An In Vitro Approach To Study RNase III Activities of Plant RTL Proteins

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Chapter 23

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ABSTRACT

RTL (RNase Three Like) proteins belong to a distinct family of endonucleases that cleave double stranded RNAs in plants. RTL1 to 3 are structurally related to the RNAse III from *E. coli* and formally belong to the class 1 of RNase III proteins. RTLs have conserved RNase III signature motif(s) and up to two dsRNA binding (DRB) domains. RTLs target and cleave coding and non-coding dsRNAs, including precursors of ribosomal (rRNA), small interference (siRNA) and micro (miRNA) RNAs. Interestingly, RTL proteins have stronger affinity than RNase III -Dicer proteins for dsRNA precursors of siRNAs, but not for miRNAs. However, very little is known of the structural and molecular bases directing and controlling RTL-RNA binding and activity. To address these questions, we have developed *in vitro* cleavage assays that combine recombinant RTL1 protein and *in vitro* transcribed or plant extracted RNAs, RT-PCR and primer extension experiments or analysis.

Keywords: RNase III, dsRBD, RNA cleavage, Arabidopsis thaliana, plants

1 Introduction

Ribonucleases III (RNases III) are endonucleases that target and cleave double stranded RNA (dsRNA) [1]. The cleavage performed by RNases III generates 5'-phosphoryl and 3'-hydroxyl ends with a two-nucleotide (nt) 3'-overhang in their dsRNA products (**Figure 1A**). Found in bacteria and eukaryotes, RNases III are universally involved in processing and maturation of almost every class of RNA precursors into functional RNAs. All members of the RNase III family contain a characteristic RNase III domain, which has a highly conserved stretch of nine amino acid residues known as the RNase III signature motif. RNase III proteins vary widely in length, from ~140 to ~2000 amino acids and have been subdivided into four classes based on domain composition [1-4].

Class I is the simplest and the smallest, including *Bacillus subtilis* Mini-III with a single RNase III domain and bacterial *Escherichia coli* and *Aquifex aeolicus* RNases III that, in addition to the single RNase III domain, also contain a DRB domain [5-7]. **Class II** is identified by the presence of a highly variable N-terminal domain extension and includes the *Saccharomyces cerevisiae* Rnt1 and *Saccharomyces pombe* Pac1 proteins [8-10]. **Class III** proteins, including Drosha, have a DRB and two RNase III domains [11-13]. **Class IV** proteins correspond to Dicer and contain a RNA helicase domain, a PAZ domain, one or two RNase III signature domains and one or two DRB domains. Dicer proteins are the only RNase III proteins that have been shown to produce small RNAs, including siRNAs and miRNAs [14-17].

In the model plant *Arabidopsis thaliana*, two families of RNases III are present. The first family corresponds to DICER-LIKE (DCL) enzymes [18, 16]. DCL1 is involved in the production of 21-nucleotide (nt) miRNAs [19, 20] while DCL2, DCL3 and DCL4 produce 22-, 24- and 21-nt siRNAs, respectively [21-24]. The 21-22-nt small RNAs produced by DCL1, DCL2 and DCL4 are loaded onto ARGONAUTE (AGO) proteins that cleave complementary RNAs to perform post-

transcriptional gene silencing (PTGS), while 24-nt small RNAs produced by DCL3 are loaded onto AGO proteins to perform RNA-directed DNA methylation (RdDM) and transcriptional gene silencing (TGS) [25-27]. [Figure 1 near here]

The second RNase III family of *A. thaliana* corresponds to RNase Three-Like (RTL) enzymes and has three members (**Figure 1B**) [28]. AtRTL1 is a ~34 kDa protein containing single RNase III and DRB domains. AtRTL1 cleaves perfectly paired dsRNAs, before they are processed by DCL2, DCL3 or DCL4 in siRNA, thus suppressing siRNA production from more than 6,000 loci [29]. AtRTL2 is a ~44 kDa protein and contains a single RNase III and two DRB domains [28, 30]. In contrast to the broad action of AtRTL1, AtRTL2 specifically modulates the expression of rDNA [28] and of ~500 loci producing 24-nt siRNAs involved in RdDM [31]. RTL3 appears to be a pseudogene whose expression cannot be detected.

In addition to DCL and RTL protein families, two *A. thaliana* chloroplast Mini-RNase III-like (RNC3 and RNC4) have been described; it is believed that RNC3 and RNC4 might cleave rRNA and participate in intron recycling in the chloroplast [32].

Modelling of AtRTL1 [33] (**Figure 2A**) and AtRTL2 [28] proteins revealed antiparallel homo-dimerization of RNase III domains, similar to *E. coli* and *A. aeolicus* RNases III [5, 6]. AtRTL1 recognizes specific rcr1sequence (for RTL1 consensus region 1) and cleaves hairpin structure located in the 3'UTR of the *A. thaliana* At3g18145 gene (**Figure 2B**). Remarkably, AtRTLs and Dicer proteins contain a conserved cysteine in the DRB domain (Cys230 in AtRTL1; **Figure 2A**) [33]. In AtRTL1 cysteine glutathionlylation inhibits RNase III cleavage activity in a reversible manner. Therefore, biotic and abiotic stresses, which affect the cellular redox environment, could modulate the activity of AtRTL1, and likely AtRTL2 and DCLs, and contribute to a fine-tuned modulation of small RNAs and subsequent gene regulation [33].

To dissect the molecular (RNA/protein) bases regulating and controlling RNase III activity of RTLs, we have established an *in vitro* RNA cleavage assay using recombinant proteins expressed in *E. coli*, total RNA extracted from plant seedlings and *in vitro* transcribed RNAs (**Figure 3**). We use the RTL1 targets 3'UTR of the *A. thaliana* At3g18145 gene [5]. Beyond our studies of AtRTL1 protein and 3'UTR of At3g18145 target, we believe that this protocol could be useful and easily adapted to study other RNases III and/or dsRNA targets from other organisms. [Figure 2 near here]

2 Materials

2.1 Production of Recombinant His-AtRTL1 Protein

1. 150 ng/µL pET16b::AtRTL1 plasmid in sterile, deionized water, prepared as described previously [33, 29] (*see* **Note 1**).
2. SoluBL21™ Competent *E. coli* cells (Genlantis) (*see* **Note 2**).
3. 1 M Isopropyl β-D-thiogalactopiraniside (IPTG). Dissolve 2.38 g of IPTG in 10 mL of deionized H_2O. Sterilize the solution by passing it through a 0.2-µm pore-size filter. Store in small aliquots at -20°C.
4. 100 mg/mL Ampicillin solution. Dissolve 1 g of ampicillin in 10 mL of deionized H_2O. Sterilize the solution by passing it through a 0.2-µm pore-size filter. Store in small aliquots at -20°C.
5. LB (Luria-Bertani) medium: Dissolve 10 g of tryptone, 5 g of yeast extract and 1 g of NaCl in 950 mL of deionized H_2O. Adjust the pH to 7 with 5 N NaOH and the final volume of the

solution to 1 L with deionized H_2O. Sterilize by autoclaving and store the medium at room temperature.

6. M9 medium: mix 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCL in 800 mL of deionized H_2O. Sterilize by autoclaving and store the medium at room temperature. Before use, add the following sterile components: 1 mL of 100 mM $CaCl_2$, 1 mL of 1 M $MgSO_4$ and 3 mL of 100% glycerol. Complete to 1L with sterile deionized H_2O.
7. LB-ampicillin medium. Add 1 mL of 100 mg/mL ampicillin to 1 L of sterile LB medium
8. M9-ampicillin medium. Add 1 mL of 100 mg/mL ampicillin to 1 L of sterile M9 medium.
9. LB-ampicillin plates: Dissolve 1.5 g of agar in 100 mL of LB medium. After autoclaving, cool down the medium (37-42 °C) and add 0.1 mL of 100 mg/mL ampicillin. Mix and pour the solution into petri dishes (use about 20 mL per dish). After solidification at room temperature, store the plates at 4°C.
10. Petri dishes 85 x 10 mm
11. Water baths set to 42°C.
12. Incubator chamber set to 37°C.
13. Shaking incubator.
14. 0.2 µm pore-size filters and 10 mL disposable syringes.
15. UV-vis spectrophotometer.
16. Centrifuge equipped with rotors holding 500 mL centrifuge bottles and 50 mL tubes.

2.2 Purification of Recombinant His-AtRTL1 Protein

1. 1 mL Gravity flow column filled with Ni-NTA His-binding resin (*see* **Note 3**).

2. Gravity flow column holder or equivalent.

3. 1X His-binding buffer: 50 mM NaH_2PO4, pH 8.0; 300 mM NaCl; 10 mM imidazole.

4. 1X His-washing buffer: 50 mM NaH_2PO4, pH 8.0; 300 mM NaCl; 20 mM imidazole.

5. 1X His-elution buffer: 50 mM NaH_2PO4, pH 8.0; 300 mM NaCl; 250 mM imidazole.

6. Protein sample buffer: 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20% (v/v) glycerol, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM Dithiothreitol (DTT).

7. Protease inhibitor cocktail (e.g cOmplete, EDTA-freeProtease Inhibitor Cocktail from Roche).

8. 1mg/mL DNase I. Stored at -20°C.

9. Protein extraction buffer: 1X binding buffer supplemented with 2.5 µM $MgCl_2$ and 1 µg/mL DNase I. Prepare 20 mL of buffer per purification and dissolve 1 tablet of cOmplete, EDTA-freeProtease Inhibitor Cocktail (Roche) right before use.

10. 10% (w/v) Ammonium persulfate (APS): Dissolve 1 g of APS in 10 mL of sterile deionized H_2O. Store in small aliquots at -20°C.

11. N,N,N,N-Tetramethylethylenediamine (TEMED). Stored at 4°C

12. 40% Acylamide/bis-acrylamide (29:1 ratio). Stored at 4°C.

13. 20% (w/v) Sodium dodecyl sulfate (SDS) in sterile deionized H_2O.

14. System for polyacrylamide gel electrophoresis (PAGE) and power supply.

15. Homemade 10% SDS-PAGE gel or commercial precast gel equivalent. For a homemade gel using 10 x 8 cm gel plates with 0.75 mm spacers, prepare 8 mL of 10% resolving mix: 3.8 mL of deionized H_2O, 2 mL of 40% Acylamide/bis-acrylamide (29:1), 2 mL of 1.5M Tris-HCl pH 8.8, 40 µl of 20% (w/v) SDS, 80 µl of 10% (w/v) APS, 8 µl of TEMED. Also prepare, 5 mL of 6% stacking solution mix: 2.9 mL of deionized H_2O, 0.75 mL of 40% Acylamide/bis-acrylamide (29:1), 1.25 mL of 0.5 M Tris-HCl pH 6.8, 50 µL of 20% (w/v) SDS, 50 µL of 10% (w/v) APS, 5 µL of TEMED. APS and TEMED should be added to each mix only right before pouring the gel.

16. 1X SDS-PAGE running buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS. The pH of the buffer should be 8.3.

17. 2X sample loading buffer (Laemmli buffer): 0.125 M Tris-HCl, pH 6.7; 4% (w/v) SDS; 20% (v/v) glycerol, 0.05% (w/v) Bromophenol blue.

18. 14.3 M β–mercaptoethanol. Stored at 4°C.

19. Commercial protein ladder covering the 10-250 kDa range. Stored at -20°C.

20. Protein gel staining solution (e.g. Symply Blue solution from Invitrogen).

21. Destaining gel solution: 10% (v/v) glacial acetic acid, 20% (v/v) methanol.

22. Protein dosage kit containing Bradford reagent and bovine serum albumin (BSA) concentration standard.

23. Size exclusion column (SEC) buffer: 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 150 mM NaCl.

24. Protein standards conalbumin (75 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) (e.g. Gel filtration calibration kit LMW from GE-Healthcare).

25. 100% Glycerol.

26. Fast Protein Liquid Chromatography (FPLC) instrument.

27. Superdex 75 10/300 GL column (GE Healthcare).

28. UV-vis spectrophotometer.

29. 8,000 Molecular weight cut-off (MWCO) dialysis bags.

30. 0.45-µm pore-size filters and 10 mL disposable syringes.

31. Benchtop centrifuge (refrigerated) and vortex mixer.

32. Dry bath set to 95°C.

33. Cell disruptor system.

2.3 Plant Total RNA Extraction and DNase treatment

1. Deionized, RNase-free water. Guaranteed high-grade (RNase/DNase-free, DEPC-treated, etc.) H_2O can be obtained from various commercial sources.
2. UV-vis spectrophotomer with microliter volume capability (e.g. NanoDrop 2000 from Thermo Scientific).
3. ~0.1g of 14-day-old *A. thaliana* seedlings, grown as described previously [33].
4. Glass beads (1.7-2.1 mm diameter).
5. Mixing device (e.g. Silamat S6 from Ivoclar Vivadent).
6. Trizol reagent, stored at 4°C.

7. Chloroform, stored at 4°C.

8. Isopropanol, stored at room temperature.

9. Chloroform:Isoamyl alcohol (24:1). Mix 24 mL of chloroform with 1 mL of isoamyl alcohol. Stored at 4°C.

10. Ethanol absolute (≥99.8%). Stored at -20°C.

11. 75% (v/v) ethanol in deionized, RNase-free water. Stored at -20°C.

12. Turbo DNA-free kit (ThermoFisher Scientific) containing Turbo DNase, 10X Turbo DNase buffer, and DNase inactivation reagent.

13. Agarose.

14. 50X TAE buffer: 2 M Tris-HCl pH 8, 1 M glacial acetic acid, 50 mM EDTA.

15. 10X MOPS buffer: 0.2 M MOPS pH 7, 50 mM sodium acetate, 10 mM EDTA

16. Dye mix: Mix and dissolve 25 mg of bromophenol blue, 25 mg of xylene cyanol FF and 3.3 mL of 100% glycerol in 6.7 mL of sterile deionized H_2O. Stored at -20°C.

17. Agarose RNA loading buffer (prepare just before use): Mix 10 µL of deionized formamide, 10 µL of formadehyde, 2 µl of 10X MOPS buffer and 1 µl of dye mix. Stored at -20°C.

18. GelRed® Nucleic Acid Gel Stain (Biorad) or equivalent.

19. Benchtop centrifuge (refrigerated) and vortex mixer.

20. Horizontal gel electrophoresis system for agarose gels with power supply.

21. Standard transilluminator (302 or 312 nm).

22. Dry baths set to 37°C.

23. Liquid nitrogen.

24. Rotating mixer.

2.4 His-AtRTL1 Cleavage Activity and RT-PCR assays

1. Items 1, 13,14 and 18-21 from Section 2.3.

2. 5X Cleavage reaction buffer: 100 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$

3. One-Step RT-PCR Kit (Qiagen) containing One Step Buffer 5X, 10 mM dNTPs, Q-Solution and One-Step RT enzyme. Stored at -20°C.

4. 160 U/µL GoScript™ Reverse Transcriptase (RT) and GoScript™ 5X reaction buffer (Promega) or equivalent. Stored at -20°C.

5. 40 U/µL RNasin (Promega) or equivalent. Stored at -20°C.

6. 10 mM dGTP, dATP, dTTP, dCTP stock solution. Stored at -20°C.

7. 10 mM $MgCl_2$

8. 6X Agarose gel loading dye: Mix and dissolve 25 mg of bromophenol blue and 3 mL of 100% glycerol in 7 mL of sterile deionized H_2O.

9. 5 U/µL Go®Taq DNA Polymerase and 5X Go®Taq buffer (Promega or equivalent). Stored at -20°C.

10. Dry baths set to 37°C and 70°C.

11. Thermocycler.

12. 10 µM UTRrt, UTRfw, UTRrev, U3fw and U3rev primer solutions. Stored at -20°C. (**Table 1**).

[Table 1 near here]

2.5 His-AtRTL1 Cleavage Activity Assay and Mapping Cleavage site

1. Items 10 and 11 from section 2.2.

2. Items 2, 5 and 6 from section 2.4.

3. Deionized, RNase-free water.

4. 50 µCi/µL, 3000 mmol/mL [γ-^{32}P]ATP. Handle and store at -20°C, following proper radio-safety regulation.

5. Plexiglas shields and containers for handling ^{32}P-containing reagents and waste.

6. T4 PolyNucleotide Kinase (PNK), with 10X PNK buffer. Stored at -20°C.

7. 0.2 µm and 0.45 µm pore-size filters with 10 mL and 50 mL disposable syringes.

8. MicroSpin G-25 columns.

9. Superscript II RT Kit (Invitrogen), containing Superscript II RT enzyme, 5X Superscript II RT buffer and 100 mM DTT. Stored at -20°C.

10. 3 M Sodium acetate pH 5.2. Dissolve 24.6 g of sodium acetate (anhydrous) in 70 mL of deionized H_2O. Adjust the pH to 5.2 by adding glacial acetic acid and complete to 100 mL with deionized H_2O. Sterilize the solution by passing it through 0.2 µm filter membrane.

11. Ethanol absolute (≥99.8%) stored at -20°C.

12. Ethanol 80% (v/v) in deionized, RNase-free water and stored at -20°C.

13. 5 mg/mL yeast tRNA. Dissolve 5 mg of yeast tRNA in 1 mL of deionized, RNase-free H_2O. Stored at -20°C.

14. Formamide loading buffer: Mix and dissolve 9.5 mL of deionized formamide, 2.5 mg of xylene cyanol FF, 2.5 mg of bromophenol blue, 100 µL of 0.5 M EDTA pH 8 and 400 µL of deionized sterile H_2O. Store at -20°C.

15. 10X TBE: 0.89 M Tris-base, 0.89 M boric acid, 20 mM EDTA.

16. 30% Acylamide/bis-acrylamide (19:1 ratio).

17. Urea, high quality grade.

18. Vertical gel electrophoresis system with 42 x 20 cm and 22 x 20 cm glass plates, 0.4 mm spacers, 20 teeth-comb, and power supply.

19. Denaturing, 6% Urea-PAGE gel: Dissolve 21 gr of urea in 20 mL of deionized H_2O, 10 mL of 30% Acrylamide/bis-acrylamide (19:1) and 5 mL of 10X TBE buffer. Warm the solution while mixing periodically to ensure complete dissolution (for instance using a 30 °C water bath). After urea is completely dissolved, complete to 50 mL with deionized H_2O. Filter the solution by passing it through a 0.45 µm filter membrane. Add 300 µL of 10% (w/v) APS and 22 µL of TEMED and immediately pour the gel within gel plates assembled following manufacturer's instructions. Then, install comb and let gel polymerize for about 60 min.

20. 100 ng/µL pBSIIk-3'UTR plasmid in sterile, deionized water, prepared as described previously [33] (*see* **Note 4**).

21. 10 µM primers pe1 and pe2 (**Table 1**).

22. Benchtop centrifuge (refrigerated) and vortex mixer.

23. Dry baths set to 37°C, 52°C, 80°C and 95°C.

24. Whatman paper 3 mm (46 x 57 cm sheets).

25. Saran Wrap.

26. Vacuum heated gel dryer.

27. Personal Molecular Imager (BioRad or equivalent), with ^{32}P imaging screen and cassette.

2.6 His-AtRTL1 Cleavage Activity Assay Using Radiolabelled RNA probes

1. Items 7-19 and 22-27 from Section 2.5.

2. 5X Cleavage reaction buffer: 100 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$.

3. 100 ng/µL pBSIIk+3'UTRrcr plasmid in sterile, deionized water, prepared as described previously [33] (*see* **Note 4**).

4. 10 U/µL Kpn1 restriction enzyme with supplied 10X buffer. Stored at -20°C.

5. DNA cleanup kit (e.g. GeneClean Turbo kit from MP biochemicals). Stored at -4°C.

6. 10 µCi/µL, 3000 Ci/mmol [α-^{32}P] CTP. Handle and store at -20°C, following proper radio-safety regulation.

7. Plexiglas shields and containers for handling ^{32}P-containing reagents and waste.

8. Scalpel blade.

9. Riboprobe® T3 transcription System (Promega) containing 5X Transcription buffer, 100 mM DTT, 10 mM rNTP stock solutions, and 20 U/µL T3 RNA Polymerase. Stored at -20°C.

10. RNA elution solution: 0.5M ammonium acetate, 1 mM EDTA, 100 mM Tris-HCl pH 8, 0.05% (w/v) SDS.

11. Phenol:chloroform:isoamyl alcohol (25:24:1), pH 5.2 premix solution.

12. Dry baths set to 37°C, 70°C and 95°C.

3 Methods [Figure 3 near here]

3.1 Production of Recombinant His-AtRTL1 Protein (Figure 3A)

1. On ice, mix 0.5 µL of the 150 ng/µL pET16b::AtRTL1 plasmid stock (*see* **Note 1**) with 50 µL of SoluBL21 Competent *E. coli* cells (*see* **Note 2**). Incubate for 20 min on ice and then for 45 s at 42°C. Add 250 µL of LB medium and incubate and shake (150 rpm) for 1 h at 37°C.

2. Streak two LB-ampicillin plates with, respectively, 20 µL and 200 µL of the cell mixture from step 1 to isolate single colonies (*see* **Note 5)**. Incubate overnight at 37°C.

3. Select a well-isolated colony from the plates and use it to inoculate 100 mL of LB-ampicillin medium. Shake (150 rpm) overnight at 37°C.

4. Transfer 50 mL of the overnight culture to 950 mL of M9-ampicillin medium. Shake (150 rpm) at 37°C and monitor the optical density at 600 nm (OD_{600}) with a spectrophotometer.

5. When the OD_{600} reaches ~ 0.4, remove a 1 mL culture aliquot (non-induced control) and store it at -20°C until further use. Then, add 1 mL of 1 M IPTG to the remaining culture and shake (150 rpm) overnight at room temperature.

6. Remove a 1 mL aliquot (induced control) and store it at -20°C. Then, transfer the overnight culture in two 500 mL centrifuge bottles. Harvest cells by centrifugation at 12,000 x *g* for 15 min at 4°C.

7. Suspend and pool cell pellets in 45 mL of M9-ampicillin medium and transfer the suspension into a 50 mL centrifuge tube. Harvest the cells by centrifugation at 12,000 x *g* for 15 min at 4°C.

8. Remove the supernatant and store the pellet at -80°C.

3.2 Purification of Recombinant His-AtRTL1 Protein (Figure 3A)

3.2.1 His-Affinity column purification

1. Prepare and equilibrate a 1 mL His-binding resin column according to the manufacturer's instructions. Use a gravity-flow column holder or equivalent.

2. Suspend the cell pellet from section 3.1, step 8 in 10 mL of Protein extraction buffer.

3. Lyse the cells and release the proteins (*see* **Note 6**).

4. Collect the lysate and centrifuge at 7,800 x *g* for 20 min at 4°C.

5. Load the ~20 mL supernatant onto the 1 mL His-binding resin column from step 1.

6. Wash the column with 10 volumes of 1X His-binding buffer and then with 6 volumes of 1X His-washing buffer.

7. Elute His-AtRTL1 with five volumes of 1X His-elution buffer. Collect up to 5 fractions (E1-5) of 1 mL.

8. Determine the protein concentration of each fraction using a Bradford protein dosage kit and following the kit's instructions.

9. Prepare samples containing 10 µL of each E1-5 fraction, 10 µL of 2X sample loading buffer, and 1 µL of β-mercaptoethanol. Heat samples in a dry bath for 2 min at 95 °C. Then spin the tube briefly in a microcentrifuge so that all the sample can be collected at the bottom.

10. Install homemade or precast 10 % SDS-PAGE gel into vertical electrophoresis system. Fill the system tank(s) with 1X SDS-PAGE running buffer.

11. Load samples from step 7 and a protein ladder aliquot into separate gel wells. Also load before- and after-induction samples from section 3.1, steps 5 and 6.

12. Perform electrophoresis at 130 V until the dye front (the bromophenol blue) reaches the bottom of the gel.

13. Remove gel plates and place the gel into a tray filled with protein gel staining solution. Gently rock at room temperature for 30-60 min. Then throwaway protein gel staining solution and refill the tray with distaining gel solution. Gently rock at room temperature until the protein bands become visible on the gel. The His-AtRTL1 protein should be visible as a band migrating around 35 kDa (*see* **Note 7**).

14. Use the Bradford concentration and SDS-PAGE analyses (*see* steps 8 and 13) to identify the fractions containing the largest and purest amounts of His-AtRTL1 protein (usually E2-E4). Combine these fractions for a second round of purification (step 15, optional) or proceed directly to step 18.

15. Load the pooled fractions into a dialysis bag and perform dialysis in 1 L of 1X His-binding buffer overnight at 4°C (*see* **Note 8**).

16. Recover the sample from the dialysis bag and centrifuge it at 18,000 x *g* for 20 min at 4°C.

17. Recover the ~5 mL supernatant and repeat steps 5 to 14, using a new His-binding resin column (step 1).

18. Load the pooled fractions into a dialysis bag and perform dialysis in 1 L of Protein sample buffer overnight at 4°C (*see* **Note 8**).

19. Recover the sample from the dialysis bag and centrifuge it at 18,000 x *g* for 20 min at 4°C.

20. Recover the supernatant and store it in 500 µL (~400 µg) aliquots at -80°C.

3.2.2 Size Exclusion Chromatography (SEC)

To purify His-AtRTL1 further and/or to remove any potential His-AtRTL1 degradation products, we recommend performing SEC using a Superdex 75 column connected to a FPLC system.

1. Connect the Superdex 75 column to a FPLC system and equilibrate it overnight with SEC running buffer. Set the FPLC system to perform SEC runs for at least 1.5 column volumes (~35 mL) at a flow rate of 0.5 mL/min.

2. Determine the elution volumes for molecular weight markers conalbumin (75 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa) by performing separate SEC runs with each protein. Use samples containing 1 mg of protein in 500 µL of SEC running buffer (*see* **Note 9**).

3. On ice, thaw a 500 µL aliquot of His-AtRTL1 from section 3.2.1, step 20 and load it on the Superdex 75 column. Perform a SEC run, collecting 0.5 mL fractions during the whole run.

4. Analyze peak fractions eluting between molecular weight markers conalbumin and carbonic anhydrase by SDS-PAGE as described in Section 3.2.1, steps 8-13 (*see* **Note 10**).

5. Combine fractions containing the most and purest His-AtRTL1 protein. Add to 500 µL sample 125 µL of 100% glycerol for a final concentration of 20% (v/v).

6. Store at -80°C as 100 µL aliquots.

3.3 His-AtRTL1 Cleavage Activity Assay Using Plant Total RNA

3.3.1 Total RNA extraction and DNase treatment

1. Mix ~0.1g of the 14-day-old *A. thaliana* seedlings in a 1.5 mL microtube with ~ 10 glass beads and freeze in liquid nitrogen.

2. Shake the samples for 8 s in a Silamat S6 mixing device and freeze in liquid nitrogen (*see* **Note 11**).

3. Repeat step 2 three times.

4. In a fume hood add to the grinded plant seedlings, 1 mL of Trizol reagent and mix thoroughly with a vortex mixer. Incubate 5 min at room temperature.

5. Add 0.2 mL of chloroform and incubate 2-3 min at room temperature.

6. Centrifuge at 12,000 x *g* for 15 min at 4°C.

7. Recover the aqueous (upper) phase and transfer it into a new 1.5 mL microtube. Add 0.5 mL of isopropanol and incubate for 10 min at room temperature.

8. Centrifuge at 12,000 x *g* for 10 min at 4°C.

9. Discard the supernatant by inverting the tubes very gently and rinse the pellet by adding 1 mL of cold 75% ethanol.

10. Centrifuge at 7,500 x *g* for 5 min at 4°C. Remove carefully the supernatant and air dry the pellet at room temperature.

11. Suspend the RNA pellet in 40 µL of deionized, RNase-free water. Estimate RNA concentration (*see* **Note 12**).

12. To remove genomic DNA contamination, set up a Turbo DNase reaction by mixing 10-15 µg of total RNA, 5 µL of 10X Turbo DNase buffer, 1 µL of Turbo DNase, and deionized, RNase-free H_2O up to a final volume of 50 µL. Incubate for 30 min at 37°C.

13. Spin the tube briefly so that all the sample can be collected at the bottom and add an extra 0.5 µL of Turbo DNase to the mix and incubate for another 30 min at 37°C.

14. Inactivate Turbo DNase by adding 5 µL of inactivating solution from the Turbo DNA-free Kit (*see* **Note 13**). Incubate 5 min at room temperature in a rotating mixer.

15. Centrifuge at 10,000 x *g*, for 1.5 min at 4°C. Recover the supernatant and estimate RNA concentration (*see* **Note 12**).

16. Prepare a 0.8% (w/v) agarose gel by mixing 0.8 g of agarose in 100 mL of 1X TAE buffer. Heat the mixture in a microwave oven until the solution is clear and the agarose is completely dissolved, add 10 µL of gel GelRed® Nucleic Acid Gel Stain, and pour it in a gel tray (use a

dedicated gel casting system or sealing tape to avoid gel leaking). Install comb and wait for the gel to solidify (~15 min). Then, install the gel in a horizontal electrophoresis system filled with 1X TAE buffer.

17. Mix 2.5 µL of RNA sample (~ 10 µg) from step 18 with 10 µL of Agarose RNA loading buffer and 7.5 µL of deionized, RNase-free H_2O. Load sample in gel well and run gel for approximately 40 min at 75 V.

18. Visualize RNA with a standard transilluminator (*see* **Note 14**).

19. Store RNA from step 15 at -80°C until further use.

3.3.2 Cleavage activity assay and One Step RT-PCR reaction (Figure 3B)

1. Set up cleavage reactions by mixing 4 µL of 5X Cleavage reaction buffer, 0.5 µg of total RNA (from Section 3.3.1, step 19), ~ 0.2 µg of His-AtRTL1 (from Section 3.2.1, step 20 or Section 3.2.2, step 6), and RNase-free H_2O up to a final volume of 20 µL. Also prepare a cleavage reaction with Protein sample buffer instead of His-AtRTL1 protein (*see* **Note 15**). Incubate cleavage reaction mixes (with and without AtHis-RTL1) for 30 min at 37°C.

2. Stop the cleavage reaction by heating samples 5 min at 70°C. Then spin the tube briefly so that all the sample can be collected at the bottom.

3. Set up One Step RT-PCR reactions by mixing 5 µL of 5X One Step buffer, 1 µL of 10 mM dNTPs, 5 µL of Q-Solution, 1.5 µL of 10 µM UTRfw, 1.5 µL of 10 µM UTRrev, 1 µL of One Step RT Enzyme, 2.5 µL of cleavage mix reactions (from step 1, with or without His-RTL1),

and RNase-free H_2O up to a final volume of 25 µL. Also prepare One Step RT-PCR mix reactions with 1.5 µL of 10 µM U3fw and 1.5 µL of 10 µM U3rev (*see* **Note 16).**

4. Perform One Step RT-PCR program with the following parameters: **step 1**, RT reaction (50°C, 30 min); **step 2**, initial denaturation (95°C, 15 min); **step 3**, second cycle of denaturation (95°C, 45 s); **step 4**, annealing (52°C, 45 s); **step 5**, extension (72°C, 90 s); **step 6**, final extension (72°C, 10 min). Repeat steps 3-5, 34 times.

5. Prepare a 1.2% (w/v) agarose gel as described in Section 3.3.1, step 16.

6. Mix ~ 2-5 µL of RT-PCR reactions from step 4 with 1.6 µL of 6X Agarose gel loading dye and deionized H_2O up to a final volume of 10 µL.

7. Load sample(s) in gel well(s) and run gel for approximately 40 min at 75 V.

8. Visualize DNA with a standard transilluminator. The size of the amplification product in the cleavage reaction without His-AtRTL1 (non-cleaved RNA) is ~100 bp with primers UTRfw and UTRrev (**Table 1**). Non amplification product is detected in the cleavage reaction with His-AtRTL1 (cleaved RNA) and primers UTRfw and UTRrev (*see* **Note 17**)

3.3.3 His-AtRTL1 Cleavage activity assay and two-step RT-PCR reaction

The two-step RT-PCR reaction can be used as an alternative to the one-step RT-PCR kit.

1. Perform cleavage activity assay as described in Section 3.3.2, steps 1-2.

2. In microtube 1, mix 1 µL of 10 µM UTRrt primer, 2.5 µL of cleavage reaction mix (with or without Hi-AtRTL1) from step 1 and RNase-free H_2O up to a final volume of 5 µL.

3. In microtube 2, mix 4 µL of GoScript™ 5X reaction buffer, 2.5 µL of 10 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 0.5 µL of 40 U/µL RNasin, 1 µL of 160 U/µL GoScript™ Reverse Transcriptase (RT), and RNase-free H_2O up to a final volume of 15 µL.

4. Incubate microtube 1 for 5 min at 70°C, spin the tube briefly so that all the sample can be collected at the bottom and then transfer the content to microtube 2. Mix thoroughly by pipetting up and down.

5. Transfer the mixture in a thermocycler and perform RT reactions with following parameters: annealing for 60 min at 42°C, extension for 10 min at 70°C, and final incubation for 10 min at 20°C.

6. Set up PCR reactions by mixing ~2 µL of the cDNA obtained from step 5 with 5 µL of 5X Go®Taq buffer, 1.5 µL of 10 µM UTRfwd, 1.5 µL of 10 µM UTRrev, 0.5 µL of 10 mM dNTP, 0.25 µL of 5 U/µL Go®Taq DNA polymerase, and RNase-free H_2O up to a final volume of 25 µL.

7. Transfer the mixtures in a thermocycler and perform PCR program with following parameters: **step 1**, initial denaturation (94°C, 3 min); **step 2**, second cycle of denaturation (94°C, 45 s); **step 3**, annealing (55°C, 45 s); **step 4**, extension (72°C, 60 s); **step 5**, final extension (72°C, 10 min). Repeat steps 2-4 for 34 times.

8. Analyze PCR products as described in Section 3.3.2, steps 5-8.

3.4 His-AtRTL1 Cleavage Activity Assay and Mapping Cleavage site

All steps involving ^{32}P-containing materials should be handled by properly trained staff, following local regulatory procedures.

3.4.1 Primer radiolabeling

1. For each relevant primer, set up a radiolabelling reaction by mixing 1 µL of 10 µM primer pe1 (or pe2; **Table 1**), 5 µL of [γ-^{32}P] ATP (50 µCi/µL; 3000 mmol/mL), 2 µL of 10X PNK buffer, 1 µL of T4 PNK, and 11 µL of sterile deionized H_2O for final volume of 20 µL. Incubate for 30 min at 37°C.

2. To isolate the [γ-^{32}P] ATP radiolabelled primers from the non-incorporated [γ-^{32}P] ATP, use a MicroSpin G-25 column or equivalent. Place the column into a 1.5 mL microtube and spin it for 1 min at 735 x *g*. Recover the microSpin G-25 column and place it into a new 1.5 microtube, load the 20 µL radiolabelling reaction and spin it for 2 min at 735 x *g*. Discard the microSpin G-25 column containing the non-incorporated [γ-^{32}P] ATP and save the radiolabelled primers collected in the bottom of the 1.5 mL microtube.

3.4.2 Primer extension analysis

1. Set up cleavage activity assay as described in Section 3.3.2, steps 1-2, using 15 µg of total RNA in each reaction (from Section 3.3.1).

2. To remove His-AtRL1 protein and recover cleaved and/or non-cleaved RNA from cleavage reactions from step 1, add to each sample 1 µL of 5 mg/mL yeast tRNA, 79 µL of RNase-free H_2O, and 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1), pH 5.2 premix solution. Mix well with a vortex mixer and centrifuge 15 min at 12,000 x *g*.

3. Recover aqueous (top) phase and add 10 µL of 3 M sodium acetate pH 5.2 and 300 µL of 100% ethanol. Incubate overnight at -20°C

4. Centrifuge at 12,000 x *g* for 15 min at 4°C. Discard the supernatant and rinse the pellet by adding 1 mL of cold 80% ethanol.

5. Suspend the RNA pellet in 6 µL of RNase-free H_2O.

6. Set up primer extension annealing mix by combining the 6 µL of RNA from step 5 with 2 µL of 5X Superscript II RT buffer and 2 µL of radiolabeled primer (from Section 3.4.1, step 2 (*see* **Note 18**). Incubate for 5 min at 80°C in a dry bath.

7. Cool down the reactions to 52°C by removing the heat block from the dry bath. Do not allow the temperature of the block to go lower than 52°C.

8. In a new 1.5 mL microtube, set up the primer extension RT mix by combining 0.5 µL of 40 U/µl RNasin, 2 µL of 10 mM dNTPs, 2 µL of 5X Superscript II RT buffer, 2 µL of 100 mM DTT, 3 µL of RNase-free H_2O and 0.5 µL of Superscript II RT enzyme. Keep the primer extension RT mix at 52°C in a dry bath.

9. Mix 10 µL of primer extension annealing (step 2) with 10 µL of primer extension RT (step 8) mixtures. Incubate 1h at 52 °C.

10. Spin the tube briefly and stop primer extension reactions by adding 2 µL of 3M sodium acetate pH 5.2, 1 µL of 5 mg/mL tRNA and 60 µL of 100% ethanol. Precipitate RNA overnight at -20°C.

11. Centrifuge at 12,000 x *g* for 15 min at 4°C. Discard the supernatant and rinse the pellet with 1 mL of cold 80% ethanol.

12. Centrifuge at 12,000 x *g* for 5 min at 4°C. Remove carefully the supernatant and air dry the pellet at room temperature.

13. Install a denaturing, 6% Urea-PAGE gel in a vertical electrophoresis system. Remove comb and fill system tanks with 1X TBE buffer. Pre-run the gel for 40-60 min to heat the gel up and to remove remaining urea from the gel. The optimal temperature should be between 45-55 °C (*see* **Note 19**). Turn off the power and flush gel wells with 1X TBE buffer to eliminate diffusing urea from the wells.

14. Suspend the pellets from step 12 in 10 µL of Formamide loading buffer and heat samples in a dry bath for 2 min at 95 °C.

15. Spin the tube briefly and load samples in gel wells and run the gel until the blue dye reaches at least 2/3 of the gel (~30 min) (*see* **Note 19**).

16. Remove gel from apparatus and pry one gel plate apart with a spatula. Transfer gel on a piece of Whatman paper and cover the free gel face with saran wrap. Dry gel on a vacuum heated gel dryer and expose it to a ^{32}P imaging screen in an imaging cassette for 5 to 12 hrs. Then screen image with a Phosphor-Imager. Primer extension reaction with primer pe1 yield products of ~42- and ~46-nt long while reaction with primer pe2 yield products of ~38- and ~42-nt long and map the RTL1 cleavage site rcr1 in each RNA strand of the 3'UTR (**Figures 2B**) (*see* **Note 20**).

3.5 His-AtRTL1 Cleavage Activity Assay Using Radiolabelled RNA probes

3.5.1 Making radiolabelled probe

1. Linearize ~1 µg of the pBSIIk+ 3'UTRrcr plasmid with Kpn1 restriction enzyme, following supplier's instructions (*see* **Note 21**). Purify linearized plasmid with a DNA cleanup kit following kit instructions (*see* **Note 22**).

2. Set up *in vitro* transcription reaction by mixing 5 µL of 5X Transcription buffer, 2.5 µL of 100 mM DTT, 5 µL of 2.5 mM rGTP, rATP, rUTP solution, 3 µL of 100 µM rCTP, ~0.2 µg of Kpn1 linearized pBSIIk+ 3'UTRrcr plasmid from step 1, 5 µL of [α-^{32}P] CTP (10 µCi/µL, 3000 Ci/mmol), 1 µL of 20 U/µL T3 RNA Polymerase and RNase-free H_2O up to a final volume of 25 µL. Incubate reaction for 120 min at 37°C.

3. Stop the T3 RNA Polymerase transcription reaction by precipitation as described in Section 3.4.2 steps 10-12.

4. Install a denaturing, 6% Urea-PAGE gel in a vertical electrophoresis system. Remove comb and fill system tanks with 1X TBE buffer. Pre-run the gel for 40-60 min before sample loading (*see* **Note 19** and Section 3.4.2 step 13). Turn off the power and flush gel wells with 1X TBE buffer to eliminate diffusing urea from the wells.

5. Dissolve pellet from step 3 in 6 µL of Formamide loading buffer and heat sample in a dry bath for 2 min at 95 °C.

6. Spin the tubes briefly and load samples in gel wells and run the gel until the blue dye reaches at least 2/3 of the gel (~30 min).

7. Remove gel from apparatus and pry one gel plate apart with a spatula. Cover the free gel face with saran wrap and expose it to a ^{32}P imaging screen in an imaging cassette for 5 min.

8. Print a highly contrasted gel image at exact size and place it under the gel plate, carefully aligning the actual gel with the gel image (*see* **Note 23**).

9. Using a clean scalpel blade, cut out the piece of gel containing the radiolabeled transcript and place it in a 1.5 mL microtube containing 500 µL of RNA elution solution. Incubate for 2 h at 37°C.

10. Centrifuge at 12,000 x *g* for 5 min at room temperature. Transfer the supernatant to a new 2 mL microtube and add 1.5 mL of 100% ethanol. Precipitate the ^{32}P-labelled RNA overnight at -20°C.

11. Centrifuge at 12,000 x *g* for 15 min at 4°C. Discard the supernatant and rinse the pellet with 1 mL of cold 80% ethanol.

12. Centrifuge at 12,000 x *g* for 5 min at 4°C. Remove carefully the supernatant, air dry the pellet and suspend it in 50 µL of RNase-free H_2O. Store the ^{32}P-labeled RNA stock solution at -20°C until further use.

3.5.2 Cleavage reaction of radiolabelled probe and analysis on denaturing PAGE

1. Mix 1 µL of ^{32}P-labeled RNA stock solution (from Section 3.5.1, step 12) with 10 µL of RNase-free, deionized water.

2. Setup cleavage reaction by mixing 4 µL of the ^{32}P-labeled RNA dilution from step 1 with 4 µL of 5X Cleavage reaction buffer, ~ 200 ng of His-AtRTL1 (from Section 3.2.1, step 20 or

Section 3.2.2, step 6) and RNase-free H_2O up to a final volume of 20 µL. Also prepare a cleavage reaction mix with protein sample buffer instead of His-AtRTL1 protein (*see* **Note 15**). Incubate for ~30 min at 37°C.

3. Stop reaction by heating samples for 5 min at 70°C. Spin the tube briefly so that all the sample can be collected at the bottom. Then, add 1 µL of 5 mg/mL yeast tRNA, 79 µL of RNase-free H_2O, and 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1), pH 5.2 premix solution. Mix well with a vortex mixer and centrifuge 15 min at 12,000 x *g*.

4. Recover aqueous (top) phase and add 10 µL of 3 M sodium acetate pH 5.2 and 300 µL of 100% ethanol. Incubate overnight at -20°C

5. Centrifuge at 12,000 x *g* for 15 min at 4°C. Discard the supernatant and rinse the pellet by adding 1 mL of cold 80% ethanol.

6. Centrifuge at 12,000 x *g* for 5 min at 4°C. Remove carefully the supernatant and suspend the pellet in 10 µL of Formamide loading buffer.

7. Install a denaturing, 6% Urea-PAGE gel in a vertical electrophoresis system as described in Section 3.5.1 step 4.

8. Heat sample from step 6 in a dry bath for 2 min at 95 °C. Spin the tube briefly and load sample in gel well and run the gel until the blue dye reaches at least 2/3 of the gel (~30 min) (*see* **Notes 19**).

9. Process and image gel as described in Section 3.4.2, step 16. The non-cleaved, ^{32}P-labelled RNA probe migrates as a ~240 nt-long RNA while cleaved ^{32}P-labelled RNA probe migrate as two ~110 and ~105 nt-long fragments (**Figure 3C**).

4. Notes

1. pET16b::AtRTL1 is a derivative of the commercial pET16b expression plasmid (Novagen) containing the full length AtRTL1 coding sequence and located downstream from a sequence encoding a hexa histidine-tag (6XHis-tag) and under control of a T7 promoter (**Figure 3A**). The plasmid allows overexpression of the N-terminal His-tagged AtRTL1 (His-AtRTL1) protein in *E. coli* cells.
2. Compared to standard BL21 cells, SoluBL21 cells yield higher amount of soluble His-AtRL1 protein.
3. Cobalt chelating resins may be used instead of Ni-NTA. Non-specific binding contaminants may be fewer but usually at the expense of yield of the expected protein. Prepacked Ni-NTA or Cobalt resin columns installed on a FPLC system can also be used.
4. pBSIIk+3'UTR plasmid contains the sequence 3'UTR-rcr1 **(Figure 2B**) cleavage site from At3g18145 gene [33].
5. We use distinct volume aliquots of the original cell culture to increase chances to obtain well-isolated bacterial colonies. Using freshly isolated colonies rather than the -80°C stock to express proteins limits risk of plasmid loss and usually increases yields of expressed proteins.
6. We use a cell disruptor from Constant Systems Limited at a pressure of 1.35 bar. Alternatively you can use sonication or a French press to disrupt *E. coli* cells and release recombinant His-AtRLT1.
7. AtRTL1 is ~34 kDa and the 6XHis-tag is ~1 Kda.
8. You can perform a second dialysis round of 5-8 h using fresh protein sample buffer.
9. In our hands and with an AKTA purifier system (GE-Healthcare), conalbumin, ovalbumin, and carbonic anhydrase elute at ~11, ~12, and ~13 mL, respectively.

10. One may also detect His-RTL1 protein by Western Blotting using anti-AtRTL1 (custom-made) or anti-His-Tag (commercial) antibodies.

11. Alternatively, frozen seedlings can be grinded in a mortar with a pestle and using liquid nitrogen to create a fine powder.

12. We use a ~1.5 µL aliquot to measure absorbance at 260 nm with a NanoDrop 2000 spectrophotometer. We then estimate RNA concentration from the absorbance using an extinction coefficient of 40 ng-cm/µL.

13. This step avoids the need DNase inactivation by heat and/or phenol extraction.

14. In the agarose gel, large (25S and 18S), small (5.8S, 5S and tRNA) as well as chloroplast RNAs are visualized. You can verify lack of DNA contamination by PCR, using primers that amplify a genomic sequence containing introns and exons (for instance eIF1α gene sequence [34]). If the RNA sample is DNA-free, no amplification should be detected. Use a genomic DNA sample as a positive control of amplification.

15. Using RNA sample buffer instead of His-AtRTL1 allows to detect absence of RNA cleavage in absence of RTL1 activity.

16. U3fw and U3rev are specific primers for the amplification of snoRNA U3 sequences which are not cleaved by AtRTL1 and then used to control RNA loading and specific RNA cleavage of At3g18145-3'UTR.

17. Primers UTRfw/UTRrev amplify non-cleaved At3g18145-3'UTR RNA sequences (97 bp) while primers U3fw/U3rev amplify U3snoRNA (215 bp) sequences.

18. As a negative reaction control, set a primer extension assays with 10 µg of yeast tRNA instead of plant total RNA in cleavage reactions with and without His-AtRTL1 protein.

19. Run settings depend on gel size. We run 0.4 mm wide gels at 18 W (22 x 20 cm gels) or 45 W (42 x 20 cm gels).

20. To estimate primer extension size products, one may load a radiolabeled DNA size ladder alongside with samples on the gel. There are various ways to prepare such ladders. For instance, one nucleotide resolution ladders can be prepared by Sanger sequencing of the pGemT-3'UTR_At3g18145 plasmid with ^{32}P-labeled pe1 and pe2 primers [35].

21. The Kpn1 site is located downstream of the 3'UTRrcr sequence and the T3 promoter is located upstream of the 3'UTRrcr sequence (**Figure 3B)**.

22. Alternatively, you may heat-inactivate Kpn1 at 65°C for 20 min and purify the plasmid by extraction with a buffer-saturated (pH 8) Phenol:chloroform:isoamyl alcohol (25:24:1) solution and, then, by ethanol precipitation.

23. Alternatively, you can stick pieces of Whatman paper to the corners of the gel and pipet 1 µL of a blue colored radioactive solution (e.g. 1 µL of labelled primer from section 3.4.1 in 10 µL of 6X Formamide loading buffer) on each piece. These highly radioactive spots will help align the gel with the gel image.

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Table 1 : DNA oligonucleotide primers		
Name	**Sequence**	**Usage**
UTRfw	AGCGTACTTGTGCAAAATGCG	One Step RT-PCR and PCR
UTRrev	GTCAGTCTGTAATAGCTC	One Step RT-PCR and PCR
UTRrt	CTTTAGTTACAAAACAAAACC	Reverse Transcription (RT)
U3fw	ACGGACCTTACTTGAACAGGATCTG	One Step RT-PCR-control
U3rev	CTGTCAGACCGCCGTGCG	One Step RT-PCR-control
pe1	GTCAGTCTGTAATAGCTC	Primer extension
pe2	GCAAAATACCAGATCCAG	Primer extension

Figure Legends

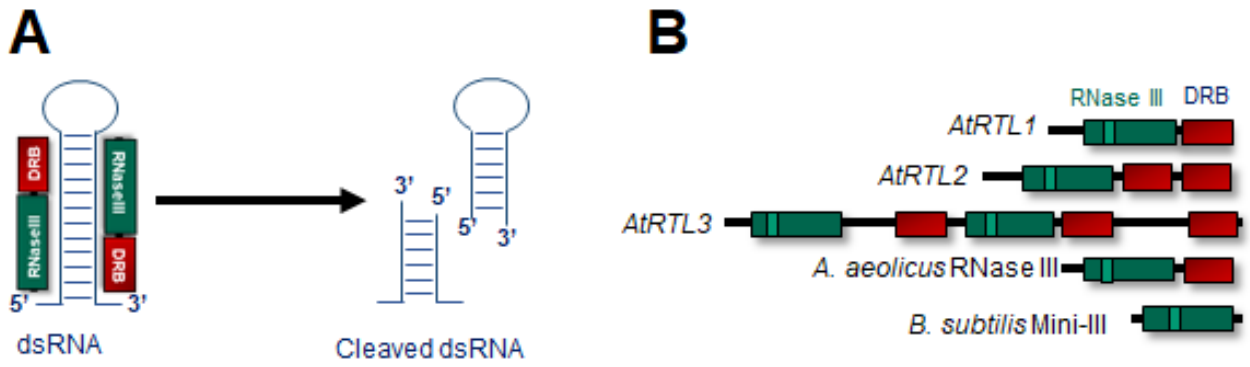

Figure 1: A) RNase III homodimer cleaves double-strand RNA (dsRNA) and generates 3' overhang ends. **B)** Schematic comparing Class I RNases III from *Arabidopsis thaliana* (AtRTL1-3), *Aquifex aeolicus* (RNase III) and *Bacillus subtilis* (Mini-RNase III). Green and red boxes correspond to RNase III and DRB domains while light green boxes correspond to the conserved RNase III motif.

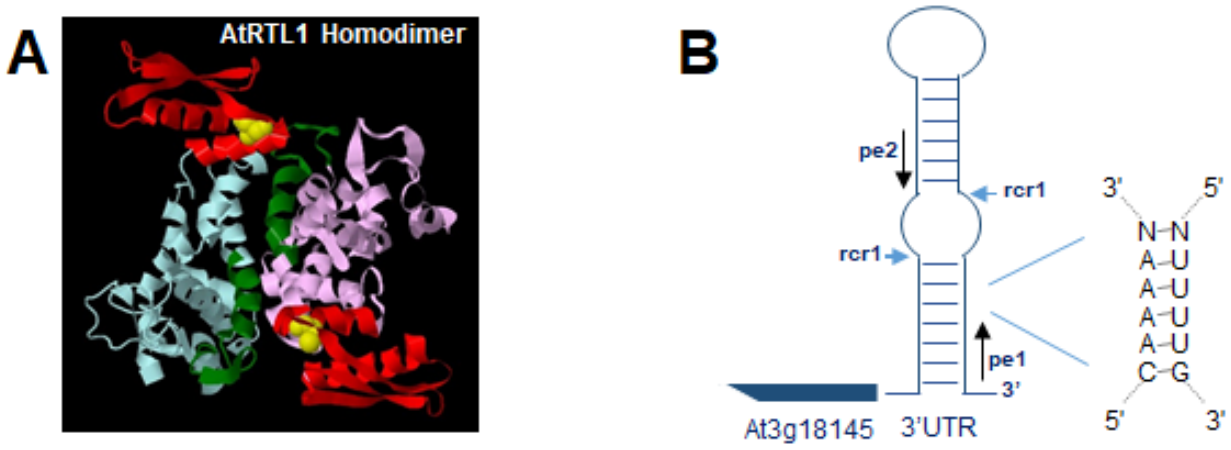

Figure 2: **A)** Modelled AtRTL1 (residues 50-284) homodimer based on *Aquifex aeolicus* (PDB: 1YYW) [5]. The RNase III motifs of two AtRTL1 molecules are shown in green while both DRBs are shown in red. Cys230 residues in the DRBs are shown in yellow. **B)** AtRTL1 cleavage site rcr1 in the At3g18145 3'UTR sequence is located near to conserved RNA duplex that directs accurate cleavage. The positions of pe1 and pe2 primers used in primer extension experiments are indicated. [7].

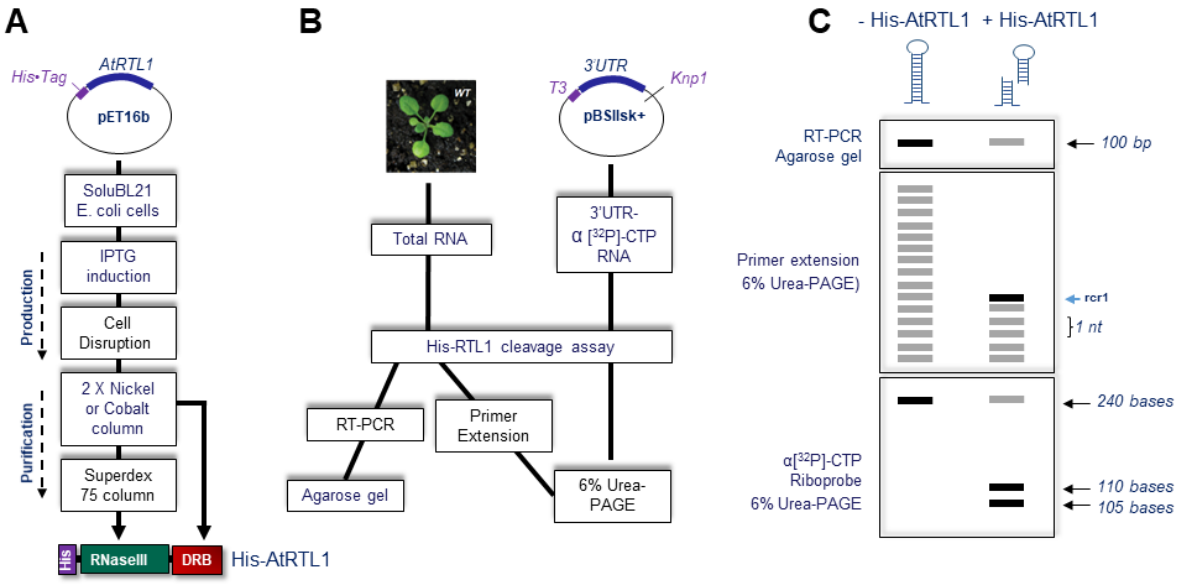

Figure 3: Schematic representation of **A)** His-AtRTL1 purification. Green and red boxes correspond to RNase III and DRB domains of AtRTL1 while the purple box corresponds to the N-terminal 6XHis-tag sequence in the His-RTL1 protein. **B)** *in vitro* cleavage assays using *Arabidopsis* total RNA or ^{32}P-labelled 3'UTR-RNA. **C)** Top, RT-PCR analysis on agarose gel: non-cleaved RNA can be amplified using specific UTRfw and UTRrev primers located upstream/downstream of AtRTL1 cleavage site rcr1 (~ 100 bp) while cleaved RNA it is not amplified. Middle, analysis on denaturing gel of primer extension reactions. Primer pe1 detects 42- and 46-nt long products while primer pe2 detects 38- and 42-nt long products. Bottom, on denaturing gel the non-cleaved radiolabeled probe migrates as a 240 nt-long RNAs while the His-AtRTL1cleaved RNAs migrate as 110 and 105 nt-long fragments.

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