**Short Communication**

**ACTIN-RELATED PROTEIN8 Encodes an F-Box Protein Localized to the Nucleolus in Arabidopsis**

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**Arabidopsis** encodes six nuclear actin-related proteins (ARPs), among them ARP8 is unique in having an F-box domain and an actin homology domain. Analysis of the **ARP8** promoter–β-glucuronidase (GUS) fusion suggests that **ARP8** is ubiquitously expressed in all organs and cell types. Immunocytochemical analysis with **ARP8**-specific monoclonal antibodies revealed that **ARP8** protein is localized to the nucleolus in interphase cells and dispersed in the cytoplasm in mitotic cells. The cell cycle-dependent subcellular patterns of distribution of **ARP8** are conserved in other members of Brassicaceae. Our findings provide the first insight into the possible contributions of plant **ARP8** to nucleolar functions.

Keywords: **ARP8** — Chromatin remodeling — Nuclear actin-related proteins — Nuclear functions — Ribosome biogenesis.

Abbreviations: ARP, actin-related protein; DAPI, 4',6-diamidino-2-phenylindole; GUS, β-glucuronidase; HAT, histone acetyltransferase; HVE, histone variant exchange; HVE complexes; and histone acetyltransferase (HAT) complexes. The NR and HVE complexes all contain a DNA-dependent ATPase subunit and use the energy of ATP hydrolysis to drive the repositioning of nucleosomes on DNA or to exchange one histone type for the other within nucleosomes. The HAT complexes, on the other hand, acetylate the N-terminal tails of histone proteins and thereby alter the chromatin structure indirectly (Meagher et al. 2007).

No single specific role has been defined for nuclear ARPs within chromatin remodeling complexes (Olave et al. 2002, Meagher et al. 2007). Although the nuclear ARPs are highly divergent from actin, they are still predicted to maintain the actin fold, which is comprised of two protein domains held together by a hinge region, creating a flexible nucleotide-binding pocket. In actin, this region undergoes a relatively large conformational change upon nucleotide binding. ATP hydrolysis or ATP/ADP exchange, which can stimulate the polymerization and depolymerization of F-actin. Hence, based on this potential ‘lock and key’ shift in structure, it has been proposed that the nuclear ARPs may aid in the assembly or modulation of chromatin remodeling complexes (Sunada et al. 2005). Moreover, yeast ARP4 and ARP8 have been shown to bind directly to core histones (Harata et al. 1999, Shen et al. 2003), thus ARPs might be required for targeting the ARP-containing complexes to the chromatin. ARPs may also play a role in stimulating core ATPase activity in NR and HVE complexes (Meagher et al. 2007).

**Arabidopsis** **ARP8**, which is the subject of the present study, shows 30 and 29% amino acid identity to yeast actin.
and Arabidopsis ACT2 in the regions of alignment, respectively (Supplementary Table S1). The presence of large regions of poor conservation in the Arabidopsis ARP8 sequence obscures its phylogenetic relationships to the other known ARPs. Because it is not closely related to yeast or human ARP8 and shows similar weak homology to yeast ARP8 and ARP9, the Arabidopsis ARP8 is considered a plant-specific orphan ARP. Moreover, Arabidopsis ARP8 has a complex gene structure encoding a novel protein with distinct F-box and actin homology domains (McKinney et al. 2002). To understand the function of this unique protein, herein we have characterized its pattern of expression using an ARP8 promoter–β-glucuronidase (GUS) reporter fusion, and examined the subcellular localization using ARP8-specific antibodies. We have shown that unlike other known eukaryotic nuclear ARPs, Arabidopsis ARP8 is localized to the interphase nucleolus, suggesting a role for ARP8 in nucleolar functions.

The ARP8 gene sequence in Arabidopsis comprises 12 exons and encodes a protein of 471 amino acids (Fig. 1A). In addition to the requisite actin-related (A) domain of 381 amino acids, the Arabidopsis ARP8 protein has unique additional domains, including an N-terminal 40 amino acid hydrophobic leader (L) and a 50 amino acid F-box (F) homology domain (Fig. 1B). Neither of which are found in fungal or animal ARP8 or other nuclear ARPs. The genomes of the evolutionarily distant dicot grape (Vitis vinifera) and monocot rice (Oryza sativa) also encode a similarly organized ARP8 homolog with 65% and 63% amino acid identity to the Arabidopsis sequence, respectively (see Supplementary Fig. S1 and Table S1). Thus, this unusual gene structure is ancient and likely pre-dating the split between monocots and dicots almost 200 million years ago. However, an ARP8 homolog is absent from the genomes of the moss Physcomitrella and green alga Chlamydomonas, suggesting that it may be specific to angiosperms. Transcripts resulting from an alternative shorter splice variant (representing exons 1–6 and exon 12) encoding a 242 amino acid protein are found in Arabidopsis flowers, but not leaves or other cDNA libraries (McKinney et al. 2002). However, this truncated ARP8 protein is not detectable on Western blots of flower or inflorescence samples, which suggests it may not be functional or may be only very weakly expressed.

Previous analysis of mRNA levels suggested that ARP8 was expressed at low levels in most plant organs (McKinney et al. 2002, Zimmermann et al. 2004). To refine our knowledge about the organ- and tissue-specific expression of ARP8, we analyzed the activity of ARP8 regulatory sequences and ARP8 protein. When transgenic plants expressing an ARP8pt::GUS fusion were incubated in X-glucuronide substrate, blue staining from GUS activity was observed in all organs and tissues at various stages of development, as shown for representative plant samples in Fig. 2. In particular, strong expression was observed in the cotyledons and hypocotyls of young seedlings (Fig. 2A) as well as in developing and mature rosette leaves and roots of relatively older seedlings (Fig. 2B, C). The reporter gene expression in the root was strongest in vascular tissues and weakest in root tips (Fig. 2A). Moreover, root hairs and trichomes showed positive staining (not shown). GUS expression was also observed in most floral organs and pollen (Fig. 2D–F). Staining was relatively weak in ovules, ovary and petals (Fig. 2D, F, G). Soon after fertilization, the developing seeds revealed strong GUS staining and the mature embryos were also positive for ARP8 expression (Fig. 2G, H).

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**Fig. 1**  ARP8 gene structure and antibody specificity. (A) ARP8 gene with exons and flanking sequence and the location of ARP8-N and ARP8-C peptides. Introns are not drawn to scale. (B) ARP8 protein with leader [L], F-box [F] and actin-homology [A] domains. (C) Western blots showing the reaction of MAbARP-N and MAbARP-C antibodies with ARP8 in Arabidopsis (A. th.) plant extracts. A Coomassie-stained gel of ARP8r is shown to the right. (D) Immunoreactivity of the MAbARP-N and MAbARP-C antibodies to recombinant ARP8 (ARP8r) and ARP8 in Arabidopsis (A. th.) plant extracts. A Coomassie-stained gel of ARP8r is shown to the right. (E) Immunoreactivity of the MAbARP-N and MAbARP-C antibodies to recombinant ARP8 (ARP8r) and ARP8 in Arabidopsis (A. th.) plant extracts. A Coomassie-stained gel of ARP8r is shown to the right. (F) Immunoreactivity of the MAbARP-N and MAbARP-C antibodies to recombinant ARP8 (ARP8r) and ARP8 in Arabidopsis (A. th.) plant extracts. A Coomassie-stained gel of ARP8r is shown to the right.
To examine ARP8 protein expression, we prepared monoclonal antibodies to ARP8 N-terminal and C-terminal peptides (Fig. 1A). These two antibodies, MAbARP8-N and MAbARP8-C, each reacted with the full-length 52 kDa recombinant ARP8 protein expressed in *Escherichia coli* and endogenous ARP8 of identical molecular weight in *Arabidopsis* plant extracts (Fig. 1C). Although MAbARP8-C reacted strongly with recombinant ARP8, it reacted relatively weakly with ARP8 protein in plant samples. Thus, MAbARP8-N was used for further analysis of tissue-specific ARP8 expression. MAbARP8-N detected ARP8 protein in all vegetative and reproductive organs examined including seedlings, roots and siliques, although higher concentrations were observed in developing flower buds and flowers within the inflorescence (Fig. 3Q, upper panel). Equal loading of total protein was confirmed by re-probing the blot with a polyclonal antibody specific for the constitutively expressed 110 kDa phosphoenolpyruvate carboxylase (PEPC; Fig. 3Q, lower panel). To confirm the ubiquitous but differential levels of ARP8 expression in vegetative and reproductive tissues, we examined the expression of its homolog in a relatively distant crucifer, *Brassica*. As in *Arabidopsis*, MAbARP8-N detected higher levels of the *Brassica* ARP8 homolog of 52 kDa in the inflorescence, compared with relatively low levels in root samples (Fig. 4A). Thus, the differential expression of ARP8 is conserved in *Arabidopsis* and *Brassica*, and its ubiquitous presence in all organs is identical to the other nuclear ARPs (ARP4, ARP6 and ARP7) characterized in plants (Kandasamy et al. 2003, Deal et al. 2005).

Plant ARP8 was classified as a potential nuclear protein based on its phylogenetic affinity for other known nuclear ARPs and its significant divergence from cytoplasmic ARPs and actin (McKinney et al. 2002, Kandasamy et al. 2004). Moreover, most of the previously characterized divergent ARP proteins of other eukaryotes, including yeast and humans, have been localized to the nucleoplasm of interphase cells. Because *Arabidopsis* ARP8 is unique in having an F-box domain, we wanted to examine whether it shows a similar subcellular distribution to other diverse nuclear ARPs or whether it may have a different subcellular localization and possibly have different function. We therefore immunolabeled fixed and dissociated *Arabidopsis* root cells with MAbARP8-N antibody. Interestingly, our immunolabeling revealed intense staining of the root cell nucleolar region within nuclei as revealed by the merged images of antibody labeling and 4',6-diamidino-2-phenylindole (DAPI) staining (Fig. 1D, left panel). The nucleolar localization of ARP8 was confirmed by labeling cells with MAbARP8-N monoclonal antibody (Fig. 1D, middle panel), which also showed a strong staining of the nucleolus. Occasionally a few cells showed faint staining in the nucleoplasm surrounding a rather densely stained nucleolus (see the upper middle panel in Fig. 1D). Apparently, ARP8 labeling with both the antibodies did not reveal any substructure in the nucleolus. To determine whether the nucleolar accumulation of ARP8 is conserved in *Brassica*, we immunolabeled root cells from *B. napus* (Fig. 4B–E) and *B. campestris* (not shown) with MAbARP8-N, which showed the same restriction of ARP8 staining to the nucleolar compartment.
A more thorough analysis of the nucleolar expression patterns was undertaken with MAbARP8-N to examine all major organs and tissues in *Arabidopsis*, shown in Fig. 3. Mature cortical root cells (Fig. 3A–C) and a file of young root apical cells (Fig. 3D–F) showed strong nucleolar staining in all interphase cells, except for the few apical initials adjacent to the quiescent center that contain very small nucleoli. In mitotic cells at metaphase, when the nuclear membrane is broken down, ARP8 staining was dispersed into the cytoplasm and not associated with the chromosomes (Fig. 3G). This cytoplasmic diffusion is very similar to the *Arabidopsis* nuclear ARP4 and ARP7 localization during metaphase and anaphase (Kandasamy et al. 2003), but contrasts sharply with human ARP8, which has recently been shown to be associated with mitotic chromosomes (Aoyama et al. 2008). However, during early telophase stage, ARP8 appeared associated with the chromatin, although there was still no distinct demarcation of a nucleolar region (Fig. 3G, H). A similar cell cycle-dependent subcellular distribution of plant ARP8 protein was also observed in *Brassica* (Fig. 4F–H). In developing *Arabidopsis* mono-nucleate microspores, ARP8 was clearly observed in the nucleolus (Fig. 3I, J). However, in trinucleate pollen, ARP8 was associated with one or more nucleolar regions of the vegetative nucleus, but no staining...
was visible in the two sperm cell nuclei (Fig. 3K, L). In the polyploid and sometimes multinucleate tapetal cells, MAbARP8-N reacted with several small nucleoli in each cell (Fig. 3M–P). Thus, plant ARP8 is localized to the nucleolar compartment within the interphase nucleus in essentially all cell types.

The nucleolus is the ribosomal factory where rRNAs are synthesized, processed and assembled with ribosomal proteins to form the ribosome subunits that are then exported to the cytoplasm (Olson et al. 2000, Hernandez-Verdun 2006). Specific complexes participate in and accomplish these different steps by interacting with the rDNA in the nucleolar organizing regions (NORs) or with rRNA. In Arabidopsis there are two active NORs located on chromosomes 2 and 4 with several hundred rDNA genes each (Copenhaver and Pikaard 1996). Considering that the nuclear ARPs function as components of various chromatin-modifying complexes and Arabidopsis ARP8 is localized to the nucleoli, it is reasonable to speculate about possible roles for ARP8 in nucleolar chromatin remodeling complexes involved in the epigenetic activation and/or silencing of the rDNA genes. This assumption is particularly appealing, because of the tight regulation of both ARP8 and rDNA activities. Although chromatin remodeling closely controls the replication timing and transcription of the rDNA genes (Grummt and Pikaard 2003, Li et al. 2005), no ARP has been identified to date as part of any nucleolar chromatin remodeling complex (e.g. NoRC). Moreover, proteomic analyses of nucleoli from humans and Arabidopsis have failed to identify any nuclear ARP component (Scherl et al. 2002, Brown et al. 2005). Thus, plant ARP8 is the first candidate ARP with the potential to participate in the epigenetic control of rDNA activity.

Moreover, the angiosperm ARP8 protein sequences contain an F-box motif upstream of the actin homology domain. The F-box motifs generally function as a site of protein–protein interaction and thus may link the resident proteins (e.g. ARP8) to the target proteins such as a ubiquitin–ligase complex for ubiquitin tagging and subsequent proteolytic degradation. F-box-mediated protein degradation has been linked to cell growth, aging and the cell cycle in diverse organisms (Itoh et al. 2003, Dong et al. 2006). In addition, F-box proteins also participate in ubiquitin tagging of histones in the nucleus, which is required for gene silencing (de Napoles et al. 2004, Baarends et al. 2005). Thus, ARP8 may have important nuclear functions, with the F-box domain contributing to its tight regulation at the level of protein turnover and/or to ubiquitination of histones, which may control gene regulation at rDNA loci.

In conclusion, we have shown that the novel plant ARP8 protein is strongly and ubiquitously expressed in all actively growing and differentiating organs and tissues. We have clearly demonstrated with two distinct monoclonal antibodies that ARP8 protein is localized to the nucleolar compartment(s) within the nucleus. Based on its unique structure and subcellular distribution, we infer that plant ARP8 has important roles in the epigenetic regulation of nucleolar genes.

Materials and Methods

To generate ARP8::GUS construct, we replaced the ADF8 promoter region in the previously characterized ADF8::GUS construct with a 1,104 bp ARP8 promoter sequence (Ruzicka et al. 2007). For recombinant ARP8 protein expression, the full-length ARP8 coding region was amplified from a flower cDNA library (Invitrogen, Carlsbad, CA, USA), cloned into the pET-15b vector (Novagen, Madison, WI, USA) and then the protein was expressed from this vector in E. coli as described earlier (McKinney et al. 2002).

Arabidopsis ecotype Columbia plants were grown at 22 °C with 16 h light and 8 h dark periods. Plants were transformed with the ARP8::GUS construct by vacuum infiltration, and the transgenic plants were selected on MS medium containing 35 μg ml⁻¹ kanamycin.

ARP8-specific monoclonal antibodies were prepared to 25 amino acid N-terminal (ARP8-N, amino acids 2–26, ILKKVWG SVWNRSNSGKDLVNHQRA) and C-terminal (ARP8-C, amino acids 447–471, SNLSIFPGPWCITRKQFRRKSRMLW) multiple antigenic peptides, as described earlier (Li et al. 2001). For preparation of protein samples, various plant organs were ground in a high salt extraction buffer [20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol and protease inhibitor cocktail (Roche, Mannheim, Germany)]. After centrifugation, the pellet proteins were extracted with sample buffer (Laemmli 1970) and assayed by Western blotting as described previously (Kandasamy et al. 2003). The recombinant ARP8 protein was prepared by boiling the bacterial pellets directly in sample buffer.

Immunocytochemistry was performed on fixed and dissociated cells as explained previously (Kandasamy et al. 2003). GUS histochemical staining and microscopic analysis of ARP8::GUS expression was performed as described earlier (Deal et al. 2005).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

Funding

The National Institutes of Health (GM 36397-21).

Acknowledgments

We are grateful to Gay Grayson and Dan Ruzicka for critical reading of the manuscript.

862 Plant nucleolar ARP8 protein
References


(Received February 7, 2008; Accepted March 27, 2008)