Chapter 9

Translating Ribosome Affinity Purification (TRAP) Followed by RNA Sequencing Technology (TRAP-SEQ) for Quantitative Assessment of Plant Translatomes

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Abstract

Translating Ribosome Affinity Purification (TRAP) is a technology to isolate the population of mRNAs associated with at least one 80S ribosome, referred as the translatome. TRAP is based on the expression of an epitope-tagged version of a ribosomal protein and the affinity purification of ribosomes and associated mRNAs using antibodies conjugated to agarose beads. Quantitative assessment of the translatome is achieved by direct RNA sequencing (RNA-SEQ), which provides accurate quantitation of ribosome-associated mRNAs and reveals alternatively spliced isoforms. Here we present a detailed procedure for TRAP, as well as a guide for preparation of RNA-SEQ libraries (TRAP-SEQ) and a primary data analysis. This methodology enables the study of translational dynamic by assessing rapid changes in translatomes, at organ or cell-type level, during development or in response to endogenous or exogenous stimuli.

Key words Ribosome immunopurification, Polysomes, Translatome analysis, Cell-type specific gene expression, RNA-seq, Alternative splicing

1 Introduction

High-throughput RNA sequencing (RNA-SEQ) technologies have extended the possibilities of transcriptome studies beyond the microarray hybridization approaches by allowing the discovery and quantification of transcript splice isoforms of all expressed genes. Quantitative RNA-SEQ data reflect gene regulation occurring at multiple strata within eukaryotic cells including chromatin-based, transcriptional and post-transcriptional events [1]. After transcription by RNA polymerase II, mRNAs are spliced, capped and polyadenylated in the nuclei and transported through the nuclear pores to the cytoplasm. Once in the cytoplasm, mRNAs can be either recruited by the translational machinery and became incorporated into 80S mono- and >80S poly-ribosomes (herein

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referred to as monosomes and polysomes, respectively), stored into translationally inactive messenger ribonucleoprotein complexes (mRNPs), or sequestered into processing bodies (PBs), which are large mRNPs characterized by the presence of decapping enzymes and 5′- to- 3′ exoribonucleases [2–4]. Due to technical limitations, most transcriptomic studies have been carried out using total cellular RNA. However, the quantification of steady-state mRNA abundance (the transcriptome) does not necessarily reflect the protein production because of post-transcriptional regulation. A more accurate approach is to quantify the abundance of mRNAs associated to actively translating ribosomes, which is referred to as the translatome [5, 6].

Isolation of mRNAs associated with ribosomes and polysomes has been conventionally achieved by differential ultracentrifugation through sucrose density gradients [7]. This methodology requires specialized equipment such as an ultracentrifuge, UV light detector and gradient fractionation system; it is time-consuming and does not allow handling of large numbers of samples in parallel. In addition, since the conventional purification and fractionation of polysomes is based on the sedimentation coefficient, other large mRNPs complexes, such as PBs, can co-fractionate with polysomes [8]. An alternative method has been developed to specifically isolate cytoplasmic ribosomal complexes, i.e., the population of mRNAs associated with at least one 80S ribosome [9]. This method, known as Translating Ribosome Affinity Purification (TRAP), is based on the expression of a tagged cytoplasmic ribosomal protein (RP) exposed to the solvent side of the ribosome. The RP is fused to a small epitope (FLAG, HA, c-myc, etc.) and becomes incorporated into ribosomes [1]. In plants, amino-terminally FLAG-epitope-tagged RPL18 has proven to be a suitable target of epitope tagging [9–11]. This small epitope does not interfere with ribosome function or the assembly of large polysomal complexes, providing a means for simple affinity purification of these complexes using antibodies conjugated to agarose or magnetic beads. mRNAs associated with ribosomes are isolated and then converted into cDNAs for quantitative PCR analysis, microarray hybridization or used for the preparation of cDNA libraries that can be sequenced using next-generation RNA sequencing technologies (RNA-SEQ) [9, 10, 12–14]. A recent report also described the use of TRAP samples to reveal translational dynamics by precisely mapping individual ribosomes to mRNAs (referred to as ribosome footprinting) [14].

The expression of a FLAG-tagged version of cytosolic RPL18 has been used in plants (e.g., *Arabidopsis thaliana*, *Solanum lycopersicum*, and *Medicago truncatula*) to affinity purify monosomes and polysomes from different organs, including mature leaves, flowers, whole seedlings, as well as shoots and roots of young seedlings [9, 10, 12, 13, 15]. Moreover, TRAP has been used

successfully to isolate mRNAs associated to polysomes from specific cell or tissue types of both vegetative and reproductive organs [5, 13, 16]. Unlike other methods that require tissue micro-dissection or sorting of cells expressing a fluorescent protein, TRAP has the advantage of reflecting the translational state of an mRNA by direct measurement of its association with ribosomes and presumed translation at the time of tissue collection. This allows the evaluation of rapid changes in mRNA association with ribosomes, which can be rapidly modulated in response to environmental signals and during development. For example, TRAP has been used to compare the translatomes in response to diverse environmental stimuli, such as low oxygen availability [5, 12], cold stress [17], pathogen infection [18], and also during photomorphogenesis [19–21] or pollen growth [16]. These changes were monitored using the Affymetrix DNA microarray technology (www.affymetrix.com). Later, Jiao and Meyerowitz combined TRAP with RNA-SEQ (referred to as TRAP-SEQ) to characterize the translatomes of three different domains of early developing flowers [13]. More recently, TRAP-SEQ was used to investigate translational regulation in seedlings of *Arabidopsis thaliana* subjected to a short period of hypoxia stress [14]. In addition, TRAP has been used to monitor changes in association with ribosomes of individual mRNAs and small RNAs in roots of the model legume *M. truncatula* at early stages of the nitrogen fixing symbiosis with *Sinorhizobium meliloti* [10]. This analysis was expanded to a genome-wide scale using Illumina RNA-SEQ technology (www.illumina.com) to quantify and resolve, at the nucleotide level, changes in the translatomes of *M. truncatula* roots upon rhizobia infection.

Herein, we present a detailed procedure for TRAP, RNA purification, and quantitative assessment of translatomes using RNA-SEQ technology. This procedure allows the generation of RNA-SEQ libraries starting with as little as 400 ng of polysomal RNA and without the introduction of a linear RNA amplification step. We also provide a guide for primary data analysis of TRAP-SEQ.

1.1 Overview of the TRAP-SEQ Protocol

The TRAP-SEQ procedure includes several steps that have been optimized for plant tissues (Fig. 1). The first step is to generate plants that express the FLAG-tagged version of the RPL18 protein in the tissue of interest and verify the incorporation of the tagged protein into the 60S ribosomal subunit, 80S monosomes and small to large polysomes [9, 10]. The tissue is collected and homogenized in a buffer that maintains the polysome integrity. After homogenization, the tissue extract is clarified by centrifugation and incubated with anti-FLAG agarose beads. Polysomes are eluted by competition with an excess of 3× FLAG peptide. Next, purification and quality check are performed on the RNA sample, which contains mRNA, rRNA and a fraction of the tRNAs.

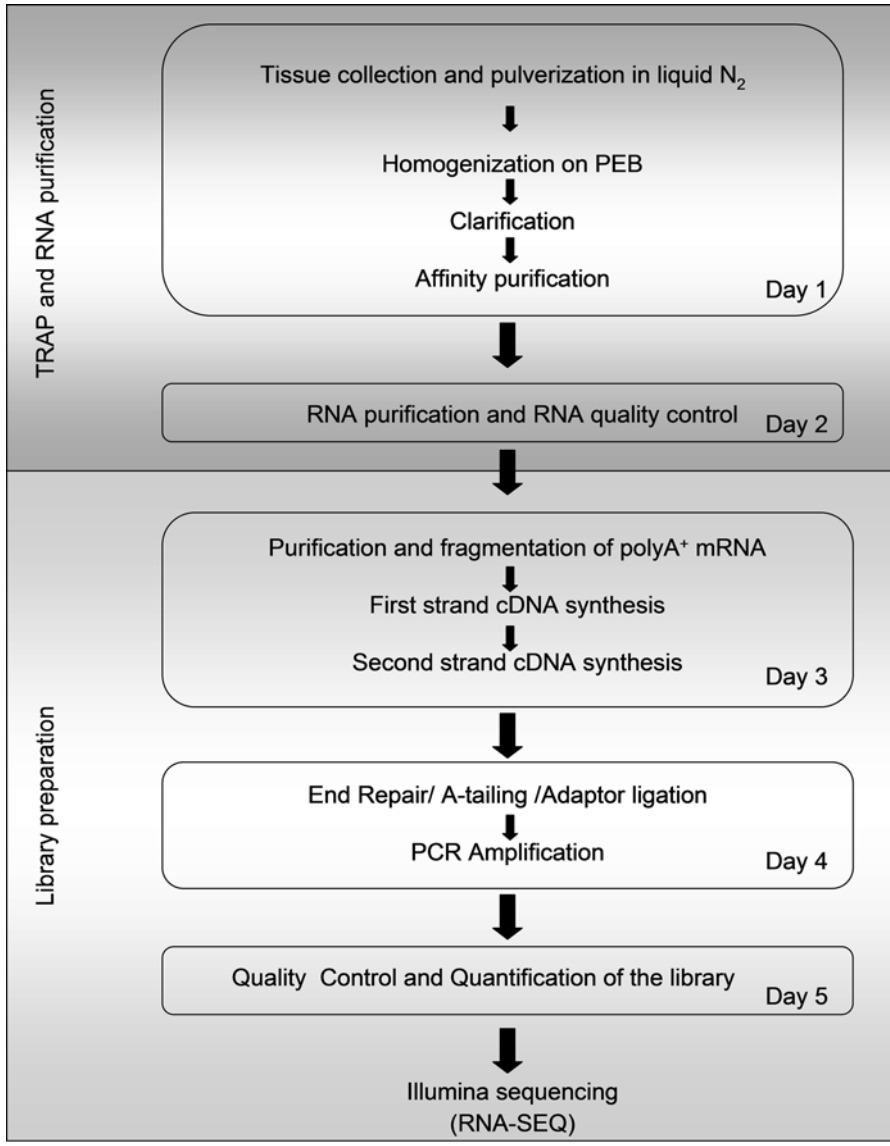

Fig. 1 Schematic representation of Translating Ribosome Affinity Purification (TRAP) and library preparation for RNA sequencing (RNA-SEQ) procedure (TRAP-SEQ). Different steps in a typical TRAP experiment, RNA purification and sample processing to obtain Illumina-compatible cDNA libraries for high-throughput RNA-SEQ are shown. PEB is Polysome Extraction Buffer

Library preparation for RNA-SEQ begins with the purification and fragmentation of the polyA+ RNA. The resulting RNA fragments are copied into cDNAs, which are then subjected to end-repair, dA tailing (addition of a single A base in their 3′ end) and ligated to specialized adapters. If more than one library is generated, different indexed (bar-coded) adapters can be used for each library, allowing the combination of multiple libraries in a single sequencing run (multiplexing). Ligation products are purified and

enriched by PCR to obtain the final cDNA library. The quality of the libraries is tested by capillary electrophoresis and quantification of the DNA library templates is performed by qPCR. In this protocol, cDNA libraries are sequenced using the Illumina technology.

1.2 Generation and Analysis of Transgenic Plants

Expression of the FLAG-tagged RPL18 in plant tissues can be achieved by stable or transient transformation methods. *Agrobacterium tumefaciens*-mediated transformation using the floral dip method was used to generate stable *Arabidopsis thaliana* lines that expressed FLAG-RPL18B in nearly all cell types (i.e., using the *CaMV35S* promoter) or in specific cell types of the root or shoot [9, 11], developing microspores and mature pollen [16]. In addition, expression of FLAG-RPL18 was achieved in hairy roots generated by *A. rhizogenes*-mediated transformation of *M. truncatula* [10] and *S. lycopersicum* [22] plants. When the technology is transferred to other plants, it is desirable to use an *RPL18* ortholog from that species. Accumulation of the tagged protein can be confirmed by Western blot analysis using a commercial anti-FLAG antibody. It is also important to confirm the incorporation of the FLAG-tagged protein into ribosomes and polysomes (*see* ref. 9, 10, 23, 24).

1.3 Tissue Harvesting, Homogenization, and Affinity Purification

The amount of tissue required to obtain enough polysomal RNA material for library preparation depends on the organ to be used and the promoter that drives the expression of the FLAG-RPL18 protein. For shoots, 1 mL of packed pulverized tissue was sufficient to obtain 1,000–1,500 ng of polysomal RNA in Arabidopsis, whereas for both Arabidopsis and Medicago roots, the same amount of tissue yielded 200–600 ng when the FLAG-RPL18 protein was expressed under the *CaMV35S* promoter [10, 25]. The tissue is then homogenized in a buffer that maintains the integrity of ribosomes and polysomes, but disrupts their association with the endoplasmic reticulum and the cytoskeleton. The homogenate is centrifuged and filtered to eliminate any insoluble material, which may represent a major source of undesirable contamination in TRAP experiments. The supernatant is the clarified cellular extract. An aliquot of this extract is saved for total RNA extraction and the rest is mixed with anti-FLAG-agarose beads. The volume of agarose beads to be used depends on the starting amount of tissue and the expression levels of the FLAG-RPL18 protein. For *A. thaliana* and *M. truncatula*, we recommend using 40 μL of beads per mL of packed tissue when the FLAG-RPL18 protein is expressed under the control of the *CaMV35S* promoter. The clarified cellular extract is incubated with the anti-FLAG agarose beads and then polysomes are eluted from the beads by the addition of a solution of 3X FLAG-peptide. As an alternative to the use of agarose beads, TRAP can be performed using magnetic

(Dyna beads, Invitrogen, www.lifetechnologies.com) or spherical microbeads (COOH-microbeads 1 μm, Polysciences, www.polysciences.com). The efficiency of the TRAP procedure can be evaluated by loading aliquots of the clarified cellular extract, the unbound fraction and the eluted material onto an SDS-PAGE followed by immunoblot with the anti-FLAG antibody [9, 10]. Levels of FLAG-tagged RPL18 in the unbound fraction should be negligible. It is also advisable to confirm that the affinity-purified RPL18 is efficiently released from the beads.

1.4 RNA Purification and RNA-SEQ Library Preparation

The RNA is purified from the clarified cellular extract or the eluted polysomes using the TRIzol reagent (Invitrogen). Alternatively, RNA can be purified using solid-phase purification systems, like glass (silica)-fiber filters; however, these systems usually do not efficiently recover small RNAs. In our hands, the TRAP procedure yielded at least 200 ng of total RNA per mL of pulverized tissue when strong promoters were used to drive the expression of the FLAG-tagged RPL18 (i.e., *CaMV35S*). However, when FLAG-RPL18 is controlled by a weak promoter or a promoter expressed just in a few cells, the yield of TRAP could be as little as 10 ng of RNA per mL of tissue (e.g., with use of promoters such as *pCO2*, *pSUC2*, *or pSultr2-1*) [25]. In this case, a significantly higher amount of tissue is required to recover at least the 100 ng of RNA recommended as starting material by the TruSeq RNA Sample Preparation kit v2 manual. We have started with as little as 400 ng of TRAP RNA, which was sufficient to obtain libraries of enough quality for RNA-SEQ (Fig. 2a).

In this protocol, preparation of the library is carried out using the TruSeq RNA Sample Preparation kit v2 essentially following manufacturer's instructions (Illumina). Some aspects of the protocol should be carefully considered during library preparation. One of them is the time and temperature of the RNA fragmentation step: eight minutes is optimal to obtain plant RNA-SEQ libraries with a median insert size of 150 bp. The second one is the final concentration of the adapters to avoid their concatenation during

Fig. 2 (continued) respectively, in the ligation reaction. DNA samples were purified with AMPure XP beads and analyzed using the DNA 1000 kit in a 2100 Bioanalyzer (Agilent). The black arrow points to the mean of the major peak at 261 bp, which corresponds to the average size of the final products of Total and TRAP RNA-SEQ libraries. Green and purple arrows point to internal markers of the DNA 1000 kit. (**b**) An example of a library prepared with 400 ng of TRAP RNA using undiluted adapters. Peaks corresponding to adapter concatemers are indicated in the electropherogram and on the right of the capillary electrophoresis with brackets. (**c**) Electrophoresis on a 1.5 % (w/v) agarose gel of a library prepared using AMPure XP beads during the DNA purification steps, which excludes DNA fragments <200 bp (*left*), and a library prepared using silica columns, which retain small DNA fragments resulting in the formation of adapter dimers that are amplified during the PCR step (*right*). The arrow points to a strong band corresponding to adapter dimers. The size of the 50 and 100 bp ladder DNA fragments are indicated

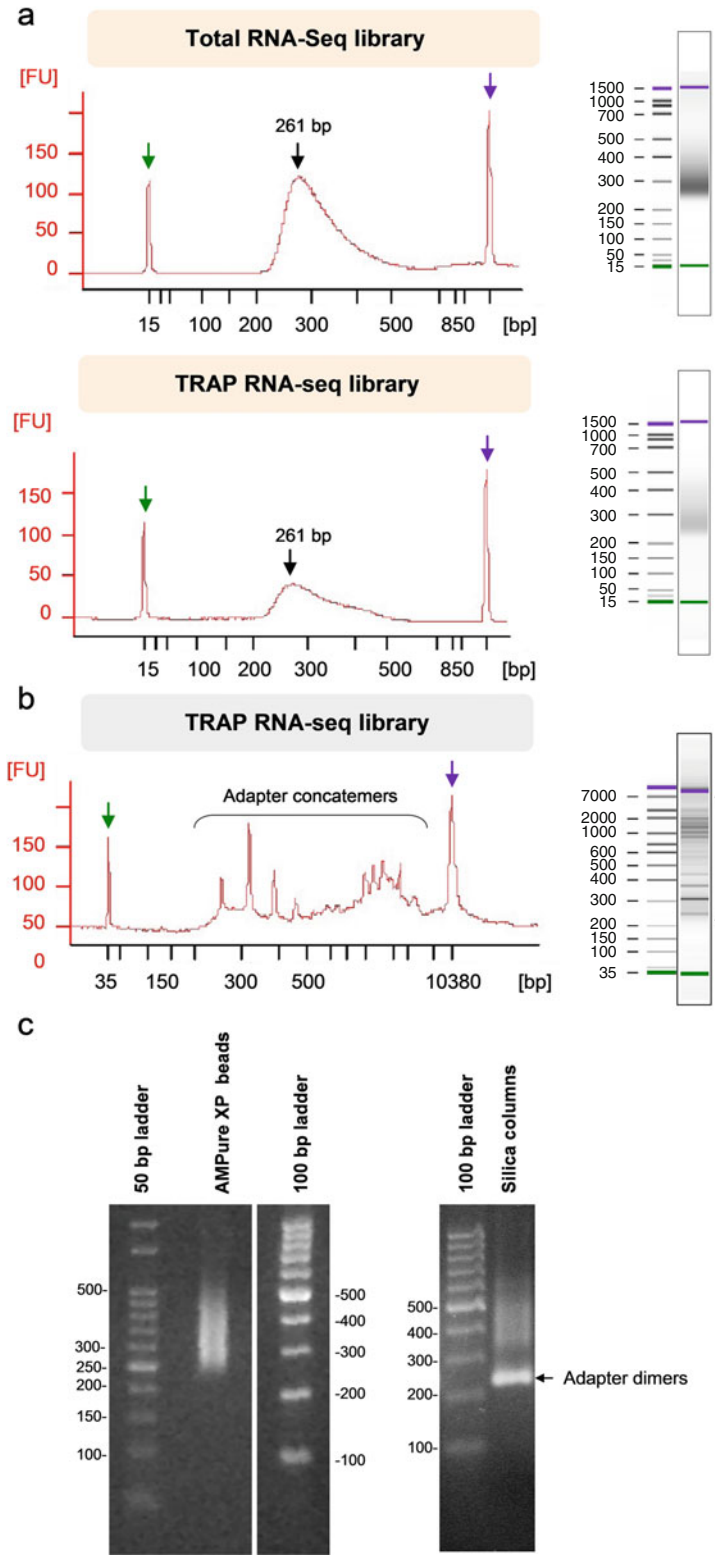

Fig. 2 Quality control of Total and TRAP cDNA libraries. (**a**) Examples of electropherograms of cDNA libraries of sufficient quality for RNA-SEQ prepared from 800 ng of Total and 400 ng of TRAP RNA samples using 1:4 and 1:8 dilutions of adapters,

the ligation step when using less than 1 μg of RNA. The TruSeq Sample Preparation kit v2 does not include a gel-based size selection step, therefore, it is crucial to use AMPure XP magnetic beads (Beckman Coulter, www.beckmancoulter.com) for DNA fragment purification in order to avoid an excess of adapters that can form dimers or concatemers (Fig. 2b, c). Unlike other kits based on column purification, the AMPure XP beads purification system excludes small DNA fragments (<200 bp). Alternatively, other Illumina-compatible kits, such as NEXTflex RNA-Seq Kit (Bioo Scientific, www.biooscientific.com), NEBNext Ultra RNA Library Prep Kit for Illumina (New England BioLabs Inc., www.neb.com), or ScriptSeq mRNA-Seq Library Preparation Kit (Epicentre, http://www.epibio.com), can be used for library preparation. In addition, small RNA sequencing kits such as TruSeq Small RNA Sample Preparation Kit (Illumina) or NEXTflex Small RNA Sequencing Kit (Bioo Scientific) can be adapted to obtain TRAP-SEQ libraries that are strand-specific, such as those described by Juntawong et al. [14]. Once the libraries are obtained, the quality is evaluated by capillary electrophoresis using the Agilent DNA1000 kit and the Agilent Technologies 2100 Bioanalyzer. It is also advisable to quantify the cDNA molecules present in the library using qPCR according to the Illumina Sequencing qPCR quantification Guide (Illumina). After quantification, libraries can be multiplexed prior to sequencing in order to be sequenced in a single lane. If multiplexing, a set of compatible adapters should be chosen during library preparation, each library should be normalized at 10 nM and then equal volumes of each library should be pooled. Multiplexed libraries can be sequenced in an Illumina NextSeq500 or HiSeq2000/2500 system.

1.5 Primary TRAP-SEQ Data Analysis

An advantage of the RNA-SEQ approach as compared with the array hybridization technology is that it allows de novo identification of transcript isoforms at single base resolution. When combined with the TRAP technology, RNA-SEQ allows the discrimination of splice isoforms that might be differentially associated to actively translating ribosomes. This requires efficient and robust algorithms for data analysis and prediction of alternative splice events. In the past years, several computational tools have been developed for RNA-SEQ analyses including read mapping, transcript assembly (including the discovery of new splice variants), and quantification of gene expression [26]. Here, we focus on the primary data analysis that begins with the quality check of reads and ends with the visualization of reads using the Integrative Genomics Viewer (IGV) [27, 28]. In this analysis, Tophat2 [29] is used to align reads to the reference genome and discover new splice variants, Cufflinks and Cuffmerge [30] to assemble transcripts, and then Cuffdiff [30] to identify genes and transcripts with differential abundance in Total or TRAP RNA samples

between two different biological conditions (e.g., non-inoculated and *S. meliloti*-inoculated roots of *M. truncatula*). The recently developed CummeRbund program [31] is used to integrate the data produced by Cufflinks package. This program helps to manage and explore gene expression by plotting and clustering expression data. Finally, we used IGV to visualize reads mapped to the reference genome and gene models [27, 28]. The comparison of TRAP samples should reveal differential regulation of transcript abundance in polysomes between two or more different biological conditions. When the *FLAG-RPL18* construct is driven by a quasi-constitutive promoter (i.e., *CaMV35S*), then the fold change of individual mRNAs in the TRAP samples can be compared with those of total cellular samples to discern changes in translation state. Such an analysis is expected to identify genes that are upregulated, downregulated, or non-regulated at the level of mRNA association with ribosomes. Figure 3 shows examples of each of these categories. It is important to note that mRNAs with

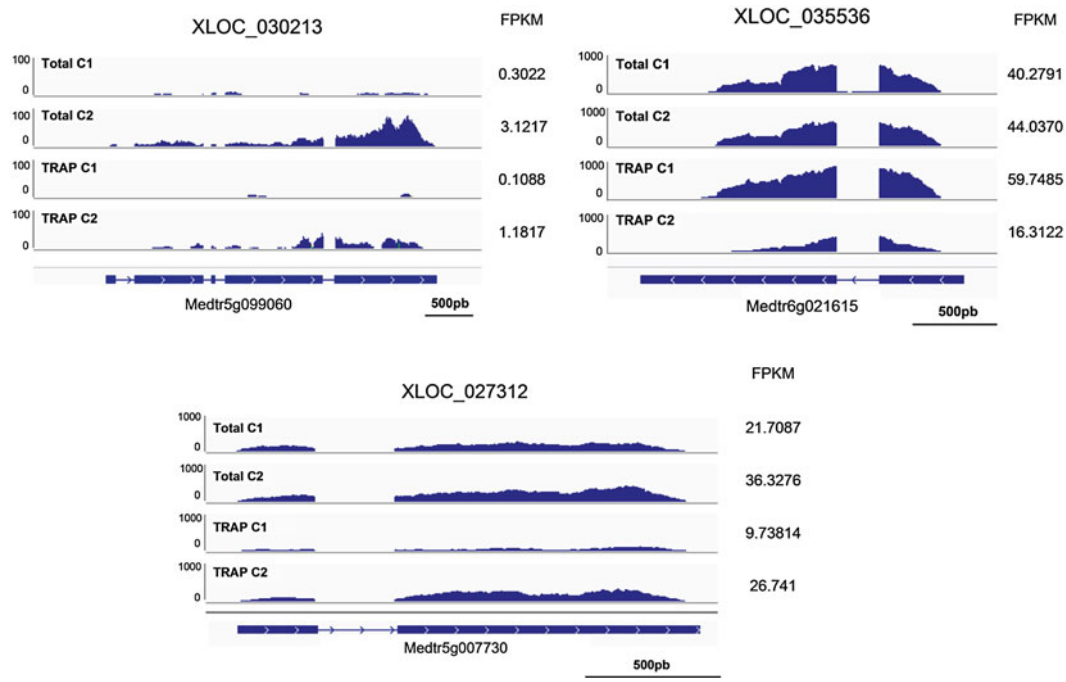

Fig. 3 Examples of Integrative Genomics Viewer visualization for three different genes in Total and TRAP samples under two different conditions, marked as C1 and C2. XLOC_030213 (Medtr5g099060) encodes the nodule inception (NIN) transcription factor and represents an example of a transcript that increases similarly in both Total and TRAP RNA samples in C2 versus C1. XLOC_ 035536 (Medtr6g021615) encodes a nodule cysteine-rich (NCR) secreted peptide and is an example of transcripts that do not significantly change in the Total RNA sample, but significantly decreases in the TRAP sample in C2 as compared with C1. XLOC_027312 (Medtr5g07730), which encodes a member of the chalcone and stilbene synthase family of proteins, is a transcript that significantly increases in TRAP sample in C2 versus C1. FPKM: fragments per kilobase of transcript per million of fragments mapped. *bp* base pairs

short upstream open reading frames (uORFs) might increase their association with translating ribosome, but this increase does not necessarily indicate a higher level of translation of the main ORF (mORF). The use of ribosome profiling analysis in *A. thaliana* confirmed that uORFs often function to limit the number of ribosomes on the mORF, therefore dampening translation of the protein-coding region [14]. Thus, an increase in the level of association with polysomes should not be considered as a strict indication of higher translational status. TRAP-SEQ can also quantify splice isoforms that are differentially associated with ribosomes under two different biological conditions. These splicing isoforms can have a contribution to gene expression that is distinct in the Total and TRAP mRNA samples in different conditions or treatments.

1.6 Experimental Design

An important consideration in the TRAP-SEQ experimental design is that the RNA extraction of total cellular or TRAP RNA samples from the different conditions, as well as library preparation from these RNA samples, should be performed at the same time in order to minimize technical variation. Once the Total and TRAP RNA have been isolated, and before library preparation, marker genes known to be induced in a particular condition or in response to an environmental stimulus can be evaluated by RT-qPCR. In order to reduce biological variability, it is important to make a robust experimental design that includes Total and TRAP libraries from two or more biological replicates of each condition. Multiplexing of libraries made with different barcoded adaptors makes it possible to combine several libraries in the same sequencing lane without increasing the sequencing costs. Single-end read (i.e., sequencing one end of the DNA fragment) or paired-end (i.e., sequencing both ends of the same DNA fragment) and the read length are also major considerations in the experimental design. Paired-end read sequencing is almost twice the cost of single-end read sequencing, but provides a more accurate transcript assembly and quantification. When the experimental design prioritizes the discovery of new splice variants or the discrimination among different members of the same gene family, longer reads (75 bp or more) are generally preferred over shorter ones. For a species with genomes less rigorously annotated than *A. thaliana* (e.g., *M. truncatula*), the use of long paired-end reads can ensure that transcript splice isoform are both discovered and quantified.

2 Materials

2.1 Plant Material, Reagents, and Kits

1. Plant material: fresh tissue expressing FLAG-RPL18 (e.g., *CaMV35S:FLAG-RPL18* roots).
2. EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, www.sigmaaldrich.com, cat. # F2426).

3. 3× FLAG® Peptide (Sigma, cat. # F4799).
4. TRIzol Reagent (Invitrogen, www.lifetechnologies.com/invitrogen, cat. # 15596).
5. Miracloth (Calbiochem, www.merckmillipore.com/life-science-research/calbiochem, cat. # 475855-1R).
6. Milli-Q water.
7. Chloroform.
8. Isopropanol.
9. 96 % (v/v) Ethanol.
10. Tris base, molecular biology grade.
11. KCl.
12. $MgCl_2$.
13. Ethylene glycol-bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.
14. Polyoxyethylene (10) tridecyl ether (PTE, Sigma, cat. # P2393).
15. Sodium deoxycholate (DOC, Sigma, cat. # D6750).
16. Polyoxyethylene (20) sorbitan monolaurate known by the common commercial brand name TWEEN® 20 (Sigma-Aldrich, cat. # P1379).
17. Triton™ X-100 (Sigma, cat. # T8787).
18. IGEPAL® CA-630 (Sigma, cat. # I8896).
19. Brij-35 (Sigma-Aldrich, cat. # P1254).
20. Sodium deoxycholate (Sigma-Aldrich, cat. # D6750).
21. Phenylmethylsulfonyl fluoride (PMSF, Sigma, cat. # P7626).
22. Cycloheximide (Sigma, cat. # O1810).
23. Chloramphenicol (Sigma, cat. # C0378).
24. Dithiothreitol (DTT, Sigma, cat. # D9779).
25. Glycogen (Invitrogen, cat. # 10814-010).
26. TruSeq RNA Sample Preparation Kit v2 (Illumina, www.illumina.com, cat. # RS-122-2001).
27. SuperScript II Reverse Transcriptase (Invitrogen, cat. # 18064-014).
28. Agencourt AMPure XP Beads (Beckman Coulter, www.beckmancoulter.com, cat. # A63881).
29. Agilent RNA 6000 Pico kit (Agilent Technologies, www.agilent.com, cat. # 5067-1511).
30. Agilent DNA 1000 kit (Agilent Technologies, cat. # 5067-1504).

2.2 Stock Buffers and Solutions

The following solutions need to be autoclaved:

1. 2 M Tris–HCl pH 9.0 adjust to pH using 36 % (v/v) HCl (*see* **Note 1**).
2. 2 M KCl.
3. 0.5 M EGTA pH 8.0, adjust to pH 8.0 using NaOH pellets (*see* **Note 2**).
4. 1 M $MgCl_2$.
5. 20 % (v/v) Detergent mix: dissolve 4 g Brij 35 by heating to about 60 °C in 4 mL of Triton X-100, 4 mL of Igepal CA-630 and 4 mL of Tween-20, fill up with Milli-Q water to 20 mL (*see* **Note 1**).
6. 20 % (v/v) PTE: shake the stock solution before pipetting PTE. Protect from light.
7. 10 % (w/v) DOC.
8. Tris-buffered saline (TBS): 50 mM Tris–HCl pH 7.4, 150 mM NaCl.
9. TE: Tris–HCl 10 mM EDTA 5 mM pH 8.0.

The following solutions are not autoclaved and are stored in aliquots at −20 °C.

1. 0.5 M DTT (*see* **Note 1**).
2. 100 mM PMSF: dissolve 87 mg of PMSF in 3.5 mL of isopropanol. Fill up with isopropanol to 5 mL (*see* **Note 1**).
3. 50 mg/mL Cycloheximide: dissolve 50 mg of cycloheximide in 1 mL of 96 % (v/v) ethanol (*see* **Note 1**).
4. 50 mg/mL Chloramphenicol: dissolve 50 mg of chloramphenicol in 1 mL of 96 % (v/v) ethanol (*see* **Note 1**).
5. 5 mg/mL 3× FLAG peptide: dissolve 4 mg in 0.8 mL of sterile TBS. Avoid successive freeze–thaw cycles.

2.3 Buffers for TRAP

The following buffers are prepared fresh the day of use from stock solutions (described in the previous Subheading 2.2) and kept on ice along the procedure.

1. Polysome extraction buffer (PEB): 200 mM Tris–HCl pH 9.0, 200 mM KCl, 25 mM EGTA pH 8.0, 35 mM $MgCl_2$, 1 % (v/v) Detergent mix, 1 % (v/v) PTE, 5 mM DTT, 1 mM PMSF, 50 μg/μL cycloheximide, and 50 μg/μL chloramphenicol. Keep the buffer on ice for 10 min before adding DTT, PMSF, cycloheximide, and chloramphenicol. Include 1 % (w/v) DOC when working with seeds or mature leaves to improve the disruption of ribosome–cytoskeleton association in these tissues (*see* **Note 3**).

2. Bead Wash buffer (BWB): 200 mM Tris–HCl pH 9.0, 200 mM KCl, 25 mM EGTA pH 8.0, 35 mM $MgCl_2$.
3. Wash buffer (WB): 200 mM Tris–HCl pH 9.0, 200 mM KCl, 25 mM EGTA pH 8.0, 35 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 50 μg/μL cycloheximide, and 50 μg/μL chloramphenicol. Keep the buffer on ice for 10 min before adding DTT, PMSF, cycloheximide, and chloramphenicol.
4. Elution Buffer: WB containing 200 ng/μL 3× FLAG peptide.

2.4 Equipment

1. Mortar and pestle. The recommended mortar size is 8 × 5 cm (diameter × height).
2. Teflon pestle and glass tube homogenizer (Sigma, cat. # P7859).
3. Standard glass centrifuge tubes, 50 mL (e.g., Pirex 50 mL round bottom centrifuge tubes, Corning, cat. #8422-50).
4. Standard microfuge tubes, 1.5 mL.
5. Standard thin-wall PCR tubes, 0.2 mL.
6. Low retention microfuge tubes, 1.5 mL (e.g., Nonstick RNAse free microfuge tubes, 1.5 mL Life technologies, cat # AM 12450).
7. Standard refrigerated centrifuges for microfuge tubes and conical tubes (e.g., Eppendorf Centrifuge 5804R).
8. Standard lab centrifuges for 50 mL tubes (e.g., Thermo Scientific Sorvall RC6 Plus).
9. Water purification system (e.g., Barnstead Easypure II RF/UV Ultrapure Water System, Thermo Scientific).
10. Standard thermocycler.
11. Back-and-forth shaker (e.g., Everlast Rocker, www.benchmarkscientific.com, cat. # BR5000).
12. NanoDrop (e.g., Thermo Scientific ND-1000 UV–Vis Spectrophotometer).
13. Agilent 2100 BioAnalyzer (Agilent Technologies, cat. # G2939AA).
14. NEXtSeq500 or HiSeq2000/2500 system (Illumina).
15. RNA-SEQ data analysis: 64-bit computer with at least 1 Tb hard disk and 16 Gb of memory.

3 Methods

All steps in this protocol are carried out with solutions and materials free of nucleases at 4 °C. Pipette tips, microcentrifuge tubes, glassware, and Miracloth must be sterilized by autoclaving.

3.1 Tissue Collection and Polysome Extraction

1. Tissue from transgenic plants expressing FLAG-RPL18 should be flash-frozen in liquid nitrogen and ground to a fine powder maintaining the tissue frozen at all time. Tissue may be collected from the whole plant, a specific organ, or a dissected region (i.e., root tips). A homogenous pulverization is accomplished with a porcelain mortar and pestle. This step takes approximately 30 min for four samples. The tissue can be kept at –80 °C until use.
2. Add approximately one volume of frozen packed pulverized tissue to two volumes of PEB and mix with a glass bar. The amount of tissue depends on the quantity of cells expressing FLAG-RPL18. In the case of strong promoters, such as *CaMV35S*, 5 mL of packed pulverized tissue is enough to get RNA for library preparation using the TruSeq RNA Sample Preparation Kit v2.
3. Let the mixture stand on ice while thawing.
4. Transfer the mixture to a glass homogenizer and make ten strokes with the teflon pestle to homogenize the mixture (*see* **Note 4**).
5. Incubate the mixture on ice for 10 min.
6. Transfer to a 50 mL centrifuge tube and centrifuge the samples at 16,000 × *g* for 15 min at 4 °C.
7. Filter the samples using Miracloth and transfer the supernatant to a new tube. It is recommended to centrifuge the samples again in the same manner to ensure removal of any insoluble material. This is the supernatant of 16,000 × *g* (SN-16).
8. Save a small aliquot (200–400 µL) of the supernatant (SN-16) to isolate total RNA. Keep the rest on ice for affinity purification of polysomes.

Steps 2–8 take approximately 1.5–2 h.

3.2 Preparation of the α-FLAG M2 Agarose Beads

These steps can be done during the centrifugation of polysomal extracts described above and take approximately 10 min.

1. Make a uniform suspension of the EZview™ Red ANTI-FLAG® M2 Affinity Gel (α-FLAG M2 agarose beads) by gently shaking the stock container from side to side by hand. Transfer 200 µL of the beads (50 % [v/v] slurry) to a new 1.5 ml microfuge tube. Use cut pipette tips to facilitate the pipetting of agarose beads.
2. Add 1.5 mL of ice-cold BWB on ice and mix thoroughly by gentle manual agitation.
3. Centrifuge at 8,200 × *g* for 1 min at 4 °C.
4. Discard the supernatant and add 1.5 mL of ice-cold BWB to repeat the wash step.
5. Centrifuge at 8,200 × *g* for 1 min at 4 °C.

3.3 Affinity Purification of Polysomes

The following steps take approximately 4 h.

1. Add 200 µL of washed α-FLAG M2 agarose beads (50 % [v/v] slurry) to the SN-16 in a 15 mL plastic conical tube.
2. Incubate for 2 h at 4 °C with gentle back and forth rocking or gyration to bind the FLAG-tagged ribosomes to the affinity beads (*see* **Note 5**).
3. Centrifuge at 8,200 × *g* for 2 min at 4 °C.
4. Transfer the supernatant to a new 15 mL plastic conical tube. This fraction is the supernatant of the affinity purification or unbound fraction.
5. Add 6 mL of WB to the beads, mix by inverting the tube, and centrifuge at 8,200 × *g* for 2 min at 4 °C (first wash).
6. Remove the supernatant with pipette and add 6 mL of WB. Incubate at 4 °C for 5 min with gently back-and-forth shaking (second wash).
7. Centrifuge at 8,200 × *g* for 2 min at 4 °C.
8. Repeat washing steps (**steps 6** and **7**) two more times to complete four washes.
9. Remove the supernatant, add 300 µL of Elution buffer to the beads and transfer to a 1.5 mL microfuge tube. Incubate for 30 min at 4 °C with back-and-forth shaking.
10. Centrifuge at 8,200 × *g* for 2 min at 4 °C. Transfer the supernatant to a new 1.5 mL microcentrifuge tube. This is the eluate of the affinity purification and can be used to isolate proteins or RNA which can be immediately used or stored at −80 °C until use (*see* **Note 6**).

3.4 RNA Extraction and Estimation of RNA Yield

This section takes about 1–2 h. Use fume hood to work with TRIzol, chloroform, and isopropanol. After use, TRIzol and chloroform should be discarded as hazardous waste following local regulations.

1. Use TRIzol for extraction of RNA from the TRAP sample and the SN-16 aliquot saved in Subheading 3.1, **step 8**.
2. Add 800 µL of TRIzol to each sample, vortex for 30 s and incubate at room temperature for 5 min to allow the dissociation of ribonucleoprotein complexes.
3. Add 200 µL of Chloroform, shake the tube vigorously by hand for 15 s and incubate at room temperature for 3 min.
4. Centrifuge at 12,000 × *g* for 15 min at 4 °C.
5. Transfer the clear upper phase to a new 1.5 mL microfuge tube.
6. Repeat the chloroform extraction **steps 3–5** to avoid TRIzol contamination that can inhibit downstream enzymatic reactions.
7. To precipitate RNA, add 500 µL of isopropanol, invert the tube gently by hand to mix, and incubate at room temperature

for 10 min or at –20 °C overnight. For samples with extremely low yields of affinity-purified polysomes (less than 100 ng), add 1 μL of 20 μg/μL glycogen along with the isopropanol, to aid in the precipitation.

8. Centrifuge at 12,000 × *g* for 15 min at 4 °C. The RNA pellet will be visible after centrifugation. Discard the supernatant as hazardous waste following local regulations.
9. Wash pellet with 1 mL of 70 % (v/v) Ethanol. Centrifuge at 7,500 × *g* for 5 min at 4 °C.
10. Discard supernatant and let the pellet air dry at room temperature for 20 min.
11. Resuspend the pellet in 50 μL of Milli-Q water by pipetting up and down. Incubate the sample at 60 °C for 10 min to allow the complete resuspension of the RNA pellet. Store at –80 °C until use.
12. Use 1 μL of each sample to estimate the yield and concentration of RNA with a NanoDrop spectrophotometer according to the manufacturer's instructions (*see* **Note 7**).
13. Evaluate the quality of the sample using Agilent 2100 Bioanalyzer with RNA 6000 Nano or Pico Assay reagent kits (*see* **Note 8**).

3.5 Construction and Sequencing of RNAseq Illumina Libraries

1. Use the TruSeq RNA Sample Preparation Kit v2 following the manufacturer's protocol, but taking into consideration that for low initial amount of RNA it is recommended to dilute adapters (e.g., starting with 400 ng of total RNA, we used a 1:8 dilution of adapters in TE buffer pH 8.0 with good results) to avoid an excess of adapters that can lead to the formation of contaminating adapter dimers (*see* Fig. 2b). Use AMPure XP Beads in the DNA purification steps to exclude non-ligated adapters; otherwise, adapter dimers can be over-represented and result in biased libraries (*see* **Note 9**). Choose a manufacturer-recommended combination of adapters to allow multiplex sequencing. The procedure takes 2 days.
2. Evaluate the quality of the libraries using Agilent 2100 Bioanalyzer with DNA 1000 Assay reagent kits (*see* **Note 9**). This step takes about 1 h.
3. Quantify the libraries according to the Sequencing Library qPCR Quantification Guide (Illumina, cat. # SY-930-1010, Part # 11322363), normalize each library to 10 nM, and pool libraries using equal volumes of each library. This step takes approximately 3 h.
4. Libraries can be sequenced in an Illumina NextSeq500 or a HiSeq2500 sequencing platform (see a comparison of different Illumina sequencing systems at www.illumina.com/systems/sequencing.ilmn). The run time ranges from 7 h to 6 days and

depends on the sequencing system, the read length, and the choice of single versus paired-end sequencing (*see* **Note 10**).

3.6 Primary Analysis of Total and TRAP RNA Sequences

In the first part of the protocol, we used the Web-platform Galaxy: https://usegalaxy.org/. This platform integrates computational tools in a Web-interface and can be accessed from a PC or a Mac computer. Galaxy's interface contains three sections. The left column shows the tools, the central panel is where the menus and data will be shown and the right column shows the history where each step of the analysis is recorded and can be accessed for viewing or downloading. A free registration to Galaxy allows the user to save results from the data analysis, create workflows and share data with other users.

1. Upload the sequence of your genome of reference in FASTA format and a file with annotation information in GFF3 or GTF format. FASTA format consist of a single-line description followed by lines of sequence data. GFF files are a tab-delimited text files describing genomic features. Enter to the section Get Data on the tools panel and select Upload File. The files can be accessed by direct uploading from your computer using the option Choose File or by specifying a URL where the file is stored. Once selected the files, click on bottom Execute. Loaded files will appear on the history panel.
2. Upload raw sequence reads in FASTQ format. This text-based format stores nucleotide sequence and its corresponding quality scores. For files bigger than 2 Gb, it is recommended to use the FTP browser Fillezilla. This FTP browser can be downloaded free of charge from the following website: https://filezilla-project.org/. In Fillezilla use the quickconnect command to the host "usegalaxy.org" by introducing your Galaxy's username and password. Once connected to Galaxy, select the files to be uploaded from your local computer and transfer them to the remote site indicated on the right. On Galaxy, select the tool Upload file where files transferred by FTP will now be listed. Select them and confirm uploading by clicking Execute.
3. Convert FASTQ reads to FASTQ Sanger format using the *FASTQ Groomer* tool which can be found in the left column folder NGS: QC and manipulation. This tool converts different FASTQ formats creating an output (FASTQ Sanger) that is compatible with the downstream analysis. Select the file to groom and the quality scores type present on the file according to the type of sequencing platform used. Press the execute bottom.
4. Check quality of raw sequence data using the *FastQ:ReadQC* tool. This tool can be found in the folder NGS: QC and manipulation. Select the converted FASTQ groomed files as input for the analysis. The reports will show a per base sequence quality score and per base sequence content, GC content,

sequence length distribution and sequence duplication levels. The summary plots obtained will indicate whether the data have any problems that you might be aware before doing further analysis.

5. Filter reads based on the quality scores using the *Filter by quality* tool. This tool can be found on the folder NGS: QC and manipulation. A cut-off value of quality (Q) score of 30 (Q30) is recommended. Ends of reads can be trimmed using the *FASTQ Trimmer* tool. The number of bases to be trimmed can be selected based on the report of per base quality scores obtained in **step 4**.
6. Map reads to the reference genome using *Tophat2*. This tool can be found in the folder NGS: RNA-seq in the left column of Galaxy. Select a FASTQ groomed file or two files depending if the library was sequenced by single-end or paired-end. Choose the FASTA file containing the genome sequence previously uploaded to Galaxy. A set of parameters can be modified by selecting the option Full Parameters list. Define the minimum length of segments from each read that will be used to find splice junctions (default is 25) and the number of mismatches allowed in the read alignment (default is 2). Set minimum and maximum distance between splice junctions which should be adjusted for plant genomes (defaults are 70 and 500,000). Activate the search for novel junctions between exons not included in the annotation file GFF3. Execute the alignments for all the conditions and replicates. A list of read alignments (BAM file) and a track of junctions (BED file) will be produced as output files (*see* **Note 11**).
7. Assemble and estimate the relative abundances of transcripts for each sample with *Cufflinks*. This tool can be found in the folder NGS: RNA-seq. Use the BAM output file of Tophat2 that contains accepted hits (i.e., a file that contains a list of read alignments) for each sample. Select a reference annotation genome as a guide (GFF3 file) to include all reference transcripts and assembly any novel genes and splice isoforms based on Tophat2 read alignments. Cufflinks will produce three outputs: a file containing assembled genes and splice isoforms as well as reference genes and transcripts (GTF file) and two files containing the estimated level of expression for each transcript and gene respectively (FPKM_Tracking files).
8. Merge assemblies with *Cuffmerge*. This tool can be found in the folder NGS: RNA-seq. Use GTF output files produced by Cufflinks and the reference genome annotation file GFF3. The purpose of using this tool is to merge annotation files to include novel genes or alternative splicing isoforms found in the experiment (in all samples and conditions) that were not included in the reference genome annotation.

9. Find significant changes between two conditions in transcript expression, splicing and promoter usage with *Cuffdiff*. This tool can be found in the folder NGS: RNA-seq. Use the GTF file containing the merged assemblies produced by Cuffmerge and the BAM output files of Tophat2 for the samples to compare. Cuffdiff outputs include files containing transcript abundance of genes (gene differential expression) and splice variants (transcript differential expression) expressed in units of fragments per kilobase of exons per million of mapped reads (FPKM), as well as results from differential expression tests between two samples at both levels. The files can be downloaded for further analysis.
10. Analyze Cuffdiff outputs with *CummeRbund* software. This package works in an R environment and can be accessed through the Bioconductor website (http://www.bioconductor.org/).
    (a) To install R, download and execute the latest release: cran.r-project.org/bin/windows/base/release.htm.
    (b) To Install CummeRbund, use the following commands in R (*see* **Note 12**): *>source("http://bioconductor.org/biocLite.R")*

        followed by

        >biocLite("cummeRbund")
    (c) Load CummeRbund commands library:

        >library (cummeRbund)
    (d) Place Cuffdiff output files in a directory and set it as working directory in R: *>setwd('C:/Example diff_out')*

        Verify the correct setting of the working directory with the command:

        >getwd()
    (e) Generate a SQlite database with Cuffdiff outputs using the command:

        >cuff<-readCufflinks()

        Verify the content of the database with command:

        >cuff
    (f) CummeRbund package offers a variety of options to manage and plot the data from Cuffdiff as well as filtering information based on the interest of the research.

        As an example, it is possible to extract groups of transcripts showing significant differences between conditions. The following commands will create a table with differentially expressed genes:

        >gene_data <- diffData(genes(cuff))

        >gene_diff <- subset(gene_data, (q_value<0.05))

>write.table(gene_diff,"gene_diff.txt", sep='\t', row.names = F, col.names = T, quote = F)

(g) Draw scatter or volcano plots to summarize the abundance of transcripts in each sample, as well as the significant differences between them:

>csVolcanoMatrix(genes(cuff), 'Sample_1', 'Sample_2')

where 'Sample_1' and 'Sample_2' should have exactly the names of the samples of interest.

>csScatter(genes(cuff), 'Sample_1', 'Sample_2',smooth=T)

11. Use the Integrative Genomics Viewer (IGV) to visualize the alignment of reads at the genome level. This software can be downloaded after a free online registration from the website: http://www.broadinstitute.org/software/igv/log-in. IGV allows the visualization of Tophat2 outputs, such as read alignments data (BAM files) and track intron/exon junction data (BED files).

(a) For genomes not included in the software database, create a genome file using the menu Genomes/Create .genome file. Choose a name as an unique identifier for the genome and select a FASTA file containing the sequence and a GFF3 or GTF file containing genome annotation.

(b) Upload genome in the menu "Genomes/Load genome from file". Select the genome file created as indicated in the previous step. In case a genome file is available online use the menu Load genome from URL and introduce the site where the file is allocated.

(c) Load the alignment files onto IGV using the menu "File/Load from file". Alignment files need to be indexed before loading to IGV. The indexed BAI files for each BAM file can be downloaded from the history panel (right column) on the Galaxy platform using the option Download bam_index.

(d) Upload annotation files (GTF), such as the Cuffmerge output, to show novel transcript isoforms or alternative spliced transcripts discovered with the Tophat2 analysis of the transcriptome/translatome samples. Exon-junction reads (BED files) can be loaded in order to guide the visualization of splicing variants in the IGV.

4 Notes

1. Use lung protection mask while weighing the following reagents: Tris base, EGTA, Brij-35, DOC, DTT, PMSF, cycloheximide, and chloramphenicol.

2. NaOH solutions can absorb carbon dioxide from the air, which will lower the pH, producing the precipitation of EGTA. To avoid this, adjust the pH using NaOH lentils.
3. PEB can include Heparin and RNAse inhibitor if working with tissue with high RNAse content such as mature maize leaves [11].
4. Make sure to grind frozen tissue to a fine powder in liquid N_2, use fresh buffer reagents including cycloheximide and DTT, and ensure tissue thaws at low temperature as these can help to release polysomes from tissue as well as to maintain their integrity.
5. The efficiency of the TRAP procedure should be evaluated by SDS-PAGE analysis of the polysomes followed by immunoblot with an anti-FLAG antibody conjugated to horseradish peroxidase. A band of approximately 25 kDa should be visualized in the samples from clarified cellular extract and the eluted material, but not in those corresponding to the unbound fraction. A low amount of FLAG-RPL18 protein in the eluted sample may indicate inefficient binding to the beads or low efficiency of elution. Possible solutions include increasing binding time and elution time using a fresh stock of beads and 3X FLAG peptide. It can also be useful to increase the amount of both of them if FLAG-RPL18 is detected in the unbound fraction.
6. To analyze the size distribution of purified polysomes in sucrose gradients, the eluted fraction should be used immediately. This requires considerably more affinity-purified complexes than the amount needed for the RNA-SEQ library construction [5, 9, 11].
7. The TRAP methodology yields about 500 ng of RNA per mL of ground tissue when FLAG-RPL18 protein is expressed in nearly all cell types. Note that the yield of TRAP RNA ranges between 10 and 150 ng per mL of tissue when the FLAG-RPL18 protein is expressed in a limited number of cells (e.g., root atrichoblast, cortical cells of the root meristematic zone, root vasculature); therefore a significantly higher amount of tissue should be processed in order to obtain ~500 ng of RNA [24].
8. The quality of the RNA starting material is crucial for the success of RNA-SEQ library preparation. The RNA integrity number (RIN) provides robust and reliable prediction of RNA integrity. RIN is calculated by a method that automatically selects features from signal measurements recorded with an Agilent 2100 Bioanalyzer and constructs regression models based on a Bayesian learning technique [32]. RIN values range from 10 (intact RNA) to 1 (totally degraded RNA). In order to proceed with RNA-SEQ library preparation, RIN should be 8 or higher, and the ratio of the 25S signal to the 18S signal should be about 1.8.

9. Total and TRAP RNA-SEQ libraries of sufficient quality for Illumina RNA-SEQ should produce a signal distribution between 200 and 500 bp with a maximum at approximately 260 bp, such as those illustrated in Fig. 2a. Prior to running DNA samples in the Bioanalyzer, they can be evaluated in an ethidium bromide-stained 1.5 % (w/v) agarose gel. A distribution of DNA fragments between 200 and 500 bp with no adapter dimers should be observed (Fig. 2c).
10. Using the HiSeq2500 system, we obtained a total of 30 Gb of data, which represents approximately 300 million paired end reads (101 bp length) per lane using four multiplexed libraries in a single lane. Each library yielded a mean of 7,765 Mb.
11. In a typical TRAP-SEQ experiment, approximately 90 % of the reads should align to the reference genome.
12. To avoid typing errors in R, use the program R Studio to introduce commands easier. This software can be downloaded free of charge from the website: http://www.rstudio.com/. For help files on any function, type:

    ?name_of_function (example *?readCufflinks)*.

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