Review

Short RNAs in environmental adaptation

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Non-coding small RNAs (19–24 nucleotide long) have recently been recognized as the important regulator of gene expression in both plants and animals. Several classes of endogenous short RNAs have partial or near perfect complementarity to mRNAs and a protein complex is guided by short RNAs to target mRNAs. The targeted mRNA is either cleaved or its translation is suppressed. Initially, short RNAs were believed to primarily regulate the normal development of plants and animals, but recent advances implicate short RNAs in environmental adaptation.

Keywords: RNA interference; post-transcriptional gene silencing; microRNA; small interfering RNA; regulation of gene expression; environmental stress

1. INTRODUCTION

Short non-coding RNA molecules acting in different gene silencing pathways have recently been recognized as important members of the gene expression regulatory network. This article gives a brief historical overview of the discovery of gene silencing and short RNAs, a detailed description of the silencing machinery and the function of this mechanism. It is generally accepted that short RNAs are important for the normal development of plants and animals and many papers have reviewed this aspect of these tiny molecules. However, a role for short RNAs in environmental adaptation is emerging thus the last section of the article will focus on this area.

2. A BRIEF HISTORY OF THE DISCOVERY OF GENE SILENCING AND SHORT RNA

The fascinating story of short RNAs started in 1990 when post-transcriptional gene silencing (PTGS) was first described in plants (Napoli *et al.* 1990). Petunia plants with purple flowers were transformed with an extra copy of the chalcone synthase (*CHS*) gene, which determines the colour of the flower. It was expected that an extra copy of *CHS* would make the colour of the flower darker due to the increased enzyme activity. However, instead of elevated CHS levels, some of the transgenic plants showed a complete lack of CHS activity and mRNA, and possessed white flowers. Since the transgenic and endogenous activity of *CHS* genes was downregulated at the same time, the phenomenon was named co-suppression (Napoli *et al.* 1990). Two important features of co-suppression were that the *CHS* mRNA level was unchanged in the nucleus, but was downregulated in the cytoplasm and that only the *CHS* mRNA was degraded. These observations suggested that the underlying mechanism was post-transcriptional and sequence-specific. Similar post-transcriptional and gene-specific RNA degradation processes also occur in other plant systems, even when there is no homologous endogenous copy of the transgene present or if it is induced by plant viruses (Baulcombe 2004). Therefore, the term PTGS was coined to describe different silencing mechanisms whether triggered by transgenes, viruses or other factors (table 1). Several years later, very similar observations were made with *Caenorhabditis elegans*. Worm genes were targeted by antisense RNA, which was thought to prevent translation by annealing to the complementary mRNA sequences (Guo & Kemphouse 1995). Antisense RNA molecules were generated by *in vitro* transcription, but unexpectedly the negative control experiments—using *in vitro*-made sense RNA—also resulted in gene inactivation. In 1998, Fire and colleagues followed upon these results and discovered that both the sense and antisense RNA batches contained some double-stranded (ds) RNA because of the way they were generated *in vitro*. Injection of dsRNA was much more effective in inhibiting gene expression than either sense or antisense RNA and the dsRNA-triggered silencing mechanism was called RNA interference (RNAi; Fire *et al.* 1998). It was later confirmed that dsRNA is the trigger of PTGS in plants and also of quelling in fungi (Waterhouse *et al.* 1998; Goldoni *et al.* 2004). In 1999, a class of short RNAs was discovered in tomato plants that exhibited gene silencing of a specific transgene (Hamilton & Baulcombe 1999). Hamilton & Baulcombe revealed that a predominantly 21 nucleotide (nt) short RNA species could be detected in all examined silenced plants, but was missing from the non-silenced plants. These 21 nt RNA molecules were sequence-specific for the gene targeted by PTGS and both sense and antisense strands were present. Later, a short RNA species of the same size was detected in animals and fungi and was called small interfering RNA (siRNA; Zamore *et al.* 2000; Catalanotto *et al.* 2002).

3. MECHANISM OF RNA INTERFERENCE/ POST-TRANSCRIPTIONAL GENE SILENCING

Long dsRNA molecules in cells can originate from different sources, e.g. *in vitro* generated and injected transcripts, viruses, transposons, etc., but they are all

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Table 1. Explanation of nomenclature of short RNAs and other terms used in the gene silencing field.

name	description
post-transcriptional gene silencing (PTGS)	an umbrella term for all gene-silencing phenomena (sequence-specific RNA degradation) in plants that act in the cytoplasm and do not affect transcription in the nucleus
virus-induced gene silencing (VIGS)	a specific case of PTGS where the trigger is a replicating viral RNA
co-suppression	a specific case of PTGS when a transgene-induced silencing mechanism suppresses the transgene and a homologous endogenous gene at the same time
RNA interference (RNAi)	the name of a sequence-specific RNA degradation mechanism in animal cells that is very similar to PTGS
microRNA (miRNA)	a single-stranded 20–22 nucleotide short RNA that is encoded by the host genome; produced from a longer precursor RNA molecule with a characteristic stem-loop structure where the miRNA is in one arm of the stem; only one double-stranded miRNA is produced from one stem-loop structure; one of the strands is called mature miRNA that is incorporated into RISC and regulates the expression of mRNAs
small interfering RNA (siRNA)	double-stranded 20–22 nucleotide short RNA molecules that are either produced from long double-stranded RNA by Dicer or synthesized *in vitro*; if generated from long dsRNA, many siRNA is produced from one dsRNA molecule; siRNAs are incorporated into RISC and mediate mRNA cleavage
trans-acting endogenous siRNA (ta-siRNA)	a specific type of 20–22 nucleotide siRNA molecules in plants; generated from non-coding transcripts encoded by the plant genome following miRNA-mediated cleavage of the primary transcript that is then turned into dsRNA and processed by DCL-4; several ta-siRNAs are produced from one locus and they are in the same phase (i.e. do not overlap with each other and there is no gap between them); also incorporated into RISC and target mRNAs
chromatin siRNA	a specific type of 24–26 nucleotide siRNA molecules in plants; generated from repeat or transposon sequences in the plant genome and act in the nucleus; they cause transcriptional gene silencing by heterochromatin formation through methylation of chromosomal DNA
natural antisense siRNA (nat-siRNA)	a specific type of 20–22 nucleotide siRNA molecules in plants; derived from naturally occurring overlapping complementary transcripts that can form dsRNA and are processed by Dicer; they are incorporated into RISC and target mRNAs

processed by the RNaseIII type dsRNA-specific endonuclease called Dicer (Bernstein *et al.* 2001). Dicer has two RNaseIII domains (a and b) and key amino acids in each domain have been identified, which are important in the formation of a catalytic site (Zhang *et al.* 2004). Aspartic and glutamic acids at positions 44 and 110, respectively, from each RNaseIII domain constitute a catalytic site, with residues from RIIIa cleaving one strand and residues from RIIIb cleaving the opposite strand (Zhang *et al.* 2004). The products are double-stranded siRNAs 19–24 nt in length with $3'$ overhangs of two nucleotides and have $3'$ hydroxy and $5'$ phosphate termini (Bernstein *et al.* 2001; Elbashir *et al.* 2001b). Duplex siRNAs are then unwound, which requires ATP (Nykanen *et al.* 2001), and one of the strands of the siRNA duplex is incorporated into a nuclease complex called the RNA-induced silencing complex (RISC; Hammond *et al.* 2000). However, the two strands of a given siRNA duplex were not found to be equally represented in RISC (Khvorova *et al.* 2003; Schwarz *et al.* 2003). The strand that is assembled into RISC is dependent upon the thermodynamic stability of the two ends of the duplex. The strand whose $5'$ end is more weakly bound to the complementary strand can be more readily incorporated into RISC (Khvorova *et al.* 2003; Schwarz *et al.* 2003). The protein, which can sense the strength of the binding between the two siRNA strands at each end is called R2D2 in *Drosophila* and RDE-4 in *C. elegans* (Tomari *et al.* 2004). Although R2D2 forms a heterodimer with Dicer, it is not required for the cleavage by Dicer, but facilitates siRNA passage from Dicer to RISC. It binds to the siRNA end with the stronger bond

between the two strands and orientates the R2D2–Dicer heterodimer on the siRNA duplex. Strong binding of R2D2 to the duplex requires the presence of a $5'$-phosphate on the siRNA strand that is not incorporated into RISC. Thus, R2D2 does not only sense the thermodynamic asymmetry of siRNA duplexes, but also checks the validity of siRNAs (Tomari *et al.* 2004).

The siRNA strand that is incorporated into RISC recognizes specific mRNAs through base pairing to complementary stretches of sequence, and in this way guides RISC to the appropriate targets. The siRNA-loaded RISC cleaves the mRNA once, at the position opposite the tenth nucleotide of the siRNA, without affecting the RISC incorporated siRNA. Other nucleases then complete the mRNA degradation process (Elbashir *et al.* 2001b). The RISC component responsible for cleaving the mRNA was originally named Slicer, without knowing the identity of the protein. Later it was discovered that Slicer is Argonaute 2 (AGO2) in animals (Meister *et al.* 2004; Liu *et al.* 2004) and AGO1 in plants (Baumberger & Baulcombe 2005). AGO1 and AGO2 are members of the Argonaute protein family, which are defined by the presence of PAZ and PIWI domains (Carmell *et al.* 2002). The Piwi Argonaute Zwilk (PAZ) domain was shown to be an RNA-binding domain (Joshua-Tor 2004) and Song *et al.* (2003) showed that the structure of the PIWI domain is very similar to the structure of ribonuclease H enzymes, which cleave the RNA strand of DNA/RNA hybrid duplexes. At the same time, a functional study of human Argonaute proteins revealed that although siRNAs bind to AGO1,2,3 and 4,

only AGO2 was required for cleavage of target mRNAs (Meister *et al.* 2004; Liu *et al.* 2004). The role of the other Argonaute proteins still remains a question.

4. NATURAL FUNCTION OF RNA INTERFERENCE/ POST-TRANSCRIPTIONAL GENE SILENCING

RNA interference and PTGS have been studied for several years without knowing the reason for its evolution. In both plants and animals, the mechanism was revealed accidentally by expressing dsRNAs. Initially, it became clear that in plants, RNAi is an effective antiviral defence mechanism (Voinnet 2001). Virus-specific siRNAs are present in virus-infected plants (Hamilton & Baulcombe 1999) and interestingly, viruses have in turn developed a counter defence strategy by producing proteins, which can suppress RNAi (Voinnet 2001). Viruses with mutated suppressor genes infect plants with a much lower efficiency (Voinnet *et al.* 1999). The best-characterized viral suppressor is P19, which can specifically bind to siRNAs recognizing their size and 3' overhangs (Vargason *et al.* 2003; Ye *et al.* 2003). P19 can also bind to siRNAs in animal cells (Lakatos *et al.* 2004). Proteins with RNAi-suppressing activity also have been identified in viruses infecting animals (Li *et al.* 2002, 2004; Delgadillo *et al.* 2004; Bucher *et al.* 2004) but no virus-specific siRNAs have been found in virus-infected animal cells (Pfeffer *et al.* 2004). However, recently it was shown that short RNAs encoded by the host genome can target viruses in human cells explaining why animal viruses also developed silencing suppressor proteins (Lecellier *et al.* 2005).

Another function of RNAi was found by analysing transposon activity in RNAi deficient *C. elegans*. Transposon activity increased dramatically in worms where some RNAi components were knocked out (Tabara *et al.* 1999). Similarly, algae cells mutant in an RNA helicase required for RNAi showed higher transposon activity than wild type (Wu-Scharf *et al.* 2000). Although the exact mechanism is not known, it is thought that transposons produce dsRNA, which is targeted by RNAi. In fact, siRNAs have been detected in wild type plants with probes specific for certain transposons (Hamilton *et al.* 2002).

RNAi is also implicated in DNA-related silencing mechanisms (for a recent review see: Lippman & Martienssen 2004). It was shown in fission yeast that siRNAs mediate heterochromatic silencing and genes homologous to known components of the RNAi machinery (AGO, Dicer and RNA-dependent RNA polymerase) were required for this silencing (Volpe *et al.* 2002). Heterochromatin is a dense chromosomal material that remains condensed throughout the cell cycle, unlike the rest of the chromosome, which decondenses between cell divisions to allow transcription. In the last few years, it has become clear that heterochromatic silencing in fungi and plants is dependent upon the processing of transcripts from repeat sequences into siRNAs, which then leads to chromatin modification (Lippman & Martienssen 2004).

Initially, the function of RNAi in mammalian cells was less clear since there was little evidence that it was a defence mechanism against viruses and transposons or that it was linked with natural heterochromatic silencing. Recently, however, it was shown that siRNAs can cause chromosomal DNA methylation and suppression of transcription in human cells if they target promoter

sequences (Morris *et al.* 2004). In fact, in the early days of RNAi discovery, somatic mammalian cells were not amenable to RNAi at all. It was thought that only long dsRNA could trigger RNAi and although long dsRNA can induce sequence-specific RNA degradation in early mouse embryos (Wianny & Zernicka-Goetz 2000), in somatic mammalian cells it also switches on several pathways which lead to non-sequence specific RNA degradation, general transcription arrest and apoptosis (for reviews see Kumar & Carmichael 1998; Gill & Esteban 2000). It was only discovered in 2001 that sequence-specific RNAi can be initiated in somatic mammalian cells by using siRNAs instead of long dsRNA (Elbashir *et al.* 2001*a*). However, the question remained: why is there Dicer expression in somatic mammalian cells if it is not required for defence against viruses and transposons, and long dsRNA activates other defence pathways? The answer was found almost 10 years ago, but it did not fall into its place in the jigsaw until recently. In 1993, a short non-coding RNA called lin-4 was identified by map-based cloning from *C. elegans* (Lee *et al.* 1993). It was classified as a short temporal RNA (stRNA) since it was not constitutively expressed and regulated the expression of heterochronic genes governing the normal development of the worm. Several years later a very similar gene, *let-7*, was identified in the same organism (Reinhart *et al.* 2000). Both genes produce a primary transcript, with a stem–loop secondary structure, from which a shorter, mature form is generated. One of the striking features of these two stRNAs was that the mature RNAs were 21 and 22 nts long, respectively (Lee *et al.* 1993; Reinhart *et al.* 2000). Their identical size to siRNAs raised the possibility that both siRNAs and the two stRNAs were produced by Dicer, which was later confirmed (Hutvagner *et al.* 2001). The stem–loop structure of the precursor stRNA transcripts seemed to be strong enough that Dicer can recognize them as dsRNAs, despite the bulges within the stem regions. The next important discovery was that there are many other short, non-coding RNAs with similar features (precursor RNA with stem–loop structure and 19–24 nt mature form) present in both plants and animals (reviewed by Pasquinelli 2002). This class of short, non-coding RNAs are now called microRNAs (miRNAs) because not all of them are expressed temporarily. More than 300 miRNA genes have been identified in the human genome, finally explaining the natural role of Dicer in somatic mammalian cells (Griffiths-Jones 2004). The precursor miRNAs, generated by another RNaseIII type enzyme Drosha in the nucleus (Lee *et al.* 2003), are cleaved by Dicer and a siRNA like molecule is released (miRNA duplex), which follows the path of an siRNA (reviewed by Bartel 2004). After ATP-dependent unwinding, one of the strands of the non-perfect duplex is incorporated into RISC and the activated complex then targets mRNAs containing complementary sequences to the miRNA. Animal miRNAs have complementary target sites at 3' untranslated regions (UTRs) of mRNAs, however, these target sequences are not perfect matches to the miRNAs (Bartel 2004). It was shown that positions 2–8 on the miRNAs must be perfectly complementary to the target, but mismatches are allowed in the rest of the duplex (Doench *et al.* 2003). Because of the mismatches between the miRNA and its target, the miRNA-loaded RISCs do not cleave the target mRNAs, but inhibit translation (Lee *et al.*

1993; Bartel 2004). In contrast, plant miRNA target sites are usually within the open reading frame and all characterized plant miRNAs mediate cleavage of their target mRNAs due to the near perfect complementarity (Baulcombe 2004). If an animal miRNA is perfectly complementary to its target, it does cleave the mRNA as shown by Yekta *et al.* (2004).

Large-scale cloning of 19–24 nts RNAs from diverse organisms has revealed several different classes of short RNAs, other than miRNAs (Llave *et al.* 2002; Ambros *et al.* 2003; Aravin *et al.* 2003; Sunkar & Zhu 2004). The main difference between miRNAs and other short RNAs is that precursors of miRNAs can be folded into a stem–loop structure, but flanking genomic sequences of other short RNAs cannot form a double-stranded structure that can be recognized by Dicer. Different names were coined for different classes of short RNAs in animals: short RNAs from repeat rich genomic regions were called repeat-associated silencing RNAs in *Drosophila* (Aravin *et al.* 2003) and repeat-associated siRNAs in zebrafish (Chen *et al.* 2005) whereas short RNAs that are not conserved even within *Caenorhabditis* species were named tiny non-coding RNAs (Ambros *et al.* 2003). The nomenclature is more unified in the plant field: short RNAs that target mRNAs in trans are called *trans*-acting endogenous small interfering RNAs (ta-siRNAs; Peragine *et al.* 2004; Vazquez *et al.* 2004) and short RNAs targeting chromosomal DNA are called chromatin siRNAs (Herr 2005). The biogenesis of animal short RNAs that are not miRNAs is very poorly understood, however, the process is much better understood in plants. Ta-siRNAs are derived from non-coding RNAs that are initially transcribed as single-stranded RNAs from five loci in *Arabidopsis* (Peragine *et al.* 2004; Vazquez *et al.* 2004). Two miRNAs (miR-173 and miR-390) can recognize these transcripts and mediate cleavages at the target sites (Allen *et al* 2005). The cleaved RNA molecules are then turned into dsRNA by RdR6 and processed by one of the four Dicer-like proteins (DCL4; Xie *et al.* 2005; Yoshikawa *et al.* 2005). The miRNA mediated cleavages set the phase for DCL4 processing, ensuring the production of specific ta-siRNAs. Very recently, a third class of plant siRNA was described by Borsani *et al.* (2006): natural antisense siRNAs (nat-siRNAs) are produced from dsRNA formed by two natural antisense overlapping mRNAs. The complexity of expressed short RNAs is much higher than previously expected, at least in plants where about 75 000 expressed short RNAs have recently been identified (Lu *et al.* 2005a). This vast number suggests that there will be further twists in the story of short RNA biogenesis.

5. SHORT RNAS AND ENVIRONMENTAL ADAPTATION

As many predicted miRNA targets are transcription factors involved in development, it was initially believed that miRNAs were mainly necessary for the normal development of plants and animals. The role of miRNAs in development and disease has been extensively reviewed (Chen 2005; Harfe 2005; Alvarez-Garcia & Miska 2005; Du & Zamore 2005; Sullivan & Ganem 2005; Wienholds & Plasterk 2005) and is not the subject of this review. Instead, this review will focus on the role of micro and other short RNAs in the adaptation of animals and plants to environmental change.

At the time of the writing of this review, there were only two reports on miRNAs and stress in animal systems (Xu *et al.* 2003; Dresios *et al.* 2005). Mir-14 was identified in a genetic screen for inhibitors of apoptopic cell death by testing P element insertion lines for their ability to enhance an eye phenotype associated with eye-specific expression of the cell death activator Reaper. Mir-14 suppresses cell death and is required for normal fat metabolism. Deletion of mir-14 causes enhanced Reaper-dependent cell death and results in animals with increased level of triacylglycerol and diacylglycerol and reduced lifespan. Loss of mir-14 is also associated with increased sensitivity to salt stress and was the first example for a miRNA involved in any stress relation process. The other report on miRNAs and stress in animals suggested that miRNAs play a role in dampening protein synthesis during cold stress (Dresios *et al.* 2005). Several genes are induced in animal cells by mild hypothermia and one of them is the RNA-binding motif protein 3 (Rbm3), a glycine-rich RNA-binding protein (Derry *et al.* 1995). Cells overexpressing Rbm3 show enhanced protein synthesis at both 37 and 32 °C and have an altered sedimentation profile on sucrose gradient compared with wild type cells. The relative abundance of a complex between the top of the gradient and 40S subunits was decreased in the presence of Rbm3. The RNA content of this complex was proved to be an RNA species around 20 nts long. Northern blot analysis confirmed that this RNA species contains at least one—but probably more-known miRNA (Dresios *et al.* 2005). This data suggests that miRNAs are part of a homeostatic mechanism in animal cells that control global protein synthesis. However, the exact mechanism of miRNAs regulating gene expression as a response to environmental changes in animal cells is not yet known. One of the reasons is that the level of complementarity between miRNAs and target mRNAs is low in animals, making it difficult to predict target genes. It is considerably easier in plants and therefore it is not a surprise that we know more about the role of plant miRNAs in environmental adaptation than in animal systems. Another explanation is that miRNAs play a more significant role in environmental adaptation in plants than they do in animals. The first such role of miRNAs in plants was described by Jones-Rhoades & Bartel (2004). They predicted novel miRNAs conserved in *Arabidopsis* and rice and after identifying almost a hundred new miRNAs they predicted the genes targeted by these new miRNAs. Many target genes were transcription factors involved in development, but some miRNAs were predicted to target genes not involved in development: superoxide dismutases, laccases and ATP sulphurylases. The expression of one particular miRNA (miR-395) showed very strong increase upon sulphate starvation, showing that miRNAs can be induced by environmental stress. The predicted target gene (*ATP sulphurylase 1*) expression showed inverse correlation with miR-395 accumulation suggesting that it was indeed targeted by miR-395 (Jones-Rhoades & Bartel 2004). The discovery of other plant miRNAs regulated by environmental stresses soon followed. Sunkar & Zhu (2004) cloned short RNAs from *Arabidopsis* seedlings exposed to several abiotic stresses and identified several miRNAs with different expression profiles. MiR-393 was

strongly upregulated by cold, dehydration, NaCl and abscisic acid treatments; miR-397b and miR-402 were slightly upregulated by all stress treatments while miR-319c was only induced by cold, but not the other treatments; however, mir-389a was downregulated by all of the stress treatments. In addition, a putative ubiquitin conjugating enzyme (UBC) was predicted to be targeted by miR-399, although this miRNA was not regulated by any of the applied stresses (Sunkar & Zhu 2004). Mir-399 was studied further and two groups showed independently that its expression is regulated by phosphate level in the media (Fujii *et al.* 2005; Chiou *et al.* 2005). Low phosphate level strongly induced the expression of miR-399 and the expression of predicted target gene was suppressed in the same condition. A peculiar feature of the target gene is that the predicted multiple miRNA target sites are at the $5'$ UTR. The expression of a transgene without the $5'$ UTR was not suppressed under low-phosphate conditions whereas a transgene containing the $5'$ UTR was suppressed in such conditions. Low phosphate-induced primary root growth inhibition was much weaker in transgenic plants expressing the miRNA-deregulated version of UBC (without the $5'$ UTR). Plants constitutively overexpressing miR-399 showed low level of accumulation of UBC even in high-phosphate condition and accumulated more phosphate than wild type plants. These data showed that miR-399 controls phosphate homeostasis in plants by regulating the expression of a proteolysis machinery component and suggest that miRNAs have an important role in the concerted response to fluctuations in mineral-nutrient level in the soil (Chiou *et al.* 2005; Fujii *et al.* 2005). However, this is not their only function in adapting to the environment, miRNAs also play a role in adaptation to mechanical stresses. Ten miRNAs have been identified in poplar that are not present in the *Arabidopsis* genome (Lu *et al.* 2005*b*) suggesting roles in tree-specific characteristics. These miRNAs were up- or downregulated in woody stems and were involved in tree-specific corrective growth against tension and compression stresses (Lu *et al.* 2005*a,b*). In addition to miRNAs, other plant short RNAs are also involved in environmental gene expression regulation. A drought stress-inducible abscisic acid biosynthesis enzyme (AAO3) is a predicted target of a short RNA identified by Sunkar & Zhu (2004). However, the best-characterized short RNA, that is not a miRNA, involved in environmental adaptation is a nat-siRNA (Borsani *et al.* 2006). Pyrroline-5-carboxylate dehydrogenase (*P5CDH*) and *SRO5*, a gene of unknown function, are overlapping antisense genes. The expression of *SRO5* is induced by salt stress and this induction is required for the accumulation of a 24 nt nat-siRNA from the overlapping region between the two mRNAs, as well as several PTGS proteins (DCL2, RdR6, SGS3 and NRPD1A). This 24 nt nat-siRNA mediates cleavage of the *P5CDH* mRNA and sets the phase for the subsequent generation of 21 nt nat-siRNAs that will mediate further cleavages of the *P5CDH* transcript. Downregulation of *P5CDH* leads to proline accumulation which is important for salt tolerance (Borsani *et al.* 2006). These examples are likely to be only the beginning of understanding the role short RNAs play in environmental adaptation. The vast number of short RNA molecules, at least in plants, has been only recently recognized (Lu *et al.* 2005*a,b*). To understand the

impact of approximately 75 000 short RNAs on gene expression is a huge task, even in *Arabidopsis*. In addition, the realization that short RNAs are not necessarily conserved between plant species implies that the number of plant short RNAs is much higher than the 75 000 found in *Arabidopsis*. It is also an attractive hypothesis that plant species possessing the ability to adapt to extreme environments may express short RNAs missing from *Arabidopsis* and those short RNAs are involved in the adaptation process. Projects to explore this idea are presently being undertaken in the author's laboratory.

REFERENCES

Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. 2005 MicroRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. *Cell* **121**, 207–221. (doi:10.1016/j.cell.2005.04.004)

Alvarez-Garcia, I. & Miska, E. A. 2005 MicroRNA functions in animal development and human disease. *Development* **132**, 4653–4662. (doi:10.1242/dev.02073)

Ambros, V., Lee, R. C., Lavanway, A., Williams, P. T. & Jewell, D. 2003 MicroRNAs and other tiny endogenous RNAs in *C. elegans*. *Curr. Biol.* **13**, 807–818. (doi:10.1016/S0960-9822(03)00287-2)

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. & Tuschl, T. 2003 The small RNA profile during *Drosophila melanogaster* development. *Dev. Cell* **5**, 337–350. (doi:10.1016/S1534-5807(03)00228-4)

Bartel, D. P. 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297. (doi:10.1016/S0092-8674(04)00045-5)

Baulcombe, D. C. 2004 RNA silencing in plants. *Nature* **431**, 356–363. (doi:10.1038/nature02874)

Baumberger, N. & Baulcombe, D. C. 2005 *Arabidopsis* Argonaute1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc. Natl Acad. Sci. USA* **102**, 11 928–11 933. (doi:10.1073/pnas.0505461102)

Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. 2001 Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366. (doi:10.1038/35053110)

Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. & Zhu, J. K. 2006 Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell* **123**, 1279–1291. (doi:10.1016/j.cell.2005.11.035)

Bucher, E., Hemmes, H., de Haan, P., Goldbach, R. & Prins, M. 2004 The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. *J. Genet. Virol.* **85**, 983–991. (doi:10.1099/vir.0.19734-0)

Carmell, M. A., Xuan, Z., Zhang, M. Q. & Hannon, G. J. 2002 The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev.* **16**, 2733–2742. (doi:10.1101/gad.1026102)

Catalanotto, C., Azzalin, G., Macino, G. & Cogoni, C. 2002 Involvement of small RNAs and role of the *qde* genes in the gene silencing pathway in Neurospora. *Genes Dev.* **16**, 790–795. (doi:10.1101/gad.222402)

Chen, X. 2005 MicroRNA biogenesis and function in plants. *FEBS Lett.* **579**, 5923–5931. (doi:10.1016/j.febslet.2005.07.071)

Chen, P. Y. *et al.* 2005 The developmental miRNA profiles of zebrafish as determined by small RNA cloning. *Genes Dev.* **19**, 1288–1293. (doi:10.1101/gad.1310605)

Chiou, T. J., Aung, K., Lin, S. I., Wu, C. C., Chiang, S. F. & Su, C. L. 2005 Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* **30**, 412–421. (www.plantcell.org/cgi/doi/10.1105/tpc.105.038943)

Delgadillo, M. O., Saenz, P., Salvador, B., Garcia, J. A. & Simon-Mateo, C. 2004 Human influenza virus NS1 protein enhances viral pathogenicity and acts as an RNA silencing suppressor in plants. *J. Genet. Virol.* **85**, 993–999. (doi:10.1099/vir.0.19735-0)

Derry, J. M., Kerns, J. A. & Francke, U. 1995 RBM3, a novel human gene in Xp11.23 with a putative RNA-binding domain. *Hum. Mol. Genet.* **4**, 2307–2311.

Doench, J. G., Petersen, C. P. & Sharp, P. 2003 siRNAs can function as miRNAs. *Genes Dev.* **17**, 438–442. (doi:10.1101/gad.1064703)

Dresios, J., Aschrafi, A., Owens, G. C., Vanderklish, P. W., Edelman, G. M. & Mauro, V. P. 2005 Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc. Natl Acad. Sci. USA* **102**, 1865–1870. (doi:10.1073/pnas.0409764102)

Du, T. & Zamore, P. D. 2005 MicroPrimer: the biogenesis and function of microRNA. *Development* **132**, 4645–4652. (doi:10.1242/dev.02070)

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. 2001*a* Duplexes of 21-nt RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498. (doi:10.1038/35078107)

Elbashir, S. M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. 2001*b* Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* **20**, 6877–6888. (doi:10.1093/emboj/20.23.6877)

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. 1998 Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811. (doi:10.1038/35888)

Fujii, H., Chiou, T. J., Lin, S. I., Aung, K. & Zhu, J. K. 2005 A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* **15**, 2038–2043. (doi:10.1016/j.cub.2005.10.016)

Gill, J. & Esteban, M. 2000 Induction of apoptosis by the dsRNA-dependent protein kinase (PKR): mechanism of action. *Apoptosis* **5**, 107–114. (doi:10.1023/A:1009664109241)

Goldoni, M., Azzalin, G., Macino, G. & Cogoni, C. 2004 Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*. *Fungal Genet. Biol.* **41**, 1016–1024. (doi:10.1016/j.fgb.2004.08.002)

Griffiths-Jones, S. 2004 The microRNA registry. *Nucleic Acids Res.* **32**, D109–D111. (doi:10.1093/nar/gkh023) (data base issue)

Guo, S. & Kemphouse, K. J. 1995 par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611–620. (doi:10.1016/0092-8674(95)90082-9)

Hamilton, A. J. & Baulcombe, D. C. 1999 A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952. (doi:10.1126/science.286.5441.950)

Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. 2002 Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671–4679. (doi:10.1093/emboj/cdf464)

Hammond, S. M., Bernstein, E., Beach, D. & Hannon, G. J. 2000 An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296. (doi:10.1038/35005107)

Harfe, B. D. 2005 MicroRNAs in vertebrate development. *Curr. Opin. Genet. Dev.* **15**, 410–415. (doi:10.1016/j.gde.2005.06.012)

Herr, A. J. 2005 Pathways through the small RNA world of plants. *FEBS Lett.* **579**, 5879–5888. (doi:10.1016/j.febslet.2005.08.040)

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. & Zamore, P. D. 2001 A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**, 834–838. (doi:10.1126/science.1062961)

Jones-Rhoades, M. W. & Bartel, D. P. 2004 Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* **14**, 787–799. (doi:10.1016/j.molcel.2004.05.027)

Joshua-Tor, L. 2004 siRNAs at RISC. *Structure* **12**, 1120–1122. (doi:10.1016/j.str.2004.06.008)

Khvorova, A., Reynolds, A. & Jayasena, S. D. 2003 Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209–216. (doi:10.1016/S0092-8674(03)00801-8)

Kumar, M. & Carmichael, G. G. 1998 Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. *Microbiol. Mol. Biol. Rev.* **62**, 1415–1434.

Lakatos, L., Szittya, G., Silhavy, D. & Burgyan, J. 2004 Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J.* **23**, 876–884. (doi:10.1038/sj.emboj.7600096)

Lecellier, C. H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saib, A. & Voinnet, O. 2005 A cellular microRNA mediates antiviral defense in human cells. *Science* **308**, 557–560. (doi:10.1126/science.1108784)

Lee, R. C., Feinbaum, R. L. & Ambros, V. 1993 The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854. (doi:10.1016/0092-8674(93)90529-Y)

Lee, Y. *et al.* 2003 The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419. (doi:10.1038/nature01957)

Li, H., Li, W. X. & Ding, S. W. 2002 Induction and suppression of RNA silencing by an animal virus. *Science* **296**, 1319–1321. (doi:10.1126/science.1070948)

Li, W. X. *et al.* 2004 Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. *Proc. Natl Acad. Sci. USA* **101**, 1350–1355. (doi:10.1073/pnas.0308308100)

Lippman, Z. & Martienssen, R. 2004 The role of RNA interference in heterochromatic silencing. *Nature* **431**, 364–370. (doi:10.1038/nature02875)

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. & Hannon, G. J. 2004 Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441. (doi:10.1126/science.1102513)

Llave, C., Kasschau, K. D., Rector, M. A. & Carrington, J. C. 2002 Endogenous and silencing-associated small RNAs in plants. *Plant Cell* **14**, 1605–1619. (doi:10.1105/tpc.003210)

Lu, C., Tej, S. S., Luo, S., Haudenschild, C. D., Meyers, B. C. & Green, P. J. 2005*a* Elucidation of the small RNA component of the transcriptome. *Science* **309**, 1567–1569. (doi:10.1126/science.1114112)

Lu, S., Sun, Y. H., Shi, R., Clark, C., Li, L. & Chiang, V. L. 2005*b* Novel and mechanical stress-responsive micro-RNAs in *Populus trichocarpa* that are absent from Arabidopsis. *Plant Cell* **17**, 2186–2203. (doi:10.1105/tpc.105.033456)

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. & Tuschl, T. 2004 Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* **15**, 185–197. (doi:10.1016/j.molcel.2004.07.007)

Morris, K. V., Chan, S. W., Jacobsen, S. E. & Looney, D. J. 2004 Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**, 1289–1292. (doi:10.1126/science.1101372)

Napoli, C., Lemieux, C. & Jorgensen, R. 1990 Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in *trans*. *Plant Cell* **2**, 279–289. (doi:10.1105/tpc.2.4.279)

Nykanen, A., Haley, B. & Zamore, P. D. 2001 ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321. (doi:10.1016/S0092-8674(01)00547-5)

Pasquinelli, A. E. 2002 MicroRNAs: deviants no longer. *Trends Genet.* **18**, 171–173. (doi:10.1016/S0168-9525(01)02624-5)

Peragine, A., Yoshikawa, M., Wu, G., Albrecht, H. L. & Poethig, R. S. 2004 SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* **18**, 2368–2379. (doi:10.1101/gad.1231804)

Pfeffer, S. *et al*. 2004 Identification of virus-encoded microRNAs. *Science* **304**, 734–736. (doi:10.1126/science.1096781)

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. & Ruvkun, G. 2000 The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**, 901–906. (doi:10.1038/35002607)

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. & Zamore, P. D. 2003 Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199–208. (doi:10.1016/S0092-8674(03)00759-1)

Song, J. J., Liu, J., Tolia, N. H., Schneiderman, J., Smith, S. K., Martienssen, R. A., Hannon, G. J. & Joshua-Tor, L. 2003 The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat. Struct. Biol.* **10**, 1026–1032. (doi:10.1038/nsb1016)

Sullivan, C. S. & Ganem, D. 2005 MicroRNAs and viral infection. *Mol. Cell* **20**, 3–7. (doi:10.1016/j.molcel.2005.09.012)

Sunkar, R. & Zhu, J. K. 2004 Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* **16**, 2001–2019. (doi:10.1105/tpc.104.022830)

Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. & Mello, C. C. 1999 The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132. (doi:10.1016/S0092-8674(00)81644-X)

Tomari, Y., Matranga, C., Haley, B., Martinez, N. & Zamore, P. D. 2004 A protein sensor for siRNA asymmetry. *Science* **306**, 1377–1380. (doi:10.1126/science.1102755)

Vargason, J. M., Szittya, G., Burgyan, J. & Tanaka Hall, T. M. 2003 Size selective recognition of siRNA by an RNA silencing suppressor. *Cell* **115**, 799–811. (doi:10.1016/S0092-8674(03)00984-X)

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J. L., Bartel, D. P. & Crete, P. 2004 Endogenous trans-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. *Mol. Cell* **16**, 69–79. (doi:10.1016/j.molcel.2004.09.028)

Voinnet, O. 2001 RNA silencing as a plant immune system against viruses. *Trends Genet.* **17**, 449–459. (doi:10.1016/S0168-9525(01)02367-8)

Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. 1999 Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl Acad. Sci. USA* **96**, 14 147–14 152. (doi:10.1073/pnas.96.24.14147)

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. & Martienssen, R. A. 2002 Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837. (doi:10.1126/science.1074973)

Waterhouse, P. M., Graham, M. W. & Wang, M. B. 1998 Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc. Natl Acad. Sci. USA* **95**, 13 959–13 964. (doi:10.1073/pnas.95.23.13959)

Wianny, F. & Zernicka-Goetz, M. 2000 Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.* **2**, 70–75. (doi:10.1038/35000016)

Wienholds, E. & Plasterk, R. H. 2005 MicroRNA function in animal development. *FEBS Lett.* **579**, 5911–5922. (doi:10.1016/j.febslet.2005.07.070)

Wu-Scharf, D., Jeong, B., Zhang, C. & Cerutti, H. 2000 Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-box RNA helicase. *Science* **290**, 1159–1162. (doi:10.1126/science.290.5494.1159)

Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. 2005 DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **102**, 12 984–12 989. (doi:10.1073/pnas.0506426102)

Xu, P., Vernooy, S. Y., Guo, M. & Hay, B. A. 2003 The *Drosophila* microRNA mir-14 suppresses cell death and is required for normal metabolism. *Curr. Biol.* **13**, 790–795. (doi:10.1016/S0960-9822(03)00250-1)

Ye, K., Malinina, L. & Patel, D. J. 2003 Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* **426**, 874–878. (doi:10.1038/nature02213)

Yekta, S., Shih, I. H. & Bartel, D. P. 2004 MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**, 594–596. (doi:10.1126/science.1097434)

Yoshikawa, M., Peragine, A., Park, M. Y. & Poethig, R. S. 2005 A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. *Genes Dev.* **19**, 2164–2175. (doi:10.1101/gad.1352605)

Zamore, P. D., Tuschl, T., Sharp, P. A. & Bartel, D. P. 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33. (doi:10.1016/S0092-8674(00)80620-0)

Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E. & Filipowicz, W. 2004 Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57–68. (doi:10.1016/j.cell.2004.06.017)