DNA METHYLATION IN PLANTS ASSOCIATED WITH ABIOTIC STRESS

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DNA METHYLATION IN PLANTS ASSOCIATED WITH ABIOTIC STRESS

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EDITORIAL
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Editorial: DNA Methylation in Plants Associated With Abiotic Stress

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Keywords: DNA methylation, epigenetics, plants, abiotic stress, RdDM RNA-directed DNA methylation

Editorial on the Research Topic

DNA Methylation in Plants Associated With Abiotic Stress

Methylation of DNA is an evolutionarily conserved modification. It is associated with heterochromatic structures. Together with histone modifications, DNA methylation generates unique patterns that support gene regulation, chromatin structuring, and repression of repetitive elements (Bhadouriya et al.). This modification provides a heritable mark that can be propagated through mitosis and meiosis. The methylated region of DNA is recognized and interpreted an epigenetic toolkit involving readers, writers and erasers. In most higher organisms, DNA methylation is restricted to symmetric cytosines. Due to the symmetry, the pattern can easily be propagated from one cell generation to the next after replication. Plants are the only organisms that display significant methylation of asymmetric cytosines, which represents a unique feature of regulation. This mechanism, defined as RNA-directed DNA methylation (RdDM), involves the presence of small regulatory RNAs as triggering molecules and was reviewed here by Liu and He and Kumar and Mohapatra.

As several of the identified regulatory components of DNA methylation respond to the environmental and developmental conditions (Kumar and Mohapatra), the pattern of methylation in the genome can also change. Some of the environmental changes can occur from minutes to hours, others can affect longer periods like days, weeks, or even years for perennial plants. These changes can result in differential methylated regions in the genome (DMRs). If a DMR is located in the regulatory region of a gene, it might influence transcriptional activity. In several cases, methylation of a promoter element leads to suppress the expression of the associated gene, a phenomenon known as transcriptional gene silencing. In other cases, such as gene body methylation, the regulatory effect is not completely understood, but maybe generated as a footprint of post-transcriptional gene silencing. During the silencing process not only are 21mer siRNA generated but 24mer heterochromatic (hc)-siRNAs can also be generated. These hc-siRNAs lead *via* the RdDM process to methylate the region homologous to the silencing trigger. Further LncRNA are capable of influencing DNA methylation during phases of abiotic stress (Urquiaga et al.).

As the origin of most small RNAs is from repetitive DNA elements and retrotransposons, it is obvious that any environmental change that might lead to transcriptional reactivation of these elements has the potential to change the DNA methylation pattern.

The presented Research Topic contains results produced with the model plants *Arabidopsis thaliana*, presented by Laanen et al. and Paul et al.. They explain the effect of Gamma radiation (Laanen et al.) on the DNA methylation landscape followed over multiple generation. Although

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abiotic stress usually applies to environmental conditions on our planet, we included also a study investigating epigenetic effects during spaceflight, which should be considered in the context of long term plans for growing plants in space or on other planets.

Therefore, for many environmental changes, differentially methylated genomic areas or sites are described. In some cases, these changes are affecting nearby genes and can cause changes in the phenotype. Although many factors involved in the molecular mechanism of DNA methylation pattern formation are identified, the complex interplay of environmentally induced DNA methylation change and phenotypic change is not always easy to address.

In the present Research Topic, results are presented for monocotyledonous species of economic and ecologic importance, such as barley (*Hordeum vulgare*) (Konate et al.), maize (*Zea mays*) (Madzima et al.) and common reed (*Phragmites australis*) (Wang et al.). In the review by Gallo-Franco et al., rice (*Oryza sativa*) was taken as model to discuss the plant epigenetic response to Aluminum toxicity.

In addition, a good selection of results is provided also for dicotyledon plants. This includes the study of the consequences of cold stress on the methylome of Tartary buckwheat (*Fagopyrum tataricum*), presented by Song et al., and the effect of UV-B radiation on the perennial herb *Glechoma longituba,* by Quan et al., where the authors found that strong UV radiation can influence the plant foraging proprieties. Another study involving a perennial plant includes sweet cherry trees (*Prunus avium*) and investigates the effect of low temperatures on the dormancy of flower buds (Rothkegel et al.).

Finally, in the paper by Srikant and Drost, the epigenetic effects of abiotic stresses are discussed and analyzed in the context of plant adaptation to stresses. The authors hypothesized that plants dynamically integrate physiological, epigenetic and genetic responses to reduce or buffer negative effects on fitness during the adaptation to a changing environment.

Collectively, this collection highlights the relevance of epigenetic response to abiotic stresses in plants in relation to develop new strategies for plant improvement, and to study mechanisms of plant adaptation and evolution. We believe that this selection of works will contribute to clarify the role of epigenetic in plants and can be of inspiration for future works in the same field.

AUTHOR CONTRIBUTIONS

MK, HJ, MC, and FJ wrote the editorial.

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ORIGINAL RESEARCH
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Dormant but Active: Chilling Accumulation Modulates the Epigenome and Transcriptome of *Prunus avium* During Bud Dormancy

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Temperate deciduous fruit tree species like sweet cherry (*Prunus avium*) require long periods of low temperatures to trigger dormancy release and flowering. In addition to sequence-based genetic diversity, epigenetic variation may contribute to different chilling requirements among varieties. For the low chill variety 'Royal Dawn' and high chill variety 'Kordia', we studied the methylome of floral buds during chilling accumulation using MethylC-seq to identify differentially methylated regions (DMRs) during chilling hours (CH) accumulation, followed by transcriptome analysis to correlate changes in gene expression with DNA methylation. We found that during chilling accumulation, DNA methylation increased from 173 CH in 'Royal Dawn' and 443 CH in 'Kordia' and was mostly associated with the CHH context. In addition, transcriptional changes were observed from 443 CH in 'Kordia' with 1,210 differentially expressed genes, increasing to 4,292 genes at 1,295 CH. While 'Royal Dawn' showed approximately 5,000 genes differentially expressed at 348 CH and 516 CH, showing a reprogramming that was specific for each genotype. From conserved upregulated genes that overlapped with hypomethylated regions and downregulated genes that overlapped with hypermethylated regions in both varieties, we identified genes related to cold-sensing, cold-signaling, oxidation-reduction process, metabolism of phenylpropanoids and lipids, and a MADS-box *SVP-like* gene. As a complementary analysis, we used conserved and non-conserved DEGs that presented a negative correlation between DNA methylations and mRNA levels across all chilling conditions, obtaining Gene Ontology (GO) categories related to abiotic stress, metabolism, and oxidative stress. Altogether, this data indicates that changes in DNA methylation precedes transcript changes and may occur as an early response to low temperatures to increase the cold tolerance in the endodormancy period, contributing with the first methylome information about the effect of environmental cues over two different genotypes of sweet cherry.

Keywords: sweet cherry, Rosaceae, DNA methylation, chilling requirement, cold acclimation

INTRODUCTION

During winter, perennial fruit trees from temperate regions face unfavorable environmental conditions like low temperatures. As a response mechanism, the tree generates protective structures called buds, which contain the meristematic tissue responsible for initiating the development of flowers and leaves (Fadón et al., 2015). Later in autumn, the tree ceases its visible growth and enters into dormancy, an adaptative process that sense the environmental cues to increase cold tolerance and to avoid flowering in winter (Lloret et al., 2018). According to Lang (1987), dormancy can be classified according to the physiological state into paradormancy, endodormancy and ecodormancy. Paradormancy refers to growth inhibition due to apical dominance, while endodormancy corresponds to an endogenous inhibition from the meristem; and growth inhibition due to unfavorable temperatures is referred to as ecodormancy (Lang, 1987).

Sweet cherry (*Prunus avium* L.) belongs to the Rosaceae family and is cultivated in areas of temperate climate, entering into dormancy in autumn to survive the low temperatures of winter. During endodormancy, in *P. avium* and other Rosaceae species, the prolonged exposition to low temperatures in winter and the fulfillment of a chilling requirement (CR), is critical to ensure an optimal flowering in spring and is considered to be specific for each variety or genotype (Campoy et al., 2011). However, with warmer winters due to climatic change, the CR of high chill varieties may not be fulfilled, leading to a delay in flowering and therefore, productivity problems (Campoy et al., 2011). Because of this, in some areas it is necessary the use of chemicals to improve the break of dormancy (Erez et al., 2008). On the other side, low chill varieties have the risk of completing this CR earlier in winter, being exposed to spring frost.

In order to adapt fruit crops to the constantly changing environment, it is necessary to understand the molecular basis of dormancy. Previous studies of dormancy have been focused in the genetic control, showing that CR is a major determinant for flowering date in peach (*Prunus persica*), sweet cherry, and almond (*Prunus dulcis*) (Fan et al., 2010; Sánchez-Pérez et al., 2011; Castede et al., 2014). In peach, quantitative trait loci (QTL) have shown that bloom date is highly variant across years because of the interaction between genotype and environment, being the chilling and heat accumulation the major sources of environmental effects (Fan et al., 2010). The authors suggested that the variable temperatures interact with different genotypes, affecting their CR and therefore, blooming.

At the transcriptomic level, studies in *Populus*, leafy spurge (*Euphorbia esula* L.), Japanese pear (*Pyrus pyrifolia* Nakai) and peach, showed changes in processes involved with cold acclimation, responses to phytohormones, cellular transport, carbohydrate metabolism, response to oxidative stress, DNA methylation and histone modifications (Horvath et al., 2008; Jiménez et al., 2010; Bai et al., 2013; Howe et al., 2015). A recent study in sweet cherry has determined that buds in the stages of organogenesis, paradormancy, endodormancy and ecodormancy, can be defined by their expression profile. In this sense, before dormancy, an increase in the expression of *DORMANCY ASSOCIATED MADS-box* genes (*DAM*), floral identity genes and developmental genes was observed. Later in endodormancy, the authors observed an overrepresentation of genes that participate in the cold-response, abscisic acid (ABA) and oxidation-reduction processes (Vimont et al., 2019).

Epigenetic mechanisms involving histone modifications, DNA methylation and small non-coding RNAs are suggested as regulators of dormancy in a similar way as vernalization in *Arabidopsis* (Amasino, 2004). In peach, histone modifications observed in *DAM6*, were associated with gene repression after dormancy release (Leida et al., 2012). In addition, peach miRNAs were found to be differentially expressed between dormant and non-dormant leaf buds, some of them colocalizing with QTLs for CR (Barakat et al., 2012). In chestnut (*Castanea sativa*), global levels of DNA methylation increased in dormant buds in comparison to non-dormant buds, while in almond, the identification of differential methylation states in response to chilling accumulation provided information about methylation markers for flowering (Santamaría et al., 2009; Prudencio et al., 2018). In plants, DNA methylation occur in three different contexts: CpG, CHG and CHH, were H can be either cytosine, thymine or adenine (Law and Jacobsen, 2010). In sweet cherry, an increase in DNA methylation in all cytosine contexts was associated with an increase in the abundance of matching siRNAs in the promoter of a *MADS-box* gene (*MADS1*), homologous to the peach *DAM* genes, at the fulfillment of CR (Rothkegel et al., 2017).

Despite this, a better understanding of the molecular control of dormancy still needs to be established. In this study, the main objective is to elucidate the global changes in DNA methylation and transcript levels during chilling accumulation in dormant buds of sweet cherry varieties contrasting for CR. For this, we used whole-genome bisulfite sequencing (MethylC-seq), followed by the additional sequencing of vectors and amplicons that comprise differentially methylated regions as validation of the methylation pattern at specific loci. We used RNA-seq to analyze transcriptomic profiles modulated by chilling accumulation and integrated MethylC-seq and RNA-seq for the identification of biological processes and molecular pathways that may participate in dormancy regulation. Finally, our work contributes with the first epigenomic data at the DNA methylation level for sweet cherry, also providing additional information about the interaction between environment and genotype in the Rosaceae family.

MATERIALS AND METHODS

Plant Material

P. avium L. var. 'Royal Dawn' was cultivated and sampled during 2015 and 2016 from the commercial orchard 'Agrícola Garcés' located at San Francisco de Mostazal, Región de O'Higgins, Chile (33° 59' 53" S; 70° 41' 38" W). Adult trees of 'Kordia', a variety that needs more chilling hours (CH) accumulation to flower, were cultivated in two different fields. During 2015, we sampled trees cultivated in Pontificia Universidad Católica de Valparaíso, Quillota (32° 53'43.6" S; 71° 12' 34"W), and during 2016 we

sampled trees from 'Agrícola Garcés: Fundo Entre Ríos', O'Higgins region, Chile (34° 41' 10.4" S; 70° 52' 23.5" W). Cuttings containing around four to six clusters of floral buds were randomly collected before chilling accumulation (0 CH) and stored at −80°C. In winter, approximately thirty cuttings, considering ˜30 trees per variety, were sampled and stored in a cold chamber at 4°C without light for chilling accumulation. Every seven days, six cuttings were collected from the cold chamber. Three of these cuttings were rehydrated by re-cutting their basal end under water and placed in a greenhouse (25°C and 16/8 h day/night) for the estimation of bud break in the BBCH (*Biologische Bundesantalt, Bundessortenamt und Chemische Industrie*) 51 stage (**Figure 1**). In parallel, from the remaining three cuttings, four to six floral buds from each cutting were considered as three biological replicates for each sample point (**Figure 1I**). Buds were stored at −80°C for later use in MethylC-seq and RNA-seq.

Measurement of Chilling Requirement (CR)

The phenological stages of dormancy and flowering of sweet cherry varieties 'Royal Dawn' and 'Kordia' were analyzed according to the

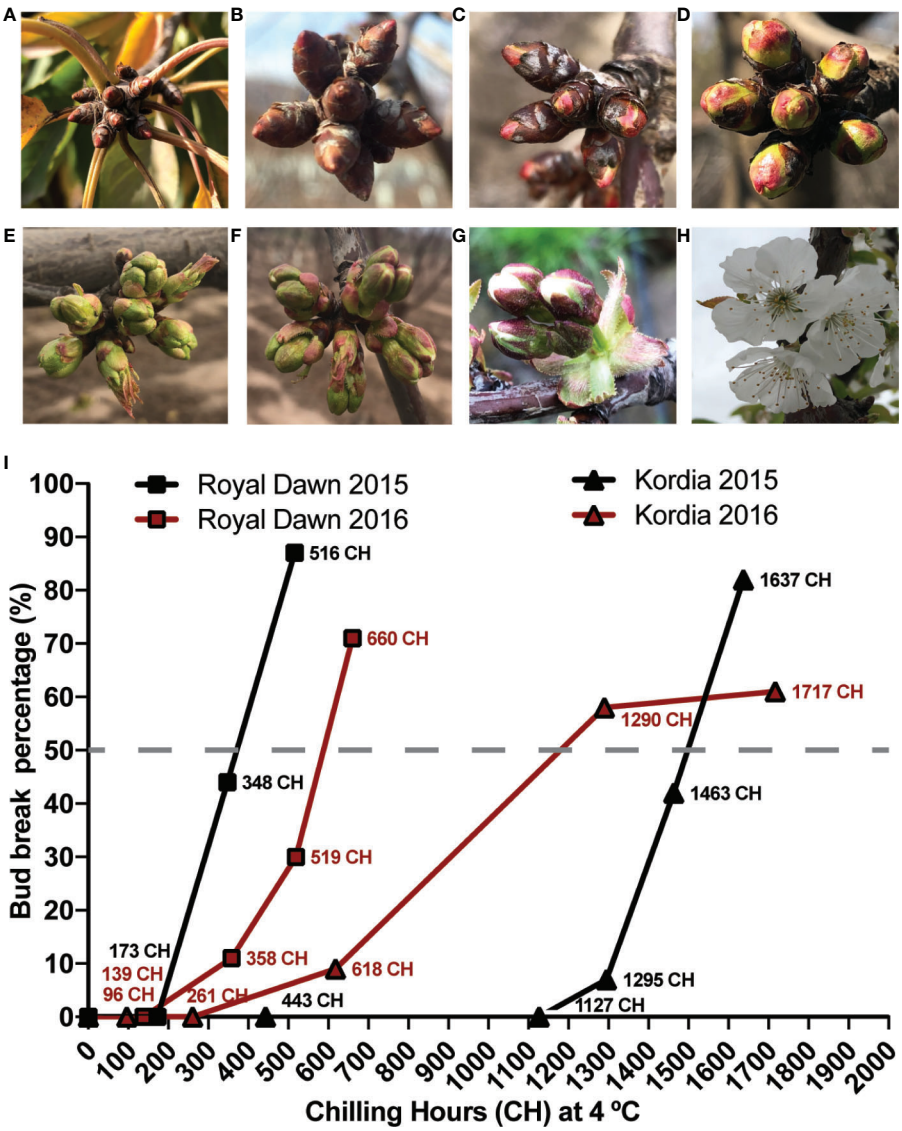

FIGURE 1 | Developmental growth stages of flower buds and sampling conditions during chilling accumulation in sweet cherry varieties according to the BBCH scale (*Biologische Bundesantalt, Bundessortenamt und Chemische Industrie*) (Fadón et al., 2015). **(A)** Paradormant buds and senescent leaves in autumn; **(B)** Endodormant buds during chilling accumulation in winter; **(C)** Inflorescence buds swelling and breaking from ecodormancy (BBCH 51 stage); **(D)** bud burst in spring; **(E)** Inflorescence enclosed by green scales; **(F)** Three to four inflorescences generated from a single flower bud; **(G)** Flower pedicel elongation; **(H)** Flowering; **(I)** Two seasons of bud break percentage during CH accumulation and sampling points for DNA methylation and transcriptome analysis in the low chill variety 'Royal Dawn' and high chill variety 'Kordia'. The dashed line represent a complete chilling requirement, indicated as a 50% or more of bud break.

BBCH scale (**Figure 1**) (Fadón et al., 2015). The CR necessary for bud break was measured for each variety as chilling hours (CH; number of hours at a temperature below 7.2°C) (Weinberger, 1950). Every seven days, 'Royal Dawn' and 'Kordia' cuttings sampled from the cold chamber were placed in water under favorable conditions (25°C and 16/8 h day/night) in a greenhouse. After 14 days in the greenhouse, the phenological state of the floral buds was analyzed and the CR was considered to be completed when at least 50% of the buds were swelling and began to show sepals in BBCH 51 stage (**Figure 1C**).

MethylC-Seq

Bisulfite treatment was carried out for season 2015 from genomic DNA of floral buds with different CH accumulation from 'Royal Dawn' (0 CH, 173 CH, 348 CH and 516 CH) and 'Kordia' (0 CH, 443 CH, 1,295 CH and 1,637 CH). DNA was extracted using the DNeasy Plant mini kit (QIAGEN, Germantown, MD, USA) according to manufacturer's instructions. DNA integrity was assessed in a 1.5% (p/v) agarose gel and concentration was determined by Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). A hundred nanograms of sample DNA was used for bisulfite treatment with the EZ DNA methylation gold kit (Zymo, Irvine, CA, USA) as previously described (Rothkegel et al., 2017). Twenty-four indexed and strand specific libraries were generated considering: two varieties, four sampling points and pooled buds from three cuttings as biological replicates. Ten nanograms of untreated DNA from 'Royal Dawn' and 'Kordia' were used as negative control libraries. All libraries were obtained with the TruSeq DNA Methylation kit (Illumina, San Diego, CA, USA) according to manufacturer's instructions. Validated libraries by Qubit Fluorometer and Fragment Analyzer (Advanced Analytical Technologies, Ankeny, IA, USA) were sequenced in HiSeq 2500, 2 × 125 bp Paired-end mode (Illumina, San Diego, CA, USA). Raw data is available at NCBI sequence read archive (PRJNA610988 and PRJNA610989).

Processing and Alignment of Bisulfite Reads

The partial genome of 'Royal Dawn' and 'Kordia' was generated with Bowtie 2 mapping the non-bisulfite treated libraries against the available data of *P. avium* as reference (Shirasawa et al., 2017) (**Table S2**). Adapters, low quality reads and clonal reads from each library were filtered with Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Filtered reads of bisulfite treated libraries were mapped to the partial genome of each variety using Bismark (Krueger and Andrews, 2011), with no mismatches. The methylation state in CpG, CHG and CHH was determined from the aligned reads using Bismark and MethylPipe (Kishore et al., 2015), and exported to Seqmonk software (Krueger and Andrews, 2011).

Methylation Level of Control Genes

To study the predictions obtained with MethylC-seq in a non-model species, we searched for a gene that should be transcriptionally active and a transposable element that should be highly methylated. Genomic DNA from floral buds of season 2015 was bisulfite treated using the EZ DNA Methylation gold kit as mentioned above, followed by amplification with ZymoTaq Polymerase (Zymo, Irvine, CA, USA) using primers for a putative *ACTIN-BINDING COMPONENT* (Pav_sc0002118.1_g070.1.mk) and a transposable element (Pav_ sc0000224.1_g040.1.br) (**Table S1**). The PCR product was cloned into a pGEM®-T vector (Promega, Madison, WI, USA), and ten clones per condition were sequenced by the Sanger method in Macrogen, Seoul, Korea. The obtained sequences were analyzed through Kismeth (http://katahdin.mssm.edu/kismeth/revpage.cl).

Identification of Differentially Methylated Regions

From cytosines covered from at least five reads, a sliding-window approach of 100 bp was used to analyze regions in the partial sweet cherry genome. The methylation state was calculated as log2 enrichment (log2 ratio of the observed base density in the region divided by the overall base density in the sample), and windows with less than 20 methylated cytosines were discarded. An ANOVA analysis (p-value <0.01) was used to obtain significant differences between windows from the four chilling conditions (0 CH, 173 CH, 348 CH and 516 CH for 'Royal Dawn'; 0 CH, 443 CH, 1,295 CH and 1,637 CH for 'Kordia'). P-value was later adjusted using a Benjamini and Hochberg correction (FDR <0.01). In addition, windows with differences of at least log2 fold-change >3 in their methylation state between two of the four conditions were identified as differentially methylated. A DMR that overlaps with a gene, including 2,000 bp upstream and downstream, was annotated as a differentially methylated gene. Subclusters of methylation profiles from DMRs were obtained considering the log2 enrichment and Kmer with MeV software.

Amplicon Bisulfite Sequencing (ABS) of Targeted Regions

Because of the lack of a reference genome, we complemented MethylC-seq with ABS using sampling points from 2015 and 2016 (**Figure 1I**). From DNA that was previously treated with bisulfite, a first PCR was performed with primers specific to each methylated region (**Table S1**) using ZymoTaq Polymerase. Twenty microliters of magnetic beads AMPure XP (Beckam-Coulter, Oakley Court, UK), were added to 25 µl of PCR product and incubated 15 min at room temperature in a magnetic stand (Thermo Fisher Scientific, Waltham, MA, USA). Supernatant was discarded and with the plate in the magnetic stand, two washes of 200 µl of freshly made ethanol 80% were added and incubated for 30 s. Ethanol was discarded and the plate was set to dry for 15 min at room temperature. The stand was removed from the magnetic plate, the pellet was resuspended in 22.5 µl of resuspension buffer (TE buffer, Tris-HCl pH 8.5) and incubated for 2 min. The stand was located in the magnetic plate and incubated for 1 min or until liquid was clear. Twenty microliters were transferred to a new tube and 2 µl were used as template for a second PCR with 10 µl of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 10 µM of Nextera indexed adapters (Illumina, San Diego, CA, USA) and nuclease free

water to a final volume of 20 µl. The PCR program considers an initial denaturation at 94°C for 1 min, followed by eight cycles at 95°C for 30 s, annealing of 68°C for 30 s, extension at 72°C for 30 s and a final extension of 5 min. Nuclease free water was added to the PCR product to reach a final volume of 50 µl. Fifty-six microliters of AMPure XP beads were added to purify the PCR product as described above but using 27.5 µl of resuspension buffer in the final step. The purified libraries were validated with Qubit Fluorometer using a High-Sensitivity DNA kit (Thermo Fisher Scientific) and Fragment Analyzer. Validated libraries were sequenced with MiSeq in 2 × 250 bp Paired-end mode (Illumina). Filtered reads were mapped to their reference with Bismark as previously described.

Total RNA Sequencing

Total RNA was extracted from floral buds of season 2015 considering three replicates of 0, 348, and 516 CH for 'Royal Dawn'; and 0, 443, 1,295, and 1,637 CH for 'Kordia'. RNA was extracted with PureLink™ Plant RNA Reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Quality of RNA was assessed by capillary electrophoresis and Qubit RNA BR Assay kit (Thermo Fisher Scientific). One microgram of RNA was used for construction of strand-specific libraries with the TruSeq Stranded mRNA kit (Illumina), and validated libraries were sequenced in HiSeq 4000, 2 × 100 bp Paired-end mode (Macrogen, Seoul, Korea). Raw data is available at NCBI sequence read archive (PRJNA611731 and PRJNA611733).

Data Analysis of RNA-Seq

Paired-end reads (100 bp) were trimmed with Trim Galore and mapped to the partial genome of *P. avium* (Shirasawa et al., 2017), using Spliced Transcripts Alignment to a Reference (STAR; Dobin et al., 2013). Filtered reads were normalized as trimmed mean of M-values (TMM) and used for differentially expressed gene (DEG) analysis with EdgeR considering a False Discovery Rate (FDR) <0.01 and a two-fold-change (Robinson et al., 2010). Subclusters of co-expressed genes (normalized with FPKM) were obtained with Kmer and MeV software.

Integration of MethylC-Seq and RNA-Seq Data

From previously obtained DMRs and DEGs, Venn diagrams were generated to obtain an overlap between the loci of methylations and transcripts. From hypermethylated regions associated with downregulated genes and hypomethylated regions with upregulated genes, we selected only the conserved patterns between varieties and represented them as a heatmap. As an additional and complementary analysis, we used conserved and non-conserved DMRs close to DEGs (upregulated and downregulated genes) for up to 2,000 bp upstream and downstream to determine the correlation value between DMR and transcript levels across all CH conditions. Those genes that presented a negative correlation value of −0.5 or less between the methylation and transcript levels were used for Gene Ontology (GO) analysis (FDR <0.01) with BiNGO from Cytoscape version 3.0.3 (http://apps.cytoscape.org/apps/bingo).

Real Time qPCR Analysis

One microgram of total RNA was treated with DNase I (Thermo Fisher Scientific), followed by cDNA synthesis with SuperScript™ first-strand synthesis system and oligo dT primers (Thermo Fisher Scientific), according to the standard protocol. Each cDNA sample was diluted 1:10 with nuclease free water before use. Master mix for RT-qPCR consisted of KAPA SYBR® FAST qPCR master mix (Kapa Biosystems, Wilmington, MA, USA), 10 µM of forward primer (**Table S1**), 10 µM of reverse primer (**Table S1**), ROX dye, template cDNA and PCR-grade water for a final volume of 10 µl. The RT-qPCR assay was performed in an AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). All RT-qPCR assays were performed using three biological and three technical replicates. Expression profiles were normalized to *Pavβ-ACTIN* gene and relative expression was obtained based in the ΔCT method.

RESULTS

Dormancy and Chilling Requirement for Contrasting Varieties of Sweet Cherry (*P. avium*)

In order to estimate the chilling requirement of 'Royal Dawn' and 'Kordia' during season 2015 and 2016, we sampled cuttings with floral buds in a paradormant state before cold accumulation (0 CH) (**Figure 1A**), and endodormant buds with different CH accumulation (**Figure 1I**). Bud break was determined when 50% or more of flower buds were in BBCH 51 stage (**Figure 1C**), which is considered to be the minimum chilling requirement (CR) for normal flowering. Considering this, for 'Royal Dawn' trees, we estimated that CR was completed at 516 CH (2015) and 660 CH (2016) (**Figure 1I**). 'Kordia' trees needed a higher chilling accumulation to complete the CR, observed at 1,637 CH (season 2015) and 1,290 CH (season 2016) (**Figure 1I**).

Genome Wide Sequencing of DNA Methylations in Contrasting Varieties for CR During Chilling Accumulation

For MethylC-seq of varieties 'Royal Dawn' and 'Kordia', we isolated genomic DNA from floral buds exposed to different CH accumulation of season 2015 (**Figure 1I**). From uniquely mapped reads (**Table S2**), we obtained the relative and absolute levels of methylated cytosines for the different contexts (CG, CHG and CHH; H = C, T or A) (**Figure 2A**, **Table S3**). The relative levels of methylated cytosines showed that in 'Royal Dawn', a 40–41% belong to the CpG context, while a 33% corresponds to CHG and a 25–27% to the CHH context (**Figure 2A**). The same tendence was observed in 'Kordia', where 38–39% corresponds to CpG, 33% to CHG and 27–29% to CHH (**Figure 2A**). To study the epigenetic variation during chilling accumulation in dormancy, we used the methylation calls and quantified them as log2enrichment to perform a sliding-window approach of 100 bp (FDR <0.01) and search for differentially methylated regions (DMRs) across the four chilling conditions of each variety.

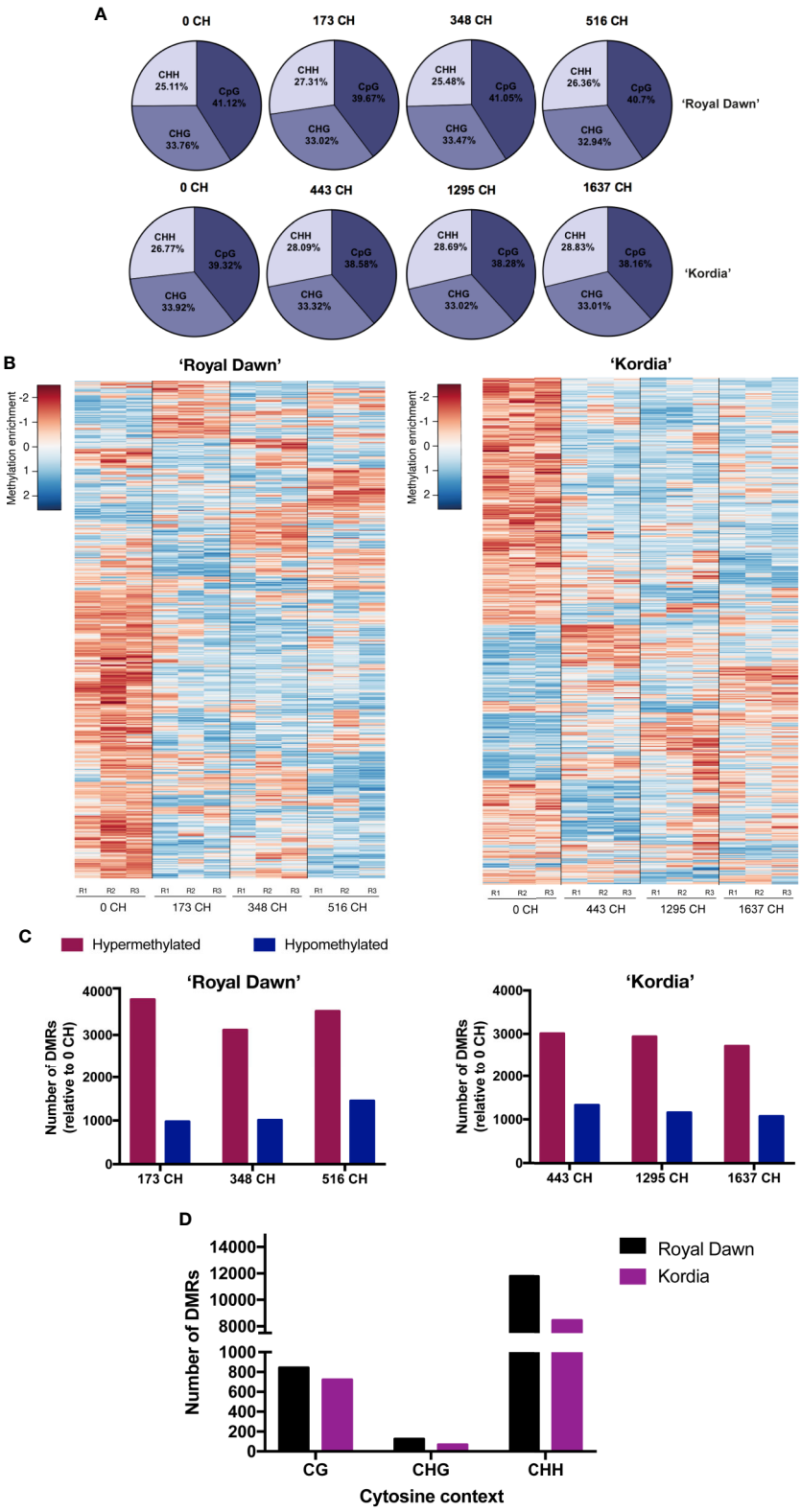

FIGURE 2 | Continued

FIGURE 2 | Methylation changes across chilling accumulation in floral buds of 'Royal Dawn' and 'Kordia'. **(A)** Relative levels of methylated cytosines in the contexts CG, CHG and CHH. **(B)** Heatmap of DNA methylation levels for 1,000 DMRs (log2FC >3; FDR <0.01; all cytosine contexts) identified from comparisons of 100-bp windows among 0, 173, 348 and 516 CH in 'Royal Dawn'; and comparisons among 0, 443, 1,295 and 1,637 CH in 'Kordia'. **(C)** Number of hypermethylated and hypomethylated regions from 173 vs 0 CH (hyper = 3,814; hypo = 975), 348 vs 0 CH (hyper = 3,115; hypo = 1,010), and 516 vs 0 CH (hyper = 3,546; hypo =1,451) in 'Royal Dawn'; and number of regions at 443 vs 0 CH (hyper = 2,990; hypo = 1,352), 1,295 vs 0 CH (hyper = 2,919; hypo = 1,179) and 1,637 vs 0 CH (hyper = 2,703; hypo = 1,094) in 'Kordia'. **(D)** Number of DMRs in each cytosine context from comparisons between 173, 348 and 516 CH against 0 CH in 'Royal Dawn' (CG, n = 843; CHG, n = 126; CHH, n = 11,786); and comparisons between 443, 1,295 and 1,637 CH against 0 CH in 'Kordia' (CG, n = 723; CHG, n = 67; CHH, n = 8,448). H = C, T or A.

Considering all cytosine contexts, we identified 9,600 DMRs in 'Royal Dawn' (**Table S4**) and 8,535 in 'Kordia' (**Table S5**). For both varieties, we grouped these DMRs in 16 subclusters according to the average methylation level and pattern (**Tables S6 and S7**). Considering only the top 1,000 DMRs that showed a highest variance value in their methylation enrichment among the four chilling conditions, changes in the methylation level were mainly between 0 CH and 173 CH for 'Royal Dawn' and between 0 CH and 443 CH for 'Kordia' (**Figure 2B**). In addition to this, a higher number of the overall DMRs was associated to hypermethylation in both varieties, however 'Royal Dawn' showed 3,115–3,814 of hypermethylated regions compared to 'Kordia' with 2,703–2,990 (**Figure 2C**). To identify the most variable cytosine context to be methylated, we searched for DMRs considering only CpG, CHG or CHH (**Figure 2D**). The highest variation in methylation was observed in the CHH context, with 11,786 (CHH), 126 (CHG) and 843 (CpG) DMRs in 'Royal Dawn'; the same tendency was observed for 'Kordia' with 723 (CpG), 67 (CHG) and 8,448 (CHH) DMRs (**Figure 2D**).

To study the results obtained with MethylC-seq, we searched for a gene that should be transcriptionally active (*e.g.* related to a housekeeping gene), and on the other hand a transposable element that should be highly methylated (**Supplementary Figure 1**). From the genomic data, we synthetized primers and with the same samples used previously, we performed bisulfite treatment followed by cloning and sequencing of 10 clones per condition (**Supplementary Figures 1 and 2**). The sequencing of clones from 'Kordia' and 'Royal Dawn' revealed that a putative *ACTIN-BINDING COMPONENT* (Pav_sc0002118.1_g070.1.mk) possess gene body methylation in the CpG context for all CH accumulations, while the transposable element (Pav_ sc0000224.1_g040.1.br) is highly methylated in the three cytosine context and maintained during chilling accumulation.

In addition, to validate targeted DMRs with higher depth, we implemented the sequencing of small size amplicons (size <350 bp) containing a DMR flanked by sequencing adapters. From the MethylC-seq data we analyzed a DMR of 180 bp that is located ˜1,600 bp downstream a gene annotated as a *2-ALKENAL REDUCTASE NADP(+)-DEPENDENT* (**Supplementary Figure 3**). From MethylC-seq data, in 'Royal Dawn' we observed an increase in the methylation level regarding to 0 CH, starting from 0.7 log2enrichment at 175 CH, increasing to 2.7 at 516 CH. In 'Kordia' there was a decrease of the levels of methylation at 443 CH, increasing later at 1,637 CH (**Supplementary Figure 3A**). Concomitant with this, the amplicon bisulfite sequencing (ABS) of the targeted region in 'Royal Dawn' showed an increase of the methylation level in the CHH context (from 76 to 82%), reaching 'Kordia' levels during chilling accumulation for two consecutive seasons (2015 and 2016). In 'Kordia', ABS showed a decrease from 87 to 84% at 1565 CH for the CpG context and a decrease from 84 to 79% at 557 CH, followed by an increase of 81% at 1565 CH for the CHG context (**Supplementary Figure 3B**). On the other side, the transcript level for *2-ALKENAL REDUCTASE NADP(+)* started with a higher expression in 'Kordia' at 0 CH, followed by a decrease in both varieties until a complete CR (**Supplementary Figure 3C**).

Sequencing the Transcriptome of Contrasting *P. avium* Varieties for CR During Chilling Accumulation

In order to identify transcripts that could be regulated by DNA methylation and chilling accumulation, we sequenced the transcriptome (RNA-seq) of dormant buds using samples of season 2015 (**Figure 1I**). In this case, 70–90% of the reads mapped uniquely, while 5–23% of the reads did not align (**Table S8**). From the uniquely mapped reads, we performed a principal component analysis (PCA) to determine if the overall gene expression was modulated by chilling accumulation (**Figures 3A, B**). For 'Royal Dawn', a 70.15% of the variability in gene expression was explained by PCA, in which all treatments were well separated according to their cold accumulation (**Figure 3A**). On the other side, for the high chill variety 'Kordia', PCA explains a 66.5% of the variability and could separate 0 CH, 443 CH and 1,295 CH, but placing together 1,295 CH and 1,637 CH, indicating a regulation that is specific for each variety (**Figure 3B**). Together with this, the expression level (log2 TMM-normalized values) of the top 1,000 transcripts were ranked according to their TMM variance value among the four chilling conditions. For 'Royal Dawn', changes in the overall gene expression were observed from 348 CH (**Figure 3C**), while in 'Kordia', differences in overall gene expression were observed from 443 CH but increasing at 1,295 CH (**Figure 3D**), indicating that gene expression is regulated depending on the variety. In addition, we performed a co-expression analysis to group the overall genes into subclusters according to their averaged expression levels (**Supplementary Figure 4**), obtaining 10 subclusters in 'Royal Dawn' and 11 in 'Kordia'. Two subclusters that represented an increase and a decrease of the transcript levels during cold accumulation are shown in **Figures 3E, F**.

For differentially expressed genes (DEGs), a two-fold difference (log2 ≥1) in the transcript counts and a false-discovery rate of 0.01 or less were used as threshold to compare the cold treatments vs 0 CH (no cold). With the DEGs generated from 348 vs 0 CH, 516 vs 0 CH ('Royal Dawn'); and 443 vs 0 CH, 1,295 vs 0 CH, 1,637 vs 0 CH ('Kordia'), we study the relationship between DNA methylation and gene expression during chilling accumulation.

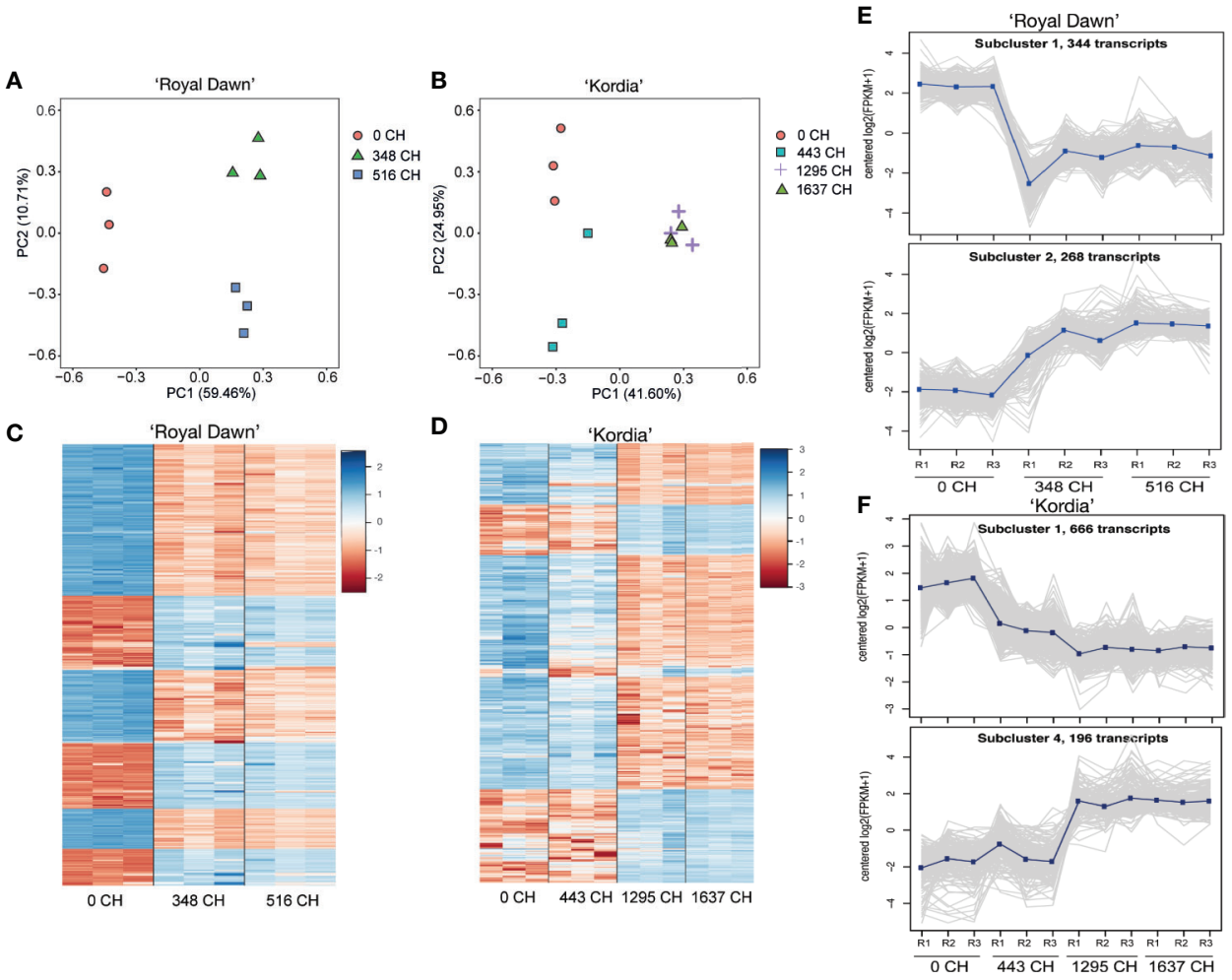

FIGURE 3 | Transcriptome profile during chilling accumulation in 'Royal Dawn' and 'Kordia'. **(A)** Principal component analysis of global gene expression with three biological replicates at 0, 348 and 516 CH in 'Royal Dawn'; and **(B)** at 0, 443, 1,295 and 1,637 CH for 'Kordia'. **(C)** Heatmap representing the 1,000 transcripts with most variance among the conditions in 'Royal Dawn' and **(D)** 'Kordia'. Color key indicates the median of log2 TMM-normalized values and red to blue represents low to high levels of transcript, respectively. **(E)** Representative subcluster plots (2 out of 10) of the overall genes that increase or decrease their expression during chilling accumulation in 'Royal Dawn' and **(F)** subcluster plots (2 out of 11) of 'Kordia'.

Initially, for 'Royal Dawn' we observed 5,083 (2,259 upregulated and 2,824 downregulated) genes that significantly change their expression at 348 CH, and 4,839 (2,141 upregulated and 2,698 downregulated) genes at 513 CH (**Figures 4A, B**). 'Kordia' started from 1,219 DEGs (447 up and 772 down), increasing to 4,291 DEGs (1,864 up and 2,427 down) at 1,295 CH and 4,387 DEGs (1,885 up and 2,502 down) at 1,637 CH, also coinciding with PCA results (**Figure 3B**).

To see if RNA-seq data was reflected in additional expression analysis, we made qPCRs in both varieties from the same RNA and studied *FLOWERING LOCUS T* as a gene associated to dormancy and flowering regulation. Genes that showed a DMR with increased methylation levels and correlated with transcript downregulation, *TRANSPARENT TESTA 1, FLAVONOL SYNTHASE*, *AMSH-like* and *β-GALACTOSIDASE*. Additionally, a gene that increased its expression with CH accumulation and was related to cold response, *COLD-SHOCK PROTEIN 2* (**Table S1**). All these genes showed similar patterns in RNA-seq and qPCR (**Supplementary Figure 5**).

Integration of the Methylome and Transcriptome During Chilling Accumulation

Afterwards, when analyzing the overlap between DEGs and DMRs, we observed for 'Royal Dawn' that 174 and 162 DMRs were associated with hypermethylation that overlapped with genes that significantly increase and decrease their expression at 348 CH, respectively. At the same time, 46 and 52 hypomethylated regions overlapped with upregulated genes and downregulated genes, respectively (**Figure 4A**). At 516 CH, 170 and 186 hypermethylations coincided with upregulated and downregulated genes respectively, while 51

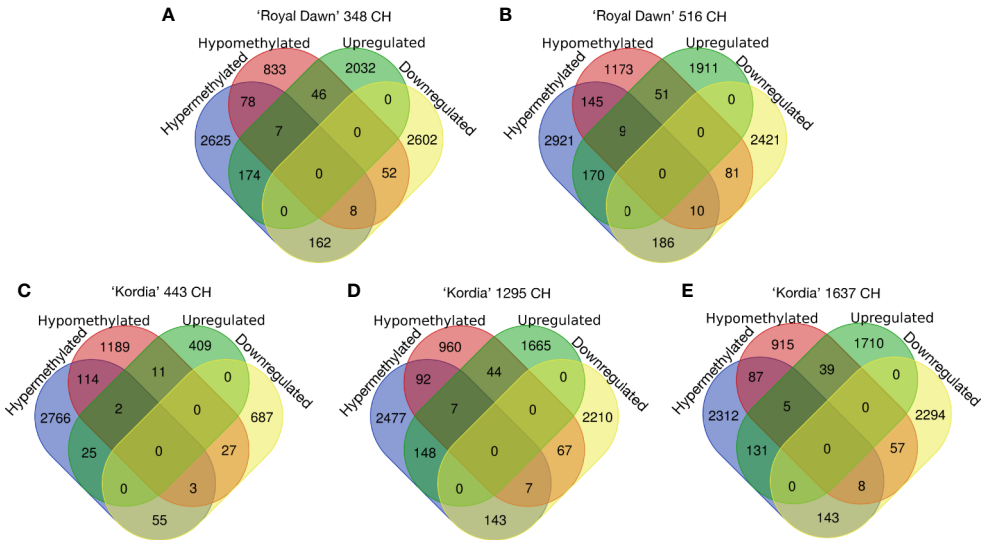

FIGURE 4 | Venn diagrams representing the number of DEGs (upregulated and downregulated genes) that overlapped with DMRs (hypermethylations and hypomethylations). **(A)** Overlapping of DMRs and DEGs at 348 CH and **(B)** 516 CH for 'Royal Dawn'. **(C)** Number of DMRs and DEGs that overlapped at 443 CH, **(D)** 1,295 CH and **(E)** 1,637 CH for 'Kordia'.

and 81 hypomethylated regions coincided with upregulated and downregulated genes (**Figure 4B**).

For 'Kordia', 25 and 55 hypermethylated regions were related to upregulated and downregulated DEGs at 443 CH, while 11 and 27 hypomethylations were associated to upregulated and downregulated genes, respectively (**Figure 4C**). With 1,295 CH, we observed an increase of 148 and 143 hypermethylations coincident with upregulated and downregulated genes, together with 44 and 67 hypomethylations associated to genes that increase and decrease their expression, respectively (**Figure 4D**). At 1,637 CH, 131 and 143 regions that increase their methylation level overlapped with genes that increase and decrease their expression, respectively. In addition, 39 and 57 regions that decreased their methylation levels overlapped with upregulated and downregulated genes, respectively (**Figure 4E**).

Regarding to DMRs that were associated to genes that do not change their expression in a significant manner (**Figure 4**), it can be observed that 2,703 hypermethylations and 911 hypomethylations were not related to DEGs in 'Royal Dawn' at 348 CH. This number increase with 3,066 hypermethylations and 1,318 hypomethylations at 516 CH. In 'Kordia', the opposite occurs were 2,880 hyper and 1,303 hypomethylated regions were observed at 443 CH, followed by a decrease of 2,569 hyper and 1,052 hypomethylations, coincident with the increase of DMRs overlapping with DEGs. Finally, at 1,637 CH, 2,399 hyper and 1,002 hypomethylated regions were observed (**Figure 4**).

DMRs and DEGs Conserved in 'Royal Dawn' and 'Kordia'

From the overlap between hypermethylated region/downregulated genes and hypomethylated region/upregulated genes (**Figure 4**), we searched for those patterns conserved in both varieties. From this analysis, we obtained thirty genes that showed a conserved pattern of methylations and expression in both varieties, indicating that most of these modifications are unique to each variety (**Figure 5**). The identified genes were related to a response to stress linked to the sensing and signal transduction of cold, such as two protein kinases, *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR 13*-like, one heat-shock protein and two proteins dependent of calcium (Ca^{+2}). For example, *CALCIUM-TRANSPORTING P-type ATPase* showed an increase in the methylation levels from a DMR located downstream the gene, starting from 173 CH in 'Royal Dawn' and 443 CH in 'Kordia', coincident with the decrease in the transcript levels at 348 CH in 'Royal Dawn' and 443 CH in 'Kordia' (**Figure 5**).

Genes associated to an oxidation-reduction process, like 3 dehydrogenases and a dioxygenase *O-DEMETHYLASE*-like, were also identified. *FOLD1* is a mitochondrial dehydrogenase that in 'Royal Dawn' showed a decrease in methylations from a DMR located upstream the gene at 348 CH, and an increase in its transcript level at the same time point. The same tendency was observed in 'Kordia' but at a different temporality, where a decrease in methylations were observed at 1,295 CH and the increase in gene expression was observed from 443 CH (**Figure 5**).

Five genes were related to the metabolism of lipids and for example a GDSL esterase/lipase increased its methylation levels from a DMR at 173 CH ('Royal Dawn') and 443 CH ('Kordia'), concomitant with the decrease in gene expression from 348 CH and 1,295 CH, respectively (**Figure 5**). From the phenylpropanoid metabolism, two genes were identified: a putative *PHENOLIC GLUCOSIDE MALONYLTRANSFERASE 1-like* and *FLAVONOID 3-O-GLUCOSYLTRANSFERASE-like*. The latter showed an increase in methylations from a DMR located in an intron at 173 CH ('Royal Dawn') and 443 CH ('Kordia'), followed by a decrease in expression from 348 CH and 1,295 CH, respectively.

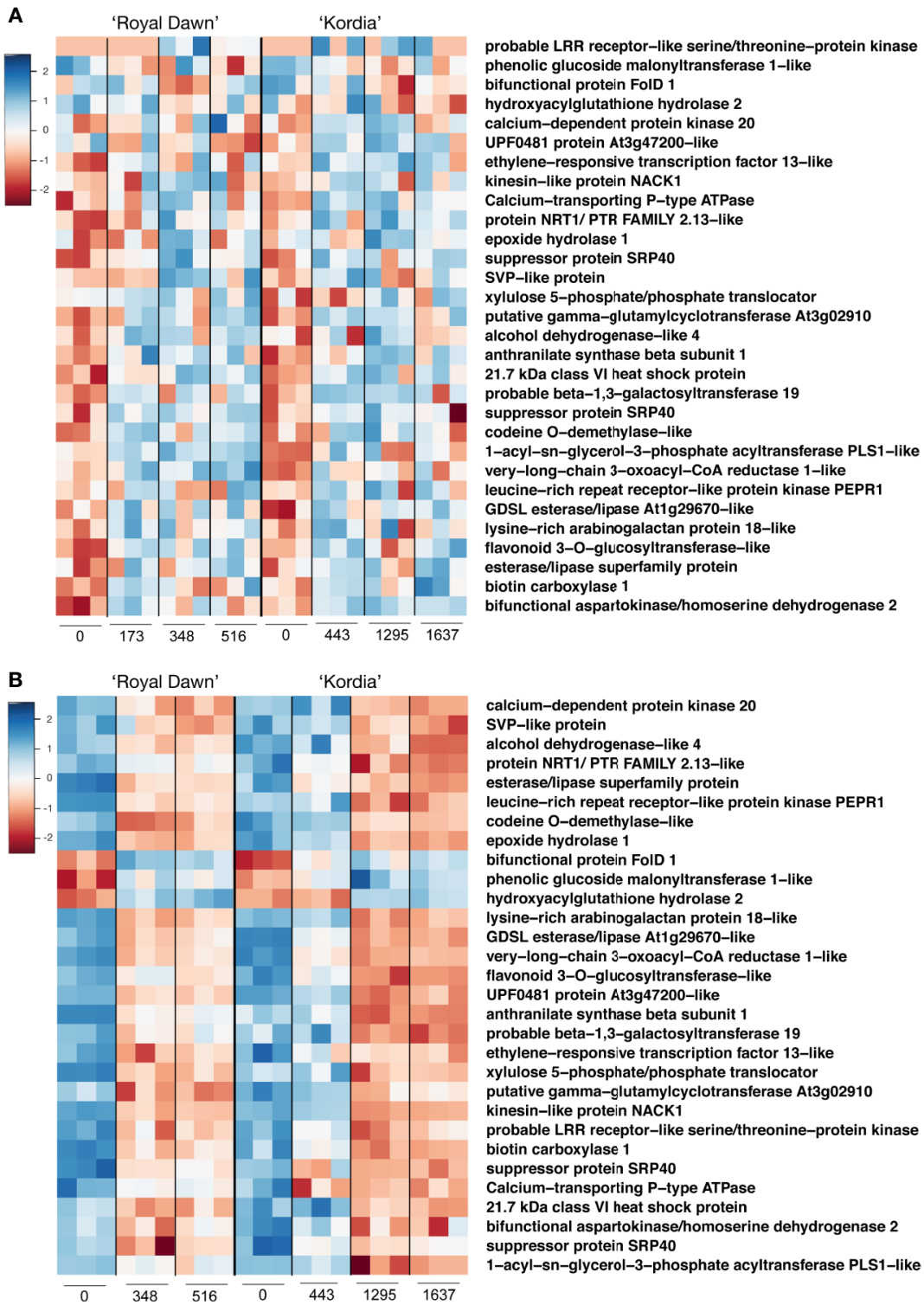

FIGURE 5 | Representation of thirty genes that showed a conserved hyper/hypomethylation pattern that overlapped with down/upregulated genes. **(A)** Methylation levels (log2 enrichment) from DMRs related to hyper and hypomethylations at different CH accumulation points for 'Royal Dawn' and 'Kordia'. **(B)** Transcript levels (log2 TMM-normalized values) from DEGs related to downregulated and upregulated genes during CH accumulation in 'Royal Dawn' and 'Kordia'. Color key from red to blue represents low to high levels, respectively.

The *SHORT VEGETATIVE PHASE* (*SVP*) gene is involved with the regulation of vernalization in *Arabidopsis thaliana* and is an orthologous of the *DAM* genes. In 'Royal Down', a DMR located downstream the gene increased its methylation levels at 348 CH, in addition to a decrease in the transcript levels at the same time point. Meanwhile in 'Kordia', this increase in methylations was observed from 443 CH and was associated to a decrease in gene expression from 1,295 CH (**Figure 5**). All these results showed that despite the conserved profile of methylations and transcript levels from the thirty genes, patterns were unique to each variety and related to their CR.

Correlation Between DEGs and DMRs Across All Chilling Conditions

To identify genes that constantly decrease their expression (in RNA-seq and qPCR analysis) together with their closest DMR (up to 2,000 bp upstream and downstream of genes) that may constantly increase its methylation level across all the chilling accumulation conditions, or vice versa, we calculated the Pearson correlation value between RNA and DNA methylation levels through 0 CH, 348 CH and 516 CH for 'Royal Dawn' and across 0 CH, 443 CH, 1,295 CH and 1,637 CH for 'Kordia'. Genes with a Pearson value of $r = -0.5$ or less were used for further study (**Tables S9** and **S10**). From these genes ($r \leq -0.05$), we obtained the GO categories and observed an overrepresentation of 60 biological processes in 'Royal Dawn', while only 15 processes were in 'Kordia' (**Figure 6A**). Afterwards, from the GO analysis we further studied three genes that presented larger DMRs, from 70 bp to 300 bp. From the GO term 'cellular process' present in both varieties, we analyzed *PavAMSH*-like (*AMSH-like UBIQUITIN THIOESTERASE 3 ISOFORM X1*) (**Figure 6B**), a gene involved with cellular trafficking. In 'Royal Dawn' and 'Kordia', the methylation level from the DMR of approximately 300 bp increased at 173 CH and 443 CH respectively. This DMR was located in the intron of *AMSH*-like and correlated with a decrease in the expression of this gene from early stages of chilling accumulation, 139 CH in 'Royal Dawn' and 96 CH in 'Kordia'.

Additionally, from the term 'response to abiotic stimulus', which was also present in both varieties, we analyzed a *PavFLAVONOL SYNTHASE-like* gene that showed a variety-specific regulation (**Figure 6C**). In 'Kordia', a DMR of approximately 300 bp located at the 3' end of the gene showed an increase in the methylation levels from 443 CH, coincident with a constant decrease in the expression levels from 96 CH. On the contrary, in 'Royal Dawn' this region was not methylated at any time point, and gene expression constantly increased until its downregulation at 519 CH, but still maintaining higher levels than 'Kordia'. Hence, this variety-specific result is complementary to the conserved profiles of *FLAVONOID 3-O-GLUCOSYLTRANSFERASE-like* and *PHENOLIC GLUCOSIDE MALONYLTRANSFERASE 1-like* mentioned previously for **Figure 5**.

Another gene named *PavPHOSPHATASE 2A* (*PP2A*), which belong to the terms of 'protein modification process', 'biological regulation' and may be related to the cold-signaling process, also showed a genotype-specific regulation (**Figure 7**). This gene possesses a DMR of 70 bp located ˜1,600 bp upstream that can be targeted and analyzed by ABS (**Figure 7A**). Initially from the MethylC-seq analysis, the methylation level of this DMR increased in 'Royal Dawn' at 173 CH, while in 'Kordia' this methylation level was maintained across all CH conditions (**Figure 7A**). An *in silico* study of the DMR sequence in The Plant ChIP-seq Database (PCBase) showed the presence of *cis*-regulatory elements for transcription factors Dof1.8 (AT1G64620), homeodomain-like protein (AT2G40260), NAC3 (AT3G15500), and a subunit of the nuclear DNA-dependent RNA polymerase V NRPE1 (AT2G40030) required for RNA-directed DNA methylation (RdDM) (**Figure 7B**). With the aminoacidic sequence of PavPP2A and the related proteins from *Arabidopsis*, we observed an 81% of identity and a closest phylogeny with AtPP2AB2 (AT1G17720), revealing that this gene is highly conserved between these species (**Figure 7C**). Later, by using ABS, this DMR also showed an increase in the methylation levels (from 27.5 to 58.7%) in the CHH context from 173 CH in 'Royal Dawn', coincident with the results of MethylC-seq and with the presence of a *cis*-element for NRPE1 (**Figure 7D**). While in 'Kordia', methylation in the CHH context was maintained from 70 to 73.5%, suggesting that the regulation of this gene is dependent on the variety (**Figure 7E**). This result also coincided with the transcript profile of *PavPP2A*, which was different for both varieties. In 'Royal Dawn', this gene was downregulated from 358 CH and in 'Kordia', the transcript level was maintained until 1290 CH, followed by a slight decrease (**Figure 7F**). These changes were also observed from validated RNA-seq data (**Supplementary Figure 5**).

DISCUSSION

The effect of the environment on epigenetic regulation can induce changes in gene expression that trigger a certain phenotype. The study of environmental epigenetics has been focused in DNA methylation due to its essential role in development, genomic imprinting and silencing of transposable elements (Feil and Fraga, 2012). As a consequence of repeated changes in temperature, epigenetic transitions can arise from individual plants. An example of this is the vernalization process in *Arabidopsis* and dormancy in perennial trees from temperate climates (Chinnusamy and Zhu, 2009; Feil and Fraga, 2012). In this study, we used an epigenomic and transcriptomic approach to elucidate how changes in DNA methylation and gene expression participate in the chilling accumulation process of dormant buds in sweet cherry.

Chilling Requirement (CR) Variability and Methylome Dynamics in Dormant Floral Buds

Temperate fruit trees can be grown in many regions with different environmental conditions and even within the same region, climate conditions can be different depending on the year, making some traits like CR and flowering date, variables across different locations and seasons (Alburquerque et al., 2008;

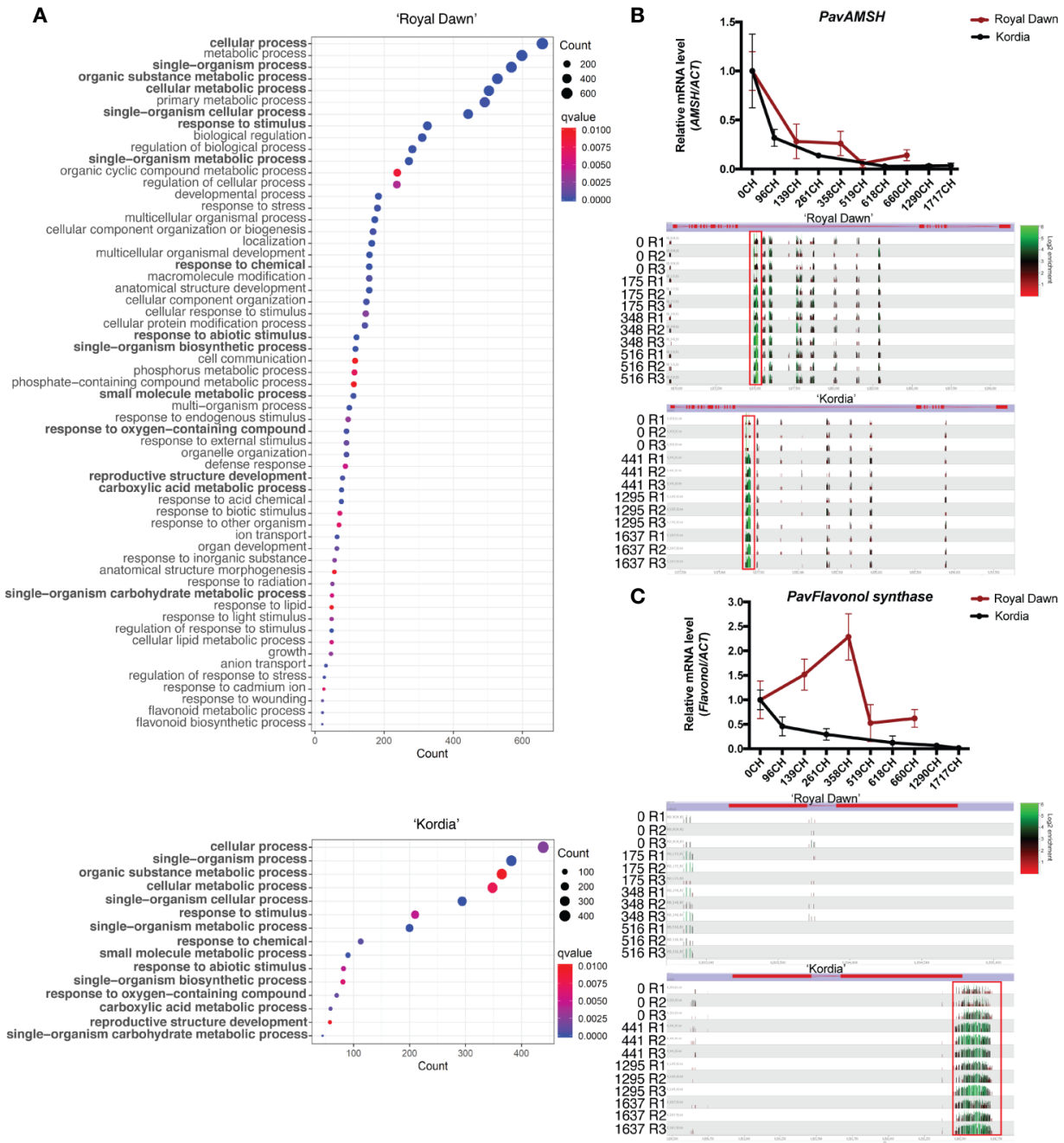

FIGURE 6 | Enrichment of differentially expressed genes that negatively correlated (r <–0.5) with methylation levels during chilling accumulation. **(A)** Gene Ontology terms (p-value <0.01) of biological processes from DEGs associated with DMRs in 'Royal Dawn' and 'Kordia'. The mechanisms in bold represent conserved mechanisms between varieties and the dot size indicates the number of genes as counts. **(B)** Transcript (above) and DNA methylation level (below) of a DMR of approximately 300 bp in an intron of *PavAMSH* (Pav_sc0001014.1_g060.1.mk), gene from the 'cellular process' mechanism that was methylated in both varieties. Transcripts are represented as qPCR analysis and gene methylation as enrichment levels from Seqmonk visualizer, where green peaks indicate high levels and black to red indicate low levels of methylation. **(C)** Differential regulation of *PavFLAVONOL SYNTHASE* (Pav_sc0000030.1_g1340.1.mk), gene from the mechanism 'response to abiotic stimulus', being methylated in approximately 300 bp of its 3' end. Transcripts are represented as qPCR (above) and DNA methylation as enrichment (below).

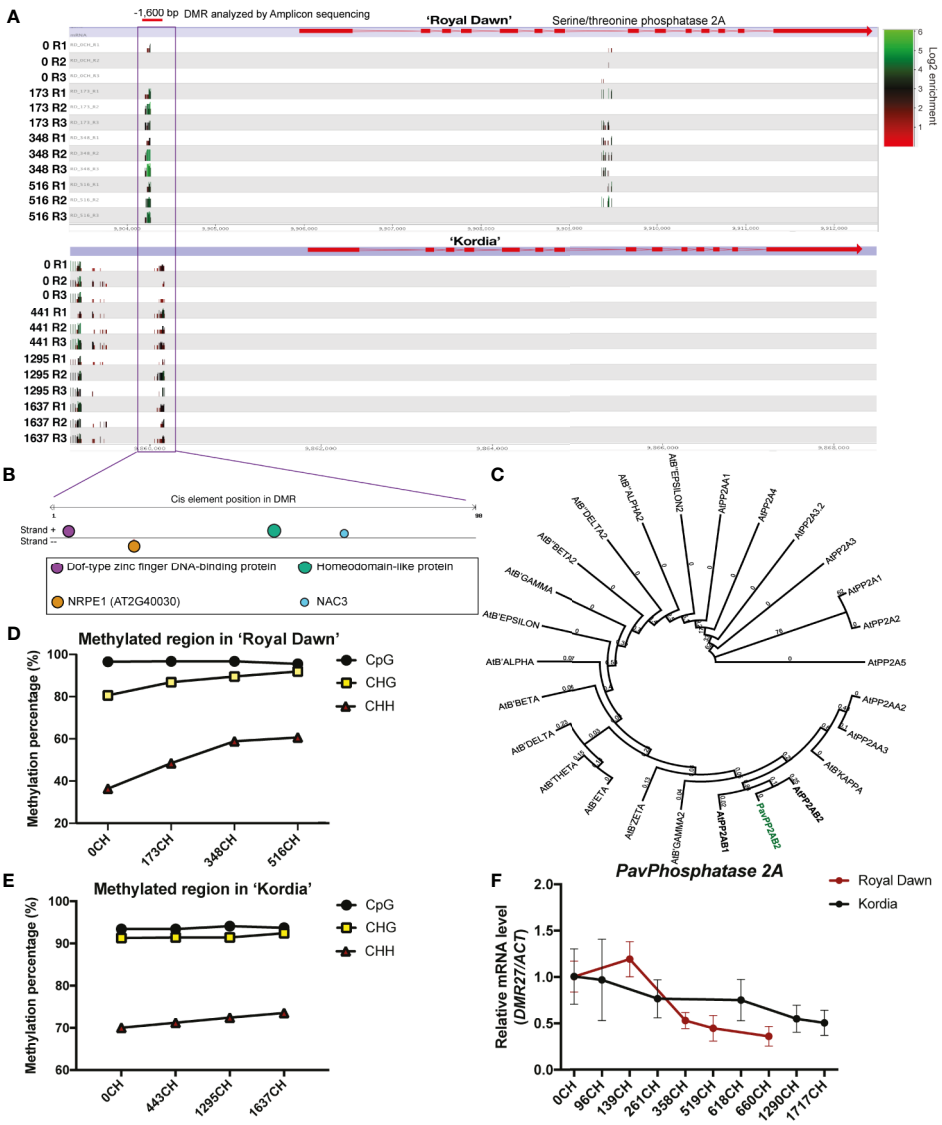

FIGURE 7 | Analysis of a DMR located –1,600 bp from a *PHOSPHATASE 2A* (*PP2AB2*) gene. **(A)** Seqmonk genome browser of the putative *PHOSPHATASE 2A* (Pav_sc0004290.1_g070.1.mk) and its level of DNA methylation including all cytosine context. Methylation levels are observed as log2 enrichment in 'Royal Dawn' and 'Kordia', where green represents high levels and red, low levels. The red line indicates the DMR analyzed by Amplicon bisulfite sequencing (ABS). **(B)** *In silico* analysis of the DMR sequence in The Plant ChIP-seq Database (PCBase), where color circles indicate the interaction sites with a zinc finger protein (AT1G64620), a homeodomain protein (AT2G40260), NRPE1 (AT2G40030) and NAC3 (AT3G15500). **(C)** Phylogenetic tree considering the aminoacidic sequence of PP2AB2 from sweet cherry (in bold-green) and 25 phosphatases from *Arabidopsis thaliana.* **(D)** Level of DNA methylation as percentage in the DMR analyzed by ABS in 'Royal Dawn' and **(E)** 'Kordia'. **(F)** Transcript levels of *PHOSPHATASE 2A* analyzed by qPCR relative to *Pavβ-ACTIN* using three biological and three technical replicates. Error bars represent standard deviation (SD).

Castede et al., 2014). During seasons 2015 and 2016, 'Royal Dawn' showed a CR that oscillated between 516 CH and 660 CH, respectively (**Figure 1**). On the other hand, 'Kordia' trees needed a higher accumulation of chilling in both seasons (1,637 and 1,290 CH), showing higher variability due to different locations of sampling and being consistent with the important effect of the environment over this trait.

The presence of epigenetic modifications related to the environmental conditions of dormancy has been previously reported in cherry, peach, apple, pear (*P. pyrifolia*), almond (*P. dulcis*), and chestnut (*C. sativa*) (Santamaría et al., 2009; Leida et al., 2012; Saito et al., 2015; Kumar et al., 2016; Rothkegel et al., 2017; Prudencio et al., 2018). In this work, the relative levels of methylated cytosines in 'Royal Dawn' and 'Kordia' at season 2015 showed that the most methylated context was CpG, followed by CHG and CHH (**Figure 2**). These patterns coincide with methylomes from model species like *Arabidopsis* and apple (*Malus domestica* borkh.), which have a higher level of CpG methylation (49–55%), continued by CHG (23–39%) and CHH (12–22%), highlighting that each type of methylation is

regulated under different pathways (Lister et al., 2008; Daccord et al., 2017). Methylation in the CG context is maintained during DNA replication by DNA METHYLTRANSFERASE 1 (MET1), and methylation in the CHG context is maintained by a reinforcing loop involving CHROMOMETHYLASE 3 (CMT3) and histone marks (H3K9). On the other side, CHH methylation is carried out by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) and RNA-directed DNA Methylation (RdDM). In this pathway, small non-coding RNAs target homologous DNA sequences for methylation by DRM2 not only on CHH cytosines, but also on CG and CHG (Law and Jacobsen, 2010).

Considering the tree cytosine contexts, we identified 9,600 DMRs in 'Royal Dawn' and 8,535 in 'Kordia' across all CH accumulation. This difference is mainly due to the genetic background of each variety, having an important effect on epigenetic modifications. In addition, we observed that most of these DMRs refers to hypermethylations between 0 CH and the following condition of CH accumulation (173 CH in 'Royal Dawn' and 443 CH in 'Kordia'), suggesting that an increase in DNA methylation occurred as an early response to cold temperatures and that the moment in which these changes occur may be even earlier than the sampled points. Concomitant with this, the highest variation in methylation for both varieties was observed in the CHH context, indicating that genes regulated by RdDM may play important roles into an abiotic stress response like temperature. In previous works, hypermethylation was reported during dormancy in chestnut, apple and almond, where the authors observed higher levels of methylation in dormant buds followed by its decrease at bud burst (Santamaría et al., 2009; Kumar et al., 2016; Prudencio et al., 2018). Regarding to another abiotic stress like salinity, in *Populus euphratica*, increases in methylation after salt treatments were dependent on the tissue and most methylations (57.4–66.2%) occurred in CHH cytosines, also suggesting the participation of RdDM as an abiotic stress response (Su et al., 2018). Moreover, RdDM can dynamically and reversibly regulate the expression of adjacent genes involved with stress responses (Matzke et al., 2015). Coincident with this, another study of sweet cherry during chilling accumulation showed an increase in DNA methylation and abundance of matching small interference RNAs, which were associated with RdDM in the promoter of a dormancy-associated MADS-box gene (Rothkegel et al., 2017).

Chilling Accumulation Modulates the Transcriptome of Sweet Cherry, Which Is Preceded by Changes in Methylation Levels

In response to low temperatures, plants can rearrange their transcriptomes and induce a large number of stress-related genes for cold acclimation. A recent work in sweet cherry showed that buds in the stages of organogenesis, paradormancy, endodormancy and ecodormancy, can be defined by the differential expression of genes involved with specific pathways. Moreover, endodormancy was characterized by pathways of cold response genes, ABA and oxidation-reduction processes (Vimont et al., 2019). In this work, we analyzed changes of the transcriptome during the chilling accumulation stage of endodormancy followed by co-expression analysis (**Figure 3**). According to the PCA, in 'Royal Dawn', the overall gene expression was reprogrammed rapidly from 348 CH in response to low temperatures. On the opposite, 'Kordia' showed that changes in gene expression started from 443 CH, increased at 1,295 CH and were later maintained until a complete CR at 1,637 CH. Coincident with this, the expression profile from the top 1,000 genes and co-expression analysis, also showed changes from 348 CH in 'Royal Dawn' and 1,295 CH in 'Kordia'. These results suggest a reprogramming that depends on the genetic background of each variety, as reported previously from QTL analysis for the trait of CR (Castede et al., 2014).

In accordance with methylation changes, which occurred from 173 CH in 'Royal Dawn' and 443 CH in 'Kordia', the reprogramming of the transcriptome was preceded by changes at the methylation level. Regarding this, in 'Kordia', 1,219 differentially expressed genes (DEGs) were observed at 443 CH, increasing to 4,291 genes at 1,295 CH and 4,387 genes at 1,637 CH (**Figure 4**). In this sense, the dynamics of DNA methylation as a transcriptional regulator has been widely studied in plants and animals. DNA methylation can regulate gene transcription by directly interfering with the binding of transcription factors to their recognition sequences (Attwood et al., 2002). However, despite this, no direct correlation can be found between MethylC-seq and RNA-seq data because usually, a specific pattern reflects a dynamic regulation of establishment and maintenance and thus, it is necessary to identify which gene was regulated due to a methylation change and which gene was previously regulated by another factor, but being maintained by a methylation change (Zhang et al., 2018).

In this work, an increase from 162 to 186 downregulated genes was associated to hypermethylated regions in 'Royal Dawn'. In 'Kordia', 25 hypermethylated regions overlapped with downregulated genes at 443 CH, increasing to 143 genes at 1,295 CH and 1,637 CH (**Figure 4**). This result showed that most of the DMRs were not associated with genes that significantly change their expression, however, they indicate a connection between DNA methylation, transcript levels and the chilling accumulation process. Hence, patterns of DNA methylation and gene expression modulated by chilling accumulation could help to establish and/or maintain the endodormancy state in sweet cherry.

Overlapping Between DMRs and DEGs Reveal Conserved Genes Associated to Cold-Sensing and Signaling, Oxidation-Reduction Process, Flowering Regulation, Phenylpropanoid and Lipid Metabolism

From the overlapping between hyper/hypomethylated regions and down/upregulated genes, we searched for those with conserved patterns between 'Royal Dawn' and 'Kordia' (**Figure 4**). Most of these genes were associated to a cold response, metabolism of lipids, genes of the oxidative-reduction process, metabolism of phenylpropanoids and flowering regulation (**Figure 5**).

Plants cultivated in temperate regions, like sweet cherry, can go through a process of cold acclimation to increase their tolerance to non-lethal low temperatures, which involves a series of physiological, biochemical and molecular changes. One hypothesis associated to the sensing of cold, is the reduction in the fluidity of the cell membrane when exposed to low temperatures, being the first barrier for the environment (Ding et al., 2019). Changes in the fluidity of the membrane are directly correlated with the proportion of desaturated fatty acids, affecting the metabolism of lipids (Martiniere et al., 2011). Another level of cold-sensing is the influx of Ca^{2+}, an important messenger of environmental cues. An increase in the Ca^{2+} influx is usually observed seconds after cold treatment and is directly correlated with the upregulation of cold regulated genes, showing that ion channels and electrophysiological responses also mediate the cold sensing (Knight et al., 1996). As an early response to cold, the downregulation of Ca^{2+} dependent proteins in our work was associated to the dissipation of these transcripts with chilling accumulation from 348 CH in 'Royal Dawn' and 443–1,295 CH in 'Kordia'.

In addition, post-translational modifications carried out by kinases and phosphatases respond to Ca^{2+} influx and membrane fluidity in an early response to cold (Sangwan et al., 2001; Teige et al., 2004), explaining the presence of receptor kinases in our results, which also decrease their expression in later CH, associated to increased methylation levels from DMRs. Additional genes identified with similar patterns were *ERF13* and a *HEAT-SHOCK FACTOR*, both downregulated by cold.

The production of reactive oxygen species (ROS) during dormancy has been of increasing interest, since oxidative and respiratory stresses are associated with bud break, suggesting that ROS molecules like H_2O_2 may also act as signaling molecules for dormancy (Beauvieux et al., 2018). Additionally, chilling alters protein stabilization, reducing the activity of ROS scavenging enzymes and increasing the oxidative stress (Orvar et al., 2000). In agreement with this, an increased expression of the dehydrogenase *FOLD1* was related to a decrease in methylation, while the opposite pattern was observed in other dehydrogenases, which may be related to the oxidative stress during dormancy.

From the phenylpropanoid metabolism, two genes were identified: a putative *PHENOLIC GLUCOSIDE MALONYLTRANSFERASE 1-like* and *FLAVONOID 3-O-GLUCOSYLTRANSFERASE-like*. Flavonoids are a specific type of phenylpropanoid and are associated with the transport and biosynthesis of auxins, which is an important phytohormone for dormancy regulation. In this sense, it was proposed that the flux of phenylpropanoids in response to environmental cues may be important for growth cessation in winter and growth resumption in spring (Conrad et al., 2019).

Regarding flowering regulation, *SVP* is a repressor of flowering described in *Arabidopsis* and is negatively regulated by low temperatures to allow flowering in spring (Kurokura et al., 2013). In this work, it was observed that *SVP-like* decrease its expression with chilling accumulation, together with an increase in the methylation levels, indicating a similar regulation to *Arabidopsis*.

Correlation Between DNA Methylation and Transcript Levels of Genes Involved With Cellular Trafficking, Flavonoid Metabolism, and Protein Phosphorylation During Chilling Accumulation

DNA methylation and modifications in the chromatin are important epigenetic marks that help to regulate gene expression, transposon silencing, chromosome interactions and inheritance of traits (Zhang et al., 2018). From genes that showed a negative correlation between their methylation state and transcript levels, we obtained their GO categories and searched for DMRs of greater size (>50 bp) that could be further studied (**Figure 6**). The most represented GO term in both varieties was 'cellular process', from which we analyzed a putative *PavAMSH*-like. This gene was downregulated from early CH accumulation and presented an hypermethylation located in an intron, that could be regulating processes like splicing and polyadenylation (Zhang et al., 2018). AMSH is a major deubiquitinating enzyme that hydrolyzes K48- and K63-linked ubiquitin chains, and is essential for vacuole biogenesis, vacuolar trafficking from the Golgi and endocytosis (Isono et al., 2010). Therefore, when exposed to low temperatures, the cellular transport and metabolic activity are decreased, which is coincident with the downregulation of *PavAMSH* (Takahashi et al., 2013).

A second gene, from the GO term 'response to abiotic stimulus', named *PavFLAVONOL SYNTHASE-like* showed a genotype-specific regulation, being highly expressed in 'Royal Dawn' and downregulated in 'Kordia', possibly by an hypermethylation at the 3' end of the gene present only in 'Kordia', indicating that flavonoids may have an epigenetic regulation (**Figure 6C**).

From the 'protein modification process' category, *PavPHOSPHATASE 2A* had an hypermethylated region in the CHH context located upstream, correlating (r = −0,98) with a decrease in its expression level in 'Royal Dawn'(**Figure 7**). The increase in the methylation levels at the CHH context was related to the presence of a putative *cis* element that interacts with NRPE1, the largest subunit of the plant-specific RNA Polymerase V that participates in the RdDM pathway (Greenberg et al., 2010), suggesting that *PavPP2A* may be regulated by this pathway during dormancy in a variety dependent manner. As mentioned previously, in response to low temperatures, protein phosphorylation and the suppression of protein phosphatases, are associated with cold sensing and signaling. For example, in *Arabidopsis*, plants that overexpressed MKK2 (MAP kinase kinase2), presented the up-regulation of proteins from the cold-sensing pathway CBF/DREB1 (C-REPEAT BINDING FACTOR/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTOR 1), increasing cold tolerance (Teige et al., 2004). Another example is the phosphorylation of ICE1 (INDUCER OF CBF EXPRESSION 1), considered as the master regulator of CBF, activating their expression in response to low temperatures (Ding et al., 2020).

As conclusion, this study provides the first methylome information in sweet cherry during the endormancy process. Changes in the levels of DNA methylation, mostly represented as hypermethylations from early CH, suggested a role for epigenetic

modifications in response to low temperatures to increase cold tolerance during endodormancy. In particular, a higher number of DMRs in the CHH context could indicate the participation of RdDM during abiotic stress as a molecular integrator of the environmental cues. In response to low temperatures, the reprograming of the transcriptome was preceded by changes in methylation levels and the integrated data revealed an increase of DEGs that overlap with DMRs during CH accumulation in a temporality that was dependent on the variety. This data established a connection between DNA methylation, transcripts and chilling accumulation during endodormancy. An important role for cold sensing and signaling pathways, lipid and phenylpropanoid metabolism, and oxidative stress was suggested for endodormancy regulation in sweet cherry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. Raw data is available at NCBI sequence read archive PRJNA610988, PRJNA610989, PRJNA611731 and PRJNA611733 and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CM, AA, and KR designed the research. KR, ES, and VL-C conducted field work and sampling. JC-M contributed with bioinformatic analysis of MethylC-seq. KR and PS performed experiments and analyzed data of MethylC-seq and Sanger sequencing. LU, ES, PS, and KR conducted amplicon bisulfite sequencing. KR, ES, and LU contributed with RNA-seq experiments, while AR performed bioinformatic analysis of RNA-seq. LU, ES, and KR validated RNA-seq by qPCR. CM conducted statistical analysis. KR wrote the original manuscript. VL-C, CM, and AA edited and reviewed the original manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.01115/full#supplementary-material

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frontiers in Plant Science

ORIGINAL RESEARCH
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Greenhouse Spatial Effects Detected in the Barley (*Hordeum vulgare* L.) Epigenome Underlie Stochasticity of DNA Methylation

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Environmental cues are known to alter the methylation profile of genomic DNA, and thereby change the expression of some genes. A proportion of such modifications may become adaptive by adjusting expression of stress response genes but others have been shown to be highly stochastic, even under controlled conditions. The influence of environmental flux on plants adds an additional layer of complexity that has potential to confound attempts to interpret interactions between environment, methylome, and plant form. We therefore adopt a positional and longitudinal approach to study progressive changes to barley DNA methylation patterns in response to salt exposure during development under greenhouse conditions. Methylation-sensitive amplified polymorphism (MSAP) and phenotypic analyses of nine diverse barley varieties were grown in a randomized plot design, under two salt treatments (0 and 75 mM NaCl). Combining environmental, phenotypic and epigenetic data analyses, we show that at least part of the epigenetic variability, previously described as stochastic, is linked to environmental micro-variations during plant growth. Additionally, we show that differences in methylation increase with time of exposure to micro-variations in environment. We propose that subsequent epigenetic studies take into account microclimate-induced epigenetic variability.

Keywords: epigenetics, positional effect, phenotypic plasticity, genome by environment, salt stress, methylation-sensitive amplified polymorphism

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INTRODUCTION

Plant epigenetic mechanisms that can alter gene expression include the actions of short-interfering RNAs (siRNAs), chemical modification of histone tails and DNA methylation (Vanyushin, 2006; Sawan et al., 2008). These have been variously implicated in orchestrating developmental processes (Kohler and Makarevich, 2006; Ishida et al., 2008; Ay et al., 2014; Jung et al., 2015; Kooke et al., 2015), cell and organ differentiation (Joyce et al., 2003; Kohler and Makarevich, 2006; Kitimu et al., 2015; Kooke et al., 2015; Konate et al., 2020), reproduction (Yaish et al., 2011; Podio et al., 2014), parental imprinting (Gehring et al., 2006), acquired transgenerational trait inheritance (Tricker P. et al., 2013; Tricker P. J. et al., 2013), and adaptation to stress (Bird and Jaenisch, 2003; Boyko and Kovalchuk, 2008; Tricker et al., 2012).

DNA methylation has emerged as the prominent epigenetic signature for past or contemporary exposure of a plant to environmental insults (e.g. Xie et al. (2017) and has been implicated in the moderation of stress response (Bird and Jaenisch, 2003; Zilberman and Henikoff, 2007; Boyko and Kovalchuk, 2008). For instance, Tricker et al. (2012) reported that *Arabidopsis thaliana* responded to high relative humidity stress by suppressing the expression of two genes that control stomatal development through DNA methylation. DNA methylation has been similarly implicated in the response of various plant species to a range of stresses, including excess salt (Karan et al., 2012; Konate et al., 2018), temperature extremes (Steward et al., 2002; Bastow et al., 2004; Hashida et al., 2006; Pecinka et al., 2010; Song et al., 2012), herbivory (Herrera and Bazaga, 2011; Herrera and Bazaga, 2013), and heterogeneous environmental pressure (Wang et al., 2016). However, the relationship between DNA methylation and the stress effect is imprecise. Many of the methylation changes observed under stress fail to occur consistently across all genotypes or populations studied, and many others are not obviously associated with exonic regions. Fewer still can be directly tied to a particular stress response gene. Such observations have been described as stochastic (Karan et al., 2012; Tricker et al., 2012), spontaneous (Raj and Van Oudenaarden, 2008; Becker et al., 2011; Van Der Graaf et al., 2015), and without clear triggering factors (i.e. occurring randomly in the genome independently of stress). Many have considered the random and spontaneous alteration of DNA methylation is an adaptive biological process in its own right; one that drives diversity and evolution in a Lamarckian-like fashion (Feinberg and Irizarry, 2010; Meyer and Roeder, 2014; Soen et al., 2015; Van Der Graaf et al., 2015; Vogt, 2015) and with the clear potential to alter fitness (Consuegra and Rodríguez López, 2016). Additionally, Soen et al. (2015) proposed a conceptual framework of random variations in the genome, instigated in response to environmental cues. They hypothesized that imposition of diverse types of stress upon individual organisms during development gives rise to an adaptive improvisation which deploys random phenotypic variations that allows some individuals to cope with unstable ambient conditions. However, the authors did not suggest an epigenetic mechanism that might be involved in the regulation of such adaptive phenotypic variation.

In a pivotal piece, Vogt (2015) provided insight into the concept of random variability. The author linked "stochastic developmental variation" to stochastic occurrence of DNA methylation (Bird and Jaenisch, 2003; Field and Blackman, 2003). However, Vogt did not consider in depth the possible role that microclimatic variation may play in this apparent stochasticity. Herrera and Bazaga (2010) suspected a role for mesoclimate in driving the epigenetic variability of natural populations but did not anticipate marked environmental differences to occur under controlled experimental conditions (greenhouse, growth room).

Moreover, since genome-by-environment interactions have been shown to be at least partially regulated by DNA methylation (Verhoeven et al., 2010), even minor perturbations of growing conditions attributable to positional effects within a controlled growing environment has the potential to introduce confounding variation in methylation patterning. One way of dealing with spatial variation, if it cannot be prevented, is to deploy an appropriate experimental design in order to distinguish treatment from positional effects (Brien et al., 2013; Cabrera-Bosquet et al., 2016). Experimental design normally accounts for such variability by combining blocking and randomization, along with appropriate statistical analyses (Addelman, 1970; Ruxton and Colegrave, 2011). Despite the usefulness of this approach, experimental design cannot entirely remove environmental variability (microclimate). This presents a potential challenge when attempting to link changes in DNA methylation to environmental stimuli. It is difficult to discriminate between the so-called stochastic methylation and position-dependent methylation due to the capacity of plants to promptly sense and epigenetically respond to subtle variation in ambient conditions (Gutzat and Mittelsten Scheid, 2012; Meyer, 2015).

In the present study, we combine methylation-sensitive amplified polymorphism (MSAP) and phenotypic analyses to assess the effect of microclimate on DNA methylation of barley plants growing under greenhouse conditions. To provide an indication of scale, we sought to compare the glasshouse positional effects on MSAP profiles and those generated after imposing mild salt stress to a replicate sample of plants grown in the same conditions. For this, nine spring barley varieties were grown in a randomized plot design under mild soil salt stress or control conditions. Environmental, phenotypic, and DNA methylation data collected at two time points are used to explore whether stochastic epigenetic may be linked to trivial environmental fluctuations. We also explore how phenotypic variability observed in these experiments correlates with differences in DNA methylation patterns.

MATERIALS AND METHODS

Plant Material and Experimental Design

Experimental Strategy

The central aim of this study was to assess the impact of microclimate (caused by differing plant positions within a

glasshouse) on above ground biomass of barley plants, and of any associated change in global leaf-blade epigenome (as detected by MSAP). In this context, the MSAP profiles are being considered as a component of the DNA methylome (phenotype of the genome) and biomass is considered a component of their physical phenotype. We also sought to assess the scale of any changes to the epigenome through comparison with responses seen to mild salt stress among biological replicates in the same experiment. We sought to control possible sources of confounding variation (independent variables) by standardizing the source of material used for DNA extraction (tissue-to-tissue variation), use of a panel of varieties with similar growth rates (genetic variation), and collecting samples at two life stages (developmental variation).

Nine varieties of spring barley (**Table 1**) were grown in a controlled temperature greenhouse at the Plant Accelerator® (Australian Plant Phenomics Facility (APPF), Waite Campus, University of Adelaide, Australia) from June 26 to October 12, 2013. Varieties with similar flowering times (Menz, 2010) were selected to minimize discrepancies in sampling times between varieties. The experiment comprised eight randomized blocks with two plants of the same variety per plot (**Figure 1**). Three seeds were sown in white pots (20 cm height × 15 cm diameter, Berry Plastics Corporation, Evansville, USA) containing 2400 g potting mixture (composed of 50% UC (University of California, Davis) potting mix, 35% coco-peat and 15% clay/loam (v/v)). Seedlings were thinned to one seedling per pot 2 weeks after sowing. Two soil salt treatments (0 and 75 mM NaCl ("control" and "salt stress," respectively, hereafter) were applied to three-leaf stage seedlings (25 days after sowing (DAS)), using the protocol described by Berger et al. (2012). Pots were watered every 2 days for up to 60 days after sowing to 16.8% (g/g) gravimetric water content, corresponding to 0.8 × field capacity. From day 61 after sowing, plants were watered daily to 16.8% (g/g) until seed set. For all samples, (50–100 mg) of leaf material was taken from the mid-point of the selected leaf blades at two time points. These comprised the 4th leaf blade after full emergence (15 days after salt treatment and 40 DAS) and from the flag leaf blade from the primary tiller at anthesis (62 days after salt treatment and 87 DAS). Samples were taken from plants growing in blocks 1, 3, 4, 6, and 8 (**Figure 1**). This sampling strategy covered all varieties in all blocks. The nine barley varieties used exhibit very similar growth and development rates and so all reached both stages over the same time period. Thus, any epigenetic variation attributable to developmental or organ-to-organ variation was minimized. All

TABLE 1 | List and description of barley genotypes used in this study.

N°	Variety	Earliness	Year* of release	Pedigree*	
				Parent 1	Parent 2
1	Barque 73	6	1997	Triumph	Galleon
2	Buloke	5	2005	Franklin/VB9104	VB9104
3	Commander	5	2009	Keel/Sloop	Galaxy
4	Fathom	6	2011	NA	NA
5	Flagship	5	2006	Chieftan/Barque	Manley/VB9104
6	Hindmarsh	6	2007	Dash	VB9409
7	Maritime	6	2004	Dampier/A14//Krisna/3/Clipper	M11/4/DampierA14//Krisna/3/Dampier/A14//Union
8	Schooner	5	1983	Proctor/PrioA (WI2128)	Proctor/CI3578 (WI2099)
9	Yarra	5	2005	VB9018/Alexis/VB9104	NA

*Earliness to flowering score is based on a 0 to 9 scale, with 0 indicating very late varieties and 9 very early ones (www.grdc.com.au/SASowingGuide2015). *Year of release and pedigree after Menz (2010). NA, not available.*

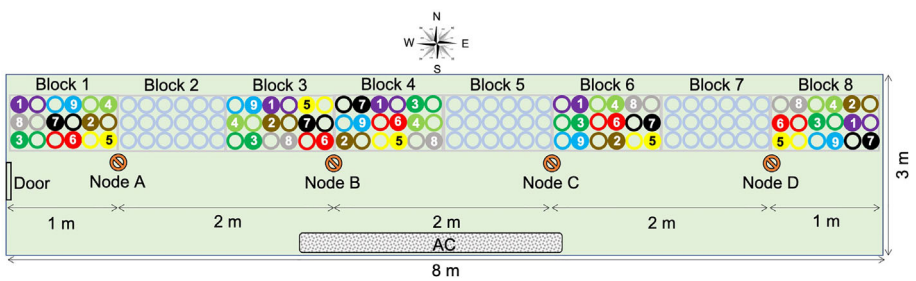

FIGURE 1 | Experimental layout and plan of the greenhouse (24 m^2). Blocks 1, 3, 4, 6, and 8 were used in this study and are, respectively, assigned to positions P1 to P5. Blocks 2, 5, and 7 contained empty pots. Four sensor nodes (nodes A, B, C, and D) were placed along benches, 2 m apart and 1 m from the east and west walls. Circles represent plant position in the block: hollow circles are control plants (0 mM NaCl) and full circles are treated plants (75 mM NaCl). Colors indicate barley varieties: 1, Barque73; 2, Buloke; 3, Commander; 4, Fathom; 5, Flagship; 6, Hindmarsh; 7, Maritime; 8, Schooner; 9, Yarra; sensor nodes. AC, air conditioning unit.

leaf samples were immediately snap frozen in liquid nitrogen and stored at −80°C until DNA extraction. Whole plants were harvested at maturity and above-ground biomass was dried and weighed.

Greenhouse Environmental Conditions

The experiment was conducted in a 24-m^2 greenhouse (~8 m × 3 m), with a gable roof 4.5 m above the floor at the lowest and 6 m at the highest point. The greenhouse (34°58′16 S, 138°38′23 E) was oriented West-East (**Figure 1**). To investigate the possible causes of position dependent variability of barley response across the greenhouse, environmental factors (temperature, relative humidity, and photosynthetic active rate) were recorded during the same period of the year (June 26, to October 12, 2015), using four sensor nodes located along the benches (**Figure 1**). Based on this period of the year, we deemed daytime to be between 7:00 AM and 6:00 PM.

The sensor nodes were positioned 2 m apart and 1 m from the east and west walls (**Figure 1**). Each node had a combination of sensors for photosynthetic active radiance (PAR) (model Quantum, LI-COR, Lincoln, Nebraska, USA) and for humidity/temperature (Probe HMP60, Vaisala INTERCAP®, Helsinki, Finland). Environmental data were recorded every minute for the duration of the experiment using wireless data loggers (National Instruments, Sydney, New South Wales, Australia). Before use for further analyses, recorded data were quality controlled to remove time slots when data were not present for all four nodes. To show the overall daily fluctuation of environmental factors between sensor nodes during the experiment, the average measure of each factor per hour was plotted for each node. Then, the vapor pressure deficit (VPD) for each time point was calculated according to Murray (1967):

$$VPD = \left(1 - \left(\frac{RH}{100}\right)\right) * \left(610.7 * 10^{\frac{7.5T}{(237.3+T)}}\right)$$

where RH = relative humidity, T = temperature, and the factor $610.7 \times 10^{7.5T/(237.3+T)}$ = saturated vapor pressure (SVP).

Pairwise comparisons of each environmental factor at sensor-node positions were performed using the Wilcoxon signed-rank test (Wilcoxon, 1945), on the R package "*ggpubr*" (Kassambara, 2019). These comparisons were performed independently for day and night periods.

DNA Extraction

Frozen plant material was homogenized in a bead beater (2010-Geno/Grinder, SPEX SamplePrep®, USA) prior to DNA extraction using a Qiagen DNeasy kit according to the manufacturer's instructions. DNA samples were then quantified in a NanoDrop® 1000 Spectrophotometer (V 3.8.1, ThermoFisher Scientific Inc., Australia) and concentrations were standardized to 10 ng/µl for subsequent MSAP analyses.

MSAP

DNA Restriction and Adapter Ligation

MSAP was used for the DNA methylation profiling of barley plants according to the method of Rodríguez López et al. (2012). To ensure marker reproducibility, DNA samples were analyzed in two technical replicates. Thus, samples were digested using a methylation insensitive restriction enzyme EcoRI in combination with either *Hpa*II or *Msp*I (isoschizomers), which show differential sensitivity to cytosine methylation at CCGG positions. Digested DNA fragments were ligated to adapters (**Table 1**) with one end cohesive with restriction products generated by *Eco*RI or *Hpa*II/*Msp*I. Digestion and ligation reactions were performed in a single solution of 11 µl comprising: 1.1 µl T4 ligase buffer; 0.1 µl *Hpa*II; 0.05 µl *Msp*I; 0.25 µl *Eco*RI; 0.05 µl T4 ligase; 0.55 µl BSA ; 1.1 µl NaCl ; 1 µl Adapter *Eco*RI; 1 µl Adapter *Hpa*II/*Msp*I; 5.5 µl DNA sample and 0.3 µl pure water. Enzymes and buffer were acquired from New England Biolabs, Australia (NEB) and oligos were produced at Sigma-Aldrich, Australia. The solution was incubated for 2 h at 37°C, then enzymes were inactivated at 65°C for 10 min.

PCR

Two PCR amplifications were performed using products of the restriction/ligation reaction. First, a pre-amplification PCR was performed, in which primers complementary to adaptors but with 3' overhangs for a unique nucleotide (*Hpa*II/*Msp*I primer +C and *Eco*RI primer +A, **Table 2**) were used in a pre-optimized PCR master mix (BioMix™, Bioline, Meridian Bioscience; Australia) following the manufacturer's instructions. DNA digestion/ligation product (0.5 µl) was used for PCR amplification, with the following profile as per Rois et al. (2013): 72°C for 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 56°C and 2 min extension at 72°C, ending with 10 min at 72°C to ensure completion of the extension.

Pre-amplification products were quality assessed by 1% w/v agarose electrophoresis (80 V for 2 h), before performing the selective amplification using two selective primer combinations, *Eco*RI_AAG vs. *Hpa*II/*Msp*I_CCA and *Eco*RI-ATG vs. *Hpa*II/*Msp*I_CAA. Amplified fragment detection through capillary electrophoresis was facilitated by labeling *Hpa*II/*Msp*I selective primers with the 6-FAM reporter molecule (6-carboxyfluorescein). Just 0.3 µl of pre-amplification product was used in the pre-optimized PCR master mix and the PCR was performed as follows (Rois et al., 2013); 94°C for 2 min, 12 cycles of 94°C for 30 s, 65°C (and decreasing by 0.7°C each cycle) for 30 s, and 72°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, ending with 72°C for 10 min.

TABLE 2 | Adapter and primer sequences used for the MSAP (Rodríguez López et al., 2012).

Oligo name	Function	Sequence
HpaII/MspI adaptor Reverse	Adapter	CGCTCAGGACTCAT
HpaII/MspI adaptor Forward	Adapter	GACGATGAGTCCTGAG
EcoRI adaptor Reverse	Adapter	AATTGGTACGCAGTCTAC
EcoRI adaptor Forward	Adapter	CTCGTAGACTGCGTACC
Pre-EcoRI	Preselective primer	GACTGCGTACCAATTCA
Pre-HpaII/MspI	Preselective primer	GATGAGTCCTGAGCGGC
EcoRI-ATG	Selective primer	GACTGCGTACCAATTCATG
EcoRI_AAG	Selective primer	GACTGCGTACCAATTCAAG
HpaII/MspI_CCA	Selective primer	GATGAGTCCTGAGCGGCCA
HpaII/MspI_CAA	Selective primer	GATGAGTCCTGAGCGGCAA

Capillary Electrophoresis

The products of the selective PCR were fractionated by capillary electrophoresis on an ABI PRISM 3730 (Applied Biosystems, Foster City, California, USA) at the Australian Genome Research Facility Ltd (Adelaide, Australia). For this, 2 µl of selective PCR products were first combined with 15 µl of HiDi formamide (Applied Biosystems) and 0.5 µl of GeneScan™ 500 ROX™ Size Standard (Applied Biosystems). The mixture was then denatured at 95°C for 5 min and snap-cooled on ice for 5 min before sample fractionation at 15 kV for 6 s and at 15 kV for 33 min at 66°C.

MSAP Data Analysis

MSAP profiles obtained using *Hpa*II and *Msp*I were used to generate; 1) a qualitative binary matrix of allelic presence/absence scores, and 2) a quantitative matrix of allelic peak height using GeneMapper Software v4 (Applied Biosystems). Qualitative epigenetic changes associated with greenhouse positional effect were analyzed using fragment sizes between 100 and 550 base pairs, which were selected to estimate epigenetic distance between individual plants (EpiGD) and subpopulations of plants (PhiPT) and perform Principal Coordinate Analyses (PCoA), using GenAlex 6.501 (Peakall and Smouse, 2012).

Quantitative analysis of peak height was used to examine the effect of position on the methylation status of individual loci. We searched for MSAP markers that were differentially methylated between experimental blocks by comparing the fragment peak heights to survey for position effects on the plant methylation profile (Rodríguez López et al., 2012). Before differential methylation analysis, model-based normalization factors were calculated for the peak height libraries using the weighted trimmed mean method of Robinson and Oshlack (2010). For each variety and sampling method, peak heights were extracted and analyzed individually using the modeling approach of Mccarthy et al. (2012). To ensure the peak heights could be compared between positions, the individual models contained a term to account for variation between blocks as well as a term to capture the differences between the control and salt stress treatments. A likelihood ratio test was then performed to determine whether estimated coefficients for the positions were equal (Mccarthy et al., 2012). The p-values from these tests were then adjusted for multiple comparisons using the false discovery rate method of Benjamini and Hochberg (1995). Analyses were conducted using the R package *edgeR* (Robinson et al., 2010), in the R statistical computing environment (R Core Team, 2019).

The extent of epigenetic divergence between salt treatments at the two developmental stages (4th leaf and anthesis) was assessed, first by performing a multiple correspondence analysis (MCA) on MSAP marker data. A linear discriminant analysis (LDA) was then performed on the MCA results. These analyses, referred to as MC-LDA thereafter, were done using the R packages FactoMineR and MASS (Lê et al., 2008; R Core Team, 2019). To visualize the results of comparisons involving more than two groups, the first two linear discriminant factors (LD1 and LD2) were plotted. Otherwise, a density plot of LD1 was performed.

Assessment of Correlations Between Epigenetic Profiles and Plant Phenotype

Epigenetic and phenotypic variability were estimated using averaged data per position for all nine barley varieties (Bishop et al., 2015). The software GraphPad Prism 6 v008 (Graph-Pad Software, San Diego, California, USA) was used to perform statistical analyses. Values of above-ground plant biomass were normalized by computing the ratio of plant biomass over the mean biomass for each individual experiencing the same treatment across all positions. The same formula was applied to grain yield. This normalization was intended to address quantitative variability between treatments and among barley genotypes. Then, biomass and yield distance matrices were generated using the difference between normalized values of any two individual plants.

We performed a Mantel Test (Mantel, 1967) to estimate the significance of the correlations between epigenetic distance and plant biomass, and position in the greenhouse. For this, we used matrices generated from epigenetic distance, physical distance and phenotypic (biomass or yield) differences estimated as described above. In all cases, the level of significance of the observed correlations was tested using 9,999 random permutations. Since both enzymes (*Hpa*II, *Msp*I) are methylation sensitive (Walder et al., 1983; Reyna-López et al., 1997), these enzymes can independently show epigenetic marks across the genome. Therefore, our inferences about plant epigenetic profile thereafter relate to results obtained using either enzyme or a combination of both.

RESULTS

Microclimatic Variability in the Greenhouse

Data quality control of climatic data provided 47,144 and 54,983 time-points of data recording for the periods of day and night, respectively. These correspond to time-points when recording was obtained simultaneously in all sensor nodes. There was clear evidence of both spatial and temporal variation for temperature, photosynthetically active radiation (PAR) and relative humidity (RH) within the experimental area (**Figures 2** and **3**).

The average dynamics of climatic data in the greenhouse showed a higher PAR between 8 AM and 10 AM at the East side than the rest of the greenhouse (node D, **Figure 1**). The PAR was also variable during the day between node positions, with sensor node B (Centre-West, **Figure 1**) recording the lowest PAR values around 12 PM (**Figure 2A**). The average temperatures evolved broadly in the same way at all node positions, with only around 1.5°C difference between the most divergent nodes at the warmest time of day (**Figure 2B**). The RH was the highest at node A (West side of the greenhouse, **Figure 1**) during both day and night, and was significantly different from the rest of the positions during the day (**Figures 2C** and **3**). The node D (East end of the greenhouse) presented the lowest RH during the day; it was not significantly different from nodes B and C (**Figure 3A**).

Although there was no clear evidence of gradient between sensor nodes for any of the climatic factors (i.e. RH, temperature, VPD and PAR, the pairwise comparison of data from sensor nodes using Wilcoxon paired signed-rank test showed significant

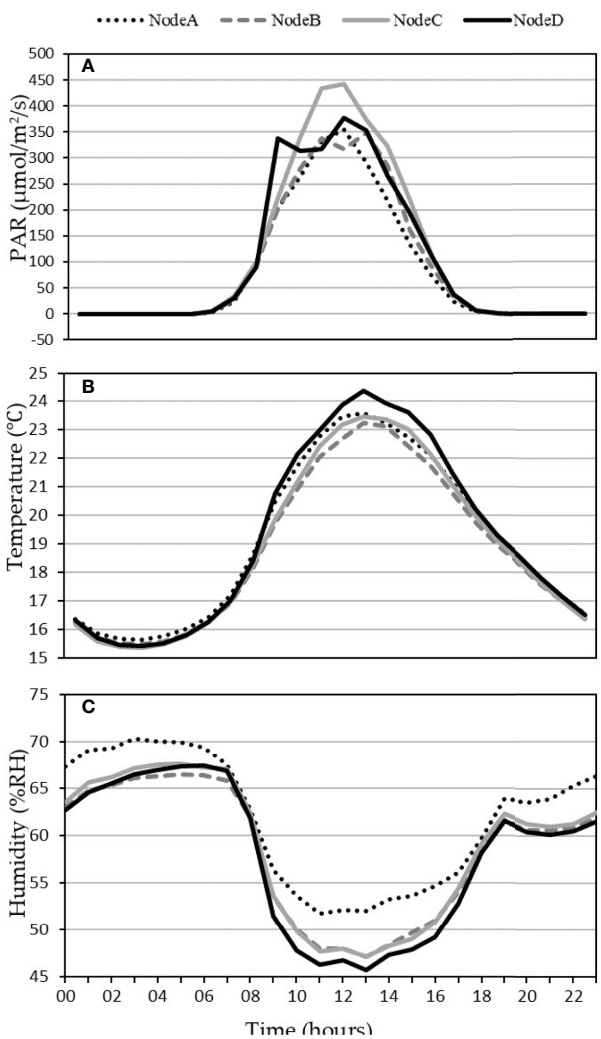

FIGURE 2 | Average daily fluctuations of climatic conditions in the greenhouse. **(A)** light, **(B)** temperature, and **(C)** relative humidity were recorded over the period from June 26 to October 12, 2015, at four positions (nodes A–D from West to East) in the greenhouse.

differences between positions for each variable (**Figures 3A–G**). Such differences were present during both day and night periods in the greenhouse. The RH appeared particularly variable at night between all positions of sensor nodes (**Figure 3B**).

Correlation Between DNA Methylation Profile and Plant Position in the Greenhouse

As expected, the variation between MSAP profiles of the nine diverse varieties used in this study led to significant confounding clustering according to genotype. In subsequent analyses, we therefore elected either to consider perturbations to MSAP across all varieties collectively or else make comparisons on a variety by variety basis. The former included all confounding variation associated with genotype but sought to provide an indication of conserved effects across the panel. The latter analyses were intended to reveal the extent to which variability in the epigenetic response is influenced by genotype.

Plant DNA methylation profiles derived from MSAP data generated 269 alleles with sizes between 100 and 550 base pairs across samples from all nine barley varieties. PCoA of MSAP profiles for barley variety at anthesis showed grouping of samples more by plant position than salt treatment, regardless of the enzyme combination used (**Figures 4A, B**). The first coordinate Eigen space matched with the position of the plants in the greenhouse in the West-East direction (**Figure 4**). The Mantel test using all treatment samples together showed weak correlations between plant epigenetic profiles and plant positions in the greenhouse at 4th leaf stage, and more significant corrections at anthesis (**Table 3**). For instance, for the variety Schooner, the Mantel test between pairwise epigenetic distance and plant position at the 4th leaf stage of barley development resulted in weak correlations for both *Hpa*II (R^2 = 0.11, P-value = 0.025, **Figure 5A**) and *Msp*I (R^2 = 0.12, P-value < 0.022, **Figure 5C**). Apart from two varieties (Buloke and Schooner), none of the remaining varieties showed a significant correlation between position and epigenetic profile at the 4th leaf stage (**Table 3**, **Figures S1**). Conversely, these correlations were stronger at anthesis for the same variety Schooner (R^2 = 0.48 and R^2 = 0.45, for *Hpa*II and *Msp*I, respectively, **Figures 5B, D**), with greater significance of the P-values (0.001). Additionally, all the remaining varieties showed significant correlation (P-value at least < 0.05) between DNA methylation profile at anthesis and the plant position in the greenhouse (**Table 3**; **Figure S1**). The correlations at anthesis were high (R^2 > 0.3) for all varieties, except Buloke and Maritime (**Table 3**).

The comparison of peak heights of MSAP markers generated from plants growing in different positions revealed significant differences between positions for some alleles (**Figure 6**). In general, significant differences in peak height were observed between plants in position P1 and the other positions (**Figure 6**). Overall, peak heights showed logarithmic trends (both positive and negative), significantly associated with the West-East distribution of the samples. A few markers were significantly different in peak heights over all positions (**Table 4**).

However, positional effect did not thwart the ability to differentiate between salt-stressed and control plants. The MC-LDA on MSAP marker data was able to separate salt stressed plants from those given control conditions (**Figures 7A, B**). Furthermore, epigenetic divergence between treatment groups increased with time, with control and stress plants consistently more similar at the 4th leaf stage than at anthesis across all varieties (**Figures 7A, B** and **S2**). MC-LDA of salt treatments could nevertheless discriminate treatments at both stages even though epigenetic divergence was strongly influenced by developmental stage (**Figures 7C** and **S2**).

Correlations Between Barley Phenotype, Epigenome, and Position

There was a clear trend in the final biomass of all nine barley varieties according to position, with a progressive increase from position P1 (west side of the greenhouse) to position P5 (East side) (**Figure 8A**). This relationship was a logarithmic trend,

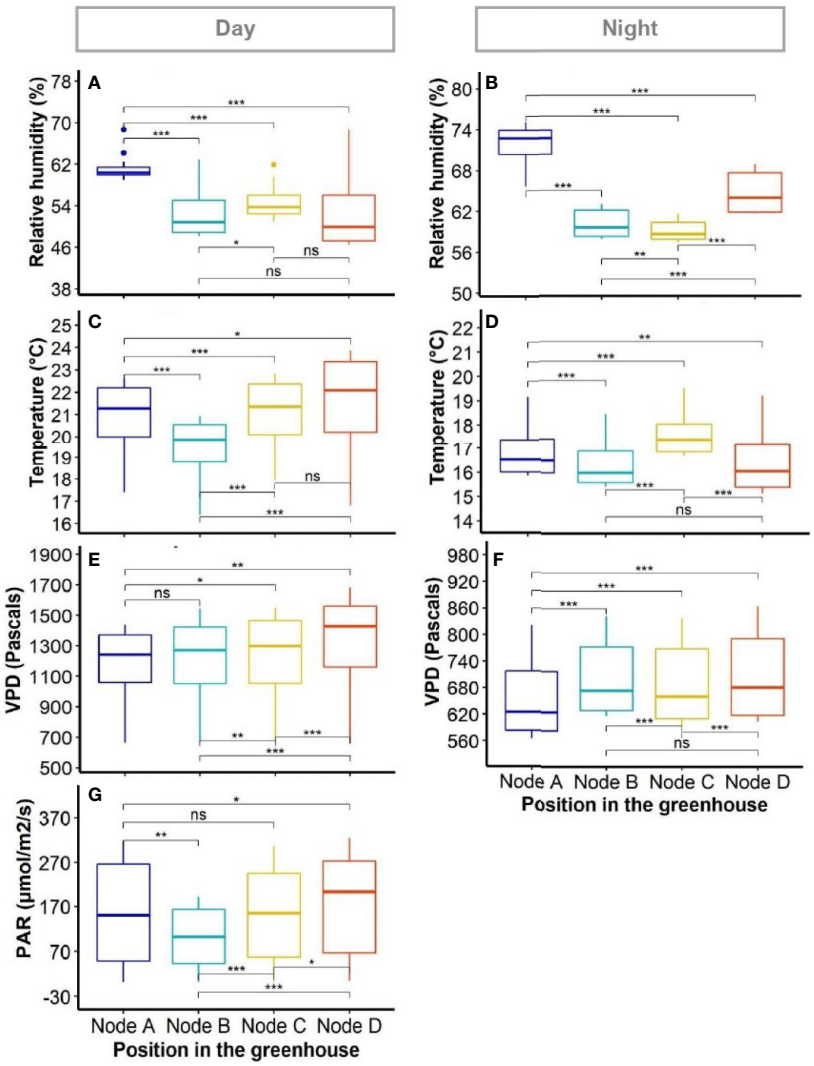

FIGURE 3 | Variability of environmental factors in the greenhouse. The boxplots show variation within positions as collected by individual sensors. Panels show diurnal **(A, C, E, G)** and nocturnal **(B, D, F)** average measurements for: relative humidity **(A, B)**; temperature (C and D); vapour pressure deficit (VPD) **(E, F)**; and photosynthetic active radiation (PAR) (G, PAR was deemed as null at night). Differences between positions were compared using Wilcoxon signed-rank test. *, ** and *** indicate the significance of the measured differences between positions for P value < 0.05, 0.01, and 0.001, respectively; ns, difference not significant.

both in the control and stressed plants. The average grain yield of the barley varieties showed the same west-east trend as the biomass (**Figure 8B**). However, when varieties were examined separately, both logarithmic and polynomial trends were observed (**Figure S3**).

Assessment of the relationship between pairwise differences in epigenetic distance and in grain yield showed significant correlations (P-values < 0.05) in control plants of six of nine varieties (Buloke, Commander, Fathom, Maritime, Schooner, Yarra), with R^2 varying between 0.247 and 0.907 (**Table 5**; **Figure S4**). Likewise, stress plants showed significant correlations (P-values at least < 0.05) between grain yield and methylation profile in six varieties (Barque 73, Buloke, Commander, Flagship, Maritime, Schooner), with R^2 between 0.164 and 0.921 (**Table 5**; **Figure S4**). An example of significant correlations between grain yield and epigenetic distance is presented in **Figures 9A–D**, for the variety Schooner.

DISCUSSION

Stochastic DNA Methylation Is Explained by Microclimatic Differences

The randomized block design aims to minimize unexplained variation between treatments, and has emerged as a preferred method in plant field trials and in controlled environment experiments (Edmondson, 1989; Guertal and Elkins, 1996; Brien et al., 2013). However, while block homogeneity is difficult to achieve, variability between blocks in the same experimental setting is often either ignored, attributed to randomness (Raj and Van

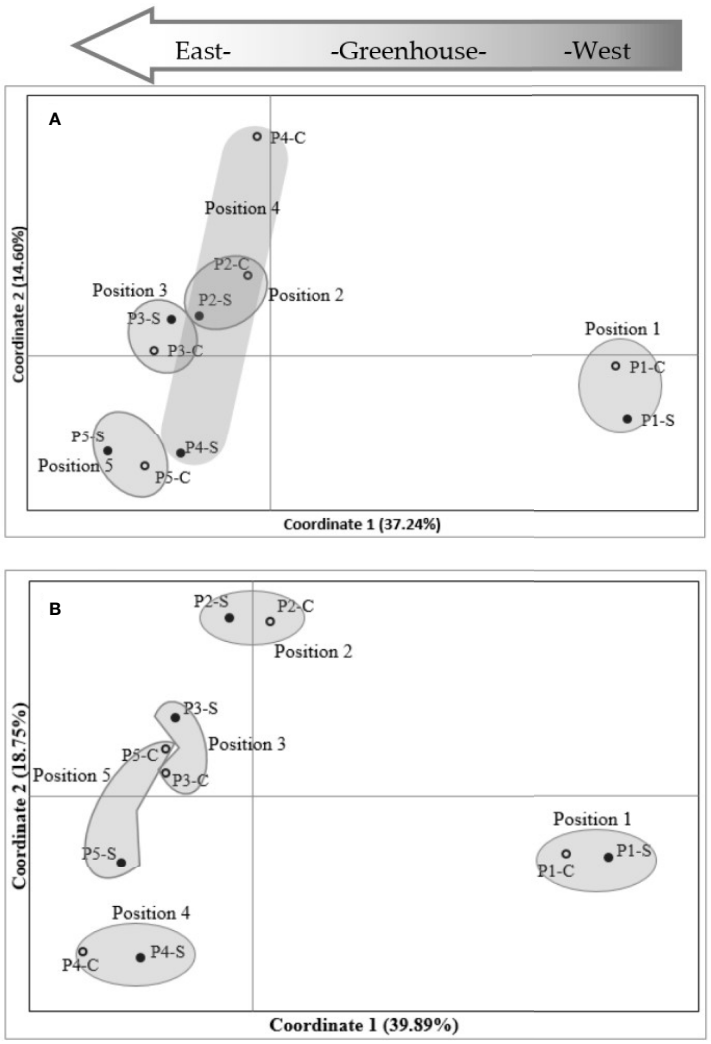

FIGURE 4 | Principal coordinates analysis (PCoA) of MSAP (methylation-sensitive amplified polymorphism) markers in barley variety Commander. MSAP markers were generated using five replicates of control (0 mM NaCl) and stress (75 mM NaCl) plant samples, for *Hpa*II **(A)** and *Msp*I **(B)**. Positions 1 to 5 indicate experimental block numbers; symbols filled in black and hollow symbols represent salt stress (–S) and control (–C) samples, respectively. The PCoAs show sample distribution in the first two principal coordinates. Numbers in brackets represent the proportion of variation explained by the coordinate.

Oudenaarden, 2008; Karan et al., 2012; Tricker et al., 2012) or in the context of epigenetic research, explained by spontaneous occurrence of the methylation (Becker et al., 2011; Baulcombe and Dean, 2014; Van Der Graaf et al., 2015).

In this study, we took care to control potentially confounding sources of variation between MSAP profiles by the selection of genetically diverse varieties with similar rates of growth and development, sourcing DNA from the same section of the same leaf from of all plants, and at two very distinct developmental stages (Konate et al., 2020). We nevertheless found evidence suggesting that microclimatic variation within a greenhouse was sufficient to trigger variability in the plant DNA methylation profile in a manner that was both independent of the experimental salt stress treatment and greater in magnitude. The clarity of the climatic variables measured across the experimental blocks, and the associated cline in methylation patterning is suggestive that each plant experienced a unique combination of climatic factors during the experimental period, and that this induces, at least partly, changes in methylation patterning. Similar observations were also reported for other greenhouse studies (Brien et al., 2013; Both et al., 2015; Cabrera-Bosquet et al., 2016). This finding is inconsistent with spontaneous DNA methylation being entirely responsible for the plant-plant variability in such experiments (Becker et al., 2011; Van Der Graaf et al., 2015), and throws into question how best to discriminate epigenetic responses to micro-environment fluctuations from those attributable to stochastic noise. Moreover, the effect of position can easily be overlooked in snap-shot exposure experiments, since the timeframe from stress exposure to induction of position-dependent methylation

TABLE 3 | Correlation between pairwise epigenetic distance and physical distance.

Varieties	Coefficient of determination (R^2)			
	*Hpa*II		*Msp*I	
	4th leaf	Anthesis	4th leaf	Anthesis
Barque73	0.003	0.320**	0.010	0.315
Buloke	0.103*	0.001	0.059	0.220*
Commander	0.052	0.332**	0.050	0.332**
Fathom	0.038	0.425****	0.079*	0.527****
Flagship	0.038	0.451***	0.001	0.214*
Hindmarsh	0.008	0.305**	0.004	0.233*
Maritime	0.014	0.130*	0.071*	0.144*
Schooner	0.112*	0.476***	0.120*	0.447***
Yarra	0.002	0.147*	0.027	0.385*
Average	0.041	0.287	0.047	0.313

Nine barley varieties were used, comprising ten individuals per variety, five replicates for control and stress plants. Samples were collected from the 4th leaf (at 4th leaf stage) and flag leaf (at anthesis). Epigenetic distances correspond to the Phi statistics of the MSAP markers between plant individuals. The coefficient of determination (R^2) was calculated according to Mantel (1967) using GenAlex 6.5. Asterisks (*), (**), (***) and (****) indicate significant correlation between treatments for P-value < 0.05, 0.01 and 0.001, respectively, estimated based on 9,999 permutations.

markers is critical but also likely to vary between loci. Support for this reasoning can be taken from our findings that it was possible to separate salt and control samples by discriminate analysis at the 4th leaf stage and at anthesis but with higher divergence at the later stage. At the same time, correlation between epigenetic differences and physical distance among plants at anthesis (87 DAS) was stronger than at the 4th leaf stage (40 DAS), indicating that exposure to the stressor and positional microclimates both have a cumulative effect on the plant epigenome. These observations are congruent with the concept that plant adaptive improvisation, through DNA methylation, is proportional to the severity and duration of the environmental cue to which the plant was exposed (Soen et al., 2015). In this sense, the scale of the effect induced by intervention stress (salt) needs to be weighed against those imposed by coincidental stresses (microenvironment effects) but also by those associated with development or ageing, as was reported in humans (Gentilini et al., 2015). Any truly stochastic DNA methylation would represent residual variation. Previous studies have observed the influence of mesoclimatic conditions (Herrera and Bazaga, 2010) and factors such as temperature (Hashida et al., 2006), humidity (Tricker et al., 2012) or light (Barneche et al., 2014; Meyer, 2015) on methylome variability. However, the current study suggests, for the first time, that even slight variations in climatic factors (temperature, humidity or light) are sufficient to induce modifications in the plant DNA methylation profile, and that this can be sufficient to mask effects of mild stresses,

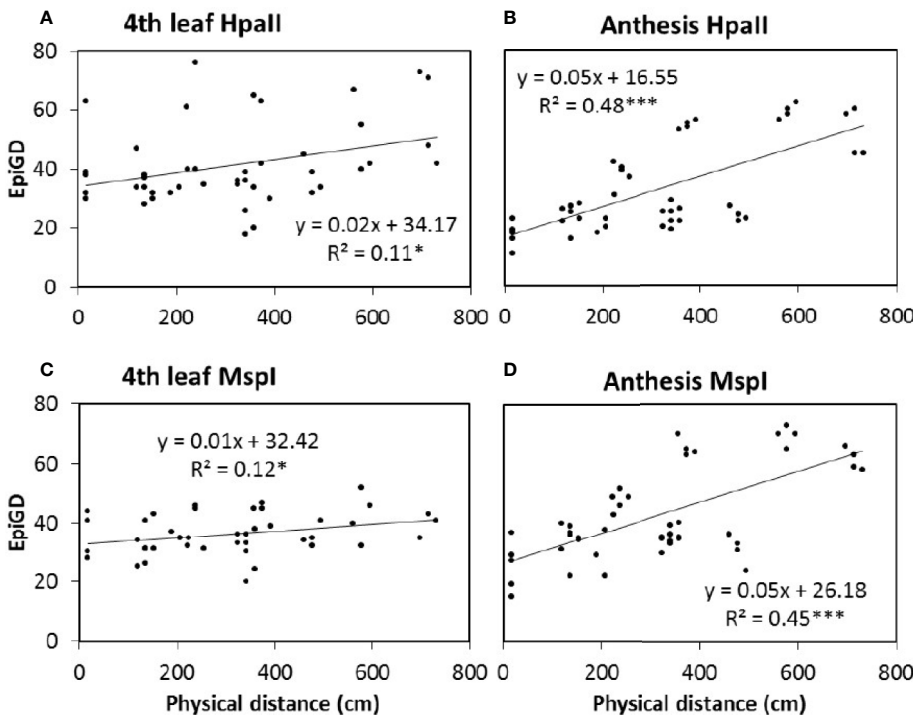

FIGURE 5 | Correlation between pairwise epigenetic distance (Epi GD) and plant position in the greenhouse. The epigenetic distance was estimated at 4th leaf stage (**A**, **C**; 40 days after sowing) and anthesis (**C**, **D**; 87 days after sowing) of barley variety Schooner, using *Hpa*II **(A, B)** and *Msp*I **(C, D)** for the MSAP (methylation sensitive amplified polymorphism) analysis. Five replicates of control (0 mM NaCl) and stress (75 mM NaCl) were analyzed together and dots represent pairwise comparisons between individual plants. Equations represent the formula of the regression line, R^2 represents the coefficient of determination, calculated according to Mantel, 1967 using GenAlex 6.5. Asterisks (*) and (***) indicate significant correlation between treatments for *P* value < 0.05 and 0.001, respectively, estimated based on 9,999 permutations.

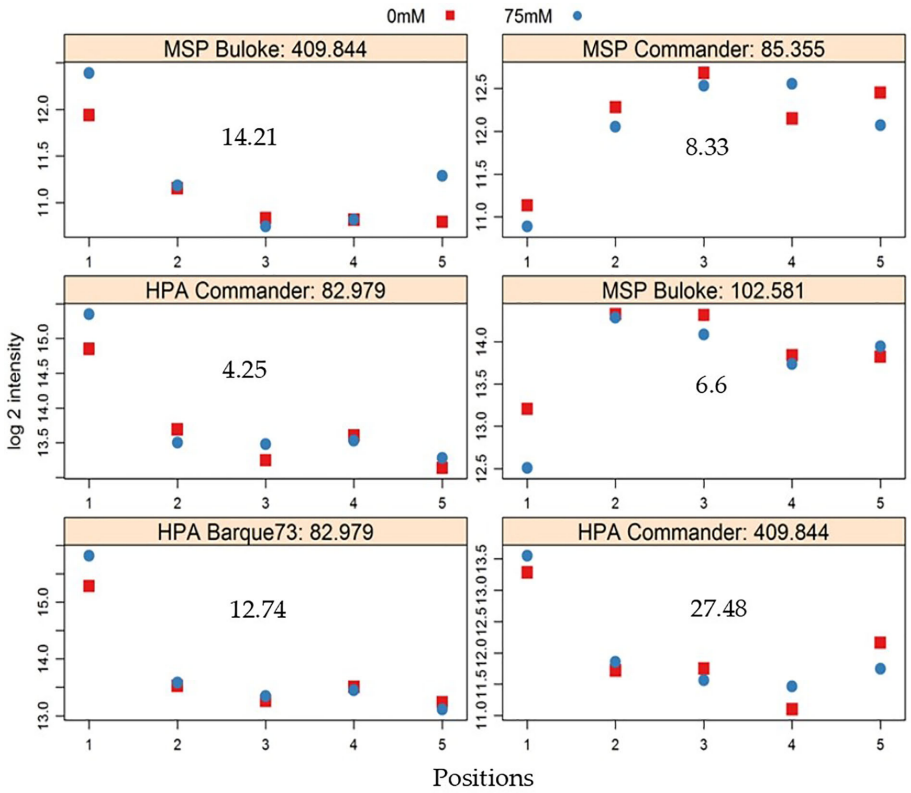

FIGURE 6 | Exemplars of MSAP (methylation sensitive amplified polymorphism) alleles that show significant differences in peak height between positions in the greenhouse. Markers were detected in control (0 mM NaCl, red symbols) and stress (75 mM NaCl, blue symbols) plants; Vertical axis shows logarithm 2 (log 2) of peak height intensity and the horizontal axis represents positions in the greenhouse, in the west to east direction. The gray number in each plot represents –log10 of *P* values. The title of each plot shows the enzyme used (either *Hpa*II (HPA) or *Msp*I (MSP), the variety, and the allele identity number.

TABLE 4 | List of salt-induced methylation marker alleles showing significant peak height differences between the five experimental blocks.

Variety	Sample tissue	Enzyme/Primer	allele	logFC	logCPM	LR	P Value	FDR
Barque73	Flag leaf	*Hpa*II/ATG-CAA	403.76	0.884	12.895	12.082	0.001	0.019
Barque73	Flag leaf	*Hpa*II/ATG-CAA	221.61	–1.749	14.043	9.817	0.002	0.032
Flagship	4th leaf	*Hpa*II/ATG-CAA	221.61	–1.202	13.901	10.507	0.001	0.036
Yarra	Leaf before flag	*Hpa*II/ATG-CAA	361.55	–0.653	12.238	10.505	0.001	0.036
Yarra	Leaf before flag	*Hpa*II/ATG-CAA	167.6	–0.796	12.866	8.726	0.003	0.040
Yarra	Leaf before flag	*Hpa*II/ATG-CAA	543.70	0.816	12.508	8.286	0.004	0.040

logFC, log fold change; logCPM, log counts per million; LR, likelihood ratio statistics; FDR, false discovery rate.

as was observed here for salt stress. We certainly do not contend that all nascent methylation arises in response to environmental or biotic effectors but we do argue that far more care is needed before discounting unaccounted epigenetic variation as stochastic noise.

Positional Effect Affects Salt Stress-Induced DNA Methylation Changes in Barley

Positional effects in greenhouse experiments are well established and if not properly accounted for can generate uncharacterized background noise that can mask the effect of the experimental treatment (Edmondson, 1989; Guertal and Elkins, 1996; Brien et al., 2013). Spatial variability in coincident environmental factors has potential to introduce variability between replicate plants' development and response to experimental treatments (Edmondson, 1989; Guertal and Elkins, 1996). Such spatial variability is liable to introduce flaws in measurements and observations between replicates that, in fact, were not experiencing exactly the same constraints (Addelman, 1970). This can compromise the search for relationships between experimentally controlled stressors (in our study, soil salt stress) and perturbations in epigenetic profiles. Indeed, in the present work the observed

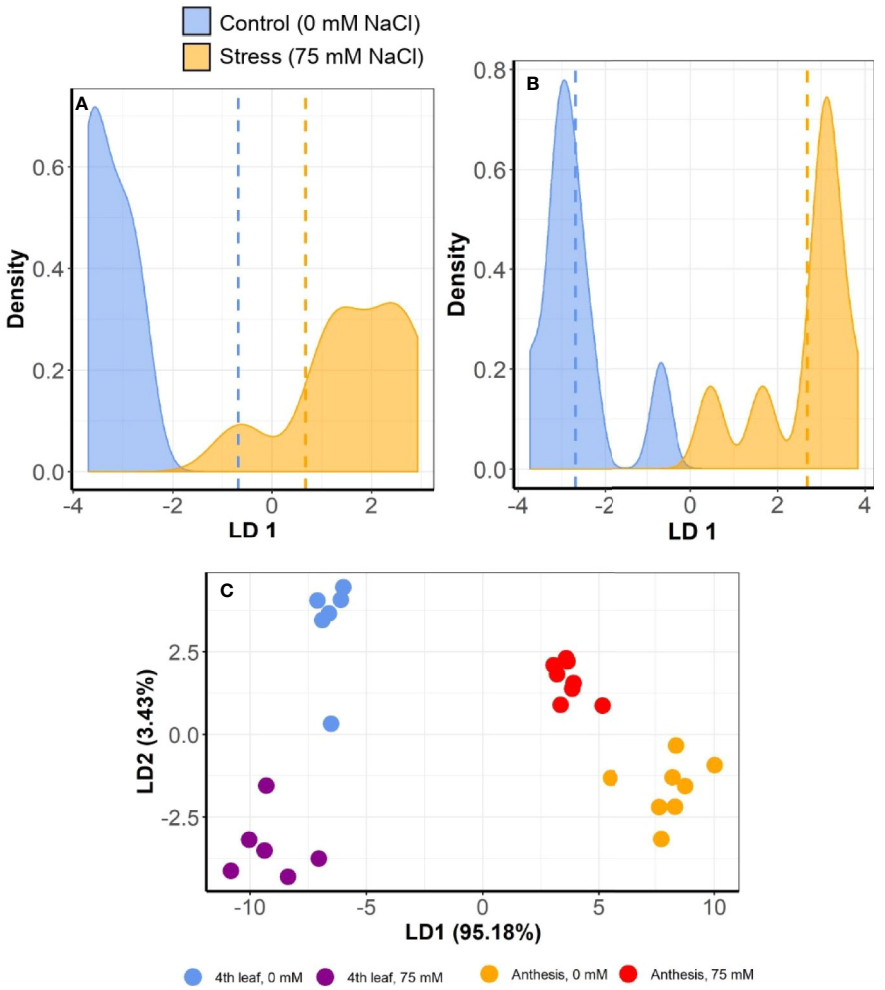

FIGURE 7 | Multiple correspondence and linear discriminant analyses (MC-LDA) of MSAP markers in barley variety Commander under salt stress (75 mM) and control (0 mM) conditions. The panel shows density plots of LD function between stress and control plants, at 4th leaf stage **(A)** and at anthesis **(B)**. Dashed vertical lines represent the mean LD1 in 2 groups' comparisons. The graph **(C)** shows MC-LDA plots comparing the salt treatment groups at both 4th leaf and anthesis stages. Similar plots for the other varieties are presented in **Supplementary Figure S2**.

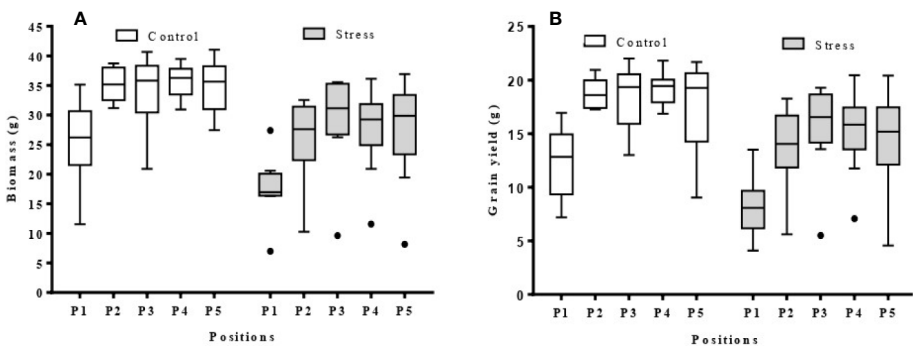

FIGURE 8 | Box plots showing biomass and grain yield range per position (P1–5) in the greenhouse (n = 9). **(A)** biomass per position for control and stress plants; **(B)** grain yield per position for control and stress plants; The average data was obtained from nine barley varieties (Barque 73, Buloke, Commander, Fathom, Flagship, Hindmarsh, Maritime, Schooner, and Yarra).

TABLE 5 | Correlation between epigenetic distance and grain yield of nine barley varieties.

Varieties	Coefficient of determination (R^2)			
	Control (0 mM NaCl)		Stress (75 mM NaCl)	
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I
Barque73	0.843	0.483	0.525	0.921*
Buloke	0.405*	0.445*	0.269*	0.164*
Commander	0.447	0.663*	0.911	0.897*
Fathom	0.030	0.247*	0.004	0.039
Flagship	0.394	0.393	0.815*	0.886
Hindmarsh	0.310	0.003	0.468	0.503
Maritime	0.271	0.902*	0.590*	0.855*
Schooner	0.907*	0.828*	0.841**	0.807*
Yarra	0.778	0.834*	0.000	0.060
Average	0.487	0.533	0.492	0.570

Epigenetic distance between plants was calculated based on MSAP data generated using HpaII and MspI. Coefficients of determination (R^2) were computed according to Mantel (1967) using five replicates for each treatment per variety. Asterisks (*) and (**) indicate significant correlation between treatments for P value < 0.05, and 0.01, respectively, estimated based on 9,999 permutations.

negative correlation between RH and differences in epigenetic differentiation between control and salt stressed pairs of plants growing in the different positions suggests that variations in environmental factors has interfered with reaction of the plant to mild salt stress. One possible mechanistic explanation is that the observed West to East decrease in RH changed the plant's requirement for water (Barnabás et al., 2008; Verslues and Juenger, 2011), and this in turn may have affected the level of salt stress experienced by each plant. In this way, plants were grown under the same salt treatment but because they experienced different RH, are likely to exhibit different responses to the salt stress; hence the inconsistent salt-induced DNA methylation profiles.

Phenotypic Differences Associated to Greenhouse Microclimates Correlate With Epigenetic Differences

Plants have been long known to exhibit phenotypic symptoms of stress in organs that are not directly exposed to the stressor (Riley et al., 2002). Indeed, it is well-established that deficiency or toxicity of plant nutrients in the soil often becomes manifest as physical symptoms in the leaves in a wide range of plants, including barley (Uchida, 2000). Similar responses have been reported in the methylation profiles of DNA extracted from organs that are equally unconnected with the source of stress. For example, Konate et al. (2018) reported that exposure of the roots to mild soil salt stress impacts on the methylation profile of barley leaves. However, it is open to question is whether phenotypic symptoms of stress co-correlate with the

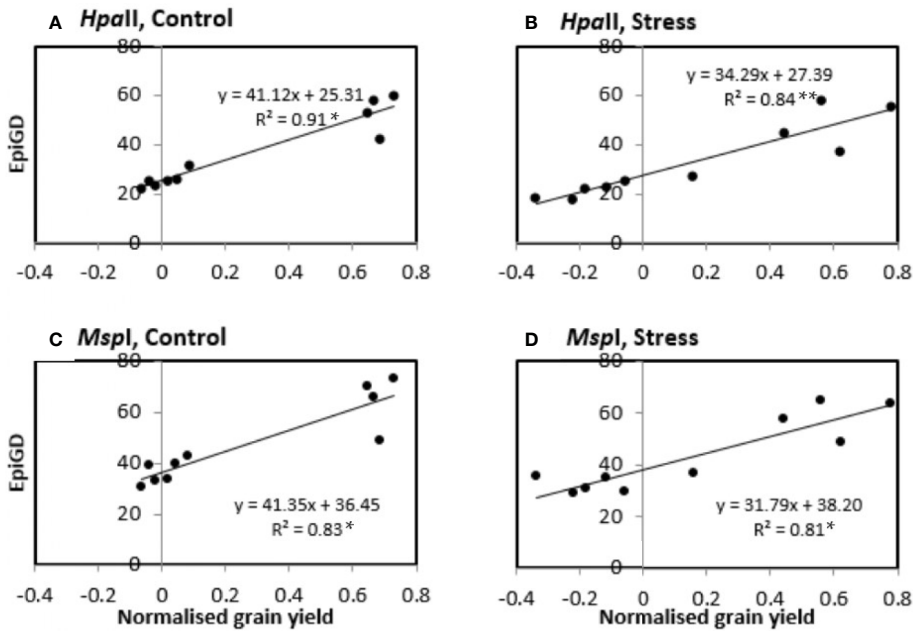

FIGURE 9 | Correlation between pairwise epigenetic distance (EpiGD) and pairwise difference in grain yield between plants of the variety Schooner. The correlation was tested according to Mantel, 1967 using GenAlex 6.5. Epigenetic distance between plants was calculated based on MSAP (methylation sensitive amplified polymorphism) data generated using *Hpa*II **(A, B)** and *Msp*I **(C, D)**. Pairwise differences in grain yield between plants were calculated separately for control **(A, C)** and stress **(B, D)** plants. Values of grain yield were normalized by computing the ratio of each individual plant grain yield over the mean grain yield for the same treatment across all positions. The dots represent pairwise comparisons between individual plants; equations represent the formulae of the regression line; R^2 represents the coefficient of determination of the Mantel test; asterisk (*) and (**) indicate significant correlation between treatments for *P* value < 0.05, and 0.01, respectively, estimated based on 9,999 permutations.

epigenomic symptoms. The finding here of a plastic response by barley plants in terms of biomass and grain yield to subtle differences associated with greenhouse position corroborates earlier work by Lacaze et al. (2008) who suggested that barley is responsive to fluctuations in ambient conditions. We postulate that the irregularity of phenotypic variability patterns across barley varieties and treatments may have emerged from two complementary factors; 1) the genetic variability among barley varieties leading to differential responsiveness to positional effect, as reported elsewhere (Lacaze et al., 2008; Kren et al., 2015), and 2) the randomness of spatial microclimatic conditions, which did not have a linear spatial gradient. The influence of a genotype-by-environment effect on plant phenotype was expected (Gianoli and Palacio-López, 2009; Aspinwall et al., 2015), but the scale of phenotypic variation induced by small-scale environmental variation was not. Our findings highlight the possibility for plants to show substantial phenotypic responses to even slight variations in ambient conditions, and that homogeneity in temperature control does not have over-riding importance. Furthermore, our discovery of a significant correlation between barley MSAP profiles and grain yield suggests that DNA methylation could at least reflect and possibly contribute toward the plastic variation in plant phenotypes. These results are in accordance with a mounting body of evidence that plant plasticity is at least partly epigenetically governed (Boyko and Kovalchuk, 2008; Rois et al., 2013; Baulcombe and Dean, 2014; Aspinwall et al., 2015). Considered together, our results demonstrate a tight interplay between plant epigenome, environment and phenotype.

CONCLUSIONS

Homogeneity of environmental conditions is practically difficult to obtain in a greenhouse (Edmondson, 1989; Guertal and Elkins, 1996; Brien et al., 2013). Awareness of plant sensitivity to microclimate is therefore important, especially in epigenetic studies, where plant epigenomes seem to be extremely responsive to small fluctuations in environmental factors. This study reveals that at least some of the DNA methylation previously considered stochastic is likely to have been, at least partially, induced by 1) positional effects on growth conditions, 2) differences in the length of plant exposure to relatively trivial variations in environment and 3) synergistic effects of stress treatment (mild salt stress in this case) and microclimatic conditions. The correlation between phenotypic DNA methylation differentiations between plants grown in different microclimates suggests that position-induced DNA methylation, previously ignored or considered as stochastic, may be a substantial source of phenotypic variability. Accordingly, we advocate that future epigenetic analyses should take into account the effect of micro-variations in environmental factors by careful experimental design and by considering position-induced DNA methylation markers as strong candidates for finely-tuned response to small environmental changes. We also propose that further research is needed to untangle microclimate-induced epigenetic variations from epigenome instability due to experimental treatment and developmental stage. We also feel the possibility of a transgenerational transmission of these effects warrants urgent attention.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

MK performed the experiments, analyzed the data and wrote the manuscript. JT performed the statistical analysis of MSAP peak heights. MW, ES, BB, and CR conceived the experiments and supervised the work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.553907/full#supplementary-material

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Roles of DNA Methylation in Cold Priming in Tartary Buckwheat

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Plants experience a wide array of environmental stimuli, some of which are frequent occurrences of cold weather, which have priming effects on agricultural production and agronomic traits. DNA methylation may act as an epigenetic regulator for the cold response of Tartary buckwheat (*Fagopyrum tataricum*). Combined with long-term field observation and laboratory experiments, comparative phenome, methylome, and transcriptome analyses were performed to investigate the potential epigenetic contributions for the cold priming of Tartary buckwheat variety Dingku1. Tartary buckwheat cv. Dingku1 exhibited low-temperature resistance. Single-base resolution maps of the DNA methylome were generated, and a global loss of DNA methylation was observed during cold responding in Dingku1. These sites with differential methylation levels were predominant in the intergenic regions. Several hundred genes had different DNA methylation patterns and expressions in different cold treatments (cold memory and cold shock), such as *CuAO*, *RPB1*, and *DHE1*. The application of a DNA methylation inhibitor caused a change of the free lysine content, suggesting that DNA methylation can affect metabolite accumulation for Tartary buckwheat cold responses. The results of the present study suggest important roles of DNA methylation in regulating cold response and forming agronomic traits in Tartary buckwheat.

Keywords: DNA metylation, cold response, agronomic trait, epigenetic regulation, Tartary buckwheat, cold priming

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INTRODUCTION

Plants can precisely perceive hypothermia through epigenetic regulation with short-term cold stress responses (Jung et al., 2013) or prolonged cold temperature changes (Luo and He, 2020). DNA methylation can function as an epigenetic regulator to potentially provide flexible genomic parameters for plants to respond to various cold stresses (Chinnusamy and Zhu, 2009; Huff and Zilberman, 2012; Sahu et al., 2013; Thiebaut et al., 2019). The stressful experiences of plants can change how they subsequently respond so that they have stronger stress tolerance when encountering sudden environmental changes in the future. In higher plants, this is known as "stress memory" or "stress imprinting" (Bruce et al., 2007), and the expression of stress memory genes is modulated by epigenetic mechanisms (D'Urso and Brickner, 2014; Chang et al., 2020). There have been many studies of stress memory, but the original report conducted by Zuther et al. (2019) proposed that cold memory can improve plant freezing tolerance by changes in gene expression and lipid and metabolite composition and defined the memory of cold acclimation as cold priming. Subsequently, there have been

several reports of the involvement of epigenetics in plant stress memory (Ding et al., 2012; Yang et al., 2020b). However, the detailed mechanism of how epigenetic memory is involved in frozen memory has not yet been described.

Tartary buckwheat (*Fagopyrum tataricum*) is a pseudocereal that belongs to the genus *Fagopyrum* within the Polygonaceae family. Tartary buckwheat is strongly adapted to growth in adverse environments (such as harsh climates and nutrient-poor soils; Zhang et al., 2017, 2018; Zhou et al., 2017b). Additionally, Tartary buckwheat is a short-generation and a diploid, with highly enriched flavonoid products, facilitating its use as a potential model species to study low-temperature adaptability in plants. Recent studies in Tartary buckwheat have focused on the functional analysis of individual genes and developmental traits (Liu et al., 2018a,b, 2019b,c; He et al., 2019; Yao et al., 2019), but there have been no reports of global epigenetic regulation.

Low temperature or repeated diurnal temperature difference promotes the accumulation of flavonoids, mainly because low temperature greatly increases the activities of enzymes in the flavonoid synthesis pathway (Christie et al., 1994; Leyva et al., 1995; Caldwell et al., 2005), suggesting that cold-induced transcriptional events can lead to desirable agronomic traits in plants. Metabolites can also protect plant cell viability during adversity. Epigenetic information in "cis-memory" is stored as a state of local chromatin (e.g., by DNA methylation or histone modification) and "trans-memory" exists as movable factors (e.g., transcriptional repressors; Dean, 2017). Some studies have confirmed that epigenetic memory formed under stress may allow for the quick adaptation of plants to ambient temperature changes. The phenotypic variation that can be induced by epigenetic memory – but not hereditary variation – is important to cope with rapid changes in the environment (Latzel et al., 2016). Few details are known of how epigenetic memory allows for a response to a changing environment, and Tartary buckwheat is an ideal plant to investigate the related mechanisms.

In this study, long-term field testing and laboratory experiments revealed that Tartary buckwheat cv. Dingku1 presents frost resistance and other agronomic traits, such as higher germination rate, higher water content of seeds, and higher flavonoid content. Experiments were designed as different cold treatments, including cold memory (cold priming) and cold shock, and phenome, methylome, and transcriptome analyses were performed to investigate the frost resistance characteristics of Dingku1. The single-base resolution DNA methylomes of Dingku1 were characterized under different cold treatments and revealed the global loss of DNA methylation, with some locally hypermethylated sites. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that DNA methylation significantly impacted the pathways of lysine degradation; pyrimidine metabolism; and the synthesis of isoquinoline alkaloids, metabolism-related genes (i.e., *FtDHE1*, *FtRPB*, and *FtCuAO*), and metabolites (i.e., lysine) to precisely regulate frozen memory. Treatment with a DNA methylation inhibitor interfered with the lysine level during the cold response, indicating that DNA methylation is critical for proper stress responses in Tartary buckwheat. These findings provide comprehensive insights into the development of cold priming and suggest guidelines for future breeding efforts in Tartary buckwheat.

MATERIALS AND METHODS

Field Experimental Site

Field experiments were conducted at the China Oat and Buckwheat Industrial Technology System Haidong Comprehensive Test Station. Tartary buckwheat varieties that were recently bred to be frost tolerant were used.

The core test base of the Haidong Comprehensive Test Station is located in Huangzhong, Qinghai Province; this site has a flat terrain and a cold and humid climate (**Figure 1**). The soil organic matter content was 98 g/kg, the content of the available N was 24.2 mg/kg, the available P (P_2O_5) was 13.43 mg/kg, the available potassium (K_2O) was 110.32 mg/kg, and the pH was 8.4 (2016–2019 data). Meteorological data were provided by the Huangzhong Meteorological Bureau. Agronomic parameters [average yield per mu, plant height, length of tillering, thousand kernel weight (TKW), and grain yield] were measured as described (Liu et al., 2019a). Tartary buckwheat seeds were collected after maturity, and total flavonoids, total protein, total starch, and crude fat were determined according to national standards (GB5009.5-2016) and technical documents of the Standardization of Shenzhen City (SZDB/Z 349-2019).

Plant Materials and Cold Treatments in Laboratory Experiments

After 4–8 h of soaking the seeds in ddH_2O, the seeds were disinfected using a 15% NaClO solution and then placed in a culture dish with two layers of gauze. The culture dish was moved to a greenhouse and cultured until germination. Three-week-old seedlings were treated with 5-aza-2'-deoxycytidine (aza-dC, Sigma) that was added to the liquid nutrient medium (7 g/ml). Seedlings were divided into two groups in cold stress experiments. The first group was to test the changes in plant morphology, plant water content, and plant temperature before and after cold stress. The experiment was set at 4°C for 24 h, followed by recovery at room temperature (21°C) for 1 day. The second group was designed to simulate the field climate and was used to assess the character of different varieties. The experiment included plants subjected to cold memory (4°C for 6 h, then at room temperature for 18 h, repeated four times, and then placed at 0°C for 6 h), cold stock (not acclimated, directly exposed to 0°C for 6 h), and control groups with normal growth conditions.

High Throughput Phenotypic Tests in Laboratory Experiments

Indoor high-throughput phenotypic observations were performed using image processing based on visible light (morphological test), infrared light (relative temperature measurement), and near-infrared light (relative water content test). A commercial

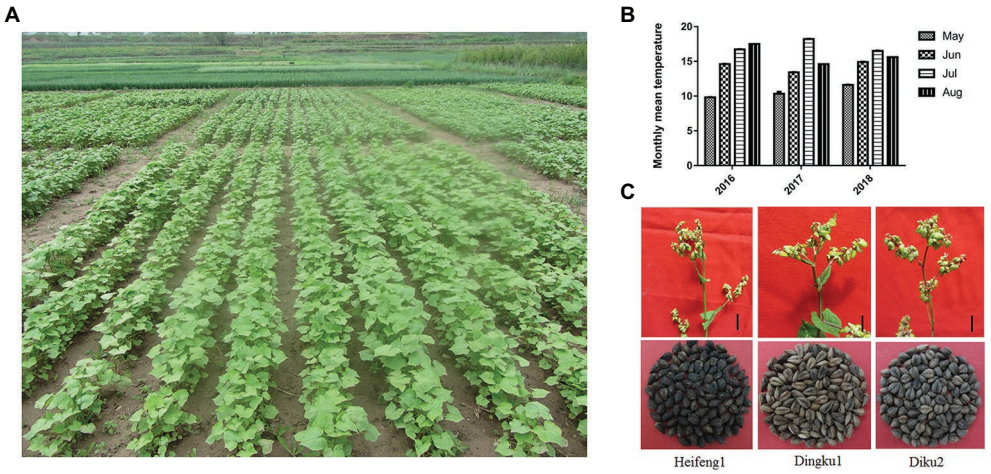

FIGURE 1 | Tartary buckwheat and experimental site climate. **(A)** Tartary buckwheat Haidong experimental site in the light raining day, average altitude: 2,620 m. **(B)** Average temperature of Tartary buckwheat Haidong experimental site in recent 3 years of growing season (°C). **(C)** Collected flower branch and seed specimens for experimental measurement. Scale bar: 7 cm.

phenotyping system (Scanalyzer3D, LemnaTec GmbH, Würselen, Germany) was used for image acquisition. Images of each plant were taken from the top and side views. Images were acquired and data preprocessing was organized into LemnaBase, a central database interface for the phenotyping system (Guo et al., 2017). Imaging, data acquisition, and data analysis of seeds and plants before and after cold treatment were carried out. Indicators of area, circumference, expansion degree, density, area of external polygons, circumferential length of external polygons, vertical length, horizontal length, and minimum diameter were performed to determine phenotypic differences in detail (An et al., 2017; Zhou et al., 2017a).

The chlorophyll values of plants were determined (Polyphenol-Chlorophyll Meter-Dualex Scientific+, Force-A, France) using the second and third fully expanded leaf near the plant center. Three indexes were measured: chlorophyll absorptivity (chlorophyll, Chl), anthocyanidin, and NBI (Cerovic et al., 2012). The one-way variance was calculated for multiple comparisons using SPSS 19.0, GraphPad Prim7 software and Microsoft Excel 2016.

The Anti-freezing Physiological Index

Electrolyte leakage tests were performed as previously described (Song et al., 2019). Leaves were transferred to 15 ml tubes and placed in a freezer (XT5201-D31-R40C, XuTemp, China). The plants were exposed to freezing temperatures ranging from 10 to −6°C, and leaves without damage were then immersed in 10 ml ultrapure water (Milli-Q Advantage) and placed on a shaker at 4°C for 2 h. Electrolyte leakage was calculated as the ratio before and after leaves were boiled *via* a conductivity meter (DDSJ-308, Leici, China; Rohde et al., 2004).

The free proline was determined according to the following method. Briefly, 0.25 g of the plant material was weighed and combined with 1.75 ml 3% sulfosalicylic acid in a test tube and incubated in a boiling water bath for 10 min before centrifugation at 5,000 rpm for 10 min. Next, 0.5 ml of water, 0.5 ml of glacial acetic acid, and 1 ml of 2.5% acid trione were added to 0.5 ml of the supernatant, and the solution was developed for 30 min in boiling water. The absorbance at the wavelength at 520 nm was measured after cooling. According to the standard curve, the average content of free proline in each gram dry weight sample was calculated (Levy, 1980; Torrecillas et al., 1984).

After freezing treatment, the seedlings were transferred to 4°C for 12 h in dark conditions, put into normal condition for recovery for 3–5 days, and the number of seedlings that generated new leaves was counted as the survival rate (Ding et al., 2019). Membrane oxidation in cold stress was assessed by measuring the MDA level (Wang et al., 2017).

Whole-Genome Bisulfite Sequencing and Analysis

Total DNA was extracted using the QIAamp Fast DNA Tissue Kit (Qiagen, Dusseldorf, Germany) following the procedure given by the manufacturer. DNA samples were fragmented using sonication and subjected to bisulfite conversion and second-generation sequencing.

The Accel-NGS Methyl-Seq DNA Library Kit (Swift, MI, United States) was utilized to attach adapters to single-stranded DNA fragments for library construction. Paired-end, 2 × 150 bp sequencing was performed at the Hangzhou Lianchuan Biotechnology Center using an Illumina Hiseq 4,000 platform. Library construction, sequencing, and bioinformatics analysis are described in the SI Appendix, **Supplementary Information Text 1**.

RNA Analysis

Total RNA was extracted with a Trizol reagent (Invitrogen, CA, United States) following the procedure provided by the manufacturer. Next, 1 μg of RNA and oligo dT primers were used to synthesize cDNA in a 20 μl reaction to create the final cDNA library using an mRNA sequence sample preparation

kit (Illumina, San Diego, CA, United States). The average insert size for the paired-end libraries was 300 bp (±50 bp). Paired-end sequencing was done on an Illumina Hiseq4000 (LC Sciences, United States) following the vendor's recommended protocol. Read mapping, transcript abundance estimation, and differential expression quantitation were performed as described in the SI Appendix, **Supplementary Information Text 1**.

Real-Time Quantitative RT-PCR

Total RNA was extracted from the frozen tissue using Tiangen's RNA prep pure plant kit (Cat.DP432, Beijing, China) according to the manufacturer's instructions. Next, 2 μg RNA was used for the first strand of cDNA synthesis using reverse transcriptase (Thermo Scientific, #EP0441). Real-time PCR amplification was carried out with the Bio-Rad CFX96 system using SYBR Green I (Takara, DRR081A, Dalian, China). PCR conditions were 3 min at 95°C followed by 40 cycles of the following: 95°C for 10 s, 60°C for 15 s, and 72°C for 15 s. The primer pairs used were *FtCuAO* 5'-ACCTCAGGTGAAGCAGTCAA-3' and 5'-GGGATTTCGCACCCTCATTC-3'; *FtRPB1* 5'-CTCACGAC AACCACCATTCC-3' and 5'-CCTCCTTGTGTGGAGTGTCT-3'; and *FtDHE1* 5'-CAGAGGAGCTTGCTTGGTTG-3' and 5'-CGCA AATGGCAGACACTGAT-3'. The *FtH3* gene was amplified as a reference gene, since its expression is unaffected by abiotic treatment (Li et al., 2019), using primers 5'-GAAAT TCGCAAGTACCAGAAGAG-3' and 5'-CCAACAAGGTATGCC TCAGC-3'.

Measurement of Rutin and Lysine Content

Fresh seedlings (1 g) of Dingku1 were frozen and ground in liquid nitrogen. The rutin content was analyzed by high-performance liquid chromatography (HPLC) from triplicate independent extractions as described previously (Zhang et al., 2017). Briefly, dried seedlings (100 mg) were ground, then mixed with 500 μl Na-S™ buffer (2% sodium citrate, 1% HCl, and 0.1% benzoic acid; Beckman, United States) for 30 min in a mixer, and extracted for 10 min *via* ultrasonication. The free lysine content was analyzed using an A200 Amino Acid Analyzer (Aminosys, Germany), from triplicate independent extractions as described previously (Yang et al., 2016).

RESULTS

Evaluation of Traits of Tartary Buckwheat Varieties by Field-Scale Experiments

To screen for high-quality Tartary buckwheat resources adapted to the high altitude and cold climate of the Tibetan Plateau, dozens of Tartary buckwheat varieties were subjected to a long-term adaptive assessment at the Haidong comprehensive test station (Qinghai Academy of Animal Science and Veterinary Medicine) using advanced field real-time detection equipment and unmanned aerial vehicle (UAV) detection managements. Through long-term observation, three varieties were focused on for further field experimental tracking and indoor experiments: Heifeng1, Diku2, and Dingku1. The experimental site was established in Huangzhong, Qinghai Province (approximately 36°28'N, 101°37'E; **Figure 1A**), with an average altitude of 2,620 m, an annual average temperature of 3.7°C, and a growing season average temperature of 14°C (**Figure 1B**). Plants were observed extensively, such as flower branch and seeds (**Figure 1C**). Thereinto, Tartary buckwheat cv. Dingku1 exhibited traits of late flowering, higher germination rate, and higher content of total flavonoids and starch in seeds (**Supplementary Figures S1**, **S2**).

Evaluation of Frost Resistance in Tartary Buckwheat Varieties by Laboratory Bench-Scale Experiments

To confirm the findings of the field data, a large-scale and accurate phenotypic analysis of three plant varieties – Heifeng1, Diku2, and Dingku1 – was carried out under laboratory control conditions (**Figure 1B**; **Supplementary Figure S2**). The morphological characteristics of seeds were observed within the 3 days before and after germination. The results showed that there was no significant difference in the morphology of the seeds from the three varieties during germination, but the seeds from Dingku1 were of slightly smaller volume than those of the other two varieties before and after germination, and the compact density was relatively larger (**Figure 1B**; **Supplementary Figures S2a**, **S3**). During germination, the seed temperature and seed water content of Dingku1 were higher compared with seeds from the other two varieties (**Supplementary Figures S1b,c**, **S4**).

An experiment of cold treatment (4°C, 24 h) followed by 1 day of recovery was carried out, and the frost resistance was assessed by observing, in detail, the morphology of seedlings with three leaves by optical imaging and image analysis technology. Compared with the other two varieties, the seedlings of Dingku1 were shorter, narrower in width, and more compact (**Figure 2A**; **Supplementary Figure S5**). Dingku1, however, did not differ significantly between groups and within groups in anthocyanin and chlorophyll test, and differences were observed in the control group only in nitrogen balance index test (**Figure 2B**). The temperature and water content of seedlings were measured before and after cold treatment in three varieties. In contrast, Dingku1 had higher water content and plant temperature after cold treatment (**Supplementary Figure S6**).

Next, experiments of cold acclimation (memory) and freezing stress (shock) were designed to simulate field weather. Through physiological and biochemical indexes, it was determined that Dingku1 exhibited characteristics for stronger frost resistance (**Figure 3**). Compared to the other varieties, Dingku1 exhibited a lower ion leakage rate (**Figure 3A**), higher free proline content (**Figure 3B**), higher survival rate (**Figure 3C**), and lower malondialdehyde (MDA) content (**Figure 3D**) during freezing stress. Therefore, Dingku1 was selected for additional study on the cold environmental adaptation mechanism.

In addition, the memory group (cold priming) with cold acclimation exhibited better performance (**Figure 3**), higher free proline content (**Figure 3B**), and higher survival rate (**Figure 3C**) after being subjected to the second freezing stress,

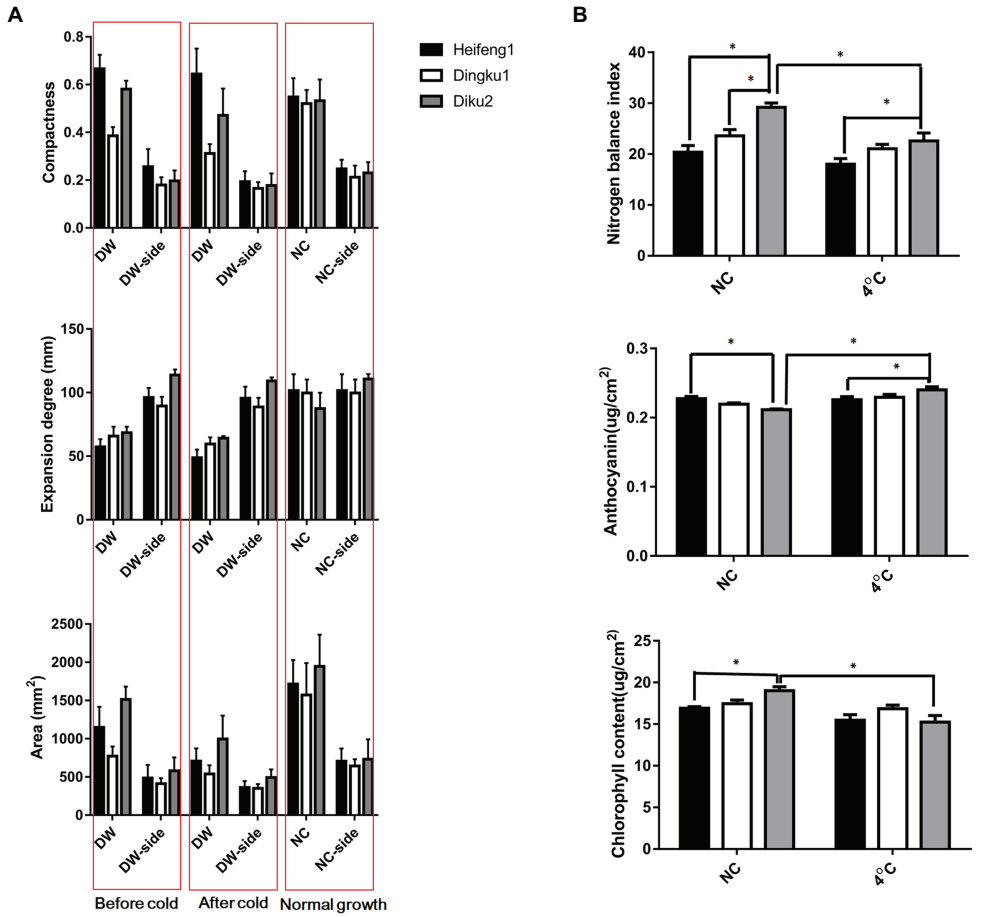

FIGURE 2 | Phenotypic observation and physiological test in cold experiment of 3-week-old seedlings. **(A)** Morphological observation before and after 4°C treatment. DW, Low temperature treatment groups from the top surface testing; DW-side, Low temperature treatment groups from the side face testing; NC, non-specific control. **(B)** Physiological test between the control and cold treatments. The every mean value was from more than 30 independent plant measurements, and error bars indicated ± SD. Analysis was performed with one-way ANOVA followed by Tukey-Kramer *post hoc* analysis. $^{*}p < 0.05$.

compared to the control plants grown at 16–21°C or the plants that experienced freezing shock without prior cold acclimation.

Tartary Buckwheat cv. Dingku1 DNA Methylomes During Cold Stress

DNA methylation is closely related to plant frost resistance (Song et al., 2015). To characterize the Tartary buckwheat Dingku1 methylome during cold stress, a whole-genome bisulfite sequencing was performed and single-base resolution maps of DNA methylation were generated for three test groups: the control group (normal temperature), cold memory group (priming: 4°C for 6 h, followed by 21°C for 18 h, repeated for 4 days, then 0°C for 6 h), and cold shock (0°C for 6 h directly, without priming).

Each methylome was sequenced to >10-fold coverage per strand, covering >52% of the genomic cytosine positions (**Supplementary Table S1**). For each sample, at least 200 M (C: 235 M; M: 218 M; S: 227 M) paired-end reads (read length = 150 bp) were produced. Approximately 75% (C: 74.96%; M:76.66%; and S:76.19%) of the reads were mapped to the reference genome using Bismark (Krueger and Andrews, 2011), covering >90% of the genome (C: 92.94%; M:92.13%; and S:92.57%; **Supplementary Table S1**).

Tartary buckwheat is the only Polygonaceae plant whose methylome has been reported to date. This analysis revealed the details of methylated base sites (**Figure 4**; **Supplementary Table S2**). The results show different 5-methylcytosine distributions in different regions for the control and the other two cold treatment groups, with most of the regions with decreasing in CpG methylation mapping to the VIII chromosome (**Figures 4A–C**). There are several functional genes with hypomethyl-modifications, such as the ubiquitin-activating enzyme E1, helicases, and hydrolases, with potential roles in the cold shock response that are regulated by DNA methylation (**Supplementary Table S4**). There was different methylation of CHH for all three treatments, with slightly higher methylation densities of CHH in the cold memory group compared to that in the other two treatments, as well as significantly lower methylation density in the cold shock group than that in the other treatments (**Figures 4A–C**).

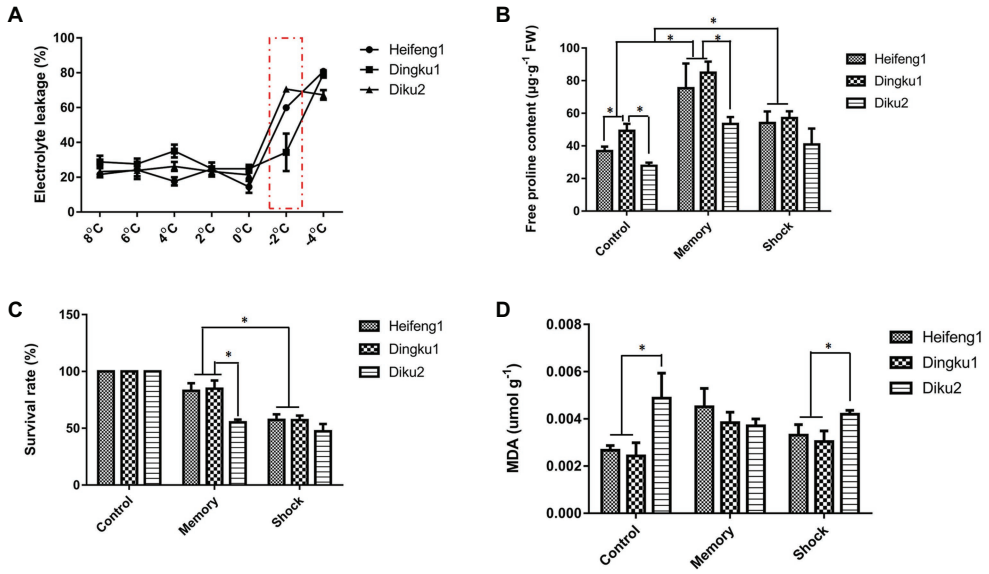

FIGURE 3 | Physiological and biochemical tests in cold acclimation and freezing treatments of Tartary buckwheat three varieties. **(A)** Electronic leakage rate of Tartary buckwheat three varieties in low temperature. **(B)** Change of proline content in different low temperature treatments. **(C)** Survival rate in different low temperature treatment. **(D)** Malondialdehyde (MDA) content in different low temperature treatments. The mean value was from more than 30 independent plant measurements, and error bars indicated ± SD. Analysis was performed with one-way ANOVA followed by Tukey-Kramer *post hoc* analysis. $^{*}p < 0.05$.

An analysis of the distribution of DNA methylation in Tartary buckwheat showed the enrichment of DNA methylation in the proximal promoter region (5' terminal of TSS) and downstream region (3' terminal of TES; **Figure 4D**), indicating increased DNA methylation in the 5' and 3' flanking regions of genes. In addition, genes were characterized by a high enrichment of CG methylation and the moderate enrichment of CHG methylation and CHH methylation (**Figure 4D**), suggesting that CG was the primary type of cytosine methylation in Tartary buckwheat gene-transcription domains. Little difference was observed in the distribution of methylation among the three test groups (**Supplementary Figure S7**), and similar trends in methylation levels were observed for the three groups. In the control group, the total DNA methylation level was 24.62%, with 78% CG methylation, 40.28% CHG methylation, and 11.1% CHH methylation (**Supplementary Table S3**).

DNA Methylation in Dingku1 Decreases During Cold Stress

As shown in **Supplementary Figure S8**, the principal component analysis (PCA) revealed significant differences among the three groups. The average DNA methylation levels were calculated and a decrease in the global cytosine methylation level from the control group to the cold treatment group (from 23 to 24.5%) was found. Differentially methylated regions (DMRs) were identified and it was found that the change of DNA methylation was mostly due to a decrease in intergenic methylation (**Figure 5A**).

To characterize the change of DNA methylation for different treatments, a method based on Fisher's exact test was used to identify DMRs between methylomes (Ausin et al., 2012). The methylome of the cold memory group or the cold shock group (M/C and S/C, respectively) was compared with that of the control group. As shown in **Figure 5**, both hyper-DMRs and hypo-DMRs were detected in similar proportion in M/C and S/C (**Figure 5B**), but there were more hypo-DMRs in all comparisons, with 526,103 intergenic hypo-DMRs identified in the M/C comparison and 664,615 in the S/C comparison, whereas only 267,055 hyper-DMRs were observed for M/C and 272,784 for S/C. These results suggest a global decrease in DNA methylation in cold treatments, especially cold shock. Hypo-methylation was mainly observed in intergenic contexts, although decreased methylation was also observed in the CGI, promoter, intron, and exon regions to a lesser extent, suggesting that they have an important regulatory function of intergenic regions in the Tartary buckwheat genome.

Correlation Between DNA Methylation and Gene Expression in Cold Stress of Dingku1

Transcriptome profiles for Dingku1 were generated by RNA-seq analysis – with three biological replicates for seedlings under the same treatments used to assay methylation – to investigate whether the observed decrease in DNA methylation during a cold response was associated with changes in gene expression. A total of 31,391 genes and 34,067 transcripts were included in the analysis, with more upregulated genes and transcripts than downregulated genes and transcripts in the two treatment groups relative to the control group (**Supplementary Figure S9**). Furthermore, 24,931 differentially expressed genes (DEGs) were identified, of which 1,893 and 1748 were significantly differentially expressed in the cold memory group and the cold shock group, respectively, relative to the control group.

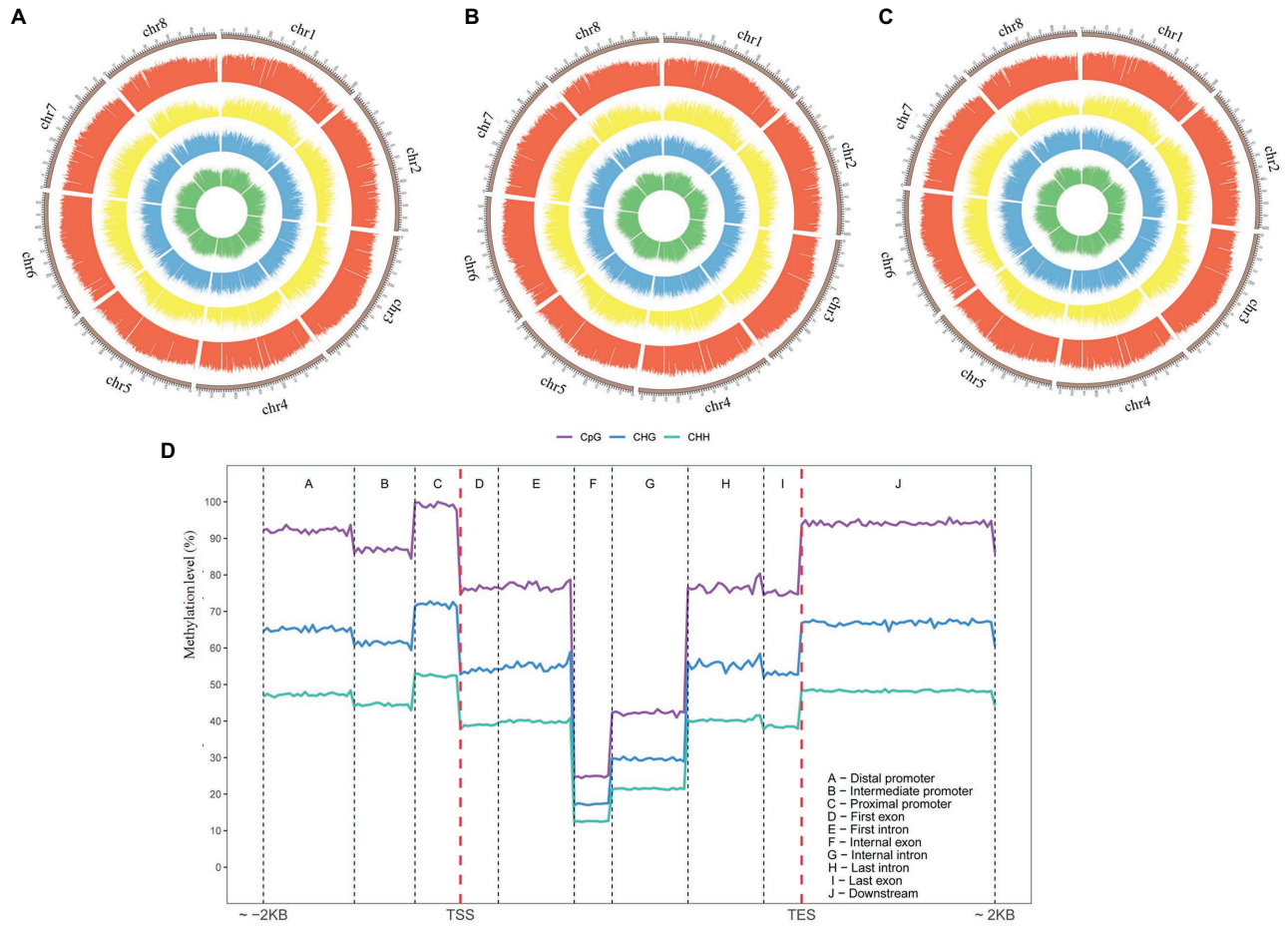

FIGURE 4 | Characterization of Tartary buckwheat cv. Dingku1 methylomes. **(A–C)** The rings indicate (from outside to inside, were quantified per 1 Mb window) density plot of 5-methylcytosine in sequence contexts in different cold treatments. The methylation of CpG (red), CpHpG (yellow), CpHpH (blue), and total C levels (green) were mCG, mCHG, and mCHH, where mC signifies 5-methylcytosine. H = A, C or T. Chromosome name and scale are indicated on the outer rim. The level of methylation at the corresponding position is represented by the height of the column on the circle map. **(A)** The control group. **(B)** The cold memory group. **(C)** The cold shock group. **(D)** The different methylation (CG, CHG, and CHH) level of cytosine in featured regions of the genome.

Considering the association between DNA methylation and gene expression, hyper- and hypo-DMR-associated genes were analyzed during cold treatments. There were 377,514 and 382,440 hyper-DMR in the cold memory and the cold shock groups, respectively, which were significantly lower than the numbers of hypo-DMRs for the two groups. There were 742,756 and 889,778 hypo-DMRs in cold memory and cold shock, respectively.

Statistical analysis revealed the potential coupling of the changes in DNA methylation and gene expression. A total of 21,335 genes were identified as DMR-associated genes in the buckwheat genome (FPKM >1). Among these genes, 336 upregulated and 217 downregulated DEGs were identified as hypo-DMR-associated genes, and 143 downregulated and 189 upregulated DEGs were identified as hyper-DMR-associated genes in the cold memory compared to the control. Additionally, 341 upregulated and 205 downregulated DEGs were found to be hypo-DMR-associated genes, and 125 downregulated and 198 upregulated DEGs were determined to be hyper-DMR-associated genes in the cold shock vs. the control (**Supplementary Table S5**). As was found previously for tomato and orange fruit ripening (Lang et al., 2017; Huang et al., 2019a), DNA hypermethylation is associated with gene activation in Tartary buckwheat cold response. The results of the present study suggest that DNA methylation may play a positive role in regulating the Tartary buckwheat response when exposed to external stresses. In the cold treatments relative to the control (M/C and S/C), there were four clusters: up-up (upregulated DEGs with hyper-DMR), up-down (downregulated DEGs with hyper-DMR), down-up (upregulated DEGs with hypo-DMR), and down-down (downregulated DEGs with hypo-DMR).

Gene Ontology (GO) analysis was performed to understand the potential role of DNA methylation in Tartary buckwheat cold tolerance. With an overall trend of hypomethylation during cold treatment, the analysis was focused on down-up genes (DNA methylation decreased and the expression increased) in M/C and S/C, which were annotated as members of 203 and 199 terms, and the significantly enriched GO terms were 29 and 38 ($p < 0.05$), respectively. The most significantly enriched

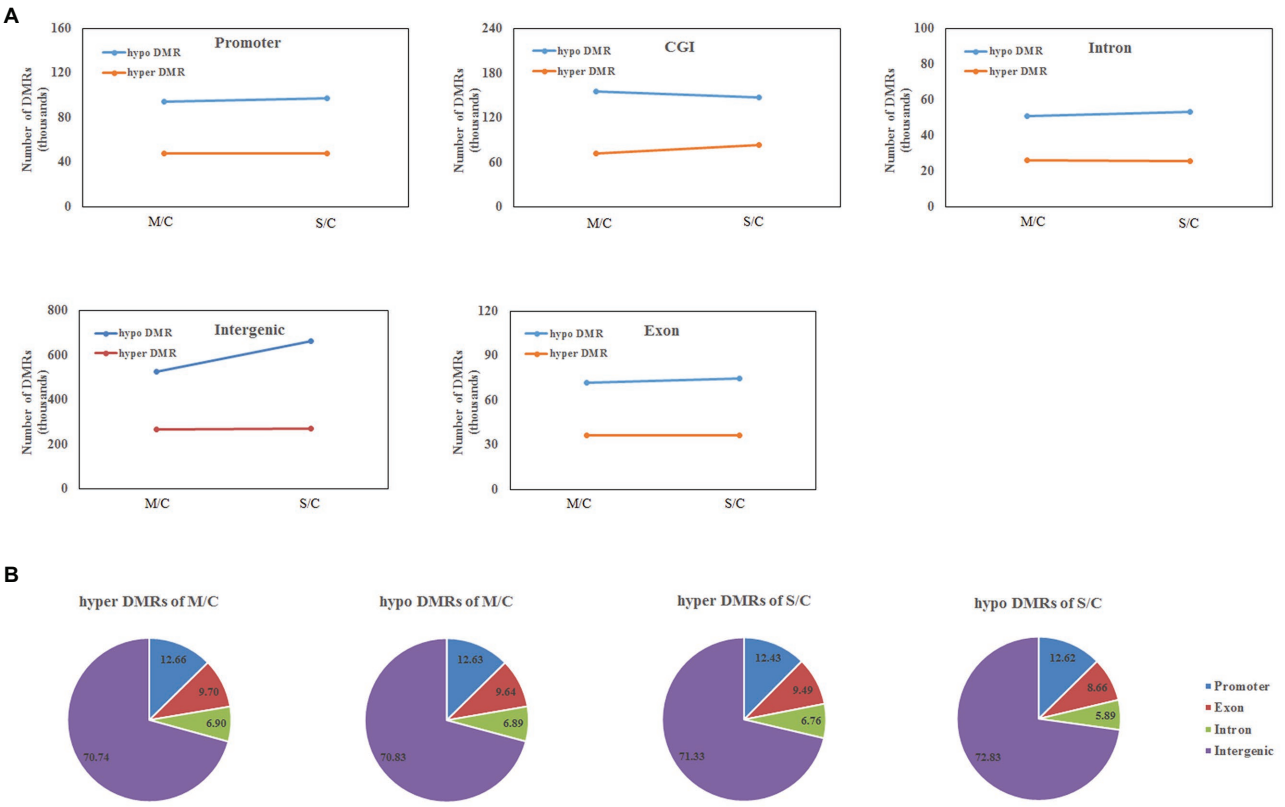

FIGURE 5 | Genome-wide decrease in DNA methylation during Dingku1 cold responses. **(A)** Numbers of hyper- and hypo-DMRs in cold memory (M) and cold shock (S) relative to the control (C) in different genomic regions. **(B)** The pie chart shows the proportion of genomic component in hyper- and hypo-DMRs.

genes in M/C are related to integral components of membranes, suggesting that membrane structure is related to the formation of frozen memory, but these genes were not significantly expressed during cold shock (**Supplementary Figure S10a**). GO analysis revealed 30 ATP binding-related genes enriched in the S/C down-up cluster, suggesting that the activation of these genes occurs by cold shock induction of DNA hypomethylation (**Supplementary Figure S10b**). ATP binding is an important physiological activity in plants. For example, ATP binding to Cryptochrome2 (cry2) and some other plant cryptochromes promotes the activation of light receptors and increases stress resistance (Eckel et al., 2018).

The same enrichment of GO terms was observed in different freezing treatments (richness factor > 0.8), including genes related to protein phosphorylation, DNA repair, endosome organization, endoplasmic reticulum organization, sulfuric ester hydrolase activity, D-alanine ligase activity, and mitotic cell cycle, suggesting that these tissue and cell activities were involved in the cold response and tolerance of plants. There were also obvious differences in the enrichment of GO terms for the different freezing treatments. For example, in the upregulated DEGs with hypo-DMR (down-up) for cold memory treatment relative to the control (M/C), dephospho-CoA kinase activity, voltage-gated calcium channel activity, and cell adhesion were significantly enriched (**Supplementary Figure S10a**). For cold shock treatment relative to the control (S/C), glycolipid biosynthetic process, regulation of cell shape, and cell division were specifically enriched (**Supplementary Figure S10b**). The enriched GO terms were compared for the two different freezing treatments (M/S). The results showed that the number of enriched genes was more different (>80%) for the metabolic process, oxidation-reduction process, and ribonucleoprotein complex, as well as the ribosome, nucleolus, intracellular, and protein binding in M/S (**Supplementary Figure S10c**). Differential enrichment was observed for some enzymes (including racemase, epimerase, and catechol oxidase) and some metabolic processes (including glycerol metabolism, cellular carbohydrate metabolism, and malate metabolism; **Supplementary Figure S10d**), indicating differences in DNA replication, RNA transcription, and the tricarboxylic acid cycle due to different freezing treatments inducing different response mechanisms by demethylation regulation.

The effect of cold memory on the adaptability of Tartary buckwheat to a cold environment wanted to be better understood, and the KEGG analysis revealed the enrichment of genes in plant metabolic pathways in comparison of different treatment groups (**Supplementary Figures S11a–c**). Glycosylphosphatidylinositol (GPI)-anchor biosynthesis and D-Alanine metabolism showed a significant increase in the cold memory group (**Supplementary Figure S11a**). Plant

GPI-anchored protein controls the cellulose content of the cell wall and guides the orientation for cell expansion (Cao et al., 2012). Frozen memory appears to activate the expression of this kind of protein to improve the freezing resistance of plants. Genes related to D-glutamine and D-glutamate metabolism, pyruvate metabolism, methane metabolism, biotin metabolism, and lipoic acid metabolism were significantly increased in the M/S cluster (**Supplementary Figure S11c**); however, little is known about the role of these pathways in countering freeze stress. There was an obvious difference between plants subjected to the slow accumulation of freezing signals and the rapid response when subjected to freezing.

A two-dimensional scatter diagram of correlation analysis was constructed to illustrate the overall relationship between differentially expressed genes and DNA methylation (**Figure 6A**; **Supplementary Table S7**). As shown in the Venn diagram, there is an overlap of the DEGs identified in the different treatments (**Figure 6B**). The numbers of down-regulated genes specifically expressed in each treatment were 294 (M/C), 200 (S/C), and 37 (M/S), and the numbers of upregulated genes were 228 (M/C), 205 (S/C), and 49 (M/S). Heatmap analysis showed a significant downregulation of transcription factors MYB108, Gcn5-related N-acetyltransferase (GNAT), and the late elongated hypocotyl (LHY), as well as a significant upregulation of WRKY40, BHLH, and JMJ30 in M/C. LHY, WAXY, and CYP were downregulated, and ERF053, WRKY40, and JMJ30 were upregulated in S/C (**Supplementary Figure S12**; **Supplementary Table S6**). The results showed shared and distinct aspects of the two cold coping strategies. The significantly different KEGG pathways were combined with metabolomics analysis (**Figure 6C**). The important genes in the pathways were demonstrated (**Figure 7**). There was a drop in methylation levels and an increase in the transcriptional level of copper amine oxidase (*CuAO*), which is a hypo-DMR-associated gene in cold treatment. This gene is involved in multiple pathways, such as the isoquinoline alkaloid biosynthesis pathway in M/C, as well as in the tropane, piperidine, and pyridine alkaloid biosynthesis pathway, and tyrosine metabolism pathway in S/C. RNA polymerase II's largest subunit (*RPB1*) and dehydrogenase E1 component (*DHE1*) showed differences in DNA methylation enrichment and transcriptional level in different cold treatments (M/S). The analysis of the present study suggests that DNA methylation can mediate the Tartary buckwheat cold response through the regulation of cold-induced genes.

Change in DNA Methylation Level Affects the Expression of Different Genes and Metabolites

The correlation between promoter methylation levels and transcript levels of genes related to lysine, pyrimidine, alkaloid, and flavonoid metabolism were further examined. Promoter methylation levels and gene expression levels were determined for individual genes for the two cold treatments. For most genes, the expression levels and promoter methylation levels

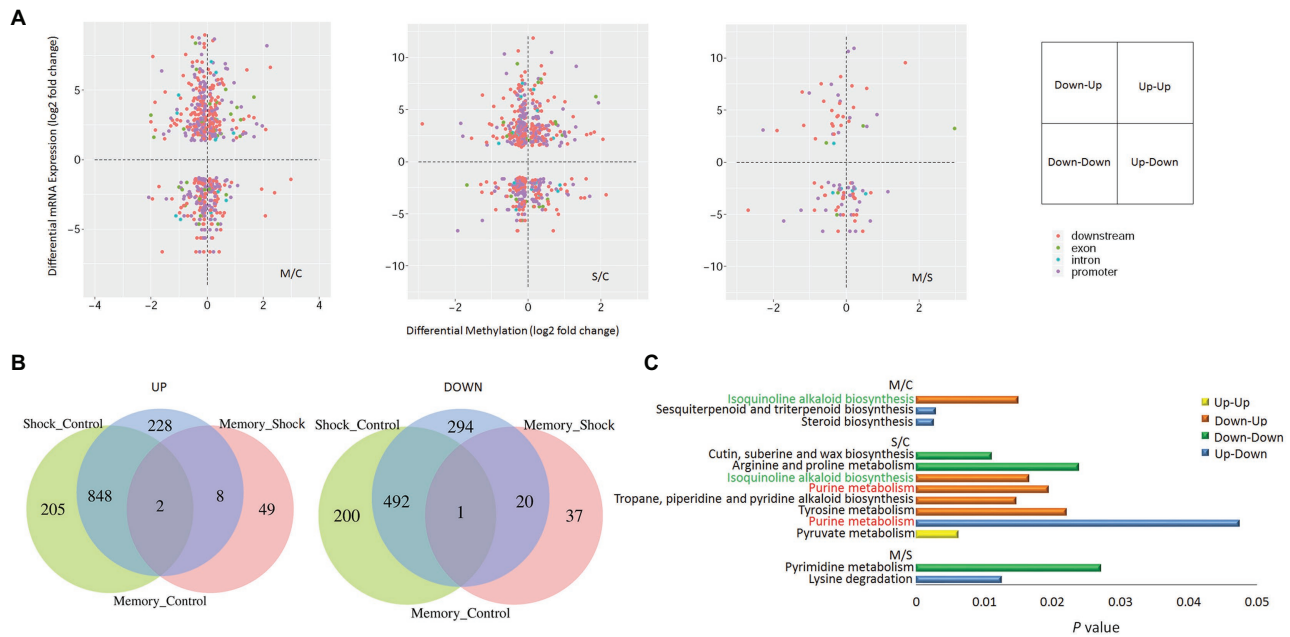

FIGURE 6 | Correlation between DNA methylation and gene expression levels during Dingku1 cold responses. **(A)** The two-dimensional scatter diagram of correlation analysis of differential gene parts and DNA methylation enrichments. The x-axis represents DNA methylation levels of gene promoter region 2 kbp (purple dots), downstream −2 kbp (pink dots), exon (green dots), and intron (blue dots). The y-axis represents expression levels of corresponding genes. **(B)** Indicated Venn diagrams of the expressed genes drew by comparative analysis of significant differential expressed genes (DEGs) with DMRs. **(C)** Significant different KEGG pathways of DEGs were illustrated ($p < 0.05$). Hypomethylated up-DEGs (down-up), hypomethylated down-DEGs (down-down), hypermethylated up-DEGs (up-up), and hypermethylated down-DEGs (down-down) are shown respectively.

exhibited a negative correlation (**Figure 8A**). For isoquinoline alkaloid biosynthesis, pyrimidine metabolism, and lysine degradation, the expression levels of *FtCuAO*, *FtRPB1*, and *FtDHE1* showed increased trends, while their promoter cytosine methylation levels were decreased in cold treatments (**Figure 8A**).

The transcript abundances were measured for three selected unigenes by qRT-PCR to validate transcriptome data. Consistent cold responses were detected between the qRT-PCR analysis and RNA-Seq data (**Figure 8A**). *CuAO* acts in the isoquinoline alkaloid biosynthesis pathway, relating to H_2O_2 production with polyamine catabolism (Rea et al., 2004) and ABA-induced stomatal closure (Fraudentali et al., 2019). *CuAO* catabolizes polyamines and is associated with stress responses (Rea et al., 2004; Fraudentali et al., 2019). *FtCuAO* showed a significant expression increase in the cold shock group by qRT-PCR, which was consistent with the observed response detected by RNA-Seq. The *FtRPB1* gene is involved in purine and pyrimidine metabolism and exhibited a significant increase by qRT-PCR in the cold shock group. *FtDHE1* participates in lysine degradation and showed a significant decrease in the memory group but a significant increase in the cold shock group based on the qRT-PCR analysis. These genes also exhibited demethylation (**Figure 8A**).

To further analyze the significance of DNA methylation for metabolic pathways, the DNA methylation inhibitor 5-aza-2'-deoxycytidine (aza-dc) was added into the Dingku1 nutrient solution. Rutin and lysine levels were detected in plants grown in this solution. The results showed that aza-dc had little effect on rutin accumulation but had a great influence on lysine level, especially in the cold memory group (**Figure 8B**), and we further examined the expression of *FtDHE1* under aza-dc treatment, the results also showed that *FtDHE1* expression has been disturbed (**Figure 8C**), suggesting a role of DNA methylation in lysine synthesis and metabolism pathway in cold priming.

DISCUSSION

Temperature is a key factor affecting growth and development in plants. However, the temperature fluctuates under natural conditions, both daily and seasonally. Different plants have evolved a variety of mechanisms to sense complex and variable temperature signals and to regulate their growth, development, or behavior to adapt to changes in environmental temperature. Plants use complex mechanisms to grow under natural conditions and perceive changes of ambient temperature and store temperature memories to better adapt and form desired agronomic traits. In this study, Dingku1 (a Tartary buckwheat variety) was identified as having a high flavonoid content and good temperature tolerance (**Figures 2, 3**). Repeated cold simulation (cold priming) was used together with high-throughput measurement to find patterns

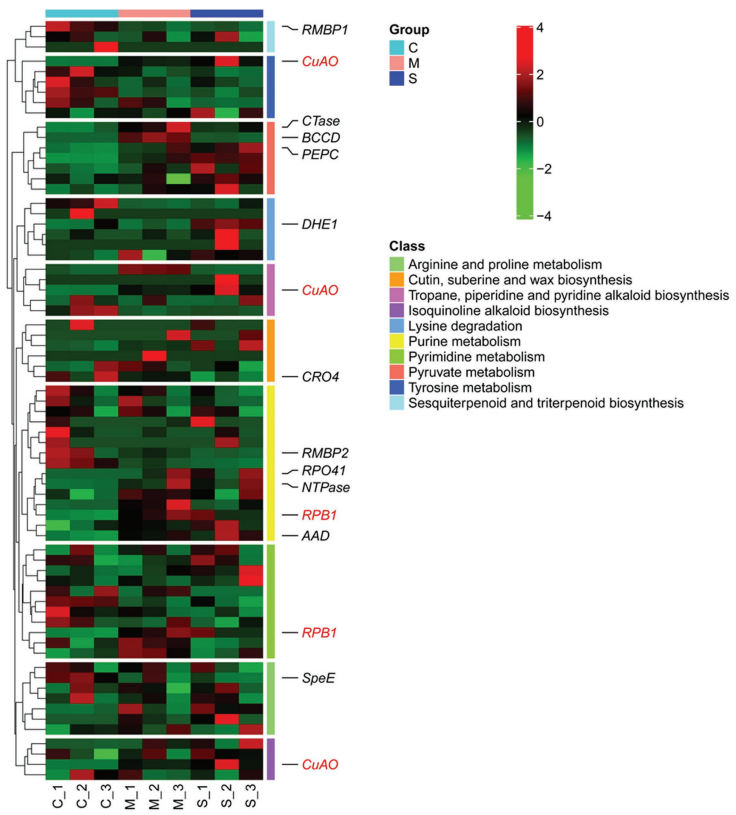

FIGURE 7 | Heatmap of differential gene expression among various treatments. Genes with an adjusted $p < 0.05$ and relative fold change|log2FC| > 0.5 are displayed. The genes marked red are involved in multiple pathways.

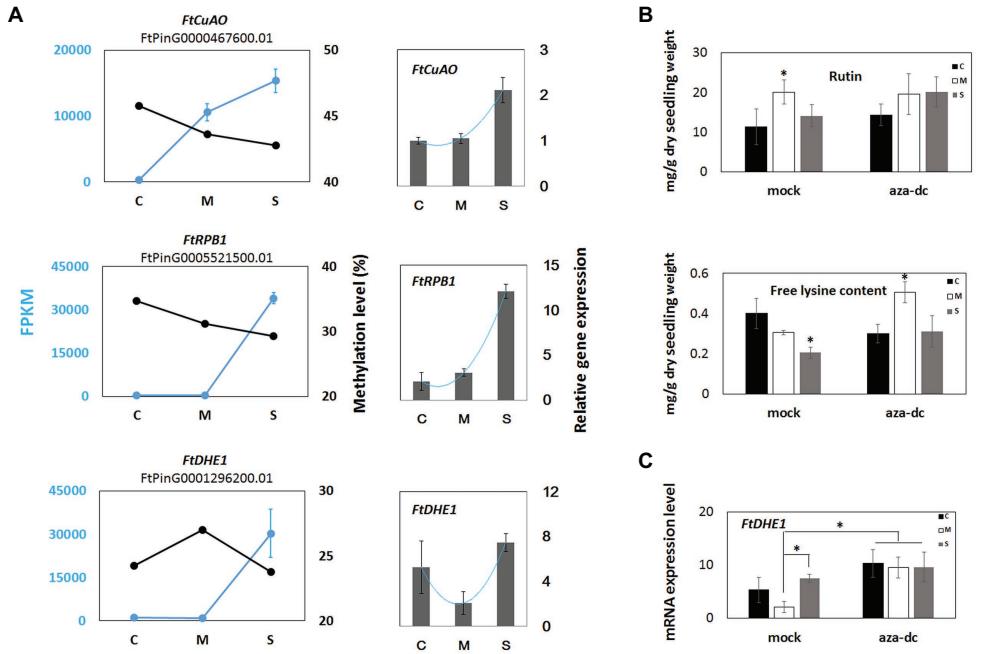

FIGURE 8 | The significance of DNA methylation for Tartary buckwheat cold responding. **(A)** DNA methylation and expression levels of cold induced differential genes during Dingku1 cold responses. Blue lines with filled dots represent transcript abundance. Black lines with filled dots represent methylation level gene promoter region (2 kb). Histograms represent qRT-PCR test results. Data shown are mean ± SE (n = 3). **(B)** Rutin and lysine tests. Dingku1 were treated with DNA methylation inhibitor, 5-aza-2'-deoxycytidine (aza-dc), or mock (ddH2O) in different treatments, the control group (C), cold memory group (M), cold shock group (S). $^{*}p < 0.05$, error bars indicate means ± SE (n ≥ 12). **(C)** mRNA of *FtDHE1* expression levels in aza-dc treatment. Data shown are mean ± SE (n = 3). Analysis was performed with one-way ANOVA followed by Tukey-Kramer post hoc analysis. $^{*}p < 0.05$.

of DNA methylation and relationships between genes and metabolites. The results showed a significant involvement of DNA methylation with enrichment changes of genes and metabolites related to isoquinoline alkaloid biosynthesis, lysine degradation, and pyrimidine metabolism (**Figures 6–8**).

Environmental temperatures affect virtually all aspects of plant growth and developmental processes. Plant cells can create various chromatin states for stress-responsive gene expression that are required for the adaptation to harsh environmental conditions (Zhao et al., 2020). The epigenetic marks are deemed as environment-dependent patterns through calculations using large populations (Alonso et al., 2019). Environmental factors modulate the epigenomic landscape and regulate adaptive responses in plants. Cytosine methylation (5-mC) is an epigenetic mark associated with developmental programs and stress responses and maintains genome stability by preventing mobilization of transposable elements (TE; Law and Jacobsen, 2010). Environmental cold temperatures can induce the transcription of transposons and lead to desirable agronomic traits in plants (Butelli et al., 2012). The 5-mC profile of a locus reflects the balance between pathways allowing accurate maintenance or a change in DNA methylation, with dynamics affected by abiotic and biotic stresses as well as developmental programs to result in phenotypic changes. This is seen by the overall change of the DNA methylation level during ripening of orange (Huang et al., 2019a), tomato (Zhong et al., 2013; Gallusci et al., 2016; Lang et al., 2017), and strawberry fruit (Cheng et al., 2018). DNA methylation may also be involved in the heterotic traits in broccoli (Li et al., 2018). These observations suggest the potential to alter the methylome to alter adaptation in plants and promote breeding (Molinier, 2020).

The results of the present study show a global loss of DNA methylation during cold treatments, with significant changes in chromosome VIII in cold shock (**Figure 4C**). No similar results were reported, and this result will continue to be validated in the future. A detailed analysis of regions in S/C showing differential expression was conducted and DEGs were classified by GO and KEGG analysis. Terms involved in the biological process were enriched, such as the "carbohydrate metabolic process," "transport," "proteolysis," "oxidation-reduction process and lipid metabolic process," "metal ion binding," and "amino acid metabolism," suggesting DEGs regulated by methylation are closely related to the metabolic activity (**Supplementary Figure S13**).

Prior studies have noted the relationship between epigenetic mechanisms and environmental changes, such as sulfur homeostasis by DNA and histone methylation (Huang et al., 2019b); salicylic acid metabolism in heterosis regulated by decreased DNA methylation 1 (DDM1; Zhang et al., 2016); indole-3-acetic acid (IAA) metabolism and transport regulated by epigenetic factors (Mateo-Bonmati et al., 2019); and the relative amounts of different forms of acetyl-CoA, which can be altered by environmental and metabolic factors. Together, these indicate that histone acetylation dynamics integrate metabolic activity to regulate plant

responses to stress (Hu et al., 2019). Differential KEGG pathways were identified through joint bisulfite sequencing and transcriptome analysis (**Figure 6C**). There was an altered expression of key genes regulated by DNA methylation in these metabolic pathways (**Figure 7**). For example, genes involved in the isoquinoline alkaloid biosynthesis, the purine metabolism pathway, and the tropane, piperidine, and tyrosine metabolism pathway were upregulated. These results suggest that carbohydrate metabolism, carbon-nitrogen budget, and secondary metabolism are constitutively promoted in cold treatment. Genes related to amino acids and pyrimidine accumulation were upregulated in the cold shock treatment, potentially due to the differential accumulation of lysine and thymidine in both cold treatments.

To the knowledge of the authors, this is the first report on the regulation of lysine metabolic pathways by DNA methylation in plants, and the result of the present study shows different regulatory mechanisms for lysine degradation under different cold treatments (cold memory group and cold shock group). Lysine is an essential amino acid, and lysine level represents crop quality. Lysine is the classic target site of epigenetic modifications (histone methylation and acetylation; Zhang, 2008), and high accumulation of free lysine in endosperm induces multiple plant stress responses (Yang et al., 2018). Lysine metabolism is involved in the plant stress response in various ways (Yang et al., 2020a), and the results showed that lysine degradation is involved in the plant freezing response, with different lysine accumulation levels under various freezing treatments affected by DNA methylation (**Figure 8B**). Rutin is a flavonoid substance that is produced from phenylalanine as a precursor. Freezing treatment affects phenylalanine biosynthesis (**Supplementary Table S2**), with changes in methylation, but there was little difference between the two cold treatments (**Figure 8B**). To test the significance of DNA methylation for lysine and rutin enrichment, the DNA methylation inhibitor 5-aza-2'-deoxycytidine was applied during cold treatment, and a significant effect of DNA methylation on lysine content was observed. Most of the differentially expressed sequences were mapped to intergenic regions, which was likely caused by incomplete genome annotation. *De novo* sequencing should be carried out in the future to deeply analyze the genome of Tartary buckwheat varieties with excellent traits.

Plant memory involves multiple physiological, proteomic, transcriptional, and epigenetic changes, with the important role of epigenetic modification in plant memory confirmed by numerous studies (Iwasaki and Paszkowski, 2014; Kinoshita and Seki, 2014; Dean, 2017; Lamke and Baurle, 2017; He and Li, 2018; Turgut-Kara et al., 2020). In this study, the relationship between the effects of repeated environmental low-temperature stimulation (cold priming) and DNA methylation patterns, as well as changes in gene expression and metabolite enrichment after cold memory generation, was investigated, which leads to changes in some agronomic traits in crops (e.g., free lysine content). Future efforts to improve crops should utilize molecular module theory based on multiomics to improve important agronomic traits and increase tolerance to extreme ambient temperatures (Zhang et al., 2019). Here, differentially expressed genes, metabolites, and the possible roles of DNA methylation modification in cold priming in Tartary buckwheat were investigated. These findings provided comprehensive insights for the role of DNA methylation in cold priming (i.e., cold memory) and facilitated the breeding of ideal agronomic traits in Tartary buckwheat varieties.

DATA AVAILABILITY STATEMENT

The sequencing data generated in this study have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through the GEO Series accession nos. GSE138547, GSE138497 (BS-Seq), and GSE138546 (RNA-seq).

AUTHOR CONTRIBUTIONS

YS, ZJ, and LA contributed to conceive, design, and coordinate the experiments. YH, XM, LL, and XJ performed experiments. YS and ZJ analyzed the data. LA participated to the manuscript revision. YS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.608540/full#supplementary-material

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REVIEW
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Small DNA Methylation, Big Player in Plant Abiotic Stress Responses and Memory

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DNA methylation is a conserved epigenetic mark that plays important roles in maintaining genome stability and regulating gene expression. As sessile organisms, plants have evolved sophisticated regulatory systems to endure or respond to diverse adverse abiotic environmental challenges, i.e., abiotic stresses, such as extreme temperatures (cold and heat), drought and salinity. Plant stress responses are often accompanied by changes in chromatin modifications at diverse responsive loci, such as 5-methylcytosine (5mC) and N^6-methyladenine (6mA) DNA methylation. Some abiotic stress responses are memorized for several hours or days through mitotic cell divisions and quickly reset to baseline levels after normal conditions are restored, which is referred to as somatic memory. In some cases, stress-induced chromatin marks are meiotically heritable and can impart the memory of stress exposure from parent plants to at least the next stress-free offspring generation through the mechanisms of transgenerational epigenetic inheritance, which may offer the descendants the potential to be adaptive for better fitness. In this review, we briefly summarize recent achievements regarding the establishment, maintenance and reset of DNA methylation, and highlight the diverse roles of DNA methylation in plant responses to abiotic stresses. Further, we discuss the potential role of DNA methylation in abiotic stress-induced somatic memory and transgenerational inheritance. Future research directions are proposed to develop stress-tolerant engineered crops to reduce the negative effects of abiotic stresses.

Keywords: cytosine methylation, N^6-methyladenine DNA methylation, abiotic stress responses, somatic memory, transgenerational inheritance

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GLOSSARY

Epigenetics:

The study of relatively stable and inheritable changes in gene expression caused by mechanisms independent of permanent changes in the underlying DNA sequence.

5-methylcytosine (5mC) methylation:

The addition of a methyl group (CH_3) to the fifth position of the pyrimidine ring of cytosine bases of DNA.

N^6-methyladenine (6mA) DNA methylation:

The addition of a methyl group (CH_3) to the sixth position of the purine ring of adenine bases of DNA.

RNA-directed DNA methylation (RdDM):

The *de novo* cytosine methylation that involves small interfering RNAs (siRNAs)-generating pathway, long non-coding RNAs (lncRNAs) synthesized by plant-specific RNA Polymerase V (Pol V), chromatin remodeling complex, *de novo* DNA methyltransferase DOMAINS REARRANGED METHYL ASE 2 (DRM2) and a set of DNA or RNA-binding proteins.

Transgenerational epigenetic inheritance:

The transmittance of epigenetic states and associated certain phenotype from one generation to at least the next offspring generation through meiotic cell divisions. The transgenerational epigenetic inheritance may offer the descendants the potential to be adaptive for better fitness.

Somatic memory:

The memories that are mitotically but not meiotically heritable and only last for one generation of organisms.

INTRODUCTION

DNA methylation is a conserved epigenetic modification in eukaryotes and prokaryotes (Law and Jacobsen, 2010; Beaulaurier et al., 2019). In plants, DNA methylation predominantly occurs by the addition of a methyl group to the fifth position of the pyrimidine ring of cytosine bases or the sixth position of the purine ring of adenine bases, which is referred to as 5-methylcytosine [5mC] or N^6-methyladenine [6mA], respectively (Liang et al., 2018a; Zhang H. et al., 2018). The 5mC occurs frequently in all three sequence contexts in plants: the symmetric CG and CHG along with the asymmetric CHH contexts (where H = A, T or C) (Zhang et al., 2006). The DNA methylation levels in plants are different in various species. In *Arabidopsis thaliana*, whole-genome bisulfite sequencing reveals that genome-wide levels of 24% CG, 6.7% CHG and 1.7% CHH contexts are methylated, which predominantly occurs on transposons and other repetitive DNA elements (Cokus et al., 2008). In rice (*Oryza sativa*), the genome-wide DNA methylation level is much higher than *Arabidopsis* with average 44.5% CG, 24.1% CHG, and 4.7% CHH methylation in the two cultivated rice subspecies and their wild ancestors (Li et al., 2012). 5mC of promoter regions usually repress gene transcription, while methylation within the gene body quantitatively impedes transcript elongation in *Arabidopsis* (Zilberman et al., 2007). However, in some genomic regions, two SU(VAR)3-9 homologs, SUVH1, and SUVH3, serve as the methyl reader and recruit two DNAJ domain-containing homologs, DNAJ1 and DNAJ2 to increase the expression of proximal neighboring genes (Harris et al., 2018). 5mC plays important roles in defending the genome against selfish DNA elements and regulating gene expression, which are essential for normal plant growth, development and reproduction as well as appropriate biotic and abiotic stress responses (Zhang H. et al., 2018).

Compared with 5mC, the 6mA abundance in plants is rather lower, ranging from 0.006% to 0.138% in 9-day-old *Arabidopsis* wild-type Col to 0.15–0.55% in rice seedlings (Liang et al., 2018b; Zhang Q. et al., 2018; Zhou et al., 2018). In *Arabidopsis* and rice, 6mA occurs most frequently at plant-specific ANYGA as well as GAGG motifs which is conserved in plantae and animalia (Liang et al., 2018b; Zhang Q. et al., 2018; Zhou et al., 2018). 6mA sites are widely distributed across the *Arabidopsis* genome and 32% of 6mA sites are located within gene bodies, while in rice, 6mA locates at about 20% of genes and 14% of transposable elements (Liang et al., 2018b; Zhou et al., 2018). 6mA seems to be positively associated with gene expression and contributes to plant developments and stress responses (Zhang Q. et al., 2018).

During their immobile lifecycles, plants are exposed to a variety of adverse abiotic stresses, such as drought (water deficiency), salinity (salt), and temperature stresses (heat and cold). These stresses not only inhibit the growth and development of plants, but also pose great threats to crop yield and food safety. Drought and extreme heat have significantly reduced national cereal production by 9–10%, according to the records from the Emergency Events Database and Food and Agriculture Organization of the United Nations during 1964–2007 (Lesk et al., 2016). From 1980 to 2008, global warming has declined the global maize (*Zea mays*) and wheat (*Triticum aestivum*) production by 3.8 and 5.5%, respectively (Lobell et al., 2011). Like other abiotic stresses, cold stress, including chilling stress (0–15°C) and freezing stress (below 0°C), also threatens crop yield and quality, and causes tremendous agricultural yield penalty and economic losses worldwide (Ding et al., 2020). Salinity is another one of the most destructive environmental factors, which affects about 20% of irrigated land and threatens different traits of crop plants, such as the growth rate, photosynthesis, transpiration, yield and quality (Negrão et al., 2017).

To survive in the adverse circumstances, plants employ diverse genetic and epigenetic strategies for regulation of plant growth, development, reproduction and immunity in response to endogenous and exogenous stress signals. The abiotic stress signaling and responses in plant have been extensively studied and recently well summarized (Zhu, 2016; Gong et al., 2020). Plants have evolved quick and sophisticated sensory mechanisms to perceive the abiotic stress cues, convert them to cellular signals and transmit the signals within cells and tissues. So far, several abiotic stress sensors have been identified, such as putative salt sensor glycosyl inositol phosphorylceramide (GIPC) sphingolipids (Jiang et al., 2019), putative cold stress sensor chilling tolerance divergence 1 (COLD1) (Ma Y. et al., 2015), hyperosmotic stress sensor OSCA1 (Yuan et al., 2014), putative heat sensor phytochrome B (phyB) (Jung et al., 2016; Legris et al., 2016), cyclic nucleotide-gated Ca^{2+} channels (CNGCs) (Saidi et al., 2009) and histone variant H2A.Z (Kumar and Wigge, 2010). Upon the perception of abiotic stress signals, these sensors are activated by altering their structure, activity or interacting partners to initiate multilayer downstream stress responses, such as the activation of stress-responsive genes, the regulation of RNA, protein, metabolism and ROS homeostasis. Although the signaling pathways underlying plant responses to different abiotic stresses vary, there are some common theme in the key downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) cascades, G-protein signaling, calcium signaling and hormone signaling (Zhu, 2016).

Besides the significant progress in elucidating the genetic basis of plant abiotic stress responses, great achievements have been made in dissecting the complicated epigenetic regulatory mechanisms in plant adaption to the adverse environments. As one of the most important epigenetic modifications, DNA methylation plays important roles in stress responses in diverse plant species. However, the roles and mechanisms of DNA methylation in plant abiotic stress responses remain largely scattered and fragmented. In this review, we briefly summarize recent progress on the establishment, maintenance and erasing of 5mC and 6mA, and present the divergent roles of DNA methylation in plant responses to different abiotic stresses. Further, we discuss the potential role of DNA methylation in abiotic stress-induced somatic memory and transgenerational inheritance. Finally, we propose some future research directions to breed crops with enhanced stress tolerances.

DNA METHYLATION

5mC Methylation

Establishment of 5mC by the RNA-Directed DNA Methylation Pathway

In 1994, for the first time, *de novo* 5mC methylation of genes is found to be induced and targeted by their own RNAs in transgenic tobacco plants infected with viroid (Wassenegger et al., 1994). This phenomenon is described as RNA-directed DNA methylation (RdDM). In the past 26 years, extensive studies have revealed an accumulating knowledge of RdDM. *De novo* 5mC methylation in all sequence contexts is directed by small RNAs and catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) in plants (Zhang H. et al., 2018). The DRM2 activity is regulated by the canonical and non-canonical RdDM pathways, which mainly differs in the small RNAs-generating pathway (**Figure 1A**; Cuerda-Gil and Slotkin, 2016). Small RNAs are 18–30 nucleotide (nt) non-protein-coding RNAs, which mediate post-transcriptional gene silencing (PTGS) through slicing or translational inhibition, or transcriptional gene silencing (TGS) by targeting chromatin for cytosine or histone methylation. According to their biogenesis and modes of regulation, small RNAs in plants can be divided into two major types: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Borges and Martienssen, 2015). In the canonical RdDM pathway in *Arabidopsis*, the plant-specific RNA polymerase IV (Pol IV) transcribes heterochromatic regions to generate 30 to 40-nt short RNAs, which are referred to as P4 RNAs (Zhai et al., 2015). RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) then converts P4 RNAs into double-stranded RNAs (dsRNAs) and typically adds an extra untemplated 3′ terminal nucleotide to the second strands. The dsRNAs are processed by RNase III-class endonuclease DICER-LIKE 3 (DCL3) to generate 24- and 23-nt heterochromatic siRNAs (hc-siRNAs) (Singh et al., 2019). The 24-nt hc-siRNAs are exported to the cytoplasm and preferentially incorporated into ARGONAUTE 4 (AGO4) or AGO6, which are re-imported to the nucleus with the help of HEAT SHOCK PROTEIN 90 (HSP90) (Ye et al., 2012). In the nucleus, target loci (mostly transposons and repeats) are transcribed by plant-specific RNA polymerase V (Pol V) to generate non-protein-coding nascent scaffold transcripts, which base-pair with the 24-nt hc-siRNAs by sequence complementarity, resulting in the DRM2 recruitment and DNA methylation at the source loci. A variety of RNA binding proteins, methylated DNA binding proteins, chromatin-remodeling complex and key enzymes responsible for histone H3 lysine 9 dimethylation (H3K9me2) also participate in the establishment of *de novo* DNA methylation (**Figure 1B**; Zhang H. et al., 2018).

In addition to this canonical Pol IV-RDR2-DCL3-dependent RdDM pathway, several types of non-canonical RdDM pathways have been reported, including Pol II-DCL3 RdDM pathway, RDR6 RdDM pathway, RDR6-DCL3 RdDM pathway, Pol IV-NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD) RdDM pathway, and dicer-independent RdDM pathway (**Figure 1A**; Cuerda-Gil and Slotkin, 2016). Pol II transcripts of some inverted repeat (IR) sequences and miRNA precursors can also be cleaved by DCL3 to produce 24-nt small RNAs, which participate in RdDM in *cis* or *trans* (Slotkin et al., 2005; Chellappan et al., 2010; Khraiwesh et al., 2010; Wu et al., 2010). In the RDR6 RdDM pathway, Pol II transcripts of *trans*-acting siRNA (*TAS*) genes and some transcriptionally active transposable elements (TEs) are cleaved by AGO1-bound small RNA-induced silencing complex (RISC), converted into dsRNAs by RDR6, and further cleaved by DCL2/4 into 21–22-nt secondary siRNAs, which are loaded onto AGO6 to initiate RdDM (Wu et al., 2012; Nuthikattu et al., 2013; McCue et al., 2015). High copy number or elevated expression of TEs such as retrotransposon *Evadé* (*EVD*) can also induce the biosynthesis of dsRNAs by RDR6, but such dsRNAs are cleaved by DCL3 to produce 24-nt siRNAs to initiate RdDM, which is referred to as the RDR6-DCL3 RdDM pathway (Marí-Ordóñez et al., 2013). As *EVD* is originally a target of PTGS, the RDR6-DCL3 RdDM pathway may be an important mechanism to silence active TEs when PTGS is saturated (Cuerda-Gil and Slotkin, 2016). In the Pol IV-NERD RdDM pathway, the transcripts of a subset of non-conserved genomic loci are produced by Pol IV but generate 21-nt siRNAs through the sequential roles of RDR6 and DCLs in *Arabidopsis*. The 21-nt siRNAs are loaded to AGO2 and initiate RdDM dependent of NERD, a GW repeat- and PHD finger-containing protein (Pontier et al., 2012). Recently, two groups have reported the dicer-independent RdDM in *Arabidopsis*, in which the dicer-independent siRNAs are generated by distributive 3′–5′ exonucleases (Yang et al., 2015; Ye et al., 2015). In summary, these diverse non-canonical RdDM pathways feed into the canonical RdDM pathways and play subsidiary roles in RdDM pathways.

Maintenance of 5mC in Different Contexts

In plants, DNA methylation in three different contexts is maintained by three different pathways. CG, CHG and asymmetric CHH methylation are maintained by METHYLTRANSFERASE 1 (MET1), CHROMOMETHYLASE 3 (CMT3)/CMT2, DRM2/CMT2, respectively (**Figure 1C**; Zhang H. et al., 2018). In *Arabidopsis*, MET1, ortholog of mammalian DNA methyltransferase DNMT1, is required for the

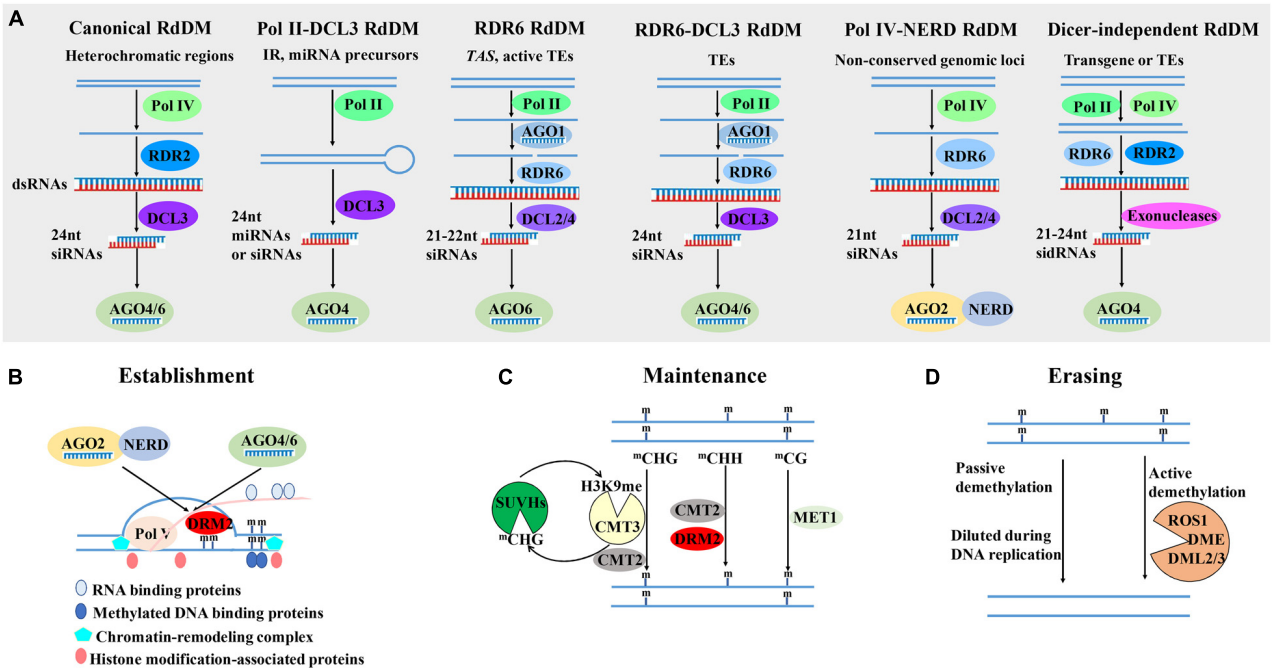

FIGURE 1 | The establishment, maintenance and erasing of 5mC in plants. **(A)** The diverse small RNAs-generating pathways involved in RNA-directed DNA methylation (RdDM). The small RNAs-generating pathways in the canonical Pol IV -RDR2-DCL3-dependent RdDM pathway, Pol II-DCL3 RdDM pathway, RDR6 RdDM pathway, RDR6-DCL3 RdDM pathway, Pol IV-NERD RdDM pathway, and dicer-independent RdDM, are briefly presented, respectively (Cuerda-Gil and Slotkin, 2016). **(B)** The establishment of 5mC in plants. 21–24-nt siRNAs or miRNAs, which are loaded onto AGO2 or AGO4/6, base-pair with Pol V-generated nascent scaffold transcript of target loci, resulting in the DRM2 recruitment and DNA methylation at the source loci with the aid of RNA binding proteins, methylated DNA binding proteins, chromatin-remodeling complex and histone modification-associated proteins (Law and Jacobsen, 2010; Zhang H. et al., 2018). **(C)** The maintenance of 5mC in different contexts. CG, CHG and asymmetric CHH methylation are maintained by MET1, CMT3/CMT2, DRM2/CMT2, respectively. It is needed to note that CMT3 and SUVH4/5/6 form a self-reinforcing feedback loop between ^{m}CHG and H3K9me (Law and Jacobsen, 2010; Du et al., 2014). **(D)** The erasing of 5mC through active and passive demethylation pathway. In the passive demethylation, 5mC is diluted during DNA replication. In the active demethylation pathway, four bifunctional 5mC DNA glycosylases-apurinic/apyrimidinic lyases, DME, ROS1 and its homologs DML2 and DML3, catalyze the active removal of 5-methylcytosine from all sequence contexts through the base excision repair pathway (Zhang and Zhu, 2012). The proteins are represented in circles. The regulatory pathways are indicated with solid arrows. Abbreviations: mC, methylated cytosine; Pol IV, Polymerase IV; RDR, RNA-DEPENDENT RNA POLYMERASE; DCL, DICER-LIKE; NERD, NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION; AGO, ARGONAUTE; MET1, METHYLTRANSFERASE 1; CMT, CHROMOMETHYLASE; DRM2, DOMAINS REARRANGED METHYLTRANSFERASE 2; SUVH4/5/6, SU(VAR)3-9 HOMOLOGUE; DME, DEMETER; ROS1, REPRESSOR OF SILENCING 1; DML, DME-LIKE 2.

maintenance of CG methylation and normal plant development (Finnegan et al., 1996). During plant mitosis and gametogenesis, MET1 recognizes the hemi-methylated templates and induces the methylation of unmodified CG dinucleotides in the daughter strand (Saze et al., 2003; Zhang H. et al., 2018). The rice genome encodes two closely related putative MET1, OsMET1-1, and OsMET1-2, but only the loss-of-function of OsMET1-2 leads to genome-wide hypomethylation and seedling lethality (Hu et al., 2014). In *Arabidopsis*, VARIANT IN METHYLATION 1-3 (VIM1-3), SRA (SET- and RING-associated) domain methylcytosine-binding proteins, play overlapping roles in the maintenance of global CG methylation in collaboration with MET1 (Woo et al., 2008; Kim et al., 2014).

In the genetic screens for reduced methylation of *Arabidopsis SUPERMAN* locus and *PHOSPHORIBOSYLANTHRANILATE ISOMERASE* (*PAI*), plant-specific methyltransferase CMT3 is found to be indispensable for the maintenance of CHG methylation (Bartee et al., 2001; Lindroth et al., 2001). CMT3-mediated CHG methylation depends on H3K9 histone methyltransferase KRYPTONITE/SUVH4 (KYP) (Jackson et al., 2002). CMT3 and KYP form a self-reinforcing feedback loop between ^{m}CHG and H3K9me. In the loop, CMT3 is recruited by H3K9me and methylate CHG DNA to create binding sites for KYP and its close homologs SU(VAR)3-9 HOMOLOGUE 5 (SUVH5) and SUVH6; in turn, KYP can methylate H3K9 to generate the binding sites for CMT3 (**Figure 1C**; Law and Jacobsen, 2010; Du et al., 2014). *Zea* methyltransferase2 (ZMET2) in maize, ortholog of AtCMT3, is also required for ^{m}CHG (Papa et al., 2001). Crystal structure analysis of ZMET2 and H3K9me2 have revealed that ZMET2 binds H3K9me2 via bromo adjacent homology (BAH) and chromo domains (Du et al., 2012).

CHH methylation is mainly maintained by the DRM2-mediated *de novo* methylation and RdDM pathway (**Figure 1C**). Besides DRM2, CMT2 mediates CHH methylation at some long TEs through binding to H3K9 methylation (Stroud et al., 2014). Moreover, CMT2 also mediates CHG methylation. Therefore, CMT2, CMT3 and DRM2 collaborate to maintain non-CG methylation, and form self-reinforcing feedback loops

with H3K9 methylation (Stroud et al., 2014). DECREASE IN DNA METHYLATION 1 (DDM1), a SWI2/SNF2-like chromatin remodeling enzyme, can facilitate CMT2 to access H1-containing heterochromatin to maintain RdDM-independent CHH methylation (Zemach et al., 2013). In maize, ZmDDM1 regulates the formation of ^{m}CHH islands through the RdDM pathway (Long et al., 2019). However, in rice, OsDDM1 antagonizes RdDM at heterochromatin and represses non-coding RNA expression from repetitive sequences (Tan et al., 2018), suggesting the distinct roles of DDM1 in different species.

Erasing of 5mC Through Active and Passive Demethylation Pathway

In plant growth, development, reproduction and stress responses, 5mC is dynamically regulated by DNA methyltransferases and demethylation pathways. There are two demethylation pathways in plants: passive and active demethylation pathways (**Figure 1D**). The passive demethylation is a process in which 5mC is diluted from the genome during DNA replication, usually due to the down-regulation of DNA methyltransferase activity or shortage of the methyl donor folate (Zhang H. et al., 2018). In *Arabidopsis* gametogenesis, loss of MET1 in the diploid central cell and the haploid egg cell as well as the loss of DDM1 and Pol IV in the vegetative cell decrease 5mC and strongly reactivate transposons, resulting in the production of siRNAs that may travel to sperm cells or egg cells to reinforce TE silencing (Bourc'his and Voinnet, 2010; Feng et al., 2010).

In the active demethylation pathway, four bifunctional 5mC DNA glycosylases-apurinic/apyrimidinic lyases, DEMETER (DME), REPRESSOR OF SILENCING 1 (ROS1) and its homologs DME-like 2 (DML2) and DML3, have been implicated in the active removal of 5-methylcytosine from all sequence contexts through the base excision repair (BER) pathway (Zhang and Zhu, 2012; Liu and Lang, 2020). ROS1 is the first identified DNA glycosylase/lyase involved in DNA demethylation (Gong et al., 2002). The recruitment of ROS1 to its target genomic regions is mediated by INCREASED DNA METHYLATION (IDM) complex (Zhang H. et al., 2018). Interestingly, the expression of ROS1 is promoted by DNA methylation and a sequence in its promoter functions as a DNA methylation monitoring sequence (MEMS) that senses DNA methylation levels and regulates ROS1 expression to fine-tune genomic DNA methylation (Lei et al., 2015; Williams et al., 2015). DME is preferentially expressed in companion cells of the female and male gametes and initiates active DNA demethylation, which is required for endosperm genomic imprinting and embryo viability (Park et al., 2017).

N^6-Methyladenine DNA Methylation (6mA)

As a new epigenetic marker in eukaryotes, the establishment, maintenance and erasing of 6mA remain largely obscure. In mammalian, N^6-mA is catalyzed by methyltransferase N6MT1 and removed by 2-oxoglutarate-dependent oxygenase AlkB homolog 1 (ALKBH1) (Xiao et al., 2018; Zhang M. et al., 2020). In rice, OsALKBH1 is proposed to function as 6mA demethylase, as the loss function of OsALKBH1 results in increased 6mA levels (Zhou et al., 2018). 6mA levels are significantly decreased in *Osddm1a*/*ddm1b* double mutants, suggesting that OsDDM1a and OsDDM1b are indispensable for 6mA modification in rice (Zhang Q. et al., 2018). Recent studies have revealed that 6mA DNA modification is positively correlated with gene activation and plays important roles in plant development, and stress responses (Liang et al., 2018b; Zhang Q. et al., 2018; Zhou et al., 2018). For better understanding of the roles of 6mA in plants, it is urgent to identify the key writer and reader of 6mA in plants.

THE DIVERGENT ROLES OF DNA METHYLATION IN PLANT ABIOTIC STRESS RESPONSES

In recent years, multiple technologies have been developed for detecting methylation levels of genome-wide DNA or specific sequence contexts, such as Chop-PCR, methylation sensitive amplification polymorphism (MSAP) technique, methylated DNA immunoprecipitation sequencing (MeDIP-Seq) or 6mA-IP-Seq, and whole genome bisulfite sequencing (WGBS). Using partial digestion by methylation-sensitive restriction enzymes followed by PCR amplification, Chop-PCR can detect the cytosine methylation at the cleavage sites that protects DNA against digestion and therefore can be amplified using PCR (Dasgupta and Chaudhuri, 2019). MSAP is widely applied for analysis of differentially methylated CCGG sites in different plant species with the use of isoschizomers with different methylation sensitivity (such as HpaII and MspI) (Guevara et al., 2017). For MeDIP-Seq and 6mA-IP-Seq, specific antibodies are used to isolate methylated DNA from genomic DNA via immunoprecipitation. WGBS is a sensitive and robust method for genome-wide analysis of 5mC at single-base resolution in plants. These techniques greatly promote the research on the roles of DNA methylation under abiotic stress conditions. The detailed roles of 5mC and 6mA in plant heat, cold, salt and drought stress responses are reviewed as follows.

Heat Stress

Most plants can only tolerate a certain range of temperature fluctuations. The elevation in temperature, which is 10–15°C beyond the ambient favorable threshold, is referred to as heat stress. There are two-tiered plant tolerance to heat stress: basal and acquired thermotolerance. The basal thermotolerance is an inherent ability for plants to respond and successfully acclimate to heat stress, while acquired thermotolerance means the ability of plants to survive in lethal heat stress after acclimatization to mild heat stress (also known as priming) (Mittler et al., 2012). The thermotolerance in plants are regulated by multiple epigenetic modifications, including DNA methylation (Liu et al., 2015).

Heat stress triggers 5mC demethylation globally or at some loci in some plant species. In cotton (*Gossypium hirsutum*) anthers, heat stress (35 to 39°C/29 to 31°C day/night) disrupts the global DNA methylation, especially CHH methylation, in a heat-sensitive line, whereas a heat-tolerant line shows higher methylation level (Min et al., 2014; Ma et al., 2018). The heat-induced down-regulation of *S-ADENOSYL-L-HOMOCYSTEINE*

HYDROLASE1 (*SAHH1*) and DNA methyltransferases *DRM1/3* may contribute to the genome-wide hypomethylation under heat stress (Min et al., 2014). The reduction of DNA methylation may result in the disruption of sugar and reactive oxygen species (ROS) metabolic pathways, leading to microspore sterility (Ma et al., 2018). In soybean (*Glycine max* L.), heat stress (40°C for 3 h) also induces the hypomethylation in all three contexts, especially the ^{m}CHH, in both root hairs and stripped roots (Hossain et al., 2017). In cultured microspores of *Brassica napus* cv. Topas, heat shock treatment (32°C for 6 h) triggers DNA hypomethylation, particularly in CG and CHG contexts (Li et al., 2016). Another research reveals that after heat stress (37°C for 2 h, and then 45°C for 3 h), more DNA demethylation events occur in the heat-tolerant genotype, while more DNA methylation events occur in the heat-sensitive genotype in *Brassica napus* (Gao et al., 2014). In rice, *OsCMT3* is repressed by heat stress, which may partly lead to the upregulation of *FERTILIZATION-INDEPENDENT ENDOSPERM 1* (*OsFIE1*), a member of Polycomb Repressive Complex 2 (PRC2). The elevated expression of *OsFIE1* may regulate seed size under heat stress (**Figure 2A**; Folsom et al., 2014). The effect of heat-induced repression of *OsCMT3* on the global 5mC remains to be investigated in rice.

In the model dicot *Arabidopsis thaliana*, the effect of heat stress on key players in 5mC, such as DNA methyltransferases, DNA demethylases, RdDM components, are distinct. These players play diverse roles in thermotolerance through modulating 5mC or other regulatory processes. Heat stress (36°C for 48 h) induced up-regulation of *DRM2*, *NUCLEAR RNA POLYMERASE D 1A* (*NRPD1A*) and *NRPD1B*, the largest subunits of PoI IV and Pol V, respectively. *nrpd1a-1 nrpd1b-1* double mutation abolished DNA methylation in the promoter of *Calmodulin-like 41* (*CML41*) and *At5g43260*, and suppressed their heat-induced increased expression, suggesting the important roles of PoI IV and Pol V in regulating gene expression under heat stress (**Figure 2B**; Naydenov et al., 2015). *Arabidopsis* plants deficient in *NRPD2*, the common second-largest subunit of PoI IV and Pol V, are hypersensitive to acute heat stress (42°C for 24–34 h). Loss-of-function of RdDM components, RDR2, DCL3 and AGO4 also dramatically decrease the basal thermotolerance (**Figure 2C**; Popova et al., 2013). In *nrpd2* mutants recovered from heat stress, the misexpression of protein-coding genes, such as auxin-responsive genes, may be affected by their adjacent transposon remnants, which are induced by heat stress (Popova et al., 2013). However, *cmt2* mutant plants and accessions with $CMT2_{STOP}$ allele display increased tolerance to heat stress (37.5°C for 24 h), natural variation in *CMT2* and associated changes in genome-wide CHH-methylation pattern contribute to the natural adaptation to variable temperatures (**Figure 2D**; Shen et al., 2014). Some new identified players involved in DNA methylation also play roles in plant heat responses. Depletion of *MutS HOMOLOGUE 1* (*MSH1*) in *Arabidopsis* results in genome-wide reprogramming of DNA methylation (Virdi et al., 2015). Intriguingly, crossing or grafting of the *msh1* mutant to wild type or hemi-complementation of mitochondrial function in the *msh1* mutant can lead to an enhancement of growth vigor and heat tolerance, which may be associated with changes in DNA methylation (**Figure 2E**; Virdi et al., 2015, 2016). The detailed roles of MSH1 in thermotolerance remain to be investigated. Above all, despite the divergent effects of different key players in 5mC on heat responses, it is no doubt that DNA methylation is important for thermotolerance in *Arabidopsis*.

Heat stress can release the TGS and PTGS of various transgenes and some endogenous loci in *Arabidopsis*, such as exogenous β-*glucuronidase* (*GUS*), 35S promoter of *Cauliflower Mosaic Virus*, endogenous imprinted gene *SDC* and several repetitive elements (transposons and retrotransposons) (Lang-Mladek et al., 2010; Pecinka et al., 2010; Tittel-Elmer et al., 2010; Zhong et al., 2013; Cavrak et al., 2014; Sanchez and Paszkowski, 2014). However, heat-induced activation of these loci occurs without loss of 5mC or seems not to be associated with changes of local 5mC. A *COPIA*-type retrotransposon *ONSEN* can be activated by heat stress and its retrotransposition confers heat-responsiveness to genes close to the new insertion site. In plants deficient in siRNA-biogenesis in RdDM, the heat-induced retrotransposition of *ONSEN* can be transmitted to the unstressed progeny. However, the heat-induced reduction of CHH methylation in *ONSEN* promoter cannot account for the activation of *ONSEN* under heat stress (Ito et al., 2011; Cavrak et al., 2014).

The 6mA levels are positively correlated with heat tolerance in rice (Zhang Q. et al., 2018). Heat stress up-regulates total 6mA levels in both *Japonica* group cultivar Nipponbare (Nip) and *Indica* group cultivar 93-11, and the fold change of 6mA level in 93-11 is 2.6-fold greater than that in Nip. In the signal transduction of heat stress, heat shock transcription factors (HSFs) and heat shock proteins (HSPs) are central players (Mittler et al., 2012). The heat-induced up-regulation of *OsHSFA1* and down-regulation of *OsHSP70* positively correlated with changes in their 6mA levels in 93-11, which may contribute to the more tolerance to heat stress of 93-11 compared with Nip (**Figure 2F**; Zhang Q. et al., 2018). Whether heat-induced up-regulation of 6mA is conserved in diverse species remains to be elucidated.

In summary, DNA methylation play some noticeable roles in plant heat stress responses. However, the exact roles of DNA methylation in the sensing and signal transduction of heat stress remain unclear in plants. Further studies should pay more attention to the possible roles of DNA methylation in the perception and signaling of heat stress in plants.

Cold Stress

In plants, the cold signal can be perceived by putative cold sensors, such as the G-protein regulator COLD1 and CBL-INTERACTING PROTEIN KINASE 7 (OsCIPK7) (Ma Y. et al., 2015; Zhang et al., 2019). The PM and ER-localized COLD1 interacts with the RICE G-PROTEIN α SUBUNIT 1 (RGA1) to activate the Ca^{2+} channel and accelerate the influx of extracellular Ca^{2+}, which confers chilling tolerance in rice (Ma Y. et al., 2015). OsCIPK7 with a point mutation at the activation loop of the kinase domain exhibits enhanced kinase activity and confers chilling tolerance through Ca^{2+} influx in rice (Zhang et al., 2019). The cold-induced cytosolic Ca^{2+} signal can initiate downstream signaling pathways, such as calcium

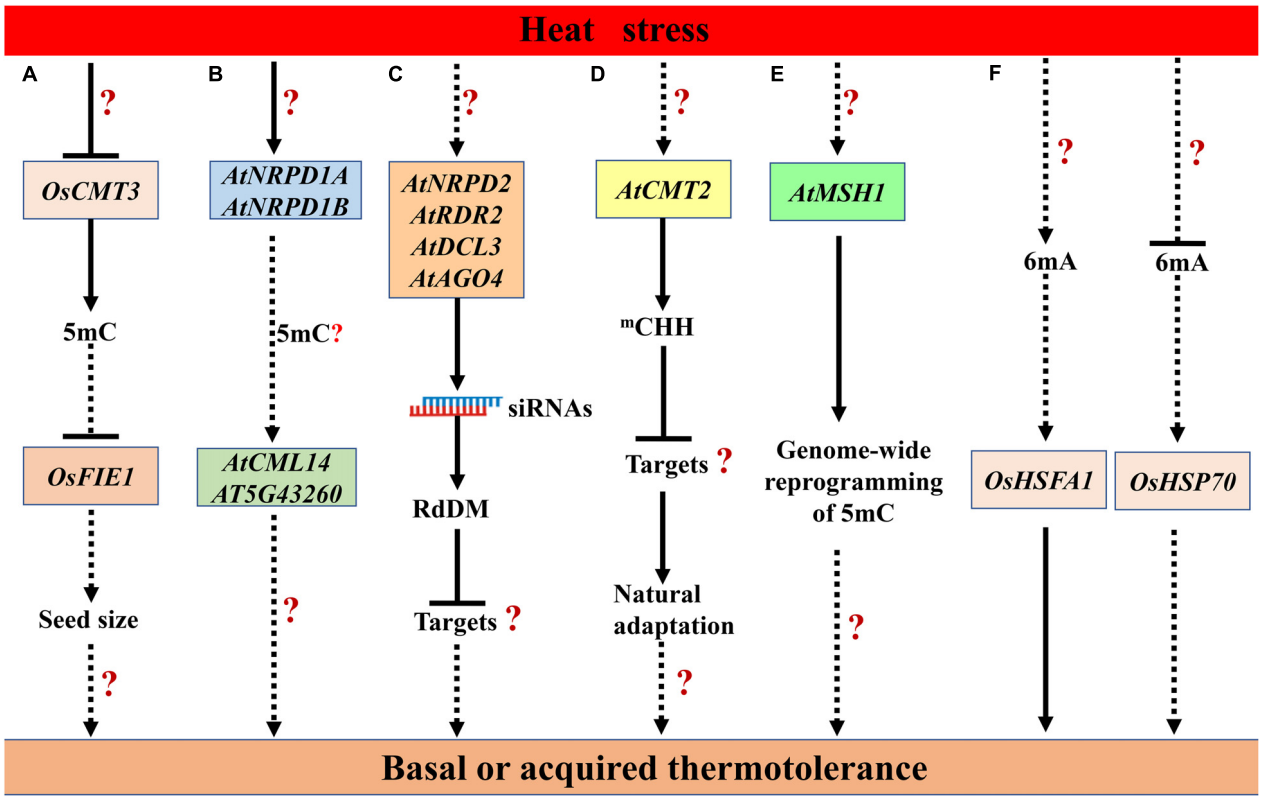

FIGURE 2 | The proposed modulation of heat stress responses by DNA methylation in plants. **(A)** The heat-mediated repression of *OsCHROMOMETHYLASE 3* (*OsCMT3*) partly leads to the up-regulation of *FERTILIZATION-INDEPENDENT ENDOSPERM 1* (*OsFIE1*), which regulates seed size under heat stress in rice (Folsom et al., 2014). **(B)** Heat stress up-regulates *NUCLEAR RNA POLYMERASE D 1A* (*NRPD1A*) and *NRPD1B*, which contributes to heat-induced increase in the expression of *Calmodulin-like 41* (*CML41*) and *At5g43260* (Naydenov et al., 2015). **(C)** The NRPD2, RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), DICER-LIKE 3 (DCL3), and ARGONAUTE 4 (AGO4)-dependent RdDM pathway is required for basal thermotolerance in *Arabidopsis* (Popova et al., 2013). **(D)** The natural variation in *AtCMT2* confers changes in genome-wide CHH-methylation pattern, which contributes to the natural adaptation to variable temperatures (Shen et al., 2014). **(E)** *MutS HOMOLOGUE 1* (*MSH1*)-mediated genome-wide reprogramming may be required for thermotolerance (Virdi et al., 2016). **(F)** Heat stress modulates 6mA levels in rice, and the changes in 6mA levels may contribute to the difference in heat tolerance between Nip and 93-11 through modulating *HEAT SHOCK TRANSCRIPTION FACTOR A 1* (*OsHSFA1*) and *HEAT SHOCK PROTEIN 70* (*OsHSP70*) (Zhang Q. et al., 2018). Genes are shown in boxes. The speculative regulatory paths are shown with broken arrows. Many unknown targets or steps (?) remain to be uncovered in this model.

signaling and MAPK cascade, which regulate the expression of key transcription factors. INDUCER OF CBF EXPRESSION 1 (ICE1), one of the central regulators in plant cold response, activates the C-repeat binding factors/Dehydration-responsive element-binding proteins (CBFs/DREBs), which then binds to the promoter of cold-responsive (COR) genes and actives their expression (Zhu, 2016; Gong et al., 2020). The ICE1-CBF-COR pathway plays a vital role in plant cold stress responses and the pathway is fine-tuned by multiple transcriptional and post-translational processes (Ding et al., 2020).

DNA demethylation has been reported to play important roles in cold stress tolerance in *Arabidopsis*, chestnut (*Castanea sativa Mill.*), poplar (*Populus tremula*), and Cucumber (*Cucumis sativus* L.) (Conde et al., 2017a,b; Lai et al., 2017; Xie et al., 2019). After treated with the DNA methylation inhibitory reagent 5-azacytidine, 30.0–78.3% increases in freezing tolerance are observed in four *Arabidopsis* populations. Similar enhancement of freezing tolerance also occurs in *drm2* mutants (Xie et al., 2019). Cold temperatures induce *CsDML* in chestnut and *PtaDML* in poplar (Conde et al., 2017a,b). In transgenic hybrid poplars overexpressing *CsDML*, apical bud formation is accelerated, alongside with the up-regulation of flavonoid biosynthesis enzymes and accumulation of flavonoids in the SAM and bud scales. The cold stress-mediated up-regulation of *CsDML* may accelerate the bud formation which is required for the survival of the apical meristem under winter (Conde et al., 2017b). In poplar, *PtaDML8/10* knock-down mutants displayed delayed bud break and the targets of *PtaDML*-dependent DNA demethylation are involved in bud break, suggesting the essential roles of chilling-responsive *PtaDML*s in the transition from winter dormancy to shoot growth in woody perennials (Conde et al., 2017a). In Cucumber, cold stress imposes a substantial and global impact on TE-related RdDM, leading to the demethylation of ^{m}CHH. Besides, cold-induced differentially-methylated regions (DMRs) may be involved in the regulation of genes in ethylene biosynthesis and signaling, which contribute to the temperature-dependent sex determination in cucumber (Lai et al., 2017). However, the loss-of-function of

MSH1 and *RNA-DIRECTED DNA METHYLATION 4* (*RDM4*), an essential player in RdDM pathway, reduce the cold tolerance in *Arabidopsis*. Cold stress poses greater influences on non-CG methylation in *msh1* mutants than in wild-type (Kenchanmane Raju et al., 2018). Surprisingly, RDM4 modulates the cold response by regulating the Pol II occupancy at the promoters of *CBF2/3*, which is independent of RdDM pathway (Chan et al., 2016). Further forward and reverse genetic approaches as well as genome-wide profiling are needed to uncover the roles of DNA methylation-related genes in plant cold stress responses.

Prolonged cold in winter induces the epigenetic silencing of floral repressors, thus ensuring plants overwinter before flowering in spring, a process known as vernalization. Early in 1993, it has been reported that cold-treated *Arabidopsis* plants and *Nicotiana plumbaginifolia* cell line have reduced 5mC in their DNA compared to non-vernalized controls (Burn et al., 1993). However, the cold-induced repression of *FLOWERING LOCUS C* (*FLC*), one of the major determinants of flowering time, is associated with changes of histone methylation but not DNA methylation within the *FLC* locus (Jean Finnegan et al., 2005). In the biennial plant sugar beet (*Beta vulgaris altissima*), the *BvFLC* locus undergoes different regulations of DNA methylation between genotypes that are resistant or sensitive to vernalization-induced bolting, while 5mC at specific cytosines of *VERNALIZATION INSENSITIVE 3* (*BvVIN3*) is correlated with bolting variables (Trap-Gentil et al., 2011). Interestingly, in *Brassica rapa*, vernalization mediates DNA demethylation and increased expression of *CASEIN KINASE II A-SUBUNIT* (*BrCKA2*) and *B-SUBUNIT* (*BrCKB4*), two subunits of the protein kinase CK2. In *BrMET1*-silenced *B.rapa* or plants treated with 5-azacytidine, DNA methylation levels in the promoter of *BrCKA2* and *BrCKB4* are reduced and the expression levels of these two genes increase, suggesting that increased expression of *BrCKA2* and *BrCKB4* could be induced through DNA demethylation. Increased expression of *BrCKA2* and *BrCKB4* confers elevated CK2 activity and results in a shortened period of the clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*BrCCA1*), which is an important player in perceiving photoperiod (**Figure 3A**; Duan et al., 2017). However, vernalization-induced demethylation is not a conserved mechanism among species. In hexaploid winter wheat, *VERNALIZATION-A1* (*VRN-A1*) gene, a floral activator in the vernalization pathway, is methylated at CG sites in gene-body region and at non-CG sites in intron 1, which contains fragments of TEs. Vernalization increases the non-CG methylation in intron 1, which can be maintained through mitosis but reset to the pretreated level after sexual reproduction (Khan et al., 2013). Whether such hypermethylation contribute to the vernalization-induced expression of *VRN-A1* remains to be dissected.

The ICE1-CBF-COR pathway is regulated by 5mC DNA methylation, which is associated with cold responses in different species. In crofton weed (*Ageratina adenophora*), the DNA methylation levels in *ICE1* coding region is negatively correlated with the cold tolerance levels among different populations (Xie et al., 2015). *Os03g0610900* is a homologous gene of protein kinase *OPEN STOMATA 1* (*OST1*), which phosphorylates and stabilizes ICE1 under cold stress. Cold stress up-regulates the expression of *Os03g0610900*, thereby enhancing ICE1-mediated cold resistance. The relationship between cold-induced 5mC demethylation in the promoter of *Os03g0610900* and its increased expression needs further investigations (**Figure 3B**; Guo et al., 2019). In *Hevea brasiliensis*, cold stress elevates the transcriptional activities of *HbICE1* as well as *HbCBF2*, which may be associated with the DNA demethylation in their promoters (**Figure 3C**; Tang et al., 2018). In *Arabidopsis,* the variation in *ICE1* 5mC methylation likely determines the phenotypic variation in freezing tolerance (Xie et al., 2019). Intriguingly, a recent study reports that a transgene locus harboring a reporter gene in *ice1-1* genome but not the loss-of-function of *ICE1* is responsible for the repression of *DREB1A* expression. The transgene induces hypermethylation in the *DREB1A* promoter through RdDM pathway, which inhibit the transcription of *DREB1A* (Kidokoro et al., 2020). Thus, the ICE1-DREB1A regulatory module in *Arabidopsis* should be validated with other evidences.

Emerging reports have demonstrated that cold stress affects the DNA methylation levels of certain loci in the genome. Under cold stress, the 5mC level in the promoter of *ALLANTOINASE* (*ALN*), a negative regulator of dormancy, is stimulated in a tissue-specific manner through non-canonical RDR6 and AGO6-dependent RdDM pathway, which represses *ALN* expression and further promotes seed dormancy (**Figure 3D**; Iwasaki et al., 2019). In *Brassica rapa*, cold acclimation decreases the DNA methylation levels in the promoter region of *MITOCHONDRIAL MALATE DEHYDROGENASE* (*BramMDH1*) and up-regulates the expression of *BramMDH1*, which enhances organic acids and photosynthesis to increase heat-tolerance and growth rate in *Arabidopsis* (Liu et al., 2017). In rose (*Rosa hybrida*), cold stress induces CHH methylation of the promoter of *RhAG*, an *AGAMOUS* homolog, which may result in the attenuated expression of *RhAG*. The enhanced suppression of *RhAG* particularly contributes to the cold-mediated increase of petal number (**Figure 3E**; Ma N. et al., 2015). Interestingly, cold stress can induce stable methylation changes of a non-coding RNA gene and regulate some cold-responsive gene expression in *Populus simonii* (Song et al., 2016).

Unlike heat stress, the 6mA level is significantly decreased in response to cold stress in rice. Following cold stress, the fold change in the 6mA level in Nip is fourfold greater than in 93-11, which may partly explain the higher tolerance of Nip to cold stress than 93-11 (**Figure 3F**; Zhang Q. et al., 2018). Overall, the roles of 5mC and 6mA in plant responses to cold stress, especially freezing stress, remain largely obscure. DNA methylation play divergent roles in different species under cold stress. High-resolution bisulphite sequencing and in-depth functional analysis are required to improve our understanding on the roles of DNA methylation in plant cold stress responses.

Salt Stress

Similar as heat stress, salt stress also up-regulates 6mA levels in Nip and 93-11. Under salt stress, the 6mA level fold change in 93-11 is 2.5-fold greater than in Nip (**Figure 4A**) (Zhang Q. et al., 2018). The roles of 6mA in salt stress remain to

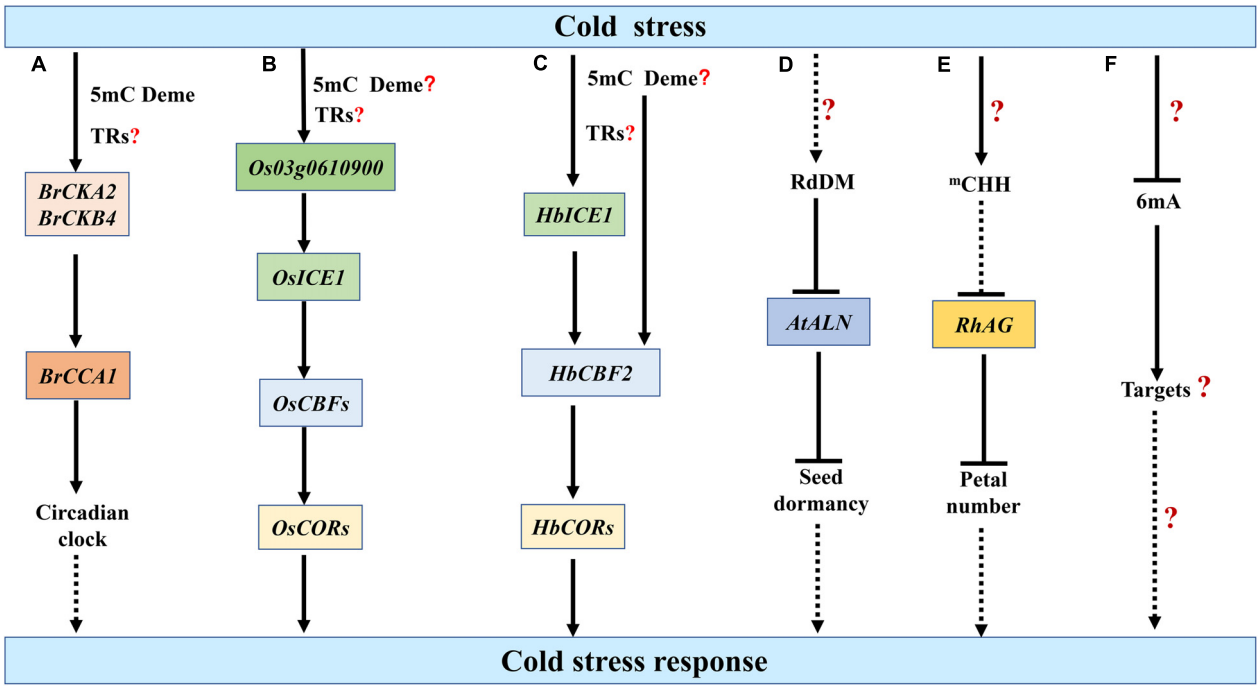

FIGURE 3 | Epigenetic regulation of plant cold stress responses by DNA methylation. **(A)** In *Brassica rapa*, prolonged cold stress mediates the demethylation and increased expression of *CASEIN KINASE II A-SUBUNIT* (*BrCKA2*) and *B-SUBUNIT* (*BrCKB4*), which may regulate cold stress response through the clock gene *CIRCADIAN CLOCK ASSOCIATED 1* (*BrCCA1*) (Duan et al., 2017). **(B)** Cold stress decreases the 5mC in the promoter of *Os03g0610900* and up-regulates its expression, thereby enhancing INDUCER OF CBF EXPRESSION 1 (ICE1)-mediated cold resistance (Guo et al., 2019). **(C)** In *Hevea brasiliensis*, cold stress elevates the transcriptional activities of *HbICE1* as well as *C-repeat binding factor 2* (*HbCBF2*), which may be associated with the DNA demethylation in their promoters (Tang et al., 2018). Whether cold-induced 5mC demethylation results in the up-regulation of *Os03g0610900*, *HbICE1* and *HbCBF2* under cold stress remains to be elucidated **(B,C)**. How cold-induced 5mC demethylation integrated with the activity of transcriptional regulators (TRs) to activate downstream gene expression also needs to be investigated **(A–C)**. **(D,E)** Cold stress up-regulates the 5mC levels of *ALLANTOINASE* (*ALN*) in *Arabidopsis* and *AGAMOUS* homolog *RhAG* in rose (*Rosa hybrida*). The resulting repressed expression of *AtALN* and *RhAG* may be associated with seed dormancy and petal number under cold stress, respectively (Ma N. et al., 2015; Iwasaki et al., 2019). **(F)** Cold stress reduces the 6mA levels in rice, but the roles of 6mA in plant responses to cold stress remain obscure (Zhang Q. et al., 2018). 5mC Deme represents the demethylation of 5mC. The speculative regulatory paths are shown with broken arrows. Many unknown targets or steps (?) remain to be investigated.

be studied. Compared with the limited research on 6mA, our knowledge on 5mC in plant salt stress responses has been accumulating. Salt stress induces diverse effects on 5mC in different species. For example, in wheat, salinity stress reduces the 5mC levels in a salinity-tolerant wheat cultivar SR3 and its progenitor parent JN177, which is less tolerant to salt stress. Among the differentially methylated salinity-responsive genes, *TaFLS1*, a flavonol synthase gene, and *TaWRSI5*, a Bowman-Birk-type protease inhibitor, can enhance the salinity tolerance of *Arabidopsis thaliana* (Wang et al., 2014). In soybean root, bisulfite sequencing reveals that 61.2% of CGs, 39.7% of CHG, and 3.2% of CHHs are methylated under durable salt stress, which was slightly lower than those under control condition (Chen et al., 2019). In Rapeseed (*Brassica napus* var. *oleifera*), salinity stress decreases the level of 5mC in the salinity-tolerant cultivar Exagone but increases the methylation levels in the salinity-sensitive cultivar Toccata (Marconi et al., 2013). However, in olive (*Olea europaea*), salt stress induces differentially methylation changes in the 5mC levels of CCGG sites in the tolerant cultivar Royal, which may contribute to plant response to salt stress by slowing down the growth (Mousavi et al., 2019).

The important roles of the key players of 5mC in salt stress tolerance have been limitedly reported. Plants carrying mutation of *RDM16*, which encodes a pre-mRNA-splicing factor 3 and functions in RdDM pathway, are hypersensitive to salt stress in *Arabidopsis* (Huang et al., 2013). *ddm1* and *met1* mutant plants also show high sensitivity to salt stress (Baek et al., 2011; Yao et al., 2012). In *Physcomitrella patens*, *PpDNMT2* accumulates in a temporal manner upon salt stress and *PpDNMT2* knockout plants are unable to recover from salt stress (Arya et al., 2016). Salt stress increases the expression of DNA demethylases in salt-tolerant rice variety Pokkali, which may be linked to the salt-induced demethylation, while in the salt-sensitive variety IR29, the induction of both DNA methyltransferases and demethylases may account for the lower plasticity of DNA methylation. However, the *osdrm2* mutant plants display slight changes of root length and biomass under salt stress as compared to wild-type (Ferreira et al., 2015). Transgenic tobacco overexpressing *AtROS1* displays enhanced tolerance to salt stress, which may be associated with enhanced expression of genes encoding enzymes of the flavonoid biosynthetic and antioxidant pathways (Bharti et al., 2015).

Numerous researches have deciphered the effect of salt stress on the DNA methylation levels of certain loci in the genome. Salt-induced demethylation events at some salt-responsive genes can enhance salt tolerance in different species. In soybean, salt stress markedly reduces the 5mC levels at the promoter of *MYB DOMAIN PROTEIN 84* (*GmMYB84*), which may be associated with its higher expression. GmMYB84 binds to the *cis*-regulatory sequences of K^+ *TRANSPORTER 1* (*GmAKT1*), thereby conferring salinity stress tolerance (**Figure 4B**; Zhang W. et al., 2020). Similarly, salt stress leads to rapid removal of 5mC from the promoter of *OsMYB91*, which may contribute to the salt-induced expression of *OsMYB91*. Plants over-expressing *OsMYB91* show enhanced tolerance with significant increases of proline levels and enhanced capacity to scavenge active oxygen (**Figure 4C**; Zhu et al., 2015). Besides, salinity stress-induced methylation events of some genome loci are also involved in salt tolerance. In the shoot and root of wheat cultivar Kharchia-65, salinity stress induces a genotype- and tissue-specific increase in 5mC levels of *HIGH-AFFINITY POTASSIUM TRANSPORTER 2;1/3* (*TaHKT2;1* and *TaHKT2;3*) that may down-regulate their expression, thereby improving the salt tolerance (**Figure 4D**; Kumar et al., 2017). Interestingly, at the 2.6 kb upstream of the ATG start codon of *AtHKT1*, a putative small RNA target region is heavily methylated, which inhibits the transcription of *AtHKT1*. The deletion of this region or the loss of 5mC in this region in *met1-3* mutants result in an altered expression pattern of *AtHKT1* and the hypersensitivity to salt stress in plants, suggesting that this putative small RNA target region is essential for maintaining *AtHKT1* expression patterns crucial for salt tolerance (Baek et al., 2011). Under salt stress, the methylation level of osa-miR393a promoter is higher in salt-tolerant genotype FL478 than that of salt-sensitive IR29, which may lead to a lower expression of osa-miR393a in FL478. As salt-responsive osa-miR393a is a negative regulator of salinity stress tolerance in rice, its down-regulation may increase the salt tolerance through up-regulation of the target *TRANSPORT INHIBITOR RESPONSE 1* (*OsTIR1*) (**Figure 4E**; Ganie et al., 2016). Maize *PROTEIN PHOSPHATASE 2C* (*ZmPP2C*), a negative regulator of ABA signaling, may be repressed by salinity-induced methylation in root, while a positive effector maize *GLUTATHIONE S-TRANSFERASES* (*ZmGST*), may be up-regulated by salinity-induced demethylation in leaf. The salt-induced alteration of 5mC at *ZmPP2C* and *ZmGST* may be involved in maize acclimation to salinity (Tan, 2010). Although salt induces expression changes of some methylated genes or TEs, the roles of salinity-induced methylation or demethylation changes in stress responses remain to be elucidated. For example, in rice, salt, heat and drought stresses can induce the expression of a long terminal repeat (LTR) retrotransposon, *HUO*, which is subjected to RdDM-mediated gene silencing. Multiple *HUO* copies may trigger genomic instability by changing global DNA methylation and small RNA biogenesis, which may result in decreased disease resistance and yield penalty (Peng et al., 2019). *SpPKE1*, a tomato proline-, lysine-, and glutamic-rich type gene isolated from abiotic-resistant species (*Solanum pennellii* LA0716), confers salt tolerance in tomato and tobacco. The detailed roles of heavy methylation in the promoter of *SpPKE1* in plant salt responses remain unclear (Li et al., 2019).

Drought Stress

The drought-induced up-regulation of 5mC methyltransferases and demethylases has been reported in apple (*Malus* × *domestica* Borkh.), tomato, chickpea (*Cicer arietinum*), barley (*Hordeum vulgare* L.), and eggplant (*Solanum melongena* L.). In chickpea roots, all methyltransferases are up-regulated by drought stress (Garg et al., 2014). Drought stress increases the expression of all cytosine-5-methyltransferases and DNA demethylases except *SmelCMT3a/3b* in leaf tissues of eggplant (Moglia et al., 2019). Similarly, potential DNA methyltransferases and demethylases are induced by drought stress in apple, and *MdCMT2* shows highest induced expression (Xu et al., 2018). *SlDRM6-8*, *SlCMT3* and *SlDNMT2* are significantly induced by dessication in tomato (Kumar et al., 2016). In a drought-tolerant barley cultivar, *HvDME* is also induced by drought stress (Kapazoglou et al., 2013). Further genome-wide analysis of DNA methylation in these species are needed to uncover the roles of drought-induced expressions changes of methyltransferases and demethylases in plant drought responses.

The differential regulation of 5mC methyltransferases and demethylases by drought stress leads to various global methylation changes in diverse species. In *Arabidopsis*, the drought stress-induced hypermethylation partly depends on histone variant H1.3, which can be up-regulated by water deficiency (Rutowicz et al., 2015). Although drought induces changes in DNA methylome in *Arabidopsis*, the methylation changes are unrelated to known transcriptome changes associated with drought stress (Ganguly et al., 2017; Van Dooren et al., 2020). Single-base resolution methylomes analysis in upland cotton by WGBS reveals that drought stress induces hypermethylation in all three sequence contexts, which are almost restored to pre-treatment levels after re-watering (Lu et al., 2017). In *Populus trichocarpa*, drought treatment significantly increases 5mC levels in upstream 2 kb, downstream 2 kb and repetitive sequences (Liang et al., 2014). However, water deficit significantly reduces global 5mC in the model grass *Brachypodium distachyon*, while plants colonized by *Bacillus subtilis* B26 exhibit an overall increase in global DNA methylation under chronic drought, which may attribute to the B26-induced up-regulation of *MET1B-like*, *CMT3-like* and *DRM2-like* genes (Gagné-Bourque et al., 2015). The *Bacillus subtilis* B26-induced methylation changes may be associated with the increased drought stress resilience of *Brachypodium*. Under water-deficiency conditions, the methylation level is high and relatively stable in barley. Drought stress mainly induces new methylations in roots but initiates equal novel methylation and demethylation events in leaves. Such organ-specific methylome changes might regulate the drought resistance in barley (Chwialkowska et al., 2016).

In the model monocot rice, drought stress induces differential 5mC methylation alterations in drought-tolerant variety and drought-sensitive variety (Wang et al., 2011, 2016; Zheng et al., 2013). Under drought conditions, hypermethylation events occur in the drought-susceptible genotypes while drought-tolerant

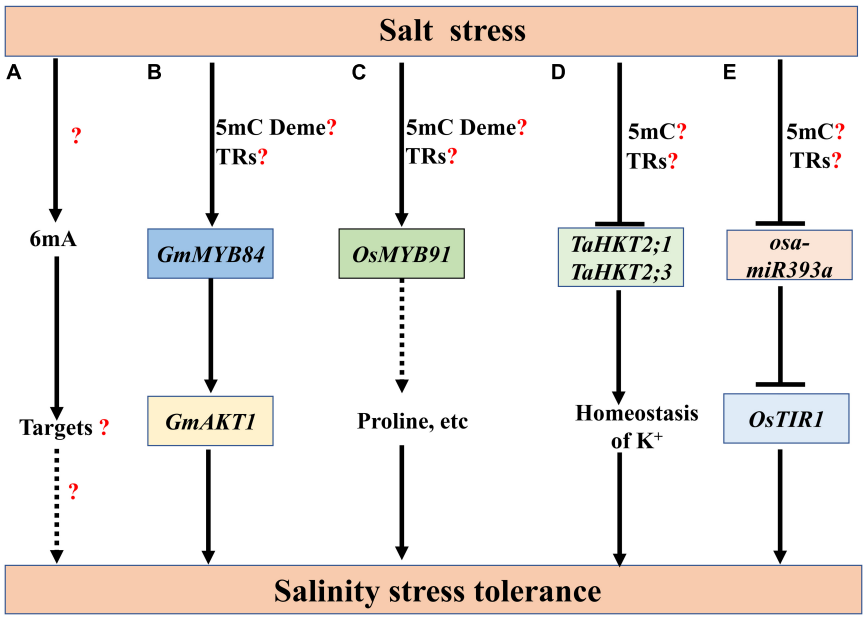

FIGURE 4 | The roles of DNA methylation in plant salt stress responses. **(A)** Salt stress increases the 6mA levels in rice (Zhang Q. et al., 2018), but the regulatory mechanisms underlying 6mA in salt stress remain to be investigated. **(B)** In soybean, salt stress induces 5mC demethylation at the promoter of *MYB DOMAIN PROTEIN 84* (*GmMYB84*), which may be associated with its higher expression. GmMYB84 activates K^+ *TRANSPORTER 1* (*GmAKT1*) to confer salinity stress tolerance (Zhang W. et al., 2020). **(C)** Salt stress leads to rapid removal of 5mC from the promoter of *OsMYB91*, which may contribute to the salt-induced expression of *OsMYB91*. Plants over-expressing *OsMYB91* show enhanced tolerance with significant increases of proline levels (Zhu et al., 2015). 5mC Deme represents the demethylation of 5mC. **(D)** Salinity stress induces an increase in 5mC levels of *HIGH-AFFINITY POTASSIUM TRANSPORTER 2;1/3* (*TaHKT2;1* and *TaHKT2;3*) that may downregulate their expression, thereby improving the salt tolerance. **(E)** Under salt stress, increased methylation levels of osa-miR393a promoter may lead to a lower expression of osa-miR393a, which may increase the salt tolerance through up-regulation of the target *TRANSPORT INHIBITOR RESPONSE 1* (*OsTIR1*). How salt-induced 5mC methylation or demethylation integrated with the function of transcriptional regulators (TRs) to activate downstream gene expression remains unclear **(B–E)**. The speculative regulatory paths are shown with broken arrows. Many unknown targets or steps (?) remain to be studied.

genotypes present hypomethylation behavior (Gayacharan and Joel, 2013). The DMR-associated genes in drought-tolerant introgression line DK151 are mainly involved in stress response, programmed cell death, and nutrient reservoir activity, which may contribute to the constitutive drought tolerance (Wang et al., 2016). Interestingly, a high proportion of multi-generational drought-induced alteration in DNA methylation status is maintained in advanced generations, which may offer the offspring improved drought adaptability in rice (Zheng et al., 2017).

Compared with heat, cold and salt stresses, our understanding regarding the drought stress-induced 5mC changes at drought-responsive TEs and genes is rather limited. Single-base methylome analysis reveals that water deficit is associated with a decrease in CHH methylation in apple cultivars, which may result in the hypomethylated status of TEs (Xu et al., 2018). In tomato, drought stress triggers the activation of a long terminal repeat (LTR) retrotransposon *Rider*, which is controlled by small RNAs and RdDM pathway under normal condition. The drought-induced *Rider* activation might be harnessed to generate genetic and epigenetic variation for crop breeding (Benoit et al., 2019). In a genome-wide association study, a miniature inverted-repeat transposable element (MITE) inserted in the promoter of *ZmNAC111* is identified to be significantly associated with natural variation in maize drought tolerance. Through the RdDM pathway, MITE represses the expression of *ZmNAC111*, which is a positive regulator of drought tolerance in maize (Mao et al., 2015). Drought stress decreases CHH methylation in the regulatory region but increases the CHG and CHH methylation in the coding region of drought-responsive gene *ABSCISIC ACID STRESS RIPENING 2* (*SlAsr2*), which functions in alleviating restricted water availability in tomato roots (González et al., 2013).

THE ROLE OF DNA METHYLATION IN ABIOTIC STRESS MEMORY

Somatic Stress Memory

Although abiotic stresses induce various chromatin changes in plants, most epigenetic changes are transient and quickly reset to pre-stressed levels when the abiotic stresses are removed. However, some chromatin changes induced by abiotic stresses can be mitotically heritable and last for several days or even the rest time of plant life in the same generation. In *Arabidopsis*, recurring dehydration stresses result in transcriptional stress memory which is featured by an increase in the rate of transcription and elevated transcript levels of some stress-response genes (Ding et al., 2012). Cold, drought and heat stress treatments can induce somatic abiotic stress memory

with a duration of 3–10 days, which mainly involve changes in histone modification, including H3K4me2/me3, H3K27me3 and H3K14ac (Lamke and Bäurle, 2017; Bäurle and Trindade, 2020). The memory of vernalization-induced *FLC* silencing can be maintained in subsequent growth and development under warm temperatures, which is associated with the establishment and maintenance of H3K27me3. In the pro-embryo, the seed-specific transcription factor LEAFY COTYLEDON1 (LEC1) promotes the H3K27me3 demethylation and activation of *FLC*, thereby erasing the vernalization memory (Tao et al., 2017; He and Li, 2018). It seems that DNA methylation is not responsible for the above stress-induced somatic memory. However, in rice, the major portion of salt-induced DNA methylation or demethylation alterations remain after recovery, suggesting that the salinity-induced DNA methylation changes can remember the environmental salt stress and transmit the stress-induced epigenetic states to daughter cells through mitotic cell divisions in the present generation (Wang et al., 2015). It remains a formal possibility that some genome-loci specific 5mC or 6mA changes may function in somatic memory of plant responses to abiotic stresses.

Transgenerational Inheritance of Stress Memory

Some abiotic stress can induce transgenerational phenotypic changes along with chromatin alterations, which can be detectable until at least one non-stressed generation (**Table 1**). In *Arabidopsis*, short-wavelength radiation (ultraviolet-C, UV-C) or flagellin treatment increases the frequency of somatic homologous recombination of a transgenic reporter, which persists in the next four untreated generations (Molinier et al., 2006). It is the first report of transgenerational epigenetic inheritance in plants. Since 2006, deciphering the transgenerational memory of plant stress responses has become a fascinating research area. Some stress responses can be only transmitted to the direct progeny, which is termed as intergenerational stress memory, while some stress responses can be memorized for at least two subsequent stress-free generations, which is known as transgenerational stress memory (Lamke and Bäurle, 2017).

The intergenerational stress memory can be triggered by multiple biotic and abiotic stresses, such as flagellin (an elicitor of plant defense), ultraviolet-C, salt, cold, heat and drought stress, β-aminobutyric acid (BABA), methyl jasmonate and the bacteria *Pseudomonas syringae* pv tomato (*PstavrRpt2*) (**Table 1**; Johnsen et al., 2005; Kvaalen and Johnsen, 2008; Sultan et al., 2009; Boyko et al., 2010; Ito et al., 2011; Scoville et al., 2011; Slaughter et al., 2012; Iwasaki and Paszkowski, 2014; Migicovsky et al., 2014; Bilichak et al., 2015; Wibowo et al., 2016; Ganguly et al., 2017; Bose et al., 2020). Interestingly, in perennial Scots pines (*Pinus sylvestris* L.), environmental memory of naturally dry conditions in the parental trees drive offspring survival and growth under hot-drought conditions (Bose et al., 2020). The stress memory may protect the immediate offspring against recurring stress or offer them the potential for local acclimation to changing environments, while the resetting in the next generation may maximize growth under favorable circumstances (Crisp et al., 2016). The intergenerational stress memory may be mediated by the direct impact of environment factors on the gametogenesis, fertilization and embryo development or maternal cues that are transported into and stored in the seeds when the progeny develops in the mother plants. It remains unclear that how much of the intergenerational stress memory is due to the environment-induced epigenetic changes. The epigenetic regulators involved in the intergenerational stress memory remain largely unidentified, except several reports of the possible roles of small RNAs and DNA methylation (**Table 1**; Boyko et al., 2010; Ito et al., 2011; Migicovsky et al., 2014; Bilichak et al., 2015; Wibowo et al., 2016). The hyperosmotic stress-induced responses are primarily maintained in the next generation through the female lineage due to widespread DNA glycosylase activity in the male germline, and extensively reset in the absence of stress (Wibowo et al., 2016). How the transient stress memory is maintained during meiosis in the stressed parental plants and removed or reset during the reproduction stage of the next generation remains to be investigated.

Increasing evidences indicate that many abiotic stress responses can exhibit transgenerational epigenetic inheritance (**Table 1**). Prolonged heat stress can induce transgenerational memory of the release of PTGS and attenuated immunity in *Arabidopsis*, which is mediated by a coordinated epigenetic network involving histone demethylases, heat shock transcription factors and *trans*-acting siRNAs (tasiRNAs) (Zhong et al., 2013; Liu et al., 2019). Cold stress and harsh UV-B treatment-induced release of TGS remain limitedly detectable for two non-stressed progeny generations (Lang-Mladek et al., 2010). The UV-C-mediated activation of some transposons can also be maintained for two generations without the presence of stress, which requires the roles of DCL proteins (Migicovsky and Kovalchuk, 2014). Upon exposure to heavy metal stress, the 5mC state of a Tos17 retrotransposon is altered and shows transgenerational inheritance in rice (Cong et al., 2019). Moreover, heavy metal-transporting P-type ATPase genes (HMAs) are up-regulated under heavy metal stress, which was transgenerationally memorized in the unstressed progeny (Cong et al., 2019). Successive generations of drought stress from the tillering to grain-filling stages induces non-random epimutations and over 44.8% of drought-induced epimutations transmit their altered DNA methylation status to unstressed progeny. Epimutation-related genes directly participate in stress-responsive pathways, which may mediate rice plant's adaptation to drought stress (Zheng et al., 2017). These transgenerational memories may offer the progeny an adaptive advantage or genomic flexibility for better fitness under diverse abiotic stresses.

Stress-induced transgenerational memory has also been reported in some asexual perennial plants. In the genetically identical apomictic dandelion (*Taraxacum officinale*) plants, various stresses triggered considerable methylation variation throughout the genome, and many modifications were transmitted to unstressed offspring (Verhoeven et al., 2010). In two different apomictic dandelion lineages of the *Taraxacum*

TABLE 1 | Examples of intergenerational and transgenerational stress memory in plants.

Plants	Abiotic stress treatment	Types of stress memory	Major effects	Possible epigenetic regulators	References
Arabidopsis thaliana	Ultraviolet-C or flagellin	Transgenerational	Increase in homologous recombination Frequency	Unknown	(Molinier et al., 2006)
Arabidopsis thaliana siRNA-biogenesis-deficient plants	37°C for 24 h	Intergenerational	Retrotransposition of *ONSEN*	siRNAs	(Ito et al., 2011)
Arabidopsis thaliana	30°C for 14 days	Transgenerational	PTGS release, early flowering and attenuated immunity	H3K27me3 demethylation and siRNAs	(Zhong et al., 2013; Liu et al., 2019)
Arabidopsis thaliana ddm1mom1 double mutants	37°C for 24 h	Intergenerational	Release of TGS	Altered positioning of nucleosome or others	(Iwasaki and Paszkowski, 2014)
Arabidopsis thaliana	50°C for 3 h/day for 5 days	Intergenerational	Fewer but larger leaves, early flowering	DCLs	(Migicovsky et al., 2014)
Arabidopsis thaliana	UV-C stress	Transgenerational	Increased transposon expression	DCLs	(Migicovsky and Kovalchuk, 2014)
Arabidopsis thaliana	Salt stress for 4 weeks	Intergenerational	Adaption to salt stress	DNA methylation machinery	(Wibowo et al., 2016)
Arabidopsis thaliana	42°C for 48 h UV-B stress	Transgenerational	Limited inheritance of TGS release	Histone acetylation	(Lang-Mladek et al., 2010)
Arabidopsis thaliana	Successive generations of drought stress	Intergenerational	Increased seed dormancy	Unknown	(Ganguly et al., 2017)
Arabidopsis thaliana	Salt, UV-C, cold, heat and flood stress	Intergenerational	Increased homologous recombination frequency	DCLs	(Boyko et al., 2010)
Arabidopsis thaliana	Grown at 30°C during reproduction for two generations	Transgenerational	Improved seed production	Unknown	(Whittle et al., 2009)
Arabidopsis thaliana	β-aminobutyric acid (BABA) or *PstavrRpt2*	Intergenerational	Improved resistance to biotic stress	Unknown	(Slaughter et al., 2012)
Arabidopsis thaliana Solanum lycopersicum	Herbivory, mechanical damage, methyl jasmonate	Transgenerational	Improved resistance to herbivory	siRNAs	(Rasmann et al., 2012)
Brassica rapa	42°C for 3 h/day for 7 days	Intergenerational	Fluctuations of smRNAome	miR168 and *braAGO1*	(Bilichak et al., 2015)
Mimulus guttatus	Simulated herbivore damage	Intergenerational	Increased trichome density	Unknown	(Scoville et al., 2011)
Oryza sativa	Hg^{2+}(50 μM/L) for 7 days	Transgenerational	Gene expression changes	DNA methylation	(Ou et al., 2012; Cong et al., 2019)
Oryza sativa	Successive generations of drought stress	Transgenerational	Improved drought adaptability	DNA methylation	(Zheng et al., 2017)
Picea abies	Daylength and temperature during seed production	Intergenerational	Adaptive plasticity	Unknown	(Johnsen et al., 2005; Kvaalen and Johnsen, 2008)
Pinus sylvestris L.	Drought stress	Intergenerational	Tolerant to hot-drought conditions	Unknown	(Bose et al., 2020)
Polygonum persicaria	Drought stress	Intergenerational	Improved drought tolerance	Unknown	(Sultan et al., 2009)
Taraxacum officinale	Drought and salicylic acid (SA) treatment	Transgenerational	Heritable DNA methylation variation	DNA methylation	(Preite et al., 2018)

officinale group (*Taraxacum alatum* and *T. hemicyclum*) under drought stress or after salicylic acid (SA) treatment, heritable DNA methylation variations are observed across three generations irrespective of the initial stress treatment (Preite et al., 2018). It is needed to note that these stress-induced transgenerational DNA methylation variations in dandelions are genotype and context-specific and not targeted to specific loci (Preite et al., 2018). Unlike most annual plants, the asexual perennial plants use clonal propagation. The stress-induced DNA methylation variations may be largely inherited during mitosis, which may enable the next-generation plants to respond accurately and efficiently to adverse environment factors in some habitats (Latzel et al., 2016). How the methylation variations contribute to the phenotypic variations in asexual perennial plants remains to be investigated.

In the germline and early embryo stage, both the paternal and maternal genomes undergo extensive DNA demethylation via both active and passive demethylation pathways in mammals, which leaves very little possibility for the inheritance of stress-induced changes in methylome (Smith et al., 2012). Some examples of stress-induced transgenerational epigenetic inheritance have been reported in some animals, such as

TABLE 2 | The divergent roles of DNA methylation in plant responses to diverse abiotic stresses.

Plants	Abiotic stress	Changes of DNA methylation levels	Major effects	References
Arabidopsis thaliana	Heat stress	Altered methylation of transposon remnants	Regulation of basal thermotolerance	(Popova et al., 2013)
Arabidopsis thaliana	Heat stress	Changes in genome-wide CHH-methylation pattern	Natural adaptation to variable temperatures	(Shen et al., 2014)
Arabidopsis thaliana	Cold stress	Enhanced methylation in *ALN* promoter	Promoting seed dormancy	(Iwasaki et al., 2019)
Arabidopsis thaliana	Drought stress	Increased 5mC methylation partly depending on H1.3	Adaptive response to water deficiency	(Rutowicz et al., 2015)
Arabidopsis thaliana *Ageratina adenophora*	Cold stress	Variation in *ICE1* methylation	Cold tolerance divergence in different accessions	(Xie et al., 2015, 2019)
Brachypodium distachyon	Drought stress	Decreased global 5mC while *B. subtilis* strain B26 inoculation increases it	Increased drought stress resilience	(Gagné-Bourque et al., 2015)
Brassica napus	Heat stress	Increased DNA methylation in heat-sensitive genotype	Adaption to heat stress	(Gao et al., 2014)
Brassica napus	Heat stress	DNA hypomethylation	Regulation of heat stress responses in cultured microspores	(Li et al., 2016)
Brassica napus	Salt stress	Decreased methylation in the salinity-tolerant cultivar but increased methylation in the salinity-sensitive cultivar	Acclimation to salt stress	(Marconi et al., 2013)
Brassica rapa	Cold stress	Decreased DNA methylation levels in the promoter of *BramMDH1*	Increased heat-tolerance and growth rate	(Liu et al., 2017)
Brassica rapa	Cold stress	Demethylation of *BrCKA2* and *BrCKB4*	Regulation of floral transition	(Duan et al., 2017)
Cucumis sativus	Cold stress	Demethylation at CHH sites	Regulation of temperature-dependent sex determination	(Lai et al., 2017)
Glycine max	Heat stress	Hypomethylation in all context	Affecting the expression of genes or TEs under heat	(Hossain et al., 2017)
Gossypium hirsutum	Heat stress	Reduced DNA methylation level in heat-sensitive line	Microspore sterility	(Min et al., 2014; Ma et al., 2018)
Gossypium hirsutum	Drought stress	Global hypermethylation in all three contexts	Acclimation to drought stress	(Lu et al., 2017)
Oryza sativa	Salt, heat and drought stresses	Activation of an LTR retrotransposon, *HUO*	Modulation of stress responses	(Peng et al., 2019)
Oryza sativa	Heat, salt, cold stress	Increased 6mA levels in heat and salt stress, decreased 6mA levels in cold stress	Regulation of plant responses to environmental stresses	(Zhang Q. et al., 2018)
Oryza sativa	Heat stress	Decreased DNA methylation levels of *OsFIE1*	Regulation of seed size under heat stress	(Folsom et al., 2014)
Oryza sativa	Salt stress	Increased methylation level of osa-miR393a promoter	Improved salt tolerance	(Ganie et al., 2016)
Oryza sativa	Salt stress	Decreased 5mC levels in the promoter of *OsMYB91*	Enhanced salt tolerance	(Xu et al., 2015)
Oryza sativa	Drought stress	Differential 5mC methylation alterations	Constitutive drought tolerance	(Wang et al., 2016)
Populus trichocarpa	Drought stress	Increased methylation of upstream and downstream 2 kb, and TEs	Regulation of drought responses	(Liang et al., 2014)
Rosa hybrida	Cold stress	Enhanced CHH methylation of the *RhAG* promoter.	Regulation of floral organ development	(Ma N. et al., 2015)
Solanum lycopersicum	Salt and drought stresses	Activation of a retrotransposon, *Rider*	Modulation of salt and drought stress responses	(Benoit et al., 2019)
Solanum melongena	Salt and drought stresses	Expression changes of C5-MTases and demethylases	Response to salt and drought stresses	(Moglia et al., 2019)
Triticum aestivum	Salt stress	Reduced methylation levels in the promoter of salinity-responsive genes	Contribute to the superior salinity tolerance	(Wang et al., 2014)
Triticum aestivum	Salt stress	Increased 5mC levels in *TaHKT2;1* and *TaHKT2;3*	Improved salt-tolerance ability	(Kumar et al., 2017)
Zea mays	Salt stress	Increased methylation of root *ZmPP2C* and demethylation of leaf *ZmGST*	Acclimation to salt stress	(Tan, 2010)
Zea mays	Drought stress	Suppression of *ZmNAC111* by MITE through RdDM	Natural variation in maize drought tolerance	(Mao et al., 2015)

Caenorhabditis elegans, the underlying epigenetic marks are mostly histone modifications or small RNAs (Skvortsova et al., 2018). However, the DNA methylation in plants is not erased but rather epigenetically inherited during plant reproduction (Feng et al., 2010; Calarco et al., 2012; Heard and Martienssen, 2014), suggesting a potential role of DNA methylation in transgenerational memory. In the successive generations of *met1-3* mutants deficient in maintaining CG methylation, the loss of mCG is found to progressively trigger new and aberrant genome-wide epigenetic patterns in a stochastic manner, such as RdDM, decreased expression of DNA demethylases and retargeting of H3K9 methylation (Mathieu et al., 2007). Upon potato spindle tuber viroid (PSTVd) infection in tobacco, the body of PSTVd transgene is densely *de novo* methylated in all three contexts. However, in the viroid-free progeny plants, only ^{m}CG can be stably maintained for at least two generations independent of the RdDM triggers (Dalakouras et al., 2012). Thus, CG methylation may function as a central coordinator to secure stable abiotic transgenerational memory. In a population of epigenetic recombinant inbred lines (epiRILs) with epigenetically mosaic chromosomes consisting of wild-type and *met1-3*, which are nearly isogenic but highly variable at the level of DNA methylation, despite eight generations of inbreeding, unexpectedly high frequencies of non-parental methylation polymorphisms are interspersed in the genome (Reinders et al., 2009). In the F5 individual plants of *ddm1* epiRILs, restoration of wild-type methylation is specific to a subset of heavily methylated repeats targeted by RNA interference (RNAi) machinery (Teixeira et al., 2009). Consistent with this, in the NRPD1 complementation *Arabidopsis* lines, the DNA methylation of a subset of RdDM target loci can also not be restored even at 20th generations. Many of these non-complemented DMRs overlap with epi-alleles defined in inbreeding experiments or natural accessions, which are functional in plant defense responses (Li et al., 2020). Under salt, drought and increased nutrient conditions in *Arabidopsis thaliana*, *ddm1* epiRILs exhibit phenotypic variations in root allocation, nutrient plasticity, drought and salt stress tolerance (Zhang et al., 2013; Kooke et al., 2015). These reports reinforce the idea that heritable variation in 5mC in epiRILs may allow the generation of epi-allelic variation, which have potential adaptive and evolutionary values. However, while the descendants of drought-stressed *Arabidopsis* lineages exhibit transgenerational memory of increased seed dormancy, the memory is not associated with causative changes in the DNA methylome (Ganguly et al., 2017).

Above all, although the potential roles of epigenetic regulations in transgenerational memory are undoubtable, the roles of stress-induced DNA methylation variations in the persistence of transgenerational inheritance remain to be further elucidated. The extent to which locus-specific methylation changes might contribute to the maintenance of stress memory also remains unclear. The *de novo* methylation of a particular region can be set up by RdDM and DNA methylation maintenance consolidates RdDM over generations in *Arabidopsis thaliana*, thereby establishing epigenetic memory (Kuhlmann et al., 2014). In *ddm1* epiRILs, several DMRs are identified as bona fide epigenetic quantitative trait loci (QTL^{epi}), accounting for 60–90% of the heritability for flowering time and primary root length (Cortijo et al., 2014). Whether the inheritance of DMRs induced by abiotic stress contributes to the transgenerational inheritance requires further investigation. In addition, whether abiotic stresses-induced 6mA changes can be inherited and their roles in stress memory remain elusive.

BOX 1 | Future research directions.

- Which enzymes or proteins are responsible for the establishment, maintenance and erasing of 6mA in plants?
- What are the roles of non-canonica RdDM pathways in plant abiotic stress response?
- How are the active and passive demethylation pathways fine-tuned by different abiotic stresses?
- What is the role of 6mA in plant somatic memory and transgenerational memory?
- How to quickly identify QTL^{epi} from epiRILs or epi-mutation library to accelerate investigation on the epigenetic regulation of abiotic stress responses in crops?
- How to efficiently identify the key DMRs responsible for the acclimation to abiotic stresses in plants?
- How are DNA methylation changes integrated with other epigenetic alterations to confer stress tolerance?
- What are the effects of abiotic stresses-induced methylation changes on the expression of key players in the sensing and signal transduction?
- How to manipulate the somatic and transgenerational memory to improve the abiotic stress tolerance of crops without sacrificing growth?

CONCLUDING REMARKS

Our knowledge on the roles of DNA methylation in plant responses to abiotic stresses is accumulating in recent years. However, these discoveries regarding the roles of 5mC in plant responses to heat, cold, drought and salt stresses are fragmented and scattered (**Table 2**). The role of 6mA in plant abiotic stress responses is largely unknown. More solid and comprehensive experiments are needed to elucidate the roles the abiotic stresses-induced 5mC and 6mA changes in stress responses through regulating the expression of downstream targets. Besides, it is urgent to investigate how the stress-induced DNA methylation changes recruit or cooperate with other transcriptional regulators to modulate gene expression under abiotic stresses.

The genome-wide DNA methylation changes induced by abiotic stresses are distinct according to the intensity and duration of stress, the developmental stages, sampled tissues, genotypes and species. The diverse global changes may be attributed to the different impacts of abiotic stresses on the key components of DNA methylation among different species. To improve the tolerance of crops under abiotic stresses, we may pay more attentions to stress-induced DMRs but not the alterations in the global methylome. The mapping of epigenetic quantitative trait loci (QTL^{epi}) will greatly accelerate the identification of causal DMRs underlying specific phenotypes or stress tolerance in plants. Several DMRs are identified as QTL^{epi} controlling the variation in growth, morphology and plasticity under normal and saline conditions in *ddm1* epiRILs (Cortijo et al., 2014;

Kooke et al., 2015). Linkage-linkage disequilibrium mapping has been used to decipher the QTL^{epi} underlying growth and wood properties in a linkage population and a natural population of *Populus* using MSAP-based analysis (Lu et al., 2020). These QTL^{epi} may be good candidates for engineering plants with better tolerance to abiotic stresses. Interestingly, two recent studies have revealed that *msh1* graft-induced enhanced growth vigor or segregation of an *MSH1* RNAi transgene produced non-genetic memory with multi-generational inheritance (Kundariya et al., 2020; Yang et al., 2020). The *msh1* graft-induced heritable phenotype is RdDM-dependent and requires DCL2-4 to generate siRNAs. In tomato, the *msh1* grafting-enhanced growth vigor in the field can be heritable over five generations, demonstrating the huge agricultural potential of epigenetic variation (Kundariya et al., 2020). The *msh1* memory produced by segregation of an *MSH1* RNAi transgene, also requires RdDM pathway, which involves the function of *HISTONE DEACETYLASE 6* and *MET1*. The *MSH1* RNAi transgene-mediated methylome reprogramming contributes to the phenotypic plasticity in the transgene-free progeny, which may offer them accelerated adaptation to changing environments (Yang et al., 2020).

Our knowledge on the roles of DNA methylation in regulating the signal transduction of abiotic stress is rather limited. Only the ICE1-CBF-COR pathway in the cold signaling has been reported to be regulated by 5mC. The influences of 5mC and 6mA on the HSF-HSP pathway in heat responses and salt-overly-sensitive (SOS) pathway in salt signaling remain to be elucidated. G-protein signaling, MAPK cascades, calcium signaling and hormone signaling are common themes in the key downstream signaling pathways under different abiotic stresses. We still need more efforts to uncover the dynamics of DNA methylation on the important players in these signaling pathways under advert abiotic circumstances.

In recent years, owing to the high efficiency and flexibility, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) has been widely used in gene editing in various plant species. CRISPR/Cas9 does not induce epigenetic changes on either the target loci, flanking DNA or off-target sites (Lee et al., 2019). A CRISPR/dCas9-based targeted demethylation system comprising the human demethylase TEN-ELEVEN TRANSLOCATION1 (TET1cd) and modified SunTag system has been developed with high specificity and minimal off-target effects, which has been successfully used to target the *FLOWERING WAGENINGEN (FWA)* promoter for demethylation to initiate a heritable late-flowering phenotype (Gallego-Bartolomé et al., 2018). Moreover, TET1-mediated demethylation has been applied for the generation of inheritable 5mC variation through random demethylation of the *Arabidopsis* genome, which results in the expression of previously silenced alleles and uncovers new phenotypic variations (Ji et al., 2018). The fusion of the catalytic domain of 5mC DNA glycosylase ROS1 to dCas9 is also able to reactivate the silenced genes and induce targeted demethylation in a replication-independent manner (Devesa-Guerra et al., 2020). These novel tools open a new window to reactivate expression of previously silenced genes or TEs, and to develop new epi-alleles for improved abiotic stress tolerance. We may take advantage of these tools to introduce epigenetic variations for improving the adaptation to abiotic stress conditions in crops.

As listed in Box 1, many questions concerning the DNA methylation in plant abiotic stress responses remain to be answered by future researches. To further elucidate the role of 6mA in plant abiotic stress responses and memory, one of the most important steps maybe the identification of the 6mA methyltransferases, demethylases and the binding proteins. Plant homologs of mammalian 6mA writers, erasers and readers may be potential targets. Forward genetic screens using reporter lines and reverse genetic approaches such as CRISPR/Cas9 technique will be helpful for identifying proteins involved in the establishment, maintenance and erasing of 6mA in plants. Among the other questions, perhaps the most important question is: how to manipulate the somatic and transgenerational memory to improve the abiotic stress tolerance of crops without sacrificing growth? To address this question, we must identify the key DMRs or QTL^{epi} responsible for the acclimation to abiotic stresses in plants. Systemic screening for DMRs or QTL^{epi} from epiRILs and natural accessions will be powerful approaches (Quadrana and Colot, 2016). The combined application of CRISPR/Cas9 techniques and alternative inducers of DMRs or QTL^{epi} may enable us to engineer crops with enhanced tolerance to abiotic stresses without yield penalty.

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JL and ZH wrote and revised the manuscript. Both authors have read and approved the final manuscript.

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REVIEW
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Epigenetic Control of Plant Response to Heavy Metal Stress: A New View on Aluminum Tolerance

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High concentrations of heavy metal (HM) ions impact agronomic staple crop production in acid soils (pH $\leq$ 5) due to their cytotoxic, genotoxic, and mutagenic effects. Among cytotoxic ions, the trivalent aluminum cation (Al^{3+}) formed by solubilization of aluminum (Al) into acid soils, is one of the most abundant and toxic elements under acidic conditions. In recent years, several studies have elucidated the different signal transduction pathways involved in HM responses, identifying complementary genetic mechanisms conferring tolerance to plants. Although epigenetics has become more relevant in abiotic stress studies, epigenetic mechanisms underlying plant responses to HM stress remain poorly understood. This review describes the main epigenetic mechanisms related to crop responses during stress conditions, specifically, the molecular evidence showing how epigenetics is at the core of plant adaptation responses to HM ions. We highlight the epigenetic mechanisms that induce Al tolerance. Likewise, we analyze the pivotal relationship between epigenetic and genetic factors associated with HM tolerance. Finally, using rice as a study case, we performed a general analysis over previously whole-genome bisulfite-seq published data. Specific genes related to Al tolerance, measured in contrasting tolerant and susceptible rice varieties, exhibited differences in DNA methylation frequency. The differential methylation patterns could be associated with epigenetic regulation of rice responses to Al stress, highlighting the major role of epigenetics over specific abiotic stress responses.

Keywords: abiotic stress, aluminum tolerance, epigenetic response, heavy metals, rice

INTRODUCTION

Plants deal with multiple challenges to adapt to different environmental conditions given their sessile lifestyle. Abiotic stresses such as drought, salinity, extreme temperatures, nutrient deficiency, and heavy metal stress, represent some of the most limiting factors for plant growth (Zhu, 2016).

Heavy metals (HMs) are elements with densities above 5g/cm^3 that belong to the Earth's crust natural components. High concentrations of heavy metals can generate cytotoxic, genotoxic, and mutagenic effects in living organisms. Under physiological conditions, HMs can be divided into two groups: (i). Essential elements that are necessary for plant growth being structural blocks in

proteins with an enzymatic function, such as iron (Fe), manganese (Mn), zinc (Zn), magnesium (Mg), molybdenum (Mo), and copper (Cu), and (ii). Non-essential elements like Cadmium (Cd), chromium (Cr), lead (Pb), aluminum (Al), and selenium (Se). While essential elements are necessary for plants in small amounts, high concentrations of both types of elements can lead to inhibition of plant growth and development (Rascio and Navari-Izzo, 2011). Heavy metals have a strong impact on acid soils, caused by the excess of cationic species such as magnesium (Mg^{2+}), calcium (Ca^{2+}), phosphorus (P), sodium (Na^{+}) and aluminum (Al^{3+}) which in turn, affect plant physiological responses leading to crop yield losses for breeders and farmers (Samac and Tesfaye, 2003; Fryzova et al., 2017).

Acid soils represent nearly 30% of worldwide arable land, with 13% of staple crops cultivated in these areas. These types of soils classified as ultisols or oxisols are characterized by a pH lower than 5.5 (**Figure 1A**; Bojórquez-Quintal et al., 2017; Rahman et al., 2018). Al toxicity on acid soils has been reported as one of the major factors limiting crop production, and becoming worse due to current fertilization practices, pasture management, and climate change (Zheng, 2010; Kochian et al., 2015).

Staple food crops such as maize, wheat, sorghum, and rice have been extensively studied to increase their Al tolerance (Famoso et al., 2010). Among these crops, rice has been used as a model thanks to its high tolerance to Al toxicity (Famoso et al., 2010; Mustafa and Komatsu, 2016). Rice is a staple crop for over half of the world population with a cultivated area of 167.25 million hectares, and with an increment of 5.55 million hectares between 2010 and 2017 period (Food and Agriculture Organization of the United Nations, 2020; **Figure 1B**). Yet, there is still a need to increase 50% of rice production by 2050 to feed a growing population (Lin et al., 2019).

Important advances in elucidating the genetic mechanisms associated with HM tolerance and, especially, the molecular network involved in Al toxicity responses, have been reported in the last decade. Several studies on different crops have focused on genetic mapping to identify either quantitative trait loci (QTLs) or up/down-regulated genes associated with the response to Al stress (Famoso et al., 2011; Zhang et al., 2019). However, an increasing number of studies highlight the role of epigenetic mechanisms in the regulation of plant stress responses (Sudan et al., 2018; Chang et al., 2020). Therefore, the aim of this review is to explore and analyze the existing scientific literature on epigenetics as an important factor that regulates HM stress responses. Additionally, the direct relationship between epigenetic and genetic elements related to HM tolerance is revised, with a special focus on Al tolerance in rice.

GENETIC MECHANISMS UNDERLYING HEAVY METAL TOLERANCE

Plants have evolved different strategies to cope with HMs, diverging according to distinct factors as the plant species or the HMs exposure time and concentrations (Horst et al., 2010). These strategies fall into two general mechanisms: (i) An exclusion mechanism, where plants exudate organic compounds to the rhizosphere to chelate HM ions, transforming them into non-toxic compounds, and avoiding their chemical intake through root cells; and (ii) A detoxification mechanism, where plants allow the entrance of HM ions for internal detoxification and sequestration (**Figure 2**; Kochian et al., 2015).

Hyperaccumulator plants have been important models to understand the possible mechanism by which plants have adapted to high HM concentrations, and to elucidate the putative genetic elements that could be involved in these processes (Yang et al., 2005; Chaudhary et al., 2018; Fasani et al., 2018). One recurrent mechanism reported in these plants as an overall HM detoxification strategy is HM chelation by a ligand, either to keep HMs out of the roots or to target them to vacuoles. Diverse metal-binding ligands have been reported in plants. The peptide ligands phytochelatins (PCs) and metallothioneins (MTs) are different classes of cysteine-rich proteins that bind to HMs and have been reported as the most important genes in HM detoxification (Chaudhary et al., 2015). Complexes of PC-HM lower the binding capacity of HMs to the cell walls while MTs control the ROS accumulation and HM sequestration. For more information see Chaudhary et al. (2018) for a review of different PC and MT genes expressed in various plants and tissues under different HM stresses. Another mechanism involved in HM tolerance is the HM transport into the cell, and later, into the vacuole. Various genes have been reported to be involved in HM transport including heavy metal ATPases and the natural resistance-associated macrophage protein (Nramp) (Yang et al., 2005; Chaudhary et al., 2015).

Several studies have reported that tolerance or hyperaccumulation of HMs in plants is related to gene transcription modulation of metal chelators or transporters that favor exclusion or detoxification of the HMs (Arbelaez et al., 2017; Gulli et al., 2018; Zhang et al., 2019). These genes are potentially regulated by a reversible epigenetic mechanism, especially on hyperaccumulator plants which can live in soils with or without high HM concentrations. In this sense, epigenetic mechanisms represent an option to modify gene expression patterns enabling a rapid adaptation to environmental stressors (Mirouze and Paszkowski, 2011; Ou et al., 2012). **Table 1** shows the main genetic players in plant responses to Al, including genes involved in the exclusion or sequestration of Al^{3+} ions.

One of the main strategies reported for Al exclusion is mediated by organic acid (OA) efflux from the root apex (Yang et al., 2013; Poschenrieder et al., 2019), a ubiquitous mechanism in all plant cells that reduces Al damage by forming stable compounds with Al^{3+} ions in the rhizosphere (Bojórquez-Quintal et al., 2017). The first genes linked to Al tolerance were malate and citrate organic acid transporters in wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), and barley (*Hordeum vulgare*) (Sasaki et al., 2004; Furukawa et al., 2007; Magalhaes et al., 2007). Subsequently, it was found that members of two transporters families, the Al-activated malate transporter (ALMT) and the OA/H + transport channel (Multi-antimicrobial extrusion protein - MATE), are responsible for the exudation of malate and citrate, respectively, from root cells to the rhizosphere in response to Al (Kochian et al., 2015). However,

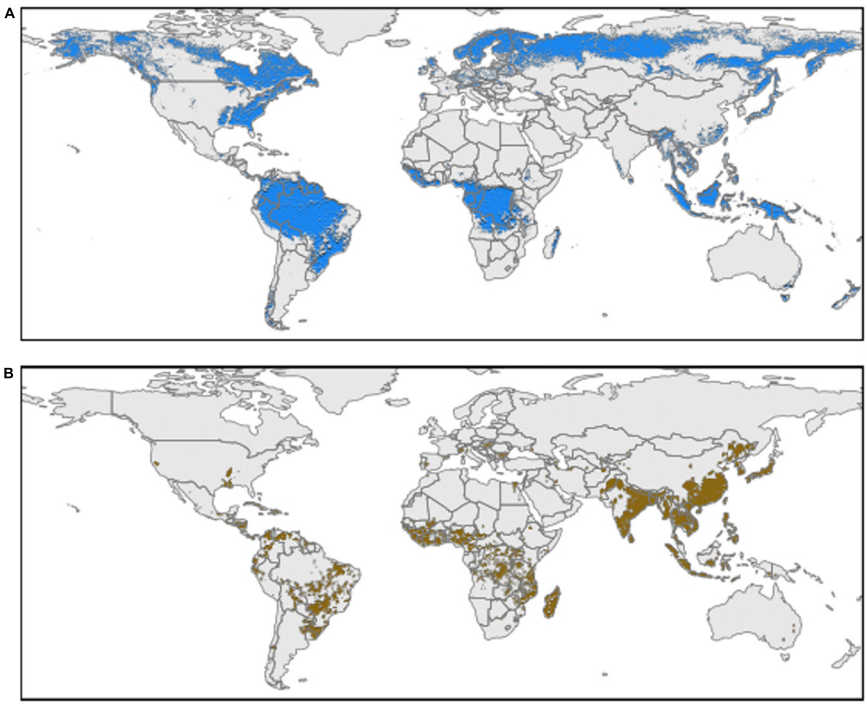

FIGURE 1 | Worldwide distribution of acidic soils and rice crop areas (1 km^2 resolution). **(A)** Areas with a weighted averaged soil pH (0-30 cm) less than or equal to 5.5 (acidic soils) using data extracted from Soilgrids (Hengl et al., 2017). **(B)** Worldwide rice crop area coverage (pixel probability > 0) (Jackson et al., 2019).

other transporters like ABC carriers and aquaporins are also required for OA transport (Liu et al., 2014).

RICE AS A GENETIC MODEL TO STUDY ALUMINUM TOLERANCE IN PLANTS

Rice is a model species to study Al tolerance being one of the plants with highest tolerance to this element (Famoso et al., 2010, 2011). Rice has a complex response against Al stress, involving a wide range of strategies and a diversity of genes (Magalhaes et al., 2004). These genes are potentially involved in the exclusion of Al^{3+} ions through OA efflux; for instance, the MATE transporters OsFRDL2 and OsFRDL4, has shown a role in OA transport (Famoso et al., 2010; Delhaize et al., 2012; Yokosho et al., 2016). Other rice Al responses include the modification of the cell wall properties (Kochian et al., 2015; Che et al., 2016), and Al^{3+} ions uptake and subsequent sequestration/translocation into the vacuole by different Al transporters like bacterial-type ABC and Nramp Al transporters (Huang et al., 2009; Xia et al., 2010; Li et al., 2014). Other genetic elements associated with Al tolerance include genes encoding transcription factors as ART1, ASR1 and ASR5 (Yamaji et al., 2009; Arenhart et al., 2016; Che et al., 2016). The upregulation of specific genes as OsMGT1, a magnesium transporter, is also linked to high Al tolerance (Chen et al., 2012). More recently, Zhang et al. (2019) reported 69 potential candidate genes related to Al tolerance, identified in a collection of 150 rice landraces using a combined GWAS-transcriptomic approach. Complementarily, several QTLs associated with Al tolerance have been identified in rice using different inter and intra-specific mapping populations (Wu et al., 2000; Ma et al., 2002; Nguyen et al., 2003; Xue et al., 2006, Xue et al., 2007; Famoso et al., 2011; Zhang et al., 2019). Famoso et al. (2011) reported 48 QTLs located on chromosomes 1, 3, 9, and 12. The QTLs were generated based on mapping populations exposed to Al stress, using relative root growth as the experimental phenotypic readout. The major QTL was found on chromosome 12, explaining 19% of the phenotypic response. Findings reported in above mentioned studies support the hypothesis that Al tolerance in rice involves multiple genes, genomic regions and mechanisms.

The previous evidence relates both, genic elements and specific genic mechanisms with the phenotypic response to cope with HMs stresses. Besides the genetic control that exists to regulate these responses, additional regulation layers might exist, being epigenetics a controlling mechanism of paramount importance in order to adapt to abiotic stresses, and specifically, to HMs restrictive conditions. In the following sections we will revise the current evidence that associates epigenetics with HMs stress responses. Giving its agronomic relevance, special attention is put on rice epigenetics as integrated strategies to cope with HMs and aluminum stresses.

EPIGENETIC MECHANISMS IN PLANTS

Epigenetics refers to the study of heritable and stable changes in gene expression without DNA sequence modifications

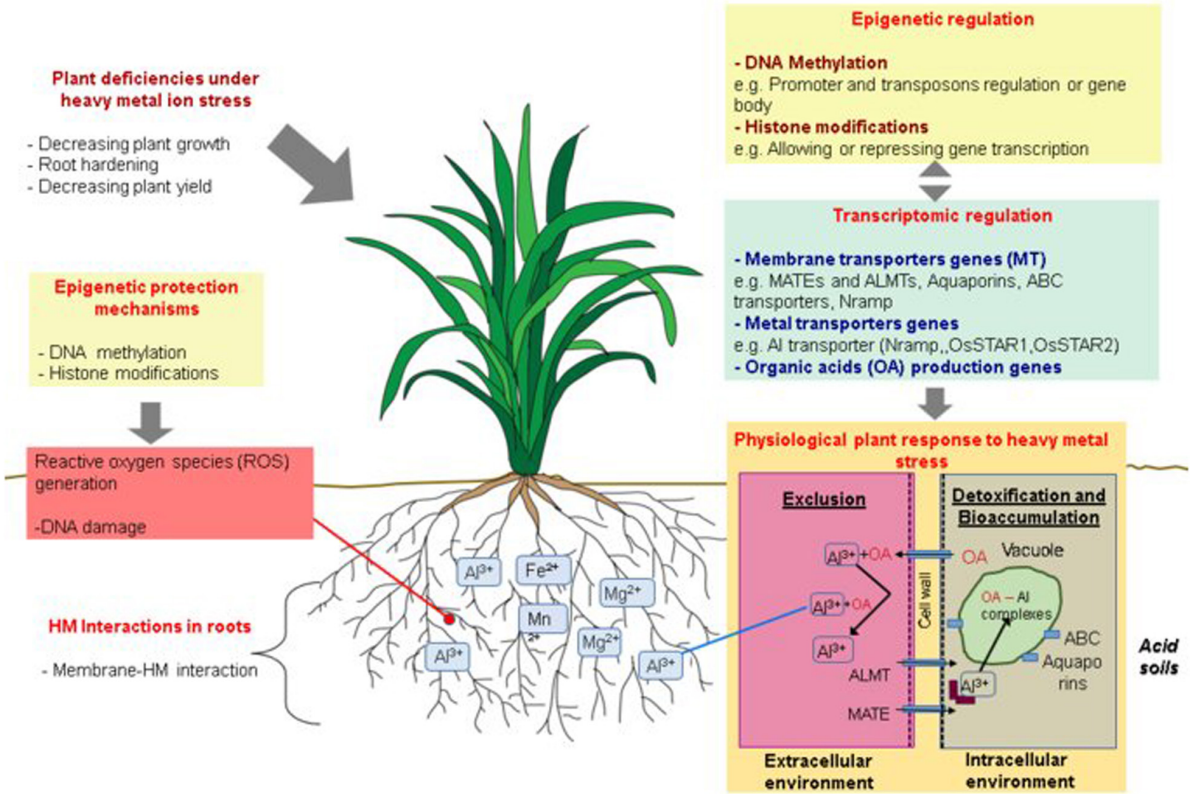

FIGURE 2 | Schematic representation of physiological, genetic, transcriptional and epigenetic mechanisms involved in plant responses to heavy metals (HM) exposure. Plant exposure to HMs induces different physiological deficiencies that could be countered by two principal tolerance mechanism shown at the bottom right of the figure: an exclusion mechanism, where the plant secretes organic acids (OAs) out of the root, avoiding the entrance of HM ions or, a detoxification mechanism and sometimes bioaccumulation, wherein plants internalize HM ions through membrane transport proteins such as ALMT or MATE carriers, and subsequently, HMs can be chelated by organic acids (OA) or translocated into the vacuoles through ABC carriers or aquaporins. The regulation of HM responsive genes has been related to epigenetic mechanisms as DNA methylation and histone modifications which can repress or activate gene expression through promoter or gene body methylation as well as avoiding transposon movement (top right). Another important epigenetic mechanism involved in the HM stress response is the hypermethylation along the genome to protect DNA from possible damages caused by metal subproducts.

(Wu and Morris, 2001). Three epigenetic mechanisms have been described in gene expression regulation: (i) DNA methylation (modifications at genomic level), (ii) histone modifications (chromatin modifications) and (iii) Small RNA modifications (RNA directed DNA Methylation-RdDM pathway) (Sudan et al., 2018; Chang et al., 2020). Currently, DNA methylation is the most documented epigenetic modification, and it is recognized as a relatively stable, and inheriting transgenerational mark involved in a set of biological processes such as the activity of transposable elements, genomic imprinting, alternative splicing, and regulation of temporal and spatial gene expression (Zhang et al., 2006; Ou et al., 2012). Mammals and plants differ in their DNA methylation patterns. In plants, DNA methylation is more widespread and complex, and occurs mainly in cytosine residues in the CG, CHG, and CHH sequence context (H can be A, C, or T), while in mammals it occurs only in a CG context (Bender, 2004; He et al., 2010). Studies on general DNA methylation profiles conducted on the model crop, *Oryza sativa* L. (cultivated rice), have shown that transposable elements and repetitive sequences are the most heavily methylated DNA regions in the rice genome (He et al., 2010; Yan et al., 2010; Li et al., 2012). Overall, gene methylation occurs mainly in the CG context, while transposon methylation occurs in all three described contexts (He et al., 2010; Yan et al., 2010; Li et al., 2012).

The methylome in plants is mainly monitored and maintained during DNA replication and cell division by DNA methyltransferases. There are three major classes of DNA methyltransferases: DNA methyltransferases (METs), which are the main CG methylases in charge of CG methylation; the plant specific enzymes chromomethyltransferases (CMTs), that are known to establish CHH and CHG methylation; and the domain rearranged methyltransferases (DRMs), that are involved in the maintenance of non-CG methylation and *de novo* methylation in all three contexts: CG, CHG and CHH (Lanciano and Mirouze, 2017). In contrast, DNA demethylation is performed by DNA glycosylases such as ROS1 (Repressor Of Silencing 1) and the DME (Demeter) enzyme (Lanciano and Mirouze, 2017).

TABLE 1 | Summary of main exclusion and tolerance mechanisms reported in plants.

Species	Genes	Mechanism	Specific mechanism	Function	References
P. vulgaris, T. aestivum, S. bicolor, H. vulgare, Zea mays, snapbean, oat, rye, *Glicine max*, *Colocasia esculenta, Triticale* sp., *Helianthus annuus*	ALMT, MATE, OSALMT4	Exclusion	Organic acid exudation	Chelate Al3+ (release of malate, citrate, or oxalate) located in the root apex	Kochian et al., 2004, 2015; Liu et al., 2018
Zea mays, *Cinnamomum camphora, Eucalyptus camaldulensis*		Exclusion	Phenolic compounds exudation	Release of other organic compounds (e.g., catechol, catechin, and quercetin), oenothein B, proanthocyanidin in roots	Kochian et al., 2015
Cucurbita pepo, wheat, tea	ATPases	Tolerance (Al detoxification)	Changes in the Rhizosphere pH	pH rhizosphere changes to induce to Al detoxification mechanisms	Bojórquez-Quintal et al., 2017
Oryza sativa, Solanum tuberosum, Arabidopsis thaliana, petunia inflata	XTH, XET, XTH31,pectin methylesterases,OsFRDL4, STAR1, STAR2, ABC transporters, HMG2, HMG3, WAK1	Tolerance (Al detoxification)	Cell wall modification	Changes in the structural properties of cell wall such as reduction of wall plasticity/elasticity, carbohydrates, methylated pectins, and reduced pectin methylesterases; increased sterols biosynthesis; negativity of apoplast to enhance Al transport	Schmohl et al., 2000; Horst et al., 2010; Kochian et al., 2015; Morkunas et al., 2018; Wagatsuma et al., 2018
Arabidopsis thaliana, Oryza sativa,	Nramp, OsNrat1, OsALS1, aquaporine family, ABC,ALMT,OsCDT3	Tolerance (Al detoxification)	Al transportation	Arrest Al from cell wall to root cell vacuole	Kochian et al., 2015; Arbelaez et al., 2017
Brassica napus, Nicotiana tabacum, wheat*, Arabidopsis thaliana, Zea mays*	ALMT,MATE,SbMATE, TaALMT1,OsFRDL4	Tolerance (Al detoxification)	ALMT/MATE proteins Al transportation	Passive efflux of malate; carriers that mediate citrate efflux coupled to H + influx	Liu et al., 2014; Kochian et al., 2015
Oryza sativa, Arabidopsis thaliana, Andropogon virginicus	Nramp,OsALS1, Nrat1	Tolerance (Al detoxification)	Nramp proteins Al transportation	Specific transporter for aluminum ions (no divalent cations) transport from cell wall to vacuoles	Yokosho et al., 2011; Ezaki et al., 2013; Kochian et al., 2015
Oryza sativa, Arabidopsis thaliana	OsSTAR1, OsSTAR2, AtALS3, OsALS1, AtALS1	Tolerance (Al detoxification)	ABC proteins Al transport	ATP-driven pumps (ABC transporters);	Huang et al., 2009; Delhaize et al., 2012; Kochian et al., 2015
Oryza sativa, Arabidopsis thaliana, Hydrangea macrophylla	Aquaporins such as HmVALT, HmPALT1	Tolerance (Al detoxification)	Aquaporins transportation	Transport and store in shots	Negishi et al., 2012; Kochian et al., 2015

The relationship among genes, mechanisms and molecular functions of the reported genes is shown.

EPIGENETIC REGULATION OF PLANT STRESS RESPONSE

Abiotic stresses can generate a diverse range of phenotypes in plants, which are a consequence of complex molecular, biochemical, and physiological changes. Plants responses and adaptation to these stress conditions vary in different ways and at various levels, including short term physiological responses such as metabolic and gene expression changes, and long-term responses such as genetic and epigenetic genome modifications (Turner, 2009). The mechanisms of signal transduction, as well as the genetic variability underlying plants responses to stress, have been widely studied and, in many cases, successfully exploited by plant breeders to improve resistance to abiotic stress through traditional breeding or marker-assisted selection (Kantar et al., 2015; Zhu, 2016). Recently, epigenetic marks have gained attention as important factors of abiotic stress-related gene control (Kumar, 2018). For example, a stress signal can promote DNA methylation changes in the promoter regions of stress-responsive genes, thus modifying their expression pattern, generating histone conformational changes, and promoting transcriptional repression by preventing transcription factors binding to their target sites (Boyko et al., 2010; Ou et al., 2012; Ueda and Seki, 2020). Since methylation affects how genes are transcribed, it is hypothesized that DNA methylation is involved in the long-term transgenerational maintenance of epigenetic changes.

DNA methylation states can be complemented by additional mechanisms such as histone modifications (Mirouze and Paszkowski, 2011). Although considered a more dynamic and transitory mechanism, because the majority of changes that occur under stress conditions revert to their initial state quickly, histone modifications could play a role in the inheritance of certain stress-tolerant phenotypes (Pecinka and Scheid, 2012). For example, Kim et al. (2012) showed that H3K4me3 and H3K9ac histone modifications were abundant in several drought-associated genes in *Arabidopsis thaliana* plants subjected to water-deficit regimes. When plants were irrigated, the H3K9ac modifications were rapidly eliminated, while H3K4me3 ones remained, indicating that the latter modification can be stably inherited through generations.

Histone modification effects on gene regulation have also been reported for other stress conditions. Sokol et al. (2007) reported transient H3Ser-10 phosphorylation, H3 phosphoacetylation, and histone H4 acetylation under salinity and cold-stress related to the expression of stress-specific genes. Likewise, the trimethylation of H3K4 and acetylation of H3K9 in *A. thaliana* was generated by exposure to drought, ABA, and salt stress, causing stress-responsive genes expression (Kim et al., 2008).

Stress-induced epigenetic changes, especially DNA methylation, occur regularly in all plant species, reinforcing the importance of this mechanism for regulating plant responses to environmental changes; most of these changes are heritable and play an important role in plant adaptation (Feng et al., 2010). Genomic sequences whose changes in their methylation status are maintained over generations, without altering the acquired methylated pattern, are known as epialleles (Kalisz and Purugganan, 2004). There is evidence that epialleles can occur over stress-related genes, however, they can also be present in genetic regions that are not directly related with the specific stress response, generating random changes across the genome. Moreover, both types of variations could be affected by natural selection according to the phenotypic effects they may cause (Verhoeven et al., 2010).

Transposons can also play a role in suppressing gene expression. This can occur due to the methylation state of a transposon located in or near a gene, which can directly affect the regulation of that gene through a methylation spread mechanism. Thus, transposon silencing through epigenetic marks contributes to the establishment of epigenetic variations affecting gene modulation in plants (Saze and Kakutani, 2007; Galindo-González et al., 2018).

Although the heritability of stress-induced methylation in plants remains poorly understood, some studies show that most of the induced variations are faithfully inherited to the offspring. For instance, Boyko et al. (2010) showed that *A. thaliana* plants exposed to salinity, cold, heat, and flooding, showed an overall increase in DNA methylation, associated with a higher stress tolerance in the progeny. In addition, Herman and Sultan (2016) reported that in *Polygonum persicaria*, DNA methylation is involved in increasing offspring drought tolerance when parental plants are subjected to this stress. Some studies have even found epialleles with direct effects on economically important traits; for instance, heritable methylation changes induced in rice due to nitrogen deficiency (Kou et al., 2011), heavy metal toxicity (Ou et al., 2012), and drought (Zheng et al., 2017) have been described. This last study showed the conservation of several non-random methylation changes generated under drought conditions (>40%) through several generations. Zheng et al. (2017) also found that these epigenetic changes are related to stress-responsive genes and they seemed to influence rice long-term adaptation to drought conditions. Thus, these studies support the potential role of epigenetic variation, and its inheritance across generations, as a relevant evolutionary process in crops. Similarly, they show that in rice, the mechanisms of epigenetic regulation of stress responses may be related to the type of stressor.

EPIGENETIC MECHANISMS INVOLVED IN HEAVY METAL TOXICITY

A recent recurring question is whether there is a general pattern of DNA methylation related to HMs exposure in plants. Evidence from previous studies suggests that DNA methylation might play a role in the regulation of plant responses to HMs through at least two mechanisms (Aina et al., 2004; Choi and Sano, 2007; Greco et al., 2012; Kumar et al., 2012; Arif et al., 2016). The first mechanism is related to a protective effect of methylation against HM-induced DNA damage through single-strand breaks or multi-copy transposition

(**Figure 2**; Bender, 1998). For example, Aina et al. (2004) compared methylation levels between clover (*Trifolium repens L.*), which is sensitive to Cr, Ni, and Cd, and hemp (*Cannabis sativa* L.), which is partially tolerant to these HMs. The study found that in the absence of HM stress, the level of methylation of hemp roots was significantly higher than in clover. Similarly, Gulli et al. (2018) found that *Noccaea caerulescens* plants (a Ni hyperaccumulator species) grown under high Ni concentrations were significantly hypermethylated at the genome level in comparison to *A. thaliana* Ni susceptible plants exposed to high Ni concentrations. These authors also showed that MET1, DRM2, and HDA8 genes, which are involved in DNA methylation and histone modification, were differentially expressed between *N. caerulescens* and *A. thaliana.* Hypermethylation has also been reported to act as a defense mechanism to counteract radiation genotoxic effect as shown by Kovalchuk et al. (2003); Volkova et al. (2018) who reported that pine trees plants (*Pinus silvestris*) adapted to survive high ionizing radiation, exhibited significantly hypermethylated loci compared to less adapted plants.

A second type of epigenetic response to HM stresses involves gene expression control (**Figure 2**). This regulation is not limited to the promoter region of genes but includes their coding regions (Choi and Sano, 2007). DNA methylation on gene promoters usually represses genetic transcription but, in some cases, it can also promote it (Zhang et al., 2006). In the meantime, exon/intron methylation occurs mainly on CG context and its function remains unclear. Gene body methylation has been related to transcriptional upregulation and has been suggested to protect genes from aberrant transcription caused by cryptic promoters (Zhang et al., 2006; Feng et al., 2016). The local acetylation of histones located near the promoter region of genes can induce transcriptional activation (Finnegan, 2001). Although there are no reports of specific histone modifications related to HM stresses in plants, some studies in animals have revealed a direct relation between HM exposition and histone modifications (Cheng et al., 2012).

Gene expression changes generated by HM exposure in rice have been described extensively in the literature and linked to variations in DNA methylation levels. For instance, Oono et al. (2016) showed a positive correlation between Cd dose-response in plants and the expression of genes coding for metal ion transporters where DNA methylation marks were detected. Similarly, using whole-genome bisulfite sequencing (WGBS), Feng et al. (2016) evaluated DNA methylation changes induced by specific Cd stress in rice plants (*Oryza sativa* ssp japonica cv. Nipponbare). The authors found specific differentially methylated regions after Cd treatment, with patterns of methylation closely associated with transcriptional differences of stress response genes involved in metal transport, metabolic processes and transcriptional regulation. Likewise, some studies have shown the heritability and stability of HM stress-induced methylation changes (Rahavi et al., 2011; Ou et al., 2012). For instance, in *A. thaliana*, improved tolerance to HMs has been observed in the progeny under the same stress experienced by parental plants (Ou et al., 2012). More recently, Cong et al. (2019) showed that specific methylation changes induced by HM stress, specifically methylation changes at the Tos17 retrotransposon, displayed transgenerational inheritance through three generations. Therefore, the evidence suggests that epigenetic mechanisms contribute to HM stress adaptation through successive plant generations.

EPIGENETIC MECHANISM INVOLVED IN ALUMINUM TOXICITY

Al exposure can trigger DNA damage and cell death through a strong binding of Al ions to pectins and other structural components of the cell wall (Murali Achary and Panda, 2010). Although there are currently few studies that have explored the relationship between epigenetic regulation and aluminum tolerance (**Table 2**), current evidence suggests that Al tolerance might be conferred through DNA methylation as specific methylation changes frequently occur after Al exposure. For example, Bednarek et al. (2017) subjected five Al-tolerant and five non-tolerant triticale lines to Al exposure. Using methylation-sensitive amplification polymorphisms (MSAP) (**Box 1**), the study showed that Al exposition in both Al-tolerant and non-tolerant plants induced demethylation. These findings are consistent with other reports that describe the

TABLE 2 | Summary of epigenetic studies related to aluminum stress responses in plants.

Plant	Variety	Epigenetic modification	Method	References
Nicotiana tabaccum	*Xan-thi* nc	DNA methylation	HPLC, direct bisulfite sequencing	Choi and Sano, 2007
Sorghum bicolor	*inbred lines, YN336 and YN267*	DNA methylation	MSAP	Kimatu et al., 2011
Zea Mays	*Kenyan tropical maize (KTM)*	DNA methylation	MSAP	Kimatu et al., 2013
Arabidopsis thaliana	*Col-0 ecotype*	DNA methylation, histone modifications	Chromatin Immuno-precipitation (ChIP), direct bisulfite sequencing.	Ezaki et al., 2016
Triticale inbred lines		DNA methylation	MSAP	Bednarek et al., 2017
Zea mays	*cultivar RX9292*	DNA methylation	CRED–RA	Taspinar et al., 2018
Triticale inbred lines		DNA methylation	metAFLP, MSAP, HPLC	Agnieszka, 2018
Triticum aestivum	*Haymana 79, Kılçıksız, and Bezostaja 1*	DNA methylation	CRED-iPBS	Pour et al., 2019

BOX 1 | Methods to study DNA methylation.

metAFLP (Amplified fragment length polymorphism) – *metAFLP* is a variation of the AFLP method. Nowadays it is poorly implemented given the emergence of genomic-scale methods. It is a cost-effective methodology that was used to elucidate methylation patterns in plants. The technique is able to detect global methylation marks throughout the studied genome. It is based on isoschizomers implementation to cut the DNA inside specific sites that display differential sensitivity to DNA methylation. A fragment comparison analysis reveals specific methylation polymorphisms. A major limitation is that it can only assess a small percentage of a global DNA methylation scenario. An important advantage is that these methods can be used for any species, even with limited or no information about their DNA sequence composition (Bednarek et al., 2007).

MSAP (Methyl Sensitive Amplified Polymorphism) – This technique is a modification of the *metAFLP* technique described above. The protocol uses the *Eco*RI restriction enzyme in combination with the methylation-sensitive enzymes *Hpa*II and *Msp*I. These last isoschizomers recognize and cleave the same tetranucleotide sequence 5′-CCGG, but differ in the sensitivity to cytosine methylation. The method can differentiate among methylated, hemimethylated, or non-methylated sites. This technique was broadly implemented because of its cost-effective advantages, but one of its principal limitations is that it cannot specify the region or gene influenced by methylation (Bednarek et al., 2017).

CRED-RA (Coupled restriction enzyme digestion and random amplification) – Similar technique as the ones previously described. It is based on the use of restriction enzymes, specifically the isoschizomers *Hpa*II and *Msp*I implemented as Random Amplified Polymorphic DNA (RAPD) (Erturk et al., 2015).

HPLC (high-performance liquid chromatography) – There are several variants for this methodology but in general it involves the enzymatic hydrolysis of DNA to its deoxyribonucleotide components and subsequent separation and quantification of the nucleotides by high-performance liquid chromatography. The system gives highly reproducible results and, under suitable conditions, it is capable of measuring 5-methylcytosine levels even at low DNA concentrations. This method is implemented by comparing control samples versus treatments to evaluate genome-wide methylated cytosines. A major drawback is that the method is incapable of determining the sequence context of the methylated cytosine (Ramsahoye, 2002).

WGBS (Whole-genome bisulfite sequencing) – It is considered as the "gold standard" method in DNA methylation studies. This technique is based on whole-genome sequencing protocols, after bisulfite conversion of DNA. The bisulfite DNA treatment mediates the deamination of non-methylated cytosines into uracil, and these converted residues will be read as thymine, after subsequent high throughput sequence analysis. The main limitations are cost and bioinformatic analysis of NGS data, which can be overcome with reduced representation bisulfite sequencing (RRBS), where only a genome fraction is sequenced (Kurdyukov and Bullock, 2016).

effects of HMs on methylation patterns (Aina et al., 2004; Filek et al., 2008; Ou et al., 2012; Feng et al., 2016). However, the opposite pattern has also been reported; for example, by using coupled restriction enzyme digestion and random amplification (CRED-RA) in corn (*Zea mays* cv. RX9292), Taspinar et al. (2018) established that exposure to Al induced mobilization of long terminal repeat retrotransposons (LTR) and triggered DNA hypermethylation as a protective response to the stress condition. Complementarily, Agnieszka (2018) compared liquid chromatography (RP-HPLC), MSAP analysis and methylation amplified fragment length polymorphisms (metAFLP) (**Box 1**) to detect DNA methylation levels of triticale lines showing contrasting tolerance to Al treatments. After Al exposure, a reduction in DNA methylation across non-tolerant lines was identified with the RP-HPLC technique, in contrast, increased methylation was seen in tolerant plants; this outcome was independent of the Al dose. When MSAP was used, increased demethylation was found in the roots of both, non-tolerant and tolerant lines, with no differences between them. Finally, metAFLP results demonstrated no differences in DNA methylation under stress conditions, suggesting that only a portion of the genome responds to Al stress.

Pour et al. (2019) used CRED_RA in three wheat cultivars (cv. Haymana79, Kılçıksız, and Bezostaja1) to evaluate genetic and epigenetic variations to different Al conditions (7.5 and 30mM). DNA hypermethylation was observed in wheat plants at higher Al concentration (30 mM) and hypomethylation at lower Al concentration (7.5 mM). These results suggest a gradual effect of Al on methylation, with concomitant cellular damages associated with increased Al toxicity. A methylation increase along the genome was concluded to confer a protective response in the affected plants. Thus, the existing evidence points to a complex influence of DNA methylation on the response to Al-induced stress in a species-dependent manner.

Methylation changes caused by Al exposure can be targeted to specific genomic locations. Choi and Sano (2007) showed a direct effect of Al over methylation changes in stress response genes in wild tobacco plants (*Nicotiana tabaccum* cv Xan-thi nc). The study showed that Al stress promotes demethylation in the coding region of the glycerophosphodiesterase-like protein gene (NtGPDL) resulting in enhanced expression. NtGPDL belongs to the glycosylphosphatidylinositol-anchored protein (GAP) family linked to the extracellular matrix. Although the function of this gene is unclear, it seems to be involved in stress responses, including Al stress in tobacco (Borner et al., 2003). Similarly, in transformed S-adenosylmethionine (SAM) *Arabidopsis* plants. The inserted gene derived from the Al-tolerant plant, *Andropogon virginicus* (AvSAMS1), conferred enhanced Al tolerance to *A. thaliana*. This enzyme represents the main methyl group donor in plants and appears to play an important role in the epigenetic stress response. Overexpression of the AvSAMS1 resulted in changes both in DNA and histone H3 methylation after plant exposure to Al. More interestingly, there were differences in the demethylation and methylation patterns at different positions in the promoter and coding regions of this gene (Ezaki et al., 2016).

Transposable elements play a role in Al stress responses. Kashino-Fujii and colleagues analyzed Al-tolerant accessions of barley derived from a multi-retrotransposon-like (MRL) insertion, located upstream of the coding region of the HvAACT1 gene. This gene is responsible for citrate efflux in roots, a mechanism involved in Al detoxification. The MRL insertion acted as a promoter and significantly enhanced HvAACT1 expression in Al-tolerant plants. This study showed that both the MRL insertion and gene expression, are due to demethylation processes, and are necessary for Al tolerance in barley. Additionally, transposon insertions close to genes have been proposed as a source of epialleles, and as a mechanism

affecting the transcriptional regulation of specific genes (Slotkin and Martienssen, 2007; Kashino-Fujii et al., 2018). Moreover, methylation would have a role in controlling genes associated with Al tolerance in plants.

DNA METHYLATION AS A REGULATORY FACTOR IN PLANT RESPONSES TO ALUMINUM STRESS: RICE AS A STUDY CASE

Epigenetics has the potential to explain mechanistically, at least part of the molecular responses to different abiotic stresses, including HM toxicity (**Figure 2**). Although there are no studies related to the epigenetic regulation of Al tolerance in rice, we hypothesize that epigenetic mechanisms, like DNA methylation, could play an important role as a regulatory factor in this response. Potentially, several of the genes mentioned in this review might be regulated through differential patterns of DNA methylation. To test this assumption, we performed a brief analysis to quantify the methylation status of specific Al responsive genes in three different rice varieties (IR64, Nipponbare, and Pokkali) with contrasting responses to Al exposure.

For this evaluation, we analyzed publicly available data from Stroud et al. (2013) obtained from the Nipponbare cultivar (highly tolerant to Al toxicity) and from Garg et al. (2015) for IR64 and Pokkali varieties (susceptible to Al toxicity). To explore the possible role of methylated cytosines over gene expression, in a set of 250 genes associated with Al tolerance in rice (Arenhart et al., 2014; Arbelaez et al., 2017), we calculated the number of methylated cytosines considering the different methylation contexts (counting was performed 1000 bps before and after the transcription initiation site). According to the reported experimental data, these 250 genes showed significant changes in expression after Al exposure (upregulated genes Log2FC ≥ 1, downregulated genes Log2FC ≤ -1) (**Supplementary Table 1**). Additionally, to increase the probability that the effects over gene expression were caused by an epigenetic regulation solely, we filtered out from this list, those genes with differences in copy number or with SNP variations in the coding region, retaining for the analysis only single-copy genes identified from the rice genes paralogous list generated by Lin et al. (2008) and without SNPs variants identified from the database Rice SNP-Seek database (Mansueto et al., 2017[1]). As a result, a group of 72 genes was kept, representing 10% of genes with the highest counts for methylated cytosines (**Supplementary Table 2**). After filtering by gene duplication and SNPs variants, we retained 26 candidate genes (**Supplementary Figure 1** and **Supplementary Table 3**). Among the three analyzed varieties, taking into account the different methylation contexts, and the localization of the methylated cytosines, Nipponbare exhibited more methylated sites than the other two varieties ($p \leq 0.01$ in an FDR analysis), while IR64 and Pokkali did not show differences in methylation (**Figure 3**). These results are interesting since Nipponbare has been extensively reported as a cultivar highly tolerant to Al (Famoso et al., 2010).

At the top of the list, representing highly methylated genes (**Table 3**), we found some genes previously reported as important players in rice Al tolerance. For example, the Calmodulin binding protein (Loc_Os09g13890) is a calcium ion-binding molecule that regulates different cellular processes, and recently, the association of the Calmodulin signal transduction pathway to Al stress has been reported (Zhang et al., 2016). This study showed that transgenic *Saccharomyces cerevisiae* strains transformed with the Calmodulin gene were more tolerant to Al toxicity, suggesting that the gene is a good candidate for improving Al tolerance in plants through transgenic approaches. Similarly, our analyses also showed the proteins STAR1 (Loc_Os06g48060) and ART1 (Loc_Os12g07280) as relevant in Al-related methylation. STAR1 encodes a nucleotide-binding domain that associates with STAR2, which encodes a transmembrane domain, to form a bacterial-type ABC transporter required for Al detoxification in roots (**Table 1**; Huang et al., 2009). On the other hand, the ART1 zinc finger protein is a transcription factor that regulates around 31 genes, probably involved in Al detoxification at different cellular levels, including STAR1 and STAR2 genes (Yamaji et al., 2009). Our results suggest that the methylation status of reported Al response genes, could play a role in Nipponbare's Al tolerance.

[1]https://snp-seek.irri.org/

TABLE 3 | Top 10 of genes with the highest methylated cytosines counts for three *O. sativa* varieties with different Aluminum tolerance levels.

Gene (MSU id)	Annotation	IR64	Nipponbare	Pokkali
Loc_Os12g32850	Cytochrome P450 71E1, putative	202	949	273
Loc_Os09g13890	Calmodulin binding protein, putative, expressed	202	1075	159
Loc_Os12g42860	Cysteine dioxygenase	161	937	219
Loc_Os03g11950	CRAL/TRIO domain containing protein, expressed	137	1059	156
Loc_Os06g48060	Protein STAR1	130	1155	175
Loc_Os05g51470	2-aminoethanethiol dioxygenase, putative, expressed	115	1053	143
Loc_Os12g07280	Zinc finger protein ART1	109	1024	99
Loc_Os12g06660	Actin-7, putative, expressed	99	990	121
Loc_Os04g33640	Glycosyl hydrolases family 17, putative, expressed	83	1357	94
Loc_Os09g37510	DUF292 domain containing protein, expressed	69	941	82

Annotations were performed using the uniprot database.

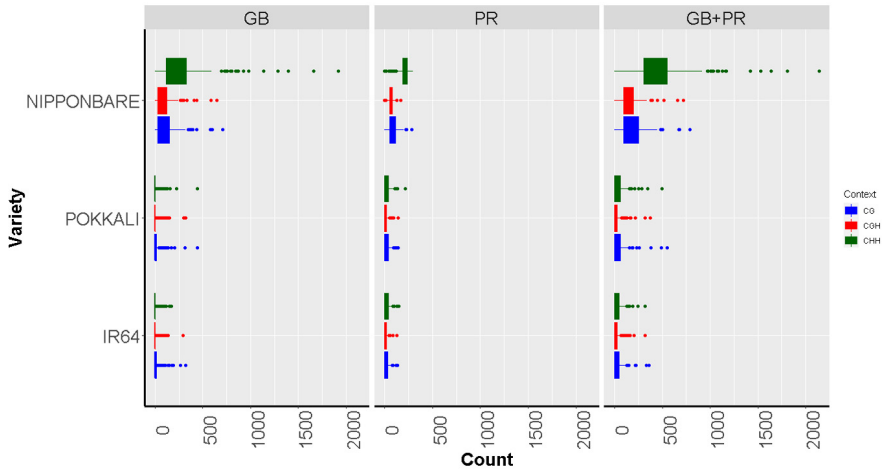

FIGURE 3 | Boxplots showing methylated cytosine frequency in three sequence contexts: CG (blue), CHG (red), and CHH (green) among three different rice varieties with contrast responses to aluminum exposure: Nipponbare (Tolerant), Pokkali, and IR64 (Susceptible). The results are discriminated according to the location of the epigenetic mark, either inside the gene body region (GB), the promoter (PR), or both the promoter and inside the gene body region of analyzed genes (PR + GB).

ALUMINUM BENEFICIAL EFFECTS FOR PLANTS

Although Al has been mainly studied for its toxic effects on plants, it can also generate benefits by inhibiting other toxic minerals, increasing defense against pathogens and by stimulating the absorption of specific nutrients as Mg, Ca, K, and P (Bojórquez-Quintal et al., 2017). Likewise, several reports show that Al can stimulate growth of both, plants adapted to acid soils (Gulli et al., 2018; Muhammad et al., 2019), and growth of commercially important crops as rice (Famoso et al., 2011) and corn (Wang et al., 2015). In plants like tea the presence of Al in soil stimulates root growth whereas its absence results in stunned plants (Fung et al., 2008). Both beneficial and negative effects are related to Al availability (Bojórquez-Quintal et al., 2017).

Some beneficial effects generated by Al are consequences of Al^{3+} cellular interactions. For example, organic acids that are exudated as a response to Al exposure, promote root growth and can increase the availability and uptake of P when it is present at limiting conditions (Muhammad et al., 2019). Currently, there are no reports of epigenetic mechanisms directly related to positive responses to Al toxic conditions, but it is possible to hypothesize that the epigenetic regulation of genes associated with the biosynthesis of organic acids, can indirectly and positively influence tolerant phenotypes in certain plants. Likewise, there are many other genes involved in metabolic processes as antioxidant enzymes, for which changes in their expression can be epigenetically regulated (Bojórquez-Quintal et al., 2017).

CONCLUSION AND PERSPECTIVES

Current knowledge of HM and Al tolerance in plants has been extensively documented with a direct focus on the physiological, and biochemical effects of these molecules, and their negative impacts on crop production. In rice, there is abundant information about genes and QTLs involved in Al tolerance in comparison with other staple cultivars such as barley or even the model plant *A. thaliana*. Nevertheless, recently, epigenetic mechanisms have emerged as important factors in the response of plants to HM stresses. Two main epigenetic strategies are relevant: (i) epigenetic marks are used as a mechanism to protect plants from possible DNA damage caused by metal ions through random DNA methylation along the genome, and (ii) epigenetic changes are used for the regulation of transposon and stress-responsive genes (**Figure 2**).

The studies carried out so far are evidence of putative epigenetic changes caused by HM exposure. However, it is necessary to evaluate the patterns of DNA methylation, as well as histone modifications occurring in precise genome regions to understand the possible epigenetic mechanisms underlying the regulation of the complex gene networks of Al tolerance responses. Likewise, there is a need for development of bioinformatics pipelines for epigenetic analyses. Future studies will be mandatory to evaluate the stability of the reported epigenetic changes through generations, given that epialleles can become permanent marks affecting genotypes and phenotypic responses. Finally, we report an overall greater abundance of methylated cytosines in an Al-tolerant rice variety, showing a contrasting methylation pattern related to differentially expressed Al responsive genes. This supports the hypothesis of DNA methylation as a fundamental key factor in the rice response to Al exposure.

AUTHOR CONTRIBUTIONS

JG-F and CS performed the methylation analysis on Nipponbare, IR64, and Pokkali, and wrote and checked the manuscript.

TG-H and MQ designed, edited, and checked the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.602625/full#supplementary-material

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From Trash to Luxury: The Potential Role of Plant LncRNA in DNA Methylation During Abiotic Stress

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Remarkable progress has been made in elucidating important roles of plant non-coding RNAs. Among these RNAs, long noncoding RNAs (lncRNAs) have gained widespread attention, especially their role in plant environmental stress responses. LncRNAs act at different levels of gene expression regulation, and one of these mechanisms is by recruitment of DNA methyltransferases or demethylases to regulate the target gene transcription. In this mini-review, we highlight the function of lncRNAs, including their potential role in RNA-directed DNA Methylation (RdDM) silencing pathway and their potential function under abiotic stresses conditions. Moreover, we also present and discuss studies of lncRNAs in crops. Finally, we propose a path outlook for future research that may be important for plant breeding.

Keywords: epigenetic, non-coding RNAs, gene regulation, environmental stresses, plant breeding

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INTRODUCTION

In 1970, the central dogma of molecular biology was proposed, suggesting that the flow of information would follow the DNA to RNA to Protein (Crick, 1970). With the sequencing of the human genome, it was found that only about 3% of the genomic DNA encoded proteins and the rest was composed of the so-called "junk" DNA, including transposable elements (TEs) and highly repetitive DNA (Nowak, 1994). They also show that despite of not encoding proteins, the vast majority of human genome is transcribed into RNA. This also occurs in plant genomes. For instance, in *Arabidopsis thaliana*, the minority of its genome has the capacity of encoding proteins (Yamada et al., 2003). Nowadays, what initially was considered trash DNA became the luxury, as researchers are unraveling important roles out of the genomic non-coding sequences.

Non-coding RNAs (ncRNAs) include a huge variety of RNAs. The regulatory ncRNAs contain small RNAs (sRNAs) and long non-coding RNAs (lncRNAs) that do not encode proteins, but can generate small peptides (BenAmor et al., 2009). The best characterized are the sRNAs: microRNAs (miRNAs) and small interference RNAs (siRNAs). Several studies have highlighted the important role of sRNAs in transcriptional and post-transcriptional regulation of gene expression in plants. Although lncRNAs were previously considered to be "transcriptional noise," emerging plant studies have Also, revealed the crucial involvement of lncRNAs in various biological processes including flowering (Fan et al., 2016), development (Zhang and Chen, 2013) and stresses responses (Sun et al., 2020).

LncRNAs are classified as ncRNAs longer than 200 nt (Ma et al., 2013). The first lncRNAs with regulatory function identified in plants was the enod40 (early nodulin 40) in Medicago, a

"riboregulator" involved in plant growth (Crespi et al., 1994). With the advance of computational methods, 503 mRNA (messenger RNA)-like transcripts that appear to not encode proteins were identified in Medicago (Wen et al., 2007). Then, an increasing number of lncRNAs have been found by computational approach in different plant species (Vieira et al., 2017; Danilevicz et al., 2018). CANTATAdb[1] is one database created to deposit these sequences, actually it collects 239,631 lncRNAs predicted in 39 species (Szcześniak et al., 2015). Although, the sequences of most lncRNAs are much less conserved than those of mRNAs, analysis of primary sequence conservation using 10 plant species revealed that the majority of lncRNAs had high sequence conservation at the intra-species and sub-species levels, in contrast to the highly diverged inter-species level (Deng et al., 2018). Moreover, lncRNAs are less expressed than mRNAs, which requires high sensitivity techniques such as RNA fluorescence *in situ* hybridization (RNA FISH), and real-time quantitative polymerase chain reaction (qRT-PCR) for the analysis of expression (Wu et al., 2020). Another feature of lncRNAs is their genomic localization, that can be located in intergenic, intronic, or coding regions, both at the sense and antisense directions (Wu et al., 2020). Interestingly, lncRNAs are regulated in response to various stimulus. Analysis of 76 lncRNAs in Arabidopsis revealed that 22 lncRNAs showed altered expression under abiotic stress (BenAmor et al., 2009). For instance, npc60 showed to be 100 times more expressed under salt stress. In cotton, lncRNA973 was increased by salt treatments and analysis by *in situ* hybridization showed that it was localized mainly to the nucleus (Zhang et al., 2019). Some studies use the subcellular localization of lncRNAs to infer their functions, since it can act both in the nucleus and cytoplasm (Karlik et al., 2019).

Although plant lncRNAs have a potential role in regulating plant responses to environmental conditions, their mechanism of function in gene regulation is poorly understood. Here, we highlight some studies that have been analyzing the importance of lncRNAs in plants. First, we included the potential roles of lncRNAs on RNA-directed DNA methylation (RdDM) silencing pathway, since many genes are methylated in response to abiotic stress. Despite showing studies on model plants, we also discuss studies of lncRNAs carried on crops, with the potential used as tools for biotechnological improvement of plants.

LncRNAs AS PRECURSORS IN RdDM SILENCING PATHWAY

LncRNAs can act as key genetic and epigenetic regulators of gene expression (Karlik et al., 2019). They may function as *cis*-acting elements by working near the site of RNA synthesis, acting directly on consecutive genes on the same strand (Zhao et al., 2020; **Figure 1A**); or as *trans*-acting factors by operating far from the site of synthesis (Suksamran et al., 2020; **Figure 1B**). LncRNAs may interfere with the binding of transcription factors to promoter regions (Csorba et al., 2014). Moreover, they can also function as miRNAs and *trans*-acting small interfering RNA (tasiRNA) precursors (Zhang et al., 2014; Fukuda et al., 2019; **Figure 1C**), miRNA target mimics (Shuai et al., 2014; **Figure 1E**) and can be processed in siRNA (Wunderlich et al., 2014). Curiously, similar to what occurs in mRNA biogenesis, the RNA polymerase II (Pol II) transcribes the majority of lncRNAs. Other RNA polymerases, such as Pol IV and Pol V that are exclusive to plants, can also act in the lncRNA generation, participating mainly in the epigenetic regulation mediated by RdDM (Wierzbick et al., 2008; Li et al., 2014). Furthermore, epigenetic mechanisms including DNA methylation (Ariel et al., 2014; **Figure 1F**) and histone modification (Heo and Sung, 2011; **Figure 1D**) are usually reported to be regulated by lncRNAs.

Plant lncRNAs play a key role in the RdDM silencing pathway. This regulatory route is based on the performance of Pol IV-dependent RNAs (P4RNAs) transcribed by Pol IV (Blevins et al., 2015; Zhai et al., 2015; Yang et al., 2016). These precursor RNAs are processed by RNA-dependent RNA Polymerase 2 (RDR2) to form double-stranded RNAs (dsRNAs), which are primarily cleaved by Dicer-like 3 (DCL3) to produce 24-nt siRNAs (Xie et al., 2004). These siRNAs are associated with Argonaute 4 (AGO4), forming AGO-siRNA complex (Holoch and Moazed, 2015). Simultaneously, lncRNAs transcribed by Pol V work as scaffold RNAs being recognized by the siRNA-AGO complex through sequence complementarity (Böhmdorfer et al., 2016). Once AGO4–siRNA–lncRNA complex is formed, it is driven to the chromatin target site together with a DNA methylation enzyme, the DNA methyltransferase domains rearranged methyltransferase 2 (DRM2) (Gao et al., 2010). This methyltransferase mediates *de novo* methylation of cytosines in all classes of sequence contexts at the target region to initiate gene silencing (Wierzbick et al., 2008). Therefore, RdDM correspond to a plant-specific *de novo* DNA methylation mechanism that requires lncRNAs as scaffold to define target genomic loci (Wierzbicki et al., 2009).

The understanding of lncRNAs role as precursors in epigenetic silencing *via* RdDM have received remarkable contributions (Chen et al., 2018, 2019). Several reports have suggested that plant lncRNAs are involved with DNA methylation performing different developmental functions such as in the regulation of embryogenesis (Chen et al., 2018), root organogenesis (Chen et al., 2019), reproduction (Ding et al., 2012), and gene silencing (Yan et al., 2018). Besides that, researchers have explored the potential of stress-regulated lncRNAs to trigger DNA methylation in response to environmental conditions. The well-characterized *AUXIN REGULATED PROMOTER LOOP (APOLO)* was identified as an auxin-induced lncRNA in Arabidopsis (Ariel et al., 2014). The double transcription of *APOLO* by Pol II and V was reported as responsible for originating a chromatin loop, which encompasses the promoter of its neighboring gene *PINOID (PID)*, a key regulator of polar auxin transport, leading to downregulation of its transcripts. Alternatively, it was proposed that *APOLO* also recognizes distant non-associated loci by R-loop formation. *APOLO*-mediated LIKE HETEROCHROMATIC PROTEIN 1 (LHP1) decoy may trigger the transcription of the target loci modulating local chromatin conformation, co-regulating auxin-responsive genes (Ariel et al., 2020). A systematic methylome

[1]http://cantata.amu.edu.pl, http://yeti.amu.edu.pl/CANTATA/

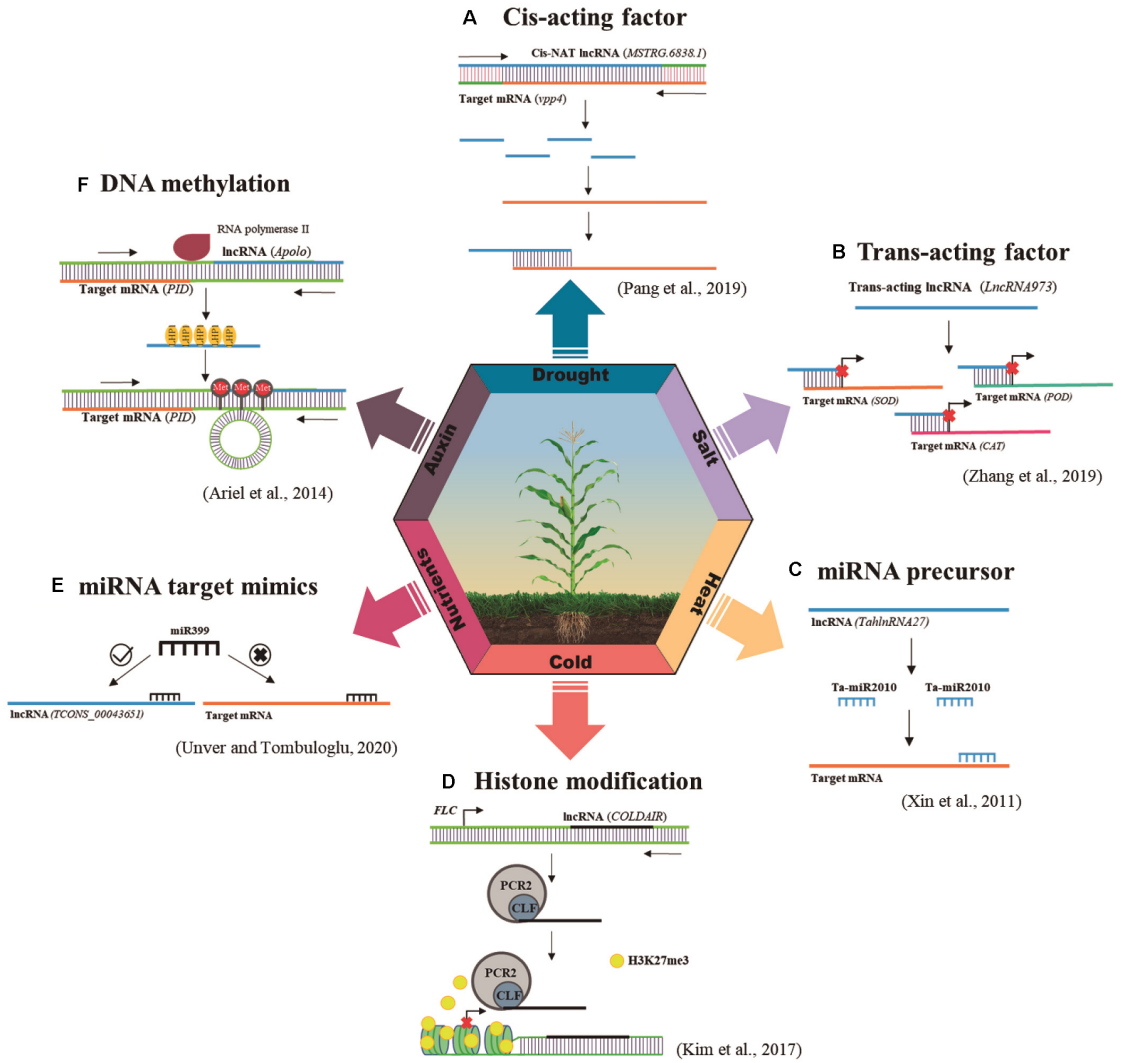

FIGURE 1 | Regulatory mechanisms of plant lncRNAs in response to abiotic stresses. The main mechanisms of action triggered by lncRNAs responsive to abiotic stresses are miRNA precursor, histone modification, target mimicry, RdDM, *cis*-acting factor and *trans*-acting factor. This figure illustrates one example of each of these mechanisms. **(A)** *Cis*-acting factor: *vpp4* encoding a vacuolar (H^+)-pumping ATPase subunit was identified as a putative target of an adjacent lncRNA *MSTRG.6838.1*. The expressions of *vpp4* and *MSTRG.6838.1* were significantly correlated in many tissues and development stages, being both repressed under drought stress, which indicates that *MSTRG.6838.1* and *vpp4* could be a promising *cis*-acting pair (Pang et al., 2019). **(B)** *Trans*-acting factor: *LncRNA973* corresponds to a *trans*-acting lncRNA responsive to salt stress, which regulates plant stress responses by modulating the expression of a series of key salt-related genes, as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (Zhang et al., 2019). **(C)** miRNA precursor: *TahlnRNA27*, a heat-induced lncRNA, can act as a miRNA precursor since it presents Ta-miR2010 family sequences. After 1 h of heat-treatment, *TahlnRNA27* expression was induced as well as Ta-miR2010 expression. The secondary structure and the corresponding expression pattern indicate that *TahlnRNA27* might be the precursor of Ta-miR2010 (Xin et al., 2011). **(D)** Histone modification: The repression of *FLC* by vernalization is accompanied by a series of changes in histone modifications at *FLC* chromatin, including the deposition of repressive histone markers, such as Histone H3 Lys 27 (H3K27me3). *COLDAIR* is up-regulated in response to cold, physically interacting with a component of PRC2, CURLY LEAF (CLF), for the increased enrichment of PRC2 at *FLC* chromatin to promote H3K27me3 accumulation at *FLC* (Kim et al., 2017). **(E)** miRNA target mimics: *TCONS_00043651* function as a potential natural miRNA sponge of miR399 sequence in response to boron-stress. Results obtained from barley roots analysis showed that *TCONS_00043651* was up-regulated (three times than that of control) upon boron-exposure, meanwhile miR399 expression was repressed (three times down-regulated) in the same stress conditions (Unver and Tombuloglu, 2020). **(F)** DNA methylation: *APOLO* can trigger RdDM in response to an auxin stimulus. In response to auxin, Pol II *APOLO* transcripts gradually recruit LHP1 to mediate loop formation, whereas Pol IV/V transcription triggers DNA methylation. Then, Pol II *APOLO*-LHP1 mediated loop is conformed and maintained by Pol IV/V-dependent DNA methylation to repress PID expression (Ariel et al., 2014).

study (Song et al., 2016) evaluated DNA methylation changes in *Populus simonii* submitted to salinity, osmotic and temperature stress, suggesting that, in association with miRNAs and lncRNAs, this regulatory mechanism can act in response to abiotic stresses in poplar. Ultimately, analysis in soybean roots continuously treated with high salinity solutions revealed that more than

75% of the lncRNAs identified were activated or induced in transcriptome sequencing (Chen et al., 2019).

The RdDM pathway constitutes an impressive extension of the transcriptional capacity of eukaryotic organisms, being considered the main epigenetic pathway mediated by siRNA in plants (Matzke and Mosher, 2014). The canonical RdDM pathway involves the recruitment of Pol IV to transcribe single-stranded RNAs (ssRNAs) at its target loci. The RDR2 copies the ssRNAs to produce dsRNAs. DCL3 processes dsRNAs to 24-nt siRNAs. Finally, *de novo* methylation occurs, which requires Pol V–dependent scaffold RNAs, AGO4-bound 24-nt siRNAs, and DRM2 (Mosher et al., 2008). Meanwhile, non-canonical RdDM pathways provides a link between PTGS of transposon transcripts and *de novo* methylation of transposon DNA, since it was reported that tasiRNAs and transposons are initially transcribed by Pol II, copied by RDR6 and processed by DCL2 and DCL4 into 21–22-nt siRNAs (Matzke et al., 2015). Additionally, experiments conducted in Arabidopsis *dcl1/2/3/4* mutants by Yang et al. (2016) demonstrated that DNA methylation at many of the RdDM target loci did not correlate with 24-nt siRNAs and it was completely independent of DCLs. Instead, it was observed that 25–50 nt RNAs were the main class of sRNAs generated from most RdDM loci in *dcl* plants. Interesting studies have contributed to broaden our understanding about RdDM biological functions of RdDM, reporting its involvement in regulating transposon silencing (La et al., 2011), gene expression (Lang et al., 2017), plant development (Kawakatsu et al., 2017), and biotic interactions (Satgé et al., 2016). Special attention has been given to the potential roles of DNA methylation in plant responses to a wide range of abiotic stresses, such as nutritional deficit (Secco et al., 2015), temperature (Liu et al., 2018), high salinity (Lira-Medeiros et al., 2010), and drought (Wang et al., 2016). Despite great efforts, issues such as the mechanism, biological roles and evolutionary importance of RdDM still remains to be fully elucidated, as well as the fundamental role that lncRNAs may be playing in regulating this silencing mechanism.

Functional investigations suggested the contributions of lncRNAs as essential modulators in plant responses to stresses (**Figure 1**). A growing body of evidence points to the great potential role for plant lncRNAs in responses to abiotic stresses *via* RdDM (Ariel et al., 2014; Yong-Villalobos et al., 2015). Given the limited number of studies, it is assumed that there is a great potential for RdDM-associated lncRNAs to be studied.

LncRNAs AS PRECURSORS TO ABIOTIC STRESS RESPONSES

Here, we briefly summarize recent examples of lncRNAs responsive to abiotic stresses in different plant species, with an emphasis on crop species, providing details of other mechanisms of action, in addition to the aforementioned epigenetic silencing *via* RdDM (**Table 1**).

A genome-wide study by Fukuda et al. (2019) reported lncRNAs that are involved in the response to low availability of nutrients in Arabidopsis, allowing the identification of 60 differentially expressed lncRNAs. Among them, *TAS3* was revealed as repressed under low-nitrogen conditions with high affinity to target *nitrate transporter 2.4* (*NRT2.4*). Similarly, a genome-wide strategy was used to identify lncRNAs differentially expressed in response to nutritional stress in poplar (Chen et al., 2016) and Arabidopsis (Franco-Zorrilla et al., 2007).

Extreme temperatures can also alter plants lncRNAs expression. In Arabidopsis, *HSFB2a* is a heat shock gene required for the gametophytic development, controlled by an antisense heat-inducible lncRNA, *asHSFB2a* (Wunderlich et al., 2014). Intriguingly, the overexpression of *asHSFB2a* represses *HSFB2a* RNA accumulation and overexpression of *HSFB2a* has a similar negative effect on *asHSFB2a* expression. Despite the lack of knowledge of the molecular mechanisms involved in this "Yin–Yang" control of sense and antisense RNA expression, the study by Wunderlich et al. (2014) showed that the vegetative and gametophytic development are impacted by this regulation of gene expression at the *HSFB2a* locus. Meanwhile, 1,614 lncRNAs were found to be differentially expressed in *Brassica juncea* under heat and drought stress conditions (Bhatia et al., 2020). Cold-responsive lncRNAs have been identified in plants such as grape (Wang et al., 2019) and Arabidopsis (Calixto et al., 2019). Both *COLDAIR* and *COOLAIR* are well-characterized examples of cold-induced lncRNAs that have been detected as regulating the vernalization process through silencing of *FLOWERING LOCUS C* (*FLC*). *FLC* encodes a MADS box transcription regulator of flowering time, repressing the induction of flowering (Heo and Sung, 2011; Marquardt et al., 2014). *COLDAIR* is transcribed from the first intron of *FLC* and physically interacts with a component of Polycomb Repressive Complex 2 (PRC2) to promote H3K27me3 accumulation at the *FLC* locus (Kim et al., 2017). *COOLAIR* is an *FLC* antisense transcript, involved in *FLC* repression by both autonomous (Tian et al., 2019) and vernalization pathways (Csorba et al., 2014), inducing H3K27me3 by recruiting plant homeo-domain (PHD)-PRC2 (Swiezewski et al., 2009).

Drought and high salinity are the main environmental conditions that adversely affect plant productivity and both can perform the same effects by overlapping genetic regulatory mechanisms. For instance, *Drought Induced lncRNA* (*DRIR*) was reported in Arabidopsis as a positive regulator of plant responses to drought and salt stress (Qin et al., 2017). Previous work identified 3 up-regulated lncRNAs under NaCl treatment (BenAmor et al., 2009) and 2,815 novel salt-responsive lncRNAs were reported in *Spirodela polyrhiza* (Fu et al., 2020). Drought-responsive lncRNAs were investigated in poplars submitted to a water deficit (Shuai et al., 2014). For example, drought induced *lincRNA2752* is a target mimic of ptc-miR169, a NF-YA transcription factor regulator. Similar results were found in drought-responsive lncRNAs identified in *Cleistogenes songorica* (Yan et al., 2019) and *B. napus* (Tan et al., 2020).

LncRNA in Crop Plants

All findings reporting lncRNAs involvement in response to environmental stresses are particularly important in the context of crop species, since abiotic stresses are a major constraint to improve agriculture yields (Halford et al., 2015). Identification

TABLE 1 | Summary of studies with abiotic stress-responsive lncRNAs in plants.

LncRNA	Stress	Plant species	Regulation mechanism	Expression	References
IPS1	Phosphate deficiency	*A. thaliana*	Target mimicry	Induced	Franco-Zorrilla et al., 2007
npc536	Salt stress	*A. thaliana*	Nat. antisense siRNAs	Induced	BenAmor et al., 2009
npc60	Salt stress	*A. thaliana*	Nat. antisense siRNAs	Induced	BenAmor et al., 2009
COLDAIR	Cold stress	*A. thaliana*	Histone modification	Induced	Heo and Sung, 2011
TahlnRNA27	Heat stress	*T. aestivum*	miRNA precursor	Induced	Xin et al., 2011
TalnRNA5	Heat stress	*T. aestivum*	miRNA precursor	Induced	Xin et al., 2011
AtR8	Hypoxic stress	*A. thaliana*	*Trans*-acting factor	Repressed	Wu et al., 2012
Cis-NAT PHO1;2	Phosphate deficiency	*O. sativa*	Translation enhancer	Induced	Jabnoune et al., 2013
Si NAT 80	Drought stress	*S. italica*	*Cis*-acting factor	Induced	Qi et al., 2013
APOLO	Auxin	*A. thaliana*	DNA demethylation	Induced	Ariel et al., 2014
asHSFB2a	Heat stress	*A. thaliana*	Nat. antisense siRNAs	Induced	Wunderlich et al., 2014
COOLAIR	Cold stress	*A. thaliana*	Histone modification	Induced	Csorba et al., 2014; Marquardt et al., 2014
Lnc-173	High-light stress	*A. thaliana*	*Cis*-acting factor	Induced	Di et al., 2014
Lnc-225	High-light stress	*A. thaliana*	*Cis*-acting factor	Induced	Di et al., 2014
LincRNA1128	Drought stress	*P. trichocarpa*	Target mimicry	Repressed	Shuai et al., 2014
LincRNA2962	Drought stress	*P. trichocarpa*	Target mimicry	Induced	Shuai et al., 2014
LincRNA1039	Drought stress	*P. trichocarpa*	Target mimicry	Induced	Shuai et al., 2014
LincRNA20	Drought stress	*P. trichocarpa*	Target mimicry	Induced	Shuai et al., 2014
LincRNA2752	Drought stress	*P. trichocarpa*	Target mimicry	Induced	Shuai et al., 2014
LincRNA2623	Drought stress	*P. trichocarpa*	Target mimicry	Repressed	Shuai et al., 2014
TCONS_00056395	Drought stress	*Z. mays*	miRNA precursor	Induced	Zhang et al., 2014
TCONS_00082174	Drought stress	*Z. mays*	miRNA precursor	Induced	Zhang et al., 2014
GRMZM2G088590_T04	Drought stress	*Z. mays*	miRNA precursor	Induced	Zhang et al., 2014
TCONS_00037470	Drought stress	*Z. mays*	miRNA precursor	Induced	Zhang et al., 2014
TCONS_00012768	Drought stress	*Z. mays*	miRNA precursor	Induced	Zhang et al., 2014
XLOC_011965	Cadmium stress	*O. sativa*	Unknown	Induced	He et al., 2015
XLOC_054416	Cadmium stress	*O. sativa*	Unknown	Induced	He et al., 2015
XLOC_001126	Cadmium stress	*O. sativa*	Unknown	Repressed	He et al., 2015
XLOC_048220	Cadmium stress	*O. sativa*	Unknown	Repressed	He et al., 2015
TCONS_00046739	Salt stress	*M. truncatula*	Unknown	Induced	Wang et al., 2015
TCONS_00100258	Salt stress	*M. truncatula*	Unknown	Induced	Wang et al., 2015
TCONS_00118328	Salt stress	*M. truncatula*	Unknown	Induced	Wang et al., 2015
Os02g0250700-01	Drought stress	*O. sativa*	Nat. antisense transcript	Repressed	Chung et al., 2016
Os02g0180800-01	Drought stress	*O. sativa*	Nat. antisense transcript	Repressed	Chung et al., 2016
TCONS_00052316	Low-nitrogen stress	*P. tomentosa*	Target mimicry	Repressed	Chen et al., 2016
TCONS_00069233	Low-nitrogen stress	*P. tomentosa*	Target mimicry	Repressed	Chen et al., 2016
TCONS_00052315	Low-nitrogen stress	*P. tomentosa*	Target mimicry	Repressed	Chen et al., 2016
TCONS_00064021	Low-nitrogen stress	*P. tomentosa*	*Cis*-acting factor	Repressed	Chen et al., 2016
TCONS_00049805	Low-nitrogen stress	*P. tomentosa*	*Cis*-acting factor	Repressed	Chen et al., 2016
TCONS_00017288	Low-nitrogen stress	*P. tomentosa*	Unknown	Induced	Chen et al., 2016
TCONS_0002186	Low-nitrogen stress	*P. tomentosa*	*Cis*-acting factor	Induced	Chen et al., 2016
TCONS_00021860	Low-nitrogen stress	*P. tomentosa*	Unknown	Induced	Chen et al., 2016
c70772_g2_i1	Drought stress	*T. turgidum*	Target mimicry	Induced	Cagirici et al., 2017
c90557_g1_i1	Drought stress	*T. turgidum*	Target mimicry	Induced	Cagirici et al., 2017
TCONS_00043651	Boron stress	*H. vulgare*	Target mimicry	Induced	Karakulah and Unver, 2017
DRIR	Drought and salt stress	*A. thaliana*	Unknown	Induced	Qin et al., 2017
AK370814	Salt stress	*H. vulgare*	*Cis*-acting factor	Induced	Karlik and Gozukirmizi, 2018
LncRNA_082364	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Induced	Ma et al., 2018
LncRNA_047461	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Induced	Ma et al., 2018
LncRNA_074658	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Repressed	Ma et al., 2018
LncRNA_000823	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Repressed	Ma et al., 2018
LncRNA_058136	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Repressed	Ma et al., 2018

(Continued)

TABLE 1 | Continued

LncRNA	Stress	Plant species	Regulation mechanism	Expression	References
LncRNA_008977	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Induced	Ma et al., 2018
LncRNA_061738	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Induced	Ma et al., 2018
LncRNA_018111	Ca^{2+} -channel blocking	*T. aestivum*	*Trans*-acting factor	Induced	Ma et al., 2018
MSTRG.4636	Heat stress	*Z. mays*	Unknown	Repressed	Lv et al., 2019
MSTRG.38321	Heat stress	*Z. mays*	Unknown	Repressed	Lv et al., 2019
MSTRG.11125	Heat stress	*Z. mays*	Unknown	Induced	Lv et al., 2019
MSTRG.15555	Heat stress	*Z. mays*	Unknown	Induced	Lv et al., 2019
MSTRG.31362	Heat stress	*Z. mays*	Unknown	Induced	Lv et al., 2019
MSTRG.63799	Heat stress	*Z. mays*	Unknown	Repressed	Lv et al., 2019
AT1G34844	Cold stress	*A. thaliana*	Nat. antisense transcript	Induced	Calixto et al., 2019
AT3G26612 I	Cold stress	*A. thaliana*	Nat. antisense transcript	Induced	Calixto et al., 2019
TAS3	Low-nitrogen stress	*A. thaliana*	*Trans*-acting factor	Repressed	Fukuda et al., 2019
LncRNA-tomato_535	Drought stress	*S. lycopersicum*	Target mimicry	Induced	Eom et al., 2019
LncRNA-tomato_146	Drought stress	*S. lycopersicum*	Target mimicry	Induced	Eom et al., 2019
LncRNA-tomato_178	Drought stress	*S. lycopersicum*	Target mimicry	Induced	Eom et al., 2019
LncRNA_tomato_467	Drought stress	*S. lycopersicum*	Unknown	Induced	Eom et al., 2019
MSTRG.6838.1	Drought stress	*Z. mays*	*Cis*-acting factor	Repressed	Pang et al., 2019
VIT_216s0100n00030	Cold stress	*V. vinifera*	*Cis*-acting factor	Induced	Wang et al., 2019
LXLOC_027751	Cold stress	*V. vinifera*	*Cis*-acting factor	Induced	Wang et al., 2019
LXLOC_010422	Cold stress	*V. vinifera*	*Cis*-acting factor	Induced	Wang et al., 2019
VIT_202s0025n00100	Cold stress	*V. vinifera*	*Cis*-acting factor	Induced	Wang et al., 2019
VIT_200s0225n00020	Cold stress	*V. vinifera*	*Trans*-acting factor	Repressed	Wang et al., 2019
MSTRG.43964.1	Drought stress	*C. songorica*	Target mimicry	Induced	Yan et al., 2019
MSTRG.4400.2	Drought stress	*C. songorica*	Target mimicry	Induced	Yan et al., 2019
LncRNA973	Salt stress	*G. hirsutum*	*Trans*-acting factor	Induced	Zhang et al., 2019
TCONS_00024229	Salt stress	*S. polyrhiza*	*Cis*-acting factor	Induced	Fu et al., 2020
TCONS_00057092	Salt stress	*S. polyrhiza*	*Cis*-acting factor	Induced	Fu et al., 2020
TCONS_00018576	Salt stress	*S. polyrhiza*	*Cis*-acting factor	Induced	Fu et al., 2020
TCONS_00023928	Salt stress	*S. polyrhiza*	*Cis*-acting factor	Induced	Fu et al., 2020
TCONS_00045028	Salt stress	*S. polyrhiza*	*Cis*-acting factor	Induced	Fu et al., 2020
TCONS_00033722	Salt stress	*S. polyrhiza*	Target mimicry	Induced	Fu et al., 2020
TCONS_00018793	Salt stress	*S. polyrhiza*	Target mimicry	Induced	Fu et al., 2020
TCONS_ 00045706	Salt stress	*S. polyrhiza*	Target mimicry	Induced	Fu et al., 2020
TCONS_00057092	Salt stress	*S. polyrhiza*	Target mimicry	Induced	Fu et al., 2020
TCONS_ 00045512	Salt stress	*S. polyrhiza*	Target mimicry	Induced	Fu et al., 2020
TCONS_00051908	Heat stress	*B. juncea*	Unknown	Induced	Bhatia et al., 2020
TCONS_00088973	Drought stress	*B. juncea*	Unknown	Induced	Bhatia et al., 2020
NcM9574	Cold stress	*M. esculenta*	*Cis*-acting factor	Induced	Suksamran et al., 2020
NcP12248	Cold stress	*M. esculenta*	*Cis*-acting factor	Repressed	Suksamran et al., 2020
NcM17949	Drought stress	*M. esculenta*	*Cis*-acting factor	Induced	Suksamran et al., 2020
NcP456	Cold stress	*M. esculenta*	*Trans*-acting factor	Repressed	Suksamran et al., 2020
NcP12197	Drought stress	*M. esculenta*	*Trans*-acting factor	Induced	Suksamran et al., 2020
NcM15664	Drought stress	*M. esculenta*	*Trans*-acting factor	Repressed	Suksamran et al., 2020
LncRNA13472	Salt stress	*S. bicolor*	Target mimicry	Induced	Sun et al., 2020
LncRNA14798	Salt stress	*S. bicolor*	Target mimicry	Repressed	Sun et al., 2020
LncRNA11310	Salt stress	*S. bicolor*	Target mimicry	Repressed	Sun et al., 2020
LncRNA2846	Salt stress	*S. bicolor*	Target mimicry	Repressed	Sun et al., 2020
LncRNA26929	Salt stress	*S. bicolor*	Target mimicry	Repressed	Sun et al., 2020
XLOC_012868	Drought stress	*B. napus*	Unknown	Repressed	Tan et al., 2020
XLOC_052298	Drought stress	*B. napus*	Unknown	Induced	Tan et al., 2020
XLOC_094954	Drought stress	*B. napus*	Unknown	Induced	Tan et al., 2020
TCONS_00043651	Boron stress	*H. vulgare*	Target mimicry	Induced	Unver and Tombuloglu, 2020
TCONS_00061958	Boron stress	*H. vulgare*	*Cis*-acting factor	Induced	Unver and Tombuloglu, 2020
MtCIR1	Cold stress	*M. truncatula*	*Cis*-acting factor	Induced	Zhao et al., 2020

of lncRNAs during crop stress responses remains largely premature, presenting few examples (Karakulah and Unver, 2017; Pang et al., 2019).

LncRNAs have been identified as involved in nutritional homeostasis in crops such as rice (Jabnoune et al., 2013; He et al., 2015) and wheat (Ma et al., 2018). Recent reports demonstrated roles of barley lncRNAs upon excessive boron-treatment (Karakulah and Unver, 2017; Unver and Tombuloglu, 2020). Both studies suggest that boron-regulation can be cooperatively controlled by the interaction of miRNA-lncRNA-coding target transcript modules. For instance, *TCONS_00043651*, a potential miRNA sponge of miR399, was positively regulated under boron-exposure (Unver and Tombuloglu, 2020). Oppositely, miR399 expression was repressed under this stress condition.

Whereas changes in temperature often causes yield loss, heat-responsive lncRNAs were identified in wheat (Xin et al., 2011) and maize (Lv et al., 2019). The lncRNA *TahlnRNA27* was induced under heat treatment and characterized as putative miRNA precursor by presenting Ta-miR2010 family sequences (Xin et al., 2011). Similarly, 182 novel cold-responsive lncRNAs are known to be differentially expressed in cassava (Suksamran et al., 2020); whereas 2,271 lncRNAs were cold-responsive in alfalfa (Zhao et al., 2020).

Salinity stress is currently an environmental factor that most constraints agricultural productivity (Song and Wang, 2015). Studies have attempted to expand knowledge about functional mechanisms of lncRNAs in response to salt stress as well as in alfalfa (Wang et al., 2015); barley (Karlik and Gozukirmizi, 2018); cotton (Zhang et al., 2019); and sorghum (Sun et al., 2020). In particular, the *lncRNA973* overexpression had increased salt tolerance, modulating the expression of cotton salt stress-related genes (Zhang et al., 2019).

To improve crop performance in regions limited by water deficit, studies have been conducted to investigate the drought-responsive lncRNAs in crop species including foxtail millet (Qi et al., 2013); maize (Zhang et al., 2014); rice (Chung et al., 2016); wheat (Cagirici et al., 2017); tomato (Eom et al., 2019); and cassava (Suksamran et al., 2020). A recent work carried out with maize identified 124 drought-responsive lncRNAs characterized as *cis*-acting factors (Pang et al., 2019). The repressed expression correlation between *vpp4*, encoding a vacuolar (H^+)-pumping ATPase subunit, and its adjacent lncRNA *MSTRG.6838.1* provides the idea that both could be a promising *cis*-acting pair.

CONCLUSION AND PERSPECTIVE

Due to the rapid progress in high-throughput sequencing, several findings have significantly expanded our knowledge of lncRNA biology. However, despite the relevant results reported recently, the biological role and mechanisms of action of plant lncRNAs remain poorly understood. Further studies on lncRNAs responsive to abiotic stresses in crop species will open paths for a better understanding of their function in various processes of plant development and management of stress. It is notable in **Table 1** that several lncRNAs regulated in response to abiotic stress have unknown regulation mechanisms. Remarkable progress has been made in elucidating the roles of plant lncRNAs in RdDM silencing pathway. The complexity of RdDM and its involvement in the activation of stress-responsive genes are undeniable, although more efforts are needed to understand RNA-induced DNA methylation and its function in plants, especially during abiotic stresses.

MiRNAs and lncRNAs are regulatory genes that can be targets for improving crop tolerance to abiotic stresses by using the currently advanced genome editing tools, as clustered regularly interspaced short palindromic repeats associated nucleases (CRISPR/Cas) (Zhang et al., 2020). A few successful reports on CRISPR/Cas9-based gene editing for miRNAs were published recently (Li et al., 2016; Zhou et al., 2017). The short sequences of miRNAs make it difficult to find a PAM sequence that is required for CRISPR/Cas genome editing. As more diversity of Cas proteins are identified and current Cas proteins are being continuously modified, the PAM requirement will be relaxed, and more genetic loci will become accessible by CRISPR/Cas system (Zhang and Zhang, 2020), including lncRNAs once they are already longer than miRNAs.

As the regulation for the use of genetically modified organisms (GMOs) and CRISPR-gene editing is still very tight in several countries, alternative approaches for crop breeding should be considered, such as the exogenous application of RNA molecules (Dalakouras et al., 2020). Based on successful examples of delivery of RNAs with the potential to trigger RNAi in plants (Cagliari et al., 2019; Werner et al., 2020), possible shortcomings of these methods might include optimization in application of several other types of RNA molecules, including lncRNAs, as well as grouped components of CRISPR/Cas to promote GMO independent editing events in lncRNA sequences.

AUTHOR CONTRIBUTIONS

MU and FT wrote the manuscript. AH reviewed the manuscript. PF contributed to design of this mini-review. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Chromatin Architecture in Plant Stress Responses: An Update

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Sessile plants possess an assembly of signaling pathways that perceive and transmit environmental signals, ultimately resulting in transcriptional reprogramming. Histone is a key feature of chromatin structure. Numerous histone-modifying proteins act under different environmental stress conditions to help modulate gene expression. DNA methylation and histone modification are crucial for genome reprogramming for tissue-specific gene expression and global gene silencing. Different classes of chromatin remodelers including SWI/SNF, ISWI, INO80, and CHD are reported to act upon chromatin in different organisms, under diverse stresses, to convert chromatin from a transcriptionally inactive to a transcriptionally active state. The architecture of chromatin at a given promoter is crucial for determining the transcriptional readout. Further, the connection between somatic memory and chromatin modifications may suggest a mechanistic basis for a stress memory. Studies have suggested that there is a functional connection between changes in nuclear organization and stress conditions. In this review, we discuss the role of chromatin architecture in different stress responses and the current evidence on somatic, intergenerational, and transgenerational stress memory.

Keywords: chromatin remodeling, transcription, nucleosome, histone variants, abiotic stress, epigenetics, intergenerational, transgenerational

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PLANTS UTILIZE EPIGENETIC AND CHROMATIN-MODIFYING STRATEGIES TO DEAL WITH STRESS

Plants utilize highly evolved mechanisms to improve their growth and development to face various biotic and abiotic stresses, in part due to their sessile nature. The plasticity of plants allows them to adapt and survive through these environmental challenges (Gratani, 2014). Chromatin modifications, often associated with alterations in gene expression, have been recognized as significant mechanisms that facilitate plant growth under challenging environments (Fan et al., 2005). The highly condensed and tightly coiled chromatin complex is composed of DNA and histone proteins (Cedar and Bergman, 2009). The tight coiling of chromatin, which is the default state, limits the access of RNA polymerase and other transcription factors to genes. To enable transcription, this compact structure must be opened: this process is termed chromatin remodeling (Bannister and Kouzarides, 2011), and it facilitates the conversion of chromatin from a transcriptionally inactive to a transcriptionally active state. The maintenance of gene activity is controlled by numerous biochemical modifications of chromatin structure, including DNA methylation (Grewal and Moazed, 2003). Some of these modifications can

be stably inherited through generations, suggesting that transgenerational adaptation to diverse stresses also has a genetic basis (Pecinka and Scheid, 2012). However, in plants, a limited number of studies have been carried out to validate this transmission of stress-induced changes in chromatin structure. Due to changes in chromatin structure, composition, and location, plants can modify transcription according to changing conditions and can maintain developmental and physiological changes for the long term (Vriet et al., 2015; Perrella et al., 2020). To cope with extreme environmental changes, plants have the power to remember the earlier stress and thus respond more efficiently when they encounter the stress again; this phenomenon is known as priming, which is often related to chromatin modification and may be maintained independently from transcription (Baurle and Trindade, 2020). It is difficult to understand chromatin folding in polyploid plants because polyploidy causes several copies of similar or related genomes in one nucleus. A study was conducted in wheat to understand chromatin architecture, which shows that there are three levels of large-scale spatial organization and concluded that for gene transcription in polyploidy plants, a three-dimensional conformation at multiple scales is the main factor (Concia et al., 2020). The use of high throughput next-generation sequencing (NGS) technologies, well-assembled genome sequences, and the availability of antibodies for a plethora of DNA and histone modifications have all benefited the studies of chromatin remodeling under stresses. This review focuses on the scope and relevance of chromatin architecture in plant stress adaptations.

CHROMATIN REMODELING ALLOWS POLYMERASES, TRANSCRIPTION FACTORS, AND OTHER NUCLEAR PROTEINS TO ACCESS DNA

In all eukaryotes, chromatin is packed into nucleosomes; the histone family of proteins makes up a large portion of the chromatin protein component. A nucleosome is a repetitive unit composed of 147 bp of DNA coiled in 1.67 left-handed turns around a histone octamer comprised of pairs of H2A, H2B, H3, and H4 histones (Luger et al., 1997). Histone proteins bear a positive charge and hence can come into close proximity with DNA. H3 and H4 are a part of core histones; they are present on the inside of the nucleosome and are bound to DNA before other histones. Variants of H2A and H2B have been found, which vary in their level of interaction with DNA. Linker DNA is a short strand of a nucleotide sequence that helps in compacting chromatin structure and gene expression regulation (Thoma et al., 1979; Lorch et al., 1999).

When highly condensed, the chromatin architecture prevents access by transcription factors, polymerases, and other nuclear proteins to DNA. Some modifications due to stress signals take place in the chromatin structure, which enables DNA to become accessible. These chromatin remodeling includes shifting or removing histones, introducing histone variants, or posttranslationally modifying existing histones (Eberharter and Becker, 2002).

There are two different strategies among many processes involving two different enzymatic mechanisms to accomplish chromatin organization: One operates through chromatin remodelers that change DNA-histone interactions *via* ATP hydrolysis, and the other utilizes specialized enzymes that methylate DNA or modify histone residues through the addition of covalent modifications (Cedar and Bergman, 2009).

CHROMATIN REMODELING COMPLEXES CONTAIN ATPASE/HELICASE OF THE SWI2/SNF2 FAMILY CATALYTIC CORE

The SWITCHING DEFECTIVE2/SUCROSE NON-FERMENTING2 (SWI2/SNF2) family of chromatin remodeling complexes (CRCs), part of a large superfamily of helicases and translocases, use the energy obtained from ATP hydrolysis to gain access to DNA sequences (Clapier and Cairns, 2009). The SWI2/SNF2 family CRCs are further subdivided into four classes/subfamilies (Clapier et al., 2017; Ojolo et al., 2018; **Table 1**).

SWI/SNF Subfamily Remodelers

The SWI/SNF subfamily remodelers comprise 8–14 subunits initially purified from *Saccharomyces cerevisiae* (Mohrmann and Verrijzer, 2005). A C-terminal bromodomain, a helicase-SANT domain, and a post-HSA domain are present in the catalytic ATPases of most SWI/SNF subfamily remodelers. Homology, dependent on arrangements of SNF2_N and HelicC areas, distinguishes two *Arabidopsis* likely proteins, At5g19310 (CHR23) and At3g06010 (CHR12), and two affirmed proteins, At2g28290 (SPLAYED or SYD) and At2g46020 (BRM), as the nearest homologs of yeast and human SWI/SNF ATPase subunits. BRM and SYD (2193 and 3574 amino acids) represent huge proteins, while CHR12 and CHR23 (1132 and 1054 amino acids) are altogether more modest. AT-hook motifs are present

TABLE 1 | The four families of chromatin remodeling proteins and their respective structural domains.

Chromatin remodelers family	Subunits	Domains	References
SWI/SNF (SWItching defective/Sucrose NonFermenting)	BAF, PBAF	HSA, DExx, HELICc, Bromo	Peterson and Workman, 2000
ISWI (Imitation SWItch)	ACF,RSF, CERF, CHRAC, NURF, NoRC, WICH, b-WICH	DExx, HELICc, HAND, SANT, SLIDE	Boyer et al., 2000
CHD (Chromodomain, Helicase, DNA binding)	CHD1, CHD2, CHD3, CHD4, CHD9, NuRD subunits	Chromo, DExx, HELICc	Boyer et al., 2000
INO80 (INOsitol requiring 80)	INO80, Tip60/p400, SRCAP	HSA, DExx, HELICc	Clapier and Cairns, 2009

in the C-terminal regions of BRM and SYD, whereas there is no such distinctive C-terminal domain in the CHR12 and CHR23. Decrease in DNA methylation 1 (DDM1) encodes a SWI2/SNF2-like protein, showing that chromatin remodeling is a crucial process for maintenance of DNA methylation (Jeddeloh et al., 1999). In *Arabidopsis thaliana*, DDM1 is one of the important plant epigenetic regulators required for maintaining cytosine methylation in genomic DNA (Dubin et al., 2015). DDM1 is found to enable methylation of DNA bound to the nucleosome. Nucleosomes are prominent barriers to DNA methyltransferases in the absence of remodeling (Lyons and Zilberman, 2017). In *Arabidopsis*, mutations in DDM1 show major methylation losses in all sequence contexts (especially in heterochromatic TEs); small losses can also be seen in genes (Ito et al., 2015). *Arabidopsis* histone H1 inactivation partially rescues the *ddm1* hypomethylation phenotype, showing that DDM1 provides methyltransferase access to H1-containing chromatin (Zemach et al., 2013). A genome-wide reduction in DNA methylation was observed in *ddm1* mutants especially in repeated regions of the genome. *ddm1* mutation induces epigenetic variation, which leads to the steady transmission of morphological phenotypes throughout generations, even if outcrossed from the original mutant backgrounds. Even though the major molecular phenotype of *ddm1* or *met1* mutants is a depletion of DNA methylation, instances of genetic variation as genomic rearrangements, copy number variants (CNVs), and successive DNA transposition have additionally been noticed and may represent a considerable amount of phenotypic variability (Zemach et al., 2013). There are four nonallelic variants of SWI3-type proteins reported in *Arabidopsis* and five in rice. The four *Arabidopsis* variations *AtSWI3A*, *AtSWI3B*, *AtSWI3C*, and *AtSWI3D*, just as their rice partners, all offer the trademark SWIRM (*Swi3p*, *Rsc8p*, and *Moira*), SANT (*Swi3*, *Ada2*, *N-Cor*, and TFIIIB), and Leucine Zipper space with yeast SWI3 and its orthologs in mouse (*Srg3*), *Drosophila* (Moira), and human (BAF170 and BAF155).

In *Arabidopsis*, only BSH (*At3g17590*) shows significant similarity to SNF5 (in yeast), which plays a key role in the organization and functioning of SWI1/SNF1 complexes. The *Arabidopsis* genome encodes two exceptionally comparable homologs of yeast SWP73: *At3g01890* (named *AtSWP73A*) and *At5g14170* (named *AtSWP73B*), which show 83.7% arrangement personality to one another. SWP73 has a functional role in transcriptional activation. The SWI2/SNF2-type ATPase domain belongs to the helicase and NTP-driven nucleic acid translocase superfamily 2 (SF2). This SF2 facilitates interaction with different targeting domains and functional modules, which activates remodeling activities in chromatin structure and thus helps in transcription regulation and DNA repair (Hopfner et al., 2012).

Imitation Switch Subfamily Remodelers

The Imitation Switch (ISWI) subfamily remodelers comprise of two to four subunits initially purified from *Drosophila melanogaster*. These remodelers consist of plant bromodomains, homeodomains, additional DNA-binding motifs, as well as DNA-binding histone fold motifs (Corona and Tamkun, 2004). In most of the eukaryotes, some specialized proteins form these ISWI family complexes using one or two different catalytic subunits. Nucleosome spacing is optimized by some ISWI family complexes like chromatin-assembly and remodeling factor (ACF) and chromatin-accessibility complex (CHRAC) promoting chromatin assembly and repressing transcription. Whereas certain complexes like nucleosome remodeling factor (NURF) can assist RNAPII activation by randomizing spacing. At the C terminus of the ISWI family, ATPases nucleosome recognition module is formed by a SANT domain (yADA2, ySWI3, hTFIIIB, and hNCoR) adjacent to a SLIDE domain (SANT-like ISWI), which binds to an unmodified histone tail and DNA. The studies on the polytene chromosomes in *Drosophila* larvae suggested the significant impact of ISWI in regulating higher-order chromatin structure.

Chromodomain Helicase DNA-Binding Subfamily Remodelers

The chromodomain helicase DNA-binding (CHD) subfamily remodelers comprise of 1–10 subunits first purified from *Xenopus laevis*. They vary in their structure due to the diversity in their chromodomains. They can act as transcriptional activators or repressors depending on CHD (Marfella and Imbalzano, 2007). In lower eukaryotes, the catalytic subunit is monomeric; however, in vertebrates, it can be in large complexes. To promote transcription, nucleosomes are ejected or slid by some CHD remodelers whereas some other CHD remodelers have repressive roles like the vertebrate Mi-2/nucleosome remodeling and deacetylase (NuRD) complex [histone deacetylases (HDAC1/2) and methyl CpG-binding domain (MBD) proteins]. CHD1 (identified as a murine protein) interacts with promoter sequences of immunoglobulin and is the founding member of the CHD family. A DNA-binding domain is present at the C-terminal of Chd1 and chd2 proteins that specifically bind to the AT-rich DNA region. The other two proteins CHD3 and CHD4 (a member of the second subfamily) do not contain standard DNA binding domains in their C terminus. However, a pair of PHD Zn-finger-like domain is present at the N-terminal of these proteins. This PHD Zn-finger-like domain is present in several nuclear proteins participating in chromatin-based transcriptional regulation. At C terminus of CHD6 to CHD9 (part of the third subfamily), additional functional motifs like SANT domain or BRK domain are present. There is a discrepancy in the identification of CHD5, as it contains both PHD fingers as well as SANT domain. PHD fingers show interaction with HDAC1 within NuRD in CHD3 and CHD4. CHD remodelers bind with enhancers and help in transcription activation.

Inositol Requiring 80 Subfamily Remodelers

The inositol requiring 80 (INO80) subfamily initially purified from *S. cerevisiae* is characterized by the presence of a split ATPase subunit with a long insertion found in the middle of the ATPase domain, which binds with the helicase-related (AAA-ATPase) Rvb1/2 proteins and one ARP protein. It is involved in transcription activation and DNA-double-strand break (DSB) repair (Bao and Shen, 2007). Higher orthologs

of the INO80 family include hINO80, hSRCAP (SNF2-related CREB-activator protein), and p400, also having HAT activity. CRCs from different subfamilies are involved in diverse plant physiological processes like cell differentiation, meristem establishment, floral morphogenesis, organ development, phytohormone signaling, and biotic and abiotic stress tolerance. RuvB-like helicases, the unique proteins for INO80 and SWR1 complexes, are related to the bacterial RuvB helicase, which takes part in DNA repair. The member of this family binds to the histone variants of H2A: H2A.X and H2A.Z. *In vivo* INO80 complex is involved in nucleosome eviction, while the SWR1 complex catalyzes the replacement of a canonical H2A-H2B dimer with an H2AZ-H2B variant dimer. The ATPase subunits of the INO80 family and other ATPases in the SNF2 helicases are different, as a long spacer region is present in the INO80 complex that splits the conserved ATPase domain. This region binds with RuVB-like subunits and Arps. The helicase-SANT domain (HAS domain) necessary for the binding Arps and actin components is also present in the motor subunits of INO80 protein. The involvement of IN080 complexes in DNA repair is suggested by the presence of RuvB-like helicases.

CHROMATIN MODIFICATIONS IN PLANT STRESS TOLERANCE

Plants exploit chromatin modification mechanisms, (i) CRCs and (ii) chromatin-modifying enzymes, to overcome various biotic and abiotic stresses (Asensi-Fabado et al., 2017). In *Arabidopsis*, during stress, RESTRICTED TO NUCLEOLUS 1 (REN1) was found to be incorporated with nucleoli and helps in pollen development (Reňák et al., 2014). STRESS RESPONSE SUPPRESSOR 1 and 2 (STRS1 and 2) are DEAD-box RNA helicases; loss-of-function mutations in these proteins result in plants resistant to various stresses (Kant et al., 2007), whereas overexpressing STRS1 or STRS2 results in stress hypersensitivity. These proteins have a transient interaction with the nucleolus during diverse stress conditions, with different kinetics. RNA-directed DNA methylation (RdDM) pathways can inactivate some genes (**Figure 1**).

In plants, histone acetyltransferases (HATs) and HDACs catalyzing histone acetylation and deacetylation show a role in cold responses (Kim et al., 2015). In *Arabidopsis*, HISTONE DEACETYLASE 6 (HDA6) is upregulated by cold stress and positively regulates freezing tolerance (Luo et al., 2017). HDACs appear to directly activate maize (dehydration responsive element binding protein 1) DREB1 (ZmDREB1) gene expression and histone hyperacetylation under cold stress (Yu et al., 2018; Ding et al., 2019). According to a recent study to regulate the expression of COR genes (COR47 and COR15A), HOS15 works together with HISTONE DEACETYLASE 2C (HD2C) by directly binding to their promoters (Park et al., 2018; **Figure 1**).

In *Arabidopsis*, salinity tolerance is determined by expression levels of DEK3 (a DEK domain-containing protein), which acts in association with DNA topoisomerase (Waidmann et al., 2014). Members of the acetylation lowers binding affinity (ALBA) family are expressed in rice plants under drought stress, but their exact mechanism in chromatin organization is not yet evident (Verma et al., 2014). According to a recent study in *A. thaliana* seedlings subjected to four abiotic stresses (heat, cold, salt, and drought), there was no change observed in a large portion of chromatin. Chromatin accessibility was increased in case of extreme temperatures, while the result for chromatin accessibility did not change much in case of drought and salt stresses (Raxwal et al., 2020).

Epigenetic regulators have been found to affect the intranuclear localization of STRSs, hence showing that they have a role to play in the silencing of stress response genes with chromatin alterations (Khan et al., 2014). Sumoylation (attachment of SUMO moiety) is one of the common posttranslational protein modifications in response to several plant stresses (Miller et al., 2013; Elrouby, 2017). During stress, SUMOylation could play an essential part in changing the messenger RNA (mRNA) profile. SUMOylation of RNA binding proteins and elements engaged with 3' pre-mRNA processing, RNA editing, transcription termination, and mRNA export (Richard et al., 2013; Lamoliatte et al., 2014) have assisted with extending the function of this modifier to the field of RNA processing and metabolism (Rouviere et al., 2013). It is found that SUMO pathway enzymes colocalize in nuclear bodies and substructures along with segments of the RNA processing machinery. A few individuals from the protein inhibitor of STAT (PIAS) family of SUMO E3 ligases localized to nuclear speckles, which are subnuclear structures advanced for pre-mRNA splicing factors (Lamond and Spector, 2003; Hall et al., 2006). According to a study, SUMO-1 and the E2-conjugating enzyme ubc9 are localized to Cajal bodies (sites of maturation of snRNPs) necessary for pre-mRNA processing (Navascues et al., 2008). Multiple putative SUMO targets are present in functional capping, splicing, polyadenylation, termination, and mRNA export processes (Richard et al., 2017). During heat stress, SUMOylation has been accounted for controlling DNA methylation patterns, which, along with the stress-up-regulated SUMOylation of *Arabidopsis* variants of histone acetylases/deacetylases, for example, GCN5/ADA2B (Sterner et al., 2006) and HDA19 (To et al., 2011), may then assist in the conversion of euchromatic regions into heterochromatic regions during stress.

The MORC family is a subfamily of microrchidia (MORC) GHKL ATPases (Gyrase, Hsp90, histidine kinase, and MutL) superfamily. MORC protein was initially isolated from mouse, which is important for meiotic nuclear division (Watson et al., 1998). Thereafter, MORC genes have been identified in mammals (Pastor et al., 2014), *Caenorhabditis elegans* (Moissiard et al., 2012), and different plant species, including *Arabidopsis* (Kang et al., 2008), tobacco, barley, and potato. In *Arabidopsis*, seven members of MORC are identified and five members in barley. Microrchidia (MORC) subfamily is highly conserved and comprises widespread domain architectures, which enables it to link with epigenetic regulation and signaling-dependent chromatin remodeling (Lorković, 2012; Li et al., 2013). The role of MORC in chromatin-based transcriptional gene silencing (TGS) is studied in *Arabidopsis* (Lorković, 2012). MORCs interacts with other proteins and derive versatility in chromatin-associated functions. Mutations in two *Arabidopsis* genes,

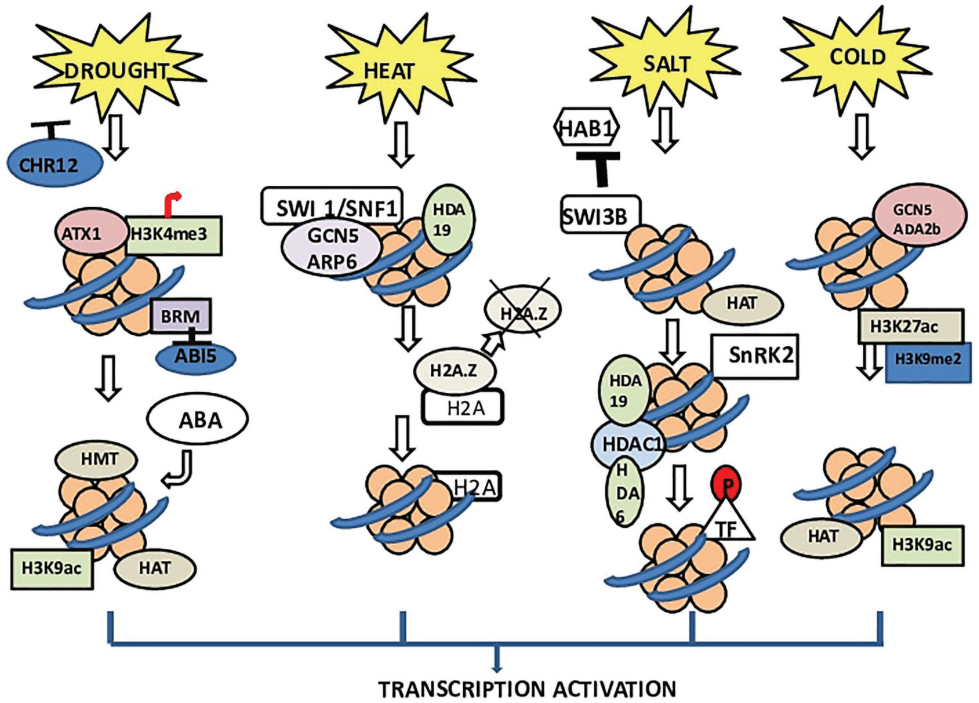

FIGURE 1 | Chromatin architecture under different stresses in plants. BRM (SNF/Brahma), CHROMATIN REMODELING 12 (CHR12) acts as a negative regulator. The receptors of drought stress deactivate CHR12 to promote plant productivity. During stress, BRM activity gets inhibited. BRM has been reported to control ABI5 expression especially by regulating the nucleosomal stability in the promoter and coding regions of this gene. BRM inhibits the expression of ABI5, thus initiating ABA biosynthesis. In heat stress, switching defective/sucrose nonfermenting (SWI1/SNF1) complex interacts with GCN5 and ARP6, which dissociates H2A.Z. The dissociation of H2A.Z causes transcription of downstream genes. Normally, the complex of ARP6 with SWI1/SNF1 plays important role in the insertion of H2A.Z into the nucleosome and replacing H2A. In the case of salinity, the receptors of salt stress inhibit the binding of SWI3B and HAB1. Due to this nonassociation, SNF1-related kinase (SnRK2) remains active, which leads to phosphorylation of transcription factors and finally transcription of genes. Under cold stress, ADA2b, which is a transcriptional activator of HATs, interacts with GCN5 (*Arabidopsis* HAT) and enhances the HAT activity of GCN5. This interaction increases the histone acetylation level.

AtMORC1 and *AtMORC6* (members of conserved MORC ATPase family), show de-repression of DNA-methylated genes and TEs. Enhanced interaction of pericentromeric regions and the genome, decondensation of pericentromeric heterochromatin, and transcriptional defects that are mainly focused on loci residing in pericentromeric regions are noticed in *atmorc1* and *atmorc6* mutants. In eukaryotes, MORC ATPases are proposed to be the conserved regulators of gene silencing (Moissiard et al., 2012). The MORC proteins are a subset of the GHKL ATPase superfamily. These proteins have been described as components involved in plant immunity in *Arabidopsis*. Resistance to *Phytophthora infestans* in solanaceous plants was compromised in silenced *StMORC1* in potato and enhanced in overexpressing lines, indicating that *StMORC1* positively affects immunity, whereas the resistance to *P. infestans* in *SlMORC1* silenced in tomato or *NbMORC1* silenced in *N. benthamiana* was increased. It was also observed that transient expression of *StMORC1* in *N. benthamiana* triggers cell death, initiated by infestin1 (INF1), while *SlMORC1* or *NbMORC1* expression represses it (Manosalva et al., 2015). *Arabidopsis MORC1*, formerly named CRT1 (compromised for recognition of TCV 1), identified as a hereditary screen to recognize components associated with the TCV resistance signaling pathway (Kang et al., 2008). *Arabidopsis* CRT1 is necessary for effector-triggered immunity. CRT1 possesses the ATPase and 5S domains, which is a characteristic of MORC proteins. These proteins are involved in DNA modification and repair (Kang et al., 2012) It has been studied that CRT1 and CRH1 (closest homolog of CRT1) are necessary for basal resistance, pathogen-associated molecular pattern (PAMP)-triggered immunity, systemic acquired resistance, and nonhost resistance. The level of CRT1 in the nucleus increases by PAMP treatment or infection with an avirulent pathogen. In *Arabidopsis*, resistance to Turnip crinkle virus (TCV) is represented by the resistance protein HRT (HR to TCV) and its related avirulence factor, the viral coat protein. Plants not having HRT fail to build up an HR after TCV infection permits systemic viral spread and results in the death of the plant. CRT1 physically interact with HRT and 10 other R proteins; these R proteins are mainly inactive. CRT1 possesses two close and four distant homologs; silencing of the two closest homologs, CRH1 (CRT1 homolog 1) and CRH2, compromised TCV resistance to a far extent in comparison to *crt1*. *crt1-1* mutation and silencing of CRT1 family members compromise cell death triggered by the R proteins. Reduced resistance to avirulent *Pseudomonas syringae* (Pst) and *Hyaloperonospora arabidopsidis* was observed in double knockout (dKO) in the Col-0 background,

crt1-2 crh1-1, which lacks CRT1 and its closest homolog. The knockout of CRT1 gene results in severe susceptibility to both virulent and avirulent *H. arabidopsidis*. These results show that CRT1 is a very crucial factor in multiple levels of plant immunity (Kang et al., 2012). GHKL ATPase motif is present in several prokaryotic and eukaryotic proteins; these proteins are involved in heat shock responses (*Hsp90*), rearranging DNA structure (gyrase or topoisomerases), signal transduction (histidine kinase), or DNA mismatch repair (MutL; Iyer et al., 2008). In *Arabidopsis*, nucleosome assembly proteins (NAPs; NRP1 and NRP2) localized in the nucleus, formed protein complexes, and acted as H2A/H2B chaperones. These protein complexes help in the regulation of chromatin organization in epigenetic inheritance, as they specifically bind to histones H2A and H2B (Zhu et al., 2006). NAP1 is evolutionary preserved from yeast to humans. In *Arabidopsis*, these NRP proteins are involved in many biological processes, for example, cell-cycle control, heat tolerance, somatic homologous recombination, DNA repair, root meristem formation, and genome defense under genotoxic stress (Gao et al., 2012). NRP proteins localized predominantly in the nucleus (Gonzalez-Arzola et al., 2017) genetically interact with the SWR1 core components and link with H2A.Z. It is proposed that, in *Arabidopsis*, NRP proteins counteract the activity of the SWR1 complex and associate with the dynamic regulation of H2A.Z (Wang et al., 2020).

Evolutionary conserved SnRK1 kinases (Snf1-RELATED KINASE1) govern metabolic adaptation during low extended darkness by controlling C/S1-bZIP signaling in *A. thaliana* (Pedrotti et al., 2018). Plants face continual environmental fluctuations because of their sessile nature, which may harm their energy storage. Plant SnRK1s adjust metabolic, developmental, and transcriptional processes due to such challenges (Hey et al., 2010; Smeekens et al., 2010). SnRK1s KIN10 and KIN11 handle energy loss by controlling the stress-responsive genes expression and signaling of abscisic acid in *Arabidopsis* (Baena-Gonzalez et al., 2007; Jossier et al., 2009). Calcineurin B-like interacting protein kinase 15 controls rice *OsSnRK1* (Lee et al., 2009) and further derepresses the expression of (glucose) Glc-repressed gene in the embryo (Lu et al., 2007) to modulate early seedling growth and seed germination. During evolution, SNF1/AMPK-related kinases proliferated and diversified to mediate the signaling of various abiotic stresses (Zu, 2016). Chromatin remodeling complexes have been found to be active during responses towards different stresses, such as *AtCHR12*, which is an SNF2/Brahma-type chromatin remodeling protein. Its paralog, *AtCHR23*, mediates growth responses under abiotic stress (Mlynárová et al., 2007; Folta et al., 2014), while SPLAYED (SWI/SNF class chromatin remodeling ATPase in *Arabidopsis*) is involved in biotic stress signaling and resistance towards pathogen (Walley et al., 2008). In the Solanaceae plants, the expression of the *SlyWRKY75* gene is induced in response to biotic stress (López-Galiano et al., 2018).

Role of Histone Chaperones in Stress Tolerance

Genome-wide responses, independent of transcriptional reactivation, inclusive of reduction in nucleosomal density, provide the first evidence of involvement of histone chaperones in poststress periods. In this context, mutants of CHROMATIN ASSEMBLY FACTOR 1 (CAF1; Pecinka et al., 2010; a histone chaperone complex facilitating H3 and H4 incorporation onto the neosynthesized DNA molecule) were impaired in nucleosome reassociation. FASCIATA 1 (FAS1), FASCIATA 2 (FAS2), and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) are three subunits of CAF1 (**Figure 2**). It is observed in *A. thaliana* that the vigor of *CAF1* mutants reduced over several generations (Kaya et al., 2001). When developmental phenotypes, transcriptomes, and DNA cytosine-methylation profiles were compared in *CAF1* mutant plants of various generations, it was seen that phenotypes related to shoot and root growth were majorly affected in successive generations of *CAF1* mutants. Limited changes in the expression of the gene were found in early and late generations of the fasciata *(fas)2-4 CAF1* mutant. The maternal participation to the phenotype severity is more than the paternal contribution when early and late generation *fas2-4* plants were crossed. It shows that the preferred maternal transmission uncovers a more prominent reprogramming of epigenetic data in the male in comparison to female germline. Epigenetic mechanisms underlie the progressive developmental phenotype aggravation in *CAF1* mutants in *Arabidopsis* (Mozgova et al., 2018).

Fasciata mutants have been reported to show pleiotropic effect in *A. thaliana*. *Arabidopsis* CAF1 is necessary for the maintenance of seedling architecture, trichome differentiation, and proper leaf size. CAF1 mutants show defects in shoot meristems. As leaf shape is primarily maintained during outgrowth of leaf primordia, the function of CAF1 is necessary for developing lateral organs and organ primordia, suggested by the strong FAS1 expression in leaf primordia (Exner et al., 2006). *fas* mutants have been observed to fail in maintaining proper expression of WUSCHEL (WUS) in SAM and SCARECROW (SCR) in RAM (Schoof et al., 2000). This shows the critical role of CAF1 in the organization of SAM and RAM during postembryonic development. In *Arabidopsis*, *fas1* and *fas2* mutants show dark green, abnormally shaped leaves, abnormal floral organs, short roots, the inability of the breakdown of meristem for distinct organs development, and thus reduced fertility (Leyser and Furner, 1992). FAS5, which is a TOP1ALPHA, a DNA topoisomerase, is not part of the CAF1 complex, and like other fasciata mutants, *fas5* mutant shows pleiotropic defects. The *fas5* mutation results in a change in the leaf and stem shape and favors the transition to the reproductive phase, leading to SAM fragmentation and tumor development on the stem. The notable increase in the SAM size in *fas5* plants in comparison with the wild-type plants suggests the role of FAS5 in WUS activity (Albert et al., 2015).

In *Arabidopsis*, MSI1 is having an important function in polycomb repressive complexes (PRC2) due to which *msi* mutants are lethal to the embryo (Köhler et al., 2003; Guitton et al., 2004 or Derkacheva et al., 2013). CAF1 plays an important role in the heterochromatin organization. It also helps in the maintenance of transcriptional gene silencing, which includes regulation of endoreduplication, homologous recombination, inactivation of certain TEs, and regulation of cell cycle duration (Mozgova et al., 2018). Stress-responsive genes mainly show progressive transgenerational upregulation in *fas2* and also affected

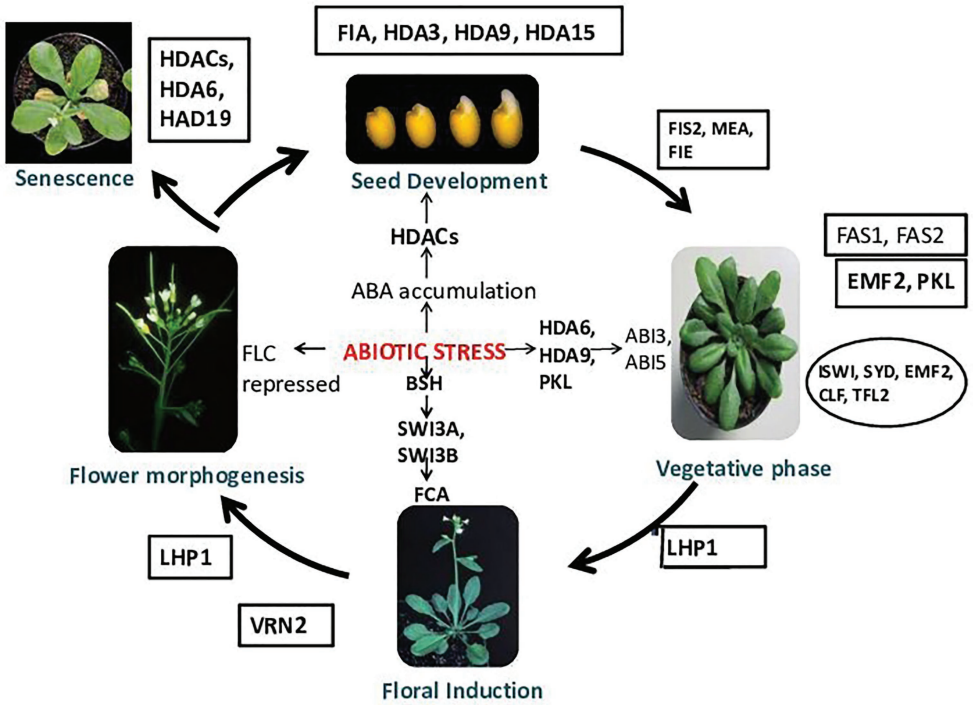

FIGURE 2 | Chromatin modifications and plant development. Chromatin remodelers FIA and HDA3 play an important role during normal seed development. During stress, because of abscisic acid (ABA) accumulation, histone deacetylases (HDACs) get activated and helps in seed germination. HDA9 shows involvement in seed dormancy and germination (Baek et al., 2020). HDA15 regulates light-controlled hypocotyl elongation and regulates seed germination in the dark (Chen et al., 2020). FAS1 (FASCIATA), FAS2, EMBRYONIC FLOWER 2 (EMF2) [EMF genes repress reproductive development by delaying the vegetative-to-inflorescence (V/IF) and inflorescence-to-flower (IF/F) transitions]. The early-flowering/terminal flower phenotypes of the transgenic plants harboring the antisense EMF2 support this hypothesis. emf2-like and tfl1-like phenotypes demonstrate the role of EMF2 in the repression of the V/IF and IF/F transitions, whereas early flowering under SD conditions suggests that EMF2-mediated, photoperiod-dependent regulation of the V/IF transition, PICKLE (PKL), Imitation Switch (ISWI), SYD, fertilization-Independent Endosperm (FIE), CLF (CURLY LEAF), and TFL2 helps in normal vegetative growth. During abiotic stress, HDA6, HDA9, and PKL activates ABI3 and ABI5. VRN2 functions during floral induction. In a stressed condition, BSH (SNF5-type protein) gets activated and binds to SWI3A and SWI3B, which activates FCA. FIS2, MEA (MEDEA), and fertilization-independent endosperm (FIE) proteins operate in the same system of control of seed development. In *Arabidopsis*, the genes MEA and FIS2 encode the polycomb group (PcG) protein. The genes MEA, FIS2, and FIE repress seed development until the double fertilization event that follows pollination provides the signals for embryo and endosperm development. After fertilization, the activity of MEA, FIS2, and FIE can be detected in the endosperm tissue, and the activity of FIE activity is also found in some other sporophytic tissues (Guitton et al., 2004). LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) has been proposed as a plant-specific subunit of PRC1 that could bind the H3K27me3, which is established by PRC2, and is required for a functional plant PcG system. LHP1 has been observed to control flowering time primarily by recognizing and binding to H3K27me3 and interacts with FLOWERING LOCUS T (FT) chromatin repression of FT expression (Feng and Lu, 2017). During stress, *FLC* gets repressed; in the senescence of plants, HDACs, HDA6, and HDA19 play vital roles (Wageningen Seed Lab, 2007).

by nucleosome depletion in *fas2*. These genes lack transcriptional repression in *fas1* and *fas2* mutant plants. Therefore, *Arabidopsis* CAF1 play a role in the stable repression of stress-responsive genes. CAF1 is necessary for effective resetting of stress-induced chromatin modifications, due to which it may be recommended that the enhanced stress responses along with inability to reset stress-induced chromatin states underlie the transgenerational aggravation of the CAF1 mutant phenotype. These histone chaperones are responsible for histone storage, assembly (Zhu et al., 2006), and eviction. Histone chaperones are differentially controlled in different plants under similar stress conditions (Tripathi et al., 2015). Stress-responsive genes are upregulated in mutants absent in ASF1 or CAF1 proteins and other H3/H4 chaperones (Schönrock et al., 2006; Weng et al., 2014). Plants lacking ASF1 or having the truncated NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1) and H2A-H2B chaperone (Weng et al., 2014) show hypersensitivity to stress (Chen et al., 2018a).

Role of Histone Modifications in Stress Tolerance

Gene expression can be affected by epigenetic factors by the addition of small functional groups (methyl, acetyl, etc.) on DNA or histones (Banerjee and Roychoudhury, 2017). Methylation of DNA by DNA methyltrasferases (DNMTs) and chromomethylases (CMTs) brings about gene silencing. Histone methylation can be a positive mark of transcription if lysine 4 of histone 3 is methylated (H3K4Me1/2/3), but methylation of lysine 9 of histone 3 is a repressive mark of transcription (H3K9m2), a hallmark of constitutive heterochromatin. A similar case is reported for H3K27me1 in plants. However, H3K27me3

deposited by the polycomb pathway is a mark of "facultative" heterochromatin, involved mostly in the repression of developmentally regulated genes. Acetylation of histones by histone acetyltransferases (HATs) increases the negative charge on protein surfaces, reducing interaction with negatively charged DNA. Acetylation of histones thus results in the loosening of condensed chromatin, facilitating transcription. On the contrary, the removal of an acetyl moiety from histones by HDACs (also referred to as lysine deacetylases) facilitates condensation of chromatin (Füßl et al., 2018; **Table 2**). In rice, OsDSI modulates histone deacetylation to repress salt stress (Julkowska, 2018).

It was shown in *Arabidopsis* and rice that, upon stress, histone variants are also differentially expressed like histone chaperons (Hu et al., 2008). The H2A variant H2A.Z is downregulated under drought or salt stress in rice and *Arabidopsis* (Nguyen et al., 2017). H2A.Z has been found to be a key element for the role as a thermosensor (Kumar and Wigge, 2010) and shows the function of H2A.Z in chromatin responses during stress (Talbert and Henikoff, 2014). H2A.W found in heterochromatin is involved in decondensation induced by stress (Yelagandula et al., 2014).

Furthermore, Plants contain a distinct subclass of variants of H1 that are stress inducible (Jerzmanowski, 2007) and, when overexpressed, confer tolerance to several abiotic stresses (Wang et al., 2014). In *Arabidopsis*, H1 variants are having a major role in the molecular and spatial chromatin organization. H1 takes part in gene expression, as it is having distinct roles in euchromatin and heterochromatin (Rutowicz et al., 2019). Three variants of H1, H1.1, and H1.2 (canonical H1 proteins that are constitutively expressed), and H1.3 (involved in plant stress tolerance) are present. H1.3 is upregulated during high or low light stress conditions. H1.3 is required for both stomatal functioning under typical growth conditions and adaptive developmental responses to combat light and water deficiency. H1.3 is expressed in stomatal guard cells and can be induced by drought or stresses that signal through abscisic acid (Rutowicz et al., 2015). Plant chromatin combats stress by modulating histones by posttranslation modifications (Kim et al., 2015; Meyer, 2015). In response to stress, changes in a specific histone modification can either be global or local. Specific changes including the formation of H3K9ac (Lee et al., 2014; Widiez et al., 2014) and H3K4me3 (Ding et al., 2019) in salt or drought-responsive genes (Tardieu et al., 2018) in various plant species are responsible for stress tolerance. Abiotic stresses result in global hyperacetylation of histones in rice and maize (Fang et al., 2014; Makarevitch et al., 2015).

Role of DNA Modifications in Stress Tolerance

DNA can also be modified by methylation in response to diverse stresses. Gene expression is maintained by the balance of methylation and demethylation at target promoters (Le et al., 2014). Modification in this equilibrium can affect the biotic stress response either negatively (Lee et al., 2014) or positively (Dowen et al., 2012). Stress conditions induce necessary changes and modifications in chromatin structure, which facilitate selective gene expression. It remains to be understood how stress signals are coordinated to drive gene activation and changes in the higher-order organization.

TABLE 2 | Chromatin-associated factors and chromatin remodeling proteins.

Chromatin-associated factors and chromatin remodeling proteins	Functions	References
HAT	Transcriptional response to various biotic and abiotic stress	Stockinger et al., 2001; Vlachonasios et al., 2003
Subunit of elongator HAT complex	Phenotypes of oxidative stress tolerance, ABA hypersensitivity, and increased accumulation of anthocyanin in the mutants of four subunits	Zhou et al., 2013; Pfab et al., 2018
HDAC	Salinity stress tolerance phenotype in transgenic plants overexpressing AtHD2C	Sridha and Wu, 2006
Homolog of human TBC	Freezing stress-hypersensitive phenotype in hos15 mutants	Zhou et al., 2013
Subunit of polycomb group protein	Drought stress tolerance phenotype in cosuppression transgenic plants of MSI1	Alexandre et al., 2009; Wang and Shen, 2018
HMG protein	Phenotype of decreased seed germination rate in transgenic plants overexpressing HMGB1, phenotypes of retarded germination and subsequent growth in transgenic plants overexpressing HMGB2	Lildballe et al., 2008
ATP-dependent chromatin remodeling factor	Phenotype of growth arrest of primary buds and stems under the drought and heat stress in transgenic plants overexpressing AtCHR12, phenotype of less growth arrest under the drought and heat stress in atchr12 mutants, phenotype of reduced sensitivity to ABA-mediated inhibition of seed germination and growth in swi3b mutants	Mlynárová et al., 2007; Saez et al., 2008
CHD4	Signaling and repair after DNA damage	Larsen et al., 2010
BRM (BRAHMA)	Modulates response to ABA by preventing premature activation of stress response pathways during germination	Buszewicz et al., 2016
CHR5	Plant immune responses and nucleosome occupancy	Zou et al., 2017
CHD3	Promotion of sporophytic and gametophytic generations	Carter et al., 2016
SWI3C	Modulates gibberellin responses	Sarnowska et al., 2013

CHROMATIN ARCHITECTURE AT PROMOTERS DURING PLANT STRESS TOLERANCE

The promoter is an array of cis-regulatory elements that helps in the expression of the gene present downstream to it. The function of the core sequences like ACGT (Mehrotra and Mehrotra, 2010; Mehrotra et al., 2013), TGAC (Dhatterwal et al., 2019),

a cis-regulatory element, and many others have revealed that cis-regulatory elements influence the gene expression either positively or negatively. Mehrotra et al. (2011) have discussed strategies to design synthetic promoter modules. Mehrotra et al. (2017) have discussed the modular nature of transcription and discussed the principles of rational combinatorial engineering; furthermore, they highlighted the importance of customized transcriptional units. A synthetic promoter is a region of DNA with a core-promoter region (or minimal promoter sequence) and multiple repeats or combinations of heterologous upstream regulatory elements (cis-motifs or TF-binding sites). Synthetic promoters are designed by the fusion of a minimal promoter to a heterologous promoter sequence at its 5' end and to a reporter gene (GUS, LUC, CAT, etc) at its 3' end (Lange et al., 2018). These synthetic constructs are introduced in plant cells by *Agrobacterium*-mediated transformation, biolistics, or, electroporation, and then, the expression of the reporter gene is studied. The core promoter region contains TATA box, which recruits RNA polymerase II, thus forming the preinitiation complex by assembling general transcription factors. The synthetic transcriptional units are the precise combination of coding and regulatory DNA sequences designed for the desired function in crop plants (Liu and Stewart, 2015). This synthetic biology is an important tool for the genetic modification of plants, thus can increase crop productivity under different environmental stresses.

SWI/SNF complexes also regulate noncoding transcription arising from promoters, enhancers, intergenic regions, and transcription termination sites (TTS) of protein-coding genes. *Arabidopsis* BRM binds to proximal promoter regions as well as the distal region of the promoter, gene bodies, and gene terminators, whereas yeast SNF2 ATPases bind specifically to promoters near the TSS site. Archacki et al. found that the binding of BRM at terminator sequences, depending on the locus, can promote or repress the transcription of antisense transcripts. Thus, it is for the effect of BRM at its gene targets that can positively or negatively regulate their transcription. In plants, SWI/SNF complex regulates promoter-centered gene function as well as controls the expression of a large number of its direct targets through their 3' ends. The regulation of noncoding RNA (ncRNA) originating from TTS by BRM does not depend on the presence of linked sense promoters, which suggests that 3'-bound BRM utilizes antisense promoters to maintain sense expression of those genes. It has been observed that the antisense transcripts arising therefrom and the TTS regions of genes have been implicated in environmental signals sensing in many systems, including cold sensing by the *FLC* 3' region and sulfur sensing by the 3' untranslated region (UTR) of SULTR2; 1 in plants, or yeast, the requirement for the 3' region of KCS1 for phosphate sensing. This suggests that a large fraction of the 3' SWI/SNF targets are stress-related genes (Archacki et al., 2017).

SOMATIC MEMORY-CHROMATIN ARCHITECTURE

Chromatin is broadly investigated as a major regulatory component for gene expression; it is also pertinent to investigate epigenetic mechanisms. *In vitro* somatic embryogenesis induced in response to external signals is an example of plant developmental plasticity developed by the chromatin-regulating molecular machinery (Fehér, 2015; Lämke and Bäurle, 2017). Plants show an interesting phenomenon that furthers our understanding of somatic inheritance vis-à-vis stress. It has been observed that treating plants with mild stress facilitates accelerated and enhanced responses to future challenges (Holeski et al., 2012), known as plant priming, of which chromatin is a part (**Box 1**). The term acquisition of thermotolerance is used when a plant is primed due to moderate heat stress (HS) and thus can tolerate high temperatures in comparison to an unadapted plant. The primed state is maintained over several days (known as maintenance of acquired thermotolerance or HS memory) after returning to normal temperatures, and this maintenance is genetically distinguished from HS priming. During HS priming, heat shock transcription factors (HSFs) get activated and increases the expression of heat shock proteins (HSPs), which then, through their chaperone activities, assist in protein homeostasis. This HS response is preserved in animals, animals, and fungi. In plants, more than 20 members of the HSP family are reported. At least eight HSFs are observed to play role in heat stress response in *Arabidopsis*. The knowledge regarding the mechanism of HS memory is not well understood. Using microarray analyses, a number of HS memory-related genes are identified, comprising genes encoding small HSPs (such as HSP21, HSP22.0, and HSP18.2) and ASCORBATE PEROXIDASE 2. The expression pattern of these genes found to be strong in the case of inducible HS when comparing with nonmemory genes (like HSP70 and HSP101). HSFA2 was reported to be the most strongly heat-induced HSF, as it is required specifically for HS memory. The

BOX 1 | Plant priming: preparing plants to tolerate future adverse conditions.

Plant priming/defense priming (Martinez-Medina et al., 2016), which is also known as hardening, can be initiated in response to environmental stress [light (Han et al., 2018), temperature (Friedrich et al., 2019), water, etc.] event that acts as a cue indicating an enhanced probability of facing that specific stress factor in the future (Filippou et al., 2013). Plants enter in the primed state (PS) following perception of the cue in which the activation of the protection responses is faster and stronger when a stress pressure is encountered (Beckers and Conrath, 2007; Conrath, 2009; Ellouzi et al., 2013; Sani et al., 2013). The impact of stress exposure on the physiology and growth of primed plants can be remarkably diminished in comparison with nonprimed plants. Plants can also enter the PS by chemical priming, which involves exposure to a natural or synthetic chemical compound that acts as a priming agent (Savvides et al., 2016). Chemical priming gives opportunities for more effective use of plant priming in plant stress physiology studies and crop stress management. There are several types of molecules having the potential to act under specific conditions as a priming agent against a range of different abiotic stresses (Islam et al., 2009). A review reveals a vast range of chemical priming agents, including amino acids [e.g., proline (Li et al., 2014)] hormones [e.g., salicylic acid (Tanou et al., 2009)], reactive oxygen-nitrogen-sulfur species [RONSS (Christou et al., 2014)] and even water [i.e., hydropriming (Casenave and Toselli, 2007)]. These agents are effective in inducing plant tolerance to several individually applied abiotic stresses or biotic stresses. Primed plants show either faster and or stronger activation of the various defense responses that are induced by either pathogens or insects, or in response to abiotic stress. If the stress recurs, the benefit to the plant being primed for that particular stress response is in facilitating a more rapid response. This provides the advantage of enhanced protection without the costs associated with constitutive expression of stress related genes (**Figure 3**).

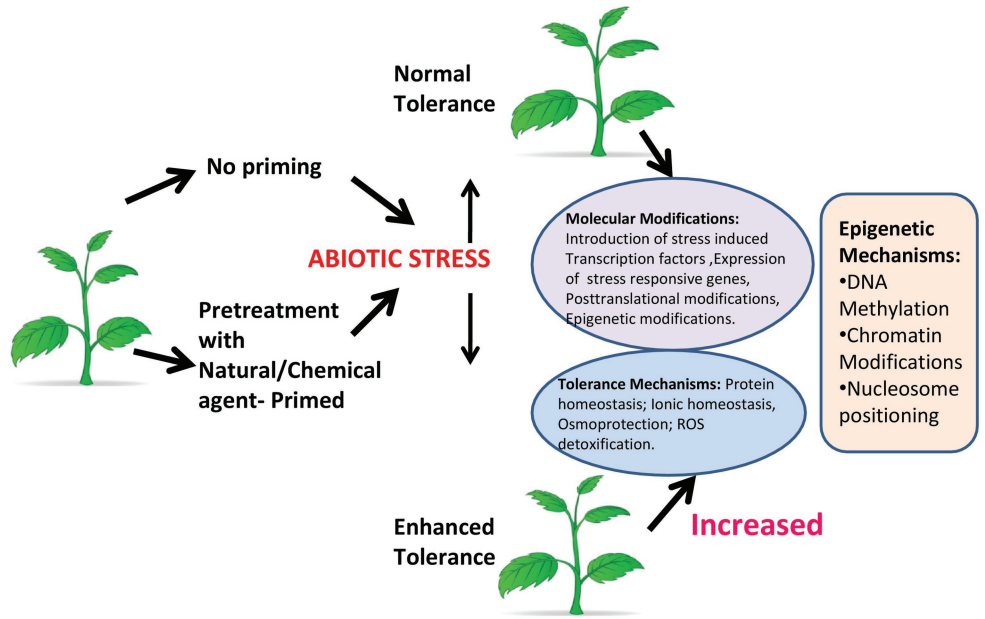

FIGURE 3 | Plant priming. Under abiotic stress, a plant that is not primed shows normal tolerance, while a primed plant shows enhanced tolerance by increasing molecular functions and inducing tolerance mechanisms. Epigenetic modifications like DNA methylation, chromatin modifications, and nucleosome positioning have a major role in response to stress in primed plants.

first reported HS memory-associated gene, specifically involved in HS memory is HSA32. It was studied that HSA32 is required for HSP101 protein stability, which suggests a similar role to chaperons. ROF1, which is the peptidyl-prolyl-isomerase and member of the FK506-binding protein family, is also seen to be specifically required for HS memory by directly interacting with HSP90.1, which further interacts with HSFA2 (Baurle, 2016).

A priming exposure of young *Arabidopsis* plant to mild salt stress, which does not affect growth, leads to enhanced salt tolerance following a subsequent exposure. This tolerance is connected with gene and tissue-specific changes that last ~2 days (Sani et al., 2013). Higher resistance to bacterial pathogens, nonspecifically primed by various abiotic stresses, is associated with histone acetyltransferase HAC1 (Singh et al., 2014; see **Box 1**). Changes in H3K4 trimethylation were observed by dehydration stress priming (Ding et al., 2019) at particular "memory genes" (Crisp et al., 2016). To understand cold-induced epigenetic changes, vernalization was studied in *Arabidopsis*, which is a mechanism in plants by which they have a memory of earlier encounter of low temperature, and the plants thus flower only in favorable condition. The flower repressor FLOWERING LOCUS C (*FLC*) is silenced during vernalization by the polycomb repressive complex 2 (PRC2), which accumulates H3K27me3 at target loci (Baurle and Trindade, 2020).

INTERGENERATIONAL AND TRANSGENERATIONAL STRESS MEMORY

Lamarck in the nineteenth century first hypothesized that traits acquired during an organism's life could be transmitted from one generation to the next generation, which is known as Lamarckism (Lamarck's theory) or the theory of "inheritance of acquired characteristics." According to Lamarck, alterations in phenotypic traits are a result of the environment and are associated with evolution. Lamarckism says that simple organisms tend to evolve into more complex ones by an adaptive force. The environment creates needs to which organisms respond by utilizing features, which are then emphasized or weakened through use and disuse; this generates characteristics that an individual organism acquires and then are pass on to its offspring. Plants have elaborate mechanisms to deal with different environmental conditions. When the memory effect is present only in the first stress-free generation, it is called intergenerational memory, while if the memory is traceable in a minimum of two stress-free generations, it is termed transgenerational memory (Tardieu et al., 2018; **Figure 4**). Transgenerational memory (TSM) likely consists of an epigenetic basis, i.e., the phenotypic traits possessed by the offspring are a result of environmental stimulus in an earlier generation but not in the parent or offspring. There are reports showing that there is an increase in somatic homologous recombination (SHR) in the parental generation when treated with the flg22 elicitor or UV-C irradiation that indicates the presence of a stress-induced transgenerational memory (Molinier et al., 2006), which remained elevated during numerous unstressed generations, showing an epigenetic basis (Kinoshita and Seki, 2014). During transgenerational memory, the DNA methylome is relatively unaffected by stress-induced changes in *Arabidopsis* (Ganguly et al., 2017). According to some studies, hyperosmotic stress priming will develop when plants were subjected to stress during their vegetative development

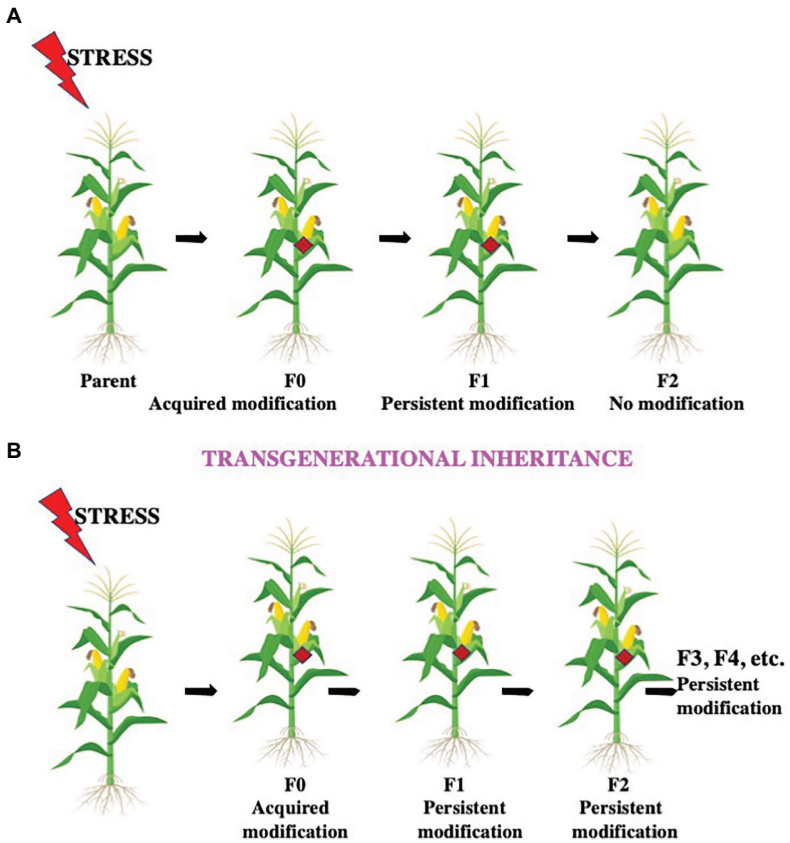

FIGURE 4 | Intergenerational and transgenerational inheritance. Environmental conditions like heat stress, temperature fluctuations, light duration and intensity, insect attack, osmotic imbalance, etc. can influence future generations by different modes. **(A)** The offspring after these conditions with the altered epigenetic structure is the F0 generation. If the modification is successfully passed on from the F0 generation only to their offspring, the F1 generation, the modification is termed as an intergenerational inheritance. **(B)** If the modification is successfully transferred from the F1 generation to the F2 generation and further generations, the change is termed as transgenerational inheritance.

for at least two generations (Pecinka et al., 2009; Murgia et al., 2015). The maternal parent is likely responsible for this intergenerational stress memory. It is suggested that, in the male gametes, DNA glycosylase DEMETER (DME) inhibits paternal inheritance, and it is restored in *dme* mutants (Choi et al., 2002). DME encodes a protein having DNA glycosylase and nuclear localization domains, and it is expressed mainly in the central cell of the female gametophyte, the progenitor of the endosperm. DME is involved in the demethylation of transposable elements (TEs) and repetitive sequences, which lead to TE upregulation and small interefering RNA (siRNA) production in endosperm and vegetative cells (Saze et al., 2012). The role of DME is also studied in genomic imprinting. Using base excision repair mechanism, DME can excise methylated cytosine bases from any sequence, which is similar to *A. thaliana* glycosylases DEMETER LIKE 2-3 (DML2-3) and REPRESSOR OF SILENCING 1 (ROS1; Ortega-Galisteo et al., 2008; Gehring et al., 2009). DME demethylating repetitive sequences, TEs, and targeted regions seem to be partially identical in the central cell and the vegetative nucleus, as it is active in both the central cell of the female gametophyte as well as the vegetative cell of pollen (Park et al., 2016). It has been proposed that the demethylation of TEs in the central cell and the vegetative cell is part of a defense mechanism so that these TEs can be silenced in the egg and sperm cells (Calarco et al., 2012; Ibarra et al., 2012). Due to the demethylation of TEs, transcriptional activation gets promoted, and thus, production of siRNAs takes place (Slotkin et al., 2009). These siRNAs can then promote DNA methylation *via* the noncanonical RNA-directed DNA methylation (RdDM) pathway, which uses them as guides and target the DNA methylation machinery to homologous sequences (Cuerda-Gil and Slotkin, 2016; Zhang et al., 2018). It is hypothesized that the siRNAs that are produced in the central cell and vegetative nucleus travel to the adjacent gametes (the egg and sperm cells) and initiate DNA methylation of TE sequences there, resulting in their silencing (Calarco et al., 2012). As imprinted genes are often found to be enriched in TEs in their flanking regions, DME-mediated methylation of these TEs may affect the expression of neighbor-imprinted genes (Hatorangan et al., 2016; Yuan et al., 2017). The role of RdDM in initiating methylation of the paternal alleles of some MEGs and the activity of DNA METHYLTRANSFERASE 1 (MET1)

and CHROMOMETHYLASE 3 (CMT3) are needed for the CG and CHG methylation levels maintenance in sperm cells, leading to epigenetic inheritance (Calarco et al., 2012). DNA methylation and H3K27me3, with some additional epigenetic modifications such as H3K9me2, are recognized to be responsible for the imprinting of some genes (Batista and Kohler, 2020). These variations occur for the transcriptional regulation of abiotic stress genes in plants. As these changes in the epigenome are stably inherited and passed to further generations, knowledge about these changes is crucial for stress management in plants. The knowledge of specific epigenetic marks with particular stressors would permit the generation of stress-tolerant plants by identification of the above-mentioned techniques.

RNA interference plays a critical role in epigenetic modification of histones and DNA, as it can repress target genes at the transcriptional level. Constitutive heterochromatin is a major source of siRNAs involved in the silencing of transposable elements. siRNAs are required for maintenance of asymmetric DNA methylation (CHH context) following mitosis and meiosis to ensure epigenetic inheritance (Law and Jacobsen, 2010). Double-stranded RNA (dsRNA), which can increase posttranscriptional silencing of cognate genes, gets cleaved by the RNase III enzyme, Dicer into siRNAs. These siRNAs guide the target effector complexes, such as the RNA-induced silencing complex (RISC), to endogenous transcripts leading to degradation or translational inhibition. These findings suggested a preserved nuclear role for RNAi in transcriptional gene silencing (TGS). As it occurs in the germline, TGS can lead to transgenerational inheritance. In *Schizosaccharomyces pombe*, a role for RNAi in TGS was observed where it is necessary for the formation of constitutive heterochromatin at pericentromeric. The release of the passenger strand from *Ago1* and dsRNA requires catalytic activity and thus is necessary for the pairing of bases between loaded siRNA and their targets. These interactions provide the RNA-induced transcriptional silencing complex (RITSC) a critical place, integrating transcription and chromatin modification, which creates a positive loop between siRNA generation, RITSC localization, and H3K9 methylation. In *S. pombe*, the coupling of transcription, production of siRNA, and silencing indicates that TGS occurs in cis; however, in plants, it has been seen that it can also occur in trans. In *A. thaliana* microRNA (miRNA)-directed siRNA biogenesis is a mechanism that particularly targets transposon transcripts and triggers epigenetic reactivation during reprogramming of the germ line (Creasey et al., 2014). In *Arabidopsis*, hetrochromatin is majorly defined by transposable elements and related tandem repeats, under the effect of the chromatin remodeling ATPase DDM1. siRNA possesses these sequences, indicating a role in guiding DDM1. The regulation of the euchromatic, imprinted gene *FWA*, as its promoter is hence can be understood by DDM1 and the DNA methyltransferase MET1, as they provide the transposable-element-derived tandem repeats that are associated with siRNAs (Lippman et al., 2004). Analysis of small RNA profiles and DNA methylation profiles identifies regions regulated by miRNA/siRNA-mediated DNA methylation, which involves the epigenetic inheritance of stress effects. Thus, the role of miRNA and siRNA in biotic and abiotic stresses in plants can be understood. The knowledge of small RNA-guided stress regulatory networks provides new insights for genetically improved plant stress tolerance. Manipulation of miRNA or siRNA-guided gene regulation can be used to engineer stress resistance in plants.

During cellular proliferation, the stable inheritance of epigenetic modifications is necessary to maintain cell identity. In plants, the transmission of H3K27me3-silenced state requires the replication-dependent histone variants H3.1 (Jiang and Berger, 2017). This H3.1 provides PRC2 function, managing proper maintenance of H3K27me3 domains and ensuring the silencing of developmental genes. In *A. thaliana*, flowering is initiated when H3K27me3 established at the floral repressor FLOWERING LOCUS C (*FLC*), which is a result of H3.1 deposition during DNA replication. At *FLC*, H3K27me3-mediated silencing finally reset in the future generation to ensure transcriptional reactivation in the early embryo (Tao et al., 2017).

The involvement of active demethylation in the loss of H3K27me3 has been suggested as implicated in the epigenetic resetting of *FLC*. The Jumonji-C family (JMJ) histone demethylases counteracts the activity of PRC2. There are three closely related JMJ H3K27 demethylases reported were EARLY FLOWERING 6 (ELF6), RELATIVE OF ELF6 (REF6), and JUMONJI 13 (JMJ13), and all are expressed in the sperm. As PRC2 is absent in the sperm, the H3K27me3 demethylation by JMJ proteins is supposed to occur globally, whereas in somatic tissues, H3K27 demethylases occupy the border of H3K27me3 domains in presence of PRC2 (Yan et al., 2018). ELF6 and REF6 play important roles in H3K27me3 and H3K27me1 homeostasis (Antunez-Sanchez et al., 2020). *elf6 ref6 jmj13* mutant showed elevated levels of H3K27me3 in the sperm when compared to wild type, suggesting the role of active demethylation by JMJ proteins in contribution to paternal H3K27me3 resetting. These JMJ proteins are found to demethylate the di-and trimethyl H3K27 but not H3K27me1 (Song et al., 2015; Zheng et al., 2019). JMJ demethylate H3K27me3 retained upon H3.10 depletion and convert it to H3K27me1 in the sperm. The *HTR10* encodes the sperm-specific histone variant H3.10 and indicates an increased level of H3K27me1, which is unlikely a result of mono-methylation by ATXR5/6, as its only substrate H3.1 is not expressed in wild type and *htr* sperm. In quadruple *elf6 ref6 jmj13 htr10* mutant sperm, a reduction in H3K27me1 levels was observed while the level of H3K27me3 was increased, suggesting that the deposition of H3.10 replaces a prominent region of H3K27me3-marked nucleosomes and H3K27me1-marked nucleosomes formed by the action of JMJ demethylases (Borg et al., 2020). During sexual reproduction, it has been seen that these chromatin marks are failed to reset, which leads to transgenerational inheritance of histone marks, resulting in loss of DNA methylation and transposon activation. Hence, in plants, JMJ-type histone demethylases help in maintaining transcriptional states through development as well as safeguard genome integrity during sexual reproduction (Borg et al., 2020).

The intergenerational memory is mediated by DNA demethylation and RNA-mediated DNA methylation pathways in case of hyperosmotic stress (Wibowo et al., 2016). Genome-wide methylation analysis helped in the identification of

differentially methylated regions (DMRs) linked with this intergenerational memory (Ferreira et al., 2019). The promoter of the gene related to stress has two such DMRs involved in priming effect on gene expression (Wibowo et al., 2016). There are reports showing the role of intergenerational and transgenerational stress memory in biotic stresses as well (Pieterse et al., 2012; Espinas et al., 2016). (Luna et al., 2012) showed that intergenerational or transgenerational memory is evidenced by increased salicylic-acid-related defense gene induction and susceptibility to biotrophic pathogens (Slaughter et al., 2012). From there, it is suggested that, for environmental challenges that plants may encounter in their life, they prime their offspring. It has been reported that, in the extremely challenging environmental conditions of a typical *Arabidopsis* habitat, transgenerational inheritance of priming may be disadvantageous over more than one generation (Luna et al., 2012; Iwasaki and Paszkowski, 2014). A full understanding of how TSM is related to seed germination and development under environmental changes could be important in research related to stress adaptation in plants and thus could help in the selection of stress-adapted genotypes.

CHROMATIN MODIFICATIONS AND PLANT DEVELOPMENT UNDER STRESS

In eukaryotic cells, cellular changes and gene expression are regulated by gene regulatory mechanisms in numerous biological processes, like a response to extracellular signals, recombination, developmental reprogramming, and genome stability (Zhu et al., 2013). Changes in DNA methylation, histone variants, and histone N-tail modifications, which are induced by stress, regulate plant development under stress and stress-responsive gene expression. Control of gene expression like this in response to endogenous and environmental stimuli in plants controlled by chromatin modifications is crucial for reproductive success and proper development (Archacki et al., 2013; Efroni et al., 2013; Sarnowska et al., 2013; Qin et al., 2014; Vercruyssen et al., 2014). A drastic change is triggered in seedling morphology when it first emerges from the soil due to rapid changes in histone modifications and gene expression including growth cessation of hypocotyls, the opening of apical hook and cotyledons, and the development of chloroplasts due to its encounter to light, which is known as photomorphogenesis. The physiology, morphology, and development of the plant thus depend on the duration and quality of light as well as the presence of competitors, which can alter the amount of light reaching the plant (Perrella et al., 2020).

The embryonic and postembryonic phases are two phases of the plant developmental cycle (Chen et al., 2018b). The postembryonic phase includes the growth of the leaf, stem, and flower meristems (Ojolo et al., 2018). The uniformity of seed germination and seedling establishment gets decreased during osmotic stress. Abscisic acid (ABA) accumulation induces several HDACs in *Arabidopsis* during seed development. *Arabidopsis* HDA6 and HDA19 have crucial roles in abiotic stress signaling through the formation of repressive complexes. HDA6 regulates the function of abiotic-stress-responsive genes (ABI1, ABI2, and ERF4) by interacting with HD2C (Luo et al., 2012b), whereas HDA19 with ERF3, ERF4, ERF7, SIN3, and SAP18 are part of chromatin remodeling complexes in abiotic stress responses. The mechanism of HDA9 function in signal transduction during abiotic stress responses is little known. A model is proposed for understanding the function of HDA9 in ABA-dependent drought stress signaling in plants (Fujita et al., 2005; Baek et al., 2020). During seed germination and plant development in wild-type plants, it was observed that, to regulate ABA homeostasis, the expression of ABA catabolism-related genes (CYP707As) changed ABA from an active to an inactive form (8′-hydroxyl ABA). Whereas, in the case of drought-stress-exposed plants, HDA9 and ABI4 together function in inhibiting the expression of CYP707As. HDA9 in association with an ABA-related transcription factor functions in inhibiting gene expression by histone deacetylation. In the drought stress response of plants, HDA9 is a crucial negative regulator in transcriptional regulation of ABA-catabolism-related genes like CYP707A1 and CYP707A2. HDA9 also plays an important role in seed dormancy and stomatal closure (**Figure 2**). It was observed that, in the case of *hda9* mutants, seed germination was significantly increased in comparison to wild type when exposed to exogenous ABA. In the presence of ABA, *hda9* mutants showed a significantly higher percentage of fully opened green cotyledons than the wild type. To suppress the effect of negative regulators of early ABA signaling, the MYB96 transcription factor associates with the histone modifier HDA15. This MYB96 TF is known as a master transcriptional regulator that mediates several plant responses to ABA, for example, seed germination, stomatal conductance, drought tolerance, anthocyanin accumulation, hormone biosynthesis, lateral root development, and cuticular wax biosynthesis. The MYB96-HDA15 complex formed interacts with the promoters of a subset of RHO GTPASE OF PLANTS (ROP) genes (ROP6, ROP10, and ROP11) and removes acetyl groups of histone H3 and H4 from the cognate regions, thus represses their expression specifically when ABA is present. A reduction in ABA sensitivity is observed in HDA15-deficient mutants, thus are affected by drought stress. Various transcription factors from bZIP, MYC, NAC, and MYB families then get activated and initiate downstream ABA responses. HDA15 represses ROP genes and acts as a positive regulator of ABA signaling by repressing (Lemichez et al., 2001). Transgenic plants overexpressing HDA15 and MYB96 possess hypersensitivity to ABA, whereas *hda15* and *myb96* mutants show reduced ABA sensitivity (Lee and Seo, 2019). Drought stress tolerance in plants as an effect of ABA was additionally affected by HDA15. During seed germination and drought tolerance, MYB96 and HDA15 act synergistically to confer ABA sensitivity (Lee and Seo, 2019). The *HDA15* gene is observed to be induced by ABA treatment. HDA15 expression enhanced under various abiotic stress factors (mainly osmotic, cold stress). HDA15 activity also affects the expression of some ABA-responsive genes. The expression of PKL (SWI/SNF type chromatin remodeling factor) is induced by seed imbibition in *Arabidopsis*, and it mediates the repression of embryonic traits during germination. Seed germination is

mediated by induction of ABI3 and ABI5 transcription factors expression in response to induced expression of PKL. This discussion suggests that there is a change in expression or activity of HDACs due to ABA accumulation, which in turn regulates growth under stress (**Figure 2**).

Plant reproduction includes flowering and seed development. Flowering is an essential part of the reproductive process as well as a critical developmental stage that can be susceptible to environmental stresses in plants (Kazan and Lyons, 2015). In appropriate environmental conditions, plants have mechanisms to flower. In *Arabidopsis*, during vernalization, low-temperature epigenetic mechanisms get induced, which repress the FLOWERING LOCUS C (*FLC*, a MADS-box protein) gene, which remains until progression to flowering. Due to prolonged cold, COOLAIR, which is a set of long noncoding RNA (lncRNA)-transcribed antisense from *FLC* in *A. thaliana*, gets induced, which is a characteristic of polycomb silencing. As discussed earlier in the review, the polycomb group (PcG) proteins are responsible for gene silencing in higher eukaryotes. PcG regulates many genes and several developmental processes. It has been found during cold conditions that the expression of *FLC* gets reduced when COOLAIR gets associated with the *FLC* locus. The synchronized replacement of H3K36 methylation with H3K27me3 gets disturbed at the *FLC* nucleation site when COOLAIR is removed during cold stress (Zeng et al., 2019). The role of COOLAIR in natural variation can be suggested by the slow repression of *FLC* in the slow vernalizing accession Var2-6 because of splicing of distally polyadenylated COOLAIR (Li et al., 2015). Two more lncRNAs, COLDWRAP and COLDAIR, are found to be responsible for the stable silencing of *FLC* by recruiting PHD-PRC2 to a specific chromatin region (Kim et al., 2017). The sequence similarity between lncRNAs across different plant species is not significant, while it has been found that they are positionally conserved. PRC2 is a very important complex in the developmental transition to flowering, which also takes part in several developmental processes in plants. In *Arabidopsis*, for PRC2-mediated H3K27me3, HISTONE DEACETYLASE 9 (HDA9)-mediated H3K27 deacetylation is necessary (Qian et al., 2012). The knowledge of COOLAIR could provide scope for understanding the mechanism of thermosensing during vernalization. LncRNAs acts as a guide for protein complexes mediating epigenetic regulation. Chromatin-associated lncRNAs maintains chromatin conformation. As lncRNAs are mobile and long, they function as bridges to mediate chromatin looping and also helps in inter- or intrachromosomal interactions. RNA hybridizes with DNA and form R-loops contributing to gene regulation. Liquid-liquid phase separation is also mediated by RNA, as it can act as a multivalent scaffold for the binding of RBPs. The role of lncRNAs in several gene regulatory networks associated with various biological processes like plant development and stress responses is studied. A few lncRNAs have been found to perform targeting functions by chromatin modification complexes, coactivation or cosuppression of trans-acting RNAs.

In *Arabidopsis*, FCA and FPA proteins downregulate flowering repressor *FLC* and form an autonomous flowering pathway. DNA methylation can be regulated by both FCA and FPA, which are RNA-binding proteins (Bäurle et al., 2007). In the compartments without membrane, the concentration of proteins and nucleic acids is a very crucial part of cellular biochemistry. The formation of these biomolecules takes place by measures including liquid-liquid phase separation, as the interactions between different multivalent macromolecules generate clear liquid-liquid-demixing phase separations, creating micrometer-sized liquid droplets in an aqueous solution. FCA involves in phase separation, as it possesses prion-like domains that phase separated *in vitro* and shows behavior *in vivo*. The construction of FCA nuclear bodies requires a coiled protein, FLL2, which enhances the proximal polyadenylation of FCA. In the *Arabidopsis* genome, this proximal polyadenylation decreases transcriptional read through (Li et al., 2012). The expression of these FLL2 has been seen to increase the number and size of FCA nuclear bodies. To increase polyadenylation at specific sites, FCA nuclear bodies compartmentalize 3'-end processing factors. It is observed that coiled-coil proteins can promote liquid-liquid phase separation (Fang et al., 2019). FCA is considered as a part of the signaling pathways mediating plant adaptation responses to high temperatures (Lee et al., 2015). FCA RNA-binding protein act as a transcriptional regulator through modifying RNA processing or chromatin modification. Various enzymes and regulators associated with the transcriptional and posttranscriptional control of plant reactions to environmental signals are mediated by FCA. FCA generally works in these processes by RNA metabolism and chromatin alteration.

The expression of PsSNF5, which is a chromatin remodeling gene, is induced by drought stress (*Pisum sativum* SNF5). PsSNF5 interacts with *Arabidopsis* SWI3-like proteins (SWI3A and SWI3B), which further interacts with FCA (Rios et al., 2007; **Figure 2**). Flowering time and stress responses are regulated by ABA-induced SNF5 and FCA by chromatin remodeling. Premature leaf senescence due to abiotic stresses leads to reduced photosynthesis. Jasmonic acid and ethylene-responsive-HDACs, HDA6 and HAD19 (Wu et al., 2008), alter leaf senescence, while HDA19 antisense transgenic plants/T-DNA mutants showed early senescence (Zhou et al., 2005; Ay et al., 2014; **Figure 2**).

EFFECT OF STRESS ON CHROMOCENTERS

Chromocenters are dense heterochromatic regions, heavily packed with DNA and proteins present in the nucleus of some cells. Emil Heitz (1928) historically identified heterochromatin as the nuclear material that remains highly condensed within the interphase nucleus. He named these regions "heterochromatin" to distinguish them from the regions showing variable staining and condensation, which he called "euchromatin." The functional properties and composition of chromatin structure came into the picture very late; however, the distinction between heterochromatin and euchromatin was provided many years back (Passarge, 1979). A major point of discussion comes from the structure of heterochromatin, which is cytologically visible upon different types of stresses within *Arabidopsis* nuclei. At a specific developmental stage or particular environmental condition, these chromocenters can be transiently decondensed. It is proposed that nuclear organization modifications and stress

responses have a functional connection (Groves et al., 2018). Stress can be accompanied by dramatic morphological alterations in the organization of plant nucleoli and the protein content. These changes are presumably related to alterations in diverse nucleolar transcriptional activity under stress conditions (Kalinina et al., 2018). The chromocenters are enriched in transposable elements, transcriptionally silent 45S and 5S rDNA arrays, and centromeric and pericentromeric satellites, which can be seen clearly in *Arabidopsis* nuclei at interphase (Fransz and Jong, 2011; Benoit et al., 2013). The formation of euchromatic loops from chromocenters has been visualized by DNA fluorescence *in situ* hybridization (FISH) experiments and more recent Hi-C analysis, revealing their role in the spatial organization of chromosomes (Ron et al., 2013; Feng et al., 2014). Thus, chromocenter organization has been extensively utilized to understand chromatin modifications under stress or during development in *Arabidopsis* (Benoit et al., 2013). Interestingly, the temporary decondensation of chromocenters that happens during the floral transition occurs in terminally differentiated leaf tissue (Tessadori et al., 2007), and it is still unclear whether it occurs in the meristem as well.

At the time of seed germination and maturation, the alteration of chromocenter structure also takes place in the nuclei of the cotyledon (Zanten et al., 2012) and postgermination development (Mathieu et al., 2003; Douet et al., 2008). Chromatin modifications are related to process linked to the development of the plant as well as external stress signals, like temperature-stress-induced dedifferentiation (van Dam, 2014), lightly shape nuclear architecture (Bourbousse et al., 2015) and gene expression (Kaiserli et al., 2018), and reprogramming of microspores. There are some reports showing how the nuclear structure is affected by abiotic stresses unrelated to specific developmental processes in the rye and rice seedlings, in which upon heat stress, the 45S rDNA (Santos et al., 2011) loci undergo decondensation. In *Arabidopsis*, it was found that the stem cell expression is mainly dependent on the developmental stage but also contain a core set of stem-cell-specific genes, some of these genes are involved in epigenetic silencing. In meristems before flower induction, increased expression of transposable elements correlates with enhanced CHG methylation during development and reduced CHH methylation, before stem cells enter the reproductive lineage (Sasaki et al., 2019). This shows the occurrence of epigenetic reprogramming at an early stage and its role in genome protection in stem cells during germline development (Gutzat et al., 2020). In the *Arabidopsis* leaf tissue, after prolonged heat stress, centromeric repeats and 5S rDNA decondensation occur (Pecinka et al., 2010). In *Arabidopsis*, HEAT INTOLERANT 4 (HIT4) was discovered for heat-stress-intolerant mutants; in excessive heat stress, it is required for chromocenter decondensation upon heat stress (Wang et al., 2013, 2015).

PLANT RESPONSE TO STRESS: THE CHROMATIN PERSPECTIVE

Plants cannot escape the myriad of biotic and abiotic stresses to which they are exposed during their life cycle. The information available highlights those changes in chromatin features; particularly, histone modifications are a key feature in plant response and adaptation to environmental insults. According to a review by Dogan and Liu (2018) and Silveira (2018), it is expected that, in the near future, there will be a wave of datasets focusing on plant epigenomes and transcriptomes in the 3D context, serving as an essential component in finding key regulators of plant chromatin folding and positioning (especially for crop plants). Changes in temperature induce specific responses modifying chromatin configurations as reported for cold (Kim et al., 2010; Roy et al., 2014) and heat (Christina et al., 2010; Kumar and Wigge, 2010; Pecinka et al., 2010) stress in higher plants and algae (Schroda et al., 2001; Lee et al., 2014). Due to global warming, guarding plants against decline due to heat stress and temperature fluctuations is becoming increasingly important (Ohama et al., 2017). Small RNAs and epigenetic regulation are involved in transcriptional regulation and heat stress memory (Kapazoglou et al., 2017). Drought signaled through abscisic acid is an extreme condition for plants and is also linked to chromatin modifications (Mehrotra et al., 2014). Experiments performed in *Coffea canephora* verified that transcriptional memory alters drought-responsive gene expression (Guedes et al., 2018). Osmotic stress or salinity is frequently associated with responses at the chromatin level. Light deficiency affects chromatin structure, signaled by light perception factors (Zanten et al., 2010, 2012). Plants exposed to chemically induced DNA damage force chromatin modifications (Braszewska-Zalewska et al., 2013; Rosa et al., 2013). Chromatin structure is also disturbed by toxic components as demonstrated by the study on seawater algae with respect to cadmium (Greco et al., 2012). In addition to these abiotic factors, it has been observed that the pathogen challenge is signaled to chromatin to induce defense gene expression (Berr et al., 2012; Schenke et al., 2014). Eventually, intrinsic responses to senescence or wounding (Gnatowska et al., 2014) can modify chromatin configurations.

Autotrophs like plants possess an impressive degree of metabolic flexibility to sense and survive under different stress conditions. Knowledge in chromatin architecture and associated modifications is important to understand varied pathways through which plants adapt themselves to various stress conditions. Chromatin organization and epigenetic modification, which can be altered by developmental or environmental stimuli, are dynamic in nature and provides a means to stabilize and condense DNA. Chromatin architecture is modulated to cope with various stresses that plants may experience. Numerous transcription factors, transcriptional memory, and small noncoding RNAs contribute towards gene expression modulation during plant stress responses (Avramova, 2015). The rearrangement of chromatin between transcriptionally inactive to transcriptionally active state facilitates access of transcription factors or other DNA binding proteins to regulate gene expression.

Stress can induce transcriptional activation as well as transcriptional repression. To bring repression of transcription, transcriptional repressor proteins counteract the activity of positively acting transcription factors. In addition, transcriptional

repression is often linked with chromatin reorganization. Numerous transcriptional repressor proteins communicate either directly or indirectly with proteins that remodel chromatin or would themselves be able to impact chromatin structure. Transcriptional repression may also display "memory" of the prior transcriptionally inactive state, which is known as transcriptional repression memory (TREM). A study conducted in yeast shows that transcriptional repression of ∼540 genes occurs at a faster rate if, during carbon source shifts, the genes have been previously repressed (Gaston and Jayaraman, 2003).

Various biochemical changes take place in chromatin structure to maintain gene activity: Some of these modifications have the capacity to be stably transmitted through cell division stages, which suggest that modifications in the chromatin state could help in coping with different biotic and abiotic stresses (Gallusci et al., 2017). Further studies may help to validate the transmission of stress-induced changes in chromatin. The information can be used to increase crop yield and thus improve agricultural systems. This information can be utilized to find out the significance of chromatin remodeling proteins in regulating transcription at each step, i.e., initiation, elongation, and termination.

OUTSTANDING QUESTIONS

- What are the kinetics of changes in histone modifications and transcripts following the stress signal perception?
- Which transcription factor interact with which coactivator or corepressors under a given stress situation and cell type?
- What is the role of cell type in determining transcriptional regulation through its chromatin status?
- What is the exact composition of native chromatin modifying complexes in different tissues, developmental stages, and stress situations?
- Can we design epigenetic switches to regulate agronomically important traits under stress conditions?
- Can we exploit the strength of epigenome modification in horticultural crops since their breeding is difficult? Can grafting change methylation and acetylation state in horticultural crops?

AUTHOR CONTRIBUTIONS

SB and SM: writing and reviewing. MB and GL: reviewing and editing. RM: conceptualizing, reviewing, writing, and editing. All authors contributed to the article and approved the submitted version.

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How Stress Facilitates Phenotypic Innovation Through Epigenetic Diversity

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Climate adaptation through phenotypic innovation will become the main challenge for plants during global warming. Plants exhibit a plethora of mechanisms to achieve environmental and developmental plasticity by inducing dynamic alterations of gene regulation and by maximizing natural variation through large population sizes. While successful over long evolutionary time scales, most of these mechanisms lack the short-term adaptive responsiveness that global warming will require. Here, we review our current understanding of the epigenetic regulation of plant genomes, with a focus on stress-response mechanisms and transgenerational inheritance. Field and laboratory-scale experiments on plants exposed to stress have revealed a multitude of temporally controlled, mechanistic strategies integrating both genetic and epigenetic changes on the genome level. We analyze inter- and intra-species population diversity to discuss how methylome differences and transposon activation can be harnessed for short-term adaptive efforts to shape co-evolving traits in response to qualitatively new climate conditions and environmental stress.

Keywords: epigenetics (DNA methylation), epigenomics, transposable element, abiotic stress, energy stress, plant engineering, methylome diversity, natural variation in plants

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INTRODUCTION

Plants grow in a variety of climatic conditions around the world, which we largely attribute to an adaptive genome that evolves over long periods of geological time. However, such extensive diversification requires thousands or up to millions of years, either induced by incremental changes in the local environment or upon sudden exposure to a new terrain (possibly as a result of seed dispersal, human intervention, domestication, etc.). These changes in local or global environment are usually perceived as stress conditions for adapted plant species. For this reason, plants have evolved complex stress response mechanisms and multiple traits as a survival buffer to generate enough plasticity when sudden environmental changes occur. Depending on the environmental cue and the plant species at hand, such mechanisms are either of physiological, epigenetic, or genetic nature and depend on the range, magnitude, and duration of the perceived stress. Whether this perceived stress and its response mechanisms are inherited through multiple generations (stress memory), however, depends on several endogenous and exogeneous factors that we review and examine in detail throughout this article. In particular, we focus on co-evolving traits and co-occurring stress response mechanisms to overcome the limited view of uncoupled stress variables. Additionally, we emphasize the need to introduce gene regulatory networks as a conceptual methodology to study the impact of rapid environmental changes such as global warming on

the survival capacity of plant ecotypes or species. This notion of interconnected stress-response mechanisms against multiple external forces acting on plants, and the heritability of these traits can stimulate the development of a new generation of plant engineering tools. We envision that such data can be used to build predictive models able to optimize biotechnological efforts, ultimately for engineering coupled traits and improved stress response mechanisms. To achieve this goal, it is crucial to gain a detailed understanding of the interplay between physiological-, epigenetic-, and genetic- mechanisms in the context of within-individual (**intra**generational) stress responses and the transgenerational manifestation (adaptation) of stress response mechanisms (**inter**generational).

We present a new hypothesis postulating that the physiological level represents a buffer zone that determines the stress response plasticity of a plant within a generation while hedging the epigenetic and genetic levels against stress-induced changes severe enough to be heritable. Only after a stress stimulus overpowers this physiological response buffer, a transgenerationally stabilized stress memory will manifest itself and induce the emergence of new (epi-)genetic stress response variants subjected to natural selection. Furthermore, the epigenetic level itself can also be seen as a buffer zone acting as an interface between the environmental and genetic levels to create a transgenerational buffer while the physiological level represents the buffer zone that determines the adaptive flexibility of a plant species within a generation. We test this hypothesis by reviewing published evidence in support of this notion and provide perspectives on experimental designs for future studies aiming to directly test this conceptual model. A mechanistic understanding of this stress-response interplay is crucial for developing the next generation of plant engineering tools.

In this context, the epigenetic level which can also respond in tandem with stress physiology by selectively reshaping the epigenetic landscape is less likely to manifest these changes transgenerationally, as long as the physiological response is sufficiently strong in buffering the stress stimulus. Once this physiological buffer is saturated, the manifestation of a restructured epigenetic landscape is mostly caused by stress-induced structural variants (SVs) and in particular, transposable element (TE) mobilization that reshuffle genetic material and thereby draw the epigenetic toolkit to new loci while changing the global distribution of epigenetic marks in progeny. However, this genetic reshuffling is only possible when a stress stimulus is strong enough to severely reshape the epigenetic landscape, reactivating previously silenced loci and triggering a mobilization burst to subsequently affect the genetic composition of the germline.

THE PLANT METHYLOME: A KEY PLAYER IN STRESS-RESPONSE AND ADAPTATION

Consider a scenario where a generalist plant species is dispersed to a new terrain with increased average temperature levels. In this environment, one form of adaptation could be to slowly increase the basal expression of heat tolerance genes for continuously reducing the impact of this subtle heat 'stress' experienced by the plant. Eventually, if the trend of temperature increase continues over several generations, these regulatory changes will be ingrained and fixed on the genetic level.

Yet, a plant that has to survive in a suddenly changing environment such as global warming where both the temperature and precipitation are drastically different and the salinity concentration of the soil increases rapidly, would need to alter several genes and pathways in tandem to maintain reproductive fitness. For most species, this would certainly be a stronger 'accumulative stress' experienced by the individual plant and requires more complex gene regulatory re-wiring for short-term adaptation. When experiencing accumulative stress, one possibility for the plant is to employ a combination of epigenetic and genetic mechanisms to maintain genome integrity without the risk of being subjected to several gene regulatory trade-offs when major physiological or developmental changes occur simultaneously. In this article, we examine the various outcomes of the interplay between epigenetic, genetic and physiological regulatory networks by first reviewing our current understanding of stress response mechanisms in plants and then providing future perspectives and applications within the (stress) epigenetics and plant engineering fields. In particular, we focus on DNA methylation as an epigenetic mark involved in gene expression regulation, and how these marks can trigger a cascade of effects that favor both short term (epigenetic response) and long term (transgenerational manifestation) adaptation to stress.

DNA methylation marks (at 5' cytosine positions) are epigenetic signatures encoding the state of a genome exposed to particular endogenous (e.g., developmental) and exogenous (e.g., environmental) factors throughout intra- and inter-generational time. A subset of these marks are sometimes closely dependent on histone modifications, and are often accompanied by other epigenomic features such as histone variants and chromatin accessibility (Lippman et al., 2004; Johnson et al., 2007; Slotkin and Martienssen, 2007; Li et al., 2018). Overall, DNA methylation marks have genome-wide distribution patterns that can be reshaped upon exposure to environmental stress.

With regards to DNA methylation, such genome-wide changes are generally observed as differentially methylated regions (DMRs) that occur in tandem with transcriptomic or other epigenetic changes under a certain condition. These marks can also be found as heritable natural epigenetic alleles (epialleles) (Schmitz et al., 2013). The methylation signatures of many such epialleles have the potential to fine-tune the expression of flanking genes or certain transposable elements based on their methylation levels. Natural and spontaneously occurring epialleles have been identified in several plant species (Weigel and Colot, 2012). Tracing the evolutionary origins and transgenerational manifestation of these marks has been expedited by the availability of large methylome datasets, some of which have recently provided evidence for the emergence of epialleles from diverse mechanisms of maintaining methylation homeostasis in natural strains (Zhang et al., 2020).

Since population diversity is largely governed by changes in the environment, a large spectrum of environmental stresses

have been studied under controlled laboratory conditions. These include biotic and abiotic stresses which are strongly experienced both above and below ground level (salinity, nitrogen and phosphate levels, pathogens, temperature, light exposure and drought, etc.).

While only a subset of all methylome signatures may directly trigger short-term stress response mechanisms, there is evidence that methylation changes are accompanied by other epigenetic changes that in turn can drive an adaptive process benefiting the plant in the long run (Fang et al., 2017; Reynoso et al., 2019; Forestan et al., 2020). Technological advances in sequencing approaches have facilitated the discovery of candidate gene-regulatory elements throughout the genome that may be conserved across strains and species. These elements are often hotspots for a combination of epigenetic marks that include DNA/histone methylation, unique histone variants, accessible chromatin regions and topologically associated domains (TADs) (Maher et al., 2017; Lu et al., 2019; Ricci et al., 2019; Karaaslan et al., 2020).

Together, the tight interplay between plant stress physiology, epigenomic regulation, and adaptive evolution requires a new focus in the light of rapid shifts in global environmental conditions such as climate change. Learning from the examples presented in this work, we propose future directions for plant stress tolerance engineering that harness the naturally occuring activation potential of transposable elements (TEs) (Paszkowski, 2015; Benoit et al., 2019) and natural variants of the methylation apparatus derived from ecotype or species comparisons (Schmid et al., 2018) to facilitate adaptive innovation in response to qualitatively new climate conditions.

CAN STRESS-RESPONSE PHYSIOLOGY HEDGE TRANSGENERATIONAL METHYLOME (IN)STABILITY?

When sudden or severe changes occur, plants which usually grow in a constant environmental niche can overcome their basal physiological response mechanisms and induce transgenerationally stable changes to the epigenetic landscape (Baulcombe and Dean, 2014). Such changes (genetic and epigenetic) can alter gene expression in order to improve the adaptive fitness of the plant upon the threat of death and ultimately population extinction. Additionally, the epigenetic landscape can be even more dynamic when genetic material is reshuffled (SVs and TEs) as a consequence of the applied stress. It is well known that structural variants in several plant species can generate wide phenotypic diversity (for example, The 1001 Genomes Consortium, 2016; Zhou et al., 2019; Alonge et al., 2020) and some of which can enable improved stress tolerance, recently demonstrated by studies such as Kalladan et al. (2017), Catacchio et al. (2019), Picart-Picolo et al. (2020). Yet, genetic mutations in methyltransferase enzymes for tuning epigenetic stability (Shen et al., 2014; Sasaki et al., 2019), or heritable genome rearrangements catalyzed by TE insertions can contain the necessary stress-response DMRs that may function to either deteriorate fitness or for adding a newly acquired stress tolerance that allows future generations to adapt to the altered environmental condition (Quadrana and Colot, 2016).

Although comprehensive evidence of the inheritance of environmentally induced changes in DNA methylation remains to be collected (Quadrana and Colot, 2016), emerging evidence suggests that epigenetic variation can be exposed to natural selection and induce rapid adaptive responses (Schmid et al., 2018). It remains to be explored whether environmentally induced epigenetic variation is a rare event violating the principles of epigenetic homeostasis (Williams and Gehring, 2020) or whether such events occur frequently enough in large populations to provide a mechanism for rapid adaptation as theoretical population genetic models suggest (Pal, 1998; Pál and Miklós, 1999; Day and Bonduriansky, 2011; Geoghegan and Spencer, 2013a,b; Jablonka and Lamb, 2015; Kronholm and Collins, 2016) and reviewed in Quadrana and Colot (2016). Here we assume that future experiments will unveil more examples of environmentally induced heritable changes to epigenomic landscapes. These insights will raise further questions regarding the association between physiological responses and epigenetic remodeling whereby physiology could act as a buffering layer before environmentally induced heritable changes to epigenomic landscapes can manifest themselves transgenerationally.

To differentiate various stress-response mechanisms and their impact on epigenetic landscapes in plants, we classify stresses by their ability to restrict energy and nutrient supply to the plant body. In this context, abiotic stresses fall under the class of energy-depleting and starvation stresses (Baena-González et al., 2007; Baena-González and Sheen, 2008; Mason et al., 2014), whereas biotic stresses can in particular cases be associated with a more complex energy housekeeping balance (for example parasitic relationships) (Alvarez et al., 2010). We believe that this distinction is vital for plant stress perception, and ultimately determines which cohort of response mechanisms will be employed when coping with respective conditions. This energy notion of stress response predicts a hierarchy of stress tolerance whereby the availability of energy determines the extent to which plants can explore their full spectrum of response mechanisms. This perspective would predict that starved plants will focus energy supply to the most vital response pathways and thereby be exposed to more dramatic epigenetic modifications in comparison with fully nutriated plants that could employ a broader spectrum of physiological responses to buffer the impact on their epigenomic landscape. Although this link between convergent energy-stress and epigenetic remodeling remains to be further explored in plants (Hauben et al., 2009; De Block and Van Lijsebettens, 2011; Ljung et al., 2015), evidence in support of this idea has been accumulated in *Drosophila* (Riahi et al., 2019). From an ecological perspective it is well established that the global availability of food/energy supply determines the reproductive fitness and thereby population size of a species (Darwin and Wallace, 1858). It remains to be established, however, to what degree and at what speed individuals within a population can evolve (epi-)genetic stress-response variants to survive under rapidly changing environments, either under high inputs of food/energy supply or during periods of starvation. Finally, we would like to point out that extensive mechanistic

studies connecting energy balance, physiology, and epigenetic remodeling are largely lacking and are only emerging for some model organisms. However, we do see an exciting opportunity in the coming years to employ multi-omics approaches to further investigate these complex relationships.

SENSING STRESS AT THE GRASSROOTS: HOW PHYSIOLOGICAL RESPONSES PREVENT OR ENABLE HERITABLE METHYLATION MARKS

Soil is a major source of nutrition for the sessile plant - it needs the right properties of pH, osmolarity, water, micro- and macro-nutrients to favor reproductive fitness and adaptability. Salinity levels for example, exhibit high variability across plant habitats ranging from deserts to marshlands and are often studied in the context of physiological and gene-level stress response (Bui, 2013). Since salinity is a property that also changes gradually alongside new climate conditions, it can be perceived as a plant stress that requires epigenetic acclimatization.

A transgenerational study in *Arabidopsis thaliana* (Wibowo et al., 2016) showed that seedlings grown in highly saline growth media carry stress-induced DMRs, a subset of which are passed on to their progeny through the female germline. Yet, these DMRs maintain their methylation state only as long as the saline conditions remain constantly present, reverting to their wild-type state when this stress no longer exists. These results raise the question: what factors govern the plasticity of the underlying stress-response methylomes, and the sensitivity with which the saline stress is perceived on the epigenetic level? While the exact threshold for this saline sensitivity (weighed by time or the strength of the applied stress) remains undetermined, there is evidence to show that epigenetic changes do get fixed over time, establishing signatures specific to the climatic condition experienced by the entire population. For example, differences in soil salinity arising from tidal water and nutrient circulation have also been shown to contribute to epigenetic diversity between salt marsh and riverside mangrove populations in Brazil (Lira-Medeiros et al., 2010). Interestingly, these populations are differentiated by genetic mutations as well (fewer in number than the epimutations), but it remains unknown if these mutations also affect the methylation machinery at the intra-species level.

Natural varieties of olive (*Olea europaea*) have been shown to be differentially tolerant to salinity (in a hydroponic system), as observed by phenotypic and metabolic changes (Mousavi et al., 2019). Although both salt-susceptible and salt-tolerant cultivars exhibit an overall increase in methylation levels upon salt stress, the susceptible cultivars harbored hypomethylated loci flanking upregulated genes involved in ionic exclusion, water and nutrient uptake. These results are in contrast with studies in rice, where salt tolerant varieties exhibit genome-wide demethylation as opposed to susceptible varieties (Ferreira et al., 2015). Methylation changes in the tolerant lines could be linked to stress-induced expression changes in demethylase and methyltransferase genes. It appears that different species (and subspecies) have evolved distinct mechanisms (such as altering the expression of stress-response genes through the methylome, or the methylation machinery as a whole) for enabling metabolic responses to this stress. A transgenerational experimental design to address the aforementioned studies would provide more insight on how these distinct mechanisms affect the germline and are fixed over evolutionary time.

Apart from natural sodium and potassium salts in water, depletion of soil nutrients can also pose as a type of stress detrimental for plant growth. Secco et al. explored the epigenetic and transcriptomic responses of plants starved of inorganic phosphate in soil (source for the vital macronutrient Phosphorus), in an attempt to resolve and compare the temporal hierarchy of stress response mechanisms between rice (*O. sativa*) and *A. thaliana* plants (Secco et al., 2015).

The study found that DMRs under phosphate starvation were more predominant in the rice genome compared to *A. thaliana*. This observation fits with genomic structural differences between the two species, especially with regards to transposable elements (TEs). TEs are mobile genetic elements that can change their genomic location by either exploiting a cut-and-paste mechanism (retro-elements) or by increasing their copy number via a copy-and-paste mechanism (DNA elements) (Feschotte, 2008). Most of the DMRs under phosphate starvation were hypermethylated in the CHH context, silencing TEs upon long-term stress and found to be flanking highly induced phosphate-homeostasis genes (Secco et al., 2015). From additional results that indicate the precedence of gene expression over methylation changes, the authors propose that TE activation may be a by-product of proximal gene transcription, eventually being silenced by *de novo* methylation. Yet, these DMRs are not transmitted meiotically, which leads us to ask whether high TE loads are an adaptive advantage or disadvantage for evolving genomes. The rice genome, for example, could harbor several TEs to provide a buffer against stress and avoid some potentially lethal methylation changes in the genome, yet would also be equally prone to the deleterious effects of chromatin reorganization during the brief activation of particular TE families. A TE-poor genome such as *A. thaliana* would presumably have other genetic/epigenetic or even physiological mechanisms to respond to this stress, which explains the reduced number of DMRs.

While we can monitor the mechanistic changes that occur over temporal scales, the intricate steps involved in instantaneous stress perception and the hierarchical order of cellular events leading to the manifestation of epigenetic marks, are still poorly explored. If we already know how DNA and RNA-level modifications occur upon stress, how do they interact with sensory and hormonal signaling networks?

In this context, some studies show that biotic stress can induce DNA demethylation and activate the expression of certain TEs and defense genes (including NLRs), possibly by interacting with pathogen-responsive elements located in gene promoters (Yu et al., 2013). Revisiting the example of salt stress in soils, there is further evidence that altered salt concentrations can be sensed by plant roots, which modulate their growth rates by accumulating reduced auxin levels. In rice, adaptation to saline soils has been attributed to altered GA (Gibberellic Acid)

levels during different stages of growth (as reviewed by Yu et al., 2020). Further studies have shown that the expression of histone acetylases and deacetylases can be influenced by hormonal cues such as ethylene and jasmonic acid signaling, and indirectly regulate auxin response pathways (Song et al., 2005; Zhou et al., 2005). Similarly, histone methylation and DNA methylation are linked with the expression of auxin efflux proteins such as PIN1, one classic example being the crosstalk between non-coding RNA, chromatin looping and H3K27me3 at the *APOLO* locus in *A. thaliana*, which is dynamically regulated upon changes in auxin concentration (Ariel et al., 2014, 2020). Chromatin remodeling proteins such as PICKLE and BRAHMA in *A. thaliana* which employ their ATPase activity to alter nucleosomal structure, can also affect accessibility to transcription factors and thereby gene expression of carrier proteins involved in ABA, GA, CK and auxin (IAA) synthesis pathways. *pkl* mutants are not only hypersensitive to salt, chilling and freezing stress (Yang et al., 2019), but also hypersensitive to germination upon abscisic acid (ABA) treatment (Perruc et al., 2007), demonstrating their strong interdependence with hormones regulating plant growth and development.

Although there is still no clear characterization of a temporal hierarchy in stress response and their impact on the epigenetic landscape, these findings certainly illustrate the possibility of hormonal signaling networks initiating genomic and epigenomic changes, but also being tightly feedback-controlled by chromatin.

BIOTIC STRESS RESPONSE: SWITCHING BETWEEN EPIGENETIC DEFENSE AND TE ACTIVATION

While natural strains in *A. thaliana* show large differences in their methylomes (Kawakatsu et al., 2016) epiRILs (epigenetic Recombinant Inbred Lines) generated from crosses between hypomethylated mutant lines (such as *met1, ddm1*) and a wild-type strain, carry a mosaic of DMRs originating from their parents. epiRILs provide a useful germplasm collection to mine for methylation variants or epialleles that influence gene expression for a desirable trait. The uniquely recombined methylation signatures harbored in individual epiRIL lines result in large phenotypic variation, possibly due to the mosaicity of epigenomes and mobilized transposable elements. This can be observed, for example, in progeny of *met1-3* mutants and wild-type plants propagated over several generations. When infected with *Pseudomonas syringae pv.* tomato(*Pst*), a subset of these lines show increased resistance or susceptibility compared to their parental lines, representing approximately 58% of the resistance variation in 127 natural accessions of *A. thaliana* (Reinders et al., 2009). It will be interesting to examine whether the subset of DMRs, or the epialleles determining the resistance phenotypes are shared between the epiRILs and natural accessions, providing clues to understand the degree of inbreeding methylation stochasticity that governs methylome heritability. Curiously, the methylation state of these epiRILs also activates the transposition of the CACTA transposon family, which is silenced in *met1-3* mutants, indicating that epigenomic recombination can trigger interaction effects that may affect select loci *in trans*.

Furci et al. (2019) used Col-0 x *ddm1* epiRIL lines in *A. thaliana* to identify genomic regions that confer methylation-mediated resistance to biotic stress by the downy mildew pathogen *Hpa* (*Hyaloperonospora arabidopsidis*). Although several epiQTL loci were identified from the varying quantitative resistance between lines, regions that were epigenetically primed by the stress and inherited in the F10 generation were also found outside these associated loci. It was found that these primed DMRs largely overlap with TEs or TE- related genes and likely regulate *in trans* the expression of *Hpa*-resistance and defense genes. When a similar *Hpa* infection is introduced in *A. thaliana* mutants of various proteins involved in DNA methylation, systemic acquired resistance to the infection is impeded from transgenerational carry-over (Luna and Ton, 2012). Most notably, it was found that hypomethylation in the CHG context catalyzed by the KYP and CMT3 proteins could be crucial for generating transgenerational memory in this pathogen species.

Biotic stresses can also induce TE-specific methylation changes, such as during *Pseudomonas syringae* pv. tomato (*Pst*) infection in *A. thaliana.* Cambiagno et al. examined whether epigenetic induction of *PRR/NLR* genes affects pericentromeric TE expression during infection (Cambiagno et al., 2018). Indeed, four TE-families (belonging to the LTR-Gypsy Superfamily) are activated upon infection triggered by hypomethylation at these loci, along with similarly induced *NLR* genes. Surprisingly, prolonged infection recruits siRNAs directed against both sets of loci, thereby eventually re-silencing them with RdDM methylation. Furthermore, the authors showed that a *mom1* (*Morpheus' Molecule-1*) mutant in which some pericentromeric TEs are expressed also activates the expression of unlinked *PRR/NLR* genes, priming these plants against *Pst.* infection. The cohort of sRNAs that commonly regulate both distal TEs and non-specific *NLR* genes upon re-establishment of methylation marks hint at the potential for transposable elements as triggers for initiating genome-wide immune responses, although the exact mechanisms remain unclear. From the plant's perspective, controlling multiple loci carrying similar genetic/epigenetic motifs may be efficient, but this could also be facilitated by tuning a single master regulator causing diverse downstream outcomes.

CONTROL VS. CHAOS: REORGANIZATION OF THE GENOME AND THE EPIGENOME UNDER HIGH TEMPERATURES

When the need arises for rapid genome transformation in response to stress, TEs can function as master regulatory switches, catalyzing a domino effect on the somatic and meiotic epigenome upon their mobilization and reintegration. One of the most seminal discoveries in the epigenetics of plant heat response is that of the *ONSEN* (*ATCOPIA78*) family of TEs in *A. thaliana* (Ito et al., 2011). When mutants defective in siRNA biogenesis, such as *nrpd1,* are subjected to heat stress

at 37°C, the *ONSEN* family of LTR elements are transcribed, extra-chromosomally replicated and re-inserted into various loci (a detailed mobilization protocol can be found at Gaubert et al., 2017; Sanchez et al., 2017). Furthermore, repeating such a heat treatment in two consecutive generations results in additional reactivation of the newly inserted elements, only to create more copies genome-wide (Matsunaga et al., 2012). It was recently shown that heat stress can also induce dispersion of the constitutive heterochromatin in canonical RdDM mutants (*nrpd1*, *rdr2, drm2*) which could potentially contribute to higher transposition rates and increased copy number of *ONSEN* elements (Hayashi et al., 2020).

Delving further into possible links between chromatin and DNA methylation, Quadrana et al. (2019) examined the locations of new TE insertions in *nrpd1* mutants under 37°C heat stress, to find that the insertion sites were largely found in proximity to the histone variant H2A.Z (Quadrana et al., 2019). The authors showed that epiRIL lines derived from a *ddm1* x Col-0 cross also display a similar enrichment for H2A.Z near insertion sites of *ATCOPIA93*. This belongs to one of three TE families that harbored the largest number of private insertions examined in 107 F8 epiRIL lines. Similarly, the *VANDAL21* family of insertions were enriched for DNaseI hypersensitivity sites (having accessible chromatin) and the *ATENSPM3*- family insertions enriched for H3K27me3 (histone methylation). Taken together, these results are indicative of the specificity with which certain TE families re-integrate their copies into the genome, preferentially favoring chromatin marks that lie within genes involved in environmental stress response.

What would be the consequences of TE mobilization in epiRILs derived from naturally hypomethylated strains? Take for example, Cvi-0, a strain that is largely hypomethylated among the 1001 sequenced strains of *A. thaliana* (Kawakatsu et al., 2016). It is tempting to speculate that genome-wide hypermethylation or hypomethylation would exhibit extreme ranges of epigenetic flexibility for facilitating stress-response. Alternatively, more robust stress resistance mechanisms at the epigenetic level may be the result of a combinatorial effect of chromatin marks and a methylation landscape determining the resistance effect rather than a uniformly high methylation level. It remains to be explored which of these scenarios is the guiding principle of epigenetic stress tolerance, but some studies started to address these questions from various angles (Tittel-Elmer et al., 2010; Mirouze and Paszkowski, 2011; Mirouze et al., 2012; Iwasaki and Paszkowski, 2014a,b; Ito et al., 2015; Hosaka and Kakutani, 2018). From the *ONSEN* example, it appears that multigenerational stress in such a hypomethylated background would only be more deleterious for the genome although recent evidence suggests that transgenic lines of hypomethylated poplars show higher tolerance to water deficit (Sow et al., 2020) hinting toward more complex mechanisms involved in this process. Furthermore, it remains unclear whether the locations of new copies (re-insertions) will remain specific to H2A.Z marks and genic loci (Gaubert et al., 2017) over consecutive generations, or eventually generate a more stochastic genome-wide pattern.

In epiRIL lines of *met1,* the activation of the mobile retrotransposon copy *EVADE* reaches a saturation point of approximately 40 copies in the F10 generation, after which siRNA-induced silencing is restarted once again (Marí-Ordóñez et al., 2013). This suggests a possible mechanism for the cell machinery to recognize TE load and prevent further gene disruption. Unlike *ONSEN*, *EVADE* (Mirouze et al., 2009) can be activated by hypomethylation alone - which means that progeny carrying the hypomethylated epialleles can continue carrying this activated TE in the next generations. *ONSEN* elements, on the other hand, can get transcriptionally silenced by methylation upon re-integration and would only reactivate in the progeny upon continuous heat stress application.

Apart from examples of heat-activated TEs in *A. thaliana*, a newly discovered giant retro-element named *MESSI* in the tomato genome has recently been identified upon long-term heat exposure. This element can potentially exploit developmentally associated escape strategies during tomato meristem development and overcome transcriptional gene silencing in vegetative tissues, thus inducing genetic variation in progeny plants (Sanchez et al., 2019).

The methylome landscape under high temperatures may also be controlled at the genetic level, driven by structural variations at methyltransferase genes, such as *CMT2* in *A. thaliana* (Shen et al., 2014). *cmt2* mutants and accessions carrying a natural knockout (*CMT2-STOP* allele) also exhibit an increased tolerance to high temperatures, resulting from CHH hypomethylation that alters gene expression for stress-response. Ultimately, the tight inter-dependence between genetic and epigenetic pathways makes it challenging to identify which of these factors were first established during the evolutionary adaptation to temperature changes.

TO WHAT EXTENT DOES MAGNITUDE OF STRESS DETERMINE THE MAGNITUDE OF RESPONSE?

It is important to note that most of the above studies examine the effects of sudden and drastic stresses on plants, which may not always reflect natural settings. In wild plants, it remains to be explored how epigenetic plasticity comes to the rescue when the plant experiences a gradual change in its environment over time. Taking the example of drought stress, it was recently shown that mild conditions of water deficit in *A. thaliana* only trigger minor changes to overall methylation patterns (Van Dooren et al., 2018). Moreover, only 2 out of 468 genes with altered expression under such a drought stress, were associated with differentially methylated positions. While there is some degree of strain-level differentiation in response to the stress (measured by reduction in rosette area), this epigenetic and transcriptomic plasticity is not inherited in the progeny. An earlier study that examined mild drought stress over 5–6 generations in *A. thaliana* came to the same conclusion regarding the transgenerational stability of the methylome, and the rare occurrence of heritable phenotypic changes (Ganguly et al., 2017). In maize however, mild drought triggers transcription of long non-coding RNAs and histone

methylation changes that can be retained in the germline as a type of stress-memory (Zhang et al., 2014; Forestan et al., 2020).

While it has been shown that polygenic architecture and loss-of-function alleles can explain differential tolerance of *A. thaliana* accessions to drought (Exposito-Alonso et al., 2018; Monroe et al., 2018), heavy drought stress response remains to be examined at the methylome level. Different strains of *citrus* plants on the other hand, have shown varied responses both in physiology as well as methylation levels to repeated cycles of water-deficit (Neves et al., 2017). Repeated drought exposure in 11 consecutive generations of two rice varieties significantly improved their adaptability, accumulating epimutations specific to stress-response genes (Zheng et al., 2017), while in tomato drought-stress response has been linked to the activity of the retrotransposon family *Rider* (Benoit et al., 2019). Since the nature of the stress may hold varied importance across species for the plant's developmental physiology, together this could possibly explain the differences in methylation changes attributed to genotypic identity and the deviation in water supply beyond optimal levels.

EXAMINING THE CONSEQUENCES OF STRESS DURING THE EPIGENETIC RESET IN EARLY EMBRYOGENESIS

In angiosperm plants, DNA methylation, histone methylation and other chromatin marks play key roles before and after double-fertilization, thereby 'resetting' the epigenome of the developing embryo. It is known that DNA methylation in the male gametes catalyze the production of TE-derived siRNAs, accompanied by chromatin changes in the female megaspore mother cell (MMC), eventually forming the endosperm and the embryo. The endosperm is largely hypomethylated compared to the embryo, resulting in the expression of several maternal epialleles (imprinted genes) and also paternally expressed genes (activated by histone methylation in their maternal alleles) (as reviewed by Wang and Köhler, 2017). These are only a few examples of the complex epigenetic dynamics that determine the fitness of the progeny.

Given the complexity of methylome response to stress, can the altered epigenetic state of individual loci during fertilization be sufficient to determine fitness levels across strains? Taking imprinting as an example, the specificity of demethylases such as DME and ROS1, in tandem with DNA and histone methyltransferases could catalyze a domino effect in impeding healthy development of the embryo and the endosperm. In *A. thaliana* for example, differential methylation of the imprinted epiallele *HDG3* in natural accessions can negatively impact embryo development and seed size (Pignatta et al., 2018), demonstrating the power of such unique loci in determining adaptive fitness.

In the context of stress, the germline plays a crucial role in maintaining the integrity of the genome and passing on the DMRs that are necessary for defense. A subset of these DMRs could also overlap with TEs, activating them in the process. In epiRILs of *met1* for example, *EVADE* transcription was observed in the L2 subepidermal adaxial layer of cotyledons, indicating transmission of this element through the female placental organs (Marí-Ordóñez et al., 2013). It is possible that post-meiotic methylation changes can activate silenced *EVADE* copies genome-wide, thereby generating genetic diversity even within seeds of the same silique.

What are the consequences of by-passing the epigenetic reset that occurs during fertilization? Wibowo et al. addressed this question by examining somatic regenerant lines from various postembryonic organs in *A. thaliana* (Wibowo et al., 2018). Their findings reveal that root-tissue derived regenerants heritably retain many root-specific methylome and transcriptome signatures not only in their roots but also their leaves. This indicates the importance of meiotic and post-fertilization processes in determining tissue identity in the growing plant.

When generating hybrids, fertilization processes involve a foreign species or strain where a cohort of homologous epigenetic marks and enzymes also interact with each other. Although this process may be successful in generating healthy seeds, successive generations may bear a negative impact of such hybridization stress. Tomato hybrids, for example, show the gradual transgressive accumulation of siRNAs indicative of epistatic epigenetic interactions between parental marks, thus generating phenotypic diversity (Shivaprasad et al., 2012). There are examples where these epigenetic clashes may be briefly beneficial, such as intra-specific hybrids in *A. thaliana* that show more immediate and predominant epigenetic effects contributing to hybrid vigor (Groszmann et al., 2011). The authors of this study also propose that reduction of hybrid vigor upon segregation may be attributed to the 'balancing' of parental epialleles and eventually reducing epigenetic diversity. Epialleles governing hybrid incompatibility, such as the histidine biosynthesis gene *HISN6B,* the folate transporter *FOLT1* and *TAD3* have also been discovered in natural *A. thaliana* accessions (Durand et al., 2012; Agorio et al., 2017; Blevins et al., 2017).

FUTURE PERSPECTIVES: HARNESSING EPIGENETIC DIVERSITY AT THE POPULATION LEVEL

In recent years, research on intra-specific variation in plants is gaining more prominence while benefiting from previous work on inter-specific variation. Intra-specific variation encompasses strain-level differences of the same species acclimatized to diverse habitats, while variation between species can be useful to study a broader picture of independently occurring evolutionary trajectories that are largely divergent from each other. Research is now focused on understanding and quantifying the degree of changes at the genetic and epigenetic level within populations (Kawakatsu et al., 2016; Quadrana et al., 2016; Stuart et al., 2016; Lanciano and Mirouze, 2018).

While large-scale sequencing of 1001 phenotypically distinct strains in *A. thaliana* has revealed significant differences in population structure (Kawakatsu et al., 2016), it has also brought to light how gene expression, regulatory marks, and repetitive elements can optimally organize their interactions to enable

fitness under diverse climatic conditions around the world. This has paved the way for research on strain-level differences across other plant species, such as agriculturally important crops, especially in the light of climate change dependent domestication. Some of these new questions are: To what extent can epigenetic variants drive domestication or speciation? How far do the genome and epigenome mold themselves to maximize their adaptive advantage while maintaining species-specific identity?

Focusing on diversity and natural variation at the level of DNA methylation, we attempt to re-define 'methylome diversity' as a metric that measures cytosine methylation in the 3-nucleotide context (CG, CHG, CHH), linked stoichiometrically with the percentage of methylation levels. While this definition does not capture the exact origin of diversification (e.g., TEs or SVs), it will allow us to determine DMR hotspots (analogous to SV-, recombination- or selection-hotspots) within the genome of a lineage. This refined definition can enable comparative analyses across species, to take into account the methyltransferase and demethylase enzymes which have evolved over time to create adapted variants of methylome landscapes and the efficiency with which they can methylate target cytosines. 'Methylation diversity,' on the other hand, is a metric we propose for studying the concentration of genome-wide methylation. This represents the relative abundance of methylated sites in each species, normalized by genome-size, and is representative of overall methylation levels, perhaps indicating the varying reliance on this epigenetic mark for gene regulation across species (**Figure 1**).

This notion of epigenetic diversity allows us to address the question whether exposure to stress within a population can affect methylome or methylation diversity in a way so that it can be stably inherited to facilitate adaptive processes. Furthermore, we can ask whether or not the population genetic concept of genic allele frequencies (Gillespie, 2004) can also be extended to methylome or methylation landscapes. One would assume that accessions within a population would have less methylation diversity, but more methylome diversity. This outcome could be the result of a mechanism aiming to maintain the balance of the species-specific epigenome, but to moderately re-organize the target cytosines under a particular context, or the extent of methylation in each cell, to fine-tune gene regulation against the stress. For example, within different strains of the rice genome that have been independently domesticated over several generations, methylome diversity is correlated with bursts of transposition in the MITE family *mPong* (Lu et al., 2017). Methylation diversity can be more apparent between species - for example two angiosperms belonging to the *Brassicaceae* family - *Eutrema salsugineum* and *Conringia planisiliqua* completely lack gene-body methylation (Bewick and Schmitz, 2017; Bewick et al., 2017), or others such as rice which harbors an increased density of transposable-elements, necessitating heavy methylation for silencing (Zemach et al., 2010; Choi and Purugganan, 2018). This metric thus gives an insight into how epigenetic networks have evolved across species, tailor-made to their underlying genome structures.

In this article, we reviewed the role of active transposable elements in reshaping epigenetic landscapes through reintegration with potential long-term effects on subsequent generations. While various environmental stresses have been associated with particular transposon families, we believe that determining potentially mobile TE families and their copy number variation between ecotypes and species will become the new focus of large-scale epigenomics studies. For this purpose, a new generation of *de novo* TE annotation and detection tools are required that do not focus on classic annotation of all types of repeats, remnant TEs, co-opted sequences, and active elements (Goerner-Potvin and Bourque, 2018; Lanciano and Cristofari, 2020), but rather specialize on the detection of intact and potentially mobile TE families in *de novo* assembled genomes derived from long-read sequencing technologies (Drost, 2020). Being aware of the distinct TE families and their respective activation cues in each strain or species may be vital for determining competitive TE interactions during hybrid generation and cross-breeding.

DISCUSSION

Plants have widely diversified in their ability to colonize varying environments, characterized by a plethora of climatic variables such as temperature, precipitation, soil nutrition, pathogens, water availability and many more.

A large body of work has focused on understanding how these factors influence plant survival and adaptation across generations. In the wild, plants are exposed to multiple stresses in parallel, in addition to circadian and seasonal climatic changes. This cross-adaptation principle whereby exposure to one stress can prepare defense pathways against another related stress requires further attention. At the physiological level, several combinatorial stress-experiments have been carried out, examining epigenetic acclimatization efficiency against a particular stress (for example, Rivero et al., 2014) or to analyze concerted signaling mechanisms in response to multiple stresses (Zandalinas et al., 2020). In particular, future studies could characterize how cross-adaptation mechanisms utilize their epigenetic repertoire to shape an optimal gene regulatory network that can buffer or respond to various combinations of stresses in tandem.

On reviewing several published works that examine the plant epigenetic landscape under stress, we realize that responses and adaptation against stress occur in parallel on various physiological and epigenetic levels of a plant. The duration, magnitude, and frequency of the applied stress determines which level has a stronger influence on shaping gene function, enzyme activity, or epigenetic marks in a transgenerational context (**Figure 2**), and may further be influenced by the availability of nutrients (energy balance) structured in a complex response hierarchy. An optimal method to employ both genetic and epigenetic responses is the mobilization and controlled reintegration of transposable elements upon consistent stress exposure. Plants store a wealth of information within their intergenic regions in the form of TEs, which may be mobilized and inserted to specific regions that may house stress-response associated or stress-response repressor genes, or perhaps located in accessible chromatin and co-occur with certain histone

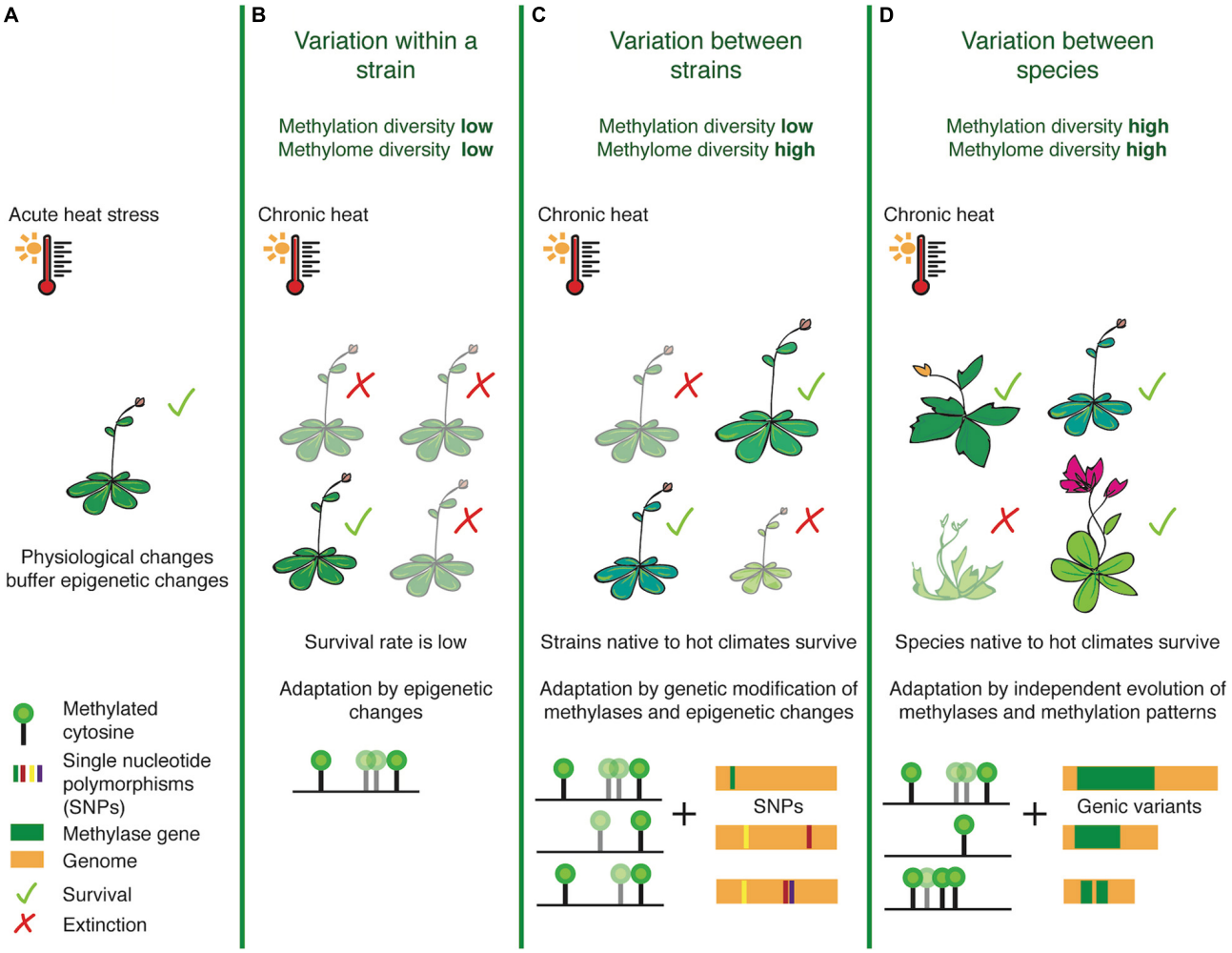

FIGURE 1 | Illustrating the differences between methylation and methylome diversity at the population, strain and species-levels. Taking the example of heat stress, physiological changes often occur as an immediate response to acute changes in temperature before epigenetic pathways and transgenerationally stable changes in epigenetic landscapes fall into place **(A)**. When the stress is chronic, populations belonging to a strain **(B)** non-native to the harsh temperature change often exhibit low survival rates, as they have rather homogenous methylation patterns and can rely only on rapid epigenetic/epigenomic changes for rapid stress-response mechanisms. Between strains however **(C)**, methylome diversity is high and this may enable survival for plants harboring heat-resistant epialleles and modified methyltransferases. At the species-level **(D)**, plants exhibit high levels of methylation and methylome diversity, due to evolutionary fixation of genetic and epigenetic marks, thereby providing an edge for heat-primed species to adapt with greater ease.

variants. Eventually, these re-inserted TE copies may be silenced by DNA methylation to regulate transcription levels. Since each TE family may have unique roles depending on their stress-activators, simultaneous but concerted activation may also facilitate flexibility to recurring stresses, and eventually fixation (**Figure 2**).

A detailed understanding of the interplay between physiological and epigenetic mechanisms during the plant adaptation process will allow us to create more powerful plant engineering tools in response to rapid changes in global environmental conditions such as global warming. While several studies have examined the co-occurrence of physiological and epigenetic changes (Fang et al., 2017; Neves et al., 2017), decoding the temporal hierarchy of interacting pathways at a high resolution and testing the heritability of these induced changes can enable better understanding of epigenetic preparation to future stress responses in plants. Gene engineering tools such as CRISPR/Cas9 are promising solutions for enabling the engineering of knockouts of key genes/enzymes for improved adaptability in several plant species. Recently, these tools have also been engineered to precisely manipulate locus-specific methylation levels (Gallego-Bartolomé et al., 2018, 2019; Papikian et al., 2019). Yet, this approach poses the limitation of inducing evolutionary trade-offs with other traits or compensatory mechanisms such as the activation of alternative pathways or undesired epigenetic remodeling at distant loci. We suggest that harnessing particular natural variants of the epigenetic toolkit derived from already adapted ecotypes in combination with controlled transposon activation will provide a more general plant engineering methodology. This

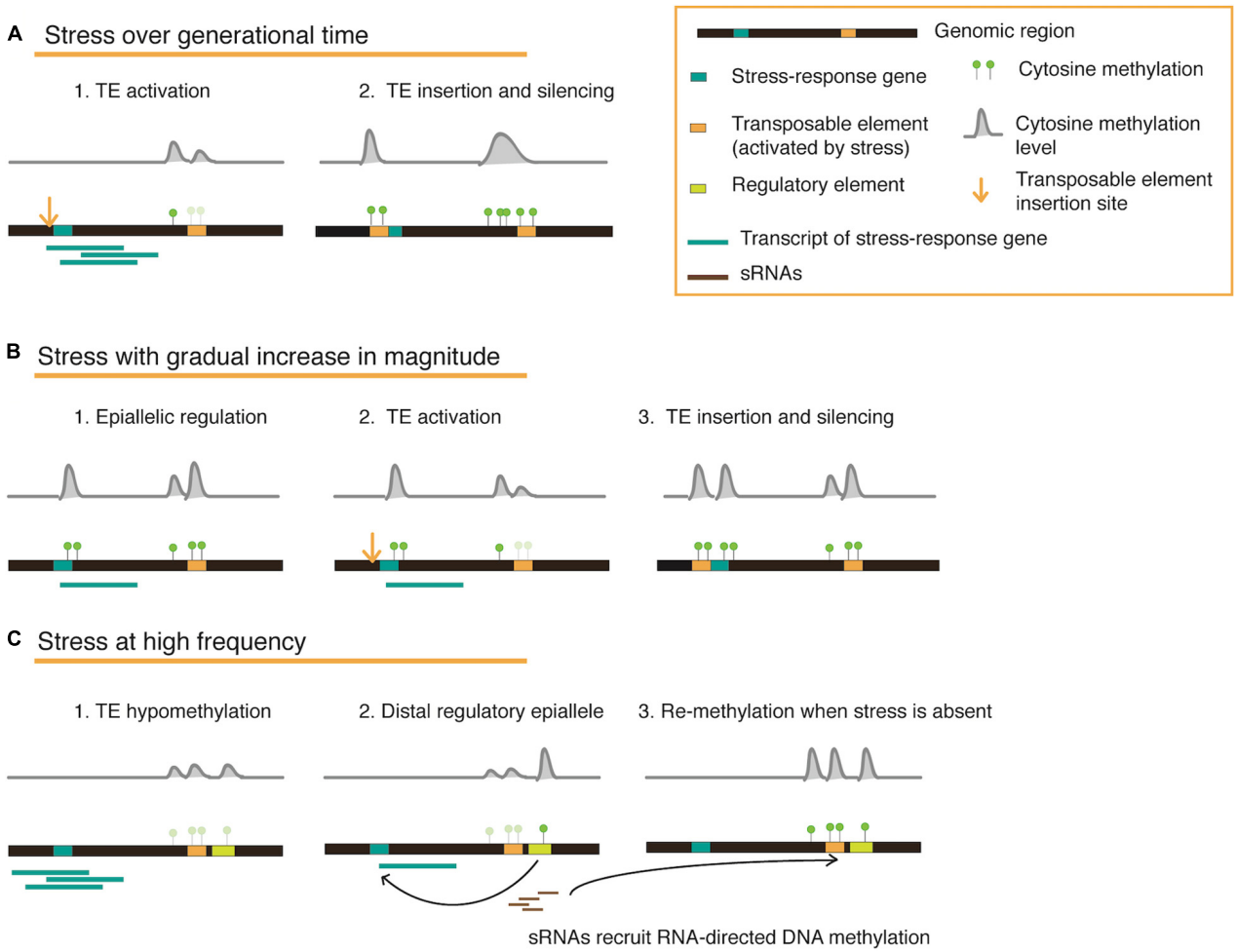

FIGURE 2 | A conceptual model for transposable element and methylation dynamics illustrated in three different stress scenarios. The cartoons represent a genomic locus that houses a stress-response gene and a methylation-silenced transposable element (TE). When a stress is constantly applied over generations **(A)**, hypomethylation at the TE locus can result in activation and re-insertion proximal to a stress-response associated/stress-response repressor gene, eventually recruiting methylation marks to fix a new regulatory mechanism for long-term stability. When a stress increases in magnitude during the lifetime of a plant **(B)**, epialleles are first created to moderately regulate gene expression. To enable stronger gene expression changes, additional methylation changes are driven by activated TEs and their re-insertion. In situations where stress occurs repeatedly for short intervals **(C)**, plants might require epigenetic switches that can be easily tuned. This can be facilitated by the presence of a distal regulatory element flanking a TE, which can also be hypomethylated upon TE activation, thus altering expression of a gene detrimental for stress-response. When TE activation is brief and does not involve copy number increase, small RNAs are recruited for methylation (through the RdDM pathway) and can spread to the neighboring element, thus switching 'off' the regulation once again when the stress is absent.

new engineering methodology is capable of shaping entire plant epigenomes that at the same time have to co-adapt to various new stress conditions resulting from rapid environmental changes such as global warming.

Physiological responses to stress are well characterized mostly providing survival buffers for rhythmic events within a particular range of environmental conditions. In contrast, the epigenomic responses of a plant can not only regulate seasonal or circadian events (such as histone-methylation mediated transcriptional silencing of the *FLOWERING LOCUS C* (*FLC*) gene in winter-annual *Arabidopsis* accessions (Berry and Dean, 2015), but can also simultaneously rewire entire gene networks and transmit this change to subsequent generations thereby enabling long-term adaptation to completely new environmental conditions.

This notion allows us to redefine plant stress adaptation as a process that combines epigenetic and genetic mechanisms to restructure gene regulatory networks and maintain genome integrity. Such network restructuring events create a fast response to a qualitatively changing environment. On a population level, variants of restructured networks within individual plants are then selected to reduce genome destabilization and to buffer negative outcomes for reproductive fitness. In contrast, the rewiring of gene networks induced by physiological stress responses will always remain within the dynamic range set by the genetic and epigenetic levels and therefore reflects a static buffering mechanism with little potential to induce transgenerational adaptive change. On an evolutionary scale, it is the epigenetic level that interfaces

between the environmental and genetic levels, thereby creating a transgenerational buffer while the physiological level represents the buffer zone that determines the adaptive flexibility of a plant species within a generation.

Applying the framework developed above, we predict that seasonal/cyclical or only slight changes to environmental conditions will have small effects on the epigenetic landscape in healthy non-starved plants and are less likely to be transmitted transgenerationally, whereas accelerating geological trends over time can be strong enough to overcome the physiological barrier and will induce significant changes to the genetic and epigenetic landscape that in turn will be exposed to natural selection and adaptation via transgenerational inheritance.

Our hypothesis further predicts that during environmental stress induction, the physiological-, epigenetic-, and genetic-levels follow a hierarchical principle of organization whereby each respective zone, gradually buffers the environmental impact over the long term. This principle generates a feedback loop between the physiological level and the epigenetic level such that environmentally induced epigenetic changes will occur most dramatically when physiological responses are insufficient whereas genetic changes are only inherited by subsequent generations when the epigenetic silencing marks are sufficiently erased leaving the genome exposed to structural variation and TE bursts.

Together, we postulate that rapid changes in global environmental conditions such as climate change in the coming years will require a new mode of plant engineering based on the control of methylation landscapes and transposon activation that can reshape entire gene regulatory networks and pathways in tandem to induce novel traits and physiological robustness to the newly emerging environmental conditions. Stabilizing these co-adapted traits transgenerationally may become the new focus of epigenetics and plant biotechnology research. Studies focusing on predicting the long-term effects of rapid environmental changes based only on a few stress variables (e.g., temperature and/or precipitation) may therefore largely overestimate the robustness of temperature/precipitation adapted ecotypes during climate change while underestimating the co-adapted traits that could either buffer or facilitate extinction events on the gene regulatory network level (e.g., compensatory pathways). As a result, future population epigenomics studies and plant engineering efforts will have to rely on new methodologies able to quantify all environmental variables in parallel to assess how they penetrate the entire gene regulatory network encoding co-adapted traits rather than relying only on a strict reductionist view of uncoupled stress variables. Epigenetic signatures such as the genome-wide distribution of DNA methylation marks or methylome/methylation diversity patterns can act as markers for selecting natural variants within populations or between ecotypes that reflect a stabilized and robustly adapted state of several co-evolved traits for which more realistic predictions of survival capacity in various environmental change conditions can be developed. The topologies of gene regulatory networks underlying particular epigenetic signatures will be more powerful predictors of future plasticity and adaptability in rapidly shifting environmental conditions than models focusing on individual SVs and their substitution frequencies. Ultimately, we envision that individuals within a population or ecotype lineages with robust co-adapted traits and gene-regulatory networks can then be used as starting material for artificial TE mobilization efforts, further fine-tuning beneficial traits to be fit for the new environmental demands.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Ultraviolet B Radiation Triggers DNA Methylation Change and Affects Foraging Behavior of the Clonal Plant *Glechoma longituba*

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Clonal plants in heterogeneous environments can benefit from their habitat selection behavior, which enables them to utilize patchily distributed resources efficiently. It has been shown that such behavior can be strongly influenced by their memories on past environmental interactions. Epigenetic variation such as DNA methylation was proposed to be one of the mechanisms involved in the memory. Here, we explored whether the experience with Ultraviolet B (UV-B) radiation triggers epigenetic memory and affects clonal plants' foraging behavior in an UV-B heterogeneous environment. Parental ramets of *Glechoma longituba* were exposed to UV-B radiation for 15 days or not (controls), and their offspring ramets were allowed to choose light environment enriched with UV-B or not (the species is monopodial and can only choose one environment). Sizes and epigenetic profiles (based on methylation-sensitive amplification polymorphism analysis) of parental and offspring plants from different environments were also analyzed. Parental ramets that have been exposed to UV-B radiation were smaller than ramets from control environment and produced less and smaller offspring ramets. Offspring ramets were placed more often into the control light environment (88.46% ramets) than to the UV-B light environment (11.54% ramets) when parental ramets were exposed to UV-B radiation, which is a manifestation of "escape strategy." Offspring of control parental ramets show similar preference to the two light environments. Parental ramets exposed to UV-B had lower levels of overall DNA methylation and had different epigenetic profiles than control parental ramets. The methylation of UV-B-stressed parental ramets was maintained among their offspring ramets, although the epigenetic differentiation was reduced after several asexual generations. The parental experience with the UV-B radiation strongly influenced foraging behavior. The memory on the previous environmental interaction enables clonal plants to better interact with a heterogeneous environment and the memory is at least partly based on heritable epigenetic variation.

Keywords: clonal plant, habitat selection, heterogeneous environment, foraging behavior, epigenetic memory, UV-B radiation

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INTRODUCTION

Essential resources like water, light, and nutrients are usually distributed patchily in natural environments often on scales of a few centimeters (Salzman, 1985; Caldwell and Pearcy, 1994; Stuefer and Hutchings, 1994; Cain et al., 1999; Hutchings and John, 2004; García-palacios et al., 2012). Research shows that, for example, clonal plants can sense the heterogeneity of their microenvironment and make choice between qualitatively different patches by placing ramets to beneficial patches (Bazzaz, 1991; Hutchings and John, 2004; Roiloa and Retuerto, 2012; Oborny and Hubai, 2014; Waters and Watson, 2015). Such foraging behavior likely belongs among the important characteristics enabling the dominance of clonal plants in many ecosystems (Wang et al., 2016; Waters et al., 2016; Dong et al., 2018; Li et al., 2018; Latzel and Münzbergová, 2018; Quan et al., 2018; Chen et al., 2019). Majority of studies explained the microhabitat foraging behavior of clonal plants by morphological plasticity and/or photosynthetic adjustments (Salzman, 1985; Evans and Cain, 1995; Wijesinghe and Hutchings, 1999; Roiloa and Retuerto, 2006a,b,c; Xiao et al., 2006, 2011; Waters and Watson, 2015; Waters et al., 2016; Ye et al., 2015; Quan et al., 2018). However, significantly less is known about the molecular mechanisms that are involved in such behavior (e.g., Latzel and Münzbergová, 2018).

Recently, it has been discovered that the behavior of plants might be altered not only by actual environment but also by previous environmental interactions (Ding et al., 2014; Latzel et al., 2016; Münzbergová and Hadincová, 2017; Latzel and Münzbergová, 2018; Tombesi et al., 2018; Virlouvet et al., 2018). Such memory on past conditions can be enabled by various mechanisms including hormonal signaling or epigenetic changes for example in DNA methylation. Interestingly, clonal plants appear to have greater ability than non-clonal plants to remember past environmental interactions via epigenetic mechanisms (Xiao et al., 2006; Latzel and Klimešová, 2010; Louapre et al., 2012; Douhovnikoff and Dodd, 2015; Dong et al., 2019). This is usually explained by the fact that epigenetic change is easier to be maintained among clonal generations (ramets) than sexual generations due to the lack of meiosis, resetting most of the environmentally induced epigenetic variation (Paszkowski and Grossniklaus, 2011), during clonal growth. It has been thus suggested that epigenetic memory triggered by environmental interactions can serve as an important mechanism contributing to the wide distribution of clonal plants in nature (Latzel and Klimešová, 2010; Verhoeven and Preite, 2014; Douhovnikoff and Dodd, 2015; González et al., 2016; Münzbergová et al., 2019). Ramets produced by clonal growth are potentially independent and genetically identical to the paternal ramet and by definition can be considered as offspring ramets, i.e., next clonal generation (Groenendael et al., 1996). Therefore, the epigenetic memory passed from parental to offspring ramet should be considered as transgenerational (Latzel and Klimešová, 2010; Douhovnikoff and Dodd, 2015).

Epigenetic memory of parental ramets could significantly influence the foraging patterns of offspring ramets and affect thus ultimately habitat selection behavior of the whole genet (Latzel et al., 2016; Latzel and Münzbergová, 2018). Sunlight is undoubtedly the most important environmental factor affecting plant growth. Ultraviolet B (UV-B) radiation (280–315 nm) represents only a small fraction of the solar radiation reaching the Earth's surface, but has significant impact on plant growth and development. The changes in plant morphology, physiology, and production of secondary metabolites induced by UV-B radiation have been elucidated by a large body of studies (Li et al., 1999; Mackerness, 2000; Frohnmeyer and Staiger, 2003; Vanhaelewyn et al., 2016; Dotto and Casati, 2017).

The radiation intensity of UV-B received by plants is affected by latitude, day time, season, cloud cover, and canopy composition, and plants in nature are thus inevitably exposed to heterogeneous UV-B radiation environment (Liu et al., 2015). Our previous studies had proved that UV-B radiation is one of the most important reasons for low-light-patch distribution of clonal plants under heterogeneous light environment (Zhang et al., 2016). However, the molecular mechanisms behind the habitat selection of clonal plants driven by UV-B radiation are still unknown. We propose that UV-B radiation induces epigenetic changes in exposed parental ramets, which can consequently alter the response of offspring ramets to UV-B patchy environment.

In this study, we explored foraging, growth, and epigenetic response to heterogeneous UV-B environment using a clonal plant, *Glechoma longituba*, and tested the following hypotheses: (1) UV-B experience of parental ramets affects subsequent foraging behavior of the growing clone in heterogeneous UV-B environment; (2) clonal plants can form epigenetic memory of their UV-B experience; and (3) epigenetic memory of UV-B radiation is transmitted among interconnected ramets.

MATERIALS AND METHODS

Plant Material

We used *G. longituba* as the model in the experiment. *G. longituba* is a perennial herb widely distributed in forests, along roadsides or creeks of tropical, subtropical, and temperate areas in China, Vietnam, South Korea, and Russian Far East (Zhang and He, 2009). The species naturally occurs under the canopy, so it experiences environment with heterogeneous UV-B distribution. In the wild, the genus *Glechoma* has two different growth forms. In its flowering phase, from March to June, it produces a vertical stem while it produces plagiotropic, monopodial stolons in vegetative phase. The monopodial stolons are able to creep on the ground and the stolons bear nodes at intervals of 5–10 cm. A pair of opposite, orbicular leaves is produced at each node. Adventitious roots may also develop at the nodes. There is a bud in the axil of each leaf, which may generate a higher-order stolon only when several younger nodes have been produced along the parent stolon (Birch and Hutchings, 1994; Liao et al., 2003). Since the *G. longituba* usually does not flower in the greenhouse, it grew only horizontal stolons in our experimental setting. The *G. longituba* was collected from Jiwozi in Qinling Mountains, Shaanxi, China (33° 47′N, 108°45′ E).

We selected a single genotype of *G. longituba* that was propagated in a greenhouse for 6 months. When we had enough plant material, we severed and replanted 64 youngest ramets (further considered as parental ramets in the study) of comparable size and planted them individually to pots ($7 \times 7 \times 7$ cm) filled with soil (25% sand, 25% organic matter, and 50% peat). We allowed the ramets to recover and root for 7 days before we started the main study. The experiment was conducted in a greenhouse from January to March of 2019.

Design of the Study

Seven days after parental ramets were planted to pots, we randomly subjected them to two light training environments, Control light environment (further referred to as Control group, $N = 32$), or UV-B radiation for 15 days (further referred to as UV-B group, $N = 32$). The UV-B treatment is described later in the text. After 15 days, the UV-B radiation of the parental ramets was terminated. The newly emerging offspring ramets developing from the parental ramets of both parental light treatments (all parental ramets had single emerging offspring ramet at this point of the study) were allowed to grow in a narrow plastic runway (2 cm wide) for 30 days. Two plastic trays (54 cm long × 28.5 cm wide × 7 cm high) filled with soil (25% and, 25% organic matter, and 50% peat) were placed on the two sides of the runway. The tray on one side of the runway was exposed to Control light environment (Control offspring environment) and the other received additional UV-B radiation (UV-B offspring environment) (**Figure 1A**). Two parental ramets (one UV-B and one Control) were sharing the same UV-B source (**Figure 1B**) and thus represented a block. At the beginning, the growth direction of all plant individuals was parallel to the runway, and newly developed interconnected ramets (further referred to as offspring ramets) faced Control light from one side and UV-B radiation from the opposite side. As the growth of the individual continued, it turned its elongating stolon either in one or the other direction, thus selecting the UV-B or Control environment. None of the main stolons remained in the runway—so each main stolon selected one or the other side. In our study, all plants produced only the main stolon but one in which we removed the secondary stolon. To prevent the UV-B radiation penetration to the side of control offspring light environment, a UV-B baffle (0.3 mm transparent polyester film, Dongguan Linuo Plastic Insulation Material Co. LTD, Guangdong, China) was settled between the two types of the light environments (**Figure 1**). The bottom of the baffle was 2-cm above the plant, ensuring that the newly emerged ramets can sense different light conditions on both sides of the baffle.

During the experiment, the mean irradiance in the greenhouse was 357 ± 20 μmol m^{-2} s^{-1}, and humidity was $40 \pm 5\%$ with a 14 h/10 h light/dark cycle and a 25/20°C day/night temperature cycle. Plants were watered every 2 days to prevent water stress.

UV-B Radiation

Following the method of Liu et al. (2015), UV-B radiation was supplied by square-wave UV-B fluorescent lamps (36 W, Beijing Lighting Research Institute, Beijing, China). The maximum output of these lamps was 313 nm. The lamps were wrapped with 0.13 mm cellulose acetate film (Grafix Plastics, Cleveland, OH, transmission down to 290 nm) for the supplemental UV-B radiation group (UV-B group). The lamps were active for 8 h per day from 9:00 to 17:00. The daily radiation dose was 2.88 kJ m^{-2} d^{-1}. Unlike the parental ramets that were exposed to UV-B for 15 days, the offspring ramets were exposed to UV-B light radiation for 45 days if they have chosen the UV-B light environment. The amount of UV-B radiation was measured using a UV radiometer (Handy, Beijing, China) every 2 days. The cellulose acetate film was replaced every 5th day.

Growth and Morphological Parameters

During the experiment, offspring ramets of *G. longituba* grew either into the Control light conditions or into the UV-B light environment (all plants had only the main stolon, so they had only one choice for UV-B or Control environment in our experimental setup). After 45 days (the end of the study), we count the number of offspring selected UV-B or Control environment to calculate the proportion of offspring ramets selection/foraging and measured a range of plant size parameters. First, we recorded biomass, leaf area, and specific leaf area of the parental ramets of every individual, i.e., the part that did not enter the pathway. For the offspring part, we recorded total biomass, total number of offspring ramets, stolon length, total leaf area, and specific leaf area of the whole offspring part of the individual. The biomass includes only above-ground biomass. To assess leaf area, fresh leaves were scanned with a scanner (EPSON Perfection V19, EPSON, China), and leaf area was calculated with Motic software (Motic Images Plus 2.0. Ink, Motic, China). Above-ground biomass was dried for 72 h at 80°C until constant weight and weighted immediately using an electronic balance (SartoriusBT25S, Beijing, China).

Methylation-Sensitive Amplification Polymorphism (MSAP) Analysis

Methylation alterations in cytosine modification of *G. longituba* were detected using methylation-sensitive amplification polymorphism (MSAP) analysis. For the analyses, we sampled leaves from parental ramet, older offspring (the third offspring ramet counted from the parental ramet on the main stolon, later referred to as older offspring), and the youngest fully developed offspring ramet (the last offspring ramet counted from the parental ramet on the main stolon, later referred to as younger offspring). For each offspring type and combination of parental and offspring environment, we had 3–6 individuals. This unbalanced design was given by the foraging decisions of the plants. The samples were scrubbed gently with 75% ethanol minimize contamination by microorganisms and then dried in silica gel for the subsequent extraction of DNA. This allowed testing whether epigenetic memory of UV-B radiation is transmitted trans-generationally among clonal offspring (ramets). Total genomic DNA was extracted from 30 mg of dry leaves using BioTeKe (Beijing, China), DNA quality was examined by electrophoresis in agarose gel 1% (w/v), and DNA concentration and purity were examined spectrophotometrically with NanoDrop2000 (Thermo Fisher

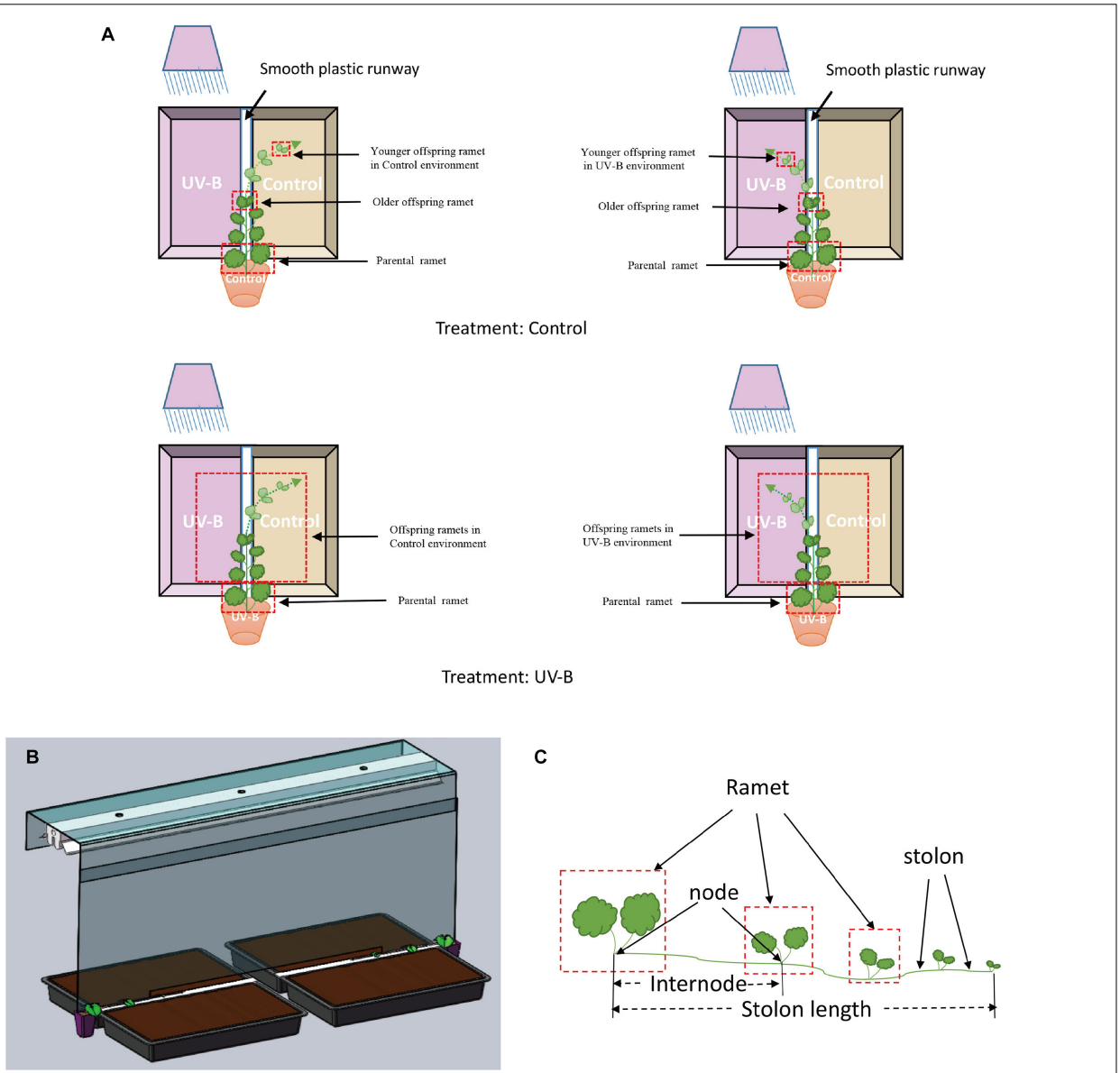

FIGURE 1 | Schema of the experiment. The experiment design consisted of two parental and two offspring light environments: **(A)** Treatment Control: the parental ramet was in control light environment; Treatment UV-B: the parental ramet was in additional 15 day UV-B radiation environment. Then, the newly emerged offspring ramets faced Control light from one side and UV-B radiation from the other side and were free to choose their growth direction (Control pot or UV-B pot, show in dotted lines and arrows). As the plant has only one stolon, each plant can only grow to one of the directions. **(B)** Installation of the experiment. **(C)** Growth pattern of *Glechoma longituba*.

Scientific, United States). The qualified DNA was diluted to the same concentration (100 ng/μl) for MSAP analysis. We used the endonuclease combination 1 μl of *Eco*RI + 1 μl of *Hpa*II (E + H) (NEB, United States) and 1 μl of *Eco*RI + 2 μl of *Msp*I (E + M) (NEB, United States) to double-enzyme genomic DNA, and the digested ends were ligated with 1 μl of *Hpa*II-Msp-adapter (50 pmol/μl), 1 μl of *Eco*RI adapter (5 pmol/μl) (Biotech, China), and 0.5 μl of T4 DNA ligase (TAKARA, Japan). Both the digestion and ligation reactions were performed in a final volume of 20 μl. The enzyme was cut at 37°C for 5 h. Connect at 8°C for 4 h.

The 2 × Taq PCR master mix and pre-amplification and selective amplification primers used in the experiments were synthesized by Shanghai Biotech (**Supplementary Appendix Table 1**). Both the reactions of pre-amplification and selective amplification were in a final volume of 50 μl. A pre-amplification step was carried out with *Eco*RI pre-amplification primers and *Hpa*II/*Msp*I pre-amplification primers. The PCR mix contained 2 μl of ligated DNA, 21 μl of double-distilled water, 1 μl of H-M pre-amplification primers (10 μM), 1 μl of *Eco*RI pre-amplification primers (10 μM), and 25 μl of 2 × Taq PCR master mix. The pre-amplification conditions were as follows: 72°C for

2 min; 94°C for 2 min; 20 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 10 min.

The pre-amplification products were diluted 10 times as a selective amplification template. A selective amplification step was carried out with 11 pairs of selective primer combinations, including the following: *Eco*RI-AAG/*Hpa*II-TGA, *Eco*RI-AAG/ *Hpa*II-TTA, *Eco*RI-AAG/ *Hpa*II-TTG, *Eco*RI-ACT/ *Hpa*II-TCC, *Eco*RI-ACT/ *Hpa*II-TTG, *Eco*RI-AGG/ *Hpa*II-TTC, *Eco*RI-AGG/ *Hpa*II-TGA, *Eco*RI-AGG/ *Hpa*II-TCC, *Eco*RI-AGG/ *Hpa*II-TTG, *Eco*RI-ACG/ *Hpa*II-TTG, and *Eco*RI-AGC/ *Hpa*II-TCC. The PCR mix contained 1 μl of pre-amplified DNA, 22 μl of double-distilled water, 1 μl of H-M selective primer (10 μM), 1 μl of *Eco*RI selective primer (10 μM), and 25 μl of 2 × Taq PCR Master Mix. The selective amplification conditions were as follows: 94°C for 2 min; 10 cycles at 94°C for 30 s; 65°C for 30 s and 72°C for 1 min (each cycle is decremented by 1°C); 23 cycles at 94°C for 30 s; 56°C for 30 s and 72°C for 1 min; and a final elongation step at 72°C for 10 min.

Before polyacrylamide gel, the selective amplification product was inactivated at 70°C for 10 min, then the selective amplification samples were separated by 10% denaturing polyacrylamide gel electrophoresis and subjected to electrophoresis at 220 V for 4 h, and the gel was applied with silver staining. Following staining of the gel, rinsing, developing, and photographing were performed, and band statistical analysis was performed. Fragments from approximately 100–500 bp were scored. The amplified MSAP products were resolved using the method described in Xu et al. (2016).

Statistical Analyses

Growth and Morphological Traits

Because we had too many possibly correlated dependent variables, we used variance inflation factor (VIF) calculated with the "vifstep" function in the R package usdm. We considered variables with VIF values less than 3 as advised by Zuur et al. (2010). While this method has been previously designed to select independent predictors, it can serve the same function when identifying sets of independent response variables. Based on this, we selected two dependent variables (biomass and leaf area) out of three initially measured for the parental plants. For offspring, we selected three dependent variables (offspring ramet biomass, specific leaf area, and ramet number) out of five initially measured. The remaining three dependent variables (specific leaf area of the parental ramet, stolon length, and total leaf area of the whole offspring part) are presented in the **Supplementary Appendix Table 2** and **Supplementary Appendix Figures 1–3**.

One-way ANOVA was used to test the effects of parental training environment (Control vs. UV-B) on biomass and leaf area of parental ramet. Generalized linear model with binomial distribution was used to test the effect of parental environment on habitat selection by the offspring (Control vs. UV-B). Two-way ANOVA was then used to test the effects of parental light environment (Control vs. UV-B) and offspring light environment (Control vs. UV-B) and their interaction on offspring ramets biomass, specific leaf area, and ramet number. Data were transformed when needed (log or square root) to meet the assumptions of homoscedasticity and normality (for details see **Table 1**). Ramet number followed Poisson distribution. The effects of paternal and offspring environment and their interaction on these variables were tested using generalized linear model with the respective distribution. All analyses were conducted using R 3.5.1. Initially, we used block as a covariate in our models. As its inclusion did not affect the results, we present results without its inclusion.

DNA Methylation Variation

From the fragment presence/absence score matrix of both enzymatic reactions, the methylated state of every locus (5′-CCGG target) was assessed: presence of both *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I products (1/1) denotes an unmethylated state, presence of only one of the *Eco*RI–*Hpa*II (1/0) or *Eco*RI–*Msp*I (0/1) products represents methylated states (hemi-methylated or internal methylation), and absence of both *Eco*RI–*Hpa*II and *Eco*RI–*Msp*I products (0/0) was considered as an uninformative state (Salmon et al., 2008; Pérez Figueroa, 2013; Wang et al., 2019). We used the "vegan" package of R (Dixon, 2003) to calculate Shannon's diversity index of each individual based on these data. Methylation level (%) was calculated by dividing MSAP bands representing methylated 5′-CCGG sites (differential presence/absence of restricted fragments in *Hpa*II and *Msp*I assays) against the total number of scored bands (Liu et al., 2012).

The binary matrix of methylated state (Loci composition) was analyzed by canonical correspondence analysis (CCA) with function capscale in the "vegan" package of R (Dixon, 2003; Bonin et al., 2007). The epigenetic diversity (Shannon's diversity index) and methylation level were tested using a generalized linear model. In all cases, we first tested the effect of parental environment (Control and UV-B), ramet age, and their interaction using the whole data, i.e., parental (Control and UV-B) and older and younger offspring ramets. Afterward, we tested the effect of parental environment, offspring environment, ramet age (old/young), and their interactions using only data from the offspring. In both cases, we accounted for the fact that the ramets of different age belong to the same individual. We did this by using the parent identity as a random factor and the individual code as random factor in the univariate analyses and by defining the individual as a hierarchical level in the multivariate analysis. Due to significant interactions with ramet age, we also repeated

TABLE 1 | ANOVA results for effects of parental environment (Control vs. UV-B) and offspring environment (Control vs. UV-B) on morphological traits of offspring ramet of *Glechoma longituba*.

	Offspring biomass[b]		Specific leaf area [a]		Ramet number	
	$F_{(1,53)}$	*P*	$F_{1,52}$	*P*	$D_{1,56}$	Pr(> Chi)
Parental (Pa)	**59.71**	**< 0.001**	**6.45**	**0.014**	2.02	0.155
Offspring (Off)	0.69	0.407	2.08	0.155	0.02	0.892
Pa × Off	2.08	0.156	2.29	0.136	0.86	0.354

Degrees of freedom (df) and F and P-values are given. Values for P < 0.05 are in bold.

[a] *log transformation.*

[b] *sqrt transformation.*

the tests separately for the ramets of different ages. All analyses were conducted using R 3.5.1.

RESULTS

The parental biomass (F = 17.95, P < 0.001) and parental leaf area (F = 36.68, P < 0.001) of *G. longituba* were both significantly lower under UV-B environment than under Control environment (**Figure 2**).

Foraging for different light environment was significantly affected by the training light environment previously experienced by the parental ramet (Residual Deviance = 6.91; P = 0.009). Plants of Control group (i.e., control training light environment) placed 58% offspring ramets in Control offspring light environment whereas the proportion increased to 88.46% in plants developed from parental ramets trained in UV-B (UV-B group, **Figure 3**). The offspring biomass and specific leaf area were significantly higher for offspring of parents trained in Control parental light than in UV-B parental light environment, while the ramet number was unaffected by parental training conditions (**Table 1** and **Figure 4**). There was no significant effect of offspring light environment or interaction between parental and offspring environment in any of the variables (**Table 1**).

MSAP Analyses

A total of 105 MSAP loci were amplified from each individual using 11 primer pair combinations. When MSAP profiles of all ramet types were analyzed together (i.e., parental and older and younger offspring ramet), loci composition, epigenetic diversity (Shannon's diversity index), and overall DNA methylation level were significantly affected by parental training environment (Control vs. UV-B, **Table 2**, Pa). Shannon's diversity index (I_{epi}) and overall DNA methylation level (L_{epi}) were significantly lower for ramets of UV-B trained parents (I_{epi} = 3.82 ± 0.22; L_{epi} = 44.34 ± 0.09%) than Control trained parents (I_{epi} = 3.97 ± 0.14; L_{epi} = 50.86 ± 0.07%). Loci composition, but not diversity and methylation level, was significantly affected by ramet type. Loci composition significantly differed between parental training environment and ramet type (**Table 2**, Pa × Ramet). Therefore, we consequently tested the effects of parental and offspring environment on each ramet type separately.

In case of the parental ramet, the loci composition (F = 1.81; P = 0.003; df = 1.16), Shannon's diversity index (Control = 4.00 ± 0.07, UV-B = 3.70 ± 0.25; F = 11.78; P = 0.003), and total DNA methylation level (Control = 52.28 ± 0.04%, UV-B = 39.58 ± 0.10%; F = 13.32; P = 0.002) significantly depended on the parental training environment.

When analyzing offspring ramets (older offspring ramet and younger offspring ramet), we found only loci composition to be significantly affected by parental training environment (**Table 3**). Offspring environment had no effect on any of the variables.

DISCUSSION

Training of Parental Ramet to UV-B Radiation Affects Foraging Behavior of a Genet

We demonstrate that the 15-day long training of parental ramets of *G. longituba* to increased UV-B radiation has a negative effect on their growth (biomass) as well as on the number and biomass of offspring ramets and hence on the fitness of the whole individual. This is in line with several other studies (Frohnmeyer and Staiger, 2003; Kakani et al., 2003; Vanhaelewyn et al., 2016; Dotto and Casati, 2017). However, our study provides an additional unique finding that the experience of parental ramet with UV-B radiation strongly affects consequent foraging behavior for light of the growing individual. Individuals with the parental ramet's experience with UV-B radiation preferentially

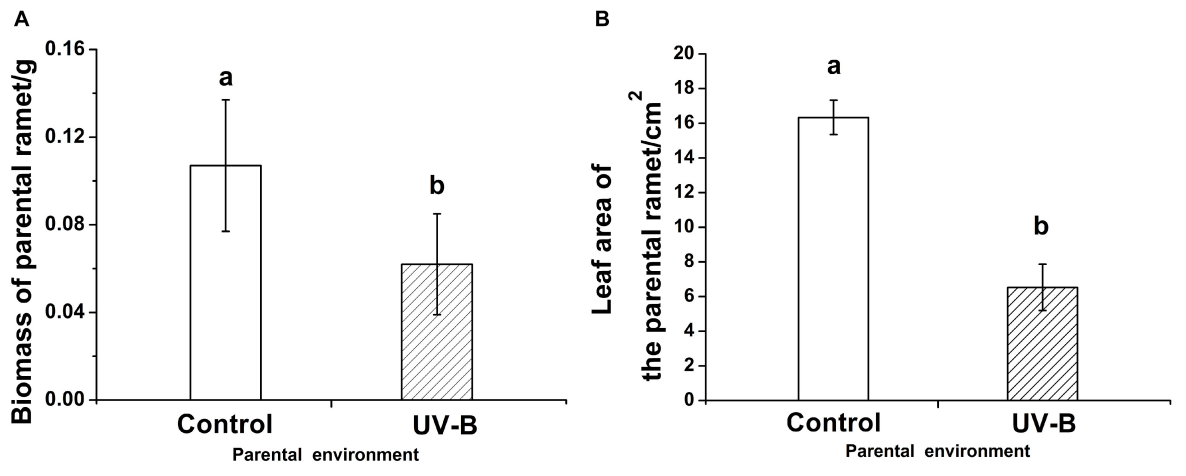

FIGURE 2 | The biomass (F = 17.95, P < 0.001) **(A)** and the leaf area (F = 36.68, P < 0.001) **(B)** of the parental ramet in different parental light environment. Control: parental ramet in control light environment, UV-B: parental ramet experienced 15 day UV-B radiation. The graphs show mean ± SE. Columns sharing the same letter are not significantly different from each other at P < 0.05.

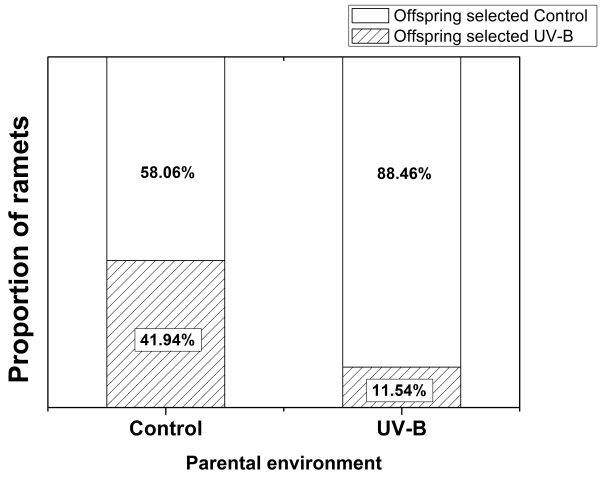

FIGURE 3 | Proportional selection for different offspring light environments by offspring ramets. Control: parental ramet trained in control light environment, UV-B: parental ramet trained in UV-B light environment (n = 32 per parental treatment; Control vs. UV-B: Residual Deviance = 6.91; P = 0.009).

placed offspring ramets to light conditions without UV-B radiation in comparison to the genetically identical individuals without the parental experience with UV-B radiation (see **Figure 3**). Such behavior probably helped to mitigate negative consequences of UV-B radiation on the individual's fitness. Avoiding patches with high UV-B levels for already weakened (offspring ramets of UV-B stressed parental ramet) individuals may reduce further negative impact of UV-B radiation on fitness of the genets. Such a behavior can be considered as an escape strategy, which has been also documented in other clonal species (de Kroon and Hutchings, 1995; Ye et al., 2006; Ikegami et al., 2007; Puijalon et al., 2008).

Our study adds to the mounting evidence that the behavior of clonal plants is not independent on their environmental interactions in the past. For example, Louapre et al. (2012) showed that the foraging behavior of clonal plants *Potentilla reptans* and *Potentilla anserina* is affected by the nutrient availability in older ramets. Latzel and Münzbergová (2018) found that clonal plant *Fragaria vesca* is able to store information on the light and nutrient availability of older ramets and based on this information decide where to place offspring ramets, which they consider as an exhibition of anticipatory behavior in clonal plants (Latzel and Münzbergová, 2018). A puzzling question is which mechanisms allow for the memory on the past environmental interactions and consequent change in foraging behavior of clonal plants.

Mechanisms Allowing for the Change in Foraging Behavior

It is very likely that the information passed from parental to interconnected offspring ramets is enabled by their connection via stolons. Some researchers suggest that the connection between ramets allows not only for transport of water with dissolved assimilates but also for transmission of signaling molecules like phytohormones (e.g., Alpert and Mooney, 1986; Hutchings, 1999; Stuefer et al., 2004; Gómez et al., 2008; Louapre et al., 2012; Waters and Watson, 2015). Hence, parental ramet can communicate with offspring ramets (and vice versa), which can alter overall behavior of the genet. Another theory suggests that it is shared epigenetic memory that can be involved in the behavior of clonal plants (González et al., 2016, 2018; Latzel et al., 2016). It has been reported that memories on the environmental interactions can be stored and transmitted to next generations via epigenetic change such as the change in DNA methylation (Molinier et al., 2006; Bossdorf et al., 2008; Boyko et al., 2010; Verhoeven et al., 2010; González et al., 2016; Latzel et al., 2016; Richards et al., 2017). Latzel et al. (2016) suggested that parental

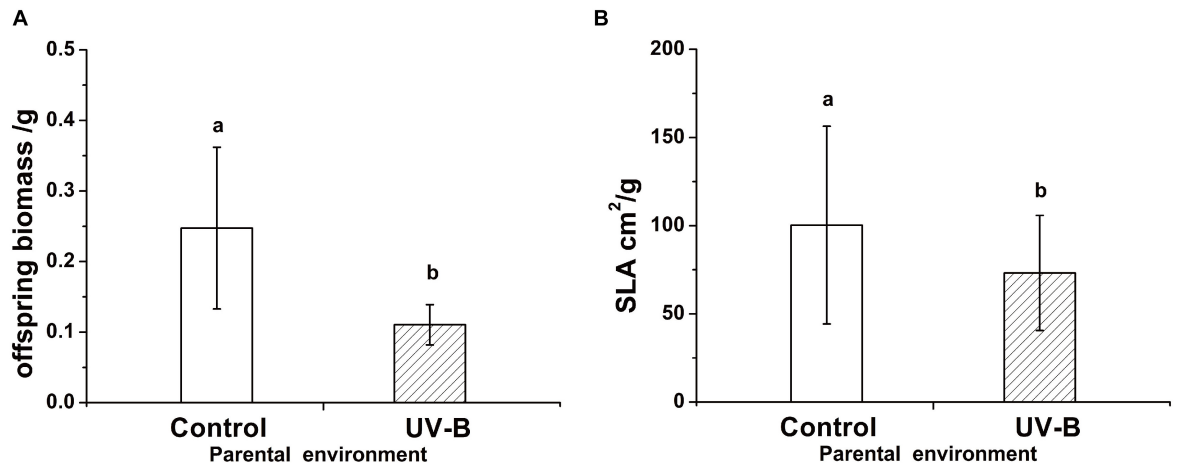

FIGURE 4 | The offspring biomass (F = 59.71, P < 0.001) **(A)** and specific leaf area (F = 6.45, P < 0.001) **(B)** in different parental light environment. Control: parental ramet in control light environment, UV-B: parental ramet in UV-B radiation. The graphs show mean ± SE. Columns sharing the same letter are not significantly different from each other at P < 0.05.

TABLE 2 | ANOVA/CCA analyses results for effects of parental environment (Control vs. UV-B), and all ramet type (parental ramet, older offspring ramet, and younger offspring ramet) on loci composition and epigenetic diversity (Shannon's diversity index) and total DNA methylation level of *Glechoma longituba*.

		Loci composition		Shannon's diversity index		DNA methylation level	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Parental environment (Pa)	1.50	**2.14**	**0.038**	**7.15**	**0.010**	**6.75**	**0.012**
Ramet type (Ramet)	2.34	**2.41**	**0.002**	1.76	0.182	1.81	0.174
Pa × Ramet	2.48	**1.65**	**0.004**	0.61	0.549	0.65	0.528

Degrees of freedom (df) and F and P-values are given. Values for P < 0.05 are in bold.

TABLE 3 | ANOVA/CCA results for effects of parental environment (Control vs. UV-B), offspring environment (Control vs. UV-B) on loci composition, epigenetic diversity (Shannon's diversity index), and total DNA methylation level of offspring ramet (older and younger offspring ramet) of *Glechoma longituba*.

		Loci composition		Shannon's diversity index		DNA methylation level	
	df	*F*	*P*	*F*	*P*	*F*	*P*
Older offspring ramet							
Parental environment (Pa)	1.15	**1.46**	**0.032**	0.22	0.643	0.25	0.622
Offspring environment (Off)	1.15	1.12	0.281	0.13	0.724	0.07	0.793
Pa × Off	1.14	1.01	0.411	3.40	0.086	3.50	0.083
Younger offspring ramet							
Parental environment (Pa)	1.15	1.55	0.052	1.30	0.274	1.36	0.264
Offspring environment (Off)	1.15	0.83	0.723	0.36	0.560	0.25	0.627
Pa × Off	1.14	0.91	0.631	0.31	0.589	0.47	0.506

Degrees of freedom (df) and F and P-values are given. Values for P < 0.05 are in bold.

ramet can carry epigenetic information about its experiences with environmental interactions and pass the information to its offspring ramets. Hence, behavior of clonal plants can be strongly influenced by epigenetic memories on the past environments (Latzel et al., 2016).

In this study, we demonstrated that UV-B radiation significantly reduced DNA methylation level and Shannon's diversity index of parental ramets. Similar reduction in DNA methylation due to increased UV radiation was reported also for *Zea mays* (Steward, 2002; Sokolova et al., 2014), *Picea abies* (Ohlsson et al., 2013), and *Artemisia annua* (Pandey and Pandey-Rai, 2015; Pandey et al., 2019). Moreover, the MSAP analysis revealed that the loci composition of parental ramets that experienced UV-B radiation significantly differed from parental ramets subjected to control light conditions. Both results suggest that DNA methylation change was involved in response to the UV-B stress. Our study also provides some evidence that the UV-B-induced DNA methylation variation can be, to some degree, passed to connected clonal offspring ramets and involved in the change of foraging behavior. Different loci composition triggered by the parental UV-B treatment was detected in both older and younger offspring ramets. In addition, the observed reduced level of DNA methylation of parental ramets was inherited by older but not younger offspring ramets (**Table 3**). A similar pattern was detected for Shannon's diversity index. This suggests that the epigenetic memory can be passed from parental to offspring ramets through mitotic cell division, but is gradually degrading during clonal growth (i.e., after several asexual generations, in our case, the younger ramet was usually the fourth clonal generation derived from the older offspring ramet). A similar conclusion was reached by Shi and colleagues (Shi et al., 2019) on a clonal plant alligator weed (*Alternanthera philoxeroides)*. They found that environmentally induced epigenetic variation is gradually resetting when plants of different populations (environments) are transplanted to a common garden. After 10 asexual generations and 2 years of cultivation in a common garden, plants of previously different epigenetic profiles become epigenetically comparable (Shi et al., 2019). Our findings thus suggest that the foraging behavior of clonal plants might be at least partly under epigenetic control, which supports the model of epigenetically coordinated advanced behavior of clonal plants described by Latzel et al. (2016). However, better insights into the role of epigenetic memory in the observed changes in foraging behavior require more sophisticated molecular methods such as whole genome bisulfite sequencing (Richards et al., 2017).

CONCLUSION

Our results demonstrated that the experience of parental ramet with UV-B radiation can affect foraging behavior of the clonal plant in an UV-B heterogeneous environment. Genets with UV-B-stressed parent adopted "escape strategy" in a heterogeneous environment by avoiding an environment with UV-B radiation and by plastic change in leaf area, stolon length, and ramets number. These results point out the importance of information sharing among parent–offspring ramets that can strongly influence behavior of clonal plants with significant impact on their overall fitness. Hence, it is evident that the behavior of clonal plants can be highly sophisticated, combining the interaction of actual environmental conditions and/or environmental heterogeneity with information from the past. Such cross-talks between actual and past experiences might provide clonal plants considerable advantage in their "understanding" of the environment. Our study also suggests that epigenetic memory can play a role in the observed change in behavior; nonetheless, more studies that employ sophisticated molecular analyses, e.g., NGS, are needed to provide unambiguous evidence.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

XL and JQ conceived and designed the experiments. JQ performed the experiments. ZM helped with the data analysis. JQ and VL wrote the manuscript and others provided editorial advice. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.633982/full#supplementary-material

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Changes in DNA Methylation in *Arabidopsis thaliana* Plants Exposed Over Multiple Generations to Gamma Radiation

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Previous studies have found indications that exposure to ionising radiation (IR) results in DNA methylation changes in plants. However, this phenomenon is yet to be studied across multiple generations. Furthermore, the exact role of these changes in the IR-induced plant response is still far from understood. Here, we study the effect of gamma radiation on DNA methylation and its effect across generations in young *Arabidopsis* plants. A multigenerational set-up was used in which three generations (Parent, generation 1, and generation 2) of 7-day old *Arabidopsis thaliana* plants were exposed to either of the different radiation treatments (30, 60, 110, or 430 mGy/h) or to natural background radiation (control condition) for 14 days. The parental generation consisted of previously non-exposed plants, whereas generation 1 and generation 2 plants had already received a similar irradiation in the previous one or two generations, respectively. Directly after exposure the entire methylomes were analysed with UPLC-MS/MS to measure whole genome methylation levels. Whole genome bisulfite sequencing was used to identify differentially methylated regions (DMRs), including their methylation context in the three generations and this for three different radiation conditions (control, 30 mGy/h, and 110 mGy/h). Both intra- and intergenerational comparisons of the genes and transposable elements associated with the DMRs were made. Taking the methylation context into account, the highest number of changes were found for cytosines followed directly by guanine (CG methylation), whereas only limited changes in CHG methylation occurred and no changes in CHH methylation were observed. A clear increase in IR-induced DMRs was seen over the three generations that were exposed to the lowest dose rate, where generation 2 had a markedly higher number of DMRs than the previous two generations (Parent and generation 1). Counterintuitively, we did not see significant differences in the plants exposed to the highest dose rate. A large number of DMRs associated with transposable elements were found, the majority of them being hypermethylated, likely

leading to more genetic stability. Next to that, a significant number of DMRs were associated with genes (either in their promoter-associated region or gene body). A functional analysis of these genes showed an enrichment for genes related to development as well as various stress responses, including DNA repair, RNA splicing, and (a)biotic stress responses. These observations indicate a role of DNA methylation in the regulation of these genes in response to IR exposure and shows a possible role for epigenetics in plant adaptation to IR over multiple generations.

Keywords: ionising radiation, DNA methylation, multigenerational, adaptation, epigenetics, whole genome bisulfite sequencing (WGBS), transposable elements

INTRODUCTION

In the last decade, the role of epigenetics in stress responses of plants, as well as their effect on gene expression has gained more attention. Epigenetic modifications, such as DNA methylation, small interfering RNA (siRNA), and histone modifications, can alter the way chromatin is packaged and can be accessed (Boyko and Kovalchuk, 2008). As such, a change in epigenetic marks can have a great impact on overall genome stability and gene expression. For example, in *Arabidopsis thaliana* DNA repeats and transposable elements (TEs) are highly correlated with cytosine methylation, which is essential for genome integrity (Vaillant and Paszkowski, 2007; Brautigam and Cronk, 2018). Stress conditions can lead to epigenetic modifications and thereby alter genome stability as well as gene expression and thus epigenetic modifications might play a role in adaptation to these stressors (Horemans et al., 2019; Schmid et al., 2018). Alterations in DNA methylation, for example, have been implicated in plant responses to several stresses (i.e., salinity, pathogen, UV, drought, water, heat stress) (Dowen et al., 2012; Sahu et al., 2013; Kinoshita and Seki, 2014).

More recently, the role of epigenetics in plant responses to ionising radiation (IR) is gaining interest. Low levels of natural background IR are present everywhere on Earth as a result of cosmic radiation and naturally occurring radionuclides in the Earth's crust. However, human activities have caused a significant increase in these dose rates and this can potentially have a negative impact on the environment (e.g., the nuclear accidents in Chernobyl and Fukushima). The IR stress responses in plants has been mainly studied on a phenotypical, physiological, biochemical, and genetic level. Some effects are still under debate such as the change in flowering induction, either by resulting in earlier or later flowering (Sax, 1955; Gunckel, 1957; Daly and Thompson, 1975; Kovalchuk et al., 2007; Hwang et al., 2016; Kryvokhyzha et al., 2018), or the effect on seed germination (Kumagai et al., 2000). Ionising radiation can have severe damaging biological effects either directly, by damaging biomolecules including DNA, or indirectly, by the production of reactive oxygen species (ROS) in the organism. These ROS are products of the radiolysis of, amongst others, water and these ROS will, when not scavenged by the plant's antioxidative defence system, lead to oxidative stress and damage to e.g., DNA molecules. As a result, DNA damage occurs often in organisms exposed to IR (West et al., 2000; Esnault et al., 2010; Dona et al., 2013; Biermans et al., 2015; Van Hoeck et al., 2017). In order to protect itself from the harmful effects of IR, processes such as oxidative stress response (i.e., increase in antioxidants) and DNA repair mechanisms will be called upon by the organism (Esnault et al., 2010; Biermans et al., 2015; van de Walle et al., 2016; Einor et al., 2016; Volkova et al., 2017). Previous research has shown that IR also affects the epigenome, of which DNA methylation has been the most studied (for an overview see Horemans et al., 2019). Pine trees from sites contaminated by the Chernobyl accident showed a dose-rate dependent increase in global DNA methylation (Kovalchuk et al., 2003; Volkova et al., 2018). A similar observation was made in soybeans that have grown in the Chernobyl exclusion zone for seven generations (Georgieva et al., 2017). However, results from *A. thaliana* sampled along radiation gradients in the exclusion zone showed some contradicting findings, demonstrating either an overall hypermethylation or hypomethylation (Kovalchuk et al., 2004; Horemans et al., 2018).

It has been established that DNA methylation of TEs is a tool to regulate their activity and it is therefore common to find high levels of DNA methylation located in these regions (Rabinowicz et al., 2003; Ikeda and Nishimura, 2015). Additionally, TE relocation has been shown to be activated by IR amongst other stressors in *A. thaliana* (Wang et al., 2016). These TEs play an important role in genetic evolution as they can result in significant genetic changes by inversion, deletion, inactivating or activating genes (Muñoz-López and García-Pérez, 2010). IR-induced hypermethylation can be seen as a defence response to prevent genome instability by prohibiting reshuffling of genetic material, such as TEs (Kovalchuk et al., 2004; Boyko et al., 2007; Horemans et al., 2018; Volkova et al., 2018).

In addition to its importance in gene regulation, DNA methylation's heritable character has recently gained interest for its potential role in acclimation and/or adaptation over generations to environmental stress conditions (Verhoeven et al., 2010; Boyko and Kovalchuk, 2011; Hauser et al., 2011; Mirouze and Paszkowski, 2011). Acclimation occurring in one generation as a method of overcoming changes in the environment or stressors has been widely studied in plants (de Azevedo Neto et al., 2005; Chinnusamy and Zhu, 2009; Chinnusamy et al., 2010). For instance, increased UV

Abbreviations: DMR, Differentially Methylated Region; GO, Gene Ontology; IR, Ionising Radiation; PAR, Photosynthetically Active Radiation; TE, Transposable Element; WGBS, Whole Genome Bisulfite Sequencing.

resistance was achieved by priming plants to low levels of chronic UV exposure (Hideg et al., 2013). This led to changes in antioxidant levels which enabled plants to cope better with increased oxidative stress induced by a second UV exposure. Similar indications of acclimation have been seen in plants in response to salinity and IR (Munns and Gilliham, 2015; Van Hoeck et al., 2017). Although the exact nature of priming is still not fully understood, previous studies have shown that epigenetics, including DNA methylation, and TEs might play a role in this priming mechanism (Espinas et al., 2016; Negin and Moshelion, 2020; Turgut-Kara et al., 2020). Adaptation over one or more generations to stress also remains under debate (Pecinka et al., 2009; Rasmann et al., 2012; Moller and Mousseau, 2016), nonetheless, a number of reports have demonstrated transgenerational adaptive stress responses in plants (Verhoeven and van Gurp, 2012; Suter and Widmer, 2013; Groot et al., 2016). However, with these studies it is important to keep in mind the difference between transgenerational studies, which explore the inherited effects over generations after exposure to stress in the first generation, and multigenerational studies, which explore the inherited effects over generations that are all exposed to a similar stress factor in each generation. Work on plant survival and reproduction in the Chernobyl exclusion zone, the Fukushima affected area, as well as studies done in lab conditions continue to contribute to the uncovering of a potential adaptation to IR exposure (Zaka, 2002; Geras'kin et al., 2005; Danchenko et al., 2009; Klubicova et al., 2012; Pozolotina et al., 2012; Rashydov and Hajduch, 2015; Georgieva et al., 2017; Kryvokhyzha et al., 2018). As mentioned, heritable epigenetic changes, such as DNA methylation, might play an important role in the adaptive responses to environmental stress (Schmid et al., 2018; Horemans et al., 2019).

To investigate the potential role of DNA methylation in plant responses to IR, exposure within one generation and over generations was performed in this study. It is hypothesised that IR induces a different cytosine DNA methylation profile in plants that are exposed compared to unexposed plants. Secondly, it is expected that plants with a different history in IR exposure will respond differently, in respect of DNA methylation, compared to plants that did not receive any prior IR exposure. In order to study this, we exposed three generations of *A. thaliana* plants [Parent (P0), Generation 1 (S1), Generation 2 (S2)] to five different dose rate conditions (natural background radiation (γ_0), 30 mGy/h (γ_{30}), 60 mGy/h (γ_{60}), 110 mGy/h (γ_{110}), and 430 mGy/h (γ_{430})) in a multigenerational experiment. First, the entire methylomes were analysed with UPLC-MS/MS to measure whole genome methylation levels. Secondly, whole genome bisulfite sequencing (WGBS) was used to identify differentially methylated regions (DMRs), including their methylation context. Based on this data, (1) both an intra- and intergenerational comparisons of the genes and TEs associated with the DMRs were made across the gamma radiation exposure conditions; and (2) a gene ontology enrichment was performed to discover the processes that might be regulated by IR-induced DNA methylation.

MATERIALS AND METHODS

Plant Material and Gamma Treatment

In order to synchronise germination, *A. thaliana* (Columbia) seeds were vernalised on moist filter paper during 3 days at 4°C. The seeds of three different generations with a different irradiation background (2 weeks exposure to either γ_0 = natural background radiation (control), γ_{30} = 30 mGy/h, γ_{60} = 60 mGy/h, γ_{110} = 110 mGy/h, or γ_{430} = 430 mGy/h) were used; P0 seeds originated from our standard seed stock and had never been irradiated, S1 seeds were harvested from the previously irradiated P0 plants and S2 seeds were harvested from previously irradiated S1 plants. This resulted in plants that had no previous history of irradiation (P0) and plants that already underwent the same gamma radiation treatments in one (S1) or two (S2) previous generations (**Figure 1**). Subsequently, the seeds were grown according to Vanhoudt et al. (2014) on cut-off plugs from 1.5 mL polyethylene centrifuge tubes filled with a Hoagland solution that was solidified with 0.6% agar and grown hydroponically in a growth cabinet (Snijders Scientific, Microclima 1000E) under a 14 h photoperiod (photosynthetically active radiation (PAR) of 200 μmol m^{-2} s^{-1} at the leaf level) with 65% humidity and a day/night temperature of 22°C/18°C. Roots were aerated during the entire course of the experiment and Hoagland solution was refreshed twice a week. When plants were 7 days old, their most vulnerable life stage for irradiation (Biermans et al., 2015), they were transferred to the irradiation unit of SCK CEN where they were exposed to gamma radiation during 14 days coming from a panoramical ^{137}Cs-source. They were exposed to different dose rates (γ_{30} = 30 mGy/h, γ_{60} = 60 mGy/h, γ_{110} = 110 mGy/h, or γ_{430} = 430 mGy/h) of gamma radiation. These dose rate conditions were chosen based on previous experiments performed in our group. Under these conditions, *A. thaliana* plants exhibited radiation-induced biochemical and physiological changes, yet were still able to recover and produce viable following generations (Vanhoudt et al., 2010, 2014; Biermans et al., 2015; van de Walle et al., 2016). For each condition 2 containers containing 36 plants each, were used. After 14 days they received a total dose of, respectively, 7, 13, 29, and 156 Gy. During the irradiation period, plants were grown at 24°C and light was supplied by LED lights for 14 h photoperiodic period with a PAR of 200 μmol m^{-2} s^{-1} at the leaf level. Control plants were grown in a separate chamber at the same temperature and light conditions. After 14 days of irradiation fresh weight of plants was measured and the plant rosettes were harvested by snap-freezing them in liquid nitrogen and stored at −80°C until further analysis. Different treatments are indicated with a generation identifier and a treatment identifier. For example, P0γ_{60} refers to plants of the P0 generation that were exposed to the second gamma treatment (60 mGy/h), while S2γ_{430} refers to plants exposed to the highest dose rate (430 mGy/h) treatment in the S2 generation.

DNA Extraction

Frozen plant samples (50–100 mg) were ground using a Mixer Mill MM 400 (Retsch) for 3 min at 30 Hz prior to the

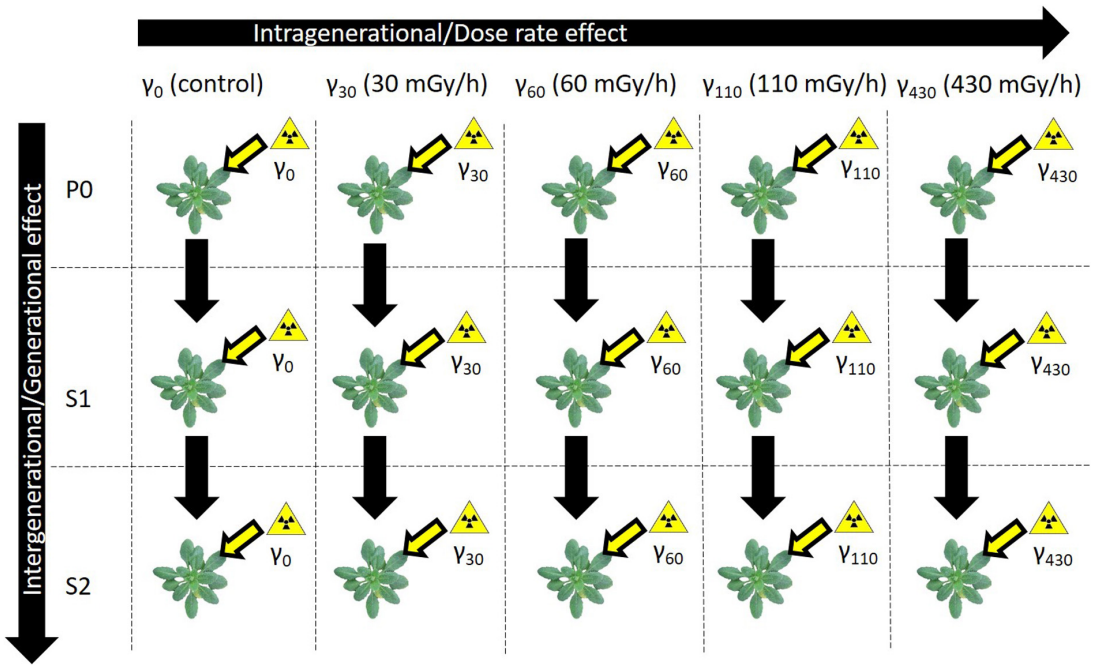

FIGURE 1 | Schematic overview of the experimental set-up. Three generations of *A. thaliana* plants [Parent (P0), Generation 1 (S1), Generation 2 (S2)] were exposed to five different gamma dose rate conditions [natural background radiation (γ_0), 30 mGy/h (γ_{30}), 60 mGy/h (γ_{60}), 110 mGy/h (γ_{110}), and 430 mGy/h (γ_{430})]. S1 came from a previously exposed generation and S2 came from a line with two previously exposed generations.

extractions. DNA was extracted from the ground material using Zymo ZR Plant/Seed DNA MicroPrep^{TM} kit according to the manufacturers' instructions. The DNA quantity and integrity were determined spectrophotometrically at 230, 260, and 280 nm (Nanodrop, Isogen Life Science, De Meern, The Netherlands) and via gel electrophoresis (Bioanalyser, Agilent Technologies, Santa Clara, CA, United States), respectively.

Global Methylation

The overall 5 methylcytosine (5mC) percentage was determined via UPLC, using five biological replicates of each (generational and dose rate) condition. One μg of extracted DNA was digested for 2 h at 37°C using the DNA Degradase Plus protocol according to Zymo Research Corporation (United States) which allows for a quick generation of single nucleotides from total DNA. Concentrations of 2′-deoxycytidine (dC) and 5-methyl-2′-deoxycytidine (5mdC) were measured with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, Milford, MA, United States) coupled to a PDA detector (Waters, Milford, MA, United States) and a Micromass Quattro Premier XE mass spectrometer (Waters, United States). Chromatograms were analysed using Masslynx software v4.1 (Waters, Milford, MA, United States). Levels of dC and 5mdC were calculated based on the corresponding standard curves. The relative content of 5mdC was expressed as a percentage (%5mdC) with respect to the total amount of cytosine (dC + 5mdC). Several quality control measures were in place during the analysis. From a home-extracted control *A. thaliana* DNA pool several samples were used to monitor the method precision. Further, control standards and method blanks were analysed. Duplicate analysis of samples was performed whenever possible.

The statistical analysis of the global DNA methylation levels was performed using the open source software package R (R i386 3.1.0, R Foundation for Statistical Computing, Vienna, Austria). The normal distribution and homoscedasticity of our data were tested with a Shapiro-Wilk and Bartlett test, respectively. A one-way ANOVA test was applied to results from one generation or one treatment to identify any statistical differences between treatments and generations, respectively. When significant differences ($p < 0.05$) were found, a Tukey HSD test was applied to identify the specific differences between groups.

Bisulfite Sequencing

Three different treatments (γ_0 = control, γ_{30} = 30 mGy/h, γ_{110} = 110 mGy/h) per generation (P0, S1, S2) were selected for sequencing. This resulted in a total of nine different conditions with five biological replicates for each condition. Concentration of the extracted DNA was measured using the "Quant-it Picogreen dsDNA assay kit" (Life Technologies, Grand Island, NY, United States). Subsequently, 400–600 ng of gDNA was fragmented to 300 bp using the Covaris S2 focused-ultrasonicator (Covaris, Woburn, Massachusetts, United States). The size of the fragmented DNA was checked on a High sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, United States). Library preparation with NEBNext Ultra II DNA library prep kit (New England Biolabs, Ipswich, MA, United States) was performed using methylated adapters, according to the manufacturer's protocol. Size selection on a 2% EX Agarose E-Gel (Thermo

Fisher Scientific, MA, United States) was performed on the resulting library, making a 300–1,000 bp gel cut followed by a purification with the Gel DNA recovery kit (Zymo Research, Irvine, CA, United States). Bisulfite conversion was performed with the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA, United States) according to the manufacturer's protocol, followed by an additional purification with AMPure XP beads (Beckman Coulter, Brea, CA, United States) (beads:sample ratio of 5:1). An enrichment PCR was performed with KAPA Hifi hotstart Uracil + Ready mix (Kapa Biosystems, Wilmington, MA, United States) in a 13 cycles PCR reaction, followed by a purification with AMPure XP beads (Beckman Coulter, Brea, CA, United States) (beads:sample ratio of 1:1). Libraries were quantified by qPCR, according to Illumina's protocol 'Sequencing Library qPCR Quantification protocol guide', version February 2011. A High sensitivity DNA chip (Agilent Technologies, Santa Clara, CA, US) was used to control the library's size distribution and quality. Sequencing was performed on 2 high throughput Illumina NextSeq 500 flow cells generating PE2 × 75 bp reads. The flowcells were clustered with 2.3 pM library and 15% Phix control library.

Differentially Methylated Region Assignment and Annotation

CLC Genomics Workbench 20.0[1] was used to analyse the data. The paired reads were mapped to the reference genome (TAIR10.31)[2] with "*Map Bisulfite reads to reference*" module, using non-directional approach (applying the default parameters). Differentially methylated regions were assigned using "*Call methylation level*" module by doing all pairwise comparison within the same generation or within the same treatment dose (resulting in 18 sets of DMRs identified for the different comparisons). The default parameters were applied (while specifying the minimum high-confidence site-coverage = 5 and minimum number of samples = 3), reporting the methylation levels for CG, CHG, and CHH contexts separately. The *p*-values produced from ANOVA statistical modelling where corrected using Benjamini Hochberg approach (using *p.adjust* in R v 3.5.0). Annotation to the nearest genes was added to each of the DMRs using "*Annotate by nearby gene*" module, using reference genome's genes set. Additionally, the annotation to the nearest transposable elements (TEs) was added with *closest* module in bedtools package, using the "TAIR10 transposable elements" data set (downloaded on 18/03/2020) from the TAIR website[2].

Differentially Methylated Region Filtering and Functional Analysis

The filtering criteria to find DMRs associated with either gene regions (promoter or gene body) or TEs, were a $p \leq 0.05$ and at least 20% difference in their methylation levels (referred to as methylation difference). This cut-off was chosen in order to ensure a definite methylation difference was being studied. Here, the methylation difference is calculated by comparing either a higher dose rate with a lower dose rate (i.e., $S1\gamma_0$ vs. $S1\gamma_{30}$), or by comparing an older generation with a younger on (i.e., $P0\gamma_{30}$ vs. $S1\gamma_{30}$). A hypermethylated DMR in $P0\gamma_{30}$ vs. $S1\gamma_{30}$ means that the region in $S1\gamma_{30}$ has a higher methylation level compared to that of $P0\gamma_{30}$.

For the filtering in DMRs associated with promoter regions, we chose to filter for DMRs with a distance to the nearest gene of $2\ \text{kbp} \leq X > 0$. For those associated with gene bodies the filter was set at a distance of 0 to the nearest gene. DMRs associated with TEs were found by filtering for a distance of 0 to the nearest TE. For the functional analysis of the genes with DMRs, either in their promoter regions or their gene bodies, a gene ontology (GO) term enrichment was done using Metascape (Zhou Y. et al., 2019). For the analysis of overlapping genes in our selected comparisons, we used an online Venn diagram tool[3] where we used the genes per comparison result as input.

RESULTS

At a Global Methylation Level the First Offspring Generation Showed the Highest Radiation Response

In the parent generation (P0) no significant dose rate dependent effects were seen on global methylation level (**Figure 2**). In the first generation (S1), however, dose rate dependent differences in comparison with control conditions were observed at the two highest dose rates γ_{110} and γ_{430}. Also in the second generation (S2) a significantly higher global methylation level compared to the control plants was present, but only after exposure to the highest dose rate. The significant increase in methylation percentage in the S1 also resulted in significant increases between the S1 and the other two generations at these same dose rates. In addition, a deeper analysis of the samples using WGBS was performed and the global methylation level was calculated using the WGBS result (**Supplementary Figure S1**). In general, these data followed the UPLC-MS/MS data but showed a higher variation and thus no significant difference were found within the WGBS global methylation levels. The global weighted methylation levels, calculated using methods described in Schultz et al. (2012), are shown in the **Supplementary Table S1**. The global weighted methylation levels in this study did not significantly vary between treatments. For control values these are on average ∼30% CG, ∼13.5% CHG, and ∼6.8% CHH, which is in line with previous studies in which DNA methylation levels in *A. thaliana* leaves is studied (Niederhuth et al., 2016; Bartels et al., 2018; Zhou M. et al., 2019).

Differentially Methylated Regions Are Predominantly Found in CG Methylation Context and at Lowest Dose Rate and Later Generations

For the WGBS analyses a limited set of three dose rates were used (γ_0 = control, γ_{30} = 30 mGy/h, and γ_{110} = 110 mGy/h).

[1] https://digitalinsights.qiagen.com

[2] www.arabidopsis.org

[3] http://bioinformatics.psb.ugent.be/webtools/Venn/

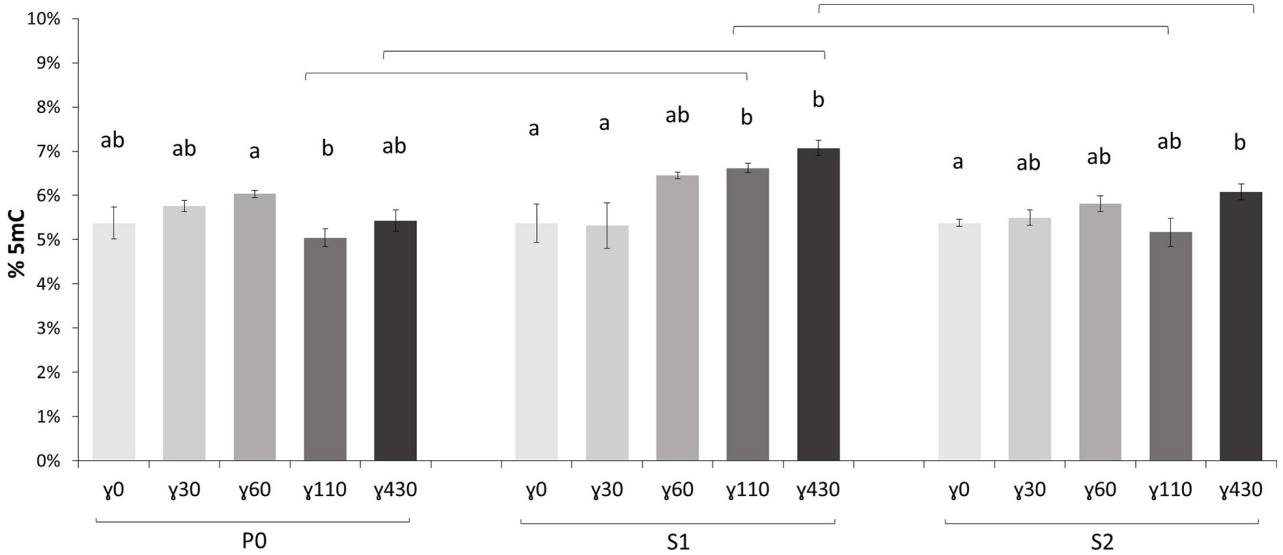

FIGURE 2 | The global methylation percentage, determined with UPLC-MS/MS, of all generations [P0 (Parent generation), S1 (generation 1), S2 (generation 2)] of *A. thaliana* plants (γ_{30} = 30 mGy/h, γ_{60} = 60 mGy/h, γ_{110} = 110 mGy/h, and γ_{430} = 430 mGy/h). Values are represented relative to P0γ_0 (effective values P0γ_0 = 5.38%, S1γ_0 = 4.88%, S2γ_0 = 6.03%). Small letters indicate significant dose rate dependent differences ($p < 0.05$) within generations, brackets indicate significant ($p < 0.05$) differences at the same dose rate between generations. Measurements are an average ± SE of 5 biological replicates.

To check for global differences between generations or treatments, a pairwise comparison was performed looking for differences only between two different radiation treatments of the same generation (intragenerational) or two different generations for the same treatment (intergenerational). A distinction was made between the different methylation contexts for which the methylated cytosine (C) was either followed by a guanine (CG) or another nucleotide by a guanine (CHG) or not followed by a guanine (CHH).

In **Tables 1**, **2**, the number of differential hyper- and hypomethylated regions (DMRs), respectively, is represented accounting for a methylation difference of >20%. This cut-off was chosen in order to ensure a definite methylation difference was being studied. A more extensive overview of DMRs with different cut-offs of methylation differences (5, 10, and 30%) can be found in the **Supplementary Tables S2–S5**. Highest differences were found for CG methylation, whereas only limited changes in CHG methylation occurred and no changes in CHH methylation were observed (**Tables 1**, **2**). Furthermore, within the generations over the different dose rates (intragenerational), the largest number of DMR changes occurred between the γ_0 and γ_{30} groups, with the majority occurring in S2 generation (i.e., 473 hypermethylated DMRs, 316 hypomethylated DMRs in S2γ_0 compared to S2γ_{30}). However, it is important to note that no significant changes are observed in the parent generation after exposure to IR. Intergenerationally, the largest number of (both hypo- as hypermethylated) DMRs occurred between generations after exposure to γ_{30}, with the major change between S1γ_{30} vs. S2γ_{30} (1,057 hypermethylated DMRs in the second generation compared to the first generation, whereas 833 DMRs were found to be hypomethylated). Here, it is also clear that no changes occur in the γ_0 group and few changes occur in the γ_{110} group.

Differentially Methylated Regions Associated With Genes and Transposable Elements

For detailed analysis, DMRs were split up into those associated with the promoter associated region (<2 kbp upstream of the gene start), gene body, or TEs. Looking into the DMRs associated with the promoter region (**Table 3**), the highest number of affected genes (either hypo- of hypermethylated) are found over the γ_{30}-exposed generations and more specifically in the second generation. Also between the control and γ_{30} dose rates, we see a higher number of DMRs associated with promoter regions. A similar observation was made in DMRs associated with the gene body i.e., DMRs that overlap, at least partially, with the gene body sequence (**Table 3**), with a strong generation effect resulting in 345 hypo- and 464 hypermethylated DMRs between S1γ_{30} and S2γ_{30}. Additionally, the biggest dose-rate dependent effect was observed in S2 with 140 and 189 hypo- and hypermethylated DMRs in S2γ_0 vs. S2γ_{30}. Again, the highest dose rate (γ_{110}) does not affect DMRs as strongly as γ_{30}. A list of genes associated with DMRs in their promoter regions and/or gene bodies can be accessed through Gene Expression Omnibus (GEO), as specified in the "Data Availability Statement" section.

The link between DMRs and TEs was studied by selecting for DMRs located within or at least overlapping with TE sequences. From this data it was observed that a large number of DMRs were associated with TEs, with the majority of them being hyper methylated (**Table 3**).

TABLE 1 | The number of hypermethylated DMRs (sorted by methylation context) that were identified after the comparison of the entire methylome of *A. thaliana*.

Hypermethylated							
Intragenerational (dose rate effects)				**Intergenerational (generation effects)**			
	CG	**CHG**	**CHH**		**CG**	**CHG**	**CHH**
$P0\gamma_0$ vs. $P0\gamma_{30}$	0	0	0	$P0\gamma_0$ vs. $S1\gamma_0$	0	0	0
$P0\gamma_0$ vs. $P0\gamma_{110}$	0	0	0	$P0\gamma_0$ vs. $S2\gamma_0$	0	0	0
$P0\gamma_{30}$ vs. $P0\gamma_{110}$	0	0	0	$S1\gamma_0$ vs. $S2\gamma_0$	0	0	0
$S1\gamma_0$ vs. $S1\gamma_{30}$	69	0	0	$P0\gamma_{30}$ vs. $S1\gamma_{30}$	92	1	0
$S1\gamma_0$ vs. $S1\gamma_{110}$	2	0	0	$P0\gamma_{30}$ vs. $S2\gamma_{30}$	176	1	0
$S1\gamma_{30}$ vs. $S1\gamma_{110}$	21	0	0	$S1\gamma_{30}$ vs. $S2\gamma_{30}$	1,057	2	0
$S2\gamma_0$ vs. $S2\gamma_{30}$	473	1	0	$P0\gamma_{110}$ vs. $S1\gamma_{110}$	0	0	0
$S2\gamma_0$ vs. $S2\gamma_{110}$	1	0	0	$P0\gamma_{110}$ vs. $S2\gamma_{110}$	0	0	0
$S2\gamma_{30}$ vs. $S2\gamma_{110}$	7	0	0	$S1\gamma_{110}$ vs. $S2\gamma_{110}$	6	0	0

Eighteen pairwise comparisons were made either between generations [P0 (Parent generation), S1 (generation 1), S2 (generation 2)] for the same dose rate {γ_{30} (30 mGy/h), γ_{110} (110 mGy/h), γ_0 [control condition (<0.1 μGy/h)] or between different dose rates of the same generation (Methylation difference of > 20%) ($p \leq 0.05$)}.

TABLE 2 | The number of hypomethylated DMRs (sorted by methylation context) that were identified after the comparison of the entire methylome of *A. thaliana*.

Hypomethylated							
Intragenerational (dose rate effects)				**Intergenerational (generation effects)**			
	CG	**CHG**	**CHH**		**CG**	**CHG**	**CHH**
$P0\gamma_0$ vs. $P0\gamma_{30}$	0	0	0	$P0\gamma_0$ vs. $S1\gamma_0$	0	0	0
$P0\gamma_0$ vs. $P0\gamma_{110}$	0	0	0	$P0\gamma_0$ vs. $S2\gamma_0$	0	0	0
$P0\gamma_{30}$ vs. $P0\gamma_{110}$	0	0	0	$S1\gamma_0$ vs. $S2\gamma_0$	0	0	0
$S1\gamma_0$ vs. $S1\gamma_{30}$	64	0	0	$P0\gamma_{30}$ vs. $S1\gamma_{30}$	95	1	0
$S1\gamma_0$ vs. $S1\gamma_{110}$	0	0	0	$P0\gamma_{30}$ vs. $S2\gamma_{30}$	103	0	0
$S1\gamma_{30}$ vs. $S1\gamma_{110}$	17	0	0	$S1\gamma_{30}$ vs. $S2\gamma_{30}$	833	2	0
$S2\gamma_0$ vs. $S2\gamma_{30}$	316	0	0	$P0\gamma_{110}$ vs. $S1\gamma_{110}$	0	0	0
$S2\gamma_0$ vs. $S2\gamma_{110}$	5	0	0	$P0\gamma_{110}$ vs. $S2\gamma_{110}$	0	0	0
$S2\gamma_{30}$ vs. $S2\gamma_{110}$	8	0	0	$S1\gamma_{110}$ vs. $S2\gamma_{110}$	8	0	0

Eighteen pairwise comparisons were made either between generations [P0 (Parent generation), S1 (generation 1), S2 (generation 2)] for the same dose rate {γ_{30} (30 mGy/h), γ_{110} (110 mGy/h), γ_0 [control condition (<0.1 μGy/h)] or between different dose rates of the same generation (Methylation difference of > 20%) ($p \leq 0.05$)}.

TABLE 3 | Number of genes with differentially methylated regions (DMRs) (CG) found in the promoter associated regions (<2 kbp upstream), gene bodies, and transposable elements (TEs) of *A. thaliana* divided in hypo- and hyper methylation.

Intergenerational (generational effect)						
	Hypo			**Hyper**		
Comparison	**Promoter associated region**	**Gene body**	**TEs**	**Promoter associated region**	**Gene body**	**TEs**
$P0\gamma_{30}$ vs. $S1\gamma_{30}$	33	34	15	31	38	12
$P0\gamma_{30}$ vs. $S2\gamma_{30}$	34	42	5	69	60	35
$S1\gamma_{30}$ vs. $S2\gamma_{30}$	255	345	95	327	464	134
$S1\gamma_{110}$ vs. $S2\gamma_{110}$	0	3	0	5	1	3
Intragenerational (dose rate effects)						
$S1\gamma_0$ vs. $S1\gamma_{30}$	31	20	10	27	26	14
$S1\gamma_0$ vs. $S1\gamma_{110}$	0	0	0	1	0	0
$S1\gamma_{30}$ vs. $S1\gamma_{110}$	7	6	4	7	12	4
$S2\gamma_0$ vs. $S2\gamma_{30}$	90	140	23	165	189	75
$S2\gamma_0$ vs. $S2\gamma_{110}$	3	2	2	1	0	0
$S2\gamma_{30}$ vs. $S2\gamma_{110}$	2	2	1	1	3	0

Methylation difference of > 20%) ($p \leq 0.05$) {γ_{30} (30 mGy/h), γ_{110} (110 mGy/h), γ_0 [control condition (<0.1 μGy/h)], P0 (Parent generation), S1 (generation 1), S2 (generation 2).

Similar as to what is observed for genes associated with DMRs (either in the promoter region or the gene body), the second generation has the highest number of TEs associated with DMRs. In addition, the plants in the γ_{30} condition have a higher number of differentially methylated TEs compared to those in the control and γ_{110} groups. However, in the case of the TEs, there is a stronger link with hypermethylation than hypomethylation than was seen in the genes. A list of the affected TEs can be accessed through Gene Expression Omnibus (GEO), as specified in the "Data Availability Statement" section. The global methylation level including the location of the DMRs per chromosome for S2γ_0 vs. S2γ_{30}, as identified in this analysis, is represented in **Figure 3**, similar representations for the comparisons P0γ_{30} vs. S1γ_{30}, P0γ_{30} vs. S2γ_{30}, and S1γ_{30} vs. S2γ_{30} can be found in the **Supplementary Figures S2–S4**. The average methylation levels over the different regions of the genome (<2 kb upstream promoter-associated region, gene body, and the region 2 kb downstream from the gene) per condition and per methylation context are presented in the **Supplementary Figure S5**. As expected, CG methylation is the biggest contributor in the gene methylation.

Genes With Differentially Methylated Regions Linked to Stress Responses

The gene ontology (GO) term enrichment of genes associated with DMRs was split between those with affected promoter regions and those with affected gene bodies. Each was also divided into hypo- and hypermethylated DMRs. The location of DNA methylation in respect to a gene is important to its regulatory function, as DMRs located in the promoter-associated region will have a different effect than those found in the gene body. By looking at these DMRs individually based on their location, it will give a better insight into the biological processes that are affected after exposure to IR in specific generations (intragenerational, different dose rates) or over three generations (intergenerational, within one dose rate).

In the intergenerational GO enrichment analysis for promotor regions (**Figure 4**), an enrichment for "ribosome biogenesis" and "rRNA processing" was observed in the hypomethylated DMRs in promotor regions between S1γ_{30} vs. S2γ_{30}. For the hypermethylated DMRs in the promoter regions, an enrichment for "RNA splicing" is observed in P0γ_{30} vs. S2γ_{30} (**Figure 4**). An enrichment for "RNA splicing" is also observed in the intragenerational analysis (S2γ_0 vs. S2γ_{30}) of hypermethylated DMRs in the promoter regions along with an enrichment for the "positive regulation of transcription by RNA polymerase II" (**Figure 4**). The intragenerational analysis of hypomethylated DMRs in the promoter-associated regions yielded no significantly enriched GO terms.

For the DMRs in gene bodies, the intergenerational GO term analysis of the hypermethylated DMRs shows an enrichment for "chromosome organisation" in the comparison between the second generation and previously unexposed plants of the parent generation (P0γ_{30} vs. S2γ_{30}) (**Figure 5**). Between the second generation and first generation (S1γ_{30} vs. S2γ_{30}), an enrichment for "cell plate formation in plant-type cell wall biogenesis," "double-strand break repair," and "trichrome branching" is observed in the hypermethylated DMRs (**Figure 5**). For the hypomethylated DMRs of the gene bodies an enrichment for "plastoquinone biosynthetic process," "cellular response to DNA damage stimulus," "negative regulation of flower development," and "tetraterpenoid biosynthesis" is observed, in the comparison between the first and second generation of γ_{30}-exposed plants (S1γ_{30} vs. S2γ_{30}) (**Figure 5**).

The intragenerational GO enrichment analysis of the hypermethylated DMRs associated with gene bodies shows an enrichment for the "response to osmotic stress," "regulation of response to stress," and "DNA repair" in the comparison within the second generation between the control plants and γ_{30}-exposed plants (S2γ_0 vs. S2γ_{30}) (**Figure 5**). In the hypomethylated DMRs in the gene bodies an enrichment for "chromosome organisation" is observed in the second generation between the control and γ_{30} group (S2γ_0 vs. S2γ_{30}) (**Figure 5**).

In the **Supplementary Figures S6**, **S8** Venn diagrams of overlapping genes (for both those affected by promoter associated DMRs and those with gene body associated DMRs) between the different comparisons within γ_{30} (P0γ_{30} vs. S2γ_{30}, P0γ_{30} vs. S1γ_{30}, S1γ_{30} vs. S2γ_{30}) can be found. Additionally, the GO enrichment can be found of those overlapping genes (**Supplementary Figures S7**, **S9**). Only a small overlap is observed over the three generations for differentially methylated promoter regions (**Supplementary Figure S6** and **Supplementary Tables S6**, **S7**), except for those in P0γ_{30} vs. S2γ_{30} and S1γ_{30} vs. S2γ_{30}. Here, 19 overlapping genes with hypomethylated DMRs in their promoter regions and 18 with hypermethylated DMRs in their promoter regions are found (**Supplementary Figures S6A,B**). This overlap of genes with hypermethylated DMRs in their promoter regions shows an enrichment for "RNA splicing," thereby showing that there is an involvement of IR-induced DNA methylation in the regulation of this process (**Supplementary Figure S7**). The hypomethylated ones are enriched for "cell differentiation," a regular day-to-day process (**Supplementary Figure S7**). The study into any overlap in genes with DMRs in their gene bodies over the three generations in the γ_{30} condition showed a similar result with most of the overlap occurring between P0γ_{30} vs. S2γ_{30} and S1γ_{30} vs. S2γ_{30} (28 hypomethylated and 47 hypermethylated) (**Supplementary Figures S8A,B**). The GO enrichment study for these genes shows an enrichment for "response to osmotic stress" and "DNA repair" (**Supplementary Figure S9**).

DISCUSSION

It was hypothesised that the exposure to IR would induce DNA methylation changes in plants and that these DNA methylation profiles differ between generations with a different IR exposure history. Based on the UPLC-MS/MS analysis a significantly increased global methylation level is observed mainly in the first generation (S1) plants, which were exposed to IR compared to the parent generation but decreased again in the following generation (S2) (**Figure 2**). Additionally, the parent generation and second generation showed no significant

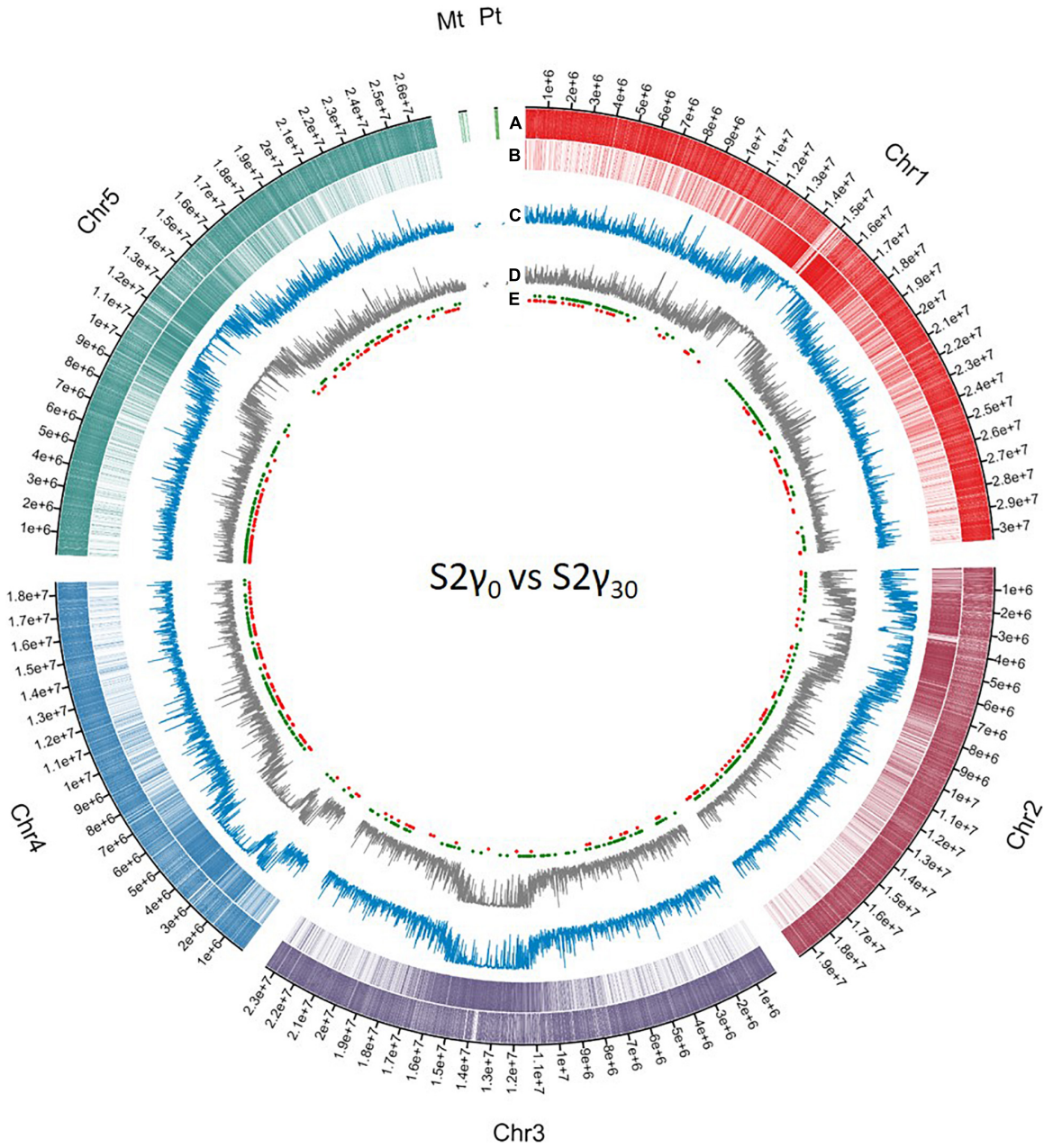

FIGURE 3 | Circos representation of DNA methylation locations on the different chromosomes and the mitochondrial genome (Mt) and the plastid (Pt) coming from whole genome bisulfite sequencing data. The first **(A)** and second layer **(B)** represent the genes and transposable elements per chromosome, respectively, differently coloured per chromosome. The third **(C)** and fourth **(D)** layer display the methylation level averaged over a window of 10,000 bp for $S2\gamma_{30}$ and $S2\gamma_0$, respectively, y-axis from 0 to 1. The fifth layer **(E)** shows the different differentially methylated regions (DMRs) as identified in the current analysis, hypermethylated and hypomethylated DMRs are represented by green dots and red dots, respectively. Circos plot was created using Circa software (http://omgenomics.com/circa).

differences compared to each other, hereby indicating that when considering global methylation levels changes in methylation percentage predominantly happen in the exposed first generation (S1). Such a strong response in S1 would fit with findings of a previous study, where oxidative stress and cell wall-related enzyme activity was also increased in the exposed first generation of *A. thaliana* plants (van de Walle et al., 2016). A study done on *Daphnia magna* exposed to chronic gamma radiation, also showed an increase in the number of DNA methylation changes in the first generations which tapered off in later generations (Trijau et al., 2018).

By analysing the number of DMRs as identified after WGBS (**Tables 1**, **2**), most DMRs were found in both IR-exposed S1 and S2 and no DMRs were observed in the non-exposed plants

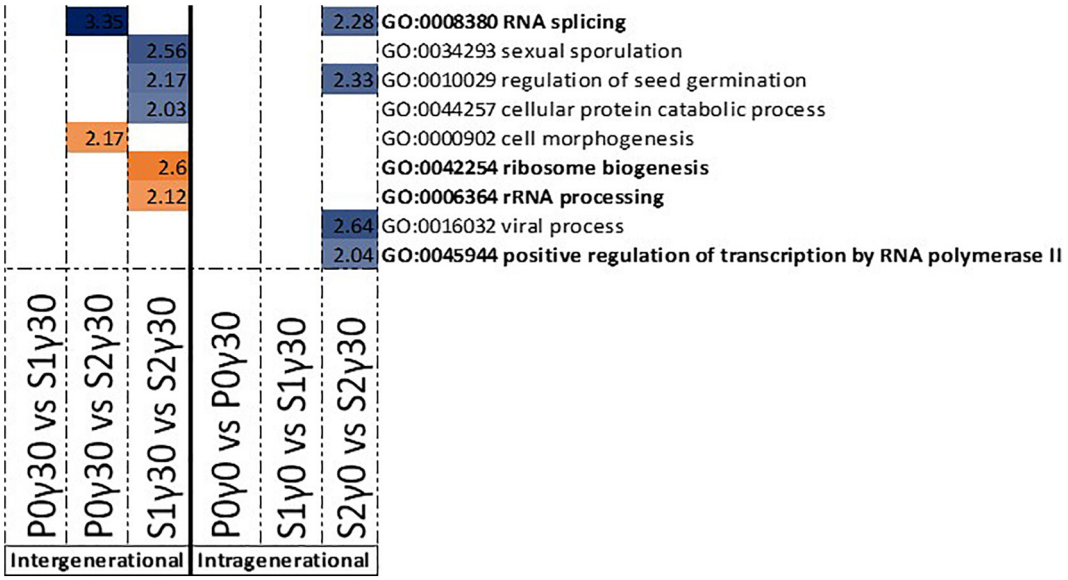

FIGURE 4 | Gene ontology (GO) term enrichment for hypo- and hypermethylated DMRs in the promoter-associated regions of *Arabidopsis thaliana* for the intergenerational comparisons P0γ_{30} vs. S1γ_{30}, P0γ_{30} vs. S2γ_{30}, and S1γ_{30} vs. S2γ_{30} (on the left), and the intragenerational comparisons P0γ_0 vs. P0γ_{30}, S1γ_0 vs. S1γ_{30}, S2γ_0 vs. S2γ_{30} (on the right) {γ_{30} (30 mGy/h), γ_0 [control condition (<0.1 μGy/h)], P0 (Parent generation), S1 (generation 1), S2 (generation 2)}. S1 came from a previously exposed generation and S2 came from a line with two previously exposed generations. The $-\log_{10}$(P) value is shown and shaded according to its value per GO term, blue represents hypermethylation, whereas orange represents hypomethylation. GO terms highlighted in bold are those discussed in this paper.

over the different generations. As no stress-induced responses are expected between control groups, these findings validate the control group. However, also no DMRs are identified in the parent generation after exposure to IR which is comparable to the results on global methylation (UPLC-MS/MS). Taken together both the data of the UPLC-MS/MS and those obtained after WGBS thus indicate that, at least within the current set-up, there is a need for an initial exposure that acts as a form of priming in which a first exposure will only elicit a response in the following generation or exposure. A similar initial priming was observed in previous studies for plants in response toward predators as well as other (a)biotic stresses (van Hulten et al., 2006; Rasmann et al., 2012; Lamke and Baurle, 2017; Thomas and Puthur, 2017; Baurle, 2018). The fact that the second generation (S2) generally shows a markedly higher number of DMRs than the other two generations (**Tables 1**, **2**) after exposure to IR, indicates the presence of a generation-dependent dose-rate effect. This could point towards an ongoing adaptive response, which will likely reach an equilibrium over a number of generations. The strong response found in the WGBS data in S2 is potentially not picked up in the UPLC-MS/MS data which only gives a global DNA methylation percentage in which local hypo- and/or hypermethylation changes will cancel each other out, and therefore are not taken into account.

For the DMRs of the S1 and S2 generation, no clear dose-rate dependent response was found (**Tables 1**, **2**) in contrast the strongest effects were observed in the lower dose rate (γ_{30}) compared to the control condition (γ_0) and very little DMRs were present in the comparisons with γ_{110} (**Tables 1**, **2**). Nonetheless, the plants in the γ_{110} group did show a normal growth (and biomass) similar to the other plants, as was also seen under the same conditions and set-up by van de Walle et al. (2016). This lack of DMRs in the highest dose rate (γ_{110}) could indicate that a certain threshold is crossed at which the plants switch to a different method of coping with the IR exposure. Kumagai et al. (2000) found some potential indications of the existence of such a threshold when studying seed germination of *A. thaliana* plants irradiated at different dose rates. They saw a gradual decrease in germination rate with increasing dose rate (2–9 kGy), however, at a certain dose rate (10 kGy) the germination rate suddenly dropped to zero. Comparably, IR-exposed *Lemna minor* plants shifted from acclimation to a survival strategy by expressing higher levels of antioxidant defence and DNA repair genes, at the higher dose rates (>232 mGy/h) (Van Hoeck et al., 2017). A transient response has also been observed in response to other stress conditions, such as salinity and UV-B irradiation (Munns and Gilliham, 2015; Mosadegh et al., 2019). Based on the current experimental design it is hypothesised that DNA methylation plays a more prominent role in the regulation of the plant response to lower dose rates than the higher ones. This hypothesis however, needs further testing for more doses and/or time points or confirming it in other plant species.

From the intra- and intergenerational comparison across different gamma exposure conditions, it is clear that most changes occur in the CG methylation context (**Tables 1**, **2**). Only a limited number of CHG DMRs are present and no changes in CHH methylation were observed. The fact that IR seems to only affect CG methylation is an interesting discovery. Research has shown that in plants the environmental stress conditions can affect each methylation context differently

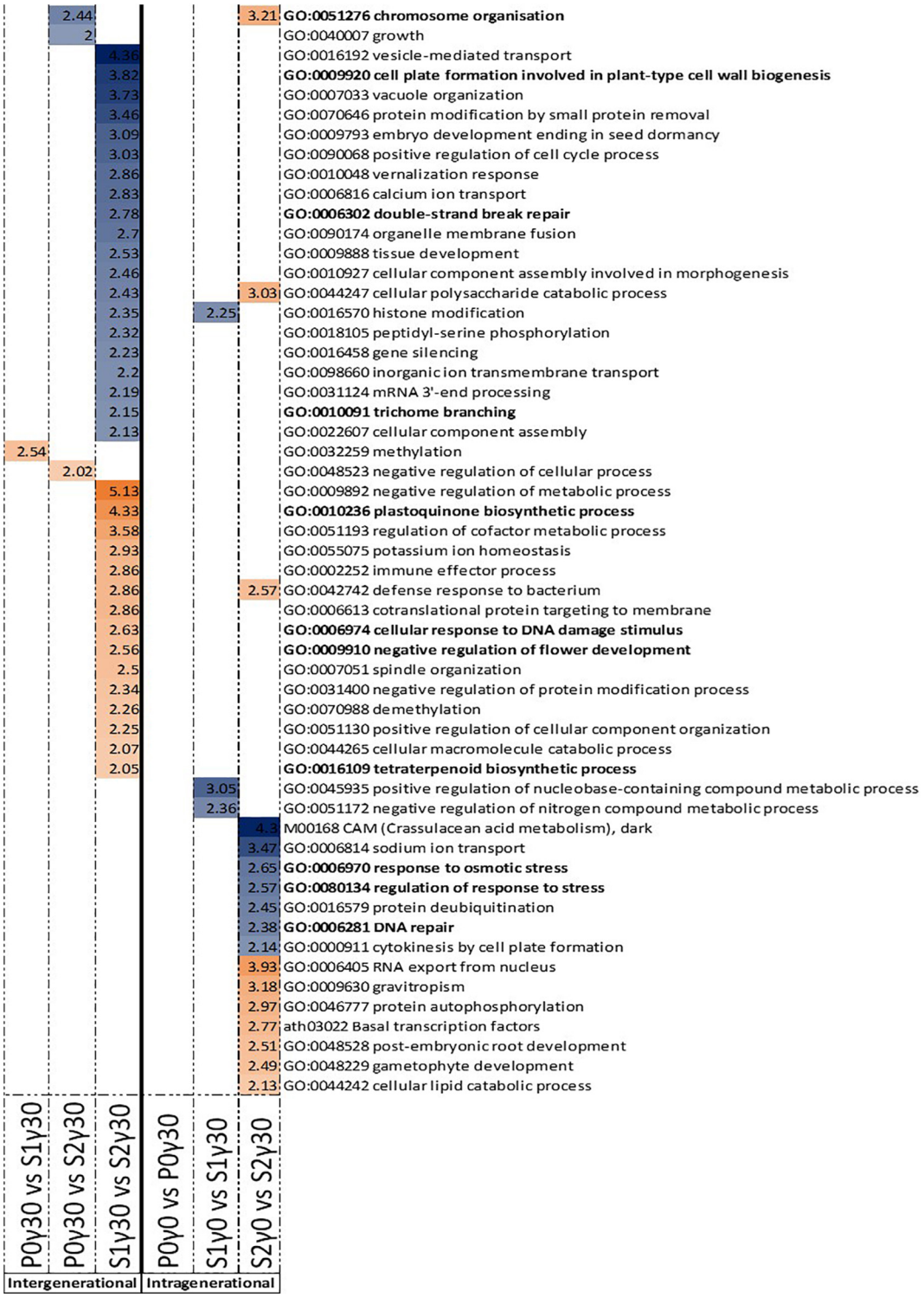

FIGURE 5 | Gene ontology (GO) term enrichment for hypo- and hypermethylated DMRs in the gene bodies of *Arabidopsis thaliana* for the intergenerational comparisons P0γ_{30} vs. S1γ_{30}, P0γ_{30} vs. S2γ_{30}, and S1γ_{30} vs. S2γ_{30} (on the left), and the intragenerational comparisons P0γ_0 vs. P0γ_{30}, S1γ_0 vs. S1γ_{30}, S2γ_0 vs. S2γ_{30} (on the right) {γ_{30} (30 mGy/h), γ_0 [control condition (<0.1 μGy/h)], P0 (Parent generation), S1 (generation 1), S2 (generation 2)}. S1 came from a previously exposed generation and S2 came from a line with two previously exposed generations. The –$\log_{10}$(P) value is shown and shaded according to its value per GO term, blue represents hypermethylation, whereas orange represents hypomethylation. GO terms highlighted in bold are those discussed in this paper.

(Niederhuth and Schmitz, 2017; Bartels et al., 2018). A study done on pathogen stress in *A. thaliana*, for instance, showed that upon infection CG and CHG levels were similar to the control group, whereas CHH methylation varied more among the samples, thereby showing CHH methylation to be more responsive to this biotic stress inducing agent (Dowen et al., 2012). Other studies showed differential DNA methylation contexts as a result of abiotic stress, e.g., differential DNA methylation in the CHH context as a result of cold stress in *Antirrhinum majus* (Hashida et al., 2006), or drought stress in *Solanum lycopersicum* (González et al., 2011), or in the CHG context as a result of salinity stress in *Mesembryanthenum crystallinum* (Dyachenko et al., 2006). This indicates that more research is needed to clarify the specific role of the cytosine methylation context in the response to stress. However, CG methylation has been shown to be very stable compared to the other methylation contexts and inheritance of CG methylation has been observed to play a key role in transferring epigenetic information to the following generations (Saze et al., 2003; Vaillant and Paszkowski, 2007). Hence, as mainly CG methylation was observed here, a potential inheritable epigenetic IR-stress response is occurring. The exact molecular mechanism behind this preference for CG methylation is yet to be studied, however, the METHYLTRANSFERASE1 (MET1) might play a role in this. MET1 is the CG methylation maintenance methyltransferase in *A. thaliana* and is also involved in *de novo* DNA methylation (Finnegan and Kovac, 2000; Gehring and Henikoff, 2008). The link with IR-stress response has already been made in a previous study where they saw an upregulation of MET1, as well as CMT3 (CHROMOMETHYLASE 3) and SUVH5 [SU(VAR)3-9 HOMOLOG 5] in *A. thaliana* plants exposed to IR (Sidler et al., 2015). In addition, there is a possibility that DNA glycosylase/AP lyase ROS1 plays a role in the active demethylation of different methylation contexts (Kim et al., 2019). However, as ROS1 does not only target CG methylation but also CHG and CHH contexts, be it at lower rates, it cannot be solely responsible for this CG methylation preference (Gong et al., 2002; Agius et al., 2006; Morales-Ruiz et al., 2006; Tang et al., 2016; Kim et al., 2019).

In general more hypermethylated than hypomethylated DMRs were observed in the current study. This corresponds with earlier reports where the offspring of stressed plants showed hypermethylation under salt stress, pathogens, and IR stress (Kovalchuk et al., 2003; Boyko et al., 2007; Bilichak et al., 2012; Volkova et al., 2018) and is consistent with the higher global methylation level as determined by UPLC-MS/MS. Zooming in on specific DNA regions, the ratio of hyper- vs. hypomethylated DMRs can, however, vary. For example, a substantial number of DMRs associated with TEs were found in the intergenerational comparisons after exposure to γ_{30} (30 mGy/h) and in the intragenerational comparison in the second generation (S2) between the control and γ_{30} conditions, with the majority of them being hypermethylated (**Table 3**). This hypermethylation will likely lead to transcriptional silencing and therefore limiting expression and mobilisation of TEs, resulting in less genomic reshuffling (Sigman and Slotkin, 2016). A hypermethylation in response to IR exposure, has been previously hypothesised to act as a protective measure to increase genome stability (Kovalchuk et al., 2004; Boyko et al., 2007; Horemans et al., 2018; Volkova et al., 2018).

As the comparisons with the highest dose rate (γ_{110}) yielded no GO term enrichments and as most significantly affected and stress related GO terms were found for the S2 generation, the focus of the following part of the discussion will lie on the comparison between the control (γ_0) and γ_{30} conditions and mostly on the second generation and γ_{30} condition, unless stated otherwise. A significant number of enriched GO terms were found that could all be linked to RNA splicing and DNA repair. It is to our knowledge the first time that a DNA methylation driven regulation of both RNA splicing and DNA damage repair mechanisms is reported in plants exposed to IR over multiple generations. Alternative RNA splicing is often used in regulating stress-related genes in order to adjust to the stressor, thereby giving the plant a dynamic tool to respond to changing environmental situations (Staiger, 2015; Calixto et al., 2018; Laloum et al., 2018; Huertas et al., 2019). Combining ribosome biogenesis, rRNA processing (P0γ_{30} vs. S2γ_{30}), and positive regulation of transcription of RNA polymerase II (S2γ_0 vs. S2γ_{30}) with the RNA splicing (P0γ_{30} vs. S2γ_{30} and S2γ_0 vs. S2γ_{30}), a potential stress (signalling) response is occurring over the three generations as well as intragenerationally between the control and γ_{30} condition. However, exactly how these mechanisms react to IR and if/how the hypo- or hypermethylation of the promoter regions affects them, needs to be studied in more detail.

A DNA repair response is regularly seen in IR-irradiated plants (Esnault et al., 2010; Gicquel et al., 2012; Dona et al., 2013; Georgieva et al., 2017). An enrichment for "DNA repair" was observed in the hypermethylated gene bodies of the second (S2) generation between the control group and the lowest dose rate. Additionally, "chromosome organisation" was found in both S2γ_0 vs. S2γ_{30} hypomethylated gene bodies and P0γ_{30} vs. S2γ_{30} hypermethylated gene bodies The latter process has previously been found to be part of the plant IR-stress response (Shirley et al., 1992; Shikazono et al., 2001), and is involved in chromatin maintenance and modifications as well as DNA repair (Kim, 2019). Further, the hypomethylated gene bodies' GO term enrichment between the first (S1) and second (S2) generation in the γ_{30} condition which contains the "cellular response to DNA damage stimulus" were found (**Figure 5**). Taken together these GO enrichments indicate DNA methylation is playing a regulating role in the DNA repair response. A few of the identified genes in our data (or homologues thereof) have been shown to be upregulated by IR in previous studies (e.g., *PARP-1*, *BRCA*) (Garcia et al., 2003; Culligan et al., 2006). In this study, a number of DNA repair and DNA damage response genes were tested (e.g., PARP1 and PARP2, data not shown). However, no direct correlation with DNA methylation levels were seen. Recently it was shown that gene associated DNA methylation resulted in a significantly delayed effect on actual gene expression (Atighi et al., 2020). In the current study we only harvested one sampling point per generation and therefore cannot corroborate this delayed effect on gene expression. Nonetheless, the fact that our data on differential DNA methylation do not directly link up with gene expression data from the harvest time point is in line with Atighi et al. (2020).

In addition to RNA splicing and DNA repair, a number of stress-related processes were found in the GO-enrichment analysis including "cell plate formation in plant-type cell wall biogenesis," "trichome branching," "plastoquinone biosynthetic process," "tetraterpenoid biosynthesis," and "negative regulation of flower development" (**Figure 5**). The fact that many DMRs are correlated with different genes and their process, including stress response, indicates that IR-induced DNA methylation is not random and indicates that regulation through changes in DNA methylation plays an important regulating role in the response of plants to IR, either by increasing genetic stability and/or regulating stress response gene expression.

Enrichment of GO-term "trichome branching" after exposure of multiple generations links IR-induced DNA methylation to the induction of trichome branching and is in agreement with a previous study that indicating the association of trichome density with epigenetic inheritance in plants (Scoville et al., 2011). Goh et al. (2014) showed that the number of trichomes increased dramatically in response to 200 Gy applied either chronically (1 week) or acutely (1 h). An enrichment between the S1 and S2 generation in the hypomethylated gene bodies was found for "plastoquinone biosynthetic process" and "tetraterpenoid biosynthesis" (**Figure 5**). The regulation of plastoquinone biosynthesis might protect plants from IR-damage to the photosynthetic apparatus which has been shown to be affected under IR (Gicquel et al., 2011; Vanhoudt et al., 2014). Induction of antioxidants and secondary metabolites including phenolic compounds, terpenoids and nitrogen-containing compounds have also been reported in this respect (Dixit et al., 2010; Popovic et al., 2013; Taheri et al., 2014; Vardhan and Shukla, 2017; Gudkov et al., 2019). In some organisms, including humans, carotenoids and lycopenes have shown a potential as radioprotectant (Islamian and Mehrali, 2015). The enrichment for terpenoid production can potentially also be linked to the aforementioned increased trichome accumulation as specific glandular trichomes have been shown to accumulate specific terpenoid molecules in response and adaptation to stress (Tang et al., 2020).

Intragenerationally, the "response to osmotic stress" was found in the second (S2) generation between the control and γ_{30} condition (**Figure 5**). Our findings correspond with the study of Rejili et al. (2008) that showed increased growth of IR-exposed *Medicago sativa* under high salinity. In addition, in *A. thaliana* plants irradiated with a gamma dose of 50 Gy, an improved tolerance to salinity, by regulating, amongst others, stress signal responses was reported (Qi et al., 2014). These studies indicate a form of priming to salinity stress by exposing the plants to IR. The more general "regulation of response to stress" includes a number of these above-mentioned osmotic stress response genes. Additionally, a significant number of the genes is associated with oxidative stress. This corresponds with the literature, in which an upregulation of certain oxidative stress response genes and antioxidant components in plants exposed to IR is observed (Van Hoeck et al., 2015; Einor et al., 2016; Volkova et al., 2017).

Lastly, an enrichment for the "negative regulation of flower development" is observed in the hypomethylated gene bodies between the first (S1) and second (S2) generation (**Figure 5**). The flowering response to IR is still under debate as studies have shown either an earlier or a later floral induction (Sax, 1955; Gunckel, 1957; Daly and Thompson, 1975; Kovalchuk et al., 2007; Hwang et al., 2016; Kryvokhyzha et al., 2018). The timing and regulation of flowering is important as it will affect the survivability of the next generation. Earlier flowering leads to quicker seed production and therefore secures the next generation. In some cases, seeds have been shown to be more stress resistant, however, under IR this is still controversial (Maity et al., 2009; Melki and Marouani, 2009; Moussa, 2011; Pozolotina et al., 2012). Alongside, premature flowering can also result in a reduced number and/or mass of the seeds (Huijser and Schmid, 2011). These studies' findings therefore add to the existing literature on flowering under IR stress and indicate for the first time a potential role of IR-induced DNA methylation in the regulation of this process.

CONCLUSION

In conclusion, our data are consistent with a potential regulating role for DNA methylation in the response of plants to IR in *Arabidopsis* plants exposed over multiple generations. The observed difference in response between γ_{30} and γ_{110}, however, also indicates that studies on the effects of low dose IR on plants, specifically chronic irradiation within and over generations, are needed for helping in environmental risk assessments. As a follow up we suggest a kinetic study to detect responses shifted in time as well as experiments in which the multigenerational set-up will be combined with a transgenerational one. By including irradiated generations stemming from non-irradiated parent generations and vice versa, the analysis would conclusively separate generational/inherited DNA methylation from IR-induced DNA methylation. Secondly, the molecular mechanism behind the DNA methylation and its preference for CG methylation as a result of IR stress should be studied, for instance, by including gene expression analysis of relevant methyltransferases.

DATA AVAILABILITY STATEMENT

The raw bisulfite sequencing data and the processed data sets generated in this study have been deposited in the Gene Expression Omnibus (GEO) under accession GSE157965.

AUTHOR CONTRIBUTIONS

ES, NH, AC, and JV conceptualised and designed the project. JV, MV, and RN performed the practical experimental work. SV and GJ performed the UPLC-MS/MS analysis. FV performed the bisulfite sequencing. MM performed the bioinformatics analysis. PL, ES, NH, and AC contributed to the data interpretation. PL did the data analysis and research on biological relevance, and drafted the manuscript. All authors contributed in the revision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.611783/full#supplementary-material

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Transcriptome Analysis of Tetraploid and Octoploid Common Reed (*Phragmites australis*)

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Polyploidization in plants is thought to have occurred as coping mechanism with environmental stresses. Polyploidization-driven adaptation is often achieved through interplay of gene networks involved in differentially expressed genes, which triggers the plant to evolve special phenotypic traits for survival. *Phragmites australis* is a cosmopolitan species with highly variable phenotypic traits and high adaptation capacity to various habitats. The species' ploidy level varies from 3x to 12x, thus it is an ideal organism to investigate the molecular evolution of polyploidy and gene regulation mediated by different numbers of chromosome copies. In this study, we used high-throughput RNAseq data as a tool, to analyze the gene expression profiles in tetraploid and octoploid *P. australis*. The estimated divergence time between tetraploid and octoploid *P. australis* was dated to the border between Pliocene and Pleistocene. This study identified 439 up- and 956 down-regulated transcripts in tetraploids compared to octoploids. Gene ontology and pathway analysis revealed that tetraploids tended to express genes responsible for reproduction and seed germination to complete the reproduction cycle early, and expressed genes related to defense against UV-B light and fungi, whereas octoploids expressed mainly genes related to thermotolerance. Most differentially expressed genes were enriched in chaperones, folding catalysts and protein processing in endoplasmic reticulum pathways. Multiple biased isoform usage of the same gene was detected in differentially expressed genes, and the ones upregulated in octoploids were related to reduced DNA methylation. Our study provides new insights into the role of polyploidization on environmental responses and potential stress tolerance in grass species.

Keywords: *Phragmites*, transcriptomics, polyploid, stress tolerance, evolution

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INTRODUCTION

Polyploidization is an important evolutionary force for shaping genetic diversity in eukaryotes (Stebbins, 1950). Polyploidizations can result in the emergence of new lineages within species, working as a driver of intraspecific diversification or even resulting in speciation. Chromosome doubling can promote novel phenotypic traits, and has therefore been proposed to greatly increase

species diversification (Crow and Wagner, 2005; Landis et al., 2018). About 70 percent of all angiosperms arose from chromosome doubling, among which nearly all Poaceae species originated from the same diploid ancestor (Masterson, 1994; Salse et al., 2008; del Pozo and Ramirez-Parra, 2015). Polyploidization, including allopolyploidization, autopolyploidization, and segmental polyploidization, is often seen among closely related plant species, and multiple polyploidization events can occur within the same species of certain genera, such as *Inga, Senna, Leucanthemum,* and *Dupontia* (Brysting et al., 2004; Figueiredo et al., 2014; Cordeiro and Felix, 2018; Wagner et al., 2019). Compared to diploids, polyploids usually have larger stomata and leaf area, increased pollen-grain size, and higher germinal pore numbers (Tamayo-Ordóñez et al., 2016; Liqin et al., 2019). These traits are considered to be advantageous in unfavorable environments, thus polyploids are often more tolerant to environmental stresses, such as drought, salinity, cold, heat, or nutrient deficiency (del Pozo and Ramirez-Parra, 2015). In addition, the evolution of polyploids is often coupled with asexual reproduction, such as apomixis (Schinkel et al., 2016; Hojsgaard and Hörandl, 2019), vegetative propagation and perennial growth, facilitating clonal spreading and increased survival rates under extreme conditions. Species featuring those traits can be highly adaptive to novel environments, and in some cases even become invasive (Te Beest et al., 2012).

Due to the high number of allelic copies, polyploids may develop unique gene expression systems to coordinate the function of multiple genomic copies, and balance the interaction between homeologs in allopolyploids (Yoo et al., 2013). Comparison of gene expression in allopolyploid and diploid *Populus* species revealed considerable differences between gene expression among different ploidy levels, resulting in overall superior phenotypic traits in polyploids. Differential expression of protein kinase genes, growth-regulating factors and hormone-related genes were largely responsible for the development of those phenotypic differences (Liqin et al., 2019). Those genes are also involved in stress-activated pathways and, hence, initiate adaptive responses to stress signaling in plant development (Golldack et al., 2014). Therefore, investigating genes expressed as a function of ploidy level is important to understand what advantages polyploidization has for plant evolution.

Phragmites australis is a cosmopolitan grass species with high intraspecific variability of ploidy levels, including 3x, 4x, 6x, 7x, 8x, 11x, 12x, x = 12 (Gorenflot, 1986). The most common seen cytology for *P. australis* in nature is tetraploid and octoploid. Tetraploids are distributed over most of the temperate region, and octoploids are found to occur mainly in South Africa, Romania, Greece and East Asia (Connor et al., 1998). *Phragmites australis* is able to tolerate extreme environmental conditions, and its suitable habitats include freshwater ponds, saline coastlines, dunes with severe aridity, and oligo- to polyhaline salt meadows (Wen-Ju et al., 1999; Song et al., 2020). Previous studies have proposed that different ploidy levels do not cause phenotypic changes (Achenbach et al., 2012) or higher tolerance to salinity (Achenbach et al., 2013). In contrast, it has been found that octoploid *P. australis* were less affected by salt stress than tetraploids (Paucă-Comănescu et al., 1999), while a recent finding showed the European lineage haplotype O (which is mainly tetraploid) was likely to be more tolerant to soil salinity than East Asian clades of haplotype P, which are more frequently octoploids (Lambertini et al., 2020; Liu et al., 2020). Ploidy has been emphasized as a key factor affecting the adaptation to new territories, for example allowing European tetraploid lineages to spread to Asian habitat (Lambertini et al., 2020), and enabling their invasion in North American environments (Pyšek et al., 2018). Despite those apparent advantages of a tetraploid genome, a large genome size may also be advantageous for certain traits of *P. australis* (Suda et al., 2015; Meyerson et al., 2016). Thus, octoploid *P. australis* have lower aphid colonization, bigger leaves, thicker shoots and taller, sturdier stems than tetraploids (Hanganu et al., 1999; Hansen et al., 2007; Lambertini et al., 2012; Meyerson et al., 2016; Eller et al., 2017). However, there is no systematic study to date investigating, how ploidy level affects gene expression of *P. australis*, which could demonstrate if underlying mechanisms determined by polyploidy control phenotypic traits.

In this study, we used transcriptomics on octoploid and tetraploid *Phragmites* individuals from a common garden to unravel potential intraspecific differences in gene expression profiles. Our aim was to understand how polyploidy affected the transcriptome in different *P. australis* genotypes grown in the same environment.

METHODS

Sampling

Leaf samples of six individuals were selected for transcriptome analysis, comprising three individuals of octoploids, and three individuals of tetraploids (**Table 1**). At least 10 healthy young leaves were collected from each individual from a common garden (Coordinates: 36.43°N, 117.43°E) at Shandong University in July, 2020. The leaves were immediately submerged into RNA-sample-preservation solution (Coolaber, Beijing, China), which keeps the RNA intact and protected from degradation. The leaf samples were then stored at 4°C in a fridge overnight, and sent to Shanghai Honsun bio Company[1] for RNA extraction and next generation sequencing. Total RNA isolated from each replicate was sequenced using the Illumina Hiseq Xten platform. The ploidy level of each plant was confirmed by flow cytometry, following the protocol in Meyerson et al. (2016). The resulting sequences were deposited in the NCBI Sequence Read Archive (SRA) database with the following identifiers: BioProject PRJNA687616.

Genome Assembly and Annotation

To facilitate the genomic mapping of transcriptomic reads, we assembled and annotated the genome of *P. australis* using next generation sequencing (NGS) reads produced by BGISEQ-500 sequencer. Whole genome sequences were obtained from NCBI SRA database (Accession: SRX4043155) (Liu et al., 2019).

[1]http://www.honsunbio.com/

TABLE 1 | Sample information of the RNA-seq data used in this study.

Species name	Sample name	Mapping rate	Number of reads (million)	Ploidy level	Origin	Coodinates	Code used in other studies
Phragmites australis	S136-1	83.63%	67.87	8	Australia	34°56′00.0″S 138°36′00.0″E	FEAU136
Phragmites australis	S150-1	84.54%	59.15	8	Australia	34°28′00.0″S 146°01′00.0″E	FEAU150
Phragmites australis	S162-1	83.98%	72.28	8	Australia	36°09′00.0″S 147°00′00.0″E	FEAU162
Phragmites australis	S191-1	84.43%	62.84	4	United States	43°16′35.0″N 77°16′40.0″W	NAint191
Phragmites australis	S207-1	84.37%	66.80	4	Italy	45°41′00.0″N 9°46′00.0″E	EU207IT
Phragmites australis	S620-1	80.09%	62.86	4	Czech Republic	48°39′00.0″N 14°22′00.0″E	EU620

The genome was assembled with MaSuRCA 3.3.3 assembler using default settings (Zimin et al., 2013). The draft genome assembly was curated with Purge Haplotigs v1.0.4 to remove wrongly assembled contigs which are heterozygous to the real reference (Roach et al., 2018). Gene prediction was performed by both homology based and *ab initio* methods. Genome assembly and annotation with *Miscanthus sinensis* were obtained from Phytozome[2] to serve as a reference for homology prediction using GeMoMa v1.6.4 (Keilwagen et al., 2019). *Ab initio* gene prediction was performed using GeneMark-ES v4.64 (Lomsadze et al., 2005), BRAKER2 v2.1.5 (Brůna et al., 2021) and PASA v2.4.1 (Haas et al., 2011). RNAseq data of one octoploid individual was aligned to the draft assembly by STAR aligner v2.7.6a (Dobin et al., 2013), and used as evidence to define intron borders in BRAKER2 prediction. *De novo* assembly of RNAseq data was performed using TRINITY v2.12.0 (Grabherr et al., 2011) and used in PASA to get a high quality dataset for *ab initio* gene predictions. We integrated all evidence of gene prediction in EvidenceModeler v1.1.1 (Haas et al., 2008) to get the consensus gene structure.

Transcriptome Assemblies

To date the divergence time between the octoploid and tetraploid, we included data of *Zea mays*, *Arundo donax*, and *Phragmites karka* to provide calibration points. RNA-seq from leaf tissue of four individuals of *P. karka* (Accession Nos.: SRR9670021, SRR9670022, SRR9670025, and SRR9670026) and one individual of *A. donax* (Accession No.: SRR8083515) were obtained from NCBI biosample database from previous studies (Evangelistella et al., 2017; Nayak et al., 2020). Transcriptome assembly of *Z. mays* was downloaded from Transcriptome Shotgun Assemblies in NCBI (**Table 1**). Transcripts of *P. australis* octoploids and tetraploids were assembled using TRINITY v2.12.0 (Grabherr et al., 2011) separately in genome-guided *de novo* mode, and RNA-seq data of *P. karka* and *A. donax* were assembled in *de novo* mode using TRINITY v2.12.0. The Open Reading Frame (ORF) of each transcriptome assembly was predicted using TransDecoder v5.5.0 (Haas et al., 2013), and the recognized protein coding sequences were used to infer orthogroups and orthologs in OrthoFinder v2.4.0 (Emms and Kelly, 2019). Both orthologs and paralogs are homologs among species, and they differ in the way that orthologs were directly descendent from the most recent common ancestor and are results of speciation, whereas paralogs within species were created from duplication of the orthologs and are often results of Whole Genome Duplication (WGD). By integrating several programs in the pipeline, OrthoFinder first inferred a rooted species tree based on the clustering the gene trees of input amino acid sequences, and then inferred orthogroups among species.

Divergence Time Between Ploidy Levels

Compared to paralogues, orthologues are the genomic regions that are directly transmitted from the most common ancestor without genomic duplications and reallocation, thus orthologues reflect the true phylogeny. We aligned 98 single copy orthologue sequences in all species using MAFFT v7.429 (Katoh et al., 2002), and calculated the divergence time of each node using BEAST2 v2.6.1 (Bouckaert et al., 2014) with a strict clock model and a Blosum62 + G (four rate categories) site model. Previous studies estimated the Most Recent Common Ancestor (MRCA) of the PACMAD clade including *Z. mays* and *P. australis* to be at 44 Million Years Ago (MYA) (Vicentini et al., 2008), so we set the parameter of TMRCA as log normal distribution, with the Mean in Real Space checked, an offset of 40.0 MY, a mean of 6.0 MY and a standard deviation of 0.5 MY. The chain length of the Markov Chain Monte Carlo was set to ten million, with sampling every 5,000 states. Tracer v1.7.1 was used to estimate the convergence of the run, and a convergence state was considered to be reached if the effective sample size (ESS) of all parameters was at least 200.

Read Mapping

RNA sequencing produced between 59.15 and 72.28 million 2 × 150b pair-end reads for each sample in this study. RNA-seq reads of each sample were cleaned and mapped on to the genome assembly to obtain the read count of each gene. Quality of the RNA-seq reads was checked with FastQC v0.11.8 (Andrew, 2010). Only reads with Phred score higher than 30 were kept, and overrepresented sequences were removed from the library using cutadapt 2.7 (Martin, 2011). The clean reads were aligned to *P. australis* draft genome using STAR aligner 2.7.1a two pass procedure (Dobin et al., 2013), and the bam files were sorted with samtools 1.10 (Li et al., 2009). Transcriptome abundance estimates were performed with StringTie v2.1.4 (Pertea et al., 2015). All transcripts were then merged and assembled to a consensus transcript set. We aligned RNAseq data of each sample to the merged transcript using command (stringtie -e -B). The

[2]https://phytozome.jgi.doe.gov/pz/portal.html, accessed at 2020 November.

TABLE 2 | Gene annotation inferred by Mercator4.

Top level bins classifying biological process	Number of leaf bins	*P. australis* occupied leaf bins	*P. australis* number of genes	Percent of the total genes (%)	Upregulate (gene number)	Downregulate (gene number)
1 Photosynthesis	230	172	404	0.285	0	0
2 Cellular respiration	130	107	303	0.214	0	0
3 Carbohydrate metabolism	110	106	376	0.265	0	0
4 Amino acid metabolism	134	127	336	0.237	0	0
5 Lipid metabolism	191	178	732	0.517	0	0
6 Nucleotide metabolism	58	58	149	0.105	0	0
7 Coenzyme metabolism	161	154	325	0.229	0	0
8 Polyamine metabolism	15	13	37	0.026	0	1
9 Secondary metabolism	100	67	202	0.143	0	0
10 Redox homeostasis	48	46	192	0.136	0	0
11 Phytohormone action	147	133	842	0.594	1	1
12 Chromatin organization	142	133	471	0.332	0	0
13 Cell cycle organization	274	264	710	0.501	1	2
14 DNA damage response	82	81	131	0.092	0	0
15 RNA biosynthesis	285	273	3,859	2.724	3	4
16 RNA processing	358	329	844	0.596	0	1
17 Protein biosynthesis	396	358	972	0.686	0	0
18 Protein modification	291	286	2,015	1.422	1	2
19 Protein homeostasis	289	283	1,665	1.175	0	3
20 Cytoskeleton	118	110	494	0.349	0	1
21 Cell wall	135	123	848	0.599	2	0
22 Vesicle trafficking	192	195	736	0.519	0	0
23 Protein translocation	141	135	325	0.229	0	0
24 Solute transport	174	171	1,860	1.313	0	3
25 Nutrient uptake	56	47	222	0.157	0	0
26 External stimuli response	116	101	357	0.252	1	0
27 Multi-process regulation	74	72	417	0.294	0	0
50 Enzyme classification	50	39	2,108	1.488	4	8

resulting coverage data were later transformed to gene count matrix by stringtie script prepDE.py.

Differential Gene Expression Across Ploidy Levels

To find out the genes that are differentially expressed between groups, rather than within group, we analyzed the read counts of each gene using R package DEseq2 v1.30.1 (Love et al., 2014) in the R environment v3.6.1. After internal normalization, DEseq2 calculate size factor for each gene in each sample to correct for library size, and uses shrinkage estimation to estimate dispersions and fold changes among biological replicates, and then fits negative binomial generalized linear models for each gene and uses the Wald test for significance testing (Love et al., 2014). Genes showing absolute values of a $\log_2$ fold-change (LFC) higher than 2 were considered as differentially expressed gene (DEG). The adjusted *P*-value was adopted to control for the false discovery rate due to multiple testing using the Benjamini and Hochberg methods in DEseq2, and a *P*-value lower than 0.001 was regarded to be statistically significant. The top 500 genes with highest row variance were selected to perform a Principal Component Analysis (PCA) to assess the effects of external variation on gene expression.

Functional Annotation of the Genome and Novel Genes

To characterize the molecular functions of the DEGs, we first blasted the genome against available protein databases to get functional annotation of each gene, and then searched the DEGs against the genome to subtract the corresponding functions. Protein function of the annotated genome was estimated through Mercator4 V2.0 (Schwacke et al., 2019). Transcription factors were predicted from the online tool plantTFDB v5.0 (Jin et al., 2016). Amino acid sequences of novel transcripts produced by StringTie were extracted using IsoformSwitchAnalyzeR v1.12.0 (Vitting-Seerup and Sandelin, 2019), and searched against pfam-A protein database using Pfamscan to obtain the domain information (Mistry et al., 2007). The novel transcripts sequences were also annotated from eggNOG-mapper v2 to get a more complete information of the genes (Huerta-Cepas et al., 2017). Annotation information including GOslim and gene association files of *Arapdopsis*

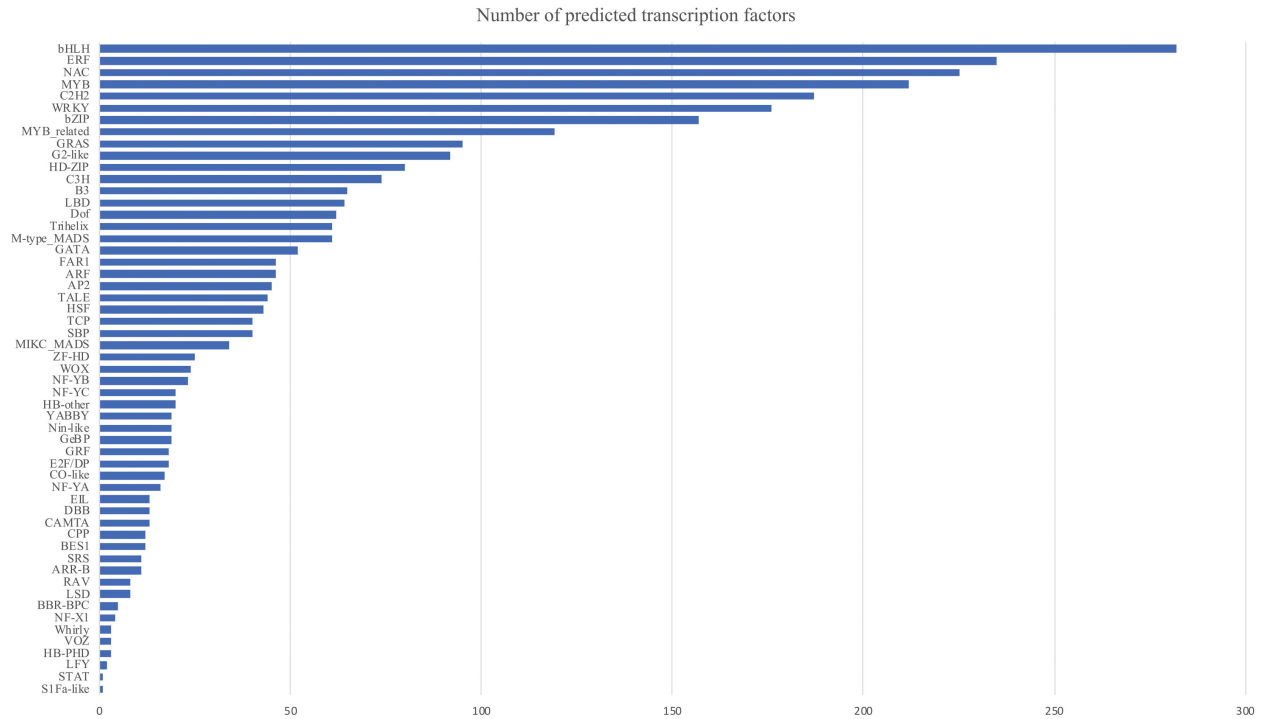

FIGURE 1 | Number of predicted transcription factors in *Phragmites australis* genome. The transcription factors were obtained by searching the annotated reference genome against the Plant TFDB V5.0. Genes annotated to the same transcription factor families were counted as one class. Details of transcription factor names can be retrieved from http://planttfdb.gao-lab.org/.

thaliana was downloaded from The Arabidopsis Information Resource[3]. The reference genome was first aligned to *A. thaliana* using BLASTp algorithms (*e*-value $< 10^{-5}$), and then to map the genes to *A. thaliana* to obtain the gene ontology (GO) terms clustered based on biological process, cellular component or molecular function. GO term enrichment analysis was conducted with GOAtools v1.0.15 using Bonferroni correction with a cut-off threshold of $P < 0.01$ (Klopfenstein et al., 2018). KEGG analysis was performed through KAAS server and gene enrichment was done in R package "clusterProfiler" v 3.18.1 (Yu et al., 2012).

Alternative Splicing

To detect whether alternative splicing has played a role in gene regulation of different ploidy levels, we performed a test on the transcriptomes using IsoformSwitchAnalyzeR (Vitting-Seerup and Sandelin, 2019). Isoform switches were predicted using DEXSeq v1.36.0 (Anders et al., 2012), with parameter set to alpha = 0.05, dIFcutoff = 0.1, in which case isoforms were only considered to be switching when there was more than 10% of the changed isoforms. Genome wide alternate splicing and potential functional consequences of the identified isoform switches between the tetraploid and octoploid sets, especially the isoforms in differentially expressed genes were predicted.

[3]https://www.arabidopsis.org, accessed at Aug 20th, 2020.

RESULTS

Genome Assembly and Functional Annotation

The genome size of *P. australis* was about 912.58 Mb, with heterozygosity of 1.31%. The N50 contig length of the new assembly was 36,770 bp. In total, 141,683 genes were annotated in the draft genome. The annotated genome was classified into 28 functional categories by Mercator4, with genes distributed in 67–100% of Mercater4 leaf bins. Among all genes in the draft genomes, 15.48% were annotated with Mercator4, and the number of genes in each top bin varied from 0.03 to 2.72% (**Table 2**). A total of 2,998 transcription factors (TFs) were predicted, specifying 55 types, and the most identified TFs (>100 genes) included bHLH (282 genes), ERF (235 genes), NAC (225 genes), MYB (212 genes), C2H2 (187 genes), WRKY (176 genes), bZIP (157 genes), and MYB related (119 genes) (**Figure 1**).

Map Efficiency of RNAseq Data and Differential Gene Expression

All samples have high percentage (>80.09%) of RNAseq data mapped on the genome draft assembly (**Table 1**). PCA showed that the first two components explain 69% of the variance, of which most of the variation (56%) was explained by PC1, which separated the samples into tetraploid and octoploid groups (**Figure 2A**). Of the 49,024 genes expressed in both octoploids

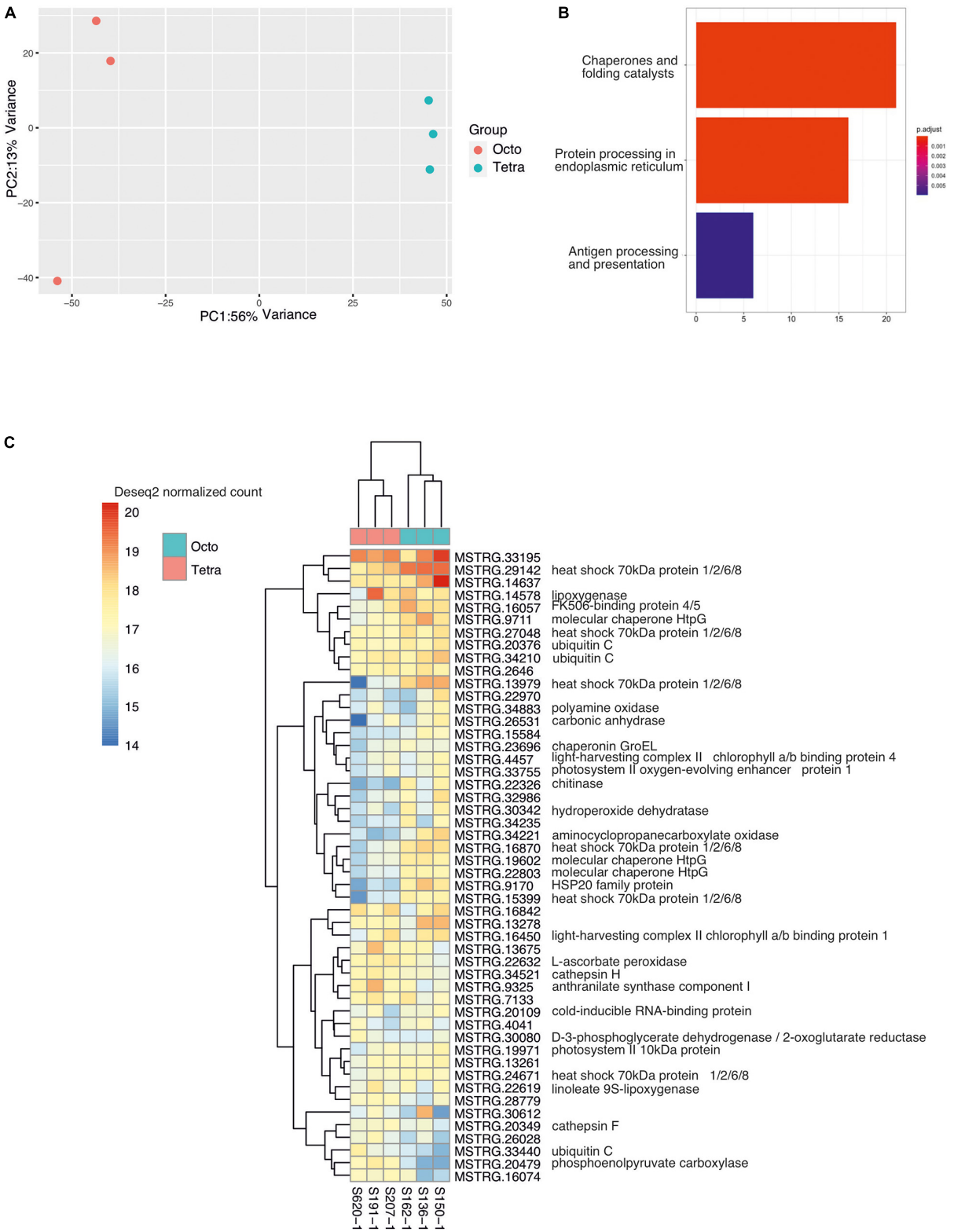

FIGURE 2 | Data visualization and differential gene expression of the transcripts between octoploid and tetraploid *Phragmites australis*. **(A)** Principal Component Analysis (PCA) of the transcript count transformed with rlog function from all samples. **(B)** Enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of the genes that are upregulated in octoploids. The horizontal axis indicates number of genes. **(C)** Hierarchical clustering of genes with the highest mean of normalized counts across all samples. Abbreviated gene names are followed by a functional annotation of that gene.

TABLE 3 | Transcription factors identified in differentially expressed genes.

Upregulated		Downregulated	
Transcript ID	**Transcription factor**	**Transcript ID**	**Transcription factor**
MSTRG.20270.1	Nin-like	MSTRG.1104.2	bHLH
MSTRG.20270.2	Nin-like	MSTRG.15405.1	SBP
MSTRG.4654.1	NAC	MSTRG.15405.3	SBP
MSTRG.25162.1	B3	MSTRG.15405.5	SBP
MSTRG.5559.1	ERF	MSTRG.15405.6	SBP
MSTRG.5559.2	ERF	MSTRG.15405.7	SBP
MSTRG.5559.3	ERF	MSTRG.15405.8	SBP
EVMevm.TU.jcf7180004141171.8	ARR-B	MSTRG.15405.9	SBP
EVMevm.TU.jcf7180004129794.10	HY5	MSTRG.1548.1	bZIP
evm.model.jcf7180004129794.10	bZIP	MSTRG.1548.2	bZIP
		MSTRG.1548.3	bZIP
		MSTRG.24846.1	bZIP
		MSTRG.24846.2	bZIP
		MSTRG.24846.3	bZIP
		MSTRG.27569.1	bZIP
		MSTRG.27569.2	bZIP
		MSTRG.27569.3	bZIP
		MSTRG.27569.4	bZIP
		MSTRG.27569.5	bZIP
		MSTRG.24613.1	ERF
		MSTRG.24613.2	ERF
		MSTRG.22481.1	HB-other
		MSTRG.22481.4	HB-other
		MSTRG.22481.5	HB-other
		MSTRG.22330.1	MYB_related
		MSTRG.22330.3	MYB_related
		EVMevm.TU.jcf7180004099680.9	BBX-DBB
		EVMevm.TU.jcf7180004112813.1	GATA
		EVMevm.TU.jcf7180004088796.12	GARP
		EVMevm.TU.jcf7180004037963.3	WRKY

and tetraploids, the expression level of 1,395 transcripts were significantly different between the two ploidy levels (Wald test, $P < 0.01$). There were 439 transcripts upregulated and 956 transcripts downregulated in tetraploids compared to octoploids (Wald test, $P < 0.01$). Altogether, protein domains of 427 out of the 439 (97.27%) upregulated transcripts and 879 out of 956 (91.95%) downregulated transcripts were annotated from the pfam-A database.

Function Enrichment of DEG

Using Mercator4 annotation, the upregulated genes were identified to be related to RNA biosynthesis, cell wall, phytohormone action, cell cycle organization, protein modification, and external stimuli response (**Table 2**). The downregulated genes were found to be involved in the biological processes including RNA biosynthesis, protein homeostasis, solute transport, cell cycle organization, protein modification, polyamine metabolism, phytohormone action, RNA processing, cytoskeleton (**Table 2**). KEGG pathway enrichment suggested downregulated genes were significantly related to "Chaperones and folding catalysts," "Protein processing in endoplasmic reticulum," and "Antigen processing and presentation" pathways (**Figure 2B**). We did not find KEGG enrichment with the upregulated genes. GO enrichment analysis assigned 174 GO terms to the upregulated genes, among which we found 24 cell components (CC), 61 molecular functions (MF), and 89 biological processes (BP) (**Supplementary Table 1**). Biological processes were mainly metabolic processes (26 GO terms), responses to stimuli (5 GO terms), responses to stress (4 GO terms), but also involved in development, reproduction and seed germination. We assigned 211 GO terms to the downregulated genes, including 28 cell components, 63 molecular function, and 120 biological process (**Supplementary Table 2**). Downregulated genes were mostly enriched in biological processes such as metabolic processes (26 GO terms), protein folding and responses to unfolded or incorrectly folded proteins (9 GO terms), responses to stimuli (9 GO terms), responses to stress (9 GO terms), telomere maintenance (4 GO terms), and response to heat stress (3 GO terms). Genes with the highest mean of normalized counts across all samples showed the most abundant expressed genes are heat shock proteins (HSP70, HSP20), chaperone (HtpG), and ubiquitin C (**Figure 2C**). Seven types of

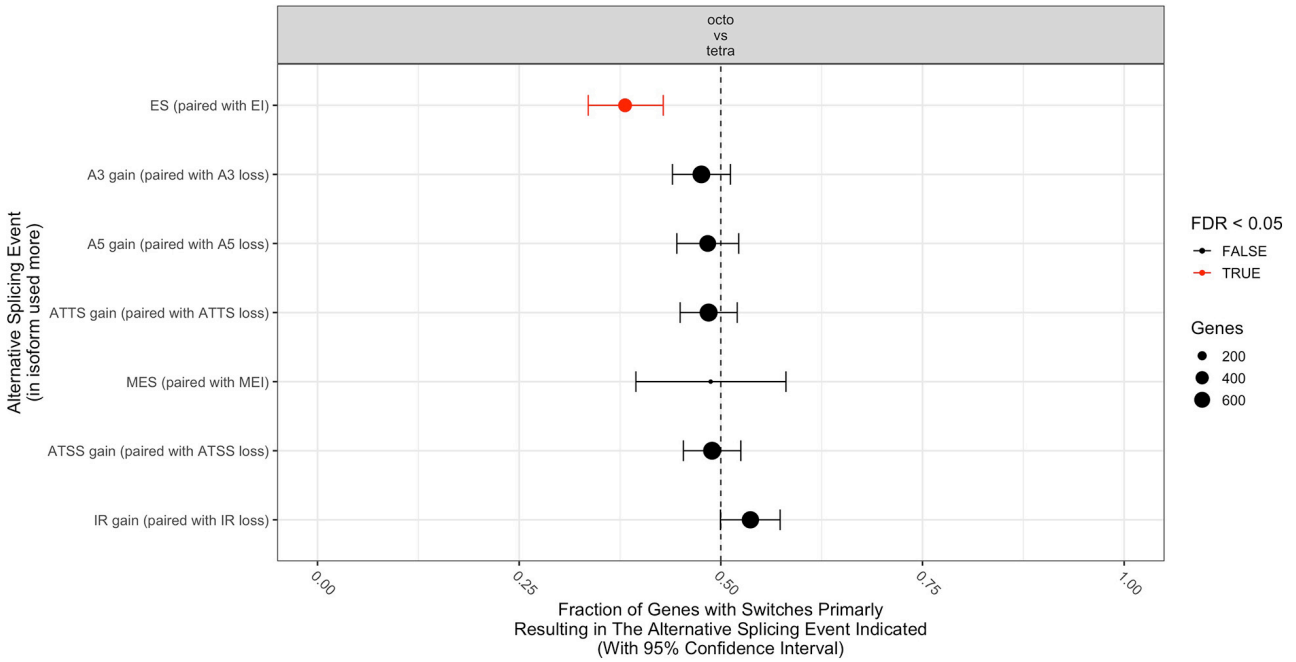

FIGURE 3 | Compared consequences of alternative splicing event between ploidy levels (octo- and tetraploid) in *Phragmites australis*, inferred from biased isoforms in each group. Fraction of genes with switches primarily resulting in the alternative splicing event were indicated with 95% Confidence Interval. Data labeled with red indicated the significant trend, with False Discovery Rate < 0.05. Significant isoform usage was indicated with an asterisk. *p-value < 0.05, ***p-value < 0.001, ns, no significant difference. Compare to octoploids, tetraploids showed higher percentage of genes (about 72%) with consequences of Exon Inclusion (EI), and only around 38% of the genes showed consequences of Exon Skipping (ES).

transcription factors were found in upregulated genes, including Nin-like (2), NAC (1), B3 (1), ERF (3), HY5 (1), bZIP (1) and ARR-B (1). Ten types of transcription factors were identified in downregulated genes, including bHLH (1), SBP (7), bZIP (11), ERF (2), HB-other (3), MYB-related (2), BBX-DBB (1), GATA (1), GARP (1) and WRKY (1) (**Table 3**).

Alternative Splicing

In total, 1,596 genes showed at least one isoform. Among these genes, 2,554 isoforms and 2,417 switches were identified. With the dIF cutoff threshold set as 0.1, we analyzed the consequences of 1,282 genes that had 2,049 isoforms, with 2,024 switches. Compared to octoploids, tetraploids showed significant biased usage of Exon Inclusion (EI) than Exon Skipping (ES) (**Figure 3**). Octoploids expressed a slightly higher level of ES than tetraploids (**Supplementary Figure 1**). Among the genes that were upregulated in tetraploids relative to octoploids, 19 genes showed a significant biased use of isoforms ($p < 0.05$), and among the genes that were downregulated in tetraploids, 31 genes were found to code for significantly biased isoforms (**Table 4** and **Figure 4**). Premature termination codons (PTCs) were frequently found in repeat regions, such as gene "MSTRG.3765" (10 isoforms) and "MSTRG.6510" (5 isoforms) in family PRR and WD40. Most of the biased usage of isoforms were Nonsense Mediated RNA Decay (NMD) insensitive, but a few isoforms were NMD sensitive (**Table 4** and **Figure 4**).

Divergence Time Between Octoploids and Tetraploids

Transcriptome assembly of octoploids contained 180,584 transcripts, with the length of contig N50 being 2,011 bp, and the assembled transcriptome of tetraploids consisted of 167,514 transcripts with the length of contig N50 being 2,046 bp. Orthologue search identified 98 single copy orthologous sequences among the five Poaceae species and dated the divergence time of tetraploid and octoploid lineage of *P. australis* to be 3.26 (95% Highest Posterior Density 2.81–3.69) Mya (**Figure 5**). Congener species *P. karka* clustered with *Zea mays*, outside of Arundineae, and diverged from Arundineae at 45.36 (95% Highest Posterior Density 41.45–50.75) Mya. *Arundo donax* diverged from *P. australis* at 27.41 (95% Highest Posterior Density 24.63–30.47) Mya. The molecular clock rate was estimated to be 2.17×10^{-9} substitutions/year.

DISCUSSION

Genome and Differentially Expressed Genes in *Phragmites*

The vast difference between the genomes of higher ploidy levels and lower ploidy levels in plants, has resulted in large gene expression bias, affecting pathways involved in flowering regulation (Braynen et al., 2021) and photosynthetic rate (Ilut et al., 2012). In this study, 1,395 transcripts were found to

TABLE 4 | Biased isoform switches in differentially expressed genes.

Condition 1	Condition 2	Upregulated			Downregulated		
		Isoform ID	Domain changed	NMD sensitivity	Isoform ID	Domain changed	NMD sensitivity
Octoploid	Tetraploid	MSTRG.31526.1, MSTRG.31526.2	DIOX_N	Insensitive	MSTRG.3253.2		Sensitive (Tetraploid)
Octoploid	Tetraploid	MSTRG.25276.2	PP2C	Sensitive (Tetraploid)	MSTRG.22620.5	HEAT (x2),HEAT_2,Importin_rep_4, Importin_rep_6	Insensitive
Octoploid	Tetraploid	MSTRG.32418.3, MSTRG.32418.4		Insensitive	evm.model.jcf718000 4089062.4		Insensitive
Octoploid	Tetraploid	MSTRG.16941.1		Insensitive	evm.model.jcf718000 4128298.6	PP2C	Insensitive
Octoploid	Tetraploid	evm.model.jcf718000 4134190.2, MSTRG.27161.1		Insensitive	evm.model.jcf718000 4108190.4	HATPase_c and HisKA decrease, Exo70 increase	Insensitive
Octoploid	Tetraploid	MSTRG.10338.3	Pribosyltran, POB3_N	Insensitive	MSTRG.33016.1	AMP-binding, AMP-binding_C increase	Sensitive (Octoploid)
Octoploid	Tetraploid	MSTRG.23807.1	Biotin_lipoyl, ACC_central (x2)	Insensitive	evm.model.jcf718000 4128736.11	DUF2048 (x2)	Insensitive
Octoploid	Tetraploid	evm.model.jcf718000 4094996.3	EF-hand_8 (x2)	Insensitive	MSTRG.4291.3		Insensitive
Octoploid	Tetraploid	evm.model.jcf718000 4127787.2	Pollen_Ole_e_1	Insensitive	evm.model.jcf718000 4098404.2, MSTRG.10410.1	Methyltransf_2 (one more domain in Tetraploid)	Insensitive
Octoploid	Tetraploid	MSTRG.28064.1, MSTRG.28064.4		Insensitive, Sensitive (MSTRG.28064.4, Octoploid)	evm.model.jcf718000 4084359.1, MSTRG.5499.2	PALP	Insensitive
Octoploid	Tetraploid	MSTRG.3765.7, MSTRG.3765.9	WD40	Sensitive (Tetraploid)	MSTRG.23823.1, evm.model.jcf718000 4130025.3	Pkinase, PK_Tyr_Ser-Thr	Insensitive
Octoploid	Tetraploid	MSTRG.6510.3	PPR (x9),PPR_2 (x4),PPR_3 (x2)	Sensitive (Octoploid)	evm.model.jcf718000 4116421.7, MSTRG.16368.3, MSTRG.16368.4		Insensitive, Sensitive (MSTRG.16368.4, Octoploid)
Octoploid	Tetraploid	MSTRG.34402.4		Sensitive (Octoploid)	MSTRG.16535.2, MSTRG.16535.3		Insensitive, Sensitive (MSTRG.16535.3, Octoploid)
Octoploid	Tetraploid	MSTRG.19828.2		Sensitive (Tetraploid)	MSTRG.29434.1, MSTRG.29434.3	zinc_ribbon_12	Insensitive
Octoploid	Tetraploid	MSTRG.15308.4	RRM_1 (x2)	Insensitive	MSTRG.5773.1	Stress-antifung	Insensitive

(Continued)

TABLE 4 | Continued

Condition 1	Condition 2	Upregulated			Downregulated		
		Isoform ID	Domain changed	NMD sensitivity	Isoform ID	Domain changed	NMD sensitivity
Octoploid	Tetraploid	MSTRG.31576.1		Sensitive (Octoploid)	evm.model.jcf718000 4139394.1, MSTRG.31634.1	Glycoside hydrolase family	Insensitive
Octoploid	Tetraploid	MSTRG.28737.2	DUF1644	Insensitive	evm.model.jcf718000 4083040.4,MSTRG.4813.2		Insensitive
Octoploid	Tetraploid	MSTRG.26492.1	Lactamase_B,Fer4_13	Insensitive	evm.model.jcf718000 4097656.2, MSTRG.9836.3	AAA_21,ABC_tran	Insensitive
Octoploid	Tetraploid	MSTRG.26732.2	zf-MYND	Insensitive	evm.model.jcf718000 4107336.3		Insensitive
					MSTRG.26324.6,MSTRG.26324.9		Sensitive (Octoploid)
					evm.model.jcf7180004138884.2, MSTRG.31121.2	PMD	Insensitive, Sensitive (MSTRG.31121.2, Octoploid)
					evm.model.jcf718000 4135647.3		Insensitive
					evm.model.jcf718000 4143412.7,MSTRG.34600.3	SMP (x3),SMP (x2)	Insensitive
					evm.model.jcf718000 4090036.1,MSTRG.7576.2, MSTRG.7576.3,MSTRG.7576.4	Sec23_BS,Sec23_helical, Sec23_trunk,zf-Sec23_Sec24	Insensitive, Sensitive (MSTRG.7576.3, MSTRG.7576.4, Octoploid)
					MSTRG.2931.1,MSTRG.2931.2		Sensitive (Tetraploid, Octoploid)
					evm.model.jcf718000 4127970.4,MSTRG.21888.2	4F5	Insensitive
					evm.model.jcf7180004098448.2, MSTRG.10464.2		Insensitive
					MSTRG.13123.3		Insensitive
					MSTRG.33255.1,MSTRG.33255.2	Retrotran_gag_2	Insensitive
					MSTRG.32200.1		Sensitive (Tetraploid)
					evm.model.jcf7180004127425.1, MSTRG.21346.2	PHD	Insensitive

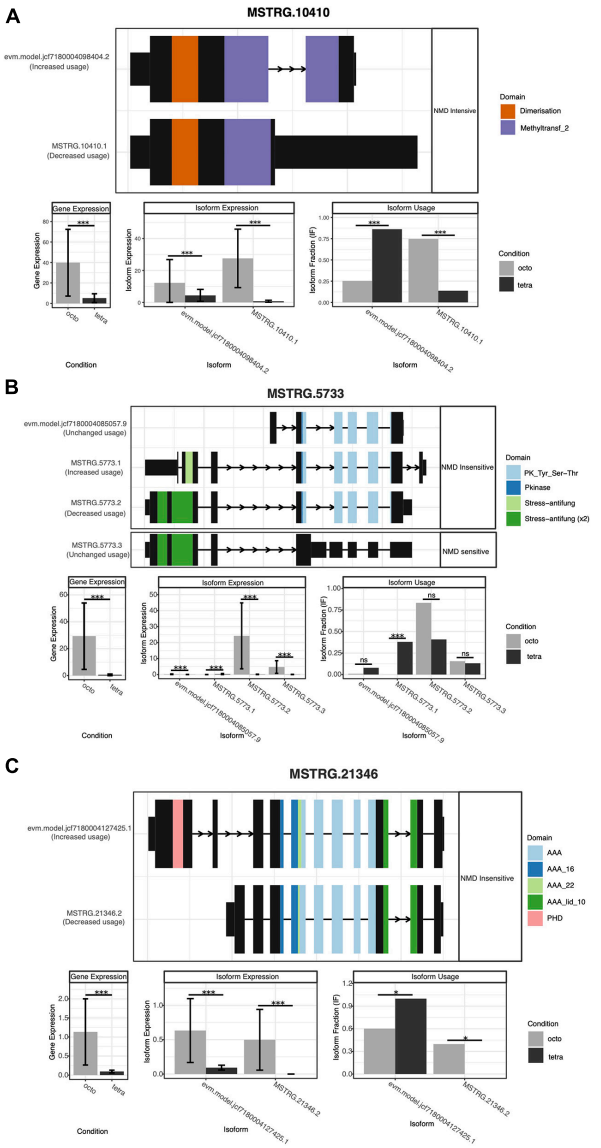

FIGURE 4 | Structural and expression analysis of gene MSTRG.10410 **(A)**, MSTRG.5733 **(B)** and MSTRG.21346 **(C)** for which with alternative splicing events has biological consequences. Isoforms insensitive and sensitive to Nonsense Mediated RNA Decay (NMD). *p-value < 0.05, ***p-value < 0.001, ns, no significant difference. For each gene, the top graph displays gene structure of the isoform, with domains annotated from Pfam database indicated. The bar graph on the left showed the differential gene expression, and the bar graph in the middle indicated the differential isoform expression, and the bar graph on the right represented isoform usage bias between octoploids and tetraploids.

be differentially expressed between *P. australis* tetraploids and octoploids, and the DEGs were classified into several functional categories which are related to reproduction and resistance to abiotic stresses. DEGs in octoploids were functioning in several pathways, including solute transport (three genes, coding for ABCC transporter, metal cation transporter, or ligand-gated cation channel), protein homeostasis (three genes, coding for cysteine-type peptidase C1 and A1 class of Papain), cytoskeleton organization, RNA processing and polyamine metabolism. Papain-like cysteine proteases are vital enzymes to numerous plant physiological activities, which also function in salt-, cold-, and drought-stress response, as evidenced in model plants such as *Arabidopsis*, wheat, sweet potato, and barley (Liqin et al., 2019). Gene ontology analysis revealed differentially expressed genes upregulated in octoploids enriched in several biological processes, mostly involved in metabolic process, and in error correction mechanisms to environmental stress, such as responses to unfold or incorrectly folded proteins and telomere maintenance. Up to 21 GO terms of these genes were assigned to responses to stress or stimuli, suggesting octoploid *P. australis* has developed many novel functions to cope with the challenging environment. Heat stress may induce abrupt and dramatic loss of telomere DNA repeats (Lee et al., 2016), and genes related to telomere maintenance were upregulated in octoploid *P. australis* to avoid damage to the plant. This is also seen in *Arabidopsis*, where a heat-shock induced molecular chaperone auxiliary maintains the integrity of telomere length under heat stress (Lee et al., 2016). Therefore, we hypothesize that octoploids probably harbor stronger tolerance to heat stress than tetraploids. However, the hypothesis draw from the transcriptomic data may not be conclusive, and more empirical evidence are needed to test the thermotolerance in each ploidy level.

Genes upregulated in tetraploids were involved in cell wall organization through monolignol conjugation and polymerization, and in external stimuli responses in reaction to UV-B light (**Table 2**). Interestingly, the GO terms were also enriched in biological processes involving development, reproduction processes and seed germination in upregulated genes in tetraploids (**Supplementary Table 1**), but not in octoploids. Therefore, it seems tetraploids were completing their life cycle faster, whereas octoploids developed more stress tolerance, especially heat resistance which may affect their natural distribution toward warmer territories in lower latitudes. This is further supported in Ren et al. (2020), where two octoploid samples and two tetraploids from this study were caught to flower in year 2017, and it took apparently longer time for the octoploids (266 days) to flower than tetraploids (220 days) (Ren et al., 2020). However, since ploidy information was not included in the study, we cannot draw a solid conclusion on the link between ploidy level and phenology. Therefore, our hypothesis based on transcriptomics data need to be interpreted with caution, and further experiments and developmental characterizations should be introduced to evaluate this theory.

Both of the upregulated and downregulated DEGs annotated in Mercator 4 included multiple genes coding for transcription factors enriched in RNA biosynthesis pathways, genes enriched in phytohormone pathways, cell cycle organization and protein modification. These transcription factors belonging to bZIP, WRKY, MYB, and C2H2 superfamilies, play a crucial role in initiating regulatory networks as response to abiotic stress, such as drought and salinity (Golldack et al., 2014; Han et al., 2020). The sampling process took place in July, the hottest month of the year in Shandong Province, with an average monthly temperature of 32°C during the day. Hence, the hot weather

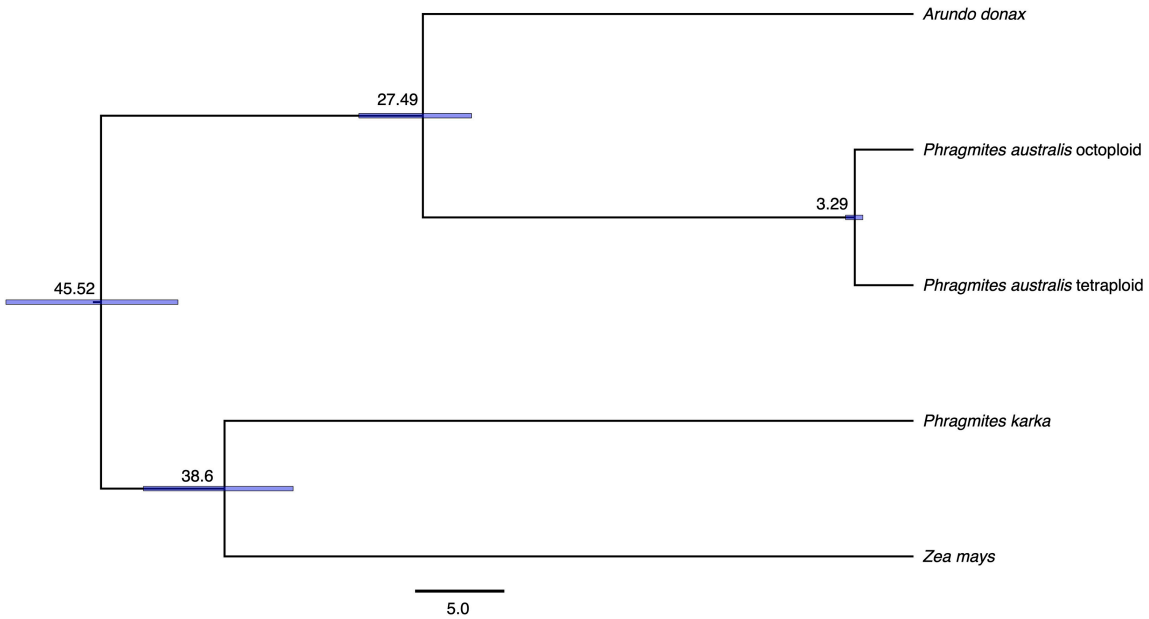

FIGURE 5 | Divergence time estimation of Poaceaespecies based on 98 single copy orthologous genes of five transcriptome assemblies including *Zea mays*, *Arundo donax*, *Phragmites karka*, *Phragmites australis* octoploid lineage, and *Phragmites australis* tetraploid lineage. The unit of the estimated divergence time is million years (MY), and the node bar indicated 95% Height Posterior Density of the node height.

may have constituted a stressful condition for the two groups, and tetraploids and octoploids may have utilized their inherently different pathways to deal with their environment.

Most of the DEGs were enriched in KEGG pathways belonging to "Chaperones and folding catalysts" and "Protein processing in endoplasmic reticulum" (**Figure 1B**). The heatmap of transcriptome profiles (**Figure 2C**) showed the most abundant transcripts to be heat shock proteins (HSP70, HSP20), chaperones (HtpG), and ubiquitin C, which are not only essential cellular components that assist with a serial of protein folding processes in cellular compartments, but also modulators of the regulatory network in a crisis of abiotic stress (Usman et al., 2014). For example, high levels of HSP 70 family protein expression have been linked to thermotolerance and resistance to high soil salinity, water stress and high temperature (Wang et al., 2004). UBC gene coding for ubiquitin C is a stress related gene, which correlates positively with higher drought tolerance in *Arabidopsis* and soybean (*Glycine max* (L.) Merr.) by conjugating ubiquitin to remove or unfold damaged proteins (Chen et al., 2020).

Biased Alternative Isoform Usage May Be Linked to Epigenetic Change

Gene differential expression could be affected by both genetic and epigenetic mechanisms. Liu et al. (2018) pointed out a trend that, as ploidy level increases in *P. australis*, the DNA methylation levels tends to be lower, although this trend was not significant (Liu et al., 2018). DNA methylation in exons or introns at the alternative splicing sites can significantly affect alternative splicing events in gene expression, but not on regularly expressed exons (Shayevitch et al., 2018). In this study, we found that the gene MSTRG.10410 (coding for proteins of the Cation-independent O-methyltransferase family), had a highly expressed isoform (evm.model.jcf7180004098404.2) with two Methyltransf_2 domains in tetraploids, while in octoploids, the gene MSTRG.10410.1 with only one Methyltransf_2 domain was highly expressed (**Figure 4A**). Methyltransf_2 domain includes a range of O-methyltransferases, which are related to DNA methylation (Keller et al., 1993). Another isoform (evm.model.jcf7180004127425.1) contains one PHD domain in tetraploids, which is responsible for binding to tri-methylated histones or demethylation of proteins, and thus affects the transcription (Schindler et al., 1993). However, the isoform in octoploids (MSTRG.21346.2) is lacking that domain (**Figure 4C**). These alternate isoforms may have contributed the different methylation level in tetraploids and octoploids. Therefore, the observed alternative splicing events in upregulated and downregulated genes are potentially a reason for, or a result of, the change of DNA methylation levels among ploidy levels. Nonsense-mediated mRNA decay (NMD) identifies cellular mRNAs carrying premature termination codons (PTC), and targets these aberrant transcripts for degradation to prevent the accumulation of potentially deleterious truncated proteins (Shaul, 2015). Moreover, it can also regulate the expression of stress responsive genes in plants, involved in pathways such as pathogen resistance, tolerance to heat shock and temperature change, as well as time-dependent flowering (Staiger and Brown, 2013). The majority of differentially expressed isoforms in *P. australis* were NMD insensitive, and only a few isoforms were NMD sensitive (**Table 4**). A high proportion of (10 out of 16) NMD sensitive isoforms were biased to be expressed in octoploids, indicating that they are either aberrant transcripts or potentially crucial in defense against environmental stress.

In addition, antistress-related isoforms were also found to be differentially expressed between tetraploids and octoploids. For example, isoform MSTRG.5773.1, containing one Stress-antifung domain, was expressed only in tetraploids, although a stronger Stress-antifung (x2) domain was found in octoploids, but this isoform was not expressed significantly higher than in tetraploids (**Figure 4B**).

Evolution of Octoploid and Tetraploid Lineages in *Phragmites* Species

The PCA plot separated individuals of the different ploidy levels apart (**Figure 1A**), suggesting the genetic variation in the samples mainly lies between ploidy level, and not between individuals. Therefore, we included all individuals in each ploidy level and built transcriptome assemblies for tetraploids and octoploids. Based on the PACMAD calibration point, we managed to estimate the divergence time between *A. donax* and *P. australis* at 27.41 Mya, concordant with previous studies which estimated the most common ancestor between *A. donax* and *P. australis* to be 29 Mya (Hardion et al., 2017). *Pragmites karka*, the congener of *P. australis*, clustered with *Zea mays* and diverged from *P. australis* from 45.36 Mya (**Figure 5**). Ancestors of *Phragmites* have been identified from the Cretaceous Period, so it was not surprising to reveal this divergence between *P. karka* and *P. australis* (Hayden, 1879). Nonetheless, the complicated phylogenetic relationship between the genera of *Phragmites* and *Arundo* suggests that the taxonomic status of Arundineae should be reconsidered.

Divergence between octoploid and tetraploid lineages of *P. australis* was estimated to be 2.81–3.69 Mya, falling at the border between Pliocene and Pleistocene (Bartoli et al., 2011). Pliocene, 5.3–2.6 million years ago, was generally characterized as a warm epoch, with only one mild glaciation cycle described. The onset of Pliocene glaciation started from 3.6 Mya, when the atmospheric CO_2 decreased transiently until between 3.4 and 3.32 Ma, sea ice volume increased and temperatures cooled down (Bartoli et al., 2011). In mid-Pliocene (3.3–3 Mya), the temperatures rose about 2–3°C higher than in the present atmosphere (Robinson et al., 2008). Pleistocene started from 2.8 Mya, when the warm climate abruptly changed, and intensive glaciation cycles repeatedly occurred. The divergence of octoploid and tetraploid happened shortly after the glaciation at 3.26 Mya, indicating that the two lineages may have experienced bottlenecks and separated in different refugia during glaciation periods which prevented gene flow between lineages, and favored recolonization to new territories during interglacial periods. It has previously been suggested in *Arabidopsis* and Alpine plants that the cool climate, which occurred during glaciation cycles, may have affected cell division during the sensitive period in meiosis, and thereby triggered the generation of polyploids (Sora et al., 2016; Novikova et al., 2018). We cannot draw such a conclusion from our study due to a paucity of information regarding the status of autopolyploidy or allopolyploidy in the investigated tetraploids and octoploids. However, it is worth noticing that there exists more than one lineage of octoploid and tetraploids in *P. australis*. We assign the octoploids to Australian (AU) lineage and tetraploids to European (EU) lineage, based on the geographic locations. Therefore, the estimated divergence time can only date to the most recent common ancestor of *P. australis* AU and EU lineages. Further studies need to be carried out to find the genetic background of different ploidy levels, so as to give a clearer explanation on the evolution of *Phragmites.*

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI SRA database with accession number, BioProject PRJNA687616.

AUTHOR CONTRIBUTIONS

CW and WG conceived the idea. CW performed data analysis. TW and MY collected the samples and coordinated with sequencing company. FE, LL, and HB maintained the common garden that supplied the samples, CW, WG, FE, TW, MY, LL, and HB led the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.653183/full#supplementary-material

Supplementary Figure 1 | Close look to genes contributed to the significant alternative splicing events Exon Skipping/Exon Inclusion (ES/EI). Genes with isoform usage of Exon Skipping (ES) were separated from genes without ES, and comparison between ploidy levels were made to both datasets. Significant isoform usage was indicated with *. *p-value < 0.05, ***p-value < 0.001, ns, no significant difference.

Supplementary Table 1 | Enriched GO terms of DEGs upregulated in tetraploids *P. australis.*

Supplementary Table 2 | Enriched GO terms of DEGs upregulated in octoploids *P. australis.*

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REVIEW
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Dynamics of DNA Methylation and Its Functions in Plant Growth and Development

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Epigenetic modifications in DNA bases and histone proteins play important roles in the regulation of gene expression and genome stability. Chemical modification of DNA base (e.g., addition of a methyl group at the fifth carbon of cytosine residue) switches on/off the gene expression during developmental process and environmental stresses. The dynamics of DNA base methylation depends mainly on the activities of the writer/eraser guided by non-coding RNA (ncRNA) and regulated by the developmental/environmental cues. *De novo* DNA methylation and active demethylation activities control the methylation level and regulate the gene expression. Identification of ncRNA involved in *de novo* DNA methylation, increased DNA methylation proteins guiding DNA demethylase, and methylation monitoring sequence that helps maintaining a balance between DNA methylation and demethylation is the recent developments that may resolve some of the enigmas. Such discoveries provide a better understanding of the dynamics/functions of DNA base methylation and epigenetic regulation of growth, development, and stress tolerance in crop plants. Identification of epigenetic pathways in animals, their existence/orthologs in plants, and functional validation might improve future strategies for epigenome editing toward climate-resilient, sustainable agriculture in this era of global climate change. The present review discusses the dynamics of DNA methylation (cytosine/adenine) in plants, its functions in regulating gene expression under abiotic/biotic stresses, developmental processes, and genome stability.

Keywords: DNA methylation, DNA modification, environmental stress, epigenetics, gene regulation, 5-methylcytosine, N^6-methyladenine, plant growth

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INTRODUCTION

Methylation of DNA bases at different positions (e.g., fifth carbon of cytosine and N^6 of adenine) plays significant roles in epigenetic regulation of gene expression in both plants and animals (Zhang et al., 2006; Xiang et al., 2010; Kumar et al., 2018). Epigenomic changes such as methylation of DNA bases, modification of histone proteins, and changes in the biogenesis of non-coding RNAs (ncRNAs) influence chromatin structure (accessibility of the genetic information to transcriptional machinery), thus gene expression, and genome integrity/stability. Methylation of DNA bases is known to be an important regulator of biological processes, and interruption in DNA methylation homeostasis leads to several developmental abnormalities in plants

(e.g., *Arabidopsis thaliana*) and animals (e.g., mice; Slotkin and Martienssen, 2007; Lang et al., 2017). While DNA methylation is catalyzed by different methyltransferases (using S-adenosyl-l-methionine as a methyl group donor), active DNA demethylation uses enzyme-catalyzed base excision repair (BER) pathway (Penterman et al., 2007; Kumar et al., 2018; Li et al., 2018a). Although the RNA-directed DNA methylation (RdDM) pathway is vital for *de novo* DNA methylation in plants, it is not so important in mammals (Matzke and Mosher, 2014). Active DNA demethylation initiates with deamination and/or oxidation of 5-methylcytosine (5-mC) in mammals, but in plants, direct excision of 5-mC takes place using methylcytosine DNA glycosylase (Law and Jacobsen, 2010; Li et al., 2018a). Besides, covalent but reversible posttranslational histone modifications and interaction with DNA play important role in regulating chromatin condensation and DNA accessibility (Ooi et al., 2006; Wei et al., 2017). Various mechanisms involved in site-specific DNA base modifications and their functions in the regulation of gene expression are being deciphered in model plants like *Arabidopsis* (Wang et al., 2016; Pecinka et al., 2019). N^6-methyladenine (6-mA) is another important modified DNA base (comparatively less abundant in plants) playing regulatory functions in animals and plants. It is considered to be essential for growth and development in *Arabidopsis* and rice (Liang et al., 2018; Xiao et al., 2018; Zhang et al., 2018b). Generally, a mutation in the gene encoding for component of DNA (de) methylation machinery or a regulatory factor does not cause lethality of the individual. Though *Arabidopsis* has been used as a model plant to understand the basic epigenetic machinery, the gathered information is validated and variations are being mapped in crop plants like rice (*Oryza sativa* L.). Efforts are also being made to identify the epigenetic marks associated with a trait of interest so that they can be utilized in crop improvement programs toward the development of climate-smart crops (Varotto et al., 2020). Nevertheless, DNA modifications appear to be crucial for developmental processes and protection from environmental stresses. Recent findings are unraveling the components (readers, writers, erasers, etc.) involved in DNA modification in plants. Such a recent understanding includes the necessity of a methylation-sensing genetic element in maintaining DNA (de)methylation homeostasis (Lei et al., 2015; Williams et al., 2015), the contribution of ncRNA in triggering *de novo* DNA methylation (Ye et al., 2016), and the role of increased DNA methylation protein in targeted DNA demethylation (Duan et al., 2017). The present review discusses the dynamics of DNA base methylation and its functions, particularly in controlling the activity of transposable elements (TEs), genome stability, regulation of gene expression during plant growth, development, and environmental stress.

DYNAMICS OF DNA METHYLATION

Variation in DNA methylation has been detected in many organisms, including viruses, prokaryotes, and eukaryotes (Berdis et al., 1998; Bell and Felsenfeld, 2000; Hoelzer et al., 2008). Methylation of DNA plays important roles in the regulation of gene expression, growth, development, and protection from environmental stresses, as well as in stabilizing the genome (Zilberman et al., 2006; Mendizabal and Yi, 2016; Kumar et al., 2017a, 2018). DNA base modification in a context-and genomic region-specific manner is catalyzed by different enzymes through distinct pathways. Methylcytosine (5-mC), also known as the fifth base of DNA, was discovered long before the DNA was recognized as genetic material in a living cell. Although more attention is given to the conventional 5-mC, recent findings on additional base modifications [e.g., hydroxymethylcytosine (5-hmC), formylcytosine (5-fC), carboxylcytosine (5-caC), and N^6-methyladenine (6-mA)] have resulted in overwhelming interest in epigenomic studies. In plants, cytosine methylation can occur in all contexts of cytosine (CG, CHG, and CHH, where H=A, C, or T; Lister et al., 2008; Wang et al., 2016). In *Arabidopsis* as well as in other plants, the heterochromatic regions are enriched with methylcytosines, generally in the repetitive sequences and TEs. However, TEs and 5-mC are also found to be interspersed in the euchromatic regions (Zhang et al., 2006; Rathore et al., 2020). The dynamics of DNA base methylation depends on the reversibility of the processes, which also controls switching on/off the gene. Diversity and complexity of epigenetic changes (DNA/histone modifications and ncRNA biogenesis) in different organisms are being discovered continuously, and the potential combinatorial interactions of epimarks indicate that epigenetic codons would be considerably more complex than it is thought today (Kumar et al., 2018).

Cytosine Methylation

Establishment, maintenance, and removal of cytosine methylation in different contexts/genomic regions in the plant genome occur through various pathways. While *de novo* cytosine methylation involves the RdDM pathway, maintenance of cytosine methylation in different sequence contexts depends on various DNA methyltransferases. Removal of 5-mC might occur either due to the malfunction of methyltransferase, scarcity of methyl donor (S-adenosylmethionine, AdoMet) during passive DNA demethylation, or by the active DNA demethylation process. In active DNA demethylation, a family of enzymes [bifunctional 5-methylcytosine DNA glycosylases–apurinic/apyrimidinic lyase (APE1L)] initiate the demethylation process *via* BER pathway (Almeida and Sobol, 2007; Li et al., 2018a). While promoter methylation is generally associated with switching-off/downregulation of the gene, methylation of the coding sequence may have negative or positive effects on gene expression (Takuno and Gaut, 2013; Williams et al., 2015; Kumar et al., 2017a).

RdDM pathway is responsible for *de novo* methylation of DNA which utilizes small-interfering RNAs (siRNAs), scaffold RNAs, and many accessory proteins (**Figure 1**). Present understanding of the RdDM pathway in *Arabidopsis* (Law and Jacobsen, 2010; Matzke and Mosher, 2014; Zhang et al., 2018a) suggests that RNA polymerase IV (Pol IV) initiates the production of 24 nt siRNA (noncoding P4 RNA) which serves as the template for RNA-dependent RNA polymerase 2-mediated generation of double-stranded RNAs (dsRNA). Sawadee Homeodomain Homolog 1

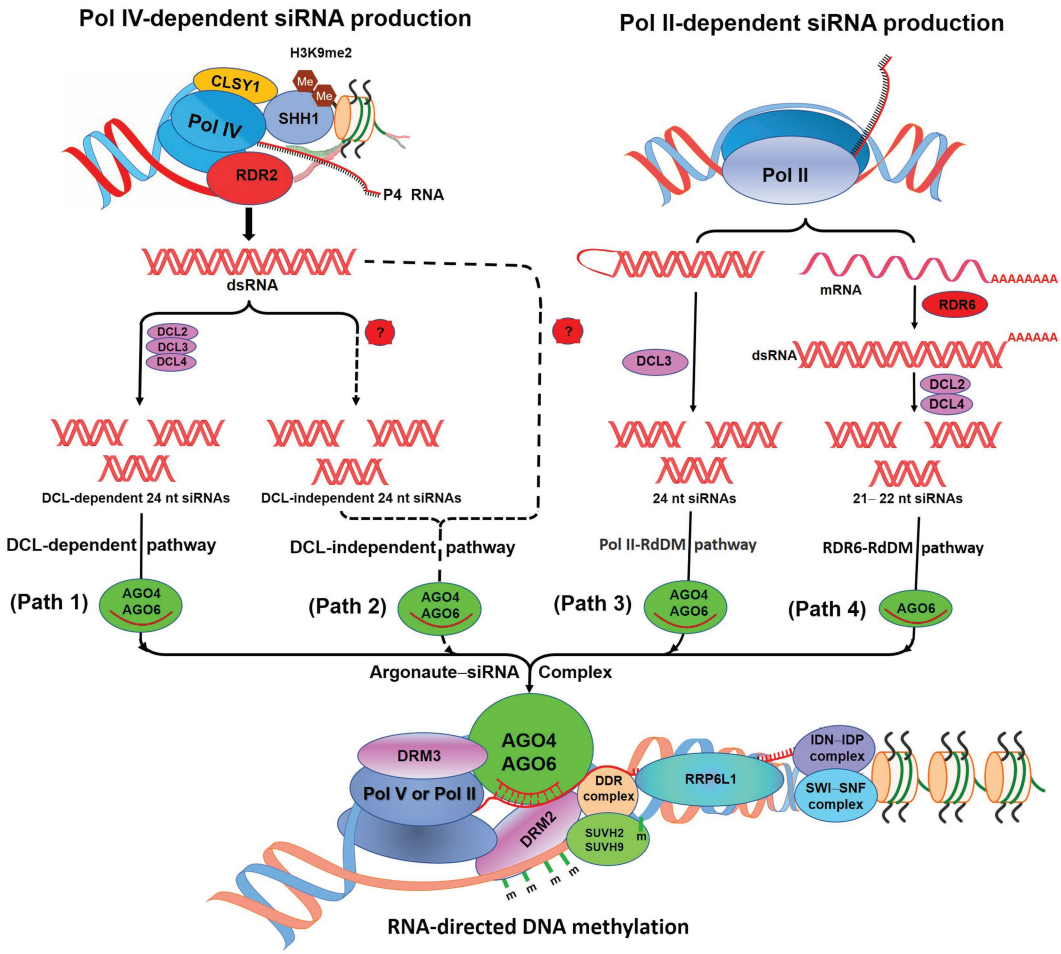

FIGURE 1 | Diagrammatic representation of the RNA-directed DNA methylation (RdDM) pathway. According to the canonical RdDM pathway, noncoding P4 RNAs are produced by RNA polymerase IV (Pol IV). SHH1 binds to dimethylated histone H3K9me2 and helps to recruit Pol IV at RdDM locus. (Path 1): P4 RNAs get converted into double-stranded RNAs (dsRNAs) by RDR2, which get cleaved into 24 nucleotide (nt) siRNAs by DICER-like protein 2 (DCL2),ss DCL3, and DCL4. These siRNAs bound with Argonaute 4 or AGO6 participate in the RdDM. (Path 2): Methylation of RdDM loci in *dcl1-dcl2-dcl3-dcl4* mutant suggests the existence of DCL-independent RdDM. (Path 3): POL II produces 24 nt siRNAs with the help of DCL3 and scaffold RNAs at some of the RdDM loci. (Path 4): For some active transposons, mRNAs get converted into dsRNAs and get cleaved into 21 nt siRNAs by DCL2, DCL4 through RDR6–RdDM pathway. Involved in *de novo* (IDN)–IDN2 Paralog (IDP) complex and RNA-binding proteins RRP6-like 1 (RRP6L1) interact with a chromatin-remodeling complex Switch/Sucrose Nonfermenting (SWI/SNF) to facilitate retention of nascent Pol V-transcribed RNA. m, methylcytosine. (Redrawn from Zhang et al., 2018a).

helps in the recruitment of Pol IV to the RdDM-targeted loci having dimethylated histone H3 lysine 9 (H3K9me2; Law et al., 2013; Zhang et al., 2013a). An SNF2 domain-containing protein Classy 1 (CLSY1), a chromatin remodeler, interacts with Pol IV, which is necessary for Pol IV-dependent siRNA production (Zhang et al., 2013a). DICER-like protein 2 (DCL2), DCL3, and DCL4 cleave the dsRNAs to generate 24 nt siRNAs (DCL-dependent siRNA production). Many of the RdDM-targeted loci were reported to remain methylated in quadruple (*dcl1-dcl2-dcl3-dcl4*) mutant; this suggests that siRNAs may also be produced by DCL-independent RdDM pathway or directly from P4 RNAs (Yang et al., 2016). At some of the RdDM-targeted loci, Pol II-dependent siRNA production starts with the production of 21–24 nt siRNAs. While transcription of some of the intergenic loci by Pol II produces 24 nt siRNAs and scaffold RNAs, transcription of some activated transposons by Pol II and RNA-dependent RNA polymerase 6 (RDR6) produces 21 or 22 nt siRNA precursors in association with DCL2 and DCL4 (Wu et al., 2012; Nuthikattu et al., 2013; McCue et al., 2015).

Subsequently, siRNA gets loaded onto Argonaute (AGO) proteins (AGO4 and/or AGO6) and directly associated with Pol V-transcribed scaffold RNAs which finally recruit domains rearranged methylase 2 (DRM2, a DNA methyltransferase) for methylation of the target locus. Interaction of AGO4 with DRM2 catalyzes *de novo* methylation of cytosine in a sequence-independent manner (Zhong et al., 2014). AGO association with Pol IV is complemented by RNA-directed DNA methylation 3 (Bies-Etheve et al., 2009). Generation of the scaffold RNAs requires DDR complex (consisted of a chromatin remodeler defective in RNA-directed DNA methylation 1, and defective in meristem silencing 3), which also associates with AGO4/AGO6, single-stranded methylated DNA, and DRM2

(Gao et al., 2010; Law et al., 2010; Zhong et al., 2012; Liu et al., 2014). The DDR complex also interacts with the suppressor of variegation 3-9 homolog protein 2 (SUVH2) and SUVH9 which bind together to the preexisting methylcytosine and help recruiting Pol V (Zhong et al., 2012; Johnson et al., 2014). SUVH2 and SUVH9 recognize methylcytosine through their RING finger-associated and SET domains which are needed for genome-wide chromatin binding of Pol V through preexisting DNA methylation. The binding of SUVH9, also having zinc finger, even to the unmethylated DNA was reported to be sufficient enough to recruit Pol V for methylation of DNA and silencing of the gene (Johnson et al., 2014). Pol V can produce ncRNAs with different 5′ ends from a locus, which indicates that it can start transcription without a promoter (Wierzbicki et al., 2008). The Pol V-generated scaffold RNAs are long enough to be detected by PCR and lack polyadenylation at 3′ end; thus, they differ from mRNA (Wierzbicki et al., 2008).

Methylation of cytosine in hemimethylated CG dinucleotide, created due to DNA replication, is performed by methyltransferase 1 (MET1), an orthologue of DNA methyltransferase 1 in mammals. It adds methyl (CH_3) group at fifth carbon of cytosine in daughter strand of the replicated DNA (**Figure 2A**). Recruitment of MET1 to the hemimethylated CG is mediated by variant in methylation proteins, which are UHRF1 orthologs (Woo et al., 2008). Methylation at CHG context in the daughter DNA strand is catalyzed mainly by chromomethylase 3 (CMT3) and to some extent by CMT2 (Stroud et al., 2014). SUVH4, SUVH5, and SUVH6 bind to the methylated CHG domain and facilitate the CMT3/CMT2 function (Du et al., 2012, 2014; Stroud et al., 2013). Mutation in SUVH4, SUVH5, and SUVH6 was reported to reduce CHG methylation in *Arabidopsis* (Ebbs and Bender, 2006; Stroud et al., 2013). Moreover, methylation at asymmetric CHH context is performed by DRM2 or CMT2 depending on the nature of the genomic region. At shorter transposons and repeat sequences in euchromatic regions, as well as at longer transposons in heterochromatin, DRM2 causes CHH methylation through the RdDM pathway (Zemach et al., 2013; Liu et al., 2014). Mutation in decreased DNA methylation 1 (DDM1), a chromatin-remodeling protein, causes impaired methylation by CMT2; DRM2 and CMT2 can also methylate cytosine in other contexts (Zhang et al., 2018a).

Demethylation of 5-Methylcytosine

Replacing 5-mC with cytosine (unmethylated) is an equally important phenomenon in the regulation of gene expression

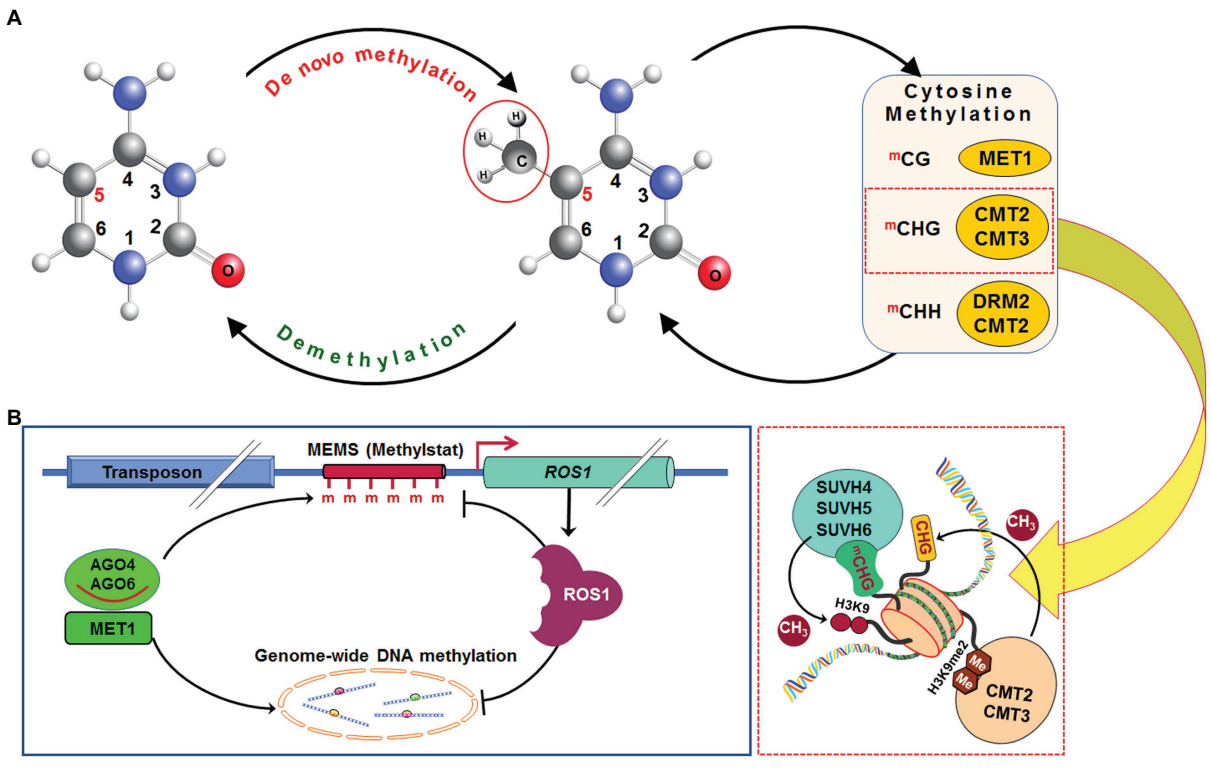

FIGURE 2 | Dynamics of DNA methylation in plants. *De novo* DNA methylation occurs in all (CG, CHG, and CHH; where H=A, C, or T) cytosine contexts. After replication of DNA, methylation in the CG context is maintained by methyltransferase 1 (MET1), while methylation in CHG context is maintained by chromomethylase 2 (CMT2) or CMT3, and methylation in CHH context is maintained by CMT2 or by DRM2 *via* RdDM pathway. Methylated CHG (^{m}CHG) attracts histone H3 lysine 9 (H3K9)-specific suppressor of variegation 3-9 homolog protein 4 (SUVH4), SUVH5, and SUVH6 and generates dimethylated H3K9 (H3K9me2), which enables CMT2 and CMT3 **(A)**. Methylation of methylation monitoring sequence (MEMS), also known as "methylstat" present in the promoter of the Repressor of silencing 1 (*Ros1*) is necessary for transcription of the *Ros1* gene. Cytosine methylation at MEMS is controlled by MET1/RdDM and Ros1 itself. This helps to sense/monitor the level of methylation and regulate DNA (de)methylation homeostasis **(B)**. CH_3, methyl group, Me/m, methylation (Redrawn from Zhang et al., 2018a).

through DNA methylation. Thus, methylation level is dynamically maintained by DNA (de)methylation. Passive (non-enzymatic) DNA demethylation occurs due to the loss of methylase activity during DNA replication (Mayer et al., 2000; Li et al., 2018a). Passive demethylation (reduced expression of MET1) was proposed to be responsible for demethylation in the central cell of female gametophyte, which develops into endosperm in seed after fertilization (Jullien et al., 2008; Kawashima and Berger, 2014). However, Park et al. (2016) recently reported maintenance of the methylation level in the central cell of *Arabidopsis* and rice. Calarco et al. (2012) reported maintenance of methylation in CG and CHG contexts during microsporogenesis which might be responsible for epigenetic inheritance in *Arabidopsis*. The vegetative nucleus in pollen shows very high methylation in CHH context, while the sperm cells show reduced CHH methylation due to reduced RdDM activity.

DNA methylation is also erased by active (enzymatic) DNA demethylation. While the active DNA demethylation process requires a family of enzymes, only one enzyme (methyltransferase) can accomplish the methylation process. In mammals, active DNA demethylation occurs through the BER pathway deploying DNA glycosylase wherein a 5-mC gets removed by TET dioxygenase-mediated oxidation of 5-hmC (Wu and Zhang, 2017). But in plants, a family of bifunctional DNA glycosylases–APE1Ls initiates the process through the BER pathway (Li et al., 2018a). Plant DNA glycosylase binds to 5-mC and removes it directly by breaking the glycosylic bond between the base and deoxyribose sugar. Subsequently, it acts as APE1Ls and breaks the DNA backbone producing an abasic site. APE1L and ZDP (a DNA polynucleotide 3′-phosphatase) generate 3′ OH; later on, the gap gets filled by the actions of DNA polymerase and ligase (Martinez-Macias et al., 2012; Lee et al., 2014; Li et al., 2015). In *Arabidopsis*, four known bifunctional DNA glycosylases include Repressor of silencing 1 (Ros1), Demeter (DME), Demeter-like protein 2 (DML2), and DML3 (Ortega-Galisteo et al., 2008). These glycosylases can remove 5-mC from any sequence context (Morales-Ruiz et al., 2006; Penterman et al., 2007; Zhu et al., 2007). DME is preferentially expressed in the companion (vegetative) cell of male and central cell of female gametes (Huh et al., 2008).

DME-favored demethylation of AT-rich TEs in euchromatin leads to changes in the expression of the nearby genes (Gehring et al., 2009; Hsieh et al., 2009; Ibarra et al., 2012). ROS1 demethylates TEs, which affects transposon activity and transcriptional silencing of the nearby gene (Tang et al., 2016). ROS1 also demethylates the RdDM-independent regions (He et al., 2009; Gao et al., 2010). The genomic regions targeted for ROS1-mediated demethylation are characterized by reduced H3K27me and/or H3K9me2, and enhanced H3K18Ac and/or H3K27me3 epimarks (Tang et al., 2016). At certain ROS1 target cites, chromatin environment legitimate for ROS1 active DNA demethylation is founded by the binding of histone acetyltransferase increased DNA methylation 1 at methylated DNA, which acetylates H3 particularly at the sites deprived of H3K4me2 and H3K4me3 (Qian et al., 2012).

The promoter of *ROS1* contains a 39 bp cytosine methylation monitoring sequence (*MEMS*), which has decreased methylation *in met1* and *RdDM* mutants (**Figure 2B**). Since hypomethylation of *MEMS* is accompanied by repression *of ROS1*, it indicates *MEMS* to function as a sensor/indicator of RdDM and MET1 activities. Thus, *MEMS* coordinates the methylation and demethylation processes through *ROS1* expression (Lei et al., 2015). *ROS1* promoter also contains a Helitron transposon upstream of the *MEMS*, which attracts cytosine methylation factors, and thus makes the promoter reactive according to the methylation level. In *ros1* mutants, hypermethylation *of MEMS* is accompanied by increased *ROS1* expression (Lei et al., 2015). Thus, like a thermostat, MEMS is considered to be a "methylstat" that senses and maintains ROS1-dependent methylation in plants (Lei et al., 2015; Williams et al., 2015). Regulation of demethylase gene by sensing methylation level has also been reported in maize (Erhard et al., 2015). Hence, the presence of such "methylstat" is considered to be an essential feature for cytosine methylation dynamics not only in plants but also in animals (Jones et al., 2015; Baylin and Jones, 2016).

Adenine Methylation

Like cytosine, adenine in DNA can also be methylated by the addition of a CH_3 group at the N^6 or N^1 position (Ratel et al., 2006; Kumar et al., 2018). Methylation of adenine at exocyclic NH_2 on the sixth position (C^6) of the purine ring forms N^6-methyladenine (6-mA). Similarly, methylation of the cyclic N at the first position (N^1) results in the formation of N^1-methyladenine (1-mA) due to the presence of endogenous or environmental alkylating agents (Sedgwick et al., 2007). The 6-mA has become a common and well-known player in the regulation of gene expression and defense against phage among the prokaryotes. *AlkB* gene of *E. coli* is considered to be an inducible factor for adaptive response to the environment. An *AlkB* homolog in humans performs a similar function and exhibits significant functional roles (Westbye et al., 2008); therefore, similar factors are expected to be present in plants also. Interestingly, N^7-methylguanine is also created in the presence of endogenous/environmental alkylating agents. A review by Law and Jacobsen (2010) suggested a certain degree of conservation in the mechanisms for the establishment and maintenance of DNA methylation between animals and plants. While conservation of some of the mechanisms has been confirmed including the role of siRNA in targeted DNA methylation and the role of methylated DNA-binding proteins, several questions regarding adenine methylation/demethylation homeostasis in plants remain to be answered.

Being detected in the lower eukaryotes at the beginning of this century, 6-mA was difficult to be detected in higher eukaryotes probably because of its lesser abundance; hence, earlier considered to be absent in most of the eukaryotes. However, recent advances in high-throughput, highly sensitive techniques, such as deep-sequencing and liquid chromatography coupled with mass spectrometry (LC–MS), have resulted in the detection of 6-mA, its localization in the genome followed by understanding its epigenetic functions in animals and plants (Huang et al., 2015; Liang et al., 2016). Even highly sensitive techniques like mass spectrometry could detect only a few

6-mA per million nucleotides in the genome of animals and plants, which suggests that the turnover (demethylation) rate of 6-mA might be faster. With more intensive studies on modified DNA bases, distribution patterns and possible functions of 6-mA in the animal system are becoming clear day by day; such information is still less known in plants.

The enzymes responsible for conversion of adenine into 6-mA in bacteria (DAMT), *C. elegans* (DAMT-1), *Bombyx mori* and mammals (METTL4), and human (N^6AMT1) have been well reported (Vanyushin, 2005), but only a little is known about adenine methyltransferase in plants (Li et al., 2019). In green algae *Chlamydomonas*, 6-mA plays an important role in the transcription of genes and nucleosome positioning. In *Chlamydomonas*, adenine-methylome was reported to contain ~85,000 6-mA in AT context mostly in the promoter and linker regions. However, it possesses a low level of 5-mC (Fu et al., 2015). A Mg^{2+}/Ca^{2+}-dependent N^6 adenine DNA methyltransferase (wadmtase) purified from wheat coleoptiles showed its potential in generating 6-mA. Wadmtase recognizes TGATCA hexanucleotide, but not GATC tetranucleotide, to methylate adenine (Fedoreyeva and Vanyushin, 2002). This stimulates examination of the presence and the potential role of 6-mA in higher eukaryotes. Analysis of the genomic DNA of *Arabidopsis* revealed the presence of 0.006% (lowest) 6-mA in root and 0.138% (highest) in rosette leaves (Liang et al., 2018). The general distribution of 6-mA in the *Arabidopsis* genome was observed to be near the transcription start site (TSS). The analysis also revealed that 6-mA, particularly those in the TSS region, positively correlates with the expression of the corresponding gene. Furthermore, the changes in 6-mA at different developmental stages of the plant were reported to be associated with gene activation. Although 5-mC and 6-mA both correlate with the transcription of genes in different manners, these epigenetic marks show a certain level of interdependence. Unfortunately, the proteins (readers and erases) that interact with 6-mA in eukaryotes have not yet been characterized.

Analysis of the rice genome revealed about 0.2% of 6-mA, a level similar to that reported in *C. reinhardtii* and *C. elegans* (Fu et al., 2015; Greer et al., 2015). Generally, 6-mA occurs in GAGG context and it was detected in 20% of the genes and 14% of TEs in rice (Zhou et al., 2018). The occurrence of 6-mA was also identified earlier in the GAGG context in *C. elegans*; however, the occurrence of 6-mA in GAGG context in rice is not palindromic, indicating its occurrence only in one strand of DNA (Zhou et al., 2018). While the presence of 6-mA in the promoter causes silencing of the gene, its occurrence in the coding region correlates with activation of the gene. Different possible functions of 6-mA include transcriptional silencing/activation, regulation of transgenerational chromatin functions, and stress response (Liang et al., 2020), as well as in other biological activities like DNA replication and mismatch repair in *E. coli* (Pukkila et al., 1983; Campbell and Kleckner, 1990; Kumar et al., 2018). However, the studies conducted so far report contrasting functions of 6-mA in different eukaryotes.

Demethylation of Methyladenine

To some extent, the mechanisms of adenine (de)methylation in animals have been understood. For example, a mutation in DNA methyladenine demethylase (*DMAD*) resulted in the accumulation of 6-mA in *Drosophila*, which revealed its role in adenine methylation/demethylation homeostasis (Zhang et al., 2015). However, an adenine methyltransferase has not been identified in *Drosophila* (Shah et al., 2019). Moreover, oxidation of the attached methyl group at 6-mA by a demethylase (e.g., AlkB dioxygenase) results in its conversion to N^6-hydroxymethyladenosine (6-hmA) and N^6-formyladenosine (6-fA), and thus causes demethylation of adenine (Kumar et al., 2018; **Figure 3**). Studies suggest that AlkB family (Fe^{2+}- and α-ketoglutarate-dependent dioxygenases involved in the removal of alkyl adducts from DNA bases by oxidative dealkylation) enzymes are important players in the demethylation of 6-mA (Fu et al., 2015; Iyer et al., 2016). Similarly, 1-mA may also get demethylated by AlkB oxidase and AlkB enzyme *via* N^1-hydroxymethyladenine (1-hmA).

Effects of Adenine Methylation

The presence of 6-mA in DNA is recognized by the binding of a specific effector molecule (reader) that may change chromatin conformation and/or transcriptional activity of the gene. Such readers, like SeqA protein, specifically bind to hemimethylated DNA with 6-mA. For example, polycomb proteins were reported as the coordinator between the accumulation of 6-mA and deactivated DMAD for transcriptionally repressing the gene (Yao et al., 2018). When present in the promoter, 6-mA generally represses the expression of the gene, but it may also function as an activator of the transcription process. These suggest that (de)methylation of adenine and cytosine takes place in a dynamic, coordinated, and context-specific manner. Therefore, it would be interesting to understand the interaction among the epimarks to investigate the complexity of epigenetic codons, which might help to answer several biological enigmas (Kumar, 2017; Kumar et al., 2018). A comprehensive understanding of such modifications and their functions in epigenetic regulation of gene expression would be essential for epigenetic manipulation of desirable traits in plants and animals (Kumar, 2019b).

FUNCTIONS OF METHYLATED DNA BASES

DNA base modifications, particularly cytosine methylation, were initially considered as a host defense mechanism in prokaryotes. Later on, it was found to play several vital functions in eukaryotes, mostly as a defense mechanism against jumping TEs to maintain genome integrity over the generations (Zhang et al., 2011). Over the last two decades, epigenetic changes in the plant genome have been reported during various developmental processes and environmental stresses (Bartels et al., 2018). Methylcytosine in the promoter region was reported to repress transcription of the gene by affecting the binding of TFs and by forming repressive-chromatin structures due to the interaction between methylated DNA-binding proteins (Bird, 2002). Regulatory flexibility is a characteristic feature of epigenetic mechanisms, particularly in response to environmental factors.

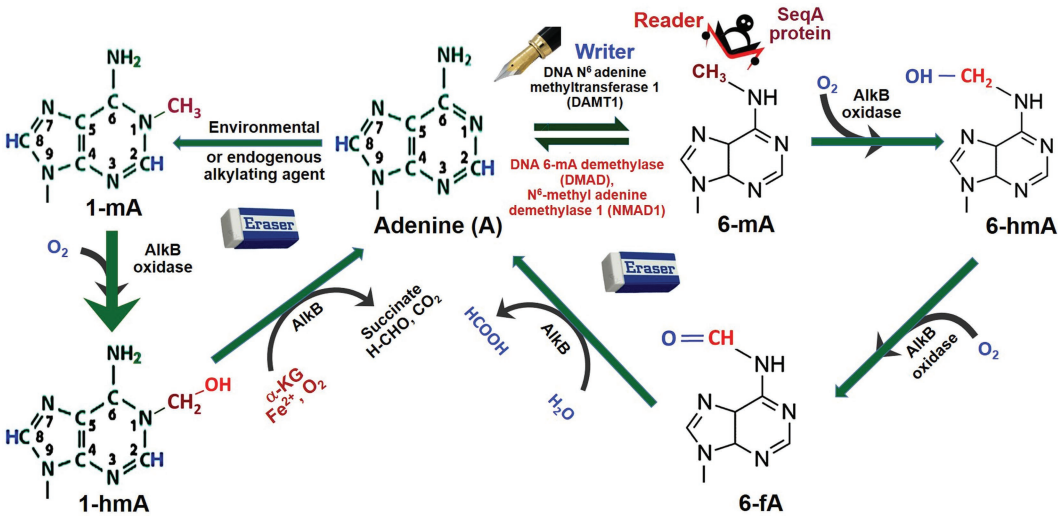

FIGURE 3 | Dynamics of adenine methylation. Adenine (A) gets methylated by the addition of CH_3 (methyl) group at N^6 position by DNA adenine methyltransferases 1, the writer, generating N^6-methyladenine (6-mA). SeqA protein, the reader, specifically binds to hemimethylated 6-mA DNA. The 6-mA gets hydroxylated (–OH) by AlkB oxidase to N^6-hydroxymethylcytosine (6-hmA). Due to the erasers like DNA 6-mA demethylase (DMAD) or N^6-methyladenine demethylase-1 (NMAD1), 6-mA gets deaminated back to Adenine. 6-hmA gets demethylated indirectly to Adenine by AlkB oxidase *via* N^6-hydroxymethyl adenine (6-hmA) and N^6-formyladenine (6-fA). Adenine may also get methyl adduct to N^1-methyladenine (1-mA) by environmental/endogenous alkylating agents. Similarly, 1-mA may also get demethylated indirectly by AlkB oxidase to Adenine *via* N^1-hydroxymethyl adenine (1-hmA). AlkB is one of the prototypic oxidative dealkylation DNA repair enzymes/dioxygenases involved in the removal of alkyl adducts from DNA base by oxidative dealkylation (Redrawn from Kumar et al., 2018).

Similarly, gene/genome imprinting (preferential expression of the gene/genome coming either from male or female parents) is also regulated through epigenetic mechanisms. Imprinted genes are silenced by DNA and/or histone modifications. Demethylation of the maternal genome and activation of the genes in endosperm have been reported in *Arabidopsis*, rice (Hsieh et al., 2009; Luo et al., 2011; Rodrigues et al., 2013), and maize (Waters et al., 2011). Knockout of *ALKBH1* (a 6-mA demethylase) resulted in a higher 6-mA level and early flowering in rice (Zhou et al., 2018), suggesting that 6-mA plays an important role in reproductive development in rice. Nucleosome remodeler (e.g., DDM1) affects 5-mC content in plants (Tan et al., 2016). Decreased 6-mA content and no change in 5-mC level were reported in CRISPR/Cas9 *ddm1a*/*ddm1b* double mutants showing dwarfing and decreased seed-setting (Zhang et al., 2018b) suggesting that 6-mA is involved in vegetative and reproductive developments in rice. Thus, DNA base and histone protein modifications in combination with the nonhistone proteins define accessibility of the genes to help regulate their expression.

Source of Diversity and Heritable Variations

Some of the epigenetic changes may persist even after the reversal of the conditions that caused such changes, and some of them may inherit to the next generation as epigenetic alleles (epialleles). Such heritable epialleles are being considered as an additional source of diversity, which may be utilized in breeding programs, particularly in those crops where genetic diversity is reported to be scarce. The creation of natural epialleles is much faster than that of alleles due to natural genetic mutation; however, the reversal rate of epialleles is also higher. Even then, epigenetics is considered to create more heritable epialleles and helps in the evolution process. Reports suggest that environmentally induced epigenetic changes in plants may be mitotically stable and meiotically inherited. Therefore, the emphasis is now given to such epigenetic changes as a source of variation. Transcriptional activation of Tos17 retrotransposon (RT) during tissue culture in rice was reported earlier, which gets repressed on plant regeneration (Liu et al., 2004). Studies demonstrate that activation of the transcription process and transposition of the RT in tissue-cultured calli are controlled through DNA hypomethylation (Cheng et al., 2006; Ding et al., 2007). Later on, it was reported that RT is demethylated by DNA glycosylase/lyase which promotes its movement during tissue culture in rice (La et al., 2011).

RdDM pathway was reported to respond to the environmental stimuli, which triggers epigenetic changes at particular loci toward the generation of heritable epialleles (Manning et al., 2006; Verhoeven et al., 2010). However, the importance of epialleles in crop breeding would require determining the extent of variation in epimarks among the individuals, the extent to which the epimarks affect the phenotype, and the heritability of the epimark-linked superior phenotypes. Moreover, several technical challenges in estimating the epigenetic variations and the level of epimark-associated phenotypic diversity do exist. With the continuously increasing understating of epigenomics, it is expected that our efficiency of exploiting epigenomic variability and deploying epigenome editing in crop improvement would become better and will have a significant impact on food security.

Regulation of Gene Expression

DNA modification in different cells/tissues is dynamically regulated during plant growth, development, and under varying environmental conditions. This indicates the important roles of DNA modifications in the regulation of gene expression and physiology. Base modifications occurring in a promoter, in the nearby regions, and/or within the gene-body, might affect the gene expression. Generally, DNA base modifications repress transcription of the gene; however, in certain cases, this may also promote transcription of the gene. Such an example is cytosine methylation in the promoter of ROS1 which enhances its transcription in *Arabidopsis* (Williams et al., 2015). Base modification may strengthen the binding of certain transcription activators, or it may inhibit the binding of transcription repressor. However, the exact mechanisms of regulating gene expression by DNA methylation in the promoter region and the gene-body are not yet clear. Since, only 5% of the genes in *Arabidopsis* are methylated in the promoter region, which indicates that DNA methylation is not the sole epigenetic regulatory mechanism for controlling the expression of genes (Zhang et al., 2018a; Kumar and Mohapatra, 2021). Crop plants with a large genome size possess a higher number of TEs, and many of them are closer to genes affecting their expression. Thus, DNA modification plays a significant role in controlling the expression of the gene in crop plants compared to that in *Arabidopsis* which contains a limited number of TEs. However, DNA demethylase targets TEs present in the promoter to regulate stress-responsive genes (Le et al., 2014). That is why DNA methylation mutants in crop plants have been reported to have severe growth/developmental defects or lethal effects (Wei et al., 2014; Liu et al., 2015; Lang et al., 2017).

In *Arabidopsis*, about one-third of the genes are methylated in the gene body (Zhang et al., 2006). In general, TEs and repeat regions are heavily methylated in all cytosine contexts, but gene body methylation sparsely occurs in non-CG context (Zhang et al., 2006; Cokus et al., 2008; Takuno and Gaut, 2013). Gene body methylation occurs in the transcribed/coding region between the transcription start and termination sites (Bewick and Schmitz, 2017). Some of the introns in a gene may harbor TE or repeat elements, which are hypermethylated in all cytosine contexts and regulate mRNA processing. Loss of DNA methylation from a retrotransposon present in an intron of the homeotic gene was reported to cause alternate splicing and premature termination of the transcript. The role of 6-mA in the regulation of gene expression appears to be conserved among the plants. 6-mA occurs in the gene-body of a transcriptionally active gene (Liang et al., 2018; Zhang et al., 2018b; Zhou et al., 2018). 6-mA and 5-mC sites might show overlap, and 6-mA-containing genes may show a high degree of nucleosome arrays (Zhou et al., 2018). This indicates that 6-mA provides an additional layer of the epigenetic regulatory mechanism of gene expression or it works along with the other epigenetic markers.

Transposon Silencing and Genome Stability

Active TEs threaten genome stability/integrity due to the jumping of transposons or repeated insertion of retrotransposons. Heterochromatins as well as transposon-or repeat-containing euchromatic regions in *Arabidopsis* are hypermethylated in all cytosine contexts (Cokus et al., 2008). CHH methylation in smaller transposons and at the ends of long transposons is established by the RdDM pathway, while it is taken care of by DDM1 and catalyzed by CMT2 at the internal positions of heterochromatin and long transposons (Zemach et al., 2013; Stroud et al., 2014). The active genes and inactive transposons in the maize genome are separated by RdDM-dependent hypermethylated CHH islands. Any loss of methylated CHH island leads to transcriptional activation of the transposon, suggesting that RdDM is needed to keep the transposons silenced (Li et al., 2015). Transposon reactivation due to DNA demethylation was observed in only a few *Arabidopsis* mutants, whereas *met1-cmt3* double mutants or *ddm1* mutants showed hypomethylation in CG and CHG contexts, and increased transposition of TEs (Mirouze et al., 2009; Tsukahara et al., 2009). DNA glycosylase/lyase 701 (a ROS1 homolog) controls the movement of retrotransposon Tos17 in rice, which indicates that DNA modification regulates transposon activity (La et al., 2011). DNA methylation also influences chromosomal interactions. In *Arabidopsis*, all the five chromosomes were reported to interact with each other forming a structure known as KNOT (Grob et al., 2014). Moreover, the chromosomal regions involved in the formation of KNOT are comprised of interactive heterochromatin islands (IHIs) containing several transposons (Feng et al., 2014; Grob et al., 2014). The KNOT engaged element represents a preferred landing site for TEs, which may be a part of the defense mechanisms for genome stability. Increased chromosome interaction between the Pol V-dependent methylation and the genes repressed by RdDM was recently reported (Rowley et al., 2017). This indicates that even chromosomal interactions might have regulatory functions in gene expression. Several studies suggest a wide variation in DNA methylation among different cells, tissues, organs, and species. The variation in DNA methylation level, GC content, and chromatin architecture among different species do not correlate with the genome size and thus serve as the source of diversity.

Genome Imprinting and Heterosis

FIS2, FWA, and MEA are some of the well-characterized genes responsible for genome imprinting in plants. While the allele from one parent is expressed, the allele from the other parent is silenced. This is known as genome imprinting (Gehring et al., 2006; Jullien et al., 2006). In flowering plants, megaspore mother cell (MMC) undergoes meiosis to form female reproductive organs. Similarly, the microspore mother cell (MiMC) undergoes meiosis to form male reproductive organs. Both MMC and MiMC undergo large-scale chromatin changes, including heterochromatin decondensation, during cell specification indicating a highly active transcriptional activity (She and Baroux, 2015). During MMC specification, CHH methylation transiently decreases and then gets restored but CG methylation remains stable. While methylation at CG and CHG contexts is maintained by MET1 and CMT3, respectively, CHH methylation is maintained by CMT2 or by the RdDM pathway (Gehring, 2019). Recently, DNA methylation was

profiled in the MiMC of *Arabidopsis*, wherein high levels of CG and CHG methylation but low level of CHH methylation were reported (Walker et al., 2018).

DME expression in the central cell before fertilization was reported to cause extensive demethylation of the maternal genome (Gehring et al., 2009; Hsieh et al., 2011) which causes the expression of the genes from the maternal genome. Zhang et al. (2011) carried out MSAP analysis and reported hypomethylation in the endosperm of *Sorghum bicolor* because of demethylation in the CG context. Genome-wide demethylation of TEs was also reported in the endosperm (Gehring et al., 2009; Hsieh et al., 2009). During male gametogenesis in plants, the transposons present in vegetative/companion cells are derepressed by transcription activator DME-mediated DNA demethylation and downregulated expression of a chromatin remodeler DDM1 (Lippman et al., 2004; Zhang et al., 2016). In the vegetative cell, the transcripts generated from TEs are processed into siRNAs and enter sperm cells to reinforce transposon silencing *via* DNA methylation (Martínez et al., 2016). In a double-fertilization system, one of the two sperm cells of pollen fertilizes the central cell of the female gametophyte (first fertilization) while the other fertilizes the egg cell (second fertilization), which produce endosperm and embryo, respectively. A sperm cell fertilizes with a central cell of the female gamete to form the endosperm wherein global demethylation, but reinforced CHH methylation at TEs, is observed (Ibarra et al., 2012). Another sperm cell fertilizes the egg cell to produce the embryo where the RdDM pathway maintains methylation (**Figure 4**). In *Arabidopsis* as well as in rice, the endosperm displays global DNA hypomethylation compared to that in the embryos (Gehring et al., 2009; Hsieh et al., 2009; Ibarra et al., 2012). Imprinting of endosperm is schemed by differential DNA methylation of the maternal and paternal genomes together with the polycomb group of genes (Hsieh et al., 2011). The maternal genomes of endosperm are less methylated (particularly in CG context) compared to that of the paternal genome (Gehring et al., 2009; Hsieh et al., 2009; Zhang et al., 2014; Klosinska et al., 2016). Certain maternally expressed genes (MEGs), for example, MEDEA in *Arabidopsis*, from the paternal genome are silenced due to repressive histone (H3K27me3) modification rather than by DNA methylation (Gehring et al., 2006; Jullien et al., 2006). In maize, the endosperm-specific MEGs are associated with H3K4me3 modification, while paternal alleles are suppressed by hypermethylation near the TSSs (Dong et al., 2017). Thus, the set of imprinted genes show that imprinting is a major epigenetic phenomenon affecting endosperm development in plants. RdDM pathway was reported to regulate parental gene imprinting at several loci in *Arabidopsis* (Vu et al., 2013). Thus, manipulation in genome imprinting through epigenome editing might help to develop a superior endosperm for improvements in seed crops (Berger, 2003; Kumar, 2019a).

Evidence suggests that F_1 hybrids are hypomethylated compared to their parental inbreds (Kovacevic et al., 2005). RNA amount polymorphism and protein amount polymorphism data in maize indicate that quantitative variations in the expression of certain loci might be responsible for the heterosis observed in the F_1 hybrid. Repeated selfing during the development of inbreds, giving more emphasis on combining ability, might result in a gradual accumulation of methylated loci (Kumar et al., 2017b). This gets repatterned and/or released

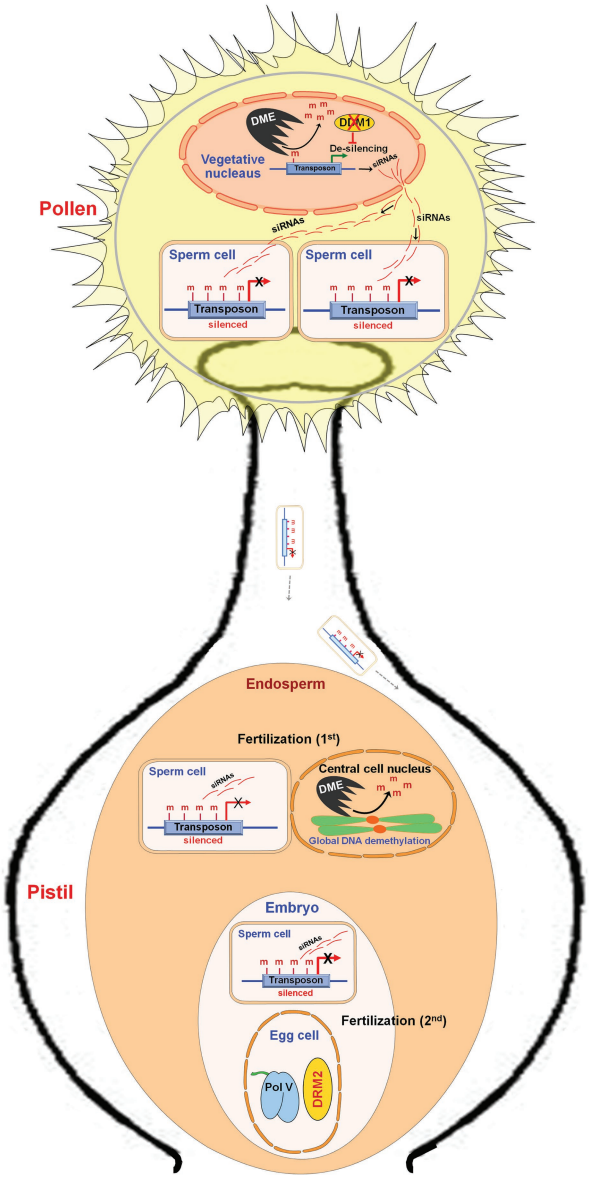

FIGURE 4 | Role of DNA methylation during reproductive development in plants. During male gametogenesis in pollen, the transposons in the vegetative cell are de-silenced due to DNA demethylation by Demeter (DME) and by downregulated expression of Decreased DNA Methylation 1 (DDM1, a chromatin remodeler). Transposon-generated transcripts are converted into siRNAs, which enter into the sperm cells and cause silencing of transposon through DNA methylation. On pollination, one of the sperm cells fertilizes a female gamete (central cell) and forms the endosperm, wherein reinforced CHH methylation at transposons (in the male genome) and DME-mediated global DNA demethylation (in the female genomes) are observed. Another sperm cell fertilizes the egg cell to form an embryo, where reinforced CHH methylation in the male genome but domain rearranged methylase 2 (DRM2) and RNA polymerase V (Pol V) derived RdDM pathway in the female genome are observed.

when the inbreds are crossed to develop a hybrid. Further, understanding the epigenetic regulation of embryo development might uncover the mysteries of apomixis (asexual reproduction through seeds) in a plant (Koltunow and Grossniklaus, 2003; Kumar, 2017; Rathore et al., 2020). If apomixis is successfully incorporated in commercial seed crops, heterosis can be preserved over the generations to overcome the current limitations of hybrid seed production.

Fruit Ripening, Seed Development, and Germination

DNA hypomethylation is reported to be a general feature at the promoter of many fruit ripening-associated genes as they contain binding sites for the ripening-associated transcription factors (Zhong et al., 2013; Lang et al., 2017). Ripening inhibitor binds to the methylated promoter of ripening genes to suppress the expression of the genes. The expression of DML2 (DNA demethylase) increases dramatically during the ripening of tomatoes. Active demethylation is required not only to activate ripening genes but also to suppress the ripening-inhibitor genes (Liu et al., 2015; Lang et al., 2017). Hypermethylation at CHH context in developing apple fruit, compared to that in the leaf, has been reported. A correlation between DNA hypomethylation and the smaller size of the fruit has also been reported (Daccord et al., 2017). Anthocyanin content in apple fruits has been reported to be negatively correlated with DNA methylation level at the promoter of MYB10 gene (Telias et al., 2011; El-Sharkawy et al., 2015).

Seed development is an essential process for food quality and productivity. During seed development in soybean, CHH methylation was reported to increase from 6% at the early stage to 11% in the late stage (An et al., 2017; Lin et al., 2017). Methylome analysis at the globular stage and seed germination in *Arabidopsis* and soybean showed a significant increase in methylation at CHH context (Lin et al., 2017). Twenty-five genes were observed to be differentially methylated during rice seed development, and endosperm cellularization was reported to be regulated by methylation dynamics (Xing et al., 2015). In *Brassica rapa*, a mutation in the RdDM pathway resulted in reproductive defects, which suggests that DNA modification is necessary for seed development (Grover et al., 2018). The maternal allele of components in the RdDM pathway was reported to be required for seed development in *Brassica rapa* (Grover et al., 2018). Demethylation of a retroelement (RE) Gy163 was observed to be associated with apomictic seed development in *Cenchrus ciliaris*. The RE Ty3-gypsy was found to be differentially methylated/expressed in the reproductive tissues of apomictic and sexual plants. Hypomethylation was observed in CHH context in reproductive (aposporous initial and mature embryo sac) tissues of apomictic plants, which was directly correlated with the activity of the RE (Rathore et al., 2020). Thus, epigenetic regulation of seed development appears to be a common process in different plant species.

Tolerance to Abiotic Stress

Abiotic stresses have been reported to cause alterations in DNA methylation in plants. Heat stress-induced accumulation of *ONSEN* retrotransposon was observed in *Arabidopsis* mutants impaired in the biogenesis of siRNAs (Ito et al., 2011). P5CS and *δ-OAT* genes were reported to be demethylated under osmotic stress in mother plants, but they restore methylation in the next generation under normal growth conditions (Zhang et al., 2013b), suggesting that epigenetic changes regulate the expression of the genes (**Figure 5A**). TEs have been reported to affect the expression of genes through their *cis-or trans*-regulatory elements, or even through serving as targets of epigenetic modifications (Seymour et al., 2014; Stuart et al., 2016; Wei and Cao, 2016). TEs being one of the targets of epigenetic machinery for controlling their activity as well as that of the nearby genes, they play important roles in the adaptation of plants to the changing global climate (Li et al., 2018b). Masuta et al., (2017) analyzed transcriptional and transpositional activation of *ONSEN* and observed heat stress-induced transposition of *ONSEN* during tissue culture. Heat stress response of soybean root showed a marginal (<10%) decrease in methylation; however, a significant change in the CHH context and TEs was observed (Hossain et al., 2017).

Several early studies on abiotic stresses indicate stress-induced DNA (de)methylation of stress-associated genes. Phosphate (Pi) starvation in rice was reported to cause more than 100 differentially methylated regions (DMRs) due to hypermethylation in CHH context mainly in the transposons near Pi-starvation-induced genes (Secco et al., 2015). Salt stress-induced changes in DNA methylation were reported to be partly inherited over the generations in *Arabidopsis*, especially through female gametes (Wibowo et al., 2016). Suppressor of DRM1, DRM2, and CMT3 (*SDC*) gene was reported to be silenced through DNA methylation of the promoter in vegetative tissues. Stress-induced activation of *SDC* due to repeated stress was also reported (Sanchez and Paszkowski, 2014). The RdDM pathway has been reported to alter TEs activity and gene expression involved in abiotic and biotic stress responses, plant development, and intercellular communication.

Recent studies suggest that 6-mA plays an important role in regulating gene expression in plants under environmental stress. 6-mA level showed dynamic changes in rice under heat, cold, and salt stress (Zhang et al., 2018b). Under heat stress, 6-mA level in the heat-tolerant rice genotype was 2.6-fold greater than that in the heat-sensitive rice genotype. 6-mA content in a heat stress master transcriptional regulator (heat shock transcription factor A1) exhibited a significant increase in heat-tolerant genotype than in heat-sensitive rice genotype. Decreased 6-mA level in HsfA1 repressor HSP70 in heat-tolerant genotype improves tolerance to heat stress (Zhang et al., 2018b). DDM1 and Morpheus molecule 1 (MOM1) in *Arabidopsis* were reported to be responsible for erasing stress-induced epigenetic marks after the stress. In double mutants for *ddm1–mom1*, stress-induced epigenetic marks are transmitted to the progeny, whereas in a single mutant either for *ddm1* or for *mom1*, stress-induced epigenetic marks are not inherited. This indicates that MOM1 and DDM1 function in checking the inheritance of stress-induced

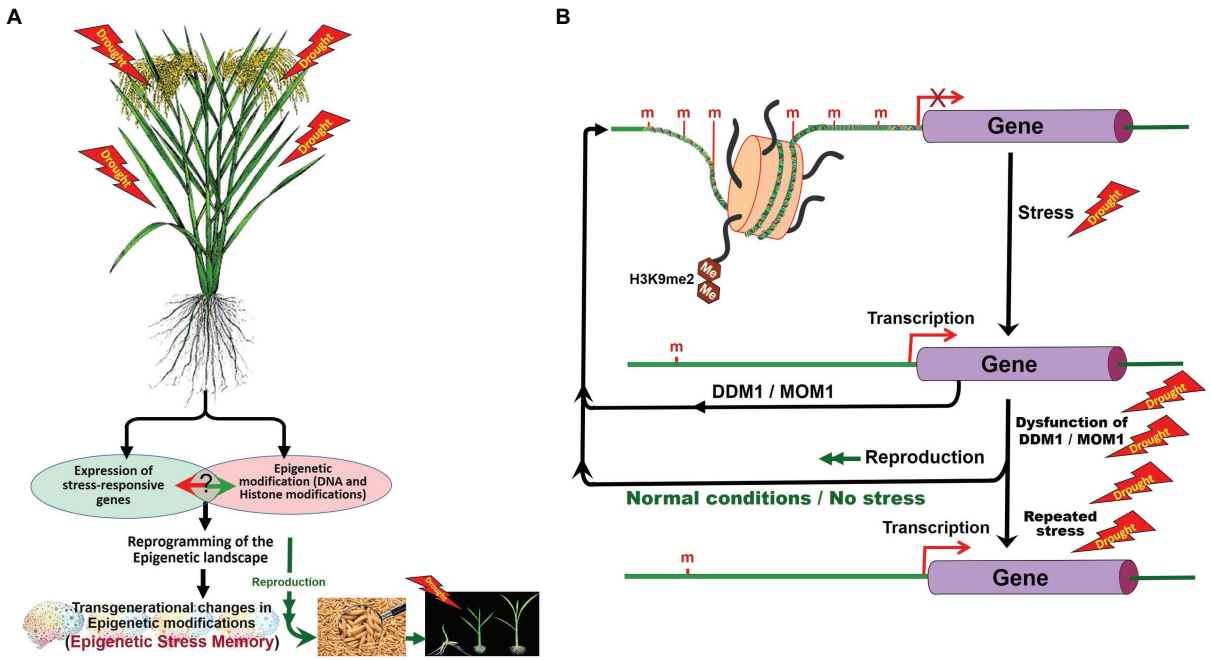

FIGURE 5 | Role of DNA methylation in stress tolerance and epigenetic stress memory in plants. Abiotic stresses may alter the expression of the stress-responsive genes and cause DNA base modifications. In stressed plants, epigenetic modifications reprogram the epigenetic landscape and some of the epigenetic changes may be inherited **(A)**. Expression of stress-responsive genes may also cause changes in epigenetic modifications. After the stress, during the recovery period, Morpheus Molecule 1 (MOM1) and DDM1 erase the stress-induced epigenetic marks. Inheritance of stress-induced epigenetic marks/de-silencing of genes can be seen in plants repeatedly exposed to the tress due to the dysfunction of DDM1 and MOM1 **(B)**. H3K9me2, dimethylated histone H3 lysine 9 (Modified from Zhang et al., 2018a).

epimarks (epigenetic stress memory; Iwasaki and Paszkowski, 2014). The stress-induced epigenetic memory may also be lost due to the passive demethylation process, if the progenies are grown under normal (without stress) conditions (Zhang et al., 2018a; **Figure 5B**).

Tolerance to Biotic Stress

In addition to the abiotic stresses, plants are also challenged by various biotic stresses like insect pests and diseases. Several studies have established the role of epigenetic variations in plant-microbe interaction mainly through gene regulation (Boyko and Kovalchuk, 2011; Diez et al., 2014; Zhu et al., 2015; Espinas et al., 2016). Another mechanism affecting the susceptibility of plants to pathogens has been reported which includes methylation of plant and viral genome (Wang et al., 2012; Sharma et al., 2013; Baulcombe and Dean, 2014). The importance of DNA methylation is emerging in transcriptional regulation of the virus-induced genes (Sahu et al., 2013; Ding and Wang, 2015; Wang et al., 2018a). Moreover, plants have evolved several defense mechanisms to cope up with viral infection mainly *via* siRNA-mediated antiviral silencing (Pumplin and Voinnet, 2013; Sharma et al., 2013). The siRNA cleaves/represses translation of the target mRNA and thus causes posttranscriptional gene silencing. Methylation of DNA base and/or histone protein causes gene silencing at the transcriptional level, which is known as transcriptional gene silencing (TGS). While DNA viruses are targeted through TGS to restrict their proliferation (Rodríguez-Negrete et al., 2009), RNA viruses are not influenced by DNA methylation. However, methylation of RNA bases, e.g., N^6-methyladenosine (m^6A; Gokhale et al., 2016; Kumar and Mohapatra, 2021), controls viral replication as well as the interaction between virus and host (Brocard et al., 2017; Dang et al., 2019). Hundreds of DMRs were identified to be influenced by a viral infection in tobacco, several of which were reported to be associated with gene expression to regulate host antiviral defense (Wang et al., 2018b). Plants use methylation of viral genomic DNA to restrict its replication, while virus encodes viral suppressor proteins to protect it from getting methylated (Raja et al., 2010). The viral suppressors might interfere with the host methylation pathways to benefit the virus. A significant variation in methylation at CHH context was observed in *Arabidopsis* in response to different biotic stresses (Slaughter et al., 2012); however, the role of such alteration in DNA methylation in priming the plants against pests/diseases is not known (Wang et al., 2019). Interestingly, evidence shows that stress priming changes the epigenetic profile of plants which may improve the stress tolerance ability of the plant (Lopez Sanchez et al., 2016; Lämke and Bäeurle, 2017; Varotto et al., 2020).

Stress Memory and Adaptation

It has been shown that plants can remember past environmental stress and use the memories to respond rapidly to the stress

when it recurs (Hilker and Schmülling, 2019). Plants have evolved various sensing and signaling mechanisms to respond appropriately to stress. Evidence gathered through various studies indicates that epigenetic variations are necessary as a part of the stress memories and adaptation in plants (Thiebaut et al., 2019). Moreover, plants have also evolved certain mechanisms by which they can memorize the events of past stress and trigger the responses to respond quickly/strongly to recurrent stress. Memorizing the events requires the storage of information, which might occur in plants (without a nervous system) in the form of chromatin architecture, transcription factor, posttranslational modifications, phytohormones, metabolites, etc., involved in the stress management (Xing et al., 2018; Hilker and Schmülling, 2019). The role of epigenetic variations in stress priming/memory is being studied (Crisp et al., 2016; Lämke and Baeurle, 2017; He and Li, 2018). Alterations to H3K4me3, which is a transcription activation mark, suggest that this epigenetic mark plays a role in transcription memory as it is enriched by drought stress and maintained at a higher level during the rehydration process (Kim et al., 2012). The small-RNA may also play a role in stress memory, as reported in the case of repeated drought stress to *Arabidopsis* (Ding et al., 2012). *Arabidopsis* plants with a mutation in *met1* were observed to be resistant to low humidity stress (Tricker et al., 2012). Moreover, a higher stomatal index was detected in *Arabidopsis* mutants for *dcl3* or *rdr6* under low humidity, indicating that RdDM pathway plays a role in remembering the stress. Transgenerational inheritance of epigenetic marks involves passing of the epigenetic changes through the germline without getting erased by the surveillance mechanisms (Lange and Schneider, 2010). Accumulating evidence indicates that short-term memory and transgenerational memories rely on epigenetic mechanisms; hence, they can be utilized in developing climate-smart crop varieties. However, many questions regarding the role of epigenetic marks in keeping the stress memory, their persistence, and stability during mitosis are still unanswered.

Many (~70%) stress-induced epigenetic alterations revert to the original state once the stress disappears, but a part of the epigenetic modifications might be carried forward as epigenetic stress memory (Crisp et al., 2016; Kumar, 2018). Therefore, utilizing such epialleles in breeding programs is still a challenging task because of the transgenerational stability of the environment-induced epigenetic alterations. Zheng et al. (2017) reported a high proportion of drought-induced DNA methylation in rice and maintenance of their pattern in successive generations under drought stress. Such findings suggested that epigenetic modifications can be utilized in improving stress responses of crop plants. Thus, epigenetic manipulation may become an efficient tool for crop improvement, as appropriate strategies are becoming available for the modulation of DNA methylation using chemicals or by genetic means, followed by the forward or reverse epigenetic approach. However, appropriate strategies would be required to ensure retention of the transferred/introduced epialleles in the new genetic environment. Moreover, epigenome editing may help achieve the desired changes and adaptive advantages without entering into the controversy of genetic engineering (Gallego-Bartolomé et al., 2018; Kumar, 2019a).

CONCLUSION AND FUTURE PERSPECTIVES

Much progress has been made in our understanding of epigenetic regulation of gene expression, particularly in model plants like *Arabidopsis* and rice. Proteins and enzymes involved in DNA and histone modifications in plants are being characterized continuously. However, we still know a little about the components controlling targeted DNA (de) methylation during the developmental process and environmental stress. Does DNA modification interplay with other epigenetic marks and affect chromatin conformation are some of the enigmatic questions that need to be answered for a better understanding of epigenomics. Increasing focus on 6-mA as an additional epigenetic mark raises several questions like (1) which AlkB family protein acts as an eraser of 6-mA? (2) Whether/how does adenine methylation-and demethylation machinery interact with histone modification and transcription machinery? (3) Moreover, the readers of the 6-mA mark are yet to be discovered. DNA methylation pattern of different plant species varies because every species possesses a different set of DNA methyltransferases (MET1, CMT2, CMT3, DRM1, and DRM2), demethylases (DME, ROS1, DML2, and DML3), and RdDM pathway to (de) methylate the TEs, repeats, and genes to switch them on/off (Bartels et al., 2018).

Toward epigenetic manipulation, a catalytically inactive SpdCas9 fused with (de)methylase (SpdCas9-Tet1 and SpdCas9-Dnmt3a) was reported to be useful in epigenome editing in a site-specific manner in mammalian cells (Liu et al., 2016). In a recent study, on the development of the tool for targeted DNA demethylation in plants, Gallego-Bartolomé et al. (2018) reported efficient and targeted demethylation with minimal off-target effects in plant. This can also be used to answer some of the basic questions in epigenomics, to develop new strategies for modulating gene expression, and to create a new epiallele for the desired trait in plants. Gallego-Bartolomé et al. (2018) utilized fusion of the catalytic domain of human demethylase (TET1cd) with an artificial zinc finger targeting promoter of the *Flowering Wageningen* (*FWA*) resulting in efficient and targeted demethylation, *FWA* upregulation, and heritable late-flowering phenotype in *Arabidopsis*. Recently (Taghbalout et al., 2016) used Casilio-ME for RNA-guided editing of 5-mC by targeting TET1 to specific genomic sites, and co-delivery of TET1 and other protein factors, to activate methylation-silenced genes. Similarly, Devesa-Guerra et al. (2020) fused the catalytic domain of ROS1 5-mC DNA glycosylase with dCas9 and reported that dCas9-ROS1 (but not the dCas9-TET1) can reactivate methylation-silenced genes by active demethylation.

With the advances in epigenomic tools and techniques, it can be expected that very soon we might be able to use epigenome editing to modulate phenotypic plasticity of plants (Kumar, 2019a) toward developing climate-smart crops for sustainable agriculture.

AUTHOR CONTRIBUTIONS

SK and TM conceived the review. SK prepared the preliminary draft. SK and TM revised the manuscript and approved the final draft. All authors contributed to the article and approved the submitted version.

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Direct and Indirect Transcriptional Effects of Abiotic Stress in *Zea mays* Plants Defective in RNA-Directed DNA Methylation

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Plants respond to abiotic stress stimuli, such as water deprivation, through a hierarchical cascade that includes detection and signaling to mediate transcriptional and physiological changes. The phytohormone abscisic acid (ABA) is well-characterized for its regulatory role in these processes in response to specific environmental cues. ABA-mediated changes in gene expression have been demonstrated to be temporally-dependent, however, the genome-wide timing of these responses are not well-characterized in the agronomically important crop plant *Zea mays* (maize). ABA-mediated responses are synergistic with other regulatory mechanisms, including the plant-specific RNA-directed DNA methylation (RdDM) epigenetic pathway. Our prior work demonstrated that after relatively long-term ABA induction (8 h), maize plants homozygous for the *mop1-1* mutation, defective in a component of the RdDM pathway, exhibit enhanced transcriptional sensitivity to the phytohormone. At this time-point, many hierarchically positioned transcription factors are differentially expressed resulting in primary (direct) and secondary (indirect) transcriptional outcomes. To identify more immediate and direct MOP1-dependent responses to ABA, we conducted a transcriptomic analysis using *mop1-1* mutant and wild type plants treated with ABA for 1 h. One h of ABA treatment was sufficient to induce unique categories of differentially expressed genes (DEGs) in *mop1-1*. A comparative analysis between the two time-points revealed that distinct epigenetically-regulated changes in gene expression occur within the early stages of ABA induction, and that these changes are predicted to influence less immediate, indirect transcriptional responses. Homology with MOP1-dependent siRNAs and a gene regulatory network (GRN) were used to identify putative immediate and indirect targets, respectively. By manipulating two key regulatory networks in a temporal dependent manner, we identified genes and biological processes regulated by RdDM and ABA-mediated stress responses. Consistent with mis-regulation of gene expression, *mop1-1* homozygous plants are compromised in their ability to recover from water deprivation. Collectively, these results indicate transcriptionally and physiologically relevant roles for MOP1-mediated regulation of gene expression of plant responses to environmental stress.

Keywords: abiotic stress, RNA-directed DNA methylation, mop1, abscisic acid, *Zea mays*, epigenetics, drought

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INTRODUCTION

The sessile nature of plants and their adaptation to terrestrial environments coincided with the evolution of whole plant and molecular responses to fluctuating environmental conditions (reviewed by Gupta et al., 2020). Extreme abiotic environments, including water scarcity, often lead to yield loss in agricultural crop plants across the globe (FAO, 2017). When osmotic stress is first detected, the initial and immediate whole plant response is often the closure of stomata, which allows the plant to conserve water within its tissues, while limiting the energy and resources expended in biological processes such as photosynthesis. More prolonged drought conditions result in responses that often limit plant growth, and are associated with developmental defects in reproductive organs, thus decreasing yield. Indeed, it has long been documented that *Zea mays* (maize) plants that experience drought stress exhibit reduced yield and the overall effects depend on the specific developmental stage at the time that stress is experienced (Claassen and Shaw, 1970). From a molecular perspective, recent studies demonstrate that whole plant responses are related to the disruption of gene regulatory networks and concomitant changes to stress-response transcriptional programs (Van den Broeck et al., 2017).

Changes in transcription at stress-responsive loci are often associated with genome-wide structural changes to chromatin that affect gene expression and can be detected as alterations in chromatin accessibility (Kim et al., 2015, reviewed by Chang et al., 2020). These changes are strongly influenced by the plant phytohormone abscisic acid (ABA), an important signaling molecule that is responsible for many processes throughout the life cycle of plants such as regulating several important stages of development, including seed germination, ABA synthesis, and signaling, and serves as a critical step in plant response to specific abiotic stress stimuli (reviewed by Ma et al., 2018, and Takahashi et al., 2020). In response to ABA, activation by phosphorylation of *trans*-acting factors initiates broad scale changes in gene expression, creating a hierarchical response that includes a combination of primary, secondary, and later stage *cis* and *trans*-acting responses at the molecular level (reviewed by Takahashi et al., 2018). It has also been observed that certain transcriptional changes in maize in response to and throughout recovery from drought stress is associated with differential enrichment for specific histone modifications (Forestan et al., 2020), and that differential DNA methylation is associated with water stress response in ABA-deficient maize mutants (Sallam and Moussa, 2021), further suggesting the overlapping regulation of ABA signaling and chromatin-mediated gene expression changes in plant stress responses. The coordinated effect of this multi-dimensional response can create whole-plant responses that originate from a molecular signal triggered by an environmental or developmental cue.

Activated *trans*-acting factors differentially regulate target chromosomal sequences, depending in part on the structure of chromatin at *cis*-regulatory elements (reviewed by Wang and Qiao, 2020). For example, evidence suggests that transcription factor binding is influenced by DNA (cytosine) methylation, although these mechanisms are not completely understood for a broad range of transcription factors (reviewed by Heberle and Bardet, 2019). Our recent investigations in maize seedlings indicates that genotypes defective in RNA-dependent DNA methylation (RdDM), a plant-specific epigenetic regulatory pathway, respond to exogenous ABA at the transcriptional level in a manner distinct from wild type plants (Vendramin et al., 2020). Genotype-specific changes in CHH (H = A, T or C) methylation were also observed at some loci transcriptionally responsive to ABA (Vendramin et al., 2020), which is consistent with prior observations for targets of RdDM (Gent et al., 2014). While this indicates that there is a relationship between transcriptional regulation by RdDM and ABA-mediated responses in maize, this association does not clearly distinguish between causality, dependence or coincidence. Interpretation is confounded by the fact that each regulatory network (ABA and RdDM) has primary and cascading indirect effects influencing to gene expression and methylation.

With regards to hormone signaling in response to environmental stress stimuli, time course experiments are a useful way to elucidate hierarchical relationships in complex regulatory networks, as the primary responses are generally expected to be triggered immediately following the stimulus, and the secondary and other downstream responses may require some time to occur. Time course analysis of ABA-regulatory networks in the model plant *Arabidopsis thaliana* suggest that ABA responsive changes in gene expression may be spread across an initial response period from 1 to 8 h after exposure to exogenously applied ABA (Song et al., 2016). To better understand the specific regulatory relationships of epigenetic gene regulation and abiotic stress responses in plants, changes in gene expression in maize plants that were either wild type or defective in RdDM were compared after 1- or 8-h exposure to exogenous ABA. Because these early responses can have long-term developmental and physiological effects on stressed plants, we also investigated the whole-plant responses of plants defective in RdDM to a severe drought simulation by withholding water for 14 days.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Maize (*Zea mays*) plants with the *mop1-1* mutation introgressed into the B73 inbred as previously described (Madzima et al., 2014) were used for this analysis. Homozygous wildtype (*Mop1* WT) and homozygous mutant (*mop1-1*) sibling progeny resulting from the self-pollination of an ear of a heterozygous plant were used. Seedlings were genotyped as previously described (Madzima et al., 2014).

For abscisic acid treatment of maize seedlings: Seedlings were grown in greenhouse conditions (16 h light period, 25°C, 50% humidity) in the Department of Biological Science at Florida State University (319 Stadium Drive) until they reached the V3 stage. At the V3 stage, maize seedlings were removed from the soil, roots were rinsed in water, dried, and then submerged in a 1 L beaker with 250 mL of liquid Murashige and Skoog (MS) media (Sigma Aldrich, M6899) with 50 μM ABA [ABA; (Sigma Aldrich, (+/–) Abscisic Acid, A1049)] or without ABA (MS) for

1 h (this study) or 8 h (Vendramin et al., 2020) in greenhouse conditions. After the incubation period, roots where removed and seedlings were immediately flash frozen in liquid nitrogen and stored at −80°C until use.

For severe drought simulation on maize plants: Plants were grown in greenhouse climate-controlled conditions (25°C, 50% humidity) at the Florida State University Mission Road Research Facility, Tallahassee, Florida, USA in January of 2017. B73 seeds were sown alongside as a control for drought response. Healthy B73, homozygous *Mop1*, and homozygous *mop1-1* seedlings were then transplanted into 300 size pots and later into 2,000 size pots ∼35 days after sowing (DAS). Plants were randomly assigned to severe drought treatment (water withheld for 14 days) or normally watered groups. B73 (11 plants), *Mop1* (11 plants), and *mop1-1* (16 plants) individuals were in the normally watered control group and *B73* (10 plants), 16 *Mop1*(16 plants), and *mop1-1* (11 plants) individuals were in the drought treated group. The non-uniformity in sample number per category was due to premature death for a few individuals. Drought-treatment began once the individual plant reached the V6 stage to control for variation in development between samples/genotypes. After 14 days, plants entered the recovery phase by application of 7.5 L of water to the soil. After recovery, plants from the drought treated group were normally watered throughout the duration of the experiment. The tip of the V8 leaf (∼4 cm) was dissected from the maize plants, immediately frozen in liquid nitrogen, and stored at −80°C until use.

Physiological Observations of Drought-Responsive Traits

All observations were made daily between the hours of 10:00 and 14:00. The growth stages of individual plants were determined using the leaf collar method (Nielsen, 2019). Plant growth was monitored beginning after seed germination (VE) and continued through the last collared leaf below the tassel ($\leq$ V18). Daily observations were made to track the emergence of ears, tassels, silks, pollen shed, and the anthesis-silking interval (ASI) which is defined as the number of days between the first pollen shed and silk emergence (Bolanos and Edmeades, 1996). We determined the "effective tassel branch score" by inspecting each tassel and determining the ratio of functional tassel branches (branches with anthers) to total tassel branches (reported as a percentage). We determined the plant height at 90 DAS by measuring the length between the first node above the soil and the tip of the longest tassel branch. We determined the average internodal length by measuring the internodal distance of the three apical internodes above the V4 leaf node.

Total RNA Isolation, RNA Library Preparation and RNA-Sequencing

Total RNA was extracted as previously described (Vendramin et al., 2020). Briefly, frozen tissue was finely ground into powder in liquid nitrogen and homogenized before total RNA extraction was performed using TRI reagent® according to manufacturer's instructions (Molecular Research Center, 18080-051). RNA samples were DNase treated (RQ1 RNase-Free DNase, Promega, M6101) and purified using RNA clean and concentrator^{TM} 25 (Zymo Research, R1018).

Three biological replicates were used for all RNA-seq experiments for each treatment and genotype, for a total of 12 samples per time point: 1 h (this study) and 8 h (Vendramin et al., 2020). The final sample concentration was quantified by Qubit. RNA library preparation (NEBNext® Ultra^{TM} II kit, NEB, E7760) and Illumina paired-end 150 bp (PE 150) sequencing were performed by Novogene Corporation (Sacramento, California). The 1 h samples were sequenced using the Illumina NovaSeq 6000 platform, whereas the previous reported 8 h samples (Vendramin et al., 2020) were sequenced using the Illumina HiSeq 2,500 platform. More than 20 million reads were obtained per library.

Read Alignment, Batch Correction and Differential Gene Expression Calling

Bioinformatics analysis was performed by Linkage Analytics, LLC (Denver, CO). To ensure a consistent and re-producible computation environment, the workflow was containerized using Singularity (3.6.4) (Kurtzer and Sochat, 2017) and the data workflow steps were defined using Snakemake (5.30.1) (Koster and Rahmann, 2018) and read quality control was assessed using fastp (0.21.0) (Chen et al., 2018). Reads from the 1 h and 8 h sequencing batches were processed simultaneously. FASTQ adapters were trimmed by Cutadapt 1.8.1 (Martin, 2011) Reads were mapped to the B73 maize genome (AGP B73v4) (Jiao et al., 2017) by HISAT2 v2.2.1 (Pertea et al., 2016). Transcripts were assembled *de novo* to allow for inclusion of transcripts that are not included in the reference genome annotation and quantified using StringTie v2.1.4 (Pertea et al., 2016). Gene count matrices were generated from this data using the prepDE.py python script available in the StringTie website (http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual). These matrices were used by the Bioconductor package edgeR 3.28.1 (Chen et al., 2016) in R for differential expression analysis in order to identify upregulated and downregulated genes for the four different genotypes under two treatments. Low-abundance counts of $<$ 0.58 cpm were removed using the DESeq2 filtering method (statquest.org/2017/05/16/statquest-filtering-genes-with-low-read-counts/); (Love and Huber, 2014) incorporated into the edgeR pipeline, and genes with an adjusted p-value of $\leq$ 0.05 and an absolute $\log_2$-fold change (FC) value of $\geq$ 0.95 were considered as differentially expressed for both upregulated and downregulated genes.

Gene Ontology Analysis and Hierarchical Clustering of Significantly Enriched GO Terms and DEGs

Singular Enrichment Analysis (SEA) was performed using the web-based tool agriGO v2.0 (Tian et al., 2017) with the B73 reference version 4 (AGOv4) gene annotations to determine enriched gene ontology terms (GO complete) associated with differentially expressed genes.

Fisher's statistical test, Hochberg (FDR) multi-test adjustment method with a significance level of $<$ 0.05 and minimum number

TABLE 1 | Summary of RNA-seq libraries and read mapping per time-point, genotype, and treatment.

Timepoint	Genotype & Treatment	Replicate	Total raw reads	HISAT2 slope filter threshold*	Mapped reads	% Mapped reads	Uniquely mapped reads	% Uniquely mapped reads
1 h (this study)	*mop1-1* mutant ABA	1	32349928	−0.2	32183321	98.2	29348167	91.19
		2	23272446	−0.2	23161089	98.29	21341515	92.14
		3	33691278	−0.2	33515782	98.3	30773769	91.82
	mop1-1 mutant control	1	35425161	−0.2	35251763	98.31	32054938	90.93
		2	36809285	−0.2	36630297	98.31	33089359	90.33
		3	33801811	−0.2	33627611	98.26	30993247	92.17
	Mop1 WT ABA	1	32652171	−0.2	32489954	98.34	29886735	91.99
		2	37341781	−0.2	37146123	98.23	34191248	92.05
		3	30156617	−0.2	30004706	98.22	27603302	92
	Mop1 WT control	1	31505710	−0.2	31337674	98.27	28676534	91.51
		2	36734172	−0.2	36545051	98.33	33554582	91.82
		3	35711598	−0.2	35537773	98.42	32637773	91.84
8 h Vendramin et al. (2020)	*mop1-1* mutant ABA	1	21775951	−0.6	21329876	97.3	19336347	90.65
		2	22373108	−0.6	21981634	96.91	19388351	88.2
		3	21583557	−0.6	21109587	96.84	18349596	86.93
	mop1-1 mutant control	1	24469219	−0.6	23915909	96.38	20602662	86.15
		2	24142476	−0.6	23706188	97.65	21382476	90.2
		3	23023844	−0.6	22521807	95.33	19520082	86.67
	Mop1 WT ABA	1	22263417	−0.6	21821952	96.02	18855244	86.4
		2	23131739	−0.6	22765701	96.49	20024820	87.96
		3	21771139	−0.6	21360341	96.87	18914163	88.55
	Mop1 WT control	1	22307497	−0.6	21875147	96.53	18844702	86.15
		2	22220268	−0.6	21837022	95.8	18620624	85.27

** HISAT2 filters reads based on a threshold defined by the slope a linear function between mapping quality score and read length. See **Supplementary Figure 1** and the HISAT2 manual entry for "–score-min" for details.*

TABLE 2 | Analysis Groups for 1 h.

Pair-wise comparison	Analysis group	Expression pattern	Significant[a] DEGs	2FC Significant[a] DEGs	Total Significant[a] DEGs	Total 2FC Significant[a] DEGs
Mutant ABA vs. Mutant MS at 1 h	A_UP	upregulated	11	9	97	72
	A_DOWN	downregulated	86	63		
WT ABA vs. WT MS at 1 h	B_UP	upregulated	22	21	66	61
	B_DOWN	downregulated	44	40		
Mutant ABA vs. WT ABA at 1 h	C_UP	upregulated	882	646	1,849	1,171
	C_DOWN	downregulated	967	525		
Mutant MS vs. WT MS at 1 h	D_UP	upregulated	413	395	604	552
	D_DOWN	downregulated	191	157		
Total DEGs					2,616	1,856
Number of DEGs in more than one analysis group					871 (33%)	737 (40%)
Number of DEGs in only one analysis group					1,745 (67%)	1,119 (60%)

[a] *Significant genes are DEGs with a p-value and FDR, 0.05.*

of mapping entries of 10 genes per GO-term. The GO term enrichment was generated by hierarchically clustering the log10 of the total GO term percentage of a set of genes that were upregulated or downregulated in wildtype or mutant in response to ABA.

RESULTS

Early ABA Treatment Is Sufficient to Induce Unique Categories of Differently Expressed Genes in *mop1-1* Mutants

To identify genes that are immediately responsive to epigenetic regulation under abiotic stress conditions, RNA from maize seedlings exposed to 1h of abscisic acid (ABA) and nutrient solution without ABA (MS) in *mop1* wildtype (WT) and mutant (*mop1-1*) genotypes was subjected to RNA-sequencing (RNA-seq) and transcriptome analysis as previously described (Vendramin et al., 2020). An average of ∼33 million 150 bp paired end raw reads were obtained per sample (**Table 1**) and mapped to the B73 maize genome (AGP B73v4) (Jiao et al., 2017). Significant differentially expressed genes (DEGs) between *mop1* genotypes and 1h ABA treatments were categorized into four pairwise comparisons designated "analysis groups" (1h Groups A–D; **Table 2**) as genes with a two-fold expression change ($\log_2$ FC $\geq$ 0.95, FDR $<$ 0.05) and identified by making direct comparisons between genotypes and treatments (**Table 2**). The DEGs in the four analysis groups were further sub-divided based on gene expression patterns (up- or down-regulated; e.g., 1h A-up and 1 h A-down) (**Table 2**; File 1) and subjected to further analysis.

The total number of significant DEGs with two-fold change (2FC) in expression identified in the four analysis groups after 1 h of ABA-induction (1 h Groups A–D) included 1,856 genes, where, 737 (40%) of these genes were found to be common to more than one group, resulting in 1,119 (60%) DEGs unique to an individual analysis group (**Table 2**; **Figure 1**). After 1 h of ABA induction, only ∼7% of the total 2FC DEGs were differentially expressed in WT and *mop1-1* genotypes relative to their own control (1 h Groups A and B). These transcriptional responses are genotype-specific as there was also almost no overlap between the DEGs identified in each of these analysis groups (1 h Groups A and B) (**Figure 1A**). The majority of DEGs (63%) were identified in comparisons that included both genotype and treatment (**Table 2** Group C*; mop1-1* ABA/WT ABA). A comparison between Group C DEGs with control plants of the same genotypes (*mop1-1* MS/WT MS from Group D), revealed 417 and 451 up- and down-regulated genes, respectively, that are uniquely responsive to ABA treatment and loss of MOP1 activity (**Figure 1B**). These 868 genes were subjected to a more in-depth analysis to identify primary and indirect MOP1 specific targets.

Gene ontology (GO) analysis was used to predict the biological processes of all annotated genes in each of the four analysis groups (1h Groups A-D; FDR $<$ 0.05). As expected, the GO term for response to stimulus (GO:0050896) was highly enriched in all 1 h analysis groups, except for the comparison constituting a genotype control of mutant and wild type plants treated with MS (Group D; **Figure 1C**). The diversity of enriched DEGs was enhanced in *mop1-1* mutants subjected to ABA (1 h Groups A and C) relative to WT plants (Group B) or the genotype control (Group D) (**Figure 1C**). These *mop1-1* ABA unique categories include biological processes associated with cell growth and size (**Figure 1C**).

In *Mop1-1* Mutants, the Most Distinct Changes in Gene Expression Occur Within the Early Stages of ABA Induction

To identify genes that respond to ABA and MOP1 in a temporal manner, the mapped reads from RNA-seq after 1 h (this study) and 8 h (Vendramin et al., 2020) of ABA induction were simultaneously, bioinformatically processed and mapped to the B73 reference genome (AGP B73v4) (Jiao et al., 2017) and used in subsequent analysis (**Tables 1**–**3**). Due to the differences in sequencing depth as a result of use of different Illumina sequencing platforms (HiSeq vs. NovaSeq) between the two timepoints, we normalized the read quality score threshold used in HISAT2 ("–score-min") between platforms. Based on consistency between replicates as well as differences in distributions of mapping qualities between sequencing platforms, the HISAT2 "–score-min" parameter was chosen to normalize the number of uniquely mapped reads across datasets (**Table 1**; **Supplementary Figure 1**).

Predictably, the overall number of DEGs increases with increasing time. Eight h of ABA treatment resulted in more genes exhibiting differential expression, but most of the genes were detected in multiple analysis groups, resulting in a lower percentage of DEGs being unique to one analysis group at 8 h (27%) compared to 60% unique DEGs observed after 1 h of ABA-induction (**Tables 2**, **3**). Consistently, while there was almost no overlap between wildtype and mutants DEGs in 1 h Groups A and B (**Figure 1**), there was more overlap between Groups A and B after 8 h of ABA treatment (**Supplementary Figure 2**; **Supplementary Table 2**). For analysis groups C, there were more significant DEGs at 1 h of ABA treatment, compared to the same comparison after 8 h (**Tables 2**, **3**). This suggests that the most distinct changes in gene expression between these genotypes occurs within the early stages of ABA induction.

Genes from the 8 h and 1 h samples were directly compared with each other (8 h/1 h) and categorized into four different pairwise comparisons, designated Groups E–H (**Table 4**). The total number of significant 2FC DEGs identified in these four 8 h/1 h analysis groups was 34,147 genes, representative of the magnitude of changes in gene expression that occur over time. However, 29,610 (87%) genes were found to be common to more than one analysis group, where only 4,537 (13%) were found to be unique to an individual group (**Table 4**; **Supplementary Figure 3**). This observation is consistent with the similarities in the enriched GO terms per group (**Figure 2**), with the least

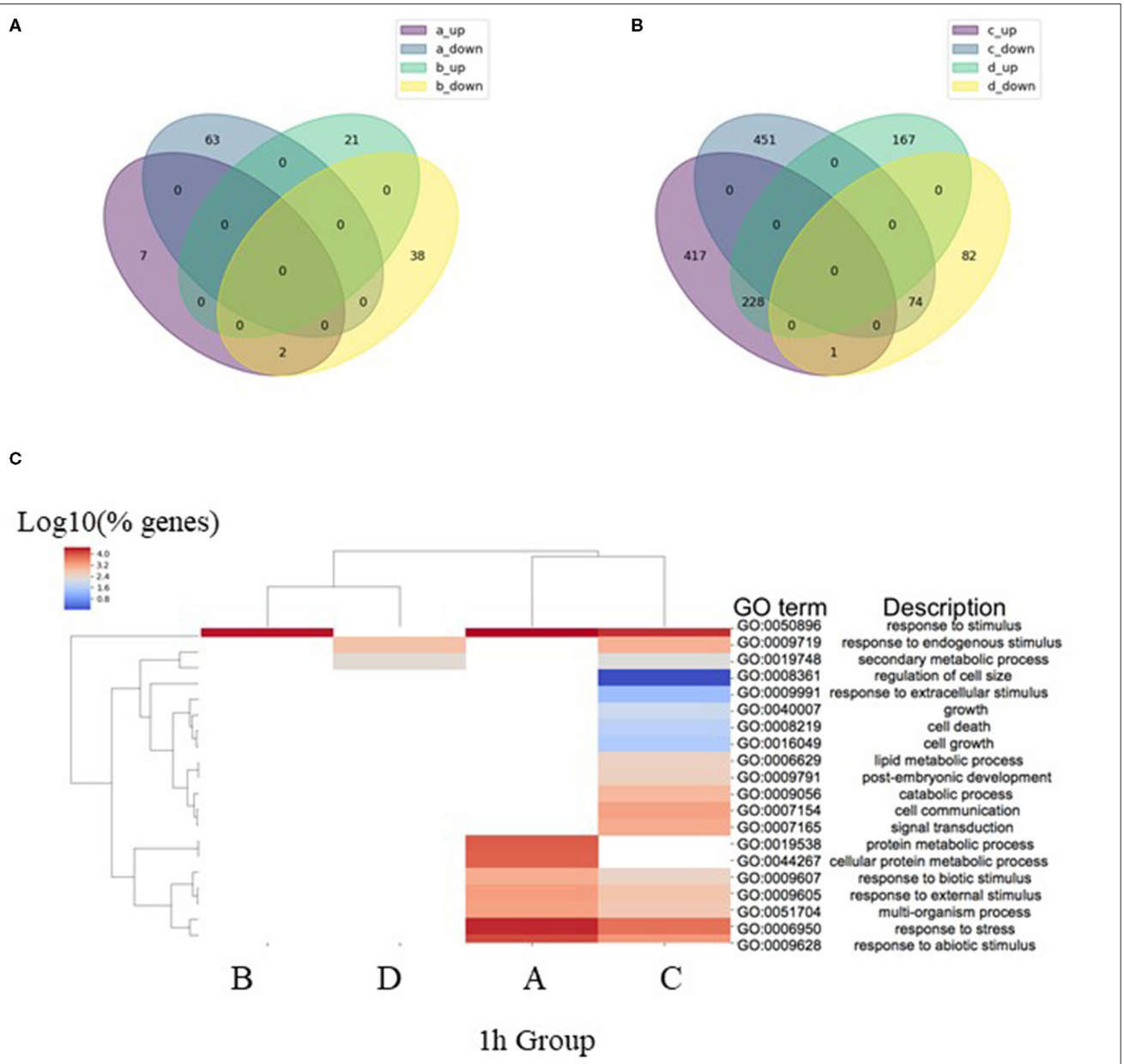

FIGURE 1 | Analysis of differentially expressed genes (DEGs) after 1 h ABA treatment. **(A)** Venn diagram of overlap in the identity of DEGs (log2 FC ≥ 0.95, FDR < 0.05) after 1 h treatment with ABA compared to control in homozygous mutant (a_up and a_down) and wild type plants (b_up and b_down). **(B)** Venn diagram of overlap in the identity of DEGs (log2 FC ≥ 0.95, FDR < 0.05) in homozygous mutant compared to wild type plants after 1 h treatment with ABA (c_up and c_down) or 1 h treatment with MS (d_up and d_down). **(C)** Comparisons of enriched biological process (BP) Gene Ontology (GO) terms in DEGs were made between 1 h analysis Groups A–D (**Table 2**), representing DEGs identified in pairwise comparisons homozygous mutants treated with ABA or MS (Group A), wild type plants treated with ABA or MS (Group B), 1 h of ABA treatment for homozygous mutant or wild type plants (Group C) and 1 h treatment with MS for homozygous mutants or wild type plants (Group D). Heatmap illustrates hierarchical clustering of log10 (% genes) of significant GO terms enriched in each expression comparison (FDR < 0.05, minimum of 10 genes per GO term). No color (white) indicates that there was no enrichment for the GO term in the dataset.

diverse biological processes observed in the wildtype control group (Group H). GO terms associated with biological regulation (GO:0065007), regulation of biological process (GO:0050789) and response to stimulus (GO:0050896) were commonly highly represented terms across all groups (**Figure 2**).

MOP1-Dependent siRNAs and Gene Regulatory Networks (GRNs) Predict Immediate and Indirect Responses to Abiotic Stress

To distinguish between primary and indirect targets of epigenetic regulation under abiotic stress conditions, the 868 genes (451

TABLE 3 | Analysis Groups for 8 h.

Pair-wise comparison	Vendramin et al. Analysis group	Analysis group	Expression pattern	Significant[a] DEGs	2FC Significant[a] DEGs	Total Significant[a] DEGs	Total 2FC Significant[a] DEGs
Mutant ABA vs. Mutant MS at 8 h	V	A_UP	upregulated	2,229	1,100	4,924	2,550
	VI	A_DOWN	downregulated	2,695	1,450		
WT ABA vs. WT MS at 8 h	I	B_UP	upregulated	1,530	957	3,145	1,903
	II	B_DOWN	downregulated	1,615	946		
Mutant ABA vs. WT ABA at 8 h	VII	C_UP	upregulated	510	448	796	609
	VIII	C_DOWN	downregulated	286	161		
Mutant MS vs. WT MS at 8 h	III	D_UP	upregulated	354	354	458	456
	IV	D_DOWN	downregulated	104	102		
	Total DEGs					9,323	5,518
	Number of DEGs in more than one analysis group					5,820 (62%)	3,986 (72%)
	Number of DEGs in only one analysis group					3,503 (38%)	1,532 (27%)

[a]*Significant genes are DEGs with a p-value and FDR, 0.05.*

TABLE 4 | Analysis Groups for 8 h vs. 1 h.

Pair-wise comparison	Analysis group	Expression pattern	Significant[a] DEGs	2FC Significant[a] DEGs	Total Significant[a] DEGs	Total 2FC Significant[a] DEGs
Mutant ABA 8 h vs. Mutant ABA 1 h	E_UP	upregulated	7,201	5,483	13,381	7,989
	E_DOWN	downregulated	6,180	2,506		
Mutant MS 8 h vs. Mutant MS 1 h	F_UP	upregulated	7,401	5,726	14,587	9,133
	F_DOWN	downregulated	7,186	3,407		
WT ABA 8 h vs. WT ABA 1 h	G_UP	upregulated	8,188	5,674	16,306	8,621
	G_DOWN	downregulated	8,118	2,947		
WT MS 8 h vs. WT MS 1 h	H_UP	upregulated	6,039	5,273	10,674	8,404
	H_DOWN	downregulated	4,635	3,131		
Total DEGs					54,948	34,147
Number of DEGs in more than one analysis group					49,465 (90%)	29,610 (87%)
Number of DEGs in only one analysis group					5,483 (10%)	4,537 (13%)

[a]*Significant genes are DEGs with a p-value and FDR, 0.05.*

up- and 417 down-regulated) identified to be uniquely associated with ABA treatment and loss of MOP1 activity (Group C; **Figure 1B**) were further analyzed for a specific connection with MOP1-mediated RdDM. MOP1 is required for the production of the majority of siRNAs at loci undergoing RdDM (Gent et al., 2014), therefore these genes were compared with a list of genes having promoter homology with MOP1-dependent siRNAs (Vendramin et al., 2020). This comparison identified 97 up- and 76 down-regulated genes from 1 h Group C that are predicted to be direct MOP1-regulatory targets based on homology with siRNAs (**Figure 3**; **Supplementary Table 3**), suggesting that MOP1-mediated RdDM is involved in early responses to ABA at these specific genes. It is plausible that these 173 genes are primary targets of MOP1 that in turn influence downstream gene expression in response to ABA. Because these genes are differentially responsive in *mop1-1* plants very early after ABA treatment, these genes were designated as MOP1-dependent immediate responsive genes (MIMs). Gene ontology analysis of these genes revealed that there were more significant (FDR < 0.05) enriched GO terms associated with the 97 up-regulated genes compared with the 76 down-regulated genes (**Table 5**). This suggests that in

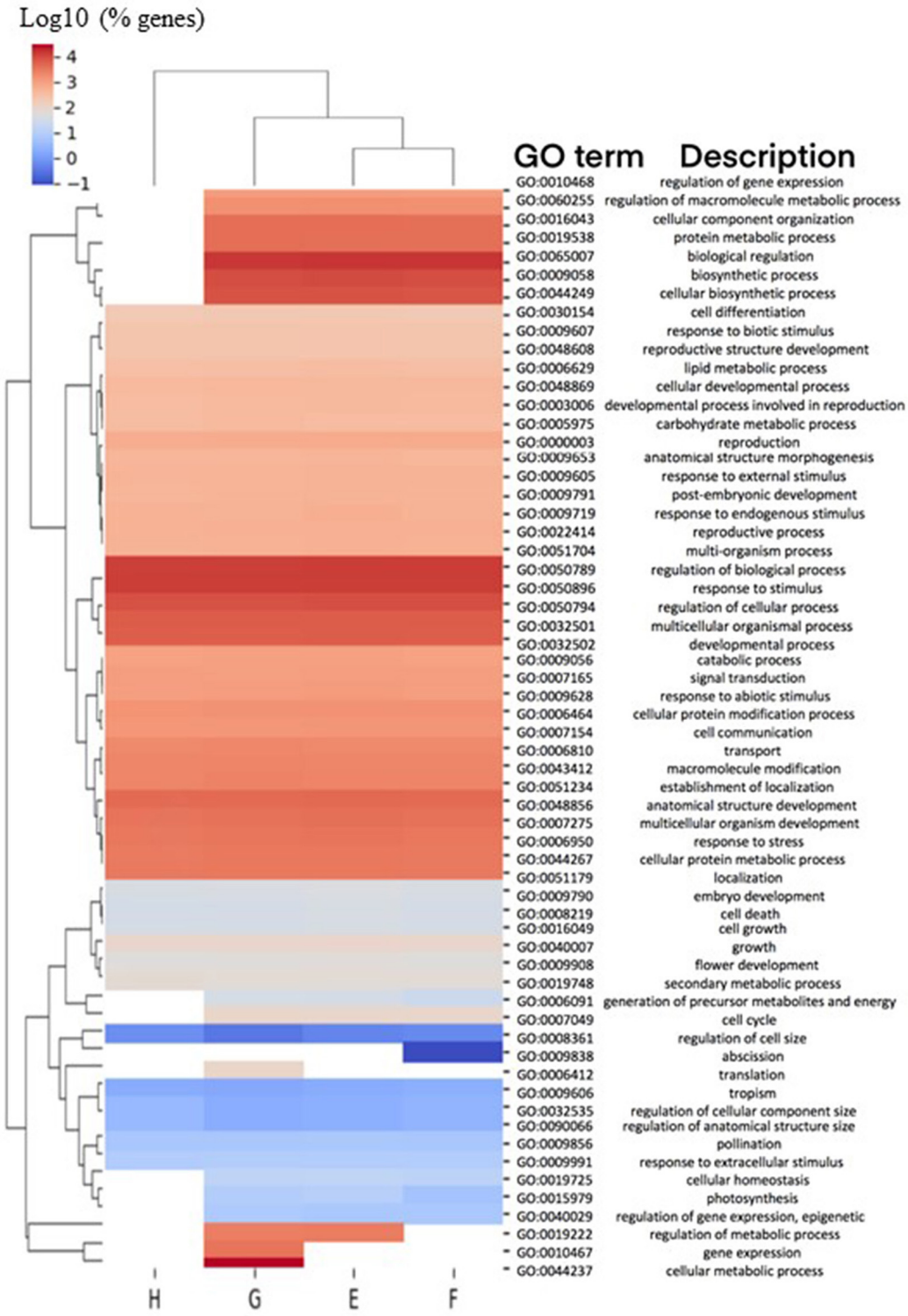

FIGURE 2 | Biological processes associated with DEGs in 8 h/1 h comparisons. Comparisons of enriched biological process (BP) Gene Ontology (GO) terms in DEGs were made between 8 h/1 h analysis Groups E-H (**Table 4**), representing DEGs from pairwise comparisons between homozygous mutant plants treated with ABA for 8 h or 1 h (Group E), homozygous mutant plants treated with MS for 8 h or 1 h (Group F), wild type plants treated with ABA for 8 h or 1 h (Group G), and wild type plants treated with MS for 8 h or 1 h (Group H). Heatmap illustrates hierarchical clustering of log10 (% genes) of significant GO terms enriched in each expression comparison (FDR < 0.05, minimum of 10 genes per GO term). No color (white) indicates that there was no enrichment for the GO term in the dataset.

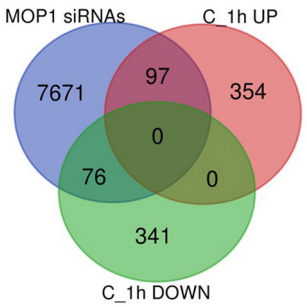

FIGURE 3 | Identification of unique Group C genes with homology with MOP1-dependent siRNAs. Venn diagram of overlap between the identities of 451 upregulated (C_1h UP) and 417 downregulated (C_1h DOWN) genes in homozygous mutant plants treated for 1 h with ABA versus wild compared with genes with homology with MOP1-dependent 24nt siRNAs within their promoters (MOP1 siRNAs; Vendramin et al., 2020).

response to ABA, MOP1-dependent activity and siRNAs are directly associated with regulation of specific biological processes, whereas the siRNAs associated with downregulated genes (MOP1-independent) may be indirect, not RdDM targets and/or have less specific biological roles in relation to ABA responses.

To understand how MIMs potentially influence downstream transcriptional responses to 1 h ABA treatment in maize, a gene regulatory network (GRN) (Huang et al., 2018) was used to predict targets of these 97 and 76 up- and down-regulated genes, respectively. Twenty one of the 97 (∼22%) upregulated genes and 5 of the 76 (∼7%) downregulated genes had predicted regulatory targets based on the GRN, and the majority of these 26 genes are transcription factors implicated in drought, ABA and stress responses based on homology and phenotypic characterization in other studies (**Table 6**). The predicted GRN targets of the 21 Group C 1 h upregulated MIMs included a total of 16,748 genes and the predicted GRN targets of the 5 Group C 1 h down-regulated MIMs included a total of 4,221 genes across all tissues types and datasets in the GRN (**Table 6**; File 3). Some of these genes (∼14%) were duplicated in the two lists of targets predicted by the GRNs and overall there were 18,014 unique target genes. These targets predicted by the GRN could be considered indirect (secondary or more downstream) targets of MOP1 responsive factors, because they are predicted to be regulated by genes with evidence of direct MOP1-mediated regulation. This group of genes were collectively designated as MOP1-dependent indirectly responsive genes (MINs).

The 18,014 MINs (**Table 6**; File 3) were compared with genes in analysis groups E and G (**Table 4**), representing genes differentially expressed in a temporal manner (8 h/1 h) after treatment with ABA in mutant and wild type, respectively (**Figure 4A**). This analysis revealed that in the mutant genotype, 54% of upregulated and 42% of downregulated genes with expression changes from 1 h to 8 h in the presence of ABA were identified as MINs (**Figure 4A**). A similar comparison in wild type identified that 52% of upregulated and 42% of downregulated were identified as MINs (**Figure 4B**). This initial observation suggests that over time, there is a similar magnitude of indirect effects in MOP1-regulated targets between mutant and wild type plants subjected to abiotic stress stimuli. However, further analysis revealed qualitative and functional differences in DEGs, as the identity of genes did not completely overlap (**Figure 4C**). Specifically, there were 689 (44% from wildtype (G) and 51% from mutant) putative indirect DEGs between 1 h and 8 h of ABA treatment common to both genotypes (**Figure 4C**; **Supplementary Table 4**). A gene ontology analysis was used to identify genes that fall into the three categories (**Figure 4D**) and GO terms associated with response to stimulus were conserved in these three categories. It appears that *mop1-1* mutants are not expressing some of the developmental genes required for MOP1-mediated responses to ABA. This comparison of DEGs between mutant and wild type may be indicative of the role of MOP1 in maize development in response to some abiotic stress stimuli. This association is also supported by recent work in other labs, indicating a role for RdDM or other chromatin-mediated regulatory events in stress response in maize (Forestan et al., 2016, Forestan et al., 2017, Forestan et al., 2020).

MOP1 Is Required for Recovery From Drought Stress

To determine the role of MOP1 in drought stress response and recovery at the whole-plant level, we characterized the vegetative and reproductive developmental consequences of a severe drought treatment (14-days without watering) on *Mop1* WT, *mop1-1*, and B73 plants. Initially, all plants were watered normally and showed no significant differences in growth rate until reaching the V6 stage (Nielsen, 2019) when drought treatment was applied to randomly selected individuals from each group (**Figure 5**). We controlled for growth rate differences between individuals by beginning the drought treatment when an individual reached the V6 stage (auricle exposed). The growth rate was significantly delayed among all drought-treated plants compared to normally-watered controls, with *mop1-1* drought-treated plants taking the longest time to reach the V7 stage (**Figure 5**). After 14 days of drought treatment, water (7.5 L) was given to each plant and plants were normally watered throughout the duration of the experiment. While B73 and *Mop1* drought-treated plants recovered rapidly and approached the growth rate curve of normally-watered controls, *mop1-1* drought-treated plants significantly lagged behind, with several plants failing to reach reproductive competency (**Figure 5A**). Normally-watered *mop1-1* plants showed no significant differences in growth rates compared to normally-watered B73 and *Mop1* suggesting that MOP1 is required to recover from drought stress.

To determine the effects loss of MOP1 during drought had on reproductive development, we made observations for plant height at maturity, internodal length, ear emergence, number of ears, effective tassel branches, and the anthesis-silking interval (ASI) (**Figure 5B**; **Supplementary Figure 4**). Because stunted plant height is indicative of severe stress during vegetative development, we measured the average internodal length and the heights of plants at 90 days after sowing (DAS). Drought-treated plants were stunted and had reduced internodal lengths compared to normally-watered

TABLE 5 | GO terms for biological processes associated with Group C 1 h genes with homology with MOP1 dependent siRNAs.

GO Accession	GO term	Number of genes	Query total	*p*-value	FDR
Group C_1h_upregulated					
GO:0009628	response to abiotic stimulus	34	97	3.50E-07	8.50E-06
GO:0009719	response to endogenous stimulus	30	97	2.00E-07	8.50E-06
GO:0050896	response to stimulus	78	97	2.00E-06	3.30E-05
GO:0006950	response to stress	50	97	1.30E-05	0.00016
GO:0019222	regulation of metabolic process	44	97	0.0014	0.014
GO:0007154	cell communication	32	97	0.0021	0.017
GO:0007165	signal transduction	29	97	0.0025	0.018
GO:0050789	regulation of biological process	71	97	0.0036	0.022
GO:0010468	regulation of gene expression	30	97	0.0068	0.037
GO:0009791	post-embryonic development	19	97	0.0076	0.037
GO:0060255	regulation of macromolecule metabolic process	34	97	0.0086	0.038
GO:0050794	regulation of cellular process	60	97	0.011	0.046
Group C_1h_downregulated					
GO:0019748	secondary metabolic process	16	73	8.20E-08	3.60E-06

TABLE 6 | 1 h Group C Genes with predicted regulatory targets based on a gene regulatory network.

Gene ID	Annotation	DE C_1h	Number of GRN predicted targets			
			Leaf	Root	SAM	Seed
Zm00001d047999	bHLH TF* 9[a]	Down	334	6	242	153
Zm00001d049173	WRKY TF 36[a]	Down	1318	1630	0	0
Zm00001d003293	NAC TF 111[a]	Down	0	49	0	743
Zm00001d017084	NAC TF 13[a]	Down	110	37	90	120
Zm00001d031728	AP2-EREBP TF 79[a]	Down	0	616	0	0
Zm00001d051239	AP2-EREBP TF 170[a]	Up	122	833	355	0
Zm00001d002025	AP2-EREBP TF 24[a]	Up	3051	293	217	1483
Zm00001d002364	AP2-EREBP TF 97[a]	Up	610	742	551	167
Zm00001d002867	AP2-EREBP TF 154[a]	Up	140	9	131	0
Zm00001d004358	ABI3-VP1 TF 28[a]	Up	109	0	145	941
Zm00001d005609	protein phosphatase 2C A5[b]	Up	486	963	507	202
Zm00001d006169	DREB 1A[c]	Up	0	31	795	0
Zm00001d011589	NAC TF 134[a]	Up	71	136	374	121
Zm00001d012285	MYB-related TF 55[a]	Up	66	1327	402	603
Zm00001d014938	trihelix TF 22[a]	Up	1700	58	784	1603
Zm00001d015521	G2-like TF 24[a]	Up	334	35	91	1679
Zm00001d017422	Homeobox TF 41[a]	Up	104	432	1167	585
Zm00001d018119	bHLH TF 161[a]	Up	1033	336	174	485
Zm00001d018178	bZIP TF 4[a]	Up	558	805	304	174
Zm00001d024200	C2C2 CO-like TF 19[d]	Up	1388	1095	740	14
Zm00001d025055	protein phosphatase 2C A9[b]	Up	20	65	1911	734
Zm00001d027901	ZIM TF 16[a]	Up	126	1221	859	136
Zm00001d028752	protein phosphatase 2C 26[e]	Up	114	231	835	91
Zm00001d041491	CCAAT-HAP2-TF 212[a]	Up	62	106	801	472
Zm00001d047732	protein phosphatase 2C 32[f]	Up	33	182	269	67
Zm00001d050195	WRKY TF 94[a]	Up	145	1060	142	198

**TF = Transcription factor.*

[a] *Yilmaz et al. (2009).*

[b] *Xiang et al. (2017).*

[c] *Qin et al. (2004).*

[d] *Song et al. (2018).*

[e] *Lu et al. (2020).*

[f] *NCBI (https://www.ncbi.nlm.nih.gov).*

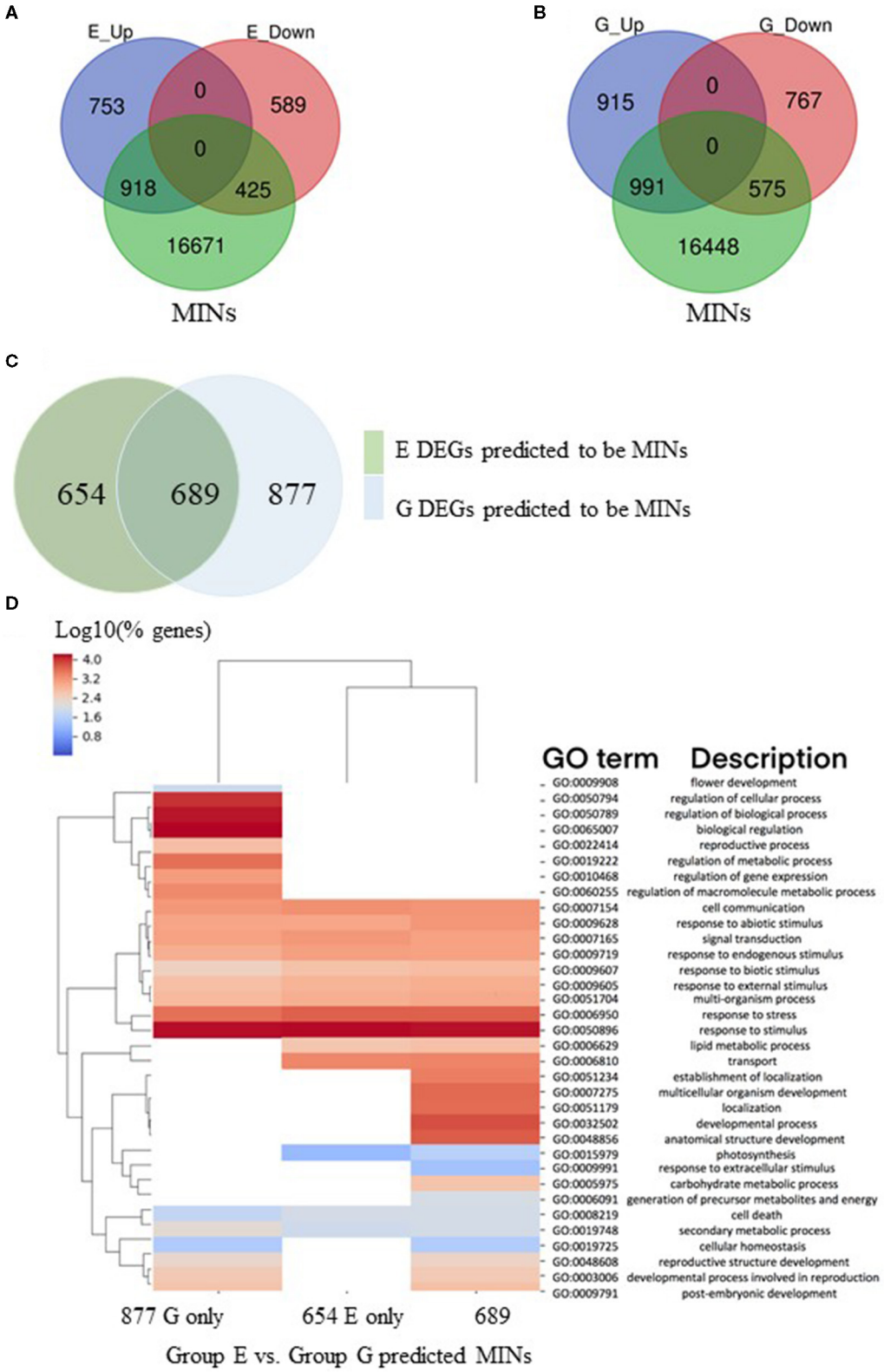

FIGURE 4 | Differentially expressed genes predicted to be downstream regulatory targets of MOP1. A gene regulatory network was used to predict the regulatory targets of genes designated as MOP1-dependent immediate responsive genes (MIMs). The predicted regulatory targets of MIMs were designated as as MOP1-dependent indirectly responsive genes (MINs). **(A)** Gene identities of MINs were compared to those of DEGs identified in a comparison between homozygous

(Continued)

FIGURE 4 | *mop1-1* plants treated with ABA for 1 h vs. 8 h (E_Up and E_Down), venn diagram illustrates overlap between these groups. **(B)** Gene identities of MINs were compared to those of DEGs identified in a comparison between wild type plants treated with ABA for 1 h vs. 8 h (E_Up and E_Down), venn diagram illustrates overlap between these groups. **(C)** The identity of MINs that were differentially expressed in homozygous *mop1-1* plants treated with ABA for 1 h vs. 8 h (E DEGs) compared to wild type plants treated with ABA for 1 h vs. 8 h (G DEGs) were compared, venn diagram illustrates the overlap between these groups. **(D)** Comparisons of enriched biological process (BP) Gene Ontology (GO) terms in DEGs were made between MINs that were identified as differentially expressed in homozygous *mop1-1* plants treated with ABA for 1 h vs. 8 h (Group E) compared to wild type plants treated with ABA for 1 h vs. 8 h (Group G) or in both analysis groups (E + G). Heatmap illustrates hierarchical clustering of log10 (% genes) of significant GO terms enriched in each expression comparison (FDR < 0.05, minimum of 10 genes per GO term). No color (white) indicates that there was no enrichment for the GO term in the dataset.

controls across genotypes (**Figure 5B**; **Supplementary Figure 4**). Reproductive development can also be affected by drought-stress and the magnitude of the effect is in some cases dependent on the stage of development in which the plant endures the stress. Because the drought-treatment in our study begins at the V6 stage, which is prior to the transition to reproductive development, we were able to determine the effects of vegetative stress on reproductive traits. To determine how drought affects tassel development, we characterized the effective tassel branches for each individual by measuring the ratio of tassel branches with functional anthers (i.e., anthers shedding pollen) to total tassel branches and found a drought-dependent decrease in effective tassel branches across genotypes, however, these differences were not significant (**Supplementary Figure 4**). The number of days until ear emergence and the number of ears per plant were also measured. It was found that drought treatment led to a significant delay in ear emergence and that *mop1-1* drought treated plants, but not B73 or *Mop1*, displayed a significant reduction in the number of ears per plant (**Figure 5B**; **Supplementary Figure 4**). In addition, *mop1-1* drought-treated plants displayed a significantly larger anthesis-silking interval compared to B73 and *Mop1,* suggesting that impaired recovery from drought stress in plants defective in MOP1 function has an effect on reproductive development and competency (**Figure 5B**).

DISCUSSION

Understanding the molecular mechanisms that contribute to plant responses to changing environments is essential to ensure that we can develop climate resistant plants that meet the increasing global demands on crop yield. RdDM and ABA-signaling are two critical gene-regulatory pathways that each influence how plants respond to environmental cues at specific developmental stages. The extent of the synergy between these two regulatory systems is largely uncharacterized in agronomically important crop plants, such as maize. To address this gap in knowledge, we recently conducted a transcriptomic analysis which demonstrated that loss of RdDM activity renders maize seedlings more susceptible to transcriptional changes as a result of ABA treatment, and that many genes were responsive to disruption of both regulatory networks after 8 h of phytohormone treatment (Vendramin et al., 2020). The differential response of the RdDM-deficient mutant to treatment with ABA and to water deprivation suggest that stressful growing conditions or exogenous of application of growth hormones like ABA might be sufficient stimuli to alter the epigenome of maize, and could be useful in crop epi-breeding platforms, which may enhance modern breeding efforts (Dalakouras and Vlachostergios, 2021). While this study identified and established synergy between these two networks in maize, interpretation of the results was confounded by the hierarchical nature of cascading transcriptional outcomes for both regulatory pathways, each dependent on varied *cis* and *trans*-regulatory elements.

Using an approach based on a temporal response to phytohormone treatment, we have identified immediate and direct MOP1-dependent transcriptional responses to ABA (MIMs) that are predicted to function upstream of genes responsive to longer periods of exposure to abiotic stress stimuli (MINs). These relatively few MIM genes, identified as unique 1 h Group C genes having homology with MOP1-dependent siRNAs, appear to be specific in their biological function. Using a GRN, we were able to establish a hierarchial relationship between predicted MIMs and MINs, where the MIMs identified in this study are predicted to regulate ∼50% of genes differentially expressed after longer exposure to abiotic stress (8 h), suggesting a substantial impact on transcriptional responses by MIMs. The lack of multiple enriched GO terms associated with the 76 down-regulated MIMs is indicative of either a lack of biological specificity of these genes or a reflection of the complexity of regulation of genes in this category that may also be targets of an active demethylaion mechanisms by DNA glycosylases. For this study, only a subsets of possible regulatory features associated with RdDM activity were used to identify MIMs, and yet the predicted regulatory impact of the identified MIMs account for almost half of the MINs, suggesting that this may in fact be an underestimation of the contribution of MOP1 in establishing responsive transcriptional profiles. Additional analysis to include other RdDM regulatory features, such as proximity to specific categories of transposable elements (TEs) (Madzima et al., 2014; Vendramin et al., 2020) and contexts of cytosine methylation that establish boundaries between the TEs and adjacent protein-coding genes in maize (Gent et al., 2014; Li et al., 2015) might identify additional specific ABA-induced MIM genes. It is likely that an extensive genome-wide analysis will need to be pursued to elucidate specific examples of direct correlation between DNA methylation, chromatin marks and differential expression in these conditions, because prior work has demonstrated that these coordinated responses are hierarchical and inter-related, and often do not involve simple relationships between differential expression and

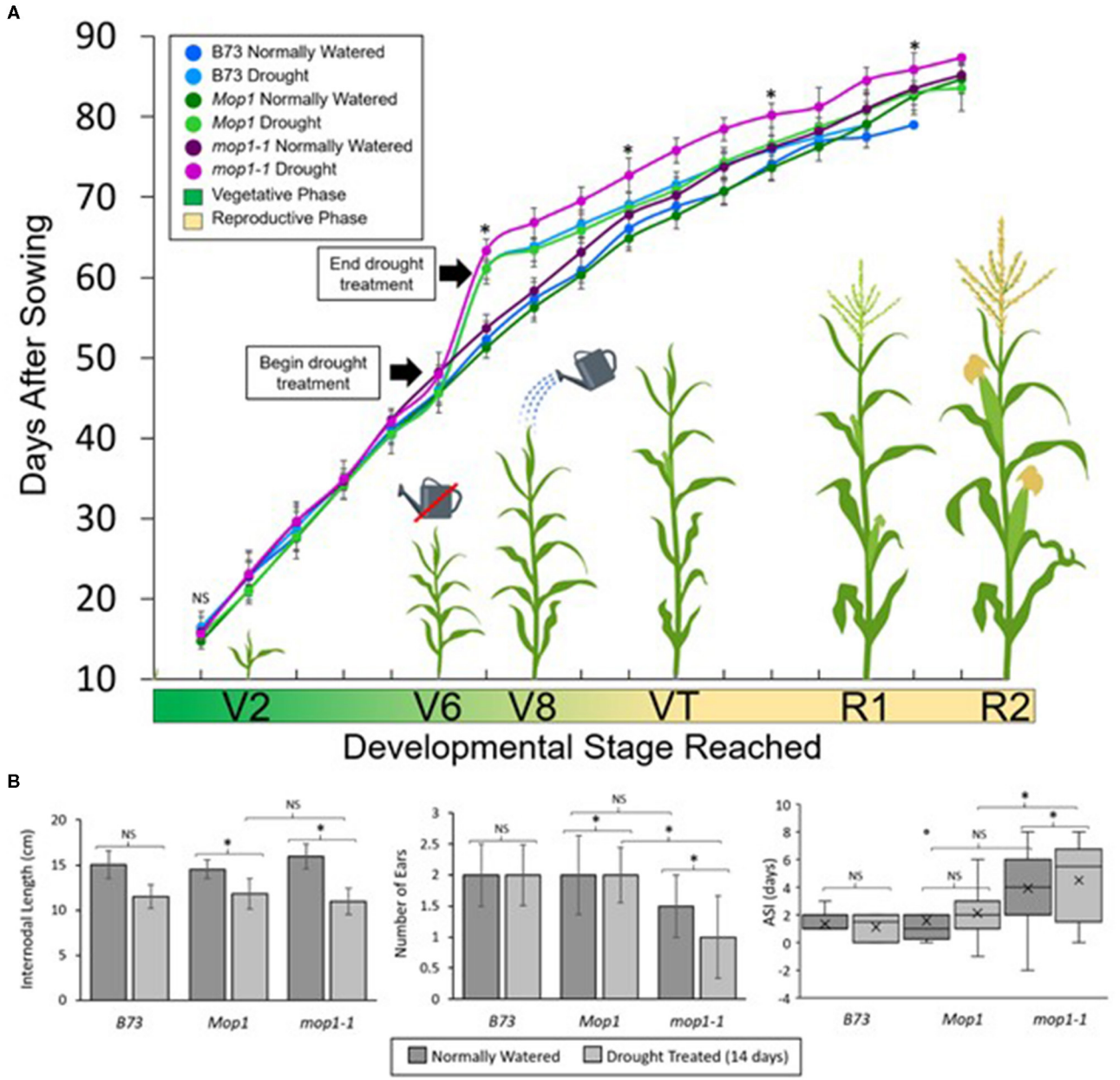

FIGURE 5 | Effects of drought treatment on growth rate and reproductive development. **(A)** The gradient on the x-axis represents the approximate vegetative to reproductive developmental transitions, from the growth stage where two vegetative leaves have emerged (V2) through the 8 vegetative leaf stage (V8), and the reproductive stages where tassels and ears are present (VT) through later stages of flowering/seed set (R1 to R2). Individual points indicate mean days after sowing (DAS) when the developmental stage was reached for the indicated genotypes and treatment groups, and error bars show standard deviation within groups. Data is shown for B73 inbred lines under different watering conditions, and wild type (*Mop1*) and homozygous *mop1-1* individuals segregating within a family. Significant differences ($p < 0.05$) between drought-treated *mop1-1* and *Mop1* plants are indicated for selected points with an asterisk (*) or NS for no significance. **(B)** Physiological observations from drought stress experiment showing average internodal length, number of ears per plant, and anthesis-silking interval (ASI). Significant differences ($p < 0.05$) between groups are indicated by an asterisk (*) or no significance (NS).

hallmarks of RdDM (Madzima et al., 2014; Vendramin et al., 2020). Thus, a locus-specific approach was not attempted in this study.

There is already compelling evidence indicating that RdDM activity in maize has consequential effects on plant growth and development, affecting the male and female inflorescences (Dorweiler et al., 2000; Hultquist and Dorweiler, 2008) and ultimately seed yield (Barber et al., 2012). The study described herein, reveals that, consistent with *mop1-1* plants misexpressing genes involved in development (Vendramin

et al., 2020), plants defective for RdDM are compromised in their growth rate recovery after water stress. This observation links the differences in transcriptional responses of maize *mop1-1* plants to differing abilities to recover from abiotic environmental influences, and highlights the physiological relevance of the gene expression phenotypes of RdDM-deficient plants.

Collectively, this data suggests that MOP1 activity is required for preparedness to respond, early response and later response to ABA signaling at the level of gene expression, and may indicate that MOP1, a component of RdDM in maize, functions in plant response to stressful growth conditions. Future work will include molecular characterization of the MIMs to identify the architecture of upstream *cis*-regulatory elements of these genes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. RNA-sequencing data is available at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (Edgar et al., 2002) through GEO Series accession number GSE179629.

AUTHOR CONTRIBUTIONS

TM, SV, JL, and KM designed the research. SV performed the seedling ABA-induction experiments and RNA-seq library preparation. JL and PL performed the drought stress experiments. TM and KM performed analysis and interpretation of seedling ABA RNA-seq data. KL assisted with bioinformatic analysis and interpretation. TM and KM wrote the manuscript. All authors edited and/or reviewed the original manuscript, and contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.694289/full#supplementary-material

Supplementary Figure 1 | Threshold normalization for uniquely mapping reads. The mapped reads from RNA-seq after 1 h (this study; Illumina NovaSeq platform) and 8 h (Vendramin et al., 2020; Illumina HiSeq platform) of ABA induction were simultaneously, bioinformatically processed and mapped to the B73 reference genome (AGP B73v4) (Jiao et al., 2017). Read quality score thresholds were normalized using HISAT2 ("–score-min") between sequencing platforms. Based on consistency between replicates as well as differences in distributions of mapping qualities between sequencing platforms, the HISAT2 "–score-min" parameter was chosen to normalize the number of uniquely mapped reads across datasets. HISAT2 filters reads based on a threshold were defined by the slope a linear function between mapping quality score and read length. HISAT2 slope filter threshold for 1 h = −0.2 and for 8 h = −0.6. Genotype-treatment samples include wild type MS (wm), wild type ABA (wa), mutant MS (mm), mutant ABA (ma).

Supplementary Figure 2 | Re-analysis of differentially expressed genes (DEGs) after 8h ABA treatment. **(A)** Venn diagram of overlap in the identity of DEGs (log2 FC $\geq$ 0.95, FDR $<$ 0.05) after 8 h treatment with ABA compared to control in homozygous mutant (a_up and a_down) and wild type plants (b_up and b_down). **(B)** Venn diagram of overlap in the identity of DEGs (log2 FC $\geq$ 0.95, FDR $<$ 0.05) in homozygous mutant compared to wild type plants after 8 h treatment with ABA (c_up and c_down) or 1 h treatment with MS (d_up and d_down).

Supplementary Figure 3 | Analysis of differentially expressed genes (DEGs) in 8h/1h comparisons. Venn diagram of overlap in the identity DEGs (log2 FC $\geq$ 0.95, FDR $<$ 0.05) in each analysis 8 h/1 h (**Table 4**) were compared. **(A)** identity of analysis groups E and F DEGs. **(B)** identity of analysis groups G and H. **(C)** identity of analysis groups E and G. **(D)** identity of analysis groups F and H.

Supplementary Figure 4 | Additional physiological observations from drought stress experiment. Graphs show **(A)** average plant height, **(B)** effective tassel branches, and **(C)** ear emergence in number of days after sowing (DAS) in normally watered plants or after water was withheld for 14 days (Drought treated). Data is shown for B73 inbred lines under different watering conditions, and wild type (*Mop1*) and homozygous *mop1-1* individuals segregating within a family. Significant differences ($p < 0.05$) between groups are indicated by an asterisk (*) or no significance (NS).

Supplementary Table 1 | List of DEGs for mop1 ABA-treated samples.

Supplementary Table 2 | List of DEGs for 1 h, 8 h and 8 h/1 h Venn diagrams.

Supplementary Table 3 | List of 1 h Group C 97 and 76 genes and GRN targets.

Supplementary Table 4 | List of secondary targets.

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Epigenomic Regulators *Elongator Complex Subunit 2* and *Methyltransferase 1* Differentially Condition the Spaceflight Response in Arabidopsis

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Background: Plants subjected to the novel environment of spaceflight show transcriptomic changes that resemble aspects of several terrestrial abiotic stress responses. Under investigation here is whether epigenetic modulations, similar to those that occur in terrestrial stress responses, have a functional role in spaceflight physiological adaptation. The Advanced Plant Experiment-04 – Epigenetic Expression experiment examined the role of cytosine methylation in spaceflight adaptation. The experiment was conducted onboard the International Space Station, and evaluated the spaceflight-altered, genome-wide methylation profiles of two methylation-regulating gene mutants [methyltransferase 1 (*met1-7)* and elongator complex subunit 2 (*elp2-5*)] along with a wild-type Col-0 control.

Results: The *elp2-5* plants suffered in their physiological adaptation to spaceflight in that their roots failed to extend away from the seed and the overall development of the plants was greatly impaired in space. The *met1-7* plants suffered less, with their morphology affected by spaceflight in a manner similar to that of the Col-0 controls. The differentially expressed genes (DEGs) in spaceflight were dramatically different in the *elp2-5* and *met1-7* plants compared to Col-0, indicating that the disruptions in these mutants resulted in a reprogramming of their spaceflight responses, especially in *elp2-5*. Many of the genes comprising the spaceflight transcriptome of each genotype were differentially methylated in spaceflight. In Col-0 the majority of the DEGs were representative of the now familiar spaceflight response, which includes genes associated with cell wall remodeling, pathogen responses and ROS signaling. However, the spaceflight transcriptomes of *met1-7* and *elp2-5* each presented patterns of DEGs that are almost completely different than Col-0, and to each other. Further, the DEGs of the mutant genotypes suggest a more severe spaceflight stress response in the mutants, particularly in *elp2-5*.

Conclusion: Arabidopsis physiological adaptation to spaceflight results in differential DNA methylation in an organ-specific manner. Disruption of Met1 methyltransferase function does not dramatically affect spaceflight growth or morphology, yet *met1-7* reprograms the spaceflight transcriptomic response in a unique manner. Disruption of *elp2-5* results in poor development in spaceflight grown plants, together with a diminished, dramatically reprogrammed transcriptomic response.

Keywords: spaceflight adaptation, DNA methylation, epigenetic, space biology, microgravity, elongator complex subunit 2, methyltransferase 1, methylation mutants

INTRODUCTION

Plants cope with environmental changes by reprogramming gene expression and metabolic processes necessary for growth, development, and survival (e.g., Hirayama and Shinozaki, 2010; Sewelam et al., 2014; Lopez-Ruiz et al., 2020). The adaptability of a genotype to changing environmental conditions is therefore determined by its genome and gene activity, both of which are in turn influenced by epigenetic factors such as DNA methylation (e.g., Dowen et al., 2012; Zhang et al., 2018). The APEX-04 EPEX spaceflight experiment investigated the role of specific epigenomic changes in determining the physiological adaptation of plants to the spaceflight environment.

DNA methylation profiles within a genome are dynamic and complex, yet integral to plant growth, development, and stress responses (reviewed in: Bartels et al., 2018). Many terrestrial abiotic stresses, such as salt stress, heat stress, drought, water stress, and phosphate starvation induce epigenetic changes that aid in the adaptation process (e.g., Labra et al., 2002; Boyko and Kovalchuk, 2010; Mirouze and Paszkowski, 2011; Bilichak et al., 2012; Colaneri and Jones, 2013; Tricker et al., 2013; Yong-Villalobos et al., 2015, 2016; Hewezi et al., 2017, 2018; Bartels et al., 2018; Kenchanmane Raju et al., 2018; Beyrne et al., 2019; Ashapkin et al., 2020; Akhter et al., 2021; Korotko et al., 2021; Laanen et al., 2021; Villagomez-Aranda et al., 2021). The genes differentially expressed in response to spaceflight share similarities with many documented terrestrial responses. Hallmarks of spaceflight responses include differential expression of genes involved in pathways associated with cell wall remodeling, reactive oxygen species (ROS), pathogen attacks, wounding, salt stress, drought stress, and hormone signaling (Hoson et al., 2002; Gao et al., 2008; Salmi and Roux, 2008; Blancaflor, 2013; Correll et al., 2013; Paul et al., 2013; Zupanska et al., 2013; Ferl et al., 2014; Inglis et al., 2014; Nakashima et al., 2014; Sugimoto et al., 2014; Kwon et al., 2015; Schüler et al., 2015; Zhang et al., 2015; Ferl and Paul, 2016; Herranz et al., 2019; Vandenbrink et al., 2019; Barker et al., 2020; Califar et al., 2020; Kruse et al., 2020; Angelos et al., 2021; Manian et al., 2021b). Plants further respond to spaceflight with changes in DNA methylation, again similarly to the epigenetic effects that occur during terrestrial stresses. Genome-wide DNA methylation and gene expression alterations occurred in plants grown for part of their life cycle in a satellite experiment (Xu et al., 2018). Arabidopsis grown from seed on orbit in the International Space Station (ISS) showed changes in specific DNA methylation contexts, with some of those changes associated with differentially expressed genes (DEGs; Zhou et al., 2019).

The patterns of spaceflight-associated DNA methylation are organ-specific. In comparison to roots, spaceflight leaves show higher methylation levels within the protein-coding genes compared to ground controls (GCs; Zhou et al., 2019). A large proportion of the genes that are differentially expressed and differentially methylated are associated with ROS signaling (Zhou et al., 2019). ROS can act as signaling molecules in responses to an array of plant stressors and there is growing evidence of an interplay between ROS metabolism and epigenetic regulation during acclimation in terrestrial environments (Huang et al., 2019). DNA methylation and other epigenetic modifications have been reported to play a role in regulating the innate immune response and pathogen response (Dowen et al., 2012; Wang et al., 2013; Yu et al., 2013; Tameshige et al., 2015; Jarosz et al., 2020). Many components of the gene networks associated with these pathogen-associated pathways are also differentially expressed by plants in spaceflight (Correll et al., 2013; Paul et al., 2013, 2017; Sugimoto et al., 2014; Kwon et al., 2015; Schüler et al., 2015; Herranz et al., 2019; Barker et al., 2020; Califar et al., 2020; Manian et al., 2021a). This commonality of terrestrial environmental responses with spaceflight responses begged the question: do plants use similar tools to regulate genes in response to spaceflight?

DNA methylation in plants occurs in three main contexts, CG, CHG, and CHH (where H = A, C, or T). Methylation in each context is directly maintained by a distinct pathway and set of enzymes which include: Methyltransferase 1 (MET1), decreased DNA methylation 1 (DDM1)1, and variant in methylation in the CG context, SUVH4-deposited H3K9me2 and CHROMOMETHYLASE 3 (CMT3) in the CHG context, and CMT2 in the CHH context (Chevalier et al., 2005; Hsieh et al., 2012; Pikaard, 2013). In addition to these direct enzymatic regulators of DNA methylation, a number of genes indirectly affect DNA methylation through RNA intermediates. RNA-directed DNA methylation facilitates the recruitment of DNA methyltransferases. In plants, small interfering RNAs (siRNAs) direct *de novo* DNA methylation and maintenance of DNA methylation at asymmetrical CHH sites through the polymerase II (Pol II)-related RNA polymerases Pol IV and Pol V (Matzke et al., 2015). In Arabidopsis, the siRNA effector ARGONAUTE4 (AGO4) exists in a complex with domains rearranged methyltransferase (DRM) for methylation of the template strand for RNA polymerase V-mediated non-coding

RNA transcripts (Zhong et al., 2014). The Elongator complex is a co-factor of RNA Pol II, and ELP2 is the most likely subunit to interact with the siRNA machinery (Woloszynska et al., 2016). To explore the effects of these two DNA methylation pathways in conditioning the spaceflight response, we chose MET1 as a representative of the proteins directly involved in methylation and ELP2 as a model regulator that affects methylation during stress responses through RNA intermediates and regulation of methyltransferases.

The cytosine DNA methyl transferase gene, Met1, is directly involved in the maintenance of cytosine methylation in Arabidopsis, particularly at CG sites (Kankel et al., 2003; Rigal et al., 2016). MET1 is one of several methylation enzymes that also modify the epigenome of plants as part of stress responses (Naydenov et al., 2015; Yong-Villalobos et al., 2015; Dhami and Cazzonelli, 2020). The role MET1 plays in maintaining the CG methylation profile is also important for the subsequent inheritance of those epigenomic changes (Saze et al., 2003). Loss of MET1 function non-specifically enhances resistance to bacterial infections (Kankel et al., 2003; Dowen et al., 2012). Many of the genes differentially regulated in methylation-mutant *met1-3* lines (**Supplementary Material**, Dowen et al., 2012) show a substantial overlap with genes that are differentially expressed in response to spaceflight (Paul et al., 2013, 2017; Zhou et al., 2019) including genes involved in defense, transcription, response to hormone stimulus and phosphorylation. These methylation mutant transcriptome profiles establish that differential methylation in the genome is central to facilitating stress responses in Arabidopsis (Dowen et al., 2012; Zhang, 2012), suggesting the hypothesis that the stress response elicited by spaceflight in Arabidopsis may also have an epigenetic component that could involve regulation by MET1.

The Elongator complex is composed of six protein subunits that are highly conserved among eukaryotes; it acts as a co-factor of RNA Pol II, and has several unique cellular functions, including tRNA modification, DNA modification, and histone acetyltransferase (HAT) activity (Nelissen et al., 2005; Glatt and Muller, 2013; Ding and Mou, 2015; Kolaj-Robin and Seraphin, 2017). Although loss of any component can compromise Elongator complex function (Ding and Mou, 2015) the subunits have independent activity and can also function as sub-complexes (e.g., Glatt and Muller, 2013; Jarosz et al., 2020). Elongator proteins were initially identified in plants as important to various aspects of growth, development, and immune response (Woloszynska et al., 2016). Elongator Proteins ELP3 and ELP2 both contribute epigenomic regulation of gene expression in response to developmental and immune response pathways in Arabidopsis. ELP3 exhibits HAT activity while ELP2 interacts with components of the siRNA machinery (Woloszynska et al., 2016). In plants, siRNAs can induce cytosine methylation by recruitment of the DNA methyl transferase DRM2 through RNA-directed DNA methylation (e.g., Xie and Yu, 2015), and it is by this mode of action that ELP2 regulates plant development and stress responses (reviewed in: Jarosz et al., 2020).

ELP2 is associated with terrestrial pathogen responses (e.g., Wang et al., 2013) and plays a role in root development (e.g., Jia et al., 2015), The role of ELP2 in plant pathogen defense is relevant to the spaceflight response in plants as many of the genes comprising the spaceflight transcriptome are also commonly associated with terrestrial pathogen responses (e.g., Paul et al., 2013, 2017; Zhou et al., 2019; Barker et al., 2020). Functionally, ELP2 is a regulator of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and accelerates the immune responses in Arabidopsis through epigenetic modification of DNA methylation (DeFraia et al., 2010; Wang et al., 2013; Silva et al., 2017). Deletion of the gene encoding ELP2 has substantial impact on pathogen-induced transcription (DeFraia et al., 2010; Wang et al., 2013; Silva et al., 2017), and also impacts fundamental processes such as root development (Jia et al., 2015) and auxin signaling (Nelissen et al., 2010). Many of the genes differentially expressed between *elp2* mutants and wild-type plants include hallmarks of abiotic stress responses (Zhou et al., 2009). Further, many of the genes differentially expressed in *elp2* mutants are also represented in Arabidopsis spaceflight transcriptomes and spaceflight methylome (e.g., Paul et al., 2013, 2017; Zhou et al., 2019). These roles for ELP2 in pathogen responses and abiotic stresses suggest that ELP2 may also play a role in the epigenetic regulation of the spaceflight response pathways in plants.

The Advanced Plant Experiment 04 – Epigenetic Expression (APEX-04-EPEX) spaceflight experiment reported herein investigated the contribution of epigenomic changes in the physiological adaptation of plants to the spaceflight environment, specifically the role of cytosine DNA methylation in the spaceflight response of Arabidopsis. The experiment was conducted onboard the ISS to evaluate the spaceflight responses of the methylation mutants *met1-7* and *elp2-5* compared to the Col-0 wild-type background of both mutant lines. Growth patterns, genome-wide methylation profiles, and differential gene expression profiles were used to develop an integrated assessment of the interplay of DNA methylation and transcriptional regulation in the response to spaceflight.

MATERIALS AND METHODS

Plant Material and Experimental Design

Three lines of *Arabidopsis thaliana* (Arabidopsis) were used: Columbia-0 (Col-0) wild-type (TAIR CS70000), and Col-0 mutants *met1-7* and *elp2-5* genotype. Mutants *met1-7* and *elp2-5* previously described in Kanno et al. (2008), Guo et al. (2013), Wang et al. (2013), and were kindly provided by Dr. Zhonglin Mou (Wang et al., 2013). Sterilized seeds from each line were sown aseptically onto Petri dishes (100 mm × 15 mm; Fisher Scientific, Pittsburgh, PA, United States), containing 50 mL of a 0.5% Phytagel-based growth medium supplemented with: 0.5 × Murashige–Skoog salts, 0.5% (w/v) sucrose, and 1 × Gamborg's Vitamin Mixture, and then sealed with breathable tape (3M Micropore®, Maplewood, MN, United States; e.g., Califar et al., 2020). Seeded plates were prepared to maintain seed dormancy with a combination of far-red light treatment and light-tight wrapping in Duvetyne™ cloth until insertion into Veggie growth facility on orbit. Dormancy preparation details described in Sng et al. (2014), Fitzgerald et al. (2016), Califar et al. (2020).

Ten plates (nine for installation in Veggie and one spare) per genotype for each environmental condition (flight and GC) were prepared for launch. The plates were stored at 4°C and remained dormant until their installation into the Vegetable Production System (Veggie) hardware on the ISS, and a comparable set was installed into the Veggie hardware in the ISS environmental simulator (ISSES) chamber at Kennedy space center. Plants in the Veggie hardware (both on the ISS and in the ISSES chamber) were exposed to constant light conditions of 100–135 μmoles/m^2s PAR for 11 days before being harvested into Kennedy space center fixation tubes (KFTs) and fixed in RNAlater™ (Ambion, Grand Island, NY, United States). All of the plants from each plate were harvested into individual KFTs. The KFTs were then stowed at −80°C in the MELFI freezer aboard the ISS. The comparable GC samples were also harvested into KFTs and stored in a standard −80°C freezer. All samples were kept frozen until delivery to the laboratory for analysis. Each plate was intended as a biological replicate and kept separate in tissue preparation operations, but the mass values drove the decision to allow a combination of two plates to comprise a replicate, which resulted in a total of four biological replicates for each of the genotypes and treatments for the analyses. Leaf and root tissues from each sample were dissected using an Olympus stereo-microscope. FromBioanalyzer and quantified with the each plate, the materials from three individual plants were pooled for RNA extraction and transcriptome analyses, and materials from 10 to 15 individual plants from that same plate were allocated for DNA extraction. Molecular analyses were performed on leaf and root tissues dissected from each of the three genotypes. One plate was lost, (*met1-7*, spaceflight C6) and so one of the spaceflight *met1-7* replicates was composed of plants from a single plate rather than two plates. Whole-genome bisulfite sequencing (WGBS) was performed for the methylome analysis and RNA sequencing (RNASeq) for the transcriptome analysis. To summarize, for each genotype under each environmental condition, a total seven (spaceflight *met1-7*) or eight (all other genotypes and treatments) plates were used to conduct WGBS and RNASeq analyses, four biological replicates for each assay.

Experiment Operations for Spaceflight and Ground Controls

The plates comprising the NASA APEX-04-EPEX experiment was launched on the SpaceX mission CRS-10 to the ISS. NASA astronauts Peggy Whitson and Shane Kimbrough (expedition 49/50) managed the experiment from insertion into the Veggie growth hardware to harvest (**Figures 1A,B**). Images and videos of ISS operations are collected in **Supplementary Files 1, 2**.

The workflow of the orbital operations was recapitulated for the GCs in the ISSES chamber with a 24 h delay. The delay enabled a precise ground replication of minor changes in daily ISS environmental parameters and crew operations scheduling. Crew operations included the de-stowing of the plates and insertion into Veggie (**Figure 1A**), daily high-resolution photography of representative plates were taken with a hand-held DSLR camera, and the harvest and fixation of the plates on the final day of growth. Photography and harvests were conducted on the maintenance work area. All plates were photographed before harvest, and representatives of each genotype presented in **Figure 1C**. The complete set of spaceflight and GC photographs are supplied in the **Supplementary File 3**. The images were used to assess the growth morphology and general health of the plants.

Genomic DNA Isolation and Whole-Genome Bisulfite Sequencing Library Preparation

DNA extraction was done using a modified phenol/chloroform protocol (LeFrois et al., 2016) and genome-wide bisulfite sequencing was performed using a similar procedure as that described by Wang et al. (2013), Zhou et al. (2019). Briefly, 700–1,700 ng of genomic DNA (>5 Kb in length) observed on the TapeStation Genomic Screen Tape (Agilent) was processed for sequencing library construction. DNA was transferred into 6 × 16 mm glass microtubes with AFA fiber and pre-slit snap caps (Cat# 520045, Covaris, Inc.) and sheared into an average fragment size of ∼400 bp using the Covaris S220 ultrasonic disruptor. Short DNA fragments (<100 bp) were removed using AMPure magnetic beads (Cat# A63881, Beckman Coulter) at a 1:1 bead to sample ratio. 100–250 ng of clean, fragmented DNA was used for the Illumina sequencing library construction. Both the NEBNext® Ultra™ II DNA Illumina construction kit (Cat# E7645S, NEB) and the Illumina-specific methylated and dual-index barcoded adaptors (Cat# E7600S NEB) were used as described in the manufacturer's guidelines. Illumina libraries (containing methylated adaptors) were subjected to sodium bisulfite treatment using the EZ DNA Methylation Direct kit (ZYMO Research, Cat #D5020) according to the manufacturer's instructions. The resulting libraries were enriched by a 13–15 cycle amplification using a uracil-insensitive polymerase (EpiMark hot start Taq polymerase, NEB, Cat #M0490S). The amplified library products were separated on a 2% agarose gel from which library fragments in the 250–500 bp range were excised (QIAquick gel extraction kit, Cat# 28704, QIAGEN) and AMPure purified (Cat# A63881, Beckman Coulter). Gel staining was done with SYBR Safe (Life Technologies) and visualized on a blue light transilluminator (Life Technologies) to avoid UV damage to the DNA. The final libraries were quantified by the RNA concentration was determined on Qubit® 2.0 Fluorometer (ThermoFisher/Invitrogen, Grand Island, NY, United States), sized on the Agilent TapeStation (DNA5000 Screen Tape) and by qPCR with the Kapa SYBR Fast qPCR reagents (Cat# KK4824, Kapa Biosystems) with monitoring on an ABI7900HT real-time PCR system (LifeTechnologies). The average library size was 350 bp. Care was taken to generate WGBS libraries that were approximately the same size as the RNASeq libraries. Sequencing was performed at the ICBR NextGen Sequencing Core[1].

Total RNA Isolation and RNASeq Library Construction

RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's

[1]https://biotech.ufl.edu/next-gen-dna/

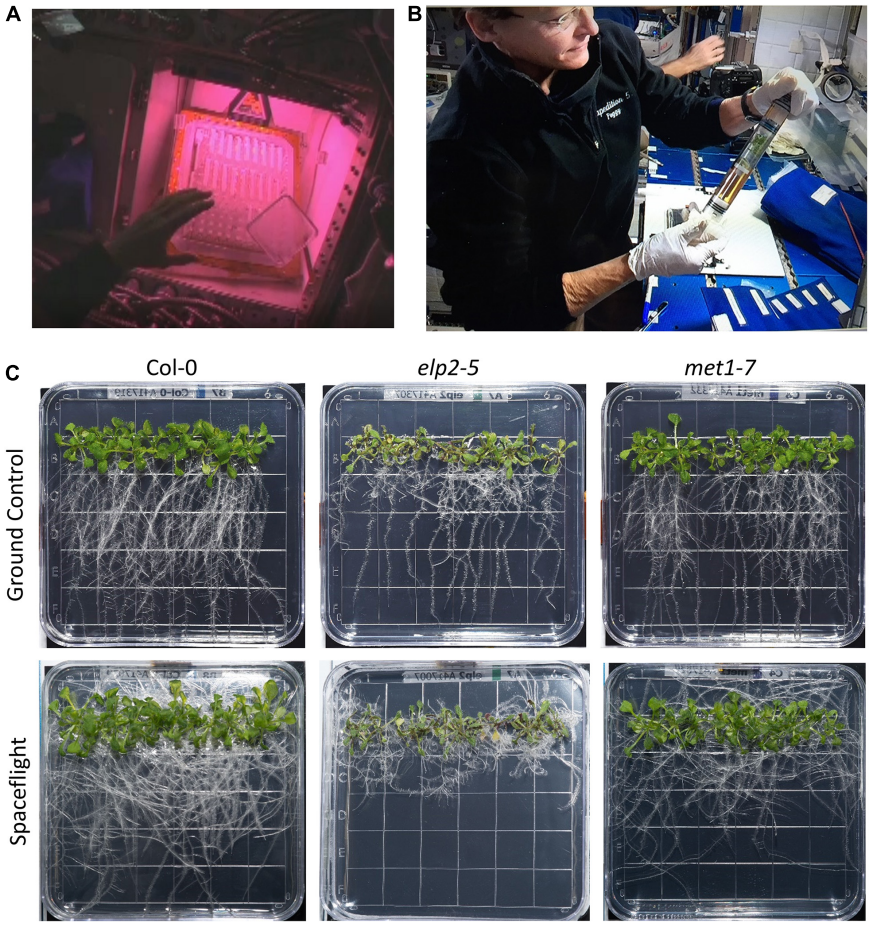

FIGURE 1 | On orbit operations and comparison of plant growth in both environmental conditions. **(A)** Astronaut Peggy Whitson inserts seeded plates into the Veggie growth hardware on the ISS. The pinkish lighting is derived from the mixture of red and blue LED growth lights (also **Supplementary File 1**). **(B)** Peggy Whitson harvests the 11 day old seedlings to RNAlater-filled KFTs at the MWA on the ISS. **(C)** Representative examples of each of the three genotypes (Col-0 wild-type, *elp2*, and *met1-7*) grown for 11 days in the Veggie hardware in the ISS Environment simulation chamber (ISSES) for the ground controls (top row) and grown for 11 days in Veggie on the ISS for the spaceflight environment (bottom row; also **Supplementary File 3**).

guidelines. RNA concentration was determined on a Qubit® 2.0 Fluorometer, RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). The RIN numbers of the total RNA used for RNASeq library construction are between 7.1 and 9.3. Basically, 2 μL of 1:200 diluted RNA spike-in ERCC (half amount of suggested in the ERCC user guide: Cat# 4456740) spike to 1,000 ng of total RNA followed by mRNA isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs, catalog # E7490). This was followed by RNA library construction with the NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs, catalog #E7420) according to the manufacturer's user guide. Briefly, RNA was fragmented in NEBNext First Strand Synthesis Buffer via incubation at 94°C for the desired time. This step was followed by first-strand cDNA synthesis using reverse transcriptase and Oligo(dT) primers. Synthesis of ds-cDNA was performed using the second strand master mix provided in the kit, followed by end-repair and adaptor ligation. At this point, Illumina adaptors were ligated to the sample. Finally, each library (uniquely barcoded) was enriched by 10 cycles of amplification, and purified with Agencourt AMPure beads (Beckman Coulter, catalog # A63881). 48 barcoded libraries were sized on the Bioanalyzer and quantified with the Qubit® 2.0 Fluorometer. Finally, these 48 individual libraries were pooled in equimolar concentration. RNASeq libraries were constructed at the UF ICBR Gene Expression Core[2].

HiSeq3000 Procedure, Pooled RNASeq, and MethylSeq

Uniquely barcoded libraries were normalized to 2.5 nM and pooled (equimolarly) for sequencing on the HiSeq3000 Illumina sequencer. Bisulfite-converted sequencing libraries were sequenced together with RNASeq libraries (uniquely barcoded) to maximize data output. The RNASeq libraries in the pool served to compensate for the low base diversity of bisulfite-converted genomic libraries. The final library was created by

[2]https://biotech.ufl.edu/gene-expression-genotyping/

mixing RNASeq vs bisulfite-converted libraries at a 60:40% ratio, with a mere 1% PhiX spike-in. Library pools were processed according to the Illumina protocol (HiSeq3000) for clustering on the cBOT machine. After denaturation, neutralization, and mixing with the ExAmp reagent, the final pool concentration for clustering was 0.25 nM. Sequencing was done using a 2 × 101 cycles format (paired-end configuration). The 48-sample project was sequenced on 12 lanes for a robust reads/lane output.

MethylSeq Bioinformatics

The short reads from the uniquely barcoded bisulfite-converted genomic libraries were trimmed using Trimmomatic v 0.36 and quality control on the original and trimmed reads was performed using FastQC v 0.11.4 and MultiQC v 1.1 (Andrews, 2010; Bolger et al., 2014; Ewels et al., 2016). The bisulfite-converted reads were aligned to the TAIR10 genome using BSMAP (Xi and Li, 2009). Methylation calling was performed with CSCALL and the differential methylation analysis was performed using the MCOMP program, which is part of the MOABS package (Abe et al., 1997; Sun et al., 2014). Cytosine sites with at least a 10x read coverage in at least two out of the four replicates were included in downstream analyses. Methylated cytosine sites for which the p-value of the difference between test and control methylation rates was below 0.01 were considered differentially methylated cytosines (DmCs). In addition, DmCs with a methylation difference > 0 were classified as hypermethylated whereas those < 0 were classified as hypomethylated. DmCs were also categorized based on the characteristics of their genomic locations including the gene body (from transcriptional start site to transcriptional termination site), promoter (2 kb upstream of transcriptional start site), and downstream (2 kb downstream of transcriptional termination site). Differentially methylated regions (DMRs) were defined following the method described in Stroud et al. (2013). Briefly, DMRs were determined by comparing the average methylation levels within a 100 bp window between spaceflight and GCs, and those with statistical significance ($p < 0.01$) were used in the analysis. The reads mapped to the chloroplast reference genome were used to calculate the bisulfite conversion efficiency as previously described (Zhou et al., 2019).

RNASeq Bioinformatics

The overall quality of the RNASeq sequence data was first evaluated using FastQC (Andrews, 2010, 2018). Low-quality bases were trimmed from the reads using Trimmomatic (Bolger et al., 2014). STAR Aligner was used to map high-quality paired-end reads to TAIR10 genome (Vandenbrink et al., 2016). Gene expression values were calculated from these alignments using RSEM (Li and Dewey, 2011). The expected read counts and fragments per kilobase of transcript per million mapped reads (FPKM) were extracted for further analysis. A generalized linear regression model was built to perform the differential gene analysis using edgeR (Robinson et al., 2010). Prior to the differential expression analysis, hierarchical clustering and principal component analysis (PCA) were conducted to identify potential outliers in the samples. The thresholds for calling significantly DEG were set at, FDR of 0.05, a fold change of greater than 2, and the average FPKM for at least one replicate of each comparison group being higher than 0. DEG lists were analyzed for overlaps using BioVenn (Hulsen et al., 2008). Processing and Analysis of the RNASeq data was performed at the UF ICBR Bioinformatics Core[3].

Functional Categories Enrichment

Arabidopsis thaliana gene IDs from each list of differentially methylated, differentially expressed genes (DmC-DEGs) output from combined methylomic and transcriptomic data were submitted to g:Profiler using the standard parameters (Raudvere et al., 2019). Lists of gene ontology (GO) terms enriched within each group of DmC-DEGs were trimmed using REVIGO (Supek et al., 2011).

Statistical Analyses

Original Student's t-tests were done with Bonferroni corrections. Two-Factor ANOVA analyses with replication were performed to demonstrate statistical differences in methylation levels between genotypes and plant organ samples.

RESULTS

Col-0 and *met1-7* Exhibited a Typical Spaceflight Growth Morphology, While *elp2-5* Displayed an Unusual Spaceflight Morphology

The three genotypes, Col-0, *met1-7*, and *elp2-5* each demonstrated different growth habits in response to spaceflight. **Figure 1C** shows a representative plate of each genotype grown on the ISS (spaceflight, FT) and comparable GC. Photos of all the harvested plates are presented in **Supplementary File 3**.

Col-0 and *met1-7* exhibited a typical growth morphology of Arabidopsis grown in Veggie during spaceflight (**Figure 1A**). The strong directional growth light gradient in Veggie produces a negative phototropism in roots that results in root growth that largely mimics terrestrial gravitropism (Zhou et al., 2019; Califar et al., 2020). For both Col-0 and *met1-7* the spaceflight (FT) roots adopt a slightly randomized growth habit compared to the GCs, yet extend well away from the site of germination and the stem, and are generally negatively phototropic in response to the "vertical" orientation of the plates relative to the light source in the Veggie growth habitat. The Col-0 and *met1-7* plants presented healthy visual phenotypes, where their leaves were fully expanded and green.

In contrast, *elp2-5* plants exhibit spaceflight growth patterns that were distinct from both Col-0 wild-type and *met1-7*, and did not appear to be responding to the environmental tropic cues directing roots away from the lights in Veggie (**Figure 1C**). The roots of *elp2-5* in spaceflight did not exhibit any directional growth. Instead, all root growth appeared to occur in random patterns that resulted in most of the root mass staying within 1 cm of the germinated seed. The spaceflight leaves of *elp2-5* tended to

[3]https://biotech.ufl.edu/bioinformatics/

be darker, with more of the plants exhibiting reddish coloration typical of anthocyanin production. Several of the *elp2-5* leaves on each of the spaceflight plates were chlorotic.

In spite of any morphological differences among the genotypes, all produced the same biomass in spaceflight as on the ground. No statistically supported differences were observed between flight and GCs in either roots or shoots, for all three genotypes (**Supplementary Figure 1**).

Spaceflight Increased Genome-Wide DNA Methylation Levels in *met1-7* and *elp2-5* but Not in the Wild-Type Col-0 Plants

In the GC plant leaves, the methylation level of *met1-7* was statistically lower than that of Col-0 and *elp2-5* plants in all three methylation contexts. Col-0 and *elp2-5* and plants showed similar genome-wide methylation levels (**Figures 2A,B**). In the GC plant roots, the methylation level of *met1-7* was statistically lower than that of Col-0 and *elp2-5* plants in the CG and CHG contexts. In the CHH context, there were no statistical differences in methylation among genotypes.

In spaceflight plants, the methylation levels varied greatly in each genotype, methylation context and tissue type. The flight *elp2-5* plants showed a higher methylation level compared to Col-0 in all methylation contexts in both leaves and roots (**Figures 2C,D**). The flight *met1-7* methylation levels were lower than Col-0 in the CG context in both leaves and roots. However, *met1-7* showed higher methylation levels than Col-0 in the CHG and CHH contexts in flight leaves while showing similar methylation levels in roots (**Figures 2C,D**).

In Col-0 the average genome-wide methylation levels within each context were not significantly different between flight and GC plants, for either leaves or roots (**Figures 2E,F**). However, *met1-7* and *elp2-5* both showed significant increases in methylation levels in spaceflight compared to GCs (**Figures 2G–J**). The *elp2-5* mutants showed significant differences in the average genome-wide methylation levels between spaceflight and GCs in each organ and all methylation contexts (**Figures 2G,H**). The *met1-7* mutants shared similar trends as the *elp2-5* mutants in their leaves, however, significant differences between FT and GC were only seen in the CG context of root tissues (**Figures 2I,J** and **Supplementary File 4**) includes a detailed breakdown of the distribution of methylation levels, divided into bins ranging from 0 to 100% methylation.

Spaceflight Changes in DNA Methylation Levels Were Associated With Protein-Coding Gene Regions

Changes in DNA methylation induced by spaceflight associated with protein-coding and flanking genic regions [2 kb upstream from the transcription start site (TSS), gene body (gold bar), and 2 kb downstream from transcription termination site] are shown in **Figure 3**. Pairwise comparison between each methylation mutant and the wild type control are depicted using plot lines color-coded to indicate the environmental conditions [flight, (**F**) and ground (**G**)], the genotype [Col-0 (**C**), *elp2-5* (**E**), and *met1-7* (**M**)] and the tissue type [leaves (**L**) and roots (**R**)], as indicated in the upper right legend of each comparison plot. Thus, the pink line in **Figure 3A**, **FCL**, reflects the percentage of average methylation for flight, Col-0 leaves across a 4 kb protein-coding region of the genome.

The *elp2-5* and *met1-7* lines differed in their methylation levels across genic regions compared to Col-0. In leaves, Col-0 and *elp2-5* showed similar CG, CHG, and CHH methylation levels across genic regions associated with protein-coding for both spaceflight and GC environments, as seen by the almost overlapping traces in **Figure 3A**. However, Col-0 and *met1-7* leaves demonstrated notable differences in the CG methylation context. The *met1-7* mutants had lower average CG methylation levels compared to Col-0 across all genic regions, with a pronounced difference within the gene body region (gold bar in graph). In spaceflight, *met1-7* leaves have a higher methylation level across all genic regions when compared to the GCs (**Figure 3B**).

The *elp2-5* roots showed higher spaceflight-associated CG, CHG, and CHH methylation levels across all genic regions compared to Col-0 (**Figure 3C**), but there were differences among the contexts with respect to the methylation levels for the other genotypes. In the CG methylation context, the GC Col-0 roots, GC *elp2-5* roots, and flight Col-0 roots all had similar methylation levels, whereas flight *elp2-5* roots showed increased methylation. In the CHG and CHH context, flight Col-0 roots had a slightly lower average methylation level across all genic regions when compared to GCs.

The *met1-7* roots demonstrated noticeable differences in the CG methylation context, with the most pronounced difference within the gene body region (**Figure 3D**). Flight Col-0 roots had a lower CG methylation level across the gene body regions when compared to the GCs. The flight *met1-7* roots had a higher CG methylation level across flanking upstream and downstream genic regions compared to their GCs. In the CHG methylation contexts, there were no obvious differences between Col-0 and *met1-7* genotypes in either flight or GCs. In the CHH context, flight Col-0 roots, GC *met1-7* roots, and flight *met1-7* roots had similar methylation levels, levels that were slightly lower than GC Col-0 roots (**Figure 3D**).

Spaceflight Altered the Distribution and Direction of DmCs in *elp2-5* and *met1-7* Leaves and Roots

The distribution of cytosines differentially methylated by spaceflight within each methylation context in both leaves and roots was assessed for each mutant relative to the wild-type genotype. DmCs were identified as those cytosines with a differential methylation that was statistically significant to $p < 0.01$ in each comparison and context (**Figure 4**). The percentage of DmCs was also mapped to different genomic regions for each methylation context. The percentage maps of Upstream (2 kb upstream of the TSS), UTRs, Exons, Introns, Downstream (2 kb downstream of the polyadenylation site), transposable element, Intergenic, and Pseudogenes are shown for each genotype in the flight vs ground comparisons of leaves and roots (**Supplementary Figure 3**).

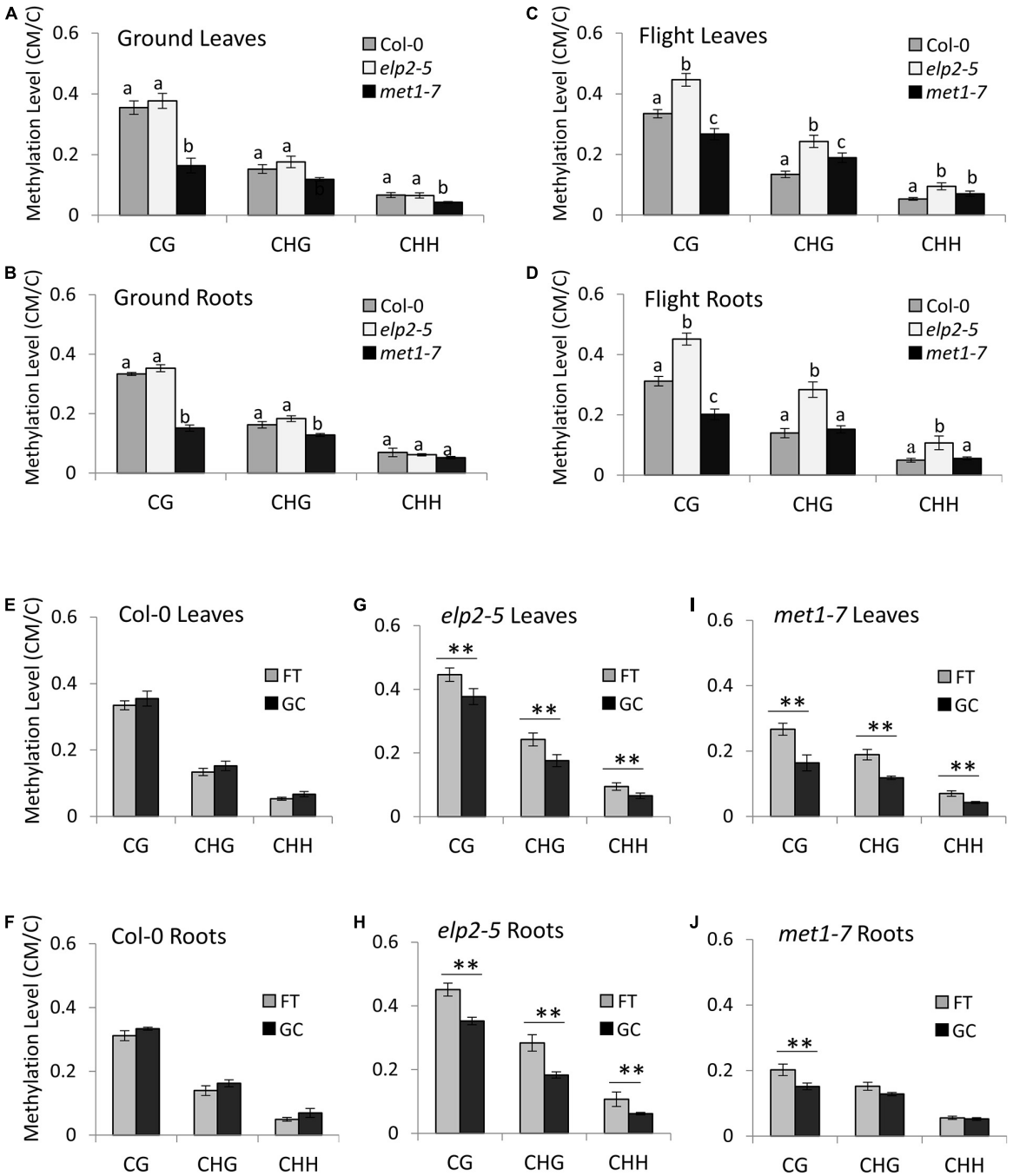

FIGURE 2 | Average genome-wide methylation level profiles in spaceflight and on the ground. **(A–D)** A comparison of the average genome-wide methylation levels (CG, CHG, and CHH) across the three genotypes is illustrated in leaves grown on the ground **(A)**, roots grown on the ground **(B)**, leaves grown in spaceflight **(C)**, and roots grown in spaceflight **(D)**. Statistical analyses were performed using two-sample *t*-test with Bonferroni corrections. Bar graphs with different letters show significant differences ($p < 0.01$). In addition, Two-Factor ANOVA analyses with replication showed that the relationships between methylation contexts were dependent on the samples ($p < 1.0E\text{-}10$). **(E–J)** Average genome-wide methylation levels are shown of each genotype (Col-0 wild-type, *elp2*, and *met1-7*) and tissue type (leaves or roots) in spaceflight (FT) and ground controls (GC). Data represent the mean of four independent samples and ** indicate significance ($p < 0.01$, two-sample *t*-test with Bonferroni corrections) difference between flight and ground controls in each of the methylation contexts (CG, CHG, and CHH).

In spaceflight, the percentage of DmCs of *elp2-5* relative to Col-0 in leaves is highest in the CHH context (53%; **Figure 4A**), whereas in the GCs the percentage was highest in the CG context (47%). Similarly, spaceflight roots (**Figure 4B**) had a higher percentage of DmCs in the CHH context (60%), while in GC roots the majority of the DmCs were divided about equally between

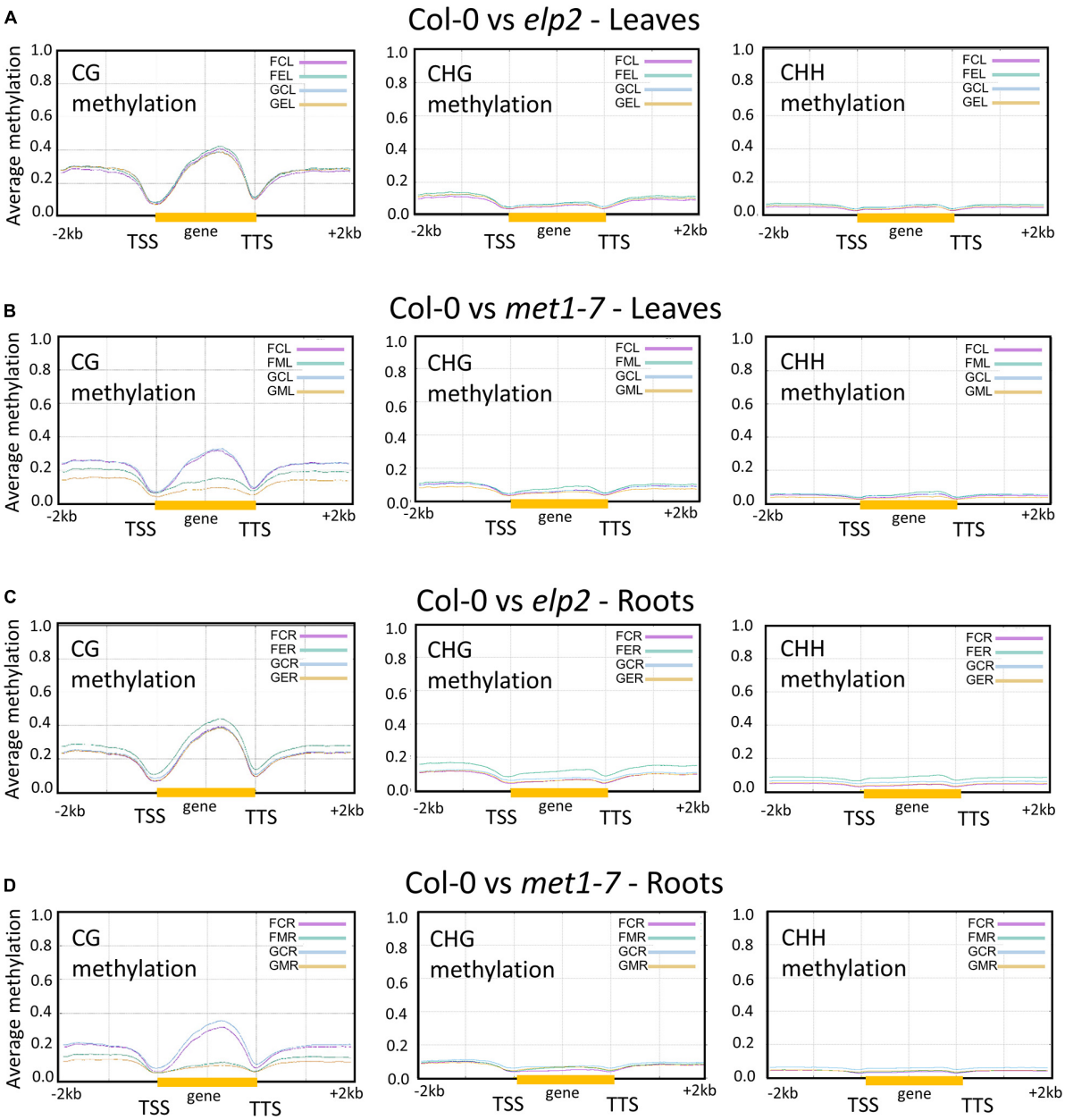

FIGURE 3 | Pairwise comparison of the average methylation levels across protein-coding genes between the genotypes in CG, CHG, and CHH contexts. Gene bodies from transcription start site (TSS) to transcription termination site (TTS; highlighted by the yellow bar) as well as the flanking upstream 2 kb and downstream 2 kb are shown. Each pairwise comparison between genotypes and environment is denoted by a three-letter code. The first letter denotes the environment [flight, (F) and ground (G)], the second letter denotes the genotype [Col-0 (C), *elp2-5* (E), and *met1-7* (M)], and the third letter denotes the specific tissue [leaves (L) and roots (R)]. The *y* axes indicate the average methylation levels in each of the CG, CHG, and CHH contexts. A pairwise comparison between the various genotypes are depicted here Col-0 vs elp2-5 leaves **(A)** and roots **(C)**, along with Col-0 vs met1-7 leaves **(B)** and roots **(D)**.

CHH (42%) and CG (43%) contexts. In all cases, the percentages of DmCs in the CHG context in the *elp2-5* plants changed very little among organs and environments (15–18%). Most of the *elp2-5* DmCs were hypermethylated (**Figures 4A,B**). In the *met1-7* and Col-0 comparison, the majority of the DmCs were in the CG context in both leaves and roots, and both organs had lower percentages of CG DmCs in FT than in GC (**Figures 4C,D**). In leaves, this decrease in the percentage of CG DmCs in FT compared to GC was larger in leaves (13%) compared to roots (3%). In addition, there was a 10% increase in the percentage of CHH DmCs in FT (29%) compared to GC (19%) in the leaves (**Figure 4C**). In roots, there was only a 1% increase in FT compared to GC (**Figure 4D**). As was seen in the *elp2-5* plants, the percentage of CHG DmCs changed very little among organs

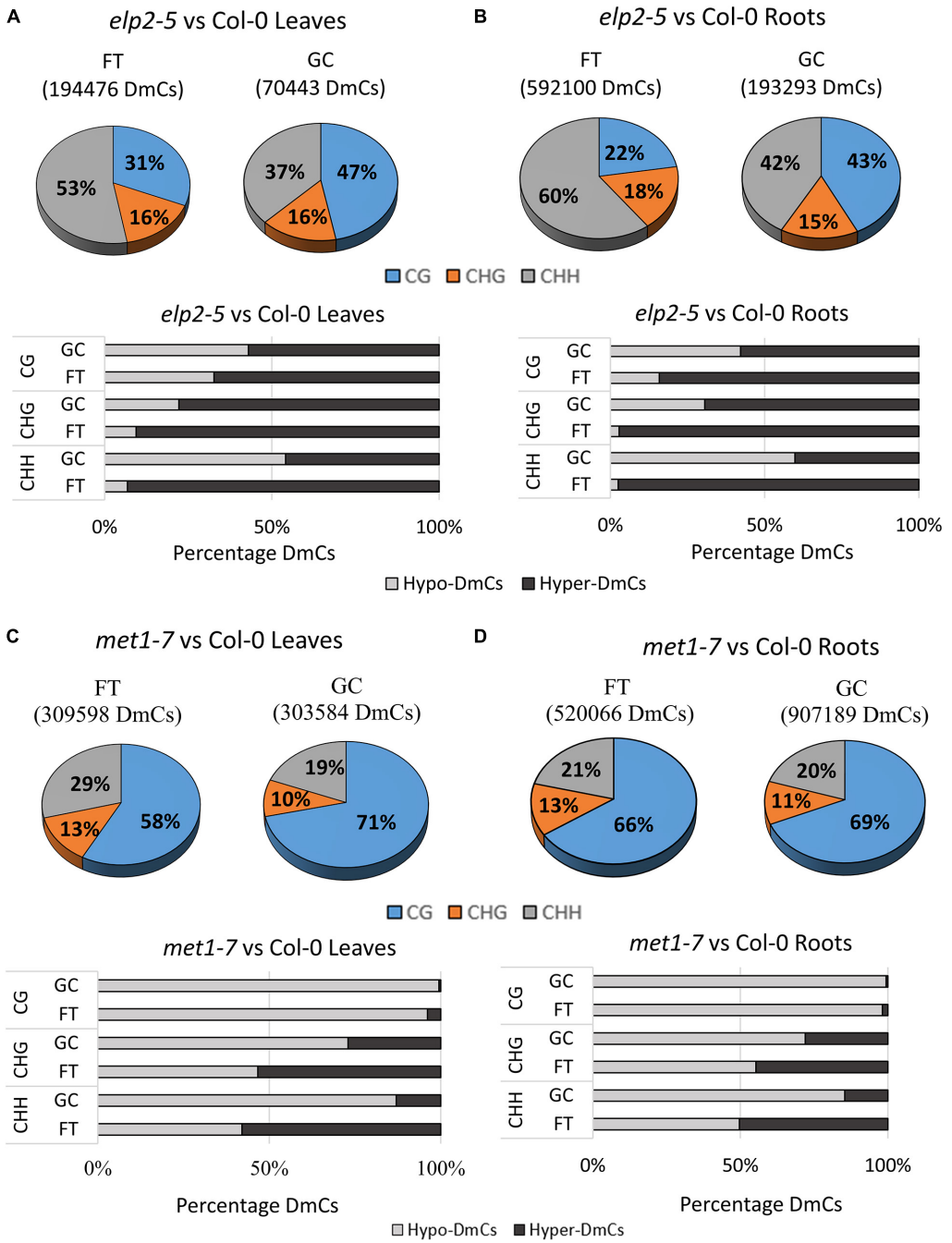

FIGURE 4 | Differentially methylated cytosines (DmCs) of leaves and roots from each genotype in response to spaceflight. **(A)** Breakdown of each methylation context for DmCs in leaves and roots of Col-0, *elp2*, and *met1-7* in spaceflight compared to ground control. **(B)** Total number of DmCs broken down in each context for all three genotypes in leaves and roots. **(C)** Breakdown of the methylation direction of total leaf DmCs in each context – Hypomethylation (Hypo) gray and Hypermethylation (Hyper) black. **(D)** Breakdown of the methylation direction of total root DmCs in each context.

and environments (10–13%). Most of the *met1-7* DmCs were hypomethylated (**Figures 4C,D**).

The distribution of gene-related DmCs in response to spaceflight was genotype- and organ-specific (**Figure 5**). In Col-0 leaves, DmCs were primarily hypomethylated. In the upstream and downstream flanking regions, the CHH context predominated, but within the gene body the CHH and CG contexts were about equally represented, and CGH methylation the least represented (**Figure 5A**). In contrast, in both *elp2-5* and *met1-7* leaves the DmCs were primarily hypermethylated (**Figures 5B,C**). In *elp2-5* leaves methylation in the CHH context predominated in loci within all genic regions (**Figure 5B**). In

met1-7 leaves the CG and CHH contexts were about equally represented in all genic regions (**Figure 5C**). In Col-0 roots, DmCs were again primarily hypomethylated, but unlike in leaves, the CHH context predominated within all genic regions (**Figure 5D**). As with leaves, the DmCs in both *elp2-5* and *met1-7* roots were predominantly hypermethylated (**Figures 5E,F**). In *elp2-5* roots, methylation of DmCs in the CHH context again predominated in loci within all genic regions, and loci with CG and CHG methylation were about equally distributed in each genic region (**Figure 5E**). In *met1-7* roots, DmCs with methylation in the CHH contexts were slightly more abundant across all genic regions, but methylation in all contexts was higher in the downstream genic region (**Figure 5F**).

Each genotype displayed distinct, organ-specific distributions of DMRs between spaceflight and the GCs (**Figure 6**). DMRs were determined by comparing the average methylation levels within 100 bp windows between FT and GC, and regions with a statistically significant difference ($p < 0.01$) were used in the analysis. In the Col-0 spaceflight response, 659 DMRs were detected in leaves, and 765 DMRs in roots. In both Col-0 tissues, an average of 51% of the DMRs were found within the CHH context, whereas the other half of the DMR were evenly distributed between the CHG and CG contexts at 24.5% each. In *elp2-5*, there were 717 DMRs in leaves and 2,974 DMRs in roots (**Figure 6A**). In both *elp2-5* tissues, an average of 63% of the DMRs were found in the CHH context, which was 12% more than Col-0. DMRs in the CHG context were distributed similarly to that of Col-0 at an average of 25%, however, DMRs in the CG context were at an average of 12.5% which was about half that of Col-0. In *met1-7*, there were 6,114 DMRs in leaves and 3,745 DMRs in roots. The majority of the DMRs in both tissues were in the CG context at an average of 83%. DMRs in the CHG context in both tissues were at an average of 8.5% whereas DMRs in the CHH context averaged 12% in the leaves and 5% in the roots (**Figure 6A**).

The spaceflight-associated DMRs in Col-0 were distributed between hypo and hypermethylation, while hypomethylated DMRs predominated in the mutants. In Col-0 leaves, 39% of the DMRs were hypomethylated and 61% hypermethylated, whereas in Col-0 roots, 67% of the DMRs were hypomethylated and 33% were hypermethylated. However, in both *elp2-5* and *met1-7* leaves and roots, DMRs were predominantly hypermethylated ranging from 97 to 99% (**Figure 6B**). Gene-related DMRs (**Figure 6C**) reflected organ-specific responses to spaceflight in all genotypes, but the largest difference in the number of DMR loci between leaves and roots was in *elp2-5*. In Col-0 leaves and roots, the number of DMRs in all genic regions and context was less than 400, with the highest number of DMRs found within the CHH context. In gene-related DMRs in Col-0 leaves hypo- and hypermethylation were fairly evenly distributed across all contexts, whereas in Col-0 roots, gene-related DMRs were mostly hypomethylated (70%). In Col-0 leaves and roots, gene-related DMRs were most abundant in the gene body region. In contrast to wild-type Col-0, the total gene-related DMRs in *elp2-5* were substantially higher in the roots compared to the leaves. In *elp2-5* roots, DMRs were primarily in the CHH context and were predominantly hypermethylated in all genic regions. The highest number of DMRs in the *elp2-5* roots were within the gene body region. In *met1-7*, DMRs were found at a higher number in the leaves compared to the roots. DMRs in both leaves and roots were predominantly hypermethylated and were distributed primarily in the CG context. In *met1-7* leaves, the highest number of DMRs were located upstream of the TSS (**Figure 6C**).

Spaceflight Affected More Genes in the Roots of *elp2-5*, and More Genes in the Leaves of *met1-7*

Spaceflight associated differential gene expression was genotype- and organ-specific; in *elp2-5* more genes were affected in roots, while in *met1-7* more genes were affected in leaves (**Figure 7**). Transcripts showing at least a twofold change ($-1 \leq \log_2 FC \geq 1$) with an FDR value of < 0.05 were identified as DEGs. PCAs of each type of tissue were performed individually, as leaves and roots have widely different patterns of gene expression (Paul et al., 2013; Zhou et al., 2019). The PCA plots of leaves and roots showed different grouping of *elp2-5* samples along with the components compared to Col-0 and *met1-7* (**Supplementary File 2** and **Supplementary Figure 1**). In the comparison of spaceflight to ground leaves, Col-0 had a total of 207 (123 up-regulated and 84 down-regulated) DEGs, *elp2-5* had 36 (26 up-regulated and 10 down-regulated) DEGs, and *met1-7* had 226 (160 up-regulated and 66 down-regulated) DEGs (**Figure 7A**). In roots, a total of 147 (28 up-regulated and 119 down-regulated), 120 (50 up-regulated and 70 down-regulated), and 47 (39 up-regulated and 8 down-regulated) DEGs were found in Col-0, *elp2-5*, and *met1-7,* respectively (**Figure 7B**). Only a few DEGs overlapped between the genotypes in both leaf and root tissues (**Figures 7C,D**). In leaves, the majority of DEGs were unique to Col-0 and *met1-7*, whereas, in roots, the majority of DEGs were unique to Col-0 and *elp2-5* (**Figures 7C,D**). The patterns of DEGs in all three genotypes were distinctly organ-specific, and of the 436 DEGs in leaves and 288 in roots, only 35 DEGs were common to both sets (**Figure 7E**). Although there were overall fewer root-specific DEGs, a greater proportion of the root DEGs were also differentially methylated in response to spaceflight compared to the DEGs of leaves. In roots, 82% of the DEGs also exhibited DmCs, whereas in leaves, 31% of the DEGs also mapped to DmCs (**Figure 7F** and **Supplementary File 5**).

The composition of DmC-DEGs in *elp2-5* compared to Col-0 was distinctive in each environmental and organ comparison (**Figure 8**). In leaves, *elp2-5* vs Col-0 on the ground had a total of 2,119 DEGs (1,322 up-regulated and 797 down-regulated) whereas in spaceflight there were a total of 2,149 DEGs (1,448 up-regulated and 701 down-regulated; **Figure 8A**). Of these, 996 DEGs were also differentially methylated in the GCs, and a total of 1,188 DmC-DEGs were observed in spaceflight (**Figure 8B**). A total of 497 DmCs-DEGs were shared between both environmental conditions. In roots, the pairwise comparison of *elp2-5* and Col-0 revealed a total of 1,667 (725 up-regulated and 942 down-regulated) DEGs on the ground and 1,626 (724 up-regulated and 902 down-regulated) DEGs in spaceflight (**Figure 8C**). Of these, 1,222 DEGs on

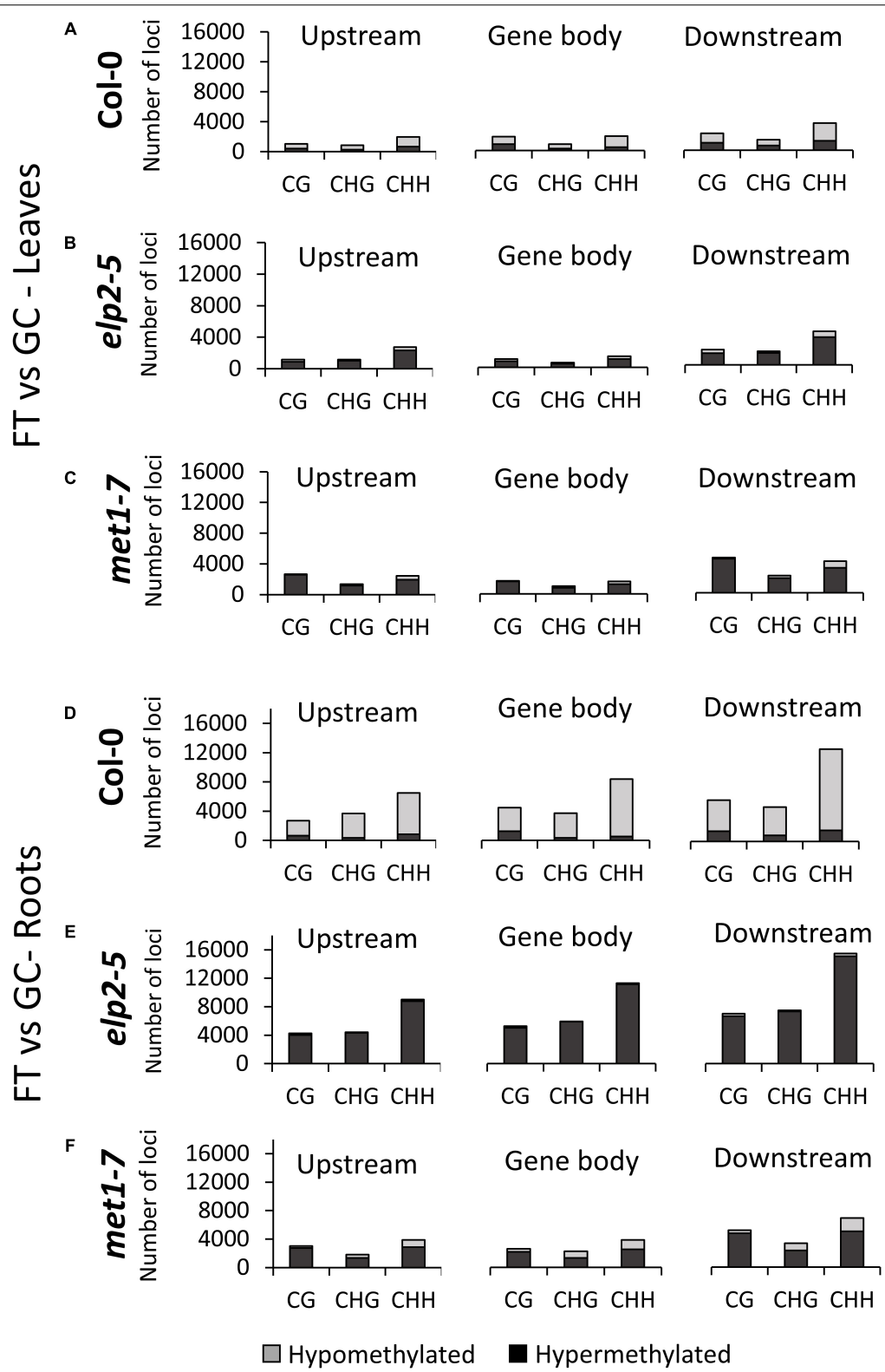

FIGURE 5 | Methylation context breakdown of DmCs mapped to different genic features in spaceflight vs ground comparisons. Leaf and Root DMCs of Col-0 **(A,D)** *elp2-5* **(B,E)**, and *met1-7* **(C,F)** in response to spaceflight are mapped to genic features such as; upstream (2,000 bp upstream of the transcription start site), gene body (exons, introns, and both 5′ and 3′ UTRs), and downstream (2,000 bp downstream of the polyadenylation site). The corresponding methylation direction in each context is also illustrated – Hypomethylation (Hypo) gray and Hypermethylation (Hyper) black.

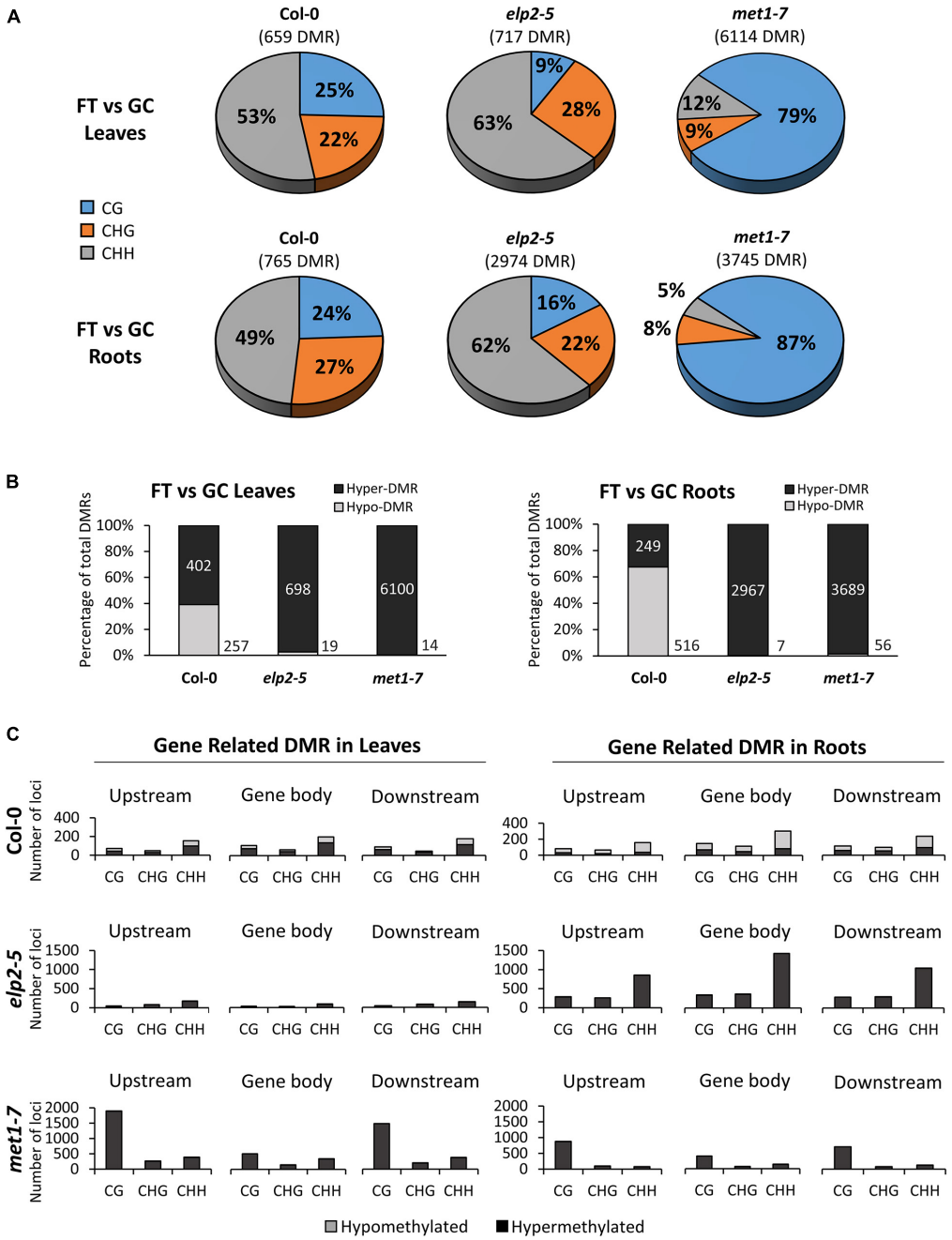

FIGURE 6 | Differentially methylated regions (DMRs) of leaves and roots from each genotype in response to spaceflight. **(A)** Breakdown of each methylation context for DMRs in leaves and roots of Col-0, *elp2-5*, and *met1-7* in spaceflight compared to ground control. **(B)** Breakdown of the methylation direction of total leaf/root DMRs in each genotype in response to spaceflight – Hypomethylation (Hypo) gray and Hypermethylation (Hyper) black. **(C)** Distribution of leaf and root gene-related DMRs of Col-0, *elp2-5*, and *met1-7* in response to spaceflight. DMRs are mapped to genic features such as; upstream (2,000 bp upstream of the transcription start site), gene body (exons, introns, and both 5′ and 3′ UTRs), and downstream (2,000 bp downstream of the polyadenylation site). The corresponding methylation direction in each context is also illustrated – Hypomethylation (Hypo) gray and Hypermethylation (Hyper) black.

the ground were also associated with DmCs and 1,481 DEGs in spaceflight were associated with DmCs. Among these, 723 DmC-DEGs were shared in both spaceflight and GC conditions (**Figure 8D**). A visual heat-map summary of the organ-specific distribution of DEGs and the DmC-DEGs for all genotypes is presented in the **Supplementary Figure 2**, which is annotated in **Supplementary File 6**.

Multiple metabolic processes appeared to be altered in *elp2-5* compared to Col-0 (**Figure 8E**). GO analysis of the DmC-DEGs of *elp2-5* compared to Col-0 showed that *elp2-5* was engaged with

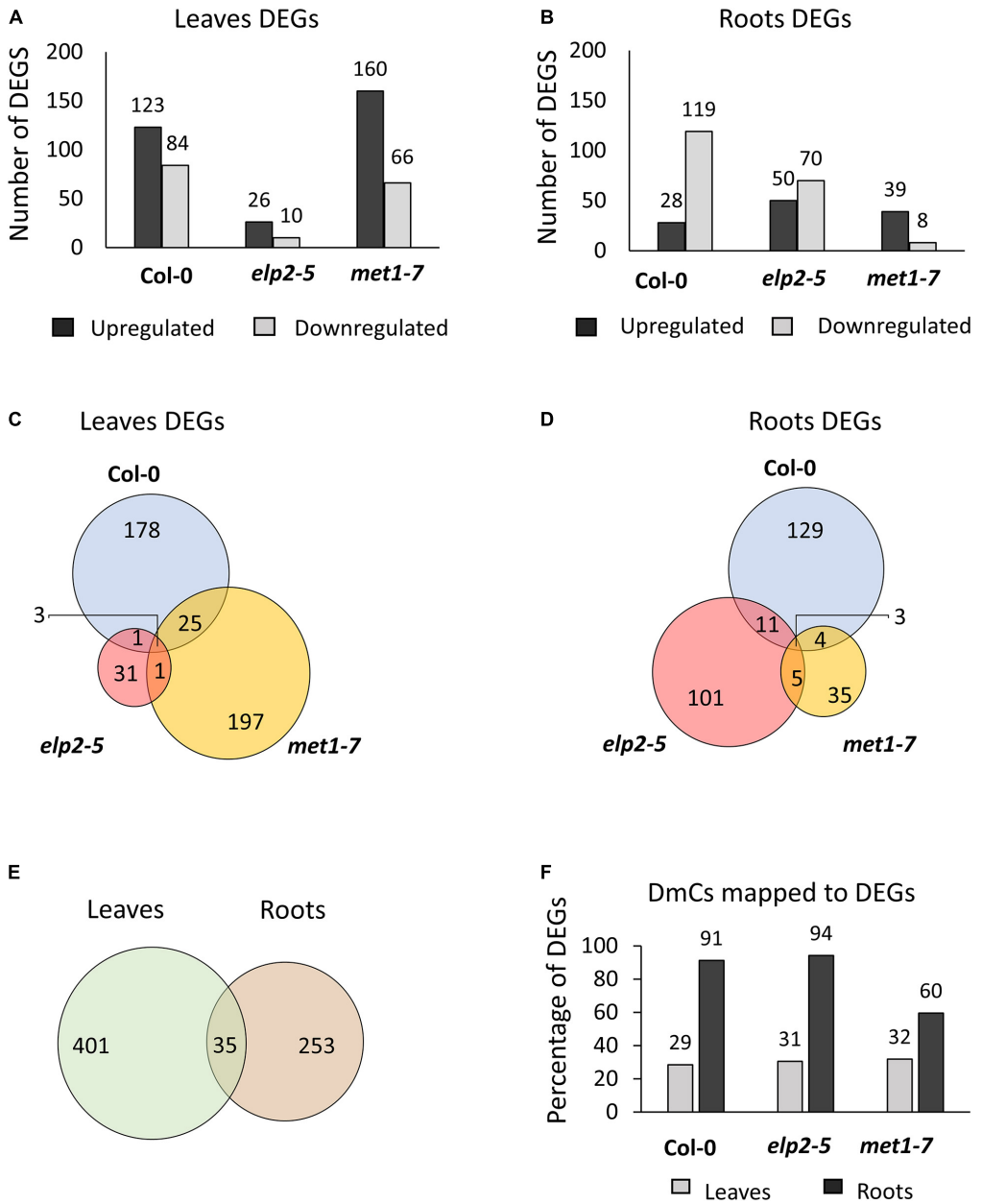

FIGURE 7 | Analysis of differential gene expression in response to spaceflight and the relationship between DmCs. Bar graph showing the number of differentially expressed genes (DEGs) in response to spaceflight (spaceflight compared to ground control) of each genotype in **(A)** leaves or **(B)** roots. Black bars indicate the number of up-regulated genes whereas gray bars indicate the number of down-regulated genes. Venn diagrams showing the DEGs that overlap between each genotype in response to spaceflight in both **(C)** leaves and **(D)** roots. **(E)** Venn diagram of the DEGs that overlap in leaves and roots. **(F)** Bar graph showing the percentage of DmCs that mapped to DEGs in leaves (gray bar) and in roots (black bars).

many processes characteristic of stress responses (**Figure 8E**). Processes shared as enriched between leaf and root DmC-DEGs included general responses to stimuli, oxidoreductase activities, and heme-binding. Leaf-specific DmC-DEGs were enriched in biosynthetic processes of flavonoids and pigments, jasmonic acid, and wounding responses in both GC and FT. DmC-DEGs in GC leaves were more specifically enriched in phenylpropanoid metabolism and the response to oxidative stress, as well as in further oxidoreducatase and peroxidase activities. DmC-DEGs in FT leaves were enriched with defense and hypoxia responses. DmC-DEGs annotated to the plasma membrane and extracellular structures were enriched among both GC and FT roots, in addition to transport processes and activities. Carbohydrate transport and binding were specifically enriched among root GC DmC-DEGs. Thus, *elp2-5* shows differential expression and methylation of stress and hormone response pathways, as well as

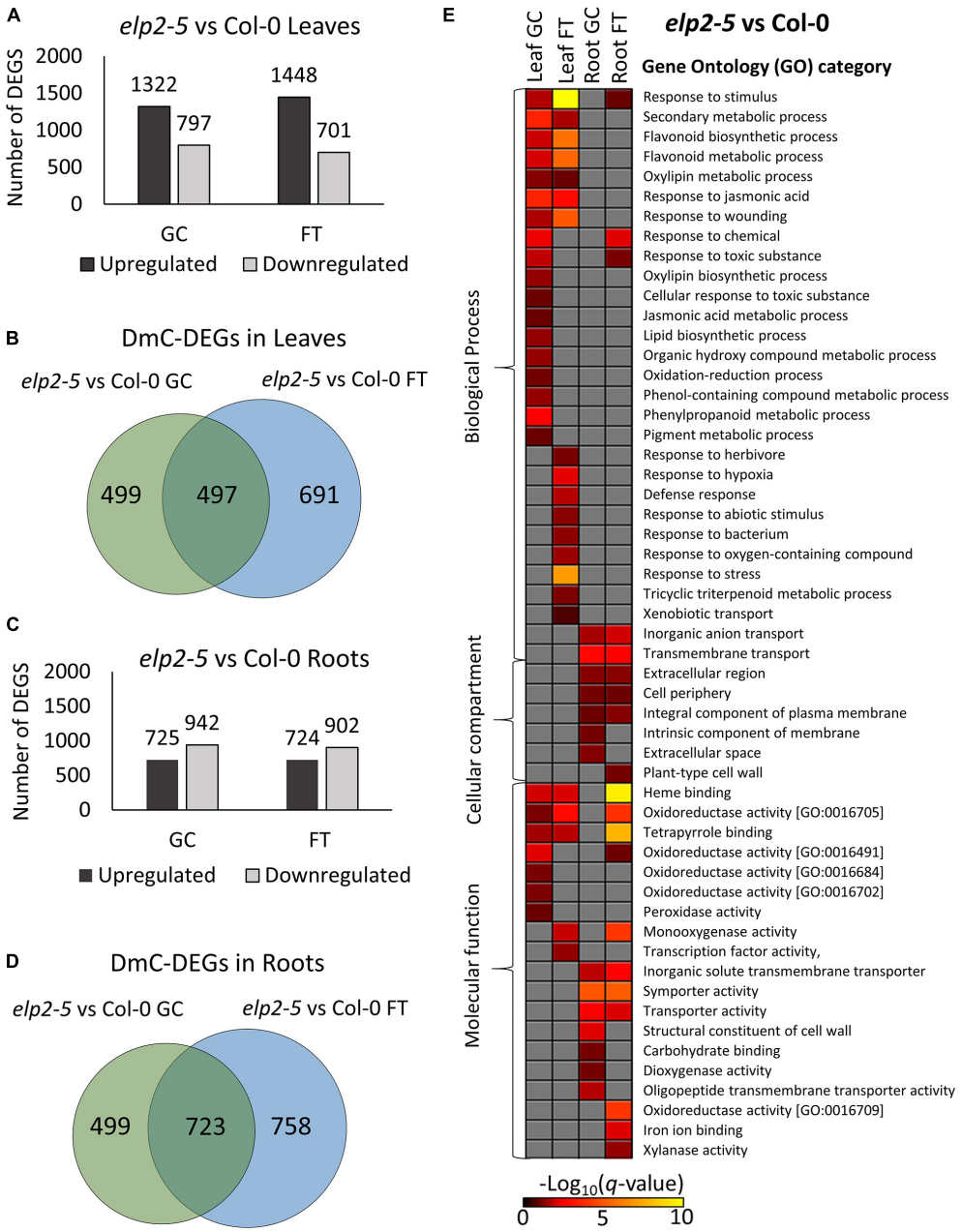

FIGURE 8 | Pairwise comparison of *elp2-5* mutant vs wild-type Col-0 DEGs-DmCs on the ground and in spaceflight. **(A)** Bar graph showing the number of DEGs between *elp2-5* and Col-0 leaves on the ground (GC) and in spaceflight (FT). Black bars indicate the number of up-regulated genes whereas gray bars indicate the number of down-regulated genes. **(B)** Venn diagrams shows the number of DEG-DmCs that overlap between *elp2-5* and Col-0 leaves on the ground and in spaceflight. **(C)** Bar graph showing the number of DEGs between *elp2-5* and Col-0 roots in GC and in FT. Black bars indicate the number of up-regulated genes whereas gray bars indicate the number of down-regulated genes. **(D)** Venn diagrams shows the number of DEG-DmCs that overlap between *elp2-5* and Col-0 roots in GC and in FT. **(E)** Gene ontology (GO) enrichment analyses of DmC-DEG genes in *elp2-5* vs Col-0 comparison of both leaves and roots in each environment (GC and FT). The heatmap shows the GO terms that were enriched within each environment and tissue combination between *elp2-5* and Col-0. The scale bar represents the negative Log_{10} of the *q*-values (corrected *p*-values) from the "test of significance of enrichment" within each list of DmC-DEGs. The *q*-value cutoff was set at 0.05. Higher values in the scaling indicate higher significance of the enrichment.

transport and metabolic pathways, when compared to the Col-0 wild-type line within each tissue and growth condition.

Relative to the Col-0 response, there were substantially more DEGs and DmCs in *elp2-5* (**Figure 8**) than were seen in *met1-7* (**Figure 9**). In leaves, *elp2-5* vs Col-0 showed a total of 2,119 (1,322 up-regulated and 797 down-regulated) DEGs in GC, whereas 2,149 (1,448 up-regulated and 701 down-regulated) DEGs were observed in FT (**Figure 8A**). DmC-DEGs in the *elp2-5* to Col-0

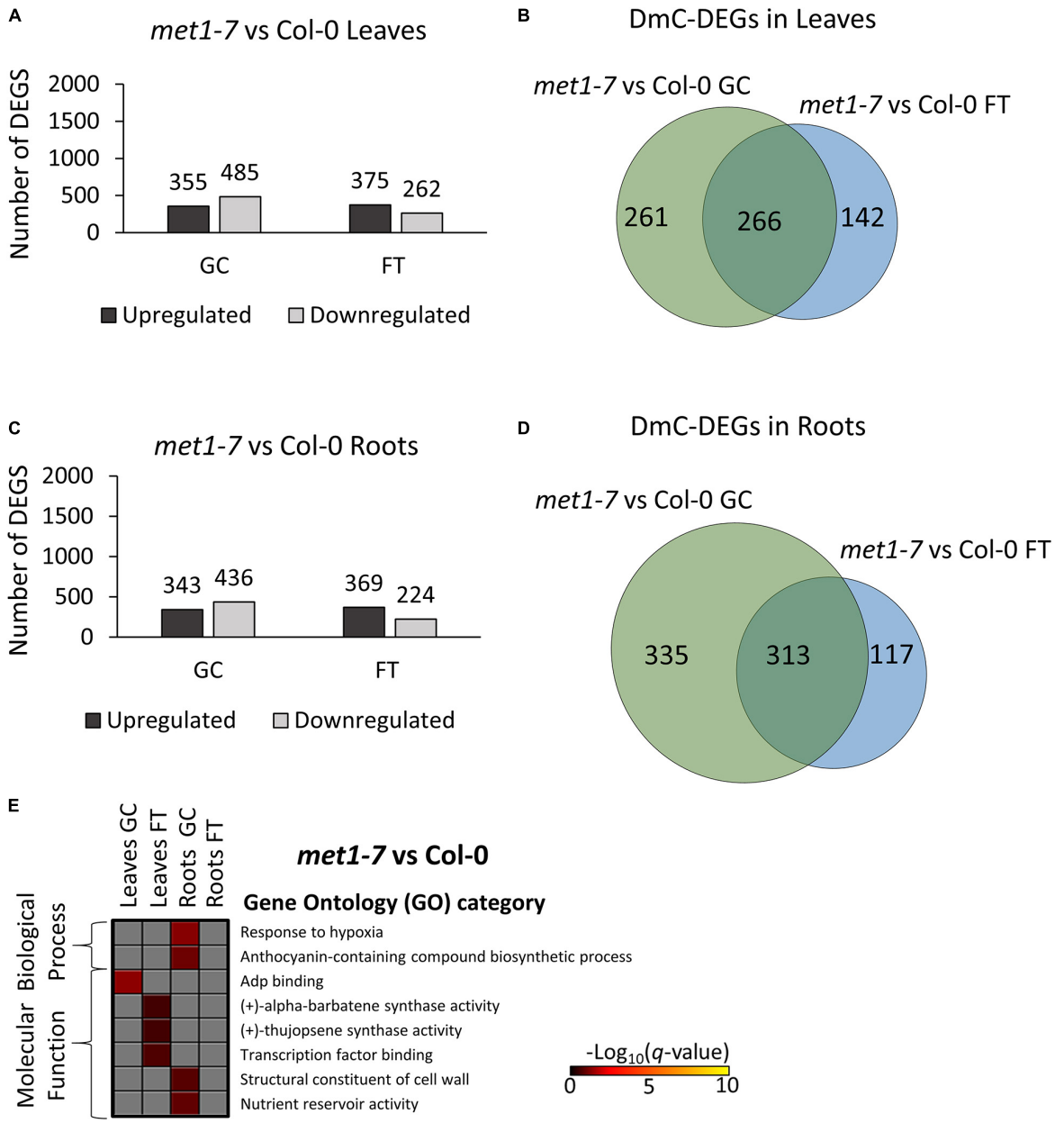

FIGURE 9 | Pairwise comparison of *met1-7* mutant vs wild-type Col-0 DEGs-DmCs on the ground and in spaceflight. **(A)** Bar graph showing the number of DEGs between *met1-7* and Col-0 leaves on the ground (GC) and in spaceflight (FT). Black bars indicate the number of up-regulated genes whereas gray bars indicate the number of down-regulated genes. **(B)** Venn diagrams shows the number of DEG-DmCs that overlap between *met1-7* and Col-0 leaves on the ground and in spaceflight. **(C)** Bar graph showing the number of DEGs between *met1-7* and Col-0 roots in GC and in FT. Black bars indicate the number of up-regulated genes whereas gray bars indicate the number of down-regulated genes. **(D)** Venn diagrams shows the number of DEG-DmCs that overlap between *met1-7* and Col-0 roots in GC and in FT. **(E)** Gene ontology (GO) enrichment analyses of DmC-DEG genes in *met1-7* vs Col-0 comparison of both leaves and roots in each environment (GC and FT). The heatmap shows the GO terms that were enriched within each environment and tissue combination between *met1-7* and Col-0. The scale bar represents the negative Log_{10} of the *q*-values (corrected *p*-values) from the "test of significance of enrichment" within each list of DmC-DEGs. The *q*-value cutoff was set at 0.05. Higher values in the scaling indicate higher significance of the enrichment.

leaves comparison revealed 996 differentially methylated and expressed transcripts on the GC and 1,188 in FT, of which 499 were unique to GC, 691 unique to FT, and 497 were shared among both conditions (**Figure 8B**). In the roots, the pairwise comparison of *elp2-5* and Col-0 in the GC revealed a total of 1,667 (725 up-regulated and 942 down-regulated) DEGs and a total of 1,626 (724 up-regulated and 902 down-regulated) DEGs in FT (**Figure 8C**). DmC-DEGs in the *elp2-5* to Col-0 roots comparison revealed a total of 1,222 DmC-DEGs in GC roots and 1,481 DmC-DEGs in FT roots, of which 723 were shared in both

conditions (**Figure 8D**). In leaves, *met1-7* vs Col-0 showed a total of 840 (355 up-regulated and 485 down-regulated) DEGs in GC, whereas in FT 637 (375 up-regulated and 262 down-regulated) DEGs were observed (**Figure 9A**). DmC-DEGs in the *met1-7* to Col-0 leaves comparison revealed 527 differentially methylated and expressed transcripts on the ground and 408 in flight, of which 261 were unique to GC, 142 unique to FT, and 266 were shared among both conditions (**Figure 9B**). In the roots, the pairwise comparison of *met1-7* and Col-0 in the GC revealed a total of 779 (343 up-regulated and 436 down-regulated) DEGs and a total of 593 (369 up-regulated and 224 down-regulated) DEGs in spaceflight (**Figure 9C**). DmC-DEGs in the *met1-7* to Col-0 roots comparison revealed a total of 648 DmC-DEGs in GC roots and 430 DmC-DEGs in FT roots, of which 313 were shared in both conditions (**Figure 9D**).

Gene ontology analysis of DmC-DEGs between *met1-7* and the wild-type line yielded few term enrichments (**Figure 8E**). Biological process enrichments associated with hypoxic responses and biosynthesis of anthocyanin-containing compounds were present in GC root DmC-DEGs. Molecular functions of ADP binding, sesquiterpene compound synthase activities, and cell wall constituents, and nutrient reservoir activity were enriched in leaf GC DmC-DEGs, leaf FT DmC-DEGs, and root GC DmC-DEGs, respectively.

Alignments of DmC-DEGs in each methylation context in the various genic regions were generated for each genotype and depicted as heatmaps (**Figure 10**). In leaves, a total of 59 DmC-DEGs were observed in wild-type Col-0, while in *elp2-5* and *met1-7*, there were 11 and 72 DmC-DEGs, respectively. Correlations between differential expression and DNA methylation were used to organize the heatmap. The top bracketed sections of the heatmaps show negative correlations where up-regulated genes aligned with hypomethylated DmCs and down-regulated genes aligned with hypermethylated DmCs are grouped. The following bracketed section shows positive correlations where the converse relationship between DEGs and DmCs was highlighted. The unbracketed section at the bottom of the heatmap shows genes that have no distinguishable correlations between DEGs and DmCs. In Col-0 leaves, 41% (24 out of 59) of genes had a negative correlation and 49% (29 out of 59) had a positive correlation. In *elp2-5* leaves, negative and positive correlation were evenly distributed at 45% (5 out of 11), whereas in *met1-7* leaves, 26% (19 out of 72) of genes showed a negative correlation and 60% (43 out of 72) had a positive correlation. In roots, there were a total of 114 DmC-DEGs in Col-0, 101 DmC-DEGs in *elp2-5*, and 28 DmC-DEGs in *met1-7* (**Figure 11**). 16% (19 out of 114) of the wild-type Col-0 root DmC-DEGs had a negative correlation between gene expression and methylation levels, whereas 66% (75 out of 114) were positively correlated. In *elp2-5* roots, 54% (55 out of 101) of genes showed a negative correlation, and 42% (42 out of 101) showed a positive correlation. In *met1-7* roots, 36% (10 out of 28) of genes showed a negative correlation, and 50% (14 out of 28) showed a positive correlation.

Gene ontology analysis of the lists of DmC-DEGs from the FT vs GC contrasts revealed that the *elp2-5* and *met1-7* lines lacked enrichment of differential expression in traditional spaceflight acclimation processes (**Figure 12**). In Col-0 leaves, DmC-DEGs were enriched in localization to glyoxosomes (**Figure 12A**). The DmC-DEGs observed in the spaceflight response of *met1-7* leaves were in pathways associated with the metabolism of pigments containing anthocyanins. The enrichments among the Col-0 root FT vs GC DmC-DEGs were primarily associated with defense pathways and responses to hypoxia, as well as the cell wall and membrane nanodomains (**Figure 12B** and **Supplementary Table 1**). Col-0 and *elp2-5* roots showed enrichments of molecular function GO terms, involving FAD and manganese ion binding, and carbonate dehydratase activity, respectively. A secondary analysis using all FT vs GC DEGs showed that the analysis of only DmC-DEGs recapitulated the majority of the terms associated with the overall transcriptomic response to spaceflight (data not shown). The GO-associated processes, localizations, and functions represented by these terms are gained or lost from the methylation-sensitive aspect of the spaceflight response dependent on the functionality of ELP2 and MET1.

DISCUSSION

DNA methylation plays a crucial role in regulating stress responses and physiological adaptation in plants (Boyko and Kovalchuk, 2010; Dowen et al., 2012; Zhang, 2012; Wang et al., 2013; Yu et al., 2013; Garg et al., 2015; Tameshige et al., 2015; Yong-Villalobos et al., 2015; Zhou et al., 2019). Physiological adaptation to spaceflight engages gene expression changes that at least mimic several terrestrial stress responses (Hoson et al., 2002; Gao et al., 2008; Salmi and Roux, 2008; Blancaflor, 2013; Correll et al., 2013; Paul et al., 2013; Zupanska et al., 2013; Ferl et al., 2014; Inglis et al., 2014; Nakashima et al., 2014; Sugimoto et al., 2014; Kwon et al., 2015; Schüler et al., 2015; Zhang et al., 2015; Barker et al., 2017; Choi et al., 2019; Kruse et al., 2020; **Supplementary Table 2**). In addition, the first whole-genome survey of DNA methylation in wild-type WS Arabidopsis plants grown entirely in the spaceflight environment showed correlated changes in DNA methylation accompanying regulation of DEGs in spaceflight, especially in stress response genes such as those associated with defense responses and ROS signaling (Zhou et al., 2019). However, such correlations do not establish a functional connection between DNA methylation and physiological adaptation to the spaceflight environment. Therefore, two methylation mutant lines, each deficient in separate and distinct functions that affect methylation, were grown on the ISS to assess their response to spaceflight compared to that of wild type. The assays for response to spaceflight were overall growth and morphology, gene expression profiles, and DNA methylation profiles.

The *elp2-5* plants failed to grow normally in spaceflight (**Figure 1A**). Previous experiments have shown that in the microgravity of spaceflight, plant root growth is altered compared to growth on the ground, yet when presented with directional light, roots generally grow away from the light source and establish a near normal growth pattern, and present a generally healthy-looking morphology (Paul et al., 2012; Zhou et al., 2019). However, the roots of the *elp2-5* plants in spaceflight did not

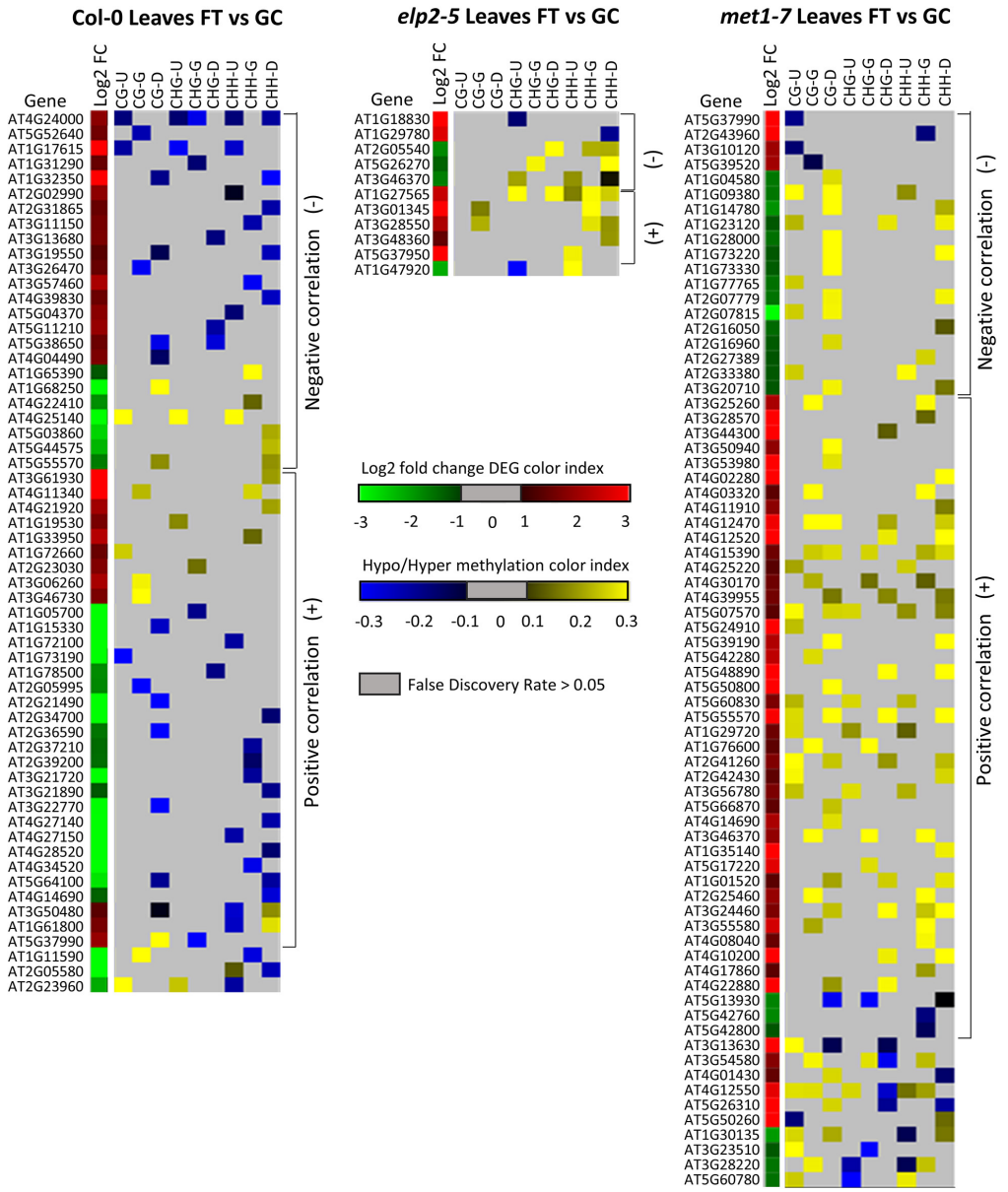

FIGURE 10 | Heatmap of spaceflight (FT) vs ground control (GC) DEGs-DmCs in Col-0, *elp2-5,* and *met1-7* leaves. Heat maps show the Log_2 (Fold-change) of differential gene expression (red: up-regulated and green: down-regulated) and differential DNA methylation (hypermethylation: yellow and hypomethylation: blue) of Col-0, *elp2-5*, and *met1-7* leaves in FT compared to GC. DmCs for each CG, CHG, and CHH methylation context in each genic region (Gene body: TSS to TTS, upstream: 2 kb from TSS, downstream: 2 kb from TTS) are denoted in the heat maps. The heatmaps show both negative and positive correlations of DEGs with DmCs. Negative correlations (–) are defined when up-regulated genes aligned with hypomethylated DmCs and down-regulated genes aligned with hypermethylated DmCs. The converse relationship is indicative of a positive correlation (+).

set up this typical spaceflight directionality, as the roots did not navigate away from the germinated seed. The total biomass of the *elp2-5* plants on orbit was similar to that of the GCs (**Supplementary Figure 1**). Thus, all of the biomass of the *elp2-5* spaceflight plants was concentrated into the area very near the seed, which may accelerate the depletion of nutrients in the immediate vicinity of germination. Nutrient depletion stress is also apparent in the *elp2-5* spaceflight transcriptome. The leaves of the spaceflight *elp2-5* plants appeared to be less expanded, more chlorotic and displayed more reddish pigmentation than the leaves of the GCs (**Figure 1C**). The *elp2-5* mutants generally exhibit over-accumulation of anthocyanin pigments, which can be further exacerbated by stress (Zhou et al., 2009). This distinct, spaceflight-dependent, morphology for *elp2-5* plants suggests that *elp2-5* plants are physiologically maladapted to spaceflight. While it has been established that ELP2 has a role in root development (e.g., Jia et al., 2015) the spaceflight data suggest that ELP2 may also play a role in gravity perception

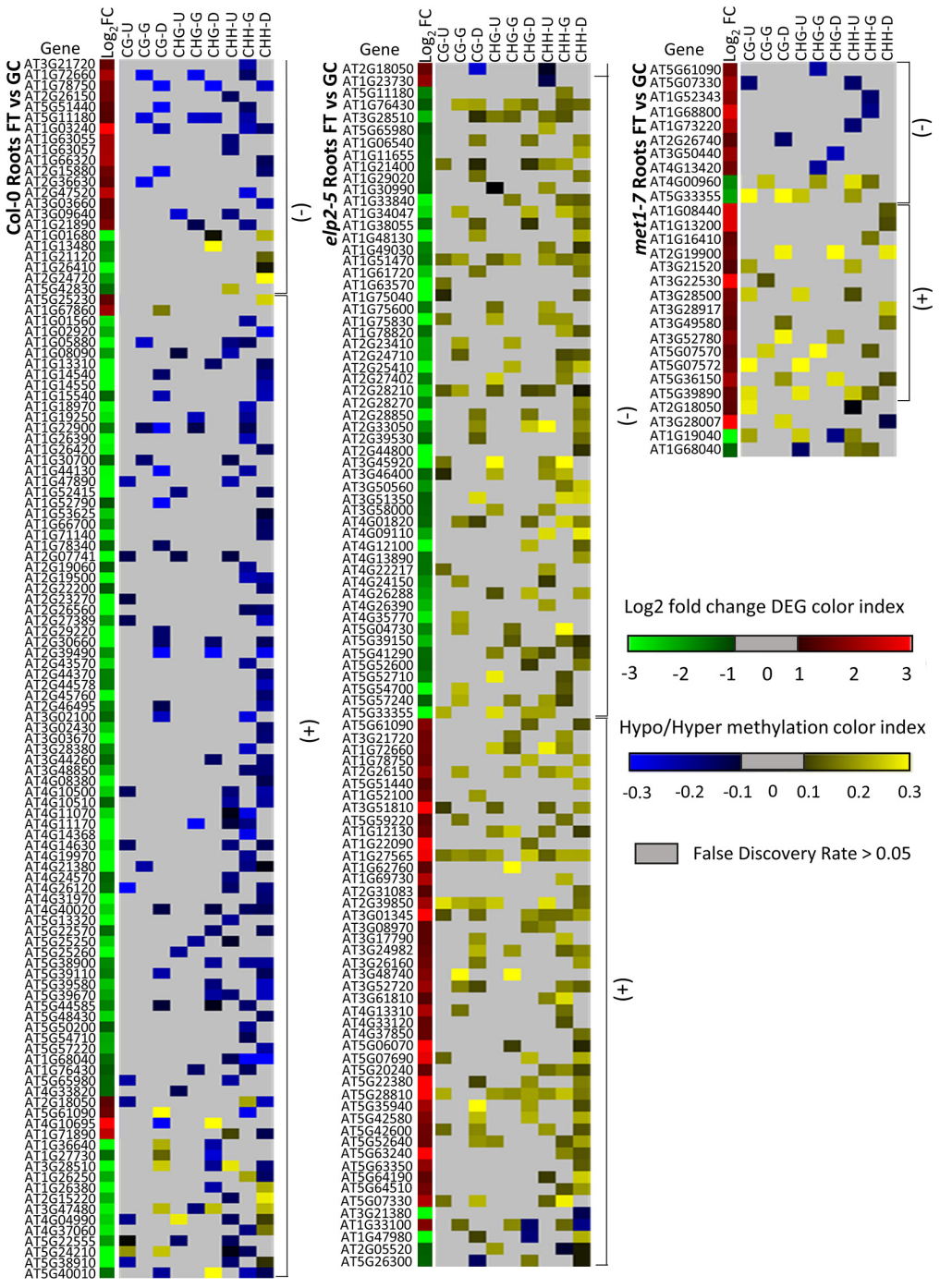

FIGURE 11 | Heatmap of spaceflight (FT) vs ground control (GC) DEGs-DmCs in Col-0, *elp2-5*, and *met1-7* roots. Heat maps show the Log_2 (Fold-change) of differential gene expression (red: up-regulated and green: down-regulated) and differential DNA methylation (hypermethylation: yellow and hypomethylation: blue) of Col-0, *elp2-5*, and *met1-7* roots in FT compared to GC. DmCs for each CG, CHG, and CHH methylation context in each genic region (Gene body: TSS to TTS, upstream: 2 kb from TSS, downstream: 2 kb from TTS) are denoted in the heat maps. The heatmaps show both negative and positive correlations of DEGs with DmCs. Negative correlations (–) are defined when up-regulated genes aligned with hypomethylated DmCs and down-regulated genes aligned with hypermethylated DmCs. The converse relationship is indicative of a positive correlation (+).

and root navigation. In terrestrial environments, where gravity can impart a tropic force, plants lacking ELP2 have shorter roots but grow reasonably well (Jia et al., 2015). However, in the absence of gravity, *elp2-5* mutant roots appear to lose the ability to navigate effectively away from the site of the germinating seed.

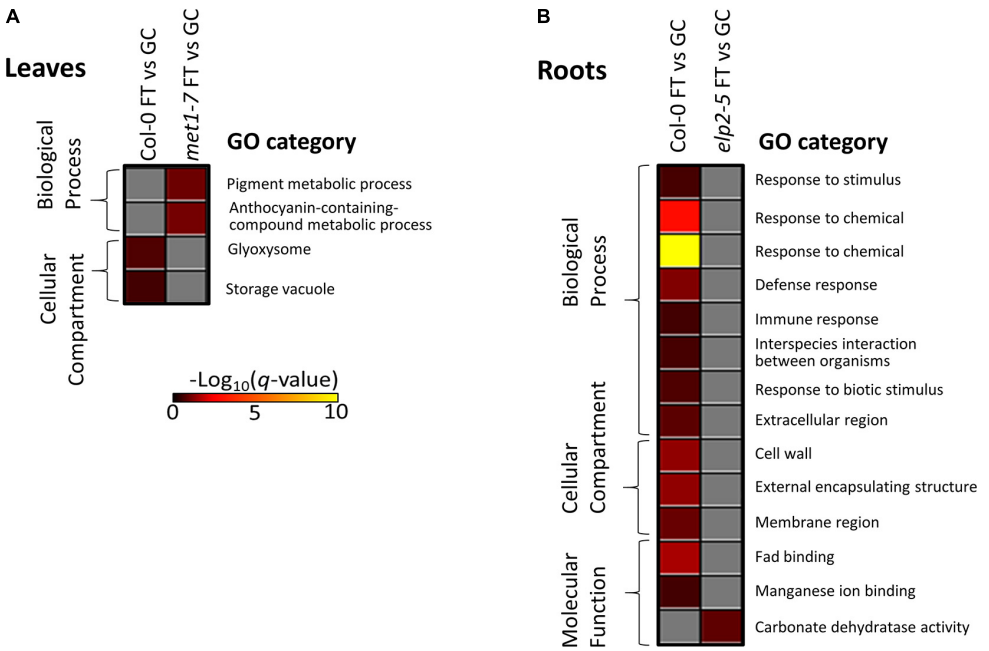

FIGURE 12 | Gene ontology enrichment analyses of spaceflight (FT) vs ground control (GC) DEGs-DmCs in each genotype. **(A)** The heatmap shows the GO terms that were enriched for in the DmC-DEG heatmap (**Figure 10**) for the leaf tissue in all three genotypes. In leaves, *elp2-5* did not have any enriched GO terms. **(B)** The heatmap shows GO terms that were enriched for in the DmC-DEG heatmap (**Figure 11**) for the root tissue in all three genotypes. In roots, *met1-7* did not have any enriched GO terms. The scale bar represents the negative Log_{10} of the *q*-values (corrected *p*-values) for the GO terms' enrichment within each list of DmC-DEGs. The *q*-value cutoff was set at 0.05. Higher values in the scaling indicate higher significance of the enrichment.

The *met1-7* plants appeared better able to cope with the absence of gravity than *elp2-5* plants, as their spaceflight root growth patterns were similar to Col-0, with no obvious deleterious morphologies seen on orbit (**Figure 1C**). The *met1-7* plants produced as much biomass on orbit as in the GCs (**Supplementary Figure 1**) and established a root growth pattern that was visually typical of spaceflight growth (Paul et al., 2012; Zhou et al., 2019) and similar to the appearance of Col-0 (**Figure 1C**).

The differential spaceflight morphologies of *elp2-5* and *met1-7* suggest that the DNA methylation events conditioned by MET1 and ELP2 differentially affect spaceflight physiological adaptation and success. Since MET1 and ELP2 represent two different mechanisms that affect methylation, these results suggest that spaceflight might affect the methylation of specific genes more than the genome-wide status of methylation.

The degree of genome-wide methylation was not statistically different between spaceflight and GCs for Col-0, but both *elp2-5* and *met1-7* were generally hypermethylated compared to the GCs (**Figure 2** and **Supplementary File 1**). This result reinforces the conclusion that changes in overall average genome methylation is not a hallmark of spaceflight adaptation in wild-type plants, as this result was also seen in the spaceflight methylome of Arabidopsis cultivar WS (Zhou et al., 2019). However, the hypermethylation of the mutant genomes suggested that the respective contributions of MET1 and ELP2 are important to maintaining an appropriate degree of genome-wide methylation in the physiological adaptation to spaceflight. The degree of DNA methylation within each context in *elp2-5* was significantly increased in response to spaceflight in both leaves and roots (**Figures 2G,H**) compared to the degree of methylation of these mutants with respect to wild type in the GCs. However, there was a distinction between the two mutant lines in the degree and context of methylation in the respective root genomes. The spaceflight *elp2-5* root genome was significantly hypermethylated in all contexts (CG, CHG, and CHH) compared to the GCs, whereas only CG methylation was elevated in the spaceflight *met1-7* genome (**Figures 2D,H,J**). Although the contribution of CHG and CHH methylation in Arabidopsis is generally small (about 14%; Niederhuth et al., 2016) it is possible that while genome-wide changes in CG methylation does not impact spaceflight root growth morphology, changes in the CHG and CHH context may contribute to the spaceflight phenotype of *elp2-5* plants.

The degree of spaceflight methylation across protein-coding regions showed organ-specificity among all genotypes. Methylation in the protein coding regions is relevant to gene expression profiles, which are also highly organ specific. Spaceflight induced more CG methylation across all protein-coding regions (upstream, gene body, and downstream) in *met1-7* leaves and only increased methylation in the upstream and downstream regions in roots (**Figure 3**). Conversely, *elp2-5* methylation was increased across all protein-coding regions and methylation contexts only in the roots in response to spaceflight (**Figure 3**). The substantial increase in CHH methylation in *elp2-5* roots (**Figures 4A,B**) suggests that ELP2 plays a dominant role

in governing the spaceflight-associated methylation changes in the roots, specifically in the CHH context. This correlates with the root growth morphology exhibited by *elp2-5*.

Col-0, *met1-7*, and *elp2-5* are each different in their spaceflight gene expression and DNA methylation profiles. The profiles of differentially expressed and differentially methylated genes in both *elp2-5* and *met1-7* were unique compared to wild-type Col-0 (**Figures 10**, **11** and **Supplementary Figure 2**). The loss of MET1 and ELP2 each had a substantial influence on the profiles of DEGs and differentially methylated genes in response to the spaceflight environment. The types and functions of the genes that are differentially expressed in the *elp2-5* spaceflight plants indicate that the spaceflight plants are under considerable stress compared to GCs. Almost a third of the genes uniquely induced in the spaceflight *elp2-5* leaves are associated with nutrient stress, and most of the rest are genes associated with heat stress, ROS and pathogen responses. These gene classes were also noticeably represented among the *elp2-5* spaceflight root DEGs. These gene expression patterns describe a highly stressed response reflecting the compact growth morphology of the spaceflight *elp2-5* plants. The types and functions of the genes that are differentially expressed in the *met1-7* spaceflight plants suggest that *met1-7* plants were better adjusted to spaceflight than the *elp2-5* plants. As with the *elp2-5* plants, the *met1-7* plants present a number of stress-associated DEGs. However, in contrast to the types of genes expressed by spaceflight *elp2-5* plants, the DEGs in spaceflight *met1-7* plants predominantly functioned in signal transduction, as membrane transporters or transcriptional activators (**Supplementary Figure 2** and **Supplementary File 6**).

While organ-specific differences in the gene expression patterns of various Arabidopsis tissue types (leaves, hypocotyls, roots, and root tips) have been previously reported in spaceflight transcriptomes (Paul et al., 2013, 2017; Zhou et al., 2019), the *met1-7* and *elp2-5* plant lines each demonstrated a different pattern of organ-specific expression and methylation. A large proportion of the differentially methylated and expressed genes in leaves were observed in Col-0 (59 out of 207) and *met1-7* (72 out of 226), whereas in roots a large proportion of differentially methylated and expressed genes were found in Col-0 (114 out of 147) and *elp2-5* (101 out of 120). Only 8% of the leaf DEGs and 12% of the root DEGs overlap between the genotypes, suggesting that unique organ-specific mechanisms were engaged for spaceflight adaptation, with MET1 playing a more important role in leaves and ELP2 having the more important role in roots, in the physiological adaptation to spaceflight.

The relationships between DNA methylation and gene expression in spaceflight were complex and differed between leaves and roots. In wild-type Col-0 leaves, the hypomethylated DmCs and hypermethylated DmCs were evenly distributed across all contexts and all genic regions (**Figure 5A**). The association of changes in methylation with gene expression showed an even proportion of positive and negative correlation. In *met1-7* leaves, DmCs were predominantly hypermethylated in the CG and CHH contexts of downstream regions, and a larger proportion of positive correlations were observed among DmC-DEGs (**Figure 10**). In *elp2-5* roots, DmCs were primarily hypermethylated in the CHH context across all genic regions, yet a larger proportion of DmC-DEGs showed a negative correlation (**Figure 11**). These observations are in contrast with other studies that report a strong negative correlation between promoter CG methylation and gene expression levels (Finnegan et al., 1996; Garg et al., 2015; Jia et al., 2015). Further investigations may clarify the relationship between leaf spaceflight transcript abundance and methylation in different contexts and genic regions. However, 80% of DEGs (across all genotypes) were also differentially methylated in roots. In leaves, that average was only 30% (**Figure 7F**). These data suggest that DNA methylation plays a significantly larger role in regulating the genes associated with the spaceflight response in roots than in leaves.

The genes differentially regulated by *elp2-5* and *met1-7* in spaceflight were from distinctly different metabolic processes than those regulated in Col-0 in response to spaceflight. Functional analyses of DmC-DEGs showed sharp contrasts among *elp2-5* and *met1-7* with the Col-0 wild-type. The mutant *elp2-5* line showed differential methylation and expression of gene classes traditionally associated with the spaceflight response and other abiotic stresses, such as metabolic processes and defense, hormone, and hypoxic responses across tissue types (**Figure 8E**; Cramer et al., 2011). The mutant *met1-7* line, conversely, demonstrated relatively few GO term enrichments, indicating that DmC-DEGs tended to result from largely untargeted changes in methylation (**Figure 9E**). ELP2 regulates root growth and development, and *elp2* loss of function mutants display shorter roots, and this phenotype may be linked to the root-specific involvement of DmC-DEGs associated with ion homeostasis, transport processes, and extracellular localization observed in this study (**Figures 1C**, 8E; Jia et al., 2015). The spaceflight-associated processes that are differentially represented in *elp2-5* are also among the primary classes enriched in DmC-DEGs involved in the spaceflight acclimation of Col-0 (**Figures 8E**, **12**). These data suggest that the spaceflight response works more directly through DNA methylation mechanisms and other mechanisms regulated by ELP2 than through the maintenance mechanisms represented by MET1.

DNA methylation profiles within a genome are dynamic and complex, yet integral to plant growth, development, and stress responses (reviewed in: Bartels et al., 2018). The response to spaceflight includes differential DNA methylation in a manner that is similar to known terrestrial stress responses, particularly those associated with pathogen attack or harsh environments. **Supplementary Table 2** provides a comparison of these spaceflight-associated DmC-DEGs with those from several terrestrial studies of environmental stress responses (Yong-Villalobos et al., 2016; Hewezi et al., 2017, 2018; Stassen et al., 2018; Korotko et al., 2021). This correlation strongly suggests that the spaceflight response, though novel, utilizes a range of mechanistic approaches that are typically employed during terrestrial environmental stress. While direct methylation mechanisms represented by MET1 certainly affect the spaceflight response in terms of gene expression profiles, the indirect DNA demethylation/methylation mechanisms associated with ELP2 have a more profound role than MET1 in the spaceflight response.

CONCLUSION

Genetic factors that influence genome DNA methylation directly impact physiological adaptation to spaceflight, affecting overall growth in space as well as the specifics of the spaceflight gene response profile. Genome methylation is therefore important for spaceflight responses in much the same way as it is important for adaptation to terrestrial stresses. In particular, processes regulated by ELP2 appear critical for proper root directional development in spaceflight. The remodeling of the Arabidopsis methylome in spaceflight, together with the negative outcomes of interfering with DNA methylation, suggests that epigenetic marking is a fundamental part of environmental responses, even during novel environmental stresses outside of the evolutionary history of plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: The RNASeq and bisulfite-seq data reported in this article have been deposited with NCBI at Gene Expression Omnibus (GEO) repository under the super series accession number GSE118503 (GSE118483 for Bisulfite-seq and GSE118502 for RNA seq).

AUTHOR CONTRIBUTIONS

A-LP and RF contributed equally and were responsible for the overall experimental design, execution of the spaceflight experiments, final data evaluations, and writing of the manuscript. NH performed RNA extraction for RNAseq analysis, data analysis, and had a substantial role in initial manuscript and figure development. BC conducted the GO analysis and contributed to the final editing and figure development. All authors contributed to the writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.691790/full#supplementary-material

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