Quantitation of Plant miRNAs by RT-PCR

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ABSTRACT

We have developed a novel, highly sensitive real-time PCR method for quantitation of miRNAs based on TaqMan® reagent chemistry. Assays have been designed and validated for over 300 known miRNAs from various species, including 50 from *Arabidopsis thaliana*. The sensitivity of this method allows detection of mouse miRNAs in as little as 0.03 ng total RNA or 100 cells and the detection of Arabidopsis miRNAs in as little as 0.07 ng of total RNA. Using the synthetic Arabidopsis miRNA, miR159a, we were able to accurately determine C_T values over seven orders of magnitude ($R^2 = 0.995$). This method allows us to discriminate between two miRNAs that differ by as little as a single nucleotide as well as between mature miRNAs and their precursors. The presence of genomic DNA or non-specific RNA does not affect the accuracy of the miRNA quantitation. Here we present data using this novel method to determine abundance and tissue-specific expression patterns of Arabidopsis miRNAs.

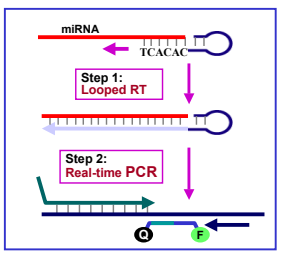
INTRODUCTION

MicroRNAs are endogenous RNAs of approximately 22 nucleotides that play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression[1]. More than 700 miRNAs have been identified across species. Their expression levels vary greatly among species and tissues[2]. Low abundant miRNAs have been difficult to detect based on current technologies such as cloning, Northern hybridization[3], and the modified Invader® assay[4]. Here, we present a new, real-time, quantitation method termed looped-primer RT-PCR for accurate and sensitive detection of miRNAs as well as other non-coding RNA (ncRNA) molecules. We have previously demonstrated the ability to accurately quantify miRNAs in human and mouse total RNA, cell lysates and whole cells. Here we demonstrate the ability to quantitate 50 miRNAs from five Arabidopsis tissue samples. For several miRNAs, we show comparative data from massively parallel signature sequencing (MPSS) and Northern analysis.

MATERIALS AND METHODS

miRNA targets: A total of 50 miRNA assays were designed against Arabidopsis miRNA sequences downloaded from the Sanger database[5].
Tissue RNA samples: Total RNA was isolated from seedling, root, callus, flower, and *rdr2* mutant flower tissues using a standard Trizol extraction method (Invitrogen).
RT-PCR: The assay includes two steps, RT and PCR (Figure 1). RT reactions (performed in duplicate) containing RNA samples, looped-primers, 1X buffer, reverse transcriptase, and RNase inhibitor were incubated for 30 minutes each, at 16ºC and at 42ºC. Real-time PCR reactions based on TaqMan® reagent chemistry were performed in triplicate on an Applied Biosystems 7900HT Fast Real-Time PCR System. No template controls (NTCs) were run for each assay under the same conditions.
Data analysis: The level of miRNA expression was measured using C_T (threshold cycle). The C_T is the fractional cycle number at which the fluorescence passes the fixed threshold. In the genomic cycle of PCR, the C_T is inversely proportional to the sample starting copy number. The ΔC_T was calculated by subtracting the C_T of a control (internal or external) from the C_T of the miRNA of interest. The $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T of the calibrator (a sample used as the basis for comparative results) with the ΔC_T of the test sample. Fold change was generated using the equation $2^{-\Delta\Delta CT}$.
Northern: RNA gel blots of small RNAs were carried out as described by Lu et al, Poster #767.
MPSS: MPSS is a RNA profiling method that has recently been adapted for the identification and quantification of small RNAs (Lu et al., P767).

Figure 1. Assay Scheme



Step 1. Looped-primer RT: Looped primers are annealed to miRNA targets and extended by reverse transcriptase.

Step 2. Real-Time PCR: MicroRNA-specific forward primer, TaqMan® probe, and universal reverse primer are used for PCR reactions. Quantitation of miRNAs is estimated based on measured C_T values.

RESULTS

Table 1. Single Base Discrimination of miRNAs Assays

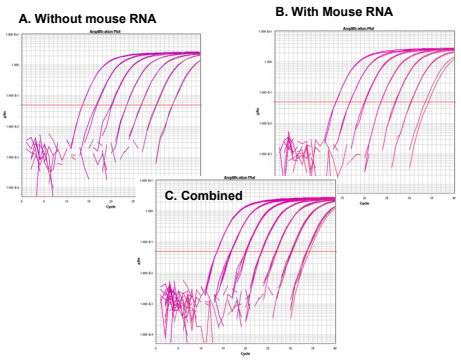
A.

miRNA assay \ Synthetic miRNA target	let-7a	let-7b	let-7c	let-7d	let-7e	Relative detection (%) *
let-7a	100.0	0.3	7.2	0.1	0.0	
let-7b	0.0	100.0	3.3	0.0	0.0	
let-7c	0.1	4.4	100.0	0.1	0.0	
let-7d	0.0	0.0	0.0	100.0	0.0	
let-7e	0.0	0.0	0.0	0.0	100.0	

B.

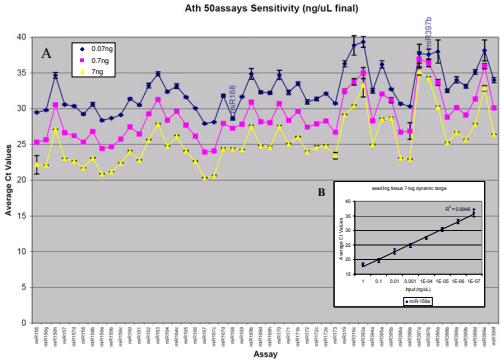
(A) Relative detection (%) calculated based on C_T difference between perfectly matched and mismatched assays. (B) Nucleotide sequences of Let-7a RNA and closely related Let-7 variants. Nucleotide differences are in red and denoted by an arrow. The number of mismatches between variants are shown to the right of the sequences.

Figure 2. Quantitiation of Synthetic Arabidopsis miR159a



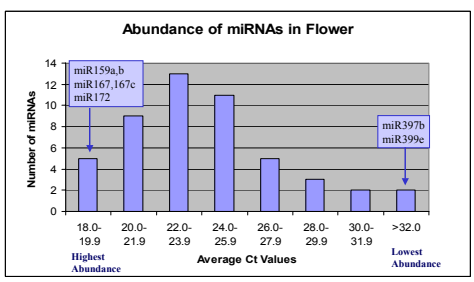
Amplification plots of fluorescence signal versus cycle number of synthetic Arabidopsis miR159a without mouse RNA (A), in the presence of 150 ng of non-specific mouse RNA (B), and combined data demonstrating the high degree of overlap (C). Estimated synthetic miRNA input (miR159a) in the RT step, based on OD, was 70, 700, 7,000, 70,000, 700,000, 7M, and 70M copies.

Figure 3. Sensitivity of miRNA Assays in Arabidopsis Seedling



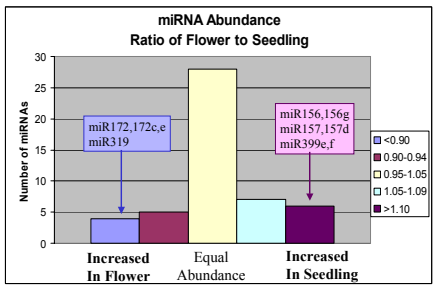
(A) Sensitivity plot of 50 miRNA assays run against 0.07, 0.7, and 7ng of total RNA isolated from seedling. (B) Inset – miR159a assay run against an estimated input of 7, 0.7, 0.007, 7E-04, 7E-05, 7E-06, and 7E-07ng (based on OD) seedling total RNA in the RT step. Detection sensitivity is $7e^{-07}$ and dynamic range extends over a 7-log range.

Figure 4. Expression of miRNAs in Arabidopsis Flower



Histogram of data from 50 miRNAs detected in total RNA isolated from flower. Expression data were binned in two C_T intervals, with the miRNAs in highest abundance grouped together in the 18.0–19.9 C_T interval and miRNAs in lower abundance grouped in the >32.0 C_T interval. The graph shows a slightly skewed distribution with the majority of miRNAs in the 20.0–25.9 C_T range.

Figure 5. Expression Ratios of miRNAs in Flower and Seedling



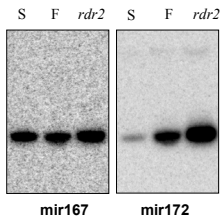
Histogram of data from 50 miRNAs detected in total RNA isolated from flower and seedling. The ratio of abundance of flower to seedling was calculated by dividing the average C_T of flower by the average C_T of seedling for each miRNA. The ratios were then binned to demonstrate the miRNAs that were most abundant in flower (<0.90 or 10% or greater C_T in flower) or most abundant in seedling (>1.10 or 10% or greater C_T in seedling). The largest number of miRNAs (bar shown in yellow) demonstrated relatively equal abundance in both flower and seedling.

Table 2. Data from MPSS Confirming RT-PCR Assays

miRNA	Flower (F)	Seedling (S)
miRNA167a,b,d	237,567	298,695
miRNA172a,b,c,d	7,492	226
mi399a,d,e,f	0	88

MPSS data from small RNA libraries prepared from flower (F) and seedling (S) are shown. miRNA167 and miRNA172 are examples of high abundance miRNAs and miRNA399 is an example of a low abundance miRNA as determined by RT-PCR of flower in Figure 4, and exhibit consistent MPSS data above. miRNA172 is more abundant in flower in both MPSS above and RT-PCR (Figure 5). miRNA399 is more abundant in seedlings in both experiments.

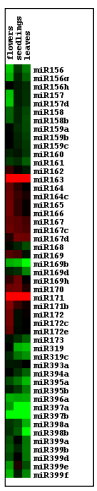
Figure 6. Northern Blot Validation of RT-PCR Assay



(Above) RNA gel blot hybridization of miRNA oligonucleotide probes to low mwt RNA from seedlings (S), flower (F), and flower from the *rdr2* mutant (*rdr2*). The abundance of miRNA167 is similar in seedlings and flower, whereas for miRNA 172 RNA abundance is much higher in flowers, similar to the RT-PCR data in Figures 4 and 5.

(Right) Heat map displaying regulation of miRNAs in flower, seedling, and leaves compared to callus. The average C_T value for miR163b was used to normalize data across callus, flower, seedling, and leaves, generating a ΔC_T for each miRNA. $\Delta\Delta C_T$ and fold changes were calculated as described in Materials and Methods using callus as the calibrator. The heat map shows miRNAs that are up- (red) and down- (green) regulated in flower, seedling and leaves as compared to callus. miR163 and miR171 showed the greatest up-regulation (>60-fold) and miR397a and miR397b demonstrated the greatest down-regulation (>12-fold) of flower, seedling, and leaves as compared to callus.

Figure 7. Heat Map Displaying Regulation of All miRNAs



REFERENCES

1. Bartel, D. 2004. *Cell* 116, 281-297
2. Kim, J. et al. 2004. *PNAS* 101:360-365
3. Lim, L. et al. 2003. *Genes & Development* 17:991-1008
4. Allawi, H. T. et al. 2004. *RNA* 10:1153-1161
5. The microRNA Registry. Griffiths-Jones S. NAR, 2004, 32, Database Issue, D109-D111
6. Lu et al., P767, PAG 2004

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TRADEMARKS/LICENSING