Chapter 10

Rapid Immunopurification of Ribonucleoprotein Complexes of Plants

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Abstract

Hundreds of RNA binding proteins posttranscriptionally regulate gene expression, but relatively few have been characterized in plants. One successful approach to determine protein function has been to identify interacting molecules and the conditions of their association. The ribonucleoprotein immunopurification (RIP) assay facilitates the identification and quantitative comparison of RNA association to specific proteins under different experimental conditions. A variety of molecular techniques can be used to analyze the enriched RNAs, whether few as in the case of highly specific interactions, or many. Identification of associated RNAs can inform hypothesis generation about the processes or pathways regulated by the target protein. Downstream analysis of associated RNA sequences can lead to the identification of candidate motifs or features that mediate the protein–RNA interaction. We present a rapid method for RIP from tissues of plants that is suitable for experiments that require immediate tissue cryopreservation, such as monitoring a rapid response to an environmental stimulus.

Key words RNA binding protein (RBP), Ribonucleoprotein immunopurification (RIP), mRNA–ribonucleoprotein complex, mRNP, Posttranscriptional regulation, Arabidopsis

1 Introduction

The field of RNA biology has drawn focus in recent years due to the high mechanistic diversity in function and regulation of RNA molecules. Aside from quantitative transfer of genetic information from the nucleus to the cytoplasm by mRNAs, a steadily expanding portfolio of RNA molecules with varying noncoding biological roles has been described in eukaryotes. The most well-known noncoding RNAs are tRNAs and rRNAs which participate in translation, as well as snRNAs involved in splicing and snoRNAs that guide processing and modification of rRNAs. Less frequently considered are the RNAs involved in telomere maintenance and secretory protein synthesis. Most recently in the limelight are small RNAs (21–24 nt) frequently bound to Argonaute proteins which direct molecular interactions via base pairing with mRNAs that can

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reduce target polyribosome (polysome) association, enhance target decay, and/or impart heritable epigenetic regulation [1]. There are also other non-protein-coding RNAs (ncRNA), generally defined as lacking an open reading frame of >100 aa [2]. These include natural antisense transcripts (NATs) that can modulate molecular activity of respective sense transcripts via base-pairing and thereby affect gene expression [3] and long noncoding RNAs (lncRNA) that mediate diverse biologically significant interactions ranging from regulation of chromatin and transcription to translation and mRNA stability [4]. In these cases, RNA primary and/or secondary structure might facilitate molecular interactions for information transfer [4]. The functions of both coding and noncoding RNAs are tightly associated with proteins with which they interact. Eukaryotic genomes encode large cohorts of RNA binding proteins (RBPs), which are often characterized by specific protein domains (e.g., RNA Recognition Motifs, K-homology, DEAD-box RNA helicases, Puf/PUM repeat, Pentatricopeptide repeat, and glycine-rich/RGG domains). These domains interact with RNAs in a sequence-specific or non-sequence-specific manner. The functions of relatively few RBPs have been characterized in plants [5]. For example, of the over 1100 RBPs in the reference plant *Arabidopsis thaliana*, the function of circa 200 is known, with most of the RBPs involved in canonical processes of splicing and translation (Fig. 1).

A tool for the study of associations between RNAs and RBPs is the ribonucleoprotein (RNP) immunoprecipitation (RIP) assay. This assay has been successfully employed to purify RNP complexes from plant tissue extracts, identify target RNAs, and demonstrate global, dynamic, and treatment-dependent RNP–RNA

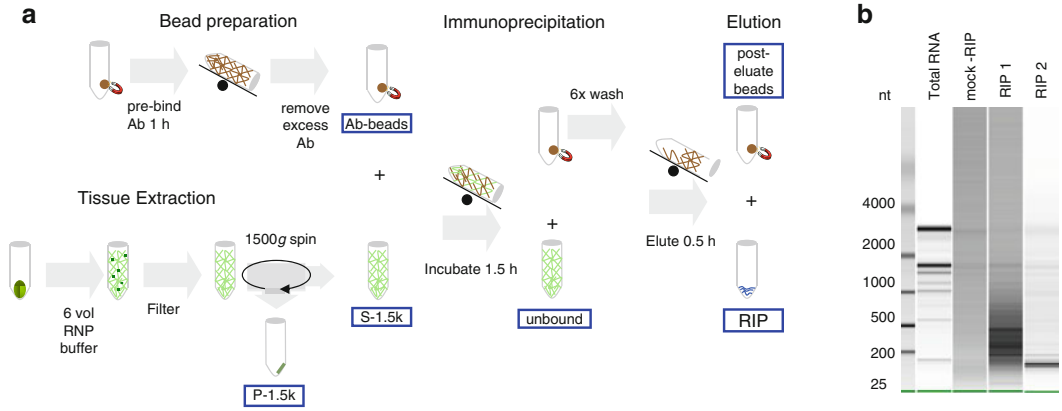

Fig. 1 RNP immunopurification. (**a**) Schematic of the RIP method, including extraction, bead preparation, immunoprecipitation, and elution. (**b**) Example Bioanalyzer RNA profile from Total RNA (Total), RNA from a mock IP using a nonspecific antibody (mock IP), and RNA extracted from RNP complexes eluted following RIP for two different RNA binding proteins (RIP 1 and RIP 2). Loading was normalized to the amount of beads used in the assay

association [6–9]. RNP–RNA associations can be by identified from both RNP complexes involved in well-conserved core RNA processes (i.e., ribosome–mRNA interactions; [10], Chapter 7), as well as in specialized processes that are active in a cell-, tissue-, condition-, or species-unique manner [11]. Moreover, the method is applicable to RNPs regardless of whether the interaction occurs in a specific subcellular location (i.e., nucleus, cytoplasm, endoplasmic reticulum (ER) membrane-associated, mitochondrion, or plastid). The RIP assay relies on a simple bead- or resin-based affinity purification step that takes advantage of a specific interaction between an antibody and its antigen (i.e., epitope). Similarly, it could utilize a biotin-labeled protein that is recognized by streptavidin. The RIP assay described here relies on several factors: (1) the availability of an antibody or antiserum that specifically recognizes an RNP, (2) an extraction procedure that maintains the mRNA–protein interaction, and (3) an efficient method of partitioning complexes from other proteins and RNAs in the extract.

RIP thus enables the enrichment of RNA molecules stably associated with a RBP that can subsequently be extracted and identified by one of several methods (e.g., quantitative Real Time Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR), DNA microarray analysis, or RNA-sequencing (RNA-seq)). There should be consideration that the associated RNAs may be molecules other than polyadenylated mRNAs (i.e., deadenylated mRNAs, tRNAs, rRNAs, miRNAs, or ncRNAs). Once the RNAs associated with an RBP are identified, sequence- or structure-specific binding elements of a particular protein might be sought using algorithms such as the Multiple Em for Motif Elicitation [12]. Enriched elements can be further assayed by independent methods. However, association with a protein of interest does not necessarily have the same effect on all RNAs. One target RNA may recruit multiple RBPs, which synergistically drive a process, whereas a second RNA may recruit a distinct ancillary binding protein, which results in another outcome. It is the dynamic sum of all interacting proteins with a particular RNA that results in its characteristic activity or regulation, which can be modulated in time and space [13]. In addition to identification of interacting RNAs, measurement of quantitative change in RNA association is also possible (i.e., in response to a stimulus or during development). For such a comparison, evaluation of changes in the steady-state abundance of the RNA is required for normalization. In addition, it may be of interest to evaluate the proteins of the complex by use of immunoblot analysis or mass spectrometry-based proteomic methods. This type of analysis can also provide additional clues about the function of the RBP. For example, associated proteins may include poly(A) binding protein and translational machinery or proteins associated with a process such as splicing, nuclear export, ribosome biogenesis, or miRNA function.

The interpretation of RIP data can be aided by an understanding of the spatial distribution of the target RBP. Many RBPs are partitioned within the cell. For example, subpopulations can be in the nucleus, nucleolus, or cytoplasm and associated with specific cellular machinery. Within the cytoplasm, the RBP may be distributed evenly, associated with the ER or cytoskeleton, or focused into large complexes such as processing bodies or stress granules. Therefore, deliberate cellular fractionation prior to RIP may be appropriate to enrich subcellular-localized interactions. Conversely, caution must be taken that unintentional fractionation does not occur during cell extract preparation through centrifugation or some other step. Some RBPs may be partitioned into multiple heterogeneous complexes. It is critical to understand that successful immunoprecipitation will be biased against any complex in which the epitope is sterically blocked, and therefore may enrich for a subpopulation of complexes that contain the RBP. For example, an RBP associated with mRNA in polysome complexes as well as in non-polysomal complexes might only be immunopurified when not associated with ribosomes.

RIP requires the availability of a specific and high affinity antibody for purification of the targeted RNP. This approach has been successfully implemented in plants using epitope-tagged proteins expressed in transgenic plants and the corresponding commercially available monoclonal antibodies [10, 14–16] or affinity-purified polyclonal antibodies raised against a unique peptide of the target RBP or the complete protein [7, 9]. Weak antibody–antigen interaction can result in poor enrichment of RBP–RNA complexes. The use of suitable negative controls in the RIP assay is critical because there may be nonspecific binding of RNA or protein to immunoglobins or the substrate used for purification of the antibodies. When targeting an epitope-tagged RBP, the negative control can be the RIP performed with an antibody to a different protein or epitope produced in the same organism (e.g., rabbit); when using an affinity-purified antiserum, the pre-immune serum is an excellent negative control. Alternatively, tissue lacking the antigen (e.g., from a knockout mutant, non-transgenic, or vector control plant) can be acceptable. A negative control can also be performed to rule out nonspecific binding of the RNA or protein from the cell extract to the magnetic beads or resin used in the purification. We find magnetic-bead facilitated purification of antibody–antigen complexes to be preferable to resin sedimentation as contamination by cellular particulates occurs more easily by co-sedimentation.

Several methods have been described for immunoprecipitation of plant RNP complexes [14–17]. These are based on the limited number of papers in which RNPs have been explored by plant biologists. The cell-lysis buffer needed to stabilize different RNPs for immunoprecipitation is likely to differ. Trial and error

may be required. Here we describe a rapid RIP method for plant cytoplasmic proteins that has proven successful in experiments considering abiotic stress response [6]. In these experiments, rapid harvest and cryopreservation of tissue was preferable, so cross-linking of RNA to proteins was not performed. We have used this approach to obtain RNAs associated with RNA Recognition Motif (RRM)-containing proteins, RNA helicase (RH)-containing proteins, and cold shock domain (CSD) proteins.

2 Materials

2.1 Stock Solutions

1. Phosphate buffered saline (PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM sodium phosphate dibasic, 2 mM sodium phosphate monobasic, adjust pH to pH 7.4 with HCl.
2. RNP extraction buffer: 200 mM Tris-HCl (made with Tris-base with the pH adjusted to 9.0 with HCl) 110 mM potassium acetate, 0.5 % (v/v) Triton X-100, 0.1 % (v/v) Tween 20, 2.5 mM dithiothreitol (*see* **Note 1**), 1.5 % (v/v) Protease Inhibitor Cocktail for Plant Cell and Tissue Extracts (Sigma P9599, St. Louis, MO), 0.04 U/μL RNaseOUT (Life Technologies) or 0.02 U/μL Superase-In RNase Inhibitor (Life Technologies).
3. Wash buffer (WB): 200 mM Tris (adjust pH to pH 9.0 with HCl), 110 mM potassium acetate, 0.5 % (v/v) Triton X-100, 0.1 % (v/v) Tween 20, 2.5 mM dithiothreitol.
4. Elution buffer: WB supplemented with 400 ng/μL freshly diluted 3 × FLAG peptide (Sigma).
5. TRIzol® (Life Technologies).
6. 3 M NaOAc, adjust pH to pH 5.2 with glacial acetic acid.
7. Isopropanol.
8. Glycogen (Ambion).

2.2 Materials

1. Plant tissue that has been rapidly cryopreserved by harvest directly into liquid nitrogen (*see* Subheading 3.1).
2. RNase-free 1.5 mL microfuge tubes (or 15 mL conical centrifuge tubes for scaled up volume).
3. Gloves.
4. Miracloth (EMD Millipore) (cut in 2 or 4 cm squares depending on tube size, autoclaved).
5. Dynabeads® Protein G or Protein A (30 mg/mL suspension; *see* **Note 2**; Life Technologies).
6. FLAG® M2 mouse monoclonal antibody (for FLAG epitope-tagged proteins; Sigma-Aldrich).

7. Disposable Pasteur pipets.
8. RNA 6000 Pico Kit (Agilent Technologies).

2.3 Equipment

1. Magnetic rack (appropriate for tube size used; Qiagen or New England Biolabs).
2. Swinging bucket clinical laboratory centrifuge (e.g., Sorvall™ ST 8 Small Benchtop Centrifuge with a HIGHConic III rotor, Thermo Scientific).
3. Two rocking tables (4 °C and RT) (e.g., Vari-Mix™ Platform Rocker, Thermo Scientific).
4. Agilent 2100 Bioanalyzer (Agilent Technologies).

3 Methods

3.1 Day 1: Tissue Preparation

1. Perform biological experiment to obtain plant material. The amount of tissue needed may differ for each protein. For an abundant protein, 100 mg frozen powdered 7-day-old Arabidopsis seedlings (~250 µL from ~100 seedlings) is sufficient (*see* **Notes 3** and **4**).
2. Harvest experimental tissue, flash-freeze and grind it in liquid nitrogen (*see* **Note 5**). For quick and efficient tissue extraction, tissue should be very finely ground (*see* **Note 6**). Store the ground tissue at −80 °C until use.

3.2 Day 2: Immunoprecipitation

1. Aliquot up to 100 µL of resuspended Dynabeads per 1.7 mL microfuge tube.
2. Magnetically separate the beads from the supernatant by placing the tube adjacent to a magnet for 3 min allowing the paramagnetic beads to aggregate against the side of the tube and discard supernatant by pipetting.
3. Wash beads one time in PBS + 0.02 % (v/v) Tween 20 as recommended by the manufacturer and repeat **step 2**.
4. Dilute antibody (1–10 µg) (commercial monoclonal or affinity-purified polyclonal antibody) in 400 µL of PBS supplemented with 0.02 % (v/v) Tween 20 for each 100 µL of resuspended Dynabeads to be used. Add the diluted antibody to the washed beads and resuspend the beads by pipetting. Incubate with gentle agitation for 1 h at RT (*see* **Note 7**).
5. While waiting, very carefully weigh out 100 mg of powdered plant tissue (*see* **Note 8**) per 1.5 mL microfuge tube (*see* **Note 9**), keeping it frozen by precooling all tubes and spatulas in liquid N_2. Handle tissue tubes with insulation and in short intervals with repeated recooling to maintain tissue in a frozen state.
6. To the frozen tissue that is maintained in tubes in a liquid nitrogen-containing Dewar up until this point of the procedure,

add 1.5 mL of ice-cold RNP extraction buffer and immediately resuspend the tissue by pipetting and let thaw slowly on ice. (With caution, very short (~2 s) warm-hand pulses to the tube after tissue resuspension can speed ice thawing.) This thawing process should take approximately 3–5 min.

7. Immediately, filter the extract through a single layer 2 cm square piece of Miracloth folded in quarters and then placed in the opening of a precooled 1.5 mL microfuge tube. Extract absorbed by the miracloth can be pressed out with gloved fingers.
8. Centrifuge the filtrate for 2 min at 1,500 × *g* in a swinging bucket clinical centrifuge at 4 °C.
9. Transfer supernatant to a new precooled 1.5 mL microfuge tube and save a 300 µL aliquot for total RNA extraction, and a 20 µL aliquot as the "input" fraction for immunoblot or analytical polyacrylamide gel electrophoresis (PAGE) analyses.
10. Magnetically separate the Dynabeads (*see* **Note 10**) from **step 3**, discard supernatant by pipetting, and precool the tube with the beads on ice. Transfer the separated beads to the remaining tissue extract from **step 9** by resuspension in a small volume of the extract and transferring back.
11. Gently agitate the suspension on a rocking table at 4 °C in the dark for 1.5 h.
12. In a 4 °C cold room, magnetically separate the beads (*see* **Note 10**) (5 min) and save 20 µL of supernatant as the "unbound" fraction and discard the remainder.
13. Briefly wash the beads and tube walls with 1 mL of cold RNP extraction buffer and magnetically separate and discard supernatant.
14. Wash beads in 0.75 mL of cold wash buffer for 5 min with gentle agitation at 4 °C, magnetically separate the beads (3 min), and discard the supernatant.
15. Repeat **step 14** three to four more times.
16. For competitive elution (e.g., with 3 × FLAG peptide for α-FLAG antibody), resuspend beads following the final wash in 100 µL of the elution buffer and incubate the suspension for 30 min at 4 °C with rocking. Following elution, separate the beads magnetically as before and transfer eluate to a new cold tube (*see* **Note 11**).
17. Alternatively, resuspend the beads bound to RNP complexes in 100 µL of cold wash buffer.
18. Aliquot 90 µL of the eluate or suspension for direct RNA extraction using TRIzol, and save 10 µL for immunoblot and/or analytical PAGE. Washed, aliquoted RNP-bound beads can be frozen at −80 °C at this point (*see* **Note 12**).

3.3 Day 3: RNA Extraction

1. To the frozen 90-µL eluate, bead aliquot, or total extract, add 1 mL of TRIzol and allow the sample to thaw, frequently mixing by shaking, and then incubate at RT for 5 min.
2. Add 200 µL of chloroform and vortex sample for 30 s.
3. Centrifuge the tube at 10,000 × *g* for 10 min.
4. Remove as much of the upper aqueous phase as possible without organic phase contamination and transfer to a new tube (~500 µL).
5. Add 25 µg of glycogen, 0.11 volumes of 3 M NaOAc pH 5.2, and 1 volume of isopropanol.
6. Incubate at −20 °C for 2 h and centrifuge at 18,000 × *g* for 30 min at 4 °C.
7. Discard supernatant and wash pellet two times with ice-cold 75 % (v/v) ethanol.
8. Dry pellet and tube for 10 min and resuspend in 15 µL of RNase-free water (*see* **Note 13**).
9. RNA quality and size distribution can be evaluated using the Agilent 2100 Bioanalyzer, RNA 6000 Nano or Pico Kit (Agilent Technologies). Capillary electrophoresis provides a high-resolution distribution of RNA sizes with small amounts of RNA and gives a first indication of the size distribution of abundant RNAs associated with the target protein.

4 Notes

1. Inclusion of dithiothreitol is optional as it can disrupt dimers/multimers of some RNP complexes. However, it is recommended in initial exploratory experiments as a reducing agent. RNaseOUT requires dithiothreitol, but can be substituted with SUPERase.In (Life Technologies), which does not require dithiothreitol.
2. Use of Protein G or Protein A can make a substantial difference as they have different affinities for a variety of antibody types from distinct species (e.g., Protein G binds mouse IgG1 with high affinity, but Protein A does not, both bind rabbit IgG well, but neither will bind IgY). See product documentation.
3. Treatment of plants (i.e., *Arabidopsis* seedlings) with the proteasome inhibitor MG-132 (for example, flooding a single plate with 10 mL 100 µM for 1 h) prior to harvesting of tissue can increase the absolute IP yield of unstable proteins.
4. Be sure to prepare sufficient tissue amount for a negative control IP (i.e., a mock immunoprecipitation). A suitable negative control may be an IP using the same tissue sample performed with a nonspecific antibody produced in the same organism as

the specific antibody. Treat similarly to evaluate nonspecific background RNA and/or protein association with the IP matrix. Alternatively, biological samples lacking the antigen but treated the same way can be used (i.e., a genotype that has a loss-of-function mutation in the gene encoding the RBP of interest). It is also possible to perform a negative control just with the cell extract and magnetic beads/resin, but no antibody.

5. Some reports note improved RIP RNA yield after crosslinking tissue with formaldehyde [7, 16], but this is incompatible with very rapid cryopreservation of tissue needed during a time-sensitive response, such as after an acute abiotic stress.
6. A ceramic mortar and pestle can be sufficient to finely grind tissue. These should be rinsed with ethanol and air dried prior to use, and precooled with 3–4 volumes of N_2 prior to grinding (so that the final volume does not rapidly boil off). An added layer of insulation to the mortar (such as a homemade cardboard sheath) slows warming and protects fingers from frostbite while grinding. To achieve very fine powder, do not over fill the pestle. Allow liquid nitrogen to evaporate completely and continue to grind 15–20 s. The cold pestle will keep the tissue frozen. Add back a few mL of liquid N_2 and agitate to obtain a paste-like consistency that forms a ball (for small tissue amounts this facilitates efficient tissue transfer) and transfer to a precooled tube.
7. Some protocols advise incubating the extract with the antibody prior to incubating the antibody with the magnetic beads. This is advantageous if the epitope is of low affinity to the target protein. If this strategy is employed, any excess antibody (i.e., not bound to antigen) will compete for bead capacity and decrease IP efficiency.
8. Beads have a maximum binding capacity. When all of the antibody-binding sites are occupied by the antigen, the beads are saturated. Once the beads are saturated, they will not IP more antigen. Therefore, it is important to perform a Western blot to compare the input extract, the post-IP unbound extract, and the IP sample. The input-to-unbound comparison can reveal if insufficient beads were used. Keep in mind that not all of the targeted protein may be purified if the epitope is not always accessible to the antibody (i.e., because of steric hindrance). The amount of beads for efficient IP depends upon the concentration of antigen in the extract. For IP of proteins with low abundance, scaling up the volume of extract (while maintaining tissue to buffer ratio) will increase the absolute IP yield to facilitate downstream analyses.
9. Continuous gentle mixing as provided by a rocking table is required for quick and efficient IP. The volume of extract in a tube must be low enough to allow an air bubble to oscillate

from one end of the capped tube to another to prevent bead settling. We use a rocking table with a rate of 0.3 Hz and an angle of 23°.

10. After bead incubation in buffer, a small amount of buffer might remain in the lid of the tube. Prior to each magnetic separation, shake down the buffer or pulse spin at a very low speed that does not sediment the beads.
11. Competitive elution increases the specificity of elution; however, it can be inefficient and expensive (e.g., in our experience 3× FLAG® peptide (Sigma) batch quality and elution efficiency can be variable). It is important in cases where there is nonspecific protein or RNA association with the bead matrix. If competitive elution is performed, we recommend evaluating the post-elution bead fraction by SDS-PAGE to determine elution efficiency. If it is not necessary because of low nonspecific contamination then it can be replaced by direct extraction of RNA or protein from the bead matrix after washing.
12. After bead washing, the RNP complexes can also be used for proteomic analysis. Wash bead-bound RNPs in the appropriate digestion buffer (e.g., 50 mM ammonium bicarbonate, 10 % (vol/vol) acetonitrile for trypsin) as final wash, magnetically separate the beads, save supernatant and freeze. Proteins can be directly digested (e.g., with trypsin) on the beads.
13. RNA yield varies depending on the biochemical properties of the protein target. A range of 50–100 ng of RNA per 200 mg powdered 7-day-old Arabidopsis seedlings can be expected for general RBPs. Highly specific RBPs can be expected to yield less. Background RNA amounts from mock IPs may yield in the range of 1–3 ng per 200 mg tissue.

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