Electronic Supplementary Information

A Three-Way Junction Structure-based Isothermal Exponential Amplification Strategy for Sensitive Detection of 3′-Terminal 2′-O-Methylated Plant MicroRNA

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1. Materials and Reagents

HPLC-purified DNA, HPLC-purified RNA, and DEPC (diethylpyrocarbonate)-treated water were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All of the DNA/RNA sequences used in this work are listed in Table S1. Bst DNA Polymerase Large Fragment and the nicking endonuclease Nt.BstNBI were purchased from New England Biolabs. SYBR Green I was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). All solutions for the 3WJ-EXPAR were prepared in DEPC-treated deionized water. The 3WJ-EXPAR and the real-time fluorescence measurements were carried out by using a StepOne Real-Time PCR System (Applied Biosystems, USA).

Table S1. Oligonucleotide sequences used in this work

Name	Sequences (5′-3′ direction)
ath-miR156a	UGACAGAAGAGAGUGAGCAC-2′me
3WJ-primer-(ath-miR156a)	GTGCTCACTCTT**GATTATG** **a** **b**
3WJ-template-(ath-miR156a)	CGACAACTTCTAATGACTCT**CATAATC**xTCTTCTGTCATTTTTp **e** **d** **b*** **c**
miR122-2′OH	UGGAGUGUGACAAUGGUGUUUG
miR122-2′me	UGGAGUGUGACAAUGGUGUUUG-2′me
3WJ-primer-(miR122)	CAAACACCATTTT**GATTATG** **a** **b**
3WJ-template-(miR122)	CGACAACTTCTAATGACTCT**CATAATC**xCACACTCCA TTTTTp **e** **d** **b*** **c**
well-performing EXPAR template for the 3WJ-EXPAR	CGACAACTTCTAATGACTCTCGACAACTTC **e** **d** **e**
Traditional EXPAR template for miR122-2′me	CAAACACCATTGTCACACTCCAAACAGACTCTCAAACACCATTGTCACACTCCAp **y** **y**
Traditional EXPAR template for ath-miR156a	GTGCTCACTCTCTTCTGTCAAACAGACTCTTGTGCTCACTCTCTTCTGTCAp **y** **y**
Let-7a	UGAGGUAGUAGGUUGUAUAGUU
miR21	UAGCUUAUCAGACUGAUGUUGA
miR24	UGGCUCAGUUCAGCAGGAACAG
miR143	UGAGAUGAAGCACUGUAGCUC
miR156a-1 (one nucleotide difference)	UGAGAGAAGAGAGUGAGCAC
miR156a-2 (two nucleotides difference)	UGAGAGAAGAGAGUCAGCAC
miR156a-3 (three nucleotides difference)	UGAGAGAACAGAGUCAGCAC
Stem-loop RT-Probe	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGT

(for the RT-PCR method)	GCTC
Forward primer (for the RT-PCR method)	GCCGCTGACAGAAGAGAGTG
Reverse primer (for the RT-PCR method)	GTGCAGGGTCCGAGGT

Note: "p" indicates a phosphate group. "2′me" in 3′-terminus indicates 2′-O-methylation. "x" indicates a C3 linker to facilitate the formation of 3WJ structure. In the traditional EXPAR templates, "**y**" sequences are complementary to the target plant miRNAs, respectively. A C3 linker between sequences **c** and **b*** in the 3WJ template, and two thymine nucleotides between sequences **a** and **b** in the 3WJ primer are necessary to reduce the structure rigidity at the junction and stabilize the miRNA-tethered 3WJ structure.

2. Extraction of total RNA from *Arabidopsis thaliana*

The *Arabidopsis thaliana* were kindly provided by College of Life Science, Shaanxi Normal University. The whole plants were removed at seedling stage (~80 mg), frozen and ground into powder with liquid nitrogen, and then 1 mL Trizol was added and kept going to grind until the liquid nitrogen was completely evaporated. The total RNA was extracted following the Trizol extraction protocols, and the amount of the extracted total RNA was quantified on a NANODROP 2000.

3. Standard procedures of the 3WJ-EXPAR system for miRNA analysis

The reaction mixtures for the 3WJ-EXPAR system were prepared as part A and part B separately. Series dilutions of target miRNA were added in part A containing NEBuffer 3.1, corresponding 3WJ primer, 3WJ template, well-performing EXPAR template, dNTPs, DEPC-treated water and incubated at 37 ^{o}C for 10 min after initial denaturation at 85 ^{o}C for 1 min. SYBR Green I were added in part B consisted of ThermoPol buffer, the nicking endonuclease Nt.BstNBI, Bst DNA Polymerase large Fragment, and DEPC-treated water. Parts A and B were mixed immediately before being placed in the Real-Time PCR System. The 3WJ-EXPAR was conducted in a final volume of 10 μL containing 0.5× NEBuffer 3.1 (25 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol), 1 nM 3WJ primer, 1 nM 3WJ template, 250 μM dNTPs, 10 nM well-performing EXPAR templates, 1× ThermoPol buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), the nicking endonuclease Nt.BstNBI (0.2 U/μL), Bst DNA Polymerase Large Fragment (0.16 U/μL), SYBR Green I (0.4 μg/mL), and DEPC-treated water. The 3WJ-EXPAR was performed at 37 ^{o}C and the real-time fluorescence intensity was monitored at intervals of 30 s.

4. Standard procedures of traditional EXPAR for miRNA analysis

The traditional EXPAR for plant miRNA analysis was performed according to the protocols reported in the previous literature.[1] Typically, the reaction mixtures for traditional EXPAR were prepared on ice separately as part A and part B. Series dilutions of target miRNA were added in part A containing NEBuffer 3.1, traditional EXPAR template, dNTPs, and DEPC-treated water. SYBR Green I were added in part B consisted of ThermoPol buffer, the nicking endonuclease Nt.BstNBI, Bst DNA Polymerase Large Fragment, and DEPC-treated water. Parts A and B were mixed immediately before being placed in the Real-Time PCR System. The traditional EXPAR was performed in a final volume of 10 μL containing 0.5× NEBuffer 3.1 (25 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol), 250 μM dNTPs, 100 nM traditional EXPAR templates, 1× ThermoPol buffer (20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$， 2 mM $MgSO_4$, 0.1% Triton X-100), the nicking endonuclease Nt.BstNBI (0.4 U/μL), Bst DNA Polymerase Large Fragment (0.008 U/μL), SYBR Green I (0.4 μg/mL), and DEPC-treated water. The traditional EXPAR was performed at 55 °C, and the real-time fluorescence intensity was monitored at intervals of 30 s.

5. Schematic illustration of the principle of traditional EXPAR for the detection of human miRNAs

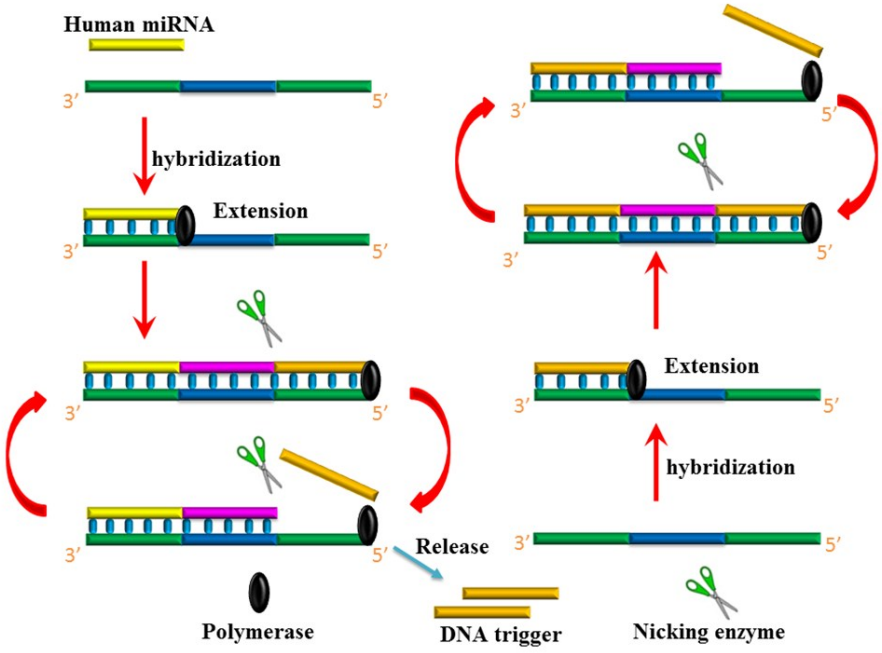

Fig. S1 Schematic illustration of the principle of traditional EXPAR for the detection of human miRNAs. It should be

noted that the sequence of the released short DNA trigger is the same as that of the miRNA target, except that the ribonucleotides and uridine in the miRNA are replaced with deoxyribonucleotides and thymine, respectively, in the DNA trigger.

6. Specificity evaluation of the 3WJ-EXPAR system towards different synthetic miRNA sequences with 1~3 nucleotides difference to ath-miR156a

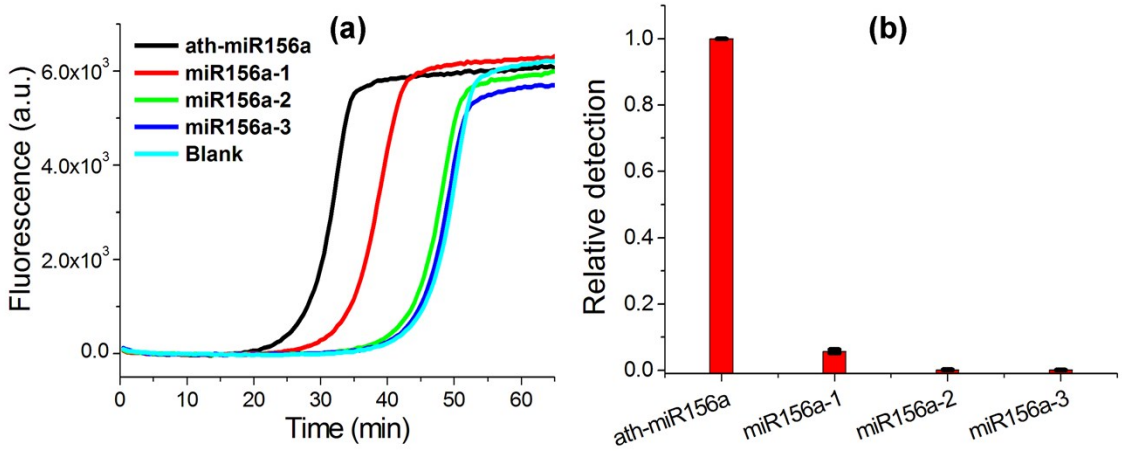

Fig. S2 Specificity evaluation of the 3WJ-EXPAR system towards different synthetic miRNA sequences with 1~3 nucleotides difference to ath-miR156a by using the ath-miR156a-specific 3WJ probes. **(a)** real-time fluorescence curves for the detection of miRNA sequences similar to ath-miR156a; **(b)** relative detection of the different miRNA targets. The concentrations of all of these miRNAs are 10 pM.

We have investigated whether similar miRNA sequences with only 1~3 nucleotides difference from ath-miR156a could interfere with the detection of ath-miR156a. It can be seen from Fig. S2 that only the ath-miR156a triggers a significant fluorescence signal. All the responses of miR156a-1 (1 nucleotide difference), miR156a-2 (2 nucleotides difference) and miR156a-3 (3 nucleotides difference) can be clearly discriminated from that of ath-miR156a. Particularly, both the miR156a-2 and miR156a-3 only arouse negligible response. Although the miR156a-1 produces positive response, its interference for the detection of target ath-miR156a is calculated to be only 5.3%, clearly demonstrating the high specificity of the 3WJ-EXPAR for discriminating similar target sequences.

In Fig. S2b, the relative detection values are calculated as follows. We suppose that the *POI* corresponding to the signals of ath-miR156a, miR156a-1, miR156a-2 and miR156a-3 are POI_0, POI_1, POI_2 and POI_3, respectively. According to the standard correlation equation for ath-miR156a determination (Fig. 4), POI_0, POI_1, POI_2 and POI_3 are corresponding to ath-miR156a amount as C_0, C_1, C_2 and C_3, respectively. The relative detection values for miR156a-1, miR156a-2 and miR156a-3 are calculated to be C_1/C_0, C_2/C_0 and C_3/C_0, respectively. Accordingly, the interference for detection of ath-miR156a amount arisen from the signals produced by the same concentration of miR156a-1,

miR156a-2 and miR156a-3 are calculated to be 5.3%, 0.1% and 0.06%, respectively.

7. Results for the detection of ath-miR156a in *Arabidopsis thaliana* extracts by the 3WJ-EXPAR method

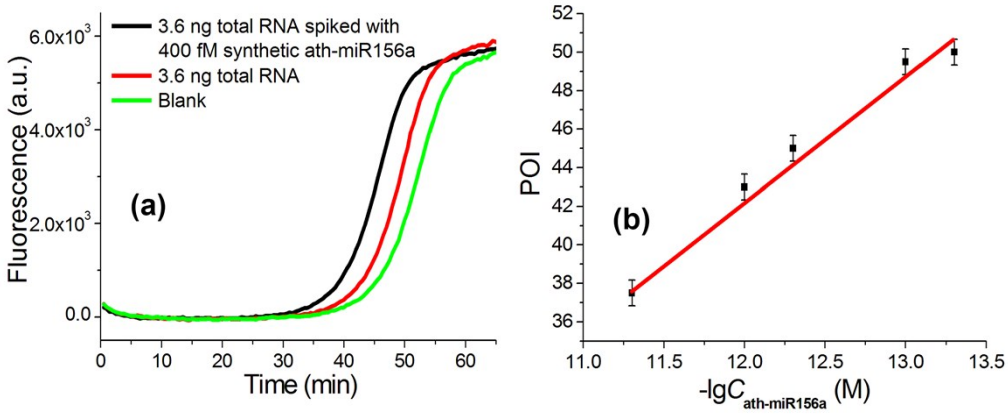

Fig. S3 Detection of ath-miR156a in total RNA sample extracted from *Arabidopsis thaliana*. **(a)** the real-time fluorescence curves for detection of 3.6 ng total RNA sample as well as 3.6 ng total RNA spiked with 400 fM synthetic ath-miR156a; **(b)** The simultaneously constructed calibration curve by using synthetic ath-miR156a as the standard. The determined concentration of ath-miR156a in total RNA sample is calculated in the 10 μL 3WJ-EXPAR system.

8. Quantification of ath-miR156a in total *Arabidopsis thaliana* RNA by stem-loop RT-PCR method

The stem-loop RT-PCR protocol is adapted from the literature methods,[2,3] which contains two processes: reverse transcription and real-time PCR. First, the stem-loop RT probe is hybridized to the target miRNA, and then inversely transcribed by reverse transcriptase. Next, the transcription products are detected by fluorescence quantitative real-time PCR using SYBR Green I as the signal reporter. It is worthy to point out that such RT-PCR-based methods for miRNA analysis are of at least two steps with lengthy assay time, and require precision temperature cycling process. The detailed experimental procedures are as follows:

Reverse transcription reaction. The reverse transcription of the target miRNA (or total RNA sample) was carried out in a mixture containing 1.2 μL of RNase-free water, 0.5 μL of 1 μM stem–loop RT-probe, 1 μL of 5 × RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 1 μL of 2.5 mM dNTPs, 0.2 μL of 200 U/μL ProtoScrip Ⅱ reverse transcriptase and 0.1 μL of 40 U/μl RNase inhibitor. The 5 μL reactions were incubated for 30

min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C.

Quantitative real-time PCR analysis. A volume of 5 μL of the transcription products was transferred to the PCR reaction mixture with a final volume of 10 μL. The PCR reaction mixture contained the forward primer and reverse primer (each 200 nM), 250 μM dNTPs, 0.4 × SYBR Green I, 0.5 U JumpStart™ Taq DNA Polymerase and 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001(w/v) gelatin, pH 8.3). The PCR reaction was carried out with a StepOne Real-Time PCR System (Applied Biosystems, USA) by using hot start of 94 °C for 2 min, followed by 50 cycles of 94°C for 15 s, 60°C for 1 min and 72 °C for 20 s.

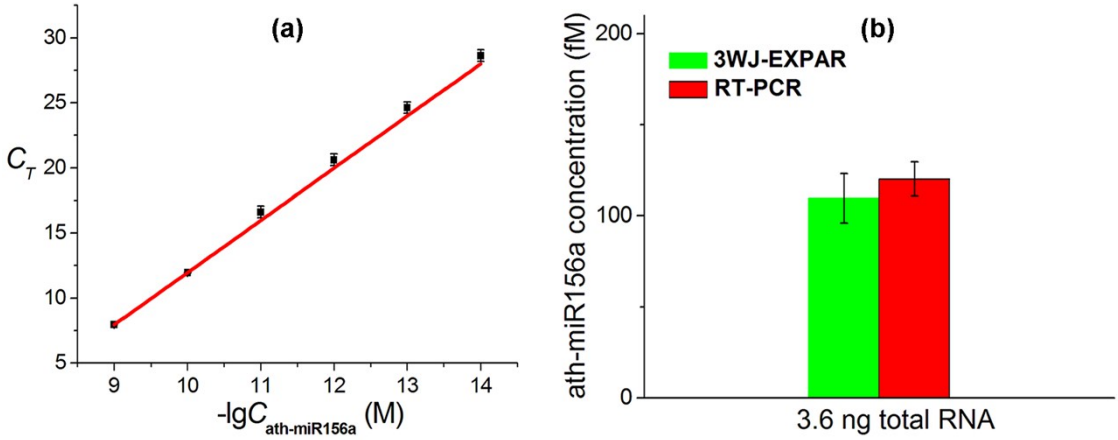

Fig. S4 (a) Standard calibration curve of the stem-loop RT-PCR protocol for the detection of ath-miR156a, which is constructed by using series dilutions of synthetic ath-miR156a standard; **(b)** the comparison of the determined amount of ath-miR156a in 3.6 ng total *Arabidopsis thaliana* RNA by using the stem-loop RT-PCR protocol and the 3WJ-EXPAR strategy, respectively.

References

[1] E. Tan, B. Erwin, S. Dames, T. Ferguson, M. Buechel, B. Irvine, K. Voelkerding and A. Niemz, *Biochemistry*, 2008, **47**, 9987-9999.

[2] C. Chen, D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Anderson, K. Q. Lao, K. J. Livak and K. J. Guegler, *Nucleic Acids Res.*, 2005, **33**, e179.

[3] F. Chen, C. Fan and Y. Zhao, *Anal. Chem*., 2015, **87**, 8758-8764.