RNA silencing: small RNAs as ubiquitous regulators of gene expression

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'RNA silencing' is the suppression of gene expression through nucleotide sequence-specific interactions that are mediated by RNA. Initially identified as an immune system that is targeted against transposons and viruses, RNA silencing is emerging as a fundamental regulatory process that is likely to affect many layers of endogenous gene expression in most, if not all, eukaryotes.

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Abbreviations

AGO	ARGONAUTE
ALG-1	ARGONAUTE-LIKE-1
CAF	CARPEL FACTORY
dsRNA	double-stranded RNA
IGR	intergenic region
miRNA	microRNA
nt	nucleotide
RDE	RNAi-DEFECTIVE
RISC	RNA-induced silencing complex
RNAi	RNA interference
siRNA	short interfering RNA
stRNA	short temporal RNA
UTR	untranslated region

Introduction

On the basis of biochemical and genetic evidence, many manifestations of RNA silencing — RNA interference (RNAi) in animals, quelling in fungi and post-transcriptional gene silencing (PTGS) in plants — appear to be mechanistically related. RNA silencing is experimentally activated by double-stranded RNA (dsRNA), and is used as a powerful technique for specific inhibition of gene expression in a variety of organisms [1–3]. In a natural context, dsRNA may be produced from rearranged loci, by transcription from converging promoters or by host- or viral-encoded RNA-dependent RNA polymerases [4–6]. A key component of RNA silencing is 21–23-nucleotide (nt) RNA known as short interfering RNA (siRNA) ([7,8•]; Box 1). In *Drosophila*, the siRNA is derived from dsRNA by the action of an RNaseIII-like enzyme named DICER [9••]. The siRNA guides a multi-subunit endonuclease, referred to as the RNA-induced silencing complex (RISC), and so ensures that it specifically cleaves RNA that shares sequence similarity with the inducing dsRNA ([10•,11•]; Figure 1b).

In addition to intracellular RNA silencing, silencing state can also be transmitted between cells and over long distances [12,13,14•]. The signal for systemic silencing is likely to incorporate a nucleic acid because it mediates a nucleotide-sequence-specific effect. Biological roles of RNA silencing include protection of the genome against mobile DNA elements [15–18] and resistance against viruses [19,20], many of which produce dsRNAs during their replication. Non-cell-autonomous RNA silencing probably represents the systemic arm of this antiviral defence [21]. Until recently, RNA silencing had been envisaged merely as a form of nucleotide-sequence-directed immunity leading to RNA degradation. However, recent studies have identified new layers of complexity in the RNA-silencing pathway and have provided evidence that other levels of gene expression, including translation and transcription, can be targeted. These studies progressively unravel a second, fundamental role for RNA silencing in the regulation of endogenous gene expression. They also provide compelling evidence of a major regulatory role for noncoding DNA in both animals and plants.

Short temporal RNA in *C. elegans*

The discovery of RNAi and the ubiquity of siRNAs have given new insights into a particular type of developmental regulation that is orchestrated by the *lin-4* and *let*-7 short temporal RNAs (stRNAs) in *Caenorhabditis elegans* (Box 1). These stRNAs do not encode proteins and are expressed at specific larval stages [22–24]. Their sequence partially complements the 3′ untranslated regions (UTRs) of several target mRNAs that are involved in the developmental-timing pathway [24–27,28•]. Expression of *lin-4* and *let*-7 coincides with the repression of translation of these mRNAs through a process that has not yet been identified, but is thought to be triggered by base-pairing between the stRNA and its targets (Figure 1a; [23,29]). Loss-of-function mutations of *lin-4* and *let-7* cause altered cell division and cell-fate determination during larval development [28•,30]. The link with RNA silencing came from several observations. First, *lin-4* and *let*-7 are 22 nt in length, which, not coincidentally, is also the size of the siRNA. Second, both stRNAs were predicted to be processed from longer, bulged and partially double-stranded precursor RNAs that resemble, at least partly, the dsRNA initiators of RNAi [26,28•]. Finally, and most compelling, knocking out the activity of DCR-1, the *C. elegans* DICER homologue, gave rise to heterochronic phenotypes similar to those caused by the *lin-4* and *let*-7 mutations. It turns out that DCR-1, which is required for dsRNA processing in RNAi, is also required for maturation of the stRNA precursors ([31••–33••]; Figure 1a).

MicroRNAs in animals

It seemed likely that the *lin-4* and *let*-7 story was not an isolated case. The search for new stRNAs was facilitated by the fact that, like siRNAs, they are produced by an RNaseIII-like protein (i.e. DICER), and thus have 2-nt-long 3′ overhangs and 5′ phosphate/3′ hydroxyl ends [8•,34]. On the basis of these unique biochemical features, a cloning procedure was developed to selectively capture endogenous RNAs of 22–25 nt in length that have the characteristics of stRNA or siRNAs. With the help of bioinformatics and the availability of full-genome sequences, three teams worked together to identify almost 100 new small RNAs from *C. elegans*, *Drosophila* and human tissues, which they termed microRNAs (miRNAs) (Box 1; [35••–37••]). Some miRNAs were highly conserved, not only between species but also across phyla. Nearly all miRNAs are predicted to be processed from precursors that are strikingly similar to the stRNA precursors. Hence, they are likely to be products of DICER, as has actually been demonstrated for two of them [37••]. Like stRNAs, some miRNAs appear to be expressed at specific life stages, suggesting that they have a developmental role. Some miRNA precursor sequences are clustered, sometimes so tightly that it is likely that several miRNAs are produced from a single transcript, potentially allowing their coordinate regulation and effect. Like *lin-4* and *let*-7, the vast majority of miRNA loci appeared to be located within intergenic regions (IGRs) where they are probably expressed as independent transcription units, previously unidentified because they do not contain an open reading frame.

Box 1. A guide to 21–25nt RNAs.

Short interfering (si)RNA
- Double stranded with 3′ overhangs of two nucleotides
- Perfectly complementary to target RNA
- Directs endonucleolytic cleavage
- Precursor:
  - Perfect or nearly perfect RNA duplex
  - Chromosomal or cytoplasmic
  - >25 base pairs in length

Micro (mi)RNA
- Single stranded
- Partially complementary to target RNA
- Unknown mechanism of action
- Often conserved between species or even across phyla
- Precursor:
  - Bulged, partially double-stranded RNA
  - Chromosomal (IGR)
  - ≦70 nucleotides in length

Short temporal (st)RNA
- Single stranded
- Partially complementary to target RNA
- Inhibits translation initiation
- Highly conserved between species and across phyla
- Precursor:
  - Bulged, partially double-stranded RNA
  - Chromosomal (IGR)
  - ≦70 nucleotides in length

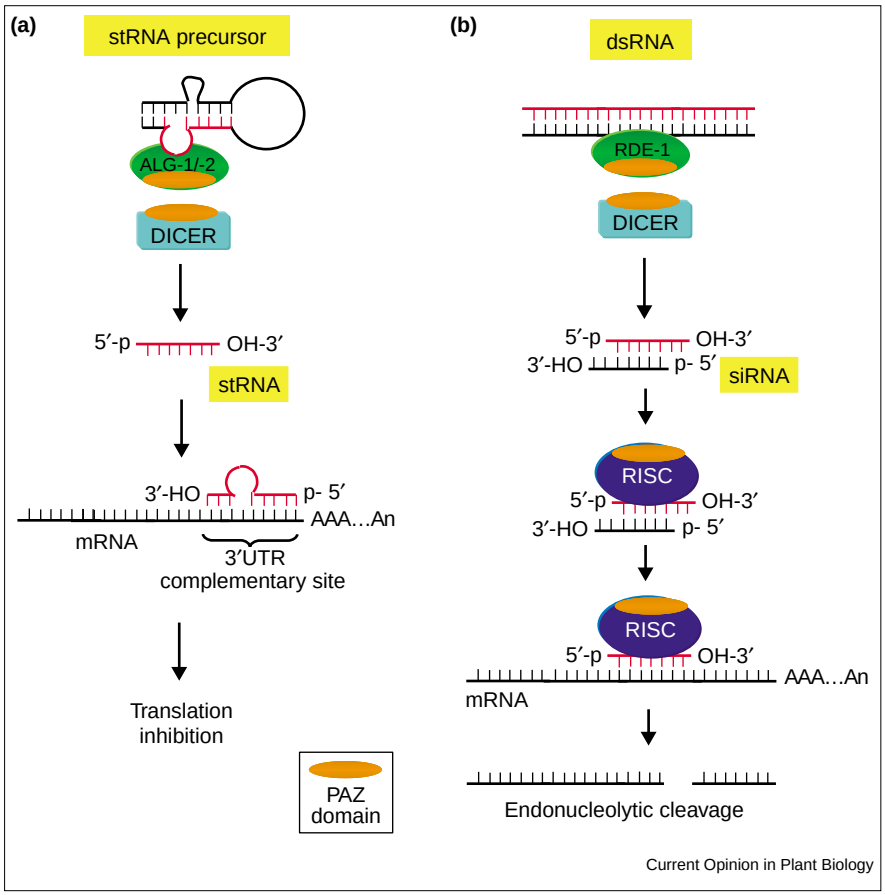
The main characteristics of each small RNA type and its precursor based on studies carried out in *C. elegans* (worm), *Drosophila melanogaster* (fly), *Homo sapiens* (human) and *A. thaliana* (plant). The only short temporal RNAs characterised to date are the *let-7* and *lin-4* RNAs in *C. elegans*. These properties of the small RNAs should not be considered as strict rules for classification. For instance, there is at least one example of a conserved miRNA in plants that is perfectly complementary to the coding sequence of three related mRNAs, suggesting that it has a siRNA-like mode of action (see text).

Endogenous small RNAs and microRNAs in plants

Endogenous RNAs with biochemical features of siRNAs have been cloned recently in *Arabidopsis*. In one study, small RNAs were extracted from inflorescence tissues, ligated to form concatemeres, amplified and subsequently sequenced. A total of 125 distinct small RNAs were identified, which showed sequence homology to more than 530 genomic loci represented equally on the five *Arabidopsis* chromosomes [38••]. As observed for the animal miRNAs, 90% of the cloned small RNAs from *Arabidopsis* matched IGR sequences and likely originated from independent transcription events. All of the small RNA analysed in this study showed tissue-specific accumulation, suggesting that the composition of the small RNA population is unique in different cell types and tissues [38••]. Some of the genomic loci of the small RNAs were highly clustered. However, in contrast to the situation in animals, they often contained overlapping small RNA sequences. Furthermore, the small RNAs derived from clustered precursor loci in *Arabidopsis* were sometimes found to occur in both sense and antisense orientation, whereas animal miRNAs are represented in only one polarity (Box 1). The predicted structures of the *Arabidopsis* IGR RNA precursors were also heterogeneous, with some containing short and simple stem-loop structures like those in animal miRNA precursors [35••–37••] whereas others were predicted to form much more extensive and longer duplexes [38••,39••]. Overall, the endogenous small RNAs in *Arabidopsis* appear to be more diverse than those in animals and may arise from multiple biosynthetic mechanisms.

While confirming this diversity, a second study focussed on *Arabidopsis* small RNAs that had features of cognate miRNAs (Box 1). Sixteen of these were identified and shown to have differential expression patterns at various developmental stages [39••]. Importantly, the accumulation of *Arabidopsis* miRNA was compromised in plants that were deficient for CARPEL FACTORY (CAF; also known as SHORT INTEGUMENT1 [SIN1]). CAF has been implicated in the regulation of stem-cell fate [40,41], and belongs to a family of at least four proteins that have amino-acid domains that are characteristic of the *Drosophila* DICER [9••]. This result strongly suggests that CAF is the plant homologue of DICER and that similar mechanisms direct miRNA processing in plants and animals. Significantly, of the sixteen miRNAs analysed in *Arabidopsis*, eight were absolutely conserved in the rice genome [39••]. This conservation, together with the known roles of CAF in *Arabidopsis* embryos, leaves and floral meristems, suggests that miRNAs play key regulatory roles in plant development.

Figure 1



Comparison of the stRNA and siRNA pathways in *C. elegans*. By recognising specific types of precursor RNAs, related factors such as ALG-1/ALG -2 and RDE-1 may partly influence processing by DICER and, more generally, may selectively control entry into the siRNA or stRNA pathways. Interactions with DICER may occur through the PAZ domain. **(a)** The stRNA, which is single-stranded, is thought to base-pair imperfectly with the 3′ UTR of the target mRNA, repressing the initiation of translation. **(b)** The siRNA, which is double-stranded and has 3′ overhangs of two nucleotides (nt), is incorporated into the RNA-induced silencing complex that may interact with DICER through the PAZ domain of one of its constituents. The siRNA serves as guide for RISC and, upon perfect base-pairing, the target mRNA is cleaved in the middle of the duplex formed with the siRNA.

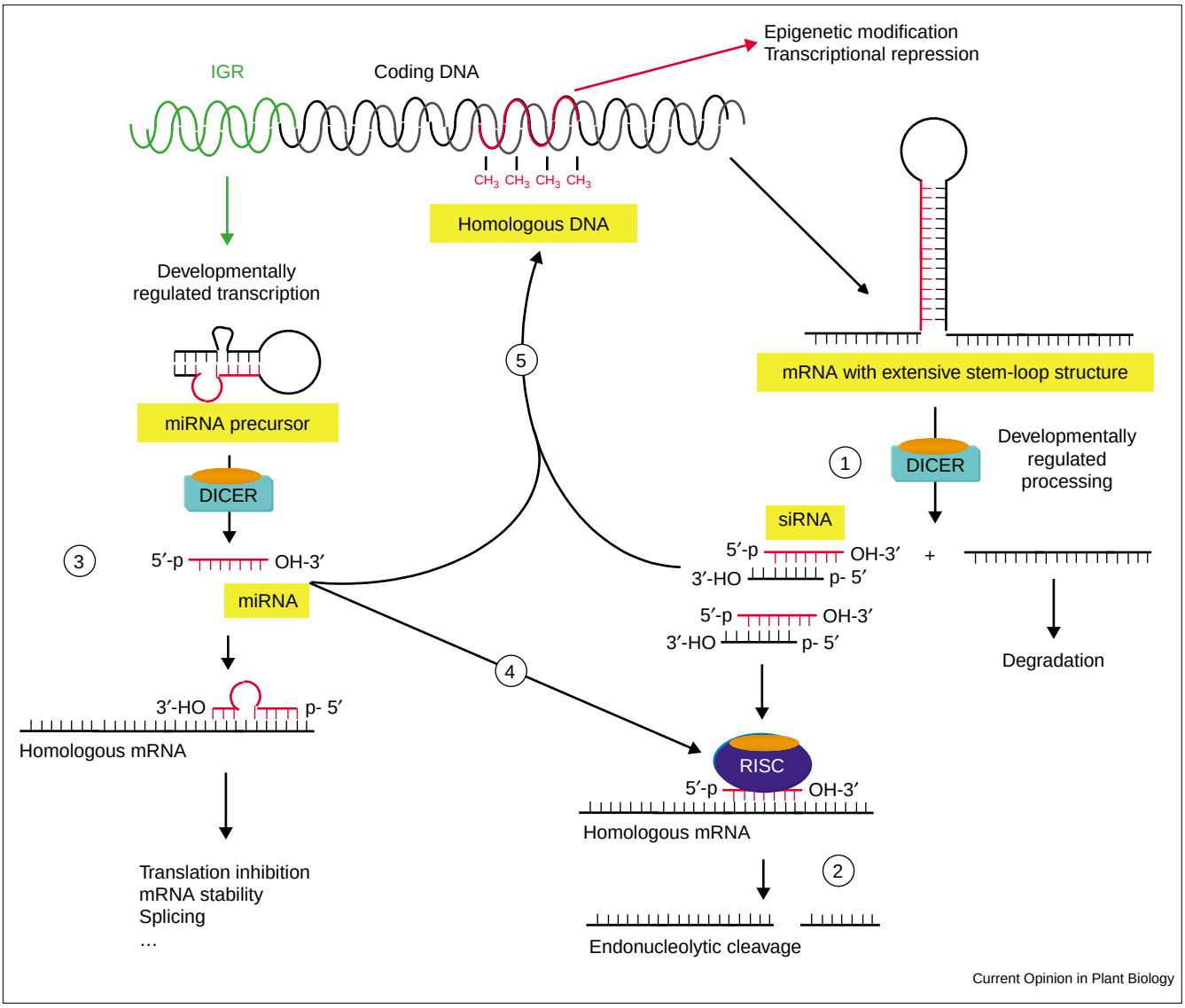
The RDE-1/ARGONAUTE family

The *lin-4* and *let*-7 stRNAs, in contrast to cognate siRNAs, are only imperfectly complementary to the 3′ UTR of their target mRNAs ([26,28•]; Box 1, Figure 1). RNA degradation that is mediated by siRNA is extremely sensitive to sequence mismatches and length alteration [34], and this feature may partly explain why stRNAs cause translational repression rather than endonucleolytic cleavage of their targets. Another difference between stRNAs and siRNAs is that, although they are both DICER products, maturation of the stRNA is asymmetric and results in the accumulation of a single RNA strand [32••] whereas the siRNA is double-stranded because of the symmetric processing of its precursor [7,9••]. Asymmetric processing also produces plant and animal miRNAs.

Processing may be influenced by the structure or sequence of the precursor itself (i.e. perfect duplex versus bulged dsRNA), its subcellular localisation or the particular factors with which it interacts. Members of the RNAi-DEFECTIVE (RDE)/ARGONAUTE (AGO) protein superfamily, which are well conserved in structure and gene-silencing function among many eukaryotes, may indeed act as specificity factors that influence the fate of precursor RNAs (Figure 1). In *C. elegans*, for instance, RDE-1 is essential for the initiation of RNAi [15] but is dispensable for maturation of the *lin-4* and *let*-7 precursors ([31••]; Figure 1b). By contrast, ARGONAUTE-LIKE-1 (ALG-1) and ALG-2, two RDE-1 homologues, are required for stRNA processing but not for RNAi ([31••]; Figure 1a). Accordingly, the *rde-1* mutant is morphologically normal, whereas reduction of ALG-1 and ALG-2 protein content causes developmental defects reminiscent of those caused by the *lin-4* and *let-7* mutations [31••].

RDE-1, ALG-1 and ALG-2 are, in fact, representatives of 24 closely related proteins in *C. elegans*, and it is conceivable that each of these proteins determines the processing of particular types of small RNAs, depending on the nature of their precursors (Figure 1). Selective processing could rely on direct protein–protein interactions between specific RDE/AGO homologues and DICER, possibly mediated by the PAZ amino-acid domain [42] that is found in both types of proteins (Figure 1). At least ten *RDE/AGO* genes have been identified in *Arabidopsis*. The product of one of them, AGO1, has been implicated in RNA silencing [43] and, like RDE-1, was genetically positioned upstream of the dsRNA-processing step [44•,45]. Although several *ago1* mutants are developmentally abnormal and sterile, point mutations have been identified recently that uncouple the

Figure 2



Several layers of regulation of gene expression possibly mediated by small RNAs in plants. (1) mRNAs with extensive stem-loop structure could be developmentally processed by DICER, which would eventually lead to degradation. (2) siRNAs produced by such processing could then be involved in endonucleolytic cleavage of related mRNAs. (3) miRNAs produced from IGR-encoded precursors could participate in a variety of regulatory processes affecting, for instance, mRNA translation, stability or splicing. (4) miRNAs that are perfectly complementary to the coding region of some mRNA could also be recruited as siRNAs. (5) siRNAs and miRNAs may direct sequence-specific epigenetic modifications of homologous DNA. For example, homology with promoter sequences could cause transcriptional gene silencing.

roles of AGO1 in silencing and in development: some mutants were hypomorphic but retained a full silencing-deficient phenotype [46•]. This suggests that AGO1 might combine the RDE-1-like silencing and ALG-1/ALG-2-like developmental functions in a single molecule.

Identifying targets

Few, if any, of the miRNAs identified in *C. elegans* and *Drosophila* have the characteristics of siRNA, that is, are double-stranded and perfectly complementary to protein-coding genes ([8•,36••]; Box 1). In fact, only siRNAs with transposon or viral sequences have been identified, suggesting that siRNAs may serve exclusively defensive purposes in these organisms. Thus, many of the animal miRNAs are likely to function as stRNAs (Box 1) by annealing imperfectly to mRNA targets and modulating their translation (Figure 1a). Several of the IGR-derived miRNAs detected in *Arabidopsis* may be similar to stRNAs because they are single stranded, accumulate in a tissue-specific manner, and are not perfectly complementary to protein-coding genes [38••,39••]. In both plants and animals, these imperfectly matched miRNAs could affect processes other than translation, such as mRNA splicing, localisation or stability (Figure 2). For instance, a large

subset of miRNAs have been identified in *Drosophila* that complement several classes of 8-nt 3′ UTR sequence motifs (the K-box, Brd-box and GY-box motifs), which are known to alter not only translation efficiency but also transcript stability [47•]. In many cases, however, the imperfect base-pairing between miRNAs and their targets precludes a straightforward computer-based identification of interacting partners from genome sequence data. Gene knockout, increased gene dosage or misexpression of the miRNA precursors may help to address this problem.

Another potential approach relies on the use of viral-encoded proteins that suppress RNA silencing [48–51]. They represent one of several strategies that viruses have evolved to counteract this defence mechanism [19,20]. Transgenic expression of some silencing suppressors in plants causes several developmental aberrations, which could be explained, at least partly, by misregulation of the miRNA pathway [52,53]. If this hypothesis is confirmed, analysis of global changes in gene expression at the RNA and protein levels in such transgenic plants could provide a powerful handle for the identification of miRNA targets. Moreover, as silencing suppressors appear to act against different stages of the silencing mechanism [51,53–55], each protein is likely to provide a unique and original set of information. Although the vast majority of suppressors have been identified in plant viruses, the isolation of a silencing suppressor that is encoded by an insect-infecting virus has been reported recently [56•], opening the possibility that a similar strategy could also be employed in animal cells.

Perfect complements

Several of the small RNAs that were identified in *Arabidopsis* have perfect complementarity to protein-encoding genes. Some correspond to genes from long terminal repeat (LTR) and non-LTR retrotransposons, as well as to transposons [38••]. A separate study also identified siRNAs that corresponded to three retroelements from tobacco and *Arabidopsis* [57••]. Taken together, these results suggest that transposons are probable targets of RNA silencing in higher plants, as shown previously in animals and *Chlamydomonas* [15–18]. In contrast to the observations made in animals, however, several *Arabidopsis* small RNAs also showed perfect complementarity to nontransposon protein-encoding genes [38••,39••].

A first type of *Arabidopsis* small RNAs corresponded to genes that were predicted to yield mRNAs or pre-mRNAs that form exceptionally stable stem-loop structures (Figure 2[1]). One such mRNA encoded a Ser carboxypeptidase-like protein and another a protein of unknown function [38••]. Small RNAs of both sense and antisense polarity that complemented the Ser carboxypeptidase-like mRNA were detected in similar abundance, a characteristic of siRNAs (Box 1). This suggests at least two possible modes of regulation in *cis* and *trans*. First, the stability of these extensively base-paired mRNAs could be directly regulated through processing by DICER/CAF, which could occur at specific developmental stages (Figure 2). For instance, some *C. elegans* miRNAs are expressed only as longer precursors at some developmental stages, suggesting that their processing rather than their transcription is regulated [36••,37••]. Second, siRNAs that are generated by the processing of these highly structured mRNAs could function in the subsequent RNA silencing of homologous or closely related sequences (Figure 2[2]).

A second type of perfectly matched small RNAs mapped to both IGRs and protein-coding genes (Figure 2). For example, an IGR located on chromosome III of *Arabidopsis* had the potential to form a stable stem structure that yielded an inflorescence-specific small RNA with the characteristics of a miRNA (Box 1). Unlike other miRNAs, however, it was completely complementary to the central region of three related mRNAs [38••,39••]. These mRNAs belong to the scarecrow-like family of plant transcription factors, which are known to be involved in several signalling and developmental processes. In this case, the IGR-derived small RNA could trigger the site-specific cleavage of its targets (Figure 2). This would require the incorporation of the small RNA into a RISC-like complex that has a sequence-specific endonucleolytic function, as shown for siRNA in *Drosophila* [10•].

Further complexity: small RNA size classes, epigenetic control and signalling

Recent work involving viral-encoded suppressors of RNA silencing and *Arabidopsis* silencing-defective mutants shows the existence of two size classes of siRNA in plants, each with distinct roles [57••]. Long (24–26 nt) siRNAs appear to be dispensable for sequence-specific mRNA degradation, but correlate with systemic silencing in *Nicotiana benthamiana* and methylation of retroelement sequences in *Arabidopsis*. Conversely, the short (21–22 nt) siRNA class correlates with mRNA degradation but not with systemic signalling or retroelement methylation. These findings reveal a further level of complexity in the RNA silencing pathway that may well apply to the many endogenous small RNAs identified in *Arabidopsis*, which were found to occur in similar size classes [38••].

The difference in the physical length of the two classes suggests that distinct small RNAs could be produced by distinct DICER homologues with, for example, specific subcellular localisation [58•]. The resulting small RNAs could then be involved in specific branches of the RNA-silencing pathway. Thus, the long siRNAs that are involved in retrotransposon methylation [57••] could be produced by a nuclear DICER homologue [58•]. Endogenous small RNAs of the long-size class may also arise from nuclear processing. They may direct the sequence-specific epigenetic modifications that are involved in plant physiology and development [59] or may trigger the transcriptional inactivation of nuclear genes (Figure 2[5]). Indeed, in plants, nuclear-encoded

dsRNAs with homology to promoter sequences trigger the transcriptional repression of the corresponding genes, which coincides with the synthesis of promoter-specific small RNAs and promoter methylation [58•,60,61•]. Small RNAs of a specific size may also be involved in cell-to-cell and long distance signalling. Local signalling near sites of small RNA synthesis could communicate epigenetic modifications, resulting, for instance, in patterning. Systemic signalling may be involved in the regulation of other processes such as commitment to flower formation [62].

Conclusions

The widespread occurrence and evolutionary conservation of miRNAs provide a new paradigm of the regulation of gene expression in eukaryotes. The developmental expression, sequence diversity and shear size of this class of tiny RNAs gives us a glimpse of the complexity and the refinement of the many biological processes that these molecules are likely to orchestrate. Other types of endogenous, noncoding small RNAs have been detected in plants, including some with features of siRNAs. Although their significance remains to be experimentally established, they are also likely to have regulatory roles, perhaps as signalling molecules or epigenetic modifiers. The initial screens for small RNAs are far from saturating, in either animals or plants, suggesting that the currently available data are just the tip of the iceberg. The discovery of so many IGR-encoded RNAs is also a stunning reminder of how little we know about the vastness of information contained in DNA. Many challenges lie ahead in answering questions such as how many types of small RNA and how many biosynthetic mechanisms exist? What are their targets? How is their expression controlled? And what is their mode of action?

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Together with the studies described in [32••] and [33••], this remarkable work identifies DCR-1 as the *C. elegans* DICER homologue and shows that it is necessary for the production of the *lin-4* and *let-7* stRNAs, paving the way to the identification of the highly abundant microRNAs. The authors also characterise two homologues of RDE-1 (known to be required for RNAi [15]), which are essential for processing of stRNAs but dispensable for RNAi. This suggests that specific RDE-1-like proteins may selectively control entry into the RNAi or stRNA pathways.

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This work shows that a human homologue of DICER is important for processing the *let-7* stRNA precursor RNA in cultured human cells, suggesting that the regulatory interactions observed in *C. elegans* [31••,33••] are highly conserved.

33. •• Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH: Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in ***C. elegans***. *Genes Dev* 2001, 15:2654-2659.
See annotation [31••].

34. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T: Functional anatomy of siRNAs for mediating efficient RNAi in ***Drosophila melanogaster*** embryo lysate. *EMBO J* 2001, 20:6877-6888.

35. •• Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T: Identification of novel genes coding for small expressed RNAs. *Science* 2001, 294:853-858.
See annotation [37••].

36. •• Lau NC, Lim LP, Weinstein EG, Bartel DP: An abundant class of tiny RNAs with probable regulatory roles in ***Caenorhabditis elegans***. *Science* 2001, 294:858-862.
See annotation [37••].

37. •• Lee R, Ambros V: An extensive class of small RNAs in ***Caenorhabditis elegans***. *Science* 2001, 294:862-864.
Together with those described in [35••] and [36••], this study describes the identification of a large number of small RNAs with potential regulatory roles in worm, fly and human cells. Most of these newly identified RNAs are encoded in intergenic or intronic regions that are predicted to form approximately 70-nt stem-loop RNAs, very similar to the stRNA precursors. Like stRNAs, they are represented in only one polarity. However, their endogenous targets and mode of action are yet to be identified. The authors collectively refer to these small RNA as micro (mi)RNAs. The work in [37••] shows that the processing of two miRNAs is DICER-dependent. None of these studies have identified a single miRNA with perfect complementarity to a protein-encoding gene.

38. •• Llave C, Kasschau KD, Rector MA, Carrington JC: Endogenous and silencing-associated small RNAs in plants. *Plant Cell* 2002, 14:1-15.
A cloning and sequencing strategy, similar to that used in [35••,36••], is employed to identify small RNAs from *Arabidopsis* inflorescence tissues. More than a hundred unique small RNAs are discovered. 90% of these small RNAs are encoded in intergenic regions and arise by transcription events that are independent of adjacent conventional genes. However, the *Arabidopsis* small RNAs and their precursors appear to be much more heterogeneous than the animal miRNAs. Several small RNAs that are identical to retroelement and transposon sequences are identified, demonstrating, together with the study in [57••], that RNA silencing is a natural defence against foreign DNA elements. Several small RNAs with perfect identity to nontransposon protein-encoding genes are found, suggesting a possible regulatory role via RISC-mediated endonucleolytic cleavage of target RNAs.

39. •• Reinhart BJ, Weinstein EG, Rhoades M, Bartel B, Bartel DP: MicroRNAs in plants. *Genes Dev* 2002, 16:1-15.
A cloning and sequencing approach is used to identify sixteen *Arabidopsis* small RNAs with characteristics of cognate miRNAs. Many are shown to have differential patterns of expression during development, and eight are perfectly conserved in the rice genome. In addition, this paper strongly suggests that CARPEL FACTORY (CAF) is the plant DICER homologue because it is required for the processing of the miRNAs.

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44. • Beclin C, Boutet S, Waterhouse P, Vaucheret H: A branched pathway for transgene-induced RNA silencing in plants. *Curr Biol* 2002, 12:684-688.
Using transgenes that encode hairpin-forming RNAs, the authors position several *Arabidopsis* mutations that were known to affect transgene-induced gene silencing in the silencing pathway. The study shows that AGO1 [43], like the *C. elegans* RDE-1, acts upstream of the dsRNA-processing step.

45. Grishok A, Tabara H, Mello C: Genetic requirements for inheritance of RNAi in ***C. elegans***. *Science* 2000, 287:2494-2497.

46. • Morel J-B, Gordon C, Mourrain P, Beclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H: Fertile hypomorphic ***ARGONAUTE*** (***ago1***) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* 2002, 14:629-639.
The authors identify additional alleles of the *ago1* mutation in *Arabidopsis*. Some of the mutations uncouple the developmental role of AGO1 from its function in RNA silencing. It is also shown that a loss-of-function mutation in *ZWILLE/PINHEAD*, a close relative of *AGO1*, causes developmental abnormalities but does not affect transgene silencing. A parallel is made with the distinct roles of RDE-1, ALG-1 and ALG-2 in RNAi and development in *C. elegans*.

47. • Lai EC: Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002, 30:363-364.
A ‘candidate’ bioinformatics approach is employed to identify potential targets for several *Drosophila* miRNAs. Three 8-nt-long 3′ UTR sequence motifs that are known to be involved in post-transcriptional regulation are analysed for possible complementarity to miRNA sequences. Many significant matches are found but, strikingly, perfect complements to the three

sequence motifs are located exclusively in the 5′ ends of all miRNAs analysed. Most of the known *lin-4* and *let-7* target sequences also involve perfect complements with the 5′ end of these stRNAs. Although these findings need to be experimentally tested, they suggest a possible pattern governing the regulatory effects of some miRNAs.

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56. • Li H, Li WX, Ding SW: Induction and suppression of RNA silencing by an animal virus. *Science* 2002, 296:1319-1321.
A suppressor of RNA silencing is identified in an animal virus for the first time. This study opens a new area of investigation in animals, in which the potential antiviral role of RNA silencing has been surprisingly under-investigated so far.

57. •• Hamilton AJ, Voinnet O, Chappell L, Baulcombe DC: Two classes of short interfering RNA in RNA silencing. *EMBO J* 2002, in press.
Using a transient expression assay in tobacco, the authors identify two size classes of siRNAs that are selectively affected by viral-encoded silencing suppressors. Inhibition of the longer siRNA class correlates with the suppression of systemic silencing but does not prevent RNA degradation in the sites of silencing initiation, suggesting that the short RNA class is sufficient to ensure RNA turnover after incorporation into RISC. siRNAs corresponding to three retroelements are found to belong exclusively to the long-size class. The long siRNA corresponding to the *AtSN1* element is absent in one silencing-defective mutant of *Arabidopsis*, correlating with the hypomethylation of the AtSN1 DNA in this mutant.

58. • Mette MF, Matzke AJM, Matzke MA: Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. *Curr Biol* 2001, 11:1119-1123.
HC-Pro, a viral suppressor of RNA silencing, is shown to be ineffective in preventing the accumulation of small RNAs produced from nuclear-encoded promoter dsRNA, which are known to trigger the transcriptional repression of homologous genes. This result contrasts with the previous demonstration that HC-Pro inhibits the production of siRNAs that are associated with transgene- and virus-induced gene silencing. This suggests that compartmentalisation and/or alternative dsRNA processing may influence the fate of small RNAs in the gene-silencing pathway in plants. The authors suggest that a nuclear-localised DICER homologue could specifically process the small RNAs that are involved in transcriptional silencing.

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Together with the studies described in [58•] and [60], this study strongly suggests that 21–23-nt RNAs have the potential to direct sequence-specific, *de novo* methylation of DNA in plants. This work also provides evidence that the promoter sequences of an endogenous gene can be affected by this process, reinforcing the notion that small RNAs have the potential to act as epigenetic modifiers of gene expression.

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