

# The nuclear and organellar tRNA-derived RNA fragment population in *Arabidopsis thaliana* is highly dynamic

Valérie Cognat<sup>1,†</sup>, Geoffrey Morelle<sup>1,2,†</sup>, Cyrille Megel<sup>1</sup>, Stéphanie Lalande<sup>1</sup>, Jean Molinier<sup>1</sup>, Timothée Vincent<sup>1</sup>, Ian Small<sup>2</sup>, Anne-Marie Duchêne<sup>1</sup> and Laurence Maréchal-Drouard<sup>1,\*</sup>

<sup>1</sup>Institut de biologie moléculaire des plantes, UPR 2357 CNRS, associated with Strasbourg University, 12 rue du Général Zimmer 67084 Strasbourg cedex, France and <sup>2</sup>Australian Research Council Centre of Excellence in Plant Energy Biology, The University of Western Australia, Crawley WA6009, Australia

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## ABSTRACT

**In the expanding repertoire of small noncoding RNAs (ncRNAs), tRNA-derived RNA fragments (tRFs) have been identified in all domains of life. Their existence in plants has been already proven but no detailed analysis has been performed. Here, short tRFs of 19–26 nucleotides were retrieved from *Arabidopsis thaliana* small RNA libraries obtained from various tissues, plants submitted to abiotic stress or fractions immunoprecipitated with ARGONAUTE 1 (AGO1). Large differences in the tRF populations of each extract were observed. Depending on the tRNA, either tRF-5D (due to a cleavage in the D region) or tRF-3T (via a cleavage in the T region) were found and hot spots of tRNA cleavages have been identified. Interestingly, up to 25% of the tRFs originate from plastid tRNAs and we provide evidence that mitochondrial tRNAs can also be a source of tRFs. Very specific tRF-5D deriving not only from nucleus-encoded but also from plastid-encoded tRNAs are strongly enriched in AGO1 immunoprecipitates. We demonstrate that the organellar tRFs are not found within chloroplasts or mitochondria but rather accumulate outside the organelles. These observations suggest that some organellar tRFs could play regulatory functions within the plant cell and may be part of a signaling pathway.**

## INTRODUCTION

Small noncoding RNAs (sncRNAs) implicated in the regulation of gene expression have been identified in evolutionarily divergent organisms (e.g. (1)). Small-interfering RNAs, microRNAs (miRNAs) and piwi-interacting RNAs

are the most well known. Many more classes of sncRNAs are now emerging. Among them, tRNA-derived RNA fragments (tRFs) (Supplementary Figure S1) have been found in eukaryotic organisms belonging to all domains of life such as fungi, protozoans, plants and metazoans (2–6). Long and short tRFs deriving from mature tRNAs have been found and a general nomenclature recently proposed is used here (5). The long (30–35 nt) tRFs are created by tRNA cleavage in the region of the anticodon and correspond to the 5' (tRF-5A) or 3' (tRF-3A) part of the mature tRNA. The two classes of short tRFs (15–28 nt) originate either from the 5' extremity of mature tRNAs after cleavage in the D region (tRF-5D) or from the 3' end of mature tRNAs after cleavage in the T region (tRF-3T). Most tRFs described so far originate from nucleus-encoded tRNAs, and only a few tRFs deriving from organelle-encoded tRNAs have been reported in the literature (e.g. (7–10)). Furthermore, tRFs deriving from the 3' trailer of nucleus-encoded tRNA precursors after cleavage by RNase Z have been found in mammals (11–13). These tRFs (called pre-tRF-3U according to (5)) usually end at the level of the short stretch of U residues where polymerase III is released.

It is suspected that tRFs are not simply tRNA degradation products due for recycling of key nutrients such as phosphate or nitrogen upon starvation. Several tRFs were recently shown to be implicated in important regulatory and biological processes. For instance, tRFs are implicated in the inhibition of protein synthesis in Archaeobacteria and in human cells (14–16). Other tRFs were found to be associated with Argonaute (AGO) complexes in *Schizosaccharomyces pombe*, in human cells and in plants, suggesting their implication in the regulation of gene expression (12,17–19). In the protozoan *Tetrahymena thermophila*, the interaction of tRF-3T with Twi12, a piwi Argonaute protein, is essential for the nuclear RNA decay pathway (20). A tRF-3T involved in the priming of a human viral reverse

\*To whom correspondence should be addressed. Tel: +333 88 41 72 40; Fax: +333 67 15 53 98; Email: laurence.drouard@ibmp-cnrs.unistra.fr

†These authors contributed equally to this work as the first authors.

transcriptase may be important for viral infection (21). The implication of tRFs in the suppression of the development of breast cancer metastasis (22) and in the regulation of the expression of retroelements (23) has also been described. Finally, Chen *et al.* recently reported that tRFs may represent a new type of paternal epigenetic factor (24).

Thus, evidence that cutting tRNAs into pieces generates tRFs with important biological functions is increasing. However, in plants, little data is available. The first report on the existence of plant tRFs is from 2008 in *Arabidopsis* cells submitted to an oxidative stress (7). In 2009, tRFs were found in the phloem of pumpkin (25) and in the same year, Hsieh *et al.* showed the overaccumulation of very specific tRFs (e.g. tRF-5D from tRNA<sup>Asp</sup>(GTC) or tRNA<sup>Gly</sup>(TCC)) upon phosphate starvation in *Arabidopsis* roots (26). Genome-wide analysis of sncRNAs from rice callus allowed the identification of several tRF-5D and tRF-3T (27). In chinese cabbage, tRFs originating from plastid tRNAs and in wheat, tRFs originating from nuclear tRNAs were shown to be created under heat stress (8,28). In barley, differential expression of sncRNAs including tRFs originating from nucleus- or chloroplast-encoded tRNAs was also observed upon phosphate deficiency (9). In 2013, several *Arabidopsis* tRFs were found to be associated with various AGO proteins or to be induced by various stresses (19) but no in depth analysis was performed, in particular concerning organellar tRFs.

The goal of the present work is to provide a thorough analysis of the tRF population present in various *A. thaliana* sncRNA libraries. Our analysis mainly concerns tRFs deriving from mature tRNAs as tRFs produced from tRNA precursors are rare. A special focus is given to plastid tRFs as they represent up to 25% of the total tRF population in leaves. Surprisingly, a few of them are found specifically associated with ARGONAUTE 1 (AGO1). The existence of mitochondrial tRFs is also addressed. We provide evidence that organellar tRFs do not accumulate within chloroplasts and mitochondria, but outside these organelles. Our work allows us to propose a specific set of tRFs to be used as a starting point to answer questions regarding their biogenesis and function in higher plants.

## MATERIALS AND METHODS

### Small ncRNA libraries

Several deep sequencing libraries were retrieved from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). In addition, homemade libraries corresponding to untreated or UV-C treated *A. thaliana* plants were used for deep sncRNA sequencing. For that purpose, *A. thaliana* plants, ecotype Columbia (Col-0), were first germinated *in vitro* on solid GM medium (MS salts (Duchefa), 1% sucrose, 0.8% Agar-agar ultrapure (Merck), pH 5.8) in a culture chamber under a 16-h-light (21°C) and 8-h-dark (19°C) photoperiod for 10 days. Afterward seedlings were transferred in soil (1 plant per pot) and put in a growth chamber (21°C/19°C; 70% humidity) for 2 weeks. For treated samples, plants (12 per replicate) were irradiated with UV-C (254 nm, 3000 J/m<sup>2</sup>) using a Stratalink (Stratagene). Thirty minutes upon UV-C exposure leaves number 3 and 4 were harvested and

rapidly frozen into liquid nitrogen. Leaves number 3 and 4 of un-irradiated control plants were also harvested. Total RNAs were prepared using TRI Reagent (Sigma-Aldrich), following manufacturer's instructions, and sent to the FASTER company (Plan-les-Ouates, Switzerland) for library preparation and Illumina HiSeq 2000 deep sequencing. For AGO1 immunoprecipitation, 3 week-old *in vitro* culture plants were exposed to UV-C (3000 J/m<sup>2</sup>). Untreated (prior UV-C treatment) and UV-C treated plants (15 min upon exposure) were collected. Total soluble proteins were extracted from 0.5 g of seedlings using 3 ml of IP buffer (29). Immunoprecipitation was performed using anti-AGO1 antibody (30). The precipitate was washed 4 times in IP buffer and RNAs were extracted from the immunoprecipitated samples by TRI Reagent (Sigma-Aldrich). Experiment was duplicated and pooled. Total RNA was also prepared from the same samples and was used as input.

The list of small RNA libraries analyzed in this study is presented in Supplementary Table S1. We are aware of several biases in the recovery and sequencing of sncRNA libraries (31). Among them, we can cite the secondary structure of RNAs, their content in modified nucleotides, the enzymatic properties of the endoribonucleases at the origin of the small RNAs impeding the ligation process or the reverse transcriptase activity, the use of various RNA-seq protocols. Consequently, we cannot exclude that a few tRFs were not correctly estimated or have escaped our detection in our various studies. To avoid most of these biases, most comparisons were done between sncRNA libraries performed with identical RNA-seq protocols.

### Bioinformatics analysis

The bioinformatics pipeline used in this work is presented in Supplementary Figure S2. The size of the available sncRNAs in the different libraries was heterogeneous (18–26 nt to 19–28 nt), so we studied tRF with a size between 19 and 26 nt. It is likely that we might be missing very short (<18 nt) or very long (>26 nt) tRFs, but doing so, the comparison between various libraries was possible. In order to retrieve the tRF population of each deep-sequenced sncRNA library, the sequenced reads were filtered through an *A. thaliana* tRNA database comprising the full set of mature tRNAs with the CCA 3' extremity from the three compartments where a genetic information is present (nucleus, chloroplast and mitochondrion). This database was extracted from the global PlantRNA database (32). Reads corresponding to tRF sequences were aligned on full-length tRNA sequences using PatMaN (33). To map the reads on mature tRNA sequences including the CCA 3' extremity is essential as without adding this triplet, more than 90% of the tRFs-3T are lost. In human, adding the CCA sequence inflates the number of false positive tRFs as shown by Telonis *et al.* (34). However, mapping the whole set of tRF sequences outside the tRNA gene sequences on the whole genome of *Arabidopsis* (TAIR10 genome release) shows that the proportion of tRF sequences that can be aligned somewhere else on the genome is very limited without any hot spots of reads. We cannot exclude the fact that small RNA sequences annotated as tRFs are false positives but according to the different analyses we have performed, this

number should be very low. Furthermore, as the same tRF sequence can derive from different *Arabidopsis* tRNA isoacceptors (32), the number of reads was weighted by the number of alignments (i.e. usually the number of tRNA isoacceptors) obtained for each read.

In order to take into account sequence bias due to the presence of modified nucleotides, two mismatches were permitted when mapping the reads sequences on the mature tRNA sequences. As shown on Supplementary Figure S3, the possibility to have two mismatches does not lead to inflate the number of false positive tRFs as this population is negligible. The population of tRFs-5D with one mismatch is also very low. By contrast, most tRFs-3T present one mismatch as compared to the corresponding tRNA sequence. The main reason for this discrepancy is the presence of m1A at position 58, a post-transcriptionally added modification highly present in *Arabidopsis* tRNAs (35) and often subjected to misincorporation by reverse transcriptase (36,37). This result shows that the possibility to permit one mismatch is essential to accurately retrieve the set of tRFs-3T.

The identification of tRFs corresponding to mitochondrial tRNAs is particularly difficult in plants, as three types of tRNAs are found in this organelle. These are the native tRNAs, the chloroplast-like (cp-like) tRNAs and the imported nucleus-encoded tRNAs. The sequences of the cp-like tRNAs are usually identical or mostly identical to true chloroplastic tRNAs. Therefore, it is impossible to differentiate between chloroplast-like mitochondrial tRFs and chloroplastic tRFs. Similarly, the imported nucleus-encoded tRNAs are shared between the cytosol and the mitochondria and their sequences cannot be distinguished (32,38). As the number of tRF reads originating from native mitochondrial tRNAs is low in the sncRNA libraries, we have assumed that the numbers of reads deriving from mitochondrial chloroplast-like and imported tRNAs are also low as compared to chloroplastic and cytosolic tRNAs respectively. Consequently, tRFs whose sequences are identical between chloroplastic and mitochondrial tRNAs were considered as originating from chloroplastic tRNAs and tRFs whose sequences are identical between cytosolic and mitochondrial tRNAs were considered to be from cytosolic nucleus-encoded tRNAs. Hence, when analyzing tRFs originating from mitochondrial tRNAs, only native tRNAs were considered.

To investigate the presence of tRFs produced from the 3' trailer of tRNA precursors after cleavage by RNase Z, a class of tRFs that we called pre-tRFs-3U (5), the sequenced reads with a size between 19 and 26 nt were mapped on the full set of 50 nt long sequences found downstream the 3' theoretical extremity of each mature tRNA obtained after cleavage by RNase Z (see Supplementary Figure S4).

For the homemade libraries, GEO database numbers are indicated in Supplementary Table S1. All excel files generated during this work are accessible upon request.

#### Northern blot analysis of *A. thaliana* total, cytoplasmic, mitochondrial and chloroplast RNA fractions

Mitochondria and chloroplasts were isolated from *A. thaliana* seedlings and from young leaves according to (39) and to (40), respectively. The cytoplasmic fraction corre-

sponds to the supernatant obtained after the first low speed centrifugation when preparing chloroplasts. It is to note that this cytoplasmic fraction contains mitochondria and is contaminated by broken chloroplasts. Total, cytoplasmic, mitochondrial and chloroplast RNA fractions were prepared 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 snc RNAs).

RNAs were separated on 15% (w/v) polyacrylamide gels, electrotransferred onto Hybond-N<sup>+</sup> nylon membranes (Amersham) and hybridized to <sup>32</sup>P radiolabeled oligonucleotide probes at 42°C in perfectHyb<sup>TM</sup>Plus (Sigma-Aldrich). Two washes (10 min) were performed at 42°C in 2xSSC followed by one wash (30 min) at 42°C in 2xSSC, 0.1% sodium dodecyl sulphate.

#### Miscellaneous

The oligonucleotides listed below were used as probes:

Plastidial and cp-like mitochondrial tRNA<sup>His</sup>(GUG), 5' TC CACTTGGCTACATCCGCC 3'.

Plastidial tRNA<sup>Gln</sup>, 5' CCGCTTGGCTACGCC 3'

Plastidial tRNA<sup>Gly</sup>, 5' CCATTCGACTATATCCCG 3'

Plastidial tRNA<sup>Asp</sup>, 5' CCAATTGAACTACAATCCC 3'

Mitochondria-imported nuclear tRNA<sup>Ala</sup>(AGC), 5' ACCA TCTGAGCTACATCCCC 3'

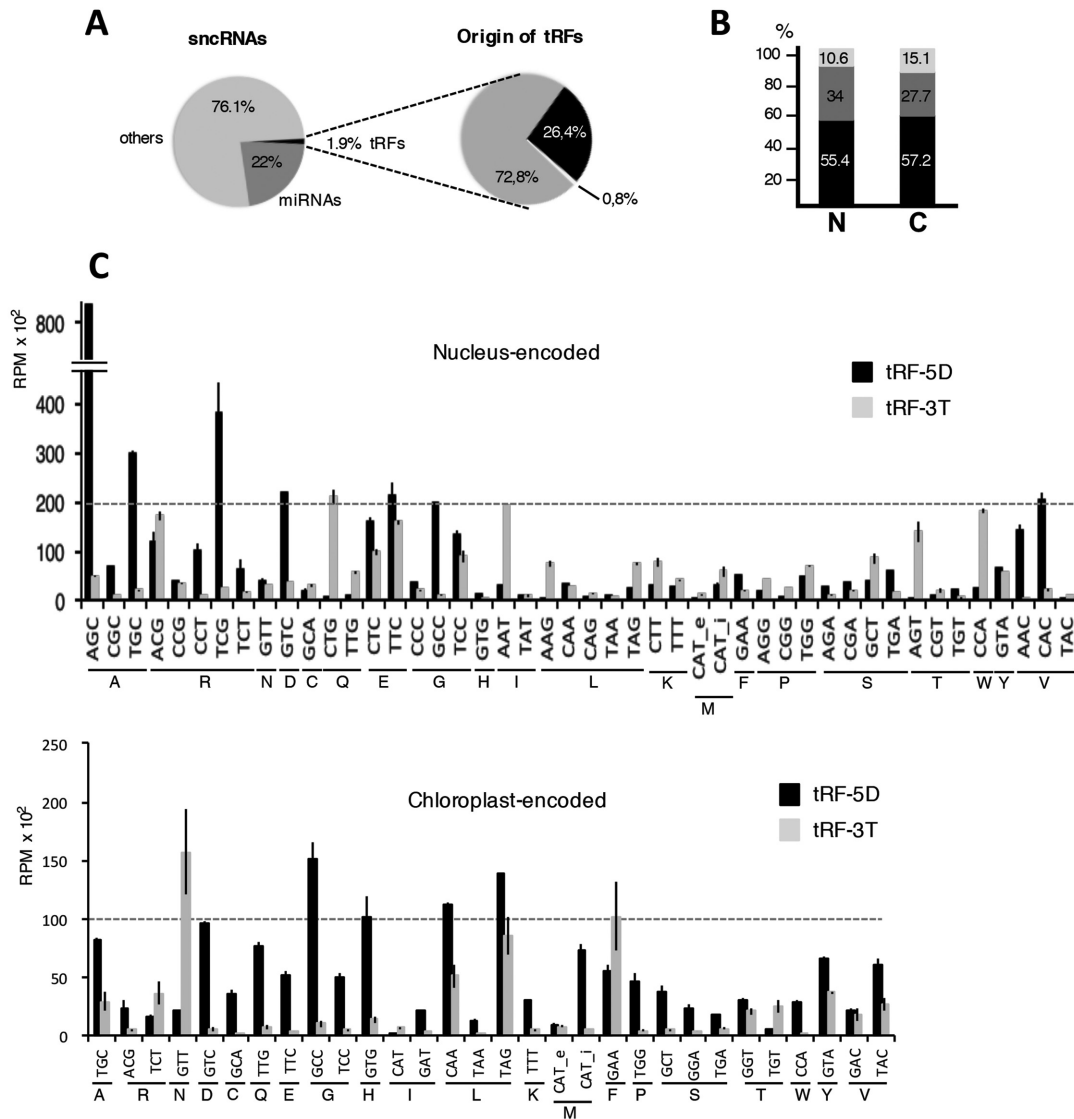
A mix of three oligoribonucleotides was used as a RNA ladder:

GGGGAUGUGCUCUAU (16 nt), GGGGAUGUGCUC AUAUGGU (20 nt), GGGGAUGUGCUCUAUUGG UAGAGCGCUCGCUU (33 nt).

## RESULTS

### One quarter of *Arabidopsis* tRFs originate from organellar tRNAs in leaves

As a first step toward a detailed analysis of plant tRFs, two sncRNA libraries prepared from *A. thaliana* leaves were analyzed. We focused most of our work on short (19 to 26 nt) tRF deriving from mature tRNAs. The two libraries (named L1 and L2, Supplementary Table S1) gave highly reproducible data (Figure 1). About 1.9% of sncRNAs corresponds to tRFs. Among them, only 0.2% of the tRFs (called pre-tRFs-3U) derives from the 3' trailers of tRNA precursors (see Supplementary Figure S4) while 99.8% comes from mature tRNAs. We thus continued our analysis on this later class of tRFs. While three quarters of them derive from nucleus-encoded cytosolic tRNAs (ntRF), around one quarter originate from plastid tRNAs (ptRF) and >1% derive from native mitochondrial tRNAs (mtRF) (Figure 1A). Most tRFs belong to the two major classes of short tRF (tRF-5D and tRF-3T) deriving from mature tRNAs. More than 55% of ntRF and ptRF belong to the tRF-5D class, meaning their sequence starts at the 5' extremity of mature tRNAs. The tRF-3T population (i.e. RNA fragments ending at the CCA extremity of mature tRNAs) represents about 34 and 28% of the total ntRF or ptRF populations



**Figure 1.** Distribution of the tRFs originating from nucleus-encoded and chloroplast-encoded tRNAs present in *Arabidopsis* leaves. (A) On the left, frequency of tRFs (in black) in the total sncRNA population of *Arabidopsis* leaves (ranging from 19 to 26 nt). Percentage of miRNAs (reads mapped on the miRNA genes of the whole *Arabidopsis* genome sequence available on TAIR10) and other sncRNAs are depicted in dark and pale grey, respectively. On the right, frequency of tRFs originating from nucleus-encoded (grey), chloroplast-encoded (black) or mitochondrion-encoded (white) native tRNAs. Percentages are given as a mean value of the data obtained in libraries L1 and L2. (B) Distribution of tRF-5D (in black) and tRF-3T (in dark grey) originating from either nucleus-encoded (N) or chloroplast-encoded (C) tRNAs. The percentage of other tRFs is in pale grey. (C) Histograms showing the abundance of tRF-5D (in black) and tRF-3T (in grey) originating from each tRNA isoacceptor (indicated using their respective anticodon; CAT<sub>e</sub> and CAT<sub>i</sub> are used to differentiate the elongator (e) and initiator (i) tRNA<sup>Met</sup>) expressed from nuclear or plastidial tRNA genes. Frequencies are given in reads per million of tRFs reads (RPM). The frequencies are the mean values of two independent sncRNA libraries and standard deviations are provided. tRF-5D from nuclear (Ala-AGC, Arg-TGC, Arg-TCG, Asp-GTC, Gly-GCC, Val-AAC and Val-CAC) or plastid (Asp-GTC, Gln-TTG, Gly-GCC, His-GTG and Met-CAT<sub>i</sub>) tRNAs are strongly enriched, but not the corresponding tRF-3T. An enrichment in tRF-3T was observed for other nuclear (Gln-CTG, Ile-AAT, Thr-AGT, Trp-CCA) or chloroplastic (Asn-GTT) tRNAs but tRF-5D were not enriched for these tRNAs. For nuclear (Arg-ACG, Glu-TTC, Gly-TCC) or plastid (Leu-TAG, Tyr-GTA, Phe-GAA) tRNAs, rather similar amounts of tRF-5D and tRF-3T are found.

respectively (Figure 1B). The other remaining tRFs (10–15%) represent RNA fragments localized elsewhere along the tRNA molecule.

As shown in Figure 1C, some tRFs are strongly enriched in the L1 and L2 libraries. Among them we find tRF-5D from nuclear or plastid tRNAs. For these tRNAs, generally no tRF-3T are found. In contrast, an enrichment in tRF-3T was observed for other nuclear or chloroplastic

tRNAs while low amounts of tRF-5D are generated from these tRNA species. In a few cases, rather similar amounts of tRF-5D and tRF-3T deriving from the same tRNA sequence are found.

As mentioned above, *Arabidopsis* tRFs are not randomly localized along tRNA molecules (Figure 1B and Supplementary Figure S5). Furthermore, a high proportion of tRFs derive from a limited set of tRNAs. Indeed, the tRF-

5D originating from 12 (out of 46) nucleus-encoded or 10 (out of 30) chloroplast-encoded tRNA isoacceptors represent 76.4% and 63.8% of their respective total tRF-5D population. Similarly, 64.5% and 82% of the tRF-3T population originate from 12 nuclear or 10 plastid tRNAs, respectively (Supplementary Figure S6). Overall, nuclear and plastid tRNAs are not expressed in the same compartments but they share rather similar profiles of tRF populations.

### Specific features of *Arabidopsis* tRFs

Concerning their size, nuclear and plastid tRF-5D of 19 and 20 nt are the most abundant and they represent 42 and 46% of tRF-5D, respectively (Figure 2A; Supplementary Table S2). For tRF-3T, no real size specificity is visible. This is in agreement with the fact that for each tRNA, not all tRF-5D or tRF-3T have the same 3' or 5' termini (Figure 2B). For some tRNAs (e.g. nuclear Ala-AGC, Gln-CTG or plastidial Asn-GTT), the termini are spread quite broadly while for others (e.g. nuclear Arg-TCG, Ile-AAT or plastid Arg-TCT), most termini correspond to a single position. We cannot exclude the existence of exonucleolytic activities able to trim the RNA fragments generated endonucleolytically, which may explain some of the diversity in the positions of the termini.

When the major site of cleavage of each tRNA is positioned on a tRNA structure (Figure 2C), globally hot spots of localization for the 3' extremity of tRF-5D or for the 5' extremity of tRF-3T emerge. These hot spots appear to be similar between nucleus- and chloroplast-encoded tRNAs. To generate tRF-5D, tRNA cleavage occurs predominantly in the D-loop and only moderately in the D-stem. By contrast, tRF-3T are generated though cleavages occurring mainly in the T-stem and much less in the T-loop. Strikingly, these two regions are closely located on the 3D L-shaped tRNA structure suggesting that the same endoribonuclease(s) may have access to both regions and adapt the positioning of the catalytic domain to cleave either in the D- or T- site. The data also show that the *Arabidopsis* endonuclease(s) responsible for tRNA cleavage can act both on single-stranded (D-loop) or double-stranded (T-stem) RNA regions. With the exception of invariant or semi-invariant nucleotides present on mature tRNAs and consequently also found in the sequences of the most abundant tRFs (Supplementary Table S2), these tRFs do not appear to share either extensive sequence homology or common secondary structure (Supplementary Figures S7 and S8).

### The root tRF population quantitatively differs from those of other tissues

To investigate whether the tRF population fluctuates depending on *Arabidopsis* tissue or developmental stage, four sncRNA libraries from roots, leaves, seedlings and flowers (R1, L3, S1, F1, Supplementary Table S1; (19)) were analyzed.

As shown in Figure 3A and B, and Supplementary Figure S9, very few fluctuations in the tRF populations deriving either from nucleus- or plastidial-encoded tRNAs were observed when comparing leaves, flowers and seedlings. Moreover, although the L3 library and the two previously an-

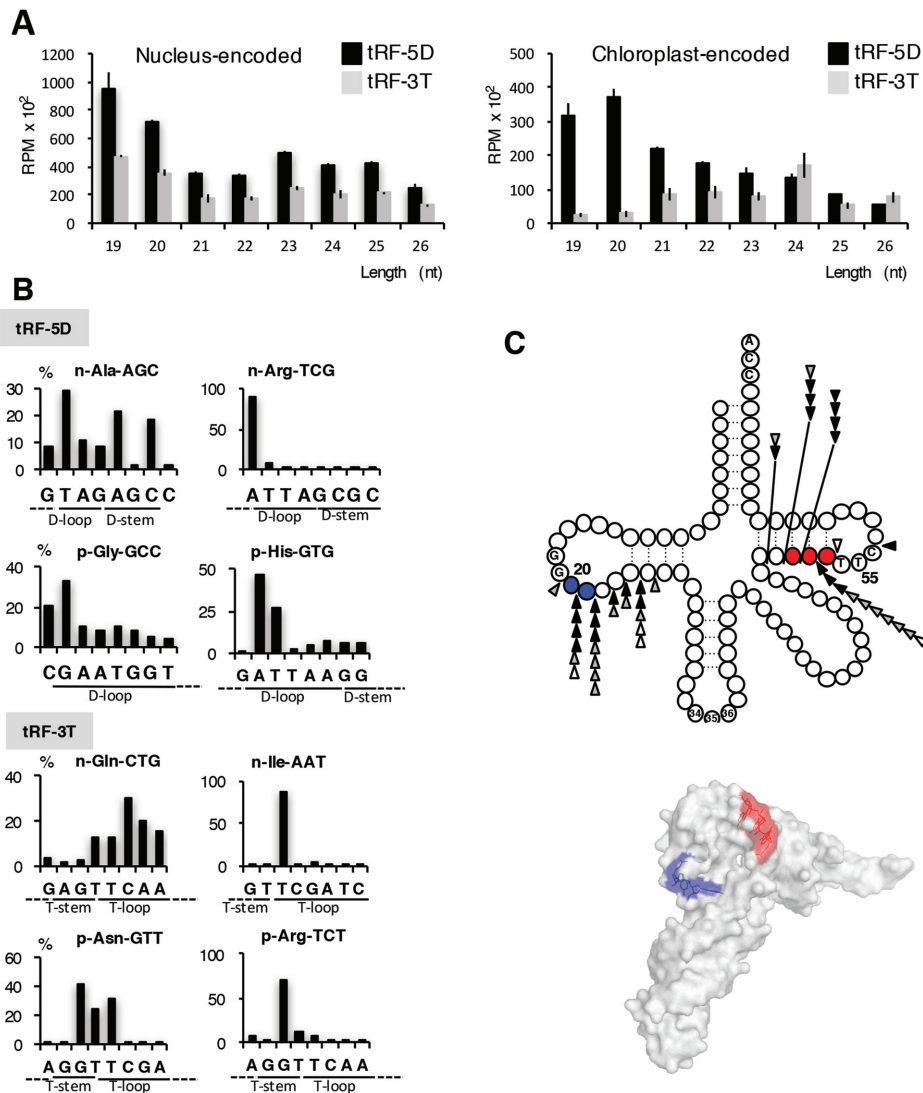
alyzed leaf libraries (L1 and L2) were prepared using neither the same library protocols and sequencing methods nor from *Arabidopsis* leaves grown under the same conditions, a comparison between the three leaf libraries shows rather similar profiles with only slight variations (Supplementary Figure S10). By contrast, more notable differences were found when comparing the three S1, F1, L3 RNA libraries with the root RNA library. While very few ntRF-3T are present in roots as compared to the three other tissues, ntRF-5D deriving from four tRNAs (Arg-ACG, Arg-TCG, Cys-GCA, Gly-TCC) are rather abundant and represent more than 75% of all ntRF-5D. Concerning ptRFs, their proportions in roots are also very low as compared to the three other tissues. This is likely because the amount of plastidial tRNAs is low in *Arabidopsis* roots as compared to green tissues. The only exception is ptRF-5D (His-GTG), which is mostly the only one found in roots. It is, however, worth to mention that the mitochondrial tRNA<sup>His</sup> is a chloroplast-like tRNA (32) and its tRF-5D would be identical to its plastidial counterpart. Thus, the presence in roots of the ptRF (His-GTG) may also be due to the cleavage of the mitochondrial tRNA<sup>His</sup>.

### Are tRFs generated from mitochondrial tRNAs?

Above, we focused the analysis on tRFs originating from either nucleus- or plastid-encoded tRNAs. The situation in mitochondria is more complex (42). First, plant mitochondria contain few 'native' tRNAs expressed from true mitochondrial tRNA genes. In addition, (i) they also possess 'chloroplast-like' tRNAs expressed from plastid genes inserted into the mitochondrial genome and (ii) they also import numerous nucleus-encoded tRNAs to compensate the lack of mitochondrial tRNA genes. Consequently, studying tRFs originating from mitochondrial tRNAs is problematic. The only tRFs we can be confident about are those deriving from true mitochondrial tRNAs. In *Arabidopsis*, they correspond to only 11 amino acids. Another difficulty resides in the low number of reads corresponding to 'native' mitochondrial tRNAs in sncRNA deep sequencing libraries, in agreement with the small proportion of 'native' mitochondrial tRNAs (around 1–2% of tRNAs, personal communication) in a plant total tRNA fraction. Indeed, the mtRF population deriving from native tRNAs fluctuates between 0.7–1.1% of total tRFs in leaves, seedlings and roots to 2.8% in flowers (Figure 3A). It is mainly in flowers that a limited set of mtRF-5D or tRF-3T was significantly found (Figure 3C). In particular, tRF-5D (Gln-TTG, Gly-GCC, Lys-TTT) and tRF-3T (Glu-TTC, Pro-TGG) are the most abundant in this library (Figure 3C and Supplementary Figure S11). Furthermore, the position of the major cleavage site on the corresponding tRNA is similar to what we already observed for nuclear and chloroplastic tRF (Figure 2C, Supplementary Table S2). Altogether, these data suggest that, as for chloroplasts, specific tRFs are also generated from mitochondrial tRNAs in *Arabidopsis*.

### Variation of tRF populations in plants submitted to environmental stresses

Long tRFs, i.e. tRFs of about 30–35 nt generated after cleavage in the region of the anticodon (mainly tRF-5A)



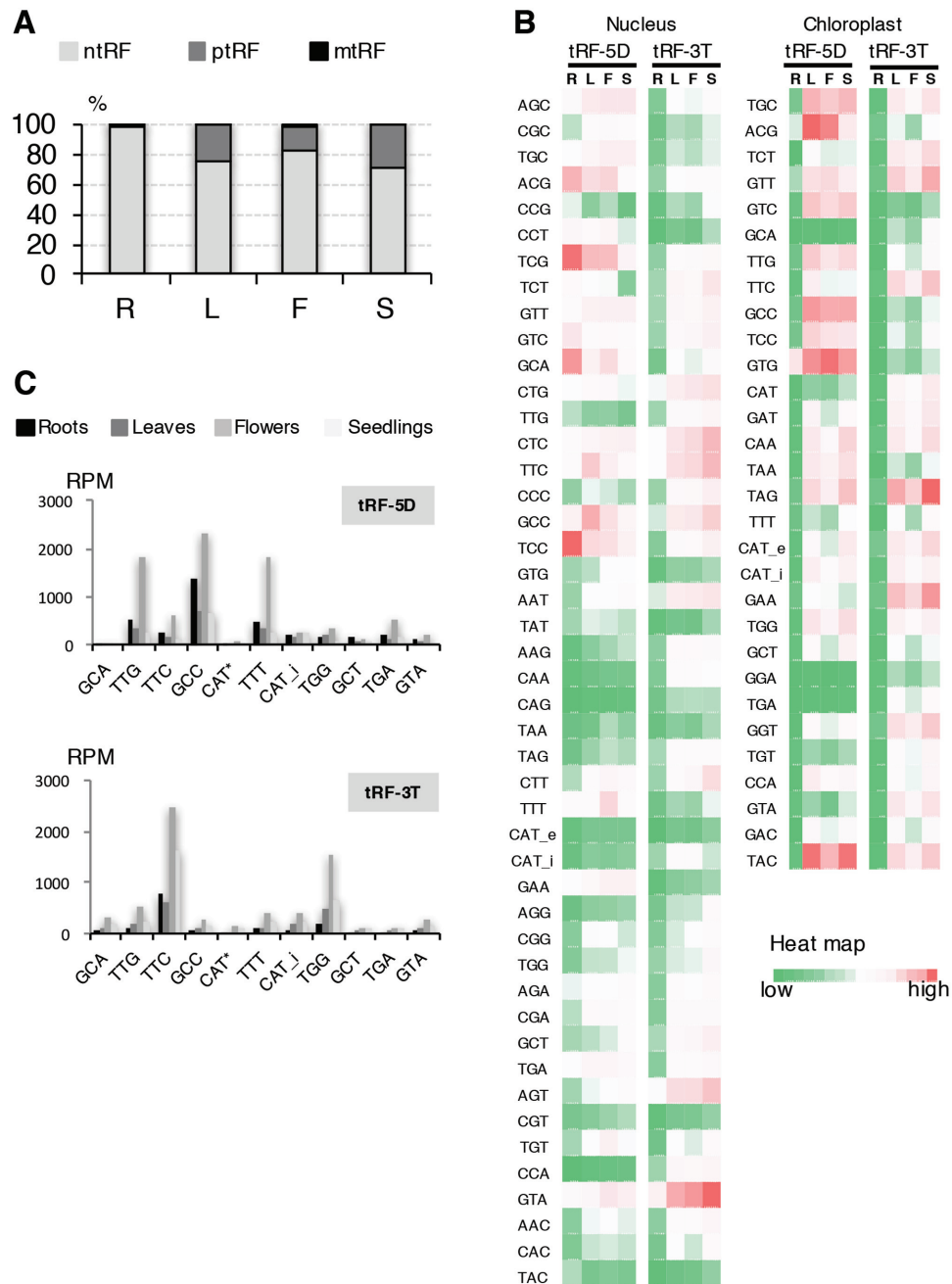
**Figure 2.** Size and tRNA cleavage sites of the major tRF originating from nucleus-encoded or chloroplast-encoded tRNAs. (A) Size distribution of tRF-5D (in black) and tRF-3T (in grey) originating from nucleus- or plastid-encoded tRNAs. (B) For eight tRNAs, the percentage of reads ending at each position is given. Four examples are given for tRF-5D and four others for tRF-3T, they are representative of all the situations found for tRFs. Their respective position on the tRNA cloverleaf structure according to the classical nomenclature is depicted below each histogram. For the eight tRNAs, the major site of cleavage and the length of the generated tRF are indicated in Supplementary Table S2. These positions are given by arrowheads. Black, grey and white arrowheads on the schematic cloverleaf structure of a tRNA (with the optional long variable loop) correspond to tRF deriving from nucleus-, plast- or mitochondrion-encoded tRNAs. Blue and red circles correspond to the positions of the most represented last nucleotides in tRFs in the D- and T- regions, respectively. These sites are also depicted in a L-shaped tRNA structure generated with Pymol (<https://www.pymol.org/>). Positions 20 and 55 on the tRNA cloverleaf structure are indicated according to the conventional nomenclature (62).

have been often shown to be induced in various organisms by a large panoply of stresses (e.g. (7,43)). However, less data exist concerning the population of stress-induced tRF-5D and tRF-3T. In order to get some insights on this question in plants, we focused our analysis on *Arabidopsis* plants submitted to various abiotic stresses.

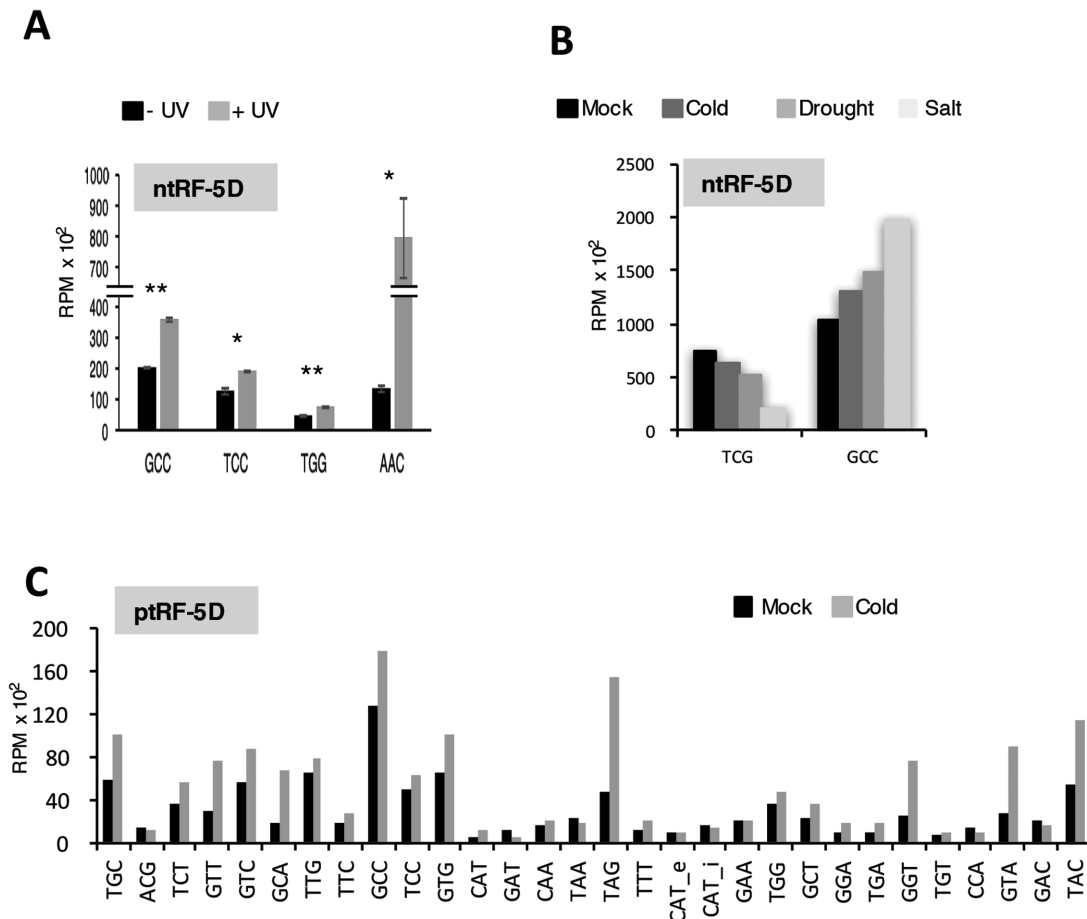
First, we compared sncRNA libraries from untreated (L1, L2) and UV-C irradiated (LU1, LU2, Supplementary Table S1) *Arabidopsis* plants, known to trigger a stress response in plants (44). For all tRF-3T and most tRF-5D there is either no change or a slight decrease of their amounts in UV-stressed plants (Supplementary Fig-

ure S12). Only very few exceptions exist, in particular tRF-5D deriving from nuclear tRNA<sup>Gly</sup>GCC, tRNA<sup>Gly</sup>TCC, tRNA<sup>Pro</sup>TGG and tRNA<sup>Val</sup>AAC that are present in significantly higher amounts in UV-stressed plants (Figure 4A).

Second, we also examined publicly available sncRNA libraries (see also (19)) to analyze the tRF profiles of *Arabidopsis* shoots submitted to drought, cold and salt stresses (D1, C1, Sa1, Supplementary Table S1, Supplementary Figure S13) as compared to control plants (Co) grown in the same conditions. A significant decrease of both nuclear and plastid tRF-3T is observed whatever the stress or the tRNA considered. Most ntRF-5D do not vary significantly,



**Figure 3.** Fluctuation of the tRF population in various tissues of *A. thaliana*. (A) Histogram showing the percentage of nucleus-encoded cytosolic tRNAs (ntRF) (pale grey), plastid tRNAs (ptRF) (dark grey) and mitochondrial tRNAs (mtRF) from native tRNAs (black) found in four *Arabidopsis* tissues or developmental stages: R = roots, L = leaves, F = flowers, S = seedlings. (B) Heat map representation of the tRF-5D and tRF-3T levels originating from either nucleus-encoded or plastid-encoded tRNAs and found in roots (R), leaves (L), flowers (F) and seedlings (S). Heat map was constructed according to the values presented in Supplementary Figure S7. Name of tRF is indicated by the anticodon sequence of its corresponding tRNA. (C) Histograms showing the abundance of tRF-5D and tRF-3T deriving from mitochondria-encoded tRNAs (CAT\_i indicates the initiator tRNA<sup>Met</sup> and CAT\* the tRNA<sup>Ile</sup>, where the CAT anticodon is changed into an isoleucine-specific tRNA by transformation of the C34 into lysidine derivative (63)). The tissues analyzed are roots (black), leaves (dark grey), flowers (middle grey) and seedlings (pale grey). Frequencies are given per RPM of reads in the snRNA libraries.



**Figure 4.** Fluctuation of the population of tRF in leaves of *Arabidopsis* submitted to environmental stresses. (A) Histogram showing the abundance of ntRF-5D originating from four affected tRNAs (tRNA<sup>Gly</sup>GCC, tRNA<sup>Gly</sup>TCC, tRNA<sup>Pro</sup>TGG and tRNA<sup>Val</sup>AAC) in leaves from untreated control plants (black) and from UV-stressed plants (grey). Histograms are the mean values of two independent experiments for both UV stressed or control *Arabidopsis* plants. Error bars are given. The name of each tRNA is indicated by its anticodon sequence. \*\* $P < 0.01$ , \* $P < 0.05$ , (Student's test). (B) Histogram showing the abundance of the two affected ntRF-5D (Arg-TCG and Gly-GCC) in leaves from untreated control plants (black) and from plants submitted to cold (dark grey), drought (grey) and salt (pale grey) stresses. Additional data are presented in Supplementary Figure S9. (C) Histogram showing the abundance of ptRF-5D in shoots from untreated control plants (black) and from plants submitted to cold (dark grey). Supplementary data are available in Supplementary Figure S9. For all histograms, frequencies are given per RPM of tRFs.

with two notable exceptions (Figure 4B). First, ntRF-5D (Arg-TCG) is less abundant under all types of stress; second ntRF-5D (Gly-GCC) is increased during the different stresses. It is to note that this tRF was already shown to be increased in UV-stressed plants (Figure 4A). Finally, while the ptRF-5D population insignificantly fluctuates under drought or salt stress, remarkably many ptRF-5D are found in higher quantities when plants are submitted to a cold stress (Figure 4C).

As a whole, the response of *Arabidopsis* to abiotic stresses in terms of production/degradation of short tRFs appears to be limited. Nevertheless, the reasons why there is an increase of ntRF-5D (Gly-GCC) under all studied stress conditions or of many ptRF-5D under cold stress needs to be addressed.

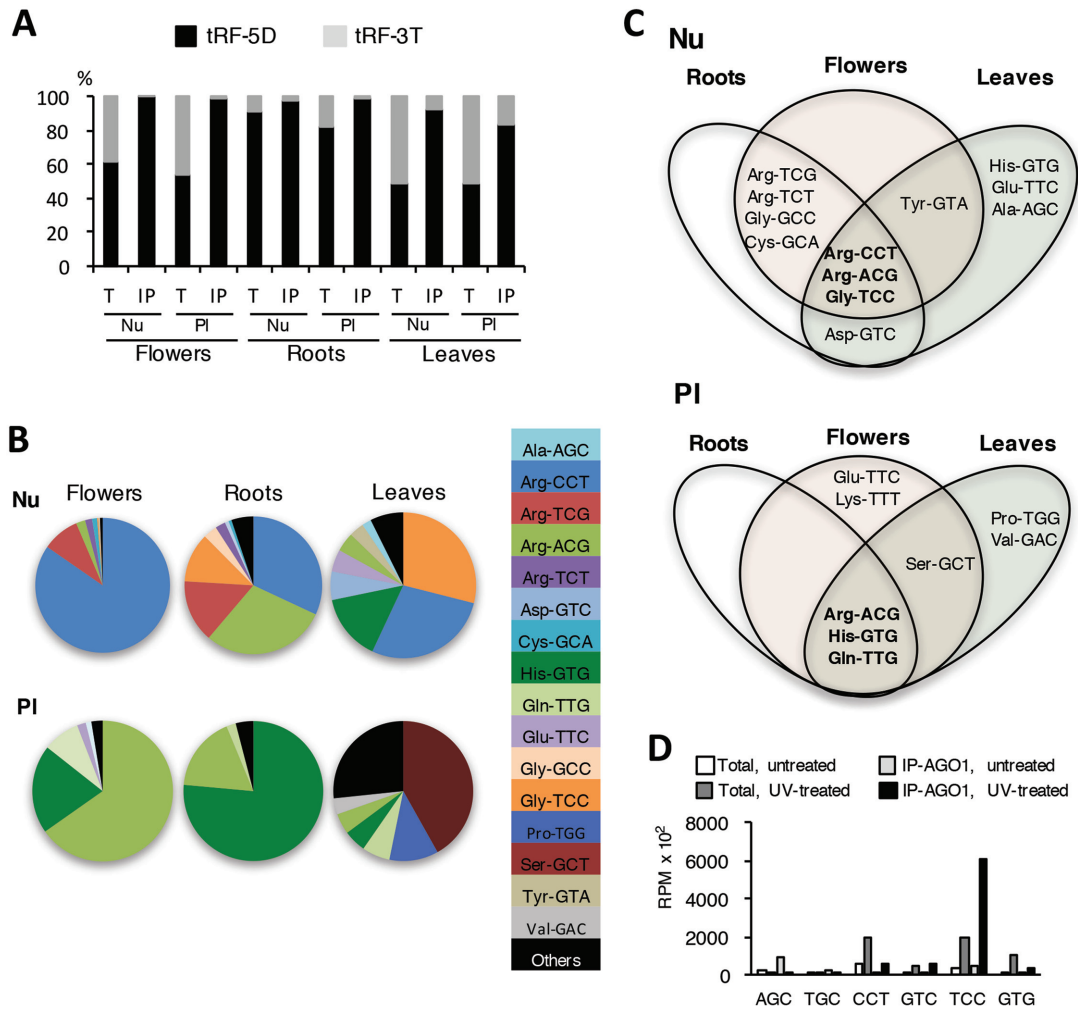
#### Nuclear but also plastid tRFs are associated with AGO1

Among the potential roles of tRFs, their implication in the regulation of gene expression *via* classical RNA silenc-

ing pathways has already been put forward. As in several other organisms, the association of plant tRFs with AGO proteins has been observed (19), but no detailed analysis was provided. We focused our work on AGO1 and analyzed the population of tRFs immunoprecipitated with this protein in roots, flowers and leaves (Figure 5). Public sncRNA libraries from roots and flowers (R1, R-AGO1, F1 and F-AGO1) and homemade sncRNA libraries (Lc3, Lc3-AGO1) were used (Supplementary Table S1).

As a mean value of the different AGO1 RNA libraries, the percentage of tRFs associated with AGO1 represents about 0.5% of the snc RNAs population immunoprecipitated with AGO1. While in the total sncRNA fractions, tRF-3T are well represented, the tRF population associated with AGO1 is primarily constituted by tRF-5D (Figure 5A). Interestingly, this population of AGO1-associated tRF-5D is constituted not only by ntRFs but also by ptRFs. Nevertheless, the set of tRFs significantly enriched in AGO1 immunoprecipitates is limited. Depending on the tissue, ntRF-5D deriving from 3 to 8 nucleus-encoded tRNAs and





**Figure 5.** Specific tRFs are associated with AGO1. (A) Percentage of tRF-5D (black) and tRF-3T (grey) in total (T) snRNAs from flowers, roots or leaves or in snRNAs from the same tissues associated with AGO1 (IP). (B) Proportion of the most abundant tRF-5D associated with AGO1 in flowers, roots and leaves. The color code is indicated on the right with the name of tRF indicated by the amino acid and anticodon sequence of the corresponding tRNA. (C) Schematic representation of the tissular localization of the most abundant tRF immunoprecipitated with AGO1. Roots, flowers and leaves are represented under white, pale pink and pale green background respectively. (D) Histogram showing the abundance of ntRF-5D in leaves upon *Arabidopsis* UV-stress treatment in total or AGO1-associated snRNA populations (see detailed analysis in Supplementary Figure S10). Total tRFs from untreated control plants (white) and from UV-treated plants (pale grey) and AGO1-associated tRF from untreated control plants (dark grey) and from UV-treated plants (black). Nu and PI: tRF originating from nucleus-encoded and plastid-encoded tRNAs, respectively.

ptRF-5D from 3 to 6 plastid tRNAs constitute up to 99% of the tRF-5D population enriched with AGO1 (Figure 5B and C). In terms of size, many tRF-5D immunoprecipitated with AGO1 are either 19 or 20 nt long (as might be expected) but longer ones (25–26 nt) are also found (Supplementary Table S3).

Importantly, the tRFs found associated with AGO1 are not necessarily the most abundant in total snRNA populations. For example, in flowers, ntRF-5D (Ala-AGC), the most abundant tRF in the total tRF-5D population, is present more than 100-fold less in the corresponding AGO1 fraction. Conversely, also in flowers, there is a 30-fold enrichment of the ntRF-5D (Arg-CCT) in the AGO1 fraction, a tRF present in very low amount in the total tRF population.

To see if the AGO1-associated tRF population varies upon abiotic stress, snRNA libraries were prepared from RNAs extracted of untreated (Lc3) or UV-C treated (LU3) *Arabidopsis* plants, and immunoprecipitated or not with AGO1 (Supplementary Table S1, Supplementary Figure S14). In the case of AGO1-associated ptRF-5D, their amount was generally decreased in UV-stressed plants and followed the downward trend observed above in the total RNA fraction. For nuclear tRFs, in most cases, no significant qualitative or quantitative variation was observed in the AGO1-associated ntRF-5D population between plants submitted or not to UV-C stress. The only notable exception concerns ntRF-5D (Gly-TCC) that was strongly enriched (Figure 5D). This tRF, also shown to be significantly increased upon the same stress in the total fraction, represents

an interesting candidate to determine the potential role(s) of tRFs in plants.

### Organellar tRFs accumulate outside the organelles

The identification of AGO1-associated tRFs originating from plastid tRNAs led us to address the question of where they are generated within the plant cell. To our knowledge, plant AGO1 has never been found within chloroplasts or mitochondria and is mainly localized in the cytosol. Thus, either the AGO1-associated ptRF are generated within the organelles and can escape their cellular compartments to reach AGO1, or tRFs are generated outside the organelles and in that case, it is the tRNAs that need to escape the organelles. To address this question, RNA fractions were extracted from total or cytosolic samples and from highly purified chloroplasts and mitochondria (Figure 6A and B). Northern experiments (Figure 6C) were performed with radiolabeled oligonucleotides complementary to the 5' extremity of the plastid tRNA<sup>His</sup>, tRNA<sup>Gln</sup>, tRNA<sup>Gly</sup> and tRNA<sup>Asp</sup>. Neither tRF-5D, nor tRF-5A can be detected in the chloroplast RNA fractions. In contrast, both tRFs are present in the cytosolic and total snRNA fractions. As mitochondria contain a chloroplast-like tRNA<sup>His</sup>, we also wondered whether these tRFs could be generated within mitochondria, but, as for chloroplasts, the RNA fragments were not detected. This was further confirmed by the absence, in the mitochondrial fraction, of tRFs deriving from the nucleus-encoded tRNA<sup>Ala</sup>, a tRNA imported into plant mitochondria (45). Thus, our data strongly suggest that organellar tRFs accumulate outside chloroplasts and mitochondria.

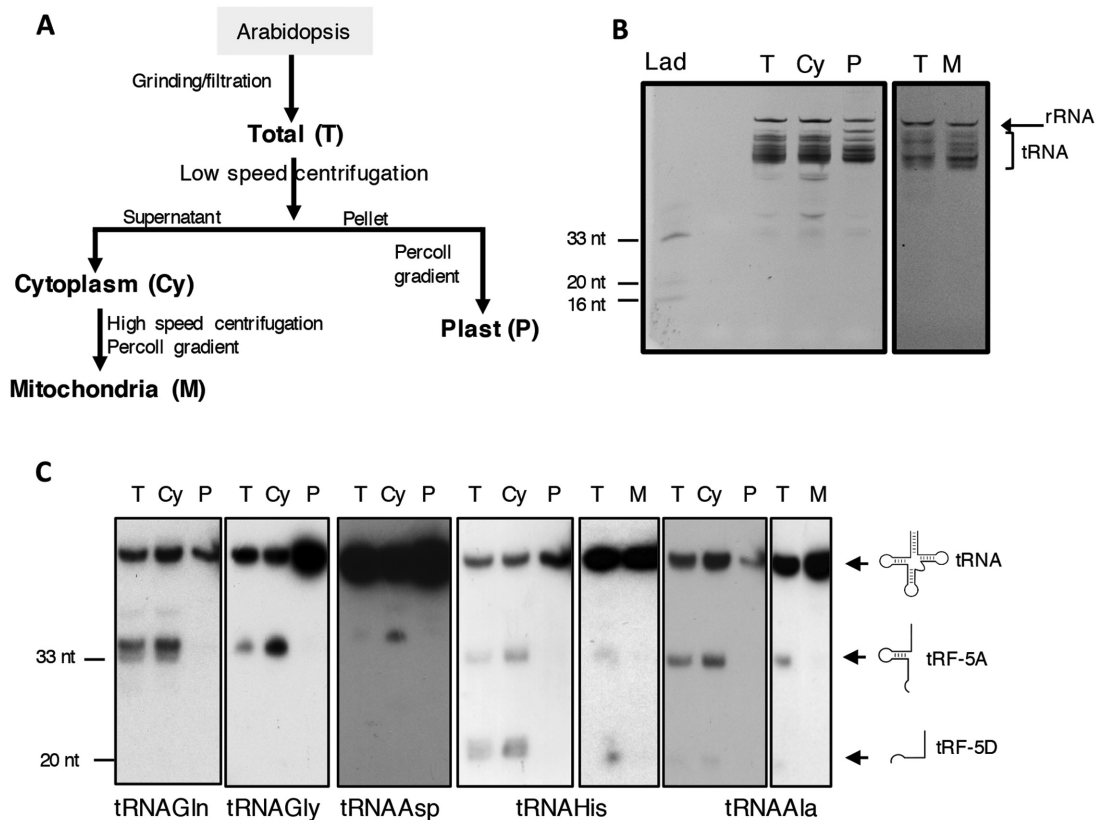
### DISCUSSION

Specific endonucleolytic cleavages of tRNAs to create tRFs have been reported in various organisms, such as protozoans, fungi and mammals. In *Arabidopsis*, only scarce data on nuclear tRFs exist (19,26,46). To decipher the potential role(s) of some of these tRFs, it is first essential to provide a strong set of data to the scientific community. In plants, tRNAs are expressed from three different genomes, in nuclei, plastids and mitochondria. Do tRFs originate from all three tRNA populations? Here, by analyzing in depth the population of tRFs found in various *Arabidopsis* tissues or organs, we first identified a major pool of nuclear tRF-5D and tRF-3T. Among the most abundant tRF-5D observed are the four tRFs (Ala-AGC, Arg-TCG, Arg-CCT and Gly-TCC) already described in (19) but also several others (Figure 1). Strikingly, the population of tRF-3T usually originates from different tRNA species (e.g. Gln-CTG, Ile-AAT, Thr-AGT and Trp-CCA). Abundant tRF-5D and tRF-3T generated from the same tRNA species are rare (e.g. Arg-ACG, Glu-TTC, Tyr-GTA). Whether this is the result of differential cleavage of each tRNA isoacceptor either in the D- and/or T- region or whether it is due to differential stabilities of tRFs *in planta* is presently unknown. The role played by nucleotide modifications in protecting or facilitating tRNA cleavage is also unknown in plants but has been described in other eukaryotes and bacteria (47,48). Cleavage sites on mature tRNAs are mainly restricted to

very specific zones (Figure 2B) and the question whether the same endonuclease(s) is responsible for the biogenesis of tRF-5D and tRF-3T remains to be addressed in plants and in other organisms. Dicer has been reported to be at the origin of some tRF-5D and tRF-3T in human cells (49,50) but other still unknown endonucleases are likely to be required (5). In our work, the double-stranded T-stem region represents a hot spot of cleavage, thus plant Dicer would also be good candidate. On the 5' side, cleavage preferentially occurs in the D-loop, thus implying a different type of endonuclease. Two endonucleases are known from other organisms that can cleave tRNAs in the anticodon loop: angiogenin, a vertebrate-specific RNase A (51), and yeast Rny1, an RNase T2 (52). Whether ribonucleases of the plant RNase T2 family (53) are able to cleave mature tRNAs in the D- or T- region is a question to be addressed in the future.

In addition to nuclear tRFs, 25% of the tRF population corresponds to chloroplast tRF-5D and tRF-3T. This is in agreement with the presence of numerous tRFs deriving from plastid tRNAs in *Brassica napra* (8). These tRFs are formed by cleavage in positions very similar to those found for nuclear tRFs. Furthermore, they accumulate outside the organelle. Unless tRNAs are cleaved within the organelles immediately prior to tRF export, this implies that chloroplast tRNAs may interact with the endonuclease responsible for their cleavage outside the organelle. Import of nucleus-encoded tRNAs into organelles is now largely documented (e.g. (45)) but export of organellar tRNAs to the cytosol has never been demonstrated. There are potentially two other ways to explain the presence of chloroplast tRNA outside the organelles. Stromules (for stroma-filled tubules) are present at the surface of chloroplasts (54) and these highly dynamic structures could represent a way to transport tRNAs from the chloroplasts to the cytosol or to another compartment of the plant cell. A more likely possibility is via chlorophagy (55). During senescence or upon stress, the delivery of chloroplasts to lytic vacuoles has been proposed (56). Chloroplastic proteins can be degraded *via* the cytosolic ubiquitin-proteasome system (57). We speculate that the cleavage of chloroplast RNAs can occur *via* non-organellar RNA degradation pathways following organelle membrane rupture during autophagy. Finally, a few tRFs deriving from native mitochondrial tRNAs have been identified. This is the first observation of their presence in plant cells, adding to observations in tadpole shrimps, *Drosophila* and humans (10,58,59), thus suggesting their widespread existence among eukaryotes.

Many tRFs have been reported to be induced by a large variety of stresses in many eukaryotes. In *Arabidopsis*, upon phosphate starvation, a group of 19 nt long tRF-5D were shown to be expressed at high levels (60). Upon oxidative stress, there is also accumulation of some *Arabidopsis* tRF-5A (7). We show here that upon UV-C irradiation, only a limited set of ntRF-5D (mainly four tRFs: Gly-GCC, Gly-TCC, Pro-TGG and Val-AAC) show a higher level of expression as compared to control plants, and mostly no differences were observed when plants were submitted to drought, cold or salt stresses, except for tRF (Gly-GCC) that also accumulates under these adverse conditions. So far, it is difficult to draw general conclusions concerning the



**Figure 6.** Organellar tRFs accumulate outside organelles. (A) Scheme of the preparation of the different *Arabidopsis* fractions. (B) Negative picture of ethidium bromide stained gels of total (T), cytosolic (Cy), plastidial (P) and mitochondrial (M) RNA fractions separated on 15% polyacrylamide gel. Lad: RNA ladder constituted by three oligoribonucleotides of 16, 20 and 33 nt in size. (C) Hybridization of plastid-encoded tRNA<sup>Gln</sup>, tRNA<sup>Gly</sup> and tRNA<sup>Asp</sup> probes and mitochondria imported nucleus-encoded tRNA<sup>Ala</sup> probe to northern blots of Total (T), Cytoplasmic (Cy), Plastidial (P) and Mitochondrial (M) RNA fractions. Note that the cytoplasmic fraction contains mitochondria and broken chloroplasts.

fluctuation of tRF populations in response to various abiotic stresses. The fluctuations observed may reflect degradation of tRNAs to recycle nucleotides and phosphate under stress conditions, but some specific tRFs may participate in regulation of gene expression in order to respond to adverse environmental conditions. This will need to be investigated in the future.

The molecular functions of plant tRFs are presently unknown. For example, tRF-5D originating from nuclear tRNA<sup>Ala</sup> are abundant, their expression fluctuates and they are not associated with AGO1. However, they start with a series of four G residues, a motif present at the extremity of human tRF-5A (Ala) and found to be essential for interaction with the translational silencer YB-1 to inhibit translation (61). Other examples of translation inhibition by tRFs have also been described (14,16). Whether plant tRFs have similar functions remains to be established. Some tRFs in several organisms were found to be associated with AGO complexes (12,17–19). Here, we found short nuclear tRFs (only tRF-5D) but also surprisingly plastidial tRFs associated with AGO1. These associations were specific because many other abundant tRFs found in total sncRNA fractions were missing from the AGO1 fractions. This raises the possibility that the tRFs associated with AGO1 may play a role in the regulation of gene expression *via* the RNA silenc-

ing pathway, and in the case of chloroplast tRFs, could be elements of a retrograde signaling pathway. Target genes for tRF-AGO1 complexes can be predicted bioinformatically (Supplementary Table S4), but it is essential now to validate the candidates. Furthermore, as the fraction of AGO1 bound to tRFs is low but enriched in a specific tRFs population, we cannot exclude the attractive hypothesis that some tRFs play a slight regulatory role in inhibiting AGO1 function.

In conclusion, several nuclear and plastid tRFs have been well characterized. They provide a strong basis for further studies dealing with (i) the identification of the endonucleases responsible for tRF biogenesis and (ii) the identification of functions attributed to these tRFs in plants.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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