# Molecular Cloning and Targeting of a Fibrillarin Homolog from Arabidopsis[1]

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Fibrillarin is a nucleolar protein known to be involved in the processing of ribosomal RNA precursors. We isolated *AtFbr1*, a cDNA encoding a homolog of fibrillarin in Arabidopsis. The cDNA is 1.2 kb in size and encodes a polypeptide of 310 amino acid residues with a molecular mass of 33 kD. *AtFbr1* is expressed at high levels in the flower and root tissue and at a slightly lower level in leaf tissue, whereas it was nearly undetectable in siliques. Expression of *AtFbr1* was compared with that of the *FLP* (fibrillarin-like protein) gene identified by the Arabidopsis genome project. Abscisic acid treatment resulted in the down-regulation of the expression of both *AtFbr1* and *FLP* genes in seedlings, although the degree of suppression was higher for *FLP* than for *AtFbr1*. In addition, the expression level of *FLP* decreased with the age of the seedlings, whereas *AtFbr1* did not exhibit any detectable change. The subcellular localization of AtFbrl was studied with an in vivo targeting approach using a fusion protein, and was found to be correctly targeted to the nucleolus in protoplasts when expressed as a green fluorescent fusion protein (GFP). Deletion experiments showed that the N-terminal glycine- and arginine-rich region is necessary and sufficient to target AtFbr1 to the nucleolus.

The nucleolus is a large structure visible in the interphase nucleus. It is the site of transcription of ribosomal DNA (rDNA), processing and modification of pre-ribosomal RNA (pre-rRNA), and the biogenesis of pre-ribosomal particles (for review, see Hadjiolov, 1985; Scheer and Venavente, 1990). The nucleolus contains a number of nucleolar proteins that function in the assembly of the ribosome, as well as in the maintenance of the structure of the nucleolus (Goessens, 1984). These proteins are associated with small nucleolar RNAs in the form of small nucleolar ribonucleoprotein complexes. One of nucleolar proteins involved in pre-rRNA processing is the most abundant protein in the dense fibrillar component of the nucleolus and has thus been named fibrillarin (Ochs et al., 1985). Fibrillarin, a basic protein of $M_r$ 34,000 to 38,000, had been originally identified as B-36 in the nucleolus of the slime mold *Physarum polycephalum*, but appears to be widespread among all organisms in nature, including mammalian cells (Christensen et al., 1977; Lischwe et al., 1985; Ochs et al., 1985; Guiltinan et al., 1988). In humans, fibrillarin is associated with the U3, U8, and U13 snRNAs, which all contain the consensus sequence elements C (UGAUGA/U) boxes and D (CUGA) boxes (Tyc and Steitz, 1989; Smith and Steitz, 1997). Recently, it has been shown that in a majority of the fibrillarin-associated C/D boxes, small nucleolar RNAs function as guide RNAs in the site-specific Rib methylation of rRNAs (Bousquet-Antonelli et al., 1997).

cDNA sequences have been determined for *Saccharomyces cerevisiae* (Schimmang et al., 1989; Henriquez et al., 1990), *Xenopus* (Lapeyre et al., 1990), human (Aris and Blobel, 1991), and *Tetrahymena* (David et al., 1997) fibrillarin. The amino acid sequences and predicted secondary structures are highly conserved among the eukaryotic fibrillarins. Most of them contain a motif rich in Gly and Arg called the GAR domain, and a consensus RNA recognition motif (RRM). Similar domains also occur in other nucleolar proteins such as nucleolin (Lapeyre et al., 1987), GAR1 (Girard et al., 1992), NSR1 (Lee et al., 1991), and SSB1 (Jong et al., 1987).

*S. cerevisiae* has a gene encoding fibrillarin that is called NOP1. Its product is essential for cell growth and required for the modification and processing of pre-rRNA (Schimmang et al., 1989; Henriquez et al., 1990). Jansen et al. (1991) demonstrated the functional conservation of fibrillarin by complementation of a yeast NOP1-disrupted strain with either the human or the *Xenopus* gene. These observations suggest that fibrillarin must have been highly conserved between species with regard to both structure and function throughout evolution. It has also been shown that antibodies against fibrillarins recognize proteins of 36 to 37 kD in plants, suggesting that fibrillarin homologs are present in plant cells (Cerdido and Medina, 1995).

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To understand the biological role of fibrillarin in plant cells, we attempted to clone a plant homolog from Arabidopsis. We report the cloning of a cDNA encoding a homolog of fibrillarin in Arabidopsis and our findings regarding the targeting mechanism of the protein to the nucleolus.

## RESULTS

### Cloning of Arabidopsis Fibrillarin cDNA

We isolated a fibrillarin homolog from Arabidopsis using the PCR approach with degenerate primers designed based on the highly conserved C-terminal region of fibrillarin. PCR was carried out using total cDNA isolated from a λZAP II leaf cDNA library and degenerate primers. Amplified PCR products were subcloned into pBluescript and the sequence of the inserts determined. One of the PCR products was 333 bp long, the expected size based on the known sequences of other fibrillarin cDNAs, and appeared to encode a fibrillarin-like protein. We therefore decided to isolate the full-length cDNA that corresponded to the 333-bp PCR product by screening the λZAP II leaf cDNA library using the 333-bp PCR product as a hybridization probe. Positive λ-clones were excised as pBluescript clones. The cloned cDNA, named *AtFbr1*, had an insert of 1.2 kb and an open reading frame for a protein of 310 amino acids, which calculates to a molecular mass of 33 kD (Fig. 1). The calculated pI of 10.5 pointed to a basic protein.

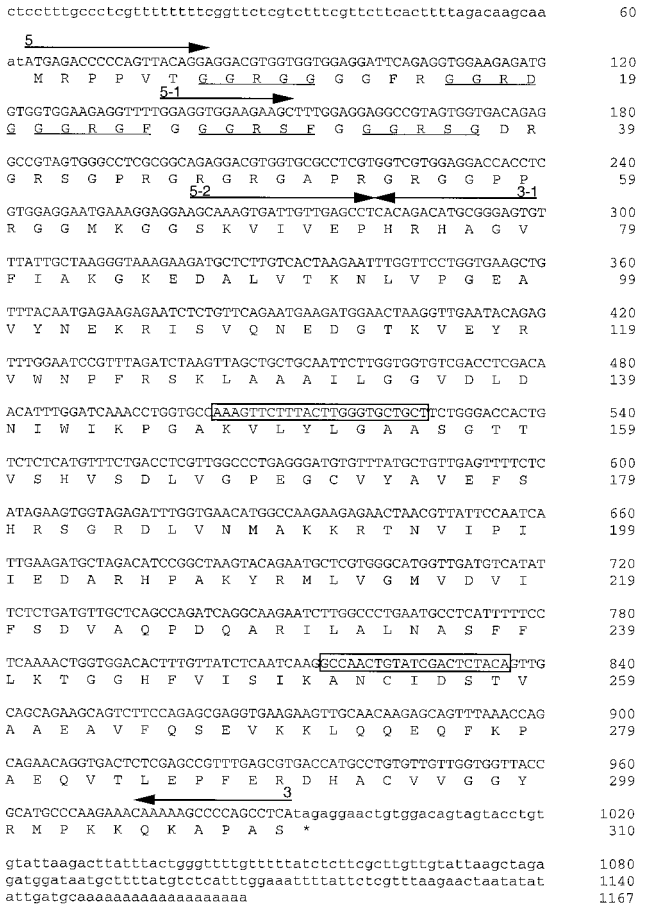


**Figure 1.** Nucleotide and deduced amino acid sequence of the fibrillarin cDNA from Arabidopsis (*AtFbrl*). Boxed sequences denote the target sequences for the oligonucleotides used in the PCR cloning procedure. The primers used in the construction of various deletion mutants are indicated by arrows. The consensus sequences of the GAR domain are underlined. The nucleotide sequence of the AtFbrl cDNA was deposited in GenBank with accession no. AF187871.

### Sequence Analysis of *AtFbr1*

The deduced amino acid sequence of *AtFbr1* revealed the typical structure of fibrillarins and fibrillarin-like proteins: a Gly- and Arg-rich N-terminal region of approximately 70 amino acids bounded by Pro residues. The N-terminal region contains the consensus sequence GGR(G/D/S)(G/F), which is repeated five times. The putative RNA-binding motif (GCVYAVCF) is present in the middle of the molecule, implying that this is a functional homolog of fibrillarin in Arabidopsis. The deduced amino acid sequence of AtFbr1 was compared with protein sequences obtained from the public databases using the BLAST program (Altschul et al., 1990). As shown in Figure 2, AtFbr1 shares a high degree of amino acid sequence homology with known fibrillarins of a variety of organisms. The C-terminal region, in particular, exhibits a higher degree of amino acid sequence similarity to other fibrillarin homologs, as in the case with a number of other fibrillarin and fibrillarin-like proteins. Overall, AtFbrl shares 88% amino acid sequence similarity with the Arabidopsis fibrillarin-like protein (accession no. CAB43694), 69% with human fibrillarin (accession no. A38712), 67.5% with *Xenopus* (accession no. P22232), and 65% with yeast fibrillarin (accession no. P15646).

### Genomic Organization of Fibrillarin Genes in Arabidopsis

To examine the genomic structure of *AtFbr1*, we performed Southern-blot analysis using the entire cDNA insert of *AtFbr1* as the hybridization probe. One strongly hybridizing band was observed, together with several minor bands in each digest under high-stringency hybridization conditions (data not shown). To get a better idea of the copy number of the gene, a 150-bp fragment from the 3′-untranslated region of *AtFbrl* was used as the hybridization probe. As shown in Figure 3A, this specific probe detected only one band in each digest, indicating that the *AtFbrl* gene is a single-copy gene, as represented by this cDNA. The BLAST search with the nucleotide sequence of the *AtFbrl* gene found a similar gene encoding FLP in the Arabidopsis genome. As shown in Figure 3B, hybridization analysis with a gene-specific probe for *FLP* showed only one band in each digest. These bands were identical to those weakly

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AtFbrl          --MRPPVT------GGRGGGGFRGGR----DGGGR---GF-------GGG   28
Fbrl-A.thaliana --MRPP1T------G--sGGGFsGGRgrggysGGRgdgGF-------sGG   33
Fbrl-human      --MkPgfsprgggfGGRGGfGdRGG------rGGR--gGFgggrgrgGGf   40
Fbrl-X.laevis   --MRPgfspr----GGRGGfGdRGG------fGGR--gGF----gdrGGf   32
Fbrl-yeast      msfRPgsr-ggsrgGsRGGfGgRGGsrggarGGSR--gGFggrggsrGGa   47

AtFbrl          RSPGGGRS--GDRGRS---GPRG--RG------RGAPR-----GR--GGP   58
Fbrl-A.thaliana Rg-GGGRg--GgRGfSd-rGgRG--RG------RGpPRggargGR--Gpa   69
Fbrl-human      R--GrGRgg-GggGgggggGgRGg--Ggfhs---GgnR---grGR----g   75
Fbrl-X.laevis   R--GGsRggfGgRGRGgdrGgRGgfRGgfsspgRGgPR---ggGRggfGg   77
Fbrl-yeast      R--GGsRg--GfgGRG---GsRG---G-----aRGgsR----gGR---Gg   75

AtFbrl          PRGGMKGGSKVIVEPHRHAGVFIAKGKEDALVTKNLVPGEAVYNEKRISV   108
Fbrl-A.thaliana gRGGMKGGSKVIVEPHRHAGVFIAKGKEDALVTKNLVPGEAVYNEKRISV   119
Fbrl-human      gkrGnqsGknVmVEPHRHeGVFIcrGKEDALVTKNLVPGEsVYgEKRvSi   125
Fbrl-X.laevis   gRGGfgaGrKVIVEPHRHeGiFIcrGKEDALVTKNLVPGEsVYgEKRISV   127
Fbrl-yeast      aaGGarGGaKVviEPHRHAGVyIArGKEDlLVTKNmaPGEsVYgEKRISV   125

AtFbrl          QNEDG------TKVEYRVWNPFRSKLAAAILGGVDLDNIWIKPGAKVLYL   152
Fbrl-A.thaliana QNEDG------TKtEYRVWNPFRSKLAAAILGGV--DNIWIKPGAKVLYL   161
Fbrl-human      segD-------dKiEYRaWNPFRSKLAAAILGGV--DqIhIKPGAKVLYL   166
Fbrl-X.laevis   edge-------vKtEYRaWNPFRSKiAAAILGGV--DqIhIKPGvKVLYL   168
Fbrl-yeast      eepskedgvppTKVEYRVWNPFRSKLAAgImGGV--DelfIaPGkKVLYL   173

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AtFbrl          GAASGTTVSHVSDLVGPEGCVYAVEFSHRSGRDLVNMAKKRTNVIPIIED   202
Fbrl-A.thaliana GAASGTTVSHVSDLVGPEGCVYAVEFSHRSGRDLVNMAKKRTNVIPIIED   211
Fbrl-human      GAASGTTVSHVSDiVGPdGlVYAVEFSHRSGRDLiNlAKKRTNiIPvIED   216
Fbrl-X.laevis   GAASGTTVSHVSDvVGPEGlVYAVEFSHRSGRDLiNvAKKRTNiIPvIED   218
Fbrl-yeast      GAASGTsVSHVSDvVGPEGvVYAVEFSHRpGReLisMAKKRpNiIPIIED   223

AtFbrl          ARHPAKYRMLVGMVDVIFSDVAQPDQARILALNASFFLKTGGHPVISIKA   252
Fbrl-A.thaliana ARHPAKYRMLVGMVDVIFSDVAQPDQARILALNASyFLKsGGHPVISIKA   261
Fbrl-human      ARHPhKYRMLiaMVDVIFaDVAQPDQtRIvALNAhtFLrnGGHFVISIKA   266
Fbrl-X.laevis   ARHPhKYRiLVGMVDVvFaDVAQPDQtRIvALNAhnFLKnGGHFVISIKA   268
Fbrl-yeast      ARHPqKYRMLiGMVDcvFaDVAQPDQARIiALNshmFLKdqGgvVISIKA   273

AtFbrl          NCIDSTVAAEAVFQSEVKKLQQEQFKPAEQVTLEPFERDHACVVGGY-RM   301
Fbrl-A.thaliana NCIDSTVpAEAVFQtEVKKLQQEQFKPAEQVTLEPFERDHACVVGGY-RM   310
Fbrl-human      NCIDSTasAEAVFaSEVKKmQQEnmKPqEQlTLEPyERDHAvVVGvY-Rp   315
Fbrl-X.laevis   NCIDSTaApEAVFaaEVKKmQQEnmKPqEQlTLEPyERDHAvVVGiY-Rp   317
Fbrl-yeast      NCIDSTVdAEtVFarEVgKLreEriKPlEQlTLEPyERDHciVVGrYmRs   323

AtFbrl          PKKQKAPAS-  310
Fbrl-A.thaliana PKKpKAataa  320
Fbrl-human      PpKvKn----  321
Fbrl-X.laevis   PpKQKk----  323
Fbrl-yeast      glKk------  327
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**Figure 2.** Alignment of the amino acid sequence of AtFbrl with other fibrillarin homologs. The deduced amino acid sequence AtFbrl was aligned with sequences obtained from public databases and refer to the following: Fbrl-Arabidopsis, Arabidopsis fibrillarin-like protein (accession no. CAB43694); Fbrl-human, human fibrillarin (accession no. A38712); Fbrl-X.laevis, *Xenopus* fibrillarin (accession no. P22232); and Fbrl-yeast, yeast fibrillarin (accession no. P15646). Gaps represented by dashes were introduced to produce the best match among the five species. Identical amino acid residues between AtFbrl and other homologs are shown in uppercase letters. Asterisks mark the RNA-binding consensus motif.

hybridizing bands detected by the full-length *AtFbr1*. These data therefore indicate that there are at least two genes encoding fibrillarin homologs in the Arabidopsis genome.

### Expression of the *AtFbr1* and *FLP* Genes

To examine the expression of the two fibrillarin genes in Arabidopsis, we performed northern-blot analyses using the specific probes for the two Arabidopsis fibrillarin homolog genes *AtFbr1* and *FLP*. Total RNA was extracted from various tissues, and RNA blots were prepared. As shown in Figure 4, *AtFbr1* was expressed at high levels in flower and root tissue and at a slightly lower level in leaf tissue, whereas it was nearly undetectable in siliques. The

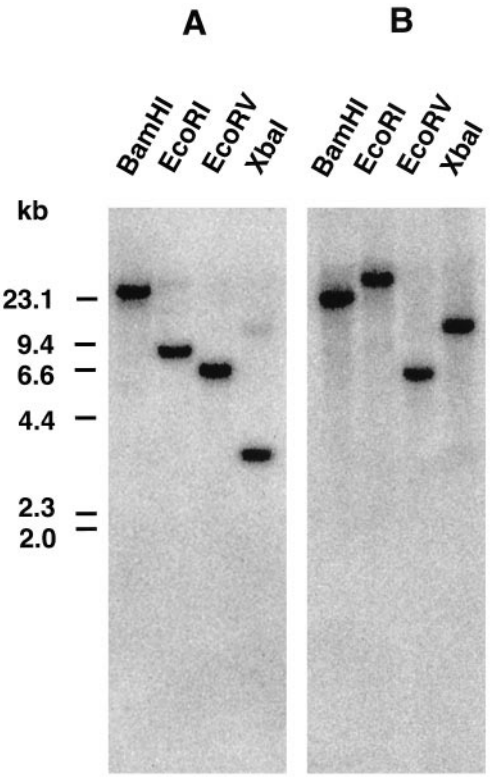


**Figure 3.** Southern-blot analysis of Arabidopsis genomic DNA. Five micrograms of genomic DNA digested with the indicated restriction enzymes was electrophoresed and transferred to a nylon membrane. Hybridizations were carried out with a 150-bp fragment from the 3′-non-coding region of *AtFbrl* (A) or *FLP* (B).

expression pattern of *FLP* was nearly identical to that of *AtFbr1* except that *FLP* was expressed overall at a slightly lower level than *AtFbr1*. The results therefore indicated that the expression of the two genes was spatially regulated.

We then examined the expression of the fibrillarin homologous genes in whole seedlings under various stress conditions to determine any functional differ-

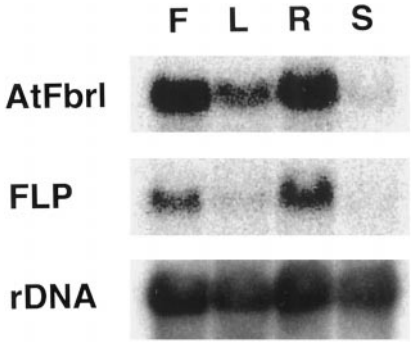


**Figure 4.** Organ-specific expression of two fibrillarin genes. Total RNA (15 $\mu$g) isolated from various tissues was size-fractionated in a formaldehyde-agarose gel and transferred onto a nylon membrane. Specific probes of *AtFbrl* and *FLP* were used as the hybridization probes. 18S rRNA was used to monitor the loading of the RNA samples. F, Flowers; L, leaves; R, roots; and S, siliques.

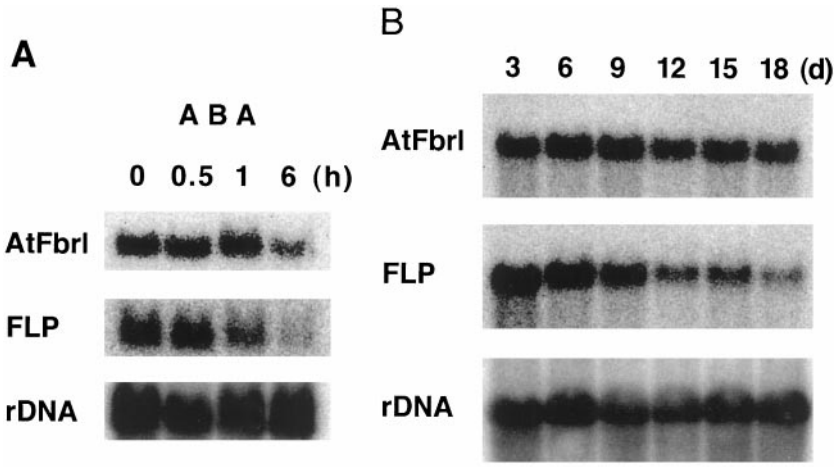


**Figure 5.** Induction of *AtFbrl* and *FLP* gene expression. A, Arabidopsis seedlings grown in MS liquid medium were treated with 100 $\mu$M abscisic acid for the indicated periods of time. B, Arabidopsis seedlings were planted on MS plates and harvested after the indicated number of days. Total RNA (15 $\mu$g) was analyzed by northern-blot analysis using the specific probes for *AtFbrl* and *FLP*. 18S rRNA was used to monitor equal loading of RNA samples.

ences. Both *AtFbrl* and *FLP* transcript levels in the seedlings decreased upon abscisic acid treatment, but the expression of *FLP* was much more affected than that of *AtFbr1* (Fig. 5A). However, other treatments such as NaCl and cold did not affect the expression of either gene (data not shown). We also examined temporal regulation of the expression of the genes. As shown in Figure 5B, the expression level of *FLP* in seedlings decreased with the age of the seedlings, while *AtFbrl* transcription remained at the same level. These results also suggest that the expression of *AtFbrl* and *FLP* is differentially regulated, and that transcription depends on environmental conditions and stage of development.

### Targeting of AtFbr1 to the Nucleolus

It has been widely observed that fibrillarin is localized at the nucleolus in all organisms examined. Furthermore, human fibrillarin is correctly targeted to the nucleolus in yeast. This suggests conservation of the targeting mechanism. To address the question of how AtFbr1 is targeted to the nucleolus, we took an in vivo approach using a GFP fusion protein. The *AtFbr1* cDNA was fused in-frame to the coding region of smGFP, and the resulting construct was introduced into Arabidopsis protoplasts by polyethylene glycol-mediated transformation (Shillito and Saul, 1988). As a control, a plain smGFP construct was also transformed into protoplasts (Fig. 6A). The protoplasts were then examined under a fluorescence microscope to look for the green fluorescent signal 24 h after transformation.

As seen in Figure 6B, a green fluorescent signal was confined to a small area within the nucleus of protoplasts transformed with the AtFbr1::smGFP fusion construct, whereas the green fluorescence in protoplasts transformed with the control smGFP was uniformly distributed throughout the cytosol. These results strongly suggested that the AtFbr1::smGFP fusion protein was correctly targeted to the nucleolus. To confirm this conclusion, the green fluorescent signal of AtFbr1::smGFP was compared with that of NLS::smGFP, a construct in which the SV40 nuclear localization signal is fused to the N terminus of the smGFP coding region (Goldfarb et al., 1986). As shown in Figure 6B, the protoplast transformed with the NLS::smGFP construct showed a green fluorescent signal covering an area that was much bigger than that seen with AtFbr1::smGFP. We also stained the protoplasts transformed with AtFbr1::smGFP with ethidium bromide (EtBr) to overlay the EtBr staining pattern with that of the green fluorescent signal of AtFbr1::smGFP. As shown in Figure 6B, the green fluorescent signal was confined to a small area within the EtBr-stained region. Thus, the results support the conclusion that AtFbr1 is correctly targeted to the nucleolus.

### The N-Terminal GAR Region Is Sufficient for Targeting

Since proteins that are translocated after translation often contain the targeting information as a sequence tag, we attempted to define the sequence necessary for the targeting of AtFbr1 to the nucleolus. For this purpose, various deletion constructs were generated from the *AtFbr1* cDNA and fused to the smGFP coding region, as depicted in Figure 7A. The deletion constructs were introduced into protoplasts, and fluorescent signals were examined with a fluorescence microscope. As shown in Figure 7B, the deletion constructs AtFbrl-dS/X, AtFbrl-d1, and AtFbrl-n79, but not AtFbrl-d2, correctly directed GFP

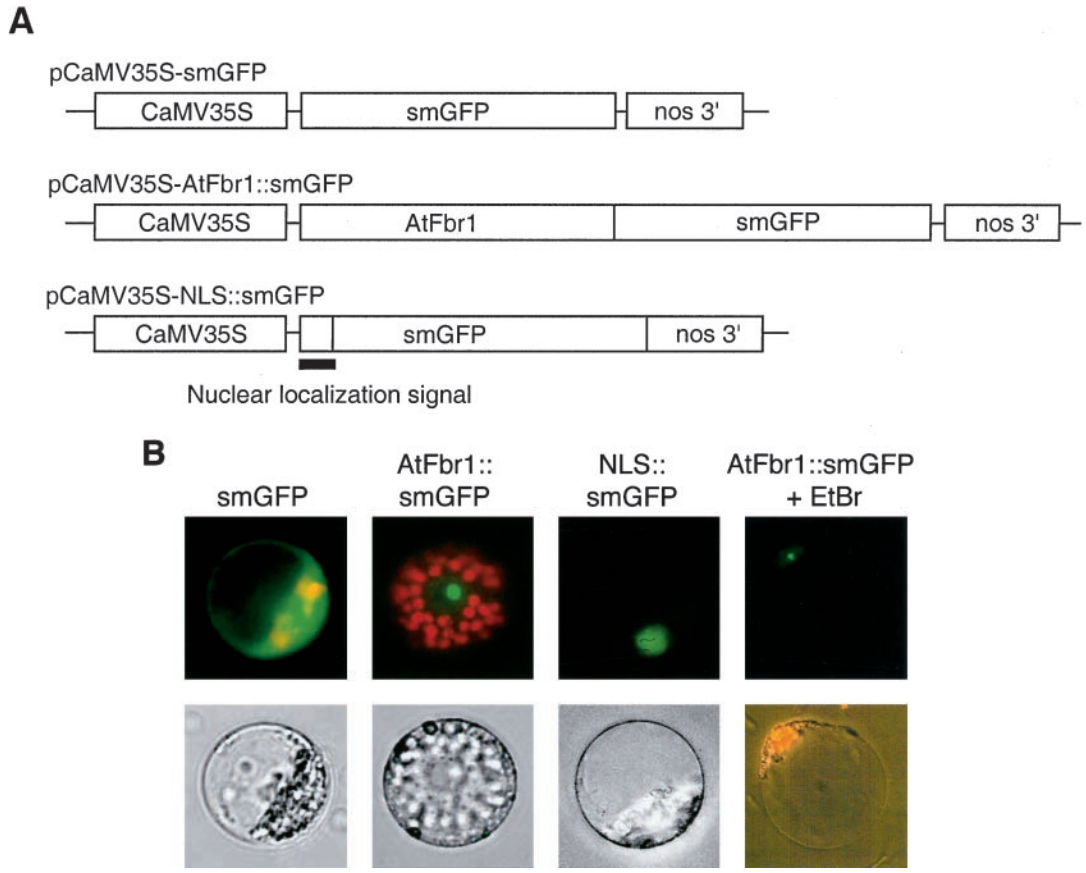


**Figure 6.** Targeting of AtFbr1 to the nucleolus. A, Schematic representation of the fusion constructs. The constructs AtFbr1::smGFP and NLS::smGFP contain the full-length AtFbr1 and SV40 nuclear localization signal, respectively. B, In vivo targeting of the GFP fusion proteins in protoplasts. The fusion constructs were introduced into Arabidopsis protoplasts prepared from whole seedlings grown in liquid MS medium for 1 to 2 weeks. The protoplasts were incubated at 22°C in the dark for 12 to 48 h. The panels AtFbrl::smGFP and NLS::smGFP show the fluorescence of the constructs transformed into protoplasts. The protoplast population contains protoplasts originated from root cells. Note that the red fluorescent signal in some protoplasts is chlorophyll. The panel AtFbr1::smGFP + EtBr shows protoplasts stained with EtBr (5 $\mu$g/mL) after transformation.

to the nucleolus, although some green fluorescent signal remained in the nucleus. Thus, the minimal region for targeting AtFbr1 seems to be is a region present in both the AtFbrl-d1 and the AtFbrl-n79 deletion constructs.

The common region present in both AtFbrl-d1 and AtFbrl-n79 contains part of the GAR domain at the N terminus. Detailed analysis of this GAR domain revealed that the region consists of a consensus sequence, GGR(G/D/S)(G/F), which is repeated five times. Since the common region present in both AtFbrl-d1 and AtFbrl-n79 has two copies of the consensus sequence, the results suggest that the N-terminal GAR region may function as the nucleolar targeting signal sequence for AtFbr1. However, in contrast to protoplasts transformed with the full-length AtFbr1, the green fluorescent signal in the protoplasts transformed with the deletion constructs was somewhat spread out through the nucleus, and the degree of spreading varied depending on the deletion construct. This observation suggests that some additional sequence may be necessary for the precise targeting of AtFbr1 exclusively to the nucleolus.

## DISCUSSION

Fibrillarin is a basic nucleolar protein involved in rRNA processing. Fibrillarin homologs have been isolated from various organisms ranging from yeasts to human, and they have been shown to be highly conserved at the level of the amino acid sequence. In addition, it has been shown that human fibrillarin can substitute for the yeast homolog in yeast cells, which further suggests functional conservation. We isolated a fibrillarin homolog, *AtFbr1*, from Arabidopsis. *AtFbr1* and the fibrillarin-like protein gene found by the Arabidopsis genome sequencing project together represent the two fibrillarin homologs of Arabidopsis. AtFbr1 is very similar to other fibrillarins and fibrillarin-like proteins found in other organisms with respect to its primary structure and organization: an N-terminal variable region with a

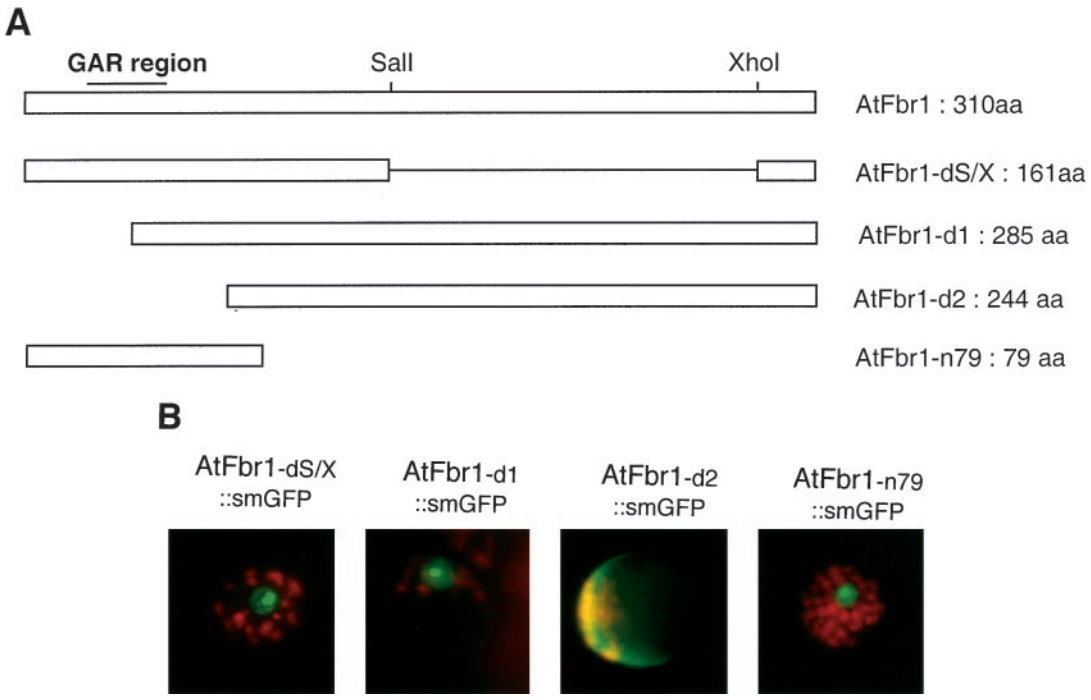


**Figure 7.** Targeting of smGFP fused to various deletion mutants of AtFbrl. A, Schematic representation of the deletion constructs. B, Protoplasts transformed with the various deletion constructs and their fluorescent signals observed through a fluorescence microscope.

high content of Gly and Arg and a highly conserved C-terminal region. As is the case with other fibrillarin and fibrillarin-like proteins, the N-terminal region of approximately 70 amino acids is rich in Gly residues, which are interspersed with Arg residues and bounded by Pro residues. Thus, the N-terminal region is similar to other fibrillarins, although the amino acid sequence is not exactly conserved.

Although fibrillarin is thought to be involved in the processing of rRNA precursor molecules in the nucleolus, the biochemical properties of the protein and its mechanism of action are still largely unknown. A prominent feature of fibrillarin is the N-terminal GAR domain. It has been observed that the GAR domain binds RNA non-specifically, destabilizing base pairing of the nucleotides. Based on these observations, it has been proposed that the GAR domain may function as a destabilizer of RNA secondary structure.

In this study we addressed the biological roles of FLPs in Arabidopsis. We first compared the expression patterns of the two FLP protein genes; *AtFbr1* was constitutively expressed in whole seedlings under the conditions at which we examined the expression, whereas the expression of *FLP* was regulated in response to environmental conditions and developmental stages. This raised the possibility that *AtFbrl* and *FLP* may be expressed in different cell types (e.g. *FLP* may be expressed in cells that are more sensitive to environmental stress and growth stages). Secondly, we investigated the targeting mechanism of AtFbr1 to the nucleolus. We used in vivo targeting using an AtFbr1::smGFP fusion protein and found that the full-length AtFbr1 was efficiently and correctly targeted to the nucleolus as a GFP fusion protein. To define the sequence necessary for this targeting, we analyzed various deletion constructs fused to the smGFP coding region. Interestingly, the N-terminal region of 79 amino acid residues, which contains the GAR domain with a few flanking amino acids, was both necessary and sufficient to direct GFP to the nucleolus. Furthermore, the deletion construct AtFbrl-d1, which contained two copies of the consensus sequence GGR(G/S/D)(G/F) of the GAR domain, also directed GFP to the nucleolus, whereas the deletion construct AtFbrl-d2, which was very similar to AtFbrl-d1 except for the additional deletion of the two copies of the consensus, failed to direct GFP to the nucleolus. The deletion construct AtFbrl-d2 has lost its nuclear localization signal in addition to the nucleolar one. These results strongly suggest that the consensus sequence GGR(G/D/S)(G/F) in the N-terminal GAR region is the targeting signal to the nucleolus. However, an additional sequence may be necessary to confine the targeted AtFbr1 protein to the nucleolus, since the green fluorescent signal diffused into other areas of the nucleus when deletion constructs were introduced into protoplasts rather than the whole protein sequence. One possibility is that the RNA-binding motif may function to hold fibrillarin into the nucleolus. It is also interesting that the location of the GAR domain is not important,

since similar domains have been found either in the center or in the C-terminal region in other nucleolar proteins. However, it is still not clear what the significance of the repetitive nature of the GAR domain is, since the AtFbrl-d1 construct, which contains only two copies of the consensus sequence, was sufficient to target GFP to the nucleolus when it alone was fused to the N terminus of smGFP. To explain fully the targeting mechanism of fibrillarin, it would be helpful to identify a protein factor that recognizes the GAR domain.

Based on this study, we propose that the GAR domain of fibrillarin may function as a nucleolar targeting signal. However, it is also possible that the GAR domain functions as an RNA-binding domain or has a function in protein-protein interaction, as has been proposed previously. Further studies are required to clarify the function of the GAR domain of fibrillarin.

## MATERIALS AND METHODS

### Plant Materials and Growth Conditions

Arabidopsis was grown either as seedlings in liquid Murashige and Skoog (MS) medium in a 250-mL flask under constant shaking at 20°C in a culture room or as plants in a greenhouse under the conditions of 70% relative humidity and a 16-/8-h light/dark cycle. Various parts of the plants were harvested and frozen immediately in liquid nitrogen for RNA preparations.

### cDNA Isolation

To isolate a cDNA encoding a homolog of fibrillarin from Arabidopsis, we designed degenerated primers (5′-AARGTNYTNTAYYTNGGNGCNGCN-3′ and 5′-NGTRGARTCRATRCARTTNGC-3′; R, A/G; Y, T/C; N, A/G/T/C) based on the nucleotide sequences of highly conserved regions of fibrillarin. PCR was carried out with the primers on DNA isolated from a λZAPII cDNA library template. One of the subcloned PCR products was of the expected length and appeared to encode a fibrillarin-like protein. To isolate a full-length cDNA clone, the Arabidopsis λZAPII cDNA library was screened under high-stringency conditions using this PCR product as a hybridization probe. Positive clones were in vivo excised as pBluescript clones, and the inserts were sequenced. Sequence analysis was performed with the BLAST sequence analysis program. We also isolated the *FLP* (fibrillarin-like protein) gene by PCR using as primers 5′-ATGAGACCTCCTCTAACTGG-3′ and 5′-CTAAGCAGCAGTAGCAGCCT-3′, which were designed based on the reported nucleotide sequence of *FLP*. The PCR product was confirmed by sequencing.

### Northern- and Southern-Blot Analyses

RNA was isolated from various tissues of soil-grown plants, except for root RNA, which was prepared from plants grown in liquid culture as described previously (Ausubel et al., 1989). Aliquots of total RNA from each tissue (15 μg) were separated in a 1.2% (w/v) formaldehyde-agarose gel and transferred onto a nylon membrane (Hybond-N+, Amersham-Pharmacia Biotech, Uppsala). After transfer, the RNA was UV cross-linked to the membrane and the blot used in northern analysis. For Southern-blot analysis, genomic DNA was prepared following a previously described protocol (Watson and Thompson, 1986). The genomic DNA (5 μg) was digested with restriction endonucleases. Hybridization and washings were carried out according to a published procedure (Church and Gilbert, 1984).

### Generation of Fusion Constructs

Various constructs for in vivo targeting experiments were generated using standard recombinant DNA technology (Sambrook et al., 1989). A full-length *AtFbr1* without the termination codon was prepared by PCR amplification using primer 5 (5′-GGCTGCAGATGAGACCCCCAGTTACAGG-3′) and primer 3 (5′-CTGGATCCTTGAGGCTGGGGCTTTTTG-3′) and fused to the N terminus of smGFP (soluble-modified green fluorescent protein; Davis and Vierstra, 1998). Deletion mutant AtFbrl-d1 was PCR amplified using primer 5-1 (5′-GCTGCAGATGGGAGGTGGAAGAAGC-3′) and primer 3, and deletion mutant AtFbrl-d2 was amplified using primer 5-2 (5′-GGCTGCAGATGAGCAAAGTGATTGTTGAGCCT-3′) and primer 3. For deletion mutant AtFbrl-n79, encoding the N-terminal 79 amino acids of AtFbrl, primer 5 and primer 3-1 (5′-GGATCCGGCACTCCCGCATGTCTGTG-3′) were used in PCR amplification. The deletion mutants were then fused to the N terminus of smGFP. Deletion mutant AtFbrl-dS/X was constructed by removing 149 amino acid residues from the C-terminal region of the AtFbr1::smGFP fusion construct utilizing the restriction sites *Sal*I and *Xho*I.

### In Vivo Targeting of GFP Fusion Constructs

Protoplasts were prepared according to the method of Abel and Theologis (1994) from 1- to 2-week-old whole Arabidopsis seedlings grown in liquid MS medium at 20°C, a 16-/8-h light/dark cycle, and under constant shaking (100 rpm) in a rotary shaker. The protoplasts were transformed with DNA as described previously (Negrutiu et al., 1987). Plasmid DNAs for transformation were purified with a column according to the manufacturer's protocol (Qiagen USA, Valencia, CA). The protoplasts were incubated at 22°C in the dark for 12 to 48 h after transformation. The green fluorescent signal was monitored using a fluorescent microscope (Axioplan 2, Zeiss, Jena, Germany). Images were processed using an automatic imaging system (FISH, Carl Zeiss).

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## LITERATURE CITED

**Abel S, Theologis A** (1994) Transient transformation of Arabidopsis leaf protoplasts: a versatile experimental system to study gene expression. Plant J **5:** 421–427

**Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ** (1990) Basic local alignment search tool. J Mol Biol **215:** 403–410

**Aris JP, Blobel G** (1991) cDNA cloning and sequencing of human fibrillarin, a conserved nucleolar protein recognized by autoimmune antisera. Proc Natl Acad Sci USA **88:** 931–935

**Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K** (1989) Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley-Interscience, New York

**Bousquet-Antonelli C, Henry R, Glunge J-P, Caizergues-Ferrer M, Kiss T** (1997) A small nucleolar RNP protein is required for pseudouridylation of eukaryotic ribosomal RNAs. EMBO J **16:** 4770–4776

**Cerdido A, Medina FJ** (1995) Subnucleolar location of fibrillarin and variation in its levels during the cell cycle and during differentiation of plant cells. Chromosoma **103:** 625–634

**Christensen ME, Beyer AL, Walker B, LeStourgeon WM** (1977) Identification of $N^G$,$N^G$-dimethylarginine in a nuclear protein from the lower eukaryote *Physarum polycephalum* homologous to the major proteins of mammalian 40S ribonucleoprotein particles. Biochem Biophys Res Commun **74:** 621–629

**Church GM, Gilbert W** (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

**David E, McNeil JB, Basile V, Pearlman RE** (1997) An unusual fibrillarin gene and protein: structure and functional implications. Mol Biol Cell **8:** 1051–1061

**Davis SJ, Vierstra RD** (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. Plant Mol Biol **36:** 521–528

**Girard J-P, Lehtonen H, Caizergues-Ferrer M, Amalric F, Tollervey D, Lapeyre B** (1992) GAR1 is an essential small nucleolar RNP protein required for pre-rRNA processing in yeast. EMBO J **11:** 673–682

**Goessens G** (1984) Nucleolar structure. Int Rev Cytol **87:** 107–158

**Goldfarb DS, Gariepy J, Schoolnik G, Kornberg RD** (1986) Synthetic peptides as nuclear localization signals. Nature **322:** 641–644

**Guiltinan MJ, Schelling ME, Ehtesham NZ, Tomas JC, Christensen ME** (1988) The nucleolar binding protein B-36 is highly conserved among plants. Eur J Cell Biol **46:** 547–553

**Hadjiolov AA** (1985) The nucleolus and ribosome biosynthesis. *In* M Alfert, W Bermann, L Goldstein, KR Porter, P Sitte, eds, Cell Biology Monographs, Vol 12. Springer-Verlag, New York, pp 1–268

**Henriquez R, Blobel G, Aris JP** (1990) Isolation and sequencing of NOP1: a yeast gene encoding a nucleolar protein homologous to a human autoimmune antigen. J Biol Chem **265:** 2209–2215

**Jansen RP, Hurt EC, Kern H, Lehtonen H, Carmo-Fonseca M, Lapeyre B, Tollervey D** (1991) Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast. J Cell Biol **113:** 715–729

**Jong AY, Clark MW, Gilbert M, Oehm A, Campbell JL** (1987) *Saccharomyces cerevisiae* SSB1 protein and its relationship to nucleolar RNA-binding proteins. Mol Cell Biol **7:** 2947–2955

**Lapeyre B, Bourbon H, Amalric F** (1987) Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. Proc Natl Acad Sci USA **84:** 1472–1476

**Lapeyre B, Mariottini P, Mathieu C, Ferrer P, Amaldi F, Almaric F, Caizergues-Ferrer M** (1990) Molecular cloning of *Xenopus* fibrillarin, a conserved U3 small nuclear ribonucleoprotein recognized by antisera from humans with autoimmune disease. Mol Cell Biol **10:** 430–434

**Lee W-C, Xue Z, Mélèse T** (1991) The NSR1 gene encodes a protein that specifically binds nuclear location sequences and has RNA recognition motifs. J Cell Biol **113:** 1–12

**Lischwe MA, Ochs RL, Reddy R, Cook RG, Yeoman LC, Tan EM, Reichlin M, Busch H** (1985) Purification and partial characterization of a nucleolar scleroderma antigen (Mr = 34,000; pI, 8.5) rich in $N^G$,$N^G$-dimethylarginine. J Biol Chem **260:** 14304–14310

**Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F** (1987) Hybrid gene in the analysis of transformation conditions. Plant Mol Biol **8:** 363–373

**Ochs RL, Lischwe MA, Spohn WH, Busch H** (1985) Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. Biol Cell **54:** 123–134

**Sambrook J, Fritsch EF, Maniatis T** (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Scheer U, Venavente R** (1990) Functional and dynamic aspects of the mammalian nucleolus. Bioassay **12:** 14–21

**Schimmang T, Tollervey D, Kern H, Frank R, Hurt EC** (1989) A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. EMBO J **8:** 4015–4024

**Shillito RD, Saul MW** (1988) Protoplast isolation and transformation. *In* CH Shaw, ed, Plant Molecular Biology: A Practical Approach. IRL Press, Oxford, pp 161–186

**Smith CM, Steitz JA** (1997) Sno storm in the nucleolus: new role for myriad small RNPs. Cell **89:** 669–672

**Tyc K, Steitz JA** (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. EMBO J **8:** 3113–3119

**Watson JC, Thompson WF** (1986) Purification and restriction endonuclease analysis of plant nuclear DNA. Methods Enzymol **118:** 57–75