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Isolation and Profiling of Protein-Associated Small RNAs

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Abstract

Small RNAs are short non-coding RNAs with important regulatory roles in many cellular processes. Small RNAs are generated by DICER or DICER-like (DCL) proteins and then incorporated into RNAi effector proteins ARGONAUTEs (AGOs) for silencing of their targets. In plants, small RNAs regulate host innate immunity against various pathogens, but their mode of action and associated protein factors that facilitate their function remain to be elucidated. Here we describe an efficient method to isolate AGO-associated small RNAs from *Arabidopsis*. This protocol can be easily adapted for the isolation of any protein-associated small RNAs. We utilized immunoprecipitation tandem with deep-sequencing to identify small RNAs with functions in plant innate immunity. Using this described protocol, we identified miR393* that play a crucial role in plant anti-bacterial defense. The distinct roles played by individual AGO proteins were observed.

Keywords

immunoprecipitation; Argonaute; protein-associated small RNAs; plant innate immunity

INTRODUCTION

Small RNAs are non-coding RNAs that regulate gene expression in a sequence-specific manner. They are generally 20-30 nucleotides (nt) in length and can be categorized as microRNAs (miRNA) or small interfering RNAs (siRNAs) according to their precursor structures and biogenesis pathways [1, 2]. Small RNAs are involved in the regulation of many growth and developmental processes, as well as stress responses, diseases and metabolic disorders [1-4].

Small RNAs are generated by DICER or DICER-like (DCL) proteins and then incorporated into effector complexes (RNA induced silencing complex [RISC]) containing an ARGONAUTE (AGO) protein to guide silencing of their target RNAs [5]. Plants have various AGO proteins with distinct functions. In Arabidopsis, 10 AGO proteins were identified [6]. AGO1 binds mainly miRNAs, while AGO4 and AGO6 bind mainly 24-nt heterochromatic siRNAs. AGO2 is highly induced by bacterial or viral pathogens and regulates plant innate immunity [7, 8]. Arabidopsis small RNAs load into corresponding AGO-containing RISC usually according to the identity of their first nucleotide [9, 10].

Most RNAs, including small RNAs, are regulated and function through RNA-binding proteins. Small RNAs need to be loaded into AGO proteins for inducing gene silencing of their targets To unveil the biological roles of small RNAs, it is necessary to investigate the expression profiles of AGO-associated small RNAs [9-11]. AGO immunoprecipitation

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(pull-down) tandem with deep-sequencing can provide a snapshot of *in vivo* small RNA populations in different AGO complexes [9, 10]. Such information is indispensable for dissecting the biological roles and functionality of small RNAs. In this chapter, we describe an efficient protocol for isolating and profiling AGO-associated small RNAs from *Arabidopsis*. We have isolated AGO1- and AGO2-associated small RNAs in healthy (mock-treated) and bacterial pathogen (*Pseudomonas syringae*) challenged *Arabidopsis* samples [12]. The deep-sequencing results allowed us to identify miR393* as a functional miRNA specifically enriched in AGO2 from bacterial pathogen-challenged plants, revealing it's important role in plant innate immunity. We demonstrate that miR393* and miR393 specifically loads into AGO2 and AGO1, respectively [7].

MATERIALS

1. Bacterial pathogen inoculation

- **A.** *Arabidopsis* Columbia-0 carrying an HA-tagged GUS gene, used as a control
- **B.** *Arabidopsis* Columbia-0 plants carrying an HA-tagged AGO2 (*AGO2::3HA:AGO2*).
- **C.** *Pseudomonas syringae* pv. *tomato* DC3000 carrying *avrRpt2* (*Pst* [*avrRpt2*]).
- **D.** Pseudomonas Agar F (PAF) medium: 19g BD Difco™ PAF powder, 5 ml glycerol. Make up to 500ml with distilled water and autoclave. Cool to 50°C then add 50 μg/ml rifampicin and 50 μg/ml kanamycin before pouring plates.
- **E.** 10mM $MgCl_2$: in sterile distilled water.

2. Protein pull-down

- **A.** IP extraction buffer (pH 7.5): 20 mM Tris-HCl, 300mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) NP40, 5 mM DTT (added prior to use), 1 tablet/50 ml protease inhibitor (added prior to use), make up to 50 ml with DEPC-treated water.
- **B.** IP washing buffer (pH 7.5): 20 mM Tris-HCl, 300mM NaCl, 5 mM $MgCl_2$, 0.5% (v/v) Triton X-100, 5 mM DTT (added prior to use), 1 tablet/50 ml protease inhibitor (added prior to use), make up to 50 ml with DEPC-treated water.
- **C.** 2× SDS loading buffer (pH 6.8): 0.125 M Tris-HCl, 4% (w/v) SDS, 20% (v/v) Glycerol, 2% (v/v) 2-mercaptoethanol, 0.05% (w/v) Bromophenol blue, and distilled water.

D. Bromophenol blue	(ICN Biomedical)
E. Bradford reagent	(BioRad)
F. Protein A-Agarose	(Roche)
G. Anti-HA Affinity matrix	(clone 3F10, Roche)
H. Glycogen blue	(Ambion)

3. RNA extraction using TRIzol reagent

A. TRIzol reagent	(Invitrogen)
B. Chloroform	(Fisher)

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C. 3M sodium acetate (pH5.2)	(Fisher)
D. 75% and 100% ethanol (RNase free)	
E. Glycogen blue	(Ambion)

4. Small RNA library construction

A. 0.3M NaCl: in DEPC-treated water

B. 3′ Linker: 5′-rAppCTGTAGGCACCATCAAT/3ddC/-3′

C. 5′ Linker: 5′-TGGAATrUrCrUrCrGrGrGrCrArCrCrArArGrGrU-3′

D. Agarose	(Fisher)
E. ATP	(NEB)
F. ATP-free T4 RNA ligase buffer	(NEB)
G. DMSO (RNase free)	(Fisher)
H. 5X First Strand Buffer	(Invitrogen)

I 10% Urea-PAGE: 2.5 ml 40% polyacrylamide (acrylamide:bisacrylamide=29:1), 1 ml 5X TBE (RNase free), 4.2 g urea (RNase free), 50 μl 10% Ammonium Persulfate (APS), 5 μl N,N,N',N'-tetramethylethylenediamine (TEMED), add water (DEPC-treated) to 10 ml.

J. dNTPs (10mM; in DEPC-treated water)	(Fermentas)
K. DTT (0.1M)	(Invitrogen)
L. Gel loading buffer II	(Ambion)
M. RNase-OUT	(Invitrogen)
N. SuperScript III Reverse Transcriptase	(Invitrogen)
O. Ethidium bromide (EtBr)	(Fisher)
P. GeneRuler 100 bp DNA ladder	(Fermentas)
Q. O'RangeRuler 10 bp DNA ladder	(Fermentas)

R Polymerase Chain Reaction (PCR) 5′ primer: 5′-AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA 3′

S RT and PCR 3′ primer: 5′-CAAGCAGAAGACGGCATACGATTGATGGTGCCTACAG-3′

T. PrimeSTAR HS DNA Polymerase	(Takara)
U. T4 RLN2	(NEB)
V. T4 RNA ligase	(NEB)

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5. mRNA detection

A. DNase I	(Invitrogen)
B. 25 mM EDTA	(Invitrogen)
C. Oligo d(T) primer	
D. *Taq* DNA polymerase	(NEB)
E. 6X DNA loading day	(Fermentas)
F. 2% agarose gel.	

METHODS

Materials and reagents prepared prior to experiments

A. Prepare IP extraction buffer and IP washing buffer without DTT and protease inhibitor cocktails. Prepare 50 mg/ml rifampicin and 50 mg/ml kanamycin stock solutions and sterilize by filtration.

B. Grow transgenic *3HA:GUS* (for AGO1-immunoprecipitation using anti-AGO1 antibody) and *AGO2::3HA:AGO2* (For AGO2- immunoprecipitation using HA antibody) *Arabidopsis* plants at a 12-h light/12-h dark photoperiod four weeks prior to starting experiments.

C. Add DTT and protease inhibitor cocktail to IP extraction buffer and IP washing buffer immediately before using.

D. Pre-equilibrate protein-A beads with IP extraction buffer immediately before using.

E. Pre-equilibrate conjugated-HA beads with IP extraction buffer immediately before using.

1. Bacteria pathogen inoculation

A. Culture *Pst (avrRpt2)* on a PAF plate supplemented with antibiotics overnight at 28°C.

B. On the second day, collect the bacteria and re-suspend in 10mM $MgCl_2$ to a final concentration of OD_{600}=0.02 (*see* **Note 1**). Prepare 10mM $MgCl_2$ solution as a mock control.

C. Syringe-infiltrate the 4-week old *3HA:GUS* and *AGO2::3HA:AGO2 Arabidopsis* plants with 10mM $MgCl_2$ (mock) and *Pst* (*avrRpt2).* A needleless syringe is used to pressure-infiltrate the abaxial (lower) side of the leaves with care such that plant tissue is not damaged.

D. Collect infiltrated leaves 12 h after inoculation and freeze immediately in liquid nitrogen. Afterward, tissue can be stored at −80°C for several months if not processed immediately.

2. Protein immunoprecipitation (*see* Note 2)

A. Pre-cool mortars and pestles with liquid nitrogen. Grind 0.25 g leaf tissue (*see* **Note 3**) into a fine powder in liquid nitrogen. Keep materials frozen all the time. Transfer

[1]Equivalent to 2×10^7 colony-forming units (cfu) per milliliter.
[2]All procedures should be carried out on ice or in a cold-room unless indicated otherwise. Cool tubes and tips in a cold room prior to use.
[3]Begin with 0.1-0.2 g plant tissue for a standard Northern blot. For constructing a small RNA library, 1-2 g tissue is needed.

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ground tissue to a 1.5 ml microcentrifuge tube (pre-cooled by liquid nitrogen prior to use) without thawing. Add 1.0 ml cold IP extraction buffer (see recipe in the Materials section) to the tube. Mix IP extraction buffer with ground plant tissue thoroughly by inverting and gentle shaking (no vortexing). Keep tubes on ice until no frozen tissue is visible.

B. Continue to mix by inverting for 10 min in a cold-room.

C. Centrifuge the homogenate at 10,000 × g, 4°C for 10 min. Pass the supernatant through a cell-strainer (*see* **Note 4**).

D. Aliquot 10 μl supernatant or IP extraction buffer (background control) to a 1.5 ml microcentrifuge tube. Add 1 ml BradFord reagent to each tube. After mixing by inverting, leave at room temperature for 10 min, then measure and record the optical density at 595 nm (OD_{595}). Adjust the volume of each sample by adding IP extraction buffer so that each sample will have equal OD units (OD_{595}×volume).

E. Save 10 μl as inputs for Western blotting detection later by adding 10 μl 2× SDS loading buffer (see recipe in the Materials section) and boiling for 5 min.

F. Pre-clear aliquots by adding 25 μl Protein A-Agarose beads to the protein extract corresponding to each 0.25 g plant tissue (pre-equilibrated). Rotate the tubes for 1h in cold room. Centrifuge at 200 × g, 4°C for 30 sec to collect the beads and transfer the supernatant to new tubes.

G. Add 25 μl Anti-HA Affinity Matrix to the protein extract corresponding to each 0.25 g of plant tissue (pre-equilibrated). Rotate in cold room for 2 h (*see* **Note 5**).

H. Collect beads by centrifuging at 200 × g, 4°C for 30 sec.

I. Remove the supernatant. Save 10 μl as flow-through by adding 10 ul 2×SDS loading buffer and boiling for 5 min.

J. Collect the Anti-HA Affinity Matrix beads into one tube if multiple tubes have been used. Add 1 ml IP washing buffer to each tube and mix by inverting 5-10 times. Collect the beads by centrifuging at 200 × g, 4°C for 30 sec and then removing the supernatant. Repeat once.

K. Add 1 ml IP washing buffer to each tube. Keep tubes in constant inverting motion for 10 min at 4°C. Collect the beads by centrifuging at 200 × g, 4°C for 30 sec. Repeat this wash step three times (*see* **Note 6**).

L. Re-suspend the beads in 1 ml IP washing buffer. Aliquot 200 μl to a new microcentrifuge tube. Collect beads by centrifuging at 200 × g, 4°C for 30 sec. Add 50 μl 1× SDS loading buffer and boil for 5 min. This fraction will be used for quality control in SDS-PAGE and Western blotting analysis.

M. Collect the remaining beads by centrifuging at 200 × g, 4°C for 30 sec. Completely remove the IP washing buffer over the beads by pipetting. The drained beads are now ready for further analysis (*see* **Note 7**).

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[4]This step is important to eliminate unwanted debris that co-precipitate with affinity beads.
[5]This step could be extended overnight in a cold room if maximum pull-down efficiency is necessary. This should be done with caution if heavy RNase activity is observed in the extraction.
[6]Washing may be repeated more times if non-specific proteins remain in the immunoprecipitate.
[7]The beads can be boiled with SDS-loading buffers for Western detection, or RNA can be extracted using TRIzol reagent or other similar reagents. This protocol should yield sufficient RNA for constructing a standard small RNA library for Illumina deep-sequencing (Section 3 and 4). The resulting RNA can also be used for Northern blotting or for investigating the target protein-associated RNAs, as described in Section 5.

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3. RNA extraction using TRIzol reagent (*see* Note 8)

A. Re-suspend the beads in 300 µl cold IP washing buffer and keep the tubes on ice.

B. In a chemical hood, add 800 µl TRIzol reagent to each tube and mix the contents for 3 min by vortexing.

C. Add 300 µl chloroform to each tube and mix for 15 sec by vortexing. Leave the tubes at room temperature for 3 min.

D. In a cold room, centrifuge the tubes at 15,000 × g for 10 min.

E. Transfer the aqueous phase to new tubes without disturbing the organic and intermediate phase.

F. Add 15 mg glycogen blue, 1/10 volume of 3M sodium acetate, and 2 volume of 100% ethanol (RNase free) to each tube. Mix the tubes by gently inverting 5-6 times.

G. Precipitate RNAs overnight at −20°C (*see* **Note 9**).

H. Spin the tubes at 15,000 × g, 4°C for 30 min. A tiny blue pellet should form at the tip of each tube.

I. Carefully remove the liquid, avoiding aspirating the pellets. Add 500 µl 70% ethanol (RNase free) to the tubes and wash the tubes by gently inverting 5-6 times.

J. Spin the tubes at 15,000 × g, 4°C for 5 min.

K. Completely remove the liquid, avoiding aspirating the pellets. Collect the remaining liquid to the bottom of the tubes by brief spinning. Remove the last drop until no liquid on the wall of the tubes visible.

L. Air-dry the pellet for 3-5 min (*see* **Note 10**).

M. Resuspend the pellet in 15 µl DEPC-treated water (*see* **Note 11**).

4. Small RNA library construction

4.1. 3’ adaptation

A. On ice, set up a 3’ adaptation reaction (add in the following order):

RNA (from immunoprecipitation; in water)	13 µl
ATP-free T4 RNA ligase buffer (NEB; 10X)	2 µl
DMSO	2 µl
3’ adapter	1 µl
T4 RLN2 (NEB)	2 µl

B. Incubate the reaction at 25°C for 1 h.

C. Bring the volume to 100 µl with DEPC-treated water.

[8] For extracting RNAs associated with specific target proteins, all the tips and tubes should be treated with DEPC prior to use; all the reagents should be RNase free.

[9] Experiment can be stopped at this step by storing reactions in a −80 °C freezer.

[10] Avoid over-drying that may cause difficulty in resuspending the sample.

[11] 1-2 µl sample can be used for quality control from this step.

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D. Follow step F to L in section 3 (RNA extraction **using TRIzol reagent**) to precipitate RNA.

E. Resuspend the pellet in 20 μl DEPC-treated water.

4.2. Gel purification of 3' adapted-small RNAs

A. Prepare a 10% polyacrylamide/urea gel as described in the Materials section [13].

B. Add equal volume of Gel loading buffer II to each sample. Heat samples at 65°C for 5 min. Immediately chill in ice for 5 min.

C. Load the samples on the gel. In a separate well, load 10 μl 10bp DNA ladder.

D. Run the gel for 2 h on constant voltage (15V/cm), or until the Bromophenol blue dye runs off the front of the gel.

E. In a clean (RNase-free) container, stain the gel with DEPC-treated water and EtBr for 10 min on a platform rocker at room temperature.

F. Under UV light (*see* **Note 12**), carefully cut the gel between 30 and 50 bp (using the marker as a guide) and transfer the gel slices into a clean 1.5 ml microcentrifuge tube.

G. Using a sterile 1 ml tip, crush the gel slices into fine debris (*see* **Note 13**)

H. Add 400 μl 0.3M NaCl to each tube.

I. Elute RNA from the gel by rotating overnight in a cold room.

J. Spin the tubes at 15,000 × g, 4°C for 10 min to pellet the gel debris.

K. Carefully pipet the liquid into new tubes without carrying over any gel debris (*see* **Note 14**).

L. Follow steps F to L in section 3 (**RNA extraction using TRIzol reagent**) to precipitate RNA.

M. Resuspend the pellet in 11 μl DEPC-treated water.

4.3. 5' adaptation

A. On ice set up a 5' adaptation reaction (add in the following order):

3'-ligated RNA (from previous step, in water)	11 μl
T4 RNA ligase buffer (10X)	2 μl
ATP (10mM)	2 μl
DMSO	2 μl
5' adapter	1 μl
T4 RNA ligase (NEB)	2 μl

[12] Use longer wavelength when possible (e.g. 365 nm instead of 302 nm). Exposure to high energy UV light may cause damage to RNA samples and could potentially jeopardize cloning efficiency.

[13] Always use autoclaved RNase-free tips. Contamination introduced from this step may cause RNA degradation during the following overnight incubation.

[14] In order to prevent carrying over gel debris to the new tubes, always leave a little bit liquid on the top of the pellet. Repeating the spinning step one time is also useful to eliminate unwanted carry over.

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B. Incubate the reaction at 37°C for 1 h.

C. Bring the volume to 100 μl by adding DEPC-treated water.

D. Follow steps F to L in section 3 (**RNA extraction using TRIzol reagent**) to precipitate RNA.

E. Resuspend the pellet in 20 μl DEPC-treated water.

4.4. Gel purification of 3'- and 5'-adapted-small RNAs

A. Prepare a 10% polyacrylamide/urea gel as described in the Materials section [13].

B. Add equal volume of Gel loading buffer II to each sample. Heat samples at 65°C for 5 min. Immediately chill in ice for 5 min.

C. Load the samples on the gel. In a separate well, load 10 μl 10bp DNA ladder.

D. Run the gel for 2 h at constant voltage (15V/cm), or until the Bromophenol Blue dye runs off the front of the gel.

E. In a clean (RNase free) container, stain the gel with DEPC-treated water and EtBr for 10 min on a platform rocker at room temperature.

F. Under UV light [12], carefully cut the gel between 50 and 100 bp (using the marker as a guide) and transfer the gel slices into a clean 1.5 ml microcentrifuge tube.

G. Using a sterile 1 ml tip, crush the gel slices into fine debris [13].

H. Add 400 μl 0.3M NaCl to each tube.

I. Elute RNA from the gel by rotating overnight in a cold room.

J. Spin the tubes at 15,000 × g, 4°C for 10 min to pellet the gel debris.

K. Carefully pipet the liquid into new tubes without carry over any gel debris [14].

L. Follow steps F to L in section 3 (**RNA extraction using TRIzol reagent**) to precipitate RNA.

M. Resuspend the pellet in 20 μl DEPC-treated water.

4.5. Reverse transcription of 3'- and 5'-adapted-small RNAs

A. On ice, set up a reverse transcription reaction (add in the following order):

3'- and 5'-ligated RNA (gel purified; in water)	11 μl
Reverse transcription primer (SBS3; 10 μM)	1 μl
dNTPs (10mM)	1 μl

B. Incubate at 65°C for 5 minutes

C. Place on ice and add:

5X first Strand Buffer	4 μl
0.1M DTT	1 μl
RNase-OUTTM (40 U/ μl)	1μl

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SuperScript III RT (200 U/µl)	1µl

D. Incubate at 50°C for 1 h then at 70°C for 15 min (*see* **Note 9**).

4.6. PCR amplification of small RNA library

A. On ice, set up a PCR reaction (add in the following order):

PrimeSTAR buffer (5X)	10 µl
dNTPs (2.5 mM)	4 µl
PCR 5’ primer (10 µM)	1 µl
PCR 3’ primer (10 µM)	1 µl
First strand cDNA (from reverse transcription)	1-5 µl
PrimeSTAR HS DNA polymerase (2.5 units/ µl)	0.5 µl
Sterile water	to 50 µl

B. Set following program on a thermocycler:

98 °C for 10 sec

55 °C for 5 sec

72 °C for 10 sec

Amplify for 22 cycles

C. Load samples onto a 10% polyacrylamide gel (0.5X TBE without urea) and separate the samples by running the gel for 2h at constant voltage (15V/cm).

D. In a clean container, stain the gel with distilled water and EtBr for 10 min on a gentle rocker at room temperature.

E. Under UV light [12], carefully cut the gel at110 bp (using the marker as a guide) and transfer the gel slices into a clean 1.5 ml microcentrifuge tube (*see* **Note 15**).

F. Using a sterilized 1 ml tip, crush the gel slices into fine debris.

G. Add 400 µl 0.3M NaCl to each tube.

H. Elute RNA from the gel by rotating overnight in a cold room.

I. Spin the tubes at 15,000 × g, 4° for 10 min to pellet the gel debris.

J. Carefully pipet the liquid into new tubes without carry over any gel debris [14].

K. Follow step F to L in section 3 (RNA extraction **using TRIzol reagent**) to precipitate RNA (*see* **Note 16**).

L. Resuspend the pellet in 10 µl DEPC-treated water.

[15]Care should be taken at this step so that only the cloned small RNAs but not the self-ligation products are collected. These two molecules are only a 20 nucleotides difference in length. Contamination at this step will affect downstream sequencing quality.

[16]The pellet in this step may invisible. Care should be taken at the washing step to avoid losing the pellet.

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