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The diversity of RNA silencing pathways in plants

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RNA silencing was discovered in plants as a mechanism whereby invading nucleic acids, such as transgenes and viruses, are silenced through the action of small (20–26 nt) homologous RNA molecules. Our understanding of small RNA biology has significantly improved in recent years, and it is now clear that there are several cellular silencing pathways in addition to those involved in defense. Endogenous silencing pathways have important roles in gene regulation at the transcriptional, RNA stability and translational levels. They share a common core of small RNA generator and effector proteins with multiple paralogs in plant genomes, some of which have acquired highly specialized functions. Here, we review recent developments in the plant RNA silencing field that have identified components of specific silencing pathways and have shed light on the mechanisms and biological roles of RNA silencing in plants.

Introduction

The paradigm of modern molecular biology, 'DNA makes RNA makes protein', predicts a role for RNA as a carrier of information, but not as a regulatory molecule. Although regulatory RNA had been sporadically observed in prokaryotes and eukaryotes, it has only recently emerged, with the discovery of RNA silencing, as a widespread and fundamental component of gene expression. We are now in a position to grasp some of what RNA silencing can do, a little about how it does it, and we use it extensively as a research tool for gene knockdown through RNA interference (RNAi).

Small RNA, Dicers and Argonautes: the biochemical core of RNA silencing

'RNA silencing' refers collectively to diverse RNA-based processes that all result in sequence-specific inhibition of gene expression, either at the transcription, mRNA-stability or translational levels. Those processes share three biochemical features: (i) formation of double-stranded (ds)RNA; (ii) processing of dsRNA to small (s) 20–26-nt dsRNAs with staggered ends; and (iii) inhibitory action of a selected sRNA strand within effector complexes acting on partially or fully complementary RNA or DNA. Although several mechanisms can generate dsRNA, the sRNA processing and effector steps have a common biochemical core. sRNAs are produced by RNase III-type enzymes called Dicers [1] with distinctive dsRNA binding, RNA helicase, RNase III and PAZ (Piwi/Argonaute/Zwille) domains. One of the two sRNA strands joins effector complexes called RNA-induced silencing complexes (RISCs), which invariably contain a member of the Argonaute (Ago) protein family. Agos have an sRNA-binding PAZ domain and also contain a PIWI domain that provides endonucleolytic ('slicer') activity to those RISCs programmed to cleave target RNAs [2,3]. In fact, sRNA-loaded human Ago2 alone constitutes a cleavage-competent RISC *in vitro*, but many additional proteins could be functional components of RISCs *in vivo* [4].

Here, we review recent evidence that several pathways built over the Dicer–Ago core execute a diverse set of sRNA-directed biological functions in higher plants. These include regulation of endogenous gene expression, transposon taming, viral defense and heterochromatin formation. Our focus is primarily on plants because they exhibit a nearly full spectrum of known RNA silencing effects, but similarities and differences with other organisms are also discussed.

Exogenously triggered RNA silencing pathways resulting in transcript cleavage

dsRNA-producing transgenes and IR-PTGS: useful but mysterious

Post-transcriptional gene silencing (PTGS) was discovered in transgenic *Petunia* as loss of expression of both transgenes (in either sense or antisense configuration) and homologous endogenous genes [5]. The transgene loci often directly produced dsRNA as a consequence of imperfect integration events that included juxtaposed sense–antisense transgenes [6,7]. Accordingly, PTGS efficacy was greatly enhanced by simultaneous sense and antisense expression [8] or by direct production of long dsRNA from inverted-repeat (IR) transgenes [9]. The latter process, IR-PTGS, currently forms the basis of experimental RNAi in plants and involves at least two distinct sRNA classes termed short interfering (si)RNAs. 21-nt siRNAs are believed to guide mRNA cleavage, whereas 24-nt siRNAs are believed to exclusively mediate chromatin modifications [10,11]. Both siRNA classes accumulate as populations along the entire sequence of IR transcripts [12].

Although widely used as a research tool, IR-PTGS remains one of the least understood plant RNA silencing processes (Figure 1a). Hence, until recently, no mutant

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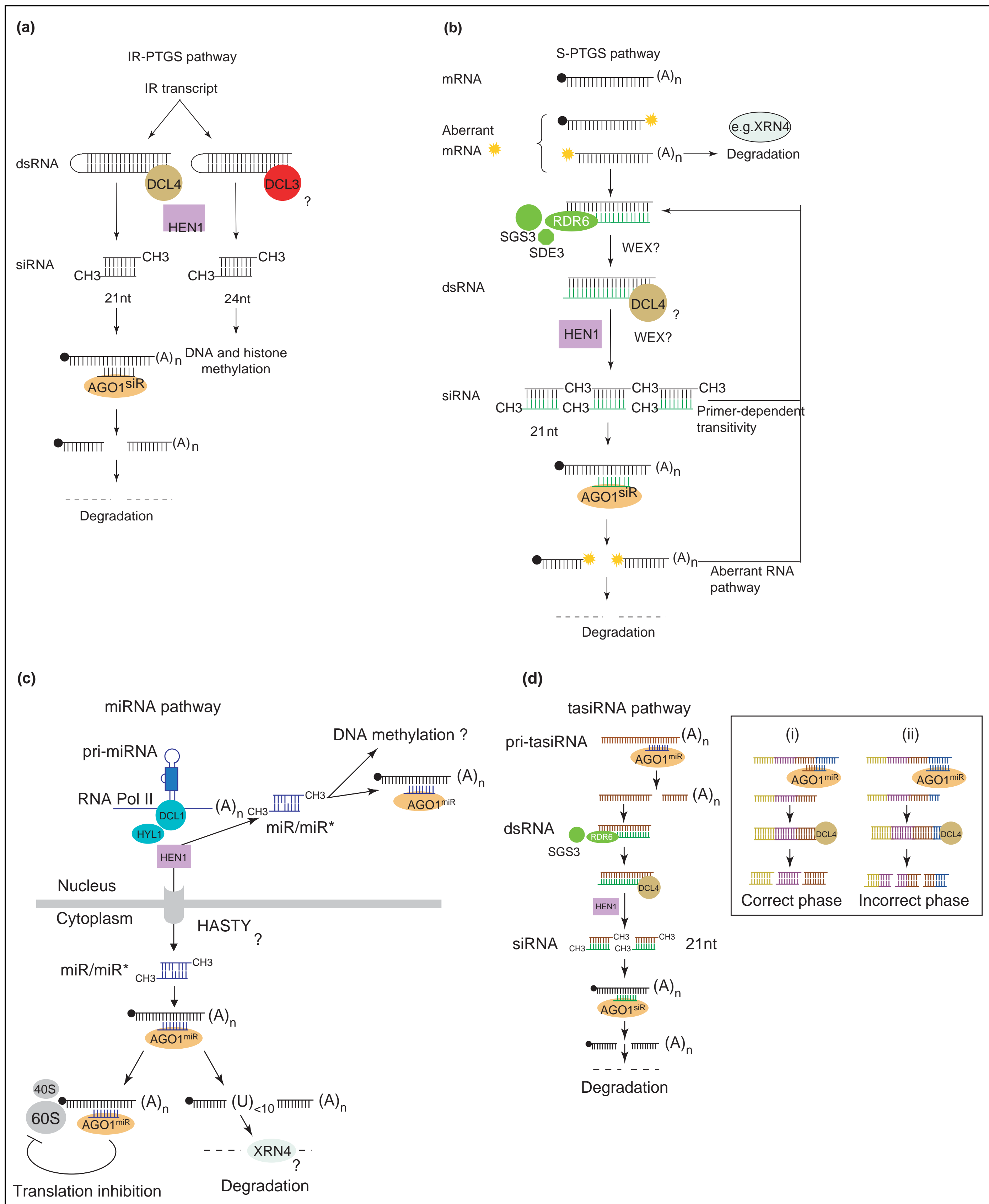

Figure 1. Post-transcriptional RNA silencing pathways in plants. **(a)** The IR-PTGS pathway. An IR transgene construct, typically employed for RNAi in plants, produces ds transcripts with perfectly complementary arms. Two distinct Dicer-like (DCL) enzymes process the ds transcripts. DCL3 probably produces siRNAs of the 24 nt size class, which can direct DNA or histone modification at homologous loci (see Figure 3) and which appear to be dispensable for RNA cleavage. DCL4 is probably the preferred enzyme for production of 21-nt siRNAs from the dsRNA. One siRNA strand incorporates into AGO1-loaded RISC to guide endonucleolytic cleavage of homologous RNA, leading to its degradation. Both siRNA species undergo HEN1-mediated methylation at their 3′ termini. **(b)** The S-PTGS pathway. The pathway is shown here as being elicited by RNAs with aberrant features, although there might be alternative triggers. The RNA aberrations could include lack of a poly(A) tail or lack of 5′ capping. The latter would normally lead to

defective in this pathway had been recovered, despite considerable efforts in several laboratories. One likely explanation is that the high dsRNA levels produced in IR-PTGS promote the activities of different Dicers and RISCs, which would normally act in distinct pathways, to mediate silencing redundantly. Recent analyses of combinatorial Dicer knockouts in *Arabidopsis* support this idea [13,14]. Nonetheless, Dicer-like 4 (DCL4) seems a preferred enzyme for IR-PTGS: it was specifically required for 21-nt siRNA accumulation and silencing from a moderately expressed, phloem-specific IR transgene [15]. DCL2 might also be involved in RNAi, because it processes some endogenous DCL4 substrates into 22 nt-long siRNAs in the absence of DCL4 [13,14], although it remains unclear whether the 22-nt molecules can functionally substitute for the 21-nt siRNA products of DCL4.

S-PTGS and transitive silencing: enter RDR

There are several examples in which single-copy transgene insertions producing sense transcripts trigger PTGS. This pathway, sense (S)-PTGS, has been dissected using *Arabidopsis* forward-genetic screens that provided insights into how dsRNA is produced (Figure 1b). These screens converged on the identification of the RNA-dependent RNA polymerase RDR6, one of six putative *Arabidopsis* RDRs [16,17]. RDR6 is thought to recognize and to use as templates certain transgene transcripts with aberrant features that include lack of 5′ capping. For example, mutation of *Arabidopsis* XRN4, a 5′–3′ exonuclease that degrades uncapped mRNAs, enhanced accumulation of uncapped transgene mRNAs. This favored their conversion into dsRNA by RDR6 and the subsequent degradation of all transgene transcripts through the S-PTGS pathway [18]. RDR6 most likely possesses RNA polymerase activity, resulting in dsRNA production, because a missense mutation in the GDD motif, which is essential for the catalytic activity of all characterized RDRs, is sufficient to alleviate S-PTGS [17].

Although the Dicer that produces siRNAs from RDR6 products remains to be formally identified, S-PTGS siRNA accumulation in *Arabidopsis* requires a coiled-coil protein of unknown function, SGS3 [17], the RNase D exonuclease WEX [19], the sRNA-specific methyl-transferase HEN1 [20] and the putative RNA helicase SDE3 [21] (Figure 1b). Unlike RDR6, SDE3 is not stringently required for transgene silencing, and so could act as an accessory to resolve the secondary structures found in some RDR templates [21]. In accordance with this hypothesis, an SDE3 homolog is part of the *Schizosaccharomyces pombe* RDR complex [22]. SDE3 could also act at other RNA silencing steps, because the homologous protein Armitage is required for RISC assembly in *Drosophila,* an organism lacking *RDR* genes [23]. WEX is related to the exonuclease domain of *mut-7,* which is required for transposon silencing and RNAi in *C. elegans,* but its role in S-PTGS remains elusive [24]. HEN1-catalyzed methylation of free hydroxy termini protects *Arabidopsis* sRNAs, including S-PTGS siRNAs, from oligo-uridylation, a modification promoting their instability (see the ‘miRNA transcription and biogenesis’ section) [25].

In one S-PTGS mutant screen, an extensive allelic series of *ago1* was recovered, suggesting that among the ten *Arabidopsis AGO* paralogs, AGO1 is specifically involved in this pathway [26,27]. Even weak *ago1* alleles completely lost S-PTGS siRNAs, initially suggesting a role for AGO1 in siRNA production rather than action [27]. However, given that AGO1 is now recognized as a slicer activity of the plant miRNA-loaded and siRNA-loaded RISCs [28,29], loss of siRNAs in *ago1* can also result from their poor incorporation into RISC, enhancing their turnover. Nevertheless, a role for AGO1 in siRNA production – possibly linked to RDR6-dependent dsRNA synthesis – cannot be excluded, because some *ago1* mutants defective in S-PTGS siRNA accumulation do not have defects in IR-PTGS [30].

RDR6, and perhaps other S-PTGS components, is also involved in the related silencing phenomenon, transitivity [31,32]. Transitivity is the ‘transition’ of primary siRNAs (corresponding to a sequence interval of a targeted RNA) to secondary siRNAs targeting regions outside the initial interval (Figure 2). In plants, this transition can occur both 5′ and 3′ to the primary interval, possibly reflecting primer-dependent and primer-independent RDR6 activities. Transitivity serves as a siRNA amplification mechanism that also accounts for extensive movement of silencing throughout transgenic plants [33]. Secondary siRNAs are exclusively of the 21-nt size class [33]. Thus, given that S-PTGS siRNAs seem to accumulate as 21-nt species [32], that DCL4 produces the 21-nt siRNAs from IR transcripts [15], and that DCL4 and RDR6 activities are coupled for 21-nt *trans*-acting siRNA biogenesis (see the next section), it is tempting to speculate that DCL4 is also the preferred Dicer for siRNA production in both S-PTGS and transitivity (Figures 1b and 2).

RNA degradation through the activity of the 5′–3′ exonuclease XRN4. Lack of XRN4 would promote accumulation of uncapped mRNAs, thereby triggering their conversion into dsRNAs by the combined action of RDR6, SGS3, SDE3 and possibly WEX. The resulting dsRNA is then processed by a DCL, probably DCL4 (see main text), producing siRNAs that are exclusively of the 21 nt size class and methylated by HEN1. These molecules can then undergo two sets of reactions. First, they can be used as primers by RDR6 to reinforce production of dsRNA from single-stranded templates, through a phenomenon known as ‘transitivity’ (see Figure 2). They can also incorporate into AGO1-loaded RISC to guide sequence-specific cleavage of homologous RNA. The resulting cleavage products could be perceived as aberrant RNAs and thus could promote further production of dsRNA, resulting in an amplified reaction. **(c)** The miRNA pathway. Pri-miRNA transcripts with fold-back structures are products of Pol II. The position of the mature miRNA (miR) is boxed. The combined nuclear action of DCL1, HYL1 and HEN1 produces a mature, methylated miRNA. On nuclear export, possibly mediated by the *Arabidopsis* exportin 5 homolog HASTY, the mature miRNA incorporates into AGO1-loaded RISC to promote two possible sets of reactions that are not mutually exclusive. A first reaction (right) would lead to endonucleolytic cleavage of homologous RNA, as directed by 21-nt siRNAs. This would result in a poly-uridylated 5′ cleavage fragment – a modification that might promote its rapid turnover – and a more stable 3′ fragment that could be degraded by the XRN4 exonuclease. The scheme also accommodates the possibility that mature miRNAs could have sequence-specific effects in the nucleus (see text). Those nuclear activities include RNA cleavage (on incorporation into a putative nuclear RISC) as well as DNA methylation. A second reaction (left) would lead to inhibition of translation, possibly at the initiation level. **(d)** The tasiRNA pathway. pri-tasiRNA transcripts are non-coding RNAs devoid of extensive fold-back structures. A miRNA incorporated into AGO1-loaded RISC guides endonucleolytic cleavage of the pri-tasiRNA. This cut generates two cleavage fragments, one of which acts as an RDR6 template, leading to the production of dsRNA. DCL4 initiates processing exclusively from the dsRNA ends corresponding to the initial miRNA cut site, to produce phased tasiRNAs that are methylated by HEN1. tasiRNAs subsequently guide cleavage of homologous mRNAs, once incorporated into AGO1-loaded RISC. The reactions depicted in the inset illustrate the importance of the initial miRNA-directed cut in determining the appropriate phase for tasiRNAs; correct phasing is shown in (i). Incorrect phasing (ii) would result in the production of off-target small RNAs.

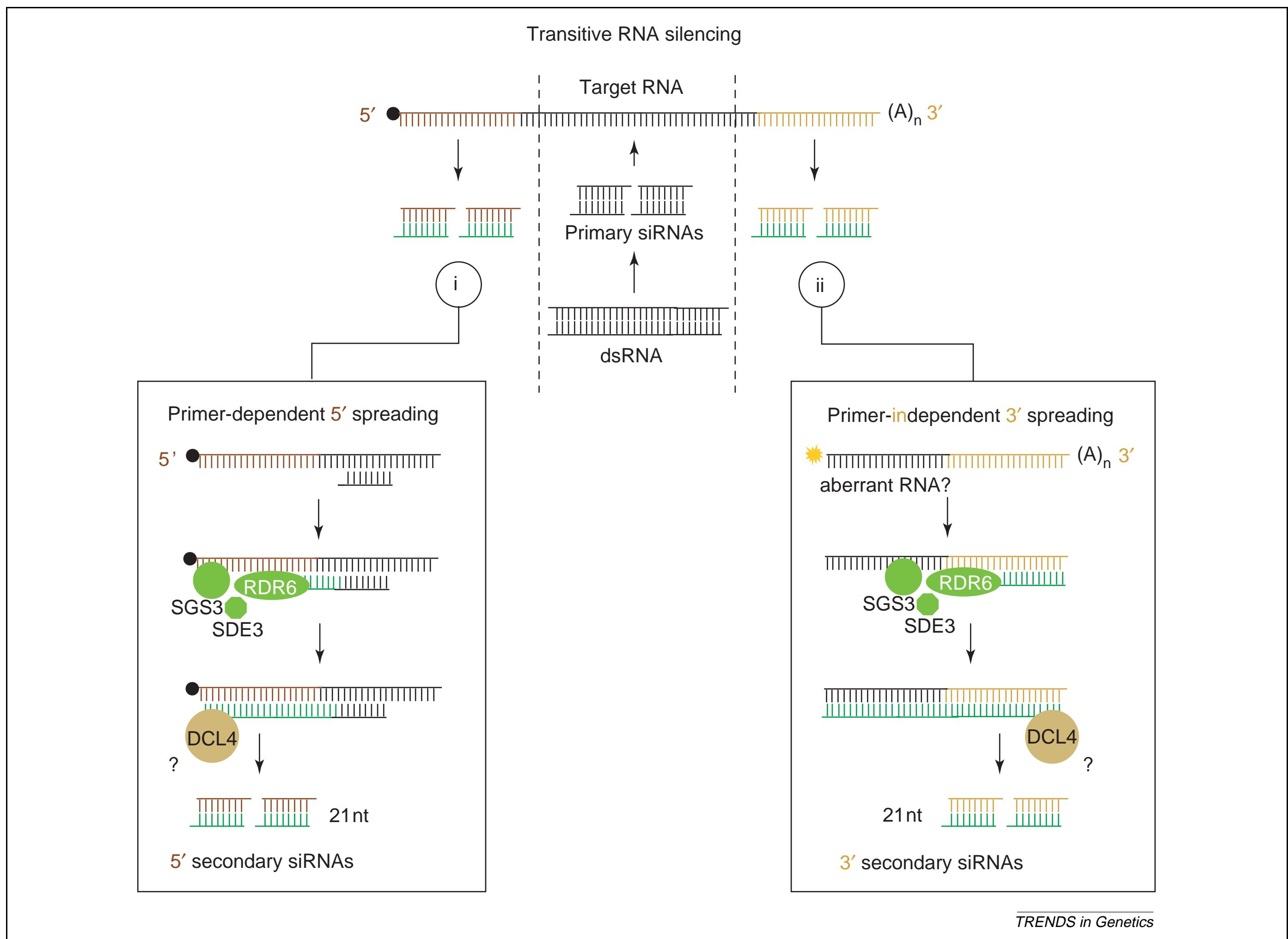

Figure 2. Transitive RNA silencing. In transitive RNA silencing, a dsRNA source of primary siRNAs promotes production of secondary siRNAs both 5′ and 3′ of the initially targeted interval of a transcript. Production of 5′ secondary siRNAs (i) can be explained by RDR6/SGS3/SDE3-dependent complementary strand synthesis that is primed by one of the primary siRNAs. Production of 3′ secondary siRNAs (ii) cannot be explained by a primed reaction, and it is possible that RNA fragments resulting from primary siRNA-directed transcript cleavage are recognized as aberrant, thereby initiating dsRNA synthesis as in S-PTGS. The 5′ and 3′ reactions should not be considered mutually exclusive, as siRNAs produced in (ii) could prime further dsRNA synthesis according to the scheme depicted in (i). DCL4 is shown as putatively involved in 5′ and 3′ secondary siRNA biogenesis. Unlike primary siRNAs (which can be 21 nt and 24 nt in size), secondary siRNA are exclusively of the 21 nt size class. It remains unclear whether 24-nt primary siRNAs can trigger transitive RNA silencing.

What would the biological function be of an amplified and non-cell autonomous pathway based on 21-nt siRNAs? At least one answer is antiviral defense. Virus-derived 21-nt siRNAs accumulate in infected cells [34], and plants with compromised RDR6 function are hypersusceptible to several viruses [17,35]. An RDR-amplified response primed by viral siRNAs (transitivity) and/or elicited by virus-derived aberrant RNAs (S-PTGS pathway) would ensure that the silencing machinery keeps pace with the high replication rates of the pathogen. The systemic nature of the response would immunize cells that are about to be infected and might result in the exclusion of the virus. Consistent with this idea, the meristems of *Nicotiana benthamiana* with compromised RDR6 activity were invaded by several viruses, whereas normally these tissues are immune to infection [36].

Endogenous RNA silencing pathways involved in post-transcriptional regulation

MicroRNAs

In plants, miRNAs are produced as single-stranded, 20–24-nt sRNA species, excised from endogenous non-coding transcripts with an extensive fold-back structure. miRNAs act *in trans* on cellular target transcripts to induce their degradation via cleavage, or to attenuate protein production (Figure 1c) [37]. Currently, ~100 *Arabidopsis MIRNA* genes falling into 25 distinct families have been identified [38], but many more are likely to exist (Box 1). miRNAs have important biological roles in plant and animal development, as shown by the strong developmental defects of several miRNA overexpression and loss-of-function mutants [37]. For instance, key regulatory elements of the plant response to the hormone auxin, which specifies organ shape and the axes of the plant body, are controlled by miRNAs [39,40]. miRNAs also regulate accumulation of transcription factors involved in defining the identity or number of floral organs [41,42], leaf shape [43], abaxial–adaxial leaf asymmetry [44,45] and lateral root formation [46]. In addition, DCL1 and AGO1, which are involved in the miRNA pathway, are themselves regulated by miRNAs [47,48]. Nonetheless, plant miRNAs with validated targets involved in primary and secondary metabolism

have been identified [39,49], indicating that their roles are not confined to developmental regulation. miRNAs might, indeed, have broad implications in plant physiology and environmental adaptation (Box 1).

miRNA transcription and biogenesis

Most plant and animal miRNA genes reside between protein coding genes or within introns [50]. Most are probably independent transcription units, and their expression patterns often show exquisite tissue specificity or even cell-type specificity, in agreement with a role in patterning and maintenance of differentiated cell states [51,52]. Nonetheless, transcription factors or post-transcriptional mechanisms that specify plant *MIRNA* expression remain unknown. Many human primary miRNA transcripts (pri-miRNAs) are synthesized by RNA polymerase II (Pol II): pri-miRNAs have typical Pol II 5′caps and poly(A) tails, their synthesis is hindered by Pol II-inhibiting drugs and Pol II is found at their promoters *in vivo* [53]. Similar, although less extensive, evidence also points to Pol II as the major polymerase producing plant pri-miRNAs [38].

Box 1. miRNA genomics: how many miRNA genes and how do they evolve?

Various abiotic stresses have been shown to induce expression of several plant miRNAs [39,109] and, although the effects of biotic stresses have not been studied so far, miRNA induction or repression by environmental cues could be a common theme of plant adaptive biology. Such induced or rare miRNAs would be under-represented in most current cloning libraries that are based on sRNA extracted from non-stressed tissues. Likewise, miRNAs expressed at specific developmental stages, in a given tissue or even in a given cell type, could be overlooked in cloning procedures involving RNA from whole organisms. Moreover, computational prediction of miRNA genes generally relies on conservation between *Arabidopsis* and rice or maize genomes, yet it now seems that some – perhaps many – miRNAs are species-specific. Accordingly, the original estimate of the miRNA gene number in human (~300 based on evolutionary conservation) has been dramatically increased to > 1000 following the discovery of many miRNAs that are not conserved beyond the primate genus [110]. Similarly, the current number of miRNA genes in plants might be vastly underestimated.

An analysis of recently evolved *Arabidopsis* miRNAs indicates that inverted gene duplication events can constitute a starting point in the evolution of fold-back structures found at *MIRNA* loci [111]. In this model, 'young' miRNA transcripts initially show extensive complementarity to their targets; they then progressively acquire nucleotide divergence to the point that only the mature miRNA sequence resembles the founder gene sequence, as seen in most older *MIRNA* genes. However, such stem-loop structures might be only one of several possible precursors, because poor RNA folding is sometimes observed at experimentally validated plant *MIRNA* loci. The insertion of transposable elements into new genomic sites also seems to be one of the driving forces that create new miRNAs during mammalian, and perhaps, plant gene evolution. For instance, in human, mouse and rat, LINE-2-derived miRNAs exhibit perfect complementarity to large families of mRNA that contain portions of miRNA and LINE-2s in their 3′ UTRs [112]. Some *MIRNA* genes might also be occasionally acquired by direct horizontal transfer through genomic integration of foreign nucleic acids. Hence, several mammalian DNA viruses encode and produce miRNAs during infection [113–115]. Likewise, phytoviruses or other plant pathogens that exploit nucleic acids to infect plants might use miRNAs as virulence factors [116], and their genes could integrate into host genomes.

Following transcription, mammalian pri-miRNAs are processed via a well-defined biosynthetic pathway. The RNase III protein Drosha and its essential cofactor DGCR8/Pasha – both constituents of the nuclear microprocessor complex – catalyze initial cuts at the base of pri-miRNA stem-loop to produce (pre)-miRNA. Following Exportin-5-dependent nuclear export, pre-miRNAs are processed by Dicer into mature miRNAs [54]. Plants have no direct equivalent of microprocessor. In *Arabidopsis*, miRNA biosynthesis depends specifically on DCL1 [55,56], required for the nuclear stepwise processing of pri-miRNAs, but whether DCL1 itself catalyzes all of the reactions involved is uncertain [57]. The plant exportin-5 homolog HASTY is involved in miRNA biogenesis [58], but its exact role is not as clear as in mammals where the microprocessor pre-miRNA is an experimentally verified cargo [59]. Mutants in *hasty* show decreased accumulation of some, albeit not all, miRNAs in both nuclear and cytoplasmic fractions [58]. These observations support the existence of HASTY-independent miRNA export systems and raise the question of whether miRNAs or miRNA-containing complexes are even direct cargoes of HASTY.

In plants and animals, Dicer processing occurs in association with specific dsRNA-binding proteins. First observed with the Dcr2–R2D2 complex required for RISC loading in the *Drosophila* RNAi pathway [60], this has now also been found for the Dcr1–Loqs complex, which is involved in the *Drosophila* miRNA pathway [61], and Dicer–TRBP and Dicer–PACT complexes in human cells [62,63]. DCL1–HYL1 is a similar complex that acts in pri-miRNA processing in the *Arabidopsis* miRNA pathway [64–67] (Figure 1c). In all of these examples, Dicer produces a duplex between the mature miRNA (miR) and its complementary strand (miR*) [68]. The miR strand is generally least stably base-paired at its 5′ end and is consequently loaded as the guide strand into RISC, whereas the miR* strand is degraded [69] (Figure 1c). In the *Drosophila* RNAi pathway, R2D2 acts as a thermodynamic asymmetry sensor of siRNA duplexes, and Loqs, TRBP, PACT and HYL1 could possibly perform similar roles.

HEN1 is an *S*-adenosyl methionine (SAM)-binding methyl-transferase that methylates the 2′ hydroxy termini of miR–miR* duplexes, a reaction apparently specific to the plant kingdom [70,71]. Methylation protects miRNAs from activities that uridylate and degrade plant sRNAs from the 3′ end [25], but it is not required for RISC-dependent miRNA-guided cleavage in *Arabidopsis* extracts [28]. All known classes of plant sRNA are methylated by HEN1 [25], but this modification seems to impact differentially on sRNA stability, perhaps reflecting variable interactions between HEN1 and distinct protein complexes or distinct sRNA populations. For example, the viral silencing suppressor Hc-Pro prevents methylation of virus-derived siRNAs, but not of miRNAs [72], and several *hen1* mutant alleles exist, in which accumulation of miRNA, but not of S-PTGS siRNAs, is impaired [20].

Plant miRNA activities

Most identified plant miRNAs have near-perfect complementarity to their targets and promote their cleavage.

This is followed by oligo-uridylation and rapid degradation of the 5′ cleavage fragment [73], and slower degradation of the 3′ cleavage fragment mediated, at least in some cases, by XRN4 [74] (Figure 1c). Animal miRNAs generally have imperfect complementarity and repress protein production from intact target mRNAs. However, it is possible that the action of both plant and animal miRNAs results from a combination of both processes (degradation and repression), whose respective contributions probably vary depending on the extent of the miRNA–target complementarity (Box 2). Although the RISC(s) acting in the plant miRNA pathway remain ill defined, AGO1 associates with miRNAs, and miRNA targets are cleaved *in vitro* by immuno-affinity-purified AGO1 [28,29]. Thus, in plants, the same Argonaute seems to function as a Slicer for both miRNA- and siRNA-loaded RISCs, contrasting with the situations in *Drosophila* and *C. elegans*. Plant RISC components other than AGO1 await identification and it could be that several alternative RISCs exist, given the number of *AGO*-like genes in *Arabidopsis*.

Box 2. miRNA activities: RNA cleavage, translational repression and many more?

The finding that mammalian RISC programmed with a fully complementary miRNA becomes cleavage-competent has prompted the widely accepted notion that the degree of miRNA–target complementarity alone largely determines whether RISC guides mRNA cleavage or translational repression in eukaryotes [117]. Most known plant miRNAs have near-perfect complementarity to their targets, and so are believed to act by guiding cleavage. This activity is indeed often experimentally validated using a modified 5′ rapid amplification of cDNA ends (5′ RACE) procedure that identifies clear cleavage sites within miRNA targets [118,119]. But is cleavage the sole mode of action of plant miRNAs?

A positive signal in the 5′ RACE assay does not indicate the extent of cleavage and appreciable levels of full-length target mRNA often remain detectable, suggesting that translational repression can contribute to the overall inhibitory effects observed. In fact, and surprisingly, only in one example, the miR172–AP2 interaction, has endogenous target protein accumulation been tested in plants [41,120]. Although miR172 has near perfect complementarity to the *AP2* transcript, the output was a combination of both cleavage and translational repression, the latter being predominant in this case. Thus, miRNA regulation purely by cleavage or purely by translational inhibition, if they exist, could represent extremes of a continuum of miRNA action that normally involves a blend of both mechanisms.

The procedures used so far to retrieve plant miRNA targets could also have strongly biased our perception of miRNA activities, precisely because they involved full or near complementarity as a common, if not exclusive, selection criterion. However, out of 20 recently isolated rice miRNAs, 13 lacked extensive complementarity to other loci in the rice genome [121]. These can be prototypic guides of translational repression, as seen with many animal miRNAs that imperfectly match the 3′ UTR of their target except in the ‘miRNA seed’, a core of 7–8 complementary 5′ nucleotides [122,123]. Nonetheless, recent evidence suggests that even the seed type of target recognition in animals could well entail RNA degradation that might not necessarily involve site-specific cleavage [124].

Altogether, those observations raise questions about the accuracy or even validity of the many rules that have been tentatively put forward to explain or predict the outcome of miRNA–target interactions in eukaryotes. This blurred picture is probably just a reflection of our limited knowledge of small RNA biology. A compelling example of this was provided in a recent report describing enhancement, not repression, of hepatitis virus C replication by a cellular miRNA in infected human cells [125]. Small RNAs are tailored to guide sequence-specific events and there is, in principle, no reason to exclude positive regulatory actions by those molecules.

Mature plant miRNAs are detected in both nuclear and cytosolic cell fractions [58]. Likewise, RISC programmed with the *let-7* miRNA can be immuno-purified from nuclear human cell fractions [75], indicating that plant and animal miRNAs might have nuclear functions (Figure 1c). These could include RNA cleavage, as suggested by the intron-targeting activity of the plant *miR173* [76], but also modifications of homologous DNA [77]. Thus, in *Arabidopsis*, recognition by *miR165* of the spliced *PHB* transcript apparently directs *cis* methylation on the *PHB* template DNA. This methylation is enigmatic, however, as it occurs several kilobases downstream of the miRNA-binding site [77]. It is conceivable that miRNA-induced cleavage of the nascent *PHB* transcript triggers dsRNA formation initiated at the 3′end of the transcript through a primer-independent RDR activity with moderate processivity. The resulting production of siRNA would thus be confined to the 3′ end and could mediate DNA methylation according to the schemes discussed below. Intriguingly, some (albeit few) siRNAs corresponding to downstream parts of several miRNA targets have been detected in *Arabidopsis*, although none was directly complementary to the methylated *PHB* sequence [78]. Direct miRNA-guided DNA methylation *in cis* and/or *in trans* has also been suggested from the observation that some 21-nt miRNAs of *Arabidopsis* accumulate as a second, 24-nt species at specific developmental stages [68].

Transacting siRNAs: mixing up miRNA and siRNA actions

Transacting (ta) siRNAs are a recently discovered class of plant endogenous sRNAs. They derive from non-coding, single-stranded transcripts, the pri-tasiRNAs, which are converted into dsRNA by RDR6–SGS3, giving rise to siRNAs produced as discrete species in a specific 21-nt phase [79,80] (Figure 1d). The RDR6–SGS3 involvement is reminiscent of siRNA biogenesis in S-PTGS, but the genetic requirements of the two pathways are not identical, because tasiRNA accumulation is normal in the hypomorphic *ago1–27* mutant and in mutants defective in SDE3 and WEX [79]. Much like plant miRNAs, mature tasiRNAs guide cleavage and degradation of homologous, cellular transcripts. To date, tasiRNA generating loci (*TAS1–3*) have been identified only in *Arabidopsis* [76], but they probably exist in other plant species and possibly in other organisms that contain RDRs, such as *C. elegans* or *Neurospora crassa*.

tasiRNA production involves an interesting mix of miRNA action and the siRNA biogenesis machinery (Box 3). Pri-tasiRNAs contain a binding site for a miRNA that guides cleavage at a defined point. The initial miRNA-guided cut has two important consequences. First, it triggers RDR6-mediated transitivity on the pri-tasiRNA cleavage products, allowing dsRNA production either 5′ or 3′ of the cleavage site [76]. Second, it provides a well-defined dsRNA terminus, which is crucial for the accuracy of a phased dicing reaction performed by DCL4, which produces mature tasiRNAs (Figure 1d).

Box 3. *Trans*-acting siRNAs: more questions than answers

miRNA-guided cleavage of pri-tasiRNA initiates tasiRNA biogenesis, which requires dsRNA formation by RDR6 and subsequent production of phased siRNAs by DCL4 (see Figure 1d). As Dicers are known to process dsRNA from both ends, it is unclear how DCL4 systematically selects the dsRNA end located at the miRNA cleavage site as its starting point of tasiRNA processing (Figure 1d). This reaction occurs both when tasiRNAs are produced from the 5′- and from the 3′-pri-tasiRNA cleavage fragments. One possible explanation is that DCL4 is directly associated with the miRNA-containing RISC that cleaves the pri-tasiRNA.

A second, intriguing question is why miRNA action triggers transitivity on pri-tasiRNA transcripts, but not on endogenous mRNAs. pri-tasiRNA cleavage can take place in the nucleus, because the tasiRNA-containing part of the *TAS1* and *TAS2* pri-tasiRNA resides within introns, and because DCL4 seems to be localized to the nucleus. Thus, it is possible that nuclear as opposed to cytoplasmic miRNA action is coupled to RDR activity, perhaps as a consequence of association of RDRs with the transcription machinery. This scenario would resemble the *S. pombe* heterochromatic RNAi pathway, where Ago1 associates with RNA Pol II, nascent transcripts and an RDR-containing complex [22,100].

Finally, it is unclear how pri-tasiRNA cleavage by mature tasiRNAs is prevented. One can argue that many tasiRNAs would be incapable of guiding pri-tasiRNA cleavage simply because they are of the same polarity. However, some tasiRNAs offset by 10–11 nt from their correct phase are occasionally detected [76], suggesting that tasiRNA-guided pri-tasiRNA cleavage can occur but that mechanisms exist to prevent such cleavage from being widespread.

What is the biological role of tasiRNAs? *Arabidopsis rdr6*, *sgs3* and *dcl4* mutants all have an accelerated juvenile-to-adult phase transition [13,14,80,81], indicating that tasiRNAs could regulate this trait. The tasiRNA targets include two auxin response factor (ARF) transcription factors and a family of pentatricopeptide repeat proteins, although there is no evidence for the involvement of the only functionally characterized target (ARF3/ETTIN) in the juvenile-to-adult phase transition [82], nor were heterochronic defects noticed in insertion mutants disrupting the *TAS1* or *TAS2* loci [79,81]. Mutants in *AGO7/ZIPPY* have a similar phase-transition defect [83], suggesting that AGO7 could be part of a specific tasiRNA-programmed RISC, although tasiRNAs co-immunoprecipitate with AGO1 to form a cleavage competent RISC [28].

Natural antisense transcript siRNAs

An example has been recently described in which a pair of neighboring genes on opposite DNA strands (*cis*-antisense genes) gives rise to a single siRNA species from the overlapping region of their transcripts [84]. This 24-nt siRNA species – dubbed natural antisense transcript siRNA (nat-siRNA) – guides cleavage of one of the two parent transcripts, and it is produced in a unique pathway involving DCL2, RDR6, SGS3 and the atypical DNA dependent RNA polymerase-like subunit NRPD1a (see the ‘Chromatin-targeted RNA silencing pathways’ section below). nat-siRNA guided cleavage triggers production of a series of secondary, phased 21-nt siRNAs, a reaction similar to tasiRNA biogenesis except that the Dicer involved is DCL1. The role of secondary nat-siRNAs is currently unclear, but primary nat-siRNA-guided cleavage contributes to stress adaptation, and, given the numerous *cis*-antisense gene pairs in plant and other genomes [85,86], this isolated example might reflect a widespread mechanism of gene regulation.

Chromatin-targeted RNA silencing pathways

In addition to acting on RNA, siRNAs can guide formation of transcriptionally silent heterochromatin in fungi, animals and plants. Plant heterochromatin is characterized by two sets of modifications: methylation of cytosines and of specific histone lysine residues (histone 3 Lys9 (H3 K9) and histone 3 Lys27 (H3 K27) in *Arabidopsis*) [87]. In some organisms, these modifications act as assembly platforms for proteins promoting chromatin condensation. *Arabidopsis* cytosine methyl-transferases include the following proteins: DRM1 and DRM2, which are all closely homologous to each other and are required for all *de novo* DNA methylation; MET1, required for replicative maintenance of methylation at CG sites; and CMT3, required for maintenance at CNG and asymmetrical CNN sites (reviewed in Refs [88,89]). Histone methyl-transferases involved in H3 K9 and H3 K27 methylation belong to the group of Su(Var)3–9 homologs and include KYP (also called SUVH4) and SUVH2 in *Arabidopsis* [90]. These and other proteins with roles in *Arabidopsis* small RNA pathways are summarized in Table 1.

In many organisms, siRNAs corresponding to several endogenous silent loci, including retrotransposons, 5S rDNA and centromeric repeats, have been found [88]. They are referred to as *cis*-acting siRNAs (casiRNAs) because they promote DNA or histone modifications at the loci that generate them. In plants, casiRNAs are methylated by HEN1 and are predominantly 24 nt in size (Box 4) [25,91]. Their accumulation is specifically dependent on DCL3 and, in many instances, on RDR2 (Figure 3) [91]. casiRNA accumulation also requires an isoform (containing subunits NRPD1a and NRPD2) of a plant-specific and putative DNA-dependent RNA polymerase, termed Pol IV [92–94], which might act as a silencing-specific RNA polymerase that produces transcripts to be converted into siRNAs by the actions of RDR2 and DCL3. However, many aspects of Pol IV silencing-related activities remain obscure. Hence, it is uncertain whether Pol IV even possesses RNA polymerase activity. Additionally, a distinct Pol IV isoform with subunits NRPD1b and NRPD2 is required for methylation directed by IR-derived siRNAs with transgene promoter homology, suggesting that the action of Pol IV complexes might not be confined to siRNA biogenesis [95]. Finally, the requirement of NRPD1a for nat-siRNA accumulation in the presence of both antisense mRNAs (produced by Pol II) suggests that Pol IV can have silencing-related functions independent of DNA-dependent RNA polymerase activity [84].

Other factors involved in IR-derived siRNA-directed promoter methylation include the chromatin remodeling factor DRD1 [96] and the putative histone deacetylase HDA6 [97] whose activity might be required to provide free histone lysines for methylation by KYP/SUVH-family enzymes (Figure 3). It is currently uncertain whether DRD1 and HDA6 are also implicated in silencing of endogenous loci. 24-nt siRNAs can act in a RISC-like complex, perhaps one akin to the RNA-induced

Table 1. Overview of proteins with roles in *Arabidopsis* small RNA pathways

Protein	Domains and motifs	Biochemical activity	Pathway	Refs
DCL1	RNase III dsRNA binding DEAD-box helicase PAZ DUF283 (unknown function)	miRNA synthesis	miRNA nat-siRNA	[55,85]
HYL1	dsRNA binding	dsRNA binding	miRNA	[64,65]
HST	RanGTP binding	Putative exportin	miRNA	[58]
AGO1	PAZ Piwi	siRNA slicer miRNA slicer	miRNA S-PTGS tasiRNA Chromatin (?)	[26–29]
HEN1	dsRNA binding Lupus La RNA binding S-adenosyl binding	sRNA methyl-transferase	All sRNA pathways	[20,25,56,70]
RDR6	RdRP	RNA-dependent RNA polymerase	S-PTGS Transitivity tasiRNA nat-siRNA	[16,17,32,33,76,79,85]
SGS3	Coiled-coil Putative Zn^{II}-binding	Unknown	S-PTGS Transitivity tasiRNA nat-siRNA	[17,79,85]
DCL4	RNase III dsRNA binding Helicase PAZ	21-nt siRNA synthesis	tasiRNA IR-PTGS S-PTGS?	[13–15]
WEX	3′–5′ exonuclease	Putative 3′–5′ exonuclease	S-PTGS	[19]
SDE3	DEAD box Helicase	Putative RNA helicase	S-PTGS Transitivity	[21,33]
DCL2	RNase III dsRNA binding PAZ	22- or 24-nt siRNA synthesis	nat-siRNA	[85]
DCL3	RNase III DEAD box helicase PAZ	24-nt siRNA synthesis	Chromatin	[28,91]
RDR2	RdRP	Putative RNA-dependent RNA polymerase	Chromatin	[91]
AGO4	PAZ Piwi	Unclear	Chromatin	[11]
NRPD1a	RNA polymerase	Putative DNA-dependent RNA polymerase	Chromatin nat-siRNA	[85,92–95]
NRPD1b	RNA polymerase	Putative DNA-dependent RNA polymerase	Chromatin	[93,95]
NRPD2	RNA polymerase	Putative DNA-dependent RNA polymerase	Chromatin	[92–95]
HDA6	Histone deacetylase	Putative histone deacetylase	Chromatin	[97]
DRD1	SNF2-related DNA and ATP binding Helicase	Putative chromatin remodel-ing	Chromatin	[96]
CMT3	Cytosine DNA methyl-transferase Chromodomain Bromo-adjacent domain	Cytosine DNA methyl-transferase	Chromatin	[88]
DRM1/2	Cytosine DNA methyl-transferase	Cytosine DNA methyl-transferease	Chromatin	[88]
MET1	Cytosine DNA methyl-transferase Bromo-adjacent domain	Cytosine DNA methyl-transferase	Chromatin	[88]
KYP	SET domain ZnII-binding domain Pre-SET domain Post-SET domain YDG domain EF-hand	H3 K9 methyl-transferase	Chromatin	[90]
SUVH2	SET domain Zn^{II}-binding pre-SET domain YDG domain	H3 K9 methyl-transferase	Chromatin	[128]

Box 4. 21-nt, 22-nt and 24-nt siRNAs: does size matter?

High-throughput cloning and case-by-case studies indicate that casiRNAs are predominantly 24 nt rather than 21 nt in size and appear to be dependent on DCL3. This prompts the question of whether there are structural and/or functional reasons that make 24-nt siRNAs better suited to guide chromatin modification and less well suited to guide transcript cleavage. Several observations indicate that this might not be true. First, 21-nt siRNAs are clearly fully competent to guide DNA methylation *in vivo*, because RDR6-dependent transitive siRNAs – exclusively 21 nt in size – have been found to direct sequence-specific DNA methylation at transgene loci [32]. Second, a 24-nt nat-siRNA directs transcript cleavage *in vivo* [84], as do 24-nt siRNAs that are artificially loaded into AGO1-containing RISC in *Arabidopsis* extracts [28]. Third, AGO1 has been implicated in 21-nt siRNA-guided DNA methylation of transgenes [27], but it is also required for TGS and H3K9 methylation at at least one transposon locus that generates 24-nt siRNAs [126]. A novel small RNA species, 22 nt in size, was recently characterized in *Arabidopsis* and its accumulation was found dependent on DCL2 [13,14]. The 22-nt siRNA accumulates during infection by at least one RNA virus and also derives from the dsRNA precursors of tasiRNA, which are normally processed by DCL4. Whether the 22-nt siRNA has any functionality remains currently unknown.

Recent crystallographic data from the *Giardia intestinalis* Dicer – an enzyme that generates 25-nt siRNAs – provides some hints as to how siRNAs of specific sizes might be generated by different Dicers [127]. It was found that the 65 Å distance between the PAZ and RNase III domains of the *Giardia* Dicer matches the length spanned by 25 bp of RNA, suggesting that Dicer is a molecular ruler that cleaves at a specified distance from the helical end. The existence of specific Dicers with specific products in plants provides a good opportunity to challenge this model experimentally, although inter- rather than intra-molecular interactions might also influence the final size of small RNAs *in vivo*. Hence, specific PAZ domain-interacting proteins could modulate the cleavage specificity of any given Dicer, for instance by acting as hinges on modification of the PAZ steric environment. This might explain, for instance, why DCL2 has been implicated in production of both 24-nt and 22-nt siRNAs in separate sRNA biosynthetic pathways in *Arabidopsis* [84,91].

transcriptional silencing complex, RITS, characterized in fission yeast [98]. This complex could contain AGO4, because *ago4* mutants have phenotypes overlapping with those of *rdr2*, *dcl3*, *nrpd1a* and *nrpd2* [11]. At loci affected by these mutations, methylation of CNG and particularly of CNN is strongly reduced, whereas loss of CG methylation is less pronounced, consistent with the observation that MET1-dependent promoter CG methylation could be maintained in the absence of a virus-encoded RNA trigger of transcriptional gene silencing (TGS) [99].

Possible targets of casiRNAs include nascent transcripts (Figure 3a) or DNA itself (Figure 3b). In the *S. pombe* heterochromatic RNAi pathway resulting in H3 K9 (but not cytosine) methylation, target transcription by Pol II is required for siRNA action, and Ago1 associates with nascent transcripts [100]. siRNA-directed histone methylation of the human *EF1A* promoter was also dependent on active Pol II transcription [101]. However, direct siRNA–DNA base-pairing cannot be excluded. For instance, in experiments involving virus-derived promoter-directed siRNAs, the methylated DNA interval on targeted promoters matched the primary siRNA source and did not extend any further into transcribed regions [99]. If siRNAs indeed interact directly with DNA, how does the double helix become available for siRNA pairing? Pol IV could facilitate this access, for instance by moving along the DNA with associated helicases.

The precise molecular mechanisms underlying sequence-specific recruitment of cytosine and histone methyl-transferases to silent loci also remain elusive, because associations between sRNA and such enzymes have been reported only once, in human cells [101]. In fact, there seems to be a self-sustaining loop in which siRNA production and DNA/histone methylation are mutually dependent at endogenous silent loci, raising the possibility that production of chromatin-directed siRNAs *in vivo* might even be a consequence, rather than a cause, of DNA or histone methylation (Figure 3).

The RDR2–DCL3–NRPD1–AGO4 pathway has clear roles in taming transposons and in maintenance of genome integrity in plants, because loss of casiRNA caused by mutations in the factors in the pathway reactivates transposon activity [11,91]. This pathway could also maintain heterochromatin at centromeric repeats, which appears mandatory for accurate chromosome segregation in *S. pombe* [102]. The 24-nt siRNA-generating machinery can also act to silence protein-coding genes. For example, expression of the key negative regulator of flowering *FLC* is maintained at a low level in an early-flowering *Arabidopsis* ecotype because of the presence of an intronic transposon that causes repressive chromatin modifications through the action of a pathway dependent of NRPD1a and AGO4 [103]. Nevertheless, several additional mechanisms, not necessarily mediated by siRNAs, account for epigenetic regulation of gene expression in plants. For example, in *Arabidopsis*, mutation of the chromatin-remodeling factor DDM1 has much broader consequences on chromatin silencing than any known single mutant in the RNA silencing machinery [104,105]. In addition, gene regulation by Polycomb-like proteins in *Arabidopsis* has not been linked to RNA silencing [106].

Future directions: integrating plant silencing pathways into the global scheme of gene expression pathways

Although the data summarized here indicate that diversification and specialization of RNA silencing pathways have contributed to specific modes of sRNA-directed gene regulation in plants, the division into pathways presented here should be merely considered as a conceptual guide. It is indeed becoming increasingly clear that diverse RNA silencing mechanisms intersect in plants, as illustrated by the sophistication of tasiRNA and nat-siRNa biogenesis. However, such intersections can also have more subtle, yet highly significant, manifestations. For instance, the chromatin silencing component NRPD1a was originally identified from the weak S-PTGS defects of *nrpd1a* mutants [92], and *rdr2*, *met1* and *ddm1* mutants show similar defects at late developmental stages [107]. This suggests that chromatin modifications might contribute to reinforce S-PTGS siRNA synthesis, for example by promoting aberrant transcription. One of the challenges facing the plant RNA silencing field will be to apprehend fully the output of such interrelated sRNA networks. A second challenge will be to tie RNA silencing into other components of gene

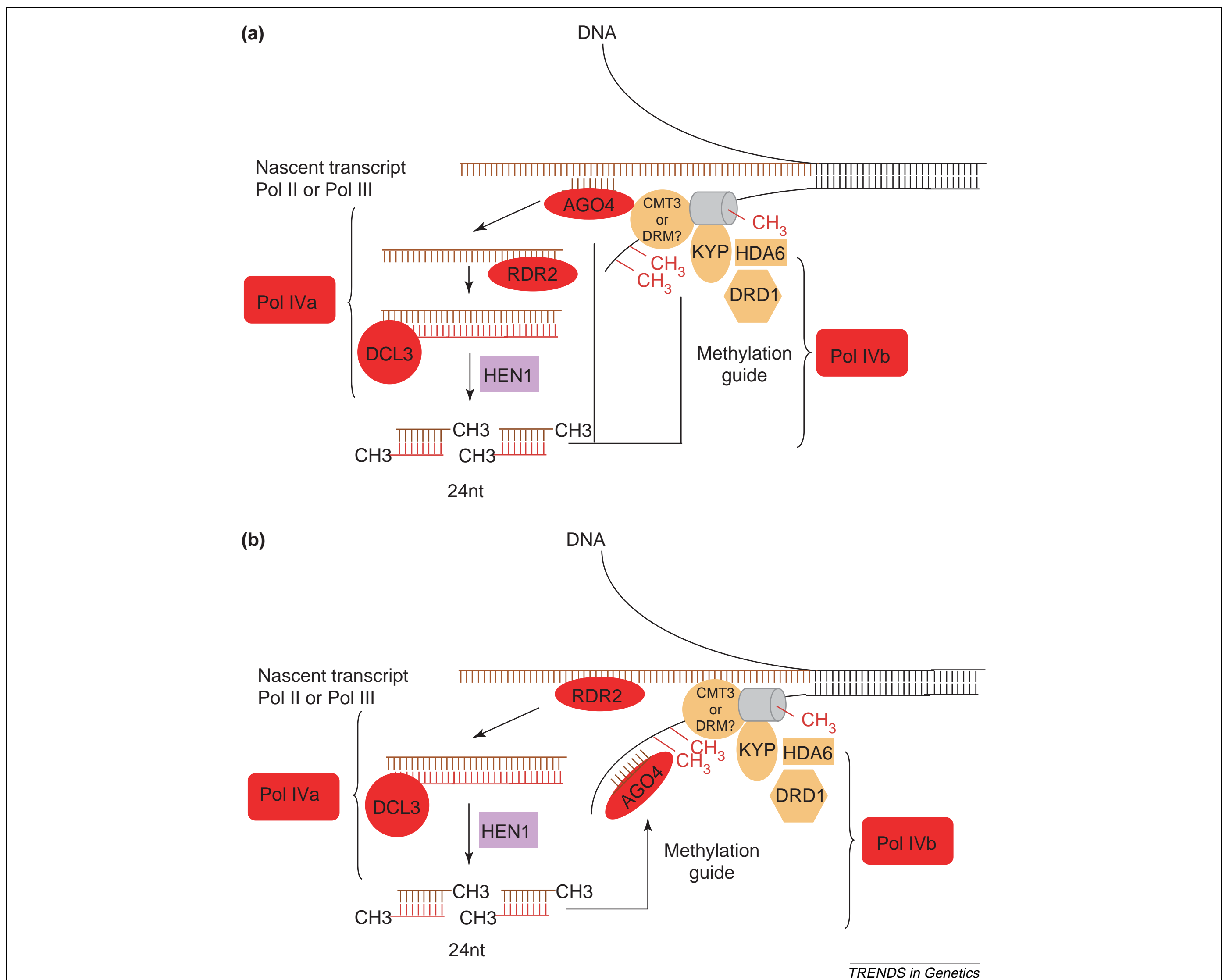

Figure 3. Chromatin-targeted RNA silencing. The scheme illustrates two of many non-mutually exclusive scenarios that possibly account for siRNA-directed chromatin modifications at endogenous loci. Note that both scenarios are based on circular and amplified schemes in which siRNA production and chromatin modification reinforce one another. **(a)** A nascent Pol II or Pol III transcript is cleaved through the action of siRNA-programmed AGO4, resulting in a truncated RNA that is converted into dsRNA by the action of RDR2. The dsRNA is then processed by DCL3 into 24-nt siRNAs that direct further cleavage of nascent transcripts and might possibly guide sequential activities of histone deacetylases (e.g. HDA6), histone methyl-transferases (e.g. KYP or SUVH2) and/or DNA methyl-transferases (CMT3 or a DRM). It is unclear whether histone modification precedes DNA methylation or not. The process might also involve siRNA-directed chromatin remodeling factors, such as DRD1. The positions of Pol IVa and Pol IVb in those reactions are currently ill defined. **(b)** The same effectors are involved but, in this scenario, RDR2 uses nascent transcripts as templates and siRNA-loaded AGO4 is recruited to guide chromatin modifications rather than RNA cleavage.

expression pathways. There are already predictable links to transcription elongation, termination and mRNA degradation, but there are also specific connections to dsRNA editing [108]. Finally, the induction of the S-PTGS pathway in *xrn4* mutants [18] suggests that the silencing machinery might have broad quality control functions in addition to its currently recognized regulatory roles.

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