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Targets of RNA-directed DNA methylation

Marjori Matzke, Tatsuo Kanno, Bruno Huettel, Lucia Daxinger and Antonius J M Matzke

RNA-directed DNA methylation contributes substantially to epigenetic regulation of the plant genome. Methylation is guided to homologous DNA target sequences by 24 nt 'heterochromatic' small RNAs produced by nucleolar-localized components of the RNAi machinery and a plant-specific RNA polymerase, Pol IV. Plants contain unusually large and diverse populations of small RNAs, many of which originate from transposons and repeats. These sequences are frequent targets of methylation, and they are able to bring plant genes in their vicinity under small RNA-mediated control. RNA-directed DNA methylation can be removed by enzymatic demethylation, providing plants with a versatile system that facilitates epigenetic plasticity. In addition to subduing transposons, RNA-directed DNA methylation has roles in plant development and, perhaps, stress responses.

Addresses
Gregor Mendel Institute of Molecular Plant Biology, Austrian Academy of Sciences, Dr. Bohr-Gasse 3, A-1030 Vienna Austria

Corresponding author: Matzke, Marjori (marjori.matzke@gmi.oeaw.ac.at), Kanno, Tatsuo (tatsuo.kanno@gmi.oeaw.ac.at), Huettel, Bruno (bruno.huettel@gmi.oeaw.ac.at), Daxinger, Lucia (lucia.daxinger@gmi.oeaw.ac.at) and Matzke, Antonius J.M. (antonius.matzke@gmi.oeaw.ac.at)

Current Opinion in Plant Biology 2007, **10**:512–519

This review comes from a themed issue on
Cell Signalling and Gene Regulation
Edited by Jian-Kang Zhu and Ko Shimamoto

Available online 16th August 2007

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2007.06.007

Introduction

RNA-directed DNA methylation (RdDM) in plants refers to a specific process in which small interfering RNAs (siRNAs) produced by the RNA interference (RNAi) pathway guide *de novo* methylation of cytosines in all sequence contexts (CG, CNG and CNN, where N is A, T or C) at the homologous DNA region [1,2]. Promoter-directed siRNAs can induce promoter methylation and transcriptional gene silencing (TGS) of transgenes [1,3] and endogenous genes [1,4,5] in different plant species.

Although the mechanism of RdDM remains incompletely understood, recent work has identified novel components of the silencing machinery and localized them to specialized subnuclear domains. Considerable information on DNA targets of RdDM has accumulated from analyses of silencing-defective mutants, the small RNA transcriptome, and the whole genome 'methylome' in *Arabidopsis thaliana*. It is increasingly apparent that RdDM contributes not only to transposon silencing but also to regulation of genes important for plant physiology and development. This often involves the antagonistic process of enzymatic demethylation, which allows dynamic control of DNA methylation in response to developmental and environmental cues. Here we describe recent research highlights pertaining to these aspects of this fast-moving field.

Components of the RdDM pathway

Forward and reverse genetics approaches in *Arabidopsis* have identified proteins acting in the RdDM pathway [1,2] (Table 1). These can be grouped into nuclear 'RNAi' proteins required for producing, stabilizing and interacting with the siRNA trigger [RNA-dependent RNA polymerase2 (RDR2), DICER-like3 (DCL3), Argonaute4 (AGO4), AGO6, Hua enhancer1 (HEN1)]; DNA cytosine methyltransferases that catalyze *de novo* and maintenance methylation [domains rearranged methyltransferase (DRM1, DRM2), chromomethylase3 (CMT3), methyltransferase1 (MET1)]; and histone modifying enzymes that help to maintain and reinforce DNA methylation [histone deacetylase6 (HDA6) and the Su(var)3-9 homologs SUVH4 (also known as kryptonite), SUVH5, SUVH6]. In addition, RdDM requires several plant-specific proteins including the putative SNF2-like chromatin remodelling factors defective in RNA-directed DNA methylation1 (DRD1) and CLASSY1 (CLSY1), as well as subunits of a novel RNA polymerase IV (Pol IV). Cytological studies have added a cell biological dimension to RdDM by demonstrating that nucleoli and associated Cajal bodies act as siRNA processing centers in this pathway [6••,7••] (Figure 1).

Pol IV

Pol IV subunits were discovered during the analysis of the *Arabidopsis* genome sequence [8]. Subsequent work revealed a requirement for these plant-specific RNA polymerase subunits in RdDM and defined two functionally diversified forms, Pol IVa and Pol IVb [9•–12•]. The two forms are distinguished by their unique largest subunits, NRPD1a and NRPD1b, which act together with the same second largest subunit, NRPD2a, at different steps of the RdDM pathway. In addition, each Pol IV isoform is associated with a specific SNF2-like factor: Pol IVa and CLSY1 [13•] are needed to produce or amplify the siRNA trigger, while Pol IVb and DRD1 generally act

Table 1

Genes implicated in RNA-directed DNA methylation and active demethylation

Gene	AGI number	Activity	Function
MET1	At5g49160	DNA methyltransferase	CG maintenance methylation
CMT3	At1g69770	DNA methyltransferase	CNG methylation
DRM2	At5g14620	DNA methyltransferase	*De novo* methylation (CG, CNG, CNN)
DRM1	At5g15380	DNA methyltransferase	A weakly active homologue of DRM2
ROS1	At2g36490	DNA glycosylase/lyase	DNA demethylation
DME	At5g04560	DNA glycosylase/lyase	DNA demethylation
DML2	At3g10010	DNA glycosylase/lyase	DNA demethylation
DML3	At4g34060	DNA glycosylase/lyase	DNA demethylation
HDA6	At5g63110	Putative histone deacetylase	Histone deacetylation, maintain CG methylation via MET1
SUVH4/KYP	At5g13960	Histone H3 lysine 9 methyltransferase	Histone methylation, maintain non-CG methylation via CMT3
SUVH5	At2g35160	Histone H3 lysine 9 methyltransferase	Histone methylation, maintain non-CG methylation via CMT3
SUVH6	At2g22740	Histone H3 lysine 9 methyltransferase	Histone methylation, maintain non-CG methylation via CMT3
RDR2	At4g11130	RNA dependent RNA polymerase	Synthesis of double stranded RNA in heterochromatic siRNA pathway
DCL3	At3g43920	RNAase III (Dicer-like)	Processes double stranded RNA to 24 nt heterochromatic siRNAs
HEN1	At4g20910	RNA methylase	Methylation of 21–24 nt siRNAs
AGO4	At2g27040	Argonaute protein	RNA cleavage, 24 nt siRNA binding, *de novo* DNA methylation
AGO6	At2g32940	Argonaute protein	DNA methylation, siRNA accumulation
CLSY1	At3g42670	SNF2-like chromatin remodelling protein	Generation of 24 nt heterochromatic siRNAs, Pol IVa partner
DRD1	At2g16390	SNF2-like chromatin remodelling protein	RNA-directed *de novo* DNA methylation (CG, CNG, CNN), Pol IVb partner
NRPD2a	At3g23780	Second largest subunit of Pol IVa and Pol IVb	Generation of 24 nt heterochromatic siRNAs, *de novo* DNA methylation
NRPD1a	At1g63020	Largest subunit of Pol IVa	Generation of 24 nt heterochromatic siRNAs
NRPD1b	At2g40030	Largest subunit of Pol IVb	*De novo* DNA methylation

downstream of this step to facilitate *de novo* methylation at the siRNA-targeted site [14] (Figure 1).

The modes of action of the Pol IV complexes in the mechanism of RdDM are unclear. It is not known whether Pol IVb transcribes extensively or simply opens chromatin at the siRNA-targeted site to expose DNA to methyltransferases [11•]. Because it is required for siRNA accumulation, Pol IVa is thought to be transcriptionally active, but the substrate is uncertain. One idea is that it transcribes methylated DNA [10•] (Figure 1A), which is consistent with the paradoxical requirement for transcription of some target loci to maintain chromatin-based silencing by RNAi-mediated mechanisms [15]. Alternatively, Pol IVa might transcribe nascent RNA at the target locus [6••] (Figure 1B) or double stranded RNA [16]. Assessing these alternatives, which are not mutually exclusive, will require in vitro transcription systems to study Pol IV activities.

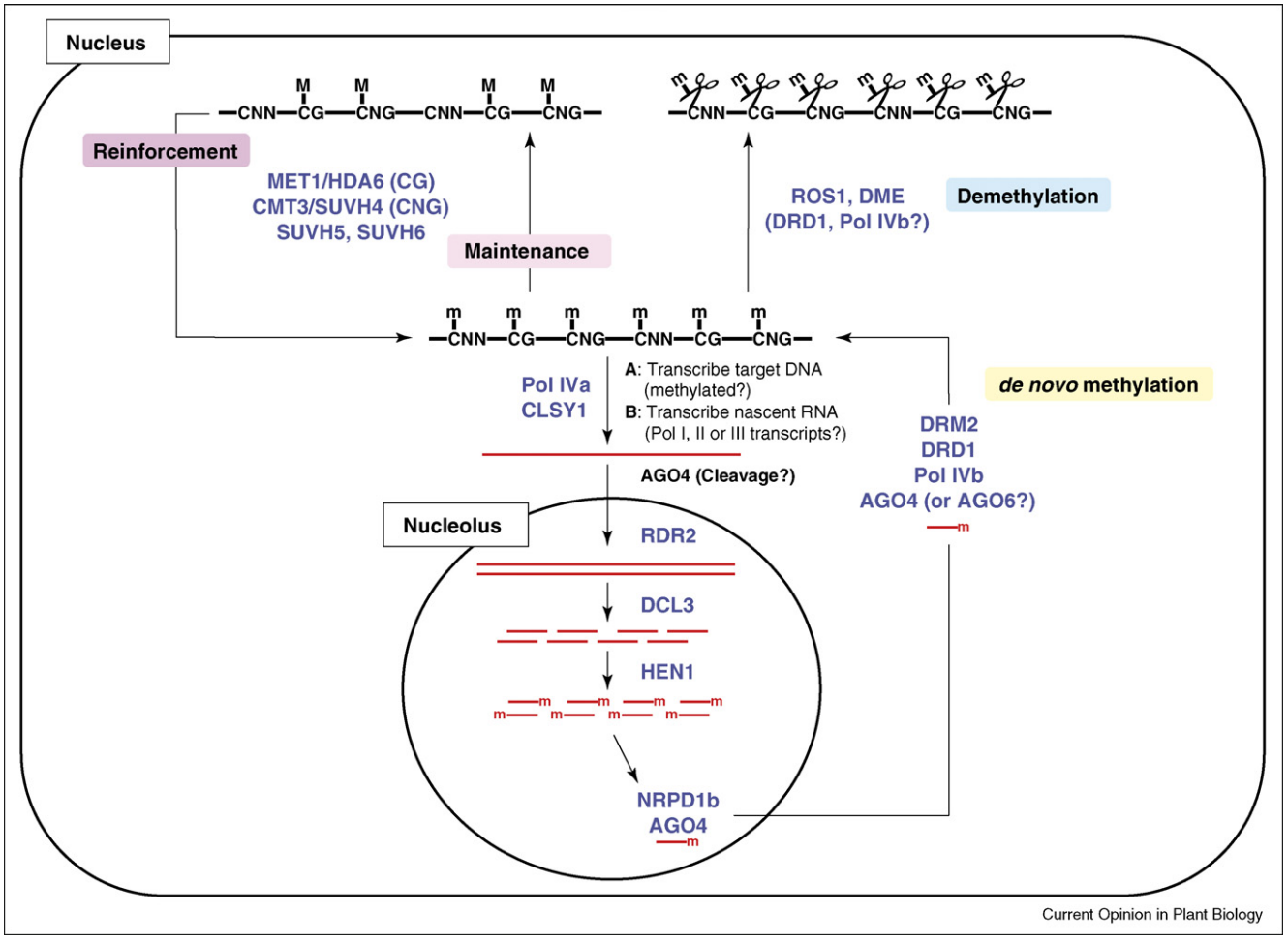
The functional distinction between the two Pol IV largest subunits presumably arises from differences in their C-terminal domain (CTD), which is about 500 amino acids longer in NRPD1b than in NRPD1a [17]. The extended CTD in NRPD1b contains an amino acid repeat with potential phosphorylation sites, which might regulate binding of factors similar to the CTD of the largest subunit of Pol II [12•]. Both proteins contain in their CTD a motif found in a group of small, plant-specific proteins called 'defective chloroplasts and leaves', which are involved in ribosomal RNA processing [18]. The function of this motif in the Pol IV largest subunits is unknown, but it may help to recognize or process RNA.

AGO proteins in RdDM

AGO proteins are core components of silencing effector complexes in all RNAi-mediated pathways. In addition to binding siRNAs through their PAZ domain, AGO proteins have a catalytic (RNA 'slicer') activity conferred by their PIWI domain, which resembles RNase H [19]. Three of the ten AGO proteins in *Arabidopsis* have been implicated in RdDM. AGO4 binds siRNAs from a number of transposons and repeats [20••] and interacts with the CTD of NRPD1b [7••], which is consistent with the involvement of AGO4 in *de novo* methylation [2] (Figure 1). While siRNA binding is sufficient for RdDM at some target loci, other targets require in addition the RNA slicer activity of AGO4 [20••]. This requirement might reflect the need to cleave a nascent transcript to produce a substrate for RDR2 (Figure 1), thus reinforcing siRNA production and/or promoting the spread of methylation [21]. In cases where AGO4 slicer activity is not required, aberrant RNAs that are substrates for RDR2 might be produced in a cleavage-independent manner, for example by premature transcription termination from a methylated DNA template.

AGO6 is required for DNA methylation and siRNA accumulation at some loci, where it may function redun-

Figure 1



A model for dynamic regulation of RdDM. Middle: Pol IVa (containing NRPD1a and NRPD2a) together with SNF2-like chromatin remodeling protein CLSY1 transcribes the target locus, which might already be lightly methylated (small 'm') or associated with specific histone modifications [47] (A). Alternatively, Pol IVa might transcribe a nascent RNA produced at the target locus by Pol I, II or III (B). The Pol IV-synthesized transcript enters the nucleolus by an unspecified mechanism, where it is copied into double stranded RNA by RDR2. For some loci the RNA slicer activity of AGO4 is required, perhaps to generate a substrate for RDR2 activity. DCL3 processes the double stranded RNA into 24 nt siRNAs, which are methylated at their 3′ termini by HEN1 [67]. The siRNA is loaded onto AGO4, which interacts with the CTD of NRPD1b. This complex moves out of the nucleolus into the nucleoplasm, where it acquires NRPD2a to form a functional Pol IVb complex. In cooperation with the DNA methyltransferase DRM2 and SNF2-like protein DRD1, the siRNA/AGO4/Pol IVb complex facilitates *de novo* methylation of cytosines in all sequence contexts at the siRNA-targeted site. Top, left: CG and CNG methylation can be maintained during DNA replication by MET1 and CMT3, respectively. Locus-specific histone modifications that are catalyzed by HDA6, SUVH4, SUVH5 and/or SUVH6 [68,69] help to maintain cytosine methylation (large 'M') and reinforce the silent state, perhaps in part by recruiting Pol IVa to amplify the siRNA trigger. Top, right: DNA methylation can be lost in nondividing cells by a base excision repair-type mechanism that involves DNA glycosylase/lyase proteins such as ROS1 and DME. Indirect evidence suggests that DRD1 and Pol IVb [14,39•] might be involved in this process. Subnuclear localizations are based on cytological data published in Refs. [6••,7••]. The nucleolar localization of HEN1 is still hypothetical.

dantly with AGO4 [22•]. A similar role has been reported for AGO1 [23], which is generally associated with siRNAs and microRNAs mediating posttranscriptional gene silencing [24]. Whether the other two members of the AGO4/AGO6 subfamily – AGO8 and AGO9 [22•] – participate in RdDM remains to be determined.

Reversible methylation

An important feature of RdDM is its potential reversibility, which is due in part to the induction of CNN methylation. In contrast to symmetrical CG and CNG methylation, which can be maintained during DNA replication in the absence of the RNA trigger (Figure 1), asymmetrical CNN methylation requires the continuous presence of the inducing RNA. Therefore, CNN methylation can be lost passively in dividing cells if the RNA signal is withdrawn [1,2].

In nondividing cells, DNA methylation can be erased through enzyme-catalyzed demethylation (Figure 1).

Biochemical studies demonstrate that proteins with DNA glycosylase/lyase activities – exemplified in *Arabidopsis* by REPRESSOR OF SILENCING1 (ROS1), DEMETER (DME) and two additional family members DEMETER-LIKE2 (DML2) and DML3 (Table 1) – can demethylate DNA by a base excision repair-type mechanism [25–27]. While DME is active only in the central cell of the female gametophyte, the other three proteins are expressed more widely, suggesting broader roles in plant physiology or development. As described below, some genes targeted for RdDM are also demethylated by DME and ROS1.

Small RNA transcriptome

Deep sequencing has revealed a vast world of small regulatory RNAs in *Arabidopsis* [28–30,31••,32••,33], rice [34,35], and maize [35] (Supplementary Table 1). Compared to other eukaryotes, flowering plants have extraordinarily large and complex populations of endogenous siRNAs. In an analysis of approximately 340 000 unique small RNAs from *Arabidopsis* [32••], more than half comprised distinct 24 nt 'heterochromatic' siRNAs, all of which can potentially trigger RdDM. Around a third of these are derived from annotated transposons and repeats, and nearly half originate from intergenic regions, indicating that *Arabidopsis* uses siRNAs extensively to regulate these portions of its genome. Most 24 nt siRNAs require Pol IVa, RDR2 and DCL3 for their generation [29,30,31••,33] (Figure 1).

Methylome

Arabidopsis mutants defective in DNA methyltransferases and other components of the RdDM pathway have been used to investigate DNA methylation and/or transcription [36,37•,38••,39•] (Supplementary Table 2). An extensive analysis exploited *Arabidopsis* whole genome tiling arrays to examine genome-wide DNA methylation (the 'methylome') as well as transcription from both DNA strands in *met1* mutants and *drm1 drm2 cmt3* triple mutants [38••]. Small RNA-generating regions were usually methylated, but around 60% of methylated regions did not colocalize with siRNAs, indicating either alternative signals for methylation or maintenance of CG methylation in the absence of the original siRNA trigger. Only around 5% of promoters were methylated, and these were often associated with tissue-specific genes. By contrast, 33% of expressed genes had methylation in the body of the gene [38••], which was found in one study to be influenced by transcription [37•]. Despite a previous report that a microRNA induces methylation of a target gene [40], microRNAs and trans-acting siRNAs do not generally appear to trigger RdDM [38••].

Targets of RdDM

Data on the methylome and transcript profiling can be combined with information on the small RNA transcriptome to provide a genome-wide perspective of RdDM-generated methylation patterns and their impact on gene expression in wild type and mutant plants. Major targets of RdDM include transposons and repeats that are present in constitutive and facultative heterochromatin [9•–12•,41,42]. Dispersed copies of these sequences that are present between and within genes in euchromatic chromosome arms are also targeted for methylation and repressive histone modifications, which can potentially be more easily reversed [36,38••,39•]. Not all transposons are regulated by RdDM [43,44] indicating alternate mechanisms for silencing these elements.

A feature that is turning up in many targets of RdDM is tandem repeats, which might attract RNAi-mediated chromatin modifications because they can sustain RDR and DCL activities in a way not possible with single copy sequences [45]. Short tandem repeats that are targets of RdDM are found in a number of retrotransposon long terminal repeats (LTRs) [17] and DNA transposons [46]. Tandem repeats are also associated with some plant genes that are regulated by the RdDM pathway (see below). However, they are not found in all targets of RdDM [39•] nor are all tandem repeats in the *Arabidopsis* genome modified by RdDM [47]. Thus, further work is required to define the full range of features that recruit the RdDM machinery.

An open question concerns the number of plant genes that are regulated by RdDM and whether conventional targets of this pathway (transposons, repeats) are always associated with these genes. A common list of target genes has not yet emerged from the data sets available so far, although the number appears to be rather modest (Supplementary Table 2). Similarly, the number of plant genes that are demethylated by ROS1 and related proteins may not be particularly high [27] (Supplementary Table 2). Additional genes that are targets of RdDM and/or active demethylation could be identified as more subtle changes of expression are assessed in different cell types and under various stress conditions. Several prominent examples illustrate that developmentally important genes can be targets of potentially reversible RdDM or siRNA-mediated chromatin modifications, either with or without an obvious transposon-related or repetitive sequence.

RdDM in development

FLOWERING WAGENINGEN (*FWA*) exemplifies a developmental gene that is regulated by RdDM and active demethylation. *FWA*, which encodes a homeobox transcription factor, is imprinted and expressed only from the maternal genome in endosperm [48]. The *FWA* promoter contains a pair of transposon-associated tandem repeats that give rise to siRNAs [17,41]. These are sufficient to induce DNA methylation and silence *FWA* expression, which is the default state [47,49•]. In the central cell of the female gametophyte, DME removes methylation, so that only the maternal allele is expressed following fertilization [48]. The maternal allele of a

second imprinted gene, *MEDEA* (*MEA*), is also demethylated by DME [50]; however, it is unknown whether siRNAs originally induce methylation of *MEA* since neither transposons nor repeats are required for imprinting of this gene [51].

The floral regulator *FLOWERING LOCUS C* (*FLC*) is a single copy gene that is subject to siRNA-mediated chromatin modifications, with different variations in two commonly used accessions. In Landsberg *erecta*, weak expression of *FLC* is due to the presence of an intronic *Mutator* transposon, which is targeted for repressive chromatin modifications by siRNAs [52]. In Columbia, a unique region of the *FLC* 3′ UTR undergoes repressive chromatin modifications induced by Pol IVa/RDR2/DCL3-dependent siRNAs, leading to suppression of expression [53].

Paramutation of the pigmentation gene *booster1* (*b1*) in maize is mediated by a bidirectionally transcribed tandem repeat that is 100 kb upstream of the transcription start site. Paramutation refers to an interaction between alleles that induces a meiotically heritable epigenetic change of one allele [54]. A mediator of paramutation, *MOP1*, encodes an RDR that is similar to *Arabidopsis* RDR2, indicating that an RNAi-related pathway is involved in maintaining heritable chromatin states in paramutation [55••] and transposon silencing [56]. *mop1* mutants also show pleiotropic developmental phenotypes [57].

RdDM in stress responses

In addition to the aforementioned examples, a role for RdDM in development is suggested by the pleiotropic developmental abnormalities observed in *drm1 drm2 cmt3* triple mutants [58]. However, the relatively normal appearance of other RdDM-defective mutants, such as *drd1*, *nrpd2a* and *nrpd1b* (T Kanno, M Matzke, unpublished data), suggests that the primary function of this pathway is not to regulate development. There are several reasons to consider a role for reversible RdDM in stress responses. A number of stress-induced small RNAs have been identified [59], at least some of which require Pol IVa for their biogenesis [60]. Targets of RdDM, such as retrotransposon LTRs [23,39•], can be derepressed by different types of stress [61,62] and provide promoter/enhancer activities for adjacent plant genes [39•,62]. Silent transposons can be maintained in a flexible epigenetic state by ROS1, which trims methylation from some of these sequences [63•]. A defense-related gene encoding the cell wall protein extensin is upregulated in a *drd1* mutant [39•] and downregulated in a *ros1* mutant [63•], illustrating the potential for dynamic control of adaptive genes through the reversible RdDM pathway.

Conclusions and outlook

Although RdDM can silence transposons, the functions of this RNAi-mediated pathway extend beyond permanently suppressing these elements. For that purpose, mitotically heritable CG methylation would suffice, as it does in mammals. Even though a process identical to RdDM does not appear to exist in mammalian somatic cells [1], testes-specific 24–31 nt Piwi-interacting (pi)RNAs, which are produced independently of Dicer, may guide methylation of retrotransposons in the male germline [64,65]. Plants have elaborated a versatile, siRNA-guided mechanism for DNA methylation that not only silences transposons and their derivatives throughout the plant but also exploits these sequences to reversibly modulate transcription of genes important for plant physiology and development. Key components of the RdDM machinery are the Pol IV subunits, which evolved in plants in a multistep process from Pol II subunits [66•]. The last and most complex subunit to evolve, NRPD1b, appeared in the ancestor of angiosperms. The epigenetic plasticity afforded by Pol IVb-mediated RdDM may have contributed to the rapid evolution of flowering plants and their ability to colonize extraordinarily diverse habitats.

Many questions about RdDM remain to be addressed in future research. What is the mode of action of Pol IV isoforms? Do siRNAs interact with target sequences through RNA–DNA or RNA–RNA recognition? How extensive is the overlap between targets of RdDM and demethylation activities, and how are these reciprocal processes channelled appropriately into pathways important for stress tolerance and development? Do siRNAs guide ROS1 and related proteins to target sequences? How does the compartmentalization of siRNA processing steps in nucleoli contribute to regulation of RdDM? Answers to these questions and a deeper understanding of the natural functions of reversible RdDM should soon emerge through the powerful combination of genetic, genomic and biochemical approaches.

Acknowledgements

Work in our lab is supported by the European Science Foundation under the EUROCORES Programme EuroDYNA, through contract No, ERAS-CT-980409 of the European Commission, DG Research, FP6, and the Austrian Fonds zur Förderung der wissenschaftlichen Forschung, grant I26-B03.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pbi.2007.06.007.

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