

# Chapter 2

## Analysis of the Uridylation of Both ARGONAUTE-Bound MiRNAs and 5' Cleavage Products of Their Target RNAs in Plants

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### Abstract

Uridylation (3' untemplated uridine addition) provides a mechanism to trigger the degradation of miRNAs and the 5' cleavage products (5' CP) that are produced from miRNA-directed ARGONAUTE (AGO) cleavage of target RNAs. We have recently shown that HEN1 SUPPRESSOR 1 (HESO1), a terminal uridylyltransferase, and its homolog UTP:RNA uridylyltransferase 1 (URT1) catalyze the uridylation of miRNAs and 5' CPs within the AGO complex in higher plants. In this chapter, we describe detailed protocols for analyzing 3' end uridylation of both AGO-bound miRNAs and 5' CP.

**Key words** miRNA, ARGONAUTE, 5' Cleavage product, Uridylation, HESO1, Rapid amplification of cDNA ends (RACE)

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### 1 Introduction

MicroRNAs (miRNAs) are ~21-nucleotide-long noncoding RNAs that mainly regulate gene expression at posttranscriptional levels. They play crucial roles in many biological processes such as development, physiology, metabolism, and immunity [1, 2]. miRNAs are first excised as duplexes with 2 nt 3' overhang from their imperfect stem-loop precursors called primary miRNA transcripts (pri-miRNAs) [3, 4]. Unlike metazoans, plant miRNA duplex is subject to 2'-O-methylation at 3' end of each strand by the small RNA methyltransferase HUA1 ENHANCER1 (HEN1) [5]. Upon production, miRNA is loaded into the effector protein called ARGONAUTE (AGO) and then guide it to interact with target RNAs through base pairing with the complementary sequence(s) within targets [6, 7]. AGO suppresses target expression through deadenylation-mediated RNA decay, translational inhibition, or target cleavage [8]. Target RNA cleavage by AGO happens at a position opposite to between 10th and 11th nucleotides of the

guide miRNA, which produces a 5' RNA fragment (5' cleavage product, 5' CP) and a 3' RNA fragment (3' CP) [9]. In plants, extensive complementarity between miRNAs and targets results in predominant target cleavage by AGO1 (the plant miRNA effector). In contrast, less complementarity in metazoans leads to predominant deadenylation-mediated RNA decay and/or translational inhibition, while target cleavage also exists [10, 11]. The AGO cleavage products need to be further removed. Otherwise, they may serve as template for secondary small interfering RNA (siRNA) formation that may cause lethality of organisms [12].

In plants lacking *hen1*, miRNAs often contain untemplated uridines at 3' end (uridylation) and become less stable [13]. Subsequently, studies have shown that uridylation regulates the stability and activity of some metazoan miRNAs [14, 15]. Interestingly, 5' CPs can also be uridylated, which triggers 5' CP degradation [16, 17]. We recently show that both miRNAs and 5' CP are uridylated by the terminal uridine transferase HEN1 SUPPRESSOR 1 (HESO1) and its homolog UTP:RNA uridylyl-transferase 1 (URT1) [16, 18–21]. Further studies have shown that HESO1 and URT1 bind AGO1, demonstrating that HESO1 and URT1 act on miRNAs and 5' CP within the AGO1 complex [16, 19]. Here, we describe the in vitro and in vivo protocols used to analyze uridylation of AGO1-bound miRNAs and 5' CP, respectively (Fig. 1).

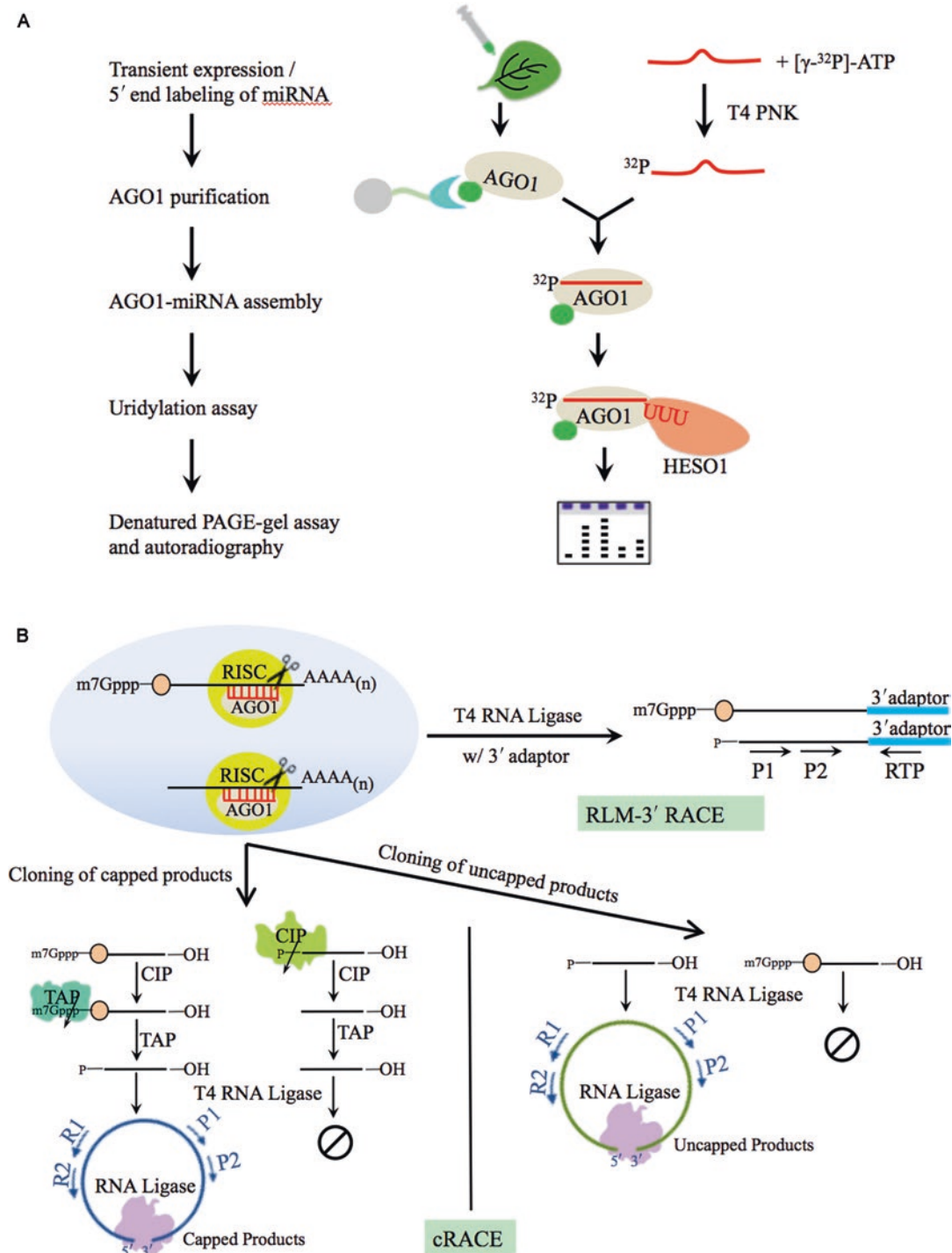
## 2 Materials and Key Equipment

### 2.1 Analysis of the 3' Uridylation of ARGONAUTE-Bound MiRNAs

#### 2.1.1 Transient Expression of GFP-AGO1 in *Nicotiana benthamiana*

1. *N. benthamiana* plants.
2. *Agrobacterium tumefaciens* strain GV3101 carrying a GFP-AGO1 or P19 plasmid [22].
3. YEB medium: 5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L magnesium chloride (MgCl<sub>2</sub>), 1% agar (plate only), with appropriate antibiotics.
4. Infiltration medium: 10 mM MgCl<sub>2</sub>, 10 mM MES pH 5.6, 200 μM acetosyringone (add freshly).
5. 3 mL syringe.

**Fig. 1** (continued) PAGE gel and autoradiography. **(b)** A schematic diagram for detecting 3' end uridylation of 5' CP in vivo. Either RLM-3' RACE or cRACE strategy was used to retrieve the 3' end signatures of 5' CP. For RLM-3' RACE, total RNA was ligated to an RNA adaptor followed by reverse transcription and two rounds of semi-nested PCR. For cRACE, total RNA was directly subject to self-ligation (for uncapped products) or successively treated with CIP and TAP before self-ligation (for capped products). After reverse transcription using R1 primer, two rounds of nested PCR were performed. For both methods, PCR products were cloned, and end signatures were obtained by Sanger sequencing



**Fig. 1** Schematic flowcharts for uridylation assays on AGO1-bound miRNAs in vitro and 5' cleavage products (CP) in vivo. **(a)** A schematic flowchart for HESO1-mediated uridylation assay on AGO1-bound miRNAs in vitro. AGO1 is obtained by transient expression of GFP-AGO1 in *N. benthamiana* followed by immunopurification using anti-GFP antibodies that were pre-coupled to protein A agarose beads. miRNA was 5' end  $^{32}\text{P}$  labeled with T4 PNK and was then loaded onto GFP-AGO1. The assembled AGO1-miRNA complex is subject to HESO1-mediated uridylation assay. After wash, AGO1-bound miRNAs were extracted and analyzed on a denatured

### 2.1.2 Purification of GFP-AGO1

1. Liquid nitrogen.
2. Mortars and pestles.
3. End-over rotator wheel.
4. Antibodies: Rabbit anti-GFP (Clontech) or GFP-Trap (ChromoTek).
5. Diethyl pyrocarbonate (DEPC)-treated H<sub>2</sub>O. Add 1 mL DEPC to 1 L ultrapure H<sub>2</sub>O (e.g., Milli-Q H<sub>2</sub>O), stir overnight, and autoclave (*see Note 1*).
6. Protein extraction buffer (prepared with DEPC-treated H<sub>2</sub>O): 50 mM Tris-HCl pH 7.5, 150 mM sodium chloride (NaCl), 5 mM MgCl<sub>2</sub>, 5% glycerol, 2 mM dithiothreitol (DTT), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 1/100 protease inhibitor (Thermo Fisher Scientific) (*see Note 2*).
7. Protein A agarose beads (Sigma-Aldrich).

### 2.1.3 Preparation of Radio-Labeled miRNA Probes

1. [ $\gamma$ -<sup>32</sup>P]ATP (10 mCi/mL, 3000 Ci/mmol, PerkinElmer).
2. miR166a RNA oligonucleotide: 5' UCGGACCAGGCUU CAUUC<sup>3</sup> 3'.
3. T4 polynucleotide kinase (T4 PNK) (New England Biolabs).
4. MicroSpin G-25 columns (GE Healthcare).
5. Phenol pH 4.5.
6. Chloroform.
7. Ethanol.
8. 3 M sodium acetate (NaOAc) pH 4.3.
9. Glycogen.

### 2.1.4 AGO1 Assembly and Uridylation Assay

1. RiboLock RNase inhibitor (Thermo Fisher Scientific).
2. Maltose-binding protein (MBP) and MBP-tagged HESO1 protein [20].
3. Reaction exchange buffer (prepared with DEPC-treated H<sub>2</sub>O): 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, pH 7.9.
4. Reaction buffer (prepared with DEPC-treated H<sub>2</sub>O): 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 100 µg/mL bovine serum albumin (BSA), 0.5 mM UTP, 1 U/µL RNase inhibitor, pH 7.9.
5. 5× Tris-borate-EDTA (TBE) buffer: 54 g/L Tris, 27.5 g/L boric acid, 10 mM EDTA, pH 8.3.
6. 16% polyacrylamide denaturing gel stock solution: 16% acrylamide/bis (29:1), 42% urea, 0.5× TBE. Dissolve the mixture in 42 °C water bath and pass through a 0.22 µm filter (*see Note 3*).

7. 10% ammonium persulfate (APS).
8. N,N,N',N'-tetramethylethylenediamine (TEMED).
9. 2× formamide RNA loading dye: mix 8 mL of formamide with 2 mL of 5× TBE, and add trace amount of xylene cyanol and bromophenol blue.
10. Bio-Rad PROTEAN II xi Cell (Bio-Rad).
11. BAS Storage Phosphor Screen (GE Healthcare).
12. Typhoon FLA 9500 scanner (GE Healthcare).

## **2.2 Analysis of the 3' Uridylation of 5' Cleavage Products**

### **2.2.1 Total RNA Extraction**

1. TRI Reagent (Molecular Research Center).
2. β-Mercaptoethanol.
3. Chloroform.
4. Isopropanol.
5. 70% ethanol.
6. DEPC-treated H<sub>2</sub>O.
7. NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

### **2.2.2 RNA Ligase-Mediated 3' Rapid Amplification of cDNA Ends (RLM-3' RACE)**

1. 3' RNA adaptor: pUUUdCdTdGdTdAdGdGdCdAdCdCdAdTdCdAdAdTdT.
2. Primers: RT primer, 5'-ATTGATGGTGCCTACAG-3'; P1 primer (For MYB33), 5'-AAGCGACTTTGGGAATCTGA-3'; P2 primer (For MYB33), 5'-AAGAATTCTCGTCGCCTGAA-3'.
3. T4 RNA ligase1 (New England Biolabs).
4. SuperScript III Reverse Transcriptase (Thermo Fisher Scientific).
5. RiboLock RNase inhibitor (Thermo Fisher Scientific).
6. 10 mM ATP.
7. pGEM-T Easy Vector (Promega).
8. Phenol pH 4.5.
9. Chloroform.
10. Ethanol.
11. 3 M NaOAc pH 4.3.
12. Glycogen.
13. DEPC-treated H<sub>2</sub>O.

### **2.2.3 Circulation-Mediated Rapid Amplification of cDNA Ends (cRACE)**

1. Alkaline phosphatase, calf intestinal (CIP) (New England Biolabs).
2. Tobacco acid pyrophosphatase (TAP) (Epicentre) (*see Note 4*).

3. Primers: P1, P2 as in Subheading 2.2.2; R1 primer (For MYB33), 5'-GCCATACGTGCCCATTCTATT-3'; R2 primer (For MYB33), 5'-TTGGCCTCAGATGATTAGCC-3'.
4. T4 RNA ligase1 (New England Biolabs).
5. Phenol pH 4.5.
6. Chloroform.
7. Ethanol.
8. 3 M NaOAc pH 4.3.
9. Glycogen.
10. DEPC-treated H<sub>2</sub>O.

### 3 Methods

#### 3.1 Analysis of the 3' Uridylation of AGO1-Bound miRNAs

##### 3.1.1 Transient Expression of GFP-AGO1 in *Nicotiana Benthamiana*

A straightforward way to obtain AGO1 is to immunoprecipitate it from *Arabidopsis* tissues using an anti-AGO1 antibody (Agrisera or produced according to [23]). Alternatively, one may express tagged AGO1 protein (e.g., GFP-AGO1) either stably in *Arabidopsis* (i.e., stable transgenic line) or transiently in *N. benthamiana* and purify it by using a commercial antibody recognizing the tag [16, 18]. In this protocol, we describe methods for the transient expression and purification of GFP-AGO1 from *N. benthamiana* leaves.

1. Grow *N. benthamiana* at 23 °C under long day condition (16 h light) for 4–5 weeks.
2. Inoculate three to four fresh colonies of *A. tumefaciens* containing either GFP-AGO1 or P19 into 10 mL of YEB medium supplemented with appropriate antibiotics.
3. Grow the culture at 28 °C with vigorous shaking (~230 rpm) to an OD<sub>600</sub> of 1.2–2.0, which usually takes 12–20 h when using fresh prepared colonies in the *A. tumefaciens* strain GV3101 (see Note 5).
4. Centrifuge the culture at 1500 × *g* for 20 min and discard the supernatant.
5. Resuspend the agrobacteria in 10 mL infiltration medium, and incubate at room temperature for 3 h with gentle shaking (~75 rpm).
6. Centrifuge the culture at 1500 × *g* for 20 min, and resuspend the agrobacteria pellet in fresh infiltration buffer, and adjust the final concentration to an OD<sub>600</sub> of 0.4–0.5 (for P19, adjust the final concentration to an OD<sub>600</sub> of 0.8–0.9).
7. Mix the GFP-AGO1 agrobacteria suspension with P19 at a ratio of 1:1 (v/v).

8. Infiltrate the third to sixth leaf (from top) using a 3 mL syringe without needle (*see* **Note 6**).
9. Collect leaf tissues 60–72 h after infiltration and quickly freeze in liquid nitrogen. Tissues can be stored at  $-80^{\circ}\text{C}$  for several months.

### 3.1.2 Purification of GFP-AGO1 with Anti- GFP Antibodies

Unless otherwise indicated, all the following procedures in this section should be conducted in cold conditions (e.g., in a  $4^{\circ}\text{C}$  cold room or on ice), and all the buffers should be precooled. Use a microfuge to centrifuge samples.

1. Grind 0.3 g of leaf tissue using a mortar and a pestle in liquid nitrogen into fine powder. Add 1 mL protein extraction buffer, and incubate the homogenate on an end-over rotator at  $4^{\circ}\text{C}$  for 1 h.
2. Centrifuge at  $16,000 \times g$  for 10 min and carefully transfer the supernatant to a new tube.
3. Repeat the centrifugation step and save a 40  $\mu\text{L}$  aliquot as input sample.
4. Antibody–beads conjugation.

Once incubation of the homogenate on an end-over rotator starts (**step 1**), conjugate the antibody to the protein A agarose beads. Take 40  $\mu\text{L}$  of protein A agarose slurry per sample (50% slurry, 20  $\mu\text{L}$  beads) using a 200  $\mu\text{L}$  tip with wide orifice. Wash the beads with 1 mL of protein extraction buffer three times. Centrifuge at  $100 \times g$  for 1 min and discard the supernatant after each wash. Resuspend the beads with 600  $\mu\text{L}$  of protein extraction buffer, and add 5–10  $\mu\text{L}$  anti-GFP antibodies (check the product datasheet for recommended antibody dilution). Incubate for 2–3 h at  $4^{\circ}\text{C}$  on an end-over rotating wheel (5–10 rpm), and collect the beads by centrifugation at  $100 \times g$  for 1 min. Wash the antibody/beads mixture with 1 mL of protein extraction buffer three times (*see* **Note 7**).

5. Preclear the protein lysate from **step 3** with 20  $\mu\text{L}$  of pre-washed protein A agarose beads.
6. Incubate 1 mL of total protein lysate with coupled antibody–beads for 2 h to overnight on the rotating wheel (5–10 rpm).
7. Sediment the beads by centrifuging at  $100 \times g$  for 1 min, and discard the supernatant. Wash the beads four to six times with 1 mL of protein extraction buffer.

### 3.1.3 Preparation of Radio-Labeled miR166a

1. Assemble the following reaction in a 1.5 mL tube, and incubate at  $37^{\circ}\text{C}$  water bath for 1 h.



DEPC-treated H <sub>2</sub> O	39 $\mu$ L
T4 PNK buffer	5 $\mu$ L
miR166a (10 $\mu$ M)	1 $\mu$ L
T4 PNK	2.5 $\mu$ L
[ $\gamma$ - <sup>32</sup> P]ATP (10 mCi/mL)	2.5 $\mu$ L
Total volume	50 $\mu$ L

2. To remove unincorporated [ $\gamma$ -<sup>32</sup>P]ATP, pass the reaction through the G25 column, and centrifuge at  $850 \times g$  for 1 min in a microcentrifuge.
3. Phenol/chloroform extraction to remove the enzyme.

Adjust the volume of elute to 120  $\mu$ L with DEPC-treated H<sub>2</sub>O. Add equal volume of acidic phenol pH 4.5/chloroform (1:1 premixed) and vortex vigorously for 30 s. Centrifuge at  $16,000 \times g$  for 5 min, and carefully transfer the aqueous phase (i.e., the top phase) to a new tube. Add 1 volume of chloroform and extract again to remove residual phenol. Carefully transfer the aqueous phase to a new tube (~100  $\mu$ L).

4. Ethanol precipitation.

Add 10  $\mu$ L of 3 M NaOAc pH 4.3, 1  $\mu$ L glycogen (20  $\mu$ g), and 300  $\mu$ L of ethanol. Mix thoroughly and incubate at  $-20^\circ\text{C}$  overnight or at  $-80^\circ\text{C}$  for at least 2 h. Centrifuge at  $16,000 \times g$  for 20 min and rinse the pellet with 70% ethanol. Note the position of the pellet and decant supernatant or remove the supernatant by pipetting. Be careful not to disturb the pellet. Remove the ethanol by centrifuging at  $7000 \times g$  for 5 min and air-dry for 3–5 min. Resolve the radio-labeled miR166a with 100  $\mu$ L of RNase-free H<sub>2</sub>O, and use an aliquot for AGO1 assembly.

### 3.1.4 AGO1 Assembly and Uridylation Assay

1. AGO1–miR166a assembly.

Add 1–5  $\mu$ L [<sup>32</sup>P]-labeled miR166a to the purified GFP–AGO1 in 0.5 mL protein extraction buffer with 20 U of RNase inhibitor. Incubate at  $4^\circ\text{C}$  for 1 h on an end-over rotating wheel (5–10 rpm), and collect the beads by centrifugation at  $100 \times g$  for 1 min in a microcentrifuge. Wash the beads with protein extraction buffer, and check the radioactivity of the supernatant with a Geiger counter after each wash. It usually requires three to five washes until the readout by the counter becomes stable.

2. HESO1-directed uridylation assay.

Methods for construction and purification of MBP–HESO1 and MBP are according to [20]. Add 0.5 mL of reaction



exchange buffer to the assembled AGO1–miR166a beads, and split the beads into two aliquots; carefully remove the exchange buffer. Add 60  $\mu\text{L}$  of reaction buffer to each aliquot. Add 1  $\mu\text{L}$  of MBP–HESO1 or MBP (~30 ng) to each aliquot, and incubate at room temperature for 30 min. Collect the beads by centrifugation at  $100\times g$  for 1 min in a microcentrifuge. Supernatant can be saved and assayed in parallel (optional). Wash the beads five times with protein extraction buffer. RNA was extracted with phenol/chloroform and precipitated with ethanol (*see steps 3 and 4* in Subheading 3.1.3). Resolve the washed and air-dried RNA in 10  $\mu\text{L}$  of DEPC-treated  $\text{H}_2\text{O}$ . Add 10  $\mu\text{L}$  of  $2\times$  formamide RNA loading dye, incubate at  $70^\circ\text{C}$  for 5 min, and leave on ice.

### 3. RNA analysis by denaturing PAGE electrophoresis.

The Bio-Rad PROTEAN II xi cell vertical electrophoresis system is used to analyze the pattern of AGO1-bound miRNA after HESO1 treatment. Assemble the glass plate and cast the gel according to the instruction manual. For 1.0 mm spacers, transfer 40 mL of 16% polyacrylamide denaturing gel stock solution into a 50 mL centrifuge tube, and if necessary, deaerate the solution under vacuum for 10–20 min. Add 420  $\mu\text{L}$  of 10% APS and 24  $\mu\text{L}$  of TEMED to the solution and mix. Pour the solution to the assembled glass plate and insert the comb in the gel sandwich. Let the gel polymerize for at least 1 h. Rinse the sample well thoroughly with  $0.5\times$  TBE running buffer. Load the samples on separate wells, and run until the bromophenol blue dye almost reaches the bottom of the gel. Remove the gel from the cassette, drain excess buffer, carefully put the gel on a Whatman paper support, and wrap it up with plastic wrap. Expose the gel using a storage phosphor screen, and the signal is monitored on a Typhoon FLA 9500 machine.

## 3.2 Analysis of the 3' Uridylation of 5' Cleavage Products of Target RNAs

### 3.2.1 Extraction of Total RNA from Wild Type and the *heso1–2* Mutant

Use a microfuge to centrifuge samples.

1. Homogenize 0.1 g tissue samples from respective genotypes in liquid nitrogen, and transfer about 100  $\mu\text{L}$  of fine powder to a 1.5 mL tube.
2. Add 1 mL of TRI Reagent (add 10  $\mu\text{L}/\text{mL}$   $\beta$ -mercaptoethanol just before use) and vortex thoroughly.
3. Place at room temperature for 5 min and centrifuge at  $16,000\times g$  for 5 min at  $4^\circ\text{C}$ .
4. Transfer the supernatant to a new 1.5 mL tube and add 200  $\mu\text{L}$  of chloroform. Vortex thoroughly and incubate at room temperature for 5 min.
5. Centrifuge at  $16,000\times g$  for 5 min at  $4^\circ\text{C}$ , and carefully transfer the aqueous phase (~600  $\mu\text{L}$ ) to a new 1.5 mL tube.

6. Add 500  $\mu\text{L}$  isopropanol, mix thoroughly, and incubate the samples at room temperature for 15–20 min.
7. Centrifuge at  $16,000\times g$  for 10 min at 4 °C. A white RNA pellet appears at the bottom of the tube.
8. Wash the RNA pellet with 70% ethanol, and recover the pellet by centrifuging at  $5,000\times g$  for 3 min.
9. Discard ethanol and air-dry the RNA for ~10 min. Dissolve the RNA in 20  $\mu\text{L}$  DEPC-treated  $\text{H}_2\text{O}$ , and check the RNA concentration with a NanoDrop spectrophotometer.

**3.2.2 Cloning of 5' Cleavage Product by RNA Ligase-Mediated 3' Rapid Amplification of cDNA Ends (RLM-3' RACE)**

1. 3' adaptor ligation.

Assemble the following mixture in a nuclease-free PCR tube:

DEPC-treated $\text{H}_2\text{O}$	Variable
3' RNA adaptor (100 pmol/ $\mu\text{L}$ )	1 $\mu\text{L}$
Total RNA (500 ng–1 $\mu\text{g}$ )	1–11 $\mu\text{L}$
Total volume	12 $\mu\text{L}$

Incubate at 70 °C for 2 min and quickly chill on ice. Add the following components to the tube and mix by pipetting. Incubate at 22 °C for 8 h.

10 $\times$ T4 RNA ligase buffer	2 $\mu\text{L}$
ATP (10 mM)	2 $\mu\text{L}$
RNase Inhibitor (40 U/ $\mu\text{L}$ )	1 $\mu\text{L}$
DMSO	2 $\mu\text{L}$
T4 RNA ligase 1 (10 U/ $\mu\text{L}$ )	1 $\mu\text{L}$
Total volume	8 $\mu\text{L}$

After incubation, perform the phenol/chloroform extraction and ethanol precipitation (*see steps 3 and 4* in Subheading 3.1.3).

2. Reverse transcription, PCR, and detection.

Resuspend RNA in 10  $\mu\text{L}$  of DEPC-treated  $\text{H}_2\text{O}$ , and use 5  $\mu\text{L}$  of ligated RNA for reverse transcription. Combine the following components in a nuclease-free PCR tube:

Ligated RNA	5 $\mu\text{L}$
RT primer (25 $\mu\text{M}$ )	1 $\mu\text{L}$
dNTP (10 mM)	1 $\mu\text{L}$
DEPC-treated $\text{H}_2\text{O}$	6 $\mu\text{L}$
Total volume	13 $\mu\text{L}$

Incubate at 65 °C for 5 min and quickly chill on ice. Add the following components to each tube and mix by pipetting. Incubate at 50 °C for 1 h and place the tube on ice.

5× first-strand buffer	4 µL
RNase inhibitor (40 U/µL)	1 µL
DTT (100 mM)	1 µL
SuperScript III Reverse Transcriptase (200 U/µL)	1 µL
Total volume	7 µL

Perform first round PCR using P1 and RT primer. Assemble the following components in a PCR tube as follows:

cDNA	1 µL
H <sub>2</sub> O	13.9 µL
10× PCR reaction buffer	2 µL
10 mM dNTP	1 µL
P1 primer (10 µM)	1 µL
RT primer (10 µM)	1 µL
Taq DNA polymerase	0.1 µL
Total volume	20 µL

Perform 25–30 cycles of PCR with default annealing temperature at 55 °C (*see Note 8*). Dilute the first round PCR product 50 times, and use 1 µL as template for second round PCR, which uses P2 and RT primer. Check the PCR product by running a 1.5% agarose gel. Perform gel purification and subclone the PCR product to a pGEM-T Easy Vector. Sequence the positive clones with the M13F sequencing primer. The 3' end signature of the cloned 5' cleavage product can be retrieved subsequently.

### 3.2.3 Cloning of 5' Cleavage Product by Circulation-Mediated Rapid Amplification of cDNA Ends (cRACE)

#### 1. 5' end RNA dephosphorylation by CIP treatment (optional).

Assemble the following reaction in a 1.5 mL nuclease-free tube:

Total RNA (~1 µg/µL)	6 µL
H <sub>2</sub> O	19 µL
10× CIP buffer	3 µL
RNase inhibitor (40 U/µL)	1 µL
CIP (10 U/µL)	1 µL
Total volume	30 µL

Mix by pipetting and incubate at 37 °C for 45 min. Perform the phenol/chloroform extraction and ethanol precipitation (*see steps 3 and 4* in Subheading 3.1.3). Resuspend CIP-treated RNA in 9 µL of DEPC-treated H<sub>2</sub>O. Use 1–2 µL to check the RNA concentration with a NanoDrop spectrophotometer.

2. 5' end RNA decapping by TAP treatment.

Assemble the following reaction in a 1.5 mL nuclease-free tube.

CIP-treated RNA or total RNA (~2–5 µg)	7 µL
H <sub>2</sub> O	26 µL
10× TAP buffer	4 µL
RNase inhibitor (40 U/µL)	1 µL
TAP (10 U/µL)	2 µL
Total volume	40 µL

Mix by pipetting and incubate at 37 °C for 2 h. Perform the phenol/chloroform extraction and ethanol precipitation (*see steps 3 and 4* in Subheading 3.1.3).

3. RNA self-circulation.

Resuspend the TAP-treated RNA in 16 µL of DEPC-treated H<sub>2</sub>O. Use 1–2 µL to check the RNA concentration with a NanoDrop spectrophotometer. Incubate the RNA at 65 °C for 5 min and chill on ice. Add the following reagent to the RNA (16 µL) and mix by pipetting. Incubate at 22 °C for 8 h.

10× T4 RNA ligase buffer	2 µL
ATP (10 mM)	2 µL
RNase inhibitor (40 U/µL)	1 µL
T4 RNA ligase 1 (10 U/µL)	1 µL
Total volume	6 µL

After incubation, perform the phenol/chloroform extraction and ethanol precipitation (*see steps 3 and 4* in Subheading 3.1.3).

4. Reverse transcription, PCR, and detection.

Resuspend ligated RNA in 15 µL of DEPC-treated H<sub>2</sub>O. Split the RNA solution into three to four aliquots for different 5' CPs. For each 5' CP, use 2 pmol of R1 primer instead of RT primer, and perform reverse transcription (*see step 2* in Subheading 3.2.2). Nested PCR is performed as

described (*see* **step 2** in Subheading 3.2.2), except that the RT primer is replaced by R1 and R2 in the first and second round PCR, respectively. PCR product subcloning and Sanger sequencing are performed as described (*see* **step 2** in Subheading 3.2.2). Both 3' end and 5' end signatures are retrieved by cRACE (*see* **Note 9**).

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## 4 Notes

1. DEPC is highly toxic. Handling of DEPC should be carried out carefully in a fume hood with good protection. The bottle cap should be tightly sealed with Parafilm after DEPC is added.
2. DTT, PMSF, and protease inhibitor should be added just before use.
3. The stock solution can be stored at 4 °C for several months without affecting performance. Old stocks of acrylamide/bis-acrylamide (29:1) may result in poor resolution (i.e., a smear rather than discrete bands appear after exposure).
4. TAP is no longer distributed. Tebu-bio's Decapping Pyrophosphohydrolase or CellScript's Cap-Clip™ Acid Pyrophosphatase could be possible replacement (<http://being-bioreactive.com/2015/09/03/two-new-enzymes-available-to-replace-tap/>).
5. When starting from an older plate or a frozen stock, recover the agrobacteria by streaking it on a YEB agar plate 3–5 days before inoculation.
6. We found that young and healthy leaves show much higher protein expression, whereas the first two top leaves are difficult to be infiltrated.
7. If using GFP-Trap or other pre-coupled antibody/agarose beads, simply wash the resin with protein extraction buffer three times before using.
8. Annealing temperature may be further optimized, and touch-down PCR may be performed if the PCR result is not satisfactory.
9. End nucleotides that match both 5' end and 3' end of the genome cannot be distinguished with this method.

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