Epitope-tagged protein-based artificial miRNA screens for optimized gene silencing in plants

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

[1]Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts, USA. [2]Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. Correspondence should be addressed to J.-F.L. (jli@molbio.mgh.harvard.edu).

Published online 27 March 2014; doi:10.1038/nprot.2014.061

Artificial miRNA (amiRNA) technology offers highly specific 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 potential target genes encoding epitope-tagged proteins are constitutively or inducibly coexpressed with individual amiRNA candidates in plant protoplasts. Accumulation of tagged proteins, detected by immunoblotting with commercial tag antibodies, 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 d. The ETPamir screens circumvent the limited availability of plant antibodies and the complexity of plant amiRNA silencing at target mRNA and/or protein levels. The method can be extended to verify predicted target genes for endogenous plant miRNAs.

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*, transfer DNA (T-DNA) insertion lines represent the most important resource for loss-of-function mutants. Targeted genome editing 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 the 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 or physically linked genes in plant genomes[7]. The 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 hairpin 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 by 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 an amiRNA precursor (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 owing 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 expressed sequence tags (ESTs)[10]. However, the *in vivo* silencing efficacy of individual amiRNA candidates can be highly variable[10,11,15–18]. This is largely due to unpredictable factors, such as amiRNA expression and processing, target mRNA structure and accessibility, and the 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* screening 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 ETPamir screen[11]. Our strategy is to constitutively or inducibly coexpress 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].

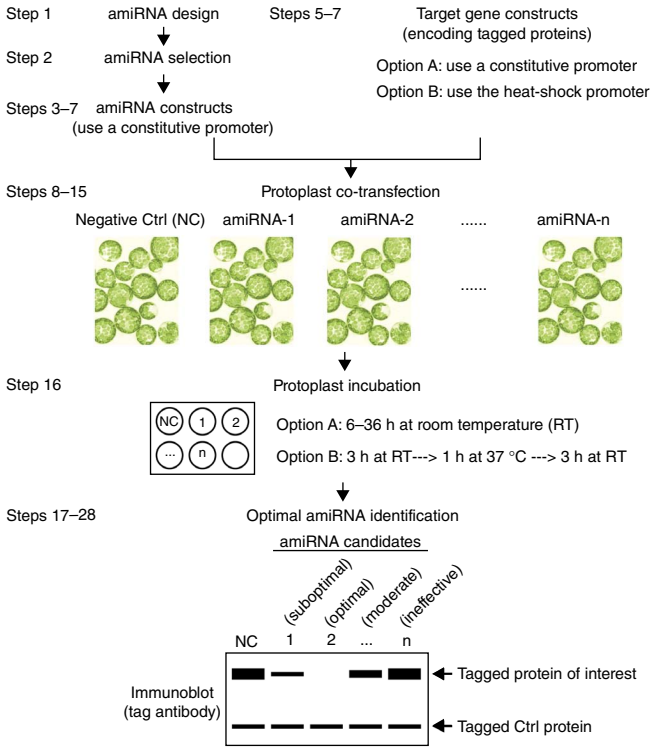

Figure 1 | Flowchart of the ETPamir screens for identifying optimal amiRNAs. Coexpression of the target gene encoding epitope-tagged proteins with different amiRNAs in plant protoplasts and subsequent immunoblot analysis of target protein accumulation using tag antibodies facilitate a quick and reliable discrimination of potent, moderate and ineffective amiRNAs from computationally designed candidates. Two coexpression strategies, options A and B (Step 16), are provided each with particular advantages. The protoplast incubation time in option A depends on the target protein stability, and unstable target proteins require a shorter incubation time (e.g., 6–12 h). An untargeted control gene is coexpressed in every transfection experiment as an indicator of equal transfection efficiency and the absence of side effects of amiRNA expression.

Transfected protoplasts are incubated for a 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 coexpression of an 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 turnover time of the tagged proteins synthesized from escaped target mRNAs at the beginning of coexpression (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. By 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 transacting siRNA[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 quantitative real-time (qRT)-PCR[11].

Comparison with other methods

Current routinely used methods for evaluating the efficacy of plant amiRNAs or miRNAs include qRT-PCR and RNA blot analyses for monitoring target transcript levels[8,9], and RNA ligase–mediated 5′ rapid amplification of cDNA ends for

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

(continued)

TABLE 1 | Plant species in WMD genome database with established protoplast transient assay (continued).

Plant Latin name	Common name	Group	Reference
Catharanthus roseus		Dicot	11
Chlamydomonas reinhardtii[a]		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

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

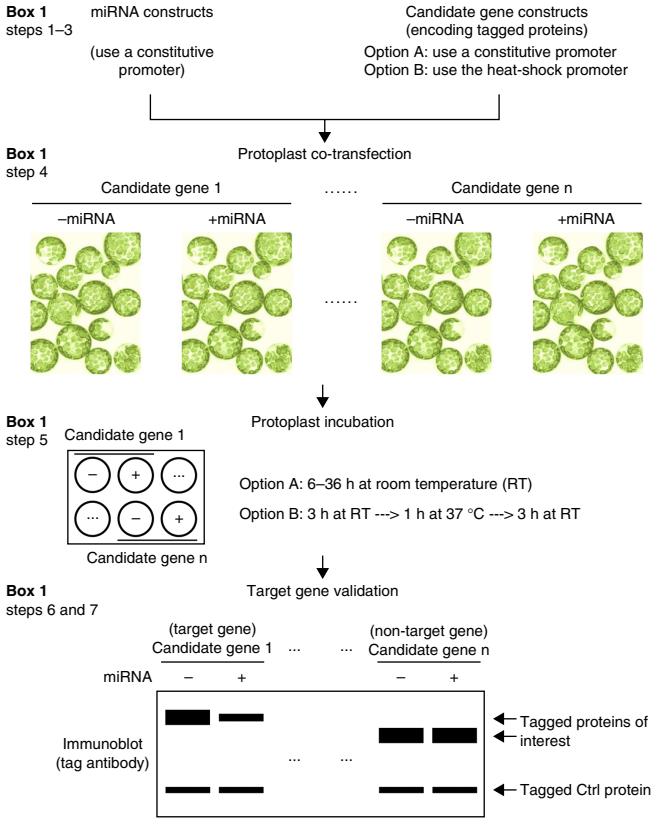

Figure 2 | Flowchart of the protein-based validation of predicted target genes for plant natural miRNAs. Coexpression of predicted candidate genes encoding epitope-tagged proteins with the miRNA of interest in plant protoplasts and subsequent immunoblot analysis of candidate protein accumulation by tag antibodies allow an easy and robust identification of authentic miRNA target genes. Two coexpression strategies, options A and 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 coexpressed in every transfection experiment as an indicator of equal transfection efficiency and absence of side effects of miRNA expression.

detecting products of amiRNA- or miRNA-mediated target mRNA cleavage[23]. However, the results of both methods do not reflect the amiRNA or miRNA action at the protein level and may lead to misinterpretation of amiRNA or 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 of amiRNA- or miRNA-mediated gene silencing at the target mRNA level and/or at the 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 coexpression 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 to several plant species, whereas the protoplast transient expression system renders the ETPamir screen applicable to a broad range of plant species (**Table 1**), thus offering higher possibility

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' page 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 the 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 9–15 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. Coexpress the miRNA and individual target candidate genes in protoplasts by using either option A or 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.

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to evaluate amiRNA/miRNA activities under 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.

Limitations of the ETPamir screens

In the ETPamir screens, optimal amiRNAs are identified on the basis of a transient expression 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 overexpression in mesophyll protoplasts.

Experimental design

The use of proper amiRNA expression backbone and experimental controls is 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 to be 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 screens, a negative control expressing the target gene alone should be conducted in parallel with other amiRNA screens to monitor target protein accumulation without amiRNA coexpression. An untargeted control gene (e.g., *GFP*) should be coexpressed 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 experiment (i.e., co-expression of a target gene with its verified optimal amiRNA) be conducted to ensure that the ETPamir screen procedure is working properly in the user's own experimental conditions (target genes and their verified optimal amiRNA constructs are available from the authors). With regard to 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 (Step 16), 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 by using a constitutive promoter for option A, or by using the heat-shock promoter for option B. In option A, 36 h of coexpression is empirically considered optimal for clearly discriminating potent, moderate and ineffective amiRNAs for most target genes, whereas shorter coexpression time (e.g., 6–12 h) is required for target genes encoding unstable proteins. For example, the *Arabidopsis* ZAT6 (zinc finger of *A. thaliana* 6) protein has a short half-life around 10 min. We found that the optimal amiRNA for the *ZAT6* gene completely blocked ZAT6-FLAG protein accumulation within 6 h of coexpression[11].

TABLE 2 | Reported amiRNA backbones in diverse plant species.

Plant Latin name	Common name	Group	amiRNA backbone	References
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 reinhardtii		Alga	cre-miR1162	64
			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

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 coexpression 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 coexpress all the target genes together plus individual amiRNA candidates. In the latter case, to monitor different silencing profiles of individual target genes, one can use 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.

MATERIALS

REAGENTS

- Plant materials: 4- to 5-week-old *Arabidopsis* ecotype Col-0 grown under conditions of 65% humidity and 75 µmol/m^2/s light intensity in photoperiods of 12 h of light at 23 °C and 12 h of dark at 20 °C (seeds available from *Arabidopsis* Biological Resource Center, http://www.arabidopsis.org/)
- 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 (PEG) 4000 (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 Biotechnology, cat. no. sc-2325)
- Bovine calf serum (HyClone, cat. no. SH30072.03)
- Anti-hemagglutinin (HA) horseradish peroxidase (HRP)-conjugated antibody (Roche, cat. no. 12013819001)
- SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific, cat. no. 34080)
- SuperSignal West Femto chemiluminescent substrate kit (Thermo Scientific, cat. no. 34095)
- Precast polyacrylamide gel, 10% (wt/vol) (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)
- Round-bottom microcentrifuge tubes, 2 ml (USA Scientific, cat. no. 1620-2700)
- Microcentrifuge tubes, 1.5 ml (USA Scientific, cat. no. 1615-5500)
- Culture plates, six-well (Falcon, cat. no. 3046)
- Storage bottle with a 0.22-µm vacuum filter, 1,000 ml (Corning, cat. no. 430517)
- Personal computer and ImageJ software

REAGENT SETUP

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

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

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

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

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

MES, 0.2 M stock (pH 5.7) Dissolve 39 g of MES in 700 ml of Milli-Q water, adjust the pH to 5.7 with KOH and bring the final volume to 1 liter 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 of Tris base in 700 ml of Milli-Q water, and adjust the pH to 6.8 with 120 ml of concentrated HCl. This solution can be stored at 25 °C for 12 months.

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

MMg solution Mix 250 ml of 0.8 M mannitol stock solution, 3.75 ml of 2 M $MgCl_2$ stock solution and 10 ml of 0.2 M MES stock solution, and adjust the final volume to 500 ml with Milli-Q water. Sterilize the solution by passing it through a 0.22-µm filter; 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 of 0.8 M mannitol stock solution, 5 ml of 2 M KCl stock solution and 10 ml of 0.2 M MES stock solution, and bring the final volume to 500 ml with Milli-Q water. Sterilize the solution by passing it through a 0.22-µm filter; 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 of 5 M NaCl stock solution, 62.5 ml of 1 M $CaCl_2$ stock solution, 1.25 ml of 2 M KCl stock solution and 5 ml of 0.2 M MES stock solution. Bring the final volume to 500 ml with Milli-Q water. Sterilize the solution by passing it through a 0.22-µm filter; 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 of PEG solution, dissolve 4 g of PEG 4000 in a mixture of 3 ml of water, 2.5 ml of 0.8 M mannitol stock solution and 1 ml of 1 M $CaCl_2$ stock solution. This solution should be freshly made before use.

SDS-PAGE loading buffer, 4× Dissolve 0.8 g of SDS and 2 mg of bromophenol blue in a mixture of 1.7 ml of 1.5 M Tris-HCl (pH 6.8), 4 ml of glycerol and 0.8 ml of β-mecaptomethanol. Bring the final 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 of Tris base, 144 g of glycine and 10 g of SDS in Milli-Q water to a final volume of 1 liter. 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 of Tris base and 14.4 g of glycine in 800 ml of Milli-Q water, add 100 ml of methanol and bring the final volume to 1 liter with Milli-Q water. This solution can be stored at 25 °C for 3 months.

Tris-buffered saline–Tween (TBST) buffer Dissolve 6.1 g of Tris base and 8.8 g of NaCl in 800 ml of Milli-Q water, and adjust the pH to 7.4 with HCl. Add 0.5 ml of Tween-20 and adjust the final volume to 1 liter with Milli-Q water. This solution can be stored at 25 °C for up to 6 months.

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

PROCEDURE

Design and selection of amiRNAs ● TIMING 1–2 d

1| Follow the detailed instructions 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 three or four amiRNA candidates satisfying all the criteria in **Table 3**.

▲ CRITICAL STEP WMD ranks amiRNA candidates on the basis of 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].

TABLE 3 | Criteria for selecting amiRNA candidates from the WMD prediction list.

Number	Criterion
1	Target site within the first 200 nucleotides of the coding sequence
2	No identical or overlapping target sequence with other selected amiRNA candidates
3	Fewer than two mismatches between the amiRNA candidate and its target mRNA
4	Mismatches are acceptable only at position 1 or positions 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 on the basis of the evaluation of 79 amiRNA-target mRNA interactions in *Arabidopsis* mesophyll protoplasts[11].

However, it is convenient that the search for 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 former 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.
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3| Input individual selected amiRNA sequences on the 'Oligo' page of WMD to design primers for generating pre-amiRNAs by PCR.

4| Assemble individual pre-amiRNAs by 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 *HA*-tagged proteins under a constitutive and strong promoter (e.g., the pHBT-HA plasmid; Step 16A) or under the heat-shock promoter[11] (e.g., the pHSP-HA plasmid; Step 16B).
▲ CRITICAL STEP The HA tag (YPYDVPDYA) and FLAG tag (DYKDDDDK) are highly recommended because of their small size 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 used for the 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 it may lead to reduced protoplast transfection efficiency.

6| Transform *Escherichia coli* and grow a single colony in 200 ml of 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 laboratory website: http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html). Alternatively, 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 in general the plasmid DNA obtained results 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 procedure[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 The use of healthy plants is crucial for achieving high-quality protoplasts that allow efficient DNA transfection and protein expression, and maintain cell integrity during prolonged (e.g., >24 h) incubations.

Co-transfection of amiRNA and target gene constructs ● TIMING 15 min for five 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 by replacing the amiRNA construct with empty vector to monitor target protein accumulation without amiRNA coexpression.

10| Add 200 µl of protoplasts (2 × 10^5 cells per ml in MMg solution) to each tube.

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

12| Incubate the samples at room temperature (~25 °C) for 5 min.

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

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

▲ **CRITICAL STEP** The supernatant (~1.2 ml) should be pipetted out by using a 1-ml pipette with caution. To avoid disturbing the protoplast pellet at the tube's bottom, leave 20–30 µl of supernatant in the tube.

15| Resuspend the transfected protoplasts with 100 µl of W5 solution per sample and transfer the cells to 1 ml of WI solution in a six-well culture plate precoated with 5% (vol/vol) calf serum; mix well.

Protoplast incubation ● TIMING 6–36 h

16| Use either option A or B for protoplast incubation, each of which has its own particular advantages (see Experimental design). Accordingly, target gene and untargeted control gene expression plasmids constructed in Step 5 should have a constitutive promoter for option A or the heat-shock promoter for option B.

(A) Constitutive coexpression 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 of light (75 µmol/m^2/s) at 23 °C and 12 h of dark at 20 °C (ref. 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 the protoplasts at 37 °C for 1 h.

(iii) Incubate the 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 six-well plate, and then transfer the 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 of WI solution and the pellet at the bottom intact.

20| Add 10 µl of 4× SDS-PAGE loading buffer to each tube, briefly vortex, and then 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% (wt/vol) 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 r.p.m.) shaking at room temperature for 30 min.

24| Incubate the membrane with the blocking buffer containing HA-specific 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 substrate kit.
? TROUBLESHOOTING

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

28| Identify the optimal amiRNA(s) whose coexpression completely blocks or leads to minimal target protein accumulation relative to the negative control.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 4**.

TABLE 4 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	WMD cannot design any single amiRNA to target multiple gene targets	Target genes do not share sufficient sequence identity	Reduce the target gene number or use multiple amiRNAs to target these genes
	Insufficient or no amiRNA candidates fulfill all the criteria in **Table 3**	The target gene has a limited number of designed amiRNA candidates targeting the first 200 nucleotides of its coding sequence	Relax the amiRNA target site requirement to include the entire coding sequence
16A(i)	Bacteria are detected at the end of the incubation step	The experimental environment is not hygienic	Add 200 μg/ml (final concentration) carbenicillin to the WI solution in Step 15
26 and **Box 1**, step 7	No target protein is detectable (even in the negative control)	Low endogenous target gene expression (because of, e.g., large protein size or codon usage bias)	Use the SuperSignal West Femto chemiluminescent substrate kit to enhance immunoblot signals
		The target protein is unstable	Use Step 16A with shorter co-expression time (e.g., 6–12 h)
28	No amiRNA candidate can sufficiently suppress target gene expression	The tested amiRNAs are not potent	Return to Step 2 to select three or four additional amiRNA candidates and repeat the ETPamir screens
		The target gene is highly expressed and its protein products are very stable	Use Step 16B

● TIMING
Steps 1–4, design and selection of amiRNAs: 1–2 d
Steps 5–7, generation of amiRNA and target gene constructs: 1–2 weeks
Step 8, protoplast isolation: 3–4 h
Steps 9–15, co-transfection of amiRNA and target gene constructs: 15 min for five samples (four amiRNA samples plus one negative control)
Step 16, protoplast incubation: 6–36 h
Steps 17–28, identification of optimal amiRNAs: 6 h

ANTICIPATED RESULTS
A typical result of the ETPamir screens is shown at the bottom of **Figure 1**. In general, at least one optimal amiRNA can be identified from three or four 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 with 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 by using a chemically inducible promoter or a tissue-specific promoter in transgenic plants to enable tight temporal and spatial controls of target gene activity during the functional study.

By 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**, in which the protein products of an authentic target gene are reduced in the presence of miRNA, whereas those of a false target gene are not affected. Even if the validated target gene and the miRNA were not coexpressed *in planta*[26], the results of this assay may still be biologically relevant when considering the possibility of intercellular movement of many plant natural miRNAs[28].

ACKNOWLEDGMENTS We thank the Weigel laboratory for developing the versatile WMD platform and members in the Sheen laboratory for their efforts to test and improve the ETPamir screens. This work has been supported by a Massachusetts General Hospital Executive Committee on Research Postdoctoral Fellowship for Medical Discovery to J.-F.L. and by grants from the US National Science Foundation (grant no. IOS-0843244) and the US National Institutes of Health (grant nos. R01 GM60493 and R01 GM70567) to J.S.

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 **Tables 1** and **2** and related references.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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