

# Chapter 2

## Identification of Plant microRNAs Using Expressed Sequence Tag Analysis

Taylor P. Frazier and Baohong Zhang

### Abstract

microRNAs (miRNAs) are a new class of small endogenous noncoding regulatory RNAs, which play an important function in plant growth, development, phase change, and response to environmental stress. Identifying miRNAs is the first step for investigating miRNA-mediated gene regulation and miRNA function. In this chapter, we describe a comprehensive comparative genomics-based expressed sequence tag (EST) analysis for identifying miRNAs from a wide range of plant species. EST analysis is based on the conservation of miRNA sequences and the stem-loop hairpin secondary structures of miRNAs. In this method, potential miRNAs will first be identified by EST analysis followed by confirmation using TaqMan® MicroRNA qRT-PCR. This method is simple and reliable with high efficiency. This method has also been widely adopted by many scientists around the world and several hundreds of miRNAs have been identified in many plant species using this method.

**Key words:** microRNA, Expressed sequence tag, Comparative genomics, BLASTn, qRT-PCR, EST

---

### 1. Introduction

microRNAs (miRNAs) are a newly discovered class of noncoding endogenous small RNAs with about 20–22 nucleotides in length (1, 2). Many investigations have demonstrated that miRNAs play a fundamental role in almost all biological and metabolic processes in plants, including plant growth and development, phase change, and response to abiotic and biotic stress factors (2, 3). Multiple stages are involved in miRNA biogenesis. First, a miRNA gene is transcribed by RNA polymerase II into a long product, called primary miRNA (pri-miRNA); pri-miRNA can form into a specific stem-loop hairpin secondary structure that is sequentially processed by several enzymes, including Dicer-like enzyme 1 (DCL1) and the miRNA methyltransferase HEN1, into the mature miRNA (3).

miRNAs act as post-transcriptional gene regulators by binding perfectly or near-perfectly to messenger RNAs (mRNAs) (4). miRNAs that are near-perfect complements to their target mRNA sequences bind and inhibit protein translation, whereas miRNAs that are perfect complements to the target mRNAs bind and target the mRNA for degradation (4–6).

There are two major approaches for identifying miRNAs in plants: experimental approaches and computational approaches. Experimental approaches include genetic screening, direct cloning, and recently developed next generation high throughput sequencing (2, 7). These methods are the most efficient for identifying miRNAs as they produce few false positive results and are particularly useful for discovering new and species-specific miRNAs. However, experimental methods are often extremely costly and technique dependent. Based on the features of miRNAs, several computational programs have been developed for predicting miRNAs, including miRcheck (8) and findMiRNA (9, 10). In the beginning, these programs were employed to predict many miRNAs in plants. However, a majority of computational programs are based on complete genome sequences which are available for only a limited number of model species, such as *Arabidopsis* and rice. The shortcomings limit the application of these computational programs on a wider range of species.

Studies on comparative genomics across vastly divergent taxa have demonstrated that many miRNAs are highly evolutionarily conserved from species to species, ranging from moss and gymnosperms to high flowering eudicot species (11, 12). This provides a powerful strategy to identify miRNAs from any species using a comparative genomic-based BLASTn search with already known miRNA sequences. Using this strategy, we developed an expressed sequence tag (EST) and a genome survey sequence (GSS) approach to identify conserved miRNAs (13, 14). There are several significant advantages for identifying miRNAs using comparative genomics-based EST analysis (11, 15): (1) EST analysis can be employed to identify miRNAs in any species for which there are previously determined EST sequences; (2) EST analysis not only can be used to identify conserved miRNA, but also provides direct evidence for miRNA expression because ESTs are derived from transcribed sequences (mRNA); (3) it is easy to identify miRNAs using EST analysis and no specialized software is needed; thus, this method is readily available for widespread usage. EST analysis is based on the conservation of miRNAs, and so it can only be used to identify conserved miRNAs. Since the development of this method in 2005 (14), EST analysis has been widely adopted by different laboratories to identify miRNAs from a variety of species, including several important crops such as apple (16), wheat (17, 18), tomato (19), cotton (20), soybean (15), oilseed (21), and maize (22). This method is also used to

identify miRNAs in animals (23) and viruses (13). Additionally, EST analysis was used to investigate the diversity and evolution of miRNAs in the plant kingdom (11). Currently, this method has been widely adopted by scientists around the world. In this chapter, we are presenting the basic protocol for the identification of miRNAs using comparative genomics-based EST analysis.

---

## 2. Materials

### **2.1. RNA Collection and Extraction from Plants Using the mirVana™ miRNA Isolation Kit**

1. *mirVana™* miRNA isolation kit (Ambion, Austin, TX)
  - (a) *miRNA wash solution 1*: Add 21 mL 100% ethanol before use. This solution contains guanidinium thiocyanate which is a potential biohazard and should be handled with caution.
  - (b) *Wash solution 2/3*: Add 40 mL 100% ethanol before use. This solution can be left at room temperature for up to 1 month. For longer storage periods, store at 4°C but warm to room temperature before use.
  - (c) *Collection tubes*: store at room temperature.
  - (d) *Filter cartridges*: store at room temperature.
  - (e) *Lysis/binding buffer*: store at 4°C.
  - (f) *miRNA homogenate additive*: store at 4°C.
  - (g) *Acid-phenol: chloroform*: store at 4°C. Phenol is a poison and an irritant and therefore gloves or other protection should be worn when handling this reagent. Dispose of phenol waste appropriately.
  - (h) *Elution solution or nuclease-free water*: preheated to 95°C when used and stored at 4°C or room temperature.
2. 100% RNase free ethanol stored at room temperature. Ethanol is flammable so handle and dispose of it accordingly.
3. Liquid Nitrogen.
4. RNase free water.

### **2.2. RT-PCR of RNA from Plant Tissues**

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA): store at -15 to -25°C. All contents should be thawed on ice and centrifuged briefly before using
  - (a) 10× RT Buffer.
  - (b) dNTP mix with dTTP (100 mM).
  - (c) RNase Inhibitor (20 U/μL).
  - (d) Multiscribe™ RT enzyme (50 U/μL).

2. Nuclease-Free water.
3. RT Primers.

### 2.3. qRT-PCR Analysis of miRNA Expression in Plant Tissues

1. TaqMan 2× Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA).
2. qRT Primers (Applied Biosystems, Foster City, CA).
3. Nuclease-Free water.

## 3. Methods

EST analysis depends on conserved plant miRNA sequences and the NCBI GenBank database in order to find potential miRNAs in other plants. Already identified and confirmed plant miRNA sequences can be obtained from the miRNA database miRBase (<http://microrna.sanger.ac.uk>) (24). Using a confirmed miRNA sequence, potential homologs can be found by BLASTn searching against the ESTs of other plant species. The resulting matches are further narrowed down by secondary structure analysis using mFold version 3.2 (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>) (25). Figure 1 summarizes the major steps for identifying miRNAs using EST analysis (11, 22). Figure 2 gives the general structure for a miRNA, including the pre-miRNA sequence and mature miRNA sequence.

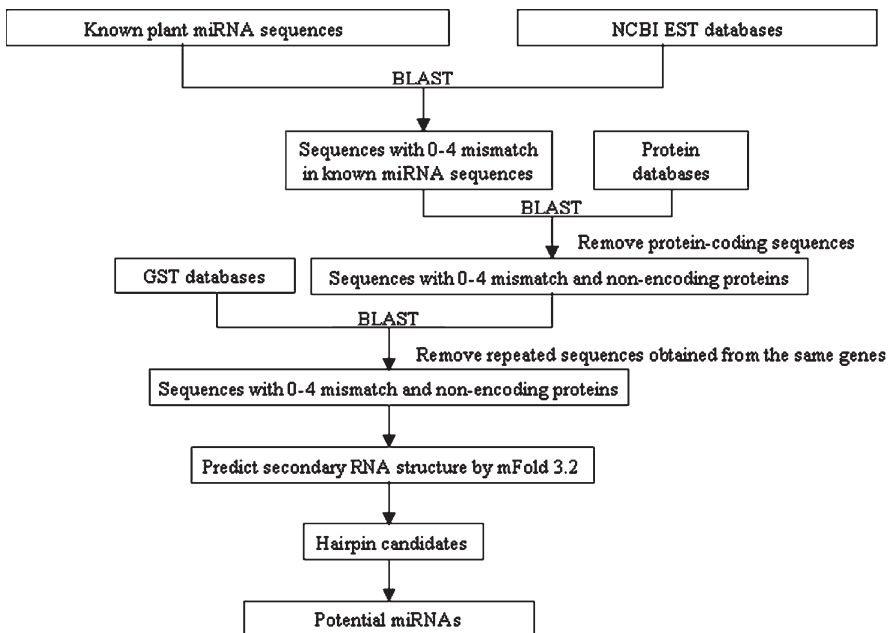


Fig. 1. Schematic representation of the miRNA gene search procedure for identifying miRNA homologs based on established miRNAs using EST analysis.

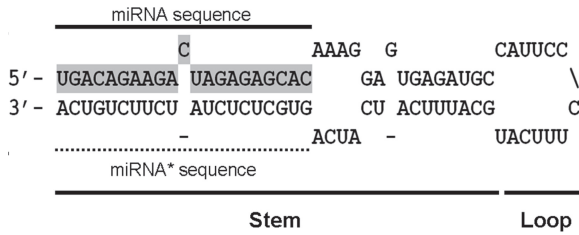


Fig. 2. An example stem-loop hairpin secondary structure of a miRNA precursor sequence.

The criteria for a potential miRNA are (11, 26): (1) predicted mature miRNAs have no more than four nucleotide substitutions compared with a known mature miRNAs; (2) an EST sequence can fold into a stem-loop hairpin secondary structure; (3) the potential mature miRNA sequence is located in one arm of the hairpin structure; (4) there are no more than six mismatches between the predicted miRNA sequence and its opposite miRNA\* sequence in the secondary structure; (5) there is no loop or break in the miRNA or miRNA\* sequence; and (6) the predicted secondary structure has a high negative minimal folding energy (MFE) and high MFE index (MFEI) value (27). Possible miRNAs that meet all of these criteria are then confirmed experimentally using reverse transcription-polymerase chain reaction (RT-PCR) followed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

### 3.1. miRNA Sequence Acquisition from the miRNA Database

1. Open a Web Browser (Internet Explorer, Mozilla, Safari, etc.) and go to the miRNA database miRBase (<http://microrna.sanger.ac.uk>).
2. Click on the Circle that says, “Sequences”. This will take you to the main home page.
3. Click on the Search tab located at the top of the page.
4. At the top of the page there will be a box that says, “By miRNA identifier or keyword”. In this box, type “plants” and click on Submit Query. This will take you to a page that contains a list of all miRNAs that have been identified in different plant species.
5. Click on the link provided under the ID column for the desired miRNA sequence. For this chapter, ath-miR156a will be used as an example for demonstrative purposes.
6. Scroll down the page until the mature miRNA sequence appears and click on the link that says, “Get Sequence”.
7. Copy the miRNA name and sequence to a word document for future use.

### 3.2. NCBI GenBank BLAST Search

1. To access the NCBI GenBank BLAST search, open a web browser and go to the NCBI homepage at <http://www.ncbi.nlm.nih.gov>.
2. Click on the BLAST link located at the top of the page.
3. Scroll down to “Basic BLAST” and click on the “nucleotide blast” link.
4. In the first box where it says to “Enter Query Sequence”, type in or copy and paste from the word document the desired miRNA sequence. In this case, the miRNA sequence listed for ath-miR156a was copied and pasted into the box.
5. Under “Choose Search Set”, change the database to “Others”. After this is done, a new tab will appear that will allow for the database to be changed. Click on the down arrow and scroll down to select “Expressed sequence tags (est).”
6. In the “Organism” box, type the scientific name of the organism that the miRNA sequence will be searched against. For the purpose of this chapter, *Nicotiana tabacum* will be used as an example organism of choice. If you wish to search for all potential miRNAs in all potential organisms, just leave the box blank.
7. Under “Programs”, make sure that the circle next to “highly similar sequences (megablast)” is selected.
8. Click the BLAST button at the bottom of the page. A minute or two will be necessary for the next page to load as the sequences are being retrieved.
9. Once the page has loaded, scroll down the page to the “Alignments” section.
10. Starting with the first result, right-click on the sequence ID (such as gb|FG164766.1) and open the link in a new tab or window.

#### BLAST Result

```
gb|FG164766.1| AGN_RNC012xi20r1.ab1 AGN_RNC
Nicotiana tabacum cDNA 3', mRNA
sequence.
Length=807
```

```
Score = 32.2 bits (16), Expect = 0.15
Identities = 19/20 (95%), Gaps = 0/20 (0%)
Strand=Plus/Plus
```

```
Query 1 TGACAGAAGAGAGTGAGCAC 20
      |||
Sbjct 430 TGACAGAAGAGAGAGAGCAC 449
```

11. Scroll down the page until “Origin” appears. This is the nucleotide sequence for this particular EST.
12. Highlight and copy up to 800 nucleotides with the targeted sequence located in the middle. This is due to the fact that the mFold software can only fold 800 nucleotide sequences for an immediately folding job. Write down on a separate piece of paper the “Query as 1–20” and the “Sbjct as 430–449”.

### **3.3. mFold to Predict miRNA Secondary Structure**

1. In a separate browser window or tab, open the mFold webpage located at <http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>.
2. Scroll down the page and where it says to “Enter the sequence to be folded in the box”, paste the nucleotide sequence copied from the BLASTn search results.
3. Scroll down to the bottom of the page and click on the button that says “Fold RNA”. The software default parameters are used to predict the secondary structures of the selected sequences. It will take a few minutes for the next page to load.
4. Once the RNA has finished folding, a new page will appear with the date and time of folding. Scroll down the page and click the link for “Structure 1”.
5. Search the page for the number “430” and look at the secondary structure of the EST between nucleotides 430 and 449.
6. If the secondary structure meets the criteria 1–5 listed above, then this sequence is selected and the end of 5′ and 3′ are determined. The selected EST fragment (potential miRNA precursor sequence) should go through another cycle of mFold.
7. For the potential pre-miRNA sequence, all mFold outputs, including free energy ( $\Delta G$  kcal/mol), the number of nucleotides (A, G, C and U), and location of the matching regions, should be recorded in an excel data sheet. The MFEI for each sequence should be calculated as previously described (27).
8. Repeat the previous steps again to continually work on other hit sequences.
9. After inspection of all hit sequences, all selected sequences now form a dataset. Perform another BLASTn search against this dataset and remove all repeated sequences found.
10. Because plant miRNAs are unlikely to be located in protein-coding genes, the third BLASTn search should be performed by searching the potential protein-coding genes using the selected sequences from step 9.
11. After removing the protein-coding sequences, the rest of the sequences will most likely be potential miRNAs.

**3.4. RNA Collection  
and Extraction  
from Plants Using  
the mirVana™ miRNA  
Isolation Kit**

Young plant leaves are harvested from the greenhouse. Total RNAs are isolated using mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacture's protocol.

1. Collect desired plant tissue using scissors and place in aluminum foil or a 1.5 mL epi-tube. Immediately freeze the tube with samples in liquid nitrogen. If not proceeding with RNA extraction, transfer tissue samples to a  $-80^{\circ}\text{C}$  freezer for storage.
2. Prechill a mortar and pestle in a  $-80^{\circ}\text{C}$  freezer for at least 30 min prior to RNA extraction.
3. Pipet 300  $\mu\text{L}$  of Lysis/Binding buffer into a 1.5 mL epi-tube and place on ice.
4. Remove the mortar and pestle from the  $-80^{\circ}\text{C}$  freezer. Take the tissue sample out of the liquid nitrogen and place in the mortar. Add liquid nitrogen slowly and using the pestle, grind the tissue sample into a fine powder. Transfer the powder, making sure it does not thaw, to the 1.5 mL tube containing the Lysis/Binding buffer making sure to keep the tube on ice.
5. Repeat the previous steps with all of the collected tissue samples.
6. Homogenize the tissue sample with a homogenizer until the tissue is thoroughly broken down.
7. Add 30  $\mu\text{L}$  (1/10 the volume of Lysis/Binding buffer) miRNA Homogenate Additive to the homogenate and mix well by vortexing.
8. Keep the tube on ice for 10 min.
9. Add 300  $\mu\text{L}$  (the volume equal to the Lysis/Binding buffer before miRNA Homogenate Additive addition) Acid-Phenol/Chloroform to each tube making sure to draw from the bottom phase of the bottle.
10. Mix thoroughly by inverting or vortex the tube for approximately 30–60 s to mix.
11. Centrifuge the tube at  $10,000\times g$  at room temperature for 5 min to separate the aqueous phase from the organic phase. If the interphase is not compact after the centrifugation, repeat with a second round.
12. Carefully remove 300  $\mu\text{L}$  of the aqueous upper phase, being careful not to disturb the lower phase, and transfer to a new 1.5 mL tube.
13. Add 375  $\mu\text{L}$  (or 1.25 volumes of the aqueous phase) of room temperature 100% ethanol to the aqueous phase. Mix well by inverting or vortexing.
14. For each sample, place a Filter Cartridge into a new collection tube provided with the kit. Pipet the lysate/ethanol mixture



- onto the filter cartridge. The maximum volume that the filter cartridge can hold is 700  $\mu\text{L}$ .
15. Centrifuge at  $10,000\times g$  for approximately 15 s. Discard the flow-through and place the filter cartridge back into the same tube. Repeat this procedure until all of the lysate/ethanol mixture has passed through the filter.
  16. Apply 700  $\mu\text{L}$  of miRNA Washing Solution 1 to the filter cartridge and centrifuge for approximately 5–10 s. Dispense of the flow-through and place the filter cartridge back into the same tube.
  17. Apply 500  $\mu\text{L}$  of miRNA Wash Solution 2/3 and pull the solution through the filter cartridge as detailed in the previous step.
  18. Repeat step 17 with a second aliquot of equal volume of miRNA Wash Solution 2/3.
  19. After discarding the flow-through from previous step, return the filter cartridge to the same collection tube and spin the assembly for 1 min at  $10,000\times g$  at room temperature. This removes residual fluid from the filter.
  20. Transfer the filter cartridge to a new collection tube. Apply 100  $\mu\text{L}$  of preheated  $95^{\circ}\text{C}$  Elution solution or nuclease-free water to the center of the filter. Let stand for 30 s–1 min.
  21. Centrifuge the tube for 20–30 s at  $10,000\times g$  to recover the RNAs.
  22. Remove the filter cartridge and mix the recovered RNAs by gently flicking the tube. Briefly centrifuge again to bring all contents to the bottom of the tube.
  23. The quality and quantity of the total RNAs are measured using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE).
  24. RNA samples are stored in a  $-80^{\circ}\text{C}$  freezer until further use.

### **3.5. RT-PCR of RNA from Plant Tissues**

qRT-PCR will be used to confirm the miRNAs identified by EST analysis. A two step assay is performed in TaqMan-based real-time quantification of miRNAs. The first step is a reverse transcription reaction in which a stem-loop RT primer is used to reverse transcribe mature miRNAs to cDNAs. The second step involves real-time PCR, in which the expression levels of miRNAs are monitored and quantified using qRT-PCR that includes miRNA-specific forward primer, reverse primer and FAM dye-labeled TaqMan probes (28).

1. Allow the TaqMan MicroRNA Reverse Transcription Kit reagents and Reverse Transcription Primers (RT primers) to thaw on ice. Briefly centrifuge to bring the reagents and primers to the bottom of the tubes.

2. In a PCR tube (0.2 mL tube), add the following amount of reagents for *one* reaction: 4.16  $\mu\text{L}$  nuclease-Free water, 0.19  $\mu\text{L}$  RNase inhibitor, 1.5  $\mu\text{L}$  10 $\times$  RT Buffer, 0.15  $\mu\text{L}$  dNTP mix (100 mM), and 1.00  $\mu\text{L}$  Reverse Transcriptase enzyme.
3. Mix the reagents gently by flicking the tube and briefly centrifuge. Place the tube back on ice.
4. The concentration of total RNA should be 1–10 ng for every 15  $\mu\text{L}$  reaction and added in a ratio such that there is 5  $\mu\text{L}$  of RNA for every 7  $\mu\text{L}$  of reagents. If necessary, add nuclease free water to the reaction tube to bring the volume to 12  $\mu\text{L}$ .
5. Add 3  $\mu\text{L}$  of RT primers to the appropriate tube bringing the total volume per tube to 15  $\mu\text{L}$ . Mix the tube gently by flicking and centrifuge briefly. Incubate for 5 min on ice or until ready to load the thermal cycler.
6. Program the thermal cycler as follows:

Step type	Time (min)	Temperature ( $^{\circ}\text{C}$ )
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	$\infty$	4

7. If not proceeding to qRT-PCR, store the RT-PCR samples in a  $-20^{\circ}\text{C}$  freezer.

**3.6. qRT-PCR Analysis of miRNA Expression in Plant Tissues**

1. Add 80  $\mu\text{L}$  of nuclease free water to the RT-PCR products from the previous method.
2. Prepare a master mix in a new 0.2 mL PCR tube by adding the following: 22.5  $\mu\text{L}$  of nuclease-free water, 37.5  $\mu\text{L}$  of 2 $\times$  PCR mixture, 9  $\mu\text{L}$  of RT-PCR products (after addition of water), and 6  $\mu\text{L}$  RT Primer. The volume of the tube should equal 75  $\mu\text{L}$ .
3. Using a 96-well PCR plate, aliquot 22  $\mu\text{L}$  of the master mix to three separate wells.
4. Centrifuge the plate briefly.
5. The reactions are incubated in a 96-well plate at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s and  $60^{\circ}\text{C}$  for 60 s. This should take approximately 2 h.
6. Analyze the miRNA expression levels from the qRT-PCR amplification results.

7. After the completion of the real-time reactions, the threshold manually sets and the threshold cycle (Ct) will be recorded. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (28).
8. Based on the qRT-PCR results, we can conclude which miRNAs are really expressed in that plant organ.

---

## 4. Notes

1. All already known miRNA datasets can be downloaded from the miRBase (<http://microrna.sanger.ac.uk/cgi-bin/sequences/browse.pl>).
2. BLASTn search can be done individually or by group.
3. BLASTn search can be done online or locally by downloading the BLASTn software.
4. If the BLASTn searches reveal partial sequence similarity to a known mature miRNA sequence, the nonaligned regions should be manually inspected and compared in order to determine the number of matching nucleotides and to assess their potential as miRNA candidates.
5. If a BLASTn search hits a sequence that is ( $\pm$ ) complementary to the known miRNA sequence, the hit sequence should be changed to the complementary sequence for final analysis.
6. If there is a greater volume of tissue sample for RNA isolation, the volume of the Lysis/Binding buffer may be increased.
7. A vacuum manifold can be used instead of a centrifuge to pull solutions through the filter cartridge.
8. When performing qRT-PCR for miRNA confirmation, the reverse transcription product needs to be diluted to avoid the potential interference of the high concentration of stem-loop primer.

---

## Acknowledgments

This work was partially supported by East Carolina University New Faculty Research Startup Funds Program and a Science and Engineering Grant from DuPont. We would like to thank Dr. Ramsey Lewis and Mr. Ted Woodlief of North Carolina State University for kindly providing tobacco seeds.

## References

1. Bartel, D. P. (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function, *Cell* 116, 281–297.
2. Zhang, B. H., Pan, X. P., Cobb, G. P., and Anderson, T. A. (2006) Plant microRNA: A small regulatory molecule with big impact, *Developmental Biology* 289, 3–16.
3. Chen, X. M. (2005) microRNA biogenesis and function in plants, *FEBS Letters* 579, 5923–5931.
4. Zhang, B. H., Wang, Q. L., and Pan, X. P. (2007) MicroRNAs and their regulatory roles in animals and plants, *Journal of Cellular Physiology* 210, 279–289.
5. Eulalio, A., Huntzinger, E., and Izaurralde, E. (2008) Getting to the root of miRNA-mediated gene silencing, *Cell* 132, 9–14.
6. Pillai, R. S., Bhattacharyya, S. N., and Filipowicz, W. (2007) Repression of protein synthesis by miRNAs: How many mechanisms? *Trends in Cell Biology* 17, 118–126.
7. Zhang, B. H., Pan, X. P., Wang, Q. L., Cobb, G. P., and Anderson, T. A. (2006) Computational identification of microRNAs and their targets, *Computational Biology and Chemistry* 30, 395–407.
8. Jones-Rhoades, M. W., and Bartel, D. P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA, *Molecular Cell* 14, 787–799.
9. Adai, A., Johnson, C., Mlotshwa, S., Archer-Evans, S., Manocha, V., Vance, V., and Sundaresan, V. (2005) Computational prediction of miRNAs in *Arabidopsis thaliana*, *Genome Research* 15, 78–91.
10. Lindow, M., and Krogh, A. (2005) Computational evidence for hundreds of non-conserved plant microRNAs, *BMC Genomics* 6, 119.
11. Zhang, B. H., Pan, X. P., Cannon, C. H., Cobb, G. P., and Anderson, T. A. (2006) Conservation and divergence of plant microRNA genes, *Plant Journal* 46, 243–259.
12. Floyd, S. K., and Bowman, J. L. (2004) Gene regulation: Ancient microRNA target sequences in plants, *Nature* 428, 485–486.
13. Pan, X. P., Zhang, B. H., San Francisco, M., and Cobb, G. P. (2007) Characterizing viral microRNAs and its application on identifying new microRNAs in viruses, *Journal of Cellular Physiology* 211, 10–18.
14. Zhang, B. H., Pan, X. P., Wang, Q. L., Cobb, G. P., and Anderson, T. A. (2005) Identification and characterization of new plant microRNAs using EST analysis, *Cell Research* 15, 336–360.
15. Zhang, B. H., Pan, X. P., and Stellwag, E. J. (2008) Identification of soybean microRNAs and their targets, *Planta* 229, 161–182.
16. Gleave, A. P., Ampomah-Dwamena, C., Berthold, S., Dejnopratt, S., Karunairatnam, S., Nain, B., Wang, Y.-Y., Crowhurst, R. N., and MacDiarmid, R. M. (2008) Identification and characterisation of primary microRNAs from apple (*Malus domestica* cv. Royal Gala) expressed sequence tags, *Tree Genetics & Genomes* 4, 343–358.
17. Dryanova, A., Zakharov, A., and Gulick, P. J. (2008) Data mining for miRNAs and their targets in the Triticeae, *Genome* 51, 433–443.
18. Jin, W. B., Li, N. N., Zhang, B., Wu, F. L., Li, W. J., Guo, A. G., and Deng, Z. Y. (2008) Identification and verification of microRNA in wheat (*Triticum aestivum*), *Journal of Plant Research* 121, 351–355.
19. Yin, Z. J., Li, C. H., Han, M. L., and Shen, F. F. (2008) Identification of conserved microRNAs and their target genes in tomato (*Lycopersicon esculentum*), *Gene* 414, 60–66.
20. Zhang, B. H., Wang, Q. L., Wang, K. B., Pan, X. P., Liu, F., Guo, T. L., Cobb, G. P., and Anderson, T. A. (2007) Identification of cotton microRNAs and their targets, *Gene* 397, 26–37.
21. Xie, F. L., Huang, S. Q., Guo, K., Xiang, A. L., Zhu, Y. Y., Nie, L., and Yang, Z. M. (2007) Computational identification of novel microRNAs and targets in *Brassica napus*, *FEBS Letters* 581, 1464–1474.
22. Zhang, B. H., Pan, X. P., and Anderson, T. A. (2006) Identification of 188 conserved maize microRNAs and their targets, *FEBS Letters* 580, 3753–3762.
23. Weber, M. J. (2005) New human and mouse microRNA genes found by homology search, *FEBS Journal* 272, 59–73.
24. Griffiths-Jones, S., Saini, H. K., van Dongen, S., and Enright, A. J. (2008) miRBase: Tools for microRNA genomics, *Nucleic Acids Research* 36, D154–D158.
25. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction, *Nucleic Acids Research* 31, 3406–3415.
26. Ambros, V., Bartel, B., Bartel, D. P., Burge, C. B., Carrington, J. C., Chen, X. M., Dreyfuss, G., Eddy, S. R., Griffiths-Jones, S., Marshall, M., Matzke, M., Ruvkun, G., and

- Tuschl, T. (2003) A uniform system for microRNA annotation, *RNA* 9, 277–279.
27. Zhang, B. H., Pan, X. P., Cox, S. B., Cobb, G. P., and Anderson, T. A. (2006) Evidence that miRNAs are different from other RNAs, *Cellular and Molecular Life Sciences* 63, 246–254.
28. Chen, C. F., Ridzon, D. A., Broome, A. J., Zhou, Z. H., Lee, D. H., Nguyen, J. T., Barbisin, M., Xu, N. L., Mahuvakar, V. R., Andersen, M. R., Lao, K. Q., Livak, K. J., and Guegler, K. J. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR, *Nucleic Acids Research* 33, e179.

Plant Reverse Genetics

Methods and Protocols

Pereira, A. (Ed.)

2011, XI, 282 p. 63 illus., 2 illus. in color., Hardcover

ISBN: 978-1-60761-681-8

A product of Humana Press