Chapter 6

Directed Gene Silencing with Artificial MicroRNAs

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Abstract

The characterization of gene function typically includes a detailed analysis of loss-of-function alleles. In model plants, such as *Arabidopsis thaliana* and rice, sequence-indexed insertion collections provide a large resource of potential null alleles that can often be easily accessed through convenient Web sites (e.g., http://signal.salk.edu). They are, however, not available for nonmodel species, require stacking for knockout of redundant homologs, and do not easily allow for partial or regulated loss of gene function, which is particularly useful when null alleles are lethal. Transgene approaches that employ directed gene silencing can substitute for null alleles and also enable refined studies of gene function, e.g., by tissue-specific and inducible gene-silencing.

This chapter describes the generation and application of artificial microRNAs (amiRNAs) as a gene silencing tool in a wide variety of different plant species.

Key words: Gene silencing, miRNA, Hairpin, Loss-of-function, Phenotypic complementation

1. Introduction

Mediators of transgene-induced gene silencing are single-stranded silencing RNAs (19–23 nucleotides) that bind to target transcripts through complementary base-pairing (1). MicroRNAs (miRNAs), one class of silencing RNAs, originate from a characteristic hairpin-containing transcript. Vectors that contain a hairpin precursor are recognized as second-generation RNAi vectors (2). Their sequence can be modified such that miRNAs of other, defined sequence, called artificial miRNAs (amiRNAs), are produced *in planta*. These vectors can serve as reverse genetics tools to direct gene silencing, also in nonmodel systems. Among their unique applications are transient and tissue-specific gene silencing, and the simultaneous

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silencing of several related genes. The latter include closely linked genes (e.g., in tandem repeats) and those that are allele- and splice form-specific, as well. In addition, they provide the possibility of phenotypic complementation with target transgenes that are no longer susceptible to amiRNA-mediated gene regulation after introduction of silent mutations into the target sites. The web application Web MicroRNA Designer (WMD) facilitates the design of suitable amiRNA sequences for a variety of different plant species, as well as the design of primer sequences needed to modify the miRNA vectors. We describe the use of the tool as well as the molecular steps necessary to engineer those vectors and generate transgenic plants.

2. Materials

2.1. Computational Prediction of amiRNA Sequences

1. Identifier (or EST name) and sequence of the target gene(s) that should be silenced.

2.2. Site-Directed Mutagenesis by PCR

1. Six oligonucleotides; two are universal to the vector (A and B; Table 1) and four are specific to the particular modification. Their sequences are the output of the amiRNA design program (3.1).
2. Template plasmid: pRS300 (containing *Arabidopsis ath-MIR319a*) or pNW55 (containing rice *osa-MIR528*).

Table 1
Oligonucleotide sequences

Template	Name	Position	Sequence 5′→3′
ath-MIR319a	I	amiRNA forward	GA $(N)_{21}$ T CTC TCT TTT GTA TTC C
	II	amiRNA reverse	GA $(N)_{21}$ T CAA AGA GAA TCA ATG A
	III	amiRNA* forward	GA $(N)_{21}$ T CAC AGG TCG TGA TAT G
	IV	amiRNA* reverse	GA $(N)_{21}$ T CTA CAT ATA TAT TCC T
osa- MIR528	I	amiRNA forward	AG $(N)_{21}$ C AGG AGA TTC AGT TTG A
	II	amiRNA reverse	TG $(N)_{21}$ C TGC TGC TGC TAC AGC C
	III	amiRNA* forward	CT $(N)_{21}$ T TCC TGC TGC TAG GCT G
	IV	amiRNA* reverse	AA $(N)_{21}$ A GAG AGG CAA AAG TGA A
	A	outside forward	CTG CAA GGC GAT TAA GTT GGG TAA C
	B	outside reverse	GCG GAT AAC AAT TTC ACA CAG GAA ACA G

3. Equipment and chemicals to perform PCR, agarose gel electrophoresis, and gel extraction of PCR bands.

2.3. Cloning of aMIRNA Precursors

1. Commercial PCR product ligation kit (e.g., TOPO kits from Invitrogen) or standard cloning plasmid (e.g., pGEM-7Z from Promega, linearized with Sma I), 10 mM ATP, restriction enzyme SmaI.
2. T4 DNA ligase.
3. Competent cells from a standard *E. coli* strain (e.g., DH5α, TOP10).
4. LB plates containing appropriate antibiotics.
5. Plasmid extraction (Miniprep) solutions or kit (e.g., QIAprep from Qiagen).
6. Restriction enzymes to test for positive clones and subclone *aMIRNAs*.
7. Binary plasmid containing a promoter of interest and a terminator.

2.4. Plant Transformation and Analysis of Transgenic Plants

1. Standard competent *Agrobacterium tumefaciens* strain (e.g., GV3101 for *A. thaliana*, LBA4404 or EHA105 for *O. sativa*).
2. Standard equipment for the generation of transgenic plants.
3. Selection marker and appropriate antibiotics (e.g., Kanamycin, Hygromycin).
4. TRIzol® reagent (Invitrogen) or commercial RNA extraction kit (e.g., RNeasy from Qiagen).
5. Reverse transcriptase kit (e.g., SuperScript III from Invitrogen) and oligonucleotides for RT-PCR.
6. Optional
   - Kit for mRNA extraction (e.g., Oligotex from Qiagen) and 5′ RACE (e.g., GeneRacer from Invitrogen).
   - Oligonucleotides for site-directed mutagenesis to engineer silent mutations in transgenes containing the target gene(s) for phenotypic complementation.

3. Methods

The method section describes in detail the design and construction of amiRNAs to silence one or several related genes of interest in a variety of different plant species. It is subdivided into four main sections: (1) the computer-assisted design of optimal amiRNA sequences, (2) the generation of *aMIRNA* precursors by site-directed mutagenesis, (3) cloning of *aMIRNA* precursors, and (4) the generation and analysis of transgenic plants.

3.1. Design of amiRNA Sequences

Sequences of amiRNAs need to be optimized for both effectiveness and specificity. The optimization for specificity (i.e., predicting and avoiding off-targets) depends on the availability of transcriptome sequence information. We have developed the Web MicroRNA Designer (WMD), which is available at http://wmd3.weigelworld.org. It predicts suitable amiRNA sequences for gene silencing in a large number of plant species for which a whole-genome annotation or significant EST/cDNA sequence information is available. Note 1 describes how to design artificial miRNA sequences for species that are not yet included in WMD.

3.1.1. Experimental Design

WMD can design amiRNAs to silence single genes as well as design amiRNAs to simultaneously silence multiple genes. These genes need to share regions of high nucleotide sequence similarity. WMD selects amiRNA candidates that are partially complementary to the target gene(s) and ensures that no other annotated gene of the respective genome/transcriptome release (species) fulfills the criteria of productive miRNA target interaction (3–5). These criteria have been empirically determined and are expected to improve as the knowledge on miRNA biology and function grows. It is important to realize that it might not be possible to design an amiRNA against one gene or several highly sequence-related genes, if all potential target sites to which the amiRNA would bind are also present in other (known) genes. Therefore, a search for highly similar genes is prudent before starting the design process. This can be done by a BLAST search implemented in the WMD webpage. In cases where targeting of highly related genes does not interfere with the experiment, these can be specified as "accepted off-targets" to increase the chances of a successful amiRNA design.

3.1.2. Using the amiRNA Design Tool (WMD-Designer Window)

WMD requires sequence information of the intended target gene(s) as input and selects 21mer amiRNA candidates from the reverse complement(s). To accommodate the different formats of sequence annotations and a variety of amiRNA applications, there are several ways to input "target genes" into the WMD-Designer:

1. Silencing single or multiple genes in fully annotated genomes: For genes that are fully annotated, it is sufficient to provide the gene identifier(s) including splice variants, e.g., At1g23450.1 or Os01g24680.1 for Arabidopsis or rice respectively. Whether all splice forms of a gene are targeted by a specific amiRNA can be quickly tested with the "WMD-Target Search tool" (*see* Note 2).
2. Silencing single or multiple genes in species for which only EST information is available:

   Locus identifiers (e.g., EST names) need to be provided, whereby the identifier has to be identical to the identifiers used

by WMD. The correct names are easily determined by a search with the WMD-BLAST tool. Multiple EST sequences often exist for a single locus. First they all need to be identified with the WMD-BLAST tool , then one of them (preferably the longest) should be used as the target gene (identifier entered in the "Target genes" field), and the identifiers of all others that likely originate from the same locus are entered as "accepted off-targets" (separated by commas). When attempting to silence multiple genes simultaneously, one sequence for each gene is entered in the "Target genes" field, and the others are added to the list of "accepted off-targets."

3. When none of the redundant ESTs comprises the full-length target transcript, several can be concatenated and serve as target for the amiRNA design. It should be given a custom identifier that distinguishes it from all annotated ESTs, and all redundant ESTs need to be specified as "accepted off-targets." The concatenated EST needs to be entered into the "Target genes" field in FASTA format.
4. Splice-form specific silencing:
   If exclusively one splice form is to be targeted, then a DNA sequence (>21 nucleotides, FASTA format) that is unique to the respective splice form should be used as target and the identifier/name of the splice form (e.g., At2g23450.2) should serve as a header.
5. Not annotated genes, and sequence variants:
   If the gene to be silenced is not contained in the WMD database for the respective species (e.g., *GUS*, *GFP*, viral genes, genes that are not yet annotated, etc.), or represents a sequence variant of an annotated gene (e.g., an allele from a different ecotype or cultivar), the target gene sequence(s) should be provided in FASTA format, headed by a custom name that is different from that of any other annotated gene. Silencing of sequence variants also requires the reference allele to be specified as an accepted off-target unless allele-specific silencing is desired.

In all cases, the respective plant species/genome release is to be selected from the drop-down menu on the WMD-Designer page to ensure specificity within this set of sequences. Specification of the "minimal number of included targets" is necessary when more than two genes are to be silenced simultaneously. In addition to finding an amiRNA that silences all genes simultaneously, WMD will attempt to generate amiRNAs that target all possible subgroups of sizes greater or equal to the number given in "minimal number of included targets."

The computation of amiRNAs will take between a few minutes and several hours. The results will be emailed to the provided email-address with the information entered in "Description" as the subject line.

Home | Target Search | Designer | Oligo | Hybridize | Blast | Downloads

Designer

Transcript library: TAIR8_cdna_20080412
Target genes: AT4G22380.1,AT4G12600.1
Description: Ribosomal Proteins
Min. number of included targets: 2
Accepted off-targets: 0
Annotated: 1
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Quality indicator	amiRNA sequence 5'->3'	Hybridiyation energy of amiRNA and a theoretical perfect match in kcal/mole	Target gene I	Hybridization energy of amiRNA/target I (kcal/mole)	Target gene II	Hybridization energy of amiRNA/target II (kcal/mole)
	TTCTGCTAACGGATATGGCTT	-43.45	AT4G12600	-31.53	AT4G22380	-36.85
	TTCTGCTAACGGATACGGCTA	-45.31	AT4G12600	-33.57	AT4G22380	-38.89
	TGGATACGCCTTAAGGTTCAG	-44.96	AT4G12600	-38.52	AT4G22380	-38.52
	TTCTGCTAACGGATACGGCTT	-44.89	AT4G12600	-33.60	AT4G22380	-38.92
	TATCTGCTAACGGATACGCAT	-43.31	AT4G12600	-35.43	AT4G22380	-40.75
	TTCTGCTAACGGATACGGCCT	-47.48	AT4G12600	-34.69	AT4G22380	-40.01
	TTCAGCTAACGGATATGGCTT	-43.45	AT4G12600	-36.85	AT4G22380	-30.96
	TTCAGCTAACGGATGCGTCTT	-44.88	AT4G12600	-41.70	AT4G22380	-35.81
	TATACGCCTTAGGGCTCACTT	-45.88	AT4G12600	-37.69	AT4G22380	-36.12
	TTCAGCTAACGGATACGCCTG	-46.70	AT4G12600	-44.56	AT4G22380	-38.67
	TTCAGCTAACGGATATGCCAT	-43.76	AT4G12600	-41.66	AT4G22380	-35.77

Fig. 1. Example of a WMD result page. Candidate amiRNA sequences are listed and ordered by efficiency and specificity criteria.

3.1.3. Processing amiRNA Design Results

The amiRNA result email contains a hyper-link to a results webpage on which the amiRNA candidate sequences are listed (Fig. 1). This list can also be downloaded for future reference (Microsoft Excel format). See Note 3 in case the results page is empty and displays the message "Unfortunately WMD3 could not design any microRNAs".

In principle, all amiRNA sequences returned by WMD fulfill the above described criteria and are expected to silence the predicted target genes successfully. However, they comply differently to the parameters that we consider optimal in terms of base pair composition, hybridization properties to the target gene(s), and specificity criteria (for details, see (6)). The possible amiRNAs are therefore ranked by a respective cumulative score. The highest ranking amiRNA candidates are presented on top of the list. Green color indicates a very favorable score, while orange and red often mark amiRNAs with potentially reduced efficiency or, more often, specificity. It is thus recommended to proceed from top to bottom of this list. The amiRNA sequences in the results page are hyperlinked to visualizations of alignments of the amiRNA to all potential target sequences in the WMD database, ordered by hybridization energy (for illustration see Fig. 2). The intended target gene ideally appears on top of the list. The WMD-Target Search tool can also show genes that align to the amiRNA

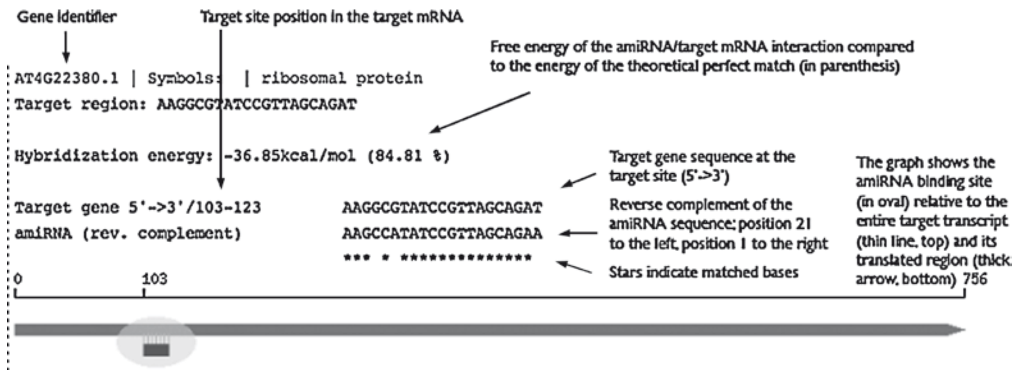

Fig. 2. Alignment of an amiRNA to its target gene. Explanation to the amiRNA-target alignment presented by WMD.

with equal or fewer than five mismatches but do not fulfill other empirical rules, as indicated with respective notes in red bars. For the subsequent selection of one (or more) amiRNAs for further experiments, we recommend the following:

1. It is preferable for all intended target genes to not have mismatches to the amiRNA at positions 2 to 12.
2. AmiRNA candidates with one or two mismatches at the 3′ end of the amiRNA (positions 18 to 21) should be preferred, since it has been suggested that perfectly matching amiRNAs might trigger so-called transitive siRNA formation, where amplification of sequences adjacent to the binding site is primed by the miRNA. These sequences could in turn themselves serve as silencing triggers and affect other, unintended genes (7).
3. The absolute hybridization energy of the binding between amiRNA and the target sequence should be less than −30 kcal/mole, and preferable be in the range between −35 and −40 kcal/mole.
4. The amiRNA binding site should be located within the coding region of the target gene, since UTRs are more likely to be misannotated.

At least two amiRNAs per target gene or group of genes should be selected for experimental work. If several are selected, the amiRNAs should bind the target mRNA at different locations, since secondary structure is suspected to influence miRNA efficacy.

3.2. Construction of aMIRNA Precursors by Site-Directed Mutagenesis

While exogenous small RNAs duplices are often directly used to transfect animal cell cultures and induce gene silencing, their accumulation in plants requires the construction and subsequent expression of a precursor RNA. AmiRNAs are engineered into vectors that contain endogenous *MIRNA* precursors by site-directed mutagenesis, such that the resulting precursor RNAs are processed by the endogenous miRNA machinery to release the

amiRNAs. Several *Arabidopsis* precursor templates have been used successfully in *Arabidopsis* (4, 8–11) and other plants (8, 26), while rice *osa-MIR528* was specifically engineered for amiRNA production in rice (12) and cre-MIR1157 and cre-MIR1162 have been used successfully in the unicellular green alga Chlamydomonas reinhardtii (24, 25). Please *see* Note 4 when working with a different plant species.

MIRNA precursors fold back on themselves to form a hairpin structure, and it is important to preserve this structure for successful processing. Therefore, engineering of amiRNAs into *MIRNA* precursor templates not only requires the exchange of the miRNA by the amiRNA sequence, but also of the pairing region in the hairpin, called the (a)miRNA*, such that pairing positions as well as G:U pairs are retained. The WMD software (WMD-Oligo window) thus generates four oligonucleotides per amiRNA sequence input: I and II to engineer the actual amiRNA, and III and IV for the amiRNA* (with wobbles). Currently, the software supports *ath-MIR319a*, *osa-MIR528*, and *cre-MIR1157* (selected from a dropdown menu) and others will be included as they become available.

3.2.1. The MIRNA Templates

Endogenous *MIRNA* precursors that have been cloned into plasmids serve as templates for PCR reactions to exchange miRNA and miRNA*. These precursors include the hairpin and short pieces of flanking sequence on either side, which are known to be part of the longer endogenous *MIRNA* transcript. Plasmids that are currently available contain *ath-MIR319a* (plasmid pRS300) and *osa-MIR528* (plasmid pNW55), which can be obtained upon sending a request to Detlef Weigel (weigel@weigelworld.org). A schematic representation of these *MIRNA* containing plasmids is shown in Fig. 3; their complete sequences are available on http://wmd3.weigelworld.org.

3.2.2. Oligonucleotides

Six PCR oligonucleotide primers are needed to produce an aMIRNA transgene. Four primer sequences are generated by WMD (WMD-Oligo window) and are given in 5′→3′ orientation. They are 40 nucleotides long and specific for the intended amiRNA. The 5′ most two and 3′ most 17 nucleotides match the template *MIRNA* precursor, while the 21 nucleotides in between do not match and will generate the amiRNA and amiRNA* in the amplicon (see Fig. 4).

An additional two general oligonucleotides (A and B; see sequences in Table 1) that match the harboring plasmid (a pBluescript derivative) outside of the *MIRNA* precursor are also required. They have been placed such that the sizes of the resulting PCR products enable convenient purification and handling. Using the six primers, the *aMIRNA* precursor is amplified in three pieces (a–c) as shown in Fig. 4. The three pieces are subsequently fused to one amplicon (d) in a single PCR reaction.

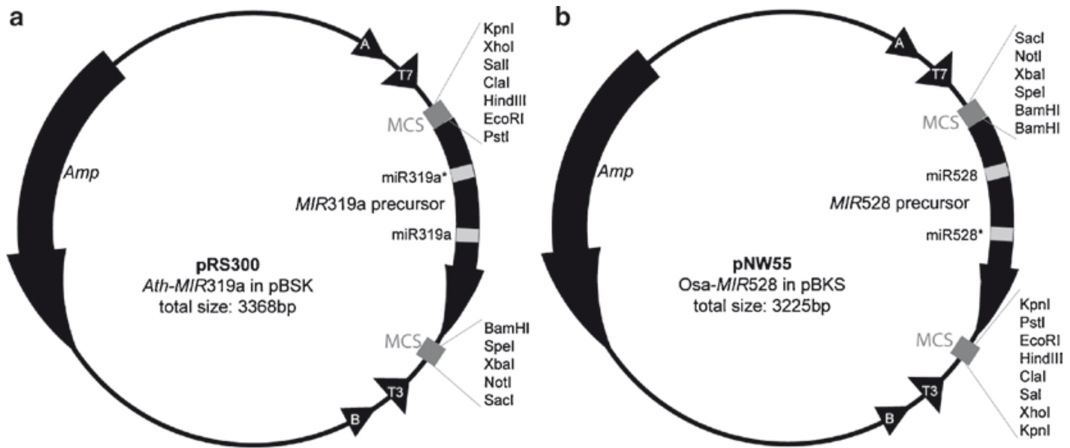

Fig. 3. Template plasmids for construction of the amiRNA precursor, the *aMIRNA* foldback. (**a**) Plasmid pRS300 containing the *ath-MIR319a* precursor in pBluescript SK (cloned via the SmaI site). (**b**) Plasmid pNW55 containing the *osa-MIR528* precursor in pBluescript KS (also cloned via the SmaI site). Complete plasmid sequences are available at http://wmd3.weigelworld.org. Abbreviations: A, B oligonucleotide binding sites; T3, T7 RNA polymerase/oligonucleotide binding sites; *Amp* Ampicillin resistance gene; MCS multiple cloning site. Sizes of the *aMIRNA* foldback and surrounding regions are indicated in Fig. 4.

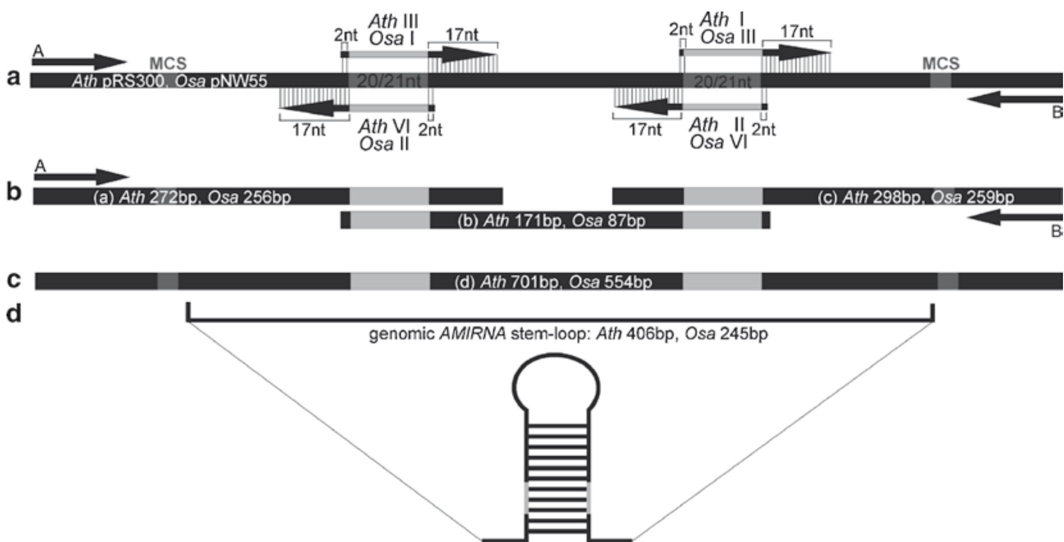

Fig. 4. Schematic representation of PCR reactions that generate *aMIRNA* precursors. (**a**) Illustration of the template plasmid (see Fig. 3) with oligonucleotide binding sites indicated. (**b**) PCR amplicons (a), (b), and (c). (**c**) (a), (b), and (c) are fused to (d) by PCR. (**d**) Only the central part encodes the *aMIRNA* precursor, which is schematically shown at the bottom. Abbreviations: *Ath Arabidopsis thaliana*; *Osa Oryza sativa* (rice); A, B, I, II, III, IV oligonucleotide identifiers (see text); MCS multiple cloning site; (a), (b), (c), (d) PCR fragments as indicated in the text.

3.2.3. Generating aMIRNA Precursors by Overlapping PCR

1. Resuspend the template plasmid upon receiving, transform into competent *E. coli* cells (standard lab strain), spread on ampicillin-containing LB plates, inoculate an overnight culture from a single colony, and isolate the plasmid again using standard plasmid isolation procedures. Prepare a 1:100 dilution.

Table 2
PCR reactions

Template	Reaction	Forward oligo	Reverse oligo	Template	Length of PCR product (bp)
ath-MIR319a	(a)	A	IV	pRS300	272
	(b)	III	II	pRS300	171
	(c)	I	B	pRS300	298
	(d)	A	B	(a) + (b) + (c)	701
osa-MIR528	(a)	A	II	pNW55	256
	(b)	I	IV	pNW55	87
	(c)	III	B	pNW55	259
	(d)	A	B	(a) + (b) + (c)	555

2. Setting up PCR reactions (a) to (c).

All PCR reactions should preferentially be carried out with a proof-reading polymerase (such as Pfu) to avoid PCR errors. Table 2 shows the oligonucleotide combinations for each PCR reaction together with the expected size of the product (see also Fig. 3).

Reactions (a) to (c):

2.0 µl	10× PCR buffer (with ~25 mM Mg^{++})
2.0 µl	dNTPs (2 mM)
1.0 µl	each oligonucleotide (10 µM; see PCR scheme)
1.0 µl	template DNA (1:100 dilution of template plasmid)
0.2 µl	polymerase
12.8 µl	water
20 µl	total

Protocol:

$$\left.\begin{array}{ll} 95^{\circ}C & 2\,min \\ 95^{\circ}C & 30\,s \\ 52^{\circ}C & 30\,s \\ 72^{\circ}C & 40\,s \\ 72^{\circ}C & 7\,min \end{array}\right\} 35\,cycles$$

3. Isolate PCR fragments from a 2% agarose gel and purify with standard gel extraction procedures. PCR fragments from reactions (a), (b), and (c) can be pooled already at this step. Elute in 20 µl of water.
4. Reaction (d): fusion of fragments (a), (b), and (c).

2.0 µl	10× PCR buffer (with Mg^{++})
2.0 µl	dNTPs (2 mM)
1.0 µl	oligonucleotides A and B (10 µM)
0.5 µl	each purified gel fragment (a), (b), and (c) or 1.5 µl of combined gel eluate
0.2 µl	polymerase
12.3 µl	water

Protocol:

$$\left.\begin{array}{ll} 95^{\circ}\text{C} & 2\,\text{min} \\ 95^{\circ}\text{C} & 30\,\text{s} \\ 52^{\circ}\text{C} & 30\,\text{s} \\ 72^{\circ}\text{C} & 90\,\text{s} \\ 72^{\circ}\text{C} & 7\,\text{min} \end{array}\right\} 35\,\text{cycles}$$

5. Isolate PCR fragment from a 1% agarose gel.

3.3. Cloning

To sequence-verify the fusion-PCR product (d), it can be blunt-end ligated into a standard cloning vector. It is important to keep in mind that this PCR fragment contains the T3 and T7 primer sites and the Multiple Cloning Sites of the template plasmid (see Fig. 2). Using T3 and/or T7 primers for sequencing may cause failed sequencing reactions, if the vector of choice also contains T3 and/or T7 sites.

3.3.1. Blunt-End Cloning Using Kits or Linearized Plasmids

(*See* Note 5 for the use of gateway-compatible plasmids.)

PCR reactions with proof-reading polymerases generate blunt-ended products. Some companies offer kits to directly clone blunt-ended DNA fragments (e.g., TOPO kits from Invitrogen), and it is recommended to follow the manufacturer's recommendations. Another simple and cheap protocol to clone blunt-ended PCR products is based on plasmids that are linearized with a restriction enzyme that produces blunt ends (e.g., SmaI). Since PCR products are not 5′ phosphorylated, the plasmid needs to retain its terminal phospho-groups after restriction and is directly used for ligations without prior dephosphorylation or purification. Re-ligation of the empty plasmid is prevented by addition of SmaI to the ligation mix.

Ligation Reaction:

1.0 µl	10× reaction buffer for SmaI
0.5 µl	ATP (10 mM)
1.0 µl	plasmid cut with SmaI, not dephosphorylated, and not purified
1.0 µl	T4 DNA ligase (10 U/µl)
0.3 µl	SmaI
6.2 µl	purified PCR fragment

The ligation mix is incubated at 16°C overnight, followed by ~2 h at 30°C (optimal temperature for SmaI restriction) prior to transformation into standard competent *E. coli* strains. If possible, blue white selection for the presence of an insert is recommended. Single colonies are cultured, and the recovered plasmid DNA should be test digested (e.g., with EcoRI and BamHI to yield a 408 bp band with the *ath-MIR319a* template, 268 bp with the *osa-MIR528* template) prior to sequence verification with standard oligonucleotides (depending on the plasmid) or oligonucleotides A or B. Sequencing at this step is strongly recommended to ensure that the new plasmid is indeed transformed. It may also be useful to know that the miRNAs in the template plasmids harbor uniquely occurring restriction sites – SacI in pRS300 (*ath-MIR319a*) and SphI in pNW55 (*osa-MIR528*) – which should (in most cases) be eliminated after successful PCR mutagenesis.

3.3.2. Sub-cloning into Binary Plasmids

aMIRNA precursors that are generated by site-directed mutagenesis do not contain a promoter or terminator; both need to be added by subsequent sub-cloning steps. For functionality tests *in planta* and initial characterizations, strong ubiquitous promoters such as cauliflower mosaic virus (35S) have been proven very helpful. More detailed analyses can be carried out with tissue-specific promoters, since amiRNAs function largely cell-autonomously (4). Because amiRNA-mediated gene silencing is quantitative (stronger promoters induce stronger effects), we do not recommend weak promoters when strongly expressed genes should be silenced efficiently. They might, however, become useful when partial silencing is intended. Inducible and transient *aMIRNA* expression was successful with ethanol and estrogen inducible systems (4, 13). Promoters are often already contained in binary vectors, or are to be inserted with standard cloning techniques. We did not observe remarkable differences in phenotypic effects with different binary plasmids in *A. thaliana*, and therefore recommend using a plasmid system that is well-established in the respective plant system.

All restriction sites of the pBluescript Multiple Cloning Sites flanking the *aMIRNA* precursor in the fusion PCR product (d) can be used to excise the amiRNA precursor (the *aMIRNA* transcript) from the sequencing plasmid. We frequently use EcoRI and BamHI for the *ath-MIR319a* backbone, but other enzymes can be used as well. It is, however, necessary to preserve the direction of the *aMIRNA* precursor, since anti-sense transcripts are not expected to form the same secondary structure. Gateway-assisted cloning is also possible, since the presence of AttB sites adjacent to the amiRNA precursor does not seem to affect its processing (*see* Note 5).

3.4. Plant Transformation and Analysis of Transgenic Plants

3.4.1. Transformation of Agrobacterium and Plants

Most protocols for the generation of transgenic plants rely on an *Agrobacterium* strain delivering the above-described binary plasmid. Transformation of competent strains (e.g., GV3101 for *A. thaliana*, LBA4404 or EHA105 for *O. sativa*) is carried out with standard transformation protocols.

Similarly, transfection of plants with the transgenic *Agrobacterium* strains should be carried out with established protocols, and primary transformants require selection with appropriate selection markers. The observation of phenotypic variation in primary transformants is expected and this might, in some cases, resemble an allelic series of the respective mutant. Gene silencing with transgenes is, in many cases, not complete such that plants resembling null mutants of the respective target gene might not be recovered. *See* Note 6 in case you do not observe phenotypic changes in primary transformants.

3.4.2. Reduced Abundance of Target Transcripts

To confirm that phenotypic changes are indeed due to reduced abundance of the intended target gene product(s), their levels should be analyzed in pools of primary transformants with similar phenotypes, or in individual plants, and compared to an untransformed or empty-plasmid-transformed control. If available, estimating target protein levels with specific antibodies should be the method of choice. Since plant amiRNAs, like many endogenous miRNAs, typically also affect the accumulation of target mRNA, RT-PCR is often indicative of successful gene silencing. RNA is preferentially isolated from tissues with strong phenotypic effects, either with commercial kits or with TRIzol® reagent (Invitrogen). Commercial reverse transcription kits can be used for cDNA synthesis. RT-PCR products preferentially span the amiRNA-guided cleavage site. *See* Note 7 when you observe phenotypic abnormalities, but no change in target mRNA levels.

To estimate the specificity of gene silencing, it is recommended to also test for the accumulation of closely related transcripts, which contain regions of partial sequence complementarity to the amiRNA (five or fewer mismatches, determined with the WMD-Target search tool; *see* Note 2). Reduced levels can be the

result of direct amiRNA targeting, but also of feedback regulation when the two genes participate in the same genetic pathway. To discriminate between the two possibilities, it is necessary to specifically test for the accumulation of cleaved targets by 5′ RACE-PCR, since (a)miRNAs trigger the cleavage of target transcripts – always opposite of positions 10 and 11 of the amiRNA.

3.4.3. Cleavage Site Mapping by RACE-PCR

RACE-PCR typically uses mRNA as a starting material, which can be isolated from total RNA with commercial kits. Standard protocols for 5′RACE typically start by de-capping full-length mRNAs, whereas this step is omitted for cleavage product detection, and mRNA is directly ligated to the RNA linker oligonucleotide. Reverse transcription is typically carried out with an oligo-dT primer. PCR amplification of cleavage products uses forward oligonucleotides that bind the introduced linker sequence and gene-specific reverse oligonucleotides complementary to a region ~200 to 300 nucleotides downstream of the putative amiRNA binding site in the gene of interest. The abundance of cleavage product can be very low, and sometimes a second, nested PCR may be necessary. Amplified products should be ligated into standard cloning vectors and sequenced to determine where the linker had been ligated and hence where the target transcript had been cleaved. Cleavage is predicted to occur at the amiRNA binding site between the two base pairs opposing positions 10 and 11 of the amiRNA.

3.4.4. Genetic Complementation

Since target sites of amiRNAs are small and distinct, it is possible to engineer silent mutations in this region of the target gene, such that the transcript is no longer susceptible to amiRNA-mediated regulation. Introducing this transgene under its endogenous or a stronger promoter should suppress the amiRNA-induced phenotypes. This approach has successfully been used to bypass regulation by endogenous miRNAs (14), and it can provide powerful evidence that the observed phenotypes are caused only by down-regulation of the intended target and not by other genes. Silent mutations are typically introduced in as many positions as possible within the amiRNA binding site by PCR-based site-directed mutagenesis, in a similar way as *aMIRNAs* are produced (3.2.3).

4. Notes

1. When the plant species of interest is not yet included in WMD, but significant sequence information is publicly available, please contact wmd@tuebingen.mpg.de to have the species added to the tool. Obviously, the specificity calculations can only take the available set of sequences into account, so there is always the possibility that amiRNAs affect additional genes

that are not annotated or only partially annotated in the current sequence release of the respective species.

2. The WMD-Target Search application rapidly identifies target genes for miRNAs and other small RNAs in a given transcript collection/genome annotation. It uses a sequence matching algorithm, based on enhanced suffix arrays (http://vmatch.de (15), and enables the identification of all genes in the collection with a defined number of mismatches to the search sequence. In addition, the WMD-Target Search applies the empirically determined parameters of miRNA target selection (5) to filter for putative target genes. The output includes an alignment of the small RNA (reverse complement) to putative targets as illustrated in Fig. 2. With default settings, WMD-Target Search output lists only one splice form per gene. All splice forms are displayed when the splice form filter is disabled ("Show only one isoform" in Advanced Search Options). This option should be used to examine whether all splice forms are targeted.
3. Failure of WMD to produce suitable aMIRNAs can have several reasons:
   (a) The input sequence might have been too short to contain suitable target sites.
   (b) The WMD-Designer is not able to compute a specific amiRNA against a target gene of interest if its nucleotide sequence is very similar to one or several other genes at all potential target sites. The similar gene(s) can be easily identified using WMD-BLAST, and one or several targets will have to be silenced together by adding them as additional targets or as "accepted off-targets." It might still be possible to conduct conclusive experiments by choosing off-target(s) that do not interfere with the experimental design, or by evaluating the effects of several amiRNA constructs *in planta* with different off-targets.
   (c) Some transcript collections contain redundant ESTs, and multiple ESTs might span the locus of interest. Here, all genes/ESTs that are highly related to the gene of interest should be identified with WMD-BLAST and included in the WMD-Designer input as "accepted off-targets."
   (d) WMD can only compute a multi-gene amiRNA that targets several genes if they share regions of high nucleotide sequence similarity. Simultaneous silencing of multiple related genes might fail if the genes are not similar enough, or one or more is/are not different enough from other genes (*see* Note 2). Try to reduce the minimal number of included target genes or silence them individually.
4. *A. thaliana MIRNA* precursors have successfully been used for amiRNA production in other plants (tomato, tobacco, and

Physcomitrella, (8, 26)), but precursor functionality across species has not yet been systematically investigated. Therefore, adapting the cloning protocol to *MIRNA* precursors endogenous to the respective plant species of interest might be the optimal approach.

MIRNA precursors have been identified and characterized in several different plants (see miRBase, http://microrna.sanger.ac.uk/, (16), often by homology to known miRNAs. As backbones for amiRNA production, we recommend either using a precursor that has been shown to be expressed and processed, i.e., by northern blot, or – when this information is not available – using a highly conserved precursor, e.g., *MIR164* or *MIR319*. Oligonucleotides I through IV will need to be adapted to reconstruct the proper hairpin structure, such that bulges remain at their respective positions.

5. Cloning with the Gateway® technology seems to not interfere with amiRNA production. In the following, we list possible cloning strategies for using the Gateway® system:
   (a) The *MIRNA* precursor fragment can be excised from the sequencing plasmid (3.3.1) with restriction enzymes and ligated into a Gateway® entry plasmid.
   (b) The fusion product (d) in 3.2.3. can be ligated into a Gateway® entry plasmid as it is.
   (c) Alternatively, the fusion PCR of the fragments (a), (b), and (c) to (d) in 3.2.3 can be carried out with oligonucleotides that already contain AttB sites at their 5′ ends. These primers do not necessarily need to bind to the primer binding sites A and B. Primers that bind to sequences in the Multiple Cloning Sites have successfully been used to obtain a short insert and to eliminate undesired restriction sites. The resulting PCR product with AttB sites at both ends can then be ligated into a vector of choice (e.g., pGEM T easy), which then serves as the entry plasmid for a subsequent recombination reaction.
6. Missing phenotypic changes in transgenic plants that (over) express amiRNAs can have several reasons:
   (a) The phenotypes might not be detectable in the growth conditions tested.
   (b) The loss-of-function phenotype of the gene of interest might be masked by redundancy. A search for related genes with similar expression patterns (see, e.g., AtGenExpress platform for *A. thaliana*; http://jsp.weigelworld.org/expviz/expviz.jsp, (17) might help to identify potentially redundant genes to be used as additional targets.
   (c) The target gene might not be sufficiently downregulated to detect phenotypic changes. It is critical to achieve

high amiRNA expression in the tissue(s) of target gene expressions, but even promoters such as the one from the CaMV35 gene are not entirely ubiquitous.

A fraction of *A. thaliana* amiRNAs generated to date (20–25%) does not silence the intended target gene(s), but the reasons are yet to be determined. It is possible that their target sites are not accessible due to extensive local secondary structures, similar to what has been observed for siRNAs in animal systems (19). Ongoing studies address this question, and we are planning to account for this effect by integrating novel tools such as "RNAup" (20) into WMD. When calculating RNA-RNA binding, "RNAup" also considers the folding of the respective RNA molecules to themselves, and can therefore be used to predict the accessibility of the target sites in the target mRNA. At present, we recommend constructing at least two amiRNAs per target gene/group of target genes, with target sites located in different regions of the target transcript(s).

(d) If even very potent amiRNAs cause only small effects on the transcript levels, the target genes might be under negative feedback regulation. Those genes may be silenced effectively on a transcriptional level (e.g., by promoter methylation, (18), however not by post-transcriptional gene silencing.

7. Typical (a)miRNA-mediated gene silencing includes the cleavage of target transcripts followed by degradation of the cleavage products, leading to a reduction in transcript abundance, which can be measured by RT-PCR. However, for some endogenous miRNAs, e.g., *ath*-miR172, translational inhibition is at least as important as miRNA-guided cleavage (21, 22, 27). Thus, phenotypic changes can be present even though mRNA levels might not have appreciably changed. When available, translational effects can be monitored on the protein level, by western blotting with target-specific antibodies. In many published cases, transcripts that were regulated on the translational level were still cleaved by the miRNAs (5, 21–23), and cleavage products were detected by RACE-PCR (*see* Subheading 3.4.3).

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