Chapter 20

Detection of MicroRNA Processing Intermediates Through RNA Ligation Approaches

Belén Moro, Arantxa M. L. Rojas, and Javier F. Palatnik

Abstract

MicroRNAs (miRNA) are small RNAs of 20–22 nt that regulate diverse biological pathways through the modulation of gene expression. miRNAs recognize target RNAs by base complementarity and guide them to degradation or translational arrest. They are transcribed as longer precursors with extensive secondary structures. In plants, these precursors are processed by a complex harboring DICER-LIKE1 (DCL1), which cuts on the precursor stem region to release the mature miRNA together with the miRNA*. In both plants and animals, the miRNA precursors contain spatial clues that determine the position of the miRNA along their sequences. DCL1 is assisted by several proteins, such as the double-stranded RNA binding protein, HYPONASTIC LEAVES1 (HYL1), and the zinc finger protein SERRATE (SE). The precise biogenesis of miRNAs is of utter importance since it determines the exact nucleotide sequence of the mature small RNAs and therefore the identity of the target genes. miRNA processing itself can be regulated and therefore can determine the final small RNA levels and activity. Here, we describe methods to analyze miRNA processing intermediates in plants. These approaches can be used in wild-type or mutant plants, as well as in plants grown under different conditions, allowing a molecular characterization of the miRNA biogenesis from the RNA precursor perspective.

Key words MicroRNA, Processing, Precursor, RNA ligation, NGS, Plants, Arabidopsis

1 Introduction

miRNAs are small RNAs present in animals and plants. They recognize target RNAs by base complementarity affecting RNA stability and mRNA translation [1]. In humans, it is considered that 60% of all genes are regulated by miRNAs [2]. In plants they control essential processes such as development, hormone signaling, and stress responses [3–6]. miRNAs can regulate gene expression in different ways, by quantitatively adjusting the proteins levels or by eliminating the RNA transcripts in the cell reassuring the inactivation of certain genes [7].

Plant miRNAs are transcribed by RNA polymerase II as longer primary transcripts, which are capped, spliced, and polyadenylated [8]. Within the primary transcript, there is a foldback with a stem-

loop structure. The miRNA is located in one arm of the stem, and the region that interacts with it is called miRNA*. miRNAs are distinguished from other classes of small RNAs by their unique biogenesis, which involves the precise excision from their foldback precursors [9]. In animals, a complex formed by DROSHA and DiGeorge Syndrome Critical Region 8 (DGCR8) recognizes the miRNA primary transcript in the nucleus and produces the first cut at the base of the miRNA/miRNA* [2]. The resulting pre-miRNA is translocated to the cytoplasm by EXPORTIN 5 and RAN-GTP. There, a complex formed by DICER and TAR RNA binding protein (TRBP) performs the second cut releasing the mature miRNA [2]. Since the processing intermediates in animals must be exported to the cytoplasm, they have a considerable half-life, which in practice means they are easily detected in blots for small RNAs [10–12].

In *Arabidopsis thaliana*, the ribonuclease type III DCL1 is the main component of the miRNA processing machinery. DCL1 interacts with the double-strand RNA domain binding protein HYL1 [13, 14] and with the type C2H2 Zn finger protein, SE [15, 16]. Several other RNA binding proteins such as CAP BINDING PROTEIN 20 and 80 [17–19], TOUGH [20], and the forkhead domain containing protein DAWDLE [21] also participate in the processing of miRNAs, although their molecular roll is not completely clear. Recently the protein KERYOPHERIN ENABLING THE TRANSPORT OF THE CYTOPLASMATIC HYL1 (KETCH1) has been reported to transport HYL1 to the nucleus [22], while the phosphatase CPL1 modulates HYL1 activity by dephosphorylation of specific residues [23]. Unlike to what happens in animals, DCL1 performs all necessary cuts to release the mature miRNA in plants [1, 7, 24].

A key step in the biogenesis of animal and plant miRNAs is the recognition of the miRNA primary transcript and the generation of the first cut. In animals, the microprocessor formed by DROSHA recognizes the transition of the ssRNA region of the primary transcript to the foldback stem and produces a cut in the dsRNA region ~11 bp away of this junction in the dsRNA region [25]. While animal miRNA stem loops have stereotypical sizes, the foldbacks in plants are variable in size and shape [26]. In turn, plant miRNA precursors have different processing modes. A group of precursors, which are processed in a base-to-loop direction, harbors a lower stem of 15–17 bp below the miRNA/miRNA* duplex, which guides the precursor processing machinery [27–30] (Fig. 1a). However, another group of plant precursors is cleaved with a first cut below the terminal loop. In this case, there is a dsRNA segment of 15–17 bp above the microRNA/microRNA* guiding the DCL1 complex [30–32] (Fig. 1c). In either case, after the initial cleavage, DCL1 performs a second cut ~21 bp of the first to release the miRNA/miRNA* duplex. In certain cases the DCL1 complex can continue to perform several cuts sequentially releasing several small RNA duplexes [26, 30, 33–35] (Fig. 1b, d).
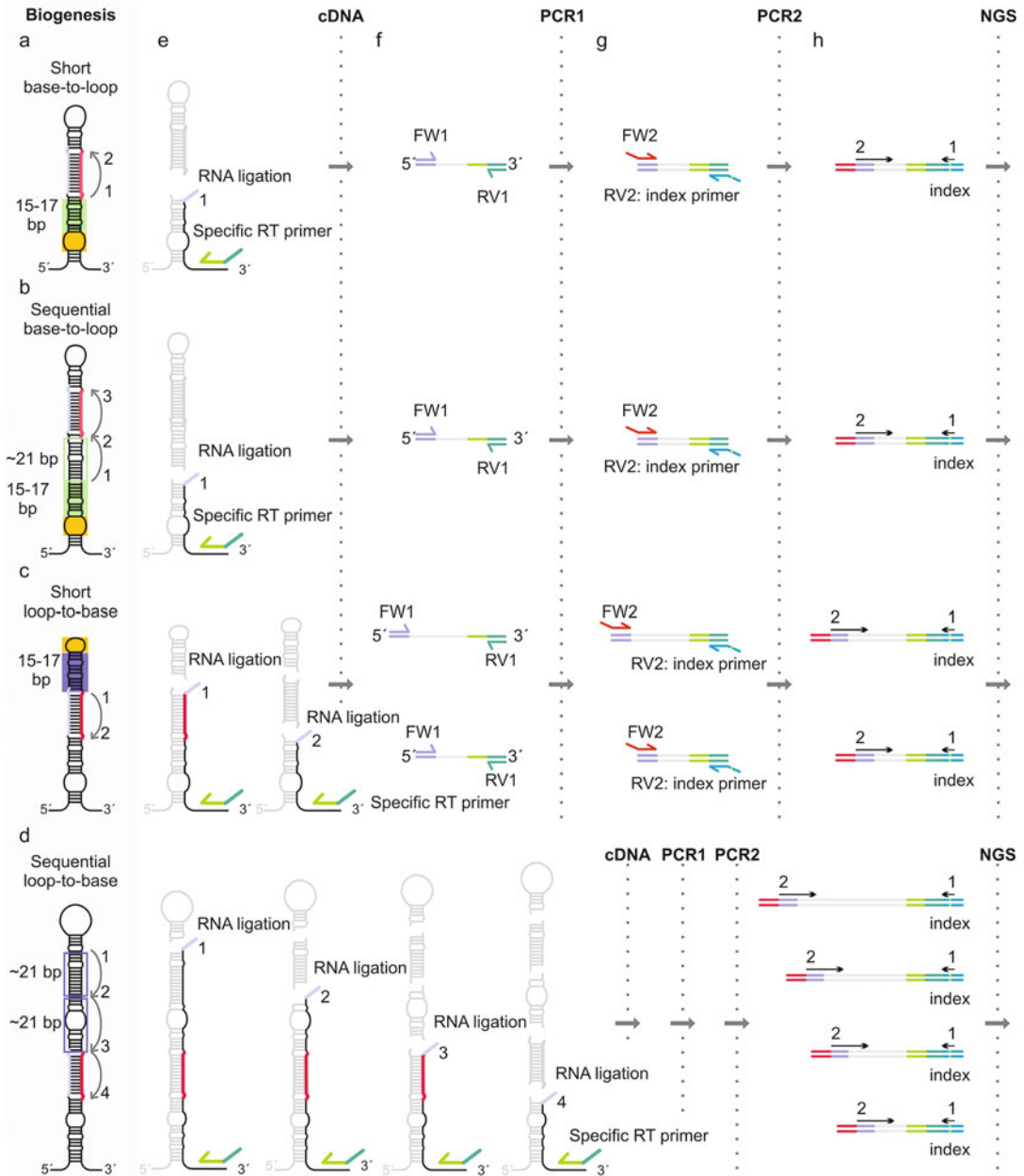
Fig. 1 Different biogenesis modes of plant miRNAs and detection of the corresponding processing inter-mediates by a modified 5′ RACE named SPARE. Processing modes of *Arabidopsis MIRNA* precursors: (**a**) short base-to-loop, (**b**) sequential base-to-loop, (**c**) short loop-to-base, and (**d**) sequential loop-to-base biogenesis. The processing determinants that guide the DCL1 complex are indicated by rectangles. The different steps of a modified 5′ RACE to detect processing intermediates are indicated by dashed lines (**e**, **h**). The 5′ end of the processing intermediate is ligated to an RNA adapter (light purple) (**e**). The cDNA is synthesized with a specific RT primer for each *MIRNA* (light green) that also harbors a common tail (dark green) (**f**). Generic primers are used to amplify the fragments (**g**). A second PCR reaction then incorporates the necessary sequences for NGS as well as a specific index to identify each library (**h**). Note that due to the relative position of the oligos, each processing mode has a different set of detectable intermediates, e.g., only the first processing intermediate is detected in the precursors processed from the base, while all processing intermediates are detected in the precursors processed from the loop

To characterize *MIRNA* processing intermediates in vivo, several approaches have been developed. A modified 5′ RACE or RNA self-ligation techniques have been used to determine the exact position of the cuts along the precursor sequence in plants [26, 30, 34, 36]. More recently, a 5′ RACE protocol was also coupled to next-generation sequencing (NGS) to detect processing intermediates for all precursors simultaneously in a method called SPARE (*S*pecific *P*arallel *A*mplification of 5′ RNA *E*nds) [30, 37]. In principle, these approaches allow to determine the exact position where the precursors are cleaved. These data are useful to characterize the different precursor processing modes in plants. However, the approaches would also allow the characterization of the miRNA biogenesis in plants growing in adverse conditions or under pathogen attack. Furthermore, analysis of processing intermediates in mutants with defects in miRNA biogenesis can provide insights into the molecular functions of these genes [38]. Here, we present an update of methods that can be used to study specific precursors on a one by one basis or coupled to methods that characterize genome-wide the biogenesis of miRNAs.

2   Materials

All reactions must be carried out under RNase-free conditions. The use of nitrile glove and filter tips is also recommended.

2.1 Plant Genotypes

Wild-type *Arabidopsis* plants (Col-0) and *FIERY1* mutants (*fiery1*; SALK_020882) were used for the construction of the SPARE libraries. Libraries can also be made from plants deficient in miRNA biogenesis such as mutants in *HYL1* (*hyl1–2*; SALK_064863) or *SE* (*se-1*; CS3257) mutants. Plants overexpressing MIRNAs, such as *35S:MIR172a* [27] or *35S:MIR166b* [31] can be used as well.

2.2 RNA Extraction

1. TRIzol™ (Thermo Fisher Scientific, 15596026).
2. Chloroform.
3. Isopropanol.
4. 70% (v/v) ethanol.

2.3 RNA Precipitation

1. Glycogen (Thermo Fisher Scientific, R0561).
2. Sodium acetate 3 M, pH 5.2.
3. Absolute ethanol, 70% ethanol.

2.4 DNase Treatment

1. DNase I (NEB M0303S).
2. 10× reaction buffer (NEB, B0303S).

3. RNaseOUT™ (Invitrogen, 10777019).

4. 0.5 M EDTA.

2.5 5′ RNA-Adapter Ligation

1. T4 RNA Ligase (Thermo Fisher Scientific, EL0021).

2. 10× reaction buffer.

3. 1 mg/mL BSA.

4. RNaseOUT™ (Invitrogen, 10777019).

5. 10 mM ATP (Thermo Fisher Scientific, PV3227).

2.6 Purification of mRNA

1. Dynabeads™ mRNA DIRECT™ Purification Kit (Thermo Fisher Scientific, 61011).

2. Magna-Sep™ Magnetic Particle Separator (Invitrogen, K1585-01) or equivalent.

2.7 Reverse Transcription

1. SuperScript™ III RT (Invitrogen, 18080093).

2. 5× first-strand buffer.

3. 0.1 M DTT.

4. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Promega, U1330).

5. RNaseOUT™ (Invitrogen, 10777019).

2.8 PCR

1. Phusion® High-Fidelity DNA Polymerase (NEB, M0530S).

2. 5× Phusion® GC Buffer Pack.

3. DMSO.

4. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Promega, U1330).

2.9 PCR Product Purification

1. UltraPure™ Agarose (Thermo Fisher Scientific, 16500100).

2. CienMarker DNA ladder (Biodynamics, B040-50).

3. 50× TAE stock solution: 2 M Tris–HCl pH = 8, 1 M acetate, and 0.05 M EDTA.

4. 6× agarose gel loading solution: 0.2% (w/v) Xylene cyanol FF, 0.2% (w/v) Bromophenol blue, 50 mM Na_2H_2EDTA (pH 8.0), and 60% (v/v) glycerol.

5. Wizard® SV Gel and PCR Clean-Up System (Promega, A9281).

6. Heat block.

2.10 Oligonucleotides

1. 5′ RNA adapter.

   RNA adapter: 5′-GUUCAGAGUUCUACAGUCCGAC-3′.

2. Precursor-specific RT primer.

   An oligonucleotide was designed to match each *MIRNA* of *Arabidopsis thaliana*. *See* Table 1 for a list of the oligos used.

Table 1
Primer sequences for SPARE and RNA self-ligation approaches

A. SPARE oligonucleotides		
Number	*MIRNA*	Sequence
1	*MIR156a*	ccttggcacccgagaattccaTTAGATCGTATCTTCTTACC
2	*MIR156b*	ccttggcacccgagaattccaACAGACGAGATGATAAGAAG
3	*MIR156c*	ccttggcacccgagaattccaTGAGAGATGAAGAACACATG
4	*MIR156d*	ccttggcacccgagaattccaTAGATGCAACATATGTATGC
5	*MIR156e*	ccttggcacccgagaattccaCTTCGACCTACTTTGATCCG
6	*MIR156f*	ccttggcacccgagaattccaAATATGCTGATTCATGTTTG
7	*MIR156g*	ccttggcacccgagaattccaTCTAACCATACACAGAGACG
8	*MIR156h*	ccttggcacccgagaattccaACGTACCTTACTTGATAGTG
9	*MIR157a*	ccttggcacccgagaattccaTTCATAACTGTTTCAATCAC
10	*MIR157b*	ccttggcacccgagaattccaATCTGCATTCTGATAGTTGC
11	*MIR157c*	ccttggcacccgagaattccaCACCCATGTTAGTATTACGC
12	*MIR157d*	ccttggcacccgagaattccaCACTTTTCTCACACCAAAAC
13	*MIR158a*	ccttggcacccgagaattccaTCTAGTTTTGAGCGAGATCC
14	*MIR158b*	ccttggcacccgagaattccaAACAAGCAGTCAGTGAAATC
15	*MIR159a*	ccttggcacccgagaattccaTCAAATTATAGCGAATAATC
16	*MIR159b*	ccttggcacccgagaattccaCTACTCAAGATCCATCATCC
17	*MIR159c*	ccttggcacccgagaattccaTGCAAATAAACATGACAACC
18	*MIR160a*	ccttggcacccgagaattccaCTACACATGATGAGGCAATG
19	*MIR160b*	ccttggcacccgagaattccaGTTATAGACAATTAGACATC
20	*MIiR160c*	ccttggcacccgagaattccaCTGTTTGCTTATTCAAATGG
21	*MIR161*	ccttggcacccgagaattccaCTAATTAAATCAAATCGATC
22	*MIR162a*	ccttggcacccgagaattccaAGTAATCGGACTTGACTCTG
23	*MIR162b*	ccttggcacccgagaattccaGCTAAAAGATGAATACTTTG
24	*MIR163*	ccttggcacccgagaattccaGGCATGAATTTAATTACATG
25	*MIR164a*	ccttggcacccgagaattccaGTCGAACACAAATGATTTAAC
26	*MIR164b*	ccttggcacccgagaattccaCGATCTAGGCTAGCTCGTAC
27	*MIR164c*	ccttggcacccgagaattccaCAATGTTAACTTCATGTCTC
28	*MIR165a*	ccttggcacccgagaattccaAAGCCATGCAAGAAAGATTC
29	*MIR165b*	ccttggcacccgagaattccaAGACGCCAATGGTAGTTACC
30	*MIR166a*	ccttggcacccgagaattccaATGAATCTGAGAAAGTAAGG

(continued)

Table 1
(continued)

31	*MIR166b*	ccttggcacccgagaattccaTATATCACATGGATTCATAG
32	*MIR166c*	ccttggcacccgagaattccaATTAATCTAATAACAAGATC
33	*MIR166d*	ccttggcacccgagaattccaGCTCTCACTTCAGGATCTAC
34	*MIR166e*	ccttggcacccgagaattccaGAAATTGAAGTTGCTTGAAC
35	*MIR166f*	ccttggcacccgagaattccaTACATTGCTGCGGATTGATG
36	*MIR166g*	ccttggcacccgagaattccaACATGGTTATACTCTAGATG
37	*MIR167a*	ccttggcacccgagaattccaAGAAAGAGAAGTAAGCTCAC
38	*MIR167b*	ccttggcacccgagaattccaTGGAGAGTGTGTCAAAGCAG
39	*MIR167c*	ccttggcacccgagaattccaAATATAATTAATCTCTGCTG
40	*MIR167d*	ccttggcacccgagaattccaCTTTCTCATGAAATGAAGTG
41	*MIR168a*	ccttggcacccgagaattccaAAACAATTTCAGATTCAAAG
42	*MIR168b*	ccttggcacccgagaattccaAACCCAATACCGAATCAATC
43	*MIR169a*	ccttggcacccgagaattccaCTTTCTGCATTGTTCCTTAG
44	*MIR169b*	ccttggcacccgagaattccaAAATACTCATACGGTCGATG
45	*MIR169c*	ccttggcacccgagaattccaCTCATTATATTAGACCATCC
46	*MIR169d*	ccttggcacccgagaattccaTATTAGCATTAGCATTCACC
47	*MIR169e*	ccttggcacccgagaattccaTATATACATTTCAACGATAC
48	*MIR169f*	ccttggcacccgagaattccaTTGAGACAAATTAAACATCG
49	*MIR169g*	ccttggcacccgagaattccaAAATCTGATCATTCAAATCG
50	*MIR169h*	ccttggcacccgagaattccaCATTGACAAAGTCCACTATG
51	*MIR169i*	ccttggcacccgagaattccaGCTCAAAGTCATCAACATTG
52	*MIR169j*	ccttggcacccgagaattccaATGCTTTCTAAATCGAATGC
53	*MIR169k*	ccttggcacccgagaattccaATCGTCAACATTCGCTCACC
54	*MIR169l*	ccttggcacccgagaattccaTCTAGTGATTCGGAAGACAG
55	*MIR169m*	ccttggcacccgagaattccaTCGAAATCATGAACATTATC
56	*MIR169n*	ccttggcacccgagaattccaACCAACTGCGAAAATTTGAC
57	*MIR170*	ccttggcacccgagaattccaATTGAGTGATCGATGAGTAC
58	*MIR171a*	ccttggcacccgagaattccaCTATAGGTAAACAATATAAC
59	*MIR171b*	ccttggcacccgagaattccaGAAATATCAAAGCCATTAATC
60	*MIR171c*	ccttggcacccgagaattccaTTGATAATACCTCATCTCTG
61	*MIR172a*	ccttggcacccgagaattccaGATATGTTAACATAAAGGTG
62	*MIR172b*	ccttggcacccgagaattccaATATGTAAACATGTTCAAAC

(continued)

Table 1
(continued)

63	*MIR172c*	ccttggcacccgagaattccaTACCTCCGATCTGTGAATTC
64	*MIR172d*	ccttggcacccgagaattccaAAGTTTCACCTCAAGTTATC
65	*MIR172e*	ccttggcacccgagaattccaGTGCATGATCAAGATATTGC
66	*MIR173*	ccttggcacccgagaattccaACCCTAATGAGATACTTTCC
67	*MIR319a*	ccttggcacccgagaattccaCAAAATGTTAATTTTACCAG
68	*MIR319b*	ccttggcacccgagaattccaACTTATTTATATTCATATCG
69	*MIR319c*	ccttggcacccgagaattccaTCCAGTTTCAGTTCAATTCG
70	*MIR390a*	ccttggcacccgagaattccaAAGATAGCTTAAATGGACAG
71	*MIR390b*	ccttggcacccgagaattccaGATTTGAACTTCAACAATTC
72	*MIR391*	ccttggcacccgagaattccaTTATGGTGTTACTATGTAAG
73	*MIR393a*	ccttggcacccgagaattccaCTGTTGTAGGCTTGAGATAC
74	*MIR393b*	ccttggcacccgagaattccaCTTGTTGATATGACTGGATC
75	*MIR394a*	ccttggcacccgagaattccaATTACCCTAGATCGAGGCTC
76	*MIR394b*	ccttggcacccgagaattccaGATAATACCTAGTTTTCTTC
77	*MIR395a*	ccttggcacccgagaattccaTTTATATCTTTAAGCCATTC
78	*MIR395b*	ccttggcacccgagaattccaATTAGCTAGTGTCATCATTG
79	*MIR395c*	ccttggcacccgagaattccaGTCCACACCATGAATCCATG
80	*MIR395d*	ccttggcacccgagaattccaTCACTCATTTTTGTGGATCG
81	*MIR395e*	ccttggcacccgagaattccaTTTTTGTGGATCGTTTAATC
82	*MIR395f*	ccttggcacccgagaattccaTCACTCATGAATGATAGATC
83	*MIR396a*	ccttggcacccgagaattccaCTACAATATAGTTGGTAGTC
84	*MIR396b*	ccttggcacccgagaattccaTCCTGTGTCTTCAATTTAGG
85	*MIR824*	ccttggcacccgagaattccaCAACAAAGTCACTGCATTAC
86	*MIR397a*	ccttggcacccgagaattccaGCCCTAAATAATATCTGATG
87	*MIR397b*	ccttggcacccgagaattccaAGAAACTAAATGTTGGAGTC
88	*MIR398a*	ccttggcacccgagaattccaAGATACAAAATAGAGGTTCC
89	*MIR398b*	ccttggcacccgagaattccaTACTACTGTGATTTCATCTG
90	*MIR398c*	ccttggcacccgagaattccaAGCCACGGGCCACGGCGTTG
91	*MIR399a*	ccttggcacccgagaattccaAGGACTTGAACATCGTCATC
92	*MIR399b*	ccttggcacccgagaattccaCAGTCTGTTCTATTCGGTCG
93	*MIR399c*	ccttggcacccgagaattccaAACCGCACTAGTTTTGTAGC
94	*MIR399d*	ccttggcacccgagaattccaAGATTCCAAGATTGATCTAG
95	*MIR399e*	ccttggcacccgagaattccaTTAATTTGAAGAGGCTCTAG

Table 1
(continued)

96	*MIR399f*	ccttggcacccgagaattccaGTTAGAACTTAGAATCGTCG
97	*MIR400*	ccttggcacccgagaattccaCTCTACCTTACCATAATCAC
98	*MIR402*	ccttggcacccgagaattccaGACTCTTTTCATGTGTATTC
99	*MIR403*	ccttggcacccgagaattccaTAGATCTTGTTTACGATTCC
100	*MIR408*	ccttggcacccgagaattccaTGAATGACAGAGAGGTAGAC
101	*MIR447a*	ccttggcacccgagaattccaATCTATGATATCGATGCAAC
102	*MIR447b*	ccttggcacccgagaattccaTGAATCTATGATATCGATGC
103	*MIR472*	ccttggcacccgagaattccaACTGAAAGTCTAGCGACTAG
104	*MIR771*	ccttggcacccgagaattccaTATCTTGACCATGGAGACAG
105	*MIR779*	ccttggcacccgagaattccaTCTCATCTCGAACGGAATGC
106	*MIR825*	ccttggcacccgagaattccaAATCCATATAGTTCTCTAGC
107	*MIR827*	ccttggcacccgagaattccaTTCGATTTGCCAGGTGATGC
108	*MIR862*	ccttggcacccgagaattccaCTGAACCGAGTGTATATGAG
109	*MIR864*	ccttggcacccgagaattccaCGCTGCTGACTTCAATATAC
110	*MIR2111a*	ccttggcacccgagaattccaGATATTCAGTCTTAAATATC
111	*MIR2111b*	ccttggcacccgagaattccaCCACTTCGAATGACTAGACC
B. RNA self-ligation oligonucleotides		
Code	***MIRNA***	**Sequence**
RT/FW	*MIR172a*	TGAATCACCACCGTCCATCAAC
RV	*MIR172a*	CTCTCCACAAAGTTCTCTATG
RT	*MIR166b*	CATCAGATCTGAATGTATTC
FW	*MIR166b*	AAATGAGATTGTATTAGAATAAGA
RT	*MIR166b*	ATTGATTAGGGTTTTAGTGT

The specific sequence for each *Arabidopsis MIRNA* is shown in upper case, and a common tail is in lower case

The primers were designed to hybridize ~100 nt downstream the miRNA/miRNA* region. The exact position was then adjusted to avoid repeats or low GC content.

3. Generic PCR1 primers.

   FW1: 5′-GTTCAGAGTTCTACAGTCCGA-3′ (contained in the FW2 primer).

RV1: 5′-TGGAATTCTCGGGTGCCAAGG-3′ (contained in the indexed primer, RV2).

4. PCR2 primers.
FW2: 5′-AATGATACGGCGACCACCGACAG<u>GTTCAGAG TTCTACAGTCCGA</u>C-3′. The underlined region matches the sequence with the primer FW1.
RV2 (index primer):
5′-<u>TGGAATTCTCGGGTGCCAAGG</u>AACTCCAGT-CACNNNNNNATCTCGTATGCCGTCTTCTGCTTG-3′.
The NNNNNN region is a variable portion of the indexed primers used to identify different libraries that will be sequenced together. The underlined region coincides in sequence with the primers RV1. Illumina indexed primers are part of the Illumina RS-200-0012 TruSeq® Small RNA Sample Prep Kit.

2.11 RNA Self-Ligation

Both plant genotypes and RNA extraction are as described in Sub-headings 2.1 and 2.2.
RNA self-ligation

1. T4 RNA ligase (10 U/μL) (Thermo Scientific, EL0021).
2. 10× buffer T4 RNA ligase ((500 mM Tris–HCl [pH 7.5 at 25 °C), 100 mM MgCl$_2$, 100 mM DTT, and 10 mM ATP).
3. 1 mg/mL BSA.
4. RNaseOUT™ (40 U/μL) (Invitrogen, 10777019).
5. 10 mM ATP (Thermo Fisher Scientific, PV3227).

RNA precipitation. As described in Subheading 2.3.
DNase treatment. As described in Subheading 2.4.
Reverse transcription

1. MMLV (200 U/μL) (Invitrogen, 28025013).
2. 5× first-strand buffer (250 mM Tris–HCl [pH 8.3 at room temperature], 375 mM KCl, 15 mM MgCl$_2$).
3. 0.1 M DTT.
4. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Promega, U1330).
5. RNaseOUT™ (40 U/μL) (Invitrogen, 10777019).

First PCR

1. Platinum™ SuperFi™ DNA Polymerase (Invitrogen, 12351250).
2. 5× SuperFi™ Buffer (includes 7.5 mM MgCl$_2$).

3. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Promega, U1330).

Second PCR

1. PFU DNA polymerase (5 U/μL) (GBT, PE2010).
2. 10× PFU DNA polymerase buffer (100 mM KCl, 160 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 200 mM Tris–HCl [pH 8.8], 1% Triton X-100, 1 mg/mL BSA).
3. dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP (Promega, U1330).

3 Methods

Subheadings 3.1 to 3.12 describe the SPARE method to detect processing intermediates in different *MIRNA* precursors, which is based on a modification of the 5′ RACE methods coupled to NGS. Subheading 3.13 describes how to detect processing intermediates by RNA self-ligation.

3.1 Plant Growth Conditions

1. Grow wild-type and mutant plants on agar plates with Murashige and Skoog [39] with continuous light at 100 mmol photons $m^{-2} s^{-1}$ and 22 °C as described [31] for 10 days.
2. Harvest forty 10-day old seedlings per sample and freeze them in liquid nitrogen (*see* **Notes 1** and **2**).

3.2 Total RNA Extraction

1. Reduce the material to a fine powder using a pylon.
2. Add 4 mL of TRIzol for a maximum of 400 mg of tissue and freeze overnight at −80 °C.
3. The next day, centrifuge the homogenate at 12,000 × *g* at 4 °C for 15 min, and transfer the supernatant (~3.6 mL) to a new tube.
4. Add 800 μL of chloroform to each tube (~0.2 volumes), and invert vigorously the mixture for 15 s.
5. Centrifuge at 12,000 × *g* at 4 °C for 15 min, and then transfer the upper phase (~1.8 mL) to a new tube.
6. Precipitate the RNA by adding one volume of isopropanol, and incubate overnight at −20 °C.
7. The next day centrifuge at 12,000 × *g* at 4 °C for 20 min.
8. Discard the supernatant, and wash the RNA pellet by adding 4 mL of 70% (v/v) cold ethanol and vortexing.
9. Centrifuge at 7500 × *g* at 4 °C for 5 min and discard the supernatant.

10. Repeat the washing step one more time.

11. Dry the precipitated RNA in an oven at 37 °C for approximately 5 min (avoid over drying of RNA).

12. Resuspend the pellet in 100 μL RNase-free water.

3.2.1 Quantification of Purified RNA

1. Spectrophotometrically quantify the RNA by measuring the absorbance (Abs) at 260. Also measure the Abs at 230 and 280 nm.

2. Estimate the purity of the preparation from the ratio of the Abs measurements to Abs260/Abs230 and Abs260/Abs280.

3. Acceptable samples must have values of at least 1.6 and 1.8, respectively; if the quality is lower, discard the RNA (*see* **Note 1**).

3.2.2 Checking the Integrity of the Purified RNA

1. Run a 1.5% (w/v) agarose gel of 5 μL of the total RNA to check the integrity of the purified RNA.

2. Stain the gel with ethidium bromide. The ribosomal RNAs should be visualized as sharp bands.

3.3 DNase Treatment

We usually use 40 μg total RNA (*see* **Note 3**).

1. Prepare the following reaction:

RNA	40 μg of RNA in 87 μL
Buffer DNase I	10 μL
RNaseOUT	2 μL
DNase I	1 μL
Final volume	100 μL

2. Incubate 30 min at 37 °C.

3. Add 1 μL of 0.5 M EDTA.

4. Heat inactivate at 75 °C for 10 min.

3.4 RNA Precipitation

1. After the DNase treatment, add 1 μL glycogen, 10 μL sodium acetate, and 220 μL absolute ethanol.

2. Incubate overnight at −20 °C.

3. Centrifuge 30 min at 4 °C at maximum speed.

4. Discard supernatant.

5. Wash with 70% ethanol.

6. Centrifuge 5 min at 4 °C at maximum speed.

7. Discard supernatant and repeat **step 5** once.

8. Dry 7 min at 37 °C.

9. Resuspend in 9 μL RNase-free water.

3.5 RNA Ligation

1. Add 1 µL RNA oligo (200 µM) to each sample of 9 µL of RNA.
2. Incubate for 5 min at 65 °C.
3. Put on ice about 2 min.
4. Add to the reaction mixture.

10× ligase buffer	2 µL
10 mM ATP	2 µL
BSA	2 µL
RNaseOUT	2 µL
T4 RNA ligase	2 µL
Final volume	20 µL

5. Incubate at 37 °C for 1 h.
6. Spin down and put on ice.

7. Inactivate the ligase by heating at 72 °C for 15 min.

3.6 Purification of mRNA with Dynabeads

1. Follow the kit instructions.
2. Resuspend in 32 µL water.

3.7 Reverse Transcription

We performed eight retro-transcription reactions for each library (Fig. 2). In each tube processing intermediates corresponding to ~14 *MIR NAs* are retro-transcribed. *See* Table 1 for the complete list of primers used.

1. Use 3.9 µL of the 32 µL purified mRNA-ligated, and prepare eight PCR tubes. For each tube prepare the following mix:

10 mM dNTP mix	1 µL
Specific primer mix (1–8)	0.5 µL

S*ee* **Note 4** for details on how to prepare the primer mix.

2. Heat at 65 °C for 5 min.
3. Incubate on ice for at least 1 min.
4. Spin down.
5. Add:

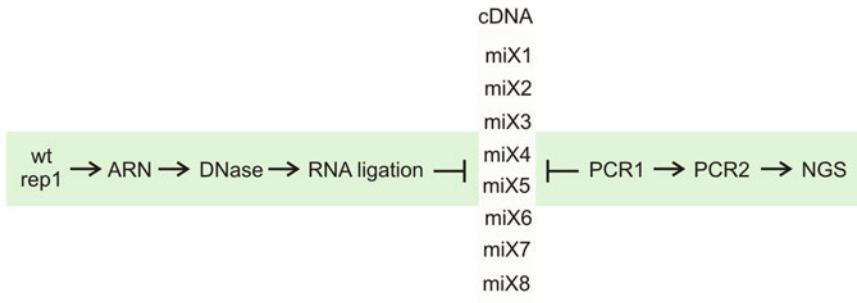|	
5× first-strand buffer	4 µL
0.1 M DTT	1 µL
RNaseOUT	1 µL
SuperScript III	0.5 µL
Total volume	20 µL

Fig. 2 Workflow for library construction. For each library one tube containing the total RNA is subject to the DNase treatment followed by the RNA ligation reaction. After that, the total volume is divided into eight tubes for the cDNA synthesis. For the PCR1 reaction, an equal volume from each cDNA mix is pooled together. Following the second PCR, the library is sequenced by NGS

6. Incubate 60 min at 50 °C.

7. Spin down.

8. Inactivate the reaction by heating at 70 °C for 15 min.

3.8 PCR1   The objective of the first PCR is to enrich the processing intermediates. Primers FW1 and RV1 are used. 5 μL of each individual mix of the cDNAs is taken, to give total 40 μL. 20 μL are used as a template for PCR1, and the remnant is reserved in case the PCR1 has to be repeated.

1. Prepare the following master mix:

5× GC buffer	10 μL
dNTPs	1 μL
Primer FW1	1.5 μL
Primer RV1	1.5 μL
DMSO	1.5 μL
Phusion	0.5 μL
H$_2$O	14 μL
Final volume	50 μL

2. PCR program:

98 °C 1 min	
98 °C 30 s	
63 °C 30 s	×20 cycles
72 °C 50 s	
72 °C 10 min	

3. Add loading solution to the PCR and run a 1.5% (w/v) agarose gel.

4. Cut the regions between 100 and 700 bp. The amount of DNA is insufficient to be visualized by staining with ethidium bromide.

3.9   DNA Purification with the Wizard SV Gel and PCR Clean-Up System Kit

1. Follow the manufacturer's instructions.

2. Resuspend the purified libraries in 35 μL of ultrapure water (*see* **Note 5**).

3.10   PCR2

The function of the second PCR is to incorporate an indexed to the product of PCR1. In PCR2, primers FW2 and RV2 are used, and both of them are nested to primers FW1 and RV1. Each library must have a different indexed primer (RV2, 0.75 μL). 10 μL of the purified PCR1 was used as template.

1. Prepare the following master mix:

5× GC buffer	10 μL
dNTPs	1 μL
Primer FW2	0.75 μL
DMSO	1.5 μL
Phusion	0.5 μL
H_2O	26.25 μL
Final volume	50 μL

2. PCR program:

98 °C for 1 min	
98 °C for 30 s	
63 °C for 30 s	×10 cycles
72 °C for 50 s	
72 °C for 10 min	

3. Add loading solution to the PCR2 products and run a 2% (w/v) agarose gels. The total volume of the reaction should be divided into two wells ~30 μL each. Figure 3 shows the results obtained for wild-type and *se* libraries.
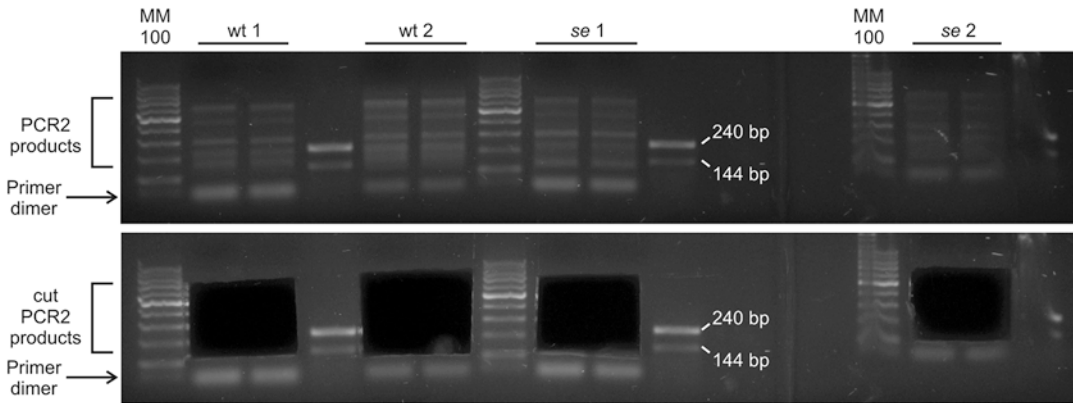
4. Cut the region from 150 to 800 bp.

Fig. 3 Separation of the processing intermediates by agarose gels. The top gel shows the results of PCR2 for wild-type and *se* duplicate samples. The gel electrophoresis was performed for 30 min, with a molecular weight marker of 100 bp and specific markers of 144 and 240 bp. The names on the lanes refer to the libraries amplified during the PCR2. The bottom gel shows the region cut between ~150 and 800 bp

3.11 DNA Purification with the Wizard SV Gel and PCR Clean-Up System Kit

1. Follow the manufacturer's instructions.
2. Resuspend the purified libraries in 22 μL of ultrapure water (*see* **Note 5**).
3. Run a 10 μL of the purified libraries in a 1.5% (w/v) agarose gel to check the efficiency of the purification (Fig. 4).

3.12 Library Quantification

1. Quantify 2 μL of the libraries by measuring absorbance at 260 nm using a NanoDrop spectrophotometer. It should be expected a DNA concentration of at least 5 ng/μL. The samples are then ready for sequencing.

3.13 Identification of Processing Intermediates Through RNA Self-Ligation

Due to the relative position of the oligos, the method described before identifies only one end of the RNA fragment and for base-to-loop precursors only detects the intermediary generated by the first cut by DCL1. A different RNA ligation approach, such as circular RT-PCR assay, can be used as a complement to the prior method (Fig. 5c–f) [40, 41]. The circular RT-PCR assay makes it possible to identify both ends of an RNA fragment, as well as both processing intermediates of a base-to-loop processed precursor (Fig. 5a–c) [13, 30]. In principle, wild-type plants or mutants in the miRNA biogenesis machinery such as *hyl1* or *se* can be analyzed by this method. It is also possible to analyze plants overexpressing *MIRNA* precursors, such as *MIR172a* or *MIR166b* [27, 31]. In the latter case, the effect of specific mutations in the precursor sequence can be analyzed.

To perform the circular RT-PCR assays, total RNA is obtained as described in Subheadings 3.1–3.4. Afterward, an RNA ligation is performed between the capless 5′ end and the 3′ end of the RNA
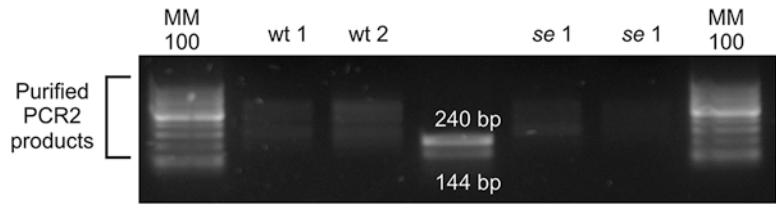
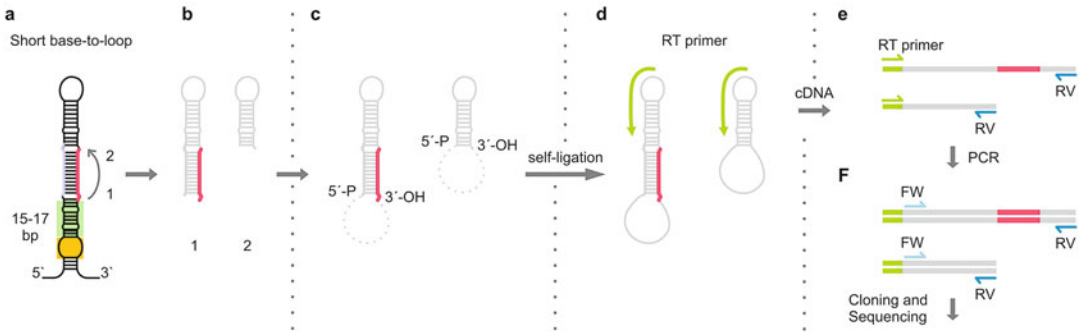**Fig. 4** Detection of processing intermediates after PCR2 purification

Fig. 5 Determination of intermediates using a circular RT-PCR assay. (**a**, **b**) Processing intermediates of a precursor processed in a base-to-loop mode. (**c**, **d**) The ends of the processing intermediates are a capless 5′ end (shown as 5′-P) and a 3′ end (shown as 3′-OH). Self-ligation of these ends yields a circular RNA molecule. (**e**) A specific oligo is used for reverse transcription of these RNA molecules (green arrows). (**f**) Then, two PCR reactions are performed followed by analysis

(Fig. 5c, d). Note that unprocessed RNAs will retain their 5′ end cap and will not be subjected to RNA ligation.

1. Add to the reaction mixture:

Total RNA	4 μg in 10 μL
10× ligase buffer	10 μL
10 mM ATP	10 μL
BSA	10 μL
RNaseOUT	2 μL
T4 RNA ligase	2 μL
H_2O	56 μL
Final volume	100 μL

2. Incubate overnight at 16 °C.

3. Precipitate RNA by adding 2 μL of 20 mg/mL glycogen, 10 μL of 3 M sodium acetate pH 5.2, and 332 μL of absolute ethanol as described in Subheading 3.4, and resuspend in 12 μL of RNase-free water. You may check the RNA amount through

quantification as described in Subheading 3.2.1. Make a 1:5 dilution of the resuspended ligated RNA prior to the DNase treatment.

4. For the DNase treatment, prepare the following reaction for each sample:

10× DNase buffer	2 μL
DNase (1 U/μL)	1 μL
Ligated RNA	5 μL
H$_2$O	12 μL
Total volume	20 μL

5. Incubate 30 min at 37 °C.
6. Add 1 μL of DNase stop solution and inactivate incubating 10 min at 65 °C.

   The cDNA synthesis has to be performed with a specific primer (0.5 μg/μL) complementary to the terminal region of the precursor analyzed (RT primer) (Fig. 5d) (*see* Table 1). This reaction was performed similar to the one described in Subheading 3.7, but MMLV was used as the reverse transcriptase (*see* **Note 6**).

7. For the reaction prepare the following mix:

Specific primer (0.5 μg/μL)	0.5 μL
10 mM dNTP mix	1 μL
RNA treated with DNase	4.5 μL
H$_2$O	6 μL

8. Heat at 65 °C for 5 min.
9. Incubate on ice for at least 1 min.
10. Spin down.
11. Add:

5× first-strand buffer	4 μL
0.1 M DTT	2 μL
RNaseOUT	1 μL
MMLV	1 μL
Total volume	20 μL

12. Incubate 50 min at 37 °C in a heat block.

13. Spin down.

14. Inactivate the reaction by heating at 70 °C for 15 min.

    Once the cDNA is synthesized, it is amplified through hemi-nested PCR (Fig. 5f). The first PCR can be performed using RT primer and a reverse primer (RV) complementary to the 3′ arm of the precursor above the last cut. After that use the product of PCR1 as template for a second PCR, with the same RV primer, but using a new forward primer (FW), nearest to the last cut in the 5′ arm. Depending on the length of the loop region of the precursor, a different set of oligonucleotides can be designed (*see* **Note** 7). The PCR products can be analyzed by gel electrophoresis and cloned for sequencing.

15. Prepare the following mix:

5× Platinum SuperFi buffer	5.0 μL
10 mM dNTPs	0.5 μL
10 μM RT primer	1.25 μL
10 μM RV primer	1.25 μL
cDNA (1:10 or 1:100 dilution)	2 μL
Platinum SuperFi (2 U/μL)	0.25 μL
H_2O	14.75 μL
Total volume	25 μL

16. PCR program:

98 °C for 30 s
98 °C for 10 s
At least 45 °C for 10 s ×30 cycles (*see* **Note 8**)
72 °C for 15 s
72 °C for 5 min

    Keep 20 μL of this first PCR for further analysis on agarose gel electrophoresis and 5 μL to prepare a dilution (1:10) to be used as a template for the second PCR.

17. Prepare the following mix:

10× PFU buffer	5.0 μL
10 mM dNTPs	1.0 μL
10 μM Fw primer	2.0 μL

(continued)

10 μM Rv primer	2.0 μL
PFU (5 U/μL)	0.5 μL
(1:10 dilution) first PCR	1.0 μL
H_2O	38.5 μL
Total volume	50 μL

18. PCR program:

95 °C for 1 min	
94 °C for 15 s	
40 °C for 30 s	×30 cycles
72 °C for 40 s	
72 °C for 5 min	

19. Samples are ready to analyze. Run 1.5% (w/v) agarose gel to visualize the PCR products, isolate the bands of interest, and clone and sequence if necessary.

4 Notes

1. The protocol presented here is designed to prepare duplicates of each genotype; however, additional independent samples can also be collected.

2. Alternatively, plant material can be collected in several Eppendorf tubes (ten seedlings per tube). Also inflorescences or a mixture of tissues can be collected.

3. Total RNA can be adjusted depending on the analysis. Less material should be required to analyze abundant miRNAs.

4. The primer stocks are 100 μM, and in each cDNA synthesis reaction, ~14 precursors are retro-transcribed in eight mixes. To prepare each primer mix, 1 μL of each primer stock, e.g., 1–14, are placed in an Eppendorf and completed with enough RNase-free water to a volume of 20 μL. From this 1:20 dilution, take 10 μL and bring it to a final volume of 100 μL, to obtain a 1:200 dilution in another tube. This procedure is repeated to obtain a 1:2000 dilution, from which 0.5 μL is used for the cDNA synthesis. The starting concentration of each primer will be 0.05 μM.

5. Take into consideration that the agarose section can be quite large. A typical purification column can bind a maximum of 350 mg of DNA at a time.

6. Other reverse transcriptase enzymes, such as SuperScript III, are not recommended due to their higher reaction temperature and also because of their strand displacement activity. This activity can lead to the over-enrichment of smaller intermediates in detriment of bigger RNA molecules.

7. The size of the loop region can be variable in plant *MIRNA* precursors. In certain cases three primers can be accommodated in this region (one for the RT reaction and two for the PCR). However, in other cases the RT and first primer might overlap.

8. Platinum™ SuperFi™ DNA polymerase enzyme has its own annealing temperature calculator: https://www.thermofisher.com/ar/es/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html.

Acknowledgments

References

1. Bologna NG, Voinnet O (2014) The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis. Annu Rev Plant Biol 65:473–503

2. Ha M, Kim VN (2014) Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15:509–524

3. Axtell MJ (2008) Evolution of microRNAs and their targets: are all microRNAs biologically relevant? Biochim Biophys Acta 1779:725–734

4. Cuperus JT, Fahlgren N, Carrington JC (2011) Evolution and functional diversification of MIRNA genes. Plant Cell 23:431–442

5. Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136:669–687

6. Rodriguez RE, Schommer C, Palatnik JF (2016) Control of cell proliferation by microRNAs in plants. Curr Opin Plant Biol 34:68–76

7. Rogers K, Chen X (2013) Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell 25:2383–2399

8. Xie Z, Allen E, Fahlgren N, Calamar A, Givan SA, Carrington JC (2005) Expression of Arabidopsis MIRNA genes. Plant Physiol 138:2145–2154

9. Meyers BC, Axtell MJ, Bartel B, Bartel DP, Baulcombe D, Bowman JL, Cao X, Carrington JC, Chen X, Green PJ, Griffiths-Jones S, Jacobsen SE, Mallory AC, Martienssen RA, Poethig RS, Qi Y, Vaucheret H, Voinnet O, Watanabe Y, Weigel D, Zhu JK (2008) Criteria for annotation of plant microRNAs. Plant Cell 20:3186–3190

10. Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21:4663–4670

11. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN (2004) The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18:3016–3027

12. Auyeung Vincent C, Ulitsky I, McGeary Sean E, Bartel David P (2013) Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing. Cell 152:844–858

13. Han MH, Goud S, Song L, Fedoroff N (2004) The Arabidopsis double-stranded

RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. Proc Natl Acad Sci U S A 101:1093–1098

14. Vazquez F, Gasciolli V, Crete P, Vaucheret H (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. Curr Biol 14:346–351

15. Lobbes D, Rallapalli G, Schmidt DD, Martin C, Clarke J (2006) SERRATE: a new player on the plant microRNA scene. EMBO Rep 7:1052–1058

16. Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis. Plant J 47:841–850

17. Gregory BD, O'Malley RC, Lister R, Urich MA, Tonti-Filippini J, Chen H, Millar AH, Ecker JR (2008) A link between RNA metabolism and silencing affecting Arabidopsis development. Dev Cell 14:854–866

18. Laubinger S, Sachsenberg T, Zeller G, Busch W, Lohmann JU, Ratsch G, Weigel D (2008) Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in Arabidopsis thaliana. Proc Natl Acad Sci U S A 105:8795–8800

19. Kim S, Yang J-Y, Xu J, Jang I-C, Prigge MJ, Chua N-H (2008) Two cap-binding proteins CBP20 and CBP80 are involved in processing primary MicroRNAs. Plant Cell Physiol 49:1634–1644

20. Ren G, Xie M, Dou Y, Zhang S, Zhang C, Yu B (2012) Regulation of miRNA abundance by RNA binding protein TOUGH in Arabidopsis. Proc Natl Acad Sci U S A 109:12817–12821

21. Yu B, Bi L, Zheng B, Ji L, Chevalier D, Agarwal M, Ramachandran V, Li W, Lagrange T, Walker JC, Chen X (2008) The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. Proc Natl Acad Sci U S A 105:10073–10078

22. Zhang Z, Guo X, Ge C, Ma Z, Jiang M, Li T, Koiwa H, Yang SW, Zhang X (2017) KETCH1 imports HYL1 to nucleus for miRNA biogenesis in Arabidopsis. Proc Natl Acad Sci U S A 114:4011–4016

23. Manavella PA, Hagmann J, Ott F, Laubinger S, Franz M, Macek B, Weigel D (2012) Fast-forward genetics identifies plant CPL phosphatases as regulators of miRNA processing factor HYL1. Cell 151:859–870

24. Axtell MJ, Westholm JO, Lai EC (2011) Vive la difference: biogenesis and evolution of

microRNAs in plants and animals. Genome Biol 12:221

25. Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN (2006) Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125:887–901

26. Bologna NG, Mateos JL, Bresso EG, Palatnik JF (2009) A loop-to-base processing mechanism underlies the biogenesis of plant microRNAs miR319 and miR159. EMBO J 28:3646–3656

27. Mateos JL, Bologna NG, Chorostecki U, Palatnik JF (2010) Identification of microRNA processing determinants by random mutagenesis of Arabidopsis MIR172a precursor. Curr Biol 20:49–54

28. Song L, Axtell MJ, Fedoroff NV (2010) RNA secondary structural determinants of miRNA precursor processing in Arabidopsis. Curr Biol 20:37–41

29. Werner S, Wollmann H, Schneeberger K, Weigel D (2010) Structure determinants for accurate processing of miR172a in Arabidopsis thaliana. Curr Biol 20:42–48

30. Bologna NG, Schapire AL, Zhai J, Chorostecki U, Boisbouvier J, Meyers BC, Palatnik JF (2013) Multiple RNA recognition patterns during microRNA biogenesis in plants. Genome Res 23:1675–1689

31. Chorostecki U, Moro B, Rojas ALM, Debernardi JM, Schapire AL, Notredame C, Palatnik J (2017) Evolutionary footprints reveal insights into plant microRNA biogenesis. Plant Cell 29:1248–1261

32. Kim W, Kim H-E, Jun AR, Jung MG, Jin S, Lee J-H, Ahn JH (2016) Structural determinants of miR156a precursor processing in temperature-responsive flowering in Arabidopsis. J Exp Bot 67:4659–4670

33. Kurihara Y, Watanabe Y (2004) Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proc Natl Acad Sci U S A 101:12753–12758

34. Addo-Quaye C, Snyder JA, Park YB, Li YF, Sunkar R, Axtell MJ (2009) Sliced microRNA targets and precise loop-first processing of MIR319 hairpins revealed by analysis of the *Physcomitrella patens* degradome. RNA 15:2112–2121

35. Bologna NG, Schapire AL, Palatnik JF (2013) Processing of plant microRNA precursors. Brief Funct Genomics 12:37–45

36. Kurihara Y, Takashi Y, Watanabe Y (2006) The interaction between DCL1 and HYL1 is important for efficient and precise processing

of pri-miRNA in plant microRNA biogenesis. RNA 12:206–212

37. Schapire AL, Bologna NG, Moro B, Zhai J, Meyers BC, Palatnik JF (2013) Construction of Specific Parallel Amplification of RNA Ends (SPARE) libraries for the systematic identification of plant microRNA processing intermediates. Methods 64:283–291

38. Moro B, Chorostecki U, Arikit S, Suarez IP, Höbartner C, Rasia RM, Meyers BC, Palatnik JF (2018) Efficiency and precision of microRNA biogenesis modes in plants. Nucleic acids research 46(20):10709–10723. https://doi.org/10.1093/nar/gky853

39. Classic Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

40. Hang R, Deng X, Liu C, Mo B, Cao X (2015) Circular RT-PCR assay using arabidopsis samples. Bio-protocol 5:e1533. https://doi.org/10.21769/BioProtoc.1533

41. Basyuk E, Suavet F, Doglio A, Bordonne R, Bertrand E (2003) Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res 31:6593–6597