Plant Molecular Biology (2005) 57:577–591
DOI 10.1007/s11103-005-0699-3

High heterogeneity within the ribosomal proteins of the *Arabidopsis thaliana* 80S ribosome

Patrick Giavalisco[1,2,3,†], Daniel Wilson[1,†], Thomas Kreitler[1], Hans Lehrach[1], Joachim Klose[2], Johan Gobom[1,*] and Paola Fucini[1,*]
[1]*Max-Planck-Institute for Molecular Genetics, Ihnestr. 73, D-14195 Berlin, Germany; *Author for correspondence; e-mails {Fucini, Gobom}@molgen.mpg.de;* [2]*Institute for Human Genetics, Humboldt University Berlin, Charité, Augustenburger Platz 1, D-13353 Berlin, Germany;* [3]*Current address: Boyce Thompson Institute for Plant Research, Tower Road, 14850 Ithaca, NY, USA;* [†]*These authors contributed equally to this work.*

Received 25 October 2004; accepted in revised form 15 January 2005

Key words: *Arabidopsis thaliana*, mass spectrometry, post-translational modification, proteosome, RACK1, ribosome

Abstract

Proteomic studies have addressed the composition of plant chloroplast ribosomes and 70S ribosomes from the unicellular organism *Chlamydomonas reinhardtii*, but comprehensive characterization of cytoplasmic 80S ribosomes from higher plants has been lacking. We have used two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) to analyse the cytoplasmic 80S ribosomes from the model flowering plant *Arabidopsis thaliana*. Of the 80 ribosomal protein families predicted to comprise the cytoplasmic 80S ribosome, we have confirmed the presence of 61; specifically, 27 (84%) of the small 40S subunit and 34 (71%) of the large 60S subunit. Nearly half (45%) of the ribosomal proteins identified are represented by two or more distinct spots in the 2-DE gel indicating that these proteins are either post-translationally modified or present as different isoforms. Consistently, MS-based protein identification revealed that at least one-third (34%) of the identified ribosomal protein families showed expression of two or more family members. In addition, we have identified a number of non-ribosomal proteins that co-migrate with the plant 80S ribosomes during gradient centrifugation suggesting their possible association with the 80S ribosomes. Among them, RACK1 has recently been proposed to be a ribosome-associated protein that promotes efficient translation in yeast. The study, thus provides the basis for further investigation into the function of the other identified non-ribosomal proteins as well as the biological meaning of the various ribosomal protein isoforms.

Introduction

Ribosomes are large macromolecular ribonucleoprotein particles responsible for the mRNA directed protein synthesis in the cell. All ribosomes consist of a small and large subunit but the composition varies considerably across the three phylogenetic kingdoms, both in terms of size, ranging from ~2.5 MDa in bacteria to >3.5 MDa in higher eukaryotes, and diversity. Bacterial ribosomes generally consist of 3 rRNAs and ~54 proteins, whereas eukaryotes have 4 rRNAs and ~80 proteins (reviewed by Wilson and Nierhaus, 2003). Studies into the composition and structure of prokaryotic ribosomes have been long-time undertakings and have culminated recently with high-resolution structures for bacterial (Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000; Harms *et al.*, 2001) and archeal (Ban *et al.*, 2000) ribosomal subunits.

The composition of the bacterial (Wittmann-Liebold, 1986) and yeast ribosomes (Link *et al*., 1999; Lee *et al*., 2002) have been fully characterised by various proteomic approaches. Proteomic analyses of ribosomes from higher eukaryotes have been limited to cytoplasmic ribosomes from rat (Wool *et al*., 1995; Louie *et al*., 1996), as well as both the small (Vladimirov *et al*., 1996) and large subunit (Odintsova *et al*., 2003) from humans. Although studies have addressed the composition of bacterial-like ribosomes, such as the chloroplast 70S ribosome from *Spinacea oleracea* (Yamaguchi and Subramanian, 2000; Yamaguchi *et al*., 2000) and *Chlamydomonas reinhardtii* (Yamaguchi *et al*., 2003), to date, little work has addressed the composition of the higher plant cytoplasmic ribosomes. In the early 1980s, two-dimensional gel electrophoresis (2-DE) analysis of cytoplasmic ribosomes from *Nicotiana tabacum* (Capel and Bourque, 1982) and Soybean (Gantt and Key, 1985) were performed. However attempts to characterize the individual ribosomal proteins by comparing the 2-DE profiles with data obtained from *E. coli* ribosomes were ambiguous due to the increased complexity of the plant cytoplasmic ribosomes (Capel and Bourque, 1982).

Proteomic studies, in turn, have benefited immensely from the rapidly increasing number of completely sequenced genomes. The complete genome sequence of *A. thaliana* (Initiative, 2000), in combination with a large number of available expressed sequence tags (ESTs), has enabled a theoretical composition of the 80S ribosome to be predicted (Barakat *et al*., 2001). This analysis identified 249 genes (including 19 pseudogenes) that encode for 80 protein families of the cytoplasmic 80S ribosome. None of the ribosomal protein genes were found to be single copy in *A. thaliana* and it seems likely based on EST accessions, that in fact the majority of these gene duplications are expressed (Blanc *et al*., 2000; Barakat *et al*., 2001). The differential expression of many gene families and high variation within specific ribosomal gene families suggests the existence of extensive ribosome heterogeneity at the intracellular level. As well as verifying the actual composition of the ribosome (as compared with a theoretical prediction), the proteomic approach can lead to the identification of novel ribosomal proteins, as was seen for chloroplast 70S (Yamaguchi and Subramanian, 2000, 2003) and yeast cytoplasmic 80S ribosomes studies (Link *et al*., 1999). In addition, a proteomic analysis can provide insight into the different protein isoforms and post-translational modifications present in the assembled ribosomal particles. This was exemplified by the determination of ribosomal proteins in the *E. coli* 70S ribosome that carry modifications (Arnold and Reilly, 1999, 2002).

The aim of the present research was to isolate and purify the cytoplasmic 80S ribosomes from *A. thaliana* leaves and to identify the protein components. For this purpose we utilized two-dimensional gel electrophoresis and matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). In addition to the identification of the large majority of the protein components of the cytoplasmic 80S ribosome, we also observed a large heterogeneity within the ribosome, both in terms of the isoforms present as well as putative post-translational modifications. Furthermore, we were able to identify a number of non-ribosomal proteins that pellet or co-migrate with the 80S ribosome. Among them, the nascent polypeptide associated complex (NAC) and RACK1, a protein found to promote efficient translation in yeast.

Materials and methods

Plant material

The plant leaves used in these experiments were sampled from *Arabidopsis thaliana* ecotype Columbia 0, provided by Dr Christina Walz from the Max Planck Institute for Plant Physiology in Golm, Germany. Green rosette leaves were harvested from 50 days old plants, shock-frozen in liquid nitrogen and stored at −80 °C until ribosome preparation.

Preparation of the 80S ribosomes

The 80S ribosomes were obtained from 500 g *Arabidopsis thaliana* rosette leaves according to the protocol established by Kruft *et al*. (2001), with few modifications. The entire procedure was performed at 4 °C in the presence of the protease inhibitor cocktail complex (Roche, Mannheim, Germany). The leaves were homogenised in 1 l of homogenisation buffer (100 mM Tris–HCl pH 7.6,

50 mM KCl, 10 mM magnesium acetate, 0.7 M sorbitol and 10 mM dithiothreitol) in a standard food processor by 5 bursts of 5 s each. The homogenate was filtered through two layers of muslin and then through two layers of Miracloth (Calbiochem, San Diego, CA, USA). The resulting filtrate was subjected to a 15 min centrifugation at $1200 \times g$ to remove cell debris and large organelles. The supernatant was then centrifuged a second time for 24 h at $45\,000 \times g$ to pellet the 80S ribosomes. The resulting pellet was rinsed and resuspended in a small volume ($\sim$500 μl) of resuspension buffer (50 mM Tris–Hcl pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, 0,5 M sucrose, 10 mM DTT and 10 mM KCO_3). This sample is referred to as the *crude* ribosome extract which was further purified to prepare the pure 80S fraction by layering it onto a 10–30% sucrose gradient in resuspension buffer, and centrifuging it in an SW28 rotor (Beckman) for 16 h at $26000 \times g$. The fraction containing the 80S ribosomes was collected and purified as described by Bommer *et al.* (1997). The purification of the 80S peak was verified by rerunning the purified 80S fraction on a sucrose gradient showing that a single high molecular weight complex was present in the preparation. In addition, the typical symmetric distribution of the ribosomal particle in the sucrose gradient indicated the likely homogeneity and integrity of the sample, which were further confirmed by the lack of a degradation pattern in the ribosomal proteins as observed in the 2D gels.

Protein extraction

For the protein extraction from the 80S ribosomal particles, 20 A_{260} units of ribosome sample were vortexed with 5 μl of 1 M magnesium acetate, 100 μl acetic acid and incubated on ice for 45 min. The sample was then centrifuged at $10\,000 \times g$, for 30 min at 4 °C. Of the supernatant, 150 μl was transferred to a new tube containing 750 μl of acetone (5x excess) and incubated overnight at −20 °C. Approximately 2 mg of proteins were pelleted at $10\,000 \times g$ for 45 min in a cooled 4 °C centrifuge and resuspended in a 100 μl volume of solubilisation buffer (2 M thiourea, 9 M urea, 2% (w/v) CHAPS, 10 mM DTT). After complete solubilisation a volume of 10 μl Servalyte® 2–4 (Serva, Heidelberg, Germany) was added to acidify the solution for the 2-DE separation.

2-DE separation and staining of 80S ribosomal proteins

Isoelectric focusing (IEF) was carried out according to Giavalisco *et al.* (2003) with some minor modifications. 20 μl protein samples, containing $\sim$500 μg total protein, were focused on 40 cm tube gels (diameter of 1.5 mm), containing 3.5% (w/v) acrylamide, 0.3% (w/v) bisacrylamide (Serva, Heidelberg, Germany) and 2% (v/v) of an ampholyte mixture. After isoelectric focusing the gels were ejected from the glass tube, cut into two 20 cm halves and applied to SDS-PAGE, which was performed on two $23.2 \times 30 \times 0.1$ cm 15% (w/v) acrylamide gels, containing 0,2% (w/v) bisacrylamide (Giavalisco *et al.*, 2003). After electrophoresis, the gels were stained with a colloidal coomassie G250 staining protocol according to Doherty *et al.* (Doherty *et al.*, 1998). In brief the gels were fixed in 50% (v/v) methanol, 2% (v/v) phosphoric acid for a minimum of 2 h. The gels were then equilibrated for 1 h in 1 l/gel of a solution containing 30% (v/v) methanol, 2% (v/v) phosphoric acid and 17% (w/v) ammonium sulphate before 660 mg crystalline coomassie G250 per gel was added. The staining proceeded for 3–5 days with constant agitation.

In situ *proteolysis of proteins isolated from 2-DE gels and protein identification by MALDI-TOF MS*

Protein spots were sampled from the 2-DE gels using an automatic spot excision workstation (Proteineer spII, Bruker Daltoniks, Bremen, Germany). The protocol for the tryptic in-gel digestion was based on the protocol of Shevchenko *et al.* (1996) with some modifications described in Giavalisco *et al.* (2004). The MALDI sample preparation was performed on MALDI AnchorChip targets (Bruker Daltoniks, Bremen, Germany), while the measurements were performed on an Autoflex (Bruker Daltoniks, Bremen, Germany) MALDI-TOF MS operated in reflector and delayed ion extraction mode as described by Giavalisco *et al.* (2004). Assignment of the first monoisotopic signals in the spectra was performed automatically using the signal detection algorithm SNAP (Bruker Daltonics, Bremen, Germany). We analysed positively charged ions in the m/z range 700–3500. Protein identification was performed using the MASCOT (Version 1.8.1) database search engine (Perkins *et al.*, 1999) with the following search

settings: 50 parts per million mass error tolerance, one permitted missed cleavage site and carbamidomethylation on cystein residues as complete- and methionine oxidations as variable modifications. The *Arabidopsis thaliana* MIPS database (http://mips.gsf.de) was employed for protein identification. A protein was considered identified if a score above the identification threshold of 59 with a minimum of five matching peptides or a sequence coverage greater than 30% was achieved.

Results

Separation of the crude and 80S ribosomal proteins using 2-DE

In this study, the preparation of the ribosomal particles from the *A. thaliana* whole leaf was performed using the protocol developed by Kruft *et al.* (2001), which is among one of the most common protocol used for the preparation of intact 80S plant ribosomes. The method employs a series of differential centrifugations, which lead to the preparation of a *crude* ribosomal fraction. This *crude* extract is then further purified using sucrose gradient centrifugation, from which a fraction containing the 80S ribosomes is recovered. The ribosomal proteins prepared from these two ribosomal fractions, hereafter referred to as *crude* ribosome extract and pure 80S fraction respectively, were then analysed using 2-DE.

In our 2-DE protein separation system we combined a non-equilibrium isoelectric focussing (IEF) step (O'Farrell *et al.*, 1977) with an extra large IEF gel (40 cm) (Klose and Kobalz, 1995) to overcome the problem of resolving the extremely basic ribosomal proteins (average *pI* of 10.7). Figure 1A and B demonstrate the protein separation achieved for the *crude* and 80S ribosomal protein fractions, allowing the separation of ~500–600 individual protein spots for each 2-DE gel.

In the acidic side of the 2-DE gel of the *crude* protein extract (left-hand side Figure 1B) several strong spots representing isoforms of the small and large subunit of the ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) (Spreitzer and Salvucci, 2002) are visible. Given that the enzyme rubisco is a large macromolecular complex formed by eight large and eight small subunits and is one of, if not the most, abundant complex in the leaves (Andersson and Taylor, 2003), their dominant presence in the *crude* ribosome fraction is not surprising. Because of the limited loading capacity of the IEF gel, these proteins, which make up a significant part of the crude protein extract, interfere with the detection of low-abundant ribosomal proteins. Further purification of the *crude* ribosome fraction by sucrose-gradient centrifugation reduced the abundance of these proteins, and as a consequence resulted in the detection of previously undetected molecules. One prominent group of proteins appeared on the acidic side of the gel (magnified region of Figure 1B), and was identified as belonging to the small ribosomal proteins P1, P2 and P3. Several new spots were also detected on the basic side of the gel (Figure 1B and 2), most of which represent ribosomal proteins.

In addition to the rubisco protein spots, a large number of protein spots with weak intensity were detected in the *crude* ribosome extract, that were either absent or present at a reduced level in the 80S ribosome fraction. These proteins were located mainly in the centre (pH 5–7) of the 2-DE gel suggesting that they are not ribosomal proteins, but rather proteins pelleted in the differential centrifugation step due to their presence in, or association with, large macromolecular complexes, such as enzymes complexes, membrane fragments or ribosomes. Passing the *crude* fraction over a sucrose gradient to obtain the *80S* ribosome fraction resulted in the loss of many of these proteins (compare Figure 1A and B), indicating that they were loosely ribosome-associated translation factors or simply contaminants. Nevertheless, the 2-DE spot patterns of both the *crude* extract and the *80S* fraction exhibited many similarities; it was therefore possible to compare the 2-DE gel images and to determine which spots were specific for the *crude* or for the *80S* fraction.

Identification of proteins from 2-DE gels

Identification of proteins separated by 2-DE was performed by MALDI-TOF MS peptide-mass fingerprinting (PMF) (Henzel *et al.*, 1993). For this purpose, 384 protein spots from the 2-DE gel of the 80S fraction were excised, digested *in situ* with trypsin and mass analysed by MALDI-TOF MS (Nordhoff *et al.*, 2001). Of these 384 proteins, 249 (65%) could be reliably identified. (The fully annotated 2-DE gels containing the complete

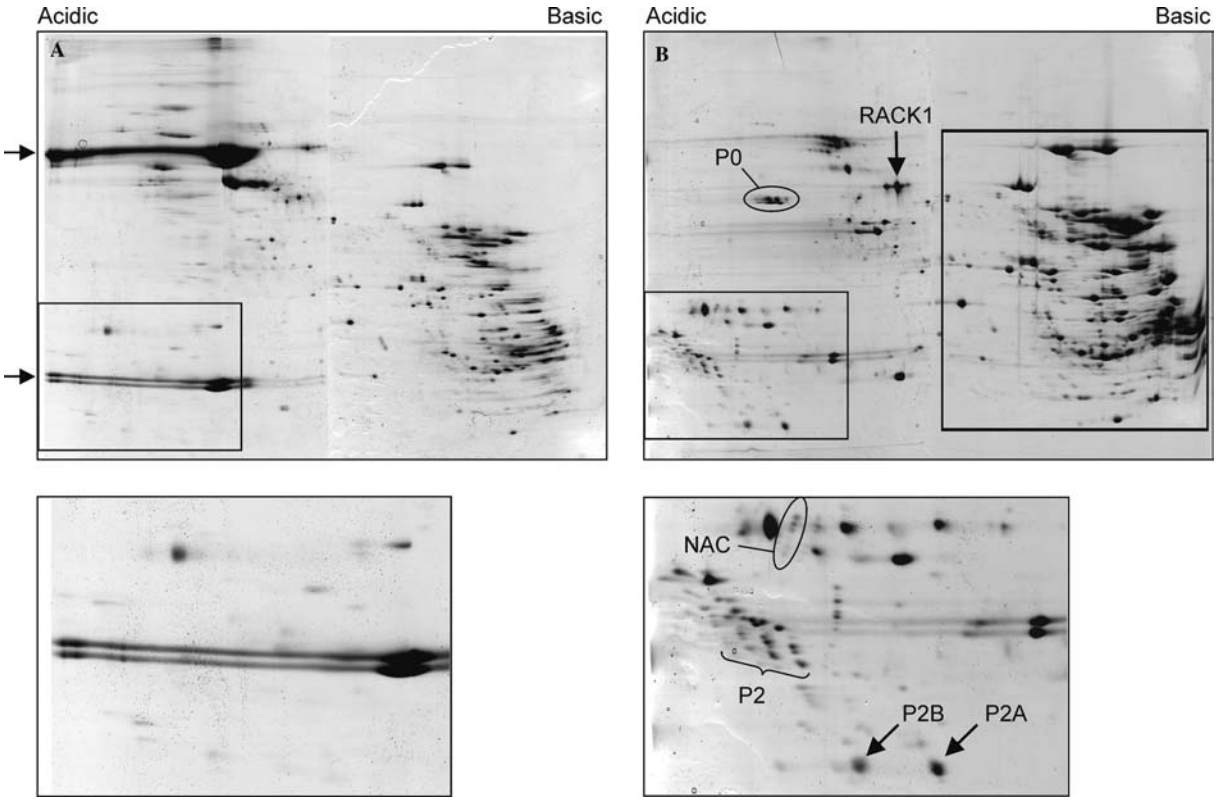

Figure 1. Separation of *crude* and pure *80S* ribosomal protein fractions by non-equilibrium IEF 2-DE. The *crude* (A) and pure *80S* (B) ribosomal protein fractions 2-DE gels stained using Coomassie blue are shown with acidic and basic ends as indicated. Arrows in (A, left side) mark the position of the rubsico subunits, ~50 kDa and ~20 kDa (upper and lower, respectively) which provide an indication for molecular weight. In (B) the protein spots identified as RACK1 and the stalk P0 proteins are indicated. The lower panels show magnifications of the low molecular weight acidic section of the *crude* (left) and pure (right) sections of the 2-DE gel. In the latter, the position of non-ribosomal protein NAC is indicated, as are the positions of the multiple isoforms for the P2 proteins, including the intense P2A and P2B protein spots. The MS data and assignments for these gels can be accessed at https://gabi.rzpd.de/cgi-bin/database/TwoDGel.pl.cgi by selecting either At 80S ribosome, crude or At 80S ribosome, pure from the drop-down menus, and clicking on the individual spots.

identification data as well as tables of all identified proteins can be directly accessed at https://gabi.rzpd.de/projects/Arabidopsis_Proteomics/). As can be seen in Table 1, the identified proteins could be classified into three major groups: The first and largest group contained 174 proteins associated to the 80S ribosomal complex, encompassing proteins of both the small- (40S) and large- (60S) subunit (MIPS database, http://mips.gsf.de/proj/thal/db/; Barakat *et al.*, 2001). In Table 2 the expected 80S ribosomal protein families from plants (MIPS database) are listed, together with the 87 proteins that were identified in this study. As can be seen from Table 2, the identified 80S ribosomal proteins cover 27 of the 32 expected protein families (84%) associated with the small 40S subunit, while 34 of the 48 expected 60S ribosomal subunit protein family members (71%) were successfully identified. Thus, in total 61 (76%) of the expected 80S ribosomal protein family members from *A. thaliana* were covered by our analysis.

There are several possible reasons why members from the missing 19 protein families could not be identified: Firstly, the molecular weight of six of these unidentified proteins is smaller than 10 kDa,

Table 1. Overview of all identified proteins from the pure 80S fraction.

Identified	Number	% of Total
80S ribosomal proteins	174	70
70S plastid ribosomal proteins	32	12
Other proteins	43	18
Total number	249	100

Table 2. Identified 80S ribosomal proteins[a].

Protein family [b]	Gene no.[c]	MW	PI	Proteins identified [d]	No. of spots identified [e]
Sa	2	32.2	5.05	At1g72370	1
S2	4	30.9	10.3	At2g41840	3
S3	3	27.5	9.57	At2g31610, At3g53870, At5g35530	6, 3, 2
S3a	2	29.9	10.6	At3g04840, At4g34670	3, 1
S4	4	29.9	10.18	At5g07090, At5g58420	2, 2
S5	2	23	9.69	At2g37270, At3g11940	4, 2
S6	2	28.2	10.83	–	–
S7	3	22	9.75	At1g48830, At3g02560, At5g16130	1, 1, 1
S8e	2	24.8	10.56	At5g59240, At5g20290	1, 3
S9	3	23.16	10.34	At5g15200	4
S10	3	27.1	10.5	At4g25740	2
S11	3	17.9	10.56	At3g48930	2
S12	3	15.3	5.62	At1g15930, At2g32060	3, 2
S13	2	17.1	10.39	At3g60770	1
S14	3	16.3	10.6	At3g11510	1
S15	6	17.1	10.3	At1g04270	1
S15a	6	14.8	9.89	At1g07770	3
S16	3	16.6	10.21	At2g09990	1
S17	4	16	10.04	At3g10610	5
S18	3	17.5	10.54	At1g34030	1
S19	3	15.8	10.14	At3g02080, At5g28060, At5g61170	1, 1, 1
S20	3	13.7	9.73	At3g47370	1
S21	3	9.5	9.06	–	–
S23	2	15.8	10.3	At5g02960	1
S24	2	15.8	10.14	At5g28060	1
S25	5	12.1	10.56	At4g39200	2
S26	3	14.8	11.09	At3g56340	1
S27	4	9.4	9.22	At2g45710, At5g47930	3, 3
S27a	3	17.67	9.72	–	–
S28	3	7.3	11.16	At3g10090	1
S29	4	6.4	10.07	–	–
S30	3	6.9	12.24	–	–
P0	3	33.6	4.93	At3g09200, At3g11250	2, 1
P1	3	11.2	4.1	–	–
P2	5	11.4	4.44	At2g27710, At2g27720	8, 6
P3	2	11.8	4.2	–	–
L3	3	44.5	10.12	At1g43170	4
L4	4	44.7	10.31	At3g09630, At5g02870	2, 2
L5	3	10.3	9.37	At3g25520, At4g01310, At5g39740	1, 5, 7
L6	3	26	10.18	At1g18540, At1g74060	4, 1
L7	4	28.2	9.94	At2g44120, At3g13580	1, 1
L7a	2	29.1	10.14	At3g62870	2
L8	3	27.9	10.91	At2g18020	2
L9	4	22	9.48	At1g33120	3
L10	3	24.9	10.5	At1g14320, At5g13510, At5g41520	2, 1, 1
L10a	3	24.4	9.89	At1g08360, At5g22440	1, 1
L11	4	20.9	9.94	At1g32990, At3g58700	2, 1
L12	3	17.9	9.06	At2g37190	1
L13	4	23.5	10.55	At3g49010, At5g23900	1, 1
L13a	4	23.5	10.41	At3g07110, At5g48760	1, 1
L14	2	15.5	10.12	At4g27090	3
L15	2	24.2	11.44	At4g16720	1

Table 2. (Continued).

Protein family [b]	Gene no.[c]	MW	PI	Proteins identified [d]	No. of spots identified [e]
L17	2	19.9	10.12	At1g67430	4
L18	3	20.9	11.62	At5g27850	1
L18a	3	21.3	11.12	At2g34480, At3g14600	1, 2
L19	3	21.8	4.73	–	–
L21	6	18.6	10.46	At1g09590	3
L22	3	14.5	9.76	At3g05560	3
L23	3	15	10.48		
L23a	2	17.4	10.2	At2g39460	1
L24	2	18.9	10.7	At3g49910	1
L26	2	16.8	11.13	–	–
L27	3	15.5	10.19	–	–
L27a	3	16.5	10.59	At1g23290	2
L28	3	15.9	10.58	At2g19730	1
L29	2	7	11.39	–	–
L30	3	12.3	9.8	At1g77940	1
L31	3	13.7	9.95	At5g56710	1
L32	2	15.5	10.89	–	–
L34	3	13.7	11.6	–	–
L35	4	14.2	10.92	At2g39390	2
L35a	4	13.7	11.6	–	–
L36	3	12.8	10.68	At3g55750	1
L36a	2	12.7	11.74	At3g53740	1
L37	3	10.8	11.86	At1g52300	1
L37a	3	10.4	10.34	–	–
L38	2	8.1	9.95	At2g43460	2
L39	3	6.4	12.31	–	–
L40	2	14.7	9.94	–	–
L41	7	3.4	12.91	–	–

[a]Summarized information on 2-DE gels of ribosomal proteins is available at https://gabi.rzpd.de/projects/Arabidopsis_ Proteomics.
[b]Ribosomal protein family.
[c]Number of genes predicted to exist for corresponding protein family.
[d]Gene of the proteins identified from the 2-DE gel (*Arabidopsis thaliana* MIPS database, http://mips.gsf.de).
[e]Number of independent spots identified for each protein family. The symbol "–" indicates that no members of the protein family were identified.

which in combination with pI values above 11, is close to or even beyond the working range of 2-DE gel systems. Secondly, most of these relatively low molecular weight proteins (<20 kDa) were predicted to yield only small numbers of peptides from proteolytic digestion with trypsin. For these proteins, identification by PMF often fails since at least 3–5 matching peptides are required for an unambiguous identification (Jensen *et al*., 1996). This problem has also been observed in a previously published yeast proteome project, where the small and basic proteins such as L41, L40 and L29 were not detected (Link *et al*., 1999). Thirdly, some proteins are likely to be heavily modified and therefore the masses of many of their tryptic peptides do not correlate with the expected primary sequence, making their identification using standard PMF impossible. The last explanation is probably valid for the ribosomal protein S6, which could not be identified in our ribosome preparation, although it is a sufficiently large protein (28 kDa). This protein is known to be phosphorylated at multiple sites in eukaryotes (Wettenhall and Morgan, 1984), a finding that was recently confirmed for a corresponding S6 protein in *A. thaliana* (Turck *et al*., 1998).

The second group of proteins identified from the 80S fraction (Table 1) contained 32 protein spots that were associated with the plastid (Yamaguchi and Subramanian, 2000; Yamaguchi *et al*., 2000) 70S ribosome (MIPS database), whereas the third group (Table 1) comprised 43 protein spots of

Table 3. Non-ribosomal proteins identified in the *crude* and the pure *80S* fractions[a].

Acc. no.[b]	Description	Gel[c]
At1g01120	Putative elongase	CB
At1g03090	Putative 3-methylcrotonyl-CoA carboxylase	CA
At1g07610	Metallothionein-like protein	CA
At1g09220	Protein T12M4.7 (unclassified)	CB
At1g11860	Aminomethyltransferase	CA
At1g16470	Proteasome subunit alpha type 2	CA
At1g20620	Catalase 3	CA
At1g20630	Catalase 1	CA
At1g27390	Putative protein import receptor	2x CB
At1g56450	Putative protein (proteasome subunit Z)	CA
At1g66200	Probable glutamine synthetase	CA
At1g67030	Putative protein (polygalacturonase)	CA
At2g21330	Probable fructose bisphosphate aldolase	CA
At2g28000	RUBISCO Subunit binding protein alpha subunit (Chaperonin 60 alpha chain)	CA
At3g09440	Heat-shock cognate 70kDa protein 3	CA
At3g09840	Cell division cycle protein 48	CA
At3g13470	GloEL protein; chaperonin	2x CA
At3g14290	Proteasome subunit alpha type 5-2	CA
At3g14410	Probable (S)-2-hydroxy-acid oxidase	CA
At3g14420	Glycolate oxidase like protein	CA
At3g22110	Proteasome subunit alpha type 4	CA
At3g51260	Proteasome subunit alpha type 7-1	2x CA
At3g62030	Peptidylprolyl isomerase ROC4	2x CA
At4g20850	Hypothetical protein T13K14.10 (tripeptidyl-peptidase II)	CA
At4g35090	Catalase 2	2x CA
At4g37930	Glycine hydroxymethyltransferase	CA
At5g02500	Heat shock cognate 70-1	CA
At5g25980	Myrosinase TGG2	3x CA
At5g26000	Myrosinase precursor (sinigrinase) thioglucosidase	CA
At5g35630	Glutamine synthetase	2x CA
At5g37600	Glutathionie S-transferase PM24	2x CA
At5g38420	Large subunit of RUBISCO	3x CA
At5g55070	2-oxoglutarate dehydrogenase E2 subunit	CB
At1g07320	Unknown protein	3x PA
At1g34430	Dihydrolipoamide S-acetyltransferase	PA
At1g55490	Rubisco subunit binding-protein beta subunit	PA
At2g07670	putative non-LTR retrolelement reverse transcriptase	PB
At3g12390	Contains similarity to alpha nascent polypeptide associated complex (NAC)	PA
At3g15200	Hypothetical protein (PPR repeat containing protein)	PB
At3g18080	Beta-glucosidase, putative	2x PB
At3g25860	Dihydrolipoamide S-acetyltransferase	PA
At4g11830	Phospholipase D-gamma-2	PB
At4g25090	Respiratory burst oxidase -like protein (cytochrome b245 homolog)	PB
At5g13850	Contains similarity to nascent polypeptide associated complex (NAC)	PA
At5g24770	Vegetative storage protein Vsp2	PA
At5g38410	RUBISCO small chain 3b precursor	2x PA
At5g55990	Calcineurin B-like protein 2	PA
At1g18080	RACK1 homologue	CA, 2x PB, 3x PA
At1g67090	RUBISCO small chain A1 precursor	2x CA, 4x PA
At3g14210	Lipase/acylhydrolase; myrosinase-associated protein	2x CA, 5x PA
At3g23990	Mitochondrial chaperonin hsp60	CA, PA
AtCg00490	Large subunit of RUBISCO	11x CA, 12x PA

Table 3. (Continued).

[a]Summarized information on 2-DE gels of ribosomal proteins is available at https://gabi.rzpd.de/projects/Arabidopsis_Proteomics/
[b]Gene code accession number of the identified protein (MIPS database, http://mips.gsf.de/proj/thal/db/).
[c]Gel in which the protein was identified from spots on the acidic or basic halves of the 2-DE gels of the *crude* (CA, CB) or pure 80S (PA, PB) fractions, as well as the number of times each protein was identified on the respective gel half.

diverse nature that are not considered to be ribosomal components. In contrast, from the 2-DE gel of the *crude* extract, 143 of the 192 excised protein spots (75%) were identified, of which 61 (43%) were non-ribosomal proteins (Table 3). The major group of these non-ribosomal proteins was, as expected, related to the rubisco complex, from which we identified three different rubisco subunits (two isoforms of the large subunit and one of the small) as well as a rubisco subunit binding protein. In addition, two other classes of non-ribosomal proteins were identified. These included chaperones, such as a mitochondrial heat shock proteins Hsp 60, two different Hsp 70 proteins (Bukau and Horwich, 1998) and a peptidyl-isomerase (Gothel and Marahiel, 1999). Apart from the mitochondrial Hsp 60 protein, these proteins were detected only in the *crude* 2-DE gel. Both, the heat shock proteins and the peptidyl-isomerase, are involved with the folding of newly translated proteins (Bukau and Horwich, 1998; Craig *et al*., 2003) and therefore their presence in the *crude* sample may reflect their transient association with the ribosome. Indeed, in *E. coli* a peptidyl-isomerase called the trigger factor has been shown to interact with the ribosome in close proximity to the nascent chain exit site (Blaha *et al*., 2003; Ferbitz *et al*., 2004). Lastly, six different proteins associated with the 26S proteasome (Parmentier *et al*., 1997; Fu *et al*., 1999) were identified from the *crude* extract. The ubiquitin/26S proteasome pathway is one of the most elaborate regulatory mechanisms in *A. thaliana*, being composed of >1300 genes and representing almost 5% of the proteome (Vierstra, 2003). The large size of the proteasome complex (~2 MDa) accounts for its presence in the *crude* fraction whereas its sedimentation coefficient of 26S explains why none of the proteins contaminate the purified 80S fraction.

Detecting proteins that associate with the plant ribosome

In order to identify proteins that putatively associate with the 80S plant ribosome we examined the non-ribosomal proteins identified from the 2-DE gel of the 80S fraction that were not present in the *crude* ribosomal extract. The number of these proteins was relatively low and they were localised mainly on the acidic side of the 2-DE gel. Of these identified non-ribosomal protein spots, 14 were unique for the 80S fraction (Table 3) indicating that they are either associated with the ribosome or that they are part of a complex with the same density of the 80S particle. As mentioned previously, a large proportion of the non-ribosomal proteins identified in both the *crude* extract and 80S fraction were represented by the large and small subunit of the rubisco complex, except for the beta-chain of the rubisco-subunit binding protein, an Hsp 60 chaperonin implicated in assembly of the rubisco complex (Spreitzer and Salvucci, 2002), which was only identified from the 80S fraction. This chaperonin is proposed to form large oligomers composed of 6 alpha and 6 beta chains, totalling to over 700 kDa (Gutteridge and Gatenby, 1995), which when bound to abundant rubisco complex (~550 kDa) could explain how it can contaminate the 80S fraction.

From the rest of the non-ribosomal proteins (Table 3), an eclectic mix of proteins was identified, the majority of which had on average a very low abundancy (<5% of the average spot intensity compared to the 80S ribosomal proteins), suggesting they are simply minor contaminants of the 80S peak. Proteins such as the respiratory burst oxidase-like protein (a cytochrome b245 beta chain homolog), the phospholipase D-gamma-2 and the hypothetical lipase may be present in the 80S peak due to their possible association with membrane complexes, whereas other proteins such as the reverse transcriptase, the two hydrolases (β-glucosidase and calcineurin), the vegetative storage protein (VSP2), the mitochondrial Hsp 60 as well as an unknown protein (At1g07320) may be components of large macromolecular complexes, accounting for their co-sedimentation with the 80S peak. Indeed, the identification of dihydrolipoamide S-acetyltransferase in our 80S peak was not surprising since this protein is a known subunit of the multi-enzyme pyruvate dehydrogenase complex

and has been shown to form 28 nm pentagonal dodecahedrons of ~2.7 MDa (Thelen *et al*., 1999).

The identification of two protein spots (out of a group of approximately seven) in close vicinity and with similar intensities to some of the ribosomal stalk proteins was more convincing for being associated with the ribosome. These two spots were identified as being a subunit of the hetero-dimeric nascent-polypeptide associated complex (NAC), a protein that has previously been shown to interact with the large subunit of the ribosome (Wiedmann *et al*., 1994). A further collection of non-ribosomal protein spots on the acidic section of the 2-DE gel was exceptional in that their intensities increased proportional to the increase of the intensities observed for the ribosomal proteins between the *crude* and the pure 80S fraction (as can be seen in Figure 1), indicating that these proteins might be factors associated with the ribosomal complex. These spots were identified as having high sequence similarity to the mammalian RACK1 protein. RACK1 homologues have been found in all eukaryotes, but not in bacteria, and were initially characterized as being a receptor for protein kinase C. However, recent evidence suggests that RACK1 associates with yeast and human 80S ribosomes (Link *et al*., 1999), binding specifically to the head of the 40S subunit (Sengupta *et al*., 2004).

The high heterogeneity of the plant 80S ribosome

A striking observation resulting from our analysis is that many of the unique protein sequences identified in the database are represented by more than one protein spot on the 2-DE gel. Of the 87 different ribosomal protein sequences that were identified, 45% were represented by 2–13 distinct protein spots (as illustrated in Figure 2). This implies that there are multiple different forms for many of the ribosomal proteins present in our ribosome population. Indeed, numerous ribosomal proteins in bacteria, rat, human and yeast have been found to be methylated, acetylated and/or phosphorylated (Louie *et al*., 1996; Vladimirov *et al*., 1996; Arnold and Reilly, 1999; Arnold and Reilly, 2002; Lee *et al*., 2002; Odintsova *et al*., 2003). We observe multiple protein spots of varying molecular weights and with different pI values from the 80S fraction derived 2-DE gel (Figure 2), which can also be indicative for several different transcriptional, translational or even post-translational modifications, such as alternative splicing (Smith *et al*., 1989; Brett *et al*., 2002), specific chemical amino acid modifications (Krishna and Wold, 1993) as well as directed protein degradation (Callis and Vierstra, 2000; Estelle, 2001). Protein degradation however can be excluded as the main reason for the large number of spots encountered in this study for most of the ribosomal protein. This kind of degenerative event would in fact affect most proteins from the sample giving rise to a spot pattern characterised by a loss of high molecular weight proteins, accumulation of low molecular weight proteins and smearing of protein spots in the vertical axis. In comparison, the general tendency of the ribosomal proteins is very different, since the various spots belonging to a distinct gene, are generally distributed evenly in the horizontal direction (Figure 2), indicating a change only in their pI and not in their MW. An exception is the slightly different pattern observed for the ribosomal proteins P1, P2 and P3, which are collectively referred to as stalk proteins because they form a stalk-like protrusion on the large subunit (reviewed by Ballesta and Remacha, 1996) and have been reported to be modified in yeast (Remacha *et al*., 1995). This is seen particularly for the spot identified as P2 (compare for example the spots for the P2 protein (caption in Figure 1B) with the ones for the S27, L11 or L5 proteins (Figure 2)). In the case of the P2 protein, a clear MW laddering of the P2 spots can be observed, indicating a possible specific degradation of this protein.

A further factor contributing to the high heterogeneity of the 80S ribosome and a peculiar feature of plant genomes, especially for *A. thaliana*, is the large number of genes (~250) encoding for the 80 ribosomal protein families, such that each ribosomal protein family is encoded by 2–7 different genes. The L41 protein family, for example is encoded by seven different genes, while the protein families L21, S15, S15a are encoded each by six different genes (Barakat *et al*., 2001). What is perhaps even more surprising is that expressed sequence tags (ESTs) for almost 200 of these ribosomal genes could be identified (Barakat *et al*., 2001) suggesting that the ribosome population is very heterogeneous within the plant cell. Indeed there is evidence suggesting that the composition of the ribosome may vary depending on the tissue type and the state of development (Barakat *et al*., 2001 and references therein).

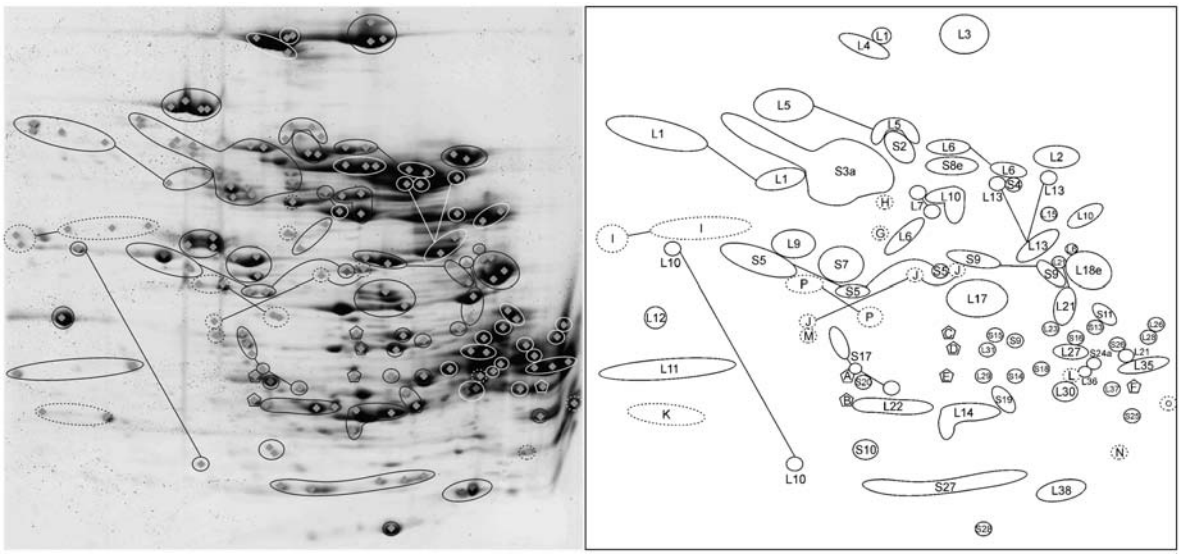

Figure 2. High heterogeneity within the *Arabidopsis thaliana* 80S ribosomes. Magnification of the 2-DE gel, basic section of the pure *80S* fraction (left) with a schematic representation of the protein spots from the gel (right). In the schematic representation the identified 80S ribosomal proteins are indicated in or near the circled areas. Hashed regions indicate chloroplast or mitochondrial ribosomal proteins and hexagonal regions indicate the presence of non-ribosomal proteins, the correlating proteins for each letter are given as follows: A, RACK1; B, Phospholipase D-gamma-2; C, RACK1; D, Cytochrome b245 beta chain homolog; E, reverse transcriptase; F, PPR repeat protein; G-P are chloroplast ribosomal proteins S4, L3, L5, L6, L13, L14, L20, L21, L23, L28 and L28.

Our 2-DE and MS-based results confirm and characterize the large heterogeneity of the *A. thaliana* cytoplasmic ribosome at the protein level, since for at least 21 of the 61 (34%) ribosomal protein families, we unambiguously identified two or more distinct proteins (Table 2). An example for the diversification of protein families is represented by the stalk proteins (magnification Figure 1B and Supplementary Table 1). In our 2-DE gels, derived from the 80S fraction, we observe multiple protein spots within the MW range of 10–12 kDa and pI range between 4–5, which are characteristic for the stalk proteins P1, P2 and P3 (magnification Figure 1B). Interestingly from a total of 14 different protein spots identified within this region we could only annotate two of the five different P2 isoform, without detecting any P1 or P3 proteins (Table 2). The ability to distinguish between different isoforms of the ribosomal proteins using MALDI-TOF MS-PMF is illustrated in Supplementary Table 1 for the P2A and P2B isoforms: Although these isoforms have a sequence identity of 94%, the seven amino acid differences are sufficient to allow the generation of a significant number of unique tryptic peptides.

Discussion

This study presents the first characterisation of the cytoplasmic 80S ribosomal proteins from the leaves of *A. thaliana*. Using the classic techniques of differential and sucrose gradient centrifugation, along with 2-DE and MALDI-TOF MS, we have identified 61 of the 80 ribosomal protein families predicted to comprise the cytoplasmic 80S ribosome from *A. thaliana*. From this analysis we observe a large heterogeneity within the ribosome population, since on average each protein is found in four different forms, indicating a high degree of post-translational modifications and a large number of expressed isoforms within the ribosomal protein families. These reflect the large-scale duplication within the *A. thaliana* genome and show that many of the large number of isoforms within each ribosomal protein family are in fact expressed and incorporated into the fully assembled cytoplasmic 80S ribosomes.

The heterogeneity in the ribosomal proteins encountered in this study, has been already observed for the ribosomal proteins of the few other organism studied so far. Interestingly, a comparison of the results indicates that the ribosomal

proteins of *A. thaliana*, are modified to a much larger extent, including for some protein families more than 10 different forms (e.g. for L5 and S3, Table 2), compared to the few modifications observed for each ribosomal protein of the other organism analysed (e.g *Escherichia coli*, (Wittmann-Liebold, 1986); yeast, (Link *et al.*, 1999; Lee *et al.*, 2002); rat, (Wool *et al.*, 1995; Louie *et al.*, 1996); human, (Vladimirov *et al.*, 1996; Odintsova *et al.*, 2003); spinach, (Yamaguchi and Subramanian, 2000; Yamaguchi *et al.*, 2000) and *Chlamydomonas reinhardtii*, (Yamaguchi *et al.*, 2003)).

An interesting model to explain the necessity and/or function of such heterogeneity in the ribosome population has been put forward by Mauro and Edelman (2002), suggesting that particular sets of ribosomal proteins or rRNA, could favour the translation of specific mRNAs. The ribosome in this respect becomes an important player for the regulation of the cellular environment. In our opinion, a further possibility of the specific use of a particular set of ribosomal proteins is the facilitation of interactions with additional factors, which again can regulate the ribosome activity depending on particular cell conditions.

Among the various isoforms observed in this study, the ribosomal stalk proteins are prime candidates for this type of translation regulation. These proteins are known to be necessary for the correct functioning of most of the translational factors (reviewed by Liljas and Gudkov, 1987; Ballesta and Remacha, 1996), so much so that if exchanged between prokaryotes and eukaryotes, to function, the ribosome will require the corresponding elongation factor from the same species used for the stalk proteins (Uchiumi *et al.*, 2002). Furthermore, the exact composition of the ribosomal stalk and the ratio of P-proteins within the stalk has been shown to be highly variable and developmentally regulated (Szick-Miranda and Bailey-Serres, 2001; Hanson *et al.*, 2004), leading to the suggestion that the composition of the stalk may be an important regulator of translation (Ballesta and Remacha, 1996).

Finally, translation regulation is under the control of multiple protein factors that interact with the ribosome at various stages of protein synthesis and under different environmental conditions (Wilson and Nierhaus, 2003). New factors that participate in this form of translation regulation are being discovered each year, for many of which the function still remains to be conclusively determined. This study has identified a number of such potential proteins, including amongst them, NAC and RACK1 homologues. NAC binds to the ribosome in the vicinity of the tunnel exit of the large subunit and modulates co-translational targeting of polypeptides (Wiedmann *et al.*, 1994). The peptide mass fingerprints acquired from the protein samples revealed the presence of at least two isoforms of alpha-NAC and the 2-DE gel pattern indicated that they may be post-translationally modified.

RACK1 is a newly discovered ribosomal protein, present in all eukaryotic genomes and has so far been demonstrated to be present in the small subunit of yeast and human ribosomes (Link *et al.*, 1999; Ceci *et al.*, 2003; reviewed by Nilsson *et al.*, 2004). RACK1 has been shown to interact with a number of different signalling molecules, such as Src, intergrin-β and PKC, and through this interaction is thought to mediate translation regulation. In yeast RACK1 has been found to control translation initiation in an mRNA specific manner and is present in the cell in differently modified forms (Shor *et al.*, 2003). This latter point is supported by our identification of RACK1 in multiple distinct spots with varying pI values but similar molecular masses. Additionally, we observed two weaker gel spots, which were identified as RACK1 homologues, in the basic pI range (pI ~9–10) despite the prediction of pI 7.6. Although much remains to be understood about the role of RACK1 in translation, the fact that we find it associated with the *A. thaliana* ribosome, suggests that the translational regulation pathways operated through this protein in yeast, could also be present in higher plants.

Acknowledgements

We would like to thank Dr Klaus-Dieter Klöppel for his constant support with the MALDI-TOF MS instruments and Renate Albrecht, Jörg Bürger, Beata Lukaszewka, Barbara Schmidt and Janine Stuwe for their excellent technical assistance. We would also like to thank Dr Svenja Meyer from the resource centre Berlin for providing the proteomics web page. A special thanks is to Prof Carol Robinson, Prof Knud Nierhaus and Dr Sean Connell for helpful and stimulating

discussions. The project was funded by the German plant genome project: Genome Analysis in the Biological System Plant (GABI) support by the German Ministry of Education and Research (BMBF/GABI Project 32P2244) and the Max Planck Society.

References

Andersson, I. and Taylor, T.C. 2003. Structural framework for catalysis and regulation in ribulose-1,5-bisphosphate carboxylase/oxygenase. Arch. Biochem. Biophys. 414: 130–140.

Arnold, R.J. and Reilly, J.P. 1999. Observation of *Escherichia coli* ribosomal proteins and their posttranslational modifications by mass spectrometry. Anal. Biochem. 269: 105–112.

Arnold, R.J. and Reilly, J.P. 2002. Analysis of methylation and acetylation in *E. coli* ribosomal proteins. Methods Mol. Biol. 194: 205–210.

Ballesta, J.P. and Remacha, M. 1996. The large ribosomal subunit stalk as a regulatory element of the eukaryotic translational machinery. Prog. Nucleic Acid Res. Mol. Biol. 55: 157–193.

Ban, N., Nissen, P., Hansen, J., Moore, P.B. and Steitz, T.A. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science 289: 905–920.

Barakat, A., Szick-Miranda, K., Chang, I.-F., Guyot, R., Blanc, G., Cooke, R., Delseny, M. and Bailey-Serres, J. 2001. The organisation of cytoplasmic ribosomal protein genes in the arabidopsis genome. Plant Physiol. 127: 398–415.

Blaha, G., Wilson, D.N., Stoller, G., Fischer, G., Willumeit, R. and Nierhaus, K.H. 2003. Localization of the trigger factor binding site on the ribosomal 50S subunit. J. Mol. Biol. 326: 887–897.

Blanc, G., Barakat, A., Guyot, R., Cooke, R. and Delseny, M. 2000. Extensive duplication and reshuffling in the Arabidopsis genome. Plant Cell 12: 1093–101.

Bommer, U.-A., Burkhardt, N., Jünemann, R., Spahn, C.M.T., Triana, F. and Nierhaus K.H. 1997. Ribosomes and Polysomes Cell Fractionation: A Practical Approach, IRL Press at Oxford University Press, Oxford. pp. 271–301.

Brett, D., Pospisil, H., Valcárcel, J., Reich, J. and Bork, P. 2002. Alternative splicing and genome complexity. Nat. Genet. 30: 29–30.

Bukau, B. and Horwich, A.L. 1998. The Hsp70 and Hsp60 chaperone machines. Cell 92: 351–366.

Callis J. and Vierstra, R.D. 2000. Protein degradation in signaling. Curr. Opin. Plant Biol. 3: 381–386.

Capel, M.S. and Bourque, D.P. 1982. Characterization of *Nicotiana tabacum* chloroplast and cytoplasmic ribosomal proteins. J. Biol. Chem. 257: 7746–7755.

Ceci, M., Gaviraghi, C., Gorrini, C., Sala, L.A., Offenhäuser, N., Marchisio, P.C. and Biffo, S. 2003. Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. Nature 426: 579–584.

Craig, E.A., Eisenman, H.C. and Hundley, H.A. 2003. Ribosome-tethered molecular chaperones: the first line of defense against protein misfolding? Curr. Opin. Microbiol. 6: 157–162.

Doherty, N.S., Littman, B.H., Reilly, K., Swindell, A.C., Buss, J.M. and Anderson N.L. 1998. Analysis of changes in acute-phase plasma proteins in an acute inflammatory response and in rheumatoid arthritis using two-dimensional gel electrophoresis. Electrophoresis 19: 355–363.

Estelle, M. 2001. Proteases and cellular regulation in plants. Curr. Opin. Plant Biol. 4: 254–260.

Ferbitz, L., Maier, T., Patzelt, H., Bukau, B., Deuerling, E. and Ban, N. 2004. Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins. Nature 431: 590–596.

Fu, H.Y., Girod, P.A., Doelling, J.H., van Nocker, S., Hochstrasser, M., Finley, D. and Vierstra, R.D. 1999. Structure and functional analyses of the 26S proteasome subunits from plants – Plant 26S proteasome. Mol. Biol. Rep. 26: 137–146.

Gantt, J. and Key, J. 1985. Coordinate expression of ribosomal protein mRNAs following auxin treatment of soybean hypocotyls. J. Biol. Chem. 260: 6175–6181.

Giavalisco, P., Nordhoff, E., Lehrach, H., Gobom, J. and Klose J. 2003. Extraction of proteins from plant tissues for two-dimensional electrophoresis analysis. Electrophoresis 24: 207–216.

Giavalisco, P., Nordhoff, E., Kreitler, T., Klöppel, K.D., Lehrach, H., Klose, J. and Gobom, J. 2004. Proteome Analysis of *Arabidopsis thaliana* by 2-D Electrophoresis and Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry. Proteomics *accepted*

Gobom, J., Mueller, M., Egelhofer, V., Theiss, D., Lehrach, H. and Nordhoff, E. 2002. A calibration method that simplifies and improves accurate determination of peptide molecular masses by MALDI-TOF MS. Anal. Chem. 74: 3915–3923.

Gobom, J., Schuerenberg, M., Mueller, M., Theiss, D., Lehrach, H. and Nordhoff, E. 2001. Alpha-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. Anal. Chem. 73: 434–438.

Gothel, S.F. and Marahiel, M.A. 1999. Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. Cell. Mol. Life Sci. 55: 423–436.

Gutteridge, S. and Gatenby, A. 1995. Rubisco synthesis, assembly, mechanism, and regulation. Plant Cell 7: 809–819.

Hanson, C.L., Videler, H., Santos, C., Ballesta, J.P. and Robinson, C.V. 2004. Mass spectrometry of ribosomes from Saccharomyces cerevisiae: implications for assembly of the stalk complex. J. Biol. Chem. 279: 42750–42757.

Harms, J., Schluenzen, F., Zarivach, R., Bashan, A., Gat, S., Agmon, I., Bartels, H., Franceschi, F. and Yonath, A. 2001. High resolution structure of the large ribosomal subunit from a mesophilic eubacterium. Cell 107: 679–688.

Henzel, W.J., Billeci, T.M., Stults, J.T., Wong, S.C., Grimley, C. and Watanabe, C. 1993. Identifying proteins from two-dimensional gels by molecular mass searching of peptide fragments in protein sequence databases. Proc. Natl Acad. Sci. USA 90: 5011–5015.

Initiative, The Arabidopsis Genome. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815.

Jensen, O.N., Podtelejnikov, A. and Mann, M. 1996. Delayed extraction improves specificity in database searches by matrix-assisted laser desorption/ionization peptide maps. Rapid Commun. Mass Spectrom 10: 1371–1378.

Klose, J. and Kobalz, U. 1995. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. Electrophoresis 16: 1034–1059.

Krishna, R.G. and Wold, F. 1993. Post-translational modifications of proteins. Adv. Enzymol. Relat. Mol. Biol. 67: 265–298.

Kruft, V., Eubel, H., Jansch, L., Werhahn, W. and Braun, H.P. 2001. Proteomic approach to identify novel mitochondrial proteins in Arabidopsis. Plant Physiol. 127: 1694–1710.

Lee,S.W., Berger,S.J., Martinovic, S., Pasa-Tolic, L., Anderson, G.A., Shen, Y., Zhao, R. and Smith, R.D. 2002. Direct mass spectrometric analysis of intact proteins of the yeast large ribosomal subunit using capillary LC/FTICR. Proc. Natl Acad. Sci. USA 99: 5942–5947.

Liljas, A. and Gudkov, A.T. 1987. The structure and dynamics of ribosomal protein L12. Biochimie 69: 1043–1047.

Link, A.J., Eng, J., Schieltz, D.M., Carmack, E., Mize, G.J., Morris, D.R., Garvik, B.M. and Yates, J.R. 1999. Direct analysis of protein complexes using mass spectrometry. Nat. Biotechnol. 17: 676–682.

Louie, D.F., Resing, K.A., Lewis, T.S. and Ahn, N.G. 1996. Mass spectrometric analysis of 40S ribosomal proteins from Rat-1 fibroblasts. J. Biol. Chem. 271: 28189–28198.

Mauro, V.P. and Edelman, G.M. 2002. The ribosome filter hypothesis. Proc. Natl Acad. Sci. USA 99: 12031–12036.

Nilsson, J., Sengupta, J., Frank, J. and Nissen, P. 2004. Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep. 5: 1137–1141.

Nordhoff, E., Egelhofer, V., Giavalisco, P., Eickhoff, H., Horn, M., Przewieslik, T., Theiss, D., Schneider, U., Lehrach, H. and Gobom, J. 2001. Large-gel two-dimensional electrophoresis-matrix assisted laser desorption/ionization-time of flight-mass spectrometry: an analytical challenge for studying complex protein mixtures. Electrophoresis 22: 2844–2855.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551–3567.

Odintsova, T.I., Muller, E.C., Ivanov, A.V., Egorov, T.A., Bienert, R., Vladimirov, S.N., Kostka, S., Otto, A., Wittmann-Liebold, B. and Karpova, G.G. 2003. Characterization and analysis of posttranslational modifications of the human large cytoplasmic ribosomal subunit proteins by mass spectrometry and Edman sequencing. J. Protein Chem. 22: 249–258.

O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133–1141.

Parmentier, Y., Bouchez, D., Fleck, J. and Genschik, P. 1997. The 20S proteasome gene family in Arabidopsis thaliana. FEBS Lett. 416: 281–285.

Remacha, M., Jimenez-Diaz, A., Bermejo, B., Rodriguez-Gabriel, M.A., Guarinos, E. and Ballesta, J.P. 1995. Ribosomal acidic phosphoproteins P1 and P2 are not required for cell viability but regulate the pattern of protein expression in Saccharomyces cerevisiae. Mol. Cell. Biol. 15: 4754–4762.

Schluenzen, F., Tocilj, A., Zarivach, R., Harms, J., Gluehmann, M., Janell, D., Bashan, A., Bartels, H., Agmon, I., Franceschi, F. and Yonath, A. 2000 Structure of functionally activated small ribosomal subunit at 3.3 angstroms resolution. Cell 102: 615–623.

Sengupta, J., Nilsson, J., Gursky, R., Spahn, C., Nissen, P. and Frank, J. 2004 Identification of the versatile scaffold protein RACK1 on the eukaryotic ribosome by cryo-EM. Nat. Struct. Mol. Biol. 11: 957–962.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. 1996. Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. Anal. Chem. 68: 850–858.

Shor, B., Calaycay, J., Rushbrook, J. and McLeod, M. 2003. Cpc2/RACK1 is a ribosome-associated protein that promotes efficient translation in *Schizosaccharomyces pombe*. J. Biol. Chem. 278: 49119–49128.

Smith, C.W.J., Patton, J.G. and Nadal-Ginard, B. 1989. Alternative splicing in the control of gene expression. Annu. Rev. Genet. 23: 527–577.

Spreitzer, R.J. and Salvucci, M.E. 2002. Rubisco: Structure, regulatory interactions, and possibilities for a better enzyme. Annu. Rev. Plant Biol. 53: 449–475.

Szick-Miranda, K. and Bailey-Serres, J. 2001. Regulated heterogeneity in 12-kDa P-protein phosphorylation and composition of ribosomes in maize (*Zea mays* L.). J. Biol. Chem. 276: 10921–10928.

Thelen, J.J., Muszynski, M.G., David, N.R., Luethy, M.H., Elthon, T.E., Miernyk, J.A. and Randall, D.D. 1999. The dihydrolipoamide S-acetyltransferase subunit of the mitochondrial pyruvate dehydrogenase complex from maize contains a single lipoyl domain. J. Biol. Chem. 274: 21769–21775.

Turck, F., Kozma, S., Thomasm, G. and Nagy, F. 1998. A heat-sensitive Arabidopsis thaliana kinase substitutes for human p70s6k function *in vivo*. Mol. Cell. Biol. 18: 2038–2044.

Uchiumi, T., Honma, S., Nomura, T., Dabbs, E.R. and Hachimori, A. 2002. Translation elongation by a hybrid ribosome in which proteins at the GTPase center of the *Escherichia coli* ribosome are replaced with rat counterparts. J. Biol. Chem. 277: 3857–3862.

Vierstra, R.D. 2003. The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. 8: 135–142.

Vladimirov, S.N., Ivanov, A.V., Karpova, G.G., Musolyamov, A.K., Egorov, T.A., Thiede, B., Wittmann-Liebold, B. and Otto, A. 1996. Characterization of the human small-ribosomal-subunit proteins by N-terminal and internal sequencing, and mass spectrometry. Eur. J. Biochem. 239: 144–149.

Wettenhall, R. and Morgan, F. 1984. Phosphorylation of hepatic ribosomal protein S6 on 80 and 40S ribosomes. Primary structure of S6 in the region of the major phosphorylation sites for cAMP-dependent protein kinases. J. Biol. Chem. 259: 2084–2091.

Wiedmann, B., Sakai, H., Davis, T.A. and Wiedmann, M. 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature 370: 434–440.

Wilson, D.N. and Nierhaus, K.H. 2003. The ribosome through the looking glass. Angewandte Chemie-International Edition 42: 3464–3486.

Wimberly, B.T., Brodersen, D.E., Clemons, W.M. Jr., Morgan-Warren, R.J., Carter, A.P., Vonrhein, C., Hartsch, T. and Ramakrishnan, V. 2000. Structure of the 30S ribosomal subunit. Nature 407: 327–339.

Wittmann-Liebold, B. 1986. In: Hardesty B, Kramer G (eds) Structure, function and genetics of ribosomes, Springer, Berlin, Heidelberg, New York. pp. 326-361.

Wool, I.G., Chan, Y.L. and Gluck, A. 1995. Structure and evolution of mammalian ribosomal proteins. Biochem. Cell Biol. 73: 933–947.

Yamaguchi, K., Beligni, M.V., Prieto, S., Haynes, P.A., McDonald, W.H., Yates, J.R. 3rd, and Mayfield, S.P. 2003.

Proteomic characterization of the *Chlamydomonas reinhardtii* chloroplast ribosome. Identification of proteins unique to the 70S ribosome. J. Biol. Chem. 278: 33774–33785.

Yamaguchi, K. and Subramanian, A.R. 2000. The plastid ribosomal proteins. Identification of all the proteins in the 50S subunit of an organelle ribosome (chloroplast). J. Biol. Chem. 275: 28466–28482.

Yamaguchi, K., Subramanian, A.R. 2003. Proteomic identification of all plastid-specific ribosomal proteins in higher plant chloroplast 30S ribosomal subunit. Eur. J. Biochem. 270: 190–205.

Yamaguchi, K., von Knoblauch, K. and Subramanian A.R. 2000. The plastid ribosomal proteins. Identification of all the proteins in the 30S subunit of an organelle ribosome (chloroplast). J. Biol. Chem. 275: 28455–28465.