



Epigenetic differences in an identical genetic background modulate alternative splicing in *A. thaliana*

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ARTICLE INFO

Keywords:

Alternative splicing
Arabidopsis
Cold stress
DNA-methylation
epiRILs
Nucleosome occupancy

ABSTRACT

How stable and temperature-dependent variations in DNA methylation and nucleosome occupancy influence alternative splicing (AS) remains poorly understood in plants. To answer this, we generated transcriptome, whole-genome bisulfite, and MNase sequencing data for an epigenetic Recombinant Inbred Line (epiRIL) of *A. thaliana* at normal and cold temperature. For comparative analysis, the same data sets for the parental ecotype Columbia (Col-0) were also generated, whereas for DNA methylation, previously published high confidence methylation profiles of Col-0 were used. Significant epigenetic differences in an identical genetic background were observed between Col-0 and epiRIL lines under normal and cold temperatures. Our transcriptome data revealed that differential DNA methylation and nucleosome occupancy modulate expression levels of many genes and AS in response to cold. Collectively, DNA methylation and nucleosome levels exhibit characteristic patterns around intron-exon boundaries at normal and cold conditions, and any perturbation in them, in an identical genetic background is sufficient to modulate AS in Arabidopsis.

1. Introduction

Plants are sessile organisms and exert firm control over their transcription and translation patterns under variable growth, development, and environmental conditions [1–3]. Plants have evolved various genetic and epigenetic strategies to control diurnal activities in response to changing environmental conditions [4,5]. Alternative splicing (AS) of pre-mRNA is one such strategy that generates multiple transcripts from a single gene and is considered to be an important gene regulatory mechanism to modulate gene expression patterns, protein diversity and/or abundance in plants [6,7]. In comparison to constitutive splicing where a selection of splice sites produces a single mRNA, AS is more complex involving alternative selections of splice sites to produce multiple mRNA isoforms from a single gene [6–8]. The sequencing data from previous studies have shown that up to 70% of multi-exon genes undergo AS in plants [9–11]. In plants and animals, AS is largely a co-transcriptional process initiated during the transcription of pre-mRNA [12–15]. Emerging evidence suggests that during transcription, RNA polymerase II (Pol II) processivity and speed are modulated by various chromatin features which in turn affects AS outcomes [16–20]. For

example, in *A. thaliana* (Arabidopsis), native elongating transcript sequencing (NET-Seq) and global run-on sequencing (GRO-Seq) data suggest that phosphorylation of Pol II C-terminal domain mediates interactions with the spliceosome machinery and the accumulation of Pol II is associated with different chromatin states [18,21]. Nascent RNA sequencing in Arabidopsis also showed that most of the introns are spliced during transcription and the efficiency of co-transcriptional splicing in protein coding genes depends on the number of exons rather than gene length [14,15]. This further suggests that exon-intron boundaries in addition to 5' splice sites (5' SS), and 3' splice sites (3' SS), and *cis*- and *trans*-regulatory splicing features also play important role in modulating co-transcriptional splicing in Arabidopsis.

DNA methylation and nucleosome occupancy are two major epigenetic marks in plants that influence chromatin structure, expression of many genes, and AS [22–26]. Plant DNA methylation occurs in all three contexts including symmetrical CpG, asymmetrical CHG, and CHH (H = A, T, or C) contexts [27]. In Arabidopsis, for instance, CpG is the most abundant methylation observed (~24%) followed by CHG (6.7%) and least CHH (~1.7%) [28,29]. DNA methylation is higher in nucleosome bound DNA compared to nucleosome-free DNA in both humans and

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<https://doi.org/10.1016/j.ygeno.2021.08.006>

Received 21 February 2021; Received in revised form 2 August 2021; Accepted 10 August 2021

Available online 12 August 2021

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Arabidopsis, thus affecting chromatin remodelling [26,30,31]. Furthermore, constitutive exons in both plants and animals show higher nucleosome occupancy levels compared to introns and alternative exons [26,31–37]. It was demonstrated that differential DNA methylation can affect the splicing patterns of a subset of genes in rice [25]. Similarly, it has been shown that changes in the methylation patterns of alternatively spliced exons in humans via CRISPR technology (dCas9 fused to enzymes that methylate or demethylate DNA) alter inclusion levels of these exons [38].

Plants exhibit variable DNA methylation and nucleosome occupancy patterns under different growth and stress conditions [2,23,39–41]. However, it remains to be seen whether these epigenetic marks provide a context through which AS patterns could be modulated under variable growth or stress conditions [2]. We previously showed differential nucleosome occupancy modulates AS under cold stress in Col-0 ecotype of Arabidopsis [41]. In the current study we analysed plants with identical genome sequences but differential DNA methylation (epigenetic Recombinant Inbred Lines - epiRILs) [42]. The epiRILs are homozygous for the *DDM1/DDM1* locus and were developed from a cross of Col-0-*DDM1* (wild type - wt) and Col-0-*ddm1* (decreased in DNA methylation via nucleosome remodelling) mutant line [42]. These epiRILs segregate for hundreds of differentially methylated regions (DMRs) across the genome, and several of these act as stable epigenetic Quantitative Trait Loci (eQTL) for flowering time and primary root length explaining 60–90% of the phenotypic variation under different environments [43]. Since these epiRILs have very little genome sequence variation, epigenetic variation because of DNA methylation differences should largely be independent of *cis*- or *trans*-acting DNA sequence changes. In our studies, we preferred *ddm1* epiRILs over *met1* epiRILs [44] because the latter have very limited influence on AS [45]. Additionally, *DDM1* methylates nucleosome bound DNA in heterochromatic regions as well as genes [46,47], hence it is intrinsically more likely to influence exon-intron definition [26] and potentially the co-transcriptional splicing process.

It has been demonstrated that cold exposure also induces changes in AS patterns to modulate the transcriptome and proteome globally [17,48–50]. Also, cold-induced DNA methylation changes are relatively rapid epigenetic regulators that mediate environmental cues and provide flexible cold responses in plants [51–54]. Recent data show that Pol II elongation kinetics [55] under cold stress in Arabidopsis modulate AS. Henceforth, we also used cold treatment to investigate whether it could generate dynamic methylation and nucleosome occupancy changes and influence AS outcomes. We demonstrate that, in plants with identical genome sequence, methylation and nucleosome occupancy differences are sufficient to modulate different types of AS events and their ratios under different temperatures.

2. Results

2.1. Epigenetic variations modulate cold dependent differential gene expression and alternative splicing

To study how epigenetic differences regulate gene expression and AS in response to normal (22 °C) and cold (4 °C) temperature, we generated transcriptome (RNA-Seq) data for epiRIL-368 and Col-0 plants [41] of Arabidopsis at both temperature conditions (see Supplementary Method S1 for details). Principal component analysis (PCA) analysis of the transcriptome data shows that temperature shift from 22 °C to 4 °C affects gene expression at the gene and transcript level. The PCA (after removing batch effects) of the gene and transcript-level expression data across all samples including replicates showed that temperature is the major contributor to gene expression variation (68.01% and 59.81% of the variance at gene and transcript level, respectively, PCA 1, Fig. S1 B). Also, epigenetic variations between Col-0 and epiRIL-368 showed contribution towards total expression variation detected at the gene (13.35%) and transcript (13.94%) levels, respectively (PCA 2; Fig. S1 B).

Collectively, PCA analysis shows that both epigenetic differences and temperature shifts from 22 °C to 4 °C affects gene expression at the gene and transcript level.

Next, differential gene expression (DGE) analysis of Col-0 versus epiRIL-368 showed differences in the expression of 875 and 474 genes at 22 °C and 4 °C, respectively (Fig. 1A a,b). To show the temperature dependent effect in individual lines, we identified 4097 and 5173 differentially expressed genes (DEGs) in Col-0 and epiRIL-368 upon their shift from 22 °C to 4 °C, respectively (Fig. 1A d-e). Interestingly, in addition to epigenetic variations, exposure to cold temperature increases the number of DEGs in both datasets. For instance, we found 5048 DEGs when comparing Col-0 at 22 °C to epiRIL368 at 4 °C (Fig. 1A c). The results from DGE analysis suggest that temperature dependent epigenetic marks are likely to be involved in differentially regulating the transcriptional responses and expression of many genes in Arabidopsis (one-way ANOVA p -value = 1.1102e-16 for DEGs).

Furthermore, the AS analysis showed that epigenetic differences between Col-0 and epiRIL-368 induced fewer but contrasting changes under similar temperature conditions (22 °C and 4 °C) (one-way ANOVA p -value = 1.7492e-09 for DAS genes). For instance, the number of identified DAS genes between Col-0 versus epiRIL-368 was 305 and 311 at 22 °C and 4 °C, respectively (Fig. 1A a,b). Furthermore, the number of DAS genes in response to temperature shift from 22 °C to 4 °C was higher but showed a similar pattern in both the lines. In response to cold, 1294 and 1255 genes in Col-0 and epiRIL-368 were found to be DAS upon the shift from 22 °C to 4 °C, respectively (Fig. 1A d,e). The number of DAS genes showed an increase due to cold stress (shift from 22 °C to 4 °C) in both Col-0 and epiRIL-368, which in line with previous studies in Arabidopsis [48]. However, more variation in terms of the number of DAS genes (1425) occurred when both epigenetic and cold variations were taken together (Col-0 at 22 °C versus epiRIL-368 at 4 °C; Fig. 1A c). Since gene expression and AS are regulated independently [25,56], we also observed that most transcriptional changes are associated with genes (DEGs) that do not display splicing changes (DAS genes), and most of the splicing changes occur in genes that are not DEGs. Interestingly, there is no overlap between DEGs and DAS genes between Col-0 and epiRIL-368 at 22 °C (hypergeometric test p -value = 0.198) and 4 °C (hypergeometric test p -value = 2.520e-05) (Fig. 1B a-b). Whereas, this number significantly increases (Col-0 at 22 °C versus epiRIL-368 at 4 °C; 7.1%; hypergeometric test p -value 0.011) when epigenetic variations and cold stress adds on together (Fig. 1B c). Further, we performed gene functional enrichment analysis for DEGs and DAS genes (Supplementary File S1) for all three gene ontology (GO) terms i.e. Biological Process (BP), Cellular Component (CC), and Molecular Function (MF) (Fig. S2). Among DEGs, the most significant (FDR <0.05) terms involved transcription regulation, Pol II processivity, cold and other such as response to abscisic acids, are highly enriched (Fig. S2 A) in different contrasting groups. Whereas, DAS genes are mainly enriched in mRNA, splicing and transcription activities, in addition to cold and other stress related genes such as salt and osmotic stresses (Fig. S2 B). Overall, RNA-Seq and GO analyses support the hypothesis that epigenetic variations regulates the expression and AS patterns (one-way ANOVA with pairwise post-hoc Tukey HSD p -value <0.01) of cold-responsive and other genes involved in multiple processes.

2.2. Alternative splicing variation between Col-0 and epiRIL-368 plants

Under stress conditions (cold in this study), plants show changes in DNA methylation patterns [57–59] and nucleosome occupancy level [41]. In Arabidopsis, a strong correlation between DNA methylation and nucleosome occupancy influencing gene expression was observed [26]. Therefore, we reasoned those variations at the methylation and nucleosome (epigenetic) levels may affect AS because of epigenetic differences between Col-0 and epiRIL-368 ecotypes under different temperatures. To further investigate these variations, we performed WGBS for epiRIL-368 plants grown at 22 °C and 4 °C. We identified high

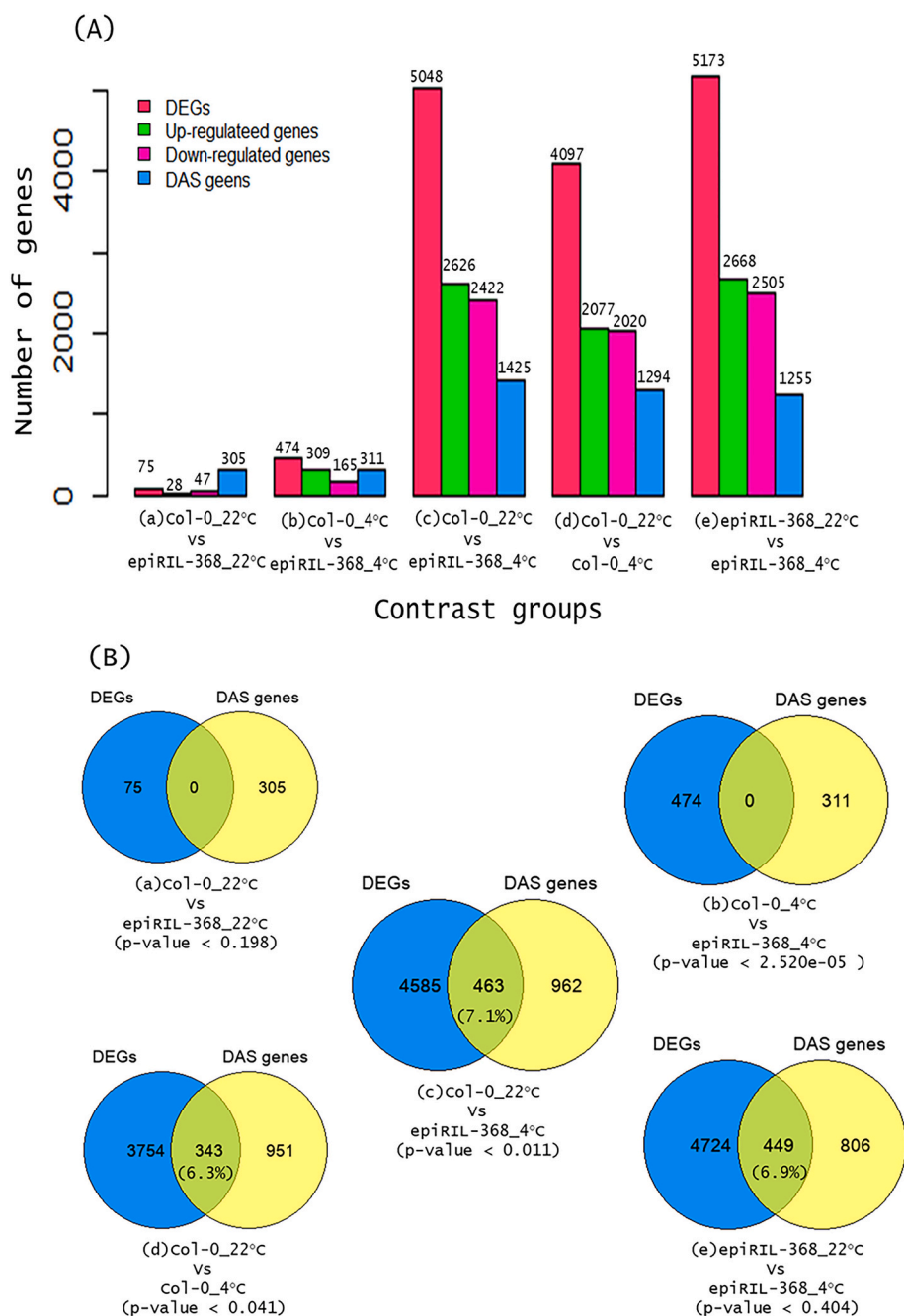


Fig. 1. Differentially expressed genes (DEGs) and differentially alternative spliced (DAS) genes in different contrasting groups. (A) Bar graph representing the number of significantly DEGs, up-regulated, down-regulated, and DAS genes in different contrasting groups (a-e), respectively (one-way ANOVA p -value = $1.1102e-16$ for DEGs and p -value = $1.7492e-09$ for DAS genes). The x-axis and y-axis represent the contrast groups and number of genes, respectively. (B) Venn diagram of DEGs (blue) and DAS genes (yellow) in each contrast group. The p -value in the parenthesis represents the significance of the overlap between DEGs and DAS genes detected by the hypergeometric distribution test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confidence differentially methylated regions (hc_DMRs) (Fisher's exact test, p -value ≤ 0.01) in comparison to 54 Columbia (Col) lines of Arabidopsis using the hc_DMR caller pipeline developed by the Jacobsen group at the University of California [60]. The high confidence hypermethylated and hypomethylated regions for all three contexts CpG, CHG, and CHH, in epiRIL-368 at 22 °C and 4 °C (Supplementary File S2). The high number of hypomethylated regions suggests relatively lower methylation levels in epiRIL-368 compared to Col ecotype (Fig. 2A). Among all hcDMRs, 22,968 were hypomethylated regions in the epiRIL-368 line as compared to Col lines (Fig. 2A). For nucleosome occupancy, we performed MNase-Seq for Col-0 (previously reported [41]) and epiRIL-368 plants at 22 °C and 4 °C. The MNase-Seq data analysis (see Materials and Methods) using improved nucleosome-positioning algorithm (iNPS) [61] and DANPOS v.2.1.2 [62] showed a high number of significant differential nucleosome positioning (DNPs) in different contrast groups (Fig. 2B; Supplementary File S3). Interestingly, a higher

number of DNPs was observed because of epigenetic variations rather than temperature differences. For example, the number of DNPs between Col-0 and epiRIL-368 at both 22 °C (27100) and 4 °C (28769) are higher than the number of DNPs observed between Col-0 at 22 °C versus epiRIL-368 at 4 °C (12041) and Col-0 at 22 °C versus Col-0 at 4 °C (12933) (Fig. 2B). Surprisingly, the highest number of DNPs was detected in the contrast group epiRIL-368 at 22 °C versus epiRIL-368 at 4 °C (31486), which might be due to the regulation of some chromatin remodelling genes under cold stress in epiRIL-368 (discussed in a later section). Overall, hcDMRs and DNPs data suggest significant variations at DNA methylation and nucleosome occupancy level in epigenetically different but genetically similar background plants observed under different temperature conditions.

Since many genes were identified that undergo AS and show differential expression (Fig. 1A) in response to epigenetic and temperature variations, we further investigated how epigenetic alterations along

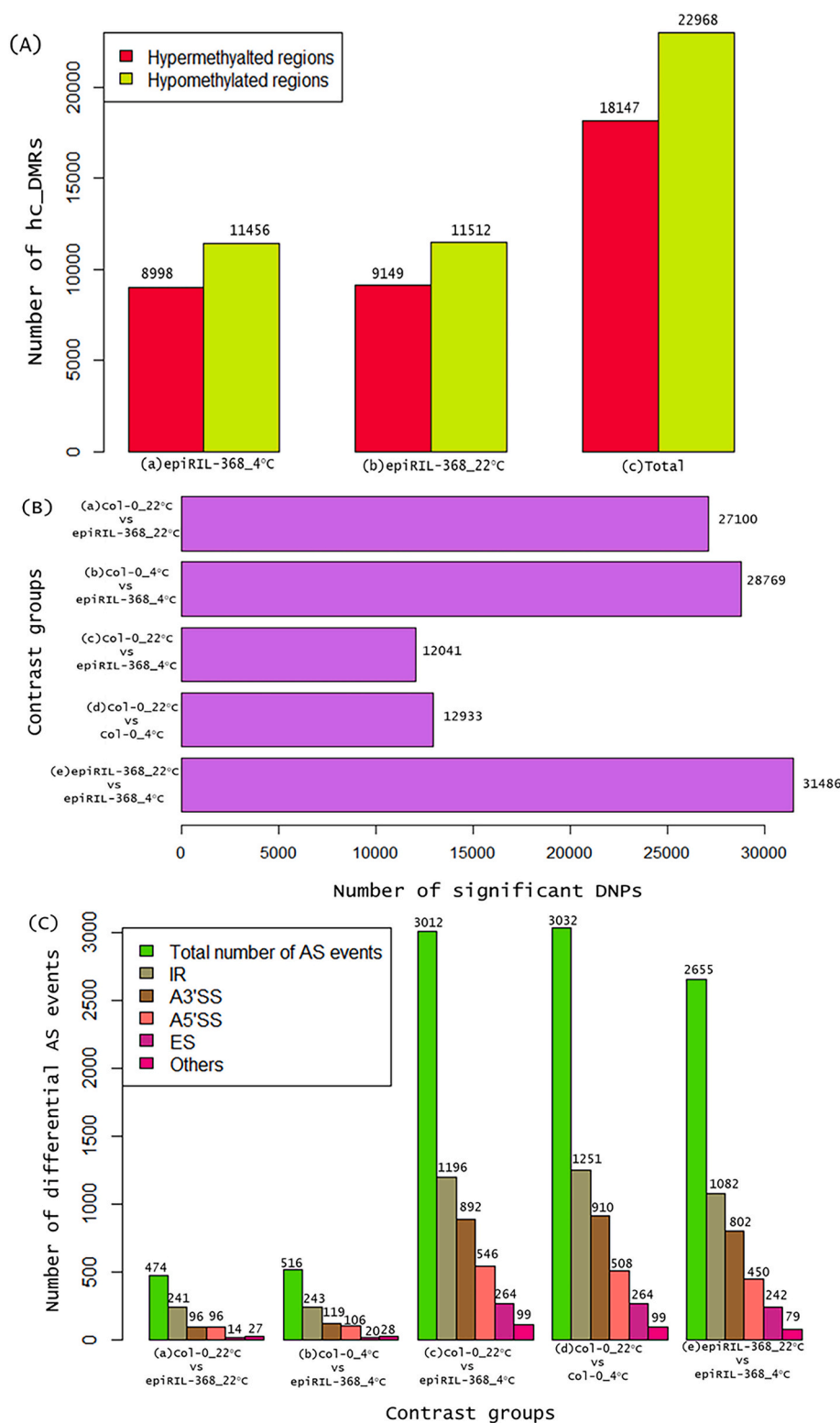


Fig. 2. High confidence differentially methylated regions (hc_DMRs), significant differential nucleosome positionings (DNPs), and differential alternative splicing (DAS) events. (A) Bar graph representing the total number of hypomethylated and hypermethylated regions (hc_DMRs) in epiRIL-368 plants under (a) normal (22 °C) and (b) cold (4 °C) conditions. (B) Bar graph representing the number of significant DNPs detected in different contrasting groups (a-e). (C) The number of differential AS events characterized as total, intron retention (IR), Alt acceptor (A3'SS), Alt donor (A5'SS), exon skipping (ES), and others, in different contrasting groups (a-e).

with cold treatment modulate different AS events in Arabidopsis. Towards this goal, AS events were identified on the basis of change in PSI (Δ PSI) using SUPPA v2.3 [63]. We first calculated AS event inclusion levels (PSI) for a total of 43,953 AS events identified in the reference annotation file of Arabidopsis, followed by the differences in their inclusion (Δ PSI) among different contrast groups. Differential AS events analysis suggests that epiRIL-368 display significant (p -value ≤ 0.05) differences in 474 and 516 AS events compared to Col-0 at 22 °C and

4 °C, respectively (Fig. 2C; Supplementary File S4). Interestingly, the impact of epigenetic variations along with cold treatment showed larger differences in the numbers of AS events. For example, we detected 3012 AS events when Col-0 at 22 °C is compared with epiRIL-368 at 4 °C whose abundance changes due to epigenetic differences and cold treatment (Fig. 2C; Supplementary File S4). Different types of AS events detected in our analysis show an overall similar distribution observed previously in Arabidopsis [9,11] where intron retention (IR) events are

the most prevalent, followed by usage of the alternative acceptor (A3'SS) and alternative donor (A5'SS) sites, and exon skipping (ES) (Fig. 2C; Supplementary File S4). However, different fractions of these AS event types are affected by epigenetic or temperature changes. Fractions of differentially regulated IRs are more than 10 times higher than differentially regulated ES events in the contrast groups Col-0 at 22 °C versus epiRIL-368 at 22 °C and Col-0 at 4 °C versus epiRIL-368 at 4 °C. Whereas, in other contrast groups fractions of differentially ES events are higher (4–5 times) or like those of IRs. These results suggest that epigenetic differences have a differential effect on AS events and are likely to regulate cold-induced AS events distribution differently. Additionally, stable epigenetic variation maintained after many generation, as in the case of epiRIL-368, showed very pronounced effect on differential AS events suggesting that epiRILs could be a useful tool to study the epigenetics of AS in plants.

2.3. DNA methylation and nucleosome occupancy differentially mark exon-intron boundaries in epigenetically different plants

In Arabidopsis, DNA methylation and nucleosome positioning are differentially marked around promoter regions, gene bodies as well as exons and introns, indicating a potential link of chromatin architecture to gene expression and splicing regulation [25,26,34,64]. Since our RNA-seq data in Arabidopsis showed genome-wide changes in gene expression and AS in epigenetically different plants, we wanted to understand whether DNA methylation and nucleosome occupancy levels differentially mark on exon-intron boundaries to modulate splicing in epiRIL-368 plants under normal and cold conditions. Towards this goal, we employed a genome-wide DNA methylation base calling for epiRIL-368 plants at 22 °C and 4 °C and nucleosome signals in Col-0 and epiRIL-

368 plants at 22 °C and 4 °C, and plot around exons and exon-intron boundaries (5' and 3' splice sites). The CpG, CHG, and CHH methylation levels generated using methylpy [65] were plotted around the donor sites (exon-intron; 5'SS), the acceptor sites (intron-exon; 3'SS), and the exons for epiRIL-368 at 22 °C and 4 °C (Fig. 3A; Fig. S3). We observed a sharp drop in CpG methylation at both splice sites (5'SS and 3'SS; Fig. 3A a-b) suggesting its role in transcription and splicing dynamics by affecting Pol II processing around 5'SS and 3'SS as compared to flanking regions. DNA methylation around exons always shows a higher methylation level and can be differentiated from their flanking regions (introns, especially splice sites) (Fig. 3A; Fig. S3A). Regardless of temperature treatment, we also found the level of methylated CpG dinucleotides (mCpG) is higher in exons as compared to flanking regions including introns and splice sites (Fig. 3A). A similar pattern was observed with mCHG and mCHH context (Fig. S3). In our data, a higher level of methylation in exons as compared to flanking regions is in agreement with the previous reports in animals [34,64] and plants [25,26].

Next, we detected genome-wide nucleosome positioning in all samples (Supplementary File S5) and the distribution of nucleosome density around the donor sites (exon-intron; 5'SS), the acceptor sites (intron-exon; 3'SS), and exons to understand how this distribution changes between Col-0 and epiRIL-368 plants at normal and cold conditions. We found a sharp nucleosome peak before the 5'SS and immediately after the 3'SS but intriguingly another peak was also present at the beginning of intron (Fig. 3B a-b). We observed a sharp drop in nucleosome occupancy at ~25 bp upstream of the 3'SS, corresponding to the location of the polypyrimidine tract in the RNA transcript (Fig. 3B b). In Arabidopsis, branch points are located –11 to –60 bp of the acceptor site, and the polypyrimidine tract downstream of the branch point is A and T rich

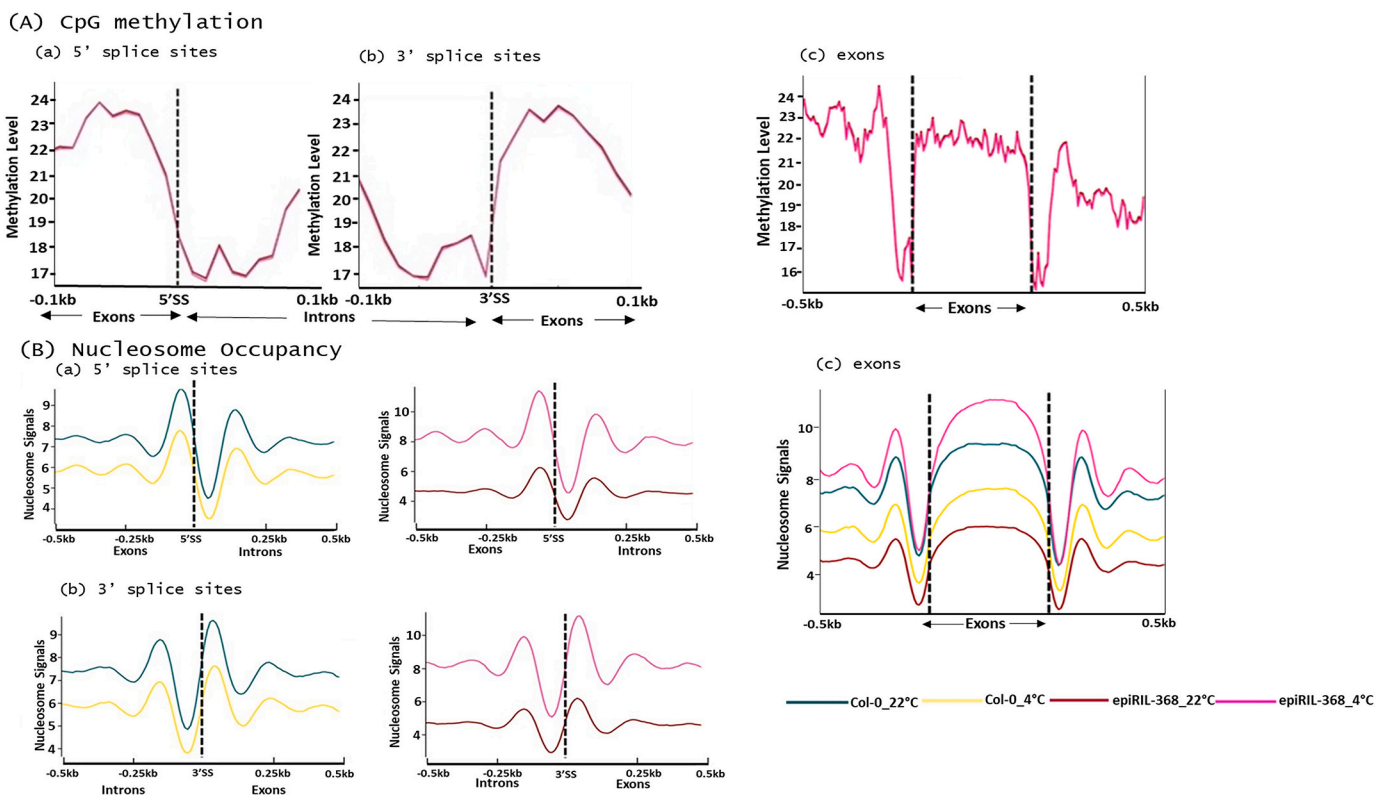


Fig. 3. DNA methylation in CpG contexts and nucleosome occupancy around splice sites (SS) and exons. (A) methylation levels in CpG context in epiRIL-368 plants under normal (22 °C) and cold (4 °C) conditions, around (a) 5'SS and (b) 3'SS alongside 100 bp upstream (– 0.1 kb) and downstream (0.1 kb) to the splice sites, and (c) represents methylation levels in CpG context around exon with 500 bp flanking regions. The y-axis represents the methylation level. (B) Nucleosome occupancy signals in Col-0 and epiRIL-368 plants under normal (22 °C) and cold (4 °C) conditions, around (a) 5'SS and (b) 3'SS alongside 500 bp upstream (–0.5 kb) and downstream (0.5 kb) to the splice sites, while (c) represents the nucleosome occupancy in Col-0 and epiRIL-368 plants under normal (22 °C) and cold (4 °C) conditions around exon with 500 bp flanking regions. The y-axis represents the nucleosome occupancy signals.

[66]. The AT-rich sequences inhibit nucleosome formation on the DNA sequence [67], which may promote the binding of SFs to their corresponding *cis*-elements. Like nucleosome DNA methylation around the splice sites also shows similar (almost identical for CpG) patterns in the three methylation contexts for epiRIL-368 plants (Fig. 3A; Fig. S3). A sharp peak of nucleosome occupancy is detected on exons, surrounded by lower density flanking regions (Fig. 3B c). Despite the similarity of

the nucleosome occupancy profile between different conditions, the change of nucleosome occupancy level upon cold stress was different between Col-0 and epiRIL-368. Although cold stress induces a genome-wide decrease in nucleosome occupancy in Col-0, epiRIL-368 displays an opposite effect manifested by increased nucleosome occupancy upon shift to low temperature. To understand the mechanism of this switch, we hypothesized that the differential expression of chromatin

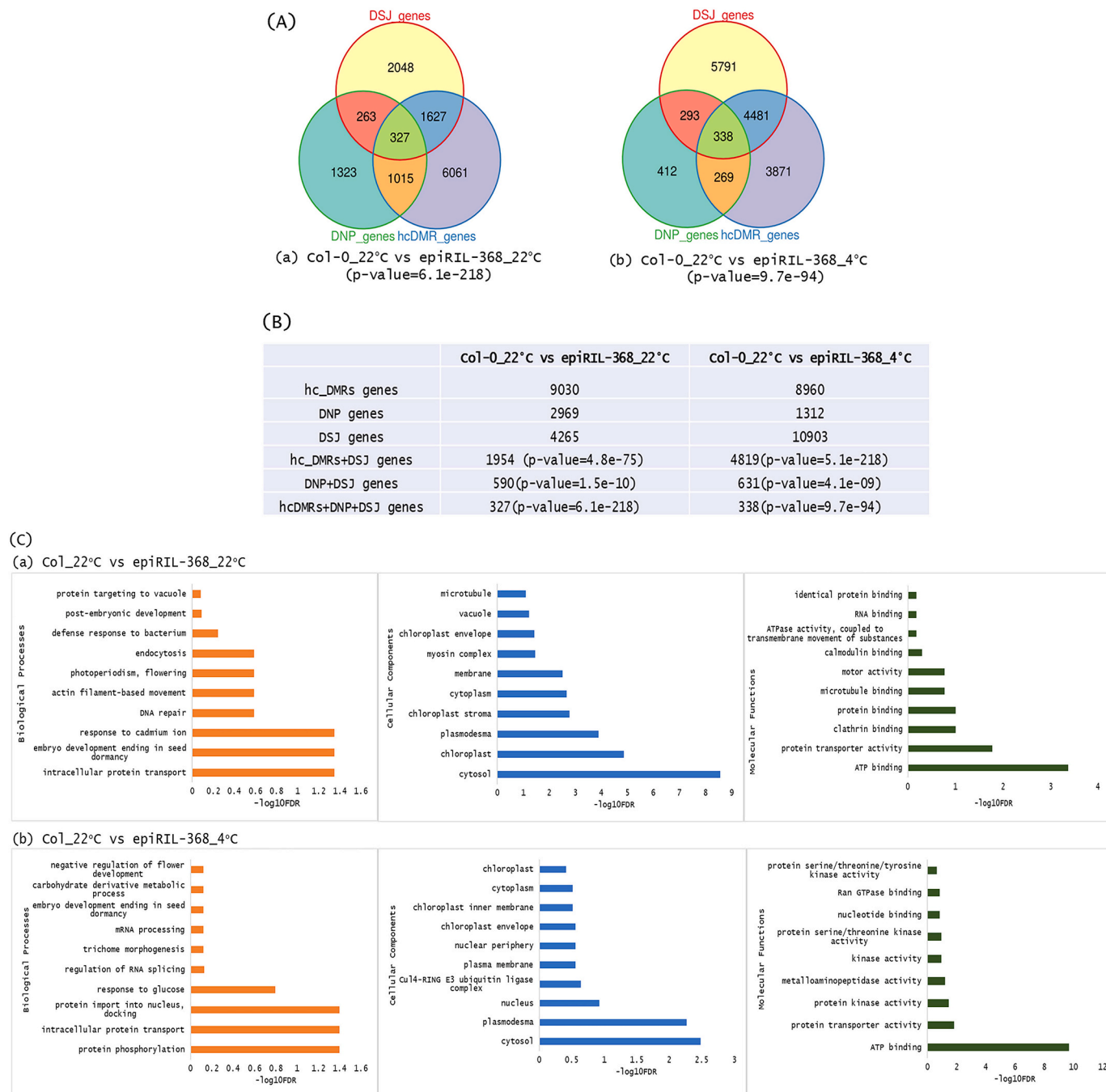


Fig. 4. Relationship between genes with high confidence differentially methylated regions (hc_DMR genes), significant differential nucleosome positioning (DNP genes), and differential splice junctions (DSJ genes). (A) Venn diagram representing the number of hc_DMR genes, DNP genes, and DSJ genes, and the overlap between them, in the contrasting groups (a) Col-0 at 22 °C versus epiRIL-368 at 22 °C, and (b) Col-0 at 22 °C versus epiRIL-368 at 4 °C. The values in parenthesis represent the p-values suggesting the significance level of overlap predicted by Fisher's exact test. (B) Table representing the number of hc_DMR genes, DNP genes, and DSJ genes and common genes between them, in the contrasting groups, Col-0 at 22 °C versus epiRIL-368 at 22 °C, and Col-0 at 22 °C versus epiRIL-368 at 4 °C. The p-value predicted from Fisher's exact test for overlap genes in each contrast group is provided in parenthesis. (C) Gene functional enrichment analysis of genes with hc_DMRs, DNPs, and DSJs. Bar graph represents the significant enrichment of genes (FDR ≤ 0.05) involved in BP: Biological Processes, CC: Cellular Components, and MF: Molecular Functions in the contrast groups (a) Col-0 at 22 °C versus epiRIL-368 at 22 °C, and (b) Col-0 at 22 °C versus epiRIL-368 at 4 °C. The x-axis represents $-\log_{10}$ FDR values.

remodelling genes may be one of the reasons behind it. We identified 26 chromatin remodelling genes showing expression differences in Col-0 and epiRIL-368 under cold stress. The expression of three genes (NUCLEOSOME ASSEMBLY PROTEIN1 [NAP1] and histone variants H2A.Z and H2A.4) were validated by qRT-PCR. The qRT-PCR results show higher expression of these chromatin re-modeller genes in epiRIL-368 compared to Col-0 when both were shifted from normal to cold conditions (Fig. S4). Our qRT-PCR data combined with our DE analysis (Col-0 and epiRIL-368) suggest the role of the selected chromatin remodellers in regulating cold stress responses. These results suggest that higher expression in the selected genes may be a driver of differential chromatin re-modelling and subsequently nucleosome occupancy levels in epiRIL-368 compared to Col-0. Remarkably, regardless of nucleosome occupancy levels in different samples, exons always show higher nucleosome occupancy and can be differentiated from their flanking regions. Overall, we reason that differences in DNA methylation and nucleosome occupancy levels between Col-0 and epiRIL-368 across splice junctions and exons are likely to regulate AS under normal growth as well as cold conditions.

2.4. Genes with differential epigenetic marks and splice junctions are involved in important biological processes

Next, we looked at the function of the genes with hcDMRs, DNPs, and differential splice junctions (DSJs) in our dataset. We first identified the genes with hcDMRs, and DNPs in addition to genes with DSJs for the contrasting groups Col-0 at 22 °C versus epiRIL-368 at 22 °C, and Col-0 at 22 °C versus epiRIL-368 at 4 °C. We divided genes into three groups including genes with hcDMRs (hcDMR genes), genes with significant DNPs (DNP genes) and genes with significant DSJs (DSJ genes). Finally, significantly overlapping (Fig. 4A) genes between hcDMR, DNP, and DSJ genes for the contrasting groups Col-0 at 22 °C versus epiRIL-368 at 22 °C, and Col-0 at 22 °C versus epiRIL-368 at 4 °C were selected for gene ontology (GO) functional enrichment analysis. Many genes with hcDMRs, DNPs and DSJs were identified in both contrasting groups (Col-0 at 22 °C versus epiRIL-368 at 22 °C, and Col-0 at 22 °C versus epiRIL-368 at 4 °C). In total, 9030 and 8960 hcDMR genes were identified in the contrasting groups Col-0 at 22 °C versus epiRIL-368 at 22 °C, and Col-0 at 22 °C versus epiRIL-368 at 4 °C, respectively (Fig. 4B). Interestingly, the number of genes with DNPs in Col-0 at 22 °C versus epiRIL-368 at 22 °C is higher (2969) when compared to Col-0 at 22 °C versus epiRIL-368 at 4 °C (1312), respectively (Fig. 4B), whereas, in the case of DSJ genes opposite trend was observed. We found 4265 DSJ genes in Col-0 at 22 °C versus epiRIL-368 at 22 °C, as compared to 10,903 in Col-0 at 22 °C versus epiRIL-368 at 4 °C (Fig. 4B). This suggests that the impact of epigenetic and temperature variation simultaneously affects the regulation of splicing by selecting differential splice junctions. The loss of nucleosome occupancy under cold as in the case of Col-0 at 22 °C versus epiRIL-368 at 4 °C, might relax the chromatin structure to modulate Pol II elongation dynamics and splice site selection. We also performed Fisher's exact test for gene overlap in all three sets of genes (hcDMR, DNP, and DSJ genes) using GeneOverlap [68] (Fig. 4). The highly significant (p-value ≤ 0.05) of overlap among the genes with hcDMRs, DNPs and DSJs suggest a strong correlation between all three sets of genes.

Next, we performed GO functional enrichment analysis for common genes having hcDMRs, DNPs, and DSJs (Fig. 4C). Genes with hcDMRs, DNPs, and DSJs, were found to be significantly enriched (FDR ≤ 0.05) for important biological functions, cellular components, and molecular functions (Fig. 4C). In the contrast group Col-0 at 22 °C versus epiRIL-368 at 22 °C, genes with hcDMRs, DNPs, and DSJs involved in biological processes such as intracellular protein transport, embryo development, cadmium stress, and DNA-repair, whereas in cellular components genes related to the cytosol, and chloroplast were highly enriched. In the case of molecular functions genes with protein transport and protein binding were found to be highly enriched. Additionally, in the contrast

group Col-0 at 22 °C versus epiRIL-368 at 4 °C, the genes involved in RNA-splicing and mRNA processing were also found to be significantly enriched. Whereas, molecular functions such as ATP binding and kinase activity were also enriched. Overall, the association between the genes with hcDMRs, DNPs, and DSJs, suggested a potential role of DNA methylation and nucleosome occupancy in the regulation of AS and involvement in various important biological, molecular and cellular processes in epigenetically different lines of Arabidopsis in a temperature dependent manner.

2.5. Epigenetic regulation of alternative splicing in plants is bidirectional

Next, we asked how epigenetic variations influence the directionality of AS in Arabidopsis. Previous reports suggest variation in DNA methylation [25] and nucleosome occupancy [41] can affect AS in both positive and negative (gain or loss of AS junctions) directions. To understand how gain or loss of AS junctions are associated with epigenetic variations which have been stabilised for more than nine generations (epiRIL-368), we identified positively, negatively, and un-affected AS junctions in all the contrasting groups (Fig. 5A). As suggested previously [25,41] we also considered AS as positively affected if $\Delta\text{PSI} \geq 0.1$ (means PSI high and more probability of inclusion of AS event) and negatively affected if $\Delta\text{PSI} \leq -0.1$ (means low PSI and probability of inclusion AS event). The AS junctions with $-0.1 < \Delta\text{PSI} < 0.1$ value are considered as un-affected AS junctions between two experimental lines (p-value ≤ 0.05). Regardless of the temperature, the percentage of negatively affected AS junctions observed is higher than the percentage of positively affected AS junctions on comparison Col-0 with epiRIL-368 (in both normal and cold conditions) (Fig. 5A; black arrows). Furthermore, upon cold shift Col-0 and epiRIL-368, showed a high percentage of positively affected than negatively affected AS junctions (Fig. 5A; green arrows), which is in agreement with a previous study suggesting that cold-stress induced AS in plants [48]. The simultaneous effect of epigenetic and temperature variations (Col-0 at 22 °C versus epiRIL-368 at 4 °C) showed very similar percentages of negatively and positively affected AS junctions (Fig. 5A c). Since in previous sections we showed both epigenetic marks and cold temperature modulate AS, these results on positive, negative, and un-affected AS junctions, further supports the notion that epigenetic marks modulate AS in both directions, which is also in line with previous findings in rice and Arabidopsis [25,41].

To further validate the bidirectionality of epigenetic variations on AS, in addition to hcDMRs genes and DNP genes, genes containing positively, negatively un-affected AS junctions were identified and the significant overlap between them was calculated (Table 1). Significant overlap has been observed between the genes in each contrasting group. However, the overlap among genes at 4 °C is more significant (Fisher's exact test p-value ≤ 0.05) in comparison with 22 °C, among genes with positively and negatively affected AS junctions under the simultaneous impact of epigenetic variations and cold stress.

We later plotted the methylation levels and nucleosome signals in positively, negatively, and un-affected AS junctions around 5'SS and 3'SS (Fig. 5B,C). In epiRIL-368 the methylation in positively and negatively affected AS junctions almost overlaps with each other (and show lower methylation as compared to un-affected AS junctions at normal temperature (22 °C) (Fig. 5B-a). Interestingly, under cold conditions (4 °C) with in epiRIL-368, a significant difference among methylation levels between positive, negative and un-affected AS junctions is clearly visible (Fig. 5B-b). The unpaired two sample *t*-test between un-affected and positively affected AS junctions, and un-affected and negatively affected AS junctions (p-value < 0.05) suggests there is a significant difference between methylation level in un-affected from positively and negatively affected AS junctions. Whereas, the non-significant p-value (p-value > 0.2) between positively and negatively affected AS junctions suggest methylation level is independently distributed among both types of AS junctions and are closely associated. Overall, these results support previous findings in animals and plants (rice), that different levels of

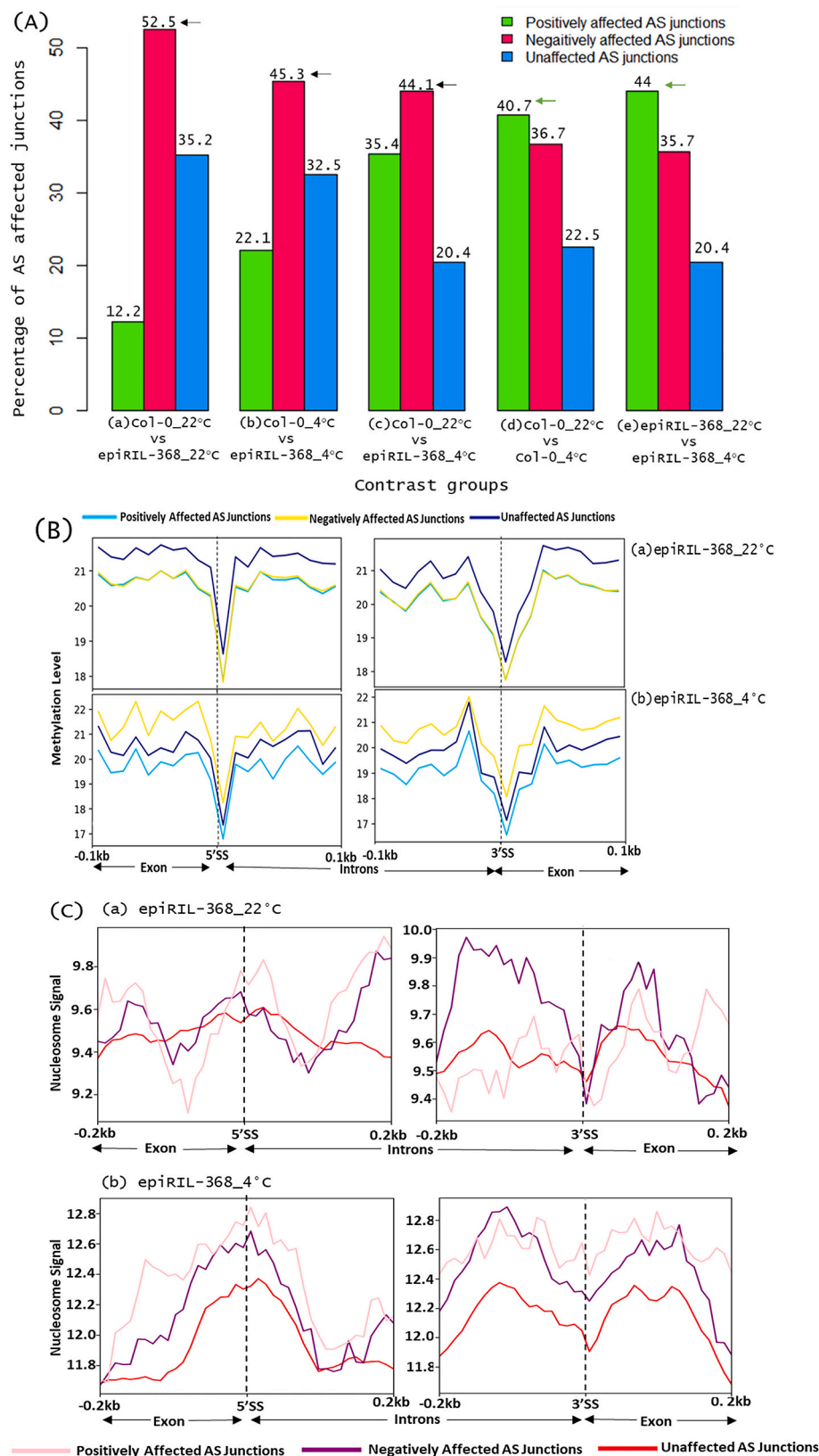


Fig. 5. DNA methylation and nucleosome occupancy of positively, negatively, and unaffected alternative splice (AS) junctions. (A) Bar graph representing the percentage of positively, negatively, and un-affected AS junctions in different contrast groups (a-e). The y-axis represents the percentage (%) of affected AS junctions, whereas the x-axis showing different contrasting groups. (B) DNA methylation level in CpG contexts on positively, negatively, and un-affected alternative splice (AS) junctions, around 5'SS and 3'SS alongside 100 bp upstream (-0.1 kb) and downstream (0.1 kb) the splice sites. The y-axis represents the methylation level in CpG context on un-affected, positively affected, and negatively affected AS junctions. The upper and lower panel showing results in (a) epiRIL-368 at 22 °C, and (b) epiRIL-368 at 4 °C, respectively. (C) Nucleosome signals on positively, negatively, and un-affected AS junctions in (a) epiRIL-368 at 22 °C and (b) epiRIL-368 at 4 °C, around 5'SS and 3'SS alongside 200 bp upstream (-0.2 kb) and downstream (0.2 kb) the splice sites.

DNA methylation are strongly associated with positive (high splicing efficiency) and negative (low splicing efficiency) AS junctions [25,69]. Also, nucleosome signals among epiRIL-3698 at 22 °C and 4 °C in positive, negative and unaffected AS junctions, around 5'SS and 3'SS, showed a similar pattern as in DNA methylation (Fig. 5C). The

significant difference between nucleosome signals among positive, negative and, unaffected AS junctions were observed, was also reported in Col-0 plants in our previous report [41].

Table 1

Details of genes associated with hc_DMRs, significant DNPs, positively, negatively, and un-affected AS Junctions. Gene overlap Fisher's exact test p-value is provided in the parenthesis.

	Col-0 22°C vs epiRIL-368 22°C	Col-0 22°C vs epiRIL-368 4°C
hc_DMR genes	9030	8960
DNP genes	2969	1312
Genes with positively affected AS Junctions	35	822
Genes with negatively affected AS Junctions	201	934
Genes with un-affected AS Junctions	143	502
hc_DMR genes+genes with positively affected AS Junctions	9 (p-value = 0.88)	388 (p-value = 2e-17)
hc_DMR genes+genes with negatively affected AS Junctions	83 (p-value = 0.012)	437 (p-value = 1.2e-18)
hc_DMR genes+genes with un- affected AS Junctions	58 (p-value = 0.044)	226 (p-value = 1.8e-08)
DNP genes+genes with positively affected AS Junctions	1 (p-value = 0.98)	41 (p-value = 0.45)
DNP genes+genes with negatively affected AS Junctions	20 (p-value = 0.69)	51 (p-value = 0.21)
DNP genes+genes with un-affected AS Junctions	22 (p-value = 0.058)	25 (p-value = 0.48)

3. Discussion

Recent evidence from Arabidopsis shows that the transcription and splicing process are coupled [2,17,21,70] and epigenetic marks such as DNA methylation and nucleosome occupancy may modulate these processes in a time- and condition-specific manner [17,71]. Epigenetic features in plants regulate transcriptional activity and differentially mark exons, introns as well as cassette and constitutively spliced exons [18]. Furthermore, Pol II elongation speed is slower in exons due to higher methylation and nucleosome occupancy, allowing more time for the splicing process to take place [18,26]. The relationship between DNA methylation and transcriptional control (gene expression and AS) in plants has been demonstrated in recent years [25,26]; however, how the epigenetic differences influence gene expression and splicing/AS processes under variable growth and stress conditions remains elusive in plants. Since splicing/AS regulation is achieved by the context of the cis-regulatory sequences as well as the chromatin environment [72], it is important to understand the relative contributions of the genetic and epigenetic landscapes. Using Col-0 and epiRIL-368 lines of Arabidopsis, we demonstrate that differential DNA methylation and nucleosome occupancy in identical genetic backgrounds are sufficient to modulate gene expression and AS in Arabidopsis. Remarkably, epiRIL-368 in comparison to their parental Col-0 ecotype behave differently in response to cold stress for gene expression, and AS, which is also observed by nucleosome occupancy profile in epiRIL-368 at 4 °C. Intriguingly, there was very little overlap between DEGs and DAS genes across all treatments and groups, indicating that despite coupling between the transcriptional and splicing machinery, SF recruitment and Pol II dynamics through differential epigenetic marks may be important in various growth conditions. Indeed, we found DAS genes between Col-0 and epiRIL-368, plants shifted from normal to cold was accompanied by epigenetics differences. Our results show that epigenetic features are not only involved in modulating AS event type in response to temperature changes but DNA methylation and nucleosome occupancy levels are also associated with the abundance of differentially spliced transcripts. The RNA-seq analysis shows that DNA methylation is more likely to modulate the transcriptome upon temperature shifts rather than steady temperature, indicating that chromatin signatures are malleable to environmental changes and modulate splicing events in Arabidopsis [73]. These results demonstrate that although the chromatin

environment provides the context through which splicing is modulated, the crosstalk of the splicing and transcriptional machinery, in a condition-dependent (cold in our case) manner, is important. For example, a recent study demonstrated that Pol II speed can be influenced by growth conditions (light quality) and affects splicing patterns in Arabidopsis [74]. Also, another recent report suggests cold-dependent differential nucleosome patterns modulates AS in Col-0 in Arabidopsis [41]. Our results are also consistent with previous findings in rice where only 7% of AS events are influenced by global changes in methylation [25], hence may play a fine-tuning role under normal conditions as is evident from the splicing pattern differences between altered DNA methylated plants in this study.

Although Col-0 and epiRIL-368 plants show pronounced variation in their DNA methylation (Fig. 2) (along with splicing differences), it is remarkable that many stress-responsive genes show differences in expression and splicing. These observations support the notion that in addition to global epigenetic differences, temperature variations also mediate expression and splicing differences in many genes. It is tempting to speculate that plants may remember previous episodes of stresses via chromatin signatures but largely modulates the expression and splicing of those genes, which are actively transcribing and/or whose expression needs to be reduced via the production of non-productive mRNA species [75,76]. Previous studies have shown that exon recognition is mainly achieved through accumulating Pol II in a context-dependent manner around splice sites to enhance SF recruitment and allowing more time for splicing to take place [18,26]. Our data support this notion and higher DNA methylation, and nucleosome occupancy may likely regulate Pol II accumulation around splice sites and enable SF recruitment to facilitate and/or modulate splicing variation.

We demonstrate that DNA methylation and nucleosome occupancy are the important controllers for the transcriptional and the AS dynamics however, growth conditions, metabolism, and physiology of plants may exert tight control over desirable expression and splicing patterns. Therefore, reprogramming and preservation of epigenetic features in multiple generations and response to diverse environmental cues may be more meaningful and provide a context to modulate gene expression patterns [2,75]. Indirectly, these results also highlight that it is the crosstalk with the transcription and the splicing machinery, in a context-dependent manner, which would ultimately influence the expression and splicing patterns. This is evident from recent findings that plants possess splicing memory for high-temperature conditions, which may be also defined by the chromatin context [76]. Recent data also shows that temperature-induced differentially spliced genes are enriched in histone H3 lysine 36 tri-methylation (H3K36me3) and any perturbation in these marks affect flowering in Arabidopsis [77]. Therefore, chromatin mapping (DNA methylation, nucleosome occupancy) for plants grown under different and recurrent growth and stress conditions needs to be undertaken to reveal the relationship between observed gene expression and splicing patterns to fully understand the underlying molecular mechanism. We envisage that chromatin mapping and splicing analyses of plants growing in diverse conditions and recurring stresses will reveal the extent to which reproducible DNA methylation and chromatin patterns are associated with observed AS patterns and have biological significance. Furthermore, the availability of genome-wide profiles of DNA methylation, nucleosome, and transcriptome profiles in stable epigenetic different lines could open new avenues to engineer desirable crop plants without changing the genetic background.

4. Materials and methods

The details of materials and methods used in the study are provided as a supplementary file (Supplementary Method S1). In brief, total RNA, and nucleosome bound genomic DNA (gDNA) was extracted from leaf tissues of Col-0 and epiRIL plants grown at 22 °C and cold treated (4 °C) for 24 h. For WGBS, genomic DNA was extracted from the leaf tissues of epiRIL plants grown at 22 °C and cold treated (4 °C) for 24 h. Illumina

paired-end sequencing for RNA-Seq, WGBS, and MNase-Seq, were performed by commercial service provider Earlham Institute, Norwich, UK. The raw reads generated from RNA-Seq, WGBS and MNase-Seq experiments were quality checked using Trimmomatic v0.35 [78]. The high quality reads were then quantified using Salmon v0.82 [79] and Arabidopsis transcriptome database, ATRTD2-QUASI [9] as reference. For DEGs and DAS, a stringent 3D-RNA-Seq [48,80] analysis was used. Gene functional enrichment analysis for DEGs and DAS was performed using DAVID v6.8 [81]. JunctionSeq v1.20.0 [82] and SUPPA v2.3 [63] were also used for differential splice junction and PSI calculation respectively. For WGBS data raw reads were trimmed and quality control using the Trim Galore tool (<https://github.com/FelixKrueger/TrimGalore>). The methylation level in CpG, CHH, CHG context was calculated using methylpy v1.4.3 [65]. Next for hcDMRs, a pipeline hcDMR caller developed by the Jacobsen group at the University of California [60] was used. For MNase-Seq high quality reads after quality check with Trimmomatic v0.35 [78], were mapped to the TAIR10 Arabidopsis reference genome using Bowtie v1.2.2 [83]. Improved Nucleosome-Positioning Algorithm (iNPS) v1.2.2 was used for accurate genome-wide nucleosome positioning as described previously by [61], whereas, differential nucleosome positioning (DNP) analysis was performed using DANPOS v2.1.2 [62].

Availability of data

All RNA-Seq, WGBS, and MNase-Seq raw data generated in this work are publicly accessible through NCBI-SRA accessions PRJNA592356.

Acknowledgements

We thank the funding agency Leverhulme Trust [RPG-2016-014] for research support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ygeno.2021.08.006>.

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