puromycin
Absorbance unit
mS/cm
Polyribosomes
Heavy
Light
80S
Free subunits
Free RNAs
B
EtBr
5S rRNA
tRNA
Ala20
C
Polyribosomes
80S – Free subunits
Arbitrary value
Fractions
Ala20
5S rRNA
tRNA
D
EtBr
5S rRNA
tRNA
16h
7d
Ala tRF
a
b
33
20
16
E
5S rRNA
a
b

Figure 6


a
Human cell
4E
YB-1
eIF4G/A
Gq
4E
7mGpppG
eIF4G/A
AAAAAAA
mRNA
7mGpppG
AAAAAAA
c
H. volcanii
mRNA
40S
d
S. cerevisiae
aaRS
b
Human cell
A. thaliana
GG
GG
MSC
mRNA
60S
40S
e
T. brucei
mRNA
60S
40S

Supplemental Figure 1



Supplemental Figure 1: Addition of GFP15 or GFP21 oligoribonucleotides have no significant effect on the *in vitro* synthesis of pGFP or pDHFR proteins. Experiments were performed as described in Figure 2B. Concentration range: 0.2, 0.4 and 1 μM for pGFP; 0.2 and 1 μM for pDHFR. Coomassie blue stainings of the gels are shown as loading controls. M: Mock experiment.

Supplemental Figure 2

A

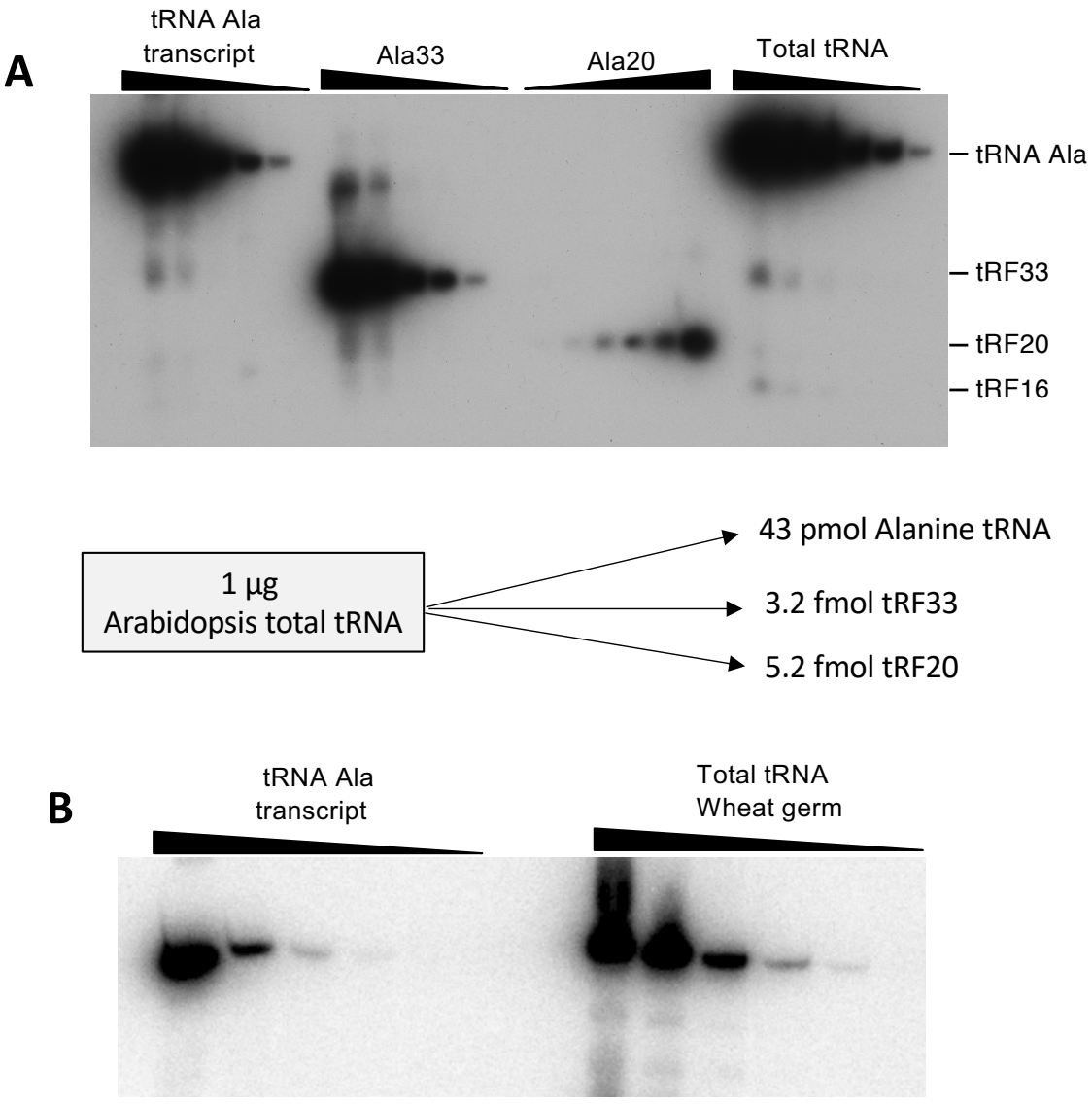
tRFs20	tRNAAla sequences	copies number
tRF-5D-Ala1	GGGGATGTAGCTCAGATGGTAGAGCGCTCGCTT**AGC**ATGCGAGAGGCACGGGGATCGATACCCCGCATCTCCACCA	12
tRF-5D-Ala1	GGGGATGTAGCTCAGATGGTAGAGCGCTCGCTT**AGC**ATGCGAGAGGTACGGGGATCGATACCCCGCATCTCCACCA	3
tRF-5D-Ala2	GGGGATGTAGCTCAAATGGTAGAGCGCTCGCTT**AGC**ATGCGAGAGGTACGGGGATCGATACCCCGCATCTCCACCA	1
tRF-5D-Ala2	GGGGATGTAGCTCAAATGGTAGAGCGCTCGCTT**TGC**ATGCGAGAGGCACGGGGTTCGATCCCCCGCATCTCCACCA	9
tRF-5D-Ala3	GGGGATGTAGCTCATATGGTAGAGCGCTCGCTT**TGC**ATGCGAGAGGCACAGGGTTCGATTCCCTGCATCTCCACCA	1
tRF-5D-Ala3	GGGGATGTAGCTCATATGGTAGAGCGCTCGCTT**CGC**ATGCGAGAGGCACGGGGTTCGATTCCCCGCATCTCCACCA	1
tRF-5D-Ala4	GGGGGTGTAGCTCATATGGTAGAGCGCTCGCTT**CGC**ATGCGAGAGGCACGGGGTTCGATTCCCCGCACCTCCACCA	5
tRF-5D-Ala4	GGGGGTGTAGCTCATATGGTAGAGCGCTCGTTT**CGC**ATGCGAGAGGCACGGGGTTCGATTCCCCGCACCTCCACCA	1

B

tRFs20	tRNAAsn sequences	copies number
tRF-5D-Asn1	GCTGGAATAGCTCAGTTGGTT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCGGAGGTTCGACCCCTCCTTCTAGCGCCA	6
tRF-5D-Asn1	GCTGGAATAGCTCAGTTGGTT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCGGAGGTTCGATCCCTCCTTCTAGCGCCA	1
tRF-5D-Asn2	GCTGGAATAGCTCAGCTGGTT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCGGAGGTTCGAACCCTCCTTCTAGCGCCA	1
tRF-5D-Asn3	GCTGGAGTAGCTCAGTTGGTT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCAGAGGTTCGACCCCTCTCTCTAGCGCCA	4
tRF-5D-Asn3	GCTGGAGTAGCTCAGTTGGTT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCAGAGGTTCGACCCCTTTCTCTAGCGCCA	1
tRF-5D-Asn4	GCTGGAATAGCTCAGTAGGTTtAGAGCGTGTGGCT**GTT**AACCTCAAGGTCGGAGGTTCGACCCCTCCTTTCAGCGCCA	1
tRF-5D-Asn5	GCTGGAATAGCTCAGATGGCT.AGAGCGTGTGGCT**GTT**AACCACAAGGTCGGAGGTTCAAGCCCTCCTTCTAGCGCCA	1

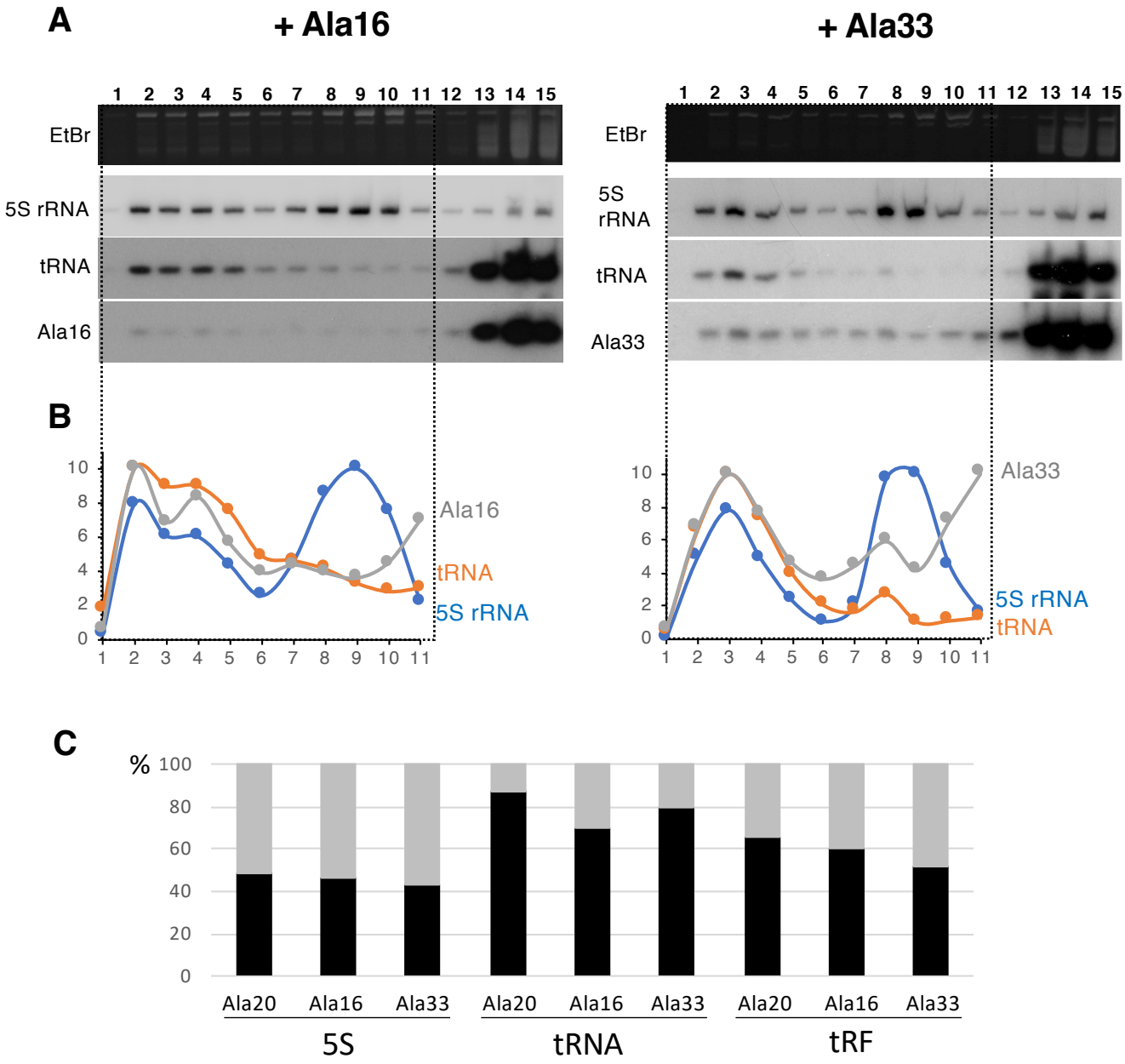
Supplemental Figure 2: Alignment of Arabidopsis tRNAIa and tRNAAsn unique sequences. The number of copies of each sequence is indicated. The tRFs of 20 nt (tRFs20) generated from these tRNAs and found in the small non coding RNA libraries are underlined. Anticodons are in bold. Nucleotide differences compared to the sequence of reference (i.e. the sequence in blue of the Ala20 and Asn20, we synthesized) are in red.

Supplemental Figure 3



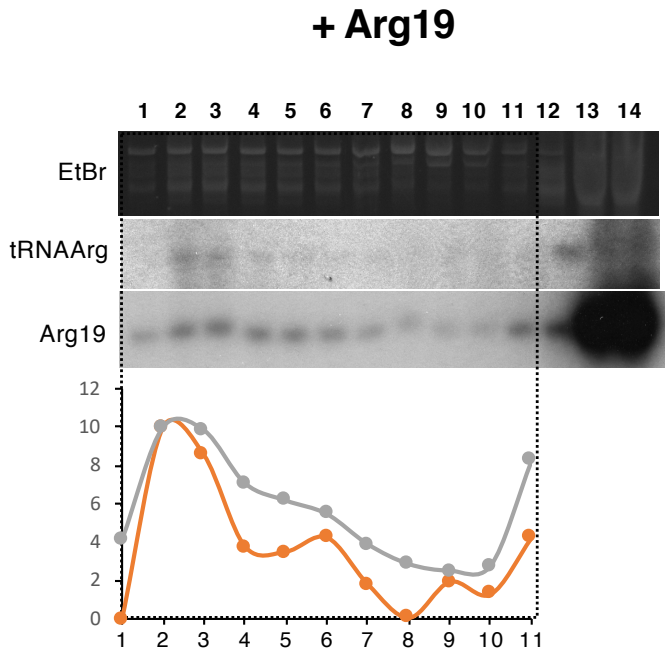
Supplemental Figure 3: (**A**) Exemple of quantification of tRFAla 20 and 33 and Alanine tRNA in an Arabidopsis total tRNA extract. Known amount of synthetic oligoribonucleotides Ala20 (10, 25, 50, 75, 100 and 250 fmol) and Ala33 (10, 50, 100, 500 and 1000 fmol) and of alanine tRNA transcript (1, 5, 10, 50 and 100 pmol) were used to obtain standard ranges and to quantify tRFs Ala and tRNA alanine in an arabidopsis total RNA fraction (10, 50, 100, 500 and 1000 ng). One example of the blot is presented here but several times of exposure were required to use non-saturated signals for the same blot. The scheme summarizes the quantification. (**B**) Example of northern blot experiment allowing the quantification of the Alanine tRNA in the TnT wheat germ extract using alanine tRNA transcript as a standard.

Supplemental Figure 4



Supplemental Figure 4: Binding experiments performed with Ala16 and Ala33 on Arabidopsis seedlings, as described for Ala20 in Figure 5A-C. **A**) After polysomal fractionation on a sucrose gradient, RNAs were extracted from each fraction and analyzed by northern blots using probes specific for 5S rRNA, $tRNA^{Ala}$ and tRF-5D (Ala). Ethidium profiles (EtBr) are also shown. **B**) Curves obtained after quantification of the signals from the northern blots (5S rRNA in blue, tRNA in orange and tRF Ala16 or Ala33 in grey). Due to saturation of the free RNA fractions with the alanine probe, quantification was done only for fractions 1 to 11. An arbitrary value of 10 was given to the highest value obtained for each curve. Note that the intensity of the signals is not comparable from one curve to the other. **C**) Global quantification of the signals obtained in polyribosomal fractions (fractions 2 to 5; black) and in 80S-free ribosomal subunit fractions (fractions 8 to 10; grey) from the experiments shown in Figure 5B (Ala20) and in Supplemental Figure 3A (Ala16 and Ala33).

Supplemental Figure 5



Supplemental Figure 5: Binding experiments performed with Arg19 on Arabidopsis seedlings, as described for Ala20 in Figure 5A-C. After polysomal fractionation on a sucrose gradient, RNAs were extracted from each fraction and analyzed by northern blots using a probe specific for, $tRNA^{Arg(CCU)}$ and tRF-5D (Arg). Ethidium profile (EtBr) profile is also shown. The curves obtained after quantification of the signals from the northern blots are presented (tRNA in grey and tRF in orange).

Table S1: Oligodeoxynucleotide and oligoribonucleotide sequences used in this work

Oligodeoxynucleotides used for Northern blots

Names	Sequences (5' to 3')
Ala	ACCATCTGAGCTACATCCCC
5S rRNA	GCATTAGTGCTGGTATGATCGCATCC
Arg	ACCACTGAGCTACAGGCGC

Oligoribonucleotides used for translation inhibition assays

Names	Sequences (5' to 3')	Description	Size
Ala16	GGGGAUGUAGCUCAGA	tRF-5D Ala(AGC)	16 nt
Ala20	GGGGAUGUAGCUCAGAUGGU	tRF-5D Ala(AGC)	20 nt
Ala33	GGGGAUGUAGCUCAGAUGGUAGAGCGCUCGCUU	tRF-5D Ala(AGC)	33 nt
Ala20-5U	UUUUAUGUAGCUCAGAUGGU	G to U mutations at the 5' extremity of tRF-5D Ala(AGC)	20 nt
Ala20-3U	GGGGAUGUAGCUCAGAUUUU	G to U mutations at the 3' extremity of tRF-5D Ala(AGC)	20 nt
Ala20 A16U	GGGGAUGUAGCUCAUUUGGU	A to U switch at position 16 of tRF-5D Ala(AGC)	20 nt
Ala20 -A16	GGGGAUGUAGCUCAUUGGU	Deletion of A16 of tRF-5D Ala(AGC)	19 nt
Arg19	GCGCCUGUAGCUCAGUGGA	tRF-5D Arg(CCU)	19 nt
Arg19 +A16	GCGCCUGUAGCUCAGAUGGA	Addition of A at position 16 of tRF-5D Arg(CCU)	20 nt
Arg19 +U16	GCGCCUGUAGCUCAGUUGGA	Addition of U at position 16 of tRF-5D Arg(CCU)	20 nt
Asn20	GCUGGAAUAGCUCAGAUGGC	tRF-5D Asn(GUU)	20 nt
Asn20-3U	GCUGGAAUAGCUCAGAUUUC	G to U mutations at the 3' extremity of tRF-5D Asn(GUU	20 nt
Gly(UCC)24	GCGUCUGUAGUCCAACGGUUAGGA	tRF-5D Gly(UCC)	24 nt
Gly(GCC)24	GCACCAGUGGUCUAGUGGUAGAAU	tRF-5D Gly(GCC)	24 nt
Glu(CUC)19	UCCGUCGUAGUCUAGCUGG	tRF-5D Glu(CUC)	19 nt
Asp(GUC)33	GUCGUUGUAGUAUAGUGGUAAGUAUUCCCGCCU	tRF-5D Asp(GUC)	33 nt
Cys(GCA)34	GGGCCCAUAGCUCAGUGGUAGAGCAUUCGACUGC	tRF-5D Cys(GCA)	34 nt
Gly(GCC)19-3'	AUUCCCGGCUGGUGCACCA	tRF-3T Gly(GCC)	19 nt
Thr(AGU)20-3'	AACUCUCAACGAAAGCACCA	tRF-3T Trh(AGU)	20 nt
GFP21	GUAAACGGCCACAAGUUCAGC	random GFP sequence	21 nt
GFP15	GUAAACGGCCACAAG	random GFP sequence	15 nt