Supporting Information

Inhibitory Impact of 3' Terminal 2'-O-Methylated Small Silencing RNA on Target-Primed Polymerization and Unbiased Amplified Quantification of the RNA in *Arabidopsis Thaliana*

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1. Oligonucleotide sequences

All the sequences are listed in Table S1. The letters highlighted in red in miR156a (2'me) and miR156a-OH (2'OH) means the 2'-O-methyl group and 2'-hydroxy group, respectively. DNA_{156a} and DNA_{156a}-mC share the same sequence as miR156a. The red letter "m" in DNA_{156a}-mC means the methyl group at the 5 position of cytosine. Molecular beacon (MB) is labeled with fluorescence group FAM at 5' end and quenched group DABCYL at 3' end. S-template and S-primer are used for assays by base-stacking hybridization, and 3-WJ primer together with 3-WJ template is employed in the design of 3-WJ structure. The 3T-PS template is the trifunctional stem-loop template containing a hemi-phosphorothioate recognition site, and the symbol (*) represents the phosphorothioate modification. 3T template shares the same sequence as 3T-PS template except the phosphorothioate modification. 3T product (ss-product in the text) is complementary with 3T-PS template. The recognition sequences of nicking enzyme Nt.BbvC I are all in bold. Reverse transcription (RT) primer, Forward primer and Reverse primer are designed for miR156a assay by stem-loop RT-PCR.

Table S1. Oligonucleotide sequences

Oligonucleotide	Sequence (5' to 3')
MiR156a	rUrGrA rCrArG rArArG rArGrA rGrUrG rArGrC rArC-2'me
MiR156a-OH	rUrGrA rCrArG rArArG rArGrA rGrUrG rArGrC rArC-2'OH
DNA_{156a}	TGA CAG AAG AGA GTG AGC AC
DNA_{156a}-mC	TGA CAG AAG AGA GTG AGC AmC
$Template_{156a}$	AGT CAG TGT CCT CAG **GCT GAG G**TT TTG TGC TCA CTC TCT TCT GTC A
MB	FAM-CCA CGA GTC AGT GT**C CTC AGC** GTG G-DABCYL
S-primer	CGT AGC TCG TAG GAT CCA GA
S-template	AGT CAG TGT CCT CAG **GCT GAG G**TT TTG TGC TCA CTC TCT TCT GTC ATC TGG
3-WJ primer	GTG CTC ACT CAT CCA AAA
3-WJ template	AGT CAG TGT CCT CAG **GCT GAG G**TT GTT TTG GTC TTC TGT CA
3T-PS template	TAT TGT GT**C* C*T*C* A*GC GCT GAG G**TT GTT TTG GTC TTC TGT CA
3T template	TAT TGT GT**C CTC AGC GCT GAG G**TT GTT TTG GTC TTC TGT CA
3T product	TCA GC**G CTG AGG** ACA CAA TA
RT primer	GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG TGC TC
Forward primer	GCC GCT GAC AGA AGA GAG TG
Reverse primer	GTG CAG GGT CCG AGG T

2. Gel electrophoresis analysis of polymerization products

Polymerization reaction by Bst 2.0 DNA polymerase was carried out in 20 μL of 1 x NEBuffer 4 containing 400 nM primers (MiR156a, MiR156a-OH, DNA_{156a} and DNA_{156a}-mC), 400 nM $Template_{156a}$, 0.25 units of Bst 2.0 DNA polymerase, 16 units of RNase inhibitor and 0.2 mM dNTPs. After incubation of 5 min at 55 ^{o}C, the mixture was heated up to 80 ^{o}C for 20 min. Products of polymerization reaction were analyzed by 3.5% agarose gel electrophoresis in 1× TAE buffer at a 70 V constant voltage for 60 min at room temperature. The gel was visualized by Syngene G:BOX Imaging System.

3. Polymerization reaction by Bst 2.0 DNA polymerase

For the analysis of polymerization reaction using SYBR Green I as fluorescence reporter, the reaction was performed in 20 μL of 1 x NEBuffer 4 containing 150 nM primers (2′-O-methylated small RNA or unmethylated one), 150 nM $Template_{156a}$, 0.5 x SYBR Green I, 0.25 units of Bst 2.0 DNA polymerase, 16 units RNase inhibitor and 0.2 mM dNTPs. The reaction temperature was set at 55 ^{o}C, and the assay was monitored by real-time fluorescence at an interval of 30 s by LightCycler 96. After that, the melting curve is recorded.

4. Impact of 3' terminal 2'-O-methylated small RNA on SDA

Using SYBR Green I as fluorescence reporter, the SDA mixture contained 10 nM primers (2′-O-methylated small RNA or unmethylated one), 150 nM $Template_{156a}$, 0.25 units of Bst 2.0 DNA polymerase, 2.5 units of Nt.BbvCI, 16 units of RNase inhibitor and 0.2 mM dNTPs in 20 μL of 1 x NEBuffer 4. All the parameters in LightCycler 96 are the same as that of polymerization reaction.

For a more quantitative analysis of inhibition, MB is used as signal reporter. The reaction was performed in 20 μL of 1 x NEBuffer 4 containing 10 nM primers (2′-O-methylated small RNA or unmethylated one), 10 nM $Template_{156a}$, 250 nM MB, 0.25 units of Bst 2.0 DNA polymerase, 2.5 units of Nt.BbvCI, 16 units of RNase inhibitor and 0.2 mM dNTPs. After incubated at 55 ^{o}C for 30 min in water bath, the fluorescence spectra were recorded by FluoroMax-4 fluorescence spectrometer. For Vent exo^{-} DNA polymerase and KF^{-}, their concentrations were 1 units and 0.62 units, respectively. Other conditions are the same as those of Bst 2.0 DNA polymerase except 37 ^{o}C for KF^{-} and the different buffer (1 x Thermoper buffer and 0.5 x NEBuffer 4) for Vent exo^{-}. To investigate the concentration-dependent effect on suppression behavior, the concentrations of target and template is increased to 50 nM without any other change.

To evaluate the effect of reaction buffer on the inhibition by this modified small RNA, Vent exo^{-} DNA polymerase is used as a sample. Three buffers including 1 x Thermoper buffer, 1 x NEBuffer 4, and the mix of 1 x Thermoper buffer and 0.5 x NEBuffer 4 are tested. Other conditions are the same as above.

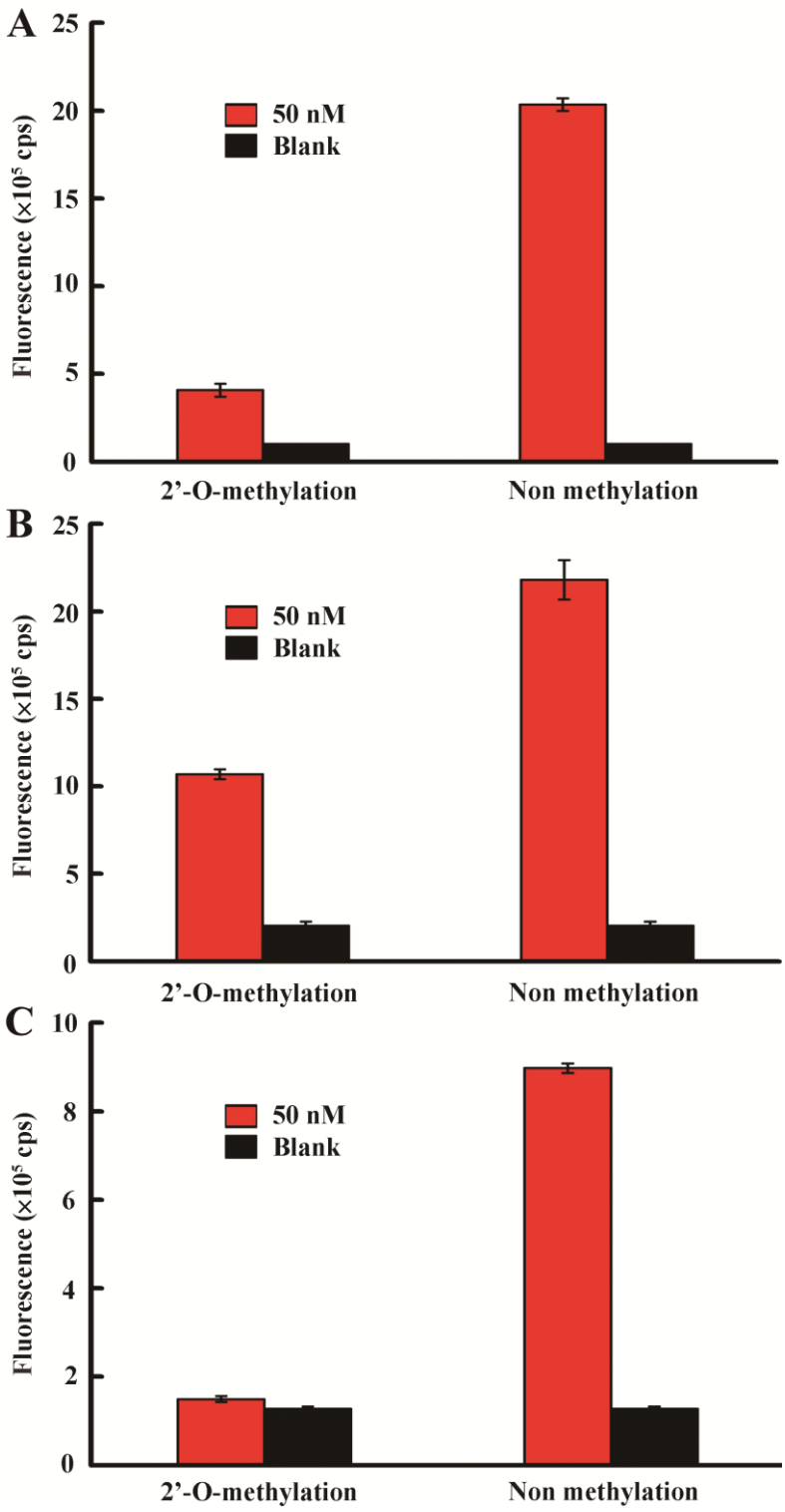

Figure S1. The fluorescence response of high target concentration (50 nM) on SDA using MB as signal reporter by three representative DNA polymerases (A, Bst 2.0 DNA polymerase; B, KF^-; C, Vent exo^- DNA polymerase). All the other conditions are the same as that of 10 nM. We expect that increasing the nucleic acid concentration may improve the polymerization efficiency. However, the ratios of signal (50 nM) to

noise (Blank) show no significant increase despite the enhanced fluorescence intensity. And the fluorescence responses of methylated target are all much lower than those of unmethylated target. These results indicate that increasing target concentration can’t weaken the inhibition by 2′-O-methylation.

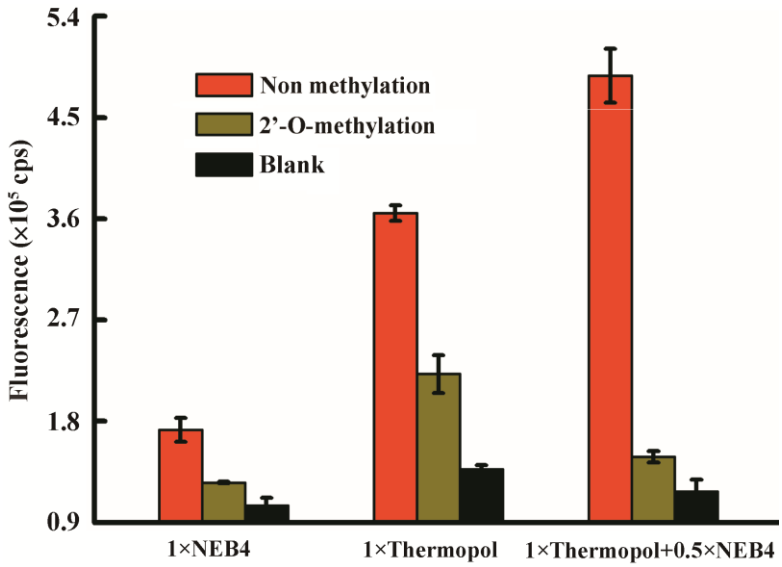

Figure S2. The fluorescence response of SDA by Vent exo$^-$ DNA polymerase in different buffers using MB as signal reporter. Obviously, the inhibition impact by 3′ terminal 2′-O-methylated small RNA is observed in all buffers despite their various fluorescence intensity. It indicates that the buffer condition doesn't affect this inhibition.

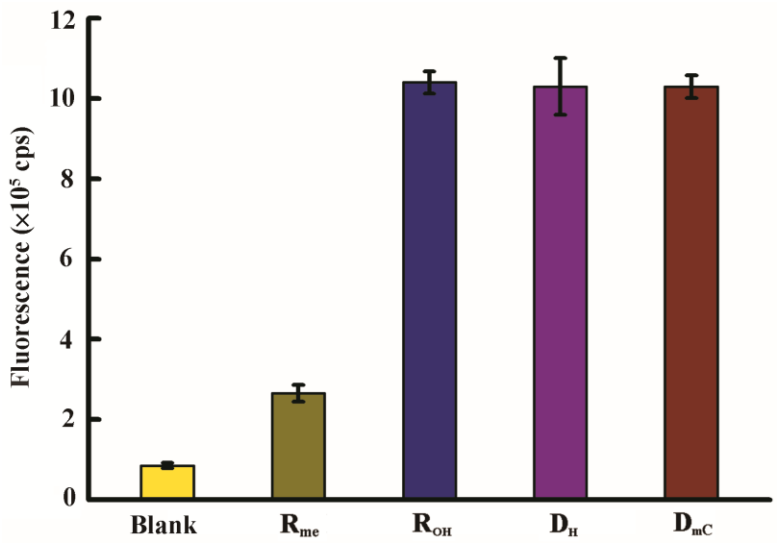

Figure S3. The fluorescence response of targets with different 3' terminal structures on SDA catalyzed by Bst 2.0 DNA polymerase using MB as signal reporter.

5. Base-stacking hybridization assisted unbiased solution

To achieve base-stacking hybridization assisted unbiased recognition, stacking primer (S-primer) are designed to be complementary (5-bp) with stacking template (S-template). The target (2′-O-methylated small RNA or unmethylated one) can hybridize with S-template adjacently to S-primer. This interaction strongly stabilizes the hybridization between S-primer and S-template, leading to polymerization reaction from 3′-OH of S-primer. Thus, the inhibition of 2′-O-methylation on polymerization can be avoided. Subsequently, the SDA reaction proceeds with the help of nicking enzyme. The fluorescence signal can be observed after the hybridization of SDA product with MB. However, in the absence of target, S-primer and S-template can't form stable duplex structure to trigger SDA.

For the unbiased detection of 2′-O-methylated small RNA, miR156a were added in 20 μL of 1×NEBuffer 4 containing 10 nM S-primer, 10 nM S-template, 250 nM MB, 0.25 units of Bst 2.0 DNA polymerase, 2.5 units of Nt.BbvCI, 16 units of RNase inhibitor and 0.2 mM dNTPs. The mixtures were incubated at 55 ^{o}C for 30 min. Subsequently, the fluorescence spectra were recorded by FluoroMax-4 fluorescence spectrometer.

6. 3-WJ structure assisted unbiased solution

For the design of 3-WJ structure, 3-WJ primer and 3-WJ template with 6 bp complementary sequences at 3′-OH termini of 3-WJ primer are used. They both are designed to be 10 bp complementary with target small RNA. In the absence of target, they can't form duplex with 3′-OH to perform SDA due to the low melting temperature ($T_m \approx 18\ ^oC$). Oppositely, when target small RNA is present, it is able to hybridize with 3-WJ primer and 3-WJ template simultaneously, forming the stable 3-WJ structure. Then, 3′-OH of 3-WJ primer in 3-WJ structure triggers the polymerization reaction ae well as subsequent SDA reaction. Thanks to the use of 3-WJ primer rather than 2′-O-methylated small RNA, the inhibition of 2′-O-methylation is avoided.

For the unbiased detection of 2′-O-methylated small RNA, miR156a were added in 20 μL of 1×NEBuffer 4 containing 10 nM 3-WJ primer, 10 nM 3-WJ template, 250 nM MB, 0.5 units of KF-, 2.5 units of Nt.BbvCI, 16 units of RNase inhibitor and 0.2 mM dNTPs. The mixtures were incubated at 37 ^oC for 30 min. Subsequently, the fluorescence spectra were recorded.

7. Demonstration of phosphorothioate-mediated protection against nicking

To verify the protection against nicking by phosphorothioate modification, 3T-PS template with phosphorothioate bases in recognition sequence for nicking enzyme is used to form stable duplex with 3T product, whereas 3T template out of phosphorothioate bases is used as control group. These duplex are incubated with nicking enzyme at 37 ^{o}C for 0.5 h, and then analyzed by melting curve using SYBR Green I as fluorescence reporter. In detail, the reaction mixture (20 μL 1×NEBuffer 4) contains 100 nM 3T product, 100 nM 3T-PS template or 3T template, and 0.5 X SYBR Green I with or without 2.5 units of Nt.BbvCI. As shown in Figure 5A, the sample of 3T-PS template with nicking enzyme still hold the similar T_m value (about 64 ^{o}C) as those of 3T-PS template and 3T template without nicking enzyme. Oppositely, no peak is observed at 64 ^{o}C in the curve of 3T template with nicking enzyme, indicating the cleavage of this duplex into two short oligos. All these results demonstrated the phosphorothioate modification in nicking site can really inhibit the cleavage by nicking enzyme.

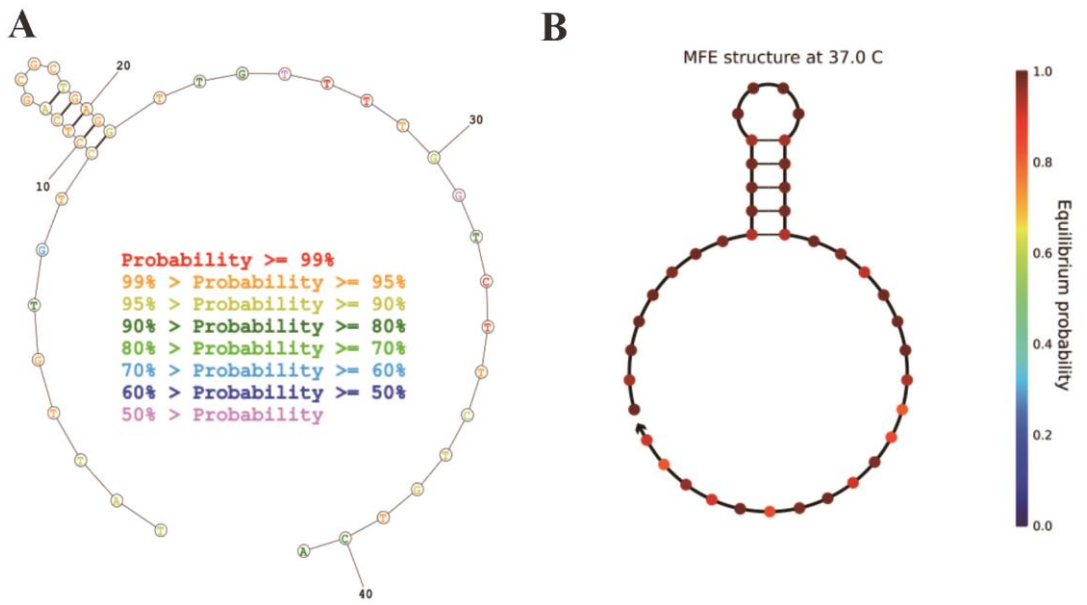

Figure S4. The lowest free energy structure of 3T-PS template obtained under 37 ^{o}C by two different tools, respectively. A is from RNAstructure, and B is achieved by NUPACK. Both of these two secondary structures are the same with a 5-bp stem-loop structure (> 95% probability) containing a hemi-phosphorothioate recognition site for nicking enzyme.

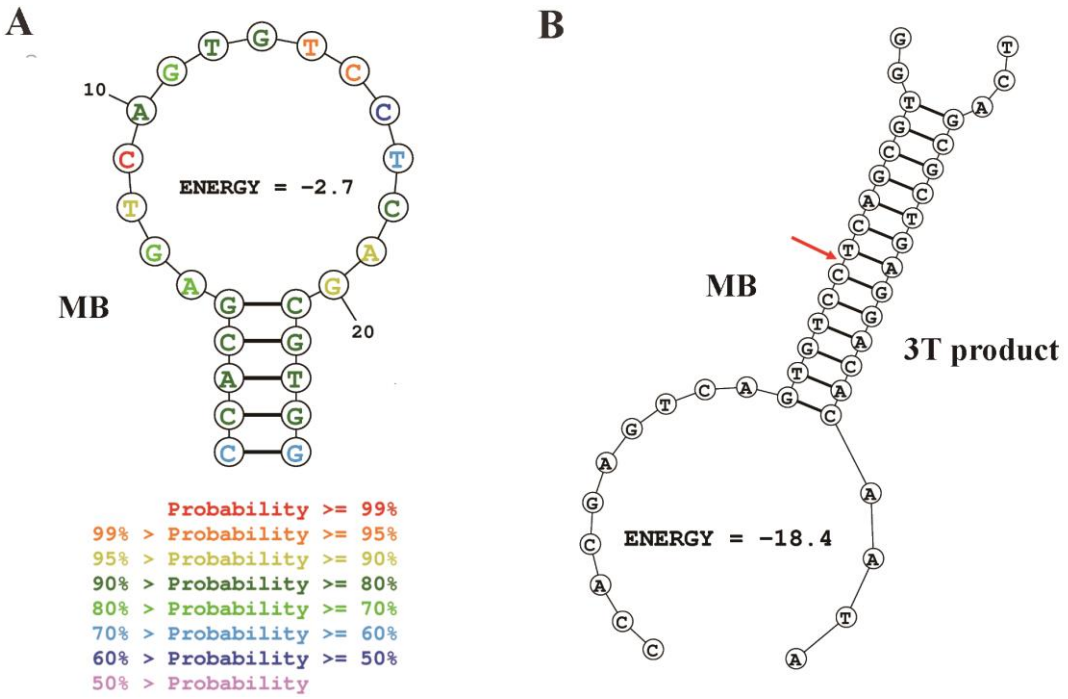

Figure S5. The lowest free energy structure of MB (A) and for MB and 3T product obtained under 37 ^{o}C from RNAstructure Web Server (http://rna.urmc.rochester.edu/RNAstructureWeb/index.html). The red arrow in (B) indicates the nicking site of MB.

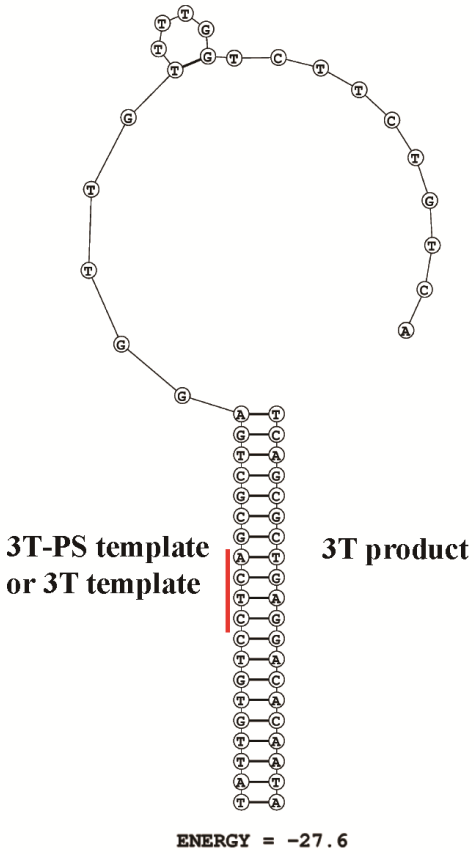

Figure S6. The lowest free energy structure for 3T-PS template or 3T template and 3T product obtained under 37 ^{o}C from RNAstructure bifold Web Server (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/bifold/bifold.html). The low free energy value indicates the stable duplex between 3T-PS template or 3T template and 3T product. The letters (CCTCAG) beside the red line represent the phosphorothioate modifications in 3T-PS template. After the cleavage of 3T template by nicking enzyme, the duplex break and can't stabilize itself at higher temperature. Oppositely, if the phosphorothioate resist the nicking reaction, the duplex of 3T-PS template and 3T product is still stable at higher temperature.

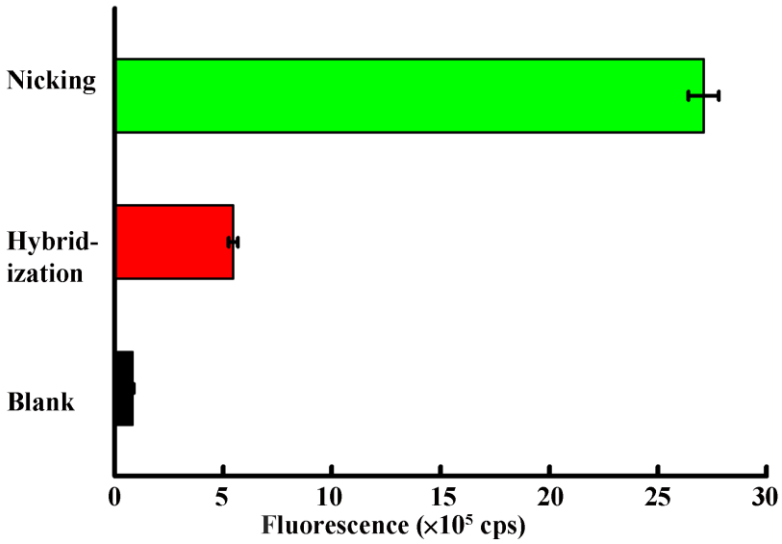

Figure S7. The fluorescence response of different system under SDA reaction conditions to demonstrate the recycling nicking of MBs. The experiments are performed in 20 μL 1×NEBuffer 4 contains 250 nM MB, 0.5 units of KF-, 2.5 units of Nt.BbvCI, 16 units of RNase inhibitor and 0.2 mM dNTPs. The Blank sample (black one) and Nicking sample (green one) indicate without and with 50 nM 3T product, respectively. And the Hybridization sample (red one) does not contain Nt.BbvCI with other conditions similar to those of Nicking sample. It can be seen that the fluorescence signal of hybridization reaction is much lower than that of nicking reaction, demonstrating the recycling cleavage of MBs. This result is important for the design of our strategy.

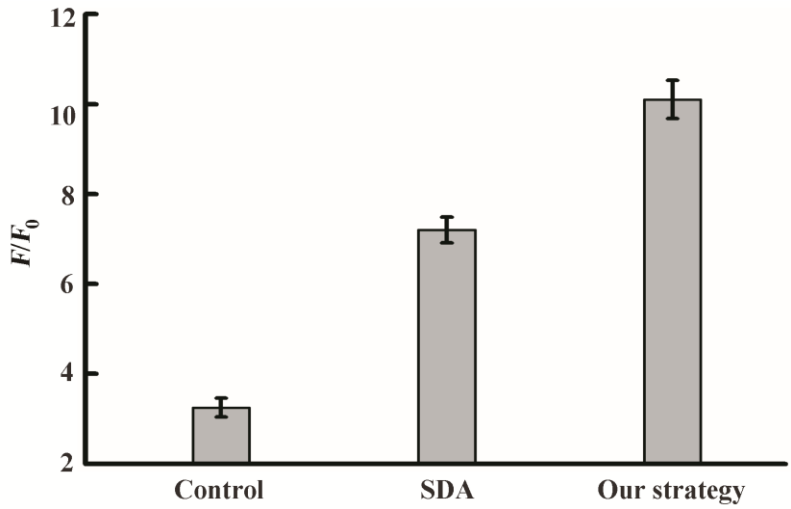

Figure S8. The fluorescence enhancement of 3T template system (Control), SDA system (SDA) and 3T-PS template system (Our strategy). The template of SDA system is 3-WJ template. The only difference of these three samples is the used template (see **Table S1**). F and F_0 represent the fluorescence intensity of signal (10 nM target) and blank (without target), respectively. The F/F_0 value of Control is about 3.5-fold lower than that of our strategy, implying the break of 3T template rather than 3T-PS template (phosphorthioate-protected). And the higher F/F_0 value of our strategy than that of SDA demonstrate the recycling cleavage of MBs by nicking enzyme. Combined these two results, our amplified strategy has been confirmed.

8. Small RNAs extracts of *Arabidopsis thaliana*

The *Arabidopsis thaliana* were kindly provided by Prof. Tian Li. The whole plants were frozen in liquid nitrogen and stored at -80 ^{o}C immediately after harvest. Small RNA was isolated by commercial kit (RNAiso for small RNA, Takara) according to the manufacturer's procedures. The purity and concentration of the small RNA sample are determined by UV-vis methods using the absorbance values at 260 nm and 280 nm.

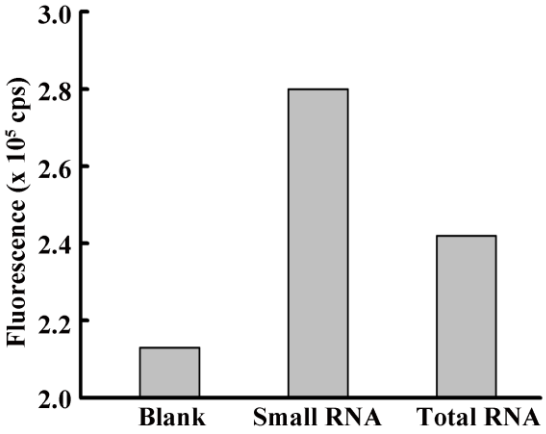

Figure S9. The fluorescence response of the proposed method upon the addition of extracted RNA samples from *Arabidopsis thaliana*. 10 μg of small RNA and 15 μg of total RNA were used, respectively.

9. Stem-loop RT-PCR for quantification of small RNA

As illustrated in Figure S10, Stem-loop RT-PCR included two processes: RT and real-time PCR. [1] First, the stem-loop RT primer is hybridized to a small RNA molecule, and then inversely transcribed by reverse transcriptase. Next, the transcription products are detected by fluorescence quantitative PCR using SYBR Green I as signal reporter. In compared with other PCR-based methods such as poly (A) PCR, [2] this one is not affected by 2′-O-methylation as it requires no enzymatic reaction at 3′ termini. It is worthy to point out that these PCR-based methods for small RNA analysis are of at least two steps with lengthy assay time, and require precision temperature cycling process in an expensive thermal cycler.

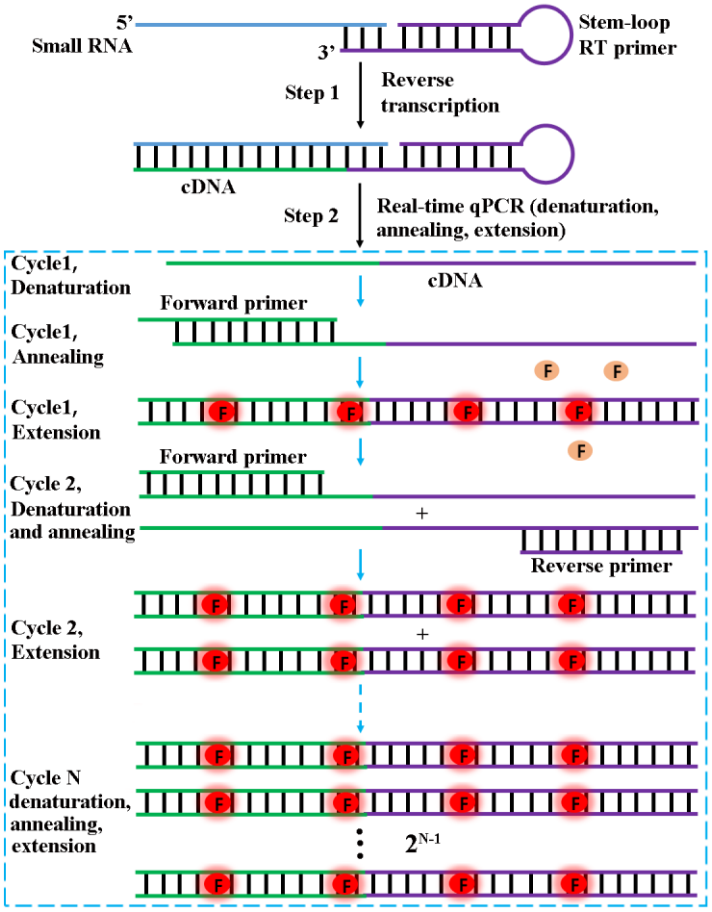

Figure S10. Scheme of stem-loop RT-PCR for small RNA. The letter 'F' indicates the fluorescent dye SYBR Green I. cDNA, generated in step 1 by reverse transcription, is acting as template for the PCR amplification in step 2.

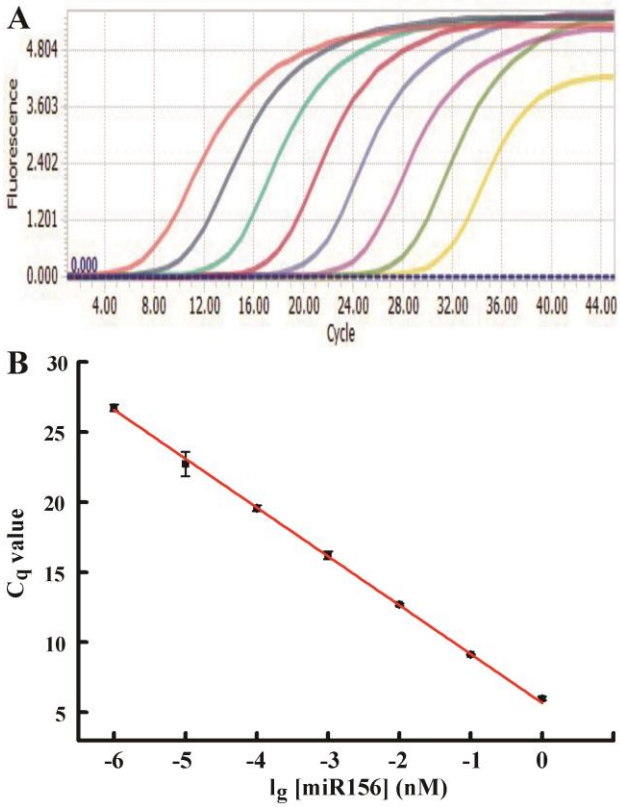

Figure S11. Amplification plot (A) and standard curve (B) of miR156a assay by stem-loop RT PCR. The miR156a concentration (A) is 1 nM, 100 pM, 10 pM, 1 pM, 100 fM, 10 fM, 1fM and 0 fM, respectively.

Reference:

1. Caifu Chen, Dana A. Ridzon, et.al. *Nucleic Acids Research*, **2005**, 33, 20, e179.
2. Rui Shi and Vincent L. Chiang. *BioTechniques*, **2005**, 39, 519-525.