Video Article

mRNA Interactome Capture from Plant Protoplasts

Zhicheng Zhang[1], Kurt Boonen[1], Meixia Li[1], Koen Geuten[1]

[1]Department of Biology, KU Leuven

Correspondence to: Koen Geuten at koen.geuten@kuleuven.be

URL: https://www.jove.com/video/56011
DOI: doi:10.3791/56011

Keywords: Plant Biology, Issue 125, leaf mesophyll protoplasts, 254 nm UV source, *in vivo* UV crosslinking, messenger RNA-binding proteins, oligo(dT) beads, messenger ribonucleoprotein complex pull-down, SDS-PAGE, silver staining, nano-LC-MS, mRNA-bound proteome

Date Published: 7/28/2017

Citation: Zhang, Z., Boonen, K., Li, M., Geuten, K. mRNA Interactome Capture from Plant Protoplasts. *J. Vis. Exp.* (125), e56011, doi:10.3791/56011 (2017).

Abstract

RNA-binding proteins (RBPs) determine the fates of RNAs. They participate in all RNA biogenesis pathways and especially contribute to post-transcriptional gene regulation (PTGR) of messenger RNAs (mRNAs). In the past few years, a number of mRNA-bound proteomes from yeast and mammalian cell lines have been successfully isolated through the use of a novel method called "mRNA interactome capture," which allows for the identification of mRNA-binding proteins (mRBPs) directly from a physiological environment. The method is composed of *in vivo* ultraviolet (UV) crosslinking, pull-down and purification of messenger ribonucleoprotein complexes (mRNPs) by oligo(dT) beads, and the subsequent identification of the crosslinked proteins by mass spectrometry (MS). Very recently, by applying the same method, several plant mRNA-bound proteomes have been reported simultaneously from different *Arabidopsis* tissue sources: etiolated seedlings, leaf tissue, leaf mesophyll protoplasts, and cultured root cells. Here, we present the optimized mRNA interactome capture method for *Arabidopsis thaliana* leaf mesophyll protoplasts, a cell type that serves as a versatile tool for experiments that include various cellular assays. The conditions for optimal protein yield include the amount of starting tissue and the duration of UV irradiation. In the mRNA-bound proteome obtained from a medium-scale experiment (10^7 cells), RBPs noted to have RNA-binding capacity were found to be overrepresented, and many novel RBPs were identified. The experiment can be scaled up (10^9 cells), and the optimized method can be applied to other plant cell types and species to broadly isolate, catalog, and compare mRNA-bound proteomes in plants.

Video Link

The video component of this article can be found at https://www.jove.com/video/56011/

Introduction

Eukaryotes use multiple RNA biogenesis regulatory pathways to maintain cellular biological processes. Among the known types of RNA, mRNA is very diverse and carries the coding capacity of proteins and their isoforms[1].The PTGR pathway directs the fates of pre-mRNAs[2,3]. RBPs from different gene families control the regulation of RNA, and in PTGR, specific mRBPs guide mRNAs through direct physical interactions, forming functional mRNPs. Therefore, identifying and characterizing mRBPs and their mRNPs is critical to understanding the regulation of cellular mRNA metabolism[2].Over the past three decades, various *in vitro* methods – including RNA electrophoretic mobility shift (REMSA) assays, systematic evolution of ligands by exponential enrichment assays (SELEX) based on library-derived constructs, RNA Bind-n-Seq (RBNS), radiolabeled or quantitative fluorescence RNA binding assays, X-ray crystallography, and NMR spectroscopy[4,5,6,7,8,9] – have been widely applied to studies of RBPs, mainly from mammalian cells. The results of these studies of mammalian RBPs can be searched via the RNA-binding Protein DataBase (RBPDB), which collects the published observations[10].

Although these *in vitro* approaches are powerful tools, they determine the bound RNA motifs from a given RNA pool of sequences and therefore are limited in their ability to discover new target RNAs. The same is true for computational strategies to predict genome-wide RBPs, which are based on the conservation of protein sequence and structure[15]. To overcome this, a new experimental method has been established that allows for the identification of the RNA motifs that an RBP of interest interacts with, as well as for the determination of the precise location of binding. This method, called "crosslinking and immunoprecipitation" (CLIP), is composed of *in vivo* UV crosslinking followed by immunoprecipitation[11]. Early studies have shown that photoactivation of DNA and RNA nucleotides can occur at excitation UV wavelengths greater than 245 nm. The reaction through thymidine seems to be favored (rank in order of decreased photoreactivity: dT ≥ dC > rU > rC, dA, dG)[12]. Using UV light with a wavelength of 254 nm (UV-C), it was observed that covalent bonds between RNA nucleotides and protein residues are created when in the range of only a few Angstroms (Å). The phenomenon is therefore called the "zero-length" crosslinking of RNA and RBP. This can be followed by a stringent purification procedure with little background[13,14].

A strategy complementary to CLIP is to combine *in vivo* UV crosslinking with protein identification to describe the landscape of RBPs. A number of such genome-wide mRNA-bound proteomes have been isolated from yeast cells, embryonic stem cells (ESCs), and human cell lines (*i.e.,* HEK293 and HeLa) using this novel experimental approach, called "mRNA interactome capture"[18,19,20,21]. The method is composed of *in vivo* UV crosslinking followed by mRNP purification and MS-based proteomics. By applying this strategy, many novel "moonlighting" RBPs containing non-canonical RBDs have been discovered, and it has become clear that more proteins have RNA-binding capacities than previously supposed[15,16,17]. The use of this method allows for new applications and for the ability to answer new biological questions when investigating

RBPs. For example, a recent study has investigated the conservation of the mRNA-bound proteome (the core RBP proteome) between yeast and human cells[22].

Plant RBPs have already been found to be involved in growth and development (*e.g.,* in the post-transcriptional regulation of flowering time, the circadian clock, and gene expression in mitochondria and chloroplasts)[24,25,26,27,28,29]. Furthermore, they are thought to perform functions in the cellular processes responding to abiotic stresses (*e.g.,* cold, drought, salinity, and abscisic acid (ABA))[31,32,33,34]. There are more than 200 predicted RBP genes in the *Arabidopsis thaliana* genome, based on RNA recognition motif (RRM) and K homology (KH) domain sequence motifs; in rice, approximately 250 have been noted[35,36]. It is notable that many predicted RBPs seem to be unique to plants (*e.g.,* no metazoan orthologs to approximately 50% of predicted *Arabidopsis* RBPs containing an RRM domain)[35], suggesting that many may serve new functions. The functions of most predicted RBPs remain uncharacterized[23].

The isolation of mRNA-bound proteomes from *Arabidopsis* etiolated seedlings, leaf tissue, cultured root cells, and leaf mesophyll protoplasts through the use of mRNA interactome capture has recently been reported[38,39]. These studies demonstrate the strong potential of systematically cataloging functional RBPs in plants in the near future. Here, we present a protocol for mRNA interactome capture from plant protoplasts (*i.e.,* cells without cell walls). *Arabidopsis thaliana* leaf mesophyll protoplasts are the major type of leaf cell. The isolated protoplasts allow optimal access of UV light to the cells. This cell type can be used in assays that transiently express proteins for functional characterization[40,41]. Furthermore, protoplasting has been applied to several other plant cell types and species[42,43,44] (*e.g.,* Petersson *et al*., 2009; Bargmann and Birnbaum, 2010; and Hong *et al*., 2012).

The method encompasses a total of 11 steps (**Figure 1A**). *Arabidopsis* leaf mesophyll protoplasts are first isolated (step 1) and are subsequently UV irradiated to form crosslinked mRNPs (step 2). When protoplasts are lysed under denaturing conditions (step 3), the crosslinked mRNPs are released in lysis/binding buffer and pulled down by oligo-d$(T)_{25}$ beads (step 4). After several rounds of stringent washes, the mRNPs are purified and further analyzed. The denatured peptides of mRBPs are digested by proteinase K before the crosslinked mRNAs are purified and the RNA quality is verified by qRT-PCR (steps 5 and 6). After RNase treatment and protein concentration (step 7), the protein quality is controlled by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (step 8). The difference in protein band patterns can easily be visualized between a crosslinked sample (CL) and a non-crosslinked sample (non-CL; the negative control sample from protoplasts that is not subjected to UV irradiation). The identification of proteins is achieved through MS-based proteomics. The proteins from the CL sample are separated by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) to remove possible background contamination, are "in-gel digested" into short peptides using trypsin, and are purified (step 9). Nano reverse-phase liquid chromatography coupled to mass spectrometry (nano-LC-MS) allows for the determination of the amount of definitive proteins in the mRNA-bound proteome (step 10). Finally, the identified mRBPs are characterized and cataloged using bioinformatic analysis (step 11).

Protocol

1. *Arabidopsis* Leaf Mesophyll Protoplast Isolation

NOTE: *Arabidopsis* leaf mesophyll protoplasts are essentially isolated as described by Yoo *et al*., 2007, with several modifications[40].

1. **Growth of plants**
    1. Soak approximately 200 *Arabidopsis thaliana* Col-0 ecotype seeds in sterilized water for 2 days at 4 °C in darkness for stratification.
    NOTE: This number of seeds is enough for one non-CL sample and one CL sample (see step 1.3).
    2. Prepare pots with a 50% (*v/v*) mixture of soil and vermiculite and soak the pots with distilled water in a tray to wet the soil.
    3. Sow and distribute the stratified seeds on wet soil. Do not cover the seeds with additional soil because *Arabidopsis* seeds need light for germination.
    NOTE: Plant growth conditions are a 12 h light/12-h dark cycle at 23 °C, with a light intensity of 100 µmol m^{-2} s^{-1}, in a growth room for 5 weeks before flowering. 4-week-old plants can also be used[40].
    NOTE: All described materials and reagents from step 1.2.1 to step 3.2.3 are for two samples (*i.e.,* one non-CL sample (the control sample that is not subjected to UV-C irradiation) and one CL sample (the research sample that is subjected to UV-C irradiation)).

2. **Preparation of isotonic enzyme solution**
    1. Make 80 mL of primary isotonic enzyme solution (400 mM mannitol, 20 mM KCl, and 20 mM MES buffer (pH 5.7)) and immediately heat the solution in a 60 °C incubator for 10 min.
    2. Add 1.2 g of Cellulase R10 (*w/v* 1.5%) and 0.32 g of Macerozyme R10 (*w/v* 0.4%) powder to the warm primary isotonic enzyme solution.
    3. Carefully bring the enzyme powder into solution by applying gentle stirring. Rapidly heat the solution in a 60 °C incubator for 10 min until that the enzyme solution is clear light brown.
    4. Cool the solution on ice for 3 min and add 800 µL of 1 M $CaCl_2$ and 800 µL of 10% (*w/v*) BSA to the solution.
    5. Homogenize the solution by pipetting and filtering through a 0.22-µm filter. Aliquot this final isotonic enzyme solution into two large-scale Petri dishes (150 mm x 20 mm).

3. **Preparation of *Arabidopsis* rosette leaf strips**
NOTE: 150 leaves for each Petri dish is recommended for one non-CL sample or one CL sample.
    1. Choose and cut a total of 300 fully expanded 2^{nd}- or 3^{rd}-pair true leaves (average: 3 - 4 per rosette) into 0.5- to 1-mm leaf strips using a new and sharp razorblade.
    2. Transfer and submerge the strips immediately into the isotonic enzyme solution.
    NOTE: To avoid any contact with light, always cover the Petri dishes with aluminum foil. Do not crush the leaves. Confirm that all strips are submerged in the solution and are not floating on the surface.

4. **Isolation of *Arabidopsis* leaf mesophyll protoplasts**

1. Place the aluminum foil-covered Petri dishes into vacuum desiccators and infiltrate the leaf strips under vacuum for 30 min. Subsequently, incubate the Petri dishes in darkness without shaking at room temperature for 2.5 h.
2. Gently and briefly shake the dishes horizontally by hand, attempting to release the protoplasts entirely into the enzyme solution.

5. **Harvesting and counting the protoplasts**
   1. Filter the protoplast suspension through a 35 to 75 µm nylon mesh. Aliquot the filtrate of each sample into two 50 mL round-bottom tubes (approximately 20 mL of filtrate in each tube).
   2. Rinse each Petri dish with 10 mL of W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES buffer (pH 5.7)) to transfer the rest of the protoplasts to the enzyme solution. Add this 10-mL filtrate of each sample to the tubes. Cover the tubes with aluminum foil and keep them on ice.
   3. Spin all four tubes for 5 min at 100 x g to pellet the protoplasts. Discard the supernatant and gently resuspend the protoplast pellets in each tube with 10 mL of W5 buffer.
      NOTE: When resuspending the protoplast pellets, gently reverse the tube upside down and gently spin the tube by hand until the pellets have disappeared. Do not pipette the pellet directly to avoid damaging the cells.
   4. Repeat step 1.5.3 once and combine the protoplast suspension from two tubes of each sample into one 50-mL round-bottom tube. Keep the tubes on ice.
   5. Count the protoplasts using a hemocytometer.
      NOTE: Each 20 cells within 25 cubes equals 2 x 10^5 protoplasts/mL. 150 leaves should yield approximately 1 x 10^7 cells. Modulate to have an equal total number of protoplasts in the non-CL and CL samples.
   6. Keep the protoplasts on ice for 30 min. Afterwards, spin the tubes for 5 min at 100 x g. Discard the supernatant and resuspend the protoplasts in each tube with 20 mL of MMg solution (400 mM Mannitol, 15 mM $MgCl_2$, and 4 mM MES buffer (pH 5.7)).

2. *In Vivo* mRNA-protein Crosslinking by UV Irradiation

NOTE: Keep the non-CL sample tube on ice. The CL sample must be immediately UV irradiated.

1. Transfer the protoplast suspension containing 1 x 10^7 cells from the CL sample tube to a new large-scale Petri dish (150 mm x 20 mm) and add 30 mL of ice-cold MMg solution to cover the plate surface. Spread the cells in the whole MMg buffer by gentle pipetting. Immediately place the Petri dish without the top cover in a UV crosslinking apparatus.
   NOTE: The height between the UV lamp and the Petri dish is 8 cm.
2. Irradiate the sample with 254 nm wavelength UV light and 0.00875 W/cm^2 UV intensity for 1 min, 3 min, or 5 min. After irradiation, rapidly aliquot the protoplast suspension into two new 50 mL round-bottom tubes. Wash the bottom of the dish with an additional 5 mL of ice-cold MMg solution to collect the remainder of the protoplasts and add this cell suspension to these two tubes.
   NOTE: 1 min was chosen as the optimal condition. The explanation can be found in the **Representative Results**.

3. Protoplast Lysis under Denaturing Conditions

1. **Preparation of protoplast pellets for lysis**
   1. Spin the non-CL sample tube together with the two CL sample tubes from step 2 at 100 x g for 5 min and discard the supernatant. Place the tube of the non-CL sample protoplast pellet back on ice.
   2. Add 10 mL of ice-cold MMg solution to each CL sample tube, gently resuspend the pellets, and combine them back into one 50 mL round-bottom tube. Spin the tube at 100 x g for 5 min. After removing the supernatant, keep the CL sample tube protoplast pellet on ice.

2. **Protoplast lysis and homogenizing protoplast lysate**
   1. Add 9 mL of ice-cold lysis/binding buffer (500 mM LiCl, 0.5% (*w/v*) Lithium Dodecyl Sulphate (LiDS), 5 mM Dithiothreitol (DTT), 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0)) to the cell pellet of each sample. Roughly lyse the protoplasts by pipetting up and down approximately 20 times until the suspension is homogenously light green.
   2. Pass the protoplast lysate two times through a 50-mL glass syringe with a narrow needle (0.9 x 25 mm^2) for homogenization. Incubate the lysate on ice for 10 min. Freeze the lysates in liquid nitrogen and store at -80 °C for up to 3 weeks.

4. mRNP Pull-down and Purification by Oligo-d$(T)_{25}$ Beads

NOTE: All the following described materials and reagents, from step 4.1 to step 4.4, are only for one sample (*i.e.,* one non-CL sample or one CL sample). The protoplast lysate must be added immediately to the tubes containing beads after the bead supernatant lysis/binding buffer is discarded.

1. **Preparation of oligo-d$(T)_{25}$ magnetic beads for oligo(dT) capture**
   1. Thaw the frozen protoplast lysate at room temperature. When the lysate is fully thawed, keep the tube of lysate on ice.
   2. Aliquot 1.8 mL of oligo-d$(T)_{25}$ magnetic beads (5 mg/mL) into 6 new 2-mL round-bottom microcentrifuge tubes on ice. In each tube, wash the bead suspension homogenously with 600 µL of lysis/binding buffer by briefly pipetting up and down and gently rotating at 4 °C for 2 min. Keep the beads on ice.

2. **Binding**
   1. Place all 6 tubes containing the beads into a magnetic rack at 4 °C for 3 min, which will result in the magnetic capture of the beads and the clearing of the suspension.
      NOTE: When the tubes are placed into the magnetic rack, wait until the beads are completely captured, at least 3 min.

2. Discard the supernatant and immediately aliquot 9 mL of protoplast lysate into these 6 tubes. Mix well by pipetting until a homogeneous brown suspension appears and incubate the beads in protoplast lysate at 4 °C for 1 h by applying gentle rotation.
   NOTE: An incubation time from 30 min to 1 h is recommended.

3. **Washing**
   1. Place the tubes back into the magnetic rack at 4 °C for 3 min. When all beads are captured on the side of the tubes, collect the protoplast lysate into 6 new 2-mL round-bottom microcentrifuge tubes and temporarily store them at 4 °C. DO NOT discard the protoplast lysate immediately; keep the lysate at 4 °C.
   2. Add 1.5 mL of ice-cold wash buffer 1 (500 mM LiCl, 0.1% (*w/v*) LiDS, 5 mM DTT, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0)) to the beads in each tube. Resuspend the beads and apply gentle rotation for 1 min. Place the tubes back into the magnetic rack at 4 °C for 3 min and discard the supernatant. Repeat this wash step with wash buffer once.
   3. Repeat the same procedure of this wash step twice using 1.5 mL of ice-cold wash buffer 2 (500 mM LiCl, 5 mM DTT, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0)) and once using 1.5 mL of ice-cold low-salt buffer (200 mM LiCl, 20 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0)).
      NOTE: If the mRNPs have been efficiently isolated from the protoplast lysate, there should be a "halo" visible around the bead pellet in the CL sample during the wash step with wash buffer 2 (**Figure 1B**).

4. **Elution**
   1. Add 500 µL of elution buffer (20 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0)) to the beads in each tube. Gently resuspend the beads and incubate at 50 °C for 3 min to release the poly(A)-tailed RNAs. Gently resuspend the beads once by pipetting. Place the tubes back into the magnetic rack at 4 °C for 5 min.
   2. Transfer and combine all eluents (approximately 3 mL in total) to a clean, sterile RNase-free, 15-mL conical-bottom tube on ice.
      NOTE: The quality and quantity of RNA can be immediately determined using a spectrophotometer device.
      NOTE: The RNA concentration of each sample is approximately 10 ng/µL, with an A_{260}/A_{280} ratio of 1.9. The A_{260}/A_{280} ratio should be between 1.6 and 2.0 because the isolated poly(A)-tailed RNAs are usually greater than 70% pure. If the RNA concentration is too low, increase the number of starting protoplasts to a maximum of 10^9. Samples can be frozen in liquid nitrogen and kept at 80 °C for long-term storage.
   3. Repeat step 4.2 to step 4.4 for an additional two rounds and re-use the oligo-d$(T)_{25}$ beads to deplete the poly(A)-tailed RNAs from the protoplast lysate.
      NOTE: After three rounds of pull-down procedure, there should be a total of 9 mL of eluent for each non-CL or CL sample. Perform all work at 4 °C to avoid the degradation of RNA. Follow step 4.5 or step 4.6 to re-use or regenerate the beads.

5. **Preparation of re-used oligo-d$(T)_{25}$ beads**
   1. Wash the beads twice with 1 mL of ice-cold elution buffer and once with 1 mL of ice-cold lysis/binding buffer to adjust the salt LiCl concentration back to 500 mM.
   2. Follow step 4.1, discard the supernatant, transfer the stored protoplast lysate in each tube, and repeat the whole procedure from step 4.2 to step 4.4 for an additional two rounds.

6. **Regeneration of oligo-d$(T)_{25}$ beads**
   NOTE: Beads can be stored and used for another experiment (maximum re-use is three times).
   1. Follow step 4.4, add 1 mL of 0.1 M NaOH to the beads, and incubate by rotating at room temperature for 5 min. For each tube, wash two times with 1 mL of wash buffer 1 and three times with 1x PBS (pH 7.4) containing 0.1% Tween-20 for equilibration. Keep the beads from each tube in 300 µL of sterile RNase-free 1x PBS (pH 7.4) containing 0.1% Tween-20 at 4 °C for long-term storage.
      NOTE: Beads used for *Arabidopsis* CL samples in this experiment can only be used for the same plant samples the next time.

5. Proteinase K Treatment and mRNA Purification

1. **Proteinase K treatment**
   1. Add 8 µL of Proteinase K solution (2 µg/µL) to 1 mL of the eluent from each sample to digest the UV-crosslinked proteins. Briefly vortex.

2. **mRNA purification**
   1. Incubate the eluent at 37 °C for 1 h. Purify the RNA using the RNA purification kit to remove residual contaminants.
      NOTE: After purification, the RNAs are ready for the RNA quality test using the qRT-PCR assay.
      NOTE: Other kits, such as RNA mini kit and TRIzol reagent, can also be used[14].

6. qRT-PCR Assay

1. **Reverse transcription**
   1. Achieve efficient synthesis of first-strand cDNA using the reverse transcription system, with 1 µg of RNA as the template. Dilute the sample to 5 ng/µL with nuclease-free water.

2. **cDNA quantification using qRT-PCR**
   1. Amplify and quantify the cDNA using the qPCR master mix and the real-time PCR cycler, with 10 ng of cDNA as the template. Use the following PCR program: 95 °C for 10 min (stage 1, one cycle) and 95 °C for 15 s and 60 °C for 1 min (stage 2, 40 cycles).

NOTE: The reference gene used here was an endogenous internal control gene, *UBQ10* (AT4G05320). qRT-PCR primers for *UBQ10* and 18S rRNA were described in Li *et al*., 2014 and Durut *et al*., 2014, respectively[45,46]. Primer sequences are listed in the **Material Table**.

7. RNase Treatment and mRBP Concentration

1. **RNase treatment**
    1. Add approximately 100 U of RNase cocktail containing RNase A and RNase T1 into 8 mL of eluent. Include a negative-control sample with RNase cocktail in which the eluent is replaced by nuclease-free water. Briefly vortex and incubate at 37 °C for 1 h.

2. **mRBP concentration**
    1. After RNase digestion, concentrate the eluent using centrifugal filter units. The end volume is 75 µL, with a total protein yield of approximately 2 µg of each sample after concentration. Place the samples at -80 °C for long-term storage.

8. SDS-PAGE and Silver Staining

1. **SDS-PAGE**
    1. Mix 25 µL of the concentrated eluent (CL sample) or the control sample (non-CL sample) with 15 µL of 2x loading dye. Heat the sample at 95 °C for 5 min and load it on an SDS-PAGE gel containing 5% stacking gel and 12% resolving gel, including a protein marker.
    2. Condense the proteins at 60 V for 40 min and separate at 160 V for approximately 1 h until the loading dye reaches the end of the resolving gel.

2. **Silver-staining assay**
    1. Wash the SDS-PAGE gel twice with ultrapure water for 5 min each time. Perform the silver-staining of the proteins using a commercial silver stain kit.
    NOTE: The protein bands must be shown within 5 min. If the bands are visible beyond 5 min with very light color, it is suggested to increase the number of starting protoplasts up to the maximum of 10^9.

9. Trypsin Digest of Protein Bands and Peptide Purification

1. **Trypsin digest**
NOTE: Run a second 1D-PAGE gel by taking another 25 µL of concentrated mRBP solution from each sample for mRBP identification; silver-stained gels cannot be used since they are not compatible with MS analysis.
    1. After running the 1D-PAGE gel, fix the gel in fixation solution (50% (*v/v*) methanol and 10% (*v/v*) acetic acid) for 1 h. Stain the gel in stain solution (0.1% (*w/v*) Coomassie Brilliant Blue R-250, 50% (*v/v*) methanol, and 10% (*v/v*) acetic acid) for 20 min with gentle shaking. Destain the gel in destain solution (40% (*v/v*) methanol and 10% (*v/v*) acetic acid) for 1 h. After destaining, keep the gel in 5% (*v/v*) acetic acid.
    NOTE: During destaining, new destain solution can be replaced until the background is completely destained.
    2. Cut the bands of interest out of the gel. Cut the bands further into pieces of approximately 1 mm^3 for the subsequent optimal trypsin digestion and extraction of peptides. Hydrate the gel pieces with 50 µL of 100 mM ammonium bicarbonate (NH_4HCO_3) for 10 min. Cover the gel pieces completely.
        1. Remove the hydrating solution carefully and dehydrate the gel pieces with acetonitrile (CH_3CN) for 10 min. Carefully remove the acetonitrile solution.
        2. Repeat the process from hydration to dehydration twice. Finally, remove the acetonitrile solution.
        NOTE: The gel run time depends on the purpose of the experiment. A long run times allows a good separation of proteins, so the stained specific gel bands can be cut out for further analysis. If the purpose is to keep the proteins and remove contaminants, a short run time is sufficient.
    3. Add 500 µL of 6.6 mM DTT solution and incubate the gel pieces for 10 min. Remove the DTT solution and wash the gel pieces twice with 500 µL of 95% (*v/v*) acetonitrile solution.
        1. After washing, remove the acetonitrile solution and incubate the gel pieces with 500 µL of 55 mM iodoacetic acid (IAA) solution for 10 min in the darkness.
        2. After incubation, remove the solution and wash the gel pieces again with 500 µL of 95% (*v/v*) acetonitrile solution. Dry the gel pieces.
    4. Embed the gel pieces completely with 25 µL of digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 6 ng/µL trypsin) and keep on ice for 45 min to let the trypsin permeate into the gel. Incubate the gel pieces overnight at 37 °C for enzymatic digestion.

2. **Peptide purification**
    1. Add 80 µL of 50 mM NH_4HCO_3 to the gel pieces in the digestion buffer and vortex repeatedly to extract the tryptic peptides. Collect the extract. Repeat this extraction step twice with 80 µL of 50% (*v/v*) CH_3CN and 5% (*v/v*) formic acid (FA).
    NOTE: The total final extract should be approximately 240 µL.
    2. Pool and concentrate the extracts into approximately 10 µL by vacuum concentration or lyophilization and add 25 µL of 0.1% (*v/v*) aqueous trifluoroacetic acid (TFA) solution. Desalt the samples with µ-C18 columns, following the manufacturer's protocol.

3. Elute the peptides with 4 - 10 µL of 60% (*w/v*) CH_3CN and 0.1% (*v/v*) FA. Lyophilize (freeze-dry) the peptides and store them at -20 °C until analysis.

10. Nano-LC-MS

1. **Nano reverse-phase liquid chromatography**
   NOTE: Perform the LC-MS analysis with a mass spectrometer that is online-coupled with a liquid chromatography instrument. This instrument should be equipped with a C18 column (2 µm particle, 100 Å pore size, and 50 µm x 15 cm in dimension).
   1. Resuspend the lyophilized peptides in 16 µL of solution (2% (*v/v*) CH_3CN and 0.1% (*v/v*) FA). Inject and load 5 µL of peptide solution at a flow rate of 5 µL/min on a C18 precolumn (3 µm particle size, 100 Å pore size, nanoviper, and 75 µm x 2 cm in dimension).
   2. 10.1.2. Separate the peptides using a 95-min gradient with a flow rate of 300 nL/min. During this separation gradient, increase the mobile phase B from 4% to 10%, 10% to 25%, and 25% to 45% in 5 min, 50 min, and 18 min, respectively. Finally, steeply increase the mobile phase B to 95% in 1 min. After each 95-min separation gradient, apply an inherent rinse step containing a 10-min gradient from 4% to 95% in 5 min.
      NOTE: Mobile phase A is 99.9% H_2O and 0.1% (*v/v*) FA, and mobile phase B is 19.92% H_2O, 80% (*w/v*) CH_3CN, and 0.08% (*v/v*) FA.

2. **Mass spectrometry assay**
   1. Operate the mass spectrometer in data-dependent mode with a mass range from 400 to 1,600 m/z.
   2. Select up to ten of the most intense ions in MS1 to generate the fragmentation spectra. Set the resolution to 70,000 full width at half maximum (FWHM) for MS1 and 17,000 for MS2, with an automatic gain control (AGC) target of 3,000,000 and 1,000,000 ions and a maximum ion injection time (IT) of 256 and 64 ms for MS1 and MS2, respectively.
   3. Set the dynamic exclusion to 10 s to avoid repeated fragmentation of the most abundant ions.
      NOTE: Here, ions with one or more than six charges are excluded for MS2.

3. **Qualitative proteomics: peptide and protein identification**
   1. Analyze fragmentation spectra using software such as Peaks studio software[47,48,49].
      NOTE: Other software packages are also available for peptide identification, such as NovoHMM[30] and PepNovo[37] for *de novo* sequencing and SEQUEST[54] and Mascot[55] for database searching.
   2. Combine spectra with the same mass (< 2 ppm difference) and retention times (< 2 min) and only retain spectra with a quality threshold higher than 0.65 to search the Swiss-Prot database (version: December 2013) for the taxonomy set to model plant *Arabidopsis thaliana*. Use the following search parameters: a precursor mass tolerance of 10 ppm through the use of the monoisotopic mass; a fragment mass tolerance of 20 mmu; trypsin as the digestion enzyme, with a maximum of 2 missed cleavages; cysteine carbamidomethylation as the fixed modification; methionine oxidation as the variable modification; and a maximum of 3 variable post-translational modifications per peptide. After identification, manually set the score thresholds for reliable peptide and protein identification so that a false discovery rate (FDR) <5% is acquired.

4. **Quantitative proteomics: statistical analysis of mass spectrometry data**
   1. Use LC-MS (*e.g.* Progenesis) for the label-free quantitation of proteomics data.
      NOTE: MS1 peptide peak areas (or intensity) are linked to the peptides identified by the studio software according to their mass, with the tolerated error at a maximum of 10 ppm.
      NOTE: This software calculates the abundance of a peptide by integrating the peak areas of all the charge states between 2^+ and 8^+. The quantitative data is linked to the PEAKS peptide identification by an inhouse script (mass matching with the tolerated error at maximum of 10 ppm).
   2. Calculate the average log2-fold changes (CL/non-CL) for each peptide. Group the fold-changes of the unique peptides by protein.
      NOTE: In each group, the final fold-change of a protein equals the average fold-changes of its peptides.
   3. Calculate the p-value using Student's *t*-test for each group to evaluate the statistical significance. Apply R software package (version 3.3.0) to correct the p-values for the FDR of all groups using the Benjamini-Hochberg (BH) method.
   4. Draw the volcano plot using the "calibrate" function package (version 1.7.2) compatible with the R software package (version 3.3.0).
      NOTE: Here, the -log10-adjusted p-values (-log10 (adj. p-value)) are a function of the log2-fold changes of all identified proteins. Proteins are treated as positive hits in the mRNA-bound proteome if their log2-fold changes are greater than 2, with or without significance.

11. Catalog of the Identified mRNA-bound Proteome

1. Classify the identified proteins from step 10 into three categories based on the items of "molecular functions and biological process" via the Gene Ontology (GO) database and "family and domain" via the InterPro database.
2. As category I, or "Ribosomal proteins," contain all detected ribosomal proteins, define proteins from category II, or "Main RBPs," as containing annotated protein domains that interact with RNAs or link to RNA binding with known or unknown functions in RNA biology.
3. As proteins with known or unknown functions in RNA biology without annotated RNA-binding domains are placed into category III, or "Candidate RBPs," define enzymes from category III based on annotations from the IntEnz database.

Representative Results

We observed a characteristic halo, which surrounds the bead pellet in the CL sample, in wash step 4.3 with wash buffer 2 (**Figure 1B**). Although it has not been investigated, this phenomenon can probably be explained by the interference of crosslinked mRNP complexes with bead aggregation during the magnetic capture, causing a more diffuse aggregate to form. It indicates that the oligo-d$(T)_{25}$ bead capture was effective[14].

The significantly higher *UBQ10* reference mRNA levels than 18S rRNA in both non-CL control and CL samples are shown in **Figure 1C**. This indicates that mRNAs are enriched in the eluent due to the capture by oligo-d$(T)_{25}$ beads, which can only bind to poly(A)-tailed mRNAs. The efficiency of the oligo-d$(T)_{25}$ bead capture was further supported by SDS-PAGE and silver staining (step 8), as shown in **Figure 1D** after RNase treatment and mRBP concentration (step 7). A difference in protein band pattern between non-CL control and CL sample lanes can clearly be observed, and the protein bands present in the non-CL control sample lane can be explained by the presence of RNase. This illustrates the strong enrichment of mRBPs in the CL sample.

The efficiency of crosslinking can be controlled by varying the time duration of UV irradiation. Sufficient crosslinking but the avoidance of protoplast damage and RNA degradation is optimal. In **Figure 1D**, specific protein band patterns in all CL samples were obtained when comparing different UV irradiation times (1, 3, and 5 min). Under the same UV intensity (0.00875 W/cm^2), the most optimal conditions were found to be 1 or 3 min, due to the indistinguishable protein band intensities. Weaker band intensities were observed with the longer crosslinking time (5 min). We considered 1 min as the optimal condition because a shorter duration of crosslinking has the additional advantage of easier transfer and collection of protoplasts from the Petri dish. Protoplasts can be rapidly precipitated to the bottom of the Petri dish during UV irradiation because they stick to the dish bottom. This makes it more difficult to collect and transfer them to the tubes after longer UV irradiation duration.

Based on the optimized conditions, the identification of proteins from three biological replicates was subsequently achieved by qualitative and quantitative proteomics, which was described in steps 9 and 10. In the analysis by qualitative proteomics (**Figure 1E**, right), a total of 341 proteins were identified in CL samples, of which 36 were found both in non-CL control and CL samples, and only 8 proteins were detected in the non-CL control sample. This enormous difference in identified protein numbers between both samples is consistent with the different protein band patterns (**Figure 1D**), supporting the idea that the SDS-PAGE and silver-staining assay are good tools to validate the efficiency of oligo-d$(T)_{25}$ bead capture. In the analysis by quantitative proteomics (**Figure 1E**, left), a total of 325 proteins (blue- and green-colored) showed a log2-fold change greater than 2. Among these proteins, 100 proteins (blue-colored) had small p-values above the significance level. Therefore, they were also defined as positive hits. It is notable that the p-values of another 225 proteins (green-colored) were greater than 0.05, below the significance level due to data sparsity. In other words, there were lowly abundant peptides present for each protein, with high variability of peptide intensities. However, because all of them were qualitatively detected only in CL samples, they were considered positive.

Based on the GO and InterPro databases[50] (step 11), these 325 proteins were further classified into category I (ribosomal proteins), category II (main RBPs), and category III (candidate RBPs) (**Figure 1F**). There were 123 ribosomal proteins in category I, while in category II, 70 annotated classical RBPs were observed. These two categories—which contain most annotated proteins linking to molecular RNA binding and RNA biology (**Figure 1F**, right) and which occupy approximately 38% and 22% of the whole mRNA-bound proteome, respectively—indicate the high efficiency of optimized mRNA interactome capture. The last 40% (132 candidate RBPs) were classified in category III due to the lack of conventional RBDs. Furthermore, most of their roles in RNA binding and RNA biological processes have not been validated (**Figure 1F**, left). We suppose that proteins from this category could reveal novel functions in RNA regulations.

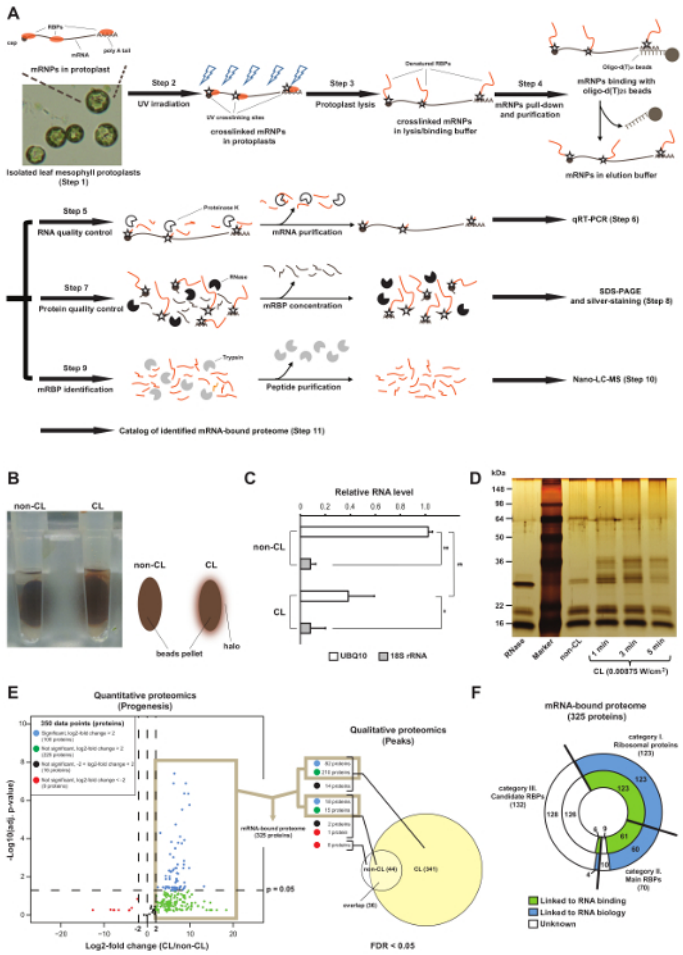

Figure 1: Flowchart and Results of the Optimized Method for Discovering the mRNA-bound Proteome from *Arabidopsis* Leaf Mesophyll Protoplasts. (**A**) Major steps of the whole method are listed from 1 to 11. Putative cellular and molecular processes are illustrated by the photo and cartoons. Details for each step have been intensively described in the Protocol. (**B**) A halo was observed surrounding the beads pellet in the CL sample, but not in the non-CL control sample, during wash step 4.3. (**C**) Comparison of relative *UBQ10* mRNA and 18S rRNA levels in non-CL control and CL samples by qRT-PCR (values are mean ± SD (n = 3); * and **: significant differences with p <0.05 and <0.01). (**D**) Concentrated protein eluent of non-CL control sample compared with CL samples irradiated by a continuous-wave UV source for 1, 3, and 5 min, with the UV intensity at 0.00875 W/cm^2. (**E**) Identification of mRNA-bound proteome by proteomics. In the quantitative proteomics performed by the Progenesis software package (left), the volcano plot displays the average log2-fold changes (CL/non-CL) and the related adjusted p-values (-log10 (adj. p-values)) of all proteins. In the qualitative proteomics performed by the Peaks software package (right), the proteins in the samples are illustrated in Venn diagrams. The quantity of the proteins is listed in numbers. The FDR at the peptide and protein levels is below 5%. The numbers of proteins inside the light-brown frames are considered positive hits. (**F**) Classification of three categories from the mRNA-bound proteome. The quantity of identified proteins is listed in numbers in each category. Green-colored regions are annotated proteins linked to RNA binding, while blue-colored regions refer to annotated proteins linked to RNA biology. The blank region indicates proteins with unknown functions linked to RNA binding or RNA biology. All figures above were modified from Zhang *et al.*, 2016. Please click here to view a larger version of this figure.

Discussion

We successfully applied mRNA interactome capture, developed for yeast and human cells, to plant leaf mesophyll protoplasts. Leaf mesophyll cells are the major type of ground tissue in plant leaves. The major advantage of this method is that it uses *in vivo* crosslinking to discover the proteins from a physiological environment.

In this protocol, we mainly present a number of optimized experimental conditions (*e.g.,* the number of protoplasts to use as starting material and the duration of UV irradiation)[50]. The captured proteins in the CL samples can only be detected by SDS-PAGE and silver staining when a minimum of 10^7 protoplasts (medium-scale) is used (step 1.5.5). Lower concentrations do not yield an observable mRBP pattern on SDS-PAGE and should probably not be used for further analysis by proteomics. Indeed, using more than 10^7 cells (*e.g.,* 10^9 in a large-scale experiment) is also possible[20]. Results from silver-staining assays suggest that UV irradiation for 1 min with 0.00875 W/cm^2 of intensity generated by a continuous-wave UV source is optimal (**Figure 1D**). High efficiency at the critical step (*i.e.,* mRNP pull-down and purification by oligo-d(T)$_{25}$ beads, step 4) is supported by the results of the RT-qPCR, silver-staining, and MS assays (**Figure 1C**; **1D**; and **1E**, right). The combination of qualitative and quantitative proteomics can help to identify the positive RBPs in the overlap region between non-CL control and CL samples (**Figure 1E**). The majority of identified proteins were RBPs not previously annotated in datasets (**Figure 1F**). We presented these previously

unknown RNA-binding proteins in category III as candidate RBPs[50] (**Figure 1F**). Exploring the binding specificities of these candidate RBPs must be done using other methods, such as the previously mentioned CLIP methods[23]. One example that investigates the binding specificity of an RBP to regulate its target mRNA transcript in *Arabidopsis* through the use of CLIP[51] can be found in Zhang *et al*., 2015. In addition, an alternative modified method from PAR-CLIP, called photoactivatable-ribonucleoside-enhanced crosslinking (PAR-CL, 365-nm UV-A) has also been previously recommended for the investigation of mRNA-bound proteomes from yeast and human cells[14,23]. PAR-CL requires 4Su, which is taken up by cells and incorporated into nascent RNAs during RNA metabolism and can be highly reactive, forming covalent bonds with amino acids[52] under UV-A (365-nm) irradiation. Currently, there are no studies focusing on the toxicity of 4sU to the plant cells and the efficient uptake of exogenous 4sU into mesophyll protoplasts[53]. However, we believe that it will become possible to use both conventional CL (254-nm UV-C) and PAR-CL (365 nm UV-A) on plants, which will contribute to the discovery and validation of diverse RBPs from the physiological environment in the future.

Disclosures

The authors have nothing to disclose.

Acknowledgements

We acknowledge the lab of Prof. Joris Winderickx, who provided the UV crosslinking apparatus equipped with the conventional UV lamp. K. G. is supported by the KU Leuven research fund and acknowledges support from FWO grant G065713N.

References

1. Jankowsky, E., Harris, M. E. Specificity and nonspecificity in RNA-protein interactions. *Nat Rev Mol Cell Biol.* **16** (9), 533-544 (2015).
2. Glisovic, T., Bachorik, J. L., Yong, J., Dreyfuss, G. RNA-binding proteins and post-transcriptional gene regulation. *FEBS Lett.* **582** (14), 1977-1986 (2008).
3. Gerstberger, S., Hafner, M., Tuschl, T. A census of human RNA-binding proteins. *Nat Rev Genet.* **15** (12), 829-845 (2014).
4. Lin, Q., Taylor, S. J., Shalloway, D. Specificity and determinants of Sam68 RNA binding. Implications for the biological function of K homology domains. *J Biol Chem.* **272** (43), 27274-27280 (1997).
5. Deo, R. C., Bonanno, J. B., Sonenberg, N., Burley, S. K. Recognition of polyadenylate RNA by the poly(A)-binding protein. *Cell.* **98** (6), 835-845 (1999).
6. Kattapuram, T., Yang, S., Maki, J. L., Stone, J. R. Protein kinase CK1 alpha regulates mRNA binding by heterogeneous nuclear ribonucleoprotein c in response to physiologic levels of hydrogen peroxide. *J Biol Chem.* **280** (15), 15340-15347 (2005).
7. Patel, G. P., Ma, S., Bag, J. The autoregulatory translational control element of poly(A)-binding protein mRNA forms a heteromeric ribonucleoprotein complex. *Nucleic Acids Res.* **33** (22), 7074-7089 (2005).
8. Song, J. K., McGivern, J. V., Nichols, K. W., Markley, J. L., Sheets, M. D. Structural basis for RNA recognition by a type II poly(A)-binding protein. *Proc Natl Acad Sci USA.* **105** (40), 15317-15322 (2008).
9. Lambert, N., Robertson, A., Jangi, M., McGeary, S., Sharp, P. A., Burge, C. B. RNA Bind-n-Seq: quantitative assessment of the sequence and structural binding specificity of RNA binding proteins. *Mol Cell.* **54** (5), 887-900 (2014).
10. Cook, K. B., Kazan, H., Zuberi, K., Morris, Q., Hughes, T. R. RBPDB: a database of RNA-binding specificities. *Nucleic Acids Res.* **39** (Database issue), D301-308 (2011).
11. Konig, J., Zarnack, K., Luscombe, N. M., Ule, J. Protein-RNA interactions: new genomic technologies and perspectives. *Nat Rev Genet.* **13** (2), 77-83 (2012).
12. Hockensmith, J. W., Kubasek, W. L., Vorachek, W. R., von Hippel, P. H. Laser cross-linking of nucleic acids to proteins. Methodology and first applications to the phage T4 DNA replication system. *J Biol Chem.* **261** (8), 3512-3518 (1986).
13. Pashev, I. G., Dimitrov, S. I., Angelov, D. Crosslinking proteins to nucleic acids by ultraviolet laser irradiation. *Trends Biochem Sci.* **16** (9), 323-326 (1991).
14. Castello, A. *et al.* System-wide identification of RNA-binding proteins by interactome capture. *Nat Protoc.* **8** (3), 491-500 (2013).
15. Nagy, E., Rigby, W. F. Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold). *J Biol Chem.* **270** (6), 2755-2763 (1995).
16. Hentze, M. W., Preiss, T. The REM phase of gene regulation. *Trends Biochem Sci.* **35** (8), 423-426 (2010).
17. Nicholls, C., Li, H., Liu, J. P. GAPDH: a common enzyme with uncommon functions. *Clin Exp Pharmacol Physiol.* **39** (8), 674-679 (2012).
18. Baltz, A. G. *et al.* The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. *Mol Cell.* **46** (5), 674-690 (2012).
19. Castello, A. *et al.* Insights into RNA Biology from an Atlas of Mammalian mRNA-Binding Proteins. *Cell.* **149** (6), 1393-1406 (2012).
20. Kwon, S. C. *et al.* The RNA-binding protein repertoire of embryonic stem cells. *Nat Struct Mol Biol.* **20** (9), 1122-1130 (2013).
21. Mitchell, S. F., Jain, S., She, M., Parker, R. Global analysis of yeast mRNPs. *Nat Struct Mol Biol.* **20** (1), 127-133 (2013).
22. Beckmann, B. M. *et al.* The RNA-binding proteomes from yeast to man harbour conserved enigmRBPs. *Nat Commun.* **6** (10127), 1-9 (2015).
23. Bailey-Serres, J., Sorenson, R., Juntawong, P. Getting the message across: cytoplasmic ribonucleoprotein complexes. *Trends Plant Sci.* **14** (8), 443-453 (2009).
24. Quesada, V., Macknight, R., Dean, C., Simpson, G. G. Autoregulation of FCA pre-mRNA processing controls Arabidopsis flowering time. *EMBO J.* **22** (12), 3142-3152 (2003).
25. Lim, M. H. *et al.* A new Arabidopsis gene, FLK, encodes an RNA binding protein with K homology motifs and regulates flowering time via FLOWERING LOCUS C. *Plant Cell.* **16** (3), 731-740 (2004).
26. Hornyik, C., Terzi, L. C., Simpson, G. G. The spen family protein FPA controls alternative cleavage and polyadenylation of RNA. *Dev Cell.* **18** (2), 203-213 (2010).
27. Stern, D. B., Goldschmidt-Clermont, M., Hanson, M. R. Chloroplast RNA metabolism. *Annu Rev Plant Biol.* **61**, 125-155 (2010).
28. Schmal, C., Reimann, P., Staiger, D. A circadian clock-regulated toggle switch explains AtGRP7 and AtGRP8 oscillations in Arabidopsis thaliana. *PLoS Comput Biol.* **9** (3), e1002986 (2013).

29. Staiger, D. RNA-binding proteins and circadian rhythms in Arabidopsis thaliana. *Philos Trans R Soc Lond B Biol Sci.* **356** (1415), 1755-1759 (2001).
30. Fischer, B. *et al.* NovoHMM: a hidden Markov model for de novo peptide sequencing. *Anal Chem.* **77** (22), 7265-7273 (2005).
31. Kwak, K. J., Kim, Y. O., Kang, H. Characterization of transgenic Arabidopsis plants overexpressing GR-RBP4 under high salinity, dehydration, or cold stress. *J Exp Bot.* **56** (421), 3007-3016 (2005).
32. Raab, S., Toth, Z., de Groot, C., Stamminger, T., Hoth, S. ABA-responsive RNA-binding proteins are involved in chloroplast and stromule function in Arabidopsis seedlings. *Planta.* **224** (4), 900-914 (2006).
33. Liu, H. H. *et al.* Molecular cloning and characterization of a salinity stress-induced gene encoding DEAD-box helicase from the halophyte Apocynum venetum. *J Exp Bot.* **59** (3), 633-644 (2008).
34. Wang, S. C., Liang, D., Shi, S. G., Ma, F. W., Shu, H. R., Wang, R. C. Isolation and Characterization of a Novel Drought Responsive Gene Encoding a Glycine-rich RNA-binding Protein in Malus prunifolia (Willd.) Borkh. *Plant Mol Biol Report.* **29** (1), 125-134 (2011).
35. Lorkovic, Z. J., Barta, A. Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant Arabidopsis thaliana. *Nucleic Acids Res.* **30** (3), 623-635 (2002).
36. Ambrosone, A., Costa, A., Leone, A., Grillo, S. Beyond transcription: RNA-binding proteins as emerging regulators of plant response to environmental constraints. *Plant Science.* **182**, 12-18 (2012).
37. Frank, A., Pevzner, P. PepNovo: de novo peptide sequencing via probabilistic network modeling. *Anal Chem.* **77** (4), 964-973 (2005).
38. Marondedze, C., Thomas, L., Serrano, N. L., Lilley, K. S., Gehring, C. The RNA-binding protein repertoire of Arabidopsis thaliana. *Sci Rep.* **6** (29766), 1-13 (2016).
39. Reichel, M. *et al.* In Planta Determination of the mRNA-Binding Proteome of Arabidopsis Etiolated Seedlings. *Plant Cell.* **28** (10), 2435-2452 (2016).
40. Yoo, S. D., Cho Y. H., Sheen, J. Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc.* **2** (7), 1565-1572 (2007).
41. Niu, Y., Sheen, J. Transient expression assays for quantifying signaling output. *Methods Mol Biol.* **876**, 195-206 (2012).
42. Petersson, S. V. *et al.* An auxin gradient and maximum in the Arabidopsis root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *Plant Cell.* **21** (6), 1659-1668 (2009).
43. Bargmann, B. O., Birnbaum K. D. Fluorescence activated cell sorting of plant protoplasts. *J Vis Exp.* **36** (1673), 1-4 (2010).
44. Hong, S. Y., Seo, P. J., Cho, S. H., Park, C. M. Preparation of Leaf Mesophyll Protoplasts for Transient Gene Expression in Brachypodium distachyon. *J Plant Biol.* **55** (5), 390-397 (2012).
45. Li, Y., Van den Ende, W., Rolland, F. Sucrose Induction of Anthocyanin Biosynthesis Is Mediated by DELLA. *Mol Plant.* **7** (3), 570-572 (2014).
46. Durut, N. *et al.* A duplicated NUCLEOLIN gene with antagonistic activity is required for chromatin organization of silent 45S rDNA in Arabidopsis. *Plant Cell.* **26** (3), 1330-1344 (2014).
47. Han, Y. H., Ma, B., Zhang, K. Z. SPIDER: Software for protein identification from sequence tags with De Novo sequencing error. *J Bioinform Comput Biol.* **3** (3), 697-716 (2005).
48. Han, X., He, L., Xin, L., Shan, B., Ma, B. PeaksPTM: Mass spectrometry-based identification of peptides with unspecified modifications. *J Proteome Res.* **10** (7), 2930-2936 (2011).
49. Zhang, J. *et al.* PEAKS DB: de novo sequencing assisted database search for sensitive and accurate peptide identification. *Mol Cell Proteomics.* **11** (4), M111 010587 (2012).
50. Zhang, Z. *et al.* UV crosslinked mRNA-binding proteins captured from leaf mesophyll protoplasts. *Plant Methods.* **12** (42), 1-12 (2016).
51. Zhang, Y. *et al.* Integrative genome-wide analysis reveals HLP1, a novel RNA-binding protein, regulates plant flowering by targeting alternative polyadenylation. *CellRes.* **25** (7), 864-876 (2015).
52. Steen, H., Jensen, O. N. Analysis of protein-nucleic acid interactions by photochemical cross-linking and mass spectrometry. *Mass Spec Rev.* **21** (3), 163-182 (2002).
53. Miller, C. *et al.* Dynamic transcriptome analysis measures rates of mRNA synthesis and decay in yeast. *Mol Syst Biol.* **7** (458), 1-13 (2011).
54. Eng, J., McCormack, A. L., Yates, J. R. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom.* **5** (11), 976-989 (1994).
55. Perkins, D. N., Pappin, D. J. C, Creasy, D. M., Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* **20** (18), 3551-3567 (1999).

