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Shaping inheritance: How distinct reproductive strategies influence DNA methylation memory in plants

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Abstract

DNA methylation is a major epigenetic mark involved in the silencing of genes and transposable elements (TEs). DNA methylation varies significantly across the plant life cycle, but is efficiently reinforced during reproduction, ensuring stable silencing of TEs. Plants are remarkably flexible in their mode of reproduction and numerous species including crops can propagate asexually, skipping one or more of these critical reinforcement steps. In this review we summarize recent advances in the characterization of DNA methylation inheritance in sexual and asexual plants. We argue that because most epigenetic reinforcement appears to occur during seed formation, methylomes of asexual seeds should resemble that of their sexual counterparts. Conversely, clonally propagated plants are expected to be hypomethylated and undergo frequent stochastic epigenetic changes. Last, we provide insights on how the use of non-model organisms will advance our understanding of epigenetic inheritance in plants.

Highlights

- Most DNA methylation reinforcement takes place during embryogenesis
- Methylome of apomictic and sexual seeds are expected to resemble
- Clonally propagated plants likely lose DNA methylation across generations
- Research on non-model organisms are key to understand epigenetic inheritance

Introduction

DNA methylation is a major epigenetic mark found in higher eukaryotes, including plants and mammals, that is involved in the silencing of genes and transposable elements (TEs). Heritable changes of DNA methylation, known as epialleles, can have phenotypic impacts, opening the interesting possibility that epialleles may underlie at least part of the phenotypic diversity observed in natural plant population [1]. Although very rare in mammals, phenotypically-relevant epiallelic variation has been recurrently observed in plants, likely because DNA methylation is not fully reset across generations [2].

In turn, plants undergo a DNA methylation reinforcement during sexual reproduction, a mechanism that plays an important role in the correction of accidental epigenetic defects. Although sexual reproduction is predominant in model plants species, such as *Arabidopsis*, rice, maize, and tomato, transitions to asexual reproduction has occurred multiple times during the diversification of flowering plants [3]. Indeed, several major crops, such as potato, citrus, strawberry, and grape vine, typically propagate asexually, providing unique opportunities to investigate how distinct reproductive strategies shape the extent and mode of epigenetic inheritance. In this review we will summarize recent advances in the characterization of the mechanisms controlling DNA methylation dynamics in sexual and asexual organisms, as well as propose plausible dynamics in asexually-propagated species. Last, we provide insights on how the use of non-model organisms has the potential to significantly advance our understanding of epigenetic inheritance in plants.

Establishment and maintenance of DNA methylation

Plant DNA methylation can occur in any possible sequence context: CG, CHG and CHH (where H is any nucleotide but G), which are faithfully maintained across cell divisions by the activity of context-specific DNA methyltransferases. In the model plant *Arabidopsis thaliana*, methylation at the symmetrical CG sites is maintained by the DNA methyltransferase MET1 during DNA replication. Indeed, symmetric CG methylation is lost during DNA replication but rapidly restored in late S/G2 phase [4]. Conversely, maintenance of non-CG methylation relies on a self-reinforcing loop between histone tail modifications and DNA methylation. Methylation of CHG and CHH sites is respectively catalyzed by the enzyme Chromomethylase 3 (CMT3) and Chromomethylase 2 (CMT2), which target DNA by recognizing the dimethylation of Lysine 3 of histone H3 tail (H3K9). In turn, methylation of CHG and CHH sites is recognized by a family of histone methyltransferases (e.g. SUVH4, 5, and 6) that catalyze H3K9 methylation [5]. This self-reinforcing loop has been proposed to delay the deposition of CHG methylation, which seems to take place during G2 or even later [4].

In addition to these DNA methylation maintenance pathways, plants can also methylate DNA *de novo* guided by small interfering RNAs (siRNAs) in a so-called RNA-directed DNA methylation (RdDM) pathway [5]. This pathway consists in the production of short non-coding RNAs from heterochromatin regions by the plant-specific RNA polymerase IV (Pol IV). These RNAs are then cutted into 24nt-long siRNA by DCL3 and loaded in the Argonaute protein AGO4, which can interact with target loci through base complementarity with scaffold RNAs transcribed by the plant-specific Pol V. This complex ultimately recruits the DNA methyltransferases DRM1/2 to methylate DNA, particularly asymmetrical

CHH sites. Methylome analysis of cycling cells revealed that RdDM-dependent DNA methylation is deposited way after DNA replication and preferentially after cell cycle arrest (G1/G0 phase) [4]. Consistent with this, CHH methylation can attain unusually high levels in quiescent cells with long G1/G0 phases, such as in late embryos [6,7] or, to a lesser extent, in some meristems [8,9]. Conversely, reduced RdDM activity was reported during early phases of germination, potentially due to the high rate of cell divisions and short G2 phases [7]. It is reasonable to speculate that perennial plants, which typically oscillate between periods of intensive and latent growth, can undergo significant DNA methylation dynamics through the year. Consistent with this hypothesis, methylome analysis of a single clone of *Arabidopsis halleri* year round revealed low and high levels of CHH methylation in Spring and Autumn, respectively [10]. Further experiments assessing methylome dynamics across life cycles and/or seasons will certainly help us to understand how environmentally-induced variation in the cell cycle rate, such as during dormancy, can alter the epigenome.

DNA methylation dynamics during plant growth

DNA methylation can vary significantly between some tissues and developmental stages. For instance, *Arabidopsis* roots are globally hypomethylated compared to shoots [11]. Notably, cell-type-specific methylome analysis of *Arabidopsis* roots show specific CHH hypermethylation in columella root cap cells, likely due to enhanced production of 24-nt siRNAs in this tissue [12]. More recently, transcriptome and methylome analysis of shoot apical meristem (SAM) revealed upregulation of genes encoding epigenetic silencing factors (*AGO5/9*, *DDM1*, *SUVH4*, *SUVR2*, *NRPD1*), transient activation of TEs, and increased CHG methylation mainly in stem cells, with lower CHG and CHH levels in surrounding cells [8]. These dynamics point to a reinforcement of DNA methylation within SAM stem cells, before their transition into reproductive cells, which at least in the case of male germline has high CG and low CHH methylation over TEs [13,14]. Supporting this hypothesis, more than half of the TEs that appear hypermethylated in rice gametes were shown to gain this methylation during the vegetative to reproductive transition of the SAM [9].

DNA methylation reinforcement during sexual reproduction

In angiosperms, female and male sexual lineages are initiated through meiotic division of the diploid megaspore mother cell (MMC) and microspore mother cell (MiMC), respectively [15]. In *Arabidopsis*, these two types of mother cells have low CHH methylation levels [14,16], which in the case of MiMC affects pericentromeric TEs preferentially. Nonetheless, few TEs become hypermethylated in

MiMCs and this hypermethylation was shown to be directed by siRNAs produced in surrounding nurse cells (tapetum) [17].

Megaspores and microspores further divide mitotically to give rise to female and male gametophytes, respectively. The female gametophyte is a seven-celled structure formed by two gametes - an haploid egg and a diploid central cell - along with other companion cells. While CHH methylation seems to remain fairly constant during *Arabidopsis* gametogenesis [16], methylomes of purified central cells from *Arabidopsis* and rice are globally hypermethylated compared to leaves [18]. Conversely, CHG is hypomethylated in rice central cells compared to vegetative tissues [18]. The contrast between *Arabidopsis* and rice suggests that methylation dynamics in female reproductive tissues can diverge significantly among species. Whether this divergence is due to differences in TE content and landscape between the two species [19,20] remains to be determined. Last, hypomethylation of CHG sites, and to some extent CG sites, occur in the rice central cell [18], likely involving the DNA glycosylase DEMETER (DME) [21].

The male gametophyte is formed by two sperm cells (SC) and a single vegetative cell (VC). VC is hypermethylated at CHH sites but undergoes active CG and CHG demethylation of TE sequences [13,14], which lead to the production of siRNAs that serve to reinforce DNA methylation of at least some TEs in the SC [22], and potentially in the developing embryo after fertilization [13]. VCs are arrested at the G1/G0 stage, when RdDM and CMT2 pathways are proposed to be more active and therefore contribute to the reestablishment of CHH methylation [6]. Conversely, SCs, which remain arrested at early S phase until fertilization [4], show higher and lower methylation levels of symmetrical (CG and CHG) and non-symmetrical sites, respectively [13,14]. Thus, differences in cell-cycle phases between both types of pollen cells might explain, at least in part, their contrasted CHH methylomes.

Methylome dynamics that take place after fertilization are of paramount importance, as all cell lineages are defined at these stages. During fertilization, SCs penetrate the female gametophyte and fuse to the haploid egg or to the diploid central cell, giving rise respectively to the embryo and the triploid endosperm, a companion nurse tissue required for embryo development [23]. Early embryos of *Arabidopsis* and soybean lose most CHH methylation, reaching the lowest levels across their life cycle [24]. In parallel, DME-mediated demethylation of CC genomes continues after fertilization, leading to CG and CHG hypomethylation of TE sequences in the maternal genomes of the endosperm [18,21]. Notably, most of these TEs gain CHH methylation during embryo development [6,18,25] in association with increased siRNAs levels in seeds [6,26]. Similar methylation dynamics were observed in soybean, chickpea, and rice [18,24,27], suggesting that incremental RdDM activity during embryogenesis likely

evolved before the radiation of flowering plants. Whether similar dynamics occur in more basal embryophytes, such as mosses and ferns, where equivalent tissues are simpler, or even ephemeral, is unknown.

siRNAs-dependent methylation of H3K9 also takes place in *Arabidopsis* embryos [28], further supporting an scenario where siRNAs produced in the endosperm migrate to the embryo to reinforce the epigenetic silencing of TEs. Indeed, the highest levels of CHH methylation are attained just after seed imbibition [7], suggesting that RdDM activity continues to increase till germination. Following imbibition, a passive demethylation of CHH sites proceeds [7,24,29], which could be explained by the high rate of cell divisions and short G2 phases during germination [4]. Future work deploying single-cell methylomes in highly replicating and quiescent cells should enable a comprehensive integration of the observed methylation dynamics with their cellular identities and cell cycle rates.

Given that DNA methylation dynamics during sexual reproduction are critical to correct epigenetic defects, one may wonder how alternative reproductive strategies impact the stability of DNA methylation. This question becomes particularly relevant in plants, especially in crops, where asexual reproduction is very frequent.

Asexual reproduction through apomixis

Plants are remarkably diverse in their mode of reproduction, ranging from the archetypal sexual reproduction, to many different flavors of asexual propagation (i.e. lack meiosis and fertilization). In particular, some plants may produce so-called apomictic seeds by a modified sexual cycle in which the MMC (diplospory apomixis), a surrounding cell (apospory apomixis), or a nucellar cell (adventitious apomixis), skips meiosis and double fertilization (reviewed in [30]).

Despite their similar development [35], transcriptomes of apomictic initial cells diverge from MMC mainly in cell cycle and epigenetic control genes [35]. An important role of siRNAs in MMC meiosis have been demonstrated in maize, where mutants of *AGO104* gene, a homologous of *Arabidopsis* *AGO9*, produce unreduced viable gametes by a diplospory-like mechanism [31]. *In situ* hybridization experiments using flowers of the apomictic grass *Eragrostis curvula* suggested that altered localization of *AGO9/AGO104*, rather than its degradation in the ovule, might be triggering diplospory in this species [32]. DNA methylation has also been linked to apomixis. For instance, maize plants carrying mutations in the homologues of the *Arabidopsis* *DRM1/2* and *CMT3* genes show features of apospory [33]. Similarly, *Arabidopsis* mutants of *AGO9*, *RNA-DEPENDENT RNA POLYMERASE 6*, and *SUPPRESSOR OF GENE SILENCING 3* genes produce aposporic embryos [34]. Notwithstanding the growing amount of evidence, the molecular mechanism underlying the epigenetic control of apomixis is still obscure.

In addition to the impact of epigenetic perturbations on apomixis, at least two loci were reported to be hypomethylated in apomictic female organs compared to sexual gametophytes of the grass *Cenchrus ciliaris* [36], suggesting that DNA methylation loss might be more severe during development of apomictic embryo sacs than gametogenesis. Nonetheless, methylome dynamics of adventitious embryos are reminiscent to the one observed during early development of sexual embryos [24], with early nucellar-derived embryos showing global CHH hypomethylation compared to surrounding maternal tissues [37]. Thus, although still incomplete, the available data suggest that embryogenesis, rather than fertilization, may be the key step where offspring's methylomes are crafted.

Clonal propagation

Vegetative propagation is an extreme case of asexual reproduction. Unlike apomixis, the parental organs that give rise to the vegetative offspring are somatic [38], initiated from meristems of differentiated and specialized organs. The lack of sexual reproduction and embryogenesis is expected to affect the stability of DNA methylation. Nonetheless, it is reasonable to speculate that some degree of DNA methylation reinforcement may take place during vegetative propagation, as SAM stem cells have higher CHG methylation levels than surrounding tissues [8]. However, species with histories of clonal propagation display very low levels of CHH methylation [39]. Hypomethylation associated with asexual propagation seems to be irreversible, as sexual offsprings of clonally-propagated *Manihot esculenta* and *Fragaria vesca* plants were found to have similarly low levels of CHH methylation than their asexual parents [39]. A plausible explanation could be that one generation of sexual reproduction may be insufficient to restore DNA methylation losses. Alternatively, siRNAs required to correct DNA methylation defects [40] are progressively lost during multiple rounds of vegetative propagation. To solve this conundrum, further studies comparing DNA methylation and siRNAs dynamics in natural vegetatively propagated and sexually reproduced individuals are therefore needed. For example, a recent work reported lower levels of CHH methylation in asexual offsprings of the tree *Robinia pseudoacacia* obtained through root sprouting or root-cutting compared to sexual ones [41]. However, because *Robinia pseudoacacia* typically outcross [42], DNA methylation changes due to genetic differences between sexual and asexual offsprings cannot be ruled out.

An artificial way to produce clonal plants is through *in vitro* culture. Studies of palm tree, rice, maize, and Arabidopsis cells have reported CHH hypomethylation of at least some TEs [43,44]. This hypomethylation could be readily explained by the fact that cells cycle faster in culture, which in turn should reduce the efficiency of RdDM [4]. In addition, because each CHH site is typically methylated in a few cells only (i.e. CHH methylation levels are low), *in vitro* amplification of cells should give rise to

clones in which most of these sites are unmethylated. Incidentally, the set of methylated and unmethylated sites should differ among clones, exposing the underlying somaclonal variation. In line with this prediction, independent maize plants regenerated from tissue cultures show somaclonal variation principally at CHH sites, as CG and CHG methylation changes are relatively consistent among regenerates [45]. Definitive evidence supporting or not this model could be obtained by performing single cell methylome analysis in tissue cultures and regenerated plants.

Hypomethylated loci are often transmitted to regenerated somatic embryos [43,44], suggesting that embryogenesis *per se* is not sufficient to reestablish DNA methylation losses. Further supporting this hypothesis, somatic embryogenesis from *Arabidopsis* roots and leaves carry over some of the methylation changes from the tissue of origin. These changes are then faithfully inherited across multiple sexual generations, in association with phenotypic variations [46]. Another study in soybean showed that somatic early embryos (at globular stage) derived from early embryonic cotyledons (likely at heart stage) are globally hypermethylated at CHH sites compared to embryonic cotyledons, and this hypermethylation was maintained after embryo differentiation [47]. Why the methylome of these regenerated embryos differ from that of early embryonic cotyledons, which are expected to be hypomethylated [24], is unknown. One possibility would be that at least part of the siRNAs required for DNA methylation reinforcement during late embryogenesis are loaded before the heart stage. Indeed, long-term culturing of these early embryos is accompanied by progressive loss of CHH methylation [47], further highlighting the importance of endosperm/maternal tissues as the source of siRNAs for epigenetic reinforcement. In conclusion, the available evidence suggests that the lack of fertilization and embryogenesis, where DNA methylation reinforcement takes place, leads to a progressive and likely irreversible loss of CHH methylation in clonally propagated plants.

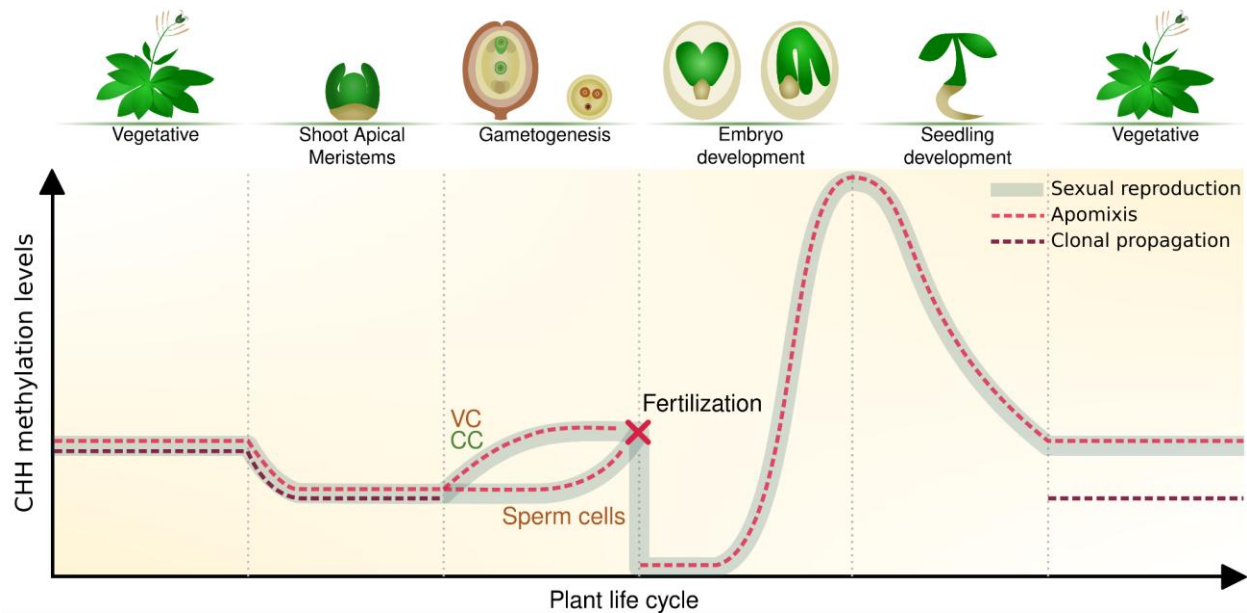


Figure 1. The mCHH dynamics during plant life cycle. Dynamic of CHH methylation observed (continuous line) and proposed (discontinuous line) in sexually and asexually propagated plants. Proposed CHH methylation profiles during apomixis (pink dashed line) and clonal propagation (violet dashed line) are based on the current knowledge of methylation dynamics during the life cycle of sexually-reproduced plants. Lack of fertilization in apomixis is indicated by a pink cross. The lack of fertilization and embryogenesis during clonal propagation is indicated by a gap in the proposed profile of CHH methylation. VC and CC indicate vegetative cell and central cell, respectively.

Conclusions

How DNA methylation is maintained across generations has been a major focus of research for the last two decades, yielding an impressive level of detail of the dynamics and mechanisms involved. Despite these advances, very little is known about how asexual reproduction shapes DNA methylation memory in plants. Here, we reviewed recent findings obtained using model plant species, particularly *Arabidopsis*, rice, and maize, to propose plausible DNA methylation dynamics in asexually-propagated species. We argue that because embryogenesis of apomictic seeds resembled that of their sexual counterparts, methylomes of apomictic embryos are expected to undergo DNA methylation reinforcement by endosperm-derived siRNAs. Conversely, most steps of sexual reproduction, including meiosis, fertilization, and embryogenesis, are skipped during clonal propagation, leading to deficient, or even absent, reinforcement of DNA methylation (Figure 1). Whether this is the case, it may have important implications for the evolution and diversification of clonally propagated species. First, lack of DNA methylation reinforcement should trigger progressive hypomethylation of TEs, leading to their reactivation and generation of TE-induced mutations. Increased transpositions may pose a threat for genome stability, yet it can also fuel the pool of genetic diversity available for evolution [48]. This TE-induced diversification could be particularly important for clonally-propagated species, as the lack of

outcrossing and recombination should reduce the amount of genetic diversity. Second, deficient reinforcement of DNA methylation likely perpetuates accidental epigenetic defects, expanding the number of epialleles in the populations. Such epigenetic variation can further contribute to the diversification of asexually-propagated plants. More studies aiming to determine the extent and type of epigenetic variation in clonally-propagated plants are critical to understand how the different modes of reproduction impact the diversification and evolution of asexual plants. In that respect, the use of non-model organisms will be of paramount importance. We anticipate that the use of long-read sequencing technologies, which greatly facilitates the assembly of genomes and epigenome studies, together with single-cell methylomes, have the potential to revolutionize our knowledge of the modes and consequences of different epigenetic inheritance systems in plants.

Conflict of interest statement

Nothing declared

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References and recommended readings

PAPERS HIGHLIGHTED (*of special interest; **of outstanding interest)

(**4) This study characterizes DNA methylation dynamics across the cell cycle using immortalized cell suspensions of *A. thaliana*. Based on comparative methylome analysis among different tissues and during cell cycle, the authors provide evidence that inefficient RdDM plays a major role in somaclonal variation.

(**8) The authors analyzed isolated stem cell nuclei from different developmental stages. They report an upregulation of genes implicated in epigenetic silencing in stem cells. In addition, transition from vegetative to reproductive stages correlates with CHH hypo methylation, suggesting that epigenetic reinforcement starts before germline development.

(**9) In this study, the analysis of vegetative and reproductive shoot apical meristems of rice showed transition from vegetative to reproductive stages, correlates with an increase in CHH methylation mostly over TE. The methylation status of some TEs were maintained in gametes, suggesting that epigenetic reinforcement starts before germline development.

(*17) Analysis of male meiocytes, tapetal cells, and sperm nuclei showed that specific TE-derived siRNAs in tapetal cells move to meiocytes to induce hypermethylation of partially matching loci.

(**37) This study revealed that CHH hypomethylation in early nucellar-derived embryos is lower than surrounding maternal tissues. In addition, they provide evidence that a gene involved in adventitious apomixis could be under epigenetic control.

(*41) This study report hypomethylation of asexual compared to sexual offsprings

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