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miSSING LINKS: miRNAs and plant development

Christine Hunter and R Scott Poethig

The discovery of hundreds of plant micro RNAs (miRNAs) has triggered much speculation about their potential roles in plant development. The search for plant genes involved in miRNA processing has revealed common factors such as DICER, and new molecules, including HEN1. Progress is also being made toward identifying miRNA target genes and understanding the mechanisms of miRNA-mediated gene regulation in plants. This work has lead to a reexamination of many previously characterized mutations that are now known to affect components or targets of miRNA-mediated pathways.

Addresses
Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018, USA

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Abbreviations

nt	nucleotide
PNH	*PINHEAD*
PTGS	post-transcriptional gene silencing
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
SAM	shoot apical meristem
UTR	untranslated region

Introduction

Over the past year the discovery of a wealth of small regulatory RNAs in *Arabidopsis*, *Caenorhabditis elegans*, *Drosophila* and humans has caused quite a stir [1]. MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) are molecules of 19–25 nucleotides (nt) that can mediate gene silencing at the transcriptional, post-transcriptional or translational level. They are required for RNA-mediated interference in animals and for post-transcriptional gene silencing (PTGS) in plants, and have been also indirectly implicated in many different developmental events (reviewed in [2,3]).

miRNAs are single-stranded molecules of 19–25 nt formed by the Dicer-mediated cleavage of endogenously encoded stem-loop RNA structures. siRNAs are also produced by Dicer, but they are double-stranded (ds) fragments cleaved from long dsRNA precursors (reviewed in [2]). In plants, siRNAs in the range of 21–22 nt mediate PTGS and co-suppression, whereas siRNAs of 24–26 nt (long siRNAs) are associated with long-range transmission of silencing signals and methylation of corresponding genomic regions (Figure 1) [4]. The role of siRNAs in plant PTGS has been reviewed recently [5,6] and so is not discussed in detail here.

The first miRNA to be identified was the *C. elegans* heterochronic gene *lin-4*, which inhibits translation by pairing with partially homologous sequences in the 3′ untranslated region (UTR) of target genes [7–11]. A second heterochronic miRNA, *let-7*, interacts with the 3′ UTR of targets and inhibits expression, although the exact mechanism by which it achieves this has not been characterized [12,13]. In humans, many miRNAs are found in a complex of about 550 kDa known as the miRNP [14], and recent work has shown that there is some similarity between this complex and the RNA-induced silencing complex (RISC), which mediates siRNA-directed target cleavage [15]. In addition, it has been shown that both siRNAs and miRNAs are capable of either cleaving or translationally repressing targets, depending on the extent of complementarity between the miRNA and the mRNA [16,17]. Thus, miRNA-mediated developmental pathways may use target cleavage in addition to the translational repression first demonstrated in *C. elegans*.

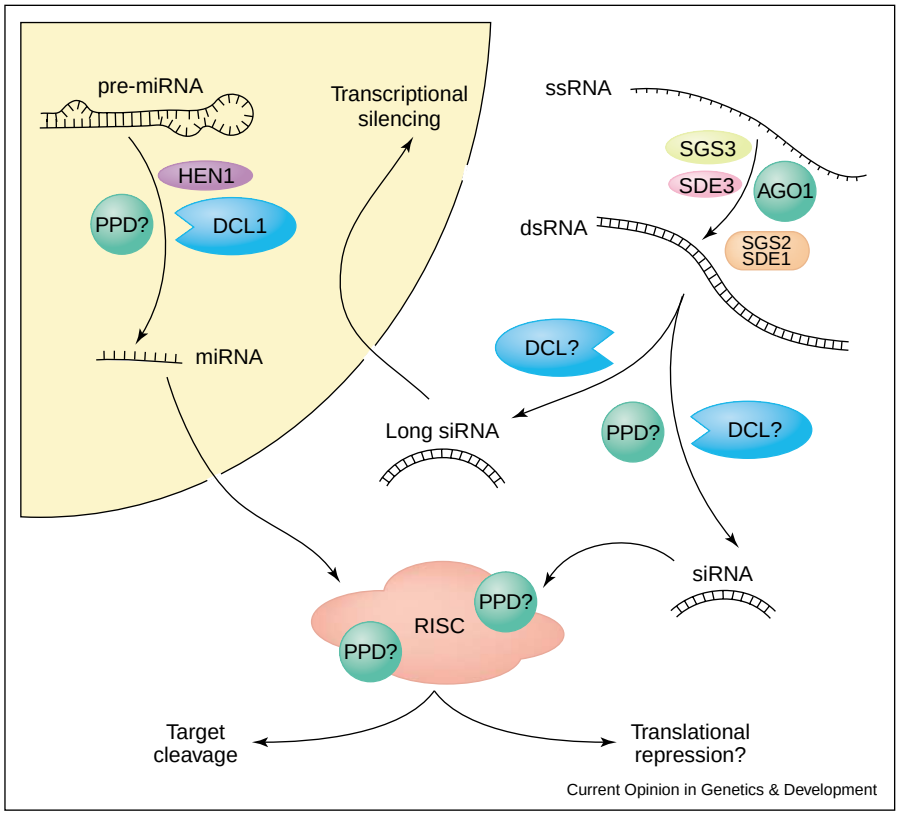
In this review, we first summarize the current understanding of miRNA-mediated gene regulation in plants. We then describe the developmental phenotypes of *Arabidopsis* genes involved in miRNA processing and activity, and comment on shared aspects of these phenotypes. Last, we discuss the intersection of the plant miRNA and siRNA pathways and its impact on our understanding of miRNA-regulated developmental events.

Plant miRNAs

In the summer of 2002, the Carrington [18•], Chen [19•] and Bartel [20•] groups reported the isolation of over 100 endogenously encoded miRNAs from *Arabidopsis* (many of which are conserved in rice, maize or tobacco). The fact that only a few of these miRNAs were isolated by more than one of the three groups suggested they represented a fraction of *Arabidopsis* miRNAs — an idea that is supported by the recent identification of another 280 miRNA sequences by the Zamore group [21••].

Detailed analysis of a subset of these small single-stranded RNAs showed that they have all of the hallmarks of miRNA: they are derived from the stem region of endogenously encoded stem-loop structures (although these structures can be larger and more complex that

Figure 1



Model of the miRNA and siRNA pathways in *Arabidopsis*. The processing of miRNA requires HEN1 and DCL1, both of which are predicted to function in the nucleus. The silencing-specific genes *SGS3*, *SDE3*, and *SGS2*/*SDE1* are predicted to function upstream of dsRNA production [41]; the dsRNA is then cleaved by an unidentified DCL protein to produce siRNA or long siRNA. Long siRNA enters the nucleus by an uncharacterized transport mechanism and mediates DNA methylation and transcriptional silencing. siRNA and miRNA may interact with the same RISC-like complex to direct target cleavage and possibly translational repression. The molecular function of the PPD proteins AGO1, PNH and AGO4 is unclear, but possible points of action are indicated, on the basis of work in other organisms.

the ~70-nt pre-miRNAs observed in animals), they range in size from 20 to 24 nt, and their production requires the activity of the *Arabidopsis* Dicer homolog, *DCL1* [18•–20•,21••,22•]. Interestingly, most of the 25 miRNAs detected by northern analysis showed temporal or spatial variations in expression, demonstrating that the transcription or processing of plant miRNAs is developmentally regulated [18•–20•,22•,23••].

All three groups also reported the identification of candidate target genes: mRNAs that contain sequences complementary to that of a miRNA [18•–20•]. An additional search for targets was carried out by the Bartel group, who found at least one mRNA target for 14 of the 16 miRNAs that they analyzed [24•]. Individual miRNAs were often complementary to multiple members of a gene family, and many of these families encoded transcription factors. In contrast to *C. elegans*, the plant mRNAs generally contained a single target site, often located in the open reading frame, with a high degree of complementarity to the miRNA. This led to the proposal that plant miRNAs mediate an siRNA-like target cleavage, rather than translational inhibition [24•].

Two groups have now demonstrated miRNA-mediated transcript cleavage in plants. The Carrington group initially examined the interaction of *Arabidopsis* miRNA39 (MIR171) with perfectly complementary targets from the *SCARECROW* family of transcription factors [25••], and then extended these studies to look at the targets of five other miRNAs [23••]. They detected mRNA cleavage products and determined that the 5′ end of these fragments corresponded to the site of miRNA:mRNA complementarity; they then co-transformed *Nicotiana benthamiana* with miRNA39 and its target gene to demonstrate miRNA-dependent target cleavage [23••,25••]. Their results also show that miRNA-directed target cleavage can tolerate some mismatches [23••].

The Zamore group [21••] also examined miRNA-directed cleavage as part of a detailed set of experiments characterizing siRNA and miRNA activity in wheat germ

extracts. They found an endogenous miRNA that cleaved the *PHAVOLUTA* (*PHV*) and *PHABULOSA* (*PHB*) transcripts but did not cleave *PHV* containing a single point mutation. Interestingly, this same sequence change is responsible for a dominant *phv* mutation in *Arabidopsis* [26•].

Developmental defects associated with miRNA processing proteins

Although the initial work on *Arabidopsis* miRNAs and targets is intriguing, no one has yet demonstrated a direct role for an miRNA during development. There are, however, plant homologs of several proteins involved in the processing and activity of small RNAs (Table 1). Examining the developmental consequences of mutations in these genes may shed some light on the requirement for miRNA-mediated gene regulation during plant development.

miRNA production: DCL1 and HEN1

Dicer molecules contain an RNA helicase domain, a PAZ domain (see below), and one or more RNase III domains. The *Arabidopsis* genome contains four predicted Dicer homologues, recently designated the Dicer-like (*DCL*) genes; of these, only *DCL1* has been characterized [27]. *dcl1* mutations that disrupt one of the two RNaseIII domains block miRNA cleavage, but do not affect siRNA production, suggesting that *DCL1* (or this domain of *DCL1*) is not involved in siRNA processing [22•].

The Waterhouse laboratory [22•] has suggested that the separate miRNA and siRNA pathways may prevent the siRNA synthesis that occurs during a viral response from disrupting miRNA-regulated developmental events. The two pathways may also operate in different cellular compartments because *DCL1* and *DCL4* have nuclear localization sequences, whereas *DCL2* and *DCL3* are predicted to be cytoplasmic [27]. The observation that long and short siRNAs may also interact with separate Dicers [21••] suggests that these enzymes may have distinct functions in plants.

Three independent isolations of *dcl1* mutations have identified a range of phenotypes associated with this gene. Null alleles of *dcl1* (originally known as *sus1* alleles) result in arrest before the heart stage of embryogenesis and cause overproliferation of cells in the suspensor [28]. A hypomorphic allele (originally known as *caf1*) that removes the carboxy-terminal dsRNA-binding domain causes narrow, occasionally filamentous, leaves and floral organs, and a loss of determinacy in the central region of the floral meristem. This mutation also results in an enlargement of the shoot apical meristem (SAM) and loss of axillary meristems [29].

Two other hypomorphic alleles with point mutations in the RNA helicase domain (originally known as *sin1* alleles) cause a reduction in plant size, delayed flowering, short internodes and female sterility owing to a failure in integument elongation [30,31]. *DCL1* is expressed widely but not ubiquitously [29,32]. Because only the maternal allele of *DCL1* is expressed after fertilization, hypomorphic mutations of the *sin-1* class have a maternal effect on embryo patterning [32,33].

The Chen laboratory [19•,34] has recently identified another mutation, *HUA enhancer 1* (*HEN1*), that results in a phenotype similar to that caused by the *caf* allele of *DCL1*. The *hen-1* mutation causes reduced plant size, pointed, upward-curled leaves, delayed flowering and

Table 1

***Arabidopsis* genes implicated in miRNA and siRNA pathways.**

Protein class	*Arabidopsis* members	Developmental phenotype	Silencing phenotype*	Small RNA processing	References
Dicer	DCL1	Null arrests in early embryogenesis; hypomorphs are late flowering with defects in organ polarity, floral morphology and floral meristem determinacy	Not known	Reduced levels of miRNA; no effect on siRNA	[17,20•,25••, 26•, 27–30]
PPD	AGO1	Defects in organ polarity; hypomorphs are late flowering	Required for PTGS	Not known	[33,34,40]
PPD	PNH	Fails to maintain SAM	None	Not known	[35,36•,37,39]
PPD	AGO4	None	Required for DNA methylation	Reduced levels of long siRNA	[42]
RdRP	SGS2/SDE1	None	Required for PTGS	Not known	[43,44]
RNA helicase	SDE3	None	Required for PTGS	Not known	[45]
Novel	HEN1	Small plants, curled leaves, late flowering, floral organ defects	Not known	Reduced levels of miRNA	[17,32]
Novel	SGS3	None	Required for PTGS	Not known	[43]

*RdRP, RNA-dependent RNA polymerase; PTGS, post-transcriptional gene silencing; SAM, shoot apical meristem.

defects in stamen and carpel specification [34]. Like *DCL1*, *HEN1* is required for the production of miRNAs [19•]. *HEN1* is expressed widely and encodes a novel protein with homologs in *Drosophila* and *C. elegans*. Interestingly, *HEN1* also has an nuclear localization sequence, suggesting that it may act in conjunction with *DCL1* to produce miRNAs in the nucleus [19•].

PPD proteins: AGO1, PNH, and AGO4

The PPD proteins (named for their shared PAZ and PIWI domains) comprise a large family of proteins that are required for developmental events in *C. elegans*, *Drosophila*, mice, humans and plants. The precise function of PPD proteins (including the role of the conserved domains) is still unclear, but members of this family are required for miRNA processing in *C. elegans* and have been found in association with *Drosophila* Dicer and RISC and with the human miRNP and RISC complexes (reviewed in [3]).

The *Arabidopsis* genome encodes ten predicted PPD proteins, of which three have been characterized. The founding member of the PPD family is the *Arabidopsis ARGONAUTE 1* (*AGO1*) gene, which is required for, among other events, establishing polarity in lateral organs [35]. *ago1* mutant plants have pointed, unexpanded cotyledons, narrow rosette leaves, radialized cauline leaves, narrow sepals and petals, and unfused carpels; similar to *dcl1* mutants, they lack axillary meristems [35]. Hypomorphic *ago1* alleles have serrate leaves and delayed flowering [36•].

AGO1 overlaps functionally with a second PPD protein encoded by the gene *PINHEAD* (*PNH*)/*ZWILLE* (*ZLL*) [37]. In *Arabidopsis*, *pnh* mutant plants terminate shoot production with a flat SAM or a single, often radialized, leaf structure; plants that escape this initial termination lack axillary meristems and produce short carpels [37–39]. Antisense constructs directed against the rice ortholog *OsPNH* have a similar phenotype, with the formation of a flat SAM that terminates in a tendril-like structure [40]. This phenotype suggests that, like *AGO1*, *PNH* is required to establish organ polarity and also has a role in maintaining undifferentiated cells in the SAM. The *pnh* and *ago1* double mutant arrests at the globular stage of embryogenesis, with overproliferation of the suspensor [37]. The Ray laboratory [32] has pointed out that *ago1;pnh* embryos phenocopy embryos containing null alleles of *dcl1*, supporting the idea that these genes function in the same pathway.

PNH is expressed in the shoot apex, vascular precursors and the adaxial leaf surface in *Arabidopsis*, and the rice homolog has a similar pattern of expression [37,39,40]. The Barton laboratory [41] has recently shown that ectopic expression of *PNH* causes a loss of determinacy in lateral organs, which supports the idea that *PNH* is involved in promoting an undifferentiated state. Unlike *PNH*, *AGO1* is broadly expressed; however, overexpression of the *AGO1* cDNA still disrupts development, causing the formation of cupped rosette leaves [35]. Interestingly, *AGO1* is a predicted target of miR168 [24•], suggesting its expression normally may be controlled by a feedback mechanism. The broad expression of *AGO1* may reflect a key difference in the function of *AGO1* and *PNH*. Whereas *AGO1* is required for PTGS and for development, *PNH* seems to have only developmental functions [36•,42,43].

In contrast to *PNH*, the most recently characterized *Arabidopsis* PPD gene, *ARGONAUTE 4* (*AGO4*), is required for PTGS but not development [44]. Isolated as a suppressor of the epigenetic *clark kent* alleles of *SUPERMAN*, the *ago4* mutation causes locus-specific reductions in DNA methylation and decreases levels of long siRNAs but has no observable affect on morphology [44]. This suggests that, similar to the DCL proteins, some plant PPD proteins may have specific functions in either the miRNA or the siRNA pathways.

PTGS proteins: SGS2/SDE1, SGS3 and SDE3

As suggested by the analysis of the Dicer and PPD proteins, some proteins (such as AGO1) operate in both the miRNA and the siRNA pathways, whereas others have only an miRNA-specific or siRNA-specific function. Several genes involved in PTGS have been identified by the Vaucheret [45] and Baulcombe [46,47] groups by screening for mutations that block the silencing of various types of transgene. Because none of these mutations has an overt effect on plant morphology, these genes are thought to be required specifically for PTGS. SGS2/SDE1 is one of six RNA-dependent RNA polymerases (RdRPs) encoded by the *Arabidopsis* genome [45].

SGS3 is a novel gene that may contain a nucleic acid binding domain [45,48]. *SDE3* encodes an RNA helicase, which is partially related to the *SMG-2* helicase of *C. elegans* [47]. As with RdRPs, the role of RNA helicases in plant miRNA-mediated pathways remains to be established. Two other genes identified in these screens, *SDE2* and *SDE4*, have yet to be cloned [46].

Conclusions: connecting miRNAs and developmental phenotypes

The phenotypes of mutations in genes implicated in miRNA regulatory pathways have certain features in common. Shoot-specific events—including the formation and maintenance of the shoot, floral and axillary meristems, the establishment of organ polarity and the timing of the vegetative to reproductive transition—all seem to be disproportionately affected by mutations in the putative miRNA signaling pathway. The Bartel group [24•] made a parallel observation about their predicted miRNA targets. They found that 34 of the 49 genes were transcription

factors, many with an established role in shoot or floral meristem development. The observation that both miRNA and siRNAs can travel over long distances has lead Jorgensen [49] to propose that miRNAs may comprise the 'florigen' that induces floral meristem production.

The next big step will be to connect the observed phenotypes to the activity of specific miRNAs and the expression of their downstream targets. Several tantalizing leads have been already reported, including the demonstration that dominant *phb* and *phv* mutations disrupt the site of miRNA complementarity [21••,24•,26•,50]. The Chen laboratory [19•] has also pointed out that loss of miR172 could increase expression of *APETALA2* (*AP2*), possibly accounting for the floral phenotype that they observed in their *hen1;hua1;hua2* mutant; supporting this theory is the observation that *ap2* mutations partially suppress the floral phenotype of this triple mutant.

Many issues remain about the mechanisms of miRNA-mediated regulation in plants. Although miRNA-mediated target cleavage no longer seems to be a plant-specific phenomenon [16], we still do not know whether plants can translationally repress targets that are not perfectly complementary to their cognate miRNA. It is also unclear how many components of the pathway are shared between plants and animals, whether homologs of *HEN1* and *SGS3* have a role in other organisms, and whether plants require counterparts like mammalian factors such as Gemins [14].

Finally, the separation of the plant miRNA and siRNA pathways requires further examination. To what extent do these two pathways use gene paralogs and do they truly function in separate cellular compartments? If there is a nuclear component of the miRNA machinery, what mediates the nuclear–cytoplasmic transport of miRNA complexes? It has been suggested recently that the human exportin 5 protein can export miRNA precursors from the nucleus [51]. This is intriguing, as mutations in the *Arabidopsis* exportin 5 gene, *HASTY*, cause defects in polarity, developmental timing and floral morphology that are reminiscent of defects resulting from mutations in genes in the miRNA pathway [52].

The intersection of the miRNA and siRNA pathways may also provide an inroad into the mechanism by which miRNA operate. A recent report has shown that the viral PTGS suppressor HcPro decreases siRNA levels but increases miRNA levels in tobacco [53]. A subsequent study in *Arabidopsis* detected the same effect of HcPro on miRNA levels, and also showed that overexpression of HcPro decreases the cleavage of the downstream targets of these miRNAs [23••]. This observation, coupled with the fact that the floral phenotype of these plants resembles that of *dcl1* mutants, suggests that HcPro interferes with the activity of miRNAs but not with their production. Why a defect in miRNA activity is associated with an increase in the amount of miRNAs is still unknown.

The discovery of miRNAs in plants and the growing evidence of their involvement in many aspects of plant growth and development has produced a great deal of excitement in plant biology. Although their specific functions remain to be identified, it is not unreasonable to think that miRNAs hold the solutions to many long-standing problems in this field. Given the current pace of research, these solutions may not be long in coming.

Update

Arabidopsis DCL1 has been shown to be subject to negative feedback regulation by the miRNA, *MIR162*, with elevated levels of *DCL1* mRNA detected in *dcl1* and *hen1* mutant plants. This result may explain the elevated levels of miRNAs observed when miRNA activity is inhibited by P1/Hc-Pro (Xie, Kasschau et al. 2003). In addition, *HEN1* has recently been shown to be required for S-PTGS. *HEN1*, *SGS2*, *SGS3*, and *AGO1* all affect the accumulation of S-PTGS-induced siRNAs, but only HEN1 is required for the production of the miRNA *MIR171* (Boutet, Vazquez et al. 2003).

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