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RESEARCH ARTICLES

Highly Specific Gene Silencing by Artificial MicroRNAs in *Arabidopsis* [W] [OA]

Rebecca Schwab, Stephan Ossowski, Markus Riester, Norman Warthmann, and Detlef Weigel[1]

Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

Plant microRNAs (miRNAs) affect only a small number of targets with high sequence complementarity, while animal miRNAs usually have hundreds of targets with limited complementarity. We used artificial miRNAs (amiRNAs) to determine whether the narrow action spectrum of natural plant miRNAs reflects only intrinsic properties of the plant miRNA machinery or whether it is also due to past selection against natural miRNAs with broader specificity. amiRNAs were designed to target individual genes or groups of endogenous genes. Like natural miRNAs, they had varying numbers of target mismatches. Previously determined parameters of target selection for natural miRNAs could accurately predict direct targets of amiRNAs. The specificity of amiRNAs, as deduced from genome-wide expression profiling, was as high as that of natural plant miRNAs, supporting the notion that extensive base pairing with targets is required for plant miRNA function. amiRNAs make an effective tool for specific gene silencing in plants, especially when several related, but not identical, target genes need to be downregulated. We demonstrate that amiRNAs are also active when expressed under tissue-specific or inducible promoters, with limited nonautonomous effects. The design principles for amiRNAs have been generalized and integrated into a Web-based tool (http://wmd.weigelworld.org).

INTRODUCTION

Two classes of small RNAs, small interfering RNAs (siRNAs) and microRNAs (miRNAs), affect gene expression in animals and plants. They interfere with normal gene function on several levels, including promoter activity, mRNA stability, and translational efficiency. Small RNAs are the specificity components of a protein machinery known as RNA-induced silencing complex (RISC), which uses the small RNAs to recognize complementary motifs in target nucleic acids (Bartel, 2004; Filipowicz, 2005).

Conventional siRNAs can be formed from endogenous as well as exogenous (e.g., transgene-derived) double-stranded RNAs through cleavage by the Dicer RNase, releasing several double-stranded intermediates of ~21 nucleotides in length, with a two-nucleotide 3′ overhang (Elbashir et al., 2001). From these intermediates, the strands with lower thermodynamic stability at their 5′ ends will be preferentially active in RISC (Khvorova et al., 2003; Schwarz et al., 2003). siRNAs then serve as specificity components of RISC, guiding it to cleave target mRNAs opposite to nucleotides 10 to 11 of the siRNA, followed often by degradation of the cleaved RNA. Before its details were known, this process was called posttranscriptional gene silencing, but today it is better known as RNA interference (RNAi).

Perfectly complementary siRNAs are widely used in animals as a tool to downregulate RNA expression of genes of interest. They can either be synthesized in vitro or by transgenic expression of a double-stranded precursor that folds back on itself as a hairpin (Hannon and Rossi, 2004). Sequence parameters that lead to particularly effective gene silencing by siRNAs have been identified through systematic analyses of siRNA effects (Reynolds et al., 2004). However, siRNAs can also affect RNAs that are not perfectly complementary, generally considered off-targets (Jackson et al., 2003; Doench and Sharp, 2004). In addition, long double-stranded precursors generate a multitude of siRNAs with varying 5′ and 3′ ends, which make the prediction of off-targets particularly difficult.

siRNAs share many properties with miRNAs, which are typically ~19 to 24 nucleotides in length and which are produced from fold-back precursors that are transcribed from imperfect inverted repeats in the genome. Sequential processing of the miRNA precursor by the double-strand specific RNases Dicer and Drosha in animals (Lee et al., 2003) and DICER-LIKE1 in plants (Kurihara and Watanabe, 2004) produces a single stable small RNA that is incorporated into the silencing complex. Animal miRNAs typically cause translational arrest of target mRNAs that have only partial complementarity to the miRNA. Complementarity to the seed region (positions 2 to 8) of the miRNA is often sufficient for effective regulation, allowing an animal miRNA to control large numbers of targets (Brennecke et al., 2005; Farh et al., 2005; Lewis et al., 2005; Lim et al., 2005). By contrast, plant miRNAs have few (zero to five) mismatches to their targets and trigger local transcript cleavage and subsequent degradation

[1] To whom correspondence should be addressed. E-mail weigel@weigelworld.org; fax 49-7071-601-1412.
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(Llave et al., 2002). The highest number of targets empirically confirmed for a specific plant miRNA is only 10 (Schwab et al., 2005), contrasting with the large number of targets of the typical animal miRNA. These observations have raised the question whether the differences are only due to intrinsic properties of the miRNA machinery or at least partially caused by selection against plant miRNAs with large numbers of targets.

To address this problem, we have generated a series of artificial miRNAs (amiRNAs) designed to target different endogenous mRNAs and compared their effects to those of natural miRNAs. We have found that amiRNAs efficiently silence both single and multiple target genes, with little evidence for the formation of secondary siRNAs, consistent with the findings for most natural plant miRNAs (Lu et al., 2005). Known determinants of target selection for natural miRNAs (Schwab et al., 2005) accurately predicted direct targets of amiRNAs, indicating that the plant silencing machinery acts with higher specificity than its animal counterpart. In support of their potential as a gene-silencing tool, we have shown that amiRNAs have only limited nonautonomous effects and are also effective when expressed under the control of an inducible promoter. In addition, we have automated the design of amiRNAs based on the input of individual or several related target sequences.

RESULTS

Design of amiRNAs

The analysis of plants that overexpress natural miRNAs, together with a reexamination of known targets, led us to propose specific sequence parameters important for target selection by plant miRNAs (Schwab et al., 2005). We found that pairing to the 5′ portion of the miRNA (positions 2 to 12) was most important, since this region often had no mismatch and rarely more than one. Similarly, mismatches at the presumptive cleavage site (positions 10 and 11) were usually not present in direct targets. In vitro experiments with mutant targets largely support these findings (Mallory et al., 2004). Clusters of more than two mismatches to the 3′ part of the miRNA were rare. In addition, perfect pairing in the 3′ portion can compensate for the presence of up to two mismatches in the 5′ portion, leading to a low overall free energy of targets paired with their corresponding miRNAs (at least 70% compared with a perfect match and a maximum of −30 kcal/mole).

The same parameters were incorporated into the design of amiRNAs. We began by selecting different target genes, most of which had known loss-of-function phenotypes that could be easily monitored. In addition, the amiRNAs were designed with uridine at position 1 and, if possible, adenine at position 10, both of which are overrepresented among natural plant miRNAs and highly efficient siRNAs (Mallory et al., 2004; Reynolds et al., 2004). We also preferred amiRNAs to display 5′ instability relative to their miRNA*, so that the correct sequence would be incorporated into RISC. amiRNA sequences fulfilling the functionality criteria were initially selected by hand from reverse complements of target genes. To reduce the likelihood that an amiRNA would act as primer for RNA-dependent RNA polymerases, and thereby trigger secondary RNAi, between one and three mismatches to the target genes were introduced in the 3′ part of the amiRNAs. See Table 1 for a list of amiRNAs and intended targets, with alignments shown in Supplemental Figure 1 online.

It was shown previously that both animal and plant miRNA precursors can be modified to express a small RNA with a sequence that is unrelated to the miRNA normally produced by the precursor (Zeng et al., 2002; Parizotto et al., 2004). We used precursors for miRNA172a and miR319a as backbones for amiRNA expression under control of the constitutive 35S promoter from *Cauliflower mosaic virus*. Using overlapping PCR, we exchanged the natural miRNA sequences with those of amiRNAs. We also modified the miRNA* region, which base pairs to the miRNA in the precursor, such that both structural and energetic features of the miRNA precursor were retained (Figure 1).

Table 1. Predicted amiRNA Sequences and Targets

amiRNA	Predicted Mature Sequence (5′–3′)	Predicted Target(s)	Known Target Functions	Reference
amiR-lfy-1	**U**AACAGUGA**A**CGUACUGUCGC	*LFY*	Master regulator of floral identity	1
amiR-lfy-2	**U**UACGAUAA**A**CGGUUGCUCGC			
amiR-white-1	**U**UAGUGAGA**A**UGUUGCGCCGG	*GUN4*	Cofactor in chlorophyll biosynthesis	2
amiR-white-2	**U**UUAACCAG**A**UUUUGCGUCGC			
amiR-ft-1	**U**AUUCUCGG**A**GGUGAGUGUUG	*FT*	Promotion of flowering	3
amiR-ft-2	**U**UGGUUAUA**A**AGGAAGAGGCC			
amiR-trichome	**U**CCCAUUCG**A**UACUGCUCGCC	*TRY*, *CPC*, *ETC2*	Trichome patterning	4, 5
amiR-mads-1	**U**UUUGGAGA**A**AGUGACUUGUC	*SOC1*, *MAF1-3*, *ANR1*, and three others	Regulation of flowering, nutrient uptake	6
amiR-mads-2	**U**UGUUCUCU**A**UCCUCUUCAGC	*SEP1-4*, *SHP1-2*, *AP1*, *CAL*, and 10 others	Patterning of floral organs	
amiR-yabby-1	**U**ACUGAAAG**C**UUCUCUGUGGG	*INO*, *YAB3*, and three others	Regulation of adaxial polarity	7
amiR-yabby-2	**U**GUAUGCUG**A**UGGGACUCUCG	*CRC*		

Positions 1 and 10 of small RNAs are marked in bold. A complete list of targets together with alignments to the respective amiRNAs is shown in Supplemental Figure 1 online. References: 1, Weigel et al. (1992); 2, Larkin et al. (2003); 3, Kardailsky et al. (1999); 4, Schellmann et al. (2002); 5, Kirik et al. (2004); 6, Becker and Theissen (2003); 7, Engstrom et al. (2004).

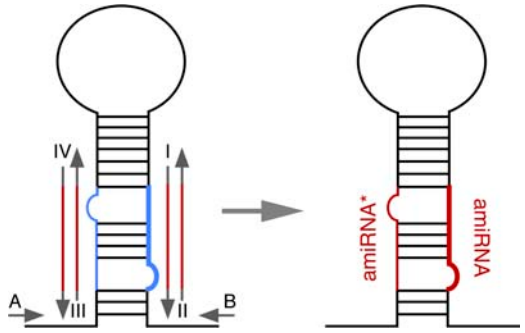

Figure 1. Engineering of amiRNAs.

Site-directed mutagenesis on precursors of endogenous miRNAs was performed using overlapping PCR. Oligonucleotide primers I to IV were used to replace miRNA and miRNA* regions (blue) with artificial sequences (red). Primers A and B were based on template plasmid sequence. Regeneration of functional miRNA precursors was achieved by combining PCR products A-IV, II-III, and I-B in a single reaction with primers A and B.

Phenotypic as well as molecular analysis of amiRNA plants was performed in primary transformants (T1 generation). The effects of amiRNA overexpression could, however, be stably inherited, as seen with the amiRNA directed against the flowering time gene *FT*.

Molecular Identity of amiRNAs

To confirm that amiRNAs accumulated in transgenic plants, we probed small RNA gel blots of inflorescence tissue from pooled T1 plants. All amiRNAs tested were efficiently expressed from both *MIR319a* and *MIR172a* backbones (Figure 2A). Differences in the mobility of the amiRNAs on polyacrylamide gels might reflect either some heterogeneity in size or might be due to sequence differences, as shown with mutant forms of miR159a (J. Palatnik and D. Weigel, unpublished data). We suggest that the majority of amiRNAs were 21 nucleotides in length, as intended. In some cases, small RNAs of different length accumulated, indicating that the position of DICER-LIKE1 cleavage was not uniform in all cases. We saw only very weak or no signals with miRNA*-specific probes, with the exception of the miRNA* for amiR-trichome. amiR-trichome was the only amiRNA without 5′ instability relative to its miRNA*, indicating that selection of the amiRNA from the double-stranded DICER-LIKE1 product was similar to siRNA strand selection.

Because ARGONAUTE (AGO) proteins cleave targets invariably opposite of positions 10 and 11 of the small RNA (Kasschau et al., 2003), the 5′ ends of small RNAs can be inferred by mapping the cleavage products of their targets (Llave et al., 2002). For targets of amiR-mads-1 (*MIR172a* backbone), amiR-mads-2 (*MIR319a* backbone), and amiR-trichome (*MIR319a* backbone), cleavage products had the expected 5′ ends (Figure 2B). Since uniform cleavage products were obtained even in cases where there was some size heterogeneity in the amiRNA, we conclude that these amiRNAs differed only at their 3′ end.

amiR-lfy-1 (*MIR172a* backbone) caused cleavage of the target two nucleotides downstream of the expected position, implying that the initial DICER-LIKE1 product was shifted by two nucleotides. Examination of the sequence surrounding the intended amiRNA in the precursor revealed that this alternative amiRNA would still be specific for its target (Figure 2B).

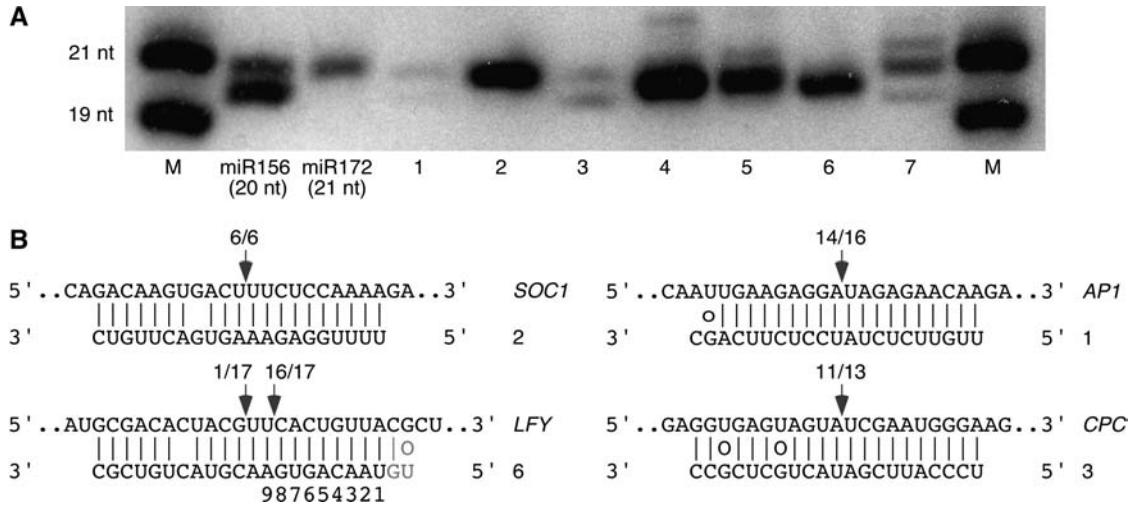

Figure 2. Expression of amiRNAs and Cleavage of Predicted Targets.

(A) RNA gel blot analysis of amiRNA overexpressers using a mixture of probes for all amiRNAs. The outermost lanes contain two standards. miR156a and miR172a overexpressers were included as controls. Lane 1, amiR-mads-2 (*MIR319a* backbone); lane 2, amiR-mads-1 (*MIR172a* backbone); lane 3, amiR-trichome (*MIR319a* backbone); lane 4, amiR-lfy-2 (*MIR319a* backbone); lane 5, amiR-lfy-2 (*MIR172a* backbone); lane 6, amiR-lfy-1 (*MIR172a* backbone); lane 7, amiR-yabby-2 (*MIR319a* backbone). M, size marker; nt, nucleotides.

(B) Mapping of target cleavage products by rapid amplification of cDNA ends using PCR. Fraction of sequenced clones with particular 5′ end indicated on top. In the case of *LEAFY* (*LFY*), only one clone had a 5′ end at the expected position, opposite nucleotides 10 to 11 of the intended amiRNA. The 5′ end of most clones was offset by two nucleotides, suggesting that most of the amiRNAs were offset as well. The sequence predicted from the aberrant processing is indicated in gray.

Taken together, amiRNAs were effectively produced from their precursors, and a high fraction was processed as the exact 21-mer that was exchanged in the backbone precursor.

Effects on Predicted Target Genes

Single Targets

Overexpression of three amiRNAs designed to target single genes resulted in robust and strong phenotypes that resembled those of plants with mutations in the respective target gene (Figure 3). In most cases, >90% of T1 plants displayed defects, but the fraction of plants that resembled null mutants varied depending on amiRNA transgenes and precursor backbones (see below). The majority of amiR-lfy-1 overexpressers had floral defects resembling *lfy* null mutants (Figure 3A), while others showed milder effects more typical of weak and intermediate *lfy* alleles (Weigel et al., 1992).

Most amiR-white overexpressers were arrested in their growth as white seedlings. Although overall similar to *gun4*-1 seedlings, the phenotype of most seedlings was more severe than that of *gun4*-1 mutants, which is likely a hypomorphic allele (Figure 3B) (Larkin et al., 2003). This conclusion was also supported by microarray data (see Supplemental Figure 2 online).

The most consistent effects were seen with amiRNAs targeted against the flowering time gene *FT*, whose loss of function results in late flowering under long days (Koornneef et al., 1991). All plants overexpressing either amiR-ft-1 or amiR-ft-2 ($n = 40$) flowered within a day of *ft* null mutants (Figure 3C).

We used RT-PCR and microarray analyses to examine the effects of amiRNAs on their targets. *FT* transcripts were decreased below detection level by RT-PCR, similar to *ft* T-DNA insertion mutants (see Supplemental Figure 3 online). *LFY* and *GUN4* transcripts were still detectable in amiR-lfy-1 inflorescences and amiR-white-1 seedlings, respectively, using Affymetrix arrays but were substantially reduced (4.5- and 5.7-fold). *GUN4* was no longer detectable in amiR-white-2 overexpressers using Affymetrix arrays (Figure 4A; see Supplemental Table 1 online).

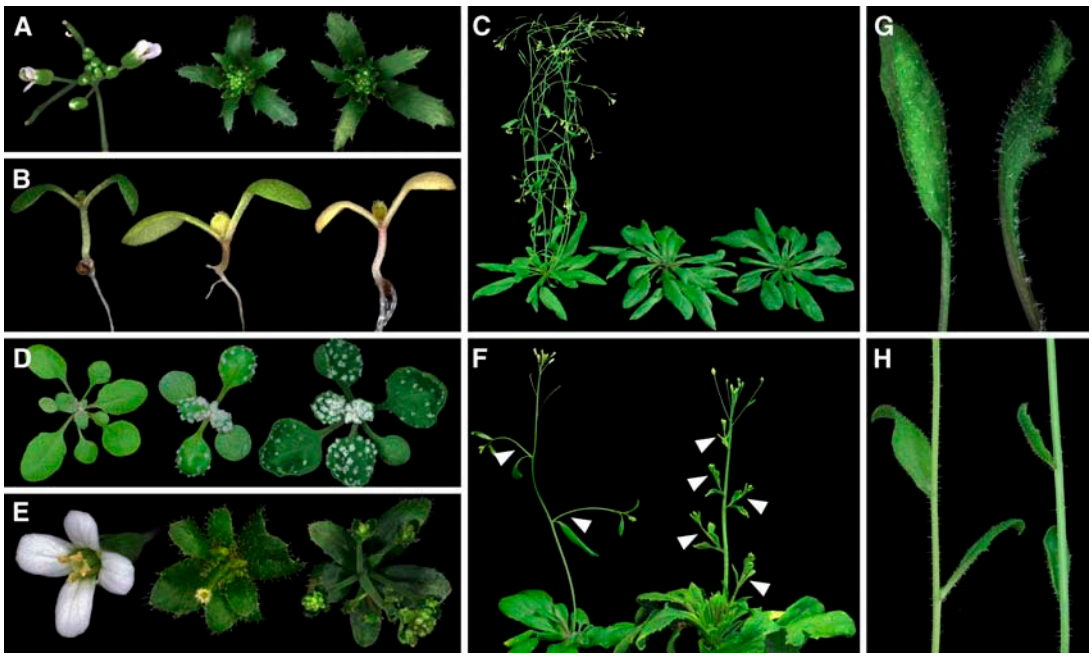

Figure 3. Phenotypes of amiRNA Overexpressers.

(A) Inflorescences. From left to right: the wild type, *lfy*-12, and amiR-lfy-1 (*MIR172a* backbone) overexpresser.
(B) Seedlings. From left to right: the wild type, *gun4*-1, and amiR-white-1 (*MIR172a* backbone) overexpresser. Bleaching of cotyledons is more pronounced in the amiR-white plants than in *gun4*-1, consistent with the more severe molecular profile of the amiR-white overexpressers.
(C) Adult plants sown on the same day. From left to right: the wild type, *ft*-10, and amiR-ft-2 (*MIR172a* backbone).
(D) Leaf rosettes. From left to right: the wild type, *try cpc* double mutants, and amiR-trichome (*MIR319a* backbone) overexpresser. Clustered trichomes are evident even at low magnification.
(E) Flowers. From left to right: the wild type, weak amiR-mads-2 (*MIR319a* backbone) overexpresser, and strong amiR-mads-2 (*MIR319a* backbone) overexpresser. In both amiR-mads overexpressers, outer whorls are transformed into leaf-like structures. In the strong line, secondary inflorescences replace the central gynoecium.
(F) Flowering plants. Left, the wild type; right, amiR-mads-1 (*MIR172a* backbone) overexpresser with increased number of cauline leaves (arrowheads).
(G) Rosette leaves of the wild type (left) and amiR-yabby-1 (*MIR172a* backbone) overexpressers. Abaxial side is at the left.
(H) Cauline leaves of the wild type (left) and amiR-yabby-2 (*MIR319a* backbone) overexpressers (right) with polarity defects.

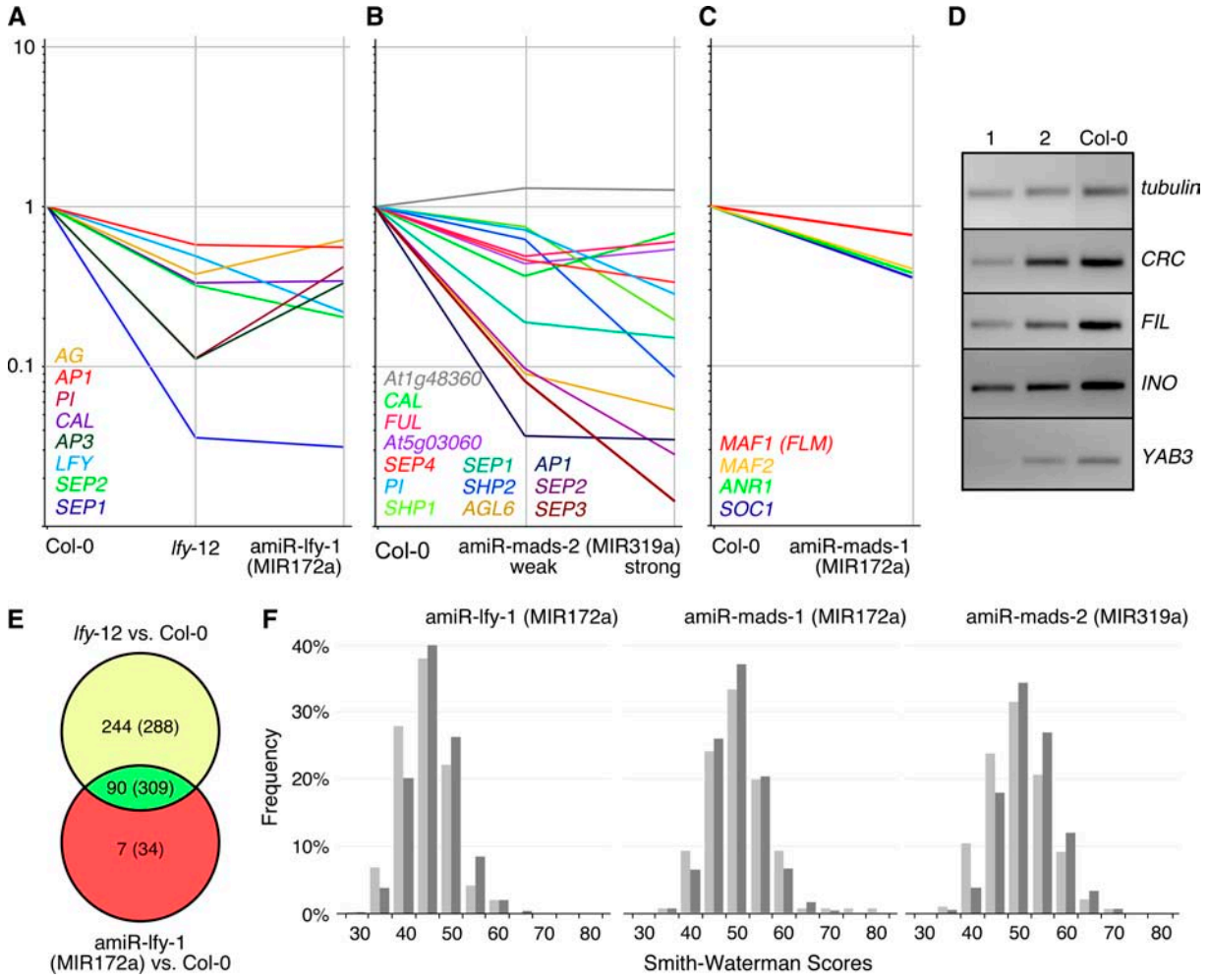

Figure 4. Expression Analyses of amiRNA Overexpressers.

(A) Microarray profiles of *LFY* and some of its direct downstream targets in inflorescences of the wild type (Columbia [Col-0]), *lfy*-12 mutants, and amiR-lfy-1 (*MIR172a* backbone) overexpressers.

(B) Microarray profiles of predicted amiR-mads-2 targets in inflorescences of the wild type (Col-0) and weak and strong amiR-mads-2 (*MIR319a* backbone) overexpressers.

(C) Microarray profiles of predicted amiR-mads-1 targets in the wild type (Col-0) and amiR-mads-1–overexpressing (*MIR172a* backbone) inflorescences.

(D) RT-PCR analysis of amiR-yabby overexpressers (inflorescence tissue). Reactions were stopped in the linear phase of amplification. Lane 1, amiR-yabby-1 (*MIR172a* backbone); lane 2, amiR-yabby-2 (*MIR319a* backbone).

(E) Overlap of significantly downregulated genes using the Linear Model for Microarray Data (LIMMA) package (Smyth et al., 2005) or logit-T (in parentheses; Lemon et al., 2003) in *lfy*-12 and amiR-lfy-1 plants indicates a very similar molecular phenotype, with amiR-lfy-1 plants being on average weaker than *lfy*-12 plants. Only genes called as present by Affymetrix algorithms in wild-type controls were considered.

(F) Distributions of Smith-Waterman scores (Smith and Waterman, 1981) are similar between genes that are significantly downregulated in response to amiRNA overexpression (light gray bars) and all genes present in the control (dark gray bars). Predicted targets have been removed.

Multiple Targets

Because many natural miRNAs have several targets, we designed three classes of amiRNAs with multiple potential targets from different transcription factor gene families. amiR-trichome targets three *MYB* genes, *CAPRICE* (*CPC*), *TRIPTYCHON* (*TRY*), and *ENHANCER OF TRIPTYCHON AND CAPRICE2* (*ETC2*), which are involved in trichome patterning (Schellmann et al., 2002; Kirik et al., 2004). amiR-mads-1 and amiR-mads-2 target 7 and 13 MADS box genes, respectively, that are mostly involved in the control of flowering time and floral patterning (Becker and Theissen, 2003), with a few additional non-MADS targets (see complete list in Supplemental Table 1 online). amiR-yabby-1 and amiR-yabby-2 target different members of the *YABBY* gene family, with two predicted targets for amiR-yabby-1 and one for amiR-yabby-2. *YABBY* genes specify the abaxial domain of

lateral organs (Engstrom et al., 2004). The target motif for amiR-trichome was located outside the region encoding the DNA binding domain, which is the typical case for natural miRNAs targeting transcription factor genes, while the target motifs for amiR-mads and amiR-yabby were in the regions encoding the DNA binding domains.

In most cases, the phenotypes of amiRNA overexpressers suggested in vivo targeting of multiple genes. Approximately 30% of amiR-trichome overexpressers phenocopied *try cpc* double mutants with highly clustered trichomes on leaf blades (Schellmann et al., 2002) (Figure 3D), while most of the remaining amiR-trichome overexpressers resembled *try* or *cpc* single mutants. A third potential target, *ETC2*, predicted to be a much less favorable target than *TRY* or *CPC*, seemed to be affected in very few plants, which had extra trichome clusters on petioles, as seen in *try cpc etc2* triple mutants (Kirik et al., 2004) (see Supplemental Table 2 online).

Potential targets of amiR-mads-1 include both floral repressors, such as the *MADS AFFECTING FLOWERING* (*MAF*) genes (Scortecci et al., 2001; Ratcliffe et al., 2003), and floral activators, such as *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000), as well as *ARABIDOPSIS NITRATE REGULATED1* (*ANR1*), which is involved in nutrient uptake (Zhang and Forde, 1998). Even though chronological flowering time was not greatly altered in overexpressing plants, cauline leaf number was increased from two or three to four or five in >90% of T1 plants grown in continuous light (Figure 3F). In addition, there were carpel defects that have not been described for any of the intended targets, although there are several MADS box genes, such as *AGAMOUS* (*AG*), *FRUITFULL* (*FUL*), *SHATTERPROOF1* (*SHP1*), and *SHP2*, that are involved in fruit patterning (Becker and Theissen, 2003). Microarray analysis of inflorescences of amiR-mads-1 overexpressers showed downregulation of the four intended targets that were present in the control, although the effects were small, between 1.4- and 2.3-fold (Figure 4C), and not statistically significant.

The targets predicted for amiR-mads-2 are mostly required for determining floral organ identity (Becker and Theissen, 2003). Flowers of overexpressers had severe changes in floral morphology with leaf-like organs in all four whorls, characteristic of *sepallata* (*sep*) multiple mutants (Pelaz et al., 2000) (Figure 3E). Defects were stronger in the two outer whorls compared with the two central whorls, which might be due to nonuniform activity of the cauliflower mosaic virus 35S promoter we used. Additional features, such as secondary flowers, indeterminacy of floral meristems, and incomplete separation of carpel valves, have been described as features of other predicted target gene mutants, such as *apetala1* (*ap1*) and *shp1/2* (Irish and Sussex, 1990; Liljegren et al., 2000). In the strongest lines, which also express more amiRNA than the weaker lines (see Supplemental Figure 4 online), carpels were replaced by a new inflorescence shoot. Expression analysis with Affymetrix microarrays showed that most predicted target genes were significantly downregulated in inflorescence tissue of both weak and strong overexpressers (Figure 4B; see Supplemental Table 1 online).

Plants overproducing amiR-yabby-1 and -2 had defects in leaf polarity, such as leaves with trichomes on both sides, indicating adaxialization (amiR-yabby-1; Figure 3G). Other phenotypes, such as polarity defects in cauline leaves (amiR-yabby-1 and -2; Figure 3H), while not described for *yabby* mutant combinations before, are likely also related to *YABBY* function in polarity establishment. RT-PCR analyses with RNA from amiR-yabby-1–overexpressing seedlings and inflorescences showed predicted targets to be downregulated. Surprisingly, expression levels of the target gene *CRABS CLAW* (*CRC*) remained unchanged in amiR-yabby-2 (Figure 4D). When we examined other *YABBYs* as potential targets of amiR-yabby-2, we found that *FILAMENTOUS FLOWER* (*FIL*) was downregulated, and mapping of cleavage sites confirmed that *FIL* was targeted by amiR-yabby-2. We had initially not considered *FIL* to be a target because there are two mismatches in the critical 5′ region of the amiRNA (positions 2 to 12). However, the mismatches are at positions 2 and 8, with only one additional mismatch at the 3′ end of the miRNA, which makes this interaction similar to the one observed for miR168 and its target *AGO1* (Rhoades et al., 2002; Vaucheret et al., 2004). This pair was one of the few exceptions that we had found to our more restrictive general rules that hold for the vast majority of natural miRNA targets (Schwab et al., 2005).

In summary, multiple mRNAs can be successfully targeted by amiRNAs. While the degree of downregulation varied for different targets, there was no clear correlation of targeting efficiency either with the extent of complementarity between amiRNA and target or with expression levels of targets in the wild type. Thus, accessibility of target sites or feedback regulation of target transcripts might play additional roles. Since the related targets had the amiRNA complementary motif at approximately the same position in the coding region, such accessibility differences would not be due to the relative position within the transcript. In support of this, both amiRNAs directed against the beginning of the coding region (+125 for amiR-white-1) and ones directed against the 3′ untranslated region (amiR-ft-2) were effective.

Effect of Backbones

Comparing the effectiveness of the different stem loop backbones used, we conclude that both *MIR319a* and *MIR172a* precursors can be used for amiRNA expression. However, more robust results were obtained with *MIR319a* derivatives, all of which led to phenotypic changes, which was not the case for *MIR172a*. For several targets, we tested more than one amiRNA (see Supplemental Table 2 online). In general, they caused similar defects, but they were not always equally effective. For example, amiR-lfy-2 caused *lfy*-like phenotypes only when expressed from *MIR319a* backbone, while amiR-lfy-1 caused strong *lfy* defects also when expressed from the *MIR172a* backbone.

Specificity and Nontransitivity of amiRNAs

Using microarray analyses, we have previously determined parameters for target selection by natural *Arabidopsis thaliana* miRNAs and found that plant miRNAs are apparently much more specific than animal miRNAs (Brennecke et al., 2005; Lewis et al., 2005; Lim et al., 2005; Schwab et al., 2005). We used a similar approach to investigate the specificity of amiRNAs. Considering that target degradation due to transcript cleavage has been

suggested as the main mode of plant miRNA action and that complementary base pairing mediates small RNA target recognition, we first asked whether downregulated genes were enriched for genes with higher complementarity to amiRNAs compared with all genes. Once predicted targets had been removed, we found that significantly downregulated genes in amiR-lfy-1, amiR-mads-1, and amiR-mads-2 overexpressers were on average not more similar to the respective amiRNAs than all genes (Figure 4F; see Supplemental Table 3 online). The same result was obtained for amiR-white-1 and amiR-white-2. The significance in this case is less clear, as we could only obtain technical replicates because lethality of the seedlings made it very difficult to collect sufficient tissue for multiple independent replicates.

Natural miRNAs have targets with up to five mismatches, but the large majority of genes with five or less mismatches are not targets. We therefore focused more specifically on mRNAs with up to five mismatches to the different amiRNAs, assuming that most, if not all, direct target genes would be found among this group. If amiRNAs were indeed specific for our intended target genes, then the fraction of genes with up to five mismatches should not be overrepresented among downregulated genes, once predicted targets have been removed. After correcting for multiple testing (Benjamini and Yekutieli, 2001), none of the amiRNAs downregulated more genes than expected (Table 2).

The highest number of downregulated genes with up to five mismatches was found for amiR-mads-1, several of which were MADS box genes that were not among the intended targets, but for which extensive cross-regulation during flowering and floral patterning is well known (Becker and Theissen, 2003). *FLOWERING LOCUS C* (*FLC*), a MADS box gene that had not been predicted as a target of amiR-mads-1 because of a mismatch to position 11, was nevertheless strongly downregulated in amiR-mads-1 overexpressers. Since its only additional mismatch to amiR-mads-1 was located at position 1, *FLC* was a candidate off-target. We tested this possibility by rapid amplification of cDNA ends using PCR but did not detect any cleavage products, suggesting that the overrepresentation of MADS box genes among downregulated genes in amiR-mads-1–overexpressing plants was indeed due to secondary effects.

Base pairing to the so-called seed region between siRNA or miRNA positions 2 and 8 is often sufficient for target recognition in animals and can also result in reduced RNA levels, although these effects are mostly not due to cleavage guided by the small RNA (Jackson et al., 2003; Bagga et al., 2005; Lim et al., 2005). We monitored expression changes of all genes with seed matches to amiR-lfy-1, amiR-mads-1, and amiR-mads-2 and did not find that more genes than expected were significantly downregulated (see Supplemental Table 7 online).

As a fourth measure for amiRNA specificity, we compared genome-wide expression profiles of amiRNA-lfy-1 overexpressers and *lfy*-12 mutants. The majority of genes downregulated in amiR-lfy-1 plants was also affected in *lfy*-12 mutants and included several direct downstream targets of *LFY* (Figures 4A and 4E). Similar conclusions can be drawn for plants overexpressing amiR-white-1 and amiR-white-2, which target different regions of *GUN4* but show considerable overlap in the downregulated genes (see Supplemental Figure 2 online). With the caveat that the amiR-white data are based merely on technical replicates, they suggest high specificity for the primary target *GUN4* as well.

Finally, we examined whether amiRNAs are likely to have indirect effects through a process called transitivity. Upon binding to target transcripts, siRNAs cannot only trigger their cleavage and subsequent destruction, but also serve as primers for RNA-dependent RNA polymerases. These extend the local RNA double strands and generate templates for production of secondary siRNAs by Dicer action (Voinnet, 2005). These secondary siRNAs, which are unrelated in sequence to the initial trigger, can in turn affect other genes not targeted by the original small RNA. For two *Arabidopsis* miRNAs, miR173 and miR390, which both bind noncoding RNAs as primary targets, similar mechanisms have been described (Allen et al., 2005). To investigate the

Table 2. Summary of Downregulated Genes with Up to Five Mismatches

		amiR-lfy-1	amiR-mads-1	amiR-mads-2
Predicted targets, represented on array		1	6	18
Predicted targets, present in control		1	4	13
Predicted targets, present in control and downregulated in amiRNA overexpressers		1	1	6
All genes present in control without predicted targets		15,367	15,364	15,355
All genes downregulated without predicted targets		342	141	287
Genes with five mismatches, present in control		32	89	311
Observed downregulated	Predicted targets	1	1	5
	Others	1	3	5
Expected downregulated without predicted targets		0.7	0.8	5.6
χ^2		0.1	6.4	0.5

Significantly downregulated genes were determined using LIMMA (Smyth et al., 2005), with an expression change of at least 1.5-fold at an estimated 1% false discovery rate (FDR) (Benjamini and Yekutieli, 2001). A χ^2 test was used to determine statistical significance of the difference between expected and observed downregulated genes among the nontargets (genes with five or less mismatches, but not predicted targets). A minimal χ^2 value of 6.63 corresponds to significant differences at $\alpha = 0.01$. Use of the logit-T algorithm produced qualitatively similar results. A total of 15,368 genes were called present in Col-0 inflorescences. For amiR-lfy-1, we used the sequence of the 21-mer deduced from the mapping of the cleavage product, which was shifted by two nucleotides from the intended amiRNA.

possibility of transitivity, we determined potential 21-mer secondary siRNAs for all amiRNA target genes from both strands of a 250-bp region, surrounding the initial binding site of the amiRNA, as described by Allen et al. (2005). Potential targets of these siRNAs were identified using our miRNA:target algorithms. Examination of microarray data did not reveal any evidence for effects on such secondary targets, except for amiR-mads-1, where these effects are likely caused by cross-regulation among MADS family members, as discussed above (see Supplemental Table 4 online). In summary, we conclude that the specificity of amiRNAs is very similar to that of natural miRNAs.

Temporally and Spatially Restricted Expression of amiRNAs

The exquisite specificity of amiRNAs suggested that they constitute an excellent gene silencing tool because of the predictability of their effects, especially when targeting multiple genes. To further explore the usefulness of amiRNAs, we first asked whether it is possible to transiently knock down gene expression, which has recently been demonstrated for conventional hairpin RNAi constructs as well (Wielopolska et al., 2005). We used an inducible expression system based on the ethanol-responsive *Alc* regulon (Roslan et al., 2001). Both amiR-white-1 and amiR-trichome produced the expected phenotypes within 3 d of ethanol application. Importantly, the effects were transient (Figures 5A and 5B), confirming that the amiRNAs do not have secondary effects due to RNAi or DNA/chromatin modification, which can be transmitted autonomously after an initial triggering event.

Next, we asked whether the effects of amiRNAs can be spatially restricted by expressing them under the control of tissue-specific promoters, similar to what has been shown for RNAi using hairpin constructs (Byzova et al., 2004). amiR-lfy-1 was expressed from the *LFY* promoter (Blázquez et al., 1997) and found to result in plants resembling *lfy* mutants (Figure 5D). amiR-mads-2, which is predicted to target several MADS box homeotic genes, and amiR-white-1, targeting *GUN4*, were expressed throughout the early flower and later in the outer two whorls using the *AP1* promoter (Hempel et al., 1997). AP1:amiR-white-1

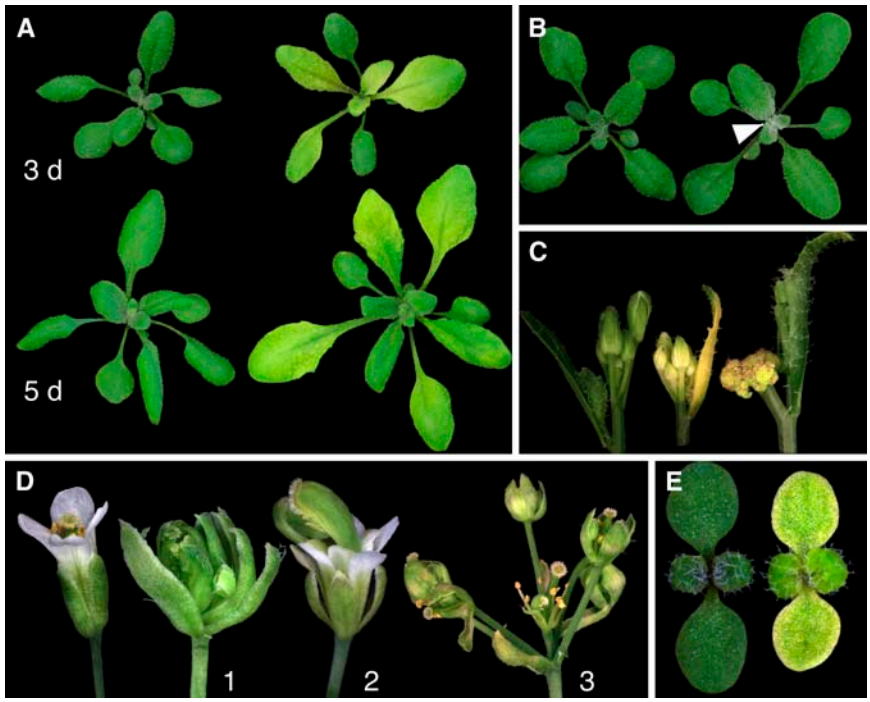

Figure 5. Inducible and Tissue-Specific Expression of amiRNAs.

Uninduced or wild-type controls are shown at the left.

(A) Ethanol-induced ubiquitous expression of amiR-white-1 3 and 5 d after induction. After 3 d, young leaves are all yellow; after 5 d, the youngest leaves are green again.

(B) Ethanol-induced ubiquitous expression of amiR-trichome (right) 3 d after induction. Clustered trichomes appear as white covering of youngest leaves (arrowhead).

(C) Inflorescences of plants expressing amiR-white-1 from the *AP1* promoter (middle) are pale yellow. Strong lines expressing amiR-mads-2 from the *AP1* promoter (right) resemble *ap1 cal* double mutants.

(D) Expression of amiR-lfy-1 from the *LFY* promoter (1) results in flowers resembling *lfy* mutants. amiR-mads-2 expressed from *AG* regulatory elements in the center of the flower (2) produces organ transformations in the central two whorls. Outer whorls remained unaffected. An opposite phenotype was seen after expression of amiR-mads-2 from the *AP1* promoter (3; weaker line), which didn't affect inner whorls but resulted in secondary flowers, resembling *ap1* mutants.

(E) Epidermal expression of amiR-white-1 from the *MERISTEM LAYER1* (*ML1*) promoter resulted in pale plants.

produced pale inflorescences (Figure 5C), and strong AP1:amiR-mads-2 lines resembled *ap1 cal* double mutants (Bowman et al., 1993), while weaker lines were more similar to *ap1* single mutants (Figures 5C and 5D). The meristem identity defects of strong lines were more severe than those of 35S:amiR-mads-2 plants (Figure 3E). amiR-mads-2 was also expressed in the inner two floral whorls using regulatory elements located in the second intron of *AG* (Busch et al., 1999). The effects of this construct were not quite as severe as seen in the strongest 35S:amiR-mads-2 plants (Figure 5D). The whorl-specific effects of amiR-mads-2 when placed under the control of *AP1* and *AG* regulatory sequences indicate that there are no long-range effects. However, effects of amiRNAs do not seem to be completely cell-autonomous, since pale yellow seedlings were obtained when amiR-white-1 was expressed from the epidermis-specific *ML1* promoter (Sessions et al., 1999) (Figure 5E). Since chloroplasts are restricted to the subepidermal mesophyll cells, we infer that this amiRNA can move across at least one cell boundary. However, the effects were much milder than with the 35S:amiR-white-1 transgene, which led to growth arrest of seedlings devoid of chlorophyll, similar to *gun4* null mutants (Figure 3B). In addition, leaf margins for ML1:amiR-white-1 plants were paler than the central part of the leaves, consistent with limited movement, since the margins contain fewer cell layers (Figure 5E).

Automated Design of amiRNAs with a Web-Based Tool

To facilitate the application of the amiRNA technology, we have developed a Web-based tool for their automated design (Web MicroRNA Designer). The program uses sequences of target genes as an input and searches for candidate 21-mers that resemble natural miRNAs in reverse complements of these genes, using the criteria described above. Target genes in the *Arabidopsis* genome are determined for individual candidates using a HyPa/vmatch search tool, which is based on a suffix array algorithm to identify sequence patterns (Gräf et al., 2001), and subsequent filtering according to rules for miRNA targeting based on our earlier work (Schwab et al., 2005). Mismatches in the 3′ part of candidate sequences are used to reduce the possibility of off-target effects. Oligonucleotide sequences for generation of amiRNA precursors through overlapping PCR are generated as a final output. Input sequences are not restricted to *Arabidopsis* sequences, so that amiRNAs that target orthologous genes from different species can be easily designed. This tool can be found at http://wmd.weigelword.org.

DISCUSSION

Specificity of amiRNAs

In contrast with miRNAs in animals, natural plant miRNAs have a very narrow action spectrum and target only mRNAs with few mismatches. We have overexpressed different amiRNAs in *Arabidopsis thaliana* and found that similar parameters of target selection apply as for natural miRNAs and that direct targets of amiRNAs can be accurately predicted using empirically derived determinants of target selection by natural miRNAs (Schwab et al., 2005). No substantial effects on genes with perfect matches to the miRNA seed regions (positions 2 to 8) could be detected, which contrasts with recent reports for animal miRNAs (Bagga et al., 2005; Lim et al., 2005). This suggests that the specificity of natural plant miRNAs is primarily due to an intrinsic property of the plant RNA silencing machinery, rather than selection against broad-spectrum miRNAs during evolution. It will be interesting to determine which components are responsible for the specificity differences between the RNA silencing machineries of plants and animals.

Mode of Action of amiRNAs

Transcript levels of most targets were substantially reduced in amiRNA-overexpressing plants, causing phenotypic changes similar to those seen in plants with mutations in the target gene(s). Some of these had strong abnormalities similar to null mutants, while others resembled weaker alleles. The strength of phenotypes for a given target was correlated with corresponding amiRNA levels, as seen in amiR-mads-2 (*MIR319a* backbone) (Figure 3E; see Supplemental Figure 4 online) and amiR-lfy-2 (*MIR172a* and *MIR319a* backbones) overexpressers (Figure 2A; see Supplemental Table 2 online). However, there was no simple correlation between amiRNA levels and phenotypes for different targets. The reason appears to be that different mRNAs differ in their susceptibility to small RNA–mediated regulation, since amiRNA expression levels, as detected by small RNA gel blotting (Figure 2A), are not proportional to the degree of target gene regulation.

To test the efficacy of amiRNA-directed gene silencing, we have chosen several target genes with previously reported mutant phenotypes. This allowed us to evaluate effects on expression of intended target genes, irrespective of the primary mode of small RNA action. Although transcript degradation initiated by miRNA-directed cleavage is the predominant mode of miRNA action in plants, there is at least one case, miR172 and its target *AP2*, in which translational inhibition plays an important role as well. Phenotypic abnormalities of miR172-overexpressing plants are consistent with reduced *AP2* function, even though mRNA levels are not affected (Aukerman and Sakai, 2003; Chen, 2004). In the case of the amiRNAs, we did not analyze protein levels, but as with natural miRNAs, translational inhibition appears to play only a secondary role. For example, we could not detect an effect of amiR-yabby-2 on transcript levels of the intended target *CRC*, but we also did not find any phenotypic evidence for reduced *CRC* function, which leads to a prominent carpel phenotype (Bowman and Smyth, 1999). Similarly, we did not find evidence for major effects of amiRNAs on mRNAs that matched the seed region, as has been demonstrated when animal miRNAs are overexpressed (Lim et al., 2005).

Small RNA–mediated effects on gene expression have been previously used to engineer directed gene silencing in plants by RNAi. While some of the original publications suggested near 100% efficiency of hairpin constructs (Chuang and Meyerowitz, 2000; Wesley et al., 2001), other publications indicate more variable effects (Kerschen et al., 2004), which is in line with anecdotal evidence from our own efforts. In any case, the availability of several complementary silencing technologies will be an advantage. In addition, we have not yet explored the simultaneous use of several amiRNAs against the same target(s), which

is commonplace with siRNAs and which may further increase the efficacy of amiRNAs.

Application of amiRNAs in Directed Gene Silencing

Our approach of expressing amiRNAs is conceptually similar to the second-generation short hairpin RNAs expressed from the precursor of miR30 in animals (Silva et al., 2005), with the important distinction that short hairpin RNAs are intended to target perfectly complementary mRNAs, while our amiRNAs preferentially avoid perfectly complementary targets in order to minimize problems caused by transitivity. Compared with conventional RNAi, amiRNAs offer several advantages. First, miRNA precursors generally generate only a single effective small RNA of known sequence. By contrast, several siRNAs with undefined 5′ and 3′ ends are produced as a silencing trigger from hairpin constructs. Therefore, potential off-targets of amiRNAs can be more accurately predicted than those of longer hairpin constructs. Second, because miRNA-insensitive variants can be generated that do not differ in the encoded protein sequence of targets (Palatnik et al., 2003), mutant defects of amiRNA-expressing plants can be complemented, which is not easily possible with RNAi plants. Third, because of their exquisite specificity, amiRNAs can possibly be adapted for allele-specific knockouts. Fourth, as with natural miRNAs, amiRNAs are likely to be particularly useful for targeting groups of closely related genes, including tandemly arrayed genes. Approximately 4000 genes in *Arabidopsis* are found in tandem arrays (Arabidopsis Genome Initiative, 2000), and no convenient tool exists for their knockout.

In addition, we anticipate that amiRNAs will be effective tools for studying forms of posttranscriptional regulation. Two important discoveries of RNA expression studies using whole-genome tiling arrays have been the previously vastly underestimated number of noncoding RNA transcripts (Kapranov et al., 2002; Yamada et al., 2003; Bertone et al., 2004; Carninci et al., 2005; Stolc et al., 2005; Li et al., 2006) as well as a complex transcriptional landscape with many overlapping transcripts (Jen et al., 2005; Wang et al., 2005). An elegant study recently identified a naturally occurring antisense transcript as an important regulator of the corresponding sense transcript (Borsani et al., 2005). amiRNAs are uniquely suited to target either the sense or antisense RNA and should therefore be very helpful for the analysis of such interactions. In contrast with conventional hairpin-mediated RNAi, in which small RNAs are generated from both strands, amiRNAs have the advantage of being strand specific. Finally, there is a substantial level of alternative splicing (Gong et al., 2004; Ner-Gaon et al., 2004), and amiRNAs have the potential to target only specific splice forms.

METHODS

Plant Material

Plants were grown in long days (16 h light/8 h dark) or continuous light at 23°C. *lfy-12*, *gun4*, and *try cpc* plants have been described (Weigel et al., 1992; Schellmann et al., 2002; Larkin et al., 2003). *ft*-10 is an *ft* null allele with a T-DNA insertion from the GABI-Kat collection (Rosso et al., 2003), isolation number 290E08. All plants except *try cpc* double mutants were in the Col-0 background.

Transgenes

amiRNAs were engineered into a 404-bp fragment containing the *MIR319a* stem loop or a 410-bp fragment containing the *MIR172a* stem loop, cloned into pBluescript SK+ as PCR templates. Oligonucleotide and stem loop sequences can be found in Supplemental Table 5 online. All fragments were sequenced and placed behind different promoters in pMLBART (Gleave, 1992). Transgenic plants were generated by *Agrobacterium tumefaciens*–mediated transformation (Weigel and Glazebrook, 2002).

Small RNA Isolation and Blot Analysis

Total RNA was isolated from inflorescences of pooled T1 plants using Trizol reagent (Invitrogen) and resolved by 17% PAGE under denaturing conditions (7 M urea). A total of ~1 pmol end-labeled synthetic RNA oligonucleotides were included as size standards. Blots were hybridized using end-labeled oligonucleotide probes (Llave et al., 2002).

RNA Analyses

Microarray analyses using the Affymetrix ATH1 platform were performed on biological (amiR-lfy-1, amiR-mads-1, and amiR-mads-2) or technical duplicates (amiR-white-1 and amiR-white-2) as described (Schmid et al., 2003). Inflorescences with oldest flowers around stage 10 (Smyth et al., 1990) (for amiR-lfy-1 and amiR-mads overexpressers) were harvested from pooled T1 plants grown in continuous light. Seedlings (for amiR-white overexpressers) were grown on 0.5× MS medium (Murashige and Skoog, 1962) without sucrose for 7 d. Total RNA processed for each array ranged from 3 μg (from seedlings) to 5 μg (from inflorescences). Twelve micrograms of labeled cRNA was used. Affymetrix microarrays were hybridized according to the manufacturer's protocol.

For RT-PCR, total RNA was isolated using the Plant RNeasy Mini kit (Qiagen) or Trizol reagent, and 2 μg was used for reverse transcription using a commercial kit (Invitrogen).

The same RNA as used for small RNA gel blot analysis was processed as described for cleavage site mapping (Schwab et al., 2005).

Statistical Analysis of Microarray Data

Normalized expression estimates were obtained using gcRMA, a modification of the RMA algorithm, in which probe intensity is modeled as a function of GC context, using an empirical Bayes estimate. We used an R-implementation of the LIMMA package to determine t-statistics for mean expression values (http://www.R-project.org; Smyth et al., 2005) and corrected P values for multiple testing according to Benjamini and Yekutieli (2001). Differentially expressed genes were required to have a FDR-adjusted P value of ≤1% and expression changes of at least 1.5-fold relative to the wild-type control.

We also determined significant changes on a per-gene level using the logit-T algorithm. Logit-t employs a logit transformation for normalization of probe intensities, followed by statistical testing on individual probe intensities across replicates, assigning P values for expression differences. While FDR cannot be easily computed for logit-T, it outperforms several other popular algorithms for Affymetrix data sets (Lemon et al., 2003).

Present calls for genes in the control (wild-type Col-0) were obtained using the algorithm implemented in Affymetrix MicroArray Suite 5.0 with default settings.

Computational Tools

Genes with specific numbers of mismatches were identified with a custom Web interface for HyPa (Gräf et al., 2001), a pattern search tool

based on enhanced suffix arrays. Smith-Waterman scores were calculated with the EMBOSS (Rice et al., 2000) implementation of the Smith and Waterman (1981) algorithm, which calculates the optimal local alignment. Statistics were calculated with the R package (http://www.R-project.org) (Ihaka and Gentleman, 1996). To identify sequence biases, position-specific score matrices were calculated for each miRNA family. Hybridization energies were calculated with mfold (Zuker, 2003) or RNAcofold (Flamm et al., 2000).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers are as follows: *AG* (At4g18960), *ANR1* (At2g13210), *CPC* (At2g46410), *CRC* (At1g69180), *ETC2* (At2g30420), *FIL* (At2g45190), *FLC* (At5g10140), *FUL* (At5g60910), *GUN4* (At3g59400), *INO* (At1g23420), *LFY* (At5g61850), *MAF1* (At1g77080), *MAF2* (At5g65050), *MAF3* (At5g54060), *SHP1* (At3g58780), *SHP2* (At2g42830), *SOC1* (At2g45660), *TRY* (At5g53200), *YAB3* (At4g00180), *MIR319a* (At4g23713), and *MIR172a* (At2g28056). Microarray data are available from ArrayExpress under experiment E-TABM-63.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Predicted Target Genes of Artificial MicroRNAs and Expression Changes in amiRNA Overexpressers Compared with Wild-Type Controls by Microarray Analyses.

Supplemental Table 2. Functionality of Artificial MicroRNAs in Different Backbones and under Different Promoters.

Supplemental Table 3. Means of Smith-Waterman Scores.

Supplemental Table 4. Potential Targets of Secondary siRNAs.

Supplemental Table 5. Sequences of miRNA Stem-Loop Backbones and Oligonucleotides.

Supplemental Table 6. Sequences of Oligonucleotides Used for RT-PCR of *YABBY* Genes in amiR-yabby Overexpressers.

Supplemental Table 7. Summary of Downregulated Genes with Seed Matches to amiRNAs.

Supplemental Figure 1. Alignments of amiRNAs to Target Genes.

Supplemental Figure 2. Overlap of Significantly Downregulated Genes (logit-T, $P < 0.01$) in *gun4*-1 and amiR-white Overexpressers.

Supplemental Figure 3. Expression of *FT* in Leaves of amiR-ft Overexpressers.

Supplemental Figure 4. RNA Gel Blot of Weak and Strong amiR-mads-2 (*MIR319a* Backbone) Overexpressers.

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