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Review

DNA methylation systems and targets in plants

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ABSTRACT

Plants contain three distinct DNA methyltransferase types that are responsible for the establishment and maintenance of cytosine methylation patterns at heterochromatic and euchromatic target regions. RNA transcripts play an important role in recruiting DNA methylation systems to specific loci, where methylation patterns are controlled by distinct epigenetic pathways that often work co-operatively and in competition with demethylation functions. DNA methylation patterns are faithfully propagated by maintenance systems that involve re-enforcing feedback effects between DNA methylation and histone marks. Our detailed knowledge about the composition of DNA methylation patterns is contrasted by a poorer understanding of the variability of DNA methylation and its contribution to gene regulation, genome evolution and adaptation to environmental changes.

1. Introduction

Cytosine methylation is an ancient modification system that has diversified into different biological roles including restriction modification systems in bacteria and epigenetic regulation of gene expression and genome structure in most eukaryotes, where cytosine methylation works in combination with histone modifications [1]. In plants, we find distinct DNA methylation patterns at the body of genes and at repeat elements, where it restricts expression of transposable elements (TEs) that represent more than 50% of many plant genomes [2]. Body methylation is an ancient and widely preserved mechanism predating the divergence of animals and plants around 1.6 billion years ago, while methylation-based control of TEs has mainly been retained in land plants and vertebrates [3,4]. Unlike animals, plants do not have a separate germ line where DNA patterns are erased and reestablished. Epigenetic changes induced in DNA methylation mutants can therefore be inherited and maintained even if the DNA methylation machinery is restored [5]. DNA methylation patterns are sensitive to stress effects, which can contribute to heritable stress adaptation that correlates with changes in genome methylation. Although these transgeneration effects do not necessarily persist over successive generations [6] even transient changes that are transmitted to the next generation can be powerful generators of epigenetic diversity. Especially plant populations living in contrasting habitats have developed a high epigenetic variability suggesting that some species use epigenetic variation to adapt to diverse environments [7]. Plants have to find a balance between keeping epigenetic patterns stable to avoid detrimental effects on gene expression and genome structure and between keeping them sufficiently flexible to induce epigenetic variation required for fast adaptation to new environmental conditions. The priorities for stable or flexible DNA methylation probably differ for individual target loci.

Depending on the sequence context of the cytosine residue to be methylated, we can define CG, CNG or CNN specific methylation types, all of which are present in plants under the control of three classes of DNA methyltransferases. CG methylation is mediated by methyltransferase 1 (MET1), a homologue of mammalian maintenance DNA methyltransferase 1 (DNMT1). CNN methylation is controlled by domains rearranged methyltransferase 1 (DRM2), a homologue of mammalian de novo DNA methyltransferase DNMT3, while the plant-specific chromomethylase 3 (CMT3) regulates CNG methylation. DNA methylation patterns at individual genomic regions are often the result of co-operative or competing interactions of the three DNA methyltransferases and the silencing pathways to which they contribute.

2. RNA-directed DNA methylation

De novo DNA methylation is predominantly controlled by DRM2 via the RNA-directed DNA methylation (RdDM) pathway and the production of 24nt short interfering RNAs (siRNAs). The siRNAs are degradation products of double-stranded (ds)RNAs, which derive from RNA polymerase IV (Pol IV)-specific transcripts that are copied into dsRNA by RNA-dependent rna polymerase 2 (RDR2). A small proportion of dsRNA is produced by RNA polymerase II (Pol II) transcribing inverted repeats or overlapping

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antagonistic transcripts. The dsRNA substrates are cleaved into 24nt siRNAs by dicer-like 3 (DCL3) nuclease, which is partially redundant with other dicer-like enzymes DCL2 and DCL4. One siRNA strand associates with members of the argonaute 4 family, especially with AGO4, in effector complexes that mediate DNA methylation and heterochromatin formation at target regions, as well as the amplification of primary siRNA signals and the production of secondary siRNA signals that cause spreading of DNA methylation [8] (Fig. 1A).

Effector complex recruitment is mediated by RNA polymerase V (Pol V), which synthesises uncapped, non-polyadenylated transcripts that act as scaffolds to attract siRNA-AGO4 complexes [9]. The ATP-dependent chromatin remodeler deficient in RNA-dependent dna methylation 1 (DRD1) facilitates Pol V transcription. An adaptor protein, KOW domain-containing transcription factor 1 (KTF1), binds to AGO4 via a region with reiterated WG/GW motifs and to Pol V scaffold transcripts via its RNA binding domain [10]. The largest Pol V subunit, NRPE1, also contains a GW/WG-rich region that binds to AGO4 assisting in the recruitment of AGO4-siRNA to target loci [11]. The RdDM effector complex also includes DRM2, which is responsible for DNA methylation of the target locus (Fig. 1B).

Recruitment of AGO4 to the RdDM target locus can also produce novel siRNAs if the slicer activity of the associated AGO4 cuts locus-specific transcripts that match the siRNA bound by AGO4. This would generate templates for RDR2 producing more dsRNA substrates for DCL3 cleavage to generate secondary siRNAs that perpetuate heterochromatin formation. At least for some loci, methylation and heterochromatin formation enhances the ability to recruit siRNAs indicative for a self-enforcement effect [12]. Pol II transcription plays an important role in promoting siRNA synthesis and gene silencing, respectively, by recruiting AGO4/siRNAs complexes, Pol IV and Pol V to distinct heterochromatic loci. Adjacent to siRNA targeted regions, Pol II synthesises non-coding scaffold transcripts. Like Pol V, Pol II interacts with AGO via a GW/WG motif region. AGO4 and Pol V associate in the peri-nucleolar processing centre, while AGO4 associates with Pol II in the nucleoplasm where most RdDM occurs [13]. This implies that Pol II and Pol V scaffold transcripts have locus-specific functions. While Pol V scaffold transcripts recruit effector complexes to RdDM targets in the peri-nucleolar processing centre, Pol II and Pol II scaffold transcripts control recruitment of Pol V and siRNA-AGO4 complexes at RdDM target loci in the nucleoplasm. Recruitment of AGO4 effector complexes to methylated regions is supported by the regulator of RdDM (RDM1), which associates with Pol V in the peri-nucleolar processing centre and with Pol II in the nucleoplasm. RDM1 binds methylated DNA, which explains how DNA methylation can be reinforced and how it can influence siRNA amplification [14] (Fig. 1C).

Re-enforcement of DNA methylation also involves the recruitment of DNA maintenance functions by DNA methylation and histone marks (Fig. 1D). This is mediated by methylcytosine-binding proteins that bind methylated Cs via a SET- or RING-associated (SRA) domain. The variant in methylation (VIM) proteins are SRA domain proteins that predominantly regulate CG methylation acting co-operatively and partly redundantly. Consequently, *vim1 vim2 vim3* triple mutants resemble the phenotype of a *met1* mutant. VIM proteins mainly control CG methylation, but also influence CNG methylation at certain loci [15]. At least some VIM family members interact with core histones and locus-specific histone variants indicating that they act as a DNA methylation-histone interface [16]. With the assistance of VIM proteins, CG methylation is maintained by MET1, which has a high affinity for hemimethylated DNA. Especially for methylation of heterochro-

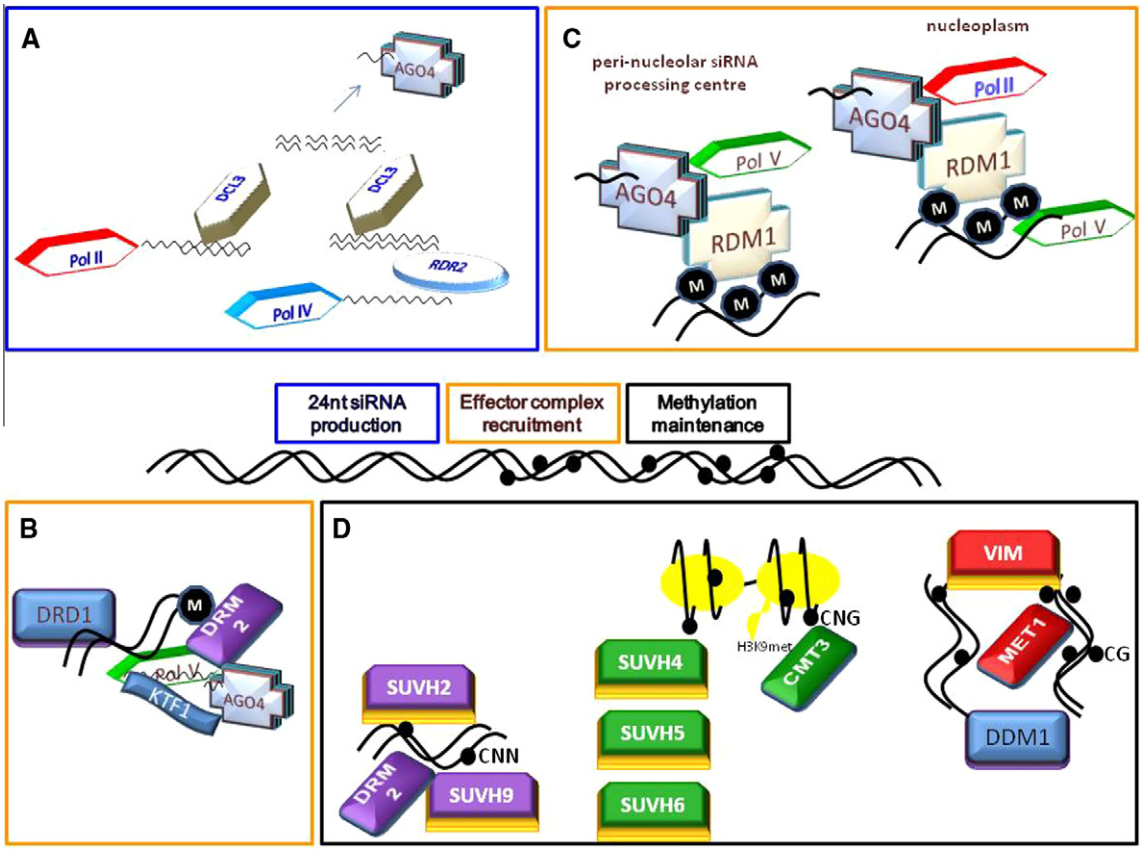

Fig. 1. Some key steps in DNA methylation and transcriptional silencing mediated by 24nt siRNAs. Details are explained in the text.

matic loci, a SWI/SNF family chromatin remodelling factor, decrease in dna methylation 1 (DDM1), is required to facilitate MET1 accessing its target region. CNG methylation, which is closely associated with H3K9 dimethylation [17], is predominantly controlled by CMT3. CMT3 targeting is assisted by three SRA domain proteins, SU(VAR)3-9 homologue 4/kryptonite (SUVH4/KYP), SUVH5 and SUVH6, which show locus-specific effects for CNG methylation and H3K9 methylation. SUVH4, SUVH5 and SUVH6 have H3-specific methylation activity in vitro and *suvh4 suvh5 suvh6* triple mutants lose H3K9me and H3K9me2 marks at CNG target loci [18]. SUVH4 and SUVH6 have been shown to bind to methylated CNG sites via their SRA domains. This establishes a H3K9me2 mark at CNG methylated regions, which binds to the chromodomain of CMT3 as part of a re-enforcement loop between H3K9 methylation and CNG methylation [19]. Self-enforcement of CNN methylation by DRM2 is also mediated by two SRA domain proteins, SUVH2 and SUVH9. SUVH2 binds methylated CG residues, and SUVH9 binds methylated CNN sites facilitating access for DRM2 to methylated regions. It is unclear if this also includes histone-specific modifications as there are conflicting report on the histone methyltransferase activity of SUVH2 and SUVH9 [20,21].

3. Histone marks involved in DNA methylation and transcriptional silencing

DNA methylation is facilitated or counteracted by histone modification marks regulated by jumonji C (JmjC) proteins with histone demethylase activity. The JMJ14 demethylase, which targets active histone H3K4me3 marks, enforces silencing at several RdDM target loci [22], suggesting that removal of active marks is required for the induction of repressive marks at certain methylation target loci. This model is in accordance with the requirement of the RPD3-type histone deacetylase AtHDA6 for maintenance of the silenced state of RdDM targets. Loss of AtHD6 results in a replacement of H3K9me2 with H3K4me3, H3K9 acetylation, H3K14 acetylation, and histone H4 tetra-acetylation [23]. AtHD6 therefore most likely erases histone acetylation as a crucial step in an epigenetic switch mechanism that creates a transcriptionally repressed state. Although being stably inherited, the repressive state is still potentially reversible [24]. Transcriptional reactivation in mutants that lack AtHDA6, is quickly reversed again when AtHDA6 is reintroduced, which suggests that histone deacetylation acts as a fast switch in repressing transcriptional activity in response to silencing RNA signals [25,26]. Another repressive histone mark, which, although not directly dependent on the RdDM pathway, influences RdDM silencing at certain loci, is H3K27me1, which is controlled by two SET-domain H3K27 monomethyltransferases, *Arabidopsis* trithorax-related protein 5 (ATXR5) and ATXR6. *Atxr5/atxr6* double mutants show reduced H3K27me1 levels and partial decondensation of heterochromatin. Neither H3K9me2 nor DNA methylation are affected in *atxr5/atxr6* double mutants [27] but H3K27me1 levels are reduced at certain RdDM target loci in *pol V* or *drd1* mutants [9,28] indicative for an indirect effect of RdDM-based silencing on H3K27 methylation.

Other histone modification functions protect defined genomic regions from silencing effects. The *IBM1* (*increase* in bonsai methylation 1) gene encodes a demethylase that influences H3K9me2 and non-CG methylation levels at several low-copy methylation targets. Over three generations, *ibm1* mutants exhibit a variety of developmental abnormalities, which are suppressed by *suvh4* or *cmt3* mutants but enhanced in a *ddm1* mutant [29]. This suggests that IBM1 balances SUVH4/CMT3-specific DNA and H3K9 methylation effects at distinct loci and that, contrary to its hypermethylation effect at repeats and transposons, DDM1 has a hypomethylating influence at some IBM target loci.

The histone variant H2A.Z acts antagonistically to DNA methylation. H2A.Z is absent from heavily methylated loci and overrepresented at unmethylated regions. Some genes and TEs that usually contain very low DNA methylation levels are hypermethylated at CG sites in the absence of H2A.Z. This implies that DNA methylation excludes H2A.Z incorporation, and that the presence of H2A.Z prevents CG-methylation [30].

4. DNA demethylation

DNA methylation is balanced by DNA demethylation functions. DME (DEMETER), ROS1 (repressor of silencing 1) and DEMETER-like proteins DML2 and DML3 are members of a group of unusually large (1100–2000 amino acids) DNA glycosylases that remove methylated cytosines via a base excision repair process. Mutations in these demethylation functions induce locus-specific DNA hypermethylation. DME expression is restricted to the two central cells of the female gametophyte and establishes genomic imprinting in the endosperm [31]. 179 loci are actively demethylated by DML enzymes [32], and many RdDM target loci show enhanced CNG methylation in a *ros* mutant, indicative for the dynamic control of methylation patterns by both methylation and demethylation [33]. It is unclear how individual loci become targets for demethylation but RNAs are likely candidates for guiding demethylation functions to distinct targets. ROS3 is a regulator of DNA demethylation that contains an RNA recognition motif, binds to small RNAs and co-localises with ROS1 in discrete loci dispersed throughout the nucleus. This suggests that ROS3 is part of a small RNA-directed demethylation system that counteracts DNA hypermethylation [34].

5. DNA methylation patterns

In all eukaryotic species tested so far, CG methylation is the most prominent methylation type. Vertebrates have very low non-CG methylation concentrations but high levels of global CG methylation, with the exception of unmethylated CpG islands, while in invertebrates, fungi and plants we find mosaic patterns of heavily methylated and methylation-free domains [3]. In contrast to animal systems, plant genomes contain significant levels of non-CG methylation with 2–3% CNN methylation and around 20% CNG methylation in rice or poplar [35]. Genome-wide single-base-pair mapping (BS-seq) of methylated cytosines in *Arabidopsis*, which has a relatively low level of heterochromatin, revealed genome-wide levels of 24% CG, 6.7% CNG and 1.7% CNN methylation. The efficiency of the different DNA methyltransferases is reflected in the level of methylation at their preferred target sites. CG methylation is most efficient with most sites being 80–100% methylated, while methylation levels at individual CNN methylation target sites rarely exceed ~10%. CNG methylation levels vary between 20% and 100% for individual sites [36].

Accessibility of genetic regions to methyltransferases influences DNA methylation efficiency. Methylation efficiency in *Arabidopsis* follows a periodicity of 167 nucleotides [36]. This may reflect the spacing pattern of nucleosomes that would facility DNA methyltransferase access to linker regions. As the 167 nucleotide periodicity is lower than the 175–185 nucleotide periodicity of plant nucleosome repeats, it was suggested that methylated regions formed a more compact chromatin structure with shorter linker regions [36]. If we interpret the 167 nucleotide periodicity as a consequence of a methylated target region, this highlights the dynamics of DNA methylation patterns. At the de novo methylation stage, the unmethylated target region would not be expected to have a compact chromatin structure, and would therefore not favour a methylation pattern with a 167 nucleotide periodicity.

This pattern could only evolve once the target region has become methylated, suggesting that methylation efficiency and patterning is not fixed but that it varies in the same way the local chromatin undergoes structural changes. If the periodicity of methylation patterns reflects the conformation of its target chromatin, and if access to linker regions improves methylation efficiency, methylation periodicity could be used to distinguish between genomic regions with fixed or sliding nucleosomes, respectively.

Most likely, enzyme conformation also influences DNA methylation patterns. CNN methylation is found at a periodicity of 10 nucleotides or one helical DNA turn [36]. A similar periodicity was observed in maternally imprinted mammalian genes where the frequencies of the distances between CG methylation sites peak periodically, with an average interval of 9.5 base pairs. This pattern appears to be specific for maternally imprinted genes as it was absent in three paternally imprinted methylation regions. The de novo CpG methyltransferase Dnmt3a and Dnmt3L, a germ cell specific enzymatically inactive factor, form a tetrameric complex with two active sites that are separated by about one helical turn. Complex formation of Dnmt3a and tissue- or stage-specific factors therefore most likely influences periodicity of DNA methylation patterns in mammals [37]. Similar interactions may regulate the activity of the Dnmt3a homologue DRM2 in plants.

Sequence context analysis shows that DNA methyltransferases have strong sequence preferences beyond the CG, CHG and CHH contexts, especially for symmetrical methylation patterns. CG, CNG and CNNG methylation of one strand correlates with a high level of methylation of the G-paired C on the opposite strand. In contrast, CGs are undermethylated when they are located in a ACGT context, and CNG and CNN methylation efficiency is poor if the target C is followed by another cytosine [36]. This may reflect a target specificity of methyltransferase complexes but it could also be the consequence of secondary structures, binding of sequence-specific factors or other effects that reduce access for methyltransferase complexes.

6. DNA methylation targets

The genome (~120 Mb) of *Arabidopsis thaliana* has been completely sequenced indicating that most of the repetitive sequences (~20 Mb) cluster in pericentromeric regions, whereas the majority of the ~27 000 protein-coding genes are distributed on the arms of the five chromosomes. CG, CNG and CNN methylation is enriched at repeat-rich pericentromeric regions, which correlates with siRNAs that regulate methylation of these regions by RdDM. About 63% of methylated regions, however, do not match to siRNAs and many of these regions contain MET1-dependent CG DNA methylation but no siRNA-targeted non-CG methylation [38]. This suggests that alternative signals to siRNA can initiate DNA methylation, or that certain methylation patterns were established by transient RdDM activity to be propagated by MET1 or other siRNA-independent maintenance systems. Promoter-specific methylation occurs in less than 5% of genes, most of which are under tissue-specific control [38]. A surprising result of genome-wide methylation profiling was that about one third of all genes contain CG-specific genic or body methylation patterns within their transcribed regions. Genic methylation is independent of DRM2 and the RdDM pathway. It involves MET1-based CG methylation that is retained in genic methylation targets and CNG methylation that is established but removed by histone demethylase IBM1 [39]. It therefore appears that IBM1 is part of a demethylation system that selectively removes CNG/H2K9me2 controlled methylation, and that works specifically at eukaryotic targets as heterochromatic methylation targets are not affected in a *ibm1* mutant. Genic DNA methylation is very likely directed by spliced mRNA [4], and transcripts or active transcription of genic methylation targets may assist in the recruitment of the CNG-specific demethylation systems. CG methylation or the presence of MET1 also appears to influence the balance between CNG methylation and removal, as CNG methylation appears at some genic methylation targets in a *met1* mutant.

A small number of genes are differentially methylated and silenced in male and female tissues. Unlike genomic imprinting in animals, however, imprinting in plants is not regulated by de novo methylation but by demethylation. An example for an imprinted gene is the *FWA* gene, which encodes a homeodomain-containing transcription factor that delays flowering. *FWA* is silenced in the sporophyte and activated by DNA demethylation in the female gamete and in extraembryonic endosperm tissue [31].

Repeats and transposons are the predominant targets of the RdDM pathway as indicated by the accumulation of matching 24nt siRNAs. Synthesis of some 24nt siRNAs requires Pol V in addition to Pol IV [40], which may be an indirect stimulating effect of Pol V-mediated heterochromatin formation on Pol IV activity. Among these type I loci are high-copy-number repeats or transposons, while low-copy-number type II repeats and intergenic sequences are Pol IV-dependent but Pol V-independent. SiRNA synthesis and local transcription are essential for heterochromatin formation of most targets. One exception is heterochromatin organisation and silencing at some pericentromeric repeats, which does not require the 24nt siRNA pathway functions POL V, RDR2, DCL3, AGO4 or DRM2. Silencing at these loci is controlled by POLV, DRD1, MET1 and DDM1 under participation of an unknown class of RNAs [41].

Single copy methylation targets usually contain repeat structures that attract DNA methylation. An example is the *suppressor of drm1 drm2 cmt3* (*SDC*) gene, which encodes an F-box protein. Its promoter contains seven tandem repeats, where non-CG DNA methylation is initiated by combined activity of DRM2 and CMT3, and from where it spreads into adjacent non-repeated sequences [42]. Repeats are, however, not always the trigger of DNA methylation. The *FWA* gene contains two extensively methylated sets of tandem repeats and a SINE-related sequence that is sufficient for imprinting, vegetative silencing, and targeting of DNA methylation. It was therefore proposed that the FWA repeats are not the cause but a consequence of epigenetic control [43]. The efficiency of initiating DNA methylation varies for individual methylation targets. Recognition of an *FWA* transgene as a de novo methylation target is restricted to the transformation phase, as an unmethylated *FWA* transgene in a *drm2* mutant is not methylated even when a functional *DRM2* gene is re-introduced [44]. In contrast, active *SDC* transgenes in a *drm2* mutant are silenced when they are crossed into a *drm2* background [42].

7. Locus-specific repression is controlled by one or several silencing systems

MET1 and DDM1 control silencing of various TEs, many of which are activated in *met1* or *ddm1* mutant backgrounds. Hallmarks for the activation of retrotransposons and DNA transposons in a *ddm1* mutant are loss of DNA methylation, 24nt siRNAs and repressive H3K9me2 marks, and an increase in active H3K4me3 marks. DDM1 also has a moderate effect on methylation at the single copy locus MHC9.7/9.8 locus [45] but body methylation does not depend on DDM1 [46]. In addition to its role in controlling CG methylation, DDM1 contributes to CNG and CNN methylation at some loci [47], and, interestingly, DDM1 may help to establish boundary functions, as it inhibits unidirectional spreading of methylation from a LINE element into an adjacent euchromatic region [48].

TEs controlled by DDM1 fall into two categories, which reflect the influence of distinct silencing pathways. At non-remethylatable DDM1 targets, like the *ATLANTYS2* TE, DNA methylation is

exclusively controlled by DDM1 and MET1, and methylation that has been lost in a *ddm1* mutant is not restored even when a functional *DDM1* allele is reintroduced. In contrast, re-methylatable DDM1 targets, like the *AtESPM5* TE, are under additional control of small RNAs and RdDM functions, which enable these methylation targets to regain their methylation marks. DDM1 and RNAi functions are both required for methylation and silencing of re-methylatable TEs (Fig. 2A), but methylation lost in a *ddm1* mutant is restored by RNAi activity over several generations when DDM1 is reintroduced. The RdDM pathway therefore serves as a protection mechanism against accidental loss of repression for certain TEs [47]. RdDM fulfils a similar stabilising role in plants with long-term loss of CG methylation. In a *met1* mutant, the loss of CG methylation is partially compensated by a combination of misdirected novel RdDM activity and demethylation repression, which leads to stochastic genome-wide de novo non-CG methylation and H3K9 remethylation [49].

DDM1 is usually necessary to facilitate maintenance of DNA methylation by MET1 but the requirement for DDM1 is most likely not determined by the methylation target but by the local chromatin environment of the target locus. This is exemplified by silencing of members of the *Sadhu* family of non-autonomous non-LTR retroposons, which are repressed by MET1, some with and some without the assistance of DDM1. *Sadhu 3-1* repression and DNA methylation is controlled by DDM1, MET1 and HDA6, and activation correlates with a switch from H3K9me2 to H3K4me3 marks. In contrast, *Sadhu 6-1* repression and methylation is predominantly under MET1 control, and H3K9me2 depletion/H3K4me3 enrichment is only observed in a *met1* mutant (Fig. 2B). *Sadhu 3-1* is embedded in a repeat-rich pericentromeric region, while *Sadhu 6-1* is located in a repeat-poor region [50]. This implies that *Sadhu 6-1* repression relies on MET1, while *Sadhu 3-1* repression is a combination of MET1 and a joint DDM1/HDA6 system, which are both required to maintain high DNA methylation and H3K9me2 levels.

The local environment may also determine the dependence of siRNA targets on MET1. The solo LTR IGT is a transcript initiated in a solo LTR that derives from a *LTR/Copia retroelement (LTRCO)* family. IG5 is a transcript initiated in the 3′ LTR of an intact *Copia-like retrotransposon* (*AtCOPIA* 95). Solo LTR repression requires siRNA production by Pol IV and RDR2, and DRD1 and Pol V for methylation, while MET1 has no or little influence on methylation and repression of solo LTR. In contrast, IG5 shows a strong dependence on MET1 for CG and CNG methylation and there is no detectable effect of Pol IV, PolV or DRD1 on IG5 methylation [28]. MET1 has a significant influence on IG5 activity, which may be a consequence of IG5 being very CG rich. Alternatively, the DRD/RNAi system that controls solo LTR may have been complemented, and partly replaced by MET1 regulation.

The two targets differ in some of their chromatin marks (Fig. 2C). Solo LTR has repressive H3K27me1 marks but no detectable H3K9me2 levels. De-repression of solo LTR in a *drd1* mutant removes repressive H3K27me1 and increases active H3K4me3 marks and overall H3 acetylation. In contrast, IG5 has H3K27me1

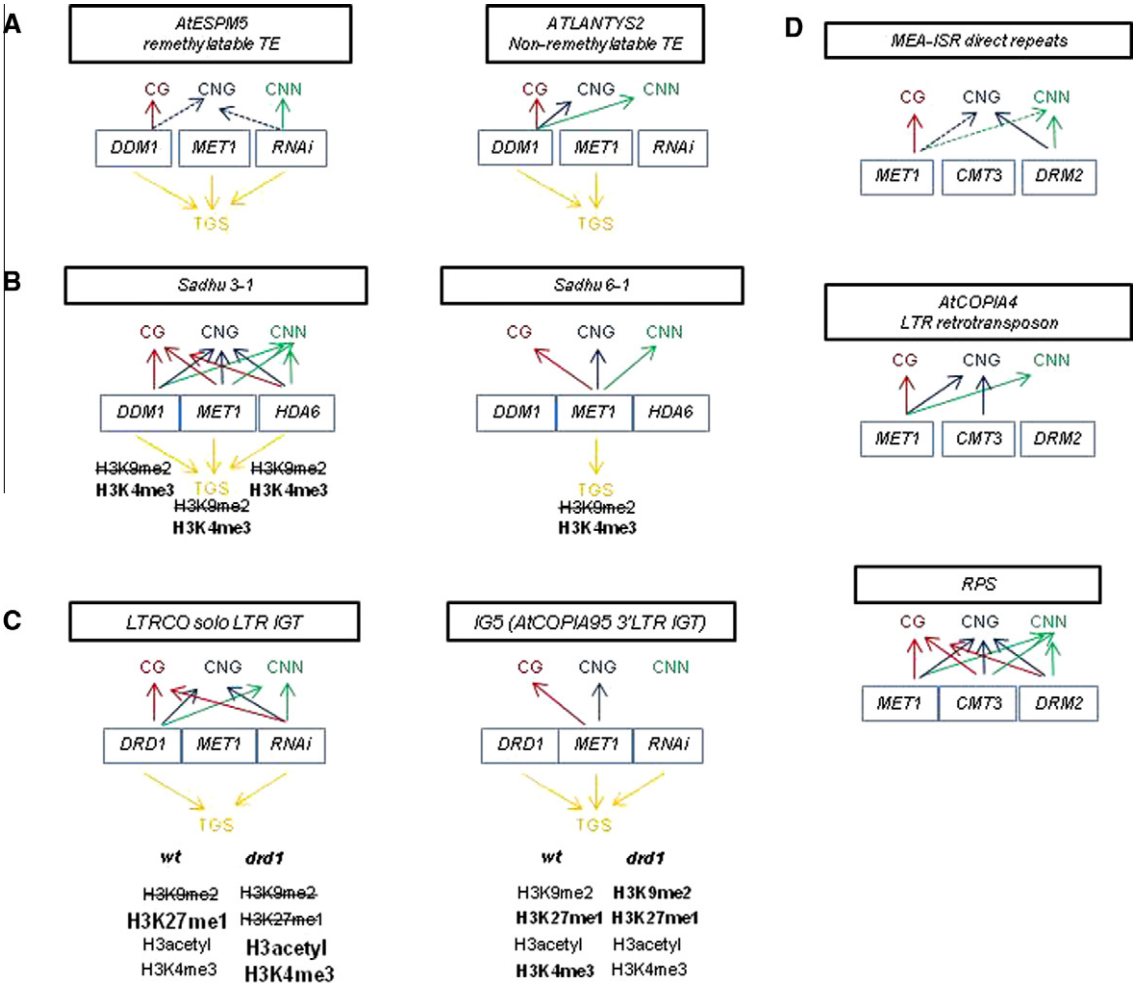

Fig. 2. Examples of methylation targets under individual or joint control of different silencing systems and DNA methyltransferases. Details are explained in the text.

marks like solo LTR but also repressive H3K9me2 marks. In a *drd1* mutant, which has a less severe influence on IG5 de-repression than a *met1* mutant, neither of these repressive marks are removed and none of the active marks increase [28]. It therefore appears that solo LTR is under sole control of a DRD1-dependent repression system that mediates reversible silencing via changes in H3K27me1, H3K4me3 and H3 acetylation, while IG5 repression involves an additional epigenetic system that regulates repression via H3K9me2, most likely under the control of MET1. The reason for these differences could be that IG5 is embedded in a large H3K9me2 marked region while soloLTR is located within a ~50kb region that is largely free of H3K9 methylation. Similar to the model discussed for *Sadhu 3-1* silencing, IG5 repression depends on two silencing systems that work co-operatively and are both required to maintain IG5 fully repressed, i.e. a IG5-specific RdDM repression system and a MET1-dependent repression system that controls DNA and H3K9 methylation of a larger chromatin domain, into which IG5 is embedded.

8. Locus-specific contributions of MET1, CMT3 and DRM2 to silencing

At a number of target loci, MET1, CMT3 and DRM2 work co-operatively or in competition, frequently with overlapping effects on CG, CNG and CNN methylation. MET1 mainly fulfils a maintenance function for CG methylation, but at some target loci, it also plays a role in CG-specific de novo methylation [51]. At certain target loci, non-CG methylation is maintained in a *drm1 drm2 cmt3* triple mutant indicative for a role of MET1 in non-CG methylation [52]. A comparison of MET1-specific effects at the *MEA-ISR, AtCOPIA4* and *RPS* loci, illustrates the direct and indirect influence of MET1 on non-CG methylation patterns (Fig. 2D). MET1 mutation reduces CNG methylation and eliminates CNN methylation at *MEA-ISR* and *FWA* but CNG or CNN methylation are lost in *drm2/cmt3* mutants. MET1 activity therefore has a positive, although indirect influence on non-CG methylation, which can not be maintained without DRM2 and the siRNA [53]. At *AtCOPIA4*, however, elimination of CMT3 and DRM2 only causes a moderate reduction in CNG methylation, which is also detectable in a *cmt3* mutant [26], while CNN methylation is even enhanced [52]. This suggest that a DRM2/CMT3-independent DNA methyltransferase, most likely MET1, regulates maintenance of CNN methylation at *AtCOPIA4*. Maintenance of CNN methylation, however, is not sufficient to maintain silencing as *AtCOPIA4* is activated in a *cmt3* mutant [26]. The *RPS* transgene is an example for the co-operative activity of all three DNA methyltransferases in establishing a DNA methylation pattern. Removal of MET1, CMT3 or DRM2, significantly reduces cytosine methylation in all sequence contexts [46], indicative for the co-operation and mutual re-enforcement of the three DNA methyltransferases.

9. Induction and heritability of epigenetic variability

One of the oldest examples for a heritable epigenetic change is a morphological mutant of flower development in *Linaria vulgaris*, which was generated more than 250 years. The mutant phenotype is due to hypermethylation and transcriptional silencing of *Lcyc*, a regulator of dorsoventral asymmetry. Occasional reversion of the phenotype correlates with demethylation and reactivation of *Lcyc* [54]. Several examples document the importance of TEs as generators of novel genes and expression patterns. TEs enhance genetic diversity via insertional inactivation and genome enlargement [55] but they can also contribute protein segments to novel genes via gene shuffling [56] and alter expression profiles of adjacent genes [57,58]. Epigenetic changes that alter the activity of TEs are therefore powerful tools to generate genetic and epigenetic variability, which probably explains why TEs are activated by drastic environmental changes [59].

In *Arabidopsis*, we find an example for tissue-specific activation of TEs. Members of different TE classes (DNA transposon, LTR-transposon, non-LTR transposon and helitron) are co-ordinately activated in the pollen vegetative nucleus (VN) that controls sperm delivery. In comparison to the heavily methylated sperm cells, VN methylation is significantly reduced at CNN sites, which is most likely the consequence of active demethylation. There is no indication for hypomethylation at CG sites, which seems not to be required for reactivation of transposable elements. Members of the RdDM pathway, especially *RDR2, DCL3, SUVH4 and CMT3* are down-regulated in pollen. *DDM1* expression is not altered but the DDM1 protein is not detectable in the VN, and, similar to *ddm1* mutant lines, activation of specific transposable elements in mature pollen correlates with a loss of 24nt si RNAs [60]. This example illustrates how quantitative changes of methylation control functions can induce widespread epigenetic change.

As the VN does not contribute DNA to the zygote, none of the epigenetic changes are transmitted to the next generation and will therefore not contribute to heritable epimutations. The analysis of DNA methylation mutants, however, demonstrates that epigenetic changes are heritable and transmitted over many generations, although transmission does not necessarily follow Mendelian predictions for random segregation. The back-crossing of CG methylation-deficient epi-alleles from a *met1* mutant line into wildtype plants revealed that plasticity, reversion and conservation rates of novel DNA methylation patterns differ for individual loci. At centromeric regions, methylation patterns were quickly restored to near-wildtype levels, most likely due to active remethylation, while many euchromatic epi-alleles were faithfully inherited over at least eight generations. Surprisingly, some loci displayed an unusually high level of epi-heterozygosity even after intensive inbreeding [5], indicative for an interaction between meta-stable epi-alleles that preserves or even enhances epigenetic variation. The target-specific differences in epigenetic variability probably reflect differences in the consequences that epigenetic changes induce at distinct loci. Plants probably tolerate or even require a high level of epigenetic variability at loci where this increases genetic and epigenetic diversity, but need to avoid epigenetic changes that compromise genome stability. This would explain why centromeric regions in particular regain their methylation pattern very quickly.

Induction and heritability of epigenetic variation will be influenced by the interaction of silencing pathways that control epigenetic patterns at individual target loci. An epigenetic pattern that is maintained by two separate silencing pathways will be less susceptible to changes if both pathways can maintain its epigenetic state independently [61]. Epigenetic control by two silencing pathways also improves resetting of epimutations as already discussed for remethylatable transposons that are under DDM1/MET1 and RNAi control [47]. In addition to regulating transcriptional silencing, some pathway functions contribute to additional layers of control that balance the impact of epigenetic variability. In a *ddm1* mutant, various TEs are mobilised in stochastic events that are independent for individual elements [62], but transcriptional activation of TEs in a *met1* mutant does not lead to transposition for most elements due to element-specific post-transcriptional regulation. The involvement of DNA methylation pathway function in this additional repressive layer was demonstrated for the É*vadé* (*EVD*) retrotransposon as repression of *EVD* transposition requires methylation-independent contributions by POL IV, POL V and SUVH4 [63].

DNA methylation mutants are useful to assess epigenetic stability and inheritance but mutations that lead to a complete loss of

silencing functions are rare and therefore will not play a significant role in natural epigenetic variation. It is more likely that temporal or conditional changes in quantity, stability and effectiveness of DNA methylation functions influence the stability of epigenetic patterns, and that these changes are especially induced by changing environmental conditions [64].

10. Outlook

While significant progress has been made in the analysis of epigenetic states and the mechanisms and pathways that control them, we are only beginning to understand the dynamics and interactions of gene silencing systems. One of the most challenging tasks will be to understand how individual genomic loci are selected for or protected from silencing. Target selection is most likely influenced by an interplay between local chromatin and transcription complexes, but there are also indications for an involvement of methylation pathway components in target selection. Single aminoacid changes in the catalytic C-terminal domain of MET1 can create interesting differential effects on methylation efficiency and target selection. The *met1-1* allele encodes a P to S mutation at amino acid 1300, and the *met1-2* allele encodes a G to S mutation at aminoacid 1101. In *met1-1*, methylation levels at TCGA sites are reduced by 70%, while the weaker allele *met1-2* produces a 50% reduction. The weaker *met1-2* allele also causes a less severe reduction than *met1-1* at CCGG sites in the 180-bp centromeric repeat arrays but, surprisingly, *met1-2* is equally efficient, or even slightly more efficient, in reducing rRNA gene repeat methylation [65]. This suggests that point mutations can alter the efficiency of MET1 in a target-specific way. This may be due to a direct role of MET1 in target selection, or it may be the consequence of changes in MET1 interaction with target-specific factors.

Target selection is also influenced by tissue- and development-specific changes in the concentration of silencing pathway components. Argonaute proteins play an important role in defining RdDM target specificity, which is partly determined by their differential expression patterns. The AGO4 family members AGO4, AGO6, and AGO9, all participate in the RdDM pathway and associate with 24nt siRNAs. They show distinct differences in their ability to promote siRNA accumulation and DNA methylation at specific target loci, indicative for a non-redundant function of the three AGO proteins. Tissue-specific differences in the expression of the AGO4 family members therefore influences DNA methylation efficiency at different loci [66]. In addition, many silencing components do not work in isolation but respond to quantitative changes of other silencing pathway components. An example is the *ROS1* demethylase, which is downregulated in *rdr2*, *drd1*, *pol IV*, *pol V*, *dcl3* and *met1* mutants [28,49]. Loss of CG methylation in a *met1* mutant induces genome-wide ectopic RdDM activity [49], and reduction of CG methylation at 5S rRNA genes in a *vim1 vim3* mutant leads to an increase in CNN methylation [15]. In conclusion, we will need a much better understanding of the regulation of silencing pathway components and their interactions in specific cell types, during specific developmental stages and in response to environmental stimuli.

Finally, we need to separate epigenetic noise from biologically meaningful epigenetic effects. We have seen significant progress in understanding the molecular and biological effects of certain epigenetic marks. Even for relatively small target regions, however, the numbers of theoretical permutations of DNA methylation and histone marks are almost endless. It will therefore be interesting to elucidate if locus-specific combinations and variations of epigenetic marks reflect epigenetic control mechanisms or merely random chance events. To fully assess the evolutionary impact of DNA methylation systems, we will also have to move outside the *Arabidopsis* model system to assess if the same epigenetic rules as detected in *Arabidopsis*, apply for other plant species.

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