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Specifications of Targeting Heterochromatin Modifications in Plants

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ABSTRACT

Plants encode a diverse repertoire of DNA methyltransferases that have specialized to target cytosines for methylation in specific sequence contexts. These include the *de novo* methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), which methylates cytosines in all sequence contexts through an RNA-guided process, the CHROMOMETHYLASES (CMTs), which methylate CHH and CHG cytosines (where H is A, T, or C), and METHYLTRANSFERASE 1 (MET1), which maintains methylation of symmetrical CG contexts. In this review, we discuss the sequence specificities and targeting of each of these pathways. In particular, we highlight recent studies that indicate CMTs preferentially target CWG or CWA/CAW motifs (where W is A or T), and discuss how self-reinforcing feedback loops between DNA methyltransferases and histone modifications characteristic of heterochromatin specify targeting. Finally, the initiating events that lead to gene body methylation are discussed as a model illustrating how interdependent targeting of different silencing pathways can potentiate the establishment of off-target epialleles.

Key Words: heterochromatin, DNA methylation, gene body methylation, epiallele, DNA methyltransferase, chromomethylase

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INTRODUCTION

Plants have evolved multiple specialized pathways to establish and maintain cytosine methylation. Plants methylate cytosines in all sequence contexts, including symmetric CG and CHG sites, as well as asymmetric CHH sites (where H is A, T, or C) (Law and Jacobsen, 2010). This proclivity in cytosine targeting is accounted for in part by plant-specific DNA methyltransferases referred to as CHROMOMETHYLASES (CMTs). CMTs mediate cytosine methylation in the CHG and CHH contexts on transposons and other repeats (Bartee et al., 2001; Jackson et al., 2002; Stroud et al., 2013, 2014; Zemach et al., 2013). CMTs in angiosperms can be divided into two clades, CMT2 and CMT1/CMT3/ZMET (Bewick et al., 2017). In *Arabidopsis thaliana*, this division is associated with sequence specificity, as CMT2 methylates cytosines predominantly in the CHH context and CMT3 methylates cytosines predominantly in the CHG context (Law and Jacobsen, 2010; Stroud et al., 2013, 2014; Zemach et al., 2013). *A. thaliana* CMT1 has no known function and is proposed to be a pseudogene (Henikoff and Comai, 1998). However, retention of CMT1 across angiosperms suggests it may have an as yet undiscovered function (Bewick et al., 2017). Specificity for CHG versus CHH contexts is not as robust in all species. For example, in *Zea mays* (maize), which has lost CMT2, enzymes related to *A. thaliana* CMT3, ZEA METHYLTRANSFERASE 2 AND 5 (ZMET2 and ZMET5), methylate DNA in CHH or CHG contexts (Li et al., 2014).

CHG is representative of three sequence motifs (CAG, CTG, and CCG) and there are nine different CHH motifs (CAA, CAT, CAC, CTT, CTA, CTC, CCC, CCA, and CCT). In the pericentromeric heterochromatin of *A. thaliana*, CMT3-dependent CHG methylation is predominantly in the CAG or CTG context opposed to CCG (Gouil and Baulcombe, 2016). Similarly, CMT2-dependent CHH methylation is predominantly found on CTA and CAA motifs. Importantly, CAG and CTG methylation are also overrepresented relative to CCG in the genomes of *Solanum lycopersicum* (tomato), *Oryza sativa* (rice), and *Z. mays* (Gouil and Baulcombe, 2016). In the CHH context, CAA and CTA are preferentially targeted in *Z. mays*, compared with CTA in *O. sativa*, and CAA and CAT in *S. lycopersicum* (Gouil and Baulcombe, 2016). Thus, CMTs are more accurately described as CWG- and CWA- (or CAW-, depending on the species) specific enzymes, rather than the more general CHG and CHH (where W is A or T).

In addition to plant-specific methyltransferases, plants also encode more broadly conserved methylation machinery. Similar

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to other organisms that methylate their DNA, plants encode a homolog of the human maintenance methyltransferase, DNA METHYLTRANSFERASE 1 (DNMT1), called MET1, and the *de novo* methyltransferase, DNMT3, called DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Law and Jacobsen, 2010). DRM2 establishes *de novo* methylation of cytosines in all sequence contexts through the RNA-directed DNA methylation pathway (RdDM) (Matzke and Mosher, 2014; Wendte and Pikaard, 2017). RdDM is mediated by two plant-specific multi-subunit RNA polymerases, NUCLEAR RNA POLYMERASE IV and V (Pol IV and Pol V) (Haag and Pikaard, 2011; Zhou and Law, 2015). Pol IV initiates the pathway by transcribing precursors for small interfering RNAs (siRNAs), which are bound by argonaute (AGO) proteins (Zilberman et al., 2003; Kanno et al., 2005; Pontier et al., 2005; Zheng et al., 2007). Pol V transcribes loci targeted for methylation to produce scaffold RNAs that are bound by AGO-siRNA complexes at chromatin (Wierzbicki et al., 2008, 2009). AGO-siRNA binding to Pol V transcripts subsequently recruits DRM2, resulting in cytosine methylation (Bohmdorfer et al., 2014; Zhong et al., 2014). MET1 maintains cytosine methylation in symmetrical CG contexts, as it is specifically recruited to hemi-methylated cytosines after DNA replication to methylate the newly synthesized, unmethylated strand (Law and Jacobsen, 2010).

DNA METHYLTRANSFERASE TARGETING FEEDBACK LOOPS ASSOCIATED WITH OTHER HETEROCHROMATIN MARKS

Cytosine methylation is correlated with other heterochromatin marks associated with transcriptional silencing, including Histone 3 hypoacetylation and di-methylated lysine 9 (H3K9me2) (Richards and Elgin, 2002; Du et al., 2015). These correlations are the result of direct or indirect physical interactions between DNA methyltransferases and chromatin modifications that result in self-reinforcing feedback loops.

CMTs are characterized by a chromo-domain and a bromo-adjacent homology domain, which mediate direct physical binding to H3K9me2 (Du et al., 2012). In *A. thaliana*, H3K9me2 is mediated predominantly by SU(VAR)3–9 HOMOLOG 4/KRYPTONITE (SUVH4/KYP) and to a lesser extent by the related methyltransferases SUVH5 and SUVH6 (Jackson et al., 2002, 2004; Stroud et al., 2013). The SET and Ring Finger Associated (SRA) domains of SUVH4/5/6 are in turn capable of binding methylated cytosines, linking these enzymes in a self-reinforcing feedback loop (Johnson et al., 2007; Rajakumara et al., 2011; Du et al., 2014) (Figure 1A). SUVH4 and SUVH6 preferentially bind methylated cytosines in the CHH and CHG contexts, whereas SUVH5 binds cytosines in all contexts equally well (Johnson et al., 2007; Rajakumara et al., 2011; Du et al., 2014). Genome-wide, losses of CHH or CHG methylation in *suvh4/5/6* triple mutants occur at the same regions as those that lose methylation in *cmt2* or *cmt3* mutants (Stroud et al., 2013, 2014). When CHG contexts are considered individually, the effect of *suvh4* is specifically on CAG or CTG sites (the preferred CWG sequence context for CMT3), and losses of methylation in the CCG context do not occur until *suvh4* is combined with *suvh5/6* (Gouil and Baulcombe, 2016). These results have led to the hypothesis that SUVH5 and SUVH6 have a redundant role in the establishment of H3K9me2 that recruits CMT3 to CCG sites, even though these sites are a minority of the CHGs targeted by CMT3. Given that SUVH4 is responsible for the majority of H3K9me2 methylation and SUVH5 and SUVH6 only play minor roles in *A. thaliana*, these results are consistent with the hypothesis that the sequence bias of CMT3 for CWG sites could be at least partially attributable to the histone methyltransferase establishing H3K9me2 binding sites (Gouil and Baulcombe, 2016).

In the RdDM pathway that targets DRM2, which is best understood in *A. thaliana*, Pol IV physically interacts with SAWADEE HOMEODOMAIN HOMOLOG 1/DNA-BINDING TRANSCRIPTION FACTOR 1 (SHH1/DTF1), which is capable of binding H3K9me2 and recruiting Pol IV to H3K9me2 sites for siRNA precursor transcription (Law et al., 2011, 2013; Zhang et al., 2013). Similarly, Pol V indirectly interacts with SUVH2 and SUVH9 through its interaction with DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) (Johnson et al., 2014; Liu et al., 2014). SUVH2 and SUVH9 are histone methyltransferases that have lost methyltransferase activity but retained their ability to bind methylated DNA (Johnson et al., 2014; Liu et al., 2014). Thus, Pol V is recruited to sites with pre-existing DNA methylation, which is reinforced by Pol V recruitment of DRM2 (Figure 1B).

MET1 methylation is dependent on VARIANT IN METHYLATION proteins 1–3 (VIM1-3), which preferentially bind hemi-methylated CG cytosines, presumably to recruit MET1 (Liu et al., 2007; Woo et al., 2007, 2008; Yao et al., 2012; Stroud et al., 2013). MET1 has also been shown to physically interact with the histone deacetylase, HISTONE DEACETYLASE 6 (HDA6), and mutation of HDA6 can cause a loss of CG methylation in a locus-specific manner, although the phenotype is not as severe genome wide as a *met1* mutant (Earley et al., 2010; To et al., 2011; Liu et al., 2012; Stroud et al., 2013; Blevins et al., 2014) (Figure 1C).

Mutation of HDA6 and MET1 can also negatively influence H3K9me2 methylation and CHG and CHH methylation at a subset of loci (Earley et al., 2010; Stroud et al., 2013; Blevins et al., 2014). This is presumably attributed to losses of DNA and histone methylation that affect recruitment of CMT2, CMT3, and/or DRM2. The mechanistic basis for MET1 influencing non-CG methylation at some loci and not others is unknown, but a recent study identified that these sites are characterized by lower levels of repetitiveness and higher densities of CG motifs than sites where non-CG methylation does not depend on MET1 (Catoni et al., 2017).

SELF-REINFORCING FEEDBACK LOOPS AND OFF-TARGET CHROMATIN MODIFICATIONS

Self-reinforcing feedback loops between multiple chromatin modifications are a robust mechanism for ensuring the proper silencing of parasitic DNA elements (Richards and Elgin, 2002; Du et al., 2015; Holoch and Moazed, 2015). However, the inter-dependencies between different pathways can also lead

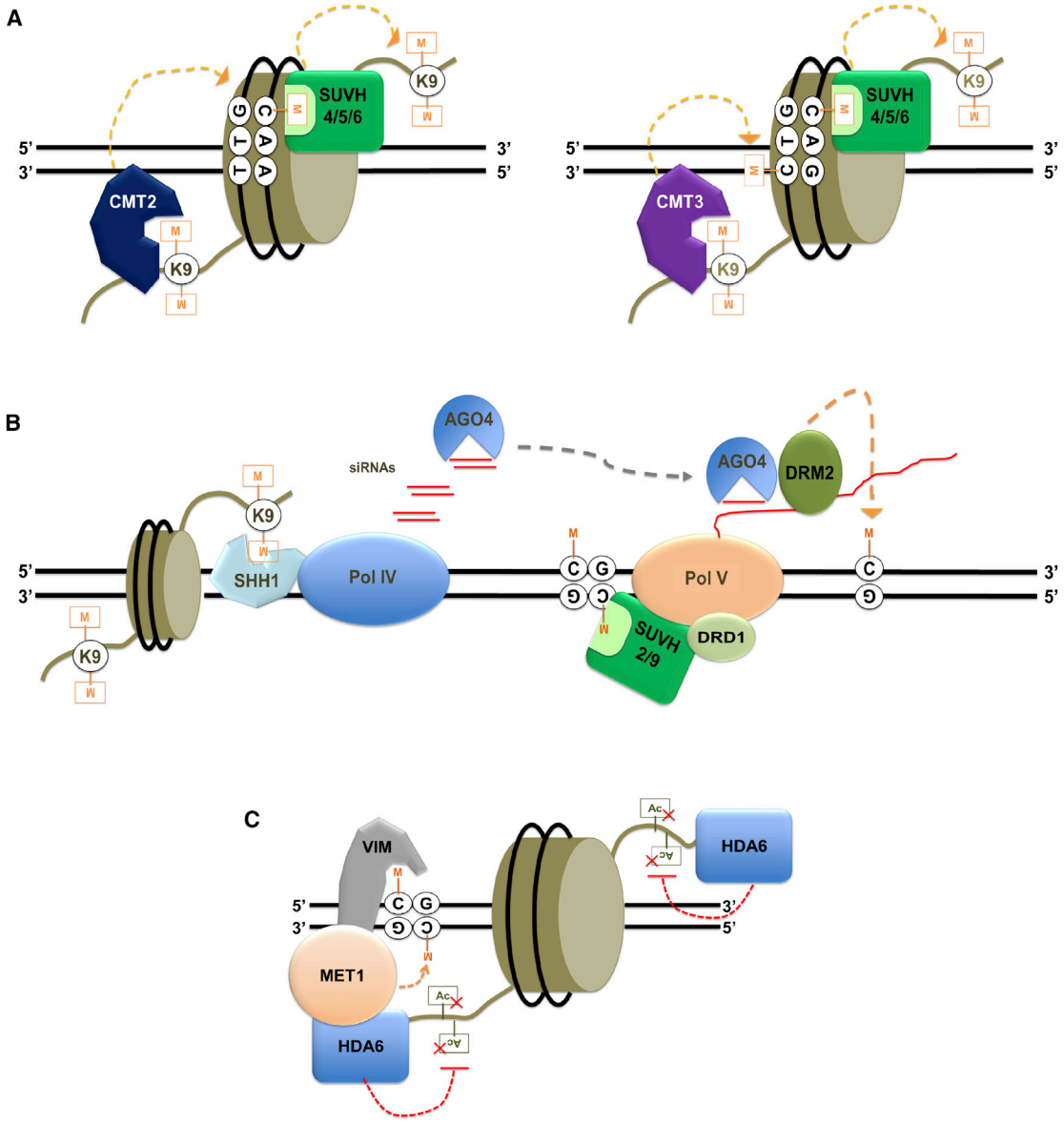

Figure 1. Specification of DNA Methyltransferase Targeting.

(A) Chromomethylases, CMT2 and CMT3, are targeted to chromatin through the binding of the CMT chromo- and bromo-adjacent homology domains to H3K9me2. CMT2 preferentially methylates cytosines in the CWA context and CMT3 preferentially methylates CWG cytosines (where W is A or T). Methylated CWA or CWG sites are bound by the histone methyltransferases, SUVH4, 5, and 6, which di-methylate Histone H3K9.

(B) The *de novo* methyltransferase, DRM2, methylates cytosines in all sequence contexts and is targeted to chromatin through RNA-directed DNA methylation (RdDM), mediated by multi-subunit RNA polymerases, Pol IV and Pol V. Pol IV forms a complex with SHH1, which binds and recruits Pol IV to sites of H3K9me2 to transcribe precursors for siRNAs. Pol V, via its interaction with DRD1, forms a complex with SUVH2 and SUVH9, which bind and recruit Pol V to sites of cytosine methylation to transcribe scaffold RNAs. Scaffold RNAs are bound by AGO4-siRNA complexes. AGO4 binding to Pol V transcripts facilitates the recruitment of DRM2, reinforcing cytosine methylation.

(C) The maintenance methyltransferase, MET1 is recruited to hemi-methylated CG sites by VIM1–3 to methylate the cytosine on the opposite strand. MET1 also interacts with the histone deacetylase, HDA6, which is required for MET1-dependent DNA methylation at a subset of loci.

to the exacerbation of negative effects resulting from mutations that lead to mis-targeting of chromatin modifications (Saze et al., 2008; Rigal et al., 2016). An example of this phenomenon in plants is the aberrant targeting of H3K9me2 and CMT3-dependent CHG methylation to gene bodies following mutation of the histone demethylase, INCREASE IN BONSAI METHYLATION 1 (IBM1) (Saze et al., 2008). In addition to targeting transposons and other repeats, H3K9me2 is presumably persistently mis-targeted to the gene bodies of several moderate to highly expressed genes. These off-target marks are removed by IBM1 in wild-type cells such that they are not detectable until IBM1 is mutated. Concurrent with the appearance of H3K9me2 in *ibm1* mutants are increased levels of cytosine methylation in the CHG context mediated by CMT3 (Saze et al., 2008; Miura et al., 2009). Thus, a mutation that is permissive to the off-targeting of one histone modification

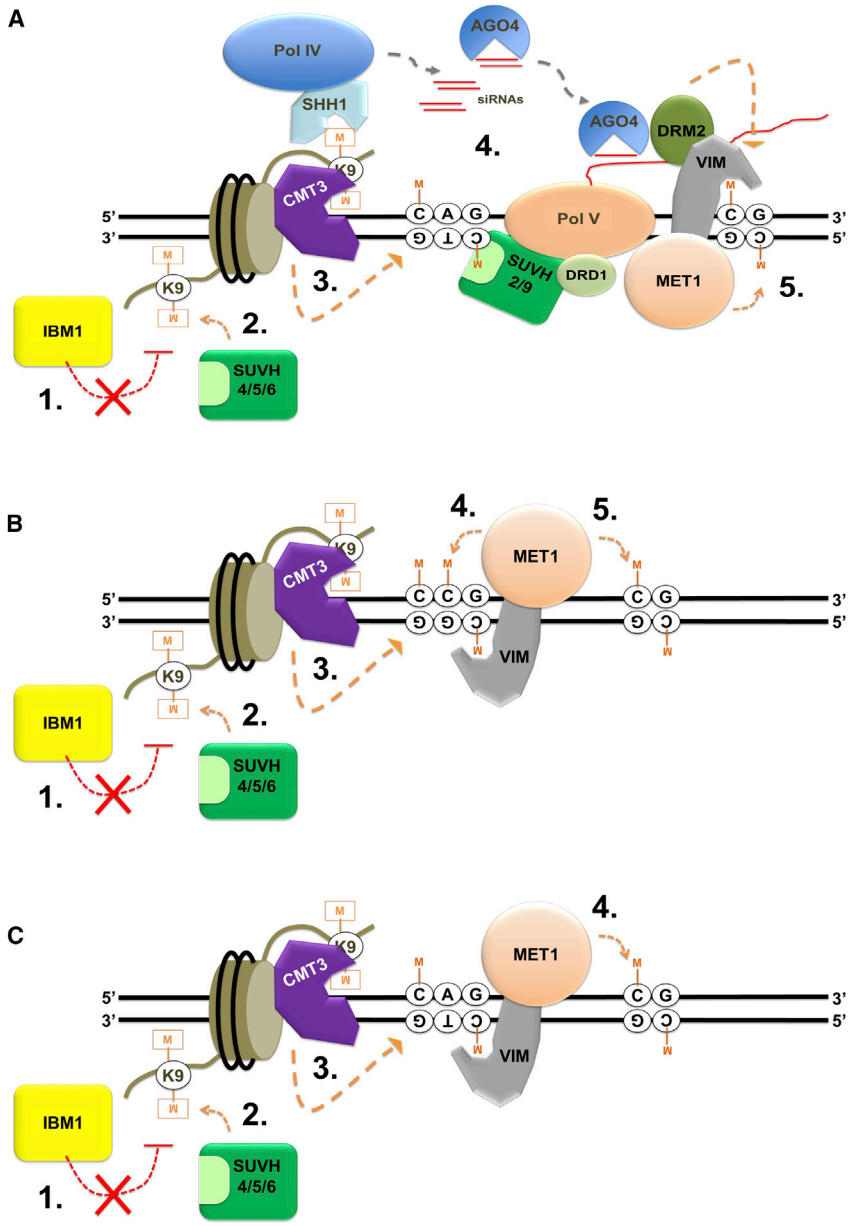

Figure 2. Hypothetical Models for the Initiation of Gene Body Methylation (gbM) by CMT3.

(A–C) In each model, gbM genes are subject to continual off-target establishment of H3K9me2 by SUVH4/5/6, but this modification is removed by the histone de-methylase, IBM1. However, on rare occasions when IBM1 fails to remove H3K9me2 (1), the H3K9me2 mark is transiently established by SUVH4/5/6 (2). Transient H3K9me2 provides a binding site for CMT3, which methylates CHG cytosines (3).

(A) The progression from CMT3-mediated CHG methylation to MET1-maintained CG methylation is unknown, but it possibly occurs through the RdDM pathway. Cytosine methylation established by CMT3 and H3K9me2 recruit Pols IV and V, promoting RdDM in all sequence contexts, including CG, by DRM2 (4). Methylated CG sites recruit VIM/MET1 and are maintained after removal of the transient H3K9me2 by IBM1 inhibits perpetuation of CHG and CHH methylation (5).

(B) CMT3 methylates the external cytosine of a CCG site, which is both a CHG and CG motif (3). This creates a hemi-methylated CG motif that recruits VIM/MET1, which methylates the internal cytosine of CCG (4). MET1 also methylates nearby CG motifs, beyond the region bound by VIM to promote spreading of CG methylation through the gene body (5).

(C) VIM binds hemi-methylated cytosines in the CHG context and MET1 methylates nearby CG motifs (4).

mark, H3K9me2, also leads to the off-targeting of CHG methylation due to the feedback loop that exists between CMT3/CHG methylation and SUVH4/H3K9me2, further reinforcing the aberrant effect.

Persistent off-targeting of heterochromatin machinery in wild-type cells may provide a plausible mechanism to explain the formation of natural epialleles. It is notable that sites of aberrant H3K9me2 and CHG methylation correspond to genes that are subject to gene body methylation (gbM) in wild-type cells (Miura et al., 2009). gbM genes are found in angiosperm species and defined as those characterized by strictly CG methylation located between the transcription start site (TSS) and transcription termination site (TTS) (Tran et al., 2005; Zhang et al., 2006; Zilberman et al., 2007; Bewick et al., 2016, 2017; Niederhuth et al., 2016; Bewick and Schmitz, 2017). GbM has been identified on orthologous genes across several species, and genes subject to gbM are generally constitutively expressed at moderate to high levels (Takuno and Gaut, 2013; Niederhuth et al., 2016; Zilberman, 2017). However, a clear functional role for gbM has yet to be identified although there have been a number of proposed functions such as regulation of expression and splicing (reviewed in Bewick and Schmitz, 2017; Zilberman, 2017). The CG methylation in gbM genes is dependent on MET1, as it is lost in *met1* mutants (Lister et al., 2008; Reinders et al., 2009; Teixeira et al., 2009; Stroud et al., 2013; Bewick et al., 2016; Catoni et al., 2017). Furthermore, upon reintroduction of wild-type *MET1*, most gbM is not restored, appearing only at a few loci after several generations of propagation following reintroduction of the wild-type allele (Reinders et al., 2009; Teixeira et al., 2009; Bewick et al., 2016; Catoni et al., 2017). Despite the dependence on MET1 for maintenance of gbM, the phenomena of aberrant targeting of CMT3 to gbM genes in *ibm1* mutants, has led to the hypothesis that CMT3 is important for the initiation of gbM. In this model, IBM1 is assumed not to be 100% effective, resulting in the rare occasions that H3K9me2 is established and CMT3 is recruited to methylate DNA. This aberrant H3K9me2 and DNA methylation is then quickly lost as IBM1 removes the H3K9me2 and thus prevents further recruitment of CMT3. However, if in this process a cytosine is methylated in the CG context, it is then consistently maintained

by MET1, whereas methylation in other contexts is lost (Figure 2). As errors by IBM1 are expected to be rare, accumulation of CG methylation at these sites would only occur slowly over many generations (Inagaki and Kakutani, 2012; Bewick et al., 2016; Bewick and Schmitz, 2017). Thus, in this model, gbM loci are epialleles that form spontaneously due to the persistent off-targeting of SUVH4/5/6 in wild-type cells. Notably, gbM loci are found to have high epiallelic potential within populations (Catoni et al., 2017).

Support for this model has come from recent comparative studies of methylomes and DNA methylation machinery across diverse plant species (Bewick et al., 2016, 2017). GbM, strictly defined as above, has only been found to be present in angiosperms, coincident with the evolution of CMT3 and IBM1 (Niederhuth et al., 2016; Bewick et al., 2017). Furthermore, two angiosperm species that have lost gbM, *Eutrema salsugineum* and *Conringia planisiliqua*, have also lost the gene encoding CMT3 (Bewick et al., 2016). Plant species in the Brassicaceae family with signatures of relaxed selection in CMT3, which can be associated with the accumulation of mildly deleterious mutations, have lower levels and numbers of gbM genes (Bewick et al., 2017). Also consistent, gbM genes have higher content of CAG and CTG motifs preferred by CMT3 than genes expressed at similar levels that do not undergo gbM (Bewick et al., 2016).

Key aspects of the model remain to be confirmed experimentally in the laboratory. Among these, an important question is how CHG methylation established by CMT3 translates to methylation in the CG context that is the substrate for MET1. One possibility is that transient establishment of H3K9me2 and DNA methylation by SUVH4/5/6 and CMT3 provides signals for recruitment of DRM2 via the RdDM pathway. RdDM then establishes cytosine methylation in all sequence contexts, yet only CG methylation is maintained via MET1 after removal of H3K9me2 by IBM1 (Figure 2A).

Another scenario, outlined in Figure 2B, involves the unique sequence context of CCG (complementary to CGG), which is both a CHG and CG sequence context. Even though CCG sites are targeted at a lower level than CWG sites, it has been noted that when these motifs are methylated in the CHG context, methylation occurs on both the external and internal cytosine ($^{m}C^{m}CG$) (Yaari et al., 2015; Gouil and Baulcombe, 2016; Zabet et al., 2017). Mutation of CMT3 causes loss of methylation of the external cytosine and mutation of MET1 causes loss of both the internal and external cytosine, suggesting crosstalk between these pathways at these sites (Yaari et al., 2015; Gouil and Baulcombe, 2016; Zabet et al., 2017). If, given a nearby binding site of H3K9me2, CMT3 can initiate CCG/CGG external cytosine methylation, the result would be a hemi-methylated binding substrate for VIM/MET1, which could then perpetuate the CG mark. This hypothesis would also invoke that MET1 could enzymatically process nearby CG cytosines beyond the CG immediately bound by VIM to spread the CG marks through the gene body. A caveat to this model is that CMT3 is apparently not capable of maintaining methylation of the external cytosine in the absence of MET1, so it is unclear whether CMT3 can initiate methylation at these sites (Yaari et al., 2015; Gouil and Baulcombe, 2016; Zabet et al., 2017). An additional, non-exclusive possibility is that VIM proteins could recruit MET1 to hemi-methylated sites in other sequence contexts, such as CHG, which is a possibility supported by *in vitro* studies (Woo et al., 2007) (Figure 2C).

An additional significant aspect for the initiation model for gbM is that it invokes the targeting of SUVH4/5/6 in the absence of pre-existing cytosine methylation (Figure 2). Currently, mechanistic details of the initiating events of the CMT3–SUVH4/5/6 feedback loop are lacking. Further understanding of gbM may be revealing in this regard and identify an alternative targeting pathway for SUVH4/5/6 and/or CMT3.

CONCLUSIONS AND FUTURE PROSPECTS

Much remains to be learned about the targeting and maintenance of DNA methylation in plant genomes. In this regard, gbM serves as an important model of how the amount and distribution of heterochromatin in a genome is a sum result of a complex interplay between self-reinforcing feedback loops that establish and maintain marks and pathways that remove off-target effects. Some important considerations for future studies are how intrinsic factors, such as genome size and transposable element content, and external factors, such as metabolic access to co-factors required for the enzymatic activity, influence the heterochromatin load of a genome, accuracy of heterochromatin targeting, and tolerance for off-target effects.

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