Review

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DNA methylation in plants: mechanisms and tools for targeted manipulation

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Summary

DNA methylation is an epigenetic mark that regulates multiple processes, such as gene expression and genome stability. Mutants and pharmacological treatments have been instrumental in the study of this mark in plants, although their genome-wide effect complicates the direct association between changes in methylation and a particular phenotype. A variety of tools that allow locus-specific manipulation of DNA methylation can be used to assess its direct role in specific processes, as well as to create novel epialleles. Recently, new tools that recruit the methylation machinery directly to target loci through programmable DNA-binding proteins have expanded the tool kit available to researchers. This review provides an overview of DNA methylation in plants and discusses the tools that have recently been developed for its manipulation.

I. Introduction

DNA methylation is a conserved epigenetic mark that regulates multiple processes, including gene expression, genome stability and gene imprinting, and consequently disruption of DNA methylation can lead to developmental abnormalities (Zhang *et al.*, 2018). In plants, DNA methylation is found in the CG, CHG and CHH sequence context (where H is A, C or T), and it is highly enriched over heterochromatic transposable elements (TEs) and repeats, where it plays a prominent role in silencing their expression at the transcriptional level (Transcriptional Gene Silencing (TGS)). DNA methylation can also trigger TGS when it is present in gene regulatory regions. Moreover, methylation of intronic TEs and repeats has been shown to affect mRNA processing mechanisms such as alternative splicing and alternative polyadenylation (Zhang *et al.*, 2018). In some instances, DNA methylation can also promote gene expression, which has recently been shown to be partially mediated by the DNA methyl-readers SU(VAR)3-9 homologs SUVH1 and SUVH3 (Harris *et al.*, 2018; Xiao *et al.*, 2019). Consequently, there are many examples of natural and induced epialleles that change their expression in response to methylation changes and that affect multiple physiological processes (Zhang *et al.*, 2018). DNA methylation, largely in the CG context, is also present over gene bodies in many plant species. Its function here is not so well understood (Bewick & Schmitz, 2017), although a recent study reported a role in suppressing intragenic antisense transcripts (Choi *et al.*, 2019).

The use of mutants and pharmacological treatments that affect DNA methylation levels has been instrumental in understanding its functions in multiple processes. However, these approaches have a

genome-wide effect on methylation, rendering the study of the direct effects of methylation in gene expression and chromatin at specific loci difficult. To bypass this issue, researchers can make use of tools for locus-specific manipulation of DNA methylation. These can also be used to generate novel epialleles with unique expression patterns, which can be made available to researchers and plant breeders. This review provides an overview of our current knowledge on DNA methylation in plants, focusing on recent advances in the development of tools for its targeted manipulation.

II. Mechanisms of DNA methylation in plants

In plants, the establishment of DNA methylation is mediated by the RNA-directed DNA methylation (RdDM) pathway, where the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) catalyzes *de novo* DNA methylation in all sequence contexts (Zhang *et al.*, 2018) (Fig. 1a). In the canonical RdDM pathway, the plant-specific RNA polymerase IV (Pol IV) synthesizes single-stranded RNAs (ssRNAs) that are converted into double-stranded RNAs (dsRNAs) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2). These dsRNAs are cut into 24-nt small interfering RNAs (siRNAs) by DICER-LIKE 3 (DCL3) which are then incorporated into ARGONAUTE 4 (AGO4) and AGO6 (Zhang *et al.*, 2018). Recruitment of Pol IV to chromatin depends on the histone reader SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) and the CLASSY family of putative chromatin remodelers (Zhang *et al.*, 2018; Zhou *et al.*, 2018). The second arm of the pathway depends on the transcription of non-coding RNAs (ncRNAs) by Pol V, which are bound through sequence complementarity by siRNA-loaded AGO4/AGO6, followed by co-transcriptional slicing (Liu *et al.*, 2018; Zhang *et al.*, 2018) (Fig. 1a). Once the AGO-siRNA-ncRNA-Pol V ribonucleoprotein complex is formed, DRM2 is recruited to target DNA methylation (Zhang *et al.*, 2018). Recruitment of Pol V to the chromatin depends on the DNA methyl-readers SUVH2 and SUVH9, which in turn recruit the DDR complex – DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and RNA-DIRECTED DNA METHYLATION 1 (RDM1) – which is also critical for Pol V's presence on chromatin (Zhang *et al.*, 2018; Gallego-Bartolome *et al.*, 2019) (Fig. 1a). The assembly of the DDR complex, whose structure has been recently revealed by cryoEM, is regulated by the Anaphase-promoting complex through control of the DMS3 protein levels (Wongpalee *et al.*, 2019; Zhong *et al.*, 2019).

In addition to the canonical RdDM pathway, which is mediated by Pol IV-dependent 24-nt siRNAs, non-canonical RdDM pathways have been reported, where small RNAs (sRNAs) from diverse origins such as viruses and Pol II transcripts can direct RdDM (Cuerda-Gil & Slotkin, 2016) (Fig. 1b). These sRNAs are generated from dsRNAs that are cut by different DCL proteins into 21–24nt sRNAs, which are incorporated into various AGO proteins to trigger post-transcriptional gene silencing (PTGS) of complementary RNAs by cleavage and/or translational repression (Martinez de Alba *et al.*, 2013). When these sRNAs are incorporated specifically into AGO4 and/or AGO6, they can trigger Pol V- and DRM2-dependent methylation of complementary DNA sequences, which might result in TGS of genes and transcriptionally active TEs (Cuerda-Gil & Slotkin, 2016) (Fig. 1b).

Following RdDM-mediated *de novo* establishment, methylation is maintained after DNA replication by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 2 and 3 (CMT2 and 3), which function in the CG, CHH and CHG contexts, respectively (Zhang *et al.*, 2018). Interestingly, a recent study proposed a *de novo* methyltransferase activity for CMT3 at genic loci to establish gene body methylation (Wendte *et al.*, 2019). Non-flowering plants present a fourth DNA methyltransferase family that has been lost in angiosperms and represents the orthologs of the mammalian *de novo* enzyme DNMT3. Yaari et al. recently reported the characterization of *Physcomitrella patens* DNMT3s and proposed a role for these enzymes in *de novo* methylation as well as maintenance of CHH methylation (Yaari *et al.*, 2019). The same study also proposed a role for PpCMT in *de novo* methylation in the CHG context.

Loss of DNA methylation can occur as a passive process after consecutive rounds of replication in the absence of functional methylation maintenance. Active demethylation in plants involves the family of glycosylases, REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2) and DML3, which prevent hypermethylation at multiple genomic locations. These enzymes can directly remove methylated cytosines irrespective of sequence context and promote demethylation through a base excision repair pathway (Zhang *et al.*, 2018). Interestingly, DNA methylation at the *ROS1* promoter has a positive effect on its own expression, thus tuning the levels of demethylase in response to changes in methylation (Lei *et al.*, 2015; Williams *et al.*, 2015). In mammals, active demethylation also occurs through glycosylases and base excision repair. However, in these organisms methylated cytosines are previously oxidized by the TEN-ELEVEN TRANSLOCATION (TET) family (Wu & Zhang, 2017). Although TET proteins are not found in plants, overexpression in Arabidopsis of the catalytic domain of the human TET3 protein (TET3-CD) triggered methylation changes at rDNA loci (Hollwey *et al.*, 2016). More recently, a comprehensive study analyzed the effect of TET1-CD overexpression in plants, which resulted in genome-wide hypomethylation, mimicking *met1* mutants (Ji *et al.*, 2018).

III. Targeted manipulation of DNA methylation in plants

There are several different tools available for the manipulation of locus-specific methylation in plant genomes, and they all rely on one of two main approaches: synthesis of siRNAs complementary to the target locus, and direct tethering of the DNA methylation machinery to the target locus through programmable DNA-binding proteins (Fig. 2).

Targeted methylation mediated by siRNAs

For many years, researchers have used different virus- and transgene-based tools to silence gene expression through the generation of siRNAs complementary to target loci, which can

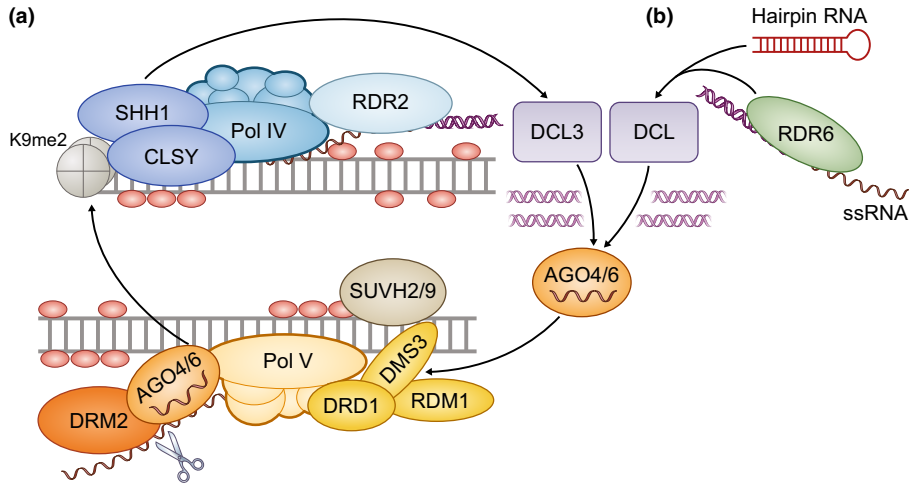

Fig. 1 RNA-directed DNA methylation in plants. (a) The canonical RNA-directed DNA methylation (RdDM) pathway. RNA polymerase IV (Pol IV) transcripts are converted to double-stranded RNAs (dsRNAs) by RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and cut into 24-nt short-interfering RNAs (siRNAs) by DICER-LIKE 3 (DCL3). These siRNAs are incorporated into ARGONAUTE 4 (AGO4) and AGO6, which interact with Pol V and trigger the recruitment of the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Zhang *et al.*, 2018). The histone reader SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) and the CLASSY (CLSY) putative chromatin remodelers are needed for Pol IV association to chromatin. The methyl-readers SU(VAR)3-9 homologs 2 (SUVH2) and SUVH9, together with the DDR complex – DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), and RNA-DIRECTED DNA METHYLATION 1 (RDM1) – are required for Pol V recruitment to chromatin. (b) RNAs from different origins, like viruses and Pol II-dependent transcripts, can serve as entry points for the generation of small RNAs to feed into non-canonical RdDM. The examples depicted represent hairpin RNAs, as well as single-stranded RNAs (ssRNAs) converted to dsRNAs by RDR6. These dsRNAs are cut by different DCL proteins into 21–24-nt small RNAs which can be loaded into the AGO4/AGO6 family. Black arrows represent the flow of the pathway. Scissors indicate RNA slicing by AGO proteins. Red ovals represent methylated cytosines. Pol IV and Pol V, which are comprised of multiple subunits, are delimited by a blue and brown thick line, respectively.

trigger RdDM-dependent methylation and TGS when targeted to promoter regions (Guo *et al.*, 2016). One of these tools, known as Virus-Induced Gene Silencing (VIGS) technology, relies on the production of siRNAs after infection of plants with modified viruses that incorporate the target sequence (Fig. 2a,b). A comprehensive study dissected the genetic components needed for VIGS-induced methylation and TGS, and showed that different sizes of virus-derived siRNAs are able to trigger Pol V- and DRM2-dependent heritable DNA methylation, while Pol IV function is dispensable (Bond & Baulcombe, 2015). The Vaucheret lab recently reported similar conclusions when characterizing PTGS-triggered methylation of a transgene (Taochy *et al.*, 2019). Thus, these results are consistent with non-canonical RdDM pathways, which use different entry points independent of Pol IV activity, such as viruses and Pol II transcripts, as sources for siRNAs, and which depend on the downstream RdDM components Pol V and DRM2 (Cuerda-Gil & Slotkin, 2016). Inverted repeat (IR) transgenes are another important source of siRNAs; they produce hairpin RNAs containing the target sequence that are processed into mostly 21-, but also 22- and 24-nt siRNAs (Guo *et al.*, 2016) (Fig. 2a,b). IR transgenes have been successfully employed to methylate and silence transgenes, and a few endogenous genes (Guo *et al.*, 2016), as well as to engineer methylated reporters used in genetic screenings to identify factors involved in methylation and gene silencing (Eun *et al.*, 2012). A recent study reported new insights into the speed by which an IR construct triggered TGS. By using an inducible IR construct, they observed targeted methylation and significant silencing of a reporter transgene just a few hours after the accumulation of IR-derived siRNAs (Pribylova *et al.*, 2019). Another recent study employed IR constructs to target methylation to distal enhancers of the flowering master regulator *FLOWERING LOCUS T* (*FT*) and trigger its down-regulation (Zicola *et al.*, 2019). IR transgenes have been also used to induce gene expression. This is the case for targeted methylation of the *ROS1* promoter, which was instrumental in confirming the positive effect of methylation on *ROS1* expression (Williams *et al.*, 2015). Hence, these recent studies advance our understanding of how siRNA-producing tools trigger methylation and present more examples of their use in manipulating gene expression.

Targeted methylation using programmable DNA-binding proteins

Different programmable DNA-binding platforms, such as artificial zinc finger proteins (ZFs), TAL effectors (TALEs) and CRISPR-dCas9, have been used in mammals to recruit components of the DNA methylation machinery directly to target loci and manipulate their methylation levels (Li *et al.*, 2007; Siddique *et al.*, 2013; Yamazaki *et al.*, 2017; Lei *et al.*, 2018; Lin *et al.*, 2018) (Table 1). In plants, the first example utilized the RdDM factor SUVH9 fused to a ZF directed against the *FLOWERING WAGENINGEN* (*FWA*) promoter, which targeted heritable methylation and *FWA* silencing (Johnson *et al.*, 2014) (Fig. 2c,d). A recent study tested the ability of other RdDM components to target methylation when fused to the same ZF and studied the genetic components required for this process (Gallego-Bartolome *et al.*, 2019) (Table 1). The ZF-induced recruitment of Pol V was able to trigger AGO- and DRM2-dependent methylation even in the absence of complementary siRNAs. These results suggest that physical interaction between Pol V and AGO, and downstream recruitment of DRM2,

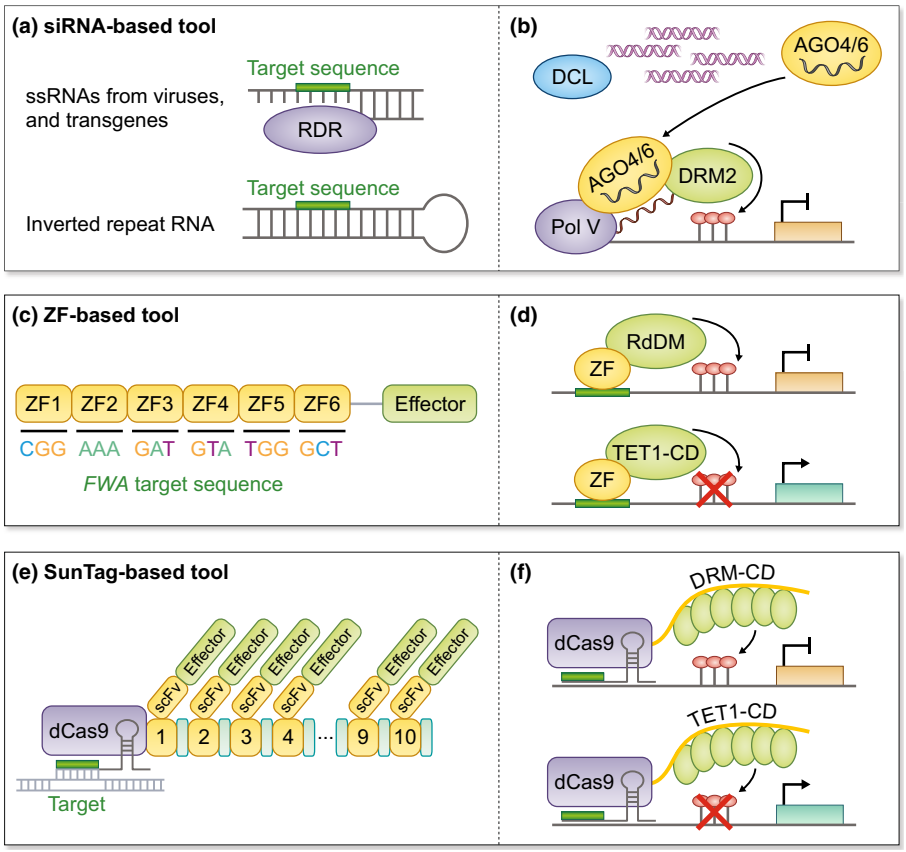

Fig. 2 Tools for targeted manipulation of DNA methylation in plants. (a) Double-stranded RNAs (dsRNAs) from different origins have been used to generate target-specific short-interfering RNAs (siRNAs) – for example, single-stranded RNAs (ssRNAs) from modified viruses and transgenes that are converted into dsRNAs by RNA-DEPENDENT RNA POLYMERASES (RDR), or hairpin dsRNAs, which can be directly generated from Inverted Repeat constructs. In every case, the target sequence is included in these RNAs (represented by thick green lines). (b) These dsRNAs are cut by DICER-LIKE 4 (DCL4), DCL2 and DCL3 into 21-, 22- and 24-nt siRNAs, respectively, which can be incorporated into ARGONAUTE 4 and/or 6 (AGO4/AGO6) and target RNA polymerase V (POL V)-dependent and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)-dependent methylation and transcriptional gene silencing (TGS) when directed against regulatory regions. (c) Representation of a translational fusion between an effector protein and an artificial zinc finger protein (ZF) designed to bind to the *FLOWERING WAGENINGEN* (*FWA*) promoter. This ZF, in particular, contains six different artificial zinc finger domains, each of them binding to a target DNA triplet. (d) Different RNA-directed DNA methylation (RdDM) components and the catalytic domain of TEN-ELEVEN TRANSLOCATION 1 (TET1-CD) can be fused to a ZF to target locus-specific methylation and demethylation, respectively. (e) Representation of a CRISPR SunTag system. A modified deactivated Cas9 (dCas9) protein with a C-terminal tail containing 10 GCN4 repeats (yellow), separated by linker regions (blue), is able to recruit up to ten single-chain variable fragment (scFv) GCN4 antibody-effector fusion proteins. dCas9-bound guide RNA (gRNA) will direct this complex to the target locus. The gRNA is shown as a grey hairpin finished with a thick green line which represents the spacer sequence that will pair with the target sequence. (f) CRISPR SunTag systems can be used together with the catalytic domain of tobacco DRM (DRM-CD) and TET1-CD to manipulate DNA methylation in plants. In (b, d, f), red ovals represent methylated cytosines.

may be sufficient to initiate methylation independent of siRNAs, at least under this artificial situation. While individual components of the Pol IV and Pol V arms of the pathway were able to target methylation, co-targeting of components from both arms greatly enhanced the efficiency of targeted methylation. Moreover, a methylation analysis after segregation of the trigger construct highlighted the importance of CG-specific targeted methylation for the efficient inheritance of this mark. Another recent study used the CRISPR SunTag system (Tanenbaum *et al.*, 2014) for targeted methylation in plants (Papikian *et al.*, 2019). SunTag consists of a three-component system that allows simultaneous recruitment of multiple effector copies to one target locus (Fig. 2e,f). This requires the addition of a C-terminal tail to dCas9 that includes tandem GCN4 peptide repeats. The modified dCas9 is co-expressed with one or more guide RNAs and a chimeric protein that contains the effector protein fused to a single-chain variable fragment (scFv) GCN4 antibody. Papikian et al. showed that a SunTag system recruiting the catalytic domain of tobacco DRM (DRM-CD) (Zhong *et al.*, 2014) could target heritable methylation and stable silencing of *FWA*. Importantly, only one major off-target was found by ChIP-seq, revealing the high specificity in target recognition of this tool. However, ectopic CHH methylation was found across the genome, indicating ectopic activity of the scFv-DRM-CD module. The ectopic CHH methylation disappeared after reducing the nuclear accumulation of this module. This tool was also reprogrammed to target specific methylation to the *SUPERMAN* gene (Papikian *et al.*, 2019).

Transcriptional silencing mediated by siRNA-producing tools has been found to be less efficient at promoters of endogenous genes compared to transgene promoters, which correlates with the failure to accumulate repressive chromatin marks in the first case (Guo *et al.*, 2016). Direct targeting of RdDM components through ZFs

Table 1 Summary of tools for targeted manipulation of DNA methylation mediated by programmable DNA-binding proteins in mammals and plants.

Organism	Activity	DNA-binding protein	Effector	Reference
Mammals	Methylation	dCas9, SunTag	DNMT3A	Lei *et al.* (2018)*
		GAL4-DBD, ZF, TALE, dCas9, SunTag	DNMT3A-CD	Li *et al.* (2007); Lei *et al.* (2018)*
		ZF, TALE, dCas9	DNMT3A-CD-DNMT3L	Siddique *et al.* (2013); Lei *et al.* (2018)*
		GAL4-DBD, ZF, dCas9	DNMT3B-CD	Li *et al.* (2007); Lin *et al.* (2018)
		ZF, TALE, dCas9	SssI	Yamazaki *et al.* (2017); Lei *et al.* (2018)*
		dCas9	Split SssI	Lei *et al.* (2018)*
		dCas9	$MQ1^{Q147L}$	Lei *et al.* (2018)*
	Demethylation	RHD, ZF	TDG	Lei *et al.* (2018)*
		ZF, TALE, dCas9, dSaCas9, MS2, SunTag	TET1-CD	Lei *et al.* (2018)*; Josipovic *et al.* (2019)
		Casilio	TET1-CD+GADD45A+NEIL2	Taghbalout *et al.* (2019)
		ZF	TET2-CD	Lei *et al.* (2018)*
		GAL4-DBD	ROS1-CD	Lei *et al.* (2018)*
Plants	Methylation	ZF	SUVH9	Johnson *et al.* (2014)
			SHH1	Gallego-Bartolome *et al.* (2019)
			NRPD1	
			RDR2	
			MORC6	
			MORC1	
			DMS3	
			RDM1	
		ZF, SunTag	DRM-CD	Gallego-Bartolome *et al.* (2019); Papikian *et al.* (2019)
	Demethylation	ZF, SunTag	TET1-CD	Gallego-Bartolome *et al.* (2018)

Examples of different tools employed for targeted manipulation of DNA methylation in mammals and plants. The suffix '-CD' in the effector list means that only the catalytic domain of this effector was used. (*) Due to space limitations, the reference provided for most mammalian tools corresponds to a comprehensive review by Lei *et al.* (2018), rather than the original papers describing the tools. In those cases where a tool was not included in the review by Lei *et al.* (2018), the original reference is provided. GAL4-DBD, GAL4 DNA-binding domain; RHD, Rel-homology domain; Casilio, combination of dCas9 and Pumilio RNA-binding protein; MS2, combination of dCas9 and MS2 RNA-binding protein; dCas9, deactivated Cas9 from *Streptococcus pyogenes*; dSaCas9, deactivated Cas9 from *Staphylococcus aureus*; ZF, artificial zinc finger protein; TALE, transcription activator-like effector; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; DNMT3L, DNA (cytosine-5)-methyltransferase 3-like; DNMT3B, DNA (cytosine-5)-methyltransferase 3B; SssI, bacterial CG-specific methyltransferase; MQ1Q147L, bacterial CG-specific methyltransferase with Q147L mutation; TDG, Thymine-DNA glycosylase; TET1, TEN-ELEVEN TRANSLOCATION 1; GADD45A, Growth Arrest and DNA-Damage-inducible Alpha; NEIL2, Endonuclease 8-like 2; TET2, TEN-ELEVEN TRANSLOCATION 2; ROS1, REPRESSOR OF SILENCING 1; SUVH9, SU(VAR)3-9 homolog 9; SHH1, SAWADEE HOMEODOMAIN HOMOLOG 1; NRPD1, DNA-directed RNA polymerase IV subunit 1 (Pol IV complex catalytic subunit); RDR2, RNA-DEPENDENT RNA POLYMERASE 2; MORC6, MICRORCHIDIA 6; MORC1, MICRORCHIDIA 1; DMS3, DEFECTIVE INMERISTEM SILENCING 3; RDM1, RNA-DIRECTED DNA METHYLATION 1; DRM, tobacco DOMAINS REARRANGED METHYLTRANSFERASE.

and CRISPR might offer the advantage of promoting the co-recruitment of other proteins associated with the RdDM component whose activities could set a more favorable chromatin environment for targeted methylation and silencing. However, a direct comparative study of the effectiveness of these tools over the same target loci has not been performed. In any case, co-targeting of siRNA-producing and Pol V-recruiting tools represents a powerful approach to enhance targeted methylation (Gallego-Bartolome *et al.*, 2019). Importantly, targeting of a methyltransferase (Suntag-DRM-CD) is the most direct way to target methylation. Future studies at different target loci will shed light on the general effectiveness and specificity of this tool.

Targeted demethylation in plants

Different tools have been developed in mammals to target demethylation using ZFs, TALEs and CRISPR-dCas9 platforms (Lei *et al.*, 2018; Josipovic *et al.*, 2019; Taghbalout *et al.*, 2019) (Table 1). Targeted demethylation has been achieved in plants by fusing TET1-CD to both ZF and SunTag systems (Fig. 2d,f) (Gallego-Bartolome *et al.*, 2018). This study reported complete, highly specific, and heritable DNA demethylation at the *FWA* promoter, which caused a strong *FWA* re-activation. It also showed targeted demethylation and reactivation of a heterochromatic TE – *CACTA1* – although contrary to the findings for *FWA*, demethylation was incomplete and re-methylation and re-silencing occurred once the trigger construct was segregated out. Although most tools tested in this study had no genome-wide effect on methylation, one of them caused global hypomethylation reminiscent of TET1-CD overexpression (Ji *et al.*, 2018). This highlights the importance of being selective with which lines to use in order to avoid off-target effects. In summary, these are the first examples of tools that can be used to target locus-specific demethylation in plants, which will complement targeted methylation tools for the further study and exploitation of DNA methylation.

IV. Conclusions

The toolset available to manipulate locus-specific DNA methylation in plants has recently been expanded with the use of programmable DNA-binding proteins fused to RdDM components and TET1. While precise and highly efficient targeted methylation/demethylation can be achieved, a significant amount of empirical investigation is still required for the optimization of these tools in order to reduce off-target effects and ensure targeting efficacy. These tools can be used to gain insight into the direct function of DNA methylation in various processes, such as gene expression, mRNA processing, gene imprinting, TE regulation and chromatin interactions. Targeted changes in methylation can also be exploited to modulate gene expression and create novel epialleles. Furthermore, these tools could be employed to fix undesired methylation changes arising, for instance, from plant regeneration through tissue culture (Stroud *et al.*, 2013). The fact that methylation could be heritable in the absence of the initial trigger construct makes targeted manipulation of this mark an attractive approach to permanently modify a locus. Importantly, the components needed to manipulate methylation can be delivered through the direct application of RNAs and proteins to plant cells (Dubrovina & Kiselev, 2019; Que *et al.*, 2019), thus bypassing the need for genetic transformation and giving access to manipulation of DNA methylation in multiple plant species and crops of agronomic interest.

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