Proteomic Characterization of Evolutionarily Conserved and Variable Proteins of Arabidopsis Cytosolic Ribosomes[1][w]

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Analysis of 80S ribosomes of Arabidopsis (*Arabidopsis thaliana*) by use of high-speed centrifugation, sucrose gradient fractionation, one- and two-dimensional gel electrophoresis, liquid chromatography purification, and mass spectrometry (matrix-assisted laser desorption/ionization time-of-flight and electrospray ionization) identified 74 ribosomal proteins (r-proteins), of which 73 are orthologs of rat r-proteins and one is the plant-specific r-protein P3. Thirty small (40S) subunit and 44 large (60S) subunit r-proteins were confirmed. In addition, an ortholog of the mammalian receptor for activated protein kinase C, a tryptophan-aspartic acid-domain repeat protein, was found to be associated with the 40S subunit and polysomes. Based on the prediction that each r-protein is present in a single copy, the mass of the Arabidopsis 80S ribosome was estimated as 3.2 MD (1,159 kD 40S; 2,010 kD 60S), with the 4 single-copy rRNAs (18S, 26S, 5.8S, and 5S) contributing 53% of the mass. Despite strong evolutionary conservation in r-protein composition among eukaryotes, Arabidopsis 80S ribosomes are variable in composition due to distinctions in mass or charge of approximately 25% of the r-proteins. This is a consequence of amino acid sequence divergence within r-protein gene families and posttranslational modification of individual r-proteins (e.g. amino-terminal acetylation, phosphorylation). For example, distinct types of r-proteins S15a and P2 accumulate in ribosomes due to evolutionarily divergence of r-protein genes. Ribosome variation is also due to amino acid sequence divergence and differential phosphorylation of the carboxy terminus of r-protein S6. The role of ribosome heterogeneity in differential mRNA translation is discussed.

The ribosome is a two-subunit ribonucleoprotein complex that catalyzes the peptidyl transferase reaction of polypeptide synthesis, an absolute requirement for cellular growth and differentiation. The structure and function of both prokaryotic and eukaryotic ribosomes have been investigated, with the eukaryotic emphasis on ribosomes of Baker's yeast (*Saccharomyces cerevisiae*) and rat (*Rattus rattus* and *Rattus norvegicus*). The cytosolic ribosomes of eukaryotes are composed of a large number of ribosomal proteins (r-proteins) and four distinct rRNAs, the 18S rRNA of the 40S subunit, and the 5S, 5.8S, and 23S-like (25–28S) rRNAs of the 60S subunit (Bielka, 1982). Early evaluation of the buoyant density and sedimentation coefficients of eukaryotic ribosomes predicted that the rat 80S ribosome has a higher mass (4.2–4.6 MD) than that of pea (*Pisum sativum*; 3.9 MD) due to distinctions in the 60S subunit (Cammarano et al., 1972). Further evidence that the plant ribosome is smaller than that of mammals was provided by the three-dimensional reconstruction of wheat (*Triticum aestivum*) and rabbit (*Oryctolagus cuniculus*) ribosomes by use of cryo-electron microscopy (Verschoor et al., 1996). Despite an overall similarity in architecture, the 60S subunit of wheat appeared approximately 20% smaller than that of rabbit. This variation in mass was attributed to differences in the length of highly variable loop regions of the 23S-like rRNA, which ranges in size from approximately 3,300 bp (25–26S) in plants to approximately 4,700 bp (28S) in mammals (Schnare et al., 1996). However, to date there has been no detailed biochemical comparison of the protein component of plant and animal ribosomes.

The systematic analysis of two-dimensional (2D) gel fractionated r-proteins of 80S ribosomes and gene sequences of animals and fungi led to the recognition of 79 eukaryotic r-proteins of common evolutionary origin (32 small subunit and 47 large subunit r-proteins; Warner, 1989; Wool et al., 1995; Goffeau et al., 1997; Planta and Mager, 1998; Veuthey and Bittar, 1998; Yoshihama et al., 2002; Nakao et al., 2004). The mouse, human, and *Drosophila melanogaster* genomes encode all 79 proteins, whereas some r-proteins are absent in the genomes of Baker's yeast (L28), and *Caenorhabditis elegans* and *Schizosaccharomyces pombe* (S27a; Nakao et al., 2004). Based on biochemical analyses of ribosomes of mammals and yeast, each

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r-protein is present in unimolar amounts with the exception of P1 and P2 of the 60S subunit, which can be either entirely absent or present as 2 or 4 monomers in yeast and 2 dimers in animals (Tsurugi and Ogata, 1985; Guarinos et al., 2003).

Recently, mass spectrometry (MS) has become an efficient technology for proteomic characterization of macromolecular complexes (for review, see Aebersold and Mann, 2003). MS analyses confirmed the presence of the 32 predicted r-proteins in the 40S subunit of rat fibroblast ribosomes (Louie et al., 1996) and 75 of the 78 putative r-proteins of yeast 80S ribosomes (Link et al., 1999; Lee et al., 2002; Inada et al., 2002). Four additional putative r-proteins were noted in the rat analysis and two ribosome-associated proteins were identified in the yeast analyses. MS analysis also allowed for the identification of plastidic r-proteins of spinach (*Spinacia oleracea*) and *Chlamydomonas reinhardtii* (Yamaguchi et al., 2000, 2002; Yamaguchi and Subramanian, 2000, 2003), as well as mitochondrial r-proteins of yeast and mammals (Koc et al., 2000, 2001; Saveanu et al., 2001; Suzuki et al., 2001a, 2001b; Gan et al., 2002). In addition, MS technology has been applied to identify covalent posttranslational modification of r-proteins. For example, the sequential phosphorylation of carboxy-terminal sites of Drosophila and maize (*Zea mays*) r-protein S6 was validated by use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) following digestion with trypsin and β-elimination of phosphate groups by treatment with barium hydroxide (Radimerski et al., 2000; Williams et al., 2003).

There have been limited biochemical analyses of the protein components of plant ribosomes. 2D gel electrophoresis was employed to resolve r-proteins of several higher plant species, including wheat, soybean (*Glycine max*), tomato (*Lycopersicon peruvianum*), maize, tobacco (*Nicotiana tabacum*), and barley (*Hordeum vulgare*; Capel and Bourque, 1982; Gantt and Key, 1983; Sikorski et al., 1983; Scharf and Nover, 1987; Bailey-Serres and Freeling, 1990; Koyama et al., 1996). The estimates of small and large subunit proteins range from 26 to 40 and 41 to 59 proteins, respectively. The strong conservation in eukaryotic r-protein primary sequence was utilized to identify orthologs of the well-characterized rat r-protein collection in Arabidopsis (*Arabidopsis thaliana*; Barakat et al., 2001). By use of expressed sequence tag and the complete genomic sequence accessions, 249 genes (including 22 apparent pseudogenes) were estimated to encode 80 putative types of r-proteins (32 small subunit and 48 large subunit), including a previously identified plant-specific r-protein of the large subunit, P3 (Bailey-Serres et al., 1997; Szick et al., 1998).

To identify the evolutionarily conserved r-proteins of plant 80S ribosomes and to determine if there are additional plant-specific r-proteins, we combined 2D gel electrophoresis and MS to characterize the ribosomal proteome of Arabidopsis. With this approach, we identified 74 r-proteins, including 2 products of evolutionarily distinct r-protein genes. We characterized a number of covalent posttranslational modifications of r-proteins, including the phosphorylation of S6. In addition, we identified a Trp-Asp (WD)-repeat domain protein associated with the 40S subunit that is a known scaffold for regulatory proteins.

RESULTS

Identification of Arabidopsis 80S R-Proteins by 2D Gel Fractionation and MS

In a previous study, we identified 249 genes that encode 80 putative types of cytosolic r-proteins in Arabidopsis (Barakat et al., 2001; an update of this analysis indicates that there are 251 genes, including 17 pseudogenes, that encode the 80 types of r-proteins; http://www.arabidopsis.org/info/genefamily/athr.html). To confirm the presence of these proteins in Arabidopsis ribosomes, a ribosome pellet fraction was isolated from dark-grown suspension culture cells by detergent extraction and differential centrifugation. The majority of r-proteins are basic and a few are highly acidic (Barakat et al., 2001). The basic proteins were optimally separated in a first dimension basic-urea gel that achieved separation of proteins ranging in pI from 9 to 13 (Williams et al., 2003). Proteins were further fractionated by apparent molecular mass in the second dimension by SDS-PAGE and stained with Coomassie Blue (Fig. 1A). The acidic and neutral proteins of the ribosome pellet fraction were optimally separated by nonequilibrium pH gel electrophoresis (NEpHGE; O'Farrell, 1975) in the first dimension and SDS-PAGE in the second dimension (Fig. 2A). The protein spots were excised and digested in-gel with trypsin and analyzed by either peptide mass fingerprinting (PMF) using MALDI-TOF, or tandem MS (MS/MS) using triple quadropole TOF (Q-TOF) with orthogonal MALDI (oMALDI) or electro-spray ionization (ESI) as the ion source.

The proteomic analyses of the ribosome pellet fraction resulted in the identification of 70 of the 80 types of putative cytosolic r-proteins (Table I; Supplemental Table I). These included 58 r-proteins identified with the basic-urea gel system, 15 with the NEpHGE system, and 3 with both systems (Table I; Supplemental Table I; Figs. 1, B and C, and 2B). None of the spots contained known proteins of plastid or mitochondrial ribosomes, indicating that organellar ribosomes were not abundant in the pellet fraction. The percentage of the total residues of each protein detected (sequence coverage) in peptide fragments by MALDI-TOF ranged from 14% to 70%, with more than one-half of the proteins identified with over 41% sequence coverage. MS/MS was used for peptide sequencing to confirm the identity of proteins with low sequence coverage. Peptides corresponding to the products of 2 or more distinct genes were detected for 33 of the 70 (approximately 47%) r-proteins (Supplemental Table I), consistent with the

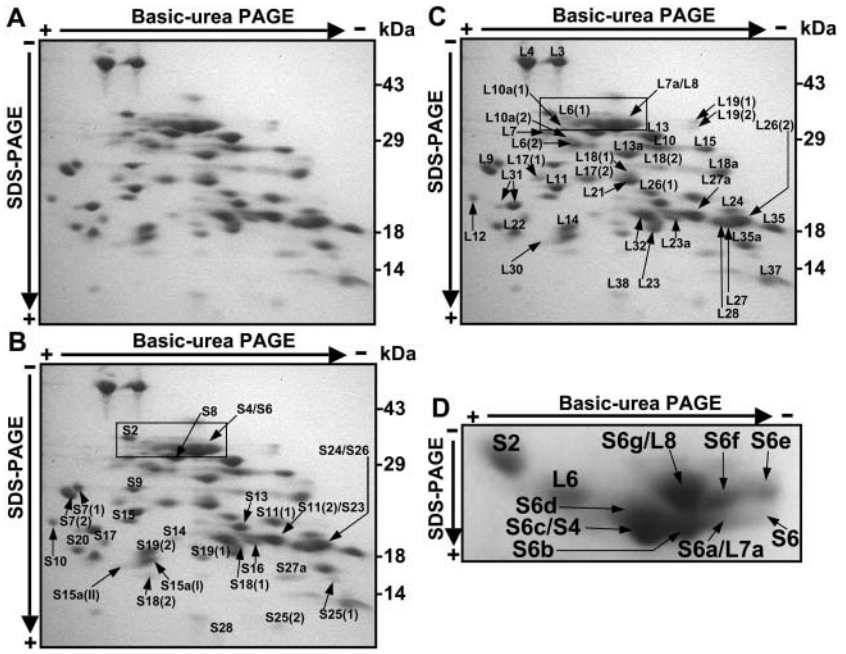

Figure 1. 2D basic-urea/SDS-PAGE of Arabidopsis r-proteins. A, Total ribosomes isolated from 6-d-old dark grown suspension cultured cells of Arabidopsis were treated with glacial acetic acid to extract rRNA and basic r-proteins were separated using 2D basic-urea/SDS-PAGE. The first-dimension fractionated proteins through a specialized basic-urea gel and the second dimension fractionated proteins by apparent molecular mass (15% [w/v] SDS-PAGE). The direction and polarity of electrophoresis is shown with arrows and charge symbols. R-proteins were visualized by staining with Coomassie Blue. The migration of molecular mass markers is indicated on the right. B and C, Identification of small (40S) subunit (B) and large (60S) subunit (C) r-proteins. Protein spots resolved in A were excised, digested in-gel with trypsin, and analyzed by MS. The r-protein designation is directly above the protein spot or by an arrow; a number or Roman numeral after a protein indicates evidence of a gene-specific or modified product. The proteins in the boxed region of the gel were further resolved in D. D, Basic-urea gel electrophoresis/SDS-PAGE of RPS6 forms. R-protein fractionation as in A, except that 12% (w/v) SDS-PAGE was used to increase protein separation. The 8 forms of r-protein S6 detected by MALDI-TOF MS are designated as S6, S6a, S6b, S6c, S6d, S6e, S6f, and S6g. Protein spots that were not reproducibly observed on gels are not identified.

finding that most r-proteins are encoded by 3 to 4 expressed genes (Barakat et al., 2001).

Biochemical variation in r-proteins was apparent from the detection of 18 r-proteins (approximately 26%) identified in more than one gel spot (Table I). Of these, peptides specific to a gene family member were identified in spots corresponding to S7, S11, S15a, S19, P2, and L10a, indicating that the difference in mobility can reflect biochemical distinctions of individual gene products. For other r-proteins found in two or more spots (S6, S25, P0, L5, L10a, L18, L19, L26, and L31), the same gene product was identified in multiple spots, suggesting that the variation in apparent charge or mass could be due to covalent posttranslational modifications. For example, the product of *RPL19A* was resolved in 2 forms with the same migration in the basic-urea gel but with different apparent molecular masses (34 and 36 kD; Fig. 1C), whereas 2 forms of L5 and P0 had slightly different pIs (Fig. 2B). Distinctions in protein migration did not appear to be due to N-terminal acetylation (+42 D), which was confirmed for nine r-proteins (Supplemental Table I). Unequal stoichiometry of some r-proteins was suggested from the Coomassie Blue staining; however, variation in the staining intensity of both basic and acidic proteins could be due to differences in avidity of proteins to the stain.

Identification of R-Proteins and Non-R-Proteins by Liquid Chromatography and MS/MS

The ribosome pellet isolated by differential centrifugation reproducibly included several acidic and neutral proteins that are not r-proteins (Fig. 2B; Supplemental Table II). These proteins included core proteasome subunits, several abundant membrane proteins, a mitochondrial chaperonin, an abundant cytosolic enzyme, and a WD-repeat domain protein. To evaluate if these proteins were present in the ribosome pellet fraction due to an association with translational complexes or cosedimentation of macromolecular complexes, the pellet was subjected to Suc density gradient fractionation, the proteins in the resulting 15 fractions were separated by one-dimensional (1D) SDS-PAGE, and visualized by Coomassie Blue staining. Immunoblot analysis with anti-maize S6 and anti-yeast L15 (ortholog of rat L12) antisera, specific for small and large ribosomal subunits, respectively,

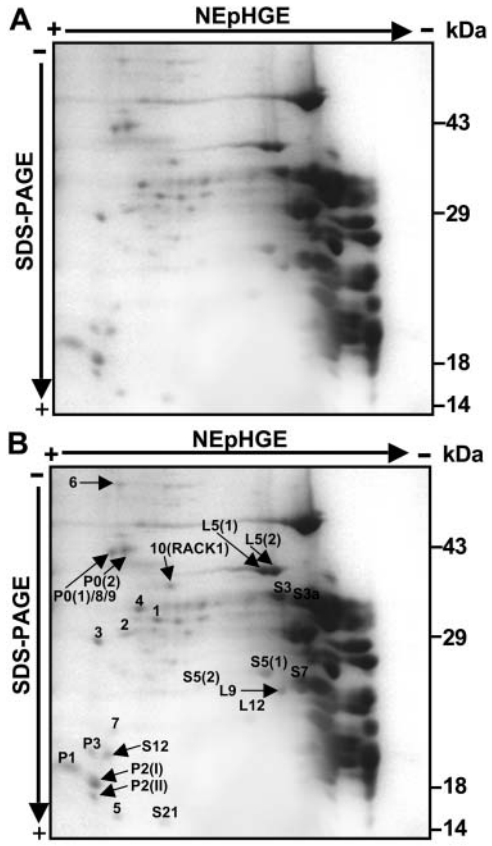

Figure 2. 2D NEpHGE of Arabidopsis r-proteins. A, Total ribosomes isolated as described in Figure 1 were separated by 2D NEpHGE/SDS-PAGE to analyze acidic and neutral proteins. The first-dimension fractionated proteins by ionic charge through a pH gradient (pH 3–10). The second-dimension fractionated proteins by apparent molecular mass (12% [w/v] SDS-PAGE). The direction and polarity of electrophoresis is shown with arrows and charge symbols. R-proteins were visualized by staining with Coomassie Blue. The migration of molecular mass markers is indicated on the right. B, Identification of proteins. Proteins resolved in A were excised, digested in gel with trypsin, and analyzed by MS. The protein designation is directly above the protein spot or by an arrow; a number or Roman numeral after a protein indicates evidence of a gene-specific or modified product. Nonribosomal proteins are identified with a number: 1, proteasome α-subunit A (At2g05840); 2, proteasome α-subunit B (At1g16470); 3, proteasome α-subunit E (At1g53850; At3g14290); 4, vacuolar H^+ ATPase subunit E (At4g11150); 5, vacuolar H^+ ATPase subunit G; 6, chaperonin heat shock protein 60 (At3g23990); 7, mitochondrial F_0 ATPase chain D (At3g52300); 8, vacuolar reversibly glycosylated protein (At3g02230; At5g15650); 9, Gln synthase (At3g17820); 10, WD-repeat domain repeat protein human RACK1 ortholog (At1g18080; At1g48630). Protein spots that were not reproducibly observed on gels are not identified.

was performed to identify fractions enriched in 40S and 60S subunits, 80S ribosomes, and polysomes (Fig. 3). The stained gel was divided into seven sections for the polysomal fraction, which were subjected to in-gel trypsin digestion followed by liquid chromatography (LC) coupled to Q-TOF ESI/MS/MS. In this manner, the polysome fraction was found to include 60 of the 70 r-proteins detected after 2D gel separation, as well as 4 additional putative r-proteins (Sa, S27, L37a, and L40; Table I; Supplemental Table I), bringing the total number of identified r-proteins to 74. Of the non-r-proteins detected on 2D gels, the abundant tonoplast membrane-associated reversibly glycosylated polypeptide was present in the nonpolysomal fraction and absent in the polysomal fraction, whereas proteasome α-subunits A, B, and E were identified in both fractions (Fig. 3; Supplemental Table II).

A 36-kD protein resolved as spot number 10 by NEpHGE/SDS-PAGE was identified as a WD-repeat domain protein related to mammalian receptor for activated protein kinase C, RACK1 (Fig. 2B; Supplemental Table II). Three Arabidopsis genes (At1g18080, At1g48630, and At3g18130) encode a protein with 65% amino acid sequence identity to human RACK1. Peptides corresponding to the product of 2 of these genes were detected in spot number 10 and in a 36-kD band observed in the 40S subunit, 80S ribosome, and polysome fractions (Fig. 3, arrow; Supplemental Table II). Consistent with the conclusion that the Arabidopsis RACK1 ortholog associates with ribosomes via an interaction with the 40S subunit, this protein was detected in the 40S, 80S, and polysome fractions at levels similar to that observed for r-protein S6 (Fig. 3).

Arabidopsis Ribosomes Contain Differentially Phosphorylated Forms of S6

The detection of r-protein S6 in multiple protein spots led to the evaluation of the phosphorylation of 2 S6 gene products (*RPS6A*, At4g31700; *RPS6B*, At5g10360). S6A and S6B are 95.2% identical in amino acid sequence but have divergent carboxy termini with 5 and 3 potential phosphorylation sites, respectively. Eight forms of S6 (S6, S6a–g) were identified among the constellation of proteins with an apparent pI of 10 to 11 and molecular mass of 28 to 30 kD (Fig. 1D; Supplemental Table II). The forms S6 and S6a to d displayed more rapid mobility in the SDS-PAGE dimension than the forms S6e to g.

MS analysis of each of the 8 S6 forms by in-gel trypsin-digestion and barium hydroxide-treatment provided evidence of carboxy-terminal phosphorylation in S6A and S6B (Table II). Phosphorylation (+80 D) of Ser or Thr decreases the charge of tryptic peptides and reduces the frequency of peptide ionization during MALDI-TOF MS analysis. To circumvent this difficulty, trypsin digestion was coupled with treatment with barium hydroxide, which causes β-elimination of H_3PO_4 (−98 D). Thus, the ions of phosphorylated peptides show an 18 mass-to-charge ratio (*m/z*) reduction for each phosphorylated residue. When this procedure was carried out with the extremely basic S6 form, only an unmodified carboxy-terminal peptide was detected. On the other hand, the *m/z* values of ions from the S6a to d digests corresponded to the predicted masses of mono-, di-, tri-, and tetra-phosphorylated S6A, respectively. Peptide ions from S6a to c were detected that also corresponded to the predicted masses

Table I. *Identification of Arabidopsis r-proteins by MS analysis*

Arabidopsis r-protein identification follows the universally accepted nomenclature as outlined in Barakat et al. (2001). R-proteins resolved in 2 gel spots are designated (1) and (2). Evolutionarily distinct r-proteins are designated (I) and (II). Gel System abbreviations: 1D, 1D SDS polyacrylamide; B, 2D basic-urea; N, 2D nonequilibrium pH. Protein identification method abbreviations: M, MALDI-TOF MS; QT, oMALDI or electro-spray ionization Q-TOF MS/MS; LC/MS/MS, ribosomal and nonribosomal protein complexes were separated by Suc density centrifugation and polysomal proteins separated by 1D SDS-PAGE were digested with trypsin and analyzed; all r-proteins were identified in the Suc gradient purified polysome fraction by LC/MS/MS except P1, P3, L3, L10a, L27a, L28, L30, L35a, L37, and L38.

Small Subunit Proteins						Large Subunit Proteins					
Ribosomal Protein ID	Gel System	ID Method	Sequence Coverage	Molecular Mass		Ribosomal Protein ID	Gel System	ID Method	Sequence Coverage	Molecular Mass	
				Calculated	Apparent					Calculated	Apparent
			%	*kD*					%	*kD*	
Sa	1D	LC/MS/MS	38	30.0–32.3	35.0–45.0[a]	P0 (1)	N	M	14	33.7–34.4	43.0
S2	B	M	27	30.1–30.9	37.0	P0 (2)	N	M	26	33.7–34.4	44.0
S3	N	M	38	27.3–27.5	36.0	P1	N	QT	[c]	11.0–11.2	17.5
S3a	N	M	34	29.8–29.9	35.0	P2 (I)	N	M	50	11.0–11.8	16.5
S4	B	M, QT	50	29.8–30.1	34.0	P2 (II)	N	M	70	11.0–11.8	15.5
S5 (1)	N	M, QT	22	22.9–23.0	26.5	P3	N	M	36	11.8–11.9	19.0
S5 (2)	N	M, QT	15	22.9–23.0	24.5	L3	B	M	45	44.5–44.6	51.0
S6 (1)[b]	B	M, QT	41	28.1–28.4	34.5	L4	B	M	44	44.7	50.0
S6 (2)[b]	B	M, QT	41	28.1–28.4	34.0	L5 (1)	N	M	30	34.2–34.4	40.5
S7 (1)	B, N	M	34	21.9–22.2	27.0	L5 (2)	N	M	21	34.2–34.4	39.5
S7 (2)	B, N	M	28	21.9–22.2	25.5	L6 (1)	B	M	37	26.0–26.2	36.0
S8	B	M	42	23.8–24.1	32.5	L6 (2)	B	M	25	26.0–26.2	30.0
S9	B	M	31	23.0–23.2	27.0	L7	B	M	35	28.1–28.5	32.5
S10	B, N	M	40	19.4–19.8	21.5	L7a	B	M	23	29.0–29.1	34.0
S11 (1)	B	M	47	17.7–18.0	21.5	L8	B	M	40	27.9–28.0	34.5
S11 (2)	B	M	33	17.7–18.0	19.0	L9	B, N	M	35	22.0	25.5
S12	N	QT	[c]	15.3–15.4	18.5	L10	B	M	45	24.1–24.9	30.0
S13	B	M	57	16.9–17.0	20.5	L10a (1)	B	M	52	24.3–24.5	34.0
S14	B	M	35	16.2–16.3	19.5	L10a (2)	B	M	43	24.3–24.5	31.5
S15	B	M	29	16.8–17.1	22.0	L11	B	M	54	20.9–21.1	22.5
S15a (I)	B	M	45	14.7–15.3	16.5	L12	B, N	M, QT	31	17.8–18.0	21.5
S15a (II)	B	M	61	14.7–15.3	15.5	L13	B	M	45	23.5–23.8	31.5
S16	B	M	56	16.6	19.0	L13a	B	M	43	23.5–23.6	23.5
S17	B	M	41	16	15.5	L14	B	M	38	15.5	18.0
S18 (1)	B	M	48	17.5	18.5	L15	B	M	32	24.2	30.0
S18 (2)	B	QT	[c]	17.5	15.0	L17 (1)	B	M	46	19.3–19.9	24.0
S19 (1)	B	M	57	15.7–15.8	16.5	L17 (2)	B	M	41	19.3–19.9	24.0
S19 (2)	B	M	53	15.7–15.8	18.0	L18 (1)	B	M	43	20.8–20.9	24.5
S20	B	M	38	13.1–13.7	14.0	L18 (2)	B	M	41	20.8–20.9	26.5
S21	N	QT	[c]	9.0–9.1	14.0	L18a	B	M	48	21.3–21.4	25.5
S23	B	M	36	15.8–16.2	19.0	L19 (1)	B	QT	[c]	23.3–24.6	36.0
S24	B	M	30	15.4	18.5	L19 (2)	B	QT	[c]	23.3–24.6	34.0
S25 (1)	B	M	34	12.1	14.5	L21	B	M, QT	15	18.7	24.0
S25 (2)	B	M	36	12.1	11.0	L22	B	M	50	14.0–14.5	17.5
S26	B	M	45	14.6–14.8	18.5	L23	B	M	63	14.5–15.0	17.5
S27	1D	LC/MS/MS	[c]	9.5	0.0–14.0[a]	L23a	B	M	45	17.4–17.9	19.0
S27a	B	QT	[c]	17.7–17.8	14.5	L24	B	M	36	18.4–18.6	19.5
S28	B	M	37	7.3–7.4	10.5	L26 (1)	B	M	64	16.8–16.9	22.0
						L26 (2)	B	M	53	16.8–16.9	19.0
						L27	B	M	28	15.5–15.6	18.5
						L27a	B	M	56	16.3–16.5	19.5
						L28	B	M	54	15.9	18.5
						L30	B	M	52	12.3–12.4	16.0
						L31 (1)	B	M, QT	21	13.7–13.8	20.0
						L31 (2)	B	M	19	13.7–13.8	20.0
						L32	B	M	32	14.5–15.5	19.5
						L34	N	M	36	13.6–13.7	20.0–25.0[a]
						L35	B	M	43	14.2–14.3	18.5
						L35a	B	M	25	12.8–12.9	15.5
						L36	N	M	39	12.2–12.7	14.0–20.0[a]
						L37	B	M	44	10.6–10.8	12.5

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Table I. (*Continued from previous page.*)

Small Subunit Proteins						Large Subunit Proteins					
Ribosomal Protein ID	Gel System	ID Method	Sequence Coverage	Molecular Mass		Ribosomal Protein ID	Gel System	ID Method	Sequence Coverage	Molecular Mass	
				Calculated	Apparent					Calculated	Apparent
									%	*kD*	
						L37a	1D	LC/MS/MS		10.0–10.4	0.0–14.0[a]
						L38	B	M	60	8.1	11.5
						L40	1D	LC/MS/MS		14.7	0.0–14.0[a]

[a]Proteins identified from the 1D gel were from gel slices that had a range in apparent molecular mass. [b]Multiple isoforms of S6 were detected in both the rapid mobility group and the slow mobility group. [c]Identification from sequencing one or more peptide.

of mono-, di-, and tri-phosphorylated S6B, respectively. By comparison of theoretical and observed mass values, we determined that S6a was phosphorylated at Ser-238; S6b to d were phosphorylated at Ser-238 and additional sites in the region R_{239}LSSAAKRSVTA. The rapid migrating S6e to g forms were sequentially di-, tri-, and tetra-phosphorylated forms of S6A. Interestingly, the detection of the ion corresponding to nonphosphorylated form of the peptide K_{236}RSR indicated that Ser-238 was skipped by the S6 kinase or dephosphorylated by an unknown phosphatase in the formation of S6e to g. Although the absence of cleavage sites in the carboxy terminus precluded identification of the exact sites of phosphorylation in some S6 forms, these results confirm extensive heterogeneity in the carboxyl-terminal phosphorylation sites of S6A and S6B.

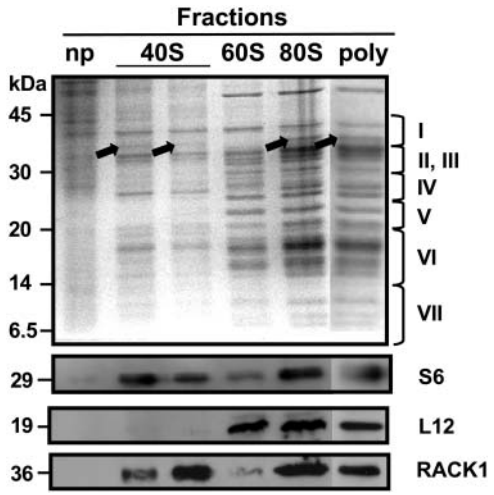

Figure 3. Suc density gradient fractionation of total ribosomes. Total ribosomes isolated from 6-d-old dark grown suspension cultured cells of Arabidopsis by high speed centrifugation were further fractionated through a Suc density gradient. The fractions were resolved by 15% (w/v) SDS-PAGE and visualized by staining with Coomassie Blue. Fractions corresponding to the nonpolysomal fraction (np), 40S subunit, 60S subunit, 80S ribosome, and polysomes (poly) are shown. The migration of molecular mass markers is indicated on the left. The SDS-PAGE gel lanes were cut into seven even sections as indicated on the right (I, 35.0–45.0 kD; II, 32.5–35.0 kD; III, 30.0–32.5 kD; IV, 25.0–30.0 kD; V, 20.0–25.0 kD; VI, 14.0–20.0 kD; and VII, 0.0–14.0 kD). Individual sections of the nonpolysomal and polysomal fractions were subjected to in-gel trypsin digestion and LC/MS/MS. Arrows indicated the position of the Coomassie Blue-stained Arabidopsis ortholog of human RACK1, as determined by LC/MS/MS. Immunological detection of protein in the gradient fractions was performed with anti-maize S6, anti-yeast L15 (ortholog of rat and plant L12), and anti-Arabidopsis RACK1.

The Arabidopsis Genome Encodes Evolutionarily Distinct S15a and P2 Types That Accumulate in Ribosomes

The percent amino acid sequence identity between the deduced Arabidopsis and rat r-protein orthologs was extremely consistent among gene family members, except for the *RPS15a*, *RPP2*, and *RPL7* multigene families (Barakat et al., 2001). Evolutionary divergence among these gene families was confirmed by use of sequence alignment, pairwise distance calculations as well as distance- and parsimony-based phylogenetic analyses. Of the 6 Arabidopsis *RPS15a* genes, 4 (*RPS15aA*, *RPS15aC*, *RPS15aD*, and *RPS15aF*) encode a protein with 73.1% to 77.7% identity and 2 (*RPS15aB* and *RPS15aE*) encode a protein with 47.6% and 48.8% identity to rat S15a, respectively (Barakat et al., 2001). Eukaryotic S15a is orthologous to S8 of eubacteria, plastids, and mitochondria of lower eukaryotes (Wool et al., 1995; Nevskaya et al., 1998; Adams et al., 2002), which binds 16S rRNA and is crucial for ribosome assembly (Held et al., 1974; Svensson et al., 1988). Conservation between plant and animal S15a includes the carboxy-terminal amino acid region that corresponds to an rRNA-binding site in eubacteria (Mougel et al., 1986; Wower et al., 1992; Davies et al., 1996; Nevskaya et al., 1998; Fig. 4A, underlined). In addition, a number of residues within this region are highly conserved in eubacterial, plastidic, and archaebacterial S8 (Fig. 4A, shaded residues).

The calculation of pairwise, uncorrected distances between Arabidopsis S8 and S15a proteins confirmed that S15a falls into 2 distinct groups: S15aA/S15aC/S15aD/S15aF (Type I) and S15aB/S15aE (Type II; Table III). This analysis revealed that evolutionary distance between Arabidopsis S15a proteins within each of the two groups was substantially less than between groups. The distance between Type I or Type II S15a to plastid S8 was quite similar, whereas the distance between *Escherichia coli* and plastid S8 was less than

Table II. *Identification of Arabidopsis S6 carboxy-terminal phosphorylation sites by MADLI-TOF MS analysis*

ID Method abbreviations: M, MALDI-TOF; asterisk indicates phosphorylated residue; single underscore indicates candidate phosphorylation sites within peptide.

S6 Form	Entry Mass	Theoretical Mass	No. Phosphates Predicted	Carboxy-Terminal Peptide Sequence	Predicted Phosphorylation Status of Carboxy-Terminal Residues from K_{236} (No. of Phosphates)
	m/z	*m/z*			
S6	547.233	546.347	0	KRSR	S6A and S6B: KRSR (0)
S6a	528.354	528.347	1	KRS*R	S6A and S6B: KRS*R (1)
S6b	528.943	528.347	1	KRS*R	
	1309.72	1309.743	2	KRSRLSSAAKRSVTA	S6A :KRS*RLSSAAKRSVTA (2)
	528.350	528.347	1	KRS*R	
	1374.52	1374.817	2	RSRLSSAPAKPVAA	S6B: KRS*RLSSAPAKPVAA (2)
S6c	527.470	528.347	1	KRS*R	
	1066.340	1066.610	2	LSSAAKRSVTA	S6A : KRS*RLSSAAKRSVTA (3)
	527.439	528.347	1	KRS*R	
	1200.130	1200.716	3	SRLSSAPAKPVAA	S6B: KRS*RLS*S*APAKPVAA (3)
S6d	528.144	528.347	1	KRS*R	
	1048.190	1066.610	3	LSSAAKRSVTA	
	1429.920	1429.845	4	RSRLSSAAKRSVTA	S6A: KRS*RLSSAAKRSVTA (4)
S6e	546.423	546.347	0	KRSR	
	1067.580	1066.610	2	LSSAAKRSVTA	S6A: KRSRLSSAAKRSVTA (2)
S6f	546.359	546.347	0	KRSR	
	1048.930	1048.610	3	LSSAAKRSVTA	S6A: KRSRLSSAAKRSVTA (3)
S6g	546.384	546.347	0	KRSR	
	1031.520	1030.610	4	LSSAAKRSVTA	S6A: KRSRLS*S*AAKRS*VT*A (4)

between any Arabidopsis or rat S15a, confirming that the *RPS15a* duplication postdates the divergence between prokaryotes and eukaryotes. Maximum parsimony and neighbor-joining evaluation of eukaryotic S15a and eubacterial, plastidic, and archaebacterial S8 amino acid sequences resolved 3 highly supported clades for eukaryotic S15a (Fig. 4B). Two clades corresponded to plants (Type I S15a and Type II S15a) and one corresponded to other eukaryotes. The plant Type I S15a grouped with S15a of rat, Drosophila, and yeast, whereas plant Type II S15a formed a strongly supported separate clade. The analysis supports the conclusion that the duplication event that led to the divergence of Arabidopsis *RPS15a* genes occurred prior to the divergence of monocots and eudicots.

The MS analysis demonstrated that both types of S15a were present in the Arabidopsis ribosome pellet fraction, strongly supporting the conclusion that the products of both evolutionarily distinct *RPS15a* gene groups encode r-proteins (Fig. 1B; Table I; Supplemental Table I). Type II S15a was more acidic and was present at lower abundance than Type I S15a, as judged by the intensity of staining with Coomassie Blue (Fig. 1B).

Evolutionary divergence was also evident within the r-protein P2 gene family. We showed previously that maize ribosomes possess a complex of approximately 12-kD acidic proteins composed of P1, P2a, P2b, and a related protein designated P3 (Bailey-Serres et al., 1997; Szick et al., 1998; Szick-Miranda and Bailey-Serres, 2001). The 5 deduced Arabidopsis P2 proteins (11.0–11.8 kD; pI of 4.2–4.4) display an unusual range in sequence identity (39.1%–57.7%) to rat P2 (Barakat et al., 2001). Figure 5A illustrates the considerable amino acid sequence divergence between the Arabidopsis P2 proteins. The divergence in the terminal 12 residues (E/KSD/EDMGFG/SLD) of P2C and P2E is especially notable since this region is nearly universally conserved among eukaryotic P1 and P2 (Ballesta and Remacha, 1996; Szick et al., 1998).

Use of methods to evaluate evolutionary relatedness confirmed the presence of 2 distinct P2 protein groups: P2A/P2B/P2D (Type I) and P2C/P2E (Type II; Table IV The pairwise uncorrected distances between proteins within each group were substantially less than distances between the two groups, supporting the Type I and Type II distinction. In addition, the distances between maize P2a and P2b to Arabidopsis Type I P2A, P2B, and P2D were less than the distances to Arabidopsis Type II P2C and P2E, suggesting that maize P2a and P2b are more closely related to the Type I P2 proteins. This conclusion was strengthened by the maximum parsimony evaluation of 15 P-protein amino acid sequences (Fig. 5B). Not unexpectedly, the analysis resolved the 3 clades of eukaryotic 12-kD P-proteins corresponding to P1, P2, and plant-specific P3. The plant P2 proteins separated into 3 distinct clusters corresponding to P2 proteins of monocotyledonous plants (maize and rice [*Oryza sativa*]) and 2 strongly supported clusters of Arabidopsis P2 proteins (Type I and Type II). The groupings of rice and maize P2a and P2b suggest the duplication event that led to 2 forms of P2 occurred prior to the divergence of rice and maize but after the divergence of monocots and eudicots. The MS analysis of the acidic proteins confirmed the presence of both evolutionary groups of P2 in the ribosome pellet (Fig.

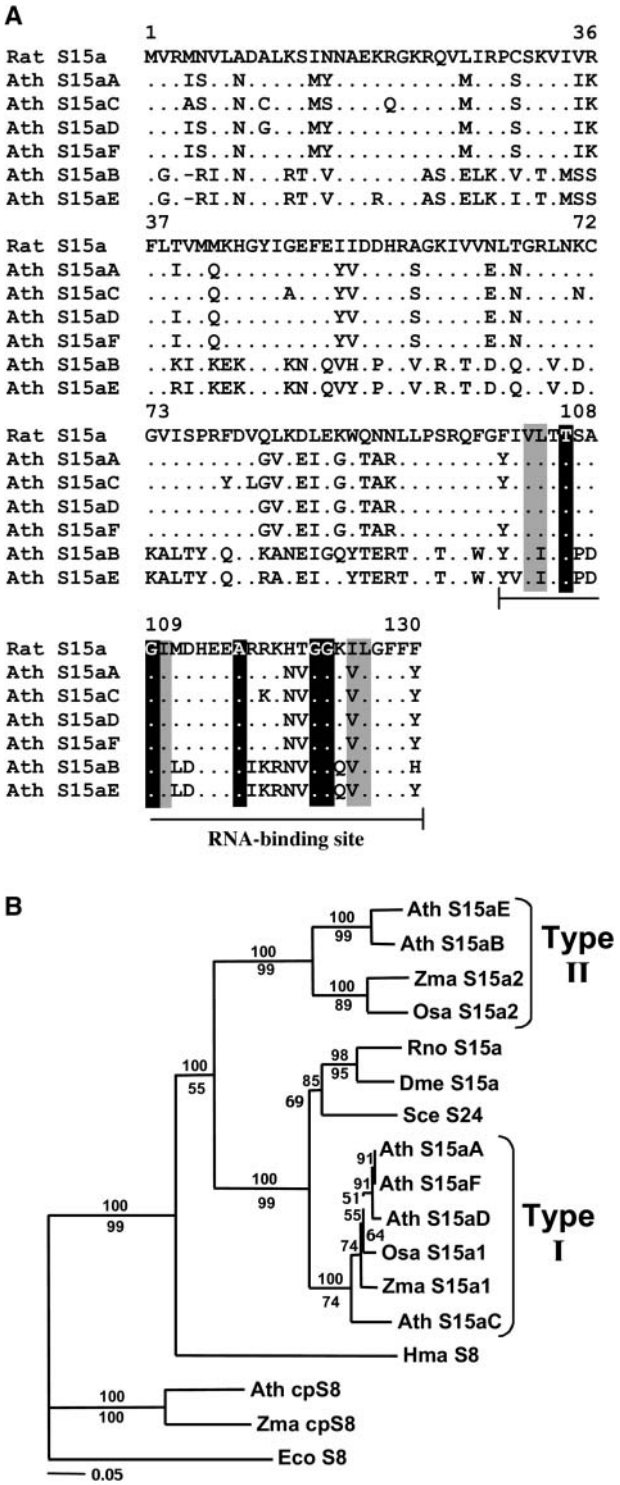

Figure 4. Arabidopsis encodes two distinct types of S15a. A, Alignment of rat and Arabidopsis RPS15a proteins. The deduced amino acid sequences for the *RPS15a* genes of rat (*Rattus norvegicus*) and Arabidopsis RPS15a were aligned by use of ClustalW. Gaps were introduced to ensure maximum homology. Amino acids that differ from rat S15a are indicated. The shaded residues within the C terminus indicate residues that are strictly conserved (shaded black) or contain conservative substitutions (V/I/L, shaded gray) in an alignment based on 12 eubacterial, 10 chloroplastic, and 3 archaebacterial sequences (data not shown). B, Amino acid phylogenetic analysis of S8 and S15a r-proteins confirms 2 distinct types of plant S15a. A phylogenetic tree was generated by the neighbor-joining method using uncorrected distances in PAUP version 4.0b10 (PPC) based on the amino acid sequences of 13 eukaryotic S15a, 2 plastidic S8, and 2 prokaryotic S8 r-proteins, respectively. Bootstrap values from 500 replicates are indicated. Parsimony bootstrap values for clades supported above the 50% level are indicated below branches, whereas neighbor-joining bootstrap values based on uncorrected distances are indicated above the branches. cp, Chloroplast; Ath, Arabidopsis; Eco, *E. coli*; Dme, *D. melanogaster*; Hma, *Halobacterium marismortui*; Osa, rice; Rno, rat; Sce, yeast; Zma, maize. GenBank accession and MIPS identifiers for sequences utilized in evolutionary analyses: Ath S15aA, At1g07770; Ath S15aB, At2g19720; Ath S15aC, At2g39590; Ath S15aD, At3g46040; Ath S15aE, At4g29430; Ath cpS8, AP000423; Eco S8:X01563; Dme S15a, Z21673; Hma S8, X58395; Osa S15a1, AK119795; Osa S15a2, AK121591; Rra S15a, NM053982; Sce S24, X01962; Zma S15a1, AY107320; Zma S15a2, AY106173.

2B; Table I; Supplemental Table I). The evolutionarily divergent Type II P2 appeared to be present in substoichiometric amounts based on the intensity of staining with Coomassie Blue.

The Arabidopsis genome encodes 3 L7-type proteins with approximately 60% identity (L7B, L7C ,and L7D) and one L7-type protein (L7A) with 38% identity to rat L7 (Barakat et al., 2001). R-protein L7 is the ortholog of eubacterial and archaebacterial L30. Distance calculations and phylogenetic analyses confirmed that L7 polypeptide sequences of plants fall into 2 evolutionarily distinct categories due to a divergence within the plant lineage that predates the monocot and eudicot divergence (Supplemental Fig. 1). The MS analysis confirmed the presence of the L7 type encoded by *RPL7B*, *RPL7C*, and *RPL7D* in the ribosome pellet fraction; however, the product of *RPL7A* was not detected in ribosomes even though the gene transcript accumulated in the cultured cells (data not shown).

Estimation of the Mass of Arabidopsis 80S Ribosomes

The predicted mass of the Arabidopsis 40S subunit is 1,159 kD, based on the presence of the 18S rRNA (At2g01010; 554 kD), a single copy of the 30 small subunit r-proteins, and the RACK1-like protein. Similarly, the predicted mass of the Arabidopsis 60S subunit is 2,010 kD, based on the presence of 3 rRNAs (26S, At2g01030, 1,038 kD; 5.8S, At2g01020, 50 kD; and 5S, AP002054, 37 kD) and a single copy of the 44 large subunit proteins, including 1 of each of the 12 kD P-proteins (P1, P2 Type I, P2 Type II, and P3). Thus, the Arabidopsis 80S ribosome is approximately 3.2 MD, with 53% of the mass contributed by rRNA. The ribosome mass could vary slightly due to covalent posttranslational modifications of r-proteins, the presence of the small r-proteins not detected by MS (S29, S30, L29, L36a, L39, and L41), and/or the absence or presence of dispensable r-proteins, such as Sa and individual P-proteins (García-Hernández et al., 1994; Szick-Miranda and Bailey-Serres, 2001).

Table III. *Divergence between S15a and S8 r-proteins of various species*

Uncorrected distances given in top matrix, total residue differences in bottom matrix.

	Arabidopsis						Rat	Ath cp	*E. coli*
	S15aA	S15aC	S15aD	S15aF	S15aB	S15aE	S15a	S8	S8
Ath S15aA		0.085	0.015	0.000	0.481	0.473	0.223	0.746	0.795
Ath S15aC	11		0.092	0.085	0.505	0.496	0.269	0.746	0.795
Ath S15aD	2	12		0.015	0.496	0.488	0.223	0.754	0.803
Ath S15aF	0	11	2		0.481	0.473	0.223	0.746	0.795
Ath S15aB	62	65	64	62		0.078	0.543	0.754	0.810
Ath S15aE	61	64	63	61	10		0.533	0.754	0.810
Rat S15a	29	35	29	29	70	69		0.738	0.779
Ath cpS8	94	95	94	94	95	95	93		0.605
E. coli S8	97	98	97	97	98	98	95	78	

DISCUSSION

Identification of 74 Distinct Proteins in Arabidopsis 80S Ribosomes

An examination of genomic and expressed sequence tag sequences of Arabidopsis confirmed the presence of gene orthologs that encode the 79 proteins of rat 80S ribosomes, as well as the plant-specific r-protein P3 (Barakat et al., 2001). Here, the combination of conventional isolation of ribosomes, 1D and 2D gel electrophoresis systems, and MS analyses resulted in the identification of 74 of the 80 predicted r-proteins of Arabidopsis ribosomes, including 30 small subunit and 44 large subunit proteins (Table I; Supplemental Table I). All but 2 of the 74 r-proteins (Type II S15a and P3) corresponded to rat and yeast 80S r-proteins. These data confirm that there is strong evolutionary conservation in the proteins of cytosolic ribosomes of Arabidopsis and other model eukaryotes.

Several putative r-proteins were not detected in the MS analyses reported here as well as those performed on ribosomes of other eukaryotes. Two putative Arabidopsis 40S subunit r-proteins (S29 [6.1–6.4 kD, 10.8–10.9 pI] and S30 [6.9 kD, 12.8 pI]) and 4 putative 60S subunit r-proteins (L29 [7.0 kD, 12.0 pI], L36a [10.0–10.4 kD, 11.0–11.1 pI], L39 [6.4 kD, 12.8 pI], and L41 [3.4 kD, 12.9 pI]) were not detected by MS after 2D gel or LC fractionation, despite strategies taken to capture the predicted peptides of these proteins (data not shown). Consistent with these results, the LC/MS/MS analysis of yeast 80S ribosomes also failed to identify L29 and L41 (Link et al., 1999). The inability to detect these small and highly basic r-proteins by MS methods could be due to the general dependence of the MS method on trypsin cleavage products of 1,000 *m/z* or higher, poor ionization of the limited number of tryptic peptides, loss of these proteins during ribosome isolation, or their bone fide absence from 80S ribosomes.

Non-R-Proteins Include a WD-Domain Repeat Protein Ortholog of Mammalian RACK1 Associated with the 40S Ribosomal Subunit and Polysomes

Our analyses identified several nonribosomal proteins in the ribosome pellet fraction of cultured Arabidopsis cells. The presence of some of these proteins may be attributed to the copurification of membrane vesicles or other macromolecular complexes with ribosomes. For example, tonoplast and mitochondrial inner membrane proteins were identified in the ribosome pellet fraction; these proteins were in complexes that were less dense than ribosomes when the pellet fraction was further fractionated on Suc density gradients. On other hand, multiple subunits of the 20S proteasome were identified in the ribosome pellet and cofractionated with polysomes after Suc density gradient centrifugation. This was not unexpected since the proteasome complex, which can include the 20S core, 2 19S regulator lids, and additional interacting proteins and complexes, can have a sedimentation coefficient similar to that of ribosomes or polysomes (Verma et al., 2000; Fu et al., 2001). When the yeast 26S proteasome was isolated by affinity purification and analyzed by MS, several large subunit r-proteins were identified (Verma et al., 2000). This association between proteasome and ribosome could be due to nonspecific interaction between 2 abundant complexes or may reflect a functional interaction due to the proteasomal degradation of approximately 30% of newly synthesized proteins (Schubert et al., 2000).

One of the nonribosomal proteins identified in the ribosomal pellet was a 36-kD WD-domain repeat protein that is the ortholog of mammalian RACK1 (Fig. 2B; Supplemental Table II). This protein was characterized in tobacco as the product of an auxin-induced mRNA (*Arca*; Ishida et al., 1996), and the mammalian ortholog is a receptor of activated protein kinase C and a scaffold for $\beta\gamma$ of the heterotrimeric G-protein complex (Ron et al., 1994; Dell et al., 2002). The analysis of the Suc density gradient fractionated ribosomes by MS and with an antiserum prepared against the Arabidopsis RACK1 ortholog supported the conclusion that this protein associates with ribosomes and polysomes via an interaction with the 40S subunit (Fig. 3; Supplemental Table II). RACK1 association with Arabidopsis ribosomes was also observed when ribosomes were isolated by an immunoaffinity purification procedure that does not involve high-speed centrifugation (M.E. Zanetti, I.-F. Chang, and

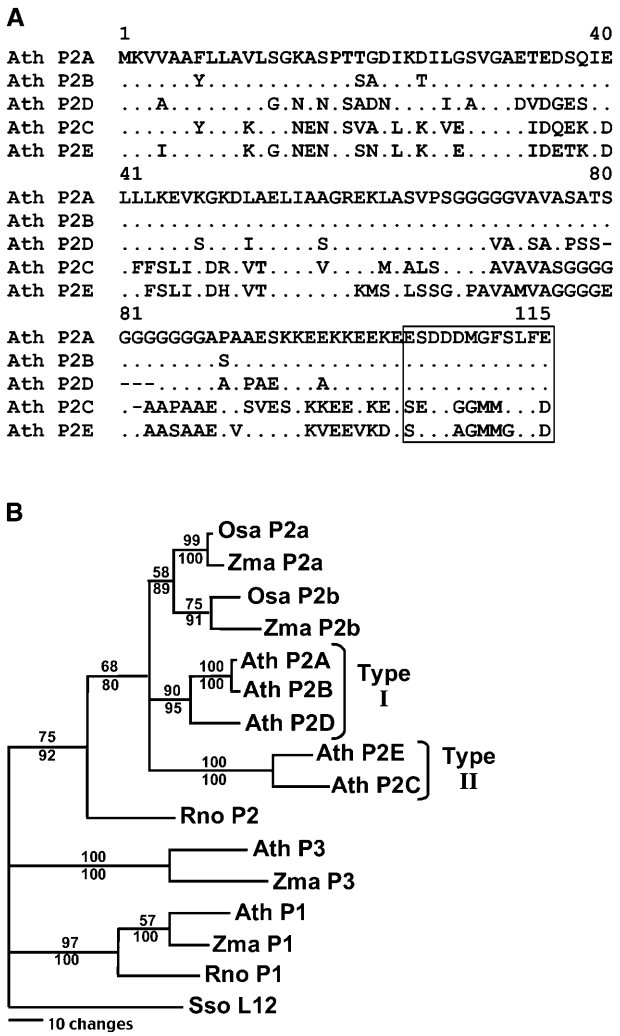

Figure 5. Arabidopsis and monocots encode 2 distinct forms of P2 that arose from evolutionarily distinct sequence divergence events. A, Alignment of Arabidopsis P2 r-proteins. The deduced polypeptides for the genes encoding Arabidopsis P2 were aligned as described in Figure 4. Amino acids that differ from P2A (At2g27720) are indicated. The C-terminal amino acids that are highly conserved among diverse eukaryotes are boxed. B, Amino acid phylogenetic analysis of 12-kD P-proteins confirms 2 distinct types of P2 in plants. A 471-step maximum parsimony phylogenetic tree was generated in PAUP version 4.0b10 (PPC) based on the amino acid sequences of 16 eukaryotic 12-kD P-proteins. Bootstrap values from 500 replicates are indicated. Parsimony bootstrap values for clades supported above the 50% level are indicated above branches, whereas neighbor-joining bootstrap values based on uncorrected distances are indicated below the branches. Ath, Arabidopsis; Osa, rice; Rno, rat; Sso, *Sulfolobus solfataricus*; Zma, maize. GenBank accession and MIPS identifiers for sequences utilized in evolutionary analyses: Ath P1, At1g01100; Ath P2B, At2g27710; Ath P2C, At3g28500; Ath P2D, At3g44590; Ath P2E, At5g40040; Ath P3, At4g25890; Osa P2a, AK058815; Osa P2b, AK121459; Rno P1, XM343403; Rno P2, XM347184; Sso L12, X59038; Zma P1, U62752; Zma P2a, U62748; Zma P2b, U62753; Zma P3, U62751.

J. Bailey-Serres, unpublished data). The association of RACK1 with the 40S subunit is a conserved feature of eukaryotic ribosomes since human and yeast (YMR116p) RACK1 also copurify with the 40S subunit (Link et al., 1999; Inada et al., 2002; Ceci et al., 2003; Sengupta et al., 2004).

RACK1 is emerging as a protein scaffold located near to the mRNA exit tunnel of the 40S ribosomal subunit that has a role in translational regulation (Sengupta et al., 2004). This WD-domain repeat protein was identified as a component of messenger ribonucleoprotein complexes associated with actively translated mRNAs in rat neurons (Angenstein et al., 2002). Moreover, studies with cultured human cells indicated that an interaction between RACK1 and translating ribosomes reflects its role in regulation of the inhibition of ribosome subunit joining by eukaryotic initiation factor 6 (Ceci et al., 2003). In yeast, the genetic disruption of RACK1 ortholog YMR116p reduced the rate of cell growth and levels of intact polysomes (Chantrel et al., 1998). Moreover, a yeast RNA-binding hnRNP K-homology domain protein (Scp160p) was shown to associate with polysomes through its interaction with RACK1 (Asc1p; Baum et al., 2004). In *S. pombe*, the deletion mutation of the RACK1 ortholog Cpc2 had no dramatic effect on the rate of cellular protein synthesis but decreased the steady-state level of a subset of cellular proteins, several of which regulate Met metabolism (Shor et al., 2003). This study also confirmed that the effect of Cpc2 was through its interaction with ribosomes. Recent cryo-electon microscopy structural studies have determined the location of RACK1 on the 40S subunit of ribosomes of the thermophilic fungus, *Thermomyces lanuginosus*, and confirmed the presence of a large structure that would support simultaneous interaction with messenger ribonucleoprotein complexes and regulatory proteins (Sengupta et al., 2004).

Distinctions in Ribosomes Due to Variation in Protein Families and Posttranslational Modifications

The Arabidopsis 80S ribosome is approximately 3.2 MD (1,159 kD 40S; 2,010 kD 60S) based on the mass of the 4 rRNAs, 74 r-proteins, and RACK1. Although the protein component of this macromolecular complex appears to be highly conserved between eukaryotes, approximately 25% of the Arabidopsis r-proteins were resolved in multiple spots by 2D gel fractionation. The detection of multiple forms of r-proteins was also reported for a subset of the components of the 30S subunit of Chlamydomonas plastid ribosomes, 60S subunit of yeast, and 40S subunit of rat (Lee et al., 2002; Yamaguchi et al., 2002). These distinct protein forms are products of specific gene family members or arise from posttranslational modifications (Table I; Supplemental Table I).

A variation in Arabidopsis ribosomes that may be of biological significance is the differential phosphorylation of the distinct carboxy termini of S6A and S6B.

Table IV. *Divergence between P2 r-proteins of various species*

Uncorrected distances given in top matrix, total residue differences in bottom matrix.

	Arabidopsis					Maize		Rat
	P2A	P2B	P2D	P2C	P2E	P2a	P2b	P2
Ath P2A		0.044	0.243	0.526	0.478	0.321	0.372	0.496
Ath P2B	5		0.270	0.526	0.460	0.321	0.372	0.487
Ath P2D	27	30		0.532	0.509	0.330	0.373	0.514
Ath P2C	60	60	59		0.254	0.527	0.478	0.614
Ath P2E	54	52	56	29		0.460	0.518	0.584
Zma P2a	36	36	36	59	51		0.321	0.438
Zma P2b	42	42	41	54	58	36		0.469
Rat P2	57	56	57	70	66	49	53	

The 8 S6 forms resolved by 2D gel electrophoresis fell into 2 groups based on mobility in the SDS-PAGE dimension, as observed for S6 of maize (Williams et al., 2003). In the fast migrating forms, phosphorylation was detected at the carboxy-terminal Ser-238, in the products of both *RPS6A* and *RPS6B*. The increasingly acidic forms appeared to be mono-, di-, tri-, and tetra-phosphorylated forms of S6A and mono-, di-, and tri-phosphorylated forms of S6B, with phosphorylation at Ser-238 and more carboxy-terminal sites. On the other hand, the slow-migrating forms, S6e to g, were di-, tri-, and tetra-phosphorylated forms of S6A that lacked phosphorylation at Ser-238. The detection of forms of S6 that differ in the presence or absence of Ser-238 phosphorylation was consistent with the detection of 2 groups of maize S6 forms: (1) those sequentially phosphorylated at Ser-238 and then Ser-241, followed by more carboxyl phosphorylation sites, and (2) those not phosphorylated at Ser-238 but phosphorylated at more carboxyl sites (Williams et al., 2003). Thus, the differential phosphorylation of S6 at Ser-238 (mammalian and Drosophila Ser-235) and more carboxyl sites results in distinctions between ribosomes in both animals and plants (Krieg et al., 1988; Radimerski et al., 2000; Williams et al., 2003).

Products of Evolutionarily Distinct Gene Family Members Are Present in Ribosomes

We determined that the products of evolutionarily distinct members of both the *RPS15a* and *RPP2* gene families are components of Arabidopsis ribosomes. By contrast, the product of a divergent member of the *RPL7* gene family was not found in ribosomes and therefore may have an extra-ribosomal function (Supplemental Fig. 1). Adams et al. (2002) demonstrated that Arabidopsis Type II S15a, which has 30% lower sequence identity to the rat ortholog than Type I S15a (Barakat et al., 2001), was imported into isolated soybean mitochondria in an in vitro system. Although this study did not address whether the imported protein was associated with mitochondrial ribosomes, it was proposed that the divergent *RPS15a* (Type II) gene encodes a mitochondrial-targeted r-protein that replaces the eubacterial-like S8, the ortholog of *RPS15a*. The import of S15a may be necessary since the mitochondrial S8 appears to have been lost in a common ancestor of angiosperms and gymnosperms. The detection of Type II S15a in the ribosome pellet fraction of Arabidopsis cells confirms that the evolutionarily divergent gene family encodes an r-protein (Fig. 1B). Since the MS analysis of the proteins in the ribosome pellet fraction from dark grown Arabidopsis cells failed to identify any known organellar r-proteins, we hypothesize that Type II S15a functions as both a mitochondrial and a cytosolic r-protein, with Type I S15a predominating in 80S ribosomes. This hypothesis might be tested by comparison of 80S cytosolic or 70S mitochondrial ribosomes purified via an epitope tagged r-protein specific to each complex.

Variation in ribosomal protein composition is also a consequence of the 2 evolutionarily distinct P2 r-proteins. P2 is a member of a group of acidic phosphoproteins that form a universally conserved lateral stalk on the large ribosomal subunit (Möller, 1990). P0 (approximately 35 kD) interacts with the 23S-like rRNA to form the base of the stalk, which is extended by the binding of 2 molecules of P1 and P2 (approximately 12 kD; Tsurugi and Ogata, 1985; Uchiumi et al., 1987; Guarinos et al., 2003). Mammals possess one type of P1 and P2, whereas *S. cerevisae* possesses 2 types of P1 and P2 (Nakao et al., 2004). The association of the dispensable 12-kD proteins with the ribosome promotes the GTPase activity of elongation factor 2 (Vard et al., 1997). In maize, there are 2 specific types of P2, P2a and P2b, which show differential expression and accumulation in ribosomes during development (Szick-Miranda and Bailey-Serres, 2001). The phylogenetic analysis presented here demonstrates that P2 of Arabidopsis is encoded by evolutionarily divergent genes (Type I and Type II; Fig. 5B). The longer branch with the Arabidopsis P2 Type II genes indicates rapid diversification of these proteins. This divergence is evident throughout the amino acid sequence and is especially notable in the carboxy-terminal region that is strongly conserved in the P-proteins of higher and lower eukaryotes. This analysis indicates that independent duplication and divergence of *RPP2* has occurred in eudicots and

monocots, giving rise to forms of this protein that may be functionally distinct.

Is There Functional Significance of Ribosome Variation?

Despite strong overall conservation of eukaryotic r-proteins, the ribosomes of cultured Arabidopsis cells can differ as a consequence of posttranslational modifications and/or variations in expression of r-proteins with distinct biochemical characteristic. Ribosome heterogeneity has been described as differences in protein composition, rRNA components, or posttranslation modifications of ribosomal components. There are examples of heterogeneity in r-protein composition in diverse eukaryotes. The slime mold *Dictyostelium discoideum* contains unique ribosomes at different stages of its life cycle (Ramagopal and Ennis, 1982). Two r-proteins were shown to be specific to vegetative amoebae (49.5 and 17.7 kD) and three to spores (43.8, 34.2 and 24.5 kD). In addition, eight proteins were found to be common in both cell types but showed stoichiometric differences. In Drosophila, quantitative and qualitative variation in r-protein accumulation was reported during larval to adult development (Lambertsson, 1975). There is also evidence of quantitative changes in r-protein composition in plants. For example, the level of two cytosolic r-proteins in etiolated barley leaves decreased during greening, whereas six r-proteins increased following illumination (Koyama et al., 1996). As mentioned, by use of antisera specific to the 12-kD P-proteins of maize, we described developmental, environmental, and subcellular heterogeneity in the level of the 2 P2 forms in ribosomes of maize (Szick-Miranda and Bailey-Serres, 2001). Distinctions between ribosomes in 12-kD P-protein composition can have ramifications on protein synthesis as demonstrated by the differential translation of mRNAs by yeast ribosomes of differing P-protein composition (Remacha et al., 1995).

Developmental and environmental regulation of posttranslational modifications of r-proteins results in ribosome heterogeneity in animals and plants. Rat r-protein L29 is methylated at Lys-4 and the amount of the methylated form varies in liver, brain, and thymus ribosomes (29%, >99%, and 95%, respectively; Williamson et al., 1997). Several additional posttranslational modifications of 40S subunit r-proteins were reported in the proteomic study of rat 40S subunits (Louie et al., 1996), although the significance and regulation or these modifications have yet to be explored. Differences in phosphorylation of r-proteins have been observed, including regulated phosphorylation of the 12-kD P-proteins in maize and yeast (Bailey-Serres et al., 1997; Zambrano et al., 1997; Szick-Miranda and Bailey-Serres, 2001) and of S6 in numerous species (Radimerski et al., 2000; Holland et al., 2004). The phosphorylation of S6, positioned in the mRNA/tRNA-binding site of the small head region of the 40S subunit, is likely to be of biological significance. In animals, the analyses of mutants of S6 kinases have indicated that S6 phosphorylation is required for reentry of quiescent cells into the cell cycle through promotion of translation of mRNAs that possess a 5′ terminal polypyrimidine track. These mRNAs predominantly encode components of the translational apparatus that are required for cell growth and proliferation (Jefferies et al., 1994; Holland et al., 2004). In plants, the phosphorylation of S6 is developmentally programmed and regulated by temperature, oxygen availability, and other growth conditions (Scharf and Nover, 1982; Perez et al., 1990; Turck et al., 1998; Williams et al., 2003). The stimulation of S6 phosphorylation upon transfer of cultured Arabidopsis cells to fresh medium containing auxin and kinetin was correlated with increased association of 2 r-protein mRNAs with polysomes (Turck et al., 2004). Conversely, a coordinate decrease in r-protein mRNAs translation was demonstrated in response to dehydration stress in Arabidopsis leaves (Kawaguchi et al., 2004). Additional studies are needed to address whether the regulation of S6 phosphorylation affects global or differential translation of mRNA in plants.

In conclusion, the systematic identification of the individual r-proteins of Arabidopsis 80S ribosomes has demonstrated strong conservation between ribosomes of plants and other model eukaryotes. This study provides a foundation for future evaluation of plant 80S ribosome function and structure. Such research could further investigate the role of ribosome heterogeneity in the fine-tuning of the translational process during development or under specific growth conditions.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Columbia ecotype suspension cultured cells derived from seedling callus tissue (gift of Dr. E. Nothnagel, University of California, Riverside [UCR]) were cultured in Gamborg's B-5 medium (Gamborg et al., 1968) in the dark at 25°C with aeration by shaking at 140 rpm and subculture every 7 d.

Ribosome Isolation and Fractionation of Proteins by 2D Gel Electrophoresis

Five days after subculture, cells (10 g fresh weight) were filtered through Miracloth (Calbiochem, La Jolla, CA) and ground to a fine powder under liquid nitrogen with a mortar and pestle. Ribosomes were isolated by use of the procedure described previously (Williams et al., 2003). Briefly, the powder was hydrated in 20 mL ribosome extraction buffer and ribosomes were obtained by centrifugation through a Suc cushion at 135,000*g* for 21 h at 4°C. The ribosome pellet was resuspended, quantified, and rRNA was removed. To fractionate basic proteins, 6 mg of proteins were separated in a basic-urea first-dimensional gel and an SDS-polyacrylamide second-dimension gel (Williams et al., 2003). To fractionate acidic proteins, 6 mg of proteins were separated by NEpHGE and then by SDS-PAGE as described previously (Bailey-Serres and Freeling, 1990). For both gel systems, the second-dimension separation was by 12% or 15% (w/v) SDS-PAGE (Laemmli, 1970) and proteins were stained with Coomassie Blue R-250.

In-Gel Digestion of Proteins and MS Analyses

Individual Coomassie Blue stained proteins (spots) were manually excised out of gels along the inside edge of the stained protein and destained as

described (Williams et al., 2003). Gel slices were dried in a vacuum and hydrated in 60 μL trypsin (Promega, Madison, WI) solution at either 20 μg/mL for proteins with an apparent molecular mass <20 kD or 40 μg/mL for proteins with an apparent molecular mass >20 kD in 25 mM ammonium bicarbonate, pH 8.0. In-gel digestion was performed for 16 h at 37°C, tryptic peptides were eluted with 50% (v/v) acetonitrile, 5% (v/v) trifluoroacetic acid, and dried under a vacuum. For the identification of phosphorylated residues in peptides, the trypsin solution was supplemented with 0.002% (w/v) barium hydroxide (Alderich, Milwaukee, WI) and peptides were processed as described previously (Williams et al., 2003).

MALDI-TOF MS (Voyager-DE STR, PerSpective Biosystems, Foster City, CA), Q-Star XL oMALDI MS/MS (PerSpective Biosystems), Q-TOF ESI LC/MS/MS (Waters, Milford, MA) were performed in the Keck Proteomics Laboratory Biological Mass Spectrometry Facility and the Analytical Chemistry Instrument Facility at UCR. MALDI-TOF MS analyses were performed in positive reflectron mode. All MS spectra were internally calibrated with two trypsin auto-cleavage peptides (*m/z* values, 842.5100 and 2211.1046) to ensure mass accuracy. MS peak signals were filtered with minimum peak intensity of 3% of the most intense ion as a cutoff. Protein identification by PMF was performed using the MS-FIT algorithm of ProteinProspector (http://prospector.ucsf.edu) to search against the National Center for Biotechnology Information nonredundant (NCBInr) protein database or a local database of Arabidopsis r-proteins. The criteria for a positive PMF hit were a minimum of 4 peptide ions matched within a mass tolerance of 80 ppm, with 2 missed trypsin cleavage sites allowed. Protein identification was based on at least two biological replica samples. Both Q-Star oMALDI MS/MS and Q-TOF LC/MS/MS were used to confirm protein identifications by PMF, particularly on spots with ambiguity. Selected individual peptide ions were analyzed by collision-induced dissociation to generate sequence-dependent MS/MS spectra. With Q-Star oMALDI MS/MS, acquisition of collision-induced dissociation spectra was performed manually to achieve optimal fragmentation and signal intensity. For mixed protein samples in gel slices from 1D-SDS PAGE gels, Q-TOF LC/MS/MS analyses were employed with a survey scan method described previously (Carter et al., 2004). MS/MS spectra were processed and queried against the NCBInr protein database using the MASCOT algorithm (http://www.matrixscience.com) for protein identification. De novo sequencing analysis was performed for some MS/MS spectra. Phosphopeptides were identified by use of an algorithm prepared to identify carboxy-terminal peptides of *RPS6A* (At4g31700) and *RPS6B* (At5g10360) with the mass expected or reduced by a multiple of 18 kD, due to barium-hydroxide modification of phospho-residues. Peptides were identified with a Δ mass value (observed mass $-$ theoretical mass) less than $\pm$ 1.0 D.

Suc Density Gradient Fractionation of Polysomes and Immunodetection of Proteins

Five days after subculture, cells (5 g fresh weight) were filtered, ground to a fine powder under liquid nitrogen, and used for fractionation of complexes from detergent-treated extracts through Suc gradients (Kawaguchi et al., 2003; Williams et al., 2003). After resuspension of the pulverized cells in 10 mL ribosome extraction buffer and centrifugation at 16,000*g* to remove cell debris, 500 μL of supernatant was loaded onto a 4.8-mL 20% to 60% (w/v) Suc gradient, centrifuged at 275,000*g* for 2 h at 4°C (Beckman, L8-M centrifuge SW-55 rotor), and passed through a UA-5 Detector and 185 gradient fractionator (ISCO, Lincoln, NE) attached to a fraction collector. Proteins in each fraction were precipitated by addition of 2 volumes of 99% (v/v) ethanol, incubation on ice for 6 h, and centrifugation at 4°C for 15 min at 16,000*g*, washed with 70% (v/v) ethanol, air-dried, and separated by 15% (w/v) SDS-PAGE. Proteins were subjected to Coomassie Blue R-250 staining or transfer to a nitrocellulose membrane for immunodetection with a 1:2,000 dilution of rabbit polyclonal antiserum against maize S6 (Williams et al., 2003), a 1:10,000 dilution of rabbit polyclonal antiserum against yeast L15 (ortholog of Arabidopsis L12; Saenz-Robles et al., 1988), or with a 1:200 dilution of affinity-purified rabbit polyclonal antiserum prepared against the peptide K_{273}VDLKAEAKADNSGPAAT291 encoded by the Arabidopsis RACK1 ortholog (At1g18080; Abgent, La Jolla, CA).

Sequence Divergence and Phylogenetic Analyses

Amino acid sequence alignments were generated using ClustalW (http://www.cmbi.kun.nl/bioinf/tools/clustalw.shtml; Thompson et al., 1994) and were adjusted manually upon visual inspection (alignments available upon request). Pairwise uncorrected distances were calculated using PAUP test version 4.0b10 (PPC/Altivec; Sinauer Associates, Sunderland, MA). The aligned polypeptide sequences were subjected to maximum parsimony and neighbor-joining analyses (p-distances) using PAUP. Parsimony analyses were conducted using the heuristic search algorithm with 500 random input orders and tree bisection-reconnection branch swapping. The reliability of the phylogenies identified by neighbor joining and maximum parsimony was estimated using bootstrapping with 500 replicates and 1 input order/replicate.

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