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Construction of short tandem target mimic (STTM) to block the functions of plant and animal microRNAs

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Abstract

Small RNAs are widespread in plants and animals. They largely include microRNAs (miRNAs) and short interfering RNAs (siRNAs), and they play key roles in gene and chromatin regulations. Here we describe in detail the method for an effective construction of the recently developed short tandem target mimic (STTM) technology to block small RNA functions in plants and animals. STTM is a powerful technology complementing the previous target mimic (TM) in plants and the miRNA sponge, as well as the recently defined endogenous competing RNA (CeRNA) in animals. We expect STTM will not only be effective in blocking small RNA functions in plants but will also become a popular approach in animals.

Keywords

Small RNA; miRNA; miR165/166; miR-30d; Short tandem target mimic (STTM); Target mimic; miRNA sponge; CeRNA

1. Introduction

Small RNAs including microRNA (miRNA) and small interfering RNA (siRNA), play an important role in regulating endogenous gene expression, DNA methylation and chromatin remodeling, silencing invaded viruses, and managing transposon activities [1–4]. The 20–24 nt small RNAs execute their functions mainly by complementarily binding to their target sites, leading to either target mRNA degradation or translational repression [5]. The regulatory roles of small RNAs cover almost all aspects of a eukaryotic organism's life cycle, such as growth, development, epigenetics, genome integrity, environmental stress resistance, and host defense [2–4]. With the help of the deep sequencing technique, large populations of small RNAs have been identified in plants and animals and the numbers are still expanding [6,7]. Previous methods for the interpretation of gene function relied much on the generation and characterization of genetic mutants [8]. However, this approach is difficult to apply to the study of small RNAs due to their small sizes and their multiple members with overlapping functions as gene families. Currently, the major approaches to small RNA investigation are to generate and analyze the transgenic lines that either express

miRNA-resistant targets [9,10] or overexpress the genes that encode small RNAs [11–13]. Whereas one small RNA usually regulates several target genes [14], this method can only uncover partial functions of a particular small RNA *in vivo*. The increasing number of small RNAs found in various organisms requires a more effective approach to unveiling their functions and pathway networks. In this report, we introduced our newly developed approach, short tandem target mimic (STTM), to explore the functions of small RNAs by simultaneously blocking all the members in a miRNA family so as to de-repress all the target genes through a single genetic transformation event [15].

The invention of STTM was based on the discovery of target mimicry (TM), which is an endogenous mechanism that modulates miR399 activity in *Arabidopsis thaliana* [16]. *INDUCED BY PHOSPHATE STARVATION 1 (IPS1)* is a non-protein-coding gene that produces transcripts partially complementary to miR399, forming a central three-nucleotide bulge in *IPS1*-miR399 complexes to prevent the cleavage of *IPS1* transcripts by miR399-associated/induced silencing complexes (RISCs) [16]. In this way, the miR399 activity in regulating its target gene *PHOSPHATE 2 (PHO2)* is subjected to the auto-modulation by *IPS1* in response to phosphate starvation. Considering the fact that miR399's abundance is uninfluenced by *IPS1*, it was suggested that miR399 was being sequestered by *IPS1* transcripts through TM [16].

While endogenous TM was first discovered in plants [16], endogenous competing RNAs (CeRNAs) were also observed in animals recently [17–19]. Similar to plant TM, CeRNAs act as pseudo-targets to sequester specific miRNAs away from their *bonafide* targets and reduce the miRNA activities [17,20]. Currently, the study of CeRNAs in animals is at its beginning [17,19], and there is no established approach to constructing an artificial CeRNA that can be applied in animals. As was demonstrated by the example of the mouse miR-30d in this study, STTM is also able to function as an artificial CeRNA to block or to down-regulate any miRNAs of interest in animals. The following protocol is described in detail for the efficient construction and expression of STTMs in plants and animals, respectively.

2. Effective construction of STTMs to be expressed in plants and animals

STTM is an artificial short (~100 nt) non-coding RNA that can be expressed through genetic engineering (Fig. 1). The central part of STTM was composed of two small RNA binding sites and a spacer/ linker (Fig. 1C) [15]. The two binding sites are complementary to target small RNAs except for the central three-nucleotide bulge (Fig. 1C) [15]. This bulge stuck out between the 10th and 11th nucleotide of the targeted small RNA, so that the binding sites could trap small RNAs without being cleaved by them. The spacer was 48–88 nt long with a weak hairpin secondary structure that has been tested essential (Fig. 1C) [15]. The spacer sequence was designed to contain a *SwaI* site in its middle for the gene construction needs (See detailed protocol section below).

To construct an STTM in an effective manner, we first generated the pOT2-Poly-Cis by introducing a 2×35S (d35S) promoter, a DNA fragment that termed Poly-Cis (for easy cloning purposes), and a 35S terminator (T35S) into the pOT2 vector (Fig. 2A). Through a polymerase chain reaction (PCR), the Poly-Cis was replaced by STTM structure, which resulted in the pOT2-STTM165/166 to be used for targeting miR165/166 in *Arabidopsis* (Fig. 2B). Primers used for the generation of pOT2-STTM165/166 were composed of a 3' complementary part and a 5' tail. The 3' complementary part was designed to bind the vector through full base pairing to initiate the PCR extension. The 5' tail of each primer was composed of half of the STTM sequences. At the far 5' end of the primer, there was an introduced *SwaI* site, protected by a three-nucleotide GCC for easy digestion by *SwaI*. During PCR, the 5' tail of each primer was amplified along with the main vector sequence

that included the promoter, terminator, the antibiotic selection marker, and the replication origin (Fig. 1A). The liner PCR product was then subjected to *SwaI* digestion and a subsequent self-ligation, which resulted in pOT2-STTM (Figs. 1B and 2B). To introduce the STTM structure into plants, such as *Arabidopsis*, through flower dipping, the STTM should be fused to a binary vector. To achieve this goal, the entire pOT2-STTM construct was firstly subjected to the pBR322 replication origin deletion through PCR. Both the forward and reverse primers used for this PCR contained a *PacI* site, which introduced the *PacI* site to both ends of the liner PCR product. This PCR product containing a d35S promoter, STTM165/166, a T35S terminator, as well as the chloramphenicol acetyltransferase (CAT) selection marker, was further sub-cloned into a binary vector such as the modified pFGC5941-*PacI* (Fig. 3A), pCambia1300-*PacI*, or pCambia2300-*PacI* through the introduced *PacI* site (Fig. 3).

To construct STTM for targeting animal miRNA, the 35S promoter and 35S terminator in the pOT2 vector can be replaced (not pursued in this study) by the EF-1 α Promoter and the SV40 polyadenylation signal that are active in animals. Alternatively, the STTM of ~100 bp can be synthesized and cloned into the pEGP-miR vector (Fig. 4A, Cell Biolabs) through *BamHI* and *NheI* sites located inside the human β -globin intron upstream of GFP-Puro, resulting in pEPG-STTM (Fig. 4B). pEPG-STTM would then be transfected into mammalian cell lines to test its effects on animal miRNA degradation.

3. Materials and reagents

1. Vectors to be used: pOT2, pFGC5941-*PacI* (Fig. 3A), pEGP-miR (Fig. 4A, Cell Biolabs), pEGP-miR Null Control (Cell Biolabs), and the modified pCambia1300-*PacI* and pCambia2300-*PacI* (not shown);
2. Primers synthesized in Integrated DNA Technology (IDT): STTM construction primers (Table 1), the origin deletion primers (Table 2) and the STTM30ad-88nt-Linker.
3. PCR amplification and DNA cloning: long amplification Tag DNA polymerase (2.5 U/ μ l), dNTPs and restriction enzymes including *SwaI*, *PacI*, *BamHI* and *NheI* from New England BioLabs, Agrose, G-25 columns (Roche Applied Science), Wizard[®] SV Gel and PCR Clean-Up kit and T4 DNA Ligase (3 U/ μ l) from Promega.
4. DNA transformation, bacterial strains, and antibiotics: *Escherichia coli* DH5- α , Agrobacterium COR308, Gentamycin (Caisson, 20 mg/ml), CAM^R (25 mg/ml), Kanamycin (25 mg/ml) and Tetracycline (5 mg/ml) from Fisher.
5. Plasmid and RNA extraction: Plasmid Miniprep kit (QIAGEN), Trizol reagent (Invitrogen)
6. Mouse cell culture and transfection: Dulbecco's modified Eagle's medium, fetal bovine serum and 1% penicillin–streptomycin from Invitrogen, Amaxa Nucleofector system (Lonza).
7. Real-time PCR and luciferase assay: TaqMan miRNA qPCR detection kit (Applied Biosystem), rDNase I (Sigma), Dual-luciferase reporter assay kit (Promega).

3.1. Protocol

3.1.1. Construction of sub-cloning vectors for STTM

- pOT2-Poly-Cis is designed for one-step production of STTM by PCR using a small (1665 bp) sub-cloning vector termed pOT2 (<http://www.fruitfly.org/about/methods/>)

[pOT2vector.html](#)). The pOT2 is modified by eliminating the *HindIII* site in the chloramphenicol acetyltransferase gene (CAT/CAM^r). To express STTM constitutively in plants, a 2×35S promoter (d35S) and a 35S terminator (T-35S) are introduced into the pOT2 vector through the *SmaI* and *HindIII*, and *EcoRI* and *XbaI* sites. *Note*: d35S may not be ideal for monocot plants such as rice and maize. Other promoters, like maize ubiquitin promoter may be optimized for use in monocot plants.

- The Poly-Cis fragment is introduced between the d35S and T-35S through the *HindIII* and *EcoRI* site to serve as a spacer for an easy promoter replacement or the convenience of STTM cloning (Fig. 2A).
- For expressing STTM in animal cells, the d35S and T-35S can be replaced by the EF-1α Promoter and the SV40 polyadenylation signal through the *SmaI* and *HindIII*, and *EcoRI* and *XbaI* sites, respectively (not pursued in this study). Alternatively, the STTM of ~100 bp can be synthesized and inserted into the human β-globin intron of pEGP-miR vector (Cell Biolabs) through *BamHI* and *NheI* sites, resulting in pEPG-STTM (Fig. 4B).

3.1.2. PCR production of STTM in pOT2-Poly-Cis vector

3.1.2.1. STTM structure: STTM, an artificial non-coding RNA that gets expressed from a short engineered DNA, has two small RNA binding sites and an artificial linker (48–88 NT) between them (Fig. 1C). Below is the backbone of the STTM (96 bp plus the 5′ end 25 bp portion identical to the 3′ end section of d35S and the 3′ end 25 bp portion identical to 5′ end section of the T-35S: 5′

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catttgagaggacagcccAAGCTTNNNNNNNNNNNNbbbNNNNNNNNNN
gttggtgtgtatggtctaatttaaatatggtctaaagaagaagaatNNNNNNNNNN
bbbNNNNNNNNNNGAATTCggtacgctgaatcaccag 3′, where the 5′ and 3′ lower case
nucleotides are from d35S and T-35S, respectively; the AAGCTT and GAATTC are HindIII
and EcoRI respectively; “N” indicates a nucleotide of the sequence that complements the
target small RNA; “bbb” indicates the tri-nucleotide “bulge” formed between the small
RNA and its binding site corresponding to the region between the 10th and 11th position of
the small RNA. The underlined 48 bp lower case nucleotides in the middle are the linker
between the two small RNA binding sites. Note: in the middle of the linker, there is a SwaI
site (atttaaat) designed for the construction purpose; the “bbb” bulge is designed to allow the
STTM capturing the targeted small RNA RISCs without being cleaved by them. Thus, the
“bbb” bulge is suggested to use 3 nucleotides that do not complement the 11th to 13th of the
small RNA [21,22].
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For example, the STTM structure targeting miR165/166 should be designed step by step as follows: The mature miR165 sequence is 5′ UCGGACCAGGCUUCAUCCCC 3′ and the miR166 sequence is 5′ UCGGACCAGGCUUCAUCCCC 3′. Therefore, the sequences for miR165 and miR166 binding sites should be: 5′ GGGGGAUGAAGC-CUGGUCCGA 3′ and 5′ GGGGAAUGAAGCCUGGUCCGA 3′, respectively. In order for the these sites to bind the mature miR165/166 without being cleaved by the miRNAs, a tri-nucleotide bulge must be designed in the region corresponding between the 10th and 11th positions of the mature miR165/166. Normally, we take the tri-nucleotide sequences (cua) from the *Arabidopsis IPS1* that corresponds to 10th and 11th positions of miR399 [16]. After being inserted with “cua”, the binding sequences become 5′ GGGGGAU-GAAGcuaCCUGGUCCGA 3′ and 5′ GGGGAAUGAAGcuaCCUGGUCC-GA 3′. We further convert these RNA sequences into DNA sequences as 5′ GGGGGATGAAGcuaCCTGGTCCGA 3′ and 5′ GGGGAATGAAGcuaCCTGGTCCGA3′. Finally, we replace the two binding sites on the backbone with the converted DNA

sequences, resulting in the following sequence of STTM165/166-48 containing a *SwaI*(ATTTAAAT) in the middle:

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5' catttgagaggacagcccAAGCTTGGGGGATGAAGctaCCTGGTCCG
AgttgtgtgttatgtctaATTTAAATatgtctaaagaagaagaatGGGGAATGA
AGctaCCTGGTCCGAGAATTCggtacgctgaatcaccag 3'
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3.1.2.2. Primer design for STTM production using pOT2-poly-cis as a template: A pair of back-to-back primers for production of pOT2-STTM165/166-48 are directly adapted from the STTM165/166-48 sequences above. The 3' half of the STTM165/166-48 including the *SwaI* site is used as the forward primer "165-166-STTMSwa48ntlink-PF" (5' gccATTTAAATatgtctaaagaagaagaatGGGGAATGAAGctaCCTGGTCCGAGAATTCggtacgctgaatcaccag 3', which has a 5' *SwaI* protecting sequence "gcc"). The 5' half of the STTM165/166-48 including the *SwaI* site is used in an anti-parallel form (reverse and complement) as the reverse primer "165-166-STTM.Swa48ntlink-PR" (5' gccATTTAAATtagaccataacaacaacaacTCGGACCAGtagCTTCATCCCCCAAGCTTggtctctctcctcaaatg 3', which also has a 5' *SwaI* protecting sequence "gcc").

Table 1 shows the two primers designed for construction of STTM165/166-48 using pOT2-Poly-Cis as a template. The binding sites of the two primers (165-166-STTMSwa48ntlink-PF and 165-166-STTM.Swa48ntlink-PR) on pOT2-Poly-Cis are labeled in red in Fig. 2A, corresponding to the STTM-Forward Primer and STTM-Reverse Primer in Fig. 1A.

3.1.2.3. Construction of STTM in pOT2-Poly-Cis

3.1.2.3.1. Amplify STTM165/166-48 in the PCR reaction below

Template (pOT2-Poly-Cis, ~0.4 ng/μl)	1 μl
STTM forward primer (165-166-STTMSwa48ntlink-PF) (10 μM):	0.5 μl
STTM reverse primer (165-166-STTMSwa48ntlink-PR) (10 μM):	0.5 μl
dNTPs (25 mM)	0.5 μl
10× Long template PCR buffer	3 μl
Long Amp Tag DNA polymerase	0.5 μl
H ₂ O	24 μl

Subject the PCR reaction to the following program on a thermal cycler: 94 °C, 2 min, [94 °C, 30 s, 58 °C, 30 s, 68 °C, 4 min (30 cycles)], 68 °C, 10 min, 4 °C ∞.

Note: The expected PCR amplification product is about 3218 bp, which needs to be confirmed on a 1% agarose gel after PCR.

3.1.2.3.2. Purification of PCR products: The PCR product is purified by passing through a Sephadex G-25 column (Roche) to remove Taq DNA polymerase, primers and salts according to the manufacturer's protocol.

3.1.2.3.3. Digestion of PCR products with *SwaI*: After purification, the PCR product is digested with *SwaI* in a 50 μl reaction: PCR product, 10 μl; NEB Buffer 3 (10×), 5 μl; BSA(100×), 0.5 μl; *SwaI* (10 U/μl), 1 μl; H₂O, 33.5 μl. The reaction is conducted at 25 °C for 3 h, followed by enzyme inactivation at 70 °C for 10 min and purification by a Sephadex G-25 column again.

3.1.2.3.4. Self-ligation of *SwaI*-cleaved PCR products: After *SwaI* digestion and purification, the DNA product self-ligates at 16 °C overnight in a 20 μl volume: *SwaI*-

cleaved PCR products, 10 μ l; Li-gase buffer (10 $\times$), 2 μ l; DNA Ligase (3 U/ μ l), 1 μ l; H₂O, 7 μ l.

3.1.2.3.5. Transformation of the self-ligated product into *E. coli* competent cells: The ligated product is transformed into chemical competent *E. coli* cells and plate on LB agar plates with 25 μ g/ml CAM for the screening of recombinant STTM clones.

3.1.2.3.6. Isolation of plasmid containing pOT2-STTM construct: The plasmid is isolated by Plasmid Mini-prep kit (QIAGEN) and verified by *Swa*I digestion. Since the original template pOT2-Poly-Cis plasmid has no *Swa*I site, only the pOT2 plasmids containing STTM recombinant can be digested by *Swa*I and generate a liner DNA fragment of 3218 bp in length.

The positive pOT2-STTM plasmid needs to be verified by DNA sequencing using the following pair of primers: STTM-common-real-PF (5' cattggagaggacagcccaag 3') and STTM-common-real-RR (5' ctggtgatttcagcgtaccgaa 3'). Only the sequencing verified pOT2-STTM construct should be used for further experiments.

3.1.3. Sub-cloning the STTM from pOT2-STTM into a binary vector for plant transformation—After the construction and verification, the structure of STTM plus the d35S promoter and the 35S terminator should be sub-cloned into a binary vector for *Agrobacterium*-mediated transformation. Normally, the sizes of the sub-cloned DNA fragments are large, which make this step inefficient. In order for us to construct STTM into a binary vector in an effective way, we invented an approach to “zero” background screening for construction of STTM into the binary vector. This approach is based on a selection with double antibiotics, which bring the STTM together with a CAM^r selection marker from pOT2-STTM into a binary vector such as pFGC5941-*Pac*I (Fig. 3A), which has a Kanamycin selection marker in bacteria. To eliminate the false positive colonies, we use a pair of primers that contain the unique *Pac*I site in each primer to delete the DNA replication origin in pOT2-STTM and generate a fragment containing the STTM structure, the d35S promoter and the 35S terminator, and the CAM^r selection marker. This fragment can be replicated in *E. coli* on a double antibiotics (CAM^r + Kanamycin) selection plate only after being integrated into the binary vector through the unique *Pac*I site. The resulting final construct will be the binary vector containing STTM and additional antibiotics selection maker (Fig. 3B). Specifically, the following steps are used for the construction:

3.1.3.1. Delete the DNA replication origin in pOT2-STTM by PCR: Prepare the PCR reaction below in a 30 μ l total volume and load the following program on a thermal cycler: 94 $^{\circ}$ C, 2 min, [94 $^{\circ}$ C, 30 s, 58 $^{\circ}$ C, 30 s, 68 $^{\circ}$ C, 4 min (30 cycles)], 68 $^{\circ}$ C, 10 min, 4 $^{\circ}$ C ∞

pOT2-STTM template (~0.4 ng/ μ l)	1 μ l
Forward primer (Origin-del- <i>Pac</i> I-PF) (10 μ M):	0.5 μ l
Reverse primer (Origin-del- <i>Pac</i> I-PR) (10 μ M):	0.5 μ l
dNTPs (25mM)	0.5 μ l
Long Amp PCR buffer (10 $\times$)	3 μ l
Tag DNA polymerase	0.5 μ l
H ₂ O	24 μ l

Table 2 lists the two primers and their sequences for the origin deletion in pOT2-STTM. The binding sites of two primers (Origin-del-*Pac*I-PF and Origin-del-*Pac*I-PR) are indicated in red in Fig. 2B. The PCR products are 2846 bp and should be verified on a 1% Agrose gel. The PCR products are purified using Sephadex G-25 columns.

3.1.3.2. Digest both STTM-CAM^r and binary vector with PacI: The binary vector pFGC5941 is modified to a short version (8608 bp) termed pFGC5941-*PacI* containing a unique *PacI* site (Fig. 3A). To sub-clone the STTM-CAM^r into the pFGC5941-*PacI*, the following digestion is assembled in a 50 µl total volume:

pFGC5941- <i>PacI</i> (200 ng/µl)	10 µl
origin-deleted PCR products (400 ng/µl)	10 µl
NEB buffer 1 (10×)	5 µl
BSA (100×)	0.5 µl
<i>PacI</i> (10 U/µl),	1 µl
H ₂ O	23.5 µl

The reaction is incubated at 37°C for 3 h, and then at 70°C for 10 min to inactivate the enzyme, followed by purification using a G-25 column.

3.1.3.3. Ligate the origin-deleted PCR products into 5941-*PacI* vector: The above purified *PacI*-digested products are subjected to ligation at 16 °C overnight in a 30 µl volume: *PacI*-digested product, 26 µl; Ligase buffer (10×), 3 µl; DNA Ligase (3 U/µl), 1 µl. Note: Specific dephosphorylation treatment of the binary vector (but never the STTM-CAM^r) increases the colonies of recombinants on the double antibiotics selection plates, but is not essential under our practice.

3.1.3.4. Transform the ligation reaction into *E. coli* and screen for recombinant pFGC5941-STTM: The ligation reaction is transformed into *E. coli* and plated on a LB Agar plate with both kanamycin (25 µg/ml) and CAM^r (25 µg/ml) for screening the colonies containing the recombinant pFGC5941-STTM construct.

3.1.3.5. Plasmid isolation for recombinant pFGC5941-STTM: The plasmid is isolated and verified by *PacI* digestion. pFGC5941-STTM construct should be cleaved by *PacI* into two DNA fragments of different sizes (one is 8608 bp and the other is 2846 bp). The constructs need to be verified by DNA sequencing and the positive one will be transformed into the *Arabidopsis* plant through *Agrobacterium*.

3.1.3.6. Plant transformation by *Agrobacterium*: The sequencing verified pFGC5941-STTM plasmid is transformed into the *Agrobacterium* strain COR308 and grown on LB plates with TKG triple antibiotics (Tetracyclin 5 µg/ml, Gentamycin 20 µg/ml, and Kanamycin 25 µg/ml) at 28 °C. The *Arabidopsis* transformation is conducted by the flower dipping method [23].

3.1.4. Blocking animal miRNA by constructing STTM into a mammalian expression vector pEGP-miR and transfecting mammalian cell line for miRNA degradation assay

3.1.4.1. Sub-cloning STTM30 to block miR-30d expression in mouse pancreatic beta cells: To evaluate if the STTM structure has a similar silencing effect on animal miRNA, we tried to block mouse miR-30d, a miRNA up-regulated by glucose, inducing insulin biosynthesis in pancreatic beta cells [24,25]. To generate STTM30 constructs, the fragment containing complementary sequences of both miR-30a and miR-30d linked by the published 88-nt spacer sequence (5' GTTGTGTTGTTATGGTCTAGTTGTTGTTGTTATGGTCTAATTTAAA TATGGTCTAAAGAAGAAGAATATGGTCTAAAGAAGAAGAAT 3') [15] was first synthesized in IDT, then sub-cloned into the pEGP-miR expression vector (Cell Biolabs; Fig. 4A) through the unique *BamHI* and *NheI* sites, resulting in pEGP-STTM30 (Fig. 4B).

Unlike the plant STTM that is expressed as a short artificial non-coding RNA, the STTM30 is expressed as part of the human β -globin intron at the 5' UTR region of a fused GFP-Puro gene driven by the constitutive EF-1 α Promoter.

3.1.4.2. Cell culture and STTM30 transfection: The insulin secreting cell line MIN6 cells were maintained in high glucose Dulbecco's modified Eagle's medium, supplemented with 15% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen) as described (24). To test the effects of STTM30 on the miR-30d activity in pancreatic beta cells, the plasmids of pEGP-STTM30 or pEGP-null empty vector (10 μ g) were electroporated into pancreatic beta cell line, MIN6 cells (1×10^6) using Amaxa Nucleofector (Lonza).

3.1.4.3. Quantitative real-time PCR for miRNA: Two days after transfection, total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions and treated with rDNase I (Sigma). TaqMan miRNA qPCR detection system (Applied Biosystem) was used for quantification of miR-30d, or the control miRNA let-7b, and normalized to the relative expression of snU6 rRNA. Real-time PCR was performed on a StepOnePlus™ System (Applied Biosystem) in the following conditions: heat activation of reverse DNA polymerase at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min.

3.1.4.4. Luciferase assays for validating STTM30 effects on miR-30d target: Mitogen-activated protein 4 kinase 4 (MAP4K4), a TNF- α activated kinase, has been identified to be a direct target of miR-30d [25]. The 3' UTR of MAP4K4 has been sub-cloned downstream of a luciferase gene (pRLTK-Map4k4-WT) and the pRLTK-Map4k4-mut construct, containing mutations in the conserved miR-30d binding site, was also sub-cloned as a control [25]. For the luciferase reporter assay, pRLTK reporter constructs (2 μ g) were co-electroporated into MIN6 cells (1×10^6) with pEGP-STTM30 or pEGP-null empty vector (10 μ g). The plasmid pGL-3 containing firefly luciferase (2 μ g) was co-transfected together to normalize for transfection efficiency. Luciferase activity was measured with a dual-luciferase reporter assay kit (Promega) two days after transfection.

4. Results

4.1. STTM165/166-48nt triggered the degradation of miR165/166 and transformed the normally developed plant into anomalous in transgenic Arabidopsis

The STTM165/166-48nt, which was designed and constructed according to the protocol, was transformed into *Arabidopsis* plants through a flower dipping approach. Compared to the control plants, the transgenic plants showed extreme anomalies (Fig. 5). Furthermore, the miR165/166 level is likely degraded and the expression of most miR165/166 target genes such as *PHB*, *PHV*, *REV* are largely up-regulated [15]. The deep sequencing also confirmed that the copy number of miR165/166 are almost close to zero in the STTM165/166-48 transgenic plants while the non-targeted miR168 has no changes [15].

4.2. STTM30 triggered reduction of mouse miR30d in MIN6 cells

To evaluate if the STTM30 also triggered the reduction of mouse miR30d, the plasmids of pEGP-STTM30 or pEGP-null empty vector were electroporated into MIN6 cells. After 48 h post electroporation, about 30% cells were transfected and displayed a GFP expression signal. Real-time PCR confirmed that the expression of miR-30d decreased by ~40% in the cells transfected with STTM30 compared to the empty vector control (Fig. 6A). In contrast, the expression of the control miRNA, let-7b level was not affected by STTM30 introduction.

To further evaluate if STTM30 triggered reduction of miR-30d, a luciferase reporter gene, containing the 3'UTR of Map4k4 (one of the miR-30d targets), was co-transfected into MIN6 cells with pEGP-STTM30 or pEGP-null empty vector. Luciferase activity of cells transfected with the pEGP-STTM30 and pRLTK-Map4k4-WT was increased ~2-fold relative to cells that were co-transfected with control pEGP-null empty vector and pRLTK-Map4k4-WT (Fig. 6B). Consistently, point mutations in the miR-30d target site (pRLTK-Map4k4-mut), reducing the complementarity between miR-30d and the Map4k4 target site, abolished the repression of endogenous miR-30d on luciferase activity (Fig. 6B). Although the reduction of miR-30d was not as dramatic (~40%, Fig. 6A) as that of miR165/166 in *Arabidopsis*, the de-repression of luciferase reporter activity was impressive in MIN6 cells containing STTM (Fig. 6B). The relatively low reduction of miR-30d by STTM may be attributed to an unidentified mechanism of STTM-triggered inhibition, rather than a complete destruction of small RNAs by STTM in mouse cells. Taken together, our data demonstrated that STTM30 significantly inhibited the repression of luciferase activity by endogenous miR-30d.

5. Conclusions

STTM is a simple but effective tool to block the function of small RNAs of interests in plant and mammalian cells. In both systems, the target small RNAs showed significant reductions upon introduction of STTM either through transgenics or transfection, suggesting a destruction of small RNA identities triggered by STTM. In plants, STTM can be expressed as a short non-coding RNA for functional blockage of miRNAs, while in animals, such an STTM structure can be expressed inside an intron and effectively blocks the function of animal miRNAs, which further de-represses the luciferase reporter gene that is fused to the 3' UTR of the miRNA target gene. We provide a comprehensive protocol for construction of STTM in an effective manner for application in plants and a novel approach for expressing STTM in animals. Allowing the simple procedures toward STTM realization, as well as the high and stable efficacies brought-by, we anticipate a wide application of the STTM technology in both plants and animals using protocols described in this study.

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References

1. Simon SA, Meyers BC. Current Opinion in Plant Biology. 2011; 14:148–155. [PubMed: 21159545]
2. Matzke M, Kanno T, Daxinger L, Huettel B, Matzke AJ. Current Opinion in Cell Biology. 2009; 21:367–376. [PubMed: 19243928]
3. Ghildiyal M, Zamore PD. Nature Reviews Genetics. 2009; 10:94–108.
4. Brosnan CA, Voinnet O. Current Opinion in Cell Biology. 2009; 21:416–425. [PubMed: 19447594]
5. Baulcombe D. Nature. 2004; 431:356–363. [PubMed: 15372043]
6. Kozomara A, Griffiths-Jones S. Nucleic Acids Research. 2011; 39:D152–D157. [PubMed: 21037258]
7. Lu C, Tej SS, Luo S, Haudenschild CD, Meyers BC, Green PJ. Science. 2005; 309:1567–1569. [PubMed: 16141074]
8. Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE, Henikoff JG, Comai L, Henikoff S. Genome Research. 2003; 13:524–530. [PubMed: 12618384]

9. Baker CC, Sieber P, Wellmer F, Meyerowitz EM. *Current Biology: CB*. 2005; 15:303–315. [PubMed: 15723790]
10. Mallory AC, Bartel DP, Bartel B. *The Plant Cell*. 2005; 17:1360–1375. [PubMed: 15829600]
11. Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, Abdellatif M, Feldman DS, Elton TS, Gyorke S. *Circulation Research*. 2009; 104:514–521. [PubMed: 19131648]
12. Zhu QH, Upadhyaya NM, Gubler F, Helliwell CA. *BMC Plant Biology*. 2009; 9:149. [PubMed: 20017947]
13. Zhang X, Zou Z, Gong P, Zhang J, Ziaf K, Li H, Xiao F, Ye Z. *Biotechnology Letters*. 2011; 33:403–409. [PubMed: 20960221]
14. Bartel DP. *Cell*. 2009; 136:215–233. [PubMed: 19167326]
15. Yan J, Gu Y, Jia X, Kang W, Pan S, Tang X, Chen X, Tang G. *The Plant Cell*. 2012; 24:415–427. [PubMed: 22345490]
16. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, Leyva A, Weigel D, Garcia JA, Paz-Ares J. *Nature Genetics*. 2007; 39:1033–1037. [PubMed: 17643101]
17. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A, Bozzoni I. *Cell*. 2011; 147:358–369. [PubMed: 22000014]
18. Tay Y, Kats L, Salmena L, Weiss D, Tan SM, Ala U, Karreth F, Poliseno L, Provero P, Di Cunto F, Lieberman J, Rigoutsos I, Pandolfi PP. *Cell*. 2011; 147:344–357. [PubMed: 22000013]
19. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. *Cell*. 2011; 146:353–358. [PubMed: 21802130]
20. Ebert MS, Sharp PA. *Current Biology*. 2010; 20:R858–R861. [PubMed: 20937476]
21. Rubio-Somoza I, Manavella PA. *Methods in Molecular Biology*. 2011; 732:131–137. [PubMed: 21431710]
22. Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D. *PLoS Genetics*. 2010; 6:e1001031. [PubMed: 20661442]
23. Clough SJ, Bent AF. *The Plant Journal for Cell and Molecular Biology*. 1998; 16:735–743. [PubMed: 10069079]
24. Tang X, Muniappan L, Tang G, Ozcan S. *RNA*. 2009; 15:287–293. [PubMed: 19096044]
25. Zhao X, Mohan R, Ozcan S, Tang X. *The Journal of Biological Chemistry*. 2012; 287:31155–31164. [PubMed: 22733810]

Fig. 1. STTM PCR construction strategy and the STTM structure. (A) The structure of STTM primers and their binding sites on the sub-cloning vector pOT2-Poly-Cis. (B) The STTM PCR and cloning strategy resulting in the expression cassette (d35S-STTM-T35S) in the vector pOT2-STTM. (C) STTM RNA transcript structure and the putative complex formed between STTM and the small RNAs. Note: The STTM linker (48–88 nt) forms a weak imperfect “stem-loop” that may play an important role in the stability of STTM in cells.

Fig. 2.

Diagram of the constructed sub-cloning vectors pOT2-Poly-Cis and the STTM165/166. (A) pOT2-Poly-Cis (3692 bp). Positions for d35S promoter, 35S terminator (T35S), chloramphenicol acetyltransferase (CAT), pBR322 origin, Poly-Cis, and the two binding sites for primers (in red) to construct STTM165/166 are shown. (B) pOT2-STTM165/166 (3218 bp). STTM165/166 is shown in red between HindIII and EcoRI sites. The binding positions for two primers (Origin-del-*PacI*-PF and Origin-del-*PacI*-PR) for subsequent PCR deletion of pBR322 origin to clone the STTM into a binary vector are shown in red.

Fig. 3.

Diagram of pFGC5941-*PacI* and the STTM165/166 constructed in this vector. (A) pFGC5941-*PacI* (modified from pFGC5941, 8608 bp). The unique sites for STTM sub-cloning are *PacI*, *XbaI*, *SmaI* and *HindIII* shown bolded in the diagram. (B) pFGC5941-*PacI*-STTM165/166 (3218 bp). The STTM165/166 expression cassette is shown between the two *PacI* sites (1352 and 4198). The orientation of the STTM165/166 expression cassette could be opposite depending on the random ligation of the two linear DNA fragments through *PacI*. Note: The STTM165/166 expression cassette fragment contains a CAT gene, which works as an antibiotic selection marker and makes the screening for the recombinants much easier using double antibiotics (chloramphenicol and kanamycin).

Fig. 4.

Diagram of pEGP-miR and the STTM30ad constructed in this vector. (A) pEGP-miR (4972 bp). The two unique sites for directional sub-cloning of STTM30ad in place of the mouse let7a inside the human β-globin intron are *Bam*HI (774) and *Nhe*I (1084). Positions for the mammalian EF-1α promoter, SV40 polyA signal site, the GFP-Puro in-frame fused protein, the human β-globin intron, and the mouse let7a inside the intron are shown in the diagram. (B) pEGP-STTM30ad-88nt-linker (4806 bp). STTM30ad-88 nt-linker is shown between the *Bam*HI and *Nhe*I sites (774 and 918). Note: After transcription, the STTM30ad-88nt-linker together with the human β-globin intron will be spliced and functionally interacting with the targeted miR30a/d.

Fig. 5. Impact of STTM165/166-48 in transgenic *Arabidopsis*. (A) The control *Arabidopsis* expressing an empty vector; (B) The transgenic *Arabidopsis* over-expressing the STTM165/166-48.

Fig. 6.

Inhibition of miR-30d by STTM30 in mouse pancreatic beta cells. (A) STTM30 reduced the expression of miR-30d in MIN6 cells. MIN6 cells were transfected with pEGP-STTM30 or pEGP-null empty vector. 48 h post transfection, total RNA was extracted to analyze the expression of miR-30d or the control miRNA let-7b by realtime PCR. (B) STTM30 inhibited the repression of luciferase activity by endogenous *miR-30d* in MIN6 cells. The reporter constructs containing either Map4k4-WT or mutant (Map4k4-mut) were co-transfected into MIN6 cells with either pEGP-STTM30 or pEGP-null empty vector. Data represent mean $\pm$ SD of three independent experiments. **, $p < 0.01$.

Table 1

Primers used for production of STTM165/166–48 using pOT2-Poly-Cis template.

Primer name	Sequences
165-166-STTM <i>Swz</i> 48ntlink-PF	gccATTAAATatggtctaaagaagaagaatGGGAATGAAGctaCCTGGTCCGAgaattcggtacgtgaaatcaccag
165-166-STTM <i>Swz</i> 48ntlink-PR	gccATTAAATtagaccataacaacaacaacTCGGACCAGGtagCTTCATCCCCaagettgggtgtcctctccaaatg

Table 2

Primers used for origin deletion in pOT2-STTM construct.

Primer name	Sequences
Origin-del- <i>PacI</i> -PF	TCCCTTAATTAAGTTTGCAAGCAGCAGATTACGCG
Origin-del- <i>PacI</i> -PR	TCCCTTAATTAAGAAAGGCGGACAGGTATCCGGTAAG