



Novel Ribonuclease Activity Differs between Fibrillarins from *Arabidopsis thaliana*

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OPEN ACCESS

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Specialty section:

This article was submitted to
Plant Cell Biology,
a section of the journal
Frontiers in Plant Science

Received: 21 August 2017

Accepted: 16 October 2017

Published: 31 October 2017

Citation:

Rodriguez-Corona U,
Pereira-Santana A, Sobol M,
Rodriguez-Zapata LC, Hozak P and
Castano E (2017) Novel Ribonuclease
Activity Differs between Fibrillarins
from *Arabidopsis thaliana*.
Front. Plant Sci. 8:1878.
doi: 10.3389/fpls.2017.01878

Fibrillarin is one of the most important nucleolar proteins that have been shown as essential for life. Fibrillarin localizes primarily at the periphery between fibrillar center and dense fibrillar component as well as in Cajal bodies. In most plants there are at least two different genes for fibrillarin. In *Arabidopsis thaliana* both genes show high level of expression in transcriptionally active cells. Here, we focus on two important differences between *A. thaliana* fibrillarins. First and most relevant is the enzymatic activity by AtFib2. The AtFib2 shows a novel ribonuclease activity that is not seen with AtFib1. Second is a difference in the ability to interact with phosphoinositides and phosphatidic acid between both proteins. We also show that the novel ribonuclease activity as well as the phospholipid binding region of fibrillarin is confined to the GAR domain. The ribonuclease activity of fibrillarin reveals in this study represents a new role for this protein in rRNA processing.

Keywords: nucleoli, fibrillarin, ribonuclease, phosphoinositides, phosphatidic acid, glycine-arginine rich domain

INTRODUCTION

The nuclear architecture and gene regulation are some of the most relevant subjects in science today. During the last few decades, the study of the molecules involved in gene regulation has revealed several proteins, DNA and RNA as the main players. Recently, other smaller molecules like phospholipids also play a crucial process in the dynamic architecture and function of the nucleus (Sobol et al., 2013; Yildirim et al., 2013). Here we focus on the nucleoli as one of the most studied nuclear structures in eukaryotic cells. Besides ribosomal RNA (rRNA) production and ribosome pre-assembly the nucleolus is also involved in many relevant aspects of the cell life including biogenesis of small nuclear and nucleolar RNA (snRNA and snoRNA, respectively), sensing cellular stress, nucleolar dysfunctions as cancer, genetic silencing, cell cycle, and viral infection progression, senescence among others (Jacobson and Pederson, 1998; Cockell and Gasser, 1999; Garcia and Pillus, 1999; Hernandez-Verdun et al., 2010; Olson and Dundr, 2015). In plants, the nucleolus consists of four components: FC, DFC, GC, and NV. Fibrillarin was first identified in fibrillar and granular regions of the nucleolus with autoimmune sera from a patient with scleroderma

Abbreviations: AtFib, *A. thaliana* fibrillarin; DFC, dense fibrillar component; FAA, formalin-acetic acid-alcohol; FC, fibrillar center; GAR, glycine arginine rich domain; GC, granular component; NV, nucleolar vacuole; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; RRM, RNA recognition motif.

(Ochs et al., 1985). Also fibrillarins in plants were first detected in onion cells in the transition zone between the FC and the DFC (Cerdido and Medina, 1995). Ultrathin sections of rat neurons have shown fibrillarins localization at the periphery of FC and in the DFC (Desterro et al., 2003). Fibrillarins are conserved S-adenosyl-L-methionine-dependent methyltransferases which are found in all eukaryotic cells and a shorter version exists in the Archaea kingdom as well (Rodríguez-Corona et al., 2015; Shubina et al., 2016). Therefore the only activity assigned to fibrillarins has been methylation of rRNA and histone H2A (Tollervey et al., 1993; Tessarz et al., 2014; Loza-Muller et al., 2015). However, this activity is not essential for life, while fibrillarins are essential proteins in eukaryotic organisms so their precise role may still need to be defined. Reduced levels of fibrillarins in *Drosophila melanogaster* exposed to mTOR resulted in lifespan prolongation and a decrease of the nucleolar size in the fat body and intestine cells (Tiku et al., 2016). Since mTOR also regulates p53 and higher levels of p53 directly reduce the amount of fibrillarins. It correlates well with several types of cancers that show the reduction of p53 and therefore an increase of fibrillarins and higher level of methylation in ribosomes causing errors during translation (Marcel et al., 2013). Human fibrillarins also form a sub-complex with splicing factor 2-associated p32 with unknown function but independent from ribosomal processing (Yanagida et al., 2004). Furthermore, it was surprising that silencing of fibrillarins in human cells shows nuclear structure alterations in a cell cycle dependent manner before the cells death (Amin et al., 2007).

Fibrillarins in plants have been found in pulldowns of the RNA pol II transcription mediator complex as subunit 36a. *Arabidopsis thaliana* has three different genes of fibrillarins (Barneche et al., 2000). It is also involved in the viral progression and long distance trafficking of viruses in plants like the Bamboo mosaic potyvirus satRNA forms a ribonucleoprotein complex with fibrillarins and this complex allows the virus phloem based movement and infection in other tissues (Chang et al., 2016). Due to the several unknowns of this protein, we therefore decided to study both fibrillarins proteins: fibrillarins 1 (AtFib1) and fibrillarins 2 (FLP fibrillarins-like protein; AtFib2) from *A. thaliana* as a model plant. In most eukaryotic cells, fibrillarins localize primarily in the FC and DFC regions of the nucleolus, where active ribosomal DNA (rDNA) transcription and rRNA processing takes place. Both proteins contain three domains; glycine-arginine rich domain (GAR domain), methyltransferase domain and alpha region. The domains are very well conserved with the exception of the GAR domain that does not exist in the Archaea. The GAR domain has been shown to be required for nucleolar localization of fibrillarins (Snaar et al., 2000), but no further studies have been carried out on the function of this domain. In human cells, recent work demonstrated how two nucleolar proteins, fibrillarins and nucleophosmin, can phase-separate into droplets similar to the subnucleolar compartments *in vitro* and *in vivo* (Feric et al., 2016). This is attributed to the physical properties of the GAR domain resulting in a disordered structure in fibrillarins. However, the combination sequence of GAR domain and at least one RRM of fibrillarins is required for proper

subnucleolar compartment formation and maintenance (Feric et al., 2016).

In the last few years, questions as to the endonuclease activity required for the proper processing of rRNA has shown to involve a complex where several proteins are, including fibrillarins (Henras et al., 2015). In yeast depleted U3 snoRNA causes affect knob formation on nascent pre-rRNA and alter as seen on the promoters by electron microscopy (Dragon et al., 2002). During our studies throughout purifications we discover that fibrillarins have a ribonuclease activity, here we show a distinction on this activity between the two fibrillarins of *A. thaliana*. Furthermore, in this study we show the interaction of both fibrillarins with phosphoinositides, which is involved in several nuclear functions (Sobol et al., 2013; Yildirim et al., 2013), and therefore may provide clues for uncovering the fibrillarins nuclear dynamics.

MATERIALS AND METHODS

Bioinformatic Analysis

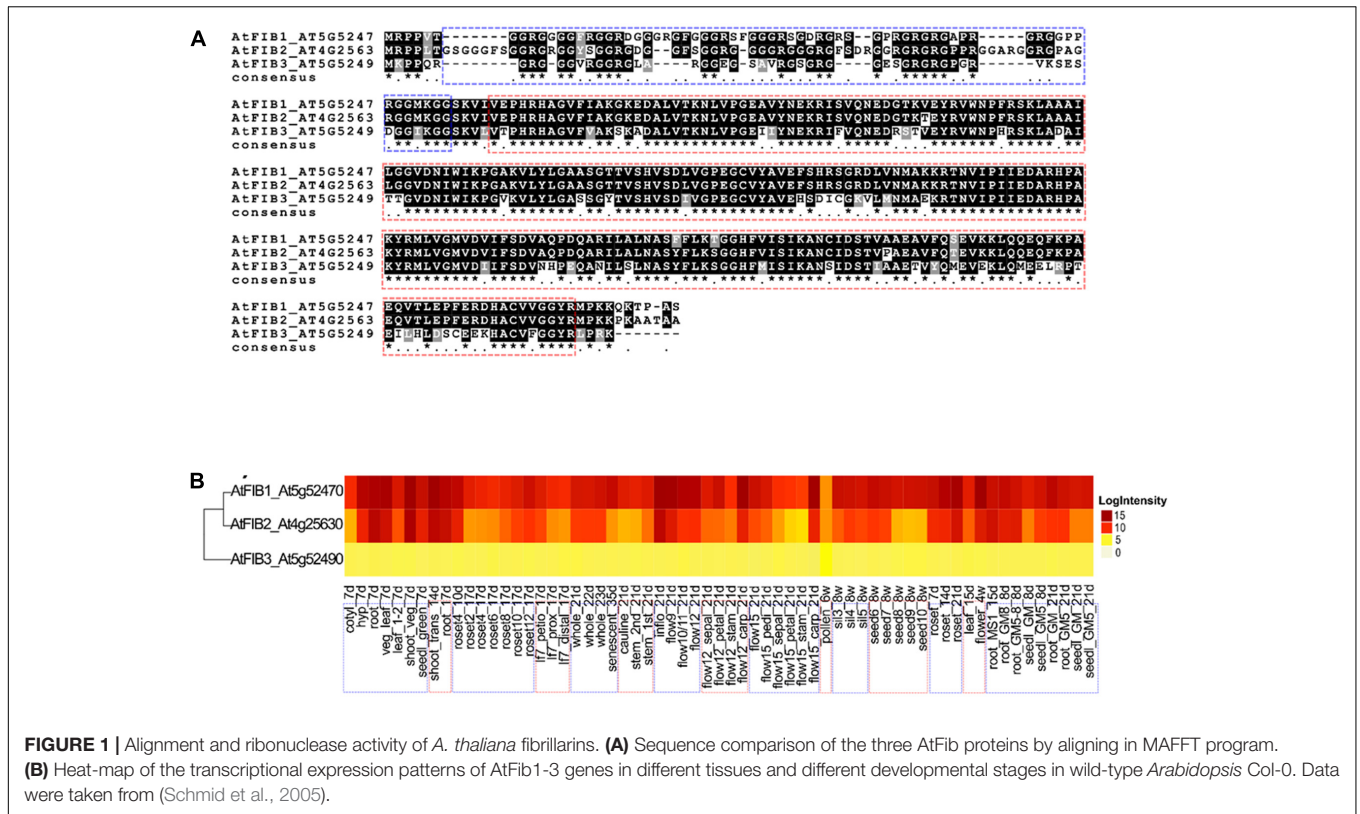
Amino acid alignment in **Figure 1A** was visualized by BOXSHADE v3.3.1C¹. Gene expression data for AtFib proteins in **Figure 1B** was taken from Schmid et al. (2005). Treatment descriptions and gene expression information can be inspected in TA^oIR (accession: ExpressionSet: 1006710873), and also can be inspected in <http://www.PLEXdb.org> (accession: AT40). The heatmap in **Figure 1B** was generated by using the ComplexHeatmap package (Gu et al., 2016) from Bioconductor project (Huber et al., 2015). Structural studies of *Arabidopsis* fibrillarins were modeled on the free software 3d-jigsaw² and edited with PyMOL v1.8.4.0.

Cloning

Arabidopsis thaliana plants were cultivated on soil in a controlled environment and photoperiod of 10–13 h light at 23°C and 11–14 h dark ***at 20°C (Yoo et al., 2007). RNA extraction was made with RNeasy[®] Plant Mini Kit (QIAGEN Sciences, Germantown, MD, United States). Sequences of AtFib1 and AtFib2 were obtained with SuperScript[™] III One-Step RT-PCR System with Platinum[™] Taq DNA Polymerase (Thermo Fischer Scientific). The specific primers to amplify AtFib1 were: forward 5′ – 3′ CATATGATGAGACCCCCAGTTACAGGA and reverse 5′ – 3′ GGATCCCTATGA GGCTGGGGTCTTTTG. To amplify AtFib2 the specific primers were: forward 5′ – 3′ CATATGATGAGACCTCCTCTAACTGGAAG and reverse 5′ – 3′ GGATCCTCTAAG CAGCAGTAGCAGCCTTTG. Forward primers have NdeI restriction enzyme sequence, reverse primers have BamHI restriction enzyme sequence for pET15b expression plasmid cloning. Same strategy was used for GAR (AtGAR2) and alpha helix (Atα2) domain cloning of AtFib2. AtGAR2 primers: forward: 5′ – 3′ CATATGATGAGACCTCCTCTA ACTGGAAG, reverse 5′ – 3′: GGATCCCAACAATCACTTTGCTTCCTCC. Atα2 primers:

¹<http://boxshade.sourceforge.net/>

²<https://bmm.crick.ac.uk/~3djigsaw/>



forward 5' – 3': CATATGCTTGTAGGCATGGTTGATGT, reverse 5' – 3': GGATCC CAAAGGCTGCTACTGCTGCTTAG.

Protein Expression and Purification

Arabidopsis thaliana fibrillarins were expressed in *E. coli* Arctic competent cells induced with 1 mM isopropyl-D-1-thiogalactopyranoside at 11°C for 24 h. Harvested cells were suspended in protein extraction buffer (500 mM NaCl, 25 mM tris pH 8, 10% glycerol, 20 mM imidazole, 0.1% triton X-100, 0.1 mM AEBSF and 0.1 mM DTT) and sonicated. After clarification by centrifugation (17400 × *g* × 15 min), the supernatant was subjected to further purification steps. The clarified supernatant was loaded onto a Ni-NTA agarose column (Thermo Fisher Scientific) and washed three times with the extraction buffer. Fibrillarins were eluted (200 mM NaCl, 25 mM tris pH 8, 20% glycerol, 0.1 mM AEBSF and 0.1 mM DTT) in a linear gradient from 20 to 200 mM of imidazole. Fibrillarins containing fractions were further purified by Q sepharose chromatography leading to single band detection of fibrillarins. Same strategy was used for AtGAR2 and Atα2 domains.

In gel RNase Activity

Proteins were separated in 15% SDS-PAGE gel. Prior to polymerization, running gel was supplied with 5 mg/mL of total RNA extracted from *A. thaliana*. After electrophoresis, gel was washed for 10 min with buffer I (10 mM Tris-HCl, 20% isopropanol, pH 7.5) and consequent incubation for 30 min in

buffer II (10 mM Tris-HCl, pH 7.5) and buffer III (100 mM Tris-HCl, pH 7.5). Gel was stained with 0.2% of toluidine blue and washed with water (Dudkina et al., 2016).

In Vitro Transcription

Arabidopsis thaliana snoRNA U3 sequence was amplified and cloned into pGEM-T® Easy Vector (PROMEGA). Once cloned, vector was linearized with NdeI enzyme for 1 h at 37°C. Transcription was made with T7 RNA polymerase (New England Biolabs Inc.) for 2 h at 37°C. Specific primers for AtsnoU3 used are: forward 5'–3' ACGACCTTACTTGAACAGGA, reverse 5'–3' CCTGTACAGACCGCGTGC GAC.

Ribonuclease Assay

Total RNA extracted from *A. thaliana* was mixed with each fibrillarins on BC200 buffer (20 mM Tris-HCl buffer, pH 8, 200 mM KCl, 0.2 mM EDTA, 10% glycerol), incubated for 1 h at 37°C and then loaded in a 1% agarose gel.

Fat Blot Assay

PIP strip with spotted phosphoinositides (Echelon Biosciences, P-6001) was probed with anti-Fib antibody. For this, the membrane was blocked with 3% BSA in PBS for 1 h followed by 3 h at room temperature of 1% BSA in PBS and 0.4 μg of each protein. After that, PIP strip was washed three times, 10-min each, with PBS-T and incubated with primary antibody for 1 h. Again washed with PBS-T and incubated with the appropriate IRDye secondary antibody for 1 h. The immunoblotting signals

were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, NE, United States).

Western Blot Assay

Fifty nanograms of each fibrillarins was loaded in a 12% acrylamide gel to perform a SDS-PAGE. Subsequently we transfer the protein to a nitrocellulose membrane and blocked with 3% of BSA in PBS at room temperature. Later was made incubation with anti-Fib rabbit antibody (1/5000) for 2 h at room temperature and a third with secondary antibody (1/4000) 1 h at room temperature, with three washes between incubations and revealed. The immunoblotting signals were analyzed by Odyssey Infrared Imager 9120 (LI-COR Biosciences, Lincoln, NE, United States).

Immunofluorescence

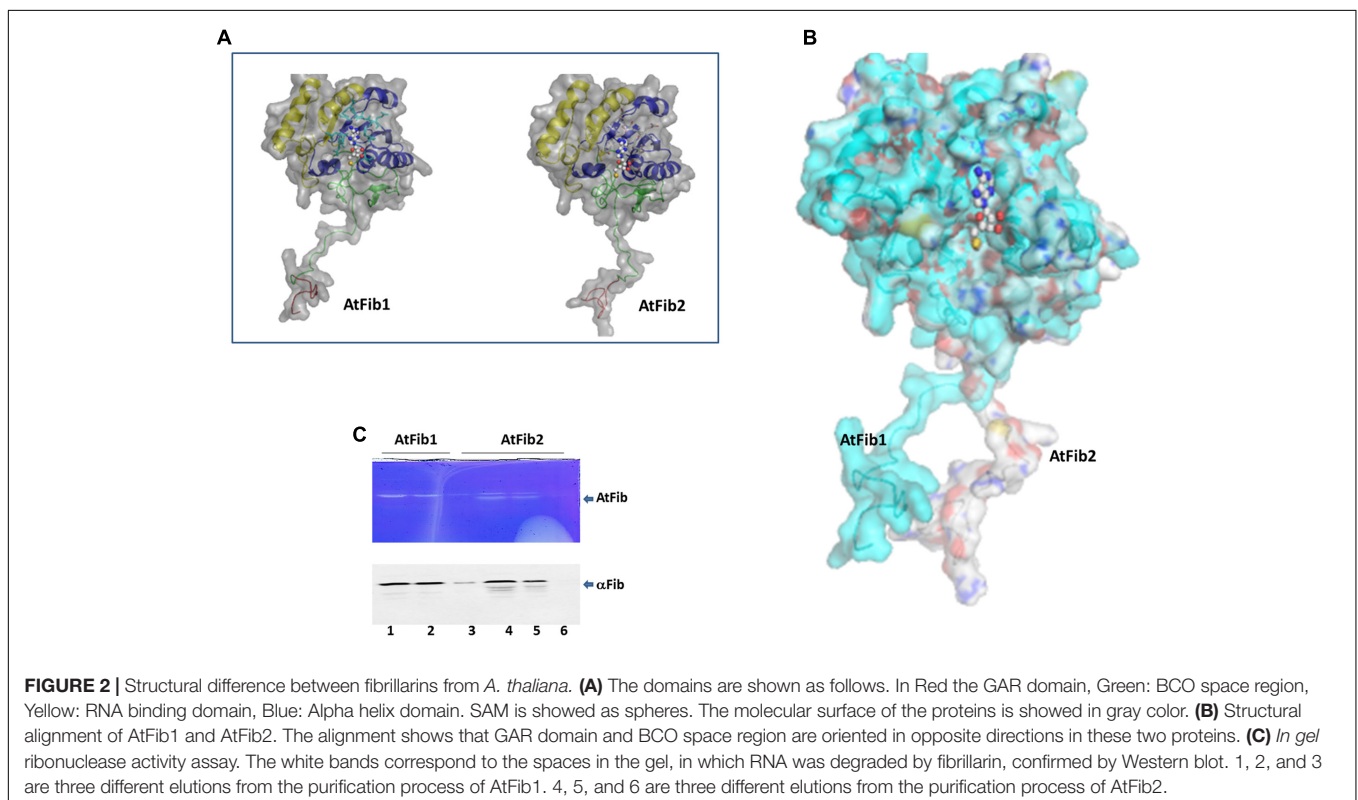
Arabidopsis thaliana callus were made according to Sugimoto and Meyerowitz (2013). Sample preparations for microscopy analysis was made as previously publish by our group (Loza-Muller et al., 2015) with callus from *A. thaliana* instead of leaves. Images were acquired in confocal microscope (Leica TCS SP5 AOBs TANDEM) and a laser-scanning microscope FV100 Olympus with 60X (NA 1.4) oil immersion objective lens.

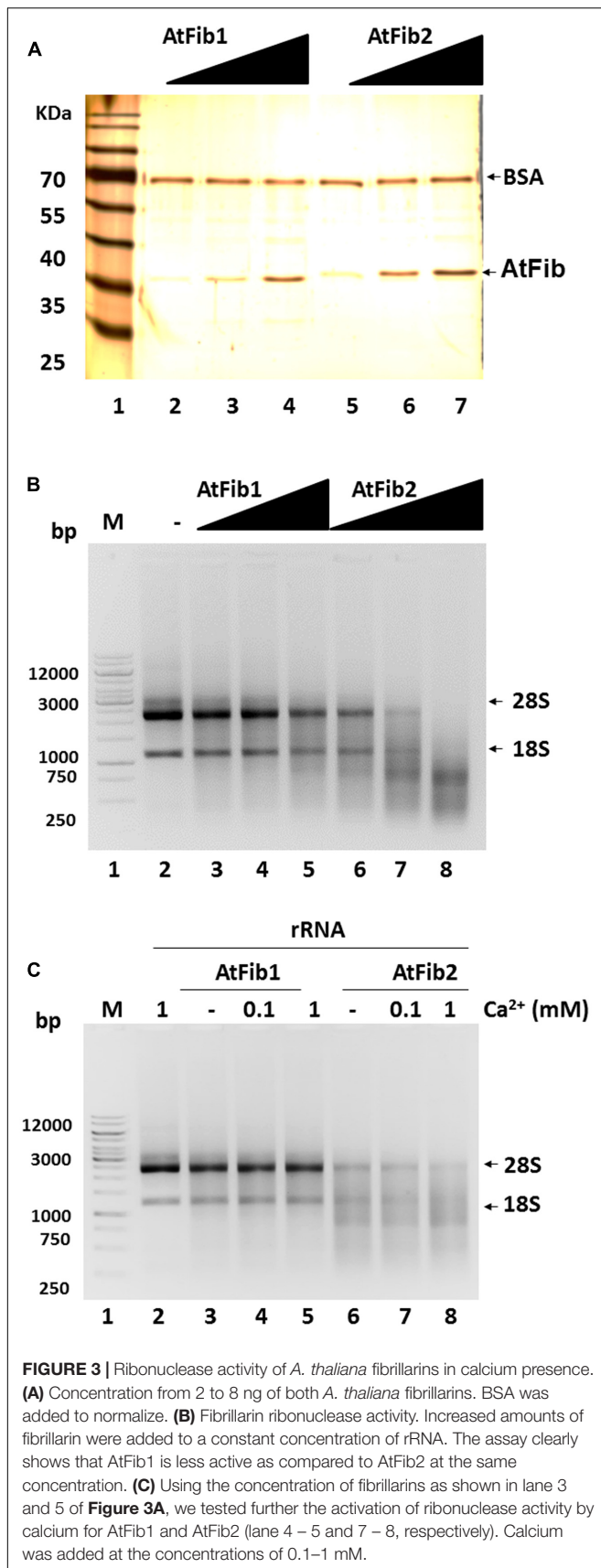
RESULTS

The comparison between the three fibrillarins genes in *A. thaliana* shows the greater amino acid difference in the GAR domain

represented by a dotted blue contour (**Figure 1A**). Considering that this part of the protein resides the main difference we check if their expression would be tissue or developmental stage specific. Transcriptional patterns of AtFib genes (**Figure 1B**) demonstrate high expression levels in the different tissues and on different developmental stages in wild-type *Arabidopsis* Col-0 (data can be inspected in <http://www.PLEXdb.org>, accession: AT40). Sequence differences between AtFib proteins (GAR domain + fibrillarins domain) could have major implications on protein activity. At transcriptional level, we found differences between AtFib genes. AtFib1 shows the highest expression levels but also little variations between treatments, while AtFib2 shows the most variation between treatments and seems to be more affected by development stages but remains expressed in all stages. AtFib3 shows the lowest level of expression values and almost no variation between tissues and development stages. We focused on AtFib1 and AtFib2 as they are expressed in almost all conditions. The *in silico* structure prediction between them (**Figure 2A**) shows that the main structural difference is due to an angle changed for the exposure of the GAR domain as can be seen in the overlay of the structures in **Figure 2B**. As in other crystal structure of fibrillarins (Rodriguez-Corona et al., 2015), the regions of methyl transferase to alpha region maintain a similar structure.

Our initial studies where to test gel mobility alterations by fibrillarins with RNA resulted in degradation of the RNA when a short incubation was carried out at room temperature. We therefore tested the purified fibrillarins with an *in gel* ribonuclease activity assay to make sure that no other protein was responsible





for this activity. The *in gel* toluidine blue staining of RNA show a white band from the lack of RNA due to its degradation at the correct molecular weight for the purified fibrillarins (**Figure 2C**). Different eluates were loaded in the ribonuclease activity gel assay and show that both fibrillarins (AtFib1 and AtFib2) have ribonuclease activity. Western blot of the bands confirmed their correspondence to fibrillarins (**Figure 2C**). AtFib2 is more susceptible to degradation as showed by Western blot and as *in gel* activity assay.

We decided to characterize this novel ribonuclease activity and purified both proteins to homogeneity (**Figure 3A**) in the exact same procedure and tested their activity under native conditions. Both fibrillarins were incubated with rRNA to test their ability to cleave rRNA. The reactions were carried out using the same amounts of fibrillarins as what is shown in the silver stained gel (**Figure 3A**). The results shown in **Figure 3B** demonstrate that AtFib2 has a potent ribonuclease activity in a dose dependent manner while AtFib1 can only show activity under the greatest amount. This correlates well with the *in gel* activity assay which shows both proteins to have activity but AtFib2 show significant rRNA cleavage. We tested if *A. thaliana* fibrillarins are activated by calcium, as other ribonucleases (Schwarz and Blower, 2014), we found that AtFib1 is not activated by calcium, while AtFib2 shows minor activation (**Figure 3C**). Interestingly, the activation of the ribonuclease activity by calcium shown for AtFib differs from that of human fibrillarins that we tested (data not shown).

Our previous studies with human fibrillarins had shown its interaction with phosphoinositides (Yildirim et al., 2013). In **Figure 4A**, AtFib1 primarily interacts with PtdIns(4)P, while AtFib2 interacts with all phosphoinositides, as well as with phosphatidic acid (PA). This is similar to what we have detected with the unique human fibrillarins (Yildirim et al., 2013 and data not shown). PA is implicated in many stress events in plants and it is also involved in phosphoinositides metabolism. Here, we detected a decrease of the ribonuclease activity by the addition of PA as seen in **Figure 4B**, lane 9. PA inactivation is reversed by the addition of calcium (**Figure 4B**, lane 10).

Nuclear phosphoinositides have been extensively studied in plant membranes but studies are lacking on the nuclear forms. To provide more information on nuclear phosphoinositides, we took advantage of the PtdIns(4,5)P₂ antibody. We carried out confocal microscopy of *Arabidopsis* callus which had membrane bound PtdIns(4,5)P₂ removed by Triton X-100 as it was done in other publications (Laboure et al., 1999) (**Figure 4C**). We show that nuclear PtdIns(4,5)P₂ has a partial colocalization with fibrillarins. Since the antibodies against fibrillarins detect both forms of fibrillarins it is impossible to discern between the two forms at this stage. We have unsuccessfully tried to raise antibodies, which would distinguish between these two fibrillarins that may lead to a better colocalization of one of them with phosphoinositides. The PtdIns(4,5)P₂ exhibits a dotted pattern in nucleoli regions and a diffuse pattern in other nuclear regions. Fibrillarins colocalizes with PtdIns(4,5)P₂ in the nucleolus but not in other regions like Cajal bodies.

In order to define the domain that has ribonuclease activity, we overexpressed two domains of the protein, which were shown to

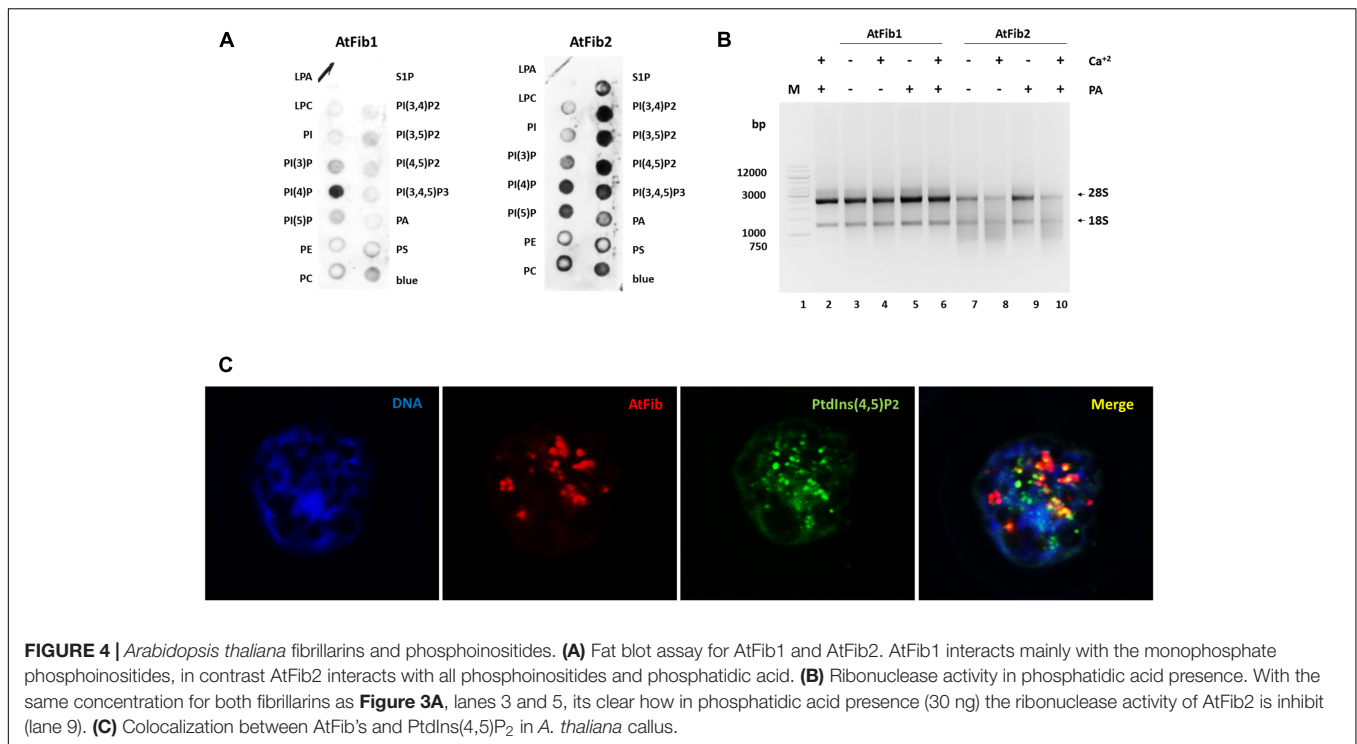


FIGURE 4 | *Arabidopsis thaliana* fibrillarins and phosphoinositides. **(A)** Fat blot assay for AtFib1 and AtFib2. AtFib1 interacts mainly with the monophosphate phosphoinositides, in contrast AtFib2 interacts with all phosphoinositides and phosphatidic acid. **(B)** Ribonuclease activity in phosphatidic acid presence. With the same concentration for both fibrillarins as **Figure 3A**, lanes 3 and 5, its clear how in phosphatidic acid presence (30 ng) the ribonuclease activity of AtFib2 is inhibited (lane 9). **(C)** Colocalization between AtFib's and PtdIns(4,5)P₂ in *A. thaliana* callus.

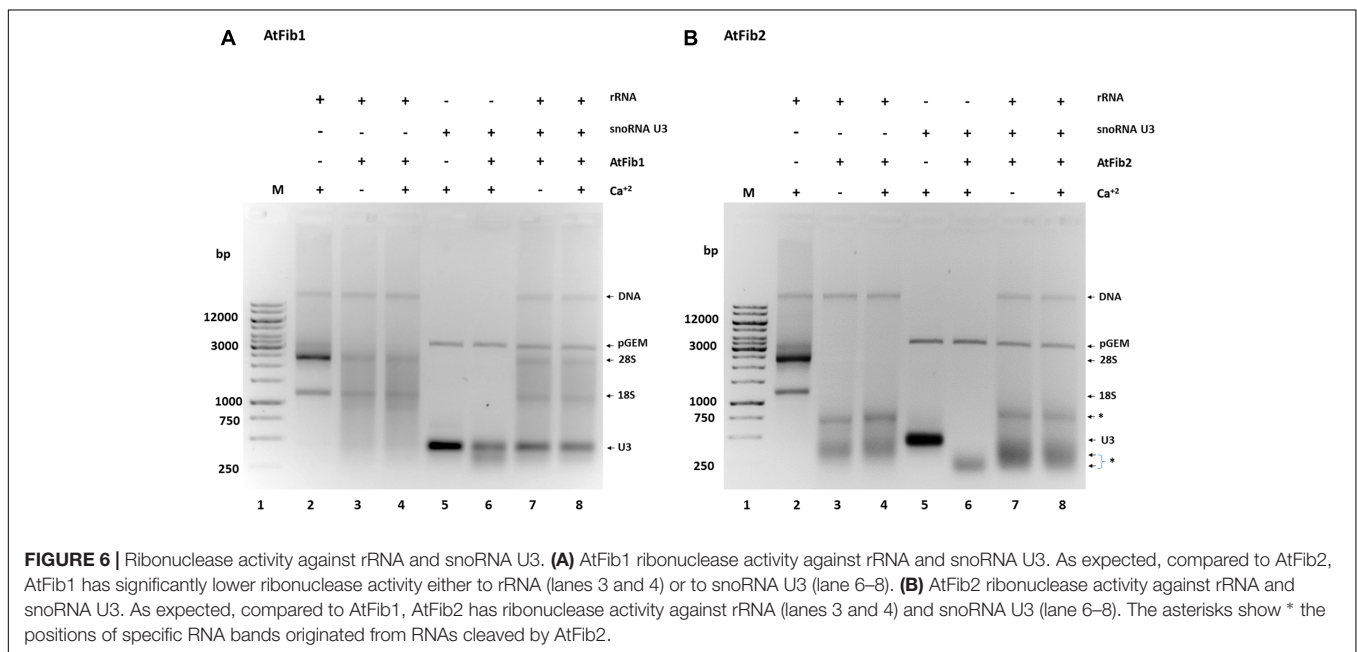
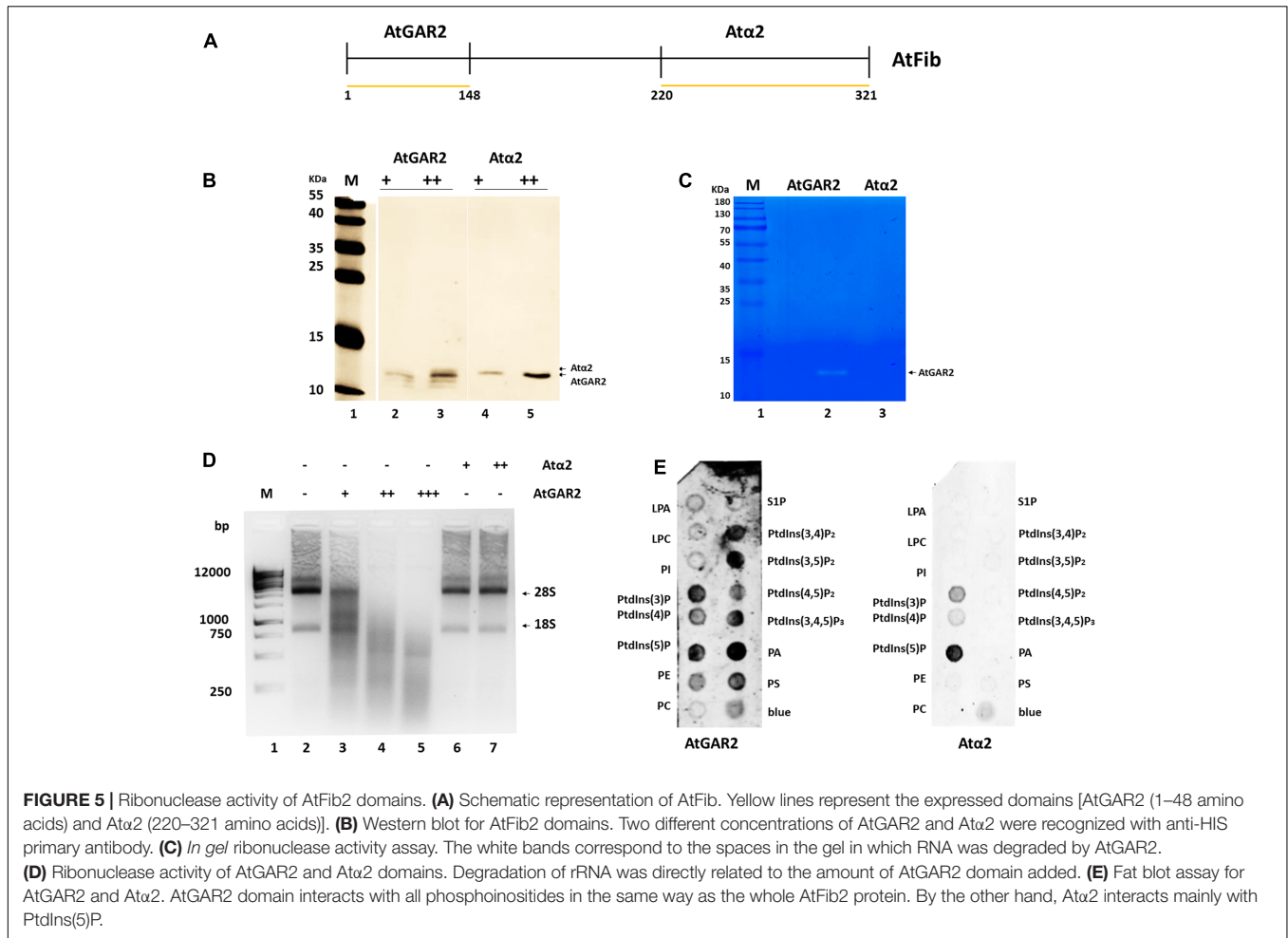
have an enzymatic activity assigned (**Figure 5A**). The N terminus contains the GAR domain and the C terminus the α domain. We purified both domains (**Figure 5B**) and tested them for activity. Only AtGAR2 domain showed high ribonuclease activity both in an *in gel* activity assay with RNA as substrate, as well as under native conditions (**Figures 5C,D**). The ribonuclease activity of AtGAR2 domain is less selective than the full fibrillar protein as it degrades both 28S and 18S simultaneously and gives a less selective pattern of bands as well; **Figure 5D**, lanes 4 and 5. The AtGAR2 domain is also the interacting domain for phospholipid binding, including all phosphoinositides species as well as PA and phosphatidylserine (PS) and resemble the full protein binding, while the alpha region of AtFib2 had no ribonuclease activity and only binds to PtdIns(5)P (**Figure 5E**). Finally, we compared AtFib1 and AtFib2 for their ribonuclease activity on U3 guide RNA and overall rRNA. We found that AtFib2 was able to cut RNA as compared between **Figures 6A,B**. AtFib1 showed only a minor reduction in the amount of rRNA but maintained the exact same pattern, while AtFib2 showed a different pattern of rRNA and U3 after interacting with this fibrillar protein as seen in **Figure 6B**, lane 6 compared to lanes 7 and 8.

DISCUSSION

The plants genomes are a mix of duplicated and triplicated regions, which results from the series of whole-genome duplication events (WGD) known as paleopolyploidy, events that occurred throughout plant evolution. These events have played a major role in Brassicaceae evolution. *A. thaliana* has undergone three paleopolyploidy events (At- α , At- β , and At- γ ;

Bowers et al., 2003; Schranz et al., 2012). These variations in gene copy number, retention of duplicated copies, and posterior sub- or neo-functionalization, increase the genetic variation (van den Bergh et al., 2016) which play an essential role in the environmental adaptation (Dassanayake et al., 2011). The major transcriptional differences between AtFib genes indicate the great importance of the functional fate of duplicated copies, which could have implications on protein activity. AtFib1 and AtFib2 are expressed in large amounts and in all tissues as seen in **Figure 1B**. Therefore, changes in the known functions can be expected for these proteins as they acquire different mutations. However, the differences are localizing to the GAR domain. Fibrillar protein is also well known to be involved in pre-rRNA processing in nucleolus in several organisms. However, the mechanism of its action is still largely unknown and a variation of function may occur during gene duplication and subsequent differential mutagenesis. Since the early experiments of Tollervey et al. (1993) with temperature sensitive mutants of Nop1 (yeast fibrillar protein), the main attributed activity of fibrillar protein was a methyltransferase for rRNA and more recent for histone H2A (Tessarz et al., 2014; Loza-Muller et al., 2015). However, even during the early experiments with mutant Nop1, the yeasts showed different phenotypes before dying at the non-permissive temperature, in particular, the *nop1.2* and *nop1.5* alleles showed a reduced level of synthesis for both 18S and 25S rRNA, moreover the production of all pre-rRNA species decreased except the main 35S primary transcript (Tollervey et al., 1993). This indicates that some mutants are not able to cut the pre-rRNA to produce the mature forms.

One of the main features of fibrillar protein is the N-terminal GAR domain. It is the least evolutionary conserved domain of the protein; however, this sequence was added in the



transition between Archaea to eukaryotic cells as it is absent in all archaeobacteria. This domain is also responsible for the phosphoinositide binding, which well correlates with the lack of it in Archaea kingdom (Amiri, 1994; Hickey et al., 2000; Wang et al., 2000). Furthermore, nucleolar localization requires the GAR domain (Snaar et al., 2000). Fibrillarins form a complex with Nop56, Nop58, a guide RNA and 15.5k, we postulate that the fibrillarins ribonuclease activity is directed by the complex to selective sites. Currently, we and others have been unsuccessful to form an active eukaryotic ribonucleoprotein complex with fibrillarins (Peng et al., 2014). These complexes have been successfully carried out in Archaea that lack the GAR domain, but not with any of the eukaryotic counterparts (Peng et al., 2014).

One elusive question in regard to ribosomal processing is the nature of the endonuclease activity involved in catalysis of the primary pre-rRNA cleavage in eukaryotic cells. Fractions carried out by Saez-Vasquez et al. (2004) showed a highly purified high-molecular-weight complex, which reproduce this cleavage *in vitro*. The authors could not discern which protein had the ribonuclease activity, but they identified nucleolin and fibrillarins as important proteins in this fraction (Saez-Vasquez et al., 2004). Other previous experiments suggested that fibrillarins is the ribonuclease protein involved in the cleavage of rRNA (Kass et al., 1990). They used specific antibodies against human fibrillarins native complex in an *in vitro* ribonuclease assay and showed a decrease in activity when the fibrillarins was blocked (Kass et al., 1990). Surprisingly the authors did not suggest that fibrillarins was involved in the cleavage of rRNA but assumed that it affected the complex. Also fibrillarins was identified in the classical RNA spreads during ribosomal transcription shown as “Christmas trees” as part of the pre-rRNA early processing complexes (Scheer and Benavente, 1990). From our work, we can speculate that AtFib2 ribonuclease activity is involved in the processing of rRNA and that when complex with Nop 56, 58, and 15.5K together with the guide RNA may direct fibrillarins for sequence specific breaks as was shown with the complex by Kass et al. (1990).

Previously we showed that human fibrillarins was able to interact with PtdIns(4,5P)₂, one of seven phosphoinositides (Yildirim et al., 2013). Amino acids 9–25 of the GAR domain of both Atfib2 and human fibrillarins are absent in Atfib1 and may explain their similarities between both of these proteins. The nuclear phospholipids, in particular phosphoinositides, can be located in nuclear speckles, intra nuclear chromatin domains as well as nucleoli. They interact with a wide range of proteins like: Star-PAP poly(A) polymerase, histone 1, TAF3, UBF, etc. (Osborne et al., 2001; Yildirim et al., 2013; Divecha, 2016). The interaction of phospholipids with such proteins can result in the activation of the protein (like Start-PAP) or affect the stability with other proteins to form particular complexes like TAF3 with H3K4me3 (Stijf-Bultsma et al., 2015). The complex nuclear environment contains large amounts of these phospholipids in a non-membrane fashion for complex formation.

Here, we show a differential binding of phospholipids to *A. thaliana* fibrillarins. Taken into account that phosphoinositides–protein interaction affects the protein ability to form new complexes it is therefore likely that both

fibrillarins in *A. thaliana* bind to different partners. This may also explain why confocal microscopy of both fibrillarins does not colocalize 100% with the PtdIns(4,5)P₂ signal as it does in human cells (Sobol et al., 2013). PA has been shown to inhibit RNase A (Hatton et al., 2015), here we show that it is also able to decrease the ribonuclease activity of fibrillarins.

It has been proposed that GAR domain can destabilize the RNA secondary structure during their interaction (Pih et al., 2000). However, it is unclear which structure can be generated when GAR domain is bound to phospholipids or during its interaction with RNA. The interaction of GAR domain with phospholipids may also explain the fibrillarins phase separation behavior for proper subnucleolar compartment formation and maintenance (Feric et al., 2016). However, the structural phase separation may be more complex involving phospholipids and their metabolism, as well as other ribonucleoproteins and guides RNA. The structure alterations of the nucleoli can be observed with different transcription inhibitors like actinomycin D. Upon transcription inhibition, the separation of nucleolar compartments forms a two phase separated system similar to what is observed with a mix of hydrophobic molecules in water (Sobol et al., 2013; Feric et al., 2016).

Several questions arise from this work including the role of fibrillarins in Cajal bodies: does it have a role in mRNA processing? Is there a ribonuclease role of fibrillarins as mediator 36a? During cell cycle, does the alteration in nuclear structure in fibrillarins depleted cells is due to degradation of structural RNA? Do viral particles require fibrillarins due to its role in RNA processing? Does GAR domain methylation by any or all of the methyltransferases (PRMT1, PRMT3, PRMT5, etc.) affect ribonuclease activity?

AUTHOR CONTRIBUTIONS

UR-C: Experiments design and conception and wrote the article. AP-S: Bioinformatics and wrote the article. MS: Microscopy related experiments. LR-Z: Experiments design and article discussions. PH: Article discussions and review. EC: Experiment design and original set of experiments that led to the research, article writing, and revisions.

FUNDING

We would like to thank for the financial support CONACYT project 2016-01-1572, FOMIX 247355, GACR (GAP305/11/2232, GA16-03346S, and GA15-08738S), TACR (TE01020118), HFSP (RGP0017/2013), project “BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University” (CZ.1.05/1.1.00/02.0109) from the European Regional Development Fund, IMG (RVO:68378050).

ACKNOWLEDGMENT

We would like to thank to Pavel Kříž, and Wilma Gonzalez for their technical help.

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- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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