

# Poly(A) Tail-dependent Exonuclease AtRrp41p from *Arabidopsis thaliana* Rescues 5.8 S rRNA Processing and mRNA Decay Defects of the Yeast *ski6* Mutant and Is Found in an Exosome-sized Complex in Plant and Yeast Cells\*

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Eukaryotic 3'→5' exonucleolytic activities are essential for a wide variety of reactions of RNA maturation and metabolism, including processing of rRNA, small nuclear RNA, and small nucleolar RNA, and mRNA decay. Two related but distinct forms of a complex containing 10 3'→5' exonucleases, the exosome, are found in yeast nucleus and cytoplasm, respectively, and related complexes exist in human cells. Here we report on the characterization of the AtRrp41p, an *Arabidopsis thaliana* homolog of the *Saccharomyces cerevisiae* exosome subunit Rrp41p (Ski6p). Purified recombinant AtRrp41p displays a processive phosphorolytic exonuclease activity and requires a single-stranded poly(A) tail on a substrate RNA as a "loading pad." The expression of the *Arabidopsis* RRP41 cDNA in yeast rescues the 5.8 S rRNA processing and 3'→5' mRNA degradation defects of the yeast *ski6-100* mutant. However, neither of these defects can explain the conditional lethal phenotype of the *ski6-100* strain. Importantly, AtRrp41p shares additional function(s) with the yeast Rrp41p which are essential for cell viability because it also rescues the *rrp41* (*ski6*) null mutant. AtRrp41p is found predominantly in a high molecular mass complex in *Arabidopsis* and in yeast cells, and it interacts *in vitro* with the yeast Rrp44p and Rrp4p exosome subunits, suggesting that it can participate in evolutionarily conserved interactions that could be essential for the integrity of the exosome complex.

A large number of eukaryotic RNA species require 3'→5' exonucleolytic activities either for processing from their respective precursor forms or for their turnover. In yeast, a large, ~300–400 kDa complex, the exosome, mediates many of these reactions (2, 21; for review, see Ref. 26). Two major forms of the exosome, nuclear and cytoplasmic, have been identified, each containing at least 10 common "core" components, Rrp4p, Rrp40p-Rrp46p, Mtr3p, and Csl4p proteins. In addition, Rrp6p appears to be associated only with the nuclear form of the

exosome. Furthermore, RNA helicases Dob1p and Ski2p are required for at least some activities of the nuclear and cytoplasmic forms of the exosome, respectively, although their physical associations with it have not been documented.

Interestingly, all but one of the core exosome subunits either have been shown to be 3'→5' exonucleases *in vitro* or were predicted to have such activity, based on sequence similarity to known exonucleases. Six of the exosome components (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p, and Mtr3p) are homologous to the *Escherichia coli* RNase PH, and the recombinant yeast Rrp41p has a phosphorolytic, processive 3'→5' exonuclease activity (21). Rrp4p and Rrp40p are homologous to each other and, along with Csl4p, contain a predicted S1 RNA binding motif. Recombinant Rrp4p is a hydrolytic, distributive 3'→5' exonuclease. Rrp44p is homologous to the *E. coli* RNase R, of the RNase II family, and exhibits a hydrolytic, processive mode of RNA degradation. Finally, Rrp6p is a member of the RNase D family, and the recombinant Rrp6p has a hydrolytic 3'→5' exonuclease activity (9). The immunopurified exosome exhibits an activity that is most similar to that of the Rrp4p subunit, but it is not known how the multiple catalytic activities in the exosome are coordinated *in vivo*.

All 10 core exosome subunits are essential for viability. This finding is most consistent with the view that an intact complex is required for the proper exosome function, and the loss of any one of the core subunits leads to a failure to assemble the complex properly. In addition, there does not appear to be a sizable pool of the uncomplexed subunits in the cell. Furthermore, many of the partial loss of function exosome mutants show very similar molecular phenotypes, for example in the case of the 5.8 S rRNA processing (1, 16, 21). Nonetheless, certain exosome mutants, particularly those affecting the Rrp6p subunit, show very distinct RNA processing defects, suggesting that multiple subunits are involved in the processing of some exosome substrates, with individual enzymes catalyzing sequential processing steps (for discussion, see Ref. 26).

Several observations suggest that exosome-like complexes are also present in other eukaryotes. A number of exosome subunit homologs could be identified in other species via BLAST searches. The human homologs of Rrp6p (PM-Scl100p) and Rrp4p can be coimmunoprecipitated, and PM-Scl100p, human Rrp4p, and human Rrp45p (PM-Scl75p) are all found in a large, exosome-sized complex (2). In addition, just like the yeast Rrp6p, its human homolog is only present in the nuclear form of the complex. Additional data supporting the notion of functional conservation of the exosome come from the complementation of the phenotypes of the partial loss of function mutations in the yeast *RRP4*, *RRP44* (*DIS3*), and *CSL4* genes

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by the corresponding human cDNAs. However, in none of these cases was complementation of null alleles achieved. Therefore, it remains possible that the partial complementation reflects the ability of these subunits to perform their function as individual proteins. Indeed, the human Rrp4p was not stably incorporated into the yeast exosome complex (21).

In this paper, we report the characterization of the *Arabidopsis thaliana* homolog of the exosome subunit Rrp4p (Ski6p). In contrast to the above examples, *AtRRP41* cDNA readily complements the null allele of the yeast *RRP41* gene. In addition, its expression in the partial loss of function yeast mutant *ski6-100* corrects the defects in the 5.8 S rRNA processing and 3'→5' mRNA degradation. Purified AtRrp41p behaves *in vitro* as a processive phosphorolytic exonuclease, and interestingly, it requires a single-stranded poly(A) tail on the substrate RNA as a "loading pad." The *Arabidopsis* AtRrp41p protein is found predominantly in a high molecular mass complex in plant cells as well as in yeast, suggesting that it could restore the integrity of the exosome complex through evolutionarily conserved interactions with the yeast exosome proteins. In support of this view, the recombinant AtRrp41p protein interacts *in vitro* with the two yeast exosome subunit proteins, Rrp44p and Rrp4p.

## EXPERIMENTAL PROCEDURES

### Materials

An *Arabidopsis* cDNA library derived from immature flower buds described in (30) was obtained from the Arabidopsis Biological Resource Center at Ohio State University. *A. thaliana* strain Columbia was from Lehle Seeds. The yeast strain P118 (*MATa his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108 GAL10::proteinA-RRP41* (21)) was a kind gift from Phil Mitchell and David Tollervey. Yeast strains yRP840 (*MATa his4-539 leu2-3, 112 trp1-Δ1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG*) and yRP1204 (*MATa his4-539 leu2-3, 112 lys2-201 trp1-Δ1 ura3-52 cup1::LEU2/PGK1pG/MFA2pG ski6::URA3 [ski6-100/LYS2]*) were kindly provided by Roy Parker. The *cup1::LEU2/PGK1pG/MFA2pG* construct contains *MFA2* and *PGK1* genes with poly(G) insertions in their 3'-untranslated regions, each driven by the *GAL1* promoter, integrated into the *CUP1* locus (15). PNPase<sup>1</sup>-deficient *E. coli* strain CA244, Pnp<sup>-</sup> (31) was a kind gift from Murray Deutscher.

### Cloning of the AtRRP41 cDNA and Plasmid Constructs

An *AtRRP41* probe used to screen the cDNA library was obtained by PCR amplification of the *AtRRP41* sequence fragment contained within EST T43164 and overlapping with it EST 116G11XP, using oligonucleotides oDB464 (5'-CAAGAGCCAGCAAAAGAAGAA-3') and oDB465 (5'-TTATGCAGCTCGGCGATACTC-3'). The library was screened using standard protocols (3). The phage with the longest insert was converted into plasmid form and sequenced. Oligonucleotides used for the 3'-RACE (12) on *Arabidopsis* poly(A)<sup>+</sup> RNA were: (dT)<sub>17</sub> adapter primer TRACER (5'-GATGGTACCTCTAGAGCTCT<sub>17</sub>-3'), adapter primer RACER (5'-GATGGTACCTCTAGAGCTC-3'), and *AtRRP41* gene-specific primer oDB471 (5'-ACGCGAGGTACTTCAAGA-3'). 5'-RACE was done using the *AtRRP41* gene-specific oligonucleotide oDB507 (5'-TGAATCGGCGACCATCTAAT-3'), but it did not produce any products that extended beyond the 5'-end of the sequence contained within the longest insert isolated by a hybridization screen of the cDNA library.

For yeast complementation studies, the *AtRRP41* open reading frame was amplified with the *Pfu* DNA polymerase using oligonucleotides oDB474 (5'-atggatccATGGAGTACGTAAACCCTGAA-3'; nucleotides that are not part of the *AtRRP41* sequence are shown in lowercase) and oDB465 (5'-TTATGCAGCTCGGCGATACTC-3'). The amplified fragment was blunt end cloned into the *SmaI* site of the plasmid p112A1NE (24), yielding the construct pDB460, in which the *AtRRP41* sense strand is transcribed from the *ADH1* promoter. To create the GST fusion for the expression in *E. coli*, the same *Pfu*-

amplified product was cut with *Bam*HI and cloned into pGEX2T that was cleaved with *Eco*RI, filled in with Klenow fragment, and then cleaved with *Bam*HI, to yield the construct pDB459. For the expression of AtRrp41p as a fusion to the maltose-binding protein (MBP), the AtRrp41p open reading frame was cloned into pMALc2 (New England Biolabs) to create pDB512.

### Phylogenetic Analysis

The following sequences were obtained from GenBank (accession numbers are given in parentheses): *Arabidopsis* PNPase (CAB43864), *E. coli* PNPase (P05055), *Spinacia oleracea* PNPase (U52048), *Bacillus subtilis* RNase PH (M85163), *E. coli* RNase PH (L10328, fragment), *Pyrococcus horikoshii* RNase PH (BAA30661), and *Saccharomyces cerevisiae* Rrp41p (CAA97221). Sequence alignment and tree building were performed using the Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI. Sequences were aligned using PileUp, edited with LineUp to exclude nonhomologous portions, distance matrix created with Distances (using Kimura correction for amino acid sequences), and the tree constructed by GrowTree using the neighbor-joining algorithm.

### Protein Expression and Purification

Expression of the GST-AtRrp41p fusion in pGEX2T (construct pDB459) was carried out in the BL21 host strain containing the *E. coli* *dnaY* gene on the plasmid pUBS520 (7). At A<sub>600</sub> = 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM, and the culture was induced overnight at 20 °C. Cells were washed in buffer A (50 mM Tris pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100) and resuspended in 0.01 volume of buffer A with 0.5 mM phenylmethylsulfonyl fluoride. Cells were lysed by sonication, and total extract was cleared by a 30-min centrifugation at 17,000 rpm in an SS34 rotor and loaded onto glutathione-agarose (Sigma). After extensive washing with buffer A, fusion protein was eluted with 10 mM reduced glutathione in buffer A. All purification steps were performed at 4 °C. Cleavage of the fusion protein with thrombin (Amersham Pharmacia Biotech) was done according to the manufacturer's instructions. MBP-AtRrp41p was expressed in a similar manner, and the purification was carried out according to the instructions of the manufacturer (New England Biolabs).

The *PAB3* open reading frame was PCR-amplified using the *Pfu* polymerase and primers oDB413 (5'-tataccatgGCCGATCAAACCCAC-CCGAAC-3') and oDB481a (5'-ccaagtcgacGTCGGTGGTAGAGAAAC-CAA-3'; nucleotides that are not part of the *PAB3* sequence are shown in lowercase). The resulting product was cut with *Sal*I and cloned into pET24a that was cut with *Nde*I, blunted with Klenow fragment, and cut with *Xho*I. The resulting construct, pDB467, encodes the Pab3p with an added hexahistidine tag at its C terminus. The Pab3p protein was expressed in BL21(DE3) coexpressing the *E. coli* *dnaY* gene. Induction was carried out for 3 h with 1 mM isopropyl-1-thio-β-D-galactopyranoside at room temperature. Cells were washed with buffer X (20 mM Tris pH 7.9, 0.1 M KCl, 2 mM imidazole, 10% glycerol), concentrated and lysed as for AtRrp41p, extract loaded onto ProBond resin (Invitrogen), washed with buffer X with 20 mM imidazole, and eluted with buffer X plus 200 mM imidazole. Purified Pab3p protein was tested in a gel shift assay with oligo(A)<sub>23</sub> and was found to have a *K<sub>d</sub>* of 10 nM (not shown).

### RNA Techniques

The RNA substrates used for exonuclease assays were generated as follows:

Oligo(A)<sub>23</sub> (from Oligo Etc.) was 5' labeled using [γ-<sup>32</sup>P]ATP and polynucleotide kinase. pSP65A<sub>38</sub>, containing a 38-nucleotide long poly(A) tract inserted between the *PsI* and *Hind*III sites of the polylinker of pSP65 (22) was linearized with *Hind*III or *Sal*I as indicated in the text and transcribed in the presence of [α-<sup>32</sup>P]UTP with SP6 RNA polymerase. Bluescript SK(+) was cut with *Not*I and transcribed in the presence of [α-<sup>32</sup>P]UTP with the T7 RNA polymerase. Where indicated, the resulting transcript was polyadenylated by yeast poly(A) polymerase (United States Biochemical) according to the manufacturer's instructions. To create templates for making transcripts with the 3'-poly(A) or poly(U) tail, a fragment of the Bluescript polylinker was amplified with the M13 "20" primer and either oDB522 (5'-TTTTTTTTTTTTTTTTTTTTTGGCCGCTCTAGAACT-3') or the oDB523 (5'-AAAAAAAAAAAAAAAAAAGGCCGCTCTAGAACT-3'). *In vitro* transcription on the resulting double-stranded products, incorporating the T7 promoter sequence, was done with the T7 RNA polymerase. The transcripts obtained from these reactions are identical in sequence to those produced from the Bluescript SK(+) template cut

<sup>1</sup> The abbreviations used are: PNPase, polynucleotide phosphorylase; PCR, polymerase chain reaction; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; Ni-NTA, nickel nitrilotriacetic acid; PABP, poly(A)-binding protein.



with *NotI*, except for the addition of the (A)<sub>20</sub> or (U)<sub>20</sub> tail. Except where noted, all *in vitro* made transcripts were purified on a denaturing gel prior to use and heated at 75 °C for 8 min, snap-cooled on ice, and added to the reaction mix. Northern blot analysis and RNase H treatment were done as described (10). Oligonucleotide probes were as follows: for the 7 S precursor, 5'-TGAGAAGGAAATGACGCT-3'; for the mature 5.8 S rRNA, 5'-GCGTTGTTCATCGATGC-3'; for MFA2pG, 5'-ATATTGAT-TAGATCAGGAATTCC-3'; and for scR1 RNA, 5'-GTCTAGCCGCGAG-GAAGG-3'. Quantitations were done on a Molecular Dynamics Storm PhosphorImager.

For the *AtRRP41* expression survey, total RNA was extracted from *Arabidopsis* tissues as described (18), reverse transcribed in a 30- $\mu$ l reaction with SuperScript II (Life Technologies) using oligo(dT) primer, and 1  $\mu$ l of the reverse transcriptase reaction was used directly for PCR with oDB474 and oDB465.

#### Yeast Techniques

Yeast techniques were according to (13). For plasmid linkage analysis of the complementation phenotype, P118 transformed with pDB460 was grown in synthetic complete galactose medium supplemented with tryptophan for 3 days, plated onto YPGal plates, and 1,000 individual colonies were streaked in a grid onto synthetic complete galactose plates with and without tryptophan. The Trp<sup>-</sup> isolates were selected and streaked onto YPGal and YPD (glucose) plates.

#### Exonuclease Assay

The exonuclease assay was performed in a 100- $\mu$ l reaction containing 10 mM Tris pH 7.6, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 100  $\mu$ g/ml bovine serum albumin, 0.8 unit/ $\mu$ l RNasin, up to 3 pmol of GST-AtRrp41p fusion protein, and a <sup>32</sup>P-labeled RNA substrate. Sodium phosphate was added to 10 mM final concentration where indicated. 15- $\mu$ l aliquots were withdrawn at 0, 5, 15, 30, 45, and 90 min and overnight incubation, added directly into 15  $\mu$ l of sequencing loading mix, and frozen immediately in liquid nitrogen. Samples were resolved by electrophoresis on 6% acrylamide, 7 M urea gels in 1  $\times$  TBE. Ascending thin layer chromatography was done on polyethyleneimine-cellulose plates (Sigma) in 1 M formic acid and 0.5 M LiCl. 100 nmol of cold 5'-UMP or 5'-UDP standards was added to the samples before loading. Standards were visualized by UV shadowing.

#### Determination of the Native Size of AtRrp41p

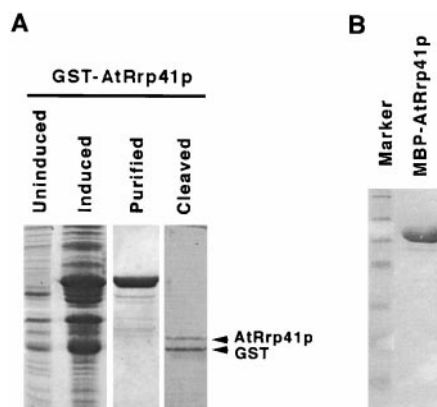
To determine the native size of AtRrp41p in yeast, antibodies were raised against a synthetic peptide with the sequence CENTKQLEYR-RAA, corresponding to the very C terminus of AtRrp41p except for the addition of the first cysteine. This peptide was coupled to maleimide-activated keyhole limpet hemocyanin (Pierce) and used as an immunogen to produce polyclonal antibody in rabbits. Yeast total protein extract and sedimentation through glycerol density gradient were as described (21), and Western analysis was done according to standard protocols (14). Total plant extracts were prepared as described (29) and resolved by sedimentation through glycerol gradients in the same way as for yeast extracts.

#### In Vitro Pulldown Assays

For initial assays, the 10 open reading frames of the core exosomal subunits were PCR amplified with primer pairs purchased from Research Genetics (YHR069C (Rrp4p), YOL142W (Rrp40p), YDL111C (Rrp42p), YCR035C (Rrp43p), YDR280W (Rrp45p), YGR095C (Rrp46p), YGR158C (Mtr3p), YOL021C (Rrp44p), YNL232W (Csl4p), and YOR001W (Rrp6p)) and cloned into Bluescript. Linearized templates were transcribed and translated *in vitro* in the rabbit reticulocyte lysate (Promega) in the presence of the [<sup>35</sup>S]methionine. The resulting radioactive polypeptides were used to challenge ~5  $\mu$ g of GST or GST-AtRrp41p fusion prebound to the glutathione resin (Sigma), in the final volume of 250  $\mu$ l of buffer A + 0.1 M KCl for several hours at 4 °C. After six washes with 0.3 ml of the binding buffer, the bound species were resolved by SDS-PAGE. For the assays shown on Fig. 10, 10  $\mu$ g of purified recombinant yeast Rrp44p or Rrp4p was prebound to Ni-NTA agarose (Qiagen) and challenged with 5  $\mu$ g of GST or the recombinant GST-AtRrp41p. After six washes with 0.3 ml of the binding buffer, bound proteins were subjected to SDS-PAGE and immunoblotting with the AtRrp41p-specific antibody. Mock-treated resin was used as negative control. No binding of GST alone to either resin was observed.

#### RESULTS

*Arabidopsis* Homolog of Rrp41p/Ski6p Is a Processive, Phosphorylolytic 3'→5' Exonuclease—A TBLASTN search of the *Ara-*



**FIG. 1. Expression and purification of the GST-AtRrp41p and MBP-AtRrp41p fusion proteins.** Panel A, GST-AtRrp41p was expressed in *E. coli* (from left, first lane, total extract prior to induction; second lane, total extract after overnight induction with 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside), purified on glutathione agarose (third lane, 5  $\mu$ g of the purified protein), and cleaved with thrombin to separate the AtRrp41p from the GST moiety (far right lane). Samples were resolved by SDS-PAGE and stained with Coomassie Blue. Panel B, 5  $\mu$ g of the purified MBP-AtRrp41p.

*bidopsis* EST data base using the amino acid sequence of the yeast Rrp41p as a query, yielded a single strong hit ( $P_N = 9.1 \times 10^{-9}$ , GenBank accession number T43164). The full-length cDNA corresponding to this EST, hereafter referred to as *AtRRP41*, was reconstructed by reverse transcriptase-PCR amplification of an *Arabidopsis* immature flower cDNA library (GenBank accession number AF191741). Southern analysis demonstrated that AtRrp41p is encoded by a single copy gene, and an RT-PCR tissue survey showed that it is expressed in all *Arabidopsis* tissues tested, including roots, stems, cauline and mature leaves, flowers, pollen, and siliques (not shown). Although these data cannot exclude qualitative or quantitative differences in the *AtRRP41* expression at the level of individual cell types, they are most consistent with the view that AtRrp41p is encoded by a single gene that is constitutively expressed. Phylogenetic analysis of several amino acid sequences related to Rrp41p (see "Experimental Procedures") supported the expectation that the AtRrp41p belongs to the family of RNase PH-like proteins (20) and is most closely related to the yeast Rrp41p (38% identity, 50% similarity at the amino acid level). All of the amino acid residues universally conserved in the RNase PH family proteins, some of which are likely to be important for catalysis, are also present in AtRrp41p (not shown).

We expressed the PCR-amplified AtRrp41p open reading frame as a fusion to GST in *E. coli* and purified it using the affinity tag (Fig. 1A). To test whether it has an exonucleolytic activity that would be expected of a *bona fide* homolog of the Rrp41p, the 5'-end <sup>32</sup>P-labeled oligo(A)<sub>23</sub> was incubated with the fusion protein or with the GST alone as a control. Only an incubation with the GST-AtRrp41p fusion protein, but not with the GST alone, resulted in the degradation of the RNA substrate (Fig. 2). A rapid accumulation of short degradation products showing a precursor-product relationship with the full-length substrate labeled at the 5'-end strongly suggests that the enzyme acts in a 3'→5' direction. Also, the fact that the binding of poly(A)-binding protein (PABP) to the 3'-terminal poly(A) tail completely protects polyadenylated RNA substrate from the AtRrp41p (below) is consistent with 3'→5', but not 5'→3', directionality. The predominance of very short products over intermediate length ones, even at those time points at which some full-length substrate still remained intact, suggests that AtRrp41p is most likely a processive enzyme (see

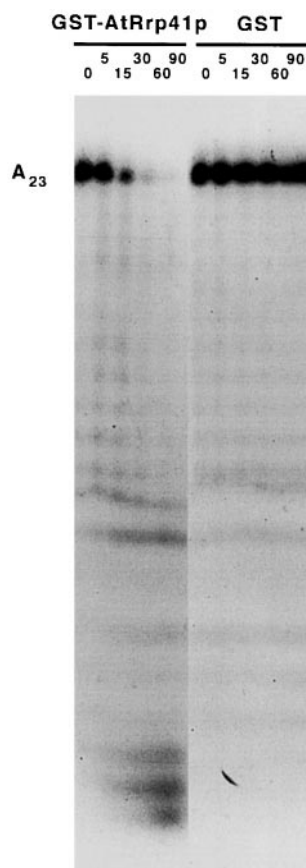


FIG. 2. **Exonuclease activity of AtRrp41p.** 3 pmol of GST-AtRrp41p fusion protein, or GST alone, was incubated with the  $^{32}\text{P}$  5'-end-labeled oligo(A)<sub>23</sub> RNA using the conditions described under "Experimental Procedures" (with 10 mM sodium phosphate), for 0, 5, 15, 30, 45, and 90 min, as indicated above the lanes. The samples were resolved by electrophoresis on 6% denaturing gel in  $1 \times$  TBE.

also Fig. 6). To exclude the possibility that our results were affected by a tendency of the GST to dimerize (19), we have also assayed the AtRrp41p that was separated from the GST moiety by thrombin cleavage. In addition, we have expressed and purified the AtRrp41p as a fusion to MBP, naturally a monomer (Fig. 1B). In both cases, a similar, processive exonucleolytic 3'→5' activity was observed (see Fig. 5 and data not shown).

The conservation of most of the amino acid residues characteristic of the RNase PH group of enzymes in the AtRrp41p suggested that it probably is a phosphorolytic exonuclease. Such enzymes use inorganic phosphate for the attack on the phosphodiester bond. Therefore, their ability to degrade RNA is phosphate-dependent, and they release mononucleoside diphosphates as the final reaction product. The assay shown in Fig. 2 was conducted in the presence of 10 mM inorganic phosphate. When phosphate was omitted, no substrate degradation occurred (Fig. 3A). The product released in the AtRrp41p reaction conducted in the presence of inorganic phosphate was identified by TLC as a mononucleoside diphosphate (Fig. 3C), confirming the expectation that the AtRrp41p is a phosphorolytic enzyme.

**Dependence of the AtRrp41p Enzyme on a Single-stranded Poly(A) Tail**—Surprisingly, when we tested the AtRrp41p using two additional substrates, very different results were obtained. First, an RNA substrate of ~100 nucleotides, including a 38-nucleotide poly(A) tail, was transcribed *in vitro* by an SP6 RNA polymerase from the pSP65(A)<sub>38</sub> template (22). As shown in Figs. 3B and 4A, it was readily digested by the AtRrp41p.

However, the transcript made with the T7 RNA polymerase from the Bluescript SK(+) template digested with *NotI* was significantly more resistant (Fig. 4B). Additional assays were performed to distinguish between the poly(A) tail dependence of the enzyme and the inhibitory effects of the particular structures or sequences present in the Bluescript-derived RNA. First, pSP65(A)<sub>38</sub> RNA was deadenylated *in vitro* using an oligo(dT) and RNase H, purified, and incubated with the AtRrp41p. Only a very slow degradation of the resulting deadenylated RNA was observed (Fig. 4C). In addition, we assayed unadenylated pSP65 RNA made from the pSP65(A)<sub>38</sub> template cut with *SalI*, which cleaves before the run of 38 Ts on the template strand. The resulting RNA substrate was also much more resistant to the AtRrp41p digestion than the substrate transcribed from the *HindIII*-cut pSP65(A)<sub>38</sub> template that had a 38-nucleotide long 3'-poly(A) tail (Fig. 4D). Finally, we have assayed the transcript of the Bluescript polylinker that was modified by an addition of poly(A) tail to the 3'-end with poly(A) polymerase. The resulting polyadenylated transcript became as susceptible to the action of the AtRrp41p enzyme as pSP65(A)<sub>38</sub> (Fig. 4E). Both the GST and MBP fusions of the AtRrp41p were used in these experiments, with identical results.

It has been reported that PNPase from *E. coli* has similar requirements for a single-stranded "foothold" to initiate processive 3'→5' degradation of the substrate (6). To eliminate the possibility of the presence of the *E. coli* PNPase as an impurity in our AtRrp41p preparation, we have also purified and assayed the GST-AtRrp41p and MBP-AtRrp41p fusion proteins expressed in a Pnp<sup>-</sup> *E. coli* strain (31). The same poly(A) tail dependence was observed (Fig. 5, A and B). Thus, the AtRrp41p activity (or more likely, its binding to the substrate), is dependent on the poly(A) tail.

To distinguish whether the AtRrp41p needs any single-stranded tail or is specific for poly(A), two more substrates were generated. A polyadenylated substrate was transcribed from the fragment of the Bluescript polylinker that was amplified by PCR using the standard M13 primer and a primer that anneals in the polylinker region and contains a (dT)<sub>20</sub> extension attached to its 5'-end. The resulting template can be transcribed by T7 RNA polymerase, and the resulting transcript is identical in sequence to the one produced from the Bluescript SK(+) template cut with *NotI*, except for the addition of the (A)<sub>20</sub> tail. An otherwise identical polyuridylated transcript was generated in a similar manner, using the PCR primer with the (dA)<sub>20</sub> extension at its 5'-end. As can be seen from Fig. 5, C and D, the polyadenylated substrate was strongly preferred by AtRrp41p. Thus, the enzyme indeed prefers a substrate ending with a monotonous stretch of adenosine residues, but not with other single-stranded homopolymeric sequences.

**Degradation of the Polyadenylated RNA by AtRrp41p Is Inhibited by PABP**—One important 3'→5' exoribonucleolytic reaction is the cytoplasmic shortening of the mRNA polyadenylate tails, which in yeast precedes decapping and subsequent 5'→3' degradation of the transcript. The deadenylation rate is message-specific and often is a determining factor of the overall half-life of the transcript. In yeast, poly(A) shortening is (directly or indirectly) stimulated by the PABP, but the identity of the actual deadenylating enzyme remains unknown. One poly(A)-specific 3'→5' exonuclease that has been cloned and characterized in yeast is poly(A) nuclease. Poly(A) nuclease has intriguing properties of not only being poly(A)-specific but also completely dependent on the PABP for its activity. Although the possible involvement of poly(A) nuclease in the cytoplasmic poly(A) tail shortening cannot be formally excluded, this enzyme is believed to be involved in the initial trimming of the

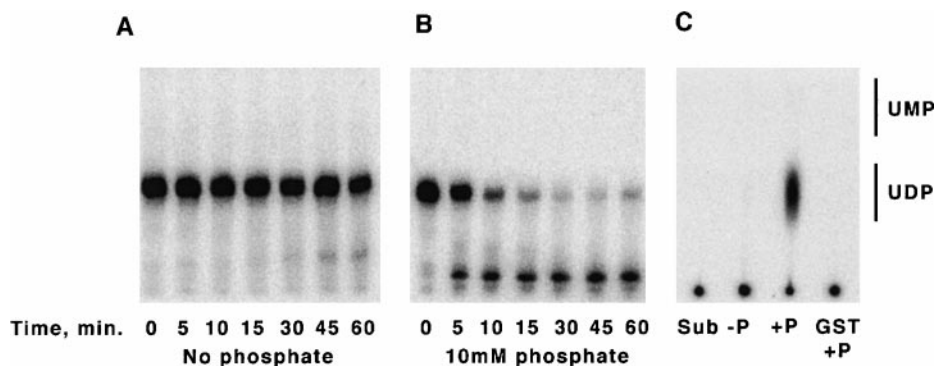


FIG. 3. **AtRrp41p is phosphorolytic exonuclease.** 3 pmol of GST-AtRrp41p fusion protein or GST was incubated with the  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled pSP65(A)<sub>38</sub> transcript for 0, 5, 15, 30, 45, or 60 min, without (panel A) or with (panel B) 10 mM inorganic phosphate. Samples were resolved by electrophoresis on 6% denaturing gel in  $1 \times \text{TBE}$ . Panel C, TLC separation of the final reaction products obtained after a 60-min incubation of the  $^{32}\text{P}$ -labeled pSP65A<sub>38</sub> substrate without any enzyme added (Sub), with the GST-AtRrp41p, without and with 10 mM phosphate, respectively ( $-P$  and  $+P$ ), and with GST in the presence of 10 mM phosphate (GST+P). Positions of the cofractionated unlabeled controls are shown on the right.

newly synthesized mRNA poly(A) tails to their mature message-specific length, most probably prior to, or concomitantly with, their export from the nucleus (8).

We were interested in examining the possible influence of PABP on the activity of the AtRrp41p. An internally  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ -labeled pSP65A<sub>38</sub> substrate was used for this assay. As with the oligo(A)<sub>23</sub> substrate, a significant fraction of the substrate RNA degraded to completion with no detectable intermediates. With the enzyme/substrate ratio used here, some of the full-length substrate still remained intact when a substantial amount of the final product had already accumulated (Fig. 6), supporting the notion that the exonucleolytic reaction occurred processively rather than distributively.

We have found that preincubation, or simultaneous addition, of the recombinant *Arabidopsis* poly(A)-binding protein Pab3p<sup>2</sup> to the assay severely inhibited degradation of the polyadenylated RNA substrate by the AtRrp41p, starting at a 25:1 molar ratio of PABP to the RNA substrate (Fig. 6). This is in contrast with the effect of PABP on poly(A) nuclease, but it is very similar to the protective effect of PABP in various mammalian *in vitro* systems (5, 11, 28) and to its effect on the purified recombinant human deadenylase (also known as deadenylating nuclease) (17). This observation is in agreement with the earlier proposal in Ref. 5 that one of the functions of PABP is to protect the mRNA poly(A) tail from the degradation by 3'→5' exonucleases such as AtRrp41p, either free or as a part of the exosome complex.

**Expression of the Arabidopsis AtRrp41p Complements the Yeast rrp41/ski6 Mutation**—To gain further insight into the function of AtRrp41p, we have attempted to complement the yeast *rrp41/ski6* null mutation with the *AtRRP41* cDNA. The yeast strain used in this experiment, P118 (21), has its *RRP41* gene replaced with the fusion of the *RRP41* gene to the open reading frame of the protein A and is driven by a *GAL10* promoter. Because the Rrp41p function is essential for viability, this strain can grow on galactose but not on glucose. We have transformed P118 with the construct pDB460, containing the *AtRRP41* cDNA under the control of the strong, constitutive yeast *ADH1* promoter. P118 cells transformed with the pDB460, but not with the empty vector, were able to grow on glucose (Fig. 7A), thus demonstrating complementation of the yeast Rrp41p function by the *Arabidopsis* AtRrp41 protein. To exclude the possibility that the growth of transformants on glucose occurred because of a spontaneous mutation that bypassed the essential requirement for the Rrp41p function, we

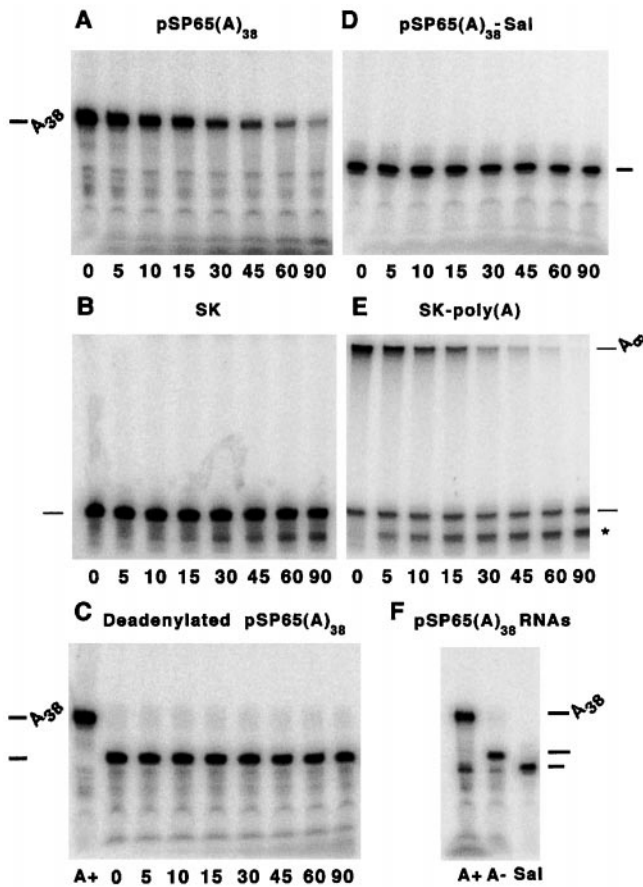
have tested whether the loss of the plasmid encoding AtRrp41p would also lead to the loss of the ability to grow on glucose. All 30 of the isolates that have lost the plasmid-encoded auxotrophic marker after a period of nonselective growth in rich galactose medium were unable to grow on glucose. Thus, we conclude that the *AtRRP41* cDNA functionally complements the yeast *rrp41/ski6* null phenotype (assuming that the P118 strain transferred to glucose is equivalent to the *rrp41/ski6* null strain).

**Expression of AtRrp41p in the Yeast Mutant ski6-100 Corrects the 5.8 S rRNA Processing Defect**—Abnormal processing of the 5.8 S ribosomal RNA is one of the consequences of perturbing the Rrp41p function in *S. cerevisiae*. Specifically, upon the thermal inactivation of the temperature-sensitive allele of *RRP41*, *ski6-100*, or upon the genetic depletion of the Rrp41p using a repressible promoter, a number of 3'-extended 5.8 S rRNA species accumulate, reflecting an abnormal exonucleolytic 3'→5' processing from the 7 S precursor. Similar manipulations of the other core subunits also lead to the accumulation of the incompletely processed species, consistent with the view that the exosome complex as a whole is required for the normal 5.8 S rRNA processing (1, 16, 21). We have addressed whether the *Arabidopsis* AtRrp41p could correct this defect. Total RNA from the wild type yeast, *ski6-100* temperature-sensitive mutant, and the *ski6-100* mutant expressing the *AtRRP41* cDNA, were isolated before and after the shift to 37 °C for 1 h and probed with the oligonucleotide targeted to the region just 3' of the mature 3'-end of the 5.8 S rRNA. Only the full-length 7 S precursor was observed in the wild type strain, whereas the *ski6-100* mutant showed a number of additional shorter products, resulting from an incomplete 3'→5' processing, as expected (Fig. 7B) (16, 21). In contrast, in the *ski6-100* strain expressing the *Arabidopsis* AtRrp41p, the amount of these products was greatly reduced, from the products/full-length precursor ratio of 2.2–2.5 in the mutant to 0.3–0.6 in the strain expressing the AtRrp41p, demonstrating its ability to restore substantially the 5.8 S rRNA processing (Fig. 7B). Hybridization of the same blot with the oligonucleotide probe targeted against the mature 5.8 S rRNA revealed that its relative amount, normalized to the levels of the scR1 RNA, was reduced by about 30% in the mutant, consistent with the published observations (21).

It is interesting to note that the strain bearing the *ski6-100* allele is as defective in 5.8 S rRNA processing at 30 °C as it is at 37 °C (Fig. 7B, lanes 5 and 6). This observation makes it very unlikely that the conditional lethality of the *ski6-100* mutant is caused by a defect in the 5.8 S rRNA processing and suggests that it is not an essential function of the Rrp41 protein. On the

<sup>2</sup> J. A. Chekanova, R. J. Shaw, and D. A. Belostotsky, manuscript in preparation.

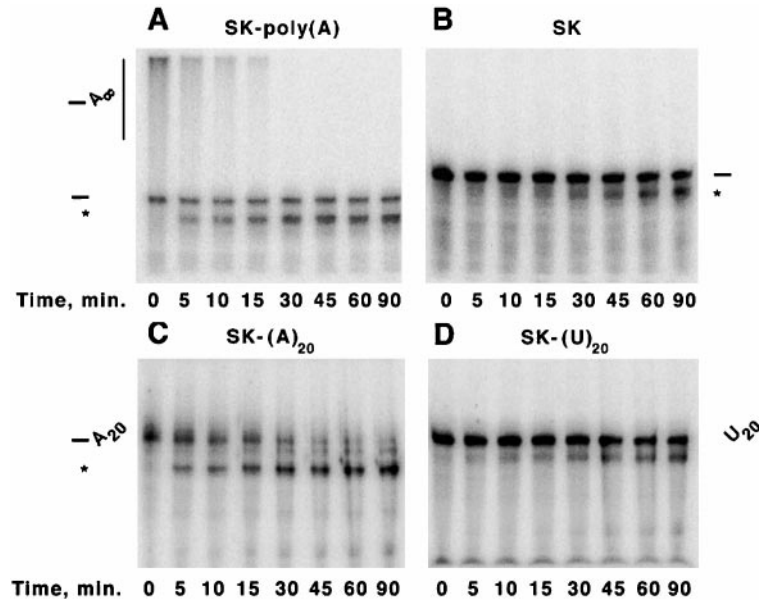




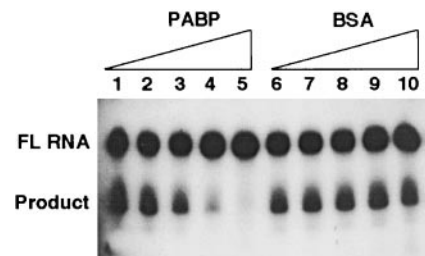
**FIG. 4. AtRrp41p is dependent on the single-stranded tail on the substrate RNA.** <sup>32</sup>P-labeled SP6 transcript of the pSP65A<sub>38</sub> polylinker (designated pSP65A<sub>38</sub>; panels A, C, and D) or T7 transcript of Bluescript polylinker (designated SK; panels B and E) were used as a substrate for GST-AtRrp41p (the RNA substrates and reaction intermediates are shown schematically along the sides of the respective panels). To make a template for *in vitro* transcription, pSP65A<sub>38</sub> was cleaved either after the encoded poly(A) tail with *Hind*III (panels A and C) or before the encoded poly(A) tail with *Sal*I (panel D). In panel C, the resulting RNA was deadenylated with oligo(dT)/RNase H (RNA prior to deadenylation is shown in the lane marked A+). Panel F compares pSP65A<sub>38</sub> transcripts made from the *Hind*III-cut template (A+) with the same RNA after the oligo(dT)/RNase H treatment (A-) and the RNA transcribed from the *Sal*I-cut template (Sal). Bluescript was cleaved with *Not*I and transcribed with T7 and either used directly (panel B) or polyadenylated with the yeast poly(A) polymerase (panel E). The product of poly(A) polymerase reaction contained a significant amount of transcript that did not get polyadenylated (lower band indicated with a thin horizontal bar). The band appearing under the unadenylated transcript at the 5-min time point and beyond (indicated by the asterisk) is the result of the inhibitory secondary structure formed by this RNA because it is greatly diminished by preheating the substrate before beginning of the reaction (data not shown).

other hand, we have found that *AtRRP41* cDNA complements the temperature-sensitive phenotype of *ski6-100* (Fig. 7C). Therefore, it must also be able to restore the essential function(s) of *SKI6/RRP41* gene product both at 30 °C (Fig. 7A) and 37 °C.

**Expression of AtRrp41p in the Yeast Mutant *ski6-100* Prevents the Accumulation of mRNA Fragments That Are Incompletely Degraded by the 3'→5' Pathway**—Another function of the exosome complex is to carry out the exonucleolytic 3'→5' degradation of mRNA. The consequences of the defects in 3'→5' decay in yeast are usually obscured by the presence of the highly active 5'→3' exonucleolytic pathway, but they can be revealed using reporter mRNAs containing poly(G) inserts that form a very stable secondary structure and thus prevent the complete 5'→3' degradation. The resulting degradation



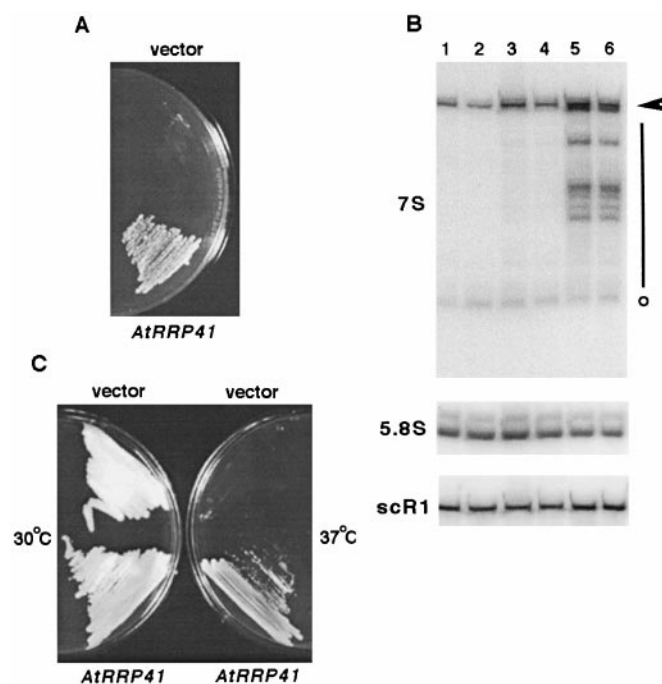
**FIG. 5. AtRrp41p purified from the PNPase-deficient *E. coli* strain is stimulated by poly(A) tail but not by poly(U) tail on the substrate RNA.** Panels A and B, MBP-AtRrp41p fusion was purified from the Pnp<sup>-</sup> strain of *E. coli* and assayed using a RNA substrate transcribed with T7 RNA polymerase from Bluescript cleaved with *Not*I and polyadenylated with the yeast poly(A) polymerase (designated SK-poly(A), panel A) or used directly, without poly(A) polymerase treatment (designated SK, panel B). Panels C and D, MBP-AtRrp41p fusion purified from the Pnp<sup>-</sup> strain of *E. coli* was assayed using RNA substrates identical to the one used in panel A except for the addition of an A<sub>20</sub> (panel C) or U<sub>20</sub> (panel D) tail. Symbols depicting the RNA substrates and reaction intermediates shown on the sides of the respective panels are as in Fig. 4.



**FIG. 6. Pab3p inhibits AtRrp41p 3'→5' exonuclease activity.** Each reaction contained 0.03 pmol of polyadenylated RNA, 0.2 pmol of GST-AtRrp41p, and 0, 0.03, 0.15, 0.75, or 3.7 pmol of either bovine serum albumin (BSA) or Pab3p, as indicated. Reactions were incubated for 60 min at 37 °C and resolved on 12% acrylamide, 7 M urea minigels in 1 × TBE. Full-length RNA substrate and final degradation product are indicated.

intermediate that is produced by the 5'→3' activity extends from the site of poly(G) insert to the 3'-end of the mRNA ("pG→3' fragment"). The pG→3' fragment must ultimately be degraded by the 3'→5' pathway, which requires the exosome complex, as well as the proteins encoded by *SKI2*, *SKI3*, and *SKI8* genes. A number of the incompletely degraded forms of the pG→3' fragment can be observed in exosome mutant strains as well as in the strains deficient in Ski2p, Ski3p, and Ski8p functions, but not in the wild type strains (16). These partially 3' trimmed species result from the inefficient 3'→5' mRNA degradation.

We asked whether the AtRrp41p can restore the proper exosome function in the 3'→5' degradation of mRNA in yeast. The strain yRP1204 used in this experiment had a temperature-sensitive *ski6-100* allele of *RRP41* gene and also contained a chromosomally integrated *MFA2* gene with the poly(G) insertion in the 3'-untranslated region (*MFA2pG*). As

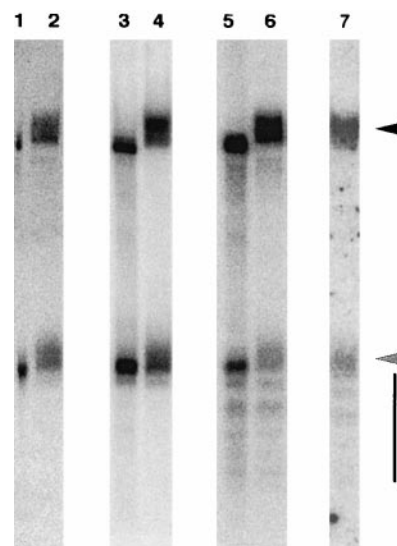


**FIG. 7. AtRRP41 complements the essential function of RRP41/SKI6 in yeast and rescues the 5.8 S rRNA processing defect of the *ski6-100* mutant.** Panel A, transformation with the AtRrp41p-expressing plasmid enables P118 to grow on glucose. Panel B, analysis of the 5.8 S rRNA processing. Total RNA was isolated and hybridized with the probes specific for the 7 S precursor (top panel), mature 5.8 S rRNA (middle panel), and scR1 RNA as a loading control (bottom panel) from the following strains: lanes 1 and 2, wild type strain yRP840; lanes 3 and 4, strain yRP1204 (*ski6-100*) transformed with pADH-AtRRP41; lanes 5 and 6, yRP1204 transformed with the empty vector. The incompletely processed 7 S rRNA species (vertical bar), full-length 7 S precursor (arrowhead), and the mature 5.8 S rRNA (open circle) are indicated on the right of the top panel. Cultures used in lanes 1, 3, and 5 were maintained at 29 °C, and those used for lanes 2, 4, and 6 were shifted to 37 °C for 60 min. Panel C, pADH-AtRRP41 complements the *ski6-100* mutant for growth at the restrictive temperature. The yRP1204 (*ski6-100*) mutant strain was transformed with the vector alone or with the pADH-AtRRP41 cDNA construct, and transformants were streaked onto YPD (2% glucose) plates and grown for 4 days at the indicated temperatures.

previously reported (16), a number of pG→3'-MFA2pG mRNA species shortened from the 3'-end could be detected in the mutant strain upon the shift to 37 °C but not in the isogenic wild type strain (Fig. 8). The introduction of the AtRrp41p-expressing plasmid into the *ski6-100* mutant strain restored the 3'→5' mRNA degradation pathway to nearly normal, as indicated by a significant reduction in the relative amount of these incompletely degraded pG→3' species (Fig. 8).

Interestingly, we have found that the relative amounts of incompletely degraded pG→3'-MFA2pG mRNA products in the *ski6-100* mutant are very similar at 30 °C and 37 °C (Fig. 8, lanes 6 and 7). Although other mRNAs have not been assayed in our experiments, this observation makes it unlikely that the defect of the *ski6-100* mutant strain in the 3'→5' mRNA degradation pathway causes the temperature-sensitive growth phenotype. Taken together with the similar observation regarding 5.8 S rRNA processing, this finding has interesting implications about the essential functions of the exosome complex (see "Discussion").

**AtRrp41p Is Found in the Exosome-sized Complex in Yeast and Plant Cell and Interacts with the Yeast Exosome Proteins in Vitro**—Our results show that the AtRrp41p rescues the apparent *rrp41/ski6* null phenotype and restores the 5.8 S rRNA maturation and mRNA 3'→5' degradation to near normal.

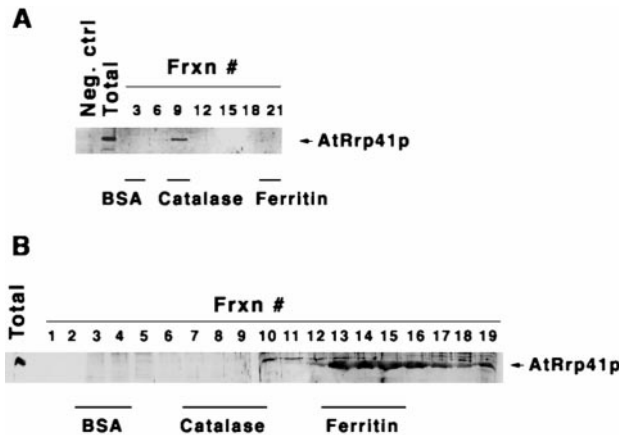


**FIG. 8. AtRRP41 rescues the 3'→5' mRNA degradation defect of the *ski6-100* mutant.** Northern blot hybridization analysis of the status of the full-length MFA2pG transcript (upper arrowhead) and its pG→3' degradation intermediate (lower arrowhead) in the strain yRP840 (wild type; lanes 1 and 2), yRP1204 (*ski6-100*) transformed with pADH-AtRRP41 (lanes 3 and 4) and yRP1204 (*ski6-100*) transformed with the empty vector (lanes 5–7). All cultures were grown at 30 °C, and those shown in lanes 1–6 were shifted to 37 °C for 60 min. The vertical bar indicates the incompletely 3'→5' degraded species in the yRP1204 (*ski6-100*) strain transformed with the empty vector. The RNA samples shown in the lanes 1, 3, and 5 were deadenylated *in vitro* by treatment with oligo(dT) and RNase H before loading.

These processes most likely require an intact exosome because mutations affecting different exosome subunits lead to accumulation of nearly identical sets of incompletely processed 5.8 S rRNA and mRNA species, respectively. Thus, our data suggest that AtRrp41p might be able to restore the integrity of the exosome complex in yeast lacking the endogenous Rrp41p. Therefore, we asked whether the AtRrp41p protein is present as a monomeric protein or as a part of a large complex in yeast cells. We produced polyclonal antibodies using the C-terminal peptide of AtRrp41p protein as an immunogen and used them in Western blot analysis of the fractionated extracts from the AtRRP41-complemented *rrp41* mutant. Total cell extracts were resolved by sedimentation through a 10–30% glycerol gradient, and every third fraction was assayed for the presence of the AtRrp41p. Bovine serum albumin (67 kDa, 4.3 S), catalase (250 kDa, 11.3 S) and ferritin (480 kDa, 65 S) were used as markers. Within the limit of resolution of this experiment, the majority of the AtRrp41p cosedimented with catalase, which is almost 10 times the size of the AtRrp41p monomer and is close to the estimated mass of the yeast exosome (Fig. 9A). These results are consistent with the view that a substantial portion of AtRrp41p is stably incorporated in an exosome-sized complex.

To provide an additional line of evidence that the high native molecular mass of the AtRrp41p truly reflects its interactions with the yeast exosome proteins, we conducted *in vitro* interaction experiments using recombinant polypeptides. Initially, 10 [<sup>35</sup>S]methionine-labeled core subunits of the yeast exosome, produced by an *in vitro* translation from the PCR-amplified, cloned open reading frames (detailed under "Experimental Procedures") were used to challenge the glutathione-Sepharose beads preloaded with either GST-AtRrp41p or with GST alone. Three of the core subunits, yRrp44p, yRrp4p, and yRrp45p, specifically bound to the GST-AtRrp41p-loaded resin in this experiment (data not shown). To confirm these interactions, these three subunits were tagged with hexahistidine, expressed and purified from *E. coli*, and used in the pulldown





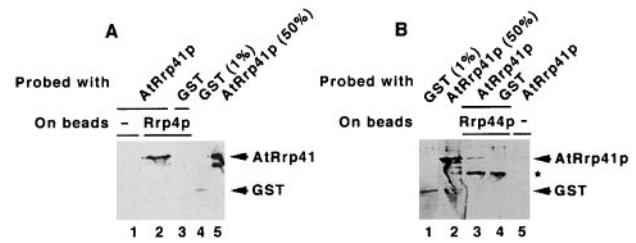
**FIG. 9. AtRrp41p is found in a large complex in yeast and *Arabidopsis* cells.** Total extract from the P118 yeast strain transformed with the pADH-AtRRP41 construct (panel A) or total extract from young *Arabidopsis* seedlings (panel B) was resolved by sedimentation through 10–30% glycerol gradient and either every third fraction (panel A) or all fractions (panel B) were analyzed by Western blotting using AtRrp41p-specific antibody. Positions of the size markers (bovine serum albumin, native molecular mass 67 kDa; catalase, 240 kDa; and ferritin, 480 kDa) were resolved in a parallel gradient as indicated. Total extract from the wild type yeast strain (Neg. ctrl) was used as a negative control in panel A.

experiments in which the orientation of interacting partners was opposite, *i.e.* the individual yeast exosome subunits proteins were immobilized on the Ni-NTA resin, and GST-AtRrp41p or GST was used as a challenging protein. Specific interactions of the AtRrp41p with the yeast Rrp44p and yRrp4p were again observed (Fig. 10). Notably, both Rrp44p and yRrp4p have close sequence homologs in other species, including *Arabidopsis*. No interaction was observed for the yRrp45p, which could indicate either that the original interaction was spurious or that the addition of the C-terminal hexahistidine tag to the yeast Rrp45p was detrimental to the protein/protein interaction.

Finally, to investigate the possibility that AtRrp41p is also a part of the exosome complex in the plant, we have resolved the total *Arabidopsis* cell extracts by sedimentation through 10–30% glycerol gradient, and we assayed the resulting fractions for the presence of the AtRrp41p protein by immunoblotting. AtRrp41p was found in the fractions with high native molecular mass (~500 kDa, Fig. 9B). This is somewhat higher than the estimated molecular mass of the yeast and human exosomes (300–400 kDa, (2, 21)), which could be caused by differences in exosome composition between the species. No signal was detected with preimmune serum (not shown). Given the ability of AtRrp41p to interact with the conserved yeast exosome proteins and to rescue its molecular functions that require the intact complex, we propose that the high molecular mass complex detected with antibodies against the AtRrp41p in the *Arabidopsis* cells is a plant exosome.

#### DISCUSSION

In this paper, we present the characterization of the *A. thaliana* homolog of the yeast exosome subunit Rrp41p (Ski6p). We find that the expression of the *Arabidopsis* RRP41 cDNA rescues the lethal phenotype of the *rrp41(ski6)* null mutant. The *Arabidopsis* protein is found predominantly in a high molecular mass complex in yeast cells, suggesting that it is incorporated into the exosome complex and probably helps to maintain its integrity. In addition, we find that the expression of the AtRrp41p in yeast corrects the known defects of the partial loss of function mutant *ski6-100* in 5.8 S rRNA processing and 3'→5' mRNA degradation. However, neither of these activities



**FIG. 10. AtRrp41p interacts with the yeast Rrp4p and Rrp44p.** Panel A, empty Ni-NTA resin (lane 1) or Ni-NTA resin preloaded with 10  $\mu$ g of purified recombinant yeast Rrp4p (lanes 2 and 3) was challenged with 5  $\mu$ g of recombinant GST-AtRrp41p (lanes 1 and 2) or GST (lane 3). Bound material was resolved by SDS-PAGE and immunoblotted with GST-specific antibody. Lanes 4 and 5 contain 1% of the supernatant from the Rrp4p/GST-AtRrp41p binding reaction and 50% of the supernatant from the Rrp4p/GST-AtRrp41p binding reaction, respectively. The band below the main band in lane 5 is a proteolytic fragment of the AtRrp41p. Panel B, empty Ni-NTA resin (lane 5) or Ni-NTA resin preloaded with 10  $\mu$ g of purified recombinant yeast Rrp44p (lanes 3 and 4) was challenged with 5  $\mu$ g of recombinant GST-AtRrp41p (lanes 3 and 5) or GST (lane 4). Bound material was resolved by SDS-PAGE and immunoblotted with GST-specific antibody. Lanes 1 and 2 contain 1% of the supernatant from the Rrp44p/GST-AtRrp41p binding reaction and 50% of the supernatant from the Rrp44p/GST-AtRrp41p binding reaction, respectively. The band marked with the asterisk is a proteolytic fragment of the Rrp44p which cross-reacts with the antibody against GST.

appears to be essential for cell viability. Thus, the Rrp41p protein must have at least one additional, essential function, which is conserved across the kingdoms' boundaries because the plant AtRrp41p readily rescues the *rrp41* lethal null phenotype.

Recombinant AtRrp41p behaves *in vitro* as a processive, phosphorolytic 3'→5' exonuclease, as would be expected of a member of the RNase PH protein family. Most of the amino acid residues that are conserved in the RNase PH/PNPase family (20) are also present in AtRrp41p. An unexpected feature of the AtRrp41p is that it requires a single-stranded poly(A) tail for the efficient degradation of the substrate. At present, it is not known whether the yeast Rrp41p is stimulated similarly by the poly(A) tails. It also remains to be seen whether the AtRrp41p still exhibits such poly(A) tail dependence when it is a part of the exosome complex. However, it is interesting to note in this regard that the yeast *ski6-100* mutant, as well as *rrp4-1*, *mtr3-1*, and *rrp6Δ* mutants, all accumulate 3'-extended polyadenylated forms of U4 small nuclear RNA and of several small nucleolar RNAs, suggesting that one of the functions of the exosome is to deadenylate these RNAs (27). On the other hand, no differences in the deadenylation rates of the two mRNAs, MFA2 and PGK1, were seen in that study, indicating that different classes of RNA could be deadenylated by different mechanisms.

The expression of the AtRrp41p in yeast complemented the essential function of the yeast Rrp41 protein and enabled rescue of some of the known molecular phenotypes of the partial loss of function, temperature-sensitive mutant *ski6-100*. Curiously, this strain shows defects in the 5.8 S rRNA processing and mRNA 3'→5' degradation which are of similar magnitude at both restrictive and permissive temperatures. This new observation strongly suggests that the reason for the temperature-sensitive lethality of this strain is not related to either 5.8 S rRNA processing or mRNA 3'→5' decay. It can be envisioned that at the nonpermissive temperature the exosome complex assembly is compromised, and thus multiple exosome-dependent processes fail simultaneously, resulting in a lethal phenotype. Alternatively, there may exist one or a few exosome-dependent reactions that are truly essential for cell viability, whereas others are dispensable. Further functional dissection of the exosome by mutational analysis and by cross-species



complementation may help resolve these alternatives.

Regardless of the precise molecular nature of the essential exosome-dependent process, it seems to require the presence of the intact complex. This idea is supported by the fact that none of the genes encoding the core exosome subunits is dispensable, that the functional inactivation of many of the core subunits results in similar molecular phenotypes, and that there is no significant amount of individual subunits in the wild type yeast cell. The exosome subunit homologs are also found in high molecular mass complexes in other species (see Refs. 2 and 23 and Fig. 9B), suggesting that similar requirement may hold for all eukaryotes. However, none of the reported instances of complementation of the yeast core subunit mutations by their homologs from other species involved complementation of the respective null phenotypes (4, 21, 25), and in none of these cases was a stable incorporation of the heterologous subunit into the yeast exosome reported (e.g. 21). The partial complementation could be the result of a transient incorporation of the heterologous subunit into the exosome complex, which nonetheless was sufficient to raise the effective concentration of the active complex above the critical threshold.

In contrast, the AtRrp41p readily complements the lethal phenotype of the complete loss of Rrp41p and is found in the high molecular mass complex in the yeast cell. We believe that this is indeed the exosome complex and that the AtRrp41p is able to participate in the evolutionarily conserved protein/protein interactions that are essential for exosome assembly and integrity, for the following reasons. First, the above arguments suggest that integrity of the exosome complex is essential for performing its essential function. Second, we find that the AtRrp41p interacts *in vitro* with the recombinant yeast Rrp44p and Rrp4p in pulldown experiments (Fig. 10). Both of these exosome subunits are conserved in other species, including *Arabidopsis*. Third, our preliminary data show that the recombinant *Arabidopsis* AtRrp41p also interacts *in vitro* with the *Arabidopsis* homolog of the yeast Rrp4p, AtRrp4p.<sup>2</sup> There is also an *Arabidopsis* homolog of the Rrp44p subunit (GenBank accession AC007584), which in the future will permit testing of the prediction that the mode of interactions between these two exosome proteins is also conserved in evolution. The cross-species complementation may therefore represent a powerful approach to the structure-function analysis of the evolutionarily conserved interactions in the exosome complex.

**Acknowledgments**—We would like to thank Phil Mitchell, David Tollervey, Murray Deutscher, and Roy Parker for the strains used in this work, Arabidopsis Biological Resource Center at Ohio State University for providing the *Arabidopsis* cDNA library, Nidhi Rumpal for help with pulldown experiments, and Richard Zitomer for a critical reading of the manuscript.

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