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Original Paper

Arabidopsis AtRRP44 has ribonuclease activity that is required to complement the growth defect of yeast *rrp44* Mutant

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Abstract The RNA exosome is a multiprotein complex responsible for 3′ to 5′ degradation and processing of various classes of RNAs in eukaryotes. Rrp44/Dis3 is the catalytic center of the RNA exosome in yeast and human. Previously, we identified *Arabidopsis thaliana* RRP44 (AtRRP44) as a single functional homolog of Rrp44/Dis3. Although AtRRP44 is potentially a catalytic center of the plant RNA exosome, the ribonuclease activity of AtRRP44 has not been tested. Here, we show that AtRRP44 has ribonuclease activity using *in vitro* translated recombinant proteins. Mutation of the aspartic acid residue D489 of AtRRP44 to asparagine (D489N) resulted in loss of ribonuclease activity, indicating that aspartic acid is at the active site. The wild-type AtRRP44 protein rescued the growth defect of *Saccharomyces cerevisiae rrp44* mutants, but the D489N mutated AtRRP44 did not. This finding suggests that the ribonuclease activity of wild-type AtRRP44 is required for yeast cell viability. We also showed that AtRRP44 was highly expressed in organs experiencing active cell turnover, such as shoot apical meristem, root apical meristem, and lateral root primordium. Along with previous studies showing that loss of RRP44 in *Arabidopsis* is lethal, our results suggest that AtRRP44 has ribonuclease activity that is related to plant development.

Key words: *Arabidopsis thaliana*, AtRRP44, DIS3, RNA degradation, RNA exosome.

RNA processing and RNA decay are critical to the survival of eukaryotic cells. After transcription, precursors of functional RNAs such as ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) are processed into functional forms in the nuclei. Unnecessary or aberrant mRNAs are potentially toxic RNAs, which are degraded rapidly in the cytoplasm. In these processing and decay reactions, various ribonucleases in 5′ to 3′ and 3′ to 5′ RNA decay pathways play essential roles (Chiba and Green 2009; Houseley and Tollervey 2009).

The 3′ to 5′ RNA degradation is catalyzed mainly by the RNA exosome (Mitchell et al. 1997). The RNA exosome is highly conserved among eukaryotes (including plants, yeasts, and humans) and is essential for viability (Belostotsky and Sieburth 2009; Houseley et al. 2006; Siwaszek et al. 2014). A vast range of RNAs is targeted by the RNA exosome from maturation through constant quality control to final decay. The characteristic structure of the RNA exosome allows it to perform its various functions.

The RNA exosome is a multi-protein complex composed of two main groups, the exosome core and cofactors (Chlebowski et al. 2013). The exosome core consists of nine components. Six of the components contain RNase PH domains (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46, and Mtr3) and are organized into a hexamer. The hexamer is capped on one side by a trimer of subunits that contain S1 RNA-binding domains (Csl4, Rrp4, and Rrp40). In human and yeast, the exosome core proteins have no RNA degradation activity, but instead, function as a scaffold of cofactors that have endoribonuclease and exoribonuclease active sites (Bonneau et al. 2009; Liu et al. 2006). Two RNA exosome cofactors, Rrp6 and Rrp44/Dis3, bind directly with the exosome core and confer the exosome's catalytic activities (Houseley and Tollervey 2009; Januszyk and Lima 2014). Rrp6 belongs to the RNase D family and functions in the degradation of rRNA maturation by-products, mRNA surveillance, and the processing of several non-coding

Abbreviations: AtRRP44, *Arabidopsis thaliana* ribosomal RNA-processing protein 44; *S. cerevisiae*, *Saccharomyces cerevisiae*; *A. thaliana*, *Arabidopsis thaliana*.

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RNAs (Allmang et al. 1999; Briggs et al. 1998; Lange et al. 2008; Mitchell et al. 1997; Shin and Chekanova 2014; Wasmuth et al. 2014). Rrp44/Dis3 is a catalytic center of the RNA exosome and has both endoribonuclease and exoribonuclease activities in yeast and human (Allmang 1999; Belostotsky and Sieburth 2009; Houseley et al. 2006; Kinoshita et al. 1991; Lebreton et al. 2008; Schneider et al. 2009; Siwaszek et al. 2014; Tomecki et al. 2010). In yeast, loss of Rrp44/Dis3 is lethal as well as loss of each subunit of the exosome core (Chlebowski et al. 2013; Lebreton et al. 2008; Schneider et al. 2009). Humans (*Homo sapiens*) have two homologs of the Rrp44/Dis3 gene, DIS3 and DIS3L, whereas yeast (*S. cerevisiae*) and plants (*A. thaliana*) have one (Bonneau et al. 2009; Dziembowski et al. 2006; Kumakura et al. 2013; Liu et al. 2006; Staals et al. 2010).

In *Arabidopsis*, well known genes encoding exosome core components and the cofactors, AtRRP44 (previously called AtRRP44A) and AtRRP6Ls, have been determined (Chekanova et al. 2007; Kumakura et al. 2013; Lange et al. 2008). Association between nine exosome core components and the cofactor AtRRP44 were detected by a combination of immunoprecipitation and mass spectrometry analysis (Lange et al. 2014). Of the nine exosome core components in *Arabidopsis*, the roles of AtRRP4, AtRRP41, AtRRP41L/MTR3, and AtRRP45B/CER7 have been investigated mainly by genetic analysis (Chekanova et al. 2000, 2007; Hooker et al. 2007; Lam et al. 2012; Yang et al. 2012). AtRRP45 and AtRRP41L/MTR3, for example, are required for the regulation of wax synthesis and early development, respectively, through the control of RNA stability. These studies indicate that characterizing the functions of the RNA exosome can also uncover more information about the regulatory mechanisms of plant development. However, the role of AtRRP44, a putative catalytic center of the RNA exosome, is still obscure.

Previously, we characterized two *Arabidopsis* Rrp44/Dis3 candidate genes, AtRRP44 (AT5G17510) and AtRRP44B/SOV/DIS3L2 (AT1G77680) (Kumakura et al. 2013). Using genetic analysis and yeast complementation assays, we showed that AtRRP44 is an *Arabidopsis* exosome component, but AtRRP44B/SOV/DIS3L2 is not. Although our results implied that AtRRP44 possessed ribonuclease activity, this speculation has yet to be confirmed experimentally.

Here, we demonstrated that AtRRP44 had ribonuclease activity using *in vitro* translated recombinant proteins and showed that the 489th aspartic acid residue (D489) in the RNA binding domain was essential for the catalytic activity of AtRRP44. Although AtRRP44 could complement the growth defect of *S. cerevisiae* inducible *rrp44* mutants, D489N mutated AtRRP44 proteins failed to complement the phenotype, suggesting that AtRRP44 ribonuclease activity is required for cell viability. Promoter *Escherichia coli* β-glucuronidase (GUS) assays showed that AtRRP44 was specifically expressed in tissues where cell division and differentiation were dynamic. Our results offer a new insight into AtRRP44 that is important for plant RNA metabolism and development.

Materials and methods

Protein domain and catalytic residue prediction

The full length amino acid sequences were obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org) and The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org): ScRrp44 (YOL021c), HsRRP44/DIS3 (AB001743.1), AtRRP44 (AT2G17510.1) and NPR1 (AT1G64280.1). Sequence similarity, domain identification, and alignment of these proteins were calculated and/or obtained from EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/), InterProScan 4 (http://www.ebi.ac.uk/Tools/pfa/iprscan/), and Tcoffee (http://www.igs.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi), respectively.

Plasmids

Plasmids used in this study are listed in Table S2. See the supplementary method for details of the plasmids.

In vitro translation and immunoprecipitation

NPR1, AtRRP44, D132ND158N, D489N and D132ND158ND489N recombinant proteins tagged with FLAG epitope tags at their C-termini were *in vitro* translated using a cell-free translation system (BioSieg, Tokushima, Japan) according to the manufacture's protocol. Then recombinant proteins were immunoprecipitated using anti-FLAG M2 monoclonal antibodies (Sigma-Aldrich, St. Louis, MO, USA) and protein G Sepharose 4 fast flow beads (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. Immunoprecipitates were analyzed by western blot (Figures 1, 2B). Buffers of the recombinant proteins combined with sepharose beads were substituted by 2×reaction buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 400 nM $MgCl_2$, and 2 mM 2-mercaptoethanol) and used for RNA decay assays.

RNA decay assay

Substrate RNAs used in Figure 1B were transcribed from PCR4_CMVQ QR1, which was digested with *Xba*I (Life technologies, Carlsbad, CA, USA), by T7 RNA polymerase. The sequence transcribed by T7 RNA polymerase, CMVQ QR1, a partial sequence derived from the internal sequence of the *cucumber mosaic virus* genome RNA1, is in the supplementary methods. Then, transcribed 286 mer RNAs were labeled with [α-^{32}P]-GTP (111 TBq mmol^{-1}, 400 MBq ml^{-1}) using the Vaccinia Capping System (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol.

Substrate RNAs used in Figure 2B, 30 mer oligoribonucleotides 16-A14 (5′-CCC ACC ACC AUC ACU

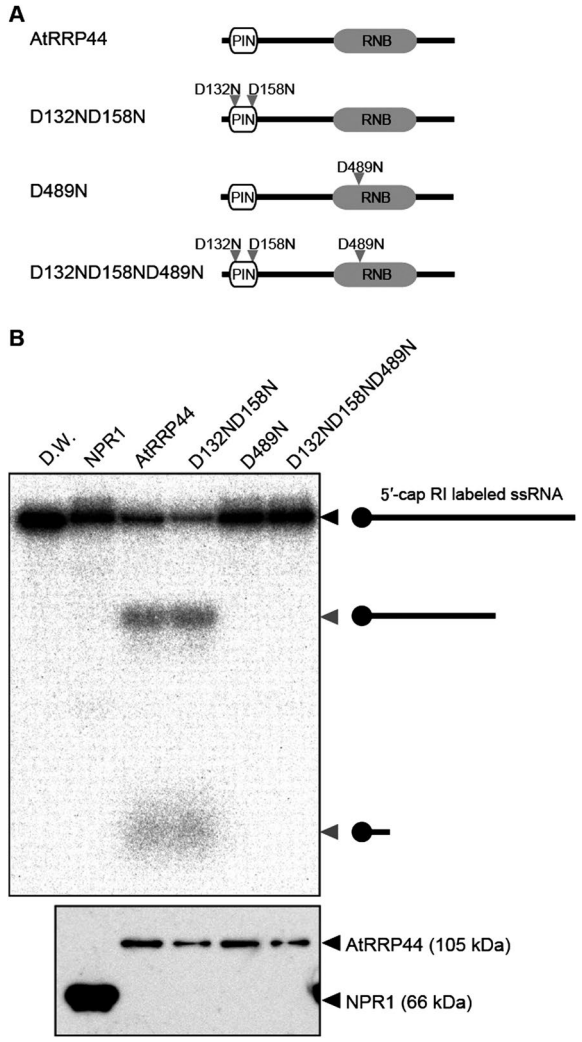

Figure 1. RNA decay assay showing AtRRP44 has ribonuclease activity *in vitro*. A. Schematic representation of wild-type AtRRP44 and mutant proteins. B. (Upper panel) RNA decay assay. Distilled water (D.W.) or recombinant proteins were mixed with substrate RNAs, incubated at room temperature, and electrophoresed on a 7 M urea and 15% polyacrylamide gel. Signals on the gel were detected using a BAS-2500 bio-imaging analyzer (GE healthcare). 5′ caps of single ssRNAs (286 mer) were radioisotope (RI)-labeled. Black circles and lines represent their 5′ RI-labeled cap and ssRNAs lengths, respectively. (Lower panel) Western blot of wild-type and mutated AtRRP44 proteins. The proteins were immunoprecipitated and detected using anti-FLAG antibodies after *in vitro* translation.

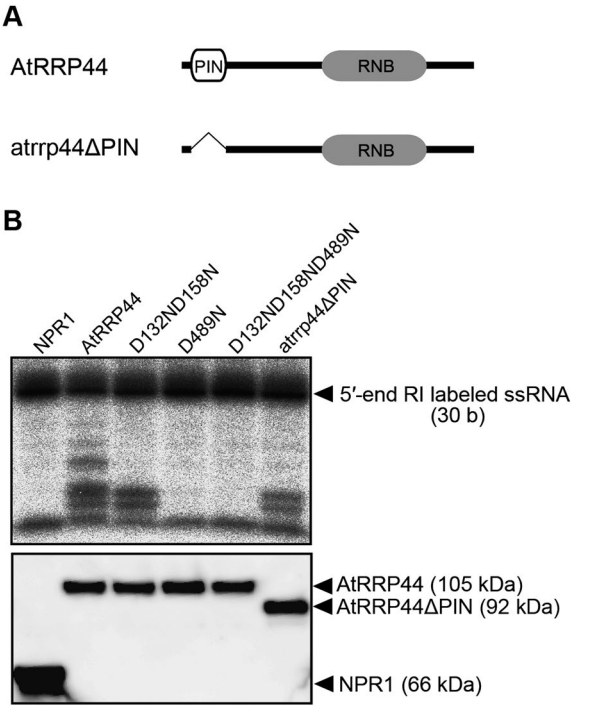

Figure 2. RNA decay assay showing D489 residue endows AtRRP44 with the ribonuclease activity *in vitro*. A. Schematic representation of AtRRP44 and atrrp44ΔPIN (lacking 114 aa from the 50th to 163rd residues of AtRRP44). B. (Upper panel) Recombinant proteins were mixed with substrate RNAs, incubated at room temperature, and electrophoresed on a 7 M urea and 20% polyacrylamide gel. All recombinant proteins were *in vitro* translated and immunoprecipitated. A short 5′-end RI labeled oligoribonucleotide 16-A14 was used as a substrate ssRNA. (Lower panel) western blot of the recombinant proteins. The proteins were immunoprecipitated and detected using anti-FLAG antibodies after *in vitro* translation.

UAAA AAA AAA AAA AA-3′), were synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan). 16-A14 was purified with a urea gel (7 M urea, 10% polyacrylamide) in the same way as mentioned above. 5′-end of 16-A14 was labeled with [γ-^{32}P]-ATP using T4 polynucleotide kinase (New England Biolabs) according to the manufacture's protocol. The labeled RNAs (5′-cap RI labeled ssRNAs and 5′-end RI labeled 16-A14) were purified on urea gels (7 M urea, 6% and 10% polyacrylamide, respectively). Bands of labeled RNAs were cut out from gels. These gels were incubated in the same weight of 2×PK buffer (200 mM Tris-HCl pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% SDS) overnight. Then supernatants were collected and ethanol precipitated. Precipitates were eluted with DEPC-treated water and used as single stranded substrate RNAs.

Immunoprecipitated AtRRP44, D132ND158N, D489N, D132ND158ND489N and atrrp44ΔPIN proteins and substrate RNAs (5′-cap RI labeled ssRNA and 5′-end RI labeled 16-A14) were mixed and incubated for 90 min at room temperature. Reactions were mixed with 2×loading buffer (95% formamide, 10 mM EDTA pH 8, 0.1% bromophenol blue, 0.1% xylene cyanol) and incubated at 65°C for 5 min. Then these mixtures were electrophoresed on 15% (Figure 1B) or 20% (Figure 2B) polyacrylamide gels as previously described (Kumakura et al. 2009; Lebreton et al. 2008). The signals on gels were detected using a BAS-2500 bio-imaging analyzer (GE Healthcare).

S. cerevisiae complementation assay

Complementation assays were performed as previously described (Kumakura et al. 2013). *S. cerevisiae* strain BSY1883 [*KanMX6: TetOFF-DIS3*] was kindly provided by Bertrand Séraphin (Lebreton et al. 2008). To express AtRRP44, D132ND158N, D489N, D132ND158ND489N, atrrp44ΔPIN and an empty vector, the pOH016, pOH027, pOH028, pOH026, and pOH029 plasmids were introduced into the BSY1883 strain, respectively.

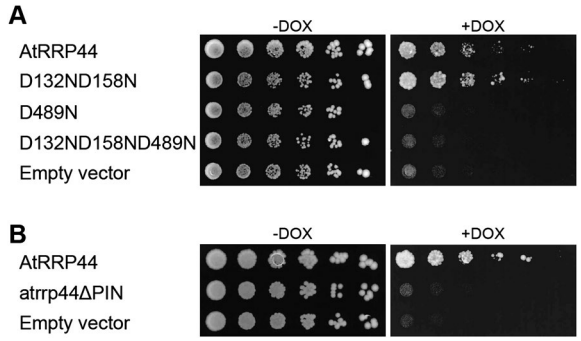

Figure 3. Yeast complementation assays. A. AtRRP44 ribonuclease activity is required for cell viability. AtRRP44 and D132ND158N complement the growth defect of the *S. cerevisiae rrp44* doxycycline (DOX) repressible mutant. Growth phenotypes resulting from the expression of AtRRP44, D132ND158N, D489N, and D132ND158ND489N in *S. cerevisiae* BSY1883 strain, and an empty vector allele were assessed in the presence (+DOX; repressed chromosomal ScRrp44) or absence (-DOX; expressed chromosomal ScRrp44) of DOX (20 μg ml^{-1}) after incubation for 110 h at 30°C in SD media lacking leucine. B. AtRRP44 PIN domain is required for cell viability. atrrp44ΔPIN does not complement the growth defect of *scrrp44* mutant. Growth phenotypes resulting from the expression of AtRRP44, atrrp44ΔPIN and an empty vector in *S. cerevisiae* BSY1883 were assessed in the same way as A.

Transformed BSY1883 strains were incubated at 30°C in SD liquid medium lacking leucine until a concentration of approximately 2×10^7 cells ml^{-1} was reached. Cultures were washed with the same medium and centrifuged. Pellets were frozen in liquid nitrogen and stored at −80°C. For RNA extraction, 250 μl hot bead buffer (15 mM NH_4OAc, 10 mM EDTA) was added to each sample and mixed with acid-washed glass beads, 25 μl 10% SDS, and 300 μl phenol acid (pH 4.5). Mixtures were blended for 1 min and incubated at 65°C for 1 min. This treatment was repeated for three times. Then, the samples were centrifuged at 18,000×*g* for 15 min and 200 μl supernatant was added with the same volume of bead buffer (15 mM NH_4OAc, 10 mM EDTA) and 400 μl of chloroform:isoamyl alcohol (24:1). These mixtures were centrifuged at 18,000×*g* for 15 min at room temperature. Then 300 μl of supernatant were blended with 600 μl isopropanol and 20.4 μl of 1.5 M NH_4OA_C. Samples were centrifuged at 18,000×*g* followed by incubation at 4°C for 30 min. Pellets were washed with RNase-free 70% ethanol and air-dried followed by elution with 16 μl RNase-free DEPC-treated water. Reverse transcription and qRT-PCR were performed as previously described (Motomura et al. 2012). The primers that were used are listed in Table S1.

Promoter GUS assay

To test the AtRRP44 promoter activity, wild-type Col-0 plants were floral-dipped and transformed with the pOH018 plasmid as previously described (Kumakura et al. 2013). Transgenic plants, which showed the predominant expression pattern, were selected from eight primary independent transformants (Figure S5) and used in Figure 4. Transformants were grown on agar solidified half-strength Murashige and Skoog (MS) medium or on mixed Jiffy mix (Sakata Seed Corp., Yokohama, Japan) and vermiculite soil at 23°C under continuous light. Plant tissues were incubated in GUS staining solution (5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 100 mM NaH2PO4 (pH 7.0), 0.1% Triton X-100, and 0.5 mg ml^{-1} X-gluc) in the decompressed state, followed by incubation at 37°C for 16 h (embryos) or 36 h (other tissues). Then, GUS staining solution was removed and the tissues were destained in 70% and 90% ethanol, and then incubated with fixation buffer (ethanol:acetic acid=9:1) at room temperature for 30 min. Samples were washed with 70%, 50%, 30%, 10% ethanol, and double-distilled water, cleared by clearing solution, and examined on MZ12 stereo microscope (Leica microsystems, Wetzlar, Germany).

Results

Arabidopsis Rrp44/Dis3 has RNA degradation activity in vitro

Arabidopsis AtRRP44, which has been found to be associated with the RNA exosome and to be able to complement growth defects of yeast *rrp44*/*dis3* mutants (Kumakura et al. 2013; Lange et al. 2014), was investigated for its RNA degradation activity. In general, Rrp44/Dis3 has two important domains, a pilT N domain (PIN) and an RNase B (RNB) domain. In yeast and human Rrp44/Dis3 proteins, both the PIN and RNB domains have ribonuclease activity (Dziembowski et al. 2006; Lebreton et al. 2008; Liu et al. 2006; Schneider et al. 2009; Tomecki et al. 2010). We aligned the amino acid sequences of *S. cerevisiae* Rrp44 (ScRrp44), *H. sapiens* RRP44 (HsRRP44) and *A. thaliana* RRP44 (AtRRP44) using ClustalW software (Figure S1). All the catalytic residues reported in ScRrp44 (D171, D198, and D551) and HsRRP44 (D146, D177, and D489) were conserved in AtRRP44 (D132, D158, and D489), implying that AtRRP44 will be catalytically active (Figure S1).

To test the catalytic activity of AtRRP44, RNA decay assays were performed *in vitro*. A series of recombinant proteins tagged with FLAG epitope tags (DYKDDDDK) at their C-termini, AtRRP44, D132ND158N (D132 and D158 residues in PIN domain mutated to N), D489N (D489 residue in RNB domain mutated to N) and D132ND158ND489N (D132, D158 and D489 residues mutated to N) (Figure 1A), were translated using cell-free translation systems. These proteins were immunoprecipitated using anti-FLAG antibody and detected by western blot analysis (Figure 1B, lower panel). Equivalent amounts of wild-type and mutant AtRRP44 proteins were detected. *Arabidopsis* non expressor of PR genes 1 (NPR1) protein (Cao et al. 1997) tagged with a FLAG epitope tag at its N terminus was used as a positive control for protein expression and immunoprecipitation. Then, the purified proteins were mixed with 5′-cap radioisotope (RI) labeled single stranded RNA (ssRNA). This ssRNA, which consists of

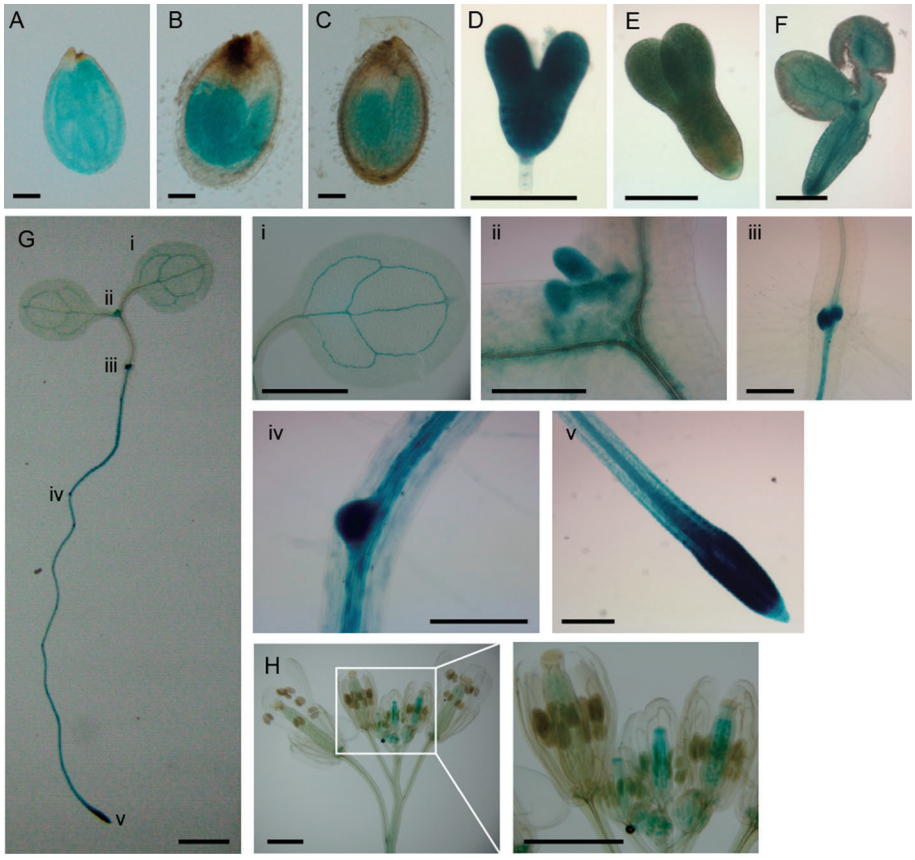

Figure 4. Promoter GUS assay showing AtRRP44 is expressed strongly in tissues actively experiencing high cell turnover. AtRRP44 promoter activity was observed by staining *Arabidopsis* transformants expressing the GUS reporter gene. The GUS reporter gene was fused to the C-terminus of AtRRP44 protein and expressed under the control of about 2.4 kb AtRRP44 promoter and 0.5 kb terminator (Figure S4). A–C. Immature seeds. Scale bars represent 0.5 mm. D–F. Heart-shaped, torpedo-shaped, and matured embryos, respectively. Scale bars represent 0.05 mm. G. Seedling and enlarged images of the seedling: cotyledons (i), shoot apical meristems (ii), base of stem (iii), lateral root primordium (iv), and root apical meristem (v). Scale bars represent 1 mm (G, G i) and 0.1 mm (G ii–v). H. Inflorescence and an enlarged image (right). Scale bars represent 1 mm.

286 bases from the *cucumber mosaic virus*, was chosen randomly as a substrate. After 90 min incubation at room temperature, the mixtures were electrophoresed and RNA bands were detected (Figure 1B, upper panel). In the AtRRP44 and D132ND158N lanes, bands of degraded RNAs were observed (gray arrow heads). These bands were not observed in the D489N and D132ND158ND489N lanes. The data suggested that AtRRP44 RNB domain has ribonuclease activity. As shown in Figure 1B, two discrete sized RNA fragments were observed. The data implied that two discrete sized RNA fragments are due to the complex secondary structure of 5′ cap RI labeled ssRNA (Figure S2).

To check whtether the ribonuclease activity is general on different substrate RNAs, we further performed the RNA decay assay experiments with an different RNA substrate harboring different sequence with possibly less secondary structure, a short 5′-end RI labeled oligoribonucleotide 16-A14 (5′-CCC ACC ACC AUCACUUA$_{14}$-3′) (Reis et al. 2013). As shown in Figure 2B, AtRRP44 and D132ND158N degraded substrate RNAs, but NPR1, D489N and D132ND158ND489N did not. Figures 1B and 2B showed that AtRRP44 has ribonuclease activity on two different RNA substrates and suggested less preference for RNA structures.

Next, to test whether the observed ribonuclease activity is due to D489 in the RNB domain, not to the PIN domain, we performed RNA decay assays with atrrp44ΔPIN protein tagged with a FLAG epitope tag at its C-terminus, which lacks the PIN domain (Figure 2A). Protein expression was confirmed by western blot (Figure 2B, lower panel). As shown in Figure 2B (upper panel), atrrp44ΔPIN degraded 5′-end RI labeled oligoribonucleotide 16-A14 as well as wild-type AtRRP44. This data showed that the measured ribonuclease activity of AtRRP44 does not come from the PIN domain.

Collectively, our results (Figures 1B, 2B) indicate that AtRRP44 has catalytic activity and that the D489 residue in the RNB domain is required for this activity.

AtRRP44 catalytic residue D489 is essential to rescue the growth defect of S. cerevisiae rrp44 mutant

Next, we tested whether the catalytic activity of AtRRP44 is correlated with the ability to complement the yeast *rrp44* mutant. We used the drug-inducible *S. cerevisiae rrp44* (*scrrp44*) mutant in which the essential chromosomal *ScRrp44* gene was under the control of a doxycycline (DOX)-repressible promoter (Lebreton et al. 2008). The AtRRP44, D132ND158N, D489N, and D132ND158ND489N proteins were expressed in this mutant. Wild-type AtRRP44 and D132ND158ND489N proteins complemented the growth defect of the *scrrp44* mutants, while D489N and D132ND158N failed to recover the phenotype (Figure 3A). The levels of the RNA transcripts, which were under the control of a *ADH1* overexpression promoter (Mumberg et al. 1995), were checked by quantitative real-time PCR (qRT-PCR) in yeast cultured without DOX. As shown in Figure S3A, sufficient amounts of the transcripts from each gene were expressed. These results indicated that AtRRP44 was a cofactor for the catalytic activity of RNA exosome and that D489 in the RNB domain was required for in vivo activity.

AtRRP44 PIN domain is required to complement the growth defect of S. cerevisiae rrp44 mutant

AtRRP44 has a PIN domain that is conserved among the Rrp44/Dis3 proteins (Figure S1A). In human and yeast, the PIN domains have been shown to have endonuclease activity and Rrp44/Dis3 associated with the exosome core subunits via the PIN domain (Schneider et al. 2009). To test whether the PIN domain of AtRRP44 was required for cell viability, atrrp44ΔPIN that lacked the PIN domain (Figure 2A) was expressed in *scrrp44* mutants. As shown in Figure 4B, atrrp44ΔPIN failed to complement the growth defect of *scrrp44* mutants in the presence of DOX, although transcripts of atrrp44ΔPIN were detected by qRT-PCR (Figure S3B). These results showed that the PIN domain of AtRRP44 was essential for AtRRP44 function in complementing the growth defect of *scrrp44* mutant.

AtRRP44 is strongly expressed in turnover active organs

Our results showed that AtRRP44 had RNA degradation activity and that this activity was essential for cell viability. To determine the tissues in which AtRRP44 was expressed, we constructed a plasmid (pOH018) to express AtRRP44 fused with a reporter gene, GUS, at the C-terminus (AtRRP44-GUS) under the control of the genomic sequence from about 2.4 kb upstream of the AtRRP44 start codon (Figure S4). In this construct, the genome sequence coding AtRRP44 was used to express the AtRRP44 protein. Further, the sequence approximately 500 bp downstream of the stop codon was used as the terminator. *A. thaliana* ecotype Col-0 was transformed with the construct. Consistent with available microarray data (Arabidopsis eFP Browser: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), GUS activity was observed in all tissues tested (Figure 4). In particular, strong signals were detected in developing organs such as embryos as well as apical and root meristems. Additionally, GUS activity was observed in the vascular tissues of leaves, roots, and stems. Collectively, our data showed that AtRRP44 was strongly expressed in tissues experiencing active turnover of cells.

We also constructed a plasmid to express the GUS protein (without AtRRP44 protein) under the control of the same AtRRP44 promoter and the terminator. No transformants showed GUS staining, though we tested eight primary independent transgenic lines. This result implies that the genomic sequence coding the AtRRP44 protein (containing introns) was somehow required for promoter activity.

Discussion

The multi-subunit RNA exosome is responsible for 3′ to 5′ degradation and processing of a wide range of RNA classes in eukaryotes. In *A. thaliana*, well known exosome core components and cofactors, AtRRP44 and AtRRP6Ls, have been determined (Chekanova et al. 2007; Kumakura et al. 2013; Lange et al. 2009, 2014). Rrp44/Dis3 proteins in human and yeast have ribonuclease activity and are the catalytic centers of the exosome (Dziembowski et al. 2006; Liu et al. 2006; Staals et al. 2010). However, whether or not plant AtRRP44 possessed catalytic activity was not known. Here, we showed that the wild-type AtRRP44 had ribonuclease activity *in vitro* by conducting assays with recombinant proteins translated with a wheat germ cell-free translation system. Though D132ND158N proteins (Figure 1A) showed catalytic activity, D489N protein and D132ND158ND489N protein did not show catalytic activity (Figures 1B, 2B). These data demonstrated the ribonuclease activity of AtRRP44 and indicated that D489 in the RNB domain was required for this activity. The RNB domain in HsRRP44 and ScRRP44 has been shown to be required for the processive exoribonuclease activity of both these proteins (Dziembowski et al. 2006; Lebreton et al. 2008; Liu et al. 2006; Schneider et al. 2009; Tomecki et al. 2010). Laddered RNA fragments were observed in AtRRP44, D132ND158N and atrrp44ΔPIN (Figure 2B), indicating that the AtRRP44 RNB domain may also have exoribonuclease activity, although our results cannot exclude the possibililty of the RNB domain possessing endoribonuclease activity. Although the wild-type AtRRP44 protein complemented the growth defect of the drug inducible *scrrp44* mutant, the D489N

proteins failed to do so, indicating that the ribonuclease activity of AtRRP44 was required for cell viability (Figure 3A).

A mutation or deletion of the RNB domain versions of yeast RRP44 can weakly complement the phenotype of the yeast *rrp44* mutant (Reis et al. 2013; Schaeffer et al. 2009). On the other hand, AtRRP44 D489N failed to complement the yeast *rrp44* mutant (Figure 3A). Previously, we showed that AtRRP44 can complement the yeast *rrp44* mutant (Figure 1B, (Kumakura et al. 2013)), but the complementation of AtRRP44 was weaker than that of ScRrp44 used as a positive control, suggesting that AtRRP44 cannot complement yeast *rrp44* mutant fully. We speculate that this resulted in the difference between published data on yeast complementation assays with versions a point mutation of the ScRrp44 RNB domain, deletion of the ScRrp44 RNB domain (Reis et al. 2013; Schaeffer et al. 2009) and our own results with AtRRP44 D489N.

Based on available co-immunoprecipitation data showing the direct interaction between the exosome core and AtRRP44 (Lange et al. 2014) and our results, we speculate that AtRRP44 endows plant RNA exosome with the catalytic activity. *Arabidopsis* Col-0 transformants expressing AtRRP44-GUS fusion proteins driven by the AtRRP44 native promoter region showed strong signals especially in organs associated with active turnover (such as shoot and root apical meristems as well as lateral root primordia), indicating that AtRRP44 has pleiotropic functions in plant development through RNA degradation and processing.

Previously, AtRRP44 was called AtRRP44A, because two Rrp44/Dis3 candidates, AtRRP44A (AT2G17510) and AtRRP44B/SOV (AT1G77680), were predicted in the *A. thaliana* genome (Chekanova et al. 2007). We analyzed these two genes and concluded that AtRRP44B/SOV worked independently from the exosome and AtRRP44 (AtRRP44A) is a functional ortholog of Rrp44/Dis3 in *A. thaliana* (Kumakura et al. 2013). Therefore, for simplicity, we think that it is suitable to call the AT2G17510 gene product AtRRP44.

In yeast and human cells, Rrp44/Dis3 proteins physically interact with the exosome core via their PIN domains (Schneider et al. 2009). In plants, Lange et al. (2014) reported that AtRRP44 also can interact with the exosome core. To determine the importance of the AtRRP44 PIN domain, we tested whether the atrrp44ΔPIN mutant could complement the *scrrp44* mutant. atrrp44ΔPIN failed to complement the growth defect of *scrrp44*, indicating that the PIN domain was essential for the AtRRP44 function in vivo. However, AtRRP44 mutants with substitutions of ribonuclease catalytic residues in the PIN domain complemented the *scrrp44* growth defect phenotype, suggesting that the ribonuclease activity of PIN domain was of minor importance for AtRRP44 function in yeast cell viability. Collectively, our results indicate that the AtRRP44 PIN domain is essential for AtRRP44 function, implying that the PIN domain is important for the interaction with the exosome core. In yeast, ScRrp44 PIN domain was shown to be necessary and sufficient for the association with the exosome core (Schneider et al. 2009).

In yeasts, humans, and plants, rRNA processing defects are common features of exosome components mutants. In previous studies, several groups including our own detected the accumulation of 5.8S rRNA processing intermediates and 5′ external transcribed spacers (ETS) in *Arabidopsis* knockdown mutants of exosome components (*rrp4, rrp41*, and *rrp44*) and T-DNA insertion mutants of *mtr4* (Chekanova et al. 2007; Kumakura et al. 2013; Lange et al. 2011). AtMTR4 is an *Arabidopsis* putative integral factor of the Trf4/Air2/Mtr4 polyadenylation complex (TRAMP), which stimulates exosome activity by adding poly (A) tails to RNA substrates (LaCava et al. 2005). All these exosome components (AtRRP4, AtRRP41, AtRRP44, and AtMTR4) localized to nuclei (Chekanova et al. 2007; Lange et al. 2011; Moreno et al. 2013; Zhang et al. 2010) and could be co-immunoprecipitated by AtMTR4-GFP (Lange et al. 2014), suggesting that these components formed the nuclear exosome. Given our present results, we propose that AtRRP44 may, at least partially, confer the RNA degradation activity of this complex.

Arabidopsis AtRRP44 mutants were reported to show severe defects in female gametophyte development and null mutants were not available (Kumakura et al. 2013; Zhang et al. 2010). These findings indicate AtRRP44 has an important role in the early stage of plant development. Consistent with these results, we demonstrated the *in vitro* ribonuclease activity of AtRRP44 (Figures 1B, 2B) and showed that this activity was essential for cell viability (Figure 3A). These data strongly suggest that the lethal phenotypes of *Arabidopsis rrp44* null mutants are because of the loss of RNA degradation activity. One of the most important questions about the biological role of AtRRP44 is what their target RNA substrates are. It was reported that Rrp44/Dis3 proteins target various substrates in yeast and human cells including ribosomal RNA intermediates, mRNAs, and non-coding RNAs (Schneider et al. 2012). In our previous study, we detected the accumulation of 5.8S rRNA intermediates and 5′ ETS in *Arabidopsis rrp44* mutants in which AtRRP44 was knocked down only in leaves, indicating that these RNAs were possible targets of AtRRP44 (Kumakura et al. 2013). However, whether AtRRP44 targets other RNAs or not is still unknown. Future studies to identify the substrate RNAs of AtRRP44 are required to address the biological role of AtRRP44.

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Supporting Information

Table S1. Oligonucleotides list

Table S2. Plasmids list

Figure S1. Aspartic acid residues in the ribonuclease catalytic centers of three Rrp44/Dis3 proteins are conserved

A. Schematic representation of the PIN and RNB domains of ScRrp44, HsRRP44 and AtRRP44. Arrows showing the catalytic residues.

B. Alignment of part of the PIN and RNB domain sequences of *S. cerevisiae* ScRrp44, *H. sapiens* HsRRP44, and *A. thaliana* AtRRP44. The ribonucelase catalytic residues in the PIN domain (upper panel) and in the RNB domain (lower panel) reported in ScRrp44 were conserved in HsRRP44 and AtRRP44 (gray boxes). The positions of the amino acid (aa) residues are indicated on the right and left sides of the sequences.

Figure S2. Secondary structure of 5′-cap RI labeled ssRNA

The image was generated using RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) (Gruber et al. 2008).

Figure S3. qRT-PCR of mRNAs in yeast complementation assays

A. Accumulation of the AtRRP44 mRNAs of AtRRP44, D132ND158N, D489N, D132ND158ND489N and an empty vector was detected by qRT-PCR in *S. cerevisiae* repressible *rrp44* mutant without DOX.

B. Accumulation of mRNAs of AtRRP44, atrrp44ΔPIN and an empty vector were detected. (A and B) mRNAs were extracted from yeast cultured at 30°C. Error bars represent standard errors. Two biological and two technical replicates were performed. *TAF10* mRNA (Teste et al. 2009) was used as an endogenous control. AtRRP44 mRNAs and *TAF10* mRNAs were detected using primer sets AtRRP44 (qRRP44-3′_F and qRRP44-3′_R) and *TAF10* (F_ScTAF10_qPCR and R_ScTAF10_qPCR), respectively. Primer sequences are listed in Table S1.

Figure S4. Schematic representation of the pOH018 plasmid used for the AtRRP44 promoter GUS assay

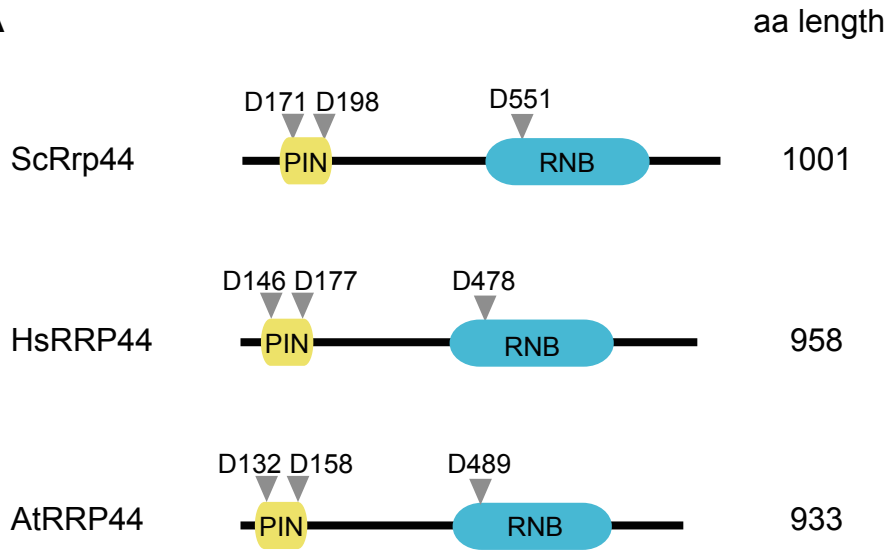
Pro:AtRRP44 represents the genomic sequence covering 2.4 kbp upstream of the AtRRP44 coding sequence. AtRRP44 genome represents the 6.9 kbp AtRRP44 coding sequence from the start to stop codons. The black box represents a linker sequence (GGGSGGG). Ter:AtRRP44 represents the approximately 0.5 kbp region downstream of the AtRRP44 coding sequence. *A. thaliana* ecotype Col-0 was transformed with this plasmid.

Figure S5. Promoter GUS assay of eight independent pOH018 transformants

To observe the predominant expression pattern of the AtRRP44, seedlings of eight independent T2 pOH018 transformants were GUS stained. The descendent of #8 was used in Figure 4.

Supplementary methods. Plasmids construction

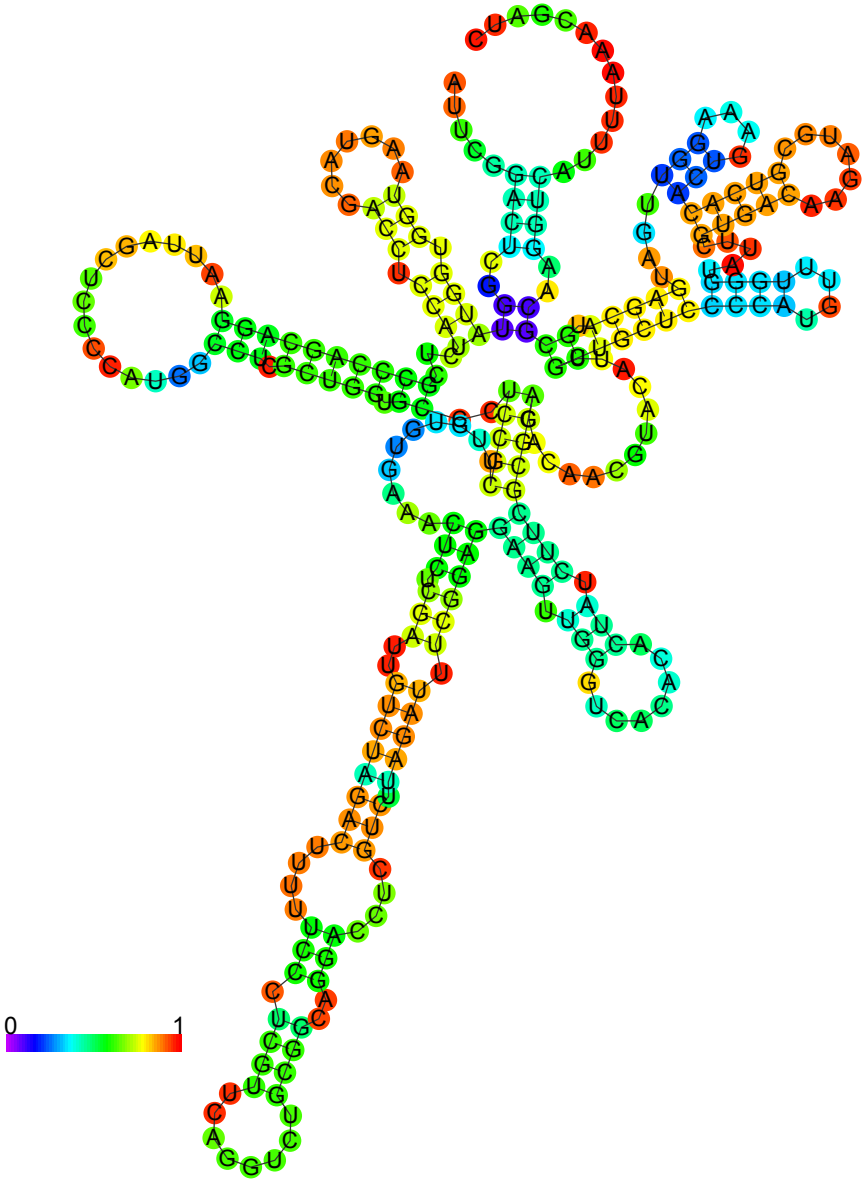
A



B

	aa		aa
ScRrp44	168	**DRNDRAIRKTCQWYSEHLKPY----DINVVLVTNDRLNRE**	203
HsRRP44	143	**DRNDRAIRVAAKWYNEHLKKMSADNQLQVIFTTNDRRNKE**	182
AtRRP44	129	**DHNDRAIRVATLWYQKHLGDT-----SQVLLVTNDRENKR**	163

	aa		aa
ScRrp44	538	**LICSIDPPGCVDIDDALHAKKLPNGNW**	564
HsRRP44	454	**CICSVDPPGCTDIDDALHCRELENGNL**	500
AtRRP44	476	**LVFSVDPPGCKDIDDALHCTSLPNGNF**	502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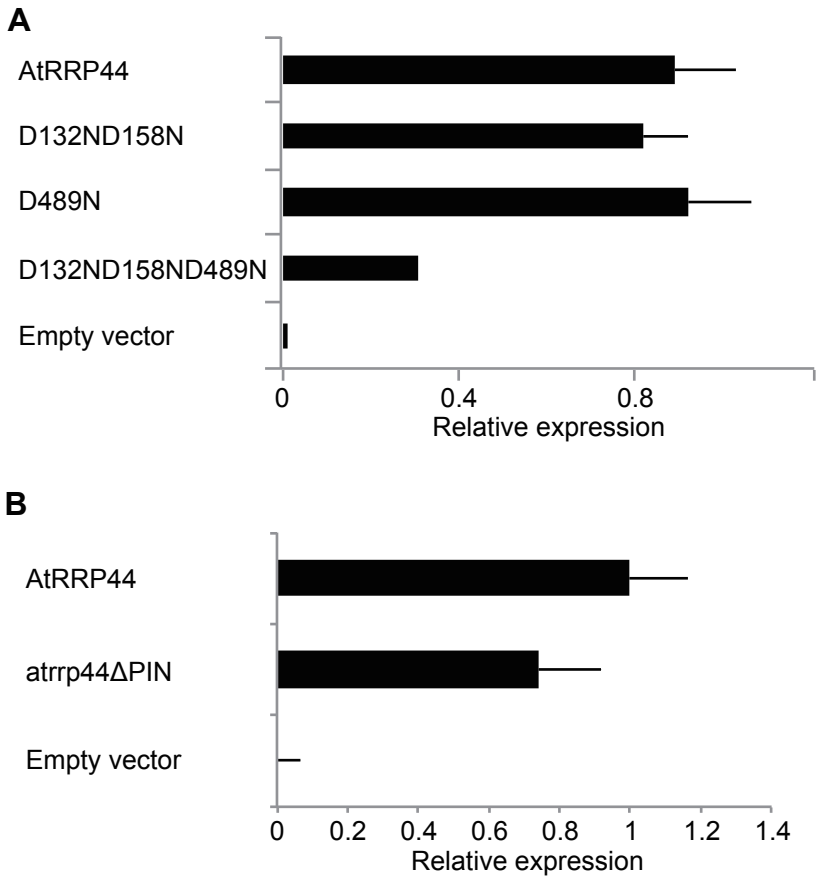
A
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D489N
D132ND158ND489N
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0.8
Relative expression
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Empty vector
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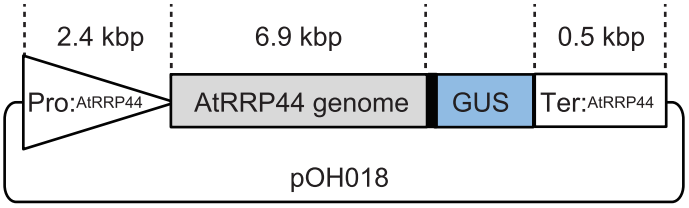
0.4
0.6
0.8
1
1.2
1.4
Relative expression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6.9 kbp
0.5 kbp
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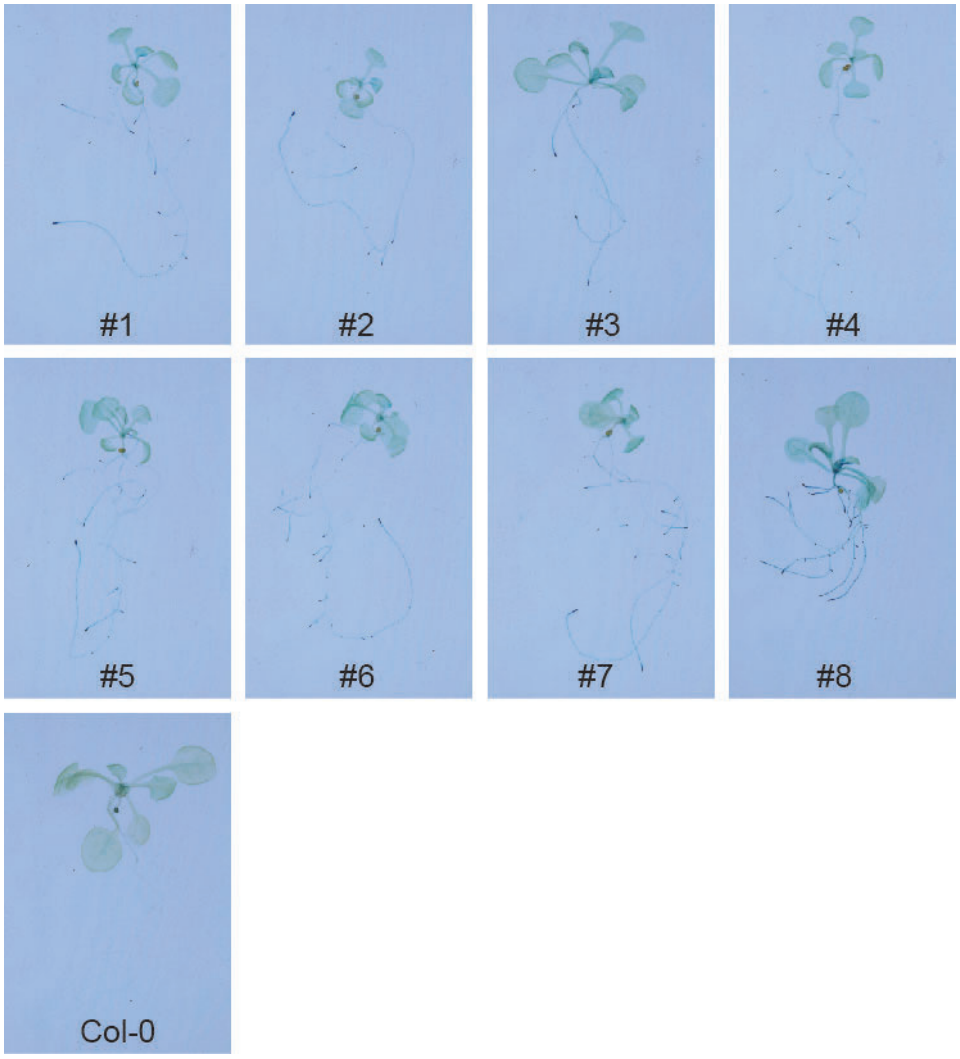
AtRRP44 genome
GUS
Ter:AtRRP44
pOH018


#1
#2
#3
#4
#5
#6
#7
#8
Col-0

Supplementary methods

Constructions

pOH027 [p415ADH:AtRRP44CDS(D132N, D158N)]

pOH027 is a vector expressing the AtRRP44 (AT2G17510.1) protein in which 132th and 158th glutamic acids (D) are mutated to glutamine (N) in *S. cerevisiae*. A PCR fragment (PCR-S1) was amplified from pOH016 [1] using the primers F_D177N_pOH005 and R_D203N_pOH005. Then, the *Nco*I-*Mfe*I fragment of the PCR-S1 was substituted for the small *Nco*I-*Mfe*I fragment of pOH016 to construct pOH027.

pOH028 [p415ADH:AtRRP44CDS(D489N)]

pOH028 is a vector expressing the AtRRP44 (AT2G17510.1) protein in which 489th glutamic acids (D) is mutated to glutamine (N) in *S. cerevisiae*. A PCR fragment (PCR-S2) was amplified from pOH016 [1] using the primers F_D556N_pOH005 and R_D556N_pOH005. Then, the *BsiW*I-*BsrG*I fragment of the PCR-S2 was substituted for the small *BsiW*I-*BsrG*I fragment of pOH016 to construct pOH028.

pOH026 [p415ADH:AtRRP44CDS(D132N, D158N, D489N)]

pOH026 is an vector expressing the AtRRP44 (AT2G17510.1) protein in which 132th, 158th and 489th glutamic acids (D) is mutated to glutamine (N) in *S. cerevisiae*. The *BsiW*I-*BsrG*I fragment of the PCR-S2 was substituted for the small *BsiW*I- *BsrG*I fragment of pOH027 to construct pOH026.

pNK026 [p415ADH:AtRRP44CDSΔPIN]

pNK026 is an vector expressing the AtRRP44 (AT2G17510.1) lacking PIN domain in *S. cerevisiae*. A PCR fragment (PCR-S3) was amplified from pOH016 [1] using the primers pOH026-A_F and pOH026-A_R and a PCR fragment (PCR-S4) was amplified from pOH016 using the primers pOH026-B_F and pOH026-B_R. We then amplified a PCR fragment (PCR-S5) from a mixture of the PCR-S3 and the PCR-S4 fragments using the primers pOH026-A_F and pOH026-B_R. The *Xba*I fragment of PCR-S5 was inserted into

the *Xba*I site on pOH016 to construct pNK026.

pOH018 [pWAT2:ProRRP44tRRP44genome-GUSA-TerRRP44]

pOH018 is an vector expressing AtRRP44-GUS fusion proteins under control of the native AtRRP44 promoter and terminator in *Arabidopsis thaliana.* About 2.4 kb upstream region of the AtRRP44 start codon was amplified from the genome DNA of *Arabidopsis thaliana* Col-0 using the primers salI+proRRP44_F and claI+RRP44_R (PCR-S6). The *Sal*I and *Cla*I fragment of PCR-S6 was substituted for the small *Sal*I-*Cla*I fragment of pWAT2 [1] to construct pWAT2-ProRRP44. Then we amplified about 0.5 kb downstream region of the AtRRP44 stop codon from Arabidopsis genome DNA by using primers *BamH*I+RRP44ter_F and *Sac*I+RRP44ter_R (PCR-S7). The *BamH*Î-*Sac*I fragment of PCR-S7 was substituted for the small *BamH*I-*Sac*I fragment of pWAT2-ProRRP44 to construct pWAT2-ProRRP44-TerRRP44. The first and latter half of AtRRP44 coding region were amplified from the Arabidopsis Col-0 genome DNA using primer sets A (F_44Ag-1_011 and R_44Ag-1_011) and B (F_44Ag-2_011 and R_44Ag-2_011, resulting AtRRP44_genome_Part1 and AtRRP44_genome_Part2, respectively. AtRRP44_genome_Part1 and AtRRP44_genome_Part2 were inserted into the large *Cla*I-*BamH*I fragment of pWAT2-ProRRP44-TerRRP44 by using IN-Fusion HD Cloning Kit (Takara, Kusatsu, Japan) according to the manufacture's protocol to construct pOH011 [pWAT2-PRRP44-RRP44genome-TRRP44]. A PCR fragment (PCR-S8) was amplified from pGWB533 [2] using primers F_GUSA_pOH018 and R_GUSA_pOH018 adding GGGSGGG amino acids at N terminus of the GUS protein as a linker. Then PCR-S8 was inserted into the large *Not*I-*Sac*II fragment of pOH011 by In-Fusion HD Cloning Kit (Takara) to construct pOH018.

PCR4_CMVQ QR1

A PCR fragment was amplified from pBinCMV [3] by using primers QR1-300F and QR1-540R. The fragment was inserted into PCR4-TOPO (Life technologies, Carlsbad, CA, USA) according to the manufacture's protocol.

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Table S1. Oligonucleotides list

Oligonucleotide	Sequence
salI+proRRP44_F	GTTGTCGACACAATTTCTGGGCCACGAAC
claI+proRRP44_R	TTATCGATTTACCTTGATCTCATAGAAATTTTTTTAATG
BamHI+RRP44ter_F	TTGGATCCAACTCTTAAACCTTGTAGTTTC
SacI+RRP44ter_R	TTGAGCTCAGCTGTACAATGTTGCTCA
F_44Ag-1_011	GATCAAGGTAAATCGATCCCGGGGGAGGAGACGTCATGATCTTAGGTATGCAATATTATCTTGCAAAAAT
R_44Ag-1_011	CTCTAGGATACGGACTCCAAGTTCAAAG
F_44Ag-2_011	GTCCGTATCCTAGAGTCCTCAGATTCTC
R_44Ag-2_011	TTTAAGAGTTGGATCCTTACCGCGGTCCAGCGGCCGCCAAGAGAGTGAGTTGGAGTTTCGGTCGGTTAGG
F_GUSA_pOH018	ACTCACTCTCTTGGCGGCCGCTGGAGGAGGATCTGGAGGAGGAATGTTACGTCCTGTAGAAACC
R_GUSA_pOH018	AGTTGGATCCTTACCGCGGTCATTGTTTGCCTCCCTGCTG
F_D177N_pOH005	CGCCCATGGAGAAAGAGAGTGCAAATGACCACAACAATAGAGCTATTC
R_D177N_pOH005	CGCGGTACCATAGAGTAGCGACGCGA
F_D203N_pOH005	CGCGGTACCAGAAGCATCTAGGGGATACATCTCAG
R_D203N_pOH005	CGCCAATTGTCTCCGCAGATATTCCCTCTTCAGTGGCCTTCCGTTTATTTTCCCTATTATTAGTTACA
F_D556N_pOH005	CGCTACGTACGACCAATCGGCAAAAT
R_D556N_pOH005	CGCTGTACAGTGCAATGCATCATTAATATCCTTGC
pOH026-A_F	TTCTCTAGAACTAGTGGATCCCCCGGG
pOH026-A_R	AGAGGAAGACAAACGAGCAGCTG
pOH026-B_F	CTGCTCGTTTGTCTTCCTCTAAGGCCACTGAAGAGGGAATATC
pOH026-B_R	TTCTCTAGAATCATCTGCATCTTCCATAG
QR1-300F	ATTCGGACTCGGTATGGTG
QR1-540R	GATCGTTTAAAATGACCTTGC
F_ScTAF10_qPCR	ATATTCCAGGATCAGGTCTTCCGTAGC
R_ScTAF10_qPCR	GTAGTCTTCTCATTCTGTTGATGTTGTTGTTG
qRRP44A-3'_F	TGGTTTTATAGTATTTGTCCCCAAG
qRRP44A-3'_R	GCTTCTCCTCATCAACATACCA

Table S2. Plasmids list

Name	Description
pOH016	p415ADH:AtRRP44CDS
pOH027	p415ADH:AtRRP44CDS[D132N_D158N]
pOH028	p415ADH:AtRRP44CDS[D489N]
pOH026	p415ADH:AtRRP44CDS[D132N_D158N_D489N]
pNK026	p415ADH:AtRRP44CDSΔPIN
pOH018	pWAT2:ProRRP44-AtRRP44genome-GUSA-TerRRP44
PCR4_CMVQ QR1	-