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DNA cytosine methylation in plant development

Meishan Zhang [a, b], Josphert N. Kimatu [a], Kezhang Xu [b], Bao Liu [a, *]

[a] *Key Laboratory of Molecular Epigenetics of MOE and Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China*
[b] *Department of Agronomy, Jilin Agricultural University, Changchun 130118, China*

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Abstract

Cytosine bases of the nuclear genome in higher plants are often extensively methylated. Cytosine methylation has been implicated in the silencing of both transposable elements (TEs) and endogenous genes, and loss of methylation may have severe functional consequences. The recent methylation profiling of the entire *Arabidopsis* genome has provided novel insights into the extent and pattern of cytosine methylation and its relationships with gene activity. In addition, the fresh studies also revealed the more dynamic nature of this epigenetic modification across plant development than previously believed. Cytosine methylation of gene promoter regions usually inhibits transcription, but methylation in coding regions (gene-body methylation) does not generally affect gene expression. Active demethylation (though probably act synergistically with passive loss of methylation) of promoters by the 5-methyl cytosine DNA glycosylase or DEMETER (DME) is required for the uni-parental expression of imprinting genes in endosperm, which is essential for seed viability. The opinion that cytosine methylation is indispensible for normal plant development has been reinforced by using single or combinations of diverse loss-of-function mutants for DNA methyltransferases, DNA glycosylases, components involved in siRNA biogenesis and chromatin remodeling factors. Patterns of cytosine methylation in plants are usually faithfully maintained across organismal generations by the concerted action of epigenetic inheritance and progressive correction of strayed patterns. However, some variant methylation patterns may escape from being corrected and hence produce novel epialleles in the affected somatic cells. This, coupled with the unique property of plants to produce germline cells late during development, may enable the newly acquired epialleles to be inherited to future generations, which if visible to selection may contribute to adaptation and evolution.

Keywords: DNA cytosine methylation; alteration; DNA methyltransferase; DNA glycosylase; chromatin structure; imprinting; plant development

Introduction

The intricate and precise regulation of gene expression in space and time is fundamental for normal development in all organisms. The spatial and temporal orchestration of gene expression trajectories is primarily controlled genetically by specific DNA sequences including *cis*- and *trans*-acting elements. However, increasing evidence suggests that many aspects of development also involve epigenetic regulations. That is, the mitotically and/or meiotically inheritable yet reversible changes in gene expression without a change in DNA sequence are intimately associated with plant development (Steimer et al., 2004). Cytosine-5 methylation is a prominent epigenetic modification, which is established and maintained by multiple, interacting cellular machineries (Chan et al., 2005; Gehring and Henikoff, 2007).

The primary role of cytosine methylation in higher eukaryotes was proposed as a genome defense system to

* Corresponding author. Tel & Fax: +86-431-8509 9822.
E-mail address: baoliu@nenu.edu.cn

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protect genomes against both endogenous selfish DNA elements (predominantly transposable elements or TEs) and exogenous virus invasions (Yoder et al., 1997; Zilberman, 2008). Indeed, TEs (including both DNA transposons and retrotransposons) are usually heavily methylated and loss of methylation at these elements may lead to their transcriptional activation and even transpositional mobilization (Miura et al., 2001; Chan et al., 2005) In addition to its role in protecting genome integrity, cytosine methylation has also been implicated in regulating gene expression across plant development and in times of stress (Bird, 2002; Zhang et al., 2006; Zilberman et al., 2007). Mounting evidence indicates that cytosine methylation plays critical roles in regulating gene expression in a tissue-specific or developmental stage-dependent manner across plant development (Chan et al., 2005; Gehring and Henikoff, 2007). For example, two well-studied but biologically unrelated phenomena, vernalization and genomic imprinting, are both found to depend on intricate epigenetic regulations at the transcriptional level during plant development. Mechanistically, methylated cytosines are capable of attracting methyl-binding proteins which in turn recruit histone deacetylases and chromatin remodeling proteins to form a complex which hinder the binding by transcription factors (Fransz and de Jong, 2002).

This short review is intended to discuss several fundamental aspects of DNA cytosine methylation in plant with emphasis on the essential roles of this epigenetic marker on plant development. Thus, issues regarding genomic distribution of cytosine methylation, establishment and maintenance of cytosine methylation, as well as the role of methylation dynamics across development by orchestrating gene transcription will be discussed. The extent to which changes in cytosine methylation are actually an integral part of the plant life cycle will also be briefly addressed.

Genomic distribution of cytosine methylation in plants

The level of methylation modification ranges from 3% to 8% of cytosines in vertebrates and from 6% to 30% in plants (Chen and Li, 2004). Cytosine methylation (^{5m}C) in a symmetrical CG dinucleotides context is an evolutionarily conserved DNA modification that is found in diverse organisms including vertebrates, plants and some fungi (Bird, 2002). A striking difference in the cytosine methylation patterns in plants from those in animals is that although methylation is predominantly occurring at the CG dinucleotides in plants, it is not confined to these sites; instead, methylation also occurs at CHG (where H is A, C or T) and asymmetric CHH (where H is A, C or T) sites (Chan et al., 2005). Each of the methylation patterns in plants requires distinct as well as overlapping cellular enzymes for establishment, maintenance, removal, restoration and perpetuation (Finnegan and Kovac, 2000; Chan et al., 2005; Zhu, 2008).

In the model plant *Arabidopsis thaliana*, most of the methylated fraction of the genome is composed of localized tandem or inverted repeats, transposons and also dispersed repeats that are often most abundant within or around the centromeric regions. Therefore, the cytosine methylation landscape of plants is of a mosaic nature, which is in contrast to that of mammalian animals, which largely exhibits a globally continuous pattern. The distribution of cytosine methylation within transposons and genes in plants is distinct: whereas transposons are usually heavily methylated along their entire length, methylation within genes is often distributed away from the 3′ and 5′ ends (Gehring and Henikoff, 2007). In the last two years, high-throughput methodologies have been developed to the point that genome-wide mapping of cytosine methylation landscapes has become both feasible and with sufficiently high-resolution (Zhu, 2008). Several recent studies have mapped the distribution of cytosine methylation in the entire genome (i.e., construction of methylome) of *Arabidopsis*, and the latest using high-throughput sequencing of bisulfite-converted DNA to achieve single base pair resolution (Zhang et al., 2006; Vaughn et al., 2007; Zilberman et al., 2007; Cokus et al., 2008; Lister et al., 2008). One of the unexpected findings from these studies is that the bodies of active genes are also specifically targeted for cytosine methylation in plants. In addition, some regions were found to be highly methylated (up to 80%), such as transcriptionally inactive heterochromatic regions including centromeres, pericentromeres, and the heterochromatic knob on chromosome 4 of *Arabidopsis*. These regions are densely packed with TEs and other repetitive sequences. Euchromatic regions, which include genes and non-repetitive intergenic regions, show lower but still significant levels of cytosine methylation. In these regions, pseudogenes and non-expressed genes show higher levels of methylation than actively expressing genes,

in line with the prevalent view that cytosine methylation associates with transcriptional silencing. Of all expressed genes, *ca.* 5% are methylated in the promoters and 33% are methylated in the transcribed regions (so-called "body-methylated genes"). Cytosine methylation in these cases is clearly biased away from gene ends, such that neither the 5′ nor the 3′ ends of transcription units are methylated. These studies also revealed that the overall genome-wide levels of CG, CHG and CHH methylation are 24%, 6.7% and 1.7%, respectively, in *Arabidopsis thaliana* (Cokus et al., 2008). Although CG, CHG and CHH methylations are highly correlated, showing enrichment in repeat-rich pericentromeric regions, as mentioned above, a marked deviation was found within gene bodies, which contained almost exclusively CG methylation (Cokus et al., 2008). A recent microarray study has uncovered new sites of CG methylation that reside predominantly at the 3′ end of genes; nonetheless, like gene-body methylation in general, the genesis and biological function of methylation in these regions are also largely unknown (Tran et al., 2005). Such detailed cytosine methylomes remain to be established for other plants. However, a recent study in two chromosomes (1 and 4) of rice (Li et al., 2008) has produced strikingly similar methylation patterns as in *Arabidopsis*.

The cytosine methylation landscape of plants with large genomes such as maize is yet to be fully established. However, structural genomic studies have shown that maize genes are often separated by long tracts of DNA segments that contain TEs and their relics; wherein genes tend to be unmethylated, and TEs heavily methylated (Rabinowicz et al., 2003). This suggests that there might also exist sharp differences between the methylation patterns of genes and those of TEs in maize, as was indeed documented recently by a tilling array epigenomic analysis in maize (Wang et al., 2009).

Establishment and maintenance of cytosine methylation in plants

Cytosine methylation in higher plants is regulated by two distinct yet complementary enzymatic activities known as "*de novo*" and "maintenance" DNA methyltransferases (MTases), together with DNA demethylases, histone-modifying enzymes, chromatin remodeling factors, and the RNA interference (RNAi) machinery. The *de novo* methylation is a process whereby previously unmethylated cytosine residues are methylated, resulting in the formation of newly methylated patterns. Maintenance methylation is the process by which the preexisting methylation patterns are maintained after DNA replication (Chen and Li, 2004). The plant DNA MTases that have been identified so far are classified into four main families (MET1, CMT, DRM and Dnmt2) based on their linear domain arrangements (Table 1). In *Arabidopsis*, establishing methylation in all sequence contexts is entirely dependent on the *de novo* methyltransferase, DOMAINS REARRANGED METHYLTRANSFERASE (DRM) (similar to the mammalian Dnmt3 family) activity (Cao et al, 2000), which functions through the RNA-directed DNA methylation (RdDM) pathway (Cao and Jacobsen, 2002b) (Table 1). The *Arabidopsis* MET1, which is a homologue of the Dnmt1 that is responsible for maintenance of CG methylation in mammals, is the most extensively studied plant DNA MTase (Table 1). In addition to its canonical role of controlling CG methylation, MET1 also likely plays a part in the maintenance of non-CG methylation (Finnegan et al., 2000; Lindroth et al., 2001). In *Arabidopsis* mutant that lacks MET1 activity, nearly 60% of the methylated regions became demethylated, and which was accompanied by transcriptional activation of TEs and pseudogenes residing in heterochromatic regions (Zhang et al., 2006). These data support the conclusion that MET1-mediated cytosine methylation is mainly responsible for the silencing of heterochromatic regions of the plant genome (Suzuki and Bird, 2008). In addition to MET1, CG methylation is also controlled by VARIANT IN METHYLATION 1 (VIM1) (Woo et al., 2008), DECREASE IN DNA METHYLATION 1 (DDM1), and HDA6 histone-deacetylase activity (Chan et al., 2005). CHG methylation is maintained by the plant-specific CHROMOMETHYLASE 3 (CMT3), and KRYPTONITE (SUVH4), a histone methyltransferase, and at some loci, is redundantly controlled by CMT3 and DRM2 (Cao and Jacobsen, 2002a). The function of CMT3 is likely conserved in other plants. For example, the maize CMT homolog ZMET2 and ZMET5 were shown to play important role in the maintenance of CHG methylation (Pavlopoulou and Kossida, 2007) (Table 1). As far as the DNA methyltransferase homologue 2 (Dnmt2) family is concerned, its role in cytosine methylation remains largely un-elucidated (Pavlopoulou and Kossida, 2007) (Table 1).

Increasing recent evidence is pointing to a crucial role played by siRNA in cytosine methylation. In plants, the

overwhelming majority of small RNAs are 24 nt siRNAs corresponding to TEs and other repetitive elements (Zhang et al., 2007b). The 24 nt siRNAs cause epigenetic silencing by directing *de novo* cytosine methylation through the RdDM pathway (Chan et al., 2005), wherein the siRNAs are generated by the action of the putative DNA-directed RNA polymerase Pol IV, RDR2 (RNA-dependent RNA polymerase 2), and DCL3 (Dicer-like 3) (Matzke et al., 2009). The siRNAs are then loaded into AGO4 and AGO6 to direct cytosine methylation by the *de novo* DNA MTase DRM2 (Matzke et al., 2009). The functioning of the siRNAs also requires another putative DNA-dependent RNA polymerase, Pol V, and the chromatin remodeling protein, DRD1 (Matzke et al., 2009) (Fig. 1). A recent study suggested that a RdDM effector, KOW DOMAIN TRANSCRIPTION FACTOR1 (KTF1), acts as an adaptor protein that binds scaffold transcripts generated by Pol V and recruits AGO4 and AGO4-bound siRNAs to form an RdDM effector complex (He et al., 2009). The *de novo* MTase DRM1/DRM2 which is presumably in the RdDM effector complex, is responsible for catalyzing cytosine methylation in all sequence contexts, CG, CHG, and CHH (Cao and Jacobsen, 2002b; Cao et al, 2003) (Fig. 1).

Two histone modifying enzymes also acting in the RdDM pathway have been identified in forward genetic screens (Huettel et al., 2007). HDA6 is a histone deacetylase that reinforces CG methylation induced by siRNA targeting. SUVH4/KRYPTONITE (KYP) is a histone H3K9 methyltransferase that stabilizes cytosine methylation, particularly in CHG trinucleotides. Although SUVH4/KYP is the major H3K9 methyltransferase in *Arabidopsis*, two related histone methyltransferases, SUVH5 and SUVH6, are needed for maintaining non-CG methylation at specific loci (Huettel et al., 2007) (Fig. 1).

In general siRNAs appear to have a fine-tuning mechanism in gene regulation because misexpression of some siRNAs may produce pleiotropic effects on development, though most *Arabidopsis* siRNA pathway mutants appeared phenotypically normal (Palatnik et al., 2003). It is expected that further deep sequencing of siRNAs in different plant tissues and developmental stages will uncover additional important roles played by them.

Table 1
Predicted function of plant DNA methyltransferases (based on Pavlopoulou and Kossida, 2007, with modifications)

Classification	Gene name	Species	Function
MET1	*AtMET1*	*A. thaliana*	Maintenance: CG, probably CHG; single-copy DNA
	NtMET1	*Nicotiana tabacum*	Maintenance
	OsMET1-1	*O. sativa*	Maintenance
	OsMET1-2	*O. sativa*	Maintenance
	ZmMET1	*Zea mays*	Maintenance
CMT	*AtCMT3*	*A. thaliana*	Maintenance: CHG in repetitive DNA and transposons in heterochromatin
	OsCMTL	*O. sativa*	Putative enzyme, function unknown
	OsMET2a	*O. sativa*	Putative enzyme, function unknown
	ZMET2	*Z. mays*	Perhaps maintenance: CHG in transposons
	ZMET5	*Z. mays*	Perhaps maintenance: CHG in transposons
DRM	*AtDRM1*	*A. thaliana*	De novo: CG, CHG, CHH Maintenance: CHG, CHH
	AtDRM2	*A. thaliana*	De novo: CG, CHG, CHH Maintenance: CHG, CHH
	NtDRM1	*N. tabacum*	De novo: CHG, CHH, some CG
	ZMET3	*Z. mays*	Putative enzyme, function unknown
Dnmt2	*AtDnmt2L*	*A. thaliana*	Putative enzyme, RNA methylation
	ZMET4	*Z. mays*	Putative enzyme, function unknown
DNA glycosylase	*DME*	*A. thaliana*	Imprinted genes, *FWA* and *MEA*
	ROS	*A. thaliana*	mCpCpG of silent transgene and homologous endogene

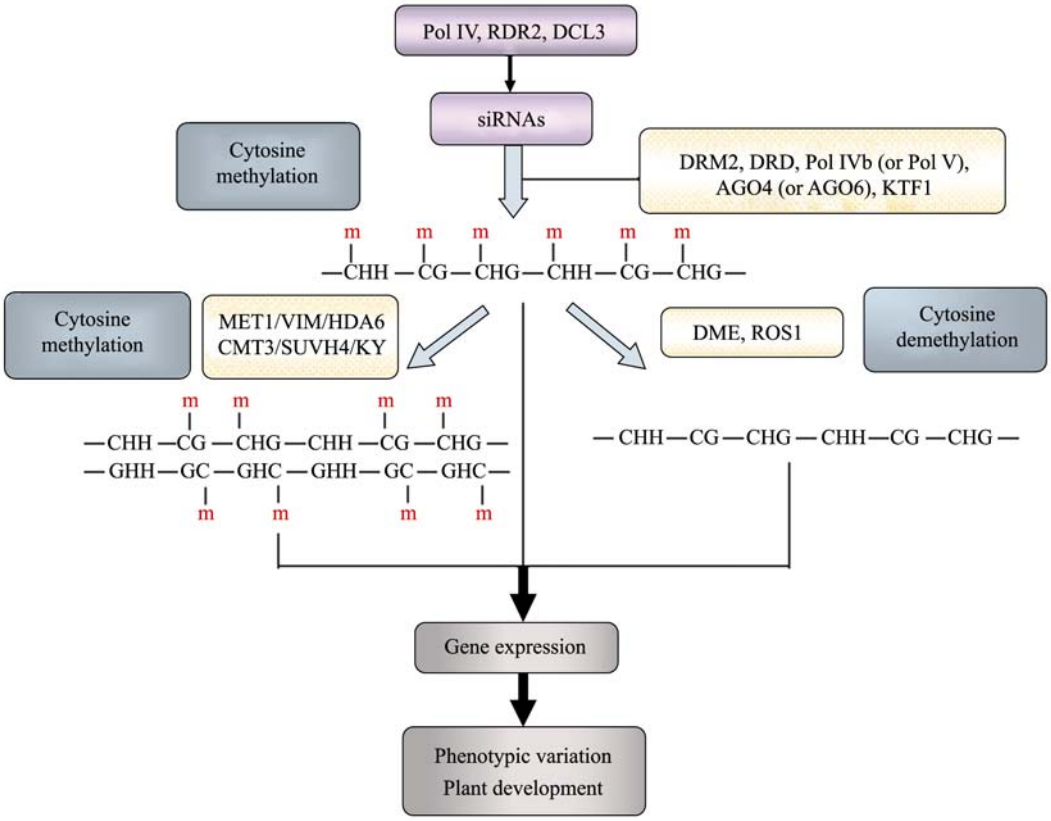

Fig. 1. A diagrammatic illustration of cytosine methylation regulating plant development. The siRNAs are generated by the concerted action of Pol IV, RDR2 and DCL3. The siRNA is then loaded onto AGO4, which interacts with the NRPD1b and form a functional Pol IVb complex. In cooperation with DRM2 and DRD1, the siRNA/AGO4/Pol IVb complex facilitates *de novo* methylation of cytosines in all sequence contexts. CG and CHG methylation can be maintained during DNA replication by MET1 and CMT3, respectively. At some loci, CHG methylation is redundantly controlled by CMT3 and DRM2. Locus-specific histone modifications that are catalyzed by HDA6 and SUVH4/KYP, and variation in methylation 1 (VIM1) and decrease in DNA methylation 1 (DDM1) help to maintain cytosine methylation. Cytosine methylation can be lost in nondividing cells by a base excision repair-type mechanism that involves DNA glycosylase/lyase proteins such as DME and ROS1. Cytosine methylation in promoter region can affect gene expression and hence may contribute to phenotypic variation and plant development.

Cytosine demethylation in plants

Intrinsic genomic cytosine methylation patterns result not only from the establishment and maintenance of methylated cytosines, but also from regulated trimming, i.e., active demethylation (Zhu et al., 2007). Intuitively, the removal of cytosine methylation patterns can be accomplished *via* either passive or active demethylation. In passive demethylation the methylated cytosines are replaced with unmethylated cytosines during DNA replication while in active demethylation the methyl mark is removed by DNA glycosylases (DME and ROS1) that are directed towards methylated cytosines even in the absence of DNA replication (Choi et al., 2002; Gong et al., 2002) (Table 1). DME has a very specific expression pattern, and hence functions, during female gametogenesis (Choi et al., 2002; Gong et al., 2002; Kinoshita et al., 2004). DME has been implicated in the regulation of parental imprinting as only maternal DME activity is required for development of viable seeds, and this reflects its expression specificity in the central cell of the female gametophyte (Choi et al., 2002). Indeed, DME is required in the central cell and endosperm for imprinted expression of the maternal genes *MEDEA* (*MEA*), *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) and *FLOWERING WAGENINGEN* (*FWA*) in *Arabidopsis* (Choi et al., 2002; Kinoshita et al., 2004). *MEA* and *FIS2* encode a polycomb protein that is required for seed viability and is expressed from the maternal allele only (a major regulator of endosperm development). DME works by activating the maternal alleles of the *MEA* gene by de-

methylating its promoter. This results in two active maternal copies and one imprinted paternal copy of the *MEA* gene, preventing over-proliferation of the endosperm (Choi et al., 2002). Methylation of the flowering time gene *FWA* at its tandem repeats silences its expression in vegetative tissues (Soppe et al., 2000). However, *FWA* has maternally imprinted expression in the endosperm during seed development (Kinoshita et al., 2004). This activation is achieved by maternal-specific expression of *DME* in the female gametophyte, accompanied by the demethylation of FWA (Kinoshita et al., 2004). Because endosperm is a terminally differentiating tissue, methylation does not need to be re-established at the imprinted loci in the next generation. This 'one-way' control of imprinting differs fundamentally from the methylation-demethylation cycles involved in mammalian imprinting (Reik and Walter, 1998). At the genetic level, DME functions antagonistically with MET1 in the control of seed development, and both are essential (Xiao et al., 2003).

A recent study demonstrated that DNA demethylation is also achieved during female gametogenesis by the MET1-mediated passive loss of DNA methylation through the Retinoblastoma pathway (Jullien, 2008). During gametogenesis, MET1 expression is repressed by the Retinoblastoma pathway involving RETINOBLASTOMA RELATED1 (RBR1) and its interacting protein MULTICOPY SUPRESSOR OF IRA1 (MSI1) (Jullien, 2008). This repression causes passive removal of DNA methylation in vegetative tissues and on the maternal allele, results in the production of hemi-methylated DNA, and then active demethylation by DME to be followed (Berger and Chaudhury, 2009).

In contrast to DME, ROS1 is expressed near ubiquitously throughout the plant developmental cycle (Gong et al., 2002). The *ros1* mutation induces hypermethylation slightly in CG but substantially in CHG and CHH compared to plants harboring the wild-type ROS1, which is concomitant with transcriptional silencing of transgenes, endogenous genes, and TEs (Gong et al., 2002; Zhu et al., 2007). This suggests that active DNA demethylation is important in pruning the genomic methylation, and even the normally 'silent' TEs are under dynamic control by both methylation and demethylation. This dynamic nature in cytosine methylation might be important in maintaining the epigenomic plasticity as a whole to enable efficient response to developmental cues and environmental stresses in a timely manner (Zhu et al., 2007).

Relationship between cytosine methylation and gene expression

The precise molecular mechanisms of transcriptional control during plant development are not fully understood. Studies have shown that the regulation of gene expression by cytosine methylation can be accomplished by having the gene itself methylated (*cis*) and/or methylating another site in the genome, which regulates the targeted gene yet physically distant from it (*trans*-action) (Chandler and Stam, 2004) (Fig. 1). A study on the methylation cycle and its functions during barley endosperm development showed that the expression of genes coding for the storage protein (prolamin) is repressed by CG hyper-methylation (Radchuk et al., 2005). The ribosomal RNA (rRNA) genes are found in tandem arrays containing hundreds of copies that are stochastically silenced. The silenced subset of rRNA genes is methylated, and treatment with 5-azacytidine, a drug that inhibits DNA methyltransferases, can reverse this silencing (Lawrence et al., 2004). In other cases, cytosine methylation controls the overall level of expression from a family of repeated genes. For example, the pathogen-resistance gene *BALL* (*BAL*) residing in a complex gene cluster is silenced by cytosine methylation (Stokes et al., 2002).

Perhaps the biggest surprise revealed by recent methylome analysis in *Arabidopsis* is the prevalence of cytosine methylation in the bodies of genes (discussed in foregoing sections). The finding that roughly a third of *Arabidopsis* genes are methylated begged answers to the question of what all this methylation might be doing. An automatic thought is that methylation is required for normal transcription. Indeed, that cytosine methylation is found in the transcribed regions of a significant fraction (> 20%) of expressed genes (Zhang et al., 2006; Zilberman et al., 2007) clearly points to a coupling relationship between transcription and cytosine methylation within genes. Thus, body-methylated genes are usually transcribed at moderate to high levels and are transcribed less tissue-specifically relative to unmethylated genes. Gene-body methylation of this kind does not shut off gene expression — the average expression level of the affected genes was significantly higher than that of either promoter-methylated or entirely unmethylated genes (62% of all expressed genes) (Zhang et al., 2006; Zilberman et al., 2007). In plants, methylation of promoter regions usually inhibits transcription, but methylation in coding regions usually either does not affect

the gene expression, or only has a moderate effect (Stam et al., 1998; Zilberman et al., 2007). Nevertheless, there are genes that do not abide to this rule, for example in the cases of *SUPERMAN* (*SUP*) and *AGAMOUS* (*AG*), cytosine methylation in the transcribed portion of the gene causes significant transcriptional down-regulation probably because there are important controlling elements in these regions (Jacobsen and Meyerowits, 1997; Sieburth and Meyerowitz, 1997; Ito et al., 2003). Interestingly, erasure of gene-body CG methylation in the *met1* mutant seems to trigger stochastic redistribution of histone modifications (e.g., H3K9me2) and hyper-CHG and CHH methylation in the transcribed region of the genes, at least some of which may result in ectopic gene silencing (Henderson and Jacobsen, 2007). There is evidence showing that intragenic methylation can influence transcription (Chawla et al., 2007). By an analogy with a yeast pathway that prevents aberrant initiation from gene bodies by ensuring proper chromatin assembly following passage of RNA polymerase (Carrozza et al., 2005), it was suggested that methylation of gene bodies might repress inappropriate transcriptional initiation and reduce transcriptional noise (Tran et al., 2005; Zilberman et al., 2007). A recent study on a *Petunia* floral gene, *pMADS3*, which is specifically expressed in the stamen and carpels of developing flowers, showed that a specific CG methylation mediated by the RdDM pathway is strongly correlated with up-regulation of the gene transcription, thus uncovered a novel regulatory mechanism by cytosine methylation in controlling gene expression (Shibuya et al., 2009).

It has been established that methylated cytosines can interact with methyl-binding domain containing proteins (MBD) which interact with various chromatin modifiers to form protein complexes (Yaish et al., 2009). Thus far, 12 putative MBD genes (*AtMBD1-AtMBD12*) have been identified in the *Arabidopsis* genome, and at least six (*AtMBD1*, *2*, *4*, *5*, *6 and 7*) of these exhibited specific binding capacity for methylated CG sequence *in vitro* (Berg et al., 2003; Springer and Kaeppler, 2005). These proteins did not show DNA demethylase activity; however, AtMBD6 showed histone deacetylation activity when a plant extract was treated with the recombinant protein (Zemach and Grafi, 2003). In *Arabidopsis*, the DECREASE IN CYTOSINE METHYLATION 1 (DDM1) protein co-localized *in vivo* and bound *in vitro* to AtMBD5–7 proteins (Zemach et al., 2005). AtMBD9 controls gene expression *via* modifying chromatin structure by acetylating histones and by decreasing the global methylation level of the DNA; loss of function of this gene led to altered expression of a number of genes related to the flowering and axillary branching pathways (Yaish et al., 2009).

Taken together, it appears that there is currently still no evidence for a direct mechanistic connection between cytosine methylation and the transcription process (Suzuki and Bird, 2008). It is likely that methylation does not intervene to silence genes that are actively transcribed, but often affects genes that have already been repressively controlled by other means (Bird, 2002). One reason why cytosine methylation does not seem to have a widespread direct role in regulating the expression of developmental genes in plants might lie in that cytosine methylation changes are often meiotically heritable in plants and does not undergo a "erasure-and-reset" cycle between generations (Jacobsen and Meyerowitz, 1997). Regulation of gene expression is complex, and the emerging evidence hints that the roles of cytosine methylation are equally diverse and likely individualized for different genes. Therefore, it may be unrealistic to expect that any unified theory will encompass all the biological consequences of cytosine methylation, but its essential role in plant development is a major one, as will be further discussed below.

Importance of cytosine methylation in plant development

Although the role of cytosine methylation as a genome defense system to maintain genome integrity is well established, it is still largely unclear how cytosine methylation is involved in the control of developmental patterning in plants. Nonetheless, substantial progress has been made in this area by using various loss-of-function methylation mutants. The first implication that cytosine methylation is important for plant development was obtained from a study using antisense *MET1 Arabidopsis* plants, which showed a number of striking phenotypes, including reduced apical dominance, alterations in flowering time, extensive floral abnormalities, and curled leaves (Finnegan et al., 1996). Similarly, tobacco antisense *MET1* plants also displayed similar phenotypes (Nakano et al., 2000), suggesting that cytosine methylation has pleiotropic effects and can affect many genes involved in plant development. This was further supported by *MET1* missense mutations

(*met1-1* and *met1-2*) which manifested delayed flowering and loss of gene silencing (Kankel et al., 2003). Both *met1-1* and *met1-2* are considered to be partial loss-of function alleles because point mutations that replace non-conserved amino acids within the MET1 catalytic domain reduce CG methylation only to 25%–50%, along with some loss of non-CG methylation (Kankel et al., 2003). However, Lister et al. (Lister et al., 2008) showed that loss of genic cytosine methylation in *met1* mutant plants is accompanied by hypermethylation of CHG sites. Recently, it was shown by using a different *met1* null allele (*met1-6*) that MET1 is essential for embryogenesis and the formation of viable seeds (Xiao et al., 2006). Self-pollinated *met1-6* homozygous plants generated siliques containing an increased number of aborted seeds compared with wild type and approximately 30% of abnormal embryos, together with the deregulation of transcription of genes regulating cell identity during early embryogenesis. Self-pollination of heterozygous *met1-6/MET1* plants and their reciprocal crosses with wild-type demonstrated that loss of CG methylation during female and male gametogenesis impairs embryogenesis and seed viability (Xiao et al., 2006).

As discussed in previous sections, methylation at non-CG sites, which is a common modification in plant DNA, is catalyzed by DRM and a chromodomain-containing plant-specific methyltransferase, CMT3 (Lindroth et al., 2001). It has been proposed that DRM and CMT3 act in a partially redundant and locus-specific manner to control non-CG methylations (Cao and Jacobsen, 2002a). Such functional redundancy was revealed by the examination of a triple *drm1/drm2/cmt3* mutant that exhibited pleiotropic phenotypes including developmental retardation, reduced plant size and partial sterility (Cao and Jacobsen, 2002a). In contrast to *met1* mutants, mutations in either DRMs or CMT3 have no apparent phenotypes, though the release of gene silencing at selected loci has been observed (Lindroth et al., 2001; Cao and Jacobsen, 2002b).

The *DDM1* gene encodes a protein related to SWI2/SNF2-like chromatin-remodeling ATPase. Similar abnormalities in inbred *ddm1* mutants as in *met1* mutations (Kakutani et al., 1996) further suggested that cytosine methylation is required for normal plant development, though unlike mice with knockout mutations in the maintenance MTase, these mutant plants were viable. However, recent work demonstrates that loss of both CG and non-CG methylation can render plants inviable in a single generation (Xiao et al., 2006), suggesting that CG methylation and non-CG methylation functionally overlap each other. The multiple abnormalities observed in methylation mutants from the earliest stages of embryogenesis through reproduction make the theory that cytosine methylation is directly required for proper plant development as nearly established. However, as cautioned by Gonzalo et al. (2006), the possibility of genomic instability resulted from decreased cytosine methylation as a cause for developmental defects needs to be tested, as this would points to an indirect role of DNA methylation for normal plant development.

Cytosine methylation dynamics during plant development

In mammals, the epigenetic status is systematically reconstructed in every individual during development, featuring erasure and reestablishment of epigenetic markers, and rendering cytosine methylation patterns to be cell-type specific (Reik et al., 2001). In plants, however, there has been no clear evidence of resetting of the epigenetic status across development, except a significant reduction in methylated cytosines in the endosperm (Lauria et al., 2004; Zhang et al., 2007a; Gehring et al., 2009; Hsieh et al., 2009). However, endosperm is terminally differentiated and hence inconsequential to heredity. Instead, the methylated patterns in several plants studied showed largely stable inheritance of cytosine methylation level and patterns over multiple generations (Zhang et al., 2007a; Zhao et al., 2007). This is also consistent with the basis for genomic imprinting in plants, which is mainly accomplished by active removal of methylated cytosines by the DNA glycosylases (Choi et al., 2002; Gong et al., 2002; Zhu et al., 2007), rather than through genome-wide erasure and followed by targeted *de novo* methylation of the paternal allele at the imprinted loci in mammals. However, a recent study by Teixeira et al. (2009) showed that cytosine methylation and gene silencing can be much more dynamic throughout plant development than previously believed. Cytosine methylation that is lost in previous generations due to mutation of a gene required for methylation can be restored in subsequent generations when the pertinent wild-type gene is reintroduced (Teixeira et al., 2009). Notably, not all hypomethylated sequences can be re-

methylaed, and in fact remethylation is restricted to loci that produce siRNAs, and depends on the RdDM pathway, leading to the important conclusion that siRNAs can selectively correct methylation defects to reinforce silencing (Teixeira et al., 2009).

Tissue-specific, differentially methylated regions (TDMs) have been identified and implicated for their indispensable involvement in mammalian development and tissue/organ differentiation (Song et al., 2005). It was estimated that 5% or more of the CpG islands in mammals are TDMs, challenging the general notion that all CpG islands are unmethylated (Song et al., 2005). TDMs are conserved between mouse and human and suggest that cytosine methylation may have played a fundamental role in regulating differentiation and tissue-/cell-specific gene expression (Kitamura et al., 2007; Igarashi et al., 2008). Indeed, studies in different human tissues indicate that tissue-specific DNA hypomethylation correlates significantly with tissue-specific transcription (Schilling and Rehli, 2007). Similar systemic investigations in plants are not yet available, but some studies have compared the overall methylation levels in different plant tissues and observed differences. For example, an earlier study measured cytosine methylation levels by HPLC on immature tomato tissues like stems, leaves, and roots, and revealed the presence of less methylation than in mature leaves like fruits and seeds (Messeguer et al., 1991). This is consistent with a more recent study showing that methylation levels in plants change during plant development with the highest methylation level being observed in senescence tissues. It was speculated that this changing trend in cytosine methylation probably functions to prevent premature DNA degradation during senescence (Brown et al., 2008). Additionally, by using the MSAP (methylation-sensitive amplification polymorphism) analysis genomic regions that are differentially methylated among several organs including cotyledons, leaves, and flowers were identified in *Arabidopsis* (Ruiz-Garcia et al., 2005). Similarly, differentially methylated bands were also found between rice seedlings and adult plants (Xiong et al., 1999; Sha et al., 2005) and among different tissues of maize (Lu et al., 2008).

In concordance with the dynamic nature of cytosine methylation in different plant tissues/organs and developmental stages, the expression of the MTase-encoding genes is also dynamic during plant development (Yamauchi et al., 2008). For example, Dai et al. (2005) investigated the expression of five genes encoding putative wheat DNA MTases (TaMET1, TaMET2a, TaMET2b, TaCMT and TaMET3), and found that although they all were expressed in developing seeds, germinating seeds and various vegetative tissues, the transcript abundance varied significantly (Dai et al., 2005). However, this does not imply that the differential spatial-temporal expressions of the DNA MTase genes are intrinsically related to the changes of DNA methylation during plant development. Indeed, a recent study on the global cytosine methylation status of rice genome during development and in various tissue cultures showed that cytosine methylation levels are not directly correlated with the DNA MTase activity (Teerawanichpan et al., 2009). It is more probably that DNA MTase activity and methylation levels across different tissues/organs and developmental stages not directly correlated, and methylation levels are consequences of a balance among DNA replication, *de novo*/maintenance DNA methylation and demethylation (Hsieh, 2000).

Conclusions and perspective

As a prominent epigenetic maker, DNA cytosine methylation is an integral part of the epigenetic controlling network to regulate gene expression trajectories, and hence likely plays essential roles in tuning tissue-, organ- and developmental stage-specific gene expression across plant development. To enable this dynamic control of gene expression across plant development, cytosine methylation itself needs to be dynamic, and which is likely materialized by the coordinated expression and hence function of the various kinds of DNA MTases, DNA glycosylases and chromatin remodeling factors. On the other hand, transgenerational fidelity of this epigenetic marker is equally crucial to ensure long-term genomic and epigenomic stability from an evolutionary perspective, and for which siRNAs appear to play an indispensable role by timely correcting any compromised methylation patterns through the RdDM pathway. Recent advances in whole genome methylation profiling technologies hold a great promise to fundamentally illuminate the functions of cytosine methylation in plant development. Thus, these technological improvements, in particular advanced sequencing technologies and computational analyses, have the potential of unlocking the complexity of epigenetic modifications just as profoundly as whole genome sequencing has impacted genetics. As more plant methylomes and transcriptomes

become available, it shall be a major priority for future research to elucidate the molecular and cellular control of cytosine methylation dynamics and inheritance, and to relate the contributions of this epigenetic modification to gene expression as well as its role in controlling plant development.

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