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MicroRNAs: something important between the genes

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Non-coding small endogenous RNAs, of 21–24 nucleotides in length, have recently emerged as important regulators of gene expression in both plants and animals. At least three categories of small RNAs exist in plants: short interfering RNAs (siRNAs) deriving from viruses or transgenes and mediating virus resistance or transgene silencing via RNA degradation; siRNAs deriving from transposons or transgene promoters and controlling transposon and transgene silencing probably via chromatin changes; and microRNAs (miRNAs) deriving from intergenic regions of the genome and regulating the expression of endogenous genes either by mRNA cleavage or translational repression. The disruption of miRNA-mediated regulation causes developmental abnormalities in plants, demonstrating that miRNAs play an important role in the regulation of developmental decisions.

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Abbreviations

AP2	*APELATA2*
dsRNA	double-stranded RNA
miRNA/miR	microRNA
miRNP	microribonucleoprotein complex
nt	nucleotide
RISC	RNA-induced silencing complex
siRNA	short interfering RNA
stRNA	small temporal RNA
UTR	untranslated region

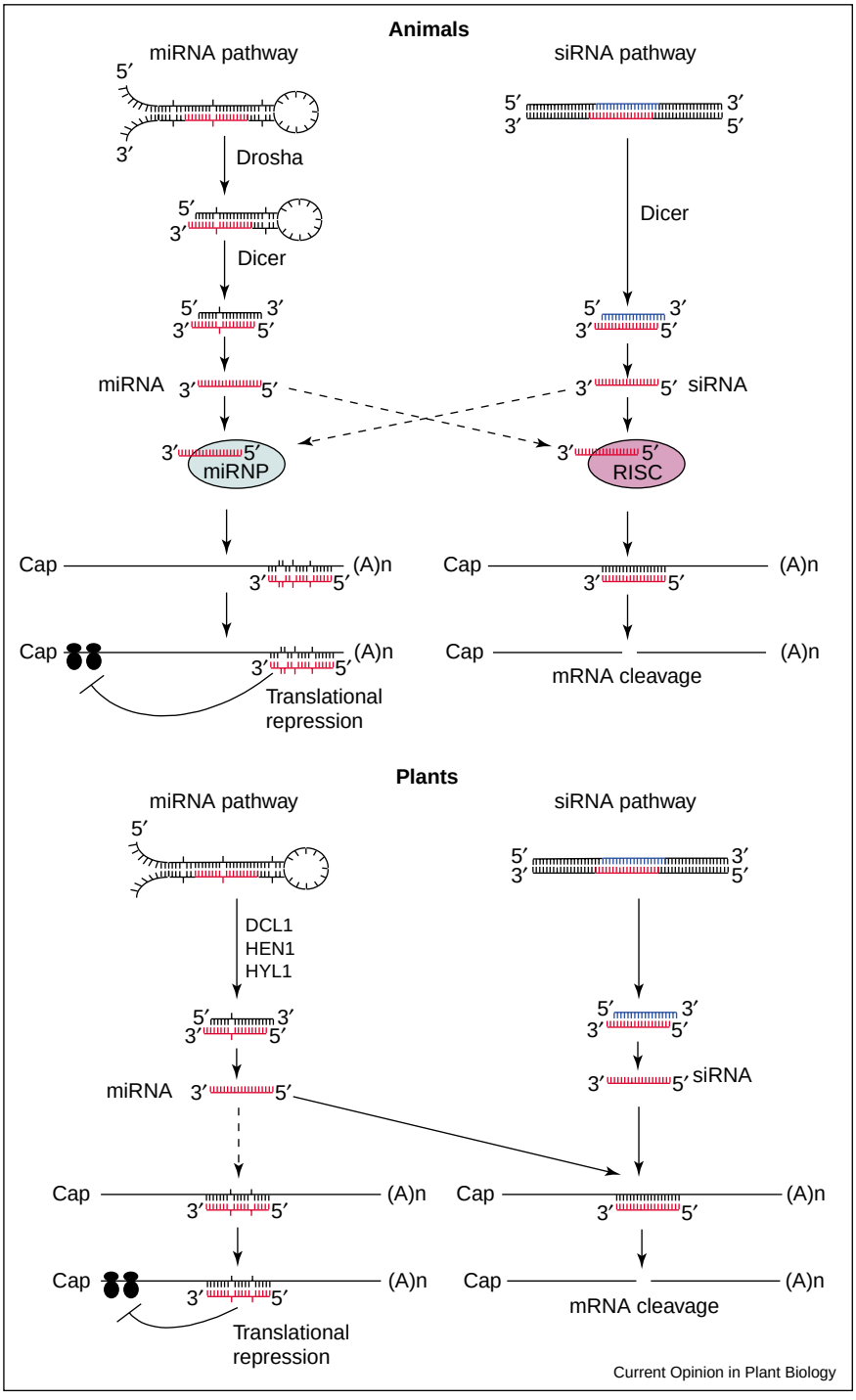
Introduction: the discovery and classification of small RNAs

Among the first small non-coding RNAs to be discovered were the small temporal RNAs (stRNAs) *lin-4* and *let-7*, which were identified from a screen of *Caenorhabditis elegans* mutants that exhibited timing defects during larval development [1,2]. These two single-stranded small RNAs are processed from imperfectly paired stem-loop precursor RNAs that are approximately 70 nucleotides (nt) in length. Once fully formed, the mature 21 nt RNAs alter gene expression by binding to multiple copies of partially complementary sites within the 3′ untranslated region (UTR) of their target mRNAs and repressing translation (for reviews see [3,4] and Figure 1).

Another important class of small RNA, the short interfering RNAs (siRNAs), was first discovered in plants exhibiting transgene- or virus-mediated RNA silencing [5]. RNA silencing is a sequence-specific RNA regulatory mechanism that is also known as post-transcriptional gene silencing or co-suppression in plants, RNA interference in animals, or quelling in fungi. It acts as an immune system to protect the genome against the deleterious effects of invading nucleic acids (i.e. viruses, transposons or transgenes) or to stabilize the different states of chromatin (for reviews see [6–10]). Biochemical data from animals demonstrated that multiple and adjacent 21-nt siRNAs are processed from exogenous, long and perfectly complementary double-stranded RNA (dsRNA) molecules. These siRNAs act as guides in a multi-component protein complex (the RNA-induced silencing complex [RISC]) to recognize, bind and induce cleavage of complementary RNAs (for reviews see [7,9] and Figure 1). Biochemical data from plants has revealed that exogenous dsRNAs are converted into two classes of RNAs, 21- and 24-nt RNAs, the latter being more abundant than the former [11••]. The antisense strand of the 21-nt RNAs probably associates, within a RISC-like complex, with mRNA targets to trigger mRNA cleavage. Conversely, the 24-nt RNAs don't seem to play a role in mRNA degradation [12,13•]. In addition, 21-, 22- and 24-nt RNAs that are derived from dsRNAs containing promoter sequences have been associated with DNA methylation and transcriptional transgene silencing [14]; however, the respective roles of these two species in this process are not known.

Following the discovery of stRNAs and siRNAs, the stage was set for the search for more non-coding small RNAs. Several laboratories successfully cloned numerous 21–24-nt RNAs from *Saccharomyces pombe*, *C. elegans*, *Drosophila melanogaster*, mice, humans and plants [15–18,19••,20•,21,22••,23,24••,25–29]. The cloned small RNAs that display the characteristics of the original *lin-4* and *let-7* stRNAs were collectively termed microRNAs (miRNAs). miRNAs derive from intergenic regions of the genome and are processed from partially folded stem-loop precursor RNAs [30]. miRNAs regulate genes that are distinct from the intergenic regions that encode them. In addition, many miRNAs are conserved across species, strongly suggesting that they have an evolutionarily conserved role in gene regulation (for review see [31]). Interestingly, the RNAs that meet the definition of miRNAs only represent a minor fraction of the cloned endogenous small

Figure 1



Similarities and differences between miRNA and siRNA pathways in animals and plants. In animals, primary miRNA precursor transcripts that are derived from intergenic regions are processed into precursor RNAs in the nucleus by the RNase3 Drosha. These precursor RNAs are transported out of the nucleus and processed by Dicer. Mature single-stranded miRNAs incorporate into an miRNP and guide the complex to the 3′ UTR of partially complementary mRNAs to repress translation. In the siRNA pathway, exogenously provided double-stranded RNAs are cleaved by Dicer to generate double-stranded siRNAs. Single-stranded siRNAs incorporate into RISCs and guide these complexes to perfectly complementary mRNA, where they mediate cleavage. miRNA can also cleave perfectly complementary mRNA, and siRNA can also repress the translation of mRNA that has short segments of complementarity in their 3′ UTR (dashed arrows). In plants, the accumulation of miRNAs depends on the activity of DCL1, HEN1 and HYL1, but it is unclear how dsRNAs are cleaved into siRNAs. microRNAs and siRNAs are thought to associate with a protein complex (miRNP/RISC) to mediate mRNA cleavage, but nothing is known about the components of the complex(es). Translational repression of one target (*AP2*) has been observed in plants that overexpress miR172, suggesting that plant miRNAs can also repress translation by binding to a single site within the mRNA coding sequence (dashed arrow). This figure was adapted from [32,41••].

RNAs. For example, among the 423 non-redundant small RNAs cloned from *Arabidopsis* [19••,20•,22••,24••], only 19 are miRNAs [32]; the remaining endogenous small RNAs from *Arabidopsis* have not yet been thoroughly classified. Interestingly, the endogenous small RNAs of *Arabidopsis* have a bimodal distribution of 21-nt (minor fraction) and 24-nt (major fraction) RNAs. This evidence suggests that, like transgene-derived small RNAs, endogenous small RNAs could include multiple classes that have different functions [11••]. To date, apart from miRNAs, only 24-nt RNAs that are derived from the endogenous transposon AtSN1 family have been shown to play a role in DNA and histone methylation, and probably in the taming of the AtSN1 transposon [12,33].

Which cellular genes control the accumulation of miRNAs?

Studies focusing on the biogenesis and action of miRNAs in human cells have demonstrated that the maturation of miRNAs is a two-step process. Briefly, long primary precursor miRNA transcripts are cleaved by the nuclear-localized RNase3 Drosha into a 70-nt precursor miRNA [34]. This 70-nt precursor is exported out of the nucleus and subsequently cleaved by another RNase3 called Dicer, which has an RNA helicase domain, to generate short-lived double-stranded miRNA intermediates [35]. One strand of this RNA accumulates as the mature miRNA and acts as a guide in a multi-component ribonucleoprotein complex (miRNP) that recognizes partially complementary mRNAs ([21] and Figure 1).

In plants, the accumulation of miRNAs is dependent upon the activity of DCL1 [22••,24••,36••,37••], HEN1 [22••,38] and HYL1 (F Vazquez, P Crete, H Vaucheret, unpublished). On the basis of its homology with animal Dicer proteins, it is likely that DCL1 has RNase3 activity. It has been proposed that HEN1 is a dsRNA methylase [39], whereas HYL1 has been shown to bind dsRNA specifically [40]. Each of the three proteins has a putative nuclear localization signal, suggesting that they act in the processing of miRNA precursors in the nucleus. However, only DCL1 and HYL1 have been shown to reside in the nucleus [37••,40].

Like animal miRNAs, many plant miRNAs do not accumulate ubiquitously but instead show preferential accumulation in specific tissue types. This suggests the existence of transcriptional and/or post-transcriptional mechanisms that spatially and temporally regulate miRNA expression [16,18,19••,21,24••,36••]. However, nothing is known about the cellular factors that regulate *MIR* gene expression or miRNA stability and action in plants.

What are the targets of miRNAs?

After the identification of miRNAs in animals and plants, the next step was to determine the identity of the mRNAs that they regulate. Given that there is at least some level of complementarity between *lin-4* and *let-7* miRNAs and their target mRNAs, it was logical to assume that miRNA targets could be predicted by scanning sequenced genomes for regions with partial complementarity to the miRNAs. To date, computational approaches have been very successful in predicting miRNA targets in plants [41••]. This success is probably due to the near perfect complementarity of plant miRNA and their predicted targets. The cloned plant miRNAs can be classified into 15 families, each comprising up to four members that differ by a single nucleotide ([32]; Table 1). Each family has at least one predicted mRNA target and some miRNAs are predicted to target multiple mRNAs (Table 1).

Intriguingly, a large fraction of plant miRNA targets are either known to be transcription factors that are involved in cell fate determination or are homologous to such transcription factors ([41••]; Table 1). This strongly suggests that the miRNA pathway is important for the proper regulation of plant development. In support of this claim, plant mutants that are impaired in miRNA accumulation, such as *dcl1*, *hen1* and *hyl1*, display interesting and dramatic developmental phenotypes that range from defects in floral development to defects in leaf morphology [38,40,42,43]. In addition, plants expressing a viral protein that alters miRNA accumulation have developmental defects [13•,36••]. The phenotypes of these plants further suggest the importance of miRNAs in regulating plant development.

How do miRNAs regulate the expression of target genes?

In animals, miRNAs alter gene expression by binding to multiple copies of partially complementary sites within the 3′ UTR of their target mRNAs and repressing the translation of these RNAs [1–4]. It is likely that the degree of complementarity between mRNAs and small RNAs determines the mode of action of small RNAs in animals. Indeed, the *let-7* miRNA can enter the RNA interference pathway and cleave a modified *let-7* target that perfectly matches *let-7* miRNA, indicating that miRNAs can function as siRNAs [44]. Similarly, an artificial siRNA can repress the translation of a target mRNA that has partially complementary binding sites in its 3′ UTR, indicating that siRNAs can function as miRNAs in animals ([45]; Figure 1).

In plants, the predicted miRNA-binding sites identified to date are present as single copies, and the majority of them are located in the open reading frame of the target mRNA [22••,41••,46••,47••]. Interestingly, the degree of complementarity between plant miRNAs and their targets is greater than that observed between animal miRNAs and their targets, although all but one of the known plant miRNAs exhibit sequence mismatches with their target mRNAs [41••]. So, plant miRNAs could potentially cleave mRNAs or repress their translation.

Table 1

Putative and validated miRNA targets in *Arabidopsis*.

Arabidopsis miRNA	*Oryza* homologs	Putative targets (number of genes)[b]	Validated targets
156/157	Yes	**Squamosa-promoter binding protein** (10)	SPL2 [36••], SPL10[a]
158	No	Unknown protein (1)	
159a/b/c/Jaw	Yes	**MYB protein** (5), **TCP protein** (5)	MYB33 [46••], MYB65 [46••], TCP2 [46••], TCP3 [46••], TCP4 [46••], TCP10 [46••], TCP24 [46••]
160	Yes	**Auxin-response factor** (3)	ARF10 [36••], ARF17 [36••]
161	No	PPR repeat protein (9)	
162	Yes	Dicer-like1 (1)	DCL1 [47••]
163	No	AtPP-like protein (5)	
164	Yes	**NAC domain protein** (5)	CUC1 [36••], CUC2 [36••]
165/166	Yes	**HD-Zip transcription factor** (4)	PHV [11••], REV [49•]
167	Yes	**Auxin-response factor** (1)	ARF8 [36••]
168	Yes	Argonaute1 (1)	AGO1[a]
169	Yes	**CCAAT-binding factor HAP2-like** (2)	
170/171	Yes	**GRAS-domain protein** (3)	SCL6-III [48••], SCL6-IV [48••]
172a/b	Yes	**AP2 and AP2-like protein** (4)	AP2 [36••], TOE1 [36••], TOE2 [36••], TOE3 [36••]
173	No	Unknown protein (1)	

This table was adapted from [32,41••]. [a] F Vazquez, P Crete, H Vaucheret, unpublished. [b] Known and putative transcription factors are in bold. Abbreviations: AGO, ARGONAUTE; ARF, AUXIN-RESPONSE FACTOR; CUC, CUP-SHAPED COTYLEDON; DCL, DICER-LIKE; PHV, PHAVOLUTA; REV, REVOLUTA; SCL, SCARECROW-LIKE; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; TCP, TEOSINTE BRANCHED1, CYCLOIDEA, PCF-2; TOE, TARGET OF EAT.

The first analyses of miRNA-mediated target regulation in plants suggested that cleavage was the rule, even if mismatches were naturally present. Indeed, truncated RNAs with 5′ ends that corresponded to the middle of the miRNA-binding site were identified for 24 miRNA targets ([11••,36••,46••–48••,49•]; Table 1). miRNA-mediated cleavage in plants seems to tolerate up to five mismatches between the miRNA and its target [46••], but the introduction of additional mismatches into the miRNA-binding site of the target reduces cleavage. This was first shown by ectopically expressing miR171 and either perfectly complementary wildtype *SCL6-III* or *SCL6-IV* mRNA targets or mutated RNA forms in *Nicotiana benthamiana* leaves using Agro-infiltration [48••]. The dramatic impact of adding a single point mutation to the miRNA-binding site of a mRNA target that already exhibited mismatches with the corresponding miRNA was demonstrated in the miR165/166-*PHV* couple [11••]. *In vitro* wheat germ assays demonstrated that miR165/R166 direct the efficient cleavage of exogenously introduced wildtype *PHV* RNA, but cleave a *phv* mRNA carrying a single-nucleotide change inefficiently. These assays support the proposal that the dominant phenotype displayed by the corresponding *phv*-mutant plants may result from altered miRNA regulation [11••,50]. Finally, the importance of miRNA-mediated cleavage of mRNA targets in the regulation of plant development was demonstrated *in vivo* by ectopically expressing mRNA targets that carried silent mutations. These mutations did not change the protein sequence but decreased the degree of complementarity between the miRNA-binding site and its miRNA. To date, dominant leaf abnormalities have been observed when expressing mutant RNA forms of *REVOLUTA* (*REV*; targeted by miR165/R166), *TCP2* and *TCP4* (targeted by miR-Jaw), and *MYB33* (targeted by miR159) [46••,49•].

Interestingly, the regulation of *APELATA2* (*AP2*) by miR172 seems to be more complex. Although *AP2* cleavage products were identified in wildtype plants, the level of uncleaved *AP2* mRNA in wildtype plants was the same as that in *hen1* and *dcl1* mutants, which exhibit reduced accumulation of miR172, and in transgenic plants that overexpressed miR172 [36••,51••,52•]. The level of AP2 protein, however, was dramatically lower in the transgenic plants that overexpressed miR172 than in wildtype plants [51••,52•]. These data led Aukerman and Sakai [51••] to suggest that there might be competition between mRNA cleavage and the inhibition of translation. Cleavage products deriving from other miR172 targets (i.e. *TOE1* and *TOE2*) were also observed in wildtype plants [36••,51••]. In contrast, increased levels of uncleaved *TOE1* and *TOE2* mRNAs were observed in *dcl1* mutants, supporting the hypothesis that miR172 regulates these two targets by cleavage [36••]. The level of complementarity between miR172 and *AP2* RNA is similar to that between miR172 and *TOE1* or *TOE2* RNAs. Hence, the reported differences in the regulation of these three targets suggest that complementarity may not be the only factor that influences the way in which miRNAs regulate their targets. Perhaps the context of the miRNA-binding site plays a role in determining its mode of action or maybe different regulation occurs in different types of cells.

Conclusions

The demonstration that endogenous miRNAs modulate gene expression by at least two mechanisms (i.e. mRNA cleavage or translation inhibition) adds a twist to the

general models of gene regulation, and has led to a flurry of activity in the field of small non-coding RNA research. However, there are still many fundamental questions that remain unanswered: How many miRNAs exist in plants? How many genes do they regulate? How are *MIR* genes regulated? Are distinct *MIR* genes that encode the same miRNA functionally redundant or differentially regulated? Where are miRNAs active? How is miRNA accumulation spatially and temporally regulated? Which cellular factors allow the action of miRNAs? How are these factors regulated? How many miRNA molecules per cell are necessary to restrict the expression of target mRNA(s)? Do miRNAs act in a stoechiometric or a catalytic manner? How do miRNAs choose between mRNA cleavage and translation inhibition in plants? Does this choice depend on the degree and/or quality of nucleotide pairing between miRNAs and their targets? Does it also depend on their respective amounts? Are miRNAs the only category of endogenous small RNAs that regulate gene expression at the post-transcriptional level?

The function of the other endogenous small RNAs is not known. Some of them are supposed to play a role in the taming of transposons and in controlling chromatin state; however, it is possible that other small RNAs could regulate different steps in gene expression such as splicing, as recently shown in yeast [53].

One can anticipate that every laboratory working on plant development, chromatin remodeling, transposon regulation or virus resistance will now look for the possible involvement of small RNAs in the processes that they are studying, thus providing further insights into the understanding of small-RNA-mediated regulation and its importance in plant biology.

Note added in proof

A list of 1823 small RNAs cloned from *Arabidopsis* has recently been made available by Carrington group at http://cgrb.orst.edu/smallRNA/.

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