Running title: Plastid Ribosome Preparation for Proteomics

Preparation and Proteomic Analysis of Chloroplast

Ribosomes

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Abstract

Proteomics of chloroplast ribosomes in spinach and *Chlamydomonas* revealed unique protein composition and structures of plastid ribosomes. These studies have suggested the presence of some ribosomal proteins unique to plastid ribosomes which may be involved in plastid-unique translation regulation. Considering the strong background of genetic analysis and molecular biology in Arabidopsis, the in-depth proteomic characterization of Arabidopsis plastid ribosomes would facilitate further understanding of plastid translation in higher plants. Here, I describe simple and rapid methods for the preparation of plastid ribosomes from *Chlamydomonas* and Arabidopsis using sucrose gradients. I also describe purity criteria and methods for yield estimation of the purified plastid ribosomes and subunits, methods for the preparation of plastid ribosomal proteins, as well as the identification of some Arabidopsis plastid ribosomal proteins by matrix-assisted laser desorption/ionization mass spectrometry.

Key Words: chloroplast ribosome, plastid ribosomal protein, sucrose density gradient ultracentrifugation, proteomics, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana*

1. Introduction

Chloroplast ribosomes, also more generally termed plastid ribosomes, are structurally related to eubacterial 70S ribosomes that are distinct from cytoplasmic 80S and mitochondrial 55-75S ribosomes (**1**). Plastid ribosomes are responsible for translation of genes encoded in the plastid genome. In green leaves, chloroplast ribosomes are present at about equimolar amounts relative to cytoplasmic ribosomes (**2**). With respect to abundance by weight, chloroplast ribosomes account for over 25% of the total leaf ribosomes (**1**). Although chloroplast ribosomes synthesize only ~80 polypeptides encoded in the plastid genome, about 50% of total protein mass in leaves comprises products of chloroplast ribosomes (**3**). Unlike in eubacteria, gene expression in plastids is regulated primarily at the translation level: i.e., the translation rate is not correlated with transcript abundance (**4**), and translation of many plastid mRNAs is activated in response to light illumination (**5, 6**). A majority of the studies on plastid translation, especially light-activated translation, has been carried out in the unicellular green alga, *Chlamydomonas* (**7, 8**).

Since two landmark discoveries published in 1962, of chloroplast ribosomes from spinach by Lyttleton (**9**) and of chloroplast DNA from *Chlamydomonas* by Ris and Plaut (**10**), the translational apparatus in chloroplasts has been mainly studied in spinach and *Chlamydomonas*; reviewed in (**2, 11-13**). Proteomic characterizations of plastid ribosomes from spinach (**14-16**) and *Chlamydomonas* (**17, 18**) have revealed that plastid ribosomes contain some plastid-specific ribosomal proteins (PSRPs) in addition to bacterial orthologs. These proteomic studies also revealed differences in protein composition and the primary structure of each ribosomal protein between higher plants and green algae. Recent cryo-electron microscopy of plastid ribosomes from spinach (**19**) and *Chlamydomonas* (**20**) has visualized the 3D-localization of PSRPs and plastid-specific domains in the ribosomes, suggesting their involvement in translation regulation. Although functional analyses of some PSRPs (PSRP-1 in spinach and PSRP-7 in *Chlamydomonas*) have been reported (**21, 22**), the physiological roles of another five PSRPs (PSRP-2 to PSRP-6) remain unclear. In addition, post-translational modifications, which may also affect translational activity of plastid ribosomes, remain to be elucidated.

Although advanced proteomic analyses of Arabidopsis cytoplasmic 80S ribosomes have been performed (**23-25**), proteomic characterization of Arabidopsis plastid ribosomes

has not been reported so far. This may be due to one or more of the following reasons:

(i) since plastid ribosomes have been well-characterized in spinach and *Chlamydomonas* as mentioned above, all the putative plastid ribosomal protein genes of *Arabidopsis* have already been annotated by sequence homology (17, 18); (ii) an isolation method for plastid ribosomes from *Arabidopsis* has not been established; (iii) compared with preparation methods for cytoplasmic ribosomes, those for plastid ribosomes are generally laborious and time consuming, and do not readily yield sufficient quantities or the purity required for proteomics. Even though purification of *Arabidopsis* plastid ribosomes may not be easy by comparison with *Chlamydomonas* or spinach, once a method is established it could be used for advanced proteomics: e.g., protein dynamics in translation regulation, post-translational modifications in translation regulation, etc. Light-dependent phosphorylation of few plastid ribosomal proteins in spinach has been reported (26, 27). Recent large-scale *Arabidopsis* phosphoproteome profiling suggested that some plastid ribosomal proteins are phosphorylated by uncharacterized chloroplast kinases (28). Therefore, detailed phosphoproteomics of plastid ribosomes must be an interesting issue.

Large-scale (over 10,000 absorbance at 260 nm [A_{260}] units) preparation methods employing zonal rotors have been established for tobacco (29, 30) and spinach (31). Although these procedures could be also applicable to *Arabidopsis*, these methods may not be practical for such a tiny plant. For proteomics and molecular biology in *Arabidopsis*, small-scale (10-100 A_{260} units) isolation methods using general ultracentrifugation rotors would be preferable. Here, I describe small-scale methods for plastid ribosome preparation, which have been established with *Chlamydomonas* (17, 18) and *Arabidopsis* (unpublished results). Methods for purity checking and yield estimation of purified ribosomal particles, as well as preparation of plastid ribosomal proteins for proteomic analysis, are also described.

2. Materials

2.1. Preparation of Total Ribosomes

2.1.1. Biological Materials

1. Plant leaves. For one isolation procedure, 20-200 g (fresh weight) of leaves would be appropriate (see **Note 1**). For example, 20 g fresh weight of leaves from 40- to 50-day-old *Arabidopsis thaliana* Columbia-0 plants (see **Note 2**).
2. Algal cells. For example, *Chlamydomonas reinhardtii* (strain CC-3395) grown at 25°C under constant light in 2 L of liquid TAP medium (32) with 50 µg/mL L-Arg to a density of $5-8 \times 10^6$ cells/mL (mid-late log phase) (see **Note 3**).

2.1.2. Buffers

1. Buffer A (all-round extraction buffer): 25 mM Tris-HCl, pH 7.6, 25 mM KCl, 25 mM MgCl₂, 5 mM dithiothreitol (see **Note 4**).
2. Buffer B (alternative extraction buffer): 25 mM Tris-HCl, pH 7.6, 25 mM KCl, 25 mM MgCl₂, 14 mM 2-mercaptoethanol (see **Note 5**).
3. Buffer C (cushion buffer): 1 M sucrose in Buffer A.

2.1.3. Homogenization

1. Laboratory blender (e.g., Waring Blender 7011HS).
2. One ice bucket, containing ice, for chilling buffers and blender during cell disruption.

2.1.4. Centrifuges, Rotors and Tubes

1. High performance centrifuge (e.g., Avanti J-E Centrifuge, Beckman Coulter, CA, USA).
2. Beckman Coulter JA-10 fixed-angle rotor or equivalent.
3. Tubes compatible with the JA-10 rotor (e.g., 500-mL Nalgene centrifuge tubes).
4. Beckman Coulter JA-17 fixed-angle rotor or equivalent.
5. Tubes compatible with the JA-17 rotor (e.g., 15-mL Nalgene centrifuge tubes).
6. Tabletop ultracentrifuge (e.g., Optima TLX Personal Benchtop Ultracentrifuge, Beckman Coulter).
7. Beckman Coulter TLA-100.3 fixed-angle rotor or equivalent.
8. Tubes compatible with the TLA-100.3 rotor (e.g., thickwall polycarbonate tubes, 3.5 mL, 13 × 51 mm, Beckman Coulter).
9. Preparative ultracentrifuge (e.g., Optima L-100 K Preparative Ultracentrifuge, Beckman Coulter).

10. Beckman Coulter Ti70.1 fixed-angle rotor or equivalent.
11. Tubes compatible with the Ti70.1 rotor (e.g., open-top thickwall polycarbonate tubes, 10 mL, 16 × 76 mm, Beckman Coulter).

2.2. Separation of Plastid Ribosomes and Subunits

2.2.1. Buffers and Solutions

1. Buffer A (*see* **Section 2.1.2**) and stock solutions of each buffer component (*see* **Note 4**).
2. Sucrose stock solution: 2 M (68.5% [w/v]) sucrose.
3. Buffer D (dissociation buffer): 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol.

2.2.2. Apparatus for Gradient Preparation/Fractionation System

1. Gradient maker, 50 mL (e.g., SG 50 Gradient Maker, Hoefer, MA, USA).
2. Magnetic stirrer and stirring bar.
3. Density gradient fractionator (e.g., Auto Densi-Flow II, Buchler, NJ, USA). This item is used for depositing a preformed sucrose gradient into an ultracentrifuge tube (i.e., preparation of sucrose gradients before ultracentrifugation), and for gentle withdrawal of centrifuged sample layers (i.e., fractionation of sucrose gradients after ultracentrifugation).
4. Peristaltic pump (e.g., Econo Gradient Pump with 0.8-mm internal diameter PharMed tubing; Bio-Rad, CA, USA).
5. UV-monitor (e.g., Model EM-1 Econo UV Monitor, Bio-Rad).
6. Tubing (e.g., 0.8-mm internal diameter Tygon tubing, Bio-Rad).
7. Fraction collector (e.g., Model 2110 Fraction Collector, Bio-Rad).
8. Chart recorder (e.g., Model 1327 Chart Recorder, Bio-Rad).

2.2.3. Centrifuges, Rotors and Tubes

1. Preparative ultracentrifuge (e.g., Optima L-100 K Preparative Ultracentrifuge, Beckman Coulter).
2. Beckman Coulter SW28 Ti swinging-bucket rotor or equivalent.
3. Tubes compatible with the SW28 rotor (e.g., open-top thinwall polyallomer tubes, 38.5 mL, 25 × 89 mm, Beckman Coulter).
4. Beckman Coulter Ti70.1 fixed-angle rotor or equivalent.

5. Tubes compatible with the Ti70.1 rotor (e.g., open-top thickwall polycarbonate tubes, 10 mL, 16 × 76 mm, Beckman Coulter).

2.3. Purity Criteria and Yield Estimation

1. Spectrophotometer (e.g., NanoDrop ND-1000, Thermo Scientific, MA, USA).
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system (e.g., Mini-Protean System, Bio-Rad).

2.4. Preparation of Plastid Ribosomal Proteins for Proteomics

1. Ribosomal protein extracting solution: 50 mM magnesium acetate in glacial acetic acid. To prepare 100 mL of this solution, dissolve 1.07 g of magnesium acetate tetrahydrate (nuclease and protease tested) in ~90 mL of acetic acid (glacial, ≥99.8%) at room temperature (20-25°C), then fill up to 100 mL with acetic acid. This solution can be stored in a Pyrex screw-cap storage bottle at room temperature for at least 6 months.
2. One ice bucket, containing ice.
3. One glass test tube (e.g., Pyrex test tube, 13 × 100 mm). An acid-stable glass tube is needed for extraction of plastid ribosomal proteins.
4. Stirring bar compatible with test tube (13 × 100 mm) and magnetic stirrer.
5. Dialysis buffers. For high performance liquid chromatography (HPLC) or SDS-PAGE, prepare 30% (v/v) acetic acid, 10% (v/v) acetic acid, and 5% (v/v) acetic acid. For two dimensional (2D)-PAGE, prepare 8 M urea containing 0.1% (v/v) 2-mercaptoethanol. Urea should be ultra-pure grade (e.g., Bio-Rad).
6. Dialysis membrane, with a molecular weight cut-off of 3.5 kDa (e.g., Spectra/Por 3 Dialysis Membrane, Spectrum, Japan).
7. Dialysis membrane closures (e.g., Spectra/Por Closures, Spectrum, Japan).
8. Solvent-absorbent powder (e.g., Spectra/Gel Absorbent, Spectrum, Japan). This is a gel powder (dehydrated polyacrylate-polyalcohol), which is used to concentrate dialysis samples (*see* **Note 6**).

2.5. Rapid Preparation of Plastid Ribosome-Rich Fraction

2.5.1. Buffers, Solutions, Reagents and Apparatus

1. Buffer A (*see* **Section 2.1.2**) and stock solutions of each buffer component (*see* **Note 4**).
2. Buffer C (*see* **Section 2.1.2**).
3. Sucrose stock solution: 2 M (68.5% [w/v]) sucrose.
4. Solid ammonium sulfate (for molecular biology, $\geq 99\%$).
5. The apparatus needed for gradient preparation and the fractionation system are the same as those listed in **Section 2.2.2**, except that the size of gradient maker is different (15 mL, instead of 50 mL).

2.5.2. Centrifuges, Rotors and Tubes/Bottles

1. Refrigerated centrifuge (e.g., Compact High Speed Refrigerated Centrifuge 7780, Kubota, Japan).
2. Kubota AG-508CA fixed-angle rotor or equivalent (*see* **Note 7**).
3. Tubes compatible with the AG-508CA rotor (e.g., 50-mL polypropylene conical centrifuge tubes, Greiner Bio-One, Germany).
4. Preparative ultracentrifuge (e.g., Himac CP 75 beta, Hitachi, Japan).
5. Hitachi P70AT2 fixed-angle rotor or equivalent.
6. Bottles compatible with the P70AT2 rotor (e.g., 8.4-mL 10PC bottle B, Hitachi).
7. Hitachi P40ST swinging-bucket rotor or equivalent.
8. Tubes compatible with the P40ST rotor (e.g., 10.9-mL 13PA tubes, Hitachi).

2.6. SDS-PAGE of Plastid Ribosomal Proteins from Sucrose Gradient-Separated Ribosome Fractions

1. 100% saturated ammonium sulfate (SAS) at 0°C. To 100 mL of RNase-free water, add 70.7 g of ammonium sulfate (for molecular biology, $\geq 99\%$) and dissolve completely by stirring. This SAS solution is saturated at 0°C (for practical use on ice). Make aliquots in 15-mL conical tubes for one-time to few-times usage, and store at 4°C.
2. Running buffer (10 $\times$): 250 mM Tris, 1.92 M glycine, 1% (w/v) sodium dodecyl sulfate (SDS).

3. SDS-sample buffer (1×): 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue; as reported by Laemmli (33).
4. Precast polyacrylamide gels (e.g., Mini-Protean TGX Gels, Bio-Rad).
5. Prestained molecular weight markers (e.g., Precision Plus Protein Dual Xtra Standards, Bio-Rad).
6. CBB staining solution: 0.1% (w/v) Coomassie Brilliant Blue-R250 (CBB), 40% (v/v) methanol, 10% (v/v) acetic acid (e.g., JIS special grade, 99.7%, Wako Pure Chemical Industries, Japan). Alternatively, use a commercial stain (e.g., Bio-Rad CBB-R250 staining solution).
7. Destaining solution: 40% (v/v) methanol, 10% (v/v) acetic acid.

2.7. Identification of Plastid Ribosomal Proteins by MALDI Mass Spectrometry

1. Utility knife for handcrafts (e.g., Art Knife AK-1/5B, Olfa, Japan) (*see Note 8*).
2. Overhead projector (OHP) sheets.
3. Destaining solution: 30% (v/v) acetonitrile in 25 mM ammonium bicarbonate.
4. Acetonitrile.
5. Vacuum centrifuge (e.g., SpeedVac, Thermo Scientific).
6. Trypsin solution. Prepare a 10 µg/mL solution of proteomics grade trypsin (e.g., Trypsin, Proteomics Grade, T6567, Sigma) in 10% (v/v) acetonitrile, 25 mM ammonium bicarbonate.
7. Microwave oven.
8. Extraction solution: 5% (v/v) formic acid, 50% (v/v) acetonitrile.
9. Cup horn sonicator (e.g., Astrason Ultrasonic Processor XL2020, Misonix, NY, USA).
10. Matrix solution. For example, 1× DHBA solution (5 mg/mL of 2, 5-dihydroxybenzoic acid [e.g., DHBA, Shimadzu, Japan]) in 33% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (TFA).
11. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometer capable of tandem mass spectrometry (MS/MS) analysis (e.g., AXIMA Resonance, Shimadzu, Japan).

3. Methods

3.1. Preparation of Total Ribosomes

Chloroplast ribosomes can be purified from either isolated chloroplasts (34) or from total cell homogenates (18, 29, 30). It has been suggested that isolation of chloroplast ribosomes from total cell homogenates results in a higher yield compared with that from isolated chloroplasts (30).

1. Harvesting plant leaves. Wear disposable examination gloves, and cut the leaves with sterile scissors. After the harvest, rinse the leaves with chilled sterile water, drain off the water with a strainer, and keep in the cold-room (4°C, in the dark) for at least 1 h. This step is required to induce polysomal run-off, yielding an accumulation of free ribosomes (35, 36). Leaves should be frozen in liquid nitrogen before homogenization (see Note 9).
2. Harvesting algal cells. Pour the *Chlamydomonas* cell culture into six 500-mL Nalgene centrifuge tubes and harvest the cells by centrifugation at 2,800 $g_{\max}$ in a high performance centrifuge (e.g., Beckman Coulter Avanti J-E) with a fixed-angle rotor (e.g., Beckman Coulter JA-10) for 5 min at 4°C. Put the tubes on ice, suspend the cells with 40 mL of chilled liquid TAP medium, and then transfer the suspension to four 15-mL Nalgene centrifuge tubes. Pellet the cells by centrifugation at 4,000 $g_{\max}$ in a fixed-angle rotor (e.g., Beckman Coulter JA-17). Cells in the tube should be frozen in liquid nitrogen before homogenization (see Note 10).
3. Homogenization. When working with plant leaves: homogenize the frozen leaves (e.g., 20 g with 40 mL of Buffer A) in a chilled Waring Blender for 1 min (three 20-s bursts). Alternatively, when working with algal cells: homogenize the frozen cells (e.g., $1.0\text{--}1.6 \times 10^{10}$ cells from 2 L culture of *Chlamydomonas reinhardtii* CC-3395 with 40 mL of Buffer A) in a chilled Waring Blender for 1 min.
4. Centrifuge the homogenates at 10,000 $g_{\max}$ for 10 min at 4°C (e.g., at 8,500 rpm in a Beckman Coulter JA-17 rotor).
5. Centrifuge the supernatant (the S-10 fraction) at 40,000 $g_{\max}$ for 30 min at 4°C (e.g., at 27,000 rpm in a Beckman Coulter TLA-100.3 fixed-angle rotor), and collect the supernatant (the S-40 fraction).
6. Layer 5 mL of the S-40 fraction over 2 mL of Buffer C (cushion buffer containing 1 M sucrose), and centrifuge at 330,000 $g_{\max}$ for 12 h at 4°C (e.g., at 60,000 rpm in a Beckman Coulter Ti70.1 fixed-angle rotor).

7. Discard the supernatant and dissolve the ribosomal pellet in a minimal volume of Buffer A.
8. Clarify the sample by centrifugation at $15,000g_{\max}$ in a microfuge for 10 min at 4°C to remove insoluble materials, and measure absorbance at 260 nm (A_{260}). The concentration of the total ribosomes should be 200-1000 A_{260} units/mL.
9. Make aliquots of the total ribosome preparation, freeze them in liquid nitrogen, and store at -80°C .

3.2. Separation of Plastid Ribosomes and Subunits

3.2.1. Preparation of Sucrose Gradients

Use the following procedure to prepare a sucrose gradient (e.g., a 10-40% [w/v] linear sucrose gradient; 36 mL) that will be used to separate plastid (70S) and cytoplasmic (80S) ribosomes.

1. Assemble a gradient preparation system. Put the gradient maker on a magnetic stirrer. Add a magnetic stirring bar to the mixing chamber. Connect the gradient maker to the inlet of a peristaltic pump and a density gradient fractionator to the outlet of the pump with tubing. Before starting sucrose gradient preparation, clean the gradient maker and tubing with RNase-free water at the maximum flow rate at room temperature for ~30 min (e.g., the maximum rate is 1.74 mL/min using the Econo Gradient Pump with 0.8-mm internal diameter PharMed tubing).
2. Prepare 10% sucrose in Buffer A and 40% sucrose in Buffer A by diluting 2 *M* sucrose stock and buffer component stocks (*see Note 4*).
3. Close the stopcocks and add 18 mL of 10% sucrose in Buffer A to the reservoir chamber of the gradient maker. Slowly open the connector stopcock and remove air in the connector channel, then close the connector stopcock.
4. Slowly add 18 mL of 40% sucrose in Buffer A to the mixing chamber. Open the connector stopcock, and start stirring.
5. Place a centrifuge tube (e.g., Beckman Coulter thinwall polyallomer 38.5-mL tube) on the tube holder of the density-gradient fractionator. Open the delivery stopcock and make the gradient by pumping at a flow rate of 1.0-1.3 mL/min for ~30 min.

3.2.2. Plastid Ribosome Separation

1. Carefully load 1-2 mL of total ribosome preparation (diluted to 25-50A₂₆₀ units/mL with Buffer A) onto the top of a 36 mL 10-40% (w/v) linear sucrose gradient made up in Buffer A.
2. Centrifuge at 91,000g_{max} for 12 h at 4°C (e.g., at 22,500 rpm in a Beckman Coulter SW28 rotor).
3. Assemble a gradient fractionation system. Disconnect tubing from the gradient maker of the gradient preparation system (*see Section 3.2.1*, step 1) and connect the tubing to the inlet of a UV-monitor. Connect the outlet of the UV-monitor to a fraction collector with tubing (e.g., 0.8-mm internal diameter Tygon tubing). Connect the UV-monitor to a chart recorder with an appropriate cable. Set the pumping direction to reverse (i.e., from the density gradient fractionator to the UV-monitor). Before fractionation, wash the tubing and probe of the density gradient fractionator, withdrawing 30-35 mL of RNase-free water from a clean ultracentrifuge tube.
4. Fractionate the gradient into ~40 microfuge tubes (1.0 mL/tube) from the top surface of the gradient using the gradient fractionation system at a flow rate of 1.0-1.3 mL/min for ~30 min at room temperature. Monitor the absorbance at 254 nm using the UV-monitor and chart recorder (chart speed set at 5-10 mm/min). After the fractionation, the tubes should be immediately placed on ice. An example sucrose gradient profile of total ribosomes from *Chlamydomonas* is shown in **Fig. 1A**.
5. Recover the 70S plastid ribosomes as pellets from appropriately-pooled fractions (*see Fig. 1A*) by centrifugation at 330,000g_{max} for 12 h at 4°C (e.g., at 60,000 rpm in a Beckman Coulter Ti70.1 fixed-angle rotor) (*see Note 11*).
6. Repurify the ribosomes by using a second sucrose gradient, if highly pure 70S ribosomes are needed (i.e., repeat steps 1-5 above) (*see Note 12*).

3.2.3. Subunit Preparation

1. Add a small volume of Buffer D to the 70S pellet, and dissolve the pellet by pipetting to dissociate the ribosomes into subunits. Adjust the subunit concentration to 20-40 A₂₆₀ units/mL with Buffer D.

2. Carefully load 1-2 mL of subunits (20-40 A_{260} units) onto a 36 mL 10-30% sucrose gradients made up in Buffer D. Centrifuge at $91,000g_{\max}$ for 20 h at 4°C (e.g., at 22,500 rpm in a Beckman Coulter SW28 rotor).
3. Fractionate the gradients as described in **Section 3.2.2** (steps 3-4). An example sucrose gradient profile of *Chlamydomonas* plastid ribosome subunits is shown in **Fig. 1B**.
4. Recover the 30S and 50S subunits as pellets from appropriately-pooled fractions (see **Fig. 1B**) by centrifugation at $330,000g_{\max}$ for 14 h at 4°C (e.g., at 60,000 rpm in a Beckman Coulter Ti70.1 fixed angle rotor) (see **Note 13**).

3.3. Purity Criteria and Yield Estimation

Fine proteomic analysis will require highly pure ribosomes. The following section describes easy and rapid methods for purity checking and yield estimation of plastid ribosomes.

1. Measure absorbance at 260 nm and 280 nm of appropriately-diluted solutions of ribosomal particles using spectrophotometer.
2. Purity criteria. Pure plastid ribosomes and subunits must show an $A_{260/280}$ ratio greater than 1.9. Although ratios in the range 1.7-1.9 for $A_{260/280}$ have been an accepted criterion for a pure plastid ribosome preparation (**31**), significant contamination of RuBisCO is usually suspected even in samples with a high $A_{260/280}$ ratio (>1.9). SDS-PAGE analysis of sucrose gradient fractions (see **Section 3.6**) can be utilized to examine the extent of RuBisCO contamination (50-kDa and 15-kDa bands correspond to the large and small subunits of RuBisCO, respectively).
3. Yield estimation. The yields of plastid ribosomes and their subunits can be estimated spectrophotometrically using the table of amount conversion (see **Table 1**). For example, 1 A_{260} unit of plastid 70S ribosomes corresponds to approximately 66 μg (26 pmol) of particles. Since protein recovery with the procedure of acetic acid extraction (**37**) is close to 100% (over 98%), the amount of extracted ribosomal proteins can be also estimated with the conversion table, without the need for a protein assay; e.g., acetic acid-extracted total protein (TP70) from 1 A_{260} unit of plastid 70S ribosomes corresponds to 26 μg (26 pmol) of protein.

4. RNA integrity. Intactness of rRNA is not a requirement for the maintaining of a full complement of ribosomal proteins, of sedimentation coefficients of the subunits, or of translation activity (30, 38), although highly-degraded rRNA does cause anomalous sedimentation peaks on sucrose gradients (30). Intactness of rRNA can be easily assessed by agarose gel electrophoresis after RNA extraction by the acid guanidinium-thiocyanate-phenol-chloroform (AGPC) method (39) using an RNA isolation reagent (e.g., TRIzol reagent, Invitrogen).

3.4. Preparation of Plastid Ribosomal Proteins for Proteomics

The following procedure is a slightly modified method of acetic acid extraction method described by Hardy et al. (37). Total proteins of plastid ribosomal particles prepared using this procedure can be used for various separation techniques in proteomics: e.g., SDS-PAGE (17, 18), HPLC, liquid chromatography-mass spectrometry (LC-MS), MALDI mass spectrometry, and 2D-PAGE (14, 15).

1. Dilute ribosomes or subunits with Buffer A and adjust the concentration to 20-100 A₂₆₀ units/mL.
2. Transfer to a glass test tube with a stirring bar and chill the ribosome solution in an ice-filled bucket. Place the bucket on a magnetic stirrer, and start stirring.
3. Slowly add 2 volumes of 50 mM magnesium acetate in glacial acetic acid. Cloudy insoluble rRNA will appear immediately.
4. Stir on ice for at least 1 h.
5. Transfer the cloudy suspension to 1.5-mL centrifuge tubes. Remove the insoluble rRNA by centrifugation at 15,000g_{max} for 10 min at 4°C (e.g., at 14,800 rpm in a Hitachi Himac RT15A3 rotor). Retain the supernatant.
6. For SDS-PAGE, HPLC, and MALDI mass spectrometry, take the supernatant (from step 5) and dialyze it against, in order: (i) 100 volumes of 30% acetic acid for 4 h; (ii) 100 volumes of 10% acetic acid for 4 h; and, (iii) 200 volumes of 5% acetic acid for 16 h; all dialysis steps should be conducted in a cold room (4°C). Make aliquots of the desalted ribosomal proteins, then lyophilize or dry using a SpeedVac. Store the protein samples at -20°C or -80°C. An example SDS-PAGE gel resolving total proteins from 30S, 50S, 70S and 80S particles from *Chlamydomonas* is shown in **Fig. 2**.

7. Alternatively, for 2D-PAGE (**40**), take the supernatant (from step 5 above) and dialyze it against: (i) 100 volumes of 8 M urea, 0.1% 2-mercaptoethanol for 4 h; and, (ii) 200 volumes of 8 M urea, 0.1% 2-mercaptoethanol for 16 h; both dialysis steps to be conducted in a cold room (4°C). Concentrate the ribosomal proteins with solvent-absorbent powder to 1-2 nmol/mL. Store the protein samples at -20°C or -80°C.
8. Protein resolution for mass spectrometry. Because many plastid ribosomal proteins possess highly basic isoelectric points ($pI > 11$), commercially available IPG (Immobilized pH Gradient)-based 2D-PAGE systems, enabling separation with a pI range of 3-10, are not suitable for proteomics of plastid ribosomal proteins. The hand-crafted acrylic plastic 2D-PAGE apparatus designed by Mets and Bogorad (**41**), and the buffer/gel system improved by Subramanian (**40**), are recommended. The RFHR (radical-free and highly reducing) method (**42**) would also be preferable to resolve plastid ribosomal proteins. Although 2D-protein mapping is a powerful approach for visualizing individual ribosomal proteins, high-throughput mass spectrometry (e.g., liquid chromatography-electrospray ionization-tandem mass spectrometry [LC-ESI-MS/MS]) allows identification of an almost complete set of plastid ribosomal proteins from protein bands separated by 1D-SDS-PAGE (e.g., **Fig. 2**) or from the whole ribosomal protein complex (**17, 18**). MALDI-quadrupole ion trap-time of flight (QIT-TOF) MS also allows identification of plastid ribosomal proteins (*see Figs. 3, 4*), post-translational processing, and modifications (data not shown) without 2D-PAGE separation. Thus, 2D-PAGE is not indispensable to conduct the proteomic characterization of plastid ribosomes.

3.5. Rapid Preparation of Plastid Ribosome-Rich Fraction

This procedure employs ammonium sulfate precipitation to concentrate total ribosomes, which allows efficient purification of intact 70S ribosomes from *E. coli* (**43**).

1. Homogenize frozen leaves (e.g., 20 g) as described in **Section 3.1**.
2. Transfer the homogenate to two 50-mL conical tubes and centrifuge at $15,000g_{max}$ for 10 min at 4°C (e.g., at 10,600 rpm in a Kubota AG-508CA rotor).
3. Transfer the supernatant (25 mL of supernatant from each tube: total 50 mL) to a 200-mL glass beaker using a disposable pipette. Put a stirring bar in the beaker and

put the beaker in an ice bucket. Place the bucket on a magnetic stirrer, and start stirring.

4. Add 24 g of solid ammonium sulfate per 50 mL of supernatant under stirring on ice.
5. Stir for 5-10 min (until solid ammonium sulfate is completely dissolved).
6. Transfer the cloudy solution to two 50-mL conical tubes, then centrifuge at $15,000g_{\max}$ for 10 min at 4°C (e.g., at 10,600 rpm in a Kubota AG-508CA rotor).
7. Discard the supernatant and add 5 mL of Buffer A to each tube.
8. Suspend the pellet by pipetting up and down (most of the pellet will be dissolved).
9. Transfer the suspension to two ultracentrifuge bottles (e.g., Hitachi 8.4-mL 10PC bottle B), and centrifuge at $40,000g_{\max}$ for 30 min at 4°C (e.g., at 20,800 rpm in a Hitachi P70AT2 fixed-angle rotor).
10. Layer 3-5 mL of the supernatant (the S-40 fraction) over 2 mL of Buffer C, and centrifuge at $230,000g_{\max}$ for 16 h at 4°C (e.g., at 50,000 rpm in a Hitachi P70AT2 fixed-angle rotor).
11. Discard the supernatant and dissolve the ribosomal pellet in a minimal volume of Buffer A.
12. Clarify by centrifugation at $15,000g_{\max}$ for 10 min at 4°C to remove insoluble material, and measure absorbance at 260 nm. The concentration of the total ribosomes should be 200-1,000 A_{260} units/mL.
13. Prepare one or more 10 mL 10-34% (w/v) linear sucrose gradients made up in Buffer A (e.g., in a Hitachi 10.9-mL 13PA tube) as described in **Section 3.2.1**.
14. Load 0.1-0.2 mL of total ribosome preparation (equivalent to 10-20 A_{260} units; the concentration should be adjusted with Buffer A if necessary) onto each sucrose gradient. Centrifuge at $111,000g_{\max}$ for 5 h at 4°C (e.g., at 25,000 rpm in a Hitachi P40ST rotor).
15. Fractionate the gradients into 12 microfuge tubes (0.8 mL/tube) from the top surface of the gradient using the density gradient fractionator by pumping at a flow rate of 0.8 mL/min through a UV-monitor and fraction collector (as described in **Section 3.2.2**, steps 3-4). Monitor the absorbance at 254 nm using a UV-monitor.

3.6. SDS-PAGE of Plastid Ribosomal Proteins from Sucrose Gradient-Separated Ribosome Fractions

This procedure is a rapid and reproducible method using a precast gel system (Bio-Rad) to separate plastid ribosomal proteins immediately after fractionation on a sucrose gradient. This method is also useful to evaluate the purity of sucrose gradient-separated ribosome fractions (*see Fig. 3*). However, attention should be paid for data interpretation in the case of proteomic analysis, since some plastid ribosomal proteins may not be appeared on gels depending on the source of ribosomal particles. For example, some plastid ribosomal proteins of *Chlamydomonas* (e.g., S2, S3, and S5) are hardly extracted by the following procedure (i.e., SDS-treatment alone) whereas most of other proteins are extracted. In such case, acetic acid extraction method (*see Section 3.4.*) should be employed.

1. Ammonium sulfate precipitation. Transfer 400 μ L of each sucrose gradient fraction to a 1.5-mL centrifuge tube, and add 1 mL of SAS to each tube. Shake vigorously by hand for few seconds, and keep them on ice for 10 min. Spin down the precipitate by centrifugation at 15,000 $g_{\max}$ for 10 min in a microfuge. Withdraw and discard the clear supernatant using a 1-mL Gilson pipette tip. Centrifuge again at 15,000 $g_{\max}$ for 1 min, and remove as much residual liquid as possible using a 200- μ L Gilson pipette tip.
2. SDS-PAGE sample preparation. Add 20 μ L of 1 $\times$ SDS sample buffer to the pellet (from step 1), and incubate the tube at 95°C for 1 min. Suspend the precipitated sample by pipetting at room temperature, and incubate the tube again at 95°C for 5 min. Centrifuge at 15,000 $g_{\max}$ for 10 min in a microfuge to remove insoluble material.
3. Assemble the gel unit and connect to a power supply.
4. Load prestained molecular weight markers and the samples. Run the SDS-PAGE at 200 V ($\sim$ 28 V/cm) until the front protein marker (2 kDa) is 1 cm from the bottom of the gel.
5. Stain with CBB staining solution for 15-30 min with constant shaking, and then destain with Destaining solution until a clear background is obtained.
6. Sandwich the gel between clear plastic sheets (e.g., clean OHP sheets) to protect the gel from physical damage and to avoid contamination (e.g., by keratin), and then

place it in a plastic zip-seal bag, and store at 4-10°C until use (e.g., the following section).

3.7. Identification of Plastid Ribosomal Proteins by MALDI Mass Spectrometry

Here described is an example procedure for conducting MALDI mass spectrometry analysis of Arabidopsis plastid ribosomal proteins using MALDI-QIT-TOF MS (*see Figs. 3, 4*). Microwave-assisted protein enzymatic digestion (**44, 45**) allows rapid and accurate identification of the plastid ribosomal proteins (*see Table 2*).

1. Place the CBB-stained gel on a clean OHP sheet.
2. Excise the protein band of interest using a sterile cutting blade (e.g., half of a band; *see Fig. 3B*) (*see Notes 8, 14*).
3. Cut the excised gel slice into several 1-mm² pieces, and transfer two pieces to a 1.5-mL tube.
4. To the tube, add 500 µL of Destaining solution and agitate for 10 min.
5. Discard the solution and repeat step 4 (usually 2-3 times) until the gels look clear.
6. Dehydration. Add 500 µL of acetonitrile to the tubes, and agitate for 10 min (during agitation, the clear gel pieces will gradually shrink and turn white). Discard the solution.
7. Dry the gel pieces by using a vacuum centrifuge (e.g., SpeedVac) for 10 min at 45°C.
8. Microwave-assisted protein enzymatic digestion. Add 10 µL of Trypsin solution per one tube and put on ice for 20-30 min (until the gel pieces look clear). Remove any excess amount of Trypsin solution using a 10-µL Gilson pipette tip, and close the caps of the tubes. Put the tubes in a plastic tube stand. In a microwave oven, place a 300-mL beaker containing 200 mL of water (*see Note 15*) and put the tube stand on top of the beaker. Microwave the samples for 5 min at 200-300 W.
9. Peptide extraction. Add 20 µL of Extraction solution to each tube, and sonicate in a cup horn sonicator (e.g., Astrason Ultrasonic Processor XL2020, Misonix, at output level 4) for 1 min. Transfer the solutions to new 0.5-mL centrifuge tubes.
10. Dry the peptide fragments by using a vacuum centrifuge (e.g., SpeedVac) for 15 min at 45°C.

11. Add 2 μL of Matrix solution (e.g., DHBA solution) and dissolve the peptides by pipetting.
12. Spot 1 μL of the sample solution onto a MALDI target plate.
13. Conduct mass spectrometry and analyze the MS/MS spectra (e.g., by MS/MS ion searching using Mascot (**46**), Matrix Science) (*see Note 16*).

4. Notes

1. For example, 20 g of *Arabidopsis* leaves yields at least 50 A₂₆₀ units of total ribosomes, and ~5 units of 70S-rich fraction at the first sucrose gradient.
2. Alternatively, leaves of *Brassicaceae* plants (closely related to *Arabidopsis thaliana*), such as *Brassica rapa* subsp. *chinensis* (bok-choy) and *Brassica rapa* var. *peruviridis* (komatsuna), are also applicable for plastid ribosome preparation and proteomics. These edible *Brassicaceae* plant leaves can easily be obtained from a fresh market (one batch is enough for one preparation). I have found that sucrose gradients of total ribosomes from bok-choy and komatsuna show similar patterns to that of *Arabidopsis* (see **Fig. 3A**), and Mascot MS/MS ion searches of in-gel digests obtained from the bok-choy 70S-rich fraction allowed efficient identification of *Arabidopsis* orthologs (unpublished observation).
3. The recipe for TAP medium is also available at the website of the Chlamydomonas Center (<http://www.chlamy.org/TAP.html>). Compared with cells of wild-type strains, those of strain CC-3395 (an arginine-requiring cell-wall deficient mutant) can be readily disrupted by the homogenization protocol described here, or by the N₂-bomb method (*17*). Steady-state cells (over 10⁷ cells/mL) contain a lesser amount of plastid ribosomes and a greater amount of cytoplasmic ribosomes than the cells in mid-late log phase (5-8 × 10⁶ cells/mL).
4. Unless otherwise stated, all solutions for ribosome extraction and purification should be prepared with RNase-free water (either diethyl pyrocarbonate [DEPC]-treated water or RNase/pyrogen-free Milli-Q water purified through an ultra-filtration cartridge [e.g. BioPak, Millipore]) and RNase-free reagents (i.e., sucrose, ammonium sulfate, other salts, etc.). For convenient and reproducible preparation, I use stock solutions of each buffer component: 1 M Tris-HCl, pH 7.6; 1 M KCl; 1 M MgCl₂ (store at 4°C, and can be used at least for six months); and, 1 M dithiothreitol (make aliquots in 0.5-mL centrifuge tubes for one-time usage and store at -20°C or -80°C). These stock solutions are also used to make other buffers (i.e., Buffer C, sucrose gradients, etc.). All the buffers should be prepared just before ribosome preparation, diluting the stock solutions with RNase-free water. Addition of polyamines (final concentrations, 0.05 mM spermine and 2 mM spermidine) to ribosome preparation buffers is helpful to prolong intactness of rRNAs (unpublished observation) and conformational stability (*47, 48*), though I

have not tested addition of polyamines for the ribosome preparation toward proteomics.

5. Dithiothreitol (final concentration, 5 mM) in Buffer A can be use in place of 2-mercaptoethanol (final concentration, 7-14 mM). I prefer to use Buffer B for mid- to large-scale preparations (>200 g leaves) instead Buffer A.
6. Alternatively, dry powder of Sephadex (e.g., Sephadex G-50, GE Healthcare, Chalfont St. Giles, UK) can be used as a solvent-absorbent powder.
7. This fixed-angle rotor is convenient, accepting 8 × 50-mL conical tubes.
8. I prefer to use a utility knife (e.g., Art Knife AK-1/5B, Olfa, Japan) rather than a generally employed scalpel or razor blade. It works nicely for precise gel excision and splitting gels into small pieces, as well as picking up and transferring gel pieces to microfuge tubes. The knife blade must be cleaned by wiping with a 70% ethanol-sprayed KimWipe tissue (Kimberly-Clark, TX, USA).
9. Liquid nitrogen-frozen leaves can be stored at -80°C until ribosome preparation.
10. Liquid nitrogen-frozen cells can be stored at -80°C until ribosome preparation.
11. 70S ribosomes can be stored at -80°C, after dissolving the ribosomal pellets in a small volume of Buffer A containing 10% (v/v) glycerol.
12. Multistep sucrose gradient ultracentrifugation is a very effective method for obtaining highly-pure chloroplast ribosomes (**18**).
13. Subunits can be stored at -80°C, after dissolving the ribosomal pellets in a small volume of Buffer A containing 10% (v/v) glycerol.
14. Half of a band is enough for tryptic peptide preparation. The residual half can be used for other peptide preparations (e.g., other enzymatic digestions with Lys-C, Arg-C, Asp-N, etc., or chemical cleavage) if required.
15. The beaker with water is to absorb excess microwave energy.
16. Identification of small (<10 kDa) and highly basic ribosomal proteins by mass spectrometry (peptide mass fingerprinting or MS/MS ion searching) is difficult or impossible (**17, 18**). In such cases, N-terminal sequencing of individual ribosomal proteins using a protein sequencer (e.g., PPSQ-33A, Shimadzu, Japan) would be effective, since most proteins in plastid 70S ribosomes possess free N-termini (**14, 15**), unlike those of cytoplasmic 80S ribosomes (**25**).

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Figure Captions

Fig. 1. Isolation of chloroplast 70S ribosomes and their subunits from the green alga *Chlamydomonas reinhardtii*.

(A) Sucrose gradient (10-40%) analysis of a total ribosome preparation (50 A_{260} units) from *C. reinhardtii* cells in Buffer A. Peaks corresponding to plastid ribosomes (70S) and cytoplasmic ribosomes (80S) are indicated by arrowheads. The underlined 70S fractions were pooled and subjected to a second sucrose gradient purification. (B) Separation of chloroplast 30S and 50S subunits in a sucrose-gradient (10-30%) after dissociation of the 70S ribosomes with Buffer D; the underlined fractions were collected for the making of total protein preparations (TP30 and TP50, respectively).

Fig. 2. SDS-PAGE profiles of total proteins from 30S, 50S, 70S and 80S ribosomal particles prepared from *Chlamydomonas reinhardtii*.

Total proteins (TP) extracted from 10 pmol of each of the following fractions were resolved by SDS-PAGE using a 1.5-mm thick, 12% acrylamide gel: chloroplast small subunits (TP30), chloroplast large subunits (TP50), chloroplast ribosomes (TP70), and cytoplasmic ribosomes (TP80). Proteins were stained with Coomassie Brilliant Blue R-250. All the protein bands from TP30, TP50, TP70 and TP80 were analyzed by LC-ESI-MS/MS (17, 18, 47).

Fig. 3. Sucrose density gradient separation of 70S chloroplast ribosomes and 80S cytoplasmic ribosomes from the leaves of *Arabidopsis thaliana*, and SDS-PAGE profiles of the sucrose gradient fractions.

(A) Ten A_{260} units of total ribosomes ($A_{260/280} = 1.98$) were loaded on a 10-40% sucrose gradient in Buffer A (10 mL) and centrifuged as described in **Section 3.5**. (B) Proteins (present in a half volume of each fraction) in fractions 1 to 8 of panel A were resolved on a Mini-Protean TGX precast gel (Any kD, 15-well comb, Bio-Rad), and stained with Coomassie Brilliant Blue R-250. Protein band sections used for mass spectrometric analysis are indicated by dotted boxes. Proteins in sections 1a (50-kDa band) and 1b (15-kDa band) were identified as RuBisCO large subunit and small subunit, respectively, by peptide mass fingerprinting. Almost invisible 50-kDa (corresponding to gel section 1a: RuBisCO large subunit) and 45-kDa (corresponding to

gel section 6a: cytoplasmic 60S ribosomal subunit proteins L3 and L4; *see Table 2*) bands in fraction 5 indicate that fraction 5 is rich in plastid 70S ribosomes with a slight contamination of RuBisCO and 80S ribosomes. This was also supported by distinct MALDI MS spectra (*see Fig. 4*) of tryptic fragments from gel sections 5a (30-kDa band of fraction 5) and 6b (30-kDa band of fraction 6).

Fig. 4. MALDI MS spectra of tryptic fragments obtained from Arabidopsis ribosome preparations.

Section 5a (30-kDa band in fraction number 5, i.e., 70S-rich fraction; upper panel) and section 6b (30-kDa band in fraction number 6, i.e., 80S-rich fraction; lower panel) of the gel shown in Fig. 3 were excised and analysed. White arrowheads indicate ion peaks assigned as plastid ribosomal proteins by MS/MS analysis; black arrowheads indicate those for cytoplasmic ribosomal proteins. Regions of relatively low signal in both spectra (m/z 1750-2300) were amplified fivefold ($\times 5$). Peptide ions annotated by numeral (m/z), and protein ID in parentheses, indicate that positive protein identification was performed by MS/MS ion searching with significant probability-based Mascot scores ($p < 0.05$). The scores, peptide sequences, and other parameters obtained by Mascot searches are listed in **Table 2**.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Table Captions

Table 1. Conversion table for 1 A₂₆₀ unit of plastid ribosomal particles of a higher plant (spinach).

^a The sum of the masses of individual ribosomal proteins (*14, 15*), taking into account that L12 protein is present in four copies per 50S subunit, and a 22-kDa protein (pRRF) is present in 70S but absent from either 30S or 50S subunits (*15, 49*).

^b Calculated from the nucleotide sequences (*50*) of 16S rRNA (1491 nucleotides [nt]) of the 30S subunit, and of 23S rRNA (2811 nt), 5S rRNA (121 nt), and 4.5S rRNA (103 nt) of the 50S subunit.

^c The sum of the total protein mass and the total RNA mass, not taking into account protein/RNA modifications, metal ions, and other ribosomal components such as polyamines.

^d Calculated from the RNA amount, using the relationship that one A₂₆₀ unit of RNA corresponds to 40 µg.

^e Calculated from the following equation: (total protein of the ribosomal particle) = (particle weight) – (RNA weight, i.e., 40 µg). TP70, TP50, and TP30 stand for total proteins present in 70S, 50S, and 30S ribosomal particles, respectively.

Table 2. Identification of Arabidopsis ribosomal proteins from the gel sections 5a, 5b, 6a and 6b in Fig. 3.

^a PRP, plastid ribosomal protein. Nomenclature of PRPs is in accordance with Yamaguchi and Subramanian (*15*).

^b Abbreviated SwissProt accession numbers omitting “_ARATH” (e.g., RK1_ ARATH is abbreviated to RK1).

^c Number of missed cleavage sites in the tryptic fragment.

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Table 1**Conversion table for 1 A₂₆₀ unit of plastid ribosomal particles of a higher plant (spinach)**

Ribosomal particle	Protein mass ^a	RNA mass ^b	Particle mass ^c	Particle amount ^d		Protein amount ^e
	(kDa)	(kDa)	(kDa)	(μg)	(pmol)	(μg)
70S	1,022	1,558	2,580	66	26	26 (TP70)
50S	571	1,040	1,611	62	38	22 (TP50)
30S	429	518	947	73	77	33 (TP30)

^a The sum of the masses of individual ribosomal proteins (**14**, **15**), taking into account that L12 protein is present in four copies per 50S subunit, and a 22-kDa protein (pRRF) is present in 70S but absent from either 30S or 50S subunits (**15**, **49**).

^b As the sodium salt, calculated from the nucleotide sequences (**50**) of 16S rRNA (1491 nucleotides [nt]) of the 30S subunit, and of 23S rRNA (2811 nt), 5S rRNA (121 nt), and 4.5S rRNA (103 nt) of the 50S subunit.

^c The sum of the total protein mass and the total RNA mass, not taking into account protein/RNA modifications, metal ions (e.g., Mg²⁺) required for RNA folding/stabilizing, and other ribosomal components such as polyamines.

^d Calculated from the RNA amount, using the relationship that one A₂₆₀ unit of RNA corresponds to 40 μg.

^e Calculated from the following equation: (total protein of the ribosomal particle) = (particle weight) – (RNA weight, i.e., 40 μg). TP70, TP50, and TP30 stand for total proteins present in 70S, 50S, and 30S ribosomal particles, respectively.

Table 2**Identification of Arabidopsis ribosomal proteins from the gel sections 5a, 5b, 6a and 6b in Fig. 3.**

Section	Protein ID ^a	Accession No. ^b	m/z	Delta	Miss ^c	Score	Sequence
5a	50S PRPL1	RK1	1817.1	0.12	1	65	K.AGTVTANIPQAIEEFKK.G
			2112.1	0.05	1	78	K.GTGQTVIVAVLAQGEKVDEAK.S
	50S PRPL2	RK2	1257.8	0.08	1	49	K.SATLKLPSGEVR.L
			1570.0	0.11	1	40	R.KKPVTPWGYPALGR.R
			1726.1	0.11	2	22	R.KKPVTPWGYPALGR.T
			2209.3	0.20	1	48	R.GVVMNPVDHPHGGGEGRAPIGR.K
	50S PRPL4	RK4	1159.7	0.04	2	38	K.DWSIKINRK.E
			1180.7	0.09	1	53	K.TKDFLAAMQR.W
			1635.1	0.15	0	51	R.TPLRPGGGVVFGRPRK.D
			1499.8	0.13	0	58	R.GFEGGQTALYR.R
5b	30S PRPS2	RR2	1838.2	0.27	0	69	R.LGTTQSHSLWFAQPK.K
	30S PRPS3	RR3	1198.7	0.11	0	34	R.GFEGGQTALYR.R
	50S PRPL15	RK15	1456.9	0.13	1	30	R.FRLDNLGPPGSR.K
			1497.7	0.13	1	30	R.FRLDNLGPPGSR.K
6a	60S RPL3	RL31	983.6	0.06	0	43	R.HGSLGFLPR.K
			1320.7	0.03	0	50	K.FIDTASIFGHGR.F
	60S RPL4	RL4A, RL4B	1149.7	0.08	1	25	R.MFAPTKIWR.R
			1444.7	0.04	0	71	K.AGHQTSAESWGTGR.A
			1572.8	0.05	1	75	K.KAGHQTSAESWGTGR.A
			1862.1	0.03	0	84	R.HAIVSAIAATAVPALVMAR.G
6b	40S RPSa	RSSA1	2066.2	0.26	0	47	R.HTPGTFTNQMQTSFSEPR.L
	40S RPS2	RS21, RS22	1783.1	0.16	1	73	K.KVLQFAGIDDVFTSSR.G
		RS23, RS24	1783.1	0.16	1	73	K.KVLQFAGIDDVFTSSR.G
	60S RPL6	RL62	1661.1	0.21	1	41	R.FSLKQGMKPHLVF.-
	60S RPL7a	RL7A1, RL7A2	1350.9	0.14	1	36	K.TLDKNLATSFLK.V
			1355.9	0.10	1	42	R.LKVPPALNQFTK.T
	60S RPL13	RL131	1019.6	0.09	0	24	K.TWFNQPAR.K
			1147.7	0.10	1	16	K.TWFNQPAR.K
			1244.8	0.14	0	7	R.SLEGLQTNVQR.L

^a PRP, plastid ribosomal protein. Nomenclature of PRPs is in accordance with Yamaguchi and

Subramanian (15).

^b Abbreviated SwissProt accession numbers omitting “_ARATH” (e.g., RK1_ ARATH is abbreviated to RK1).^c Number of missed cleavage sites in the tryptic fragment.