MicroRNAs: At the Root of Plant Development?[1]

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Although most genes use RNA in the form of mRNA as a coding intermediate for protein production, there are many genes whose final products are RNA. These noncoding RNAs range from the familiar transfer and ribosomal RNAs to the more recently discovered regulatory RNAs. One type of regulatory RNA was first discovered during the study of nematode larval development. Two approximately 22-nucleotide (nt) RNAs (the *lin-4* and *let-7* RNAs) control developmental timing by binding to their respective mRNA targets and preventing productive use of these messages, perhaps by attenuating translation (Lee et al., 1993; Pasquinelli and Ruvkun, 2002). The *let-7* RNA was found broadly throughout bilateral animals, including humans, suggesting that these two riboregulators were more than oddities of worm larval development (Pasquinelli et al., 2000). In 2001, it was discovered that these two RNAs are members of a large class of 21- to 24-nt noncoding RNAs, called microRNAs (miRNAs), found in nematodes, fruitflies (*Drosophila melanogaster*), and humans (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Recent computational and molecular analyses indicate that humans have over 200 miRNA genes, nearly all of which are conserved in mice, and at least 80% of which are conserved in fish (Lim et al., 2003a). Although the functions of nearly all metazoan miRNAs are unknown, their abundance and evolutionary conservation, together with the analogy to the two founding nematode miRNAs, which are posttranscriptional gene regulators, suggests that an important mode of metazoan gene regulation had gone virtually undetected until just recently.

miRNAs were also ripe for discovery in plants. In mid-2002, four groups reported RNAs with miRNA characteristics among the tiny RNAs present in Arabidopsis (Llave et al., 2002a; Mette et al., 2002; Park et al., 2002; Reinhart et al., 2002). One difference between plant and animal miRNAs is that the regulatory targets of plant miRNAs can be convincingly predicted simply by identifying mRNAs with near-perfect complementarity (Rhoades et al., 2002). Evolutionary conservation of the miRNA:mRNA pairing in Arabidopsis and rice (*Oryza sativa*), together with experimental evidence showing that miRNAs can direct the cleavage of the targeted mRNAs, supports the validity of these predictions (Llave et al., 2002a, 2002b; Park et al., 2002; Reinhart et al., 2002; Rhoades et al., 2002; Kasschau et al., 2003; Tang et al., 2003). With this ability to confidently predict regulatory targets for plant miRNAs, there is already an arguably broader understanding of the regulatory roles and biochemical actions of miRNAs in plants than in animals, despite the fact that the first plant miRNAs were reported less than a year ago.

miRNAs: SIMILAR TO BUT DISTINCT FROM siRNAs

Not all endogenous tiny RNAs are miRNAs. Understanding miRNA biogenesis and function has been greatly facilitated by analogy and contrast to a related class of tiny RNAs known as short (or small) interfering RNAs (siRNAs), first identified because of their association with posttranscriptional gene silencing (PTGS) in plants (Hamilton and Baulcombe, 1999). During the animal PTGS-like phenomenon, known as RNAi (for review, see Hutvágner and Zamore, 2002b), long double-stranded RNA is processed by Dicer, an RNase III enzyme, into many siRNAs. Although these siRNAs are initially short double-stranded species with 5′ phosphates and 2-nt 3′ overhangs characteristic of RNase III cleavage products, they eventually become incorporated as single-stranded RNAs into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC; Fig. 1B; Carmell et al., 2002; Hutvágner and Zamore, 2002b; Martinez et al., 2002; Schwarz and Zamore, 2002). The RISC identifies target messages based on perfect (or nearly perfect) antisense complementarity between the siRNA and the mRNA, and then a RISC endonuclease cleaves the mRNA near the middle of the siRNA complementarity region (Hutvágner and Zamore, 2002b). Similar pathways have been proposed for gene silencing in plants and fungi, with PTGS-associated siRNAs directing mRNA cleavage (Vaucheret et al., 2001) and heterochromatic siRNAs targeting chromatin for histone methylation, trigger-

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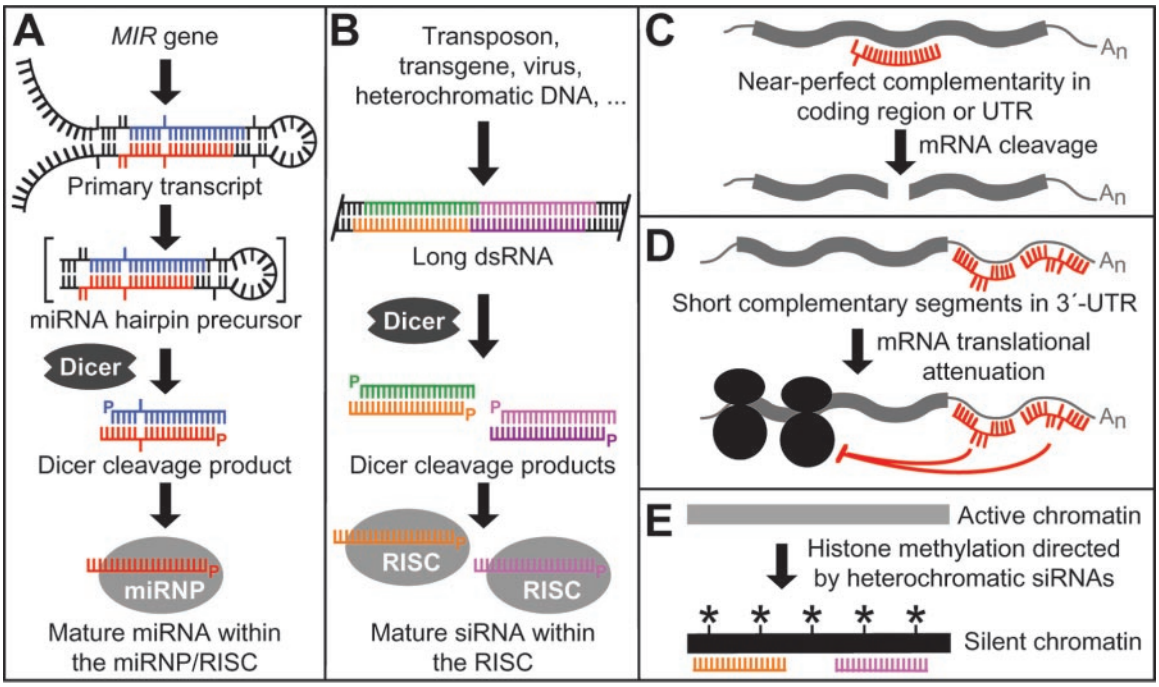

Figure 1. Current models for the biogenesis and possible roles of miRNAs and siRNAs. See text for references. A, The portion of the primary transcript that contains the miRNA sequence (red) resides on one arm of a predicted stem-loop precursor structure. The transcription start and stop sites for miRNA primary transcripts have not yet been defined. In animals, the hairpin precursor (in brackets) is processed from the primary transcript, but such intermediates have not been detected in plants. Either the primary transcript or this processed hairpin is cleaved by Dicer to yield paired approximately 21-nt RNAs with 2-nt 3′ overhangs, 5′ phosphates, and 3′ hydroxyls. One strand of this short-lived double-stranded intermediate accumulates as the mature miRNA (in red), which acts as a guide RNA within the miRNP/RISC complex. B, Long dsRNA is processed into many different siRNA species. siRNAs from both strands of the precursor accumulate within RISC complexes. C, The near perfect pairing between many plant miRNAs and their mRNA targets directs the RISC to cleave the target near the center of the complementarity site. This is also the classical mode of action for siRNAs during RNAi. D, Characterized animal miRNAs appear to recognize multiple sites in the 3′-untranslated region (UTR) of target mRNAs. Because they bind to their targets with numerous mismatches, the miRNP/RISC does not cleave the message. Although the message levels remain constant, protein levels decrease, perhaps from translational attenuation. Whether any plant miRNAs act via this mechanism is not known. E, Some endogenous siRNAs, known as heterochromatic siRNAs, are thought to direct histone methylation, which is correlated with transcriptional silencing of the modified regions. Many of the non-miRNA small RNAs that have been cloned from Arabidopsis might act similarly.

ing heterochromatin formation, and consequent transcriptional gene silencing (Allshire, 2002; Fig. 1E).

Like siRNAs, miRNAs are processed by Dicer and thus are the same length and possess 5′-phosphate and 3′-hydroxyl termini (Hutvágner and Zamore, 2002b). The miRNAs are also incorporated into a ribonucleoprotein complex, known as the miRNP, which is similar, if not identical, to the RISC (Hutvágner and Zamore, 2002a; Mourelatos et al., 2002). As discussed below, miRNAs can also direct the cleavage of their mRNA targets, as if they were functioning as siRNAs within the RISC complex. Despite these chemical, biochemical, and mechanistic similarities to siRNAs, there are key differences between miRNAs and siRNAs in origin (Fig. 1, A and B) and evolutionary conservation: (a) miRNAs derive from genomic loci distinct from other recognized genes, whereas siRNAs derive from mRNAs, transposons, viruses, or heterochromatic DNA. (b) miRNAs are processed from transcripts that can form local RNA hairpin precursor structures, whereas siRNAs are processed from long bimolecular RNA duplexes or extended hairpins. (c) a single miRNA molecule ultimately accumulates from one arm of each miRNA hairpin precursor molecule, whereas many different siRNAs accumulate from both strands of siRNA precursors. (d) miRNA sequences are nearly always conserved in related organisms, whereas siRNA sequences are rarely conserved in related organisms. These differences have been used to develop practical guidelines for distinguishing and annotating newly discovered miRNAs (Ambros et al., 2003).

Although there is still much to learn about miRNAs and siRNAs, their differences can be rationalized by the following functional distinction: siRNAs mediate the silencing of the same (or very similar) genes from which they originate, whereas miRNAs are encoded by their own genes and regulate different genes. This idea readily explains the greater evolutionary conservation of miRNAs compared with siRNAs. Because

siRNAs come from the genes that they target, a mutational event that changes the sequence of the siRNA would also change the sequence of its regulatory target such that siRNA regulation would be maintained. In contrast, a mutation in a miRNA would rarely be accompanied by simultaneous compensatory changes at the loci of its targets, and thus selection pressure would preserve the miRNA sequence.

miRNAs IN PLANTS

Biochemical approaches for identifying small RNAs involve ligation of size-selected RNAs to adaptors, reverse transcription, PCR amplification, concatamerization, and sequencing (Elbashir et al., 2001). When variations of this technique were applied to plants, only a small fraction of the hundreds of small RNA species thus identified turned out to be miRNAs. For example, Llave et al. (2002a) identified 125 small RNAs between 16 and 25 nts; only four of these meet the definition (Ambros et al., 2003) of miRNAs (Table I). Similarly, Park et al. (2002) identified 230 unique sequences of which five appear to be miRNAs (Table I). Reinhart et al. (2002) enriched for miRNAs and siRNAs by using a protocol that preferentially clones RNAs with chemical features of Dicer products. Of the 18 small RNAs that were cloned multiple times, 16 were bona fide miRNAs, whereas only a few of the approximately 200 singly cloned species in this collection represent miRNAs. Two related miRNAs were identified by Mette et al. (2002) as the most frequently isolated nonstructural RNAs in a project isolating 17- to 27-nt RNAs. The fact that multiple clones have been found for nearly all the reported miRNAs suggests that each of these miRNAs is more abundant than each of the non-miRNA species, most of which have only been found once. In animals, individual miRNA species are typically present at greater than 1,000 molecules per cell—some are as abundant as 50,000 molecules per cell (Lim et al., 2003b). Although the same is likely to be true in Arabidopsis, there appear to be so many different non-miRNA species, which are either endogenous siRNAs or classes of tiny RNAs remaining to be characterized, that in aggregate they make up a sizable majority of the tiny RNAs.

Because four miRNAs were found by more than one of the four groups, a total of 19 unique Arabidopsis miRNAs have been reported among the hundreds of small RNAs cloned (Table I). Because some appear to be derived from multiple genomic loci, these 19 miRNAs represent up to 41 Arabidopsis genes (Table I). Estimates for the total number of miRNA genes in nematodes (105 ± 15) and vertebrates (230 ± 30) have relied on computational gene-finding tools (Lim et al., 2003a, 2003b). Although this approach has not yet been adapted to obtain a similarly comprehensive accounting of plant miRNAs, it is likely that the 41 miRNA genes identified in Arabidopsis are only a small subset of the total.

The loci that encode plant miRNAs, the *MIR* genes, are clearly distinct from previously annotated genes, but their promoters, primary transcripts, and responsible RNA polymerase remain to be identified. As

Table I. *MicroRNAs identified in Arabidopsis*

miRNA	*At* Genes	miRNA Sequence (Related miRNAs Bracketed)	miRNA Length	Rice Homolog[a]	Fold-Back Arm	Fold-Back Size
	n		*nt*			*nt*
miR156[b]	6	[UGACAGAAGAGAGUGAGCAC	20–21	Yes	5′	80–96
miR157[b]	4	[UUGACAGAAGAUAGAGAGCAC	20–21	Yes	5′	91–173
miR158[b]	1	UCCCAAAUGUAGACAAAGCA	20	No	3′	64
miR159a[b,c,d,f]	1	[UUUGGAUUGAAGGGAGCUCUA	21	Yes	3′	182
miR159b[d,g]	1	[UUUGGAUUGAAGGGAGCUCUU	21	Yes	3′	186
miR160[b]	3	UGCCUGGCUCCCUGUAUGCCA	21	Yes	5′	78–81
miR161[b,e,h]	1	UUGAAAGUGACUACAUCGGGG	20–21	No	5′	90
miR162[b]	2	UCGAUAAACCUCUGCAUCCAG	21	Yes	3′	85–88
miR163[b,c]	1	UUGAAGAGGACUUGGAACUUCGAU	24	No	3′	303
miR164[b]	2	UGGAGAAGCAGGGCACGUGCA	21	Yes	5′	78–149
miR165[b]	2	[UCGGACCAGGCUUCAUCCCCC	20–21	Yes	3′	101–136
miR166[b]	7	[UCGGACCAGGCUUCAUUCCCC	21	Yes	3′	90–136
miR167[b,c,e,i]	2	UGAAGCUGCCAGCAUGAUCUA	20–21	Yes	5′	90–101
miR168[b]	2	UCGCUUGGUGCAGGUCGGGAA	21	Yes	5′	89–104
miR169[b]	1	CAGCCAAGGAUGACUUGCCGA	21	Yes	5′	190
miR170[b]	1	[UGAUUGAGCCGUGUCAAUAUC	21	Yes	3′	64
miR171[b,e,j]	1	[UGAUUGAGCCGCGCCAAUAUC	21	Yes	3′	92
miR172[c]	2	AGAAUCUUGAUGAUGCUGCAU	21	Yes	3′	93
miR173[c]	1	UUCGCUUGCAGAGAGAAAUCAC	22	No	5′	88

[a] Rice homologs defined as potential miRNAs with 0- to 3-nt changes from an Arabidopsis miRNA that are potentially encoded in fold-back structures. [b] Reinhart et al. (2002). [c] Park et al. (2002). [d] Mette et al. (2002). [e] Llave et al. (2002a). [f] Alternate name, miR40a. [g] Alternate name, miR40b. [h] Alternate name, small RNA 111. [i] Alternate name, small RNA 5/small RNA 35. [j] Alternate name, small RNA 39/miRNA39.

with animal miRNAs, plant miRNAs appear to be processed from a portion of the miRNA transcript that can fold into a stable hairpin (Fig. 1A). However, the predicted hairpin precursors of the plant miRNAs are more variable in size than their animal counterparts, ranging from 64 to 303 nt (counting the miRNA residues, those pairing to the miRNA, and the intervening segment, but excluding more distal base pairs; Table I), whereas animal miRNA hairpins are typically 60 to 70 nt (Lim et al., 2003b). Plant miRNAs also pair to the opposite arm of their precursor hairpin with fewer mismatches and bulges than do the animal miRNAs (for plant miRNA predicted hairpins, see supplemental material of Reinhart et al., 2002).

One of the defining characteristics of miRNAs is their evolutionary conservation. Eight of the 19 Arabidopsis miRNAs have at least one perfect match in the rice genome in the context of homologous stem-loop precursors (Reinhart et al., 2002), and an additional seven have paralogs with three or fewer mismatches (Table I). Although there is no striking sequence bias in the body of the mature miRNA, almost all plant miRNAs begin with a U residue (Table I), as is seen with most animal miRNAs (Lau et al., 2001). Mature plant miRNAs are equally likely to be encoded in the 5′ or 3′ arm of the hairpin (Table I). However, when a miRNA is encoded by multiple *MIR* genes, the miRNA is always encoded in the same arm of the hairpin in all members of the gene family, and this conservation extends to the rice homologs as well (Reinhart et al., 2002). This conservation in both sequence and structure implies that many of the plant miRNAs have been playing important roles since before monocots and dicots diverged approximately 250 million years ago. Because plants and animals are thought to have evolved multicellularity independently (Meyerowitz, 2002) and because both possess miRNAs, it appears that miRNAs have been modulating gene expression since before the emergence of multicellular life (Reinhart et al., 2002).

PROTEINS INVOLVED IN PLANT miRNA ACCUMULATION AND FUNCTION

Double-stranded RNA (dsRNA)-specific nucleases in the Dicer family are needed to process both animal miRNAs from their stem-loop precursors and siRNAs from long dsRNA precursors (Hutvágner and Zamore, 2002b). Dicer enzymes contain an N-terminal RNA helicase domain followed by a Piwi/Argonaute/Zwille (PAZ; see below) domain, tandem RNase III motifs, and one or two C-terminal dsRNA-binding domains. Whereas *Caenorhabditis elegans* has one and fruitfly has two Dicer genes, there are four Arabidopsis Dicer-like genes, *DCL1*, *DCL2*, *DCL3*, and *DCL4* (Schauer et al., 2002). The in vitro products of Dicer cleavage are dsRNA molecules with 2-nt 3′ overhangs (Elbashir et al., 2001), but only one of the two cleavage products accumulates as the mature miRNA. Therefore, the fragment from the other arm of the precursor (known as the miRNA* fragment) is rarely cloned and generally not detected on northern blots (Lim et al., 2003b). In the two cases in which an Arabidopsis miRNA* was cloned, it was offset by 2 nt, as predicted for a Dicer product (Reinhart et al., 2002; Fig. 1A).

The Arabidopsis *carpel factory* (*caf*) mutant contains a T-DNA insert in *DCL1*. *caf* (*dcl1-9*) was identified because of its proliferation of inner whorl floral organs, but it confers pleiotropic phenotypes as well (Jacobsen et al., 1999). The T-DNA in *caf* is near the 3′ end of the *DCL1*-coding sequence and would presumably result in a truncated caf/dcl1-9 protein, with all of the protein except one of the two dsRNA-binding domains remaining intact in the mutant (Jacobsen et al., 1999). Because some Dicer enzymes contain only a single dsRNA-binding domain, caf/dcl1-9 might still retain partial function. A complete loss of *DCL1* function confers embryo lethality, as seen in the *suspensor1* alleles, which are disrupted further upstream in the *DCL1*-coding sequence (Schauer et al., 2002).

In the *caf/dcl1-9* mutant, mature miRNAs accumulate to lower levels than in wild type (Park et al., 2002; Reinhart et al., 2002; Kasschau et al., 2003). Unlike in animals (Hutvágner and Zamore, 2002b), larger RNAs the size of predicted hairpin precursor RNAs do not accumulate in wild-type or *caf/dcl1-9* plants (Reinhart et al., 2002). In animals, the hairpin precursor is processed from the primary transcript in the nucleus, then is exported to the cytoplasm for Dicer processing (Lee et al., 2002). Perhaps in plants, Dicer processing occurs in the nucleus cotranscriptionally, such that there is no need to process the hairpin precursor for nuclear export. If a certain size is needed for precursor export in animals, the lack of this constraint for nuclear processed miRNAs would also explain a tolerance in plants for larger hairpin precursors.

The observations that the null *dcl1* allele (*sus1*) is an embryo lethal (Schauer et al., 2002) and a partial disruption (*caf*) has reduced accumulation of all tested miRNAs (Park et al., 2002; Reinhart et al., 2002; Kasschau et al., 2003) indicate that the other three Arabidopsis *DCL* genes do not compensate for *DCL1* function. This lack of functional redundancy suggests that other Dicers might act in metabolism of other RNAs, which is also consistent with biochemical evidence (Tang et al., 2003). PTGS and siRNA production from a long dsRNA hairpin proceeds normally in the *caf/dcl1-9* plants (Finnegan et al., 2003), implying that distinct Dicer enzymes process miRNA and siRNA precursors, or that the second dsRNA binding domain of DCL1 is required for miRNA but not for siRNA production.

The Argonaute family is a second family implicated in both miRNA and siRNA functioning (for

review, see Carmell et al., 2002). Argonaute and its homologs are approximately 100-kD proteins that share two domains, the PAZ and the PIWI domains. Intriguingly, Dicer enzymes also contain a PAZ domain; it is possible that PAZ domains mediate homo- or heterotypic protein-protein associations. In nematodes, Argonaute family members AGL-1 and AGL-2 are required for miRNA biogenesis and function. Other Argonaute family members are genetically implicated in other processes involving short RNAs, including RNAi (Carmell et al., 2002). Moreover, Argonaute family members are found in the RISC and the RISC-like miRNP (Carmell et al., 2002; Martinez et al., 2002; Schwarz and Zamore, 2002).

In Arabidopsis, the *argonaute* (*ago1*) mutant displays pleiotropic shoot architecture defects (Bohmert et al., 1998) and *PINHEAD/ZWILLE* is a related gene that is necessary to maintain undifferentiated stem cells in the shoot apical meristem (Moussian et al., 1998; Lynn et al., 1999). The 10 Arabidopsis AGO-like proteins might contribute to the functional specificity of various RISC-like complexes. For example, AGO1, but not PINHEAD/ZWILLE, is required for post-transcriptional gene silencing (Fagard et al., 2000; Morel et al., 2002), and AGO4 is required for accumulation of certain heterochromatic siRNAs (Zilberman et al., 2003). The roles of Arabidopsis AGO family members in miRNA accumulation have not been reported.

The *hen1* mutant, which is defective in a novel protein, displays similar morphological phenotypes to *caf/dcl1-9* and also is defective in miRNA accumulation (Park et al., 2002), suggesting that HEN1 might also act in miRNA metabolism. Uncharacterized *HEN1* homologs are found in animals (Park et al., 2002). Future experiments will reveal whether *hen1* provides another example (like *dcl1* and *ago1*) in which components important in small RNA processing in animals are first uncovered by cloning Arabidopsis genes responsible for mutant phenotypes.

PLANT miRNAs BIND TO TARGET mRNAs WITH NEAR-PERFECT COMPLEMENTARITY

The severe developmental defects of plant mutants defective (*dcl1* and *hen*) or implicated (*ago1* and *pinhead/zwille*) in miRNA processing suggest that these phenotypes might result directly from altered miRNA function in the mutants. The paradigm for miRNA function comes from the study of nematode *lin-4* and *let-7* RNAs (Pasquinelli and Ruvkun, 2002). These miRNAs pair to multiple sites within the 3′-UTR of specific target mRNAs, which results in reduced protein from the target message through a mechanism that is unknown but follows translation initiation (Olsen and Ambros, 1999; Fig. 1D). The base pairing between the miRNA and its target is limited, which has hindered reliable computational identification of other animal miRNA targets. The observation that certain Arabidopsis miRNAs have near-perfect antisense matches in protein-coding sequences or UTRs suggested that target prediction for plant miRNAs might be more straightforward (Park et al., 2002; Rhoades et al., 2002). In fact, miR171 has perfect antisense complementarity to three mRNAs encoding SCARECROW-like (SCL) transcription factors (Llave et al., 2002a; Reinhart et al., 2002).

A systematic search for annotated mRNAs with complementarity to miRNAs (Rhoades et al., 2002) yielded potential targets for most of the known plant miRNAs (Table II). Potential regulatory targets with three or fewer mismatches were identified for 14 of the 16 miRNAs queried, and targets for two other miRNAs, miR162 and miR163, have been identified by allowing a 1-nt gap in the search algorithm (Table II). Unlike the situation with animal miRNAs, there are substantially more antisense hits to authentic miRNAs than to their corresponding randomized sequences, indicating that the identified plant targets are relevant (Rhoades et al., 2002). Moreover, the biological significance of the matches between the miRNAs and the identified targets is further supported by the conserved pairing of orthologous miRNA:mRNA partners in rice (Rhoades et al., 2002). There are more potential miRNAs targets than those listed in Table II, but targets with more than three mismatches are more difficult to computationally distinguish from false positives (Rhoades et al., 2002).

The miRNAs have a remarkable propensity to target messages of transcription factors (Rhoades et al., 2002). MiRNA complementary sites with three or fewer mismatches are found in 74 cases, which, due to overlap between similar miRNAs, represent 61 unique mRNAs (Table II). Of these 61 predicted targets, 40 are known or putative transcription factors (Table II), even though transcription factors apparently represent only 6% of Arabidopsis protein-coding genes (Riechmann et al., 2000). Furthermore, most of the targeted transcription factors are known to regulate development or are related to genes with known developmental roles, suggesting that miRNAs help coordinate a wide range of cell division and cell fate decisions throughout the plant (Rhoades et al., 2002). For example, miR165 apparently targets *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), which encode homeodomain-Leu zipper transcription factors that regulate axillary meristem initiation and leaf development (McConnell et al., 2001), and miR172 is predicted to target *AP2*, which specifies floral organ identity (Bowman et al., 1989). MiR164 apparently targets *CUC1* and *CUC2*, which act in organ separation (Aida et al., 1997), and NAC1, a related gene that promotes lateral root development (Xie et al., 2000).

It is also intriguing that several of the non-transcription factor miRNA targets have links to RNA metabolism, and in some cases that of miRNAs. Most notably, *DCL1* and *AGO1* are predicted targets of miR162 and miR168, respectively (Table II); their

ties to miRNAs and plant development are discussed above. The prediction of *DCL1* and *AGO1* as miRNA targets suggests a negative feedback mechanism controlling their expression. Moreover, miR157 might target an uncharacterized DEAD-box RNA helicase (Table II). MiR161 is predicted to target a family of uncharacterized pentatricopeptide repeat-containing proteins; several members of this family from other organisms have RNA associations (Small and Peeters, 2000), including a fruitfly protein that binds to the 3′-UTR of the *bicoid* mRNA (Mancebo et al., 2001). Finally, miR163 apparently targets uncharacterized members of a plant family of *S*-adenosyl-L-Met dependent methyltransferases (Ross et al., 1999); it will be interesting to learn whether these enzymes methylate nucleic acids, proteins, or small-molecule substrates.

PLANT miRNAs CAN DIRECT mRNA CLEAVAGE

The observation that miR171 has perfect antisense complementarity to three mRNAs encoding SCL transcription factors led to the suggestion that miR171 acts in the same manner as siRNAs to target these mRNAs for cleavage (Llave et al., 2002a; Reinhart et al., 2002). Inflorescence tissue, where miR171 is abundant, contains two *SCL6-III* and *SCL6-IV* transcripts, full-length versions and uncapped 3′-cleavage products with 5′ ends that map to the middle of the miR171 complementarity sites (Llave et al., 2002b). In contrast, only the full-length *SCL6-III* and *SCL6-IV* transcripts are detected in stem tissue, which does not accumulate high miR171 levels (Llave et al., 2002b). Direct support for the hypothesis that miR171 targets *SCL6-III* and *SCL6-IV* for cleavage was obtained when various miR171 and *SCL6* constructs were expressed in *Nicotiana benthamiana* leaves after *Agrobacterium tumefaciens* inoculation. Coexpression of miR171 and *SCL6-IV* leads to *SCL6-IV* mRNA cleavage, whereas mutating three residues in the target site is sufficient to abolish cleavage (Llave et al., 2002b).

The observation that most other plant miRNAs match targets with near perfect antisense complementarity led to the hypothesis that they also might act as if they were siRNAs and guide target cleavage (Rhoades et al., 2002). This would require an RNAi-like pathway that tolerates the mismatches that can occur between a miRNA and its predicted targets (Table II; Fig. 1C). Support for this idea has come from in vitro studies using a wheat (*Triticum aestivum*) germ system (Tang et al., 2003). The endogenous miR165 or miR166 in the extract directs cleavage of an mRNA containing the *PHB* or *PHV* target site (Tang et al., 2003). However, when an additional mismatch is incorporated into the target, the cleavage rate is reduced 14-fold (Tang et al., 2003). Moreover, in vivo mRNA cleavage products with 5′ ends within the

Table II. *Arabidopsis microRNA targets*

microRNA	Target Family	Predicted Target Genes	No. of Mismatches[a]	Validated Cleavage Targets
miR156	SQUAMOSA-PROMOTER BINDING PROTEIN (SBP)-like proteins	10 *SPL* genes[b]	1–2	*SPL2*[g]
miR157	SBP-like proteins	9 *SPL* genes[b]	1–3	*SPL2*[g]
	Putative DEAD-box RNA helicase	At5g08620 (= *AtRH25*)[b]	3	
	Unknown proteins	At1g22000, At3g47170[b]	3	
miR158	Unknown protein	At1g64100[b]	3	
miR159a	MYB transcription factors	5 *MYB* genes[b,c]	2–3	
	Unknown protein	At1g29010[b]	3	
miR159b	MYB transcription factors	3 *MYB* genes	3	
miR160	Auxin Response Factors	*ARF10*, *ARF16*, *ARF17*[b]	1–3	*ARF10*, *ARF17*[g]
miR161	Pentatricopeptide repeat proteins	9 genes[b]	3	
miR162	DICER	*DCL1* (=*CAF*=*SIN1* = *SUS1*)	1-nt bulge	
miR163	SAM-dependent methyltransferases	5 genes[b]	0–2 with 1-nt bulge	
miR164	NAC domain proteins	*CUC1*, *CUC2*, *NAC1*, 2 others[b]	2–3	*CUC1*, *CUC2*[g]
miR165	HD-Zip transcription factors	*PHV*, *PHB*, *REV*, *ATHB-8*[b]	3	*PHV*[h]
miR166	HD-Zip transcription factor	*ATHB-15*[b]	3	
miR167	Auxin response factors	*ARF6*[b,c], *ARF8*[c]	3–4	*ARF8*[g]
miR168	ARGONAUTE	*AGO1*[b]	3	
miR169	CCAAT-binding factor (CBF)-HAP2-like proteins	At1g17590, At1g54160[b]	3	
miR170	GRAS domain transcription factors (SCARECROW-like)	*SCL6-II*, *SCL6-III*, *SCL6-IV*[b]	2	
miR171	GRAS domain transcription factors (SCARECROW-like)	*SCL6-II*, *SCL6-III*, *SCL6-IV*[b,d,e]	0	*SCL6-III*, *SCL6-IV*[f]
miR172	APETELA2-like transcription factors	*AP2*, 3 *AP2-like* genes[c]	1–3	*AP2*, 3 *AP2-like* genes[g]

[a] G:U wobbles are included as mismatches in this analysis. [b] Rhoades et al. (2002) [c] Park et al. (2002) [d] Reinhart et al. (2002) [e] Llave et al. (2002a) [f] Llave et al. (2002b) [g] Kasschau et al. (2003) [h] Tang et al. (2003)

miRNA complementary sites have been detected for 10 of the other predicted targets with less-than-perfect complementarity (Kasschau et al., 2003; Table II).

Although these experiments demonstrate that perfect complementarity between a miRNA and its target is not required for efficient target cleavage (Kasschau et al., 2003; Tang et al., 2003), a detailed analysis of the number and positions of tolerated mismatches has not been conducted. In several cases, particular miRNA-target mismatches have been maintained through the evolutionary distance that separates Arabidopsis and rice (Rhoades et al., 2002), suggesting that certain mismatches are not only tolerated, but are under positive selective pressure. It has been suggested that properly placed miRNA-target mismatches might allow more rapid release of the cleaved RNA from the RISC, which might improve the enzyme turnover rate (Tang et al., 2003).

Some plant viruses interfere with host PTGS to enable effective replication (Vance and Vaucheret, 2001); these viruses might interfere with miRNA function as well. Expressing a viral RNA silencing suppressor in plants inhibits accumulation of certain siRNAs but promotes miRNA accumulation (Mallory et al., 2002; Kasschau et al., 2003). Rather than reducing target mRNA levels, these higher miRNA levels are associated with accumulation of miRNA targets, implying that the accumulated miRNAs are not fully functional (Kasschau et al., 2003). Moreover, the plants display floral defects similar to those of the *caf/dcl1-9* mutant. These observations suggest that some aspects of virus-induced disease result from reduced miRNA function.

A ROLE FOR PLANT miRNAs IN CLEARING REGULATORY TRANSCRIPTS

Plants carrying dominant mutations in the *PHB* or *PHV* genes, which encode related homeodomain-Leu zipper transcription factors, make leaves in which abaxial leaf fates are transformed into adaxial leaf fates (McConnell et al., 2001). In wild-type plants, *PHB* mRNA is initially found throughout the meristem and leaf primordium at low levels before becoming localized to the adaxial side of organs. In the *phb-1d* mutant, however, *PHB* RNA remains present throughout the primordium (McConnell et al., 2001). Thus, the dominant mutations in *PHB* and *PHV* may cause constitutive adaxialization as a result of this expanded expression domain. These dominant mutations, mostly point substitutions, cluster in a short segment of the coding sequence (McConnell et al., 2001), which happens to correspond to the miR165 complementary site (Rhoades et al., 2002). Thus, a simple explanation for the mutant phenotypes is that miR165 (and its close relative, miR166) negatively regulates *PHB* and *PHV*, and point substitutions that weaken this regulation lead to dominant developmental phenotypes because these messages are no longer cleared from abaxial regions (Rhoades et al., 2002). The point mutation that confers dominant phenotypes on *phb-1d* plants also greatly reduces miR165/166-directed cleavage of the *PHB* mRNA (Tang et al., 2003).

The model in which plant miRNPs act to clear regulatory messages from specific daughter cell lineages would enable rapid daughter cell differentiation without requiring constitutively unstable messages (Rhoades et al., 2002). In this respect, miRNA regulation is analogous to ubiquitin-dependent degradation, except that particular mRNAs rather than proteins are targeted for degradation. The idea that clearing regulatory transcripts during differentiation is a crucial role for miRNAs may explain why miRNAs target so many transcription factor messages responsible for developmental decisions (Table II).

FUTURE PROSPECTS

We anticipate that in the near future, more plant miRNAs will be discovered and numerous mRNAs will be validated as miRNA cleavage targets using the *A. tumefaciens* infiltration assay (Llave et al., 2002b), the wheat germ in vitro assay (Tang et al., 2003), or in vivo cleavage product detection (Kasschau et al., 2003). Showing that miRNAs can direct cleavage of their predicted targets is important but is only the beginning. The next step will be to understand the biological significance and roles of miRNA regulation during plant development. For this, we will need to know how the miRNAs themselves are regulated at the transcriptional and posttranscriptional levels, and the developmental consequences of disrupting miRNA regulation.

In the discovery and study of RNAi and related gene-silencing mechanisms, including miRNAs, pioneering work in plants has provided fundamental insights to those working in both plant and animal systems. For example, the first analysis of posttranscriptional gene silencing was in petunia (*Petunia hybrida*; Napoli et al., 1990; van der Krol et al., 1990), the first observation of siRNAs was in tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicon esculentum*; Hamilton and Baulcombe, 1999), and the first demonstrations that Dicer and Argonaute proteins are needed for proper development was in Arabidopsis (Bohmert et al., 1998; Jacobsen et al., 1999; Schauer et al., 2002). The first evidence that some miRNAs direct cleavage of their target mRNAs was also in plants (Llave et al., 2002b; Tang et al., 2003), which together with the finding that a human miRNA can direct cleavage of an artificial target (Hutvágner and Zamore, 2002a) has prompted a reassessment of the molecular mechanisms of miRNA action and their relationship to siRNAs. The field of small RNAs has been one in which studies in plants and animals have repeatedly informed and enriched each other; it is likely that this interplay will continue as the breadth

of the distribution and functions of these tiny riboregulators are more fully elucidated in the coming years.

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