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Original Paper

Expression and subcellular localization of pre-rRNA processing factor homologues in higher plants

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Abstract *Rpf2* (Ribosome Production Factor 2) is involved in ribosome biogenesis, and functions as one of the pre-rRNA processing factors. Although properties of the Rpf2 protein in yeast have been described in detail, there have been no reports on *Rpf2* homologues in higher eukaryotes. To investigate the function of *Rpf2* homologues in higher plants, we identified genes encoding Rpf2-like proteins from *Arabidopsis* and rice and designated them *AtRpf2* and *OsRpf2*, respectively. The predicted amino acid sequence revealed that both genes contain the Brix domain, a characteristic domain associated with ribosome biogenesis, and a sigma 70-like RNA binding motif, a eukaryotic RNA binding domain unique to a superfamily of proteins required for ribosome biogenesis. Both gene transcripts were detected in all organs and tissues tested and the expression patterns were consistent with the typical expression pattern of previously described ribosomal protein genes. Transient expression of GFP::AtRpf2 and GFP::OsRpf2 revealed predominant nucleolar localization within the nucleus of both fusion proteins in onion epidermal cells and tobacco BY-2 cells. These results suggest that Rpf2-like proteins in higher plants are also involved in ribosome biogenesis and function as pre-rRNA processing factors.

Key words: Nucleolar protein, ribosome biogenesis, Rpf2, rRNA processing.

Ribosomes are responsible for translating the RNA into proteins and essential for all living organisms. Eukaryotic ribosome, composed of 40S and 60S subunits containing four rRNAs and about 80 ribosomal proteins, is synthesized mainly in the nucleolus through many processes (Warner 1999; Woolford and Warner 1991). In *Saccharomyces cerevisiae*, three of four rRNAs are transcribed as a single 35S pre-rRNA and are cleaved to the 25, 18, and 5.8S rRNA sequences. During rRNA processing, ribosomal proteins are transported into the nucleolus and are assembled with rRNAs to form preribosomal particles. Many of the steps in ribosome biogenesis have been studied in yeast, and most of these mechanisms are conserved throughout evolution (Kressler et al. 1999; Venema and Tollervey 1999). However, the function of each factor has not been elucidated. Previous studies have identified *Rpf2* (Ribosome Production Factor 2) as an essential gene for growth in *S. cerevisiae*. The Rpf2 protein is a pre-rRNA processing factor responsible for the processing of 27Spre-rRNA to 25SrRNA in ribosome biogenesis and interacts with Rrs1, an essential nuclear protein of 203 amino acids with an important function in the maturation of 25S rRNA in *S. cereviseae* (Morita et al. 2002). Rpf2 family proteins show similarity to the Imp4 protein, a specific component of the U3 snoRNP, and constitute the Imp4 superfamily in combination with Imp4, Rpf1, Brx1 and Ssf1 protein family. All of the Imp4 superfamily member proteins possess a 17-amino acid consensus sequence that constitutes a sigma-70 like RNA binding motif (Wehner and Baserga 2002).

In Arabidopsis and tobacco, previous studies indicated that disruption or down-regulation of the ribosomal protein gene is associated with various morphological abnormalities, but the mechanisms involved in phenotypic changes have not been fully clarified (Van Lijsebettens et al. 1994; Williams and Sussex 1995; Ito et al. 2000; Weijeres et al. 2001; Nishimura et al. 2005). On the other hand, there are few reports available concerning the rRNA processing factors in plants (Brown and Shaw 1998).

To investigate the functions of rRNA processing factors in plants, we conducted a series of experiments using *Rpf2* homologous genes from *Arabidopsis* and rice because of the availability of the genome sequence information and their versatility. Here we report some results on the database analysis, cDNA isolation and partial characterization of the Rpf2 homologues from

Abbreviations: DAPI, 4′,6-Diamidino-2-phenylindole; GFP, green fluorescence protein; rRNA, ribosomal RNA; Rpf2, ribosome production factor 2; Rrs1, regulator of ribosome synthesis 1; RT-PCR, reverse transcription polymerase chain reaction

This article can be found at http://www.jspcmb.jp/

Arabidopsis and rice.

Materials and methods

Bioinformatic methods

BLASTN and BLASTP analyses were conducted with Genome net (http://www.genome.jp/). Multiple sequence alignment analysis and construction of a phylogenetic tree were performed with the CLUSTAL W program (http://align.genome.jp/).

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Colombia) and rice (*O. sativa* L. ssp. *japonica* cv. Nipponbare) were sterilized by incubation for 10 min in 2% hypochlorite/0.01% Tween-20 followed by rinsing five times with sterilized water, and then germinated on MS plates. Seeds were stratified in darkness at 4°C for 2 d before being transferred to a greenhouse. Two weeks later, *Arabidopsis seedlings* were transferred to soil and grown in growth chambers at 23°C under a 12-h-light/12-h-dark cycle. Opened flowers, flower buds, stems, cauline leaves, rosette leaves, roots and siliques were harvested for RNA preparation. Two weeks after germination rice seedlings were transferred to soil and grown in an air-conditioned room under an 11-h-light, 25°C/13-h-dark, 18°C cycle. Panicles before heading, panicles after heading, leaves, culms, and roots were harvested for RNA preparation.

Semi-quantitative RT-PCR analysis

For semi-quantitative RT-PCR, total RNA from *Arabidopsis* and rice were isolated with TRI reagent (Sigma, product number; T9424) according to the protocol recommended by the manufacturer. We used 400 ng of total RNA as a template for RT-PCR amplification with an RNA PCR Kit (AMV) Ver.3.0 (Takara). The cDNA fragments were amplified with specific primers AtM532-Nde5 (5′-GGGCATTATGATGGAAATACGAACTCCGAAGACT-3′) and AtM532-Xho3 (5′-GGGCTCGAGTCAGAAGACACCTTCATCTTTT-3′) for *AtRpf2* and OsRpf2-5 long (5′-GGTCTAGAATGGTGGCGGCCATCAGGGTGCCCA-3′) and OsRpf2-3 (5′-GGTCTAGATCACTCGGGATTTATCTTTTGC-3′) for *OsRpf2*. The primer combination F-EF1α (5′-TCGAGACCACCAAGTACTACTGC-3′) and R-EF1α (5′-ATCATACCAGTCTCAACACGTCC-3′) of the *EF1*α gene in *Arabidopsis* and OsActin-5 (5′-AGAGCTACGAGCTTCCTGATGGAC-3′) and OsActin-3 (5′-GAGAGATGCCAAGATGGATCCTCC-3′) of the *Actin1* gene in rice were used for RT-PCR amplification as an internal control. After 22cycles of PCR in *Arabidopsis* and 27cycles in rice, amplified products of internal control genes were detected by ethidium bromide staining of 1% agarose gel. PCR products of *AtRpf2* and *OsRpf2* were blotted onto Hybond N+ membranes and detected with AlkPhos Direct Labeling and Detection System (Amersham) with corresponding cDNA probes.

Reporter gene constructions and transient expression of the GFP fusion proteins

The cDNA of *AtRpf2* was cloned using a 3′ and 5′ RACE system (Gibco BRL) with *AtRpf2*cDNA specific primers AtM532/1 (5′-GGTTGAAACTGGGAAGAAGACG-3′), MDB1 (5′-CTCTGTAATGACGGAACTCTAC-3′), MDB2 (5′-CTTTGTTCCTTCATGAGGTGC-3′), and NMDB2 (5′-CGCCCGCAAGGATTTAAAGTTC-3′), and subcloned into *Eco*RV site of the pT7-Blue vector (Novagen). Because of the availability of restriction enzyme sites, a cDNA fragment corresponding to *AtRpf2* (69-314aa) was excised as an *Stu*I-*Sal*I fragment from the pT7-Blue and inserted into *Xho*I/blunt-*Sal*I site of 221-EGFP-C1 vector (Bayarmaa et al. 2003). The full-length coding region of OsRpf2 was amplified with primers OsRpf2-5Kpn (5′-GGGGGTACCATGGTGGCGGCCATCAGGGTGCCCA-3′) and OsRpf2-3Sma (5′-GGGCCCGGGTCACTCGGGATTTATCTTTTGC-3′), and the amplified fragment was digested with *Kpn*I-*Sma*I. The digested fragment was subcloned into *Kpn*I-*Sma*I site of 221-EGFP-C1 vector to produce the GFP::OsRpf2 fusion protein.

Onion epidermal cells and tobacco BY-2 cells were transformed transiently with 1.6 μm gold particles coated with each plasmid using a model PDS-1000/He particle delivery system (Bio-Rad). The GFP localization was visualized using a fluorescent microscope, E800 (Nikon), equipped with a color CCD camera system (Hamamatsu Photonics).

Results

Identification of Rpf2 homologues and isolation of cDNAs

We identified *Rpf2* homologous genes from *A. thaliana* (locus ID: At3g23620) and *O. sativa* (locus ID: R01-AP002970-40060R http://cdna01.dna.affrc.go.jp/cDNA/), by a database search using the yeast Rpf2 amino acid sequence (GenBank accession number; CAA82160) as the query sequence. To investigate whether Rpf2 proteins are conserved in evolution, we searched the database and identified Rpf2 homologues from eight species. The amino acid sequence of the eight Rpf2 homologues were aligned and displayed as a phylogenetic tree showing that the gene products from plant species are clustered (Figure 1). These results suggest that Rpf2 proteins are conserved throughout evolution and that the *Rpf2* homologous gene exists as a single copy in most cases including *A. thaliana*. On the other hand, sequence information for two closely related *Rpf2*-like genes were obtained from the rice genome sequence.

Because alignment of the predicted amino acid sequence revealed high homology to the sequence of *Rpf2*, especially in the sigma70-like motif region, and the Brix domain, a characteristic domain involved in ribosome biogenesis (Eisenhaber et al. 2001), we designated these two genes *AtRpf2* and *OsRpf2*, respectively (Figure 2). The predicted amino acid sequence of these two genes from genome annotation corresponded well to that of the full-length cDNA sequence information (accession number: At3g23620: AY122939 and AY070410; R01-AP002970-40060R: AK100776, respectively). We could isolate both cDNA clones by RT-PCR using specific primers designed based

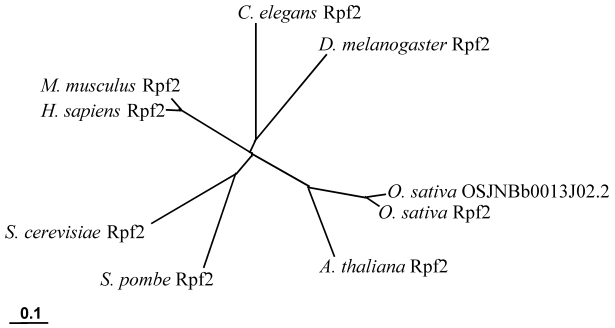

Figure 1. Phylogentic tree of Rpf2 homologues in various organisms. The distance scale represents the evolutionary distance expressed as the number of substitution per amino acid. GenBank accession number; *O. sativa* Rpf2, BAB32971; *O. sativa* OSJNBb0013J02.2, AAT07597; *A. thaliana* Rpf2, AAM67472; *S. cerevisiae* Rpf2, CAA82160; *H. sapiens* Rpf2, BAB14983; *M. musculus* Rpf2, BAA95117; *D. melanogaster* Rpf2, AAF55514; *C. elegans* Rpf2, AAF60765; *S. pombe* Rpf2, CAB54156.

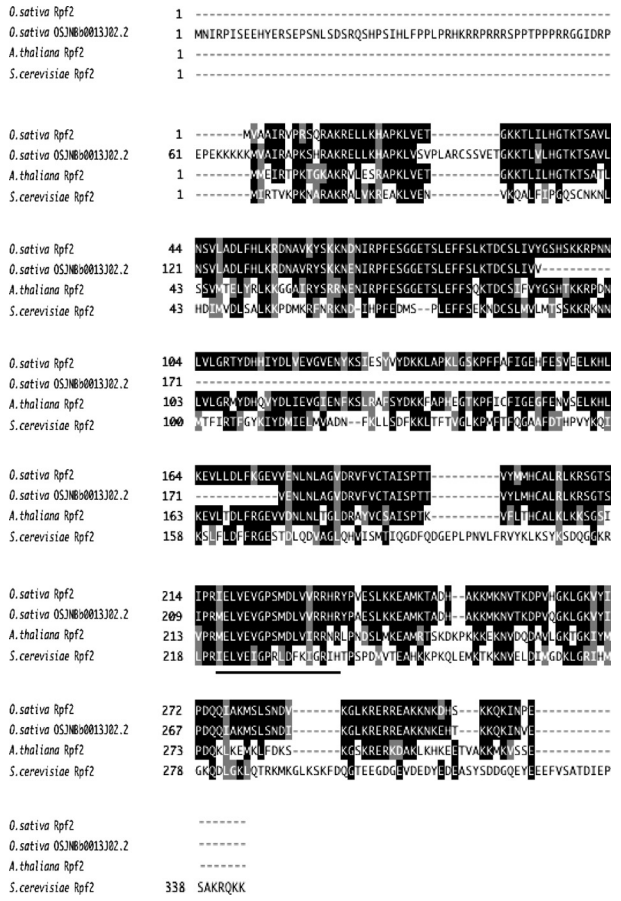

Figure 2. Multiple sequence alignment of Rpf2 homologues. White letters on black boxes indicate identical amino acids, and similar ones are indicated with shading boxes. The underlined amino acid sequences show a sigma-70 like RNA binding motif (216-233aa). GenBank accession number; *O. sativa* Rpf2, BAB32971; *O. sativa* OSJNBb0013J02.2 , AAT07597; *A. thaliana* Rpf2, AAM67472; *S. cerevisiae* Rpf2, CAA82160.

on the predicted sequence of the corresponding cDNAs. The *AtRpf2* cDNA contained an ORF of 945 bp encoding a protein of 314 amino acids and the *OsRpf2* cDNA contained an ORF of 933 bp encoding a protein of 310 amino acids, respectively.

By the sequence homology search based on the Rpf2 amino acid sequence, we also identified a putative *Rpf2*-like gene from the rice genome. The predicted gene product encoded by the OSJNBb0013J02.2 (accession number: AC129719) contains a sigma-70 like RNA binding motif, a characteristic motif of the Rpf2 family. However, from the database information, the predicted mRNA sequence of the OSJNBb0013J02.2 differed from that of the full-length cDNA information (accession number: AK106697) that is actually transcribed from the genomic region (locus ID: R05-AC129719-40020R; http://cdna01.dna.affrc.go.jp/cDNA/).

To investigate whether the mRNA sequence from OSJNBb0013J02.2 is transcribed, we attempted to obtain the cDNA derived from the predicted mRNA sequence of OSJNBb0013J02.2 (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=34732726&itemID=2&view=gbwithparts). Amplification of the predicted cDNA by the oligonucleotide primers was confirmed by PCR using genome DNA as a template and we performed RT-PCR using total RNA samples from various organs including panicles before heading, panicles after heading, leaves, stems, and roots. However, we could not detect the amplification of corresponding cDNA fragment predicted from the OSJNBb0013J02.2. These results suggest that the *Rpf2*-like gene products encoded by the R05-AC129719-40020R is not expressed, if not at all, under the experimental conditions used in this study.

To obtain expression profiles, we analyzed the RNA accumulation in various samples to determine the transcription levels of *AtRpf2* and *OsRpf2* genes. Analysis of semi-quantitative RT-PCR demonstrated that the transcripts from both genes were detected in all samples tested (Figure 3). Relatively higher expression of *AtRpf2* mRNA was observed in buds, open flowers, stems and roots of *Arabidopsis*. Similarly, expression levels of *OsRpf2* mRNA were also higher in panicles before heading, panicles after heading and leaves in rice. Although there are morphological differences between *Arabidopsis* and rice, the expression profiles observed in this study suggest that the expression patterns of *AtRpf2* and *OsRpf2* are essentially the same, and prominent tissue-specific expression was not observed under the conditions of this study.

Subcellular localization of GFP fusion proteins

To identify the subcellular localization of AtRpf2 and OsRpf2 proteins, we made plasmid constructs expressing an in-frame N-terminal translational fusion with GFP. These plasmid constructs encoding GFP fused to the 69-314aa of AtRpf2 (GFP::AtRpf2) and the full-length protein of OsRpf2 (GFP::OsRpf2), respectively,

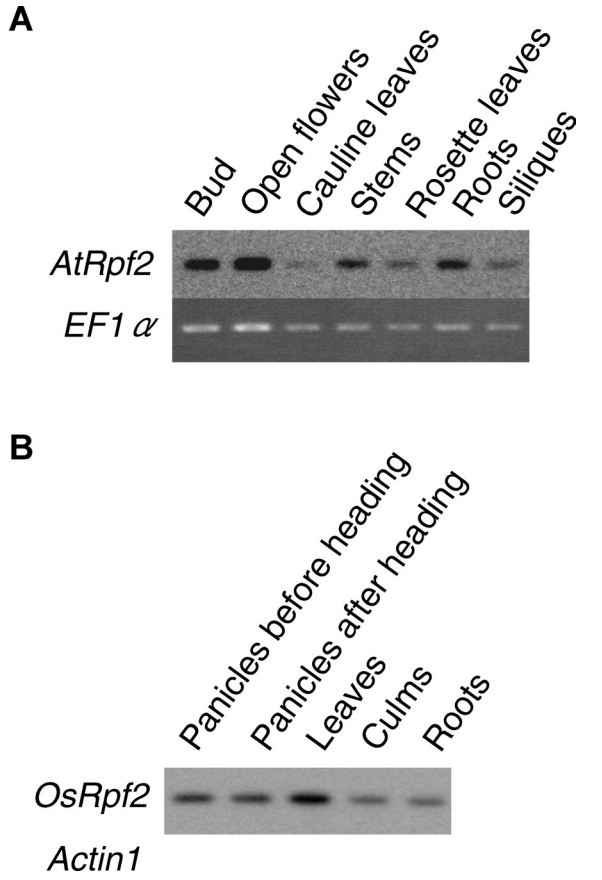

Figure 3. Detection of mRNA expression by RT-PCR. (A) RT-PCR analysis of *AtRpf2* in various organs of *Arabidopsis*. RT-PCR products of the Arabidopsis *EF1α* gene were used as internal standard. (B) RT-PCR analysis of *OsRpf2* in various organs of rice. RT-PCR products of the rice *Actin 1* gene were used as internal standard.

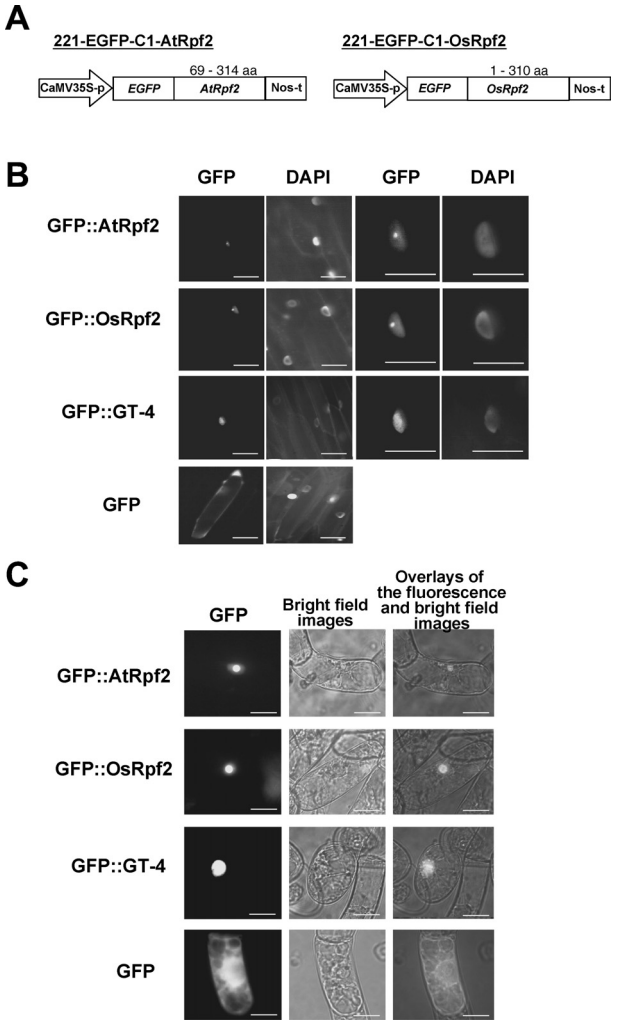

Figure 4. Subcellular localization of GFP::AtRpf2 and GFP::OsRpf2. (A) Schematic representation of plasmid constructs for GFP::AtRpf2 (221-EGFP-C1-AtRpf2) and GFP::OsRpf2 (221-EGFP-C1-OsRpf2). (B) Intranuclear localization of GFP-fusion proteins in onion epidermal cells. Epifluorescence micrographs show localizations of GFP::AtRpf2, GFP::OsRpf2, GFP::GT-4 and GFP only (left), and DAPI images (right), respectively. Bar=100 μm. (C) Transient expression of GFP::AtRpf2, GFP::OsRpf2, GFP::GT-4 and GFP only in tobacco BY-2 cells. Epifluorescence micrographs on the left show GFP fluorescence alone. The central images show bright field images. Overlays of the bright field and the fluorescence images are shown in the right. Bar=10 μm.

were introduced into plant cells by microprojectile bombardment and expressed under control of the CaMV35S promoter (Figure 4A). Plasmid constructs expressing GFP alone and GFP::GT-4 that express a GFP fusion protein were used as controls. The GT-4 is a putative transcription factor that localized to the nucleus (Murata et al. 2002). Transient expression of GFP::AtRpf2 and GFP::OsRpf2 fusion proteins in onion epidermal cells was observed by fluorescence microscopy 9 h after bombardment. The fluorescent signals from GFP::AtRpf2 and GFP::OsRpf2 were detected predominantly in the nucleolus and there were obvious differences in their intracellular localization compared with GFP alone. Notably, compared with GFP::GT-4 that localizes predominantly in the nucleus, nucleolar localization of GFP::AtRpf2 and GFP::OsRpf2 was evident (Figure 4B). To further confirm the nucleolar localization of both GFP fusion proteins, we conducted a transient expression assay using tobacco BY-2 cells. As shown in Figure 4C, we could observe characteristic intracellular localization of GFP fusion proteins. As in onion epidermal cells, the nucleolar localization of GFP::AtRpf2 and GFP::OsRpf2 was clearly within the nucleus in tobacco BY-2 cells.

Discussion

The *Rpf2* was originally identified as an essential gene for vegetative growth in yeast and database analyses indicated that Rpf2 homologues are conserved throughout evolution (Wehner and Baserga 2002). In this study, we also confirmed that the Rpf2 homologues are ubiquitous across the eukaryotes. Because evolutionary conservation often indicates functional importance, we speculate that the *Rpf2* genes are also essential for plant growth

and development. In fact, our provisional results for characterization of a T-DNA insertion mutant indicate this possibility (Asada et al. unpublished data).

Although genes encoding Rpf2 homologues exist as a single copy gene in most organisms, two *Rpf2* homologous sequences were found within the rice genome. High similarity between two rice homologues suggested that these genes had arisen by gene duplication. Our RT-PCR analysis indicated that only one of the two genes, R01-AP002970-40060R, is expressed. However, further investigation is necessary to confirm the expression of mRNA encoded by the other locus. Analysis of mRNA expression levels of *AtRpf2* and *OsRpf2* showed ubiquitous expression of both genes with some elevated expression in tissues where active protein synthesis is required. These expression profiles of both genes are similar to the typical expression pattern of ribosomal protein genes described previously (Van Lijsebettens et al. 1994; Williams and Sussex 1995; Ito et al. 2000; Weijeres et al. 2001; Nishimura et al. 2005).

In yeast, Rpf2 and its interacting protein, Rrs1, have been shown to be localized predominantly in the nucleolus (Morita et al. 2002; Wehner and Baserga 2002; Tsuno et al. 2000). Since the nucleolus is the site of ribosome biogenesis, the nucleolar localization of these proteins indicates that the Rpf2 and related proteins are involved in ribosome biogenesis. We investigated the intracellular localization using a transient expression assay of GFP-fusion proteins and demonstrated that the Rpf2 homologues from *Arabidopsis* and rice are able to direct nucleolar localization when fused to GFP. These results are consistent with a previous study on proteomic analysis of nucleolar proteins in *Arabidopsis*. The *Arabidopsis* cDNA derived from At3g23620 that encodes AtRpf2 was identified as a cDNA encoding a nucleolar protein and the GFP fusion with the protein was targeted to the nucleolus and nuclear bodies of *Arabidopsis* suspension cells (Pendle et al. 2005, http://germinate. scri. ac.uk/cgi-bin/atnopdb/ get-all-data?type=more&value=at3g23620). On the other hand, we could observe nucleolar localization of GFP::AtRpf2 and GFP::OsRpf2 proteins in onion epidermal cells and tobacco BY-2 cells, respectively. These results show that the nucleolar localization capability of AtRpf2 and OsRpf2 is also active in heterologous species. Although the mechanisms involved in nucleolar localization of proteins are not well understood, we could observe prominent nucleolar localization of GFP-fusion proteins by transient expression assay by microprojectile bombardment. This versatile assay system is an ideal experimental approach for the dissection of nucleolar protein molecules and the identification of signals involved in nucleolar targeting of the proteins.

The findings obtained in this study suggest that the Rpf2 homologues in plants are also involved in ribosome biogenesis as a ribosomal RNA processing factor as has been reported for Rpf2 in yeast. However, further studies on biochemical as well as biological properties of AtRpf2 and OsRpf2 proteins are needed to confirm the function of Rpf2 homologues in higher plants.

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