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Mimicry technology, a versatile tool for small RNA suppression

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Abstract

A decade ago the discovery of the target mimicry regulatory process on the activity of a mature micro RNA (miRNA), enabled for the first time the customized attenuation of miRNA activity in plants. That powerful technology was named MIMIC and was based on engineering the IPS1 long non-coding transcript to become complementary to the miRNA under study. In order to avoid IPS1 degradation, the predicted miRNA-mediated cleavage site was interrupted by three additional nucleotides giving rise to the so called MIMIC decoy. Since then, MIMIC technology has been used in several plant species and in basic and translational research. We here provide a detailed guide to produce custom designed MIMIC decoys to facilitate the study of sRNA functions in plants.

Keywords: Target mimicry, miRNA, small RNA attenuation.

1. Introduction

Small RNAs (sRNAs) are regulatory molecules embedded within the gene networks that orchestrate both plant development and stress responses. Micro RNAs (miRNAs) are a special class of sRNAs that negatively regulate their targets post-transcriptionally through cleavage and translational inhibition and in a sequence homology manner. Traditionally, one of the major handicaps to address the role of miRNAs in the different processes they were involved in was their multigenic nature. Thus, the same functional miRNA is produced from different loci within plant genomes. The advent of the Mimicry technology (MIMIC technology, hereafter) constituted a key inflection point on unravelling the molecular role of different miRNAs by enabling the specific down-regulation of entire miRNA families. MIMIC technology is based on a natural regulatory event controlling the Phosphate starvation response in Arabidopsis thaliana. As part of that starvation response, the long-non-coding RNA IPS1 is able to outcompete PHO2 transcripts from miR399-loaded RNA silencing complexes (RISC) preventing their down-regulation and prompting their translation [1] (Figure 1). A decade ago, we harnessed that natural layer of regulation by replacing the miR399 complementary sequence within the IPS1 transcript by one that mimics the target of other miRNAs. Later on, we generalized the MIMIC approach to accomplish the first systematic characterization of the roles of miRNAs in plant development [2]. Since then, MIMIC
technology has been applied in a wide variety of plant species including those of agricultural importance such as rice [3]. Additionally, MIMIC-based approaches have allowed the surgical inhibition of miRNA action uniquely in specific cell types by choosing the appropriate promoter to drive the engineered IPS1 transcript [4, 5]. Here, we provide an easy and complete guide to generate customized MIMICs to study the role of any sRNA.

2. Material

2.1 Generating MIMIC constructs, cloning, plant transformation and validation.

1. Kit or protocol for genomic DNA isolation from A. thaliana plants.
2. PCR reagents (Fusion DNA polymerase (NEB), dNTPs, specific primers detailed in the next chapter) and Thermocycler.
3. Equipment to perform DNA electrophoretic assays (BioRad), agarose, TAE buffer and kit for agarose gels DNA isolation (Gelpure, NZY).
4. Reagents for A-tail addition (Taq DNA polymerase (NEB), dATP).
5. PCR acceptor vector (PCR8/GW/TOPO, Invitrogen)
6. Engineered plasmid with specific promoter to drive MIMIC expression
7. Escherichia coli and Agrobacterium tumefaciens competent bacterium for plasmid amplification and plant transformation respectively.

3. Methods

3.1 Primer design for MIMIC building

MIMIC technology is suitable for targeting known miRNAs and for the functional characterization of newly discovered ones. Mature miRNA sequences from a variety of plants species can be found at publicly available repositories, such as miRBAse (www.mirbase.org).

The first step to obtain a customized MIMIC construct is to replace the original miRNA399 target within the IPS1 sequence by the one that recognizes the sRNA under study. MIMIC sequence is the reverse complementary to the one from the sRNA to attenuate. It is necessary to add a three-nucleotide sequence within the predicted miRNA cleavage site in order to generate a functional MIMIC construct that is resistant to miRNA regulation avoiding its degradation. That three-nucleotide sequence must be introduced between bases 10th and 11th from the miRNA 5’ end and must not contain a possible cleavage reconstitution site.

A recommended tool to easily obtain the complementary reverse sequence of the sRNA to be decoyed is the Reverse complement tool on-line (www.bioinformatics.org/sms/rev_comp.html).

1- Choose the option “Convert the DNA sequence into its reverse-complement counterpart” and paste the sRNA sequence in the white box at the web tool and just click submit.
2- Insert three nucleotides within the retrieved sequence after the nucleotide in position 10th from the 3’ outermost nucleotide. In order to prevent undesired reconstitution of the cleavage site and failure to downregulate the sRNA
under study, it is important that the chosen nucleotides are different to the ones flanking their insertion (positions 10th and 11th on the original sequence; see Note 1).

3- To design the MIMIC forward primer (MIM.fw), take the resulting sequence from the step above and paste it in the underlined X in the following sequence:

MIM.fw: 5’-CTAGAAAAAGCTTCGGTTCCC-3’

4- To design the MIMIC reverse primer (MIM.rev), replace the underlined X in the following sequence with the reverse complementary sequence obtained in the above step 2.

MIM.rev: 5’-GGGGAACCGAAGCTTTTCTAGAGG-3’

3.2. Amplifying IPS1 sequence for MIMIC template

1- After isolating DNA from Arabidopsis plants, set a PCR using 200ng of that DNA as template in a final volume of 20 µl. Use the following primers in the reaction:

IPS1.fw: 5’-AAAACACCACAAAAACAAAAAG-3’
IPS1.rev: 5’-AAGAGGAATTCTAATAAGAG-3’

2- Mix the PCR reaction with loading buffer and load it in an agarose-TAE gel (1%w/v) along with a suitable DNA size marker (the expected band is 542 bp long, see Note 2).

3- Under UV light, and wearing protective equipment, isolate the corresponding band from the agarose gel using a scalpel. Purify the DNA using a commercial kit (i.e.NZY GelPure) following the manufacturer’s instructions.

4- Prior to clone the IPS1 sequence in an acceptor plasmid, an overhang “A” must be added to the insert (Note 3). Set a 10 µl reaction in a PCR tube including Taq polymerase buffer, dATP, IPS1 DNA and Taq polymerase enzyme. Incubate the tube in a thermocycler at 72ºC for 45 minutes.

5- Clone the IPS1 DNA into an acceptor plasmid (i.e. pCR8/GW/TOPO) to generate the template that will be used in subsequent steps.

3.3. Customizing MIMIC sequence

1. Set up two independent PCR reactions using as template 1µl of the IPS1-containing plasmid in a 20 µl final reaction volume.

2. In order to generate the 5’ region, use the following primer combination in PCR1:

IPS1.fw: 5’-AAAACACCACAAAAACAAAAAG-3’
MIM.rev: 5’-GGGGAACCGAAGCTTTTCTAGAGG-3’

3. To generate the 3’ region in PCR2, use the following primers combination:

MIM.fw: 5’-CTAGAAAAAGCTTCGGTTCCC-3’
IPS1.rev: 5’-AAGAGGAATTCTAATAAGAG-3’

4. Add loading buffer to each PCR reaction and proceed to load it and run it in two separate wells in an agarose-TAE gel (1%w/v), along with a suitable DNA size marker (the expected products are 314 bp from PCR1 and 273 bp from PCR2).

5. Isolate DNA fragments separately using the same commercial kit and procedure used in the step 3 of the former section.
6. Set up a final PCR in a single tube to reconstitute the full length MIMIC sequence using as template 1 µl from each of the purified products from PCR1 and PCR2, and with primers IPS1fw and IPS1rev.

7. Run the PCR reaction in an agarose.TAE gel (1% w/v) along with a suitable DNA size marker. The expected size of the final PCR product is 542 bp.

8. Isolate the 542bp DNA band from gel repeating the step 3.2.3.

9. Add the A-tail to the MIMIC sequence by repeating the procedure described in step 3.2.4.

10. Clone the MIMIC into a Gateway compatible acceptor vector as done above in step 3.2.5. Verified the sequence by Sanger sequencing and proceed to clone into a final binary vector for plant transformation.

3.4. Choosing the binary vector to use.

Currently, there are several collections of plasmids publicly available providing options for constitutive (i.e. viral 35S and plant ubiquitin derived promoters), inducible (estradiol or ethanol based responding systems) or cell-type specific expression (such as vectors containing the AP3 promoter). We have used the three kinds with different purposes. It is noteworthy that the constitutive attenuation of the action of a given miRNA, can result in pleiotropic effects that affect developmental processes others that the ones in which the miRNA under study directly participates. In addition, we have found that in some cases constitutive suppression of a miRNA family leads to sterility and reduced fitness limiting the lines than can be recovered to those with low levels of expression of the MIMIC transcript and small reduction on the function of its decoyed miRNA. We have also found that an ethanol inducible system successfully allows a reversible sequestration of the targeted miRNA, which constitutes a clear advantage to overcome the possible sterility problems mentioned above. Finally, we have also successfully used cell- type specific promoters in order to surgically attenuate the function of a given miRNA and finely study its regulatory roles in a defined cellular niche [4, 5].

The MPSS public database (https://mpss.danforthcenter.org) encompasses extensive sRNA expression data from several plant species, including Arabidopsis. The information available in there can be of great help to assist on the election of the most suitable promoter to drive MIMIC constructs.

3.5. Validating MIMIC impact on sRNA regulation

The successful suppression of sRNA function by the MIMIC technology is not always accompanied by an eye-catching phenotype. In order to know whether your plants have reduced activity of the sRNA under study, there are two complementary approaches that can be taken. First, monitor the expression of the sRNA targeted genes in MIMIC plants and compare those to their expression levels in wild type plants. To that end, performing a quantitative real-time PCR (QRT-PCR) using primers specifically binding to those sequences flanking the predicted sRNA binding site within the target transcripts might be very instructive. sRNA target levels should be higher in the MIMIC line than in the control where sRNA regulation is not affected. Nevertheless, such approach is only able to detect one of the two co-existing mechanisms in which miRNAs operate, target cleavage, neglecting the regulation through translational inhibition. A second approach is based on the observation that expression of MIMICs triggers the degradation of the corresponding miRNAs in a SDN1-dependent manner.
Therefore, quantification of mature miRNA levels, by small RNA blots or QRT-PCR, is a good proxy to validate the efficiency of the MIMIC construct.

4. Notes

1. The vast majority of plant miRNAs cleave their targets between the positions 10th and 11th from the outermost 5' base of the miRNA. Nevertheless, there are also some exceptions described. Since that is a crucial parameter for MIMIC design, we strongly recommend confirming that instance in the available literature. In the case that information is not available, we recommend performing 5'RACE on predicted sRNA targets. In case a shifting from the canonical cleavage site is observed, the three extra nucleotides need to be relocated between the two bases where cleavage occurs.

2. Shorter MIMICs, using 124 bp from the IPS1 have also been successfully assayed.

3. This step is just necessary when using Pfu derived polymerases. If you used a Taq-derived enzyme, the resulting PCR products will already bear an A-tail.

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References

1. Franco-Zorrilla JM, Valli A, Todesco M, Mateos I, Puga MI, Rubio-Somoza I, et al. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat Genet. 2007;39(8):1033-7. doi: 10.1038/ng2079. PubMed PMID: WOS:000248446900023.
2. Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D. A collection of target mimics for comprehensive analysis of microRNA function in Arabidopsis thaliana. PLoS Genet. 2010;6(7):e1001031. doi: 10.1371/journal.pgen.1001031. PubMed PMID: 20661442; PubMed Central PMCID: PMCPMC2908682.
3. Gao F, Wang K, Liu Y, Chen Y, Chen P, Shi Z, et al. Blocking miR396 increases rice yield by shaping inflorescence architecture. Nat Plants. 2015;2:15196. doi: 10.1038/nplants.2015.196. PubMed PMID: 27250748.
4. Knauer S, Holt AL, Rubio-Somoza I, Tucker EJ, Hinze A, Pisch M, et al. A protodermal miR394 signal defines a region of stem cell competence in the Arabidopsis shoot meristem. Dev Cell. 2013;24(2):125-32. doi: 10.1016/j.devcel.2012.12.009. PubMed PMID: 23333352.
5. Rubio-Somoza I, Weigel D. Coordination of flower maturation by a regulatory circuit of three microRNAs. PLoS Genet. 2013;9(3):e1003374. doi: 10.1371/journal.pgen.1003374. PubMed PMID: 23555288; PubMed Central PMCID: PMCPMC3610633.
Fig. 1 Target mimicry mechanism. Target mimic transcripts are similar to natural miRNA targets except by the presence of three extra nucleotides interrupting the miRNA cleavage site. That results in the specific sequestration of RISC complexes, reducing miRNA levels within the cells and thus preventing mRNA target degradation allowing their translation.
