Developmental Cell, Vol. 8, 517–527, April, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.devcel.2005.01.018

Specific Effects of MicroRNAs on the Plant Transcriptome

Rebecca Schwab,[1,3] Javier F. Palatnik,[1,3] Markus Riester,[1] Carla Schommer,[1] Markus Schmid,[1] and Detlef Weigel[1,2,*]
[1]Department of Molecular Biology
Max Planck Institute for Developmental Biology
72076 Tübingen
Germany
[2]Plant Biology Laboratory
The Salk Institute for Biological Studies
La Jolla, California 92037

Summary

Most plant microRNAs (miRNAs) have perfect or near-perfect complementarity with their targets. This is consistent with their primary mode of action being cleavage of target mRNAs, similar to that induced by perfectly complementary small interfering RNAs (siRNAs). However, there are natural targets with up to five mismatches. Furthermore, artificial siRNAs can have substantial effects on so-called off-targets, to which they have only limited complementarity. By analyzing the transcriptome of plants overexpressing different miRNAs, we have deduced a set of empirical parameters for target recognition. Compared to artificial siRNAs, authentic plant miRNAs appear to have much higher specificity, which may reflect their coevolution with the remainder of the transcriptome. We also demonstrate that miR172, previously thought to act primarily by translational repression, can efficiently guide mRNA cleavage, although the effects on steady-state levels of target transcripts are obscured by strong feedback regulation. This finding unifies the view of plant miRNA action.

Introduction

MicroRNAs (miRNAs) are a class of small regulatory RNAs derived from foldback structures that are part of longer precursor transcripts (Bartel, 2004). MiRNAs can reduce the protein levels of their targets through two mechanisms, translational repression and transcript cleavage. Both require complementary base pairing of miRNAs to target mRNAs. Translational inhibition is found mainly in animals, where miRNAs often bind several motifs in the 3′ UTRs of their targets, with the target motifs typically having several mismatches to the miRNA. In plants, miRNA target motifs have normally few mismatches and are mostly found in coding sequences. Recognition by plant miRNAs appears to lead predominantly to transcript cleavage through a mechanism closely related to RNA interference (RNAi), which is associated with siRNAs.

Because of the large number of mismatches in most animal miRNA binding motifs, statistical approaches are needed for sequence-based identification of animal miRNA targets. These methods are reasonably successful, particularly when comparative genomic information is included, but the prediction of false positives remains a problem (Enright et al., 2003; Kiriakidou et al., 2004; Lai, 2002; Lewis et al., 2003; Rajewsky and Socci, 2004; Rehmsmeier et al., 2004; Smalheiser and Torvik, 2004; Stark et al., 2003).

In contrast, many plant miRNA targets have been successfully predicted simply based on the perfect or near-perfect complementarity with their targets (Llave et al., 2002a; Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002; Sunkar and Zhu, 2004; Wang et al., 2004). Computational tests along with conservation of target sites in two distantly related species, *Arabidopsis* and rice, supported the validity of many of these predictions (Rhoades et al., 2002). All predicted targets that have since been shown to be affected by a specific miRNA have three or fewer mismatches to the corresponding miRNA (Achard et al., 2004; Aukerman and Sakai, 2003; Chen, 2004; Juarez et al., 2004; Laufs et al., 2004; Vaucheret et al., 2004; Xie et al., 2003).

Experimental indication that plant miRNAs can affect targets with an even greater number of mismatches came from an analysis of the first identified plant miRNA mutant, in which miR319a (miR-JAW) is overexpressed (Palatnik et al., 2003). MiR319a targets five members of the *TCP* family of transcription factor genes, which have up to five mismatches to miR319a, or up to four mismatches when counting G:U pairs as 0.5 mismatches. Such targets would be missed by simple mismatch predictions, because increasing the number of allowed mismatches leads to unacceptably high numbers of false positives. To overcome these limitations, an elegant addition to the computational rules has been to consider less-than-perfect conservation of potential miRNA target sites between members of gene families, which allows for prediction of targets that would not be found with the original rules (Jones-Rhoades and Bartel, 2004). However, criteria such as similarity to other targets cannot be used by the miRNA itself for target recognition. In addition, not all miRNAs are conserved between distantly related species (Allen et al., 2004).

Here, we use genome-wide expression profiling to experimentally establish parameters for target cleavage guided by plant miRNAs. Because these parameters take only the interaction of a single miRNA with a single target into account, they address directly the mechanistic basis of plant miRNA:target recognition. Although the empirically inferred parameters share several features with those described for artificial siRNAs (Haley and Zamore, 2004; Reynolds et al., 2004), miRNAs appear to be more specific than siRNAs (Jackson et al., 2003), which likely reflects the selection history of miRNAs, which have coevolved with the mRNA complement encoded in the genome.

Results and Discussion

To study whole-genome effects of miRNAs, we generated transgenic plants with increased levels of miR159,

*Correspondence: weigel@weigelworld.org
[3]These authors contributed equally to this work.

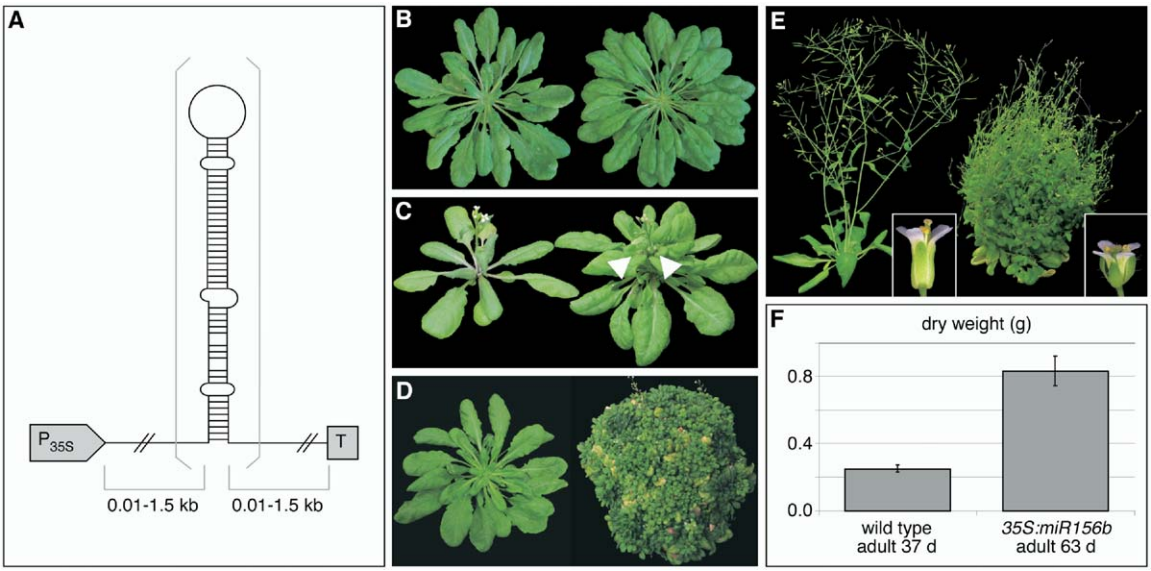

Figure 1. Overexpression of miRNAs

(A) Overexpression strategy: fragments of different sizes were placed between the RNA polymerase II promoter of the cauliflower mosaic virus *35S* gene (P_{35S}) and an *ocs* terminator (T).

(B–F) Phenotypes of plants overexpressing miR156b. Left, wild-type controls; right, *35S:miR156b* plants. (B) Plants grown in short days for 56 days. Note the increased leaf number in *35S:miR156b*. (C) Plants grown in long days, shown shortly after main inflorescence has started to elongate. Precocious release of side shoots (arrowheads) is apparent in *35S:miR156b*. (D) Short day-grown plants shortly after the first open flowers have become visible. The *35S:miR156b* plant, which is about 7 months old, has many more, but smaller, leaves. (E) Long day-grown, mature plants at the stage that fruits are fully developed. Insets show flowers, highlighting the squashed appearance of the *35S:miR156b* flower. (F) Average dry weight of 15 plants each, including those shown in (E). Error bars indicate standard deviations.

miR164, and miR319/JAW, for which other sets of overexpressers have been described (Achard et al., 2004; Laufs et al., 2004; Mallory et al., 2004a; Palatnik et al., 2003), and for miR156. All four miRNAs are encoded by more than one locus, which can potentially give rise to the same or slightly different miRNAs. We overexpressed precursors for several members of each family under the control of the viral 35S promoter in the Columbia background (Figure 1A). The phenotypes were always similar for each family, with some variation in severity of defects caused by the different members. Furthermore, overexpression of larger genomic fragments had very similar effects as overexpression of smaller fragments limited to the fold-back structures of the miRNA precursor genes.

Phenotypes and Transcriptome Analysis of miRNA-Overexpressing Plants

miR156 has been predicted to target members of the *SPL* (*SQUAMOSA PROMOTER BINDING PROTEIN LIKE*) family, which encodes a class of plant-specific transcription factors. Ten of the 16 members of the *SPL* family have been predicted to be miR156 targets (Rhoades et al., 2002). Knockout phenotypes have not been described for any of these, but *SPL3* has been shown to accelerate flowering when overexpressed (Cardon et al., 1997). Consistent with a positive role of *SPL* genes in floral induction, miR156 overexpression causes a moderate delay in flowering from 19 (n = 12) to 25 days (n = 39) under long days. In addition, miR156 overexpressers initiate rosette leaves faster than wild-type (2.2 versus 1.4 leaves per day, n = 40, in short days; Figures 1B–1F). Increased miR156 levels also cause a severe decrease of apical dominance, and the first flowers often arise from side shoots. Combination of these traits leads to a substantial increase in total leaf number on main and side of shoots, which can be more than ten times higher than in wild-type.

Overexpression of miR164, miR159a, and miR319 affects members of the *NAC*, *MYB*, and *TCP* families of transcription factor genes, respectively (Achard et al., 2004; Laufs et al., 2004; Mallory et al., 2004a; Palatnik et al., 2003). Our overexpressers have phenotypes similar to those described (Figures S1 and S2; see the Supplemental Data available with this article online), with the exception of our miR159a overexpressers, which have anther defects, but normal flowering time. This may be due to less efficient overexpression or to a genetic background that is different from the one used previously (Achard et al., 2004).

As before (Palatnik et al., 2003), we used expression profiling with duplicate Affymetrix ATH1 arrays to explore the spectrum of genes that might be subject to miRNA-induced transcript cleavage. To this end, we focused on tissues with maximal expression levels of predicted or known miRNA targets (Figure 2). We complemented the array analyses with quantitative, real-time RT-PCR (qRT-PCR) for additional tissues and for putative targets not detected on arrays. An obvious limitation of this approach is that it ignores targets subject to translational inhibition, as proposed for miR172 (Aukerman and Sakai, 2003; Chen, 2004).

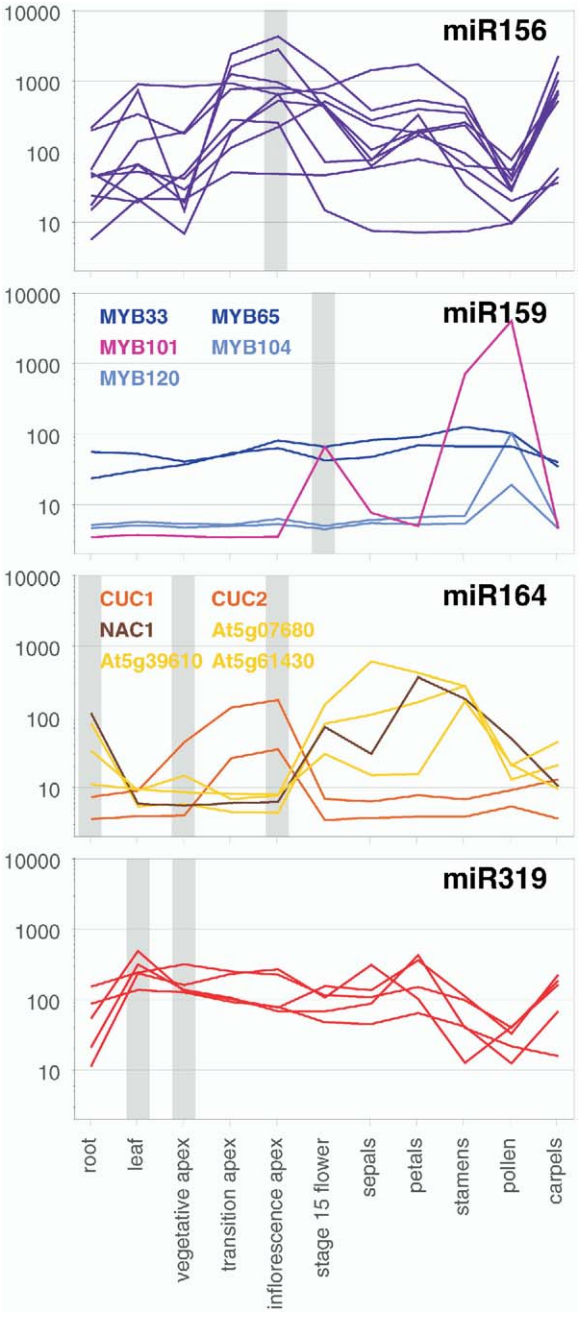

Figure 2. Expression Patterns of Predicted miRNA Targets in Wild-Type

Expression estimates by gcRMA are from the AtGenExpress expression atlas, based on Affymetrix ATH1 analyses (Schmid et al., 2005). Gray bars indicate tissues analyzed in miRNA overexpressers. Floral organs are from stage 15 flowers. See Table S3 for gene identifiers.

Effects of miRNAs on Genes with Limited Number of Mismatches

There have been several attempts to identify plant miRNA targets from first principles. Rhoades et al. (2002) initially predicted targets based on the observation that there were statistically significantly more mRNAs with three or fewer mismatches to authentic miRNAs than to randomized miRNAs. These predictions have been recently refined by, for example, counting G:U pairs as 0.5 mismatches. Additional genes are considered as targets if they belong to the same gene family as a previously predicted target and share sequences closely related to the miRNA complementary motif (Jones-Rhoades and Bartel, 2004). Even so, the *TCP* genes only conform to these predictions if one treats the closely related miRNAs miR159 and miR319 as interchangeable, which is inconsistent with in vivo data (this work; Achard et al., 2004; Palatnik et al., 2003). At the same time, genes sharing the same mismatch limitations are generally not considered as targets if the presumptive target sites are not conserved in other species (Jones-Rhoades and Bartel, 2004).

While miRNA effects on the transcriptome can be indirect due to secondary effects induced by altered expression of primary targets, direct targets should all have at least some sequence complementarity to the overexpressed miRNA. Across all four miRNA overexpressers, transcripts with up to four mismatches to the respective miRNA are enriched among downregulated genes (Table 1; statistical significance cannot be determined because of the small numbers of expected and observed cases). The excess of observed downregulated genes is readily explained by the presence of known or previously predicted targets in this group. Only in the case of miR159, two additional downregulated genes are found in the class of genes with up to four mismatches (Table 1).

Specific effects of miRNA overexpression are particularly apparent in miR156 overexpressers: of the 15 *SPL* genes represented on the ATH1 array, 10 genes with predicted miR156 target sites are substantially reduced in expression, while the remaining genes without miR156 target sites are unaffected (Figure 3).

Six *NAC* family members have been predicted to be targeted by miR164, including *At5g39610* with four mismatches (Jones-Rhoades and Bartel, 2004). It has been suggested that only four of these are efficiently guided to cleavage by miR164 (Laufs et al., 2004), although cleavage site mapping in wild-type is consistent with all of them being miR164 targets (data not shown; Mallory et al., 2004a). Using qRT-PCR, we found that expression of all four, including *At5g39610*, is substantially reduced in roots of miR164 overexpressers (Figure 3).

MYB33 has been shown to be downregulated in leaves of miR159 overexpressers (Achard et al., 2004). The male sterility of these plants is consistent with the genetic evidence for genes related to *MYB33* being required for proper stamen development (Kaneko et al., 2004). Surprisingly, we did not detect a change in *MYB33* or *MYB65* RNA expression in flowers, even though both genes are easily detected in the controls. The arrays show only one predicted target, *MYB101*, to be significantly reduced. qRT-PCR revealed that another target, *MYB120*, which is not detected on the arrays, is downregulated as well (Figure 3). While these observations confirm that miR159a is capable of guiding target RNA cleavage, they also suggest that higher levels of miR159a than present in our overexpressing lines are required for significant reduction of *MYB33*

Table 1. Summary of Downregulated Genes in Four miRNA Overexpressers

	Mismatches			Perfect Match
	≤3	4	5	Positions 2–12
Present in control	17	31	299	87
Observed downregulated				
All	13	4	7	18
Conserved targets	13	2	3	15
Others	0	2	4	3
Expected downregulated without conserved targets	0	1	8	3

Across all four comparisons, 59,395 genes are called present in the controls, of which 1,906 (3.2%) are significantly downregulated in the miRNA overexpressers. For miR164b and miR319a, only one tissue is included in this summary (inflorescence and vegetative apices, respectively).

and *MYB65* mRNAs. Notably, *MYB33* and *MYB65* transcript abundance is much higher and more even throughout development than that of the other miR159 targets (Figure 2), suggesting that miR159 and its targets *MYB33* and *MYB65* are closer to equilibrium than other pairs of miRNAs and targets.

A Transcript Unrelated to Conserved Targets Can Be Cleaved by miR159

In addition to *MYB101* and *MYB120*, two genes with four or fewer mismatches, At5g55930 (*OPT1*) and At4g37770 (*ACS8*), are downregulated in flowers of miR159 overexpressers. Both are unrelated to the conserved *MYB* targets. We decided to focus on *OPT1*, because the reduction in RNA levels is particularly dramatic (Figure 3). In flowers of miR159a overexpressers, *OPT1* mRNA is cleaved at a site opposite of position 10–11 of the miRNA (Figure 3), which is typical for miRNA- or siRNA-guided cleavage (Elbashir et al., 2001; Kasschau et al., 2003). We did not detect any cleavage products in wild-type, where *OPT1* expression is highest in pollen, similar to the pattern of

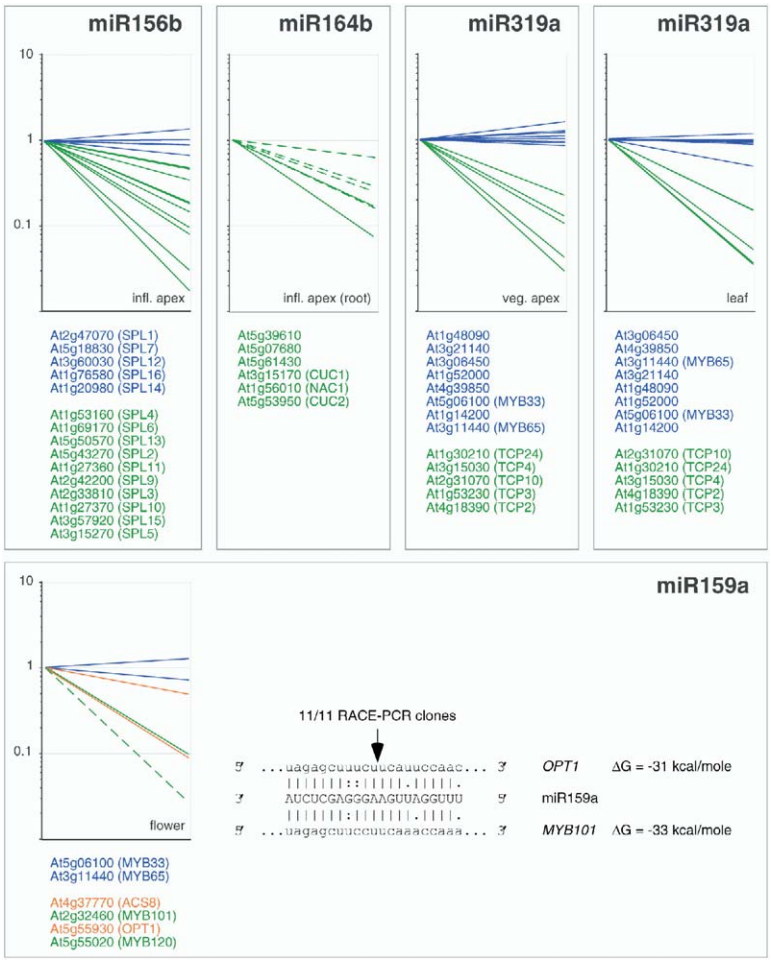

Figure 3. Expression Analysis of miRNA-Overexpressing Plants

Gene identifiers are in the same order from top to bottom as the lines in the graphs above. Wild-type is always on the left, and miRNA overexpressers are on the right; expression values are normalized to the wild-type control. Solid lines indicate microarray data, and dashed lines indicate real-time RT-PCR data. Genes that change significantly in expression are indicated in green. Blue indicates genes that do not change significantly and include the following: for miR156b, *SPL* genes that do not contain miR156 target sites; for miR319a, eight genes with four mismatches; for miR159a, two *MYB* genes related to the genes that are significantly downregulated. For miR159a overexpressers, ochre indicates two genes with four mismatches that are not conserved targets, but are significantly downregulated. One of these is *OPT1*, for which an alignment of the target site is shown in comparison with the *MYB101* target site. The 5′ ends of *OPT1* cleavage products are indicated by an arrow. miR164b data are from inflorescence apices (*CUC1* and *CUC2*), and the remainder are from roots.

MYB101 expression (Figure S3). These findings suggest that the *OPT1* transcription pattern in flowers does not normally overlap with miR159 expression. Alternatively, higher miR159 levels than present in wild-type may be required for efficient *OPT1* cleavage.

Effects of Plant miRNAs on Transcripts with Limited Sequence Complementarity

Because validated plant miRNA targets are highly complementary to the respective miRNAs, we initially focused on genes with a limited number of mismatches. However, it has been reported for siRNAs, which act in a similar manner as most plant miRNAs, that perfect complementarity to siRNA positions 2–12 can be sufficient to trigger cleavage in vitro (Haley and Zamore, 2004). Based on this finding, it has been predicted that artificial siRNAs may have substantial effects not only on their intended targets, to which they are complementary in their entire sequence, but also on unrelated mRNAs with more limited complementarity. Downregulation of such so-called off-targets has been observed by expression profiling of cultured human cells transfected with synthetic siRNAs (Jackson et al., 2003). Recent reviews have highlighted the problems that may arise from off-target effects in therapeutic applications of siRNAs (Anonymous, 2004; Couzin, 2002; Hannon and Rossi, 2004).

To detect effects of plant miRNAs on transcripts with limited sequence complementarity, we first analyzed genes that are perfectly complementary to nucleotides 2–12 of the miRNAs, but are not conserved targets. Across the four overexpressers, 3.2% of such genes are downregulated in response to the corresponding miRNA, as would be expected by chance (Table 1).

The first eight nucleotides of the miRNA are the core region of the miRNA-target interaction in animals (Doench and Sharp, 2004; Lewis et al., 2003), and along with the residues around the cleavage site, they also include the ones most critical for plant miRNA function (Mallory et al., 2004b; Parizotto et al., 2004; Vaucheret et al., 2004). We therefore considered next transcripts that are perfectly complementary to the first eight nucleotides of each miRNA. Among genes with at most 5 mismatches to the respective miRNAs, there are 16 genes with perfect matches to positions 1–8 of the miRNAs under investigation. Of these, only one gene is affected by miRNA overexpression, At4g37770 (*ACS8*), which we had already identified as potentially regulated by miR159 (see above).

We also examined all genes with up to five mismatches that are downregulated in each overexpresser. Of a total of 24 genes with 5 or fewer mismatches, only 6 downregulated genes are not conserved targets (9 expected; no significant difference in χ^2 test; Table 1). These general findings do not change when one counts G:U as 0.5 mismatches (not shown).

Finally, we asked whether downregulated genes that are not conserved targets are overall more similar in sequence to the overexpressed miRNA than random genes. This is not the case. Surprisingly, the downregulated genes are on average even less similar to the miRNA, although this difference is not statistically significant (Figure 4; Table S2).

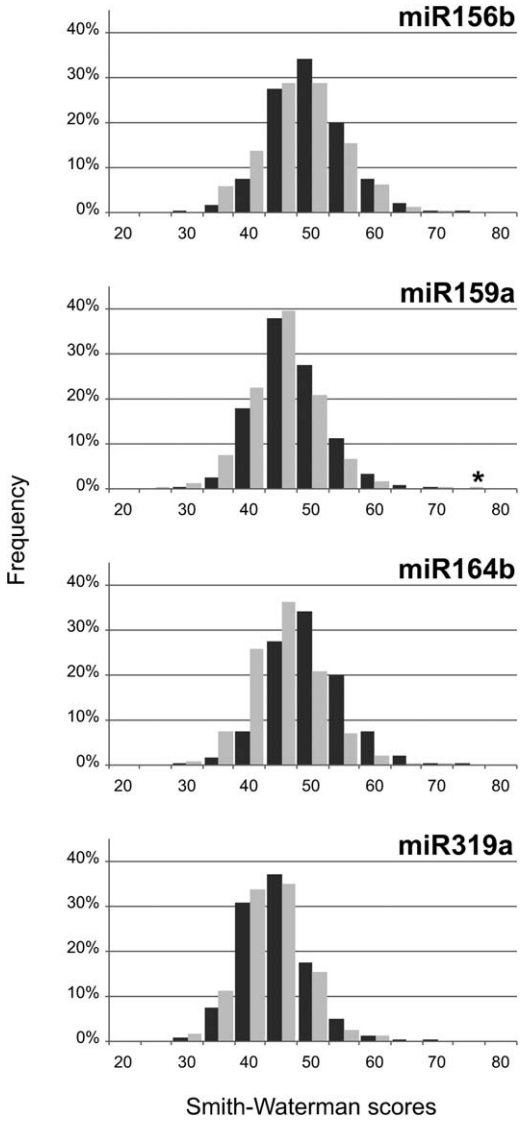

Figure 4. Absence of Major Off-Target Effects of Plant miRNAs

Distributions of Smith and Waterman (1981) scores are similar between genes that are significantly downregulated in response to miRNA overexpression (light gray) and all genes present in the control (dark gray). Authentic, conserved targets are excluded from this analysis. For miR159a, the asterisk indicates At5g55930 (*OPT1*), which is guided to cleavage by miR159a (Figure 3).

In conclusion, while evolutionary conserved targets appear to constitute the vast majority of genes directly affected by overexpression of four different miRNAs, we have identified at least one transcript, *OPT1*, that is not an evolutionarily conserved target, but can be cleaved by miR159. Although *OPT1* is otherwise unrelated to the conserved *MYB* targets of miR159, the sequence complementarity in the miRNA target site is as high as that of conserved targets. This finding makes it likely that not only the evolution of miRNAs is an ongoing process (Allen et al., 2004), but also that of miRNA targets.

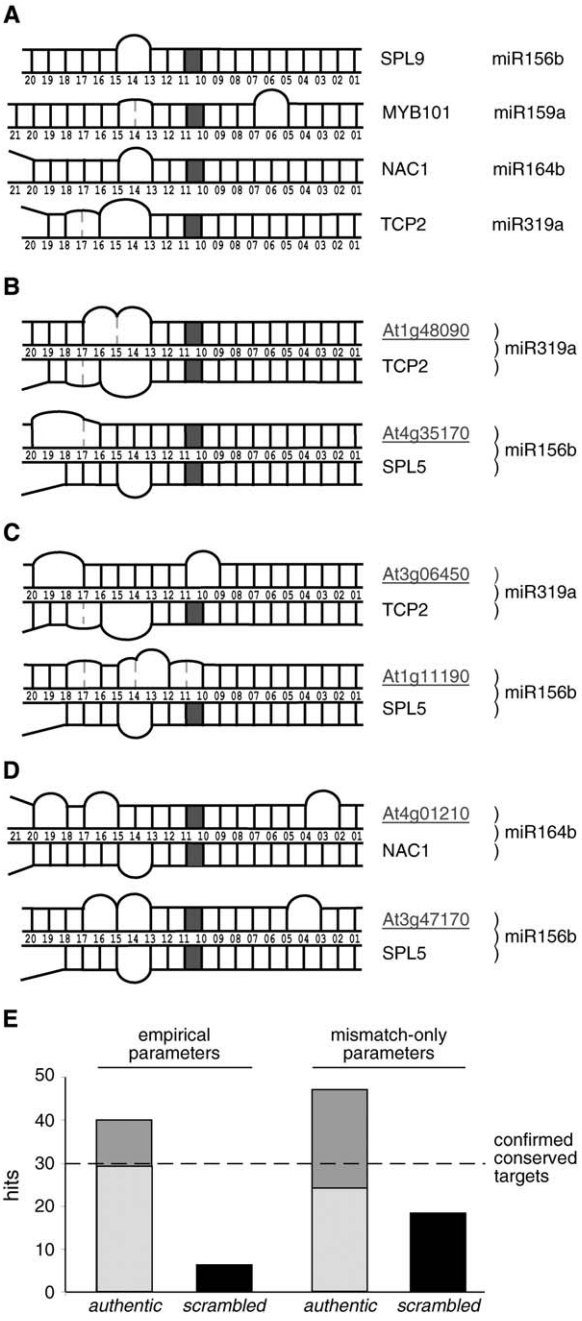

Figure 5. Sequence Features that Determine miRNA Target Selection

Empirical parameters are: no mismatch at positions 10 and 11; no more than one mismatch at positions 2–12; no more than two consecutive mismatches downstream of position 13; at least 72% of free energy compared to a perfectly complementary target.

(A) Validated targets that conform to these rules. Numbering begins at the 5′ end of the miRNA. The cleavage site is marked in gray. Dashed lines indicate G:U mismatches.

(B–D) Comparison of miRNA interaction with nontargets (top, underlined, gray) and authentic targets (bottom). (B) Requirement of internal stretches of less than three contiguous mismatches, even when the region complementary to the 5′ end of the miRNA matches perfectly. (C) Requirement of perfect matches surrounding a potential cleavage site. (D) Mismatches in the region complementary to the 5′ end of the miRNA often have adverse effects on targeting if there are also mismatches to the 3′ region.

(E) Comparison of empirical parameters with ad hoc rules using only mismatches (maximum of 3.0 mismatches to the 20-mer that gives the most hits in the genome, counting G:U as 0.5 mismatches) (Jones-Rhoades and Bartel, 2004). Light gray boxes indicate hits to conserved targets, and dark gray boxes indicate hits to nonconserved potential targets. Black boxes show hits with cohorts of ten randomized miRNAs with the sequences of miR156, miR159a, miR164a, and miR319a.

Sequence Requirements for miRNA: Target Recognition

Rhoades et al. (2002) suggested that at least some transcripts with four mismatches are miRNA targets, but reliable prediction of true targets in this group by computational means was not possible. Experimental evidence that miRNAs can target genes with four or even five mismatches came from our work on miR319a (Palatnik et al., 2003). Even when counting G:U pairs only as 0.5 mismatches, the *TCP3* target has 4 mismatches to miR319a and exceeds the 3.5 proposed mismatch limit for dependable prediction of conserved miRNA targets (Jones-Rhoades and Bartel, 2004).

To identify parameters that unambiguously distinguish targets from nontargets regardless of sequence conservation in other species or other gene family members, we inspected all genes with up to five mismatches to each of the four miRNAs. By this definition, 317 potential target sequences are found in 305 genes detected in the wild-type controls (176 for miR156, 47 for miR159, 29 for miR164, and 65 for miR319). Among these, we compared genes that are not affected by miRNA overexpression ("nonfunctional sequences") with genes that are ("functional sequences").

Free Energy of miRNAs Paired with Potential Target Sites

We aligned potential target sequences with the corresponding miRNA and calculated their free energy (ΔG) (Zuker, 2003). When we sort the potential target sites according to free energy, the functional motifs always rank among the top of the lists, but free energy alone does not distinguish functional and nonfunctional sequences (Table S1).

A computational analysis of mammalian miRNAs has indicated that pairing to nucleotides 2–8 of the miRNA is very important for target recognition (Lewis et al., 2003), and experimental analysis has shown that interaction with the first eight nucleotides of the miRNA is crucial for translational inhibition in cultured cells (Doench and Sharp, 2004). Sorting of potential target sequences according to their free energy when paired only with the first eight nucleotides of our set of plant miRNAs results in validated targets appearing at the top of the respective lists again; but, as with the complete miRNAs, this procedure does not unambiguously discriminate between targets and nontargets.

Position of Mismatches

Most functional miRNA target sequences have long stretches of perfectly matching nucleotides, especially to the 5′ portion of the miRNA. In contrast, runs of internal mismatches are limited to two contiguous mismatches (Figures 5A and 5B). We found several nontargets that have free energies similar to those of functional targets, but with mismatches at positions 10

or 11, which flank the cleavage site in functional miRNA targets (Figure 5C). We conclude that mismatches at these positions are not allowed. This contrasts with a recent report that suggested allowable mismatches at position 11, when monitoring known miRNA target interactions. Mismatches at the cleavage site, however, do substantially reduce both in vitro cleavage and in vivo phenotypic effects (Mallory et al., 2004b), in agreement with our observations. Reports on siRNAs in animals are in line with nucleotides immediately flanking the cleavage site being particularly important, even though there are some cases in which individual mismatches are tolerated (Boutla et al., 2001; Elbashir et al., 2001; Holen et al., 2002).

Consistent with experimental analyses of animal targets and mutational analysis of plant targets (Doench and Sharp, 2004; Laufs et al., 2004; Lewis et al., 2003; Mallory et al., 2004b; Parizotto et al., 2004; Vaucheret et al., 2004), we found the region pairing with the 5′ portion of the miRNA to be specifically sensitive to mismatches. We can extend the mismatch-sensitive region from positions 1–8, which are critical for animal miRNAs, to positions 2–12, as we did not find a functional target sequence with more than one mismatch in this region. Several genes that have otherwise low free energy, including no mismatches at the presumptive cleavage site and no runs of more than two contiguous mismatches in the region complementary to the 3′ end of the miRNA, are insensitive to miRNA overexpression if they have mismatches in the 5′ region (Figure 5D). Conversely, even if a potential target sequence has ten or more consecutive matches in the 5′ region, it is not functional if there is a stretch of three mismatches toward the 3′ portion of the miRNA (Figure 5B).

There remain a few genes that do not change in transcript abundance in our overexpressers, although they conform to the sequence criteria discussed above. This set comprises *MYB33* and *MYB65*, which are cleaved in wild-type in a position consistent with miR159a targeting (Palatnik et al., 2003). MiR159a overexpression causes efficient downregulation of the related *MYB101* gene, even though the free energy of *MYB101* paired with miR159a is less favorable than that of *MYB33* or *MYB65*. It seems unlikely that flanking sequences are important in this context, since miR159a efficiently cleaves *OPT1*, which is unrelated to *MYB101* outside of the target motif.

Summary of Empirical Parameters for Target Recognition

Authentic miRNA target motifs are distinguished by low overall free energy when paired with the corresponding miRNA (in the set examined, at least 72% compared to perfect match). Only one mismatch is tolerated in the region complementary to nucleotides 2–12 of the miRNA, but not at the cleavage site. Information outside the miRNA complementary motif does not seem to be very important for efficient transcript cleavage, as indicated by the *MYB* genes and the unrelated *OPT1* gene, both of which are guided to cleavage by miR159a. This conclusion is confirmed by the two classes of miR156 targets. Both comprise *SPL* genes, but one group has the target motif in the coding region, while the other has the target motif in an unrelated sequence context in the 3′ UTR.

A final issue that should be taken into account is the relative ratio of an miRNA and its target (Doench and Sharp, 2004). As discussed above, the higher basal levels of *MYB33* and *MYB65* expression may explain why these genes respond very little to miR159a, whereas *MYB101* and *MYB120* respond strongly, even though the latter genes have less favorable free energy than *MYB33* and *MYB65* when paired with miR159a.

Validation of Sequence Parameters for Target Recognition

We used two approaches to validate the sequence parameters deduced from analysis of plants overexpressing four different miRNAs. First, we randomized the miRNA sequences, an approach that has been used to gauge the power of target predictions based on sequence complementarity (Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002). In comparison to previously used rules (Jones-Rhoades and Bartel, 2004), the experimentally inferred parameters produce fewer hits with randomized miRNA sequences (Figure 5E).

Next, we asked whether the parameters inferred from the analysis of four miRNA overexpressers were consistent with targets of other miRNAs. We focused initially on miR160, miR165–168, miR170/171, miR393–R395, and miR397, which can be aligned with their targets without bulges. We confirmed that the predicted or confirmed miRNA:target pairs have low free energies, no mismatches at the cleavage sites, long stretches of perfect matches to the 5′ portion of the miRNA, and no strings of more than two mismatches to the 3′ portion of the miRNA. *AGO1*, which is targeted by miR168 (Vaucheret et al., 2004), is the only target with two mismatches between positions 2 and 12. The same rules also apply to targets of miR162 and miR396, if one considers a bulge in the mRNA as a mismatch, as well as two of the three proposed miR398 targets. (The bulges, if they are present, are always found opposite of the 5′ region of the miRNA). Only the potential miR398 target *CSD2*, with two mismatches and a bulge in nucleotides 2–12, is an exception (Jones-Rhoades and Bartel, 2004). Finally, predicted targets of miR161 are cleaved in wild-type plants, despite a mismatch at position 11 (Vazquez et al., 2004). However, it needs to be confirmed that these predicted targets are indeed regulated by the proposed miRNA, and not one that is closely related in sequence. In addition, it is unknown how efficient the miRNA-induced cleavage is.

The small number of exceptions demonstrates that the experimentally inferred parameters are broadly applicable. We therefore revisited known plant miRNAs, and we found new or additional potential targets for three of them (Table S4). We detected products consistent with miR408-guided cleavage for a potential miR408 target, which has four mismatches to miR408 (Figure S4).

As plant miRNAs are similar to siRNAs in their mode of action, we compared highly potent siRNAs, as defined by Reynolds et al. (2004), with all plant miRNAs known or proposed to cause cleavage. Most of the criteria for siRNAs apply also to plant miRNAs; low internal stability at the 5′ end of the small RNA, which can be partially attributed to U being the most common

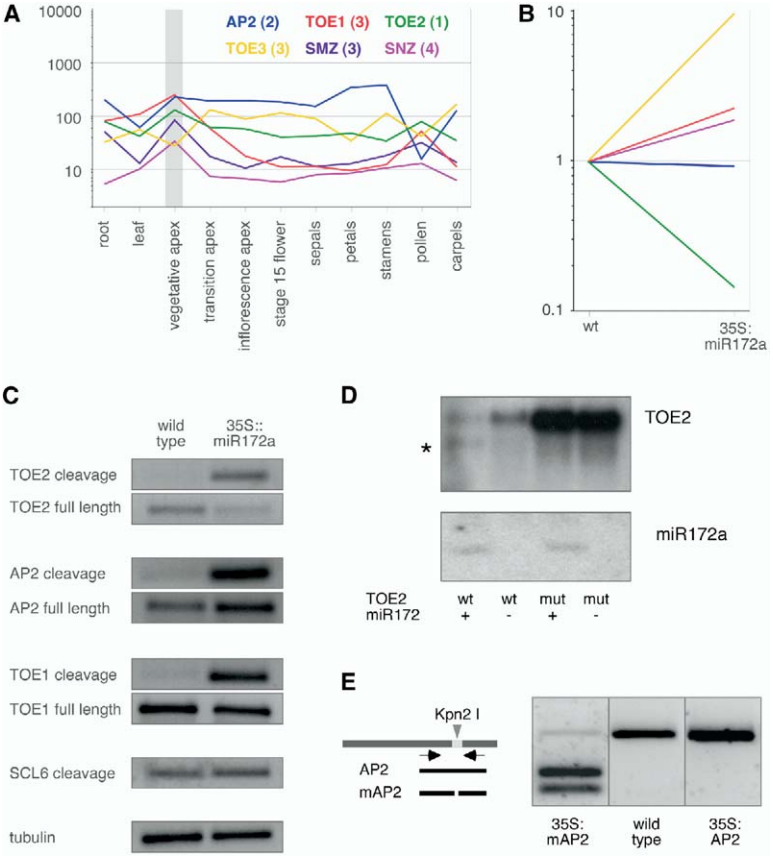

Figure 6. Analysis of miR172 Targets

(A) Expression profile of predicted miR172 targets in wild-type (see Figure 2). The gray bar indicates tissue analyzed in miR172a overexpressers. Numbers indicate mismatches of predicted targets to miR172a.

(B) Response of predicted targets to miR172a overexpression. The apparent increase in *SNZ* expression is not statistically significant.

(C) RT-PCR analysis of uncleaved transcripts and cleavage products (see also Figure S5). Cleavage products of the *SCL6* gene (Llave et al., 2002b) and tubulin cDNAs were amplified as controls.

(D) RNA blot analysis of *N. benthamiana* leaves infiltrated with *TOE2* and miR172a overexpression constructs. The asterisk indicates the cleavage product in the presence of miR172a. Transcripts from the miRNA-resistant form of *TOE2* ("mut") are more stable.

(E) Feedback regulation of *AP2*. Wild-type and miRNA-resistant ("mAP2") transcripts were amplified by RT-PCR and were distinguished by digestion with the restriction enzyme Kpn2I, which cuts only the mutant form. Wild-type transcript is increased in *35S:AP2* plants, as expected, but strongly decreased in plants that presumably overproduce AP2 protein because they express an miRNA-resistant version of AP2 ("35S: mAP2").

nucleotide at position 1, is among these criteria. Among specific sequence biases, Reynolds et al. (2004) found an A at position 10 to be the most important one. A total of 50.9% of plant miRNAs (adjusted for frequency within a family) have an A at this position. Without a mismatch, the target has a U at position 10, consistent with endonucleases preferring to cleave 3′ of a U (Donis-Keller, 1979; Reynolds et al., 2004).

Reexamination of miR172 Targets

While cleavage seems to be the predominant mode of plant miRNAs, an exception appears to be miR172, which has been reported to act primarily by translational inhibition (Aukerman and Sakai, 2003; Chen, 2004). miR172 has been predicted to target *APETALA2* (*AP2*) along with five other members of the *AP2* family, *TOE1–3*, *SMZ*, and *SNZ* (Park et al., 2002; Schmid et al., 2003). If miR172 acts similarly to the other miRNAs we examined, all six genes should be subject to miR172-guided cleavage. In wild-type, products consistent with miR172-guided cleavage can be detected for at least four of these genes (Aukerman and Sakai, 2003; Kasschau et al., 2003), but it has been suggested that these cleavage products are rare and that they do not contribute significantly to normal miR172 function (Aukerman and Sakai, 2003; Chen, 2004).

We generated plants overexpressing miR172a, and we analyzed vegetative apices, which have the highest levels of target mRNA expression in wild-type (Figure 6A). Only a single gene with up to five mismatches to miR172a is significantly downregulated in miR172a overexpressers: *TOE2* (Figure 6B). Interestingly, *TOE3* levels are substantially increased.

Because arrays measure only steady-state levels of mRNA, we wanted to confirm that the substantially reduced levels of *TOE2* are indeed due to increased cleavage. To this end, we developed an assay for quantification of miRNA-induced cleavage (Figure S5). We found that *TOE2* cleavage products increase substantially in miR172a-overexpressing seedlings (Figure 6C). A transient assay with *Nicotiana benthamiana* as host and *Agrobacterium tumefaciens* as delivery vehicle has been developed to study miRNA-guided cleavage (Llave et al., 2002b; Palatnik et al., 2003; Xie et al., 2003). In this system, cotransfection with a construct designed to overexpress miR172a reduces the levels of full-length *TOE2* transcript and leads to accumulation of a shorter mRNA indicative of cleavage (Figure 6D). An miRNA-resistant version of *TOE2* is unaffected by miR172a, and, in addition, it accumulates to much higher levels even in the absence of exogenous miR172a (Figure 6D). Together, these experiments demonstrate that *TOE2* is efficiently guided to cleavage by miR172a, leading to much reduced steady-state levels of *TOE2* in miR172a overexpressers. We do not know why this effect has not been detected by Aukerman and Sakai (Aukerman and Sakai, 2003).

We also detect substantial increases in the cleavage products of *AP2* and *TOE1* (Figure 6C). Since there is no commensurate decrease in *AP2* or *TOE1* steady-

state levels, the simplest explanation for these findings is that miR172 targets are under direct or indirect feedback regulation by their products. In this scenario (which differs from the type of feedback regulation proposed for miR159, Figure S6; Achard et al., 2004), miR172 both causes cleavage and translational repression of its targets, and the ensuing reduced protein accumulation leads to increased transcription of targets.

As a test for the hypothesis of feedback regulation, we compared *AP2* RNA levels in wild-type and in plants that overexpress *mAP2*, a mutated, miRNA-resistant form of *AP2*. RT-PCR demonstrated a substantial decrease in the levels of endogenous, wild-type *AP2* mRNA when *mAP2* is overexpressed (Figure 6E). This result confirms that AP2 protein directly or indirectly represses its own transcription (see Figure S6). We suggest that the efficiency of the feedback regulation differs for the different targets, explaining why *AP2* levels do not respond to miR172a overexpression, while the levels of other targets increase. In addition, the extent of cleavage versus translational inhibition may be affected by the number of mismatches to the miRNA, which may account for the strong miR172a-induced reduction in steady-state levels of *TOE2*, the only target with a single mismatch.

During wild-type development, expression of at least one of the miR172a precursors increases upon flowering (Schmid et al., 2003), and miR172 can be detected at high levels in developing flowers (Chen, 2004). While in our miR172a overexpressers only *TOE2* decreases, the levels of all miR172 targets but *TOE3* decline upon floral induction in wild-type (Schmid et al., 2003). This response, while moderate, has been confirmed in several independent experiments (M.C. Kim, A. Singh, J. Lempe, M.S., and D.W., unpublished data). These observations suggest either further modulation of the feedback regulation of miR172 targets, or effects of other miR172 isoforms. Notably, *TOE3* expression, which is increased in vegetative apices of miR172a overexpressers, responds in the same manner to floral induction in wild-type (Schmid et al., 2003). Constitutive expression of a *TOE3* variant with a mutation in the miR172 complementary motif causes a floral phenotype similar to that reported for miRNA-resistant *AP2* (Figure S7; Chen, 2004), indicating that *TOE3* is miRNA regulated.

Finally, having discovered that feedback regulation obscures the effects of miR172 on steady-state levels of most of its targets, we asked whether a similar scenario explains why only some of the miR159 targets are reduced in our miR159 overexpressers. However, there is no obvious increase in *MYB33* or *MYB65* cleavage products (Figure S8), suggesting again that, at least in our miR159a overexpressers, cleavage of *MYB33* and *MYB65* is already saturated.

Conclusions

We have studied global transcriptome changes in a series of transgenic plants overexpressing different miRNAs, and we have made the following main findings:

(1) Expression profiling is a powerful method for empirical identification of mRNAs that are guided to cleavage by an miRNA. So far, there is only a single report for an animal miRNA causing cleavage of its targets (Yekta et al., 2004). Our approach should be used for other animal miRNAs, to explore whether this particular miRNA constitutes an exception, or whether cleavage is more common in animals than usually thought.

(2) Plant miRNAs have limited effects on transcripts that do not conform to a narrow set of sequence parameters, which contrasts with the extensive off-target effects reported for siRNAs (Jackson et al., 2003). This may reflect that natural miRNAs have coevolved with the remainder of the transcriptome, and that there has been selection against off-target effects. Alternatively, natural miRNAs might have endogenous properties that make them more specific than artificial siRNAs. The latter case would be very exciting vis à vis the dangers of off-target effects in therapeutic applications of siRNAs (Anonymous, 2004; Hannon and Rossi, 2004). Of course, we cannot exclude that there are miRNA targets that have a higher number of mismatches, but are regulated by translational repression.

(3) The sequence parameters for miRNA:target recognition that we have deduced here should guide future mechanistic studies of miRNA action. The presence of G:U versus other mismatches appears to play only a minor role in plant miRNA:target interaction, consistent with reports on animal miRNAs, in which G:U mismatches behave the same as other mismatches, at least in the region complementary to the first eight nucleotides of the miRNA (Doench and Sharp, 2004).

(4) The effects of miRNAs on steady-state levels of target mRNAs can be complicated by feedback regulation, as shown for miR172. Feedback regulation of miRNA targets may provide a sensitive mechanism for fine tuning the expression of target genes and, coupled with translational repression, for fine tuning the expression of proteins encoded by target genes. Feedback mechanisms appear to be a general theme in miRNA function (Baulcombe, 2004). It would be desirable to compare steady-state transcript levels with transcription rates on a genome-wide basis, which will hopefully become technically feasible in the future.

(5) Together with previous work (Aukerman and Sakai, 2003; Chen, 2004; Kasschau et al., 2003), there is now compelling evidence for RNA cleavage and translational repression being similarly important in miR172 function. Experiments to shift the bias in the miR172 and other miRNA systems to either RNA cleavage or translational repression should be informative as to whether there is an advantage in having both mechanisms operate at the same time.

Experimental Procedures

See the Supplemental Data for additional information on experimental procedures.

Plant Material

Plants were grown on soil in long days (16 hr light/8 hr dark) or short days (8 hr light/16 hr dark) under fluorescent lights at 23°C. *jaw*-D plants have been described (Weigel et al., 2000). All plants were in the Columbia (Col-0) background.

Microarray Analyses

Microarray analyses were performed as described (Schmid et al., 2003). Inflorescence apices (for miR156b and miR164b overexpressers) were from long day-grown plants, with the oldest flowers being around stage 7 (Smyth et al., 1990). Vegetative apices (for miR164 and miR172a overexpressers) were from 7-day-old, short day-grown plants. Hypocotyls and cotyledons were removed by hand dissection. Stage 15 flowers (for miR159 overexpressers) were from long day-grown plants. Leaves (for miR319a overexpressers) were from 6-week-old plants grown in short days. We also reexamined previously published array data from vegetative apices of miR319a overexpressers (Palatnik et al., 2003). Normalized expression estimates were obtained by using gcRMA (http://www.bioconductor.org) (Irizarry et al., 2003), and significant changes were calculated by using logit-T (Lemon et al., 2003). Microarray data have been deposited with the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/; series GSE2078-2081).

Quantification of Cleavage Products

This method is based on the template cDNA libraries for mapping target cleavage sites (Kasschau et al., 2003). We designed chimeric oligonucleotides, with the 5′ portion hybridizing to the RNA adaptor and the 3′ region hybridizing to 4–6 nucleotides immediately downstream of the previously mapped cleavage site in the gene of interest (Figure S5). Reverse gene-specific oligonucleotides targeted regions 200–250 bases downstream of the ligated adaptor. To amplify full-length transcripts, forward oligonucleotides that hybridized upstream of, but close to, the cleavage site were used.

Supplemental Data

Supplemental Data including additional information on experimental procedures and sequence characteristics of miRNA targets, as well as additional figures, are available at http://www.developmentalcell.com/cgi/content/full/8/4/517/DC1/.

Acknowledgments

We thank M. Jones-Rhoades, Y. Kobayashi, A. Maizel, H. Vaucheret, X. Wu, and especially D. Bartel for critical reading of the manuscript and discussion; E. Allen and J. Carrington for teaching us the *N. benthamiana* assays and small RNA blots; and J. Berger for help with scanning electron microscopy. J. Fitz and S. Henz provided invaluable computational assistance. We acknowledge the use of microarray data from the AtGenExpress project, coordinated by L. Nover, T. Altmann, and D.W. and supported by funds from the Deutsche Forschungsgemeinschaft (DFG) and the Max Planck Society.. This work was supported by a postdoctoral fellowship from the HFSPO (J.F.P.); grants from the DFG (SFB 446) and the National Institutes of Health (GM62932) to D.W.; and by the Max Planck Society. D.W. is a Director of the Max Planck Institute.

Received: December 17, 2004
Revised: January 7, 2005
Accepted: January 12, 2005
Published: April 4, 2005

References

Achard, P., Herr, A., Baulcombe, D.C., and Harberd, N.P. (2004). Modulation of floral development by a gibberellin-regulated microRNA. Development *131*, 3357–3365.

Allen, E., Xie, Z., Gustafson, A.M., Sung, G.H., Spatafora, J.W., and Carrington, J.C. (2004). Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana*. Nat. Genet. *36*, 1282–1290.

Anonymous (2004). How to interfere with RNA. Nature *431*, 229.

Aukerman, M.J., and Sakai, H. (2003). Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. Plant Cell *15*, 2730–2741.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281–297.

Baulcombe, D. (2004). RNA silencing in plants. Nature *431*, 356–363.

Boutla, A., Delidakis, C., Livadaras, I., Tsagris, M., and Tabler, M. (2001). Short 5′-phosphorylated double-stranded RNAs induce RNA interference in *Drosophila*. Curr. Biol. *11*, 1776–1780.

Cardon, G.H., Hohmann, S., Nettesheim, K., Saedler, H., and Huijser, P. (1997). Functional analysis of the *Arabidopsis thaliana* SBP-box gene *SPL3*: a novel gene involved in the floral transition. Plant J. *12*, 367–377.

Chen, X. (2004). A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. Science *303*, 2022–2025.

Couzin, J. (2002). Breakthrough of the year. Small RNAs make big splash. Science *298*, 2296–2297.

Doench, J.G., and Sharp, P.A. (2004). Specificity of microRNA target selection in translational repression. Genes Dev. *18*, 504–511.

Donis-Keller, H. (1979). Site specific enzymatic cleavage of RNA. Nucleic Acids Res. *7*, 179–192.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. *20*, 6877–6888.

Enright, A.J., John, B., Gaul, U., Tuschl, T., Sander, C., and Marks, D.S. (2003). MicroRNA targets in *Drosophila*. Genome Biol. *5*, R1.

Haley, B., and Zamore, P.D. (2004). Kinetic analysis of the RNAi enzyme complex. Nat. Struct. Mol. Biol. *11*, 599–606.

Hannon, G.J., and Rossi, J.J. (2004). Unlocking the potential of the human genome with RNA interference. Nature *431*, 371–378.

Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., and Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. *30*, 1757–1766.

Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B., and Speed, T.P. (2003). Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. *31*, e15.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. *21*, 635–637.

Jones-Rhoades, M.W., and Bartel, D.P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell *14*, 787–799.

Juarez, M.T., Kui, J.S., Thomas, J., Heller, B.A., and Timmermans, M.C. (2004). microRNA-mediated repression of *rolled leaf1* specifies maize leaf polarity. Nature *428*, 84–88.

Kaneko, M., Inukai, Y., Ueguchi-Tanaka, M., Itoh, H., Izawa, T., Kobayashi, Y., Hattori, T., Miyao, A., Hirochika, H., Ashikari, M., and Matsuoka, M. (2004). Loss-of-function mutations of the rice *GAMYB* gene impair alpha-amylase expression in aleurone and flower development. Plant Cell *16*, 33–44.

Kasschau, K.D., Xie, Z., Allen, E., Llave, C., Chapman, E.J., Krizan, K.A., and Carrington, J.C. (2003). P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA function. Dev. Cell *4*, 205–217.

Kiriakidou, M., Nelson, P.T., Kouranov, A., Fitziev, P., Bouyioukos, C., Mourelatos, Z., and Hatzigeorgiou, A. (2004). A combined computational-experimental approach predicts human microRNA targets. Genes Dev. *18*, 1165–1178.

Lai, E.C. (2002). Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat. Genet. *30*, 363–364.

Laufs, P., Peaucelle, A., Morin, H., and Traas, J. (2004). MicroRNA

regulation of the *CUC* genes is required for boundary size control in *Arabidopsis* meristems. Development *131*, 4311–4322.

Lemon, W.J., Liyanarachchi, S., and You, M. (2003). A high performance test of differential gene expression for oligonucleotide arrays. Genome Biol. *4*, R67.

Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W., Bartel, D.P., and Burge, C.B. (2003). Prediction of mammalian microRNA targets. Cell *115*, 787–798.

Llave, C., Kasschau, K.D., Rector, M.A., and Carrington, J.C. (2002a). Endogenous and silencing-associated small RNAs in plants. Plant Cell *14*, 1605–1619.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002b). Cleavage of *Scarecrow-like* mRNA targets directed by a class of *Arabidopsis* miRNA. Science *297*, 2053–2056.

Mallory, A.C., Dugas, D.V., Bartel, D.P., and Bartel, B. (2004a). MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Curr. Biol. *14*, 1035–1046.

Mallory, A.C., Reinhart, B.J., Jones-Rhoades, M.W., Tang, G., Zamore, P.D., Barton, M.K., and Bartel, D.P. (2004b). MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. EMBO J. *23*, 3356–3364.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425*, 257–263.

Parizotto, E.A., Dunoyer, P., Rahm, N., Himber, C., and Voinnet, O. (2004). In vivo investigation of the transcription, processing, endonucleolytic activity, and functional relevance of the spatial distribution of a plant miRNA. Genes Dev. *18*, 2237–2242.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. (2002). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. Curr. Biol. *12*, 1484–1495.

Rajewsky, N., and Socci, N.D. (2004). Computational identification of microRNA targets. Dev. Biol. *267*, 529–535.

Rehmsmeier, M., Steffen, P., Hochsmann, M., and Giegerich, R. (2004). Fast and effective prediction of microRNA/target duplexes. RNA *10*, 1507–1517.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. (2002). MicroRNAs in plants. Genes Dev. *16*, 1616–1626.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., and Khvorova, A. (2004). Rational siRNA design for RNA interference. Nat. Biotechnol. *22*, 326–330.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. (2002). Prediction of plant microRNA targets. Cell *110*, 513–520.

Schmid, M., Uhlenhaut, N.H., Godard, F., Demar, M., Bressan, R., Weigel, D., and Lohmann, J.U. (2003). Dissection of floral induction pathways using global expression analysis. Development *130*, 6001–6012.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Schölkopf, B., Weigel, D., and Lohmann, J. (2005). A gene expression map of *Arabidopsis* development. Nat. Genet. *37*, in press.

Smalheiser, N.R., and Torvik, V.I. (2004). A population-based statistical approach identifies parameters characteristic of human microRNA-mRNA interactions. BMC Bioinformatics *5*, 139.

Smith, T.F., and Waterman, M.S. (1981). Identification of common molecular subsequences. J. Mol. Biol. *147*, 195–197.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell *2*, 755–767.

Stark, A., Brennecke, J., Russell, R.B., and Cohen, S.M. (2003). Identification of *Drosophila* MicroRNA targets. PLoS Biol. *1*, E60.

Sunkar, R., and Zhu, J.K. (2004). Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell *16*, 2001–2019.

Vaucheret, H., Vazquez, F., Crátá, P., and Bartel, D.P. (2004). The action of *ARGONAUTE1* in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev. *18*, 1187–1197.

Vazquez, F., Gasciolli, V., Crete, P., and Vaucheret, H. (2004). The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. Curr. Biol. *14*, 346–351.

Wang, X.J., Reyes, J.L., Chua, N.H., and Gaasterland, T. (2004). Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. Genome Biol. *5*, R65.

Weigel, D., Ahn, J.H., Blázquez, M.A., Borevitz, J., Christensen, S.K., Fankhauser, C., Ferrándiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., et al. (2000). Activation tagging in *Arabidopsis*. Plant Physiol. *122*, 1003–1013.

Xie, Z., Kasschau, K.D., and Carrington, J.C. (2003). Negative feedback regulation of *Dicer-Like1* in *Arabidopsis* by microRNA-guided mRNA degradation. Curr. Biol. *13*, 784–789.

Yekta, S., Shih, I.H., and Bartel, D.P. (2004). MicroRNA-directed cleavage of *HOXB8* mRNA. Science *304*, 594–596.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. *31*, 3406–3415.

Note Added in Proof

It has recently been shown that animal miRNAs reduce the transcript abundance of a large number of genes with limited sequence complementarity, reminiscent of the so-called off-target effects of siRNAs (Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. [2005]. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769–773). These findings suggest that the high specificity of miRNAs in plants could be due to the plant miRNA machinery rather than being a consequence of sequence selection during evolution.