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Ribosomal Protein S6, a Target of Rapamycin, Is Involved in the Regulation of rRNA Genes by Possible Epigenetic Changes in *Arabidopsis*[*] [S]

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Background: Ribosomal protein S6 has been known to be a key downstream effector of the TOR signaling pathway.
Results: We demonstrated that ribosomal protein interacts with a histone deacetylase and binds to rRNA gene promoter.
Conclusion: The TOR signaling controls rRNA synthesis via interaction of RPS6 to rRNA genes.
Significance: This study links the environmental signals via TOR kinase to control growth of an organism by regulating ribosome biogenesis.

The target of rapamycin (TOR) kinase pathway regulates various biological processes, including translation, synthesis of ribosomal proteins, and transcription of rRNA. The ribosomal protein S6 (RPS6) is one of the well known downstream components of the TOR pathway. Ribosomal proteins have been known to have diverse functions in regulating cellular metabolism as well as protein synthesis. So far, however, little is known about other possible role(s) of RPS6 in plants, besides being a component of the 40 S ribosomal subunit and acting as a target of TOR. Here, we report that RPS6 may have a novel function via interaction with histone deacetylase 2B (AtHD2B) that belongs to the plant-specific histone deacetylase HD2 family. RPS6 and AtHD2B were localized to the nucleolus. Co-expression of RPS6 and AtHD2B caused a change in the location of both RPS6 and AtHD2B to one or several nucleolar spots. ChIP analysis suggests that RPS6 directly interacts with the rRNA gene promoter. Protoplasts overexpressing both *AtHD2B* and *RPS6* exhibited down-regulation of pre-18 S rRNA synthesis with a concomitant decrease in transcription of some of the ribosomal proteins, suggesting their direct role in ribosome biogenesis and plant development. This is consistent with the mutation in *rps6b* that results in reduction in 18 S rRNA transcription and decreased root growth. We propose that the interaction between RPS6 and AtHD2B brings about a change in the chromatin structure of rDNA and thus plays an important role in linking TOR signaling to rDNA transcription and ribosome biogenesis in plants.

Ribosome biogenesis is central to the growth and development of eukaryotic cells and organisms. Accordingly, rapidly growing cells invest most of the cells' transcriptional/translational capacities into the syntheses of rRNAs and the ribosomal proteins (1, 2). Ribosome biogenesis in eukaryotic cells involves coordinated syntheses of the four ribosomal RNAs (rRNAs) and more than 70 ribosomal proteins, and transcription/translation of each component is tightly regulated in response to the physiological status of the cell (3). Transcription of ribosomal DNA (rDNA) depends on multiple signaling pathways responding to the external environmental cues, including, stress, nutrients, hormones, and mitogens (1, 4).

Three of the four ribosomal RNAs (18 S, 5.8 S, and 25 S) are transcribed as a single precursor (pre-rRNA)[4] by RNA polymerase I (pol I), which is an important rate-limiting step in the biogenesis of ribosomes. Processing of pre-rRNA begins at the 5′ external transcribed spacer (ETS). Subsequent cleavages occur at the 5′ end of the 18 S rRNA and the internal transcribed spacer 1 (ITS1) to generate 18 S rRNA and a precursor containing the 5.8 S and 25 S rRNAs. Final cleavage in the ITS2 and the 3′-ETS generates mature 5.8 S and 25 S rRNAs (5, 6).

Target of rapamycin (TOR) kinase (7, 8) signaling coordinates many cellular metabolic activities under varying energy and stress conditions (3). In yeast, the pol I-dependent transcription of 35S rRNA precursor is directly controlled by TOR, which binds to the rDNA promoter via its helix-turn-helix motif (9). More recently, association of mammalian TOR and the *Arabidopsis* TOR to their respective rDNA promoters has also been reported, and the binding of mammalian TOR was

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[4] The abbreviations used are: pre-rRNA, precursor rRNA; pol, polymerase; TOR, target of rapamycin; BiFC, bimolecular fluorescence complementation; ETS, external transcribed spacer; 5′-TOP, 5′-terminal oligopyrimidine tract; N35, nodulin-35.

shown to be sensitive to rapamycin treatment (10, 11). TOR has also been implicated in the transcriptional activation of a number of ribosomal protein genes that is mediated by the activities of its downstream effector kinase (ribosomal protein S6 kinase) and the c-Myc transcription factor (Sch9 in animals and Sfp1 in yeast, respectively) (3). Activation of the pol I-mediated transcription by TOR is indirectly controlled by ribosomal protein S6 kinase, impinging on the general transcription factor UBF1 (Hmo1 in yeast) (12). Evidence suggests that the activity of TOR is required in derepressing the epigenetic silencing of the rDNA promoter (13, 14), and a possible role of histone deacetylases has been suggested in epigenetic silencing of the rRNA genes (15).

Ribosomal protein S6 (RPS6), a component of the 40 S ribosomal subunit, has been known to be a key downstream effector of the TOR signaling pathway, which is conserved among yeast, mammals, insects, and plants (16, 17). The phosphorylation status of RPS6, which reflects the activity of S6K, has been recognized as a hallmark of actively proliferating cells (18–20). The phosphorylation of RPS6 plays a role in the translational up-regulation of mRNAs containing the 5′-terminal oligopyrimidine tract (5′-TOP), which are found in many mRNAs encoding the proteins involved in ribosome biogenesis (21). However, the RPS6 phosphorylation-defective cells did not show a dramatic reduction in global protein translation as well as in translation of the 5′-TOP mRNAs (19). Thus, the exact role of RPS6 in the regulation of ribosome biogenesis and the identities of the factors involved in this process remain a subject of scrutiny.

To obtain a better insight into the possible role of RPS6 in the mechanism of regulation of ribosome biogenesis in plants, we attempted to identify novel interacting partners of RPS6 from *Arabidopsis* by GST pulldown followed by LC/MS protein identification. A plant-specific histone deacetylase AtHD2B (also known as HDT1) was identified as one of the interacting partners of RPS6. Here, we present evidence for a specific interaction of RPS6 with AtHD2B and demonstrate a possible role of this complex in transcriptional regulation of rRNA genes. We propose a new paradigm for controlling rDNA transcription in plants in which TOR may control a silencing mechanism of the rDNA transcription via its downstream signaling component RPS6, the mechanism of which involves interaction of the RPS6 with AtHD2B. Such an interaction can provide a direct link between stress signals and the regulation of translation and transcription (particularly rDNA) machineries controlling plant growth.

EXPERIMENTAL PROCEDURES

Plant Materials and Hormone Treatments—The *Arabidopsis thaliana* ecotype Columbia or Columbia-0 was used in BiFC and protoplast transformation assay and in mutant analysis of *rps6b*, respectively. Seeds were sterilized by gentle shaking in 70% ethanol for 5 min, followed by treatment with 50% hypochlorite and 0.01% Triton X-100 for 15 min and then with distilled water. Sterile seeds were grown on aseptic solid media (0.8% plant agar) containing ½ MS salts (Sigma) and 1% sucrose in a growth chamber at 22 °C with a 16-h photoperiod. For root elongation assays, wild-type, mutant, and transgenic seeds were germinated on MS agar media, and seedlings were transferred to plates with or without kinetin after 4 days and grown vertically. Root size was measured after an additional 10 days of growth. To apply plant hormones, 14-day-old seedlings were transferred to sterile liquid growth medium containing hormones and salt and harvested after 24 h. The concentrations of hormones used were as follows: 20 μM indole-3-acetic acid, 20 μM kinetin, 20 μM abscisic acid, and 300 mM NaCl.

Plasmid Construction for Transient Expression Studies—To make various constructs of *RPS6*-GFP fusion (S6FL-GFP, S6CT-GFP, or S6NT-GFP), the full-length and C- or N-terminal regions of RPS6 were amplified by PCR with *Pfu* DNA polymerase (Takara, Japan) using forward and reverse primers (see supplemental Table S1) with a SacI site and a BamHI site, respectively, and fused in-frame with *GFP* of the 326-smGFP vector.

Transgenic Plants with P_{35S}-AtHD2B or P_{rDNA}-GUS—The DNA fragment of *AtHD2B-HA* or P_{rDNA}-*GUS* were transferred into pK7WGF2.0. The resulting fusion constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101, and *Arabidopsis* plants were transformed by the floral dip method (22). To make a construct of the rDNA minimal promoter fused to a *GUS* reporter gene, a 500-bp upstream region of rDNA was amplified using the following primers: forward, 5′-AGAATTCGTCGACCAGGACGGCGGAAC-3′, and reverse, 5′-AGACTCCCTCAACACCCACCCCCCTATA-3′.

Real Time Quantitative PCR—Real time (RT)-quantitative PCR was set up with SYBR Green PCR master mix (Takara, Japan) using Rotor-Gene 3000 (Qiagen, Germany). All reactions were normalized using *ACTIN2* gene as an internal control. Primers used for various fragments are listed in supplemental Table S1.

Identification and Complementation of the T-DNA Insertion of RPS6B—Seeds of a T-DNA insertion line SALK_012147 of *RPS6B* were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). To confirm the insertion of T-DNA of *RPS6B*, genomic PCR was performed, and the PCR product was sequenced. Primers used were as follows: T-DNA left border-specific forward primer (LBb1), 5′-GCGTGGACCGCTTGCTGCAACT-3′; *RPS6B* forward primer (S6Bf), 5′-CAATGACCAAGTTAAGAACAGACAGGTCA-3′, and *RPS6B* reverse primer (S6Br), 5′-CTGCGTTGGTCTGATATATAACCAGTTC-3′. To search whether *RPS6B* expression is decreased in the T-DNA insertion *rps6b* mutant, RT-quantitative PCR was performed to examine the *RPS6B* expression in the mutant. For complementation of the mutant, the *RPS6B* promoter was fused with a full-length *RPS6B* cDNA. The recombinant plasmid was introduced into a homozygous mutant, *rps6b-3,* by the floral dip method (22).

Protein Pulldown Assay—GST protein was immobilized onto the CNBr-activated Sepharose resin and used for pre-clearing the cell extract. Then 100–300 mg of *Arabidopsis* total soluble proteins were applied to the columns of different GST fusion protein substrates arranged in tandem as outlined in supplemental Fig. S1. The protein sample passed through the columns was re-circulated at least three times to facilitate specific interaction between the GST fusion proteins and the cellular proteins. The columns were then washed with 1000 volumes of

wash buffer A (50 mM Tris·Cl, pH 7.5, 125 mM NaCl, 5 mM benzamidine, 1 mM PMSF, 1 mM DTT, 10% glycerol), 100 volumes of wash buffer B (50 mM Tris·Cl, pH 7.5, 500 mM NaCl, 5 mM benzamidine, 1 mM PMSF), and then 500 volumes of wash buffer A. For the samples to be eluted by thrombin digestion, the wash step was completed with an additional 500 volumes of wash buffer C (50 mM Tris·Cl, pH 7.5, 125 mM NaCl, 1 mM DTT) to remove the traces of the protease inhibitors from the resins before they were subjected to thrombin digestion. Isolated proteins were visualized by Coomassie staining after SDS-PAGE, and the protein band identification by peptide mass fingerprinting by MALDI-TOF mass spectrometry was conducted at the proteomics facility at Ohio State University and at Genomine Inc. His-fused proteins were purified as described above and incubated with total soluble proteins extracted from 3HA-RPS6 transgenic plant for 16 h. After incubation, His-AtHD2B and 3HA-RPS6 were washed and resolved by SDS-PAGE.

BiFC Assay—The BiFC assay was performed according to the method described by Hu *et al.* (23). Full-length or the N-terminal region of AtHD2B and full-length cDNA of *N35* were cloned into the binary BiFC vectors, p2YN and p2YC, respectively. The resulting fusion constructs were verified by sequencing and introduced into *Arabidopsis* protoplasts by transfection (24). After 16 h of incubation, fluorescence and DAPI staining were visualized by Olympus fluorescence microscope.

Chromatin Immunoprecipitation (ChIP)—The ChIP experiments were performed as described (11) with minor modifications. 1.5 g of 12-day-old *Arabidopsis* seedlings were fixed with 37 ml of 1% formaldehyde solution for 10 min until seedlings turn translucent for cross-linking proteins to DNA. The reaction was stopped by the addition of 2.5 ml of 2 M glycine. The chromatin was isolated and fragmented by sonication followed by immunoprecipitation with HA antibody (Santa Cruz Biotechnology). The chromatin solution was precleared with protein-agarose beads with sheared salmon sperm DNA. Co-precipitated DNA was amplified using Ex-Tag polymerase (Takara, Japan) and the primers listed in supplemental Table S2.

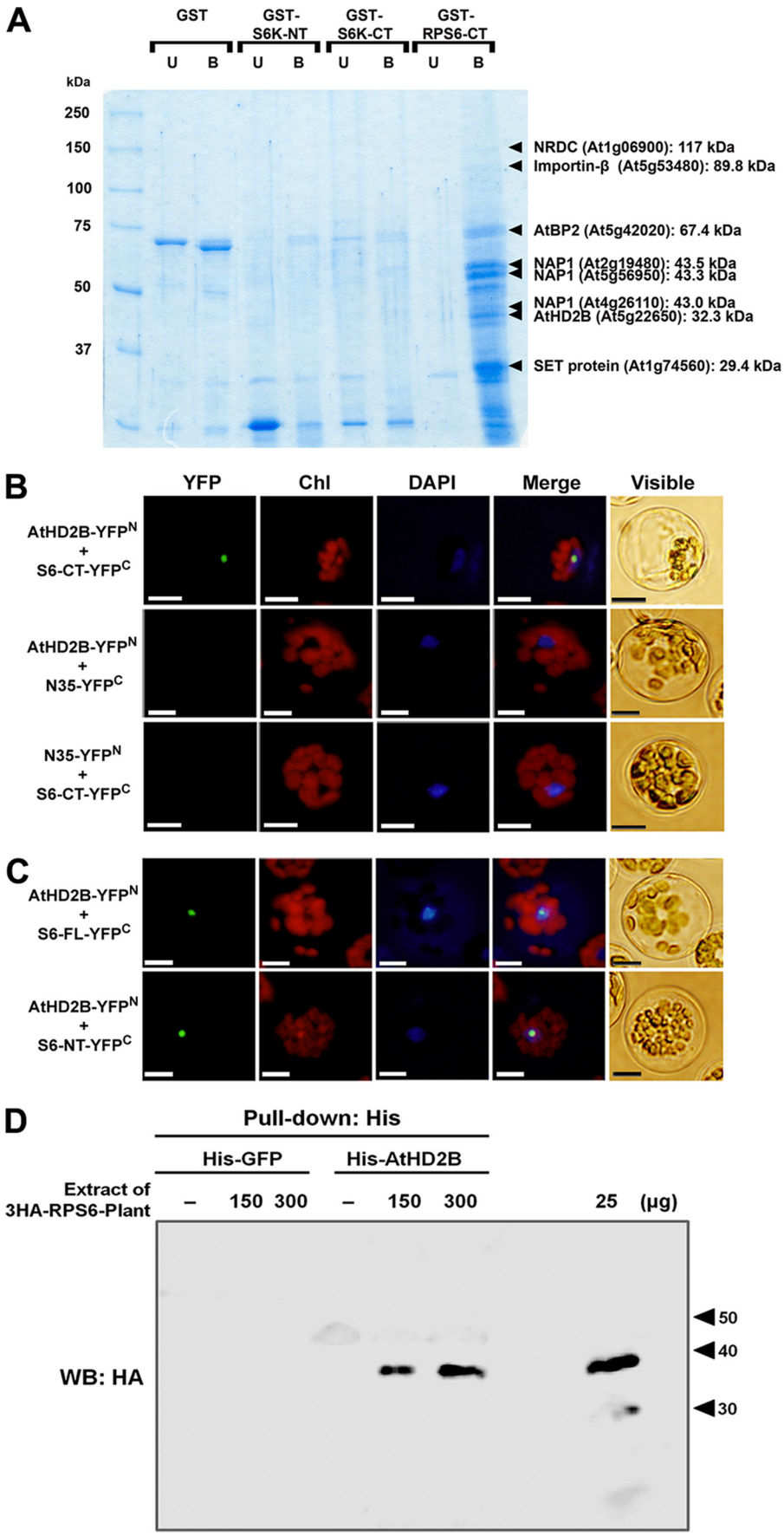
RESULTS

Identification of Proteins Interacting with the RPS6 C Terminus—To isolate potential candidates for the interacting partners of RPS6, total soluble proteins extracted from *Arabidopsis* suspension cells were applied to an affinity column containing GST-fused RPS6 protein (used as an affinity bait). To reduce nonspecific interaction and focus on the candidates that are relevant to the TOR signaling, only the C-terminal 100 amino acids of the RPS6 (RPS6-CT), which includes the region of the putative phosphorylation sites by S6 kinase (19), was used as a bait. The GST-fused AtS6K1 N-terminal fragment (GST-S6K-NT) and the C-terminal fragment (GST-S6K-CT) were also prepared and used as controls together with the full-length of GST protein, in affinity purification (supplemental Fig. S1). The experiment was repeated several times, and in each case, about 100–300 mg of total soluble proteins extracted from actively growing *Arabidopsis* suspension culture cells were applied to the columns containing glutathione-agarose resin to which GST fusion proteins were bound by affinity interaction. After rigorous washing, the affinity-bound proteins were released by in-column thrombin digestion. Following size-exclusion fractionation to remove the GST fusion proteins co-eluted during thrombin digestion, the proteins were visualized by SDS-PAGE (Fig. 1*A*) and two-dimensional gel electrophoresis (data not shown). In several repeated experiments, GST fusion protein of RPS6-CT consistently pulled down a number of specifically interacting proteins (indicated by *arrowheads* in Fig. 1*A*) that were subjected to identification through both MALDI-TOF and LC-MS spectrometry analyses.

The identities of the proteins (and their corresponding gene accession numbers in parentheses) are shown in Fig. 1*A*. All of the identified proteins, with the exception of protease subtilisin homologue, have been functionally implicated in the chromatin-related activities, including the nucleosome-mediated regulation of gene expression. Of particular interest among these was the presence of a histone deacetylase, AtHD2B, a paralogue of which (AtHD2A) had been implicated in silencing of rDNA transcription in *Arabidopsis* (25). Because the physical interaction of AtHD2B with RPS6 presents an interesting perspective with regard to the role of RPS6 phosphorylation in the activation of translation and possibly ribosome biogenesis, we focused on uncovering the functional significance of the RPS6-AtHD2B interaction *in vivo*.

In Vivo Interaction of AtHD2B with RPS6—AtHD2A belongs to the plant-specific HD2 family of histone deacetylases that have been suggested to be involved in rDNA gene silencing and nucleolar dominance (15, 25). This means that AtHD2B might also be involved in the regulation of rDNA transcription. To confirm the physical interaction between the RPS6-CT and AtHD2B *in vivo*, we tested the formation of this complex using BiFC analysis using *Arabidopsis* protoplasts. The C-terminal regions of AtHD2B and RPS6 were fused to the complementary N- and C-terminal fragments of YFP (YFP^N and YFP^C), respectively. Soybean nodulin-35 (N35) (26) fused to either the N- or the C-terminal fragment of YFP was used as a negative control. Whereas no fluorescence was observed in protoplasts expressing pairs of N35 + AtHD2B or N35 + RPS6 proteins (Fig. 1*B, middle* and *bottom panels*), protoplasts transfected with AtHD2B-YFP^N and RPS6-CT-YFP^C constructs showed fluorescence signal that was concentrated into a smaller region within the nucleus, resembling the nucleolus (Fig. 1*B, middle* and *bottom panels*). These data suggest that AtHD2B specifically forms a complex with RPS6 in the nucleolar region of plant cells, and such a complex may be involved in the transcription of rDNA or processing of rRNAs.

To determine whether the interaction between the RPS6 and AtHD2B only occurs through the C-terminal region of the RPS6, the N-terminal fragment of RPS6 (RPS6-NT) that was excluded in the original GST pulldown experiment, by which AtHD2B was identified as an interacting partner, was also tested for interaction using the BiFC assay. Because fluorescence in protoplasts co-expressing a pair of AtHD2B-YFP^N and RPS6-NT-YFP^C was also detected in the nucleolus (Fig. 1*C, bottom panels*), these results suggest that more than one region of RPS6 might be associated with AtHD2B, forming a complex. To confirm that AtHD2B and AtRPS6 interact with each other, we performed an *in vitro* pulldown assay using His-AtHD2B-


A
GST
GST-S6K-NT
GST-S6K-CT
GST-RPS6-CT
U B U B U B U B
kDa
250
150
100
75
50
37
NRDC (At1g06900): 117 kDa
Importin-β (At5g53480): 89.8 kDa
AtBP2 (At5g42020): 67.4 kDa
NAP1 (At2g19480): 43.5 kDa
NAP1 (At5g56950): 43.3 kDa
NAP1 (At4g26110): 43.0 kDa
AtHD2B (At5g22650): 32.3 kDa
SET protein (At1g74560): 29.4 kDa
B
YFP
Chl
DAPI
Merge
Visible
AtHD2B-YFP^N + S6-CT-YFP^C
AtHD2B-YFP^N + N35-YFP^C
N35-YFP^N + S6-CT-YFP^C
C
AtHD2B-YFP^N + S6-FL-YFP^C
AtHD2B-YFP^N + S6-NT-YFP^C
D
Pull-down: His
His-GFP
His-AtHD2B
Extract of 3HA-RPS6-Plant
– 150 300 – 150 300 25 (μg)
WB: HA
50
40
30

fused and RPS6-GST-fused proteins isolated from *Escherichia coli*. However, no interaction using proteins from *E. coli* was observed. Subsequently, transgenic plants expressing HA-tagged RPS6 driven by the CaMV-35S promoter was used for pulldown assay to test the possibility that AtHD2B may interact indirectly with RPS6 forming a complex. Proteins from the *3HA-RPS6*-expressing plants were incubated with His-AtHD2B, which was used as a bait, and HA-RPS6 was found to interact with His-AtHD2B (Fig. 1*D*). These results suggest that AtHD2B and RPS6 may associate indirectly with each other, forming a multiprotein complex.

Interaction of RPS6 and AtHD2B Facilitates Their Nucleolar Localization—In contrast to the results obtained from the BiFC analyses described above, we observed that both GFP-tagged AtHD2B and RPS6 were localized in the nucleus when they were overexpressed alone in protoplasts (Fig. 2*A, top two panels*). The possibility of interaction-dependent nucleolar translocation of these proteins was also tested by co-expressing them in *Arabidopsis* protoplasts. Indeed, co-expression of RPS6-GFP with AtHD2B caused a change in localization of RPS6 to one or several putative nucleolar spots (Fig. 2*A, bottom two panels*). Although both the N-terminal (RPS6-NT) and the C-terminal (RPS6-CT) fragments of RPS6 were shown to interact with AtHD2B in the BiFC analyses, this nucleolar translocation was not observed when the GFP fusion construct of RPS6-NT was co-expressed with AtHD2B (Fig. 2*B, bottom two panels*). The interaction-dependent nucleolar translocation of AtHD2B was also confirmed by reciprocally monitoring the GFP fluorescence after overexpressing RPS6 in protoplasts prepared from transgenic *Arabidopsis* expressing GFP-AtHD2B (data not shown). These results indicate that formation of the RPS6-AtHD2B protein complex is critical for these proteins to be targeted to the nucleolus and also suggest that the protein motif present in the RPS6-CT may provide a tighter interaction with AtHD2B.

The *Arabidopsis* genome contains four isoforms of the HDT-type histone deacetylases, namely AtHD2A, AtHD2B, AtHD2C, and AtHD2D, among which AtHD2A and AtHD2B share the highest sequence homology and the apparent functional similarities (27–29). To test the specificity of interaction between RPS6 and the AtHD2B, GFP fusion construct of a full-length RPS6 (S6FL-GFP) was co-expressed with AtHD2A in protoplasts (Fig. 2*C*). In this case, localization of the GFP fluorescence was not altered by co-expression of AtHD2A, supporting the idea that RPS6 may play a role as a specific functional component of the AtHD2B silencing complex, in regulating the synthesis and/or processing of rRNAs in plant nucleolus.

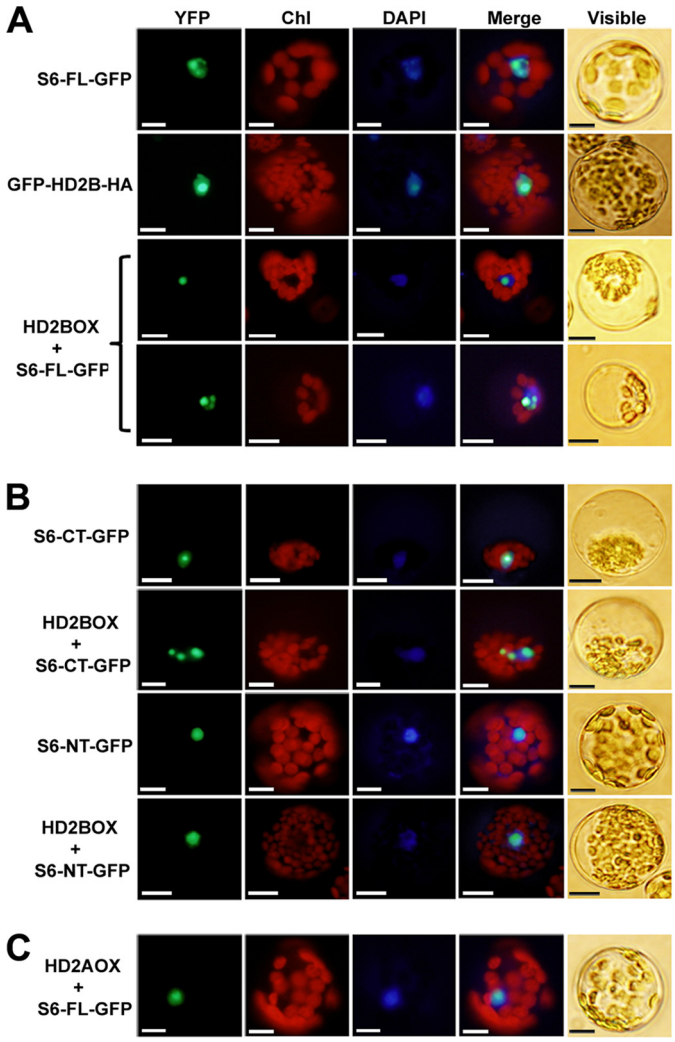


FIGURE 2. **Interaction-dependent localization of AtHD2B and RPS6.** *A*, subcellular localization of full-length RPS6 and AtHD2B. In the *top panels*, protoplasts were transfected with full-length *RPS6* fused to *GFP* (*S6FL-GFP*). The *2nd panels* show protoplasts isolated from transgenic plants with GFP-AtHD2B fusion (*GFP-HD2B-HA*). In the *two bottom panels*, transgenic protoplasts with S6FL-GFP were transfected with P_{35S}-*AtHD2B*-NOS. *B*, subcellular localization of C- and N-terminal fragments of RPS6 and AtHD2B. Protoplasts were transfected with truncated *RPS6* fused to *GFP* (*S6CT-GFP* or *S6NT-GFP*) or co-transfected with truncated *RPS6* and *AtHD2B*-expressing plasmids (*S6CT-GFP* or *S6NT-GFP* and *HD2BOX*). *C*, subcellular localization of RPS6 in *AtHD2A*-expressing protoplasts. Protoplasts were co-transfected with *RPS6* and *AtHD2A*-expressing plasmids (*S6FL-GFP* and *HD2AOX*). Transfected protoplasts were stained with DAPI to visualize the nucleus. These experiments were replicated three times with similar results. *Bar,* 10 μm.

Effect of RPS6/AtHD2B Overexpression on rDNA Transcription—Several plant histone deacetylases have been reported to be involved in the silencing of rDNA genes (25, 30). We tested the

FIGURE 1. ***In vivo* interaction of AtHD2B and RPS6.** *A*, protein profiles after thrombin elution of the pulldown products. The cellular proteins bound with GST fusion protein substrates were released by thrombin digestion and run on SDS-polyacrylamide gels. For *U lanes*, the substrate resin was not incubated with the cellular proteins; for *B lanes,* substrate pulldown resins were incubated with the cellular proteins. Protein bands potentially representing the product of specific interaction with RPS6 were marked with *arrowheads* and were further identified. *B*, BiFC analyses of interaction between RPS6-CT and AtHD2B as follows: co-expression of P_{35S}-AtHD2B-YFP^N and 35S-S6-CT-YFP^C (*top panels*); co-expression of P_{35S}-AtHD2B-YFP^N and P_{35S}-N35-YFP^C (*middle panels*); co-expression of P_{35S}-N35-YFP^N and P_{35S}-S6-CT-YFP^C (*bottom panels*). *C*, BiFC analyses of interaction between RPS6-NT and AtHD2B as follows: co-expression of P_{35S}-AtHD2B-YFP^N and P_{35S}-S6-FL-YFP^C (*top panels*); co-expression of P_{35S}-AtHD2B-YFP^N and P35$_S$-S6-NT-YFP^C (*bottom panels*). Transfected protoplasts were stained with DAPI to visualize the nucleus. These experiments were replicated three times with similar results. *Bar,* 10 μm. *D*, interaction between AtHD2B and RPS6 by a pulldown assay with His tag followed by a Western blot with anti-3HA antiserum. Protein extracts from plants expressing 3HA-RPS6 were incubated with different amounts (150 and 300 μg) of His-GFP or His-AtHD2B fusion proteins, respectively. *WB,* Western blot.

possible effect of the RPS6-AtHD2B complex on rDNA gene transcription by examining pre-rRNA transcription levels in *AtHD2B*- or *RPS6*-expressing protoplasts. *Arabidopsis* pre-rRNA transcript contains the 18 S, 5.8 S, and 25 S rRNAs as well as the 5′ and 3′ external transcript spacer (ETS) and two internal transcript spacers between the three rRNAs (ITS1 and ITS2). Mature 18 S, 5.8 S, and 25 S rRNAs are generated after processing pre-rRNA (5, 6). Total RNA was isolated from the protoplasts transfected with *AtHD2A*-, *AtHD2B*-, or *RPS6*-overexpressing constructs, and DNA contamination was removed with DNase I digestion. Real time RT-PCR was performed with pre-18 S rRNA forward primer (position in 5′-ETS) and pre-18 S rRNA reverse primer (position in 18 S ribosomal RNA). *AtHD2A*-expressing protoplasts were used as a positive control for the silencing effect, as it has been reported that AtHD2A is associated with the silencing of rRNA genes (25).

These results directly demonstrated that an increase in the *AtHD2A* expression caused a decreased pre-rRNA transcript level (Fig. 3*A*). Co-expression of both *AtHD2A* and *RPS6* in the protoplasts resulted in a more dramatic suppression of the pre-18 S rRNA transcript level (Fig. 3*A*), raising the possibility of a functional association of RPS6 with AtHD2A in the regulation of pre-rRNA transcription. However, as shown in Fig. 2, RPS6 might be specifically associated with AtHD2B, but not AtHD2A. Thus, the elevated inhibition of pre-18 S rRNA transcription observed in RPS6/AtHD2A-overexpressing protoplasts is likely to be caused by an independent synergistic effect of AtHD2A and RPS6 on rDNA transcription. Similar to the inhibitory effect observed from *AtHD2A* overexpression, protoplasts overexpressing *AtHD2B*, *RPS6*, or both *AtHD2B* and *RPS6* constructs all exhibited down-regulation of pre-18 S rRNA synthesis with a concomitant decrease in some of the ribosomal protein transcriptions (Fig. 3*B*). This indicates that both AtHD2B and RPS6 are negatively involved in regulating rDNA transcription, probably as a single functional entity-protein complex. Co-expression of both AtHD2B and RPS6 did not result in an additional decrease in transcript levels of pre-18 S rRNA compared with those in AtHD2B- or RPS6-expressing protoplasts.

Transcription of an mRNA gene driven by rDNA promoter has been successfully demonstrated previously in frog oocyte (31). To establish a convenient assay system for the transcriptional activity of rDNA in plants, we made transgenic plants expressing the P_{rDNA}-*GUS* construct, which contains a 500-bp fragment, including the minimal *Arabidopsis* rDNA promoter region (32) fused with the GUS reporter. Transcription of this chimeric construct was confirmed to produce mRNA of GUS coding sequences with a poly(A) tail (supplemental Fig. S2*C*). To test if GUS expression driven by P_{rDNA}-*GUS* is correlated to an endogenous rRNA transcription level, we made a comparison between GUS expression in P_{rDNA}-*GUS* transgenic plants and the transcription level of endogenous pre-18 S rRNA after the treatment with several plant hormones and salt stress. The endogenous pre-18 S rRNA level was about two times higher after auxin, cytokinin, or both auxin and cytokinin treatments, but it was significantly lower with abscisic acid or NaCl treatments (supplemental Fig. S2). GUS expression in P_{rDNA}-*GUS* transgenic plants was also increased in the presence of auxin or cytokinin but was decreased by abscisic acid. This means that P_{rDNA}-*GUS* transgenic plants can be used to monitor transcrip-

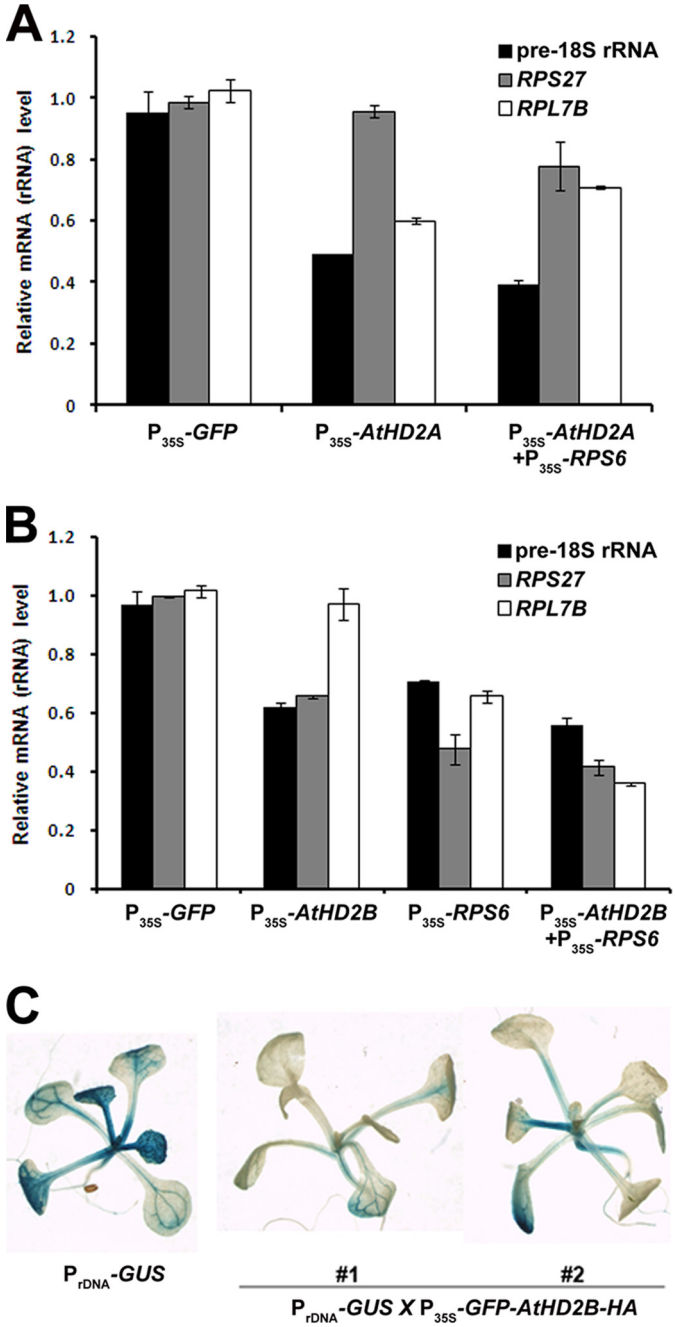


FIGURE 3. **rDNA transcription regulated by AtHD2s and RPS6.** *A*, transcript levels of rDNA and a few ribosomal protein genes in *AtHD2A*-expressing *Arabidopsis* protoplasts. Protoplasts were transfected with control plasmid (P_{35S}-*GFP*), *AtHD2A*-expressing plasmid (P_{35S}-*AtHD2A*), or both *AtHD2A*- and *RPS6*-expressing plasmids (P_{35S}-*AtHD2A* and P_{35S}-*RPS6*). *Error bars* represent standard deviations ($n = 3$). *B*, transcript levels of rDNA and a few ribosomal protein genes in *AtHD2B*-expressing *Arabidopsis* protoplasts. Protoplasts were transfected with control plasmid (P_{35S}-*GFP*), *AtHD2B*-expressing plasmid (P_{35S}-*AtHD2B*), *RPS6*-expressing plasmid (P_{35S}-*RPS6*), or both *AtHD2B*- and *RPS6*-expressing plasmids (P_{35S}-*AtHD2B* and P_{35S}-*RPS6*).RNAs were extracted from the transfected protoplast, and then real time RT-PCRs were performed. Expression level of *ACTIN2* was used as an internal control. *Error bars* represent standard deviations ($n = 3$). *C*, GUS expression in transgenic plants with P_{rDNA}-*GUS* and in F2 progeny of a cross between P_{rDNA}-*GUS* transgenic plants and P_{35S}-*AtHD2B*-expressing plants.

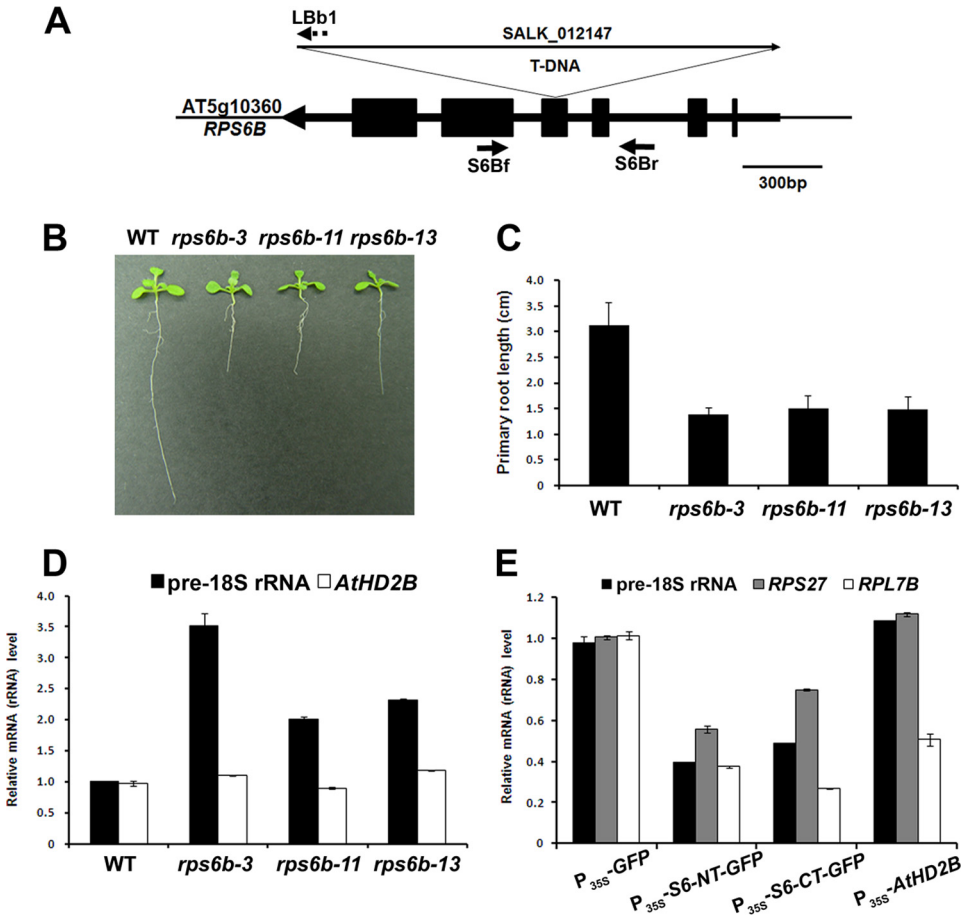


FIGURE 4. **Root development in *rps6b* mutant.** *A*, scheme of the T-DNA insertion in the *RPS6B* gene. Exons are indicated by *filled boxes*. Gene-specific primers (*S6Bf* and *S6Br*) and a T-DNA border primer (*LBb1*) are indicated by *arrows* and a *dotted arrow*, respectively. *B*, root growth in 14-day-old seedlings of wild type and those of *rps6b* mutant lines. *C*, quantitative comparison of primary root lengths of wild type and *rps6b* mutants shown in *B*. *Error bars* represent standard deviations ($n \geq 10$). *D*, expression of pre-18 S rRNA and *AtHD2B* in wild-type and *rps6b* seedlings examined by real time RT-PCR. The results shown are representative of more than three independent experiments. Data shown represent the mean ± S.D. ($n > 3$). *E*, expression of pre-18 S *rRNA*, *RPS27*, and *RPL7B* in *Arabidopsis* protoplasts with RPS6-expressing plasmids examined by real time RT-PCR. *Arabidopsis* protoplasts were isolated from *rps6b* mutant plants and then transfected with control plasmid (P_{35S}-*GFP*), *RPS6*-expressing plasmids (P_{35S}-*S6NT-GFP* and P_{35S}-*S6CT-GFP*), or *AtHD2B*-expressing plasmid (P_{35S}-*AtHD2B*). The results shown are representative of more than three independent experiments. Data shown represent the mean ± S.D. ($n > 3$).

tion of rRNA and related genes. To further confirm whether rDNA transcription is negatively regulated by AtHD2B in *Arabidopsis* utilizing this system, P_{rDNA}-*GUS* transgenic plants were crossed with transgenic plants containing GFP-tagged AtHD2B construct. Consistent with the results in AtHD2B-overexpressing protoplasts, the GUS expression level of P_{rDNA}-*GUS* construct was significantly decreased in *GFP-HD2B-HA*-containing F2 transgenic plants compared with that of wild type (Fig. 3*C*). These results further support the negative role of the AtHD2B/RPS6 interaction in the regulation of rDNA transcription.

Effect of RPS6 Expression on rDNA Transcription and Root Growth—There are two copies of nearly identical ribosomal protein S6 genes, *RPS6A* (AT4g31700) and *RPS6B* (AT5g10360), in *Arabidopsis* (33). The deduced amino acid sequences of RPS6A and RPS6B predict 251 and 250 amino acids, respectively, with 95% sequence identity between them at the amino acid level, which strongly suggest the possible interaction of AtHD2B with RPS6A as well. To evaluate the function of the *RPS6* gene in *Arabidopsis* development, we used a T-DNA insertion mutant having the decreased expression of *RPS6B*. The T-DNA insertion line was obtained from ABRC, and the position of the T-DNA insertion in this line was confirmed by genomic PCR and RT-PCR (Fig. 5*C* and data not shown). Based on genomic PCR and sequencing of PCR products, the *rps6b* mutant line was found to have T-DNA inserted in exon 4 of the *RPS6B* gene and was confirmed to be a homozygous line (Fig. 4*A*). RT-PCR analysis revealed that the expression level of *RPS6B* was dramatically reduced in three *rps6b* mutant lines compared with the wild-type Columbia-0 (data not shown). Although no apparent abnormality was observed in leaves and shoot apices of *rps6b* mutant seedlings, significant differences in root length were found in 14-day-old seedlings (Fig. 4*B*). The average root length of *rps6b* seedlings was about 50% shorter than that of WT (Fig. 4*C*). These results suggest that down-regulation of RPS6B significantly inhibits root growth apparently affecting ribosome biogenesis.

To determine whether the lack of expression of *RPS6B* was directly responsible for the *rps6b* mutant phenotype, the *rps6b* mutant was transformed with a full-length *RPS6B* cDNA under the control of native promoter (P_{RPS6B}-*RPS6B*). The full-length *RPS6B* cDNA rescued the root length to wild type in T2 gener-

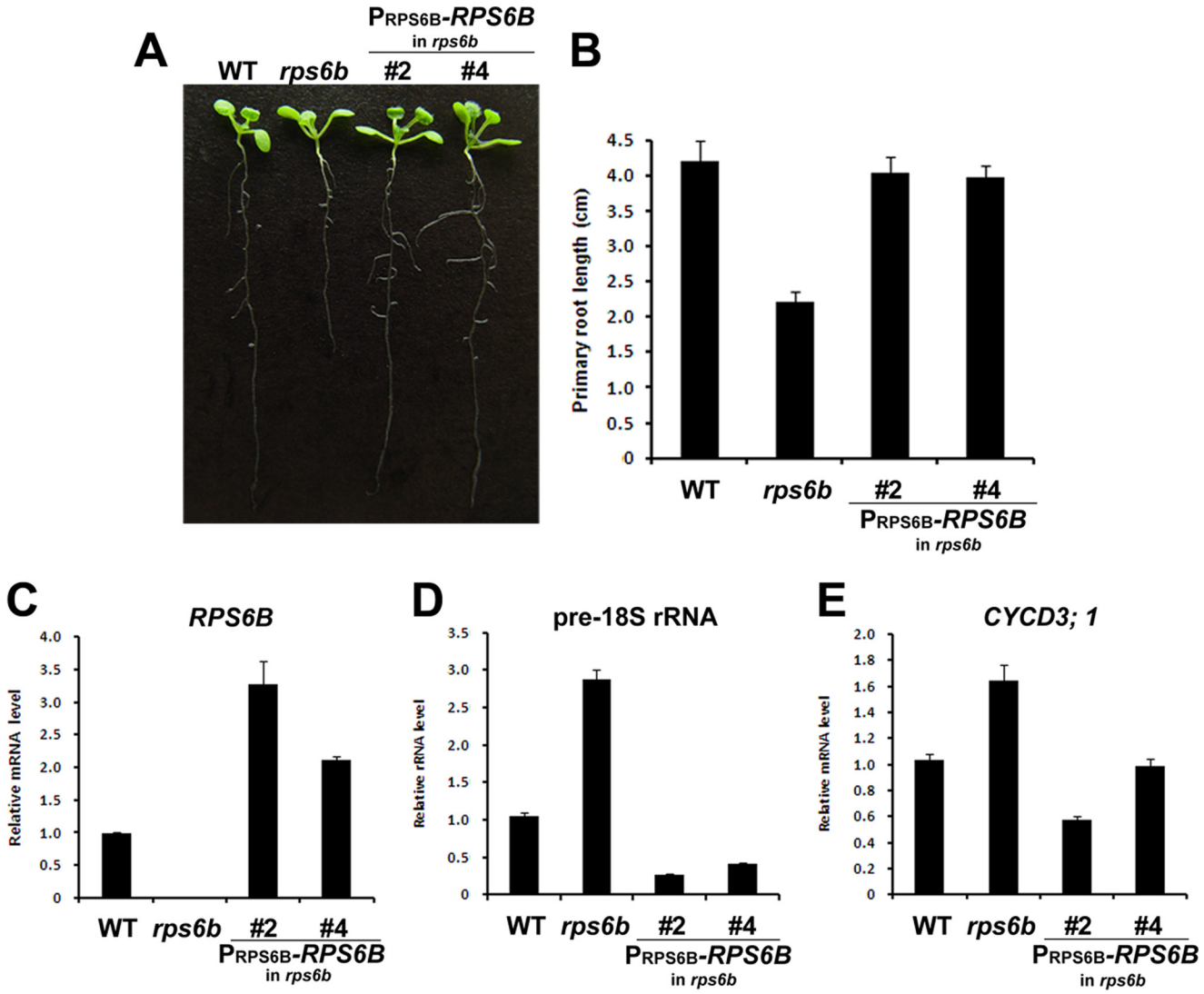


FIGURE 5. **Transgenic complementation of the *rps6b* mutant.** The *rps6b* plants were transformed with P_{RPS6B}-*RPS6B*, a complementation construct, to check if the mutant phenotype was caused by defective expression of *RPS6B*. *A*, root length of wild type (*WT*), *rps6b* mutant, and transformants with P_{RPS6B}-*RPS6B*. *B*, comparison of primary root lengths of wild type, *rps6b* mutant, transformants with P_{RPS6B}-*RPS6B* shown in *A*. *Error bars* represent standard deviations ($n \geq 10$). *C–E*, expression of *RPS6B* (*C*), pre-18 S *rRNA* (*D*), and *CYCD3;1* (*E*) in seedlings of wild type (*WT*), *rps6b* mutant, transformants with P_{RPS6B}-*RPS6B*, respectively. The results shown are representative of more than three independent experiments. Data shown represent the mean ± S.D. ($n > 3$).

ation plants carrying P_{RPS6B}-*RPS6B* construct in a T-DNA insertion mutant of *rps6b* (Fig. 5, *A* and *B*), indicating that a functional copy of *RPS6B* can complement the observed *rps6b* phenotype.

A possible mechanism by which RPS6 could control rRNA gene transcription was explored by measuring the expression level of pre-18 S rRNAs in *rps6b* mutants. Increased transcript level of pre-18 S rRNA was observed in three *rps6b* mutants (Fig. 4*D*), providing the evidence that RPS6 participates in rRNA biogenesis in plants. However, the expression level of AtHD2B was not affected in the *rps6b* mutant indicating that RPS6B did not have any effect on AtHD2B transcription. Consistent with the phenotypic complementation in the *rps6b* mutant background, the full-length *RPS6B* cDNA restored the transcript level of pre-18 S rRNA (Fig. 5*D*).

To test if the increased level of pre-18 S rRNA in the *rps6b* mutant resulted from down-regulation of *RPS6B*, protoplasts isolated from *rps6b* mutant were transfected with the RPS6 N-terminal region or RPS6 C-terminal region-expressing plasmids. As expected, RPS6-NT or RPS6-CT overexpression resulted in decreased pre-18 S rRNA and some ribosomal protein gene expressions as compared with those in the *rps6b* mutant. However, in the *rps6b* mutant, *AtHD2B* overexpression did not result in decreased rRNA transcript levels (Fig. 4*E*), which suggests that functional RPS6 may be necessary for exerting the negative effect of AtHD2B on rDNA transcription. The expression levels of pre-18 S rRNA and *CYCD3.1*, both of which are known to be induced by cytokinin, were found to be increased in the *rps6b* mutant (supplemental Fig. S3). This is consistent with the above phenotypic complementation by *RPS6B*, which also reduced the transcript levels of pre-18 S rRNA and *CYCD3:1* (Fig. 5, *D* and *E*).

Binding of RPS6 to rDNA Promoter—To determine whether the down-regulation of rDNA transcription by the RPS6-AtHD2B complex is mediated through a physical interaction of the complex with the rDNA promoter, thereby regulating

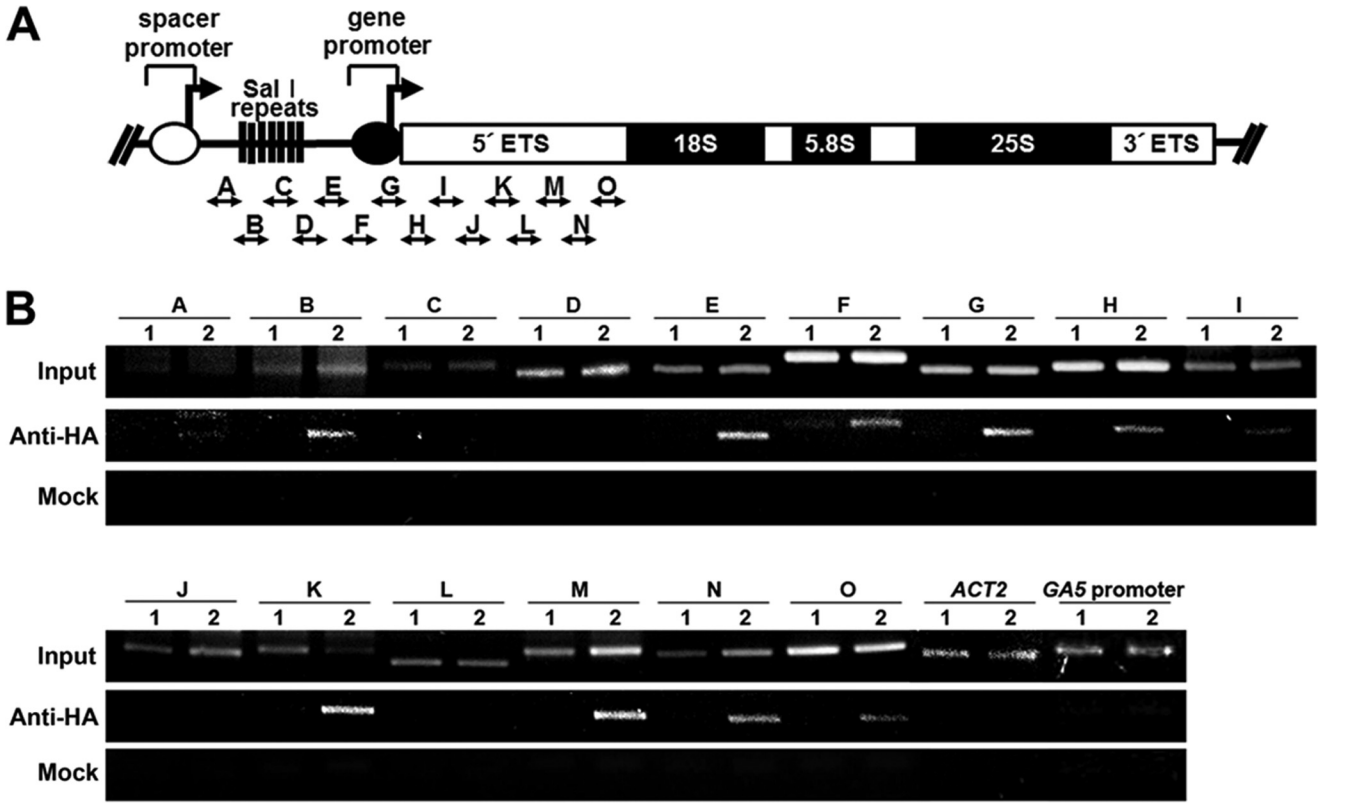


FIGURE 6. **Interaction of RPS6 with rDNA promoter and its 5′-ETS.** *A*, structure of ribosomal RNA genes, including an intergenic spacer, the gene promoter, the ETS, and the coding region for the 18 S, 5.8 S, and 25 S rRNA. DNA fragments used in chromatin precipitation (*ChIP*) (fragments *A*–*O*) are shown. *B*, ChIP with anti-HA antiserum followed by PCRs. *Input* is chromatin before immunoprecipitation. *Mock* is ChIP product with no antibody. *Lane 1,* ChIP product using chromatin of wild type. *Lane 2,* ChIP product using chromatin of transgenic plants with P_{35S}-*3HA-RPS6*.

rDNA transcription (9–11), chromatin immunoprecipitation (ChIP) was performed using chromatin from transgenic plants expressing HA-tagged RPS6B. Eighteen primer pairs covering the 45 S rRNA promoter and 5′-ETS regions (32, 34) were used in amplifying chromatin fragments after isolating DNA associated with RPS6 by immunoprecipitation with anti-HA antibody (Fig. 6*A*). Regions B, E–I, K, and M–O were significantly amplified using chromatin fragments extracted from *3HA-RPS6*-expressing transgenic plants, whereas no specific band was observed using those from wild-type control (Fig. 6*B, middle section*). A mock experiment without adding anti-HA antibody in ChIP reaction also did not produce any specific band (Fig. 6*B, bottom section*). In addition, nonspecific binding of RPS6 to an actin gene (*ACT2*) or *GA5* promoters was not observed, ensuring specificity of the RPS6 binding to the rDNA promoter. These results suggest that RPS6 is involved in the control of rDNA transcription by binding to the promoter and 5′-ETS of the ribosomal RNA gene in *Arabidopsis*. The binding of RPS6 with the rDNA promoter and specific interaction of RPS6 with HDA2B may bring HD2B in close contact with the rDNA chromatin affecting the acetylation state of histones and thus controlling transcriptional activation of these genes.

DISCUSSION

Regulation of rRNA Genes via Histone Deacetylases—Multiple mechanisms have been implicated in the regulation of rRNA gene transcription (1, 4). Based on the result of this study, we suggest that histone deacetylase AtHD2B is involved in the regulation of rDNA transcription via the interaction of its promoter with the RPS6B protein. AtHD2B was identified as one of the interacting proteins using a pulldown assay (Fig. 1 and supplemental Fig. S1).

Maintenance of nucleolar dominance, a phenomenon in hybrids or allopolyploids, requires that only rDNA genes inherited from one parent are transcribed (34–37). Histone deacetylases and methylases control rDNA genes and thus regulate the epigenetic on/off switch (gene dosage), providing an important mechanism in the biogenesis of ribosomes (25, 27). It has been demonstrated that the activity of the yeast RPD3 is required for nucleolar reorganization and RNA pol I delocalization via its association with the rDNA chromatin, which can be antagonized by TOR (13). In *Arabidopsis*, two histone deacetylases, HD2A and HDA6 that are localized to the nucleolus, have been reported to directly interact with the rDNA promoter to repress rRNA transcription via epigenetic alteration of rDNA chromatin acetylation on histone H3, and knockdown of these histone deacetylases results in increased transcription of the rDNA genes (25, 30).

Our results identified AtHD2B as a new regulator of the rDNA transcription, in addition to the aforementioned two histone deacetylases. Of particular interest in this finding is that the regulatory activity of AtHD2B involves interaction with RPS6, a component of the 40 S ribosome and a substrate of the S6 kinase, AtS6K (Figs. 1–3). This observation was further substantiated by our ChIP results as shown in Fig. 6, which dem-

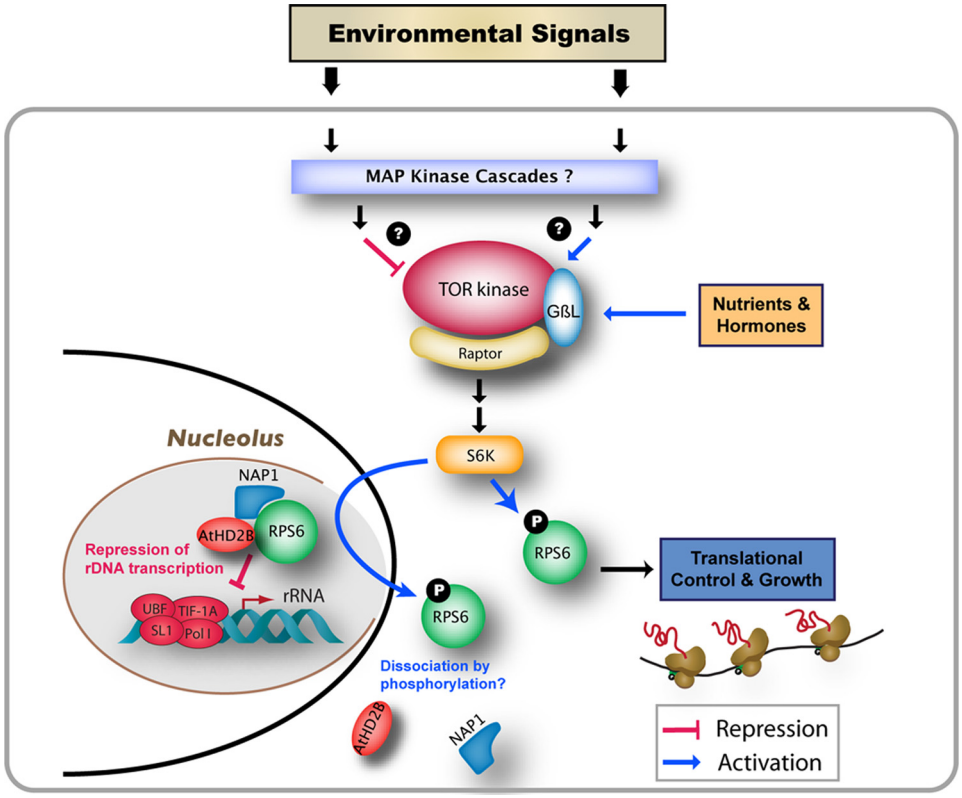


FIGURE 7. **Model for plant growth control by TOR complex via the interaction between AtHD2B and RPS6.**

onstrated specific binding of the RPS6 to the *Arabidopsis* rDNA promoter region and thereby suggested a possible role of RPS6 as a component of the repressor complex for the rDNA transcription. It is likely that the down-regulation of pre-18 S rRNA synthesis observed in *RPS6*-overexpressing protoplasts (Fig. 3) as well as in *rps6b* mutant plants complemented with transgene expression of *RPS6b* (Fig. 4), is a result of the RPS6 binding to the rDNA promoter, thereby recruiting AtHD2B to the proximity and thus repressing rDNA transcription. The ChIP assay data showed the binding footprints of RPS6 over multiple regions of the rDNA promoter at regular intervals, which is consistent with the proposed role of the RPS6-AtHD2B complex in chromatin modification. In this regard, determining how TOR is involved in this process would be a key to fully understand the mechanisms by which the TOR pathway controls rDNA transcription and ribosome biogenesis.

It is possible that a multimeric protein complex containing RPS6 recruits AtHD2B to rDNA promoter to repress rRNA transcription. Among the proteins identified as interacting proteins (Fig. 1*A*), it seems that nucleosome assembly protein 1 (NAP1) is a strong candidate for acting as a bridging factor between the RPS6 and AtHD2B. This is consistent with the fact that several isoforms of NAP1 were isolated as interacting proteins with RPS6 (Fig. 1*A*). In addition, NAP1 has been shown to serve as a bridge between Kap114p and histone H2A and H2B in mediating chromatin assembly (38). Interestingly, the HD2B protein, which is about 39 kDa, was originally identified in maize as a nucleolar targeted phosphoprotein and was purified as an enzyme with a native molecular mass of about 400 kDa (39), suggesting the nature of the RPS6-AtHD2B protein complex as a massive multiprotein complex. Thus, our working model for the RPS6-AtHD2B complex in the control of rDNA transcription illustrated in Fig. 7 portrays NAP1 acting as an adaptor protein bringing the RPS6 and AtHD2B together.

If TOR also binds to rDNA and protein phosphatase 2A (PP2A) is a part of the TOR complex (40), it may regulate the phosphorylation state of RPS6 under stress conditions. We have shown that one of the AtS6Ks is localized in the nucleolus (17), and RPS6 phosphorylation in the nucleolus occurs during active ribosome biogenesis. The phosphorylation of RPS6 in the cytoplasm promotes translation (41), and this phosphorylation state is directly linked to stress signals (17, 42). Phosphorylation of RPS6 by AtS6K may cause dissociation of this complex, directly linking rRNA biogenesis to the TOR signaling pathway. This is consistent with the negative effect of AtHD2B on the regulation of rDNA transcription that was abolished in the *rps6b* mutant, and it also offers a possible explanation on the correlations between dephosphorylation of RPS6 and repression of rDNA transcription observed in our previous work (17) and this study, suggesting that AtHD2B regulates rRNA transcription levels through the activity of RPS6, which may be dependent on its phosphorylation state.

Similar to yeast and animals (9, 10), binding of the *Arabidopsis* TOR to the rDNA promoter has recently been reported (11). However, our ChIP data suggest that the region of the rDNA promoter to which RPS6 binds is largely different from the binding sites for *Arabidopsis* TOR reported by Ren *et al.* (11). In contrast to the fact that the activity of TOR has been positively implicated in transcriptional regulation of rDNA in the study of Ren *et al.* (11), as well as in those of yeast and animals (9, 10), our

results show an RPS6-dependent mechanism in transcriptional repression of the rDNA. However, it is possible that the RPS6 exists as a component of the nucleolar TOR complex and plays a negative regulator for the rDNA transcription, antagonizing the positive effect provided by the TOR kinase.

Extraribosomal Function of RPS6 Links Stress Signals to Ribosome Biogenesis—It has been recognized that many ribosomal proteins have additional functions besides their role as components of the ribosome, namely the extraribosomal functions, including mRNA processing and translational control of their own genes (42, 43). In animal cells, RPS6 has also been implicated in the regulation of protein synthesis through association with 5′-TOP tract mRNA, such as RPL11 and RPS16 mRNAs, suggesting that RPS6 has a role of a negative regulator in the translation of 5′-TOP mRNAs (21). With the apparent lack of the 5′-TOP motif in plant mRNA structures, it is not certain whether the same mode of translational control by RPS6 is conserved in plants. Our findings revealed a novel extraribosomal function of RPS6 in regulating rRNA transcription via interaction with HD2B. Because the levels of rRNA transcripts and the amount of ribosomal proteins for ribosome assembly are tightly regulated according to the need for protein synthesis in eukaryotic cells, the mechanism proposed here may allow shutting down proliferative activities of cells to minimize wasting cellular resources in response to the environmental signals.

The data presented here provide evidence for the involvement of RPS6 in possible control of plant rRNA gene expression via its interaction with AtHD2B, although given that the catalytic activity of AtHD2B has not been verified unlike that of AtHD2A (25), it cannot be ruled out that the repression of rDNA by AtHD2B could be achieved through a mechanism not involving the chromatin modification. RPS6 has been identified as the first protein to be phosphorylated in response to various signals (44, 45). The presence of S6K in the nucleolus and demonstration that TOR phosphorylates RPS6 via S6K, which is sensitive to osmotic stress (17), and the direct binding of TOR to rDNA promoter (11), may control optimum transcription of rRNA according to the environmental conditions and gene dosage. Such a mechanism would allow environmental signals to be transduced via TOR to control not only translation but also transcription (particularly of rRNA) for optimum growth of the plant under a given environment (Fig. 7). AtHD2B may play a critical role in linking the translation and transcription machineries to the transduction of such signals. Further studies may resolve the exact roles of AtHD2B, S6K, and TOR in this process.

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