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Invited Review

Diversity and dynamics of DNA methylation: epigenomic resources and tools for crop breeding

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DNA methylation is an epigenetic modification that can affect gene expression and transposable element (TE) activities. Because cytosine DNA methylation patterns are inherited through both mitotic and meiotic cell divisions, differences in these patterns can contribute to phenotypic variability. Advances in high-throughput sequencing technologies have enabled the generation of abundant DNA sequence data. Integrated analyses of genome-wide gene expression patterns and DNA methylation patterns have revealed the underlying mechanisms and functions of DNA methylation. Moreover, associations between DNA methylation and agronomic traits have also been uncovered. The resulting information may be useful for future applications of natural epigenomic variation, for crop breeding. Additionally, artificial epigenome editing may be an attractive new plant breeding technique for generating novel varieties with improved agronomic traits.

Key Words: DNA methylation, epigenome, transposable element, small RNA.

Introduction

Cytosine DNA methylation is a chemical modification of the fifth position of the cytosine base. In plants, DNA methylation occurs in three distinct sequence contexts, symmetrical CG and CHG as well as asymmetrical CHH, where H is either C, A, or T (Law and Jacobsen 2010). These DNA methylation patterns are stably inherited through cell division. Changes in DNA methylation can occur spontaneously and may be induced by genetic factors and environmental stimuli. Additionally, stress conditions can alter DNA methylation patterns (Dowen *et al.* 2012, Hossain *et al.* 2017, Secco *et al.* 2015, Wibowo *et al.* 2016). There are two types of DNA methylation patterns in plants, namely the CG-only gene body methylation (gbM), which is DNA methylation within transcriptional regions, and non-CG as well as CG transposable element (TE)-like methylation (teM). gbM is associated with mild constitutive expression levels, whereas teM is associated with the repression of TE activities (TE silencing) and gene expression (gene silencing) (Coleman-Derr and Zilberman 2012, Tran *et al.* 2005, Zhang *et al.* 2006, Zilberman *et al.* 2007). Transposable element activities

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Abbreviations: ACS: 1-Aminocyclopropane-1-carboxylate synthase, AGO4/6: ARGONAUTE 4/6, AtLIG1: ARABIDOPSIS DNA LIGASE 1, b1: booster 1, CLSY1-4: CLASSY 1-4, CMT2/3: CHROMOMETHYRASE 2/3, CNI1: CARBON/NITROGEN INSENSITIVE 1, CNR: COLORLESS NON-RIPENING, CYC: CYCLOIDEA, DCL3: DICER-LIKE 3, DDM1: DECREASED IN DNA METHYLATION 1, df: DWARF AND FLOWER ABERRANT, DME: DEMETER, DML1-3: DEMETER LIKE 1-3, DMR: Differentially methylated region, DMS3: DEFECTIVE IN MERISTEM SILENCING 3, DNAJ: DnaJ-domain containing chaperone protein, DRD1: DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1, DRM2: DOMAINS OF REARRANGED METHYLTRANSFERASE 2, dsRNA: Double-stranded RNA, FIE1: FERTILIZATION-INDEPENDENT ENDOSPERM1, FIS2: FERTILIZATION-INDEPENDENT SEED 2, FIP1: FRIGIDA INTERACTING PROTEIN1, FOLT1: FOLATE TRANSPORTER1, FWA: FLOWERING WAGENINGEN, gbM: Gene body methylation, GFP: Green fluorescent protein, H3K4/9/27: Histone H3 lysine 4/9/27, KYP: KRYPTONITE, lncRNA: Long non-coding RNA, MEA: MEDEA, MeGI: MALE GROWTH INHIBITOR, MET1: DNA METHYLTRANSFERASE 1, MOP: MEDIATOR OF PARAMUTATION, MPS1/PRD2: MULTIPOLAR SPINDLE 1/PUTATIVE RECOMBINATION INITIATION DEFECTS 2, MYB20: MYB DOMAIN PROTEIN 20, OGI: OPPRESSOR OF MEGI, PAI: Phosphoribosylanthranilate isomerase, PE gene: Poly-epiallelic gene, PRC2: Polycomb repressive complex 2, r1: red color 1, RdDM: RNA-directed DNA methylation, RDM1: RNA-DIRECTED METHYLATION 1, RDR2: RNA-DEPENDENT RNA POLYMERASE 2, RIL: Recombinant inbred line, RIN: RIPENING INHIBITOR, RMR: REQUIRED TO MAINTAIN REPRESSION, ROS1: REPRESSOR OF SILENCING 1, SHH1: SAWADEE HOMEODOMAIN HOMOLOGUE 1, siRNA: Small interfering RNA, SNP: Single nucleotide polymorphism, SP11/SCR: S-locus protein 11/S-locus cysteine-rich, SRK: S-receptor kinase, SUVH2/4/5/6/9: SU(VAR) HOMOLOGUE 2/4/5/6/9, TE: Transposable element, teM: TE-like methylation, TET1: Ten-eleven translocation methylcytosine dioxygenase 1, VIM1: VARIATION IN DNA METHYLATION 1, VTE: Vitamin E

increase genomic diversity, which is applicable for breeding as well as functional genomics investigations. However, TE silencing is important for stable crop cultivation and production because excessive TE activities can cause variable phenotypes, including deleterious ones. Moreover, changes in gene expression can lead to visible phenotypic alterations. Therefore, DNA methylation must be appropriately regulated for effective crop breeding.

High-throughput DNA sequencing has enabled transcriptome and epigenome profiling at single-base resolution, as well as genome re-sequencing (Lister *et al.* 2008). Integrating these omics-based data has resulted in the accumulation of information regarding the biological roles of epigenomes. Importantly, there are considerable intra- and inter-species variabilities in DNA methylation patterns (Kawakatsu *et al.* 2016a, Niederhuth *et al.* 2016). The associated data have not been restricted to model plant species with compact genomes, but have been extended to agronomically important crops with large and complex genomes (Daccord *et al.* 2017, Regulski *et al.* 2013, Schmitz *et al.* 2013a, Turco *et al.* 2017, Zhong *et al.* 2013). Natural genomic variation, such as single nucleotide variants and structural variations, has been exploited for plant breeding (Morrell *et al.* 2011). Recent studies suggest that it may also be possible to exploit natural epigenomic variation as a new tool for breeding.

In this review, we describe the DNA methylation machinery, diversity, and dynamics in the model plant Arabidopsis (*Arabidopsis thaliana*) as well as the agronomic traits associated with DNA methylation. Heterosis is one of the best-known agronomic traits associated with DNA methylation. Please refer to Fujimoto *et al.* (2018) in the same review series for details regarding heterosis and related epigenetics, including the transgenerational inheritance of DNA methylation or the epigenetics of recombinant inbred lines (epiRILs) derived from hybrids between DNA methylation deficient mutants and wild type.

DNA methylation machinery

CG methylation is maintained by MET1 and VIM1 (Kankel *et al.* 2003, Woo *et al.* 2007). VIM1 recognizes hemimethylated DNA and recruits MET1 to replication foci. The recruited MET1 catalyzes DNA methylation on newly synthesized hemimethylated DNA strands. Thus, CG methylation is maintained in a semi-conservative manner during DNA replication. CHG methylation is catalyzed by CMT3, which binds to methylated histone 3 lysine 9 (H3K9) (Bartee *et al.* 2001, Lindroth *et al.* 2001). Additionally, CHG and CHH methylation within heavily heterochromatic regions is regulated by CMT2, which binds to dimethylated H3K9 (H3K9me2) (Stroud *et al.* 2014, Zemach *et al.* 2013).

DNA methylation within heterochromatic regions also depends on the chromatin remodeling factor DDM1, which removes histone H1 linker proteins from densely packed chromatin to enable MET1, CMT3, and CMT2 to methylate the DNA in heterochromatic regions (Zemach *et al.* 2013). Furthermore, RNA-directed DNA methylation (RdDM) mediates all types of cytosine methylation within short TEs in euchromatin and along the edges of long TEs in heterochromatin (Zemach *et al.* 2013). In canonical RdDM, two plant-specific RNA polymerases Pol IV and Pol V, which are the result of Pol II duplications, play critical roles in small interfering RNA (siRNA) biogenesis and *de novo* methylation during RdDM, respectively (Matzke and Mosher 2014). Pol IV is recruited to target regions through a direct association with the SHH1, which recognizes H3K9me2, and CLSY proteins (Law *et al.* 2013, Zhou *et al.* 2018).

Pol V is recruited to target regions through an indirect association with the inactive histone methyltransferases SUVH2 and SUVH9, which recognize methylated DNA (Johnson *et al.* 2014). DDR (DRD1-DMS3-RDM1) complex mediates the association between Pol V and SUVH2/9 (Matzke and Mosher 2014). Pol IV synthesizes short RNAs [approximately 30–40 nucleotides (nt)] that are converted to double-stranded RNA (dsRNA) by RDR2 (Blevins *et al.* 2015, Zhai *et al.* 2015). The dsRNAs are diced into 24-nt siRNAs by DCL3 (Xie *et al.* 2004). AGO4 binds to these siRNAs, and the resulting AGO4-siRNA complex is guided to Pol V target loci, with Pol V transcripts as scaffolds (Gao *et al.* 2010, Havecker *et al.* 2010). DRM2 is recruited to target regions through an indirect association with AGO4 and catalyzes methylation reactions in all contexts (Gao *et al.* 2010).

There are several non-canonical RdDM pathways (Cuerda-Gil and Slotkin 2016). Once RdDM is initiated by non-canonical RdDM pathways, it is then established by canonical RdDM pathways (McCue *et al.* 2015, Stroud *et al.* 2014). Additionally, the histone methyltransferases KYP/SUVH4, SUVH5, and SUVH6 recognize methylated CHG and CHH *via* the SRA domain and catalyze the dimethylation of H3K9 (Du *et al.* 2014, Ebbs *et al.* 2005, Ebbs and Bender 2006, Rajakumara *et al.* 2011). Hence, non-CG DNA methylation, histone modification, and nucleosome positioning form self-reinforcing loops.

DNA demethylation machinery

DNA demethylation is initiated by a bi-functional DNA glycosylase that exhibits both DNA glycosylase activity and apurinic/apyrimidinic (AP) lyase activity, through a base excision repair mechanism (Zhang and Zhu 2012). Specifically, DNA glycosylase excises methylated cytosines, while AP lyase nicks the AP site. Additionally, DNA phosphatase ZDP, AP endonuclease APE1L, DNA polymerases, and the DNA ligase AtLIG1 cooperatively fill the single nucleotide gap with unmethylated cytosine (Li *et al.* 2015, Martínez-Macías *et al.* 2012). Arabidopsis has four DNA glycosylases: DME, ROS1/DML1, DML2, and DML3. The *DME* gene is predominantly expressed in the central cell of the female gametophyte before fertilization, where DME induces global hypomethylation, leading to maternal allele-

specific demethylation and expression of imprinting genes as well as some transposons (Choi *et al.* 2002, Hsieh *et al.* 2009). These imprinting genes include *FWA*, *FIS2* and *MEA* (Kinoshita *et al.* 1999, 2004, Luo *et al.* 2000, Vielle-Calzada *et al.* 1999). *FWA* encodes a homeodomain-containing transcription factor that controls flowering. *FIS2* and *MEA* encode components of the PRC2 that catalyzes the repressive H3K27me3 modification. Because PRC2 is required for endosperm cellularization, DME-dependent demethylation in the central cell is indispensable (Köhler *et al.* 2003). *DME* is also expressed in the vegetative cell of the male gametophyte, and is required for demethylation of imprinting genes and transposons (Ibarra *et al.* 2012). Moreover, *ROS1*, *DML2*, and *DML3* are expressed in vegetative tissues, and are required for the demethylation of thousands of discrete loci, including TEs within the promoters of stress-responsive genes (Calarco *et al.* 2012, Le *et al.* 2014, Tang *et al.* 2016). The overlap between RdDM target regions and ROS1 target regions reveals the antagonism between active DNA methylation and demethylation. Interestingly, a TE located in the *ROS1* promoter region is a target of RdDM and ROS1. DNA methylation in this TE promotes the expression of *ROS1*. Therefore, the balance between DNA methylation and demethylation in the TE may be critical for fine-tuning the genome-wide methylation level (Lei *et al.* 2015, Williams *et al.* 2015).

Finally, because nascent DNA being synthesized during DNA replication is not methylated, cell division itself can induce "passive" demethylation by diluting DNA methylation in the absence of maintenance DNA methylation or *de novo* DNA methylation. Nucleoside analogs of cytidine, such as 5-azacitidine and zebularine, can be incorporated into DNA and substituted for cytosine. The 5-azacitidine- or zebularine-substituted DNA inhibits DNA methyltransferase activity, leading to genomic DNA demethylation.

Lessons from the reference plant Arabidopsis

Cell type-specific DNA methylation in Arabidopsis

Mature pollen grains are the final form of male sexual lineage cells. Meiosis produces haploid microspores from diploid meiocytes. Two rounds of mitosis result in the production of mature pollen grains comprising two sperm cells and a vegetative cell. Sperm cells initiate a simultaneous "double fertilization" process: one fusing with haploid egg cell and the other with the diploid central cell. The vegetative cell which supports growth of the pollen tube does not transmit its genomic information to the next generation. Active RdDM induces locus-specific hypermethylation in the sperm cell genomes, but not in the vegetative cell genome. The male sexual lineage-specific methylation within intron 9 of *MPS1/PRD2*, which is crucial for meiosis, is required for the proper splicing of this intron (Walker *et al.* 2018). In RdDM-deficient *drm1 drm2* meiocytes, approximately 30% of *MPS1/PRD2* mRNAs retain intron 9, which introduces a premature stop codon, suggesting that proper *MPS1* expression (and meiosis) is regulated by RdDM. Indeed, the meiocytes of *drm1 drm2* and *rdr2* mutants do not undergo normal meiosis, forming triad, tetrad, and pentad microspores.

Transposable elements are typically silenced by DNA methylation; however, they are explicitly reactivated in the vegetative cell nucleus (Slotkin *et al.* 2009). The chromatin in the vegetative nucleus is decondensed, whereas the sperm nuclei are compact (Slotkin *et al.* 2009). The DNA glycosylase genes *DME*, *ROS1*, *DML2*, and *DML3* are expressed in the vegetative nucleus, but not in the sperm (Schoft *et al.* 2011). These DNA glycosylases mediate the demethylation of small AT-rich euchromatic TEs in the vegetative nucleus, which reactivates these TEs (Calarco *et al.* 2012, Ibarra *et al.* 2012). In contrast, in sperm, these TEs undergo DME-dependent hypermethylation suggesting there is a link between demethylation and de-condensation in the vegetative nucleus and hypermethylation along with compact chromatin in the sperm (Calarco *et al.* 2012, Ibarra *et al.* 2012). The transcripts of reactivated TEs are degraded in the RNAi pathway, like *trans*-acting siRNA-generating *TAS* transcripts (Creasey *et al.* 2014). The vegetative nucleus-specific expression of a truncated *GFP* gene fused to miRNA173 or an endogenous 21-nt transposon siRNA target site produces a 21-nt siRNA that can target *GFP*, leading to non-cell autonomous silencing of the sperm cell-specific expression of *GFP* (Grant-Downton *et al.* 2013, Martínez *et al.* 2016, Slotkin *et al.* 2009). These results suggest that the 21-nt siRNAs produced from reactivated TEs in the vegetative nucleus are transported to the sperm (**Fig. 1**). Such non-canonical RdDM 21-nt siRNAs may reinforce TE silencing in the sperm germline. It is noteworthy that several protein-coding genes silenced by DNA methylation are also reactivated specifically in the vegetative nucleus, and are important for pollen tube growth and development (Schmitz *et al.* 2013b).

During double fertilization, the sperm cells fertilize the egg cell and the central cell to produce the embryo and the endosperm. The endosperm genome is globally hypomethylated in all contexts, relative to the embryo genome (Gehring *et al.* 2009, Hsieh *et al.* 2009). The hypomethylation of the endosperm genome occurs only in maternal chromosomes. Additionally, the hypomethylation in the endosperm depends on DME activity, similar to that in pollen grains (Hsieh *et al.* 2009, Ibarra *et al.* 2012). The *DME* gene is expressed in the central cell before fertilization, and the demethylation is initiated in the central cells (Park *et al.* 2016). Maternal-specific demethylation contributes to the maternal-specific expression of imprinted genes. Hypomethylation in the endosperm has also been observed in rice and maize (Wang *et al.* 2015, Zemach *et al.* 2010). In rice, the both CG and CHG hypomethylation in the endosperm are associated with the endosperm-specific expression of some seed storage protein genes and starch synthase genes. As described above, DME targets AT-rich euchromatic TEs. Hypomethylated TEs in the endosperm are hypermethylated

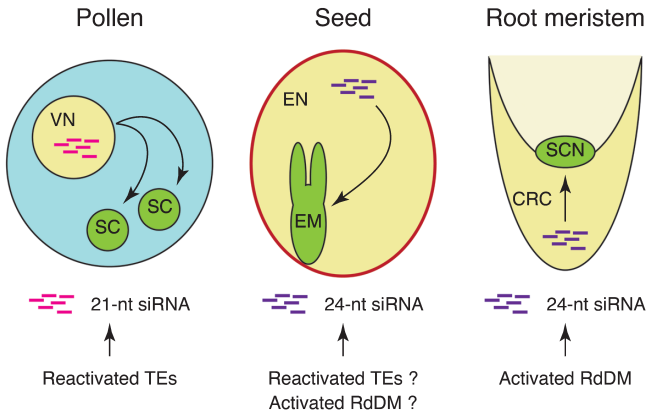

Fig. 1. Epigenetic silencing of transposable elements (TEs) reinforced by siRNAs produced in companion cells. In developing pollen grains, TEs are reactivated via demethylation in the vegetative nucleus. The TE transcripts are converted to dsRNAs, then degraded into 21-nt siRNAs. These 21-nt siRNAs are transported to sperm cells and reinforce the DNA methylation within TEs. In developing seeds, 24-nt siRNAs are produced by activated RdDM or from reactivated TEs in the endosperm. These 24-nt siRNAs are transported to the embryo and reinforce TE silencing in the embryo. In the root meristem, 24-nt siRNAs are over-produced in the columella cells by activated RdDM. These 24-nt siRNAs may be transported to the stem cell niche, where they reinforce TE silencing. VN: vegetative nucleus, SC: sperm cell, EN: endosperm, EM: embryo, CRC: columella root cap, SCN: stem cell niche.

in the embryo, suggesting the non-cell autonomous regulation of TE silencing in the embryo is due to siRNAs produced in the endosperm (Ibarra *et al.* 2012). The expression of an endosperm-specific artificial microRNA targeting *GFP* results in silencing of embryo-specific expression of *GFP*, suggests transfer of siRNAs from the endosperm to the embryo. Some TEs are weakly reactivated in the developing seed suggesting that, like in pollen grains, epigenetically activated TE transcripts are the source of 21-nt siRNAs (Hsieh *et al.* 2009, Slotkin *et al.* 2009). However, 24-nt Pol IV-dependent siRNAs, rather than 21-nt siRNAs, are explicitly produced from the maternal chromosomes in the endosperm suggesting that, like in pollen grains, endosperm-derived 24-nt siRNAs are transported to the embryo and reinforce TE silencing (Lu *et al.* 2012, Mosher *et al.* 2009). A link between the weak reactivation of TEs and the increased production of 24-nt siRNAs in the endosperm has not been established.

Plants have stem cell niches in the shoot apical meristem (SAM) and root apical meristem (RAM). These stem cells are responsible for shoot and root architecture patterning and are affected by TE activities. Hypermethylation in the SAM and the RAM due to the increased abundance of RdDM factors and DDM1 likely reinforces the TE silencing in the meristems (Baubec *et al.* 2014). To elucidate the dynamics underlying the TE silencing mediated by DNA methylation in the RAM, DNA methylation patterns for specific cell types within the RAM needs to be clarified. Manually collecting specific cell types from somatic tissues is not feasible. Thus, dissociating single cells from somatic tissues by enzymatically digesting the cell wall, and a subsequent fluorescence-activated cell sorting analysis enables researchers to distinguish cells producing fluorescent proteins (e.g., GFP) from the various cells of somatic tissues. Among the six major cell types of the RAM (epidermis, cortex, endodermis, stele, whole columella root cap, and lower columella), the whole columella root cap is globally hypermethylated in all contexts, but especially in the CHH context (Kawakatsu *et al.* 2016b). Global CHH hypermethylation has also been observed in the lower columella, indicating that CHH hypermethylation is a signature of columella cells.

Among all of the Arabidopsis cell types that have been analyzed, CHH hypermethylation is greatest in columella cells. Columella CHH hypermethylation occurs primarily in the pericentromeric regions, in which TEs are abundant, but also occurs in chromosomal arms. Local DNA methylation changes were identified as CG-only differentially methylated regions (CG-DMRs), non-CG only DMRs (CH-DMRs), and CG and non-CG DMRs (C-DMRs). The CH-DMRs are the major DMRs among root cell types and more than 70% of the CH-DMRs overlap with all classes of TEs. The DNA methylation patterns in CG- and C-DMRs and the transcriptional profiles are more similar between cell types originating from the same initial cells than between cell types derived from different initial cells. However, DNA methylation patterns in CH-DMRs are more dependent on the physical position in the RAM, suggesting that positional information or cell-to-cell communication also influence the regulation of DNA methylation patterns. The CHH hypermethylation in the columella is accompanied by an over-accumulation of 24-nt siRNAs, likely due to the upregulated expression of siRNA biogenesis machinery genes. The DNA methylation within TE bodies is primarily dependent on either RdDM or CMT2, and, in leaves, 24-nt siRNAs do not accumulate within CMT2-dependent TE bodies. In the columella, RdDM-dependent TEs as well as CMT2-dependent TEs exhibit CHH hypermethylation with an over-accumulation of 24-nt siRNAs, indicating that an enhanced RdDM is responsible for a genome-wide CHH hypermethylation in the columella. The downregulated expression of heterochromatin-related component genes may suggest that heterochromatin is loosened in the columella. Decondensed heterochromatin in the columella may be responsible for the enhanced production of 24-nt siRNAs within heterochromatin, where CMT2, but not RdDM, is responsible for DNA methylation. The biological importance of enhanced RdDM in the columella is unclear because these cells are sloughed into the soil soon after differentiating from initial cells but likely does not involve extensive TE silencing. Columella cells are adjacent to the stem cell niches in the RAM, which are presumably vulnerable to TE activities. One attractive hypothesis is that excessive amounts of 24-nt siRNAs produced in the columella are transported into the stem cell niches to

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reinforce TE silencing, analogous to the cell non-autonomous TE silencing in the reproductive cells (**Fig. 1**).

Developmentally regulated DNA methylation

During embryogenesis, plants form the basis of their architecture with two apical meristems and a few leaves. Meanwhile, the embryo and/or endosperm store energy and amino acid reserves for germination. After maturing, dry seeds can remain dormant for an extended period until conditions are favorable for germination. In developing seeds, CHH methylation of TEs increases, but not CG or CHG methylations (Bouyer *et al.* 2017, Kawakatsu *et al.* 2017, Lin *et al.* 2017, Narsai *et al.* 2017).

Additionally, CHH hypermethylation decreases in the dry seeds of the *drm1 drm2 cmt3* triple mutant, and is absent in the dry seeds of the *drm1 drm2 cmt2 cmt3* quadruple mutant, suggesting that both RdDM and CMT2 are responsible for the CHH methylation occurring in developing seeds. In contrast, the CHH methylation of TEs drastically decreases during germination. The global demethylation resets the hypermethylation in dry seeds. A lack of DNA demethylases does not affect the global demethylation during germination. Therefore, the global demethylation during germination likely occurs passively, in which methylation is diluted because of repeated cell divisions. Intriguingly, both RdDM components and CMT2 are produced, and 24-nt siRNA levels are relatively unchanged during germination. This suggests that unknown factor(s) inhibit *de novo* re-methylation or that cells are dividing so quickly that *de novo* re-methylation cannot compensate for the passive demethylation.

Many of the genes exhibiting upregulated expression upon germination are associated with cell division and cell wall organization. These genes tend to have nearby DMRs that are methylated during seed development and demethylated during germination. This raises the possibility of the epigenetic regulation of germination and the existence of a positive feedback loop between passive demethylation and induction of cell division-related genes (**Fig. 2**; Kawakatsu *et al.* 2017). These dynamic changes to CHH methylation have also been observed in rice and soybean, implying that the epigenomic reconfiguration during seed development and germination is widely conserved in the plant kingdom. The *drm1 drm2 cmt2 cmt3* quadruple mutant exhibits normal seed development, with minor transcriptome changes, although TEs are reactivated, suggesting that in Arabidopsis, CHH hypermethylation during seed development is a failsafe mechanism for TE silencing (Lin *et al.* 2017). A maternally transmitted defect in RdDM increases the seed abortion rate and severely decreases seed size in *Brassica rapa*, which produces seeds that are much larger than those of the related Arabidopsis (Grover *et al.* 2018). Closer examination of an Arabidopsis mutant with defective RdDM, also revealed a decrease in the weight of seeds, although the extent was much smaller than that observed for *B. rapa* seeds. The diversity in the embryo and endosperm sizes in aborted seeds may reflect an asynchronous seed abortion in

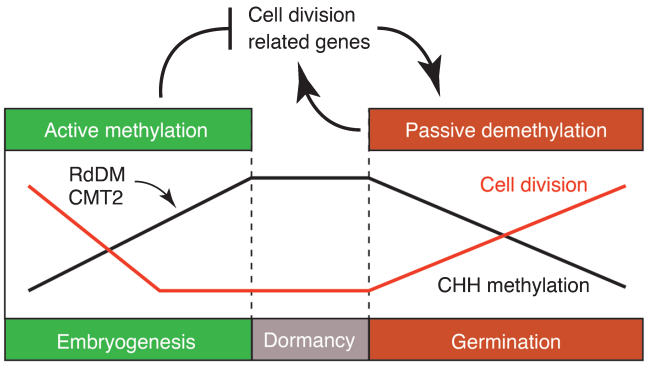

Fig. 2. Hypothesized epigenetic regulation of seed germination. During seed development, global CHH methylation levels (black line) increase, whereas the cell division rate (red line) decreases toward maturation. Under conditions that are favorable for germination, cell division is induced, leading to passive CHH demethylation. Cell division-related genes whose expression levels are upregulated in response to germination are often located near regions affected by CHH methylation reconfigurations. According to this model, reconfiguration of CHH methylation may help to regulate the seed germination process.

B. rapa RdDM mutants, suggesting that a sudden increase in TE activities due to TE reactivation terminates normal seed development at random times.

Population-wide DNA methylation diversity

In addition to genetic variations, natural epigenetic variation might also be shape phenotypic diversity and adaptation. Analyses of DNA methylomes from more than 1000 Arabidopsis accessions revealed extensive epigenomic variation with 38% of the reference genome differentially methylated among these accessions (Dubin *et al.* 2015, Kawakatsu *et al.* 2016a, Schmitz *et al.* 2013b). While CG-DMRs mainly overlap with protein-coding genes related to housekeeping processes, the CH-DMRs overlap with TEs and intergenic regions, and the C-DMRs overlap with TEs and/or protein-coding genes whose expression levels vary across tissues or environments.

Earlier investigations involving the reference Arabidopsis accession Col-0 indicated that gbM may be associated with the exclusion of the histone variant H2A.z from gene bodies, leading to constitutive gene expression (Tran *et al.* 2005, Zhang *et al.* 2006, Zilberman *et al.* 2007). Across accessions, genes that undergo gbM tend to be more highly expressed than unmethylated genes or genes that undergo teM. However, gbM variation is significantly larger than transcriptome variation among accessions. Additionally, global gene expression levels in accessions that nearly lack gbM are similar to those of accessions with higher gbM levels, which is consistent with the observation that the loss of gbM in the *met1* mutant does not affect gbM gene expression patterns (Bewick *et al.* 2016). Moreover, gbM is conserved between orthologous genes, but two Brassicaceae species completely lack gbM, presumably because of the absence of CMT3 (Bewick *et al.* 2016). Similar global

expression patterns have been observed for orthologs in Arabidopsis and these two Brassicaceae species. Therefore, gbM is associated with mild constitutive gene expression, but there is no clear evidence of its impact on transcriptomes, at least under normal growth conditions.

Arguably the most striking finding from the 1000 epigenomes population study is that one-quarter of all protein-coding genes (7,524 genes) are poly-epiallelic (PE) genes, which undergo gbM in some accessions and teM in other accessions (Kawakatsu *et al.* 2016a). The ratio of teM epialleles of PE genes is much lower than that of gbM epialleles, and only one accession has teM epialleles in approximately 30% of the PE genes, suggesting that many teM epialleles of PE genes are newly formed in gbM genes. There are several possible explanations for the emergence of poly-epialleles. First, RdDM may have spread from nearby newly inserted TEs. Second, siRNAs produced from the newly formed inverted repeats at unlinked loci (e.g., *PAI* loci) may reinforce DNA methylation (Luff *et al.* 1999). Third, aberrant mRNA from gbM genes may be subjected to non-canonical RdDM (Cuerda-Gil and Slotkin 2016). Last, purely spontaneous reversions may occur, as gbM may have evolved from teM (Bewick *et al.* 2016). The PE genes are enriched with genes involved in signaling and metabolic pathways, with an emphasis on phosphorylation-related and immune response-related genes. Among the analyzed PE genes that have gbM and teM epialleles in at least five accessions, 10% of the genes exhibit an association between DNA methylation and gene expression, with the expression levels of teM epialleles significantly lower than those of gbM epialleles under normal growth conditions. Thus, the epigenetic regulation of PE genes may provide a mechanism for increasing phenotypic diversity and plant adaptation.

The correlation between genome-wide methylation and the place of origin suggests there is a genetic basis for methylation variation (Kawakatsu *et al.* 2016a). A genome-wide association study revealed the associations between RNA silencing or DNA methyltransferase activities and genome-wide methylation levels. The methylation levels within RdDM-targeted TEs are associated with SNPs linked with *AGO1*, *NRPD1B*, and *AGO9*, whereas those of CMT2-targeted TEs are associated with SNPs linked with *CMT2* and *AGO9*. Natural variation in *AGO9* expression patterns may help to regulate TE methylation (Rodriguez-Leal *et al.* 2015). Additionally, gbM levels are reasonably associated with SNPs linked with *MET1*. Relatively high DNA methylation levels within TEs likely repress TE expression, resulting in increased genomic integrity and homogeneity within the population. However, relatively low DNA methylation levels may allow an increase in TE expression and transposition. When TEs are expressed, mobilized and inserted into genes, the affected gene may be knocked out, or the expression of nearby genes may be positively or negatively altered. In some cases, TE insertions may cause nearby genes to become responsive to stress (Naito *et al.* 2009). Therefore, decreasing DNA methylation levels potentially leads to genomic diversity and possibly enable adaptations to environmental changes. Natural variation in identified genes may provide a balance between adaptation and population homogeneity (**Fig. 3**).

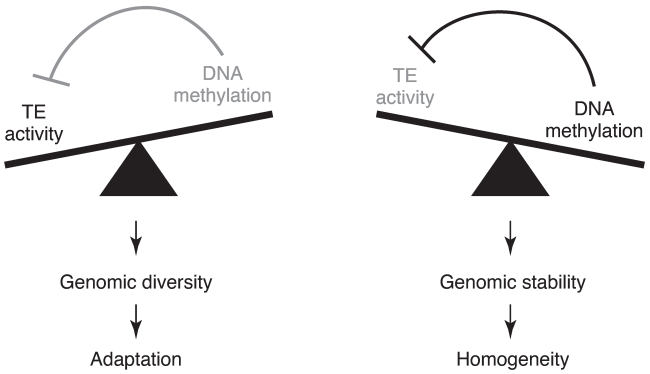

Fig. 3. The balance between global DNA methylation levels and transposable element (TE) activities potentially influences population diversity. Natural variation in several components involved in the DNA methylation pathway are associated with global DNA methylation levels. Lower DNA methylation levels are conducive for TE activation, whereas higher DNA methylation levels can silence TEs. Because TE activities are associated with genomic integrity, such natural variation may function as biological rheostat.

DNA methylation and adaptation

Both abiotic and biotic stress can change DNA methylation patterns globally or locally. However, transgenerational inheritance of stress-induced epigenetic variation is controversial, because there have been few comprehensive analyses. Phosphate starvation increases DNA methylation around highly induced genes, especially in the adjacent TEs in rice (Secco *et al.* 2015). Interestingly, this process unlikely depends on RdDM, because DCL3 is dispensable for TE hypermethylation. These DNA methylation changes occur after transcriptional changes, possibly reflecting the failsafe system to inactivate TEs in the vicinity of accessible chromatin. However, the hypermethylation is recovered in the following generations.

Repeated hyper-osmotic stress for over 5 generations confers tolerance against osmotic stress in Arabidopsis (Wibowo *et al.* 2016). This stress memory is transmitted to the next generation, suggesting epigenetic regulation. Indeed, repetitive hyper-osmotic stress induces both hypermethylation and hypomethylation within TEs and 2-kb upstream regions of protein coding genes in non-CG contexts, although hypermethylation is dominant. One stress-induced hyper-DMR locates upstream of *MYB20*, that is involved in abscisic acid signaling and stress tolerance (Wibowo *et al.* 2016). Progenies with the stress memory show the decreased expression of *MYB20* under salt stress condition, whereas the down regulation of *MYB20* is not observed in progenies without stress memory. On the other hand, one stress-induced hypo-DMR locates downstream of *CNI1*

(Wibowo *et al.* 2016). Salt stress induces the expression of lncRNA including antisense *CNI1*, which downregulates the expression of *CNI1* under hyperosmotic stress. DNA methylation at the *CNI1*-downstream hypo-DMR represses the expression of the lncRNA under normal condition, but hypermethylation allows its expression under salt stress. Reciprocal crossing revealed the stress memory is transmitted through the female germline (Wibowo *et al.* 2016). This biased sexual transmission is likely caused by DME-dependent resetting of stress-induced DNA methylation changes in the male germline, because repeatedly osmotic stressed *dme* is more tolerant than stressed wild type. Stress-induced DNA methylation changes are inherited to the next generation, however, they are not further inherited to their offspring without stress. Another study also identified repetitive hyper-osmotic stress for over 10 generations induced DNA methylation changes, especially in CG context (Jiang *et al.* 2014). The rate of accumulated epimutation is significantly higher in progenies of repeatedly salt stress-treated plants than progenies of control plants. Among CG-DMRs accumulated during 10 generations with stress and one generation without stress, over 75% of CG-DMRs are inherited to the next generation, suggesting that some stress-induced DMRs can be inherited to subsequent multi-generational progenies even without stress.

Growing seeds in discreate patches and collecting only dispersed seeds creates the artificial dynamic landscape, therefore simulates selection (Fakheran *et al.* 2010). Starting with 19 RILs between Cvi and L*er*, selected populations after five rounds of selection experiments showed later flowering and increased number of branches and siliques, accompanied by significantly reduced genetic variations, in which only 2 genotypes dominated the all populations (Schmid *et al.* 2018). Epigenetic variation, that is, single DNA methylation polymorphisms, among these populations were also reduced, compared to their ancestors, suggesting that epigenetic variation is also subjected to selection. Although there is no global correlation between differentially methylated cytosines accumulated during selection and gene expression, the expression of a lncRNA *At2g06002* was negatively associated with DNA methylation (Schmid *et al.* 2018). Selected populations tended to lose DNA methylation within *At2g06002* and showed the higher expression of *At2g06002*. Association of lower DNA methylation, higher gene expression and delayed flowering time was also observed among natural accessions. *At2g06002* locates upstream of *FIP1*, and the expression levels of *At2g06002* and *FIP1* were correlated. Since FIP1 interacts with FRIGIDA involved in flowering time regulation, this association may suggest a possible link between selection-associated hypomethylation of *At2g06002* and FIP1-FRIGIDA-mediated delayed flowering, thus can contribute to rapid adaptation (Schmid *et al.* 2018).

DNA methylation-associated gene activation

As described above, DNA methylation is associated with gene silencing. However, in some cases, DNA methylation can also be associated with gene activation (Harris *et al.* 2018). A forward genetic screen identified SUVH1 as an anti-silencing factor (Li *et al.* 2016). SUVH1 is required for both transgene and endogenous genes with methylated promoters. SUVH1 and its close homolog SUVH3 were also identified as methylated DNA binding proteins (Harris *et al.* 2018). SUVH1 and SUVH3 colocalize with RdDM target regions. SUVH1 and SUVH3 form a protein complex with chaperone proteins DNAJ1 and DNAJ2. DNAJ1 and DNAJ2 are essential for SUVH1/SUVH3 anti-silencing activities. Additionally, recruiting DNAJ1 to promoters enhanced reporter gene expression. Finally, constitutive expression of DNAJ1 upregulated the expression of genes proximal to DNAJ1 binding sites. Interestingly, *FWA*, which is stably silenced in the wild type plants, could not be reactivated by DNAJ1, suggesting that DNAJ1 activity is effective only on expressed genes. The underlying mechanisms of DNAJ1's preference for expressed genes are unknown, but are keys to enhanced expression of proximal genes, whereas TE expression remains silent.

Agronomic traits associated with DNA methylation

Previous studies have characterized the association between DNA methylation and observable phenotypes that are potentially important for crop yield or quality. Some of these phenotypes were initially described several decades ago. The peloric toadflax (*Linaria vulgaris*), which is a naturally occurring epi-mutant that was initially described in 1744, has radially symmetrical flowers, whereas the wild-type plant produces bilaterally symmetrical flowers (Gustafsson 1979). Mutations in the homeobox gene *CYC* lead to a similar phenotype in snapdragon (*Antirrhinum majus*), suggesting that a mutation in *Lcyc*, which is a toadflax homolog of *CYC*, is responsible for the peloric phenotype (Cubas *et al.* 1999, Luo *et al.* 1999). Indeed, *Lcyc* is transcriptionally silenced in the *peloric* mutant; however, no mutation was observed in the coding region or in the approximately 1-kb upstream region. Interestingly, the link between the peloric phenotype and *Lcyc* was initially identified by a restriction fragment length polymorphism, likely not by a genetic variant but because of the use of a DNA methylation-sensitive enzyme *Sau*3AI. Thus, a heavily methylated *Lcyc* is likely the basis for the peloric phenotype. Indeed, the occasional reversion of the peloric phenotype appears to be correlated with the demethylation of *Lcyc*.

Paramutation

Paramutation, which is also a classical epigenetic phenomenon (Chandler 2007), refers to an interaction between a paramutagenic allele and a paramutable allele. Both alleles can have identical DNA sequences. The paramutagenic allele induces a heritable change in the paramutable allele, which becomes paramutagenic. The paramutagenic state is heritable even after the original paramutagenic allele is lost

during segregation. The paramutagenic *R-stippled* (*R-st*) allele at the *r1* locus confers the spotted pigmentation of pericarps, whereas the paramutable *R-r* allele is responsible for full pigmentation (Kermicle *et al.* 1995). Although F_1 pericarps with *R-r/R-st* are fully pigmented, the pericarps of their progenies with the *R-r* allele exhibit decreased pigmentation. Loss of pigmentation is associated with the downregulated expression of the *r* gene and increased DNA methylation at the *r* locus (Walker 1998). A silenced *R-r* allele (*R-r'*) is inherited by the progenies, which revert to *R-r* phenotypes in a few generations (Brown and Brink 1960). The paramutagenic *B'* allele at the *b1* gene, involved in anthocyanin biosynthesis, confers the light pigmentation of the whole plant body, whereas the paramutable *Booster-Intense* (*B-I*) allele confers the dark pigmentation (Stam *et al.* 2002b). Seven tandem repeats of approximately 850 bp spanning about 6 kb located 100 kb upstream of *b1* are required for the paramutation of *b1* (Stam *et al.* 2002b). A transcriptionally active *B-I* allele is associated with increased chromatin accessibility and DNA methylation, compared to those in silenced *B'* allele (Stam *et al.* 2002a). In contrast to the frequent reversion from *R-st* to *R-r*, a newly established *B'* from *B-I* is stable, with no reports describing the reversion from *B'* to *B-I*. Several other paramutations have been reported in maize and in other plant species (Das and Messing 1994, Hollick *et al.* 1995, Pilu *et al.* 2009). A forward genetic screen identified genes required for paramutations in maize, including *MOP* genes and *RMR* genes (Chandler 2007). These genes are required for siRNA production, and contribute to RdDM, except for *RMR2*, implying that RdDM regulates paramutation. However, the transcriptional gene silencing (TGS) induced by RdDM in transgenic plants is less stable than that induced by paramutation when the trigger T-DNA is lost during segregation. Additionally, the alleles silenced by RdDM are not paramutagenic. Therefore, DNA methylation cannot solely explain paramutation.

Incompatibility

Sterility and inviability may result from crossing of distinct accessions (hybrid incompatibility) or the same accessions (self-incompatibility). Hybrid incompatibility produces reproductive barriers, whereas self-incompatibility leads to outcrossing. In Arabidopsis, the hybrid incompatibility between Col-0 and Shandara (Sha) is caused by the combined effects of the duplicated genes *FOLT1* and *FOLT2* (Durand *et al.* 2012). Both Col-0 and Sha possess *FOLT1*, whereas only Sha carries *FOLT2* along with two truncated *FOLT2* copies. The complete *FOLT2* sequence and the rearranged truncated *FOLT2* copies produce siRNAs that induce RdDM at the *FOLT1* locus that silences *FOLT1* in Sha. In contrast, the Col-0 *FOLT1* allele is actively expressed. Additionally, *FOLT2* is actively expressed in Sha. Recombinant inbred lines with insufficient *FOLT* transcripts (i.e. silenced *FOLT1* and lack of *FOLT2*) are not viable.

Histidine biosynthesis is essential for viability. Arabidopsis possesses two histidinol-phosphate aminotransferase genes (*HISN6A* and *HISN6B*). The Col-0 *HISN6A* allele is actively expressed, whereas the Cvi *HISN6A* allele is mutated and non-functional. The Col-0 *HISN6B* allele is silenced by CG and CHG methylation, whereas the Cvi *HISN6B* allele is actively expressed (Blevins *et al.* 2017). Recombinant inbred lines with the non-functional Cvi *HISN6A* allele and the silenced Col-0 *HISN6B* allele are not viable because of inhibited histidine biosynthesis.

In *Brassica* species, the recognition of self or non-self is controlled by haplotypes of the *S* locus encoding *SP11/SCR* and *SRK* (Kitashiba and Nasrallah 2014). The encoded SP11/SCR and SRK proteins function as the pollen-derived ligand and the stigmatic receptor, respectively, and the identical haplotype at the *S* locus causes self-incompatibility (Fujii *et al.* 2016). Additionally, *SP11/SCR* is expressed in the tapetum, and the dominance relationships between *SP11/SCR* determine the self-incompatibility phenotype in pollen grains (Kusaba *et al.* 2002, Shiba *et al.* 2002). In heterozygotes with both dominant and recessive *SP11/SCR*, the recessive *SP11/SCR* is silenced by the methylation within the promoter region, leading to the monoallelic expression of the dominant *SP11/SCR* allele (Shiba *et al.* 2006). The dominant *S* haplotype includes the inverted repeat(s) similar to those in the promoter region of the recessive *SP11/SCR* allele in the vicinity of the dominant *SP11/SCR* allele (Tarutani *et al.* 2010). The inverted repeat encoded by the dominant *S* haplotype is also expressed in the tapetum and produces 24-nt siRNAs targeting the promoter region of the recessive *SP11/SCR* allele in *trans*.

Sex determination

Most flowering plants, including crops, produce bisexual flowers with pistils and stamens. In addition to self-incompatibility, unisexual flowers that have either pistils or stamens enhance outcrossing. Consequently, sex determination is related to the expansion of genetic diversity. In the female flowers of melon (*Cucumis melo*), ethylene produced in the carpel primordia by the 1-aminocyclopropane-1-carboxylate synthase CmACS-7 represses stamen development (Boualem *et al.* 2008). In male flowers, the C2H2 zinc-finger transcription factor CmWIP1 arrests carpel development and indirectly represses *CmACS-7* expression. Moreover, the insertion of a *hAT* DNA transposon into the *CmWIP1* promoter converts a male flower to a female flower because of the dispersion of DNA methylation due to the *hAT* transposon and the subsequent silencing of *CmWIP1* (Martin *et al.* 2009).

Diploid persimmon (*Diospyros lotus*) is a dioecious species, in which an individual plant has either male or female flowers, whereas hexaploid persimmon (*Diospyros kaki*) is a monoecious species, in which an individual plant has both male and female flowers. Homeodomain transcription factor genes *MeGI* and *OGI* help mediate the sex determination in persimmon (Akagi *et al.* 2014). The encoded MeGI protein represses anther development in female flowers. The

Y-chromosome-encoded pseudogene *OGI* includes inverted repeats and produces 21-nt siRNAs targeting *MeGI*. In *D. lotus*, these 21-nt siRNAs post-transcriptionally silence *MeGI*, resulting in male flowers with fertile stamens. In *D. kaki*, *OGI* expression is suppressed by the insertion of a *Kali*-type SINE retrotransposon in the promoter region. Sex determination in *D. kaki* depends on the expression of *MeGI* (Akagi *et al.* 2016). Specifically, *MeGI* is silenced in male flowers because of DNA methylation at the *MeGI* locus, and the spontaneous conversion to female flowers is associated with demethylation at this locus. Additionally, zebularine treatment inhibits anther development in *D. kaki* male flower buds, likely because of the associated re-activation of *MeGI* expression.

Fruit ripening

Many fruits are edible and are important components of human/animal diets. Ripening alters fruit texture, flavor, taste, color, and nutrition. In addition to the plant hormone ethylene, the *SQUAMOSA* promoter-binding protein-like transcription factor CNR and the MADS-box transcription factor RIN are essential for fruit ripening in tomato (*Solanum lycopersicum*) (Eriksson *et al.* 2004, Thompson *et al.* 1999, Vrebalov *et al.* 2002). The dominant mutant *Cnr* and the semi-dominant mutant *rin* exhibit pleiotropic phenotypes, including the production of colorless fruits and delayed softening. In mature *Cnr* fruits, the *CNR* promoter is methylated in CG and CHG contexts, which silences *CNR* (Manning *et al.* 2006). In contrast, the same region is demethylated during ripening in wild-type fruits. Rare, but occasional revertant sectors in *Cnr* fruits are consistent with the epigenetic regulation of *CNR* (Zhong *et al.* 2013). Artificial global demethylation induced by a 5-azacitidine treatment causes premature ripening. During fruit ripening, the promoters of various ripening-related genes that are directly targeted by RIN are frequently demethylated by the DNA demethylase SlDML2 (Lang *et al.* 2017, Liu *et al.* 2015). In the fruits of *SlDML2* knock-down/knock-out transgenic plants, the demethylation of ripening-related genes is inhibited, which results in downregulated expression.

Vitamin E (VTE) is a valuable nutrient for humans. The VTE content in ripe fruits is higher for the wild tomato species *Solanum pennellii* than for the cultivated tomato *S. lycopersicum*. A 2-methyl-6-phytylquinol methyltransferase, VTE3(1), catalyzes the final steps of tocopherol biosynthesis, and *VTE3*(1) expression is correlated with VTE content (Almeida *et al.* 2011). In *S. lycopersicum*, downregulated *VTE3*(1) expression has been associated with the insertion of a SINE retrotransposon in the promoter and hypermethylation of the inserted SINE (Quadrana *et al.* 2014). The spontaneous demethylation of the *VTE3*(1) promoter leads to the upregulated expression of *VTE3*(1) and an increase in fruit VTE content.

Harvested tomato fruits are stored under cool conditions to extend their shelf-life, leading to a loss of flavor due to altered volatile synthesis. Cold storage downregulates the expression of *RIN* and some of its targets involved in fruit maturation and volatile synthesis. The expression of these genes resumes when plants are exposed to normal temperatures. The transient repression of these genes induced by a cold treatment is accompanied by increased DNA methylation within their promoters (Zhang *et al.* 2016). The expression of *SlDML2* is also transiently downregulated during cold storage but is immediately upregulated at normal temperatures, suggesting that SlDML2 contributes to changes to chilling-responsive DNA methylation and gene expression.

Somaclonal variation

Tissue cultures are widely used for clonal propagations and the generation of transgenic crops. However, abnormal phenotypes often arise after tissue culture processes related to dedifferentiation and regeneration. These phenomena are called somaclonal variations and have been applied for mutagenesis studies aimed at improving agronomic traits. Single nucleotide variants and small insertions/deletions are sources of somaclonal variations. In rice, reactivation of the *Copia*-type LTR retrotransposon *Tos17* located on chromosome 7 reportedly results in somaclonal variations (Hirochika *et al.* 1996). Additionally, *Tos17* is methylated throughout the life cycle but is demethylated by the DNA demethylase DNG701 in calli (La *et al.* 2011). The knockout of *DNG701* decreases *Tos17* activity, while the overexpression of *DNG701* has the opposite effect, indicating that DNA methylation has important effects on *Tos17* silencing.

Aberrant DNA methylation reprogramming is also a source of somaclonal variation. In rice calli grown under tissue culture conditions, loss of DNA methylation may occur stochastically. The loss of DNA methylation phenotype is randomly inherited by regenerated plants and can affect the expression of nearby genes. Notably, there are rice genome regions particularly susceptible to the loss of DNA methylation. In the dominant *df* mutant, hypomethylation in the promoter region of the rice homolog of *FIE1*, which encodes an Esc-like component of PRC2, induces the ectopic expression of *FIE1* (Zhang *et al.* 2012). Indeed, the constitutive expression of *FIE1* results in the same phenotype as that of the *df* mutant. Another representative example of the link between aberrant DNA methylations and somaclonal variations is the mantled phenotype of African oil palm (Ong-Abdullah *et al.* 2015), which is important for the production of edible oils and biofuels. Clonal propagation is widely used to improve yields. However, tissue culture techniques often induce the hypomethylation of a LINE retrotransposon in the intron of the homolog of the B-class MADS box gene *DEFICIENS*, resulting in alternative splicing and premature termination of expression. These changes result in the conversion of stamens and staminodes to pseudocarpels, leading to the production of parthenocarpic flowers and decreased oil yields.

Conclusions and perspectives

Plants potentially employ epigenome regulatory processes as a survival strategy, including for the maintenance of genome stability in germline cells and adaptation during cell differentiation and under long-term or transient stress conditions. Studies mainly involving Arabidopsis have revealed the mechanisms underlying DNA methylation regulation and dynamics. However, DNA methylation patterns and the set of DNA methylation associated genes are different among plant species, suggesting the importance of methylome analysis in individual crops (Bewick *et al.* 2016, Li *et al.* 2014, Niederhuth *et al.* 2016, Stroud *et al.* 2013). Advances in high-throughput sequencing techniques have enabled the identification of agronomic traits controlled by epigenetic regulation. Applying methods for creating even greater epigenomic variation may help breeders develop crops with new properties such as better qualities or those better enable to adapt to global environmental changes. Both targeted and global methods for epigenome editing represent an attractive new approach for plant breeding. Targeted *de novo* DNA methylation and gene silencing can be induced by expressing siRNAs. In addition, tethering SUVH2 to target gene promoters by an engineered zinc-finger induces DNA methylation and results in gene silencing (Johnson *et al.* 2014). Conversely, recruiting human TET1 by an artificial zinc-finger cause DNA demethylation of target genes and their reactivation (Gallego-Bartolomé *et al.* 2018). Other genome editing tools, such as transcription activator-like effector and dead Cas9 (dCas9) that loses nuclease activity, might also be applied to targeted epigenome editing in crops (Luo *et al.* 2018). Although DNA methylation deficit mutants are viable in Arabidopsis, they are often lethal in crops (Hu *et al.* 2014, Li *et al.* 2014, Moritoh *et al.* 2012, Yamauchi *et al.* 2014). Constitutive expression of TET1 randomly induces DNA demethylation so that resulting individual transgenic plants have distinct methylomes, leading to phenotypic variation (Ji *et al.* 2018). Since induced DNA demethylation is relatively mild, it is feasible to apply TET1-mediated epimutagenesis to crops. One anticipated problem of epigenome-edited crops involves the reversion of methylation status. Further characterizing the mechanisms underlying DNA methylation and demethylation may help researchers overcome the problems associated with epigenome-edited crops to improve sustainable agricultural practices.

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