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Plant RNases T2, but not Dicer-like proteins, are major players of tRNA-derived fragments biogenesis

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ABSTRACT

RNA fragments deriving from tRNAs (tRFs) exist in all branches of life and the repertoire of their biological functions regularly increases. Paradoxically, their biogenesis remains unclear. The human RNase A, Angiogenin, and the yeast RNase T2, Rny1p, generate long tRFs after cleavage in the anticodon region. The production of short tRFs after cleavage in the D or T regions is still enigmatic. Here, we show that the Arabidopsis Dicer-like proteins, DCL1-4, do not play a major role in the production of tRFs. Rather, we demonstrate that the Arabidopsis RNases T2, called RNS, are key players of both long and short tRFs biogenesis. Arabidopsis RNS show specific expression profiles. In particular, RNS1 and RNS3 are mainly found in the outer tissues of senescing seeds where they are the main endoribonucleases responsible of tRNA cleavage activity for tRFs production. In plants grown under phosphate starvation conditions, the induction of RNS1 is correlated with the accumulation of specific tRFs. Beyond plants, we also provide evidence that short tRFs can be produced by the yeast Rny1p and that, *in vitro*, human RNase T2 is also able to generate long and short tRFs. Our data suggest an evolutionary conserved feature of these enzymes in eukaryotes.

INTRODUCTION

Beyond their role in translation, transfer RNAs (tRNAs) play many other essential roles within the cell (1). One of them is to provide a huge repertoire of tRNA-derived fragments (tRFs). First believed to be only tRNA degradation products, tRFs now appear associated with specific and important biological functions in all branches of life (2,3). For instance, they were shown to regulate gene expression, to promote RNA degradation, and to inhibit protein synthesis in various manners. They can be involved in stress responses, in the regulation of cell proliferation, be used as primer for viral reverse transcription and were recently demonstrated to be involved in transgenerational paternal heredity (4), in the regulation of retro-elements expression (5) and in ribosome biogenesis (6).

Although tRFs can originate from tRNA precursor molecules (7), most are generated after cleavage of mature tRNAs. These later tRFs can be classified into two classes according to the region of cleavage and their size. As several nomenclatures exist, we will use the one we have proposed in (8). The long tRFs (circa 30−35 nt) originate from tRNA cleavage in the anticodon region, the short tRFs (<28 nt) from cleavage either in the D or T regions. Many tRFs identified so far originate from nucleus-encoded tRNAs but organellar tRFs have also been found. Indeed, we recently identified mitochondrial and plastidial tRFs through a detailed analysis of the Arabidopsis tRFs population (9). This population varies upon stress and plant development. Moreover, some tRFs were specifically enriched in ARGONAUTE (AGO) proteins immunoprecipitates, suggesting a role in the regulation of gene expression (9).

Beyond the question of the multiple functions of tRFs, it is also crucial to gain insight on their biogenesis. Angiogenin, an RNase A, is involved in the biogenesis of long tRFs in human (10). In the yeast *Saccharomyces cerevisiae* and in the protozoan *Tetrahymena thermophila*, long tRFs are produced through the cleavage of tRNAs by enzymes in the RNase T2 family, Rny1p (11) and Rnt2 (12) respectively. Concerning short tRFs, only few data exist. So far, they have not been described in *S. cerevisiae*. In human, DICER1 was shown to cleave in the D or T regions of some tRNAs (13) but more recent data present evidence that the production of short tRFs can be DICER1-independent (14,15). Similarly, contradictory results were obtained in Arabidopsis. Indeed, indirect evidence of the implication of Dicer-like 1 (DCL1) to generate tRFs in pollen has been proposed (16)

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but Alves and collaborators present data where plant DCLs are not involved in tRF biogenesis (17).

Here, using RNA-seq analysis and *in vitro* tRNA cleavage assays, we first demonstrate that in Arabidopsis, DCLs are likely not the major actors of tRFs biogenesis. Rather, we show that RNases T2, namely RNS1, RNS2 and RNS3, are essential endoribonucleases for the production of both long and short tRFs. We observed a strong reduction of the tRNA cleavage ability of senescing silique enzymatic extracts obtained from single *rns1, rns3* and double *rns1rns3* knock-out mutant lines. As demonstrated by RT-qPCR experiments, reporter gene assays and western blots, RNS1 and RNS3 exhibit a very specific spatiotemporal expression pattern in the seed coat and endosperm of senescing silique seeds. The constitutively expressed enzyme RNS2 is the major RNS present in old leaves and the tRNA cleavage activity of the corresponding enzymatic extract prepared from the single *rns2* mutant line is affected. *In planta*, we established that RNS1 is responsible of the accumulation of specific tRFs under phosphate starvation. Furthermore, *in vivo* and *in vitro* studies support the idea that not only long but also short tRFs can be produced in yeast. Finally, we also provide evidence that the human RNase T2 is able to generate the two classes of tRFs *in vitro* thus suggesting that, *in vivo*, this enzyme may act in addition to Angiogenin and DICER. Altogether, our study supports the idea that the RNase T2 family has the capability to generate all types of tRFs and likely plays crucial roles during development and stress responses in a large variety of organisms.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana T-DNA insertion lines *rns1* (FLAG_566A08) and *rns3* (FLAG_164A04), from Wassilewskija (Ws) ecotype, were obtained from the Versailles Arabidopsis stock Centre. Double mutant *rns1rns3* was obtained by crossing single mutants. The *rns2* mutant of Columbia (Col-0) ecotype (Salk_69588) was obtained from the Arabidopsis Biological Resource Center. *Arabidopsis thaliana dcl1234* mutant line (triple KO for *dcl2, 3, 4*; hypomorph for *dcl1*) was provided by T. Blevins (18). Arabidopsis Col-0 or Ws ecotypes and related mutants were grown on soil at 22°C under a 16h light photoperiod. For *in vitro* culture, surface-sterilized seeds stratified 2 days at 4°C were sown on agar plates containing MS255 (Duchefa) supplemented with 3% (wt/vol) sucrose and grown at 22°C under a 16 h light photoperiod. For phosphate starvation study, surface-sterilized seeds stratified 2 days at 4°C were grown in phosphate containing medium (MSP01, Caisson Labs UT, USA) during 4 days at 22°C under continuous light, then transferred into medium containing phosphate or not (MSP01 and MSP11 respectively) for a period of 1–2 days.

Accession numbers

Sequence data can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: RNS1, At2g02990; RNS2, At2g39780; RNS3, At1g26820; RNS4, At1g14210; RNS5, At1g14220.

Small ncRNA libraries and tRFs analysis

Deep sequencing libraries (GSM1919557, GSM1919558, GSM1919559, GSM1919560, GSM1919561, GSM1919562) from RNA of three-week-old Arabidopsis seedlings were retrieved from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and libraries (SRX1070790, SRX1070791, SRX1070795, SRX1070796) from RNA of leaves of two-week-old plants were retrieved from SRA (https://www.ncbi.nlm.nih.gov/sra/). Bioinformatics analysis of tRFs was performed as described in (9).

RNS constructs

RNS1, *RNS2* and *RNS3* coding sequences were amplified from RNA extracted from *A. thaliana* leaves by RT-PCR using specific primers overlapping the initiation and stop codons and cloned into pGEM®-T Easy vector (Promega Madison WI). For overexpression in *E. coli*, the *RNS1*, *2* and *3* coding sequences deleted of their signal peptide were amplified by PCR and cloned into the p0GWA Gateway vector using the Gateway technology (19). All constructs were expressed in *E. coli* with a His-tag at the C-terminal extremity of the proteins. For expression in *S. cerevisiae rny1δ* mutant line, *RNS1*, *2* and *3* coding sequences amplified by PCR using appropriate oligonucleotides were cloned into pGEM®-T Easy vector (Promega Madison WI). After digestion with appropriate restriction enzymes, the insert was cloned into pESC-Trp® vector (Agilent Technologies) by using the DNA Ligation Kit <Mighty Mix> (Takara) following manufacturer's instructions. Constructs corresponding to mutated versions of *RNS1* and *RNS3* in the catalytic sites I or II were obtained using classical oligonucleotide-directed mutagenesis (20).

Expression in *E. coli* and purification of recombinant proteins

His-tagged RNS1, RNS2 and RNS3 mutants with deleted signal peptide were produced in BL21(DE3) *E. coli* strain. For antibodies production, recombinant proteins were purified by classical immobilized metal affinity chromatography on Ni-NTA resin under denaturing conditions in the presence of 8M urea according to manufacturer's recommendation (Qiagen, Valencia, CA, USA).

In vitro synthesized tRNA transcript

Plasmids containing *A. thaliana* cytosolic $tRNA^{Ala}$(UGC) gene sequence was obtained previously (21,22). *In vitro* transcript was synthesized with T7 RNA polymerase using Ribomax™ transcription kit (Promega, Madison, WI, USA) as described in (20).

S. cerevisiae complementation

S. cerevisiae wild-type yRP 840 strain and *rny1δ* strain lacking Rny1p protein (11) (obtained from R. Parker, The University of Arizona, USA) were grown under conditions furnished with pESC-Trp vector® (Agilent Technologies). Complementation of the *rny1Δ* strain with wild-type RNS1, RNS2 or RNS3 and with catalytically inac-

tive RNS1-1 or RNS3-2 constructs was performed following the same protocol (Supplementary Figure S1). The different RNS were expressed under the control of GAL promoter (pGAL). Due to their ability to degrade RNAs, the recombinant RNS expressed in yeast cells are highly toxic. Consequently, we adopted a strategy in which yeast strains were grown until log-phase, induced by 2% (w/v) galactose and then harvested only 2 h later.

RNA extraction, Reverse Transcription and quantitative RT-PCR analysis

For all *Arabidopsis* tissues except siliques, RNAs were extracted using TRI reagent (Sigma-Aldrich), following manufacturer's instructions. For siliques samples, total RNAs fractions were prepared according to (23). Prior to reverse transcription, RNAs samples were treated by DNase RQ1 (Promega, Madison WI). Quantitative RT-PCR (RT-qPCR) analyses were performed as in (24). For each extract, three biological replicates were done. The qPCR efficiency for each primer's couple was determined by serial dilutions. Results were normalized with the EXP (At4g26410), TIP1 (At4g34270) and F-BOX (At5g15710) mRNAs.

RNS promoter-GUS fusion

The 5′ region upstream of the ATG initiation codon of *RNS1* (2180 bp), *RNS2* (1486 bp) and *RNS3* (2006 bp) were cloned in the binary vector pGWB633 upstream of the GUS gene. Transgenic *Arabidopsis* plants were produced. Young seedlings grown either in liquid medium or on agar plate and tissues from plants grown on soil were stained with 5-bromo-4-chloro-3-indolyl-b-glucuronic acid or observed on transmission and stereo microscope. For each analysis, 10–15 independent plants, from three independent Arabidopsis lines were used.

Antibodies and western blot analysis

For immunoblots, protein extracts from various tissues of Arabidopsis were prepared and analyzed as described in (25). Antisera specific to *E. coli* recombinant RNS1, RNS2 and RNS3 produced by Covalab (Villeurbanne, France) were purified on HiTrap Protein A HP (GE Healthcare). They were used at a 1/1000. Specificity of antisera was checked by western blots (Supplementary Figure S2). Antibody against maize eEF1α was kindly provided by B. Hunter (The University of Arizona, USA) and used at 1/2000. The relative amount of RNS1, 2 and 3 in senescing siliques was obtained by immunoblot quantification using standard curves obtained with the respective purified recombinant proteins (Supplementary Figure S3).

Crude enzymatic preparations

The *S. cerevisiae* protein extracts were prepared from 50 ml of culture. After centrifugation (2500g, 10 min), yeast pellets were resuspended in 500 μl of YeastBuster™ Protein Extraction Reagent (Novagen) supplemented with 10 mM $MgCl_2$, 1x tris(hydroxypropyl)phosphine (Novagen), 1 mM PMSF, 1× protease inhibitor cocktail Complete® (Roche). After a 30 min incubation at 20°C under shaking, extracts were centrifuged 5 min at 4°C and 10 000 × g and supernatants were used for *in vitro* cleavage assays. For each plant enzymatic extract, 200 mg of tissue was harvested from 20 independent Arabidopsis plants. The powder obtained by grinding in liquid nitrogen was 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 μM PMSF, 1× protease inhibitor cocktail Complete® (Roche)) and shaked at 4°C for 20 min. After centrifugation (10 000 × g, 15 min, 4°C) supernatant was used for *in vitro* cleavage assays. For tRNA cleavage assay in the presence of 2.5 ng of the human RNase T2, the enzyme expressed and purified as described in (26) was kindly provided by Prof. Robert Steinfeld (University Medical Center Goettingen, Germany).

In vitro tRNA cleavage assay

Assays were performed at 20°C in a 35 μl per time point reaction volume containing 800 ng of synthesized tRNA transcript and 0.5,10 μg of total proteins extract. Each mix contains the same final proportion of extraction buffer and is completed with water (final pH 7, unless otherwise stated). At each time point, 30 μl aliquot was rapidly transferred into a microtube containing 200 μl of water saturated phenol and 170 μl of tRNA extraction solution (10 mM Tris–HCl pH7.5, 10 mM $MgCl_2$, 1% SDS) and vortexed for 10 min. After centrifugation (2300 × g, 10 min, 20°C), the aqueous phase was ethanol precipitated.

Small RNA fractions and Northern blots

Total RNA fractions from *S. cerevisiae* were prepared as described in (27). Total tRNA fraction from Arabidopsis was prepared from leaves as described in (28). 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 small non-coding RNAs). Northern blots were performed with 4 μg of RNAs essentially as described in (9).

Miscellaneous

The oligonucleotide sequences used in this study are listed in Supplementary Table S1.

RESULTS

Dicer-like proteins are not major players of tRFs biogenesis in plants

Currently, data on the enzymes involved in the production of short tRFs are scarce. In human, the RNase III DICER1, known to be essential for the production of small interfering RNAs (siRNAs) and microRNAs (miRNAs), seems implicated in the production of a specific set of short tRFs of 18−22 nt in size (13) but this has been challenged in more recent articles (14,15). In Arabidopsis, four Dicer-like (DCL1-4) proteins exist (29). As in human, these RNase III enzymes cleave double-stranded RNA sequences and are involved in small RNA pathways. DCL2, DCL3 and DCL4

have partial redundant functions and are mainly involved in the production of siRNAs of 22, 24 and 21 nt respectively. DCL1 is essential and produces miRNAs of 21 nt. Recent data suggest the implication of DCL1 to generate tRFs in pollen (16); by contrast, another work suggests that tRF biogenesis does not rely on DCL nucleases (17). Therefore, we also addressed the question of *Arabidopsis* DCL implication in short tRFs biogenesis. Analysis of small RNAs libraries shows no substantial variations in tRFs profiles between *Arabidopsis* wild type and *dcl234* mutant lines (Figure 1A and B), suggesting that these 3 DCL enzymes are not involved in tRF biogenesis. In addition, in *dcl1234* (triple KO for *dcl2,3,4;* hypomorph for *dcl1*) mutant line, the abundance of tRFs is not reduced as compared to *dcl234* mutant line (Figure 1A and C), thus likely DCL1 is also not strongly involved in tRFs biogenesis. To strengthen these results, *in vitro* cleavage assays of a tRNA transcript were performed in the presence of protein extracts from flowers, a tissue where DCLs are abundant in plants (30), from wild-type Col-0 and *dcl1234* mutant line. As shown in Figure 1D, no tRFs are produced when no protein extract is added while both long and short tRFs are generated when protein extracts from flowers of both genotypes are used. Importantly, no difference of tRNA cleavage activity exists between wild-type Col-0 and *dcl1234* mutant plants (Figure 1D and E), in agreement with the absence of variation in the tRFs profiles observed above (Figure 1A–C).

Altogether, these data suggest that the four *Arabidopsis* DCLs do not play major roles in the biogenesis of tRFs in plants. Therefore, other endoribonucleases must be involved. Among the other potential candidates, Angiogenin, an RNase A known to cleave tRNAs in the anticodon region in human, has no homologues in plants. In the yeast *S. cerevisiae* and in the protozoan *T. thermophila*, tRNAs can be cleaved in the anticodon region by RNases T2 called Rny1p (11) and Rnt2 (12) respectively. However, these endoribonucleases were never shown to be involved in the production of short tRFs. Nevertheless, as plants also possess RNase T2 homologues, we focused our attention on the Arabidopsis RNases T2 family.

Recombinant Arabidopsis RNS complement *rny1δ* yeast mutant and generate long and short tRFs *in vitro* and *in vivo*

Five putative RNases T2 are encoded by the Arabidopsis nuclear genome (RNS1-5; Supplementary Figure S4) (31). Arabidopsis RNS possess the two conserved RNases T2 catalytic sites, CAS I and CAS II (Supplementary Figure S4 and Figure 2A), essential for ribonuclease activity (32). No expression of RNS5 was detected by RT-qPCR (data not shown) and RNS4 mRNA is almost only present in roots at a very low level (Supplemental Figure S5A). Thus we focused our work on the three others. Arabidopsis RNS2 was shown to be involved in ribosomal RNAs (rRNAs) degradation (33) and we hypothesized that this RNS may also cleave tRNAs to give rise to tRFs. As the two other endoribonucleases, RNS1 and RNS3, are structurally similar to RNS2 (Supplementary Figure S6), we wondered whether they can also generate tRFs.

As a first attempt to demonstrate the involvement of plant RNases T2 in tRFs biogenesis, Arabidopsis RNS1, RNS2 and RNS3, were expressed from the GAL promoter in the yeast *rny1δ* strain (Supplementary Figure S1). As expected, complementation of this strain by one of the three Arabidopsis RNS leads to the production of long tRFs from endogenous yeast $tRNA^{His}$ or $tRNA^{Arg}$ (Figure 2B). Interestingly, only long tRFs were generated from yeast $tRNA^{His}$ while both long and short ones were produced from $tRNA^{Arg}$. Although weak, the biogenesis of short tRFs (Arg) was observed not only in the *rny1δ* strain expressing an Arabidopsis RNS but also in the wild-type yeast strain. By contrast either long or short tRFs were barely or not detectable in the mutant *rny1δ* strain. Altogether our data show first that *Arabidopsis* RNS can complement the yeast Rny1p for the production of long tRFs and second that, depending on the endogenous tRNA considered, both the Arabidopsis and the *S. cerevisiae* enzymes can also cleave in the D-region to generate short tRFs. So far, only the existence of long tRFs was reported in yeast. Thompson *et al* showed that long tRFs production by this nuclease can be increased upon stress (11) and Bakowska-Zywicka *et al.* (34) reported the presence of long tRFs under numerous yeast growth conditions, even in the absence of stress. To our knowledge, the presence of short tRFs in *S. cerevisiae* has never been described. In agreement with these results, *in vitro* cleavage of the Arabidopsis cytosolic $tRNA^{Ala}$ transcript was observed in the presence of a protein extract prepared from either wild-type yeast (thus expressing Rny1p) or *rny1δ* strain complemented with one of the RNS (Figure 2C). By contrast, no cleavage was observed when the tRNA transcript was incubated with a protein extract prepared from the *rny1δ* complemented with catalytically inactive RNS where histidine residues of either the CAS I or the CAS II catalytic sites were replaced by alanine residues (Figure 2C). Therefore, all Arabidopsis RNS are likely able to cleave tRNAs to produce the two main classes of tRFs, i.e. the long and the short ones, and interestingly, the yeast RNase T2, Rny1p, is able to produce both classes of tRFs as well.

Activity of RNases T2 is usually optimal at acidic pH (35). Hillwig *et al*. using ribonuclease activity in gel assays (33) demonstrated that RNS1 and RNS3 are indeed acidic nucleases but found an optimal pH of 7.5 for RNS2. Here, in the presence of yeast-expressed RNS1, RNS2 or RNS3, almost no *in vitro* cleavage of $tRNA^{Ala}$ transcript was obtained under basic conditions while efficient cleavage was observed at pH 7 or below (Supplementary Figure S7). Thus, as in RNS1 and RNS3, our studies indicate that RNS2 is likely also an acidic ribonuclease, which is more relevant with the vacuolar localization found in Arabidopsis for this endoribonuclease (33).

RNS are differentially expressed during plant development and in senescing tissues

As described above, Arabidopsis RNS1, RNS2 and RNS3 are able to produce long and short tRFs in a heterologous system. To prove more convincingly their involvement in tRFs production *in planta*, we first had to analyze their expression profile. Based on phylogenetic and expression studies, plant RNases T2 are divided into three classes (36). No class III RNases T2, proteins involved in gametophytic self-

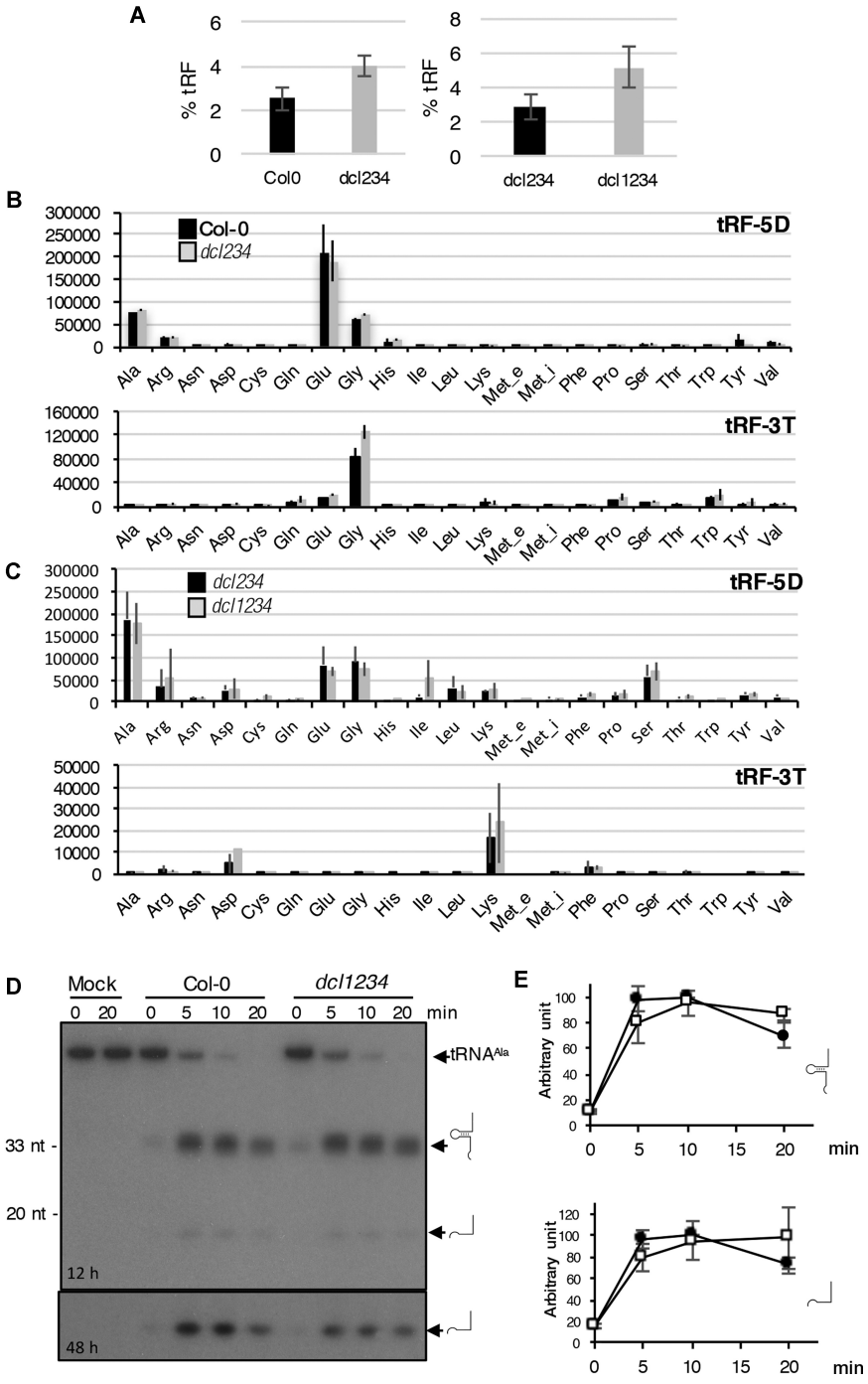

Figure 1. Arabidopsis DCLs are not major players of tRFs biogenesis. (**A**) Percentage of tRFs (19-26 nt) related to total small RNAs of the same size in Col-0 versus *dcl234* triple KO mutant lines and in *dcl234* versus d*cl1234* (triple KO for dcl2,3,4; hypomorph for dcl1) mutant lines. Note that the higher percentage of tRFs observed in *dcl234* versus Col-0 and in d*cl1234* versus *dcl234* is likely a consequence of a lower amount of siRNAs and miRNAs in the *dcl234* and d*cl1234* respectively. The histograms show the distribution of tRF-5D and tRF-3T of 19−26 nt originated from **B**) nucleus-encoded tRNAs in wild-type (black) or in *dcl234* mutant (gray) and (**C**) nucleus-encoded tRNAs in *dcl234* mutant (black) or in *dcl1234* mutant (grey). Isoaccepting tRNAs for given amino acids have been combined and are presented by their specific amino acids on the X-axis. Frequencies are given in reads per millions of tRF reads (RPM). Standard deviations are presented. Analyzed libraries are indicated in the material and methods section. For Col-0 versus dcl234, $n = 2$; for dcl234 versus dcl1234, $n = 3$; n = number of independent libraries. (**D**) Kinetics of cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$ transcript upon incubation in the presence of 10 μg of total proteins prepared from flowers of wild-type Col-0 or *dcl1234* mutant line. After incubation, RNAs were analyzed by northern blot analysis with a radiolabeled oligonucleotide specific of the 5′ extremity of cytosolic *Arabidopsis* $tRNA^{Ala}$ (see Supplementary Table S1 for sequence). Mock: control experiment without protein extract; Tr: *in vitro* synthesized $tRNA^{Ala}$ transcript. Two exposure times for the production of short tRFs are shown. (**E**) Histograms showing the relative amount of long and short tRFs generated in experiments depicted in (D). Error bars represent standard errors of mean of three independent biological experiments. An arbitrary value of 100 was given to the maximum of cleavage efficiency obtained in the presence of wild-type Col-0 protein extract after 10 min of incubation. According to a two-way ANOVA test, there is no significant difference in term of cleavage efficiency between wild-type and *dcl1234* genotypes ($P > 0.8$)

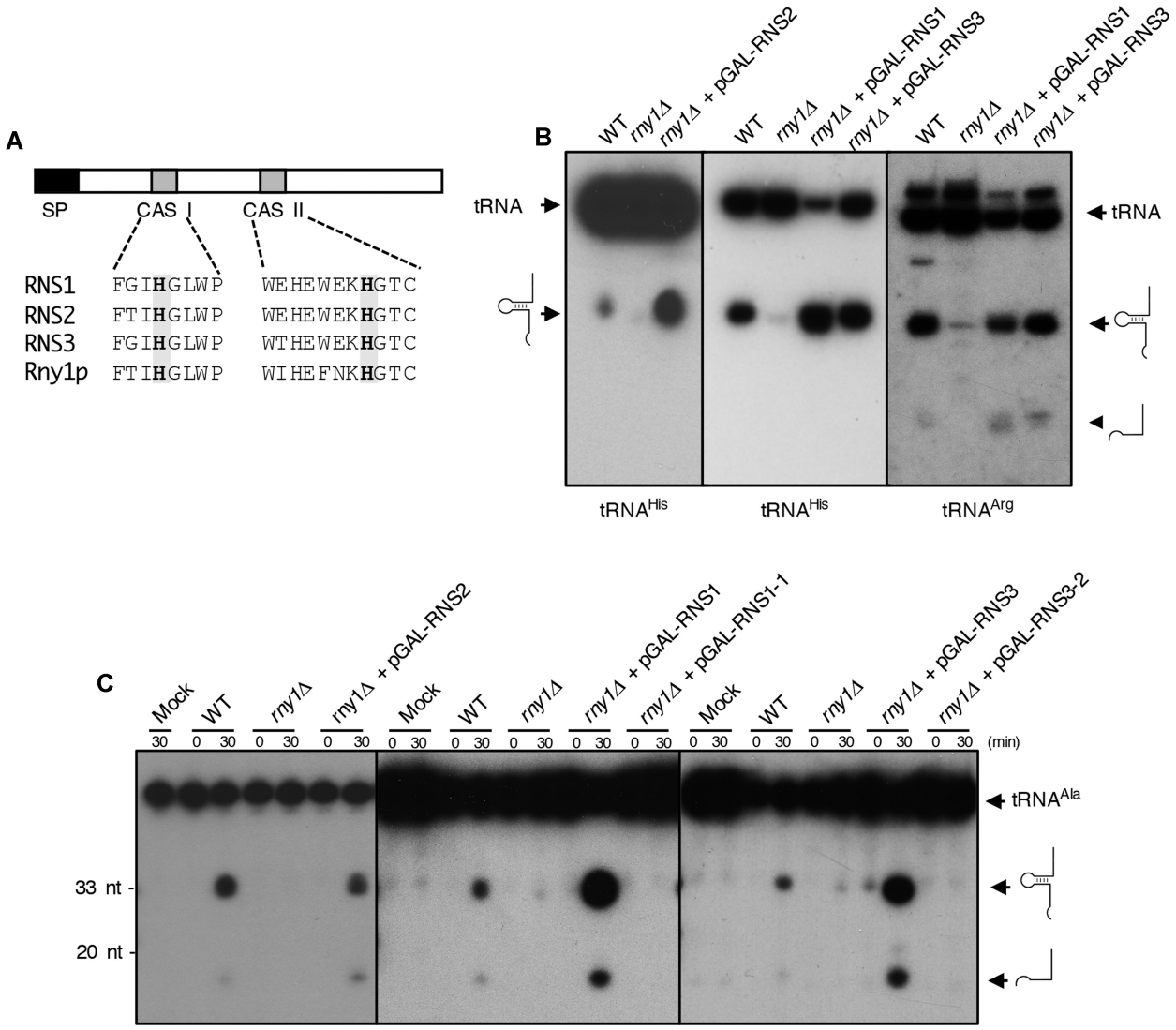

Figure 2. Arabidopsis RNS1, RNS2 and RNS3 complement the *rny1δ* yeast mutant and generate long and short tRFs. (**A**) Schematic representation of Arabidopsis RNS1, RNS2 and RNS3. As for the yeast RNase T2, Rny1p, they possess a predicted signal peptide (SP) and two catalytic sites (CAS I and CAS II) with conserved Histidine (bold H) residues depicted under grey background. (**B**) Hybridization of specific probes (see Supplementary Table S1 for sequences) complementary to the 5′ extremity of endogenous yeast $tRNA^{His}$(GUG) or $tRNA^{Arg}$(ACG) to total RNA fractions from *S. cerevisiae* wild-type, *rny1δ* mutant and *rny1δ* mutant complemented with RNS1, RNS2 or RNS3 expressed under the control of pGAL promoter. (**C**) Kinetics of cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$(UGC) transcript upon incubation in the presence of *S. cerevisiae* protein extracts prepared from wild-type strain (WT), *rny1δ* mutant, *rny1δ* mutant complemented with wild-type RNS1, RNS2 or RNS3 or catalytically inactive RNS1-1 or RNS3-2 expressed from the pGAL promoter. In RNS1-1 and RNS3-2, the H residue of CAS I and CAS II respectively was replaced by an A residue. Mock: control experiment without protein extract. After incubation, RNAs were phenol extracted and fractionated on 15% polyacrylamide gel, followed by northern blot analysis with a radiolabeled oligonucleotide specific of the 5′ extremity of cytosolic Arabidopsis $tRNA^{Ala}$.

incompatibility, are present in Arabidopsis. The only class II *Arabidopsis* RNS, RNS2, has been proposed to be a housekeeping enzyme expressed in all tissues and developmental stages (36,37). The other RNS genes code for class I RNases T2 (32,36).

Quantification by RT-qPCR (Supplemental Figure S5a) indeed shows that RNS2 messenger RNA (mRNA) is constitutively expressed (although at a very low level in roots). We found that while RNS1 and RNS3 transcripts are present at low levels in various tissues, their amount is strongly increased in siliques. More specifically, in senescing siliques at stage Si2 (15–18 days after pollination (DAP)), transcripts levels of RNS1 and RNS3 are about 10-fold and 6-fold higher respectively compared to the levels observed in flowers. In contrast to RNS1 and RNS3, the level of stable RNS2 mRNA remains constant during silique development. The expression profile of RNS1, 2 and 3 was further studied in the reproductive tissues of Arabidopsis lines stably transformed with the GUS gene under the control of their own promoter. High expression of the three RNS promoters was observed in inflorescences (Supplemental Figure S5b). No promoter activity was observed in seeds from siliques at stages Si1 (mature silique) and Si3 (dried silique). The expression of RNS1 and RNS3, but not RNS2, was

only detected in the senescing seeds of siliques at stage Si2 (yellowing silique). More precisely, RNS1 promoter was active in the chalazal seed coat and RNS3 in the endosperm but none of them in embryo.

Finally, at the protein level (Figure 3, Supplementary Table S2), western blots performed on proteins from different tissues showed that RNS2 is mainly present in old leaves and flowers. In siliques, RNS2 was also detected but mostly only at stage Si1. RNS1 and RNS3 are also found in flowers and remarkably, in agreement with RT-qPCR and RNS promoter-GUS fusion analyses, both are primarily and strongly present in Si2 siliques. The determination of their relative amount in Si2 protein extracts shows that RNS1 and RNS3 are 5- and 7-fold more abundant than RNS2 respectively (Supplementary Figure S3). At this stage, while RNS3 is present both in the valves of siliques and in seeds, RNS1 was primarily found in seeds. In these last, both RNS1 and RNS3 were found exclusively in outer tissues but not in embryos, confirming the data presented in Supplementary Figure S5b. A few discrepancies are nevertheless observed between GUS staining that reflect promoter activities, RT-qPCR that measure stable RNAs, and western blot that quantify the protein levels, suggesting a regulation of RNS expression at the post-transcriptional and/or translational levels. As a whole, data indicate a very specific spatiotemporal distribution pattern of RNS1 and RNS3 expression (Supplementary Table S2) and accumulation in the seeds of Arabidopsis yellowing siliques.

Arabidopsis RNS produce long and short tRFs in a tissue-specific manner

On the one hand RNS can generate tRFs *in vitro* and *in vivo* in a heterologous yeast system and on the other hand each Arabidopsis RNS has a specific spatiotemporal expression profile. These findings were used to address the question of RNS activity *in planta*. Enzymatic extracts were prepared from siliques, where RNS1 and RNS3 are the most abundant, and from old leaves where mostly only RNS2 is found (Figure 3A). The tRNA cleavage activity from wild-type and mutant plants was then analyzed.

In order to correlate the abundance of RNS1 and RNS3 in seeds with a tRNA cleavage activity, *in vitro* cleavage assays of a tRNA transcript were performed in the presence of protein extracts from Si1 and Si2 siliques of four genotypes: wild-type Ws, *rns1* and *rns3* single knock out (KO) Ws mutant lines and *rns1rns3* double KO Ws mutant line (Figure 4A). The absence of RNS1 and/or RNS3 in the mutant lines was validated by western blots (Figure 4B). As shown in Figure 4C, no tRFs are produced when no protein extract is added while both long and short tRFs are generated when protein extracts from wild-type siliques are used. In accordance with western blot analysis (Figure 3A), the tRNA cleavage efficiency is about 2-fold higher when the incubation is performed in the presence of Si2 as compared to Si1 wild-type extract. Furthermore, in the mutant line *rns3*, the absence of RNS3 strongly affects the cleavage activity and the biogenesis of both long and short tRFs. In the mutant line *rns1*, the biogenesis of tRFs is only weakly affected. Finally, in the double *rns1rns3* mutant lines, there is a 5-fold decrease of the tRNA cleavage activity in senescing Si2 siliques, thus demonstrating that most of the tRNA cleavage activity found in this tissue is due to RNS1 and RNS3. The comparison between tRNA cleavage assays performed in the presence of *rns1* or *rns3* mutant lines protein extracts show that, in siliques, RNS3 activity is stronger as compared to RNS1 activity (Figure 4C and D). This may reflect differences either in their intrinsic catalytic activity or in their relative abundance in this organ. Indeed, in the protein extracts of Si2, RNS3 is ~1.4-fold more abundant than RNS1 (Supplemental Figure S3), thus supporting the idea that the decrease of activity in Si2 in the *rns3* mutant as compared to *rns1* is due to a larger amount of RNS3 than RNS1.

RNS1 and RNS3 are the main endoribonucleases responsible of the production of long and short tRFs in siliques, but a low activity still persists in the double *rns1rns3* mutant line (Figure 4C). RNS2 is also expressed in siliques at stage Si2, even though at a lower level as compared to RNS1 and RNS3 (Figure 3A). It is therefore likely that the remaining tRNA cleavage activity present in the double mutants is due to RNS2. To confirm the RNS2 activity *in planta*, activity tests were performed with old leaves, where RNS2 was found in higher amount while RNS1 and RNS3 are present in very low abundance (Figure 3A). Comparison between tRNA cleavage assays performed in the presence of enzymatic extracts from wild-type Col-0 or KO *rns2* mutant line (Figure 4B) must reflect RNS2 activity. As shown in Figure 4E, two long tRFs of very similar size (called 'a' and 'b') are generated in the presence of the leaves extract. While the production of the longer RNA fragment 'a' seems unaffected when RNS2 is absent, the production of the second product 'b' is slightly reduced of ~30% after 30 min (Figure 4F). By contrast, in the absence of RNS2, the production of short tRFs is strongly affected as mostly no short tRFs are visible upon incubation (Figure 4E and F). This demonstrates that, in this tissue, RNS2 is the main endoribonuclease responsible for the production of short tRFs.

Altogether, our data demonstrate the ability of Arabidopsis RNS expressed in a tissue-specific manner (e.g. RNS1 and RNS3 in siliques, and RNS2 in leaves) to generate both long and short tRFs *in vivo*. This again contrasts with the inability of DCLs to produce such tRNA fragments.

RNS1 produces tRFs *in planta*

To further demonstrate that RNS can indeed cleave tRNAs to generate tRFs in Arabidopsis cells, we used the following approach. It has been previously shown by independent studies that under phosphate (Pi) starvation, on the one hand RNS1 is induced (32) and on the other hand, very specific tRFs (i.e. from nuclear $tRNA^{Gly}$(UCC) and $tRNA^{Asp}$(GUC)) accumulate (38). Thus, we asked the question whether the accumulation of tRFs under Pi starvation is indeed due to RNS1 endoribonuclease activity. When Arabidopsis seedlings are grown in Pi deficiency conditions, we observed both a higher activity of RNS1 promoter in secondary roots and cotyledons (Figure 5A) and an increase in RNS1 protein expression level (Figure 5b). This confirms that RNS1 expression is induced in Arabidopsis young seedlings under Pi deprivation. In parallel, the small

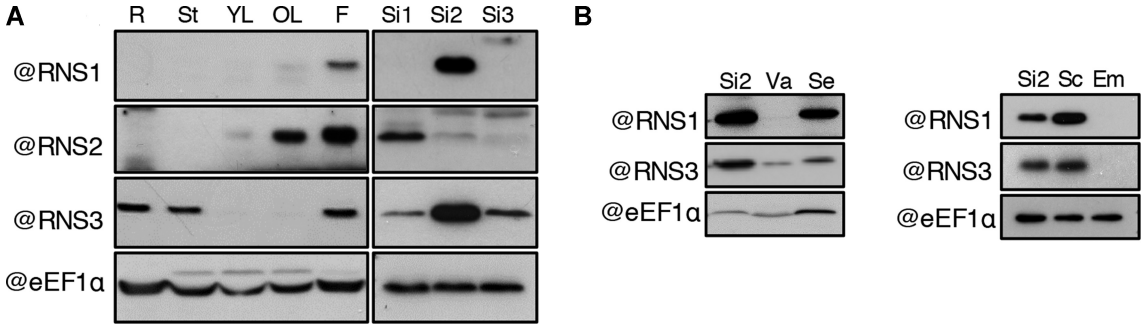

Figure 3. RNS display different protein expression profiles. (**A**) Immunodetection of RNS1, RNS2 and RNS3 in Arabidopsis Col-0 roots (R), stems (St), young leaves (YL), old leaves (OL), flowers (F), siliques (Si) at stage Si1, Si2 and Si3. (**B**) Immunodetection of RNS1 and RNS3 in valves (Va), seeds (Se), seed coats and endosperms (Sc) and embryos (Em) from siliques at stage Si2. Immunodetection of eEF1α is shown as a loading control.

RNAs extracted from the same Pi-starved seedlings were analyzed by Northern blots (Figure 5C). We showed that short and long tRF-5 (Gly) and long tRF-5 (Asp) accumulate under Pi deprivation and in agreement with (38), no clear increase in the amount of tRFs (Ala) was observed. By contrast, in the Arabidopsis KO *rns1* mutant line (Figure 4A) where no RNS1 is present (Figure 4B), no increase in the amount of tRF-5 (Gly) or tRF-5 (Asp) was observed. This shows that specific tRFs accumulation is linked to RNS1 activity *in planta*. Whether, *in planta*, upon Pi deficiency, RNS1 enhances the cleavage of all tRNAs and only some specific tRFs are stabilized or whether RNS1 essentially affects the cleavage of specific tRNAs is currently unknown.

Human RNase T2 generates long and short tRFs *in vitro*

In human, the RNase A, Angiogenin, was shown to generate only long tRFs (10). As RNases T2 from protozoans, yeast and now plants are able to produce tRFs, we can wonder whether the human RNase T2, can also cleave tRNAs. As a first attempt to address this question, Arabidopsis $tRNA^{Ala}$ transcript was incubated in the presence of purified human RNase T2 (26). As shown in Figure 6, the human enzyme is able to generate *in vitro* not only long but also short tRFs. Presently, on the one hand the involvement of the RNase III Dicer has been described for the production of short tRFs (12). On the other hand, evidence that their biogenesis is Dicer-independent have also been obtained (13,14). Thus, human RNase T2 appears to be a good candidate for future *in vivo* studies in order to decipher how short tRFs are generated in mammalian cells.

DISCUSSION

The number of tRFs functions is expanding rapidly, but many questions still remain concerning their biogenesis. This is particularly true for the production of short tRFs where almost nothing is known. Here, we first confirm that plant DCLs are likely not the major nucleases involved in tRNA cleavage and second, we demonstrate that at least three members of the Arabidopsis RNase T2 family, RNS1, RNS2 and RNS3, can generate both long and short tRFs.

Although we cannot exclude the possibility of a Dicer-dependent tRFs biogenesis in plants as argued by the work of Martinez *et al*. (16), we propose that the implication of DCLs in small tRFs production could occur, but in very particular conditions and for only a subset of tRNAs. In contrast, and in agreement with the work of Alves *et al.* (17), both RNA-seq data and tRNA cleavage assays we performed here strongly support the idea that DCLs are not the major players of tRFs production in plants.

Arabidopsis RNS represent the first example of endoribonucleases able to generate both classes of tRFs from the same tRNA species. The class II RNase T2, RNS2, the constitutively expressed and housekeeping enzyme of the family, was also shown to be induced during senescence (37). Here, we provide evidence that, class I RNS1 and RNS3 have very specific expression pattern. These two endoribonucleases can be highly expressed in seeds, in particular during their senescence, as compared to RNS2. Their spatiotemporal regulation during seed development is very accurate and must correspond to a strong biological need. Interestingly, RNS1 was also shown to be regulated by abscissic acid (ABA) (39), a hormone involved in seed dormancy and the ABA profile in seeds (40) is similar to the expression profile of RNS1 we have observed. This may suggest a link between ABA metabolism and tRFs production. Under normal growth condition, the *rns1rns3* KO mutant plants do not show any eye-visible phenotype, seeds have no apparent defects and have no reproductive deficiency, therefore studies under stress conditions appear crucial to better understand the role of RNS and tRFs in plants. RNases T2 likely regulate the overall homeostasis of rRNAs and tRNAs and their importance in tRFs production will likely come from further studies, for instance on tRFs(Gly) or tRFs(Asp) that strongly accumulate under phosphate starvation upon RNS1 induction. It is also worth mentioning that tRFs can circulate *via* the phloem (41). Thus, the place where tRFs are produced is not necessarily always the location where they play biological functions. Future research aiming at a better understanding of the tRFs production by plant RNases T2 under stress conditions or during cell development will unravel their key role in the regulation of gene expression.

The *in vivo* tRFs population is likely the result of interplay between tRNA cleavage efficiency and tRFs stability due to interaction with protein partners. RNases T2 are nonspecific single-stranded RNA nucleases, raising the

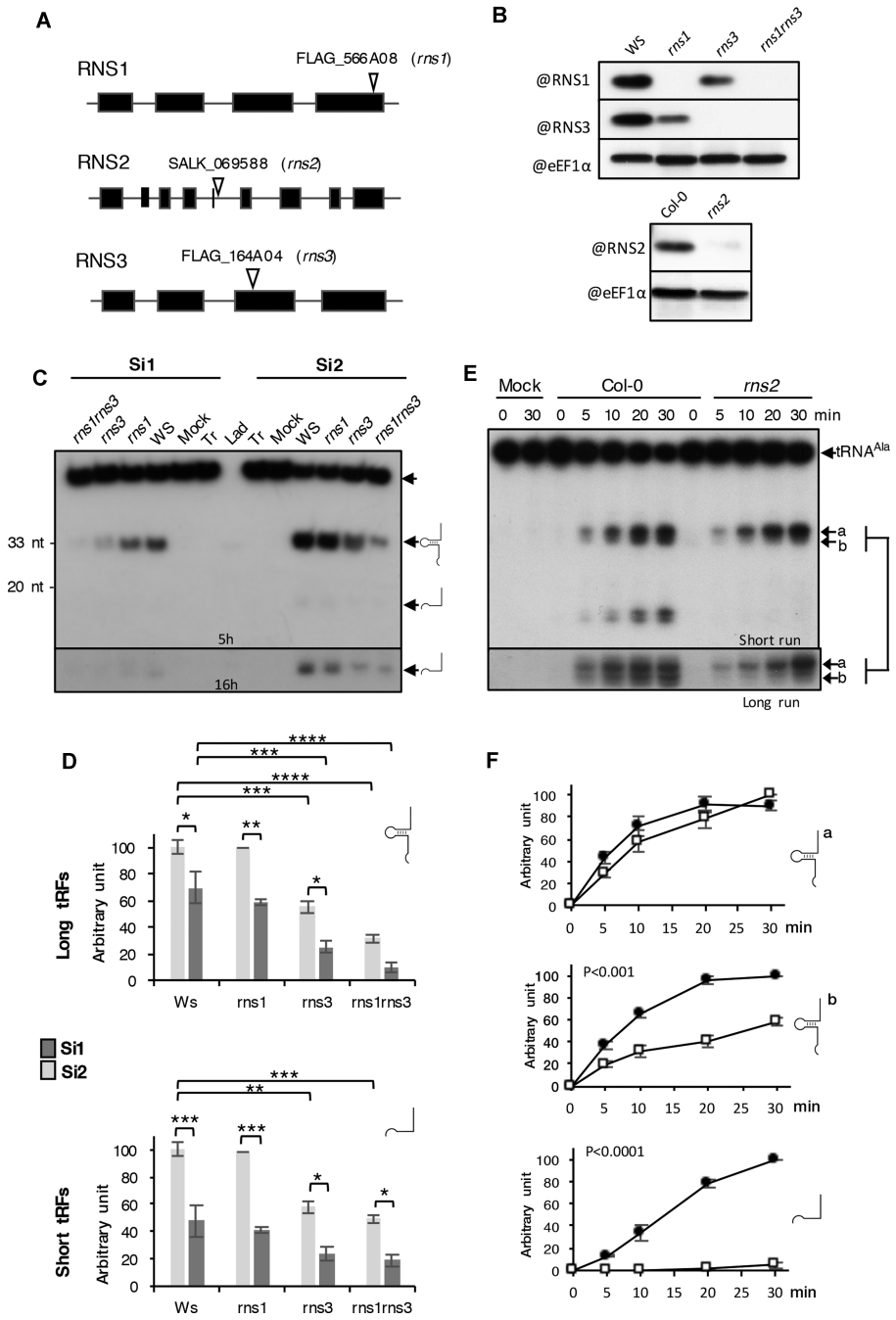

Figure 4. Arabidopsis RNS possess tRNA cleavage activities. (**A**) Representation of the Arabidopsis RNS1, RNS2 and RNS3 genes. Coding sequences are depicted in black boxes, introns with thin lines. The position of the T-DNA insertions in the mutant lines *rns1, rns2* and *rns3* is depicted by arrowheads. (**B**) Immunodetection of Arabidopsis RNS1 and RNS3 in siliques (stage Si2) of Ws, *rns1*, *rns3* and *rns1rns3* lines and of RNS2 in old leaves of wild-type Col-0 and *rns2* mutant lines. Immunodetection of eEF1α is shown as a loading control. (**C**) Cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$(UGC) transcript upon incubation, in the presence of 0.3 μg of total proteins prepared from siliques (stages Si1 or Si2) of Ws, *rns1*, *rns3* or *rns1rns3* mutant lines. After a 15 min incubation, RNAs were analyzed by northern blot with a radiolabeled oligonucleotide specific of the 5′ extremity of cytosolic *Arabidopsis* $tRNA^{Ala}$. Mock: control experiment without protein extract; Tr: *in vitro* synthesized $tRNA^{Ala}$ transcript; Lad: RNA ladder. Two exposure times for the production of short tRFs are shown. (**D**) Histograms showing the relative amount of long and short tRFs generated in experiments depicted above with siliques Si1 (dark grey) or Si2 (pale grey). Error bars represent standard errors of mean of three independent biological experiments. An arbitrary value of 100 was given to the cleavage efficiency obtained in the presence of wild-type Ws protein extract of siliques at stage Si2. Two-way ANOVA tests were used to calculate p-values. Asterisks indicate statistically significant differences (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.1$). (**E**) Kinetics of cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$ transcript upon incubation in the presence of 3 μg of total proteins prepared from senescing leaves of wild-type Col-0 or *rns2* mutant line. Experiment was performed as described in C). (**F**) Kinetics curves showing the amount of long (a or b length) and short tRFs generated in experiments shown above. Amounts of tRFs obtained after 30 min of cleavage in the presence of wild-type Col-0 protein extracts were taken as references with arbitrary values of 100. Error bars represent standard errors of mean of three independent biological experiments. According to two-way ANOVA tests, there is no significant difference in the production of long tRFa between wild-type and *rns2* while the difference is significant for the production of long tRFb ($P < 0.001$) and short tRF ($P < 0.0001$).

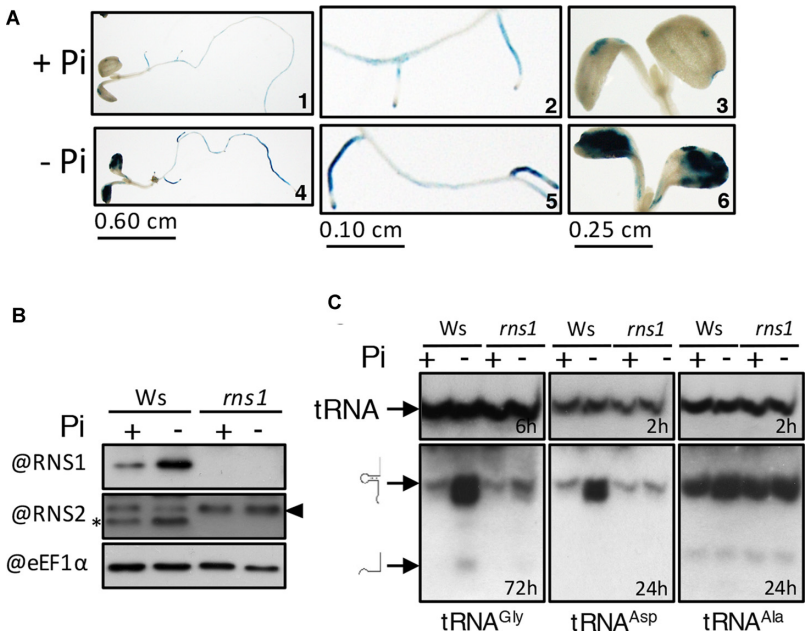

Figure 5. RNS1 is responsible for specific tRFs accumulation in Arabidopsis seedlings grown under phosphate deprivation. (**A**) Expression of RNS1 in Arabidopsis Col-0 seedlings grown in the presence (1) or absence (4) of phosphate, according to promoter-GUS fusion. In 2 and 5, primary and secondary roots and in 3 and 6, cotyledons, seen in 1 and 4, are enlarged respectively. Pictures are representative of three independent Arabidopsis lines. (**B**) Immunodetection of RNS1 and RNS2 (indicated by an arrowhead) in Arabidopsis Ws seedlings of wild type or *rns1* mutant lines grown in the presence (+) or absence (−) of phosphate. The star indicates an unspecific band. Immunodetection of eEF1α is shown as a loading control. (**C**) Hybridization of specific probes (see Supplementary Table S1) complementary to the 5′ extremity of Arabidopsis $tRNA^{Gly}$, $tRNA^{Asp}$ and $tRNA^{Ala}$ to total tRNA fractions from Arabidopsis Ws seedlings of wild type or *rns1* mutant lines grown in the presence (+) or absence (−) of phosphate. As the amount of tRNAs is high as compared to the amount of the corresponding tRFs, short and long exposure times (in hours) of the same blot are presented for each probe to visualize tRNAs and tRFs respectively.

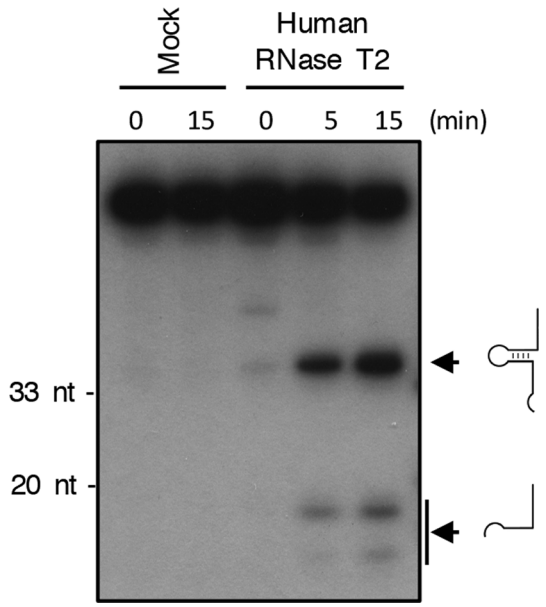

Figure 6. The human RNase T2 is able to generate long and short tRFs *in vitro*. Kinetics of cleavage of Arabidopsis *in vitro* synthesized $tRNA^{Ala}$ transcript upon incubation in the presence of pure recombinant human RNase T2. Mock: control experiment without protein extract. Experiment has been performed as described in Figure 4.

question as to whether all tRNAs are cleaved with the same efficiency? In *in vitro* experiments, long and short tRFs were always observed when Arabidopsis $tRNA^{Ala}$ was used as a substrate, but only long tRFs were generated from $tRNA^{Asp}$ transcript. This correlates with the *in vivo* situation where upon phosphate starvation, only tRF-5A (Asp) are visible whereas both classes of tRFs are found for $tRNA^{Ala}$ (Figure 5C). Similar observations were made in yeast where short tRFs are detected for $tRNA^{Arg}$ but not $tRNA^{His}$ (Figure 2B). The implication of some promoting/repressive post-transcriptional modifications in different conditions adds another layer in the understanding of tRNAs cleavage. Bacusmo *et al* (42) demonstrated that the threonylcarbamoyl adenosine t^6A modification at position 37 of the $tRNA^{Lys}$(UUU) is a positive determinant for the cleavage by the bacterial ribotoxin Prrc. On the contrary, methylation of $tRNA^{Asp}$(GUC), $tRNA^{Val}$(AAC) and $tRNA^{Gly}$(GCC) by the Dnmt2 enzyme protects them from several stress-induced endonucleolytic cleavages in *Drosophila* (43). The regulation of tRNA cleavage by post-transcriptional tuning seems to be a widespread occurrence and may play an even larger role in plants. Furthermore, *in vivo*, tRNAs are not naked molecules but complexed with proteins such as aminoacyl-tRNA synthetases, elongation factors or engaged in ribosomes. How mature tRNA molecules can escape such channeling to be cleaved in order to produce smaller RNA fragments is currently unknown. This leads to intriguing questions regarding the molecular mechanisms involved. Can the same tRNA molecule be only processed once (e.g. either in the anticodon region or in the D region) or be cut twice with a specific order of

cleavage? In L-shaped tRNA structure, the anticodon region appears to be the most accessible cleavage site and could serve as direct precursors of short tRFs. Indeed, long tRFs are present in higher amount as compared to short tRFs. By contrast, the D- or T-regions are stacked together and much less accessible, thus potentially implying the need for factors to help unfold tRNAs. To address this question, structure/function analysis to understand how tRNAs interact with RNS needs to be performed, a work that has never been done so far.

The ability of RNases T2 to produce all types of tRFs from mature tRNAs appears to be conserved in eukaryotic evolution. Indeed, we also provide evidence here that the *S. cerevisiae* Rny1p has the ability not only to produce tRNA halves as already demonstrated by several researchers (11,34) but unexpectedly also short tRFs *in vitro* and *in vivo*. Whether these short tRFs have a biological role or whether they are just degradation products warrants investigation, as yeast does not have silencing machinery and the observed concentration was relatively low. However, *in vitro*, we show that the mammal specific RNase T2 enzyme is capable of generating long and short tRFs. And we argue that the conserved tRNA cleavage activity of RNase T2 enzymes across eukarya are at the basis of tRNA fragmentation, not only to maintain RNA homeostasis and recycle nutrients, but also to produce small non coding RNAs with proper biological functions. In human cells, the presence of different types of short tRFs have been implicated in some cases of cancer and disease (44). Considering that, until now, Angiogenin has only been shown to produce long tRFs, the short ones have plausibly to be considered as the product of Dicer and RNase T2 activities. Since human RNase T2 exhibits anti-tumoral properties (26), it would be interesting to point out a causal connection with tRFs. Finally, specific tRFs were found associated with ARGONAUTE proteins in human and plants (9,13,45,46) or with a PIWI protein, Twi12, in *T. thermophila* (47). At least in plants, if RNS are the major contributors of tRFs production, the questions of how and where they are loaded on AGO proteins will need to be addressed. Thus, whether Dicer-dependent and RNase T2-dependent pathways coexist for the production of tRFs remains to be established.

DATA AVAILABILITY

Sequence data can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: RNS1, At2g02990; RNS2, At2g39780; RNS3, At1g26820; RNS4, At1g14210; RNS5, At1g14220.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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