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Review

Recent Advances in DNA Methylation and Their Potential Breeding Applications in Plants

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Abstract: Traditional plant breeding encompasses repetitive crossing and selection based on morphological traits, while phenotypic selection has been complemented by molecular methods in recent decades. Genome editing with techniques like the CRISPR-Cas9 system is still a novel approach that is being used to make direct modifications to nucleotide sequences of crops. In addition to these genetic alterations, an improved understanding of epigenetic variations such as DNA methylation on the phenotype of plants has led to increased opportunities to accelerate crop improvement. DNA methylation is the most widely studied epigenetic mark in plants and other eukaryotes. These epigenetic marks are highly conserved and involved in altering the activities and functions of developmental signals by catalyzing changes in the chromatin structure through methylation and demethylation. Cytosine methylation (5mC) is the most prevalent modification found in DNA. However, recent identification of N6-methyladenosine (6mA) in plants starts to reveal their critical role in plant development. Epigenetic modifications are actively involved in creating the phenotype by controlling essential biological mechanisms. Epigenetic modifications could be heritable and metastable causing variation in epigenetic status between or within species. However, both genetic and heritable epigenetic variation has the potential to drive natural variation. Hence, epigenome editing might help overcome some of the shortcomings of genome editing (such as gene knockout), which can have significant off-target effects and only enables the loss of a gene's function. In this review, we have discussed the mechanism underlying DNA methylation and demethylation in plants. Methyltransferases and demethylases are involved in catalyzing specific types of modification. We also discuss the potential role of DNA modifications in crop improvement for meeting the requirements of sustainable and green agriculture.

Keywords: epigenetics; DNA methylation; 6mA; 5mC; epigenetic breeding

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1. Introduction

Epigenetics is one of the most fascinating and topical fields of genetics, adding to classical knowledge about how genes interact with phenotypes, which has perplexed scientists for decades [1]. The concept of epigenetics was proposed in the mid-twentieth century by Waddington, by integrating epigenesis and genetics to elucidate the phenotypic

traits of plants as a result of the causal interaction between genes and their products [2]. However, our present understanding of molecular biology has led us to a narrower definition that comprises the study of molecular processes in and around DNA that regulate genome-related activity and phenotype, regardless of DNA sequence. It can be inherited via mitosis or meiosis, and several studies have shown that stress-induced epimutations are successfully passed down to the next generation [3–7]. Epigenetics, as defined by Arthur Riggs and his colleagues, is the study of mitotically and meiotically inherited changes in gene function that cannot be explained by changes in DNA sequence [8]. Epigenetics has had considerable success due to its applications in plant breeding, which has been used to examine the transmission of epigenetic marks through generations to enhance desirable traits in crops [9]. Epigenetics has the potential to be employed as a crop improvement tool. Epigenetic modifications, including DNA methylation, histone modifications, chromatin remodeling, and activity of small RNAs (sRNAs), are thus inherited but do not follow the known inheritance patterns [1].

Because of its heritability and potential to influence plant phenotypes, DNA methylation seems to be a viable source of variation contributing to crop fitness and production. Variations in DNA methylation impact agronomically important traits including seed dormancy, flowering time, and yield, and the partial heritability of DNA methylation patterns reveals epigenetics had a role in plant domestication and evolution [10–12]. Understanding DNA methylation modifications would enable breeders to concurrently create favorable variations while limiting undesirable epigenetic changes that can be caused by breeding practices such as tissue culture [13–17]. Various approaches have previously been established for this purpose, and efficient induction of epigenetic modifications for plant breeding necessitates a technical grasp of the molecular mechanisms involved in both its introduction and its maintenance over successive generations.

DNA methylation being a conserved epigenetic mark regulates various cellular processes in eukaryotes including plants. Several mechanisms have been reported associated with this epigenetic mark such as genomic imprinting, X-chromosome inactivation, cell type differentiation during embryonic development, genome stability, chromatin architecture, transcription activation, embryogenesis, chromosome stability; it is associated with gene and transposon silencing [18–23]. The methylation patterns are heritable and have transgenerational effects [24]. However, the methylation marks are dynamic and show variations during reprogramming stages throughout the life cycle of an organism [25]. Moreover, methylation is significantly involved in stem cell pluripotency and cellular differentiation to distinct behaviour, memory and ageing [26–28]. At the population level, DNA methylation participates in the biodiversity of natural populations, disease susceptibility and response to environmental stimuli [29].

5mC has a significant role in regulating gene expression and TE silencing [30–32]. DNA methylation starts by stabilizing the DNA double helix, strongly affecting the binding of various proteins on DNA including regulatory proteins. Moreover, sometimes it prevents the binding of certain nuclear proteins which are vital for transcription and play significant roles in cellular pathways [33]. In contrast, some proteins arrange an entire ensemble on a methylated DNA sequence by specifically binding on it and regulate gene expression. Hence, DNA methylation can be defined as a factor of positive or negative control of transcription. The proliferation and accumulation of TE sequences are also influenced by epigenetic mechanisms. TEs are targeted for DNA methylation by small RNA (sRNA)–mediated mechanisms in several eukaryotic lineages [34]. DICER-LIKE RNase enzymes in plants generate 24-bp sRNA that directs ARGONAUTE and other downstream proteins to complementary DNA sequences, promoting and sustaining DNA and histone methylation [35,36]. In *Arabidopsis*, TE methylation has been found to silence transposition, as evidenced by drastically increased levels of TE transcription in *met1* methylation mutants [37,38]. Silencing TEs near genes may also inhibit the generation of aberrant transcripts via read-through transcription beyond TE termini [39], in addition to preventing the proliferation of new TE sequences. However, methylated sequences can influence the

expression of nearby genes, typically reducing expression [40]. Expression of flowering time gene FWA has been found to be correlated with the methylation status of nearby SINE-like TE. Hence, changes in gene expression caused by methylation of neighboring TEs may have a deleterious impact on gene and genome function.

Differences in cytosine DNA methylation patterns can contribute to phenotypic variability because they are inherited through both mitotic and meiotic cell divisions. High-throughput sequencing technologies have made it possible to generate a large amount of DNA sequence data. The underlying mechanisms and functions of DNA methylation have been discovered by integrated investigations of genome-wide gene expression patterns and DNA methylation patterns. There have also been associations discovered between DNA methylation and agronomic characteristics. The results could be relevant for future crop breeding applications of natural epigenomic variation. Artificial epigenome editing could also be a promising new plant breeding strategy for developing novel varieties with improved agronomic traits [41]. With its potential for disease resistance enhancement, DNA methylation presents a new direction to both scientists and breeders by providing a new source of variation [42]. Moreover, a recent study discovered that DNA methylation can cause epigenetic modifications in plants in response to abiotic stresses [43].

In this review, we explain the DNA 5mC methylation machinery, diversity and dynamics, molecular and biological functions in plants. We also discuss the newly identified N6-methyladenosine (6mA) dynamic distribution and responses to developmental signals in model plants such as *Arabidopsis thaliana* and rice (*Oryza sativa*). We consider DNA methyltransferases and demethylases that catalyze specific DNA modifications (5mC, 6mA), recent advances and understanding in DNA methylation and demethylation in genetic regulation and functions with emphasis on DNA methylation in plants. We also highlight knowledge gaps, and discuss challenges and opportunities for exploitation of DNA methylation in breeding applications.

2. DNA 5mC in Plants

Methylation marks in DNA show dynamic regulation of establishing, maintaining, and active removal of their activities. Among all the methylation modifications discovered to date, 5mC is the well-known and well-studied DNA mark and has been widely identified in plants and other eukaryotes. 5mC has been located in all sequence contexts such as CG, CHG and CHH (where H = A, T or C) [44]. *De novo* methylation on all sequence contexts is regulated by production of small interference RNAs (siRNA) comprising ~21–24 nucleotides [45]; it is an intricate process that is highly controlled by the RNA directed DNA methylation (RdDM) pathway. RdDM is a transcriptional gene silencing pathway that is specific to plants only, and is directed by siRNA molecules leading to DNA *de novo* methylation. The multistep pathway consists of polymerase IV-dependent siRNA biogenesis, polymerase V mediated *de novo* methylation and chromatin alteration [23,45,46]. It initiates with the production of siRNA, which consists of specific DNA dependent RNA polymerase Pol IV and V, the Pol IV interacting Sawadee homeodomain homolog 1 (SHH1), double-stranded RNA endonuclease DCL3, RDR2, and Argonaute proteins (AGO4 and AGO6) with the effector kow-domain containing transcription factor 1 (KTF1) [45,47–49].

The family of methyltransferases catalyze the establishment of 5mC by transferring a methyl group from S-adenosylmethionine (SAM) to the substrate (Figure 1) [28], while the maintenance of pre-existing methylation continues particularly on CG and CHG motifs, which are known as symmetrical sites as they are read in 5′ to 3′ direction. This symmetry after each round of replication provided a base for cytosine methylation on a mother strand and transfer to daughter strands. Following the DNA repair mechanism, methylation can take place, allowing newly synthesized strands to be methylated. Comparable to CHG and CHH methylation, CG methylation also takes place over gene bodies, while the CHG and CHH sequence context is fully involved in transcriptional gene silencing (TGS) and heterochromatin formation [50].

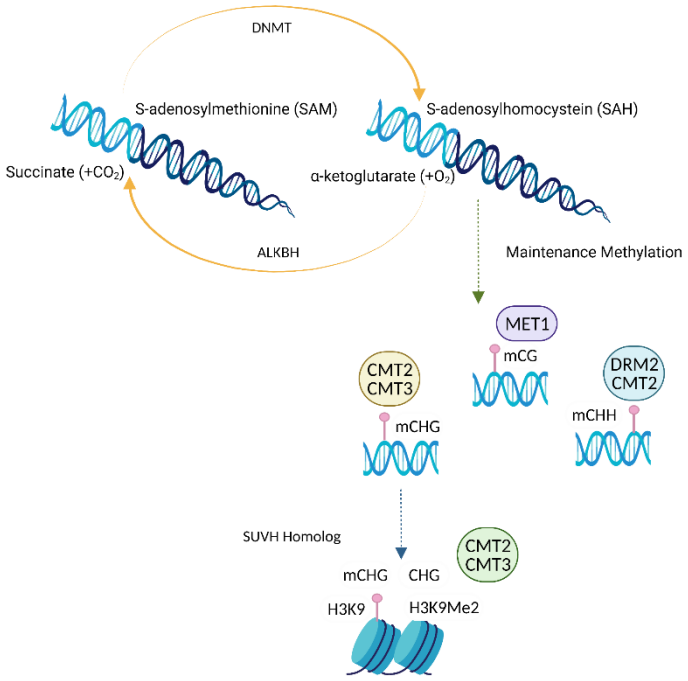

Figure 1. Maintenance of methylation and demethylation in plants.

2.1. Distribution of 5mC in Plant Genome

So far, DNA methylation has been detected in a variety of plant species, including algae, cereal crops, vegetables and trees [51]. The importance of DNA methylation cannot only be described by its abundance, but is also dependent on its location on different parts of the gene and its positioning such as CH, CHG and CHH. A widespread diversity in the type, amount, and location of DNA methylation in a gene has been observed at different developmental stages in various plant species. In mammals, 5mC is mostly found on the CG sequence context, while in plants its presence in all sequence contexts has been observed. In *Arabidopsis*, gene bodies are mainly targeted by CG methylation; while CHG and CHH methylation mostly occur on TEs and repeats, CG methylation is also found on TEs. Genome-wide methylation in *Arabidopsis* shows that heterochromatin is a highly methylated region, which is enriched by transposons and other repetitive sequences [52].

Phylogenetic investigations of methylomes of more than 30 eukaryotes, a majority being plant species, have revealed the significant variation in CG and non-CG methylation among different species [51,53]. The lowest methylation levels, 5.4% CG, 2.6% CHG and 2.5% CHH were found in *C. reinhardtii* [51], while the highest levels were observed in Beta vulgaris, where 92.6% CG, 81.2% CHG and 18.9% CHH methylation were present. In *Arabidopsis* leaf, CG, CHG and CHH methylations were reported as 30.5%, 10.0% and 3.9%, respectively. Rice leaves displayed intermediate levels of CG, CHG and CHH methylation as 54.4%, 31.0 and 5.1% respectively. In the maize genome, 86% CG, 74% CHG and 5% CHH methylation have been reported [51,53]. Hence, methylation levels in all three sequence contexts vary significantly among different organisms.

Compared to CHG and CHH methylation, CG methylation has been found predominant. CG methylation in angiosperms contributes to more than 50% of total cytosine methylation [53]. Higher variation in CHG and CHH levels is found as compared to CG methylation, suggesting that DNA methylation patterns are diverse in various plant species. In plants, non-CG methylation plays vital role in exogenous DNA silencing via the RdDM pathway [21]. In *Arabidopsis*, during the early stage of plant development, higher invariable CG methylation was found on TEs and repeats while increasing levels of CHG and CHH methylation were observed. This non-CG methylation culminates in the mature embryo, where it reaches higher levels as compared to seedlings or adult plants [54].

2.2. Derivatives of 5mC

Apart from 5mC, the most common modification, several other types of changes on the same position have also been found to exist, for instance, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC); these modifications have also been considered the most critical marks [55]. Active demethylation through ten eleven translocation cytosine dioxygenases (TETs) oxidize 5mC to 5hmC and successive oxidation produces 5fC and 5caC, which are found in lower abundance in the genome and can be removed by BER proteins [56–58]. 5hmC is also regarded as the sixth nucleobase in DNA; its presence is reported in mammals and it is found to play key functions in gene regulation [59]. Recent studies revealed that 5hmC regulates various cellular and developmental pathways such as neuron development in mammals, tumorigenesis and embryonic stem cells pluripotency [55].

DME and ROS1 purified from *E. coli* have the ability to excise 35-mer oligonucleotides comprising of Cytosine, 5mC, 5hmC, 5fC and 5caC, suggesting that they can cleave both the 5mC and 5hmC in vitro. Previously, several attempts have been made for the detection of 5hmC in plants [59–63]. However, the *Arabidopsis* genome does not contain 5hmC [64]. Based on these studies, it can be concluded that either a lower abundance of 5hmC is present in plants or there is a distinct mechanism and independent evolution of DNA demethylation in plants as compared to animals. The presence of 5hmC in plants is still under debate. Recently, 5hmC has been detected in a plant genome. Wang et al. (2015) conducted a study using three rice cultivars and found low levels of 5hmC at 1.39 ± 0.16 and 2.17 ± 0.03 per million nucleotides in leaf and panicle, respectively. Levels of 5hmC varied significantly among different tissues. 5hmC sites were mainly enriched in heterochromatin regions and TEs, particularly around retrotransposons [59]. These findings will provide a base for studying, detecting and understanding 5hmC in plants and their functions in plant development and DNA demethylation.

2.3. Writers, Erasers and Readers of DNA Methylation

In plants, the DNA methylation mechanism is highly complicated and much richer as compared to animals [65]. These methylation modifications are enzymatically installed by: writers, that introduce various chemical modifications on DNA (i.e., methyltransferases); erasers, the unique set of enzymes capable of erasing these chemical tags (i.e., demethylases); and readers, the specialized domain comprising proteins that recognize and interpret such alterations (Table 1). Several DNA methyltransferases (DNMTs) have been reported in plants. However, some are limited to for specific organisms and possess a ubiquitin binding domain. Loss or knockdown of methyltransferases in plants is not as lethal as in animals [66]. In *Arabidopsis*, methyltransferase including *Chromomethylase 1* (*CMT1*), *CMT2* and *CMT3*, *Domain rearrange DNA methylase 1* (*DRM1*) and *DRM2*, *methyltransferase 1* (*MET1*) and *MET2* have been identified as catalyzing DNA methylation [21]. Maintenance of cytosine methylation is carried out by MET1, a homolog of mammalian methyltransferase DNMT1, which recognizes hemimethylated CG sites and maintains symmetrical CG methylation along with different methylation proteins during DNA replication [67]. In contrast, CMT3, being a plant-specific methyltransferase, targets particular sequences and maintains methylation at CHG sites by interacting with KRYPTONINE (KYP), SUVH5 and SUVH6 [21,68]. Also, *de novo* DNA methylation levels in all sequence contexts are regulated by DRM2. DRM2 or CMT2 regulate the maintenance of CHH sequence contexts through different pathways [21,69,70]. These consist of nucleosomes remodelers DRD1 and DDM1, respectively [46,69]

Table 1. Summary of writers, erasers and readers of 5mC and 6mA.

Modification	Substrate Specificity	Putative Functions	Example Proteins/Domains		
			Human	Arabidopsis	Rice
5mC	Cytosine	Repression			
Writer			DNMT1	MET1	
			DNMT3	CMT3	
				CMT2	
				DRM2	
Eraser			TET1	ROS1	
			TET2	DME	
			TDG	DML2	
				DML3	
Reader			MECP2	SUVH2	SUVH7
			MBD	SUVH9	
6mA	Deoxyadenosine	Activation			
Writer			N6AMT1	AtN6AMT1	OsN6AMT1
Eraser			ALKBH1	AtALKBH1	OsALKBH1

Diverse and dynamic DNA methylation patterns in different plant species contain different sets of methyltransferases and demethylases [51,71]. For example, *Arabidopsis* possesses MET1; maize contains ZmMET1, peach has PsMET and rice OsMET1-1 and OSMET1-2 [72–74]. Also, *Arabidopsis* possesses CMT2 and CMT3 [75] and Brassica rapa BrCMT [76,77], while maize and rice contain ZMET2, ZMET5 and OsCMTL, OsMET2a respectively. Likewise, for the DRM family, *Arabidopsis* possesses DRM1 and DRM2 [78,79] and maize ZmDMT106 and Zmet3, while rice has OsDMT106 and OsZmet3 [80]. However, their biological functions are still unclear.

Demethylation complementary to DNA methylation causes the removal of methylation from CpG sites [81–83]. It is a complicated process that can be achieved actively by demethylases or passively during DNA replication [84,85]. Demethylation mediated by either deamination or oxidation of cytosine, results in the formation of primary sites, which is followed by the base excision repair (BER) pathway. TET proteins catalyze the oxidation pathway and thymine DNA glycosylase (TGD), while apolipoprotein B mRNA editing enzyme (APOBEC) and activation-induced deaminase (AID) catalyze the deamination pathway [83,86,87].

DNA glycosylase lyases can catalyze identification and active demethylation on specific loci (DNA-GL), a DNA demethylase, which removes 5mCs forming non-methylated cytosines [21,81]. The active removal of methylation using the BER pathway is associated with ROS1 and other DME family members such as DME, DML2 and DML3, apyrimidinic (AP) lyase and the bifunctional DNA glycosylase [88–90]. They are crucial for inhibiting hypermethylation at thousands of genomic regions and play a vital role in regulating transposable elements, transgenes and numerous endogenous gene expressions [91–93]. The removal of 5mC by DME forms a primary site which is subjected to the BER pathway [94]. In *Arabidopsis*, DME functions in gene imprinting in the endosperm [88], where DML2 and DML3 carried out removal of improperly placed methylation [71,95]. Hypermethylation on both endogenous genes and transgenes is prevented by ROS1 [96,97], which consequently affects the gene expression during developmental process such as, maternal imprinting [81,98], epidermal cell differentiation [93], development of male gametophytes [99] and response to pathogen attack [100]. ROS activity and function seems to be regulated by *INCREASE IN DNA METHYLATION 1* (*IDM1*), *IDM2*, *Histone 3* (*H3*) acetyltransferase, an alpha protein and methyl-binding protein7 (MBD7) [92,101,102]. Recent studies re-

vealed that the total level of DNA methylation is determined by the combined action of DNA methyltransferases and demethylases in the regulatory loop where methylation level determines the expression of ROS1 gene [103,104].

Phylogenetic studies have revealed that the rice genome has six bifunctional DNA demethylases that are responsible for demethylating cytosine, two DML3 orthologs (DML3a and DML3b), and four ROS1 orthologs (ROS1a, 1b, 1c and 1d), while no DME ortholog has been reported in rice yet [105]. *DNA GLYCOSYLASE 701* (DNG 701) also known as ROS1c, a functional DNA glycosylase, carried out demethylation and is involved in controlling the activity of retrotransposon Tos17 [84]. The knockout mutants of *dng701* exhibit retrotransposon Tos17 transposition in calli compared to wildtype, while overexpression of DNG701 displays reduced methylation level and frequent transposition of Tos17 in calli, indicating that TEs repression is regulated by DNA methylation [84]. It has been revealed by a qRT-PCR investigation that DMAL3, ROS1a and ROS1d are expressed in diverse plant tissues [85]. By constructing the knock in null allele of ROS1a, it has been revealed that failure of early-stage endosperm and embryo development is caused by the maternal *ros1a* allele and neither paternal nor maternal allele is transmittable to progeny, showing that ROS1a demethylation plays a vital role in both female and male gametophytes [85].

The epigenetic information of DNA methylation can thus be further translated by various reader proteins to direct downstream functions. To a certain extent, reader proteins may ultimately affect the biological outcome. For readers of DNA 5mC, recent discoveries have shown that these proteins with a methyl-CpG (mCpG)-binding domain (MBD) can recognize and further bind to 5mC sites (Table 1). In mammals, the MBD family has five members, including methyl-CpG-binding protein 2 (MeCP2), MBD1, MBD2, MBD3 and MBD4 [106]. Among these MBD proteins, MBD3 does not bind to methylated DNA, while other MBD proteins bind to methylated DNA. In plants, these MBD proteins have also been identified in various pant species, such as *Arabidopsis*, soybean, watermelon, and *Brassica napus* [107]. Remarkably, recent reports have presented evidence that plant SU(VAR)3-9 homologs were among the methylated-DNA reader candidates. In *Arabidopsis*, the transcriptional anti-silencing factor SUVH1, and SUVH3, bound methylated DNA in vitro, were associated with euchromatic methylation in vivo, and formed a complex with two DNAJ domain-containing homologs, DNAJ1 and DNAJ2 [108]. Rice OsSUVH7 acts as a DNA 5mC reader forming a complex of BCL-2-ASSOCIATED ATHANOGENE4 (OsBAG4)-OsMYB106-OsSUVH7 expression in response to salt stress [109]. Thus, the SUVH proteins may also function as 5mC readers that bind to methylated DNA sites in plants.

3. DNA Adenine Methylation 6mA in Plants

Although 6mA was discovered during the same time period as 5mC a few decades ago, its existence was thought to be limited to prokaryotes only. However, 6mA could not achieve the same level of research attention in eukaryotes as 5mC due to its low abundance and technological limitations. 6mA is a widely known prokaryotic DNA modification which involves critical regulatory functions in eukaryotes [110–115]. Recent studies have demonstrated its potential epigenetic role in cellular processes with biological consequences in diverse eukaryotes [116], which are dynamic and probably even contradictory to 5mC, a well-known epigenetic mark. The recent discovery and characterization of 6mA in different eukaryotes including plants such as *Caenorhabditis elegans* (nematode) [117], *Chlamydomonas reinhardtii* (algae) [118], *Drosophila melanogaster* (insect) [119], *Tetrahymena* (Protozoan) [120], fungi [121], yeast [122], *Bombyx mori* (silkworm) [123], vertebrates such as *Xenopus laevis* (Frog) [124], mouse [125,126], human [127], pig and zebrafish [128], plants such as *Arabidopsis thaliana* [129,130], and rice [131,132] have been reported, suggesting its potential regulatory functions in all organisms [133,134].

6mA is a vital element of the gene regulatory system in mammals and plays an essential role in gene silencing. Unlike animals, adenine DNA-methyltransferases have been found associated with mitochondria such as in wheat. However, cytosine DNA-methyltransferase

is absent in mitochondria. An enzyme that was isolated from mitochondria of wheat methylates an internal adenine residue in the TGATCA sequence in DNA [135] and seems to regulate mitochondrial DNA replication. Surprisingly, it has also been reported that both adenine and cytosine methylations can coincide on the one and same gene. Cytosine DNA methylation can affect adenine methylation and vice versa. Hence, adenine methylation may regulate DNA replication and gene expression such as like cytokine methylation. Plants are known as masters of epigenetic regulation. All of the significant epigenetic controls that are known to occur in eukaryotes are used by plants [50]. Because of its involvement in diverse cellular functions, it has captivated the attention of researchers globally.

DNA 6mA has been revealed as an important and potential epigenetic mark in eukaryotes including plants, such as *Arabidopsis* and rice. Moreover, the conservation, distribution and functions of 6mA methylomes in plants have also been reported very recently [131,132,136]. Methylomes analysis suggest that 6mA distribution and function is rather conserved among *Arabidopsis* and rice. 6mA is found to be enriched around the transcriptional start site (TSS). In *Arabidopsis* [136], *Chlamydomonas* [137] and fungi [138], it is correlated with active transcription. The distribution and function of 6mA has been found divergent in different organisms. For example, 6mA is evenly distributed in the *C. elegans* genome [117], while in *Drosophila* it is found mainly on transposable elements [139]. In *Tetrahymena* it is not associated with active transcription, but is correlated with RNA polymerase II transcribed gene [120] and in the frog it is depleted in gene exons [140], while in mouse it is associated with gene expression and is depleted in exons [126,141].

Although 6mA was found in plants long ago [142–144], its genomic distribution and function remained elusive for a long time. Very recently, with advances in detection methods, finally the detection of 6mA in plant genomes has been made possible. In *Arabidopsis*, 6mA is widely distributed across the genome and more enriched on gene bodies [136] while its function and regulatory mechanism in *Arabidopsis* is yet unknown. Moreover, the distribution and genomic function of 6mA has also been reported in the rice genome, where 0.2% of all adenines were 6mA methylated and its presence was mostly found on GAGG motifs that was mapped to around 20% of genes and 14% of TEs. 6mA in gene bodies was found to be correlated with gene activity while in promoters it marked silent genes. In the rice genome, 6mA was also found to overlap with 5mC and in some regions it was also found complementary to 5mC. Additionally, OsALKBH1 was also proposed to be potentially involved in 6mA demethylation (Table 1) [131], suggesting distinct roles of 6mA. In another study among two rice cultivars *Japonica* and *Indica*, Zhang et al. (2018) revealed that 6mA was broadly distributed across genomes of both cultivars, while most abundantly found on exons and promoter regions. 6mA was positively correlated with key genes related to plant development and stress response. Additionally, DDM1 was found to be crucial for 6mA modification and decreased 6mA levels; defects in plant growth were observed due to *DDM1* mutations, and expression of vital genes involved in plant growth was also affected by DDM1 [132]. This suggests that 6mA is conserved as a DNA methylation modification, is positively correlated with gene expression and significantly contributes to important agronomic traits in plants [132].

4. Molecular and Biological Function of DNA Methylation

4.1. Molecular Functions of DNA Methylation

DNA methylation has been found to be involved in the regulation of various cellular processes in plants and animals, playing a leading role in genome functioning, stability and development, TE silencing, genomic imprinting and X-chromosome inactivation [21,51,145,146]. It is also involved in both gene expression and repression. For example, methylation in promoter regions is associated with gene expression and ultimately regulates growth and development in plants and other eukaryotes. Methylation of cytosine residues strongly affects DNA binding with several proteins including regulatory proteins. Usually, it blocks the binding of certain nuclear proteins involved in transcription

and multiple other pathways. In contrast, some proteins specifically bind to methylated DNA sequences and assemble an entire group of proteins on DNA regulating gene expression [147]. 5mC in plants is mainly found in transposons and repetitive sequences, preventing their transcription and transposition, and therefore is vital for gene silencing and genome stability. Thus, DNA methylation in plants and animals is a relatively stable but reversible epigenetic mark regulating gene expression.

In somatic cells of multicellular organisms, genome-wide epigenetic modifications are stable, while in early embryos and germ cells genome-wide epigenetic reprogramming takes place involving DNA demethylation and remodelling of histones. This epigenetic reprogramming drives various important cellular processes such as epigenetic inheritance across generations, TEs control and imprinting [148]. In plants and mammals, cytosine methylation is crucial for gene regulation. This epigenetic mark is extensively reprogrammed in the germline and early embryos of mammals, while in plants the extent by which it is reset between generations remains largely unknown [54]. Histone modifications and small RNAs may also play role in epigenetic inheritance and reprogramming [148].

DNA methylation in association with histone modification and non-histone proteins defines chromatin structure and accessibility. DNA methylation is therefore involved in gene expression and various other cellular processes. In plants and mammals, DNA methylation in promotor regions or within a transcribed gene body is associated with gene expression and plays vital role in growth and development. The accessibility of genomic regions to regulatory proteins and protein complexes is affected by cytosine methylation, subsequently affecting the chromatin structure and transcription rate of the gene [149]. Cytosine methylation in promotor and enhancer regions usually represses gene transcription; hence, in some cases it also promotes gene transcription, for instance, ROS1 in *Arabidopsis* and numerous genes in tomato that inhibit fruit ripening [103,104,150]. DNA methylation on promotor regions either directly suppresses gene transcription by inhibiting the binding of transcription promotors or by promoting the binding of transcription repressors or indirectly represses transcription by promoting repressive histone modifications like H3K9 and inhibiting permissive histone modifications such as histone acetylation [66,151,152].

Moreover, genome stability can also be threatened by transposons through their relocation or the insertion of new copies of retrotransposons. Cytosine methylation is crucial for transposon silencing and regulation of endogenous genes. In *Arabidopsis*, heavily methylated cytosines are found in all three contexts on pericentromeric heterochromatin regions and some transposon or repeat containing euchromatic regions and regions producing siRNA. More than one third of expressed genes are methylated within transcribed regions and promotor methylation was found on only 5% of the genes. Remarkably, genes methylated on transcribed regions are highly expressed and constitutively active, while the expression of promotor methylated genes is mostly tissue specific [52,153].

4.2. Dynamic DNA Methylation in Plant Development

DNA methylation is conserved in plants and animals, as its genome-wide precise patterns are crucial for growth and development. It has been revealed that along with silencing TEs and repeats, DNA methylation regulates transition to flowering, vernalization, leaf morphology, developmental reprogramming, fertility, floral organ identity and embryogenesis, seed development, response to environmental stimuli and genome protection [154–162]. Recent investigations have revealed that DNA methylation plays a crucial role in controlling agronomical relevance traits, such as heterosis [163], ripening in tomato and other fleshy fruits [164–167] and response to biotic and abiotic stress [168,169]. Recent studies revealed that DNA methylation is dynamic during embryogenesis and early vegetative development. Higher levels of CHH methylation have been found in embryos compared to seedlings or adult plants, suggesting that DNA methylation has a vital role to play in embryogenesis [54]. Mutations in MET1 and DDM1 have been shown to affect seed size and development [170], indicating that DNA methylation is crucial for seed development.

Recent studies have shown that DNA methylation is dynamic during plant development. In plants, experimentally induced hypomethylations in plant genomes have shown numerous developmental defects confirming that DNA methylation is vital for proper growth and development. CMT2, CMT3, DRM and MET1 mutants in plants are viable, even in combination with one or another, in contrast to mammals where DNMT1 and DNMT3 mutants show lethality during embryo development. Thus, DNA methylation in plants is involved in a variety of processes such as fertilization, gametogenesis, vegetative and reproductive development and crosstalk between DNA methylation and histone modification [171,172]. Seed development has also been found to be affected by DDM1, an ATP dependent SWI2/SNF2 chromatin remodelling factor and MET1. DNA methylation profiles in rice have shown strong fluctuations in DNA methylation levels in embryo and endosperm during seed development, indicating the association of controlled and complex DNA methylation with seed development [173]. In soybeans, DNA methylation levels can change in gene promotors during different stages of seed maturation [174]. Moreover, methylation levels vary in different cell types in gametophytes that have a similar origin and are separated by a few cell divisions. Nevertheless, the molecular mechanism regulating these dynamic methylation patterns remains to be elucidated.

Likewise, DNA methylation has been shown to regulate the growth progress of cell division and cell expansion within a growing organ of maize plants. Maintenance of methylation is transcriptionally regulated throughout the growth zone of maize leaf (division zone, transition zone, elongation zone and mature zone). Interestingly differentially methylated sequences mainly exist at or near gene bodies; numerous other genes involved in chromatin remodelling, cell cycle progression and growth regulation were also differentially methylated [162], suggesting an essential role of DNA methylation in leaf development in maize. In *Arabidopsis*, dysfunction of ROS1 has been found to cause hypermethylation of promoters and repression of the gene encoding *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*), which represses stomata formation; this leads to stomatal lineage cells overproliferation, showing the important role of DNA methylation in pattern formation of some leaf epidermal cells [93]. DME DNA glycosylase expression is found significantly in vegetative cells of male gametophyte and central cells of male gametophyte, suggesting that DME mediated demethylation in *Arabidopsis* is vital for endosperm development. In many fruit ripening genes, active DNA methylation is found, as their promoter regions contain binding sites for *RIPENING INHIBITOR* (*RIN*), that is a major ripening transcription factor [150,165]. RIN binding to target promoters has been confirmed in most of the known ripening genes whose expression is negatively correlated with promoter DNA methylation levels [165].

4.3. DNA Methylation Heritability in Plants

In contrast to genomic variance, the potential of epigenetic markers to transmit acquired environmentally adapted traits to offspring is extremely advantageous. Several examples of DNA methylation pattern heritability in plant species such as *Nicotiana tabacum*, *A. thaliana*, *Taraxacum officinale* and *Picea abies*, as well as crop species such as rice and wheat, have been described [175–180]. DNA methylation modification transmission in *A. thaliana* has been witnessed for at least eight generations [11]. Stress-induced epigenetic modifications can be passed down to offspring, but their long-term stability and impact on a population are unknown [178]. A recent study on epigenetic changes that occurred during cotton domestication indicated that epialleles were passed between generations, resulting in phenotypic diversity and allowing the widespread geographic expansion of species [181,182]. Intergenerational transfer of phenotypic traits induced by DNA methylation modifications, such as delayed flowering time, drought stress adapted roots, and modified plant architecture, has also been reported [183,184]. Because DNA methylation is heritable and can result in phenotypic alterations, inducing/removing DNA methylation has become a focus of research with the goal of altering the expression of genes already present in crop genomes. The increased interest in epigenomics has resulted in the devel-

opment of approaches to induce DNA methylation, first at the genome-wide level using non-specific approaches, and now at the base-specific level using new epigenetic editing tools [185].

4.4. DNA Methylation in Response to Stresses

Plant cells can sense environmental changes or stressors, which cause epigenetic modifications such as methylation pattern modification. At a late embryonic stage, germ cells are derived from somatic tissues. As a result, epigenetic modifications caused by environmental changes or stress, such as changes in methylation pattern, can be transferred down through generations [186,187]. This is crucial for understanding phenotypic variation in nature and biological stress adaptation [188], and it also indicates that methylation changes could be useful in breeding. This potential benefit is clearly expressed by the impact of DNA methylation variations on important agronomic traits such as flowering time and plant height [189,190], pathogen resistance [191,192], and yield [193]. Methylation polymorphisms have been reported in many varieties, biotypes, and intra-species of rice [194], *Arabidopsis* [195], *Brassica oleracea* [196], and cotton (*Gossypium hirsutum* L.) [196]. This methylation diversity may result in phenotypic changes [197,198], which provide the raw materials for plant breeding. Breeding selection, on the other hand, can be effective only if the methylation alleles are heritable. The amount of total and strand-specific methylation in DNA from different batches of seeds varies significantly. This difference is assumed to be caused during seed production, perhaps as a result of changing environmental conditions. Seeds grown in a more stressful environment produce seedlings with increased strand-specific methylation. Such patterns are likely to emerge early in development and are heritable during several days of seedling growth [199]. Recent studies on TMV-infected tobacco [200], blight pathogen-infected rice [201], and MSAP polymorphism in Brassica oleracea populations [202] have supported the inheritability of methylation modifications generated from environmental stresses. Crops like rice and corn are bred primarily to exploit heterosis, highlighting the relevance of inherited methylation diversity patterns in F1 hybrids. Rice hybrids had lower levels of methylation at some loci than their parents, but higher levels at others [203]. Although the majority of methylated loci in corn can be inherited by hybrid progeny, 6.59–11.92% of loci in hybrids differ from their parents, and these loci represent a variety of functional proteins or ESTs. These modifications can be directed or stochastic [204], implying that methylated locus inheritance is complicated and that methylation variations could be beneficial in plant hybrid breeding.

5. Epigenetics Prospective for Plant Breeding

The applications of epigenome editing tools have facilitated the use of methylation modification in plant breeding. Although TALE-based systems are simpler to construct, the first-generation tool, ZFs, remains important due to its tiny size and binding properties. TALEs have a problematic sensitivity to DNA methylation, which hampers their usage in epigenome editing. The CRISPR-Cas9 approach has comparable efficiency, but it is easier and less expensive to implement, and it can target several sites (multiplex). CRISPR-Cas9 has already been widely used in genome editing and is rapidly becoming the most popular technique for inducing methylation in plants [184]. CRISPR-Cas9 has already been efficiently employed to edit a variety of crop genomes, including maize, rice, cotton, potato, tomato, soybean and sweet orange [205–211]. Endonucleases, on the other hand, run the danger of producing off-target double-strand breaks (DSBs), which can result in unanticipated changes elsewhere in the genome. Because the effects of CRISPR-Cas9 epigenome editing are gradual and proportionate to target binding, it produces less dramatic off-target activity than genome editing. Furthermore, most off-target impacts will occur in non-regulatory regions, rendering them silent [212]. Furthermore, unlike most genetic variations, which tend to produce a loss of function, epigenetic mutagenesis might result in a genetic gain of function [213].

Plant breeding could benefit from epigenome editing on multiple levels. Firstly, as with genetic alterations, it has the potential to speed the process of domestication of wild plants by modifying traits involved in growth habit, flowering time, yield, nutrition, and seed and fruit size and number in a single generation. In contrast to gene knockout, however, induced (de)methylation has a variable impact and can affect both gene expression and repression. Induced DNA methylation may also enhance hybrid breeding and plant propagation via tissue culture, which can create new gene expression patterns in their offspring [214–217], and thus help manage offspring phenotypic. It could be used to remove undesirable traits or to introduce desirable traits to crops, such as biotic and abiotic stress resistance (Table 2). Finally, it has the potential to generate plant resistance to viruses or even modify a pathogen population, making it susceptible to the plant's defenses [218]. The extensive use of induced DNA methylation in plant breeding is hindered by a number of limitations. On a biological level, the selection of genes to target, the durable maintenance of induced epialleles, and the occurrence of off-target effects. On the other hand, improper legislation involving new breeding techniques has been introduced, which has restricted research and the adoption of sophisticated breeding approaches [219]. Numerous GMO regulations are based on out-of-date definitions that do not take into account new plant breeding techniques and are incompatible with modern genome editing technology. The European Court of Justice ruled in July 2018 that plants resulting from genome editing methods, including ones that do not employ recombinant DNA, are subject to current GMO regulations [220]. Some nations, such as Canada, define the safety of novel crops based on the end product's attributes (product-based) rather than the technology used to create it (process-based). The United States has implemented a hybrid system in which only plants featuring novel traits are subject to stringent regulations [221]. The Australian government deregulated "DNA free" gene editing methods in 2019. It is critical to amend regulations governing novel plant breeding techniques, since it has both scientific and commercial implications [222].

5.1. Applications of DNA Methylation in Plant Breeding

Plant species, unlike animals, have certain methylation loci that can be inherited for numerous generations [245,246]. Treatment with 5-azacytidine, for example, reduces methylation in flax (*Linum usitatissimum*) and produces dwarfism and early maturity traits [189]. The methylation level is likewise lower in F2 and F3 progenies resulting from crossings between wild type and mutant plants selected for the phenotype of early maturity [188]. This shows that 5-azacytidine-induced hypomethylation can be passed down for at least three generations. Resistance to the blight pathogen, which is caused by variations in rice methylation, can be passed on for at least nine generations [191]. Because the market vitality of new rice varieties is often fewer than 5 years, this is a good option for commercial development. Some methylation differences can be passed down for hundreds of years, having an impact on evolution. Linnaeus 250 years ago discovered a toadflax (*Linaria vulgaris*) mutant in which the flower's fundamental symmetry has been altered. This mutant's Lcyc gene, which is crucial in floral development, has been discovered to be extensively methylated and silenced [197]. These stably transmitted DNA methylation alterations are identical to DNA sequence mutations, implying that they have genetic behavior similar to that specified by Mendelian genetics [247]. For example, traditional hybridization and segregation approaches revealed that three methylation alleles govern the phenotypic of early maturity in flax, two of which are recessive and one dominant or codominant [248]. Because of the similarities to classical genetics, traditional breeding processes such as hybridization, selection, and purification can be used to create new plant types containing novel methylation alleles.

Table 2. Known epigenetic mechanisms involved in different abiotic stresses in plants.

Abiotic Stress	Crop	Epigenetic Mechanism(s)	Reference
Drought	Rice	Hypomethylation	[208]
		Up-regulation of miR408 expression	[209]
		Site-specific DNA methylation	[210,211]
	Maize	Modifications of H3K4me3 and H3K9ac dynamics	[212]
		Enrichment in H3K36me3, H3K9ac, and H3K4me3	[213]
	Soybean	miR1514a modulation of a NAC transcription factor transcript	[214]
		Up-regulation of isomiRNAs	[215]
	Barley	Hc-siRNA-mediated hyper-methylation at CYTOKININ-OXIDASE 2.1 promoter	[216]
		Increase in H3 and loss in H3K9me2	[217]
		Accumulation of miR408 transcripts	[218]
	Tomato	RNA-dependent DNA methylation	[219]
		Increased Asr1 and Asr2 expression due to demethylation of putative regulatory and transcribed regions	[220,221]
	Pea	Hypermethylation of cytosine residues	[221]
	Cowpea	Increase of P5CS transcripts and very low expression of vun-miR5021 and vun-miR156b-3p	[222]
	Bean	Dicistronic arrangement of miR398a and miR2119	[223]
	Faba bean	Increased DNA demethylation	[224]
	Alfalfa	Overexpression of miR156	[225]
	Chickpea	Accumulation of miR408 transcripts	[226]
		Accumulation of miRNAs at root apex	[227]
Salinity	Wheat	Hypermethylation of cytosines at HKT genes	[228]
		5-mC depletion	[229]
	Rice	Demethylation at promoter region of OsMYB91 gene and rapid histone modifications at OsMYB9 locus	[230]
		DNA methylation	[231,232]
	Rapeseed	Increased DNA demethylation	[233]
	Chickpea	Accumulation of miRNAs at root apex	[227]
	Arabidopsis	Increased acetylation of histone H4 at AtSOS1 due to inhibition of de-acetylase	[234]
Heat	Maize	H3K4me2 and H3K9ac alterations	[235]
		Increased histone acetylation and decreased H3K9me3	[236]
	Wheat	Increased histone demethylation of the various genes	[237]
	Soyabean	Hypomethylation of cytosine	[238]
	Rapeseed	Increased DNA demethylation	[239]
Cold	Maize	Enrichment in H3K9ac and decrease in DNA methylation and H3K9me2	[240]
		Reduction in histone acetylation in euchromatin-associated gene regions	[241]
		DNA demethylation	[242]
	Tomato	Increased DNA methylation	[243]
	Arabidopsis	Non-CG hypermethylation under cold and low light stress	[244]

Methylation patterns are dynamic and reversible during eukaryotic life cycles [249] and various stages of cancer development [250], it also occurs in plants sometimes [251]. The distribution patterns of methylated loci in individual clover and flax plants are largely

identical before and after metal stress [252], implying that methylation pattern alterations may occur with stress and then dissipate once the stress is eliminated. Similar situation has also been observed in *Bryonia dioica* and *Antirrhinum majus*. When *Bryonia dioica* is exposed to rubbing stress, the level of methylation drops, but it returns to normal after the stress is removed [253]. Low temperature-induced methylation changes in *Antirrhinum majus* are reversed when the temperature is returned to normal even within the same generation [254]. Lower methylation levels are generally associated with higher levels of gene expression. Gene overexpression and energy loss are inescapable if a plant's methylation level does not rebound when the stress is removed. We deduce that alterations in methylation that are reversed once the stress is removed are more common in nature, since they allow for more rational use of biological energy, just as the resistance response to pathogens occurs only when the disease is attacking. This emphasizes the possible significance of methylated alleles in breeding. This type of methylation may be studied in the same way that pathogen resistance is. Stress is first induced, either naturally or artificially, and then the resulting methylation variations in the plant genome are discovered. The methylation pattern must be assessed at multiple time points, and the amount of methylation must also be considered. A numerical approach can be used to represent the level of methylation variation in disease resistance responses. With the help of molecular markers, the methylation variations that are shown to be induced reversibly in response to stress by several rounds of multidrop experiments can be identified. Classic hybrid breeding strategies can be used to produce new varieties if the loci are closely related to stress resistance [255].

5.2. Potential Applications of RdDM in Crop Improvement

RdDM is a biological mechanism in which non-coding RNA molecules direct the addition of DNA methylation to specific sequence [256]. The mechanism underlying the sequence-specificity of RdDM is well understood. RdDM can be 'tricked' into targeting and silencing endogenous genes in a highly specific, which offers a variety of potential biotechnological and breeding applications. Numerous methods can be employed to induce RdDM based DNA methylation and silencing of specific genes. One approach, known as virus-induced gene silencing (VIGS), involves introducing a portion of the target gene's promoter region into a virus [257]. As the virus replicates its own RNA, it will generate the chunk of promoter sequence as part of its own RNA, which is foreign to the plant. The viral RNA being foreign will be targeted for PTGS and processed into sRNAs, some of which will be complementary to the promoter of the original target gene. Certain sRNAs add DNA methylation to the target gene by recruiting RdDM machinery. Various studies have used this approach such as fruit ripening gene in tomato and a gene affecting flower pigmentation in Petunia have been silenced using Cucumber Mosaic Virus to recruit RdDM to silence the gene [258]. *Arabidopsis* FWA locus has also been silenced using VIGS, which resulted in delayed flowering [257]. RdDM-induced changes can occasionally be retained and inherited through multiple generations without outside intervention or manipulation, implying that RdDM can be a useful tool for targeted epigenome editing.

5.3. DNA Methylation and Heterosis

Heterosis, often known as hybrid vigor, refers to hybrid offspring outperforming their parents. It is a significant biological phenomenon that is often used to enhance agricultural productivity. Heterosis can be seen in plants in traits including environmental adaption yield, and growth [259]. Heterosis' molecular foundation is mainly unknown, which limits its application in plant development. Recent omics research has demonstrated that substantial changes in gene activity between hybrids and their parents produce gene expression complementation and consequently hybrid vigor [259]. A variety of biological processes are affected by these changes, including, stress responses, light and hormone signaling, ageing and energy utilization and metabolism [260]. Epigenetic variation is also implicated in heterosis, as it regulates gene expression and stabilizes the genome [261]. As an epigenetic alteration, DNA methylation at cytosine occurs in all sequence contexts,

including CG, CHG, and CHH (where H = A, T, or C). Numerous studies have demonstrated that hybrids of many crops, such as rice [262], maize [263], and pigeonpea [264], undergo DNA methylation rearrangements, leading their methylome to differ from their parental lines. Additionally, loss genome methylation of TE in hybrids resulted in loss of phenotypic superiority [265,266]. According to research in rice, paternal CHG methylation divergence may play a role in allelic-specific expression and hybrid vigor [267]. In pigeon pea, there was a substantial association between expression levels and methylation for certain heterosis-related genes [264]. Dissecting the significance of DNA methylation in heterosis, on the other hand, is difficult since genome-wide DNA methylation levels differ depending on developmental stages and environmental factors. Increased DNA methylation levels in reciprocal hybrids were found in studies employing *Arabidopsis* and pigeon pea seedlings [264,265]. In contrast to their parents, rice developing seeds revealed that overall DNA methylation of reciprocal hybrids decreased [268]. Changing DNA methylation in hybrids did not generate significant changes in target gene expression in 6-week-old rice seedling leaf or root [269]. In maize, there was no association between expanded leaves at the 7–8 leaf stage and grain yield heterosis identified for parental variations in CG or total (CG + CHG) methylation levels [270].

The accumulation of various phenotypic advantages at distinct developmental stages can lead to heterosis of a trait, which can start as early as double fertilization, as seen in maize hybrid embryos with phenotypic dominance at 6 days after fertilization [271]. When reciprocal hybrids and their parents are examined, embryos and endosperm experience synchronized significant alterations in the DNA methylome, which is thought to be the cause of heterosis of immature seed-related characteristics [268]. During soybean seed development, DNA methylation is altered differently in different cytosine contexts: CHH increases significantly throughout the seed and drops precipitously within the germinating seedling, whereas only minor changes in the global level of CG/CHG occur during the same developmental period [173,272]. Furthermore, in *Arabidopsis*, loss of CHH and CHG methylation has no effect on seed development, and the key genes for soybean seed development are found in non-methylated genomic regions [272]. According to the information above, methylation profiles produced during seed development may have other functions. Hybridization, on the other hand, can cause DNA methylation changes in hybrids, which are maintained in the hybrid progenies [273]. Additionally, epigenetic modifications that arise during seed development can be transmitted via cell division to exert their effect once the seed reaches a certain stage of development [274]. A recent study was carried out on reciprocal hybrids of soyabean. They established three soybean reciprocal hybrid combinations and then utilized MethylRAD-seq to identify CCGG and CCWGG (W = A or T) methylation in the whole genome of these hybrids and their parents during the middle development stage of contemporary seed. The study demonstrated that alterations in DNA methylation patterns occurred in immature hybrid seeds, and that parental variation had a role in differential expression between reciprocal hybrids. In hybrids, non-additive differential methylation sites (DMSs) were also found in considerable numbers. Surprisingly, the majority of these DMSs were hyper-methylated and localized in gene regions, contrary to the natural distribution of methylation sites. Further investigation of the non-additive DMSs found in gene regions revealed their involvement in a variety of biological processes, including those linked to transcriptional control and hormone function. These findings demonstrated a pattern of DNA methylation reprogramming in hybrid soybeans, which is linked to phenotypic variance and heterosis initiation [275].

5.4. Challenges and Opportunities for DNA Methylation in Breeding Applications

Plant genomic methylation modification can respond quickly to stress. For example, rubbing stress can reduce methylation levels to zero in 1 h [253]. However, some methylation alterations can only be noticed after comparatively prolonged periods of stress, such as 12 h for wounding and chilling stresses [276]. Methylation, on the other hand, varies dynamically [251]. Because of the dynamic and flexible nature of DNA methylation

changes, we can precisely specify the time points at which observations are made. Errors of a few hours can lead to vastly divergent conclusions. To assess if the alterations in methylation are reversed after the stress is removed, multiple time points of observation and repeated analysis under several rounds of stress are required. For example, treatment of flax with 5-azacytidine causes the reduced level of methylation and early maturity phenotype to be reversed partly in the first generation and completely in the fourth generation of one line [248]. Under stress, quantitative changes in methylation variation can also be detected. For example, the degree of decrease in DNA methylation stressed clover and hemp, which ranges from 20% to 40%, is clearly dependent on the concentration of metal ions to which they are exposed [252]. In Stellaria longipes subjected to red/far red light, a similar phenomenon was found [277]. Quantitative analysis is far more difficult than qualitative analysis. Bisulfite sequencing and Southern blotting, the two most used approaches, are now time-consuming and expensive procedures, making their application to breeding challenging.

In the field of breeding, the utilization of methylation alleles is currently uncommon. Treatment with 5-azacytidine has been used in all previous studies to generate a decline in plant methylation levels and produce unique phenotypes that will be studied in future breeding studies. Early maturity and dwarfism in flax [188,189] and resistance to the blight pathogen in rice [191] are two examples. We believe that 5-azacytidine-induced methylation variation in breeding has numerous drawbacks. First, while the changes in methylation may not be stochastic [188], the approach does not allow for directed variation, akin to breeding with radiation-induced mutations. Second, the entire genome is influenced. As a result, favorable phenotypes are inextricably associated to a significant number of unfavorable phenotypes. The majority of plants do not survive the treatment [191], reducing its usefulness in breeding. For example, 5-azacytidine-induced early maturity has numerous impacts [278] and is associated with dwarfism [189]. To allow breeding selection with a clear target for such differences in methylation pattern, an approach comparable to molecular marker-assisted breeding in classical genetics can be developed. As a result, in the quest for strategies that can be applied to these aims, conventional genetics and breeding procedures should be examined.

Classical genetics has advanced from DNA sequencing to the post-genomic period, which is focused on the biological roles of genes. For major crops including rice, corn, and wheat, molecular linkage maps have been created, providing a platform for molecular-assisted selection and gene function analysis. These linkage maps may be employed in gene mapping and cloning, allowing the DNA sequence, gene function, and phenotype to be linked together. The approach of constructing genomic methylation maps is similar to that of DNA sequencing in classical genetics. This effort, however, is still in its early phases. The *Arabidopsis* high-resolution methylation map created by Zhang et al. [52] and Cokus et al. [152] is now the only example accessible. There hasn't been a map like this for other plant species, including crops. One factor for this is that we place far too much emphasis on the DNA sequence, resulting in a lack of understanding of the critical role of DNA methylation in genetics and evolution. The absence of appropriate technologies for genome methylation mapping is another concern. The bisulfite sequencing was the initial step. It is, however, far too time and labor intensive to be used in a large-scale genomic study. Zhang et al. used chromatin immunoprecipitation and a tiling microarray approach to discover the distribution of methylated cytosines in the *Arabidopsis* genome for the first time in 2006. They were able to attain a resolution of as high as 35 bp [52]. However, most plant species, including crops, do not have commercial tiling microarrays, which severely restricts the technique's potential uses. Peng et al. [278] proposed to replace chip hybridization with Solexa sequencing to generate high-resolution genome methylation maps in order to solve this issue and the limitations of microarrays. This high-throughput sequencing-based design potentially enhances the accuracy and detail of the generated map [279]. The key benefits of Solexa sequencing over microarrays are the digital signal, the lack of hybridization interference, and the removal of the need for PCR amplification [280].

Molecular marker-assisted selection was widely used in breeding practice even before the advent of genome sequencing. It is theoretically plausible that it can also be used to select methylated alleles. However, challenges exist in breeding practice. The MSAP marker, which is derived from the amplified fragment length polymorphism (AFLP) marker, is currently the most commonly used type of molecular marker for methylated alleles. The primary distinction is that MSAP employs restriction enzymes that are variably sensitive to methylation. The use of MSAP in markers assisted selection has numerous drawbacks. First, in a given sequence context, a single enzyme can only detect one type of methylation. Distinct stresses, on the other hand, may result in different methylation variations. Salt stress, for example, causes hypermethylation in CCWGG sequences in Mesembryanthemum crystallinum but leaving CCGG sequences untouched [281]. Second, the AFLP technique is time-consuming and difficult, making it only ideal for theoretical research, implying a bleak future for MSAP in breeding practices. Finally, the MSAP approach makes it difficult to quantify methylation variation.

Methyl cytosine immunoprecipitation separates methylated and unmethylated DNA into two pools (mCIP). Primers are designed to allow for target gene amplification and are used to amplify the target gene in both pools. The methylation level of the target gene is represented by the ratio of the quantity of PCR product produced from each pool. This design's detection process is comparable to that of microsatellite (SSR: simple sequence repeat) markers in classical genetics for qualitative determination. It is comparable to RT-PCR in terms of quantitative analysis. The advantages of this design over MSAP include the creation of a single band, the capacity to investigate any locus, and the ease of detection by agarose gel electrophoresis. To identify several genes in a single sample, just one round of mCIP is required. However, numerous rounds of mCIP are required to detect a single gene in several samples, resulting in a high cost at present.

6. Conclusions and Future Perspectives

Currently sustainable agriculture has been regarded as a concept for feeding an increasing population on our planet to face the undergoing climactic changes. The continuous increases in crop yields with traditional breeding strategies meets on a bottleneck in agricultural production, constantly raising questions about the capacity of traditional genetic improvement to face emerging needs. Thus, novel breeding strategies and new technologies will be required for filling the predicted yield gaps. Benefiting from the progresses in biological and agricultural research, we are better known that DNA methylation beyond genome, have vital functions during plant growth, development and response to environmental factors. In addition to genetic factors, DNA methylation has been found to excitingly regulate a variety of genes that are vital for many important processes ranging from plant growth and development to biotic and abiotic stress response in plants having more complex genomes than *Arabidopsis*. These and numerous other discoveries expand our knowledge of understanding the epigenetic regulations especially DNA methylation dynamics not only in plants but other prokaryotes and eukaryotes including mammals. Further studies in future are required for undoubtedly revealing new DNA methylation modifications, their derivatives and functions in plants, novel mechanisms for targeting DNA methyltransferases and demethylases and the mechanisms by which DNA methylation patterns are generated, maintained, converted and erased. It will also be interesting to understand whether there is any interaction between the two different methylation modifications such as 5mC and 6mA, if yes, how they coordinate and affect each other. Very recently, an expansion in our understanding about how these epigenetic mechanisms control plant development and stress responses has unveiled to likely develop improved crops, heralding the promise of "synthetic epigenetics" [282]. This strategy will enable to breed engineered smart crops that can actively monitor and rapidly respond to environmental cues for meeting the requirements of sustainable and green agriculture.

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