Chapter 7

Ribosome Profiling: A Tool for Quantitative Evaluation of Dynamics in mRNA Translation

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Abstract

Translational regulation is important for plant growth, metabolism, and acclimation to environmental challenges. Ribosome profiling involves the nuclease digestion of mRNAs associated with ribosomes and mapping of the generated ribosome-protected footprints to transcripts. This is useful for investigation of translational regulation. Here we present a detailed method to generate, purify, and high-throughput-sequence ribosome footprints from *Arabidopsis thaliana* using two different isolation methods, namely, conventional differential centrifugation and the translating ribosome affinity purification (TRAP) technology. These methodologies provide researchers with an opportunity to quantitatively assess with high-resolution the translational activity of individual mRNAs by determination of the position and number of ribosomes in the corresponding mRNA. The results can provide insights into the translation of upstream open reading frames, alternatively spliced transcripts, short open reading frames, and other aspects of translation.

Key words Translating ribosome affinity purification (TRAP), Polysomes, Ribosome-protected fragment, Ribosome footprint, Initiation, Elongation, Termination, Alternative splicing, Upstream open reading frame, Translatome, Ribo-seq, mRNA-seq, Cell-type specific gene expression, Translational regulation

1 Introduction

Nuclear gene expression is dynamically controlled by numerous processes that occur in the nucleus and cytoplasm of plant cells [1]. The regulation of a given gene is dependent upon the gene allele, cell type, developmental stage, and environmental conditions. Within the nucleus, the transcription of protein coding genes is influenced by chromatin structure, the histone code, and DNA methylation, as well as transcription factor activity and the RNA polymerase II machinery. Once transcription has commenced, there are a multitude of posttranscriptional steps ranging from nuclear pre-RNA processing and splicing, to cytoplasmic mRNA

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translation, sequestration, localization, and degradation. All these mechanisms involve RNA binding proteins, which are often components of large mRNA–ribonucleoprotein (mRNP) complexes, including the ribosomes. For many years, spatial and temporal regulation of gene activity leading to phenotype has relied on the measurement of steady-state abundance of the polyadenylated mRNA transcript. It is frequently assumed that transcript level, often termed "gene expression." accurately reflects protein abundance. However, methods including DNA microarray hybridization and liquid chromatography-tandem mass spectrometry (LC-MS/MS) have shown significant inconsistencies between the abundance of an mRNA and the protein it encodes, respectively [2, 3]. These discrepancies can reflect regulated mRNA translation or differential turnover of proteins. A number of studies have demonstrated that translational regulation is an important level of gene regulation in plants (reviewed by [4–8]). Here we describe a method that provides a high-resolution assessment of the translational activity of individual mRNAs by determination of the position and number of ribosomes on gene transcripts.

1.1 The Importance of Posttranscriptional Gene Regulation in Plants

Actively translating mRNAs are associated with the most well-characterized mRNP complex, the ribosome. Typically, translated mRNAs are associated with multiple ribosomes in a complex called a polyribosome (polysome). In plants, the translational activity of an mRNA is frequently evaluated by fractionation of a cell extract containing stabilized polysomes through a sucrose gradient by differential centrifugation, followed by detection of the amount of mRNA in monosomes (a single ribosome) and polysomes of increasing density. In some cases, total mRNA abundance (the transcriptome) has been compared to the amount of mRNAs associated with ribosomes (monosomes and polysomes). By comparison to the transcriptome, this quantitative assessment of gene activity has been referred to as the translatome. To accomplish mRNA–ribosome complex isolation from the model plant *Arabidopsis thaliana* without the use of an ultracentrifuge, we developed the translating ribosome affinity purification (TRAP) technology [9]. TRAP involves the incorporation of a FLAG epitope-tagged ribosomal protein L18 (RPL18) into the large (60S) subunit of the cytosolic ribosome, which enables immunopurification of ribosome–mRNA complexes from crude cell extracts [9, 10]. An advantage of this method over traditional differential centrifugation techniques is the reduction in contamination of ribosomes preparations with mRNPs of similar density.

By use of either conventional differential centrifugation or TRAP to obtain polysomes, numerous studies have shown that translation machinery mutants, abiotic stress, biotic stress, symbiotic interactions, hormones and developmental processes modulate the ribosome association of individual mRNAs (*see* Table 1). For

Table 1
Selected examples of analyses of ribosome-associated mRNAs in plants

Isolation method	Focus of study	References
Arabidopsis thaliana—translatome analyses		
Differential centrifugation	Abiotic stress: hypoxia, water deficit, heat, cold	[13, 15, 26–30]
	Toxic ions: cadmium	[31]
	Light and carbon: photomorphogenesis, carbon availability, diurnal cycle, re-illumination	[3, 16, 17, 33, 34]
	Hormone action: auxin	[35, 36]
	Translational machinery mutants: *eIF3h*, *rpl24b*, *pab2 pab8*	[11, 12]
TRAP with 35S:His_6FLAG-RPL18	Abiotic stress and light: hypoxia, unanticipated darkness, reillumination	[14, 32, 37]
	Hormone action: gibberellin	[38]
	Intergenic polyadenylated mRNAs	[39]
Arabidopsis thaliana—Organ and cell-specific translated mRNAs		
TRAP with cell- and region-specific promoters	Seedling cell-types under control and hypoxia conditions	[40, 41]
	Leaf bundle sheath	[42]
	Floral meristem cell-types	[43]
	Microspore and elongating pollen tubes	[44]
Arabidopsis thaliana—proteome analyses		
TRAP with 35S:His_6FLAG-RPL18	Ribosome proteome	[45]
Oryza sativa—translatome analyses		
Centrifugation	Abiotic stress: cold, drought, salinity	[46]
Hordeum vulgare		
Centrifugation	Response to singlet oxygen	[47]
Medicago truncatula—translatome and miRNA analyses		
TRAP with *Agrobacterium rhizogenes* transformed roots	Symbiosis with *Sinhorhizobium meliloti*	[48]
Lycopersicon esculentum		
TRAP with *Agrobacterium rhizogenes* transformed roots	Tool development for root cell types	[49]

most transcripts, ribosome occupancy (amount of mRNA in polysomes relative to total abundance; translatome / transcriptome) or translation state (amount of mRNA in polysomes relative to non-polysomal complexes) is less than 100 % [3, 11–17] due to mRNA localization in mRNPs other than ribosomes (i.e., complexes involved in sequestration [18] or degradation [19–25]). Despite the increased knowledge about gene activity gained through the

evaluation of ribosome-associated mRNAs, the methodology does not provide insight into the number of ribosomes per length of mRNA (ribosome density) or the actual distribution of ribosomes along a transcript. This is because an mRNA–ribosome complex obtained by differential centrifugation or TRAP may include one to tens of ribosomes. This variation in ribosome number can be due to factors such as differential initiation, elongation or termination of translation and differences in mRNA and open reading frame length.

1.2 The Development of Ribosome Profiling Technology in Yeast and Mammals

Methods to determine the position and number of ribosomes on an individual eukaryotic cellular mRNAs were first developed over 25 years ago, but have undergone a recent renaissance due to high-throughput methods. A pioneering study reported in 1988 used micrococcal nuclease to digest bovine *preprolactin* mRNA undergoing translation in cell-free systems derived from wheat germ or rabbit reticulocytes [50]. The resultant monosome (80S) complexes were isolated by sucrose density centrifugation and the ~30 nt regions of mRNA protected from the digestion by the ribosome were purified following dissociation of the two ribosome subunits. To survey the positioning of individual ribosomes on the *preprolactin* transcript, the ribosome-protected fragments were hybridized to single-stranded phage DNA that corresponded to the antisense transcript and used as primers in a DNA polymerase extension reaction. The length and abundance of the extension products were used as proxy to map the position of ribosomes. This elaborate method revealed that initiation and termination are the rate-limiting steps in *preprolactin* mRNA translation.

The development of global-scale mRNA abundance evaluation technologies, such as DNA microarrays, facilitated advancement of ribosome occupancy analyses. In one study of yeast (*Saccharomyces cerevisiae*), ribosome–mRNA complexes were separated by velocity sedimentation through sucrose gradients and mRNA abundance was analyzed in 14 fractions across the gradient using microarrays [51]. As expected, the number of ribosomes on a given mRNA increased with the length of the mRNA. However, the density of ribosomes along individual mRNAs was well below the theoretical maximum packing density, suggesting that initiation or another step limits ribosome loading. The sequel to this study was a ribosome density mapping analysis in which site-specific digestion of polysomal mRNA with RNase H was followed by sucrose density gradient centrifugation and RNA blot analysis [52]. This revealed no significant decline in ribosome density between the 5′ to 3′ end of open reading frames, suggesting that the processivity of elongation is generally high. In contrast to the earlier study [51], there was no evidence of slower initiation on longer mRNAs. These findings were insightful, but yielded no information about nuances of gene regulation involving small upstream open reading frames

(uORFs), non-AUG start codons, miRNAs, or mRNA isoforms generated by selection of alternative transcription start, splice acceptor/donor or poly(A) addition sites, which occur in diverse eukaryotes including plants (reviewed by 7, 53, 54). These would require a method to map ribosomes along entire transcripts that was accurate, facile, and high-throughput.

A major breakthrough for studies of translational regulation came in 2009 when RNA-seq technology was applied to ribosome-protected mRNA fragments (ribosome footprints) obtained from yeast (*Saccharomyces cerevisiae*) cultures grown in rich or amino acid depleted medium [55]. RNA-seq is the method of rapid high-throughput sequencing of cDNA generated from RNAs of various origins (mRNA, miRNAs, and other small RNAs) [56]. Ingolia et al. [55] incubated crude cell extracts from yeast cultures with *Escherichia coli* RNase I to digest unprotected RNA. They then isolated 80S monosomes obtained by sucrose density gradient centrifugation and purified the ~28 nt ribosome-protected fragments from a polyacrylamide gel [55]. For each condition, a ribosome footprint library was constructed using an elaborate procedure and deep-sequenced using the RNA-seq technology, with the reads mapped back to the genome. This revealed that translation efficiency, defined as the ratio of ribosome footprints to mRNA abundance, differed ~100-fold between individual genes, suggesting that translational regulation substantially contributes to gene expression control in yeast. Higher ribosome density at the 5′ end of mRNAs was found to be independent of transcript length. When corrected for protein synthesis rates, high ribosome density correlated with an increased rate of elongation or termination. This "ribosome profiling" analysis also identified over 1,000 candidate uORFs and translation initiation events that occur at non-AUG codons, including confirmation of two UUG initiation codons in two *tRNA synthetase* transcripts. This pioneering study also showed that translation efficiency is strongly affected by amino acid starvation [55].

1.3 Ribosome Profiling (Ribo-seq) in Plants and Future Applications

Ribosome profiling (also known as Ribo-seq) analyses has been employed to study translation in animal cell lines (i.e., human cervical cancer cell line (HeLa) [57], human prostate cancer cell line (PC3) [58], embryonic mouse cells [59]). Ribosome profiling of HeLa-cells investigating the function of two miRNAs (miR1 and miR155) determined that destabilization of the mRNA was the predominant reason for reduced synthesis of protein from the targeted transcript [57], although ribosome profiling in zebrafish indicated that miRNA inhibition of translation preceded mRNA degradation [60]. In human PC3 cells treated with the mTOR ATP site inhibitor PP242, ribosome profiling identified mRNAs encoding proteins associated with proliferation, metabolism and metastasis of prostate cancer as candidates of mTOR regulation [58].

Thousands of ribosome pause sites and previously unannotated ribosome-associated RNAs were found in embryonic mouse cells that were treated with translation initiation inhibitor harringtonine [59]. A major advantage of Ribo-seq over traditional mRNA-seq analysis is the information gleaned on ribosome number and distribution per transcript. In some cases the ribosome footprinting can provide codon-level accuracy of ribosome positions.

Motivated by the evidence for translational regulation in plants in response to abiotic stress such as hypoxia and developmental programs such as photomorphogenesis, ribosome profiling technology was applied to *Arabidopsis thaliana* [33, 37], with minor modifications from the procedure of Ingolia [61]. In the study of Liu et al., cell extracts were incubated with RNase I, 80S monosomes were isolated by sucrose gradient centrifugation and the ribosome-protected fragments were isolated following fractionation on polyacrylamide gels [33]. Juntawong et al. [37] used two approaches, the first included pre-purification of polysomes, followed by RNase I digestion and 80S monosome isolation. The second entailed incubation of cell extracts with RNase I followed by immunopurification of FLAG-tagged ribosomes by the TRAP method [10]. The construction of libraries from the ribosome-protected fragments in both studies was adapted from the methods developed by Ingolia and colleagues [55], with strand-specific sequencing performed by Juntawong et al. [37]. Consistent with findings in yeast and animals, the *A. thaliana* studies confirmed that both translation initiation and termination are rate limiting, uORFs impede translation of downstream coding regions, and translation efficiency is modulated by environmental stimuli. Lui et al. [33] reported that miRNA-targeted mRNAs have lower than average translational efficiency and that a CUG triplet can serve as an initiation codon. As in mammals [59], some plant ribosome footprints mapped to annotated noncoding RNAs (ncRNA) [37]. Ribosome footprinting was also applied to plastids of maize, where translation is highly regulated [62].

In addition to key insights in the differential translation of individual mRNAs, ribosome profiling has proven important in the consideration of the relationship between miRNAs and translation. The RNA-induced silencing complex (RISC) is recruited to its target by base pairing between a miRNA guide and the target mRNA. This interaction is associated primarily with translational repression followed by mRNA cleavage in animals, but primarily with mRNA cleavage in plants [63]. This has largely been explained by differences in the target recognition of miRNA-RISC (miRISC) between animals and plants [63]. However, several studies suggest that miRNAs inhibit translation on a handful of plant mRNAs [64–68]. Recently, miRNA-mediated translational repression was demonstrated to occur at the endoplasmic reticulum (ER) in ALTERED MERISTEM PROGRAM 1 (AMP1)-dependent manner in *Arabidopsis thaliana* [66]. AMP1 is associated with the ER

and was shown to be required for miRNA-mediated translational repression specifically on ER-bound polysomes. Ribosome profiling could well be applied to membrane-bound polysomes to gain insights into the mechanisms of miRNA-mediated translational inhibition in plants.

Ribosome profiling has revealed that long noncoding RNAs (lncRNAs) are bound to ribosomes in mammals and plants [37, 59] suggesting they might contain short ORFs that are translated or play a role in translation regulation. Guttman et al. [69] reexamined lncRNA association with ribosomes in a published ribosome footprint dataset [59] and developed new metrics for lncRNA analysis in Ribo-seq data. In particular, they used a ribosome release score (RRS) that measured the decrease of ribosome density at the stop codon to demonstrate that the vast majority of the lncRNAs are unlikely to be translated. This highlighted the value of Ribo-seq data in transcript annotation and suggested a role for lncRNAs in the regulation of translation. Ribo-seq performed with genotypes altered in noncoding RNAs (i.e., miRNAs and lncRNAs) biogenesis may facilitate determination of their roles in translational regulation.

Here we describe a method for ribosome profiling analysis with *A. thaliana*. This method extends protocols for polysome isolation by use of differential centrifugation or TRAP [9, 10, 70] to the generation, isolation and sequencing of ribosome-protected fragments (Fig. 1). The procedure is complex and some troubleshooting may be required depending upon the starting material and whether or not TRAP is used to obtain ribosomes. This chapter complements the TRAP-seq methodology described in Chapter 9 [contribution by Reynoso et al.].

2 Materials

2.1 General Remarks

- This Ribo-seq protocol is based upon the ribosome profiling protocols developed by Ingolia and Ingolia et al. [61, 71, 72] and is adapted to plants. In addition, material and methods have been adapted from Mustroph et al. [10, 70] with minor modifications.
- This protocol requires standard practices to avoid RNAse contamination. All glassware, Miracloth and solutions should be autoclaved or baked unless indicated otherwise. All tubes should be RNase-free and have low binding capacity (i.e., nonstick), and all extractions, incubations, and reactions should be carried out on ice or at 4 °C unless indicated otherwise.
- Plant tissue must be harvested directly into liquid nitrogen, pulverized using a porcelain mortar and pestle under liquid nitrogen and stored at −80 °C. Tissue must not thaw until the extraction procedure.

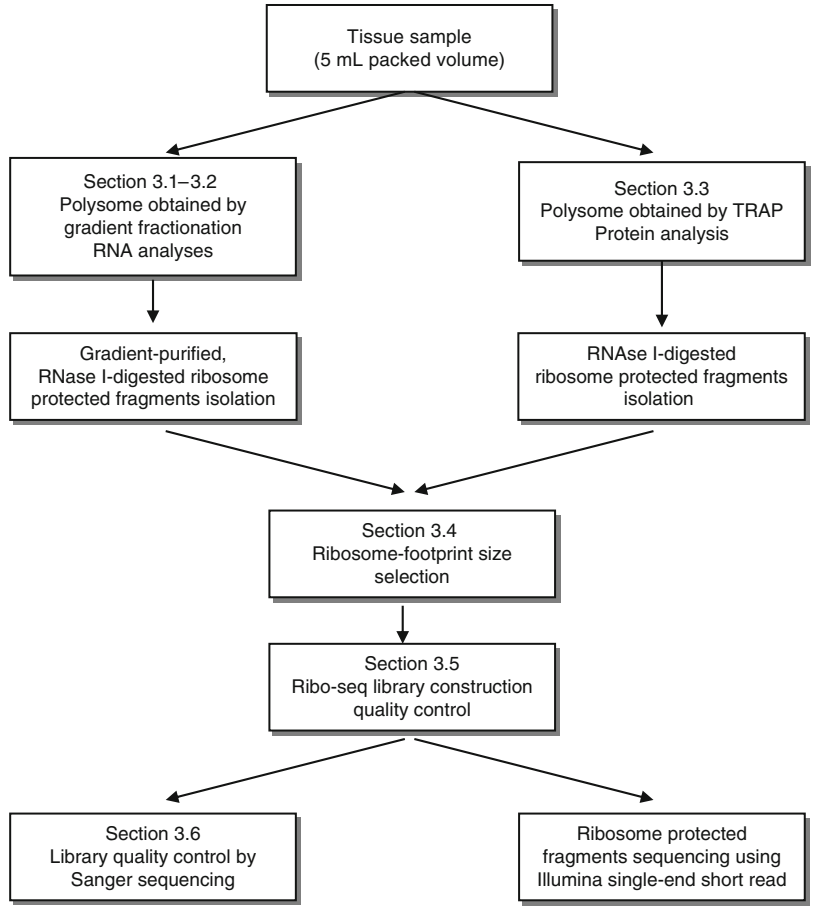

Fig. 1 Ribosome profiling experimental overview. Ribosome-protected fragments (ribosome footprints) can be isolated from ribosomes obtained by differential centrifugation or by immunopurification using transgenic lines that express an epitope-tagged version of ribosomal protein L18. The acronym used for the latter method is TRAP (translating ribosome affinity purification)

2.2 Conventional Isolation of Polysomes by Use of Differential Centrifugation

2.2.1 Equipment

1. Eppendorf or other benchtop microcentrifuges capable of centrifugation at 16,000 × *g*.
2. Preparative centrifuge with fixed angle or swinging bucket rotor accommodating 30 mL tubes (e.g., Beckman J2-21 high-speed centrifuge and JA-20 rotor, fitted with rubber inserts to accommodate 15 or 30 mL Corex tubes).
3. Ultracentrifuge with fixed angle rotor accommodating 30 mL thick walled polycarbonate tubes (e.g., Beckman L8-M ultracentrifuge, TY 70Ti rotor).
4. Thick-walled polycarbonate tubes (e.g., Beckman centrifuge tubes #355654).

2.2.2 Solutions and Chemicals

1. Sucrose (ultracentrifuge grade; Fisher BP220-212).
   The following stock solutions need to be autoclaved for 15 min and stored at room temperature.
2. 2 M Tris, adjust to pH 9.0 with HCl.
3. 2 M KCl.
4. 0.5 M ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), adjust to pH 8.0 with NaOH.
5. 1 M $MgCl_2$.
6. 20 % (v/v) polyoxyethylene 10 tridecyl ether (PTE).
7. 10 % (w/v) sodium deoxycholate (DOC).
8. 20× Detergent mix: 20 % (w/v) polyoxyethylene(23)lauryl ether (Brij-35), 20 % (v/v) Triton X-100, 20 % (v/v) octylphenyl-polyethylene glycol (Igepal CA 630, Sigma I8896), 20 % (v/v) polyoxyethylene sorbitan monolaurate (Tween-20).
9. Sucrose cushion solution (*see* **Note 1**).
   (a) Dissolve 1.75 M sucrose in 400 mM Tris, pH 9.0, 200 mM KCl, 5 mM EGTA, 30 mM $MgCl_2$. While heating to about 60 °C, adjust to desired volume, autoclave for 15 min and store at 4 °C.
   (b) *Important*: *add the following reagents to this solution just before use*: 5 mM dithiothreitol, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol.

The following solutions should *not* be autoclaved and need to be stored at −20 °C in aliquots

1. 0.5 M dithiothreitol (DTT).
2. 50 mg/mL cycloheximide, dissolved in ethanol.
3. 50 mg/mL chloramphenicol, dissolved in ethanol.
4. 0.5 M phenylmethylsulfonyl fluoride (PMSF), dissolved in isopropanol.

2.2.3 Buffers

Buffers should be prepared on the day of each experiment and kept on ice. The amounts indicated are at their final concentration. All buffers are optimized for isolation of polysomes from plants and are prepared according to Mustroph et al. [10] with minor modifications.

1. Polysome Extraction Buffer (PEB, for plants).
   200 mM Tris–HCl, pH 9.0, 200 mM KCl, 25 mM EGTA, 35 mM $MgCl_2$, 1× Detergent mix, 1 % PTE, 5 mM DTT, 1 mM PMSF, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol (antibiotics are added from 50 mg/mL stocks dissolved in 100 % (v/v) EtOH and stored at −20 °C).

2. RNase I Digestion Buffer (RNID).
   20 mM Tris–HCl, pH 8.0, 140 mM KCl, 35 mM $MgCl_2$, 50 µg/mL cycloheximide, 50 µg/mL chloramphenicol.

2.3 Isolation of Polysomes by Use of TRAP Technology

2.3.1 Equipment

1. Eppendorf or other benchtop microcentrifuge capable of 16,000 × *g*.
2. Rotating table, capable of 20 rpm/min. A rocking platform table is also acceptable.
3. Low-speed benchtop centrifuge with swinging buckets for 15 or 50 mL Falcon tubes.

2.3.2 Solutions and Chemicals

1. The same stock solutions are used as described in Subheading 2.2.2.
2. EZview FLAG M2 Agarose beads (Sigma, F 2426).
3. *A. thaliana* plants expressing epitope-tagged *RPL18* under a constitutive, region or cell-type specific promoter (i.e., *35S:His$_6$-FLAG-RPL18* [9] or cell-type-specific promoter-driven *His$_6$-FLAG-RPL18* lines [41]).

2.3.3 Buffers

1. Polysome Extraction Buffer (*see* Subheading 2.2.3, buffer 1).
2. RNase I Digestion Buffer (RNID) (*see* Subheading 2.2.3, buffer 2).

2.4 Analysis of Sucrose Gradient Fractionated Polysomes

Method is the same as described in detail by Mustroph et al. [10].

2.4.1 Equipment

1. Ultracentrifuge with a swinging bucket rotor accommodating 5 mL tubes (e.g., Beckman L8-M ultracentrifuge and Sw55Ti rotor).
2. Polypropylene tubes for gradients (e.g., Beckman centrifuge tubes #326819).
3. ISCO UA-5 UV detector, 185 Gradient Fractionator (ISCO Lincoln, NE).
4. Optional: A computer with a DAS-8 compatible data acquisition card connected to the data integrator output devise of the UA-5 detector unit [10].

2.4.2 Solutions and Chemicals

1. 10× Sucrose Salts.
   400 mM Tris, adjust to pH 8.4 with HCl, 200 mM KCl, 100 mM $MgCl_2$, autoclave for 15 min, store at –20 °C.
2. 2 M sucrose, autoclave for 15 min.
3. Displacement fluid (i.e., Fluorinert FC-40 (PC-FC40), ACROS Organics).

2.4.3 Preparation of Sucrose Gradients

Sucrose gradients are prepared in polypropylene tubes in a stepwise manner, with each new sucrose layer frozen for 1 h at –80 °C before adding the next layer. The tubes need to be kept on ice during the addition of the individual layers. Avoid air bubbles and thawing of layers to get a more uniform gradient. The indicated volumes are for 50 gradients (*see* Table 2). Gradients are stored at –80 °C. On the day of use they are removed from the freezer and thawed at 37 °C for exactly 1 h and then cooled at 4 °C for 1–1.5 h before use. Gradients must be handled with care to avoid mixing.

2.5 Isolation of Ribosome-Protected Fragments

2.5.1 Equipment

1. Ultra-4 centrifugal filters with Ultracel-100 membranes (Amicon UFC810008).
2. Eppendorf or other benchtop microcentrifuge capable of centrifugation at 16,000 × *g*.
3. Rocking shaker, capable of about 25 rpm/min.
4. Low-speed benchtop centrifuge with swinging buckets for 15 or 50 mL Falcon tubes.
5. Clinical centrifuge capable of centrifugation at 1,900 × *g* (e.g., IEC, Cat. No. 428 with rotor 215 at speed 7).
6. Polyacrylamide mini gel system (e.g., Bio-Rad mini Protean electrophoresis system).
7. NanoDrop™ spectrophotometer and Agilent Bioanalyzer or similar instrumentation.

2.5.2 Solutions and Chemicals

The following solutions are autoclaved and stored at room temperature.

1. 2 M Tris, adjust to pH 8.0 with HCl.
2. 2 M KCl.

Table 2
Sucrose gradient layer composition and volumes in gradient

Percent sucrose (w/v)	2 M Sucrose	10× Sucrose salts	Sterile water	Chloramphenicol and cycloheximide (50 mg/mL)	Volume per gradient step
	Mix reagents and use the volume indicated for each gradient step				
60 %	44 mL	5 mL	1 mL	5 µL	0.75 mL
45 %	49.5 mL	7.5 mL	18 mL	7.5 µL	1.5 mL
30 %	33 mL	7.5 mL	34.5 mL	7.5 µL	1.5 mL
15 %	11 mL	5 mL	34 mL	5 µL	0.75 mL

3. 250 mM ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), adjust to pH 8.0 with NaOH.
4. 3 M NaOAc, adjust to pH 5.2 with glacial acetic acid.
5. 1 M NaOH.
6. 10× TBE buffer. To make, dissolve 108 g of Tris-base, 55 g of boric acid, and 40 mL of 0.5 M EDTA (pH 8) in 1 L of ddH_2O.

The following solutions and chemicals need to be stored according to the manufacturer's recommendations.

1. RNase I (100 U/μL; Ambion AM2294).
2. SUPERase·In RNase inhibitor (20 U/μL; Ambion AM2694).
3. RNase-free water (Invitrogen AM9930).
4. Isopropanol.
5. TRIzol (Invitrogen 15596-026).
6. T4 polynucleotide kinase (10 U/μL; New England BioLabs M0201S).
7. 10 bp DNA ladder (1 μg/μL; Invitrogen 10821-015).
8. SYBR Gold (Invitrogen S11494).
9. T4 RNA ligase 2, truncated (New England BioLabs M0242S), supplied with PEG 8000 50 % (w/v) and 10× T4 Rnl2 buffer.
10. Superscript III (Invitrogen 18080-051).
11. CircLigase (Epicentre Biotechnologies CL4111K).
12. Phusion HF polymerase (New England BioLabs M0530S).
13. Glycogen (5 mg/mL, Ambion AM9510).
14. Poly(A) polymerase (5 U/μL; New England BioLabs M0276S).
15. Dynabeads® MyOne™ Streptavidin C1 (Invitrogen 65001).
16. Minelute Gel Extraction kit (Qiagen 28604).
17. Deoxynucleotide (dNTP) Solution Set (NEB N0446S), diluted to 10 mM each with deionized water
18. Adenosine 5′ triphosphate (ATP), 10 mM (NEB P0756).
19. 40 % (w/v) Acrylamide/Bis-acrylamide Solution, 19:1 (Bio-Rad 161-0144).
20. TEMED (Bio-Rad, 161-0801).
21. 10 % (w/v) ammonium persulfate (Sigma 215589); store at 4 °C for no longer than 1 month.
22. Urea molecular grade (Sigma, U5378).
23. RNA loading dye 2× (2× denaturing loading dye) (NEB B0363S).
24. Gel loading dye 6× (NEB B7021S).

25. SSC (20×), RNase free (Invitrogen AM9763).
26. Optional: pGEM®-T Easy Vector System II (Promega A1380).

2.5.3 Buffers

Buffers should be prepared on the day of each experiment and kept on ice. The final concentration of the reagent is indicated. All buffers are optimized for isolation of polysomes from plants.

1. DNA gel extraction buffer.
   300 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
2. RNA gel extraction buffer.
   300 mM NaOAc pH5.2, 10 mM Tris–HCl pH 8.0, 1 mM EDTA
3. 10× Tris–borate–EDTA buffer (TBE).
   1 M Tris, 0.9 M boric acid, 0.01 M EDTA.
4. RNase I Digestion Buffer (RNID): *see* Subheading 2.2.3, buffer 2.

2.6 Oligo and Linker Sequences Used in Library Preparation (See Table 3)

3 Methods

Two methods are presented for the isolation of polysomes and generation of RPFs. The first one (*see* Subheadings 3.1 and 3.2) uses conventional differential centrifugation and can be performed with any genotype. The second one (*see* Subheading 3.3) utilizes the translating ribosome affinity purification (TRAP) method that requires a transgenic plant expressing a tagged version of a ribosomal protein that allows for mRNA–ribosome complex purification (e.g., *35S:His$_6$-FLAG-RPL18* [9] or tissue-specific promoter:*His$_6$-FLAG-RPL18* [41]). We present one method for isolation of the RPFs (*see* Subheading 3.4), along with two variations on the construction of small RNA libraries of RPFs are presented (*see* Subheadings 3.5.2 and 3.5.3).

3.1 Conventional Isolation of Polysomes (Estimated Time, 4.5 h)

This method is adapted from Mustroph et al. for ribosome profiling [10]; *see* Subheading 3.3 for a more specialized method of polysome isolation.

1. Plant tissues are harvested immediately into liquid nitrogen. The material is pulverized in a prechilled mortar with a pestle, maintaining a frozen state at all times. The frozen pulverized tissue is transferred into a 50 mL Falcon tube and placed in liquid nitrogen or stored at −80 °C.

Table 3
Oligonucleotides and linker sequences used in library preparation

Oligonucelotide name	Working concentration (μM)	5′ to 3′ nt sequence plus modifications
Marker		
oNTI199 (RNA)—28 nt	2.5	AUGUACACGGAGUCGACCCGCAACGCGA
Linkers		
oNTI225—oligo dT Reverse transcription primer from Ingolia [55] for polyadenylated RPFs	0.24	5′-/5PhosGATCGTCGGACTGTAGAACTCTGAACCTGTC GGTGGTCGCCGTATCATT-iSp18-CACTCA-iSp18-CAAG CAGAAGACGGCATACGATTTTTTTTTTTTTTTTTTTVN
oNTI226 Reverse transcription primer from Ingolia [55]	0.24	5′-/5PhosAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAG ATCTCGGTGGTCGC-iSp18-CACTCA-iSp18TTCAGACGTGTGC TCTTCCGATCTATTGATGGTGCCTACAG
oNTI227 (RNA) (miRNA cloning linker 1; IDT 11-04-02-08)	7.5	5rAppCUGUAGGCACCAUCAAU-3ddC
Library amplification		
oNTI200	1.3	CAAGCAGAAGACGGCATA
oNTI201	1.3	AATGATACGGCGACCACCGACAGGTTC AGAGTTCTACAGTCCGACG
rRNA subtraction		

rRNA1: targets 18S rRNA (AT2g01020, AT3G41768)	1	5BioTEG-CATAAACGATGCCGACCAGGGATCAGCGG
rRNA2: targets 18S rRNA (AT3G41768)	1	5BioTEG-TTTATTAGATAAAAGGTCGACG
Multiplexing		
Universal forward primer	0.24	ATGATACGGCGACCACCGAGATCTACAC
Index primers		
ACGACT	0.24	CAAGCAGAAGACGGCATACGAGAT**AGTCGT**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
ATCAGT	0.24	CAAGCAGAAGACGGCATACGAGAT**ACTGAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
CAGCAT	0.24	CAAGCAGAAGACGGCATACGAGAT**ATGCTG**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
CGACGT	0.24	CAAGCAGAAGACGGCATACGAGAT**ACGTCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
GCAGCT	0.24	CAAGCAGAAGACGGCATACGAGAT**AGCTGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
TACGAT	0.24	CAAGCAGAAGACGGCATACGAGAT**ATCGTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
CTGACG	0.24	CAAGCAGAAGACGGCATACGAGAT**CGTCAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCG
GCTACG	0.24	CAAGCAGAAGACGGCATACGAGAT**CGTAGC**GTGACTGGAGTTCAGACGTGTGCTCTTCCG

All oligonucleotides (oligos) are listed in the 5′ to 3′ orientation. All oligos are DNA unless otherwise indicated. For the index primers, the index name is provided in the reverse orientation within the primer sequence. Oligos should be synthesized and HPLC or PAGE purified by the supplier (www.idtdna.com). Modifications: 5Phos = 5′ phosphorylation, 5BioTEG = 5′ Biotinylation with TEG (triethylene glycol) linker, 5rApp = 5′A and 2 phosphates, iSp18 = internal 18-atom hexa-ethyleneglycol spacer, and 3ddC = 3′ Dideoxy-C (3′ blocking). Index sequence of index primers is shown in *bold font*.

2. To begin the preparation, place the amount of pulverized tissue powder to be used in a Falcon tube (e.g., 5 mL of rosette or seedling tissue powder) to which two volumes (i.e., 10 mL) of ice-cold freshly prepared Polysome Extraction Buffer (PEB) are added using a plastic transfer pipette.
3. Let the mixture thaw slowly on ice. During thawing, stir gently with a glass rod.
4. Transfer tissue to glass homogenizer with a Teflon or glass pestle using a plastic transfer pipette.
5. Homogenize the mixture with the help of the glass homogenizer in five strokes; keep on ice.
6. Let the mixture stand on ice for 10 min (or until all samples are prepared).
7. Pour the homogenate into 15 mL Corex tube (tubes will need to be balanced to within 0.05 g).
8. Centrifuge the samples at 4 °C, 16,000 × *g*, for 15 min in a preparative centrifuge; use a fixed angle or swinging bucket rotor with rubber Corex tube adapters.
9. Put a fresh Corex tube on ice. Place a piece of Miracloth (EMD Millipore; cut in 2 or 4 cm squares, autoclaved) in the opening to form a small funnel.
10. Using a transfer pipette, filter the supernatant into the new tube, avoiding the pellet. If any of the pellet has been transferred, repeat the centrifugation step to ensure removal of all material that pellets at 16,000 × *g*. Keep the clarified extract.
11. Label a 1.5 mL microfuge tube and save 5 % of the clarified extract in a separate tube to be used to isolate total RNA as a control.
12. Arrange thick walled polycarbonate tubes in an ice bucket or rack and put 8 mL of ice-cold sucrose cushion solution into each tube.
13. In a cold room, layer gently and slowly with a pipette the clarified extract (above) on top of this solution, avoiding mixing of the sample and sucrose cushion solution.
14. Balance the weight of the tubes within 0.05 g with PEB; install the two unit cap on each tube, set them on ice if the ultracentrifuge is not yet at 4 °C.
15. Place the tubes into the fixed angle rotor (70Ti rotor, Beckman) that has been prechilled at 4 °C according to the manufacturer's specifications.
16. Centrifuge samples at 4 °C, 170,000 × *g* (50,000 rpm) for 3 h.
17. After centrifugation, transfer tubes to ice, mark the pellet side on the tube.

18. Carefully remove the supernatant and then the sucrose cushion with a 10 mL pipette, taking care not to disturb the pellet. The polysome pellet (P170) should be clear and sticky, of a translucent light yellow to brown color. The supernatant is discarded.
19. Wash the tube walls with 0.5 mL sterile water (*see* **Note 2**).
20. Resuspend the pellet in 300 μL of ice-cold RNID buffer by pipetting the solution up and down near the marked pellet region (*see* **Note 3**).
21. Let sit on ice for 30 min; it is important to resuspend the pellet carefully.
22. Transfer the resuspended sample to a prechilled 1.5 mL microfuge tube and centrifuge for 1 min, 12,000 × *g* at 4 °C. Transfer the supernatant to a new sterile prechilled microfuge tube and discard the pellet. Keep the sample on ice.
23. Measure the OD_{260} of the sample using a NanoDrop spectrophotometer (*see* **Note 4**).
24. The suspension contains ribosomal subunits, ribosomes and polysome complexes to be used for two samples, an RNase I digested sample, and a non-digested control sample.
25. Continue with Subheading 3.2.

3.2 Generation of Ribosome-Protected Fragments

This protocol is optimized for isolation of RPFs from plants and adapted from Ingolia and Ingolia et al. [61, 71] and uses polysomes isolated in Subheading 3.1. Preliminary experimentation may be needed to optimize the digestion before proceeding to the RFP library construction.

3.2.1 RNase I Digestion (Estimated Time 2.5 h)

1. This process requires 4,000 unit OD_{260} of polysomes (obtained from 5 mL of packed tissue powder from rosette tissue or whole seedlings). The RNase I digest is performed with 2,000 unit OD_{260} of polysomes; the same amount is used for a non-digested control sample. *See* **Note 4** for procedures that describe estimation of sample OD_{260} units per μL.
2. To one sample, add 5 μL of the RNAse inhibitor SUPERase·In, mix by pipetting, adjust the volume to 250 μL with RNID and hold on ice. This is the untreated control sample.
3. To the RNase digested sample, add RNase I (10 U per 10 OD_{260} unit polysomes) and adjust the volume to 250 μL with (RNID). This is the treated sample.
4. Incubate the treated sample (RNase I digest) at room temperature for 2 h with rotation.
5. The amount of RNase I used and digestion time can be adjusted based on the amount of polysomes present in each sample (*see* **Note 5**).

3.2.2 Polysome Profiling (Estimated Time 2.5 h)

This method is adapted from Mustroph et al. [10] with minor modifications. It can be used to evaluate the RNase I digested polysomes by comparison to an untreated control (non-digested polysome sample). The monosomes (80S) obtained by the RNase I digestion of the polysomes are separated from other complexes by this procedure and subsequently used for RPF library construction.

1. For analysis of the absorbance profile of polysomes, load the resuspended polysomal pellet (untreated sample) and RNAse I digested polysomes (treated sample) from Subheading 3.2.1 (prepared in **steps 2** and **5**) on top of two sucrose gradients prepared as indicated in Subheading 2.4.3, following also the specific instructions on the thawing and chilling of the gradients. One gradient per sample is used. The polysome samples are layered onto the top of the gradient by pipetting on the side of the tube to avoid mixing of the sample and gradient.
2. Balance all tubes to within 0.05 g with RNID buffer.
3. Place tubes in the swinging buckets that have been prechilled at 4 °C. Hang buckets on rotor according to the manufacturer's specifications. All buckets must be placed onto the rotor even if some of the paired buckets do not contain a sample.
4. Perform ultracentrifugation at 4 °C, 237,000 × *g* (50,000 rpm, SW55.1 rotor) for 1.5 h. If desired, the run length can be increased or decreased by 10–15 min to optimize the separation of the ribosome complexes.
5. While the gradient is spinning, prepare the ISCO absorbance detector according to the manufacturer's specifications (model # UA-5, ISCO (Teledyne), Lincoln, NE) so that it is linked to a fraction collector (e.g., Foxy R1, Teledyne ISCO). Switch on 20 min prior to use to warm up the UV lamp. Assemble the peristaltic pump and gradient holder according to the manufacturer's instructions. Adjust the absorbance detector to 0.2 or 1.0 sensitivity for small and large-scale preparations, respectively Use 150 cm/h chart speed. If the option is available, collect absorbance data (A_{254} nm) with the chart recorder and digitally for later quantitative analysis.
6. Prepare two "blank" sucrose gradients with 250 μL of RNID loaded on top of the gradients for the centrifuged samples (*see* **Note 6**). This volume of RNID should equal the RNase I digest and untreated control sample volumes.
7. Assemble the gradient in the UV detector holder. Puncture the tube bottom with the piercing apparatus of the fractionator and run the displacement fluid (Fluorinert FC40) from the syringe controlled by the peristaltic pump through the tubing and the whole at the bottom of the tube at a flow rate of 0.75 mL/min. Record the A_{254} nm profile (Fig. 2) with chart recorder and using a data acquisition device if available.

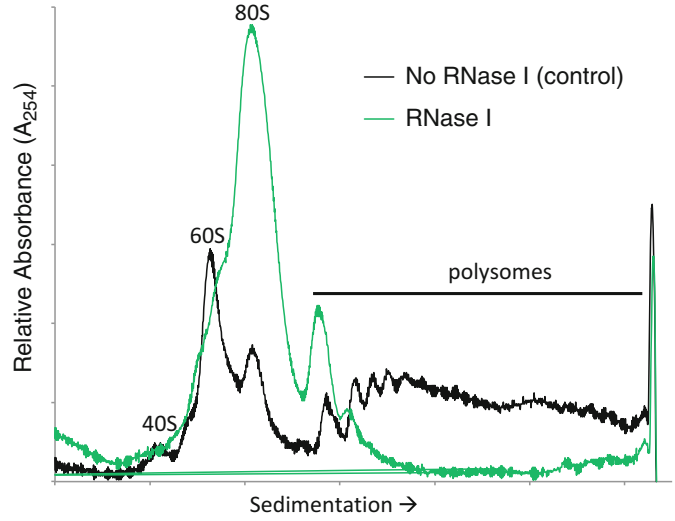

Fig. 2 Absorbance (A254 nm) profile of sucrose density gradient fractionated ribosomes from RNase I-treated or the undigested control sample. The RNase I digestion reduces the polysomes to 80S monosomes and some larger ribosome complexes. Some disomes (a two-ribosome complex) are detected even after digestion with higher concentrations of nuclease or for longer incubation times

8. After centrifugation, carefully remove the rotor from the centrifuge, place the buckets on ice, and remove the first gradient to be analyzed.
9. While running the gradient through the UV absorbance detector to the fraction collector, collect individual fractions (usually 12 fractions of 0.4 mL) into 1.5 mL Eppendorf tubes, in order to obtain the 80S monosome fractions. Close the cap of each tube and place on ice immediately to avoid RNA degradation.

3.2.3 Isolation of Ribosome-Protected Fragments (Estimated Time 3.5 h)

1. From the RNase I treated samples, combine the gradient fractions that contain monosomes (80S ribosomes), usually about two fractions in a 1.5 mL Eppendorf tube (*see* **Note 7**). The 80S monosomes have the desired ~30 nt protected fragments. Most but not all polysomes are reduced to monosomes after RNase I digestion (*see* Fig. 2).
2. Perform TRIzol RNA extraction on the monosomes according to the manufacturer's protocol. For example, to 400 μL of monosomes, add 1 mL TRIzol, mix and incubate for 5–15 min at room temperature. Add 200 μL chloroform, vortex 1 min, centrifuge at 16,000 × *g*, 4 °C for 15 min, and transfer the upper phase (~700 μL) to a new 1.5 mL Eppendorf tube on ice.
3. For RNA precipitation, use 1 volume ice-cold isopropanol, 300 mM NaOAc pH 5.2, and 20 μg glycogen (e.g., to 700 μL

of supernatant, add 140 μL of 3 M NaOAc pH 5.2, and 4 μL of 5 mg/mL glycogen, mix and incubate at −20 °C for at least 30 min).

4. Centrifuge at 16,000 × *g*, 4 °C for 30 min.
5. Wash RNA pellet with 75 % (v/v) EtOH.
6. Air-dry pellet for 5 min and resuspend in 100 μL of RNase-free water.
7. Quantify the amount of RNA in 1 μL by use of a NanoDrop spectrophotometer; a typical yield is ~80 μg RNA from seedling or rosette tissue processed as described.
8. Add 401 μL of RNase-free water to the 99 μL RNA sample, mix by pipetting.
9. In the cold room, load the 500 μL of RNA onto prechilled Ultra-4 centrifugal filters with Ultracel-100 membrane. This will separate <200 nt small RNAs, which include the RPFs, from larger RNAs, including most intact larger rRNA molecules.
10. Add 2 μL of SUPERase·In RNase inhibitor to the sample (top) and collection (bottom) sections of the tube.
11. Centrifuge at 1,900 × *g* for 30 min at 4 °C to isolate RPFs (in a clinical centrifuge, speed 7 or 1,900 × *g*). The small RNAs including RPFs will go through the Ultracel-100 membrane, while larger RNAs remain in the top part of the tube.
12. Precipitate the flow-through (usually ~450 μL) by addition of 1 volume of isopropanol, 45 μL of 3 M NaOAc, pH 5.2, and 20 μg glycogen, mix well and incubate for 30 min at −20 °C.
13. Centrifuge at 16,000 × *g*, 4 °C for 30 min.
14. Wash pellet with 0.5 mL of ice-cold 75 % (v/v) EtOH, air-dry for 5 min. Repeat **step 13** if the pellet detaches from the wall of the centrifuge tube during the wash step.
15. Resuspend in 10 μL of RNase-free water; this is the RPF RNA.
16. Keep on ice or freeze at −80 °C until ready for Subheading 3.4.

3.3 Isolation of Ribosome-Protected Fragments by Use of TRAP Technology

This is an alternative to isolation of polysomes by centrifugation (Subheadings 3.1 and 3.2).

3.3.1 Tissue Extraction (Estimated Time 1 h)

1. Perform tissue extraction as described in Subheading 3.1. For transgenic *35S:His$_6$FLAG-RPL18* tissue, 5 mL of pulverized frozen whole seedling tissue or 10 mL of seedling root tissue in 2 volumes of PEB can be used in this step. For lines that use cell-type specific promoters expressed in few cells, the amount of starting material will need to be increased; see [70] for guidance.

2. Estimate the amount of RNA per mL of clarified extract by performing a TRIzol extraction on 500 µL of the extract from Subheading 3.1, **step 11**, according to the manufacturer's protocol, Follow by quantification of the RNA yield by use of a NanoDrop spectrophotometer (*see* **Note 8**). This sample obtained in this step can be used as Total RNA control in mRNA-seq library construction. [*See* Chapter 9—Reynoso contribution to the same volume].

3.3.2 Preparation of the Anti-FLAG M2 Agarose Beads (Estimated Time 0.5 h)

1. In a 15 mL tube, resuspend 500 µL of anti-FLAG M2 agarose gel, pipetted with a 0.5 mL disposable pipette tip, in the 5 mL of RNID buffer.
2. Centrifuge at 4 °C, 1,900 × *g* for 30 s.
3. Carefully aspirate the supernatant using a pipette, add 5 mL of ice-cold RNID buffer and vortex briefly to resuspend the beads.
4. Centrifuge at 4 °C, 1,900 × *g* for 30 s.
5. Carefully aspirate the supernatant using a pipette and keep the prewashed beads on ice until ready.

3.3.3 Immunoprecipitation of Polysomes (Estimated Time 3 h)

1. Combine the clarified extract containing polysomes (corresponding to the volume containing 60 to 80 µg of total RNA as determined in Subheading 3.3.1, **step 2**) with prewashed anti-FLAG M2 agarose beads in a 15 mL tube (*see* **Note 9**).
2. To bind FLAG-tagged ribosomes to the anti-FLAG matrix, incubate for 2 h at 4 °C with gentle rocking (e.g., 10 back-and-forth oscillations per minute).
3. Centrifuge for 30 s at 1,900 × *g* at 4 °C.
4. Carefully aspirate the supernatant using a pipette.
5. Add 6 mL of RNID buffer to the beads, wash by gentle rocking at 4 °C for 5 min, centrifuge at 1,900 × *g* at 4 °C, and carefully aspirate the wash using a pipette.
6. Repeat **step 5** three times, maintaining the washed bead sample on ice until ready for Subheading 3.3.4.

3.3.4 On-Bead RNase I Digestion to Generate Ribosome Footprint Fragments (Estimated Time 2.5 h Plus Time for RNA Isolation)

1. Add 400 µL of ice-cold RNID buffer to the washed beads and transfer everything into a 1.5 mL tube, maintaining sample on ice.
2. Add 37.5 U of RNase I per µg of RNA to the mixture; incubate for 2 h at room temperature (~23 °C) with gentle rocking (e.g., 10 back-and-forth oscillations per minute).
3. Purify the RNA by use of TRIzol reagent according to the manufacturer's protocol.
4. Resuspend in 10 µL of RNase-free water; keep sample on ice.

5. Quantify the amount of RNA in 1 µL of sample by use of a NanoDrop spectrophotometer. A typical yield from *35S:His$_6$FLAG-RPL18* is ~40 µg of RNA starting from extract containing 60–80 µg of total RNA (as measured in Subheading 3.3.1, **step 1**); this is the RPF RNA.
6. Keep on ice or freeze at −80 °C until ready for Subheading 3.4.

3.4 Ribosome-Protected Fragment Size Selection (Estimated Time 2.5 h Plus Overnight Soaking Followed by 1.5 h Precipitation)

This protocol is optimized for isolation of RPFs from plants and adapted from the procedures of Ingolia [61, 71, 72].

1. Prepare a 17 % (w/v) 1.5 mm vertical urea polyacrylamide gel with 0.5× TBE (e.g., 8 cm mini gel, Bio-Rad mini Protean 3 electrophoresis system). For 10 mL of gel solution, add 1 mL of 10× TBE, 4.2 g of urea, 4.25 mL of 40 % (w/v) acrylamide/bis acrylamide mix, and 0.5 mL ddH_2O in a flask. Mix until urea is completely dissolved. Assemble gel glass plates according to the manufacturer's instructions. Add 10 µL of TEMED and 200 µL of 10 % (w/v) APS to 10 mL of gel solution and pour the gel into the glass plate assembly. Immediately add the comb without trapping any bubbles. Wait 20 min for the gel to polymerize before removing the comb. Pre-run the gel in 0.5× TBE for 15 min at 150 V and wash urea from the wells by pipetting up and down with 0.5× TBE.
2. Add 10 µL of 2× denaturing loading dye (*see* Subheading 2.5.2) to 10 µL of the resuspended RNA.
3. Set up a sample with 0.5 µL of 10 bp DNA ladder with 9.5 µL water and 10 µL 2× denaturing loading dye.
4. Set up a separate sample with 1 µL of oNTI199 control RNA oligo (28 nt RNA, *see* Table 3) at 50 µM with 9 µL of water and 10 µL of 2× denaturing loading dye.
5. Denature samples for 2 min at 75 °C, then place them immediately on ice.
6. Load samples into the gel wells and run in 0.5× TBE for 65 min at 200 V.
7. Stain gel for 5 min in a 1:10,000 dilution of SYBR Gold in 0.5× TBE.
8. Photograph gel.
9. Excise the 28mer region using the oNTI199 control RNA oligo samples as a guide (*see* Fig. 3a). The region excised is the investigator's decision; we recommend cutting the gel piece corresponding to the sizes above and below the 28mer to obtain the ~24–34 nt fragments; this is the RPF sample.
10. Optional: Excise the 28mer control oligo band and use as a control for all subsequent reactions in the library synthesis.
11. Photograph the cut gel.

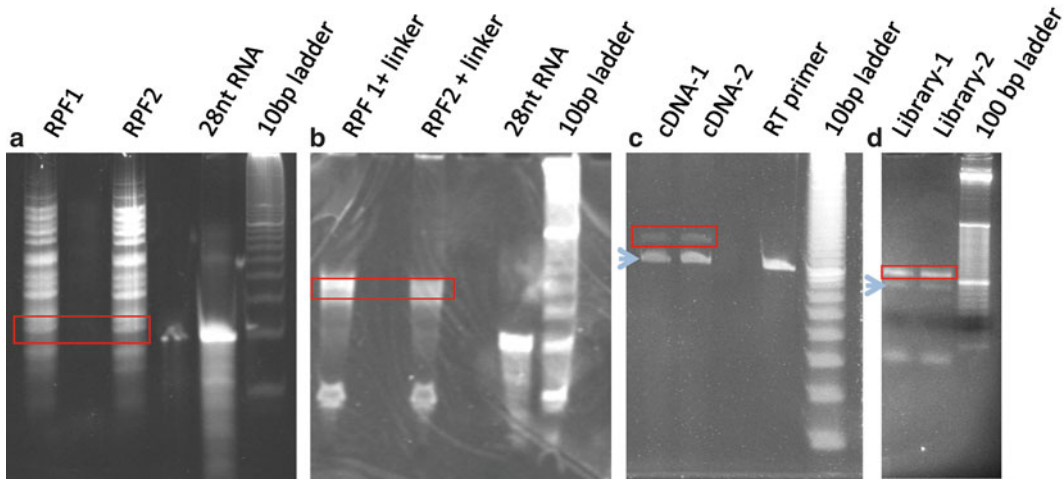

Fig. 3 Representative gel images from ribosome-protected fragment (RPF) library construction. (**a**) Size selection of RPFs. The ribosome footprint RNA fragments are derived from ribosomes obtained by TRAP from two *Arabidopsis* rosette leaf samples expressing the transgene *35S:His$_6$-FLAG-RPL18* [9]. The starting amount of RNA following TRAP that was used for the RNase I digestion was 60 μg. The *red rectangle* indicates the gel region with the ~30 nt footprints to be excised and further processed. Other abundant bands are of rRNA origin. (**b**) Purification of ligation products (Subheading 3.5.3). Ligated RPFs are 20 nt larger than the 28 nt RNA marker. The lower band represents free linker. The *red rectangle* indicates the gel region to be extracted. (**c**) Purification of reverse transcription products. The *blue arrow* indicates the reverse transcription primer that should not be excised. The *red rectangle* indicates the gel region to be excised. (**d**) Purification of PCR products. The *red rectangle* indicates the ~120 nt product band that should be purified (from method including polyadenylation of RPFs, Subheading 3.5.2). The *blue arrow* indicates the ~90 nt band derived from the residual reverse transcriptase primer that should be avoided. If the linker-ligation method is used (Subheading 3.5.3), the expected PCR product size is around ~175-nt and unextended RT primers background is around 145 nt

12. Recover RNA from each gel slice by use of the crush-and-soak method (*see* below) and precipitate RNA with isopropanol. Punch the bottom of 0.5 mL tubes five times using a 21 gauge needle to create small holes. Put gel slice inside. Place the 0.5 mL tube into a clean 1.5 mL collection tube.
13. Centrifuge 16,000 × *g* at 4 °C for 10 min to crush gel slices (the crushed gel will be in the 1.5 mL collection tube).
14. Add 500 μL of ice-cold RNA gel elution buffer to the crushed gel in the collection tube (Subheading 2.5.3, buffer 2); incubate at 4 °C overnight on a rotating or rocking table.
15. Centrifuge gel pieces to the bottom (5 min, 16,000 × *g* at 4 °C) and transfer the supernatant into a new tube.
16. Add 500 μL of isopropanol and 4 μL of 5 mg/mL glycogen to the supernatant from **step 15**. Mix and incubate at −20 °C for 30 min.
17. Centrifuge 16,000 × *g* at 4 °C for 30 min.

18. Add 1 mL of 75 % (v/v) EtOH and vortex quickly. Centrifuge 16,000 × *g* at 4 °C for 10 min, carefully remove all the ethanol and air-dry the pellet (approximately 5 min).
19. Resuspend the pellet of gel-purified RNA in 10 μL of RNase-free water.

3.5 Library Generation

We provide two methods for generating the RPF libraries. One is adapted from the procedure of Ingolia [61, 71, 72] that involves modification of the RPFs by 3′ dephosphorylation, 3′ polyadenylation, annealing of an oligo-dT primer-linker, reverse transcription, circularization of the single-stranded cDNA, removal of cDNAs of rRNA origin by subtractive hybridization, amplification of the cDNAs by PCR, and size selection (*involves* Subheadings 3.5.1 and 3.5.2). The alternate procedure requires the 3′-dephosphorylation but removes the requirement for 3′ polyadenylation by use of a linker with a 5′ Adenylation (5′-App) modification that promotes efficient 3′ ligation without the requirement for ATP addition (*involves* Subheadings 3.5.1 and 3.5.3).

3.5.1 Dephosphorylation (Estimated Time 2.5–3 h)

1. Denature RNA from **step 19** of Subheading 3.4 for 2 min at 75 °C, then place immediately on ice.
2. On ice, add 15 μL of water, 3 μL of 10 × T4 polynucleotide kinase buffer (without ATP), 1 μL of SUPERase · In, and mix well by pipetting.
3. Add 1.5 μL of T4 polynucleotide kinase, mix and incubate for 1 h at 37 °C.
4. Heat-inactivate the enzyme by incubation for 10 min at 70 °C and then place on ice.
5. Add 56 μL of water, 4 μL of glycogen, and 10 μL of 3 M NaOAc pH 5.2 to each dephosphorylation reaction and mix well by pipetting.
6. Add 120 μL of isopropanol to each reaction, mix and incubate at −20 °C for 30 min.
7. Precipitate the dephosphorylated RPFs as described for RNA in Subheading 3.4, **steps 16–17**.
8. Add 500 μL of 75 % (v/v) EtOH and vortex quickly. Centrifuge 16,000 × *g* at 4 °C for 10 min, carefully remove all the ethanol and air-dry the pellet for 5–10 min.
9. Continue with either Subheadings 3.5.2 or 3.5.3.

3.5.2 Polyadenylation (Estimated Time 1.5–2 h) (See **Note 10**)

1. Resuspend dephosphorylated RPFs from **step 8** of Subheading 3.5.1 in 11.25 μL of RNase-free water.
2. Prepare 2× tailing reaction mix and keep on ice. For each sample, use 2.5 μL of 10× Poly(A) Polymerase buffer, 2.5 μL of 10 mM ATP, 0.625 μL of SUPERase · In, and 6.875 μL of water.

3. Prepare Poly(A) polymerase at 0.5 U/μL in the following mix: 1.5 μL of 2× reaction mix, 1.2 μL of water, and 0.3 μL of Poly(A) polymerase (5 U/μL stock). Keep the mix on ice until use.
4. Denature dephosphorylated RPF sample for 2 min at 75 °C, then return to ice. On ice, add 11.25 μL of 2× tailing reaction mix and 2.5 μL of Poly(A) polymerase mix to each RNA sample.
5. Incubate for 10 min at 37 °C.
6. Quench the tailing reaction by adding 1.3 μL of 250 mM EDTA.
7. Precipitate the polyadenylated RPFs with isopropanol as described for RNA in Subheading 3.4, **steps 16–18**.
8. Continue with Subheading 3.5.4, **step 1**.

*3.5.3 Small RNA Linker-Ligation Alternative for Polyadenylation (Estimated Time 3–3.5 h) (See **Note 10**)*

1. Resuspend the dephosphorylated RPFs from **step 19** of Subheading 3.4 for in 8.5 μL of 10 mM Tris (pH 8). Add 1.5 μL of oNTI227 linker (100 μM, *see* Table 3), denature the sample for 90 s at 80 °C, and then cool to room temperature.
2. Add 2 μL of T4 Rnl2 buffer (10×), 6 μL of PEG 8000 (50 %, w/v), 1 μL of SUPERase·In (20 U/μL), 1 μL of T4 Rnl2 (200 U/μL) and mix by pipetting.
3. Incubate for 2.5 h at room temperature.
9. Precipitate linker-ligated RNA as described as described for RNA in Subheading 3.4, **steps 16–18**.
4. Resuspend the RNA pellet in 10 μL of RNase-free water
5. Prepare a 17 % (w/v) 1.5 mm vertical urea polyacrylamide gel with 0.5× TBE (8 cm mini gel, Bio-Rad mini Protean 3 electrophoresis system). Refer to Subheading 3.4 for gel preparation.
6. Add 10 μL of 2 × denaturing loading dye (*see* Subheading 2.5.2) to 10 μL of the resuspended RNA.
7. Set up a sample of 0.5 μL of 10 bp DNA ladder (Invitrogen) mixed with 9.5 μL of water and 10 μL of 2× denaturing loading dye.
8. Set up a marker sample with 1 μL oNTI199 control RNA oligo (28 nt RNA, *see* Table 3) at 50 μM with 9 μL water and 10 μL 2× denaturing loading dye.
9. Denature samples for 2 min at 75 °C, then place them immediately on ice.
10. Load samples onto the gel and run in 0.5× TBE for 65 min at 200 V.
11. Stain gel for 5 min in a 1:10,000 dilution of SYBR Gold in 0.5× TBE.

12. Photograph gel.
13. Excise the band corresponding to the linker-ligated RPF region that is ~20 nt larger than the oNTI199 control RNA oligo (*see* Fig. 3b).
14. Recover linker-ligated RPFs from the gel slice as described in Subheading 3.4, **steps 12–18**.
15. Continue with Subheading 3.5.4, **step 4**.

3.5.4 cDNA Synthesis and Size Selection with Denaturing Polyacrylamide Gel (Estimated Time 4 h Plus Overnight Soaking, Followed by 1.5 h Precipitation)

If the RPFs are enzymatically polyadenylated in the manner described in Subheading 3.5.2 then proceed with the next three steps:

1. Resuspend polyadenylated RPFs from **step 7** of Subheading 3.5.2 in 12 μL of RNase-free water.
2. Prepare template mix by combining in a new 0.5 mL tube 11 μL of polyadenylated RPFs, 1 μL of dNTPs at 10 mM each, and 1 μL of oNTI225 (*see* Table 3) at 50 μM. The remaining ~1 μL of RPFs is not used.
3. Continue with the denaturation **step 6** (below).

If the RPFs have been modified by linker ligation as described in Subheading 3.5.3 then proceed with **steps 4** and **5** below:

4. Resuspend linker-ligated RPFs from **step 14** of Subheading 3.5.3 in 12 μL of RNase-free water.
5. Mix 11 μL of linker-ligated RPFs, 1 μL of dNTPs at 10 mM each, and 1 μL of oNTI226 at 50 μM. The remaining ~1 μL of RPFs is not used.

For either polyadenylated or linker-ligated RPFs, perform the reverse transcription reaction and purify cDNA:

6. Denature the sample by heating for 5 min at 65 °C, then place on ice for 1 min.
7. Add 4 μL of 5× First Strand Buffer, 1 μL of SUPERase·In, 1 μL of 0.1 M DTT, and 1 μL of SuperScript III.
8. Incubate for 30 min at 48 °C for the reverse transcriptase reaction.
9. Add 2.3 μL of 1 M NaOH and mix to hydrolyze the RNA template.
10. Incubate for 15 min at 98 °C to inactivate the reverse transcriptase.
11. Add 22.5 μL of 2× denaturing loading dye to each reaction.
12. As a control for size selection of the cDNA product, prepare a sample of 0.5 μL oNTI225 (for polyadenylation method) or oNTI226 (for linker ligation method) at 50 μM, 9.5 μL of water, and 10 μL of 2× denaturing loading dye (*see* **Note 11**).
13. Set up a gel ladder sample by combining 0.5 μL of 10 bp ladder, 9.5 μL of water and 10 μL of 2× denaturing loading dye.

14. Set up a 1.5 mm vertical 10 % TBE-urea polyacrylamide gel in 0.5× TBE (8 cm mini gel, Bio-Rad mini Protean 3 electrophoresis system). Refer to Subheading 3.4, **step 1** for gel preparation, but adjust the amount of acrylamide used to achieve 10 %.
15. Denature samples for 2 min at 75 °C and load on the gel. Each RT reaction will require two lanes, each loaded at ~20 μL per well.
16. Run gel in 0.5× TBE for 65 min at 200 V.
17. Stain for 5 min in SYBR Gold 1:10,000 in 0.5× TBE.
18. Photograph gel.
19. Excise the extended RT product; it should be ~30 nt larger than the RT primer (Fig. 3c).
20. Photograph the cut gel.
21. Recover reverse-transcribed RPF cDNA from each gel slice using the crush-and-soak method as described for RNA (*see* Subheading 3.4, **steps 9–18**), except that DNA gel extraction buffer is used (*see* Subheading 2.5.3, buffer 1).

3.5.5 Circularization of RPF cDNA (Estimated Time 1.5 h)

1. Resuspend pellets from gel-extracted reverse-transcribed RPFs cDNAs from **step 21** of Subheading 3.5.4 in 15 μL of RNase-free water.
2. Add 2 μL of 10× CircLigase buffer, 1 μL of 1 mM ATP, 1 μL of 50 mM $MnCl_2$, and 1 μL of CircLigase (100 U/μL)
3. Incubate for 1 h at 60 °C.
4. Heat inactivate for 10 min at 80 °C.
5. Store circularized RFP cDNA at −20 °C

*3.5.6 rRNA Subtractive Hybridization (Optional) (Estimated Time 2–2.5 h) (See **Note 12**)*

1. Prepare a pool of sense-strand rRNA oligos for the subtraction hybridization by combining 2 μL of each biotinylated oligo (rRNA1 and rRNA2, *see* Table 3) from a 200 μM stock and water to a final volume of 40 μL. This pool can be stored at −20 °C.
2. Combine 5 μL of the circularization reaction from **step 5** of Subheading 3.5.5 with 1 μL of rRNA oligo pool, 1 μL of 20× SSC, and 3 μL of water.
3. In a thermal cycler, denature for 90 s at 100 °C and cool down (0.1 °C s^{-1}) to 37 °C to anneal.
4. Prepare 2× Bind/Wash buffer: 400 μL of 5 M NaCl, 2 μL of 0.5 M EDTA, 10 μL of 1 M Tris pH 7.5, 590 of μL water. Dilute a 500 μL aliquot with 500 μL of water to make 1× Bind/Wash buffer and keep the remainder of the buffer as 2×.
5. Prepare 25 μL of Dynabeads® MyOne™ Streptavidin C1 (*see* **Note 12**) at 10 mg/mL for each subtraction reaction; all beads are prewashed together.

6. Add one volume 1× Bind/Wash buffer.
7. Resuspend in 2× Bind/Wash buffer so that the volume is 10 µL per subtraction reaction.
8. Take one 10 µL aliquot per subtraction reaction, transfer to a new 1.5 mL tube, and equilibrate at 37 °C in a heating block.
9. Add 10 µL of hybridization mix from **step 3** to 10 µL of washed Dynabeads.
10. Incubate for 20 min at 37 °C in a shaking incubator set at 300 rpm.
11. Collect the Dynabeads by placing the tubes in the magnetic rack for 2 min and recover 20 µL of eluate by carefully pipetting subtracted cDNA. Transfer to a new 1.5 mL tube.
12. Add 4 µL of glycogen, 6 µL of 5 M NaCl, and 70 µL of water and mix well.
13. Add 150 µL of isopropanol, mix by pipetting and incubate at −20 °C for 30 min.
14. Centrifuge 16,000 × *g* at 4 °C for 30 min.
15. Add 1 mL of 75 % (v/v) EtOH and vortex quickly. Centrifuge 16,000 × *g* at 4 °C for 10 min, carefully remove all the ethanol and air-dry the pellet (approximately 5 min).
16. Resuspend in 5 µL of water.

3.5.7 PCR Amplification (Estimated Time 2 h)

Two alternative reactions are provided based on our experience. Method I is used when samples are not multiplexed; it was developed for the polyadenylated RPFs (*see* Subheading 3.5.2). Method II was developed for the linker-ligated RPF method (*see* Subheading 3.5.3) and can be employed to multiplex samples for Illumina sequencing.

Method I: PCR reaction for non-multiplexed samples:

1. 16.7 µL of 5× Phusion HF buffer.
2. 1.7 µL of dNTPs, 10 mM each.
3. 0.8 µL of library primers, 50 µM each.
4. 58.4 µL of water.
5. 0.8 µL of Phusion HF polymerase.
6. 5 µL of circularized template from **step 5** of Subheading 3.5.5 or rRNA-subtracted circularized template from **step 16** of Subheading 3.5.6.

Method II: PCR reaction for linker-ligated RPFs and sample multiplexing:

1. 16.7 µL of 5× Phusion HF buffer.
2. 1.7 µL of dNTPs, 10 mM each.
3. 2 µL of universal forward primer, 10 µM.

4. 2 μL of index primer, 10 μM (*see* **Note 13**).
5. 58.8 μL of water.
6. 0.8 μL of Phusion HF polymerase.
7. 5 μL of circularized template from **step 5** of Subheading 3.5.5 or rRNA-subtracted circularized template from **step 16** of Subheading 3.5.6.

For Method I and II

1 Perform PCR amplification using the mix prepared for Method I or Method II with the following process: 30 s at 98 °C; 12–15 cycles of {10 s at 98 °C, 10 s at 60 °C, and 5 s at 72 °C}.

2 Run PCR products on a 3 % (w/v) low-melting agarose gel in 1× TBE. The amplified library should run around 120–125 bp for libraries made with polyadenylation (Subheading 3.2.2) or 170–175 bp for libraries made with linker ligation (Subheading 3.2.3) (Fig. 3d).

3 Gel-purify PCR products with the Qiagen Minelute Gel Extraction kit (28604) according to the manufacturer's protocol. Elute with 10 μL Elution buffer. This eluate is the RPF library.

4 Perform a Bioanalyzer 2011 Expert High Sensitivity DNA Assay to determine the size of the product and quantity. The library product peak should be around 120 nt for the polyadenylation method and 170 nt for the linker-ligation method with indexed primers.

5 Deep sequencing analysis with an Illumina sequencer can be performed on the RFP library DNA in single-end 50 nt sequencing reactions. Analysis of ribosome-protected reads (Ribo-seq dataset) can be accomplished by a series of bioinformatic analyses as described by Ingolia and colleagues [61, 71, 72] and applied to plants [33, 37].

3.6 Library Quality Control by Sanger Sequencing (See Note 14)

This requires dA tailing and cloning into the pGEM T-Easy vector

1. To 1 μL of RPF library DNA, add 1 μL of 10× PCR buffer, 1 μL of 2 mM dATP, 1 μL of Taq polymerase, and 6 μL of water.
2. Incubate for 30 min at 70 °C.
3. Perform standard cloning into pGEM T-Easy according to the manufacturer's recommendations.
4. Select colonies for plasmid isolation (*see* **Note 15**). Isolate plasmid from about ten individual colonies and perform Sanger sequencing.
5. Use BLAST (or TAIR BLAST, http://arabidopsis.org/Blast/) to confirm that ~30 nt mRNA fragments have been obtained and not only rRNA fragments.

4 Notes

1. Autoclave the sucrose cushion solution for maximum of 15 min to keep it clear. It should not turn yellow after autoclaving. Keep the solution at 4 °C for a maximum of 12 weeks.
2. After obtaining the polysome pellet by centrifugation, suction off all of the tube contents except for the pellet. The tube walls should be washed carefully with 0.5–1 mL of RNase-free water, avoiding the pellet. This can be done by rolling the tube on surface of the ice in the bucket. Remove all of the wash solution by suction.
3. The resuspension of polysomes must be done slowly and completely so that the complexes go into solution. This step may take 10 min. It is important to remove any insoluble material that is in the pellet fraction by centrifugation prior to the RNase I digestion step. The sample should remain cold at all times. Poorly resuspended polysomes could result in incomplete RNase I digestion.
4. To measure the OD A_{260}, read the absorbance of 1 μL of resuspended polysomes using a Nanodrop spectrophotometer. The raw value of A_{260} read by the NanoDrop corresponds to the number of OD_{260} units per μL sample. We typically use 2,000 OD_{260} per RNase I reaction. The same amount of polysomes is required for an untreated (non-RNase I digested) sample as a control. It is possible to proceed with less material but it is harder to visualize the RPFs on the gel in Subheading 3.4, **step 7**.
5. Differences in the size of the ribosome footprints and the amount of RPFs that map to untranslated regions may depend upon the efficiency of the RNase I digestion. We recommend performing a sucrose density gradient analysis of the digested polysomes to determine the proportion of monosomes relative to larger polysome complexes to assess the efficiency of digestion. Adjustment of the RNAse I to polysome ratio and digestion time may need to be optimized. For optimization we have used 500–1,000 OD_{260} units of resuspended polysome. The sensitivity of the UV detector can be increased to evaluate these digests.
6. The blank sucrose gradients do not need to be centrifuged. These are analyzed with the gradient fractionator before the centrifuge run ends. They are processed to ensure the UV detector is properly set up and to establish a baseline absorbance profile for the experimental samples.
7. The A_{254} nm UV absorbance profile of the treated and untreated samples is analyzed to identify the fractions containing 80S monosomes (Fig. 2). To determine which fractions correspond to the 80S monosomes in the RNAse I digested polysome

sample after sucrose gradient centrifugation, typically 5 μL of each fraction is diluted in 0.5 mL water the absorbance of the sample is analyzed at A_{260} nm in a standard spectrophotometer. The plotted data should resemble the gradient profile allowing the 80S fractions to be identified.

8. For the isolation of RPFs by use of TRAP, we recommend starting with the amount of extract corresponding to 60–80 μg of total RNA, as measured in Subheading 3.3.1. The procedure of RPF isolation is described for isolation from 60 to 80 μg of digested polysomal RNA (includes rRNA, mRNA, and some tRNA) obtained by either differential centrifugation or TRAP. The same amount of plant extract yields on the order of 2.5–3 mg of total RNA. We have not determined a lower limit of starting ODA_{260} of pelleted polysomes that can be used for RPF library construction, but little as 20 μg of RNA obtained by TRAP from roots of a *35S:His$_6$FLAG-RPL18* transgenic can be used for RPF library construction. The amount of tissue needed will increase when using a genotype that expresses *His$_6$FLAG-RPL18* in a low abundance cell type [10, 70].
9. TRAP will work when the initial extraction is performed with 2–6 mL of PEB to 1 mL of pulverized frozen tissue and in the purification of epitope-tagged ribosomes from low abundance cell types [10, 70]. It is important that the bead binding capacity exceed the number of epitopes (i.e., FLAG-tagged protein) in the sample.
10. Use either Subheading 3.5.2 or 3.5.3 after deadenylation of the RFPs. In Subheading 3.5.2, RPFs are 3′ polyadenylated by an enzymatic reaction to facilitate library construction; in Subheading 3.5.3, a linker that is typically used in small RNA library construction (i.e., miRNA) is directly ligated to the RNA.
11. Use the same primer that was used in the reverse transcriptase reaction; this differs for the polyadenylated versus linker-ligated method of RPF preparation.
12. We observe 60–80 % rRNA sequence reads in RPF libraries generated from whole seedling tissues. Most of the contamination is from fragments of 18S rRNA, but there are also contaminating fragments from chloroplast and mitochondrial rRNAs. This is the main limitation to the Ribo-seq technology. To reduce the rRNA fragments in the library, we perform a subtractive hybridization step that is based on the abundant ~30 nt rRNA fragments that we observed in seedling and rosette tissue RPF libraries. The method utilizes biotinylated primers that hybridize to the rRNA fragments and Streptavidin coated magnetic beads. Commercial products for subtraction of rRNA from plant RNA samples have not worked effectively

for removal of the contaminating fragments. Users may need to determine the prevalent rRNA contaminating fragments in their samples, especially if the method is applied to other plant species. Although the Sanger sequencing of cloned library constructs will provide information about prevalent rRNA fragments (*see* **Note 14**), the identification of the prevalent rRNAs is better assessed from deep sequencing. Once contaminating rRNAs are identified, oligos can be designed to improve the rRNA subtraction. As implemented, we use only 25 % of the RPF library. However, the amount used might be increased if the amount of starting material or RPF yield is lower than suggested in **Note 8**.

13. If samples are to be multiplexed, use a different index linker primer from Table 3 for each independent RPF sample.
14. Sequencing of about ten clones per library using Sanger sequencing before performing the high-throughput sequencing of the Ribo-seq libraries. This number of clones is sufficient for a rough idea of ratio of mRNA footprint to rRNA contamination. Typically the mRNA footprints found in these ten clones are from abundant transcripts.
15. In the blue/white colony selection, we have seen some light blue colonies containing a library insert.

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