Assessment of stress-induced and developmentally-induced DNA methylation changes in barley (Hordeum vulgare L.)

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Moumouni Konaté

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ஓ *This thesis is dedicated with love to my mother, Tene Mema Cisse and the memory of my dad Zakaria Konate who passed away while this thesis was under review* ୧

Thesis abstract

DNA methylation is involved in both plant development and adaptation to environmental stress. Changes in DNA methylation can affect the expression of genes that are important for both plant tissue differentiation and stress response. Characterisation of tissue and stress specific methylation markers generates an invaluable tool for epiallele discovery that can be used for future functional and crop improvement studies.

We used barley as a plant model, and salinity as a stress model, to study methylation markers that discriminate the plant tissues and that are specific to salinity stress. This choice presented the advantage of using a crop plant with a reference genome sequence, which allows for genomic analyses; and an abiotic stress factor that is relatively easy to control.

Nine barley varieties subjected to mild salt stress (75 mM NaCl) were studied for their response to the stress by measuring phenotypic traits, such as biomass, yield and ion accumulation in the leaves. Then, Methylation Sensitive Amplified Polymorphisms (MSAP) were used to analyse changes induced by salt stress in their DNA methylation profiles, which were tested for correlation with the phenotypic data from the same plants. This study revealed that, although the MSAP approach can detect differentially methylated markers induced by a mild salt stress in barley, it presented a limitation in the number of differentially methylated markers (DMMs) detected. This study also revealed that the detection of DMMs by MSAPs was significantly influenced by genotypic differences among varieties. Finally, analysis of the epigenetic variability detected by MSAP indicated that microclimatic differences experienced by different plants in the study contributed to what was previously considered to be stochastic variability.

The results from the MSAP suggested an alternative approach was required to identify DMMs that are conserved across barley varieties. Using the high throughput DNA sequencing approach methylation-sensitive genotyping by sequencing (ms-GBS), we detected thousands of salt-induced DMMs and similar numbers of tissue-specific DMMs. Ms-GBS-generated DMMs were potentially universal, since they were conserved in five barley varieties used in the study. Sequence analysis of the ms-GBS generated DMMs indicate that both tissue-specific and salt-

induced changes in DNA methylation happen preferentially in repeat regions, but also target other gene types, such as protein-coding and Transfer RNA genes. Ontology analysis of differentially methylated protein-coding genes revealed that many are likely to play a role in stress response and organ-specific functions. However, further studies, including expression analyses, are needed to link gene methylation to gene expression.

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Abbreviations

µl	microlitre(s)
µM	micromolar
ACPFG	Australian Centre for Plant Functional Genomics
AFLP	Amplified Fragment Length Polymorphism
AGRF	Australian Genome Research Facility
ANOVA	Analysis of variance
AP2/DREB	Activating Protein 2 / dehydration-responsive element-binding
bp	Base pair(s)
b-ZIP	Basic Leucine Zipper Domain
BSA	Bovine Serum Albumin
cm	Centimetre(s)
DAS	Day(s) after sowing
DE	Differentially expressed
DF1, DF2,	Discriminant Factor 1, 2
DM	Differentially Methylated
DMM	Differentially Methylated Marker
DNA	deoxyribonucleic acid
dNTP	Dinucleotide tri-phosphate
FDR	False Discovery Rate
GO	Gene Ontology
HCA	Hierarchical Cluster Analysis
HKT	High affinity potassium transport
HNO3	Nitric acid
ICRISAT	International Crop Research Institute for Semi-Arid Tropics
INERA	Environment and Agriculture Research Institute (Burkina Faso)
K^+	Potassium ion
Kb	Kilo base pair(s)
L	Litre(s)
logFC	Logarithm 2 of fold-change
LSD	Fisher's Least significant difference
m	Metre(s)

mg	Milligrams(s)
ml	Millilitre(s)
mM	Millimolar(s)
MSAP	Methylation-Sensitive Amplification Polymorphism
ms-GBS	Methylation-Sensitive Genotyping By Sequencing
Na^+	Sodium ion
NaCl	Sodium Chloride
NEB	New England Biolabs
ng	Nanogram(s)
NHX	Na+/H+ exchanger
Pa	Pascal(s)
PAR	Photosynthetic active radiance
PC-LDA	Principal Components – Linear Discriminant Analysis
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
Phi-ST	Phi Statistics
pmol	Pico Mole(s)
REVIGO	Result Visualisation of Gene Ontology
RGB	Red Green Blue
RH	Relative humidity
SEM	Standard Error of the Mean
SVP	Saturated Vapour Pressure
TE	Transposable elements
TES	Transcription End Site
TSS	Transcription Start Site
UC Davis	University of California at Davis
UTR	Untranslated Region
v/v	volume/volume
VPD	Vapour pressure deficit
w/w	weight/weight
WRKY	a protein starting with amino-acids Tryptophan (W)- Arginine (R)- Lysine (K)- Tyrosine (Y).

Chapter 1: Literature review and research aims

1.1.   Introduction

Epigenetics has been subject to mounting curiosity in the scientific community, in recent years. This mounting interest in epigenetics is not only for simple scientific curiosity. Genetics alone can hardly explain a number of biological facts such as the differences in the quality of clonally propagated crops (Javierre *et al.*, 2010, Rodriguez Lopez & Wilkinson, 2015), stress priming (Conrath, 2011, Luna *et al.*, 2012) and many other circumstances of differential gene expression in plants that have identical genomes (Chandler *et al.*, 2000). Moreover, cell differentiation and plant developmental processes are dependent on epigenetic regulation (Cokus *et al.*, 2008, Ruiz-García *et al.*, 2005, Zilberman *et al.*, 2007), suggesting, without overemphasis, that genome expression requires epigenetic control.

The importance of epigenetic mechanisms in plant genomics has raised much attention in the study of epigenetic variants, of which DNA methylation appears to be the best studied (Bossdorf *et al.*, 2010, Boyko & Kovalchuk, 2008, Brandeis *et al.*, 1993, Chan *et al.*, 2005, Choy *et al.*, 2010, Doerfler, 1983, Finnegan *et al.*, 2000). These remarkable studies allow us to understand how DNA methylation markers occur, and their implication in biological processes, including responses to stressful conditions (Alvarez *et al.*, 2010, Bossdorf *et al.*, 2010, Boyko & Kovalchuk, 2008). However, there is still an important gap in the knowledge concerning the stability of DNA methylation markers in plants. Plant methylomes are unstable during development (Boyko & Kovalchuk, 2011, Brandeis *et al.*, 1993). This instability has been attributed to factors such as changing environmental conditions and developmental stages, due to continuous readjustment of the plant methylome (Boyko & Kovalchuk, 2011, Brandeis *et al.*, 1993). This made it difficult to untangle DNA methylation changes due to a specific condition (e.g. a stress, or age), from all other factors susceptible to altering methylation in time and space.

DNA methylation changes due to specific condition such as stress, have been reported before (Ferreira *et al.*, 2015, Karan *et al.*, 2012, Tan, 2010, Tricker *et al.*, 2012), leading to the thought that any single methylation change should have a trigger. Therefore, reporting a plant's methylation profile may show a pattern that correlates with the inducing factor. However, many instances of unexplained changes in DNA methylation were reported, suggesting that some methylation markers are just random (Karan *et al.*, 2012, Tricker *et al.*, 2013a, Vogt, 2015). This view was further blurred by the fact that, apart from DNA methylation, there are other epigenetic mechanisms (such as histone variants, or interfering RNAs) that contribute to plant response to stress. Therefore, without a clear association between differentially methylated markers (DMMs) with specific conditions, it is difficult to decipher the functional significance of DNA methylation in plants (Ferreira *et al.*, 2015). This requires both appropriate experimental settings and appropriate data analyses, to unambiguously detect DMMs and eventually attribute them with specific functions.

This literature review will first cover the concept of epigenetics, including epigenetic mechanisms, with a special focus on DNA methylation markers and functions in plant biology. After a brief description of epigenetic profiling methods, salt-induced DNA methylation in plants will be presented, before introducing the project objectives.

1.2. Concepts and mechanisms of epigenetics

Genetic information is encoded in the DNA sequence of the genome (Avery *et al.*, 1944). However, individuals with a common genome, such as plant cuttings and monozygotic twins, can develop different phenotypes, especially when they are exposed to different environmental conditions (Kaminsky *et al.*, 2009, Levenson & Sweatt, 2005). This observation suggests that the information stored in the DNA is utilised circumstantially, depending on the broad environmental conditions (internal and external). A remarkable example of a phenotypic trait controlled by environmental factors is temperature-dependent sex determination in some reptiles and fish species (Ellison *et al.*, 2015, Griffiths, 2001, Pieau *et al.*, 1999). In these species, the offspring's sex is not molecularly defined at fertilisation, but rather by the temperature at which the egg is incubated. In recent years, a number of traits in diverse organisms across all kingdoms have been found to be associated with molecular mechanisms that do not affect the underlying nucleotidic sequence, namely epigenetic mechanisms (Berger *et al.*, 2009, Gourcilleau *et al.*, 2010, Riggs, 1975, Rodriguez Lopez & Wilkinson, 2015).

The term epigenetics evolved from the original concept of "epigenesis", coined by Aristotle to indicate that the development of organisms results from a "series of causal interactions between various components" (Tsaftaris *et al.*, 2003). Conrad Waddington subsequently coined the term "epigenetics" in the 1940s, to show that other molecular factors above the genotype are implicated in defining phenotypes during development (Waddington, 2012). Riggs (1975) defined epigenetics as 'heritable factors affecting development or gene functions that are not associated with a DNA nucleotidic sequence'. This definition currently includes all modifications that occur in genomic structures, affecting gene expression and subsequent phenotype, be these transient (Boyko & Kovalchuk, 2008) or stable (Calarco *et al.*, 2012). Such modifications are controlled by a number of interdependent mechanisms including histone modifications, DNA methylation, and small RNA-interference (Sawan *et al.*, 2008, Vanyushin, 2006).

1.2.1. Histone modifications

Histones are proteins that together with DNA form complex structures referred to as nucleosomes. Each nucleosome is composed of eight histones (hence octamer), and rolled with 147 bp of DNA (Levenson & Sweatt, 2005). A series of nucleosomes makes up chromatin. Histones are highly alkaline nuclear proteins which determine the compactness of the DNA within the nucleus and provide a platform for the regulation of gene transcription (Levenson & Sweatt, 2005). Histone modifications (Figure 1.1a) shape chromatin conformation to euchromatin, relaxed and open to transcription factors, and heterochromatin, which is compact and blocks transcription (Levenson & Sweatt, 2005, Zhu *et al.*, 2008). This structural modification involves the so-called *histone code*, such as the diverse modifications of amino-acids in the histone tail (Tsaftaris *et al.*, 2003, Levenson & Sweatt, 2005). The most common modifications occur in the lysine residues, which may acquier diverse molecules, such as acetyl or methyl groups (acetylation and methylation, respectively) (Levenson & Sweatt, 2005). Histone modification may also occur in other amino-acids such as phosphorylation in serine and methylation in arginine, due to the acquisition of a phosphorus and a methyl group, respectively. These modifications take place following environmental and intracellular signalling (Levenson & Sweatt, 2005), and are profoundly involved in modulating the expression of genes responsive to environmental conditions. For instance, the acetylation of lysine 9 of histone 3 (H3K9) is known to regulate many biological processes, including meiotic DNA double-strand break

formation, which favours recombination in fission yeast (Yamada *et al.*, 2013), the activity of the Flowering Locus C gene (*FLC*) to allow plant vernalisation (Bastow *et al.*, 2004, Zhu *et al.*, 2008), and the activity of the sirtuin-like gene *OsSRT1* in rice, which regulates the expression of many stress and metabolism related genes (Zhong *et al.*, 2013b). In the same way, phosphorylation of a serine residue was reported to regulate cotton *Di19* (*drought-induced protein 19*) activity during high salinity stress (Qin *et al.*, 2016) and there are many other examples of alterations of gene expressions due to histone modification.
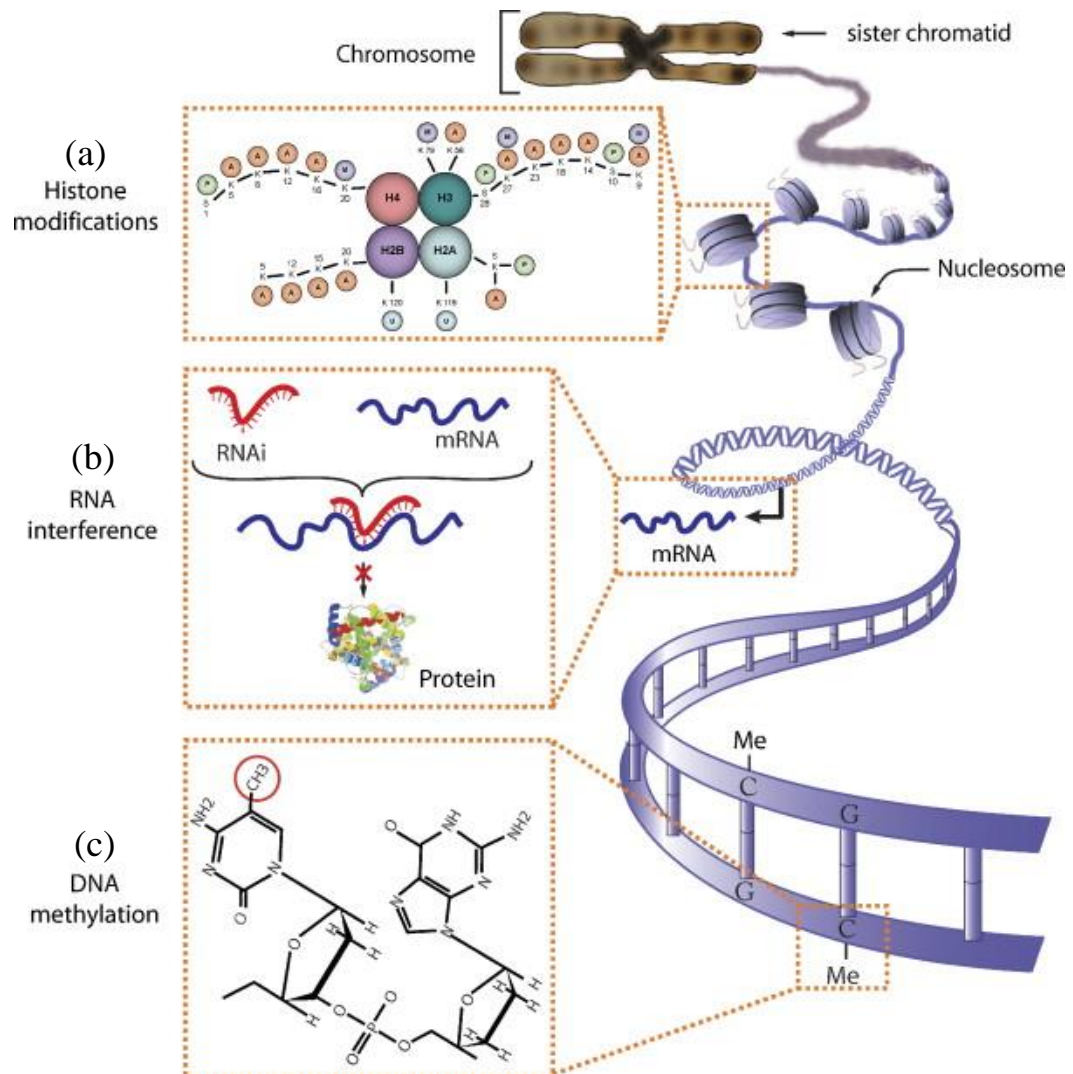
Figure 1.1: Different types of epigenetic mechanism.
(a) Histone modifications, (b) RNA-mediated gene silencing and (c) DNA methylation constitute three distinct mechanisms of epigenetic regulation. DNA methylation is a covalent modification of the cytosine (C) that is located 5′ to a guanine (G). Histone (chromatin) modifications refer to covalent post-translational modifications of N-terminal tails of four core histones (H3, H4, H2A, and H2B) (Sawan *et al.*, 2008).

1.2.2. Small interfering RNA

RNA interference (Figure 1.1b) is an essential mechanism of gene regulation in eukaryotes (Poulsen *et al.*, 2013). It refers to a process in which small interfering RNAs (siRNA) align with target messenger RNA (mRNA) after transcription to block their translation into proteins (Lewsey *et al.*, 2016). The biogenesis of siRNA, 20-25 nucleotides in size, is instigated by RNA-dependent RNA polymerase factors, DICER-LIKE 3 (DCL3) and Argonaut family proteins (i.e. ARGONAUT 4, AGO4) (Law & Jacobsen, 2010, Zilberman *et al.*, 2003). Molecules of siRNA are particularly active in the plant's defence system, by suppressing the multiplication of abnormal cells and foreign nucleic acids from attackers, such as viruses (Baylin, 2005, Fire *et al.*, 1998, Poulsen *et al.*, 2013). They perform this role as part of the post-transcriptional gene silencing (PTGS) machinery, acting as guides for the degradation of specific mRNAs, thereby causing gene repression (Poulsen *et al.*, 2013).

Transposable elements (TEs) in the genome are often targeted by siRNAs to trigger DNA methylation that can fine-tune the expression of adjacent genes (Slotkin *et al.*, 2009, Wei *et al.*, 2014). One such example was control of the expression of the *Arabidopsis thaliana HIGH-AFFINITY K⁺ TRANSPORTER 1* (*AtHKT1*), reported to be controlled by an siRNA, targeting a region 3.9 Kb upstream of the gene (Baek *et al.*, 2011). In this case, small RNAs directed non-CG methylation at this region to maintain a methylation state required for *AtHKT1* expression. This RNA-directed DNA methylation (RdDM) is instigated by the alignment of siRNA with homologous DNA sequences in the genome, thereby providing a substrate for cytosine methyltransferases to act upon (Bender, 2004). Required enzymes include the DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) (Cao *et al.*, 2003) and two plant-specific RNA polymerases (POLYMERASE IV and POLYMERASE V) (Law & Jacobsen, 2010). Additionally, RNAs may also trigger histone tail methylation (Popova *et al.*, 2013, Zilberman *et al.*, 2003), so that siRNAs are able to program both chromatin dependent gene silencing and DNA methylation dependent gene silencing. Furthermore, it has been suggested that siRNAs can also regulate genes that are involved in the plant's spatio-temporal development, nutrition and response to stress (Borsani *et al.*, 2005, Khraiwesh *et al.*, 2012).

1.2.3. DNA methylation

DNA methylation is proposed as the best characterised (Cao & Jacobsen, 2002, Cokus *et al.*, 2008, Zilberman *et al.*, 2007) and the most commonly occurring epigenetic mechanism in plants (Choi & Sano, 2007, Doerfler, 1983, Dowen *et al.*, 2012). DNA methylation is a chemical modification of the DNA caused by the addition of a methyl group in the cyclic carbon-5 of cytosine (Figure 1.1c). 5-methylcytosine is present in plants and animals (Law & Jacobsen, 2010), but is much richer and more complex in plants (Schmitz *et al.*, 2013, Vanyushin, 2006). This may be partly attributable to the fact that plants are naturally sessile and have limited capacity to avoid environmental insults imposed on them (Lang-Mladek *et al.*, 2010). It is speculated that 30-50% of cytosines in plant genomes are methylated (Doerfler, 1983). However, the level of DNA methylation is highly variable within and among species (Zhong *et al.*, 2009), and in time and space in the same individual organism (Kitimu *et al.*, 2015, Rodríguez López *et al.*, 2012, Sha *et al.*, 2005).

Cytosine methylation takes place primarily in symmetric CG and CHG sequences (where H is a nucleotide other than G) (Finnegan *et al.*, 2000), but may also occur at non-symmetric CHH sites (Cokus *et al.*, 2008, Steward *et al.*, 2002). This reversible change in DNA methylation is generally triggered by internal or external stimuli (Grativol *et al.*, 2012), and is correlated with the activity of a methyltransferase (Bender, 2004). Distinct methyltransferases catalyse methylation in different cytosine contexts in the genome (Bender, 2004, Finnegan *et al.*, 1996) or in histones (He *et al.*, 2015). The METHYLTRANSFERASE 1 (MET1) family plays little role in *de novo* methylation but rather seems to maintain CG methylation (Kankel *et al.*, 2003, Lister *et al.*, 2008). However, non-CG methylation is maintained by the DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) (Cao *et al.*, 2003, Cokus *et al.*, 2008) and the CHROMOMETHYLASE 3 (CMT3) (Lister *et al.*, 2008). The chromomethylase protein family is unique to plants and propagates DNA methylation preferentially in transposons and heterochromatin (Lin *et al.*, 2015, Lindroth *et al.*, 2001). The expression of methyltransferases is controlled by multiple genes (Genger *et al.*, 1999) and is responsive to ambient conditions (Steward *et al.*, 2000). The loss of capacity to synthesise methyltransferase may lead to some phenotypic and developmental abnormalities, such as plant growth, fitness and delay in flowering time, due to reduced DNA methylation levels (Bossdorf *et al.*, 2010, Finnegan *et al.*, 1996). Hence, many biological functions in plant existence have been attributed to DNA methylation.

1.3. Biological functions of DNA epigenetic variations

DNA methylation is involved in gene repression in such a way that a remarkably high negative correlation was found between the level of methylation and the level of gene expression (Aceituno *et al.*, 2008, Boyko & Kovalchuk, 2008, Zilberman *et al.*, 2007). This effect of DNA methylation results from competition with transcription factors (TFs) to modulate gene activity (Domcke *et al.*, 2015). Despite common agreement that DNA methylation is repressive of gene activity, instances of moderate influences of stress-induced DNA methylation on expression, such as that observed in the *Arabidopsis* transcriptome (Aceituno *et al.*, 2008), suggest that the functional significance of DNA methylation is yet to be entirely deciphered (Ferreira *et al.*, 2015, Jones, 2012). This difficulty is due to the dependence of the effect of DNA methylation on several factors such as sequence context, tissue types, and environment (Aceituno *et al.*, 2008), each of which may lead to a different functional consequence. Therefore, modulating contextual gene expression is not the sole role of DNA methylation, and the implications of DNA methylation in transcriptional gene silencing is becoming more and more nuanced when compared with earlier claims (Jones, 2012, Suzuki & Bird, 2008). Nonetheless, a large body of work has linked epigenetic variation to diverse biological processes such as development (germination to flowering), responses to stress, and genome maintenance among others (Ay *et al.*, 2014, Brandeis *et al.*, 1993, Ishida *et al.*, 2008, Zhu *et al.*, 2008). This functional importance of DNA methylation is discussed below.

1.3.1. DNA methylation as a developmental script

Despite the presence of identical genomic DNA across all cells of the plant, the phenotypic outcome can diverge substantially from one tissue to another. There must be mechanisms that are permissive of differential readings of the same genomic DNA during cell divisions and tissue differentiation (Zhang *et al.*, 2011, Zhu *et al.*, 2008). Such a flexibility of the genome is instructed by epigenetic mechanisms in order to match spatial and temporal gene expression to developmental stages (Ay *et al.*, 2014, Brandeis *et al.*, 1993, Ishida *et al.*, 2008) and ambient conditions (Bird & Jaenisch, 2003, Boyko & Kovalchuk, 2008, Finnegan *et al.*, 2000).

Many remarkable impacts of epigenetic mutations on developmental processes have been reported (Bossdorf *et al.*, 2010, Cubas *et al.*, 1999, Finnegan *et al.*, 1996, Manning *et al.*, 2006,

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Podio *et al.*, 2014). Stem cell renewal, gametogenesis and embryogenesis are all contexts known to be partially or entirely regulated by DNA methylation (Podio *et al.*, 2014, Schmitz *et al.*, 2013, Zhong *et al.*, 2013a). For instance, demethylation at the maternal *MEA* allele (*MEDEA*, an *Arabidopsis* Polycomb group gene) in the central cell is indispensable to the formation of viable seeds in angiosperms (Xiao *et al.*, 2006). Other noteworthy illustrations of the role of DNA methylation in plants include the alteration of flower architecture in *Linaria vulgaris* (Cubas *et al.*, 1999), the activation of the Colourless non-ripening (*Cnr*) locus in tomato (Manning *et al.*, 2006) and the control of parthenogenesis in apomictic *Paspalum* (Podio *et al.*, 2014) among others. Therefore, the status of genomic DNA methylation is a fundamental component of plant development programmes. Additionally, DNA methylation can also contribute to plant adaptation to stress.

1.3.2. DNA methylation as a defence mechanism

The many stresses that plants experience during their sessile existence require response mechanisms that should be quick and effective to face the unfavourable condition. Plant survival relies on its capacity to differentially regulate gene expression and protein function as soon as they meet a stressful condition. Thus, stress responsive genes and other adaptive genes are regulated using a rapid switch mechanism: epigenetic gene regulation (Aceituno *et al.*, 2008, Zilberman *et al.*, 2007). Proceeding via transcriptional and posttranscriptional gene silencing (Spoel & Dong, 2012, Wang *et al.*, 2013) or activation of silent genes (Secco *et al.*, 2015, Wada *et al.*, 2004), epigenetic control provides flexibility to the genome to face unpredictable challenges (Boyko & Kovalchuk, 2008, Dowen *et al.*, 2012). The speed of methylation response to stress (from a few minutes to a few hours) (Ferreira *et al.*, 2015, Mastan *et al.*, 2012, Wada *et al.*, 2004) shows that epigenetic mechanisms are at the forefront of plant defence and adaptation systems.

Abundant studies have clearly established the fact that stress perpetrates alterations of the genome methylation profile. Instances of stress-induced modifications of DNA methylation were reported for salinity (Ferreira *et al.*, 2015, Karan *et al.*, 2012, Mastan *et al.*, 2012, XueLin *et al.*, 2009), heavy metals (Aina *et al.*, 2004, Labra *et al.*, 2004), pathogens (Mason *et al.*, 2008, Sha *et al.*, 2005) and climatic conditions (Tricker *et al.*, 2012). This role of DNA methylation in plant adaptation to stress is supported by the fact that a high proportion of genes influenced by methylation (62.5%) appear to be closely involved in biotic or abiotic stress responses (Wada

et al., 2004). Furthermore, it has been hypothesized that epigenetic variations in response to environmental cues can speed up plant adaptation (Consuegra and Rodriguez Lopez, 2016) to the same (Tricker *et al.*, 2013a) or novel cues (Tricker *et al.*, 2013b). These studies provide evidence that DNA methylation is a prominent epigenetic signature of stress, and usually targets transcribed regions of stress responsive genes (Aceituno *et al.*, 2008, Zilberman *et al.*, 2007). This process may also involve methylation changes in other genomic features such as transposons.

1.3.3.   DNA methylation as a regulator of transposons and plant plasticity

Transposable elements (TEs or transposons) constitute a considerable proportion of plant genomes (Lisch, 2009), reaching, for example, about 84% of the barley genome (Mayer *et al.*, 2012). These mobile genetic units exhibit a broad diversity in their structure and transposition mechanisms (Feschotte & Pritham, 2007), and are typically hypermethylated (Zhang, 2008). The profusion of evidence correlating DNA methylation with TEs activity (Dowen *et al.*, 2012, Hashida *et al.*, 2006, Slotkin *et al.*, 2009, Vaillant *et al.*, 2006), shows that DNA methylation is the key factor in the repression of transposition and TE-derived promoters (Dowen *et al.*, 2012). This transcriptional silencing is instigated to prevent the generation of abnormal RNAs and proteins that could undermine host cell conformity and thus retain genome integrity (Bender, 2012).

Furthermore, epigenetic regulation of TE activity seems to promote plant phenotypic plasticity (Dowen *et al.*, 2012, Rubio-Somoza & Weigel, 2011). Phenotypic plasticity refers to the capacity for a given genotype to express variable phenotypes under different environmental conditions (Schlichting, 2002). This aptitude for environment-contingent trait expression is common in plant species (Rubio-Somoza & Weigel, 2011, Schlichting, 2002) and the mechanistic way by which plastic gene expression occurs has been the subject of much research in recent years. There is a mounting evidence that phenotypic plasticity is fundamentally epigenetically regulated (de la Paz Sanchez *et al.*, 2015, Dowen *et al.*, 2012, Kitimu *et al.*, 2015, Rubio-Somoza & Weigel, 2011, Tricker *et al.*, 2012, Wang *et al.*, 2016). This implies that during growth and successive generations, the plant constantly implements cycles of sensing-accommodating with environmental cues (Figure 1.2), which are triggers of epigenetic mechanisms (de la Paz Sanchez *et al.*, 2015) that often target transposons (Hashida *et al.*, 2006,

Vaillant *et al.*, 2006). Consequently, the expression of adjacent genes is altered, with an impact on the phenotype (Baek *et al.*, 2011, Hashida *et al.*, 2006, Vaillant *et al.*, 2006).

The best-known examples of the implication of epigenetic mechanisms in plasticity concern temperature dependent gene regulation. This includes epigenetic silencing of the gene encoding floral repressor FLOWERING LOCUS C (FLC) in *Arabidopsis* to allow vernalisation (Bastow *et al.*, 2004, Song *et al.*, 2012a), cold stress activation of *ZmMI1* in maize (Steward *et al.*, 2002), low temperature induced flower pigmentation in *Antirrhinum* (Hashida *et al.*, 2006), and heat induced gene activation in *Arabidopsis* (Pecinka *et al.*, 2010). Beside the regulation of transposon activity, DNA methylation is also known to contribute to the evolution of genomes.
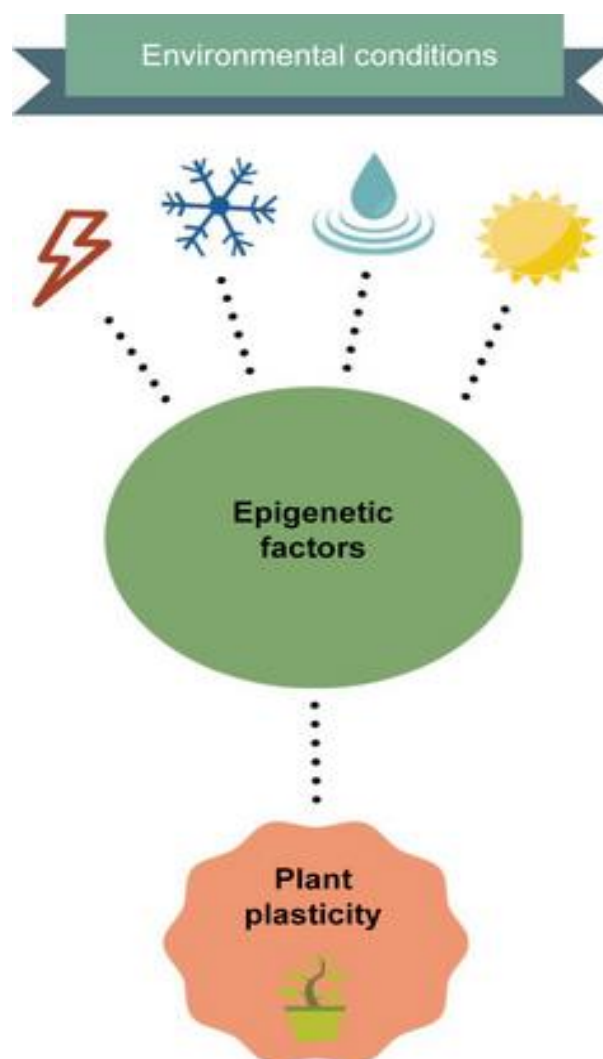
Figure 1.2: Involvement of epigenetic mechanisms in plant plasticity.
Environmental cues such as light, temperature (cold/heat) and humidity are triggers of epigenetic factors, which in turn may alter gene expression to adjust plant development, generating plant plasticity (de la Paz Sanchez *et al.*, 2015).

1.3.4. DNA methylation as a driver of evolution

There is a consensus that evolution is driven by the environment (Bradshaw & Hardwick, 1989, Lenormand *et al.*, 2009, Paenke *et al.*, 2007, Price *et al.*, 2003). Environmental cues can lead to stochastic genetic events, including gene flow and genetic mutations that build up over evolutionary times (Lenski & Mittler, 1993, Soen *et al.*, 2015). However, genetic mutations alone do not fully explain evolution, suggesting that changes driving it can also be epigenetic in nature (Herb, 2014).

Indeed, evolution involves interactions between genetic and environmental components (Bradshaw & Hardwick, 1989, Lenormand *et al.*, 2009, Paenke *et al.*, 2007, Price *et al.*, 2003, Rois *et al.*, 2013). Genetic mutations are randomly induced by environmental constraints (biotic and abiotic), creating both genetic and phenotypic diversity which are then subject to adaptive selection across generations (Bradshaw & Hardwick, 1989, Coyne *et al.*, 1997, Lenski & Mittler, 1993, Soen *et al.*, 2015). As phenotypic diversity and plasticity are adaptive in nature (Paenke *et al.*, 2007), they constitute, more than genotype, determining factors of evolution (Price *et al.*, 2003, Schlichting, 2002).

Epigenetic mechanisms and other cellular strategies are involved in keeping gene expression in tune with physiological needs dictated by the environment (Bradshaw & Hardwick, 1989, de la Paz Sanchez *et al.*, 2015), therefore promoting both short and long term phenotypic adaptation (Lopez-Maury *et al.*, 2008). This soft adaptation appears more plausible than that due to very rapid and abrupt changes in environmental conditions (Steffensen *et al.*, 2008). As such, epigenetic regulation predisposes plants to phenotypic plasticity (de la Paz Sanchez *et al.*, 2015), which may lead to genetic assimilation over time (Hauben *et al.*, 2009, Paenke *et al.*, 2007, Price *et al.*, 2003). Environmental stress-responsive genes are the first targets in which epialleles are induced (Pecinka *et al.*, 2010, Schmitz & Amasino, 2007, Song *et al.*, 2012a), hence are potentially subject to selection in evolutionary processes (Bräutigam *et al.*, 2013, Ruden *et al.*, 2015). The view that phenotypic stochasticity underlies evolution has gained much credit in recent years (Lenormand *et al.*, 2009, Raj & van Oudenaarden, 2008, Soen *et al.*, 2015, Vogt, 2015), supporting the randomness of factors contributing to evolution. However, whilst the role of epigenetic mechanisms triggered by random environmental experiences is often overlooked, yet such epigenetic modifications might be crucial in initiating evolutionary changes.

1.4. Epigenetic profiling methods

Due to the functional importance of DNA methylation in many eukaryotic species, DNA methylation profiling across the genome was essential to broaden our knowledge of epigenetic mechanisms. Hence, many methods have been developed to detect DNA methylation markers, including High Pressure Liquid Chromatography (HPLC), Bisulfite treatment and Methylation Sensitive Amplified Polymorphism (MSAP).

The HPLC approach associates the quantification of methylated DNA cytosines and the use of a methyltransferase to incorporate labelled methyl groups, which are subsequently quantified to provide a measure of the sample methylation level (Jaligot *et al.*, 2000). However, the low sensitivity and the anonymous detection of DNA methylation may constitute a limitation for the HPLC method. The Bisulfite treatment method is also an indirect method of DNA methylation analysis. Bisulfite treatment of sample DNA results in the conversion of unmethylated cytosines to uracils by deamination, leaving methylated cytosines unchanged. The methylation level of such treated samples can then be evaluated through sequencing and comparison with control untreated samples (Karan *et al.*, 2012, Tricker *et al.*, 2012). Bisulfite treatment is considered as the standard method to assess DNA methylation, but relies on the efficiency of cytosine conversion, and is thought to be costly and labour-demanding when whole-genome coverage is needed (Xia *et al.*, 2014, Zhang *et al.*, 2006).

The cognition of isoschizomer enzymes presenting differential sensitivity to methylation at restriction sites, has enabled the development of the MSAP method (Reyna-López *et al.*, 1997) from the fingerprinting method AFLP (Amplified Fragment Length Polymorphism) (Vos *et al.*, 1995). The MSAP uses isoschizomer enzymes such as *Hpa*II and *Msp*I to reduce genomic DNA complexity and generate anonymous marker fragments, which are PCR amplified and can be detected following gel or capillary electrophoresis (Rodríguez López *et al.*, 2012). This PCR based technique has proved to be suitable for methylation profiling even of non-model genomes (Paun & Schönswetter, 2012), providing polymorphic and reproducible markers (Perez-Figueroa, 2013, Rodríguez López *et al.*, 2012). These advantages of the MSAP made it a popular technique, especially in plants, for the detection of DNA methylation due to natural variations (Fang & Chao, 2007, Fang *et al.*, 2010, Herrera & Bazaga, 2010) and environmental cues (Karan *et al.*, 2012, Li *et al.*, 2008, Marconi *et al.*, 2013, Mason *et al.*, 2008). One limitation of this technique though, is that it detects DNA methylation in the enzyme recognition motif only, such as CCGG for *Hpa*II and *Msp*I (Figure 1.3).

Nevertheless, the development of high-throughput DNA sequencing has opened new possibilities for DNA methylation profiling. Analyses of DNA methylation can now be performed on a genome-scale, and entire methylomes can be characterized at single-base-pair resolution (Laird, 2010). Interestingly, current high throughput DNA analyses can harness many previously low throughput methods to achieve better coverage of the genome. For instance, Bisulfite conversion (Secco *et al.*, 2015) and the methylation sensitive genotyping by sequencing (ms-GBS) (Kitimu *et al.*, 2015, Xia *et al.*, 2014), can now be used routinely for genome-wide methylation profiling in plants.
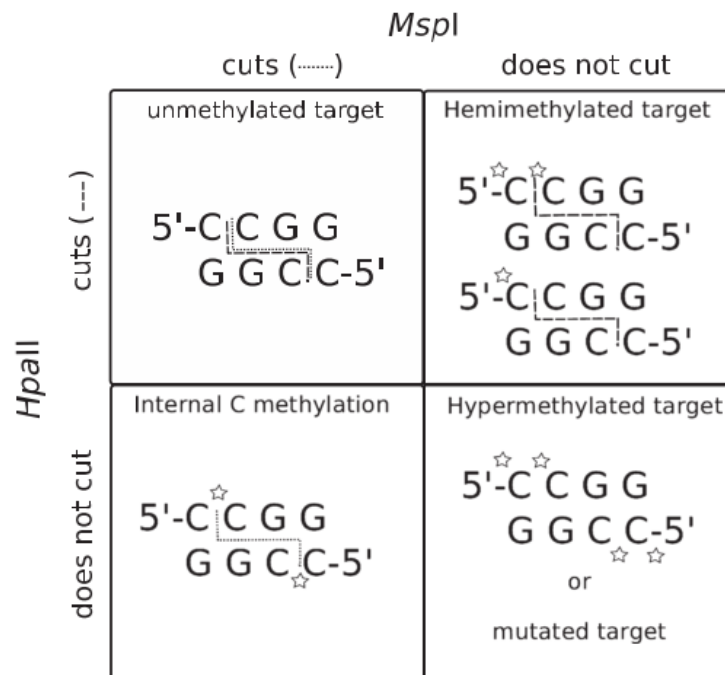
Figure 1.3: Sensitivity of isoschizomers *Msp*I and *Hpa*II to DNA cytosine methylation in their recognition site 5'-CCGG-3'.
Methylation in represented by stars; dotted lines show the cleavage pattern for *Msp*I and dashed lines for *Hpa*II (Perez-Figueroa, 2013).

1.5. Plant responses to stress

Plants may recurrently experience diverse stresses during which they readily deploy defence responses, sporadically or continuously, to cope with the challenging conditions (Figure 1.4). This is done through the activation of cascades of complex molecular networks involved in stress perception, signal transduction, and differential expression of specific stress-responsive genes (Mahajan *et al.*, 2008, Tuteja, 2007).

Stress can be triggered in the plant by biotic (e.g. pathogens, pests or weeds) and abiotic (e.g. drought, heat, frost or salinity) factors (Figure 1.4) (Arnholdt-Schmitt, 2004, Madlung & Comai, 2004). Of the abiotic stresses, salinity is one of the most damaging to crop production in the world (Munns & Tester, 2008). Like many other stress types, salinity causes a deviation from optimal plant development and reproduction (Munns & Tester, 2008, Roy *et al.*, 2014). Salinity elicits responses and disturbances at functional levels of the plant, resulting in immediate and long-term tuning of its metabolism to fit the present condition (Asai *et al.*, 2002).

Currently, there is great understanding of stress-induced physiological changes in plants (Asai *et al.*, 2002, Lam *et al.*, 2001, Mahajan *et al.*, 2008, Tuteja, 2007). Also, much knowledge on the stress-induced differential expression has been generated, showing how stress responsive genes contribute to stress tolerance in plants (Hill *et al.*, 2016, Walia *et al.*, 2006, Walia *et al.*, 2007, Ziemann *et al.*, 2013). Molecular mechanisms of plant salt stress responses involve epigenetic variants, such as stress-induced generation of siRNAs, histone modification and DNA methylation (Baek *et al.*, 2011, Borsani *et al.*, 2005, Chen *et al.*, 2010, Choi & Sano, 2007, Xu *et al.*, 2015). Such stress-induced molecular changes influence specific mRNA abundance, translation efficiency and protein activity (Hill *et al.*, 2016, Walia *et al.*, 2006, Walia *et al.*, 2007, Ziemann *et al.*, 2013), and underlie the repair of disturbed functions, hardening and/or adaptation to the stress (Baek *et al.*, 2011). The principle of epigenetic mechanisms' involvement in plant responses to stress has been well documented (Baek *et al.*, 2011, Borsani *et al.*, 2005, Boyko & Kovalchuk, 2008, Chen *et al.*, 2010, Dowen *et al.*, 2012, Xu *et al.*, 2015) and DNA methylation seems to be an integral epigenetic marker of plant responses to stress.

The next sections give an overview of salt-induced DNA methylation in plants and its subsequent effect on gene regulation. Numerous studies have examined the association between salt stress and changes in the pattern of DNA methylation in diverse plant species such as cotton (XueLin *et al.*, 2009), wheat (Wang *et al.*, 2014, Zhong *et al.*, 2009), rapeseed (Lu *et al.*, 2007, Marconi *et al.*, 2013), rice (Karan *et al.*, 2012, Wang *et al.*, 2011b), barley (Demirkiran *et al.*, 2013, Katsuhara & Kawasaki, 1996). It appears that excess salt alters the plant's DNA methylation profile in a tissue-specific manner.
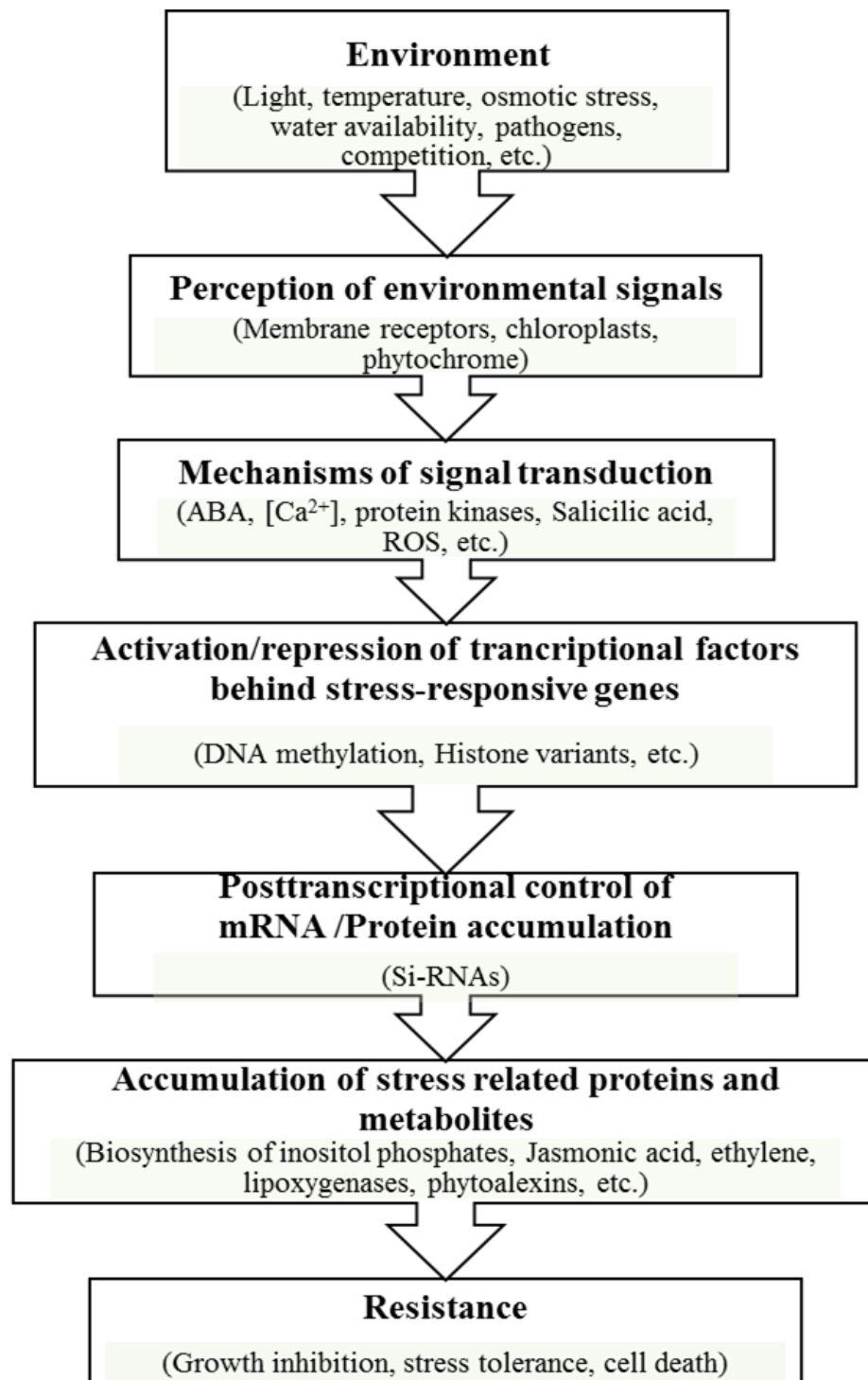
Figure 1.4: Schematic representation of mechanisms controlling plant responses to stresses
(adapted from Cattivelli *et al.* (2010) and Mahajan *et al.* (2008)).

1.6.   Salinity induced alteration of plant methylation patterns

1.6.1.   Salinity induces DNA hypomethylation in roots

Consistent findings show that, under salt stress, root tissues relax their DNA methylation levels, regardless of the plant species (Demirkiran *et al.*, 2013, Ferreira *et al.*, 2015, Karan *et al.*, 2012, Wang *et al.*, 2015c, Wang *et al.*, 2011b, Zhong *et al.*, 2009), even in halophyte plant species (Gao *et al.*, 2013). Such overall hypomethylation in roots can be explained by the need to up-regulate genes that are essential to the root response to this salinity. Root response to salt relates primarily to physiological functions involved in restoring the osmotic balance and regulating water uptake (Munns, 2002). Therefore, changes in DNA methylation in genes involved in this process are expected to boost their expression (Baek *et al.*, 2011).

However, the activation of genes responsive to salinity is not the sole reason for DNA hypomethylation in roots. Indeed, salt induced oxidative stress in plants not only disturbs normal metabolism (Dionisio-Sese & Tobita, 1998, Katsuhara & Kawasaki, 1996), but also leads to DNA demethylation due to 8-hydroxyguanosine, which has the property of being able to inhibit the methylation of adjacent cytosine residues in CG sequences (Weitzman *et al.*, 1994). This salt induced inhibition of DNA methylation has been demonstrated experimentally previously (Choi & Sano, 2007), and suggests that hypomethylation in plant roots under salinity stress might be mediated by oxygen radicals (Zhong *et al.*, 2009).

Furthermore, it has been reported that high salt concentrations may result in apoptosis-like DNA fragmentation in root cells (Katsuhara & Kawasaki, 1996) or mutation in DNA sequences (Lu *et al.*, 2007). Although the extent of such DNA degradation has not yet been detailed, DNA fragmentation or mutations may introduce bias in DNA templates, thus affecting the epigenetic profile. This is especially crucial when using restriction enzyme-based techniques.

Besides these arguments, salt induced DNA hypomethylation in roots needs to be considered with caution. Even in the absence of stress, plant methylomes are highly tissue specific (Aceituno *et al.*, 2008). Also, although root hypomethylation under salt stress has proved to be common in most plant species (barley, rice, wheat, cotton or *Arabidopsis*), there are exceptions in which root DNA is hypermethylated in response to salt. Hypermethylation in roots has been reported in salt stressed *Jatropha curcas*, though the percentage of polymorphic markers was low in root tissues, when compared with leaves (Mastan *et al.*, 2012). In addition, different studies on the same species may give contradictory epigenetic responses to salt, as was the case

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for cotton, in which both root hypomethylation (Qian *et al.*, 2014, Wang *et al.*, 2015a, Zhao *et al.*, 2010) and hypermethylation (Lu *et al.*, 2015) were reported. If this is not an exception, it suggests that plant epigenome instability (Boyko & Kovalchuk, 2011, Brandeis *et al.*, 1993) may distort the tissue specificity of salt induced DNA methylation. It is noteworthy that roots respond more strongly than leaves to the high pH in a saline environment (Gao *et al.*, 2013). Since the pH was not measured in most of the reported studies, contradictions in root hypermethylation under salt stress may be attributable to substrate alkalinity.

1.6.2. Salinity induces hypermethylation in shoots

Contrary to what has been observed in roots, plants respond to salinity by increasing cytosine methylation in shoot cells (Demirkiran *et al.*, 2013, Karan *et al.*, 2012, Zhong *et al.*, 2009). Limited information exists about the implication of DNA methylation in the regulation of specific genes in shoot tissues, but the known negative correlation between gene expression and methylation levels (Bird & Jaenisch, 2003, Boyko & Kovalchuk, 2008), suggests that hypermethylation in the plant shoot will result in total or partial repression of many genes.

Salt-induced stress and drought instigate similar stresses in plant, such as the osmotic stress in in both roots and leaves (Munns, 2002). Therefore, salt stress can lead to alterations of stomata development, proven to be associated with an epigenetic repression of the genes FAMA and SPEECHLESS by *de novo* cytosine methylation (Tricker *et al.*, 2012). Additionally, osmotic stress during salt stress perpetrates an inverse circadian rhythm of stomatal operation (closing pores during the day time and opening them at night) in order to limit water loss, possibly using the Crassulacean acid metabolism (CAM) pathway of carbon dioxide assimilation (Bohnert *et al.*, 1988, Dyachenko *et al.*, 2006). The salt stress-induced adoption of CAM has been proven to be paralleled with the hypermethylation of satellite DNA and a significant reduction of plant photosynthetic activities (Dyachenko *et al.*, 2006). These examples support the view that shoot DNA hypermethylation is correlated with down-regulation of genes; the activity of which could be detrimental to the plant's adaptation to salinity.

1.6.3. Factors affecting salinity-induced alteration of DNA methylation

Salt sensitive and tolerant genotypes have contrasting methylation profiles in response to salt stress. Varieties tolerant to salinity proceed to an increase of their overall DNA methylation compared with more sensitive ones (Feng *et al.*, 2012, Wang *et al.*, 2015a, Zhong & Wang,

2007). This behaviour looks like a common response feature to salt stress in plants. For instance, a salt-tolerant wheat variety maintained a higher level of methylation than salt-sensitive wheat after 10 days under salinity (Zhong & Wang, 2007). A similar correlation between methylation and plant tolerance level was reported in rice (Feng *et al.*, 2012) and cotton (Wang *et al.*, 2015a). The prevalence of hypermethylation in tolerant varieties suggests that during times of stress, there may be a down-regulation of non-vital genes in order to focus energy on functions essential for tolerance. This also suggests that salt-tolerant varieties may 1) have a predisposition to shut down more genes than salt-sensitive varieties, and 2) inherently possess active genes with functions in salt stress adaptation, in osmotic and ion homeostasis or in a metabolism that may be missing in salt sensitive genotypes (Diédhiou *et al.*, 2009a). The presence of such genes in their active form in salt-tolerant varieties before the stress, reduces their need to resort to DNA methylation changes during salt stress.

However, preferential hypomethylation in salt-sensitive plant varieties during salt stress imposition (Feng *et al.*, 2012, Wang *et al.*, 2015a), does not always apply. There are exceptions where salt-sensitive varieties gained higher methylation compared with their salt-tolerant counterparts. Higher DNA hypermethylation was found in salt-sensitive cotton varieties compared with the more salt-tolerant ones (Lu *et al.*, 2015, Zhao *et al.*, 2010). This particularity in the methylation pattern in cotton under salt stress evokes all the complexity of an epigenetic response to stress. Plant epigenome susceptibility to salt stress is highly dependent on species and genotype responses to the environment (Gao *et al.*, 2013, Lu *et al.*, 2015, Verhoeven *et al.*, 2010). Therefore, the overall estimation of DNA methylation does not always provide a credible indication of the plant's resistance level. Plant species and varieties often implement different mechanisms of adaptation to salt stress (Diédhiou *et al.*, 2009b, Popova *et al.*, 2008, Volkov *et al.*, 2004). Since both demethylation and methylation can be deployed in coding regions (Wang *et al.*, 2014), the overall genome methylation has limited qualitative value for salt tolerance.

Besides the variety-specificity of salt induced DNA methylation, it has been difficult to establish a linear correlation between methylation changes and salt stress intensity. Differential plant responses to levels of salt stress have been established using global methylation changes based on qualitative data (i.e. the presence/absence of a marker) (Lu *et al.*, 2015, Marconi *et al.*, 2013, Mastan *et al.*, 2012, Wang *et al.*, 2015a, Zhao *et al.*, 2010). This anonymous-marker approach gives a picture of salt induced DNA methylation changes compared with a control condition (salt stress free) (Fulnecek & Kovarik, 2014), but does not necessarily provide a quantification of the methylation per locus, nor the function of specific loci. Based on this approach, salt-

induced changes in DNA methylation were reported as dose-dependent, but counterintuitively, low salt concentrations induced higher DNA methylation than high salinity concentrations (Lu *et al.*, 2007, Mastan *et al.*, 2012, Zhang *et al.*, 2014). For example, epigenetic profiling of *Jatropha curcas* subjected to two salinity levels, revealed higher methylation under 25 mM compared with 75 mM sodium chloride (Mastan *et al.*, 2012). Similarly, it was found in the ornamental species *Camptotheca acuminata*, that salt induced ISSR (Inter-Simple Sequence Repeat) markers displayed a negative correlation between DNA methylation and salt concentration (Zhang *et al.*, 2014). It is not yet clear whether these findings can be generalized, but they suggest a possible association between salt concentrations and specific epigenetic loci, so that plants show a more qualitative response to variable salt concentrations. Furthermore, soil pH constitutes another factor able to influence the plant's epigenetic profile under salinity (Gao *et al.*, 2013, Lu *et al.*, 2015). Thus, DNA methylation has proven to be more impacted by alkaline salts than neutral ones, and this effect was greater in roots than in leaves (Gao *et al.*, 2013). Therefore, salt-induced DNA methylation can be concomitantly influenced by many factors, including salt concentration, pH and potentially other, currently unidentified factors. Such methylation changes may affect gene regulation during salt stress.

1.6.4. Epigenetic regulation of gene expression during salinity stress

There are numerous studies of plant epigenetic responses to salinity (Karan *et al.*, 2012, Song *et al.*, 2012b, Wang *et al.*, 2015b, Wang *et al.*, 2014, XueLin *et al.*, 2009, Zhong *et al.*, 2009). Although only a few amongst those studies investigated in depth the mechanism by which salt responsive genes are epigenetically regulated, these studies demonstrated that salt responsive genes may be under the control of salt stress-induced DNA methylation (Karan *et al.*, 2012, Wang *et al.*, 2014, Zhu *et al.*, 2015b, Zilberman *et al.*, 2007), which is a kind of relay between the stress perception and plant adaptation.

1.6.4.1. *DNA methylation: a relay between salt stress sensing and adaptive gene expression*

The involvement of epigenetic regulation in plant responses to salinity has become clear through extensive documentation in the literature, especially describing salt-induced DNA methylation (Karan *et al.*, 2012, Song *et al.*, 2012b, Wang *et al.*, 2015b, Wang *et al.*, 2014, XueLin *et al.*, 2009, Zhong *et al.*, 2009). Additionally, it has been shown that, within promoter and coding

regions of the genome, DNA methylation typically leads to transcriptional gene silencing (Matzke & Mosher, 2014, Popova *et al.*, 2013, Tricker *et al.*, 2013a).

Salinity perception is paralleled with changes to DNA methylation (Choi & Sano, 2007, Wang *et al.*, 2011b), including both *de novo* methylation and demethylation (Qian *et al.*, 2014). These salt-induced modifications of DNA methylation are thought to underlie the on-off switch of quantitative traits during plant responses to salinity (Marconi *et al.*, 2013, Sun *et al.*, 2016, Tan, 2010, Wang *et al.*, 2014, Zhong *et al.*, 2009). In this way, salt-induced methylation was reported to potentially regulate the expression of a large numbers of genes (Marin *et al.*, 2003), including stress specific ones (Wada *et al.*, 2004, Wang *et al.*, 2014).

However, most studies reporting salt induced epigenetic markers affecting transcriptional level of salt responsive genes, rely on fragment homology with reference genes (Karan *et al.*, 2012, Song *et al.*, 2012b, Wada *et al.*, 2004, Wang *et al.*, 2014). This has the limitation of not describing the mechanistic involvement of DNA methylation; but rather shows a definite correlation between the change in methylation status and expression. Wang *et al.* (2014) conducted an interesting study showing the role of the plant methylome in the regulation of salt stress responsive genes. Exploring 24 genes known for their differential expression under salt stress in two salt tolerant wheat varieties, Wang *et al.* (2014) found a change in cytosine methylation in 7 coding regions (~30%) and more than half (56.25%) of the studied promoter regions of target sequences. They found that salt-induced demethylation and *de novo* methylation were correlated with up to 4-fold changes in expression of these genes (Wang *et al.*, 2014).

Many salt stress responsive genes have been characterised (Bohnert *et al.*, 1988, Kore-eda *et al.*, 2004, Lai *et al.*, 2014, Song *et al.*, 2012b, Waditee-Sirisattha *et al.*, 2012, Zhu *et al.*, 2015b), some of which have had detailed assessment of DNA methylation alterations that may affect their expression. It appeared that salt stress mainly affects methylation of promoters and transcription factors of salt responsive genes.

The MYB (<u>my</u>elo<u>b</u>lastosis) family genes are transcription factors responsive to stress and involved in coordinating plant tolerance to salt stress (Song *et al.*, 2012b, Zhu *et al.*, 2015b). For soybean, Song *et al.* (2012b) observed an alteration of DNA methylation in some MYB transcription factors in response to salt stress, suggesting a possible effect on adjacent genes' expressions. The expression of the analogue gene, OsMYB91, in rice confers enhanced tolerance to salt by increasing proline levels and reducing the accumulation of reactive oxygen species and malondialdehyde (Zhu *et al.*, 2015b). A prompt DNA demethylation at the

OsMYB91 locus upon salt stress results in enhanced expression of the gene (Zhu *et al.*, 2015b). Changes in cytosine methylation in MYB and other transcription factors, such as b-ZIP (Basic Leucine Zipper Domain), AP2/DREB (Activating Protein 2 / dehydration-responsive element-binding) and WRKY (a protein starting with amino-acids Tryptophan- Arginine- Lysine-Tyrosine), and subsequent activation of these genes, correlates with histone modifications of the promoter and coding regions (Ramamoorthy *et al.*, 2008, Song *et al.*, 2012b, Zhu *et al.*, 2015b), thus highlighting a tight interplay between DNA methylation and histone modification during epigenetic gene regulation (Mathieu *et al.*, 2005, Song *et al.*, 2012b, Zhu *et al.*, 2015b).

Salt-induced DNA methylation also influences the synthesis and accumulation of glycine betaine, an "osmoprotectant", the presence of which in roots supports osmotic balance during salt stress (Lai *et al.*, 2014, Waditee-Sirisattha *et al.*, 2012). The synthesis of choline (a precursor of glycine betaine) is triggered by salt stress under the catalytic effect of light, and it is suggested that it is regulated by DNA methylation (Waditee-Sirisattha *et al.*, 2012, Weretilnyk *et al.*, 1995). Another context in which salt induced DNA methylation was determined was reported for the facultative halophyte *Mesembryanthemum crystallinum*. In this species, salt stress is paralleled with the up-regulation of CAM-related enzymes (Bohnert *et al.*, 1988, Kore-eda *et al.*, 2004), and the down-regulation of light-harvesting and C3 photosynthetic enzymes (Kore-eda *et al.*, 2004). Using an enzymatic approach, Dyachenko *et al.* (2006) showed that under salt stress, the transition from C3 to the CAM metabolism, is concomitant with a hypermethylation of CCWGG (with W = A or T) motifs in satellite DNA associated with salinity induced water stress in leaf tissues.

Furthermore, the benefit of heterosis in conferring superior salt tolerance to hybrid plants seems to be correlated with changes in DNA methylation due to the genetic stress imposed by hybridization. This was observed by Zeng *et al.* (2015) in hybrid F1 offspring of *Fraxinus spp.* where salt tolerance was associated with a relaxation of DNA methylation in hybrids, compared with both parents. Similar observations were made in wheat, although it was argued that the enhanced salinity tolerance shown by the progeny was due to hybridization-induced methylation modifications independent of salt stress (Wang *et al.*, 2014).

1.6.4.2. *Epigenetic regulation of many salt responsive genes is unknown*

This review led to the conclusion that DNA methylation remodelling upon the imposition of salt stress is critical in plant conditioning and adaptation to salt stress. Plant adaptation to salt stress is associated with the on-off status of quantitative expression of key salt responsive genes (Lu

et al., 2015, Walia *et al.*, 2007). Although the description of plant global methylation profiles is a good indication of plant epigenetic response to salinity, this blind approach has minor importance in demonstrating which genes are in play. We now have a greater understanding of the correlation between gene expression and DNA methylation (Aceituno *et al.*, 2008, Boyko & Kovalchuk, 2008), but there have only been limited studies that went beyond the description of the methylation pattern to assess coding or promoter regions that are influenced by salt induced epigenetic marks to date. The cognition of the tight correlation between salt-induced epigenetic modifications and specific genes, suggests that epigenetic profiling can be used as a forward and reverse genetics tool (Amoah *et al.*, 2012).

Salt stress regulates the expression of large numbers of genes (Marin *et al.*, 2003), including those involved in plant energy metabolism, ionic transmembrane transport, photosynthesis, signal transduction and many other pathways (Kumar *et al.*, 2013, Lu *et al.*, 2015). Key genes responsive to salinity include NHX-type Na^+/H^+ transporters (Bassil & Blumwald, 2014, Shi & Zhu, 2002), HKT1 (Baek *et al.*, 2011, Munns *et al.*, 2012), *AVP1* (Schilling *et al.*, 2014) and *AtCIPK16* (Roy *et al.*, 2013), and salt overly sensitive (SOS) genes (Mahajan *et al.*, 2008, Zhu, 2001). However, few or no studies have been reported on the epigenetic regulation under salt stress of such important genes that confer salinity tolerance. The best known example of key salt responsive genes that are epigenetically regulated is the cation transporter AtHKT1, involved in the Na+ uptake in roots (Baek *et al.*, 2011). AtHKT1 expression confers salt tolerance, and was enhanced by non-CG methylation in a putative small RNA target region, about 2.6 Kb upstream of the ATG start codon (Baek *et al.*, 2011). The need for methylation near this gene to allow its expression shows how important epigenetic marks are for plant response to salt.

1.7. Project objectives

Our comprehension of salt induced modifications of DNA methylation is still limited by the gaps in the research literature. Genotype and tissue specificity of salt induced DNA methylation needs to be further examined in order to untangle epigenetic variations due to salt stress from the background noise. Background noise may arise from micro-environmental conditions including climate factors, pH, and plant internal signals. Further, it is not established to date if methylation sites that are affected by salinity occur at random or are conserved across varieties and species. This offers a considerable opportunity for exploration of the role of salt-induced remodelling of DNA methylation in plants. Answers to these questions are essential to further

understand gene regulation under salt stress conditions, in order to allow crop improvement strategies to be deployed, based on molecular understanding.

The overall aim of this research project is to expand our understanding of DNA methylation dynamics in plants, using barley as a model plant, under stress-free and variable salt stress conditions. In order to achieve this, we will assess plant DNA methylation profiles, taking into consideration background noises such as genotype and positional effect.

Specifically, the following research aims will be addressed:

1. To validate salt-induced alteration of the plant epigenome in barley;

2. To characterise salt-induced DNA methylation markers and their correlation with the expression of salt responsive genes in barley;

3. To characterise tissue-specific DNA methylation markers and their correlation with specific genes in barley;

4. To assess the effect of position of barley plants in the greenhouse on their methylation profile.

1.8. Linking statement

This thesis is organised into six chapters, including four research chapters, three of which were written in journal article format, according to the instructions for authors of each target journal.

Chapter 1 presents an introduction to the thesis project and a review of the literature relative to epigenetic mechanisms and how these are involved in plant responses to stress, with a special focus on salt stress. The research objectives are also presented at the end of that chapter.

Chapter 2 reports the effect of mild salt stress on the barley phenotype and epigenome, using nine varieties. This chapter reveals some contrasting phenotypic and epigenetic responses to salt, alongside the difficulties of assessing the response of barley, a salt-tolerant crop, to mild salt stress.

Chapters 3 and 4 are manuscripts formatted to be submitted for review to the special issue "Plant Epigenome Dynamics" of the journal "Epigenomes". These chapters report the results of two ms-GBS projects: A characterisation of salt-induced DMMs in barley (Chapter 3), which reveals that salt-induced DMMs are both highly tissue specific and are more abundant in leaves than roots; and an Atlas of tissue and age specific patterns of DNA methylation during barley early development (Chapter 4).

In Chapter 5, a manuscript is presented, reporting on the variability of plant methylation profiles due to their positional effect in the greenhouse. Instead of attributing stochastic DNA methylation to randomness, this manuscript shows that at least part of such methylation is induced by microclimatic variations across the experimental environment. The manuscript is formatted to be submitted for review to the journal 'Plant Cell and Environment'.

Chapter 6 is a general discussion of the main findings of this thesis, and considers future research directions arising from the work already undertaken.

Chapter 2:   Assessment of the effect of mild salt stress on barley phenotypes and epigenomes

2.1.   Introduction

Soil salinity is a major cause of yield loss in barley and other crops across the world. Salt concentration in the soil changes with location, depth, seasonal progression and farm management and can impact on yield, even at relatively low concentrations. During growth, plants need to adapt to these variations in salt levels in a dynamic manner. This requires physiological responses, including osmotic adjustment, tolerance to excess Na^+ and Na^+ exclusion from the leaves (Munns & Tester, 2008, Roy *et al.*, 2014).

Physiological adaptations to stress are associated with an alteration of the expression of stress specific genes (Causevic *et al.*, 2005, Choi & Sano, 2007), through mechanisms of enhancement of gene expression (Wada *et al.*, 2004), transcriptional (TGS) and post-transcriptional gene silencing (PTGS) (Wang *et al.*, 2013, Zilberman *et al.*, 2007). DNA methylation is considered to be the primary epigenetic mechanism deployed upon stress perception in the plant and is associated with gene regulation by affecting the local chromatin structure (Boyko & Kovalchuk, 2008, Choi & Sano, 2007, Wada *et al.*, 2004). This role of DNA methylation in plant adaptation to stress conditions has been of interest in recent years (Bossdorf *et al.*, 2010, Boyko & Kovalchuk, 2008, Chinnusamy & Zhu, 2009). It has been demonstrated that the DNA methylation pattern varies relative to the stress exerted on the plant (Boyko & Kovalchuk, 2008, Chinnusamy & Zhu, 2009). Stress factors that alter DNA methylation include heavy metals (Aina *et al.*, 2004), temperature extremes (Liu *et al.*, 2015, Pecinka *et al.*, 2010, Song *et al.*, 2012a), nutrient deficiencies (Secco *et al.*, 2015, Yong-Villalobos *et al.*, 2015) and salinity (Karan *et al.*, 2012, Marconi *et al.*, 2013, Wang *et al.*, 2014, Zhong *et al.*, 2009).

Salinity induced DNA methylation has been the subject of several studies conducted on the model plant Arabidopsis (Baek *et al.*, 2011, Suter & Widmer, 2013) and also on crops such as cotton (Lu *et al.*, 2015), rapeseed (Lu *et al.*, 2007, Marconi *et al.*, 2013), rice (Ferreira *et al.*, 2015, Karan *et al.*, 2012), wheat (Wang *et al.*, 2014, Zhong *et al.*, 2009), maize (Tan, 2010) and barley (Demirkiran *et al.*, 2013). It appears that salt stress significantly alters the plant's epigenetic profile (Karan *et al.*, 2012, Lu *et al.*, 2015, Marconi *et al.*, 2013, Wang *et al.*, 2014, Zhong *et al.*, 2009), although the minimum level of stress that triggers methylation changes is not clear. Nevertheless, this acute alteration of the plant epigenome in response to salt stress suggests that, hypothetically, the DNA methylation pattern may reflect the plant's stress condition. Therefore, plant epigenetic profiling offers an opportunity to identify DNA methylation markers associated with salinity stress responses in plants.

Molecular markers have been employed in crop improvement for many years. Marker assisted selection in plant breeding has made use of diverse marker types, which were fundamentally based on the plant's genetic make-up (Mohan *et al.*, 1997). However, due to genotype by environment interactions (referred to as G × E or plasticity), lines from DNA sequence-based marker selection may show significant phenotypic variability across variable environments (Fernández-Pascual & Jiménez-Alfaro, 2014, Wellstein *et al.*, 2013). One of the ways to overcome this issue has been to implement multi-location trials before the commercial release of new varieties (Narh *et al.*, 2014, Yan *et al.*, 2016). Since plant plasticity is known to involve epigenetic regulation (Bossdorf *et al.*, 2010, Herrera & Bazaga, 2010, 2013), applying epigenetics in plant breeding has potential benefits (Rodriguez Lopez & Wilkinson, 2015).

Studies on DNA methylation and related molecular dynamics have been increasing in number in recent years (Boyko & Kovalchuk, 2008, Crisp *et al.*, 2016, Dowen *et al.*, 2012, He *et al.*, 2011, Turck & Coupland, 2014). One of the techniques widely used for the assessment of genome wide DNA methylation is methylation sensitive amplification polymorphism (MSAP). This is an enzyme based technique in which a selective PCR amplification is performed on DNA fragments generated by digestion by isoschizomers such as *Hpa*II and *Msp*I, in association with *Eco*RI (Reyna-López *et al.*, 1997). Based on the differential sensitivity to methylation, the pattern of digestion by *Hpa*II and *Msp*I provides information about the state of methylation at the target CCGG sites across the genome. MSAPs has been used extensively to study methylation patterns in genomes and has proved to be both very effective and reproducible in differentiating plant populations (Fang *et al.*, 2010, Li *et al.*, 2008, Rois *et al.*, 2013), tissue

types (Rodríguez López *et al.*, 2012) and various stress conditions (Cao *et al.*, 2011, Mason *et al.*, 2008).

While the MSAP is in principle appropriate for epigenetic profiling (Li *et al.*, 2008, Rodríguez López *et al.*, 2012), it is not clear whether methylation changes during a stress such as salinity are consistent and specific within a species. The aim of this research is to compare the methylation profiles of barley plants from multiple cultivars under control and mild stress conditions and to identify from these profiles a set of differentially methylated markers (DMMs) that are associated with salt stress.

2.2.   Material and methods

2.2.1. Plant material and greenhouse conditions

This experiment was conducted from June to October 2013, in a greenhouse 8 m long and 3 m wide (24 m^2) at The Plant Accelerator (34°58'16 S, 138°38'23 E) at the University of Adelaide's Waite Campus. The greenhouse temperature was set at 22℃/15℃ (day/night), with natural light throughout the experiment.

Eight barley varieties were used in this study (Barque 73, Buloke, Commander, Flagship, Hindmarsh, Maritime, Schooner and Yarra) in a randomised block design including five replicates and two salt treatments: control (0 mM) and 75 mM NaCl(i.e. five blocks, each with eight varieties and two treatments). Barley varieties were grown in GL potting mixture (50% UC mix (University of California Davis), 35% coco-peat and 15% clay/loam (v/v)). White pots, 20 cm height × 15 cm diameter, were filled to weight with soil, to ensure controlled salt application and watering. Pots were lightly watered before sowing three evenly sized seeds per pot. Two weeks after sowing, the barley seedlings were thinned to one per pot.

To calculate the water and NaCl amounts needed for salinity treatments, the soil dry weight per pot was calculated based on the soil dry weight of four randomly selected pots (without plants) and dried in an oven at 65℃ until a constant weight was reached. Based on the soil dry weight and field capacity of the soil mix, the amount of NaCl required to impose 0 mM and 75 mM NaCl in the soil was calculated, according to the method described by Berger *et al.* (2012). Salt treatments were applied 25 days after sowing and the pots were watered to 0.8 x field capacity

(16.8% (g/g)) every two days, up to 60 days after sowing. Then the plants were watered to target weight daily, until the seeds were set.

2.2.2. Measurement of phenotypic parameters

The stress and control plants were monitored throughout development and developmental data were recorded to assess possible salt stress impacts. Automated imaging was performed at 41, 87 and 119 days after sowing (DAS), using fixed-optics cameras at The Plant Accelerator® (see Section 2.2.2.3 below). These digital colour images provided an estimate of the plant height and projected shoot area of each plant (Berger *et al.*, 2010, Rajendran *et al.*, 2009). After the first imaging (41 DAS), the 4th leaf blade of each plant was sampled for analysis of its sodium (Na^+) and potassium (K^+) contents.

2.2.2.1. *Leaf ion content [Na^+, K^+]*

The sodium and potassium ion content in the 4th leaf blades were measured according to the method described by Shavrukov *et al.* (2010). Dried leaf samples were digested for 4h, in 10 ml of 1% nitric acid (HNO_3) at 85°C in a 54-well HotBlock (Environmental Express, Mount Pleasant, SC, USA). A flame photometry (Model 420, Sherwood, UK) was used to measure the concentrations of Na^+ and K^+ in the digested samples, by applying the following formula: standard solutions × [(total volume of digest) ÷ (fresh − dry weight of leaf sample)], with standard solution at the concentration of 500 mM for both sodium (Na^+, Cl^-) and potassium (K^+, Cl^-) (Shavrukov *et al.*, 2010). In this way, ion concentrations were expressed as concentrations in the plant sap.

2.2.2.2. *Plant developmental parameters*

In addition to the ion content in the 4th leaf, other phenotypic data were recorded in order to evaluate the impact of salinity on plant development. These data included the dimensions of the 4th leaf blade, the flag leaf, the flag leaf minus one (F-1) and the third awn; the number of tillers and spikelets, and yield components such as biomass, seed number and seed weight. The biomass and grain were weighed using an electronic balance model UW4200H (Shimadzu Scientific Instruments, Japan) and seed counting was carried out using an automated seed counter (Contador, Pfeuffer GmbH, Germany).

2.2.2.3. Automated colour imaging

Plant size was estimated using the automated phenotyping system at The Plant Accelerator® facility (Scanalyzer 3D, LemnaTec, Aachen, Germany). This was done by loading pots manually onto the conveyer belt, which ensured automatic movement to the image capture stations to ensure standardised lighting and imaging conditions. Then, three high resolution visible light (RGB) digital images were taken, including two side (90° from each other) and one top view, thus providing an estimate of plant height and projected shoot area (Berger *et al.*, 2010). The imaging was performed at three time points, corresponding respectively to full emergence of 4th leaves (41 DAS), anthesis (87 DAS) and pollination (119 DAS).

2.2.2.4. Phenotypic data analysis

Phenotypic data were analysed using ANOVA in GraphPad Prism Version 6.07 (GraphPad Prism Software Inc., La Jolla, CA 92037 USA). Fisher's LSD was used at the probability level of P-value < 0.05 to compare stress and control plants and Tukey's multiple comparisons test was used to compare varieties.

2.2.3. MSAP analysis

2.2.3.1.   *DNA restriction and adapter ligation*

The MSAP (Reyna-López *et al.*, 1997, Rodríguez López *et al.*, 2012) was used to perform DNA methylation profiling of barley plants. To ensure marker reproducibility, DNA samples were analysed in two technical replicates. Samples were digested using a combination of a methylation insensitive restriction enzyme *Eco*RI and one of the isoschizomers that shows differential sensitivity to DNA methylation at CCGG sites (*Hpa*II and *Msp*I). Double stranded DNA adapters (Table 2.1) complementary to the restriction products generated by *Eco*RI or *Hpa*II/*Msp*I were ligated to restricted DNA, which was then used as a template to perform two successive PCR amplifications. DNA digestion and ligation of adapters were done in a single reaction, as outlined in Table 2.2. The reaction was incubated in a Bio-Rad T100™ Thermal Cycler (#1861096, Bio-Rad Laboratories, Inc. Australia) for 2h at 37ºC, followed by enzyme inactivation at 65ºC for 10 minutes.

Table 2.1: Adapter and primer sequences used for the MSAP (Rodríguez López *et al.*, 2012).

Oligo name	Function	Sequence
*Hpa*II/*Msp*I adaptor Reverse	Adapter	CGCTCAGGACTCAT
*Hpa*II/*Msp*I adaptor Forward	Adapter	GACGATGAGTCCTGAG
*Eco*RI adaptor Reverse	Adapter	AATTGGTACGCAGTCTAC
*Eco*RI adaptor Forward	Adapter	CTCGTAGACTGCGTACC
Pre-*Eco*RI	Preselective primer	GACTGCGTACCAATTCA
Pre-*Hpa*II/*Msp*I	Preselective primer	GATGAGTCCTGAGCGGC
*Eco*RI-ATG	Selective primer	GACTGCGTACCAATTCATG
*Eco*RI_AAG	Selective primer	GACTGCGTACCAATTCAAG
*Hpa*II/*Msp*I_CCA	Selective primer	GATGAGTCCTGAGCGGCCA
*Hpa*II/*Msp*I_CAA	Selective primer	GATGAGTCCTGAGCGGCAA

Table 2.2: Composition of the master mixture for restriction of genomic DNA and ligation of adapters.
The restriction enzyme *Hpa*II and *Msp*I were used in two separate reactions. The DNA and master mixtures were kept on ice during preparation.

Reagents	Concentration		Quantity per sample	Catalogue No	Provider
	Stock	per reaction			
T4 ligase buffer	10×	1×	1.1 µl	M0202S	New England Biolabs, Australia
*Hpa*II	10000 U/ml	90.90 U/ml	0.1 µl	R0171L	
*Msp*I	20000 U/ml	90.90 U/ml	0.05 µl	R0106S	
EcoRI	20000 U/ml	454.54 U/ml	0.25 µl	R0101S	
T4 ligase	20000 U/ml	90.90 U/ml	0.05 µl	M0202S	
BSA	1 mg/ml	50 µg/ml	0.55 µl	B900S	
NaCl	0.5 M	50 mM	1.1 µl	S5150	Sigma-Aldrich, Australia
Adapter *EcoRI*	10 µM	0.9 µM	1 µl	-	
Adapter *Hpa*II/*Msp*I	10 µM	0.9 µM	1 µl	-	
Reverse osmosis water	Quantity sufficient for 5.5 µl				
DNA sample	10 ng/µl	5ng/ µl	5.5 µl		
Total reaction/sample			11 µl		

2.2.3.2.   *PCR amplifications*

Products of the restriction/ligation were used as DNA templates to perform two consecutive PCR amplifications. In the first PCR amplification (referred to as pre-amplification), primers complementary to adaptors but with unique 3' overhangs (*Hpa*II/*Msp*I primer +C and *Eco*RI primer +A, Table 2.1) were used in a pre-optimised PCR master mix (BioMix™, Bioline,

Meridian Bioscience; Australia) as in Table 2.3. Just 0.5 µl of DNA digestion/ligation product was used for PCR amplification, performed in a Bio-Rad T100™ Thermal Cycler (#1861096, Bio-Rad Laboratories, Inc. Australia). The PCR reactions were performed with the following profile after Rois *et al.* (2013): 72℃ for 2 min, 29 cycles of 30 s denaturing at 94℃, 30 s annealing at 56℃ and 2 min extension at 72℃, ending with 10 min at 72℃ to ensure completion of the extension.

Table 2.3: Composition of the solution for the pre-amplification PCR

Reagents	Concentration		Quantity per sample	Catalogue No	Provider
	Stock	per reaction			
Biomix™	2×	1×	6.25 µl	BIO-25011	Bioline, M B; Australia
*Hpa*II/*Msp*I primer +C	10 µM	0.9 µM	0.25 µl	-	Sigma-Aldrich, Australia
*Eco*RI primer +A	10 µM	0.9 µM	0.05 µl	-	
BSA	1 mg/ml	50 µg/ml	0.1 µl	B900S	N E Biolabs, Australia
Reverse osmosis water	Quantity sufficient for 12.5 µl		5.35 µl		
DNA (restrict. ligation)	5 ng/µl	0.2 ng/µl	0.5 µl		
Total /sample			12.5 µl		

After checking the pre-amplification product for the presence of a smear of fragments (100-500bp in size) by agarose electrophoresis, for example, the second amplification was performed using two selective primer combinations, *Eco*RI_AAG vs. *Hpa*II/*Msp*I_CCA and *Eco*RI-ATG vs. *Hpa*II/*Msp*I_CAA (Table 2.8). *Hpa*II/*Msp*I selective primers were end labelled using a 6-FAM reporter molecule (6-CarboxyFluorescein) for fragment detection during capillary electrophoresis. The remaining components of the reaction were the same as in the pre-amplification reaction, with the exception that the DNA template was 0.3 µl of pre-amplification product (Table 2.8). The selective amplification PCR was carried out in a Bio-Rad T100™ Thermal Cycler (#1861096, Bio-Rad Laboratories, Inc. Australia), with the following cycling conditions (Rois *et al.*, 2013): 94℃ for 2 min, 12 cycles of 94℃ for 30 s, 65℃ (decreasing by 0.7℃ each cycle) for 30 s, and 72℃ for 2 min, followed by 24 cycles of 94℃ for 30 s, 56℃ for 30 s, and 72℃ for 2 min, ending with 72℃ for 10 min.

Table 2.4: Composition of the solution for the selective amplification PCR

Reagents	Concentration		Quantity per sample	Catalogue No	Provider
	Stock	per reaction			
Biomix™	2×	1×	6.25 µl	BIO-25011	Bioline, Australia
*Hpa*II/*Msp*I_CAA or *Hpa*II/*Msp*I_CCA	10 µM	0.9 µM	0.25 µl	-	Sigma-Aldrich, Australia
*Eco*RI_ATG or *Eco*RI_AAG	10 µM	0.9 µM	0.05 µl	-	
BSA	1 mg/ml	50 µg/ml	0.1 µl	B900S	New England Biolabs, Australia
Reverse osmosis water	Quantity sufficient for 12.5 µl		5.55 µl		
DNA (pre-amplification)	-		0.3 µl		
Total /sample			12.5 µl		

2.2.3.3. *Capillary electrophoresis*

Once the selective amplification PCR was completed, MSAP products were separated by capillary electrophoresis on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA) at the Australian Genome Research Facility Ltd (Adelaide node). To perform sample fractionation, 2 µl of the labelled MSAP products were first combined with 15 µl of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5 µl of GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Foster City, CA). This product was then denatured at 95 °C for 5 min and snap-cooled on ice for 5 min. Sample fractionation was performed at 15 kV for 6 s and at 15 kV for 33 min at 66 °C.

2.2.3.4. *MSAP data analysis*

Plant epigenetic profiles were analysed using MSAP fragment sizes between 100 and 550 base pairs. Comparisons of epigenetic profiles of stress and control plants were carried out using both presence/absence and peak height analyses (Rodríguez López *et al.*, 2012).

In the presence/absence analysis, a peak height threshold was set at 150 relative fluorescence units (rfu), and a matrix of DMMs was generated with scores of 1 (present) and 0 (absent). To minimise user bias, peak calling was carried out using unnamed samples and only markers that were consistent in both technical replicates were retained in the matrix to estimate the epigenetic

distance between individual plants, and visualise the data through Principal Coordinate Analyses (PCoA) in

the software package *msap* in R (Perez-Figueroa, 2013, R Core Team, 2016). From the epigenetic distance between barley plants, a pairwise Phi statistic (Phi-ST) (Cramer, 1946, Michalakis & Excoffier, 1996) was performed to determine the proportion of variation amongst and within treatment groups, then the significance of the Phi-ST values was estimated by an Analysis of Molecular Variance (AMOVA) in *msap*, calculating the probability of a null hypothesis (Phi-ST = 0) estimated over 9999 permutations (Perez-Figueroa, 2013). We next selected candidate salt-specific DMMs (i.e., present or absent in one of the salt treatments) based on the presence of differential alleles in at least four out of five samples (frequency ≥ 0.8).

Peak height analysis was performed using fragment fluorescence intensity in the capillary electrophoresis. When the raw peak intensity of fragment fluorescence is converted to a binary matrix in GeneMapper v4 software, any values below the acquisition threshold are returned as an absent locus. As a consequence, presence/absence analyses alone can miss useful information, due to the omitted values and zeros assigned to samples with peak intensity below the threshold (Rodríguez López *et al.*, 2012). The quantitative data analysis aimed at accounting for such software related variability and also methylation patterns in samples due to different cells types (Zhang *et al.*, 2011), which may be a source of variation in monomorphic bands (Rodríguez López *et al.*, 2012).

To account for peak height variations between monomorphic fragments, raw intensity scores were compared between salt treated and control samples. Thus, peak heights were normalised from the model based weighted trimmed mean method derived in Robinson and Oshlack (2010). Normalised peak heights of salt treated and control groups were extracted and compared using the approach described in Robinson and Smyth (2007, 2008). With this method, normalized peak heights were assumed to be distributed as a negative binomial with a common dispersion calculated across the complete set of epialleles for the compared groups. After calculating dispersions of epialleles using the empirical Bayes methods of Robinson and Smyth (2007), a statistical test was then conducted for each epialleles to determine differences in peak heights between salt and control groups (Robinson and Smyth, 2008). The p-values obtained from this statistical analysis were adjusted for multiple comparisons using the false discovery rate (FDR) method of Benjamini and Hochberg (1995). Salt-induced differentially methylated markers (DMMs) were selected at a significance cut-off of FDR < 0.05.

2.3. Results

2.3.1. Effect of mild salinity on barley varieties

2.3.1.1. *Leaf Na$^+$ and K$^+$ contents*

There was a higher Na$^+$ concentration in leaves of barley plants exposed to salt stress compared with the control plants in all eight varieties (Figure 2.1a). The difference between the two treatments was significant at P-value < 0.0001 for all varieties, except Yarra (P-value < 0.001) and Barque 73 (P-value < 0.01) (Figure 2.1a). Regarding leaf K$^+$ content, there was a reduction in salt stress plants compared with the control in all varieties, although this reduction was significant in only three varieties (Maritime, Buloke and Flagship) (P-value < 0.05; Figures 1b and c).

The ratio of [K$^+$]/[Na$^+$] in the leaves was also significantly different between the control and the stress plants (P-value < 0.05, Figure 2.1c). Using one way ANOVA (Tukey's multiple comparisons test) to compare varieties, these ratios were similar across most varieties under control conditions, except that Schooner differed from Commander (P-value < 0.01) (Figure 2.1c). Under stress conditions, Barque 73 stands out as significantly different (P-value < 0.05) from three varieties (Flagship, Hindmarsh, and Schooner; Figure 2.1c), and Schooner differed from two varieties (Barque 73 and Commander, P-value < 0.05; Figure 2.1c).
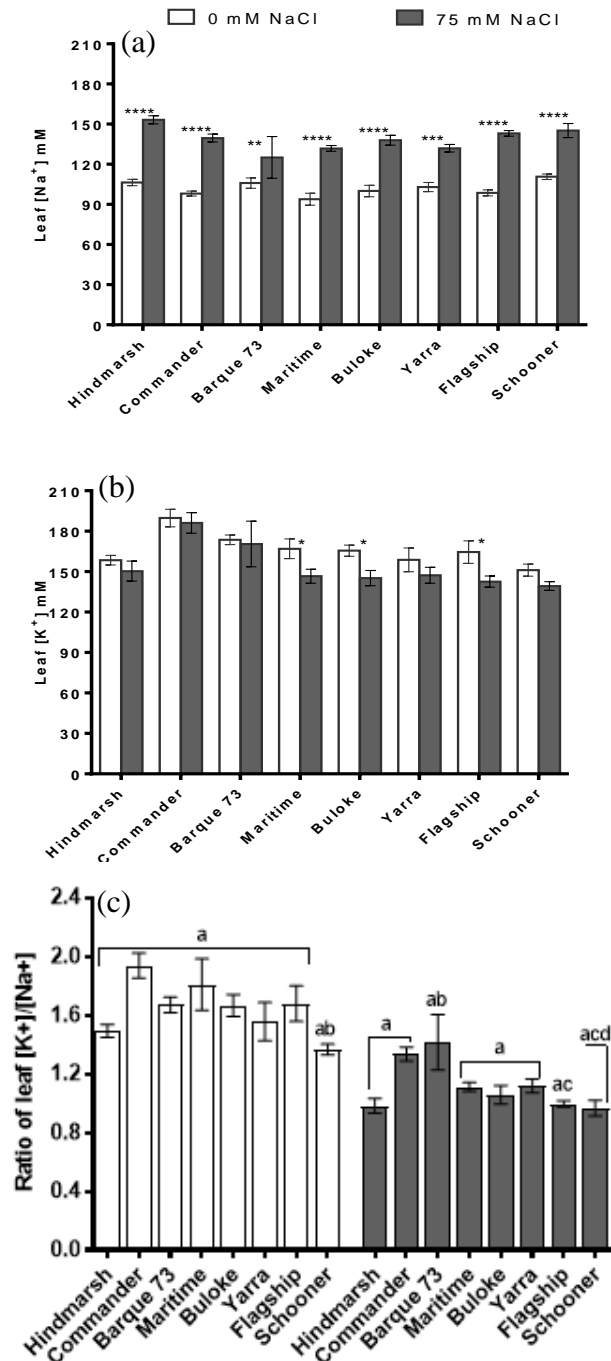
Figure 2.1 Leaf [Na$^+$] and [K$^+$] of eight barley varieties.
(a) Na$^+$ and (b) K$^+$ concentrations (mM plant sap) in the 4th leaf blade of control (0 mM NaCl, white bars) and salt stress (75 mM NaCl, grey bars) plants. (c) Ratio of [K+]/[Na+] in the 4th leaf of the same barley varieties. Varieties with the same letter are not significantly different according to the LSD test (P-value < 0.05). Salt stress was imposed at the barley three-leaf stage (27 days after sowing) in two increments of 37.5 mM NaCl over two days. The 4th leaf blades were sampled 14 days after salt application for measurement of Na$^+$ and K$^+$ concentrations. Values are the mean ± SEM (n = 5). Asterisks (*), (**), (***) and (****) indicate significant differences between treatments at P-value < 0.05, 0.01, 0.001 and 0.0001, respectively (2-way ANOVA, Fisher's LSD).

2.3.1.2. *Projected shoot area, biomass and yield components*

Imaging of barley plants at three time points (41, 87 and 119 days after sowing) showed that the difference in projected shoot area between salt treated (75 mM NaCl) and control (0 mM NaCl) individuals depended on both the variety and the developmental stage (Figure 2.2a-c). At 41 DAS, two varieties (Barque 73 and Maritime) showed a significant difference (P-value < 0.05, n = 3) between plant treatments (Figure 2.2a). At 87 DAS, there were three varieties (Barque 73, Commander and Maritime) that showed significant salt effects on shoot development (P-value < 0.05, n = 3; Figure 2.2b). However, at 119 DAS (anthesis), none of the eight barley varieties showed a significant difference (P-value < 0.05, n = 3) between the stress and the control plants (Figure 2.2c).

Plant height was only moderately affected by 75 mM NaCl (Tables 2.S3 and 2.S4) At 41 DAS (2 weeks after salt application), there was no significant difference between the height of the control and the salt treated plants, regardless of the variety. Unexpectedly, Barque 73, Buloke and Flagship, had treated plants taller than the controls, although the difference was not statically significant at P = 0.05. Commander and Hindmarsh showed a significant difference (P-value < 0.01, n = 3) in plant height between treatments at 87 DAS (Tables 2.S3 and 2.S4).

Shoot biomass at plant maturity revealed that the salt effect on the dry weight differed across varieties (Figure 2.3a). A significant difference (P-value < 0.05, n = 3; Figure 2.3a) between treatments was found in varieties such as Barque 73, Commander, Hindmarsh and Maritime, whereas Flagship, Schooner and Yarra did not produce significantly different shoot biomass under salt and control conditions (Figure 2.3a). As with the results from the biomass, the grain yield was variety dependent, but only Hindmarsh and Commander were significantly affected by salt stress (P-value < 0.05, n = 3; Figure 2.3b). Head production per plant was significantly reduced (P-value < 0.05 n = 3) due to salinity in varieties Barque 73 and Hindmarsh, while this reduction was not significant in the remaining varieties (Buloke, Commander, Flagship, Maritime, Schooner and Yarra).

Relative salinity tolerance was deduced from the biomass and grain yield produced under salt stress relative to the biomass and grain yield produced under control conditions (Munns, 2002). This estimation showed variety specific salt tolerance, which varied between 0.69 (Hindmarsh)

and 1.08 (Schooner) (Figure 2.3c). Based on their relative salt tolerance, varieties were divided into two groups: a group of salt-sensitive varieties with a relative salt tolerance < 1 (Hindmarsh, Commander, Barque 73, Maritime and Buloke) and a group of salt-tolerant varieties with a relative salt tolerance ≥ 1 (Yarra, Flagship and Schooner) (Figure 2.3c).
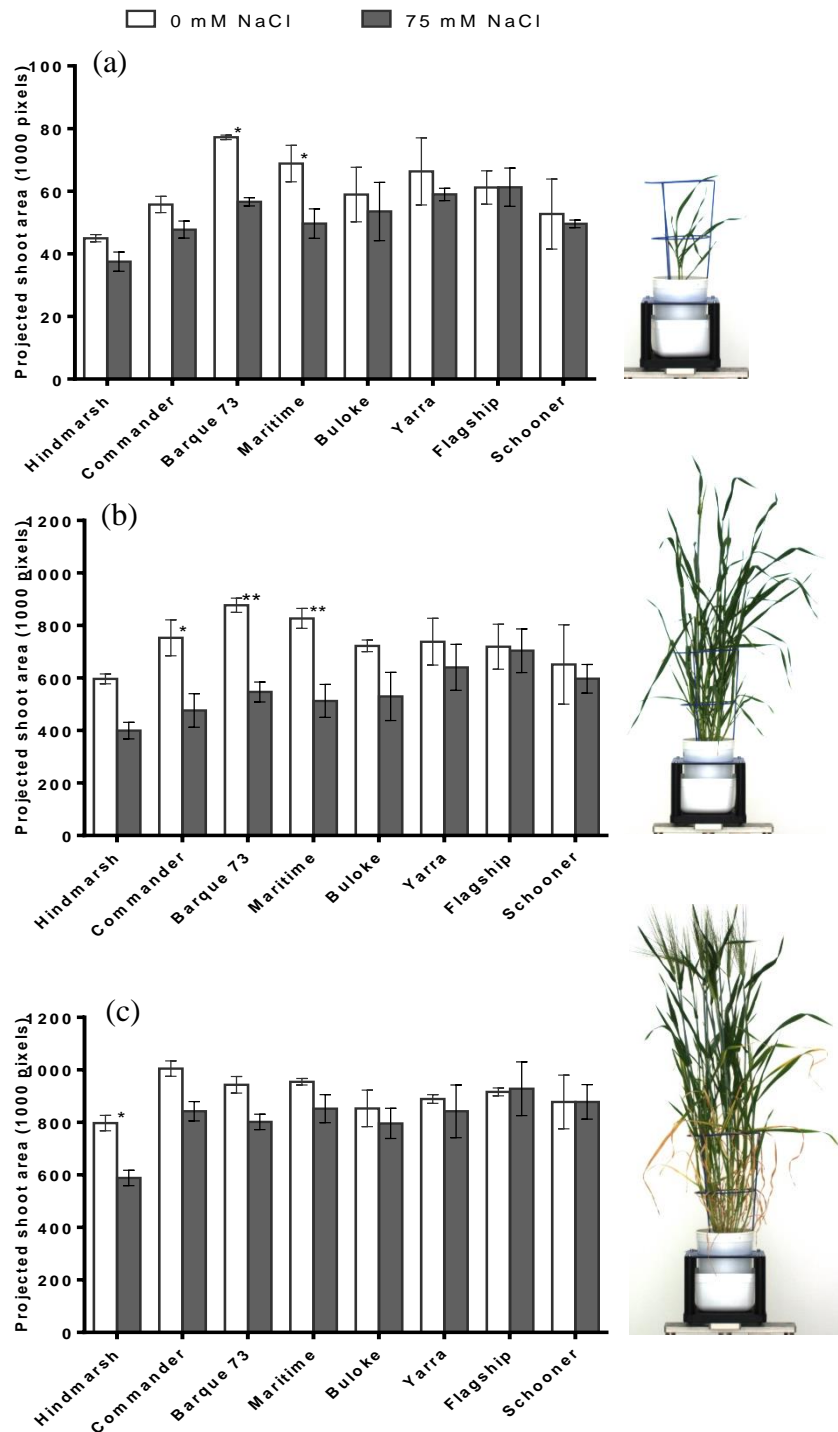
Figure 2.2: Projected shoot area of eight barley varieties under control (0 mM NaCl, white bars) and stress (75 mM NaCl, grey bars) conditions.

(a) at 41, (b) 87 and (c) 119 days after sowing (DAS). The projected shoot area (pixels) was derived from visible light (RGB) images taken at the Plant Accelerator®. Values are the mean ± SEM (n = 3) with asterisk (*) and (**) indicating significant difference between treatments at $p < 0.05$ and < 0.01, respectively (2-way ANOVA, Fisher's LSD). Plant images (variety Commander), exemplar images showing the relative size of barley plants at 41, 87 and 119 DAS.
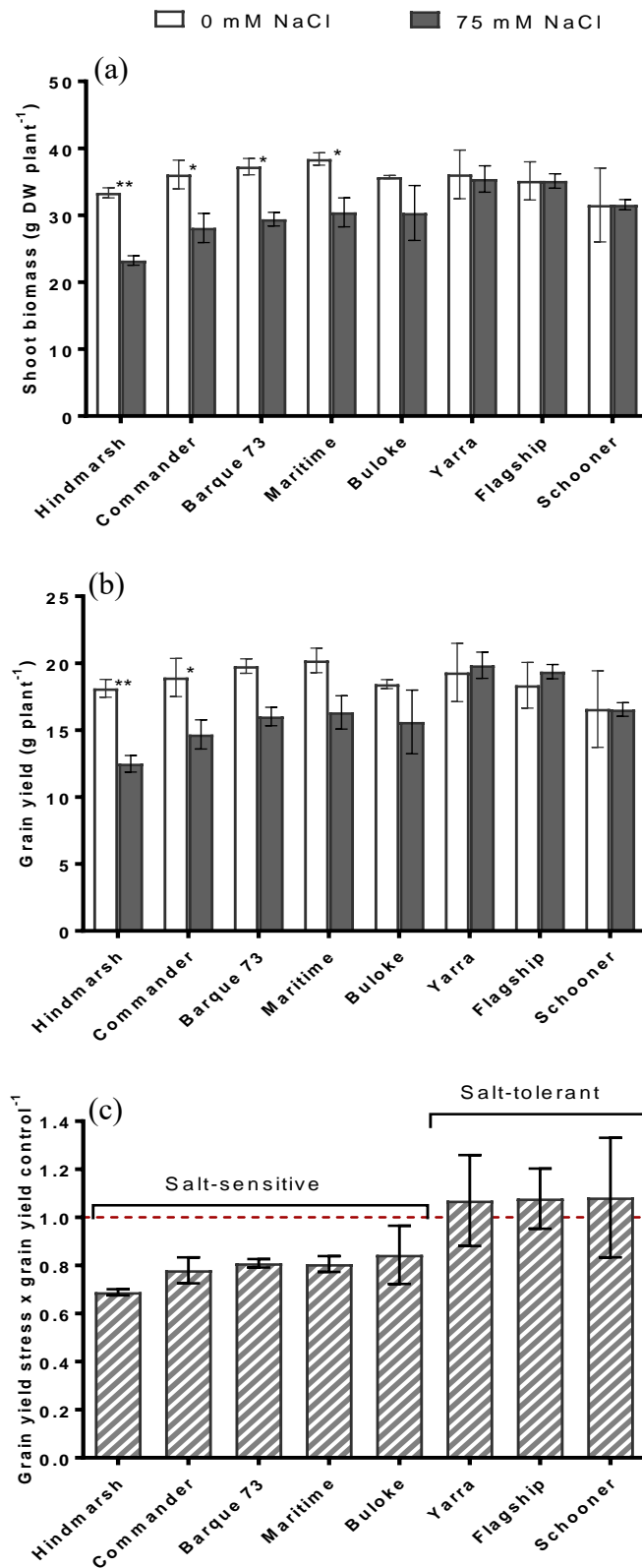
Figure 2.3: Salt tolerance of eight barley varieties.

(a) shoot biomass (g DW per plant); (b) grain yield of eight barley varieties after harvest at maturity in condition of control (0 mM NaCl, white bars) and salt stress (75 mM NaCl, grey bars). Values are the mean ± SEM (n = 3) with asterisk (*) and (**) indicating significant difference between treatments at P-values < 0.05 and 0.01, respectively (2-way ANOVA,

Fisher's LSD). (c) Relative salt tolerance of the varieties, based on the ratio of grain yield of salt stress plant over the grain yield of control plant. Varieties with relative salt tolerance above 1 were considered as salt-tolerant, otherwise they were considered as salt-sensitive.

2.3.2. Salt-induced DMMs

Plant DNA methylation profiles were derived from MSAP data obtained from two primer combinations, *Hpa*II/ *Msp*I-CCA + *Eco*RI-AAG and *Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG, which generated 144 and 125 alleles, respectively, across samples from all eight barley varieties. Of these MSAP fragments, 223 were polymorphic (82.9%). Salt-induced DMMs were obtained from comparison between epigenetic profiles of control and stress samples. To be considered as salt-induced DMM, the presence/absence marker allele should be present in at least four of five samples in the same treatment group and absent in the opposite group. In this way, we identified 19 salt-induced epigenetic markers amongst which nine were from *Hpa*II digestion and 10 from *Msp*I (Table 2.5). The variety Schooner had the highest number of presence/absence DMMs (5) whereas Commander did not have any (Table 2.5). The proportion of qualitative DMMs found in the tissue types were eight in the 4th leaf blade samples, six in F-1, four in the flag leaf and one in the first tiller (Table 2.5).

To account for salt-induced DMMs resulting from variations in peak intensity between control and stress plants, monomorphic alleles were compared and markers that showed a false discovery rate (FDR) below 0.05 were selected at P-value < 0.05. In this way, 24 salt-induced DMMs were found from both *Hpa*II (16) and *Msp*I (8) digestions (Table 2.6).

Of the 43 salt-induced DMMs (including presence/absence and peak height DMMs), 20 were found in the 4th leaf samples (Tables 2.5-6), whereas twelve, eight and three salt-induced DMMs were found respectively in samples from the F-1, flag leaf and tiller 1 (Tables 2.5-6). These epigenetic markers were also variety specific, since there was no DMM that was conserved across all organ types and varieties (Table 2.5-6). Shared salt-induced DMMs were only three presence/absence markers (ATG-CAA_m-356 in Hindmarsh and Yarra, ATG-CAA_m-402 in Maritime and Schooner, and ATG-CAA_m-534 in Schooner and Yarra) and one peak height marker (ATG-CAA_m-500 in Maritime and Schooner) (Tables 2.S1 and 2.S2). The highest number of salt-induced DMMs (14) was found in Yarra, whereas Commander had no salt-induced DMM (Table 2.5-6). The list and fragment size of all salt-induced DMMs are in supplementary Tables 2.S1 and 2.S2.

Table 2.5: Number of qualitative salt-induced DMMs in barley.
Salt-induced epigenetic markers were selected based on their presence in at least four samples of five (frequency ≥ 0.8) while absent in all the opposite treatment (frequency = 0). 4th L = 4th leaf; Til-1 = first leaf of tiller 1; F-1 = flag leaf minus one; FL = flag leaf. Varieties: Barq = Barque 73, Bulo = Buloke, Comd = Commander, Flag = Flagship, Hind = Hindmarsh, Mari = Maritime, Scho = Schooner, Yara = Yarra.

Varieties		Barq	Bulo	Comd	Flag	Hind	Mari	Scho	Yara	Total
HpaII	4th L	0	0	0	0	1	0	2	0	3
	Til-1	0	0	0	0	0	0	0	0	0
	F-1	1	0	0	1	0	0	0	1	3
	FL	1	1	0	0	0	0	0	1	3
MspI	4th L	0	0	0	0	1	1	3	0	5
	Til-1	0	1	0	0	0	0	0	0	1
	F-1	0	0	0	0	0	1	0	2	3
	FL	0	0	0	0	1	0	0	0	1
Total		2	2	0	1	3	2	5	4	19

Table 2.6: Number of quantitative salt-induced DMMs in barley.
Quantitative salt-induced DMMs were detected based on MSAP fragment peak heights in eight barley genotypes (FDR (false discovery rate) < 0.05). The MSAP was performed using DNA samples collected 14 days after salt stress imposition from barley 4th leaf blades (4th L) and first leaf of tiller 1 (Til-1 = 1); and 87 days after salt stress imposition from flag leaf minus one (F-1) and flag leaf (flag leaf). Varieties: Barq = Barque 73, Bulo = Buloke, Comd = Commander, Flag = Flagship, Hind = Hindmarsh, Mari = Maritime, Scho = Schooner, Yara = Yarra.

Varieties		Barq	Bulo	Comd	Flag	Hind	Mari	Scho	Yara	Total
HpaII	4th L	0	0	0	2	0	0	0	7	9
	Til-1	0	1	0	0	0	0	0	0	1
	F-1	0	0	0	0	0	1	0	3	4
	FL	2	0	0	0	0	0	0	0	2
MspI	4th L	0	0	0	0	1	1	1	0	3
	Til-1	0	0	0	0	1	0	0	0	1
	F-1	1	1	0	0	0	0	0	0	2
	FL	1	0	0	1	0	0	0	0	2
Total		4	2	0	3	2	2	1	10	24

2.3.3. Estimation of epigenetic differentiation between salt treatments

Based on qualitative epigenetic markers, pairwise Phi-ST between salt stress and control plants showed a poor differentiation between treatments of barley varieties, regardless of the primer combination used (Table 2.3-4). Only Schooner showed a significant difference between stress and control plants, with a Phi-ST = 0.117 (P = 0.031; Table 2.8). The principal coordinate analyses (PCoA) of qualitative MSAP data did not show a clear grouping of samples according to salt treatments, regardless of the genotype (Figure 2.S1).

Table 2.7: Pairwise Phi-ST (Phi statistics) and P-value (in brackets) between control and salt stress samples (respectively 0 mM and 75 mM NaCl).
The MSAP was performed using the primer combination *Hpa*II/*Msp*I-CCA + *Eco*RI-AAG and DNA samples from barley 4th leaf blades collected 14 days after salt stress imposition. Data were analysed using *msap* software package in R. Pop, population; Phi-ST.

Varieties	Pop	Phi-ST		2.3.3.1.1. Polymorphic loci of 144 alleles			
		*Hpa*II	*Msp*I	*Hpa*II		*Msp*I	
				Samples	Loci	Samples	Loci
All varieties	**2**	0.006 (P= 0.875)	0.005 (P= 0.211)	90	76	88	82
Barque73	2	0.048 (P= 0.758)	0.069 (P= 0.876)	10	41	10	46
Buloke	2	0.036 (P= 0.757)	0.030 (P= 0.300)	10	44	10	43
Commander	2	0.097 (P= 0.986)	0.129 (P= 1)	10	45	8	38
Flagship	2	0.074 (P= 0.914)	0.018 (P= 0.430)	10	42	10	37
Hindmarsh	2	0.028 (P= 0.757)	0.033 (P= 0.265)	10	34	10	44
Maritime	2	0.002 (P= 0.552)	0.005 (P= 0.417)	10	41	10	49
Schooner	2	0.065 (P= 0.114)	0.073 (P= 0.146)	10	53	10	40
Yarra	2	0.042 (P= 0.200)	0.069 (P= 0.931)	10	54	10	64

Table 2.8: Pairwise Phi-ST (Phi statistics) and P-value (in brackets) between control and salt stress samples (respectively 0 mM and 75 mM NaCl).
The MSAP was performed using the primer combination *Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG. And DNA samples from barley 4th leaf blades collected 14 days after salt stress imposition. Data were analysed using *msap* software package in R. Pop, population.

Varieties	Pop	Phi-ST		2.3.3.1.2. Polymorphic loci of 125 alleles			
				*Hpa*II		*Msp*I	
		*Hpa*II	*Msp*I	Samples	Loci	Samples	Loci
All varieties	**2**	0.001 (P= 0.525)	0.004 (P= 0.245)	88	92	88	83
Barque73	2	0.027 (P= 0.693)	0.061 (P= 0.900)	9	58	10	68
Buloke	2	0.068 (P= 0.760)	0.011 (P= 0.349)	10	58	10	62
Commander	2	0.000 (P= 0.468)	0.005 (P= 0.374)	9	63	8	58
Flagship	2	0.087 (P= 0.953)	0.115 (P= 1)	10	60	10	57
Hindmarsh	2	0.027 (P= 0.668)	0.088 (P= 0.071)	10	62	10	57
Maritime	2	0.013 (P= 0.581)	0.006 (P= 0.521)	10	67	10	66
Schooner	2	0.081 (P= 0.065)	**0.117 (P= 0.031)**	10	70	10	58
Yarra	2	0.118 (P= 0.984)	0.086 (P= 0.884)	10	66	10	68

2.3.4. Correlation between salinity symptoms and DNA methylation

The Pearson correlation coefficient was used to estimate the relationship between the epigenetic distances between control and stress plants and salt-induced variations in phenotypic parameters such as $[Na^+]$, $[K^+]$, biomass and yield. The correlation was deemed significant when the absolute value of the coefficient r was ≥ 0.3 ($R^2 \geq 0.09$) (Mukaka, 2012) for at least one of the enzymes used to digest sample DNA (*Hpa*II or *Msp*I). In any case, the highest value between *Hpa*II or *Msp*I was considered for each variety.

The variation in leaf Na^+ concentration between the salt stress and control plants correlated with their epigenetic distance for most barley varieties that have been trialled (Table 2.9), except Commander ($R^2 = 0.012$, Table 2.9). The highest correlation between plant epigenetic profiles and leaf Na^+ concentrations was found in varieties Hindmarsh and Schooner ($R^2 = 0.757$ and 0.656 respectively, Table 2.9). Likewise, there were correlations ($R^2 \geq 0.09$) between epigenetic distances between treatment plants and salt-induced variations in leaf $[K^+]$. Here, Commander also displayed a high coefficient of determination between epigenetic distances and the leaf $[K^+]$

($R^2 = 0.980$, Table 2.9). Biomass and grain yield variations between treatments also correlated with epigenetic distances in a variety dependent manner, with Commander showing the lowest coefficient of determination ($R^2 = 0.110$, Table 2.9).

Table 2.9: Coefficient of determination (R^2) between epigenetic distance and salt-induced variation in leaf [Na^+], [K^+], biomass (Biom) and grain yield (Yield).
R^2 values were estimated from the Pearson coefficient of correlation, computed using the epigenetic distance between control and stress plants, at the 4th leaf stage for [Na^+] and [K^+]. For the biomass and grain yield, the correlation coefficient was calculated using epigenetic distances between treatments at anthesis. Moderate to high correlations are shown in bold; na indicates missing data.

		Barque 73	Buloke	Commander	Flagship	Hindmarsh	Maritime	Schooner	Yarra
[Na^+]	*Hpa*II	**0.608**	**0.563**	0.012	**0.314**	**0.757**	**0.436**	**0.656**	**0.423**
	*Msp*I	**0.360**	**0.212**	na	0.144	**0.757**	0.005	**0.221**	**0.360**
[K^+]	*Hpa*II	0.008	**0.096**	**0.980**	0.026	**0.774**	0.012	0.036	**0.325**
	*Msp*I	**0.810**	**0.176**	na	**0.348**	**0.563**	**0.203**	**0.185**	**0.397**
Biom	*Hpa*II	0.048	0.001	0.017	0.036	**0.706**	**0.774**	**0.240**	**0.090**
	*Msp*I	**0.185**	**0.810**	0.044	**0.922**	0.005	**0.372**	**0.116**	**0.281**
Yield	*Hpa*II	0.002	0.014	**0.090**	0.020	**0.578**	**0.608**	**0.176**	**0.185**
	*Msp*I	**0.203**	**0.706**	**0.110**	**0.706**	0.0361	**0.230**	0.053	**0.490**

2.4. Discussion

2.4.1. The effect of mild salt stress is genotype dependent

In this study, we found that mild salinity impacted on several traits in barley, including Na^+ and K^+ concentrations in leaves (Figure 2.1a-b), projected shoot areas (Figure 2.2), shoot biomass, grain yields and salt tolerances (Figure 2.3) and numerous other phenotypic parameters (Tables 2.S3 and 2.S4). Despite significant accumulation of Na^+ in the leaves of barley under salt stress, compared with control conditions for all varieties (P-value at least < 0.01, Figure 2.1a), their projected shoot area, biomass and grain yield were not affected in the same way (Figure 2.2, Figure 2.3a-b). For instance, six varieties did not show significant differences (P-value < 0.05) in grain yields between treatments (Figure 2.3b), amongst which three (Flagship, Schooner and Yarra) were deemed salt-tolerant (Figure 2.3d). Therefore, it appears that leaf Na^+ content at low salinity levels may not be a good estimate of salt tolerance, which is in accordance with previous studies (Genc *et al.*, 2007, Zhu *et al.*, 2015a). This is particularly true for barley which, compared with other cereals, is known to have a higher tissue tolerance to excess Na^+ (Colmer *et al.*, 2005, Gorham *et al.*, 1990) and a higher ability to selectively partition Na^+ into older leaves and leaf sheaths, and K^+ into growing tissues (Gorham *et al.*, 1990).

Furthermore, leaf K^+ concentration in stressed plants tended to be lower than that in the control plants, however, differences in leaf K^+ concentration between the two treatments were not always significant (Figure 2.1b-c), which may be attributable to the following possible reasons: 1) the level of salt stress applied was too mild for some barley varieties, so that Na^+ did not compete significantly with K^+ uptake; or 2) the barley varieties tested, had the capacity to maintain K^+ uptake despite the salt stress. Yet, in this study, there was no evidence that K^+ uptake was correlated to varietal salt tolerance, as none of the varieties deemed salt-tolerant (Flagship, Schooner and Yarra) showed a significant difference in K^+ concentration between stress and control plants, apart from Flagship (P-value < 0.05, n = 5; Figure 2.1b-c).

Since high levels of salinity can inhibit K^+ uptake (Kronzucker *et al.*, 2006), salt-tolerance has often been estimated based on varieties' aptitudes to maintain K^+ uptake under salt stress compared with salt-sensitive ones (Ali *et al.*, 2012, Munns & James, 2003). This aptitude has been correlated with a relatively high ratio of K^+/Na^+ (Shavrukov *et al.*, 2009), although this has been reported elsewhere to not always result in salt tolerance (Genc *et al.*, 2007, Gorham *et al.*, 1990). In the current study, as the sensitive and tolerant varieties showed roughly similar K^+/Na^+

ratios (Figure 2.1c), it can be argued that estimating salt tolerance based on the K^+/Na^+ ratio alone could be misleading, probably because this is not appropriate for all crops.

Moreover, caution must be observed when screening barley varieties for salt tolerance under mild salinity. It has been reported previously that mild salinity can be beneficial to plant growth, as Na^+ is required in cellular activity, to ensure osmotic potential and maintain turgor (Pardo & Quintero, 2002), especially in conditions of potassium (K^+) deficiency (Maathuis, 2013). In this way, it has been shown that salinity improves barley biomass and grain yield up to 120 mM NaCl, above which the biomass and grain yield declined (Hassan *et al.*, 1970). This salt-induced enhancement was also reported in rapeseed grown in up to a 100 mM NaCl condition (Lu *et al.*, 2007). Therefore, it is difficult to conclude whether varieties deemed salt-tolerant in this study (Flagship, Schooner and Yarra, Figure 2.3a-c) were showing intrinsic salt tolerance or salt-induced improvement of biomass and yield, as reported in previous studies (Hassan *et al.*, 1970, Lu *et al.*, 2007). However, while some barley varieties were enhanced by up to 120 mM NaCl (Hassan *et al.*, 1970), an even lower salt level (70-80 mM NaCl) resulted in significant yield reduction in other barley varieties (Katerji *et al.*, 2006). These contrasting results indicate that there is a varietal determinism in barley's response to salt stress as observed in this study (Figure 2.3a-d), despite barley's overall salt tolerance (Ayers, 1952, Munns & Tester, 2008). This also shows that the effects of salt can depend on the experimental conditions, which may vary considerably between studies.

2.4.2. Salt stress induces both qualitative and quantitative DMMs in barley

Salt stress induced epigenetic changes in plant genomes have been reported frequently (Karan *et al.*, 2012, Lu *et al.*, 2007, Wang *et al.*, 2015a, Wang *et al.*, 2014, Wang *et al.*, 2011a). Salt stressed plants reorganise their methylation patterns as a means to adapt to the external stress (Alvarez *et al.*, 2010, Angers *et al.*, 2010, Boyko & Kovalchuk, 2008). This commonly accepted assumption suggests a clear epigenetic differentiation of stressed plants compared with non-stressed plants.

Using a mild salinity level (75 mM NaCl) to stress barley plants, we found little epigenetic differentiation between control and stressed plants, based on qualitative epigenetic markers (Table 2.7-8, Figure 2.S1). The only variety (Schooner) that showed significant epigenetic differentiation between treatments (Phi-ST = 0.117, P = 0.031, Table 2.8) only had three salt-

induced DMMs (Table 2.5). These results may be due to the low stress levels imposed on the plants (Hassan *et al.*, 1970, Lu *et al.*, 2007), which did not trigger significant epigenetic responses. Similar results were reported by Demirkiran *et al.* (2013), who observed that low salt stress (50 mM NaCl) did not induce any epigenetic signature in barley cultured *in vitro*.

The detection of few salt-induced DMMs seemingly contrasts with previous studies (Marconi *et al.*, 2013, Wang *et al.*, 2015c, Zhong *et al.*, 2009), but highlights the dependence of plant epigenetic responses on the stress intensity and duration (Lu *et al.*, 2007, Soen *et al.*, 2015). In contrast with this study, previous studies generally used high salinity levels to assess salt stress alterations of their epigenetic profiles (Karan *et al.*, 2012, Lu *et al.*, 2007, Marconi *et al.*, 2013, Wang *et al.*, 2015c, Zhong *et al.*, 2009). Such salt levels imposed acute stress on the respective species, to the extent that in some cases sensitive lines died (Karan *et al.*, 2012, Lu *et al.*, 2007). Such high salt stress conditions resulted in the epigenetic divergence previously reported between control and stressed plants (Lu *et al.*, 2015, Marconi *et al.*, 2013, Zhong *et al.*, 2009). In barley, salt-induced DNA methylation was found only at 100 mM NaCl, but not at lower salt concentrations (Demirkiran *et al.*, 2013), as we noticed in some varieties assessed in this study (e.g. Commander) (Table 2.5 and 2.6).

However, qualitative DMMs do not show the entire picture of salt-induced adjustments of plant epigenomes. We found that quantitative epigenetic markers prevail in plant responses to salt stress over qualitative (presence/absence) markers (Table 2.5 and 2.6), suggesting that peak intensity analysis of MSAP markers is necessary in estimating salt-induced DNA methylation changes in the plant. The importance of quantitative markers (peak intensity) in explaining biological states has been demonstrated before (Rodríguez López *et al.*, 2012, Verhoeven *et al.*, 2010), and relies on, 1) the principle of variegation by which a proportion of cells in a given tissue type does not inherit the original epigenetic state through mitotic divisions (Rakyan *et al.*, 2002, Secco *et al.*, 2015); 2) quantitative DNA methylation, which modulates the level of gene expression (not a complete turn-off/on) (Secco *et al.*, 2015). As a consequence, DNA methylation levels will be altered differentially in more or fewer cells depending on the stress intensity and duration (Johannes *et al.*, 2008, Secco *et al.*, 2015).

2.4.3. No universal salt-induced DMMs in barley under mild salinity

In this study, 43 salt-induced epigenetic markers were identified in eight barley varieties (Table 2.5-6). However, none of these DMMs were common to all varieties (Table 2.5-6). There was not enough density in this study to ensure that even the three DMMs present in at least two varieties (Table 2.S1-2), were necessarily identical, due to the anonymous nature of MSAP markers (Reyna-López *et al.*, 1997). Additionally, tissues sampled at the same time-point (4th leaf – tiller or flag leaf -flag leaf minus one) did not share the same salt signatures (Tables 2.S1-2). This might mean that there are specific DMMs in barley organs in response to salt stress. Although organ-specific salt-induced DNA methylation was reported previously in many crops, especially between shoot and roots (Karan *et al.*, 2012, Wang *et al.*, 2015a, Wang *et al.*, 2014, Wang *et al.*, 2011a), it was surprising to detect DMMs between different leaves (Table 2.S1-2). Nevertheless, the DMMs detected support the view that the plant epigenome is responsive to stress, in a variety-dependent fashion. The variety-specificity of salt-induced DMMs undermines their use as universal salt DMMs. It is possible that the salt stress imposed on plants in the current study was too mild to trigger many DMMs, so that the markers that were detected might relate more to genetic rather than epigenetic diversity.

2.4.4. Correlation between salinity symptoms and DNA methylation

Previous studies have demonstrated that modification of DNA methylation resulted in subsequent phenotypic changes (Bossdorf *et al.*, 2010, Cao & Jacobsen, 2002, Finnegan *et al.*, 1996, Zilberman *et al.*, 2007). Alteration of plant methylation profiles upon salt stress (Table 2.5-6), along with physiological adjustments (Figure 1a and b), suggest that DNA methylation may be integral to plant adaptive responses to stress (Boyko & Kovalchuk, 2008, Chinnusamy & Zhu, 2009). In this study, the correlations between salt-induced variations in phenotypic parameters and the epigenetic distance between control and stress plants (Table 2.9), suggest that the aptitude to alter the epigenome during salt stress is a trait that is a function of both the genotype and the environment (Gao *et al.*, 2013, Lu *et al.*, 2015, Verhoeven *et al.*, 2010). Therefore, salt-induced DNA methylation may contribute to the regulation of trait expression, including salt accumulation in leaves, growth rate, biomass and the grain yield (Figure 2.3b).

Additionally, although alteration in one epigenetic locus would suffice to induce adaptive responses to stress (Baek *et al.*, 2011, Tricker *et al.*, 2012), the presence of more than a single

salt-induced DMM in a variety suggests that salinity alters several loci simultaneously (Tables 2.5-6), as previously reported (Karan *et al.*, 2012, Wang *et al.*, 2015a, Wang *et al.*, 2014, Wang *et al.*, 2011a, Zhong *et al.*, 2009). Due to the multi-genic nature of salt tolerance (Flowers, 2004, Roy *et al.*, 2014) and the known involvement of DNA methylation in the regulation of stress-responsive genes (Baek *et al.*, 2011, Hashida *et al.*, 2006, Zhong & Wang, 2007), it is plausible that salt stress instigates methylation changes in numerous genomic positions simultaneously.

Furthermore, despite the relatively low epigenetic differentiation of stressed plants from controls (Tables 2.7-8), there were significant differences in some phenotypic variables such as ion accumulation in leaves (Figure 2.1a-b) and grain yield (Figure 2.3b). Although phenotypic alteration does not necessarily require a high number of DMMs (Cubas *et al.*, 1999, Hashida *et al.*, 2006, Manning *et al.*, 2006, Tricker *et al.*, 2012), DMMs identified here may not represent the cause of phenotypic change. In this way, there was not enough density to rule out the presence of a higher number of DMMs than reported. It has to be remembered that the MSAP inherently detects only a subset of potential markers, through the use of selective primers during PCR amplifications.

2.5.   Conclusion

Physiological and metabolic stress responses in plants, rely on complex epigenetic interactions to mitigate the effect of the external stress (Boyko & Kovalchuk, 2008). On the basis of fragment length analysis, several salt-induced epigenetic markers were found in barley, but these were not constant across varieties. The lack of universal salt-induced DMMs in this study can be due to several reasons. The low salinity level chosen may have resulted in cultivar specific responses, rather than a universal salt-induced epigenetic change. In addition, the use of only two enzyme combinations in the MSAP, which intrinsically captures only a subset of methylation markers (Reyna-López *et al.*, 1997, Rodríguez López *et al.*, 2012), leaves out many markers that could be salt signatures in the plant. Further investigations are required to characterise fragments corresponding to such epigenetic markers, and ultimately determine their functions in the barley genome. To achieve this, one of the best approaches would be to use a Next Generation Sequencing technique, such as the methylation-sensitive Genotyping-by-sequencing (ms-GBS).

Chapter 3:   Patterns of salt-induced differentially methylated markers in barley (*Hordeum vulgare*) genome as revealed by Methylation-sensitive Genotyping-By-Sequencing.

This Chapter contains a manuscript to be submitted for review to the journal "Epigenomes". Therefore, the Chapter is formatted according to the instruction for authors of this journal, except for figure numbers and page headers, which were formatted to facilitate referencing and navigation across the thesis.

Statement of Authorship

Title of Paper	Methylation-sensitive GBS reveals non-CG methylation in gene-body induced by salt stress in barley seedlings (*Hordeum vulgare*)
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	This manuscript describes the use of methylation-sensitive genotyping by sequencing to characterise salt-induced differentially methylated markers (DMMs) in barley seedlings, with the aim to use these markers for discovery of salt-responsive genes. The results showed that salt stress leads to alteration of DNA methylation patterns in a tissue specific manner, and that salt-induced DMMs were associated with over a thousand genes which enriched gene ontology terms associated with plant response to stress.

Principal Author

Name of Principal Author (Candidate)	Moumouni Konate
Contribution to the Paper	Conceived and designed the experiments, conducted the experiments, analysed the results in consultation with co-authors, wrote the manuscript
Overall percentage (%)	50%
Certification	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 27-2-2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Patterns of salt-induced differentially methylated markers in barley (*Hordeum vulgare*) genome as revealed by Methylation-sensitive Genotyping-By-Sequencing.

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Abstract

Salinity in ground water or soil can negatively impact on crop growth and yield. Excess salt concentrations evoke various physiological and molecular responses in the plant to better enable survival and growth under this adverse condition. At a molecular level, *de novo* DNA methylation is known to occur when plants are challenged by various stress conditions and has been increasingly implicated in the regulation of some stress-response genes. However, the role of DNA-methylation in mediating the expression of genes in response to salt stress has been relatively poorly studied among the important food crops, including barley. In this study, we therefore assess the extent of salt-induced alterations of DNA methylation in barley, and examine the correlation between DNA methylation markers and the expression of stress responsive genes. Using methylation-sensitive Genotyping-By-Sequencing, we screened the leaf and root methylomes of five barley varieties grown under control and three salt concentrations (75, 150 and 200 mM NaCl), to seek salt-induced Differentially Methylated Markers (DMMs). Perhaps surprisingly, DMMs that were induced by all salt concentrations, was higher in the leaves than in the roots that were in direct contact with the salt solutions (5,593 and 528 respectively). Furthermore, salt stress increased methylation in leaves but a decrease in methylation in the roots. Taken together, these results indicate that changes to global methylation patterns following exposure occur in a tissue specific manner. DMMs were mostly located in close proximity to repeat elements but included 1094 genes, of which many possessed GO terms associated with plant responses to stress.

Key words: Epigenetics, DMMs, leaves, roots, gene expression, ontology, salinity stress.

3.1. Introduction

Barley is an important crop for food, feed and brewing [1,2], and is used as a model plant for research in temperate cereals [3,4]. Although considered relatively tolerant to salinity [5], barley yield losses can nevertheless be substantial when the crop is grown under saline conditions [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 by activating processes that function in coordination to 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 mechanisms [11].

Epigenetic mechanisms of gene regulation evoke changes to gene expression independently of any change to DNA sequence [12-14]. Of the many epigenetic mechanisms, DNA methylation often plays a critical role in gene expression [13,15,16]. Indeed, DNA methylation is known to be involved in an array of key biological functions, most notably including various aspects of plant development and adaptation to stress [13,17-20]. *De novo* DNA methylation is generally associated with gene repression, while demethylation usually enhances gene expression [16], although exceptions to this rule are known [16,21,22]. There are grounds for characterising changes to the 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 the potential to allow diagnosis of the level of plant exposure to salt stress across its entire root system, based on its molecular response to the stress. This would be difficult to measure in natural soils and allows for differential exposure (for instance through differing root architectures) or sensitivities to the stress to be identified from different individuals. At the same time, knowledge of which genic regions are likely to be methylation-regulated in response to salt stress provides a useful starting point from which to build understanding of the molecular mechanisms in play that influence plant resilience to saline stress; something that has the potential to open up new avenues for crop breeding [11].

Several studies have demonstrated that salt stress can perturb plant methylation profiles [23-26]. Numerous works have correlated stress-induced modifications to DNA methylation to changes in gene regulation across a range of species [14,18,27,28], although some controversy remains over the consistency of the identity of DNA methylation [26,29]. In general, most salt-induced changes to DNA methylation seem to occur within or in proximity to stress response genes [7,23,30,31]. In maize, salinity induced methylation and demethylation, respectively to *zmPP2C* in roots and to *zmGST* in leaves, leading to changed expression levels [32]. 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 [32]. In barley, acute salt stress has been similarly shown to evoke methylation-modulated changed expression of several genes involved in metabolic and physiological processes implicated in the plant's ability to cope with the stress [3,9,33]. However, to date there has been a marked lack of reports linking salt-induced gene expression to global changes in DNA methylation across a representative sample of any crop species.

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 [34]. For this reason, most works on stress-induced methylome change have elected to either target particular loci [27,35] or else to survey only a proportion 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 [23,25,31], in part because of the reproducible reputation of the technique [36-38]. However, the MSAP method only generates relative small numbers of anonymous markers [39,40] and so has limited utility for studies aiming to establish links between changes in methylation and altered gene expression. While some workers have sought to overcome this limitation by targeted sequencing of MSAP amplicons [7,23,30,31], others have argued that this amendment of the method is still cumbersome, costly and time consuming [41]. 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 [41,42]. By using methylation-sensitive restriction enzymes to reduce genome complexity during library preparation, differentially methylated fragments are

produced and appropriate for high throughput sequencing [41,42]. 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 [41].

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 to characterize the genomic locations of such changes. 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.

3.2. Results

3.2.1. 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 platform. A high proportion of the raw reads passed the filter for the presence of the barcoded adapter, the MspI restriction product site and the EcoRI adapter (1,004,318,258; 98.87%). However, when these reads were filtered further to identify those uniquely mapping to the barley reference genome [4], the numbers 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 reference genome (2,828,642; Table 3.1).

Table 3.1: 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

3.2.2. 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 salt treatments (Figure 3.1 and Figure 3.2a). Curiously, the number of leaf DMMs increased with salt concentration (75, 150 and 200 mM NaCl), whereas there was no such correlation from the roots (Figure 3.1). The fold-change in the read counts was next computed between markers in salt-stressed and control plants to study the directionality of DNA methylation flux (hypomethylation or hypermethylation). This revealed that soil salt induces more hypermethylation than hypomethylation in both leaves and roots, regardless of concentration (Figure 3.1). Although the number of salt-induced DMMs was higher in leaves than roots, the intensity of the change evoked by salt stress was higher in roots for both P-values (Figure 3.2a) and the fold-change in read counts (Figure 3.2b-c). Furthermore, comparison of the median fold-change of methylation across all markers in the two organs revealed that overall, salt induces hypomethylation in roots and hypermethylation in leaves (Figure 3.2a-c).
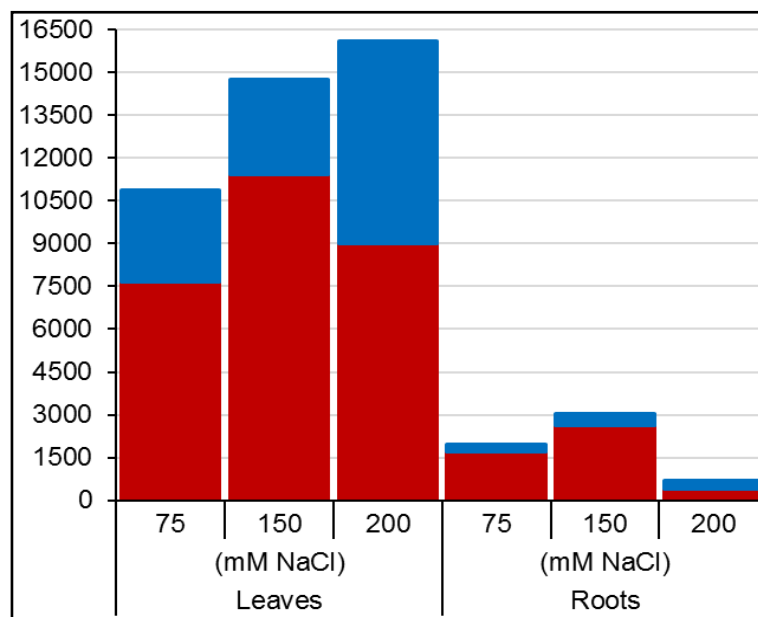
Figure 3.1: Number of salt-induced differentially methylated markers (DMMs) in barley leaves and roots.
Samples from barley plants exposed to 75, 150 and 200 mM NaCl were compared with salt-free control plant samples. The red and blue sections in the bar chart represent the proportion of salt-induced 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).
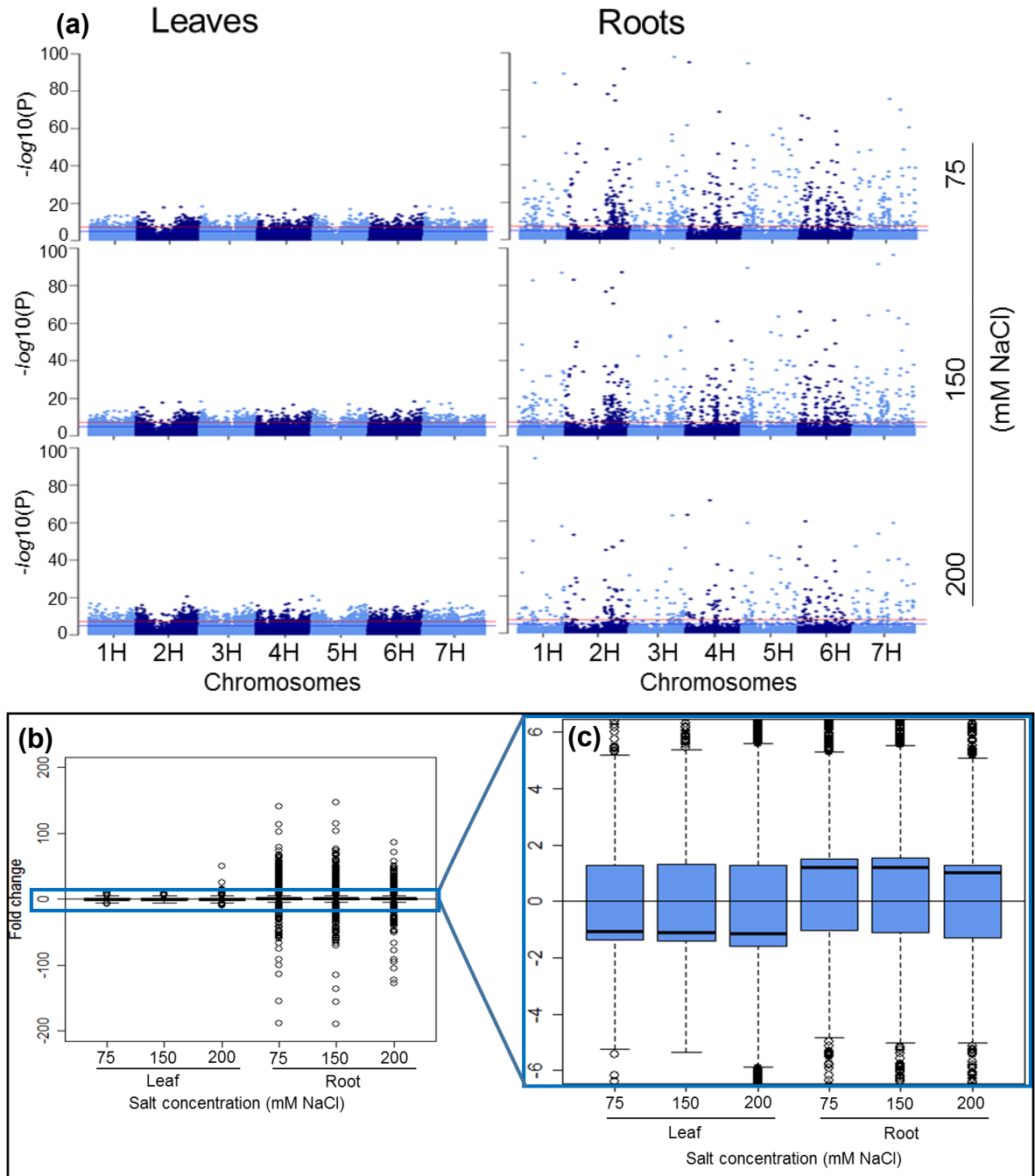
Figure 3.2: Tissue-specific response intensity and directionality of salt-induced DNA methylation changes.
(a) Distribution of salt-induced epigenetic markers in the barley genome. Each point represents the genomic location (horizontal axis) of a marker and its associated negative *log*10 P-value (vertical 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 7, 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 markers were obtained from sequencing of *Msp*I restriction products, which provides more reads when the locus is unmethylated. 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).

3.2.3. Stability of salt-induced DMMs across treatments

We next investigated the stability of DMMs across treatments and organs. A high proportion of DMMs failed to appear across all salt concentrations (Figure 3.3a-b). Moreover, the 24,395 salt-induced DMMs detected in leaf samples included 2,390, 4,070 and 6,202 that were specific to 75 mM, 150 mM and 200 mM NaCl respectively (Figure 3.3a), suggesting a positive association between the increased number of salt concentration-specific DMMs and increasing salt concentration. In roots, there were 633, 1,642 and 88 salt-concentration-specific DMMs for 75 mM, 150 mM and 200 mM NaCl, respectively (Figure 3.3b). In this case, there was no positive correlation between the number of concentration-specific DMMs and salt levels.

There were nevertheless stable markers that appeared in all salt concentrations but were absent from the control treatments. 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 found from roots (Figure 3.3a-b). These dose-insensitive DMMs invariably presented the same directionality of methylation change (i.e. always hyper- or hypomethylated) following exposure to any salt concentrations (Figures 3.4a-b). Dose-insensitive DDMs that exhibited hypomethylation following exposure to salt predominated in both leaves (4744, 84.82%) and roots (329, 62.31%). Just 22 of the dose -insensitive DMMs were shared between leaf and root samples (Figure 3.4c). These markers invariably shared the same directionality of methylation change following salt exposure within organs and 20 of the 22 were also conserved between organs. However, two markers ("2:1:467135271" and "6:1:259709553") became hypermethylated in leaves but hypomethylated in roots following exposure to salt (Figure 3.4c).
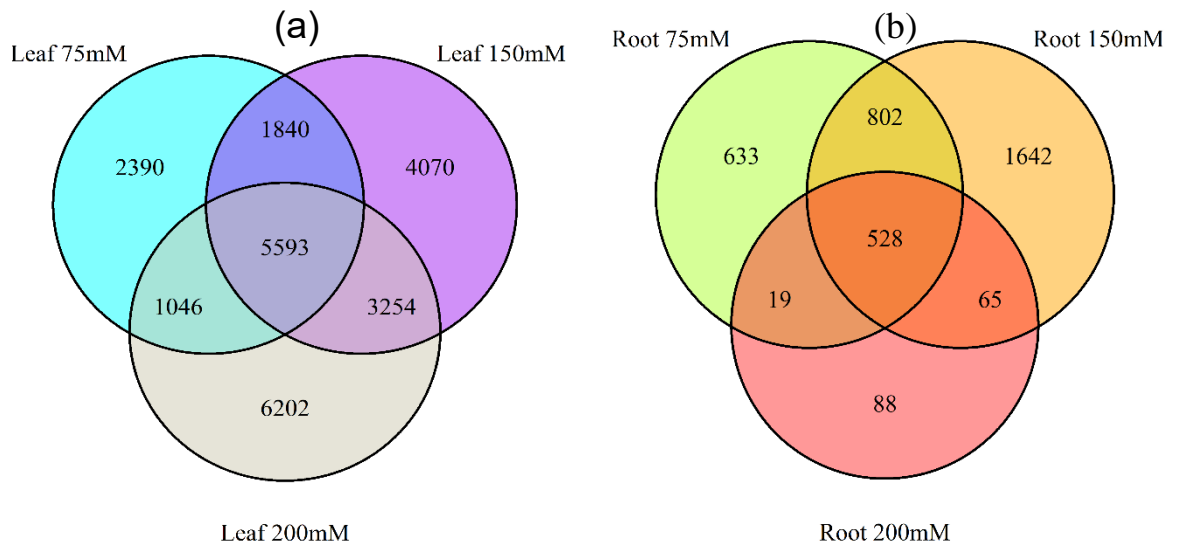
Figure 3.3: Venn diagram showing the number of differentially methylated markers (DMMs) induced by different salt concentrations in barley leaves and roots.

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.
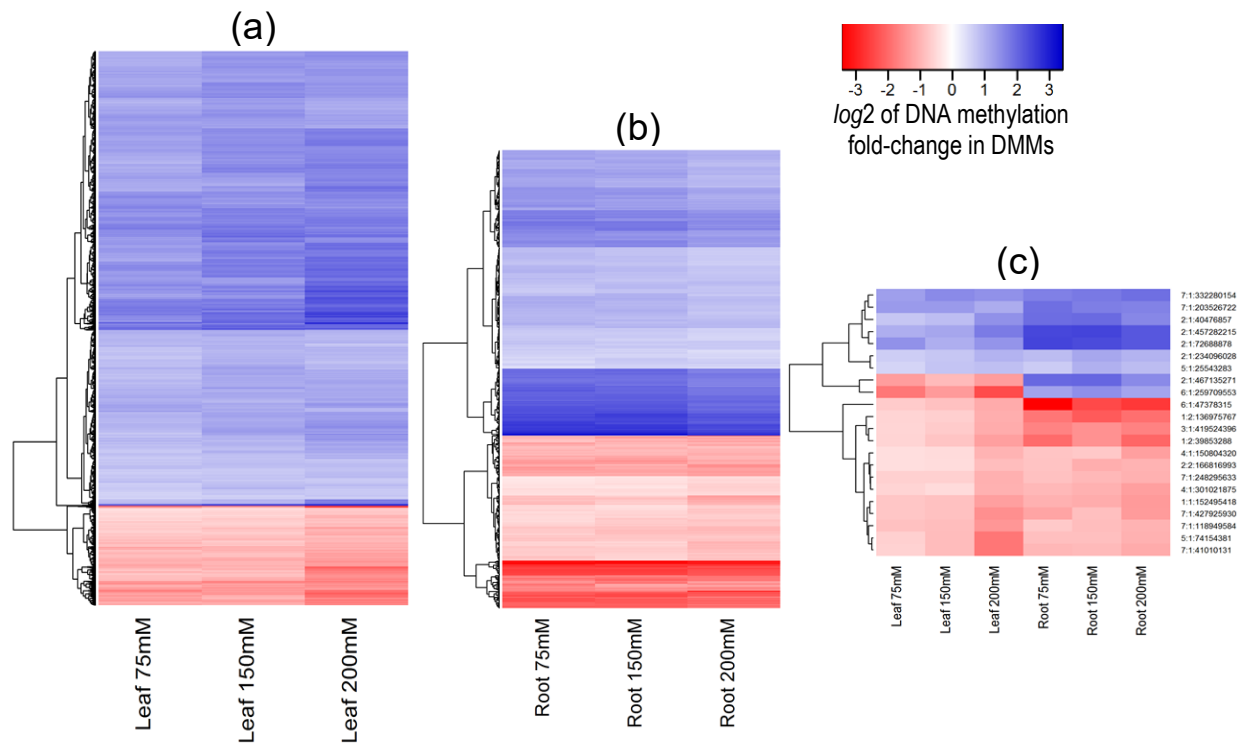
Figure 3.4: Hierarchical clustering of the fold changes in read counts of DMMs stable across salt concentrations.
(a) in leaves (5593 DMMs); (b) in roots (528 DMMs); (c) shared by both leaf and root tissues (22 DMMs). Stable DMMs refer to those conserved across the three salt treatments (75, 150 and 200 mM NaCl). 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).

3.2.4. Distribution of salt-induced DMMs around repeat[1] regions and genes

We assessed the distribution of DMMs relative to annotated features (e.g. protein coding genes, repeats, tRNAs etc) of the barley genome. To do so, DMMs induced by 150 mM NaCl were used. It appeared that proximity to a repeat sequence was a strong factor determining the distribution of DMMs induced by salt. Indeed, 96.5 % of DMMs induced by salt in leaves and 99.8% in roots occurred either within repeats themselves or within 1 Kb of them (Figures 3.5a-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 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 3.5c; Supplemental Data 2), with the majority located within the gene-body itself (56.4%, 603 DMMs; Figure 3.5c). 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 3.5d; Supplemental Data 2). Additionally, it is worth mentioning that of the 22 dose-insensitive DMMs shared in leaves and roots (Figure 3.4c), 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 [16,43], 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 3.6a). Outside these windows, DMMs occurred in the range 40-65 DMMs per Kb (Figure 3.6a). 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), decreasing gradually to reach background levels (50-70 DMMs per KB) after 4 Kb (Figure 3.6b). 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.

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[1] We used "repeat regions" as defined in the barley reference genome "ASM32608v1" in Ensembl database, and may include TEs, SSRs, telomeres, centromeres and minisatellites (http://plants.ensembl.org/Hordeum_vulgare/Info/Index).

The majority of DMMs within gene-bodies from leaf samples lay within exons (81.4%, 498 of 612; Figure 3.6e). The remaining DMMs were generally within 1 Kb near an exon (Figure 3.6e). Three out of the five gene-body DMMs from roots were similarly exonic or within 1Kb (Figure 3.6f). 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 3.6ef). Additionally, there were 41 DMMs from leaves and two DMMs from roots DMMs that clustered around tRNA genes (Figure 3.6gh). 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) (Figure 3.6g). The two DMMs near tRNA genes in roots were within 1 and 4 Kb downstream (Figure 3.6h).
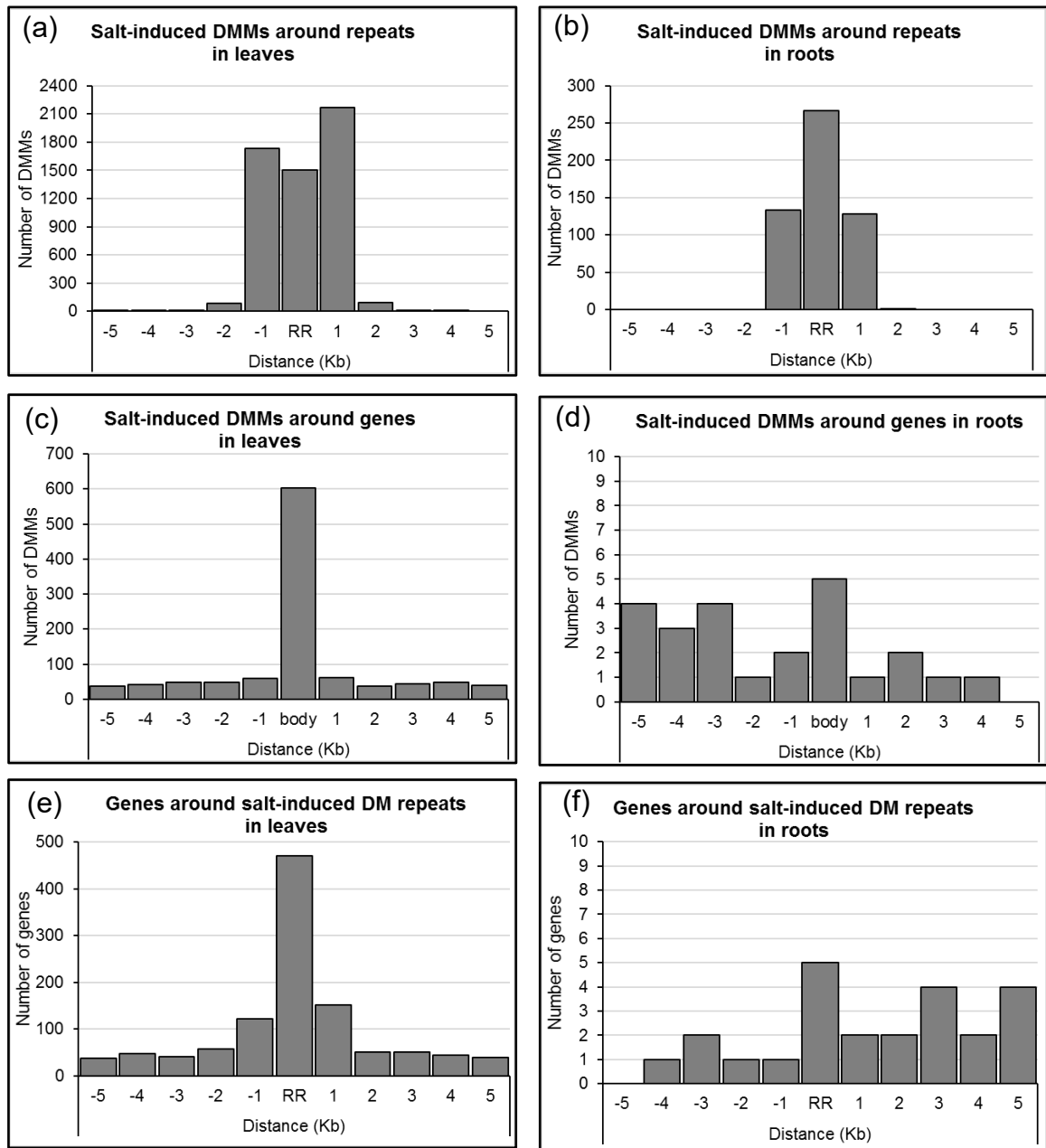
Figure 3.5: Distribution of salt-induced differentially methylated markers (DMMs) around repeat regions and genes.
(a, b) distribution of DMMs distance from the closest repeat in leaves and roots, respectively; (c, d) distribution of DMMs distance from the closest gene in leaves and roots, respectively; (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.
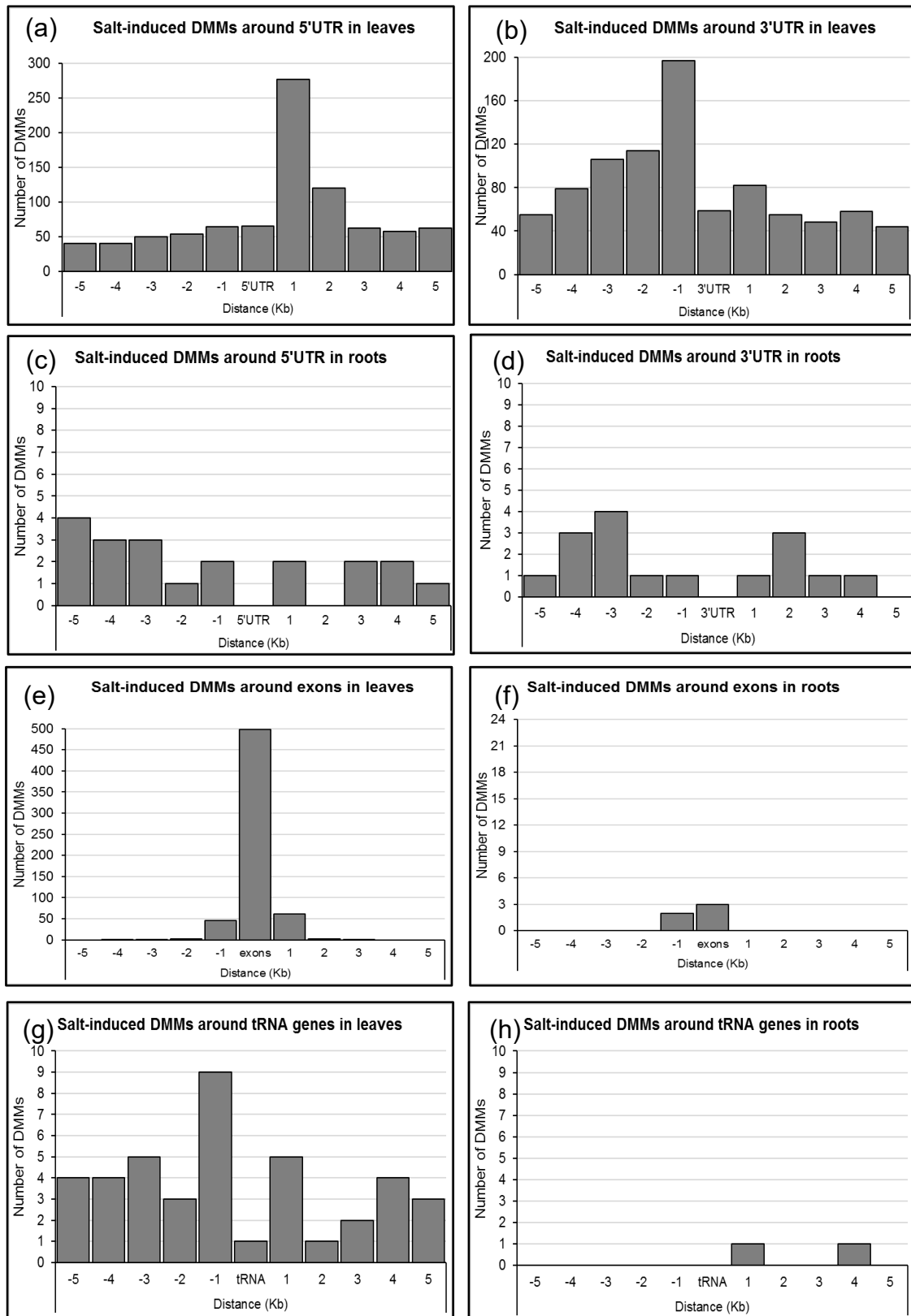
Figure 3.6: Distribution of salt-induced differentially methylated markers (DMMs) around UTRs, exons and tRNA genes.
(a, b) 5`UTRs and 3`UTRs in leaves; (c, d) 5`UTRs and 3`UTRs in roots; (e) exons in leaves; (f) exons in roots; (g) tRNA genes in leaves; (h) tRNA genes in roots; The distance of each

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.

3.2.5. Gene ontology analysis of salt-induced DMMs

Gene Ontology (GO) analysis was performed for all salt-induced differentially methylated genes from both leaf 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 3.1). 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 3.7a, Appendix 2). 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 3.7b, Appendix 2).

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 3.2). 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 3.8a, Appendix 2). 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 Appendix 2.

These GO terms, enriched from differentially methylated genes, gave 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" and so forth, among others (Appendix 2).

Table 3.2: 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/).

	Genes	GO terms per GO group			Total GO terms
		Biological process	Cellular component	Molecular function	
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
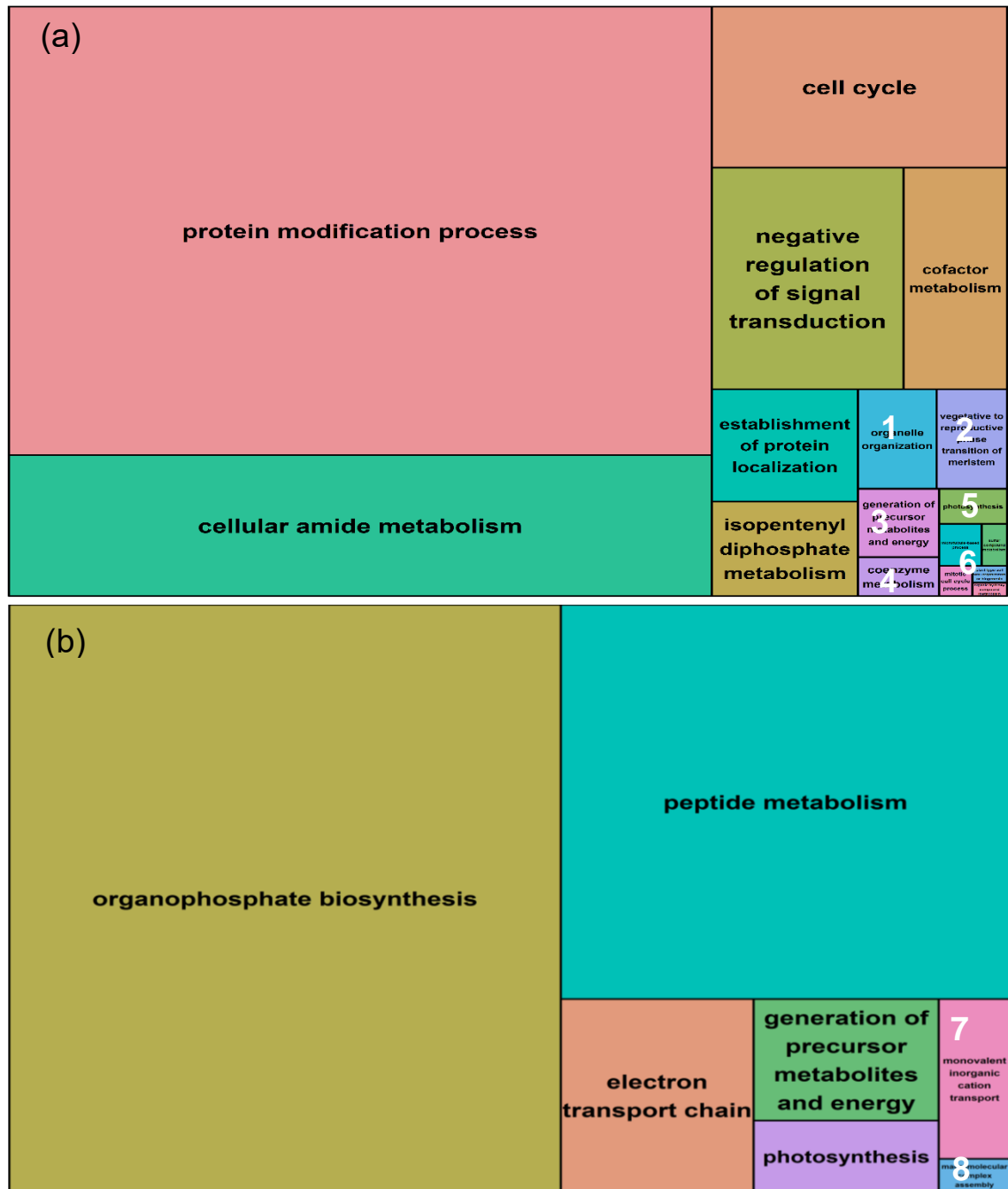
Figure 3.7: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially methylated genes in barley leaves. (a) Representatives of GO terms enriched by hypomethylated genes in leaves; Numbers represent GO term representatives with invisible 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/). The detailed list of terms in the background of GO representatives is provided in the Appendix 2.
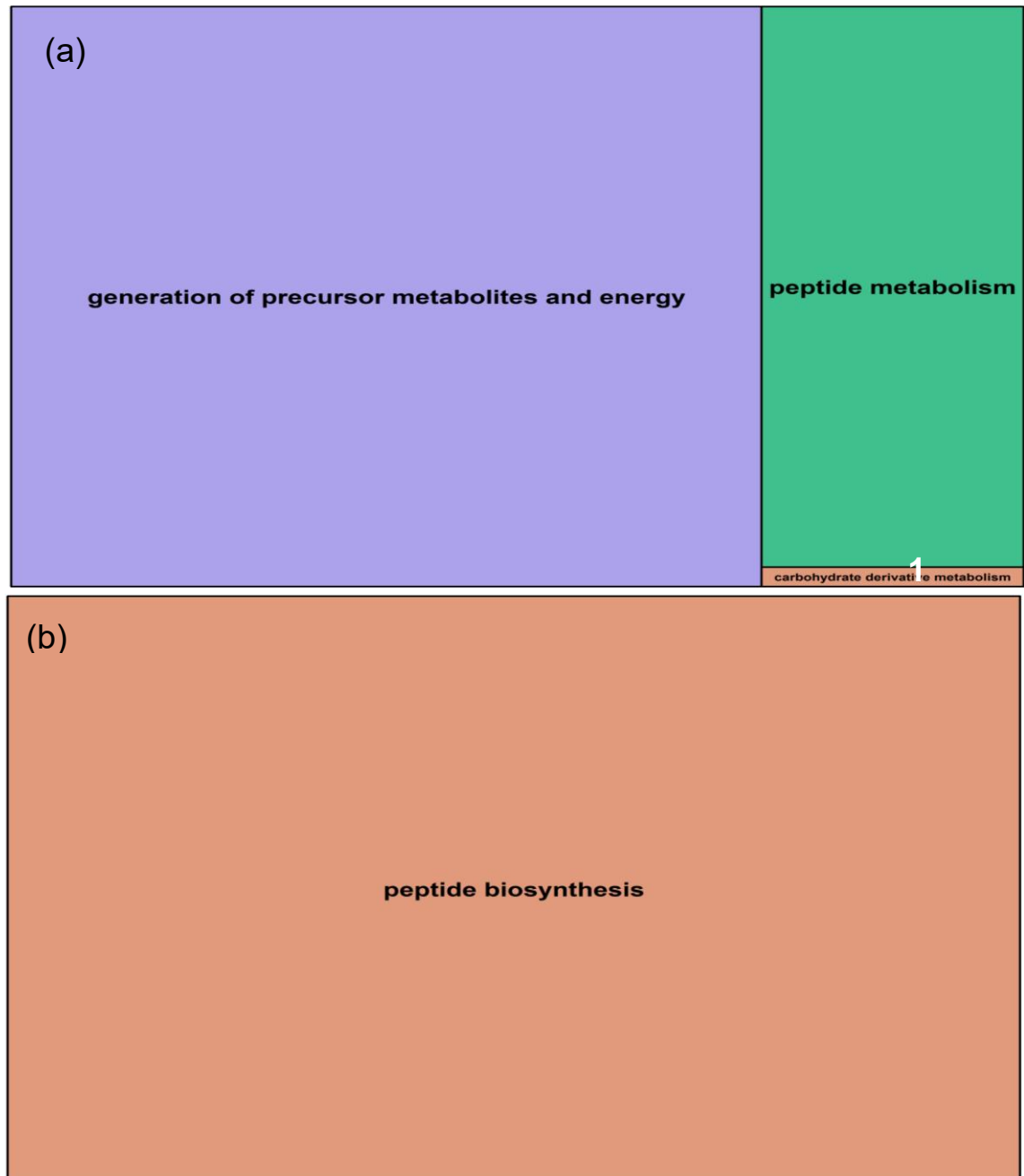
Figure 3.8: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially methylated genes in barley roots: (a) Representatives of GO terms enriched by hypomethylated genes in roots; 1 = carbohydrate derivative metabolism; (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 Appendix 2.

3.2.6. Differentially expressed genes in barley roots

To investigate whether salt-induced DMMs correlated with publicly available gene expression responses to salt exposure. Datasets of these samples, included four biological replicates and two genotypes (Sahara and Clipper) (see material and methods). Differential gene expression between salt treatments revealed 124 upregulated and 34 downregulated transcripts (Appendix 5), 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 3.9, Appendix 5). 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 searched for differentially expressed genes that presented DMMs in the current study. This was assessed by seeking DE genes within 5 Kb flanking DMMs, either side of the marker. Since there were no differentially methylated genes amongst DE genes with FDR below 5%, we extended the gene list by reducing the stringency of the FDR cut-off to 10%. With this setting, seven DE genes were found differentially methylated, one of which contained two DMMs (MSTRG.43260, one hypo- and one hypermethylated) (Table 3.4). However, there was no correlation between their 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 3.4). 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 (Appendix 5).

Table 3.3: 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 GO terms
	Total transcripts	Annotated	Biological process	Cellular component	Molecular function	
Upregulated	124	76	94	22	29	145
Downregulated	34	18	23	12	0	53
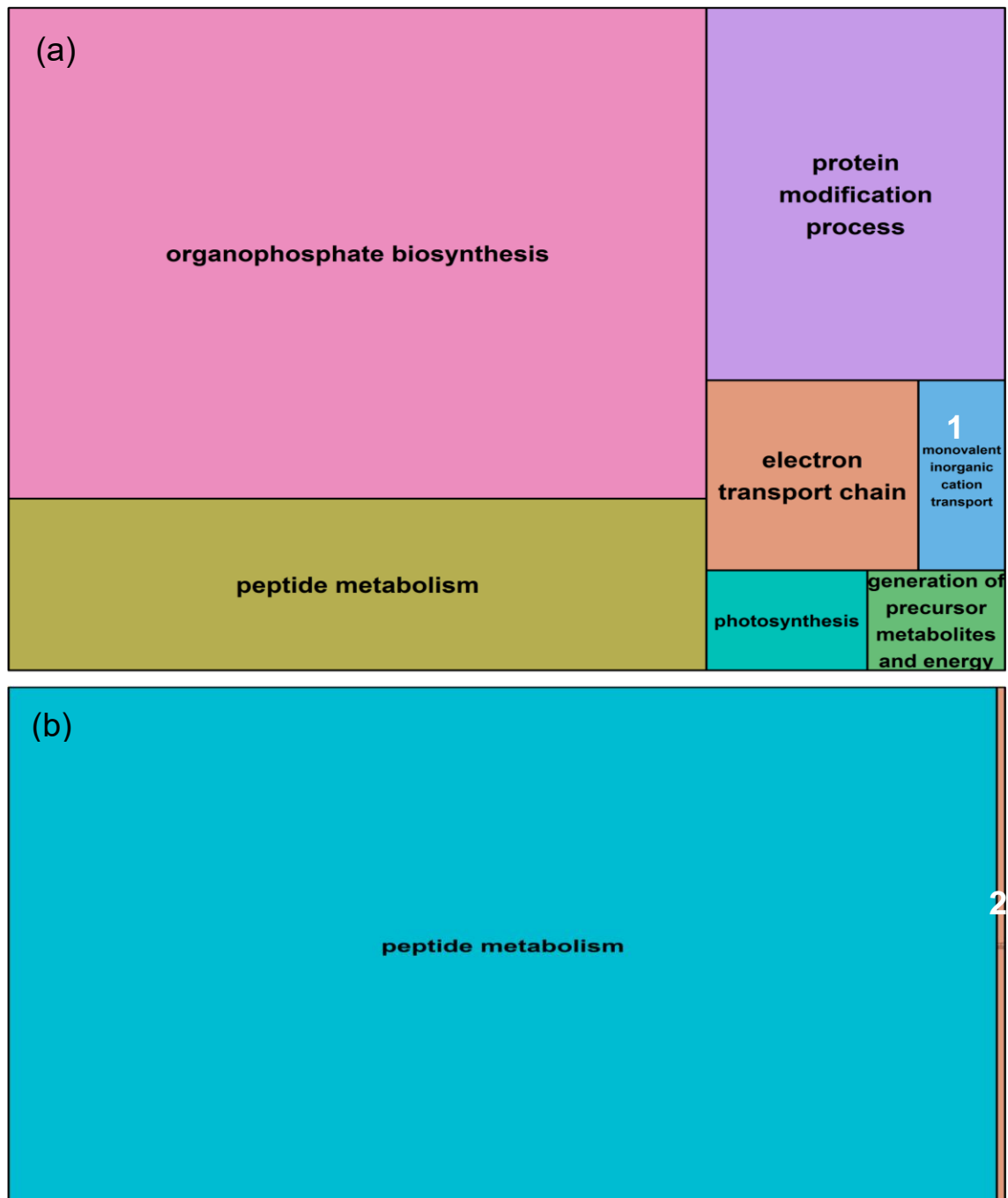
Figure 3.9: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from salt-induced differentially expressed genes in barley roots. (a) Representatives of GO terms enriched by upregulated genes in roots; 1 = monovalent inorganic cation transport; (b) 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 Appendix 5.

Table 3.4: 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

3.3. Discussion

A growing number of studies highlight the role of DNA methylation in mediating the adaptive response of plants to stress [14,28,29]. The primary challenge, particularly for crops with large genomes, rests 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 [7,23-25,31,44]. 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 representative portion of the genome (Figure 3.2). Application of this approach allowed us to characterize salinity-induced methylation flux in both leaf and root samples, and then to relate the pattern of Differentially Methylated Markers to specific genomic features.

3.3.1. Salt-induced DMMs are not that stochastic

Of the salt-induced DMMs identified in barley seedlings, only a small proportion were dose-insensitive (and so conserved) across all salt treatments, with the remaining markers being either concentration-specific or shared between two salt treatments (Figure 3.3a-b). The prevalence of concentration-specific DMMs in barley leaves (18,802 out of 24,395 DMMs, 77%) and roots (3,249 out of 3,777 DMMs, 86%) (Figures 3.3a-b) could imply that these salt-induced DMMs occur stochastically, consistently with observations made in previous studies [23,26]. However, the positive correlation between concentration-specific DMMs and salt levels in leaves (Figure 3.3a) suggests that there is some structure to the appearance of these DMMs. The pattern of increasing abundance of DMMs with increasing salt concentration rather suggests that a large number of DMMs only become activated above a threshold concentration of salt; a theory previously hypothesized by Soen and co-workers [45]. 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. Additionally, we also identified a substantial number of DMMs that were insensitive to salt concentration but whose identity was organ-specific (Figure 3.3). It could be postulated that if such markers are hypersensitive and so change methylation status in response to low salt thresholds, then they would appear in all salt treatments but not in the controls. These DMMs would therefore provide a robust indication of exposure to salt. This shows that salt-

induced markers (at least a substantial number) target specific loci, and do not appear stochastically. Instead, they are likely to accumulate in a progressive fashion and we speculate that many could fulfil a function in the plant's adaptation to different levels of salt stress.

3.3.2. Salt-induced DMMs are more abundant in leaves but more intense in roots

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 compared with roots [23-25,31,46-49]. 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 [23]. 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 3.2), 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 [8,50]. Nevertheless, as salinity also imposes an increasing stress in leaves, due to ion accumulation after prolonged exposure to salt [5,51], the response in leaves is more widespread, although more measured.

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 [7,23-25,31,44]. However, in barley we found that the proportion of *de novo* methylation and demethylation events varied in the same manner in both roots and leaves, with a prevalence of hypermethylation in both organs, albeit at different frequencies (Figure 3.1). It is possible that divergence between our findings and those of previous studies [7,23-25,31,44] may simply be a feature of the crop. However, it is also possible that the trend towards hypermethylation 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 [23-25]. Most studies of salt-induced DNA methylation have relied

on MSAPs to assess flux in DNA methylation [7,23-25,31,44]. However, MSAP generates anonymous markers and lacks resolution in showing whether there is a gain or loss of methylation in markers [39].

Beside organ-specific methylation levels, there was a positive linear correlation between the salt concentration and the abundance of salt stress related DNA methylation in leaves, but not in roots (Figure 3.1). This gradual epigenetic response to salt stress in leaves is concordant with a previous study, showing that salt concentrations correlated with differential DNA methylation in rapeseed [31]. Roots seemingly lacked this relationship, possibly because of the low number of loci involved but equally plausibly because of DNA fragmentation at high salt concentrations [31,52-54]. 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 genetic mutations and/or DNA fragmentation have previously been reported in Arabidopsis [52], onion [53], rapeseed [31] and barley [54], suggesting that this is not an isolated phenomenon. Resolution of these alternatives requires further investigation.

3.3.3. Salt-induced DNA methylation may be involved in gene regulation

DNA methylation regulates genomic activity in three ways: *de novo* methylation (hypermethylation), methylation maintenance, and methylation removal (hypomethylation) [55]. Modification of DNA methylation in response to stress is thought to be directed (at least partially) to specific genomic regions where the methylation status of the DNA acts to regulate genes implicated in the plant's response to the stress [18,28,56,57]. Our results provide some support for this assertion since salt-induced DMMs in barley clustered around repeat regions (Figure 3.5ab) but also genes (Figure 3.5cd), 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 plant molecular responses to salinity [57]. There is indeed 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 [23,24,27,32,56-58]. The implications of DMMs in mediating metabolic responses to saline conditions depends partly on the position of specific DMMs relative to the target genes [19,59-61]. The clustering of DMMs around Un-Translated Regions (UTRs) and exons (Figure 3.6) in the present work is therefore consistent with the possibility that salt-induced DMMs may have a role in mediating a functional response to the stress. This possibility

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has been highlighted previously by others. For instance, the high frequency of salt-induced DMMs in gene extremities (towards 5`UTR and 3`UTR) has been shown to influence gene regulation by affecting through 5`UTRs' and 3`UTRs' closed-loop regulation systems, which generate inactive transcripts [62,63]; or through independent gene regulation by each UTR type [64]. Karan et al. [23] similarly observed that salt-induced DNA methylation changes generally occurred in exon and UTR regions and could affect diverse biological functions in the plant [23]. There is also a strong body of evidence suggesting that gene-body methylation can affect gene expression [19,59,60], by enhancing or inhibiting transcription and translation processes [62-64].

It has been claimed that, of all cytosine contexts, only ^{m}CG methylated occurs within gene-bodies [60,65-67]. Our results 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 3.5cd). It is still open to question whether these markers, like ^{m}CG, play a role in regulating gene expression [68]. We also observed salt-induced DMMS associated with tRNA genes (Figure 3.6d), perhaps supporting the suggestion of a role for methylation dependent regulation to support the RNA quality control system and protein synthesis [69-71].

3.3.4. Salt-induced DMMs correlate with stress related genes

Salt stress in barley has been shown previously to alter the expression pattern of genes involved in diverse 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", suggests that salt-induced DMMs target genes that play crucial functions for a plant under salt stress [27,72-74]. Some of the DM genes enriched GO terms such as hydrolase activity, oxidoreductase activity, nucleic acid binding, and translation factor activity (Figure 3.7; Appendix 2), which were reported before as from genes differentially methylated by salt stress in rice [23,24].

This study also revealed a presumed role of DNA methylation in the expression of genes involved in organophosphate biosynthesis process (Figure 3.7). This result aligns with previous

studies showing that salt stress induced 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 [75,76]. Furthermore, it was reported that salinity induced inorganic phosphate toxicity when Pi exceeds 0.10 mM in the substrate [77,78]. 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 solutes [75,77].

Therefore, even uncoupled from expression analysis, the presence of DMMs near a gene may be an indication of responsiveness to salt stress. However, this is not sufficient evidence of DMM involvement in the process of gene regulation [21,61]; gene expression analysis is required to assess the link between DNA methylation and gene activity under salt stress.

Contrary to expectations, only seven differentially expressed genes in roots were differentially methylated under salt stress (FDR < 10%, Table 3.4). This result may be attributable to the fact that different biological samples were used for methylation profiling and gene expression analyses. While DM genes were characterised in roots of three weeks old barley seedlings grown in soil substrate, DE genes were identified in roots from three-day old germinating seeds in a saline nutritive solution in vitro [3]. Also, this limited number of DM genes among differentially expressed genes in roots might result from the reduced number of DMMs in roots, perhaps biased by salt-induced DNA degradation [31,52-54]. Nevertheless, an interesting finding was the enrichment of plastid regulation pathways by these DM genes in roots. As prior studies showed that salt-stress impaired amyloplast (root plastid) development, resulting in atrophy of thylakoids and their starch grain contents, due to osmotic stress in roots [79-81], the occurrence of salt-induced DMMs in a gene encoding amyloplast suggests that DNA methylation may be involved in gene regulation. Therefore, the regulation of amyloplast development in roots during salt stress is a requisite for the plant to adapt to the stress [79-81], and this seems to involve DNA methylation.

3.3.5. Conclusion

This study has shown that salinity acutely alters the plant methylation profiles of barley DNA samples secured from leaves and roots. The number and scale of salt-induced DMMs varied according to organ identity, and their appearances were either dependent on salt concentration or salt concentration-independent. These observations of flux in the plant methylation profile in

response to salt stress are at least consistent with the presence of a methylation-based molecular mechanism for sensing and responding to salt stress. Salt-induced DMMs seem to favour repeat regions, and genes whose function accords with that needed for metabolic adjustment of the plant to accommodate for the presence of salt. Many of these DMMs, which were in ^mCCGG context, overlapped with genes, indicating that gene-body methylation is not restricted to ^mCGs.

However, one limitation of this study was the use of different datasets to investigate the role of DMMs in gene expression. However, this limitation was considered minimal because genetic differences between barley varieties have been found to be minor [3]. Also, since barley lacked a completely annotated map of the reference genome, the gene ontology analysis for differentially expressed transcripts in roots could be performed via the use of orthologs in other model plant species, to attempt to validate salt-induced differential methylation in known salt-responsive genes. In future studies, DNA methylation profiling and gene expression analysis should be performed on the same individual plants to ensure a strong correlation between methylation markers and DE genes.

3.4.   Material and methods

3.4.1. Plant material and stress treatment

Five 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 [82]. 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) [82]. 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)) [82] until sampling.

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).

3.4.2. 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℃ 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.

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

The methylation-sensitive genotyping by sequencing (ms-GBS) was performed using a modified version [41,42] of the original GBS technique [83,84]. 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℃ for 2 hours, followed by enzyme inactivation at 65℃ 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℃ for 2 hours followed by an enzyme inactivation step at 65℃ 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 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).

3.4.4. 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.

3.4.5. 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 (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) (Robinson *et al.*, 2010). 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) 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 and therefore reduce the number of sequences generated for that locus [36].

3.4.6. 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 [42,85], using the shell module *bedtools /2.22.0* [86]. The same procedure was repeated to estimate the number of DMMs around exons and UTRs of differentially methylated genes, and tRNA genes.

3.4.7. 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* [87,88]. Significant GO terms were selected based on Bonferroni adjusted P-values [89] 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 [90].

3.4.8. 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* [91], followed by a second round of quality control.

After alignment using *hisat2-2.0.4* in bash [92], salt-induced differential gene expression analysis was performed, using a custom GTF file from Ensembl and created by the tool *StringTie 1.3.1c* [93]. 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* [94], 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

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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.

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 [95], 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 [90], to show the main GO representatives.

Supplemental data

Appendix 1: Ontology of salt-induced differentially methylated genes in barley

Appendix 2: List and ontology of salt-induced differentially expressed genes in barley roots

Author Contributions:

M.K. conceived and performed the experiments, analysed the data and wrote the manuscript; B.J.M. performed ms-GBS data alignments; S.M.P. performed bioinformatic analysis of publicly available RNA-Seq data; M.J.W., E.S.S., B.B., C.M.R.L. conceived the experiments and supervised the work. All authors read and commented on the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Chapter 4: Atlas of tissue and age specific patterns of DNA methylation during early development of barley (*Hordeum vulgare*)

The Chapter 4 contains a manuscript to be submitted for review to the journal "Epigenomes". Therefore, the Chapter is formatted according to the instruction for authors of this journal, except for figure numbers, which were formatted to facilitate referencing and navigation across the thesis. Also, to avoid repetitions where relevant, we referred to methods that were described in previous Sections.

Statement of Authorship

Title of Paper	Root- and leaf-specific DNA methylation targets repeat regions and genes with tissue-specific functions in barley (Hordeum vulgare)
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Publication Details	This manuscript describes the use of methylation-sensitive genotyping by sequencing to characterise salt-induced differentially methylated markers (DMMs) in barley seedlings, with the aim to use these markers for discovery of salt-responsive genes. The results showed that salt stress leads to alteration of DNA methylation patterns in a tissue specific manner, and that salt-induced DMMs were associated with over a thousand genes which enriched gene ontology terms associated with plant response to stress.

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Contribution to the Paper	Conceived and designed the experiments, conducted the experiments, analysed the results in consultation with co-authors, wrote the manuscript
Overall percentage (%)	50%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

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Atlas of tissue and age specific patterns of DNA methylation during early development of barley (*Hordeum vulgare*)

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Abstract

The barley genome comprises over 32,000 genes, and differentiated cells in organs such as roots and leaves, express only a subset of these genes, the remainder being silent. Mechanisms by which tissue-specific genes are regulated are not entirely understood, but DNA methylation is proposed to be involved. The DNA methylation pattern is not static during plant development. However, there is still a debate concerning the distinctiveness of methylation profiles with respect to plant organs. Here we used methylation-sensitive genotyping by sequencing to generate DNA methylation profiles for root and leaf (sheath and blade) in five barley varieties, using seedlings at the three-leaf stage. Differentially Methylated Markers (DMMs) were characterised by pairwise comparisons of roots, blades and sheaths. While a large number of DMMs were found between roots and each of the leaf parts, only a few DMMs were identified between blades and sheaths, these differences increasing with leaf age. Organ-specific DMMs appeared to target mainly repeat regions of the genome, suggesting that organ differentiation partially relies on the spreading of DNA methylation from repeat regions to the promoter of adjacent genes. Furthermore, the biological functions of differentially methylated genes in the different organs correlated with functional specialisation. Our results suggest that DNA methylation controls gene regulation by two mechanisms and is important for both differentiation and organ function.

Keywords: Epigenetics, tissue specific DNA methylation, root, blade, sheath, ms-GBS, repeats, gene expression.

4.1.   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]. Genome-wide methylation patterns are not static during development [4], as they can undergo specific changes, which are involved in biological processes such as transposon silencing, control of gene expression, and organisation of chromatin structure [3,5-10]. This flux of DNA methylation patterns has been proposed to regulate developmental shifts during plant growth and development [4,11]. The effect of DNA methylation variants on plant development has been demonstrated through methylation alteration tests, which showed that the lack of DNA methylation can result in plant abnormalities [12-14].

DNA methylation has been reported to vary from tissue to tissue in many plant species [15-19], and these methylation changes proved to be essential for the plant normal development [11,20]. Tissue-specific DNA cytosine methylation contributes to tissue identity via spatial and temporal changes to specific genes through methylation changes in their regulatory regions [21]. Therefore, the study of DNA methylation patterns in plant tissues is important for a better understanding of how these epigenetic markers determine tissue differentiation.

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, reported to be epigenetically controlled [22], and differential gene expression between organs attributed to differentially methylated regions in soybean [23] and sorghum [17]. These studies extended our understanding of the functional importance of tissue-specific DNA methylation, including its role in setting developmental trajectories [16,22,24]. To this extent, it has been noted that a substantial proportion of developmentally expressed genes have multiple promoters, which initiate different regulatory programmes [25]. Promoters that regulate the same gene, referred to as "alternative promoters", were proposed to be controlled by intragenic DNA methylation [26]. This developmental gene regulation relies on transposon activity [25], suggesting that silencing of transposons due to DNA methylation may be central to tissue-specific gene expression. Furthermore, tissue-specific gene expression has also been associated with methylation changes in promoter regions [21,27,28], especially CG islands within promoters [29]. 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.

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However, the magnitude of differential methylation between tissues has been the subject of controversy. It was believed that substantial distinctive DNA methylation existed only between specialised tissues such as endosperm, pollen, leaves and roots [16,17,30-32]. Yet, many of these studies also showed that differential DNA methylation between organs, such as roots and leaves, was minor in rice [31], maize [33], sorghum [17] and Arabidopsis [16]. DNA methylation differences between roots and leaves were small in both [m]CG and [m]CHG contexts [16,17], with about 1% and 5% divergence, respectively, reported in Arabidopsis [16]. While these studies of differential DNA methylation between tissues generally compared the overall methylation levels [16,17,33], these results differ from comparisons made with DMMs between the same tissues [17], 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 study approach. Thus, further investigation to clarify organ specificity of cytosine methylation and the distribution patterns of tissue-specific DNA methylation markers in the plant genome is warranted.

To undertake such an investigation, we used barley, a globally important cereal crop, the genome of which has been sequenced recently [34]. The availability of a reference genome made barley a model for the study of cereal crops such as wheat, oat or rye grass. Also, with over 5 gigabase pairs in size, barley offers an opportunity to study complex genomic events such as cytosine methylation in DNA. In this study, we assessed differential DNA methylation between two barley organs (roots and leaves), using methylation-sensitive GBS (ms-GBS). For the sake of simplicity and consistency with the literature, roots and leaves or leaf parts (sheath, blade) are referred to here as tissues and not organs. Assessing methylation at [m]CCGG sites, we found that roots, leaf blades and sheaths each displayed a specific methylation profile. Although differentially methylated markers (DMMs) were preferentially concentrated in and around repeat regions, some DMMs were close to genes that had tissue-specific functions.

4.2.   Results

4.2.1.   Methylation-sensitive genotyping by sequencing (ms-GBS)

To assess the genome-wide cytosine methylation at CCGG sites, we performed ms-GBS using DNA samples from roots, leaf blades, and leaf sheaths of seedlings of five barley varieties at

the three-leaf stage (21 days old). 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. Ms-GBS sequencing libraries were prepared from five biological replicates of each variety. Five samples did not meet the DNA quality criteria, resulting in a total of 170 samples for sequencing in an Illumina HiSeq 2500 platform. Sequencing yielded, as summarised in Table 4.1, over 900 million raw reads, with more than 91% bases above Q30 (99.9% accuracy of base call [35]) across all samples. 99.27% of these reads contained the barcode and *Eco*RI/*Msp*I adaptors 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%), which averaged 2,637,916 high quality reads per sample. These high-quality reads accounted for 913,697 sequence tags, representing 32.30 % of CCGG sites (2,828,642 CCGG) in the barley genome. Of these sequence tags, 748,594 (80.62%) were polymorphic for methylation changes at ^{m}CCGG sites.

Table 4.1: Data yields from ms-GBS, generated using the Illumina HiSeq 2500 platform.

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

4.2.2. Estimation of "tissue and age"-dependent epigenetic differentiation

To estimate the epigenetic differentiation between root, blade and sheath samples, harvested from the same individuals, a principal component - linear discriminant analysis (PC-LDA) was performed using normalized read counts per million. Plotting of the first two discriminant factors (DF1 and DF2) showed a clear clustering according to tissue types, roots, blades and sheaths (Figure 4.1a). However, there was no obvious age-dependent sub-grouping within blade and sheath clusters. Therefore, we tested for age-dependent differentiation between tissues by performing a hierarchical cluster analysis (HCA) of the distances between sample group centres, based on the Mahalanobis distance [36,37]. This analysis supported the tissue-specific clustering

as in the PC-LDA plot, and presented a better group separation according to the sample rank of appearance for both blades and sheaths (Figure 4.1b). Samples closer in appearance clustered together (i.e., 1 and 2 or 2 and 3, but not 1 and 3 (Figure 4.1b)).

We further assessed age-dependent DNA methylation differences between tissues by comparing the methylation profiles of blades and sheaths of different rank of appearance. No DMMs were observed between the three leaf blades, whereas sheaths 1 and 3 presented 18 DMMs (Table 4.2).
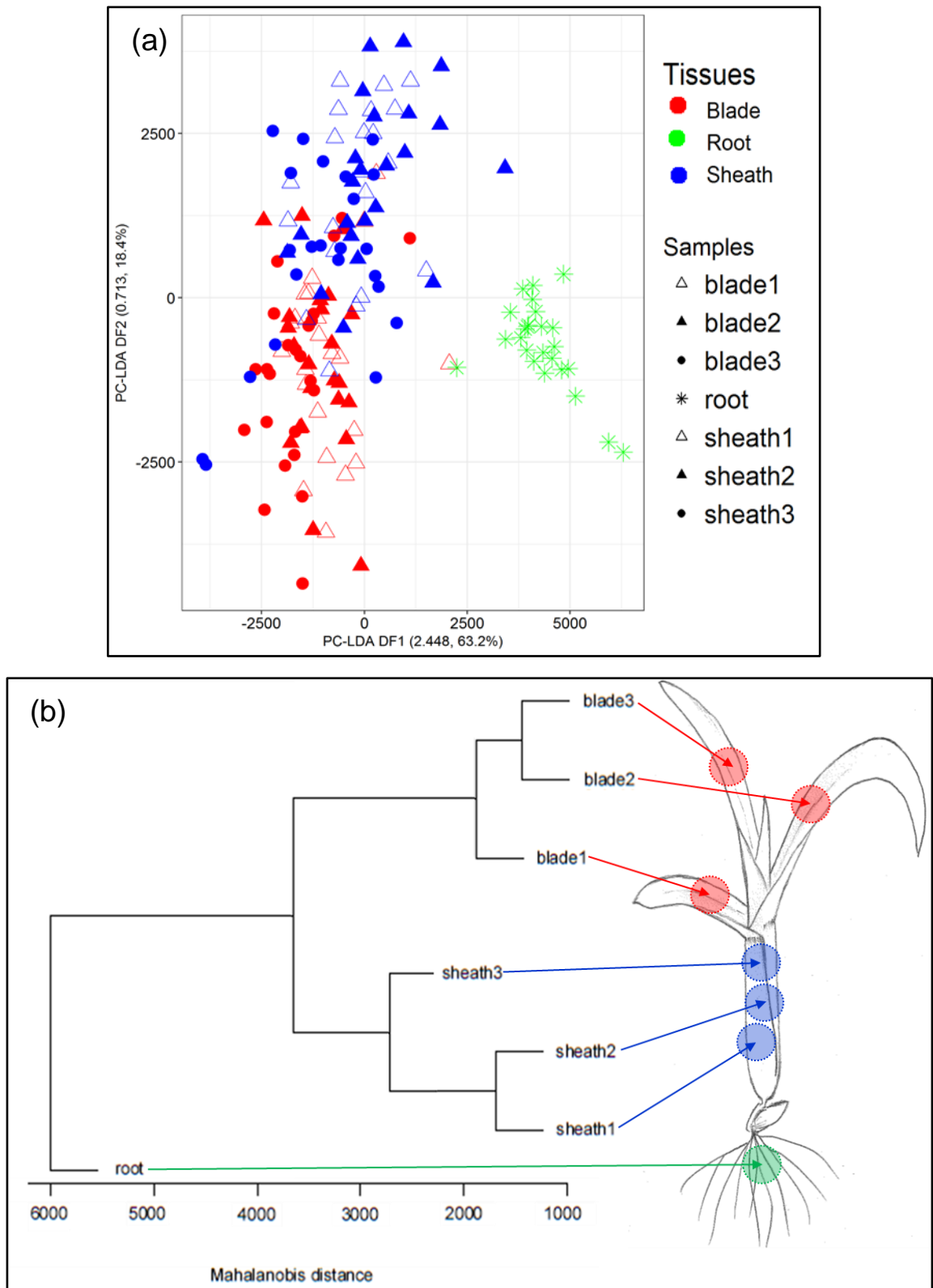
Figure 4.1: Analysis of the differentiation of DNA methylation profiles of barley roots, leaf sheaths and leaf blades.

(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

three-leaf stage (21 days after sowing), including five varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra). (b) 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.

Table 4.2: Number of Differentially Methylated Markers in barley tissues of different ages. Significant differentially methylated markers (FDR <0.05) were obtained from 913,697 ms-GBS DNA methylation markers 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 organ type, first, second and third, respectively, on seedlings.

	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	-

4.2.3. Analysis of DNA methylation differences between roots and leaves

The variation in DNA methylation between barley root and leaf samples was assessed by comparing the read count per million of organ types, independently of genotypes. DMMs were identified based on false discovery rates (FDR) lower than 5%, obtained from adjustment of Bonferroni P-values. This assessment revealed substantial DMMs for both roots vs. blades and roots vs. sheaths (Figure 4.2a). For all pairwise comparisons, DMMs were predominantly hypomethylated (95-98%) in leaf parts (sheath or blade) compared to roots (Figure 4.2a). This result was further supported by the median fold-change intensity, which indicated an overall DNA hypomethylation in leaves (Figure 4.3ab).

The number of DMMs between roots and leaf blades decreased with their rank of appearance, whereas DMMs between roots and leaf sheaths did not show any relationship with such a rank (Figure 4.2a). In addition, there were more DMMs between roots and blades (6510 DMMs, Figure 4.2b) than between roots and sheaths (4116 DMMs, Figure 4.2c). 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 4.4a). From here on, the

3266 DMMs common of roots vs. sheaths and roots vs. blades will be designated as stable markers between roots and leaves.
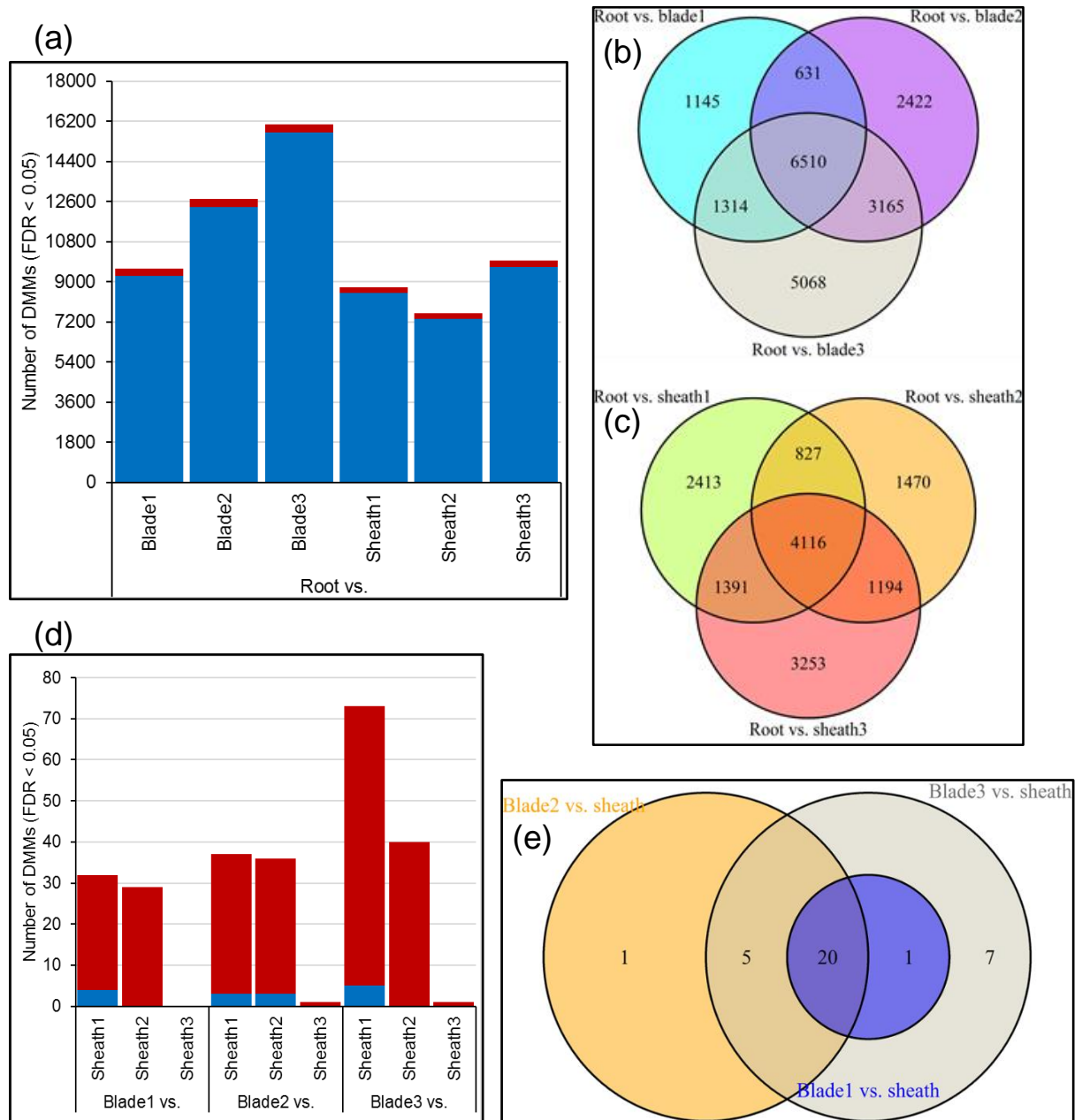
Figure 4.2: Analysis of the number of DMMs among three barley tissues.
(a) Number of DMMs between roots and leaf blades (Root vs. blade) or roots and sheaths (Roots vs. sheaths). Histogram colour indicates whether the DMMs are hypomethylated (blue) or hypermethylated (red) in leaf parts compared to roots. (b-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 to 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, are designated as markers between roots and leaves.
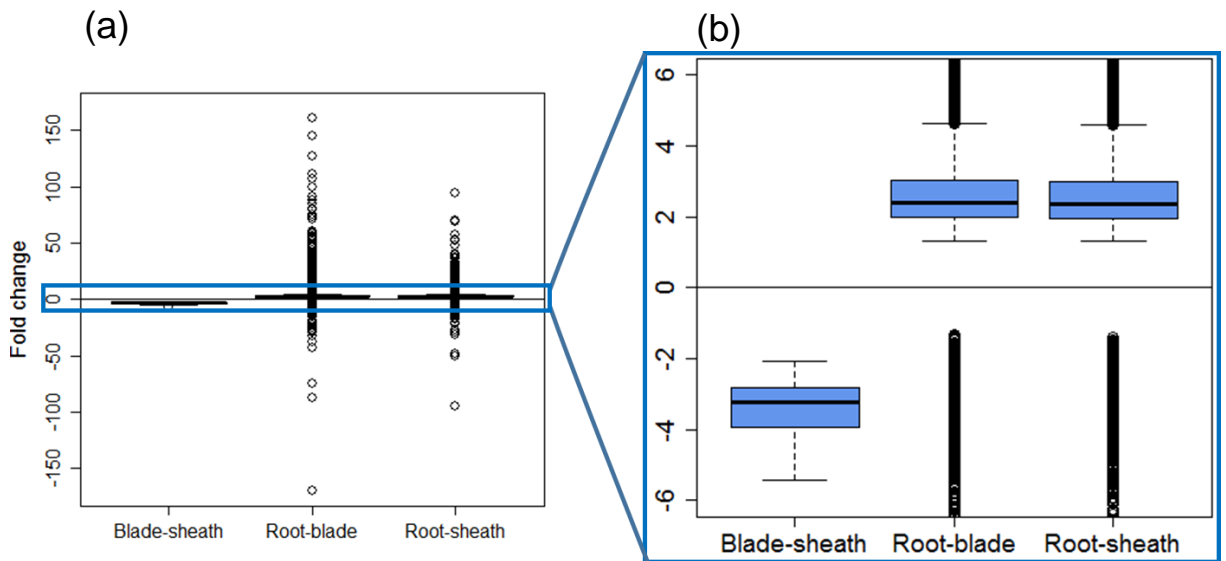
Figure 4.3: Directionality of the methylation in tissue-specific DNA methylation markers.
(a) Boxplots showing the spread of the fold-change of read counts of DMMs between blades and sheaths, roots and blades, and roots and sheaths. (b) Detail of boxplots, highlighting the median of methylation fold-change of all samples in each comparison. The fold-change of DNA methylation was estimated by computing $2^{(log2FC)}$, with $log2FC$ = logarithm 2 of fold-change in read counts per DMM 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.
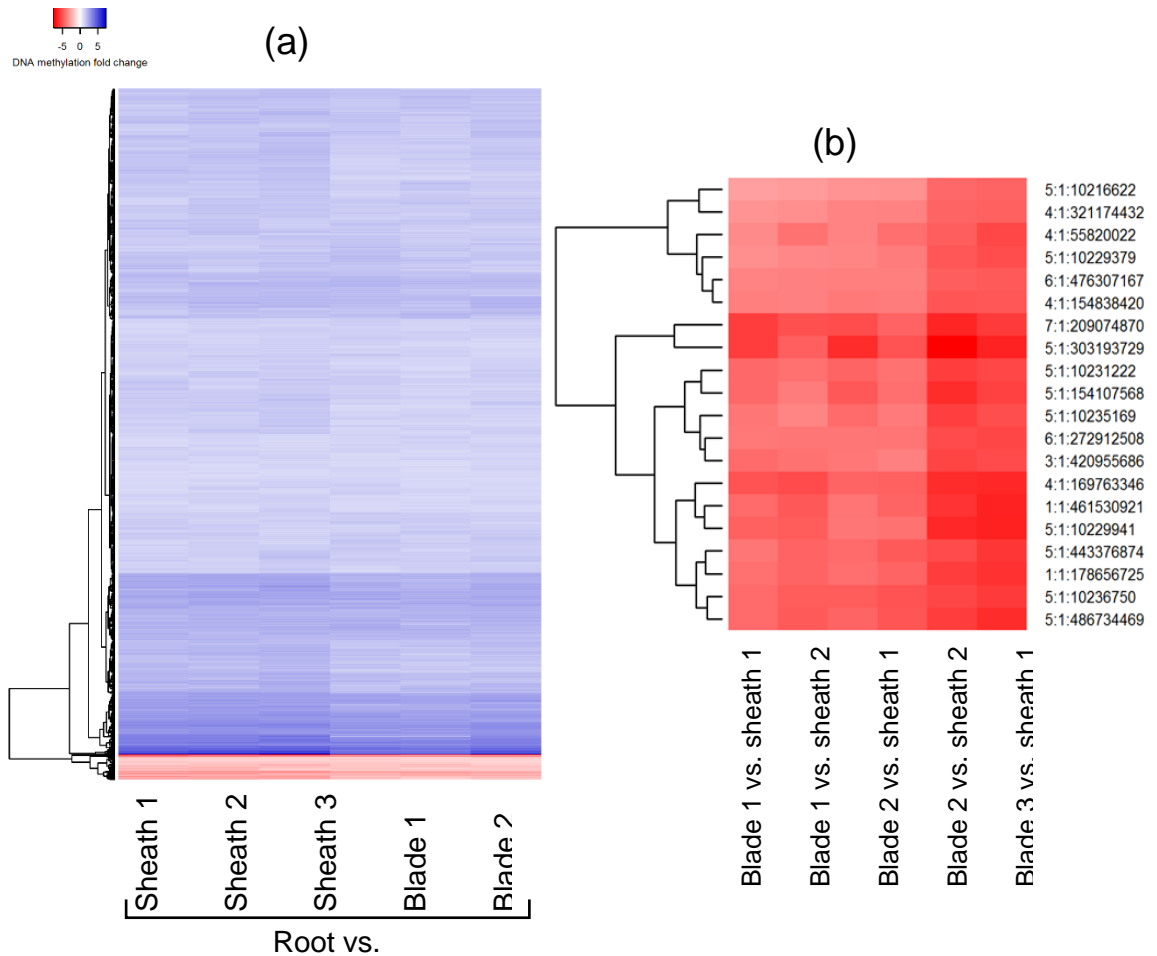
Figure 4.4: Hierachical clustering analysis of the DMMs.
(a) the 3266 common DMMs between roots and all leaf parts (sheath 1-3, blade 1-3). The colours in the heat map indicate whether the DMM is hypomethylated (blue) or hypermethylated (red) in leaf parts compared to roots. (b) Hierarchical clustering of the 20 stable DMMs between blades and sheaths. In this heat map the red colour shows hypermethylation of DMMs in sheaths compared to 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.

4.2.4. Analysis of DNA methylation differences between leaf blades and sheaths

There was a small number of significant DMMs between leaf blades and sheaths (0 to 73 DMMs, Table 4.2; Figure 4.2d). However, these DMMs were essentially between leaf blades and sheaths 1 and 2; and there no significant DMMs between blade 1 and sheath 3, while blades 2 and 3 showed only 1 DMM each, with sheath 3 (Table 4.2; Figure 4.2d). Pairwise comparisons between blades 1-2 and sheaths 1-2 revealed 20 common DMMs, which were all

hypermethylated in sheaths compared to blades (Figure 4.2e and Figure 4.4b). Half of the 20 common DMMs between blades and sheaths were located on chromosome 5H, suggesting that this chromosome may carry important loci for blade and sheath identities. Furthermore, there were no significant 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 4.2). These results suggest that, once organs are differentiated and mature, similar tissues do not have significant differences in methylation profiles, regardless of age differences, at least at this seedling stage.

4.2.5. Distribution of tissue-specific DMMs around genes

To determine the distribution pattern of distinctive methylation markers in pairwise comparison of root, leaf blade and leaf sheath samples, the number of DMMs was estimated within genes and 5 Kb flanking regions either side. In this way, we found that DMMs were relatively scarce around gene transcript sequences. Of the 3266 stable DMMs between root and leaf samples, only 60 (1.8%) were 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 4.5a). Apart from the absence of DMMs within 1 Kb upstream of transcription start sites, there was no particular tissue-specific DMM distribution pattern around the genes (Figure 4.5a).

Applying the same process, we assessed the distribution of blade-specific and sheath-specific DMMs near genes. We found that, as with common DMMs, only a small proportion of blade-specific DMMs (44 of 3246, 1.3%) was near a gene (Figure 4.5b). Of these, 15 DMMs overlapped with a gene transcript, whereas the remaining 29 DMMs were distributed within 5 Kb of the gene without any obvious pattern (Figure 4.5b), 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 4.5b). Additionally, the number of sheath-specific methylation markers within 5 Kb from genes was even smaller than that of blade-specific markers (13 of 2391 DMMs, 0.5%) (Figure 4.5c). The majority of these (10 out of 13 DMMs) were within 3 Kb upstream and downstream of a gene, and no DMMs were present after 3 Kb downstream genes (Figure 4.5c). We further explored the positions, relative to exons, of tissue-specific DMMs that overlapped with genes. These gene-body DMMs were mapped with exons of genes overlapping with DMMs. Of 37 total gene-body DMMs in all comparisons (Figure 4.5a-c), 27 overlapped with an exon and the remaining 10 markers were in intergenic

regions, 70 to 604 bp upstream of exons, except 1 DMM, which was 62 bp downstream an exon. (Appendix 3).
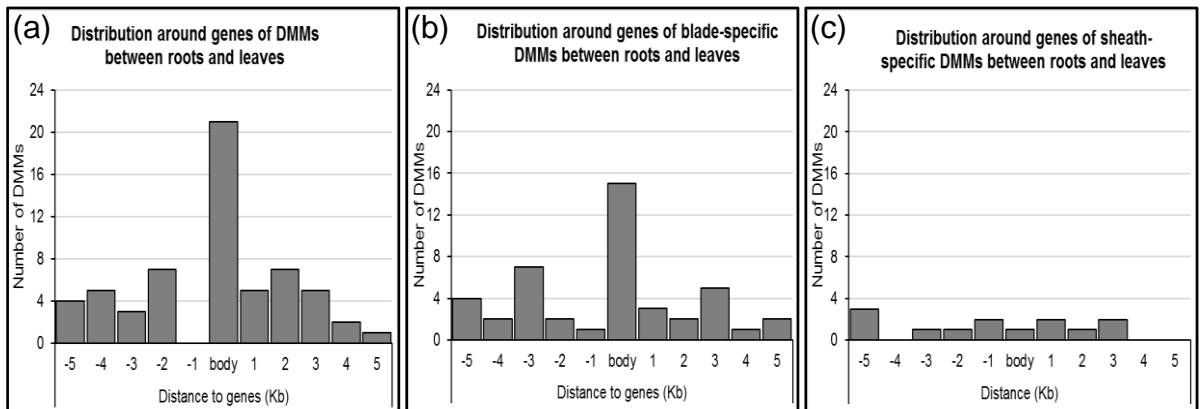
Figure 4.5: Distribution of tissue-specific differentially methylated markers (DMMs) around genes.
(a) DMMs between roots and leaves, present in both blades and sheaths as in Figure 4.2b-c; (b) Blade-specific DMMs between roots and leaves and (c) Sheath-specific DMMs between roots and leaves. The y axis 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.

4.2.6. Distribution of tissue-specific DMMs near repeat regions

As for genes, we estimated the distribution of DMMs between roots and leaves, and then, blade-specific and sheath-specific DMMs around repeat regions in the barley genome (repeats as defined in the Ensembl database (http://plants.ensembl.org/biomart/martview/)). Many more DMMs were detected near repeats than near genes. DMMs between roots and leaves around repeat regions were concentrated within repeat sequences and 1 Kb before and after the repeat regions (Figure 4.6a). A similar distribution pattern was obtained with both blade-specific and sheath-specific DMMs between roots and leaves, showing more DMMs overlapping with repeats than 1 Kb downstream or upstream (Figure 4.6bc). Also, 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 4.6d). These results revealed that stable tissue-specific DMMs occur preferentially within repeats and 1 Kb flanking regions, with higher frequency within 1 Kb downstream than within 1 Kb upstream, regardless of whether markers are between roots and blades, roots and sheaths, or blades and sheaths (Figure 4.6a-d).
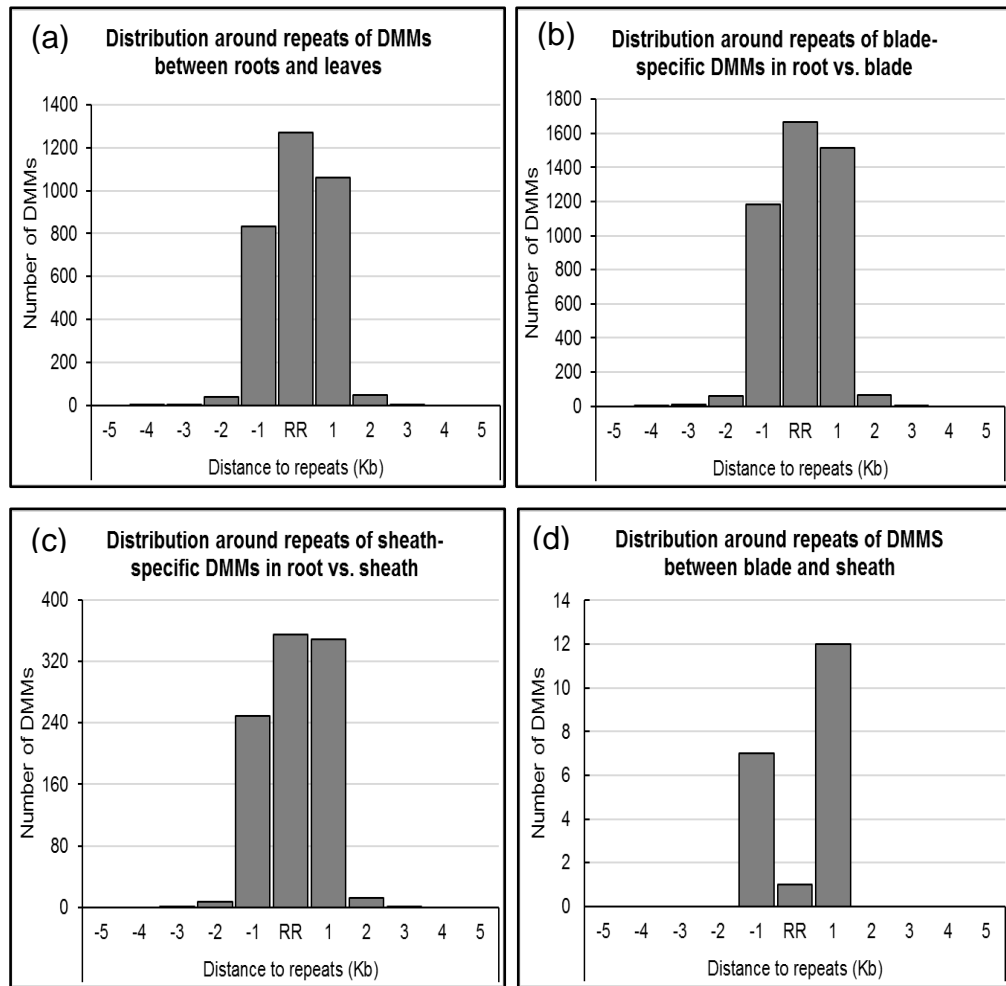
Figure 4.6: Distribution of tissue-specific differentially methylated markers (DMMs) around repeats.
(a) DMMs between roots and leaves, present in both blades and sheaths as in Figure 4.2bc; (b) blade-specific DMMs between roots and leaves; and (c) sheath-specific DMMs between roots and leaves; (D) DMMs 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.

4.2.7. Distribution of genes around differentially methylated (DM) repeats)

To investigate a possible interaction between DM repeats and genes, the distance of genes from differentially methylated 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 of the repeat (Figure 4.7). The number of DM repeats surrounded in this way by genes represented a small proportion of the total repeats that were differentially methylated between roots and leaves (105 out of 3266 DM

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repeats, 3.21%). Genes around DM repeats are listed in Supplementary Data 4.S2. About half of these genes near DM repeats (52 of 105 genes) were also differentially methylated, whereas the other half (53 genes) was not.
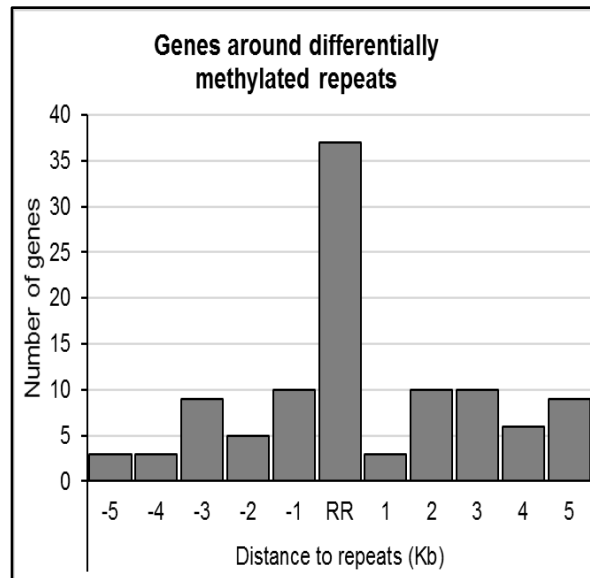
Figure 4.7: Distribution of genes around differentially methylated repeat regions.
The x axis indicates the distance to repeats in kilo base pairs (Kb) on both flanking regions. Negative and positive values indicate upstream and downstream repeat regions, respectively. RR, repeat regions. The y axis shows the number of genes per 1 Kb window.

4.2.8. Gene ontology of differentially methylated genes

Genes differentially methylated between root and leaf samples (107 genes, Table 4.3) comprised 44 blade-specific (three hypermethylated and 41 hypomethylated genes), two sheath-specific (one hypermethylated and one hypomethylated genes), and 60 genes present in both blades and sheaths (ten hypermethylated and 50 hypomethylated genes). These DM genes were described by 213 GO terms (Table 4.3) within the three main categories, "biological process", "cellular component" and "molecular function". While 121 GO terms were common in leaf parts, 88 GO terms were specific to blades and 4 GO terms to sheaths (Table 4.3).

The GO analysis provided a picture of the role of DM genes in barley physiology and metabolism. Genes that were differentially hypermethylated in leaves compared with roots related to GO terms predominantly represented by "organonitrogen compound metabolism" and "generation of precursor metabolites and energy" (Figure 4.8a, Appendix 5). The top five

GO term representatives of hypomethylated genes in leaves relative to roots were; "organophosphate biosynthesis", "peptide metabolism", "monovalent inorganic cation transport", "electron transport chain", and "generation of precursor metabolites and energy" (Figure 4.8b). It is worth noting that photosynthesis-associated GO terms (GO:0015979) were enriched among genes hypomethylated in leaf tissues. Likewise, cellular components that set apart roots and leaves concerned chloroplast thylakoid (GO:0009534 and GO:0009579) and cytochrome complex (GO:0070069), which are part of the photosynthetic machinery, and were all derived from genes hypomethylated in leaves (Appendix 5). Furthermore, there was a high frequency of the GO term "plastid" (GO:0009536), which was enriched in both hypermethylated and hypomethylated genes, regardless of whether they were common or specific to either blades or sheaths (Appendix 5).

Some of the GO terms from differentially hypomethylated genes in leaves, were related to molecular functions represented by; "tetrapyrrole binding", "monovalent inorganic cation transmembrane transporter activity", "transition metal ion binding", "hydrolase activity" and "quinone activity" (Appendix 4). While no GO terms belonging to molecular function enriched by DM genes specific to sheaths, blade-specific DM genes enriched GO terms around "monovalent inorganic cation transmembrane transporter activity", "ATPase activity coupled", and "adenyl-ribonucleotide binding" (Appendix 5).

Table 4.3: Number of differentially methylated DM genes and associated gene ontology (GO) terms.
DM genes between roots and leaves common to both blade and sheath (Root vs. blade + sheath), specific to blade (Root vs. blade specific) and specific to sheath (Root vs. sheath specific). Hyper and hypo refer to hypermethylation and hypomethylation in roots compared with the other tissue (blade and sheath, respectively). GO terms were selected based on difference between their frequency in DM genes and non-DM genes, with adjusted P-value < 0.01.

	DM genes			GO terms		
	Hyper	Hypo	*Total	Hyper	Hypo	*Total
Root vs. blade + sheath	10	51	61	23	100	123
Root vs. blade specific	3	41	44	3	85	88
Root vs. sheath specific	1	1	2	2	2	4
*Total	14	92	107	28	187	215

*Totals may include duplicates, since the same gene can be both hypo- and hypermethylated, and the same GO term may be present in both groups.

Figure 4.8: Summary treemaps of GO (gene ontology) term representatives for the category "biological process" obtained from differentially methylated genes between roots and leaves. (a) Representatives of GO terms enriched by common differentially hypermethylated genes in blades and sheaths; 1 = generation of precursor metabolites and energy; (b) Representatives of GO terms enriched by common differentially hypomethylated genes in blades and sheaths; 2= photosynthesis; (c) Representatives of GO terms enriched by blade-specific differentially hypomethylated genes; 3 = generation of precursor metabolites and energy, 4 = photosynthesis, 5 = macromolecular complex subunit. 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 Appendices 4 and 5.

4.2.9. Gene ontology of genes near differentially methylated repeats

Since some of the genes around DM repeats were also differentially methylated and analysed as such for GO enrichment above, only non-DM genes around DM repeats (53 of 105) were used for further GO analysis. This analysis generated 97 significantly enriched GO terms in the three categories "biological process", "molecular function", and "cellular component". Strikingly, most of the GO terms enriched by non-DM genes around DM repeats (93 of 97 GO terms, 95.88%) were also enriched in DM genes.

The top GO term representatives in the "biological process" category were; "organophosphate biosynthesis", "peptide metabolism", electron transport chain", "monovalent inorganic cation transport", "generation of metabolites and energy" and "photosynthesis" (Figure 4.9a). In the GO category "cellular component", the GO term "plastid" predominated, along with "thylakoid" and "thylakoid membrane" (Figure 4.9b). GO terms enriched in the category "molecular function" belonged to the following five sub-categories in order of importance; "tetrapyrrole binding", "cation transmembrane transporter activity", "transition metal ion binding", "hydrolase activity", and "NADH dehydrogenase (quinone) activity" (Figure 4.9c).
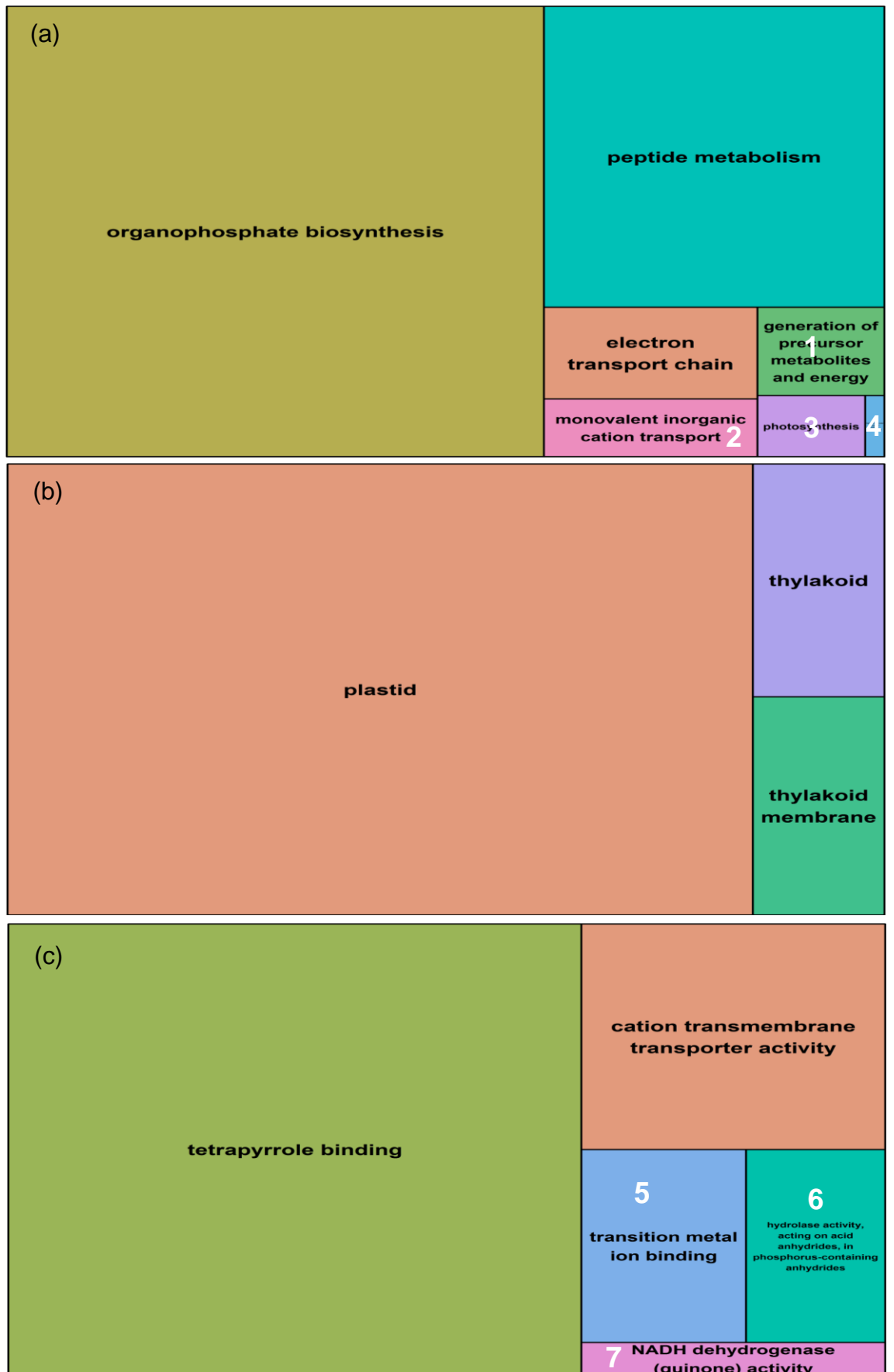
Figure 4.9: Representative GO enrichment summary treemaps obtained from genes near DM repeats between roots and leaves.
(a) Representatives of GO terms enriched in the category "biological process"; 1 = generation of precursor metabolites and energy; 2 = monovalent inorganic cation transport; 3 = photosynthesis; 4 = macromolecular complex subunit organisation; (b) Representatives of GO terms enriched in the category "cellular component"; (c) Representatives of GO terms enriched in the category "molecular function"; 5 = transition metal ion binding; 6 = hydrolase activity, acting on acid anhydrides, in phosphorus containing anhydrides; 7 = NADH dehydrogenase (quinone) activity. 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 Appendices 4 and 5.

4.3.   Discussion

4.3.1. Extensive DMMs between roots and leaves

In this study, we detected extensive DMMs between roots and leaves of barley seedlings (Figure 4.2bc, Figure 4.3b and Figure 4.4a). These genotype-independent DMMs in ^{m}CCGG sequence context were predominantly hypomethylated in leaves compared to roots. Also, we showed that differential DNA methylation occurred even between tissues of the same organ, such as leaf blade and sheath, despite their closeness in both function and structure (Figure 4.2e and Figure 4.4b). However, the number of stable DMMs between blades and sheaths, all hypermethylated in sheaths, was relatively small (20 DMMs, Figure 4.4b). These findings are in general agreement with previous studies, which reported differential DNA methylation between variable tissues (e.g. endosperm, pollen, leaves, roots) in plant species such as sorghum, rice and Arabidopsis [16-19]. However, the absence of significant differentially methylated markers between blades and sheath 3 was unexpected (Figure 4.2d). This may be due to variability in growth stages between sheath 3 samples, thus affecting statistical significance. For instance, during sampling, the third leaves were not always fully emerged as the two first leaves (Figure 4.1b).

Previous studies detected relatively little differential methylation between roots and leaves [16-19,33]. For example, little difference was found in the methylation levels of both ^{m}CG and ^{m}CHG motifs between roots and leaves in Arabidopsis [16] and sorghum [17]. We suspect that the lack of numerous tissue-specific makers in previous studies is attributable to factors such as experimental plant species and the methylation profiling method. That is, plant species can show specific DNA methylation profiles that may reflect on differences between their tissues [33]. In contrast to these studies, our results revealed that plant organs can display a substantial

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proportion of tissue-specific DNA methylation. However, since the same technique was not always used to assess plant methylation profiles, the comparison of results may be biased. The results of DMM analyses can be influenced by factors such as 1) the depth of the methylation profiling method which may overlook many markers (e.g. MSAP); and 2) the analysis approach which can compare either global methylation levels (percent methylation, e.g. [16]) or methylation loci (DMMs, e.g. [38]). 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. This was noted before by Zhang et al. [17], who acknowledged that, despite few differences in the relative level of methylation among six sorghum tissues, pairwise comparisons revealed that each tissue had a distinct methylation pattern, due to locus-specific changes in the genome. Our findings advance previous studies [16-19,33], in that we assessed DMMs across five barley varieties (comprising five biological replicates), thus providing statistically and biologically robust markers.

Furthermore, the present study demonstrates that the CHG context (at least ^{m}CCGGs) is a major niche of tissue-specific DNA methylation in barley (Figure 4.2a-c). This is consistent with prior studies reporting the same cytosine context (CHG) as predominant in differential DNA methylation between leaves and flower buds in *Brachypodium distachyon* [19]. Also, it has been observed hitherto in sorghum, that differential gene expression between organs correlated more with methylation changes in CHG than all other cytosine contexts [17]. Therefore, CHG methylation is likely to play a significant role in tissue-specific gene expression. Although tissue-specific DNA methylation also occurs in other cytosine contexts [16,17], our results and other studies [17,19] suggest that ^{m}CCGG is a primary motif of epigenetic distinctiveness of plant organs. Additionally, while tissue-specific DMMs were mostly hypomethylated in leaves compared to roots in the present study (Figure 4.3b), in Arabidopsis, Widman et al. [16] 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 due, again, to the methylation profiling method implemented. While we performed a pairwise comparison between epigenetic loci, Widman et al. compared the levels of methylation in the two organs tested [16]. Variability in data analysis approaches makes it difficult to compare results.

4.3.2. Minor association of DNA methylation with organ ageing in barley seedlings

Data generated in the current study showed clustering of samples according to organ type and age (Figure 4.1b), suggesting occurrence of differential DNA methylation between organs and between ages of organs. Additionally, a considerable portion of DMMs between roots and leaves, was also specific to the leaf age (Figure 4.2a-c), in that there was a steady decrease in the number of DMMs between roots and leaf blades with age of the latter (Figure 4.2a). That is, older blades were epigenetically closer to roots than younger ones. It was observed that epigenetic profiles of leaves become more and more similar as they mature. Therefore, we hypothesise that there is a default epigenetic profile for mature leaves, assuming that some of the DMMs result from developmental progression [11,14], not necessarily from organ identity. Such developmental DMMs should shrink at organ maturity, while organ-specific markers become more prominent. Thus, the number of DMMs decreased between older leaves and roots (Figure 4.2a-c), because it evolves in the same way as DMMs between leaf ages. That is, DMMs between leaves and roots will become differences between the default epigenetic profiles of the two organs, without markers of developmental progression.

As mentioned earlier, the extent of differential DNA methylation between similar tissues was minor or non-existent. The few DMMs between sheath 1 and sheath 3 (18 DMMs, Table 4.2) may be attributable to differences in leaf growth stages, including cell division, elongation, and maturation, each of which may carry a specific epigenetic profile [39]. In this way, the methylation profile varies progressively as the organ develops [3,10,40] before reaching, at maturity, a "default" methylome which may conserve similar patterns across varieties [33]. Our results suggest that DNA methylation differences are important during tissue formation, but these differences disappear once the tissue is differentiated. Therefore, the tissue-specific DNA methylation profile may not be stable before tissue maturity is reached.

This view point contrasts with several lines of evidence indicating that ageing of plant organs is controlled by DNA methylation [11,41]. However, this difference may have originated from the biological material used. Previous studies commonly compared organs with contrasting ages, such as young vs. senescent organs, or in multi-year plants, samples from different plant individuals having several years difference in age [42-44] (see Dubrovina and Kiselev [41] for review). To the best of our knowledge, differential methylation between organs with only a few days' age difference on the same seedlings has not been investigated. Assuming that age-dependent DMMs are plausible, this phenomenon may require a large age difference before it

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becomes evident. Therefore, we suspect that age-dependent difference in methylation profiles reported previously [11,42-44] would correlate more logically with developmental stages, rather than with age itself.

4.3.3. Tissue-specific DNA methylation preferentially targets repeat regions in the barley genome

Having characterised tissue-specific DMMs between root and leaf tissues, we investigated the distribution pattern of these around repeat regions and genes in the barley genome. Compared to repeat regions, we found fewer DMMs between roots and leaves around genes (Figure 4.5a-c). Some of these tissue-specific DMMs overlapped with exons (27 DMMs) and gene introns (10 DMMs) (Appendix 3), showing that CHG methylation is not exclusively assigned to repeats and intergenic regions, as extensively claimed previously [3,5,29,30,45]. These patterns of gene-body methylation suggest that tissue-specific DMMs can influence gene expression by enhancing gene transcription [16] and alternative splicing [46] or through repression due to immediate proximity to transcription start site [47] in a tissue-specific manner.

The predominance of DMMs around repeats was coupled with their concentration within repeats and 1 Kb from repeat regions (Figure 4.6a-c). This result shows that repeat regions are likely to play a central role in the definition of organ identity in barley, and validates previous findings in *Brachypodium distachyon*, where most of the differential methylation between leaf and bud tissues occurred within repeats [19]. Although this result does not prove that DM repeats impact on tissue-specific development, it can be linked to at least three well-known phenomena in plant genomes. First, repeat regions were previously proposed to be involved in alternative promoters, a substantial proportion of which (>40%) was reported to shape tissue differentiation [25]. Therefore, tissue-specific differential methylation in repeats may contribute to alternative promoters, and thus influence organ type in this way. Second, differential gene expression between roots and leaves [34,48] implies a firm regulatory system, including epigenetic mechanisms to guarantee tissue-specific cell development. Tissue-specific DNA methylation in repeats shows that these are not the so-called "selfish parasites" of the genome [49], but can directly or indirectly affect tissue-specific gene expression through methylation [10,28,50,51]. Finally, it has been suggested that transposons coordinate splice variants, a genomic event that occurs in more than 60% of plant genes [52,53], thus generating multiple mRNA transcripts

from a single gene [54,55]. Many splice variants are tissue-specific and coordinated by transposons, a form of repeat sequences [56]. Therefore, DMMs in repeats are likely to affect alternative splicing and subsequent gene expression. Also, as some DM genes were near DM repeats, these genes might potentially be simultaneously regulated by both gene methylation and adjacent repeat methylation. Additionally, repeats have been reported previously to be involved in the regulation of distant gene expression [57,58]. Therefore, tissue specific alterations of DNA methylation in and around repeats may influence this function of repeat elements [57,58]. Although this was not tested in the present study, a pivotal experiment by Baek and colleagues showed that the *AtHKT1* gene expression was controlled by the methylation of a region proximal to repeats upstream of the ATG start codon of the gene [58]. This finding highlights the importance of DNA methylation in gene cis-regulation through methylation changes in repeat and enhancer sequences.

4.3.4. DMMs between roots and leaves, target genes that are relevant to plant tissue identity

Based on the principle that differential DNA methylation between tissues correlates with differential gene expression [17,23,59], we performed gene ontology analysis on DM genes in roots and leaves. In this way, we found over 60 genes that were differentially methylated between leaves and roots, most of which were hypomethylated in leaves (Table 4.3). The dominance of hypomethylated genes in leaves correlates with more upregulated than downregulated genes in barley leaves compared to roots [34,48]. This GO analysis was extended to genes around DM repeats, considering that the level of methylation and activity of repeats [60,61] can potentially regulate nearby genes [8,10,50,62]. DM genes (which were also mainly near DM repeats) enriched GO terms that correlated with some of the known functions of roots and leaves. Photosynthesis-associated GO terms (GO:0015979) were enriched among genes hypomethylated in leaf tissues. These included those related to chloroplast thylakoid (GO:0009534 and GO:0009579) and cytochrome complex (GO:0070069), which were differentially methylated between roots and leaves (Figure 4.8, Supplemental Data 4.S2). For instance, in leaves, some of the hypomethylated genes enriched "photosynthesis (GO:0015979)", whereas hypermethylated genes contributed to the term "organonitrogen compound metabolism", which relates to processes occurring in roots, such as nitrogen assimilation [61,62]. Also, a high frequency of the GO term plastid (GO:0009536) was noticed among DM genes, indicating that this cellular component is crucial in defining plant tissue

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identity. This observation is supported by the fact that plastids play diverse purposes in green plants, including in organ structure and function [65].

Furthermore, ontology analysis of genes near DM repeats but not differentially methylated revealed GO terms that were similar to those enriched by DM genes between the same organs (95.88% identical GO terms) (Figure 4.9). This similarity of GO pathways enriched by DM genes and genes near DM repeats suggests that tissue-specific DMMs occur in specific genomic locations that contribute to the regulation of precise biological functions. All together, these results are supportive of the view that tissue-specific DMMs are likely to be involved in the differential regulation of genes in the tissues compared [27,28], to fulfil functions that are specific to either tissue [16]. This possible role of tissue-specific DMMs remains plausible, whether DMMs are directly around the gene or around repeat regions that are close to genes, in which case the genes can be indirectly regulated due to proximity with transposons [8-10,50].

4.3.5. Conclusion

We have shown that roots and leaves of barley seedlings, displayed substantial epigenetic divergence. There were also distinctive DMMs between leaf blades and sheaths, suggesting a possible role of DMMs in defining organ identity. There were DMMs that separated barley developmental stages, however, these DMMs seem to require tissue maturity before they appear significantly. Findings that DMMs between barley tissues target repeat regions suggest that repeats are important in determining organ identity, possibly through regulation of nearby genes. Furthermore, the abundance of stable tissue-specific methylation in ^{m}CCCG sites suggests that this context is a determining factor in organ differentiation.

It is noteworthy that a major limitation of this study is the lack of expression analysis on the same samples to test the gene ontology. However, indexed markers were generated, allowing us to directly map DMMs with barley reference gene transcripts. Although this cannot prove that genes identified as differentially methylated are also differentially regulated between organs, it has the merit of specifying the locations of DMMs in the genome. Nevertheless, ontology of DM genes and those near DM repeats suggests that the tight correlation between differentially methylated genes and their expected tissue-specific function is not incidental. That is, differentially methylated genes enriched GO terms related to physiological processes that fit with the specialised functions of roots and leaves.

4.4. Material and methods

4.4.1. Plant material and growth conditions

Five spring barley varieties (Barque73, Flagship, Hindmarsh, Schooner and Yarra) were grown in potting mix comprising 50% UC (University of California at Davis), 35% coco-peat and 15% clay/loam (v/v) 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) (0.8 × field capacity) [66] until sampling 21 days after sowing (three-leaf stage, Zadok stage 13-14 [67]). About 50 mg of plant material was cut from the middle section of each leaf blade and each leaf sheath and snap frozen in liquid nitrogen 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. Samples were frozen in liquid nitrogen, and then all samples were stored at -80°C until DNA extraction.

Prior to DNA extraction, frozen plant material was homogenized in a bead beater (2010-Geno/Grinder, SPEX SamplePrep®, USA). DNA isolation was performed using a Qiagen DNeasy kit 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.

4.4.2. Methylation sensitive genotyping by sequencing (ms-GBS)

The libraries for the methylation-sensitive genotyping by sequencing (ms-GBS) were prepared using DNA samples from roots, leaf blades (1-3) and leaf sheaths (1-3) collected from the three-leaf stage barley seedlings (7 samples x 5 varieties x 5 replicates). The procedures for library preparation for ms-GBS and bioinformatic data analysis are described in Chapter 3, section 3.4.3. and section 3.4.4. of the present thesis.

4.4.3. Principal component – linear discriminant analysis (PC-LDA)

Grouping of organ type samples was explored by performing a principal component – linear discriminant analysis (PC-LDA) and a hierarchical cluster analysis using the R package *FIEmspro 1.1-0* [37]. To visualise the result, a scatter plot of the first two discriminant factors was performed and a hierarchical cluster tree was built based on Mahalanobis distance [36].

4.4.4. DMMs detection in barley

Differential DNA methylation was assessed in ^{m}CCGG motifs, between barley leaf (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 at minimising genotype-dependent methylation markers. DMM detection was carried out as described in Chapter 3, section 3.4.5. of this thesis, with the exception that FDR significance threshold was set < 5% for the difference in read count per million between sample groups. DMMs were selected based on Bonferroni adjusted P-values [68,69] for the difference in read counts per million between salt-free control and salt treatments (75 mM, 150 mM or 200 mM NaCl). The selection of the marker also fulfilled the condition that the read counts reached at least 1 CPM (count per million reads) 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 organs.

4.4.5. Distribution of DMMs around genomic features and gene ontology

The distribution patterns of tissue-specific DMMs around genomic features (e.g. genes and repeat regions as defined in Ensembl database (http://plants.ensembl.org/biomart/martview/) were investigated. DMMs stable between organs were mapped to the barley reference genome, and their distribution around genomic features (genes or repeats) was assessed as described in Section 3.4.6. of Chapter 3. Then, we explored the functions of tissue-specific DM genes and genes near DM repeats, by performing gene ontology analysis as described in the previous Chapter, Section 3.4.6.

Supplementary Materials:

Appendix 3: List of differentially methylated exons in barley roots and leaves

Appendix 4: List of GO terms enriched for "biological process" using differentially hypomethylated genes between roots and leaves

Appendix 5: Lists of gene ontology terms from differentially methylated genes between barley roots and leaves and specific to leaf blades and leaf sheaths

Author Contributions:

M.K. conceived and performed the experiments, analysed the data and wrote the manuscript; B.J.M. performed ms-GBS data alignments; M.J.W., E.S.S., B.B., C.M.R.L. conceived the experiments and supervised the work. All authors read and commented on the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Chapter 5: Greenhouse spatial effects detected in the barley (*Hordeum vulgare* L.) epigenome may underlie the stochasticity of DNA methylation

This Chapter contains a manuscript to be submitted for review to the journal "Plant, Cell & Environment". Therefore, the Chapter is formatted according to the instruction for authors of this journal, except for figure numbers and page headers, which were formatted to facilitate referencing and navigation across the thesis. Also, to avoid repetitions where relevant, we referred to methods that were described in previous Sections.

Statement of Authorship

Title of Paper	Greenhouse spatial effects detected in barley (Hordeum vulgare L.) epigenome may underlie stochasticity of DNA methylation
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	This manuscript reports the effect of spatial variations in greenhouse on the DNA methylation profile of barley under both salt stress and stress-free conditions. Combining environmental, phenotypic and epigenetic data analyses, we found that at least part of the epigenetic variability, previously described as stochastic, is linked to environmental micro variations during plant growth. We propose that subsequent epigenetic studies consider microclimate-induced epigenetic variability

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Overall percentage (%)	50%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii.   permission is granted for the candidate in include the publication in the thesis; and

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Greenhouse spatial effects detected in the barley (*Hordeum vulgare* L.) epigenome underlie the stochasticity of DNA methylation

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ABSTRACT

Environmental cues are known to drive changes in DNA methylation, which is a part of the mechanisms of adaptation to stress in plants. Methylation alterations target stress responsive loci in order to adjust gene expression to respond to the environment. However, some of the stress induced methylations have been reported to be, at least partially, stochastic in nature, even under controlled conditions. The effects of position on plant growth and performance have been well documented, and constitute an additional layer of variability, generally addressed through careful experimental design and spatial analyses of data. Here we assessed the role of positional effects on the plant DNA methylation during a greenhouse experiment, using methylation-sensitive amplified polymorphism (MSAP) analysis and phenotypic analyses. Nine spring barley varieties were grown in a randomized plot design, including two salt treatments (0 mM and 75 mM NaCl). Combining environmental, phenotypic and epigenetic data analyses, we show that at least part of the epigenetic variability, previously described as stochastic, is linked to environmental micro-variations during plant growth. We propose that subsequent epigenetic studies take into account microclimate-induced epigenetic variability.

Key words: epigenetics, positional effect, phenotypic plasticity, genome by environment, salt stress, MSAP.

INTRODUCTION

Epigenetics is concerned with the study of molecular modifications to the genome and associated proteins and their consequences for gene expression and phenotype. Epigenetic gene control includes non-coding RNAs (short-interfering RNAs (siRNAs)), histone variants and DNA methylation (Sawan *et al.*, 2008, Vanyushin, 2006). In plants, such mechanisms are involved in a range of biological contexts, including developmental processes (Ay *et al.*, 2014, Ishida *et al.*, 2008, Jung *et al.*, 2015, Kohler & Makarevich, 2006), cell and organ differentiation (Joyce *et al.*, 2003, Kitimu *et al.*, 2015), reproduction (Podio *et al.*, 2014, Yaish *et al.*, 2011), parental imprinting (Gehring *et al.*, 2006), acquired trait inheritance (Tricker *et al.*, 2013a, Tricker *et al.*, 2013b) and adaptation to stress (Bird & Jaenisch, 2003, Boyko & Kovalchuk, 2008, Tricker *et al.*, 2012).

In particular, DNA methylation has proved to be a prominent epigenetic signature of environmental stress and can readily affect the expression of stress responsive genes (Bird & Jaenisch, 2003, Boyko & Kovalchuk, 2008, Zilberman & Henikoff, 2007). For instance, two genes controlling stomatal development in Arabidopsis, FAMA and SPEACHLESS, were found to be regulated by DNA methylation in response to changes in atmospheric relative humidity (Tricker *et al.*, 2012). Other examples of the involvement of DNA methylation in the regulation of stress response include adaptation to salt stress (Karan *et al.*, 2012), temperature stress (Bastow *et al.*, 2004, Hashida *et al.*, 2006, Pecinka *et al.*, 2010, Song *et al.*, 2012, Steward *et al.*, 2002), herbivory (Herrera & Bazaga, 2011, Herrera & Bazaga, 2013) and heterogeneous environmental pressure (Wang *et al.*, 2016).

However, not all methylation changes observed under stress happen consistently across the populations studied or can be linked to genomic regions associated with stress response. For this reason, such changes have been considered stochastic in nature (Karan *et al.*, 2012, Tricker *et al.*, 2012). Furthermore, stochastic epigenetic mutations have been shown to be spontaneous (Becker *et al.*, 2011, Raj & van Oudenaarden, 2008, van der Graaf *et al.*, 2015), requiring no triggering factors (i.e. occurring randomly in the genome independently of stress). This random and spontaneous alteration of DNA methylation has been considered a biological process that drives diversity and evolution in a Lamarckian-like fashion (Feinberg & Irizarry, 2010, Meyer & Roeder, 2014, Soen *et al.*, 2015, van der Graaf *et al.*, 2015, Vogt, 2015).

Moreover, Soen *et al.* (2015) proposed a conceptual framework of random variations in the genome, initiated in response to environmental cues. They hypothesized that imposition of

diverse types of stress upon individual organisms during development gives rise to an adaptive improvisation which deploys random phenotypic variations to cope with unstable ambient conditions. However, the authors did not suggest an epigenetic mechanism that might be involved in the regulation of such adaptive phenotypic variation.

In a recent review, Vogt (2015) provided greater insight into the concept of random variability. In this pivotal literature analysis, the author linked 'stochastic developmental variation' to stochastic occurrence of DNA methylation (Bird & Jaenisch, 2003, Field & Blackman, 2003). However, Vogt did not consider in depth the role of microclimatic conditions in stochasticity. Although scientists have suspected a role for mesoclimate in epigenetic variability in natural populations (Herrera & Bazaga, 2010), such marked environmental differences were not expected in controlled experimental conditions (e.g. greenhouse or growth room).

Further, genome-by-environment interactions have been shown to be at least partially regulated by DNA methylation (Verhoeven *et al.*, 2010). Therefore, uniformity of environmental conditions is important during plant growth, to minimize variation caused by spatial effects. One way of dealing with spatial variation, if it cannot be prevented, is appropriate experimental design in order to distinguish treatment and positional effects (Brien *et al.*, 2013, Cabrera-Bosquet *et al.*, 2016). Experimental design normally accounts for such variability using blocking and randomization, along with appropriate statistical analyses (Addelman, 1970, Ruxton & Colegrave, 2011). Despite the usefulness of this approach, experimental design cannot entirely remove environmental variability (microclimate). This presents a challenge in the study of DNA methylation. Due to the capacity of plants to promptly sense and epigenetically respond to variation in ambient conditions (Gutzat & Mittelsten Scheid, 2012, Meyer, 2015), it is difficult to discriminate between the so-called stochastic methylation and position-dependent methylation.

In the present study, we used methylation-sensitive amplified polymorphism (MSAP) analysis and phenotypic analyses to assess the effect of microclimate on DNA methylation during a greenhouse experiment. Nine spring barley varieties were grown in a randomized plot design under control and mild salt stress conditions. Environmental, phenotypic and DNA methylation data were collected. We show that at least part of the previously described stochastic epigenetic variability observed during plant experiments is linked to exposure to trivial environmental variations. Moreover, we show how the phenotypic variability observed in these experiments correlates with differences in DNA methylation patterns. Consequently, there is a need to

formally account for microclimate-induced epigenetic variability in future epigenetic and functional studies.

MATERIAL AND METHODS

Plant material and experimental design

Nine varieties of spring barley (Table 5.1) were grown in a controlled temperature greenhouse at the Plant Accelerator® (The Australian Plant Phenomics Facility (APPF)) at the Waite Campus, University of Adelaide from 26 June to 12 October 2013. Varieties with similar flowering times (Menz, 2010) were selected in order to minimize discrepancies in sampling time between varieties. The experiment was designed in eight randomized blocks with two plants of the same variety per plot (Figure 5.1). Three seeds were sown in white pots (20 cm height × 15 cm diameter, Berry Plastics Corporation, Evansville, USA) containing 2400 g potting mixture (composed of 50% UC (University of California at Davis) potting mix, 35% coco-peat and 15% clay/loam (v/v)). Seedlings were thinned to one seedling per pot 2 weeks after sowing. Two salt treatments (0 mM and 75 mM NaCl ('control' and 'salt stress', respectively, hereafter) were applied to three-leaf stage seedlings (25 days after sowing (DAS)), using the protocol described by Berger *et al.* (2012). Pots were watered every 2 days for up to 60 days after sowing to 16.8% (g/g) gravimetric water content, corresponding to 0.8 × field capacity. From day 61 after sowing, plants were watered daily to 16.8% (g/g) until seed set. Leaf samples (50-100 mg) were taken for DNA extraction from blocks 1, 3, 4, 6 and 8 (Figure 5.1) at two time points, *viz.*: 4th leaf blade after full emergence (15 days after salt treatment and 40 DAS) and flag leaf blade from the primary tiller at anthesis (62 days after salt treatment and 87 DAS). Samples were immediately snap frozen in liquid nitrogen and stored at -80 °C until DNA extraction. Whole plants were harvested at maturity and above-ground biomass was dried and weighed.

Table 5.1: List and description of barley genotypes used in this study

Nº	Variety	Earliness	Year[*] of release	Pedigree[*]	
				Parent 1	Parent 2
1	Barque 73	6	1997	Triumph	Galleon
2	Buloke	5	2005	Franklin/VB9104	VB9104
3	Commander	5	2009	Keel/Sloop	Galaxy
4	Fathom	6	2011		
5	Flagship	5	2006	Chieftan/Barque	Manley/VB9104
6	Hindmarsh	6	2007	Dash	VB9409
7	Maritime	6	2004	Dampier/A14//Krisna/3/Clipper	M11/4/DampierA14//Krisna/3/Dampier/A14//Union
8	Schooner	5	1983	Proctor/PrioA (WI2128)	Proctor/CI3578 (WI2099)
9	Yarra	5	2005	VB9018/Alexis/VB9104	

Earliness to flowering score is based on a 0-9 scale, with 0 indicating very late varieties and 9 very early ones (SARDI, 2015). *Year of release and pedigree after Menz (2010).
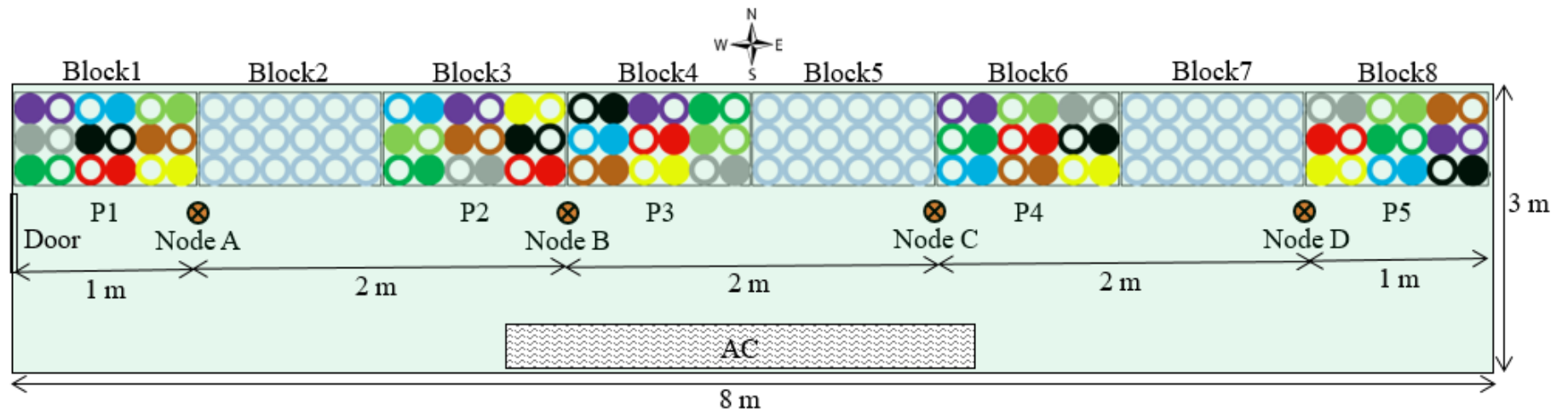
Figure 5.1: Experimental layout and plan of the greenhouse (24 m^2).

Blocks 1, 3, 4, 6 and 8 were used in this study and are respectively assigned to positions P1 to P5. Four sensor nodes (Node A, B, C, and D) were placed along benches, 2 metres apart and one metre from the east and west walls. Circles represent plant position in the block: hollow circles are control plants (0 mM NaCl) and full circles are treated plants (75 mM NaCl). Colours indicate barley varieties: ◐● = Barque73; ◐● = Buloke; ◐● = Commander; ◐● = Fathom; ◐◐ = Flagship; ●● = Hindmarsh; ◐● = Maritime ◐● = Schooner; ◐● = Yarra; ⊗ = Sensor nodes. AC = air conditioning unit.

Greenhouse environmental conditions

The experiment was conducted at the Plant Accelerator®, in a 24 m² greenhouse (~8 m x 3 m), with a gable roof 4.5 m above the floor at the lowest and 6 m at the highest point. The greenhouse (34°58'16 S, 138°38'23 E) was oriented West-East (Figure 5.1), with night/day temperatures controlled at 15°C and 22°C, respectively. To investigate the possible causes of position dependent variability of barley response across the greenhouse, we mimicked environmental conditions of 2013 in 2015 during the same period of the year (26 June to 12 October) in the same greenhouse. Possible differences in environmental factors between the two years (Figure 5.S1) were minimised by the controlled conditions in the greenhouse. Then, environmental factors (temperature, relative humidity and photosynthetic active radiance) were recorded, using four sensor-nodes located along the benches (Figure 5.1), which were growing barley plants, at similar density as in 2013, to minimise any bias. Based on this period of the year, we considered as day, the time between 7 a.m. to 6 p.m., whereas between 6 p.m. and 7 a.m. was considered as night.

The sensor-nodes were two metres apart from each other and one metre from the east and west walls (Figure 5.1). Each node had a combination of sensors for photosynthetic active radiance (PAR) (model Quantum, LI-COR, Lincoln, Nebraska, USA) and for humidity/temperature (Probe HMP60, Vaisala INTERCAP®, Helsinki, Finland). Environmental data was recorded every minute for the period of the experiment using wireless data loggers (National Instruments, Sydney, New South Wales, Australia). Before use for further analyses, this recorded data was quality controlled to remove time slots when data were not present for all four nodes. To show the overall daily fluctuation of environmental factors between sensor-nodes during the experiment, the average measure of each factor per hour was plotted for each node. Then, the vapour pressure deficit (VPD) for each time point was calculated according to Murray (1967): VPD = $(1 - (RH/100))*(610.7*10^{7.5T/(237.3+T)})$, where RH = relative humidity, T = temperature, and the factor $610.7*10^{7.5T/(237.3+T)}$ = saturated vapour pressure (SVP). VPD measures were used to perform analysis of variance (ANOVA), to test the significance of the average differences between the VPD at each sensor-node position in the greenhouse, for day and night separately. In the same way, an ANOVA was also performed for the light integral, to test the significance of the average difference in PAR between each sensor-node, for day and night. A summary statistics of these environmental data at sensor nodes was generated using the R package *compareGroups* (Subirana *et al.*, 2014).

DNA extraction

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

MSAP

The MSAP was used for plant DNA methylation profiling. The method has been described in Chapter 2, Section 2.2.3, of this thesis.

MSAP data analysis

MSAP profiles obtained using *Hpa*II and *Msp*I were used to generate; 1) a qualitative binary matrix of allelic presence/absence scores, considering a peak height threshold of 150 (label relative fluorescence), and 2) a quantitative matrix of allelic peak height using GeneMapper Software v4 (Applied Biosystems). Qualitative epigenetic changes associated with greenhouse positional effect were analysed using fragment sizes between 100 and 550 base pairs, which were selected to estimate epigenetic distance (PhiPT) between individuals and perform Principal Coordinate Analyses (PCoA), using GenAlex 6.501 (Peakall & Smouse, 2012). Then, quantitative analysis of peak height was used to examine the effect of position on the methylation status of individual loci (Rodríguez López *et al.*, 2012).

To examine the effect of position on the plant methylation profile we searched for MSAP markers that were differentially methylated between experimental blocks by comparing the fragment peak heights (Rodríguez López *et al.*, 2012). Before differential expression analysis, model based normalization factors were calculated for the peak height libraries using the weighted trimmed mean method of Robinson and Oshlack (2010). For each variety and sampling method the peak heights were extracted from the data and the MSAP markers were analysed individually using the modelling approach of McCarthy *et al.* (2012). To ensure the peak heights could be compared between positions, the individual models contained a term to

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account for variation between blocks as well as a term to capture the differences between the control and salt stress treatments. A likelihood ratio test was then performed to determine whether estimated coefficients for the positions were equal (McCarthy *et al.*, 2012). The p-values from these tests were then adjusted for multiple comparisons using the false discovery rate method of Benjamini and Hochberg (1995). Analyses was conducted using the differential expression analysis R package *edgeR* (Robinson *et al.*, 2010), in the R statistical computing environment (R Core Team, 2016).

Assessment of correlations between epigenetic profiles and plant phenotypes

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

To estimate the significance of the correlations observed between epigenetic distance and plant biomass and position in the greenhouse, we performed a Mantel Test (Mantel, 1967) in GenAlex 6.501, using matrices generated from epigenetic distance, physical distance and phenotypic (biomass or yield) differences estimated as described above. In all cases, the level of significance of the observed correlations was tested using 9,999 random permutations. Since both enzymes (*Hpa*II, *Msp*I) are methylation sensitive (Reyna-López *et al.*, 1997, Walder *et al.*, 1983), these enzymes can independently show epigenetic marks across the genome. Therefore, our assumptions about plant epigenetic profile thereafter relies on results obtained using either enzyme or a combination of both.

RESULTS

Microclimatic variability in the greenhouse

Recording of greenhouse environmental conditions showed the existence of spatial and temporal disparities for temperature, PAR and humidity (Figure 5.2; Table 5.2). The east side (node D, Figure 5.1) showed, on average, a higher PAR between 8 a.m. and 10 a.m. than the rest of the greenhouse. The PAR was also variable during the day between node positions (Figure 5.2a), with sensor-node B (centre-west, Figure 5.1) recording the lowest PAR values around 12 p.m. (Figure 5.2a).

Average temperatures were similar at all node positions, with about 1℃ differences between nodes around 1 p.m. (Figure 5.2b). No temperature gradient could be observed (Figure 5.2b). However, RH showed a west to east decreasing gradient, with node A (west side) (Figure 5.1), showing the highest RH during both day and night (Figure 5.2c). While nodes B, C and D showed similar RH at night, during the day, the lowest RH was recorded in node D (east end of the greenhouse), and nodes B and C were similar (Figure 5.2c).

A comparison of calculated VPDs per sensor-node showed that node positions experienced significantly different VPDs during both day and night ($P < 0.001$, Figure 5.2). Similar comparison using light integrals also showed significant difference between node positions during the day ($P = 0.000$) and the night ($P < 0.001$) (Figure 5.2; Table 5.S1; Figure 5.S5)).
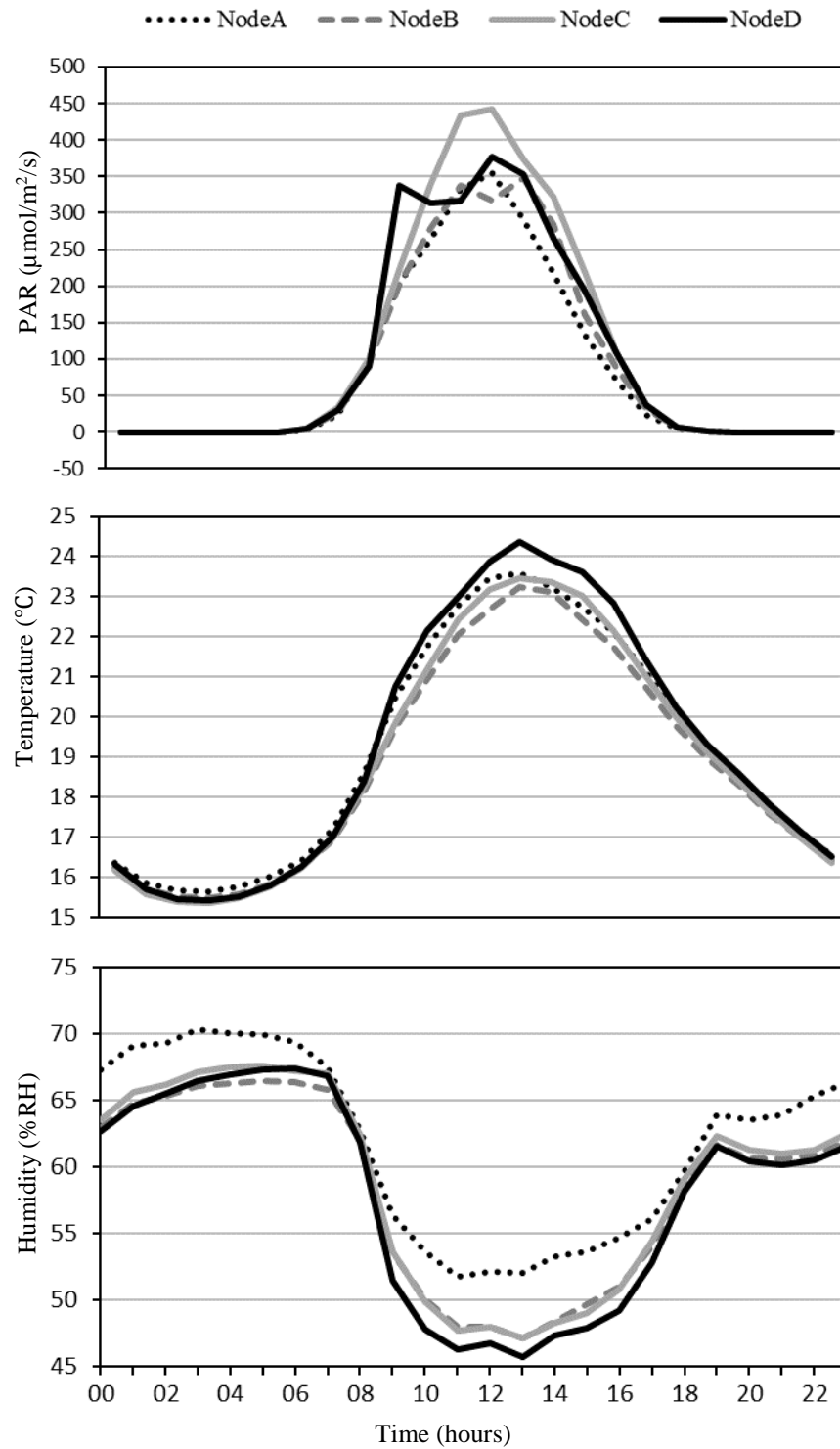
Figure 5.2: Average daily environmental conditions in the greenhouse.
(a) temperature, (b) light and (c) relative humidity were recorded over the period from 26 June to 12 October 2015, at four positions (Node A-D from West to East) in the greenhouse.

Table 5.2: Summary descriptives of the Vapour Pressure Deficit (VPD) and light integral by sensor node (Node A-D).

Environmental data was recorded over the period from 26 June to 12 October 2015, at four positions (Node A-D from West to East) in the greenhouse. VPD_day and LI_day represent day time data (7 a.m. to 6 p.m.) and VPD_night, LI_night represent night time data (6 p.m. to 7 a.m.). SD, standard deviation; P. overall, probability of overall difference between nodes.

	Node A		Node B		Node C		Node D		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	P. overall
VPD_day (Pa)	1284	514	1259	505	1249	486	1339	531	<0.001
VPD_night (Pa)	724	363	696	311	711	311	730	308	<0.001
LI_day $(mol\ m^{-2}\ d^{-1})$	11326	11076	13309	12329	14497	13211	13206	13350	0.000
LI_night $(mol\ m^{-2}\ d^{-1})$	39.7	260	56.9	353	56.3	370	56.7	375	<0.001

Correlation between DNA methylation profile and plant position in the greenhouse

Plant DNA methylation profiles were deduced from MSAP data, which generated 269 alleles with sizes between 100 and 550 base pairs across samples from all nine barley varieties. These markers were used to compare plant epigenetic profiles and determine epigenetic distances between groups. PCoA of epigenetic profiles of barley varieties at anthesis showed grouping of samples by plant position rather than salt treatment, regardless of the enzyme combination used (Figure 5.3a and b). The first coordinate Eigen space matched with the position of the plants in the greenhouse in the West-East direction (Figure 5.3).

The Mantel test using all treatment samples together showed weak correlations between plant epigenetic profiles and plant positions in the greenhouse at 4th leaf stage, and more significant corrections at anthesis (Table 5.3). For instance, for the variety Schooner, the Mantel test between pairwise epigenetic distance and plant position at the 4th leaf stage of barley development resulted in weak correlations for both *Hpa*II ($R^2 = 0.11$, P-value = 0.025, Figure 5.4a) and *Msp*I ($R^2 = 0.12$, P-value < 0.022, Figure 5.4c). Apart from two varieties (Buloke and Schooner), none of the remaining varieties showed a significant correlation between position and epigenetic profile at the 4th leaf stage (Table 5.3, Figure 5.S2). Conversely, these correlations were stronger at anthesis for the same variety, Schooner ($R^2 = 0.48$ and $R^2 = 0.45$, for *Hpa*II and *Msp*I, respectively, Figure 5.4b and d), with greater significance of the P-values

(0.001). Additionally, all the remaining varieties showed significant correlation (P-value at least < 0.05) between DNA methylation profile at anthesis and the plant position in the greenhouse (Table 5.3; Figure 5.S2). The correlations were high ($R^2 > 0.3$) for all varieties, except Buloke and Maritime (Table 5.3).

Additionally, the comparison of peak heights of MSAP markers generated from plants growing in different positions revealed large differences between positions for some alleles (Figure 5.5). In those case, there was generally a big difference in peak height between position P1 and the other positions (Figure 5.5). Peak height differences between positions were not necessarily linear, but showed strong significance.
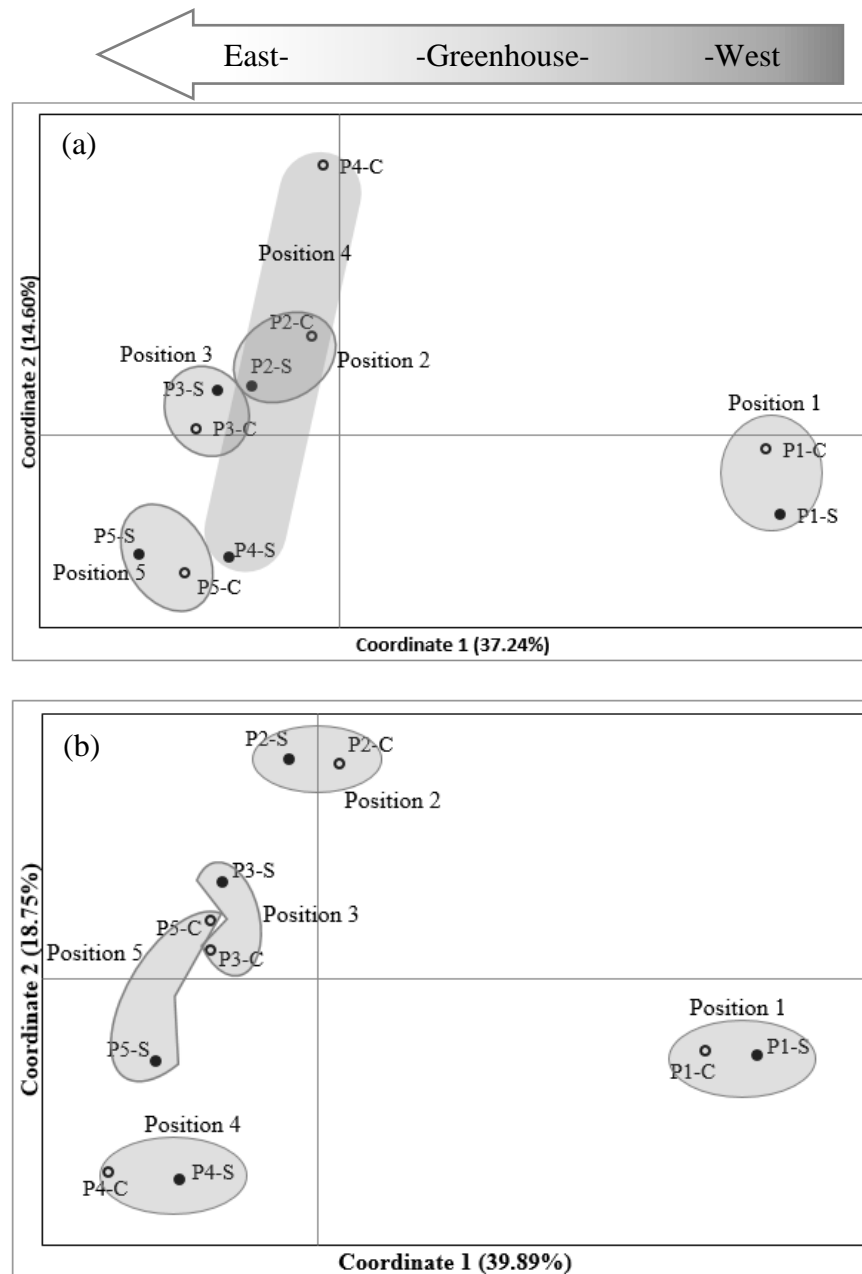
Figure 5.3: Principal coordinates analysis (PCoA) of MSAP (methylation sensitive amplified polymorphism) markers in barley variety Commander.
MSAP markers were generated using five replicates of control (0 mM NaCl) and stress (75 mM NaCl) plant samples, for *Hpa*II (a) and *Msp*I (b). Positions 1 to 5 indicate experimental block numbers; Symbols filled in black and hollow symbols represent salt stress (-S) and control (-C) samples, respectively. The PCoAs show sample distribution in the first two principal coordinates. Number in brackets represent the proportion of variation explained by the coordinate.

Table 5.3: Correlation between pairwise epigenetic distance and physical distance.

Nine barley varieties were used, including ten individuals per variety, five replicates for control and stress plants. Samples were collected from the 4th leaf (at 4th leaf stage) and flag leaf (at anthesis). Epigenetic distances correspond to the Phi statistics of the MSAP markers between plant individuals. The coefficient of determination (R^2) was calculated according to Mantel (1967) using GenAlex 6.5. Asterisk (*), (**) and (***) indicate significant correlation between treatments for P-value < 0.1, 0.01 and 0.001, respectively, estimated based on 9999 permutations.

| Varieties | Coefficient of determination (R^2) | | | |
| | *Hpa*II | | *Msp*I | |
	4th leaf	Anthesis	4th leaf	Anthesis
Barque73	0.003	0.320**	0.010	0.315
Buloke	0.103*	0.001	0.059	0.220*
Commander	0.052	0.332**	0.050	0.332**
Fathom	0.038	0.425****	0.079*	0.527****
Flagship	0.038	0.451***	0.001	0.214*
Hindmarsh	0.008	0.305**	0.004	0.233*
Maritime	0.014	0.130*	0.071*	0.144*
Schooner	0.112*	0.476***	0.120*	0.447***
Yarra	0.002	0.147*	0.027	0.385*
Average	0.041	0.287	0.047	0.313
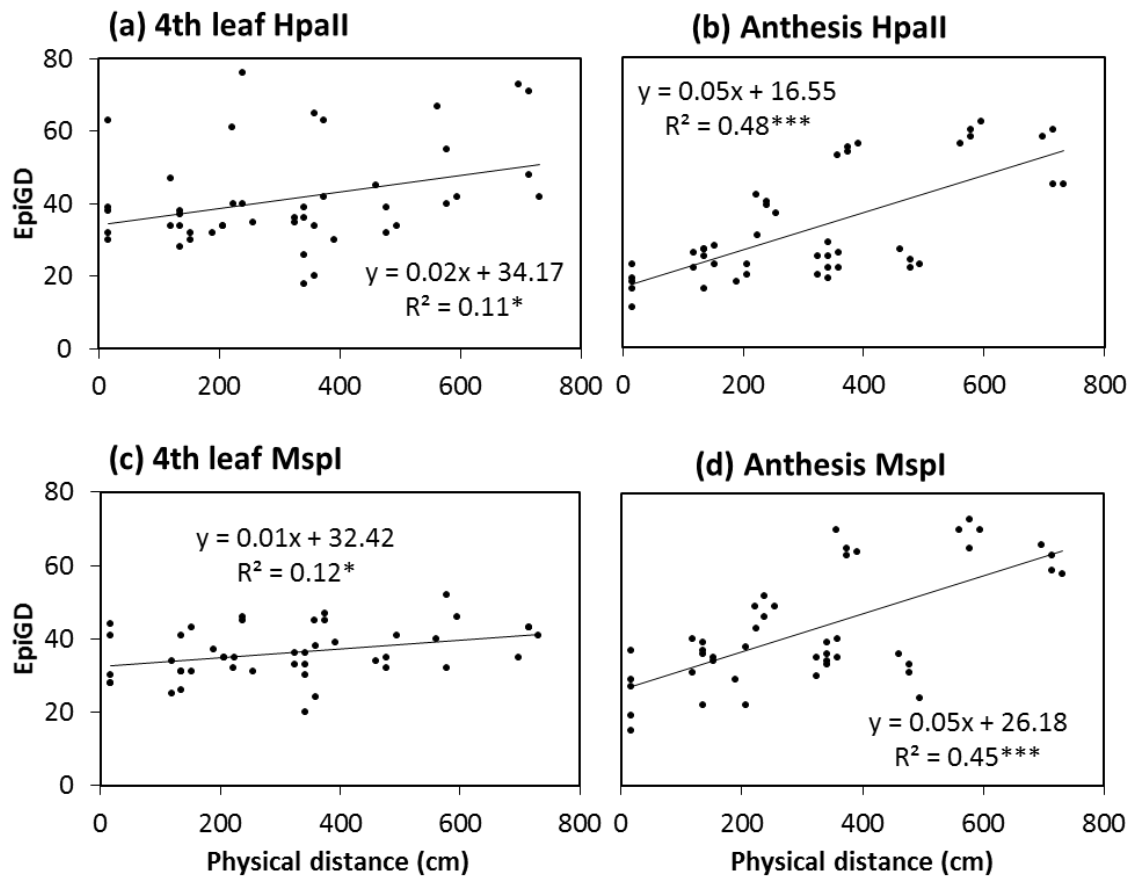
Figure 5.4: Correlation between pairwise epigenetic distance (Epi GD) and plant position in the greenhouse.

The epigenetic distance was estimated at four-leaf stage (a, c; 40 days after sowing) and anthesis (c, d; 87 days after sowing) of barley variety Schooner, using *Hpa*II (a, b) and *Msp*I (c, d) in the MSAP (methylation sensitive amplified polymorphism). Five replicates of control (0 mM NaCl) and stress (75 mM NaCl) were analysed together and dots represent pairwise comparisons between individual plants. Equations represent the formula of the regression line, R^2 represents the coefficient of determination, calculated according to Mantel (1967) using GenAlex 6.5. Asterisk (*) and (***) indicate significant correlation between treatments for P-value < 0.05 and 0.001, respectively, estimated based on 9999 permutations.
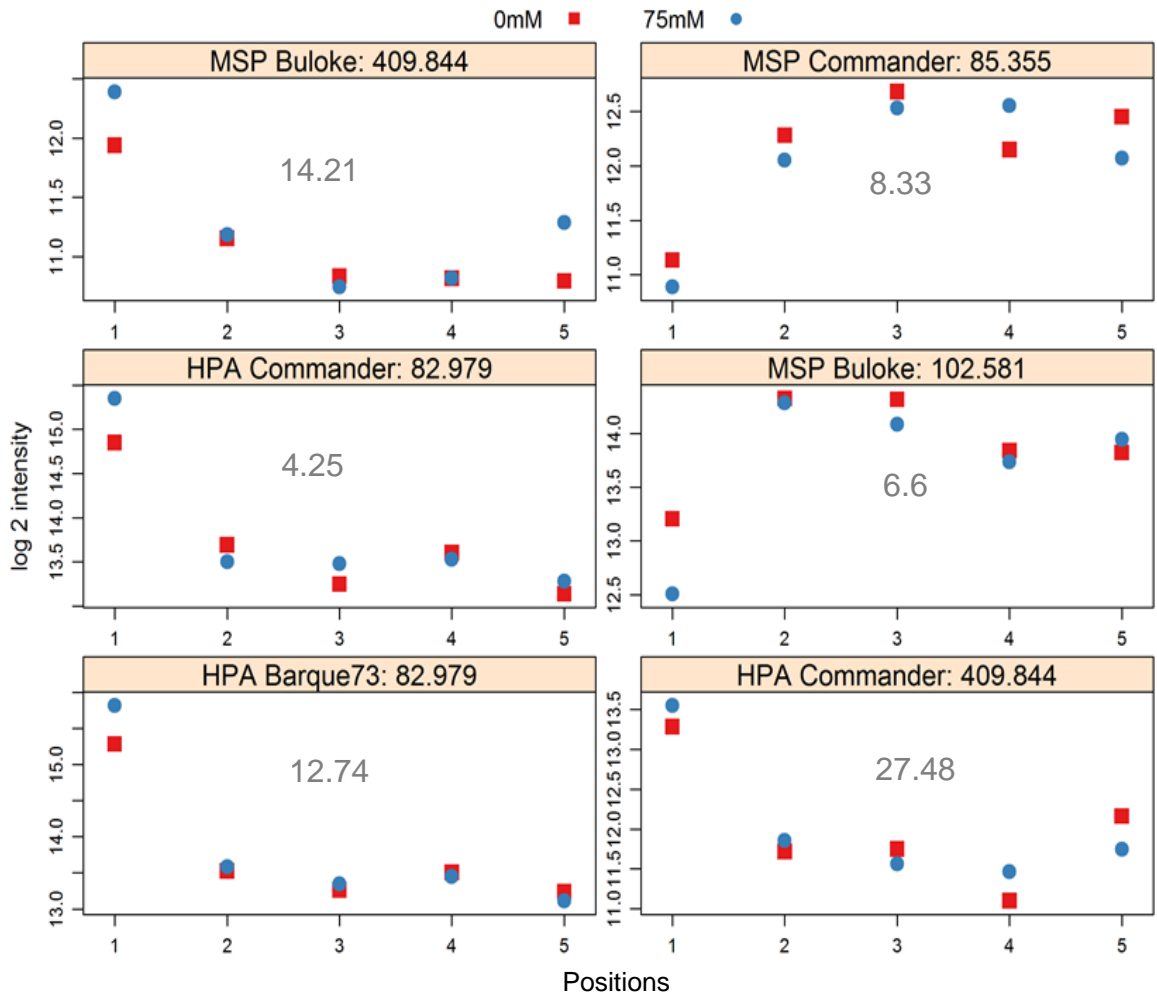
Figure 5.5: Exemplars of MSAP (methylation sensitive amplified polymorphism) alleles that show significant differences in peak height between positions in the greenhouse.

Markers were detected in control (0 mM NaCl, red symbols) and stress (75 mM NaCl, blue symbols) plants; Vertical axis shows logarithm 2 (log 2) of peak height intensity and the horizontal axis represents positions in the greenhouse, in the West to East direction. The grey number in each plot represents -log10 of p-values. The title of each plot shows the enzyme used (either *Hpa*II (HPA) or *Msp*I (MSP), the variety, and the allele identity number.

Correlations between barley phenotype, epigenome and position

The average biomass production of the nine barley varieties increased from position P1 (west side of the greenhouse) to position P5 (east side) (Figure 5.6a and b, Figure 5.S3) and this trend was independent of the plant stress condition. The average grain yield of the barley varieties showed the same West-East trend as the biomass. However, when varieties were examined separately, some displayed diverse patterns of phenotypic variability that did not necessarily match a West-East linear trend observed when combining all data (Figure 5.S3).

Assessment of the relationship between epigenetic profile and grain yield by Mantel test showed significant correlations (P-values < 0.05) between barley grain yield and epigenetic profile in control plants of six of nine varieties (Buloke, Commander, Fathom, Maritime, Schooner, Yarra), with R^2 varying between 0.247 and 0.907 (Table 5.4; Figure 5.S4). Likewise, stress plants showed significant correlations (P-values at least < 0.05) between grain yield and methylation profile in six varieties (Barque 73, Buloke, Commander, Flagship, Maritime, Schooner), with R^2 between 0.164 and 0.921 (Table 5.4; Figure 5.S4). An example of significant correlations between grain yield and epigenetic distance is presented in Figure 5.7a-d, and concerns the variety, Schooner.
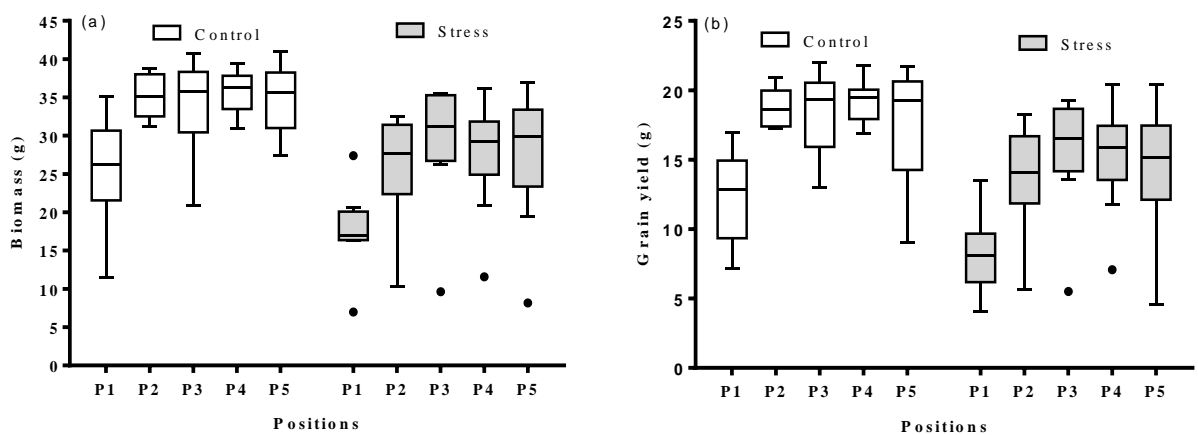
Figure 5.6: Box plots showing biomass and grain yield range per position (P1-5) in the greenhouse (n = 9).
(a) biomass per position for control and stress plants; (b) grain yield per position for control and stress plants; The average data was obtained from nine barley varieties (Barque 73, Buloke, Commander, Fathom, Flagship, Hindmarsh, Maritime, Schooner and Yarra).
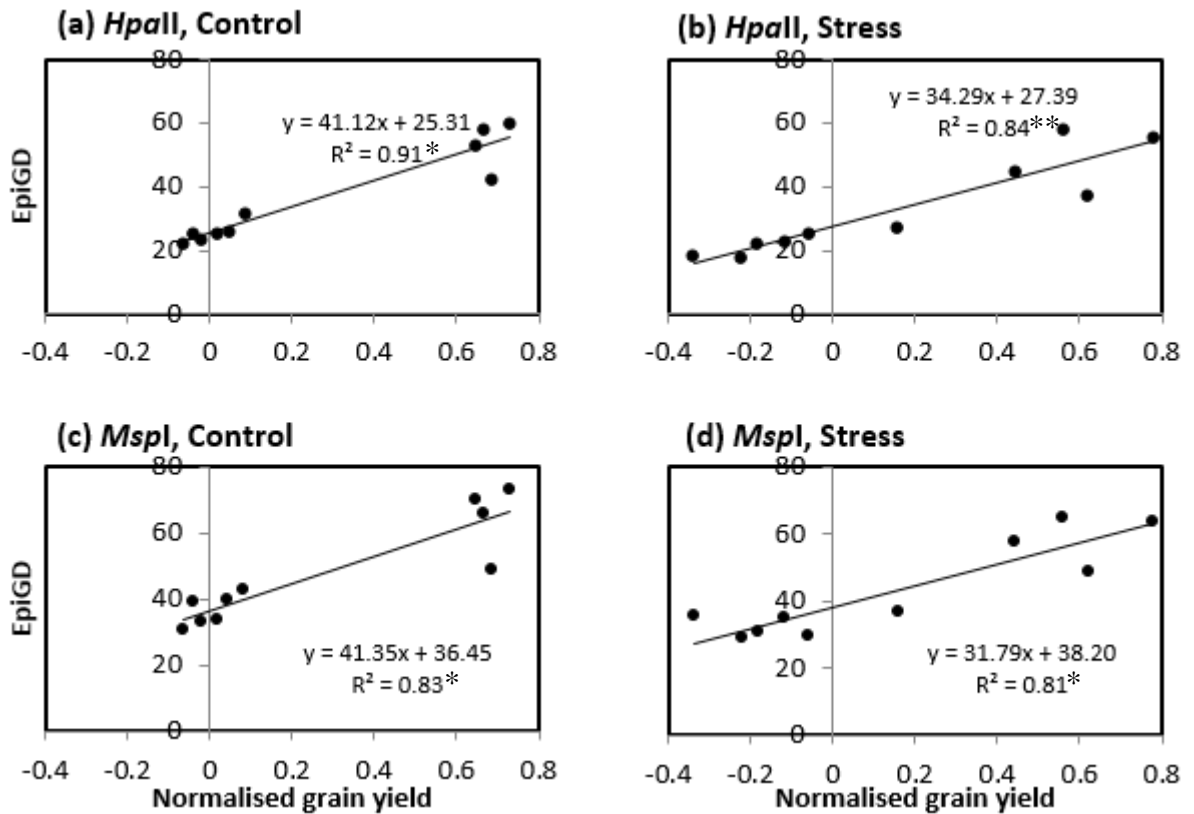
Figure 5.7: Correlation between pairwise epigenetic distance (EpiGD) and pairwise difference in grain yield between plants of the variety Schooner.

The correlation was tested according to Mantel (1967) using GenAlex 6.5. Epigenetic distance between plants was calculated based on MSAP (methylation sensitive amplified polymorphism) data generated using *Hpa*II (a, b) and *Msp*I (c, d). Pairwise differences in grain yield between plants were calculated separately for control (a, c) and stress (b, d) plants. Values of grain yield were normalized by computing the ratio of each individual plant grain yield over the mean grain yield for the same treatment across all positions. The dots represent pairwise comparisons between individual plants; equations represent the formula of the regression line; R^2 represents the coefficient of determination of the Mantel test; asterisk (*) and (**) indicate significant correlation between treatments for P-value < 0.05, and 0.01, respectively, estimated based on 9999 permutations.

Table 5.4: Correlation between epigenetic distance and grain yield of nine barley varieties. Epigenetic distance between plants was calculated based on MSAP data generated using *Hpa*II and *Msp*I. Coefficient of determination (R^2) were computed according to Mantel (1967) using five replicates for each treatment per variety. Asterisk (*) and (**) indicate significant correlation between treatments for P-value < 0.05, and 0.01, respectively, estimated based on 9999 permutations.

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

DISCUSSION

Stochastic DNA methylation is explained by microclimatic differences

The main aim of the randomized block design is to minimize unexplained variation between treatments, and has been a preferred method in plant experiments in the field and in controlled environments (Brien *et al.*, 2013, Edmondson, 1989, Guertal & Elkins, 1996). However, while block homogeneity is difficult to achieve, variability between blocks in the same experimental setting is often either ignored or attributed to randomness (Karan *et al.*, 2012, Raj & van Oudenaarden, 2008, Tricker *et al.*, 2012). For example, DNA methylation not explained by experimental treatments has been proposed to be stochastic (Baulcombe & Dean, 2014, Karan *et al.*, 2012, Raj & van Oudenaarden, 2008, Tricker *et al.*, 2012) and due to spontaneous occurrence of the methylation (Becker *et al.*, 2011, van der Graaf *et al.*, 2015).

In this research, we found that microclimatic variation within a greenhouse (Figure 5.2, Figure 5.2) was sufficient to trigger variability in the plant DNA methylation profile, independent of experimental treatment (Figure 5.5). The variability in environmental data across the greenhouse (Figure 5.2) demonstrates that each plant experienced a unique combination of climatic factors during the experimental period (Figure 5.2a-c); similar observations were also reported for other greenhouse studies (Both *et al.*, 2015, Brien *et al.*, 2013, Cabrera-Bosquet *et al.*, 2016). Such unique environmental conditions may induce position-specific changes in DNA methylation (Figure 5.4 and 5.5), suggesting that spontaneity of DNA methylation (Becker *et al.*, 2011, van der Graaf *et al.*, 2015) is not solely responsible for stochastic DNA methylation.

However, an effect of position can easily be overlooked for short time experiments, since exposure time seems to be critical in the appearance of position-dependent epigenetic markers. The higher correlation between epigenetic differences and physical distance among plants at anthesis (87 DAS) than at the 4th leaf stage (40 DAS) supports the view that exposure to positional microclimates has a cumulative effect on the plant epigenome (Figure 5.3; Figure 5.3-5; Figure 5.S2). This observation is in line with the concept that plant adaptive improvisation, through DNA methylation, is proportional to the severity and duration of the environmental cue to which the plant was exposed (Soen *et al.*, 2015). Therefore, despite the expected influence of mesoclimatic conditions (Herrera & Bazaga, 2010) and factors such as temperature (Hashida *et al.*, 2006), humidity (Tricker *et al.*, 2012) or light (Barneche *et al.*, 2014, Meyer, 2015) on epigenetic variability, the current study suggests, for the first time, that even slight variations in climatic factors (temperature, humidity or light) (Figure 5.2a-c) are sufficient to induce

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modifications in the plant DNA methylation profile (Figure 5.5), as supported by the correlation between plant epigenetic profiles and plant positions in the greenhouse (Figures 5.3-5.5). This observation suggests that stochastic DNA methylation is at least partly attributable to the microclimate in which each plant developed.

Positional effect affects salt stress-induced DNA methylation changes in barley

Positional effects in greenhouse experiments are well known and, if not properly accounted for, can generate background noise that can confound the effect of the experimental treatment (Brien *et al.*, 2013, Edmondson, 1989, Guertal & Elkins, 1996). For instance, spatial variability in environmental factors (Figure 5.2), may introduce variability between replicate plants' development and response to experimental treatments (Edmondson, 1989, Guertal & Elkins, 1996). In the case of salt stress, such spatial variability is liable to introduce flaws in measurements and observations between replicates that, in fact, were not experiencing exactly the same conditions (Addelman, 1970). In this way, the observed negative correlation between RH (Figure 5.2c) and differences in epigenetic differentiation between control and salt stressed pairs of plants growing in the different positions of the greenhouse (Figure 5.6), suggests that variations in environmental factors (Figure 5.2) can stimulate variations in the perception of salt stress by the plant. That is, the observed West to East decrease in RH may affect the plant's requirement for water (Barnabás *et al.*, 2008, Verslues & Juenger, 2011), which, in turn, affects the level of salt stress experienced by each plant. Therefore, plants grown under the same salt treatment but experiencing different RH, are likely to exhibit different response to the salt stress, including different DNA methylation profiles (Boyko & Kovalchuk, 2011, Steward *et al.*, 2002, Tricker *et al.*, 2012).

Phenotypic differences associated to greenhouse microclimates correlate with epigenetic differences

In this study, barley plants exhibited a plastic response to microclimatic conditions, as shown by the variability of biomass and grain yield at different positions in the greenhouse (Figure 5.6). This result corroborates earlier findings by Lacaze *et al.* (2008) that barley is a crop particularly responsive to ambient conditions. Additionally, the irregularity of phenotypical variability patterns across barley varieties and treatments (Figure 5.S3) may have emerged from

two complementary factors; 1) the genetic variability among barley varieties leading to differential responsiveness to positional effect (Kren *et al.*, 2015, Lacaze *et al.*, 2008), and 2) the randomness of spatial microclimatic conditions, which did not have a linear spatial gradient (Figure 5.2a-c, Figure 5.5). The influence of a genotype-by-environment effect on plant phenotype was expected (Aspinwall *et al.*, 2015, Gianoli & Palacio-López, 2009), but the scale of phenotypical variation induced by environmental variation was unclear. Our findings highlight the possibility for plants to show phenotypic responses to even slight variations in ambient conditions. Furthermore, the correlation observed between barley epigenetic profiles and grain yield (Figure 5.4; Figure 5.7a-d; Figure 5.S4), suggests that DNA methylation could have contributed the variation in the plant phenotypes. These results are in accordance with a mounting body of evidence that plant plasticity is epigenetically governed (Aspinwall *et al.*, 2015, Baulcombe & Dean, 2014, Boyko & Kovalchuk, 2008, Rois *et al.*, 2013). Altogether, these results show a tight interplay between plant epigenome, environment and phenotype.

CONCLUSION

Whilst homogeneity of environmental conditions is difficult to obtain in a greenhouse in practical terms (Brien *et al.*, 2013, Edmondson, 1989, Guertal & Elkins, 1996), awareness of plant sensitivity to microclimate is important, especially in epigenetic studies, where plant epigenomes seem to be responsive to small fluctuations in environmental factors. This study reveals that DNA methylation markers which might previously have been considered stochastic are likely to have been at least partially induced by 1) positional effects in growth conditions, 2) differences in the length of plant exposure to relatively trivial variations in environment and 3) synergistic effects of stress treatment (mild salt stress in this case) and microclimatic conditions. Moreover, the correlation between phenotypic DNA methylation differentiation between plants grown in different microclimates suggests that, position-induced DNA methylation, previously ignored or considered as stochastic, may be the source of the phenotypic variability. Therefore, future epigenetic analyses need to take into account the effect of micro-variations in environmental factors, by careful experimental design and by considering position-induced DNA methylation markers as strong candidates for fine-tuned response to small environmental changes.

However, although these results should be viewed with some caution due to data collection in two different years, they are comforted by the evidence that spatial variability within

greenhouses has been recurrently reported. Therefore, possible difference in greenhouse external environmental conditions is not expected to abolish spatial variability. In any case, further research is needed to untangle microclimate-induced epigenetic variations from epigenome instability due to experimental treatment and developmental stages.

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AUTHOR CONTRIBUTIONS:

M.K. performed the experiments, analysed the data and wrote the manuscript; J.T. performed the statistical analysis of MSAP peak heights; M.J.W., E.S.S., B.B., C.M.R.L. conceived the experiments and supervised the work. All authors read and commented on the manuscript.

CONFLICTS OF INTEREST:

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in Appendix 6 of this thesis.

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Chapter 6: General discussion

6.1.   Summary of the thesis project

Since genomic DNA is similar in all cells in a given individual plant, it is difficult to explain spatial and temporal differential gene expression by genetic information alone (Zhang, 2008). While it has now become clear that epigenetic mechanisms are involved in plant development and response to environmental cues, the discovery and characterisation of epigenetic markers diagnostic of specific environments has remained challenging, because of the difficulty in dissociating developmental differences (Ay *et al.*, 2014, Brandeis *et al.*, 1993, Yaish *et al.*, 2011) from purely environmental responses (Boyko & Kovalchuk, 2008, Grativol *et al.*, 2012, Gutzat & Mittelsten Scheid, 2012, Pan, 2013, Sahu *et al.*, 2013).

In this project, we explored DNA methylation differences associated with salt stress and organ differentiation in barley. The study of this prominent epigenetic mechanism provides new insights into the way DNA methylation fluctuates under salt stress and during plant developmental progression. The use of salinity to study stress-induced DMMs presented the advantage of being easy to control, since salt concentrations can be manipulated at will, provided that the environmental conditions are strictly maintained as homogenous. Methylation profiles of plant samples were generated using two approaches: MSAP and ms-GBS.

Using MSAPs, we detected a relatively low number of DMMs, which were not conserved across the barley varieties analysed (Chapters 2). It was also found that microclimatic variations between plant growing positions (Chapters 5) can significantly affect the plant methylation profile. This finding suggests that at least some of the changes in DNA methylation previously deemed as random may be in fact be due to positional effect. Although results generated using MSAPs are reliable in detecting changes in DNA cytosine methylation (Li *et al.*, 2013, Li *et al.*,

2008, Marconi *et al.*, 2013, Rodríguez López *et al.*, 2012), they present two main limitations. First, due to the nature of the technology used, the number of markers generated is low, and therefore represents only a small subset of the potential methylation markers. Second, the generated markers are anonymous, that is, MSAPs only inform the researcher if a marker is present or not, but does not provide a genetic sequence to put the marker into a genomic context. (Fulnecek & Kovarik, 2014, Reyna-López *et al.*, 1997, Rodríguez López *et al.*, 2012). It can be understood that these limitations of the MSAP, are explanatory of the low detection rates of DMMs that are stable in all the barley varieties tested. Therefore, we sought an appropriate option for detecting and characterising site specific DNA methylation as ms-GBS. With these study tools (MSAP and ms-GBS), we achieved conclusions from our experiments, that are summarised in the following Sections.

6.1.1. Salt-induced and tissue specific- DMMs in barley

An appropriate DNA methylation profiling method is necessary to identify DMMs between samples. The widespread use of the MSAP supposes that it is reliable in detecting polymorphic methylation markers in a reproducible manner. However, it is difficult to cover all possible primer combinations to amplify all restriction fragments (*Hpa*II and *Msp*I), as this may result in high volume of labour and costs (Xia *et al.*, 2014). Therefore, only two primer combinations were used in this study (Chapter 2). Although these primers detected polymorphic bands, they provided only a few salt-induced DMMs, which were not conserved across barley varieties. However, these MSAP markers gave an indication that exposure to low levels of salinity can induce specific DNA methylation in barley. The MSAP results also showed that DNA methylation profiles in barley are influenced by both genotype (Chapter 2) and ambient microclimatic conditions (Chapter 5). With these results in mind, we performed ms-GBS projects, using samples from experiments designed to minimise epigenetic variability due to positional effects (Chapters 3 and 4). Five barley varieties, including sensitive and tolerant genotypes (Chapter 2, Figure 2.3) were used to cover possible genetic diversity in these ms-GBS projects, as an affordable and rapid methylation profiling method that harness next generation sequencing on Illumina HiSeq 2500 platform (Kitimu *et al.*, 2015, Xia *et al.*, 2014).

Ms-GBS showed at least two benefits over the MSAP: 1) it generated nearly one million unique sequence tags per experiment, covering 32% of 5`-CCGG-3` sites in the barley genome; 2) Detected DMMs could be mapped to the barley reference genome, to assess correlations with

genomic features such as genes, repeats and regulatory regions (e.g. promoters, UTRs) (Chapters 3 and 4). In contrast with the MSAP results, the ms-GBS, allowed us to detect thousands of DMMs between plant tissues and between salt treatments. Indexed DMM detection was performed using the *msgbsR* package in R (https://github.com/BenjaminAdelaide/msgbsR), which improved the way results were analysed in earlier ms-GBS studies (Kitimu *et al.*, 2015). In this way, and due to the use of diverse varieties, including both sensitive and tolerant varieties (Chapter 2, Figure 2.3), the DMMs detected are potentially universal.

Here, DMMs were mapped to the barley genome to establish their correlation with known genomic features, thus providing a more complete marker characterisation (Chapters 3 and 4). Moreover, we applied some flexibility in the selection of DMMs, based on the FDR values. Although the general rule is to use DMMs with an FDR below 0.05, this threshold may vary depending on the project. The use of variable FDR cut-off threshold is common and relies on the need to obtain markers, therefore decreasing FDR stringency; or to control marker abundance, therefore increasing FDR stringency (e.g. (Secco *et al.*, 2015)).

As we used *Msp*I in the ms-GBS, the restriction fragments were either from non-methylation at CCGG or internal cytosine methylation (C^{m}CGG), since *Msp*I does not cleave ^{m}CCGG. Therefore, differences in read counts arose from methylation changes at ^{m}CCGG, part of the CHG context. The detection of DMMs in the CHG context and their mapping with the barley genome, revealed two important results: 1) CHG methylation occurs in the gene-body, contrary to previous views that only CG methylation was fund in the gene-body (Bewick *et al.*, 2016, Cokus *et al.*, 2008, Deaton & Bird, 2011, Illingworth *et al.*, 2008), and 2) CHG methylation is involved in discriminating barley tissues and salt stress states. Tissue-specific methylation changes in CHG were previously reported in sorghum, in which CHG was more prominent in distinguishing tissues than the CG and CHH contexts (Zhang *et al.*, 2011). However, it should be remembered that the current study reports only ^{m}CCGG methylation analysis, therefore it did not provide any methylation information about CG, CHH and even CHG that differs from CC̲GG.

6.1.2. Stochasticity of DNA methylation

One of the challenges in the analysis of stress-induced, tissue-specific and age-specific DNA methylation markers is the occurrence of DMMs that are independent of the treatment in question. This has led to the common belief that many epigenetic markers are stochastic and unpredictable (Becker *et al.*, 2011, Massicotte *et al.*, 2011). DMMs were considered as stochastic when they could not be related to the experimental treatment (Feinberg & Irizarry, 2010, Meyer & Roeder, 2014, Vogt, 2015). Although scientifically valid approaches were used to reach the conclusion that some of the DNA methylation modifications acquired during the development or under stress are stochastic, these studies underestimated the effect of microclimatic differences affecting individual plants during experimentation, on the plant DNA methylation profile. Unravelling possible causes of stochastic DNA methylation constitutes an important advance in our understanding of the instability of plant epigenomes. Moreover, microclimatic conditions experienced by individual plants in experimental settings can significantly affect plant methylation profiles, to the point of even overriding salt-induced alterations of DNA methylation (Chapter 5) (Pecinka *et al.*, 2010). Our results improve the current understanding of plant sensitivity to small variations in ambient conditions, and suggest that future epigenetic studies should be observant of rigorous standardisation, to minimise background noise, in order to generate robust DMMs across compared sample groups.

6.1.3. Generating genotype-independent DMMs

In this study, attention was paid to the genotype dependent variability of salt stress induced DMMs. Plant genetic background is known to affect its methylation profile (Fang *et al.*, 2010, Garg *et al.*, 2015), and this has an implication for the selection of DMMs in a given species, be they salt- or developmentally-induced. Some DMMs in a genotype may disappear in another one, simply due to a mutation in the *Msp*I recognition site. Thus, it was expected that sets of DMMs may be genotype specific (Karan *et al.*, 2012). To detect potentially universal DMMs, we selected those conserved in five barley varieties (Barque 73, Flagship, Hindmarsh, Schooner and Yarra), for detection of both salt-induced and tissue-specific DMMs. This approach provided statistical power and robustness to the detected DMMs. More importantly, selecting genotype-independent DMMs, offers the possibility of using these for diagnostic purposes in barley, as such DMMs can diagnose both salt stress and tissue types in the plant. Although DNA methylation markers have been used previously for the diagnosis of medical conditions such as

cancers (Stebbing *et al.*, 2006, Van Neste *et al.*, 2012), this possibility was yet to be applied to plants. Therefore, our results open a great opportunity for the use of DMMs as diagnostic tools for plant stresses.

However, using genotype-independent DMMs leads to the loss of some makers that may show significant differential methylation within varieties. In the case of salt-induced DMMs, genotype-specific DMMs could highlight the response to salt of barley varieties having different salt tolerance levels. This would present a way to characterise barley epigenetic response to salt stress, based on salt tolerance levels. It has been previously shown that sensitive and tolerant crop plants adapt their DNA methylation profiles differently to salt stress (Poulsen *et al.*, 2013). Data analyses are underway to uncover barley methylation profiles relative to salt tolerance levels. For tissue-specific DMMs presenting genotype specificity, these might underlie phenotypic characteristics of barley varieties. Therefore, it will be important to identify genotype-specific tissue DMMs and investigate their correlation with varietal traits.

6.1.4. DMMs induced simultaneously by salt stress and tissue identity

The influence of environmental factors on the variability of plant DNA methylation profiles, and salt-induced DMMs coupled with tissue specificities of plant methylomes, constitute layers of complexity in epigenetic profiling. This study achieved the conclusion that salt-induced DNA methylation was tissue specific, and that roots and leaves also display thousands of distinctive DMMs. However, it was a surprise that some salt-induced DMMs were differentially methylated between the two organs (roots and leaves) under stress-free conditions. Of 6099 salt-induced DMMs (in both roots and leaves), 561 were also differentially methylated between root and leaf tissues of salt-free plants. These "double markers" were predominantly found in leaves (504 DMMs) rather than in roots (57 DMMs). This observation shows that the same marker can change simultaneously for two independent reasons; in this case, salt stress and tissue identity. This is also a possible cause of the tissue-specificity of salt-induced DMMs, as extensively reported (Chapter 3; (Karan *et al.*, 2012, Lu *et al.*, 2007, Zhong *et al.*, 2009)). The existence of DMMs that show multiple inducing factors, suggests that there is a kind of functional unity in the plant genome, which deploys epigenetic mechanisms to modulate simultaneously, various types of functions (Colot & Rossignol, 1999). However, further study is needed to clarify how and why "double markers" occur in the genome.

6.1.5. DMMs target repeat regions of the barley genome

The majority of salt-induced and tissue specific DMMs were found concentrated around repeat regions of the barley genome (Chapters 3 and 4). This shows an important role for repeat regions in plant response to stress and in setting plant tissue identity. Among repeat regions, transposable elements, also referred to as 'jumping genes', influence genes expression (Hollister & Gaut, 2009, Martienssen, 1998, Selker, 1999). Methylation of transposons prevents transposition, and it has been proposed that this occurs after the required nearby genes have been activated (Secco *et al.*, 2015). The authors suggested that transposon methylation is a consequence of the activation of nearby genes (Secco *et al.*, 2015). It can be inferred that gene silencing may be accompanied by demethylation of transposons and that the methylation status of transposons can inform about the activity of nearby genes. Therefore, considering transposons as genomic parasites or sequences only reserved to allow genome evolution, seems too restrictive. Although further investigation is needed to establish the correlation between the methylation pattern in repeat regions and plant response to stress or tissue identity, this genomic feature seems to be essential. For instance, DMMs that differentiated leaf blades and sheaths, were all near repeats, and none was around a gene. In this case, should there be genes that are essential to these tissues' definition, they are likely to be influenced by DM repeats. Therefore, gene regulation by DNA methylation may not always be direct, but may instead occur indirectly through methylation changes in adjacent repeats, and this possible role of DM repeats was valid for both salt-induced and tissue-induced DMMs. This view point corroborates with that suggesting that methylation in repeats determines chromatin conformation, which is known to affect gene expression (Bird & Wolffe, 1999, Hollister & Gaut, 2009, Kass *et al.*, 1997, Martienssen, 1998).

Furthermore, it appeared that the extent of the plant response to stress is proportional to the abundance of repeat regions in the genome. While *Arabidopsis* grown under stressful conditions showed very limited changes in DNA methylation, a massive alteration of DNA methylation was observed in barley and rice under stress (Secco *et al.*, 2015). This contrast may be attributable to the fewer transposons in *Arabidopsis,* compared with rice (Secco *et al.*, 2015).

6.1.6. DNA methylation profiling for gene discovery

Furthermore, this study revealed that many DM genes correlate with functions that are likely to set apart the samples that were compared to generate DMMs. Thus, while salt-induced DM genes enriched GO terms correlated with plant response to stress (Chapter 3), tissue-induced

DM genes enriched GO terms that related to tissue-specific functions (Chapter 4). In this way, the present study paved the way for a gene discovery method, based on the identification of DM genes, most of which are likely to correlate with the plant's response to the factor that induced the DMM. This approach had been evoked before by Mason *et al.* (2008), who thought that MSAP markers could detect genes involved in tomato response to viral infection. Therefore, the identification of DM genes provides a valuable gene discovery approach, although this needs to be refined, due to the complexity of gene activity in response to methylation.

In spite of the suggestion that the DNA methylation status can predict gene activity (Wiench *et al.*, 2011), it should be borne in mind that DNA methylation within and around a gene does not necessarily lead to gene repression, as commonly described in the literature (Berdasco *et al.*, 2008, Bird & Jaenisch, 2003, Bird & Wolffe, 1999, Jones, 2012, Kass *et al.*, 1997). Previous studies have shown that DNA methylation is responsible for both gene up-regulation (Zilberman *et al.*, 2007) and down-regulation (Choi & Sano, 2007). Therefore, DMMs in a gene do not always explain whether such a gene will be subsequently silenced or enhanced (Li *et al.*, 2015). The effect of DNA methylation on gene regulation seems to be dependent on the methylation context (CG, CHG or CHH) and position relative to the gene and transcription factors (Jones, 2012). DNA methylation in the immediate proximity of the TSS supresses transcription, whereas gene-body methylation might enhance transcriptional elongation (Jones, 2012). Hence, gene discovery through differential methylation analysis, will still require gene expression analysis to address the question as to whether DM genes are also DE genes, and whether methylation and expression levels correlate. Refining this correlation is needed, to apply DNA methylation profiling as a gene discovery method and eventually use this approach as a surrogate for forward and reverse epigenetics and genetics (Amoah *et al.*, 2012).

6.2.   Outlook work

As this PhD project comes to an end, there were a few topics that needed further research to complement our findings. The identification of salt-induced and tissue-specific DMMs, and DM genes in barley was a valuable achievement in this thesis. However, as discussed above (Chapters 3 and 4), it was difficult to tell whether methylation affected the expression of DM genes. Therefore, gene expression analysis along with DNA methylation profiling is necessary to determine the correlation between DMMs and the expression of DM genes. Also, there is a need to investigate the effect of DMMs in precise genomic features (e.g. introns, exons, repeats)

on the expression of nearby genes. This is important in order to understand the complex interrelationships between these genomic features (Emami *et al.*, 2013, Li-Byarlay *et al.*, 2013, Zhang *et al.*, 2006).

In the present project, central to DNA methylation profiling of barley in various conditions, was the prospect of using DMMs, at least a subset, for diagnostic purposes (epidiagnostic). Although many salt-induced DMMs were characterised in this study, further analyses may determine qualitative diagnostic markers that show the presence or absence of salt stress, and quantitative diagnostic markers able to show the relative salt concentrations that induced the stress. Epidiagnostic of salt stress requires the identification of robust DMMs, preferably conserved across barley genotypes. In the same way, the diagnostic of tissue identity can be explored from tissue-induced DMMs. Previous studies have already introduced the idea of epidiagnostic, and succeeded in detecting tissue types and disease conditions (Doi *et al.*, 2009, Esteller *et al.*, 2001, Rodríguez López *et al.*, 2012). These works should be capitalised with the DMMs detected here for salt stress and tissue identity, to further to further strengthen the diagnostic value of those DMMs.

Furthermore, future work should investigate the transmissibility of salt-induced markers. Observed phenomenon such as priming or hardening in plants suggests that plant response to stress may be imprinted in the genome to permit quicker and/or improved response to later similar stress (Conrath, 2011, Luna *et al.*, 2012, Tricker, 2015). The transmissibility of this 'memory' of the known stress attracts interest as this provides an additional tool to breeders for plant improvement (Rodriguez Lopez & Wilkinson, 2015). This can be investigated by seeking salt-induced DMMs in the next generation of plants having experienced salt stress. Also, as the evolutionary significance of salt-induced changes in DNA methylation relies on the plant capacity to pass acquired epigenetic states to progeny, therefore, it is important to know how altered states of DNA methylation are perpetuated between generations.

Appendices

Appendix 1: Ontology of salt-induced differentially methylated genes in barley

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensabi	representa	eliminated
GO:0009968	negative regulation of signal transduction	0.18%	2.592	-5.641	4.944	-104.387	0.773	0	9968	0
GO:0031324	negative regulation of cellular metabolic process	0.72%	null	null	5.552	-10.7011	0.802	0.819	9968	1
GO:0035556	intracellular signal transduction	2.72%	null	null	6.131	-10.762	0.726	0.912	9968	1
GO:0045934	negative regulation of nucleobase-containing compound metabolic process	0.54%	null	null	5.431	-6.2441	0.752	0.92	9968	1
GO:0010629	negative regulation of gene expression	0.59%	null	null	5.47	-7.0419	0.789	0.928	9968	1
GO:0010558	negative regulation of macromolecule biosynthetic process	0.50%	null	null	5.398	-3.3565	0.755	0.913	9968	1
GO:0007165	signal transduction	3.80%	null	null	6.277	-42.5452	0.715	0.653	9968	1
GO:0033554	cellular response to stress	2.34%	null	null	6.065	-5.4461	0.884	0.796	9968	1
GO:0032101	regulation of response to external stimulus	0.32%	null	null	5.196	-13.0501	0.856	0.817	9968	1
GO:0009890	negative regulation of biosynthetic process	0.51%	null	null	5.408	-6.2441	0.807	0.915	9968	1
GO:0006974	cellular response to DNA damage stimulus	1.98%	null	null	5.993	-4.9957	0.886	0.722	9968	1
GO:0036211	protein modification process	2.90%	7.72	-1.062	6.158	-192.544	0.846	0	36211	0
GO:0018193	peptidyl-amino acid modification	0.77%	null	null	5.586	-17.4647	0.838	0.819	36211	1
GO:0044267	cellular protein metabolic process	8.78%	null	null	6.64	-247.513	0.796	0.669	36211	1
GO:0032446	protein modification by small protein conjugation	0.12%	null	null	4.785	-12.9469	0.864	0.552	36211	1
GO:0043414	macromolecule methylation	1.18%	null	null	5.768	-13.4609	0.847	0.708	36211	1
GO:0006412	translation	4.70%	null	null	6.369	-9.5654	0.745	0.578	36211	1
GO:0006464	cellular protein modification process	2.90%	null	null	6.158	-115.038	0.812	0.798	36211	1
GO:0009451	RNA modification	1.92%	null	null	5.979	-8.0969	0.773	0.754	36211	1
GO:0006508	proteolysis	3.71%	null	null	6.266	-17.062	0.852	0.537	36211	1
GO:0034645	cellular macromolecule biosynthetic process	17.20%	null	null	6.932	-49.4389	0.719	0.592	36211	1
GO:0045184	establishment of protein localization	1.68%	-4.032	-3.953	5.922	-37.9031	0.89	0	45184	0
GO:0015031	protein transport	1.60%	null	null	5.9	-32.284	0.888	0.972	45184	1
GO:0070727	cellular macromolecule localization	0.67%	null	null	5.523	-26.4168	0.888	0.847	45184	1
GO:0033365	protein localization to organelle	0.17%	null	null	4.925	-5.6126	0.895	0.773	45184	1
GO:0006886	intracellular protein transport	0.52%	null	null	5.413	-15.2027	0.881	0.969	45184	1
GO:0008104	protein localization	1.72%	null	null	5.931	-40.3251	0.892	0.945	45184	1
GO:0072666	establishment of protein localization to vacuole	0.00%	null	null	3.269	-6.6038	0.918	0.764	45184	1
GO:0006605	protein targeting	0.38%	null	null	5.277	-6.1643	0.837	0.94	45184	1
GO:0010876	lipid localization	0.10%	null	null	4.687	-9.857	0.916	0.699	45184	1
GO:0072594	establishment of protein localization to organelle	0.15%	null	null	4.882	-15.2027	0.894	0.756	45184	1
GO:0034613	cellular protein localization	0.67%	null	null	5.523	-28.9508	0.882	0.879	45184	1
GO:0071669	plant-type cell wall organization or biogenesis	0.01%	0.677	-3.062	3.728	-4.9586	0.964	0.018	71669	0
GO:0044036	cell wall macromolecule metabolic process	0.89%	null	null	5.648	-2.6808	0.861	0.642	71669	1
GO:0006996	organelle organization	0.93%	-2.231	-1.683	5.666	-63.1433	0.883	0.026	6996	0
GO:0000280	nuclear division	0.06%	null	null	4.503	-8.466	0.893	0.746	6996	1
GO:0071822	protein complex subunit organization	0.87%	null	null	5.635	-23.1427	0.876	0.777	6996	1
GO:0071826	ribonucleoprotein complex subunit organization	0.11%	null	null	4.732	-56.9393	0.895	0.524	6996	1
GO:0022613	ribonucleoprotein complex biogenesis	1.06%	null	null	5.72	-5.2168	0.92	0.759	6996	1
GO:1902589	single-organism organelle organization	0.64%	null	null	5.5	-21.1349	0.82	0.619	6996	1
GO:0061024	membrane organization	1.01%	null	null	5.701	-9.5918	0.883	0.684	6996	1
GO:0051276	chromosome organization	0.34%	null	null	5.223	-10.762	0.883	0.861	6996	1
GO:0006325	chromatin organization	0.11%	null	null	4.733	-20.8665	0.885	0.78	6996	1
GO:0022607	cellular component assembly	1.47%	null	null	5.865	-31.1681	0.876	0.678	6996	1
GO:0051783	regulation of nuclear division	0.02%	null	null	3.922	-6.9957	0.801	0.903	6996	1
GO:0070271	protein complex biogenesis	0.57%	null	null	5.449	-14.7077	0.924	0.708	6996	1
GO:0065003	macromolecular complex assembly	0.68%	null	null	5.527	-16.1778	0.877	0.923	6996	1
GO:0010965	regulation of mitotic sister chromatid separation	0.01%	null	null	3.659	-2.2447	0.796	0.87	6996	1
GO:0043933	macromolecular complex subunit organization	1.06%	null	null	5.723	-22.6289	0.882	0.688	6996	1
GO:0033043	regulation of organelle organization	0.10%	null	null	4.685	-7.6819	0.821	0.772	6996	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP) (Continued

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensab	representa	eliminated
GO:0007010	cytoskeleton organization	0.15%	null	null	4.876	-12.0482	0.89	0.802	6996	1
GO:0045229	external encapsulating structure organization	0.90%	null	null	5.652	-10.6517	0.884	0.643	6996	1
GO:0051128	regulation of cellular component organization	1.02%	null	null	5.705	-2.2268	0.792	0.685	6996	1
GO:0034622	cellular macromolecular complex assembly	0.50%	null	null	5.399	-20.0301	0.879	0.896	6996	1
GO:0006461	protein complex assembly	0.56%	null	null	5.448	-17.5867	0.878	0.948	6996	1
GO:0006091	generation of precursor metabolites and energy	3.22%	1.009	1.169	6.205	-47.0506	0.907	0.035	6091	0
GO:0010228	vegetative to reproductive phase transition of meristem	0.01%	-1.035	-3.604	3.674	-59.7305	0.87	0.036	10228	0
GO:0090567	reproductive shoot system development	0.02%	null	null	3.939	-12.6308	0.86	0.951	10228	1
GO:0048580	regulation of post-embryonic development	0.01%	null	null	3.694	-9.5817	0.809	0.824	10228	1
GO:0048608	reproductive structure development	0.06%	null	null	4.449	-37.9031	0.859	0.892	10228	1
GO:0007275	multicellular organismal development	0.46%	null	null	5.36	-52.0635	0.86	0.661	10228	1
GO:0048437	floral organ development	0.01%	null	null	3.689	-4.4685	0.863	0.967	10228	1
GO:0048447	sepal morphogenesis	0.00%	null	null	2.824	-33.1096	0.876	0.945	10228	1
GO:0048449	floral organ formation	0.00%	null	null	3.225	-35.6536	0.87	0.779	10228	1
GO:2000026	regulation of multicellular organismal development	0.10%	null	null	4.714	-8.1113	0.793	0.79	10228	1
GO:0048831	regulation of shoot system development	0.01%	null	null	3.61	-4.857	0.812	0.836	10228	1
GO:0048827	phyllome development	0.02%	null	null	3.872	-8.0969	0.869	0.943	10228	1
GO:0030154	cell differentiation	0.28%	null	null	5.146	-5.1487	0.837	0.754	10228	1
GO:0048367	shoot system development	0.03%	null	null	4.118	-17.1952	0.875	0.711	10228	1
GO:0010374	stomatal complex development	0.00%	null	null	3.331	-9.719	0.877	0.788	10228	1
GO:0048316	seed development	0.01%	null	null	3.697	-10.0937	0.868	0.895	10228	1
GO:0009790	embryo development	0.08%	null	null	4.621	-6.0511	0.869	0.777	10228	1
GO:0009791	post-embryonic development	0.05%	null	null	4.388	-34.0182	0.873	0.745	10228	1
GO:0009793	embryo development ending in seed dormancy	0.01%	null	null	3.6	-3.5884	0.87	0.983	10228	1
GO:0009908	flower development	0.02%	null	null	3.931	-12.1568	0.859	0.925	10228	1
GO:0022412	cellular process involved in reproduction in multicellular organism	0.02%	null	null	4.076	-4.4123	0.856	0.7	10228	1
GO:0061458	reproductive system development	0.06%	null	null	4.451	-39.1746	0.87	0.753	10228	1
GO:0048731	system development	0.35%	null	null	5.246	-44.7305	0.855	0.878	10228	1
GO:1901615	organic hydroxy compound metabolic process	0.99%	1.037	-4.005	5.694	-3.9867	0.933	0.062	1901615	0
GO:0015979	photosynthesis	0.34%	0.096	-2.001	5.232	-20.2782	0.928	0.063	15979	0
GO:0043603	cellular amide metabolic process	0.91%	5.938	6.38	5.656	-40.8729	0.874	0.07	43603	0
GO:1903047	mitotic cell cycle process	0.08%	-1.127	0.002	4.582	-8.466	0.898	0.075	1903047	0
GO:0000278	mitotic cell cycle	0.09%	null	null	4.637	-8.466	0.9	0.591	1903047	1
GO:0098813	nuclear chromosome segregation	0.03%	null	null	4.166	-2.9202	0.906	0.682	1903047	1
GO:0051726	regulation of cell cycle	0.15%	null	null	4.864	-2.1812	0.821	0.619	1903047	1
GO:0006790	sulfur compound metabolic process	1.67%	2.08	-1.819	5.92	-9.1586	0.914	0.076	6790	0
GO:0046490	isopentenyl diphosphate metabolic process	0.17%	-5.155	4.836	4.919	-48.2182	0.784	0.079	46490	0
GO:0044255	cellular lipid metabolic process	2.58%	null	null	6.108	-42.4698	0.789	0.67	46490	1
GO:0006650	glycerophospholipid metabolic process	0.34%	null	null	5.227	-4.475	0.769	0.87	46490	1
GO:0030258	lipid modification	0.25%	null	null	5.099	-2.4541	0.831	0.699	46490	1
GO:0046488	phosphatidylinositol metabolic process	0.15%	null	null	4.87	-2.1561	0.785	0.904	46490	1
GO:0008654	phospholipid biosynthetic process	0.86%	null	null	5.63	-15.3625	0.687	0.815	46490	1
GO:0006720	isoprenoid metabolic process	0.47%	null	null	5.365	-10.762	0.821	0.746	46490	1
GO:0006721	terpenoid metabolic process	0.28%	null	null	5.14	-2.757	0.826	0.949	46490	1
GO:0008299	isoprenoid biosynthetic process	0.46%	null	null	5.362	-10.0937	0.756	0.746	46490	1
GO:0009240	isopentenyl diphosphate biosynthetic process	0.17%	null	null	4.919	-9.5817	0.728	0.908	46490	1
GO:0006644	phospholipid metabolic process	1.01%	null	null	5.703	-4.2916	0.748	0.817	46490	1
GO:0006631	fatty acid metabolic process	0.87%	null	null	5.635	-7.6819	0.745	0.801	46490	1
GO:0007017	microtubule-based process	0.17%	-2.93	0.158	4.932	-15.2027	0.907	0.079	7017	0
GO:0006732	coenzyme metabolic process	2.74%	1.686	0.376	6.135	-26.7959	0.892	0.081	6732	0
GO:0006733	oxidoreduction coenzyme metabolic process	0.81%	null	null	5.604	-5.6676	0.905	0.761	6732	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP) (Continued

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-val	uniqueness	dispensability	representative	eliminated
GO:0006739	NADP metabolic process	0.43%	null	null	5.334	-7.3526	0.723	0.915	6732	1
GO:0019362	pyridine nucleotide metabolic process	0.67%	null	null	5.521	-23.1427	0.711	0.744	6732	1
GO:0051188	cofactor biosynthetic process	2.76%	null	null	6.137	-12.9469	0.826	0.891	6732	1
GO:0051186	cofactor metabolic process	3.61%	0.354	1.702	6.254	-36.8761	0.906	0.084	51186	0
GO:0007049	cell cycle	1.41%	-4.761	-0.503	5.846	-52.71	0.887	0.095	7049	0
GO:0006081	cellular aldehyde metabolic process	0.47%	-5.715	0.74	5.365	-15.3143	0.861	0.104	6081	0
GO:0046148	pigment biosynthetic process	0.45%	-0.061	6.384	5.352	-4.6345	0.832	0.105	46148	0
GO:0042440	pigment metabolic process	0.49%	-4.296	1.527	5.387	-8.466	0.906	0.105	42440	0
GO:0043170	macromolecule metabolic process	32.80%	5.666	0.253	7.213	-300	0.889	0.109	43170	0
GO:0006793	phosphorus metabolic process	16.89%	3.142	3.494	6.924	-158.222	0.881	0.112	6793	0
GO:0022900	electron transport chain	1.16%	-6.305	1.461	5.763	-13.15	0.839	0.115	22900	0
GO:0009060	aerobic respiration	1.37%	null	null	5.832	-5.7352	0.835	0.899	22900	1
GO:0045333	cellular respiration	2.26%	null	null	6.051	-7.3526	0.826	0.853	22900	1
GO:0015980	energy derivation by oxidation of organic compounds	2.41%	null	null	6.079	-4.8697	0.825	0.884	22900	1
GO:0022904	respiratory electron transport chain	0.88%	null	null	5.642	-9.6576	0.843	0.724	22900	1
GO:0019684	photosynthesis, light reaction	0.09%	null	null	4.627	-7.3072	0.919	0.577	22900	1
GO:0005975	carbohydrate metabolic process	5.98%	3.686	-1.83	6.474	-27.7077	0.905	0.129	5975	0
GO:0007154	cell communication	4.36%	-5.859	-0.196	6.336	-46.9172	0.87	0.136	7154	0
GO:0032787	monocarboxylic acid metabolic process	2.31%	-4.729	5.153	6.061	-22.6596	0.763	0.146	32787	0
GO:0072330	monocarboxylic acid biosynthetic process	0.82%	null	null	5.611	-7.6819	0.73	0.756	32787	1
GO:0016053	organic acid biosynthetic process	4.67%	null	null	6.366	-19.9393	0.676	0.629	32787	1
GO:0046394	carboxylic acid biosynthetic process	4.62%	null	null	6.361	-17.8268	0.674	0.917	32787	1
GO:0019752	carboxylic acid metabolic process	9.04%	null	null	6.653	-15.9626	0.717	0.897	32787	1
GO:0043648	dicarboxylic acid metabolic process	1.12%	null	null	5.748	-4.0106	0.782	0.53	32787	1
GO:1901607	alpha-amino acid biosynthetic process	3.03%	null	null	6.179	-13.4609	0.667	0.863	32787	1
GO:1901605	alpha-amino acid metabolic process	4.27%	null	null	6.327	-9.3134	0.713	0.691	32787	1
GO:0008652	cellular amino acid biosynthetic process	3.70%	null	null	6.265	-17.0958	0.659	0.839	32787	1
GO:0006520	cellular amino acid metabolic process	6.44%	null	null	6.505	-3.3945	0.699	0.837	32787	1
GO:0043436	oxoacid metabolic process	9.21%	null	null	6.661	-35.3401	0.716	0.788	32787	1
GO:0006090	pyruvate metabolic process	0.73%	null	null	5.557	-19.9788	0.789	0.503	32787	1
GO:1901135	carbohydrate derivative metabolic process	11.65%	4.918	-1.729	6.763	-2.3083	0.908	0.155	1901135	0
GO:0072524	pyridine-containing compound metabolic process	0.89%	4.474	6.853	5.646	-20.3197	0.84	0.155	72524	0
GO:0006629	lipid metabolic process	3.09%	-5.295	2.489	6.187	-16.4389	0.851	0.155	6629	0
GO:0097659	nucleic acid-templated transcription	0.77%	5.888	4.506	5.584	-104.893	0.761	0.178	97659	0
GO:0005976	polysaccharide metabolic process	1.08%	7.498	-0.907	5.73	-51.7645	0.844	0.186	5976	0
GO:0044723	single-organism carbohydrate metabolic process	3.60%	null	null	6.253	-46.767	0.8	0.674	5976	1
GO:0044724	single-organism carbohydrate catabolic process	1.20%	null	null	5.777	-3.8662	0.79	0.746	5976	1
GO:0016052	carbohydrate catabolic process	1.35%	null	null	5.827	-12.6289	0.842	0.694	5976	1
GO:0016051	carbohydrate biosynthetic process	1.54%	null	null	5.884	-28.8477	0.743	0.706	5976	1
GO:0000271	polysaccharide biosynthetic process	0.89%	null	null	5.647	-7.5498	0.717	0.936	5976	1
GO:0044264	cellular polysaccharide metabolic process	0.78%	null	null	5.59	-16.4724	0.808	0.91	5976	1
GO:0044262	cellular carbohydrate metabolic process	1.84%	null	null	5.96	-21.7447	0.844	0.723	5976	1
GO:0044042	glucan metabolic process	0.24%	null	null	5.074	-11.8239	0.855	0.812	5976	1
GO:0030243	cellulose metabolic process	0.05%	null	null	4.357	-2.4095	0.848	0.878	5976	1
GO:0009250	glucan biosynthetic process	0.15%	null	null	4.865	-3.294	0.739	0.959	5976	1
GO:0006006	glucose metabolic process	0.74%	null	null	5.567	-4.2782	0.826	0.706	5976	1
GO:0006073	cellular glucan metabolic process	0.24%	null	null	5.074	-9.0526	0.825	0.873	5976	1
GO:0005996	monosaccharide metabolic process	1.21%	null	null	5.781	-8.8477	0.817	0.747	5976	1
GO:0034637	cellular carbohydrate biosynthetic process	0.74%	null	null	5.568	-4.2612	0.737	0.831	5976	1
GO:0005984	disaccharide metabolic process	0.19%	null	null	4.976	-7.3526	0.823	0.698	5976	1
GO:0033692	cellular polysaccharide biosynthetic process	0.68%	null	null	5.53	-16.3325	0.703	0.556	5976	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP) (Continued

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensab	representa	eliminated
GO:0052249	modulation of RNA levels in other organism involved in symbiotic interactior	0.00%	4.15	-5.713	2.953	-45.2366	0.93	0.188	52249	0
GO:0044265	cellular macromolecule catabolic process	1.11%	8.085	0.225	5.742	-16.4724	0.826	0.191	44265	0
GO:0044257	cellular protein catabolic process	0.22%	null	null	5.039	-9.857	0.835	0.761	44265	1
GO:0043632	modification-dependent macromolecule catabolic process	0.14%	null	null	4.828	-5.4802	0.857	0.728	44265	1
GO:0051603	proteolysis involved in cellular protein catabolic process	0.22%	null	null	5.036	-5.8013	0.835	0.916	44265	1
GO:0034641	cellular nitrogen compound metabolic process	33.43%	4.52	5.613	7.221	-293.211	0.807	0.201	34641	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	null	null	7.173	-237.371	0.757	0.507	34641	1
GO:0090304	nucleic acid metabolic process	20.16%	null	null	7.001	-191.33	0.727	0.528	34641	1
GO:1901137	carbohydrate derivative biosynthetic process	3.92%	1.092	7.619	6.29	-16.699	0.812	0.218	1901137	0
GO:0044272	sulfur compound biosynthetic process	1.34%	1.928	8.101	5.825	-3.9326	0.856	0.22	44272	0
GO:0044281	small molecule metabolic process	21.50%	-5.597	3.137	7.029	-119.903	0.85	0.225	44281	0
GO:1901360	organic cyclic compound metabolic process	33.91%	4.868	0.504	7.227	-197.706	0.888	0.23	1901360	0
GO:0032970	regulation of actin filament-based process	0.03%	4.352	-5.212	4.198	-37.8153	0.851	0.239	32970	0
GO:0006725	cellular aromatic compound metabolic process	33.05%	2.796	3.982	7.216	-244.733	0.866	0.259	6725	0
GO:0046483	heterocycle metabolic process	33.33%	3.371	4.209	7.22	-147.928	0.865	0.26	46483	0
GO:0016311	dephosphorylation	0.78%	-4.376	6.044	5.587	-10.2262	0.859	0.265	16311	0
GO:0046031	ADP metabolic process	0.00%	-1.806	7.101	3.017	-9.857	0.808	0.271	46031	0
GO:0006165	nucleoside diphosphate phosphorylation	0.04%	null	null	4.336	-8.5591	0.738	0.699	46031	1
GO:0009135	purine nucleoside diphosphate metabolic process	0.00%	null	null	3.072	-5.1831	0.82	0.704	46031	1
GO:0006757	ADP phosphorylation	0.00%	null	null	1.531	-3.4664	0.808	0.852	46031	1
GO:0009132	nucleoside diphosphate metabolic process	0.15%	-1.372	6.355	4.881	-10.7011	0.765	0.29	9132	0
GO:0008380	RNA splicing	0.10%	7.702	2.826	4.683	-4.4685	0.84	0.291	8380	0
GO:0050790	regulation of catalytic activity	0.65%	4.842	-5.069	5.51	-15.6383	0.87	0.291	50790	0
GO:0016192	vesicle-mediated transport	0.38%	-4.255	-4.338	5.277	-2.878	0.934	0.298	16192	0
GO:0043604	amide biosynthetic process	0.55%	3.88	7.476	5.439	-41.266	0.818	0.309	43604	0
GO:0043043	peptide biosynthetic process	0.21%	null	null	5.017	-38.4535	0.809	0.819	43604	1
GO:0006518	peptide metabolic process	0.32%	null	null	5.206	-33.1013	0.851	0.852	43604	1
GO:0044260	cellular macromolecule metabolic process	28.59%	6.661	1.436	7.153	-300	0.786	0.313	44260	0
GO:0046907	intracellular transport	0.74%	-4.567	-4.198	5.566	-34.4881	0.897	0.32	46907	0
GO:0032940	secretion by cell	0.62%	null	null	5.487	-4.475	0.807	0.829	46907	1
GO:0007034	vacuolar transport	0.01%	null	null	3.498	-6.0209	0.891	0.569	46907	1
GO:0006405	RNA export from nucleus	0.07%	null	null	4.552	-2.8962	0.902	0.682	46907	1
GO:0051649	establishment of localization in cell	1.49%	null	null	5.87	-30.8041	0.891	0.887	46907	1
GO:0055114	oxidation-reduction process	15.04%	-6.056	2.952	6.874	-24.3063	0.858	0.328	55114	0
GO:0009416	response to light stimulus	0.08%	-0.346	-5.762	4.616	-15.2027	0.937	0.33	9416	0
GO:0009266	response to temperature stimulus	0.15%	null	null	4.863	-14.0742	0.934	0.851	9416	1
GO:0009314	response to radiation	0.09%	null	null	4.659	-16.7305	0.936	0.817	9416	1
GO:0006972	hyperosmotic response	0.02%	null	null	3.904	-4.0146	0.939	0.727	9416	1
GO:0009409	response to cold	0.02%	null	null	4.074	-2.9202	0.938	0.745	9416	1
GO:0071495	cellular response to endogenous stimulus	0.09%	-0.333	-5.785	4.656	-66.7852	0.937	0.332	71495	0
GO:0032870	cellular response to hormone stimulus	0.06%	null	null	4.488	-4.475	0.899	0.951	71495	1
GO:0009755	hormone-mediated signaling pathway	0.05%	null	null	4.363	-6.2441	0.792	0.924	71495	1
GO:0009725	response to hormone	0.08%	null	null	4.584	-18.5969	0.928	0.957	71495	1
GO:0051156	glucose 6-phosphate metabolic process	0.04%	-2.217	5.013	4.331	-3.1345	0.855	0.346	51156	0
GO:0072522	purine-containing compound biosynthetic process	1.59%	3.046	7.199	5.899	-6.2441	0.76	0.349	72522	0
GO:0009163	nucleoside biosynthetic process	1.67%	null	null	5.918	-3.0576	0.652	0.978	72522	1
GO:0046129	purine ribonucleoside biosynthetic process	1.07%	null	null	5.728	-3.4837	0.667	0.932	72522	1
GO:0042455	ribonucleoside biosynthetic process	1.65%	null	null	5.913	-6.2441	0.652	0.523	72522	1
GO:0043412	macromolecule modification	5.09%	7.87	-0.225	6.404	-210.32	0.87	0.354	43412	0
GO:0010033	response to organic substance	0.29%	-0.34	-6.321	5.155	-25.4989	0.933	0.363	10033	0
GO:1901700	response to oxygen-containing compound	0.31%	null	null	5.191	-21.4609	0.933	0.7	10033	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP) (Continued

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensabi	representa	eliminated
GO:0070887	cellular response to chemical stimulus	0.50%	null	null	5.392	-10.2262	0.893	0.734	10033	1
GO:0071310	cellular response to organic substance	0.21%	null	null	5.021	-10.7011	0.896	0.677	10033	1
GO:0044249	cellular biosynthetic process	28.21%	2.496	6.949	7.147	-273.703	0.779	0.369	44249	0
GO:0009059	macromolecule biosynthetic process	17.67%	null	null	6.944	-97.5031	0.771	0.554	44249	1
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	null	null	6.895	-198.104	0.75	0.534	44249	1
GO:1901576	organic substance biosynthetic process	28.89%	null	null	7.157	-249.845	0.797	0.663	44249	1
GO:0019438	aromatic compound biosynthetic process	15.56%	null	null	6.889	-110.925	0.759	0.532	44249	1
GO:0018130	heterocycle biosynthetic process	16.15%	null	null	6.905	-146.141	0.756	0.538	44249	1
GO:1901362	organic cyclic compound biosynthetic process	16.58%	null	null	6.916	-10.8665	0.773	0.543	44249	1
GO:0010604	positive regulation of macromolecule metabolic process	0.31%	6.256	-3.325	5.182	-12.3778	0.791	0.379	10604	0
GO:1902680	positive regulation of RNA biosynthetic process	0.20%	null	null	4.989	-7.3526	0.704	0.992	10604	1
GO:0031328	positive regulation of cellular biosynthetic process	0.26%	null	null	5.103	-8.0969	0.767	0.971	10604	1
GO:0031325	positive regulation of cellular metabolic process	0.32%	null	null	5.197	-10.6326	0.802	0.989	10604	1
GO:0051254	positive regulation of RNA metabolic process	0.20%	null	null	4.993	-2.2822	0.735	0.993	10604	1
GO:0045935	positive regulation of nucleobase-containing compound metabolic process	0.21%	null	null	5.012	-9.5817	0.759	0.954	10604	1
GO:0010557	positive regulation of macromolecule biosynthetic process	0.25%	null	null	5.094	-5.8013	0.756	0.994	10604	1
GO:0009891	positive regulation of biosynthetic process	0.26%	null	null	5.107	-3.0714	0.805	0.971	10604	1
GO:0051173	positive regulation of nitrogen compound metabolic process	0.21%	null	null	5.015	-10.9508	0.813	0.954	10604	1
GO:0019682	glyceraldehyde-3-phosphate metabolic process	0.15%	-3.398	5.92	4.878	-13.4609	0.805	0.39	19682	0
GO:0043102	amino acid salvage	0.06%	-0.251	7.825	4.484	-2.2511	0.774	0.392	43102	0
GO:0090407	organophosphate biosynthetic process	4.46%	0.315	6.979	6.346	-31.4815	0.736	0.393	90407	0
GO:0032774	RNA biosynthetic process	9.29%	null	null	6.665	-47.1379	0.675	0.712	90407	1
GO:0006796	phosphate-containing compound metabolic process	16.69%	null	null	6.919	-190.105	0.789	0.603	90407	1
GO:0016310	phosphorylation	6.30%	null	null	6.496	-118.77	0.817	0.649	90407	1
GO:0009116	nucleoside metabolic process	8.23%	null	null	6.612	-19.2457	0.643	0.896	90407	1
GO:0009124	nucleoside monophosphate biosynthetic process	1.56%	null	null	5.89	-3.9326	0.642	0.895	90407	1
GO:0009123	nucleoside monophosphate metabolic process	6.48%	null	null	6.508	-3.911	0.645	0.767	90407	1
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	null	null	6.476	-22.2418	0.644	0.699	90407	1
GO:0009127	purine nucleoside monophosphate biosynthetic process	1.10%	null	null	5.737	-3.9326	0.655	0.856	90407	1
GO:0006163	purine nucleotide metabolic process	7.64%	null	null	6.58	-17.2197	0.626	0.896	90407	1
GO:0009144	purine nucleoside triphosphate metabolic process	6.48%	null	null	6.508	-22.059	0.642	0.751	90407	1
GO:0009141	nucleoside triphosphate metabolic process	6.72%	null	null	6.524	-24.2823	0.643	0.767	90407	1
GO:0009150	purine ribonucleotide metabolic process	7.46%	null	null	6.569	-23.1818	0.62	0.89	90407	1
GO:0009161	ribonucleoside monophosphate metabolic process	6.36%	null	null	6.5	-4.1713	0.641	0.919	90407	1
GO:0009167	purine ribonucleoside monophosphate metabolic process	6.01%	null	null	6.475	-2.1722	0.644	0.922	90407	1
GO:0009165	nucleotide biosynthetic process	2.78%	null	null	6.141	-7.0419	0.621	0.558	90407	1
GO:0006753	nucleoside phosphate metabolic process	9.90%	null	null	6.692	-22.9469	0.627	0.805	90407	1
GO:0046034	ATP metabolic process	5.37%	null	null	6.427	-7.9788	0.628	0.893	90407	1
GO:1901657	glycosyl compound metabolic process	8.26%	null	null	6.614	-15.7122	0.795	0.755	90407	1
GO:0009201	ribonucleoside triphosphate biosynthetic process	0.59%	null	null	5.467	-4.4685	0.677	0.795	90407	1
GO:0009199	ribonucleoside triphosphate metabolic process	6.56%	null	null	6.514	-5.7825	0.641	0.922	90407	1
GO:0046128	purine ribonucleoside metabolic process	7.32%	null	null	6.561	-18.2182	0.645	0.887	90407	1
GO:0042278	purine nucleoside metabolic process	7.36%	null	null	6.564	-24.2823	0.645	0.738	90407	1
GO:0009205	purine ribonucleoside triphosphate metabolic process	6.48%	null	null	6.508	-16.2211	0.642	0.92	90407	1
GO:0009260	ribonucleotide biosynthetic process	1.77%	null	null	5.944	-6.2441	0.631	0.91	90407	1
GO:0009259	ribonucleotide metabolic process	7.84%	null	null	6.591	-25.4225	0.628	0.909	90407	1
GO:0019637	organophosphate metabolic process	11.97%	null	null	6.775	-65.8041	0.782	0.755	90407	1
GO:0044283	small molecule biosynthetic process	5.87%	null	null	6.466	-33.2899	0.734	0.568	90407	1
GO:0019693	ribose phosphate metabolic process	7.90%	null	null	6.595	-26.8962	0.747	0.683	90407	1
GO:0034654	nucleobase-containing compound biosynthetic process	13.02%	null	null	6.811	-55.3706	0.71	0.559	90407	1
GO:0006082	organic acid metabolic process	9.36%	-2.859	4.724	6.668	-73.3298	0.74	0.393	6082	0
GO:0006812	cation transport	3.98%	-4.739	-3.872	6.297	-32.2916	0.843	0.394	6812	0

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: biological processes (BP) (Continued

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensab	representa	eliminated
GO:0055085	transmembrane transport	9.40%	null	null	6.67	-13.2636	0.775	0.811	6812	1
GO:0006818	hydrogen transport	1.98%	null	null	5.992	-8.466	0.864	0.507	6812	1
GO:0006810	transport	17.38%	null	null	6.937	-152.137	0.899	0.579	6812	1
GO:0006811	ion transport	5.99%	null	null	6.474	-40.9626	0.845	0.631	6812	1
GO:0044765	single-organism transport	12.59%	null	null	6.797	-41.1746	0.829	0.753	6812	1
GO:0071702	organic substance transport	5.49%	null	null	6.436	-22.9431	0.913	0.619	6812	1
GO:0015985	energy coupled proton transport, down electrochemical gradient	0.45%	null	null	5.35	-17.2226	0.83	0.613	6812	1
GO:0098662	inorganic cation transmembrane transport	2.31%	null	null	6.06	-10.7011	0.799	0.839	6812	1
GO:0098655	cation transmembrane transport	2.86%	null	null	6.153	-6.3862	0.795	0.864	6812	1
GO:0098660	inorganic ion transmembrane transport	2.58%	null	null	6.108	-6.0535	0.798	0.769	6812	1
GO:0030001	metal ion transport	1.47%	null	null	5.864	-14.1512	0.857	0.791	6812	1
GO:0015672	monovalent inorganic cation transport	2.71%	null	null	6.13	-14.1586	0.847	0.775	6812	1
GO:0034220	ion transmembrane transport	4.00%	null	null	6.298	-6.9747	0.79	0.821	6812	1
GO:0016128	phytosteroid metabolic process	0.00%	-4.767	4.107	3.093	-10.055	0.859	0.396	16128	0
GO:0071496	cellular response to external stimulus	0.51%	-0.233	-5.999	5.406	-9.4123	0.939	0.398	71496	0
GO:0006396	RNA processing	2.78%	6.889	3.298	6.141	-33.4306	0.773	0.406	6396	0
GO:0071705	nitrogen compound transport	2.07%	-3.964	-4.794	6.013	-8.0325	0.922	0.407	71705	0
GO:0046939	nucleotide phosphorylation	0.26%	-1.106	6.566	5.114	-7.1349	0.752	0.413	46939	0
GO:0006952	defense response	0.57%	-0.282	-6.142	5.452	-7.3526	0.936	0.424	6952	0
GO:0045087	innate immune response	0.14%	null	null	4.832	-2.4541	0.943	0.52	6952	1
GO:1901564	organonitrogen compound metabolic process	19.57%	5.996	5.503	6.988	-94.71	0.84	0.428	1901564	0
GO:0032268	regulation of cellular protein metabolic process	0.69%	6.656	-2.56	5.536	-2.1331	0.762	0.431	32268	0
GO:0046903	secretion	0.63%	-4.351	-4.507	5.494	-6.6038	0.879	0.437	46903	0
GO:0051246	regulation of protein metabolic process	0.88%	6.524	-2.856	5.639	-11.8239	0.781	0.443	51246	0
GO:0019538	protein metabolic process	12.33%	7.474	0.273	6.788	-268.47	0.842	0.448	19538	0
GO:0042592	homeostatic process	0.97%	4.557	-5.392	5.683	-22.0044	0.889	0.451	42592	0
GO:0019725	cellular homeostasis	0.80%	null	null	5.602	-14.0742	0.804	0.742	42592	1
GO:1901566	organonitrogen compound biosynthetic process	9.30%	3.316	6.868	6.665	-96.2526	0.77	0.457	1901566	0
GO:0072521	purine-containing compound metabolic process	8.06%	null	null	6.603	-8.8794	0.785	0.536	1901566	1
GO:0016070	RNA metabolic process	13.71%	6.035	3.291	6.834	-117.939	0.728	0.463	16070	0
GO:0006259	DNA metabolic process	6.34%	null	null	6.499	-34.0675	0.759	0.539	16070	1
GO:0010256	endomembrane system organization	0.03%	-1.876	-1.499	4.156	-3.3565	0.911	0.47	10256	0
GO:0006399	tRNA metabolic process	2.53%	6.95	3.163	6.1	-6.0209	0.779	0.473	6399	0
GO:1903506	regulation of nucleic acid-templated transcription	1.45%	5.768	2.777	5.857	-45.5591	0.661	0.474	1903506	0
GO:0080090	regulation of primary metabolic process	9.60%	null	null	6.679	-112.284	0.776	0.874	1903506	1
GO:0031323	regulation of cellular metabolic process	9.52%	null	null	6.675	-113.971	0.751	0.623	1903506	1
GO:0031326	regulation of cellular biosynthetic process	9.00%	null	null	6.651	-96.9788	0.691	0.909	1903506	1
GO:0051252	regulation of RNA metabolic process	8.53%	null	null	6.628	-89.1952	0.636	0.873	1903506	1
GO:2001141	regulation of RNA biosynthetic process	8.44%	null	null	6.623	-88.0209	0.589	0.898	1903506	1
GO:0019219	regulation of nucleobase-containing compound metabolic process	8.82%	null	null	6.642	-88.4609	0.673	0.905	1903506	1
GO:0006355	regulation of transcription, DNA-templated	8.42%	null	null	6.622	-16.3757	0.575	1.978	1903506	1
GO:0006351	transcription, DNA-templated	9.01%	null	null	6.652	-92.9393	0.655	1.453	1903506	1
GO:0010556	regulation of macromolecule biosynthetic process	8.98%	null	null	6.65	-90.6144	0.673	0.909	1903506	1
GO:0010468	regulation of gene expression	9.33%	null	null	6.667	-48.1397	0.725	0.881	1903506	1
GO:2000112	regulation of cellular macromolecule biosynthetic process	8.95%	null	null	6.649	-97.4306	0.645	0.862	1903506	1
GO:0060255	regulation of macromolecule metabolic process	9.70%	null	null	6.683	-88.2541	0.73	0.878	1903506	1
GO:0009889	regulation of biosynthetic process	9.00%	null	null	6.651	-42.5467	0.751	0.863	1903506	1
GO:0051171	regulation of nitrogen compound metabolic process	8.83%	null	null	6.643	-100.967	0.759	0.859	1903506	1
GO:0044282	small molecule catabolic process	1.35%	-3.849	4.455	5.826	-13.8356	0.816	0.48	44282	0
GO:0044712	single-organism catabolic process	7.76%	null	null	6.587	-35.7258	0.83	0.858	44282	1
GO:0016054	organic acid catabolic process	1.14%	null	null	5.755	-3.3565	0.753	0.537	44282	1
GO:0009057	macromolecule catabolic process	1.64%	null	null	5.912	-7.2604	0.856	0.503	44282	1
GO:0044248	cellular catabolic process	7.95%	null	null	6.597	-40.3251	0.853	0.862	44282	1
GO:1901575	organic substance catabolic process	9.32%	null	null	6.666	-44.3439	0.868	0.653	44282	1
GO:0046700	heterocycle catabolic process	6.22%	null	null	6.49	-2.1812	0.808	0.821	44282	1
GO:0015849	organic acid transport	1.35%	-4.027	-4.459	5.828	-17.2426	0.864	0.481	15849	0
GO:0034660	ncRNA metabolic process	3.21%	6.675	3.216	6.203	-5.1818	0.772	0.488	34660	0
GO:0010467	gene expression	16.70%	7.549	0.579	6.92	-200.544	0.843	0.493	10467	0
GO:0009069	serine family amino acid metabolic process	0.64%	-1.005	7.466	5.505	-4.4685	0.769	0.496	9069	0
GO:0044711	single-organism biosynthetic process	12.46%	-2.28	6.675	6.792	-105.21	0.787	0.497	44711	0

REVIGO analysis for GO enrichment from differentially hypermethylated genes in leaves: biological processes (BP)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-va	uniquenes	dispensabi	representa	eliminated
GO:0034641	cellular nitrogen compound metabolic process	33.43%	4.009	2.477	7.221	-59.7423	0.659	0.201	34641	0
GO:0043170	macromolecule metabolic process	32.80%	1.124	-4.642	7.213	-54.4306	0.805	0.12	43170	0
GO:1901564	organonitrogen compound metabolic process	19.57%	2.87	-0.196	6.988	-53.4056	0.693	0.428	1901564	0
GO:0019538	protein metabolic process	12.33%	3.699	-6.071	6.788	-51.7986	0.776	0.158	19538	0
GO:0044249	cellular biosynthetic process	28.21%	6.935	-0.404	7.147	-51.7986	0.641	0.38	44249	0
GO:1901566	organonitrogen compound biosynthetic process	9.30%	6.188	0.571	6.665	-50.7212	0.588	0.457	1901566	0
GO:0044260	cellular macromolecule metabolic process	28.59%	3.677	-3.689	7.153	-49.8013	0.699	0.448	44260	0
GO:1901576	organic substance biosynthetic process	28.89%	6.461	-2.02	7.157	-49.8013	0.652	0.663	1901576	0
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	5.78	1.083	6.895	-48.2565	0.575	0.534	44271	0
GO:0044267	cellular protein metabolic process	8.78%	4.323	-5.093	6.64	-45.7077	0.747	0.331	44267	0
GO:0010467	gene expression	16.70%	3.309	-5.966	6.92	-37.0477	0.781	0.493	10467	0
GO:0006518	peptide metabolic process	0.32%	5.145	6.317	5.206	-36.2976	0.719	0	6518	0
GO:0034645	cellular macromolecule biosynthetic process	17.20%	5.332	-2.4	6.932	-34.5436	0.603	0.55	34645	0
GO:0043603	cellular amide metabolic process	0.91%	3.326	6.56	5.656	-30.8125	0.767	0.14	43603	0
GO:0006091	generation of precursor metabolites and energy	3.22%	-2.876	-2.582	6.205	-27.8539	0.847	0.062	6091	0
GO:0009059	macromolecule biosynthetic process	17.67%	5.758	-3.16	6.944	-26.9747	0.64	0.554	9059	0
GO:1901360	organic cyclic compound metabolic process	33.91%	1.815	-4.841	7.227	-18.8125	0.803	0.23	1901360	0
GO:0006725	cellular aromatic compound metabolic process	33.05%	0.701	1.026	7.216	-18.8125	0.784	0.259	6725	0
GO:0055114	oxidation-reduction process	15.04%	-0.233	6.761	6.874	-17.4737	0.783	0.177	55114	0
GO:0015979	photosynthesis	0.34%	-4.668	-3.974	5.232	-16.3925	0.877	0.063	15979	0
GO:0006796	phosphate-containing compound metabolic process	16.69%	3.73	4.563	6.919	-15.6716	0.561	0.603	6796	0
GO:0022900	electron transport chain	1.16%	-2.225	5.868	5.763	-14.3595	0.779	0.072	22900	0
GO:1901135	carbohydrate derivative metabolic process	11.65%	-0.816	-6	6.763	-14.219	0.836	0.155	1901135	0
GO:0044281	small molecule metabolic process	21.50%	-0.612	6.37	7.029	-14.219	0.772	0.328	44281	0
GO:0072521	purine-containing compound metabolic process	8.06%	4.939	2.999	6.603	-14.219	0.614	0.536	72521	0
GO:0009199	ribonucleoside triphosphate metabolic process	6.56%	3.686	3.461	6.514	-14.219	0.387	0.655	9199	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	3.931	0.382	7.173	-12.8356	0.578	0.507	6139	0
GO:0015672	monovalent inorganic cation transport	2.71%	-5.141	1.016	6.13	-10.054	0.789	0	15672	0
GO:0046129	purine ribonucleoside biosynthetic process	1.07%	6.265	2.612	5.728	-10.054	0.433	0.256	46129	0
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	0.51%	6.128	3.201	5.401	-10.054	0.464	0.445	9206	0
GO:0090407	organophosphate biosynthetic process	4.46%	7.828	0.457	6.346	-9.7122	0.497	0.087	90407	0

REVIGO analysis for GO enrichment from differentially hypermethylated genes in leaves: biological processes (BP) (continued)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-val	uniqueness	dispensabi	representa	eliminated
GO:0006811	ion transport	5.99%	-4.882	1.608	6.474	-8.4802	0.802	0.631	6811	0
GO:0006810	transport	17.38%	-5.215	0.279	6.937	-8.118	0.878	0.537	6810	0
GO:0043412	macromolecule modification	5.09%	2.694	-7.05	6.404	-7.0195	0.818	0.295	43412	0
GO:1901137	carbohydrate derivative biosynthetic process	3.92%	8.555	-1.251	6.29	-6.3605	0.631	0.489	1901137	0
GO:0015985	energy coupled proton transport, down electrochemical gradient	0.45%	-4.745	1.194	5.35	-5.2255	0.765	0.687	15985	0
GO:0019684	photosynthesis, light reaction	0.09%	-3.555	5.334	4.627	-4.2857	0.874	0.577	19684	0
GO:0018130	heterocycle biosynthetic process	16.15%	6.743	0.439	6.905	-4.1543	0.592	0.538	18130	0
GO:0055086	nucleobase-containing small molecule metabolic process	10.84%	2.784	2.636	6.732	-4.0615	0.531	0.357	55086	0
GO:0006818	hydrogen transport	1.98%	-5.208	0.56	5.992	-4.0526	0.823	0.481	6818	0
GO:0065003	macromolecular complex assembly	0.68%	-3.818	-5.483	5.527	-2.9414	0.919	0.026	65003	0
GO:0043604	amide biosynthetic process	0.55%	null	null	5.439	-36.2976	0.671	0.852	6518	1
GO:0006753	nucleoside phosphate metabolic process	9.90%	null	null	6.692	-15.6716	0.373	0.816	9199	1
GO:0009259	ribonucleotide metabolic process	7.84%	null	null	6.591	-15.109	0.362	0.795	9199	1
GO:0009141	nucleoside triphosphate metabolic process	6.72%	null	null	6.524	-15.109	0.393	0.768	9199	1
GO:0009116	nucleoside metabolic process	8.23%	null	null	6.612	-14.219	0.378	0.824	9199	1
GO:0009167	purine ribonucleoside monophosphate metabolic process	6.01%	null	null	6.475	-13.8539	0.38	0.753	9199	1
GO:0009205	purine ribonucleoside triphosphate metabolic process	6.48%	null	null	6.508	-12.2373	0.387	0.922	9199	1
GO:0046034	ATP metabolic process	5.37%	null	null	6.427	-12.2373	0.339	0.922	9199	1
GO:0098655	cation transmembrane transport	2.86%	null	null	6.153	-9.7122	0.722	0.864	15672	1
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	null	null	6.476	-9.4763	0.381	0.91	9199	1
GO:0006164	purine nucleotide biosynthetic process	1.47%	null	null	5.864	-9.4134	0.381	0.93	46129	1
GO:0009127	purine nucleoside monophosphate biosynthetic process	1.10%	null	null	5.737	-8.4802	0.415	0.707	9206	1
GO:0098662	inorganic cation transmembrane transport	2.31%	null	null	6.06	-8.2924	0.725	0.839	15672	1
GO:0019693	ribose phosphate metabolic process	7.90%	null	null	6.595	-7.1791	0.46	0.71	9199	1
GO:0042451	purine nucleoside biosynthetic process	1.07%	null	null	5.728	-6.7235	0.433	0.93	46129	1
GO:0009152	purine ribonucleotide biosynthetic process	1.38%	null	null	5.836	-6.1198	0.373	0.958	46129	1
GO:0046390	ribose phosphate biosynthetic process	1.82%	null	null	5.958	-5.3354	0.444	0.729	46129	1
GO:0009161	ribonucleoside monophosphate metabolic process	6.36%	null	null	6.5	-5.2652	0.377	0.919	9199	1
GO:0009124	nucleoside monophosphate biosynthetic process	1.56%	null	null	5.89	-4.6968	0.401	0.799	9206	1
GO:0009168	purine ribonucleoside monophosphate biosynthetic process	1.10%	null	null	5.737	-4.4101	0.415	0.908	9206	1
GO:0009163	nucleoside biosynthetic process	1.67%	null	null	5.918	-3.9229	0.425	0.929	46129	1
GO:0009150	purine ribonucleotide metabolic process	7.46%	null	null	6.569	-2.9967	0.348	0.909	9199	1
GO:0009156	ribonucleoside monophosphate biosynthetic process	1.45%	null	null	5.857	-2.4307	0.4	0.936	9206	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: cellular components (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	4.012	3.162	5.573	-149.7375	0.374	0	9536	0
GO:0009507	chloroplast	1.45%	null	null	5.531	-12.5591	0.305	0.805	9536	1
GO:0044391	ribosomal subunit	1.36%	null	null	5.504	-6.1694	0.289	0.664	9536	1
GO:0031967	organelle envelope	2.48%	null	null	5.765	-7.4461	0.299	0.607	9536	1
GO:0005773	vacuole	0.11%	null	null	4.402	-18.5969	0.481	0.403	9536	1
GO:0031976	plastid thylakoid	0.22%	null	null	4.71	-34.2976	0.217	0.985	9536	1
GO:0005634	nucleus	2.81%	null	null	5.819	-29.9914	0.357	0.573	9536	1
GO:0070013	intracellular organelle lumen	0.50%	null	null	5.073	-17.5867	0.389	0.514	9536	1
GO:0031984	organelle subcompartment	0.23%	null	null	4.738	-58.6655	0.406	0.445	9536	1
GO:0031981	nuclear lumen	0.44%	null	null	5.012	-10.762	0.36	0.982	9536	1
GO:0044435	plastid part	0.28%	null	null	4.817	-92.7212	0.336	0.441	9536	1
GO:0044434	chloroplast part	0.28%	null	null	4.815	-78.5171	0.295	0.692	9536	1
GO:0009534	chloroplast thylakoid	0.22%	null	null	4.71	-15.8297	0.217	0.995	9536	1
GO:0044427	chromosomal part	0.45%	null	null	5.024	-8.0969	0.382	0.508	9536	1
GO:0044428	nuclear part	0.65%	null	null	5.18	-28.8182	0.358	0.486	9536	1
GO:0009526	plastid envelope	0.04%	null	null	3.954	-3.3311	0.362	0.853	9536	1
GO:0043231	intracellular membrane-bounded organelle	8.85%	null	null	6.318	-300	0.296	0.767	9536	1
GO:0009532	plastid stroma	0.05%	null	null	4.07	-18.719	0.359	0.87	9536	1
GO:0005739	mitochondrion	3.81%	null	null	5.952	-7.3526	0.329	0.646	9536	1
GO:0005740	mitochondrial envelope	2.33%	null	null	5.738	-6.2441	0.287	0.729	9536	1
GO:0005794	Golgi apparatus	0.27%	null	null	4.794	-2.2685	0.45	0.439	9536	1
GO:0055035	plastid thylakoid membrane	0.21%	null	null	4.697	-32.9788	0.217	0.974	9536	1
GO:0031461	cullin-RING ubiquitin ligase complex	0.05%	3.431	-5.481	4.08	-3.3644	0.747	0.08	31461	0
GO:0042651	thylakoid membrane	0.31%	-4.973	-3.643	4.859	-56.5058	0.572	0.094	42651	0
GO:0009521	photosystem	0.21%	null	null	4.701	-33.2262	0.514	0.927	42651	1
GO:0009523	photosystem II	0.16%	null	null	4.566	-5.1487	0.523	0.903	42651	1
GO:0044436	thylakoid part	0.40%	null	null	4.97	-39.8182	0.569	0.967	42651	1
GO:0009579	thylakoid	0.41%	-5.365	4.185	4.985	-37.109	0.785	0.097	9579	0
GO:0005737	cytoplasm	38.16%	-1.456	0.598	6.952	-298.5214	0.68	0.183	5737	0
GO:0005938	cell cortex	0.09%	3.205	6.305	4.344	-7.3526	0.66	0.31	5938	0
GO:0044444	cytoplasmic part	13.61%	-0.728	2.616	6.505	-291.062	0.684	0.316	44444	0
GO:0030529	ribonucleoprotein complex	6.09%	1.602	-2.902	6.155	-17.9355	0.657	0.319	30529	0
GO:0005856	cytoskeleton	0.71%	6.338	1.6	5.224	-12.6289	0.467	0.342	5856	0
GO:0015630	microtubule cytoskeleton	0.39%	null	null	4.967	-9.857	0.473	0.452	5856	1
GO:0005840	ribosome	5.76%	null	null	6.131	-9.857	0.307	0.835	5856	1
GO:0005694	chromosome	0.97%	null	null	5.359	-10.7011	0.455	0.495	5856	1
GO:0044430	cytoskeletal part	0.58%	null	null	5.134	-6.821	0.357	0.918	5856	1
GO:0043232	intracellular non-membrane-bounded organelle	7.68%	null	null	6.256	-29.8182	0.358	0.629	5856	1
GO:0030120	vesicle coat	0.04%	4.875	-0.697	4.001	-25.8508	0.386	0.372	30120	0
GO:0098588	bounding membrane of organelle	0.40%	null	null	4.968	-9.6459	0.417	0.526	30120	1

REVIGO analysis for GO enrichment from differentially hypermethylated genes in leaves: cellular components (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	4.864	-0.111	5.573	-108.475	0.42	0	9536	0
GO:0070069	cytochrome complex	0.05%	-3.71	5.019	4.056	-2.8042	0.756	0	70069	0
GO:0009579	thylakoid	0.41%	-3.605	-5.407	4.985	-73.8013	0.744	0.097	9579	0
GO:0005737	cytoplasm	38.16%	0.237	-2.531	6.952	-143.2069	0.651	0.183	5737	0
GO:0044444	cytoplasmic part	13.61%	1.989	-3.96	6.505	-139.9957	0.644	0.316	44444	0
GO:0030529	ribonucleoprotein complex	6.09%	-2.305	1.594	6.155	-25.7471	0.551	0.317	30529	0
GO:0009534	chloroplast thylakoid	0.22%	5.937	0.991	4.71	-76.0726	0.191	0.431	9534	0
GO:0044436	thylakoid part	0.40%	null	null	4.97	-72.0039	0.419	0.967	9534	1
GO:0044434	chloroplast part	0.28%	null	null	4.815	-77.4989	0.312	0.976	9534	1
GO:0016469	proton-transporting two-sector ATPase complex	1.00%	null	null	5.369	-10.054	0.637	1.051	9534	1
GO:0031976	plastid thylakoid	0.22%	null	null	4.71	-76.0726	0.191	0.987	9534	1
GO:0009521	photosystem	0.21%	null	null	4.701	-23.8097	0.306	0.927	9534	1
GO:0042651	thylakoid membrane	0.31%	null	null	4.859	-69.1463	0.421	0.919	9534	1
GO:0055035	plastid thylakoid membrane	0.21%	null	null	4.697	-60.2299	0.189	0.995	9534	1
GO:0031984	organelle subcompartment	0.23%	5.198	2.626	4.738	-76.0726	0.459	0.436	31984	0
GO:0044391	ribosomal subunit	1.36%	2.347	1.986	5.504	-11.9508	0.313	0.512	44391	0
GO:0043232	intracellular non-membrane-bounded organelle	7.68%	3.156	0.287	6.256	-30.8125	0.408	0.659	43232	0
GO:0005840	ribosome	5.76%	null	null	6.131	-6.7122	0.308	0.835	43232	1
GO:0043231	intracellular membrane-bounded organelle	8.85%	3.85	0.11	6.318	-117.767	0.352	0.666	43231	0
GO:0044435	plastid part	0.28%	5.44	1.442	4.817	-77.4989	0.364	0.678	44435	0
GO:0009507	chloroplast	1.45%	null	null	5.531	-72.1972	0.315	0.805	44435	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: molecular functions (NF)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0016773	phosphotransferase activity, alcohol group as acceptor	4.04%	-6.235	-1.784	6.281	-113.9208	0.813	0	16773	0
GO:0016772	transferase activity, transferring phosphorus-containing groups	9.19%	null	null	6.638	-144.3526	0.829	0.42	16773	1
GO:0016301	kinase activity	5.08%	null	null	6.381	-99.475	0.809	0.701	16773	1
GO:0016779	nucleotidyltransferase activity	2.93%	null	null	6.142	-16.4724	0.819	0.643	16773	1
GO:0004672	protein kinase activity	1.88%	null	null	5.948	-76.8125	0.826	0.603	16773	1
GO:0034062	RNA polymerase activity	0.90%	null	null	5.627	-8.466	0.837	0.547	16773	1
GO:0050662	coenzyme binding	4.57%	-6.109	3.727	6.334	-10.7011	0.91	0	50662	0
GO:0016879	ligase activity, forming carbon-nitrogen bonds	1.68%	-1.831	-8.128	5.901	-8.466	0.943	0.031	16879	0
GO:0016616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	1.93%	4.477	1.45	5.96	-6.4976	0.914	0.031	16616	0
GO:0016820	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	2.03%	4.668	-3.791	5.983	-65.762	0.632	0.032	16820	0
GO:0070035	purine NTP-dependent helicase activity	0.50%	null	null	5.374	-3.6465	0.757	0.519	16820	1
GO:0022804	active transmembrane transporter activity	3.87%	null	null	6.263	-3.1336	0.841	0.703	16820	1
GO:0015405	P-P-bond-hydrolysis-driven transmembrane transporter activity	2.06%	null	null	5.99	-2.5689	0.835	0.969	16820	1
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	7.51%	null	null	6.551	-7.5622	0.682	0.912	16820	1
GO:0015399	primary active transmembrane transporter activity	2.06%	null	null	5.99	-9.5817	0.839	0.641	16820	1
GO:0043492	ATPase activity, coupled to movement of substances	1.91%	null	null	5.955	-9.5817	0.716	0.745	16820	1
GO:0004386	helicase activity	1.17%	null	null	5.743	-12.0482	0.737	0.572	16820	1
GO:0016887	ATPase activity	5.23%	null	null	6.394	-4.466	0.692	0.86	16820	1
GO:0042626	ATPase activity, coupled to transmembrane movement of substances	1.90%	null	null	5.954	-2.4541	0.612	0.87	16820	1
GO:0042623	ATPase activity, coupled	2.88%	null	null	6.134	-12.983	0.707	0.642	16820	1
GO:0015077	monovalent inorganic cation transmembrane transporter activity	2.61%	null	null	6.091	-4.2692	0.842	0.805	16820	1
GO:0016462	pyrophosphatase activity	7.43%	null	null	6.546	-3.4517	0.682	0.92	16820	1
GO:0008324	cation transmembrane transporter activity	3.90%	null	null	6.265	-14.1938	0.837	0.64	16820	1
GO:0017111	nucleoside-triphosphatase activity	7.07%	null	null	6.525	-15.7595	0.683	0.777	16820	1
GO:0022891	substrate-specific transmembrane transporter activity	6.72%	null	null	6.502	-30.0325	0.831	0.816	16820	1
GO:0008092	cytoskeletal protein binding	0.16%	-1.312	-6.695	4.885	-7.0218	0.92	0.055	8092	0
GO:0015631	tubulin binding	0.07%	null	null	4.495	-6.2441	0.923	0.524	8092	1
GO:0032561	guanyl ribonucleotide binding	1.74%	-3.311	5.552	5.915	-8.767	0.786	0.073	32561	0
GO:0017076	purine nucleotide binding	15.99%	null	null	6.879	-204.6556	0.729	0.695	32561	1
GO:0030554	adenyl nucleotide binding	14.35%	null	null	6.832	-237.1113	0.723	0.675	32561	1
GO:0035639	purine ribonucleoside triphosphate binding	15.48%	null	null	6.865	-226.2741	0.749	0.689	32561	1
GO:0000166	nucleotide binding	20.35%	null	null	6.983	-285.9393	0.716	0.744	32561	1
GO:0032559	adenyl ribonucleotide binding	13.95%	null	null	6.82	-220.5045	0.706	0.793	32561	1
GO:0032550	purine ribonucleoside binding	15.54%	null	null	6.866	-245.1355	0.719	0.817	32561	1
GO:0032549	ribonucleoside binding	15.66%	null	null	6.87	-256.5654	0.718	0.839	32561	1
GO:0032555	purine ribonucleotide binding	15.59%	null	null	6.868	-230.0031	0.701	0.817	32561	1
GO:0032553	ribonucleotide binding	16.29%	null	null	6.887	-267.1637	0.704	0.512	32561	1
GO:0001883	purine nucleoside binding	15.55%	null	null	6.867	-258.2958	0.719	0.837	32561	1
GO:0001882	nucleoside binding	15.74%	null	null	6.872	-256.1675	0.719	0.819	32561	1
GO:0051536	iron-sulfur cluster binding	2.61%	-0.399	-1.828	6.092	-4.4685	0.916	0.077	51536	0
GO:0043169	cation binding	15.81%	2.405	4.346	6.874	-207.8729	0.876	0.106	43169	0
GO:0008135	translation factor activity, RNA binding	0.84%	4.329	5.42	5.597	-2.9202	0.882	0.112	8135	0
GO:0046906	tetrapyrrole binding	1.91%	1.471	6.833	5.955	-7.7167	0.882	0.123	46906	0
GO:1901265	nucleoside phosphate binding	20.35%	-0.634	5.966	6.983	-188.6253	0.835	0.178	1901265	0
GO:0019787	ubiquitin-like protein transferase activity	0.01%	-3.934	-3.087	3.712	-7.6819	0.897	0.191	19787	0
GO:0008170	N-methyltransferase activity	0.45%	-6.193	-0.588	5.33	-12.5229	0.87	0.273	8170	0
GO:0016758	transferase activity, transferring hexosyl groups	0.60%	-5.377	-3.909	5.453	-14.0742	0.854	0.282	16758	0

REVIGO analysis for GO enrichment from differentially hypomethylated genes in leaves: molecular functions (NF) (Continued)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0008194	UDP-glycosyltransferase activity	0.19%	null	null	4.945	-6.0209	0.865	0.698	16758	1
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	1.16%	4.3	-3.511	5.739	-7.0419	0.806	0.286	4553	0
GO:0022835	transmitter-gated channel activity	0.00%	1.092	-7.392	2.55	-3.2608	0.908	0.291	22835	0
GO:0016798	hydrolase activity, acting on glycosyl bonds	1.61%	4.101	-4.935	5.881	-25.4225	0.8	0.297	16798	0
GO:0003723	RNA binding	5.86%	-1.14	5.387	6.443	-20.9393	0.852	0.313	3723	0
GO:0003677	DNA binding	13.92%	null	null	6.819	-18.5969	0.832	0.497	3723	1
GO:0003676	nucleic acid binding	21.75%	-0.135	5.643	7.012	-165.3706	0.833	0.317	3676	0
GO:0070011	peptidase activity, acting on L-amino acid peptides	3.02%	4.695	-4.706	6.155	-16.1221	0.773	0.323	70011	0
GO:0004175	endopeptidase activity	1.83%	null	null	5.937	-11.7077	0.784	0.854	70011	1
GO:0016757	transferase activity, transferring glycosyl groups	1.75%	-5.866	-3.147	5.917	-16.1778	0.854	0.324	16757	0
GO:0004518	nuclease activity	2.63%	5.202	-4.333	6.095	-7.3526	0.762	0.334	4518	0
GO:0016791	phosphatase activity	0.75%	null	null	5.551	-2.4709	0.781	0.819	4518	1
GO:0042578	phosphoric ester hydrolase activity	1.06%	null	null	5.701	-3.5237	0.781	0.701	4518	1
GO:0004721	phosphoprotein phosphatase activity	0.23%	null	null	5.032	-6.0209	0.8	0.59	4518	1
GO:0016747	transferase activity, transferring acyl groups other than amino-acyl groups	2.54%	-5.475	-2.224	6.08	-3.659	0.849	0.342	16747	0
GO:0008233	peptidase activity	3.61%	5.567	-3.718	6.232	-9.1518	0.782	0.35	8233	0
GO:0016741	transferase activity, transferring one-carbon groups	3.19%	-5.714	-1.416	6.179	-11.8239	0.846	0.353	16741	0
GO:0016746	transferase activity, transferring acyl groups	3.24%	-6.154	-2.437	6.185	-15.2027	0.845	0.354	16746	0
GO:0016655	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	0.86%	5.566	1.826	5.608	-3.0576	0.904	0.376	16655	0
GO:0003954	NADH dehydrogenase activity	0.79%	null	null	5.573	-2.757	0.904	0.885	16655	1
GO:0016788	hydrolase activity, acting on ester bonds	4.90%	5.719	-3.227	6.365	-3.7532	0.775	0.377	16788	0
GO:0016817	hydrolase activity, acting on acid anhydrides	7.56%	null	null	6.553	-77.4737	0.763	0.426	16788	1
GO:0019001	guanyl nucleotide binding	1.74%	-3.508	5.135	5.915	-9.5817	0.799	0.38	19001	0
GO:0046872	metal ion binding	15.49%	1.335	3.587	6.865	-150.7645	0.867	0.395	46872	0
GO:0046914	transition metal ion binding	7.34%	null	null	6.54	-121.1284	0.879	0.694	46872	1
GO:0016651	oxidoreductase activity, acting on NAD(P)H	1.35%	6.29	2.041	5.805	-4.8013	0.916	0.397	16651	0

REVIGO analysis for GO enrichment from differentially hypermethylated genes in leaves: molecular functions (MF)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0015077	monovalent inorganic cation transmembrane transporter activity	2.61%	-3.977	4.986	6.091	-12.767	0.492	0	15077	0
GO:0015075	ion transmembrane transporter activity	5.34%	null	null	6.402	-11.3152	0.496	0.816	15077	1
GO:0019829	cation-transporting ATPase activity	0.77%	null	null	5.562	-2.5187	0.434	0.733	15077	1
GO:0008324	cation transmembrane transporter activity	3.90%	null	null	6.265	-10.7959	0.489	0.805	15077	1
GO:0046906	tetrapyrrole binding	1.91%	3.146	6.2	5.955	-13.7144	0.73	0	46906	0
GO:0050136	NADH dehydrogenase (quinone) activity	0.78%	-4.514	-2.153	5.569	-2.2213	0.846	0	50136	0
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	7.51%	-0.959	-5.499	6.551	-6.1331	0.77	0.03	16818	0
GO:0046914	transition metal ion binding	7.34%	4.514	-4.919	6.54	-7.2526	0.772	0.079	46914	0
GO:0003676	nucleic acid binding	21.75%	4.173	2.078	7.012	-21.2716	0.691	0.18	3676	0
GO:0003723	RNA binding	5.86%	6.187	4.374	6.443	-15.109	0.711	0.226	3723	0
GO:0017076	purine nucleotide binding	15.99%	5.683	1.056	6.879	-5.1415	0.59	0.294	17076	0
GO:0015399	primary active transmembrane transporter activity	2.06%	-4.295	4.237	5.99	-4.8633	0.521	0.607	15399	0
GO:0032555	purine ribonucleotide binding	15.59%	6.123	1.489	6.868	-3.988	0.576	0.687	32555	0
GO:0032553	ribonucleotide binding	16.29%	null	null	6.887	-2.8801	0.575	0.817	32555	1

**REVIGO analysis for GO enrichment from differentially hypomethylated genes
in roots: biological process (BP)**

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:1901576	organic substance biosynthetic process	28.89%	4.221	-0.697	7.157	-8.71	0.546	0.224	1901576	0
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-3.78	2.481	7.221	-6.4776	0.487	0.161	34641	0
GO:0009059	macromolecule biosynthetic process	17.67%	5.076	0.004	6.944	-5.2062	0.556	0.559	9059	0
GO:0043043	peptide biosynthetic process	0.21%	3.1	3.967	5.017	-4.1707	0.528	0	43043	0
GO:0034645	cellular macromolecule biosynthetic process	17.20%	2.609	-0.487	6.932	-3.1149	0.5	0.554	34645	0
GO:0055086	nucleobase-containing small molecule metabolic process	10.84%	-2.077	3.674	6.732	-2.9127	0.385	0.352	55086	0
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	-2.896	0.799	6.476	-2.8257	0.282	0.599	9126	0
GO:0019637	organophosphate metabolic process	11.97%	-2.208	-3.443	6.775	-2.7467	0.445	0.177	19637	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-1.189	1.727	7.173	-2.1647	0.405	0.507	6139	0
GO:0046483	heterocycle metabolic process	33.33%	-5.398	-1.26	7.22	-2.0231	0.631	0.26	46483	0
GO:0009116	nucleoside metabolic process	8.23%	null	null	6.612	-2.7467	0.301	0.757	9126	1
GO:0009123	nucleoside monophosphate metabolic process	6.48%	null	null	6.508	-2.7467	0.277	0.751	9126	1
GO:0006163	purine nucleotide metabolic process	7.64%	null	null	6.58	-2.2041	0.255	0.785	9126	1

**REVIGO analysis for GO enrichment from differentially hypermethylated genes
in roots: biological process (BP)**

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:1901576	organic substance biosynthetic process	28.89%	4.22	0.698	7.157	-8.71	0.546	0.224	1901576	0
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-3.779	-2.482	7.221	-6.4776	0.487	0.161	34641	0
GO:0009059	macromolecule biosynthetic process	17.67%	5.076	-0.002	6.944	-5.2062	0.556	0.559	9059	0
GO:0043043	peptide biosynthetic process	0.21%	3.101	-3.966	5.017	-4.1707	0.528	0	43043	0
GO:0034645	cellular macromolecule biosynthetic process	17.20%	2.609	0.488	6.932	-3.1149	0.5	0.554	34645	0
GO:0055086	nucleobase-containing small molecule metabolic process	10.84%	-2.076	-3.675	6.732	-2.9127	0.385	0.352	55086	0
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	-2.896	-0.8	6.476	-2.8257	0.282	0.599	9126	0
GO:0019637	organophosphate metabolic process	11.97%	-2.209	3.443	6.775	-2.7467	0.445	0.177	19637	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-1.188	-1.727	7.173	-2.1647	0.405	0.507	6139	0
GO:0046483	heterocycle metabolic process	33.33%	-5.399	1.259	7.22	-2.0231	0.631	0.26	46483	0
GO:0009116	nucleoside metabolic process	8.23%	null	null	6.612	-2.7467	0.301	0.757	9126	1
GO:0009123	nucleoside monophosphate metabolic process	6.48%	null	null	6.508	-2.7467	0.277	0.751	9126	1
GO:0006163	purine nucleotide metabolic process	7.64%	null	null	6.58	-2.2041	0.255	0.785	9126	1

REVIGO analysis for GO enrichment from differentially hypomethylated genes
in roots: cellular cmpoenents (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	-2.4	1.055	5.573	-27.0061	0.305	0	9536	0
GO:0044444	cytoplasmic part	13.61%	2.357	3.22	6.505	-28.8601	0.534	0.194	44444	0
GO:0030529	ribonucleoprotein complex	6.09%	3.697	-2.46	6.155	-5.2062	0.53	0.195	30529	0
GO:0005737	cytoplasm	38.16%	3.158	0.983	6.952	-24.8962	0.558	0.316	5737	0
GO:0009534	chloroplast thylakoid	0.22%	-4.068	0.552	4.71	-5.2062	0.163	0.431	9534	0
GO:0009507	chloroplast	1.45%	null	null	5.531	-3.2107	0.234	0.786	9534	1
GO:0042651	thylakoid membrane	0.31%	null	null	4.859	-4.9547	0.399	0.919	9534	1
GO:0055035	plastid thylakoid membrane	0.21%	null	null	4.697	-4.451	0.163	0.995	9534	1
GO:0044391	ribosomal subunit	1.36%	-1.123	-1.343	5.504	-5.032	0.279	0.509	44391	0
GO:0031984	organelle subcompartment	0.23%	-4.105	-1.152	4.738	-4.5031	0.339	0.512	31984	0
GO:0043231	intracellular membrane-bounded organelle	8.85%	-1.5	0.262	6.318	-28.8601	0.249	0.666	43231	0

REVIGO analysis for GO enrichment from differentially hypermethylated genes
in roots: cellular cmpoenents (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	2.404	1.047	5.573	-27.0061	0.305	0	9536	0
GO:0044444	cytoplasmic part	13.61%	-2.344	3.232	6.505	-28.8601	0.534	0.194	44444	0
GO:0030529	ribonucleoprotein complex	6.09%	-3.708	-2.442	6.155	-5.2062	0.53	0.195	30529	0
GO:0005737	cytoplasm	38.16%	-3.155	0.999	6.952	-24.8962	0.558	0.316	5737	0
GO:0009534	chloroplast thylakoid	0.22%	4.07	0.537	4.71	-5.2062	0.163	0.431	9534	0
GO:0009507	chloroplast	1.45%	null	null	5.531	-3.2107	0.234	0.786	9534	1
GO:0042651	thylakoid membrane	0.31%	null	null	4.859	-4.9547	0.399	0.919	9534	1
GO:0055035	plastid thylakoid membrane	0.21%	null	null	4.697	-4.451	0.163	0.995	9534	1
GO:0044391	ribosomal subunit	1.36%	1.117	-1.345	5.504	-5.032	0.279	0.509	44391	0
GO:0031984	organelle subcompartment	0.23%	4.1	-1.167	4.738	-4.5031	0.339	0.512	31984	0
GO:0043231	intracellular membrane-bounded organelle	8.85%	1.5	0.258	6.318	-28.8601	0.249	0.666	43231	0

Appendix 2: Lists of salt-induced differentially expressed genes in barley roots

List of differentially expressed genes in barley roots in response to salt stress (100 mM NaCl)

GeneID	ref_gene_id	Chr	Start	End	logFC	AveExpr	t	P.Value	adj.P.Value
MSTRG.14676		3H	58248883	58253432	3.29	4.10	12.65	1.12E-09	3.42E-05
MSTRG.12231		2H	601501169	601502106	3.14	0.95	10.31	2.03E-08	3.11E-04
MSTRG.26594	MLOC_62978	5H	19842073	19846557	-1.94	5.41	-9.74	4.42E-08	4.51E-04
MSTRG.28505	MLOC_72489	5H	179733646	179736907	1.38	5.28	9.41	7.14E-08	5.46E-04
MSTRG.44690		7H	570271809	570274117	2.98	-1.47	8.05	5.59E-07	0.00317235
MSTRG.44681	MLOC_57585	7H	569716111	569721057	2.03	6.83	7.94	6.67E-07	0.00317235
MSTRG.2760	MLOC_54790	1H	308938797	308940068	4.00	3.55	7.89	7.26E-07	0.00317235
MSTRG.6749		2H	36503642	36505061	1.63	1.10	7.19	2.31E-06	0.008264874
MSTRG.6210	MLOC_1260	2H	7962408	7964264	3.95	-0.21	7.07	2.86E-06	0.008264874
MSTRG.10250		2H	530403290	530404172	2.61	3.19	7.06	2.89E-06	0.008264874
MSTRG.7653	MLOC_11838	2H	232819209	232822068	2.02	3.69	7.05	2.97E-06	0.008264874
MSTRG.31009	MLOC_50968	5H	486131265	486133992	1.71	2.60	6.98	3.35E-06	0.008320851
MSTRG.23353	MLOC_74282	4H	306353587	306355887	1.50	0.85	6.90	3.86E-06	0.008320851
MSTRG.39761	MLOC_37167	7H	22263804	22310471	-4.57	2.32	-6.89	3.90E-06	0.008320851
MSTRG.26701	MLOC_16950	5H	32045808	32046894	3.95	6.35	6.86	4.08E-06	0.008320851
MSTRG.12351		2H	606353759	606355046	-1.58	0.72	-6.62	6.28E-06	0.010970611
MSTRG.6802	MLOC_36363	2H	40118813	40120408	4.65	1.55	6.52	7.53E-06	0.010970611
MSTRG.4007		1H	428417475	428418457	1.32	0.21	6.43	8.82E-06	0.012264599
MSTRG.7917	MLOC_55525	2H	408462332	408465102	-1.82	2.40	-6.37	9.78E-06	0.01301926
MSTRG.37424	MLOC_62430	6H	464665142	464666059	3.19	-1.44	6.33	1.06E-05	0.013388969
MSTRG.24670	MLOC_57462	4H	501235168	501238590	2.43	-1.25	6.31	1.10E-05	0.013388969
MSTRG.42186		7H	271552873	271554622	2.49	0.41	6.24	1.24E-05	0.014026849
MSTRG.44647	MLOC_53958	7H	566170266	566173320	2.26	-1.21	6.23	1.28E-05	0.014026849
MSTRG.4691		1H	456850040	456851247	-3.55	-1.51	-6.21	1.33E-05	0.014059093
MSTRG.39805		7H	24921621	24922713	-4.59	0.99	-6.15	1.48E-05	0.015125905
MSTRG.24490	MLOC_70158	4H	488664822	488666564	2.03	3.38	6.10	1.62E-05	0.016012905
MSTRG.18627	MLOC_3645	3H	503839820	503842108	2.83	1.78	6.02	1.87E-05	0.016363598
MSTRG.19683	MLOC_79868	3H	556785275	556785749	2.01	0.51	6.02	1.87E-05	0.016363598
MSTRG.2774	MLOC_73684	1H	309135533	309138458	1.03	0.79	5.94	2.19E-05	0.017439062
MSTRG.32435	MLOC_70842	5H	543286555	543287891	2.37	-1.41	5.91	2.31E-05	0.017439062
MSTRG.4740	MLOC_81871	1H	458202279	458204366	2.43	0.08	5.91	2.31E-05	0.017439062
MSTRG.30127	MLOC_40020	5H	437578739	437579903	-1.41	1.09	-5.91	2.32E-05	0.017439062
MSTRG.24192	MLOC_63275	4H	447447955	447450280	1.10	5.96	5.87	2.48E-05	0.017439062
MSTRG.9308	MLOC_13104	2H	475724633	475726552	-3.60	3.83	-5.87	2.50E-05	0.017439062
MSTRG.30849		5H	480492611	480492735	-1.24	1.95	-5.86	2.52E-05	0.017439062
MSTRG.43122	MLOC_36919	7H	360541305	360544493	2.02	-0.65	5.85	2.59E-05	0.017439062
MSTRG.27368		5H	79945573	79946836	2.07	1.09	5.84	2.62E-05	0.017439062
MSTRG.19337	MLOC_13908	3H	538096842	538098495	3.83	-1.10	5.84	2.64E-05	0.017439062
MSTRG.44594		7H	563123578	563124350	1.60	1.71	5.82	2.74E-05	0.017439062
MSTRG.13830		3H	8913899	8914451	1.79	0.70	5.78	2.96E-05	0.018129314
MSTRG.42780		7H	329580684	329581403	1.60	-1.68	5.75	3.16E-05	0.018642473
MSTRG.16416		3H	376696186	376697842	2.23	-0.62	5.74	3.17E-05	0.018642473
MSTRG.32560	MLOC_5439	5H	549371194	549372944	3.11	0.42	5.71	3.38E-05	0.019174842
MSTRG.15976	MLOC_61831	3H	365965904	365970743	1.15	2.93	5.69	3.52E-05	0.019349991
MSTRG.36945	MLOC_60168	6H	412454172	412458108	1.63	2.90	5.69	3.54E-05	0.019349991
MSTRG.40542		7H	90637333	90638079	2.67	-2.29	5.64	3.84E-05	0.020598722
MSTRG.17924		3H	456892734	456893598	2.68	0.27	5.62	4.00E-05	0.020836755
MSTRG.39809	MLOC_5665	7H	25146404	25146853	1.97	-0.01	5.62	4.04E-05	0.020836755
MSTRG.9459	MLOC_34619	2H	483888836	483890332	2.79	0.08	5.61	4.08E-05	0.020836755
MSTRG.8468		2H	436079272	436080633	-1.76	1.40	-5.57	4.44E-05	0.02225432
MSTRG.30260	MLOC_61924	5H	444510564	444512753	2.07	-0.45	5.55	4.58E-05	0.02225432
MSTRG.37207		6H	446559276	446560600	1.16	0.80	5.51	5.01E-05	0.02313345
MSTRG.12023		2H	590017802	590020161	-3.30	2.08	-5.49	5.14E-05	0.02313345
MSTRG.21468	MLOC_64685	4H	139271133	139274563	2.68	8.21	5.48	5.23E-05	0.02313345
gene:MLOC_60587	MLOC_60587	2H	578457397	578470675	1.78	0.00	5.48	5.29E-05	0.02313345
MSTRG.6872	MLOC_55663	2H	45887892	45889505	3.07	4.89	5.48	5.29E-05	0.02313345
MSTRG.12888		2H	625473691	625474112	5.33	-2.12	5.44	5.67E-05	0.024095237
MSTRG.425		1H	19266004	19271183	1.68	5.37	5.42	5.90E-05	0.024730604
MSTRG.40489	MLOC_8139	7H	84206108	84206859	1.01	3.20	5.40	6.10E-05	0.025220426
MSTRG.34119		6H	47602766	47603314	-2.38	-2.06	-5.39	6.30E-05	0.025356974
MSTRG.15797	MLOC_54606	3H	353259065	353261433	1.95	0.68	5.38	6.34E-05	0.025356974
MSTRG.27115		5H	62528305	62529251	-1.56	1.72	-5.38	6.39E-05	0.025356974
MSTRG.44095	MLOC_13204	7H	533315344	533318093	-2.51	3.11	-5.34	6.96E-05	0.026357623
MSTRG.16814		3H	407790746	407791297	1.86	-0.23	5.34	6.98E-05	0.026357623

GeneID	ref_gene_id	Chr	Start	End	logFC	AveExpr	t	P.Value	adj.P.Value
MSTRG.3450	MLOC_71021	1H	383645364	383647364	4.01	1.14	5.31	7.30E-05	0.027227798
MSTRG.3749		1H	413688014	413689295	2.54	2.26	5.30	7.53E-05	0.027738149
MSTRG.37249	MLOC_58678	6H	448968381	448972361	1.05	1.82	5.27	8.01E-05	0.028392176
MSTRG.37488		6H	471390260	471391114	1.70	-1.23	5.26	8.16E-05	0.028392176
MSTRG.16239	MLOC_211	3H	371382111	371383338	1.85	-0.78	5.26	8.16E-05	0.028392176
MSTRG.11658	MLOC_13009	2H	579508462	579511920	3.03	5.54	5.22	8.77E-05	0.029308685
MSTRG.12297	MLOC_52569	2H	603568722	603570632	2.00	-1.91	5.22	8.80E-05	0.029308685
MSTRG.17370	MLOC_7422	3H	429161408	429162048	-1.49	-1.42	-5.22	8.80E-05	0.029308685
MSTRG.33497	MLOC_8028	6H	5087380	5089205	3.42	-1.95	5.18	9.54E-05	0.030338545
MSTRG.24986	MLOC_12009	4H	519520124	519526895	1.61	5.33	5.18	9.56E-05	0.030338545
MSTRG.11981		2H	589100628	589101819	-3.69	0.78	-5.18	9.56E-05	0.030338545
MSTRG.16791	MLOC_21654	3H	406464961	406467656	1.57	3.45	5.17	9.66E-05	0.030338545
MSTRG.24436	MLOC_15171	4H	481667551	481668750	1.54	3.55	5.16	9.79E-05	0.030338545
MSTRG.32778	MLOC_32569	5H	559331751	559333707	2.47	4.03	5.16	9.80E-05	0.030338545
MSTRG.12817		2H	623742710	623743251	5.03	-2.40	5.16	9.81E-05	0.030338545
MSTRG.4251		1H	435786064	436191924	-2.40	3.09	-5.16	9.92E-05	0.030355004
MSTRG.14326	MLOC_22463	3H	36412750	36414865	1.37	2.32	5.15	1.01E-04	0.03046918
MSTRG.4561		1H	455101668	455104921	2.39	0.38	5.14	1.02E-04	0.030697119
MSTRG.30771	MLOC_74265	5H	478346055	478570384	-1.08	5.91	-5.13	1.05E-04	0.031093042
MSTRG.15014	MLOC_81303	3H	142412259	142412864	3.21	1.00	5.11	1.08E-04	0.031788013
MSTRG.30159	MLOC_44441	5H	442089791	442091666	-1.16	2.74	-5.09	1.13E-04	0.032216238
MSTRG.44484		7H	558546047	558549347	-1.51	7.87	-5.09	1.14E-04	0.032216238
MSTRG.19283		3H	537145455	537146139	1.42	-1.47	5.08	1.17E-04	0.032216238
MSTRG.34383		6H	64581697	64583452	-1.07	2.33	-5.07	1.19E-04	0.032216238
MSTRG.19712		3H	557107913	557108248	2.58	2.53	5.06	1.21E-04	0.032216238
MSTRG.36635	MLOC_11331	6H	358650249	358651753	-1.70	2.70	-5.05	1.22E-04	0.032216238
MSTRG.19568		3H	549924476	549925082	1.50	-0.76	5.05	1.22E-04	0.032216238
MSTRG.27629		5H	87368808	87370817	1.00	-0.96	5.05	1.23E-04	0.032216238
MSTRG.32188		5H	535206237	535206489	1.05	-1.27	5.03	1.29E-04	0.032329203
MSTRG.15489		3H	271965793	271966544	1.62	1.33	5.02	1.30E-04	0.032329203
MSTRG.6770		2H	38167193	38167722	1.26	-1.45	5.02	1.30E-04	0.032329203
MSTRG.19338		3H	538137879	538139489	1.63	6.82	5.02	1.32E-04	0.032329203
MSTRG.1290	MLOC_58690	1H	129358954	129363065	1.37	2.86	5.01	1.32E-04	0.032329203
MSTRG.8318	MLOC_72858	2H	429783971	429785223	1.05	3.97	5.00	1.35E-04	0.032433692
MSTRG.2487	MLOC_78260	1H	275451994	275454685	-2.24	5.62	-4.99	1.38E-04	0.032433692
MSTRG.17434		3H	430731068	430732120	1.86	-0.43	4.99	1.39E-04	0.032433692
MSTRG.30861	MLOC_55919	5H	480999805	481006965	2.61	-1.74	4.97	1.44E-04	0.033396455
gene:MLOC_43280	MLOC_43280	5H	180013369	180015748	1.01	0.46	4.94	1.52E-04	0.034030083
MSTRG.21135	MLOC_18654	4H	42915430	42916023	1.68	0.96	4.90	1.67E-04	0.0364929
MSTRG.33694		6H	14490959	14493448	1.68	2.77	4.89	1.71E-04	0.036586654
MSTRG.29302		5H	360313640	360315047	3.58	3.39	4.86	1.79E-04	0.037772034
MSTRG.45355	MLOC_69078	7H	599958354	599962176	1.86	3.92	4.85	1.83E-04	0.037772034
MSTRG.20987	MLOC_57218	4H	27537013	27539637	1.64	4.68	4.84	1.88E-04	0.038299201
MSTRG.32156		5H	533609038	533612641	1.87	0.63	4.84	1.89E-04	0.038299201
MSTRG.18485	MLOC_61339	3H	496420360	496425367	1.26	5.76	4.82	1.94E-04	0.03878174
MSTRG.9590	MLOC_56998	2H	493303994	493307967	3.41	-0.19	4.82	1.95E-04	0.03878174
MSTRG.20988	MLOC_72638	4H	27546682	27547683	-1.11	2.74	-4.80	2.03E-04	0.039029444
MSTRG.14678		3H	58752137	58753235	1.39	1.30	4.78	2.08E-04	0.040049771
MSTRG.23174		4H	289648934	289649666	2.72	-1.49	4.78	2.13E-04	0.040578994
MSTRG.19633	MLOC_52070	3H	553088427	553493800	3.05	4.45	4.77	2.16E-04	0.040641773
MSTRG.25519		4H	543739034	543741111	4.50	-1.73	4.77	2.16E-04	0.040641773
MSTRG.17522		3H	432884163	432886215	1.07	3.86	4.76	2.21E-04	0.040913743
MSTRG.6570		2H	20368404	20368984	1.60	1.52	4.76	2.22E-04	0.040913743
MSTRG.812	MLOC_58100	1H	61930071	61933332	1.02	2.18	4.75	2.23E-04	0.040928609
MSTRG.40507	MLOC_64254	7H	88617434	88621703	-1.25	6.49	-4.74	2.30E-04	0.04139871
MSTRG.2555	MLOC_61818	1H	284329846	284334509	-1.27	6.48	-4.73	2.34E-04	0.04157952
MSTRG.25293	MLOC_5021	4H	533453286	533459057	-1.17	4.72	-4.73	2.35E-04	0.04157952
MSTRG.33402	MLOC_46472:MLOC_2	6H	29673	268851	2.67	1.06	4.73	2.36E-04	0.04157952
MSTRG.38472	MLOC_58163	6H	530782286	530958982	2.29	5.28	4.72	2.41E-04	0.041839108
MSTRG.4024	MLOC_66415	1H	428647812	428697328	1.91	10.69	4.71	2.43E-04	0.042002709
MSTRG.6765		2H	38029779	38030838	1.70	-0.89	4.70	2.48E-04	0.042138817
MSTRG.22494		4H	248673922	248674709	2.11	-0.23	4.69	2.52E-04	0.042138817
MSTRG.31786		5H	518129262	518131992	1.42	-0.66	4.69	2.52E-04	0.042138817
gene:MLOC_75827	MLOC_75827	5H	132426143	132429247	2.42	-1.55	4.69	2.52E-04	0.042138817
MSTRG.11579	MLOC_29498	2H	575704748	575706016	4.08	-1.29	4.69	2.56E-04	0.042138817
MSTRG.43177		7H	404965066	404966859	1.34	5.30	4.68	2.57E-04	0.042138817

GeneID	ref_gene_id	Chr	Start	End	logFC	AveExpr	t	P.Value	adj.P.Value
MSTRG.24639		4H	499083090	499123062	1.45	4.55	4.68	2.58E-04	0.042138817
MSTRG.32529		5H	548437622	548438116	2.33	0.82	4.68	2.59E-04	0.042138817
MSTRG.23907		4H	416800531	416801796	2.64	-0.29	4.67	2.62E-04	0.042436448
MSTRG.45268		7H	595628991	595631259	2.57	-0.43	4.66	2.70E-04	0.043082071
MSTRG.31490	MLOC_63969	5H	506324567	506327901	1.77	4.65	4.65	2.76E-04	0.043549306
MSTRG.32025	MLOC_80912	5H	528302034	528305319	1.18	2.68	4.63	2.86E-04	0.043624366
MSTRG.18414	MLOC_19670	3H	489725374	489727559	1.07	1.40	4.63	2.87E-04	0.043624366
MSTRG.37668		6H	482672184	482675879	-1.70	2.43	-4.63	2.88E-04	0.043624366
gene:MLOC_70409	MLOC_70409	3H	424149321	424151862	1.92	-1.46	4.63	2.89E-04	0.043624366
MSTRG.23921	MLOC_10899	4H	417627494	417629292	3.77	-0.20	4.62	2.91E-04	0.043624366
MSTRG.43641		7H	472921291	472922151	1.57	-0.83	4.62	2.94E-04	0.043624366
MSTRG.35458	MLOC_10990	6H	237984258	237985595	1.83	2.54	4.62	2.95E-04	0.043624366
MSTRG.10717	MLOC_69129	2H	546434355	546434775	-2.27	-0.63	-4.61	2.98E-04	0.0438744
MSTRG.22621	MLOC_39183	4H	253291250	253293308	2.28	-0.58	4.58	3.15E-04	0.04599558
MSTRG.30488	MLOC_65161	5H	457039799	457044219	1.22	0.21	4.58	3.20E-04	0.04599558
MSTRG.39357	MLOC_74611	7H	4736698	4737814	-2.55	0.75	-4.57	3.24E-04	0.04599558
MSTRG.3444		1H	383304475	383306043	1.75	-0.69	4.57	3.24E-04	0.04599558
MSTRG.10806	MLOC_11562	2H	548557768	548558756	-1.02	4.81	-4.57	3.24E-04	0.04599558
MSTRG.37845	MLOC_5716	6H	498140223	498142706	1.75	4.98	4.57	3.24E-04	0.04599558
MSTRG.29627		5H	386207007	386208218	-2.11	0.60	-4.57	3.26E-04	0.04599558
MSTRG.15822	MLOC_71129	3H	353972359	353974145	1.31	0.06	4.55	3.35E-04	0.046822123
MSTRG.40851	MLOC_72166	7H	118419391	118420912	2.14	-0.89	4.55	3.37E-04	0.046822123
MSTRG.12898	MLOC_43077	2H	626365965	626366451	5.53	-1.72	4.55	3.38E-04	0.046822123
MSTRG.37751	MLOC_76480	6H	492323674	492325584	1.49	2.58	4.53	3.52E-04	0.048523871
MSTRG.28162		5H	121195215	121195812	1.13	-0.95	4.51	3.66E-04	0.049496112
MSTRG.18386	MLOC_37763	3H	487546601	487555710	2.19	5.83	4.50	3.71E-04	0.049644142
MSTRG.37084		6H	430802820	430803293	-1.10	-0.74	-4.50	3.72E-04	0.049644142
MSTRG.3601		1H	403988593	403988720	-1.95	-1.59	-4.50	3.73E-04	0.049644142

REVIGO analysis for GO enrichment from salt-induced downregulated genes in roots: biological process (BP)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0006518	peptide metabolic process	0.32%	-5.269	-5.39	5.206	-18.2118	0.621	0	6518	0
GO:0006091	generation of precursor metabolites and energy	3.22%	-0.935	7.355	6.205	-2.2823	0.77	0.062	6091	0
GO:1901564	organonitrogen compound metabolic process	19.57%	-0.819	-4.342	6.988	-30.1791	0.554	0.158	1901564	0
GO:0044260	cellular macromolecule metabolic process	28.59%	2.065	0.872	7.153	-23.8297	0.576	0.175	44260	0
GO:0043170	macromolecule metabolic process	32.80%	4.252	-4.359	7.213	-23.8297	0.714	0.214	43170	0
GO:0006725	cellular aromatic compound metabolic process	33.05%	-3.602	4.004	7.216	-2.9779	0.7	0.242	6725	0
GO:0009123	nucleoside monophosphate metabolic process	6.48%	-4.177	-2.248	6.508	-4.1925	0.342	0.267	9123	0
GO:0009144	purine nucleoside triphosphate metabolic process	6.48%	null	null	6.508	-2.0616	0.342	0.761	9123	1
GO:0046128	purine ribonucleoside metabolic process	7.32%	null	null	6.561	-4.1925	0.301	0.741	9123	1
GO:0009117	nucleotide metabolic process	9.83%	null	null	6.689	-4.0788	0.309	0.824	9123	1
GO:0009150	purine ribonucleotide metabolic process	7.46%	null	null	6.569	-4.0788	0.291	0.889	9123	1
GO:0042278	purine nucleoside metabolic process	7.36%	null	null	6.564	-2.8463	0.3	0.89	9123	1
GO:0009167	purine ribonucleoside monophosphate metabolic process	6.01%	null	null	6.475	-2.8052	0.348	0.751	9123	1
GO:0006163	purine nucleotide metabolic process	7.64%	null	null	6.58	-3.2904	0.289	0.896	9123	1
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	-2.982	-1.194	6.895	-26.9788	0.465	0.335	44271	0
GO:1901566	organonitrogen compound biosynthetic process	9.30%	-2.379	-3.507	6.665	-25.0191	0.453	0.394	1901566	0
GO:0044267	cellular protein metabolic process	8.78%	3.516	2.147	6.64	-22.4881	0.635	0.407	44267	0
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-3.984	-0.007	7.221	-29.3665	0.519	0.428	34641	0
GO:0019538	protein metabolic process	12.33%	5.308	0.694	6.788	-22.4881	0.668	0.448	19538	0
GO:0010467	gene expression	16.70%	5.431	-0.401	6.92	-13.0453	0.674	0.493	10467	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-1.176	-1.147	7.173	-3.2327	0.433	0.507	6139	0
GO:1901576	organic substance biosynthetic process	28.89%	1.614	-3.005	7.157	-17.7375	0.61	0.539	1901576	0
GO:0044249	cellular biosynthetic process	28.21%	-2.495	1.792	7.147	-11.3936	0.601	0.663	44249	0

REVIGO analysis for GO enrichment from salt-induced downregulated genes in roots: cellular components (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	4.63	-2.483	5.573	-55.2733	0.371	0	9536	0
GO:0009579	thylakoid	0.41%	-4.11	2.862	4.985	-13.7011	0.65	0.097	9579	0
GO:0044444	cytoplasmic part	13.61%	0.708	-5.526	6.505	-55.2733	0.555	0.194	44444	0
GO:0030529	ribonucleoprotein complex	6.09%	-2.587	-5.051	6.155	-18.2118	0.555	0.195	30529	0
GO:0005737	cytoplasm	38.16%	-0.692	-2.595	6.952	-49.4342	0.56	0.316	5737	0
GO:0031984	organelle subcompartment	0.23%	5.505	0.235	4.738	-9.2676	0.434	0.428	31984	0
GO:0043232	intracellular non-membrane-bounded organelle	7.68%	2.789	-1.963	6.256	-14.109	0.386	0.464	43232	0
GO:0009507	chloroplast	1.45%	4.546	-1.447	5.531	-16.5086	0.336	0.528	9507	0
GO:0009521	photosystem	0.21%	null	null	4.701	-3.8777	0.405	0.892	9507	1
GO:0044436	thylakoid part	0.40%	null	null	4.97	-12.067	0.423	0.939	9507	1
GO:0009534	chloroplast thylakoid	0.22%	null	null	4.71	-16.5086	0.222	0.786	9507	1
GO:0043231	intracellular membrane-bounded organelle	8.85%	3.123	-1.295	6.318	-54.2733	0.308	0.666	43231	0

REVIGO analysis for GO enrichment from salt-induced upregulated genes in roots: biological process (BP)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0006518	peptide metabolic process	0.32%	-6.726	-2.594	5.206	-44.7825	0.71	0	6518	0
GO:0043604	amide biosynthetic process	0.55%	null	null	5.439	-36.0414	0.66	0.852	6518	1
GO:0043043	peptide biosynthetic process	0.21%	null	null	5.017	-43.7235	0.642	0.782	6518	1
GO:0015672	monovalent inorganic cation transport	2.71%	3.473	-3.757	6.13	-13.6108	0.756	0	15672	0
GO:0015992	proton transport	1.97%	null	null	5.992	-14.1726	0.758	0.822	15672	1
GO:0006812	cation transport	3.98%	null	null	6.297	-11.4535	0.757	0.775	15672	1
GO:0098662	inorganic cation transmembrane transport	2.31%	null	null	6.06	-9.6635	0.691	0.839	15672	1
GO:0098655	cation transmembrane transport	2.86%	null	null	6.153	-3.93	0.687	0.864	15672	1
GO:0098660	inorganic ion transmembrane transport	2.58%	null	null	6.108	-6.2865	0.695	0.728	15672	1
GO:1902600	hydrogen ion transmembrane transport	1.80%	null	null	5.951	-12.0273	0.695	0.969	15672	1
GO:0015985	energy coupled proton transport, down electrochemical gradient	0.45%	null	null	5.35	-13.1355	0.735	0.825	15672	1
GO:0015979	photosynthesis	0.34%	5.598	-1.296	5.232	-35.4248	0.885	0.05	15979	0
GO:0036211	protein modification process	2.90%	3.152	7.103	6.158	-7.1851	0.821	0.054	36211	0
GO:0006091	generation of precursor metabolites and energy	3.22%	5.54	0.74	6.205	-30.3063	0.853	0.063	6091	0
GO:0022900	electron transport chain	1.16%	-1.975	-5.561	5.763	-26.2	0.768	0.072	22900	0
GO:0006119	oxidative phosphorylation	1.17%	null	null	5.764	-2.7248	0.404	0.747	22900	1
GO:0090407	organophosphate biosynthetic process	4.46%	-6.711	2.306	6.346	-13.6108	0.5	0.087	90407	0
GO:0043170	macromolecule metabolic process	32.80%	2.441	5.304	7.213	-76.1421	0.812	0.12	43170	0
GO:0046483	heterocycle metabolic process	33.33%	0.74	0.749	7.22	-40.0878	0.784	0.136	46483	0
GO:0043603	cellular amide metabolic process	0.91%	-5.212	-2.723	5.656	-11.4034	0.769	0.14	43603	0
GO:1901135	carbohydrate derivative metabolic process	11.65%	4.961	3.808	6.763	-14.3768	0.844	0.155	1901135	0
GO:0055114	oxidation-reduction process	15.04%	-3.924	-5.141	6.874	-22.9208	0.77	0.177	55114	0
GO:1901564	organonitrogen compound metabolic process	19.57%	-2.407	6.602	6.988	-70.3915	0.698	0.184	1901564	0
GO:0006793	phosphorus metabolic process	16.89%	2.561	0.677	6.924	-23.9136	0.811	0.198	6793	0
GO:1901360	organic cyclic compound metabolic process	33.91%	1.618	6.153	7.227	-30.0376	0.81	0.23	1901360	0
GO:1901659	glycosyl compound biosynthetic process	1.69%	-8.102	1.363	5.925	-12.7212	0.537	0.246	1901659	0
GO:0046129	purine ribonucleoside biosynthetic process	1.07%	null	null	5.728	-8.3969	0.405	0.905	1901659	1
GO:0006164	purine nucleotide biosynthetic process	1.47%	null	null	5.864	-12.3565	0.366	0.93	1901659	1
GO:0042455	ribonucleoside biosynthetic process	1.65%	null	null	5.913	-12.7212	0.394	0.932	1901659	1
GO:0009152	purine ribonucleotide biosynthetic process	1.38%	null	null	5.836	-5.9208	0.349	0.958	1901659	1
GO:0009163	nucleoside biosynthetic process	1.67%	null	null	5.918	-10.7447	0.394	0.978	1901659	1
GO:0006754	ATP biosynthetic process	0.46%	null	null	5.363	-11.2007	0.383	0.822	1901659	1
GO:0042451	purine nucleoside biosynthetic process	1.07%	null	null	5.728	-12.7212	0.406	0.723	1901659	1
GO:0009201	ribonucleoside triphosphate biosynthetic process	0.59%	null	null	5.467	-7.2487	0.439	0.945	1901659	1
GO:0006725	cellular aromatic compound metabolic process	33.05%	0.549	0.153	7.216	-39.068	0.785	0.259	6725	0
GO:0072522	purine-containing compound biosynthetic process	1.59%	-6.852	2.936	5.899	-13.6108	0.552	0.266	72522	0
GO:0019538	protein metabolic process	12.33%	0.51	7.979	6.788	-57.5751	0.786	0.266	19538	0
GO:0043412	macromolecule modification	5.09%	2.211	7.935	6.404	-8.9431	0.828	0.295	43412	0
GO:0055086	nucleobase-containing small molecule metabolic process	10.84%	-3.627	0.671	6.732	-4.8153	0.508	0.303	55086	0
GO:0044281	small molecule metabolic process	21.50%	-4.06	-5.185	7.029	-22.3747	0.756	0.328	44281	0
GO:0097659	nucleic acid-templated transcription	0.77%	-4.678	5.511	5.584	-2.924	0.621	0.349	97659	0
GO:0044249	cellular biosynthetic process	28.21%	-5.729	3.652	7.147	-78.6198	0.634	0.38	44249	0
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-2.101	1.221	7.221	-64.8665	0.656	0.428	34641	0
GO:0044260	cellular macromolecule metabolic process	28.59%	-0.171	5.591	7.153	-57.3125	0.709	0.448	44260	0
GO:1901566	organonitrogen compound biosynthetic process	9.30%	-5.963	3.804	6.665	-60.7721	0.576	0.457	1901566	0
GO:0006818	hydrogen transport	1.98%	3.449	-3.925	5.992	-8.9706	0.804	0.481	6818	0
GO:0010467	gene expression	16.70%	0.75	7.769	6.92	-54.5391	0.791	0.493	10467	0
GO:0044711	single-organism biosynthetic process	12.46%	-7.275	1.047	6.792	-14.8239	0.651	0.497	44711	0
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-2.211	3.313	7.173	-40.0878	0.565	0.507	6139	0
GO:1901137	carbohydrate derivative biosynthetic process	3.92%	-8.23	3.848	6.29	-8.5031	0.608	0.519	1901137	0
GO:0090304	nucleic acid metabolic process	20.16%	-1.824	4.515	7.001	-10.6478	0.54	0.528	90304	0
GO:0019438	aromatic compound biosynthetic process	15.56%	-5.429	3.281	6.889	-18.8297	0.581	0.532	19438	0

REVIGO analysis for GO enrichment from salt-induced upregulated genes in roots: biological process (BP)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	-4.986	2.654	6.895	-38.3726	0.57	0.534	44271	0
GO:0072521	purine-containing compound metabolic process	8.06%	-3.237	2.282	6.603	-23.0721	0.596	0.536	72521	0
GO:0018130	heterocycle biosynthetic process	16.15%	-5.462	3.057	6.905	-21.8069	0.58	0.538	18130	0
GO:1901362	organic cyclic compound biosynthetic process	16.58%	-5.558	4.581	6.916	-16.5157	0.594	0.543	1901362	0
GO:0034645	cellular macromolecule biosynthetic process	17.20%	-3.252	5.125	6.932	-49.7077	0.602	0.55	34645	0
GO:0009059	macromolecule biosynthetic process	17.67%	-3.9	6.053	6.944	-41.8041	0.643	0.554	9059	0
GO:0009165	nucleotide biosynthetic process	2.78%	-5.229	1.289	6.141	-13.1355	0.358	0.558	9165	0
GO:1901293	nucleoside phosphate biosynthetic process	2.83%	null	null	6.148	-12.0273	0.37	0.832	9165	1
GO:0009260	ribonucleotide biosynthetic process	1.77%	null	null	5.944	-4.9101	0.354	0.91	9165	1
GO:0009127	purine nucleoside monophosphate biosynthetic process	1.10%	null	null	5.737	-8.1918	0.404	0.936	9165	1
GO:0009142	nucleoside triphosphate biosynthetic process	0.67%	null	null	5.525	-3.8525	0.436	0.808	9165	1
GO:0009156	ribonucleoside monophosphate biosynthetic process	1.45%	null	null	5.857	-12.7212	0.388	0.886	9165	1
GO:0034654	nucleobase-containing compound biosynthetic process	13.02%	-4.412	3.364	6.811	-24.6162	0.493	0.559	34654	0
GO:0019637	organophosphate metabolic process	11.97%	0.765	1.987	6.775	-23.9136	0.561	0.565	19637	0
GO:0006796	phosphate-containing compound metabolic process	16.69%	null	null	6.919	-11.1135	0.564	0.755	19637	1
GO:0019684	photosynthesis, light reaction	0.09%	1.38	-5.08	4.627	-17.1158	0.876	0.577	19684	0
GO:0016310	phosphorylation	6.30%	-0.99	-2.26	6.496	-4.4023	0.622	0.605	16310	0
GO:0044267	cellular protein metabolic process	8.78%	0.114	7.135	6.64	-59.0701	0.751	0.614	44267	0
GO:0044765	single-organism transport	12.59%	1.036	-5.101	6.797	-13.6108	0.753	0.633	44765	0
GO:0055085	transmembrane transport	9.40%	null	null	6.67	-14.1726	0.682	0.811	44765	1
GO:0006810	transport	17.38%	null	null	6.937	-11.2007	0.857	0.753	44765	1
GO:0009167	purine ribonucleoside monophosphate metabolic process	6.01%	-3.959	0.036	6.475	-22.3747	0.368	0.652	9167	0
GO:0046128	purine ribonucleoside metabolic process	7.32%	null	null	6.561	-21.2457	0.345	0.73	9167	1
GO:0042278	purine nucleoside metabolic process	7.36%	null	null	6.564	-21.2457	0.347	0.891	9167	1
GO:0009205	purine ribonucleoside triphosphate metabolic process	6.48%	null	null	6.508	-23.9136	0.364	0.761	9167	1
GO:0009116	nucleoside metabolic process	8.23%	null	null	6.612	-19.2358	0.348	0.909	9167	1
GO:0009117	nucleotide metabolic process	9.83%	null	null	6.689	-22.3747	0.342	0.857	9167	1
GO:0009119	ribonucleoside metabolic process	7.93%	null	null	6.596	-22.3747	0.351	0.89	9167	1
GO:0009259	ribonucleotide metabolic process	7.84%	null	null	6.591	-23.9136	0.336	0.789	9167	1
GO:0009123	nucleoside monophosphate metabolic process	6.48%	null	null	6.508	-23.9136	0.377	0.751	9167	1
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	null	null	6.476	-5.3036	0.369	0.919	9167	1
GO:0019693	ribose phosphate metabolic process	7.90%	null	null	6.595	-20.7696	0.439	0.755	9167	1
GO:0006163	purine nucleotide metabolic process	7.64%	null	null	6.58	-20.7696	0.336	0.894	9167	1
GO:0009144	purine nucleoside triphosphate metabolic process	6.48%	null	null	6.508	-10.8633	0.365	0.922	9167	1
GO:0009141	nucleoside triphosphate metabolic process	6.72%	null	null	6.524	-7.9245	0.374	0.795	9167	1
GO:0009150	purine ribonucleotide metabolic process	7.46%	null	null	6.569	-20.7696	0.316	0.909	9167	1
GO:0009161	ribonucleoside monophosphate metabolic process	6.36%	null	null	6.5	-22.3747	0.365	0.919	9167	1
GO:0006753	nucleoside phosphate metabolic process	9.90%	null	null	6.692	-18.618	0.351	0.816	9167	1
GO:0046034	ATP metabolic process	5.37%	null	null	6.427	-18.9172	0.315	0.922	9167	1
GO:1901657	glycosyl compound metabolic process	8.26%	null	null	6.614	-21.2457	0.596	0.744	9167	1
GO:0009199	ribonucleoside triphosphate metabolic process	6.56%	null	null	6.514	-18.3363	0.364	0.922	9167	1
GO:1901576	organic substance biosynthetic process	28.89%	-5.52	5.298	7.157	-42.0052	0.652	0.663	1901576	0

REVIGO analysis for GO enrichment from salt-induced upregulated genes in roots: cellular components (CC)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0009536	plastid	1.59%	5.557	-1.411	5.573	-198.9136	0.43	0	9536	0
GO:0042651	thylakoid membrane	0.31%	-2.103	4.642	4.859	-98.3936	0.383	0.094	42651	0
GO:0044436	thylakoid part	0.40%	null	null	4.97	-76.1561	0.38	0.967	42651	1
GO:0045259	proton-transporting ATP synthase complex	0.88%	null	null	5.316	-12.7212	0.428	1.21	42651	1
GO:0016469	proton-transporting two-sector ATPase complex	1.00%	null	null	5.369	-14.1726	0.518	1.051	42651	1
GO:0009521	photosystem	0.21%	null	null	4.701	-24.1851	0.248	0.927	42651	1
GO:0009523	photosystem II	0.16%	null	null	4.566	-15.7773	0.26	0.903	42651	1
GO:0009579	thylakoid	0.41%	-5.228	-3.107	4.985	-97.5229	0.742	0.097	9579	0
GO:0005737	cytoplasm	38.16%	0.152	-1.784	6.952	-198.9136	0.643	0.183	5737	0
GO:0030529	ribonucleoprotein complex	6.09%	1.972	-6.519	6.155	-42.4306	0.535	0.248	30529	0
GO:0044444	cytoplasmic part	13.61%	0.272	-3.776	6.505	-197.6021	0.64	0.316	44444	0
GO:0031984	organelle subcompartment	0.23%	6.527	0.087	4.738	-103.9788	0.469	0.428	31984	0
GO:0044391	ribosomal subunit	1.36%	4.855	-3.169	5.504	-14.0696	0.315	0.512	44391	0
GO:0009507	chloroplast	1.45%	5.04	-0.381	5.531	-111.8239	0.328	0.528	9507	0
GO:0044435	plastid part	0.28%	null	null	4.817	-104.3686	0.377	0.805	9507	1
GO:0009534	chloroplast thylakoid	0.22%	null	null	4.71	-80.9031	0.175	0.995	9507	1
GO:0044434	chloroplast part	0.28%	null	null	4.815	-110.4271	0.327	0.805	9507	1
GO:0031976	plastid thylakoid	0.22%	null	null	4.71	-103.9788	0.175	0.985	9507	1
GO:0055035	plastid thylakoid membrane	0.21%	null	null	4.697	-103.9788	0.173	0.974	9507	1
GO:0043232	intracellular non-membrane-bounded organelle	7.68%	3.972	-1.898	6.256	-42.4306	0.416	0.659	43232	0
GO:0005840	ribosome	5.76%	null	null	6.131	-8.4776	0.306	0.835	43232	1
GO:0043231	intracellular membrane-bounded organelle	8.85%	3.952	-1.022	6.318	-189.4078	0.362	0.666	43231	0

REVIGO analysis for GO enrichment from salt-induced upregulated genes in roots: molecular functions (MF)

term_ID	description	frequency	plot_X	plot_Y	plot_size	log10 p-value	uniqueness	dispensability	representative	eliminated
GO:0022890	inorganic cation transmembrane transporter activity	3.19%	-4.52	-4.066	6.179	-19.3726	0.471	0	22890	0
GO:0008324	cation transmembrane transporter activity	3.90%	null	null	6.265	-9.2676	0.466	0.829	22890	1
GO:0022891	substrate-specific transmembrane transporter activity	6.72%	null	null	6.502	-19.3726	0.465	0.789	22890	1
GO:0015075	ion transmembrane transporter activity	5.34%	null	null	6.402	-10.0343	0.468	0.861	22890	1
GO:0015077	monovalent inorganic cation transmembrane transporter activit	2.61%	null	null	6.091	-17.4191	0.48	0.879	22890	1
GO:0046906	tetrapyrrole binding	1.91%	1.879	-7.149	5.955	-20.8297	0.835	0	46906	0
GO:0050136	NADH dehydrogenase (quinone) activity	0.78%	0.346	6.201	5.569	-14.1726	0.835	0	50136	0
GO:0003954	NADH dehydrogenase activity	0.79%	null	null	5.573	-14.1726	0.835	0.877	50136	1
GO:0042623	ATPase activity, coupled	2.88%	-5.056	1.894	6.134	-10.6478	0.68	0.027	42623	0
GO:0044769	ATPase activity, coupled to transmembrane movement of ions, r	0.38%	null	null	5.249	-10.6478	0.433	0.814	42623	1
GO:0015405	P-P-bond-hydrolysis-driven transmembrane transporter activity	2.06%	null	null	5.99	-9.2668	0.468	0.969	42623	1
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-con	7.51%	null	null	6.551	-4.3726	0.677	0.778	42623	1
GO:0042626	ATPase activity, coupled to transmembrane movement of substa	1.90%	null	null	5.954	-10.6478	0.38	0.744	42623	1
GO:0042625	ATPase activity, coupled to transmembrane movement of ions	0.98%	null	null	5.667	-10.6478	0.39	0.905	42623	1
GO:0043169	cation binding	15.81%	5.843	1.229	6.874	-26.2	0.821	0.09	43169	0
GO:0003676	nucleic acid binding	21.75%	5.022	-2.88	7.012	-42.4306	0.791	0.18	3676	0
GO:0003723	RNA binding	5.86%	4.049	-5.859	6.443	-24.6021	0.817	0.226	3723	0
GO:0001883	purine nucleoside binding	15.55%	5.159	-4.063	6.867	-8.6021	0.696	0.291	1883	0
GO:0032559	adenyl ribonucleotide binding	13.95%	null	null	6.82	-7.7932	0.702	0.784	1883	1
GO:0032549	ribonucleoside binding	15.66%	null	null	6.87	-5.4377	0.696	0.839	1883	1
GO:0032553	ribonucleotide binding	16.29%	null	null	6.887	-5.6253	0.696	0.817	1883	1
GO:1901265	nucleoside phosphate binding	20.35%	3.91	-3.299	6.983	-11.0255	0.792	0.317	1901265	0
GO:0016651	oxidoreductase activity, acting on NAD(P)H	1.35%	-0.523	6.086	5.805	-6.9914	0.869	0.358	16651	0
GO:0016817	hydrolase activity, acting on acid anhydrides	7.56%	-4.693	2.606	6.553	-3.0328	0.783	0.389	16817	0
GO:0046872	metal ion binding	15.49%	5.178	1.853	6.865	-21.6402	0.797	0.395	46872	0
GO:0016820	hydrolase activity, acting on acid anhydrides, catalyzing transme	2.03%	-4.858	-1.433	5.983	-3.9616	0.438	0.642	16820	0
GO:0022853	active ion transmembrane transporter activity	0.91%	-4.445	-4.519	5.635	-8.4976	0.501	0.681	22853	0
GO:0022804	active transmembrane transporter activity	3.87%	-4.83	-3.772	6.263	-6.6716	0.505	0.682	22804	0
GO:0046914	transition metal ion binding	7.34%	5.273	2.847	6.54	-12.7212	0.807	0.694	46914	0

GO enrichment from salt-induced hypomethylated DE genes in roots

Term	Description	DECount	notDECoun	p	adjP	FDR
GO:0043231	intracellular membrane-bounded organelle	3	10	6.41E-10	2.56E-09	2.67E-09
GO:0009536	plastid	3	21	4.53E-09	1.81E-08	1.26E-08
GO:0044444	cytoplasmic part	3	32	1.47E-08	5.86E-08	3.05E-08

GO enrichment from salt-induced hypermethylated DE genes in roots

Term	Description	DECount	notDECoun	p	adjP	FDR
GO:0005737	cytoplasm	2	5	2.18E-07	8.71E-07	8.07E-07
GO:0043231	intracellular membrane-bounded organelle	2	6	2.90E-07	1.16E-06	8.07E-07
GO:0009536	plastid	2	10	6.84E-07	2.74E-06	1.43E-06

Appendix 3: List of exons differentially methylated in barley roots and leaves

Exons					Tissue-specific DMMs				Distance to exon
Chromosome	start	end	ID	rank	Chromosome	start	end	Tissue	(base pair)
3H	256588863	256589313	exon:MLOC_37071.2:3	3	3H	256588258	256588258	leaf	-604
1H	173809114	173809167	exon:MLOC_44613.1:2	2	1H	173808619	173808619	leaf	-494
2H	427507334	427507612	exon:MLOC_61110.4:1	1	2H	427506881	427506881	blade	-452
7H	584462328	584462663	exon:MLOC_6930.1:4	4	7H	584461984	584461984	blade	-343
3H	48188588	48188710	exon:MLOC_36518.3:9	9	3H	48188256	48188256	leaf	-331
4H	531043445	531043540	exon:MLOC_66787.2:5	5	4H	531043255	531043255	leaf	-189
3H	282775878	282775978	exon:MLOC_57866.1:2	2	3H	282775689	282775689	leaf	-188
2H	507101612	507102232	exon:MLOC_57766.1:6	6	2H	507101429	507101429	blade	-182
3H	451801679	451801792	exon:MLOC_4568.8:12	12	3H	451801608	451801608	blade	-70
1H	295869691	295869957	exon:MLOC_57040.1:1	1	1H	295869907	295869907	blade	0
1H	372664328	372665243	exon:MLOC_11591.1:1	1	1H	372665217	372665217	leaf	0
1H	398203764	398206694	exon:MLOC_52730.3:1	1	1H	398204886	398204886	leaf	0
2H	436039625	436040167	exon:MLOC_16240.2:1	1	2H	436040156	436040156	leaf	0
2H	550574223	550574658	exon:MLOC_7365.2:1	1	2H	550574622	550574622	leaf	0
3H	141116151	141117572	exon:MLOC_70576.2:1	1	3H	141116946	141116946	blade	0
4H	428185287	428190462	exon:MLOC_52907.1:1	1	4H	428185685	428185685	leaf	0
5H	449547966	449548309	exon:MLOC_66740.1:1	1	5H	449548006	449548006	blade	0
6H	5471445	5474755	exon:MLOC_54256.1:1	1	6H	5473235	5473235	leaf	0
6H	247447067	247450327	exon:MLOC_7517.2:1	1	6H	247448194	247448194	blade	0
7H	96048516	96048816	exon:MLOC_36488.1:1	1	7H	96048734	96048734	leaf	0
7H	440064807	440067513	exon:MLOC_72767.1:1	1	7H	440065330	440065330	leaf	0
7H	544501261	544504310	exon:MLOC_39738.1:1	1	7H	544501865	544501865	sheath	0
6H	69839676	69839776	exon:MLOC_11882.4:2	2	6H	69839743	69839743	leaf	0
7H	331094393	331097017	exon:MLOC_54330.1:2	2	7H	331096165	331096165	blade	0
1H	61790876	61791279	exon:MLOC_66388.8:3	3	1H	61791253	61791253	leaf	0
3H	421991486	421991892	exon:MLOC_18521.3:3	3	3H	421991580	421991580	leaf	0
7H	96049105	96050237	exon:MLOC_36488.1:3	3	7H	96049134	96049134	leaf	0
3H	516390233	516390451	exon:MLOC_37766.1:4	4	3H	516390244	516390244	blade	0
4H	434415593	434415838	exon:MLOC_58529.1:4	4	4H	434415773	434415773	blade	0
2H	578608506	578608551	exon:MLOC_54514.1:5	5	2H	578608549	578608549	blade	0
5H	484203288	484203413	exon:MLOC_73139.2:5	5	5H	484203386	484203386	blade	0
2H	2183704	2183865	exon:MLOC_57446.2:9	9	2H	2183753	2183753	leaf	0
7H	41386814	41387497	exon:MLOC_57450.2:9	9	7H	41387134	41387134	leaf	0
4H	434420196	434420586	exon:MLOC_58529.6:13	13	4H	434420355	434420355	blade	0
3H	541205210	541205401	exon:MLOC_37244.3:16	16	3H	541205351	541205351	leaf	0
7H	570620131	570620572	exon:MLOC_14604.2:16	16	7H	570620258	570620258	blade	0
7H	583930566	583930636	exon:MLOC_62970.1:2	2	7H	583930697	583930697	leaf	62

Appendix 4: Lists of gene ontology terms from differentially methylated genes in barley roots and leaves

List of GO terms enriched for "**biological process**" using differentially HYPOmethylated genes between roots and leaves

% WARNING - This is exported REVIGO data useful only for the specific purpose of constructing a TreeMap visualization.
% Do not use this table as a general list of non-redundant GO categories as it sets an extremely permissive
% threshold to detect redundancies (c=0.10) and fill the 'representative' column while normally c>=0.4 is recommended.
% To export a reduced-redundancy set of GO terms go to the Scatterplot & Table tab   and export to CSV from there.

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0006518	peptide metabolic process	0.32%	-31.5045	0.723	0	peptide metabolism
GO:0055086	nucleobase-containing small molecule metabolic process	10.84%	-4.1945	0.536	0.357	peptide metabolism
GO:0046129	purine ribonucleoside biosynthetic process	1.07%	-10.1586	0.44	0.256	peptide metabolism
GO:1901564	organonitrogen compound metabolic process	19.57%	-48.7258	0.698	0.428	peptide metabolism
GO:1901137	carbohydrate derivative biosynthetic process	3.92%	-6.4634	0.636	0.489	peptide metabolism
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-13.1574	0.582	0.507	peptide metabolism
GO:0043603	cellular amide metabolic process	0.91%	-26.0419	0.77	0.14	peptide metabolism
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-54.9281	0.663	0.201	peptide metabolism
GO:0006725	cellular aromatic compound metabolic process	33.05%	-18.9747	0.786	0.259	peptide metabolism
GO:0015672	monovalent inorganic cation transport	2.71%	-10.1586	0.792	0	monovalent inorganic cation transport
GO:0006818	hydrogen transport	1.98%	-4.1864	0.826	0.481	monovalent inorganic cation transport
GO:0006810	transport	17.38%	-8.2218	0.88	0.537	monovalent inorganic cation transport
GO:0006811	ion transport	5.99%	-8.585	0.804	0.631	monovalent inorganic cation transport
GO:0015985	energy coupled proton transport, down electrochemical gradient	0.45%	-5.3279	0.768	0.687	monovalent inorganic cation transport
GO:0043933	macromolecular complex subunit organization	1.06%	-2.9917	0.899	0.028	macromolecular complex subunit organization
GO:0065003	macromolecular complex assembly	0.68%	-2.9917	0.902	0.632	macromolecular complex subunit organization
GO:0006091	generation of precursor metabolites and energy	3.22%	-28.0975	0.848	0.062	generation of precursor metabolites and energy
GO:0015979	photosynthesis	0.34%	-16.5935	0.878	0.063	photosynthesis
GO:0022900	electron transport chain	1.16%	-14.52	0.782	0.072	electron transport chain
GO:0019684	photosynthesis, light reaction	0.09%	-4.3778	0.875	0.577	electron transport chain
GO:0044281	small molecule metabolic process	21.50%	-14.3615	0.775	0.328	electron transport chain
GO:0055114	oxidation-reduction process	15.04%	-17.6364	0.786	0.177	electron transport chain
GO:0090407	organophosphate biosynthetic process	4.46%	-9.8182	0.502	0.087	organophosphate biosynthesis
GO:1901576	organic substance biosynthetic process	28.89%	-45.0655	0.657	0.663	organophosphate biosynthesis
GO:0019538	protein metabolic process	12.33%	-46.9914	0.78	0.158	organophosphate biosynthesis
GO:0006796	phosphate-containing compound metabolic process	16.69%	-15.8153	0.565	0.603	organophosphate biosynthesis
GO:0009206	purine ribonucleoside triphosphate biosynthetic process	0.51%	-10.1586	0.471	0.445	organophosphate biosynthesis
GO:1901566	organonitrogen compound biosynthetic process	9.30%	-45.9508	0.594	0.457	organophosphate biosynthesis
GO:0010467	gene expression	16.70%	-32.3449	0.784	0.493	organophosphate biosynthesis
GO:1901135	carbohydrate derivative metabolic process	11.65%	-14.3615	0.838	0.155	organophosphate biosynthesis
GO:0018130	heterocycle biosynthetic process	16.15%	-4.2899	0.596	0.538	organophosphate biosynthesis
GO:0072521	purine-containing compound metabolic process	8.06%	-14.3615	0.619	0.536	organophosphate biosynthesis
GO:1901360	organic cyclic compound metabolic process	33.91%	-18.9747	0.806	0.23	organophosphate biosynthesis
GO:0044260	cellular macromolecule metabolic process	28.59%	-45.0655	0.702	0.448	organophosphate biosynthesis
GO:0009059	macromolecule biosynthetic process	17.67%	-22.9172	0.645	0.554	organophosphate biosynthesis
GO:0043412	macromolecule modification	5.09%	-7.1051	0.82	0.295	organophosphate biosynthesis
GO:0044249	cellular biosynthetic process	28.21%	-46.9914	0.645	0.38	organophosphate biosynthesis
GO:0043170	macromolecule metabolic process	32.80%	-49.6198	0.808	0.12	organophosphate biosynthesis
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	-43.5901	0.58	0.534	organophosphate biosynthesis
GO:0044267	cellular protein metabolic process	8.78%	-41.0501	0.75	0.331	organophosphate biosynthesis
GO:0009199	ribonucleoside triphosphate metabolic process	6.56%	-14.3615	0.394	0.655	organophosphate biosynthesis
GO:0034645	cellular macromolecule biosynthetic process	17.20%	-29.8508	0.607	0.55	organophosphate biosynthesis

List of GO terms enriched for "**biological process**" using differentially HYPERmethylated genes between roots and leaves

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0006091	generation of precursor metabolites and energy	3.22%	-3.2835	0.695	0	generation of precursor metabolites and energy
GO:1901564	organonitrogen compound metabolic process	19.57%	-11.6757	0.518	0.014	organonitrogen compound metabolism
GO:0009126	purine nucleoside monophosphate metabolic process	6.01%	-2.6132	0.353	0.599	organonitrogen compound metabolism
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-6.9101	0.512	0.428	organonitrogen compound metabolism
GO:0009059	macromolecule biosynthetic process	17.67%	-4.8861	0.536	0.465	organonitrogen compound metabolism
GO:0019637	organophosphate metabolic process	11.97%	-2.5342	0.491	0.135	organonitrogen compound metabolism
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	-7.2967	0.415	0.335	organonitrogen compound metabolism
GO:1901135	carbohydrate derivative metabolic process	11.65%	-2.4619	0.689	0.134	organonitrogen compound metabolism
GO:0044267	cellular protein metabolic process	8.78%	-3.8502	0.553	0.124	organonitrogen compound metabolism
GO:0034645	cellular macromolecule biosynthetic process	17.20%	-2.6079	0.479	0.506	organonitrogen compound metabolism

List of GO terms enriched for "**cellular components**" using differentially HYPOmethylated genes between roots and leaves

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0009536	plastid	1.59%	-104.4724	0.42	0	plastid
GO:0044435	plastid part	0.28%	-78.3546	0.364	0.678	plastid
GO:0044391	ribosomal subunit	1.36%	-12.0726	0.313	0.512	plastid
GO:0009534	chloroplast thylakoid	0.22%	-76.8447	0.191	0.431	plastid
GO:0043232	intracellular non-membrane-bounded organelle	7.68%	-26.3979	0.408	0.659	plastid
GO:0005737	cytoplasm	38.16%	-138.4045	0.651	0.183	plastid
GO:0043231	intracellular membrane-bounded organelle	8.85%	-113.4976	0.352	0.666	plastid
GO:0031984	organelle subcompartment	0.23%	-76.8447	0.459	0.436	plastid
GO:0044444	cytoplasmic part	13.61%	-135.2388	0.644	0.316	plastid
GO:0070069	cytochrome complex	0.05%	-2.8545	0.756	0	cytochrome complex
GO:0030529	ribonucleoprotein complex	6.09%	-21.8153	0.551	0.317	cytochrome complex
GO:0009579	thylakoid	0.41%	-74.5719	0.744	0.097	thylakoid

List of GO terms enriched for "**cellular components**" using differentially HYPERmethylated genes between roots and leaves

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0009536	plastid	1.59%	-32.9393	0.258	0	plastid
GO:0009534	chloroplast thylakoid	0.22%	-10.2874	0.11	0.436	plastid
GO:0031984	organelle subcompartment	0.23%	-11.4134	0.308	0.428	plastid
GO:0044444	cytoplasmic part	13.61%	-18.1319	0.507	0.316	plastid
GO:0005737	cytoplasm	38.16%	-30.5272	0.543	0.183	plastid
GO:0043231	intracellular membrane-bounded organelle	8.85%	-34.9957	0.206	0.666	plastid

List of GO terms enriched for "**molecular function**" using differentially HYPOmethylated genes between roots and leaves

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0015077	monovalent inorganic cation transmembrane transporter activity	2.61%	-12.8894	0.492	0	monovalent inorganic cation transmembrane transporter activity
GO:0015399	primary active transmembrane transporter activity	2.06%	-4.9508	0.521	0.607	monovalent inorganic cation transmembrane transporter activity
GO:0046906	tetrapyrrole binding	1.91%	-13.8761	0.73	0	tetrapyrrole binding
GO:0017076	purine nucleotide binding	15.99%	-5.2097	0.59	0.294	tetrapyrrole binding
GO:0003676	nucleic acid binding	21.75%	-18.9747	0.691	0.18	tetrapyrrole binding
GO:0003723	RNA binding	5.86%	-12.8894	0.711	0.226	tetrapyrrole binding
GO:0032555	purine ribonucleotide binding	15.59%	-4.0555	0.576	0.687	tetrapyrrole binding
GO:0050136	NADH dehydrogenase (quinone) activity	0.78%	-2.2874	0.846	0	NADH dehydrogenase (quinone) activity
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing a	7.51%	-6.2182	0.77	0.03	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
GO:0046914	transition metal ion binding	7.34%	-7.3382	0.772	0.079	transition metal ion binding

Appendix 5: Lists of gene ontology terms from differentially methylated genes in barley roots and leaves and specific to leaf blades and leaf sheaths

List of GO terms enriched for "**biological process**" using differentially HYPOmethylated genes between roots and leaves and specific to leaf blades

% WARNING - This is exported REVIGO data useful only for the specific purpose of constructing a TreeMap visualization.
% Do not use this table as a general list of non-redundant GO categories as it sets an extremely permissive
% threshold to detect redundancies (c=0.10) and fill the 'representative' column while normally c>=0.4 is recommended.
% To export a reduced-redundancy set of GO terms go to the Scatterplot & Table tab   and export to CSV from there.

term_ID	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
GO:0043603	cellular amide metabolic process	0.91%	-26.8996	0.758	0	cellular amide metabolism
GO:1901564	organonitrogen compound metabolic process	19.57%	-42.8996	0.679	0.184	cellular amide metabolism
GO:0006518	peptide metabolic process	0.32%	-27.7423	0.671	0.14	cellular amide metabolism
GO:0006139	nucleobase-containing compound metabolic process	29.92%	-11.8041	0.567	0.507	cellular amide metabolism
GO:0009116	nucleoside metabolic process	8.23%	-10.4078	0.37	0.693	cellular amide metabolism
GO:0072522	purine-containing compound biosynthetic process	1.59%	-8.0846	0.537	0.554	cellular amide metabolism
GO:0006164	purine nucleotide biosynthetic process	1.47%	-7.8239	0.396	0.264	cellular amide metabolism
GO:0034641	cellular nitrogen compound metabolic process	33.43%	-22.6073	0.652	0.428	cellular amide metabolism
GO:0042455	ribonucleoside biosynthetic process	1.65%	-5.51	0.405	0.517	cellular amide metabolism
GO:1901137	carbohydrate derivative biosynthetic process	3.92%	-8.0846	0.603	0.009	carbohydrate derivative biosynthesis
GO:1901576	organic substance biosynthetic process	28.89%	-40.1831	0.613	0.371	carbohydrate derivative biosynthesis
GO:1901566	organonitrogen compound biosynthetic process	9.30%	-37.9957	0.54	0.46	carbohydrate derivative biosynthesis
GO:0044711	single-organism biosynthetic process	12.46%	-7.8239	0.639	0.5	carbohydrate derivative biosynthesis
GO:0019438	aromatic compound biosynthetic process	15.56%	-3.3392	0.57	0.536	carbohydrate derivative biosynthesis
GO:0072521	purine-containing compound metabolic process	8.06%	-10.7496	0.583	0.536	carbohydrate derivative biosynthesis
GO:0009059	macromolecule biosynthetic process	17.67%	-19.9547	0.596	0.559	carbohydrate derivative biosynthesis
GO:0044249	cellular biosynthetic process	28.21%	-36.8794	0.607	0.663	carbohydrate derivative biosynthesis
GO:0044271	cellular nitrogen compound biosynthetic process	15.79%	-37.9957	0.548	0.539	carbohydrate derivative biosynthesis
GO:0034645	cellular macromolecule biosynthetic process	17.20%	-27.7423	0.563	0.554	carbohydrate derivative biosynthesis
GO:0098660	inorganic ion transmembrane transport	2.58%	-8.0846	0.741	0.034	inorganic ion transmembrane transport
GO:0055085	transmembrane transport	9.40%	-5	0.709	0.7	inorganic ion transmembrane transport
GO:0006818	hydrogen transport	1.98%	-3.2534	0.816	0.478	inorganic ion transmembrane transport
GO:0006810	transport	17.38%	-6.7011	0.864	0.533	inorganic ion transmembrane transport
GO:0006811	ion transport	5.99%	-7.0132	0.794	0.631	inorganic ion transmembrane transport
GO:0044281	small molecule metabolic process	21.50%	-10.4078	0.776	0.109	inorganic ion transmembrane transport
GO:0055114	oxidation-reduction process	15.04%	-2.5128	0.786	0.328	inorganic ion transmembrane transport
GO:0015979	photosynthesis	0.34%	-13.1518	0.873	0.055	photosynthesis
GO:0006091	generation of precursor metabolites and energy	3.22%	-19.163	0.843	0.07	generation of precursor metabolites and energy
GO:0046483	heterocycle metabolic process	33.33%	-9.5867	0.783	0.243	generation of precursor metabolites and energy
GO:0019538	protein metabolic process	12.33%	-38.4401	0.76	0.448	generation of precursor metabolites and energy
GO:0010467	gene expression	16.70%	-21.6253	0.763	0.493	generation of precursor metabolites and energy
GO:1901135	carbohydrate derivative metabolic process	11.65%	-8.9872	0.827	0.149	generation of precursor metabolites and energy
GO:0044260	cellular macromolecule metabolic process	28.59%	-41.1599	0.683	0.122	generation of precursor metabolites and energy
GO:0043412	macromolecule modification	5.09%	-2.8965	0.801	0.354	generation of precursor metabolites and energy
GO:0043170	macromolecule metabolic process	32.80%	-43.8996	0.798	0.214	generation of precursor metabolites and energy
GO:0044267	cellular protein metabolic process	8.78%	-34.0329	0.733	0.407	generation of precursor metabolites and energy

List of GO terms enriched for "**cellular component**" using differentially HYPOmethylated genes between roots and leaves and specific to blades

% WARNING - This is exported REVIGO data useful only for the specific purpose of constructing a TreeMap visualization.

% Do not use this table as a general list of non-redundant GO categories as it sets an extremely permissive

% threshold to detect redundancies (c=0.10) and fill the 'representative' column while and export to CSV from there.

% To export a reduced-re	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
term_ID	plastid	1.59%	-84.2916	0.371	0	plastid
GO:0009536	plastid part	0.28%	-57.0964	0.312	0.692	plastid
GO:0044435	ribosomal subunit	1.36%	-12.7721	0.298	0.521	plastid
GO:0044391	chloroplast part	0.28%	-57.0964	0.259	0.441	plastid
GO:0044434	intracellular non-membrane-bounded organelle	7.68%	-16.5638	0.359	0.659	plastid
GO:0043232	cytoplasm	38.16%	-113.9066	0.617	0.183	plastid
GO:0005737	intracellular membrane-bounded organelle	8.85%	-116.4067	0.301	0.666	plastid
GO:0043231	organelle subcompartment	0.23%	-55.057	0.412	0.444	plastid
GO:0031984	cytoplasmic part	13.61%	-111.8928	0.609	0.316	plastid
GO:0044444	ribonucleoprotein complex	6.09%	-21.6289	0.572	0.248	plastid
GO:0030529	thylakoid membrane	0.31%	-55.057	0.376	0.094	thylakoid membrane
GO:0042651	thylakoid	0.41%	-53.0585	0.718	0.097	thylakoid
GO:0009579						

List of GO terms enriched	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
term_ID	plastid	1.59%	-7.7423	0.006	0	plastid
GO:0009536	cytoplasmic part	13.61%	-7.2314	0.133	0.194	plastid
GO:0044444	intracellular membrane-bounded organelle	8.85%	-8.5918	0.011	0.666	plastid
GO:0043231						

List of GO terms enriched	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
term_ID	cation transmembrane transporter activity	3.90%	-9.3768	0.475	0	cation transmembrane transporter activity
GO:0008324	P-P-bond-hydrolysis-driven transmembrane transporter acti	2.06%	-5.4295	0.48	0.641	cation transmembrane transporter activity
GO:0015405	adenyl ribonucleotide binding	13.95%	-4.4342	0.5	0	adenyl ribonucleotide binding
GO:0032559	purine nucleotide binding	15.99%	-3.7527	0.529	0.667	adenyl ribonucleotide binding
GO:0017076	nucleoside phosphate binding	20.35%	-3.377	0.687	0.277	adenyl ribonucleotide binding
GO:1901265	transition metal ion binding	7.34%	-2.3719	0.764	0.114	adenyl ribonucleotide binding
GO:0046914	ATPase activity, coupled	2.88%	-7.3799	0.556	0	ATPase activity, coupled
GO:0042623	hydrolase activity, acting on acid anhydrides, catalyzing trans	2.03%	-6.7011	0.367	0.642	ATPase activity, coupled
GO:0016820						

List of GO terms enriched	description	frequencyInDb	log10pvalue	uniqueness	dispensability	representative
term_ID	plastid	1.59%	-5.5622	0.105	0	plastid
GO:0009536	cytoplasmic part	13.61%	-6.6038	0.222	0.316	plastid
GO:0044444	cytoplasm	38.16%	-6.06	0.287	0.183	plastid
GO:0005737	intracellular membrane-bounded organelle	8.85%	-5.9355	0.097	0.666	plastid
GO:0043231						

Appendix 6: Supporting information (Chapter 5)
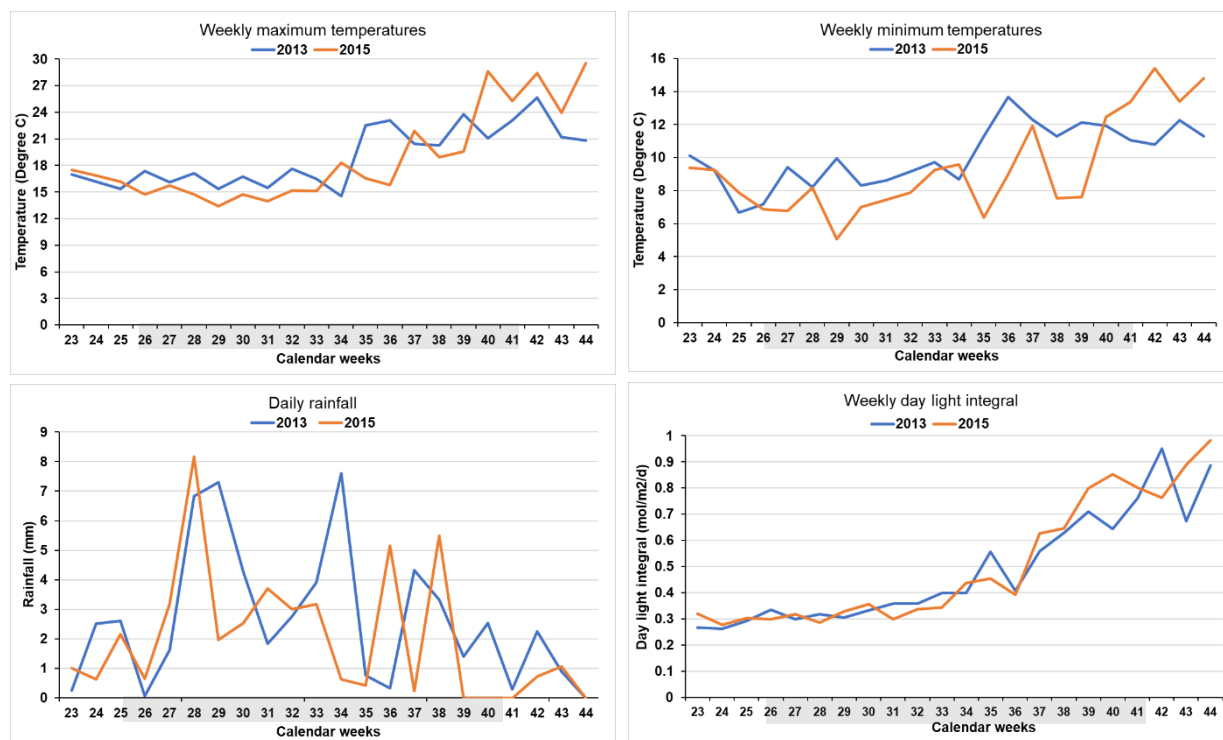
Figure 5.S1: Average climatic factors per calendar week in 2013 (blue curves) and 2015 (orange curves) from data collected in Kent Town, the closest Bureau of Meteorology station to the Waite campus . Shadowed sections on the x-axis indicate the period in which the experiment was conducted in the greenhouse (June to October). Data source: http://www.bom.gov.au/climate/data/

1. Barque73

(a) 4th leaf *Hpa*II
y = -0.001x + 36.75
R² = 0.00

(b) Anthesis *Hpa*II
y = 0.02x + 30.71
R² = 0.32

(g) 4th leaf *Msp*I
y = -0.004x + 43.57
R² = 0.01

(h) Anthesis *Msp*I
y = 0.05x + 34.93
R² = 0.31

(c)) 4th leaf *Hpa*II, Control
y = -0.005x + 40.21
R² = 0.06

(d) Anthesis *Hpa*II, Control
y = 0.02x + 29.12
R² = 0.12

(i) 4th leaf *Msp*I, Control
y = -0.003x + 45.54
R² = 0.02

(j) Anthesis *Msp*I, Control
y = 0.04x + 31.41
R² = 0.33

(e)) 4th leaf *Hpa*II, Stress
y = -0.01x + 40.55
R² = 0.14

(f) Anthesis *Hpa*II, Stress
y = 0.04x + 28.51
R² = 0.48

(k) 4th leaf *Msp*I, Stress
y = -0.007x + 46.47
R² = 0.05

(l) Anthesis *Msp*I, Stress
y = 0.09x + 23.68
R² = 0.54

EpiGD

Physiscal distance (cm)

204

2.   Buloke

3. Commander

(a) 4th leaf *Hpa*II

y = -0.01x + 44.26
R² = 0.05

(b) Anthesis *Hpa*II

y = 0.05x + 24.06
R² = 0.33

(g) 4th leaf *Msp*I

y = -0.01x + 42.93
R² = 0.05

(h) Anthesis *Msp*I

y = 0.05x + 23.86
R² = 0.33

(c) 4th leaf *Hpa*II, Control

y = -0.03x + 60.84
R² = 0.25

(d) Anthesis *Hpa*II, Control

y = 0.04x + 26.92
R² = 0.22

(i) 4th leaf *Msp*I, Control

y = 0.002x + 40.91
R² = 0.01

(j) Anthesis *Msp*I, Control

y = 0.04x + 26.47
R² = 0.17

(e) 4th leaf *Hpa*II, Stress

y = -0.006x + 38.49
R² = 0.04

(f) Anthesis *Hpa*II, Stress

y = 0.06x + 20.37
R² = 0.37

(k) 4th leaf *Msp*I, Stress

y = -0.09x + 71.50
R² = 0.80

(l) Anthesis *Msp*I, Stress

y = 0.06x + 24.33
R² = 0.35

Physiscal distance (cm)

EpiGD

206

4. Fathom

(a) 4th leaf *Hpa*II

y = 0.01x + 36.98
R² = 0.03

(b) Anthesis *Hpa*II

y = 0.05x + 25.703
R² = 0.42

(g) 4th leaf *Msp*I

y = 0.01x + 35.74
R² = 0.07

(h) Anthesis *Msp*I

y = 0.07x + 21.37
R² = 0.53

(c) 4th leaf *Hpa*II, Control

y = 0.003x + 39.48
R² = 0.00

(d) Anthesis *Hpa*II, Control

y = 0.06x + 20.48
R² = 0.45

(i) 4th leaf *Msp*I, Control

y = 0.003x + 41.05
R² = 0.00

(j) Anthesis *Msp*I, Control

y = 0.07x + 17.95
R² = 0.50

(e) 4th leaf *Hpa*II Stress

y = -0.02x + 47.83
R² = 0.17

(f) Anthesis *Hpa*II, Stress

y = 0.05x + 24.25
R² = 0.41

(k) 4th leaf *Msp*I, Stress

y = -0.003x + 41.81
R² = 0.00

(l) Anthesis *Msp*I, Stress

y = 0.08x + 18.18
R² = 0.60

Physiscal distance (cm)

207

5. Flagship

(a) 4th leaf *Hpa*II
y = 0.007x + 35.60
R² = 0.04

(b) Anthesis *Hpa*II
y = 0.03x + 24.27
R² = 0.45

(g) 4th leaf *Msp*I
y = -0.001x + 35.17
R² = 0.00

(h) Anthesis *Msp*I
y = 0.05x + 27.08
R² = 0.21

(c) 4th leaf *Hpa*II, Control
y = -0.005x + 41.72
R² = 0.03

(d) Anthesis *Hpa*II, Control
y = 0.04x + 21.20
R² = 0.42

(i) 4th leaf *Msp*I, Control
y = 0.003x + 37.25
R² = 0.01

(j) Anthesis *Msp*I, Control
y = 0.05x + 27.28
R² = 0.20

(e) 4th leaf *Hpa*II, Stress
y = 0.01x + 34.58
R² = 0.09

(f) Anthesis *Hpa*II, Stress
y = 0.03x + 25.88
R² = 0.44

(k) 4th leaf *Msp*I, Stress
y = -0.01x + 38.00
R² = 0.16

(l) Anthesis *Msp*I, Stress
y = 0.06x + 21.55
R² = 0.20

Physiscal distance

Physiscal distance

Physiscal distance (cm)

Physiscal distance (cm)

6.   Hindmarsh

(a) 4th leaf *Hpa*II
y = 0.003x + 33.42
R² = 0.00

(b) Anthesis *Hpa*II
y = 0.04x + 24.37
R² = 0.30

(g) 4th leaf *Msp*I
y = 0.003x + 36.55
R² = 0.00

(h) Anthesis *Msp*I
y = 0.03x + 23.15
R² = 0.23

(c) 4th leaf *Hpa*II, Control
y = 0.03x + 26.35
R² = 0.33

(d) Anthesis *Hpa*II, Control
y = 0.03x + 32.71
R² = 0.18

(i) 4th leaf *Msp*I, Control
y = 0.006x + 34.82
R² = 0.01

(j) Anthesis *Msp*I, Control
y = 0.02x + 26.97
R² = 0.07

(e) 4th leaf *Hpa*II, Stress
y = 0.005x + 32.72
R² = 0.01

(f) Anthesis *Hpa*II, Stress
y = 0.05x + 18.06
R² = 0.32

(k) 4th leaf *Msp*I, Stress
y = -0.0003x + 35.29
R² = 0.00

(l) Anthesis *Msp*I, Stress
y = 0.04x + 21.93
R² = 0.23

7. Maritime

(a) 4th leaf *Hpa*II
y = 0.004x + 38.13
R² = 0.01

(b) Anthesis *Hpa*II
y = 0.03x + 34.99
R² = 0.13

(c) 4th leaf *Hpa*II, Control
y = 0.02x + 32.45
R² = 0.35

(d) Anthesis *Hpa*II, Control
y = 0.005x + 51.11
R² = 0.00

(e) 4th leaf *Hpa*II, Stress
y = -0.01x + 41.99
R² = 0.05

(f) Anthesis *Hpa*II, Stress
y = 0.04x + 22.16
R² = 0.52

(g) 4th leaf *Msp*I
y = 0.01x + 38.44
R² = 0.07

(h) Anthesis *Msp*I
y = 0.03x + 29.09
R² = 0.14

(i) 4th leaf *Msp*I, Control
y = 0.01x + 37.00
R² = 0.10

(j) Anthesis *Msp*I, Control
y = 0.02x + 32.54
R² = 0.05

(k) 4th leaf *Msp*I, Stress
y = 0.02x + 33.35
R² = 0.24

(l) Anthesis *Msp*I, Stress
y = 0.06x + 24.96
R² = 0.27

8.　Schooner

(a) 4th leaf *Hpa*II
y = 0.02x + 34.17
R² = 0.11

(b) Anthesis *Hpa*II
y = 0.05x + 16.55
R² = 0.48

(g) 4th leaf *Msp*I
y = 0.01x + 32.42
R² = 0.12

(h) Anthesis *Msp*I
y = 0.05x + 26.18
R² = 0.45

(c) 4th leaf *Hpa*II, Control
y = 0.04x + 31.19
R² = 0.20

(d) Anthesis *Hpa*II, Control
y = 0.053x + 17.80
R² = 0.44

(i) 4th leaf *Msp*I, Control
y = 0.002x + 33.22
R² = 0.00

(j) Anthesis *Msp*I, Control
y = 0.05x + 29.61
R² = 0.37

(e) 4th leaf *Hpa*II, Stress
y = 0.01x + 28.68
R² = 0.18

(f) Anthesis *Hpa*II, Stress
y = 0.0461x + 16.493
R² = 0.35

(k) 4th leaf *Msp*I, Stress
y = 0.03x + 24.61
R² = 0.35

(l) Anthesis *Msp*I, Stress
y = 0.04x + 27.38
R² = 0.36

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9. Yarra

Figure 5.S2: Correlation between epigenetic distance (EpiGD) and physical distance between plants (cm, centimetre) using the Mantel test, which was performed on data from 9 barley varieties (Barque 73, Buloke, Commander, Fathom, Flagship, Hindmarsh, Maritime, Schooner and Yarra) and methylation sensitive enzymes *Hpa*II (a-f) and *Msp*I (g-l). Analyses involved control and stress plants together (a, b, g and h), control plants only (c, d, i and j) or stress plants only (e, f, k and l)
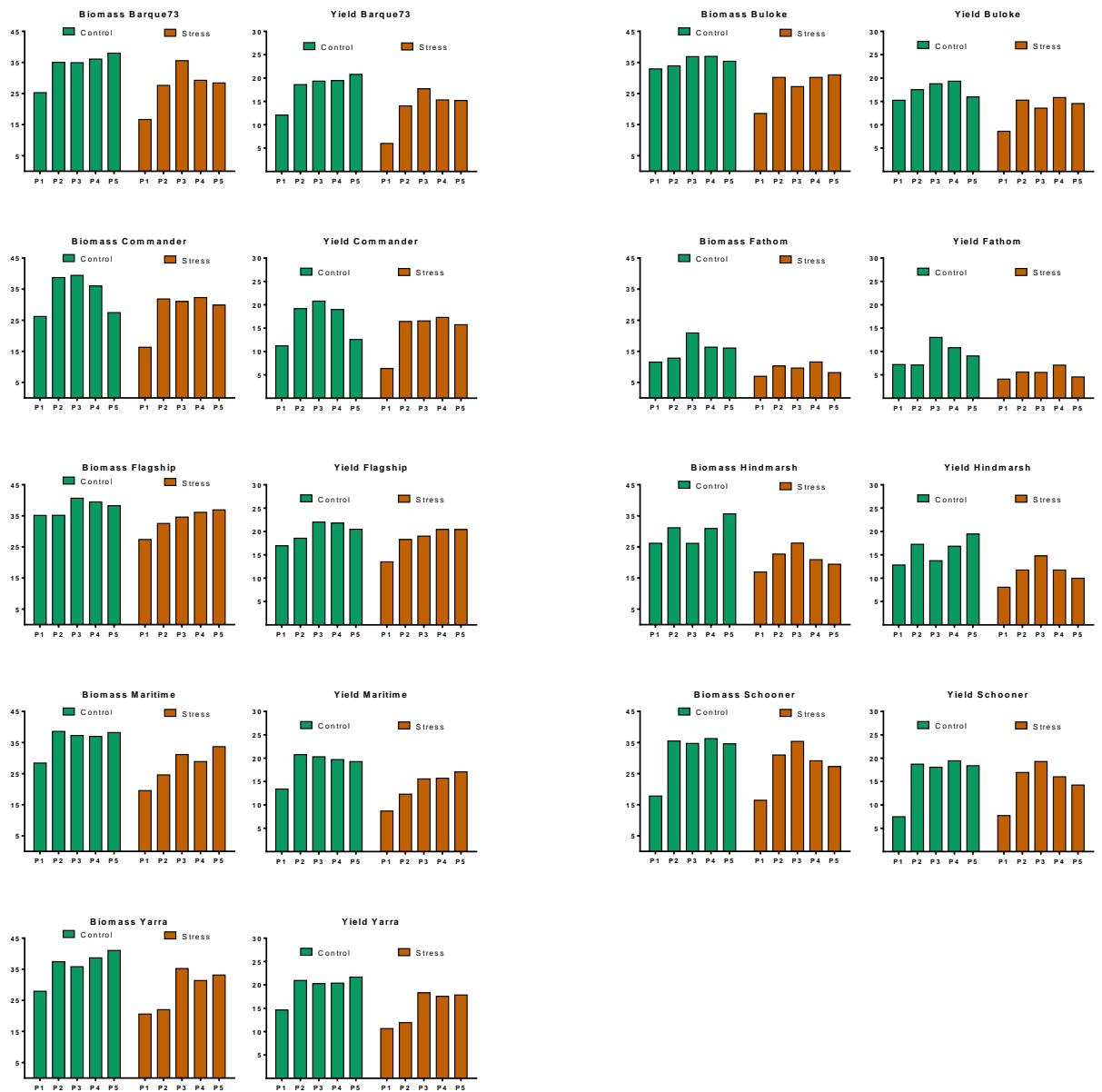
Figure 5.S3: Variability of biomass and yield (grams) between plant positions (P1-5) in the greenhouse for the nine barley varieties: Barque73; Buloke; Commander; Fathom; Flagship; Hindmarsh; Maritime; Schooner and Yarra.

1. Barque73

(a) *Hpa*II, Control
y = 50.53x + 25.35
R² = 0.84

(b) *Hpa*II, Stress
y = 20.57x + 36.28
R² = 0.52

(c) *Msp*I, Control
y = 40.41x + 35.27
R² = 0.48

(d) *Msp*I, Stress
y = 48.00x + 32.64
R² = 0.92

2. Buloke

(a) *Hpa*II, Control
y = 40.10x + 41.58
R² = 0.40

(b) *Hpa*II, Stress
y = -19.31x + 29.69
R² = 0.27

(c) *Msp*I, Control
y = 76.56x + 33.14
R² = 0.44

(d) *Msp*I, Stress
y = 25.04x + 13.21
R² = 0.16

3. Commander

(a) *Hpa*II, Control
y = 27.53x + 39.96
R² = 0.45

(b) *Hpa*II, Stress
y = 47.66x + 28.67
R² = 0.91

(c) *Msp*I, Control
y = 38.712x + 39.621
R² = 0.6632

(d) *Msp*I, Stress
y = 50.74x + 32.83
R² = 0.89

4. Fathom

(a) *Hpa*II, Control
y = 7.25x + 38.66
R² = 0.03

(b) *Hpa*II, Stress
y = 3.27x + 42.51
R² = 0.00

(c) *Msp*I, Control
y = 23.23x + 36.75
R² = 0.25

(d) *Msp*I, Stress
y = -12.95x + 48.15
R² = 0.04

5. Flagship

(a) *Hpa*II, Control
y = 53.27x + 30.03
R² = 0.3944

(b) *Hpa*II, Stress
y = 55.88x + 26.85
R² = 0.81

(c) *Msp*I, Control
y = 100.29x + 25.35
R² = 0.39

(d) *Msp*I, Stress
y = 137.34x + 11.586
R² = 0.89

6. Hindmarsh

(a) *Hpa*II, Control
y = 38.72x + 36.58
R² = 0.31

(b) *Hpa*II, Stress
y = 31.15x + 30.89
R² = 0.47

(c) *Msp*I, Control
y = 4.84x + 33.82
R² = 0.00

(d) *Msp*I, Stress
y = 32.06x + 32.43
R² = 0.50

Axis labels: EpiGD (y-axis), Normalised grain yield (x-axis)

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Figure 5.S4: Correlation between epigenetic distance using *Hpa*II (a, b) or *Msp*I (c, d) profiles and yield from control (a, c) and stress (b, d) plants (varieties: Barque73, Buloke, Commander, Fathom, Flagship, Hindmarsh, Maritime, Schooner and Yarra).

Table 5.S1: Summary statistics of climatic data between 26 June and 12 October 2015 in the greenhouse: Std err =standard error; Std Dev = standard deviation; Var.S =Sample variance; Skew. = skewness; Min = minimum Max = maximum.

		Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
Vapour Pressure Deficit (VPD)	NodeA_day	202.8	872.1	1319.2	1283.6	1672.5	2768.1
	NodeB_day	213.6	862.6	1263	1258.5	1625.5	2947.7
	NodeC_day	136.4	874.3	1255.1	1249.2	1600.2	2661.2
	NodeD_day	292.2	918.7	1360.6	1339.3	1751.7	2904
	NodeA_night	193.7	447.1	613.4	723.9	902.4	2292.5
	NodeB_night	168.9	456.9	612.8	696.2	855.7	1814.4
	NodeC_night	182.7	473.7	624.1	710.7	870.5	1836.1
	NodeD_night	198.4	493.3	650.2	729.7	888.7	1898.9
Light integral	NodeA_day	0	3164	7672	11326	16219	180000
	NodeB_day	0	3585	9190	13309	19670	142554
	NodeC_day	0	4060	10255	14497	22103	75633
	NodeD_day	0	3779	9359	13206	18056	113934
	NodeA_night	0	0	0	39.67	0	4889.98
	NodeB_night	0	0.776	1.357	56.942	1.987	7185.13
	NodeC_night	0	0	0	56.3	0	7628.5
	NodeD_night	0	0	0	56.7	0	7407.7

(a) Normality plots of 'VPD_day'

(b) Normality plots of 'VPD_night'

(c) Normality plots of 'Lint_day'

(d) Normality plots of 'Lint_night'
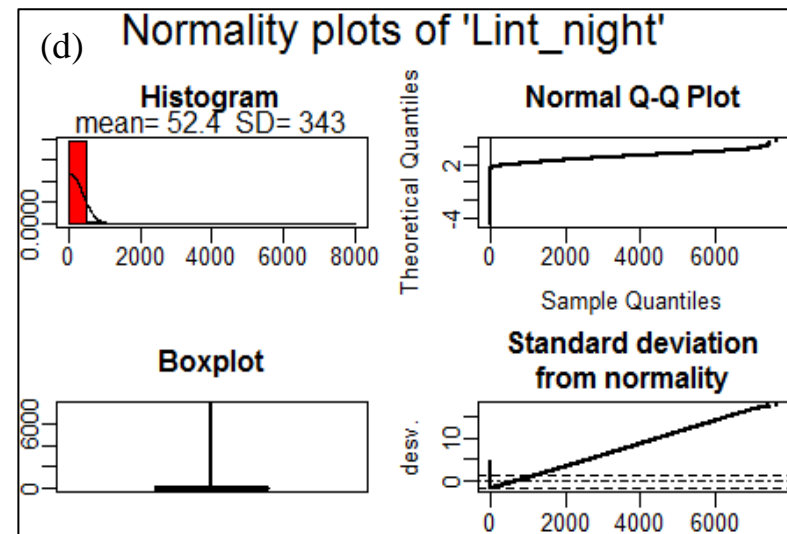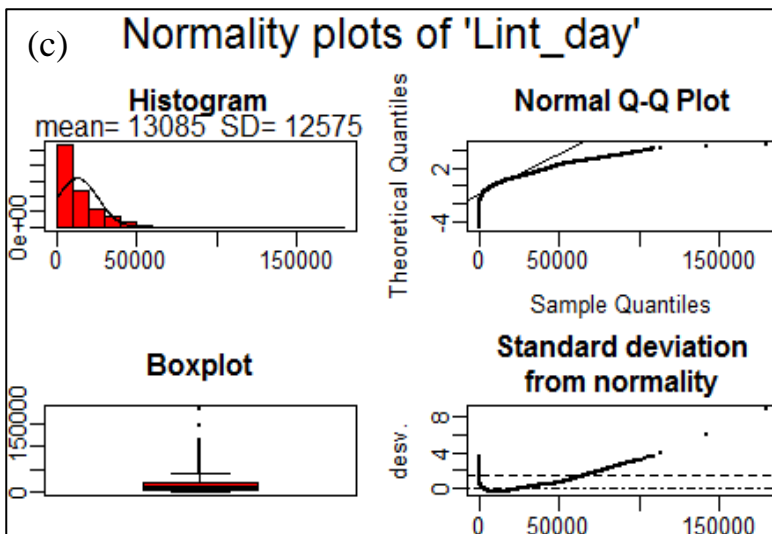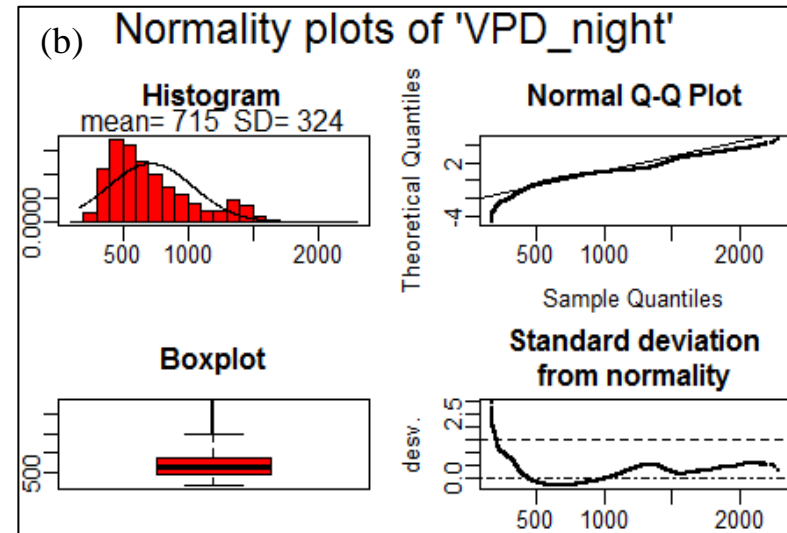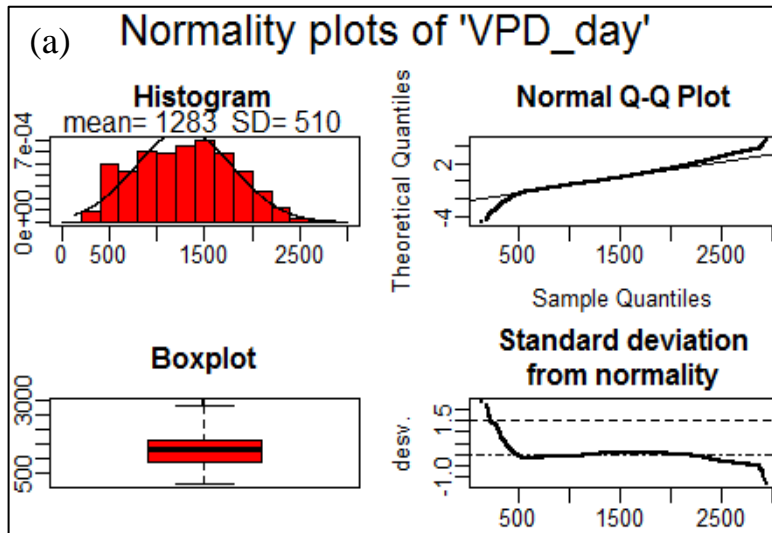
Figure 5.S5: Normality plots of environmental factors calculated from data recorded in the greenhouse from 26 June to 12 October 2015, without missing data due to lack of recording in at least one sensor node: (a) Vapour Pressure Deficit during the day (VPD_day); (b) Vapour Pressure Deficit during the night (VPD_night); (c) Light integral during the day (LI_day); (d) Light integral during the night (LI_night). The timepoint of recording was N=47144 and N=54983 for day and night, respectively.

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