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MicroRNAs and Their Regulatory Roles in Plants

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Abstract

MicroRNAs (miRNAs) are small, endogenous RNAs that regulate gene expression in plants and animals. In plants, these ~21-nucleotide RNAs are processed from stem-loop regions of long primary transcripts by a Dicer-like enzyme and are loaded into silencing complexes, where they generally direct cleavage of complementary mRNAs. Although plant miRNAs have some conserved functions extending beyond development, the importance of miRNA-directed gene regulation during plant development is now particularly clear. Identified in plants less than four years ago, miRNAs are already known to play numerous crucial roles at each major stage of development—typically at the cores of gene regulatory networks, targeting genes that that are themselves regulators, such as those encoding transcription factors and F-box proteins.

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INTRODUCTION

Multicellular organisms depend on complex networks of gene regulatory pathways. MicroRNAs (miRNAs), which went unnoticed until recently, are key components of these networks. Initially discovered as regulators of developmental timing in *Caenorhabditis elegans* (67, 114), miRNAs are now known to play a variety of important regulatory roles in both plants and animals.

MicroRNAs are short, endogenously expressed, nontranslated RNAs that are processed by Dicer-like proteins from stem-loop regions of longer RNA precursors (**Figure 1**, reviewed in 13). MicroRNAs are chemically and functionally similar to small interfering RNAs (siRNAs, see Small Interfering RNAs sidebar), which can mediate the related phenomena of RNA interference (RNAi), post-transcriptional gene silencing (PTGS), and transcriptional gene silencing (TGS). Like miRNAs, siRNAs are processed by the Dicer RNaseIII family of enzymes, but instead of deriving from local stem-loop structures, siRNAs are processed from long, double-stranded precursors (either from much longer stems or from bimolecular duplexes). Both miRNAs and siRNAs are incorporated into silencing complexes that contain Argonaute proteins, wherein they can guide repression of target genes.

Although miRNAs are deeply conserved within both the plant and animal kingdoms, there are substantial differences between the two lineages with regard to the mechanism and scope of miRNA-mediated gene regulation; several of these differences have been instrumental in the rapid increase in understanding of plant miRNA biology. Plant miRNAs are highly complementary to conserved target mRNAs, which allows fast and confident bioinformatic identification of plant miRNA targets (53, 116). As expected from this extensive complementarity to their

targets, plant miRNAs guide cleavage of their targets, an activity readily assayed in vitro and in vivo, which allows facile confirmation of predicted targets (55, 79, 128). In addition, *Arabidopsis* is a genetically tractable model organism, which enables study of the genetic pathways that underlie miRNA-mediated regulation and the phenotypic consequences of perturbing miRNA-mediated gene regulation. In this review, we describe the flurry of exciting results revealing the biological functions of these tiny riboregulators that have been made possible by the convergence of these factors.

GENOMICS OF PLANT MicroRNAs

MicroRNA Gene Discovery: Cloning

MicroRNAs have been discovered using three basic approaches: direct cloning, forward genetics, and bioinformatic predictions followed by experimental validation. The most direct method of miRNA discovery is to isolate and clone small RNAs from biological samples, and several groups have used this approach to identify small plant RNAs (78, 92, 107, 115, 125, 127, 143). The cloning methods were adapted from those first used to identify large numbers of animal miRNAs (62, 64) and involve isolating small RNAs, ligating adaptor oligonucleotides, reverse transcription, amplification, and sequencing. Some protocols incorporate methods to enrich for Dicer cleavage products (i.e., molecules with 5′ phosphates and 3′ hydroxyls) and to concatemerize the short cDNAs so that several can be identified in a single sequencing read (64). The initial cloning experiments in *Arabidopsis* identified 19 miRNAs, which fell into 15 families (78, 92, 107, 115), although the hundreds of other small RNAs also cloned, which included degradation fragments and endogenous siRNAs, sometimes complicated classification of the miRNAs. Subsequent cloning experiments have expanded our knowledge of small RNAs in *Arabidopsis* (127, 141, 142), *Oryza sativa* (rice) (125, 126), and *Populus trichocarpa* (81), and new, high-throughput sequencing methods have recently been employed to dramatically expand the depth of small RNA cloning coverage in *Arabidopsis* (80a).

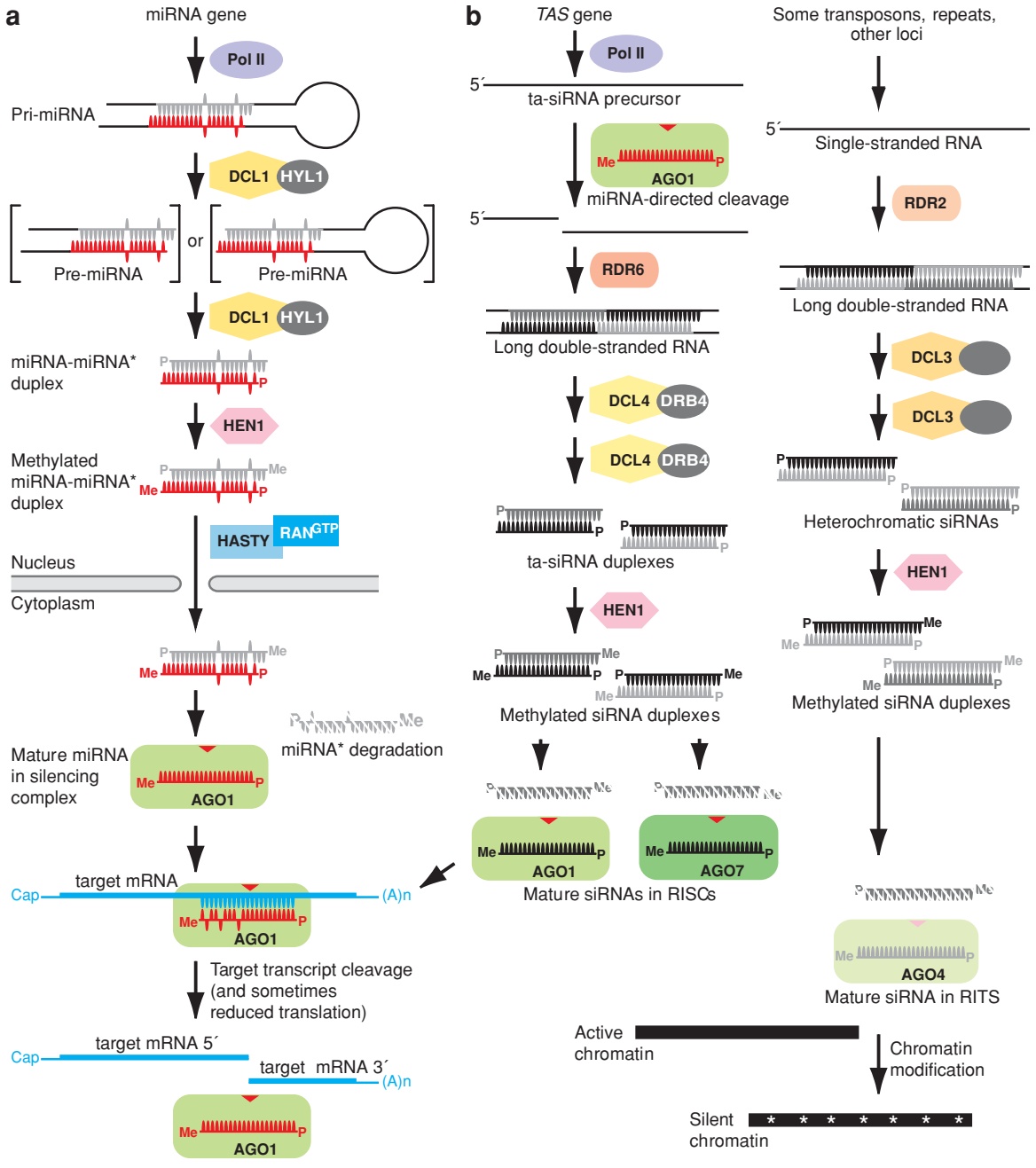
SMALL INTERFERING RNAs

siRNAs were first observed in plants (47), and in *Arabidopsis*; most small RNAs are siRNAs (78, 115, 127, 141, 142). They are implicated in a variety of processes, including defense against viruses, establishment of heterochromatin, silencing of transposons and transgenes, and post-transcriptional regulation of mRNAs (reviewed in 15). MicroRNAs and siRNAs have much in common (**Figure 1**). Both are 20–24 nucleotides long and processed from longer RNA precursors by Dicer-like ribonucleases (19, 44, 47, 48, 52, 67, 147a), and both are incorporated into ribonucleoprotein silencing complexes in which the small RNAs, through their base-pairing potential, guide target gene repression (37, 48, 67, 96a, 138, 147a).

The fundamental difference between the two small RNA classes is the nature of their precursors; siRNAs are processed from long, double-stranded RNAs (37, 147a), whereas miRNAs are processed from single RNA molecules that include an imperfect stem-loop secondary structure (62, 64, 66, 115). Several additional characteristics distinguish most miRNAs from most siRNAs. Many miRNAs are conserved between related organisms, whereas most endogenously expressed siRNAs are not (62, 64, 66, 115). Many (but not all) siRNAs target the gene from which they are derived or very closely related genes. In contrast, miRNAs regulate genes unrelated to loci encoding the miRNAs. In fact, the imperfect base pairing in the miRNA precursor stem-loop may help prevent the miRNA locus from undergoing silencing by the miRNA that it encodes. Finally, although the proteins required for siRNA and miRNA biogenesis are related and sometimes overlap, the genetic requirements for miRNA and siRNA function are partially distinct in many organisms. For example, many *Arabidopsis* siRNAs require RNA-dependent RNA polymerases for their biogenesis, whereas miRNAs do not (17, 31, 97, 142). Moreover, most *Arabidopsis* miRNAs are processed by *DICER-LIKE1* (*DCL1*) (61, 107, 115), one of four Dicer-like genes in *Arabidopsis* (117), whereas many endogenous siRNAs require *DCL3* or *DCL4* (41, 141a, 142, 146a).

MicroRNA Gene Discovery: Genetics

Although miRNAs were first discovered through forward genetic screens in round worms (67, 114), no miRNA gene families have been discovered by this method in plants, and miRNA involvement in plant mutant phenotypes was not inferred until after cloning experiments had established that plant genomes contained numerous miRNAs



(107, 115, 116). To date, only a single plant miRNA loss-of-function allele has been identified in forward genetic screens; *early extra petals1* is caused by a transposon insertion ~160 bp upstream of the predicted *MIR164c* stem-loop, and results in flowers with extra petals (9).

The fact that loss-of-function miRNA mutants have been recovered so rarely using forward genetics may reflect small target size for mutagenesis coupled with redundancy; nearly all evolutionarily conserved plant miRNAs are encoded by gene families (**Table 1**). Family members are likely to have overlapping functions, buffering against loss at any single miRNA locus. Overexpression screens can circumvent redundancy limitations. At least three plant miRNAs, *miR319* (also known as *miR-JAW*), *miR172* (also known as *EAT*), and *miR166*, were isolated in overexpression screens for dominant mutants with developmental abnormalities (6, 59, 103, 139). Mutations in miRNA target sites, which can prevent the entire family of miRNAs from repressing a target gene, can also circumvent redundant functions of miRNA family members. The dominant mutations in the HD-ZIP genes *PHB*, *PHV*, and *REVOLUTA* (*REV*) in *Arabidopsis* and *ROLLED LEAF1* (*RLD1*) in maize result in adaxialization of leaves and/or vasculature (89, 90, 100, 148) and are all caused by mutations in miR166 complementary sites (39, 54, 87, 116, 148).

MicroRNA Gene Discovery: Bioinformatics

In both plants and animals, cloning was the initial means of large-scale miRNA discovery (62, 64, 66, 115). However, cloning is biased toward RNAs that are expressed highly and broadly. MicroRNAs expressed at low levels or only in specific cell types or in response to certain environmental stimuli are more difficult to clone. Sequence-based biases in cloning procedures might also cause certain miRNAs to be missed. Because of these limitations, bioinformatic approaches to identify miRNAs have provided a useful complement to cloning.

A straightforward use of bioinformatics has been to find homologs of known

Figure 1

RNA-silencing pathways in plants. (*a*) A model for microRNA (miRNA) biogenesis in *Arabidopsis*. Following transcription, the pri-miRNA is processed by DCL1, perhaps with the aid of HYL1 and other factors, to a miRNA:miRNA* duplex with 5′ phosphates (P) and two-nucleotide 3′ overhangs. Pre-miRNAs, which are readily detectable in animals, appear to be short-lived in plants (*brackets*). The 3′ sugars of the miRNA:miRNA* duplex are methylated (Me) by HEN1, presumably within the nucleus. The miRNA is exported to the cytoplasm by HST, probably with the aid of additional factors. The mature, methylated miRNA is incorporated into a silencing complex that can include AGO1, and the miRNA* is degraded. Complex maturation is depicted after nucleocytoplasmic export, but might occur before. Within the silencing complex, the miRNA is capable of targeting complementary RNAs for cleavage by AGO1, and perhaps also for translational repression. (*b*) Models for biogenesis of trans-acting small interfering RNAs (ta-siRNA; *left*) and heterochromatic siRNAs (*right*) in *Arabidopsis*. Other endogenous siRNAs and siRNAs from transgenes or viral RNA are generated through similar or partially overlapping pathways. Long double-stranded RNA, generated through the action of RNA-dependent RNA polymerases (RDRs), is iteratively processed by Dicer-like (DCL) proteins to yield multiple siRNA duplexes. The phase of the ta-siRNA duplexes can be set by miRNA-directed cleavage of the *TAS* transcript. One strand from each siRNA duplex is stably incorporated into a silencing complex [RNA-induced silencing complex (RISC) or RNAi-induced transcriptional silencing (RITS) complex], and the other is degraded. siRNAs in RISCs guide cleavage of complementary RNAs, whereas those in RITS complexes are associated with the establishment or maintenance of heterochromatin. Pol IV is involved in heterochromatic siRNA production in plants, either transcribing the genomic DNA to produce the single-stranded RNA or transcribing the double-stranded RNA to amplify the single-stranded RNA (49a, 54a, 101a).

MicroRNA (miRNA): 20- to 24-nucleotide silencing RNA processed from a stem-loop region of a longer transcript by Dicer-like enzymes

Dicer-like (DCL) proteins: RNaseIII-like enzymes that process siRNAs from long double-stranded RNA precursors or miRNAs from local stem-loop secondary structures of primary transcripts

Small interfering RNA or short interfering RNA (siRNA): silencing RNA, typically 20- to 24-nucleotides in length, processed from long double-stranded RNA by Dicer-like enzymes

RNA interference (RNAi): The phenomenon by which exogenous double-stranded RNA directs the post-transcriptional silencing of homologous

Argonaute (AGO) proteins: members of a protein family that contain a PAZ small RNA-binding domain and a Piwi RNase H-like domain

Table 1 MicroRNA gene families conserved in plants

miRNA family (reference)		*Arabidopsis*	*Oryza*	*Populus*
miR156	(115)	12	12	11
miR159/319	(78, 92, 103, 107, 115)	6	8	15
miR160	(115)	3	6	8
miR162	(115)	2	2	3
miR164	(115)	3	5	6
miR166	(115)	9	12	17
miR167	(78, 107, 115)	4	9	8
miR168	(115)	2	2	2
miR169	(115)	14	17	32
miR171	(78, 115)	4	7	10
miR172	(107)	5	3	9
miR390	(2, 127, 141)	3	1	4
miR393	(53, 127)	2	2	4
miR394	(53)	2	1	2
miR395	(53)	6	19	10
miR396	(53)	2	5	7
miR397	(53, 127)	2	2	3
miR398	(53, 127)	3	2	3
miR399	(53, 127)	6	11	12
miR408	(127)	1	1	1
miR403	(127)	1	0	2
miR437	(125)	0	1*	0
miR444	(125)	0	1*	0
miR445	(125)	0	9*	0
Total		92	127	169

All known miRNA families that are conserved between more than one plant species are listed together with the number of genes identified in the sequenced genomes. Rice miRNA families that have orthologs in maize but do not appear to have orthologs in the eudicots (*Arabidopsis* and *Populus*) are marked with an asterisk. The following families contain miRNA genes annotated with more than one number: miR156 (miR156 and miR157), miR159/319 (miR159 and miR319), miR166 (miR165 and miR166), miR171 (miR170 and miR171), and miR390 (miR390 and miR391).

miRNAs, both within the same genome and in the genomes of other species (62, 64, 66, 108, 115). A more difficult challenge is to identify miRNAs unrelated to previously known miRNAs. This was first accomplished for vertebrate, nematode, and fly miRNAs, using algorithms that search for conservation of sequence and secondary structure (i.e., miRNA stem-loop precursors) between species, searching for patterns that are characteristic of miRNAs (63, 72, 74). Although these methods identified numerous potential animal miRNAs, many of which were subsequently confirmed experimentally, they have not been directly useful in finding plant miRNAs because of the longer and more heterogeneous secondary structures of plant miRNA stem-loops.

To address this need, several groups devised bioinformatic approaches specific to plant miRNA identification (2, 22, 53, 137). Like algorithms for identifying animal miRNAs, these approaches use conservation of secondary structure as a filter, but are

necessarily more relaxed in terms of allowed structures. Some approaches take advantage of the high complementarity of plant miRNAs to target messages, implementing the requirement that the candidate has conserved complementarity to mRNAs (2, 53). This additional filter has been useful for distinguishing authentic plant miRNAs from false positives and recently has been extended to mammalian miRNA gene prediction (140).

The Conserved miRNAs in Plants

In aggregate, cloning, genetics, and bioinformatics have resulted in the annotation of 118 potential miRNA genes in *Arabidopsis* (miRBase, release 7.0) (43). These 118 loci can be grouped into 42 families, with each family comprised of stem-loops with the potential to produce identical or highly similar mature miRNAs. Twenty-one families represented by 92 genes are clearly conserved in species beyond *Arabidopsis* (**Table 1**; miRBase, release 7.0). These families are somewhat expanded in the other sequenced plant genomes, *Oryza sativa* (rice), and *Populus trichocarpa* (cottonwood), where they are represented by 116 and 169 potential miRNA genes, respectively (**Table 1**). The number of members per family in a genome ranges from 1 to 32. With the exception of the miR-430 family, which is represented by a cluster of ~80 loci in zebrafish (42), and a related family, which is represented by a cluster of 43 loci in human (18), animal miRNAs typically fall into smaller families that have much more diverse members; metazoan family members sometimes share only a common 5′ seed region (13). In plants, the number of members in each family correlates among examined species; certain families contain numerous members in all three species (e.g., miR156, miR166, miR169), whereas others consistently contain only a few genes (e.g., miR162, miR168, miR394) (**Table 1**). Although it is unclear why a plant would need, for example, 12 genes encoding miR156, this correlation suggests functional significance of the various miRNA family sizes.

Twenty miRNA families identified to date are highly conserved between all three sequenced plant genomes: *Arabidopsis*, *Oryza sativa*, and *Populus trichocarpa* (**Table 1**). Several additional miRNA families are conserved only within specific lineages; miR403 is present in the eudicots *Arabidopsis* and *Populus* but absent from the monocot *Oryza* (127), and three families identified by cloning in *Oryza* are conserved in other monocots such as maize, but are not evident in either sequenced eudicot (125). Within each family, the mature miRNA is always located on the same arm of the stem-loop (5′ or 3′), as would be expected if the genes share common ancestry (**Figure 2**). Although the sequence of the mature miRNA and, to a lesser extent, the segment on the opposite arm of the hairpin to which it pairs, are highly conserved between members of the same miRNA family (both within and between species), the sequence, secondary structure, and length of the intervening "loop" region can be highly divergent between family members (**Figure 2**). The pattern of pairing and nonpairing nucleotides within the mature miRNA is often conserved between homologous miRNA stem-loops from different species (**Figure 2**). The significance of the conserved mismatches is unknown; perhaps they help guide DCL1 to cleave at the appropriate positions along the stem-loop.

Most efforts to clone small RNAs in plants have focused on the eudicot *Arabidopsis* or the monocot *Oryza*, and bioinformatic methods have focused on miRNAs conserved between these two species. These flowering plants diverged from each other ~145 million years ago (27). Growing evidence shows that many angiosperm miRNA families, and their complementary sites in target mRNAs, are conserved in more basal land plants. A cDNA containing a miR166 stem-loop has been cloned from the lycopod *Selaginella kraussiana*, and a miR159 stem-loop is present in an expressed sequence tag (EST) from the moss *Physcomitrella patens* (40, 53). Furthermore, a survey of miRNA expression

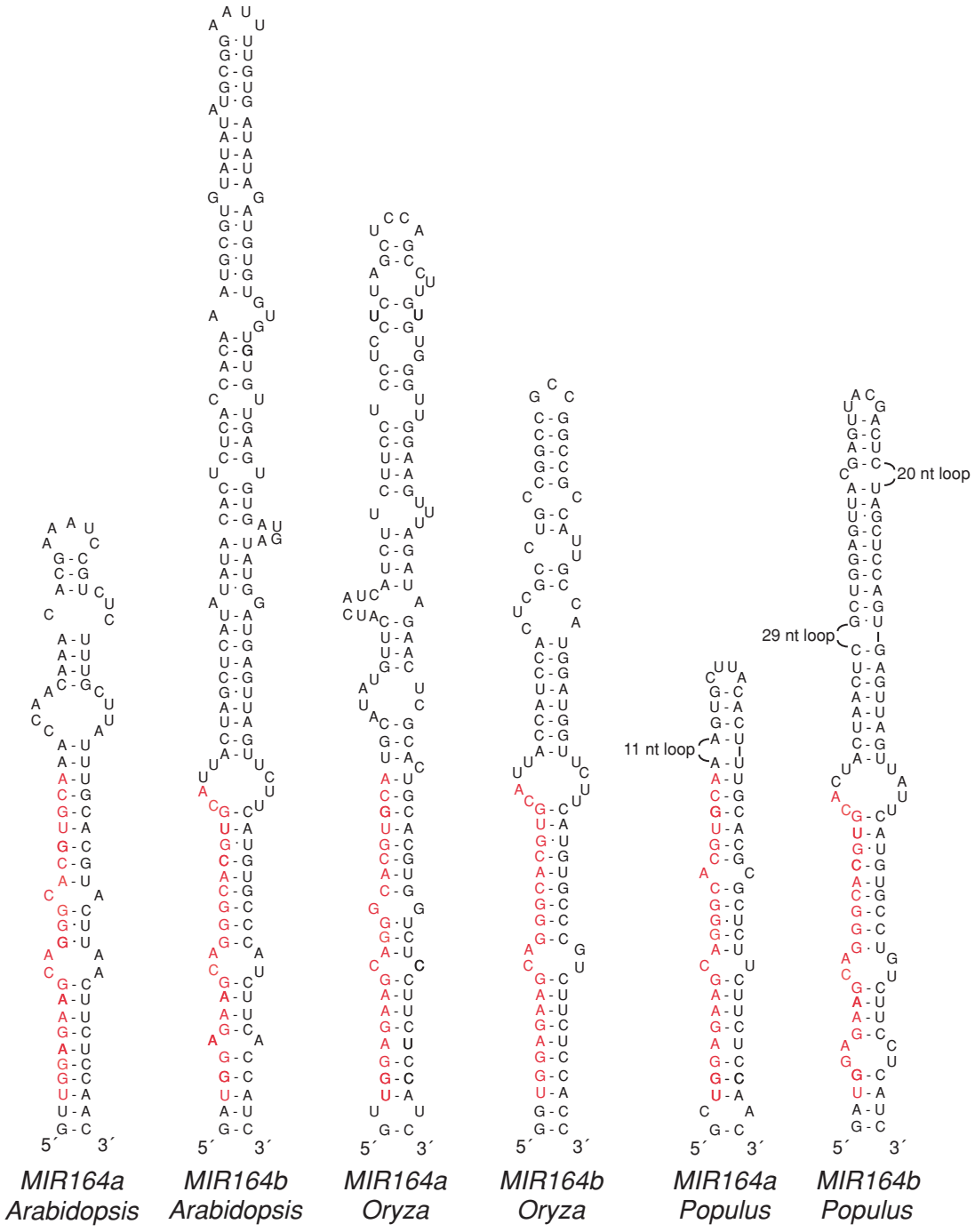


Figure 2

Representative miR164 stem-loops from *Arabidopsis*, *Oryza*, and *Populus*. Segments corresponding to the mature miRNAs are shown in red.

using microarray technology revealed at least 11 miRNA families with detectable expression in gymnosperms; at least two (miR160 and miR390) are detectable in moss (7). Ten miRNA families have conserved target sites in ESTs from gymnosperms or more basal plants (53), and five miRNA families (miR160, miR166, miR167, miR171, and miR172) in gymnosperms, ferns, lycopods, or mosses direct cleavage of target mRNAs that are homologous to the verified *Arabidopsis* targets (7, 40). More recently, direct cloning of small RNAs from moss has identfied several homologs of Arabidopsis miRNAs (5a). Some of these deeply conserved miRNA families regulate development in *Arabidopsis* and are necessary for processes such as proper specification of floral organ identity (miR172) or leaf polarity (miR166). It is interesting that these miRNAs regulate homologous mRNAs in basal plants that have very different reproductive structures and leaf morphology, leading to the speculation that these miRNAs are parts of ancient, conserved regulatory modules underlying seemingly different developmental outcomes (7).

Nonconserved MicroRNAs and the Challenges of Definitive miRNA Classification

Although most annotated miRNAs are conserved throughout flowering plants, others are found only in a single sequenced genome, and thus could be of a more recent evolutionary origin (**Table 1**). The extended homology between nonconserved miRNA precursors and target genes provides strong evidence that some of these potentially "young" miRNAs arose from duplications of target gene segments (4). Although several nonconserved miRNAs, including miR161, miR163, miR173, miR447, miR475, and miR476, are known to direct cleavage of target transcripts (3, 4, 81, 125, 132), it is difficult to confidently predict targets for many because it is not possible to use complementary site conservation as a filter against false-positive target predictions.

In fact, it is difficult to be confident that all annotated nonconserved miRNAs are miRNAs rather than siRNAs. The established minimal standard for miRNA annotation is a small RNA with detectable expression and the potential to form a stem-loop when joined to flanking genomic sequence (5). In practice, these requirements are too loose to definitively categorize many small RNAs cloned from plants. Many plant siRNAs are detectable on blots (109, 133, 142), and hundreds of thousands of non-miRNA genomic sequences can be predicted to fold into secondary structures that resemble structures of plant miRNA precursors (53). Therefore, without conservation of both sequence and secondary structure, it is difficult to be confident that a given cloned RNA originated from a stem-loop (i.e., is a miRNA) rather than from a double-stranded RNA (i.e., is a siRNA). In fact, many of the thousands of small RNAs cloned from *Arabidopsis* (46, 80a) would probably meet the literal requirements for annotation as miRNAs. A few of these sequences might be miRNAs, but others that meet the literal criteria probably are not.

The challenges for annotating nonconserved small RNAs cloned from plants are illustrated by three related small silencing RNAs that were originally annotated as miRNAs but turned out to be among the founding members of the *trans*-acting siRNAs (ta-siRNAs) (109, 133). As is typical of plant miRNAs, these ta-siRNAs direct cleavage of mRNA targets, are detected on northern blots, and require many of the same proteins as do miRNAs for their accumulation. Although these three silencing RNAs also fall within predicted stem-loop structures, the predicted hairpins are not as extensively paired as plant miRNAs and in two cases are not part of the optimal fold of this genomic region. Current evidence supports a model in which these small RNAs are generated by the sequential DCL4-mediated cleavage of a long double-stranded product of RDR6—a model in which

***Trans*-acting siRNA (ta-siRNA):** siRNA that negatively regulates mRNA distinct from and unrelated to the locus from which the siRNA is encoded

the predicted stem-loops are fortuitous and unrelated to the biogenesis of the ta-siRNAs (41, 109, 133 141a, 146a). For these and other ta-siRNAs, the cleavage occurs at ~21-nt increments in essentially a single register (3, 133). This register appears to be determined by miRNA-directed cleavage of the ta-siRNA precursor, which explains why all the proteins needed for miRNA biogenesis and function are also needed for ta-siRNA accumulation (3).

The challenge in confidently identifying miRNAs is not limited to small cloned RNAs; informatic predictions face even greater difficulties. For example, recent bioinformatic screens for miRNAs conserved between *Arabidopsis* and *Oryza* (22, 137) yielded dozens of miRNA gene candidates that had not been reported in previous cloning and bioinformatic efforts. Most *Arabidopsis* genes conserved with rice, a monocot, would be expected to also be present in other eudicots. Indeed, members of each of the 20 miRNA families listed in **Table 1** that are conserved in *Arabidopsis* and *Oryza* are also found in ESTs from other plant species and in the genome of *Populus*, a recently sequenced eudicot (53). However, none of the newly reported informatic candidates (i.e., those not listed in **Table 1**) are found in the sequenced ESTs from other species or in the *Populus* genome (M.W. Jones-Rhoades, unpublished), raising questions as to whether any of these candidates identified based on their putative conservation between *Arabidopsis* and *Oryza* are bona fide miRNAs. Probing for nine of these newly reported candidates (miR413 – 420 and miR426) gave weak apparent hybridization signals on northern blots (137), thereby providing experimental evidence for expression, as required for classifying conserved stem-loops as miRNAs genes. However, their apparent absence in genomes outside of *Arabidopsis* and *Oryza*, the observation that these putative miRNAs are generally less paired within their stem-loops than is typical of plant miRNAs with more experimental support (141; M.W. Jones-Rhoades, unpublished), the absence of confirmed targets of these miRNAs, and the possibility of false-positive detection on northern blots all suggest that these sequences might be bioinformatic false positives rather than bona fide miRNAs. In sum, confident annotation of poorly conserved miRNAs in plants appears to require evidence more stringent than that originally specified in the annotation guidelines (5). (See Potential Guidelines for Confident miRNA Annotation sidebar.)

Although it appears that most miRNAs that are broadly conserved among flowering plants are now identified and experimentally validated (53; **Table 1**), the challenges and ambiguities for classifying nonconserved miRNAs preclude meaningful estimates of the total number of miRNA genes in *Arabidopsis* and other plant genomes. It is possible to imagine that miRNAs in each species have escaped detection because they are both nonconserved and expressed in only a few cells or conditions. On the other hand, the possibility that some of the currently annotated miRNAs might be false positives leaves open the prospect that the actual number of authentic miRNAs in *Arabidopsis* is less than the number currently annotated.

MicroRNA BIOGENESIS

Transcription of icroRNA Precursors

Because plant miRNAs are primarily found in genomic regions not associated with protein-coding genes (115), it appears that most, if not all, plant miRNAs are produced from their own transcriptional units. This contrasts with animal miRNAs, which sometimes appear to be processed from introns of protein-coding genes (14). Plant miRNA genes are occasionally clustered near each other in the genome, suggesting transcription of multiple miRNAs from a single primary transcript [e.g., the miR395 cluster (44a, 53)], but this polycistronic arrangement of miRNA genes appears far less frequently in plants than in animals (13). Northern, EST, and mapping evidence indicate that plant miRNA primary

transcripts (sometimes called pri-miRNAs), as in animals, are longer than needed to encompass the miRNA stem-loops (6, 53, 103, 141). At least some of these pri-miRNA transcripts appear to be spliced, polyadenylated (6, 61), and capped (141). Two rice miRNAs are contained within transcripts that contain exon junctions within the presumptive stem-loop precursor, implying that in these cases, splicing is a prerequisite for Dicer recognition (125). The observations that plant pri-miRNAs can be over 1 kb in length, that they are usually preceded by typical TATA box motifs, and that they can undergo canonical splicing, polyadenylation, and capping, indicates RNA polymerase II is probably responsible for transcribing most plant miRNAs (141), as is the case for several animal miRNAs (69). Relatively little is known about the regulation of miRNA transcription in plants, but there is no reason to suspect that this regulation would differ from that of protein-coding transcripts.

MicroRNA Processing and Export

A central step in miRNA maturation is excising the mature miRNA from the pri-miRNA by RNaseIII-type endonucleases, such as Dicer (**Figure 1*a***). This processing has important differences between plants and animals. In animals, miRNAs are processed from the pri-miRNA by a pair of enzymes in a stepwise manner. Drosha, a nuclear-localized RNaseIII enzyme, makes the initial cuts (one on each arm of the stem-loop) in the pri-miRNA to liberate the miRNA stem-loop, the "pre-miRNA," from the flanking sequence of the pri-miRNA (68). After export to the cytoplasm, Dicer makes a second set of cuts, separating the miRNA, duplexed with its near reverse complement, the miRNA*, from the loop region of the premiRNA (68). The resulting miRNA/miRNA* duplex typically has two-nucleotide 3′ overhangs (74), similar to the overhangs of siRNA duplexes produced by Dicer from long double-stranded RNA (19, 37, 38).

POTENTIAL GUIDELINES FOR CONFIDENT miRNA ANNOTATION

Confident annotation of poorly conserved miRNAs in plants appears to require evidence more stringent than that originally specified in the annotation guidelines (5). First, it seems reasonable to require that the extent of pairing within the precursor stem-loop resembles that of the conserved miRNAs. Plant miRNA stem-loops generally have no more than 7 unpaired nucleotides in the 25 nucleotides centered on the miRNA, of which no more than 3 are consecutive and no more than 2 are without a corresponding unpaired nucleotide in the miRNA*, and they have analogous pairing constraints for the 25 nucleotides centered on the miRNA*. Nearly all (96%) of the hundreds of conserved miRNA genes listed in **Table 1** fulfill these criteria, and there is little reason to suspect that authentic nonconserved miRNAs would not. Second, expression evidence would ideally include evidence that expression depends on miRNA pathway genes (such as DCL1) but does not depend on genes unique to the ta-siRNA pathway (such as RDR6 or DCL4) or heterochromatic siRNA pathway (such as RDR2 or DCL3). This second criterion is currently more difficult to satisfy in plants other than *Arabidopsis* because other species do not have mutants defective in the silencing pathways, although RNAi can be used to deplete miRNA pathway genes (75a). For these other species, high-throughput sequencing (yielding hundreds of thousands of reads per sample) is often sufficiently thorough to identify both the miRNA and miRNA* segments (**Figure 1*a***). The cloning of candidates representing both strands of the miRNA duplex to the exclusion of other small RNAs from the locus, whereby the cloned RNAs are positioned within a predicted hairpin such that they are paired with two-nucleotide 3′ overhangs, is diagnostic of a miRNA locus. Another tractable criterion would be evidence of silencing function, for example, detection of cleavage fragments of predicted miRNA targets that end precisely at the nucleotide expected for miRNA-directed cleavage (79). This functional criterion, which has now been satisfied for virtually all the confidently identified *Arabidopsis* miRNAs, was recently used to provide strong support for the authenticity of nonconserved miRNAs in *Oryza* (126) and *Populus* (81). Although the possibility remains that relying on this functional criterion would occasionally annotate other silencing RNAs (such as ta-siRNAs) as miRNAs, this possibility would be largely avoided with stringent adherence to the pairing criterion described above. Moreover, at the end of the day, evidence that a small RNA guides RNA cleavage, be it siRNA or miRNA, would provide the most useful biological information.

MicroRNA* (miRNA*): The non-miRNA strand of a miRNA duplex generated by a Dicer-like enzyme from a miRNA stem-loop precursor

In plants, DCL1 is required for miRNA accumulation, yet processing intermediates do not appear to overaccumulate in DCL1 mutants, suggesting that DCL1 has the Drosha-like activity responsible for the first set of cuts (61, 107, 115). None of the other three Dicer-like enzymes in *Arabidopsis* is required for miRNA biogenesis (41, 142), suggesting that DCL1 also makes the second set of cuts. Supporting the idea that DCL1 has both Drosha and Dicer functions in plant miRNA maturation is the observation that in plants the two sets of cuts that liberate the miRNA/miRNA* duplex both occur in the nucleus, which is the predominant location of DCL1 (104, 106, 142). RNAs corresponding to the premiRNAs of animals are detected only rarely for plant miRNAs, suggesting that both sets of cleavage events happen in rapid succession. The scarcity of detected premiRNAs might also indicate that the initial set of cuts is frequently proximal to the loop rather than the base of the stem-loop, although the recent detection of the miR168 pre-miRNA-like intermediate in *Arabidopsis* indicates that some pri-miRNAs can be cut first at the base of the stem-loop (H. Vaucheret, A.C. Mallory, D.P. Bartel, unpublished).

Although there can be some length heterogeneity at both the 5′ and 3′ ends of plant miRNAs, it is clear that DCL1 cuts preferentially at specific positions in the miRNA stem-loop precursor that result in accumulation of the appropriate mature miRNA (115). The mechanism by which DCL1 recognizes where to cut is largely a mystery. As would be expected from the diversity of miRNA sequences, the secondary structure rather than the primary sequence appears to be most important within the miRNA region of the stem-loop, in that functional miRNAs are still produced when substitutions are made within the miRNA, provided that compensatory changes on the other arm of the stem-loop are introduced to maintain the same pattern of paired and unpaired residues (105, 131). However, the resulting artificial miRNA can be of a slightly different length, indicating that primary sequence or geometry of the mismatched residues plays a role in determining the cleavage sites (131). This recognition appears to involve the dsRNA-binding domain of DCL1 because the dcl1–9 allele, which disrupts the dsRNA-binding domain, cuts the miR163 stem-loop at aberrant positions (61). In addition, the *HYPONASTIC LEAVES1* (*HYL1*) (80) gene product probably collaborates with DCL1 during substrate recognition and subsequent functions. Metazoan Dicer-like (DCL) proteins each appear to partner with dsRNA-binding proteins, which can help recognize cleavage substrates and help load the silencing RNA into the silencing complex. A family of five dsRNA-binding proteins may play this role in *Arabidopsis* (50). Genetic, molecular, and biochemical evidence all indicate that HYL1 is the member of this family that preferentially partners with DCL1 during miRNA biogenesis (49, 50, 132).

In addition to *DCL1* and *HYL1*, *HUA ENHANCER1* (*HEN1*) is also important for miRNA maturation. Mutations in *HEN1* result in 3′-end uridylation of miRNAs and siRNAs, which apparently leads to reduced miRNA accumulation and function (23, 71a, 107, 143). HEN1 contains a methyltransferase domain, and can methylate miRNA/miRNA* duplexes in vitro (147). The 3′-terminal nucleotide of endogenous miRNAs is methylated on its 2′ hydroxyl group in wild-type plants, but not in *hen1* mutants or in animals (36a, 147) (M. Axtell & D.P. Bartel, unpublished). End-methylation of miRNAs does not enhance silencing activity in vitro (112) and instead appears to protect the 3′ ends of silencing RNAs from uridylation and associated destabilization (71a).

After DCL1-mediated cleavage and HEN1-mediated methylation, most miRNA molecules exit the nucleus and enter the cytoplasm (**Figure 1*a***). This export into the cytoplasm is facilitated by *HASTY* (*HST*), a member of the importin β family of nucleocytoplasmic transporters (21). A similar pathway exists in animals; Exportin-5, the mammalian HST ortholog, exports premiRNA hairpins

from the nucleus to the cytoplasm (82, 146). *hst* mutants have reduced accumulation of most, but not all, miRNAs, suggesting that HST is important for miRNA export, but that other components or pathways can substitute (106). As pre-miRNAs appear to be very short-lived in plants, it is likely that HST transports either miRNA/miRNA* duplexes or single-stranded miRNAs after they are fully excised by DCL1. Northern blot analysis suggests that miRNAs are primarily single stranded in the nucleus (106), suggesting either that a fraction of functional miRNAs are located within the nucleus or that miRNAs are already single stranded before transport to the cytoplasm by HST. It is unknown whether plant miRNAs are already associated with components of the silencing complex when transported to the cytoplasm, or if transport occurs before loading into the silencing complex.

MicroRNA Incorporation into the Silencing Complex

The miRNA-programmed silencing complex is often referred to as an RNA-induced silencing complex (RISC) to emphasize the functional parallels between the miRNA-programmed silencing complex and the siRNA-programmed complex that mediates RNAi (48, 79, 128). MicroRNAs are processed from their pri-miRNA precursors as duplexes, still paired with their miRNA* strands. However, cloning and expression data indicate that the miRNA strand of this duplex accumulates to much higher levels in vivo than does the miRNA* (74, 115). This accumulation asymmetry is achieved by preferential loading of the miRNA strand into the silencing complex, where it is protected from degradation, whereas the miRNA* strand is preferentially excluded from the silencing complex and consequentially subject to degradation (**Figure 1*a***).

Key insight into the asymmetry of RISC loading came from bioinformatic and biochemical studies of functional siRNA duplexes. The siRNA duplex strand with less stable 5′ end pairing is selectively loaded into RISC, where it guides silencing, whereas the strand with the more stable 5′ end pairing is excluded from RISC (57, 119). These two strands of the siRNA duplex are called the guide and passenger strands and are analogous to the miRNA and miRNA* strands, respectively. Most miRNA/miRNA* duplexes also appear to have energetic asymmetry; the 5′ ends of most miRNAs are less stably paired than are the 5′ ends of the corresponding miRNA*s (57, 119). The mechanism by which the silencing RNA is incorporated as a single strand into the silencing complex is not fully known, but a model is emerging for siRNA incorporation into RISC, based primarily on biochemical studies in *Drosophila*: The asymmetry of an siRNA duplex is first sensed by the Dicer2–R2D2 heterodimer, wherein R2D2 is the dsRNA-binding-domain protein that partners with Dicer2. R2D2 binds the end of the duplex that is more stably paired, and Dicer binds the other end (130). Dicer2-R2D2 then loads the siRNA duplex into the Argonaute protein such that the guide strand of the siRNA directs Argonaute-catalyzed cleavage of the passenger strand (87a). Cleavage of the passenger strand facilitates its dissociation, thereby liberating the guide strand to pair to target transcripts and direct their cleavage. An analogous mechanism involving DCL1 and HYL1 might operate to load the plant miRNAs into AGO1 to form plant RISCs. Whether passenger-strand cleavage is important for plant miRNA RISC assembly is not known; cleavage-assisted loading is less important for siRNAs with mismatches to the passenger strand and appears to be bypassed altogether for miR-1 (87a), which has many mismatches to its miRNA* strand at its 5′ end. Even in this bypass scenario, the observation that plant miRNAs follow the asymmetry trends seen for siRNAs implies a function for DCL1 and HYL1 in RISC loading, which raises the question of whether plant RISC maturation occurs in the nucleus, the predominant site of DCL1 and HYL1

RNA-induced silencing complex (RISC): originally defined as the ribonucleoprotein complex that cleaves messenger RNAs during RNAi, RISC is now frequently used to refer to any silencing complex that includes an Argonaute protein and an siRNA or miRNA guide strand

proteins, or whether it occurs in the cytoplasm, where these proteins are less abundant but might still be present.

The final product of the miRNA/siRNA biogenesis pathway is a single-stranded RNA incorporated into a silencing complex (**Figure 1**). There are several varieties of these silencing complexes that vary at least partially in their composition and function; apart from the RISC mediating PTGS, a related silencing complex important for chromatin modification and TGS is typically referred to as an RNAi-induced transcriptional silencing (RITS) complex (134). A central component of all these silencing complexes is a member of the Argonaute protein family. Argonaute proteins, which have been implicated in a broad range of RNAi-related mechanisms, contain two conserved regions, the PAZ and Piwi domains (25). The PAZ domain appears to be an RNA-binding domain (75, 122, 144), and the Piwi domain has structural and functional similarity to RNase H enzymes (76, 123). Many organisms contain multiple members of the Argonaute family; in some cases, there is evidence for functional diversification of the different Argonautes. For example, only 1 of 4 mammalian Argonautes, Ago2, can mediate RNA cleavage in vitro (76). *Arabidopsis* contains 10 Argonaute proteins, 4 of which have been investigated genetically. AGO4 is involved in targeting some transposons and inverted-repeat transgenes for DNA methylation (149, 150). *PNH/ZLL/AGO10* and *ZIPPY/AGO7* are required for proper development (51, 83, 98, 99), but the mechanism by which they act is not known. AGO7 has been linked to the function of some ta-siRNAs, in that the *ago7* phenotype resembles that of *rdr6* and *sgs3*, two genes needed for ta-siRNA biogenesis (109, 133) (**Figure 1*b***), and levels of messages targeted by *TAS3* (but not *TAS1*) ta-siRNAs are elevated in *ago7* (3, 109, 133). *AGO1* is the only Argonaute gene known to be required for miRNA function in *Arabidopsis*. *Arabidopsis* AGO1 binds miRNAs and catalyzes target cleavage in vitro (16, 112), and *ago1* mutants have elevated levels of miRNA targets in vivo (131). A null allele of *AGO1* shows a sharp decrease in accumulation of most miRNAs compared to wild type, presumably because miRNAs are less stable before they enter the silencing complex than after (131).

Plant MicroRNA Expression

Transcription, processing, and RISC incorporation together determine mature miRNA levels found in a cell. Some miRNAs are among the most abundant RNAs; individual animal miRNAs are present at up to 10,000–50,000 copies per cell (74). Although the expression levels of plant miRNAs have not been similarly quantified, it is clear that many are abundantly expressed. Certain miRNAs have been cloned hundreds of times, and most are readily detectable by Northern blot (46, 113). More recently, microarray technology was adapted to rapidly survey expression profiles of plant miRNAs (7). Some miRNAs are broadly expressed, whereas others are expressed most strongly in particular organs or developmental stages (7, 115). More precise data on the localization of a few plant miRNAs has come from in situ hybridization (29, 54, 58) or from miRNA-responsive reporters (105). Little is known about the transcriptional or post-transcriptional regulation of miRNA expression, although expression patterns of miRNA promoter reporter constructs have been described for miR160 (136) and miR171 (105). Levels of several miRNAs are responsive to phytohormones or growth conditions; miR159 levels are enhanced by gibberellin (1), miR164 is transiently induced by certain auxin treatments (45), and miR393 levels are increased by a variety of stresses (127). The dependence of miR395 and miR399 levels on growth conditions is particularly striking. A regulator of sulfate-assimilation enzymes and sulfate transporters (2, 3, 53), miR395 is undetectable in plants grown on standard medium, but induced over 100-fold in sulfate-starved plants (53). Similarly, miR399 is strongly and

specifically induced in plants grown on low-phosphate medium (40a).

MECHANISMS OF MicroRNA FUNCTION

MicroRNA-Directed RNA Cleavage

Small silencing RNAs regulate gene expression by three basic mechanisms: RNA cleavage, translational repression, and transcriptional silencing. Directing target cleavage is the best-understood mode of action used by small RNAs to regulate gene expression. In this mechanism, the small silencing RNAs guide the Argonaute component of RISC to cleave ("slice") a single phosphodiester bond within complementary RNA molecules. The cleavage fragments are then released, freeing the RISC to recognize and cleave another transcript.

Plant miRNAs have been implicated in both target cleavage and translational repression. Lines of evidence indicating that plant miRNAs generally guide the cleavage of complementary or nearly complementary mRNAs are as follows: MicroRNA-guided slicer activity is present in wheat germ and *Arabidopsis* lysates (112, 128). Many miRNA targets are expressed at higher levels in plants that have impaired miRNA function as the result of mutations in the miRNA pathway (e.g., *hen1*, *ago1*, and *hyl1*) (23, 131, 132). Similarly, the expression of certain viral suppressors of RNA silencing causes overaccumulation of miRNA target messages (26, 28, 36, 55, 86), whereas overexpression of miRNAs can cause reduction of target messages (1, 40a, 45, 59, 103, 118, 136, 139). These results imply that miRNAs negatively regulate stability of their targets. Moreover, the 3′ cleavage products of many miRNA targets are detectable in vivo, either by Northern blot (55, 79, 84, 124) or 5′ RACE (3, 28, 53, 55, 79, 84, 85, 87, 103, 125, 132, 143). The fragments mapped by 5′ RACE correspond to cleavage between the target nucleotides that pair to nucleotides 10 and 11 of the miRNA—precisely the position expected for RISC-mediated cleavage (38, 79). The "slicer" activity guided by the miRNAs appears to reside in the Piwi domains of certain Argonaute proteins (76, 123), including *Arabidopsis* AGO1 (16, 112).

Additional Mechanisms of miRNA-Directed Repression

RISC-mediated cleavage does not explain all the repression attributed to silencing RNAs, particularly in animals, in which only a single endogenous target is known to be subject to this type of cleavage (145). The first miRNAs identified, the *lin-4* and *let-7* RNAs, regulate the expression of heterochronic genes that are critical for timing certain cell divisions in *C. elegans* larval development (67, 95, 114, 121, 138). The original experiments with *lin-4* RNA and two of its targets, *lin-14* and *lin-28*, indicated that *lin-4* RNA repressed the amount of target proteins without a substantial decrease in the amount of target mRNA, and it was generally thought that the same would be true for most metazoan miRNA targets, including *lin-41*, a target of *let-7* (102, 121, 138). However, a recent report describes substantial decreases in *lin-14*, *lin-28*, and *lin-41* mRNA levels, which could largely explain the repression previously reported at the protein level (8). These results from worms are consistent with previous cell-culture experiments showing that introducing a miRNA into mammalian cells can reduce the levels of ~100 mRNAs targeted by the miRNA (73). The nonextensive miRNA:target pairing and the nature of the target degradation fragments suggest that mRNA destabilization observed in worms and mammalian cell culture does not occur through the slicer mechanism (8, 73). Although these findings show that transcript destabilization plays more of a role in metazoan miRNA action than previously appreciated, evidence from reporter assays continues to implicate translational repression as a component of miRNA-directed repression, in that partial complementarity to silencing RNAs (or tethering Argonaute proteins to mRNAs

by some other means) can decrease protein output of mRNAs without corresponding decreases in message levels (34, 35, 110, 111).

What then might be the mechanism of silencing when the miRNA:target pairing is not sufficient to trigger efficient RISC-mediated cleavage? A potential clue came with the observation that Argonaute proteins and miRNA targets are localized to cytoplasmic foci known as Processing bodies (P bodies), which are sites for storage and degradation of mRNAs (77, 120). This suggests that miRNA binding directs the message to the P body, where it can be sequestered from the translation machinery and destabilized (77, 120). Whether this repression appears as translation repression or message destabilization or a combination of the two would depend on the message and its relative degradation kinetics outside the P body.

In plants, the degree of repression apart from RISC-mediated cleavage is unknown, but is likely less than that seen in animals. RISC-mediated cleavage is an important component of the repression for every plant target examined, as expected from the extensive complementarity between these targets and the plant miRNAs. The original experiments investigating repression of APETALA2 (AP2) showed that miR172 appears to affect accumulation of target protein but not that of target mRNA (6, 29). More recent experiments revealed that RISC-mediated mRNA cleavage represents a large component of miR172-directed AP2 repression, but that the *AP2* message remains relatively constant because of compensatory transcriptional activation triggered by lower AP2 protein (118). Nonetheless, in addition to RISC-catalyzed cleavage, animportant component of repression operates at the level of translation (or nacsient protein destabilization). The same could be true for many other established plant targets. Assessing the extent of miRNA-directed translational repression in plants awaits additional cases in which target proteins, rather than only target mRNAs, are monitored in response to changing miRNA levels.

Small RNA-Directed Transcriptional Silencing

Segments of transcriptionally silent DNA, known as heterochromatic regions, are associated with certain covalent modifications of DNA and histones. Evidence from several organisms has demonstrated that small RNAs are important for establishing and/or maintaining these heterochromatic modifications. In fission yeast, Dicer produces small RNAs corresponding to heterochromatic repeats (113), and deletion of Dicer or Argonaute disrupts heterochromatin silencing (135). This transcriptional repression involves the RITS complex, which, like RISC, contains Argonaute and a single-stranded Dicer-produced siRNA, as well as Chp1 and Tas3, proteins that are not thought to be present in RISC (96, 101, 134). Small RNAs also guide repressive modifications of DNA and histones in plants (reviewed in 88). For example, *AGO4* is required for siRNA-guided transcriptional silencing of the *SUPERMAN* gene and the maintenance of transcriptional repression triggered by inverted repeats (149, 150).

Do miRNAs guide transcriptional silencing in plants? Recent evidence raises the possibility that they might (10). Dominant mutations within the miR166 complementary sites of *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) mRNAs result in abnormal leaf development (90) that correlates with reduced miR166-guided mRNA cleavage (87). Curiously, these *phb* and *phv* mutations also correlate with reduced DNA methylation within the coding region of the mutant alleles (10). This reduced methylation occurs only in *cis*; in heterozygous plants, only the mutant copy of *PHB* is affected, whereas the wild-type copy is not (10). Because the miRNA complementary site in these mRNAs spans an exon junction, miR166 is presumably not able to interact with the genomic DNA, suggesting that interaction between miR166 and the nascent, but spliced, *PHB* mRNA somehow results in local DNA methylation (10). Although intriguing, the functional significance of this methylation

change is not yet clear. Whereas methylated promoter regions are often associated with transcriptional silencing (reviewed in 91) the observed methylation in *PHB* and *PHV* is near the 3′ end of the coding regions (10), and it is unknown what effect this methylation is having on *PHB* or *PHV* transcription. Further, it is not known if reduced miRNA complementarity generally correlates with reduced target gene methylation.

REGULATORY ROLES OF PLANT MicroRNAs

Identification of Plant MicroRNA Targets

The challenge in miRNA target prediction has been to capture most of the regulatory targets without bringing in too many false predictions. Progress has been made on this front in animals, particularly in the past year (24, 60, 70), but this was more than three years after the abundance of miRNAs in animals was first discovered. In contrast to the delay in animals, the high degree of complementarity between *Arabidopsis* miRNAs and their target mRNAs allowed the confident prediction of targets soon after the discovery of the plant miRNAs themselves (116), at a time when only three targets were known for animal miRNAs. The first clue to the general paradigm for miRNA target recognition in plants came from mapping miR171 to the genome. This miRNA has four matches in the *Arabidopsis* genome: one is located between protein-coding genes and has a predicted stem-loop structure, whereas the other three are all antisense to *SCARECROW-LIKE* (*SCL*) genes and lack stem-loop structures, leading to the idea that the intergenic locus produces a miRNA that guides the cleavage of the complementary *SCL* mRNAs (78, 115).

Although other *Arabidopsis* miRNAs are not perfectly complementary to mRNAs, most are nearly so. An initial genome-wide screen for miRNA targets identified mRNAs containing ungapped, antisense alignments to miRNAs with 0–3 mismatches, a level of complementarity highly unlikely to occur by chance (116). Using this cutoff, targets were predicted for 11 out of 13 miRNA families known at the time, comprising 49 target genes in total (116). For conserved miRNAs, more sensitive predictions allowing gaps and more mismatches can be made by identifying cases where homologous mRNAs in *Arabidopsis* and *Oryza* each have complementarity to the same miRNA family (53). Moreover, including EST information in addition to annotated genes has yielded additional targets, which include ta-siRNA precursors (3).

Because plant miRNAs affect stability of their targets, mRNA expression arrays can be used in genome-wide screens for miRNA targets. For example, expression array data showed that five mRNAs encoding TCP transcription factors are downregulated in plants overexpressing miR319 (103). Expression arrays may be especially useful in identifying miRNA targets that have been missed by bioinformatic approaches, i.e., targets with more degenerate or nonconserved complementarity that are nonetheless subject to miRNA-guided cleavage or destabilization (73). Such an experiment has been done for four plant miRNAs, carefully examining the expression profiles of plants overexpressing each miRNA (118). Perhaps surprisingly, no new direct targets were identified beyond those found previously through bioinformatics. Two new target candidates were found, but evidence for miRNA-guided cleavage of these targets was not detected by 5′ RACE in wild-type plants, suggesting that these mRNAs may only be cleaved in plants that ectopically express miRNAs (118).

The Scope of MicroRNA-Mediated Regulation in Plants

The observation that the expression-array experiment did not reveal new targets (118) suggests that most cleavage targets have already been found for known plant miRNAs. Nonetheless, other types of targets might be

missed both in the informatic predictions and in the array experiment. For example, targets repressed only at the level of translation would not be detected in the array experiment, and if such targets had less extensive complementary to the miRNAs, like that observed for many of the metazoan targets, they also would not be confidently predicted with the computational methods previously applied to plants. One approach for finding such targets would be to apply methods that have successfully detected metazoan targets above the noise of false predictions (24, 60, 70, 71). These methods search for conserved Watson-Crick complementarity between the 3′ UTRs and the 5′ seed region of the miRNA, a region that is also most important for miRNA target recognition in plants (87). However, applying the animal methods to plants has not yielded more predictions than expected by chance (M.W. Jones-Rhoades, unpublished data), suggesting that such animal-like target recognition is relatively rare, or might not even exist in plants, which again supports the notion that most targets for the known plant miRNAs have been found.

Many predicted miRNA targets encode regulatory proteins, suggesting that plant miRNAs are master regulators. The 21 miRNA families conserved in eudicots (*Arabidopsis* and *Populus*) have 95 confirmed or confidently predicted conserved targets in *Arabidopsis* (**Tables 2** and **3**). Sixty-five (68%) of these encode transcription factors, pointing to a role for miRNAs at the core of gene regulatory networks (**Table 2**). These transcription-factor targets have a remarkable propensity to be involved in developmental patterning or stem cell identity, leading to the proposal that many plant miRNAs function during differentiation to clear regulatory gene transcripts (116). Such miRNA-assisted reprogramming provides an attractive alternative to mechanisms in which regulatory genes have constitutatively unstable messages.

Among the nontranscriptionfactor targets (**Table 3**), six (6%) encode F-box proteins or ubiquitin-conjugating enzymes implicated in targeting selected proteins for proteasomal degradation, indicating a role for miRNAs in regulating protein stability. *DCL1* and *AGO1* are also miRNA targets, suggesting that plant miRNAs play a role in tuning their own biogenesis and function. Other conserved miRNA targets, such as ATP-sulfurylases, superoxide dismutases, and laccases have less clear regulatory roles, and although in vivo miRNA-mediated cleavage has been shown for many of these targets, the biological significance of their regulation by miRNAs is not yet known.

All 20 miRNA families that are conserved among *Arabidopsis*, *Populus*, and *Oryza* have complementary sites in target RNAs that also are conserved in all three species (**Tables 2** and **3**). Although these miRNAs may have additional nonconserved targets, this target-site conservation suggests that these miRNAs play similar roles in different plant species. Indeed, mutations in class III HD-ZIP target genes that reduce miR166 complementarity in *Arabidopsis* and maize confer similar phenotypes (54, 90, 116). However, the expansion of certain miRNA families and target classes in different species suggests that some of these families may have species-specific roles. For example, the miR397 family is complementary to 26 putative laccase mRNAs in *Populus*, whereas it has comparable complementarity to only three in *Arabidopsis*. Laccases are speculated to be involved in lignification (11), a process that may be more critical in woody plants such as *Populus* than in the herbaceous *Arabidopsis*.

Experimental Confirmation of Plant MicroRNA Targets

A growing number of plant miRNA targets predicted through bioinformatics have been experimentally confirmed. One means of confirmation uses *Agrobacterium* infiltration to observe miRNA-dependent cleavage of targets in *Nicotiana benthiama* leaves (55, 79). Another assays endogenous miRNA-mediated cleavage activity in wheat-germ lysate (84,

Table 2 Transcription-factor targets of plant miRNAs

miRNA family	Target family	*A.t.*	*O.s.*	*P.t.*	Confirmed targets	Confirmation method
miR156	SBP (116)	11	9	16	*SPL2*, *SPL3*, *SPL4*, *SPL10* (3, 28, 55, 133)	5′ RACE
miR159/319	MYB (107, 116)	8	6	5	*MYB33*, *MYB65* (1, 93, 103)	5′ RACE, Agro-infiltration, miRNA-resistant target
miR159/319	TCP (103)	5	4	7	*TCP2*, *TCP3*, *TCP4*, *TCP10*, *TCP24* (103)	5′ RACE, miRNA-resistant target
miR160	ARF (116)	3	5	9	*ARF10*, *ARF16*, *ARF17* (3, 55, 84, 136)	5′ RACE, in vitro cleavage, Agro infiltration, miRNA-resistant target
miR164	NAC (116)	6	6	6	*CUC1*, *CUC2*, *NAC1*, *At5g07680*, *At5g61430* (45, 55, 65, 85)	5′ RACE, in vitro cleavage, Agro infiltration, miRNA-resistant target
miR166	HD-ZIPIII (116)	5	4	9	*PHB*, *PHV*, *REV*, *ATHB-8*, *ATHB-15* (39, 59, 87, 128, 139)	5′ RACE, in vitro cleavage, miRNA-resistant target
miR167	ARF (107, 116)	2	4	7	*ARF6*, *ARF8* (3, 55)	5′ RACE
miR169	HAP2 (116)	8	7	9	*At1g17590*, *At1g72830*, *At1g54160*, *At3g05690*, *At5g06510* (53)	5′ RACE
miR171	SCL (78, 115)	3	5	9	*SCL6-III*, *SCL6-IV* (55, 79)	5′ RACE, Agro infiltration
miR172	AP2 (107)	6	5	6	*AP2*, *TOE1*, *TOE2*, *TOE3* (6, 29, 55)	5′ RACE, miRNA-resistant target
miR393	bZIP* (53)	1	1	1	*At1g27340* (53)	5′ RACE
miR396	GRF (53)	7	9	9	*GRL1*, *GRL2*, *GRL3*, *GRL7*, *GRL8*, *GRL9* (53)	5′ RACE
miR444	MADS (125)	0	1	0	*Os02g49840* (125)	5′ RACE
Total		65	66	93		

Predicted and confirmed targets of *Arabidopsis* miRNAs that encode known or suspected transcription factors are listed. For each target family, the number of predicted target genes in each of three plant species with sequenced genomes (*A.t.*, *Arabidopsis thaliana*; *O.s.*, *Oryza sativa*; *P.t.*, *Populus trichocarpa*) is indicated. To be counted, a potential target must contain a complementary site to at least one member of the indicated miRNA family with a score of 3 or less (as described in 53), with the exception of the bZIP family (marked with an asterisk), for which some targets with more relaxed complementarity were included. Abbreviations: SBP, SQUAMOSA-promoter binding protein; ARF, AUXIN RESPONSE FACTOR; SCL, SCARECROW-LIKE; GRF, GROWTH REGULATING FACTOR.

87, 128). Perhaps the most useful method of miRNA target validation uses 5′ RACE to detect in vivo products of miRNA-mediated cleavage (3, 28, 53, 55, 79, 84, 85, 87, 103, 125, 132, 143). An adaptor oligonucleotide is ligated to the 5′ end of the uncapped 3′ portion of a cleaved miRNA target, followed by reverse transcription and PCR with a gene-specific primer (79). Sequencing the resulting PCR product reveals the precise position of target cleavage, expected to be between nucleotides that pair to positions 10 and 11 of the miRNA (38). This method is particularly useful because, unlike the infiltration or in vitro methods, 5′ RACE detects miRNA-directed mRNA cleavages that occur endogenously in the plant, a necessary prerequisite for biological relevance. Analysis of the biological significance of the miRNA-mediated regulation of that target can be explored by using reverse genetic approaches. As summarized below, these approaches are revealing the in vivo relevance of a growing number of miRNA-target interactions.

Table 3 Non-transcription-factor targets of plant miRNAs

miRNA family	Target family	*A.t.*	*O.s.*	*P.t.*	Confirmed targets	Confirmation method
miR161	PPR (116)	9	0	0	*At1g06580* (4, 133)	5′ RACE
miR162	Dicer (143)	1	1	1	*DCL1* (143)	5′ RACE
miR163	SAMT (107, 116)	5	0	0	*At1g66690*, *At1g66700*, *At1g66720*, *At3g44860* (4)	5′ RACE
miR168	ARGONAUTE (116)	1	6	2	*AGO1* (131, 133)	5′ RACE, miRNA-resistant target
miR173	ta-siRNA (3)	4	0	0	*TAS1a*, *TAS1b*, *TAS1c*, *TAS2* (3)	5′ RACE
miR390	ta-siRNA (3)	1	2	3	*TAS3* (3)	5′ RACE
miR390	receptor-like kinase (125)	4	15	21	*Os02g10100* (125)	5′ RACE
miR393	F-box (53, 127)	4	2	5	*TIR1*, *ABF1*, *ABF2*, *ABF3*, *At3g23690* (53)	5′ RACE
miR394	F-box (53)	1	1	2	*At1g27340* (53)	5′ RACE
miR395	APS (53)	3	1	2	*APS1*, *APS4* (53)	5′ RACE
miR395	SO_2 transporter (2)	1	2	3	*AST68* (3)	5′ RACE
miR396	Rhodenase (53)	1	1	1		
miR397	Laccase (53, 127)	3	15	26	*At2g29130*, *At2g38080*, *At5g60020* (53)	5′ RACE
miR398	CSD* (53)	2	2	2	*CSD1*, *CSD2* (53)	5′ RACE
miR398	CytC oxidase(53, 127)	1	1	0	*At3g15640* (53)	5′ RACE
miR399	PO_4 transporter (53)	1	4	4		
miR399	E2-UBC (127)	1	1	2	*At2g33770* (3)	5′ RACE
miR403	ARGONAUTE (3)	1	0	1	*AGO2* (3)	5′ RACE
miR408	Laccase (118)	3	2	3	*At2g30210* (81, 118)	5′ RACE
miR408	Plantacyanin (127)	1	3	1	*Os03g15340* (125)	5′ RACE
miR436	Unknown (125)	0	1	0	*Os12g42390* (125)	5′ RACE
miR447	2-PGK (3)	1	0	0	*At5g60760* (3)	5′ RACE
miR475	PPR (81)	0	0	24	4 PPR genes (81)	5′ RACE
miR476	PPR (81)	0	0	20	1 PPR gene (81)	5′ RACE
Total		49	60	123		

Predicted and confirmed targets of *Arabidopsis* miRNAs that do not encode known or suspected transcription factors are listed. For each target family, the number of predicted target genes in each of three plant species with sequenced genomes (*A.t.*, *Arabidopsis thaliana*; *O.s.*, *Oryza sativa*; *P.t.*, *Populus trichocarpa*) is indicated. To be counted, a potential target must contain a complementary site to at least one member of the indicated miRNA family with a score of 3 or less (as described in 53), with the exception of CSD (marked with an asterisk), for which some targets with more relaxed complementarity were included. Nonconfirmed target families are listed only if they score well in all three species Abbreviations: SAMT, SAM-dependant methyl transferase; APS, ATP-sulfurylase; CSD, COPPER SUPEROXIDE DISMUTASE; E2-UBC, E2 ubiquitin-conjugating protein; ta-siRNA, *trans*-acting short interfering RNA; 2-PGK, 2-phophoglycerate kinase.

Regulatory Roles of Plant MicroRNAs

The first evidence that small RNAs play roles in plant development came from mutants impaired in small RNA biogenesis or function. Indeed, several genes central to miRNA function, including *DCL1*, *AGO1*, *HEN1*, and *HYL1*, were first identified in plants based on the developmental consequences of their mutations even before they were known to be

important for small RNA biogenesis or function. Multiple groups isolated *dcl1* mutants; the most severe mutations result in early embryonic arrest, and even partial loss-of-function mutants result in pleiotropic defects, including abnormalities in floral organogenesis, leaf morphology, and axillary meristem initiation (reviewed in 117). *ago1*, *hen1*, *hyl1*, and *hst* mutants all have pleiotropic developmental defects that overlap with those of hypomorphic *dcl1* plants (20, 30, 80, 94, 129). In addition, plants that express certain viral inhibitors of small RNA processing or function, such as HC-Pro and P19, exhibit developmental defects reminiscent of *dcl1* mutants (26, 28, 36, 55, 86). Although many or all of these developmental defects may result from impaired miRNA activity, they may also reflect disruption of other pathways in which these genes act, such as in the generation and function of siRNAs. However, in contrast to mutations in genes needed for miRNA biogenesis, mutations in genes required for the accumulation of various siRNAs, such as *AGO4*, *RDR6*, *DCL2*, *DCL3*, and *DCL4* (**Figure 1*b***), result in few, if any, developmental abnormalities (31, 41, 97, 132, 141a, 142, 146a 149), with the only severe abnormalities appearing stochastically after several generations, consistent with the loss of epigenetic modifications (41). This distinction suggests that disrupting miRNA-based regulation has more severe immediate consequences than does disrupting siRNA-based regulation.

Mutations that impair a fundamental step in miRNA biogenesis result in misregulation of numerous miRNA targets (23, 132), making it difficult to assign the observed phenotypes to any particular miRNA family. Fortunately, the ease with which transgenic *Arabidopsis* can be generated has allowed investigation of particular miRNA/target interactions through two reverse genetic strategies. The first is to make transgenic plants that overexpress a miRNA, typically under the control of the strong 35S promoter (**Table 4**; **Figure 3**). This approach can potentially downregulate all mRNAs targeted by the overexpressed miRNA. The second strategy is to make transgenic plants that express a miRNA-resistant version of a miRNA target, in which silent mutations have been introduced into the miRNA complementary site that disrupt miRNA-mediated regulation without altering the encoded protein product (**Table 5**; **Figure 4**). For seven miRNA families that have been investigated in vivo by these strategies, perturbing miRNA-mediated regulation results in abnormal development. Taken together, these studies confirm that miRNAs are key regulators of many facets of *Arabidopsis* development.

Table 4 Consequences of miRNA overexpression

miRNA	Target family	Consequences of overexpression
miR156	SPL transcription factors	Increased leaf initiation, decreased apical dominance, delayed flowering time (118)
miR159	MYB transcription factors	Male sterility, delayed flowering time (1)
miR319	TCP transcription factors	Uneven leaf shape and curvature, late flowering (103)
miR160	ARF transcription factors	Agravitropic roots with disorganized root caps, increased lateral rooting (136)
miR164	NAC domain transcription factors	Organ fusion (65, 85), reduced lateral rooting (45)
miR166	HD-ZIP transcription factors	Seedling arrest, fasciated apical meristems, female sterility (59, 139)
miR172	AP2-like transcription factors	Early flowering, lack of petals, transformation of sepals to carpels (6, 29)
miR399	Ubiquitin-conjugating enzyme	Phosphate accumulation (40a)

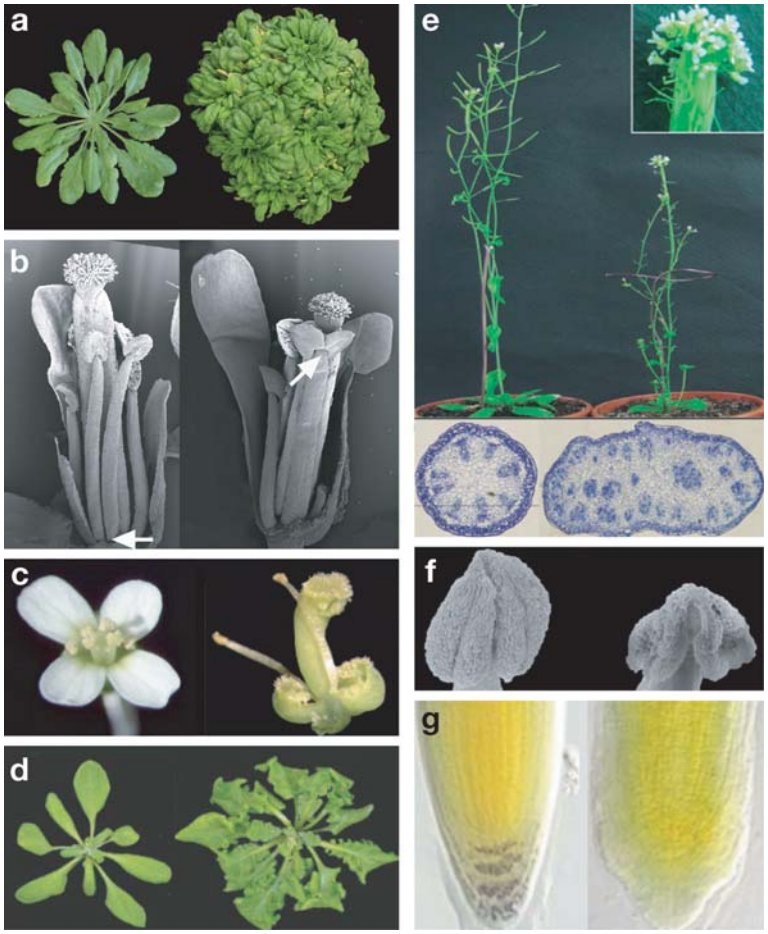


Figure 3

A sampling of phenotypes resulting from microRNA (miRNA) overexpression in *Arabidopsis*. Each panel depicts wild type (*left*) compared to a corresponding specimen from a miRNA-overexpressing plant (*right*). (*a*) miR156-overexpressing plants have increased leaf initiation and decreased apical dominance, resulting in dramatically bushier plants. Images reprinted from Reference 118, copyright 2005 by Elsevier. (*b*) The stamens of miR164-overexpressing plants are frequently fused together rather than distinct (*arrows* denote point of stamen separation). This phenotype is similar to that of loss-of-function mutants in miR164 targets *CUC1* and *CUC2*. Images reprinted from Reference 85, copyright 2004 by Elsevier. (*c*) The outer floral organ whorls of miR172-overexpressing flowers are transformed into carpeloid tissue rather than having four sepals and four petals. This phenotype is similar to that of loss-of-function mutants in miR172 target *AP2*. Images reprinted with permission from Reference 29, copyright 2004, AAAS. (*d*) The *jaw-D* mutant phenotype, which includes severely affected leaf morphology, results from miR319 overexpression (103). Images copyright by Nature Publishing Group, used with permission. (*e*) Plants overexpressing miR166 have decreased stature and fertility (*top*) and fascinated, enlarged inflorescence stems (*inset*) with highly abnormal vasculature (*bottom*) (59). Images copyright by Blackwell Publishing, used with permission. (*f*) miR159a-overexpressing plants have reduced male fertility and altered anther morphology. Images reprinted from Reference 118, copyright 2005 by Elsevier. (*g*) miR160-overexpressing plants have disorganized root tips and fewer starch granules (*purple staining*) (136). Images copyright by American Society of Plant Biologists, used with permission.

Table 5 Consequences of disrupting miRNA-mediated regulation of specific targets

miRNA family or subfamily	miRNA-resistant target	Promoter	Phenotype
miR159	*MYB33*	35S	Upwardly curled leaves (103)
miR159	*MYB33*	Endogenous	Upwardly curled leaves, reduced stature, shortened petioles (93)
miR319	*TCP4*	Endogenous and 35S	Arrested seedlings, fused cotyledons, lack of SAM (103)
miR319	*TCP2*	35S	Long hypocotyls, reduced stature, and apical dominance (103)
miR160	*ARF16*	Endogenous and 35S	Fewer lateral roots, small plants with reduced fertility, increased basal expression of auxin-induced genes (136)
miR160	*ARF17*	Endogenous	Extra and lobed cotyledons, short roots with decreased branching, small plants with reduced fertility, altered basal expression of auxin-induced genes (84)
miR164	*CUC1*	Endogenous	Short petioles, aberrant leaf shape, extra petals, missing sepals (85)
miR164	*CUC2*	Inducible and 35S	Aberrant leaf shape, extra petals, increased sepal separation (65)
miR164	*NAC1*	35S	Increased number of lateral roots (45)
miR166	*REV*	Endogenous	Radialized vasculature, strands of leaf tissue attached to stem (39)
miR166	*PHB*	35S	Adaxialized leaves, ectopic meristems (87)
miR168	*AGO1*	Endogenous	Curled leaves, disorganized phyllotaxy, reduced fertility (131)
miR172	*AP2*	35S	Late flowering, excess of petals and stamens (29)
miR399	*UBC*	35S	Reduced response to low phosphate (40a)

One of the best-studied families of miRNA targets is the class III HD-ZIP transcription factor family. The importance of miR166 for the proper regulation of this gene class is underscored by the large number of dominant gain-of-function alleles that map to the miR166 complementary sites of HD-ZIP mRNAs (39, 54, 89, 90, 148). Dominant *phb* and *phv* mutations adaxialize leaves and overaccumulate *phb* or *phv* mRNA (89, 90), whereas dominant *rev* mutations result in radialized vasculature (39, 148). Similarly, mutations within the miR166 complementary site of the maize HD-ZIP gene *RLD1* adaxialize leaf primordia and cause overaccumulation of *rld1* mRNA (54). All of these gain-of-function HD-ZIP mutations isolated through forward genetics change the amino acid sequence of the conserved START domain. Before the discovery of miR166 it was hypothesized that the HD-ZIP mutant phenotypes resulted from the loss of negative regulatory interaction mediated by the START domain (90), but after the discovery of miR166 and realization that the mutations map to the miRNA complementary site, it was hypothesized that the phenotypes resulted from the loss of miRNA-directed repression (116). Transgenic plants expressing an miR166-resistant version of *PHB*, *PHV*, or *REV* with unaltered coding potential resemble the respective gain-of-function mutants, whereas transgenic plants containing additional wild-type copies of these genes have essentially wild-type phenotypes (39, 87) (**Figure 4*b***). This demonstrates that changing the RNA sequence, rather than the amino acid sequence, is sufficient to account for the developmental abnormalities observed in HD-ZIP gain-of-function mutants, indicating that the disrupted regulatory interaction is mediated at the RNA level rather than via the encoded protein.

miR172-mediated regulation of *APETALA2* (*AP2*) and related *AP2*-like genes is needed for proper specification of organs during flower development (6, 29). Plants that overexpress miR172 have floral defects that resemble *ap2* loss-of-function mutants, such as the absence of petals and

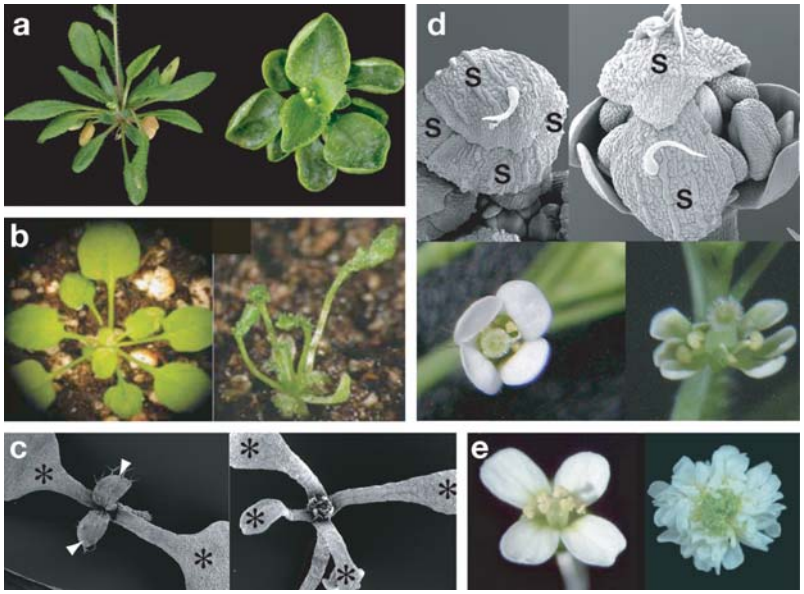


Figure 4

Phenotypes of transgenic *Arabidopsis* expressing miRNA-resistant targets. Each panel depicts a control plant, either wild type or a plant expressing a miRNA-sensitive transgene (*left*), compared to a plant expressing a miRNA-resistant transgene (*right*). (*a*) Plants expressing miR159-resistant *MYB33* have reduced stature and upwardly curled leaves (93). Images copyright of American Society of Plant Biologists, used with permission. (*b*) Plants expressing miR166-resistant *PHB* have radialized, reduced leaves with adaxial characteristics all around the circumference of the leaf (87). Images copyright of EMBO, used with permission. (*c*) Seedlings expressing a wild-type *ARF17* transgene have two cotyledons (*asterisks*) and two emerging true leaves (*arrowheads*), whereas miR160-resistant *ARF17* seedlings have up to four cotyledons, with a leaf emerging between each pair of cotyledons (84). Images copyright of American Society of Plant Biologists, used with permission. (*d*) Flowers expressing a wild-type *CUC1* transgene have the expected four sepals (*S*, *top*) and four petals (*bottom*), whereas flowers expressing a miR164-resistant *CUC1* transgene often display two sepals (*top*) and six petals (*bottom*). Images reprinted from Reference 85, copyright 2005 by Elsevier. (*e*) miR172-resistant *AP2* transgenic flowers have variable numbers of floral organs; this flower has numerous petals and lacks inner whorl organs. Images reprinted with permission from Reference 29, copyright 2004, AAAS.

sepal transformation into carpels (6, 29) (**Figure 3*c***). As described above, these plants provide evidence that plant miRNAs can direct translation repression in addition to mRNA cleavage (6, 29, 118).

Although most miRNA families appear to target a single class of targets, the miR159/319 family regulates both MYB and TCP transcription factors (103, 116). Overexpression of miR319, which specifically downregulates *TCP* mRNAs, results in plants with uneven leaf shape and delayed flowering time (103) (**Figure 3*d***). Expression of miR319-resistant *TCP4* results in aberrant seedlings that arrest with fused cotyledons and without forming apical meristems (103). Overexpression of miR159a specifically reduces *MYB* mRNA accumulation and results in male sterility (1, 118) (**Figure 3*f***), whereas plants that express miR159-resistant *MYB33* have upwardly curled leaves, reduced stature, and shortened petioles (93, 103) (**Figure 4*a***).

Thus, miR159a and miR319, which differ at only three nucleotides, are related miRNAs that can target unrelated mRNAs (103).

The signaling pathway that mediates responses to the phytohormone auxin is particularly densely packed with miRNA regulation. For example, miR393 targets mRNAs encoding TIR1 and three closely related F-box proteins (53, 127). These F-box proteins are auxin receptors (32, 33, 56) that target short-lived repressors of ARF transcriptional activators for ubiquitin-mediated degradation in response to auxin. Intriguingly, not only the receptors, but also at least seven of the 23 *Arabidopsis ARF* mRNAs are either directly or indirectly subject to miRNA-mediated regulation (**Table 2**). miR167 targets *ARF6* and *ARF8*; miR160 targets *ARF10*, *ARF16*, and *ARF17* (55, 116), and miR390 directs cleavage of *TAS3*, leading to the production of ta-siRNAs that target *ARF3* and *ARF4* mRNAs (3). Freeing *ARF17* or *ARF16* from miR160 regulation results in dramatic morphological changes and alters basal levels of auxin-induced transcripts (84, 136) (**Figure 4*c***). The deep conservation of some of these miRNAs (miR160 and miR390 are detectable in moss, one of the most evolutionarily basal land plants) implies that miRNAs have been modulating auxin signaling since very early in the development of multicellularity in plants (7). Beyond influencing auxin signaling, miRNAs are likely to aid in signal integration. For example, miR164 targets *CUC1*, *CUC2*, and *NAC1*, and perturbing this regulation disrupts root, leaf, and flower development (45, 65, 85) (**Figures 3*b*** and **4*d***).

In addition to the miRNAs that target transcription factors, two miRNA families target genes central to miRNA biogenesis and function; miR162 targets *DCL1* (143), and miR168 targets *AGO1* (116, 131). Moreover, although the biological and biochemical roles of *Arabidopsis* AGO2 are not known, it is intriguing that *AGO2* mRNA is targeted by miR403 (3). miRNA targeting of *DCL1* and *AGO1* suggests a feedback mechanism whereby miRNAs negatively regulate their own activity. Curiously, although plants expressing miR168-resistant *AGO1* overaccumulate *AGO1* mRNA as expected, they also overaccumulate numerous other miRNA targets and exhibit developmental defects that overlap with those of *dcl1*, *hen1*, and *hyl1* loss-of-function mutants (131). This suggests that a large overabundance of AGO1 inhibits, rather than promotes, RISC activity (131). In any case, the fact that miR162 and miR168 family members also target *DCL* and *AGO* family members in *Oryza* and *Populus* (**Table 3**) suggests an important in vivo role for this regulation.

Protein-coding messages are not the only targets of plant miRNAs. At least two miRNAs, miR173 and miR390, target precursors of a special class of siRNAs, the trans-acting siRNAs (ta-siRNAs) (3). Unlike most siRNAs, which target loci closely related to the loci encoding them, ta-siRNAs direct cleavage of targets encoded at distinct loci (109, 133), thereby acting similarly to plant miRNAs (reviewed in 12). ta-siRNAs are encoded by *TAS* genes (**Figure 1*b***). The miRNA complementarity sites in *TAS* transcripts are in register with the 21-nucleotide ta-siRNAs derived from the locus (3), suggesting a model in which *TAS* transcripts undergo miRNA-directed cleavage prior to reverse transcription by the RDR6 RNA-dependent RNA polymerase, producing a double-stranded RNA that is processed by DCL4 into 21-nucleotide siRNAs (3, 41, 109, 133, 141a, 146a). Some of these siRNAs go on to direct cleavage of target mRNAs, including those encoding a subset of ARF transcription factors and several proteins of unknown function (3, 109, 133).

MicroRNAs: PLANTS VERSUS ANIMALS

As understanding of miRNA genomics and function in plants and animals has grown, so has the realization that there are numerous differences between the kingdoms in the ways

miRNAs are generated and carry out their regulatory roles. Indeed, the evolutionary relationship between plant and animal miRNAs is unclear. Did the last common ancestor of plants and animals possess miRNAs from which modern miRNAs are descended, or did the plant and animal lineages independently adapt conserved RNAi machinery (including Dicer and Argonaute) to use endogenously expressed stem-loop RNAs as transregulators of other genes? Although miRNAs are deeply conserved within each kingdom (7, 40, 108), no miRNA family is known to be conserved between kingdoms. Moreover, there are several kingdom-specific differences in miRNA biogenesis. For example, the stem-loop precursors of plant miRNAs are markedly longer and more variable than their animal counterparts (115). Furthermore, the cellular localization of miRNA processing appears to differ between plant miRNAs, which are entirely processed within the nucleus (104, 106, 142), and animal miRNAs, which are sequentially processed in the nucleus and cytoplasm (68). Perhaps most interestingly, the scope and mode of regulation carried out by miRNAs appears to be drastically different between the two kingdoms. Most plant miRNAs guide the cleavage of target mRNAs (53, 55, 79, 128), and the predicted targets of *Arabidopsis* miRNAs, which comprise less than 1% of protein-coding genes, are highly biased toward transcription factors and other regulatory genes (53, 116). Although at least some animal miRNAs guide cleavage of endogenous targets (145), most appear to act through other mechanisms. Furthermore, the analysis of conserved, reverse-complementary matches to the 5′ seed regions of animal miRNAs suggests that a large percentage (20–30% or more) of animal protein-coding genes are conserved miRNA targets (24, 60, 70, 140). With these striking differences in biogenesis and function, we speculate that miRNAs arose at least twice, once in early plants and once in early animals. Perhaps in both lineages, and each in its unique way, the availability of this post-transcriptional layer of gene regulation enabled the emergence of more robust and specialized gene expression programs, thereby facilitating the emergence of the many cell types and developmental programs needed to build a complex plant or animal.

SUMMARY POINTS

1. MicroRNAs are endogenously expressed, ~21-nucleotide RNAs that do not encode proteins. A miRNA is initially expressed as a precursor RNA containing an imperfect stem-loop, from which a miRNA/miRNA* duplex is excised by DCL1. The miRNA strand of this duplex is subsequently incorporated into a silencing complex, where it guides targeting of complementary RNAs.
2. Plant miRNA genes are generally not located within protein-coding genes but comprise their own RNA polymerase II-dependent transcriptional units.
3. Plant miRNAs occur in gene families, each family contains 1–32 loci within a single genome, each potentially encoding identical or nearly identical mature miRNAs. At least 20 miRNA families are broadly conserved in flowering plants, and many are conserved to more deeply diverged plant lineages. In addition, there are an unknown number of lineage- and species-specific miRNA families.
4. Plant miRNAs primarily have been discovered through direct cloning and sequencing of small cellular RNAs or through comparative genomics. Only a handful of mutations at miRNA loci have been identified in genetic screens.

5. Nearly all plant miRNAs are highly complementary to target mRNAs, which the miRNAs repress through directed RNA cleavage and perhaps other mechanisms. The majority of known plant miRNA targets encode transcription factors or other regulatory proteins, such as components of the ubiquitin and RNAi pathways. Some miRNAs guide the cleavage of ta-siRNA precursors.
6. Bioinformatic approaches have identified targets for nearly all plant miRNAs. Several experimental methods have been used to confirm miRNA-target interactions and explore the biological significance of miRNA-mediated regulation.
7. Plant miRNAs are high-level regulators of gene expression that affect numerous aspects of plant biology, especially developmental patterning. Mutants impaired in miRNA biogenesis exhibit severe, pleiotropic abnormalities, and plants that overexpress particular miRNAs or express miRNA-resistant versions of particular miRNA targets exhibit a wide array of unusual phenotypes.

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