Article

Salt stress induces non-CG methylation in coding regions of barley seedlings (*Hordeum vulgare*)

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Abstract: Salinity can negatively impact crop growth and yield. Changes in DNA methylation are known to occur when plants are challenged by stress and have been associated to the regulation of stress-response genes. However, the role of DNA-methylation in moderating gene expression in response to salt stress has been relatively poorly studied among crops such as barley. Here we assess the extent of salt-induced alterations of DNA methylation in barley, and their putative role in perturbed gene expression. Using Next Generation Sequencing, we screened the leaf and root methylomes of five divergent barley varieties grown under control and three salt concentrations, to seek genotype independent salt-induced changes in DNA methylation. Salt stress caused increased methylation in leaves but diminished methylation in roots with a higher number of changes in leaves than in roots, indicating that salt induced changes to global methylation are tissue specific. DMMs were mostly located in close proximity to repeat elements but also 1094 genes, of which many possessed GO terms associated with plant responses to stress. Identified markers identified have potential value as sentinels of salt stress and provide a start point to understand the functional role of DNA methylation in facilitating barley's response to this stressor.

Keywords: Epigenetics, Differentially Methylated Markers (DMMs), leaves, roots, DNA methylation, salinity stress, barley.

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1. Introduction

Barley is an important crop for food, feed and brewing [1,2], and is used as a research model for temperate cereals [3,4]. Although considered relatively tolerant to salinity [5], barley grown under saline conditions often suffers substantial yield losses [6]. In recognition of a global increase in saline soils worldwide [5], there are continuing efforts to improve the salt-tolerance of barley varieties to maintain current levels of production. As with other plant species, barley responds to salt stress through the coordination of processes that alleviate both osmotic stress and ion toxicity [7]. Acclimation to saline conditions requires the stimulation of multiple molecular networks, including stress sensing, signal transduction, and the expression of stress-specific genes and metabolites [3,7-9]. Modern genetic improvement strategies aimed at improving salt tolerance require characterisation of genes activated in response to saline stress [10], and ideally, better understanding of their interactions and of any plasticity in their expression afforded by epigenetic regulation [11].

Epigenetic mechanisms that control gene regulation act independently of any change to DNA sequence [12-14], although one, DNA methylation, does alter its chemistry. The term DNA methylation describes the addition of a methyl group onto a specific cytosine base in the DNA and this change often plays a critical role in moderating gene expression [13,15,16]. Indeed, DNA methylation has been implicated in several critical aspects of plant development and in regulating a plant's adaptation to stress [13,17-20]. Change in DNA methylation status can occur via *de novo* DNA methylation, which is generally associated with gene repression, or by demethylation, which usually enhances gene expression [16], although numerous exceptions to this rule are known [16,21,22]. There are several reasons for characterising changes to the global methylation status of the genome that occur in response to a stress such as excessively saline soil. At the simplest level, identifying salt-induced methylation changes to specific sites has potential to diagnose the presence and level of salt stress experienced by roots, based solely on the methylation status of key epimarkers. Salt concentration in saline soils varies on both spatial and temporal scales [23,24] and so measuring the timing and extent of exposure of an individual plant can be difficult in field conditions. The possibility of being able to characterise fine scale salt exposure of individual (sentinel) plants based on changes to the methylome is therefore an attractive prospect, and one that may also facilitate ready identification of genotypes exhibiting differential responses (e.g. avoidance, ion exclusion or tolerance). At the same time, better knowledge of which genic regions most likely to be methylation-regulated in response

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to salt stress provides a useful starting point from which to identify candidate genes that may be implicated in plastic salt stress responses and to build broader understanding of the molecular mechanisms in play that confer plant resilience to saline stress; something that has the potential to open up new avenues for crop breeding [11].

Several studies have demonstrated that exposure to salt stress can significantly perturb plant methylation profiles [25-28]. Others have correlated stress-induced modifications to DNA methylation to changes in gene regulation across a range of species [14,18,29,30], although some controversy remains over the consistency of the DNA methylation sites described [28,31]. In general, most salt-induced changes to DNA methylation seem to occur within or in proximity to known stress response genes [7,25,32,33]. In maize, salinity induced *de novo* methylation to *zmPP2C* in roots but demethylation to *zmGST* in leaves, with both changes seemingly linked to altered expression levels [34]. *De novo* methylation significantly repressed the expression of *zmPP2C* in roots, whereas demethylation of *zmGST* enhanced its expression in leaves, implying that DNA methylation changes in response to salt stress might contribute to stress acclimation [34]. In barley, acute salt stress has been similarly shown to evoke methylation-modulated changes to the expression of several genes involved in metabolic and physiological processes implicated in the plant's ability to cope with the stress [3,9,35]. However, to date there has been a marked lack of reports linking salt-induced gene expression to global changes in DNA methylation or of methylation-associated changes that apply across a representative sample of any crop species [36].

For food crops with large genomes, the use of genome-wide Bisulfite sequencing to characterise genome-wide flux in methylation from a representative range of genotypes is effectively precluded by cost and the complexity of bioinformatics [37]. For this reason, most works on stress-induced methylome change have elected to either target particular loci [29,38] or else to survey only a subset of the genome. Of the many methods available, Methylation Sensitive Amplification Polymorphism (MSAP) analysis has proved particularly popular to study stress-induced changes to genome-wide methylation patterns [25,27,32], in part because of the reproducible reputation of the technique [39-41]. However, the MSAP method only generates relatively small numbers of anonymous markers [42,43] and so has limited utility for studies aiming to establish links between changes in methylation and altered gene expression. While some works have sought to overcome this limitation by targeted sequencing of MSAP amplicons [7,25,32,33], others have argued that this amendment of the method is still cumbersome, costly and time-consuming [44]. The ability of Next Generation Sequencing to analyse large numbers of loci in multiple methylomes in parallel provides the opportunity to overcome these limitations. The use of methylation-sensitive GBS (ms-GBS) provides workers with the possibility of identifying differentially methylated markers (DMMs) with a better depth and coverage of the genome [44,45]. By using methylation-sensitive restriction enzymes to reduce genome complexity during library preparation, differentially methylated fragments are produced and appropriate for high throughput sequencing [44,45]. This approach presents the advantage of detecting methylated sites that are dispersed across the genome, and is particularly appealing for species with a large genome such as barley [44].

In this study, we used ms-GBS to assess the level of salt-induced changes to methylation site distribution patterns in roots and leaves of five diverse barley genotypes and match against the reference barley genome to characterize the genomic locations of changed loci. We then combined these results with publicly available data about the gene expression of barley roots under salt to postulate on the possible functional implications of DNA methylation flux on gene regulation in barley under salt stress.

2. Results

2.1. Methylation-sensitive Genotyping-By-Sequencing (ms-GBS)

Overall, we generated in excess of 1 billion raw reads (1,015,703,602) from ms-GBS libraries, sequenced on a HiSeq 2500. A high proportion of the raw reads passed the filter for the presence of the barcoded adapter, the *Msp*I restriction product site and the *Eco*RI adapter (1,004,318,258; 98.87%). However, when these reads were filtered further to identify those uniquely mapping to the draft barley reference genome [4], the numbers

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fell substantially to 496,960,365 reads (i.e. 49.48% of raw reads). This yielded an average of 2,484,801 high quality reads per library and represented 892,859 unique sequence tags. Tags represented in this set amounted to 31.56% of the *Msp*I recognition sites (5`-CCGG-3`) estimated for the barley reference genome (2,828,642; Table 1).

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

Raw reads	1,015,703,602
Reads that matched barcodes	1,004,318,258
Reads aligned to barley reference genome	496,960,365
Samples	200
Average reads per sample	2,484,801
Total unique tags	892,859
Polymorphic tags	645,297

2.2. Salt-induced DNA methylation changes is tissue and concentration specific

In total, 24,395 and 3,777 unique sequence tags were deemed 'significantly Differentially Methylated Markers (DMMs)' (FDR < 0.01) in leaf and root samples respectively across all five varieties and salt treatments (Figure 1 and Figure 2a). Curiously, the overall number of leaf DMMs increased progressively with salt concentration (75, 150 and 200 mM NaCl), whereas there was no such pattern seen in the roots (Figure 1). Soil salt was found to induce more hypomethylated DMMs than hypermethylated DMMs in both leaves and roots, regardless of concentration (Figure 1). Although the number of salt-induced DMMs was higher in leaves (24,395 DMMs) than roots (3,777), the scale of the change evoked by salt stress was far higher in roots for when measured by P-values (Figure 2a) and the fold-change in read counts (Figure 2b-c). Comparison of the median fold-change of methylation across all markers in the two organs revealed that salt induces net hypomethylation in roots and hypermethylation in leaves (Figure 2a-c), even though the number of salt induced hypomethylated sites exceeds those of hypermethylated sites in both organs (Figure 1).

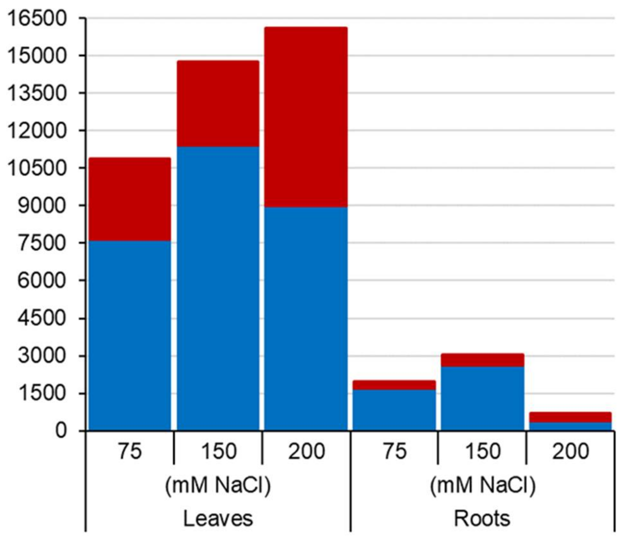
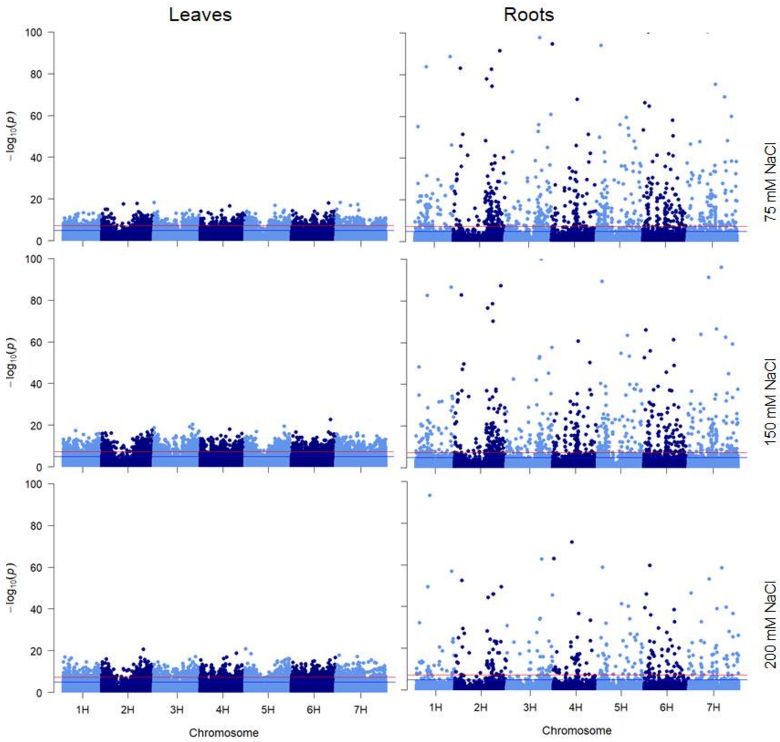
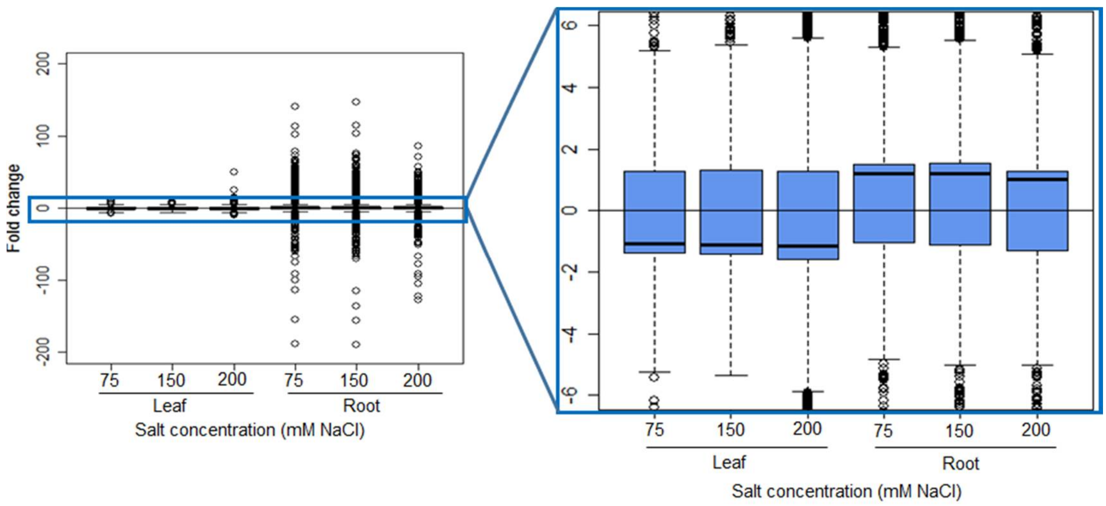


Figure 1: Number of salt-induced differentially methylated markers (DMMs) in barley leaves and roots. Samples from barley plants exposed to 75, 150 and 200 mM NaCl were compared with salt-free control plant samples. The red and blue sections in the bar chart represent the proportion of salt-induced hypermethylated (red) and hypomethylated (blue) DMMs. DMMs were identified by comparing 25 samples per treatment, each composed of five replicates of five barley varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra).



(a)



(b) (c)

Figure 2: Tissue-specific response intensity and directionality of salt-induced DNA methylation changes. (a) Distribution of salt-induced epigenetic markers in the barley genome. Each point represents the genomic location (horizontal axis) of a marker and its associated negative *log*10 P-value (vertical axis), for the three salt treatments (75, 150 and 200 mM NaCl) in leaf and root samples compared with the control in the respective tissue. The red line represents the genome-wide threshold (p = 5e-8); the blue line indicates the suggestive threshold (p = 1e-5). (b, c) Directionality of the methylation in salt-induced DNA methylation markers. Boxplots show the distribution of the intensity of changes in DNA methylation level, represented here as the fold-change (2 power *log*2FC) in read counts between samples exposed to 75, 150 and 200 mM NaCl compared with those grown in control condition, in leaves and roots. (c) Enlarged area shows the direction of the methylation flux at a whole genome level in each tissue/salt treatment combination (i.e. positive medians indicate a global decrease in DNA methylation (hypomethylation) while negative medians indicate a global increase in DNA methylation induced by salinity stress). The methylation changes were obtained from msGBS sequencing data

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from 25 samples per salt treatment were compared with 25 control samples, and each treatment was composed of five replicates of five barley varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra).

2.3. Stability of salt-induced DMMs across treatments

We next surveyed the appearance of DMMs across treatments and organs. Only a small proportion of DMMs appeared across all salt concentrations (Figure 3a-b). Moreover, of the 24,395 salt-induced DMMs detected in leaf samples, 52% were specific to 75 mM, 150 mM or 200 mM NaCl (2,390, 4,070 and 6,202 respectively) (Figure 3a), implying a positive association between salt concentration and the number of loci affected by methylation changes. In roots, there was no obvious relationship with salt concentration, with 633, 1,642 and 88 salt-concentration-specific DMMs for 75 mM, 150 mM and 200 mM NaCl, respectively (Figure 3b) (62% of the total).

There were nevertheless many stable DMMs that appeared in all salt concentrations. These dose-insensitive DMMs accounted for 22.9% (5,593 of 24,395) of all salt-induced DMMs recovered from leaves and 14% (528 of 3,777) of those recovered from roots (Figure 3a-b, Supplemental Data Set S1). These dose-insensitive DMMs invariably presented the same directionality of methylation change across all concentrations (i.e. always hyper- or hypomethylated) (Figures 4a-b). The dose-insensitive DDMs followed the global trend (see above) and so mostly became hypomethylated following salt exposure in both leaves (4744, 84.82%) and roots (329, 62.31%). Of these, just 22 were shared between leaf and root samples, most of which again became hypomethylated following salt exposure (Figure 4c). Of these, 20 markers shared the same directionality of methylation change following salt exposure between organs but two markers ("2:1:467135271" and "6:1:259709553") became hypermethylated in leaves but hypomethylated in roots following exposure to salt (Figure 4c).

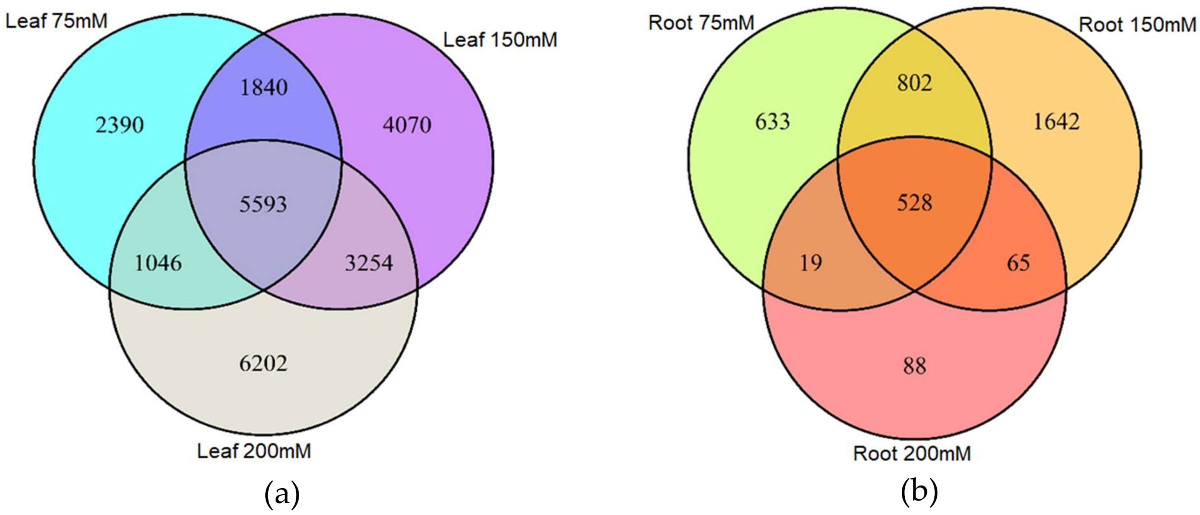

Figure 3: Venn diagram showing the number of differentially methylated markers (DMMs) induced by different salt concentrations in barley leaves and roots. DMMs in leaves (a) and roots (b) were obtained from barley plants exposed to 75mM, 150 mM and 200 mM NaCl, compared with a non-saline control. DMMs (FDR < 0.01) were identified by comparing 25 samples per treatment, each composed of five replicates of five barley varieties. FDR, false discovery rate.

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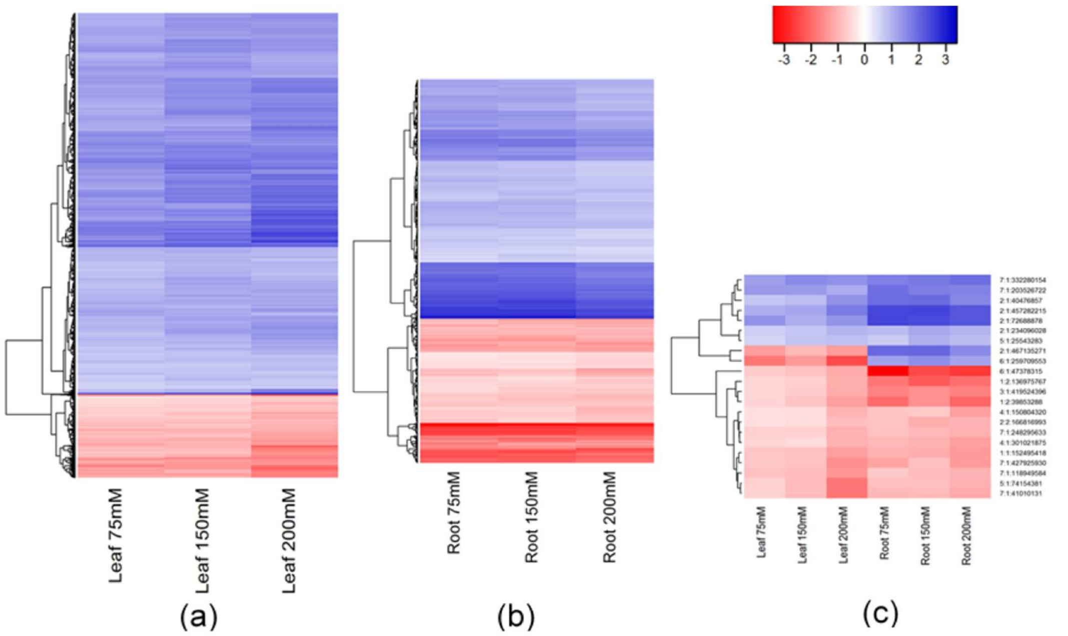

Figure 4: Hierarchical clustering of the fold changes in read counts of DMMs stable across all salt concentrations (75, 150 and 200 mM NaCl) when compared to control plants. Hypermethylated (red) and hypomethylated (blue) DMMs (a) in leaves (5593 DMMs); (b) in roots (528 DMMs); (c) shared by both leaf and root tissues (22 DMMs). Colour legend represent the fold-change (2 power *log*2FC) in read counts between samples exposed to 75, 150 and 200 mM NaCl compared with those grown in control condition. DMMs (FDR < 0.01) were identified by comparing 25 samples per treatment, each composed of five replicates of five barley varieties (Barque 73, Flagship, Hindmarsh, Schooner, and Yarra).

2.4 Distribution of salt-induced DMMs around annotated genomic features

We assessed the distribution of DMMs induced by 150 mM NaCl relative to annotated features of the barley genome (e.g. protein coding genes, repeats, tRNAs etc). Proximity to a repeat sequence appeared to be a strong predictor of the location of DMMs induced by salt. Indeed, 96.5 % of DMMs induced by salt in leaves and 99.8% in roots occurred either within the repeats themselves or within 1 Kb of them (Figures 5a-b).

We next sought to identify genes positioned within the proximity of the dose-insensitive salt-induced DMMs. The expression of these genes was considered most likely to be consistently influenced by salt-induced methylation flux. In leaves, 19.1% (1070/5,593) of dose-insensitive DMMs were located within 5 Kb of genes (Figure 5c; Supplemental Data Set S2), with the majority located within the gene-body itself (56.4%, 603 DMMs; Figure 5c). In roots, just 24 (i.e. 4.5%) of the dose-insensitive DMMs lay within 5Kb of a gene, five of which were located within the gene-body, 14 were upstream and five were downstream (Figure 5d; Supplemental Data Set S2). Additionally, it is worth mentioning that of the 22 dose-insensitive DMMs shared in leaves and roots (Figure 4c), only one was positioned within 5 Kb of a gene (3994 bp upstream MLOC_63677 on chromosome 2H).

Given that the effect of DNA methylation on gene expression may depend on the position of the change relative to the transcribed sequences [16,46], we further investigated DMM distance to 5`UTRs, 3`UTRs, and exons of differentially methylated genes in leaves and in roots. In leaves, it appeared that salt-induced DMMs near 5`UTRs were most abundant within 1 Kb (277 DMMs) of the 5`UTR in the downstream direction, with those falling between 1 and 2kb being the second most common (120 DMMs; Figure 6a). Outside these windows, DMMs occurred in the range 40-65 DMMs per Kb (Figure 6a). DMMs were more common in the upstream direction of 3`UTRs, with the 1 Kb bin immediately upstream containing the highest number of DMMs (197 DMMs),

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decreasing gradually to reach background levels (50-70 DMMs per KB) after 4 Kb (Figure 6b). In comparison, there were insufficient gene-associated DMMs from root samples to provide strong evidence of clustering around either the 5`UTRs or 3`UTRs.

The majority of DMMs within gene-bodies from leaf samples lay within exons (81.4%, 498 of 612; Figure 6e). The remaining DMMs were generally within 1 Kb of an exon (Figure 6e). Three out of the five gene-body DMMs from roots were similarly exonic or within 1Kb of the nearest exonic region (Figure 6f). Considered collectively, gene-body DMMs were most commonly associated with the first exons (57.5%; 355/617), and included 296 overlaps, 45 downstream and 14 upstream (Figure 6ef). Additionally, there were 41 DMMs from leaves and two DMMs from roots DMMs that clustered around tRNA genes (Figure 6gh). While only one DMM overlapped with a tRNA in leaves, 14 out of the 41 DMMs were within 1 Kb upstream (nine DMMs) and downstream (five DMMs) of a tRNA gene (Figure 6g). The two DMMs nearest tRNA genes in roots were located within 1 and 4 Kb downstream of the gene respectively (Figure 6h).

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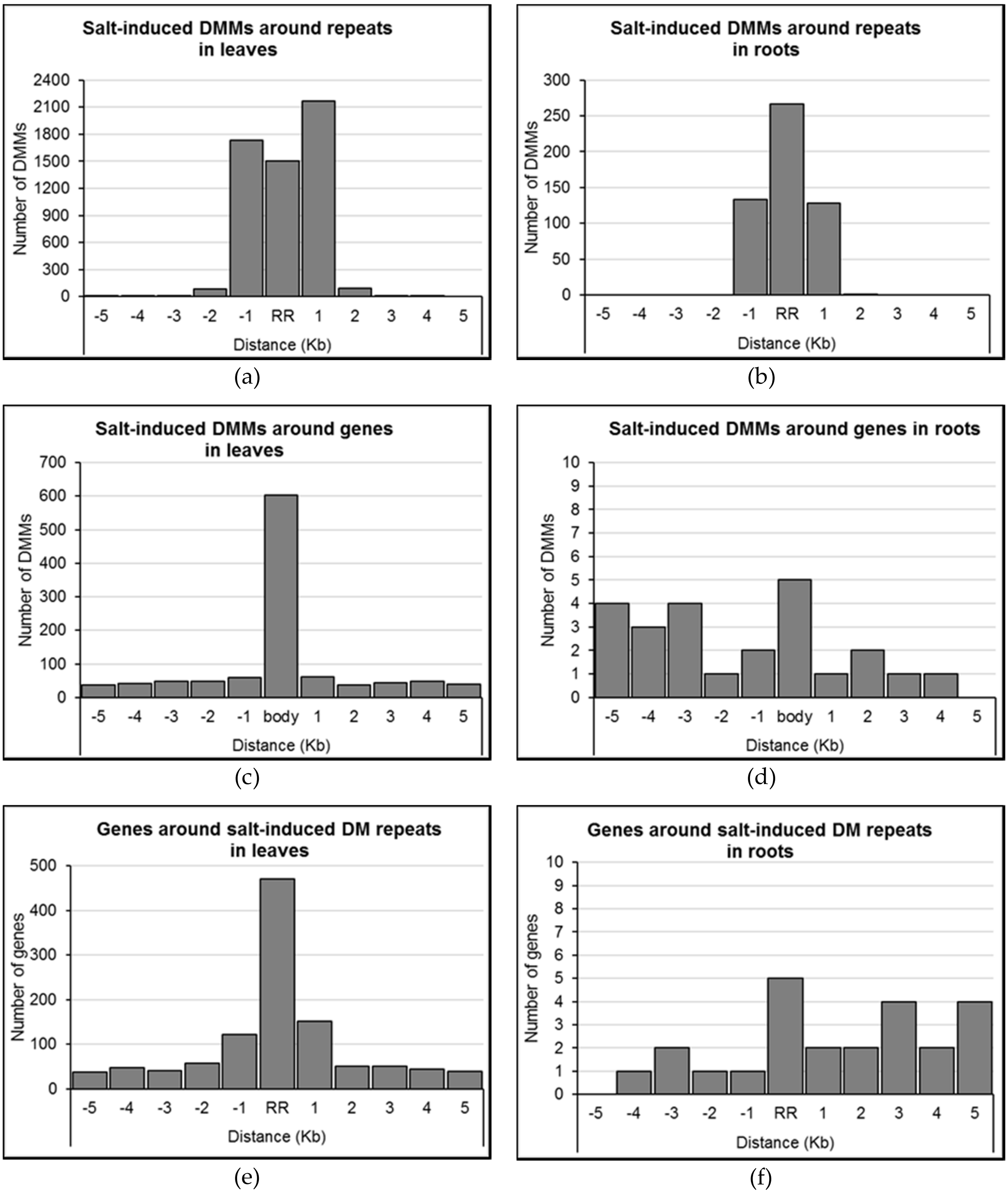

Figure 5: Distribution of salt-induced differentially methylated markers (DMMs) around repeat regions and genes. (a, b) distribution of DMMs distance from the closest repeat in leaves and roots, respectively; (c, d) distribution of DMMs distance from the closest gene in leaves and roots, respectively; (e, f) distribution of genes' distance from the closest differentially methylated (DM) repeats in leaves and roots, respectively. The distance of each DMM was calculated to the genomic feature, and DMMs were counted within repeats and genes, and five consecutive 1 Kb wide bins upstream and downstream. DMMs induced by 150 mM NaCl were used to show DMM distribution pattern around genomic features. body, gene-body. RR, repeat region.

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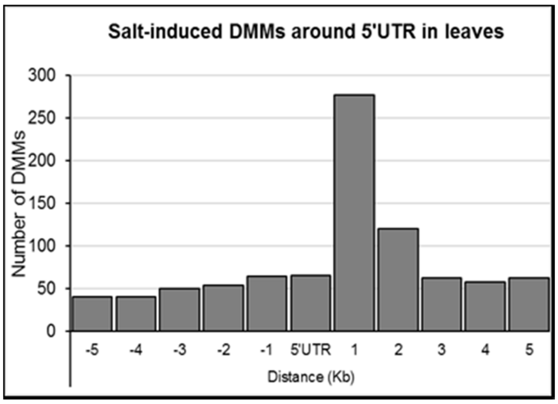

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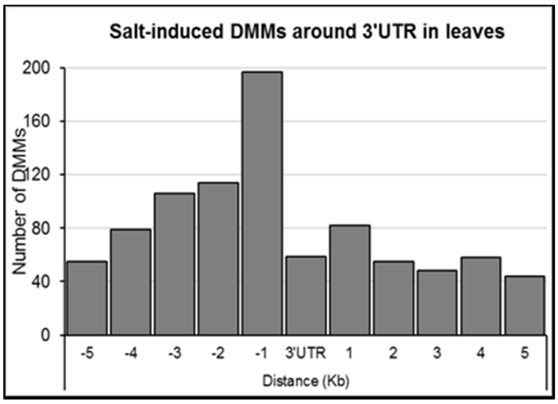

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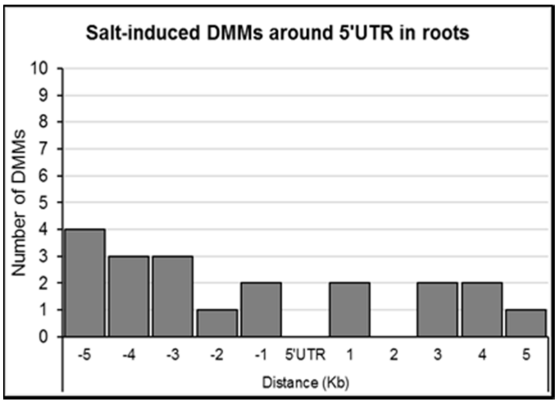

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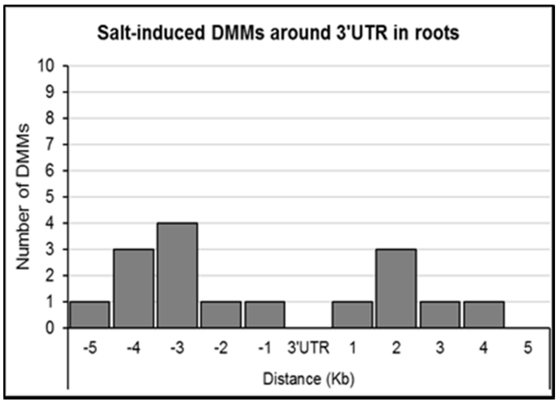

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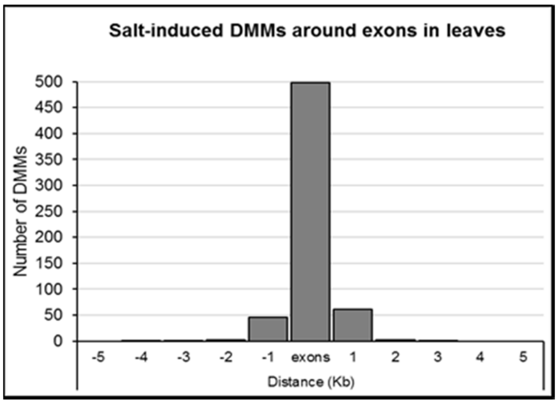

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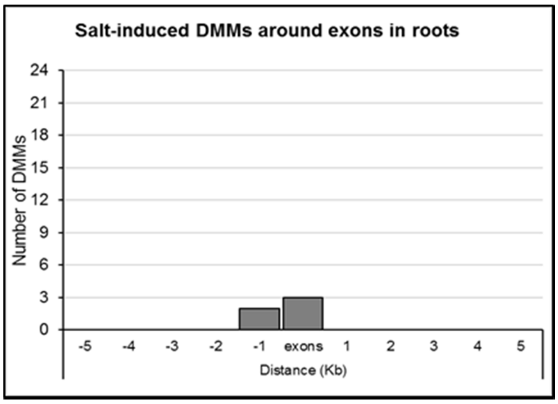

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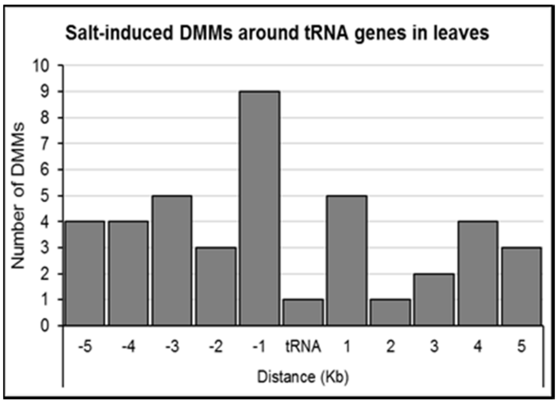

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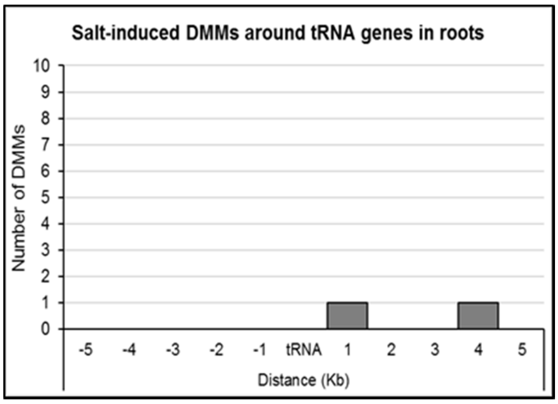

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Figure 6: Distribution of salt-induced differentially methylated markers (DMMs) around UTRs, exons and tRNA genes. (a, b) 5`UTRs and 3`UTRs in leaves; (c, d) 5`UTRs and 3`UTRs in roots; (e) exons in leaves; (f) exons in roots; (g) tRNA genes in leaves; (h) tRNA genes in roots; The distance of each DMM was calculated to the genomic feature (respectively, 5`UTR 3`UTR, exons and tRNA genes), and the number of DMMs was counted within these genomic features, and in five consecutive 1 Kb wide bins upstream and downstream. Kb, kilo base pair. DMMs induced by 150 mM NaCl were used to show DMM distribution pattern around genomic features.

2.5. Gene ontology analysis of salt-induced DMMs

Gene Ontology (GO) analysis was performed for all differentially methylated genes (i.e. within 5kb of a salt-induced DMM) from both leaves and roots. The 1,070 DM genes identified from leaves included 1,017 that were hypomethylated and 53 hypermethylated following salt exposure. These genes yielded 433 and 99 high level GO terms, for the hypomethylated and hypermethylated groups respectively (Table 1). The top five function groups retrieved from the hypomethylated genes in leaves were the "protein modification process", "cellular amide metabolism", "cell cycle" and "negative regulation of signal transduction" (Figure 7a, Supplemental Data Set S3). Hypermethylated genes were enriched with GO terms that associated with "organophosphate biosynthesis", "peptide metabolism", "peptide metabolism transport chain", "generation of precursor metabolites and energy", and "photosynthesis" (Figure 7b, Supplemental Data Set S3). In roots, salt-induced hypomethylated markers were associated with 15 genes whereas hypermethylated DMMs were in or proximal to nine genes. These genes were significantly enriched for 29 (hypomethylated) and 24 (hypermethylated) GO terms (Table 2). The GO terms derived from hypomethylated genes in roots fell into three main function groups: "generation of precursor metabolites and energy", "peptide metabolism" and "carbohydrate derivative metabolism" in this order (Figure 8a, Supplemental Data Set S3). Hypermethylated genes enriched GO terms that were related to one main biological function: "peptide biosynthesis". The details concerning all GO terms enriched by differentially methylated genes in roots are listed in Supplemental Data Set S3.

These GO terms, enriched from differentially methylated genes, give an indication of the biological pathways which activity might be modified in response to salinity. Some GO terms, although not dominant, related to functions essential for plant responses to salt stress, such as "ion transmembrane transport", "potassium ion transport", "cation transmembrane transporter activity", "response to osmotic stress, "response to chemical stimulus", "oxidation-reduction process", "regulation of innate immune response", "cellular response to stress", "defence response", among others (Supplemental Data Set S3).

Table 2: Number of genes differentially methylated and associated GO terms in barley leaves and roots. GO, gene ontology; hypo, hypomethylated genes; hyper, hypermethylated genes. GO groups were determined using REVIGO (http://revigo.irb.hr/).

		GO terms per GO group			
	Genes	Biological process	Cellular component	Molecular function	Total GO terms
Leaf hypo	1017	315	40	73	433
Leaf hyper	53	64	21	14	99
Root hypo	15	19	10	0	29
Root hyper	9	13	11	0	24

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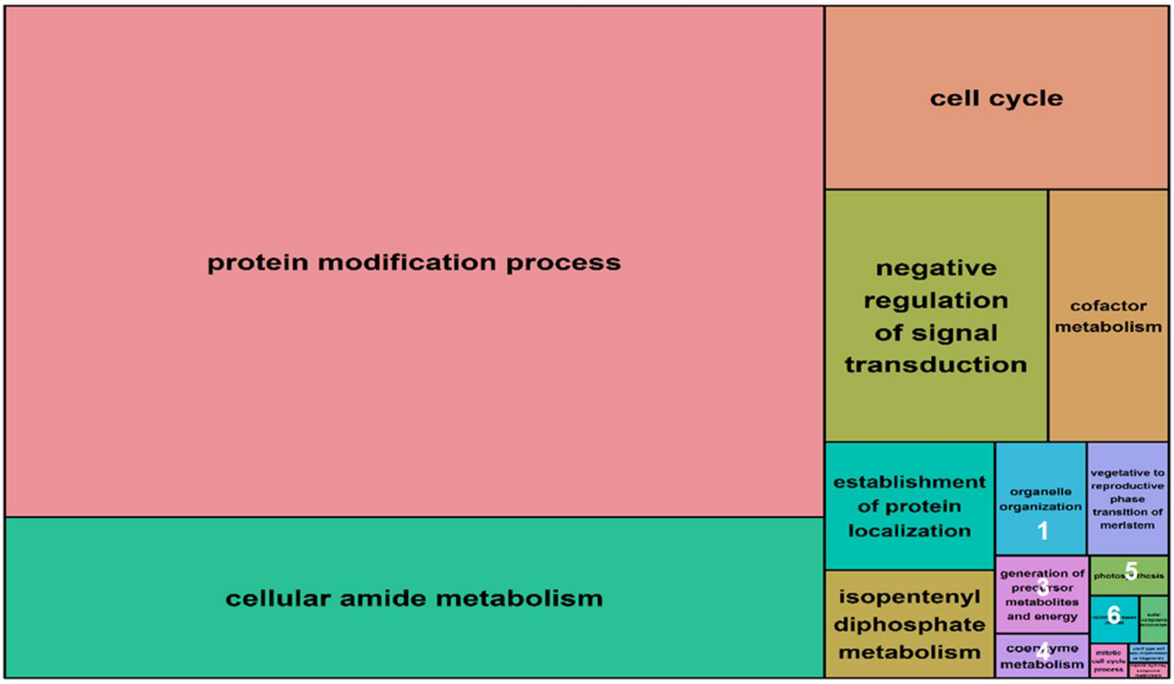

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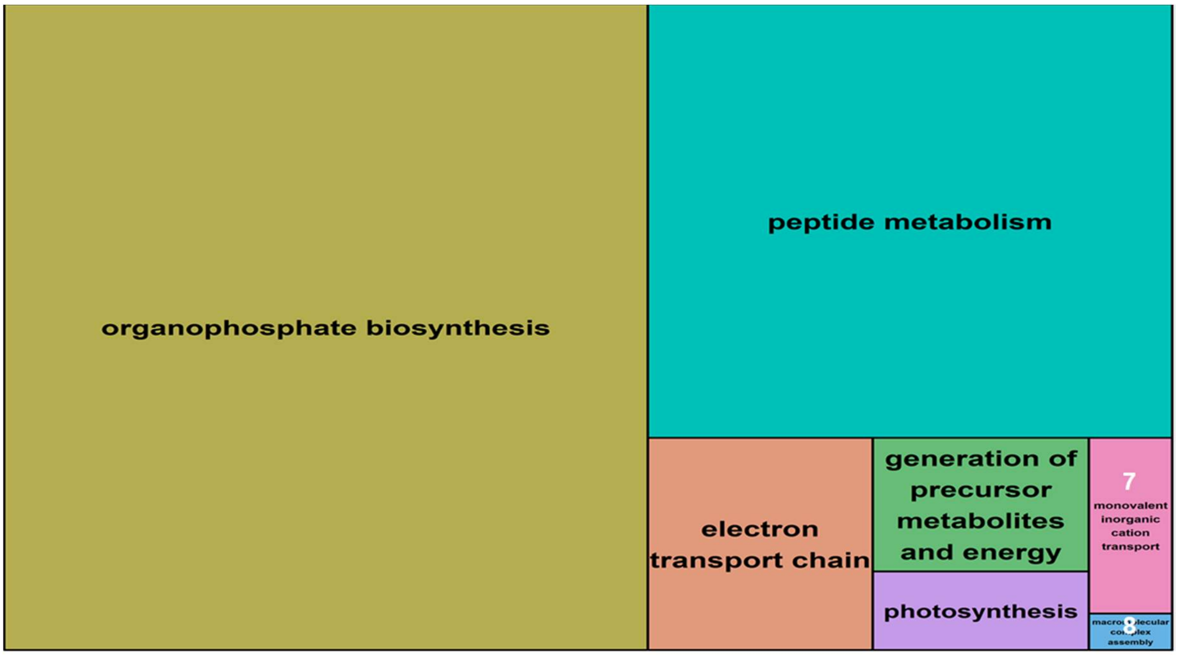

(b)

Figure 7: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially methylated genes in barley leaves. (a) Representatives of GO terms enriched by hypomethylated genes in leaves; Numbers represent GO term representatives with very small font size: 1 = organelle organization; 2 = vegetative to reproductive phase transition of meristem; 3 = generation of precursor metabolites and energy; 4 = coenzyme metabolism; 5 = photosynthesis; and 6 = microtubule-based process, sulfur compound metabolism, mitotic cell cycle process, plant-type cell wall organization or biogenesis, organic hydroxy compound metabolism, in order. (b) Representatives of GO terms enriched by hypermethylated genes in leaves; 7 = monovalent inorganic cation transport; 8 = macromolecular complex assembly. Treemaps were constructed using R scripts produced by the REVIGO server (http://revigo.irb.hr/).

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The detailed list of terms in the background of GO representatives is provided in the Supplemental Data Set S3.

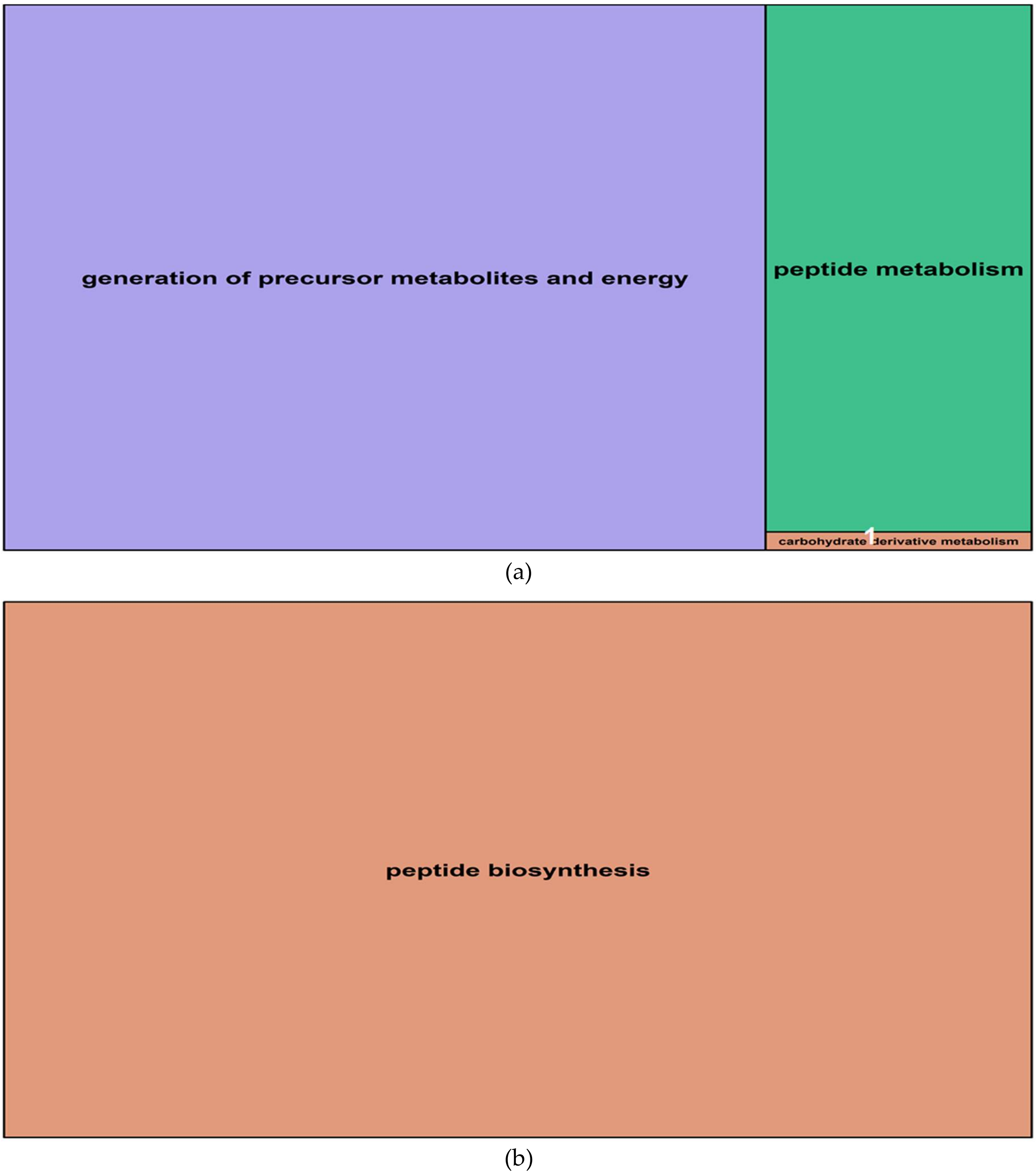

Figure 8: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially methylated genes in barley roots: (a) Representatives of GO terms enriched by hypomethylated genes in roots; 1 = carbohydrate derivative metabolism; (b) Representative of GO terms enriched by hypermethylated genes in roots. Treemaps were constructed using R scripts produced by the REVIGO server (http://revigo.irb.hr/). The detailed list of terms in the background of GO representatives is provided in the Supplemental Data Set S3.

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2.6. Differentially expressed genes in barley roots

To investigate whether observed changes in DNA methylation could be associated to changes in gene expression, salt-induced DMMs were compared to publicly available gene expression responses to salt exposure. These datasets related to two genotypes (Sahara and Clipper) and included four biological replicates of each variety (see material and methods). Differential gene expression between salt treatments revealed 124 upregulated and 34 downregulated transcripts (Supplemental Data Set S4), among which 76 and 18 transcripts, respectively, matched barley reference genes in the public database "Ensembl" (http://plants.ensembl.org/biomart/martview). Ontology of these annotated genes revealed many pathways that were regulated by salinity in barley roots. The top five gene representatives of significantly enriched GO terms in upregulated genes were "organophosphate biosynthesis", "peptide metabolism", "protein modification process", "electron transport chain", "monovalent inorganic cation transport" and "photosynthesis" (Figure 9, Supplemental Data Set S4). Downregulated genes enriched GO terms which clustered around the functional pathway "peptide metabolism" and to a small extent, around "generation of precursor metabolites and energy".

We then cross-referenced the differentially expressed genes against DMMs identified in the current study. This was achieved by searching for DE genes within 5 Kb of DMMs. There were no differentially methylated genes amongst DE genes with a false discovery rate (FDR) below 5%, and so we extended the gene list by reducing the stringency of the FDR cut-off to 10%. With this setting, seven DE genes were found to be differentially methylated, one of which contained two DMMs (MSTRG.43260, one hypo- and one hypermethylated) (Table 4). However, there was no correlation between gene methylation status and the direction of gene expression. Some hypomethylated genes were downregulated whereas others were upregulated; and vice versa for hypermethylated genes (Table 4). Only four of these differentially methylated transcripts matched with annotated barley genes in public databases. Gene ontology of these genes revealed that hypomethylated and hypermethylated genes enriched functionally close GO terms, which were all related to cellular components: plastid, cytoplasmic part and intracellular membrane-bounded (Supplemental Data Set S4).

Table 3: Number of genes differentially expressed (DE genes) and associated GO terms in barley roots. GO, gene ontology; GO groups were determined using REVIGO (http://revigo.irb.hr/).

	DE Genes		GO terms per GO group			
	Total transcripts	Annotated	Biological process	Cellular component	Molecular function	Total GO terms
Upregulated	124	76	94	22	29	145
Downregulated	34	18	23	12	0	53

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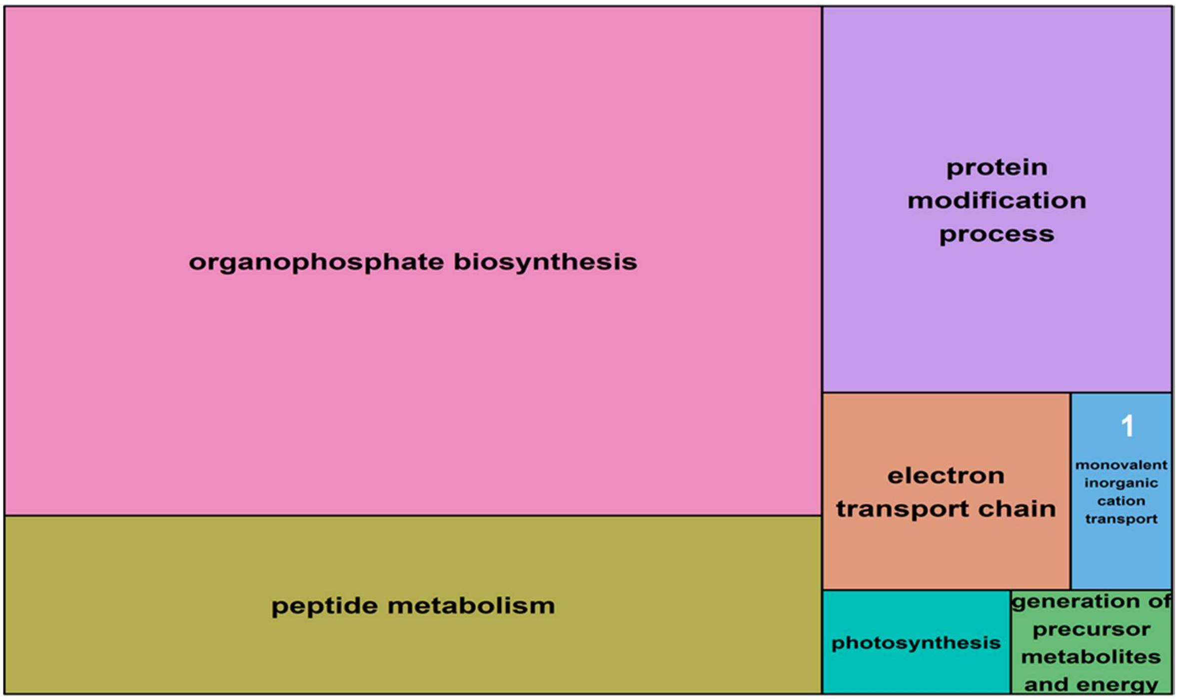

(a)

(b)

Figure 9: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially expressed genes in barley roots. (a) Representatives of GO terms enriched by upregulated genes in roots; 1 = monovalent inorganic cation transport; (b) Representatives of GO terms enriched by downregulated genes in roots; 2 = generation of precursor metabolites and energy. Treemaps were constructed using R scripts produced by the REVIGO server (http://revigo.irb.hr/). The detailed list of terms in the background of GO representatives is provided in the Supplemental Data Set S4.

Table 4: List of differentially methylated DE genes in barley roots. DE, differentially expressed gene; DMM, differentially methylated markers, Chrom, chromosome; FDR, false discovery rate; dist2Gene, DMM position relative to gene.

DE Genes		DMMs			Statistics				Annotation
GeneID	Range	Chrom	Position	Methylation	logFC	P.Value	FDR	dist2Gene	
MSTRG.4246	1:435681474-435731845	1H	435689351	hyper	-1.76	0.000	0.053	0	-
MSTRG.31525	5:507135444-507397451	5H	507332872	hypo	-1.47	0.002	0.083	0	MLOC.2917
MSTRG.43260	7:427906474-427974581	7H	427925930	hyper	-1.05	0.006	0.093	0	MLOC.73155
MSTRG.43261	7:427906474-427974581	7H	427948871	hypo	-1.05	0.006	0.093	0	MLOC.73155
MSTRG.10572	2:543673444-543674117	2H	543678039	hypo	-1.05	0.006	0.095	3922	-
MSTRG.6485	2:17425326-17624569	2H	17517122	hypo	1.39	0.007	0.095	0	-
MSTRG.6418	2:15418194-15419914	2H	15414469	hypo	1.53	0.004	0.089	3725	MLOC.24124
MSTRG.10644	2:545135370-545135958	2H	545131372	hyper	3.43	0.003	0.086	3998	MLOC.48766

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3. Discussion

A growing number of studies highlight the role of DNA methylation in coordinating the adaptive response of plants to stress [14,30,31]. The primary challenge, particularly for crops with large genomes, resides in assembling a genome-wide picture of the role of methylation in orchestrating the molecular response to the stressor. As with the study of other stresses, works on methylation-based responses to salt stress have therefore largely relied on low throughput targeted approaches, low genome coverage or anonymous markers applied to a low number of genotypes [7,25-27,33,47]. However, our use here of methylation sensitive Genotyping-By-Sequencing (ms-GBS) to study salt-induced changes to in DNA methylation in ^{m}CCGG contexts has allowed us to survey methylome flux across a reasonably representative portion of the genome (Figure 2). Application of this approach allowed us to characterize genotype independent salinity-induced methylation flux in both leaf and root samples, and then to relate the pattern of Differentially Methylated Markers to specific genomic features.

Consistency of salt-induced DMMs

Of the salt-induced DMMs identified in all barley genotypes, 23% and 14% were dose-insensitive (and so conserved) across all salt treatments in leaves and roots respectively, with the remaining markers being either concentration-specific or shared between two salt treatments (Figure 3a-b). The prevalence of concentration-specific DMMs in barley leaves (52%) and roots (62%) (Figures 3a-b) could imply that these DMMs occur stochastically, consistent with observations in previous studies [25,28]. However, all DMMs described here were conserved across all five diverse barley genotypes and five biological replicates (per variety), with the direction of salt-induced methylation flux being conserved in all cases. This element of the experimental pipeline was introduced to minimize the effect of stochastic noise and was intended to strongly enrich for conserved responses to salt stress. There are several aspects of the resulting data to suggest that this action did uncover at least some consistent epimarks of salt exposure. At a simplistic level, the progressive increase in the number of leaf DMMs as the salt concentration increased could be taken as suggestive of an incremental response as the salt concentration rises. This pattern would appear to accord with the established theory that a large number of DMMs only become activated above a threshold concentration of salt, as hypothesized by Soen and co-workers [48]. Following this reasoning, as the salt concentration increases, so more thresholds are exceeded and so more DMMs become recruited into the global methylation flux. In this way DMM abundance increases proportionally to the salt concentration. However, it is important to note that the use of only three concentration points in the titration series provides somewhat limited scope for confidence and that this pattern was not repeated among the root materials. Greater confidence can be taken from examining the 22% (6,121/28,172) of DMMs that were conserved across all salt concentrations. The fact that these dose-insensitive DMMs invariably exhibited the same directionality of methylation change across all concentrations (i.e. always hyper- or hypomethylated) despite differing between DMMs is compelling evidence that most are truly salt-induced DMMs that appear across diverse genotypes and respond to salt exposure in a consistent manner.

Salt induces different changes to the DNA methylation of leaves and roots

The vast majority of salt-induced, dose-insensitive DMMs were also organ-specific (Figure 3). One plausible explanation for the relative paucity of DMMs shared between roots and leaves can be taken from the ability of the crop to effectively compartmentalize excess Na+ in the roots and inhibiting its transport to the leaves [49]. This response gives rise to very different Na+ environments in the two organs, with Na+ concentrations being far higher in the roots than the shoots. We reason that the dose-insensitive DMMs identified in the present study are all likely to response to low salt thresholds, and so appear in all salt treatments across the titration. However, because the internal Na+ environments are very different between roots and leaves [50], the dose-insensitive DMMs found in the two organs are highly likely to have very different induction thresholds (presumably higher in the roots). Viewed in this way, it is unsurprising that the abundance and scaling of leaf and root DMMs were so different, and that so few dose-insensitive DMMs were shared by both organs. Indeed, this divergence is in accordance with the known physiological response of the species to saline exposure of the roots, and implies that both sets of DMMs would therefore provide a robust indication of exposure to salt. Certainly, it has been widely reported that salinity imposes extensive, genome-wide modification of the DNA methylation patterns, with more methylation changes being reported in leaves when compared with roots [25-27,33,50-53]. This trend accords with the finding here of a higher number of stable salt-induced DMMs in leaves than in roots (5,593 vs. 528 DMMs respectively; Figures 1 and 3). In rice, about 50% of CCGG site methylation were altered under salt stress in leaves, whereas less than 15% changed in roots [25]. Taken at face value, the detection of more salt-induced methylation changes in leaves than in roots appears counterintuitive, since roots are the primary organ of contact with the salt stress. That said, the scale of the change in methylation was greater in roots than in leaves (Figure 2), suggesting that although salt evokes change in fewer loci, the effect on these sites is greater. Provided these changes are associated with concurrent changes to expressions of key genes involved with responses to salt stress, these observations can accommodate root-specific epigenetic responses to saline environments, while plants are undergoing osmotic stress and salt toxicity [8,54]. Should at least one of these processes be focused on repressed transport of Na+, then more mild ion accumulation in the leaves will slowly increase stress [5,55] at a lower level because of 'leakiness' of the system, evoking a widespread but more measured response.

Previous studies have reported that the overall level and direction of methylation flux in response to salinity varies according organ types, with a tendency towards hypomethylation in roots and hypermethylation in leaves [7, 25-27,33,47]. Similarly, we found that that salt induces net hypomethylation in roots and hypermethylation in leaves (Figure 2a-c). However, the proportion of *de novo* methylation and demethylation events varied in the same manner in both roots and leaves, with a prevalence of hypomethylated events in both organs, albeit at different frequencies (Figure 1). It is possible that divergence between our findings and those of previous studies [7, 25-27,33,47] may simply be a feature of the crop. However, it is also possible that the trend towards hypomethylation is a more general one and our findings diverge because of methodological differences in the present work such as 1) the high-through put sequencing used to generate methylation profiles, 2) the level of stringency in selecting DMMs (FDR < 0.01), and 3) the diversity of barley varieties used in this study, to account for genotype-dependent DNA methylation [25-27]. Most studies of salt-induced DNA methylation have relied on MSAP analysis of a single variety to assess flux in DNA methylation [7, 25-27,33,47]. However, MSAP generates anonymous markers and lacks resolution in showing whether there is a gain or loss of methylation in markers [42].

Beside organ-specific methylation levels, there was a progressive increase in both the salt concentration and the abundance of salt-related DMMs in leaves, but not in roots (Figure 1). This gradual additive response to salt stress in leaves is concordant with a previous study, showing that salt concentrations correlated with differential DNA methylation in rapeseed [33]. Roots seemingly lacked this relationship, possibly because of the low number of loci involved but possibly also because DNA fragments naturally at high salt concentrations [33,56-58] affecting the effectiveness of the msGBS approach. DNA degradation may have occurred at salt concentrations above 150 mM NaCl, leading to a decrease in salt-induced DMMs in roots. High salinity-induced DNA fragmentation have previously been reported in Arabidopsis [56], onion [57], rapeseed [33] and barley [58], suggesting that this is not an isolated phenomenon. Resolution of these alternatives requires further investigation.

Salt-induced changes in DNA methylation may influence gene regulation

DNA methylation is modulated in the genome in three ways: *de novo* methylation (hypermethylation), methylation maintenance, and methylation removal (hypomethylation) [59]. Modification of DNA methylation in response to stress is hypothesised to be at least partially directed to specific genomic regions where DNA methylation status influences the expression of stress-response genes [18,30,60,61]. Our results provide limited support for this assertion. We found salt-induced DMMs in barley primarily clustered around repeat regions (Figure 5ab) but also some genes (Figure 5cd), with most DMMs occurring within 1 Kb of repeats and within gene-bodies. Overall, we found that dose-insensitive salt-induced DMMs appear more common in sites that could facilitate perturbation in the expression of genes that hypothetically could be part of a molecular response to salinity, as reported elsewhere [61]. There is evidence from previous studies suggesting that salt-induced DMMs can play an important role in evoking metabolic differences between seedlings growing under control and saline conditions [25,26,29,34,60-62], although the markers found in these works were either few in number or else identified from a single genotype. The provision here of a robust list of consistent salt-responsive DMMs therefore provides a useful starting point from which to gather a more holistic picture of DNA methylation-mediated regulation of molecular responses to salt exposure.

Proof of a functional link between change of methylation status in these DMMs and associated alteration in the expression of proximal stress response genes is beyond the scope of the current study. Nevertheless, there are several grounds for reasoning that at least some of the markers identified here will indeed be functionally important. Certainly, others have argued that the close proximity of DMMs relative to the target genes is at least one requirement for such a relationship [19,63-65]. Viewed in this context, the observed clustering of DMMs around Un-Translated Regions (UTRs) and exons (Figure 6) is at least

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consistent with salt-induced DMMs mediating a functional response to the stress. Others have shown a high frequency of salt-induced DMMs in gene extremities (towards 5`UTR and 3`UTR) can influence gene regulation through 5`UTRs' and 3`UTRs' closed-loop regulation systems, which generate inactive transcripts [66,67]; or through independent gene regulation by each UTR type [68]. Karan et al. [25] similarly observed that salt-induced DNA methylation changes generally occurred in exon and UTR regions and could affect diverse biological functions in the plant. There is also a strong body of evidence suggesting that gene-body methylation in general can affect gene expression [19,54,64], by enhancing or inhibiting transcription and translation processes [67,68].

It has been claimed that, of all cytosine contexts, only ^{m}CG methylated occurs within gene-bodies [64,69-71]. Ours and previous results [72] do not support this stance, with non-CG methylation such as ^{m}CCGG found frequently in transcribed regions from DNA isolated from both leaves and roots of barley (Figure 5cd). It is, however, still open to question whether these markers, like ^{m}CG, play a role in regulating gene expression [73]. We also observed salt-induced DMMS associated with tRNA genes (Figure 6d), perhaps inviting further study of the suggestion of a role for methylation dependent regulation to support the RNA quality control system and protein synthesis [74-76].

Salt-induced DMMs correlate with stress related genes

There is circumstantial support to argue that at least some of the DMMs identified here may play a functional role in the expression of salt-response genes. Salt stress in barley alters the expression pattern of genes involved in a diverse range of physiological and regulatory pathways [3,9]. Given that salt-induced DMMs have the potential to regulate gene expression, the functions of differentially methylated genes were explored for possible correlations with stress responsive genes. The correlation of DM genes with GO terms that are related to plant responses to stress, such as "negative regulation of signal transduction", "photosynthesis", "response to osmotic stress" and "ion transmembrane transport", is at least consistent with the possibility that salt-induced DMMs target genes could play active functions in the plant's response to salt, and in broad accordance with previously expression studies of salt response [29,77-79]. In addition, some of the DM genes identified here were enriched for GO terms such as hydrolase activity, oxidoreductase activity, nucleic acid binding, and translation factor activity (Figure 7; Supplemental Data Set S3). This agrees with similar reports of differentially methylated genes associated with salt stress in rice [25,26]. This concordance of DM gene idententity between species could be taken to imply that a functional role is more likely. The present study also revealed differential DNA methylation of genes implicated in organophosphate biosynthesis (Figure 7). Should the change in methylation state alter expression of this gene, it would accord with previous studies showing that salt stress induces an increase of the amount of intra-cellular organophosphate solutes such as di-myo-inositol-phosphate, Inositol (1,4,5) trisphosphate, b-mannosylglycerate, b-mannosylglycerate and Glutamate [80,81]. Furthermore, it was reported that salinity induced inorganic phosphate toxicity when Pi exceeds 0.10 mM in the substrate [82,83]. This salt-induced phosphate toxicity may arise from excess of phosphate not only due to P uptake, but also salt-induced increase of intracellular organophosphate

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solutes [81,82]. However, it is important to recognise that the presence of DMMs near a gene may be an indication of responsiveness to salt stress, but not provide sufficient evidence of a functional role of DNA methylation in the regulation of the gene [21,65]; gene expression analysis is required to assess the link between DNA methylation and gene activity under salt stress.

Difficulty in extrapolating a functional link is highlighted by the fact that only seven of the genes previously reported to be differentially expressed in roots [3] were also differentially methylated under salt stress here (FDR < 10%, Table 4). This result may imply that few of the marks found here are functionally important or alternatively be attributable the use of different biological samples for methylation profiling and gene expression analyses, different growing conditions [3], to the partial coverage of the barley reference genome used here [4] or to the possibility of biased by salt-induced DNA degradation [33,56-58].

Conclusions

To our knowledge, this study has provided the most comprehensive set of robust leaf and root epimarkers to indicate the exposure of barley to salt stress. These markers were conserved in both identity and direction across five diverse genotypes, biological replicates and all salt concentrations used. The leaf markers have potential value as epigenetic sentinels of the exposure of individual plants to soil salt stress. Viewed collectively, the root and leaf markers provide a useful start point from which to assemble a more comprehensive picture of the functional role of DNA methylation in facilitating the plastic molecular response of barley to this important stressor.

4. Materials and Methods

4.1. Plant material and stress treatment

Five diverse spring barley varieties were used in this investigation: Barque 73, Flagship, Hindmarsh, Schooner and Yarra. Seeds were kindly provided by the Salt Focus Group at the Australian Centre for Plant Functional Genomics (ACPFG, Adelaide, South Australia). The experiment was designed in randomized blocks of five replicates and four salinity treatments: control (0), 75, 150 and 200 mM NaCl.

Seeds were germinated, and seedlings grown in 3.3 L free-draining pots, placed on saucers, containing 2915 g of growth substrate (50% UC (University of California at Davis) potting mix, 35% coco-peat, and 15% clay/loam (v/v)). The five barley varieties were sown per pot and variety positions were randomized in each pot to minimize block effect. Two seeds were sown per variety and thinned to one seedling 8 days after sowing. Salinity treatments were applied 10 days after sowing in four increments over 4 consecutive days, to minimise osmotic shock [84]. The required amount of NaCl for each salt concentration was calculated based on the substrate soil dry weight and the target gravimetric water content of 16.8% (g/g) [84]. At the time of salt application, the water content reached 26.4% and dropped down to the final concentration through evapotranspiration. Pots were watered to weight every 2 days to maintain the target gravimetric water content (16.8% (g/g)) [84] until sampling.

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This 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 and temperature was set at 22°C/15°C (day/night).

4.2. DNA extraction

At day 11 after the first salt stress imposition to barley seedlings (21 days after sowing, three leaves stage), 50 mg samples were collected from middle sections of the 3rd leaf blades and roots. In total, 200 samples were collected (five varieties, four treatments and two tissue types), and were snap frozen in liquid nitrogen, then stored in a -80°C freezer until needed for DNA extraction. Prior to DNA extraction, frozen plant material was disrupted in a bead beater (2010-Geno/Grinder, SPEX SamplePrep®, USA). Genomic DNA was isolated using a Qiagen DNeasy kit following 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 ms-GBS library preparation.

4.3. Methylation Sensitive genotyping by sequencing (ms-GBS)

The methylation-sensitive genotyping by sequencing (ms-GBS) was performed using a modified version [44,45] of the original GBS technique [85,86]. Genomic DNA was digested using the combination of a rare cutter, *Eco*RI (GAATTC), and a frequent, 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, 8U of HF-*Eco*RI (High-Fidelity) and 8 U of *Msp*I (New England BioLabs Inc., Ipswich, MA, USA). The reaction was performed in a BioRad 100 thermocycler at 37°C for 2 hours, 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 (NEB T4 DNA Ligase #M0202) in a total volume of 40 µl. Ligation was carried out at 24°C for 2 hours 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 (#A63880, Beckman Coulter, Australia) at a ratio of 0.85 and 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 reactions (25 µl each) containing 10 µl of library DNA, 5 µl of 5x Q5 high fidelity buffer, 0.25 µl polymerase Q5 high fidelity, 1 µl of each Forward and Reverse common primers at 10 µM, 0.5 µl of 10 µM dNTP and 7.25 µl of pure sterile water. PCR amplification was performed in a BioRad T100 thermocycler consisting of DNA denaturation at 98°C (30 s) and ten cycles of 98°C (30 s), 62°C (20 s) and 72°C (30 s), followed by 72°C for 5 minutes. 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

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instructions. Bead-captured fragments were eluted in 35 µl of water and 30 µl of elution were collected in a new labelled microtube. Next, libraries were 125bp paired-end sequenced in an Illumina HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA) at the Australian Genome Research Facility (AGRF, Melbourne node, Australia).

4.4. Data analysis

The ms-GBS data was analysed following a workflow requiring bioinformatics tools in both Linux bash shell and R environments. Fastq files from the Illumina sequencing platform were first de-multiplexed and checked for read quality by the sequencing service provider, reporting read quality encoded in symbolic ASCII format in Phred-like quality score + 33. Only fragments with at least 95% of the reads having Phred > 25 were retained. Reads that did not have a barcode were put into undetermined files and removed from any downstream analyses. Prior to demultiplexing, Illumina adaptor sequences used for library construction, were also removed. The second step consisted of preparing the reads for alignment in the barley reference genome. As this was pair-end read sequencing data, both strands were merged together in a single read, using the module *bbmap* in bash. Merged reads were next aligned to the barley reference genome downloaded from the Ensembl database (http://plants.ensembl.org/Hordeum_vulgare/). This required the module *bowtie/2-2.2.3* to build a bowtie2 index for the barley genome, and the module *samtools/1.2* to perform alignments. As paired reads were merged into single reads, therefore only those that overlap were retained, to allow proper map. This alignment step yielded bam files containing only reads that matched with the reference genome. Next, a read count matrix was generated using only marker sequence tags that matched with *Msp*I cut sites on known chromosomes (1H to 7H) and those on contigs were discarded. This count matrix was then used as source data to perform subsequent analyses using R packages.

4.5. Salinity induced differentially methylated markers in barley

Alteration of DNA methylation in barley seedlings exposed to salinity was assessed in ^{m}CCGG contexts by the use of *Msp*I during sample preparation. Differentially methylated markers (DMMs) were identified using the package *msgbsR* developed by Mayne et al [87] (https://github.com/BenjaminAdelaide/msgbsR, accessed on 26/08/2016), fitting a generalised linear model to the design, with the trimmed mean of M-values normalisation option (TMM). Then, Benjamini-Hochberg method was used for P-values. Then, DMMs were selected based on FDR < 0.01 for differences in read counts per million between salt-free control and salt treatments (75 mM, 150 mM or 200 mM NaCl), with at least 1 count per million (CPM) reads. To obtain robust salt-induced markers, we selected DMMs that were conserved in all barley genotypes, and present in at least 20 samples per treatment. The *log*FC (logarithm 2 of fold-change in CPM reads) was computed to evaluate the intensity of salt treatment-induced alteration of DNA methylation and infer whether the change was a *de novo* methylation or demethylation event. This approach of 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 restriction products for that locus [39] and therefore reduce its number of CPM reads.

4.6. Distribution of salt-induced DMMs around genomic futures

To determine whether there was a correlation between salt-induced DNA methylation and genomic features in barley, the distribution of DMMs was assessed around genes and repeat regions as defined in the Ensembl database (http://plants.ensembl.org/biomart/martview/). This was done by mapping stable salt-induced DMMs with repeats and genes in the barley reference genome. Then, we tallied the number of DMMs within genomic features (repeats, genes, exons) and per 1 Kb bins within 5 Kb flanking regions both up- and down-stream [47,88], using the shell module *bedtools /2.22.0* [89]. The same procedure was repeated to estimate the number of DMMs around exons and UTRs of differentially methylated genes, and tRNA genes.

4.7. Gene ontology of differentially methylated genes

Genes within 5 Kb of a DMM were referred to as differentially methylated genes (DM) genes. These genes were used for gene ontology analysis, to investigate whether salt-induced changes in DNA methylation correlated with salt responsive genes. DM genes were grouped in hypermethylated and hypomethylated genes per organ (leaf or root), which were next used separately for GO terms enrichment, using two R packages: *GO.db* and *annotate* [90,91]. Significant GO terms were selected based on Bonferroni adjusted P-values [92] at a significance threshold of 0.01 and a total GO enrichment of DM and non-DM genes at least equal to 10. The results of GO analysis were visualized in treemaps generated in REVIGO [93].

4.8. Gene expression and ontology analysis of root transcriptome

We further investigated whether differentially methylated genes were known to be differentially expressed in the plant. To do so, we used as an exemplar, a dataset of root transcriptome of two barley varieties (Clipper and Sahara-3771) grown under salt stress (100 mM NaCl) and control conditions [3]. The raw data was downloaded from https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-4634/, and samples from the root maturation zone, as defined by the authors [3], were used. The data contained four biological replicates of two varieties and two salt treatments (control and 100 mM NaCl), for a total library size over 390 million reads. A quality control was performed on these reads, which were then merged to form a single large *fastq* file for each sample. Merged read pairs were trimmed using *AdapterRemoval* [94], followed by a second round of quality control.
After alignment using *hisat2-2.0.4* in bash [95], salt-induced differential gene expression analysis was performed, using a custom GTF file from Ensembl and created by the tool *StringTie 1.3.1c* [96]. This GFF file was restricted to transcripts on the known chromosomes (1H to 7H). Read counts were assigned to genes in the GTF file using *featureCounts v1.5.1* [97], and loaded as *DGEList* object in R. As the data contained paired end reads, the parameters were set to only count fragments (i.e. template molecules), instead of individual reads. This dataset was next filtered to keep only genes with CPM > 0.5 in at least four samples. Gene transcripts passing these conditions and present on chromosomes 1H to 7H, were retained for differential expression analysis.

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Before comparing treatments, the dataset was explored for sample variability using the MDS plot. Differential gene expression was then estimated using the *lmFit* function in *limma::voom*, a gene-wise linear model [98], and differentially expressed genes were defined as having an absolute fold-change > 2, with an FDR adjusted P-value < 0.05. Differentially expressed genes were first used "as are" for gene ontology analysis as described above (previous section). Differentially expressed genes were then assessed for proximity to salt-induced DMMs within 5 Kb in both directions. Genes found in this proximity with DMMs and referred to as differentially methylated DE genes, were used for another GO analysis. Results of these GO enrichments were visualized in treemaps produced in REVIGO [93], to show the main GO representatives.

Supplementary Materials: The following are available online at www.mdpi.com/link. Supplemental Data Set S1. List of stable salt-induced differentially methylated markers in leaves and roots. Supplemental Data Set S2. List of salt-induced differentially methylated genes. Supplemental Data Set S3. Annotation of salt-induced differentially methylated genes in barley leaves and roots. Supplemental Data Set S4. List and annotation of salt induced DE genes and differentially methylated DE genes in roots of Clipper and LR Sahara.

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