Chapter

Atlas of Age- and Tissue-Specific DNA Methylation during Early Development of Barley (*Hordeum vulgare*)

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Abstract

The barley (*Hordeum vulgare*) genome comprises over 32,000 genes, with differentiated cells expressing only a subset of genes; the remainder being silent. Mechanisms by which tissue-specific genes are regulated are not entirely understood, although DNA methylation is likely to be involved. To shed light on the dynamic of DNA methylation during development and its variation between organs, methylation-sensitive genotyping by sequencing (ms-GBS) was used to generate methylation profiles for roots, leaf-blades and leaf-sheaths from five barley varieties, using seedlings at the three-leaf stage. Robust differentially methylated markers (DMMs) were characterised by pairwise comparisons of roots, leaf-blades and leaf-sheaths of three different ages. While very many DMMs were found between roots and leaf parts, only a few existed between leaf-blades and leaf-sheaths, with differences decreasing with leaf rank. Organ-specific DMMs appeared to target mainly repeat regions, implying that organ differentiation partially relies on the spreading of DNA methylation from repeats to promoters of adjacent genes. Identified DMMs indicate that different organs do possess diagnostic methylation profiles and suggest that DNA methylation is important for both tissue differentiation and organ function and will provide the basis to the understanding of the role of DNA methylation in plant organ differentiation and development.

Keywords: epigenomics, *Hordeum vulgare*, leaf, root, tissue-specific methylation, developmental epigenomics

1. Introduction

DNA methylation is an important characteristic of plant genomes [1, 2], and can occur in all cytosine contexts (CG, CHG and CHH, where H = A, C or T) [3]. The effect of DNA methylation variants on plant development has been demonstrated through methylation alteration tests, which led to plant abnormalities [4, 5]. Furthermore, DNA methylation has been reported to vary from tissue to tissue in many species [6–10], and these methylation changes seemed to be essential for normal plant development [11, 12].

Additionally, tissue-specific methylation was proposed to have a strong correlation with the differential expression of some tissue-specific genes. Examples include tissue-specific pigmentation in maize, which is reported to be under epigenetic control [13], and differential gene expression between organs attributed to differentially methylated regions in soybean [14] and sorghum [10]. These studies extended our understanding of the functional importance of tissue-specific DNA methylation, including its role in setting developmental trajectories [9, 13, 15].

A substantial proportion of developmentally expressed genes have alternative promoters (multiple promoters that regulate the same gene) which are under different regulatory programmes [16]. Maunakea et al. [17] proposed that alternative promoters are, at least sometimes, controlled by intragenic DNA methylation. This form of developmental gene regulation is reasoned to be dependent on transposon activity [16] and by implication would mean that silencing of transposons due to DNA methylation may be central to tissue-specific gene expression. Also, tissue-specific gene expression has been associated with methylation changes in promoter regions [2, 18, 19], especially CG islands within promoters [20]. These studies indicate that tissue-specific gene expression does not rely on a single methylation pattern in the genome but, probably, on a combination of variable DNA methylation features.

The magnitude of differential methylation between tissues has been the subject of controversy. It was believed that significant distinctive DNA methylation existed only between specialised tissues such as endosperm, pollen, leaves and roots [9, 10, 21, 22]. Nevertheless, many of these studies also showed that differential DNA methylation between organs, such as roots and leaves, was minor in rice [23], maize [24], sorghum [10] and Arabidopsis [9]. DNA methylation differences between roots and leaves were small in both ^{m}CG and ^{m}CHG contexts [9, 10], with about 1% and 5% divergence, respectively, reported in Arabidopsis [9]. While these studies of differential DNA methylation between tissues generally compared the overall methylation levels [9, 10, 24], these results differ from comparisons made with differentially methylated markers (DMMs) between the same tissues [10], probably due to differences in methylation profiling methods, making it difficult to compare results from different studies. Therefore, it is difficult to know whether differences in the results concerning tissue-specific DNA methylation are due to the plant species or to the approach taken. The study of DNA methylation patterns in plant tissues is important for a better understanding of how these epigenetic markers determine tissue differentiation. Thus, further investigation is warranted to clarify organ specificity of cytosine methylation and the distribution patterns of tissue-specific DNA methylation markers in the plant genome.

To undertake such an investigation, we used barley, a globally important cereal crop, the genome of which has been sequenced recently [25]. The availability of a reference genome made barley a model for the study of cereal crops such as wheat, oats or rye. In this study, we assessed differential DNA methylation between two barley (*Hordeum vulgare*) organs (roots and leaves), using methylation-sensitive genotyping by sequencing (ms-GBS) on five genetically distinct varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra). For the sake of simplicity and consistency with the literature, roots and leaves or leaf parts (sheath, blade) may be referred to here as tissues and not organs.

2. Materials and methods

2.1 Plant material and growth conditions

Five spring barley varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra), were selected based on their similarity in phenology in order to minimize epigenetic variability between varieties associated with developmental differences. Seeds from all varieties were provided by the Salt Focus Group at the Australian Centre for Plant Functional Genomics (ACPFG, Adelaide, South Australia), and planted at the same time in potting mix comprising 50% UC (University of California at Davis), 35% coco-peat and 15% clay/loam ($v\ v^{-1}$) in 3.3 L pots, 17.5 cm deep, free-draining and placed on saucers. The experiment was conducted from 30th January to 20th February 2015 in a greenhouse at the Waite Campus, University of Adelaide, South Australia (34°58′11″S, 138°38′19″E). The seedlings were grown under natural photoperiod, while temperatures were set at 22°C/15°C (day/night). The experiment consisted of five randomized blocks of five varieties (25 seedlings per block). Pots were watered to weight every 2 days to a gravimetric water content of 16.8% ($w\ w^{-1}$) (0.8 × field capacity) [26] until sampling 21 days after sowing, when seedlings were at three-leaf stage (Zadok stage 13 [27]). Blades and sheaths of leaves 1–3 were sampled separately. Leaves 1 and 2 were fully expanded prior to sampling, whilst leaf 3 had just completed growth. About 50 mg of plant material was cut from the middle section of each leaf blade and each leaf sheath and collected in 2 ml micro tubes. Roots were cut from the seedlings and washed using tap water to remove soil particles, then blotted dry with paper towels before sampling 50 mg of root tissue. All samples were snap frozen in liquid nitrogen, and then stored at −80°C until DNA extraction. In total, 175 tissue samples were collected, including 25 root samples (i.e. 5 plants per each of the five varieties used in the study), 75 leaf blade samples (i.e. from leaves 1, 2 and 3 from each of the 5 plants per variety used in the study) and 75 leaf sheath samples (i.e. from leaves 1, 2 and 3 from each of the 5 plants per variety used in the study).

2.2 DNA isolation

Prior to DNA extraction, frozen plant material was homogenized in a bead beater (2010-Geno/Grinder, SPEX SamplePrep®, USA). DNA isolation was performed from pulverised plant samples using a Qiagen DNeasy kit and following the manufacturer's instructions. DNA samples were quantified using a NanoDrop® 1000 Spectrophotometer (V 3.8.1, ThermoFisher Scientific Inc., Australia) and concentrations were standardized to 10 ng/μl for subsequent library preparation.

2.3 Methylation-sensitive genotyping by sequencing (ms-GBS)

The ms-GBS was performed using a modified version [28] of the original GBS technique [56]. Genomic DNA was digested using the combination of a methylation-insensitive rare cutter, *Eco*RI (GAATTC), and a frequent and methylation-sensitive cutter, *Msp*I (CCGG). Each sample of DNA was digested in a reaction volume of 20 μl containing 2 μl of NEB Smartcut buffer, 8 U of HF-*Eco*RI (High-Fidelity) and 8 U of *Msp*I (New England BioLabs, Australia). The reaction was performed in a BioRad 100 thermocycler at 37°C for 2 h, followed by enzyme inactivation at 65°C for 10 min.

Then the ligation of adapters to individual samples was achieved in the same plates by adding 0.1 pmol of the respective barcoded adapters with an *Msp*I cut site overhang, 15 pmol of the common Y adapter with an *Eco*RI cut site overhang, 200 U of T4 Ligase and T4 Ligase buffer (New England BioLabs, Australia) in a total volume of 40 µl. Ligation was carried out at 24°C for 2 h followed by an enzyme inactivation step at 65°C for 10 min.

DNA samples were allocated to plates, 81 samples each, including the negative control, water. Prior to pooling plate samples into a single 81-plex library, the ligation products were individually cleaned up to remove excess adapters using an Agencourt AMPure XP purification system (Beckman Coulter, Australia) at a ratio of 0.85 (AMPure magnetic beads/ligation product), following the manufacturer's instructions. Individual GBS libraries were produced by pooling 25 ng of DNA from each sample. Each constructed library was then amplified in eight separate PCR (25 µl each) containing 10 µl of library DNA, 5 µl of 5× Q5 high fidelity buffer, 0.25 µl polymerase Q5 high fidelity, 1 µl each of Forward and Reverse common primers at 10 µM, 0.5 µl of 10 µM dNTP and 7.25 µl of sterile pure water. PCR amplification was performed in a BioRad T100 thermocycler, consisting of DNA denaturation at 98°C (30 s) and 10 cycles of 98°C (30 s), 62°C (20 s) and 72°C (30 s), followed by 72°C for 5 min. PCR products were next pooled to reconstitute libraries. DNA fragments between 200 and 350 bp in size were captured using AMPure XP magnetic beads following the manufacturer's instructions. Bead-captured fragments were eluted in 35 µl of water, of which 30 µl were collected in a new labelled microtube. Libraries were next paired-end sequenced in an Illumina HiSeq 2500 (Illumina Inc., USA) at the Australian Genome Research Facility (AGRF, Melbourne Node, Australia). Sequencing results were deposited in the European Nucleotide Archive (ENA) (Study Accession Number: PRJEB27251).

2.4 Analysis of global differences in DNA methylation between samples

Differences in ms-GBS profiles between samples were explored by performing principal component-linear discriminant analysis (PC-LDA) (a supervised clustering approach for high dimensional data), using different levels of hierarchy between samples as the putative drivers in DNA methylation differences (i.e. grouping samples by organ (root vs. leaf), tissue (root vs. blade vs. sheath, and tissue) and age (root vs. leaf 1 vs. leaf 2 vs. leaf 3 vs. sheath 1 vs. sheath 2 vs. sheath 3)). PC-LDA was implemented using the R package FIEmspro 1.1-0 [29] on the standardized coverage, the count per million reads (CPM) of the 913,697 ms-GBS markers generated. PC-LDA results were visualized by a scatter plot of the first two discriminant factors (DFs), and a 3D plot using the first three DFs. Finally we used an unsupervised hierarchical cluster analysis to generate a dissimilarity tree based on Mahalanobis distance [30] generated also based on the standardized coverage (CPM) of the 913,697 ms-GBS markers.

2.5 Detection of DMMs in barley

Differentially methylated DNA was assessed in ^{m}CCGG motifs (recognised by *Msp*I), between barley leaf parts (blade and sheath) and roots. To do so, samples were grouped according to organ type (root, blade and sheath) regardless of the genotype of origin, making 25 samples per organ. This approach aimed to minimise genotype-dependent methylation markers. DMMs were identified using the package, *msgbsR*, developed by Mayne et al. [31]. DMMs were selected based on FDR adjusted P-values with a threshold of 0.05 [32, 33]. The significance of the marker also fulfilled the condition that the read counts reached at least 1 CPM and was present in at least 20 samples per organ type (maximum sample per group = 25). The *log*FC (logarithm 2 of fold-change) was computed to estimate the intensity and directionality of differential DNA methylation between tissues. Determining the directionality of DNA methylation uses the fold change as an inverse proxy for change in the methylation level. That is, higher methylation levels on a specific locus will reduce the number of *Msp*I restriction products and therefore reduce the number of sequences generated for that locus [34].

2.6 Distribution of DMMs around genomic features

To test whether there was a relationship between tissue-specific DMMs and particular genomic features (e.g., genes and repeat regions as defined in Ensembl database (http://plants.ensembl.org/biomart/martview/)), DMM distribution was assessed in the barley genome. Therefore, DMMs stable between tissues were mapped to the barley reference genome. Then, the number of DMMs within genomic features (repeats, genes, exons, UTRs and tRNA genes) and per 1 kb bins within 5 kb flanking regions [24, 28] was tallied using the shell module, *bedtools/2.22.0* [35].

3. Results

3.1 Methylation-sensitive genotyping by sequencing

The sequencing of the 170 samples of barley tissue which met DNA quality requirements yielded over 900 million raw reads, with more than 91% bases above Q30 (99.9% accuracy of base call [36]) across all samples (**Table 1**). Of these reads, 99.27% contained the barcode and *Eco*RI/*Msp*I adapters ligated during library construction. Further filtering was performed to retain reads that strictly aligned with the barley reference genome. In this way, we obtained nearly 450 million reads (50.10%), with a mean of 2,637,916 high quality reads per sample. These high-quality reads accounted for 913,697 sequence tags, representing 32.30% of the 2,828,642 CCGG sites in the barley genome. Of these sequence tags, 748,594 (80.62%) showed some form of polymorphism for methylation between samples.

3.2 Estimation of tissue- and tissue rank-dependent epigenetic differentiation

The PC-LDA plots revealed clear evidence of structuring of methylation between samples (**Figure 1a**). A 3D plot using the first three discriminant factors

Sequencing parameters	Yield
Raw reads	901,617,058
Reads that matched barcodes	895,013,295
Reads aligned to barley reference genome	448,445,748
Samples	170
Average reads per sample	2,637,916
Total unique tags	913,697
Polymorphic tags	748,594

Table 1.
Data yields from ms-GBS, generated using the Illumina HiSeq 2500 platform.

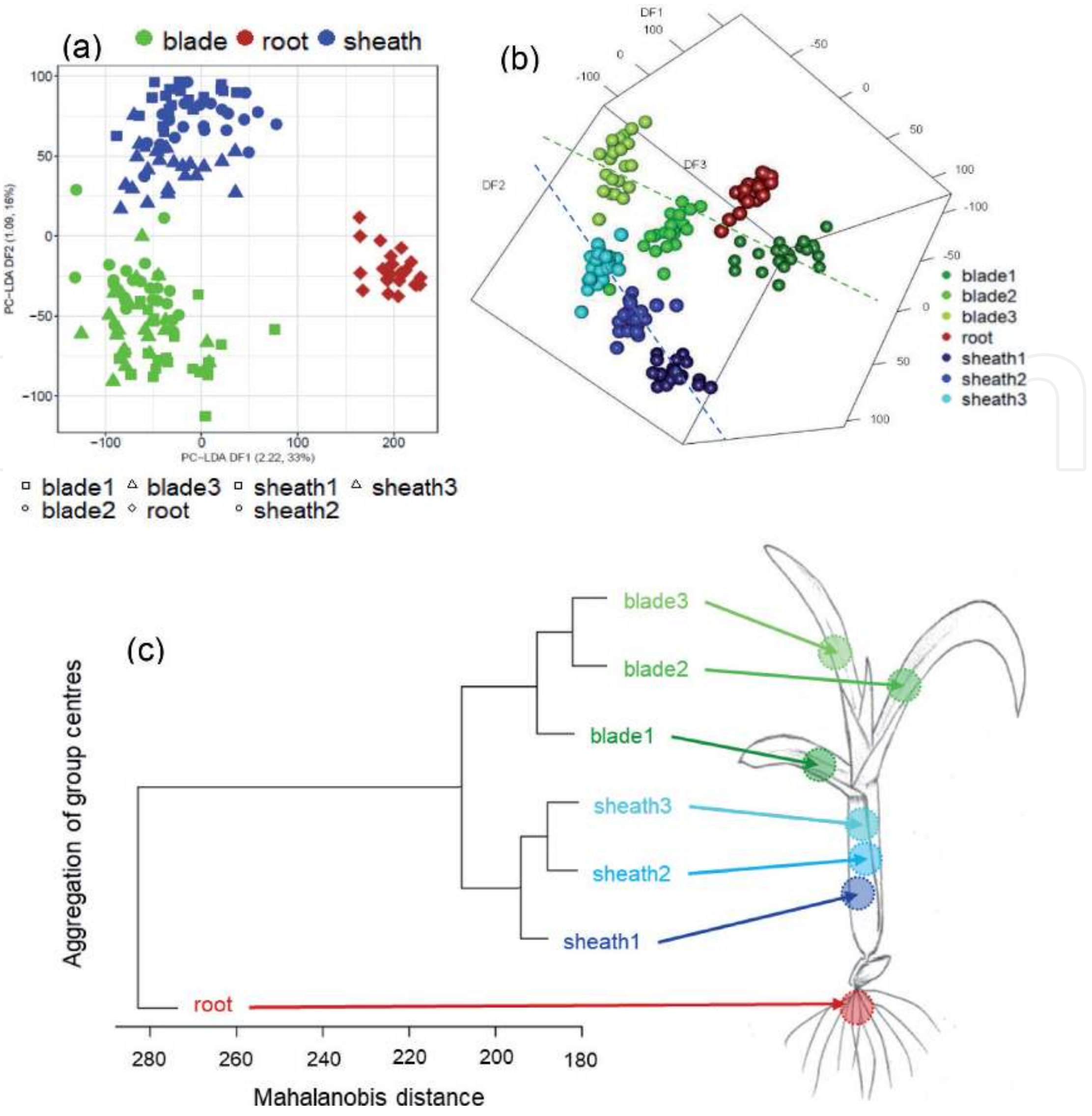


Figure 1.

Analysis of the differentiation of DNA methylation profiles of barley roots, leaf sheaths and leaf blades. (a) Scatter plot of the first two discriminant factors of the principal component-linear discriminant analysis (PC-LDA) (DF1 and DF2) using 913,697 ms-GBS markers generated from genomic DNA of roots, leaf sheaths and leaf blades, collected from 25 barley plants at the three-leaf stage (21 days after sowing), comprising five varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra). (b) Three-dimensional plot of the first three discriminant factors of the PC-LDA of the same ms-GBS data. (c) Hierarchical cluster of the distances between sample group centres, based on Mahalanobis distance. Blade 1-3 and sheath 1-3 indicate the rank of the organ type, first, second and third leaf of seedlings, respectively.

(DF1, DF2 and DF3) revealed that blades and sheaths were further grouped according to the rank of the leaf from which they were harvested. The distance between blades and sheaths seems to shrink with leaf rank (**Figure 1b**). This leaf rank-dependent grouping was also supported by hierarchical cluster analysis (HCA) of the distances between sample group centres (**Figure 1c**), based on the Mahalanobis distance [29, 30], and sample clusters matched the leaf developmental age (**Figure 1c**). Leaf rank-dependent DNA methylation differences were further assessed between tissues by comparing the methylation profiles of blades and sheaths for each rank of leaf appearance. No DMMs were observed between the three leaf blades, whereas sheaths 1 and 3 presented 18 DMMs (**Table 2**).

3.3 Differentially methylated DNA markers between roots and leaves

DMMs between barley roots and leaves were obtained through comparison of the read count per million of tissue types, independently of genotypes.

	Blade 1	Blade 2	Blade 3	Sheath 1	Sheath 2	Sheath 3
Blade 1	—					
Blade 2	0	—				
Blade 3	0	0	—			
Sheath 1	32	37	73	—		
Sheath 2	29	36	40	0	—	
Sheath 3	0	1	1	18	0	—

Differentially methylated markers (FDR <0.05) were obtained from 913,697 ms-GBS tags generated from genomic DNA of barley roots, leaf sheaths and leaf blades, collected from 25 plants at three-leaf stage (21 days after sowing) of five barley varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra). Blade 1–3 and sheath 1–3 indicate the rank of the leaf; first, second and third, respectively, on seedlings.

Table 2.
Number of differentially methylated markers in barley tissues of different ages.

This comparison revealed substantial DMMs between both roots vs. blades and roots vs. sheaths (**Figure 2a**), and there were more DMMs between roots and blades (6510 DMMs **Figure 2b**) than between roots and sheaths (4116 DMMs **Figure 2c**). Of these markers, 3266 DMMs were present in both blades and sheaths when compared to roots, and their methylation changed consistently in the same direction in each comparison (**Figure 3a**). The number of DMMs between roots and leaf blades increased with leaf-rank, whereas DMMs between roots and leaf sheaths did not show any relationship with rank (**Figure 2a**). Tissue-specific DMMs were predominantly hypomethylated (95–98%) in leaf parts (sheath or blade) compared to roots (**Figure 2a**). This result was in line with the median of the fold-changes of DMMs, which indicated an overall DNA hypomethylation in leaves (**Figure 4a** and **b**). From here on, DMMs consistently present in roots vs. sheaths and roots vs. blades will be designated as stable markers between roots and leaves.

3.4 Differentially methylated DNA markers between the leaf blade and sheath

There was only a small number of DMMs between leaf blades and sheaths (0–73 DMMs, **Table 2** and **Figure 2d**). These DMMs were basically between leaf blades and sheaths 1 and 2; and there was none between blade 1 and sheath 3. There was only 1 DMM between sheath 3 and blades 2 and 3 (**Table 2** and **Figure 2d**). Pairwise comparisons between blades 1–2 and sheaths 1–2 revealed 20 common DMMs, which were all hypermethylated in sheaths compared to blades (**Figures 2e** and **4b**). Half of the 20 common DMMs between blades and sheaths were located on chromosome 5H. Furthermore, there were no DMMs in pairwise comparisons among blades 1–3 and among sheaths 1–3, except between sheath 1 and sheath 3 which had 18 DMMs (**Table 2**). However, comparing blades and sheaths of the same leaf rank showed 32 DMMs between blade 1 and sheath 1, 36 DMMs between blade 2 and sheath 2 and 1 DMM between blade 3 and sheath 3.

3.5 Distribution of tissue-specific DMMs around genes

Relatively few of the tissue-specific DMMs were located around gene exons. Indeed, of the 3266 stable DMMs between root and leaf samples, only 60 (1.8%) were located within 5 kb of a gene, including 21 overlaps with genes and 39 DMMs that were spread within 5 kb upstream and downstream of genes (**Figure 5a**). Apart from the absence of DMMs within 1 kb upstream of transcription start sites, there was no obvious tissue-specific DMM distribution pattern around the genes

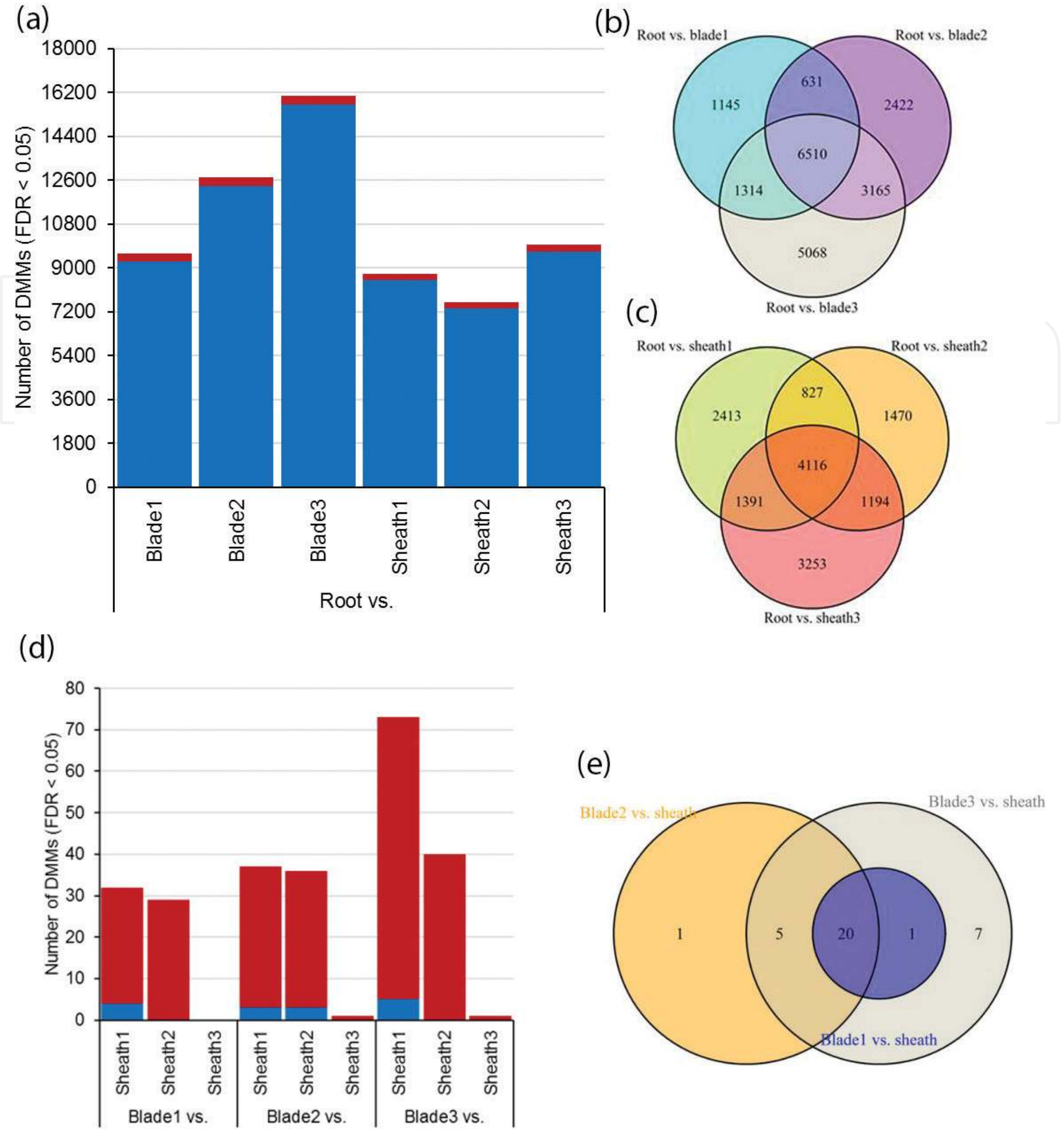


Figure 2.
Analysis of the number of DMMs among three barley tissues. (a) Number of DMMs between roots and leaf blades (root vs. blade) and roots and sheaths (roots vs. sheaths). Histogram colour indicates whether the DMMs are hypomethylated (blue) or hypermethylated (red) in leaf parts compared to roots. (b and c) Venn diagram showing the number of DMMs stable between root and blade tissues (b) and between root and sheath tissues (c). (d) Number of DMMs from pairwise comparison between leaf blades 1–3 and sheaths 1–3. Histogram colour indicates whether the DMMs are hypomethylated (blue) or hypermethylated (red) in sheaths compared with blades. (e) Venn diagram showing the number of DMMs common in pairwise comparisons between leaf blades 1–3 and sheaths 1–2. Tissue samples were collected from seedlings at the three-leaf stage of five barley varieties grown in five replicates for 21 days after sowing. Blade 1–3 and sheath 1–3 indicate the rank of the organ type; first, second and third, respectively, on seedlings. DMMs were selected based on the significance of the false discovery rate, FDR, <0.05. DMMs present in both sheaths and blades when compared with roots, have been designated as markers between roots and leaves.

(**Figure 5a**). The same assessment process showed that, as with common DMMs, only a small proportion of blade-specific DMMs (44 of 3246, 1.3%) was positioned close to a gene (**Figure 5b**). Of these, 15 DMMs overlapped with a gene transcript, whereas the remaining 29 DMMs were distributed within 5 kb of the gene without any clear pattern (**Figure 5b**), except that the number of DMMs located between 2 and 3 kb bins was higher both upstream and downstream, than any other 1 kb bin within the 5 kb flanking regions (**Figure 5b**). There were fewer sheath-specific methylation markers within 5 kb from genes than blade-specific markers (13 of

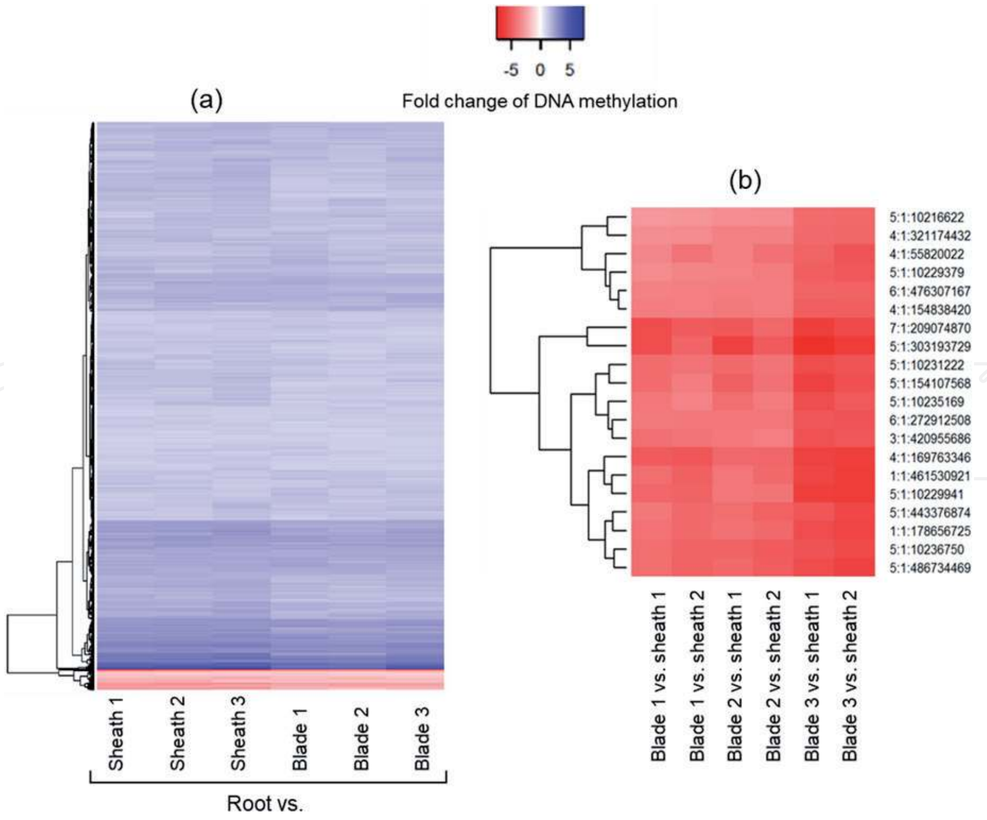


Figure 3.
Hierarchical clustering analysis of the DMMs. (a) The 3266 common DMMs between roots and all leaf parts (sheath 1–3, blade 1–3). The colours in the heat map indicate whether the DMM is hypomethylated (blue) or hypermethylated (red) in leaf parts compared to roots. (b) Hierarchical clustering of the 20 stable DMMs between blades and sheaths. In this heat map the red colour shows hypermethylation of DMMs in sheaths compared with blades. Blade and sheath samples were collected from seedlings at three-leaf stage of five barley varieties grown in five replicates for 21 days after sowing. Blade 1–3 and sheath 1–3 indicate the rank of the leaf on seedlings, first, second and third, respectively. The first number of the marker label on the y axis indicates the chromosome number on which the marker is located.

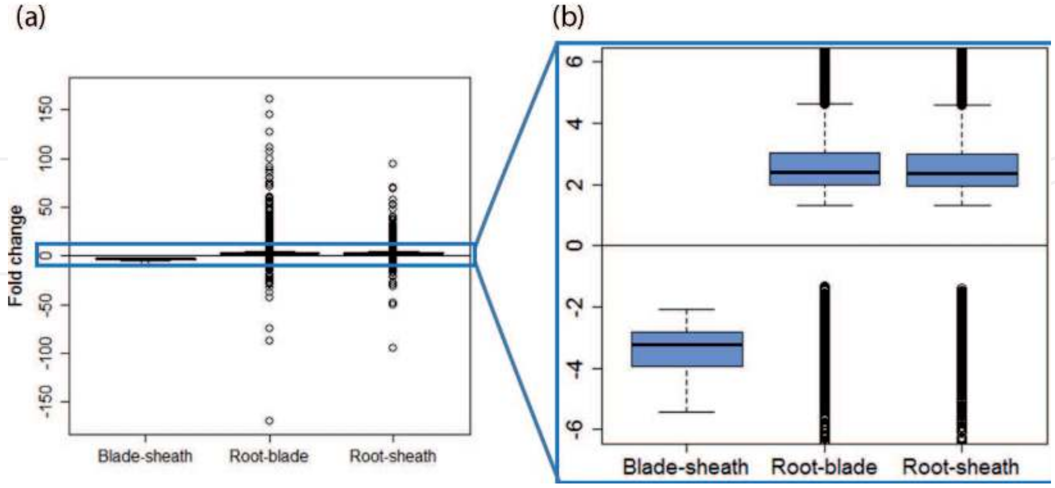


Figure 4.
Directionality of the methylation in tissue-specific DNA methylation markers. (a) Boxplots showing the spread of the fold-change of locus read counts between blades and sheaths, roots and blades, and roots and sheaths. (b) Detail of boxplots, highlighting the median of methylation fold-change of all loci in each comparison. The fold-change of DNA methylation was estimated by computing $2^{(\log^{2FC})}$*, with log2FC = logarithm 2 of fold-change in read counts for each sequenced locus between pairwise comparisons of tissues collected from three-leaf stage barley seedlings. Leaf blades were the reference state for blade-sheath comparison, whereas roots were the reference for root-blade and root-sheath comparisons. Negative and positive values on the y axis indicate respectively, hypermethylation and hypomethylation of the tissue that is compared to the reference. Locus coverage was estimated for each tissue by using 25 replicates for roots and 75 for blades and sheaths (5 plants from each of the 5 varieties included in the study (DNA was extracted from 1 single root and from 3 independent leaves per plant)).*

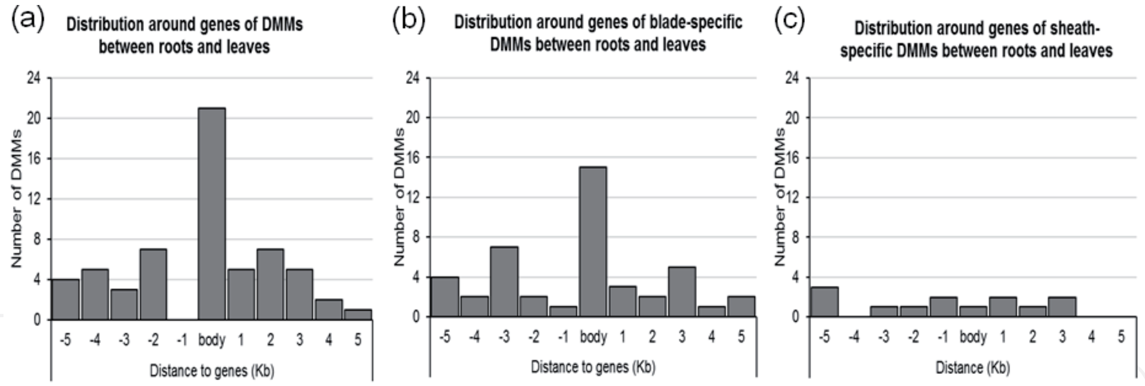


Figure 5.
Distribution of tissue-specific differentially methylated markers (DMMs) around genes. (a) DMMs between roots and leaves, present in both blades and sheaths as in **Figure 2b** *and* **c***; (b) blade-specific DMMs between roots and leaves and (c) sheath-specific DMMs between roots and leaves. The y axis indicates the distance to genes in kilo base pairs (kb) on both flanking regions. Negative and positive values indicate upstream and downstream of genes, respectively. DMMs overlapping with genes are considered as changes in gene-body methylation (body). The x axis shows the number of DMMs per 1 kb window.*

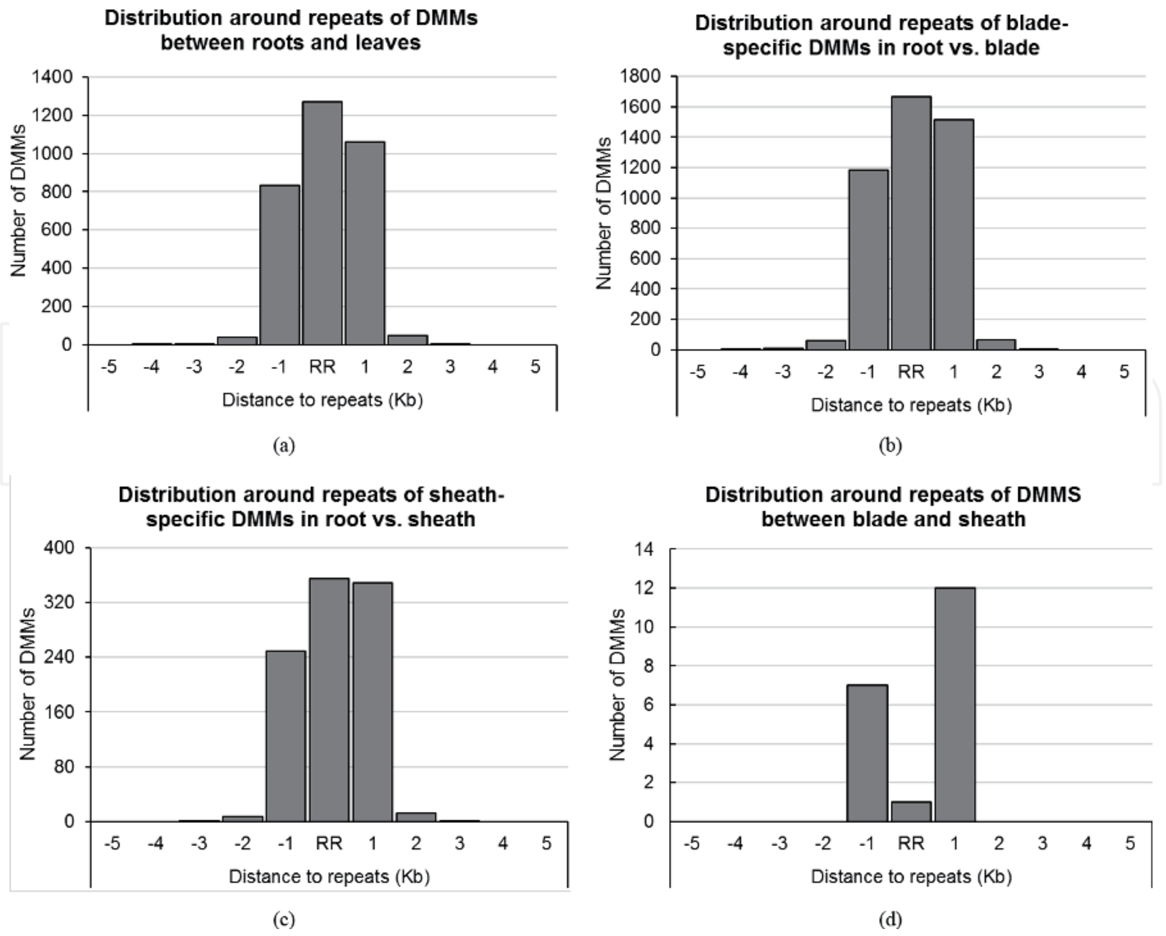
2391 DMMs, 0.5%) (**Figure 5c**). The majority of these (10 out of 13 DMMs) were sited within 3 kb of a gene, and no DMMs were present 3–5 kb from transcription margins (**Figure 5c**). Of 37 gene-body DMMs detected across all comparisons (**Figure 5a–c**), 27 overlapped with an exon and the remaining 10 markers were in intronic regions, 70–604 bp upstream of exons, except 1 DMM, which was 62 bp downstream an exon (Appendix A).

3.6 Distribution of tissue-specific DMMs near repeat regions

Many more tissue-specific DMMs were detected near repeats than near genes. The DMMs around repeat regions (as defined in the Ensembl database (http://plants.ensembl.org/biomart/martview/)) were concentrated either within the repeats or within 1 kb of their margins (**Figure 6a**). A similar distribution pattern was obtained with both blade-specific and sheath-specific DMMs when contrasted with roots, with more DMMs overlapping with the repeats themselves than in the 1 kb stretches flanking their margins (**Figure 6b** and **c**). The few markers that were differentially methylated between blades and sheaths (20 DMMs in total) were all located within 1 kb of a repeat (**Figure 6d**). Therefore, stable tissue-specific DMMs appeared to occur preferentially within repeats and 1 kb flanking regions, with higher frequency within 1 kb downstream than within 1 kb upstream, regardless of tissue types (**Figure 6a–d**).

3.7 Distribution of genes around differentially methylated (DM) repeats

To investigate a possible interaction between differentially methylated (DM) repeats and genes, the distance of genes from DM repeats between root and leaf samples was evaluated. In this way, we found 105 genes near repeats (up to 5 kb either side), of which 37 overlapped with a repeat and the remaining genes were scattered up- and downstream from the repeat (**Figure 7**). The number of DM repeats surrounded by genes thus represented only a tiny proportion of the total repeats that were differentially methylated between roots and leaves (105 out of 3266 DM repeats, 3.21%). About half of genes near DM repeats (52 of 105 genes) were also differentially methylated, whereas the remainder (53 genes) were not.



(a) (b)

(c) (d)

Figure 6.
Distribution of tissue-specific differentially methylated markers (DMMs) around repeats. (a) DMMs between roots and leaves, present in both blades and sheaths as in **Figure 2b** *and* **c***; (b) blade-specific DMMs between roots and leaves; (c) sheath-specific DMMs between roots and leaves; (d) DMMs between blades and sheaths. The x axis indicates the distance to repeats in kilo base pairs (kb) on both flanking regions. Negative and positive values indicate upstream and downstream repeat regions, respectively. RR: repeat regions. The y axis shows the number of DMMs per 1 kb window.*

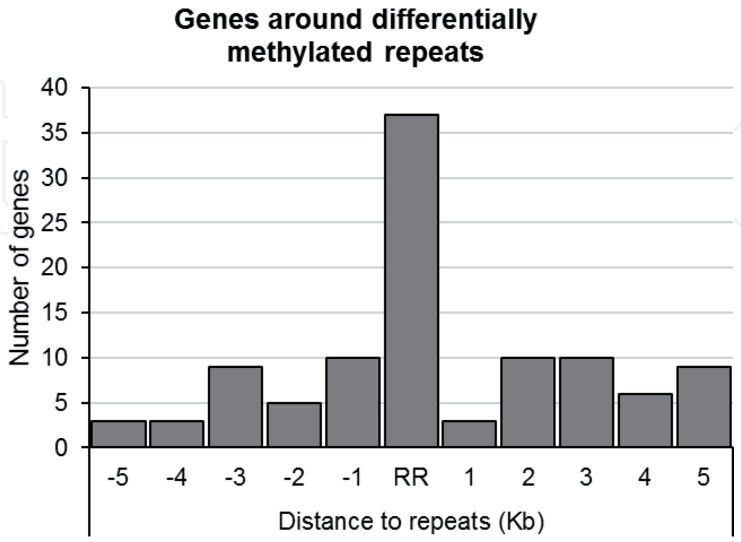


Figure 7.
Distribution of genes around differentially methylated repeat regions. The x axis indicates the distance to repeats in kilo base pairs (kb) on both flanking regions. Negative and positive values indicate upstream and downstream repeat regions, respectively. RR, repeat regions. The y axis shows the number of genes per 1 kb window.

4. Discussion

4.1 Extensive epigenetic differentiation between roots and leaves

In this study, we detected large numbers of DDMs between roots and leaves that were conserved across a diverse array of barley genotypes, and so were deemed far more likely to be organ-specific than genotype-dependent. Of these, hypomethylation of the ^{m}CCGG motif predominated in leaves (**Figures 2b** and **c**, **3b** and **4a**). More surprisingly, we also detected similarly conserved DMMs between leaf-blades and leaf-sheaths (**Figures 2e** and **4b**). The number of conserved DMMs between blades and sheaths (20 DMMs), all hypermethylated in sheaths, was relatively consistent with the closeness of these structures in position and function. These findings are broadly congruent with previous studies, which reported differential DNA methylation between variable tissues (e.g. endosperm, pollen, leaves, and roots) in diverse plant species [7–10], but additionally hint that the developmental closeness of structures being compared may also be reflected in the distinctiveness of their methylation profiles. However, controversy over the extent and validity of organ-specific DMMs [9, 10, 21–23] could cast doubt over their utility for organ diagnosis or as a tool to gain greater insight into the genes responsible for organ development/identity. Here, we sought to mitigate against the possibility of type I errors in DMM assignment through the unprecedented use of five diverse varieties and five biological replicates of each variety in the identification of these marks. In contrast to our findings, previous workers have reported little difference in the methylation levels of both ^{m}CG and ^{m}CHG motifs between roots and leaves in Arabidopsis [9] and sorghum [10]. Further, no significant difference was detected at all for ^{m}CG and ^{m}CHG methylation levels between tissues in cotton [37]. These divergences may simply reflect genuine biological differences between taxonomic groups. However, it is also important to recognise that such differences may also arise from the approach used to identify organ-specific DMMs. Variability in the techniques used to assess plant methylation profiles may introduce different forms of bias and preclude or complicate comparison among studies. DMM detection can be influenced by factors such as (1) the genome coverage of the methylation profiling method (low coverage methods such as MSAP are likely to miss many markers) [7], and (2) the data analysis approach used, which can compare either global methylation levels (e.g. percent methylation) [9] or methylated loci (e.g. DMMs) [28]. We contend that relying solely on global methylation levels can be misleading in comparing tissue profiles, because similar methylation levels may show completely different patterns and so vital information content is lost.

The current study revealed that tissue-specific DNA methylation occurred abundantly in the ^{m}CHG context (in particular ^{m}CCGGs) (**Figure** 2a and **c**). This concurs with reports of the CHG context similarly dominating differential DNA methylation between organs in *Brachypodium distachyon* [8] and sorghum [10]. Although tissue-specific methylation also occurs in other cytosine contexts [10], our results and other studies [10, 22] suggest that ^{m}CCGG is a primary motif of epigenetic distinctiveness of plant organs. However, since *Msp*I activity is affected by the presence of cytosine hydroxymethylation on its recognition sequence [38], some of the markers identified here as being cytosine methylation induced, could be due to the presence of (de) hydroxymethylation events instead. Additionally, while tissue-specific DMMs were mostly hypomethylated in leaves compared to roots in the present study (**Figure 3b**), in Arabidopsis, Widman et al. [9] found that hypermethylation prevailed in leaves compared with roots. This apparent contradiction in the directionality of methylation in DMMs between roots and leaves may be a reflection a difference in the polarity of early divisions in the monocotyledonous barley and the dicotyledonous Arabidopsis embryos or else the methylation profiling method implemented.

4.2 DNA methylation flux is tissue-specific during barley seedlings development

In addition to tissue-specificity of methylation profiles, one notable finding in the current study was that leaf cohorts exhibited a strong tendency to co-cluster. This suggests that the nature of methylation divergence between organs is not absolutely fixed and instead appears to change with developmental progression. This observation accords with previous reports that genome-wide methylation patterns are not static during plant development [39]. Additionally, a considerable portion of DMMs between roots and leaves was also specific to the leaf rank, due to the steady decrease in the number of DMMs between roots and leaf blades with the rank of the latter (**Figure 2a–c**). In this case, therefore, the slow but progressive accumulation of additional methylation marks in the leaves increases their divergence from root profiles and enables the separation of leaf cohorts. However, the small number of DMMs distinguishing between leaf blades and leaf sheaths ran counter to this trend such that there were no DMMs capable of discrimination between these leaf parts among the oldest cohort studied (leaf 1) (**Figure 2d** and **Table 2**). It seems intuitively improbable that older cohorts of leaves would simply lose differentiation between structurally distinct parts, especially if these marks had a functional role in defining function. Perhaps the most plausible biological explanation for the apparent erosion of divergence lies in the different chronological ages of the leaf cohorts that were sampled. Put simply, the third leaves were the least mature of the three cohorts collected and so it is entirely possible that the blade-sheath differential marks had yet to appear in these samples. Thus, it is important to consider the developmental and ageing progression chronology when assigning DMMs and that some organ- or structure-specific marks may only become organ-specific late in their development. Such late-emerging developmental DMMs should mean that the cumulative number of tissue-specific markers increases and so the organs or structures become more distinct, through leaf growth stages [40], each of which may carry a specific epigenetic profile. Certainly, others have noted that methylation profiles vary progressively as the organ develops [3, 41, 42] before reaching, at maturity, a “default” methylome, which may be conserved across varieties [24]. These results suggest that, once leaves are differentiated and mature, they do not show significant differences in DNA methylation profiles, regardless of their rank of appearance. Additionally, the location of half of the 20 common DMMs between blades and sheaths on chromosome 5H implies that this chromosome carries loci important for blade and sheath identities.

4.3 Tissue-specific DNA methylation preferably occurs in repeat regions of the barley genome

Organ-specific DMMs identified here were primarily associated with repeat regions. No significant difference was observed between the frequency of CCGG sites in and around genes and repeats. However, 84% of the barley genome is comprised of mobile elements or other repeat structures [25, 43], indicating that the fact that the majority of detected DMMs are located within or in the proximity of a repeat is due to the intrinsic repetitive nature of the studied genome. Nevertheless, the fact that 27 DMMs overlapped with exons and 10 were located in introns (Appendix A) contradicts previous claims that CHG methylation marks are exclusively restricted to repeat regions and intergenic regions [20, 21, 44, 45]. The possible regulatory significance of such gene body CHG methylation marks requires further investigation [46]. However, it is already well-established that tissue-specific DMMs can influence gene expression by enhancing gene transcription [9] and alternative splicing [47] or through repression due to immediate proximity to transcription start site [48].

The predominance of DMMs around and within repeats leads us to speculate that they could play an important role in defining organ identity in barley, and accords with previous findings in *Brachypodium distachyon* [8]. This flux of DNA methylation patterns in repeats [8, 42, 49] has been proposed to regulate [44] developmental shifts during plant growth and development [11, 39]. Nevertheless, the association between DMMs in/around repeat regions and organ identity described here does not establish a causal link between the two. However, there are grounds for reasoning that this may be the case and that the possibility warrants further study. First, repeat regions were previously proposed to be involved in alternative promoters, a substantial proportion of which (>40%) was reported to shape tissue differentiation [16]. Therefore, tissue-specific DMMs in repeats may contribute to alternative promoters, and thus determine organ identity. Second, differential gene expression between roots and leaves [25, 50] implies a firm regulatory system, including epigenetic mechanisms to guarantee tissue-specific cell development. Tissue-specific DMMs in repeats show that repeats are not the so-called "selfish parasites" of the genome [51], but can directly or indirectly affect tissue-specific gene expression [42, 52, 53]. Finally, it has been suggested that transposons coordinate splice variants, a genomic event that occurs in more than 60% of plant genes [54, 55], thus generating multiple mRNA transcripts from a single gene [56, 57]. Many splice variants are tissue-specific [58], suggesting that it is entirely possible that tissue-specific DMMs in repeats affect alternative splicing and subsequent gene expression. Also, some DM genes might potentially be regulated simultaneously by their own methylation and that of repeats [53, 59], due to proximity with DM repeats.

5. Conclusions

This study provides a comprehensive set of robust tissue specific epimarkers which were conserved in all barley genotypes tested and can therefore be considered genotype independent. Such markers have potential to be converted into locus-specific methylation sensitive cleaved amplified polymorphic sequence markers (ms-CAPS) to be used as diagnostic of sample origin. Moreover, these markers provide a basis for the understanding of the role of DNA methylation in plant organ differentiation and development. Our data illustrates that during tissue development, DNA methylation evolves to reach a default profile once the tissue is completely differentiated at maturity. It is possible that the plant organ formation and maturation is under at least partial control of DNA methylation changes. In addition, repeats could play an important role in tissue definition. The existence of tissue-specific mCCGG sites suggests that this context carries important factors of tissue differentiation. Expression analysis of tissue samples would conclusively demonstrate the role of tissue-specific DMMs in gene regulation. These markers will provide a basis for future studies of the role of DNA methylation in plant organ differentiation and development.

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Conflict of interest

“The authors declare no conflict of interest.”

Author contributions

MK conceived and performed the experiments, analysed the data and wrote the manuscript; BJM performed ms-GBS data alignments; MJW, ESS, BB, CMRL conceived the experiments and supervised the work. All authors read and commented on the manuscript.

Appendix

Chrom.	Exons				DMMs			Tissue
	Start	End	ID	Rank	Start	End	bp to exon	
3H	256588863	256589313	exon:MLOC_37071.2:3	3	256588258	256588258	−604	leaf
1H	173809114	173809167	exon:MLOC_44613.1:2	2	173808619	173808619	−494	leaf
2H	**427507334**	**427507612**	**exon:MLOC_61110.4:1**	**1**	**427506881**	**427506881**	**−452**	**blade**
7H	584462328	584462663	exon:MLOC_6930.1:4	4	584461984	584461984	−343	blade
3H	48188588	48188710	exon:MLOC_36518.3:9	9	48188256	48188256	−331	leaf
4H	531043445	531043540	exon:MLOC_66787.2:5	5	531043255	531043255	−189	leaf
3H	282775878	282775978	exon:MLOC_57866.1:2	2	282775689	282775689	−188	leaf
2H	507101612	507102232	exon:MLOC_57766.1:6	6	507101429	507101429	−182	blade
3H	451801679	451801792	exon:MLOC_4568.8:12	12	451801608	451801608	−70	blade
1H	295869691	295869957	exon:MLOC_57040.1:1	1	295869907	295869907	0	blade
1H	372664328	372665243	exon:MLOC_11591.1:1	1	372665217	372665217	0	leaf
1H	398203764	398206694	exon:MLOC_52730.3:1	1	398204886	398204886	0	leaf
2H	436039625	436040167	exon:MLOC_16240.2:1	1	436040156	436040156	0	leaf
2H	550574223	550574658	exon:MLOC_7365.2:1	1	550574622	550574622	0	leaf
3H	141116151	141117572	exon:MLOC_70576.2:1	1	141116946	141116946	0	blade
4H	428185287	428190462	exon:MLOC_52907.1:1	1	428185685	428185685	0	leaf
5H	449547966	449548309	exon:MLOC_66740.1:1	1	449548006	449548006	0	blade
6H	5471445	5474755	exon:MLOC_54256.1:1	1	5473235	5473235	0	leaf
6H	247447067	247450327	exon:MLOC_7517.2:1	1	247448194	247448194	0	blade
7H	96048516	96048816	exon:MLOC_36488.1:1	1	96048734	96048734	0	leaf
7H	440064807	440067513	exon:MLOC_72767.1:1	1	440065330	440065330	0	leaf
7H	544501261	544504310	exon:MLOC_39738.1:1	1	544501865	544501865	0	sheath
6H	69839676	69839776	exon:MLOC_11882.4:2	2	69839743	69839743	0	leaf
7H	331094393	331097017	exon:MLOC_54330.1:2	2	331096165	331096165	0	blade
1H	61790876	61791279	exon:MLOC_66388.8:3	3	61791253	61791253	0	leaf
3H	421991486	421991892	exon:MLOC_18521.3:3	3	421991580	421991580	0	leaf

Chrom.	Exons				DMMs			Tissue
	Start	End	ID	Rank	Start	End	bp to exon	
7H	96049105	96050237	exon:MLOC_36488.1:3	3	96049134	96049134	0	leaf
3H	516390233	516390451	exon:MLOC_37766.1:4	4	516390244	516390244	0	blade
4H	434415593	434415838	exon:MLOC_58529.1:4	4	434415773	434415773	0	blade
2H	578608506	578608551	exon:MLOC_54514.1:5	5	578608549	578608549	0	blade
5H	484203288	484203413	exon:MLOC_73139.2:5	5	484203386	484203386	0	blade
2H	2183704	2183865	exon:MLOC_57446.2:9	9	2183753	2183753	0	leaf
7H	41386814	41387497	exon:MLOC_57450.2:9	9	41387134	41387134	0	leaf
4H	434420196	434420586	exon:MLOC_58529.6:13	13	434420355	434420355	0	blade
3H	541205210	541205401	exon:MLOC_37244.3:16	16	541205351	541205351	0	leaf
7H	570620131	570620572	exon:MLOC_14604.2:16	16	570620258	570620258	0	blade
7H	583930566	583930636	exon:MLOC_62970.1:2	2	583930697	583930697	62	leaf

DMMs: differentially methylated markers; Chrom: chromosome; bp: base pair

Table A1.
List of differentially methylated exons. Bolded value is the only first exon methylated upstream 452 bp from a transcription start.

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