NOVEL ASPECTS OF NUCLEOLAR FUNCTIONS IN PLANT GROWTH AND DEVELOPMENT

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NOVEL ASPECTS OF NUCLEOLAR FUNCTIONS IN PLANT GROWTH AND DEVELOPMENT

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Nucleolus-localization of GFP-OLI2 fusion protein in the root apical meristem of *Arabidopsis thaliana* carrying a *pOLI2::GFP-OLI2* construct. OLI2 is a ribosome biogenesis factor involved in 60S ribosome subunit biogenesis. Photo: Gorou Horiguchi.

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EDITORIAL
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Editorial: Novel Aspects of Nucleolar Functions in Plant Growth and Development

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Keywords: nucleolus, ribosome biogenesis, plant, growth, development, environmental response

Editorial on the Research Topic

Novel Aspects of Nucleolar Functions in Plant Growth and Development

The nucleolus is a prominent nuclear body that is common to eukaryotes. Since the nucleolus was first described in the 1830s, its identity had remained a mystery for longer than 100 years. Major advances in understanding of the nucleolus were achieved through electron microscopic and biochemical studies in the 1960s to 1970s followed by molecular biological studies. These studies finally established the view of the nucleolus that it is a large aggregate of RNA-protein complexes associated with the rRNA gene region of chromosome DNA, serving mainly as a site of ribosome biogenesis, where pre-rRNA transcription, pre-rRNA processing, and assembly of rRNAs and ribosomal proteins (r-proteins) into ribosome subunits occur. This function of the nucleolus appears to indicate that the nucleolus plays a constitutive and essential role in fundamental cellular activities by producing ribosomes. Recent research has shown, however, that the nucleolus is more dynamic and can have more specific and wider functions.

In plants, nucleolar functions have been lately implicated in developmental regulations and environmental responses from experimental evidence obtained mostly from genetic studies of nucleolus-related mutants. Detailed and comprehensive analysis of nucleolar components and molecular cytological characterization of sub-nucleolar domains have also provided new insights into functions and behaviors of the plant nucleolus. This Research Topic has collected articles concerning recent findings from the plant nucleolar research, with a primary focus on physiological and molecular links of the nucleolus to growth and development in plants.

In the model plant Arabidopsis, a number of mutants have been reported for genes encoding r-proteins and ribosome biogenesis factors (RBFs). Previous works showed that most of these mutants share developmental phenotypes, one of the most typical examples of which is a pointed leaf shape. Many of these mutations are also known to affect leaf polarity and cause severe leaf abaxialization in a sensitized genetic background such as *asymmetric leaves2* (*as2*). Kojima et al. characterized two pointed-leaf mutants, *oligocellula2* (*oli2*) and *g-patch domain protein1* (*gdp1*) and demonstrated that these mutations synergistically repress cell proliferation in leaf primordia and that either of them do not strongly enhance leaf abaxialization in *as2*. Both OLI2 and GDP1 proteins were shown to localize in the nucleolus and participate in ribosome biogenesis. These results suggest that the leaf cell proliferation defect and leaf abaxialization triggered by mutations in r-protein genes or RBF genes may be mediated by different mechanisms.

In animals, it is well known that perturbations of ribosome biogenesis in the nucleolus cause a particular type of stress called nucleolar stress (or ribosomal stress) activating specific signaling pathways to induce cell cycle arrest or apoptosis. In plants, however, a corresponding stress response pathway had long been unrecognized. Very recently,

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loss-of-function mutations in the NAC transcription factor gene *ANAC082* were found to suppress growth defects and developmental abnormalities in various ribosome biogenesis-impaired mutants, which has led to the hypothesis that plants respond to nucleolar stress via a plant-unique, ANAC082-dependent signaling pathway. Ohbayashi and Sugiyama outline these studies along with their research background and introduce the concept of plant nucleolar stress response as a new face of NAC-dependent cellular stress responses.

Phenotypic studies of mutants impaired in genes encoding r-proteins or RBFs have also indicated important roles of the nucleolus in various environmental responses in plants. Liu and Imai present a mini-review focusing on DExD/U-box RNA helicases among RBFs, in which information about plant DExD/U-box RNA helicases with functions in ribosome biogenesis is surveyed to highlight their involvement in adaptation to environmental stresses such as high and low temperatures.

Molecular biological reexamination of nucleolar components is another trend in exploring the hidden functions of the plant nucleolus. Fibrillarin is a major conserved nucleolar protein, which localizes at the boundary between fibrillar center and fibrillar component of the nucleolus as well as in the nucleolus-related nuclear domain Cajal body and is considered to act as a methyltransferase in the initial stage of pre-rRNA processing. Notably, most of plants have two or more different fibrillarin genes. Rodriguez-Corona et al. investigated two Arabidopsis fibrillarins AtFib1 and AtFib2 and detected a novel ribonuclease activity only in AtFib2. This discovery suggests an unidentified role of plant fibrillarin.

Fibrillarin and several other nucleolar components have been found to associate with pathogen-derived factors such as plant viral proteins, and today the role of the nucleolus in plant-pathogen interaction has become an important topic. With a special emphasis on this aspect, Kalinina et al. summarize a broad range of findings to depict a comprehensive view of the multifaceted functions of the nucleolus in growth, development, disease, and stress responses of plants.

Nucleolar proteome analysis has contributed very much to our understanding of the nucleolus. Montacié et al. carried out proteome analysis of highly purified nucleoli of Arabidopsis by mass spectrometry and identified many RBFs and also proteins non-related to ribosome biogenesis, which interestingly contain proteins of 26S proteasome. By further experiments, they demonstrated the nucleolar localization of 26S proteasome subunits and an interplay between proteasome activity and nucleolar organization.

Small nuclear ribonucleoproteins (snRNPs), complexes of proteins and a specific class of non-coding RNAs involved in RNA processing events such as pre-mRNA splicing, are contained not only in the nucleoplasm but also in the nucleolus and Cajal bodies. It is now considered that critical steps of snRNP biogenesis occur in the nucleolus and Cajal bodies. Ohtani provides an overview of the current knowledge about the roles of the nucleolus and Cajal bodies in snRNA biogenesis and discusses its possible relation to plant development and environmental responses.

Imaging techniques are nowadays essential in molecular cell biology research. Labeling of RNA with 5′-ethynyl uridine (EU) is one of such techniques recently established. Dvořáčková and Fajkus present a protocol of EU labeling optimized for visualization of plant nucleoli, which is very useful for studying nucelolar behavior and activities in plant cells.

We hope that these articles arouse interest in expanding aspects of the nucleolar functions in plants beyond the classical view of the nucleolus and inspire new research on the nucleolus across various fields of plant science.

AUTHOR CONTRIBUTIONS

MS wrote the draft and YM edited it. MS and YM revised the manuscript.

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ORIGINAL RESEARCH
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Two Nucleolar Proteins, GDP1 and OLI2, Function As Ribosome Biogenesis Factors and Are Preferentially Involved in Promotion of Leaf Cell Proliferation without Strongly Affecting Leaf Adaxial–Abaxial Patterning in *Arabidopsis thaliana*

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Leaf abaxial–adaxial patterning is dependent on the mutual repression of leaf polarity genes expressed either adaxially or abaxially. In *Arabidopsis thaliana*, this process is strongly affected by mutations in ribosomal protein genes and in ribosome biogenesis genes in a sensitized genetic background, such as *asymmetric leaves2* (*as2*). Most ribosome-related mutants by themselves do not show leaf abaxialization, and one of their typical phenotypes is the formation of pointed rather than rounded leaves. In this study, we characterized two ribosome-related mutants to understand how ribosome biogenesis is linked to several aspects of leaf development. Previously, we isolated *oligocellula2* (*oli2*) which exhibits the pointed-leaf phenotype and has a cell proliferation defect. *OLI2* encodes a homolog of Nop2 in *Saccharomyces cerevisiae*, a ribosome biogenesis factor involved in pre-60S subunit maturation. In this study, we found another pointed-leaf mutant that carries a mutation in a gene encoding an uncharacterized protein with a G-patch domain. Similar to *oli2*, this mutant, named *g-patch domain protein1* (*gdp1*), has a reduced number of leaf cells. In addition, *gdp1 oli2* double mutants showed a strong genetic interaction such that they synergistically impaired cell proliferation in leaves and produced markedly larger cells. On the other hand, they showed additive phenotypes when combined with several known ribosomal protein mutants. Furthermore, these mutants have a defect in pre-rRNA processing. *GDP1* and *OLI2* are strongly expressed in tissues with high cell proliferation activity, and GDP1-GFP and GFP-OLI2 are localized in the nucleolus. These results suggest that OLI2 and GDP1 are involved in ribosome biogenesis. We then examined the effects of *gdp1* and *oli2* on adaxial–abaxial patterning by crossing them with *as2*. Interestingly, neither *gdp1* nor *oli2* strongly enhanced the leaf polarity defect of *as2*. Similar results were obtained with *as2 gdp1 oli2* triple mutants although

they showed severe growth defects. These results suggest that the leaf abaxialization phenotype induced by ribosome-related mutations is not merely the result of a general growth defect and that there may be a sensitive process in the ribosome biogenesis pathway that affects adaxial–abaxial patterning when compromised by a mutation.

Keywords: OLI2, GDP1, ribosome biogenesis, cell proliferation, adaxial–abaxial polarity regulation, leaf development, Arabidopsis

INTRODUCTION

Ribosome biogenesis drives cellular growth, and, in principle, individual cells must grow twofold before division during the cell cycle. To do so, a large proportion of the gene expression machinery in a cell is devoted to ribosome biogenesis (Warner, 1999). In multicellular organisms, behavior of individual cells is under the control of developmental programs to establish the appropriate shape and function of tissues and organs. Therefore, ribosome biogenesis is expected to be important to developmental pattering. However, the details of this putative connection are not well understood.

In eukaryotes, cytosolic ribosomes consist of a 60S subunit and a 40S subunit. Ribosome biosynthesis initiates in a specialized membraneless nuclear subcompartment, the nucleolus. Pre-ribosomal RNA [pre-rRNA: 45S rRNA in *Arabidopsis thaliana* (Arabidopsis) and 35S rRNA in *Saccharomyces cerevisiae* (yeast)] is transcribed by RNA polymerase I (Pol I) as a polycistronic transcript from rDNA repeats and contains 25–28S, 5.8S, and 18S rRNA sequences that are flanked by a 5′ external transcribed spacer (5′-ETS) and a 3′-ETS and separated by internal transcribed spacer 1 (ITS1) and ITS2 (Layat et al., 2012). Ribosome biogenesis requires more than 200 ribosome biogenesis factors (RBFs) and small nucleolar RNA (snoRNA) species in addition to ribosomal proteins (r-proteins), and has been best characterized in yeast. The 90S pre-ribosome, also designated as the small subunit (SSU) processome, is a huge ribonucleoprotein complex in which a nascent pre-rRNA and a subset of r-proteins for SSU are encapsulated by U three protein (UTP) complexes and U3 small nucleolar ribonucleoprotein (snoRNP). Numerous RBFs dynamically join and dissociate from the 90S pre-ribosome in a hierarchical manner and carry out folding, cleavage, and trimming of rRNA precursors, as well as assembly of r-proteins with rRNAs to produce pre-40S ribosomes (Grandi et al., 2002; Pérez-Fernández et al., 2007; Kornprobst et al., 2016; Chaker-Margot et al., 2017; Sun et al., 2017). The remaining 3′ part of the pre-rRNA forms the large subunit (LSU) processome and produces pre-60S ribosomes (Konikkat and Woolford, 2017). The 5S rRNA is transcribed separately by Pol III and forms a complex with RPL5 and RPL11, then joins to a pre-60S particle (Zhang et al., 2007). Final maturation of pre-ribosomal subunits takes place after export from the nucleus into the cytoplasm (Nerurkar et al., 2015).

Approximately 250 ribosome biogenesis factors have been identified in yeast by genetic and proteomics analyses. Similarly, 286 ribosome biogenesis factors have been identified in human cells, but among them, 74 do not have a yeast ortholog (Tafforeau et al., 2013). For land plants, orthologs to about 75% of yeast ribosome biogenesis factors were identified by bioinformatics methods (Ebersberger et al., 2013). However, only a small fraction of these molecules have been functionally characterized in Arabidopsis (Weis et al., 2015a). These studies suggest that molecular mechanisms for ribosome biogenesis are largely conserved, but that some processes are mediated by species-specific factors. A recent nucleolar proteomics analysis of Arabidopsis supported this interpretation (Palm et al., 2016).

A plant-specific feature of ribosome biogenesis is also seen in two routes of rRNA processing (Weis et al., 2015a). In Arabidopsis, transcription of 45S rRNA is terminated by endonucleolytic cleavage at site B_0 in the 3′-ETS by RIBONUCLEASE THREE LIKE2 (atRTL2; Comella et al., 2007). Then endonucleolytic cleavage at site P within the 5′-ETS takes place by the action of EXORIBONUCLEASE2 (XRN2) and U3 snoRNP to yield 35S rRNA (Sáez-Vasquez et al., 2004; Zakrzewska-Placzek et al., 2010). There are two alternative routes for further processing of 35S rRNA accompanied by simultaneous removal of the 3′-ETS (Missbach et al., 2013). In one pathway, the 5′-ETS is removed before cleavage within ITS1 (5′-ETS-first pathway) while in the other, cleavage within ITS1 of 35S takes place prior to 5′-ETS cleavage (ITS1-first pathway). In yeast, processing of pre-rRNA strictly follows the 5′-ETS-first pathway, while the ITS1-first pathway is the major route in mammals (Fernández-Pevida et al., 2015; Henras et al., 2015).

In Arabidopsis, impaired function of RBFs affects normal processing of pre-rRNAs as well as diverse developmental processes, such as auxin response, cell proliferation, root epidermal patterning, vascular patterning, leaf shape regulation, callus formation, and development of the gynoecium, embryo, and female gametophyte (Shi et al., 2005; Griffith et al., 2007; Petricka and Nelson, 2007; Lange et al., 2008, 2011; Fujikura et al., 2009; Li et al., 2009; Abbasi et al., 2010; Huang et al., 2010; Liu et al., 2010; Ohbayashi et al., 2011; Wieckowski and Schiefelbein, 2012; Cho et al., 2013; Kumakura et al., 2013; Missbach et al., 2013; Hang et al., 2014; Weis et al., 2014, 2015b). Many of these phenotypes are also shared by mutants defective in a gene for an r-protein (for review, see Byrne, 2009; Horiguchi et al., 2012; Machida et al., 2015). These findings suggest that production and/or function of ribosomes may be associated with developmental regulation. Recently, impaired ribosome biogenesis in mutants, such as *root initiation defective2* (*rid2*), was shown to induce ribosomal stress (Ohbayashi et al., 2017). Explants from *rid2* are unable to form callus at high temperature and produce pointed leaves, but these phenotypes are suppressed by mutations in the NO APICAL MERISTEM, ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR1/2 and, CUP-SHAPED COTYLEDON2

(NAC) transcription factor gene *SUPPRESSOR OF RID TWO1* (*SRIW1*)/*ANAC082* (Ohbayashi et al., 2017). Notably, *rid2* mutants are defective in the processing of rRNAs, but this phenotype is not suppressed by *sriw1*, indicating that *SRIW1* mediates ribosomal stress to induce developmental alterations (Ohbayashi et al., 2017).

The potential developmental roles of ribosomes have been further expanded by observations that mutants defective in r-protein significantly enhance the leaf polarity defect of *asymmetric leaves1* (*as1*) and *as2* (Pinon et al., 2008; Yao et al., 2008; Horiguchi et al., 2011; Szakonyi and Byrne, 2011; Casanova-Sáez et al., 2014) and *revoluta* (*rev*) (Pinon et al., 2008). In the double mutants, leaves fail to expand into a flat laminar structure, but form in a trumpet- or needle-like shape. Normally, the vascular tissues are organized such that xylem and phloem tissues face the adaxial (upper) and abaxial (lower) sides of the leaves, respectively (Waites and Hudson, 1995). In contrast, vascular tissues in radialized leaves are organized with the phloem tissues surrounding the vascular tissues, or in extreme cases the vascular tissues are absent (Pinon et al., 2008; Yao et al., 2008; Horiguchi et al., 2011). These double mutants have increased or expanded expression of abaxially expressed genes (Pinon et al., 2008; Yao et al., 2008; Horiguchi et al., 2011), suggesting that mutations in r-protein genes enhance leaf abaxialization in the *as* mutant backgrounds. A similar leaf polarity defect was also found in mutants defective in *Arabidopsis PUMILIO23* (*APUM23*), which encodes a member of the Nop9 family involved in regulation of rRNA processing (Thomson et al., 2007; Abbasi et al., 2010; Droll et al., 2010; Huang et al., 2014; Zhang and Muench, 2015; Zhang et al., 2016). More recently, mutations in *RNA HELICASE10* (*RH10*), *NUCLEOLIN-LIKE1* (*NUC-L1*), and *RID2* were also shown to enhance the leaf polarity defect of *as2* (Matsumura et al., 2016). RH10 is a member of the DEAD-box RNA helicases, putative orthologs of which are Rrp3 in yeast and DDX47 in human (O'Day et al., 1996; Matsumura et al., 2016). NUC-L1 is an ortholog of nucleolin in human and plays multiple roles in ribosome biogenesis (Sáez-Vasquez et al., 2004). RID2 is a homolog of Bud23 in yeast, which is a methyltransferase that catalyzes the methylation of G1575 in 18S rRNA (Ohbayashi et al., 2011; Létoquart et al., 2014). Yeast and human orthologs of RH10, NUC1, and RID2 are components of, or are associated with, the SSU processome (Turner et al., 2009; Phipps et al., 2011; Sardana et al., 2013). These findings imply that correct ribosome production and/or function are required for leaf adaxial/abaxial patterning (Matsumura et al., 2016).

To understand the mechanisms of ribosome biogenesis in plants and how it is connected to developmental programs, we have been trying to genetically identify individual RBFs and have characterized their molecular and developmental phenotypes. Although ribosome biogenesis is highly complicated, comparison of phenotypes among different RBF mutants would provide information concerning the general and specific functions of each factor. The purpose of this study is to find differential requirement of RBFs in two major leaf developmental processes, namely cell proliferation and abaxial–adaxial pattering. In this study, we found a novel gene for a nucleolar protein, G-PATCH DOMAIN PROTEIN1 (GDP1), and characterized it together with previously identified OLIGOCELLULA2 (OLI2; Fujikura et al., 2009), which is a homolog of yeast nucleolar protein Nop2 involved in formation of 5-methylcytosine (m5C) at C2870 in 25S rRNA (Sharma et al., 2013). Loss-of-function mutants of *GDP1* and *OLI2* exhibited a range of phenotypes frequently found in mutants defective in r-proteins or RBFs. Interestingly, *gdp1*, *oli2*, and double mutants between them did not show strongly enhanced *as2* leaf polarity defects despite their association with significantly impaired cell proliferation in leaves. These results suggest that the roles of ribosome biogenesis/function in leaf adaxial/abaxial patterning and other developmental processes are at least partially separate.

MATERIALS AND METHODS

Plant Materials

The wild type accession of Arabidopsis used in this study was Columbia-0 (Col-0). T-DNA insertion lines (Salk_065904 [*gdp1-1*], Salk_041661 [*gdp1-2*]) were obtained from the Arabidopsis Biological Resource Center (ABRC). *gdp1-3* was previously identified as #416 (Horiguchi et al., 2006a,b), and *oli2-1* and *oli2-2* (Salk_129648) were reported previously (Fujikura et al., 2009). Seeds were sown on rockwool and grown at 22°C under a 16-h light/8-h dark photoperiod for quantitative characterization of leaf phenotypes and RNA preparation. Seedlings were watered daily with 0.5 g L^{-1} of Hyponex (Hyponex Japan). For fluorescence imaging of root, seedlings were grown for 5 days on half-strength Murashige and Skoog (MS) medium supplemented with Gamborg B5 vitamins and 3% (w/v) sucrose, and solidified with 0.5% (w/v) gellan gum at 22°C under a 16-h light/8-h dark photoperiod. Oligonucleotide pairs for genotyping of *gdp1* alleles are listed in Supplementary Table S1.

Generation of Transgenic Plants

An approximately 1.6-kb *GDP1* promoter region with or without the *GDP1* transcribed region was amplified and cloned into pDONR201 using BP clonase (Thermo) followed by transfer of the insert into pBGGUS (Horiguchi et al., 2005) or pHWG (Horiguchi and Tsukaya, unpublished) with LR clonase II (Thermo) to yield p*GDP1*::*GUS* and p*GDP1*::*GDP1-GFP* constructs, respectively. For construction of p*OLI2*::*GUS*, an *OLI2* promoter DNA fragment and *GUS* cDNA were cloned into pDONR P4P1R and pENTR/D-TOPO (Thermo), respectively, and combined into the Gateway binary vector, pGWB501 (Nakagawa et al., 2007), with LR clonase II plus (Thermo). For construction of p*OLI2*::*GFP-OLI2*, a 3.6-kb promoter region of *OLI2*, *GFP* cDNA, and a transcribed region plus a 4.1-kb 3′ untranscribed region of *OLI2* were amplified and cloned into pSMAH621 digested with HindIII and SacI using an In-Fusion HD cloning kit (Clontech). These constructs were introduced into Arabidopsis by the floral dip method (Clough and Bent, 1998). Transgenic plants with a single T-DNA insertion were selected and homozygous T3 plants were used.

Oligonucleotides used in the construction of these vectors are listed in Supplementary Table S1.

RNA Analyses

Total RNA was prepared from shoots of wild type, *gdp1*, *oli2*, and *gdp1 oli2* using Trizol reagent (Thermo). Isolated RNA was treated with DNase I (Thermo) followed by first-strand cDNA synthesis with SuperScript III reverse transcriptase (Thermo) primed either with oligo (dT) (for messenger RNA) or random hexamers (for rRNAs). The cDNAs were subjected to semi-quantitative and quantitative RT-PCR (RT-qPCR). RT-qPCR was carried out using GoTaq qPCR master mix (Promega) with an ABI7500 real-time PCR system (Thermo) by the $\Delta\Delta$*C*t method. The expression level of *ACTIN2* (*ACT2*) was used as an endogenous control for mRNAs, while 18S rRNA was used as an endogenous control to determine rRNA intermediate levels. Oligonucleotide pairs used to detect *AUXIN RESPONSE FACTOR3* (*ARF3*), *KANADI2* (*KAN2*), *YABBY5* (*YAB5*), *PHABOLUSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REV*, and *ACT2* were described previously (Iwakawa et al., 2007). Other oligonucleotide pairs are listed in Supplementary Table S1.

For detection of rRNA intermediates by Northern hybridization, aliquots of 3 μg of total RNAs isolated from 12-day-old shoots were separated by formaldehyde-agarose gel electrophoresis (1.2%, w/v) and transferred onto nylon membranes by downward capillary transfer. After ultraviolet crosslinking of RNA to the nylon membranes, hybridization was carried out using a DIG Northern starter kit (Sigma–Aldrich) and hybridization signals were detected using a digital imaging system (LAS 4000 mini; GE Healthcare). RNA probes were generated using 5′-ETS, ITS1, and ITS2 DNA fragments amplified with the appropriate oligonucleotide pairs (Supplementary Table S1) and T7 RNA polymerase.

Microscopic Observation

Quantitative analyses of leaves were carried out as described (Horiguchi et al., 2005). Plants were gron for 25 days and first leaves were fixed in a formalin/acetic acid/alcohol [FAA, formalin: acetic acid: 70% (v/v) ethanol = 1:1:18] solution, cleared using a chloral hydrate solution (200 g chloral hydrate, 20 g glycerol, and 50 ml dH_2O) and observed by stereomicroscope (M165FC; Leica) and differential contrast interference microscope (DM2500; Leica), respectively. Leaf blade area and the projection area of palisade cells in adaxial subepidermal layer was determined using Image J[1]. For each leaf, the palisade cell area was determined by mearuing at least 20 cells. Average palisade cell size was determined using data from 10 leaves. Cell density in adaxial subepidermal layer was manuary determined by counting cells in a unit area. The number of cells in adaxial subepidermal layer per leaf was estimated by dividing the leaf blade area with the cell density. For fluorescence imaging of p*OLI2*::*GFP-OLI2*/*oli2-1* and p*GDP1*::*GDP1-GFP*/*gdp1-1* lines, roots of 5-day-old seedlings were fixed in phosphate buffered saline (PBS, pH 7.0) containing 4% (w/v) paraformaldehyde, washed twice with PBS, and stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Merck) or Calcofluor White M2R (Merck) and cleared with TOMEI-II (Hasegawa et al., 2016). For fluorescence imaging of leaves, 14-day-old shoots grown on rock wool were fixed in PBS [pH 7.0 containing 4% (w/v) paraformaldehyde, washed twice with PBS, and treated with TOMEI-II]. Fluorescent signals were observed with a confocal laser scanning microscope (LSM710 or LSM800; Zeiss). Histochemical staining of promoter::*GUS* lines was carried out according to Donnelly et al. (1999).

[1]https://imagej.nih.gov/ij/

RESULTS

Identification of *gdp1* Mutants and Characterization of Their Vegetative Phenotypes

During the course of studying mutants with altered leaf size and shape, we found that two T-DNA insertion lines of At1g63980 (Salk_065904 and Salk_041661) exhibited the "pointed-leaves" phenotype that is typically observed in mutants defective in r-protein genes and ribosome biogenesis genes (**Figures 1A–C,E**). An additional allele was also found in our mutant collection reported previously (#416; Horiguchi et al., 2006a,b) that had a 26-bp deletion in the third exon and a point mutation in the third intron (**Figures 1A–C**). As At1g63980 was not a characterized gene and it was apparent that At1g63980 does not encode an r-protein, we decided to characterize these mutants in relation to ribosome biogenesis.

The At1g63980 gene product has a G-patch domain near its amino-terminal region (**Figure 1D**). The G-patch domain is about 48 amino acid residues in length and contains several conserved glycine residues (Supplementary Figure S1). This domain is often found in RNA processing proteins with or without known RNA-binding motifs (Aravind and Koonin, 1999). Therefore, we named At1g63980 *G-PATCH DOMAIN PROTEIN1* (*GDP1*), and Salk_065904, Salk_041661, and #416 were designated as *gdp1-1*, *gdp1-2*, and *gdp1-3*, respectively (**Figure 1C**). The Arabidopsis genome includes at least 15 G-patch domain-containing protein genes. The structures of individual G-patch domain proteins differ in the location and number of G-patch domains, combination of RNA-binding motifs, and amino acid lengths (Supplementary Figure S1). Among them, *GDP1* appears to be a single-copy gene.

The two T-DNA insertion alleles of *gdp1* did not accumulate *GDP1* transcripts at a detectable level, while *gdp1-3* showed *GDP1* transcript accumulation at a lower level than wild type, as determined by RT-PCR using an oligonucleotide pair that amplifies the whole coding region (**Figure 1F**). When oligonucleotide pairs designed to amplify partial *GDP1* cDNA fragments corresponding to the 5′ and 3′ regions of the coding sequence were used, the 5′ fragment was amplified in *gdp1-1* at a level similar to the wild type, but neither the 5′ nor 3′ regions were detectable in *gdp1-2* (**Figure 1F**). In *gdp1-3*, both fragments were detected at lower levels than in wild type (**Figure 1F**). These results suggest that *gdp1-2* is a null allele. We also considered both

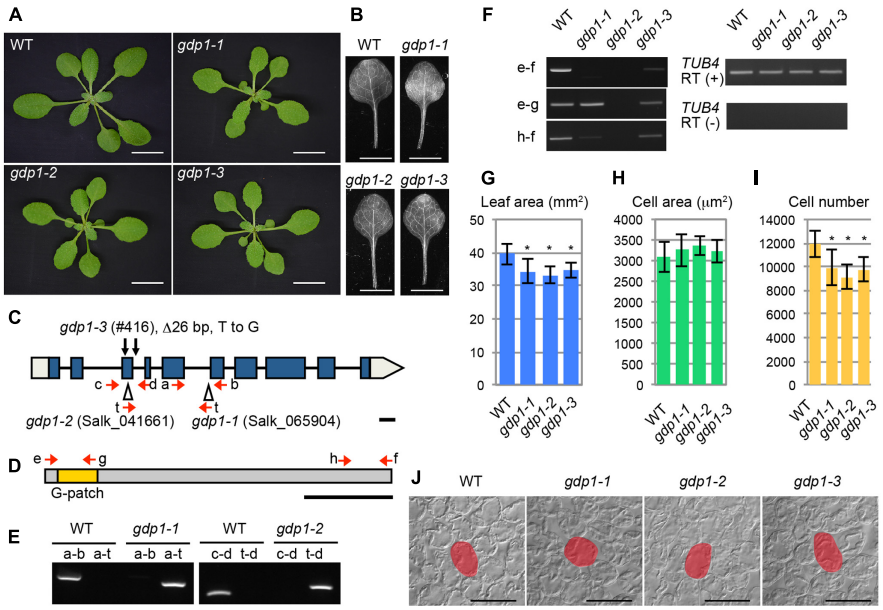

FIGURE 1 | Genetic analyses of *gdp1* alleles. **(A)** Shoots of wild type (WT) and *gdp1-1*, *gdp1-2*, and *gdp1-3* alleles grown for 25 days. Bars, 1 cm. **(B)** The first leaves of WT and *gdp1-1*, *gdp1-2*, and *gdp1-3* alleles. Bar, 5 mm. **(C)** Schematic diagram of mutation points of *gdp1* alleles. Red arrows indicate oligonucleotides used for genotyping of *gdp1* alleles. Black arrows indicate the mutation points in *gdp1-3*. T-DNA insertions in *gdp1-1* and *gdp1-2* are indicated by triangles. Bar, 100 bp. **(D)** Schematic diagram of GDP1 protein. Arrows indicate the positions of oligonucleotides on the corresponding *GDP1* transcript used for RT-PCR analysis. Bar, 100 amino acid residues. **(E)** Genotyping of *gdp1-1* and *gdp1-2* alleles. Oligonucleotide pairs indicated by letters were used to amplify genomic DNA fragments from the WT and the two *gdp1* alleles. **(F)** RT-PCR analysis of *GDP1* transcripts. Total RNAs were isolated from 10-day-old shoots and subjected to reverse transcription followed by PCR with oligonucleotide pairs indicated by letters. **(G–I)** Quantitative analyses of leaf phenotypes. The leaf blade area **(G)**, the projection area of leaf mesophyll cells **(H)**, and the number of leaf mesophyll cells **(I)** in the adaxial subepidermal layer of palisade tissues of first leaves are shown. First leaves were harvested from 25-day-old plants. $n = 10$, mean ± SD. Asterisks indicate statistically significant differences compared to WT ($P < 0.05$, Student's *t*-test). **(J)** Leaf palisade cells observed from a paradermal view. Bar, 100 μm. Representative cells are highlighted.

gdp1-1 and *gdp1-3* to be strong loss-of-function alleles as their phenotypes were almost identical to *gdp1-2*, as shown below.

We examined the leaf phenotypes of *gdp1* at the cellular level. Both the area of the leaf blade and the number of palisade cells in the subepidermal layer of the first leaves were reduced by about 20% in *gdp1* compared to the wild type (**Figures 1G,I**). On the other hand, the projection area of palisade cells was similar in both *gdp1* and wild type (**Figures 1H,J**). These phenotypes were similar to those observed in *oli2*, *oli7*, and *oli5*, the latter two of which are defective in paralogous r-protein genes, *RPL5A* and *RPL5B* (Fujikura et al., 2009). These *oli* mutants showed strongly enhanced cell enlargement in leaves of *angustifolia3* (*an3*), which is defective in a transcription coactivator (Horiguchi et al., 2005) by further reducing the leaf cell number (Fujikura et al., 2009). Similar to these mutants, when *gpd1-1* was crossed with *an3-4*, the resultant double mutant showed a further decrease in number of leaf palisade cells in the subepidermal layer (86% fewer than wild type) and triggered excessive cell enlargement (**Figure 2**; 237% larger than wild type), which is known as compensated cell enlargement (Horiguchi and Tsukaya, 2011; Hisanaga et al., 2015).

The above developmental phenotypes are quite similar to those observed in mutants defective in r-protein genes and ribosome biosynthesis genes. To examine potential genetic interactions between *gdp1* and these ribosome-related mutants, *gdp1-1* was crossed with various r-protein defective mutants reported previously (**Figure 3**; Horiguchi et al., 2011). Generally, these double mutants showed an additive phenotype as determined from the number of leaf palisade cells (**Figure 3D**). The double mutants showed reduction of leaf palisade cells in the subepidermal layer by 13–39% when compared with respective parental r-protein mutants (**Figure 3D**). In relation to the projection area of palisade cells, the double mutants tended to have a larger cell size than parental r-protein mutants, showing the occurrence of compensated cell enlargement (**Figure 3C**). Consequently, the overall shoot size and first leaf size were less significantly reduced from single r-protein mutants (**Figures 3A,B**). A unique exception was found in *rps6a-2*. Strikingly, *gdp1-1 rps6a-2* had 66% fewer leaf palisade cells in the subepidermal layer when compared with *rps6a-2*, suggesting a synergistic interaction between these two mutations (**Figure 3D**).

We also examined genetic interactions between *gdp1-1* and *oli2-1* (**Figure 4**). The double mutants between *gdp1-1* and *oli2-1* produced smaller shoots and narrower leaves than the respective parental mutants (**Figures 4A,B,D**). The double mutants frequently produced monocots or tricots that were rarely observed in the single mutants (Supplementary Figure S2). Although the effect of each single mutation on the number of leaf palisade cells in the subepidermal layer was relatively weak

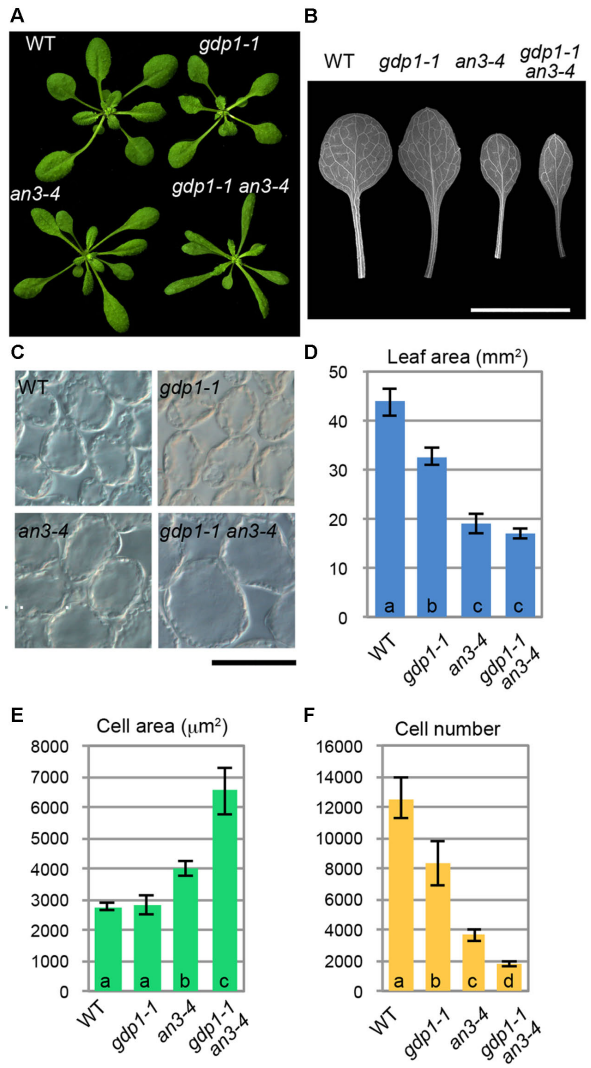

FIGURE 2 | Genetic interaction between *gdp1* and *an3*. **(A)** Shoots of 25-day-old WT, *gdp1-1*, *an3-4*, and *gdp1-1 an3-4*. **(B)** The first leaves of WT, *gdp1-1*, *an3-4*, and *gdp1-1 an3-4*. Bar, 1 cm. **(C)** Leaf palisade cells observed from a paradermal view. Bar, 100 μm. **(D–F)** Quantitative analyses of leaf phenotypes. Leaf blade area **(D)**, the projection area of leaf mesophyll cells **(E)**, and the number of leaf mesophyll cells **(F)** in the adaxial subepidermal layer of palisade tissues of first leaves are shown. First leaves were harvested from 25-day-old plants. $n = 10$, mean ± SD. Statistically significant differences were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$).

(only 10–30% reduction), the double mutants showed a reduction by about 65% compared to the wild type level (**Figure 4F**). The projection area of leaf palisade cells was more than 83% larger in *gdp1-1 oli2-1* than the wild type, indicating compensated cell enlargement (**Figures 4C,E**). These phenotypes were found in different allelic combinations, *gdp1-3 oli2-2* (**Figure 4**). As *OLI2* encodes a putative m5C methyltransferase and likely participates in ribosome biogenesis (Fujikura et al., 2009), the strong genetic interaction between *gdp1* and *oli2* suggests a role of GDP1 in ribosome biogenesis.

Tissue-Specific *GDP1* and *OLI2* Expression and Subcellular Localization of Their Gene Products

When *GDP1* expression was observed in a p*GDP1*::*GUS* transgenic line, relatively strong GUS staining was detected in the shoot tip, young leaf primordium, root tip, and floral buds (**Figures 5A–D**). Active ribosome biogenesis takes place in the proliferating cell population. Therefore, *GDP1* expression in actively developing tissues is consistent with the expected function of GDP1 in ribosome biogenesis. On the other hand, p*OLI2*::*GUS* transgenic lines showed GUS staining in guard cells and the basal parts of lateral roots rather than in these tissues (**Figures 5E,F**). We considered this result to indicate that the promoter region used in the transgenic line was insufficient to show the authentic expression pattern of *OLI2*. To overcome this problem, we generated p*OLI2*::*GFP-OLI2*/*oli2-1* lines. These transgenic lines fully complemented the leaf shape of *oli2-1* (**Figures 6D–F**). In relation to cell proliferation, the two p*OLI2*::*GFP-OLI2*/*oli2-1* lines had even greater numbers of leaf palisade cells in the subepidermal layer than the wild type (**Figure 6F**). In contrast to the p*OLI2*::*GUS* lines, strong GFP-OLI2 signals were observed in leaf primordia and root apical meristem (**Figures 5H,J**). We also generated two p*GDP1*::*GDP1-GFP*/*gdp1-1* lines that also complemented the *gdp1* leaf phenotypes (**Figures 6A–C**). GDP1-GFP signals were also found in root tips and leaf primordia (**Figures 5G,I**). In addition, strong expression levels of *GDP1* and *OLI2* were found using the electronic Fluorescent Pictograph (eFP) Browser (Winter et al., 2007). These results suggest that both *GDP1* and *OLI2* are strongly expressed in actively growing tissues that have a high demand for ribosome production.

No information is available regarding the subcellular localization of GDP1 even in nucleolar proteomics analyses (Brown et al., 2005; Pendle et al., 2005; Palm et al., 2016), while OLI2 was detected as a nuclear/nucleolar protein (Palm et al., 2016). We examined the subcellular localization of GDP1-GFP and GFP-OLI2 in root tips stained with DAPI. DAPI stains nuclear chromosomal DNA, and the nucleolus is recognized as a round and dark region in the center of the nucleus. Both GDP1-GFP and GFP-OLI2 signals were found in DAPI-negative nuclear regions (**Figures 6G,H**). As the nucleolus is the center of ribosome biogenesis, the nucleolar localization of GDP1 and OLI2 further supported their roles in ribosome biogenesis.

Processing of rRNAs in *gdp1*, *oli2*, and *gdp1 oli2*

We next examined the effects of *gdp1* and *oli2* mutations on rRNA processing. A brief overview of rRNA processing intermediates relevant to this experiment is shown in **Figure 7A**. We first examined whether the levels of rRNA intermediates were altered in these mutants. RT-qPCR analysis suggested that rRNA intermediates containing 5′-ETS, ITS1, or ITS2 accumulated at higher levels in *gdp1-1*, *oli2-1*, and *gdp1-1 oli2-1* than in the wild type (**Figure 7B**). The *oli2-1* mutation seemed to have a stronger negative effect on rRNA processing of ITS2-containing intermediates than those containing 5′-ETS or ITS1. On the other

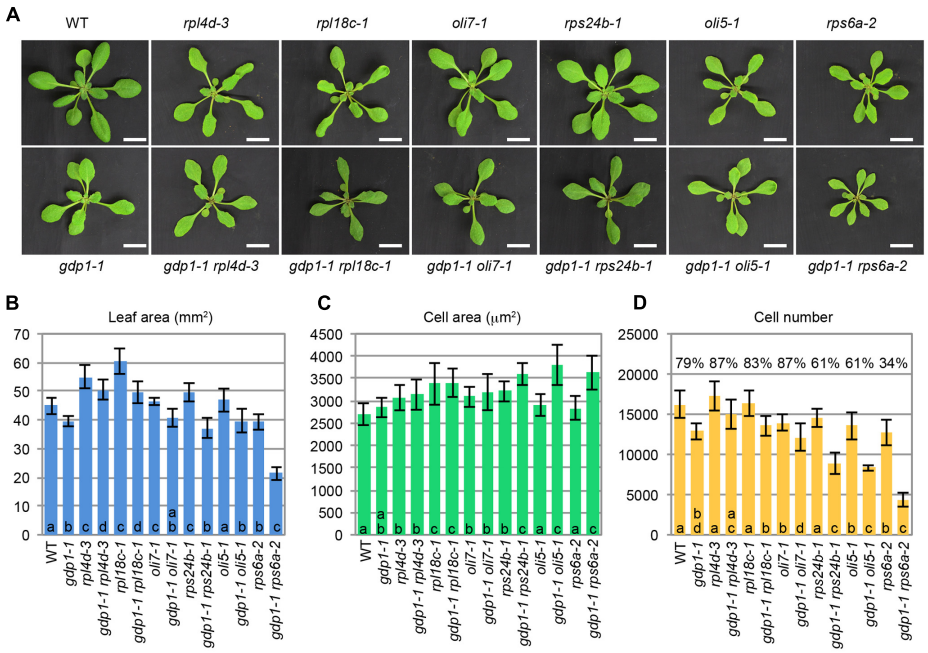

FIGURE 3 | Genetic interactions between *gdp1* and various r-protein mutants. **(A)** Shoots of 25-day-old WT, *gdp1*, r-protein mutants, and double mutants between them. Bar, 1 cm. **(B–D)** Quantitative analyses of leaf phenotypes. Leaf blade area **(B)**, the projection area of leaf mesophyll cells **(C)**, and the number of leaf mesophyll cells **(D)** in the adaxial subepidermal layer of palisade tissues of first leaves are shown. First leaves were harvested from 25-day-old plants. $n = 10$, mean ± SD. Statistically significant differences among four plant lines namely, wild type, *gdp1-1*, single r-protein mutants, and double mutants between *gdp1* and the r-protein mutants were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$). In **(D)**, relative cell number in *gdp1* and double mutants on the basis of WT and parental single r-protein mutants, respectively, are shown.

hand, *gdp1-1* had a broader impact on the accumulation of rRNA intermediates compared to *oli2-1* as the levels of 5′-ETS-, ITS1-, or ITS2-containing rRNA intermediates accumulated by more than twofold compared to the wild type. Unexpectedly, the 5′-ETS-, ITS1-, and ITS2-containing intermediates accumulated at similar levels in *gdp1-1 oli2-1* compared to those found in *gdp1-1*, despite their synergistic negative effect on cell proliferation (**Figure 7B**). Next we examined the patterns of rRNA intermediate accumulation by Northern hybridization (**Figure 7C**). In *gdp1-1*, *oli2-1*, and *gdp1-1 oli2-1*, 35S rRNA accumulated at higher levels than in the wild type. These mutants also accumulated 27SA, 27SB, P-A3, and 18SA3 rRNAs at higher levels than those seen in the wild type. Similar to the results of RT-qPCR analyses (**Figure 7B**), *gdp1-1 oli2-1* showed only modest increases, if any, in levels of these rRNA intermediates compared to their parental mutants (**Figure 7C**). These results indicate that OLI2 and GDP1 are required for normal progression of rRNA processing.

Effects of *gdp1* and *oli2* on the Leaf Polarity Defects of *as2*

As many mutants defective in an r-protein gene show the enhanced leaf abaxialization phenotype of *as2*, we examined whether *gdp1* and *oli2* also have similar developmental effects. In this experiment, we included *as2-1 rpl4d-3*, which was reported previously to show very strong leaf abaxialization (Horiguchi et al., 2011). The *as2-1 oli2-1* plants showed only moderately enhanced leaf abaxialization, as determined from the frequencies of formation of needle and trumpet-like leaves (**Figures 8A,B**). On the other hand, *gdp1-1* mutation had an even weaker effect on the leaf polarity defect of *as2* than *oli2-1*; *as2-1 gdp1-1* only occasionally produced trumpet- and needle-like leaves (**Figures 8A,B**). We also generated *as2-1 gdp1-1 oli2-1* triple mutants. In contrast to the synergistic negative effect of *gdp1-1* and *oli2-1* on cell proliferation (**Figure 4**), the triple mutant showed only slight enhancement of the leaf polarity defect compared to *as2-1 oli2-1* (**Figures 8A,B**). Next, we examined the expression levels of leaf polarity genes. Abaxially expressed genes, such as *ARF3*, *KAN2*, and *YAB5*, were slightly upregulated in *gdp1-1*, *oli2-1*, and *as2-1*, and the degree of upregulation increased progressively in double mutant combinations among *as2-1*, *gdp1-1*, and *oli2-1* and *as2-1 gdp1-1 oli2-1* triple mutants (**Figure 8C**). On the other hand, the expression levels of adaxially expressed genes, such as *PHB*, *PHV*, and *REV*, were relatively constant between wild type and both single and multiple mutants examined (**Figure 8C**). These results suggest that *gdp1* and *oli2* upregulate the expression levels of *ARF3*, *KAN2*, and *YAB5*, and slightly enhance leaf abaxialization of *as2*. However, the effects of *gdp1-1* and *oli2-1* on leaf abaxialization were much weaker than those of *rpl4d-3* (**Figures 8A,B**). One possible explanation for this observation is that a ribosome biosynthesis defect has the potential to induce leaf abaxialization in *as2-1*, but at the same time, GDP1 and OLI2 also play roles in the promotion of leaf abaxialization. To examine this possibility, we

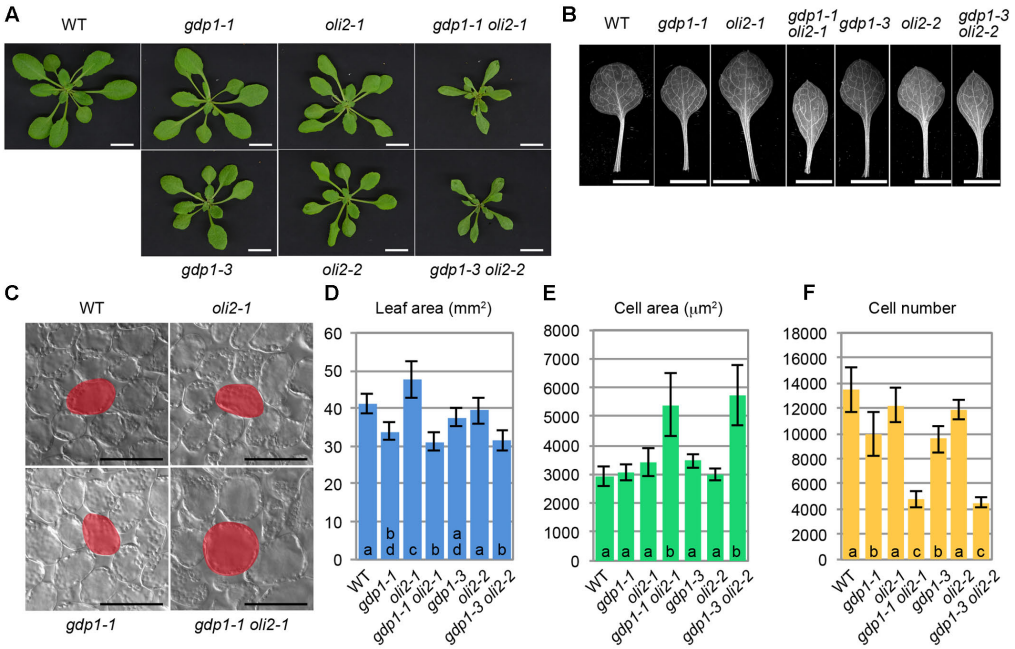

FIGURE 4 | Genetic interaction between *gdp1* and *oli2*. **(A)** Shoots of 25-day-old WT, *gdp1* alleles, *oli2* alleles, and double mutants between them. Bars, 1 cm. **(B)** First leaves. Bars, 5 mm. **(C)** Leaf palisade cells observed from a paradermal view. Bar, 100 μm. **(D–F)** Quantitative analyses of leaf phenotypes. Leaf blade area **(D)**, the projection area of leaf mesophyll cells **(E)**, and the number of leaf mesophyll cells **(F)** in the adaxial subepidermal layer of palisade tissues of first leaves are shown. First leaves were harvested from 25-day-old plants. $n = 10$, mean ± SD. Statistically significant differences were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$).

generated *as2-1 rpl4d-3 gdp1-1* and *as2 rpl4d-3 oli2-1*. These triple mutants did not show alleviation of the leaf polarity defect of *as2-1 rpl4d-3*, but had more severe developmental defects judging from their smaller shoot size and formation of filamentous first and/or second leaves (**Figure 8D**). This result suggests that *GDP1* and *OLI2* are dispensable for leaf polarity establishment and/or maintenance even in the *as2* background.

DISCUSSION

Involvement of GDP1 and OLI2 in Ribosome Biogenesis

In this study, we found that *GDP1* and *OLI2* are strongly expressed in developing tissues and encode different nucleolar proteins. Their loss-of-function mutations cause developmental phenotypes that are often observed in RBF-defective mutants and affect rRNA processing. These results suggest that the two nucleolar proteins are involved in ribosome biogenesis.

OLI2 is a homolog of yeast Nop2, which has methyltransferase activity and modifies 25S rRNA at a specific cytosine residue (m5C2870; Sharma et al., 2013). This modification is evolutionarily conserved at an equivalent site in animals (Maden, 1988) and plants (Burgess et al., 2015). However, m5C2870 is dispensable in yeast as catalytically inactive Nop2 is able to complement a *nop2* deletion mutant (Bourgeois et al., 2015). Nop2 is a component of the LSU processome (McCann et al., 2015) and acts as one of the B factors necessary to carry out the processing of 27S rRNA into 25S and 5.8S rRNAs (Hong et al., 1997; Talkish et al., 2012). Depletion of Nop2 results in increased accumulation of 27S rRNA and corresponding decreases in levels of mature 25S and 5.8S rRNAs (Hong et al., 1997).

The conserved m5C methylation site in 25S rRNA in plants indicates the presence of a functional ortholog of Nop2 in Arabidopsis. However, this modification normally presents in *oli2* mutant alleles (named *nop2a* in Burgess et al., 2015). This may be due to functional redundancy among the *OLI2* gene family; double mutants between *nop2a* and *nop2b* are lethal (Burgess et al., 2015). Therefore, direct evidence for OLI2 as an m5C methyltransferase is lacking at present. In addition, while both *nop2* in yeast and *oli2* show overaccumulation of precursors that retain the intact ITS2, and *nop2* reduces the 25S rRNA level, *oli2* does not detectably reduce the 25S rRNA level (**Figure 7C**). This suggests that the overall processing mechanisms involving OLI2 and Nop2 are conserved in eukaryotes, but their details may differ. Taken together, these results indicate that OLI2 plays a role as an RBF; however, whether it is a genuine Nop2 ortholog remains to be determined.

In this study we also identified a novel nucleolar RBF named GDP1, which contains a G-patch domain. In yeast, two G-patch domain proteins, Gno1 and Pfa1, are involved in ribosome biogenesis (Guglielmi and Werner, 2002; Lebaron et al., 2005;

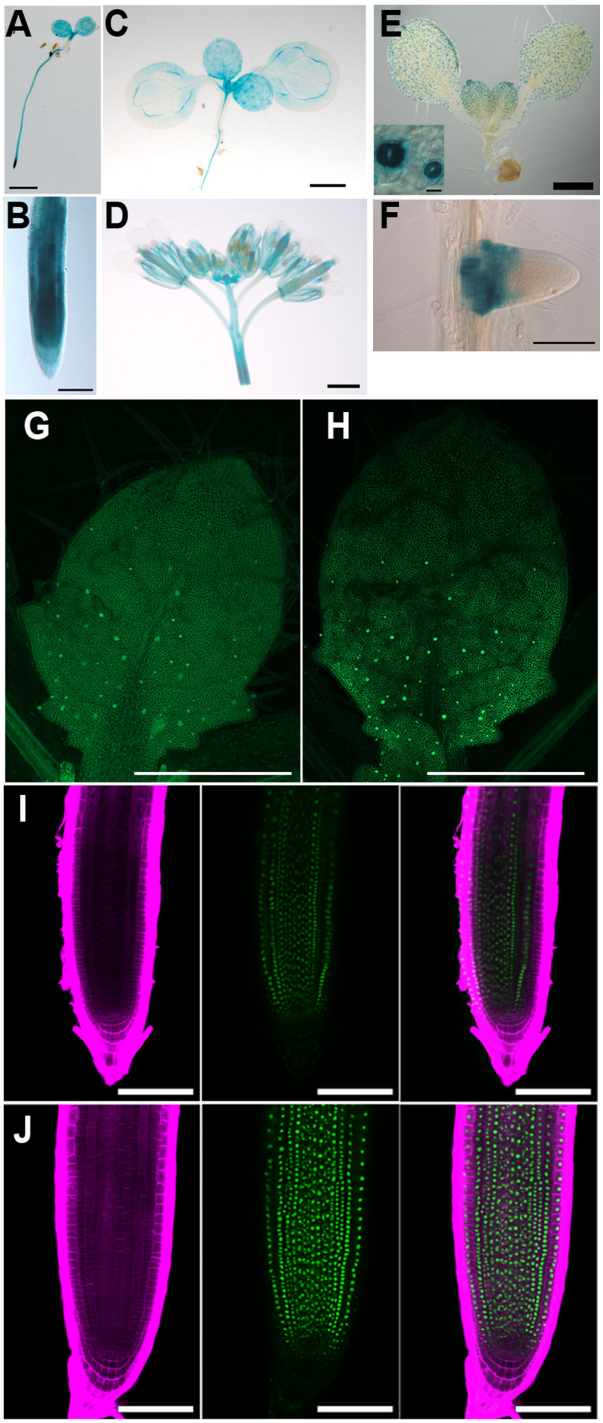

FIGURE 5 | Tissue-specific expression analysis of *GDP1* and *OLI2*. **(A–D)** Histochemical staining of p*GDP1*::*GUS* plants. **(A)** A 4-day-old seedling. **(B)** Primary root tip. **(C)** A 12-day-old seedling. **(D)** Inflorescence. **(E,F)** Histochemical staining of p*OLI2*::*GUS* plants. **(E)** A 5-day-old seedling. The insert shows a close-up view of guard cells in a cotyledon. **(F)** A lateral root. **(G,H)** Confocal images of 5-day-old p*GDP1*::*GDP1-GFP*/*gdp1-1* **(G,I)** and p*OLI2*::*GFP-OLI2*/*oli2-1* **(H,J)**. **(G,H)** Leaf primordia. **(I,J)** Primary root tips. Calcofluor fluorescence, GFP fluorescence, and merged images are shown from left to right. Bars in **(A,C–E)**: 1 mm, **(B,F,I,J)**: 100 μm, insert in **(E)**: 50 μm, **(G,H)**: 0.5 mm.

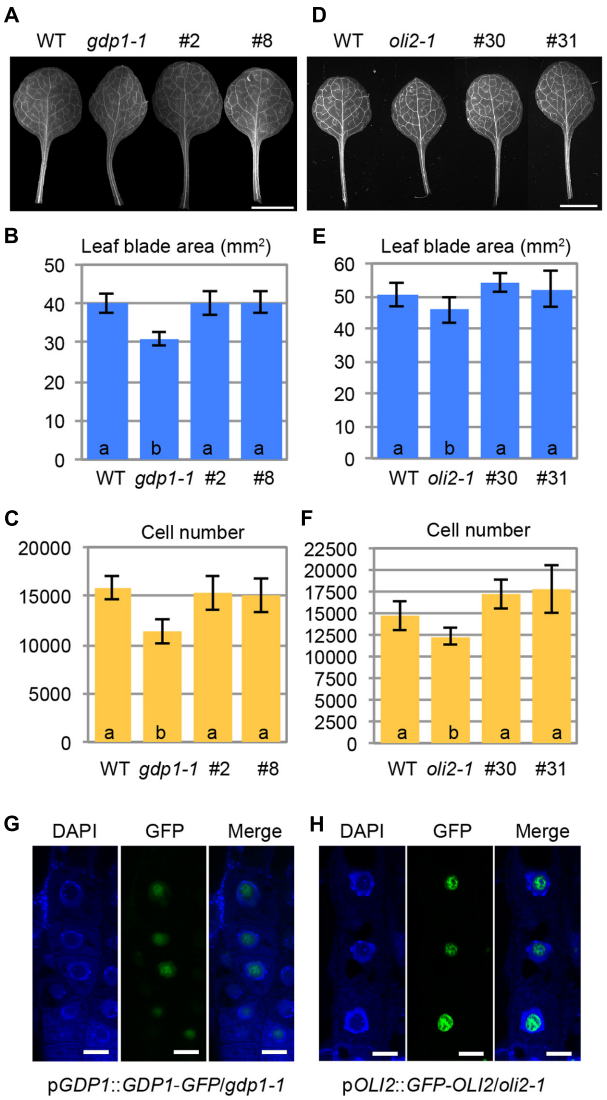

FIGURE 6 | Intracellular localization of GDP1-GFP and GFP-OLI2. **(A,D)** First leaves of transgenic *gdp1-1* lines harboring a p*GDP1*::*GDP1-GFP* construct (#2 and #8) **(A)** and a p*OLI2*::*GFP-OLI2* construct (#30 and #31) **(D)** grown for 25 days. **(B,C,E,F)** Quantitative analyses of leaf phenotypes of the *gdp1* **(B,C)** and *oli2* **(E,F)** transgenic lines. Leaf blade area **(B,E)**, and the number of leaf mesophyll cells **(C,F)** in the adaxial subepidermal layer of palisade tissues of first leaves are shown. In **(B,C,E,F)**, data are means ± SD (n = 10). Statistically significant differences were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$). **(G,H)** Subcellular localization of GDP1-GFP **(G)** and GFP-OLI2 **(H)** in cells stained with DAPI. Fluorescence images of DAPI, GFP, and merged images are shown from left to right. Bars, 10 μm.

Chen et al., 2014). According to PANTHER version 12.0[2] (Mi et al., 2016), GDP1 was classified as a protein family (PTHR23149) that also contains Gno1 and its ortholog in human, PINX1. These three proteins harbor a G-patch domain in their amino-terminal side. On the other hand, Pfa1 has a G-patch domain in its carboxy-terminal side and also has an

[2]http://www.pantherdb.org/publications.jsp

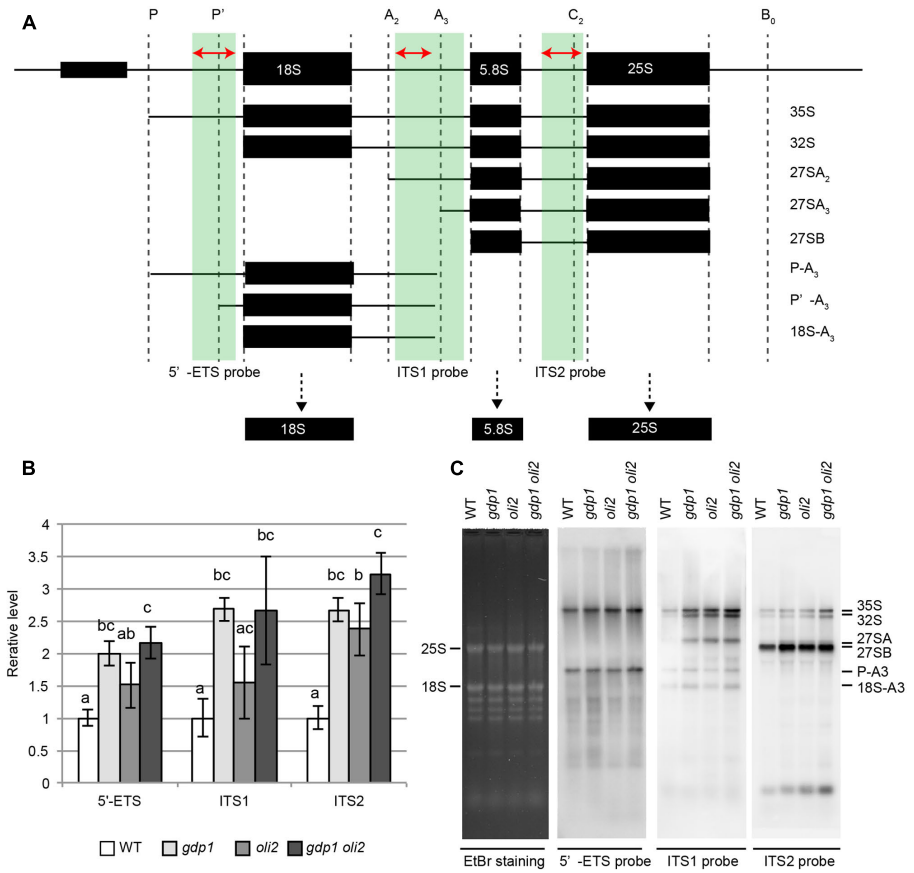

FIGURE 7 | Accumulation of rRNA intermediates. **(A)** Brief overview of rRNA intermediates detected by either RT-qPCR or Northern hybridization. Parts of rRNA intermediates amplified by RT-qPCR and those detected by Northern hybridization are indicated by red arrows and green boxes, respectively. **(B)** RT-qPCR analysis of rRNA intermediates. $n = 3$, mean ± SD. Statistically significant differences were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$). **(C)** Northern hybridization of total RNAs. Shoots of 12-day-old WT, *gdp1-1*, *oli2-1*, and *gdp1-1 oli2-1* were used.

R3H domain. Gno1 associates with both 90S pre-ribosomes and early pre-60S ribosomes, but only transiently with early pre-40S ribosomes (Chen et al., 2014). The lack of Gno1 leads to processing defects within the 5′-ETS and ITS1 (Chen et al., 2014). Downstream rRNA intermediates, such as 27S rRNAs and mature 18S and 25S rRNAs are reduced in *gno1*. In *gdp1*, 35S and 32S rRNAs increased, but in contrast to *gno1*, 27SA/B rRNAs increased without detectably affecting the steady-state levels of 25S and 18S rRNAs (**Figure 7C**). Thus, *gdp1*, like *gno1*, seems to affect multiple steps of rRNA processing, but it has a partially different effect on rRNA processing. Thus, whether GDP1 has an orthologous function to Gno1 remains to be elucidated. An emerging molecular role of G-patch domain protein is the activation of DEAH/RHA helicases (Robert-Paganin et al., 2015). Both Gno1 and Pfa1 function in ribosome biogenesis in close association with the DEAH/RHA helicase, Prp43. Future characterization of an Arabidopsis homolog of Prp43 may provide additional insight into the function of GDP1.

Relationships between R-Proteins and RBFs

In a series of genetic crosses, we found an additive or synergistic genetic interaction depending on the combination of single mutants examined. The combination between *gdp1* and most r-protein mutants examined resulted in an additive phenotype. This suggests that processes of GDP1-dependent ribosome biogenesis and those involving these r-proteins occur largely independently. On the other hand, the synergistic genetic interaction between *gdp1* and *oli2* suggests the interdependence of GDP1 and OLI2 functions in ribosome biogenesis. In yeast, Gno1 acts at multiple steps of ribosome biogenesis in close association with Prp43 (Lebaron et al., 2005, 2009; Chen et al., 2014). Prp43 is expected to function in rearrangement of pre-ribosomal particles through its RNA helicase activity (Lebaron et al., 2005, 2009). Nop2 is an essential protein and is required for hierarchical recruitment of the B factors (Hong et al., 1997; Talkish et al., 2012). Thus, successive changes in the geometry of pre-ribosomes are important for the progression of subsequent processes. The interdependence of GDP1 and OLI2 functions may have arisen from the dynamic nature of ribosome biogenesis.

Unexpectedly, *gdp1 oli2* did not show marked increases in the levels of rRNA intermediates compared to its parental single mutants (**Figure 7**). This discrepancy may have been due to the nature of the experiments, in that our

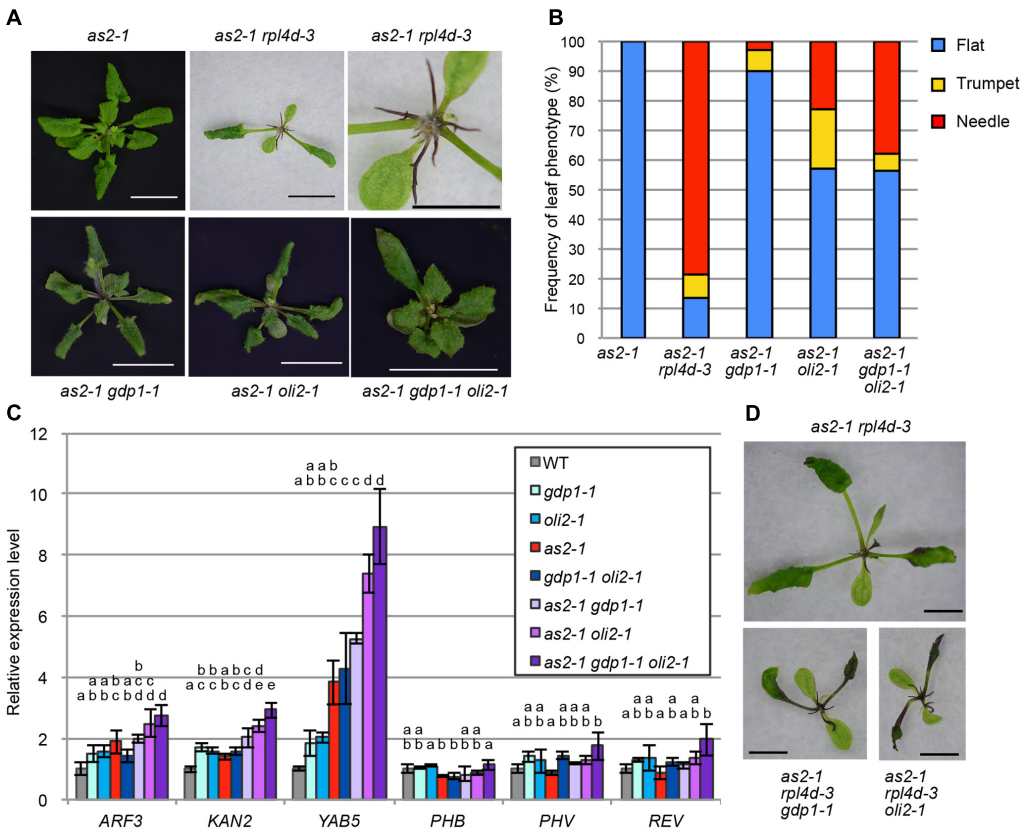

FIGURE 8 | The effects of *gdp1* and *oli2* on leaf development in the *as2* background. **(A)** Shoots of *as2-1*, *as2-1 rpl4d-3*, *as2-1 gdp1-1*, *as2-1 oli2-1*, and *as2-1 gdp1-1 oli2-1* grown for 25 days. Bars indicate 1 cm except for the close-up view of *as2-1 rpl4d-3* where it shows 5 mm. **(B)** Frequencies of flat, trumpet-like, and needle-like leaves. More than 13 plants were examined and all rosette and cauline leaves were scored. **(C)** Expression levels of leaf polarity genes. Shoots of 10-day-old seedlings were used for RNA extraction. $n = 3$, mean ± SD. **(D)** Shoots of *as2-1 rpl4d-3*, *as2 rpl4d-3 gdp1-1*, and *as2 rpl4d-3 oli2-1* grown for 25 days. Bars, 1 mm. Statistically significant differences were indicated by different letters (one-way ANOVA with Tukey HSD test, $p < 0.05$).

RNA analyses determined the steady-state levels of rRNA intermediates. Further characterization of GDP1 and OLI2 and measurement of rRNA processing rate could resolve this issue.

Another notable interaction was found between *gdp1* and *rps6a*. Despite being a component of the 40S subunit, RPS6A was shown to interact with the histone deacetylase HD2B and NUCLEOSOME ASSEMBLY PROTEIN1 (NAP1), and to regulate transcription of 45S rRNA (Kim et al., 2014; Son et al., 2015). Thus, the exceptionally strong interaction of *rps6a* with *gdp1* among other r-protein mutants may have been due to the role of RPS6A in ribosome biogenesis. Similar synergistic relationships were also reported between another pair of RBF mutants, *apum23* and *nuc-l1* (Abbasi et al., 2010). In the same work, double mutants between *apum23* and *as1/2 enhancer5* (*ae5*)/*rpl28a* or *ae6*/*rpl5a* were reported not to show more severe phenotypes than the respective single mutants (Abbasi et al., 2010). As *ae6* is an allele of *oli5*, and *gdp1 oli5* shows an additive phenotype in relation to the cell proliferation defect (**Figure 3**), evaluation of genetic interactions between ribosome-related mutants based solely on shoot size could miss a more fundamental interaction. In summary, our genetic experiments suggested that a combination of weak defects in RBFs may cause a marked reduction of the flow of ribosome biogenesis, resulting in a strong cell proliferation defect.

Genetic Defects in RBFs Do Not Always Induce Strong Leaf Abaxialization in *as2*

Several reports described enhanced leaf abaxialization in *as2* by r-protein mutants (Byrne, 2009; Horiguchi et al., 2012; Machida et al., 2015). Recently, mutations in three RBF genes, *NUC-L1*, *RH10*, and *RID2*, were also shown to strongly enhance leaf abaxialization in *as2* (Matsumura et al., 2016). In yeast, orthologs of these three proteins are components of, or RBFs associated with, the SSU processome. These findings suggested a tight link between the SSU processome and AS2-dependent cell fate decision (Matsumura et al., 2016). In addition, *APUM23* encodes a homolog of yeast Nop9, and *apum23* also strongly enhances the *as2* leaf polarity phenotype (Huang et al., 2014). Although the precise functions of APUM23 and Nop9 could differ, APUM23 is able to partially complement the *nop9* mutant phenotype in yeast (Huang et al., 2014). Interestingly, Nop9 is also a component of the SSU processome

(Zhang et al., 2016). In contrast to reports regarding these RBFs (Huang et al., 2014; Matsumura et al., 2016), *gdp1*, *oli2*, and even *gdp1 oli2* had little or very mild effects on the enhancement of leaf abaxialization in the *as2* background (**Figure 8**). These results suggest that not all of the RBFs are tightly linked with leaf adaxial/abaxial polarity. Gno1 was found in the SSU processome, pre-40S, and pre-60S ribosomes (Chen et al., 2014). If GDP1 is also a component of the SSU processome, this argues against a link between the SSU processome and leaf abaxial/adaxial polarity regulation. A possible explanation for this discrepancy is that there may be a key structure or a subcomplex within the SSU processome, dysfunction of which is linked to leaf abaxialization in *as2*. This is a likely scenario, as the SSU processome is a 2.2-MDa ribonucleoprotein composed of modular subcomplexes (Pérez-Fernández et al., 2007). On the other hand, Nop2 is a component of the LSU processome in yeast, and OLI2 is a putative ortholog of Nop2. Several scenarios may explain the limited effect of *oli2* on leaf abaxialization. First, the LSU processome may not have a link to the regulation of adaxial/abaxial polarity. However, we consider this to be unlikely, because we reported previously that mutations in *RPL4D* encoding a 60S r-protein have a very strong effect on leaf abaxialization in *as2* (Horiguchi et al., 2011), and, in yeast, RPL4 is incorporated during an early stage of pre-60S formation (Gamalinda et al., 2014; Stelter et al., 2015; Chen et al., 2017). Similar to the SSU processome, the LSU processome may also have a link to adaxial/abaxial patterning through its local structures. As ribosome biogenesis is a highly dynamic process, surveillance of ribosome biogenesis defects at every step is probably impossible. Consistently, in yeast cells with specific genetic backgrounds, aberrant ribosomal subunits can escape from the surveillance systems and engage in translation (Rodríguez-Galán et al., 2015). On the other hand, surveillance at multiple key checkpoints to sense local defects of nascent ribosomes represents an easier strategy. The differential contributions of RBFs to leaf abaxial/adaxial polarity regulation may be correlated with their relative importance to such checkpoints.

Our genetic analyses on *gdp1* and *oli2* illustrated that a genetic defect in ribosome biogenesis leads to cell proliferation defect but it does not have a strong effect to enhance leaf abaxialization when it is introduced into the *as2* background. A contrasting example is found in *rpl4d* where *rpl4d* single mutants do not have a clear cell proliferation defect yet it causes very strong leaf abaxialization in the *as2* background (Horiguchi et al., 2011). These examples suggest there may be a critical point during ribosome biogenesis that is linked to the regulation of leaf adaxial/abaxial polarity. It is noteworthy that *oli2* has a statistically significant effect to upregulate *YAB5* expression when compared to *as2* (**Figure 8C**) but it did not result in strong leaf abaxialization. In *as2 rh10*, strong leaf abaxialization is dependent on *ARF3* (Matsumura et al., 2016). The expression levels of *ARF3* in *as2 rh10* and *as2 gdp1 oli2* were four and threefold higher than that in the wild type plants, respectively (Matsumura et al., 2016; **Figure 8C**). Although plant samples used in these studies were different (shoot tips or whole shoots), the differential sensitivities of leaf abaxialization in these mutants might be attributable to the degree of enhanced *ARF3* expression. An increasing number of characterized ribosome biogenesis mutants of Arabidopsis should offer an opportunity to localize such a point through quantitative and comparative analyses of these mutants in a future study.

A critical issue concerning the above discussion is whether there is actually such a checkpoint in plants. In mammals, ribosome biogenesis defects lead to activation of the tumor suppressor, p53, which acts as a transcriptional activator and triggers stress responses, such as cell cycle arrest, senescence, and apoptosis (Kastenhuber and Lowe, 2017). Under normal conditions, p53 is ubiquitinated by the E3 ubiquitin ligase, MDM2, and subjected to proteolysis. On the other hand, ribosome biogenesis defects inhibit MDM2 activity through direct interaction between the 5S rRNA-RPL5-RPL11 complex and MDM2 (Sun et al., 2010; Sloan et al., 2013; Bursac et al., 2014). However, plants have orthologs of neither p53 nor MDM2. Recently, Ohbayashi et al. (2017) showed that the NAC transcription factor gene, *SRIW1*, is upregulated in *rid2*. Notably, *rid2 sriw1* still suffers from the rRNA processing defects, but the developmental phenotypes, such as impaired callus formation and pointed leaf formation, were suppressed (Ohbayashi et al., 2017). These results indicated that the observed developmental phenotypes are indirect consequences of rRNA processing defects, and are more directly mediated by the ribosomal stress response in which SRIW1 plays a central role, similar to p53 in animals.

In this study, we showed that *gdp1 oli2* double mutants had a very strong cell proliferation defect, but enhanced the leaf polarity defect of *as2* less strongly. How a defect in a general cellular process such as ribosome biogenesis interferes with a specific developmental process is an important emerging issue in plant developmental and cell biology (Tsukaya et al., 2013). We suggest that the cell proliferation defect in leaf primordia and leaf abaxialization triggered by a mutation in RBF or an r-protein gene could be mediated by different mechanisms. It will be interesting to examine whether these developmental phenotypes are mediated by SRIW1.

AUTHOR CONTRIBUTIONS

GH and HT designed and conducted the experiments. KK, JT, HC, KF, and GH performed the experiments. GH and HT wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.02240/full#supplementary-material

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Plant Nucleolar Stress Response, a New Face in the NAC-Dependent Cellular Stress Responses

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The nucleolus is the most prominent nuclear domain, where the core processes of ribosome biogenesis occur vigorously. All these processes are finely orchestrated by many nucleolar factors to build precisely ribosome particles. In animal cells, perturbations of ribosome biogenesis, mostly accompanied by structural disorders of the nucleolus, cause a kind of cellular stress to induce cell cycle arrest, senescence, or apoptosis, which is called nucleolar stress response. The best-characterized pathway of this stress response involves p53 and MDM2 as key players. p53 is a crucial transcription factor that functions in response to not only nucleolar stress but also other cellular stresses such as DNA damage stress. These cellular stresses release p53 from the inhibition by MDM2, an E3 ubiquitin ligase targeting p53, in various ways, which leads to p53-dependent activation of a set of genes. In plants, genetic impairments of ribosome biogenesis factors or ribosome components have been shown to cause characteristic phenotypes, including a narrow and pointed leaf shape, implying a common signaling pathway connecting ribosomal perturbations and certain aspects of growth and development. Unlike animals, however, plants have neither p53 nor MDM2 family proteins. Then the question arises whether plant cells have a nucleolar stress response pathway. In recent years, it has been reported that several members of the plant-specific transcription factor family NAC play critical roles in the pathways responsive to various cellular stresses. In this mini review, we outline the plant cellular stress response pathways involving NAC transcription factors with reference to the p53-MDM2-dependent pathways of animal cells, and discuss the possible involvement of a plant-unique, NAC-mediated pathway in the nucleolar stress response in plants.

Keywords: nucleolar stress response, nucleolus, NAC transcription factor, ribosome biogenesis, ribosomal protein, Pre-rRNA processing, cell proliferation, development

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The core processes of ribosome biogenesis, such as ribosomal RNA (rRNA) transcription, pre-rRNA processing, and ribosome assembly, take place in the nucleolus, which is the most prominent nuclear domain. These processes are finely controlled by many nucleolar factors, to build precisely small and large ribosome particles. Perturbations of any of the steps of ribosome biogenesis in the nucleolus cause a type of stress called nucleolar stress or ribosomal stress, which stimulates a specific signaling pathway in animal cells. Recent studies have implicated a plant-unique pathway in the nucleolar stress response of plant cells. In this mini review, we will discuss how plant cells sense and respond to nucleolar stress, with reference to the p53-dpeendent nucleolar stress response pathway of animal cells.

RIBOSOME BIOGENESIS IN THE NUCLEOLUS

The nucleolus is formed around the nuclear rDNA regions where tandem repeats of rRNA genes lie, and serves as a site for the main part of biogenesis of the ribosome, a huge ribonucleoprotein machinery that executes translation of the nucleotide sequence of mRNAs into the amino acid sequence of proteins. In the nucleolus, ribosome biogenesis starts with the transcription of pre-rRNAs from rRNA genes, followed by their processing and assembly with ribosomal proteins (RPs) into two ribosome subunits, the small subunit (SSU) and the large subunit (LSU).

Among the four species of rRNA, i.e., 5S, 5.8S, 18S, and 25–28S rRNAs, only 5S rRNA is transcribed separately, whereas the other three rRNAs are transcribed as a single precursor molecule. This precursor undergoes sequential processing events, such as base modification, cleavage, and trimming, which are tightly linked with the stepwise assembly of RPs onto pre-rRNAs. These events are guided by ribosome biogenesis factors (RBFs), which are enriched in the nucleolus, including small nucleolar RNAs (snoRNAs), snoRNA-associated proteins, and many other proteins. The processing pathway of pre-rRNAs and the repertoire of RBFs are partly different but largely common between yeast, animals, and plants (Henras et al., 2015; Weis et al., 2015). The nucleolar activities of pre-rRNA processing and RP assembly produce near-complete forms of the SSU and LSU. The SSU comprises 18S rRNA and 33 types of RPs, whereas the LSU comprises 5S, 5.8S, and 25–28S rRNAs and 46–48 types of RPs. The SSU and LSU RPs are highly conserved throughout eukaryotes, with only a few exceptions.

NUCLEOLAR STRESS RESPONSE PATHWAY IN ANIMAL CELLS

The elaborately regulated biogenesis of ribosomes is disturbed under unhealthy conditions for various reasons, such as nutrient starvation, hypoxia, heat shock, chemical suppression of ribosome biogenesis, genetic impairments of RBFs, and deficiencies of RPs (Mayer and Grummt, 2005; Boulon et al., 2010). In animal cells, such perturbations of ribosome biogenesis, regardless of origin, are mostly accompanied by structural disorders of the nucleolus, although they are not always obvious, and cause a particular type of stress called nucleolar stress or ribosomal stress, which stimulates specific signaling pathways leading to cell-cycle arrest, senescence, or apoptosis (Olausson et al., 2012; James et al., 2014). These stress signaling pathways are classified into two types, p53-dependent and -independent ones (Olausson et al., 2012; James et al., 2014; Russo and Russo, 2017).

The canonical and most extensively studied pathway of animal nucleolar stress response is the p53-dependent pathway. The well-known antitumor transcription factor p53 and its destabilizer MDM2 play a pivotal role in this pathway. In normal conditions, the activity of p53 is suppressed to a low level under the control of MDM2, which acts as a component of E3 ubiquitin ligase and targets p53, leading to the ubiquitination and proteasomal degradation of p53. Upon nucleolar stress, RPs are released from the nucleolus into the nucleoplasm. Several of the RPs released, including RPL5, RPL11, RPL23, and RPS7, bind directly to MDM2 as effectors, thus preventing its action on p53 (Lohrum et al., 2003; Dai and Lu, 2004; Jin et al., 2004; Chen et al., 2007). As a result, p53 becomes stable and active in regulating the expression of genes that are involved in cell-cycle arrest, senescence, and apoptosis (Zhang and Lu, 2009; Deisenroth and Zhang, 2010; Golomb et al., 2014). Genes that are expressed at relatively high levels of p53 activity induce senescence or apoptosis, whereas genes that are expressed at low p53 levels induce cell-cycle arrest (Lai et al., 2007).

In recent years, increasing evidence has indicated that additional mechanisms not involving p53 participate in nucleolar stress response. For instance, RPs released from the nucleolus upon nucleolar stress, such as RPL11 and RPS14, were shown to repress the activity of the oncoprotein transcription factor c-Myc, which is crucial for the expression of many genes involved in cell growth and proliferation, through direct binding to the c-Myc protein and/or controlling microRNA-induced silencing complex (miRISC)-mediated turnover of *c-Myc* mRNA (Dai et al., 2007; Challagundla et al., 2011; Zhou et al., 2013). It was also reported that in response to nucleolar stress, released RPs can induce cell cycle arrest via regulation of the E2F transcription factor E2F-1 or CDK inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$, independently of p53 (Iadevaia et al., 2010; Donati et al., 2011; Russo et al., 2013).

EFFECTS OF THE PERTURBATION OF RIBOSOME BIOGENESIS IN PLANTS

In plants, especially in the model plant Arabidopsis, many ribosome-related mutants, which are impaired in an RBF- or RP-encoding gene, have been isolated and characterized. Most of these mutants exhibit a similar spectrum of phenotypes, including a narrow and pointed leaf shape and retardation of root growth (Byrne, 2009; Horiguchi et al., 2012; Tsukaya et al., 2013; Weis et al., 2015). A severe loss of the adaxial–abaxial polarity of leaves under the genetic background of *asymmetric leaves 1* (*as1*) or *as2* is also a notable phenotype that is shared by the ribosome-related mutants (Pinon et al., 2008; Horiguchi et al., 2011; Huang et al., 2014; Matsumura et al., 2016). These common phenotypic features of various ribosome-related mutants imply the existence of a common mechanism that regulates growth and development in response to various perturbations of ribosome biogenesis.

At the subcellular level, structural changes of the nucleolus have been reported for various RBF mutants (**Table 1**). In most cases, enlargement of the nucleolus was observed in association with excessive accumulation of intermediates of pre-rRNA processing. This nucleolar enlargement is sometimes linked with the development of a nucleolar cavity, i.e., nucleolar vacuolation (Ohbayashi et al., 2011). Moreover, in the Arabidopsis mutant that carries a disrupted nucleolin gene (*AtNUC-L1*), nucleolar disorganization and decondensation of the rDNA chromatin structure were shown to co-occur (Pontvianne et al., 2007). These findings indicate that perturbation of rRNA biogenesis generally

TABLE 1 | List of Arabidopsis genes that have been shown to participate in the function and/or structural integrity of the nucleolus.

Gene symbol	AGI code	Protein product	Protein localization	Mutant allele	Mutation type	Mutant effects on			Mutant suppression by *sriw1*	References
						Nucleolar structure	Nucleolar functions	Growth and development		
RID2	At5g57280	Methyltransferase	Nucleolus	*rid2-1*	Base substitution (missense)	Enlargement accompanied by vacuolation	Impairment of pre-rRNA processing	Temperature-dependent defects in leaf blade development and root growth in seedlings, and in callus formation, adventitious root formation and shoot regeneration in tissue culture, and severe loss of leaf abaxial–adaxial polarity in *as1* and *as2*	Yes	Konishi and Sugiyama, 2003; Ohbayashi et al., 2011, 2017; Shinohara et al., 2014; Matsumura et al., 2016
				rid2-2, *rid2-3*	T-DNA insertion	—	—	Female gametophyte lethality, maternal sterility, or maternal embryonic lethality	—	Ohbayashi et al., 2011
RID3	At3g49180	WD40 repeat protein	Nucleus	*rid3*	Base substitution (missense)	Enlargement	Impairment of pre-rRNA processing	Temperature-dependent defects in leaf blade development and root growth in seedlings, and in shoot regeneration in tissue culture	Yes	Tamaki et al., 2009; Shinohara et al., 2014; Ohbayashi et al., 2017
RH10	At5g60990	DEAD-box RNA helicase	Nucleolus	*rh10-1*	Base substitution (missense)	Enlargement	Impairment of pre-rRNA processing	Temperature-dependent defects in leaf blade development and severe loss of leaf abaxial–adaxial polarity in *as1* and *as2*	Yes	Matsumura et al., 2016; Ohbayashi et al., 2017
ATNUC-L1	At1g48920	Nucleolin	Nucleolus	*Atnuc-L1-1*	T-DNA insertion	Disorganization (rDNA heterochromatin decondensation)	Impairment of pre-rRNA processing (blockage of cleavage at least at the P site) and increased transcription of specific rRNA variants	Pleiotropic defects in growth and development, including abnormal leaf morphogenesis, and severe loss of leaf abaxial–adaxial polarity in *as1* and *as2*	—	Pontvianne et al., 2007, 2010; Matsumura et al., 2016
				parl1-1	2 bp insertion	—	—	Abnormal vein patterning, pointed narrow leaf, retarded root growth, and reduced fertility	—	Petricka and Nelson, 2007
				parl1-2 (*Atnuc-L1-2*)	T-DNA insertion	Disorganization (rDNA heterochromatin decondensation)	Impairment of pre-rRNA processing and increased transcription of specific rRNA variants	Abnormal vein patterning, pointed narrow leaf, retarded root growth, reduced fertility, and severe loss of leaf abaxial–adaxial polarity in *as1* and *as2*	—	Petricka and Nelson, 2007; Pontvianne et al., 2010; Matsumura et al., 2016
DOMINO1	At5g62440	Small plant-specific protein	Nucleus	*domino1*	T-DNA insertion	Enlargement	Inactivation of ribosome biogenesis	Retarded embryogenesis	—	Lahmy et al., 2004
NOF1	At1g17690	DUF1253-containing protein	Nucleolus	*nof1-1*	T-DNA insertion (in the promoter region)	Enlargement	Increase in rRNA transcription	Embryonic lethality (irregular pattern and/or additional cell division, and lack of cell adhesion)	—	Harscoët et al., 2010
				nof1-2	T-DNA insertion	—	—	Female gametophyte lethality	—	Harscoët et al., 2010

(Continued)

TABLE 1 | Continued

Gene symbol	AGI code	Protein product	Protein localization	Mutant allele	Mutation type	Mutant effects on			Mutant suppression by *sriw1*	References
						Nucleolar structure	Nucleolar functions	Growth and development		
APUM23	At1g72320	Pumilio protein	Nucleolus	*apum23-1*, *apum23-2*	T-DNA insertion	Enlargement	Impairment of processing/degradation of pre-rRNAs	Pleiotropic defects in growth and development, including abnormal leaf morphogenesis	–	Abbasi et al., 2010
				apum23-3	Base substitution (missense)	–	Impairment of pre-rRNA processing	Pleiotropic defects in growth and development, including abnormal leaf morphogenesis and severe loss of leaf abaxial–adaxial polarity in *as1* and *as2*	–	Huang et al., 2014
TOZ	At5g16750	WD40 repeat protein	Nucleolus	*toz*	Ds insertion	Enlargement	Impairment of pre-rRNA processing	Embryonic lethality (aberrant cell division)	–	Griffith et al., 2007
ATLa1	At4g32720	La motif protein	Nucleoplasm and nucleolar cavity (?)	*atla1-1*, *atla1-2*	T-DNA insertion	Enlargement	Interference with ribosome biogenesis (?)	Embryonic lethality (arrest at the early globular stage)	–	Fleurdépine et al., 2007
ATREN1	At1g77570	Heat-shock transcription factor	Nucleolus	*atren1*	T-DNA insertion	Enlargement	Interference with ribosome biogenesis (?)	Defects in male gametophyte development	–	Reňák et al., 2014

induces structural disorders of the nucleolus in plant cells, as well as in animal cells (Nishimura et al., 2015).

Taking together the morphological and cytological characteristics of ribosome-related mutants, we can speculate that perturbations of ribosome biogenesis and the resultant structural disorders of the nucleolus affect several aspects of plant growth and development via a specific signaling pathway. If this is true, then what is the pathway? By analogy with animal cells, it seems reasonable to assume the presence of a nucleolar stress response pathway at work in plant cells. However, as plants have no homologs of p53 or MDM2 (Huart and Hupp, 2013), the plant pathway should not involve a p53- and MDM2-dependent mechanism and might be distinct from the nucleolar stress response pathway of animal cells.

IMPLICATION OF ANAC082 AS A MEDIATOR OF THE NUCLEOLAR STRESS RESPONSE IN PLANTS

Very recently, the important evidence of the nucleolar stress response pathway in plants came from molecular genetic studies of Arabidopsis mutants, *root initiation defective 2* (*rid2*) and *suppressor of rid two 1* (*sriw1*). *rid2* is a temperature-sensitive mutant that is impaired in pre-rRNA processing because of a missense mutation in the gene encoding a putative RNA methyltransferase and is characterized phenotypically by severe defects in cell proliferation and a striking enlargement and vacuolation of the nucleolus at high temperatures (Ohbayashi et al., 2011). *sriw1* is a *rid2* suppressor mutant that was isolated from a mutagenized population of *rid2* (Ohbayashi et al., 2017). The *sriw1* mutation markedly alleviated the cell proliferation defects of *rid2* without relieving the impaired pre-rRNA processing. It was further shown that the *sriw1* mutation had the abilities to restore growth and development not only in *rid2* but also generally in various ribosome-related mutants, including both RBF mutants and RP mutants, and to confer weak resistance to chemicals that interfere with ribosome biogenesis or ribosomal function. *sriw1* was identified as a loss-of-function mutation of the gene encoding ANAC082, which belongs to the plant-specific transcription factor family NAM/ATAF/CUC (NAC). The expression level of *ANAC082* was temperature-dependently increased in temperature-sensitive RBF mutants. These findings collectively implicate ANAC082 as a key mediator that works downstream of perturbations of ribosome biogenesis and nucleolar disorders, thus leading to growth defects and developmental alterations in plants; i.e., plant cells are considered to respond to nucleolar stress via a plant-unique signaling pathway mediated by ANAC082.

POSSIBLE MECHANISMS OF SENSING NUCLEOLAR STRESS IN PLANTS

To understand the nucleolar stress response in plants, how plant cells sense perturbed ribosome biogenesis and nucleolar disorders is one of the most critical questions. Among the fragmentary pieces of information available currently regarding

the plant pathway of the nucleolar stress response, a clue to one of its early steps can be found in the elevated expression of *ANAC082* in RBF mutants (Ohbayashi et al., 2017). Transcriptional regulation and/or post-transcriptional regulation may contribute to this upregulation of *ANAC082* expression. With respect to post-transcriptional regulation, the existence of a conserved upstream open reading frame (uORF) in the upstream region of the main ORF in the *ANAC082* mRNA is of considerable note (Takahashi et al., 2012). This uORF was demonstrated to have an amino-acid-sequence-dependent, negative effect on the expression of the downstream main ORF (Ebina et al., 2015). Most of the regulatory uORFs studied to date cause ribosome stalling at their termination codon, which impedes the access of ribosomes to the main ORF and often induces nonsense-mediated mRNA decay (Gao and Geballe, 1996; Law et al., 2001; Gaba et al., 2005; Uchiyama-Kadokura et al., 2014). Such uORF-dependent control can be affected by ribosomal defects, as was reported for the expression of uORF-containing genes that encode auxin signaling factors in the Arabidopsis RP mutant *rpl24b* (Nishimura et al., 2004, 2005; Zhou et al., 2010). Taking these findings into consideration, the uORF of *ANAC082* might be a candidate site of nucleolar stress sensing. A possible underlying mechanism is that, under nucleolar stress, because of a shortage of functional ribosomes, imbalance of ribosomal subunits, or some other abnormal situation of ribosomes, the constraint of *ANAC082* expression by ribosome stalling at the uORF is loosened, resulting in the activation of ANAC082.

It is also possible that the activity of ANAC082 is regulated via a protein–protein interaction upon nucleolar stress, given the knowledge that NAC transcription factors generally form a homodimer or heterodimer through interaction at the N-terminal NAC domain (Ernst et al., 2004). ANAC082 has a potential of binding to several species of NAC transcription factors, including NAC1, CUP-SHAPED COTYLEDON 2 (CUC2), ANAC103, and VASCULAR-RELATED NAC-DOMAIN (VND) proteins (Yamaguchi et al., 2015). Moreover, ANAC082 is part of the list of transcription factors that can bind to the WWE domain-containing, non-NAC proteins RADICAL-INDUCED CELL DEATH 1 (RCD1), and SIMILLAR TO RCD-ONE 1 (SRO1) (Jaspers et al., 2009). If some of these proteins act as a partner of ANAC082, it is also possible that the partner, instead of ANAC082, is responsible for the process of nucleolar stress sensing.

MULTIPLE NACs IN PLANT CELLULAR STRESS RESPONSES CORRESPONDING TO MULTIPLE ROLES OF P53 IN ANIMAL CELLULAR STRESS RESPONSES

In animal cells, p53 participates as a nodal regulator not only in the nucleolar stress response pathway but also in pathways that respond to several other cellular stresses, such as oncogenic stress, DNA damage stress, and oxidative stress (Serrano et al., 1997; Horn and Vousden, 2007; Hu et al., 2012). Oncogenic stress, which is caused by the inappropriate expression of oncogenes or proto-oncogenes, is transduced to the activation of p53 mainly through the transcriptional upregulation and protein stabilization of the tumor suppressor $p14^{ARF}$ and the consequent inhibition of MDM2 activity by $p14^{ARF}$ (Sherr and Weber, 2000; Gallagher et al., 2006; Chen et al., 2010). Another route connecting oncogenic stress to p53 activation depends on the binding of the 5S ribonucleoprotein complex (5S RNP), consisting of 5S rRNA, RPL5, and RPL11, to MDM2 (Nishimura et al., 2015). In the pathway of DNA damage stress response, sensing DNA double-strand breaks, persisting single-stranded DNA, or stalled replication forks, the PI3K-related protein kinase ATM or ATR is activated and phosphorylates specific sites of p53 and MDM2, which results in the activation and stabilization of p53 (Shiloh, 2001; Maréchal and Zou, 2013). Oxidative stress caused by reactive oxygen species (ROS) induces the activation of p53 via the DNA damage stress response pathway, which is triggered by oxidation damage of DNA and via direct activation of ATM by oxidation (Barzilai and Yamamoto, 2004; Guo et al., 2010). Importantly, the ROS-induced p53 signaling acts toward generating ROS through the downstream effector $p66^{shc}$, which comprises a positive feedback loop (Migliaccio et al., 1999; Nemoto and Finkel, 2002).

Among the cellular stresses with responses that rely on p53 in animal cells, DNA damage stress and oxidative stress also occur in plant cells. Therefore, as is the case for the nucleolar stress response, the question arises regarding how plant cells respond to these stresses in the absence of p53 homologs. In Arabidopsis, it was demonstrated by molecular genetic studies that the DNA damage stress response is governed by SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1)/ANAC008, a transcription factor of the NAC family (Preuss and Britt, 2003; Yoshiyama et al., 2009). Upon DNA damage stress, the SOG1 protein is hyperphosphorylated in an ATM-dependent manner and thereby activated to regulate gene expression, which resembles the regulatory mechanism of p53 by ATM and ATR in animal cells (Yoshiyama et al., 2013). Based on these findings, SOG1 is often discussed as a functional counterpart of p53, although SOG1, and p53 have no sequence similarity (Yoshiyama, 2015). Oxidative stress induces DNA damage and elicits the DNA damage stress response in plant cells, as well as in animal cells. Moreover, in plants, oxidative stress promotes leaf senescence independently of DNA damage. Several NAC transcription factors, such as ARABIDOPSIS THALIANA ACTIVATION FACTOR 1 (ATAF1)/ANAC002 and ANAC092, have been reported as mediators of this process (Balazadeh et al., 2010; Garapati et al., 2015). Of these, the most notable one is the membrane-bound NAC protein NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 4 (NTL4)/ANAC053, as this transcription factor is activated through oxidative proteolysis by ROS and promotes ROS production, thus representing a positive feedback loop, similar to that of the p53-dependent oxidative stress response pathway in animal cells (Lee et al., 2012, 2014).

In summary, in the context of cellular stress responses, animal cells utilize p53 to regulate responses to nucleolar stress, DNA damage stress, and oxidative stress, while plant cells employ multiple NAC transcription factors for these roles of

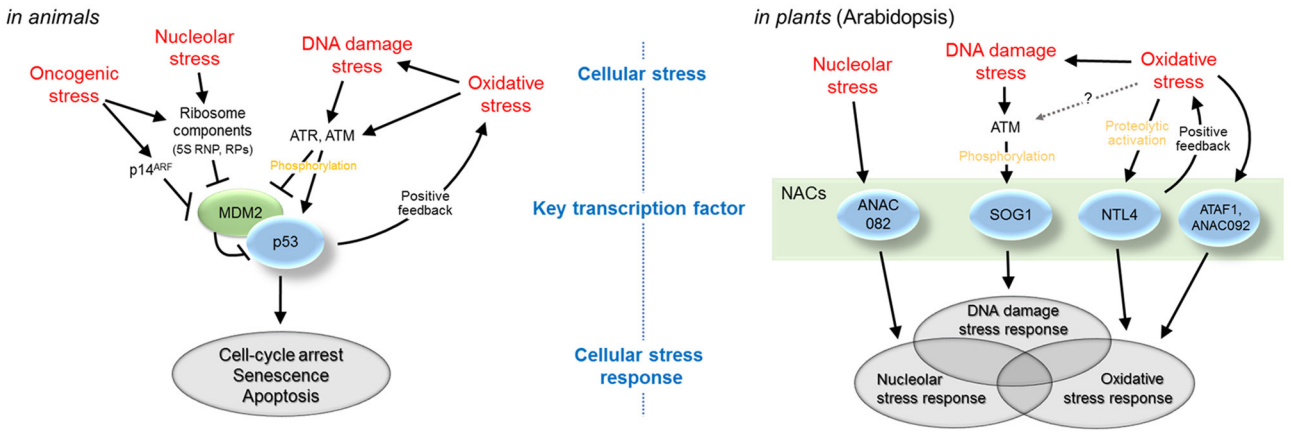

FIGURE 1 | NAC-dependent pathways of plant cellular stress responses and p53-dependent pathways of animal cellular stress responses. Animal cells utilize p53 to regulate responses to nucleolar stress, DNA damage stress, and oxidative stress, while plant cells employ multiple NAC transcription factors for these roles of p53.

p53 (**Figure 1**). Cellular stresses such as nucleolar stress, DNA damage stress, and oxidative stress are both extrinsic and intrinsic to basic cellular activities of ribosome biogenesis, genome replication, and energy metabolism; thus, they are unavoidable in all organisms. To sense and cope with cellular stresses, animals have evolved signaling systems that share a limited number of transcription factors, including p53, as central regulators. In contrast, plants have evolved stress signaling systems that use different transcription factors for different stresses. This strategy seems related to the diversification of transcription factors of particular groups in plants. In this respect, the NAC family has received increasing attention. NAC is one of the largest families of plant transcription factors and includes more than 100 members (Zhu et al., 2012). Many of the NAC-family members have been previously implicated in responses to abiotic and biotic stresses triggered by external causes, such as drought, salt, cold, and pathogen infection (Nakashima et al., 2012; Puranik et al., 2012; Nuruzzaman et al., 2013). Recently discovered NAC-dependent pathways of intrinsic cellular stress responses have added more emphasis to the variety of the NAC transcription factors in plant stress responses.

FUTURE PERSPECTIVES

In conclusion, recent studies collectively suggest that plant cells use a specific pathway for nucleolar stress response involving ANAC082, a member of the plant-unique transcription factor family NAC. Considering the possible role of uORF in the regulation of *ANAC082* expression, the molecular mechanism of this pathway may be quite different from that of the p53-dependent pathway of nucleolar stress response in animal cells; nevertheless, ANAC082 might be regarded as a counterpart of p53 because of their common role as critical transcriptional regulators of the nucleolar stress response. During plant evolution, NAC transcription factors have been highly diversified, and different NAC factors have been assigned to different pathways of stress responses in plants, several of which correspond to the different roles of p53 in the stress responses of animal cells. In this sense, ANAC082 might also be considered a player of the NAC team that is responsible for the tasks carried out by p53 in animal cells.

The ANAC082-dependent pathway underlying the plant nucleolar stress response is a recent concept, and its molecular details remain totally unknown. A particularly important issue is the elucidation of how nucleolar stress is sensed in plants to activate the ANAC082 pathway. Further studies focusing on this problem may lead to the understanding of nucleolar surveillance in plant cells and open new horizons in plant nucleolar biology.

AUTHOR CONTRIBUTIONS

IO and MS conceived this review and wrote the manuscript.

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Function of Plant DExD/H-Box RNA Helicases Associated with Ribosomal RNA Biogenesis

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Ribosome biogenesis is a highly complex process that requires several cofactors, including DExD/H-box RNA helicases (RHs). RHs are a family of ATPases that rearrange the secondary structures of RNA and thus remodel ribonucleoprotein complexes. DExD/H-box RHs are found in most organisms and play critical roles in a variety of RNA-involved cellular events. In human and yeast cells, many DExD/H box RHs participate in multiple steps of ribosome biogenesis and regulate cellular proliferation and stress responses. In plants, several DExD/H-box RHs have been demonstrated to be associated with plant development and abiotic stress tolerance through their functions in modulating pre-rRNA processing. In this review, we summarize the pleiotropic roles of DExD/H-box RHs in rRNA biogenesis and other biological functions. We also describe the overall function of the DExD/H-box RH family in ribosome biogenesis based on data from human and yeast.

Keywords: nucleolus, DExD/H-box RNA helicase, ribosome, development, stress response

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INTRODUCTION

The ribosome is an important molecular machine where mRNA is translated into protein. In eukaryotes, the ribosome consists of a small 40S and a large 60S ribonucleoprotein (RNP) subunit. Ribosome biogenesis is a highly complex and fundamental process that comprises the maturation of rRNAs and their assembly with ribosomal proteins (RPs; Panse and Johnson, 2010). Biosynthesis of rRNA initiates with the transcription of 35S pre-rRNA and 5S rRNA by RNA polymerase I and RNA polymerase III respectively, followed by multiple steps of pre-rRNA processing, modification and folding to yield mature rRNAs (Henras et al., 2008; Woolford and Baserga, 2013). A large number of non-coding RNA and ribosome biogenesis factors (RBFs), such as endo- and exoribonuclease, rRNA-modifying enzymes, and RNA helicases (RHs), participate in these processes (Kressler et al., 2010; Lafontaine, 2015). In human cells, mutations in RBFs and RPs usually cause defects in pre-rRNA processing, resulting in various genetic disorders (Narla and Ebert, 2010; Tafforeau et al., 2013). In plants, RBFs and RPs modulate growth and development, affecting processes including leaf morphology, gametogenesis, and embryo development (Byrne, 2009; Weis et al., 2015).

RHs are enzymes that rearrange the secondary structure of RNA and RNP complexes in an energy-dependent manner (Tanner and Linder, 2001). RHs are classified into six subclasses, SF1–SF6, based on specific motif sequences (Gorbalenya and Koonin, 1993; Singleton et al., 2007). SF2 is the major subclass of RHs, and its members share 12 conserved motifs that are involved in ATP hydrolysis, RNA binding, and intramolecular interactions (Fairman-Williams et al., 2010; Linder and Fuller-Pace, 2013). The related DEAH-, DExD-, and DExD/H-Box families are often referred to as the DExD/H family. They, together with the

DEAD-Box family, constitute the main group of SF2 RHs (Gorbalenya and Koonin, 1993; Fairman-Williams et al., 2010). DExD/H-box RHs are involved in most cellular events associated with RNA, such as ribosome biogenesis, spliceosome assembly, RNA decay, and RNA editing (Jarmoskaite and Russell, 2014). Recent research has revealed the importance of DExD/H-box RHs in many aspects of plant RNA metabolism and physiological processes. In this review, we will focus on the emerging roles of plant nucleolus DExD/H-box RHs in rRNA biogenesis, and summarize the current research on their functions in growth and stress responses.

YEAST AND HUMAN DExD/H-BOX RHs IN RIBOSOME BIOGENESIS

Over the past several decades, the functions of DExD/H-box RHs have been widely studied in yeast and human cells. Approximately half of the characterized RHs are associated with various steps in ribosome biogenesis, from rRNA transcription to final ribosome subunit maturations (Martin et al., 2013; Rodríguez-Galán et al., 2013). Most yeast DExD/H-box RHs involved in ribosome biogenesis are essential for cell viability (Rocak and Linder, 2004). However, some are non-essential and their mutants display specific phenotypes. The yeast mutant of *Dbp2*, which functions in both nonsense-mediated mRNA decay and 25S rRNA biogenesis (Bond et al., 2001), displays growth retardation under cold conditions (Barta and Iggo, 1995). Disruptions of *Dbp3* and *Dbp7*, two additional RHs involved in pre-rRNA processing, render a slow growth phenotype under both optimal and cold conditions (Weaver et al., 1997; Daugeron and Linder, 1998). In human cells, deregulation of ribosome-biogenesis-related DExD/H-box RHs is associated with tumorigenesis. DDX21 is highly expressed in breast carcinomas; it can promote tumorigenesis by enhancing RNA processing in breast cancer cells (Zhang et al., 2014). DDX10 is associated with the inv(11)(p15q22) chromosome abnormality which is found in some myeloid leukemia patients (Arai et al., 1997). The NUP98–DDX10 chimeric protein may promote tumorigenesis through altered ribosome assembly or aberrant mRNA transportation (Arai et al., 1997). DDX5 and DDX17 have pro-proliferation or oncogenic functions in cancer development. They are up-regulated in different types of cancer cells, such as colon, prostate, and cutaneous squamous cell carcinoma (Fuller-Pace and Moore, 2011; Fuller-Pace, 2013). Together, these studies suggest that yeast and human DExD/H-box RHs that are involved in ribosome biogenesis play important roles in maintaining cell proliferation as well as stress adaptation.

BIOLOGICAL ROLES OF RIBOSOME BIOGENESIS RELATED DExD/H-BOX RHs IN PLANTS

Plant genomes encode a larger and more diverse DExD/H RH family than is found in other organisms (Linder and Owttrim, 2009; Xu et al., 2013). Recently, increasing numbers of plant DExD/H-box RHs have been functionally characterized and their roles in biotic and abiotic stresses as well as plant development have been extensively studied (**Table 1**). Among these DExD/H-box RHs, only a small fraction is known to be involved in ribosome biogenesis. AtRH36/SWA3, a homolog of yeast Dbp8p, is the first ribosome biogenesis-related RH that has been functionally analyzed. Knockdown mutants of *AtRH36* display higher accumulations of immature rRNA precursors than WT (Huang et al., 2010a). Loss-of-function of *AtRH36* resulted in a disrupted progression of mitosis during female gametophyte development, whereas the RNAi knock-down mutants displayed several defects in growth and development, such as short roots and abnormal leaves (Huang et al., 2010a; Liu et al., 2010). AtRH18 is another essential DExD/H-Box RH involved in ribosome biogenesis. AtRH18 is a homolog of yeast Sbp4p which participates in the biogenesis of the 60S ribosomal subunit (de la Cruz et al., 1999). Plants with reduced AtRH18 activity show chlorosis, while the knockout mutants are embryo-lethal (Plötner et al., 2017).

Knocking out of non-essential RHs resulted in pleiotropic phenotypes. AtMTR4 is a predominantly nucleolar localized DExD/H protein that associates with the RNA exosome complex and functions in rRNA maturation and surveillance (Lange et al., 2011, 2014). A mutation in *AtMTR4* increases accumulation of rRNA precursors and rRNA maturation by-products, resulting in several developmental defects, such as delayed embryogenesis, abnormal cotyledons, shorter root, etc. (Lange et al., 2011, 2014). It has been reported that AtRH57 is an ATP-independent RH, and that it can be induced by glucose, ABA, and salt (Hsu et al., 2014). Functional analyses using *atrh57* knockout mutants indicated that AtRH57 negatively regulates glucose-mediated ABA accumulation and signaling in germination and early seedling development (Hsu et al., 2014). In addition, *AtRH57* mutations cause accumulation of abnormal rRNA precursors, hampering small ribosomal subunit formation, which becomes more significant with high levels of glucose (Hsu et al., 2014). Recently, the relationship between DExD/H-box RHs and temperature stress tolerance has been reported in *Arabidopsis*. AtRH10 is a homolog of human DDX47 and yeast Rrp3p, both of which are involved in ribosome biogenesis (Matsumura et al., 2016). The missense *rh10* mutant, impaired in pre-rRNA processing, shows defects in primary root elongation and leaf development under high temperature (Matsumura et al., 2016; Ohbayashi et al., 2017). AtRH7/PRH75 is a bifunctional RH with RNA unwinding and rewinding activities (Nayak et al., 2013) and is required for pre-rRNA processing (Huang et al., 2016; Liu et al., 2016). The *rh7* mutants display pleiotropic phenotypes in growth and development including pointed leaves and disturbed vascular patterns which are also found in ribosome-related mutants (Huang et al., 2016; Liu et al., 2016). The functions of AtRH7 are associated with low-temperature stress. *rh7* mutants exhibit severe retardation in germination and defects in leaf morphogenesis under a mild cold stress condition (12°C), and cannot survive under prolonged 4°C treatment (Huang et al., 2016; Liu et al., 2016). Genetic analysis showed that the abnormal accumulation of

TABLE 1 | List of characterized DEAD-box RHs in *Arabidopsis*, rice, and maize.

Plant	Symbol	RNA metabolism	Physiological function	Reference
Arabidopsis	AtRH3	Chloroplast ribosome biogenesis	Chloroplast development	Asakura et al., 2012
		roup II intron splicing	ABA response	Gu et al., 2014
			Stress tolerance	Lee et al., 2013
	AtRH7	Ribosome biogenesis	Plant development	Huang et al., 2016
			cold tolerance	Liu et al., 2016
	AtRH10	Ribosome biogenesis	High-temperature tolerance	Matsumura et al., 2016
	AtRH22	Chloroplast ribosome biogenesis	Chloroplast development	Chi et al., 2012
			Seed oil biosynthesis	Kanai et al., 2013
	AtRH36	Ribosome biogenesis	Female gametogenesis	Huang et al., 2010a
				Liu et al., 2010
	AtRH39	Chloroplast ribosome biogenesis	Chloroplast development	Nishimura et al., 2010
	LOS4	mRNA export	Stress tolerance	Gong et al., 2002, 2005
	PMH2	Group II intron splicing	Unidentified	Köhler et al. (2010)
	RCF1	mRNA splicing	Freezing tolerance	Guan et al., 2013
	STRS1	Gene silencing	Stress tolerance	Kant et al., 2007
	STRS2			Khan et al., 2014
	ESP3	mRNA splicing	Embryonic development	Herr et al., 2006
	RID1	mRNA splicing	Root development	Ohtani et al., 2013
	AtRH57	Ribosome biogenesis	Glucose and ABA response	Hsu et al.,2014
	ABO6	Mitochondrial RNA splicing	ABA and auxin signaling	He et al., 2012
	HEN2	RNA surveillance	Flower development	Western et al., 2002;
				Lange et al., 2014
	AtMTR4	Ribosome biogenesis	Plant development	Lange et al., 2011
	ISE2	Chloroplast ribosome biogenesis	Plasmodesmata regulation	Carlotto et al., 2016
		Group II intron splicing		Bobik et al., 2017
	AtRH2	Unidentified	Tombusvirus defense	Kovalev and Nagy, 2014
	AtRH8	Unidentified	Potyviruses defense	Huang et al., 2010c
	AtRH9	Unidentified	Potyviruses defense	Li et al., 2016
	AtRH18	Unidentified	Embryonic development	Plötner et al., 2017
	AtRH20	Unidentified	Tombusvirus defense	Kovalev et al., 2012
	ISE1	Unidentified	Plasmodesmata regulation	Stonebloom et al., 2009
	AtHELPS	Unidentified	K^+ tolerance	Xu et al., 2011
Rice	TOGR1	Ribosome biogenesis	Thermotolerance	Wang et al., 2016
	OsRH36	Unidentified	Female gametogenesis	Huang et al., 2010b
	OsBIRH1	Unidentified	Biotic and abiotic stress tolerance	Li et al., 2008
	OsSUV3	Unidentified	Salt tolerance	Tuteja et al., 2013
Maize	ZmRH3	Chloroplast ribosome biogenesis	Unidentified	Asakura et al., 2012
		Group II intron splicing		
	ZmDRH1	Ribosome biogenesis	Unidentified	Gendra et al., 2004

rRNA precursors in *rh7* was elevated when these plants were exposed to cold (Huang et al., 2016; Liu et al., 2016); thus, cold may trigger the abnormal phenotypes in the mutant. Interestingly, AtRH7 can physically interact with AtCSP3 (Cold Shock Domain Protein 3), an RNA chaperone involved in cold adaptation (Kim et al., 2013), suggesting that AtRH7 may complex with AtCSP3 to regulate the secondary structure of pre-rRNA, and thus ensure proper pre-rRNA processing in *Arabidopsis*.

Functions of DExD/H-box RHs as RBFs were also investigated in crop plants. OsRH36 complemented the homologous *atrh36-1* mutant and was required for either gametogenesis or fertilization during reproduction in rice (Huang et al., 2010b). Recently, another rice DExD/H-box RH, TOGR1 (Thermotolerant Growth Required 1), was isolated by map-based cloning from a thermosensitive dwarf *indica* mutant (Wang et al., 2016). The *togr1* mutant exhibited high-temperature-dependent dwarf phenotypes. Overexpression of *TOGR1* resulted in enhanced thermotolerance as well as increased plant height and yield under high-temperature conditions (Wang et al., 2016). Molecular analyses on the *togr1* mutant demonstrated that TOGR1 is associated with U3 snoRNA and is involved in pre-rRNA homeostasis (Wang et al., 2016). When the temperature was raised from 25 to 38°C, rRNA precursor *P-A3* accumulated to a higher level in *togr1* mutants than in WT, suggesting a crucial role of TOGR1 in maintaining pre-rRNA processing at high

temperatures (Wang et al., 2016). In maize, the DExD/H-box RH, ZmDRH1, has been shown to interact with MA16 and fibrillarin to form a RNP complex involved in rRNA metabolism (Gendra et al., 2004). Collectively, the recent studies of plant DExD/H-box RHs have revealed multiple roles for these enzymes in plant development and stress adaptation.

POTENTIAL PLAYERS IN RIBOSOME BIOGENESIS

Considering the fact that about half of the identified DExD/H-box RHs from human and yeast are RBFs (Rocak and Linder, 2004; Jankowsky et al., 2011; Rodríguez-Galán et al., 2013), we reasoned that there are more DExD/H-box RHs associated with rRNA metabolism in plants than have previously been identified. We thus conducted a database search using *Ensembl Plants*[1] to identify the homologs of human and yeast DExD/H-box RHs from *Arabidopsis*, rice and maize genomes. Twenty-eight potential candidates, whose homologs in human and yeast were involved in ribosome biogenesis, were identified in the *Arabidopsis* genome, whereas 27 and 29 were found in rice and maize, respectively (**Table 2**). A recent proteomic analysis in *Arabidopsis* identified 1,602 nucleolar proteins, and 519 potential RBFs (Palm et al., 2016). Among these RBFs, 31 were identified as DExD/H-box RHs (Palm et al., 2016). This research together with our database search indicated that over 20 DExD/H-box RHs might participate in ribosome biogenesis in plants, suggesting that many steps in their ribosome biogenesis are RHs dependent, as has been shown in human and yeast.

[1]http://plants.ensembl.org

TABLE 2 | List of rice, maize, and *Arabidopsis* DExD/H-RHs with homology to human and yeast DExD/H-box RHs which function as RBFs.

Human	Yeast	Rice	Maize	*Arabidopsis*
DDX3X	Dbp1p	OS03G0805200 C H	Zm00001d007757	AT3G58510 (AtRH11)*
DDX3Y	Ded1p	OS11G0599500 D	Zm00001d007755	AT2G42520 (AtRH37)* C
		OS07G0202100	Zm00001d048924 C D H	AT3G58570 (AtRH52)* C
DDX5	Dbp2p	OS01G0197200 D	Zm00001d039452	AT1G55150 (AtRH20)*
DDX17		OS01G0911100 C D	Zm00001d042416	AT5G63120 (AtRH30)* C
DDX10	Dbp4p	OS07G0517000	Zm00001d006497 D	AT5G54910 (AtRH32)* C H
DDX18	Has1p	OS03G0802700 H	Zm00001d013056 C	AT3G18600 (AtRH51)* C H
		OS06G0535100	Zm00001d023501	AT5G65900 (AtRH27)* H
DDX21	N/A	OS09G0520700 C D H	Zm00001d006160 C D	AT5G62190 (AtRH7) C
DDX50			Zm00001d021196	
DDX24	Mak5p	OS04G0510400 D H	Zm00001d003031 C	AT3G16840 (AtRH13)* C H
DDX27	Drs1p	OS12G0481100 H	Zm00001d006113 D	AT4G16630 (AtRH28)*
			Zm00001d021127	
DDX31	Dbp7p	OS05G0110500 D	Zm00001d010225	AT2G40700 (AtRH17)* C H
DDX41	N/A	OS02G0150100	Zm00001d047502	AT5G51280 (AtRH35)*
		OS06G0697200 D		AT4G33370 (AtRH43) C
DDX47	Rrp3p	OS03G0669000 (TOGR1) D H	Zm00001d014787 C	AT5G60990 (AtRH10)* H
		OS07G0660000		
DDX48	Fal1p	OS01G0639100	Zm00001d018542	AT3G19760 (AtRH2)*
		OS03G0566800	Zm00001d051840	AT1G51380 (AtRH34)* C H
DDX49	Dbp8p	OS07G0633500	Zm00001d022246	AT1G16280 (AtRH36) C
DDX51	Dbp6p	OS02G0795900 H	Zm00001d018375	AT4G15850 (AtRH1)
DDX52	Rok1p	OS07G0647900 H	Zm00001d022356	AT3G09720 (AtRH57)* H
			Zm00001d022360 C D	
DDX54	Dbp10p	OS08G0416100 C H	Zm00001d050315	AT1G77030 (AtRH29)* C
DDX55	Sbp4p	OS01G0164500 D H	Zm00001d039746	AT5G05450 (AtRH18)*
				AT1G71370 (AtRH49) S
DDX56	Dbp9p	OS03G0728800 H	Zm00001d013358	AT4G34910 (AtRH16)* C H
DHX15	Prp43p	OS03G0314100 D	Zm00001d028923	AT3G62310 (DEAH2)*
			Zm00001d047601	AT2G47250 (DEAH3)*
DHX37	Dhr1p	OS02G0736600	Zm00001d017967 D	AT1G33390 (DEAH13)* C
SkiV2L2	Mtr4p	OS12G0279000 D H	Zm00001d045590 D	AT1G59760 (AtMTR4)*

The amino acid sequences of human and yeast DExD/H-box RHs were BLAST-searched against the protein databases of Arabidopsis, rice, and maize available at Ensembl Plants (http://plants.ensembl.org). The protein sequences showing the highest similarity were considered as homologs. Data for abiotic stress-responsiveness were obtained from eFP Brower (http://bar.utoronto.ca/efp_arabidopsis/) for Arabidopsis and GENEVESTIGATOR (https://genevestigator.com) for rice and maize. The genes showing a fold change >2 are indicated as C, cold inducible; D, drought inducible; S, salt inducible; and H, heat inducible following the annotation numbers. Asterisk "" indicates the Arabidopsis nucleolar DEAD-box RHs reported as human and yeast RBFs orthologs (Palm et al., 2016).*

Recent studies on AtRH7 and TOGR1 have suggested that stress inducible RBF-type RHs play an important role in modulating plant stress adaptation (Huang et al., 2016; Liu et al., 2016; Wang et al., 2016). This led us to search two plant expression databases, eFP Brower[2] and GENEVESTIGATOR[3], to analyze the stress-responsive expression pattern of previously identified candidates. As shown in **Table 2**, many RH genes are potentially up-regulated by at least one type of stress, implying that these DExD/H-box RHs may participate in plant stress responses. Ribosome biogenesis is known to be highly coupled with stress stimuli (Boulon et al., 2010); DExD/H-box RHs are critical players in this life process. Therefore, it will be interesting to investigate how these helicases modulate rRNA maturations and how they contribute to plant morphogenesis under stress conditions in future studies.

CONCLUSION AND PERSPECTIVES

This mini-review has focused on the DExD/H-box RHs which function in pre-rRNA processing in plants. Recent studies have revealed physiological roles of these RHs in plant reproduction, development, and stress responses. However, their precise molecular functions in rRNA biogenesis still remain unclear. Thus, in future studies, it will be necessary to investigate which steps of the pre-rRNA processing these DExD/H-box RHs are involved in, and how they recognize their target rRNA sequence.

In human cells, single DExD/H-box RHs function in a variety of steps in ribosome biogenesis. For instance, DDX47 has a potential role in rRNA transcription in addition to pre-rRNA processing (Sekiguchi et al., 2006; Zhang et al., 2011); DDX21, a homolog of AtRH7, not only regulates pre-rRNA processing but also modulates transcription of rRNA, snoRNAs and RP mRNA (Henning et al., 2003; Calo et al., 2014; Xing et al., 2017). Therefore, it will be interesting to determine if plant DExD/H-box RHs participate in the transcription of ribosome biogenesis components. Moreover, many DExD/H-box RHs are still uncharacterized but are predicted to be involved in ribosome biogenesis (**Table 2**). Therefore, future work should be focused on characterizing these potential candidates and revealing their functions in plant growth and stress adaptation. Together, these investigations will provide further insights into the complexity of plant ribosome biogenesis and the intrinsic connection between ribosome biogenesis and plant physiological processes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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[2] http://bar.utoronto.ca

[3] https://genevestigator.com

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Novel Ribonuclease Activity Differs between Fibrillarins from *Arabidopsis thaliana*

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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 confine 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

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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 fibrillarin 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)P_2, phosphatidylinositol 4,5-bisphosphate; RRM, RNA recognition motif.

(Ochs et al., 1985). Also fibrillarin in plants was detected for first time in onion cells in the transition zone between the FC and the DFC (Cerdido and Medina, 1995). Ultrathin sections of rat neurons have shown fibrillarin localization at the periphery of FC and in the DFC (Desterro et al., 2003). Fibrillarin is a conserved *S*-adenosyl-L-methionine-dependent methyltransferase which is found in all eukaryotic cells and a shorter version exists in the Archaea kingdom as well (Rodriguez-Corona et al., 2015; Shubina et al., 2016). Therefore the only activity assigned to fibrillarin 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 fibrillarin is an essential protein in eukaryotic organism so its precise role may still need to be defined. Reduced levels of fibrillarin 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 fibrillarin. It correlates well with several types of cancers that show the reduction of p53 and therefore an increase of fibrillarin and higher level of methylation in ribosomes causing errors during translation (Marcel et al., 2013). Human fibrillarin also forms 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 fibrillarin in human cells shows nuclear structure alterations in a cell cycle dependent manner before the cells death (Amin et al., 2007).

Fibrillarin in plants has been found in pulldowns of the RNA pol II transcription mediator complex as subunit 36a. *Arabidopsis thaliana* has three different genes of fibrillarin (Barneche et al., 2000). It is also involved in the viral progression and long distance trafficking of viruses in plants like the Bamboo mosaic potexvirus satRNA forms a ribonucleoprotein complex with fibrillarin 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 fibrillarin proteins: fibrillarin 1 (AtFib1) and fibrillarin 2 (*FLP* fibrillarin-like protein; AtFib2) from *A. thaliana* as a model plant. In most eukaryotic cells, fibrillarin localizes 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 fibrillarin (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, fibrillarin 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 fibrillarin. However, the combination sequence of GAR domain and at least one RRM of fibrillarin 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 for rRNA has shown to involve a complex were several proteins are, including fibrillarin (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 fibrillarin has 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 fibrillarin nuclear dynamics.

MATERIALS AND METHODS

Bioinformatic Analysis

Amino acid alignment in **Figure 1A** was visualized by BOXSHADE v3.3.1C[1]. 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°IR (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-jigsaw[2] 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′: GGATCCCACAATCACTTTGCTTCCTCC. Atα2 primers:

[1]http://boxshade.sourceforge.net/

[2]https://bmm.crick.ac.uk/~3djigsaw/

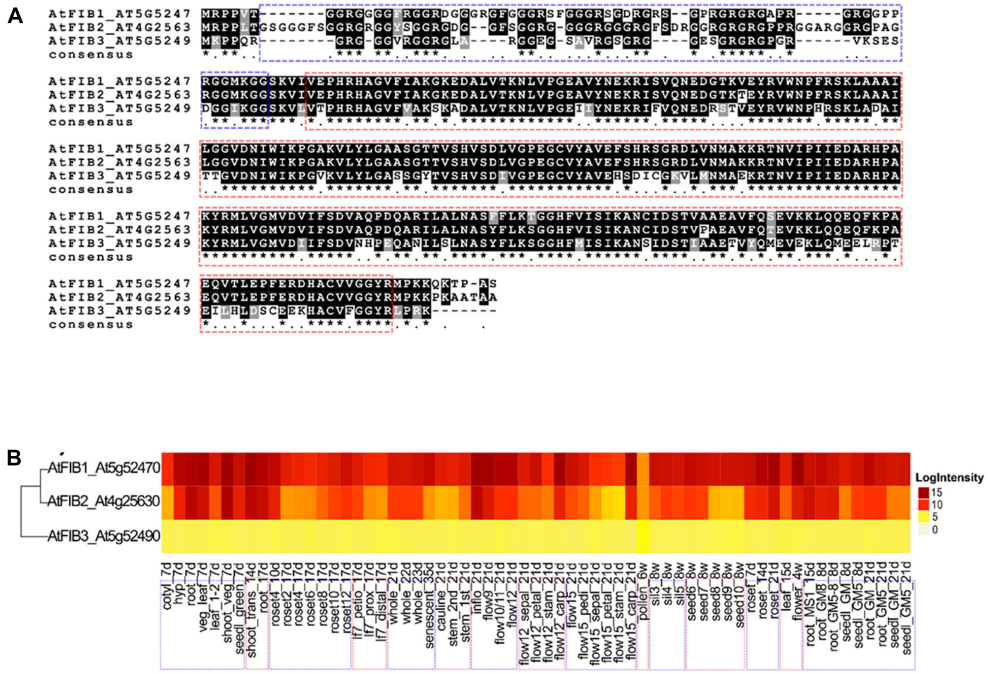

FIGURE 1 | Alignment and ribonuclease activity of *A. thaliana* fibrillarins. **(A)** Sequence comparison of the three AtFib proteins by aligning in MAFFT program. **(B)** Heat-map of the transcriptional expression patterns of AtFib1-3 genes in different tissues and different developmental stages in wild-type *Arabidopsis* Col-0. Data were taken from (Schmid et al., 2005).

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

Protein Expression and Purification

Arabidopsis thaliana fibrillarins were expressed in *E. coli* Artic 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 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 1h 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′ CCTGTCAGACCGCCGTGC GAC.

Ribonuclease Assay

Total RNA extracted from *A. thaliana* was mixed with each fibrillarin 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 fibrillarin 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 fibrillarin 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 + fibrillarin 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 fibrillarin (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 fibrillarin with RNA resulted in degradation of the RNA when a short incubation was carried out at room temperature. We therefore tested the purified fibrillarin with an *in gel* ribonuclease activity assay to make sure that no other protein was responsible

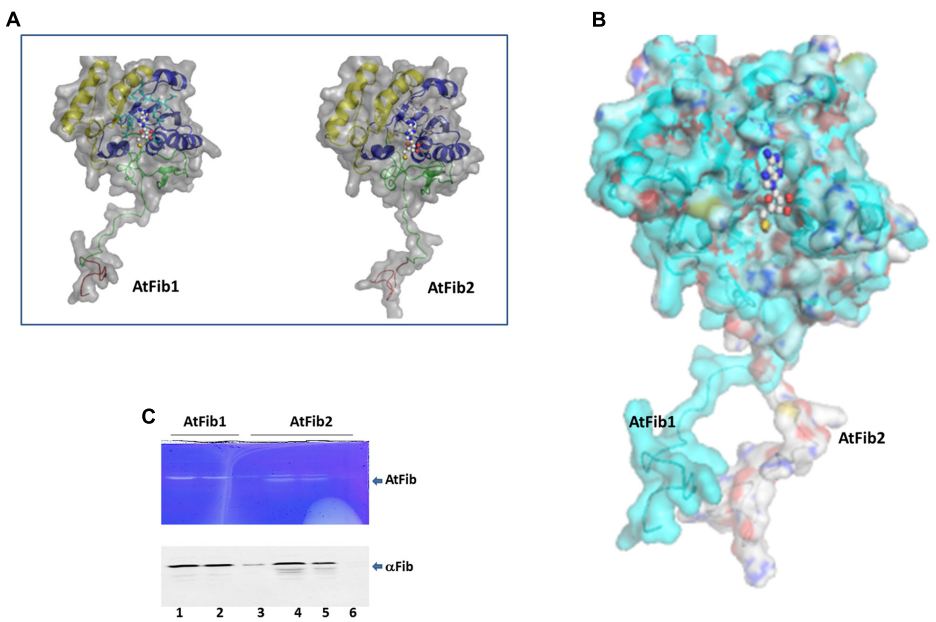

FIGURE 2 | Structural difference between fibrillarins from *A. thaliana*. **(A)** The domains are shown as follows. In Red the GAR domain, Green: BCO space region, Yellow: RNA binding domain, Blue: Alpha helix domain. SAM is showed as spheres. The molecular surface of the proteins is showed in gray color. **(B)** Structural alignment of AtFib1 and AtFib2. The alignment shows that GAR domain and BCO space region are oriented in opposite directions in these two proteins. **(C)** *In gel* ribonuclease activity assay. The white bands correspond to the spaces in the gel, in which RNA was degraded by fibrillarin, confirmed by Western blot. 1, 2, and 3 are three different elutions from the purification process of AtFib1. 4, 5, and 6 are three different elutions from the purification process of AtFib2.

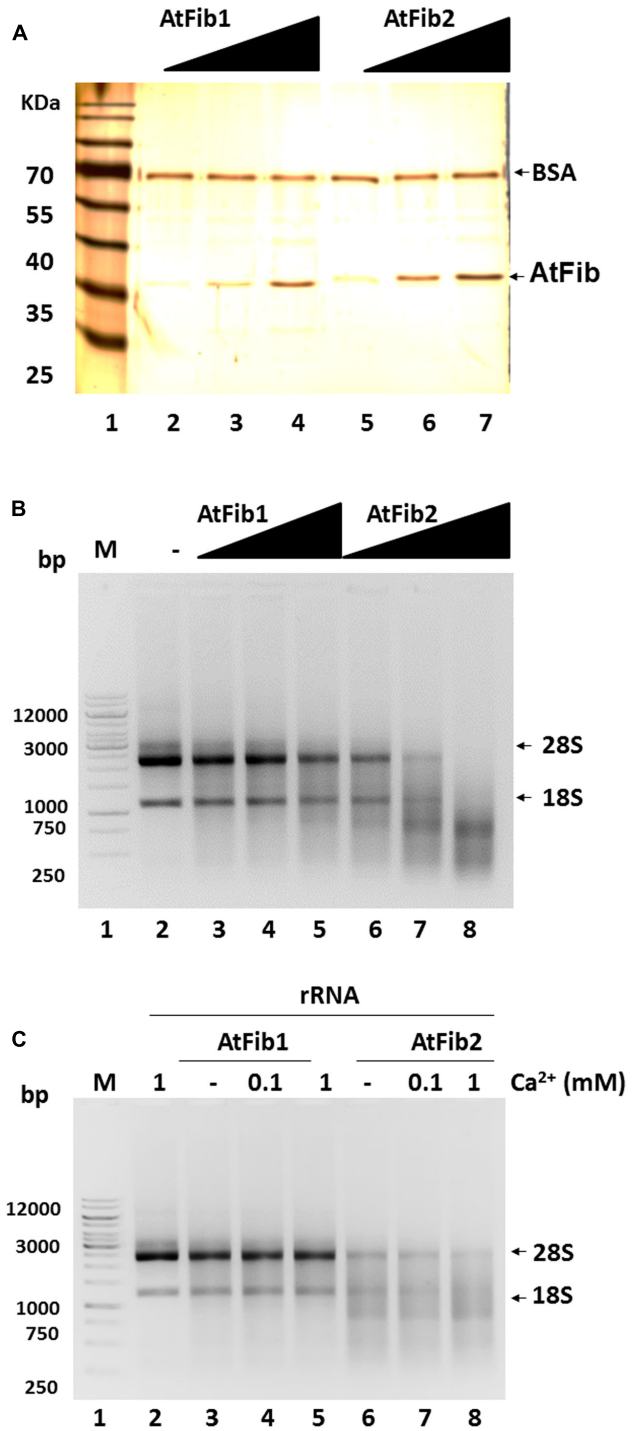

FIGURE 3 | Ribonuclease activity of *A. thaliana* fibrillarins in calcium presence. **(A)** Concentration from 2 to 8 ng of both *A. thaliana* fibrillarins. BSA was added to normalize. **(B)** Fibrillarin ribonuclease activity. Increased amounts of fibrillarin were added to a constant concentration of rRNA. The assay clearly shows that AtFib1 is less active as compared to AtFib2 at the same concentration. **(C)** Using the concentration of fibrillarins as shown in lane 3 and 5 of **Figure 3A**, we tested further the activation of ribonuclease activity by calcium for AtFib1 and AtFib2 (lane 4 – 5 and 7 – 8, respectively). Calcium was added at the concentrations of 0.1–1 mM.

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 fibrillarin (**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 fibrillarin (**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 fibrillarin that we tested (data not shown).

Our previous studies with human fibrillarin 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 fibrillarin (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)P_2 antibody. We carried out confocal microscopy of *Arabidopsis* callus which had membrane bound PtdIns(4,5)P_2 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)P_2 has a partial colocalization with fibrillarin. Since the antibodies against fibrillarin detect both forms of fibrillarin 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)P_2 exhibits a dotted pattern in nucleoli regions and a diffuse pattern in other nuclear regions. Fibrillarin colocalizes with PtdIns(4,5)P_2 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

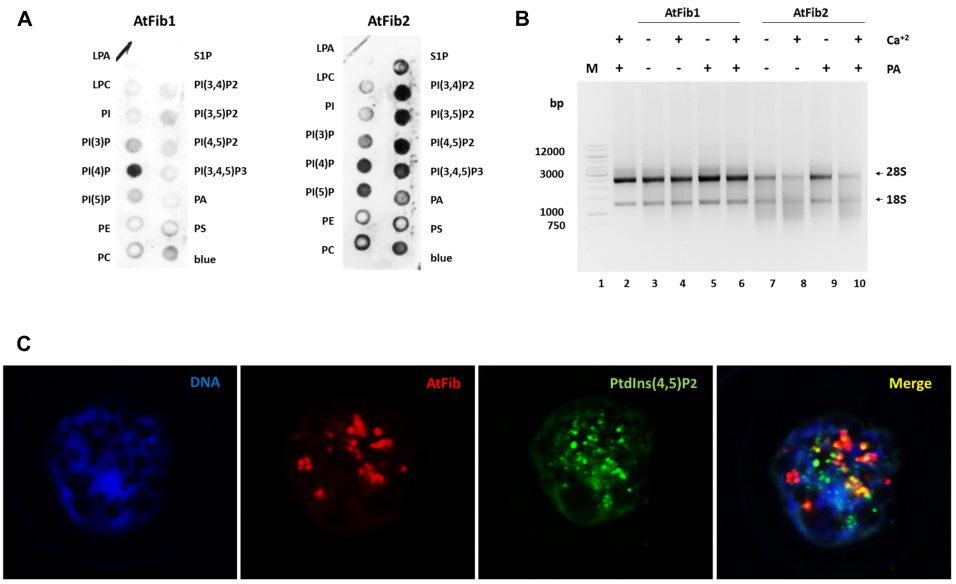

FIGURE 4 | *Arabidopsis thaliana* fibrillarins and phosphoinositides. **(A)** Fat blot assay for AtFib1 and AtFib2. AtFib1 interacts mainly with the monophosphate phosphoinositides, in contrast AtFib2 interacts with all phosphoinositides and phosphatidic acid. **(B)** Ribonuclease activity in phosphatidic 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 inhibit (lane 9). **(C)** Colocalization between AtFib's and PtdIns(4,5)P_2 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 fibrillarin 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 fibrillarin 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 environment 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. Fibrillarin 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 fibrillarin), the main attributed activity of fibrillarin 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-RNA to produce the mature forms.

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

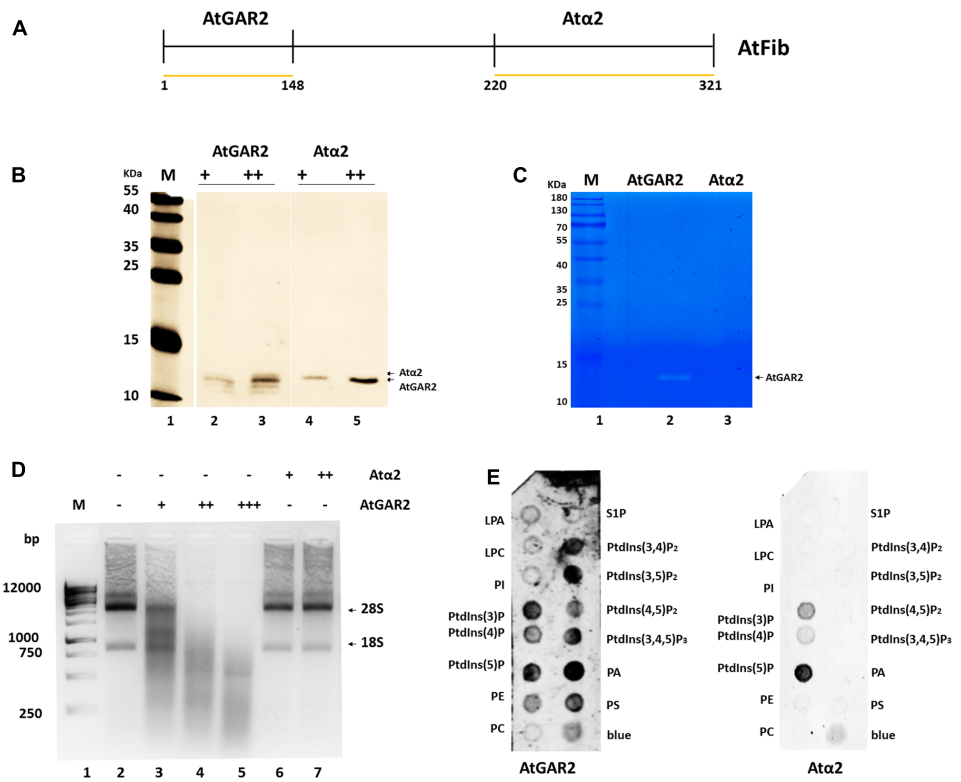

FIGURE 5 | Ribonuclease activity of AtFib2 domains. **(A)** Schematic representation of AtFib. Yellow lines represent the expressed domains [AtGAR2 (1–48 amino acids) and Atα2 (220–321 amino acids)]. **(B)** Western blot for AtFib2 domains. Two different concentrations of AtGAR2 and Atα2 were recognized with anti-HIS primary antibody. **(C)** *In gel* ribonuclease activity assay. The white bands correspond to the spaces in the gel in which RNA was degraded by AtGAR2. **(D)** Ribonuclease activity of AtGAR2 and Atα2 domains. Degradation of rRNA was directly related to the amount of AtGAR2 domain added. **(E)** Fat blot assay for AtGAR2 and Atα2. AtGAR2 domain interacts with all phosphoinositides in the same way as the whole AtFib2 protein. By the other hand, Atα2 interacts mainly with PtdIns(5)P.

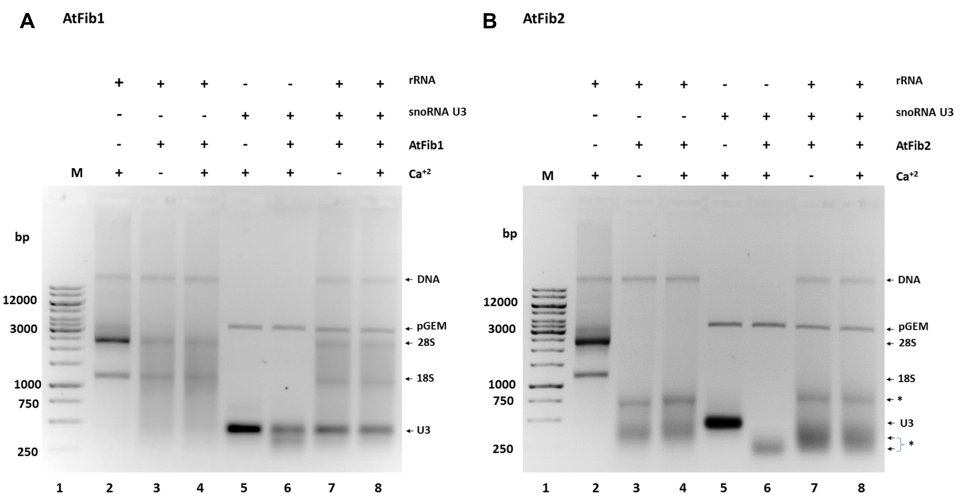

FIGURE 6 | Ribonuclease activity against rRNA and snoRNA U3. **(A)** AtFib1 ribonuclease activity against rRNA and snoRNA U3. As expected, compared to AtFib2, AtFib1 has significantly lower ribonuclease activity either to rRNA (lanes 3 and 4) or to snoRNA U3 (lane 6–8). **(B)** AtFib2 ribonuclease activity against rRNA and snoRNA U3. As expected, compared to AtFib1, AtFib2 has ribonuclease activity against rRNA (lanes 3 and 4) and snoRNA U3 (lane 6–8). The asterisks show * the positions of specific RNA bands originated from RNAs cleaved by AtFib2.

transition between Archaea to eukaryotic cells as it is absent in all archaebacteria. 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). Fibrillarin forms a complex with Nop56, Nop58, a guide RNA and 15.5k, we postulate that the fibrillarin 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 fibrillarin (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-RNA 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 fibrillarin as important proteins in this fraction (Saez-Vasquez et al., 2004). Other previous experiments suggested that fibrillarin is the ribonuclease protein involved in the cleavage of rRNA (Kass et al., 1990). They used specific antibodies against human fibrillarin native complex in an *in vitro* ribonuclease assay and showed a decrease in activity when the fibrillarin was blocked (Kass et al., 1990). Surprisingly the authors did not suggest that fibrillarin was involved in the cleavage of rRNA but assumed that it affected the complex. Also fibrillarin 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 fibrillarin for sequence specific breaks as was shown with the complex by Kass et al. (1990).

Previously we showed that human fibrillarin 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 fibrillarin 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)P$_2$ 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 fibrillarin.

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 fibrillarin 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 whit 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 fibrillarin in Cajal bodies: does it have a role in mRNA processing? Is there a ribonuclease role of fibrillarin as mediator 36a? During cell cycle, does the alteration in nuclear structure in fibrillarin depleted cells is due to degradation of structural RNA? Do viral particles require fibrillarin 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.

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REVIEW
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The Multiple Functions of the Nucleolus in Plant Development, Disease and Stress Responses

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The nucleolus is the most conspicuous domain in the eukaryotic cell nucleus, whose main function is ribosomal RNA (rRNA) synthesis and ribosome biogenesis. However, there is growing evidence that the nucleolus is also implicated in many other aspects of cell biology, such as regulation of cell cycle, growth and development, senescence, telomerase activity, gene silencing, responses to biotic and abiotic stresses. In the first part of the review, we briefly assess the traditional roles of the plant nucleolus in rRNA synthesis and ribosome biogenesis as well as possible functions in other RNA regulatory pathways such as splicing, nonsense-mediated mRNA decay and RNA silencing. In the second part of the review we summarize recent progress and discuss already known and new hypothetical roles of the nucleolus in plant growth and development. In addition, this part will highlight studies showing new nucleolar functions involved in responses to pathogen attack and abiotic stress. Cross-talk between the nucleolus and Cajal bodies is also discussed in the context of their association with poly(ADP ribose)polymerase (PARP), which is known to play a crucial role in various physiological processes including growth, development and responses to biotic and abiotic stresses.

Keywords: the nucleolus, plant development, plant stress responses, virus, plant–pathogen interactions

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Abbreviations: rRNA, ribosomal RNA; RNP, ribonucleoprotein; FC, fibrillar centre; DFC, the dense fibrillar component; GC, the granular component; snoRNA, small nucleolar RNA; snRNAs, small nuclear RNAs; NoV, nucleolar vacuole; NAD, nucleolus-associated chromatin domain; TE, transposable element; NOR, nucleolus organizer region; Pol II-RNA polymerase II; Pol I, RNA polymerase I; snoRNP, small nucleolar ribonucleoprotein; EJC, Exon junction complex; NMD, nonsense-mediated mRNA decay; scaRNAs, small Cajal bodies-specific RNAs; CB, Cajal body; siRNA, small interfering RNA; ncRNA, non-coding RNA; sRNA, small RNA; ta-siRNA, trans-acting small interfering RNA; natsiRNA, natural cis-antisense siRNA; miRNA, microRNA; hc-siRNA, heterochromatic small interfering RNA; TGS and PTGS, transcriptional and post transcriptional gene silencing; DCL, DICER-like enzyme; RDR, RNA-dependent RNA polymerase; TR, telomerase RNA; TERT, telomerase reverse transcriptase; RBFs, ribosome biogenesis factors; TOR, target of rapamycin; SIN, septation initiation network; TAC1, telomerase activator 1; ABA, abscisic acid; GRV, groundnut rosette virus; PLRV, potato leaf roll virus; RSM, rice stripe virus; BaMV, bamboo mosaic virus; satBaMV, satellite RNA associated with bamboo mosaic virus; SNI1, suppressor of NPR1-1inducible 1; CP, coat protein; PVA, potato virus A; TGBp1, protein 1 encoded with triple gene block; PSLV, poa semilatent virus; S6K, S6 kinase; RPS6, ribosomal protein S6; HD2B, histone deacetylase 2; NAP1, nucleosome assembly protein 1; TuMV, *turnip mosaic* virus; TuCV, turnip crinkle virus; TMV, tobacco mosaic virus; CMV, cucumber mosaic virus; AlMV, Alfalfa mosaic virus; TF, transcription factor; TBSV, tomato bushy stunt virus; STRS, stress response suppressor; RdDM, RNA-directed DNA methylation; LEA, late embryogenesis abundant protein; TRV, tobacco rattle virus; BSMV, *barley stripe mosaic* virus; TBRV, tomato black ring virus; TGMV, tomato golden mosaic virus; TVCV, turnip vein clearing virus; PVY, potato virus Y; PARP, poly(ADP ribose)polymerase; PAR, ADP-ribose polymer; PCD, programmed cell death; SOG, suppressor of gamma response; DDR, DNA damage response; DSB, double-strand breaks; TDP, tyrosyl-DNA phosphodiesterase; RTEL, regulator of telomere elongation helicase.

PLANT NUCLEOLAR ORGANIZATION

The nucleolus is the largest and most prominent domain in the eukaryotic interphase cell nucleus. Nucleoli vary in size in different cells, for example in small cells like yeast they are $<1\,\mu m$ diameter, whereas in larger cells such as pea they are $>10\,\mu m$ in diameter (Shaw, 2015). The nucleolus is a dynamic membrane-less structure whose primary function is ribosomal RNA (rRNA) synthesis and ribosome biogenesis. However, there is mounting evidence that the nucleolus is also implicated in many other aspects of cell biology, such as differentiation, cell cycle regulation, growth and development, senescence, gene silencing, telomerase activity, responses to biotic and abiotic stresses, and biogenesis of various ribonucleoprotein (RNP) particles (Olson and Dundr, 2005; Boisvert et al., 2007; Hiscox, 2007; Sirri et al., 2008; Greco, 2009; Taliansky et al., 2010; Stepinski, 2014; Brighenti et al., 2015; Lafontaine, 2015; Weis et al., 2015b).

The plant nucleolus has a well-defined architecture with prominent functional compartments such as fibrillar centers (FC), the dense fibrillar component (DFC), the granular component (GC), nucleolar chromatin, nucleolar vacuoles, and nucleolonema (**Figure 1**; Stepinski, 2014). It is largely formed of proteins (85–90%) and RNA (5–10%), with rDNA comprising a minor component (Gerbi and Borovjagin, 1997; Shaw and Brown, 2012).

Interestingly the DFC and FC nucleolar components are typically organized into an important nucleolar substructure called the nucleolonema, which is composed of a DFC matrix punctuated with spherical or tubular FCs, and may also contain FC condensed chromatin and harbor rDNA. This structure has several functional domains such as rDNA transcription, transcript processing and ribosome assembly zones, which are consistent with the activities associated with DFC and FC (Yano and Sato, 2002; Sato et al., 2005).

Plant FCs are assumed to be the assembly sites of complexes containing transcription-associated factors which can either be ready for transcription or be in an inactive state (de Carcer and Medina, 1999; González-Camacho and Medina, 2006). Plant FCs also contain rDNA, which are not yet engaged in transcription but may later be deployed to this process in specific circumstances (Shaw, 1996; McKeown and Shaw, 2009). The number and sizes of FCs depend on the cell cycle phases: cells in G1 phase normally possess much fewer numbers of FCs than those at the G2 phase (Grummt, 2003; González-Camacho and Medina, 2006).

In plant nucleoli the DFC occupies the majority of the nucleolar volume (up to 70%) and provides an environment for transcription of precursor rRNAs (pre-rRNAs); a process which can occur simultaneously at multiple sites (200–400) within this region. The produced pre-rRNAs subsequently undergo further processing in the DFC and then in the associated GC. For example, localization studies of pre-rRNAs and different small nucleolar RNAs (snoRNAs) and proteins have elucidated that early and late pre-rRNA cleavage events can occur in the DFC and GC, respectively, suggesting a vectorial model for the production and maturation of rRNAs (Brown and Shaw, 1998). It is thought that in the GC the final steps of assembly of small and large ribosomal subunits from mature rRNAs and ribosomal proteins occurs, and that the GC could participate in the transit of the assembled ribosomes through the extranucleolar nucleoplasm to the cytoplasm (Shaw et al., 1995; Shaw, 1996).

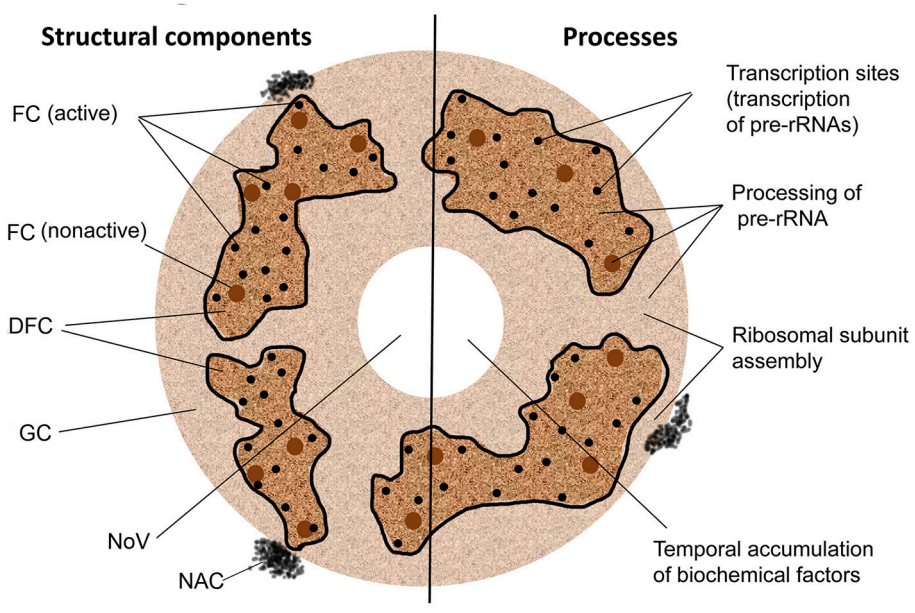

FIGURE 1 | Structural and functional domains of the nucleolus. FC, fibrillar center; DFC, the dense fibrillar component; GC, the granular component; NoV, nucleolar vacuole; NAC, nucleolus-associated chromatin. Nucleolonema is encircled with black lines.

Plant nucleoli also usually contain so called nucleolar cavities or vacuoles (NoV) often located in the central part of the nucleolus (**Figure 1**; Brown and Shaw, 1998; Shaw and Brown, 2004). Although the function of the NoVs is currently unknown, it could be suggested that these structures may be regions of temporal sequestration and storage of some biochemical factors such as elements of the ubiquitin-proteasome system (Stepinski, 2012), snoRNAs (snoRNPs) and spliceosomal small nuclear RNAs (snRNAs/snRNPs) (Beven et al., 1996; Lorković and Barta, 2008), which may be released into the nucleoplasm depending on specific physiological requirements incurred during stress responses or at particular developmental stages (Mineur et al., 1998).

Chromatin clusters which are often associated with the nucleolus (nucleolus-associated chromatin domains, NADs) primarily have a heterochromatic nature, and comprise sub-telomeric regions, transposable elements (TEs), and largely inactive protein-coding genes (Pontvianne et al., 2016). However, NADs also include active rRNA genes, which are typically arranged in tandem DNA arrays, (known as nucleolus organizer regions, or NORs). In Arabidopsis, NORs are located on the left arms of chromosomes 2 and 4 (NOR2 and NOR4, respectively; Chandrasekhara et al., 2016). In the wild type plants only NOR4 and the adjacent entire short arm of chromosome 4 were shown to be associated with the nucleolus (Pontvianne et al., 2016). In contrast NOR2 and its neighboring region in chromosome 2, were excluded from the nucleolus and had inactive rRNA genes (Chandrasekhara et al., 2016; Pontvianne et al., 2016); this may suggest that although NOR2 may have structural similarity to NOR4, its activity may differ depending on the experimental/environmental conditions. Interestingly there are indications that NOR2 and NOR4 may share some regulatory mechanisms as suggested by null mutants for the *NUCLEOLIN 1* gene (encoding one of the major nucleolar proteins, nucleolin 1–see below). In these null mutants both NOR4 and NOR2 localized to the nucleolus, and the NOR2 rRNA genes which are usually silenced during development in wild-type leaves, became active (Pontvianne et al., 2010). Among the genes found to be localized to the nucleolus were functional genes, tRNA genes, and pseudogenes (Pontvianne et al., 2016). Since RNA polymerase II (Pol II) is not present in the nucleolus, it can be assumed that those NAD-genes which are normally transcribed by Pol II would likely not be expressed; this may constitute a novel mechanism of gene expression regulation.

NORs constitute sites on metaphase chromosomes where nucleoli become organized during reinitiation of transcription in postmitotic cells as they enter interphase. After cell division, nucleoli are reconstituted on NOR sites that contain rDNA genes which were transcriptionally active during the previous interphase but remained comparatively decondensed during mitosis (Heliot et al., 1997; Mais et al., 2005; Prieto and McStay, 2008). The newly organized nucleoli are rebuilt from rDNA gene products, such as primary pre-ribosomal RNAs undergoing different steps of processing, constituents of transcriptional and processing machineries which include U3 snoRNA, and major nucleolar proteins such as nucleolin, fibrillarin, Nop52 and B23. These components, which are derived from the previous interphase nucleoli, first form perichromosomal compartments, then prenucleolar bodies and, finally culminate in the formation of nucleolus-derived foci (Dundr and Olson, 1998; Hernandez-Verdun, 2011; Carron et al., 2012). At the end of mitosis (late telophase) the formation of one or more nucleoli at each active NOR occurs, and these small nucleoli often fuse together to form a single nucleolus (this frequently occurs in plant cells) as interphase progresses (Shaw and Jordan, 1995).

THE NUCLEOLUS AND RIBOSOME PRODUCTION

The major activities of the nucleolus are associated with ribosome production (**Figure 1**). In the nucleolus, RNA polymerase I (RNA Pol I) mediates the transcription of the pre-rRNA, which takes the form of 45S rRNA. This pre-rRNA can either be co- or post-transcriptionally processed by snoRNPs (small nucleolar ribonucleoproteins) to produce 5.8S, 18S, and 28S rRNAs (Nazar, 2004; Russell and Zomerdijk, 2005) which may also be 2′-O-methylated and pseudouridinylated (Matera et al., 2007). After processing, suitable rRNA species assemble with ribosomal proteins into small and large pre-ribosomal subunits (Fromont-Racine et al., 2003) which are exported separately to the cytoplasm where they are modified further to form mature 60S and 40S ribosome subunits. These three activities of the nucleolus (pre-rRNA synthesis, processing, and ribosomal RNP assembly) are well consistent with its FC, DFC, and GC derived "tripartite" internal structure mentioned above. Indeed, pre-rRNA appears to be transcribed from rDNA in the FC or at its border with the DFC. For example, FCs are enriched in RNA Pol I machinery components (such as UBF), and the DFC contains factors involved in pre-rRNA processing, such as fibrillarin, snoRNAs, snoRNP proteins and Nop58. The FC and DFC are both surrounded by the GC, where pre-ribosome subunits are assembled (Boisvert et al., 2007; Sirri et al., 2008; Boulon et al., 2010).

PROTEIN COMPOSITION AND PLURIFUNCTIONALITY OF THE NUCLEOLUS

The three most abundant and major rRNA-associated nucleolar proteins involved in ribosome biogenesis are fibrillarin, nucleolin, and B23. Fibrillarin is a key component of box C/D snoRNP particles and has methyltransferase activity which directs 2′-O-ribose methylation of rRNA and spliceosomal snRNAs, and is required for pre-rRNA processing and splicing of snoRNA (Warner, 1990; Tollervey et al., 1993). Nucleolin plays an important role in regulating chromatin structure-mediated rDNA transcription and processing of pre-rRNA (Ginisty et al., 1998; Roger et al., 2003; Pontvianne et al., 2007), the assembly of ribosome particles and their nucleocytoplasmic transport (Bouvet et al., 1998). B23 (nucleophosmin) plays a crucial role in maintaining nucleolar structure, rDNA transcription, rRNA maturation, ribosome assembly and export (Murano et al., 2008). While much is known about the ribosome biogenesis

functions of these proteins, it is becoming clear that they are also involved in a broad range of processes other than ribosome synthesis. Moreover, accumulating evidence shows that many other proteins and RNAs completely unrelated to ribosome production are present in the nucleolus. Protein and RNA localization studies and comprehensive proteomic analyses of both human and plant nucleoli enabled identification of these macromolecules (Andersen et al., 2002; Pendle et al., 2005; Ahmad et al., 2009; Lewandowska et al., 2013). Thus, many non-conventional functions have been attributed to the nucleolus (Pederson, 1998; Olson and Dundr, 2005). In plants, these functions include surveillance (nonsense-mediated decay) of mRNA, metabolism, modifications, assembly, or transport of various small nuclear and nucleolar RNAs (snRNAs and snoRNAs) and regulatory RNAs (siRNAs and miRNAs) (Pendle et al., 2005; Brown and Shaw, 2008; Kim et al., 2009, 2010; Shaw and Brown, 2012; Pontes et al., 2013), interactions with DNA and RNA viruses (see for reviews Hiscox, 2007; Greco, 2009; Taliansky et al., 2010) and other pathogens (Jones et al., 2009; Leonelli et al., 2011; Stam et al., 2013; Petre et al., 2015; Boevink et al., 2016), stress sensing, signaling and defense pathways (Lewandowska et al., 2013), DNA damage responses (Yoshiyama et al., 2014; Yoshiyama, 2016); Manova and Gruszka (2015) and developmental control (Weis et al., 2015a). These non-canonical functions of the nucleolus will be discussed in detail below (see **Tables 1**, **2**).

NEW NUCLEOLAR FUNCTIONS IN RNA REGULATORY PATHWAYS

Exon Junction Complex (EJC)

The most striking finding from the proteomic analysis of the Arabidopsis nucleolus is that this sub-nuclear organelle comprises six proteins of the EJC: UAP56, MAGO, ALY/REF, RNPS1, Y14, and the translation initiation factor eIF4A-III, whereas in animals these proteins localize to cytoplasmic processing bodies (Pendle et al., 2005). Components of EJC mark splice junctions in mRNAs after mRNA splicing and play key roles in various post-splicing processes such as mRNA export from the nucleus to its cytoplasmic location, and the nonsense-mediated mRNA decay (NMD) pathway of mRNA surveillance (Dreyfuss et al., 2002; Maquat, 2004). The NMD surveillance system recognizes and degrades aberrant (truncated) mRNAs that contain a premature termination codon. It has been shown that in plants there is a greater abundance of aberrant mRNAs in the nucleolus, while in the nucleoplasm fully spliced products are more abundant. Moreover, direct correlation between aberrant mRNA accumulation in the nucleolus and their NMD-mediated turnover has been demonstrated using Arabidopsis *upf* mutants, which are known to be impaired in NMD, whereby mRNAs that are typically degraded by NMD will accumulate in nucleoli (Kim et al., 2009). This suggests that the plant nucleolus is directly involved in recognizing aberrant mRNAs and NMD. A possible role of the EJC components in plant development is discussed later in the chapter.

Novel Small Nucleolar RNAs

With regard to snoRNAs, they form an abundant class of non-coding small RNAs that guide 2 main types of modifications of other RNAs, such as rRNAs, tRNAs, and snRNAs (Love et al., 2017). The C/D box snoRNAs are associated with fibrillarin (methyltransferase) and other additional proteins to form snoRNPs which direct 2′-O- methylation of RNA targets. Whereas, the H/ACA box snoRNAs forms a complex with dyskerin (pseudouridine synthase) which guides pseudouridylation of specific nucleotides. Another group of small RNAs which are structurally related to snoRNAs are small Cajal bodies-specific RNAs (scaRNAs), and these are found in abundance in Cajal bodies (CBs), sub-nuclear structures functionally and physically connected to the nucleolus (Love et al., 2017). scaRNAs contain either or both of the boxes together and modify certain snRNAs. Using an RNomics approach on Arabidopsis, 188 different scaRNA/snoRNA genes and 294 scaRNA/snoRNA gene variants were identified. In addition to snoRNA and scaRNAs, some novel "orphan" snoRNAs have also been found which do not have complementarity to rRNA or snRNAs but are expressed (e.g., Kim et al., 2010). Orphan snoRNAs have previously been found in other eukaryotes, and bioinformatic analysis of possible mRNAs which could be targets of orphan human snoRNAs revealed a potential connection with genes that are alternatively spliced; suggesting a function in regulating alternative splicing (Bazeley et al., 2008). Thus, it is possible that in plants orphan snoRNAs (besides modifying rRNAs and snRNAs) may target mRNA, which could affect gene regulation and influence plant development and growth. Another potential activity of snoRNAs may be attributed to the mechanism by which snoRNA can be processed to microRNAs (miRNAs) in human cells: DICER can process box H/ACA snoRNA to produce small RNAs which in association with Argonaute proteins cause depletion of target gene expression (Ender et al., 2008). It is thus intriguing to speculate whether these novel snoRNA functions described for other organisms also occur in plants and to what extent they could control plant growth and development; potential pathways which warrant future research.

Gene Silencing Pathways and Small RNAs

In addition to rRNAs, tRNAs, snRNAs, and snoRNAs, several other classes of small non-coding RNAs (small ncRNAs or sRNAs), namely silencing RNAs, have been implicated in regulatory functions in eukaryotes. Silencing RNAs constitute an exquisite and complex mechanism which are required for controlling plant development, determining epigenetic modifications (e.g., histone and DNA methylation) and defense against viruses (Mallory and Vaucheret, 2010), for example. The major types of sRNAs include microRNA (miRNA), natural cis-antisense siRNA (natsiRNA), trans-acting small interfering RNA (ta-siRNA), and heterochromatic small interfering RNA (hc-siRNA) (Mallory and Vaucheret, 2010). These RNA species effectively regulate various transcriptional and post-transcriptional gene silencing (TGS and PTGS) pathways by modulating mRNA production or degradation. These pathways are invoked by the presence of aberrant mRNA structures

TABLE 1 | Role of selected proteins in plant development and stress responses.

Protein	Function / process	Loss-of-function / gain-of-function phenotype	References
atNucleolin	Various steps of ribosome biogenesis	Gene disruption (Δ*AtNuc-L1-1* plants): reduced growth rate, prolonged life, bushy growth, pointed leaves, and defective vascular patterns and pod development Induction of AtNuc-L1-1: growth resumption	Kojima et al., 2007
atBRX-1-1 and atBRX-1-2	Maturation of the large pre-60S ribosomal subunit	*brx1-1brx1-2:* delay in development *1brx1-2*: pointed leaves	Weis et al., 2015a
atRPS18A; atRPS13A; atRPS5A; atRPL24B	Ribosomal proteins	Gene disruptions: phenotypes are similar to those observed for Δ*AtNuc-L1-1* plants	Van Lijsebettens et al., 1994; Ito et al., 2000; Weijers et al., 2001; Nishimura et al., 2005
atRPL23a	Ribosomal protein; ribosome biogenesis	*RPL23aA* RNAi: growth delay, irregularities in morphology of leaves, roots, phyllotaxy and vasculature, and loss of apical dominance	Degenhardt and Bonham-Smith, 2008
RBFs, ribosome biogenesis factors	Pre-rDNA transcription, pre-rRNA processing, modification, folding, and assembly with RPs	Gene disruptions: infertility, embryo lethality, impaired growth and gametophyte development, aberrant cotyledon, leaf and root development	Weis et al., 2015b
atTHAL: SAS10/C1D family protein	Processing of precursor rRNAs, and expression of the major rDNA variant (*VAR1)*	*thal-1* and *thal-2*: lethal early in reproductive development; enlarged nucleoli in arrested embryos THAL overexpression: multiple nucleoli	Chen et al., 2016
osNMD3	Non-sense mediated decay; 60S pre-ribosome export and maturation	Overexpression of $osNMD3^{\Delta NLS}$: dwarfism and decrease in the internode length	Shi et al., 2014
atSGP1/2 and bnMAP4Ka	Homologous to fission yeast spg1p and sid1p, respectively - septation initiation network (SIN)	Overexpression in yeast complements mutant spg1-B8 and sid1-239 proteins and induces multisepta in wild-type yeast, suggesting the existence of plant SIN-related cell cycle network	Champion et al., 2004
MAGO and Y14	Components of EJC: nonsense mediated decoy	RNAi: male infertility, defects in pollen grain maturation, spermatogenesis, floral and vegetative growth and stamen development; defects in root, shoot and seed development	Chen et al., 2007; He et al., 2007; Park et al., 2009; Boothby and Wolniak, 2011; Gong and He, 2014.
RID1	a DEAH-box RNA helicase; splicing	*rid1-1*: abnormalities in meristem maintenance and leaf and root morphogenesis	Ohtani et al., 2013
TERT	Catalytic subunit of telomerase; interacts with dyskerin	TERT activity is developmentally regulated in plants (high in reproductive organs but low in vegetative tissues)	Procházková Schrumpfová et al., 2016
STRS1 and STRS2	DEAD-box RNA helicases; negative regulators of stress-induced gene expression	*strs* mutants: enhanced tolerance to salt, osmotic and heat stress *STRS* overexpression: diminished tolerance to salt and heat stress	Khan et al., 2014
atRab 28 LEA	unknown	Overexpression: increased leaf and root areas, higher relative water content and reduced chlorophyll loss when grown under osmotic stress	Amara et al., 2013
atREN1	Strongly homologous to the heat shock transcription factor gene *HSFA5*	*ren*: abnormalities in male gametophyte and pollen grain development, and perturbed heat stress responses	Renák et al., 2014
Coilin	The signature protein of CB; essential for CB formation and function	RNAi: enhanced salt stress tolerance.	Love et al., 2017
Poly (ADP-ribose) polymerase (PARP)	PARP modifies the function of a variety of nuclear "target" proteins by attaching chains of ADP ribose them and itself	*atPARP2* overexpression: diminished incidence of DNA nicks at high H_2O_2 concentration and increased incidence of DNA nicks at low H_2O_2 concentration atPARP1/PARP2 knock down: enhanced tolerance to drought, oxidative and high light stress	Reviewed in Briggs and Bent, 2011
SOG1	Functional analog of animal p53: master regulator of DNA damage response (DDR) including stimulation of transcriptional response, cell cycle arrest and PCD	*sog1-1*: increased resistance of root growth to zeocin; no cell cycle arrest and PCD in response to DNA double-strand breaks (DSB)	Yoshiyama et al., 2014; Yoshiyama, 2016
RMI2 and RTEL1	Stabilization of plant 45S rDNA repeats	*rmi2-2 rtel1-1*: male infertility	Röhrig et al., 2016
TDP1	Tyrosyl DNA phosphodiesterase - DNA repair	*tdp1*: dwarfism, diminished cell number, developmental cell death (Arabidopsis) RNAi: reduced cell division, perturbed plant growth and early leaf senescence; impaired rRNA processing and ribosome biogenesis and disruption of the nucleolus (*M. truncatula*)	Lee et al., 2010; Donà et al., 2013

TABLE 2 | Selected plant pathogen-nucleolar interactions.

Pathogen	Non-host factor	Host factor	Function	References
Groundnut rosette virus (GRV, umbravirus)	ORF3	fibrillarin	Association required for long-distance virus movement	Canetta et al., 2007; Kim et al., 2007a,b
Potato leaf roll virus (PLRV, polerovirus)	Capsid protein (CP) and CP read-through protein	fibrillarin	Association required for long-distance virus movement	Haupt et al., 2005; Kim et al., 2007b
Bamboo mosaic virus (BaMV, potexvirus)- associated satRNA (satBaMV)	p20 satBaMV	fibrillarin	Association required for long-distance virus movement	Chang et al., 2016
Rice stripe virus (RSV, tenuvirus)	p2 protein (silencing suppressor protein)	fibrillarin	Association required for long-distance virus movement	Zheng et al., 2015
Potato virus A (PVA, potyvirus)	VPg domain of nuclear inclusion protein a (NIa)	fibrillarin	Depletion of fibrillarin reduces accumulation of PVA; this may operate through association of VPg with fibrillarin	Rajamäki and Valkonen, 2009
Poa semilatent virus(PSLV, hordeivirus)	TGBp1 (Triple gene block protein 1)	fibrillarin	Functions of this association remain to be elucidated	Semashko et al., 2012
Barley stripe mosaic virus (BSMV, hordeivirus)	TGBp1 (Triple gene block protein 1)	fibrillarin	Association required for cell-to-cell virus movement	Li et al., 2017
Potato virus A (PVA) and *turnip mosaic* virus (TuMV) (potyviruses)	VPg	S6K (protein S6 kinase)	Silencing of the S6K gene in *N. benthamiana* decreases accumulation of PVA and TuMV	Rajamäki et al., 2017
Cucumber mosaic virus (CMV, cucumovirus)	2b, silencing suppressor	Argonaute4	Functions of this association remain to be elucidated	González et al., 2010; Du et al., 2014
Alfalfa mosaic virus (AlMV, alfamovirus)	CP	ILR3 (transcription factor of a basic helix–loop–helix family of TFs)	The AlMV CP–ILR3 interaction leads to activation of plant hormone responses, which forms a hormonal balance optimal for plant viability and virus production	Aparicio and Pallás, 2017
Tomato bushy stunt virus (TBSV, tombusvirus)	P19 (silencing suppressor protein)	ALY proteins	ALY proteins may interfere with the silencing suppressor activity of P19, which could constitute a novel antivirus defense response	Canto et al., 2006
Globodera pallida (potato cyst nematode)	two protein effectors 22E10 and 13G11		Suppresses host defense	Jones et al., 2009
Phytophtora infestans (oomycete plant pathogen)	effector Avr3a protein	E3 ligase CMPG1	Association regulates host resistance	Bos et al., 2010; Gilroy et al., 2011
Hyaloperonospora arabidopsidis (obligate biotrophic oomycete pathogen)	HaRxL44	Mediator subunit 19a (MED19a)	Pathogen effector modulates host transcription to enhance invasion	Caillaud et al., 2013
Hyaloperonospora arabidopsidis	ATR13 Emco5	RPP13-Nd	interaction triggers hypersensitive response which limits pathogen spread	Leonelli et al., 2011
P. infestans	Pi04314	phosphatase 1 catalytic (PP1c) isoforms	Promotes late blight disease by attenuating transcription of host plant defense genes	Boevink et al., 2016

(such as RNA with hairpin loops or double stranded RNA (dsRNA), which may arise for instance from endogenous genes). Each pathway typically starts with the conversion of aberrant RNAs into dsRNA (if not already double stranded) by viral or endogenous RNA-dependent RNA polymerases (RDRs) (Mallory and Vaucheret, 2010) before their cleavage into 21–24 nucleotide (nt) dsRNA duplexes by specific DICER or DICER-like (for plants) enzymes (DCL). The sRNA duplexes are unraveled, with one strand binding an ARGONAUTE (AGO) protein, which then targets the RNA for cleavage, and mediates repression of translation or the establishes epigenetic modifications (Vaucheret, 2008).

Arabidopsis contains four DCLs, 10 AGOs, and six RDRs that operate in concert with various sRNAs in different combinations, forming a complex variety of silencing pathways. How unique pathways are determined for each individual sRNA is generally unknown. Recent localization studies have indicated that many proteins involved in the miRNA, hc-siRNA and ta-siRNA silencing pathways accumulate within sub-nuclear structures in the nucleolar periphery (Pontes et al., 2013). Cytological analysis of these structures indicated that these may be CBs or CB-related structures, which suggests that CBs may be a site for assembly, re-cycling and storage of RNA silencing components, and also a site for specific sRNA silencing pathways (Pontes et al., 2006,

2013). However, in mutant Arabidopsis plants which contain no conventional CBs, changes in siRNA accumulation or in DNA methylation patterns have not been detected. It can therefore be speculated that RNA silencing functions may still be fulfilled by other multiple CB-related bodies present in eukaryotic cells, e.g., pre-CB structures which may be produced by some CB components at the early stages of their formation (Love et al., 2017).

CBs (as well as CB-related bodies) are dynamic structures, with major roles in RNA metabolism and formation of RNPs involved in transcription, splicing and ribosome biogenesis, and which are closely associated with the nucleolus. This, therefore, suggests a role for the nucleolus in RNA silencing pathways (Pontes and Pikaard, 2008). Indeed, some mature and precursor miRNAs are enriched in mammalian cell nucleoli (Politz et al., 2009; Scott et al., 2009). There is also cross-talk between snoRNAs and miRNA precursors, in which snoRNA precursors may be processed into some miRNAs, which may retain snoRNA features (Saraiya and Wang, 2008; Ono et al., 2011). In addition, sRNAs derived from snoRNAs were reported to associate with the AGO proteins of RNA silencing pathways in both Arabidopsis and animals (Taft et al., 2009), and it was observed that sRNAs derived from a human snoRNA could reduce expression of target genes (Ender et al., 2008). A challenge for future research is to give further insights into the precise molecular functions localized within the nucleolus and CBs (CB-related bodies) in regulating miRNA and siRNA pathways.

PLANT GROWTH AND DEVELOPMENT

Nucleolus-related ribosome production, spliceosome formation, gene expression regulation (e.g., transcriptional/post-transcriptional gene silencing), mRNA surveillance (EJC-mediated intron-based NMD pathway) and telomere maintenance (with links to aging) can be expected to play essential roles in plant growth and development. Indeed, several recent reports provide emerging evidence that these nucleolar activities are involved in various developmental processes. Since these data will be discussed in more detail in other papers of this Research Topic, this section on developmental regulation will only briefly cover these aspects, with a later emphasis on functional implications of nucleolar responses to pathogens and stress described further below (**Tables 1**, **2**, **Figure 2**).

Ribosome Biogenesis and Growth and Development

The varied impacts of ribosome biogenesis on plant growth and development are illustrated in **Table 1**.

Two Arabidopsis proteins atBRX-1-1 and atBRX-1-2, which are highly similar in sequence, are mainly localized to the nucleolus and are implicated in maturation of the large pre-60S ribosomal subunit (Weis et al., 2015a). Plant lines deficient in both these factors showed significant developmental delays, and also pointed leaves were observed in the *brx1-2* mutant. Taken together this suggests a strong link between plant development and ribosome biogenesis.

Nucleolin is an abundant multifunctional nucleolar protein involved in various stages of ribosome biogenesis. Disruption of its gene in Arabidopsis (AtNuc-L1) led to reduced pre-rRNA processing and resulted in prolonged life, reduced growth rate, pointed leaves, bushy growth, and defective development of vascular patterns and pods, which are similar to those phenotypes reported for several RP gene mutants (Kojima et al., 2007). In contrast, induction of *AtNuc-L1* expression with glucose normalized plant growth. The reduced growth rate of nucleolin-deficient plants was presumably caused by reduced cell division due to a shortage of ribosomes. These data suggest that the rates of ribosome production in meristem tissues may have a significant effect on growth and plant architecture.

Like RPs, many other ribosome biogenesis factors (RBFs) are also involved in plant developmental processes (Weis et al., 2015b; **Table 1**, **Figure 2**). It is tempting to speculate that RBFs may take part in some specific modifications of RPs and rRNAs, which may facilitate remodeling of ribosome pools in response to developmental stimuli and environmental conditions (Lafontaine, 2015; Weis et al., 2015b). Such ribosome reprogramming may be closely related to nucleolar organization. In this respect, it is worth noting that, for example, the Arabidopsis THAL protein belonging to the SAS10/C1D family is involved in the processing of precursor rRNAs, specifically regulating expression of the major rDNA variant (*VAR1*). It was found that defects in *THAL* significantly increase nucleolar size in arrested embryos (Chen et al., 2016). On the other hand, *THAL* overexpression results not only in recovery of *VAR1* expression but also promotes formation of multiple nucleoli per nucleus, possibly linking changes in nucleolar organization with regulation of ribosome biogenesis.

Changes in ribosome biogenesis may also affect global protein synthesis which would inevitably affect plant growth and development. NMD3 is a nucleo-cytoplasmic shuttling protein which has been previously characterized as a component of the NMD pathway. It is also involved in transport and maturation of large ribosomal (60S) subunits (Shi et al., 2014). In rice, overexpression of an NMD3 mutant which contains a deleted nuclear localization site, was found to be retained in the cytoplasm and produced abnormalities in plant growth and development (dwarfism and decrease in the internode length, grain size and weight); these effects are possibly due to changes in ribosome biogenesis and consequent decreases in mRNA translation efficiency (Shi et al., 2014).

It is also worth noting that expression of many RP genes are controlled and activated by the target of rapamycin (TOR), a master cell cycle regulator. Plants overexpressing TAP46, an important factor of the TOR signaling network, demonstrated significant increases in production of some RPs (Weis et al., 2015b), corroborating the functional cross-talk between ribosome biogenesis and plant development and cell cycle.

Plant Nucleolus and Cell Cycle

Cytokinesis is the final phase of the cell cycle when the cell is divided into two daughter cells via formation of a cell plate between them, which is later converted into a proper

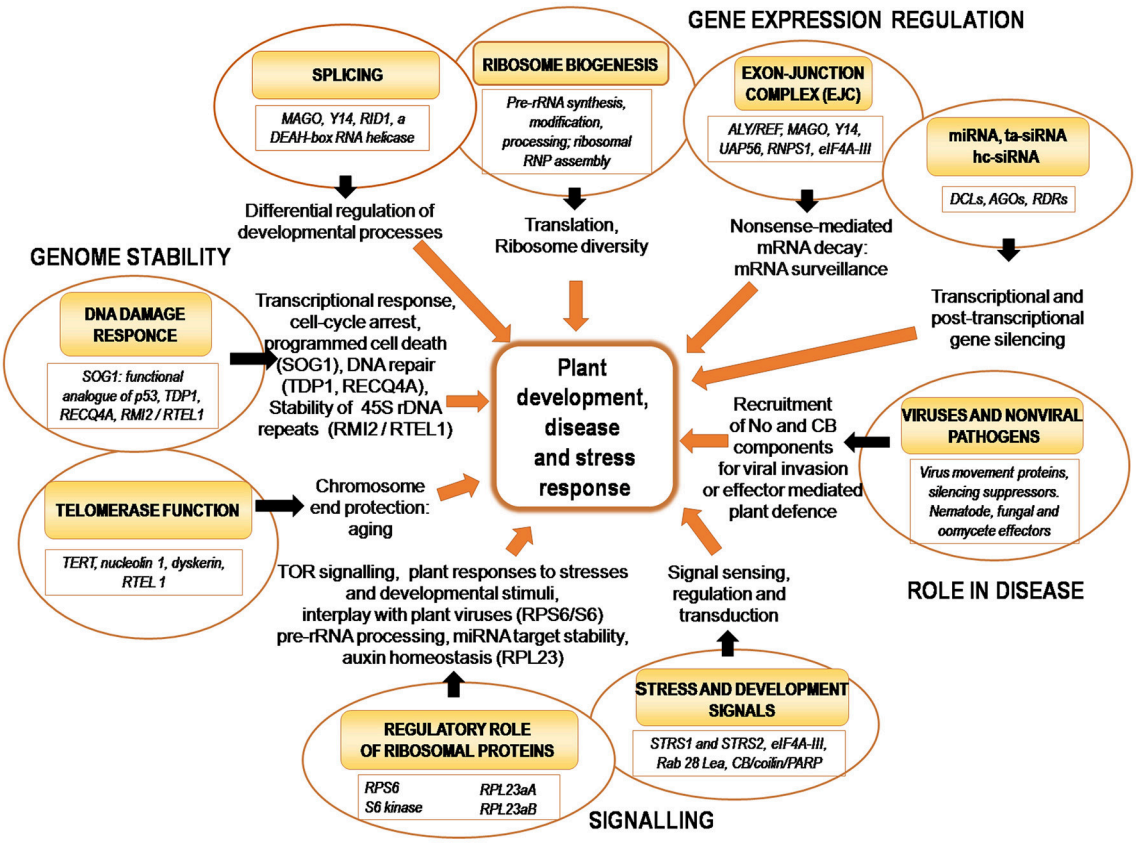

FIGURE 2 | The role of the nucleolus and its molecular activities in regulating plant physiology.

cell wall. Generation of the cell plate (septum) in yeasts (*Schizosaccharomyces pombe*) involves several proteins (kinases and GTPase) which form a so called septation initiation network (SIN). Plants also contain proteins which are homologous to the yeast SIN proteins which are localized in nucleoli. Remarkably, some of these proteins (such as Arabidopsis SGP1/2 and *Brassica napa* MAP4Ka2) have been shown to complement yeast mutants defective in homologs of these genes, as evidenced by formation of multisepta during their overexpression in *S.pombe*. These data suggest the existence of a plant-specific nucleolar SIN-like network with important roles in the cytokinesis and cell cycle regulation (Champion et al., 2004).

Pre-mRNA Splicing and Growth and Development

Pre-mRNA splicing is crucial to the regulation of gene expression in eukaryotes. As mentioned above, the nucleolus and particularly CBs play important roles in snRNP synthesis, which are essential for the formation of spliceosomes (reviewed in Love et al., 2017). The EJC and particularly two of its core subunits such as MAGO and Y14, are widely known to have essential multiple developmental roles in animals, whereas information for such roles in plants is more limited (Gong and He, 2014; Yang et al., 2016). It has been shown that MAGO proteins are responsible for male fertility (*Physalis floridana*; He et al., 2007), pollen grain development (Arabidopsis; Park et al., 2009) and spermatogenesis (*Marsilea vestita*; Boothby and Wolniak, 2011). The MAGO and Y14 proteins in rice also appear to be involved in floral and vegetative growth, stamen development and pollen maturation. In addition, one of two rice isoforms of Y14 has been shown to be involved in embryogenesis (Gong and He, 2014). The growth and development of other plant organs are also affected by MAGO and Y14: roots, shoots, seed and root hairs (Chen et al., 2007; Park et al., 2009). Interestingly, Y14 and MAGO have been shown to selectively bind pre-messenger RNA of UNDEVELOPED TAPETUM1 (OsUDT1), which is a key controller of stamen development. Down regulation of MAGO and Y14 leads to abnormalities in the OsUDT1 transcript splicing, suggesting that rice EJC subunits may regulate this process (Gong and He, 2014).

Another nucleolar protein which is essential for plant development is RID1, a DEAH-box RNA helicase. Studies on a root initiation defective1-1 Arabidopsis mutant (*rid1-1*) (Ohtani et al., 2013) have implicated this protein in a certain subset of splicing events which may differentially regulate specific developmental programmes, such as root and leaf morphogenesis and meristem maintenance.

Further research is required to explore if nucleolar localization of RID1, MAGO and Y14, are completely required for their role in plant growth, development and reproduction.

Telomerase Maintenance and Plant Growth and Development

Telomeres, are specific DNA–protein structures located at the ends of eukaryotic chromosomes which contain repetitive nucleotide sequences that protect chromosomes from in appropriate attack by endogenous DNA nucleases. Telomere shortening can lead to chromosomal degradation which can culminate in aging and ultimately cell death. To counteract this, plants and other organisms have evolved strategies to maintain telomere length, which predominantly operates via the activity of telomerase, an RNP-based enzyme which consists of telomerase reverse transcriptase, telomerase RNA (TR), and other associated proteins (reviewed in Procházková Schrumpfová et al., 2016). The catalytic subunits of this complex (TERTs) possess multiple nuclear export/localization signals and have been shown to localize to the nucleus and the nucleolus. Furthermore, a preferential nucleolar accumulation was also shown for telomere binding proteins and the telomerase RNA-binding protein, dyskerin (Dvorácková et al., 2010; Dvoráčková et al., 2015). Finally, telomeres as well as subtelomeric regions (flanking the telomeres) also tend to associate with the nucleolus (Pontvianne et al., 2016). The concentration of various telomere-related components in the plant nucleolus strongly suggests a functional link between this sub-nuclear structure and telomere biology. This suggestion is supported by recent observations showing that in Arabidopsis null mutants for the *NUCLEOLIN 1* gene, which have altered rRNA gene expression and overall nucleolar structure, telomeres were shortened and had reduced association with the nucleolus (Pontvianne et al., 2016). Moreover, it was found that NUCLEOLIN 1 physically interacts with a macromolecular complex possessing telomerase activity. These data strongly implicate the nucleolus (and its protein, nucleolin 1) in plant telomere maintenance.

TERT is developmentally regulated in plants (Procházková Schrumpfová et al., 2016). In Arabidopsis plants, the activity of telomerase is low in vegetative tissues but high in reproductive organs. However, application of exogenous auxin, can overcome this developmental regulation and potentiates telomerase activity in mature leaves (Ren et al., 2007); a phenomenon which may be regulated by the Arabidopsis transcription factor TELOMERASE ACTIVATOR1 (TAC1). It has also been shown that telomerase activity in tobacco suspension cells significantly increases at early S-phase of the cell cycle due to auxin, but interestingly abscisic acid (ABA), a plant hormone which can induce the cyclin-dependent protein kinase inhibitor, readily abolishes this effect. These results suggest that antagonistic functions of ABA and auxin in the cell cycle-dependent modulation of telomerase activity in tobacco may be governed via reciprocal phosphorylation and dephosphorylation of telomerase complexes (Yang et al., 2002). A major future challenge is to elucidate the role of nucleolar environment in the cross-talk between plant telomerase and developmental pathways.

Another observation which may link nucleolar functions and plant growth and developmental pathways is that Arabidopsis TR is able to interact with dyskerin which is known to be a component of nucleoli and CBs (Procházková Schrumpfová et al., 2016).

Nucleolar miRNAs and Development

In plants, small 21–24 nucleotide miRNA molecules play important "roles in post-transcriptional gene regulation by base pairing with their complementary mRNA targets" (Li and Zhang, 2016). Mutations in the genes involved in biogenesis and the regulatory roles of miRNAs produce strong effects on development; implicating miRNAs in a broad range of physiological and developmental processes (Li and Zhang, 2016). Taking into account that the nucleolus is involved in RNA silencing pathways it looks natural that such an involvement may be important for plant growth and development. Moreover, in human cells, several miRNAs are highly and specifically localized in nucleoli relative to other compartments. The presence of miRNAs in the nucleolus is independent of DICER and the RNA polymerase I transcription activity of the nucleolus, however it is dependent on CRM1, which is known to be related to nucleolar trafficking of snoRNAs. These data demonstrate the spatial arrangement and complexity of miRNA regulation (Bai et al., 2014). It is enticing to theorize that there might also be specific variability in nucleolar miRNA content which may be dependent on cell type and physiological state, and which could regulate developmental processes.

Fibrillarin and Systemic Macromolecular Trafficking in Plants

Plants have evolved a specific network interconnected by plasmodesmata (PD), which are cytoplasmic channels between cells that permit local movement of various molecules. In addition, plants can rapidly transfer nutrients, assimilates and macromolecules over longer distances via the phloem, a specific plant transport system composed of enucleated sieve elements and neighboring companion cells. The phloem and PD form a continuous symplastic connection which can link distant plant organs, and likely play a key role in transmitting macromolecules, such proteins and RNAs including siRNAs/miRNAs, as components of integrated signaling pathways which are central to plant development and controlling responses to both biotic and abiotic stresses (reviewed in Lough and Lucas, 2006; Buhtz et al., 2010). However, to govern such signaling pathways, plants have evolved stringent control systems to prevent molecules other than those that perform necessary functions from trafficking throughout the plant by cell-to-cell (PD) and long-distance (phloem companion cell-sieve element junctions) movement. Indeed, the plant transport network is fully permeable only to some low-molecular weight compounds, but specific "transport" proteins are able to increase the permeability of the control systems, which permits entry of larger macromolecules or macromolecular complexes. The most studied example of active plasmodesmatal transport involves the movement of plant viruses, which use designated virus-encoded movement proteins (Lough and Lucas, 2006) to hijack and

manipulate the PD to allow viral particles or their transport forms to pass between cells; thus facilitating systemic invasion. Plant proteins such as fibrillarin (discussed in more detail below) which is an abundant nucleolar protein, is also able to facilitate long distance movement and has been implicated in the systemic spread of various plant viruses, such as groundnut rosette virus (GRV), potato leaf roll virus (PLRV), rice stripe virus (RSV) (Kim et al., 2007a; Zheng et al., 2015) and subviral bamboo mosaic virus-associated satellite RNA (satBaMV; Chang et al., 2016). It would be difficult to expect that plant fibrillarin, whose main function is in rRNA processing and modification, has evolved specifically to assist viruses; it is more likely that viruses hijack fibrillarin's role in phloem RNA trafficking. The phloem transport system and RNAs play a critical role in plant survival and together they likely operate as a complex, multifunctional and regulatory long-distance RNA signaling system. Recently in addition to siRNAs and miRNAs, small non-coding RNA molecules of sizes between 30 and 90 bases have been identified and characterized in pumpkin phloem sap. In addition to fragments of rRNAs and tRNAs, the identified RNAs include phloem-specific spliceosomal RNAs, which also have nucleolar steps in their formation (Zhang et al., 2009).

Effect of Mammalian p53 on Plant Development

p53 is a key mammalian nucleolar tumor suppressor which plays a pivotal role in molecular stress responses, developmental processes and guarding the genome from DNA damage (Boulon et al., 2010). However, p53 has not been found in plants. It is therefore striking that p53 transgenically expressed in Arabidopsis induced early senescence and excessive inflorescence branching (fasciation) (Ma et al., 2016). This effect in plants is presumed to be due to the elevated homologous DNA recombination directed by p53. SUPPRESSOR OF NPR1-1 INDUCIBLE 1 (SNI1) is a negative regulator of plant homologous recombination (operating with RAD51D downstream of SNI1), which is not present in animals. Interestingly, *sni1* mutants have a fasciated phenotype in the presence of p53, whereas *rad51d* mutants are able to fully suppress the p53-induced phenotype; implicating the SNI1-RAD51D signaling pathway as a regulator of p53 (Ma et al., 2016). The underlying molecular mechanisms of how this signaling pathway is activated by p53, and what nucleolar functions are involved remain to be explored.

As indicated in the above sections, nucleolar components are key to many facets of plant development and growth, moreover in recent years they have also been implicated in modulating responses to exogenous biotic and abiotic stresses.

VIRUS INFECTIONS

Considering the diverse functional roles of the nucleolus, it is unsurprising that this structure is a common target of many types of viruses, including plant viruses (Hiscox, 2007; Greco, 2009; Taliansky et al., 2010). Interestingly, the repertoire of viruses that interact with nucleoli includes not only "nuclear viruses" that replicate within the nucleus, but also "cytoplasmic viruses" (containing mainly positive-strand RNA) in which cytoplasm is an exclusive site of their replication. Since the early studies on plant viruses and their association with the nucleolus (Taliansky et al., 2010), a plethora of proteins from RNA- and DNA-containing viruses which can enter the nucleus and target nucleoli have been described. Moreover, several recent studies have expanded beyond these phenomenological observations and have elucidated the molecular mechanisms underpinning various virus-nucleolar associations and their roles in the plant viral life cycle and disease with potential links to a broader range of biological processes including growth and development. This section will therefore focus on functional and mechanistic implications of the virus–nucleolar interactions (**Table 2**).

Direct Roles of Fibrillarin in the Life Cycle of Plant Viruses

Due to its integral role in various RNA processing and RNP assembly events, the nucleolus has evolved as an attractive target for many viruses to exploit its functions in production and transport of viral RNPs. Plant viruses can hijack nucleolar proteins for production of viral RNP particles, replication and movement, and to counter antivirus defense.

For example, in the nucleus, the ORF3 long-distance movement protein of GRV (an umbravirus) targets CBs causing their re-organization into multiple CB-like structures which move to and coalesce with the nucleolus. ORF3 subsequently recruits and uses fibrillarin for assembly of cytoplasmic viral RNP complexes able to move long-distance systemically through the phloem (Canetta et al., 2007; Kim et al., 2007a,b). Localization of ORF3 to the nucleolus is essential for successful systemic infection. In another example, the coat protein (CP) and CP read-through protein of PLRV (polerovirus) are targeted to the nucleolus and although the implications of this from an infection perspective are unelucidated, it is known that nucleolar components such as fibrillarin are required for long-distance movement of PLRV and subsequent systemic plant infections (Haupt et al., 2005; Kim et al., 2007a). A different scenario of fibrillarin-dependent long-distance movement has been recently described for satBaMV (satellite RNA). While the helper virus, bamboo mosaic virus (BaMV, potexvirus) utilizes virus-specific transport machinery composed of movement and capsid proteins for invasion, it does not require fibrillarin (Chang et al., 2016). In contrast, for the BaMV satellite virus (satBaMV) to establish long-distance movement, satBaMV-encoded p20 protein is required to interact with fibrillarin and form RNP complexes with satBaMV, which are competent for trafficking in the phloem.

Plant viruses usually encode silencing suppressor proteins which can counteract RNA-silencing-based defense mechanisms induced by infection. Protein p2 of RSV (tenuivirus) is a weak silencing suppressor and is able to interact with fibrillarin within the nucleolus. Furthermore, fibrillarin depletion (using RNAi knock down) abolished the systemic movement of RSV, suggesting that interaction of fibrillarin with the p2 silencing suppressor facilitates the long-distance RSV movement (Zheng

et al., 2015). However in studies of some other viruses, it has been shown that interaction of fibrillarin with viral silencing suppressors modulates virus functions other than the long-distance movement. Indeed, the VPg/NIa silencing suppressor protein of potato virus A (PVA, potyvirus) has activity which depends on VPg localization to CBs and the nucleolus. The NIa interacts with fibrillarin in the nucleolus and CBs via its VPg domain, and depletion (RNAi knock down) of fibrillarin lowers PVA accumulation, but does not compromise the process of virus long-distance movement *per se* (Rajamäki and Valkonen, 2009). These data raise the question as to whether VPg/NIa targets components of RNA silencing pathways that are localized in the nucleolus and CBs (Pontes and Pikaard, 2008) to inhibit silencing machinery and consequently enhance infection.

The poa semilatent virus (PSLV, hordeivirus) movement protein which is encoded by the first gene of the triple gene block (TGBp1) has also been shown to be nucleolar localized and interact with fibrillarin (Semashko et al., 2012). Similar interactions have been observed between fibrillarin and TGB1 encoded by another hordeivirus, *barley stripe mosaic* virus (BSMV), which were found to facilitate cell-to cell movement of the virus (Li et al., 2017).

Collectively these data demonstrate how diverse taxonomic virus groups and functional categories of viral proteins can interact with fibrillarin, to control the disease process. Thus fibrillarin may have new unrecognized activities which are exploited by plant viruses, but which may also be involved in other biological processes (such as growth and development) in healthy plants.

Role of Ribosomal Proteins

A growing body of evidence demonstrates that the ribosomal proteins (r-proteins) are not only scaffolds required to maintain the structural integrity of mature ribosomes, but rather, some of them are involved in regulatory activities in various cell cycle, cell death and developmental processes (e.g., Lindström, 2009). In addition, the accumulation of different sets of r-proteins has been shown to be enhanced by many viruses, including various plant viruses (Dardick, 2007; Yang et al., 2007, 2009; Rajamäki et al., 2017). Moreover, some r-proteins have been found to interact with plant viral proteins implicating them as important components of plant-virus interactions.

Of all the r-proteins involved in interactions with viruses RPS6 is the most studied. RPS6 is partially regulated by ribosomal protein S6 kinase (S6K) which, in turn, is a downstream component of TOR signaling pathway and a key modulator of plant responses to stresses and developmental stimuli (Xiong Sheen and Sheen, 2014; Son et al., 2016). In addition to its well-known role as a structural component of the 40S ribosomal subunit, RPS6 is also involved in regulation of rDNA transcription via nucleolar interactions with histone deacetylase 2b (HD2B) and nucleosome assembly protein 1 (NAP1), which is a histone chaperone (Kim et al., 2014; Son et al., 2015). The RPS6-HD2b complex functions as a negative regulator of rRNA synthesis via its binding to and blocking rDNA promoter sites, but this negative effect may be de-repressed by NAP1. Interestingly, S6K-mediated phosphorylation of RPS6 may also activate rDNA transcription presumably causing dissociation of the rDNA transcriptional repression complex.

With regards to virus infection it has been shown that silencing of the *RPS6* and *S6K* genes in *N. benthamiana* decreased accumulation of CMV, PVA and *turnip mosaic* virus (TuMV, potyvirus), which is in contrast to turnip crinkle virus and tobacco mosaic virus (TuCV and TMV respectively; tobamoviruses). This suggests differential requirements for RPS6 and S6K for different virus groups (Rajamäki et al., 2017). While the underpinning mechanisms are still to be elucidated, these observations indicate that there might be interplay between RPS6/S6K activities and plant virus infections. A possible activity of these proteins may be to directly interact with viral proteins and this has recently been shown for potyviral VPg, which is able to form a complex with S6K in the nucleus, nucleolus and cytoplasm. This suggests that potyviruses may recruit S6K to modulate downstream proteins (RPS6 in particular) for enhancing viral invasion. For example, in the nucleolus such an interaction may lead to stimulation of rDNA transcription whereas cytoplasmic interaction may facilitate protein translation. Additional studies are still necessary to explore the role of the nucleolar activities of RPS6 and S6K in relation to virus infections, which may also have implications for other stress responses, growth and development.

Nucleolar Sequestration, Storage, and Compartmentalization of Virus Proteins

The nucleolus was demonstrated to be a region of molecule sequestration which may also have activities outside this organelle, such as in the nucleoplasm or cytoplasm (Sirri et al., 2008). The regulation of various nucleolar functions is controlled by the compartmentalization or effusion of specific proteins; a mechanism which may also be hijacked by viruses for localization and storage of viral proteins. For example, cucumber mosaic virus (CMV, cucumovirus) 2b protein is a silencing suppressor that is involved in virus accumulation and virulence (Du et al., 2014), and it is found distributed between the nucleus/nucleolus and the cytoplasm. In the nucleolus this protein interacts with the Argonaute 4 silencing machinery (González et al., 2010), however, neither of these interactions nor nucleolar localization are sufficient for suppression of RNA silencing (González et al., 2012). Instead, it has been shown that it is the cytoplasmic portion of 2b that predominantly possess silencing suppressor activity. It was found that enhanced nuclear and nucleolar 2b accumulation increases virulence and accelerates symptom production, which is independent of its effect on RNA silencing. Thus, it has been suggested that nuclear/nucleolar and cytoplasmic partitioning of the 2b protein between these compartments permit CMV to regulate the equilibrium between damage to the plant and virus accumulation, presumably to optimize virus accumulation (Du et al., 2014).

Alfalfa mosaic virus (AlMV, alfamovirus) CP is a multifunctional protein required not only for virion assembly but also for translation, cell-to-cell and systemic movement (Bol, 2005). The AlMV CP localizes in both the nucleus/nucleolus and cytoplasm. The data shows that the nucleolar import signal

masks the RNA-binding activities of AlMV CP, which are required for viral translation and replication; this suggests a model in which the virus life cycle is precisely regulated by the balance between the cytoplasmic/nuclear localization of the CP (Herranz et al., 2012).

In more recent work (Aparicio and Pallás, 2017), AlMV CP has been shown to interact with transcription factor (TF) ILR3, a basic helix–loop–helix family member of TFs, which were suggested to operate in a number of metabolic pathways (Toledo-Ortiz et al., 2003). ILR3 can regulate NEET in Arabidopsis, a key protein in plant senescence, development, reactive oxygen species (ROS) modulation and iron metabolism (Nechushtai et al., 2012). The AlMV CP–ILR3 interaction causes partial re-distribution of this TF from the nucleus to the nucleolus and this re-distribution may cause down-regulation of NEET, which can induce plant hormone responses, which may form a hormonal balance optimal for plant viability and virus production (Aparicio and Pallás, 2017).

Another viral protein which is sequestered into the nucleus to regulate its activity is the P19 silencing suppressor encoded by the tomato bushy stunt virus (TBSV, tombusvirus). For example, P19 silencing suppressor activity is realized in the cytoplasm, however host plant ALY proteins (mRNA-processing-export factors) can potentially interfere with this function by redistributing P19 to the nucleus/nucleolus, where it cannot reach its target silencing RNA (Canto et al., 2006); this constitutes a novel plant defense mechanism which blocks silencing suppression.

Taken together, these data show that various plant host nucleolar proteins, in addition to their traditional roles, may have other diverse natural functions which are widely exploited by viruses for their own benefits. Interestingly, some of these proteins as mentioned above are also involved in plant perception and responses to environmental and developmental cues. Further detailed investigation of molecular mechanisms underlying the virus-nucleolus interactions will provide new insights into our understanding of intriguing multifunctional complexity of the nucleolus including its role in plant growth and development.

PATHOGENS OTHER THAN VIRUSES

Several lines of evidence demonstrate that plant pathogens other than viruses, also target the nucleolus (**Table 1**). In particular, some effector proteins expressed by plant pathogens which aid infection and favor parasitism, have been shown to localize to nucleoli (Chaudhari et al., 2014). For example, *Globodera pallida*, a potato cyst nematode, delivers two protein effectors encoded by gene members of the SPRYSEC family (*22E10* and *13G11*), into the nucleolus presumably to suppress host defense (Jones et al., 2009). Nucleolar localization has also been demonstrated for several effectors encoded by filamentous pathogens (oomycetes and fungi), such as the poplar leaf rust fungus *Melampsora larici-populina* (Petre et al., 2015) and the broad-host-range oomycete *Phytophtora capsici* (Stam et al., 2013). Interestingly, a plant E3 ligase (CMPG1) which is involved in host resistance against *P. infestans*, also accumulates in the nucleolus when stabilized by the Avr3a effector protein (Gilroy et al., 2011). These data, although merely descriptive, suggests that the nucleolus may be an important controller of host defense mechanisms against a broad range of pathogens.

This hypothesis is supported by more functional and mechanistic studies carried out using *Hyaloperonospora arabidopsidis* and *P. infestans*. Several RXLR effectors encoded by the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* (the causal agent of downy mildew), have been shown to localize to the nucleolus of plant cells (Leonelli et al., 2011; Caillaud et al., 2012) and regulate plant responses. For example, the effector HaRxL44 interacts with nuclear and nucleolar Mediator subunit 19a (MED19a), a component of the Mediator complex involved in the association between RNA polymerase II and transcriptional regulators. This interaction leads to MED19a degradation in a proteasome-dependent manner, which switches transcription of plant defense genes from the salicylic acid-responsive pathway to the jasmonic acid and ethylene-responsive pathways; demonstrating that this nucleolar pathogen effector alters host transcription to enhance susceptibility to infection (Caillaud et al., 2013). In contrast, other nucleolar *H. arabidopsidis* effectors, such as ATR13 Emco5, may interact with host RPP13-Nd (a cognate R-gene product) which triggers programmed cell death and limits pathogen spread (Leonelli et al., 2011).

The *P. infestans* RXLR effector Pi04314 enhances leaf colonization through its nuclear activity which can regulate activation of salicylic and jasmonic acid-responsive defense pathways. Pi04314 associates with three isoforms of the nucleolar host protein phosphatase 1 catalytic (PP1c) unit, inducing their re-distribution from the nucleolus to the nucleoplasm. A model has been proposed whereby Pi04314 interacts with PP1c isoforms to form holoenzymes, which attenuate transcriptional responses of host plant defense genes to promote late blight disease (Boevink et al., 2016).

In light of these findings it could be proposed that plant pathogens deliver effectors to alter host processes *via* activities in the host nucleolus. Understanding of how effectors target and manipulate host proteins and elucidating the function of the nucleolus in these processes is a critical area which needs further exploration.

THE PLANT NUCLEOLUS UNDER STRESS

In mammalian cells, various types of stress often affect the nucleolus by inducing complex and diverse changes in its size, structure and protein composition. Proteomic analysis has revealed a broad network comprising different nucleolar proteins involved in stress responses (Boulon et al., 2010) and suggested that the mammalian nucleolus can function as a key regulator in stress sensing, perception and response. Although in plants, cross-talk between nucleolar functions and stress signaling pathways has been less well studied, there are multiple sources of evidence which suggests that the plant nucleolus also has a direct role in sensing stresses such as drought, salinity and inclement temperature and responding by modulating a variety of different pathways, which may increase stress tolerance.

It has been shown that stress can be accompanied by dramatic morphological alterations in the protein content and organization of plant nucleoli. These changes are presumably related to alterations in diverse nucleolar transcriptional activity under stress conditions. For example, in soybean meristemic root cells exposed to low temperature stress, transcriptional activity is reduced and this is reflected in the looser structure and size increase of nucleoli, but decrease in the number of FCs and DFCs (Stepinski, 2009). In this study decreases in the amounts of important nucleolar proteins (fibrillarin and B23) were also observed.

As mentioned above, in mammalian cells, one of the major regulators of cellular responses to diverse stresses including genome damage (DNA damage response, DDR) is the p53 transcription factor (Boulon et al., 2010). DDR is a key process to maintain genome stability and protect DNA from damage caused by numerous endogenous and exogenous DNA damaging agents. It has been found that disintegration of the nucleolus, by drugs or UV irradiation can mediate activation of p53 pathways, and suggested that the nucleolus itself can act as an upstream stress sensor. p53 has not been found in plants indicating that plants may possess their own, unique system(s) for stress responses and genome stability maintenance in particular, and recent evidence has implicated several plant nucleolar proteins in such responses (**Table 1**, **Figure 2**).

Plant SUPPRESSOR OF GAMMA RESPONSE 1 (SOG1) operates as a transcription factor and master regulator of DDR that has many similarities with p53. Like p53, SOG1 activates transcription of more than 100 genes that control cell cycle, DNA repair and programmed cell death (Yoshiyama et al., 2014; Yoshiyama, 2016). The *sog1-1* mutant exhibits increased resistance of root growth to zeocin and no cell cycle arrest and PCD in response to DNA double-strand breaks (DSB) (Yoshiyama, 2016).

There are many other plant proteins that are involved in plant DDR which have been extensively reviewed by Manova and Gruszka (2015), Yoshiyama et al. (2014), Yoshiyama (2016), and Donà and Mittelsten Scheid (2015). Among them are TDP1, RECQ4A, and RTEL1. Like its human or yeast homologs, Arabidopsis tyrosyl-DNA phosphodiesterase 1 (TDP1) for example, is able to repair DNA damage in which topoisomerases can occasionally covalently bind the ends of DNA strand breaks, by hydrolyzing the 3′-phosphotyrosyl bond between topoisomerase and DNA (Lee et al., 2010). Interestingly, inability of the Arabidopsis *tdp* mutants to repair DNA damage results in plant dwarfing and extensive cell death during development (Lee et al., 2010). This is consistent with later studies which show that RNAi silencing of the *TDP1* gene in *Medicago truncatula* results in significant changes in gene expression, which is manifested in reduced cell division, perturbed plant growth and early leaf senescence (Donà et al., 2013). *TDP1* knockdown is known to impinge on rRNA processing and ribosome biogenesis and disrupts the structure and architecture of the nucleolus, as revealed by electron microscopic analysis (Donà et al., 2013). It is also worth noting that deficiency of the TDP1 protein leads to telomere shortening (Donà et al., 2013). Taken together these findings strongly implicate the nucleolus as a central component of TDP-dependent DDR and developmental processes.

Arabidopsis REQ4A helicase is involved in dissolution of Holliday junctions, aberrant DNA structures that are formed during DNA replication and recombination, by suppressing homologous recombination and producing non-crossover recombinants (Hartung et al., 2007). However, to achieve this activity RECQ4A usually acts as a part of the RTR protein complex also containing topoisomerase TOP3α and structural proteins RMI1 and RMI2. Interestingly, another Arabidopsis protein, Regulator of Telomere ELongation helicase 1 (RTEL1), which is normally involved in telomere maintenance is also able to reduce efficiency of homologous recombination (Röhrig et al., 2016). Moreover, both RMI2 and RTEL1 have recently been shown to safeguard stability of 45S rDNA repeats. Plants defective in both these proteins exhibit male infertility, implicating functional links between suppression of homologous recombination and plant developmental defects especially in highly proliferative tissues such as the male germline, where reduction of 45S rDNA repeats seems to be most pronounced (Röhrig et al., 2016). Intriguingly, plant REQ4B helicase which is closely related to RECQ4A, has an antagonistic function and promotes formation of crossovers; exhibiting a role which has not been recognized for any other eukaryotic RECQ-like helicases (Hartung et al., 2007).

Although some DDR and DNA repair factors (such as TDP1) are associated with nucleoli, the precise role of the nucleolus in DDR remains to be elucidated.

DEAD-box RNA helicases, STRESS RESPONSE SUPPRESSOR1 (STRS1) and STRS2 proteins function as negative regulators of stress-induced gene expression. They are typically active in unstressed plants and serve to reduce high level constitutive expression of stress-responsive genes which may be detrimental to plant growth and development (Khan et al., 2014). Under normal conditions, the STRS proteins are mainly localized in the nucleolus and chromocentres, which is suggestive of their site of function. However, in response to salt or heat stresses, the STRS proteins exhibit rapid relocalization into nucleoplasm, presumably activating stress responses. STRS defective mutants (*strs*) exhibit enhanced tolerance to salt, osmotic and heat stress whereas *STRS* overexpression leads to diminished tolerance (Khan et al., 2014). Interestingly, in Arabidopsis mutants that are defective in RNA-mediated gene silencing, such as the RNA-directed DNA methylation (RdDM) pathway, the STRS proteins have been shown to mis-localize. Furthermore, it has been found that heterochromatic RdDM target loci have enhanced expression and lowered DNA methylation in the *strs* mutants, which indicates that the STRS proteins could participate in the epigenetic silencing of stress-responsive gene expression (Khan et al., 2014).

Another protein which is redistributed in response to stress conditions (for example, hypoxia) is eIF4A-III, one of the core EJC components (Koroleva et al., 2009). However, in contrast to STRSs, eIF4-III has been shown to concentrate in the nucleolus and splicing speckles under this kind of stress. It is possible that in such hypoxia conditions, the eIF4A-III may retain certain

mRNAs in the nucleolus, which could prevent their movement to the cytosol and subsequent translation.

Several other plant proteins involved in stress responses have also been relocalized to the nucleolus and have been implicated in growth and development. For example the Rab 28 Late Embryogenesis Abundant (LEA) protein has been observed to accumulate in nucleoli, and transgenic overexpressors of this protein exhibit higher relative water content, increased root and leaf areas, and reduced chlorophyll loss compared with wild-type plants when grown under osmotic stress (Amara et al., 2013). As an additional example, AtREN1 protein (an early male gametophytic gene, At1g77570, which is strongly homologous to the heat shock transcription factor gene *HSFA5*) has been shown to accumulate in the nucleolus, and plants mutated in this gene have structural and functional abnormalities in male gametophyte development, pollen grain development and perturbed heat stress responses relative to wild-type plants (Renák et al., 2014). Taken together this is indicative of a complex integrated signaling mechanism which links nucleolar functions, pollen development and heat stress in Arabidopsis.

FUNCTIONAL ASSOCIATION OF THE NUCLEOLUS, CAJAL BODIES AND POLY(ADP-RIBOSE) POLYMERASE

In physical and functional coordination with nucleoli, CBs play many important roles in RNA metabolism and formation of RNPs involved in transcription, splicing, ribosome biogenesis, and telomere maintenance (reviewed by Love et al., 2017). In addition, like the nucleolus, plant CBs participate in various other non-canonical functions, such as modulating plant responses to virus infections and abiotic stresses (Shaw et al., 2014). For example it was previously observed that coilin (the hallmark protein of CBs) could differentially affect the interactions of plants with viruses of diverse taxa. In this study it was found that coilin deficiency and/or CB depletion could increase accumulation and systemic infection by some viruses like *barley stripe mosaic* virus (BSMV, hordeivirus), tobacco rattle virus (TRV, tobravirus), tomato black ring virus (TBRV, nepovirus) and tomato golden mosaic virus (TGMV, begomovirus), while for other viruses such as potato virus Y (PVY, potyvirus) and turnip vein clearing virus (TVCV; tobamovirus) the opposite phenomenon was observed (Shaw et al., 2014). These data clearly show that coilin/CBs are important in regulating virus pathogenesis in plants. Coilin gene suppression in plants could also confer salt tolerance (Love et al., 2017), which *in toto* implicates CBs in plant perception and responses to stress.

While these underlying processes remain poorly elucidated, a possible mechanism may lie within recent studies which show an intimate association between the nucleolus, CBs and the poly (ADP-ribose) polymerase (PARP) family member, PARP1. In dissected *Drosophila* salivary gland cells, coilin and fibrillarin are known to interact with PARP1 (Kotova et al., 2009), a nuclear protein which has important regulatory functions in DNA repair and genotoxic stress tolerance, transcription, cell cycle control and programmed cell death (PCD) (Kotova et al., 2009; Briggs and Bent, 2011; Luo and Kraus, 2012; Ji and Tulin, 2013; Schulz et al., 2014). PARP1 modifies the function of a variety of nuclear "target" proteins by attaching chains of ADP ribose (PAR) to them and itself. Although most of the PARP1 molecules bind chromatin and accumulate in the nucleolus, automodified PARP1 has been shown to interact non-covalently via PAR polymers with some nucleolar and CB components, including fibrillarin and coilin respectively (Kotova et al., 2009). These associations may mediate the shuttling of PARP1 and PAR-containing protein complexes from the nucleolus and chromatin into CBs. This movement is presumed to be required for PAR removal and recycling, which may act as a molecular switch which modulates the functional activities of PARP1.

In plants, such activities are thought to be involved in the regulation of several physiological processes, including responses to abiotic and biotic stresses, differentiation and cell cycle control (reviewed in Briggs and Bent, 2011; Love et al., 2017). For example, it has been shown that PARP deficiency in Arabidopsis and *Brassica napus* (oilseed rape) plants leads to increased tolerance to drought and heat stress (De Block et al., 2005). On the other hand, overexpression of *PARP2* in Arabidopsis decreased the number of DNA nicks at high concentrations of H_2O_2 but increased their number at low H_2O_2 concentrations (reviewed in Briggs and Bent, 2011). In addition, PARP inhibitors reduce damage to tobacco and soybean cells from oxidative stress and heat shock (Amor et al., 1998; Tian et al., 2000). PARP also markedly affects plant-pathogen interactions. In particular, PARP knockout mutants of Arabidopsis have been found to display increased susceptibility to *Pseudomonas syringae*, indicating that PARP is required for antibacterial resistance. In agreement with these observations, PARP inhibitors have been shown to block some basal plant defense responses such as cell wall reinforcement with callose and lignin, which are induced by microbe-associated molecular patterns, such as bacterial flagellin or EF-Tu epitopes (Adams-Phillips et al., 2010). It has been shown that callose deposition provides a physical barrier that blocks spread of virus infection through the plasmodesmata (Li et al., 2012), and it would be interesting to speculate whether PARP may be involved in this process. While the evidence indicates that PARP is a central factor which controls resistance to various plant pathogens, plant PARP-mediated activities have also been implicated in differentiation and cell cycle control pathways, which are known to overlap with components of plant stress signaling pathways (reviewed in Briggs and Bent, 2011). Alterations in the poly(ADP-ribosyl)ation level induced by extrinsic (biotic or environmental) or intrinsic (genetic/physiological) cues play an important role in plant stress signaling and developmental processes (Briggs and Bent, 2011). It is possible that in such cases, the PARP levels could be controlled via coilin and fibrillarin induced trafficking and redistribution of automodified PARP and other PARylated proteins from the nucleolus to CBs for recycling. Although this mechanism may transduce responses to developmental and stress cues, it is also an intriguing possibility that they may underpin at least some of the architectural and protein content changes in CBs and the nucleolus. Future work will be required to elucidate these possibilities further.

PERSPECTIVES

The nucleolus is involved in coordinating many major biological processes such as ribosome production, spliceosome formation, gene expression regulation (e.g., transcriptional/post-transcriptional gene silencing), mRNA surveillance and telomere maintenance (**Figure 2**). It is therefore unsurprising that this prominent sub-nuclear domain has been repeatedly implicated as an important regulator of signaling pathways which control plant growth and development, disease and stress responses (**Tables 1**, **2**, **Figure 2**). This is particularly intriguing especially considering that in the last decade it has been found that there is frequent cross-talk between components or facets of these pathways, which may in turn regulate or be regulated by the nucleolus and associated CBs. In spite of this we are still far from comprehensively understanding the molecular mechanisms underpinning such control systems, and much remains uncharacterized.

For instance, it is becoming particularly interesting to explore if plant cells can produce more than one type of ribosome. What are the external (stress and/or disease) or internal (developmental) factors that may cause modification of RPs or rRNAs, giving rise to formation of distinct "specialized" ("renegade"; Lafontaine, 2015) ribosomes? Can such ribosome reprogramming be tissue- or organ-specific and differentially affect translation in response to stress or disease to provide mechanisms underpinning developmental regulation and biotic and environmental stress defense?

An increasing number of viral proteins and proteins encoded by other pathogens (effectors) have been shown to target nucleoli and CBs of infected plants. What are the mechanisms of such targeting and what are the molecular consequences of this targeting with respect to the host defense response?

This review has provided various examples demonstrating that some viruses have evolved to be able to exploit some nucleolar proteins (fibrillarin in particular), for their own benefits (e.g., virus movement throughout the infected plant). It would be especially intriguing to elucidate the molecular mechanisms of this phenomenon, given the essential role of fibrillarin in absolutely different process of modification and processing of rRNA. Is fibrillarin also involved in other processes of macromolecular trafficking in plants or interaction with other pathogens, e.g., controlling or assisting transport of regulatory or signaling mRNAs or ncRNAs in plants? What are the other nucleolar proteins interacting with pathogen effectors?

In future, uncovering the large-scale protein-protein interactome networks will be required to elucidate the contextual mechanisms and molecular switches which underpin nucleolar activities and physiological control. Such work will identify key nucleolar regulators of different plant signaling responses, which shall provide real targets for crop improvement by allowing us to tailor how plants respond to particular forms of environmental stress.

AUTHOR CONTRIBUTIONS

MT and NK contributed to the review conception, literature analysis and writing. AL contributed to the review editing, writing and data evaluation. SM and AM were involved in writing the Chapter "Virus Infection" and designing Figures and Table.

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Nucleolar Proteome Analysis and Proteasomal Activity Assays Reveal a Link between Nucleolus and 26S Proteasome in *A. thaliana*

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In all eukaryotic cells, the nucleolus is functionally and structurally linked to rRNA synthesis and ribosome biogenesis. This compartment contains as well factors involved in other cellular activities, but the functional interconnection between non-ribosomal activities and the nucleolus (structure and function) still remains an open question. Here, we report a novel mass spectrometry analysis of isolated nucleoli from *Arabidopsis thaliana* plants using the FANoS (Fluorescence Assisted Nucleolus Sorting) strategy. We identified many ribosome biogenesis factors (RBF) and proteins non-related with ribosome biogenesis, in agreement with the recognized multi-functionality of the nucleolus. Interestingly, we found that 26S proteasome subunits localize in the nucleolus and demonstrated that proteasome activity and nucleolus organization are intimately linked to each other. Proteasome subunits form discrete foci in the disorganized nucleolus of *nuc1.2* plants. *Nuc1.2* protein extracts display reduced proteasome activity *in vitro* compared to WT protein extracts. Remarkably, proteasome activity in *nuc1.2* is similar to proteasome activity in WT plants treated with proteasome inhibitors (MG132 or ALLN). Finally, we show that MG132 treatment induces disruption of nucleolar structures in WT but not in *nuc1.2* plants. Altogether, our data suggest a functional interconnection between nucleolus structure and proteasome activity.

Keywords: proteasome, nucleolus, *Arabidopsis*, nucleolin, FANoS

INTRODUCTION

The nucleolus is the most prominent structural and functional nuclear compartment of eukaryotic cells. The main function of the nucleolus is linked with ribosome biogenesis, intimately associated with cell metabolism, proliferation and stress response (Lam et al., 2005; Saez-Vasquez and Medina, 2008; Boulon et al., 2010; Pederson and Powell, 2015). Functional biochemical and proteomic analyses have revealed that the nucleolus is involved in other important biological processes beyond ribosome biogenesis, including RNA metabolism, gene regulation, cell cycle regulation, DNA repair

and cell aging (Pendle et al., 2005; Padeken and Heun, 2014; Tsai and Pederson, 2014; Pederson and Powell, 2015; Bensaddek et al., 2016; Palm et al., 2016). Notably the nucleolus plays a role in the cellular response to intrinsic and environmental changes as well as in genome stability and organization (Saez-Vasquez and Medina, 2008; Boulon et al., 2010; Lewinska et al., 2010; Nalabothula et al., 2010; Audas et al., 2012a; Grummt, 2013).

An important property of the nucleolus is that it sequesters a large number of nuclear genes from which RNA polymerases II and III are normally excluded and hence it might play a key role in regulating gene expression (Németh et al., 2010; Németh and Längst, 2011; Padeken and Heun, 2014; Pontvianne et al., 2016b). The nucleolus has also novel and poorly characterized functions in protein sequestering via interaction with other proteins and/or long non-coding RNAs (Audas et al., 2012a,b; Jacob et al., 2012; Lin et al., 2017). The nucleolar retention of specific proteins can potentially suppress or inhibit diverse cellular activities by recruiting general transcription or RNA processing factors or other proteins involved in protein dynamic and activities. Nucleolar sequestering may therefore directly affect post-translational protein modifications and their turnover.

In *Arabidopsis thaliana*, two proteomic studies of the nucleolus have been performed using nucleolar fractions purified from cell cultures (Pendle et al., 2005; Palm et al., 2016). The first analysis identified around 217 proteins in the nucleolus (Pendle et al., 2005). This work revealed several proteins related to the exon-junction complex as well as other non-ribosomal and even "non-nucleolar" proteins. The more recent proteome extends the initial work and identified 1602 proteins in the nucleolar fraction (Palm et al., 2016). Both studies demonstrated also nucleolar localization of spliceosomal proteins and proteins involved in non-sense mediated mRNA decay (NMD) among many others. Splicing factors have been already characterized for their role in the processing of rRNAs (Yoshikawa et al., 2011; Gupta et al., 2014), however, it is not yet known how the nucleolus might impact the activity of spliceosomal or NMD factors.

To have a more precise view of the nucleolar protein content in entire full growing plants, we isolate nucleoli from leave cells by the recently established FANoS (Pontvianne et al., 2013, 2016a). This strategy yielded the identification of most of the factors and complexes involved in rRNA transcription and processing, and in the first steps of ribosome biogenesis. In addition, we identified proteins not observed in the previous approaches using *Arabidopsis* cell cultures (Pendle et al., 2005; Palm et al., 2016). This might be linked to additional nucleolar activities in an entire and growing plant or to cell type specific variations of the nucleolar proteome.

The proteasome is a nuclear-cytoplasmic proteolytic complex involved in nearly all regulatory pathways in eukaryotic cells (Kurepa and Smalle, 2008; Collins and Goldberg, 2017). In particular, the proteasome-ubiquitin system is required for degradation of ribosomal proteins produced in excess (Sung et al., 2016b) or unassembled (Sung et al., 2016a). Furthermore, impaired proteasome function has been correlated with disease in human (Collier et al., 2017; Voutsadakis, 2017) and the stress response in plants (Gladman et al., 2016; Kang et al., 2017; Misas-Villamil et al., 2017). A functional interplay between proteasome and nucleolar activities is in line with the integration of the nucleolus in multiple pathways and its established role as stress sensor (Boulon et al., 2010; Tsai and Pederson, 2014; Pederson and Powell, 2015). Accordingly, we show that nucleolus organization is required for optimal proteasome activity and vice-versa, that inhibition of proteasome activity affects the structure and organization of the nucleolus. The role and biological significance of proteasome subunits in the nucleolus are discussed.

RESULTS

Proteomic Analysis of the Nucleolus

We previously reported the FANoS method to purify nucleolar DNA and RNA (Pontvianne et al., 2013, 2016b; Durut et al., 2014). Here, we applied FANoS to investigate the nucleolus proteome of 3 weeks-old *Arabidopsis thaliana* plants. Two nucleoli purifications from leaves of independently grown plants were performed (exp-1 and exp-2). We obtained ∼9.83 × 10^5 and ∼8.25 × 10^5 nucleoli in exp-1 and in exp-2 respectively, and by nanoLC-MS/MS analysis, we identified 1,001 (exp-1) and 778 (exp-2) different proteins (**Figure 1A** and **Tables S1**, **S2**). Comparative analysis revealed that 562 proteins were consistently identified in both experimental data sets (**Figure 1A** and **Table S3**). This subset of 562 common proteins identified in both biological replicates was considered in the subsequent analysis, if not otherwise specified. 99 and 409 proteins out of these 562 proteins have been previously identified by Pendle et al. (2005) and Palm et al. (2016) respectively (**Figure S1A**).

Based on functional characterization and cellular localization studies reported in the literature, we determined that ∼35% of the proteins found in nucleolus fractions have been assigned as ribosome biogenesis factors (RBF); including 45S rRNA transcription and processing factors, 90S processome, 40S and 60S assembly factors and ribosomal proteins from large (RPL) and small (RPS) ribosome subunits. ∼26% of the 562 proteins have been described as nucleolar proteins, but not yet characterized as RBFs while the remaining 39%, to our knowledge, have not been described as nucleolar and/or having a related nucleolar function (**Figure 1B**). Then, we assessed in which other subcellular compartments these (562) proteins could be also (transiently or not) localized. We therefore used the subcellular protein distribution report recently published in Palm et al. (2016). We determined that ∼75.6% of these proteins have already been reported as nucleolar components with either nucleolar, nucleolar/nuclear or nucleolar/nuclear/cytoplasmic localization revealing the highly proteomic dynamic nature of the nucleolus. ∼22.8% were reported as nuclear or nuclear/cytoplasmic and ∼1.7% of them were only detected in the cytoplasmic fraction. None of the proteins are localized both in the nucleolus and in the cytosol fractions (**Figure S1B** and **Table S4**).

To distinguish particular extra ribosomal biogenesis functions of the nucleolus from *Arabidopsis* leaves, we assessed the enrichment of specific categories of proteins identified in the nucleolar fractions. For that, we compared the 562

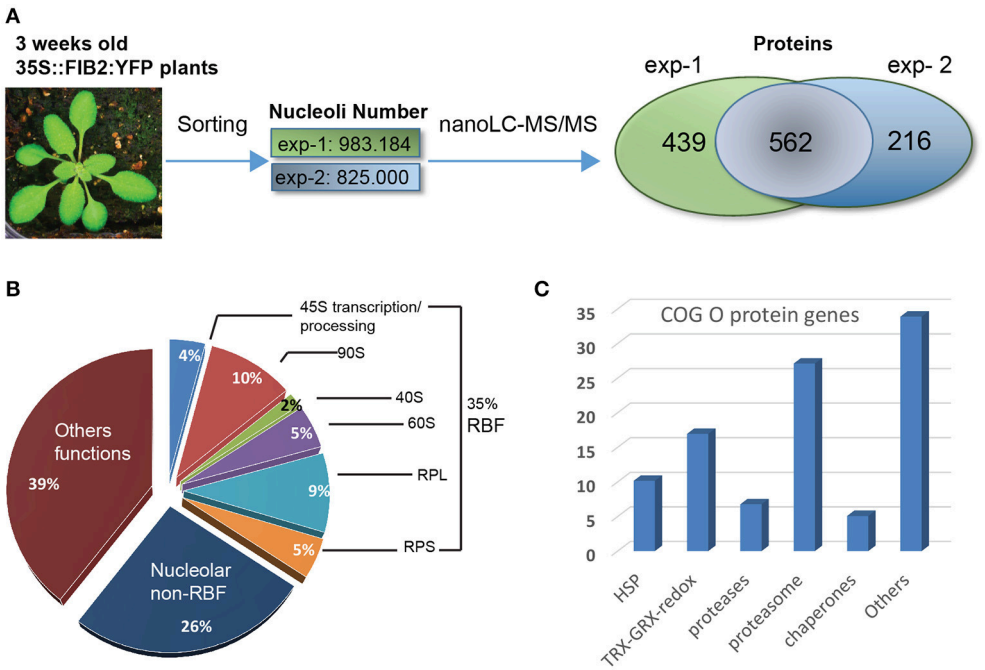

FIGURE 1 | Proteomic analysis of *A. thaliana* nucleoli. **(A)** Nucleoli extraction and nanoLC-MS/MS analysis from biological uplicates: exp-1 and exp-2. From left to right: picture of a 3 week-old leaves from WT FIB2:YFP *A. thaliana*, number of sorted nucleoli per experiment and Venn Diagram showing the number of proteins identified in exp-1 (1,001), exp-2 (778), and both (562). **(B)** Pie graph shows categories of proteins found in the nucleolar fractions. Proteins are classed in three major categories: Ribosome Biogenesis Factors or RBF (35%), nucleolar non-RBF (26%) and others functions (39%). RBF are detailed in sub-categories: 45S transcription/processing (4%), 90S processome (10%), 40S (2%) and 60S (5%) assembly factors, large (9%) and small (5%) ribosome sub-units (RPL and RPS). **(C)** The histogram shows the percentage of nucleolar proteins found in the Cluster of Orthologous Group (COG) O.

protein accessions with a proteomic dataset we obtained from an *Arabidopsis thaliana* whole cell protein extract fraction (**Table S5**). For comparative purposes, the MS/MS spectrometry analysis of this fraction was performed in a similar manner to that with the nucleolar fractions. A Gene Ontology (GO) analysis revealed that in the nucleolar fractions there is an enrichment of ∼3.8X of proteins linked to ribosome biogenesis, ∼3.3X of RNA processing factors and ∼3.5X of proteins related to Ribo Nucleo Protein (RNP) Complexes compared to whole cell protein extracts (**Figure S2A**). Additionally, we performed a Cluster of Orthologous Groups (COG) analysis and four major functional categories came up (**Figure S2B**): COG J (for ribosome biogenesis structure and translation) (17%), COG A (for RNA processing and modifications) (14%), COG O (for post-translational modification, protein turn over and chaperones) (13%) and COG R (for general function prediction only) (12%). These two analyses clearly show that nucleolus of *Arabidopsis* plants is enriched in proteins linked to ribosome biogenesis, to RNA processing and modifications and to RNP complexes. The COG analysis revealed also nucleolar enrichment of proteins linked to protein dynamics. More precisely, in the COG O category, 27% of the proteins correspond to 26S proteasome subunits while the 73% of the remaining proteins include redox activities (17%), HSP (10%), proteases (7%), chaperones (5%), and others (34%) (**Figure 1C** and **Table S6**).

All together these results demonstrate that FANoS methodology allows to obtain purified nucleoli from *Arabidopsis* leaves for proteasome analysis. This analysis indicates that in addition to ribosome biogenesis and RNA related factors the nucleolus is enriched in proteins/factors involved in enzymatic reactions and/or gene expression regulation. Because proteasome subunits are the most abundant proteins in the post-translational modification category, protein turn over and chaperones (COG O), we decided to study the functional relevance of the nucleolar localization of these subunits of the 26S proteasome.

20S Proteasome Localization in *Arabidopsis* Protoplasts

The proteasome is a sophisticated complex that selectively degrades protein substrates marked by ubiquitin covalent linkage (Kurepa et al., 2009; Liepe et al., 2014; Bach and Hegde, 2016). The 26S proteasome complex is composed of the 19S regulatory and the 20S catalytic subunits. The 19S subunit is organized in two sub-complexes: the lid built of 8 Rpn proteins and the base composed of 3 Rpn proteins (Regulatory Particle Non-ATPase) as well as 6 Rpt (Regulatory Particle Triple-A or Regulatory Particle Triphosphatase) proteins. The 20S subunit is built of 7 alpha and 7 beta proteins (Vierstra, 2003; Kurepa and Smalle, 2008).

Among the 562 proteins identified in our nucleolar fractions, we found components of the lid (Rpn5, Rpn7), the base (Rpt1 and Rpt5), and the alpha (α1, α3-α6) and beta (β3, β5, β7)

subunits (**Figure S3A**, blue labeled). We also detected additional proteins from the lid (Rpn6, Rpn8, Rpn9, and Rpn11), the base (Rpn1 and 2, Rpt2, 3, and 4) and the alpha (α2 and 7) and beta (β1, 2, 4, and 6) subunits in the individual exp-1 or exp-2 data sets (**Figure S3A**, orange labeled). Accordingly, several of these proteins were also reported in the nucleolus proteome of *Arabidopsis* cells (Palm et al., 2016; **Table S7**). To verify to which extend proteasome subunits are localized in the nucleolus, we analyzed the subcellular localization of 20S (Rpn5a and Rpt5b) and 19S (PBC1/β3 and PBG1/β7) proteins fused to GFP in *A. thaliana* protoplasts (**Figure S3B**). We noted that nucleolar localization of Rpn5a and PBG1/β7 is weak and dependent on the N- or C-terminal position of the GFP, while Rpt5b and PBC1/β3 do not show nucleolar localization with none of the constructs, suggesting either that only a small fraction of individual "tagged" proteins are assembled into proteasome and/or that they localized transiently in the nucleolus.

20S Proteasome Localization and Activity Are Altered in *Nuc1* Mutant Plants

Therefore, to investigate a potential functional relationship between nucleolus and 26S proteasome, we determined proteasome localization and activity in *nuc1.2* mutant plants which display a complete structural disorganization of the nucleolus (Pontvianne et al., 2007). *A. thaliana* contains two nucleolin protein genes NUC1 and NUC2, previously named AtNUC-L1 and AtNUC-L2 (Pontvianne et al., 2007, 2010). *nuc1.2* plant corresponds to a T-DNA insertion mutant line described in Pontvianne et al. (2010) and Durut et al. (2014).

We first analyzed the cellular localization of proteasome subunits in *nuc1.2* mutant plants (**Figure 2**). Immunolocalization experiments revealed that, Rpn1 and Rpn10 proteins localize in the cytoplasm and mostly in the nucleoplasm of root apex from WT plants (WT panels). However, Rpn10 might also localize in nucleolar subdomains (**Figure S4**), which are reminiscent of the nucleolar cavity also called nucleolar vacuoles (Saez-Vasquez and Medina, 2008; Stepinski, 2014). Interestingly, in *nuc1.2* mutants, Rpn1 and Rpn10 proteins form discrete foci (**Figure 2**, white arrows) in the nucleolus suggesting that localization of proteasome subunits is closely linked to the nucleolus structure.

Secondly, we performed an *in vitro* 20S proteasome activity assay using total protein extracts from WT and *nuc1.2* plants (**Figures 3A,B**). The results show that proteasome activity in *nuc1.2* (∼26 RFU) is reduced compared to WT (∼39 RFU) plants (**Figure 3A**). Proteasome activity measured in proteasome subunit mutant plants *rpt2* and *rpt5* does not show significant variations (∼35 and ∼40 RFU, respectively) when compared to WT plant extracts, in agreement with previous reports (Lee et al., 2011; Sakamoto et al., 2011). Then, we investigated 26S proteasome activity in protein extracts from WT and *nuc1.2* plants treated or not with proteasome inhibitors MG132 and ALLN (**Figure 3B**). WT plants treated with MG132 or ALLN show proteasome activity decrease (∼22 RFU for each inhibitor) compared with untreated plants (∼37 RFU). Remarkably, in *nuc1.2* mutants, treatment with either MG132 or ALLN inhibitors slightly reduces the proteasome activity (∼21 and ∼20 RFU, respectively) compared to untreated conditions (∼27 RFU). Interestingly, similar proteasome activity is observed between *nuc1.2* (treated or not) and WT treated plants. These results show that *nuc1*.2 is hyposensitive to proteasome inhibitors.

To verify that lower proteasome activity in *nuc1.2* protein extracts is not due to a reduced amount of proteasome protein in *nuc1.2* plants, we determined the amount of two proteasome subunits (Rpn1 and Rpn10) in WT and *nuc1.2* plants, treated or not with the MG132 inhibitor (**Figure 3C**). Western blot analysis does not reveal significant changes in protein level in WT and *nuc1.2* plants treated or not with MG132, suggesting that proteasome complex amount is not affected in *nuc1.2* plants. Similarly, we checked if NUC1 protein level could be affected by MG132 treatment. However, we do not observe detectable variations of NUC1 protein level in WT plants, suggesting that lower proteasome activity in these plants is not due to changes in the NUC1 protein level after MG132 treatment. If altered post-translational protein modifications of the 26S proteasome complex occur in *nuc1.2* plants, remains to be determined.

Because proteasome activity can be detected both in the nucleus and cytoplasm (Kurepa and Smalle, 2008), we

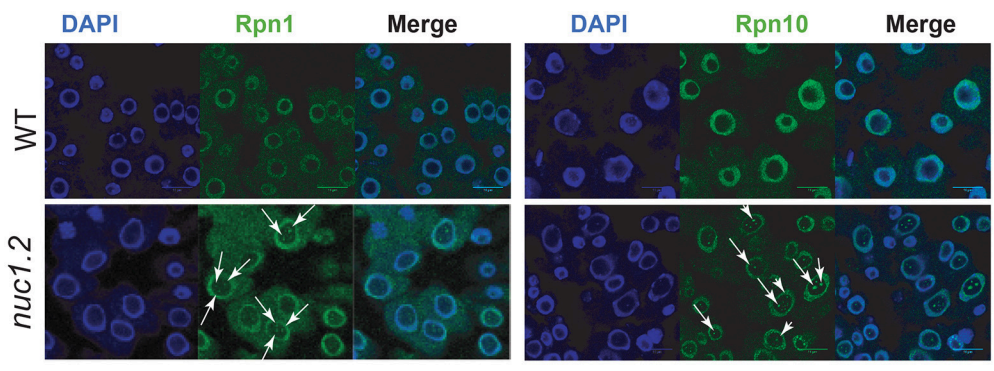

FIGURE 2 | Localization of 26S proteasome subunits in WT and *nuc1.2* plants. Immuno-localization of Rpn1a **(Left)** and Rpn10 **(Right)** proteasome protein subunits in WT and *nuc1.2* root tip cells. Arrows point foci of Rpn1a and Rpn10 proteins in nucleoli of *nuc1.2* mutant cells. DAPI staining was used to visualize nucleoplasm and distinguish the nucleolus.

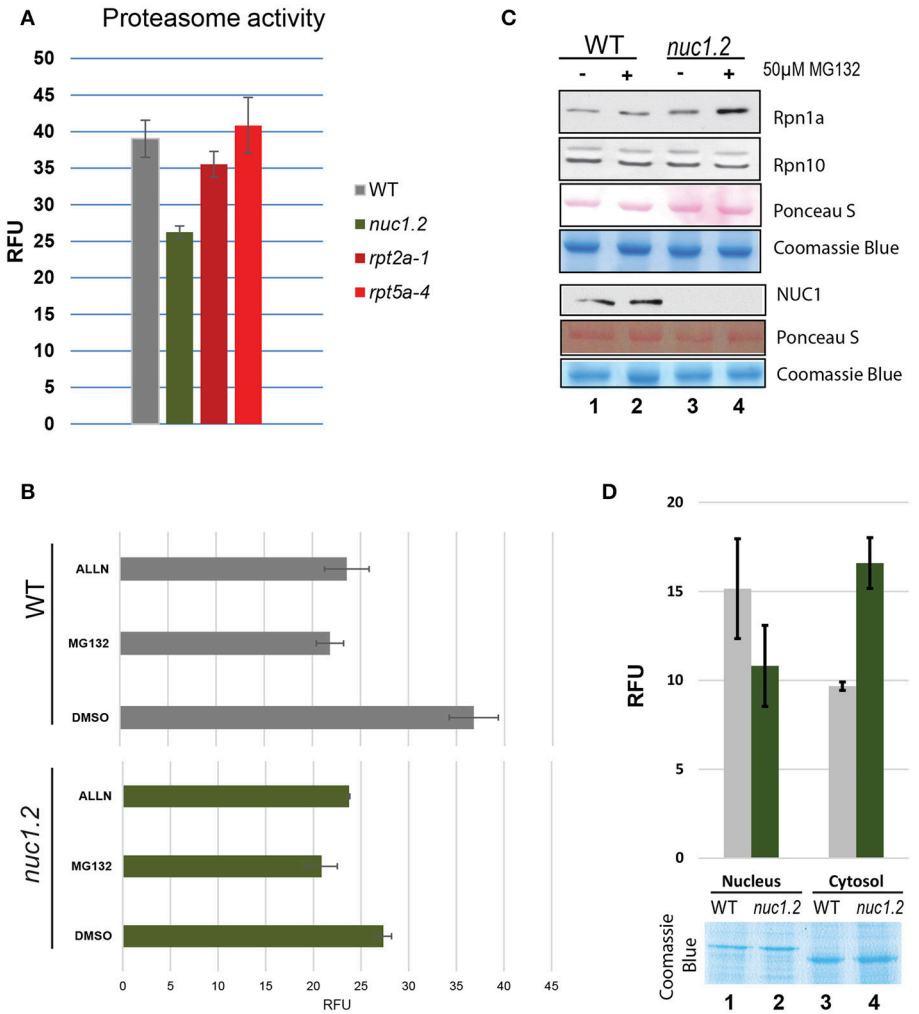

FIGURE 3 | Proteasome 26S activity in plant protein extracts. **(A)** The bar graph shows the proteasome activity in WT (gray), *nuc1.2* (green), *rpt2-1* (dark red) and *rpt5-4* (red) plant protein extracts. The 20S proteasome activity is shown in Relative Fluorescence Units (RFU). **(B)** Histogram of proteasome activity in WT (gray) and *nuc1.2* (green) protein extracts from plants treated with proteasome inhibitors MG132 or ALLN. Reactions without proteasome inhibitors (DMSO only) were used as control. **(C)** Western blot analysis to determine the protein level of Rpn1a and Rpn10 proteasome subunits in WT and *nuc1.2* mutant plants. Gels and membranes were stained with Coomassie blue or S- Ponceau respectively to verify similar amount of protein in each sample. **(D)** Histogram of proteasome activity in nuclear and cytoplasmic fractions from WT (gray) and *nuc1.2* (green) protein extracts. Gel was stained with Coomassie blue to verify similar amount of protein in each sample. Standard deviation of 3 independent experiments in **(A,B)** and **(D)** is indicated.

decided to determine proteasome activity in the nuclear and cytoplasmic fractions of *nuc1.2 mutant* plants (**Figure 3D**). The results show that the proteasome activity is reduced in *nuc1.2* (∼10.8 RFU) compared to WT (∼15.2) plants in the nuclear fractions, while in cytosolic fractions, proteasome activity is higher in *nuc1.2* (∼16.6 RFU) compared to WT (∼9.7) plants. Coomassie blue staining shows similar amount of nuclear and cytosolic proteins from *nuc1.2* and WT protein fractions. Detection of H3 histone protein and absence of the cytosolic protein PRXII validates the purity of nuclear fractions (**Figure S5**).

Altogether these results suggest that functionally structured nucleolus and/or nucleolin protein is required for optimal proteasome dynamics and activity in plants.

Inhibition of Proteasome Activity Induces Nucleolus Disruption

It is not known if it is the absence of NUC1 protein or the nucleolar disorganization phenotype observed which is responsible of proteasome localization and/or activity previously observed in *nuc1.2* mutant plants. Thus, we analyzed if inhibition of proteasome activity could have an impact on nucleolus functional organization. Three major structures are visualized in the nucleolus: the Fibrillar Centers (FC), the Dense Fibrillary Component (DFC) and the Granular Component (GC). rDNA transcription localizes to the periphery of the FC, pre-rRNA processing initiates in the DFC and later pre-rRNA processing and ribosome assembly occurs in the GC (Raska et al., 2006; Saez-Vasquez and Medina, 2008).

We investigated nucleolar structure in response to MG132 in WT and *nuc1.2* plants expressing the Fib2:YFP nucleolar marker construct (Fibrillarin2:Yellow Fluorescent Protein) which allows to visualize nucleolus organization through the fluorescence of the YFP (**Figure 4** and Picart and Pontvianne, 2017). The green signal of the Fib2:YFP protein reveals 3 distinct states of the nucleolus in WT plants: *Structured*, in which the FC and DFC are clearly recognized, *Unstructured*, in which the FC and the DFC are practically undetectable, and *Intermediate*, where nucleoli cannot be classed in the two previous categories. These three different states are observed in both WT and *nuc1.2* plants treated or not with MG132, although the ratios are clearly different (**Figures 4A,B**). In untreated WT plants (DMSO only), ∼54% of nucleoli appeared to be structured, ∼17% are unstructured and ∼29% are in an intermediate state (**Figure 4B**). In contrast, MG132 treatment increases the proportion of unstructured (∼22%) and intermediate (∼48%) nucleolus states, concomitant with a decreased proportion of structured nucleoli (∼30%). In untreated *nuc1.2* mutants (DMSO only), ∼9% of the nucleoli are structured, while the others present unstructured (∼32%) or intermediate states (∼59%), which is in agreement with previous observations (Pontvianne et al., 2007; Picart and Pontvianne, 2017). Remarkably, this analysis shows that MG132 treatment does not result in further unstructured nucleoli in *nuc1.2*, in contrast to WT plants. The fraction of structured, unstructured and intermediate states in *nuc1.2* plants treated with MG132 (∼5, ∼33, ∼62%) remains similar to those observed in untreated plants (∼9, ∼32, ∼59%). This result is also reminiscent to the analysis of proteasome activity in *nuc1.2* showing minimal threshold to MG132 and ALLN (**Figure 3B**).

Altogether, these data show that inhibition of proteasome activity affects nucleolus structure/organization which might impact on rRNA transcription and processing.

Proteasome Inhibition Affects Accumulation of Pre-RNA in *nuc1.2*

In all eukaryotic cells, nucleolus formation and structure depend essentially on 45S rRNA synthesis and ribosome assembly (Hannan et al., 1998; Grummt, 2003; Sáez-Vásquez and Gadal, 2010). The 45S rRNA genes (encoding 18S, 5.8S, and 25S rRNAs) are transcribed in the nucleolus by RNA polymerase I (RNA pol I) as a single precursor (or pre-rRNA) containing internal (ITS1 and ITS2) and external transcribed spacers (5′ETS and 3′ETS). Pre-rRNA processing depends on the conserved U3 small nucleolar ribonucleoprotein particle (snoRNP) containing fibrillarin and on other transiently associated proteins such as nucleolin (Turner et al., 2009; Phipps et al., 2011; Henras et al., 2015). In *Brassicaceae*, we have shown that the nucleolin-U3 snoRNP complex binds both 5′ETS rDNA and the 5′end of nascent pre-RNA, suggesting coupling of transcription and processing of pre-rRNA (Sáez-Vasquez et al., 2004a,b). Furthermore, we demonstrated that 26S RPN subunits co-purified with the nucleolin-U3snoRNP complex suggesting that 26S proteasome might affect 45S rRNA gene expression (Samaha et al., 2010).

To investigate if 26S proteasomal activity can affect rRNA transcription and or processing, we measured the accumulation of (1) primary pre-rRNA precursor produced by RNA Pol I and (2) processed pre-rRNA at the primary cleavage site (P) in WT and/or *nuc1.2* mutant plants treated or not with MG132 (**Figure 5**). Primer *tis* maps the transcription initiation site (TIS) (Saez-Vasquez and Pikaard, 1997) while primer *p* maps the P

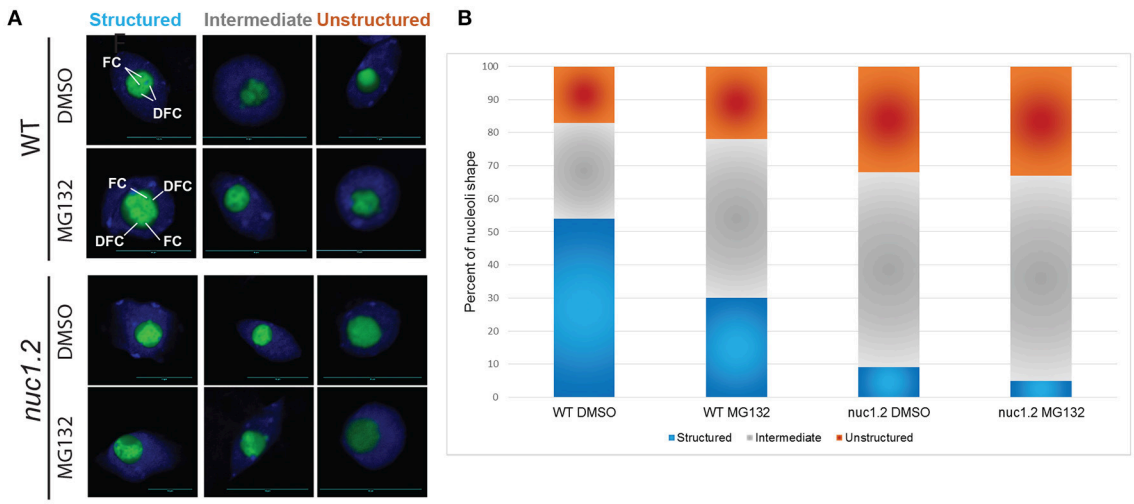

FIGURE 4 | MG132 proteasome inhibitor affects nucleolus structure. **(A)** Nucleoli from WT and *nuc1.2* plants, expressing the Fib2:YFP constructs, treated or not with 50 μM MG132. 100 nucleoli were analyzed in each sample. Green fluorescence of the Fib2:YFP, is used to visualize nucleolus organization with the FC (Fibrillar Centers), and the DFC (Dense Fibrillary Component) components in Structured, Intermediate and Unstructured forms. DAPI staining was used to visualize nucleoplasm and distinguish the nucleolus. **(B)** The bar graph depicts the percentage of structured (blue), intermediate (gray) and unstructured (orange) nucleoli in WT and *nuc1.2* plants treated or not with 50 μg MG132. Reactions without proteasome inhibitors (DMSO only) were used as control. 100 nuclei for each plants and conditions were analyzed. Scale bar = 10 μm.

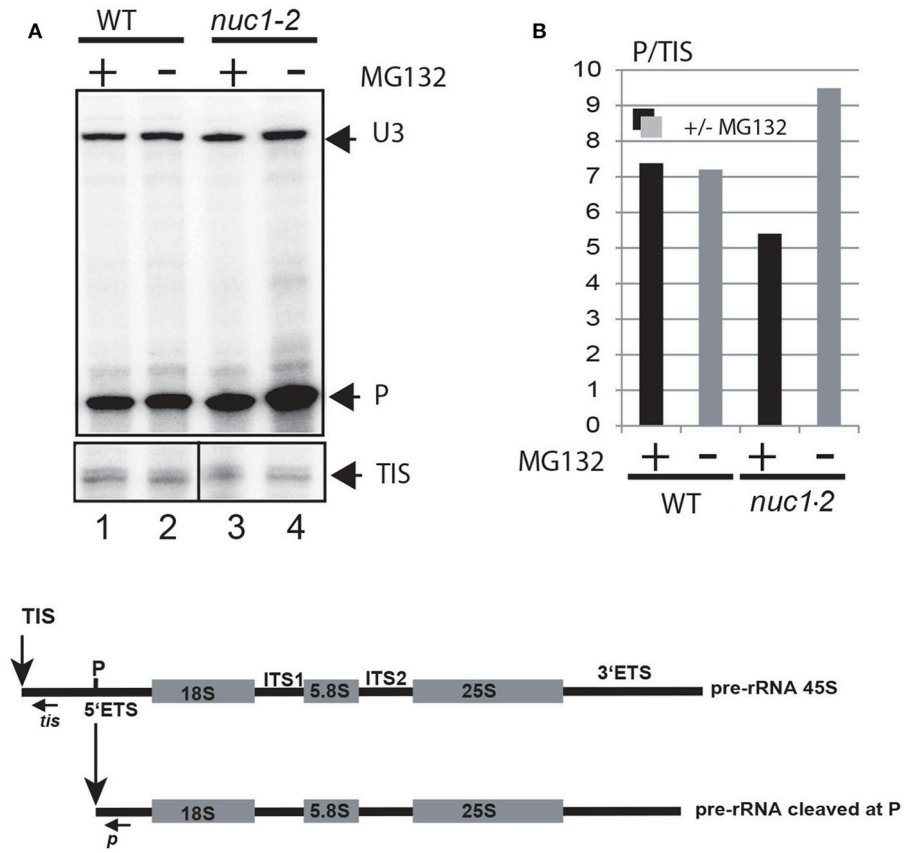

FIGURE 5 | Proteasome inhibitor affects accumulation of pre-rRNA in *nuc1.2* plants. **(A)** Primer extension experiments were performed using total RNA extracted from WT (lanes 1 and 2) and *nuc1.2* (lanes 3 and 4) plants treated or not with MG132. The relative amount of pre-rRNA initiated at the transcriptional initiation site (TIS) and cleaved at the primary cleavage site (P) was determined using primers *tis* and *p* respectively. Mapping of U3snoRNA (U3) was performed to verify similar amount of RNA in each sample. **(B)** Histogram show ratio of P/TIS signals in each sample. Black and gray bars represent respectively treated and untreated samples with MG132. Below, the scheme shows the 45S pre-rRNAs, containing the external transcribed spacers (5′ETS and 3′ETS), and the structural rRNA sequences (18S, 5.8S, and 25S rRNA in gray boxes) separated by internal transcribed spacers (ITS1 and ITS2). The vertical arrows show the Transcription Initiation Site (TIS) and primary cleavage site (P) in the 5′ETS. Positions of primers used to detect rRNA initiated at the TIS and cleaved at P sites are indicated.

primary cleavage site (Sáez-Vasquez et al., 2004b). To compare the ratio between primary and cleaved pre-rRNA (TIS/P) in WT and *nuc1.2* plants (treated or not with MG132), *tis* and *p* primers were added simultaneously to the same primer extension reactions (lanes 1-4). As previously reported, we observed an accumulation of pre-rRNA precursors in the *nuc1.2* mutant compared to WT plants (**Figure 5A**, lanes 2 and 4), suggesting a defect in primary pre-rRNA processing in mutant plants (Pontvianne et al., 2007). Interestingly, while P/TIS ratio remains similar in WT plants treated or not with MG132, in *nuc1.2* mutants, P/TIS ratio decreases after MG132 treatment (∼5) compared to untreated conditions (∼9); signifying an increase of ∼2-fold of pre-rRNA cleaved at the P site, or alternatively a decrease of pre-RNA initiated at the TIS, in the *nuc1.2* plants.

Altogether these observations indicate that proteasome inhibition does not affect RNA Pol I transcription or co-transcriptional cleavages of pre-rRNA, but rather later cleavages events taking place in the nucleolus. The data suggest also a role of NUC1 in proteasome activity or complex organization.

DISCUSSION

We report a proteomic analysis of the nucleolus from *Arabidopsis thaliana* leaves. We identified most of the proteins required for ribosome biogenesis, including rRNA transcription and processing factors, ribosomal proteins and assembly factors, indicating that the FANoS strategy allows the purification of integral nucleoli for nucleolus proteomic analysis (**Figure 1** and **Tables S1**–**S4**). We also reported ∼100 new proteins not identified in previous nucleolar proteome analyses from *Arabidopsis* cell cultures (Pendle et al., 2005; Palm et al., 2016). These novel identified proteins might be cell type, tissue or development specific and they might play a role either in ribosome biogenesis or in other central functions of the nucleolus. Moreover, and in contrast to the previous studies, we obtained the nucleolar proteome from growing plants. Therefore, all signals perceived by the plants are integrated and might impact the nucleolar proteome content, as we know that the nucleolus might also function as a stress sensor (Tsai and Pederson, 2014; Pederson and Powell, 2015).

The nucleolus from *Arabidopsis* leaves contains several factors related to RNA metabolism in the nucleoplasm, in agreement with previous reports in mammalian cells (Ahmad et al., 2009; Bensaddek et al., 2016) and *Arabidopsis* protoplasts (Pendle et al., 2005; Palm et al., 2016). We showed that nucleolus from *Arabidopsis* plants contains also proteins linked to protein metabolism, especially to protein modification and turnover (**Figure 1C**, **Figure S2B** and **Tables S6**, **S7**). We identified proteasome subunits from the regulatory 20S and the catalytic 19S subunits in the nucleolar fractions, suggesting that 26S proteasome subunits or complexes localize in the nucleolus (**Figure 1** and **Figures S1–S3**). Protein subunits from the 26S proteasome have already been reported in the nucleolus from animal cells (Arabi et al., 2003; Fátyol and Grummt, 2008; Latonen et al., 2011; Jitsukawa et al., 2012; Galimberti et al., 2016), however the functional significance of this localization remains to be completely understood.

Even if most of the subunits of the 26S proteasome complex were identified in the nucleolus from *Arabidopsis* leaves, slight or none nucleolar localization of tested proteasome subunits were observed in roots or mesophyll protoplast nucleoli. Localization of Rpn10 in the nucleolar cavity (NoC) of WT root apical cells (**Figure S4**) is interesting. The role of these nucleolar subdomains, is not yet clear. NoC are rather characteristic of plants and appear mainly in the actively transcribing nucleoli (Saez-Vasquez and Medina, 2008; Stepinski, 2014). Other proteins showing NoC localization are the AtLa1 protein, demonstrated to bind RNA Pol III primary transcripts (Fleurdépine et al., 2007) and the AtRRP6L1, required for RNA degradation (Lange et al., 2008). In addition, small nuclear and nucleolar RNAs were also shown to localize in this nucleolar subdomain (Shaw and Brown, 2004). It would be then interesting determining more precisely nucleolar localization of the proteasome in leave cells and in other plant tissue and organs.

Nucleolar localization of 26S proteasome might be required for instance for degradation of protein factors involved in transcription and processing of rRNA and/or ribosome assembly. Earlier studies showed a direct role of proteasome in controlling RNA polymerase I transcription and the presence of ubiquitinated pre-rRNA processing factors in the nucleoli in human cells (Stavreva et al., 2006; Fátyol and Grummt, 2008). In plants, 26S proteasome subunits co-purified with the U3snoRNP complex which is required for nucleolar transcription and processing of pre-rRNA (Sáez-Vasquez et al., 2004b; Samaha et al., 2010). In other hand, 26S proteasome dependent degradation of transcriptional regulator c-Myc (Arabi et al., 2003) and protein deubiquitination (Khan et al., 2015; Sun et al., 2015) in the nucleolus of mammalian cells suggest that ubiquitination/deubiquitination might regulate activity of the proteasome in the nucleolus. We cannot exclude neither the possibility that proteasome subunits in the nucleolus might also have activities non-related to proteolyse function, including transcription, DNA repair or chromatin remodeling (Tanaka, 2009).

Our results also indicate that NUC1 protein and/or a structured nucleolus is required for optimal 26S proteasome activity (**Figure 3**). Remarkably, similar 20S proteasome activities are observed between WT plants treated with proteasome inhibitors and *nuc1.2* mutants. We do not know yet why proteasome activity is reduced in *nuc1.2* protein extracts, nonetheless this is not due to a deregulation of proteasome gene expression, because the level of proteins encoding proteasome subunits is not affected in *nuc1.2* plants (**Figure 3C**). Interestingly, we observed that the proteasome activity is lower in the nucleus, while it is higher in the cytoplasm of *nuc1.2* plants compared to the WT (**Figure 3D**). One explanation for these results could be that a putative factor might control negatively proteasome activity or assembly. In this case, in WT plants, this factor should be present in the cytoplasm and down regulates proteasomal activity. In *nuc1.2* mutant, this factor might move to the nucleus, together with the proteasomal subunits (**Figure 2**), to reduce the proteasomal activity in the nucleus with a concomitant increase in the cytoplasm. Because in whole cell extracts, proteins from nucleus and cytosol are together in a single fraction, the potential proteasome inhibitor factor could globally reduce proteasome activity. This hypothesis is in agreement with a study reporting the involvement of a proteasome inhibitor protein (PAAF1) controlling assembly/disassembly of proteasome in Hela cells (Park et al., 2005). Nevertheless, the involvement of a similar factor and its potential role on proteasome activity in plants remains to be further investigated.

In contrast, we demonstrated that proteasome subunits form discrete foci in the nucleolus of *nuc1.2* plants (**Figure 2**). Formation of these proteasome-foci might probably affect 26S proteasome assembly or potential modifications required for optimal proteasome activity. The nucleolus is involved in the confinement of nuclear proteins through interactions with long non-coding RNAs (Audas et al., 2012a) or with the ribosomal protein pNON40 (Lin et al., 2017). Thus, it is reasonable to consider that nucleolus might be involved in the regulation of proteasome assembly or activity through a RNA or protein dependent nucleolar sequestering mechanism. Likewise, we cannot exclude that reduced 26S proteasome activity observed in *nuc1.2* protein extracts is due to the absence of NUC1 protein in these plants. Indeed, NUC1 co-purifies with an affinity purified 26S proteasome complex from *Arabidopsis* plants (Sako et al., 2014), while in Hela cells nucleolin might also regulate ubiquitination/deubiquitination status of proteasome targets in response to DNA damage (Lim et al., 2015).

In mammalian cells, proteasome inhibitors induce accumulation of proteasome subunits in the nucleolus (Mattsson et al., 2001; Arabi et al., 2003), nucleolar aggregation of proteasome targets and polyadenylated RNAs (Latonen et al., 2011), increase considerably oocytes nucleolus diameter (Jitsukawa et al., 2012) and accumulation of the stress-inducible transcription factor ATF4 in the DFC and Granular Component (GC) (Galimberti et al., 2016). We showed that inhibition of 26S proteasome activity has a major impact on nucleolus organization, which is reminiscent to the nucleolus disorganization observed in *nuc1.2* mutants (**Figure 4** and Pontvianne et al., 2007) and clearly linking proteasome activity with the nucleolar localization of proteasome subunits and functional structures of the nucleolus. Interestingly, inhibition

of proteasome activity did not induce significant changes in the accumulation of 45S pre-RNA precursors transcribed by RNA pol I and/or cleaved at the P site in WT plants. In contrast, inhibition of proteasome activity in *nuc1.2* plant mutants affects these primary events. Then, it is reasonable to suggest a fonctional interaction of the 26S proteasome with NUC1 protein activities, since proteasome subunits and nucleolin co-purified with the nucleolin-U3snoRNP complex (Samaha et al., 2010). It would be interesting to investigate how 26S proteasome activity could affect rRNA transcription and processing and more generally ribosome biogenesis to better explain the altered FC and DFC organization observed in WT after proteasome inhibition (**Figure 4**) but also under different cellular and environmental conditions that might disrupt nucleolus organization.

To conclude, we propose that nucleolar localization of the 26S proteasome is intimately linked to nucleolar activity that is connected with protein synthesis, cell growth and proliferation. Thus, we suggest that 26S proteasome localizes in the nucleolus to control ribosome biogenesis and maybe other cellular processes associated with the nucleolar functions. Nucleolar transit (of 20S or 19S particles or individual protein subunits) might be also required for specific post-translational protein modifications and hence for regulation of proteasome activity (Yedidi et al., 2016). Indeed, the regulation of 26S proteasome activity involves different mechanisms, including post-translational modifications, substitution of catalytic subunits, binding of regulatory complexes and proteasome conformational modifications (Kurepa and Smalle, 2008; Liepe et al., 2014). Investigating, the proteasome nucleolar retention mechanisms, potential proteasome modifications in the nucleolus and how the proteasome might regulate nucleolar functions should be the next steps to better understand the functional link between the nucleolus and proteasome in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All lines were derived from *Arabidopsis thaliana* Columbia (Col 0) ecotype. Plants expressing Fib2:YFP nucleolar marker construct were described in Pontvianne et al. (2013) and Picart and Pontvianne (2017). The *nuc1.2, rpt2a-1,* and *rpt5a-4* T-DNA insertion mutant lines were reported previously in Pontvianne et al. (2007, 2010), Wang et al. (2009), Sakamoto et al. (2011). Seeds were sown either on soil or on 1X Murashige and Skoog medium (MS containing 1% sucrose) and left for 2 days at 4°C to synchronize. Plants were then grown in controlled growth chambers under a 16 h light/8 h dark cycle at 21°C for 3 weeks (FANoS) or 2 weeks *(in vitro* activity assay) or under continuous light for 14 days (Western blot). For treatment with proteasome inhibitors MG132 and ALLN (Sigma), 15-days-old plant seedlings were transferred to petri dishes containing 6 mL of liquid MS medium complemented with 50 μM MG132 and/or 50 μM ALLN for 24 h before harvesting.

Purification of Nucleolus by FANoS

Leaves (without petiole) from 3-weeks-old Fib2-YFP plants, were fixed for 20 min in 4% formaldehyde in cold Tris buffer (10 mM Tris-HCl pH7,5, 10 mM EDTA, 100 mM NaCl), and washed twice for 10 min with Tris buffer. Then, leaves were chopped with a razor blade in FACS buffer (45 mM MgCl2, 20 mM MOPS pH7, 30 mM Sodium citrate, 0.1% TritonX-100) containing protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail from Roche) and filtered through a 30 μM PARTEC CellTrics membrane. Filtrates were sonicated using a Bioruptor (Diagenode) with parameters setted up as follows: three 5-min pulse ON/30-s OFF at Medium intensity. Samples were kept on ice and protected from light until sorting experiment was performed using BD FACS ARIA II (Biosciences), at the IGMM institute, Montpellier (MRI platform), and with parameters described previously in Pontvianne et al. (2016a).

Nuclear and Cytosolic Cellular Fractionation

Fifteen-days-old plant seedlings were collected, shock-frozen in liquid nitrogen and grinded in fine powder. Samples were homogenized in three volumes of Extraction buffer (20 mM HEPES/KOH, 10 mM MgCL2, 0.5M Hexylene glycol), filtrated through a Miracloth (EMD Millipore Corporation) and a bolting cloth (Sefar AG, 31 μm). Then, Triton X-100 was added (0.5% final) and samples incubated on a rotor for 15 min at 4°C. To obtain cytosolic and nuclear fractions, whole cell extract samples were centrifuged at 1,000 g during 10 min at 4°C. The supernatant corresponds to the cytosolic fraction. The pellet was washed with 1 mL of Extraction buffer containing 0.5% Triton X-100, centrifuged at 1,000 g for 10 min at 4°C and finally resuspended in 150 μL of Extraction buffer (supplemented with 0.5% Triton X-100). This corresponds to the nuclear fraction. For each proteasome activity assay, 1 μg of protein of each fraction was used.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

Electrophoresis and in Gel Trypsin Digestion

Purified nucleoli fractions were resuspended in Laemmli buffer containing Tris HCL pH 6.8, EDTA 1 mM, 5% of βmercaptophenol, 5% of SDS and protease inhibitors before being separated on an in-house poured 4–10% acrylamide gel. Gel was stained with Coomassie Blue and the lanes were manually cut into six bands of similar size each. Proteins in the gel slices were then reduced, alkylated and digested overnight at 37°C with modified trypsin in a 1:100 enzyme:protein ratio (Promega, Madison, USA). Peptides were extracted during 45 min with 100 μL of 60% acetonitrile, 0.1% formic acid and 15 min with a solution of 100% acetonitrile.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analyses

LC-MS/MS analyses of nucleoli peptide extracts were performed on a NanoAcquity LC-system (Waters, Milford, MA, USA) coupled to a Q-Exactive plus Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer equipped with a nanoelectrospray ion source. Mobile phase A (99.9% water and 0.1% FA) and mobile phase B (99.9% acetonitrile and 0.1%

FA) were delivered at 450 nL/min. Samples were loaded into a Symmetry C18 precolumn (0.18 × 20 mm, 5 μm particle size, Waters) over 3 min in 1% buffer B at a flow rate of 5 μL/min. This step was followed by reverse-phase separation at a flow rate of 450 nL/min using an ACQUITY UPLC® BEH130 C18 separation column (200 mm × 75 μm id, 1.7 μm particle size, Waters). Peptides were eluted using a gradient from 1 to 8% B in 2 min, from 8 to 35% B in 43 min, from 35 to 90% B in 1 min, maintained at 90% B for 5 min and the column was reconditioned at 1% B for 20 min.

The Q-Exactive plus Orbitrap instrument was operated in data dependent acquisition mode by automatically switching between full MS and consecutive MS/MS acquisitions. Survey full scan MS spectra (mass range 300–1,800) were acquired with a resolution of 70,000 at 200 m/z with an automatic gain control (AGC) fixed at 3 × 106 ions and a maximum injection time set at 50 ms. The 10 most intense peptide ions in each survey scan with a charge state ≥2 were selected for MS/MS fragmentation. MS/MS scans were performed at 17,500 resolution at 200 m/z with a fixed first mass at 100 m/z, AGC was fixed at 1 × 105 and the maximum injection time was set to 100 ms. Peptides were fragmented by higher-energy collisional dissociation (HCD) with a normalized collision energy set to 27. Peaks selected for fragmentation were automatically put on a dynamic exclusion list for 60 s and peptide match selection was turned on. MS data were saved in.raw file format (Thermo Fisher Scientific) using XCalibur.

LC-MS/MS Data Interpretation and Validation

Raw files were converted to.mgf peaklists using msconvert and were submitted to Mascot database searches (version 2.5.1, MatrixScience, London, UK) against an *Arabidopsis thaliana* protein sequences database downloaded from The Arabidopsis Information Resource TAIR site (TAIR10 version), common contaminants and decoy sequences were added. The concatenated database contains 70994 protein entries. Spectra were searched with a mass tolerance of 5 ppm in MS mode and 0.07 Da in MS/MS mode. One trypsin missed cleavage was tolerated. Carbamidomethylation of cysteine residues and oxidation of methionine residues were set as variable modifications. Identification results were imported into Proline software (http://proline.profiproteomics.fr/) for validation. Peptide Spectrum Matches (PSM) with pretty rank equal to one, with peptide length equal to or above seven amino acids and with a Mascot ion score above 25 were kept. False Discovery Rate was then optimized to be below 1% at PSM level.

Cloning of 26S Proteasome Subunits and Subcellular Localization in *Arabidopsis* Protoplasts

Subcellular localization was performed as described in Sommer et al. (2011) and Palm et al. (2016). In brief, the coding sequence of Rpn5a (At5g09900), Rpt5b (At1g09100), PBC1/β3 (At1g21720), and PBG1/β7 (At1g56450) genes was amplified using *Arabidopsis thaliana* cDNA and specific oligonucleotides (**Table S8**). Then, amplified fragments were cloned in the pRTds vector to generate C- and N-terminal GFP fusion constructs. As a nucleolar localization control, atFIB2 (At4g25630) was cloned in front of mCherry into the same vector and co-transformed with the GFP-fusion constructs (Missbach et al., 2013).

Leaves of 4-weeks-old *Arabidopsis thaliana* plants were rubbed on K240 sandpaper and then incubated in 25 mL of extraction buffer [1% (w/v) cellulase R10, 0.3% (w/v) macerozyme in MCP (29 mM MES-KOH pH 5.6, 500 mM sorbitol, 1 mM CaCl2)] for 2 h at 30°C to isolate protoplasts from mesophyll cells. After incubation, the released protoplasts were filtered through a 75 μm nylon mesh and underlayed with 2.5 mL of 100% (v/v) Percoll MCP (pH 5.6 containing 5 mM MES, 500 mM sorbitol, 1 mM CaCl2). After centrifugation at 405 g for 8 min, the clear supernatant of around 20 mL was removed and the remaining protoplast fraction was mixed with the Percoll cushion, followed by overlaying with 7.5 mL 25% (v/v) Percoll in MCP and 5 mL MCP. The mixture was centrifuged at 270 g for 8 min and the green protoplast fraction between MCP and 25% (v/v) Percoll was collected in a new tube. After centrifugation at 100 g for 5 min, the protoplast pellet was diluted in MMg (5 mM MES-KOH pH 5.6, 400 mM sorbitol, 15 mM MgCl2) to a cell number of 106 cells per mL. For transfection, 100 μL protoplasts were mixed with 10 μg pDNA per construct. 100 μL PEG-solution [40% (w/v) PEG-4000, 100 mM Ca(NO3)2, 400 mM sorbitol] was added to the protoplasts. After incubation for 20 min at room temperature, the reaction was stopped with K3-solution (20 mM MES-KOH pH 5.6, 400 mM sucrose, 1 mM CaCl2, MS salts). The protoplasts were incubated over night at room temperature and under constant light condition. The expression analysis was done by confocal laser scanning microscopy (CLSM) using a HCX PL APO CS 40× 1.25 NA 1.25 oil objective. Transformed protoplasts (around 10 μL) were spotted on an object slide. Fluorescence was excited and detected as follows: GFP 488 nm/505–525 nm, mCherry 568 nm/580–610 nm, chlorophyll fluorescence 514 nm/650–750 nm.

Proteasome Activity Assays

Fifteen-days-old plant seedlings treated or not with MG132 or ALLN were collected, shock-frozen in liquid nitrogen and grinded in fine powder. Samples were incubated on ice in Extraction buffer (50 mM HEPES/KOH, 2 mM MgCl2, 150 mM NaCl, 10% Glycerol, 1% Triton X-100) for 30 min with vortexing steps every 10 min. Then samples were centrifuged at 22,000 g for 20 min at 4°C and supernatant recovered. Activity assay was performed using the kit "20S Proteasome Activity Assay Kit" (Chemicon® International) according to the manufacturer's instructions. For each assay, 2 μg of protein extract was used. Fluorescence was determined using "Fluoroskan Ascent FL" (Thermo Scientific), with light excitation at 355 nm and emission at 460 nm.

Western Blot

Plant material (100 mg) treated or not with MG132, was homogenized and extracted in protein extraction buffer [50 mM Tris-HCl pH8, 150 mM NaCl, 10 mM EDTA, 50 mM NaFluoride, 1% NP40, 0.5% Deoxycholate, 0.1% SDS and protease inhibitors (cOmplete, Mini Protease Inhibitor Cocktail from Roche)]. Samples were cleared by centrifugation at 13,000 g for 20 min at 4°C and proteins extracted in 1X SDS-Laemmli buffer. Western

blot was performed as described previously (Durut et al., 2014) using, α-H3 (CT, pan from Millipore) α-NUC1 (Pontvianne et al., 2010), α-RPN1a (Wang et al., 2009), α-RPN10 (Lin et al., 2011), and α-PRXII (Bréhélin et al., 2003) antibodies.

Cytology Analysis

Immunofluorescence was performed on roots apex from 8 day –old seedlings as previously described in Durut et al. (2014). Briefly, treated roots were incubated overnight at 4°C with α-RPN1 (1:1,000) and α-RPN10 (1:1,000) and then with anti-rabbit coupled with Alexa 488 (1:1,000, Invitrogen), for 3 0h at room temperature. Slides were then mounted in Vectashield medium containing DAPI solution. For nucleolus structural studies, 2 week-old Fib2-YFP plants (WT and *nuc1.2,* treated or not with MG132), grown on MS medium, were fixed for 20 min in 4% formaldehyde in cold Tris buffer (10 mM Tris-HCl pH7,5, 10 mM EDTA, 100 mM NaCl) and washed twice for 10 min with Tris buffer. Then plants were chopped with a razor blade in LB01 buffer (15 mM Tris-HCl pH 7,5, 2 mM NaEDTA, 0.5 mM spermine, 80 mM KCl, 20 mM NaCl, 0.1% Triton X-100) and filtered through a 30 μM PARTEC CellTrics membrane. Filtrates were completed with an equal volume of sorting buffer (100 mM Tris-HCl pH7,5, 50 mM KCl, 2 mM MgCl2, 0.05% Tween-20, 5% sucrose, filtered through 0.45 μm filter) before spreading on a polysine slide. After air-drying, slides were post-fixed in 2% formaldehyde in phosphate buffer (PBS) for 5 min and washed twice with 1X PBS. Slides were mounted in Vectashield medium containing DAPI solution. Observations and imaging were performed using a confocal microscope LSM 700 from Zeiss.

Primer Extension

Total RNAs from *A. thaliana* WT and *nuc1.2* plant mutants were extracted using Trizol reagent (Invitrogen), according to manufacturer's instructions. Then, all samples were then treated with RQ-DNase (Promega) to eliminate contaminant genomic DNA. Primer extension analysis to detect TIS and P sites was done using 5–10 μg of RNAs and specific 5′end labeled primers, as previously described (Sáez-Vasquez et al., 2004b; Pontvianne et al., 2007). Products of the reaction were analyzed on 8% polyacrylamide/ 7 M urea sequencing gel.

AUTHOR CONTRIBUTIONS

CM, ND, AO, DP, PC, and CP performed the experiments. MC analyzed data. ND took part in writing the manuscript. FP, CC, and ES supervised experiments. JS conceived, designed the study and wrote the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2017.01815/full#supplementary-material

Figure S1 | *A. thaliana* nucleolar proteomes. **(A)** Venn diagram of *A. thaliana* of the overlaps of nucleolar proteomes from Palm et al. (2016) (blue), Pendle et al. (2005) (purple) and the present study (green). **(B)** Histogram showing the subcellular distribution of identified nucleolar proteins from *Arabidopsis* leaves according to Palm et al. (2016). Each protein can be localized in only one of the following categories: Cytosol or Nucleus or Nucleus and Cytosol or Nucleolus or Nucleolus and Cytosol or Nucleolus and Nucleus or Nucleolus and Nucleus and Cytosol.

Figure S2 | Analysis GO (Gene Ontology) and COG (Cluster of Orthologous Genes) of proteins found in the nucleolus. **(A)** The Table Shows values for the five top GO categories for proteins identified in nucleolar fractions. No, Nucleolar proteins; WCE, Whole Cell Extract proteins. *P*-values and adjusted *p*-values, obtained with a Fisher test, are provided to indicate significant enrichment of specific proteins in the nucleolus compared to proteins detected in WCE. **(B)** The bar graph shows the percentage of each COG categories of proteins identified in the nucleolus. The GO and COG analysis were performed using the website servers http://www.genome.jp/tools/kaas/ and https://www.ncbi.nlm.nih.gov/COG/ respectively.

Figure S3 | Subcellular location of 26S proteasome proteins in *A. thaliana* **(A)** Scheme of the 26S proteasome complex showing the 19S Regulatory Particle (RP) and the 20S Core Particle (CP). In blue are represented protein subunits identified in both replicates (exp-1 and exp2) and in orange protein subunits only present in one of the replicate (exp1 or exp-2). In gray are indicated protein subunits non-identified in the nucleolar fractions. **(B)** Immunolocalization of Rpn5a-GFP, GFP-Rpn5a, PBC1/β3-GFP, GFP-PBC1/β3, Rpt5b-GFP, GFP-Rpt5b, and PBG1/β7-GFP and GFP-PBG1/β7 fused proteins in protoplasts of *A. thaliana*. The green signal shows the localization of Rpn5a, PBC1/β3, Rpt5b, and PBG1/β7 proteins. The red signal shows the signal emitted by the nucleolar marker Fibrillarin fused to mCherry (Palm et al., 2016). AUF corresponds to auto fluorescence signal. Scale bar 5 μm.

Figure S4 | Immuno-localization of Rpn10 in root tip cells from WT FIB2:YFP plants. Arrows point subnucleolar structures called nucleolar cavities (NoC). Green fluorescence of the Fib2:YFP, is used to visualize nucleolus and DAPI staining to visualize nucleoplasm.

Figure S5 | Western blot analysis of nuclear and cytoplasmic protein fractions. Specific antibodies were used to detect nuclear (Histone H3) or cytoplasmic (PRXII) proteins. NUC1 detection serves to verify the absence of NUC 1 protein in *nuc1.2* mutant plants. Membrane was stained with Ponceau-S to verify similar amount of protein in each sample.

Table S1 | List of Proteins found in exp-1 nucleoli isolation.

Table S2 | List of Proteins found in exp-2 nucleoli isolation.

Table S3 | List of Proteins found in both exp-1 and exp-2.

Table S4 | Localization of proteins found in both exp-1 and exp-2.

Table S5 | List of proteins found in *A. thaliana* whole cell extracts.

Table S6 | List of proteins found in exp-1 and exp-2 and belonging to COG O.

Table S7 | 26S proteasome proteins and accessions in *A. thaliana.*

Table S8 | List of oligonucleotides used to clone CDS of Rpn5a, Rpt5b, PBC1/β3, and PBG1/β7 into pRTds vector.

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REVIEW
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Plant snRNP Biogenesis: A Perspective from the Nucleolus and Cajal Bodies

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Small nuclear ribonucleoproteins (snRNPs) are protein–RNA complexes composed of specific snRNP-associated proteins along with small nuclear RNAs (snRNAs), which are non-coding RNA molecules abundant in the nucleus. snRNPs mainly function as core components of the spliceosome, the molecular machinery for pre-mRNA splicing. Thus, snRNP biogenesis is a critical issue for plants, essential for the determination of a cell's activity through the regulation of gene expression. The complex process of snRNP biogenesis is initiated by transcription of the snRNA in the nucleus, continues in the cytoplasm, and terminates back in the nucleus. Critical steps of snRNP biogenesis, such as chemical modification of the snRNA and snRNP maturation, occur in the nucleolus and its related sub-nuclear structures, Cajal bodies. In this review, I discuss roles for the nucleolus and Cajal bodies in snRNP biogenesis, and a possible linkage between the regulation of snRNP biogenesis and plant development and environmental responses.

Keywords: Cajal bodies, nucleolus, nucleus, pre-mRNA splicing, snRNA, snRNP

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INTRODUCTION

In eukaryotes, protein-coding genes contain non-coding sequence regions, called introns, as well as the coding regions, or exons. After transcription, the cellular machinery removes introns from primary transcripts and splices together the exons to generate the mature messenger RNA (mRNA). This process of pre-mRNA splicing is a critical step in mRNA metabolism and is carried out in nucleoplasmic regions by the spliceosome (reviewed by Will and Lührmann, 2011; Matera and Wang, 2014). Spliceosomes are large molecular machinery composed mainly of small nuclear ribonucleoproteins (snRNPs), which are protein–RNA complexes comprising small nuclear RNAs (snRNAs), a class of non-coding RNA molecules abundant in the nucleus, with specific snRNP-associated proteins. Major and minor forms of the spliceosome vary based on the type of snRNPs present and their target introns (reviewed by Patel and Bellini, 2008; Will and Lührmann, 2011; Matera and Wang, 2014; Lanfranco et al., 2017). The major spliceosome contains U1, U2, U4, U5, and U6 snRNPs as core components, while the minor spliceosome contains U11, U12, U4atac, U5, and U6atac snRNPs (Will and Lührmann, 2011; Matera and Wang, 2014). These UsnRNPs recognize intron sequences and cleave and join pre-mRNA by esterification reactions, resulting in the release of introns and splicing of exons into a complete mRNA (Will and Lührmann, 2011; Matera and Wang, 2014).

Other types of snRNPs regulate different aspects of RNA metabolism, such as the modification and processing of pre-ribosomal RNA (rRNA), and the modification of spliceosomal snRNAs (reviewed by Maxwell and Fournier, 1995; Kiss, 2004). The snRNPs for these RNA metabolic

processes contain small nucleolar RNAs (snoRNAs) or small Cajal body-specific RNAs (scaRNAs) (**Figure 1**). These specific snRNAs contain conserved motifs, including box C, box D, box H, and box ACA; thus, snRNPs containing these snRNAs are also called snoRNPs or scaRNPs (Maxwell and Fournier, 1995; Jády et al., 2003; Kiss, 2004). The modifications and processing steps that are mediated by snoRNPs occur in the nucleolus and the steps mediated by scaRNPs occur in Cajal bodies, sub-nuclear structures that are physically and functionally associated with the nucleolus (**Figure 1**; reviewed by Bassett, 2012; Shaw and Brown, 2012; Love et al., 2017). snoRNPs or scaRNPs mediate the biogenesis of functional ribosomes and spliceosomal snRNPs. In addition, several species-specific snRNPs are known to have specific molecular functions: U7 snRNP functions in the 3′ processing of histone mRNA in metazoan cells (reviewed by Dominski and Marzluff, 2007) and 7SK snRNP is a critical regulator of the homeostasis and activity of P-TEFb, a key regulator of RNA polymerase ll transcription, in vertebrates (reviewed by Quaresma et al., 2016).

As snRNPs play fundamental roles in the regulation of gene expression, snRNP biogenesis is a critical regulatory step in determining cellular activity. The accumulated data indicate that snRNP biogenesis is a complex process and that the nucleolus, and Cajal bodies specifically, are pivotal elements of snRNP biogenesis (Patel and Bellini, 2008; Fischer et al., 2011; Matera and Wang, 2014; Lanfranco et al., 2017). Roles for such distinct nuclear compartments and subnuclear domains in the regulation of gene expression, cellular signaling, and stress responses have attracted attention over the years not only in animal cells (reviewed by Boulon et al., 2010), but also in plant cells (reviewed by Shaw and Brown, 2012; Ohtani, 2015; Love et al., 2017). However, it has been shown that the structures of nucleolus are different between animal and plant cells; for instance, plant nucleoli contain a specific structure known as the nucleolar cavity, which contains spliceosomal snRNAs and accumulates snoRNAs (**Figure 1**; Beven et al., 1995, 1996; reviewed by Shaw and Brown, 2012; Stępiński, 2014), suggesting that plant nucleoli could organize snRNP biogenesis in a plant-specific manner. Here, I provide an overview of current knowledge regarding spliceosomal snRNP biogenesis mechanisms, focusing on the nucleolus and Cajal bodies. I further discuss the linkage between snRNP biogenesis and plant development and environmental responses, from the viewpoint of nucleolus-based regulation of snRNP biogenesis.

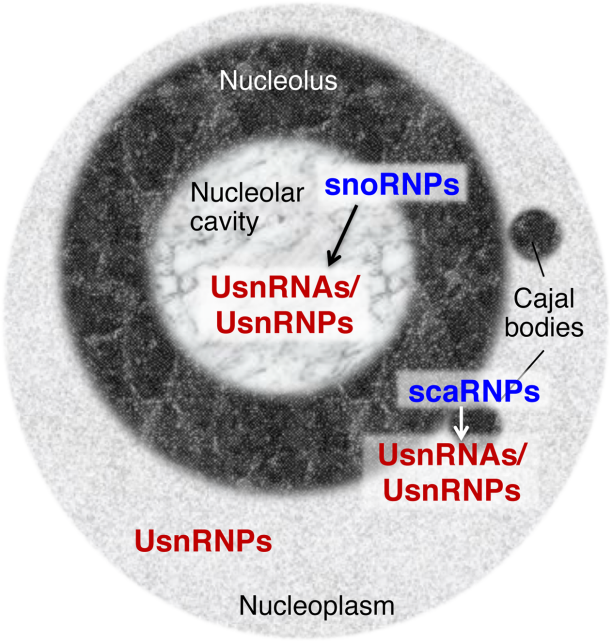

FIGURE 1 | Small nuclear ribonucleoproteins (snRNPs) found in the plant nucleus. snRNPs distributed in specific nuclear domains have specific functions, such as the guidance of chemical modifications of UsnRNAs/UsnRNPs by snoRNPs or scaRNPs (indicated by arrows between snoRNPs and UsnRNAs/UsnRNPs, and scaRNPs and UsnRNAs/UsnRNPs), and pre-mRNA splicing by mature UsnRNPs in nucleoplasmic regions. UsnRNAs, uridylate-rich small nuclear RNAs, UsnRNPs, uridylate-rich small nuclear ribonucleoproteins, snoRNPs, small nucleolar ribonucleoproteins, scaRNPs, small Cajal body-specific ribonucleoproteins.

CURRENT MODEL OF SPLICEOSOMAL snRNP ASSEMBLY BASED ON MAMMALIAN STUDIES

Spliceosomal snRNP biogenesis has been extensively studied in mammalian cells (Patel and Bellini, 2008; Will and Lührmann, 2011; Matera and Wang, 2014; Lanfranco et al., 2017), and is known to vary for different snRNP species. For example, snRNPs containing RNA polymerase II-transcribed uridylate-rich small nuclear RNAs (UsnRNAs), such as U1, U2, U4, U5, U11, U12, and U4atac (also known as Sm class snRNAs based on sequence features and protein cofactors), undergo both nuclear and cytoplasmic maturation steps (**Figure 2**). By contrast, maturation of snRNPs containing RNA polymerase III-transcribed UsnRNAs, such as U6 and U6atac (also known as Sm-like class snRNAs), is completed within the nucleus (Patel and Bellini, 2008; Will and Lührmann, 2011; Matera and Wang, 2014; Lanfranco et al., 2017).

Figure 2 shows a current model of Sm class snRNP assembly in mammalian cells. snRNP biogenesis is initiated by transcription of snRNAs through a complex called SNAPc (snRNA activating protein complex) (**Figure 2**; reviewed by Hernandez, 2001; Will and Lührmann, 2011; Matera and Wang, 2014; Ohtani, 2017). As for mRNAs transcribed by RNA polymerase II, the 5′ capping and 3′ cleavage of Sm class UsnRNAs occurs in a co-transcriptional manner. The transcriptional termination of Sm class UsnRNAs requires a large multiprotein complex, called the Integrator complex (reviewed by Chen and Wagner, 2010). The Integrator complex is thought to participate in the cleavage and polyadenylation of pre-snRNAs, acting with the 3′ box sequence, the Sm class UsnRNA-specific processing signal (Chen and Wagner, 2010; Matera and Wang, 2014). The 5′-linked *N*7-methyl guanosine ($_{m1}$G) cap of transcribed pre-snRNA molecules is first recognized by the cap binding

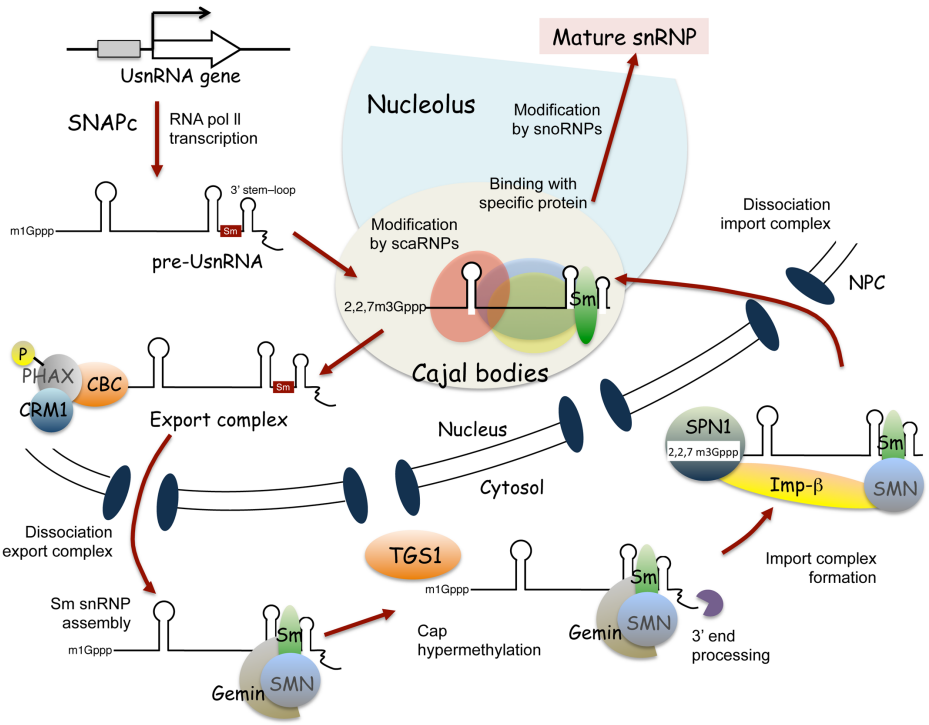

FIGURE 2 | Current model of Sm class snRNP biogenesis in mammalian cells. Maturation of Sm class snRNPs involves nuclear and cytoplasmic steps. SNAPc, snRNA activating protein complex; CBC, cap-binding complex; PHAX, phosphorylated adapter RNA export; CRM1, chromosome region maintenance 1; TGS1, trimethylguanosine synthase 1; SPN, Snurportin; Imp-β, importin-β; NPC, nuclear pore complex.

complex (CBC) (Izaurralde et al., 1994), followed by binding with the phosphorylated adaptor for RNA export (PHAX) (Ohno et al., 2000; Segref et al., 2001). In the nucleoplasm, the export receptor, chromosome region maintenance 1 (CRM1) (Fornerod et al., 1997), and the GTP-bound form of RAN GTPase interact with PHAX, to translocate the pre-snRNA molecules to the cytosol through the nuclear pore complex (Askjaer et al., 1999) (**Figure 2**). Interestingly, transcribed pre-snRNAs seem to pass through Cajal bodies before their nuclear export; pre-snRNA molecules with unprocessed 3′ extensions were found in Cajal bodies (Smith and Lawrence, 2000), and injection of pre-snRNA molecules into the nucleus showed the temporal localization of pre-snRNAs in Cajal bodies (Suzuki et al., 2010). Moreover, PHAX and CRM1 accumulate in Cajal bodies (Frey and Matera, 2001), suggesting that Cajal bodies also function in the formation of the export complex of pre-snRNA (Matera and Wang, 2014; Lanfranco et al., 2017).

In the cytoplasm, the export factors dissociate from the pre-snRNA molecule (Kitao et al., 2008). Next, the SMN-Gemins complex, which contains the SMN protein and seven Gemin proteins (Gemin 2–8), mediates the assembly of seven Sm core proteins at the Sm site of snRNA (Meister et al., 2001; Massenet et al., 2002; Narayanan et al., 2002; Pellizzoni et al., 2002; and the articles reviews in Lanfranco et al., 2017), to form Sm core snRNP, a stable ring-like structure (Raker et al., 1996; Kambach et al., 1999; Leung et al., 2011). The SMN–Gemins complex mediates the hypermethylation of the 5′ cap structure of snRNA by bridging it to trimethylguanosine synthase Tgs1 (Mouaikel et al., 2002, 2003), as well as the 3′ end trimming of snRNA (Matera and Wang, 2014; Lanfranco et al., 2017). The hypermethylated cap structure, known as the 2,2,7-trimethylguanosine cap structure, is recognized by the importer adaptor, Sniurpotin (SPN), that recruits the import receptor, Importin-β (Palacios et al., 1997; Huber et al., 1998). Sm core snRNPs are imported into the nucleus via importin-mediated transport through the nuclear pore complex. Following nuclear import, the SMN complex is immediately released from Sm core snRNPs (Matera and Wang, 2014; Lanfranco et al., 2017). Then, Sm core snRNPs accumulate in Cajal bodies, where additional modifications of the snRNAs and binding of snRNP-specific proteins occurs (Bassett, 2012; Matera and Wang, 2014; Lanfranco et al., 2017). *De novo* assembly and reassembly of U4-U6/U5 tri-snRNPs are thought to occur in Cajal bodies (Jády et al., 2003; Nesic et al., 2004; Schaffert et al., 2004; Matera and Wang, 2014). Finally, the matured spliceosomal snRNPs localize to nucleoplasmic speckle structures, called nuclear speckles, which facilitate pre-mRNA splicing (Will and Lührmann, 2011; Matera and Wang, 2014).

In the case of Sm-like class snRNP biogenesis, the pre-snRNA transcripts are localized in the nucleolus and then processed by 3′ trimming (Patel and Bellini, 2008). Further modification of the pre-snRNAs by snoRNPs and the binding of Lsm (like Sm) core proteins to the pre-snRNAs to yield stable ring-like structures also occur in the nucleolus (Achsel et al., 1999). U6 snRNAs

transiently localize in the nucleolus after transcription, and then translocate into Cajal bodies (Lange and Gerbi, 2000), where U6 snRNPs are combined with U4 and U5 to form U4/U6.U5 tri-snRNPs (Schaffert et al., 2004). Thus, the maturation of Sm-like class snRNPs takes place in the nucleolus and Cajal bodies (Patel and Bellini, 2008; Matera and Wang, 2014).

In plants cells, snRNP biogenesis is thought to proceed via similar pathways as described in mammalian cells (Lorković and Barta, 2004; Shaw and Brown, 2012). However, experimental evidence pertaining to snRNP biogenesis processes in plant cells is limited, partly due to the absence of a suitable experimental system in which to examine the assembly and translocation of snRNAs and related proteins in plants, analogous to the *Xenopus* oocyte injection system (Cohen et al., 2009). In humans, spliceosome disorders have been linked to severe inherited diseases, such as spinal muscular atrophy, which is caused by reduced levels of SMN proteins (Matera and Wang, 2014; Lanfranco et al., 2017). Plant molecular genetics studies revealed that genes involved in snRNP biogenesis are important for plant development (Ohtani et al., 2008, 2010, 2013; Swaraz et al., 2011), circadian clock regulation (Deng et al., 2010; Hong et al., 2010; Sanchez et al., 2010; Schlaen et al., 2015), stress tolerance (Xiong et al., 2001; Zhang et al., 2011; Gao et al., 2017), and plant organ regeneration (Ohtani and Sugiyama, 2005; Ohtani et al., 2010, 2013) (reviewed by Staiger and Brown, 2013; Tsukaya et al., 2013; Shang et al., 2017; for the details, please see below), suggesting that snRNP biogenesis has indispensable roles in the differentiation and function of cells that are conserved between animals and plants.

ROLES FOR THE NUCLEOLUS AND CAJAL BODIES IN SPLICEOSOMAL snRNP BIOGENESIS IN PLANT CELLS

As described above, the nucleolus and Cajal bodies have pivotal functions in snRNP assembly in mammalian cells (**Figure 2**). Here, I provide an overview of what we know about the roles for the nucleolus and Cajal bodies in plant snRNP biogenesis.

Chemical Modification of snRNAs Guided by snoRNAs and scaRNAs

Post-transcriptional modifications of snRNAs, guided by snoRNAs and scaRNAs, occur in the nucleolus and Cajal bodies (**Figure 1**; reviewed by Bassett, 2012; Love et al., 2017; Meier, 2017). These modifications are conserved among eukaryotes, including plants (Huang et al., 2005; Bassett, 2012), and are thought to convey the binding affinity of snRNPs for their substrate pre-mRNAs (Darzacq et al., 2002). After work in animal systems revealed scaRNAs, which carry a CB box that directs them to Cajal bodies, in addition to snoRNAs, which carry conserved box C, box D, box H, and box ACA (Jády and Kiss, 2001; Kiss et al., 2002), a genomic survey identified candidate scaRNAs in plant species (Marker et al., 2002). Experimental approaches also confirmed the localization of scaRNAs in Cajal bodies (Kim et al., 2010), suggesting that scaRNA-guided modification of snRNAs has important functions in plant cells (Bassett, 2012; Love et al., 2017).

In plant genomes, most snoRNA genes occur as polycistronic clusters (Leader et al., 1997; Brown et al., 2003; Chen et al., 2003). *In situ* hybridization analysis detected such polycistronic precursors of snoRNAs in the nucleolus and Cajal bodies (Shaw et al., 1998). Therefore, plant nucleoli function in the maturation of snoRNAs and in snoRNP assembly, to generate functional snoRNPs that modify snRNAs.

Assembly of snRNA and snRNP-Specific Proteins

Early studies in *Pisum sativum* (pea) showed that complexes harboring spliceosomal snRNPs were localized in the nucleolus and associated with sub-nuclear structures in close proximity to the nucleolus, later shown to be Cajal bodies (Beven et al., 1995, 1996). These observations have subsequently been supported by transient reporter assays in *Arabidopsis thaliana* (Arabidopsis) using fluorescent protein-tagged snRNP proteins, such as the U2 snRNP-specific protein U2B" (**Figure 3**; Boudonck et al., 1999; Collier et al., 2006) and U1 snRNP-specific proteins U1-70K, U1A, and U1C (Lorković and Barta, 2008). These snRNP-specific proteins have distinct distributions in nuclear regions; although all U2B", U1-70K, U1A, and U1C proteins accumulate in both the nucleolus and Cajal bodies, but they co-localize only in Cajal bodies, U1-70K also localizes in nuclear speckles, the sites of pre-mRNA splicing, whereas the other U1 snRNP-specific proteins do not (Lorković and Barta, 2008). This observation suggests that the nuclear assembly pathway differs for different snRNP-specific proteins. Recently, Hyjek et al. (2015) described the dynamic assembly of U4 snRNPs during the first meiotic prophase in European larch microsporocytes. They showed that U4 snRNAs and Sm proteins have two distinct spatial distributions in the cytoplasm—diffuse or within distinct foci—which depend on the rate of *de novo* snRNP formation relative to the expression of U4 snRNAs and Sm proteins (Hyjek et al., 2015). Furthermore, they found that the distribution of snRNPs change dynamically in the nucleus; the size and number of Cajal bodies with U4 snRNP signals varied during meiotic prophase (Hyjek et al., 2015). Cajal body dynamics have been well established in Arabidopsis. Reporter analyses using the Cajal body marker proteins U2B" and/or Coillin (Boudonck et al., 1999; Collier et al., 2006) revealed that the number and size of Cajal bodies present varied depending on the cell cycle stage, cell type, and biotic and abiotic stresses present (**Figure 3A**; Shaw and Brown, 2012; Love et al., 2017), as shown in animal cells (Boulon et al., 2010). For example, the number of Cajal bodies was decreased in newly divided cells, and as the G1 phase progressed, the size of Cajal bodies increased (Boudonck et al., 1999). Cajal bodies were also disappeared immediately by heat shock treatment (Boudonck et al., 1999). In addition, Cajal bodies are reorganized into Cajal bodies-like structures by the infection of groundnut rosette virus (Kim et al., 2007a,b). These observations would partly reflect changes in *de novo* snRNP biogenesis activity under different cellular conditions.

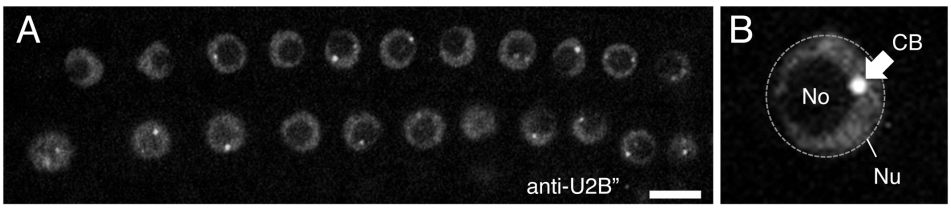

FIGURE 3 | (A) Confocal image of Arabidopsis root nuclei with Cajal bodies, as visualized by immunostaining with anti-U2B" protein. **(B)** Shows a close-up view of the nucleus. Nu, nucleus (shown by a dotted line), No, nucleolus, and CB, Cajal body (shown by arrow). Scale bars, 10 μm.

The central role of the nucleolus in snRNP assembly was additionally supported by proteomics analysis of Arabidopsis nucleoli. Pendle et al. (2005) identified 217 proteins as nucleolar proteins containing proteins with ribosome-related functions, including ribosomal proteins, rDNA transcription regulators, and ribosome biogenesis-related proteins. In addition to these expected proteins, snRNP proteins and other spliceosomal proteins were identified (Pendle et al., 2005). A recent detailed proteome analysis of the nucleus and nucleolus of Arabidopsis identified 86 proteins annotated as pre-mRNA splicing-related factors, with snRNP proteins among them, and demonstrated that 49 of these were localized in both the nucleus and nucleolus (Palm et al., 2016). Some of such pre-mRNA splicing-related factors, e.g., RSZp22 (Tillemans et al., 2006) and eIF4A-III (Koroleva et al., 2009), have been shown to shuttle between the nucleus and nucleolus in response to cellular stresses. Thus, the pre-mRNA splicing-related factors detected in both the nucleus and nucleolus could be also under the regulation of active trafficking between the nucleus and nucleolus in plant cells. It was also suggested that post-translational modifications of nuclear proteins, including acetylation and phosphorylation, differ based on the localization of each protein. This implies that an important role of trafficking nuclear domains could be the regulation of post-translational modifications, and thus the regulation of protein activities, as shown for the phosphorylation-depending mobility of RSZp22 between nuclear domains (Tillemans et al., 2006). These proteome data also identified many "unknown proteins" and/or "plant-specific nucleolar proteins," some of which were annotated as RNA-binding proteins (Pendle et al., 2005; Palm et al., 2016). It is possible that an unknown plant-specific regulatory system may contribute to snRNP assembly in the nucleolus. Arabidopsis ROOT INITIATION DEFECTIVE 1 (RID1), a nucleolus-localized DEAH-box RNA helicase, is a candidate to be one such factor. Although RID1 itself did not seem to be a direct part the spliceosome, pre-mRNA splicing was significantly affected in the *rid1-1* mutants (Ohtani et al., 2013). Future studies should examine the plant-specific aspects of snRNP assembly.

Linkage between the Regulation of snRNP Biogenesis and Development, Growth, and Stress Responses in Plants

Since pre-mRNA splicing is critical for gene expression, severe disorders of snRNP biogenesis are expected to be lethal for eukaryotic cells. Indeed, knock-out mutations of snRNA biosynthesis genes and essential components of snRNPs result in gametophyte or embryo lethality in Arabidopsis (Tsukaya et al., 2013). However, molecular genetic studies have revealed that snRNP biogenesis-related genes could be related to specific physiological processes in Arabidopsis (Tsukaya et al., 2013; Ohtani, 2015), such as circadian clock regulation, abiotic and biotic stress responses, and plant regeneration (Staiger and Brown, 2013; Tsukaya et al., 2013; Ohtani, 2015; Shang et al., 2017). For example, Arabidopsis homologs of PROTEIN ARGININE METHYLTRANSFERASE 5 (PRMT5) and GEMIN2, the genes essential for the formation of the SMN-Gemins complex, which is required for the binding of Sm core proteins to snRNAs, are important for circadian clock regulation (Deng et al., 2010; Hong et al., 2010; Sanchez et al., 2010; Schlaen et al., 2015). Mutations of LSM4 and LSM5, which encode essential core proteins of Sm-like class snRNPs (**Figure 2**; Achsel et al., 1999), and of Tgs1, which hypermethylates the 5′ cap of snRNAs (**Figure 2**; Mouaikel et al., 2002), were reported to enhance plant sensitivities to abiotic stresses, such as salt, drought, and cold stress (Xiong et al., 2001; Zhang et al., 2011; Gao et al., 2017). Moreover, SHOOT REDIFFERENTIATION DEFECTIVE 2 (SRD2), a subunit of the snRNA-specific transcription activator complex, SNAPc (**Figure 2**), is required for *in vitro* dedifferentiation and organogenesis (Ohtani and Sugiyama, 2005; Ohtani et al., 2015; Ohtani, 2015, 2017). The disorders of the corresponding mutants can be explained by the misregulation—due to altered RNA processing—of specific genes involved in key processes, i.e., circadian rhythms, stress responses, and auxin polar transport (Xiong et al., 2001; Deng et al., 2010; Hong et al., 2010; Ohtani et al., 2010; Sanchez et al., 2010; Zhang et al., 2011; Schlaen et al., 2015).

Recent studies using pre-mRNA splicing inhibitors targeting a subunit of U2 snRNPs indicated that the global inhibition of pre-mRNA splicing primarily triggered transcriptomic changes resembling those in abiotic stress responses. These changes were partly mediated by a disturbance in the signaling pathway of abscisic acid, a key phytohormone in the stress response. The inhibitor was expected to affect all splicing events equally; however, the effects of inhibitor treatment were shown to differ for different genes (AlShareef et al., 2017; Ling et al., 2017). Thus, the impact of pre-mRNA splicing inhibition has to be interpreted from the wider perspective of mRNA

turnover, considering factors such as transcriptional kinetics and/or mRNA stability. Notably, poly(A)-mRNA molecules accumulate in Cajal bodies long after their synthesis in plants (Niedojadło et al., 2014), implying that Cajal bodies function in the (pre-)mRNA metabolism, possibly serving as a storage site and/or quality control checkpoint. Notably, the plant nucleolus contains the proteins involved in nonsense-mediated decay/mRNA surveillance (Pendle et al., 2005), as well as aberrantly spliced mRNAs (Kim et al., 2009). Thus, it can be speculated that Cajal bodies might fine-tune the rate of snRNP biogenesis in response to mRNA usage and/or might function in quality control during the translation. Detailed analyses of such possibilities are required, to further obtain clues as to why specific molecular pathways place high demands on *de novo* snRNP biogenesis.

CONCLUSION AND PERSPECTIVES

The functions of snRNPs, such as in pre-mRNA splicing, are essential for gene expression in eukaryotic cells. The biogenesis of snRNPs is a highly complicated process, including both nuclear and cytoplasmic steps for snRNA modification and protein–snRNA interaction (**Figures 1**, **2**). Molecular genetic work has indicated that eukaryotic cells place high demands on *de novo* snRNP biogenesis for specific molecular pathways, such as the differentiation of motor neurons in human (Lanfranco et al., 2017), and environmental responses in plants (Shang et al., 2017), suggesting that each step of snRNP biogenesis could function as a kind of molecular node between cellular activity and cellular circumstance. Advanced visualization studies have suggested that there are dynamic changes in distributions of snRNP-related factors, depending on the rate of *de novo* snRNP biogenesis, in plant cells (Lorković and Barta, 2008; Hyjek et al., 2015). Thus, the spatiotemporal regulation of snRNP biogenesis could be a critical aspect of a cell's response to its needs.

As discussed in this review, the nucleolus plays central roles not only in ribosome biosynthesis, but also in snRNP biogenesis. Current work has expanded our knowledge of the functions of the nucleolus. Now we know that the nucleolus is not a static structure, but an active and dynamic functional structure (Smoliński et al., 2007; Boulon et al., 2010; Shaw and Brown, 2012; Love et al., 2017). In addition to ribosome biosynthesis and snRNPs biogenesis, the nucleolus is involved in mRNA surveillance, i.e., mRNA quality control by the nonsense-mediated decay system (Pendle et al., 2005; Kim et al., 2009) and in microRNA biogenesis (Pontes and Pikaard, 2008). Thus, the nucleolus is filled with all sorts of functional RNAs and their interacting proteins. Within the nucleolus, these RNAs and proteins must be well assembled to properly function, according to cellular conditions. In line with this idea, we can consider the nucleolus as a center of RNA processing that links cellular conditions with cellular activity (Shaw and Brown, 2012).

From the perspective of the evolution of eukaryotic cellular partitioning, the establishment of the nucleolus as a center of RNA processing represents a major innovation that prevents the diffusion of RNP macromolecules, leading to the enhancement of RNA processing efficiency (Collins et al., 2009). We know that many features of the nucleolus are conserved between animals and plants. However, plant-specific features of nucleolus also exist; for example, the abundant accumulation of mRNAs within the nucleolus might be a plant-specific phenomenon (Shaw and Brown, 2012). The structure of the nucleolus also greatly differs between animals and plants (Shaw and Brown, 2012; Stępiński, 2014). Thus, after the separation of the animal and plant lineages, the functionally specialized compartmentation within the nucleolus might have progressed differently between these lineages.

The view that the plant nucleolus is a center of RNA processing raises the question as to how plant nucleoli manage to organize multiple, complicated processes, including ribosome biosynthesis, snRNP biogenesis, mRNA surveillance, and microRNA biogenesis. One solution could be the partitioning of specific RNA processing activity via sub-nuclear structures. If these sub-nuclear structures are flexibly formed and demolished, as shown in Cajal bodies, plant nucleoli can accelerate specific activity of RNA processing when necessary (Kanno et al., 2016). Future work on RNA processing mechanisms within the nucleolus will answer this important question.

AUTHOR CONTRIBUTIONS

MO designed this review work, assembled and analyzed the related papers, performed the experiments, and wrote the manuscript.

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METHODS
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Visualization of the Nucleolus Using Ethynyl Uridine

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Thanks to recent innovative methodologies, key cellular processes such as replication or transcription can be visualized directly *in situ* in intact tissues. Many studies use so-called click iT chemistry where nascent DNA can be tracked by 5-ethynyl-2′-deoxyuridine (EdU), and nascent RNA by 5-ethynyl uridine (EU). While the labeling of replicating DNA by EdU has already been well established and further exploited in plants, the use of EU to reveal nascent RNA has not been developed to such an extent. In this article, we present a protocol for labeling of nucleolar RNA transcripts using EU and show that EU effectively highlights the nucleolus. The method is advantageous, because the need to prepare transgenic plants expressing fluorescently tagged nucleolar components when the nucleolus has to be visualized can be avoided.

Keywords: nucleolus, nucleus, transcription, *Arabidopsis thaliana*, click iT

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INTRODUCTION

The nucleus, as the most prominent cellular component, represents an important research target, and thus considerable effort has been put into establishing reliable detection methods to track nuclear processes. The most prominent structure in the plant nucleus is the nucleolus, the region where transcription of rRNA genes and processing of their transcripts occur (Stoykova et al., 1985; French and Miller, 1989; Raska et al., 1989; Scheer et al., 1997; Koberna et al., 2002). Due to their unique structure, plant and animal nucleoli have represented an attractive object for microscopy. This can be documented in a number of studies focusing on its structure, metabolism, or DNA and protein components (Jacob and Sirlin, 1964; Bernhard, 1966; Jordan and Luck, 1976; Zankl and Bernhardt, 1977; Ochs et al., 1985; Fakan and Hernandez-Verdun, 1986; Biggiogera et al., 1989; Beven et al., 1995; Kopecny et al., 1996; de Carcer and Medina, 1999).

This article will introduce a method to label nucleolar RNA in the plant model *Arabidopsis thaliana*. The first protocols exploring transcription took advantage of using radioactively labeled [^{3}H] uridine detected by autoradiography (Uddin et al., 1984; Wassermann et al., 1988). With the development of halogenated nucleoside triphosphates such as 5-bromouridine-5′-triphosphate (BrUTP) which are detected by specific antibodies, a wide range of possible downstream applications emerged (Gratzner, 1982; Dundr and Raska, 1993; Jensen et al., 1993; Wansink et al., 1993; Chang et al., 2000). However, BrUTP is not absorbed well by living cells, and thus it has to be applied on isolated nuclei in so-called run-on assays (Thompson et al., 1997; Dhoondia et al., 2017), or introduced into cells via transfection, injection, or electroporation (Waksmundzka and Debey, 2001). Molecules such as 5-bromouridine (BrU), 5-iodouridine (IU), or 5-fluorouridine (FU), on the other hand, are efficiently taken up by living organisms. Direct incubation of fish in FU containing sea water or its

injection into rats enabled tracking of RNA transcription *in vivo* (Casafont et al., 2006; So et al., 2010). Similar approaches also led to the development of genome-wide analyses of nascent RNA, isolated via chromatin immunoprecipitation using anti-BrU antibody. This method is called the BrU immunoprecipitation chase (BRIC) assay and involves deep sequencing of the obtained RNA moieties (Tani and Akimitsu, 2012; Imamachi et al., 2014).

In *A. thaliana*, the application of BrU has not been reported so far and the above-mentioned applications remain to be explored further. One of the few studies using BrU in plants by the run-on method was implemented in *Brassica napus* to describe nuclear transcription (Straatman et al., 1996). BrU combined with electron microscopy helped to uncover transcription in particular domains inside the nucleolus of garden peas (Thompson et al., 1997) and recently it has been successfully applied in tobacco (Singh et al., 2017).

Because BrU, FU, or IU are detected indirectly by immunofluorescence, the novel derivative 5-ethynyl uridine (EU), which can be revealed by a click iT reaction, brought a great improvement by reducing the number of steps in the detection procedure (Dimitrova, 2011). EU was shown to be incorporated efficiently into all kinds of RNA, and HPLC revealed that every 35th nucleotide is substituted by EU (Jao and Salic, 2008). Also, relatively short pulses (∼10 min) were sufficient to obtain visible signal in cultured cells.

The click iT reaction is a selective alkyne-azide cycloaddition where the ethynyl group of EU is covalently connected to azide-containing molecules under Cu (I) catalysis (Rostovtsev et al., 2002; Tornoe et al., 2002). Individual components of click iT reactions are small, which enables their use even in whole tissues including fixed whole root tips. Because the free copper (I) present in the click iT can affect other fluorescent molecules and precludes protocols where multiple labeling is needed (Kennedy et al., 2011; Dvorackova et al., 2018), picolyl azide in combination with copper chelates without any side effects were developed, as discussed previously (Kuang et al., 2010; Uttamapinant et al., 2012).

The click iT chemistry is nowadays widely used to label replication sites by ethynyl deoxy uridine (EdU). EdU was already successfully applied in *Arabidopsis*, first to visualize the DNA replication phase in cultured cells (Kotogany et al., 2010; Mickelson-Young et al., 2016), and later to track S phase progression in root meristems (Hayashi et al., 2013; Yokoyama et al., 2016; Dvorackova et al., 2018) or to detect proliferation capacity (Kazda et al., 2016). It was also demonstrated that EdU persists in plant material during growth and that it could be used to track cell fate (Watson et al., 2016).

As mentioned above, the use of EU remains to be explored in plants, and here we suggest to implement EU as an *in situ* marker of plant nucleoli. The nucleolus delimits the nuclear territory of transcriptionally active and mostly de-condensed ribosomal genes, corresponding to approximately 1 Mb in *A. thaliana* (Pruitt and Meyerowitz, 1986; Beven et al., 1995; Pontvianne et al., 2013). More traditional methods to label the plant nucleolus implement tagging of specific nucleolar proteins by fluorescent tags or raising antibodies against nucleolar proteins (e.g., Pendle et al., 2005; Pontvianne et al., 2007; Chandrasekhara et al., 2016). In addition, due to its relatively low DNA density the nucleolus does not stain well with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and it appears as a black hole inside the nucleus. Thus, the visualization of the nucleolus using EU is a relatively easy and fast approach, advantageous over many more demanding protocols and time-consuming protocols.

MATERIALS AND METHODS

WT-Col0 plants and plants expressing fibrillarin-YFP (kindly provided by F. Pontvianne, CNRS, Perpignan, France) were used. Plants were grown on ½ Murashige–Skoog (MS; Duchefa 0255) plates with 1% plant agar (Duchefa) and 1% sucrose. The growth conditions were: 8 h/16 h light/dark, 22°C, and light intensity 100 μmol m^{-2} s^{-1}.

EU Labeling

Two types of EU were used in this study, product CLK-N002-10 (Jena Bioscience, 200 mM in sterile water) and E-10345 (Life Technologies, 100 mM in DMSO). Four days old *A. thaliana* seedlings were transferred into 12-well plates (Greiner Bio-One). Each well contained 2 ml of the liquid growth medium (½ MS). When CLK-N002-10 product was used, 20 µl of DMSO was added to the media to keep the same conditions as for the E-10345 product. Although DMSO is not required for efficient EU labeling, when the product E-10345 (diluted in DMSO) is used, seedlings are always exposed to 1% DMSO. Thus, when developmental studies or long EU incubation are performed, DMSO should be kept as low as 0.1% to avoid its potential side effects on the root growth (Shibasaki et al., 2009; Zhang et al., 2016). In such cases, the use of CLK-N002-10 product (diluted in water) is recommended. Alternatively, product E-10345 can be prepared as 0.5 M stock solution to decrease the DMSO content in the media.

Seedlings were labeled by adding 10 µM, 50 µM, 500 µM, or 1 mM EU into the liquid growth medium and incubated for the required time. The incubation was performed avoiding direct light.

Fixation

5-Ethynyl uridine-labeled seedlings were fixed in freshly made 4% formaldehyde/1× PBS/0.5% Triton X-100 solution for 20 min, followed by 4% formaldehyde/1× PBS/1% Triton X-100 for an additional 25 min. The first 2 min of fixation was performed under vacuum (0.7 bar) in a plastic desiccator (Kartell). The 1× PBS buffer contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4. Formaldehyde solution (8%) was made by dissolving 0.8 g of paraformaldehyde (Sigma P6148) in 10 ml of distilled water containing 100 µl of 1 M NaOH and heated up to 60°C in the exhaust hood; the pH was then adjusted to 8.0 and the solution was filtered using Whatman filter paper. After fixation, seedlings were washed for 1 × 10 min in 1× PBS, 1 × 10 min in 1× PBS/135 mM glycine, and 10 min in 1× PBS, and proceeded directly to the click-iT reaction.

Click iT Reaction

5-Ethynyl uridine-labeled and fixed seedlings were incubated with 500 μl–1 ml of click iT mixture containing 1× PBS, 4 mM $CuSO_4$, 5 μM AF488 azide (Thermo Fisher Scientific, A10266), and 40 mM sodium ascorbate (Applichem A5048.0100, freshly prepared as a 400 mM solution and added into the click iT mixture at the required amount). The reaction was incubated for 1 h at room temperature in the dark and followed by two 5 min washes in 1× PBS. Alternatively, an Alexa Fluor 488 picolyl azide 488 toolkit (Thermo Fisher Scientific C10641) was used instead of the Alexa Fluor 488 azide protocol. This toolkit was developed to avoid quenching of fluorescent molecules caused by free copper present in the click iT reaction. It employs picolyl azide instead of azide and a protected copper solution. We efficiently used the picolyl azide provided in the C10641 kit as well as picolyl azide sulfo Cy5 (Jena Bioscience, CLK-1177). The reaction mixture was prepared according to the manufacturer's protocol, using a copper:protected copper ratio of 1:1.

DAPI Staining

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, 1 mg ml^{-1}; Serva) was added to the seedlings after performing the click iT reaction to a final concentration of 1 μg $ml^{-1}$and incubated overnight in the refrigerator in the dark. The excess of DAPI was removed by two washes in 1× PBS. Shorter incubation with DAPI is recommended when the overnight incubation produces a high background noise.

Nuclei Preparation

Overnight EU-labeled root tips were excised and fixed in freshly made ice-cold ethanol:acetic acid (3:1) fixative for 24 h. The fixative was exchanged once during this time. Roots were then washed 1 × 5 min in distilled water, 2 × 5 min in 10 mM citrate buffer (4 mM citric acid and 6 mM sodium citrate, pH 4.5), and digested by a mixture of cellulase (Onozuka R10, Serva 16419.03), pectolyase (Duchefa, P8004.0001), and cytohelicase (Sigma, C8274), 0.3% (w/v) each in 10 mM citrate buffer, for 25 min at 37°C. Digested root tips were washed once in citrate

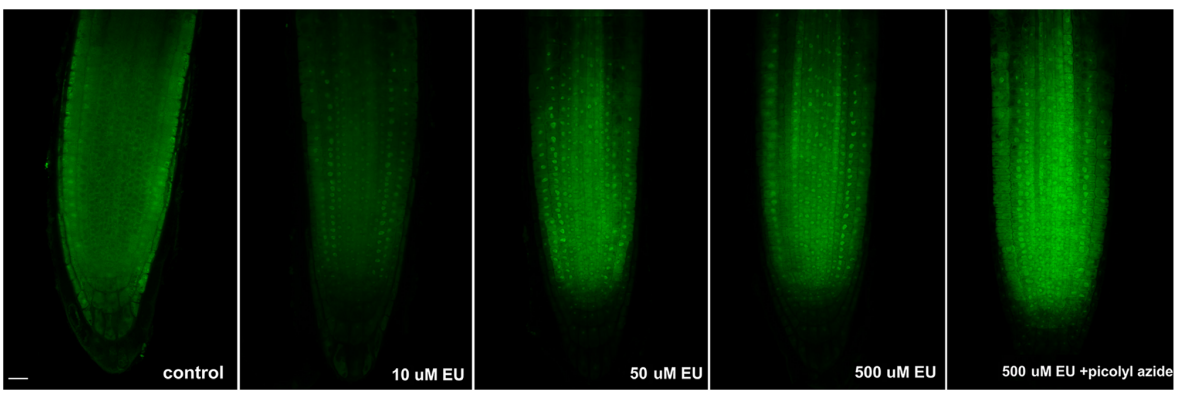

FIGURE 1 | Long EU pulses. *Arabidopsis* 4 days old seedlings were incubated overnight avoiding direct light with an increasing concentration of EU (Jena Bioscience) and EU-containing RNA was detected by the click iT reaction. The last image in the row shows the result of a modified detection protocol using AF488 picolyl azide instead of AF488 azide. Confocal sections in the middle part of the root are shown. Bar = 10 μm.

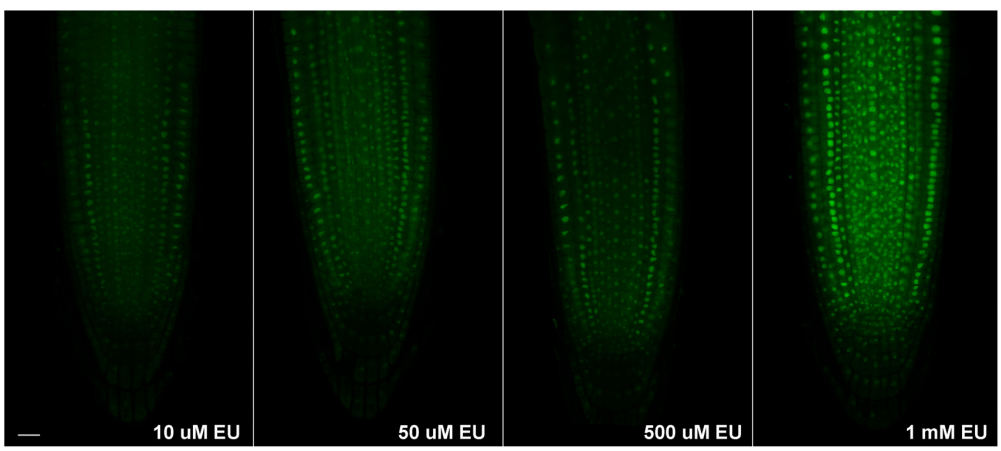

FIGURE 2 | Short EU pulses. *Arabidopsis* 4 days old seedlings were incubated avoiding direct light for 2 h with an increasing concentration of EU (Jena Bioscience) and EU-containing RNA was detected by the click iT reaction. Confocal sections in the middle part of the root are shown. Bar = 10 μm

buffer and transferred to slides. After complete removal of the citrate buffer, root tips were squashed in a drop of 50% acetic acid. Cover slips were removed in liquid nitrogen, and slides were re-fixed in fresh ethanol:acetic acid fixative and air dried. The click iT reaction to detect EU by fluorescence was performed as described above, 200 μl of click iT mixture was applied on each slide. Slides were then washed 3 × 5 min in 1× PBS and stained with DAPI in Vectashield (1 μg ml^{-1}, Vector Laboratories, H100).

Actinomycin D Treatment

To inhibit transcription, Actinomycin D (ActD, Sigma, A1410, 1 mg ml^{-1} in DMSO) was added to the ½ MS/0.5% sucrose liquid medium in 6-well plates (Greiner Bio-One) to a final concentration of 25 μg ml^{-1}. Four days old seedlings were incubated for 2 h with ActD, then for 2 h with 1 mM EU, and processed as stated above.

Microscopy

Root tips were transferred onto slides with a drop of water, covered with coverslips, and imaged on a Zeiss LSM780 confocal microscope using a 40× C-Apochromat/1.20 W objective and *Z*-stacks of 1.0–1.4 μm step size, pinhole 66–68 μm. Alternatively, a Zeiss Axioimager Z1 with filters corresponding to DAPI and AF488 excitation and emission spectra (AHF Analysentechnik[1]) was used. Image processing was done in ImageJ[2].

RESULTS AND DISCUSSION

EU Labeling of Nucleolar Processes

Visualization of Nucleolar Transcription

The majority of RNA transcripts in the plant nucleus correspond to the rRNA genes. RNA polymerase I, the enzyme responsible for rRNA transcription inside the nucleolus, can be efficiently blocked by ActD leading to the re-distribution of nucleolar proteins and nucleolar fragmentation (Yung et al., 1990; Chen and Jiang, 2004). Efforts to detect rRNA synthesis by qPCR after ActD treatment are biased, likely due to pleiotropic effects of ActD on other RNAs including transcripts of the reference genes, as discussed (e.g., Turner et al., 2012). Here, we present an assay to detect rRNA transcription *in situ* using 5-EU and test the protocol on ActD-treated seedlings.

Overnight EU Labeling

Initially, EU was applied on 4 days old seedlings at different concentrations (10, 50, and 500 μM), incubated overnight, and detected by the click iT reaction (**Figure 1**). The fixation step in the protocol included incubation of seedlings with higher concentration of Triton X-100 (compared to standard protocols) to facilitate the penetration of the click iT components into the nucleolus. All labeling pulses showed a similar labeling pattern, and a small round area inside the nucleus was observed in each cell, as expected for a nucleolar signal (**Figure 1**). A better signal-to-noise ratio was achieved when lower EU concentrations were used, probably indicating that an excess of EU contributes to the background noise signal or that the signal becomes re-distributed. The presence of cytoplasmic signal was also observed in other tested species after long incubation likely reflecting the RNA dynamics in the cell (Jao and Salic, 2008).

We next tested whether the modified version of the click iT reaction using picolyl azide and protected copper (instead of azide and reactive copper species) that is required when quenching has to be inhibited (e.g., in double labeling protocols including fluorescently tagged proteins or flow cytometry) was similarly efficient in EU detection. As shown in **Figure 1**, the modified click iT reaction produced satisfactory signal intensity similar to the standard click iT detection method. This shows that in addition to visualization of the nucleolus, the method could be efficiently used, e.g., in flow cytometry or for double labeling protocols. Since the size of nucleoli differs in different cell types, the protocol could be further exploited to measure, for example, the size of the nucleoli. Also, in combination with fluorescence-activated cell sorting, rDNA transcription can be further evaluated at the single cell level.

Short EU Pulses

To allow for detection of ongoing transcription, shorter EU pulses were necessary. Thus, the EU labeling was repeated again with three different concentrations of EU (10, 50, and 500 μM) and the labeling time shortened to 2 h. While

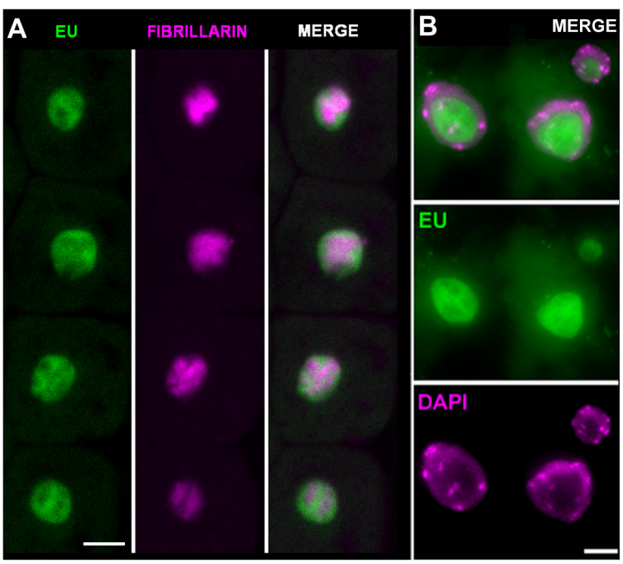

FIGURE 3 | Ethynyl uridine-containing RNA accumulates in the nucleolus. **(A)** *Arabidopsis* seedlings expressing fibrillarin-YFP (magenta) were labeled with 1 mM EU (Invitrogen, green) for 2 h and EU-containing RNA was detected by the click iT reaction. Selected sections from confocal *Z*-stacks are shown. Bar = 5 μm. **(B)** Cytological spread of EU (green) and DAPI (magenta) labeled nuclei from root tip incubated avoiding direct light with EU overnight. The detailed nucleolar structure is detectable. Bar = 5 μm.

[1]http://www.ahf.de/

[2]http://imagej.nih.gov/ij/

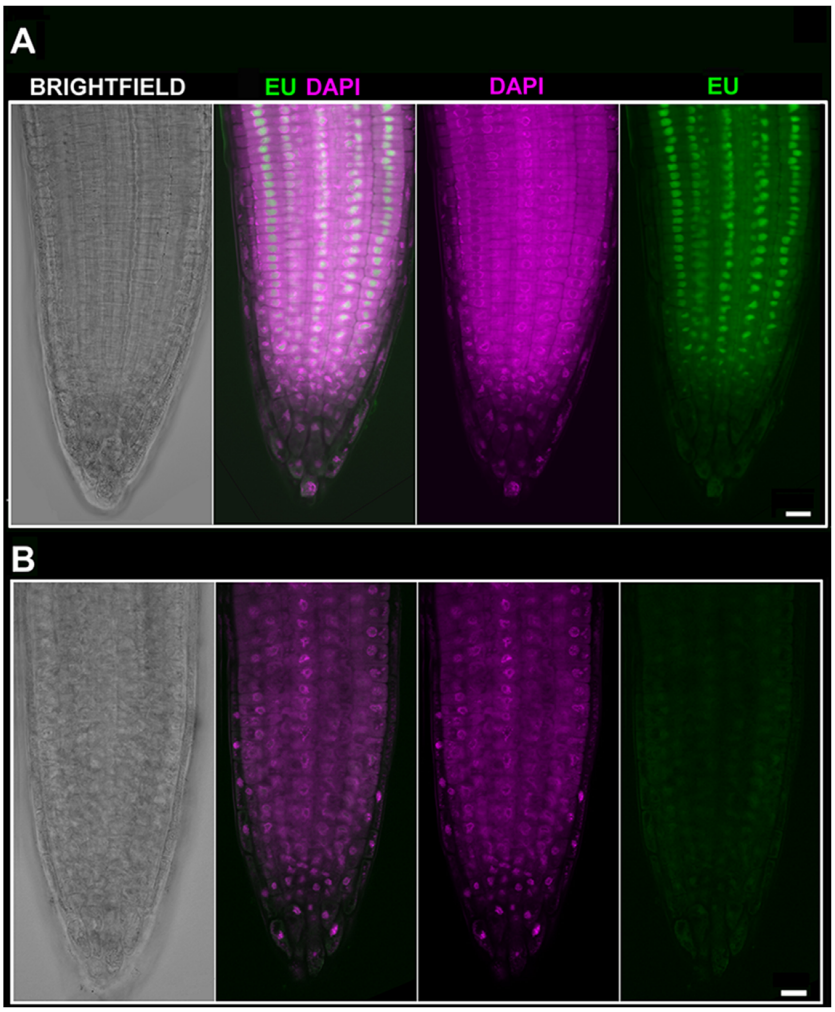

FIGURE 4 | EU signal diminishes after Actinomycin D (ActD) treatment. **(A)** *Arabidopsis* 4 days old seedlings were incubated without pre-treatment for 2 h with 1 mM EU (Invitrogen, green) avoiding direct light, detected by click iT reaction and stained with DAPI (magenta). Confocal sections in the middle part of the root are shown. Bar = 10 μm. **(B)** *Arabidopsis* 4 days old seedlings were treated for 2 h with ActD prior to EU labeling. DAPI (magenta), EU (green). Selected sections from confocal *Z*-stacks are shown. Bar = 10 μm.

10 μM EU produced a rather weak signal, 50 and 500 μM EU were brighter (**Figure 2**). The signal-to-noise ratio, however, was not satisfactory and needed improvement. Finally, the optimal concentration for short EU pulses was set to 1 mM (**Figure 2**) which provided the expected result. The EU signal appeared not only in the root tip, but also in some leaf cells as shown in Supplementary Figure 1. Since the signal was not seen in hypocotyls, it is likely that leaves can absorb EU via stomata.

To confirm in more detail where the observed EU signal accumulates, two additional experiments were performed. First, fibrillarin-YFP expressing plants were EU labeled and signal overlap between the fibrillarin and EU was assessed (**Figure 3A**). Second, double EU/DAPI was applied along with EU labeling (**Figure 3B**). The best DAPI signal was achieved by overnight incubation of fixed seedlings with a low DAPI concentration (1 μg ml^{-1}) followed by two washes with 1× PBS.

Both experiments show that the majority of the detectable signal occurs in the nucleolus, and the DAPI staining confirmed that signal in the nucleoplasm cannot be detected. The nucleolar signal does not entirely overlap with fibrillarin. In fact, it is largely accumulated in areas with a lower fibrillarin density and expands outside the area delimited by the fibrillarin. This EU distribution seems to reflect compartmentalization of the processes in the nucleolus. Pre-rRNA is mostly transcribed at the periphery of the fibrillar centers, while fibrillarin occurs in the dense fibrillar component (see e.g., Jordan, 1984; Ochs et al., 1985; Beven et al., 1995; de Carcer and Medina, 1999; Raska et al., 2006; Montanaro et al., 2011).

It is interesting that a similar labeling pattern is achieved using short or long EU pulses and that the EU signal is detectable exclusively inside the nucleolus and in its vicinity. These results are contrary to the EU pattern observed in cell cultures, where nuclear signal is detected along with strong nucleolar labeling

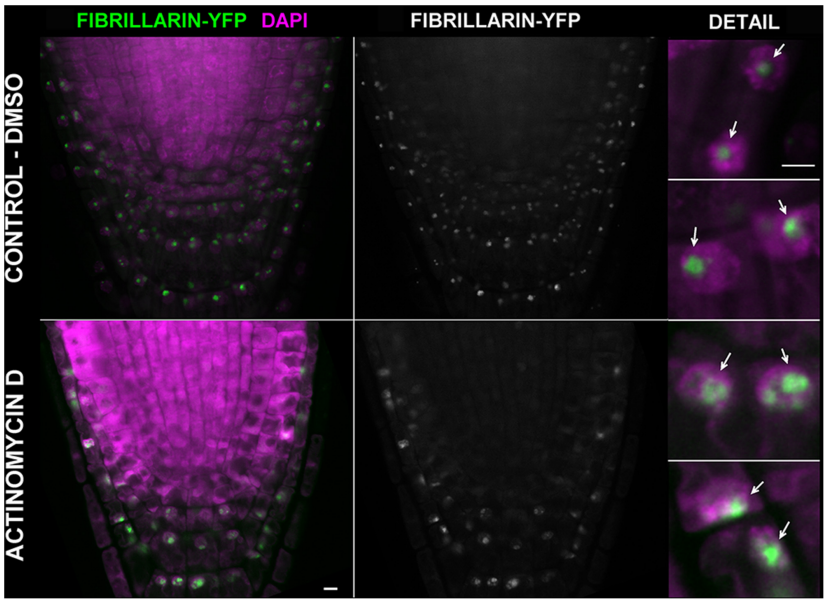

FIGURE 5 | Actinomycin D causes re-distribution of fibrillarin. *Arabidopsis* 4 days old seedlings expressing fibrillarin-YFP (green) were treated with 25 μg ml^{-1} of ActD for 2 h (the bottom panel). In the upper panel, a control incubated in the solvent (2.5% DMSO) is shown. Roots were fixed, stained with DAPI (magenta), and imaged on a confocal microscope. Selected sections from confocal *Z*-stacks are shown. Arrows point to the nucleoli. Bar = 5 μm.

(Jao and Salic, 2008). Also, the same study reported that the rRNAs are labeled with a lower efficiency, while labeling of poly(A)-containing mRNAs was more profound. In our *in situ* experiments, it seems that the rRNA fraction is the only labeled RNA. This might suggest a relatively high turnover of labeled RNA in *Arabidopsis* cells, or a sensitivity issue in the protocol. It may be necessary to amplify fluorescent signal by biotin–streptavidin system or antibodies to reveal remaining RNA transcripts. Also, during the fixation, higher amounts of Triton X-100 were used, which could possibly cause the re-distribution of nuclear RNAs into the cytoplasm. It is possible that signal would be more stabilized if the detection is performed on isolated nuclei instead of the whole root, requiring stronger permeabilization step in the protocol. We confirmed that RNA turnover was very fast by pulse-chase experiment. When we incubated seedlings for 2 h in EU followed by 3 or 6 h incubation in EU-free ½ MS medium, no signal was detected (Supplementary Figure 2). Although decreased stability of EU-containing RNA in plant tissue has not been reported, it could not be completely neglected. We observed, for example, that when EU-labeled material is stored, after some time the signal diminishes, but when similar material is stored after EdU labeling, this phenomenon does not occur. We also tested whether light could affect the EU stability. However, incubation of seedlings in dark or light does not seem to have any strong effect on EU labeling (Supplementary Figure 3).

Actinomycin D Blocks Nucleolar Transcription

We next asked whether inhibition of transcription could be monitored by using EU. The effect of ActD on RNA pol I which further changes the nucleolar structure has been long known (Unuma et al., 1972; Kramer, 1980). Thus, we treated *Arabidopsis* seedlings with ActD prior to the EU incubation. When EU was added after 2 h of ActD treatment, no RNA transcripts were detected indicating that ActD acted in the expected manner (**Figure 4**). To further characterize the effect of ActD on plant nucleoli, nucleolar integrity was monitored by fibrillarin-YFP after ActD treatment (**Figure 5**). After 2 h of ActD, fibrillarin started to re-localize from the nucleolus, confirming the sensitivity of the nucleolus to this drug as well as proving that the EU signal corresponds to the nascent RNA transcripts.

As we demonstrate here, labeling of the nucleolus by 5-EU represents a reliable protocol applicable to monitor nucleolar transcription directly in the root tip. The protocol can be used to track potential rRNA transcription inhibitors or rRNA metabolism under various stress conditions. Due to the elevated protein density inside the nucleolus the procedure requires relatively high detergent concentrations. In addition, high EU concentrations are required when shorter EU pulses are used. Thus, possible side effects need to be considered in each experimental set-up.

CONCLUSION

Developments in microscopic approaches and their combinations with tissue- and cell-type-specific labeling and nuclei sorting allow for description of previously unknown details of key cellular processes *in situ* or *in vivo* at a high spatiotemporal resolution. This new knowledge is obtained at the cost of three factors: the increasing complexity of experiments, the high cost of instrumentation, and the need for careful optimization of methods for a given purpose and model system.

While the first two factors can be efficiently managed in current well-established research centers, optimization remains challenging and the most time-consuming part of these experiments. Therefore, we describe here the optimized approach to visualize transcription in nucleoli of *A. thaliana in situ* to share this experience with the plant science community.

AUTHOR CONTRIBUTIONS

MD designed the experiments and performed the optimization of the protocol and all experiments. JF and MD contributed to the concept of the project and data interpretation, prepared the manuscript, and co-supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00177/full#supplementary-material

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