Real-Time PCR Quantification of Plant miRNAs Using Universal ProbeLibrary Technology

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MicroRNAs (miRNAs) are naturally occurring, highly conserved families of transcripts (18–25 nt in length) that are processed from larger hairpin precursors. Both plant and animal genomes encode miRNAs, and to date, there are over 4,000 mature miRNA transcripts annotated. Although miRNAs represent a relatively abundant class of transcripts, their expression levels vary greatly among species and tissues. Less abundant miRNAs routinely fail detection using technologies such as cloning, northern hybridization and microarray analysis. Here, we present a real-time quantification method for accurate, sensitive, and economical detection of plant miRNAs based upon the stem-loop RT primer approach using an miRNA-specific primer coupled with a stem-loop specific Universal ProbeLibrary (UPL) probe. This approach represents a highly specific, sensitive, and easy-to-apply system to detect miRNAs.

Introduction

MicroRNAs (miRNAs) are short, highly conserved families of non-coding transcripts, arising from larger hairpin precursors [1]. MicroRNAs, together with short interfering RNA (siRNAs), belong to a class of 18- to 25-nucleotide (nt) small RNAs that are essential for genome stability, development and differentiation, cellular communication, and adaptive responses to biotic and abiotic stresses [1,2]. Currently, over 4000 miRNA sequences from vertebrates, flies, worms, plants, and viruses are annotated in the Sanger Institute miRNA sequence database (miRBase; Release 9.0, October 2006) [3].

Efficient and reliable detection of miRNAs is an essential step towards understanding their roles in specific tissues and cells. Less abundant miRNAs routinely escape detection with standard technologies, such as cloning, northern hybridization, and microarray analysis. Furthermore, the high complexity of the small RNA population in plants, comprising both miRNAs and endogenous siRNAs [4,5], can affect conventional detection methods. Sensitive qRT-PCR techniques are now available, including a real-time method for accurate and sensitive detection of mammalian miRNAs, using stem–loop RT followed by a TaqMan® PCR analysis [6]. However, the cost of specific TaqMan® probes required for each miRNA sequence is prohibitive when screening large numbers of miRNAs. Here we describe an accurate, sensitive, and economical miRNA quantification method based on stem-loop RT-PCR procedure combined with the Universal ProbeLibrary (UPL) technology, optimized to detect and quantify expression levels of plant miRNAs.

Table 1: miRNA and primer sequences. Highlighted in red is the Universal ProbeLibrary Probe #21 binding site.

miR156a	miRNA sequence	UGACAGAAGAGAGUGAGCAC
	RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGTGCTC
	Forward primer	GCGGCGGTGACAGAAGAGAGT
miR159a	miRNA sequence	UUUGGAUUGAAGGGAGCUCUA
	RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTAGAGC
	Forward primer	CGGCGGTTTGGATTGAAGGGA
miR166a	miRNA sequence	UGAAGCUGCCAGCAUGAUCUA
	RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGGGGAA
	Forward primer	TCGCGTGAAGCTGCCAGCAT
miR167a	miRNA sequence	UGAUUGAGCCGCGCCAAUAUC
	RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTAGATC
	Forward primer	TTCCTTGATTGAGCCGCGCC
Universal	Reverse primer	GTGCAGGGTCCGAGGT

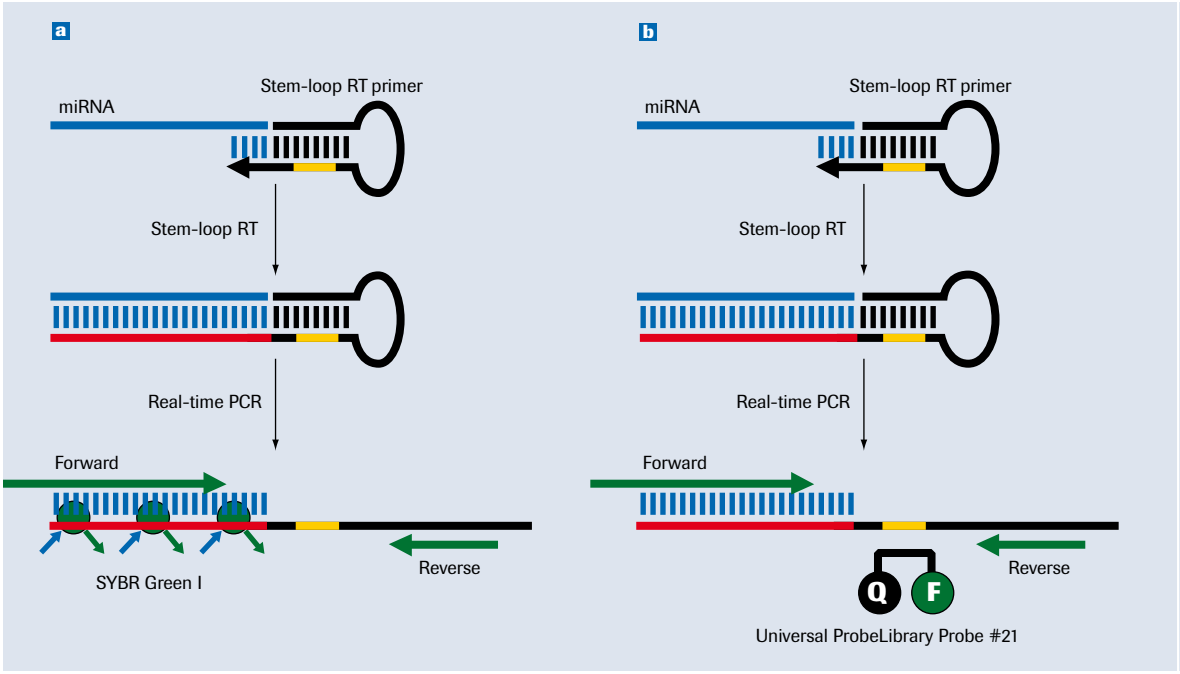

Figure 1: Real-time PCR miRNA assays. miRNA assays include two steps, an initial stem–loop RT and a subsequent real-time PCR. Stem–loop RT primers bind to the 3' portion of miRNA molecules, initiating reverse transcription of the miRNA. Highlighted in yellow is the Universal ProbeLibrary probe binding site. Then the RT product is quantified using PCR with a miRNA specific forward primer and the universal reverse primer. **(a)** SYBR Green I assay. **(b)** Universal ProbeLibrary probe assay.

Materials and Methods

miRNA sequences and primers

Plant miRNA genes were selected from the Sanger Institute miRBase Sequence Database at www.microrna.sanger.ac.uk/sequences/index. Universal ProbeLibrary Probe #21 was obtained from Universal ProbeLibrary database. Stem-loop RT primers were designed according to Chen *et al.* [6], with a modification to include the Universal ProbeLibrary Probe #21 sequence binding site into the primer stem region. Sequence data are presented in Table 1.

Preparation of RNA and cDNA

Total RNA was isolated from shoots of 4-week-old *Arabidopsis thaliana* seedlings grown in a greenhouse under natural daylight. Reverse transcription reactions (10 µl) contained RNA, 50 nM stem–loop RT primer, 0.25 mM each of dNTPs, 50 units reverse transcriptase, 1x reverse transcriptase buffer, 10 mM DTT, and 4 units RNase inhibitor. The reactions were incubated for 30 minutes at 16°C, followed by pulsed RT of 60 cycles at 30°C for 30 seconds, 42°C for 30 seconds, and 50°C for 1 second. Pulsed RT reactions provide better detection sensitivity compared with non-pulsed reactions [7]. Reactions were terminated by incubating at 85°C for 5 minutes to inactivate the reverse transcriptase. All reverse transcriptase reactions included no-template and minus-RT controls.

PCR

Both the SYBR Green I assay and the Universal ProbeLibrary probe assay were performed using the LightCycler® 1.5 Instrument. For the SYBR Green I assays, the final reaction volume was 10 µl consisting of 1x LightCycler® FastStart DNA Master^{PLUS}, SYBR Green I, 0.5 µM of each forward and reverse primer, and 1 µl cDNA. Amplification curves were generated with an initial denaturing step at 95°C for 5 minutes, followed by 45 cycles of 95°C for 5 seconds, 60°C for 5 seconds, and 72°C for 8 seconds. Melting curves were generated using the following program: PCR products were denatured at 95°C and cooled to 65°C at 20°C per second. The fluorescence signals at a wavelength of 530 nm were then collected continuously from 65°C to 95°C as the temperature was increased at 0.2°C per second.

For a Universal ProbeLibrary probe assay, a final reaction volume of 20 µl consisted of 0.5 µM of each forward and reverse primer, 0.1 µM Universal ProbeLibrary Probe #21, 1x LightCycler® TaqMan® Master, and 1µl of cDNA. Amplification curves were generated with an initial denaturing step at 95°C for 10 minutes, followed by 45 cycles of 95°C for 5 seconds, 60°C for 10 seconds, and 72°C for 1 second.

SYBR Green I and Universal ProbeLibrary probe PCR products were visualized on 4% agarose gels by ethidium bromide staining.

Results and Discussion

The expression of four plant miRNAs was detected using a two-step process. In the first, the stem–loop RT primer was hybridized to an miRNA molecule, and then reversely transcribed in a pulsed RT reaction. In the second, the RT product was amplified and quantified using the SYBR Green I assay (Figure 1a) or the Universal ProbeLibrary probe assay

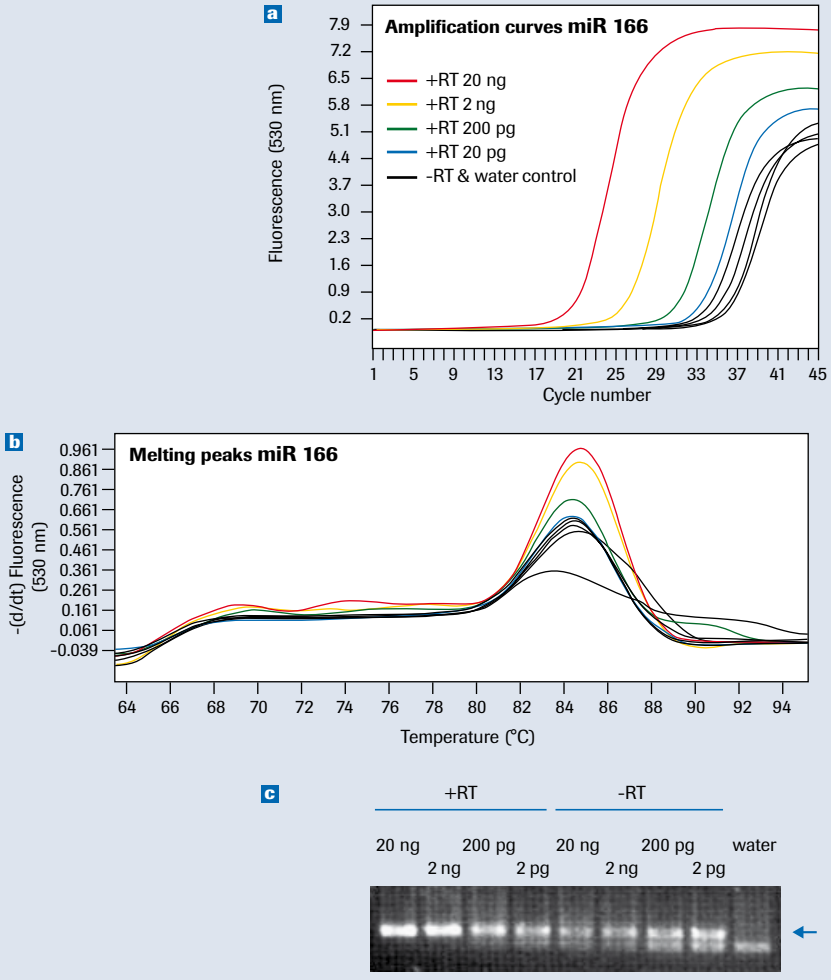

Figure 2: SYBR Green I master mix. Analysis of miR166. Amplification curves were generated using SYBR Green I master mix. **(a)** Amplification curves for 20 ng to 20 pg total RNA of plus-RT, minus-RT and water control reactions. **(b)** Melting curves. **(c)** Real-time PCR products separated by gel electrophoresis (4% agarose) and visualized by ethidium bromide staining. Arrow indicates the size of the expected PCR products.

(Figure 1b). One Universal ProbeLibrary probe was used to detect the four miRNAs.

SYBR Green I assay

SYBR Green I is a fluorescent dye which non-specifically intercalates into double-stranded DNA. Figure 2 is a representative example of one of the four miRNAs reactions using SYBR Green I detection. In addition to the fluorescence from the desired PCR products, the SYBR Green I assay also detected fluorescence from non-specific products found in the minus-RT control and primer-dimers in the water control (Figure 2a). Melting-curve analysis was unable to distinguish between the specific and non-specific PCR products due to the small size of these products (Figure 2b). The sensitivity of this assay could be as low as 2 ng of RNA added. Gel analysis confirmed the presence of amplification products of the expected size as well as smaller fragments in both the plus-RT reactions and minus-RT controls (Figure 2c). Cloning and sequencing of products from the plus-RT reaction confirmed that these were the expected miRNAs. Cloning and sequencing of minus-RT control amplification products revealed the amplification of concatenated primer sequences (data not shown).

Universal ProbeLibrary probe assay

A Universal ProbeLibrary probe assay utilizes a short hydrolysis probe of 8–9 nucleotides, one of which is a locked nucleic acid (LNA). The reproducibility of the Universal ProbeLibrary probe assay was examined by repeating the analysis with four different miRNAs and a dilution series from 20 ng to 20 pg total RNA (Figure 3). Amplification curves correlated to the concentration of the RNA template and spanned four orders of magnitude. Neither of the negative controls (minus-RT and water) for miR166 and miR167 gave a detectable signal (Figures 3a and 3b), though non-specific amplification bands from minus-RT control could be seen on the agarose gels (Figures 3c and 3d). Minus-RT controls for miR156 and miR159 produced amplification curves after 38 cycles, 15 cycles more than with the same amount of RNA template in plus-RT reaction (Figures 3e and 3f). These results suggest that the contribution of the background signal in this assay is negligible. Cloning and sequencing confirmed that plus-RT reaction products were the expected amplicons. Sequencing of the minus-RT control products revealed that the amplicons were the result of non-target priming. In the minus-RT reactions, the amplicons did not contain the Universal ProbeLibrary detection site. The larger background amplicons were concatenated primer sequences and contained the stem-loop primer sequence without a Universal ProbeLibrary probe binding site (data not shown).

Conclusions

The stem-loop RT-PCR assay described here using the Universal ProbeLibrary probe technology, is a rapid, sensitive, specific, and reproducible method that can be used to detect miRNAs from very small amounts of plant RNA. Consequently, a large number of miRNAs can be screened quickly and inexpensively. It is envisaged that this approach will have application to other species, including animals. ■

References

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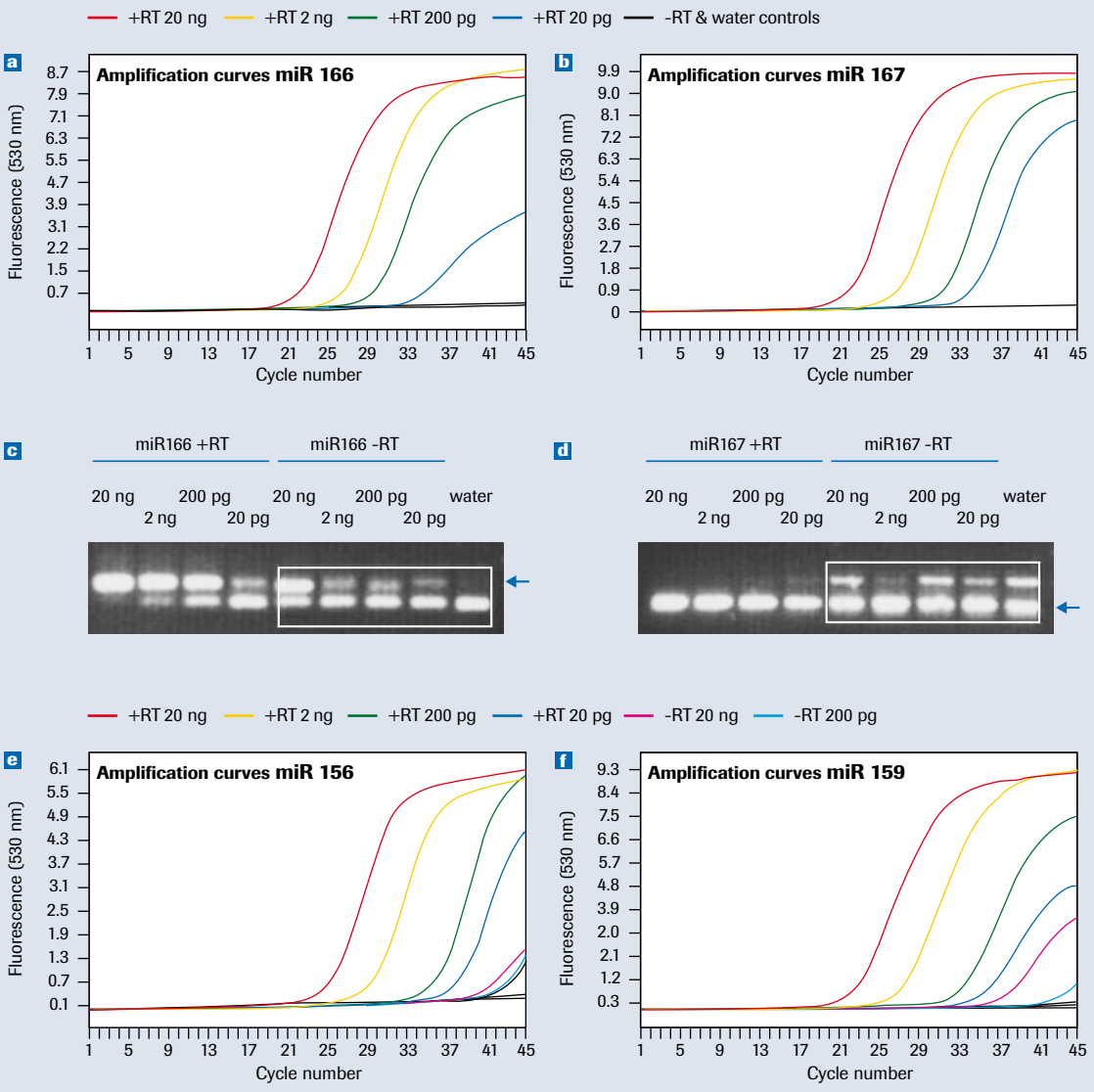

Figure 3: Universal ProbeLibrary probe assay. Amplification curves were detected using Universal ProbeLibrary Probe #21 for **(a)** miR166 and **(b)** miR167. Amplification products of **(c)** miR166 and **(d)** miR167 separated by gel electrophoresis (4% agarose) and visualized by ethidium bromide staining. Arrows indicate the size of the expected PCR products. The bands in white boxes are non-specific PCR products and primer dimers. Amplification curves as detected by Universal ProbeLibrary Probe #21 for **(e)** miR156 and **(f)** miR159. Limited non-specific amplification, during the minus-RT control reactions, was detected 15 cycles after the amplification of the target.

Order INFO

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LightCycler® FastStart DNA Master^{PLUS} SYBR Green I	1 kit (96 reactions)	03 515 869 001
LightCycler® TaqMan® Master	1 kit (96 reactions)	04 535 286 001
Universal ProbeLibrary Probe #21	2 vials (2 x 625 reactions of 20µl)	04 686 942 001