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Priming effect of menadione sodium bisulphite against salinity stress in *Arabidopsis* involves epigenetic changes in genes controlling proline metabolism

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ABSTRACT

Plants are able to develop numerous defence strategies to face stress. Amongst these, higher plants are capable of demonstrating stress imprint, a mechanism related with the phenomenon of priming. This is usually defined as genetic or biochemical modifications induced by a first stress exposure that leads to enhanced resistance to a later stress. Menadione sodium bisulphite (MSB), a water-soluble addition compound of vitamin K3, was first studied as a plant growth regulator and has been later widely shown to function as plant defence activator against several pathogens in a number of plant species. We recently reported that treating Arabidopsis seeds with MSB primes salt tolerance by inducing an early acclimation to salt stress. Here we describe the analysis of the effect of MSB on cytosine methylation in a salt stress background demonstrating that one of the mechanisms underlying this early acclimation to salt stress is an epigenetic mark. Specifically, MSB leads to a hypomethylation state at the promoter region of genes involved in the biosynthesis (*P5CS1*) and degradation (*ERD5*) of proline, affecting mainly CHG and CHH sites(where H is any nucleotide except G). The epigenetic changes detected are correlated with the observed expression patterns of *P5CS1* (upregulation) and *ERD5* (downregulation) genes and the increase in proline accumulation.

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1. Introduction

Due to their sessile nature, plants cannot avoid exposure to environmental stresses, but they must continuously face survival challenges. They have evolved strategies to defend themselves against a plethora of hostile factors, both biotic and abiotic, through the acquisition of physiological adaptations, molecular mechanisms and biochemical responses to allow completion of their life cycle. One of these adaptive strategies, which has attracted growing interest from the plant research community, is the priming phenomenon. In this sensitization process, an early perception of a stressful event modifies the response to a second stimuli in such a way that this is faster or/and more intense than in unprimed plants (Conrath, 2011; Pastor et al., 2013). Experimental evidence collected over the last decade has indicated that priming processes involve various molecular mechanisms, which are not yet fully understood (Conrath, 2011; Pastor et al., 2013). They include: (i) intracellular accumulation of regulatory proteins; (ii) accumulation of defence compounds inactivated by conjugation; (iii) epigenetic mechanisms. Through changes in chromatin structure, such as variations in composition and position of nucleosomes, post-transcriptional histone modifications or alteration of DNA methylation patterns, epigenetic mechanisms are the best candidate for explaining a long-term primed state (Chinnusamy and Zhu, 2009), especially the latter type because it is the most stable (Henderson and Jacobsen, 2007).

DNA methylation at 5-position of cytosine is a common modification found in genomes of plants, fungi and animals, which affects DNA transcription, replication and repair, and also controls cellular differentiation and transposon activity (Suzuki and Bird, 2008; Jones, 2012). Among eukaryotic organisms, plants show the highest levels of DNA methylation, affecting up to 50% of

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cytosine in some species (Montero et al., 1992). This high percentage of methylcytosine in plant genomes is related with the high content of repetitive DNA, a major target of methylation machinery. Notably, cytosine methylation in plant DNA can be found in each possible sequence context (CG, CHG and CHH, where H is any nucleotide except G), although most of them are localized at the CG dinucleotide. For example, a massive sequencing project revealed that up to 5% of cytosines are methylated in the *Arabidopsis thaliana* genome, with the following distribution: 55%, 23% and 22% in CG, CHG and CHH contexts, respectively (Lister et al., 2008). From a classical point of view, cytosine methylation is an epigenetic mark that suppresses gene expression and the transposition of mobile elements. However, recent studies have indicated that the relationship between DNA methylation and expression is complex, and depends on the affected genetic element, although the repressor effect on gene expression of cytosine methylation in the promoter sequence seems to be very consistent (Lauria and Rossi, 2011; Jones, 2012).

An interesting aspect from an applied perspective is the induction of priming through the exposure of plants to synthetic or natural compounds, both in vegetative tissues (Ton et al., 2005; Alcázar et al., 2010; Molassiotis et al., 2010; Molassiotis and Fotopoulos, 2011; Li and Zhou, 2012; Aranega-Bou et al., 2014) or seeds (Pushpalatha et al., 2007; Worrall et al., 2012). In this context, our research group has demonstrated the properties of menadione sodium bisulphite (MSB) as priming elicitor. MSB is a water-soluble addition compound derived from vitamin K3 that can induce priming against biotic and abiotic stress (Borges et al., 2009; Jiménez-Arias et al., 2015). Interestingly, soaking *A. thaliana* seeds in MSB establishes a priming mark that confers higher tolerance to salt stress on adult plants, specifically by inducing a faster accumulation of proline (Jiménez-Arias et al., 2015). Since innumerable cell divisions have occurred between seed treatment with MSB and plant exposure to salinity stress, we suggest that an epigenetic change may act as "stress imprint". To confirm this, we analysed the effect of seed exposure to MSB on cytosine methylation in adult *Arabidopsis* plants in a salt-stress background. The study was focused on the promoter region of two genes encoding key enzymes involved in the proline metabolism. *P5CS1* (Pyrroline-5-Carboxylate Synthetase 1) encodes an enzyme involved in proline biosynthesis under stress conditions, playing a fundamental role in plant acclimation to salt stress, while *ERD5* (Early Responsive to Dehydration 5) is involved in proline degradation (Kishor et al., 2005; Szabados and Savouré, 2009). The epigenetic changes detected during a time-course analysis were compared with the expression patterns of *P5CS1* and *ERD5* genes and the kinetics of proline accumulation.

2. Material and methods

2.1. Growth conditions and sample collection

Stratification of seeds from *A. thaliana* Col-0 (Leshlee Seed, USA) was performed by soaking in distilled water or in 20 mM MSB, for 2 days at 4 °C in dark conditions. After germination, seedlings were grown in a hydroponic system (Araponics®, Liège, Belgium) with 18 plants each container. The nutritive solution used was: KNO_3 1.25 mM, KH_2PO_4 0.5 mM, $MgSO_4$ 0.75 mM, $Ca(NO_3)_2$ 0.75 mM, H_3BO_3 50 μM, $MnSO_4 \times H_2O$ 10 μM, $ZnSO_4$ 2 μM, $CuSO_4$ 1.5 μM, $(NH_4)_6Mo_7O_{24}$ 0.075 μM, Sequestrene® 44 μM. This solution was renewed every 7 days. The hydroponic system was placed in a growth chamber at 22 °C ±2, 60% relative humidity, 16 h light (100–110 μmol m^{-2} s^{-1}). Gentle aeration was applied every day for 1 h.

After 28 days of growth, NaCl was added to the nutritive solution at a final concentration of 50 mM, defining four experimental groups: plants without treatment (Control); plants treated with NaCl or MSB (Salt treatment and MSB treatment, respectively); and plants exposed to both treatments (MSB-Salt treatment). At each sampling time, whole rosettes were collected, immediately frozen in liquid nitrogen and conserved at −80 °C. The time just before salt addition was set as the 0 h experimental point.

2.2. Measures of proline concentration

The proline concentration at each experimental time-point was calculated as the average of 18 plants from 3 independent experiments. Proline content was determined as described by Bates et al. (Bates et al., 1973) with minor modifications. Samples of 50–100 mg fresh tissue were ground in liquid nitrogen and extracted with 4 ml of 3% sulphosalicylic acid. Two μl of extract was mixed with 2 ml of acidic ninhydrin and incubated at 100 °C for 60 min. This reaction was stopped in an ice bath. After extraction with 4 ml of toluene, the absorbance of the organic phase was read at 520 nm in an Aquarius CE7200 Double Beam Spectrophotometer (Cecil Instruments, Cambridge, England). Proline concentration was calculated from a standard curve, and normalized to the fresh weight.

2.3. Relative quantification of gene expression by real-time RT-qPCR

Relative quantification of mRNA levels was carried out as described by (Borges et al., 2009). Each experimental time-point was analysed as four independent biological replicas, each from 100 mg leaf samples. Sequences of amplification primers for *P5CS1*, *ERD5* and reference genes (*PSBR* and *NPK1*) are shown in Supplementary Table 1.

2.4. Bisulphite sequencing of genomic DNA

Samples for analyses of cytosine methylation patterns were taken from the four experimental groups in four independent replicas at the indicated times. After grinding tissues in liquid nitrogen, genomic DNA (gDNA) was purified with E.Z.N.A. ®SP Plant DNA Kit (OMEGA bio-tek). Concentration of gDNA preparations was measured with a spectrophotometer (NanoDrop® Thermo-scientific). A total of 16 g DNA samples, 250 ng each, were bisulphite treated with the EZ DNA Methylation-Gold™ Kit (Zymo Research), following the manufacturer's recommended protocol except for the thermal profile of bisulphite incubation in a thermocycler that was as follows: initial gDNA denaturation at 95 °C for 2 min, and bisulphite attack at 55 °C for 8 h. During this latter long incubation, denaturation pulses at 95 °C for 1.5 min were applied every 1 h.

Amplification and sequencing primers suitable for analysis of cytosine methylation by bisulphite sequencing (Supplementary Table 2) were designed according to recommendations of various authors (Clark et al., 2006; Henderson et al., 2010). In addition to the CG island close to the transcription start site of *P5CS1* and *ERD5* genes, we also developed primers targeted to the coding region of *ZAT12* gene. This latter sequence was used as internal control for estimating the efficiency of conversion of cytosine to uracil by bisulphite treatment, because the available epigenomic data indicate that *ZAT12* gene is completely unmethylated (Cokus et al., 2008). PCR reactions were performed in a final volume of 20 μl, including 2 μl of gDNA (approximately 10 ng), 200 μM of each dNTP, 0.2 μM of each primer, 0.8 units of FastStart Taq DNA polymerase (Roche), 2 mM of $MgCl_2$, and 1X reaction buffer. The thermal profile was as follows: initial denaturation at 95 °C for 2 min, followed by 45–55 amplification cycles consisting of denaturation at 95 °C for 30 s, annealing at optimized temperature (Supplementary Table 2) for 40 s, and extension at 72 °C for 50 s. Final extension was at 72 °C for 5 min. The optimal annealing

temperature for each primer pair was sought using a thermal gradient (53-60 °C), selecting the lower temperature that completely discriminated DNA untreated with bisulphite. Amplicons were purified with the Illustra ExoStar 1-Step (GE Healthcare).

Amplicon sequencing was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using a3500 Series Genetic Analyzer (Applied Biosystems). Sequencing data files were generated with the KB™ Basecaller because this software does not over-normalize the base least represented in the sequencing read-out as a consequence of bisulphite conversion.

2.5. Quantitative analysis of cytosine methylation at single-base resolution

The methylation level of each cytosine in the selected genomic region was estimated with a methylation analysis tool implemented in the Mutation Surveyor software (SoftGenetics, LLC, 2010). In this applet, sequencing electrophoregrams from bisulphite-treated DNA samples are compared with a 100% converted reference sequence generated by the software, which allows identification of positions affected by methylation. In the case of a partially methylated cytosine, areas of the co-migrating peaks for cytosine and thymine were calculated and expressed as percentage of cytosine (methylation). This operation was manually performed for cytosine outside the standard CG context (Khan et al., 2013). The significance of differences detected in cytosine methylation level at the different sequence context or particular positions were estimated using a nonparametric one-way analysis of variance (Kruskal–Wallis test; IBM SPSS Statistics 20).

2.6. Other bioinformatics tools

The visualization tool from the Athena program (O'Connor et al., 2005) was used to identify CG islands in the upstream region of *P5CS1* and *ERD5* genes. PLACE software (Higo et al., 1999) was used to find on selected CG islands putative DNA-binding motifs for transcriptional factors related to abiotic stress (Supplementary Fig. 1).

3. Results

3.1. Influence of MSB on short-term proline accumulation under salinity stress

First at all, we performed a short term analysis during the first 48 h (Table 1). Like the Control group, endogenous proline levels remained stable throughout the first 48 h in plants which underwent MSB treatment. Addition of salt to the hydroponic system triggered proline accumulation as expected, however this change began earlier in MSB-Salt treatment. After 6 h of exposure to 50 mM NaCl, the amount of proline in plants derived from seeds soaked in MSB was two-fold compared with the other treatment. This higher concentration of proline in MSB-Salt treatment compared to Salt was maintained during the remaining 42 h of the experiment, while in Salt treatment a plateau was observed after 24 h of treatment.

3.2. Priming effect of MSB on proline metabolism at transcriptional level

To find out how MSB enhances proline accumulation in response to salt, the expression levels of *P5CS1* and *ERD5* genes involved in synthesis and degradation of proline, respectively, were estimated in the four experimental groups over a 24-h period after salt addition (Fig. 1). The stable proline levels in Control and MSB treatment groups are in accordance with the lack of significant variations in the quantities of the two transcripts in these plants. In contrast, addition of 50 Mm of NaCl altered the expression of *P5CS1* and *ERD5*. In the case of MSB-Salt treatment, upregulation of *P5CS1* was detected 9 h sooner than in Salt treatment, and reached an expression level approximately 8-fold

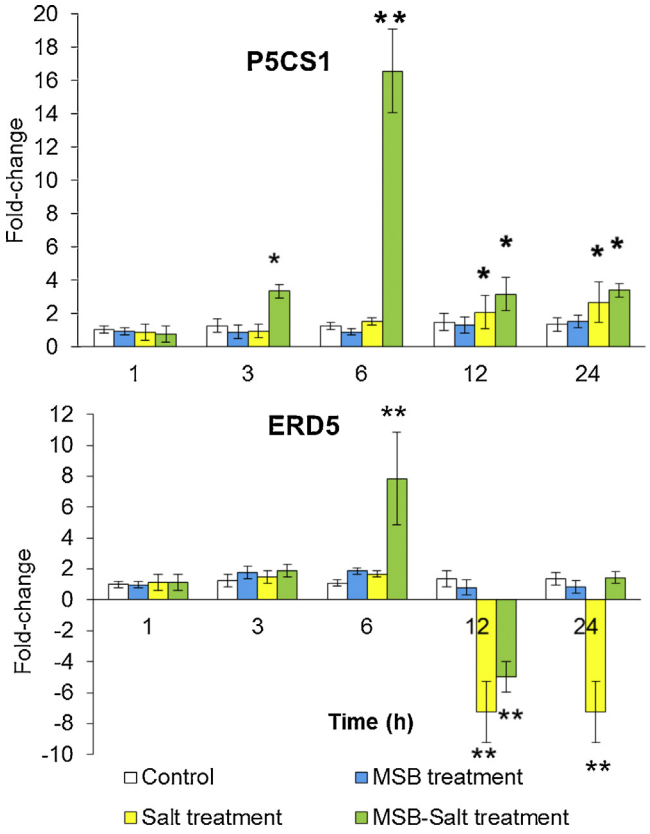

Fig. 1. Expression analysis of *P5CS1* and *ERD5* genes.
Changes in expression of genes associated with proline metabolism over the first 24 h of exposure to 50 mM NaCl. Expression levels have been relativized respect to Control group at 1 h. Expression stability values of the reference genes (*NPK1* and *PSBR*) in the whole sample panel were $M = 0.25$ and CV = 0.086. * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, between Control and Salt treatment, or MSB and MSB-Salt treatments at the same time-point.

Table 1
Kinetic of proline accumulation. Proline levels are presented as μM of proline per mg of fresh plant weight. * and ** indicate significant differences between Salt and MSB-Salt treatments at $p < 0.05$ and $p < 0.01$, respectively.

Treatment	Time after salt addition					
	0 h	3 h	6 h	12 h	24 h	48 h
Ctrl	0.43 ± 0.09	0.49 ± 0.13	0.39 ± 0.17	0.44 ± 0.09	0.42 ± 0.1	0.52 ± 0.18
MSB	0.29 ± 0.07	0.28 ± 0.04	0.3 ± 0.07	0.27 ± 0.07	0.23 ± 0.21	0.28 ± 0.06
Salt	–	0.43 ± 0.07	0.31 ± 0.08	0.41 ± 0.18	1.44 ± 0.13	1.23 ± 0.7
MSB-Salt	–	0.42 ± 0.1	0.61 ± 0.16*	0.81 ± 0.15*	1.71 ± 0.12*	2.75 ± 0.46**

higher after 6 h of NaCl addition. Regarding the *ERD5* gene, the 5-fold downregulation observed under salinity conditions at later time-points (12-24 h) was in agreement with the catabolic nature of the encoded protein and the requirement of an increased proline level to counterbalance osmotic pressure.Contrastingly, a 4-fold increase of *ERD5* expression was detected at time 6 h in MSB-Salt treatment.

3.3. Effect of MSB and salinity on cytosine methylation patterns in the upstream region of P5CS1 and ERD5 genes

Since in the genome of *A. thaliana* most of the 5-methylcytosines are located in the CG context (Lister et al., 2008), we focused our analysis on the CG island closer to *P5CS1* and *ERD5* genes. After bisulphite treatment of 56 gDNA samples representing the four experimental groups at different time-points, conversion

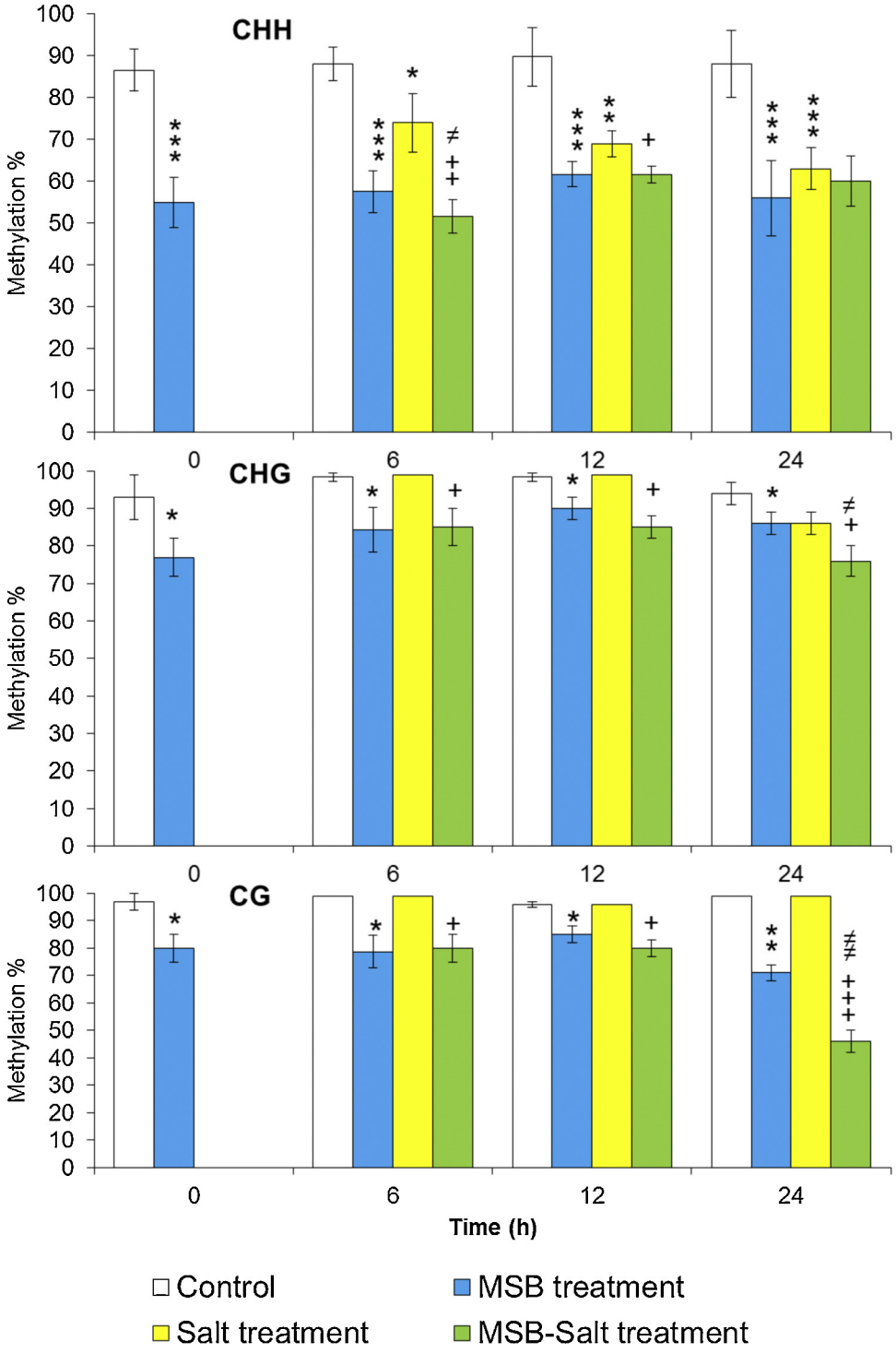

Fig. 2. Level of 5-methylcytosine in the CG island proximal to *P5CS1* gene.
Cytosines unaffected by any treatment were eliminated in this analysis. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, between Control and Salt or MSB treatments. # and ## indicate significant differences at $p < 0.05$ and $p < 0.01$ between MSB and MSB-Salt treatments. +, ++ and +++ indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, between Salt and MSB-Salt treatments.

efficiency of cytosine to uracil was assessed by sequencing the ZAT12 amplicon, whose cytosine content and frequency of CG dinucleotide was similar to the P5CS1 amplicon but higher than ERD5 amplicon. These control analyses revealed 100% cytosine conversion in all gDNA samples and conferred a high confidence level to the results summarized in Figs. 2 and 3.

In regard to *P5CS1* locus (Fig. 2), Control plants showed a high level of cytosine methylation (>85%) in all sequence contexts. The three treatments induced the demethylation of cytosines, which occurred mostly in the CHH context and were concentrated in the region proximal to the transcription initiation site (detailed information is provided in Supplementary Fig. 2). In this sequence context a highly significant reduction in methylation level (around 35%) was detected at 0 h in MSB plants treated at seed stage (4 weeks earlier), compared with Control group (Fig. 2). This drop in methylation level at CHH sites was maintained during the time-course study. Incorporation of NaCl into the nutritive solution led to a progressive decrease in methylcytosine in the CHH context, reaching 28% demethylation 24 h after salt addition. However this treatment did not significantly affect cytosine methylation in plants previously exposed to MSB. The loss of methyl groups at CHG sites induced by MSB treatment was modest (average 12%) but statistically significant at all experimental points. Salinity stress only affected CHG methylation level at the 24 h time-point, when a synergic action of MSB and salt treatments was observed (19% demethylation). Finally, it is noteworthy that a single CG site was demethylated in our assays, solely in plants pre-treated with MSB and reinforced by salinity stress after 24 h of NaCl treatment.

At the *ERD5* locus, only cytosines in symmetrical sites showed a high level of methylation in Control plants (>80%), while this level was moderate (<50%) in the CHH context (Fig. 3). A remarkable difference with respect to *P5CS1* was the predominant response of CHG at *ERD5* (Supplementary Fig. 3), while CHH sites were preferentially affected in the other locus (Supplementary Fig. 2). In addition, CG sites were not affected by any treatment at this locus. Again, methylcytosine contents were reduced by MSB treatment. The reduction in this epigenetic mark was about 31% and 18% at CHH and CHG sites respectively, and was essentially unaltered in MSB treated plants along the time-course (Fig 3). Interestingly, salt exposure did not alter the methylation status of cytosines in the CG island of the *ERD5* locus, with the exception of CHH sites at 6 h after salt addition, which underwent an increase in methylation in both Salt and MSB-Salt plants. Moreover, the methylcytosine level in Salt group was even higher (17%) than in control plants.

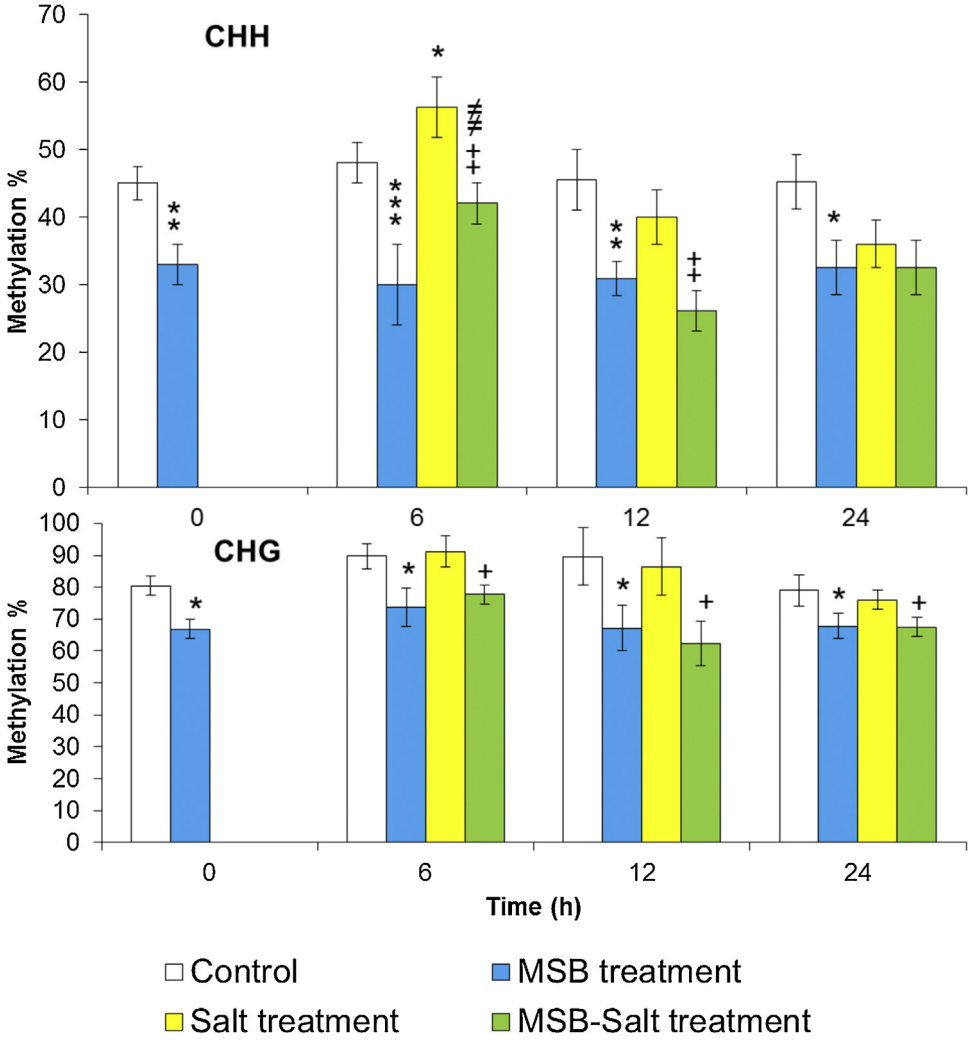

Fig. 3. Level of 5-methylcytosine in the CG island proximal to *ERD5* gene.
Cytosines unaffected by any treatment were eliminated in this analysis. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively, between Control and Salt or MSB treatments. ## indicates significant differences at $p < 0.01$, between MSB and MSB-Salt treatments. + and ++ indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively, between Salt and MSB-Salt treatments.

3.4. DNA-binding motifs and cytosine demethylation events

Using a bioinformatics tool (PLACE) we searched for DNA sequences that can act as binding sites for transcription factors implicated in response to salinity and osmotic stress (Golldack et al., 2011). In the CG island of P5CS1 we found two DNA-binding motifs for MYB factors, separated by only 25 bp (Supplementary Fig. 1), that were demethylated by the experimental treatments.. Although the methylation status of these motifs were analysed during the time course (Supplementary Fig. 4), only the results for 6 h after salt addition are shown in Fig. 4. This is because the most drastic change in expression of *P5CS1* gene was observed at that time point. In this regard, methylcytosine levels were significantly lower (44% and 21% for WACCA and CNGTTR motifs, respectively) in MSB-Salt plants compared with Salt plants.

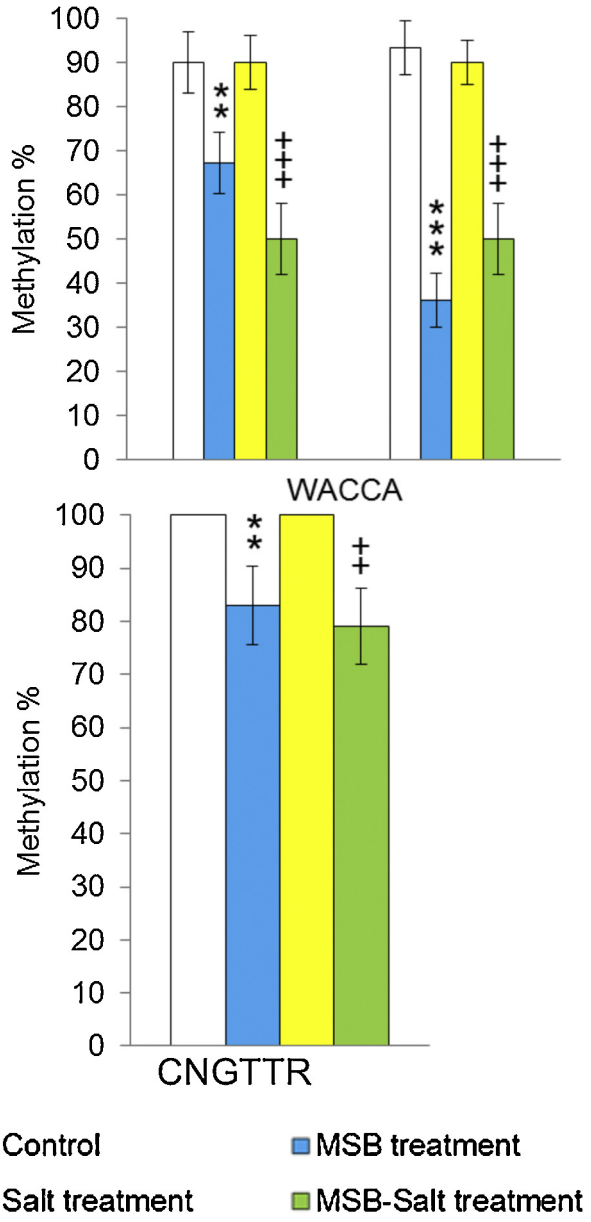

Fig. 4. Effect of NaCl and MSB on the methylation level of cytosines included in putative DNA-binding motifs for transcriptional factors belonging to the MYB family in the *P5CS1* promoter.
Only results obtained at 6 h after salt addition are shown. (A) Two CHH sites in the WAACCA consensus. (B) One CHG site in the CNGTTR consensus. Exact locations of these motifs are indicated in Supplementary Fig. 1. ** and *** indicate significant differences at $p < 0.01$ and $p < 0.001$ respectively, between Control and MSB treatments. ++ and +++ indicate significant differences at $p < 0.01$ and $p < 0.001$, respectively, between Salt and MSB-Salt treatments.

4. Discussion

Numerous studies have demonstrated that adaptive strategies displayed by plants to counteract adverse environmental conditions include the modification of gene expression through regulatory epigenetic mechanisms (Grativol et al., 2012; Sahu et al., 2013). These often lead to a long-term response and sometimes to a trans-generational effect (Sano, 2010; Holeski et al., 2012). Reported chromatin alterations induced by stressing factors include chemical modifications of histones and changes in the cytosine methylation pattern. The specific type of altered epigenetic mark and its consequences on gene expression depend on the nature of the stress and the genomic region analysed (Grativol et al., 2012; Sahu et al., 2013). For example, in *Arabidopsis* leaves after 24 h of salt stress, we found a significant demethylation of cytosines in the CHH (28.5%) and CHG (8.5%) contexts within the promoter region of *P5CS1*, while the upstream region of *ERD5* was unaffected under the same experimental conditions (Figs 2 and 3). Consistently, the reduction of 5-methylcytosine level was correlated with a two-fold upregulation of *P5CS1* transcription in the Salt treatment, whereas expression of *ERD5* was not increased in the same plants (Fig. 1). This is a well-known response of these genes to salinity stress (Szabados and Savouré, 2009). The results we report here about epigenetic changes affecting *P5CS1* gene in a context of salinity stress are in agreement with data published by Zhang et al. (2013) regarding the response of rice to osmotic stress, obtained with a low-resolution technique for methylation analysis.

Although epigenetic mechanisms are clearly implicated in the phenomenon of defence priming, little progress has been made in revealing interconnections among priming-eliciting stimuli, epigenetic changes, gene expression patterns and physiological responses (Pastor et al., 2013; Oosten et al., 2014). In the present report, we further explore the molecular basis of the priming effect against salt stress that is set up in *Arabidopsis* plants after soaking seeds in MSB (Jiménez-Arias et al., 2015). Here, we demonstrate that demethylation of cytosines in the promoter region of *P5CS1* underlies the accelerated accumulation of proline observed under salinity stress conditions in adult plants obtained in this way (Table 1). The maximum activation of *P5CS1* expression induced by salt took place 12 h earlier and was 8-fold higher in MSB-Salt than in Salt plants (Fig. 1), which is correlated with the imprinting left by MSB at the gene promoter as a reduction in methylcytosine content (Fig. 2). These results can explain the subsequent faster and higher accumulation of proline, observed in the MSB-Salt treatment in comparison to Salt treated plants on the basis of epigenetic regulation.

The loss of methyl groups from 5-methylcytosine in the promoter region usually has a positive effect on gene transcription by inducing a more open chromatin conformation, although the effectiveness of this predisposition to expression can rely on specific transcription factors that may or may not be available, depending on other stimuli (Lauria and Rossi, 2011; Pastor et al., 2013). This last mechanism seems to operate in the case of *P5CS1* and its interaction with MSB and NaCl. The epigenetic imprint left by MSB on the upstream region of *P5CS1* in the seeds was similar to the changes in the same DNA sequence induced by NaCl exposure in adult plants (Fig. 2 and Supplementary Fig. 2), both in magnitude (ca. 35% of demethylation), sequence context (preferentially CHH sites) and number of affected sites. However, while NaCl exposure induced a modest but significant upregulation of *P5CS1* with respect to Control treatment, MSB pre-treatment was not sufficient by itself to activate *P5CS1* transcription (Fig. 1).

MSB pre-treatment also affected the methylation status of the region upstream of *ERD5*, decreasing the level of 5-methylcytosine at non-CG sites (Fig 3). Similar to *P5CS1*, exposure to NaCl was necessary to alter the transcription rate of *ERD5* (Fig. 1). Although we also observed the decline in *ERD5* expression under salinity stress described by other authors (Szabados and Savouré,20019), a transient but very significant rise in *ERD5* mRNA was detected in MSB-Salt plants at 6 h after NaCl addition. This may be explained by the lower methylation level of cytosine observed in the promoter region of *ERD5* in MSB-Salt (42%) in comparison with Salt plants (56%) at that time-point, and the fact that increased proline can induce *ERD5* expression (Szabados and Savouré, 2009). In any case, this increment in *ERD5* expression was not high enough to halt proline accumulation in the MSB-Salt group (Table 1).

Interestingly, cytosine demethylation induced by the separate or combined exposure to MSB and NaCl occurred almost exclusively in CHH and CHG contexts within the two analysed DNA regions (Supplementary Figs. 2 and 3). It has been proposed that the dynamic methylation of cytosines in CHH and CHG sites plays an important role in regulating the expression of structural genes in *Arabidopsis* (Zhang et al., 2006). This is because certain mutations that impair methyltransferase activities and cause a hypomethylation state in the non-CG context (*drm1drm2cmt3* triple mutant) lead to the activation of many genes scattered throughout the genome. It is widely accepted that cytosine methylation negatively affects gene expression through the recruitment of chromatin remodelling complexes that make DNA less accessible to transcriptional machinery (Zemach and Grafi, 2007; Lauria and Rossi, 2011). Nevertheless, in some genetic scenarios a methyl group in DNA can directly interfere with binding of regulatory proteins to their target sequences (Tate and Bird, 1993). In this sense, it is important to point out that MSB treatment induced a reduction up to 44% in cytosine methylation at two possible binding sites of MYB and MYC proteins on the *P5CS1* promoter sequence (Fig. 4). These transcription factors are synthesized *de novo* under osmotic damage and cooperatively activate a number of stress-induced genes (Abe et al., 2003).

The generation of reactive oxygen species (ROS) is one of the early events during the establishment of priming against stressing environmental factors in plants (Pastor et al., 2013; Borges et al., 2014). Indeed, ROS-mediated signalling seems to be a common feature of plant response to stress (Pitzschke et al., 2006; Suzuki et al., 2012), although the mechanisms linking ROS generation and final response are so far not well established. As an example, exposure to heavy metals, salt or low temperature cause a similar demethylation in the *NtGPDL* locus of tobacco, and this hypomethylated state is mimicked by treatment with paraquat, a well-known ROS generator (Choi and Sano, 2007). Indeed, menadione has been used as an oxidant agent in a variety of organisms, including plants (Sun et al., 1999). Our research group has demonstrated that MSB activates the expression of many genes related to ROS detoxification in *Arabidopsis* (Borges et al., 2009). Hence, the oxidant activity of menadione is probably involved in the ability of MSB to promote stable epigenetic modifications in seed embryos that would be responsible for tolerance to salinity stress exhibited by adult plants. It would be interesting to investigate with a next-generation sequencing approach how many and which genomic sequences are epigenetically imprinted by MSB, and also the potential relationships of these genetic loci with the different types of resistance against biotic and abiotic stresses induced by this priming compound (Borges et al., 2014).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2015.07.003.

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