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Chromatin-based silencing mechanisms

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Eukaryotic genomes are organized into regions of transcriptionally active euchromatin and transcriptionally inactive heterochromatin. In plant genomes, heterochromatin is marked by methylation of cytosine and methylation of histone H3 at lysine 9. Heterochromatin formation is targeted to transposons as a means of defending the host genome against the deleterious effects of these sequences. Heterochromatin is directed to transposon sequences by transposon-derived aberrant RNA species and functions to prevent unwanted transcription and movement. Formation of heterochromatin at rRNA-encoding genes and centromere-associated repeats might also involve an RNA-based mechanism that is designed to stabilize these potentially labile structures.

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Current Opinion in Plant Biology 2004, **7**:521–526

This review comes from a themed issue on
Cell signalling and gene regulation
Edited by Jen Sheen and Steven Kay

Available online 28th July 2004

1369-5266/$ – see front matter

DOI 10.1016/j.pbi.2004.07.003

Abbreviations

CMT	chromomethylase
DMTase	DNA methyltransferase
dsRNA	double-stranded RNA
HMTase	histone methyltransferase
HP1	heterochromatin protein 1
RdDM	RNA-directed DNA methylation
RdRP	RNA-dependent RNA polymerase
RNAi	RNA interference
siRNA	small interfering RNA

Introduction

In eukaryotes, DNA is packaged into the nucleus of the cell by assembly into chromatin. The fundamental unit of chromatin is the nucleosome, consisting of an octamer with two subunits each of histones H2A, H2B, H3 and H4, which provides a globular core that is wrapped by roughly 150 bp of DNA. Nucleosomal DNA is further packaged by higher-order folding and association with other proteins. Along each chromosome, chromatin is organized into transcriptionally active, less-condensed euchromatin, and transcriptionally inactive, highly condensed heterochromatin.

In some eukaryotes, including plants and mammals, DNA in regions of heterochromatin is marked by methylation at the 5-position of cytosines. This covalent DNA modification can be inherited on the template strand during DNA replication to guide the maintenance of the mark. In both plants and animals, heterochromatin is also marked by histones carrying particular posttranslational modifications on their amino-terminal tails, which extend outwards from the core of the nucleosome. These modifications include methylation of histone H3 at lysine 9 (H3 mK9), and a lack of acetylation on histones H3 and H4. By contrast, euchromatin is marked by methylation of H3 at lysine 4 (H3 mK4) and acetylation of histones H3 and H4. These combinations of histone modifications have been proposed to constitute a 'histone code' that guides chromatin interactions with appropriate chromatin remodeling factors (reviewed in [1,2]). In this review, I discuss recent results that elucidate how heterochromatin is targeted to appropriate regions of the plant genome, and how interplay between DNA methylation and histone modification acts to maintain the heterochromatin state.

RNA-directed DNA methylation

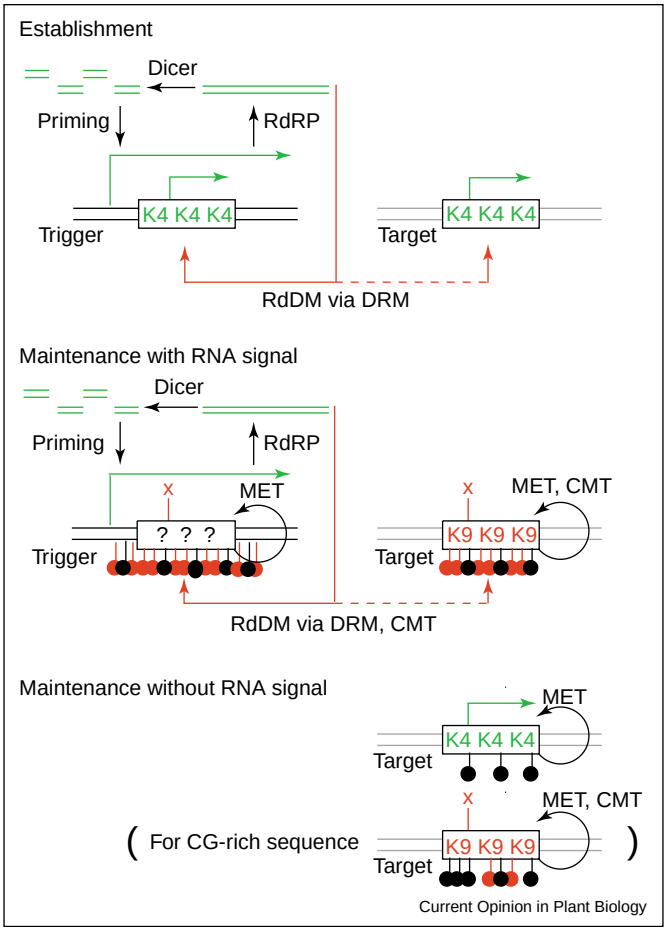
Some, if not all, heterochromatin formation in plant genomes is directed by aberrant RNA species. This RNA-based heterochromatin formation is interrelated with RNA interference (RNAi), another RNA-based silencing mechanism (reviewed in [3]). In RNAi, double-stranded RNA (dsRNA) provides a trigger for the degradation of transcripts that have shared sequence identity. The dsRNA trigger is first diced into small interfering RNAs (siRNAs) of 21–26 nt by Dicer ribonucleases. At least some species of these siRNAs are subsequently taken up by an RNA-induced silencing complex and used to guide the degradation of target transcripts by sequence complementarity. The observation from plant systems that produce high levels of dsRNA, such as RNA virus infections [4,5••] or highly transcribed inverted repeat transgenes [6,7•,8], is that dsRNA and/or siRNAs can also trigger the methylation of DNA with identical sequences. This RNA-directed DNA methylation (RdDM) is highly sequence-specific, methylation does not significantly spread beyond the boundary of the RNA trigger [9,10].

In plant genomes, DNA methylation is maintained preferentially at cytosines in the symmetric dinucleotide context 5′-CG-3′ (hereafter referred to as CG) but

methylation can also occur in CNG (where N is any nucleotide) and asymmetric contexts. By contrast, in mammalian genomes, methylation is maintained almost exclusively in CG contexts. RdDM typically involves dense methylation of both CG and non-CG cytosines in the target regions. Forward and reverse genetic approaches in *Arabidopsis thaliana* have implicated two plant-specific types of DNA methyltransferase (DMTase) in non-CG methylation: DRM DMTases are primarily involved in initiating new RdDM imprints and can methylate asymmetric cytosines [11,12,13•,14], whereas the CMT3 DMTase maintains the RdDM imprint preferentially at CNG cytosines ([12,14–16]; Figure 1). A third type of DMTase, MET1, is related to the mammalian DMTase Dnmt1 and maintains methylation in CG contexts [17,18•]. Thus, the relative contribution of each of type of DMTase to the overall methylation status of a locus depends on the sequence composition of the locus. The CMT 'chromomethylase' class carries a 'chromo-domain' interaction motif embedded in its methyltransferase catalytic domain. As the chromo-domains found in the animal heterochromatin protein 1 (HP1) and Polycomb protein bind to H3 mK9 and H3 mK27, respectively [19–21], it is attractive to speculate that the CMT chromo-domain is similarly involved in interactions with methylated histones. In fact, the genetic screens in *Arabidopsis* that identified *cmt3* mutations also identified mutations in the KYP/SUVH4 H3 K9 histone methyltransferase (HMTase) that confer deficiencies in non-CG methylation, indicating a direct link between the H3 mK9 mark and CMT3-mediated DNA methylation [22•,23•].

In the fungus *Neurospora crassa*, DNA methylation is maintained by the DMTase DIM-2, which lacks a chromo-domain [24], but also requires a separate

Figure 1



Establishment and maintenance of heterochromatin. The box represents a duplicated transposon sequence, which is either transcribed (green arrows) or silenced (red crosses), depending on whether DNA methylation occurs on promoter sequences. The histone H3 methylation status corresponding to transcriptional activity is indicated by K4 for methylation on Lys4 (active) or by K9 for methylation on Lys9 (silenced). Question marks indicate unknown histone-modification state for densely methylated but transcribed sequence. DNA methylation is indicated by filled circles, with methylated CG cytosines in black and methylated non-CG cytosines in red. The unbroken and broken arrows for RdDM indicate, respectively, strong and weak interactions of the RNA signal. CMT, DRM and MET are different classes of DNA methyltransferases.

chromo-domain-containing HP1-related protein [25] and H3 mK9 [26]. These observations suggest that the DMTase/chromo-domain/H3 mK9 relationship is conserved among eukaryotes, with variations in whether the functional protein domains are located in separate polypeptide chains. At present, however, there is no evidence that the CG methylation maintained by MET1 in plants is directed by patterns of histone methylation. In addition, the *Arabidopsis* HP1 homolog LHP1 is not needed to maintain DNA methylation [23•]. Instead, the histone deacetylase HDA6 is needed to reinforce CG methylation patterning in *Arabidopsis* [27,28,29•].

Transposon defense via RdDM

Transposon sequences present a threat to the integrity of the host plant genome because of their movement to new insertion sites. Even transposon-derived duplications that are no longer capable of movement can potentially promote unwanted rearrangements by recombination-based mechanisms. A primary line of host defense against these dangerous sequences is to target them for heterochromatin formation, which acts to suppress their transcription, movement and recombinational activity. Heterochromatin targeting involves RdDM, as indicated by patterns of CG and non-CG methylation, and transposon-derived siRNAs [28,30,31••,32].

Analysis of the mobile *CACTA* transposons in *Arabidopsis* shows that CG and non-CG methylation patterning function as redundant reinforcements in transposon suppression. When *CACTA* elements are partially demethylated by mutation of the MET1 CG DMTase or mutation of the CMT3 non-CG DMTase, they show partial transcriptional reactivation but no movement [31••]. Only when the elements are completely demethylated by a double *met1 cmt3* mutation can they transpose. Thus, the different pathways of methylation patterning in plants provide different layers of transposon defense.

A puzzling aspect of transposon methylation is how transposons, but not normal host genes, generate the dsRNA triggers for RdDM. Part of the answer to this puzzle comes from genetic studies in *Arabidopsis* showing that an RNA-dependent RNA polymerase (RdRP) and other putative RNA-processing factors are needed for both RdDM and RNAi triggered by aberrant RNA species that are not themselves double-stranded [7•,33–37]. These results suggest that some single-stranded RNAs act as templates for RdRP-mediated synthesis of a second strand to form dsRNA. Furthermore, when a particular template RNA is converted into dsRNA and diced siRNAs, the siRNAs then have the potential to amplify the pathway either by directly priming RdRP-mediated synthesis or by annealing to and causing structural changes in the template RNA that indirectly facilitate RdRP-mediated synthesis ([5••,38]; Figure 1).

What features, then, would distinguish transposon-derived transcripts as preferred templates for RdRP? A key aspect of transposons (or transgenes) is that they integrate at random sites in the host genome and, thus, can be potentially transcribed from fortuitous nearby promoters. Such 'read-through' transcripts are likely to have disrupted splice sites and translational open reading frames across the junction of host and transposon sequences, which might create cues that shunt them into the RdRP pathway. Another possibility is that independent sense and antisense strand read-through transcripts have the potential to pair with each other to form transposon dsRNA. Read-through RNA triggers of RdDM that encompass transposon sequences from end to end can impair movement at two levels: by silencing internal transposon promoters and by blocking interactions of transposon-encoded proteins with transposon sequences. RNAi provides an additional line of defense against externally driven, transposon-derived transcripts that might otherwise yield protein products that promote transposon movement.

What level of dsRNA would be required to maintain transposon methylation, especially if the transposon sequence were present around the genome in multiple copies, of which only one or a few were the source of dsRNA? Studies on the methylated endogenous *PAI* genes in *Arabidopsis*, which are arranged as an inverted repeat and transcribed from a fortuitous external promoter, suggest that extremely low levels of dsRNA can be potent RdDM signals. Only a few *PAI* transcripts extend beyond a polyadenylation site at the center of the *PAI* inverted repeat to make dsRNA, and diced siRNAs are below the limit of detection by RNA gel blot [8]. Nonetheless, the inverted repeat promotes dense CG plus non-CG methylation of itself plus two unlinked duplicated *PAI* sequences through RdDM. Moreover, a rearrangement in the center of the inverted repeat that further suppresses dsRNA levels causes reduced but still very stable methylation of *PAI* sequences [39]. The *PAI* genes thus illustrate two key points relevant to transposon defense: even very low levels of aberrant RNAs can efficiently promote RdDM, and a single 'bad' member of a family can potentially generate enough RNA to suppress the whole family *in trans*.

Transposon duplications might consist of two classes: one in which transposon sequences are transcribed from external promoters to make RdDM triggers, and another in which transposon sequences are transcriptionally silent targets (Figure 1). This idea fits in with another observation regarding non-CG methylation, that is, that the KYP/SUVH4 H3 K9 HMTase, which makes the H3 mK9 mark diagnostic of transcriptional silencing, is needed only to maintain non-CG methylation at a subset of methylation target sequences. For example, the transcribed *PAI* inverted repeat is not demethylated in a *kyp/suvh4* mutant

background [23•]. Only the unlinked transcriptionally silent *PAI* genes are affected in this background. In contrast, all three *PAI* loci are demethylated in a *cmt3* mutant background. These results suggest that, at transcriptionally active loci that produce RNA triggers of RdDM, CMT3 can respond to KYP/SUVH4-independent cues, such as H3 mK9 catalyzed by another HMTase, a different histone modification, and/or direct interactions with accumulated aberrant RNAs. Conversely, at transcriptionally silenced target loci where aberrant RNA interactions might occur more rarely, CMT3 is strongly dependent on the KYP/SUVH4-catalyzed H3 mK9 mark.

rRNA-encoding and centromere-associated repeats

Like transposon repeats, the rRNA-encoding gene (rRNA gene) repeats in plant genomes carry both cytosine methylation and the H3 mK9 mark, and yet these sequences produce high levels of rRNA transcripts. An explanation for this apparent paradox comes from recent *Arabidopsis* studies [40•,41] of the rRNA genes in nucleolus-organizer regions, which are transcribed by RNA polymerase I, and the 5S rRNA genes, which are transcribed by RNA polymerase III. These studies show that rRNA repeats consist of two populations: one that is methylated and enriched for the H3 mK9 mark, and one that is unmethylated and enriched for the H3 mK4 mark.

For the nucleolus-organizer-region repeats, which lie in subtelomeric regions, chromatin immunoprecipitation was used to show that the fraction enriched in H3 mK9 contains cytosine-methylated rRNA genes, whereas the fraction enriched in H3 mK4 contains unmethylated rRNA genes [40•]. For the 5S rRNA repeats, which lie in pericentromeric regions, immunocytology was used to show that a presumably transcriptionally active population of repeats lacking H3 mK9 emerges as a loop away from the heterochromatic population [41]. Thus, although methylation of rRNA genes might be an inevitable consequence of producing high levels of RNA, it could be that these sequences can assemble active domains of repeats that are resistant to methylation by virtue of a higher-order organization within the nucleus. In addition, those repeats that are assembled into heterochromatin might represent a storage form of rRNA sequences that are resistant to rearrangement by recombination, but that can be activated if necessary to maintain appropriate levels of rRNA.

Centromere-associated repeats (centromere repeats) represent another principal target for DNA methylation, H3 mK9 modification and heterochromatin formation [17,42,43•,44,45]. A unique feature of these repeats is that they associate with a centromere-specific histone H3 variant, CenH3, which is necessary for centromere function [46,47]. Because of the complexity of centromere repeats, it remains to be determined whether they are organized into subdomains, in which functional regions are interspersed with inactive regions. siRNAs corresponding to centromere sequences have been detected in *Arabidopsis* [32], arguing that at least a subset of the centromere repeats are transcribed to make signals for RdDM. In addition, centromere repeat sequences in a *kyp/suvh4* mutant background are only partially demethylated at CNG residues in comparison with those in a *cmt3* mutant background [22•,23•], suggesting that only a subset of the repeats are transcriptionally silent and carry the H3 mK9 mark of transcriptional silencing.

Condensed centromeric heterochromatin could be required for correct chromosome segregation and could also stabilize centromere repeats against recombination; however, methylation-deficient mutant plants with strong demethylation and decondensation of centromere sequences are viable [43•,45]. Thus, either the tight compaction of centromere repeats is not a requirement for function or deleterious effects of decondensed centromeres are not evident for plants grown under laboratory conditions for only a few generations.

Conclusions

Although it is clear that dsRNA provides a sequence-specific signal for heterochromatin formation in plants, we are only beginning to understand the underlying mechanisms. Key issues include defining the features that make some transcripts preferred substrates for processing into dsRNA, elucidating how dsRNA-derived species align with cognate DNA sequences, and identifying the components of the RNA–DNA complex that recruit DNA- and histone-modifying enzymes. Heterochromatin directed at transposon sequences prevents the deleterious effects of unchecked transcription and movement; however, the functions of heterochromatin formation at rRNA gene repeats and centromere repeats remain unclear. Mutations that disrupt heterochromatin will provide key tools with which to dissect these functions.

Acknowledgements

J Bender's research on gene silencing is supported by National Institute of Health (NIH) grant GM-61148.

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The authors use RNA viruses to deliver dsRNA that corresponded to specific sequences into the plant. Notably, when the viral dsRNA trigger corresponds only to 5′ 'GF' sequences of the green fluorescent protein gene (*GFP*), which is expressed in full length from a transgene in the tobacco or *Arabidopsis* genomes, *GFP* siRNAs and *GFP* DNA methylation spread from the trigger into the 'P' segment of the target. Additional experiments show that spreading is dependent on both transcription of the resident *GFP* transgene and the *sde1/sgs2* RdRP. These findings argue that siRNAs located anywhere along a target transcript can potentially activate the transcript as a template for RdRP-catalyzed synthesis of dsRNA. Two endogenous tobacco transcripts are shown to be resistant to RNAi spreading, however, indicating that not all transcripts can be similarly activated.

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Transgene-induced methylation and silencing of the endogenous *Arabidopsis SUPERMAN* gene provide a reporter system for mutations that are defective in silencing. This screen yields mutations in the KYP/SUVH4 H3 K9 HMTase [22•], the CMT3 DMTase [15], and the AGO4 RNA-processing factor [37] that effects the control of non-CG methylation, particularly in the CNG context that is enriched in *SUPERMAN* 5′ sequences. This paper provides the first link in a plant system between the H3 mK9 modification and DNA methylation. *In-vitro* evidence suggests that the LHP1 chromodomain protein functions as a bridge between H3 mK9 and CMT3; however, this model has subsequently been disproved by an *lph1* mutation that does not disrupt DNA methylation patterns (see [23•]).

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A methylated and silenced endogenous *Arabidopsis PAI* gene is used as a reporter to isolate silencing-defective mutations. Like the *SUPERMAN* screen [22•], this screen yields mutations in the KYP/SUVH4 H3 K9 HMTase [23•] and the CMT3 DMTase [16]. Although mutations in both genes reduce non-CG methylation on the silenced *PAI* gene reporter, they have different effects on the transcribed *PAI* inverted repeat that triggers methylation on *PAI*: the inverted repeat is demethylated by *cmt3* but not by *kyp/suvh4* mutations. Furthermore, a new *PAI* methylation imprint cannot be established in a *cmt3* mutant background but can be established in a *kyp/suvh4* mutant background. Together, these results argue that only a subset of CMT3-mediated methylation relies on the KYP/SUVH4-mediated H3 mK9 mark. Notably, in contrast to the *SUPERMAN* screen, the PAI screen does not yield *ago4* mutations; this difference might reflect the need for processing a dsRNA signal in the *SUPERMAN* system that differs from the direct production of dsRNA in the *PAI* system (see [8]).

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Mutations in the *Arabidopsis* histone deacetylase gene *HDA6* have been previously isolated in screens for the reactivation of silenced methylated transgenes (e.g. [27]). In this paper, *hda6* alleles isolated from two other screens that were based on transgene silencing are tested for their effects on endogenous repetitive sequences, including the nucleolar rRNA genes. This analysis shows that the *hda6* mutations cause loss of CG methylation, enrichment for acetylated histone H4, and enrichment for H3 mK4 on rRNA sequences. These mutations have little or no effect on DNA methylation at other sequences, such as the centromere repeats, or on bulk levels of acetylated H4 and H3 mK4. A probable null *hda6* mutation also confers decondensation of rRNA but not of centromeric heterochromatin. Together, these results suggest that HDA6 might have a specific function in rRNA chromatin organization; however, the *hda6*-induced chromatin changes are not sufficient to increase rRNA expression. The authors speculate that HDA6 might work together with HDT1, another histone deacetylase implicated in rRNA regulation (see [40•]).

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