Published in final edited form as:
*Nat Protoc*. 2014 April ; 9(4): 939–949. doi:10.1038/nprot.2014.061.

# Epitope-tagged protein-based artificial microRNA (ETPamir) screens for optimized gene silencing in plants

**Jian-Feng Li**[1,2], **Dandan Zhang**[1,2], and **Jen Sheen**[1,2]

Jian-Feng Li: jli@molbio.mgh.harvard.edu; Dandan Zhang: ddzhang@molbio.mgh.harvard.edu; Jen Sheen: sheen@molbio.mgh.harvard.edu

[1]Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA

[2]Department of Genetics, Harvard Medical School, Boston, Massachusetts 02114, USA

## Abstract

Artificial microRNA (amiRNA) technology offers highly specific and versatile gene silencing in diverse plant species. The principal challenge in amiRNA application is to select potent amiRNAs from hundreds of bioinformatically designed candidates to enable maximal target gene silencing at the protein level. To address this issue we developed the epitope-tagged protein-based amiRNA (ETPamir) screens, in which single or multiple target genes encoding epitope-tagged proteins are constitutively or inducibly co-expressed with individual amiRNA candidates in plant protoplasts. Accumulation of tagged proteins, detected by immunoblotting with a commercial tag antibody, inversely and quantitatively reflects amiRNA efficacy *in vivo*. The core procedure, from protoplast isolation to identification of optimal amiRNA, can be completed in 2-3 days. The ETPamir screens circumvent the widespread shortage of plant antibodies and the complexity of plant amiRNA silencing at target mRNA or/and protein levels. This method can be extended to verify predicted endogenous target genes for plant natural miRNAs.

### Keywords

Plant artificial microRNA; Gene silencing; Plant microRNA; Target gene validation

## INTRODUCTION

The rapidly expanding genomic information across the plant kingdom stresses an urgent need for reliable and versatile tools to decipher the functions of newly discovered genes and their regulatory networks. Determination of gene functions often requires examination of loss-of-function phenotypes. In the model plant *Arabidopsis thaliana*, T-DNA insertion lines represent the most important resource for loss-of-function mutants. Targeted genome editing

Correspondence should be addressed to J.F.L. (jli@molbio.mgh.harvard.edu), Tel: 001-617 6433314, Fax: 001-617 6433050.

**AUTHOR CONTRIBUTIONS**

J.F.L. developed the protocol under the guidance of J.S. The article was written by J.F.L. and J.S. D.Z. prepared the Tables 1 and 2 and related references.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

tools, including zinc finger nucleases[1], transcription activator-like effector nucleases[2,3], and RNA-guided Cas9 endonucleases[4–6], have recently opened up promising new avenues for generating targeted loss-of-function mutants for *Arabidopsis* genes lacking T-DNA insertion mutants and for genes in other plant species. However, lethality and complex long-term physiological and developmental consequences associated with stable mutants have imposed limitations in functional characterization of most genes essential for plant growth and reproduction. It is also more challenging to use T-DNA insertion mutants to study functionally redundant and physically linked genes in plant genomes[7]. The artificial microRNA (amiRNA)-based method for targeted gene silencing provides an invaluable alternative approach for conditional, reversible and multiplex control of gene activities for systematic functional genomic analyses in plants.

Targeted gene silencing in plant research has been obtained mostly by hair-pin RNAs (hpRNAs), amiRNAs and virus-induced gene silencing (VIGS). The amiRNA technology exploits the biogenesis and silencing machineries of natural miRNAs for silencing one or multiple genes of interest. A desired amiRNA can be easily generated using a native miRNA precursor (pre-miRNA) backbone by replacing its original mature miRNA sequence with a custom sequence that base-pairs with and triggers cleavage, decay or/and translational inhibition of target mRNAs of interest[8–13]. The homogeneity of a single silencing amiRNA produced by a pre-amiRNA and the prerequisite of a near-perfect complementarity between plant amiRNAs and target mRNAs ensure the superb silencing specificity of plant amiRNAs[8–13], whereas hpRNAs and VIGS often exhibit off-target effects due to the unpredictable heterogeneity of the siRNAs produced. In addition, the amiRNA-targeted genes can be easily modified to resist amiRNA activities and then used for functional complementation in transgenic mutant plants with amiRNA-mediated gene silencing, to establish a solid genotype-phenotype correlation[9,10].

Although manual design of plant amiRNAs is feasible[14], the resourceful web-based miRNA designer (WMD) facilitates an automatic design of gene-specific amiRNA candidates for over 100 plant species with fully sequenced genomes or extensive databases of ESTs[10]. However, the *in vivo* silencing efficacy of individual amiRNA candidates can be highly variable[10,11,15–18]. This is largely owing to unpredictable factors, such as amiRNA expression and processing, target mRNA structure and accessibility, and effects of potential target mRNA binding proteins[11,18,19]. Therefore, optimal amiRNAs for gene silencing are not readily recognizable among dozens to hundreds of candidates in the WMD prediction list. Without rapid *in vivo* screen and quantitative evaluation of the performance of selected amiRNA candidates, tremendous time and labor investment in generating and screening amiRNA-expressing transgenic plants could lead to ineffective or partial rather than complete silencing of the target gene(s) at the protein level. Therefore, a facile and robust method for identifying optimal amiRNAs in a broad range of plant species will facilitate highly efficient gene silencing in plants and promote scientific advances and discoveries in plant research.

## Development of the ETPamir screens

To pinpoint the most potent amiRNAs from bioinformatically designed candidates for silencing single or multiple target genes, we have developed a straightforward and widely adaptable method, the epitope-tagged protein-based amiRNA (ETPamir) screen[11]. Our strategy is to constitutively or inducibly co-express full-length target genes encoding epitope-tagged proteins with individual amiRNA candidates in plant mesophyll protoplasts, which are freshly isolated leaf cells lacking cell walls that support highly efficient DNA transfection[20]. Transfected protoplasts are incubated for sufficient time to allow each amiRNA to accumulate and exert its inhibitory effect on target mRNAs, through a combination of cellular mechanisms, to suppress the production of tagged proteins. This suppression is quantified by immunoblotting with the suitable tag antibody. One option for co-expression of amiRNA and its target gene(s) is to use a constitutive promoter to drive the expression of both. This option requires longer protoplast incubation time (e.g., 36 h) to determine the amiRNA efficacy, considering the turn-over time of the tagged proteins synthesized from escaped target mRNAs at the beginning of co-expression (i.e., in the absence of sufficient amiRNA activity). An alternative option is to allow sufficient amiRNAs to be produced under a constitutive promoter for 3 h before a 1 h heat induction of target mRNA expression, which is driven by the heat shock promoter. The amiRNA efficacy is then distinguishable after another 3 h of protoplast incubation. Using either option, the accumulation of tagged proteins from target mRNAs quantified by immunoblotting is inversely correlated with the *in vivo* silencing efficacy of each amiRNA. We have observed excellent consistency between the amiRNA efficacy determined by the ETPamir screen in protoplasts and its corresponding silencing phenotypes in transgenic plants[11]. The protocol presented here is a streamlined procedure covering steps from the selection of computationally designed amiRNA candidates to the identification of an optimal amiRNA for a single target gene (Fig. 1).

## Applications of the ETPamir screens

Our protocol for the ETPamir screens can be used to identify optimal amiRNAs for silencing single or multiple target genes in *Arabidopsis* and other plant species listed in Table 1, all of which have established protocols for protoplast-based transient gene expression and have been included in the WMD genome database for computational amiRNA design. If amiRNA candidates are manually designed according to the procedure of Eamens and co-workers[14], our protocol in principle can be adapted to any plant species amenable to protoplast isolation and DNA transfection. The protocol can also be used to screen potent amiRNAs for the silencing of viral mRNAs to confer enhanced viral resistance in transgenic plants expressing these amiRNAs[13]. By replacing amiRNA candidates with hpRNA or trans-acting small interfering RNA (tasiRNA)[21] candidates, this protocol can also be used to rapidly evaluate the *in vivo* efficiency of other post-transcriptional gene silencing techniques. The key concept of the ETPamir screen can be further extended to validate *in silico* predicted target genes for natural miRNAs from plants or interacting organisms including fungal pathogens and pests[22] (Fig. 2; this procedure is described in Box 1). In addition, this protocol can be used to determine the silencing specificity of amiRNAs or other gene silencing methods, and the fates of target mRNAs in plant cells by parallel quantification of proteins by immunoblotting and of mRNAs by RT-qPCR (see ref. 11).

**BOX 1**

**PROTEIN-BASED VALIDATION OF PREDICTED TARGET GENES OF ENDOGENOUS PLANT MIRNAS**

The key strategy of the ETPamir screen can be extended to validate computationally predicted target genes of endogenous plant miRNAs (Fig. 2).

**PROCEDURE**

**1|** Input the sequence of the miRNA of interest on the "Target Search" webpage of the WMD website (http://wmd3.weigelworld.org) to predict its endogenous target genes.

**2|** Clone the miRNA and its individual target candidate genes according to Step 5 of the main Procedure.

**3|** Extract plasmid DNA according to Steps 6 and 7 of the main Procedure.

**4|** Co-transfect protoplasts with the miRNA and individual candidate gene constructs expressing epitope-tagged proteins, as described in Steps 8-15 of the main Procedure. For each target candidate gene, set up a negative control, in which the miRNA construct is replaced by empty vector as described in Step 9 of the main Procedure.

**5|** Co-express the miRNA and individual target candidate genes in protoplasts using either Option A or Option B according to Step 16 of the main Procedure.

**6|** Monitor candidate protein accumulation by SDS-PAGE and immunoblotting, as described in Steps 17-28 of the main Procedure.

**7|** Identify authentic target genes whose expression is reduced in the presence of the miRNA.

? TROUBLESHOOTING

## Comparison with other methods

Current routinely used methods for evaluating the efficacy of plant amiRNAs or miRNAs include RT-qPCR and RNA blot analyses for monitoring target transcript levels[8,9], and RNA ligase mediated-5′ rapid amplification of cDNA ends (RLM-RACE) for detecting products of amiRNA/miRNA-mediated target mRNA cleavage[23]. However, the results of both methods do not reflect the amiRNA/miRNA action at the protein level and may lead to misinterpretation of amiRNA/miRNA activities given the complexity of the potential silencing mechanisms[11,24,25]. The ETPamir screen directly examines the ultimate outcome of gene silencing at the protein level, bypassing the complexity that amiRNA/miRNA can mediate gene silencing at the target mRNA or/and protein level[11,24,25]. The use of epitope tags and tag antibodies in the screens not only circumvents the technical obstacle of plant antibody paucity but also offers enhanced sensitivity and flexibility. Although translational repression has been analyzed by co-expression of plant miRNA and the GFP fusion to a

specific target gene through agroinfiltration of *Nicotiana benthamiana* leaves and microscopic visualization[26], our protoplast-based ETPamir screen offers four advantages over that method. First, the leaf agroinfiltration-mediated transient assay is only amenable in several plant species, whereas the protoplast transient expression system renders the ETPamir screen applicable in a broad range of plant species (Table 1), thus offering higher possibility to evaluate amiRNA/miRNA activities in near-native cellular contexts in the plant species of interest. Second, leaf agroinfiltration has relatively lower efficiency and higher variability in DNA co-delivery than the protoplast transient assay[20]. Third, GFP visualization is not as sensitive and quantitative as protein blot analyses. Fourth, the large size of the GFP protein may interfere with the stability, function and regulation of target proteins.

## Experimental design

Proper amiRNA expression backbone and experimental controls are key for identifying optimal amiRNAs in a conclusive and reliable manner. An appropriate endogenous miRNA backbone from the plant species of interest or its close relatives should be used to express amiRNA precursors to avoid potential problems associated with amiRNA expression and processing. Table 2 summarizes miRNA backbones that have been proven useful for amiRNA expression in dicot, monocot, tree or alga species. If a native or species-related miRNA backbone is not readily available, the *Arabidopsis* miR319a (ath-miR319a) backbone or the rice miR528 (osa-miR528) backbone can be used as an alternative for amiRNA expression in dicots and monocots, respectively (see many examples in Table 2). In the ETPamir screen, a negative control with the target gene expression alone should be conducted in parallel with other amiRNA screens to monitor target protein accumulation without amiRNA co-expression. An untargeted control gene (e.g., *GFP*) should be co-expressed with the target gene in every transfection experiment (including in the negative control) to indicate comparable transfection efficiencies between samples, as well as the absence of nonspecific silencing effects of amiRNA expression. The protein products of the untargeted control gene should be clearly distinguishable in size from the proteins of interest. On the user's first attempt of the ETPamir screen, we recommend that a positive control (i.e, co-expression of a target gene with its verified optimal amiRNA) may be conducted to ensure the ETPamir screen procedure is working properly in user's own experimental conditions (target genes and their verified optimal amiRNA constructs are available from the authors). Regarding the target gene validation for endogenous plant miRNAs, the miRNA expression backbone is not an issue because the endogenous pre-miRNAs of interest will be expressed. However, the same requirements on the control setup should be followed.

For protoplast incubation in the ETPamir screens, users can choose Option A (i.e., constitutive co-expression of amiRNA and target mRNAs) if less hands-on manipulation is preferred or if the protein products of the target gene are relatively unstable. Alternatively, users can choose Option B (i.e., constitutive expression of amiRNA but inducible expression of target mRNAs) if a quicker identification of optimal amiRNAs is desired. Accordingly, target gene and untargeted control gene should be expressed using a constitutive promoter for Option A, or using the heat shock promoter for Option B. In Option A, 36 h of co-

expression is empirically considered optimal for clearly discriminating potent, moderate and ineffective amiRNAs for most target genes, while shorter co-expression time (e.g., 6-12 h) is required for target genes encoding unstable proteins. For example, the *Arabidopsis* ZAT6 (Zinc Finger of *Arabidopsis thaliana* 6) protein has a short half-life around 10 min. The optimal amiRNA for the *ZAT6* gene was found to completely block ZAT6-FLAG protein accumulation within 6 h co-expression[11].

The procedure presented in this protocol is specific for identifying an optimal amiRNA for a single target gene. When applying the ETPamir screen to identifying a single optimal amiRNA for multiple target genes, one can conduct the co-expression of each target gene with each amiRNA candidate in a pairwise manner and determine the optimal amiRNA that is able to potently silence all the target genes. Alternatively, one can co-express all the target genes together plus individual amiRNA candidates. In the latter case, to monitor different silencing profiles of individual target genes, one can employ the same tag for all the target genes if their proteins are well distinguishable by size, or use different tags for different target genes if the proteins migrate too closely in SDS-PAGE.

### Limitations

In the ETPamir screens, optimal amiRNAs are identified based on a transient assay. Therefore, we cannot completely rule out the possibility that reduction of the endogenous target gene expression by these optimal amiRNAs in transgenic plants can trigger enhanced target gene transcription to counterbalance the silencing effects, as some gene expression is controlled by transcriptional regulatory loops *in planta*[10]. In those cases, more potent amiRNA may be required. In terms of target gene validation of endogenous plant miRNAs by the ETPamir screen, one needs to be aware that target validation in this assay is conducted in conditions of miRNA over-expression in mesophyll protoplasts.

## MATERIALS

### REAGENTS

4-morpholineethanesulfonic acid (MES, Sigma, cat. no. M3671)

Mannitol (MP Biomedicals, cat. no. 102248)

$MgCl_2$ (Sigma, cat. no. M9272) KCl (Sigma, cat. no. P3911)

NaCl (Sigma, cat. no. S9888)

$CaCl_2$ (Sigma, cat. no. C7902)

CsCl (American Bioanalytical, cat. no. AB00300)

Polyethylene glycol 4000 (PEG4000, Sigma, cat. no. 81240)

Tween-20 (Sigma, cat. no. P7949)

Phusion DNA polymerase (NEB, cat. no. M0535)

PVDF membrane (Immobilon, cat. no. IPVH304F0)

Nonfat dry milk (Santa Cruz Biotechology, cat. no. sc-2325)

Bovine calf serum (HyClone, cat. no. SH30072.03)

HA HRP-conjugated antibody (Roche, cat. no. 12013819001)

SuperSignal West Pico chemiluminescent kit (Thermo Scientific, cat. no. 34080)

SuperSignal West Femto chemiluminescent kit (Thermo Scientific, cat. no. 34095)

10% precast polyacrylamide gel (Bio-Rad, cat. no. 456-1034)

Terrific broth (American Bioanalytical, cat. no. AB01966)

Custom oligo primers for PCR generating amiRNA precursors (Oligo synthesis service provider)

pHBT-HA constitutive expression vector (Available from the authors upon request)

pHSP-HA heat shock inducible expression vector (Available from the authors upon request)

pHBT-ath-miR319a constitutive expression plasmid (Available from the authors upon request)

## EQUIPMENT

CL2 clinical centrifuge (Thermo Scientific, cat. no. 004260F)

Mini-PROTEAN Tetra system (Bio-Rad, cat. no. 165-8006)

Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad, cat. no. 170-3940)

Fisher Scientific Isotemp heating block (Fisher Scientific, cat. no. 11-715-305Q)

2-ml round-bottom microcentrifuge tubes (USA Scientific, cat. no. 1620-2700)

1.5-ml microcentrifuge tubes (USA Scientific, cat. no. 1615-5500)

6-well culture plates (Falcon, cat. no. 3046)

1000-ml storage bottle with 0.22 μm vacuum filter (Corning, cat. no. 430517)

## REAGENT SETUP

**Mannitol, 0.8 M stock—**Dissolve 146 g mannitol in Milli-Q water to a final volume of 1 L. This solution can be stored at 25°C for 6 months.

**NaCl, 5 M stock—**Dissolve 292.2 g NaCl in Milli-Q water to a final volume of 1 L. This solution can be stored at 25°C for 12 months.

**$CaCl_2$, 1 M stock—**Dissolve 111 g $CaCl_2$ in Milli-Q water to a final volume of 1 L. This solution can be stored at 25°C for 6 months.

**KCl, 2 M stock—**Dissolve 149.1 g KCl in Milli-Q water to a final volume of 1 L. This solution can be stored at 25°C for 6 months.

**$MgCl_2$, 2 M stock—**Dissolve 190.4 g $MgCl_2$ in Milli-Q water to a final volume of 1 L. This solution can be stored at 25°C for 6 months.

**MES, 0.2 M stock (pH 5.7)—**Dissolve 39 g MES in 700 ml Milli-Q water, adjust to pH 5.7 with KOH, and finalize the volume to 1 L with Milli-Q water. This solution can be stored at 4°C for 12 months.

**Tris-HCl, 1.5 M stock (pH 6.8)—**Dissolve 181.65 g Tris base in 700 ml Milli-Q water, adjust to pH 6.8 with 120 ml concentrated HCl. This solution can be stored at 25°C for 12 months.

**Calf serum, 5% (vol/vol)—**Mix 25 ml bovine calf serum with 475 ml sterile Milli-Q water. This solution can be stored at 4°C for up to 6 months.

**MMg solution—**Mix 250 ml 0.8 M mannitol stock solution, 3.75 ml 2 M $MgCl_2$ stock solution and 10 ml 0.2 M MES stock solution, and finalize the volume to 500 ml with Milli-Q water. Sterilize by passing through a 0.22 μm filter and collect the flow-through into a storage bottle. This solution can be stored at 4°C for 6 months.

**WI solution—**Mix 312.5 ml 0.8 M mannitol stock solution, 5 ml 2 M KCl stock solution and 10 ml 0.2 M MES stock solution. and finalize the volume to 500 ml with Milli-Q water. Sterilize by passing through a 0.22 μm filter and collect the flow-through into a storage bottle. This solution can be stored at 4°C for 6 months.

**W5 solution—**Mix 15.4 ml 5 M NaCl stock solution, 62.5 ml 1 M $CaCl_2$ stock solution, 1.25 ml 2 M KCl stock solution and 5 ml 0.2 M MES stock solution. Finalize the volume to 500 ml with Milli-Q water. Sterilize by passing through a 0.22 μm filter and collect the flow-through into a storage bottle. This solution can be stored at 4°C for 6 months.

**PEG solution—**To make 10 ml PEG solution, dissolve 4 g PEG4000 in a mixture of 3 ml water, 2.5 ml 0.8 M mannitol stock solution and 1 ml 1 M $CaCl_2$ stock solution. This solution should be freshly made before use.

**SDS-PAGE loading buffer (4×)—**Dissolve 0.8 g SDS, 2 mg bromophenol blue in a mixture of 1.7 ml 1.5 M Tris-HCl, pH 6.8, 4 ml glycerol and 0.8 ml β-mecaptomethanol. Finalize the volume to 10 ml with Milli-Q water. This solution can be stored at 25°C for up to 6 months.

**Tris-Glycine-SDS Running buffer—**To make a 10× stock solution, dissolve 30.3 g Tris base, 144 g glycine, 10 g SDS in Milli-Q water to a final volume of 1 L. This stock solution can be stored at 25°C for 12 months and can be diluted to 1× before use.

**Transfer buffer—**Dissolve 3 g Tris base, 14.4 g glycine in 800 ml Milli-Q water, add 100 ml methanol, and finalize the volume to 1 L with Milli-Q water. This solution can be stored at 25°C for 3 months.

**TBST buffer—**Dissolve 6.1 g Tris base, 8.8 g NaCl in 800 ml Milli-Q water, adjust to pH 7.4 with HCl. Add 0.5 ml Tween-20 and finalize the volume to 1 L with Milli-Q water. This solution can be stored at 25°C for up to 6 months.

**Blocking buffer—**Dissolve 5 g nonfat dry milk in 100 ml TBST buffer. This solution should be freshly made before use.

## PROCEDURE

### Design and selection of amiRNAs ● TIMING 1-2 days

**1|** Follow the detailed instruction on the WMD website (http://wmd3.weigelworld.org) to obtain a list of predicted, gene-specific amiRNA candidates for the gene(s) of interest. In the "Designer" webpage of WMD, the user can either input the gene identification number or the gene sequence in the fasta format as "Target genes", and select the intended plant genome from the WMD genome database as "Genome", and input "0" as "Accepted off-targets" to ensure that the designed amiRNA candidates are specific to the gene(s) of interest.

**2|** Select 3-4 amiRNA candidates satisfying all the criteria in Table 3.

**CRITICAL STEP** WMD ranks amiRNA candidates based on sequence complementarity and small RNA properties[10]. The amiRNA ranking on the WMD prediction list may or may not be correlated with its experimentally determined efficacy[11]. However, it is convenient that the search of suitable amiRNA candidates starts from the top candidate on the list. By clicking into each amiRNA candidate on the list, the user can access detailed characteristics about the candidate, including the target site location, mismatch number and position, hybridization energy and potential off-targets. It should be noted that potential off-targets are different from the "defined" off-targets excluded in Step 1, as the formers may have considerable sequence complementarity with a given amiRNA but the mismatch positions or/and hybridization energy parameters prohibit the WMD algorithm from making a clear judgment.

**? TROUBLESHOOTING**

**3|** Input individual selected amiRNA sequences on the "Oligo" webpage of WMD to design primers for generating amiRNA precursors (pre-amiRNAs) by PCR.

**4|** Assemble individual pre-amiRNAs using an appropriate endogenous miRNA backbone (see **Experimental design** and Table 2) by overlapping PCR according to the detailed instructions on the WMD website.

### Generation of amiRNA and target gene constructs ● TIMING 1-2 weeks

**5|** Clone individual pre-amiRNAs into a transient expression plasmid (e.g., the pHBT-ath-miR319a plasmid) containing a constitutive and strong promoter and the *NOS* terminator. Meanwhile, clone the target gene of interest or an untargeted control gene (see **Experimental design**) into a transient expression

plasmid encoding hemagglutinin (HA)-tagged proteins under a constitutive and strong promoter (e.g., the pHBT-HA plasmid; for Option A in Step 16) or under the heat shock promoter[11] (e.g., the pHSP-HA plasmid; for Option B in Step 16).

**CRITICAL STEP** The HA tag (YPYDVPDYA) and FLAG tag (DYKDDDDK) are highly recommended due to their small sizes and excellent antibody resources. Their 27-bp and 24-bp coding sequences, respectively, can be easily fused with the target gene coding sequence as part of the primer sequence through PCR. Other epitope tags and fluorescent proteins (e.g., GFP) with commercial antibodies available can also be used. A binary plasmid can also be used instead of the transient expression plasmid but may lead to reduced protoplast transfection efficiency.

**6|** Transform *E. coli* and grow a single colony in 200 ml Terrific broth with appropriate antibotics at 37°C for 16 h.

**7|** Purify the DNA of the plasmids expressing amiRNAs and target genes.

**CRITICAL STEP** Obtaining high-quality and concentrated (2 μg/μl) plasmid DNA is crucial for high transfection efficiency in protoplasts, and we highly recommend using CsCl gradient ultracentrifugation for this purpose (its protocol is provided on the Sheen lab website: http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html). DNA preparation by homemade silica resin[27] or by commercial DNA maxiprep kits is acceptable. The commercial DNA maxiprep kits are more convenient but expensive, and their plasmid DNA yields in general result in lower protoplast transfection efficiency.

**PAUSE POINT** Purified DNA can be stored at −20°C until use.

### Protoplast isolation ● TIMING 3-4 h

**8|** Follow the detailed protocol[20] for isolating mesophyll protoplasts from 4-week-old *Arabidopsis* plants. We used this protocol successfully, with no modification, to isolate protoplasts from, but not limited to, 4-week-old tobacco, 3-week-old *Catharanthus roseus*, and 2-week-old tomato or sunflower[11].

**CRITICAL STEP** Using healthy plants is very critical for achieving high quality protoplasts that allow efficient DNA transfection and protein expression, and maintain cell integrity during prolonged (e.g., >24 h) incubation.

### Co-transfection of amiRNA and target gene constructs ● TIMING 15 min for 5 samples

**9|** For each co-transfection, mix DNA of the following three plasmids in a 2-ml round-bottom microcentrifuge tube to generate a 21 μl DNA cocktail (2 μg DNA/μl): 16 μl (32 μg) of the amiRNA construct, 4 μl (8 μg) of the target gene-*HA* tag construct, and 1 μl (2 μg) of control gene (e.g., *GFP*)-*HA* tag construct. For a negative control, prepare an additional DNA cocktail replacing the amiRNA construct with empty vector to monitor target protein accumulation without amiRNA co-expression.

**10|** Add 200 µl protoplasts ($2 \times 10^5$ cells/ml in MMg solution) to each tube.

**11|** Add 220 µl PEG solution to each tube and mix well by gently tapping on the tube bottom 15 times.

**12|** Incubate the samples at room temperature for 5 min.

**13|** Quench the transfection by adding 800 µl W5 solution and inverting the tube twice.

**14|** Pellet the protoplasts by centrifugation at 100 × *g* for 2 min at room temperature using a CL2 clinical centrifuge, and remove the supernatant.

**CRITICAL STEP** The supernatant (~1.2 ml) should be pipetted out using a 1 ml pipette with caution. To avoid disturbing the protoplast pellet at the tube bottom, there is no need to completely remove the supernatant and 20-30 µl of supernatant can be left in the tube.

**15|** Resuspend the transfected protoplasts with 100 µl W5 solution per sample and transfer cells to 1 ml WI solution in a 6-well culture plate pre-coated with 5% calf serum, and mix well.

### Protoplast incubation ● TIMING 6-36 hr

**16|** Use either Option A or Option B for protoplast incubation, each with particular advantages (see **Experimental design**). Accordingly, construct target gene and untargeted control gene expression plasmids using a constitutive promoter for Option A, or the heat shock promoter for Option B (see Step 5).

**A.** Constitutive co-expression of amiRNA and target mRNAs:

**i.** Incubate the transfected protoplasts under normal plant growth conditions for 6-36 h (see **Experimental design**). Normal plant growth conditions are photoperiods of 12 h light (75 µmol$m^{-2}s^{-1}$) at 23°C and 12 h dark at 20°C[20].

**? TROUBLESHOOTING**

**B.** Constitutive expression of amiRNA but inducible expression of target mRNAs:

**i.** Incubate the transfected protoplasts under normal plant growth conditions for 3 h.

**ii.** Incubate protoplasts at 37°C for 1 h.

**iii.** Incubate protoplasts under normal plant growth conditions for another 3 h.

### Identification of optimal amiRNAs ● TIMING 6 h

**17|** Resuspend the protoplasts by gently swirling the 6-well plate and transfer cells to 1.5 ml microcentrifuge tubes.

**18|** Pellet the protoplasts by centrifugation at 100 × *g* for 2 min at room temperature using the CL2 clinical centrifuge.

**19|** Remove most of the supernatant and leave ~30 µl WI solution and the pellet at the bottom intact.

**20|** Add 10 µl 4 × SDS-PAGE loading buffer to each tube, briefly vortex, and boil the samples at 95°C for 5 min.

**PAUSE POINT** Protein samples can be stored at −20°C until further analysis.

**21|** Resolve all protein samples (~40 µl each) in a 10% precast polyacrylamide gel until the dye is running out.

**22|** Transfer the proteins from the gel to a PVDF membrane.

**23|** Incubate the membrane with the blocking buffer under gentle (70 rpm) shaking at room temperature for 30 min.

**24|** Incubate the membrane with the blocking buffer containing HA HRP-conjugated antibodies (1:10,000 dilution) under gentle shaking at room temperature for 2 h.

**25|** Wash the membrane three times (10 min each time) with the TBST buffer under gentle shaking.

**26|** Detect tagged proteins with the SuperSignal West Pico chemiluminescent kit.

**? TROUBLESHOOTING**

**27|** Quantify the immunoblot signals by densitometric analysis using the Image J program (the program can be downloaded at http://rsbweb.nih.gov/ij/download.html).

**28|** Identify the optimal amiRNA(s) whose co-expression completely blocks or leads to minimal target protein accumulation relative to the negative control.

**? TROUBLESHOOTING**

## TIMING

Step 1-4, design and selection of amiRNAs: 1-2 days

Step 5-7, generation of amiRNA and target gene constructs: 1-2 weeks

Step 8, protoplast isolation: 3-4 h

Step 9-15, co-transfection of amiRNA and target gene constructs: 15 min for 5 samples (4 amiRNA samples plus 1 negative control)

Step 16, protoplast incubation: 6-36 h

Step 17-28, identification of optimal amiRNAs: 6 h

## ? TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.

## ANTICIPATED RESULTS

A typical result of the ETPamir screen is shown at the bottom of Figure 1. In general, at least one optimal amiRNA can be identified from 3-4 selected amiRNA candidates for a single target gene by following this protocol. The optimal amiRNAs should be able to reduce the target protein accumulation by over 90% compared to the negative control, given that the expression of the untargeted control gene is comparable between samples. Although constitutive expression of moderate to suboptimal amiRNAs can generate target gene knockdown phenotypes, constitutive expression of those optimal amiRNAs would very likely lead to “functional knockout” of target gene expression, conferring silencing phenotypes resembling genetic null mutants[11]. Optimal amiRNAs can also be expressed using a chemically inducible promoter or a tissue-specific promoter in transgenic plants to gain tight temporal and spatial control of target gene activity during the functional study.

Using the key strategy of the ETPamir screen, bioinformatically predicted target genes for a given endogenous plant miRNA can be experimentally validated as illustrated in Figure 2, where the protein products of an authentic target gene will be reduced in the presence of miRNA, while those of a false target gene will not be affected. Even if the validated target gene and the miRNA were not co-expressed *in planta*[26], the results of this assay may still be biologically relevant considering the possibility of intercellular movement of many plant natural miRNAs[28].

## Acknowledgments

The authors thank the Weigel lab for developing the versatile WMD platform and members in the Sheen lab for their efforts to test and improve the ETPamir screens. This work has been supported by Massachusetts General Hospital ECOR Postdoctoral Fellowship for Medical Discovery to J.F.L. and the National Science Foundation Grant IOS-0843244 and the National Institutes of Health Grants R01 GM60493 and R01 GM70567 to J.S.

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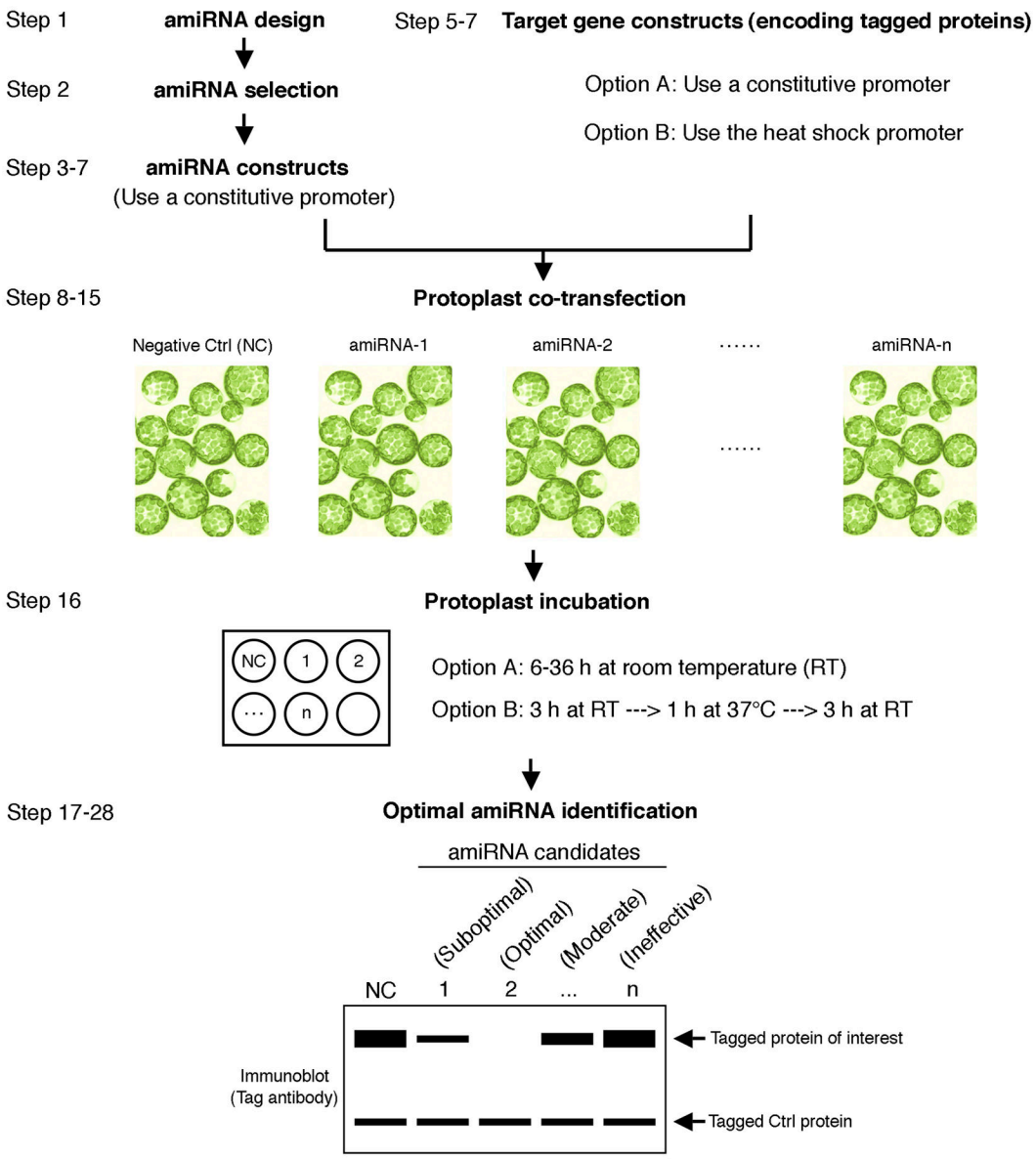


**Figure 1.**

Flow chart of the ETPamir screens for identifying optimal amiRNAs. Co-expression of the target gene encoding epitope-tagged proteins with different amiRNAs in plant protoplasts and subsequent immunoblot analysis of target protein accumulation using tag antibodies facilitate a quick and reliable discrimination of potent, moderate and ineffective amiRNAs from computationally designed candidates. Two co-expression strategies, Option A and Option B, are provided each with particular advantages. The protoplast incubation time in Option A depends on the target protein stability - unstable target proteins require a shorter incubation time (e.g., 6-12 h). An untargeted control gene is co-expressed in every transfection experiment as an indicator of equal transfection efficiency and absence of side effects of amiRNA expression.

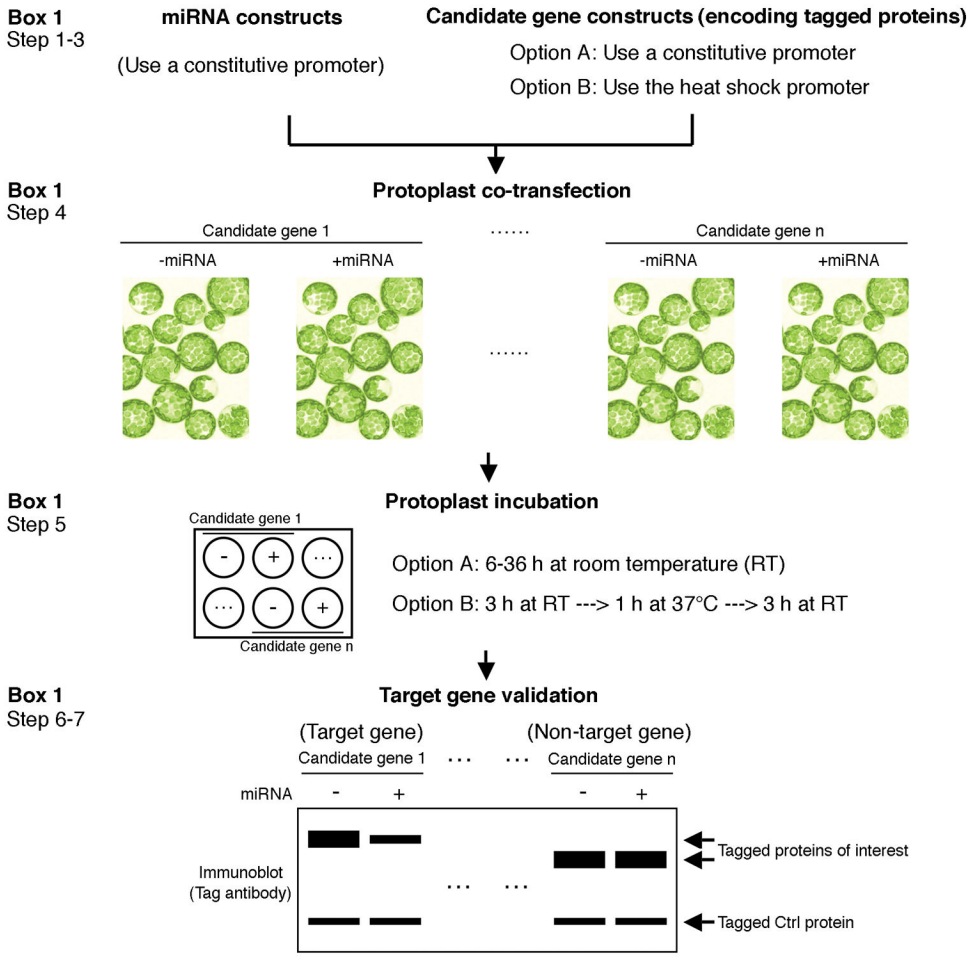


**Figure 2.**

Flow chart of the protein-based validation of predicted target genes for plant natural miRNAs. Co-expression of predicted candidate genes encoding epitope-tagged proteins with the miRNA of interest in plant protoplasts and subsequent immunoblot analysis of candidate protein accumulation by tag antibodies allow an easy and robust identification of authentic miRNA target genes. Two co-expression strategies, Option A and Option B, are provided each with particular advantages. The protoplast incubation time in Option A depends on the candidate protein stability. An untargeted control gene is co-expressed in every transfection experiment as an indicator of equal transfection efficiency and absence of side effects of miRNA expression.

## TABLE 1

Plant species in WMD genome database with established protoplast transient assay.

| Plant Latin name | Common name | Group | Reference |
|---|---|---|---|
| *Actinidia deliciosa* | Kiwifruit | Dicot | 29 |
| *Arabidopsis thaliana* | | Dicot | 20 |
| *Arachis hypogaea* | Peanut | Dicot | 30 |
| *Avena sativa* | Oat | Monocot | 31 |
| *Brassica napus* | Rapeseed | Dicot | 32 |
| *Brassica oleracea* | | Dicot | 33 |
| *Capsicum annuum* | Pepper | Dicot | 34 |
| *Carica papaya* | Papaya | Dicot | 35 |
| *Catharanthus roseus* | | Dicot | 11 |
| *Chlamydomonas reinhardtii** | | Alga | 36 |
| *Citrus sinensis* | Sweet orange | Dicot | 37 |
| *Cucumis sativus* | Cucumber | Dicot | 38 |
| *Festuca arundinacea* | Tall fescue | Monocot | 39 |
| *Glycine max* | Soybean | Dicot | 40 |
| *Gossypium hirsutum* | Cotton | Dicot | 41 |
| *Helianthus annuus* | Sunflower | Dicot | 11 |
| *Hordeum vulgare* | Barley | Monocot | 42 |
| *Lactuca sativa* | Lettuce | Dicot | 43 |
| *Medicago sativa* | Alfalfa | Dicot | 44 |
| *Nicotiana benthamiana* | | Dicot | 11 |
| *Nicotiana sylvestris* | | Dicot | 45 |
| *Nicotiana tabacum* | Tobacco | Dicot | 46 |
| *Oryza sativa* | Rice | Monocot | 47 |
| *Panicum virgatum* | Switchgrass | Monocot | 48 |
| *Petunia hybrida* | | Dicot | 49 |
| *Phaseolus vulgaris* | Bean | Dicot | 50 |
| *Physcomitrella patens* | | Bryophyte | 51 |
| *Pinus pinaster* | Maritime pine | Pinophyta | 52 |
| *Pisum sativum* | Pea | Dicot | 53 |
| *Populus tremula × alba* | Poplar | Dicot | 54 |
| *Saccharum officinarum* | Sugarcane | Monocot | 55 |
| *Selaginella moellendorffii* | | Lycophyte | 56 |
| *Solanum lycopersicum* | Tomato | Dicot | 11 |
| *Solanum tuberosum* | Potato | Dicot | 57 |
| *Taraxacum officinale* | Dandelion | Dicot | 58 |
| *Triticum aestivum* | Wheat | Monocot | 59 |
| *Vigna unguiculata* | Cowpea | Dicot | 60 |
| *Vitis vinifera* | Grapevine | Dicot | 61 |
| *Zea mays* | Maize | Monocot | 62 |

* *Chlamydomonas reinhardtii* is transformed by the glass-bead method[36] instead of protoplast transfection.

## TABLE 2

Reported amiRNA backbones in diverse plant species.

| Plant name | Common name | Group | amiRNA backbone | Reference |
|---|---|---|---|---|
| *Arabidopsis thaliana* | | Dicot | ath-miR159a | 13 |
| | | | ath-miR164 | 8 |
| | | | ath-miR169d | 63 |
| | | | ath-miR172a | 9 |
| | | | ath-miR319a | 9, 11 |
| *Catharanthus roseus* | | Dicot | ath-miR319a | 11 |
| *Chlamydomonas* | | Alga | cre-miR1162 | 64 |
| *reinhardtii* | | | cre-miR1157 | 65 |
| *Glycine max* | Soybean | Dicot | ath-miR319a | 66 |
| *Gossypium hirsutum* | Cotton | Dicot | ghi-miR169a | 67 |
| *Helianthus annuus* | Sunflower | Dicot | ath-miR319a | 11 |
| *Medicago sativa* | Alfalfa | Dicot | ath-miR319a | 68 |
| *Medicago truncatula* | | Dicot | mtr-miR159b | 69 |
| *Nicotiana benthamiana* | | Dicot | ath-miR319a | 11 |
| *Nicotiana tabacum* | Tobacco | Dicot | ath-miR164b | 8 |
| *Oryza sativa* | Rice | Monocot | osa-miR528 | 11, 15 |
| *Physcomitrella patens* | | Bryophyte | ath-miR319a | 70 |
| *Populus tremula × alba* | Poplar | Dicot | ptc-miR408 | 71 |
| *Solanum lycopersicum* | Tomato | Dicot | ath-miR319a | 11, 72 |
| | | | ath-miR164 | 8 |
| *Solanum melongena L.* | Eggplant | Dicot | ath-miR319a | 73 |
| *Solanum tuberosum* | Potato | Dicot | ath-miR168a | 18 |
| *Triticum aestivum* | Wheat | Monocot | osa-miR395 | 74 |
| *Vitis vinifera* | Grapevine | Dicot | vvi-miR166f | 75 |
| *Zea mays* | Maize | Monocot | zma-miR396 | 76 |
| | | | ath-miR319a | 11 |

## TABLE 3

Criteria for selecting amiRNA candidates from the WMD prediction list.

| Number | Criterion |
|---|---|
| 1 | Target site within the 5′ 200 nucleotides (nt) of the coding sequence (CDS) |
| 2 | No identical or overlapping target sequence with other selected amiRNA candidates |
| 3 | Less than 2 mismatches between the amiRNA candidate and its target mRNA |
| 4 | Mismatch can be acceptable only at the position 1 or 15-21 of an amiRNA candidate |
| 5 | Hybridization energy between the amiRNA candidate and its target sequence should be above 80% of that between the amiRNA and a perfect complement |
| 6 | No potential off-target is predicted by WMD |

The criteria were empirically determined based on the evaluation of 79 amiRNA-target mRNA interactions in *Arabidopsis* mesophyll protoplasts[11].

## TABLE 4

Troubleshooting table.

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 2 | WMD cannot design any single amiRNA to target the multiple gene targets<br>Insufficient or no amiRNA candidates fulfill all the criteria in Table 3 | Target genes do not share sufficient sequence identity<br>Target gene has a limited number of designed amiRNA candidates targeting its 5′ 200 nt of CDS | Reduce target gene number or use multiple amiRNAs to taregt these genes<br>Relax the amiRNA target site requirement to include the entire CDS |
| 16A(i) | Bacteria are detected at the end of the incubation step | Experimental environment is not hygienic | Add 200 μg/ml (final) carbenicillin to WI solution in Step 15 |
| 26 and Box 1, step 7 | No target protein is detectable (even in the negative control) | Target gene is expressed low due to inherent reason (e.g., large protein size or codon usage bias)<br>Target protein is unstable | Use the SuperSignal West Femto chemiluminescent kit to enhance immunoblot signals<br>Use Option A in step 16 with shorter co-expression time (e.g., 6-12 h) |
| 27 | No amiRNA candidate can sufficiently suppress target gene expression | Tested amiRNAs are not potent<br>Target gene is highly expressed and its protein products are very stable | Return to Step 2 to select 3-4 additional amiRNA candidates and repeat the ETPamir screen<br>Use Option B in Step 16 |