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Composition and formation of heterochromatin in *Arabidopsis thaliana*

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Abstract

The term heterochromatin has been applied to both large-scale, microscopically visible chromocentres and small-scale, silent genes located outside chromocentres. This may cause confusion in the interpretation of epigenetic marks for both features. The model plant *Arabidopsis thaliana* provides an excellent system to investigate composition and function of chromatin states at different levels of organization. In this review we will discuss recent developments in molecular networks underlying gene silencing and the relationship with visible heterochromatin in *Arabidopsis*.

Introduction

Heterochromatin plays an important role in maintaining thousands of genes and other DNA elements selectively repressed in order to allow a structured sequence of transcriptional events during development. Central to the process of gene silencing is the epigenetic network of molecular interactions, including DNA methylation and histone modifications, as well as recruitment of protein complexes that maintain the repressed state of chromatin throughout subsequent cell divisions. Most of the silencing processes occur in or lead to a heterochromatin environment. DNA methylation is one of the major events causing gene repression in plants where 5–25% of all cytosines is methylated (Rangwala & Richards 2004). Plants have developed several ways to initiate and maintain methylation of cytosines in different contexts. For example, plants establish not only CpG methylation but also significant non-CpG methylation throughout plant development. In comparison, mammals mainly have CpG methylation, except in embryonic stem cells where non-CpG methylation is prevalent (Ramsahoye *et al*. 2000). The inactivation of genes and transposons by DNA methylation involves a number of key players, including methyltransferases, chromatin remodelling proteins and histone modification enzymes. Several of these proteins and their specific role in gene silencing have been elucidated in the past few years. The emerging picture is an intricate molecular network of interactions between DNA methylation, histone modification and RNA-directed silencing complexes. Yet a number of basic questions are still to be answered. Who are the targets for DNA methylation? How is DNA methylation directed to its targets? Who are the co-players? How do they interact with DNA methyltransferases? What is the functional difference between CpG-, CpNpG- and CpNpN-methylation? How is DNA methylation linked to chromatin structure? Here we focus on the central role of DNA methylation in gene silencing and describe a possible mechanistic link between molecular networks of gene silencing and the

appearance of heterochromatin at both local and large-scale level.

Molecular networks underlying heterochromatin

DNA methyltransferases in plants

The Arabidopsis genome encodes four classes of DNA methyltransferases. DRM1 and DRM2 (domains rearranged methyltransferase) are *de-novo* methyltransferases, responsible for virtually all *de-novo* DNA methylation. They are the orthologues of the mammalian DNMT3. MET1 (methyltransferase), the equivalent of DNMT1, is a maintenance DNA methyltransferase, responsible for CpG methylation at hemimethylated sites. However, MET1 may also contribute to *de-novo* methylation (Aufsatz *et al.* 2004). CMT3 (chromomethyl-transferase), which contains a chromodomain, is unique to plants. CMT3 is involved in *de-novo* methylation at non-CpG positions and also maintains CpNpG methylation. Finally, like mammals, plants have a DNMT2 gene, encoding a putative methyltransferase with unknown function. With the exception of the latter the described methyltransferases have been demonstrated to be involved in all known methylation-directed silencing.

De-novo DNA methylation follows an RNA-dependent pathway

In general, DNA methylation can be classified into two events. *De-novo* methylation initiates the silent status, while maintenance of methylation ensures the repressed state throughout cell division (Figure 1). *De-novo* methylation probably occurs at selected positions in the genome and requires a specific targeting mechanism in order to methylate the correct genomic site. The mechanism may recognize either the DNA sequence or the chromatin state. For example, *de-novo* methylation of the mouse major satellite repeats by DNMT1 is dependent on H3K9 trimethylation (H3K9me3) and HP1 (Lehnertz *et al.* 2003), indicating that histone H3K9 methylation directs DNA methylation. A similar control of DNA methylation has been reported for *Neurospora crassa* (Tamaru & Selker 2001) and in *Arabidopsis* at non-CpG sites (Jackson *et al.* 2002, Malagnac *et al.* 2002). A more direct sequence-based targeting of DNA methylation involves siRNA-generating pathways. The sequence specificity of siRNAs provides an excellent mechanism to recognize any DNA sequence. RNA-dependent DNA methylation (RdDM) is well documented in plants and a number of key components in this process have been identified (Chan *et al.* 2004, Kanno *et al.* 2004). These include RDR2 (RNA-dependent RNA polymerase), DCL3 (Dicer-like RNase), AGO4 (Argonaute protein), Pol IV (RNA polymerase IV, unique to plants), DRD1 (defective-in-RNA-directed DNA methylation, a putative SNF2-like chromatin remodelling protein) and DRM2.

RdDM-mediated silencing requires a repeat transcript

The molecular triggers for RNA-directed *de-novo* methylation are generated by repetitive elements. If an inverted repeat is transcribed, it can generate a dsRNA molecule by forming a hairpin structure through a foldback. The dsRNA is subsequently degraded to siRNA by DCL3. The siRNAs then guide components of the RNA-dependent DNA methylation (RdDM) machinery to target sequences complementary to the siRNA. The prime candidate protein to link siRNAs with the DNA methylation complexes is Argonaute, which contains a PAZ-PIWI domain and can bind RNA. Arabidopsis has 10 AGO-like genes, of which AGO4 is involved in many RdDM cases (Zilberman *et al.* 2003, Chan *et al.* 2004). How the RdDM complex is assembled to activate DNA methylation at the target locus is unknown. Two plant-specific proteins, DRD1 and PolIVb, are essential for RNA-directed *de-novo* methylation. DRD1 facilitates *de-novo* methylation of target sequences in the CpG and non-CpG context (Kanno *et al.* 2004, Kanno *et al.* 2005b). Interestingly, DRD1 can also effect loss of methylation when the trigger locus is no longer present. The authors speculate that DRD1 opens chromatin to facilitate both *de-novo* methylation by methyltransferases and demethylation by DNA glycosylases. These antagonistic functions of DRD1 are reminiscent of the SWI/SNF complex Brahma in animals, which facilitates repression or activation of TCF target genes, depending on the presence of regulatory proteins Groucho or b-catenin respectively (Klochendler-Yeivin *et al.* 2002). The other essential complex, RNA PolIVb, which contains the DRD3 subunit, co-

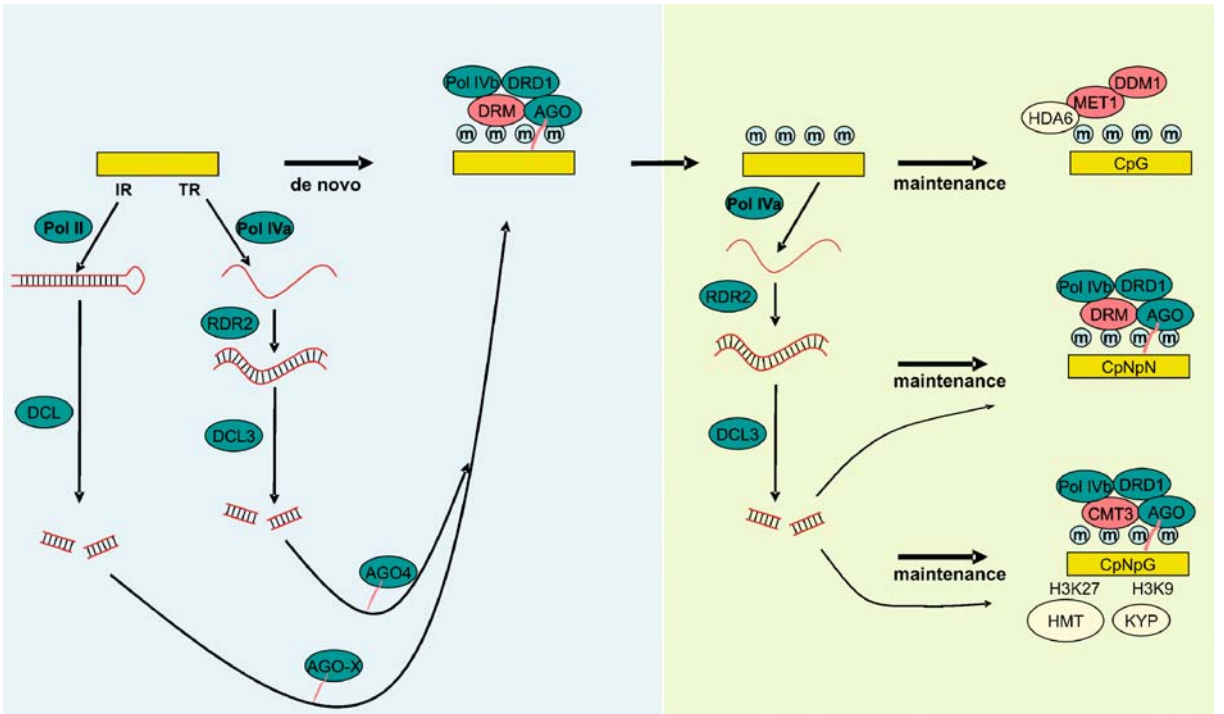

Figure 1. Simplified scheme of *de-novo* and maintenance of DNA methylation. Non-methylated repetitive sequences are transcribed and generate dsRNA, which is degraded to siRNA by DICER-like proteins. In association with AGO, the siRNAs guide the RdDM-mediated machinery to target sequences, which become *de-novo* methylated by DRM. Maintenance of DNA methylation may occur in three different ways. CpG methylation is established during DNA replication by MET1 under supervision of DDM1 and HDACs. Non-CpG sites require siRNAs to recruit RdDM components. RNA polymerase IVa is responsible for transcribing methylated sequences into RNA that is processed to the siRNA. The RdDM machinery employs either DRM or CMT3 to methylate CpNpN or CpNpG, respectively. CMT3-mediated methylation is under the control of histone methylation at H3K9 and possibly H3K27. DDM1 is also required for non-CpG methylation (Johnson *et al.* 2002); however, its position in this pathway is not clear. IR, inverted repeat; TR, tandem repeat.

operates with DRD1 to enable methylation of the siRNA target (Kanno *et al.* 2005a). Both DRD1 and PolIVb do not appear to affect large-scale CpG methylation levels, but rather selected sequences. These observations reinforce the idea that *de-novo* methylation acts locally and does not take place on a large scale. This hypothesis was postulated some years ago, based on the fact that remethylation of CpG sites in repeats occurs slowly in DNA methylation mutants after several crosses to wild type (Finnegan *et al.* 1996, Kakutani *et al.* 1999).

In comparison to inverted repeats, direct repeats follow a different pathway to siRNA production. First, they are transcribed by RNA PolIVa (Herr *et al.* 2005, Kanno *et al.* 2005a, Onodera *et al.* 2005), which contains the subunit SDE4 (silencing defective 4), a functionally diversified homologue of DRD3. Second, transcripts of direct repeats do not fold back to form dsRNA but are converted to dsRNA by the action of RDR2. After degradation to siRNA by DCL3, the same RNA-mediated methylation process as described above for inverted repeats can take place. The RdDM pathway can silence transposons because they contain repeats or are arranged as repeats and they can be transcribed. Likewise, RdDM can silence genes if repeats are in or near the promotor of a gene or if copies of the gene are arranged as repeats. For example, many transgenes but also endogenous genes such as the *PAI1-4* locus in ecotype Wassilevsky (Melquist & Bender 2004) and rDNA genes are arranged as repeats. Other genes such as *FWA* (Soppe *et al.* 2000) or *FLC* in ecotype Landsberg *erecta* (Liu *et al.* 2004) contain repeat elements or a transposon near the promotor. These genes are subject to RdDM-mediated silencing. Moreover, genes encoding miRNAs control expression of other genes that are often part of regulatory networks. The mode of silencing is to target the RISC

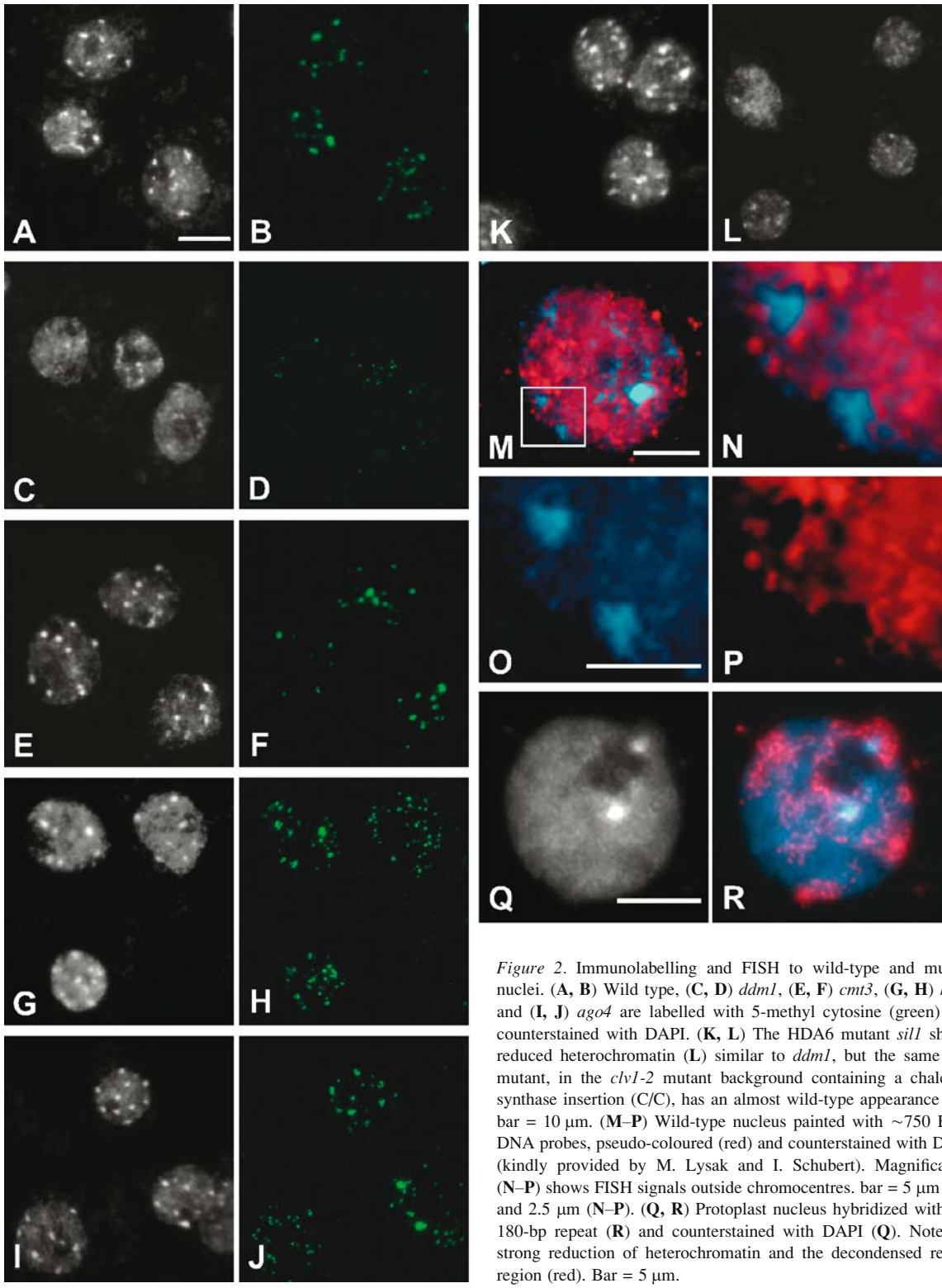

Figure 2. Immunolabelling and FISH to wild-type and mutant nuclei. (**A, B**) Wild type, (**C, D**) *ddm1*, (**E, F**) *cmt3*, (**G, H**) *kyp2* and (**I, J**) *ago4* are labelled with 5-methyl cytosine (green) and counterstained with DAPI. (**K, L**) The HDA6 mutant *sil1* shows reduced heterochromatin (**L**) similar to *ddm1*, but the same *sil1* mutant, in the *clv1-2* mutant background containing a chalcone synthase insertion (C/C), has an almost wild-type appearance (**L**). bar = 10 μm. (**M**–**P**) Wild-type nucleus painted with ~750 BAC DNA probes, pseudo-coloured (red) and counterstained with DAPI (kindly provided by M. Lysak and I. Schubert). Magnification (**N**–**P**) shows FISH signals outside chromocentres. bar = 5 μm (**M**) and 2.5 μm (**N**–**P**). (**Q, R**) Protoplast nucleus hybridized with the 180-bp repeat (**R**) and counterstained with DAPI (**Q**). Note the strong reduction of heterochromatin and the decondensed repeat region (red). Bar = 5 μm.

complex to the mRNA. Recent studies have demonstrated that miRNAs can also establish DNA methylation of the transcription factor genes *PHB* and *PHV* by interacting with complementary sequences in the corresponding mRNAs (Bao *et al*. 2004).

Maintaining DNA methylation with and without an siRNA pathway

Once methylation has been initiated, it needs to be maintained during cell division. The majority of maintenance of CpG methylation is assumed to take place during or after DNA replication at hemimethylated sites. This model is supported by the fact that the mammalian maintenance methyltransferase (Dnmt1) can physically interact via its N-terminal region with DNA-bound PCNA complexes (Iida *et al*. 2002). In *Arabidopsis*, the maintenance of CpG methylation requires the activity of at least MET1 and the decrease in dna methylation 1 (DDM1) protein, an ATP-dependent chromatin-remodelling factor with homology to SWI-SNF. DDM1 is probably involved in enhancing chromatin accessibility by nucleosome repositioning, thus facilitating methylation by MET1 (Brzeski & Jerzmanowski 2004). In addition, the histone deacetylase, HDA6, may also be involved in maintaining CpG methylation, at specific loci (Aufsatz *et al*. 2002, Probst *et al*. 2004). However, to retain methylation at non-CpG sites, not only are other methyltransferases (CMT3 and DRM) necessary, but also a DNA replication-independent system. Similar to *de-novo* methylation, RdDM is crucial in maintaining methylation of cytosines in a non-CpG context. This RNA-dependent pathway was confirmed by the erasure of non-CpG methylation in mutants defective in RNA silencing pathway components: *rdr2*, *dcl3*, *sde4* and *ago4* (Chan *et al*. 2004, Xie *et al*. 2004). Using specific trigger and target transgenes the authors could discriminate not only CpG from non-CpG, but also *de-novo* from maintenance methylation. DRM methylates CpNpN targets at the siRNA target locus, while CMT3 is responsible for CpNpG methylation. In contrast to DRM and MET1, CMT3 activity appears to be dependent on histone methylation by kryptonite (or SUVH4). It has been shown that the chromodomain of CMT3 can interact *in vitro* with histone H3 when it is simultaneously trimethylated at two histone residues, H3K9 and H3K27 (Lindroth *et al*. 2004).

The role of repeat-derived siRNAs in *de-novo* and maintenance methylation provides a satisfactory explanation of how genes and transposons are specifically targeted for silencing. However, a paradoxical situation seems to exist because, in order to maintain the methylated state via siRNA, these small RNA molecules have to be produced from transcribed sequences, which is not possible if they are silenced. The current idea is that PolIV, unlike other RNA polymerases, is resistant to silent chromatin and still produces transcripts from methylated DNA. These transcripts are degraded to siRNA (Herr *et al*. 2005, Kanno *et al*. 2005a, Onodera *et al*. 2005). Alternatively, PolIV could transcribe single-stranded RNA from the RDR2-generated dsRNA, thus perpetuating siRNA production without transcribing the methylated locus (Vaucheret 2005). Both models would maintain the silenced state of the siRNA targets through subsequent cell divisions.

Nuclear organization of heterochromatin

CpG methylation affects chromocentre structure

Visible heterochrochromatin in *Arabidopsis* is organized in 6–10 conspicuous chromocentres, each of which can be identified (Fransz *et al*. 2002). Chromocentres accommodate all tandem repeats and the majority of dispersed repeats (among others transposable elements). They are also the regions that are enriched with methylated DNA, H3K9me2 (Jasencakova *et al*. 2003) and Methyl-CpG-Binding-Domain proteins (Zemach *et al*. 2005). In contrast, gene-rich regions are located outside chromocentres. This sharp discrimination between euchromatin and visible heterochromatin makes *Arabidopsis* an attractive system for studying chromatin proteins in different chromatin states. For example, leaf nuclei in DNA methylation mutants such as *ddm1-2* (SWI/SNF-like remodelling protein) and *met1-1* (DNA methyltransferase), show a 70–75% reduction in heterochromatin (Figure 2a–d; Soppe *et al*. 2002). The loss of heterochromatin is due to relocation of pericentric non-transposon elements and activated transposons away from chromocentres. Tandem repeats and silent transposons, however, remain in visible heterochromatin. Interestingly, restoration of DNA methylation activity by crossing the mutant (*met1* or *ddm1*) with a wild-type plant does not lead

to reformation of heterochromatin in the mutant-derived chromosomes (Soppe *et al*. 2002, Fransz *et al*. 2003). Considering that remethylation of repeats is slow after restoration of DNA methylation activity (Finnegan *et al*. 1996, Kakutani *et al*. 1999), these findings indicate that a methylated CpG mark at pericentric repeats is required to maintain visible heterochromatin. An even more severe nuclear phenotype has been reported for the *ddm1-5* mutant, which is allelic to *ddm1-2*, but lacks detectable levels of *DDM1* transcripts. Nuclei of this mutant show decondensation of pericentric heterochromatin even from the region containing the centric 180-bp repeat (Mittelsten Scheid *et al*. 2002, Probst *et al*. 2003). Plants defective in CMT3, responsible for maintaining methylation at CpNpG, display a normal nuclear phenotype (Figure 2e–f) and distinct methylation of chromocentres. This accounts also for mutants lacking KYP and AGO4, both of which control CMT3 activity (Figure 2g–j and O. Kulikova, personal communication). This indicates that CpG methylation is responsible for the large-scale DNA methylation pattern in interphase nuclei. Furthermore, chromocentre formation does not appear to depend on CpNpG methylation. The conclusion is that CpG hypomethylation affects the organization of visible heterochromatin. However, DNA methylation is dispensable for its formation, since all DNA hypomethylation mutants, even the severe *ddm1*-5 mutant, contain a fraction of nuclei that show a wild-type appearance and normal levels of heterochromatin (Mittelsten Scheid *et al*. 2002). This suggests that other factors can maintain normal levels of visible heterochromatin in the absence of DNA methylation. This is clearly illustrated in the met1-3 mutant, which is allelic to *met1-1*, and considered a null mutant. Nuclei of *met1-3* show a wild-type appearance, with distinct chromocentres (Tariq *et al*. 2003), despite having reduced DNA methylation. Why *met1-1* and *met1-3* display different nuclear phenotypes remains unclear. It is possible that certain domains of MET1, other than the catalytic region affect the stability of chromocentres.

Nucleosome assembly and heterochromatin

Reduced heterochromatin is not only observed in DNA methylation mutants, but has also been found in plants with other chromatin protein defects. MSI1 is a member of a highly conserved subfamily of WD40 repeat proteins and implicated in histone function in several organisms. MSI1 is a subunit of the chromatin assembly factor 1 (CAF1). In *Arabidopsis* plants with co-repressed AtMSI1, the amount of heterochromatin is reduced by a factor two (Hennig *et al*. 2003). The authors conclude that *Arabidopsis* MSI1 (AtMSI1) is involved in the efficient formation of heterochromatin. This is consistent with the hypothesis that CAF-1 ensures stable propagation of epigenetic states by facilitating rapid reformation of chromatin structure after passage of a replication fork (Kaya *et al*. 2001). This idea is further supported by studies in human cells showing that, during replication, the heterochromatin protein HP1 interacts with the p150 subunit of CAF-1 to promote delivery of HP1 at heterochromatic domains (Quivy *et al*. 2004). However, FAS1, another component of the CAF1 complex, does not seem to affect formation of visible heterochromatin (Takeda *et al*. 2004 and O. Kulikova, personal communication).

The binding property of MSI1 proteins to histone deacetylases (HDA) may connect MSI1 to heterochromatin, since aberrant heterochromatin phenotypes have been found in mutants defective in HDA6. Depending on genetic background, the phenotype varied from a local decrease in heterochromatin at the 45S rDNA locus (Probst *et al*. 2004) to a reduction in all visible heterochromatin (Figure 2k–l). Furthermore, the *BRU* (*BRUSHY*) gene encodes a novel nuclear protein with protein–protein interacting domains involved in DNA damage and gene silencing (Takeda *et al*. 2004). The phenotype of the *bru1-1* mutant resembles the *Arabidopsis* mutants in CAF1 subunits, including significant diffusion of chromocentres, suggesting reduced heterochromatin. The data further reinforce the concept that the machinery for nucleosome assembly propagates the organization of chromocentres.

Histone methylation marks for chromocentres

Both DNA methylation and histone methylation at H3K9 are prominent features of chromocentres in *Arabidopsis* (Fransz *et al*. 2002, Soppe *et al*. 2002, Jasencakova *et al*. 2003, Probst *et al*. 2003). Recently, three different laboratories have examined additional marks of visible heterochromatin (Lindroth *et al*. 2004, Mathieu *et al*. 2005, Naumann *et al*. 2005). They independently demonstrated distinct

colocalization of H3K9me1, H3K9me2, H3K27me1, H3K27me2 and H4K20me1 with chromocentres. Conversely, immunocytological marks for euchromatin are acetylated histones, H3K4me2, H3K9me3, H3K27me3, H4K20me2 and H3K20me3. Surprisingly, a comparison between histone methylation patterns in nuclei of *Arabidopsis* and mouse, which also contain chromocentres, reveals the opposite distribution patterns of five histone modification marks (Table 1). H3K9me1, H3K9me2 and H3K27me2 are predominantly in *Arabidopsis* chromocentres, but not in mouse chromocentres, whereas H3K9me3 and H4K20me3 are in mouse chromocentres, but largely absent from visible heterochromatin in *Arabidopsis*. Other chromatin modifications, such as 5-methylcytosine and acetylated histones, show similar distribution patterns in the two species. From these data, we can distinguish two features of histone methylation and chromatin state. Firstly, for each lysine residue, there is at least one methylation mark associated with euchromatin or heterochromatic chromocentres. The second striking aspect is that the change from mono- to trimethylation in all cases corresponds to a difference in the chromatin appearance. In *Arabidopsis*, all three monomethylated lysine residues (H3K9, H3K27 and H4K20) reside predominantly in chromocentres, whereas trimethylation-rich modifications localize in euchromatin. In comparison, of the three trimethylated histones in mouse, only H3K27m3 shows up in euchromatin. Apparently, mouse and *Arabidopsis* have developed different strategies to utilize the different methylation levels at H3K9 and H4K20 in chromatin function.

Histone methylation and HP1 in Arabidopsis

One of the main components of heterochromatin in flies and mammals is the heterochromatin protein 1 (HP1), characterized by the presence of a chromodomain (CD) and a chromoshadow domain (CSD). This chromatin protein can interact with histone H3 methylated at lysine 9 via its CD and forms hetero- and homodimers via the CSD. The only HP1 homologue found in *Arabidopsis*, LHP1 (like-heterochromatin-protein 1), is involved in several developmental processes (Gaudin *et al*. 2001). Surprisingly, unlike the animal HP1, the *Arabidopsis* LHP1 is absent from visible heterochromatin (Libault *et al*. 2005, Nakahigashi *et al*. 2005). Plants that are defective in LHP1 show no change in chromocentres. It is therefore suggested that LHP1 is involved in gene-rich euchromatin regions, reminiscent of the mammalian HP1-γ function. Interestingly, H4K20 trimethylation in mouse depends on HP1 and SUV39H, but H4K20 is not a target of SUV39H (Schotta *et al*. 2004). After trimethylation of H3K9 by SUV39H, a molecular interaction between H3K9me3, HP1 (α and β) and the HMTase SUV4–20 results in trimethylation of H4K20. The recruitment of SUV4–20 to mouse chromocentres is regulated by the non-catalytic C-terminal end, since trunca-

Table 1. Nuclear distribution of euchromatin and heterochromatin marks in *Arabidopsis* and mouse and their SUVH mutant.

			H3K9			H3K27			H4K20		
Species/mutant (refs)	Domain	5m-C	mono	di	tri	mono	di	tri	mono	di	tri
Arabidopsis(3,4,5)	Chromocentres	high[a]	high	high	low	high	high	low	high	low	low
	Euchromatin	low	low	low	high[c]	low	low	high	low	high	high
suvh2(3)	Chromocentres	low	high	high[b]	low	medium	medium	low	low	low	low
	Euchromatin	low	low	low	high	low	low	high	low	high	high
Mouse(1,2,6)	Chromocentres	high	low	low	high	high	medium	low	low	low	high
	Euchromatin	low	high[e]	high	low	medium	high	high	high	high	low
suv39h dn(1,2,7)	Chromocentres	low[d]	high	low	low	low	low	high	low	low	low
	Euchromatin	low	medium	high	high	high	high	medium	high	high	high

[a]The indications 'high' and 'low' are relative for chromocentres compared to euchromatin. [b]Less than in wild type. [c]There is some controversy about the presence of trimethylated H3K9 in *Arabidopsis* nuclei. [d]At major satellite repeats. [e]Transient according to ref. 1.
References: 1: Schotta *et al*. 2004; 2: Peters *et al*. 2003; 3: Naumann *et al*. 2005; 4: Mathieu *et al*. 2005; 5: Lindroth *et al*. 2004; 6: Lehnertz *et al*. 2003; 7: Lachner *et al*. 2001.

tion of this part results in accumulation of H4K20 trimethylation in euchromatin domains. Likewise, in *Arabidopsis*, truncation of the non-catalytic N-terminal domain in SUVH2, the HMTase for H4K20, abolishes ectopic nuclear distribution of SUVH2 in an overexpressing SUVH2 transformant (Naumann *et al*. 2005). It is tempting to speculate that trimethylated H3K9 and LHP1 interact with trimethylation of H4K20me3 outside chromocentres.

Independent histone methylation events in euchromatin and chromocentres?

In *Arabidopsis*, four classes of histone methyltransferases (HMTs) can be distinguished in analogy to *Drosophila*: E(Z), TRX, ASH1 and SU(VAR)3–9 (Baumbusch *et al*. 2001). The *Arabidopsis* SUVHs 1 to 10 are SU(VAR)3–9 homologues and are possibly involved in methylation of H3K9. The question arises of what the causal relationship is between the methylation level of a histone and its spatial position relative to visible heterochromatin. Three situations may occur for the change from di- to tri-methylation in *Arabidopsis*: (1) trimethylation of H3K9me2 takes place at chromocentres and causes the methylated region to move away from chromocentres into euchromatin, or (2) under certain epigenetic conditions, dimethylated H3K9 regions move into euchromatin domains, where they become trimethylated, or (3) histone trimethylation in euchromatin domains occurs independently from histone mono- and di-methylation in visible heterochromatin. In the last situation, H3K9 becomes trimethylated in the euchromatic domains but not in chromocentres. The spatial separation of histone methylation can be explained if there are different HMTs for euchromatin and heterochromatin or if the chromatin environment restricts the level of histone methylation. In a chromocentre environment, an HMT would methylate only to the mono- or dimethylation level. Conversely, in euchromatin, the same or another HMT establishes mono-, di- and trimethylation. Such an HMT activity has been demonstrated for the mouse SUV4–20h, which can trimethylate *in vitro* non-methylated H4K20 (Schotta *et al*. 2004).

A number of HMTs have been demonstrated to affect H3K9 methylation. These include SUVH1, SUVH2, SUVH4 (or KYP) and SUVH6, of which the last three have HMTase activity for H3K9 (Jackson *et al*. 2004, Naumann *et al*. 2005). The histone methyltransferase SUVH4 (or KYP), methylates H3K9 up to the dimethylated level, a mark for chromocentres. However, nuclei of the mutant *suvh4* (or *kyp*) display a normal phenotype with distinct chromocentres, which implies that SUVH4 activity is not required for visible heterochromatin formation. The chromocentres in *kyp* nuclei show reduced levels of H3K9me2 (Jasencakova *et al*. 2003, Zemach *et al*. 2005), suggesting that chromocentre formation is not controlled by H3K9 methylation. This is supported by the observations in the *met1*-3 mutant, which shows normal chromocentres that lack H3K9m2 labelling (Tariq *et al*. 2003).

SUVH2 methylates H3K9, but it also has histone H4 methylation activity (Naumann *et al*. 2005). Considering that, in *suvh2* mutants, monomethylation of H4K20 is strongly affected, SUVH2 is a good candidate for H4K20 monomethylation. This is further supported by the specific localization of SUVH2 in chromocentres. In addition, plants lacking SUVH2 display reduced heterochromatin. Therefore, SUVH2 activity or H4K20 monomethylation may be associated with chromocentre appearance. In fact, this was shown by the ectopic formation of heterochromatin regions in plants with overexpressed SUVH2. It remains to be resolved which of the other HMTs are located in or outside chromocentres.

From these data, we may conclude that histone methylation in euchromatin occurs independently from histone methylation in visible heterochromatin, at least for H3K9 and H4K20. In this view, it is interesting to report that reduction of H3K9 methylation from chromocentres did not decrease the overall H3K9me2 content in *ddm1* or *met1*-3 (Gendrel *et al*. 2002, Tariq *et al*. 2003), indicating a relocation of H3K9 dimethylation activity. Furthermore, the *Arabidopsis* SUVH2 and the mammalian SUV4–20 contain a domain that recognizes specific nuclear regions, since absence of these domains leads to ectopic methylation (see above). This suggests that the same HMTs can be active in both euchromatin and visible heterochromatin depending on the targeting mechanism.

Epigenetically silent heterochromatin and visible DAPI-positive large-scale heterochromatin in Arabidopsis

A clear link between the molecular level of gene silencing and the spatial distribution of epigenetic

modifications in interphase nuclei is difficult to establish because of the limited resolution of the fluorescence microscope. Some chromatin marks are microscopically observed in visible heterochromatin but their presence in euchromatic domains is not excluded. Consequently, some confusion exists in discriminating visible DAPI-positive large-scale heterochromatin from epigenetically silent heterochromatin at local positions. The organization of chromosomes in *Arabidopsis* nuclei may exemplify the two situations. By far the majority of repeats are in distinct chromocentres (Fransz *et al.* 2002), whereas gene-rich regions are in so-called euchromatin. The latter is well illustrated in a nucleus painted with ~750 chromosome-specific BACs (130–180 per chromosome), that cover all euchromatic chromosome arms (Figure 2m–p). The euchromatin BAC signals localize outside chromocentres, which indicates that most of the 25 500 *Arabidopsis* genes are outside visible heterochromatin. That does not imply that all these genes are active or potentially active. Assuming that only 10% of the genes is active, it follows that the majority of genes are in silent chromatin at local positions outside visible heterochromatin. The histone modification marks for silencing, however, are predominantly in chromocentres (Table 1), which represent only 10–20% of the genomic sequence (Tessadori *et al.* 2004). This corresponds with the percentage of repetitive DNA (transposons, rDNA and centromere) in *Arabidopsis* (The_Arabidopsis_Initiative 2000). Apparently, the repeats, but not the silent genes, are heavily decorated with heterochromatin marks. This is well demonstrated in a chromatin profile assay using micro-arrays containing sequential 1-kb segments of a 1.5-megabase region of chromosome arm 4S (Lippman *et al.* 2004; see also http://chromatin.cshl.org/ddm1/). This ChIP-on-chip approach bridges the gap between molecular studies at local positions and large-scale microscopic studies. It shows that repetitive sequences are heavily methylated, whereas gene-rich sequences show a low level of methylation, regardless of their expression. Obviously, gene repression by DNA methylation occurs in local or 'cryptic' (Lippman *et al.* 2004) heterochromatin and depends on defined sites of methylated cytosines (e.g. relative to the promotor). The *FWA* gene is a good example. Silencing of *FWA* is established via non-CpG methylation by CMT3 (see above), which is directed via H3K9 dimethylation (Lindroth *et al.* 2004). Moreover, the *FWA* gene region contains two sets of tandem repeats in and near the promotor. These are associated with H3K9 methylation when *FWA* is silent in the wild-type, but not in the *ddm1* mutant, when *FWA* is active (Lippman *et al.* 2004). Surprisingly, in both situations the *FWA* locus localizes outside chromocentres in more than 90% of the cases (Soppe *et al.* 2002). In contrast, transposable elements, such as *CAC1*, are generally in chromocentres but move to a euchromatic environment upon reactivation in the *ddm1* mutant.

The emerging picture from the RdDM studies is that repetitiveness, either low or high, directs DNA methylation. We propose that the degree of repetitiveness determines the level of chromatin condensation. The more repeats there are, the higher the chance of finding the repeat sequence in visible heterochromatin. Chromocentres are formed by two repeat classes with a highly repetitive nature: tandemly arranged (180 bp and rDNA) and dispersed transposable elements. A third class of less repetitive (pericentric) repeats is in chromocentres of WT nuclei, but outside visible heterochromatin under hypomethylated conditions. A fourth class of low repetitive sequences is generally outside chromocentres, irrespective of their cytosine methylation state. Why regions with highly repetitive DNA become visible as chromocentres is unclear. The fact that hypomethylated 180-bp rDNA and transposons can still form chromocentres indicates that methylation is dispensable for chromocentre formation.

Yet, visible heterochromatin is not a permanent structural characteristic of *Arabidopsis* chromosomes. We recently discovered a dramatic decrease in visible heterochromatin in leaf nuclei, after dedifferentiation to totipotent protoplasts, without significant loss of DNA methylation (Figure 2q–r, Tessadori *et al.* submitted). The reduction of heterochromatin was accompanied by decondensation of all tandem repeats, including the 180-bp centromeric repeat. Apparently, an as-yet-unknown factor that recognizes the repetitive nature, is responsible for chromocentre formation. The level of repetitiveness seems to be important, since reformation of chromocentres during the first days of protoplast culture was accompanied by successive condensation of 45S rDNA 180-bp-repeat 5S rDNA transposons. This order of repeat elements matches the rank of repetitiveness, thus supporting the relationship between degree of repetitiveness and level of chromatin condensation.

Conclusions

Gene silencing is triggered by repeat sequences, which are transcribed and processed to siRNAs. Guided by the RdDM machinery, the distinct methyltransferases determine which cytosines at the target become or remain methylated. Genes do not have to contain tandem or inverted repeats in order to trigger their own silencing. If the gene region includes a sequence with homology to a siRNA, the gene is a potential target for the RdDM machinery. In *Arabidopsis*, the decision to silence genes can be carried out via different transcriptional and post-transcriptional mechanisms. The siRNA pathway already shows extensive variation as revealed by the specificity of DNA methyltransferases, RNA polymerases, the number of DCL and AGO proteins and multiple SUV39 homologues. A number of these proteins are unique to plants. The recently discovered role of miRNA in transcriptional gene silencing further expands the repertoire of gene-silencing pathways in the plant cell. Apparently, *Arabidopsis* has developed several alternative ways to selectively repress thousands of genes in a sequence-specific manner. Enzymes that modify chromatin probably contain specific domains, apart from the catalytic site, that facilitate not only recognition of subnuclear targets but also recruitment of other effector proteins. Variation in these domains may determine where these enzymes operate, i.e. in or outside chromocentres, and who their co-players are. Our knowledge of these domains and their interactions is very limited and in most cases absent.

The molecular, genetic and microscopic studies of chromatin in *Arabidopsis* provide an excellent view of how chromatin states are affected by epigenetic interactions at the molecular level and indirectly by the linear organization of the sequence. At the microscopic level, *Arabidopsis* chromosomes are partitioned into visible heterochromatin (chromocentres) and visible euchromatin domains, based on the repeat content. Highly repetitive regions remain in chromocentres even in hypomethylation mutants, while less-repetitive sequences become dispersed in certain epigenetic mutant backgrounds. How these repeat regions come together and form chromocentre structures is unknown. The visible euchromatin domains are gene rich and consist of cryptic heterochromatin (silent genes) and euchromatin (active genes). This nuclear organization reflects the relatively simple organization of the linear genomic sequence in *Arabidopsis*. Highly repetitive sequences are clustered only at the centromeres and the subtelomeric NORs in chromosome arms 2S and 4S. Hence, the number of chromosomes (2n = 10) corresponds to the maximum number of chromocentres (Fransz *et al*. 2002). Outside pericentric heterochromatin, the chromosomes are generally euchromatic and gene dense (one gene every 5 kb) with few repeats. Consequently, the majority of genes localize outside visible heterochromatin regardless of their transcriptional activity. This includes silent genes, which reside in cryptic heterochromatin. It will be interesting to find out whether cryptic heterochromatin coincides with the speckled patterns of LHP1 and trimethylation of H3K9, H3K27 and H4K20.

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