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PRH75, a New Nucleus-Localized Member of the DEAD-Box Protein Family from Higher Plants

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The putative RNA helicases of the DEAD-box protein family are involved in pre-mRNA splicing, rRNA maturation, ribosome assembly, and translation. Members of this protein family have been identified in organisms from *Escherichia coli* to humans, but except for the translation initiation factor 4A, there have been no reports on the characterization of other DEAD-box proteins from plants. Here we report on a novel member of the DEAD-box protein family, the plant RNA helicase 75 (PRH75). PRH75 is localized in the nucleus and contains two domains for RNA binding. One is located at the C terminus and is similar to RGG RNA-binding domains of nucleus-localized RNA-binding proteins. The other one is located between amino acids 308 and 622, a region containing the conserved motif VI characteristic of DEAD-box proteins and known as the RNA-binding site of eIF-4A. The N-terminal 81 amino acids are sufficient for nuclear targeting of the protein. Northern and Western blot analyses show that PRH75 is mainly expressed in young and rapidly developing tissues. The purified recombinant PRH75 has a weak ATPase activity which is barely stimulated by RNA ligands. The fractionation of spinach whole-cell extracts by glycerol gradient centrifugation and gel filtration on a Superdex 200 column shows that the protein exists in a complex of about 500 kDa. Possible biological functions of PRH75 as well as structure-function relationships in the context of its modular primary structure are discussed.

In recent years it became apparent that posttranscriptional processes are as important as transcriptional processes in the overall control and regulation of eukaryotic gene expression. These regulatory processes are mediated by a growing number of different RNA-binding proteins. As the pre-mRNA emerges from the transcriptional complex, it is bound by a large number of proteins, termed heterogeneous nuclear ribonucleoproteins (hnRNPs) (7). At least two different sets of RNA-binding proteins are involved in pre-mRNA splicing, those that are constitutively associated with small nuclear ribonucleoprotein particles (snRNPs) and those that interact only transiently with the spliceosome during distinct steps of the splicing process (19). Besides RNA-protein interactions, the secondary structure of the RNA molecule is equally important for splicing, ribosome assembly, and translation of transcripts (44). In all these processes, various members of the so-called DEAD-box protein family are involved (21, 41, 72). These proteins share seven highly conserved sequence motifs, including the sequence Asp-Glu-Ala-Asp (DEAD), first identified in the eukaryotic translation initiation factor eIF-4A, and are supposed to have ATP-dependent RNA helicase activities (41). The importance of these proteins is underlined by their presence in all prokaryotes and eukaryotes analyzed to date. Based on differences in the conserved motifs (DEAH versus DEAD, GRAGR versus HRIGR, and PRRVAA versus PTRELA), the members of the DEAD-box protein family can be classified into the DEAD- and DEAH-box proteins (60, 72). Both of them contain the conserved sequence motif (G/A)X_4GKT, which resembles ATP-binding domains, consistent with the observation that some members of the DEAD/H-box protein family exhibit RNA-dependent ATPase activity (72). Although helicase activity has been demonstrated for only a few DEAD-box proteins (17, 20, 38, 56, 63), they are good candidates for melting RNA secondary structures in all processes described before (19, 72). For example, the *PRP* yeast genes required for pre-mRNA processing (PRP), a process involving numerous RNA-RNA interactions (for reviews, see references 19 and 62), encode DEAD/H-box proteins (3, 4, 6, 60, 67). PRP5 and PRP28 are DEAD-box proteins that are required early in spliceosome assembly and for the first step of splicing, respectively (6, 67). PRP2 and PRP16 are required for the first and second steps of the splicing reaction (3, 60), whereas PRP22 promotes the release of the spliced RNA from the spliceosome (4). PRP2, PRP16, and PRP22 belong to the DEAH-box protein family, which seems to be more specific for pre-mRNA splicing. In addition to pre-mRNA splicing, rRNA processing, translation, and ribosome assembly, the members of the DEAD/H-box protein family are involved in cellular processes as diverse as germ line development (18, 41, 55, 72), embryogenesis (36, 41, 72), nucleocytoplasmic transport of mRNA (40), RNA degradation (43, 52), and mRNA stability (25).

Most current knowledge about posttranscriptional processes in eukaryotic cells derives from studies on yeast and metazoa. Posttranscriptional processes in higher plants are still poorly understood, mainly because of the lack of suitable in vitro systems. Progress has been made by characterizing *cis*-acting elements involved in pre-mRNA splicing and polyadenylation (for reviews, see references 9 and 23). These analyses indicate that some processes in higher plants are similar to those in vertebrates (9, 23, 37, 64, 65), while others exhibit unique features and may involve plant-specific protein factors (16).

Except for the translation initiation factor 4A, which has been cloned from many plant species (reference 49 and references therein), and two other genes encoding DEAD-box proteins, which were not further characterized (26, 69), there are no other reports on plant DEAD-box proteins. We describe

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the isolation and molecular characterization of a cDNA which encodes a nucleus-localized DEAD-box protein from spinach.

MATERIALS AND METHODS

cDNA cloning and recombinant DNA work. A spinach cDNA library (42) was screened (57) with a 400-bp α-^{32}P-labeled probe encoding a glycine-arginine-rich part of a spinach RNA-binding protein containing three RGG domains (29), each consisting of four consecutive RGG repeats (42a). Hybridization was performed in 5× SSPE (1× SSPE is 10 mM NaH_2PO_4, pH 7.4, plus 150 mM NaCl and 1 mM EDTA), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 μg of herring sperm DNA per ml at 62°C for 18 h. Positive phages were plaque purified, and the inserts were recloned into pBSC$^-$ (Stratagene) and sequenced (58) with Sequenase version 2.0 (U.S. Biochemicals). Recombinant DNA work was performed according to Sambrook et al. (57) or according to protocols provided by the enzyme suppliers (Boehringer Mannheim and New England Biolabs). The oligonucleotides were obtained from MWG-BIOTECH (Ebersberg, Germany).

RNA isolation and analysis. For Northern blot analysis, total RNA was isolated from different spinach organs with the RNeasy Plant Total RNA kit (Qiagen). Equal amounts of RNA were separated on 1% formaldehyde-containing agarose gels. After transfer to nitrocellulose membranes, the RNA filters were hybridized with the α-^{32}P-labeled 1.8-kb *Nde*I-*Pst*I fragment from pRSET-PRH75 (see below). Hybridization and washing of the filters were performed under stringent conditions.

Preparation of spinach whole-cell extracts. Protein extracts were prepared by two different methods. For denatured extracts, tissue samples were homogenized in liquid nitrogen and subsequently thawed in 100 mM Tris-HCl buffer, pH 8.0, supplemented with 100 mM NaCl, 50 mM EDTA, 1% SDS, and 0.07% β-mercaptoethanol. To achieve complete lysis, samples were incubated for 20 min at 60°C and then centrifuged (30,000 × *g* for 30 min at 30°C). The same buffer volume/fresh weight ratio was used for all samples. For native extracts, tissue samples were homogenized in liquid nitrogen and subsequently thawed in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100, 5 mM β-mercaptoethanol, 1 μg of leupeptin per ml, 1 μg of antipain per ml, and 1 mM phenylmethylsulfonyl fluoride (WCE buffer). Samples were centrifuged at 40,000 × *g* for 30 min at 4°C, and protein concentrations were measured by the dye-binding method, with bovine serum albumin as a standard.

Construction of plasmids for nuclear localization: protoplast isolation, transformation, and GUS assay. The β-glucuronidase (GUS) coding sequence under the control of the 35S RNA cauliflower mosaic virus (CaMV) promoter in pUC19 (35S-GUS-nos cassette) was used for the construction of the N-terminal translational fusions between GUS and PRH75 and its deletions. The plasmids p35S-PRH75::GUS and p35S-81NPRH75::GUS were derived from the 2.1-kb *Nde*I-*Hin*dIII and 0.24-kb *Nde*I-*Xmn*I fragments of pRSET-PRH75 (see below), respectively. Prior to cloning, all PRH75 DNA fragments were filled in with Klenow enzyme and gel purified before ligation into the filled-in *Xma*I site of the pUC19 derivative. This procedure allows the synthesis of DNA constructs with correct reading frames of all PRH75 fragments relative to the GUS coding region. The p35S-PRH75::GUS construct generates a fusion protein with five additional amino acid residues (LNSKL) at the C terminus of PRH75, while the plasmid p35S-81NPRH75::GUS encodes an L81I exchange. The sequence at the PRH75-GUS junction in each of these plasmids was verified by DNA sequence analysis.

Tobacco leaf protoplasts were isolated from aseptically grown plants (30). Protoplasts were transformed by the polyethylene glycol method (30), with 30 μg of supercoiled plasmid DNA per 10^6 protoplasts. The GUS activity was assayed 20 h after transformation essentially as described previously (22). Protoplasts were inspected on a Zeiss Axiovert microscope with Nomarski optics.

Construction of plasmids expressing the PRH75 deletion mutants. Overexpression of PRH75 deletions was performed in the pRSET vectors (68). The entire coding region of *PRH75* was amplified by PCR from pBSC$^-$-PRH75, with gene-specific oligonucleotides. The 5′ primer (5′-ATCTTGAAAC<u>CATATG</u>CC TTC-3′) was designed to contain an *Nde*I restriction site (underlined) at the ATG initiation codon. The 3′ primer (5′-CAGATGGGA<u>CTTAAG</u>CGACGAC CA-3′) generated an *Afl*II restriction site (underlined) at the position of the stop codon. The PCR product was cloned into pCR^{TM}II (Invitrogen) and sequenced before use. The insert was then isolated as an *Nde*I-*Afl*II fragment and inserted into pRSET 5b, which had been linearized with *Nde*I and *Eco*RI. The recessive ends of both the insert and the vector were filled in with the Klenow enzyme prior to ligation. The resulting construct, pRSET-PRH75, was used for the production of the PRH75 deletion mutants. Del1 was constructed by subcloning an *Nde*I-*Pst*I fragment (encoding amino acid residues 1 to 622) into the corresponding restriction sites of pRSET 5b. Del2 was generated by subcloning a *Bst*BI fragment (encoding amino acid residues 124 to 685) into a *Bst*BI site of pRSET 5d, and Del4 was obtained by subcloning the *Nde*I-*Bst*BI fragment (encoding amino acid residues 1 to 110) into corresponding sites of pRSET 5c. For the generation of Del5, a *Xho*I-*Eco*RV fragment (encoding amino acid residues 124 to 353) was removed from Del2, and the ends were filled in prior to ligation. The construct Del6 was obtained by removing an *Eco*RV-*Hin*dIII fragment (encoding amino acid residues 309 to 622) from Del3 (see below), again after a fill-in reaction with the Klenow enzyme. Del3 was obtained by subcloning an *Msc*I-*Pst*I fragment (encoding amino acids 182 to 622) from pBSC$^-$-PRH75 into pRSET 5b. For the latter construct, pRSET 5b was linearized with *Nhe*I, filled in with the Klenow enzyme, and digested with *Pst*I. For more detailed information on PRH75 deletion mutants, see Results. All deletion mutant plasmids were checked by sequence analysis before overexpression in *Escherichia coli* BL21.

Construction of plasmids expressing GST-PRH75 and GST-GYR fusion proteins. The coding region of PRH75 was fused in frame to the glutathione *S*-transferase (GST) gene in the vector pGEX 2T (Pharmacia) to obtain the construct pGST-PRH75. For this purpose, the DNA region was amplified with the oligonucleotides 5′-CTTGAAACT<u>GAATTC</u>CTTCAATCT-3′ (5′ end) and 5′-ACAGATGGG<u>GAATTC</u>GACGACC-3′ (3′ end). These oligonucleotides introduce *Eco*RI restriction sites (underlined) into the coding region exactly at the positions of the start and stop codons. The *Eco*RI fragment was then cloned into the corresponding site of pGEX 2T. The plasmid pGST-GYR contains sequences for the 57 C-terminal amino acids encoded by PRH75, the GYR domain plus 14 preceding codons (encoding amino acids 629 to 685; see also Results). A DNA fragment encoding this region was amplified by PCR with the same 3′ primer and the 5′ primer 5′-TGGATG<u>GAATTC</u>AAGCCTGCCTCC-3′. Again, the resulting *Eco*RI fragment was inserted into pGEX 2T. Both constructs were sequenced before overexpression experiments.

Overexpression and purification of GST fusion proteins. The plasmids harboring GST-PRH75 fusions were transformed into *E. coli* BL21(DE3)/pLysS. Overnight cultures were grown at 37°C in the presence of 40 μg of chloramphenicol per ml and 100 μg of ampicillin per ml, then diluted 100-fold, and again grown at 30°C to an optical density at 600 nm of 0.6. The expression of the transgenes was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration, 0.5 mM) at 26°C. The cells were harvested by centrifugation (5,000 × *g* for 10 min at 4°C) 4 h later, resuspended in 1× phosphate-buffered saline, and sonicated. After centrifugation (12,000 × *g* for 15 min at 4°C), aliquots of the supernatant were directly applied onto a freshly prepared glutathione Sepharose 4B column and further processed according to the manufacturer's instructions (Pharmacia). The GST (overexpressed from the pGEX 2T) and GST-GYR proteins were used for RNA-binding experiments directly without further purification. In contrast, the GST-PRH75 fusion protein was contaminated by degradation products which were removed by gel filtration on a Superdex 200 HR 10/30 column (Pharmacia). All steps of purification were done at 4°C, and the buffers used contained 2 μg of aprotinin per ml, 2 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride.

RNA-protein interaction (Northwestern assay). Bacterial lysates containing overexpressed PRH75 deletion proteins were separated on an SDS-polyacrylamide gradient gel (10 to 17% acrylamide) (32). Gel slices containing the overexpressed proteins were excised after staining, loaded onto a second gradient gel, run as before, and transferred to nitrocellulose. The Northwestern analysis was performed as described previously (8) with a randomly chosen RNA template (*psbW* gene) (42) produced by in vitro transcription in the presence of [α-^{32}P]UTP.

ATP hydrolysis assay. ATP hydrolysis assays were carried out in a 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 1 μM [γ-^{32}P]ATP in a total volume of 20 μl. Each reaction mixture contained 100 ng of protein. The reactions were performed at 25°C for 45 min in the presence of total spinach RNA, total *Arabidopsis* RNA, spinach poly(A)$^+$ RNA, total spinach DNA, M13 single-stranded DNA, and poly(A), poly(U), poly(G), and poly(C) ribohomopolymers. The reactions were stopped by the addition of 1 volume of 0.5 M EDTA, pH 8.0. Two microliters was analyzed by thin-layer chromatography on polyethyleneimine cellulose plates (Macherey-Nagel) as described previously (11). The products were visualized by autoradiography.

Fractionation of spinach whole-cell extracts on glycerol gradients and by gel filtration. The whole-cell extracts were prepared as described before. The 11-ml 10 to 35% glycerol gradients prepared in WCE buffer were overlaid with 300 μl of spinach whole-cell extract (total protein amount, 2 mg) and centrifuged at 4°C for 24 h at 27,000 rpm (110,000 × *g*) in a Beckman SW 40 Ti rotor. Parallel gradients were loaded with molecular mass standards (thyroglobulin, 669 kDa; catalase, 232 kDa; and albumin, 67 kDa) (all from Pharmacia). Gradients were divided into 28 fractions of 0.4 ml and were analyzed by SDS-polyacrylamide gel electrophoresis (32) and Western blotting for the presence of PRH75 and the large subunit of ribulose 1,5-bisphosphate carboxylase (RuBisCO) (500 kDa). The position of molecular weight standards was determined by measuring absorption at 280 nm.

For the gel filtration analysis, the whole-cell extracts were precipitated stepwise by ammonium sulfate. The protein fractions containing PRH75 (as determined by Western blotting) were diluted twice with WCE buffer and then loaded onto a Superdex 200 HR 10/30 column equilibrated with WCE buffer containing 0.4 M ammonium sulfate. The column was eluted with the same buffer at a flow rate of 0.3 ml/min (for more details see also Results).

Nucleotide sequence accession numbers. Sequences of the *Spinacia oleracea* and *Arabidopsis thaliana PRH75* genes are deposited in the EMBL nucleotide sequence database under accession numbers X99937 and X99938.

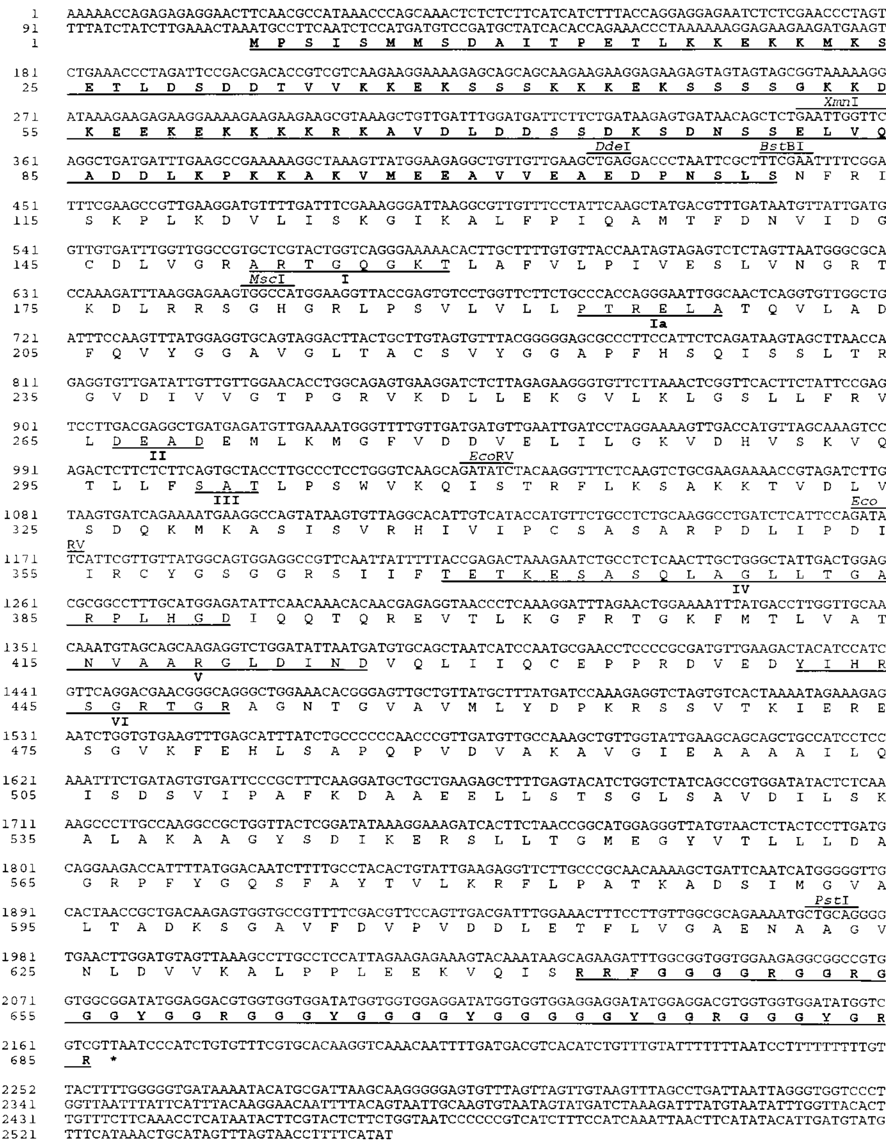

FIG. 1. The nucleotide and predicted amino acid sequences of spinach PRH75 cDNA. The seven helicase motifs are underlined and numbered I to VI. Restriction sites used for construction of overexpression and nuclear import plasmids are indicated. The N-terminal KDES and C-terminal GYR domains are underlined and in bold.

RESULTS

cDNA cloning and primary structure of PRH75. Using one of the three glycine-arginine-rich segments of an RNA-binding protein from spinach (42a), we have isolated a phage with an insert of 2,556 bp. Sequence analysis revealed that this cDNA encodes a protein belonging to the DEAD-box protein family (41). The nucleotide and deduced amino acid sequences of this cDNA are presented in Fig. 1. The cDNA encodes a polypeptide of 685 amino acid residues with a calculated molecular mass of 75 kDa. We named this protein PRH75 (plant RNA helicase 75). The proposed translation initiation codon at nucleotide 111 is preceded by four in-frame stop codons, and the in vitro-transcribed and translated PRH75 product comigrates with the immunoreactive protein from spinach whole-cell extract, confirming that the cDNA is full length (data not shown). The nucleotide sequence surrounding the first methionine codon (CTAAATGCCT; methionine codon is underlined) is not in agreement with the consensus sequence proposed by Kozak (31). However, the same translation initiation context was found in the *Arabidopsis* cDNA, which we isolated as well, again indicating that the cDNAs are full length. Southern analysis indicates that *PRH75* originates from a single-copy gene in spinach (data not shown).

PRH75 is a basic protein with a calculated pI of 9.9. The PRH75 sequence can be divided into three distinct domains (Fig. 1): the N terminus (amino acids 1 to 110), which is enriched in lysine (24%), glutamic acid (12%), aspartic acid (12%), and serine (17%) residues (KDES domain); the central part (amino acids 111 to 490), harboring the conserved helicase motifs; and the C terminus (amino acids 491 to 685), with the characteristic 43 amino acids at the very end, referred to as the GYR domain, which contains 67% glycine residues interrupted by either arginines (18%) or tyrosines (12%).

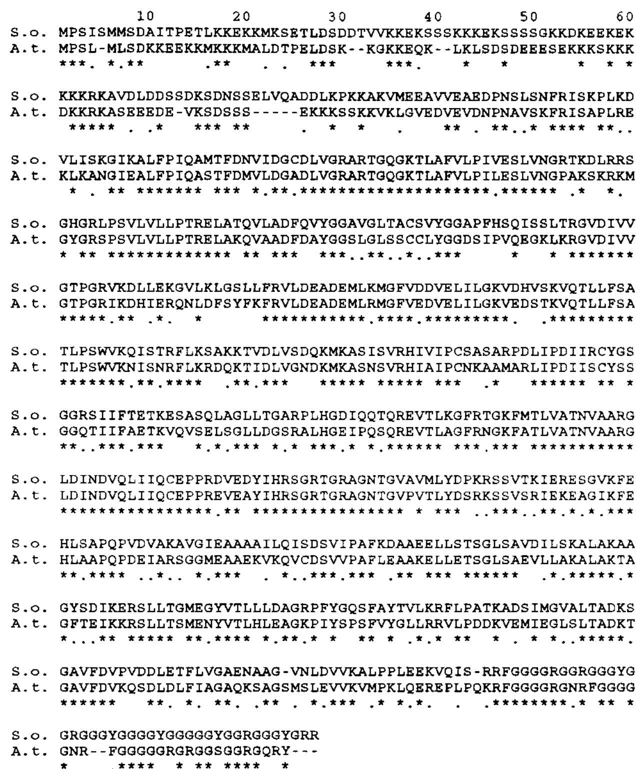

FIG. 2. Alignment of the amino acid sequences of PRH75 from spinach (S.o.) and *Arabidopsis* (A.t.). Conserved amino acids are indicated by asterisks, and conservative changes are indicated by dots.

PRH75 and other DEAD-box proteins. The homology of PRH75 to various members of the DEAD-box protein family (41) is restricted to the central domain, which contains seven highly conserved helicase motifs. The only difference concerns motif VI (YIHRIGRTGR), in which the conserved isoleucine residue at position 5 (underlined) is replaced by a serine residue. This seems to be characteristic for PRH75, since the same exchange exists in the *A. thaliana* homolog (Fig. 2). The central part of PRH75 exhibits 29 and 38% identity to the corresponding segment of other DEAD-box proteins, whereas no significant homology could be found in the N- and C-terminal domains, indicating that they are unique to PRH75. The only protein sequence showing a higher overall identity with PRH75 (41% versus 29 to 34% with others) is the human Gu protein (71), which also shows a similar domain structure. The Gu protein contains the DEVD sequence in motif II, in contrast to PRH75, which contains DEAD. Finally, the human protein is 15 kDa larger than PRH75, and it is not clear if the cDNA is complete (71). A similar domain structure has been shown for several other DEAD-box proteins, such as human p68 (10) and *Xenopus* An3 (18). However, they do not exhibit sequence similarities to PRH75 in the respective N- and C-terminal domains. Furthermore, the DEAD/H-box proteins which are supposed to be homologs are at least 55 to 84% identical, even when they are from very distantly related organisms (18, 24, 48). Thus, we conclude that PRH75 represents a new member of the DEAD-box protein family.

Expression of *PRH75* during plant development. *PRH75* is expressed in all spinach organs tested, albeit at quite different levels (Fig. 3). The highest levels were detected in hypocotyls, roots, and apical buds. During leaf development, the *PRH75* mRNA level drops substantially. In addition, the mRNA levels in etiolated and green tissues do not differ significantly (Fig. 3, lanes 1 and 2). To extend this study, we performed the same analysis on the protein level. As shown in Fig. 4A, irrespective of the extraction method used (see Materials and Methods), one prominent band of the expected size (75 kDa) was detectable on Western blots. Cross-reactivity of antibodies was found only with one protein, of 40 kDa (Fig. 4A). To analyze the expression pattern on the protein level, we prepared native whole-cell extracts (see Materials and Methods) from the tissues used for Northern analysis. Figure 4B, lanes 3 and 9, indicates high protein levels in apical buds and inflorescences. Finally, as shown for RNA, the protein level drops during leaf development, indicating that PRH75 is required for rapid cell growth and division.

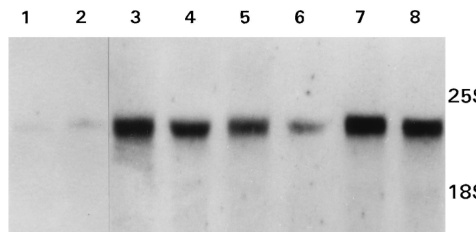

FIG. 3. Organ- and development-specific expression of PRH75. Each lane was loaded with 15 μg of total RNA isolated from green cotyledons (lane 1), etiolated cotyledons (lane 2), apical buds (lane 3), not completely opened leaves (lane 4), leaves of approximately 0.5 cm (lane 5), completely developed leaves (lane 6), hypocotyls (lane 7), and roots (lane 8). The positions of 25S and 18S rRNA are indicated.

Subcellular localization of PRH75. With the exception of eIF-4A (the cytoplasmic translation initiation factor 4A), Suv3p (a DEXH-box protein which is a component of a yeast mitochondrial 3′-to-5′ exoribonuclease [43]), and MSS116 (a polypeptide involved in mitochondrial splicing [61]), all other known eukaryotic DEAD-box proteins are located in the nucleus. A computer search with the PRH75 sequence, however, did not reveal any significant homology to nucleus localization sequences (NLS) (28, 53), despite a lysine-rich N terminus (Fig. 1). Therefore, the entire coding region of *PRH75* was fused 5′ to the *uidA* reporter gene (encoding GUS). The chi-

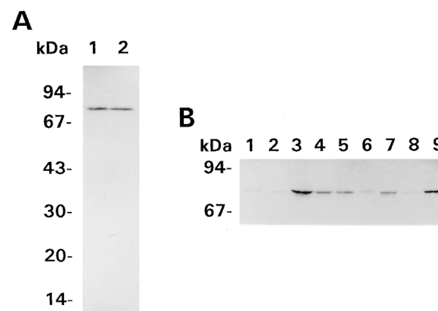

FIG. 4. Western blot analysis of spinach whole-cell extracts. After electrophoresis on an SDS-polyacrylamide gradient gel (10 to 17% acrylamide) (32), proteins were transferred to nitrocellulose and probed with rabbit polyclonal antiserum raised against Del3 of spinach PRH75 (see Materials and Methods) (Fig. 6C) at a 1:1,000 dilution. The secondary antibody was anti-rabbit immunoglobulin G alkaline phosphatase conjugate at a 1:30,000 dilution. (A) Protein extracts were prepared as native (lane 1) and denatured (lane 2) and were tested for the presence of PRH75. (B) Native protein extracts were prepared from the same samples as in Fig. 3 and are in the same order on the gel. Lane 9 was loaded with extract from inflorescences. Each lane contained the same quantity of protein (50 μg).

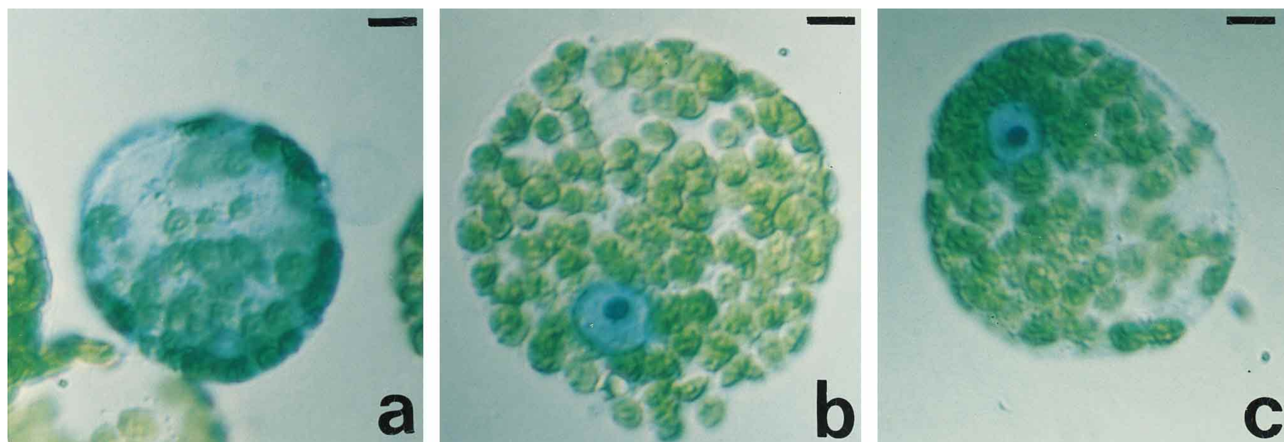

FIG. 5. PRH75 directs GUS to the nucleus. Tobacco leaf protoplasts were transiently transformed with the *uidA* reporter gene (encoding GUS) (a), PRH75::GUS (b), and 81NPRH75::GUS translational fusions under the control of the 35S RNA CaMV promoter (c). The transformation was repeated at least four times with each construct. Bars, 10 μm.

meric gene construct and the *uidA* gene alone were transiently expressed in tobacco protoplasts under the control of the 35S RNA CaMV promoter, and the GUS activities were determined histochemically. As expected, the GUS protein alone did not accumulate in tobacco nuclei, whereas the translational *PRH75-uidA* fusion was found exclusively in nuclei (Fig. 5). To identify the determinants responsible for the nuclear targeting, a series of PRH75 deletions (see also Materials and Methods) was fused to the N terminus of GUS and transfected into the tobacco protoplasts. This analysis demonstrates that the N-terminal 81 amino acids are sufficient to direct the GUS protein into the nucleus (Fig. 5). This segment still contains several possible candidates for NLS (KKEKKMK, KKEK, KKKEK, and KKKKRK). Based on its similarity to simian virus 40 NLS (28), the KKKKRK sequence is the most likely candidate. A combination of mutation and deletion approaches should allow precise determination of the motifs.

PRH75 contains two RNA-binding sites. The fact that all DEAD/H-box proteins examined to date are involved in different aspects of RNA metabolism prompted us to investigate whether PRH75 possesses RNA-binding activity in vitro. Unlike hnRNPs (7), SR proteins (74), and some members of the helicase superfamily II (21), PRH75 does not contain a consensus sequence RNA-binding domain (CS-RBD) (for reviews, see references 2, 7, and 44). Inspection of the PRH75 primary structure suggests two possible RNA-binding motifs, the helicase motif VI (HRIGRTGR), which is the RNA-binding domain in some viral RNA helicases (8, 54) and in the DEAD-box protein eIF-4A (50, 51), and the C-terminal glycine-rich GYR domain. The latter resembles to some extent a recently characterized RNA-binding RGG box (Fig. 6) (29).

Several PRH75 deletion mutants (Fig. 7C) (see also Materials and Methods) were overexpressed in *E. coli* (Fig. 7A), the proteins were gel purified, transferred to nitrocellulose membranes, and incubated with a ^{32}P-labeled RNA probe. As shown in Fig. 7B, all deletion mutants containing the GYR domain are capable of RNA binding. Deletion of the GYR domain substantially reduced RNA binding. Interestingly, deletion mutants containing only the helicase motif VI interacted weakly with RNA (Fig. 7B). The Del4 and Del6 mutants, both lacking the GYR domain and the helicase motif VI, no longer bound to RNA (Fig. 7B and C) (see also Discussion). This indicates the presence of a strong (GYR domain) and a weak (located between amino acids 308 and 622) RNA-binding domain in PRH75. In addition, RNA binding could not be competed with single-stranded or double-stranded DNA, suggesting that it is RNA specific (data not shown).

The C-terminal GYR domain is an independent RNA-binding module. To determine whether the GYR domain is sufficient for RNA binding, this motif was fused to the C terminus of the GST (see Materials and Methods). The fusion protein, as well as GST alone, was produced in *E. coli*, purified, and used for RNA-binding assays (Fig. 8A and B). As a control, we used the full-length PRH75 fused to the GST (Fig. 8A and B, lane 1). As expected, GST alone did not bind RNA, whereas the GST-GYR fusion protein bound RNA with an efficiency comparable to that of GST-PRH75 (Fig. 8B, lane 2). This indicates that the GYR domain is an independent RNA-binding module. In addition, RNA binding is at least in part ATP independent, since no ATP was added to the binding buffer (see also Discussion).

PRH75 is present in a large complex, of about 500 kDa. After glycerol gradient centrifugation, PRH75 was always detected in high-molecular-weight fractions, together with the most abundant plant protein RuBisCO (500 kDa). This molecular weight is six to seven times higher than that proposed from the cloned cDNA (data not shown). To prove this, we performed ammonium sulfate precipitation to obtain an enriched fraction containing PRH75 suitable for gel filtration. Proteins were precipitated stepwise, and the PRH75 was found at 40 to 50% ammonium sulfate saturation. These fractions

650	**RGG**	----	**RGG**	G-	YGG	-	**RGG**	G--	**YGG**	666	PRH75
696	**RGG**	NF--	**RGG**	--	APG	N	**RGG**	YN-	**RGG**	715	hnRNP U
15	**RGG**	-FGD	**RGG**	--	**RGG**	-	**RGG**	FGG	GRG	35	fibrillarin
645	**RGG**	----	**RGG**	--	**RGG**	G	**RGG**	FGG	**RGG**	663	nucleolin
34	**RGG**	DNHG	**RG**R	G-	**RG**R	G	**RGG**	--G	**R**P**G**	55	EBNA-1
331	**RGG**	S--G	GRG	--	**RGG**	S	GGR	--G	**RGG**	349	EBNA-1
4	**RGG**	N---	**RGG**	--	**RGG**	F	**RGG**	F--	**RGG**	21	GAR1
147	**RGG**	ASMG	**RGG**	S-	**RGG**	F	**RGG**	---	**RGG**	167	GAR1
	RGG	---**G**	**RGG**	--	**RGG**	-	**RGG**	**F-G**	**RGG**		**Consensus**

FIG. 6. Identification of RGG motifs in PRH75. The glycine-rich C terminus of PRH75 is aligned with RGG box-containing proteins with known RNA-binding activity (human hnRNP U protein, human fibrillarin, chicken nucleolin, (EBNA-1), and yeast GAR1). The numbers indicated correspond to the first and last amino acid residues of each sequence, respectively. The RGG repeats are boxed and in bold. The alignment is based on that in the work of Kiledjian and Dreyfuss (29).

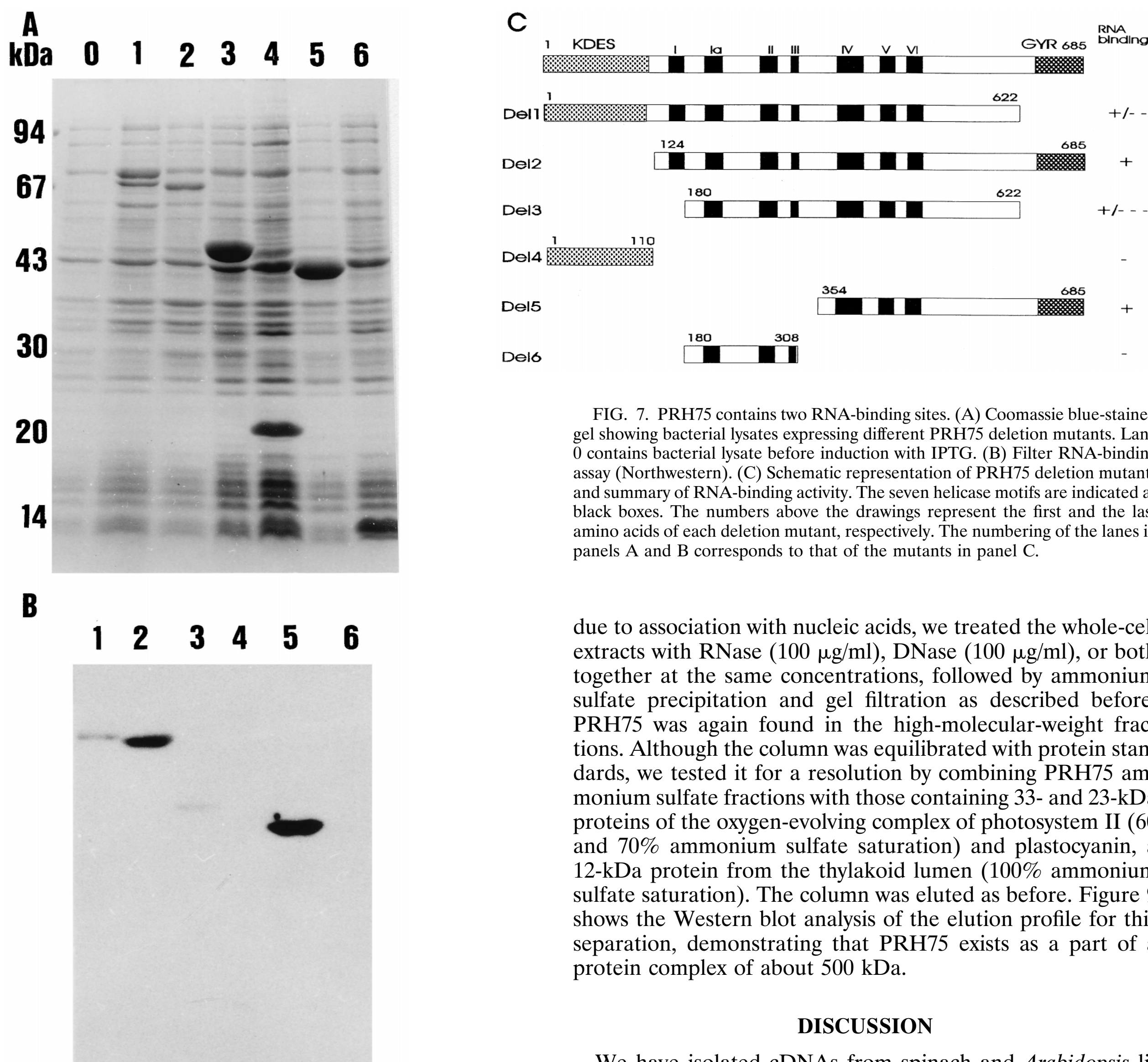

FIG. 7. PRH75 contains two RNA-binding sites. (A) Coomassie blue-stained gel showing bacterial lysates expressing different PRH75 deletion mutants. Lane 0 contains bacterial lysate before induction with IPTG. (B) Filter RNA-binding assay (Northwestern). (C) Schematic representation of PRH75 deletion mutants and summary of RNA-binding activity. The seven helicase motifs are indicated as black boxes. The numbers above the drawings represent the first and the last amino acids of each deletion mutant, respectively. The numbering of the lanes in panels A and B corresponds to that of the mutants in panel C.

were pooled, diluted twice with WCE buffer, and loaded onto a Superdex 200 HR 10/30 column equilibrated with the same buffer containing 0.4 M ammonium sulfate. PRH75 was eluted in the same fractions as RuBisCO, confirming the result obtained by glycerol gradient centrifugation. These effects could be explained by (i) nonspecific protein-protein interactions; (ii) self-aggregation; (iii) association with nucleic acids, since we have shown that PRH75 interacts with RNA (Fig. 7 and 8); or (iv) the fact that PRH75 is a part of a large complex. Elution profile on a Superdex 200 column gave a single peak for PRH75, about 500 kDa, devoid of any monomeric form, which indicates that such behavior is not due to self-aggregation or to nonspecific protein-protein interactions. Otherwise, one would expect the protein to be in different forms throughout the whole elution profile. Furthermore, the possibility of a nonspecific protein-protein interaction is also excluded by the fact that we loaded the ammonium sulfate fraction directly onto a Superdex 200 column preequilibrated with a buffer containing 0.4 M ammonium sulfate, conditions which do not favor nonspecific interactions. To find out if such behavior was due to association with nucleic acids, we treated the whole-cell extracts with RNase (100 μg/ml), DNase (100 μg/ml), or both together at the same concentrations, followed by ammonium sulfate precipitation and gel filtration as described before. PRH75 was again found in the high-molecular-weight fractions. Although the column was equilibrated with protein standards, we tested it for a resolution by combining PRH75 ammonium sulfate fractions with those containing 33- and 23-kDa proteins of the oxygen-evolving complex of photosystem II (60 and 70% ammonium sulfate saturation) and plastocyanin, a 12-kDa protein from the thylakoid lumen (100% ammonium sulfate saturation). The column was eluted as before. Figure 9 shows the Western blot analysis of the elution profile for this separation, demonstrating that PRH75 exists as a part of a protein complex of about 500 kDa.

DISCUSSION

We have isolated cDNAs from spinach and *Arabidopsis* libraries which encode PRH75, a new member of the large family of putative RNA helicases. These proteins are characterized by seven conserved sequence motifs (41). The homology of PRH75 to other DEAD-box proteins can be found only within the central part of the protein (320 amino acids) containing these motifs. The spinach and *Arabidopsis* proteins are 62% identical and 74% similar (Fig. 2). Characteristic of PRH75 is its domain (or modular) structure (Fig. 1), typical of many nuclear regulatory proteins. We have demonstrated that each of these domains in PRH75 has a unique role and function.

DEAD-box proteins are involved in quite different aspects of RNA metabolism in both the cytosol and the nucleus; however, most of them lack a CS-RBD (2, 7, 44). This raises the questions of how they interact with RNA and whether they contain as-yet-unidentified RNA-binding domains (50, 51, 70). The fact that almost all biochemically studied, highly purified DEAD-box proteins exhibit RNA-dependent ATPase activities strongly argues for a direct interaction with RNA. Potential RNA-binding domains are glycine-rich regions, which are usually interrupted by arginines or hydrophobic residues such as tyrosine or phenylalanine (18, 24, 36, 55). These domains

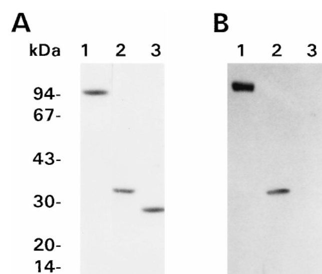

FIG. 8. The C-terminal GYR domain of PRH75 converts the non-nucleic-acid-binding protein GST to an RNA-binding protein. (A) Coomassie blue-stained gel showing purified GST-PRH75 fusion protein (lane 1), GST-GYR fusion protein (lane 2), and GST alone (lane 3). Two micrograms of purified protein was loaded per lane. (B) Filter RNA-binding assay (Northwestern). Protein quantities per lane were as in panel A.

could also be involved in protein-protein interactions (5). On the other hand, it has been shown that the helicase motif VI (HRIGRTGR) is crucial for RNA binding of eIF-4A, the prototype and best-studied member of the DEAD-box protein family (50, 51). Like other DEAD-box proteins, PRH75 does not contain a CS-RBD but harbors the C-terminal glycine-rich GYR domain (Fig. 1 and 6). Secondary-structure predictions suggest that the GYR domain forms an extended structure with β-turns which may be extremely flexible due to its high glycine content and that it could adopt different conformational states, as has been demonstrated for nucleolin RGGF tetrapeptides (12). Deletion analysis demonstrates that two segments are involved in RNA binding; the domain containing the helicase motif VI interacts weakly with RNA, whereas the C-terminal glycine-rich GYR domain interacts strongly with RNA (Fig. 7B and C). The latter is also capable of conferring RNA-binding activity on the non-nucleic-acid-binding protein GST (Fig. 8B). Except for eIF-4A and two viral DEXH-box helicases (8, 54), to our knowledge, PRH75 is the only DEAD-box protein for which RNA-binding sites have been identified. The RGG repeats or GAR domains (15) were first identified in the nucleolar proteins nucleolin (35), fibrillarin (34, 59), SSB1 (27), NSR1 (39), and GAR1 (15) and are supposed to have functions as diverse as RNA binding, nucleolar targeting, and protein-protein interactions. The involvement of GAR domains in non-sequence-specific RNA binding has recently been demonstrated for nucleolin (12), the hnRNP U protein (29), and Epstein-Barr nuclear antigen 1 (EBNA-1) (66).

PRH75 is a nucleus-localized protein, with the crucial region for the translocation into this organelle being located within the 81 N-terminal amino acids (Fig. 5). Apparently, the C-terminal part harboring the GYR domain is not required for translocation. This is reminiscent of the nucleolar protein GAR1, which lacks a classical nuclear (nucleolar) targeting signal (14), but different from nucleolin, which contains a bipartite NLS (53). In addition to the NLS, the targeting of nucleolin to the nucleolus requires both the CS-RBD and the GAR domains (45). The precise location of PRH75 within the nucleus is not known. In protoplast experiments, a stronger GUS staining is always found in the nucleolus, suggesting that PRH75 might be involved in the maturation of rRNAs or the assembly of preribosomes. However, this can also be caused by differences in the structure of the nuclear and nucleolar matrices. The first attempts to answer this question by immunolocalization in situ were unsuccessful, most probably because the antibodies do not recognize the protein in its native form or because the epitopes recognized by the antibodies are buried by another protein or RNA factor(s) interacting with PRH75 in vivo. This could also explain why immunoprecipitation experiments with the same antibodies did not work (data not shown). The organ-specific and developmentally regulated expression of PRH75 (Fig. 3 and 4B) may argue against its involvement in general processes in the nucleus. This can also be explained by the requirement of higher PRH75 activity in the nucleus during developmental processes which require higher overall metabolic activity. Here it should be mentioned that the protein and mRNA levels in the roots did not correlate (Fig. 3 and 4B). This discrepancy may have been caused by differences in translation efficiency or protein stability in particular cells or tissue types.

It also became clear that PRH75 interacts with RNA, at least partially, in an ATP-independent manner, since no ATP was added to the binding buffer. PRP2, a yeast protein involved in pre-mRNA splicing, also interacts with spliceable mRNA in an ATP-independent manner (70). The RNA binding of eIF-4A, in contrast, is very inefficient in filter binding assays (1) and could be detected only by cross-linking studies (1, 50). Unlike PRH75 and PRP2, protein-RNA interaction was detectable only in the presence of ATP in both assays used (1, 50). The possibility that the interaction with RNA of Del1 and Del3 of PRH75 could be stimulated by ATP was not excluded. Nevertheless, comparison of our results with those from eIF-4A and PRP2 studies clearly indicates that there are at least two different modes of action of DEAD-box proteins which are reflected in their primary structure.

PRH75 shows a very weak ATPase activity which is barely stimulated by different RNA ligands (data not shown). Most ATPases of the DEAD/H-box protein family are active in the presence of a large variety of RNA ligands, and even homopolymers like poly(A), poly(U), poly(C), and poly(G) are efficient stimulators (references 38, 60, and 71 and references therein). The low ATPase activity of PRH75 may indicate that the enzyme requires a very specific RNA ligand, as shown for *E. coli* DbpA (11) and yeast Slt22 (73) proteins preferentially stimulated by 23S rRNA and annealed U2/U6 snRNAs. The yeast Rrp3 protein involved in 18S rRNA processing exhibits a weak ATPase activity which is not specific for rRNA (47). The other possible explanation for the weak ATPase activity of PRH75 is that it requires an additional protein factor(s) like the *E. coli* RhlB protein, which is active as ATPase only as a part of RNA degradosome, not as a free protein (52). In

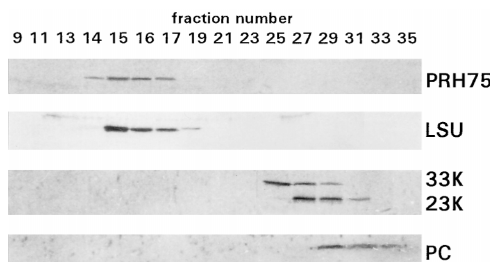

FIG. 9. Superose 200 gel filtration analysis demonstrates the presence of PRH75 in a large protein complex, of about 500 kDa. The spinach whole-cell extract was treated with RNase (100 μg/ml) and DNase (100 μg/ml) and precipitated stepwise by ammonium sulfate. The fractions containing PRH75, 33- and 23-kDa proteins of the oxygen-evolving complex of photosystem II, and the 12-kDa thylakoid luminal protein plastocyanin were pooled and subjected to gel filtration on a Superdex 200 column (for more details, see Results). Proteins were separated by SDS-polyacrylamide gel electrophoresis (32) and transferred to nitrocellulose, and the positions of PRH75, the RuBisCO large subunit (LSU), the 33-kDa protein, the 23-kDa protein, and plastocyanin (PC) were determined by Western blotting (for more details about the 33-kDa, 23-kDa, and plastocyanin proteins, see reference 42 and references therein). Only fractions 9 to 35 are shown, since little protein eluted in fractions 1 to 8 and 36 to 50.

addition, RhlB binds RNA in Northwestern assays (52), as does PRH75, indicating the uncoupling of RNA-binding and ATP hydrolysis activities (see also above). The interaction with RNA of PRH75 in filter binding assays seems to contrast with its low ATPase activity. The most likely explanation is that the GYR domain interacts with RNA in a non-sequence-specific manner. The specificity for a distinct RNA species may be provided by another protein factor(s) through protein-protein interactions. Once the protein has been brought into the right environment via protein-protein interactions, it binds RNA through its GYR domain, which in turn stabilizes the whole complex and allows the protein to perform the specific function, most probably conformational change in the RNA substrate followed by ATP hydrolysis. The fact that PRH75 purifies as a high-molecular-weight complex (see Results) (Fig. 9) strongly supports this idea. In this respect it is interesting that *E. coli* RhlB protein exhibits ATPase and RNA helicase activities only as a part of RNA degradosome (52).

Although the members of the DEAD/H-box protein family are often referred to as RNA helicases, so far, this activity has been demonstrated for only two DEAD-box proteins in a monomeric form, p68 (20) and An3 (17); one DEAH-box RNA helicase (38); and two viral helicases, NPH-II (63) and PPV CI (33). Monomeric DEAD-box proteins such as p68 and the RNA helicase A contain two possible RNA-binding domains (although not experimentally determined), motif VI and a second domain, which is presumably required for a stable protein-RNA interaction (13). In the case of p68, for example, a basic (arginine-rich) region is present at the C terminus (10, 20), and in the case of RNA helicase A, a double-stranded RNA-binding motif was identified at the N-terminal part of the polypeptide (13). In contrast, eIF-4A, which lacks a second RNA-binding domain, binds RNA but exhibits helicase activity only in the presence of the second protein, eIF-4B (50, 51, 56), which contains CS-RBD (46). Apparently, the weak interaction of RNA with motif VI, common to all helicases of the DEAD-box family, is required for the catalytic activity of the enzyme (50) but is not sufficient for stable protein-RNA interaction (13). Although PRH75 contains two distinct RNA-binding sites (Fig. 7 and 8), the low ATPase activity as well as the fact that PRH75 purifies as a large complex (Fig. 9) argues against the possibility that PRH75 is a monomeric helicase.

Besides biochemical analysis, the most important question concerning PRH75 is its biological function. The availability of the *Arabidopsis PRH75* homolog should allow this question to be addressed by using the reverse genetic approach in combination with further biochemical analyses. In this context we are currently trying to purify the protein and to identify the interaction partners by in vitro studies (pull-down experiments) with different GST-PRH75 fusions.

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