Polysomal-mRNA Extraction from *Arabidopsis* by Sucrose-gradient Separation

Shih-Yun Lin and Guang-Yuh Jauh*

Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan
*For correspondence: jauh@gate.sinica.edu.tw

[Abstract] mRNAs surrounded by polysomes are ready for translation into proteins (Warner *et al.*, 1963); these mRNAs are defined as polysomal-mRNAs (Mustroph *et al.*, 2009). The process is affected by various growth conditions or surrounding situations. Microarray analysis is a powerful tool for detecting genome-wide gene expression. Therefore, using polysomal-mRNAs for microarray analysis can reflect the gene translation information (the translatome) under different developmental stages or environmental conditions from eukaryotes. Polysomal-mRNAs can be collected from the polysomal fraction by sucrose-gradient separation for further quantitative PCR or microarray assay. We modified a protocol (Mustroph *et al.*, 2009) for collecting polysomal-mRNAs via sucrose-gradient separation to eliminate monosomal-mRNA contamination from *pLAT52:HF:RPL18 Arabidopsis*. This transgenic Arabidopsis uses a pollen-specific promoter (*ProLAT52*) to generate epitope-tagged polysomal-RNA complexes that could be purified with a specific antigen (Lin *et al.*, 2014). The polysomal-mRNAs we obtained via sucrose-gradient separation and antibody purification underwent *in vivo* translation in pollen tubes grown from self-pollinated gynoecia of *Arabidopsis thaliana*.

Material and Reagents

1. *pLAT52: HF-RPL18* transgenic *Arabidopsis*
2. RNase-free water
3. Tris buffer (Sigma-Aldrich, catalog number: T1378)
4. KCl (Sigma-Aldrich, catalog number: P9541)
5. EGTA (Sigma-Aldrich, catalog number: E3889)
6. $MgCl_2$ (Sigma-Aldrich, catalog number: M8266)
7. β-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
8. Cycloheximide (Sigma-Aldrich, catalog number: C7698)
9. Chloramphenicol (Sigma-Aldrich, catalog number: C0378)
10. Polyoxyethylene 10 tridecyl ether (PTE) (Sigma-Aldrich, catalog number: P2393)
11. Sodium deoxycholate (DOC) (Sigma-Aldrich, catalog number: D6750)
12. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)
13. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)

14. Triton X-100 (Sigma-Aldrich, catalog number: X-100)
15. Polyoxyethylene(23)lauryl ether (Brij -35) (Sigma-Aldrich, catalog number: P6938)
16. Tween-20 (Sigma-Aldrich, catalog number: P1379)
17. NP-40 (Sigma-Aldrich, catalog number: NP 40)
18. Polyoxyethylene (Sigma-Aldrich, catalog number: P2393)
19. Deoxycholic acid (Sigma-Aldrich, catalog number: D2510)
20. ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich, catalog number: A2220)
21. RNAsin (Promega Corporation, catalog number: N2511)
22. 3x FLAG peptide (Sigma-Aldrich, catalog number: F4799)
23. Sucrose (Sigma-Aldrich, catalog number: 84097)
24. Polysomal extraction buffer preparation (see Recipes)
25. 20% detergent mixture (see Recipes)
26. 20% PTE and 10% DOC (see Recipes)
27. Sucrose gradient layer preparation (see Recipes)
28. 10x sucrose salts (see Recipes)
29. Preparation of the FLAG M2 Agarose beads (see Recipes)

Equipment

1. QIAGEN QIA shedder columns (QIAGEN, catalog number: 27115)
2. 15-ml tube (Labcon, catalog number: 9205-946CB-946C)
3. Hitachi ultracentrifuge himac CP100WX (Hitachi, catalog number: CP100WX)
4. Hitachi P40ST swing rotor (Hitachi, model: P40ST)
5. 13PA tubes (Hitachi, catalog number: 332901A)
6. Spectrophotometer (Beckman Coulter, catalog number: DU648B)
7. UV monitor (GE Healthcare, catalog number: UVIS-920)
8. Pump (GE Healthcare, model: P-50)
9. Fraction collector (Gilson Products, catalog number: FC80)
10. UV detector system (GE Healthcare, catalog number: monitor UVIS-0912)
11. Recorder (GE Healthcare, catalog number: pharmaria LKB REC102)

Procedure

Pre-treatment: All glass materials were heated in 180 °C for 12 h and all plastic materials were treated with non-sterile DEPC-H_2O overnight, then autoclaved at 121 °C for 20 min. RNase-free water was used to prepare all buffers.

1. Collect 0.5-1 mg plant tissue sample into a mortar and then add liquid nitrogen immediately.
2. After grinding the plant tissue finely, add 1,250 µl polysome extraction buffer into the mortar.
3. Transfer the crude extract in an Eppendorf tube and let it sit on ice for 10 min.
4. Centrifuge the crude extract for 10 min at 13,000 rpm at 4 °C.
5. Transfer the supernatant into a QIA shedder column (700 µl/column). The column is spun for 1 min at 13,000 rpm at 4 °C, then the flow-through is collected.
6. Detect the OD_{260} unit for the flow-through by spectrophotometry.
7. Prepare the four different sucrose gradient layers in the Hitachi centrifuge column.
8. Carefully add 800 µl sample (20-25 OD_{260} unit; the nucleic acid concentration for 1 OD_{260} unit is equal to 40 µg/ml RNA) onto the top sucrose layer (the 20% layer). Balance tubes and buckets to 0.03 g by adding sample or polysome extraction buffer.
9. Ultracentrifuge for 225 min at 39,000 rpm at 4 °C in an Hitachi P40AT swing rotor.
10. Link the pump with a UV detector and the fraction collector. Link the UV detector with the recorder.
11. Put the 260-nm filter into the UV detector system and turn it on at least 20 min before polysome profile analysis.
12. Put the probe into a sample separated in sucrose gradient after ultracentrifugation, turn on the pump to suck the sample, and turn on the recorder to create the sucrose gradient profile.
13. According to the sucrose gradient profile, combine the polysomal fractions together into a 15-ml tube.
14. Add the washed FLAG beads to the 15-ml tube and incubate for 2 h at 4 °C on a rocking platform.
15. Centrifuge the mixture for 3 min at 3,000 rpm at 4 °C. Remove the supernatant.
16. Add 2 ml wash buffer to the tube and mix gently by inverting it for 5 min at 4 °C, then centrifuge the mixture for 3 min at 3,000 rpm at 4 °C. Remove the supernatant (first wash).
17. Add 2 ml wash buffer to the tube and mix gently by inverting it for 5 min at 4 °C, then centrifuge the mixture for 3 min at 3,000 rpm at 4 °C. Remove the supernatant. Repeat 3 times.
18. Transfer the mixture to a new Eppendorf tube.
19. To elute the affinity-purified polysomes, add the 300 µl FLAG peptide (400 ng/µl) with RNAsin (20 U/ml) to the Eppendorf tube and incubate for 30 min at 4 °C on a rocking platform.

20. Centrifuge for 1 min at 8,200 *x g* at 4 °C. Transfer the supernatant to a new Eppendorf tube. If the supernatant still contains the beads, centrifuge again for 5 min at 13,000 rpm at 4 °C.
21. Add additional wash buffer to the eluent to reach 500 µl volume.
22. Add phenol/chloroform (1:1) in an equal volume (500 µl) to the eluent and mix by inverting.
23. Centrifuge for 10 min at 13,000 rpm at 4 °C.
24. Transfer the supernatant to a new Eppendorf tube and add an equal volume of chloroform.
25. Centrifuge for 10 min at 13,000 rpm at 4 °C.
26. Transfer the supernatant to a new Eppendorf tube and add 0.1 volume 3 M NaOAC (pH 5.2) and 2.5 volume 100% EtOH to precipitate the RNA overnight at -20 °C.
27. Centrifuge for 25 min at 13,000 rpm at 4 °C.
28. Pour out the supernatant, then wash the pellet with 70% EtOH twice.
29. Air-dry the RNA pellet and use 20 µl RNase-free water to suspend the RNA pellet.

Representative data

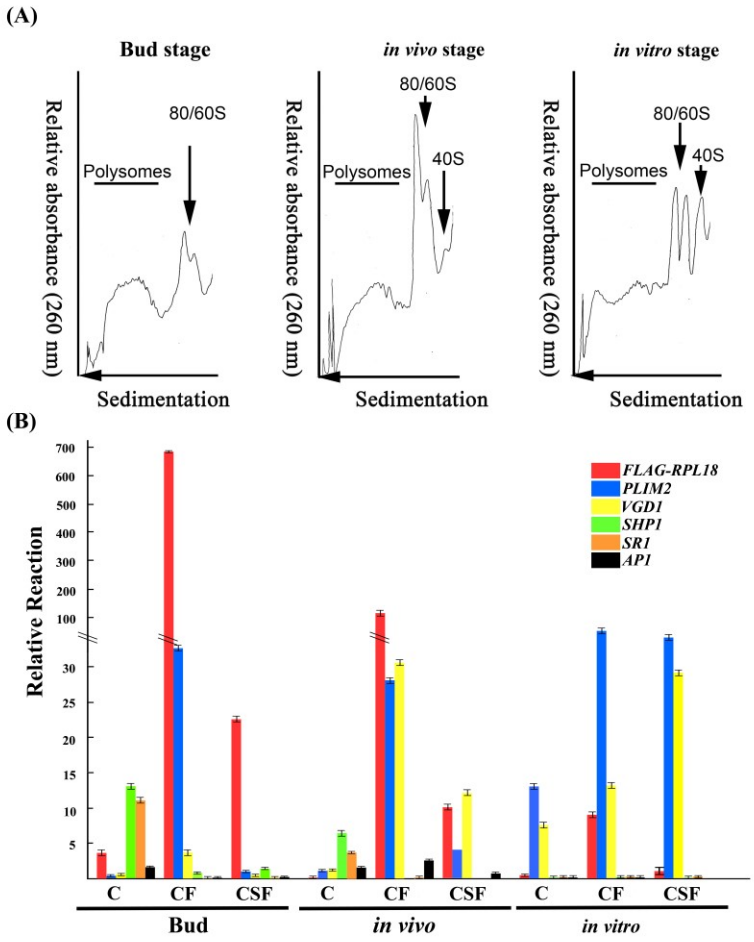

Figure 1. Polysomal profiles and validation of the specificity of immunopurification of mRNAs associated with pollen from pollinated floral buds, *in vivo*-pollinated pollen tubes, and *in vitro*-cultured pollen tubes of *LAT52:HF-RPL18* transgenic plants. (A) Typical sucrose-gradient absorbance (A 260) profiles of ribosome complexes obtained from Bud stage, *in vivo* stage, and in vitro-cultured pollen. Positions of peaks corresponding to polysomes (line), 40S ribosomal subunits and 60S ribosomal subunit/80S monosomes (arrowheads) are indicated. The arrow for the sedimentation reflects the sucrose gradient from 20% to 60% (top to bottom). (B) Quantitative RT-PCR with primer sets targeting the *FLAG-RPL18* transgene and organ-specific genes to confirm that the RNA extracted from purified polysomal mRNAs examined by sucrose-gradient separation and further purified by FLAG-agarose beads was all male gametophyte-specific. Primers span both the *His6-FLAG* tag and the *RPL18* sequence (*FLAG-PRL18*); pollen-specific *PLIM2* and *VGD1*, female-specific *SHP1* and *SR*, and petal/sepal-specific *AP1*. *ACT2* was an internal control (Lin *et al.*, 2014).

Table1. Primer pairs for Q-PCR in Figure 1 (Lin *et al.*, 2014)

FLAG-RPL18 QF	TATGCGAGACGCCTATGATCG
FLAG-RPL18 Q R	CGTGCACAACAGAATTGAAAGC
AP1 QF	TACTCTTACGCCGAAAGACAGC
AP1 QR	TGTATTGACGTCGGACTCAGGT
CRP1 QF	AGGTGCGAACAAGAGGTGTCAC
CRP1 QR	GCAAGCCTTGTTCCCATCATT
SHP1 QF	GTACCTGCGAGCAAAGATAGCC
SHP1 QR	TCACACTCGATTCCTGCTGGTC
SR1 QF	TGATATCTGGTGTGGCGCTTG
SR1 QR	TGGTTCAGCGGCTATAATCCG
VGD1 QF	GTTAAACTCAGCCCTGGAACCA
VGD1 QR	AACCTGGACTGTGCCACTAAGG
PLIM2 (CRP1) QF	GACTTGCTTCAGGTGCACACAC
PLIM2 (CRP1) QR	AGGACGCCATTAAGAGAAGCG

Notes

1. Prepare enough samples for sucrose gradient profile presentation.

Table 2. Number of flowers used for the polysomal-RNA extraction for *LAT52:HF-RPL18* Arabidopsis (Lin *et al.*, 2014)

Sample ID	No. of flowers	RNA level (ng/μl)	aRNA level (ng/μl)
Bud	800-900	8.761	198.1
in vivo	500-700	0.455	1,159.1
in vitro	7,200	2.897	1,036.4

aRNA: antisense RNA synthesized

Recipes

1. Polysomal extraction buffer preparation

2 M Tris-HCl (pH 9.0)	1 ml
2 M KCl	1 ml
0.5 M EGTA (pH 8.3)	0.5 ml
1 M $MgCl_2$	0.36 ml
dd H_2O	To 8 ml
Mix well	
β-mercaptoethanol	80 µl
50 mg/ml cycloheximide	10 µl
50 mg/ml chroramphenicol	10 µl
20% detergent mixture	0.5 ml
2% PTE, 10% DOC	1 ml
0.5 M DTT	20 µl
0.5 M PMSF	20 µl
ddH_2O	To 10 ml

2. 20% detergent mixture

Triton X-100	10 ml
Brij 35	10 g
Tween-20	10 ml
NP-40	10 ml
ddH_2O	To 50 ml

3. 20% PTE and 10% DOC

Polyoxyethylene 10 trydecyl ether	4 ml
Deoxychollic acid	2 g
ddH_2O	To 20 ml

4. Sucrose-gradient layer preparation

   Add 0.1 µl per 1 ml volume of cyclohexamide (50 mg/ml) and chloramphenicol (50 mg/ml) to each layer

% sucrose	2 M (68.5%) sucrose	10x sucrose salt	ddH_2O	Vol/gradient
60	44 ml	5 ml	1 ml	1.5 ml
45	66 ml	10 ml	24 ml	3.0 ml
30	44 ml	10 ml	46 ml	3.0 ml
20	14.5 ml	5 ml	30.5 ml	1.5 ml

5. 10x sucrose salts

Tris-base	2.43 g
KCl	0.75 g
$MgCl_2$	1.02 g
ddH_2O	To 50 ml
Adjust pH value with HCl	8.4
Autoclave 20 min, 121 °C	Stored at -20 °C

6. Preparation of the FLAG M2 Agarose beads
   a. Use cut pipette to transfer 100 µl FLAG M2 from a bottle into an Eppendorf tube, then centrifuge for 1 min at 8,200 *x g* at 4 °C.
   b. Remove the supernatant and add 500 µl wash buffer to resuspend the beads. Centrifuge for 1 min at 8,200 *x g* at 4 °C.
   c. Repeat the above step once.
   d. Wash buffer.

2 M Tris-HCL (pH 9.0)	1 ml
2 M KCl	1 ml
0.5 M EGTA (pH 8.3)	0.5 ml
1 M $MgCl_2$	0.36 ml
50 mg/ml cycloheximide	10 µl
50 mg/ml chroramphenicol	10 µl
0.5 M DTT	20 µl
0.5 M PMSF	20 µl
RNAsin	20 U/ml
ddH_2O	To 10 ml

Acknowledgments

We thank Dr. Julia Bailley-Serres (Department of Botany and Plant Sciences, University of California, Riverside) for the *pLAT52-HF-RPL18* Arabidopsis and for sharing the polysomal-mRNA extraction protocol. This work was funded by Academia Sinica (Taiwan), the Taiwan National Science and Technology Program for Agricultural Biotechnology (Lin et al 31; NSTP/AB, 098S0030055-AA) and the Taiwan National Science Council (99-2321-B-001-036-MY3 and 102-2321-B-001-040-MY3) to G.-Y. Jauh.

References

1. Lin, S. Y., Chen, P. W., Chuang, M. H., Juntawong, P., Bailey-Serres, J. and Jauh, G. Y. (2014). Profiling of translatomes of *in vivo*-grown pollen tubes reveals genes with roles in micropylar guidance during pollination in *Arabidopsis*. *Plant Cell* 26(2): 602-618.
2. Mustroph, A., Juntawong, P. and Bailey-Serres, J. (2009). Isolation of plant polysomal mRNA by differential centrifugation and ribosome immunopurification methods. *Methods Mol Biol* 553: 109-126.
3. Warner, J. R., Knopf, P. M. and Rich, A. (1963). A multiple ribosomal structure in protein synthesis. *Proc Natl Acad Sci U S A* 49: 122-129.