Detection of differentially methylated markers induced by mild salt stress in barley using the methylation sensitive amplified polymorphism (MSAP)

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Abstract

Soil salinity is a major cause of yield loss in barley and other crops across the world. Salt stress induces quick and dynamic physiological adjustments in the plant, to maintain osmotic balance, tolerate Na^+ toxicity or instigate Na^+ exclusion through leaves. The possibility to diagnose salt stress in barley using epigenetic profiling was examined. Global epigenetic profiles of salt stress plants generated by methylation sensitive amplification polymorphism (MSAP) were mostly influenced by genotype. Analysis of qualitative and peak heights of fragment fluorescence showed that there were a small number of epigenetic markers that were significantly induced by salt treatments. Phenotypic data of control and stress plants showed that moderate salinity affects leaf Na^+ and K^+ contents, the projected shoot area, shoot biomass and grain yield in a variety dependent manner. Furthermore, a correlation was found between epigenetic distance and some phenotypic treats such as leaf Na^+ content, shoot biomass and grain yield. These results show an acute implication of DNA methylation in plant response to salinity. However, further investigation is required to characterize fragments corresponding to epigenetic markers and their possible role in gene regulation.

Keywords: MSAP, markers, barley, epigenetics, salinity, diagnostic.

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Détection de marqueurs de méthylation différentielle induits par un stress salin modéré chez l'orge en utilisant la technique du polymorphisme d'amplification sensible à la méthylation (MSAP)

Résumé

La salinité du sol est une cause majeure de perte de rendement chez l'orge et d'autres cultures dans le monde. Le stress salin induit des ajustements physiologiques chez la plante afin de maintenir la balance osmotique, de tolérer la toxicité des ions Na^+ ou de les exclure à travers les feuilles. La possibilité de diagnostiquer le stress salin dans la plante d'orge a été examinée en utilisant le profilage épigénétique. Le profile épigénétique général des plantes sous stress salin par la méthode MSAP (polymorphisme d'amplification sensible à la méthylation) était essentiellement influencé par le génotype. L'analyse des marqueurs qualitatifs et de la hauteur des courbes de fluorescence des fragments montre qu'il y un petit nombre de marqueurs épigénétiques significativement induits par le traitement salin. Les données phénotypiques des plantes témoins et stressées ont montré que la salinité même modérée affecte la concentration des ions Na^+ et K^+ dans les feuilles, le port de la plante, la biomasse foliaire et le rendement grain en fonction des variétés. En plus, une corrélation a été trouvée entre la distance épigénétique et certains traits phénotypiques tels que la teneur en ions sodium des feuilles, la biomasse foliaire et le rendement grain. Ces résultats montrent une possible implication de la méthylation de l'ADN dans la réponse de l'orge à la salinité. Cependant, des investigations complémentaires sont nécessaires pour caractériser les fragments de marqueurs épigénétiques et leur rôle éventuel dans la régulation génique.

Mots-clés : MSAP, marqueurs, orge, épigénétique, salinité, diagnostique.

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), which are associated with an alteration of the expression of stress specific genes (CHOI & SANO, 2007), through mechanisms of enhancement of gene expression (WADA *et al*., 2004), transcriptional (TGS) and post-transcriptional gene silencing (PTGS) (ZILBERMAN *et al*., 2007 ; WANG *et al*., 2013).

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 (CHOI & SANO, 2007 ; BOYKO & KOVALCHUK, 2008). In this way, the DNA methylation pattern varies relative to the stress exerted on the plant (BOYKO & KOVALCHUK, 2008), including but not limited to heavy metals (AINA *et al*., 2004), temperature extremes (PECINKA *et al*., 2010 ; LIU *et al*., 2015), nutrient deficiencies (SECCO *et al*., 2015 ; YONG-VILLALOBOS *et al*., 2015) and salinity (WANG *et al*., 2014 ; KONATE *et al*., 2018). Salinity induced alteration of the plant epigenome suggests that, hypothetically, the DNA methylation pattern may reflect the plant stress condition. Therefore, plant epigenetic profiling offers an opportunity to identify DNA methylation markers associated with salinity stress in the plant.

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 *Ms*pI, in association with *Eco*RI (REYNA-LÓPEZ *et al*., 1997). Based on the differential sensitivity to methylation, the pattern of digestion by HpaII and *Ms*pI 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 ; ROIS *et al*., 2013), tissue types (RODRÍGUEZ LÓPEZ *et al*., 2012) and various stress conditions (MASON *et al*., 2008 ; LIU *et al*., 2015 ; SECCO *et al*., 2015).

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 given species. In this study, barley (*Hordeum vulgare* L.) was used as a model crop plant to understand plant epigenetic response to mild salt stress in a controlled environment. Thus, the methylation profiles of barley plants from multiple cultivars under control and mild stress conditions were compared to identify from these profiles a set of differentially methylated markers (DMMs) that are associated with salt stress. Then, we explored the correlation between the plant epigenetic profile and salt impact.

I. Material and methods

1.1. Plant material and greenhouse conditions

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 NaCl) and 75 mM NaCl. 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 with soil at target weight, to ensure controlled salt application. Pots were lightly watered before sowing three evenly sized and healthy barley seeds per pot. Two weeks after sowing, the barley seedlings were thinned to one per pot.

The soil dry weight and field capacity were used to compute the amount of NaCl required to imposing salinity levels of 0 mM and 75 mM NaCl (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. 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. The greenhouse day/night temperatures were set at 22°C/15°C, with natural light throughout the experiment. The stress and control plants were monitored throughout development to assess the impact of salt stress.

1.2. Measurement of phenotypic parameters

1.2.1. Leaf ion content [Na^+, K^+]

Sodium and potassium ion contents in the 4th leaf blades were measured according to the method described by Shavrukov *et al*. (2010). The 4th leaf blades were harvested 41 days after sowing (DAS), and immediately weighted using and electronic balance. After drying in oven at 65°C for 48 hours, each leaf sample was 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 photometer (Model 420, Sherwood, UK) was used to measure the concentrations of Na^+ and K^+ in the digested samples, using the following formula: standard solutions × [(total volume of digest) ÷ (fresh – dry weight of leaf sample)], with standard solutions at the concentration of 500 mM for both sodium (Na^+, Cl^-) and potassium (K^+, Cl^-) (SHAVRUKOV *et al*., 2010).

1.2.2. Automated colour imaging and plant developmental parameters

Automated imaging was performed at three time points, corresponding respectively to full emergence of 4th leaves (41 DAS), anthesis (87 DAS) and pollination (119 DAS). Fixed-optics cameras (Scanalyzer 3D, LemnaTec, Aachen, Germany) were used for this imaging at The Plant Accelerator (The University of Adelaide, Australia), under standardised lighting 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 (RAJENDRAN *et al*., 2009 ; BERGER *et al*., 2010). At maturity, grain yield and above ground biomass 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).

1.2.3. Phenotypic data analysis

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

1.3. MSAP analysis

1.3.1. DNA restriction and adapter ligation

DNA was first extracted from frozen tissue samples collected on 4th leaf blades, first tillers, flag leaf minus one and flag leaves. Frozen plant samples were ground in a bead beater (2010-Geno/Grinder, SPEX SamplePrep®, Metuchen, NJ, USA). DNA isolation was performed using the Qiagen DNeasy kit (Qiagen, Dusseldorf, Germany), according to manufacturer's instructions. Then, the concentration of DNA samples was standardised to 10 ng/μ1, and stored at 20°C until needed for subsequent analyses.

The MSAP 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 isoschizomers that shows differential sensitivity to DNA methylation at CCGG sites (*Hp*aII and *Msp*I).

Double stranded DNA adapters (table 1) complementary to the restriction products generated by *Eco*RI or *Hpa*II/*Msp*I were ligated to the digested DNA. DNA digestion and adapter ligation were carried out in a single reaction solution of 11 μl, including 1.1 μl T4 ligase buffer; 0.1 μl HpaII; 0.05 μl *Msp*I; 0.25 μl *Eco*RI; 0.05 μl T4 ligase; 0.55 μl BSA ; 1.1 μl NaCl ; 1 μl Adapter *Eco*RI; 1 μl Adapter *Hpa*II/*Msp*I; 5.5 μl DNA sample and 0.3 μl reverse osmosis water. The buffer and all enzymes were from New England Biolabs, Australia (NEB) and oligos were synthesised at Sigma-Aldrich, Australia. The reaction was incubated in a Bio-Rad T100™ Thermal Cycler (Bio-Rad Laboratories, Inc. Australia) for 2h at 37ºC, followed by enzyme inactivation at 65ºC for 10 minutes.

Table 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

1.3.2. PCR amplifications

Products of the restriction/ligation reaction were used to perform two successive PCR amplifications. In the first PCR amplification (pre-amplification), primers complementary to adaptors but with unique 3' overhangs (*Hpa*II/*Msp*I primer +C and EcoRI primer +A, table 1) were used in a pre-optimised PCR master mix (BioMix™ , Bioline, Meridian Bioscience; Australia) following the manufacturer's instructions. Just 0.5 μl of DNA digestion/ligation product was used for PCR amplification, performed in a Bio-Rad T100™ Thermal Cycler. The PCR reactions were performed with the following profile after Rois e*t al*. (2013): 72°C for 2 min, 29 cycles of 30 s denaturing at 94°C, 30 s annealing at 56°C and 2 min extension at 72°C, ending with 10 min at 72°C to ensure completion of the extension.

After checking the pre-amplification product for amplification using agarose electrophoresis, the selective 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. *Hpa*II/*Msp*I selective primers were end labelled using a 6-FAM reporter molecule (6-CarboxyFluorescein) for fragment detection during capillary electrophoresis. The PCR was performed in a solution containing 0.3 μl of pre-

amplification product in the pre-optimised PCR master mix using a Bio-Rad T100™ Thermal Cycler and the following cycling conditions (ROIS *et al*., 2013): 94°C for 2 min, 12 cycles of 94°C for 30 s, 65°C (decreasing by 0.7°C each cycle) for 30 s, and 72°C for 2 min, followed by 24 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min, ending with 72°C for 10 min.

1.3.3. Capillary electrophoresis

The MSAP products were next separated by capillary electrophoresis on an ABI PRISM 3730 (Applied Biosystems, Foster City, CA) at the Australian Genome Research Facility Ltd (Adelaide, Australia). 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.

1.3.4. 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). To minimise user bias, peak calling was carried out using unnamed samples at 150 relative fluorescence units (rfu) threshold. Then, only markers that were consistent in both technical replicates were retained in the marker matrix.

The epigenetic distance between treatments was computed by performing a pairwise Phi statistic (Phi-ST) (MICHALAKIS & EXCOFFIER, 1996) using *msap* package, then the significance of the Phi-ST values was estimated by an Analysis of Molecular Variance (AMOVA) calculating the probability of a null hypothesis (Phi-ST = 0) estimated over 9999 permutations (PEREZ-FIGUEROA, 2013). Salt-induced DMMs were selected based on the presence of differential alleles in at least four out of five samples (frequency ≥ 0.8).

To account for peak height variations in monomorphic fragments, raw intensity scores were compared between salt treated and control samples. Then, 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 & 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 DMMs were selected at a significance cut-off of FDR < 0.05.

II. Results

2.1. Effect of mild salinity on barley varieties

2.1.1. 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 (P-value between 0.0001 and 0.01) (figure 1a). K^+ ions were lower in salt stress plants compared with the control in all varieties. However, this reduction was statistically significant in only three varieties (Maritime, Buloke and Flagship) (P-value < 0.05; figure 1b). The ratio of $[K^+]/[Na^+]$ in the leaves was also significantly different between the control and the stress plants (P-value < 0.05, figure 1c). There was no signification difference between genotypes for these ratios across varieties under control conditions, except Schooner which showed a slight difference with the rest, under control conditions (figure 1c). Also, a pairwise comparison between genotypes showed a significant difference only between Skooner and Commander (P-value < 0.01). Under stress conditions, Barque 73 stands out with the highest ratio (figure 1c) and the genotypes were into four overlapping groups based on the LSD. (figure 1c).

2.1.2. Projected shoot area, biomass and yield components

Imaging of barley plants at three time points (41, 87 and 119 DAS) showed that the difference in projected shoot area between salt treated and control individuals depended on both the variety and the developmental stage (figure 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 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 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 2c).

Shoot biomass at plant maturity revealed that the salt effect on the dry weight differed across varieties (figure 3a). A significant difference between treatments (P-value < 0.05, n = 3; figure 3a) 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 3a). Similarly to the results from the biomass, the grain yield was variety dependent, and only Hindmarsh and Commander were significantly affected by salt stress (P-value < 0.05, n = 3; figure 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 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 3c).

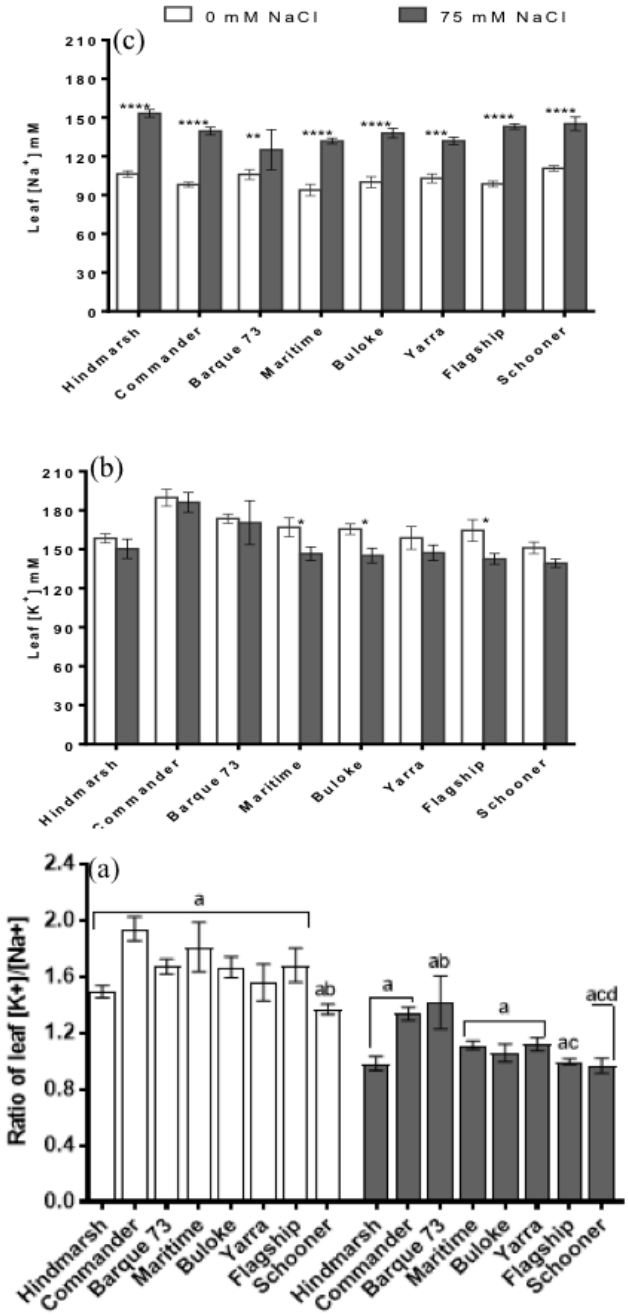

Figure 1: Leaf [Na+] and [K+] of eight barley varieties. (a) Na+ and (b) K+ concentrations (mM plant sap) in the 4th leaf blade of control (0 mM NaCl, white bars) and salt stress (75 mM NaCl, grey bars) plants. (c) Ratio of [K+]/[Na+] in the 4th leaf of the same barley varieties. Varieties with the same 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). (*), (**), (***) 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).

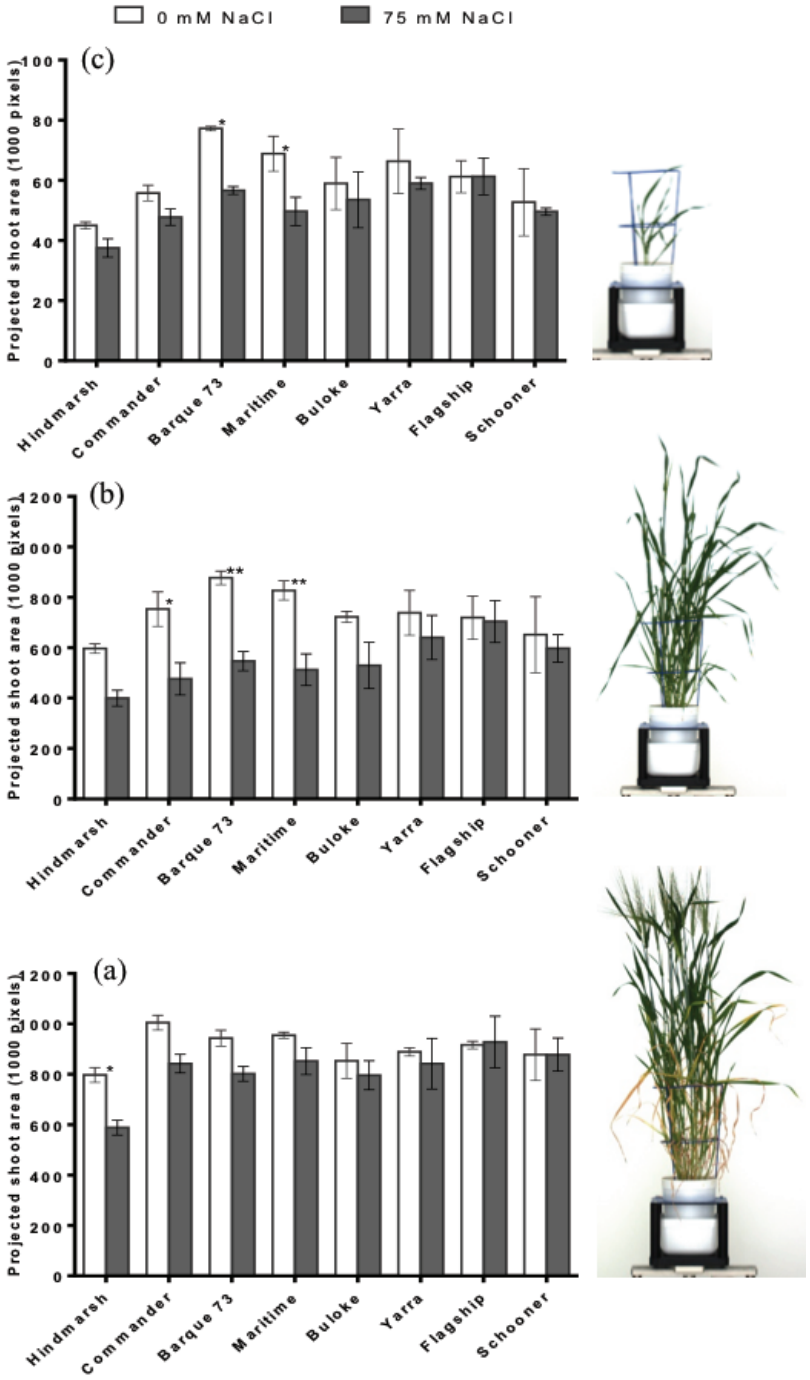

Figure 2: Projected shoot area of eight barley varieties under control (0 mM NaCl, white bars) and stress (75 mM NaCl, grey bars) conditions. (a) at 41, (b) 87 and (c) 119 days after sowing (DAS). The projected shoot area (pixels) was derived from visible light (RGB) images taken at the Plant Accelerator. Values are the mean ± SEM (n = 3) with (*) and (**) indicating significant difference between treatments at $p < 0.05$ and < 0.01, respectively (2-way ANOVA, Fisher's LSD). Plant images: exemplar images (variety Commander) showing the relative size of barley plants at 41, 87 and 119 DAS.

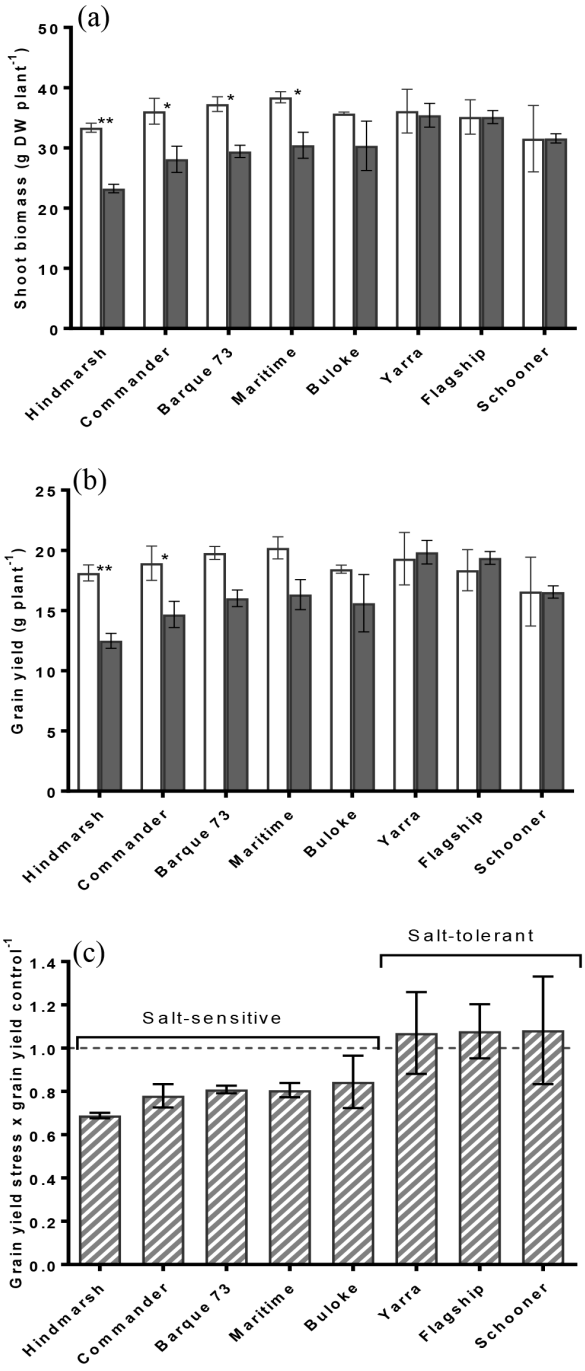

Figure 3: Salt tolerance of eight barley varieties. (a) shoot biomass (g DW per plant); (b) grain yield of eight barley varieties after harvest at maturity in condition of control (0 mM NaCl, white bars) and salt stress (75 mM NaCl, grey bars). Values are the mean ± SEM (n = 3) with asterisk (*) and (**) 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.

2.2. 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%) and 19 were salt-induced differentially methylated markers (DMM) (table 3). The variety Schooner had the highest number of DMMs (5) whereas Commander did not have any (table 3). 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 3). Additionally, 24 salt-induced quantitative DMMs were detected from variations in peak intensity of monomorphic alleles between control and stress plants, from both HpaII (16) and MspI (8) digestions (table 3).

Of the 43 salt-induced DMMs (including qualitative and quantitative DMMs), 20 were found in the 4th leaf samples, whereas twelve, eight and three salt-induced DMMs were found respectively in samples from the F-1, flag leaf and tiller 1 (table 3). Shared salt-induced DMMs were only three qualitative 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 S1 and S2). The highest number of salt-induced DMMs (14) was found in Yarra, whereas Commander had no salt-induced DMM (table 3). The list and fragment size of all salt-induced DMMs are in supplementary Tables S1 and S2.

2.3. Estimation of epigenetic differentiation between salt treatments

Based on qualitative DMMs, 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 4). Only Schooner showed a significant difference between stress and control plants, with a Phi-ST = 0.117 (P = 0.031; Table 3) for the primer combination *Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG.

2.4. 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 variables 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). Then, the highest value between *Hpa*II and *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 trialled, except Commander ($R^2 = 0.012$, table 4). 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 4). Likewise, there were correlations ($R^2 \geq 0.09$) between epigenetic distances between plant treatments 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 4). 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 4).

Table 2: Number of qualitative (Qual) and quantitative (Quant) salt-induced DMMs in barley. Qualitative salt-induced 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). 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); and 87 days after salt stress imposition from flag leaf minus one (F-1) and flag leaf (flag leaf).

Varieties		Barque 73		Buloke		Commander		Flagship		Hindmarsh		Maritime		Schooner		Yara		Total
		Qual	Quant	Qual	Quant	Qual	Quant	Qual	Quant	Qual	Quant	Qual	Quant	Qual	Quant	Qual	Quant	
HpaII	4th L	0	0	0	0	0	0	0	2	1	0	0	0	2	0	0	7	12
	Til-1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
	F-1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	3	7
	FL	1	2	1	0	0	0	0	0	0	0	0	0	0	0	1	0	5
MspI	4th L	0	0	0	0	0	0	0	0	1	1	1	1	3	1	0	0	8
	Til-1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	2
	F-1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	2	0	5
	FL	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	3
Total		2	4	2	2	0	0	1	3	3	2	2	2	5	1	4	10	43

Table 3: 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 DNA samples were from barley 4th leaf blades collected 14 days after salt stress imposition and primer combinations *Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG (125 loci) and *Hpa*II/*Msp*I-CCA + *Eco*RI-AAG (144 loci); Data were analysed using msap software package in R. Pop, population.

Varieties	Pop	*Hpa*II		*Msp*I	
		*Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG	*Hpa*II/*Msp*I-CCA + *Eco*RI-AAG	*Hpa*II/ *Msp*I-CAA + *Eco*RI-ATG	*Hpa*II/*Msp*I-CCA + *Eco*RI-AAG
Barque73	2	0.027 (P= 0.693)	0.048 (P= 0.758)	0.061 (P= 0.900)	0.069 (P= 0.876)
Buloke	2	0.068 (P= 0.760)	0.036 (P= 0.757)	0.011 (P= 0.349)	0.030 (P= 0.300)
Commander	2	0.000 (P= 0.468)	0.097 (P= 0.986)	0.005 (P= 0.374)	0.129 (P= 1)
Flagship	2	0.087 (P= 0.953)	0.074 (P= 0.914)	0.115 (P= 1)	0.018 (P= 0.430)
Hindmarsh	2	0.027 (P= 0.668)	0.028 (P= 0.757)	0.088 (P= 0.071)	0.033 (P= 0.265)
Maritime	2	0.013 (P= 0.581)	0.002 (P= 0.552)	0.006 (P= 0.521)	0.005 (P= 0.417)
Schooner	2	0.081 (P= 0.065)	0.065 (P= 0.114)	0.117 (P= 0.031)	0.073 (P= 0.146)
Yarra	2	0.118 (P= 0.984)	0.042 (P= 0.200)	0.086 (P= 0.884)	0.069 (P= 0.931)
All varieties	2	0.001 (P= 0.525)	0.006 (P= 0.875)	0.004 (P= 0.245)	0.005 (P= 0.211)

Table 4: 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 = 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**

III. Discussion

The symptoms of salinity were variety dependent and no correlation could be establish between grain yield and salt effect on the plant, except for the accumulation of Na^+ in the leaves of barley under salt stress (P-value at least < 0.01, Figure 1a). Therefore, accumulation of sodium ions in leaves does not necessarily reveal the level of salt tolerance of varieties, as noticed in previous studies (GENC *et al*., 2007, ZHU *et al*., 2015). Furthermore, there was no evidence that K^+ uptake was correlated with varietal salt tolerance, since none of the varieties deemed salt-tolerant (Flagship, Schooner and Yarra) showed a significant difference in K^+ concentration between stress and control plants, except Flagship (P-value < 0.05, n = 5; Figure 1b-c). Generally, salt tolerance manifests in the capacity to maintain K^+ uptake under salt stress (ALI *et al*., 2012), often assessed through the K^+/Na+ ratio. In the current study, sensitive and tolerant varieties showed roughly similar K^+/Na^+ ratios (Figure 1c), probably due to the mildness of the salt stress. The low ability to detect salt tolerance level from ions concentrations in leaves (Na^+ and K^+) might be due to the moderate salinity treatment coupled with the overall salt tolerance of barley. This crop is known to have a high tissue tolerance to excess Na^+ (GORHAM *et al*., 1990 ; COLMER *et al*., 2005) and, a high ability to selectively partition K^+ into growing tissues and Na^+ into older leaves and leaf sheaths (GORHAM *et al*., 1990). Additionally, 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 (HASSAN *et al*., 1970 ; MAATHUIS, 2013). Therefore, future studies should show whether varieties considered salt-tolerant (Figure 3a-c) were showing intrinsic salt tolerance or mild salt-induced improvement of biomass and yield.

The low stress level imposed on the plants may explain the weak epigenetic differentiation between control and stressed plants (HASSAN *et al*., 1970). Similar result was reported in a previous study where low salt stress (50 mM NaCl) did not induce any epigenetic signature in barley cultured *in vitro* (DEMIRKIRAN *et al*., 2013). Therefore, the plant epigenetic response is dependent on the stress intensity and duration (DEMIRKIRAN *et al*., 2013 ; SOEN *et al*., 2015). Furthermore, the anonymous nature MSAP markers (REYNA-LÓPEZ *et al*., 1997) and variety specificity of the DMMs make it difficult to validate these as exclusively salt-induced. Nevertheless, our results support the view that barley epigenome is responsive to salt stress, in a variety-dependent manner. The presence of more than a single salt-induced DMM in a variety suggests that salinity can alter several loci at numerous genomic positions simultaneously (WANG *et al*., 2015).

The correlations between salt-induced variations in phenotypic parameters and the epigenetic distance between control and stress plants, suggest that the aptitude to alter the epigenome during salt stress is a function of both the genotype and the environment (GAO *et al*., 2013; LU *et al*., 2015). Therefore, salt-induced DNA methylation may contribute to the regulation of trait expression (KONATE *et al*., 2018), including salt accumulation in leaves, growth rate, biomass and the grain yield (Figure 3b). Although the epigenetic differentiation between stressed plants and controls was relatively low, it has to be remembered that the MSAP inherently detects only a subset of potential markers, due to use of a few selective primers during PCR amplifications.

Conclusion

Salinity, even mild, alters barley physiology and phenotype in a variety dependent fashion. In the same way, salinity induces DMMs, although these were not conserved across varieties. The lack of conserved salt-induced DMMs in this study is attributable to several reasons, including the low salinity level, which may have resulted in cultivar specific responses. The detection of conserved salt-induced DMMs may require higher salt stress or increased number of primer combinations during the MSAP. This technique intrinsically captures only a subset of methylation markers, thus missing many markers that could be salt signatures in the plant. Further investigations are needed to characterise fragments corresponding to salt induced markers, and ultimately determine their functions in the barley genome. One of the ways to achieve this would be to use a Next Generation Sequencing technique, such as the methylation-sensitive Genotyping-by-sequencing (ms-GBS).

Acknowledgments

The authors are grateful to the AusAID for supporting MK with an Australian Awards Scholarship. We acknowledge the staff of the Plant Accelerator® and the Salt Focus Group at ACPFG for their assistance during the study. MJW was paid by the BBSRC (BBS/E/W/0012843C).

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