10 Lee, S-S. (1987) Bolting in radish. In *Improved Vegetable Production in Asia* (Series no. 36) (Asian and Pacific Council, eds), pp. 60–70, Food & Fertilizer Technology Center, Taipei, Republic of China
11 Park, D.H. *et al.* (1999) Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGANTEA* gene. *Science* 285, 1579–1582
12 Fowler, S. *et al.* (1999) *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *EMBO J.* 18, 4679–4688
13 Redei, G.P. (1962) Supervital mutants of *Arabidopsis*. *Genetics* 47, 443–460
14 Koornneeff, M. *et al.* (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Mol. Gen. Genet.* 229, 57–66
15 Curtis, I.S. *et al.* (2002) Expression of an antisense *GIGANTEA* (*GI*) gene fragment in transgenic radish causes delayed bolting and flowering. *Transgenic Res.* 11, 249–256
16 Wang, L. *et al.* (2002) Effects of peroxidase on hyperlipidemia in mice. *J. Agric. Food Chem.* 50, 868–870
17 Nakamura, Y. *et al.* (2001) 4-(Methylthio)-3-butenyl isothiocyanate, a principal antimutagen in daikon (*Raphanus sativus*; Japanese white radish). *J. Agric. Food Chem.* 49, 5755–5760

1360-1385/03/$ - see front matter
doi:10.1016/S1360-1385(03)00127-4

RNA silencing bridging the gaps in wheat extracts

Olivier Voinnet

Institut de Biologie Moléculaire des Plantes du CNRS 12, Rue du Général Zimmer 67084 Strasbourg, Cedex, France

In plants, RNA silencing plays important roles in antiviral defence, genome integrity and development. This process involves nucleotide sequence-specific interactions that are mediated by small RNA molecules of 21–25 nucleotides. Although the core biochemical reactions of RNA silencing have been well characterized in animals, such information was crucially missing in plants. Recent work now addresses this question and reveals an overall similarity between the plant and animal RNA-silencing pathways, as well as some intriguing plant-specific aspects.

Eukayotic cells have developed a powerful system to wipe out foreign nucleic acids such as transposons and viruses. Several manifestations of this defence, collectively referred to as 'RNA silencing', are triggered by double-stranded RNA (dsRNA), a replication intermediate of many viruses. Experimentally, this molecule can be produced through transcription of inverted repeat transgenes or delivered directly into cells. In *Drosophila*, an Rnase III-like enzyme named Dicer cleaves the dsRNA into 21 nucleotide-long RNA duplexes, the 'short interfering' RNAs (siRNAs) [1,2]. The siRNA is then incorporated into a multi-subunit endonuclease, the RNA-induced silencing complex (RISC), and so ensures, upon base-pairing, that it specifically cleaves RNA sharing sequence identity with the dsRNA [3]. In terms of defence, the logic behind this two-step degradation process is impeccable because it not only targets the initially unwelcome dsRNA but also any potential sibling viral molecules, thanks to the activity of RISC. Small RNAs correlating with RNA silencing were originally discovered in plants [4] whose genomes encode several Dicer-like proteins [5]. Thus, the reactions characterized in *Drosophila* were also likely to be the core of the RNA-silencing mechanism in plants. However, there was no biochemical proof for Dicer or RISC activities in plants, nor was there a direct indication that the plant small RNAs were bona fide siRNAs. This has now been established in recent work by Guiliang Tang and colleagues from Phillip Zamore's group [6].

Separate Dicer activities generate two species of siRNAs in plants

Tang *et al.* [6] used an *in vitro* silencing assay based on wheat germ extracts, in which they incubated labelled dsRNA. The dsRNA became rapidly processed into a discrete species of small RNAs, with no intermediate products, a reaction characteristic of the activity of purified Dicer from *Drosophila* or humans [1,7]. As for animal siRNAs [1,8], the occurrence of the small RNAs depended on the presence of ATP in the wheat germ extract. Moreover, RNase protection indicated that the plant small RNAs, just like their animal counterparts, have a double-stranded body with 2 nt-long 3′ overhangs, the hallmarks of Dicer cleavage products [9]. Plant siRNAs arose as two distinct species of 25 nt and 21 nt in the wheat extract (Fig. 1), which confirmed earlier *in vivo* observations made in tobacco and *Arabidopsis* [10]. By contrast, incubation of labelled dsRNA in fly embryo extracts led exclusively to 21 nt-long siRNAs. Because Dicer is encoded by a single gene in *Drosophila,* whereas at least four homologues are found in the rice and *Arabidopsis* genomes [5], Tang *et al.* reasoned that the two siRNA classes in plants were probably produced by two distinct Dicers. They tested the hypothesis by adding 25 nt-long, cold siRNA duplexes as competitor molecules in the extract. Synthesis of 25 nt-long siRNA from labelled dsRNA was strongly inhibited by this treatment, whereas the production of 21 nt species remained unaffected. The simplest explanation for this contrasting sensitivity to competitor siRNAs is that distinct Dicer-like enzymes generate each class of siRNA [6].

More dsRNA makes more siRNAs

Historically, plant scientists' interest in RNA silencing originated from observations made in transgenic plants

Corresponding author: Olivier Voinnet (olivier.voinnet@ibmp-ulp.u-strasbg.fr).

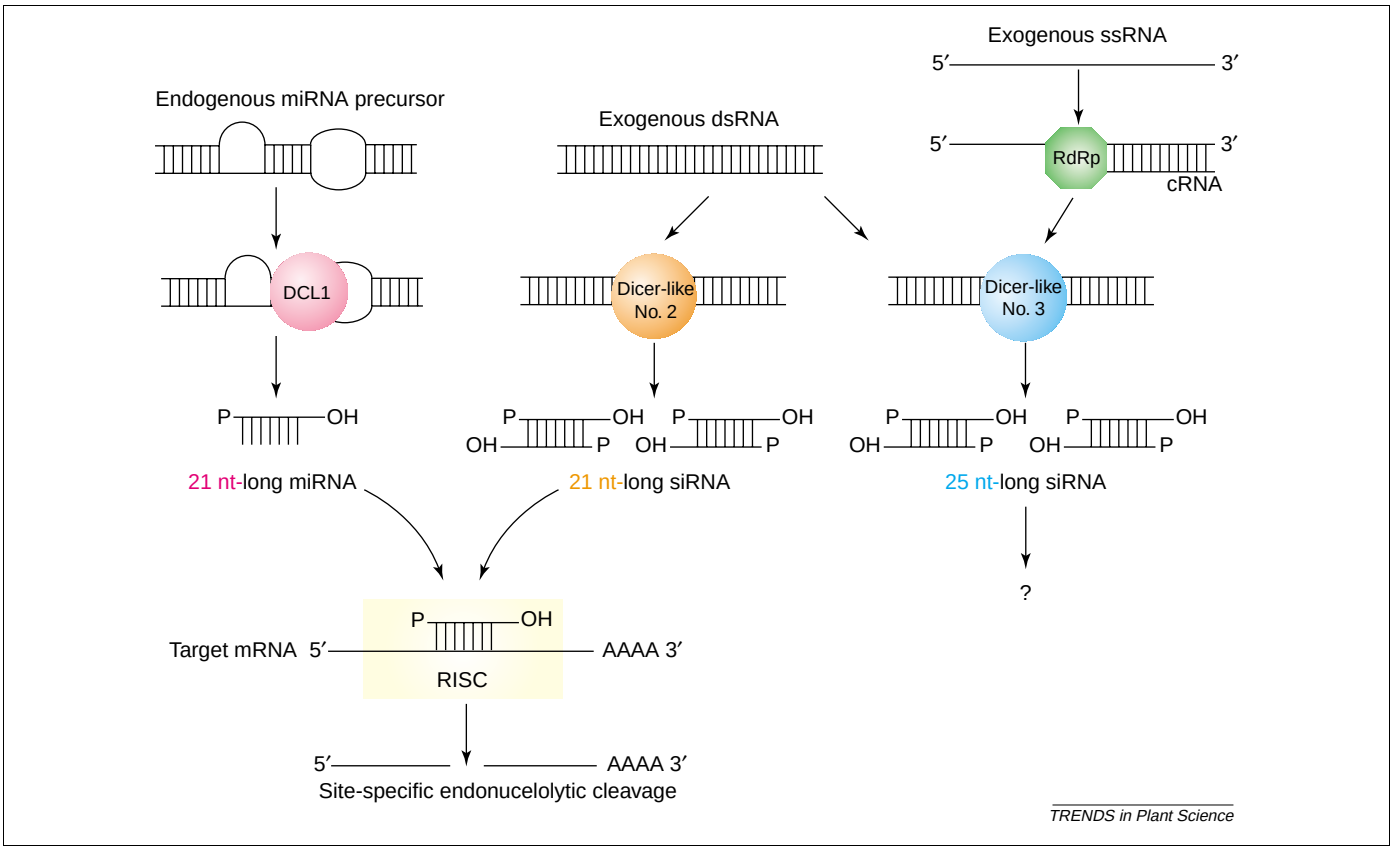

Fig. 1. A biochemical framework for RNA silencing in wheat germ extracts, as defined by the work of Guiliang Tang *et al.* [6]. Two distinct Dicer-like enzymes process exogenous double-stranded RNA (dsRNA) into 21 nucleotide (nt)-long and 25 nt-long short interfering RNAs (siRNAs) duplexes with characteristic 2 nt 3′ overhangs. Exogenous single-stranded RNA (ssRNA) is converted into complementary RNA (cRNA) by one or several endogenous RNA-dependent-RNA polymerases (RdRps). The resulting dsRNA is then preferentially processed into 25 nt-long siRNAs duplexes, presumably by the same Dicer-like enzyme that processes dsRNA into 25 nt-long siRNAs. The fate of this siRNA species remains unclear, although experiments in tobacco and *Arabidopsis* suggest that it could be involved as guide RNA for epigenetic modifications of homologous DNA [10]. Endogenous precursors of micro RNA (miRNA), which are bulged and partially dsRNAs, are processed by a third type of Dicer-like enzyme called DCL1. This results in the production of single-stranded, 21 nt-long miRNAs. Both 21 nt-long siRNA and miRNA are then incorporated into an RNA-induced silencing complex (RISC) that retrieves and cleaves cellular mRNAs that are homologous in sequence. Cleavage occurs upon base-paring, at the precise site of siRNA and miRNA interaction with mRNA. For clarity, siRNAs and miRNAs have been represented as being incorporated into the same RISC, although this remains to be experimentally tested.

engineered to over-express endogenous proteins. Against expectations, some transgenic lines exhibited knockout phenotypes resulting from the elimination of the transgene and the corresponding endogenous gene mRNAs (reviewed in Ref. [11]). This phenomenon, termed sense-transgene silencing, was a likely manifestation of RNA silencing because it was later found to be associated with the production of 21–25 nt-long RNA duplexes with sequence of the silenced transgene [4]. Although dsRNA was the likely precursor for these RNA duplexes, the transgene constructs used to engineer the plants had not been designed to produce such a molecule. To reconcile the data, it was proposed that some single-stranded transcripts produced in the silenced lines had distinctive, ‘aberrant’ features that triggered their conversion into dsRNA through the action of an elusive, plant-encoded, RNA-dependent RNA polymerase (RdRp) [12]. Plant RdRp activities had been described in the past [13]. More compellingly, an *Arabidopsis* protein with RdRp signatures named SDE1/SGS2, was identified in two independent genetic screens as being essential for sense-transgene silencing [14,15].

Tang and colleagues now provide direct biochemical evidence for the involvement of RdRp activity in RNA silencing in plants [6]. Again, they used the wheat germ extracts, but instead of incubating labelled dsRNA, they provided cold, single-stranded (ss)RNA of varying length together with labelled oligonucleotides. In the absence of any exogenous nucleic acid primer, the ssRNA was copied into an approximately full-length complementary (c)RNA, the expected product of an RdRp with high processivity. The resulting labelled dsRNA acted as a Dicer template but it was preferentially cleaved into 25 nt-long siRNAs (Fig. 1). Presumably, the Dicer-like enzyme involved was the same as the one that generated the 25 nt species from exogenous dsRNA because it was also sensitive to competition by cold siRNA [6]. The involvement of one or several RdRp means that RNA silencing can be amplified in plants through *de novo* synthesis of dsRNA and siRNAs. Amplification can be an important part of a silencing-based antiviral response because, having detected only a few pathogenic RNAs, the plant can mount a large response against the invader.

Plant microRNAs program a RISC activity

As well as a defence mechanism, RNA silencing plays crucial roles in endogenous gene regulation. Indeed, 21–25 nt-long endogenous RNAs have been detected in plants and animals. These 'micro (mi)RNAs' are single-stranded and are processed by Dicer-like enzymes from stem–loop precursor RNAs (Fig. 1) that are transcribed from intergenic regions (reviewed in Ref. [16]). In plants, the Dicer homologue responsible for miRNA processing, but not siRNA processing, is Carpel Factory, now referred to as Dicer-like 1 (DCL1) (Fig. 1) [5]. Animal miRNAs are thought to act as translational repressors by pairing to the 3′UTR of target mRNA with imperfect complementarity [17]. By contrast, many plant miRNAs resemble siRNAs in that they exhibit complete or near complete complementarity with the coding regions of their predicted targets, many of which are transcription factor (TF) mRNAs [18]. miRNA-directed cleavage of Scarecrow-like TF mRNAs has been recently documented [19], suggesting that plant miRNAs (or at least some of them) can program a plant-encoded RISC complex to retrieve and destroy endogenous transcripts.

One miRNA (miR165) predicted to target the mRNA of the PHAVOLUTA TF [18] was found in abundance in wheat germ extracts, which prompted Tang and colleagues to assay for a plant RISC activity. A labelled RNA with a stretch of *PHAVOLUTA* sequence was readily cleaved in the extract. Cleavage occurred at the precise site of predicted miRNA–mRNA interaction, in a multiple turnover reaction typical of the activity of the *Drosophila* RISC [3]. The same reaction could be reproduced in fly extracts supplied with synthetic miR165 [6]. Moreover, in plant and fly systems, cleavage failed upon pre-treatment with micrococcal nuclease, a known inhibitor of RISC activity. Thus, miR165 was incorporated into an RISC complex that specifically cleaved its cognate target mRNA (Fig. 1).

Conclusion and future challenges

The outstanding paper from Zamore's laboratory has been long awaited by the plant-silencing community. This paper is (1) reassuring because it validates the prevalent assumption that similar core reactions account for RNA silencing in plants and animals. (2) This work provides a solid basis for further investigation by bringing together separate findings and incidental observations into a coherent set of biochemical data. (3) It emphasizes that, besides an apparent conformity, there are also complex aspects of RNA silencing in plants that remain to be addressed.

- What is the functional relevance of the two siRNA species?
- Which Dicer homologues are involved in their respective synthesis?
- Is SDE1/SGS2 responsible for the RdRp activity detected in the wheat extract and what is the feature of ssRNA that prompts such activity?
- Is the RISC programmed by miRNA the same as the RISC programmed by siRNA?

To answer these questions, it is now essential to confront the available genetic data with the results obtained from *in vitro* assays.

References

1 Bernstein, E. *et al.* (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366
2 Elbashir, S.M. *et al.* (2001) RNA interference is mediated by 21-and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200
3 Hammond, S.M. *et al.* (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts. *Nature* 404, 293–296
4 Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in post-transcriptional gene silencing. *Science* 286, 950–952
5 Finnegan, E.J. *et al.* (2003) Posttranscriptional gene silencing is not compromised in the *Arabidopsis* Carpel Factory (Dicer-Like1) mutant, a homolog of Dicer-1 from *Drosophila*. *Curr. Biol.* 203, 500–502
6 Tang, G. *et al.* (2003) A biochemical framework for RNA silencing in plants. *Genes Dev.* 17, 49–63
7 Billy, E. *et al.* (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. U. S. A.* 98, 14428–14433
8 Zamore, P.D. *et al.* (2001) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25–33
9 Elbashir, S.M. *et al.* (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 20, 6877–6888
10 Hamilton, A.J. *et al.* (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J.* 21, 4671–4679
11 Vaucheret, H. *et al.* (1998) Transgene-induced gene silencing in plants. *Plant J.* 16, 651–659
12 Dougherty, W.G. and Parks, T.D. (1995) Transgenes and gene suppression – telling us something new? *Curr. Opin. Cell Biol.* 7, 399–405
13 Schiebel, W. *et al.* (1993) RNA-directed RNA polymerase from tomato leaves. I. Purification and physical properties. *J. Biol. Chem.* 268, 11851–11857
14 Dalmay, T. *et al.* (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543–553
15 Mourrain, P. *et al.* (2000) *Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* 101, 533–542
16 Voinnet, O. (2002) RNA silencing: small RNAs as ubiquitous regulators of gene expression. *Curr. Opin. Plant Biol.* 5, 444–451
17 Olsen, P.H. and Ambros, V. (1999) The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* 216, 671–680
18 Rhoades, M.W. *et al.* (2002) Prediction of plant microRNA targets. *Cell* 110, 513–520
19 Llave, C. *et al.* (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* 297, 2053–2056

1360-1385/03/$ - see front matter
doi:10.1016/S1360-1385(03)00129-8