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Topical Review

Population epigenetics: DNA methylation in the plant omics era

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Abstract

DNA methylation plays an important role in many biological processes. The mechanisms underlying the establishment and maintenance of DNA methylation are well understood thanks to decades of research using DNA methylation mutants, primarily in Arabidopsis (*Arabidopsis thaliana*) accession Col-0. Recent genome-wide association studies (GWASs) using the methylomes of natural accessions have uncovered a complex and distinct genetic basis of variation in DNA methylation at the population level. Sequencing following bisulfite treatment has served as an excellent method for quantifying DNA methylation. Unlike studies focusing on specific accessions with reference genomes, population-scale methylome research often requires an additional round of sequencing beyond obtaining genome assemblies or genetic variations from whole-genome sequencing data, which can be cost prohibitive. Here, we provide an overview of recently developed bisulfite-free methods for quantifying methylation and cost-effective approaches for the simultaneous detection of genetic and epigenetic information. We also discuss the plasticity of DNA methylation in a specific Arabidopsis accession, the contribution of DNA methylation to plant adaptation, and the genetic determinants of variation in DNA methylation in natural populations. The recently developed technology and knowledge will greatly benefit future studies in population epigenomes.

Introduction

DNA methylation, the addition of methyl groups onto a DNA molecule, was first discovered in the DNA of animals and plants in 1950 (Wyatt 1950), 3 yr before the double helix structure of DNA was determined. During the past 70 yr, extensive studies on DNA methylation have revealed its important roles in various biological processes, including regulating gene expression, maintaining genome integrity, and conferring adaptation to the environment. Several DNA methyltransferases have been identified in Arabidopsis (*Arabidopsis thaliana*), including DNA METHYLTRANSFERASE 1 (MET1; Kankel et al. 2003), CHROMOMETHYLASE 3 (CMT3; Lindroth et al. 2001), CHROMOMETHYLASE 2 (CMT2; Zemach et al. 2013), and DOMAINS REARRANGED METHYLTRANSFERASE 1/2 (DRM1/2; Cao et al. 2000). The knockout of all 5 methyltransferase genes yielded methylation-free plants with serious developmental defects (He et al. 2022).

The demethylation process, involving the removal of methyl groups from cytosines, is controlled by several DNA glycosylases, including DEMETER (DME; Choi et al. 2002), REPRESSOR OF SILENCING 1 (ROS1; Gong et al. 2002), and DEMETER-LIKE 2/3 (DML2/3; Penterman et al. 2007). The pathways and mechanisms of the establishment, maintenance, and removal of DNA methylation involving these methyltransferases and demethylases have been reviewed (Matzke and Mosher 2014; Matzke et al. 2015; Zhang et al. 2018a; Chakraborty et al. 2022; Leichter et al. 2022; To and Kakutani 2022). At the population level, DNA methylation shows great diversity: differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) among the Arabidopsis 1001 Epigenomes account for 78% of total methylated cytosines and 38% of the reference genome, respectively (Kawakatsu et al. 2016a). However, the genetic basis underlying this great variation is largely unknown. In this review, we focus on recent advances in methods for quantifying DNA

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ADVANCES

- The development of new bisulfite-free methods (i.e. third-generation sequencing and 5-letter sequencing) enables the simultaneous detection of genotypes and the quantification of genome-wide DNA methylation.
- The dynamic DNA methylation patterns illustrate the phenotypic plasticity within cells, tissues, individuals, and accessions.
- The genetic basis of the variation in DNA methylation within natural populations has been revealed by GWAS.

methylation, the plasticity of DNA methylation in Arabidopsis accession Col-0, and the genetic basis of variation in DNA methylation in natural populations.

Technology for genome-wide quantification of DNA methylation

Bisulfite-based next-generation sequencing

The first step in DNA methylation research is to detect and quantify methylation levels at either the individual locus or whole-genome scale. Dozens of approaches have been developed to achieve this goal (Fraga and Esteller 2002; Harrison and Parle-McDermott 2011; Kurdyukov and Bullock 2016). Among these, bisulfite-based methods are the most widely used. Whole-genome bisulfite sequencing (WGBS) has become the gold standard for quantifying DNA methylation at single-base resolution. While bisulfite treatment of DNA turns the unmethylated cytosines into uracil, the methylated cytosines remain unchanged. Following PCR amplification, the unmethylated cytosines will appear as thymines upon sequencing. After aligning the WGBS data to a reference, the methylation level of each cytosine is determined based on the proportion of thymines at a given locus (Frommer et al. 1992). To accurately quantify methylation levels, the coverage requirement of WGBS is usually higher than that of whole-genome sequencing (WGS) for single nucleotide polymorphism identification (Schmitz et al. 2022). This hinders the use of WGBS at the population level for species with large genomes, such as maize (*Zea mays*) (2.1 Gb; Hufford et al. 2021) and wheat (*Triticum aestivum*, 14.5 Gb; International Wheat Genome Sequencing 2018). To overcome this disadvantage, several alternative strategies have been developed to capture DNA methylation at target genomic regions, such as reduced representation bisulfite sequencing (RRBS; Meissner et al. 2005, 2008), methylated DNA immunoprecipitation sequencing (MeDIP-seq; Weber et al. 2005), genome-wide DNA methylation microarray (Bibikova et al. 2011), and the convert then capture of modified cytosines (Li et al. 2015).

Bisulfite-free long-read sequencing

One limitation of bisulfite-based sequencing approaches is the bias inherent in library preparation and methylation mapping. This is largely due to the harsh sodium bisulfite treatment conditions during the cytosine conversion reaction and the difficulty in uniquely aligning short reads to genomic regions harboring repetitive DNA sequences. The origin of the bias can be, but is not limited to, faster degradation of genomic regions enriched for unmethylated cytosines, incomplete cytosine conversion, and PCR bias caused by skewed base content (Olova et al. 2018). In recent years, many bisulfite-free strategies have been developed to reduce this bias via third-generation sequencing or enzymatic reaction-based sequencing techniques (Table 1).

Third-generation sequencing, also known as long-read sequencing, yields long reads with lengths ranging from 10 kb to several megabases. Nanopore sequencing records changes in current, which are used to distinguish among different types of nucleobases, when single-stranded DNA/RNA goes through a voltage-based nanoscale pore (Deamer et al. 2016). After training a computational model, the current signal can be used to accurately distinguish methylated from unmethylated cytosines, enabling the direct detection of DNA methylation (Fig. 1A; Rand et al. 2017; Simpson et al. 2017; Liu et al. 2019a). Single molecule real-time (SMRT) sequencing, an alternative third-generation sequencing technology, utilizes surface chemistry to immobilize a DNA polymerase into a nanostructure called zero-mode waveguides. SMRT determines the DNA sequence by detecting the distinguishable fluorescent pulse signal when a fluorescently labeled dNTP is incorporated during PCR (Eid et al. 2009). Given that methylation modification can affect the kinetics of DNA polymerase, SMRT sequencing has been used to detect cytosine methylation genome-wide. This method builds a "holistic kinetic" computational model from training data based on 3 parameters—the time needed for the incorporation of 1 base, the duration between 2 consecutive base incorporations, and the sequence context flanking the cytosine. This model is then applied to real SMRT sequencing data to detect methylated cytosines (Fig. 1B; Tse et al. 2021). These third-generation sequencing techniques directly profile the methylation of DNA without bisulfite treatment. The resulting long reads can easily span highly repetitive regions of less than 100 kb.

Bisulfite-free enzyme-based sequencing

In addition to long-read sequencing, 2 bisulfite-free methods are largely dependent on the enzymatic activities of ten-eleven translocation (TET) dioxygenase, beta-glucosyltransferase (β-GT), and APOBEC3A deaminase. TET oxidizes methylated cytosine to 5-carboxylcytosine (He et al. 2011), while APOBEC3A deaminates cytosines that are not oxidized by TET (Fig. 1C; Schutsky et al. 2017). TET-assisted pyridine borane sequencing (TAPS) utilizes TET and pyridine borane reduction to convert methylated cytosines to dihydrouracils, which are subsequently converted to thymines

Table 1. Comparison of methods for genome-wide quantification of DNA

Method	DNA input[a]	Bisulfite treatment	Enzyme treatment	Read length	Genetic variants	Training model	Cost
WGBS	100 ng	Y	N	<500 bp	ND	N	$
Nanopore	50 ng	N	N	>10 kb	D	Y	$$$$
SMRT	300 ng	N	N	>10 kb	D	Y	$$$$
TAPS	1 ng	N	Y	<500 bp	ND	N	$
EM-seq	100 pg	N	Y	<500 bp	ND	N	$
MethylSaferSeqS	30 ng	Y	Y	<500 bp	D	N	$$$
Five-letter seq	2 ng	N	Y	<500 bp	D	N	$$

[a]Minimum DNA input.
Y, required; N, not required; D, detectable; ND, not detectable.

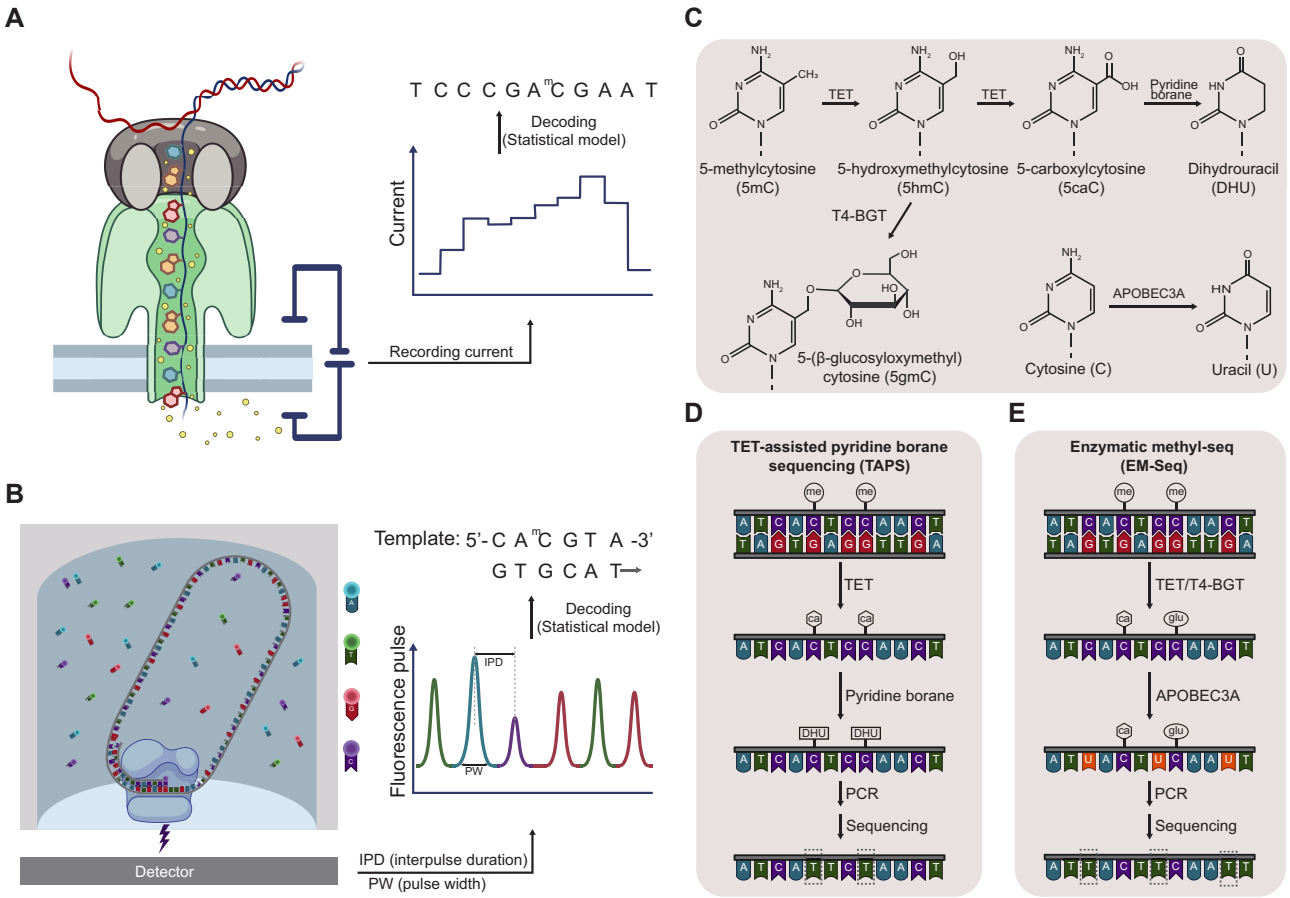

Figure 1. Bisulfite-free methods for the quantification of DNA methylation. **A)** Nanopore long-read sequencing. **B)** SMRT long-read sequencing. **C to E)** Two enzyme-based sequencing methods for the quantification of DNA methylation. The enzymatic reactions used in 2 bisulfite-free enzyme-based sequencing methods are shown in **C)**. **D and E)** are schematic diagrams of TAPS and EM-seq, respectively. Some elements in this figure were created with BioRender (BioRender.com).

during PCR (Fig. 1D; Liu et al. 2019b). Enzymatic methyl-seq (EM-seq) uses 3 enzymes in 2 consecutive reactions—TET and β-GT in the first reaction to convert methylated cytosines into products that cannot be oxidized by APOBEC3A, and APOBEC3A in the second reaction to deaminate unmethylated cytosines into uracils (Vaisvila et al. 2021). During subsequent PCR, products of the TET/β-GT reaction are converted to cytosines, while uracils are converted to thymines (Fig. 1E). Remarkably, EM-seq can be successfully performed with as little as 100 pg DNA as input.

Simultaneous detection of genetic and epigenetic information

Besides long-read sequencing technologies, MethylSaferSeqS and 5-letter seq are important techniques that can simultaneously determine genetic information and methylation status. Compared to traditional bisulfite library preparation, MethylSaferSeqS separates the original DNA template and amplification products after PCR, which are used as input for subsequent WGBS and WGS, respectively (Wang et al. 2023). Unlike MethylSaferSeqS, which stores genetic and

epigenetic information in 2 separate libraries, the 5-letter seq retains both genetic and epigenetic information in a single library (Fullgrabe et al. 2023). In this method, both ends of fragmented DNA are ligated to hairpin adapters containing a uracil residue. The 2 DNA strands are then separated by treatment with uracil-specific excision reagent. Upon synthesis of the cDNA strand, the resulting amplicons form a hairpin structure, with 1 strand containing the original epigenetic information and the complimentary strand maintaining the genetic information. Following ligation with sequencing adapters, methylated cytosines are protected from oxidation by APOBEC3A with TET and BGT, and unmethylated cytosines are converted to uracils with APOBEC3A and UvrD helicase. The methylated cytosines remain unchanged, and the unmethylated cytosines are converted to thymine in the final sequencing reads. Following the decoding rules, methylated and unmethylated cytosines are distinguished from sequencing reads for each hairpin (Fullgrabe et al. 2023). The 5-letter seq can determine genetic sequences and DNA methylation levels in a single library, dramatically reducing the cost of methylome studies in a population. These methods hold great potential for application in the field of population epigenomics, which requires information about both genetic variations and methylation patterns, especially for species with large genomes.

Profiling DNA methylation in a single cell or single cell type

The cytosines in DNA should be in 1 of 2 states (i.e. either methylated or unmethylated) in a living cell. However, the methylation level often quantitatively varies from unmethylated to fully methylated due to cell heterogeneity and the pooling of samples from tissues and individuals. More than a dozen methods have been developed to profile DNA methylation in mammals from a single cell during the past decade (reviewed by Karemaker and Vermeulen 2018; Vandereyken et al. 2023). These include single-cell reduced representation bisulfite sequencing (scRRBS; Guo et al. 2013), single-cell bisulfite sequencing (scBS-seq; Smallwood et al. 2014), single-cell CpG island methylation sequencing (scCGI-seq; Han et al. 2017), and Smart-RRBS for DNA methylation and transcription in a single cell (Gu et al. 2021). Single-cell multiomics sequencing technology (scNOMeRe-seq) is used for genome-wide profiling of chromatin accessibility, DNA methylation, and RNA expression simultaneously from the same single cell (Wang et al. 2021). Compared to the rapid development of these techniques in mammals, the progress in profiling single-cell DNA methylation in plants has been much slower. While the majority of single cell studies in plants have focused on the transcriptome, 1 study revealed the single-cell DNA methylomes of 16 microspores from 4 tetrads in maize using bisulfite-converted randomly integrated fragments sequencing (BRIF-seq; Li et al. 2019). WGBS, combined with fluorescence-activated cell sorting, has allowed the DNA methylomes of specific cell types to be revealed, including 6 different root meristem cell populations (Kawakatsu et al. 2016b), sperm and vegetative cells (Hsieh et al. 2016), and stem and nonstem shoot meristem cells (Gutzat et al. 2020). These studies have shed light on the heterogeneity of plant cells and the dynamics of DNA methylation among various cell types.

The plasticity of DNA methylation

Variation in DNA methylation within Arabidopsis accession Col-0

At the individual level, DNA methylation is dynamic and can vary among generations of plants from the same ancestor (Becker et al. 2011; Schmitz et al. 2011), from different laboratories (Fig. 2A; Zhang et al. 2018b), in tissues from the same plant (Fig. 2B; Widman et al. 2014; Williams et al. 2022), and in cells from the same tissue (Fig. 2C; Kawakatsu et al. 2016b; Gutzat et al. 2020). The difference of methylation levels of Col-0 genomic DNA from different labs reaches up to 10% for CG and 7% for non-CG methylation (Fig. 2A). DNA methylation patterns show context-dependent differences, with stable CG methylation, increased CHG methylation, and decreased CHH methylation observed during various stages of development (Gutzat et al. 2020). Notably, most development-associated changes in methylation occur in centromeric transposable elements (TEs).

In contrast, the differences in methylation patterns between tissues (i.e. roots and shoots) are dynamic, with great variations in CG methylation in euchromatic regions and non-CG methylation in centromeric regions (Widman et al. 2014). This epigenome plasticity is largely attributed to spontaneous epigenetic mutation. Compared to genetic mutation, the rate of epigenetic mutation is several orders of magnitude higher (Becker et al. 2011; Schmitz et al. 2011; Yao et al. 2023). Interestingly, epimutation hotspots have been identified in the Arabidopsis genome. Although these regions only cover ~12% of CG sites in the genome, ~63% of the epimutation events were observed in these hotspots (Hazarika et al. 2022). Another important factor in differences in methylation patterns is the tissue/cell-specific expression of the methylation machinery. Ten genes are known to participate in epigenetic regulation, including *DECREASE IN DNA METHYLATION 1* (*DDM1*), *ARGONAUTE 9* (*AGO9*), and *SU(VAR)3-9 HOMOLOG 4* (*SUVH4*), which are upregulated in stem cells compared to nonstem cells (Gutzat et al. 2020). CLASSY family genes exhibit a tissue-specific expression pattern; these proteins regulate 24-nt siRNA production and DNA methylation in a tissue-specific matter (Zhou et al. 2022). Active DNA demethylation is another contributor to the differential methylation among tissues. In Arabidopsis, DNA demethylation is controlled by 4 DEMETER family members—*DME*, *DML2*, *DML3*, and *ROS1* (Law and Jacobsen 2010). Many tissue-specific changes in methylation identified in the wild type were absent in quadruple mutants of these 4 genes (Williams et al. 2022).

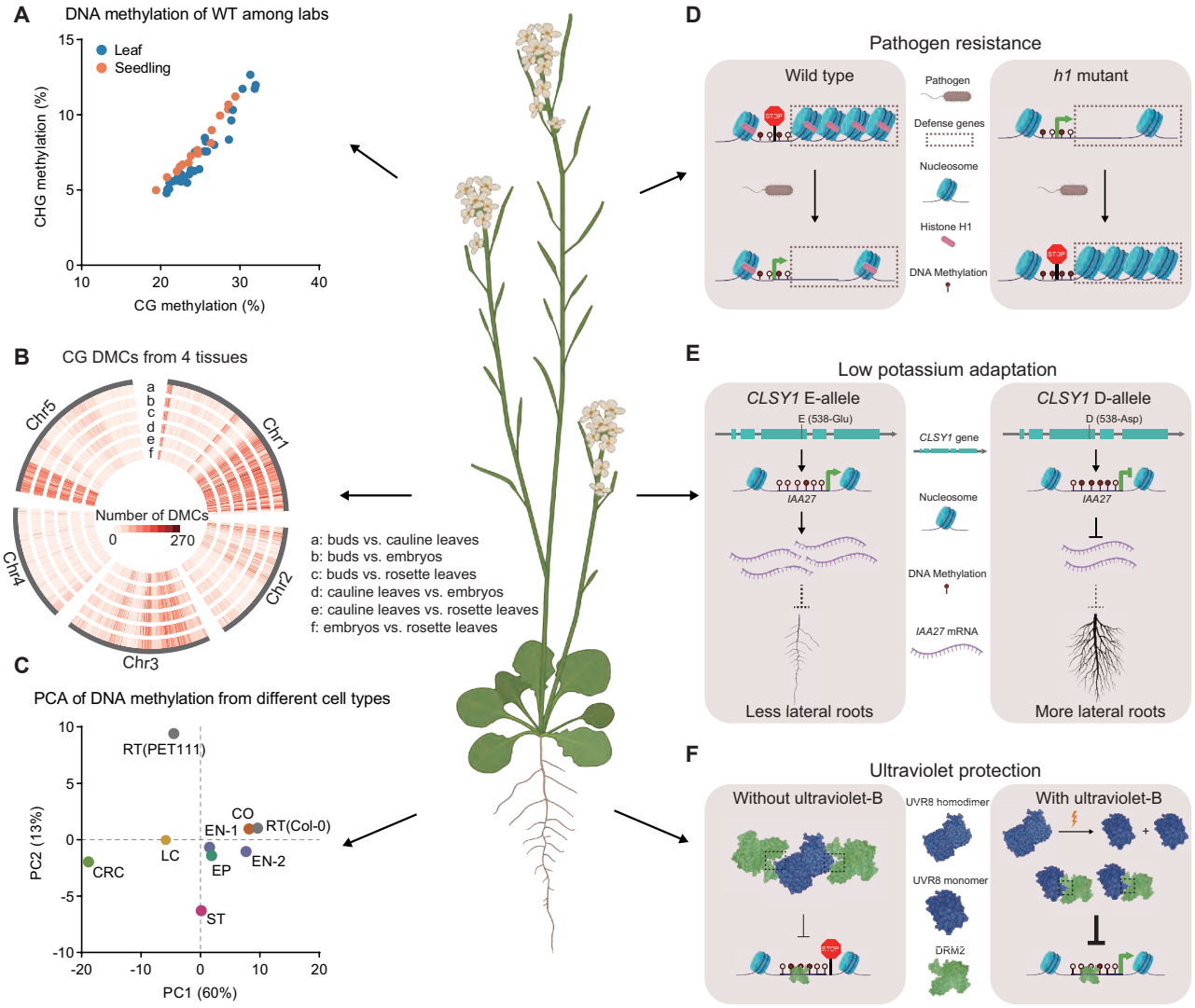

Figure 2. The plasticity of DNA methylation and the contribution of DNA methylation to plant adaptation. **A)** The genome-wide average CG and CHG methylation levels of 50 high-quality WGBS data sets from wild-type (WT) Arabidopsis Col-0 from different labs. The data used to construct this plot can be found in Zhang et al. (2018b). **B)** The distribution of CG DMCs identified by comparing DNA methylation data from 4 tissues. The data used to construct this plot can be found in Williams et al. (2022). **C)** Principal component analysis (PCA) of CG, CHG, and CHH methylation levels in 100 kb windows from 9 root cell types. The data used to construct this plot can be found in Kawakatsu et al. (2016b). RT, root tip; EP, epidermis; CO, cortex; EN, endodermis; ST, stele; CRC, columella root cap; LC, lower columella. **D)** DNA methylation is important for plant sensitivity to immunity priming. **E)** Changes in DNA methylation of *IAA27* coupled with the genetic variation of *CLSY1* affect lateral root development and confer adaptation to a low-potassium environment. The "T"-shaped solid line indicates the repression of the expression of *IAA27* by increased DNA methylation. The "T"-shaped dashed lines indicate the negative regulation of lateral root development by the expression of *IAA27*, with the thicker one having stronger repression due to higher expression. **F)** The decrease in DNA methylation induced by the UVR8-DRM2 module following exposure to UVB activates downstream genes/TEs. The dashed boxes indicate the physical interaction sites between UVR8 and DRM2. The "T"-shaped solid lines indicate the inhibition of DRM2-mediated DNA methylation, with the thicker one having stronger inhibition. The diagram of Arabidopsis and some elements in **D to F)** were created with BioRender (BioRender.com).

Variation in DNA methylation in response to the environment

Accumulating studies have demonstrated that DNA methylation, a heritable modification of cytosines, can respond and adapt to various biotic and abiotic stress factors. This topic has been the focus of several reviews (Feil and Fraga 2012; Meyer 2015; He and Li 2018; Zhang et al. 2018a; Alonso et al. 2019; Lloyd and Lister 2022). In this section, we focus on the latest studies in Arabidopsis describing the important roles of DNA methylation in the adaptation of plants against biotic and abiotic stress.

Whereas genetic mutations confer long-term plant adaptation, DNA methylation can rapidly respond environmental challenges and quickly enhance fitness. Sheikh et al. (2023)

recently discovered the important roles of the linker histone H1 and DNA methylation in plant defense. An Arabidopsis triple mutant of 3 histone H1 variants (*h1.1 h1.2 h1.3*) showed increased resistance to plant pathogen infection due to the altered expression of plant defense genes. The priming process involving pretreatment with 22-amino-acid flagellin (flg22) prior to pathogen infection enhanced the resistance of wild-type plants to subsequent pathogen infection. However, the *h1* mutant was insensitive to priming. This could be partially explained by the observation that flg22 treatment resulted in increased DNA methylation in the promoters of defense genes in *h1*, leading to their repression (Fig. 2D; Sheikh et al. 2023). This study suggests that DNA methylation can quickly respond to pathogen infection and affect plant defense by regulating the expression of defense genes.

DNA methylation can also enhance plant adaptation during long-term evolution, dependent or independent of genetic mutations. A recent study by Shahzad et al. (2020) showed that changes in DNA methylation coupled with genetic mutation allow plants to cope with potassium deficiency. Sensing and responding to nutrient elements in the soil are essential for plants. The deficiency of potassium, an essential nutrient element, can lead to poorly developed roots (Tsay et al. 2011). Different Arabidopsis accessions have evolved 2 strategies to overcome low potassium stress by increasing the growth of main or lateral roots (Kellermeier et al. 2013). Using genome-wide association study (GWAS), Shahzad et al. (2020) identified CLSY1 as a regulator of lateral root development under low-potassium conditions. Low potassium prevents the degradation of INDOLE-3-ACETIC ACID INDUCIBLE 27 (IAA27), which negatively regulates root branching via the auxin signaling pathway. In parallel, CLSY1 can silence *IAA27* through DNA methylation. The change of aspartate to glutamate at position 538 of CLSY1 is significantly associated with lateral root development under low-potassium conditions in natural Arabidopsis accessions. Accessions harboring the *CLSY1* aspartate-encoding allele showed significantly higher DNA methylation of the *IAA27* promoter and lower expression of *IAA27* than accessions harboring the glutamate-encoding allele (Fig. 2E; Shahzad et al. 2020). These findings demonstrate that DNA methylation can coordinate genetic changes and facilitate lateral root development to enable plants to overcome a challenging environment.

Although DNA methylation has been implicated in plant adaptation, the evidence is mostly from studies showing the association of changes in DNA methylation with environmental changes. Jiang et al. (2021) provided direct evidence to illustrate how exposure to UV light can suppress DNA methylation. DNA methylation in Arabidopsis responded to UVB light through the physical interaction between UV RESISTANCE LOCUS 8 (UVR8, a UVB photoreceptor) and DRM2 (a de novo DNA methyltransferase). UVB irradiation induced genome-wide DNA hypomethylation and derepression of TEs via a UVR8-dependent pathway (Fig. 2F). This UVR8-DRM2–mediated TE reactivation mechanism could directly or indirectly regulate the expression of key genes involved in plant protection against UV exposure.

The genetic basis of variation in DNA methylation in natural populations

Studies of both specific genomic loci and genome-wide methylomes of different Arabidopsis accessions have shown great variations in DNA methylation among natural accessions (Vaughn et al. 2007; Schmitz et al. 2013; Kawakatsu et al. 2016b). In contrast to the well-characterized regulators of the establishment and maintenance of DNA methylation in an individual accession (Fang et al. 2021, 2022; He et al. 2022; Leichter et al. 2022), the genetic and mechanistic basis of the variation in DNA methylation among natural accessions is poorly understood. Several contributors to this process have been identified by GWAS (Baduel and Sasaki 2023). Among these, *CMT2* was mapped by multiple GWASs based on non-CG methylation levels of the whole genome, DMRs, and CMT2-targeted TEs (Shen et al. 2014; Dubin et al. 2015; Kawakatsu et al. 2016a; Sasaki et al. 2019; Hüther et al. 2022; Sasaki et al. 2022). This aligns well with the function of CMT2 as a DNA methyltransferase responsible for maintaining CHH methylation (Zemach et al. 2013) and the strong correlation between CHH and CHG methylation (Sasaki et al. 2022). Another major determinant of CHH methylation at TEs is *NRPE1*, which encodes the largest subunit of RNA polymerase V and a key component of the RNA-directed DNA methylation (RdDM) pathway (Kawakatsu et al. 2016a; Sasaki et al. 2019). The role of *NRPE1* in controlling the mobilization of TEs was also revealed via GWAS (Baduel et al. 2021).

In a GWAS, *miR823A* was found to be frequently associated with CHG DMRs (Hüther et al. 2022). Another GWAS of CHG methylation of RdDM/CMT2-targeted TEs identified both *CMT3* and *miR823A* after setting CHH methylation as a covariate (Sasaki et al. 2022). *CMT3* encodes a DNA methyltransferase responsible for CHG methylation maintenance (Lindroth et al. 2001), while *miR823A* encodes microRNA823A, which is predicted to target *CMT3*. These findings point to possible *miR823A-CMT3* module-mediated regulation of CHG methylation, although further validation is required.

ARGONAUTE genes, including *AGO9* and *AGO1*, have been identified as regulators of CHH methylation of RdDM-targeted TEs in natural populations (Kawakatsu et al. 2016a; Sasaki et al. 2019). The functions of these genes in these accessions may differ from the function of *AGO9* in Col-0, as the *ago9* mutant showed no changes in methylation in RdDM-targeted hypo-DMR regions (Stroud et al. 2013). This suggests that AGO proteins that are not involved in the RdDM pathway in Col-0 may participate in DNA methylation in other accessions.

MULTICOPY SUPPRESSOR OF IRA1 (*MSI1*) encodes a subunit of Polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of histone 3 at lysine 27 (H3K27me3; Kohler et al. 2003; Bemer and Grossniklaus 2012). In 2 recent GWAS, *MSI1* was shown to be associated with CHG methylation at 44 DMR regions, as well as RdDM- and CMT2-targeted TEs (Hüther et al. 2022; Sasaki

et al. 2022). In the Col-0 background, H3K27me3 has been shown to be independent of DNA methylation (Zhang et al. 2007), unlike H3K9me2, which is required for CHG methylation by CMT3 (Fang et al. 2022). These findings point to a possible relationship between DNA methylation and H3K27me3 in other accessions, which remains to be characterized.

Although not repeatedly identified, genetic variations of *JUMONJI26* (*JMJ26*)—a homolog of *INCREASE IN BONSAI METHYLATION 1* (*IBM1*)—were reported to be associated with CHG methylation of RdDM-targeted TEs in conditional GWAS using CHH as a covariate (Sasaki et al. 2022). Knockout of *JMJ26* resulted in increased CHG methylation of RdDM-targeted TEs (Sasaki et al. 2022). The key for the identification of *JMJ26* is conditional analysis in which CHH methylation is included as a covariate when performing GWAS of CHG methylation. This finding highlights the potential for innovations in GWAS, including improved methods or population construction, in population epigenetic studies.

Besides *trans* regulators, a substantial number of genetic variations, whether nearby or at a considerable distance, have been shown to be associated with variations in DNA methylation at the target sites (Schmitz et al. 2013; Dubin et al. 2015; Sasaki et al. 2019; Hüther et al. 2022). Still, little is known about the causal genetic changes, as there has been no validation of the genetic changes underlying these associations. Several studies have demonstrated that genetic variations, such as TE insertions and structural variations (SVs), can influence DNA methylation at nearby sites. Ahmed et al. (2011) found that hundreds of TEs with no matching 24-nt siRNA acquired DNA methylation through the spreading of the methylation from adjacent densely methylated TEs with matching 24-nt siRNA. Another study identified the recent TE transpositions in 211 Arabidopsis accessions and found that half of the new TE insertion sites are highly methylated and spread to adjacent region in accessions with TE insertions (Quadrana et al. 2016). Direct evidence comes from the de novo deposition of CEN180 repeats into a euchromatic target site, which induced the establishment and spreading of local DNA methylation in the *ibm1* mutant (Liu et al. 2023). In the Arabidopsis 1001 Methylomes, a large proportion of SVs (22% to 50%) are differentially methylated (Kawakatsu et al. 2016a), highlighting the impact of SV on the DNA methylation of flanking sequences. The best example showing how SV affects DNA methylation is the *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*) gene family (*PAI1* to *PAI4*) in Arabidopsis. Some accessions contain unmethylated *PAI* genes, such as Col, while others contain methylated *PAI* genes, such as WS. Col contains 3 unlinked *PAI* genes (*PAI1*, *PAI2*, and *PAI3*), while WS contains 4 *PAI* genes with a *PAI1* to *PAI4* inverted duplication. This inverted repeat leads to methylation of all 4 *PAI* genes (Bender and Fink 1995; Luff et al. 1999; Melquist et al. 1999).

OUTSTANDING QUESTIONS

- To what extent does epigenetic variation contribute to plant adaptation?
- How can the functions of epialleles be discovered and validated in a high-throughput manner?
- How can epialleles efficiently and effectively be applied in crop breeding programs?

Concluding remarks

Recent GWASs have provided important information about the genetic basis of the natural variation in the non-CG methylation of TEs. However, our understanding of the genetic basis of CG methylation and the DNA methylation of other genomic features, such as promoter regions and protein-coding genes, remains largely unknown. The discovery and validation of epiallele functions in a high-throughput manner and the application of epialleles in crop breeding programs represent important future endeavors (see "OUTSTANDING QUESTIONS"). Furthermore, the availability of a genome-wide chromatin atlas with a multiomics data set at the population level will undoubtedly enhance our understanding of epigenetic variations. The use of combination of big data analysis with epigenetic editing technologies for the mining and application of epigenetic variations will greatly enhance crop breeding programs.

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Author Contributions

J.L. and X.Z. wrote the paper.

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Conflict of interest statement. None declared.

Data availability

No new data were generated in this research.

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